

POLLEN AND ANTHOR DEVELOPMENT IN *NELUMBO* (NELUMBONACEAE)¹

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The Nelumbonaceae are a small family of aquatic angiosperms comprising *Nelumbo nucifera* and *Nelumbo lutea*. Historically, the genus has been considered to be closely related to Nymphaeales, however new systematic work has allied *Nelumbo* with lower eudicots, particularly *Platanus*. In recent years, studies of pollen development have contributed greatly to the understanding of phylogenetic relationships, but little has been known about these events in *Nelumbo*. In this paper, pollen and anther development are morphologically described for the first time in *N. lutea*. A comprehensive ontogenetic sequence is documented, including the sporogenous tissue, microspore mother cell, tetrad, free spore, and mature pollen grain stages. The deposition of a microspore mother cell coat and callose wall, the co-occurrence of both tetrahedral and tetragonal tetrads, the formation of a primexine in tetrads, and primexine persistence into the late free spore stage are shown. The majority of exine development occurs during the free spore stage with the deposition of a tectate-columellate ectexine, a lamellate endexine, and an unusual granular layer below and intermixed with the endexine lamellae. A two-layered intine forms rapidly during the earliest mature pollen stage. Major events of anther development documented include the degradation of a secretory-type tapetum during the free spore stage and the rapid formation of U-shaped endothelial thickenings in the mature pollen grain stage. The majority of mature pollen grains are tricolpate, however less common monosulcate and diaperturate grains also develop. Co-occurring aperture types in *Nelumbo* have been suggested to be an important transition in angiosperm aperture number. However, aperture variability in *Nelumbo* may be correlated with the lateness of aperture ontogeny in the genus, which occurs in the early free spore stage. This character, as well as other details of pollen and anther ontogeny in *Nelumbo*, are compared to those of Nymphaeales and *Platanus* in an effort to provide additional insight into systematic and phylogenetic relationships. Although *Nelumbo* is similar to both groups in several characters, the ontogenetic sequence of the genus is different in many ways.

Key words: anther; development; morphology; *Nelumbo*; Nelumbonaceae; pollen; ultrastructure.

The Nelumbonaceae are a small family of aquatic angiosperms comprising *Nelumbo nucifera* Gaertn. and *Nelumbo lutea* (Willd.) Pers. *Nelumbo nucifera*, the Indian or sacred lotus, is found throughout Asia and Australia, whereas *N. lutea*, the American lotus or water chinquapin, occurs in eastern and southern North America (Williamson and Schneider, 1993). More recently, *Nelumbo lutea* has been considered to be a subspecies of *N. nucifera* (Borsch and Barthlott, 1994).

Historically, many authors have considered *Nelumbo* to be closely related to Nymphaeales (water lilies; Moseley, 1958; Ueno and Kitaguchi, 1961; Meyer, 1964; Walker, 1976; Batyгина and Shamrov, 1983; Ito, 1987;

Cronquist, 1988). This taxonomic position was based primarily on similarities in floral and vegetative morphology, as well as in habitat (e.g., Cronquist, 1988). However, there has been disagreement regarding this classification, and several studies have attempted to resolve the phylogenetic relationship of lotuses and water lilies. Past investigations have included studies of floral anatomy (e.g., Moseley and Uhl, 1985; Ito, 1986), leaf anatomy and alkaloids (e.g., Goleniewska-Furmanowa, 1970; Kristen, 1971; Rao and Banerjee, 1979; Barthlott et al., 1996), and seed anatomy (e.g., Collinson, 1980). One especially important difference between lotuses and water lilies is the triaperturate pollen of *Nelumbo*, which differs from the monoaperturate pollen grains of most Nymphaeales (Ueno and Kitaguchi, 1961; Walker, 1976; Kuprianova, 1979; Osborn, Taylor, and Schneider, 1991). The aforementioned suite of characters has supported the placement of *Nelumbo* in its own family and order, distinct from Nymphaeales (Williamson and Schneider, 1993, and references therein). Williamson and Schneider (1993) have suggested that the similarities between *Nelumbo* and water lilies are attributed either to a shared ancestor or to convergent evolution.

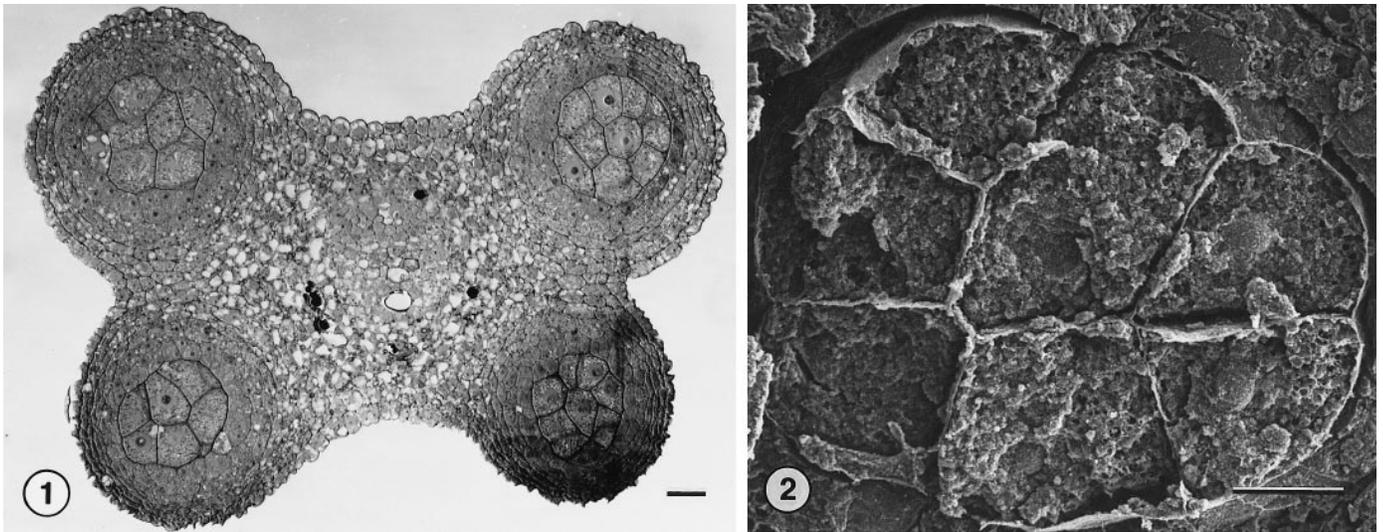
Recent studies have offered support for the convergence hypothesis. Phylogenetic analyses based on chloroplast *rbcl* gene sequence data have indicated that *Nelumbo* may only be remotely related to water lilies (Les, Garvin, and Wimpee, 1991; Chase et al., 1993; Qui et al., 1998). Studies of 18S ribosomal DNA sequences have also found *Nelumbo* and *Nymphaea* to be only distantly related (Soltis et al., 1997). Most molecular studies

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Figs. 1–2. Sporogenous tissue stage. **1.** Section of an entire anther showing four locules. Note that the sporogenous cells have relatively large nuclei and that a distinguishable tapetum has not yet differentiated. Bar = 64 μm . **2.** Detail of single locule showing polygonal shape of cells. Bar = 10 μm .

have placed *Nelumbo* among lower eudicots, although there is no consensus on its exact taxonomic position within this group. *Nelumbo* has been placed in the Hamamelidae, closely related to *Platanus* (Chase et al., 1993; Qui et al., 1998), as well as among the Ranunculids (Soltis et al., 1997). Additional phylogenetic analyses, based on both molecular and nonmolecular characters, have also placed *Nelumbo* among the Hamamelids or Ranunculids (Donoghue and Doyle, 1989; Nandi, Chase, and Endress, 1998).

Another line of evidence for assessing phylogenetic relationships among plants comes from studies of pollen development, specifically ontogeny of the pollen wall (e.g., Blackmore and Crane, 1988; Blackmore and Barnes, 1990; Gabarayeva, 1991; Zavada, 1991). However, no published studies have documented a complete pollen developmental sequence in the genus *Nelumbo*, and none has addressed any aspect of pollen ontogeny in *Nelumbo lutea*. One study examined pollen wall development in *Nelumbo nucifera* using transmission electron microscopy (TEM; Flynn and Rowley, 1971a), but these authors observed samples only in the tetrad stage specifically with respect to primexine and aperture formation (Flynn and Rowley, 1971a; see also Rowley, 1975). Furthermore, only four studies have focused on anther development in *Nelumbo*. Each of these used light microscopy (LM) exclusively and examined the single taxon *N. nucifera* (Khanna, 1965; Gupta and Ahluwalia, 1979; Batygina, Kravtsova, and Shamrov, 1980; Batygina and Shamrov, 1983).

In contrast to the few studies on pollen development, more is known about the morphological aspects of mature pollen grains of *Nelumbo*. Ueno and Kitaguchi (1961) used TEM to examine ultrastructural features of the mature pollen wall from nine species of Nymphaeaceae, including *Nelumbo nucifera*. Walker (1976) studied pollen of *Nelumbo lutea* using scanning electron microscopy (SEM), and several other studies have used LM and SEM to focus on the morphology of both extant and fossil

Nelumbo pollen in a comparative context (e.g., Kuprianova and Tarasevich, 1983; Skawinska, 1985; Zetter and Keri, 1989).

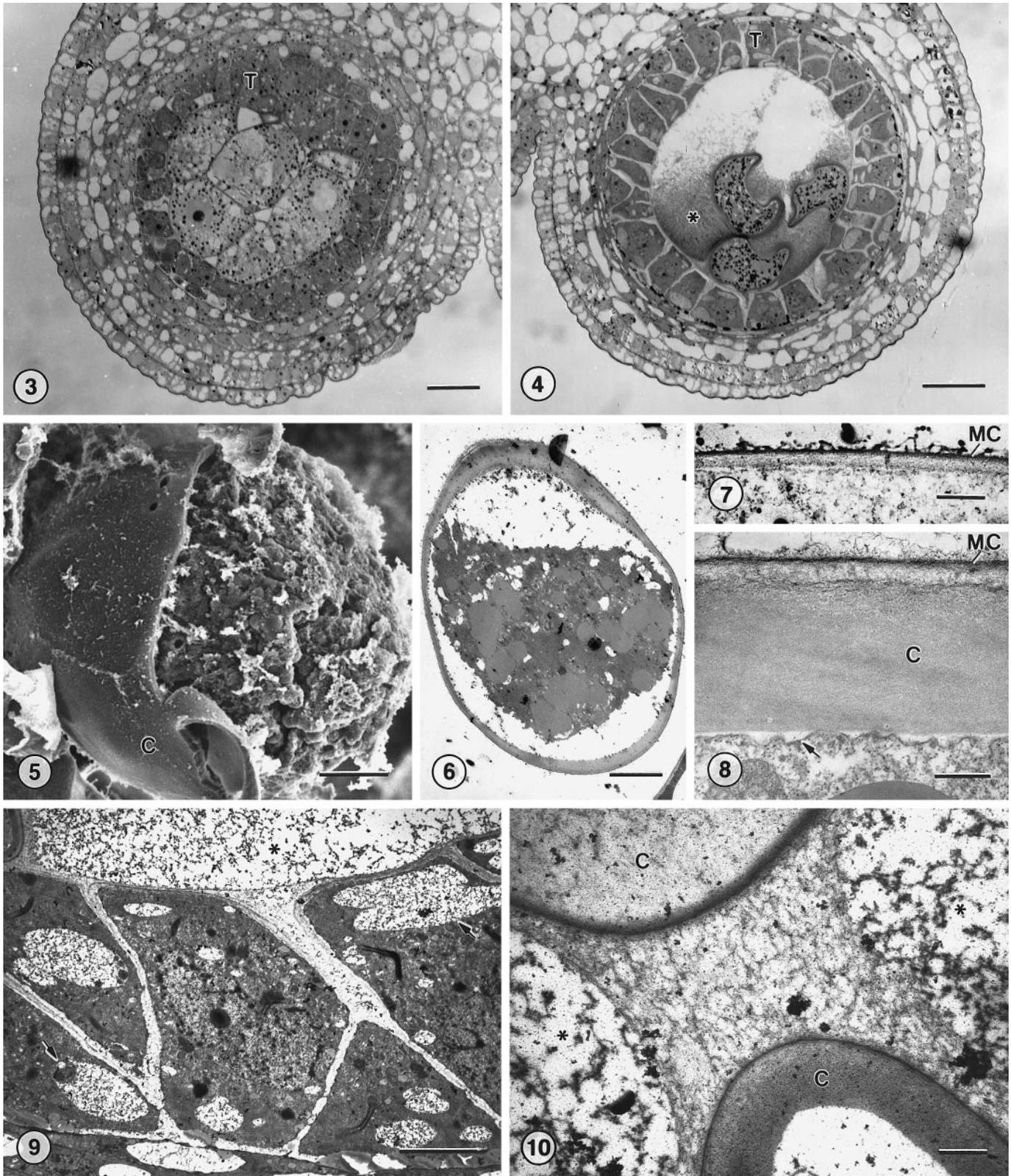
The primary objective of this paper was to document the events of pollen and anther development in *Nelumbo lutea* using LM, TEM, and SEM. Furthermore, the details of pollen and anther ontogeny in *Nelumbo* were compared to those of Nymphaeales and lower eudicots, primarily *Platanus*, in an effort to provide additional insight into systematic and phylogenetic interpretations.

MATERIALS AND METHODS

Floral buds of *Nelumbo lutea* extend above the water surface on long peduncles and are easily accessible. Within the buds, numerous stamens surround a central, cone-shaped receptacle in which the gynoecium is embedded. The stamens of *Nelumbo* consist of a thin filament, an elongate anther, and a vegetative tip appendage that has thermogenic properties (capable of raising bud and floral temperatures an average of 2.3°–4.8°C above ambient; see Schneider, Williamson, and Whitenberg, 1990, and references therein). Anthers were measured from the base of the thermogenic tip to the top of the filament.

Ten floral buds were collected from Lilypons aquatic nursery in Brookshire, Texas, and an additional 49 buds were collected from Lake Springfield in Springfield, Missouri. Anthers were dissected from the buds in the field and were fixed in 3% glutaraldehyde (in 0.2 mol/L phosphate buffer, pH 7.4) for 24 h and then buffer-washed at least four times. Specimens were postfixed in 1% osmium tetroxide (in 0.2 mol/L phosphate buffer, pH 7.4) for 3 h and buffer-washed four times.

The anthers for LM and TEM were dehydrated in a series of ethanol and acetone rinses and then gradually infiltrated and embedded in Spurr epoxy resin. Embedded anthers were sectioned on an ultramicrotome with glass or diamond knives. Thick sections (850 nm) were stained with either Richardson's Stain (Azure II and Methylene Blue) or Toluidine Blue O and examined/imaged with bright-field and differential interference contrast illumination on an Olympus BHS Compound Light Microscope. Thin sections (90–100 nm) were collected on 1 \times 2 mm slot grids and dried on formvar-support films (Rowley and Moran, 1975). Grids were stained with 1% potassium permanganate (0–2 min), 1% uranyl acetate (10 min), and lead citrate (8 min; Venable and Cog-



Figs. 3-10. Microspore mother cell stage. **3.** Section of a single locule showing early microspore mother cells and initially differentiated tapetum (T). Note that the microspore mother cells are tightly appressed and fill the locular space. Bar = 60 μ m. **4.** Section of a single locule showing three late microspore mother cells. Note that the microspore mother cells are separated and each is surrounded by a callose coat. The locule is lined by a well-differentiated tapetum (T) and contains a matrix (*). Bar = 60 μ m. **5.** Detail of a single microspore mother cell with a portion of the callose (C) missing due to removal during specimen preparation. Bar = 5 μ m. **6.** Section through a single microspore mother cell. Note that

geshall, 1965) and then examined/imaged using a JEOL JEM-100SX transmission electron microscope at 80 kV.

Anthers for SEM were dehydrated in a graded ethanol series, critical point dried, and mounted onto aluminum stubs with colloidal graphite. To view morphological features of the anther wall and the locular contents, some anthers (while in 70% ethanol) were either transversely fractured with a double-edged razor blade at room temperature, or were frozen in liquid nitrogen and then fractured. Additional anthers were transversely fractured after critical point drying. To view individual pollen grains, dried anthers were macerated using a syringe needle and mounted onto aluminum stubs with double-sided adhesive tape. All stubs were sputter-coated with gold-palladium and evaluated/imaged using a JEOL JSM-6100 scanning electron microscope at 5 kV.

RESULTS

Within a single floral bud, anther and pollen development typically vary in timing among the stamen whorls (personal observation). Furthermore, pollen development is not synchronous within individual anthers, but varies along the length of the individual anthers. The results presented below have been divided into sections based on the following major stages of pollen ontogeny: sporogenous tissue, microspore mother cells, tetrads, free spores, and mature pollen grains.

Sporogenous tissue stage—The sporogenous tissue completely fills the locular space. Individual cells tightly abut and have somewhat polygonal shapes (Figs. 1–2). The cell cytoplasm stains densely with chromatic stains (Fig. 1), and the nuclei are relatively large (Figs. 1–2). Anthers in the sporogenous stage range in length from 2.5 to 7 mm. The tapetum is undifferentiated at this stage (Fig. 1).

Microspore mother cell stage—Following division of the sporogenous cells and dissociation of the combined cell walls, individual microspore mother cells are identifiable (Figs. 3–6). Early microspore mother cells are relatively large, remain somewhat appressed (Fig. 3), and are surrounded by an electron-dense microspore mother cell coat (Fig. 7). A thin layer of callose is then deposited beneath the microspore mother cell coat (Figs. 3, 7). At this early stage, the plasmalemma is appressed against the callose (Fig. 7).

In the late microspore mother cell stage, cells are completely separated from one another (Fig. 4). Late microspore mother cells are characterized by a thickened callose wall (Figs. 4, 8) that appears two layered, with the inner layer more dense (Fig. 8). At this stage, the plasmalemma is slightly undulated beneath the callose (Fig. 8). In some late microspore mother cells the plasmalemma pulls away from the callose coat (Fig. 6).

Anthers in the microspore mother cell stage range in

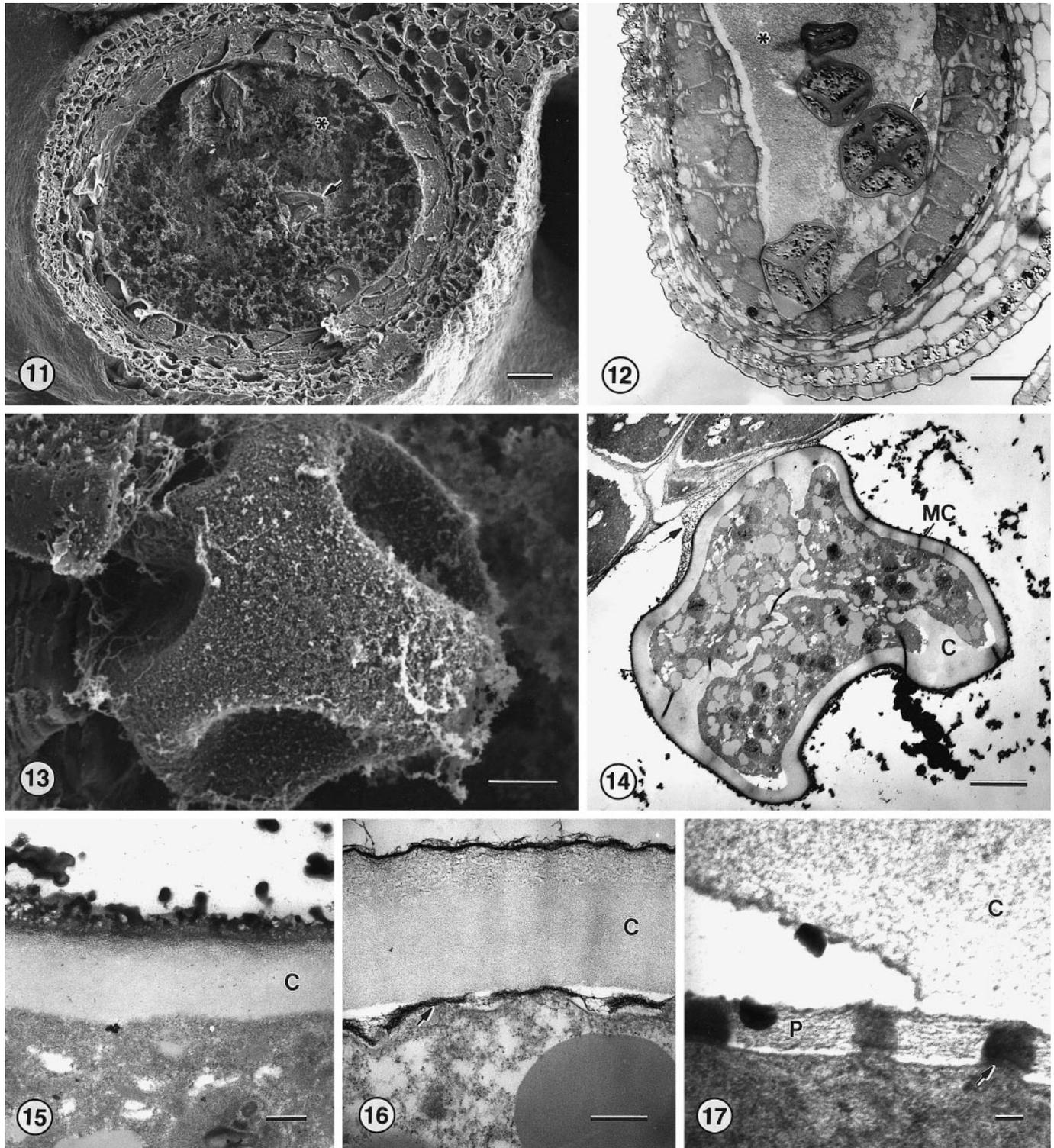
length from 8 to 11 mm. The tapetal cells tightly abut, are multinucleate, and contain abundant endoplasmic reticulum (Figs. 3–4, 9). The tapetal cells have well-defined primary cell walls at this stage (Figs. 4, 9). Large vacuoles are also present in many tapetal cells. These vacuoles contain an electron-dense substance resembling a locular matrix that surrounds the microspore mother cells (Fig. 9) and often completely fills the locular space (Figs. 4, 9). Another, more dense substance is also present in the locules. This second locular matrix is concentrated in regions of contact between adjacent microspore mother cells (Fig. 10), as well as between microspore mother cells and tapetal cells.

Tetrad stage—The majority of the tetrads occur in a tetrahedral arrangement (Figs. 11–14), but a small proportion have been documented in a tetragonal configuration (Fig. 12). The tetragonal tetrads were not found in all anthers, but rather occurred in specific anthers. The microspore mother cell coat (Figs. 14–16) and underlying callose (Figs. 11–17) are persistent through the duration of the tetrad stage. Both layers surround the entire tetrad, whereas only callose separates individual members of the tetrad (Figs. 12, 14). In early tetrads, the microspore plasmalemma is tightly appressed against the callose (Fig. 15). At the middle tetrad stage, the microspore plasmalemma pulls away from the callose and a primexine begins to develop (Fig. 16). The primexine has a lamellar appearance and uniformly surrounds each of the four microspores within the tetrads. In late tetrads, the primexine thickens and numerous electron-dense procolumellae become distinct and span the height of the primexine (Fig. 17).

Anthers in the tetrad stage range in length from 10 to 11 mm. The tapetal cells at this stage also closely abut and contain abundant endoplasmic reticulum, as well as numerous vacuoles (Figs. 11–12). The tapetal vacuoles at this stage continue to contain a substance that is similar in ultrastructure and electron density to that of the locular matrix (Fig. 12). The second, more dense, locular substance is also present between tetrads and between tetrads and the tapetum (Fig. 14).

Free spore stage—Breakdown of the callose wall marks the transition from the tetrad stage to the free spore stage. In the earliest free spore stage, anthers contain microspores still in a tetrad arrangement, but with residual callose present between free spores (Figs. 18–20). During this stage, the initial tectum forms over the primexine in nonapertural regions. In surface view, the tectum of early free spores appears as a loose reticulum (Figs. 21–22). In transverse section, the tectal elements are widely spaced, a foot layer is established in the lowermost region of the

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the cell membrane is pulled away from the callose. Bar = 5 μ m. **7.** Detail of an early microspore mother cell wall showing the microspore mother cell coat (MC) and thin layer of callose. Bar = 0.5 μ m. **8.** Detail of a late microspore mother cell wall showing the microspore mother cell coat (MC) and a thick, two-layered callose wall (C) below. Note also the slightly undulated plasmalemma (arrow) below the callose. Bar = 0.5 μ m. **9.** Transverse section of the tapetum at the late microspore mother cell stage; the cells have distinct cell walls and contain several large vacuoles (arrows). Note that the vacuoles contain a material similar in structure and electron density to the locular matrix (*). Bar = 5 μ m. **10.** Detail of two late microspore mother cells in close proximity; the callose (C) layers are visible. The locular matrix (*) seen in Figs. 4 and 9 is visible, as is a more dense matrix in the region between the two microspore mother cells. Bar = 0.5 μ m.



Figs. 11–17. Tetrad stage. **11.** Single locule showing the locular matrix (*) completely filling the locular space and surrounding the tetrads (arrow). Bar = 20 μm . **12.** A single locule containing two tetrahedral tetrads and one tetragonal tetrad (arrow). Note also the abundant locular matrix (*) and well-defined tapetal layer. Bar = 80 μm . **13.** Detail of the external morphology of a single tetrahedral tetrad. Bar = 20 μm . **14.** Section through three cells of a tetrahedral tetrad; this early tetrad shows a persistent microspore mother cell coat and callose (C) surrounding the entire tetrad, as well as callose separating each microspore. The dense locular matrix is present between the tapetum and the tetrad (arrow). The highly electron-dense globules in the locule represent stain contamination. Bar = 5 μm . **15.** Detail of the outer tetrad wall seen in Fig. 14 showing callose (C). Note that the plasmalemma is tightly appressed against the callose. The electron-dense globules on the surface of the tetrad are stain contaminants. Bar = 0.5 μm . **16.** Detail of the tetrad wall in a later stage of development than seen in Fig. 15 showing early primexine development (arrow). Note also the persistent microspore mother cell coat and two-zoned callose layer (C). Bar = 0.5 μm . **17.** Detail of the tetrad wall in a later stage of development than seen in Fig. 16. The primexine (P) has thickened below the callose (C) and initial, electron-dense proclumellae (arrow) have formed. Bar = 0.1 μm .

primexine, and columellae begin to form on the procolumellae (Fig. 23).

Aperture formation is first detectable during the early free spore stage. Most grains are tricolpate, and the apertures initiate as indented areas at the equatorial region of individual free spores (arrows, Fig. 21). The three apertures later extend from the equator to each pole (Fig. 22). In transverse section, the apertural wall of early free spores is characterized by a thickening of the foot layer and the absence of tectal elements (Fig. 24). The first detectable lamellae of the endexine are present in apertural regions of early free spores (Fig. 24).

Anthers in the early free spore stage range in length from 11 to 15 mm. By this stage, the locular matrix has disintegrated and free spores completely fill the locular space. During the early free spore stage the tapetum begins to dissociate; the primary cell walls begin to degrade and individual cells initially pull apart (Fig. 25). The cytoplasm of the tapetal cells remains relatively dense and contains pre-orbicular bodies and abundant lipid globules (Fig. 25).

At the middle free spore stage, tectum deposition continues in nonapertural regions and forms a more reticulate surface sculpture. Deposition does not occur uniformly in all grains, as patchy areas of a more dense ornament may form (Figs. 26–27). At nonapertural walls, more columellae form and widen, the foot layer becomes much thicker, and endexine lamellae develop (Fig. 29). At the apertural regions, the columellae widen, additional foot layer is deposited, and the endexine lamellae thicken and anastomose (Fig. 28). Furthermore, the apertures widen (Fig. 26). At both apertural and nonapertural regions, the primexine begins to dissociate and a layer of minute granules develops intermixed with the endexine lamellae (Figs. 28–29).

In the late free spore stage, all components of the ectexine (tectum, columellae, and foot layer) become more complete (Figs. 30–31). In nonapertural regions the endexine lamellae become compressed against the foot layer and appear thinner as the underlying granular layer thickens (Fig. 30). At this stage a layer of larger granules develops below the minute granules (Fig. 30). At apertural regions, the endexine lamellae remain substantial and are only slightly intermixed with the granules (Fig. 31).

Anthers in the late free spore stage range in length from 15 to 17.5 mm. The tapetal cell walls have completely degraded at this stage, and the cells have a bulbous appearance (Figs. 32–34). Exocytosed orbicules are present around the periphery of the tapetal cells and are especially abundant on the locule-facing, inner periclinal walls (Figs. 32–34). The cytoplasm of the tapetal cells is less dense than in earlier free spore stages, but still contains well-defined nuclei and lipid globules (Fig. 34). At this stage, endothelial cells do not yet exhibit any cell wall thickenings (Fig. 32).

Mature pollen grain stage—The majority of mature pollen grains are tricolpate (Figs. 35, 38) and have a uniformly dense reticulate ornamentation (Figs. 35–36). The nonapertural wall is characterized by a well-developed ectexine, a compressed layer of endexine lamellae and granules, and a two-layered intine (Fig. 39). In surface

view, the apertures are intectate, but have well-defined ectexine elements present (Fig. 37). The endintine is thicker at apertural regions and causes the entire pollen wall to bulge slightly (Figs. 38, 40).

In addition to the common tricolpate grains, several other apertural conditions have been occasionally documented (Figs. 41–43). These less common grains seemed to be clustered in certain anthers. Of these, monoaperturate pollen, in which a single aperture encircles the grain, was found most frequently (Fig. 41). Examples of less frequently occurring pollen grains include those with two apertures (one equatorial colpus and one aperture that encircles the grain in a spiral pattern; Fig. 42) and those with a single spirally encircling aperture (Fig. 43).

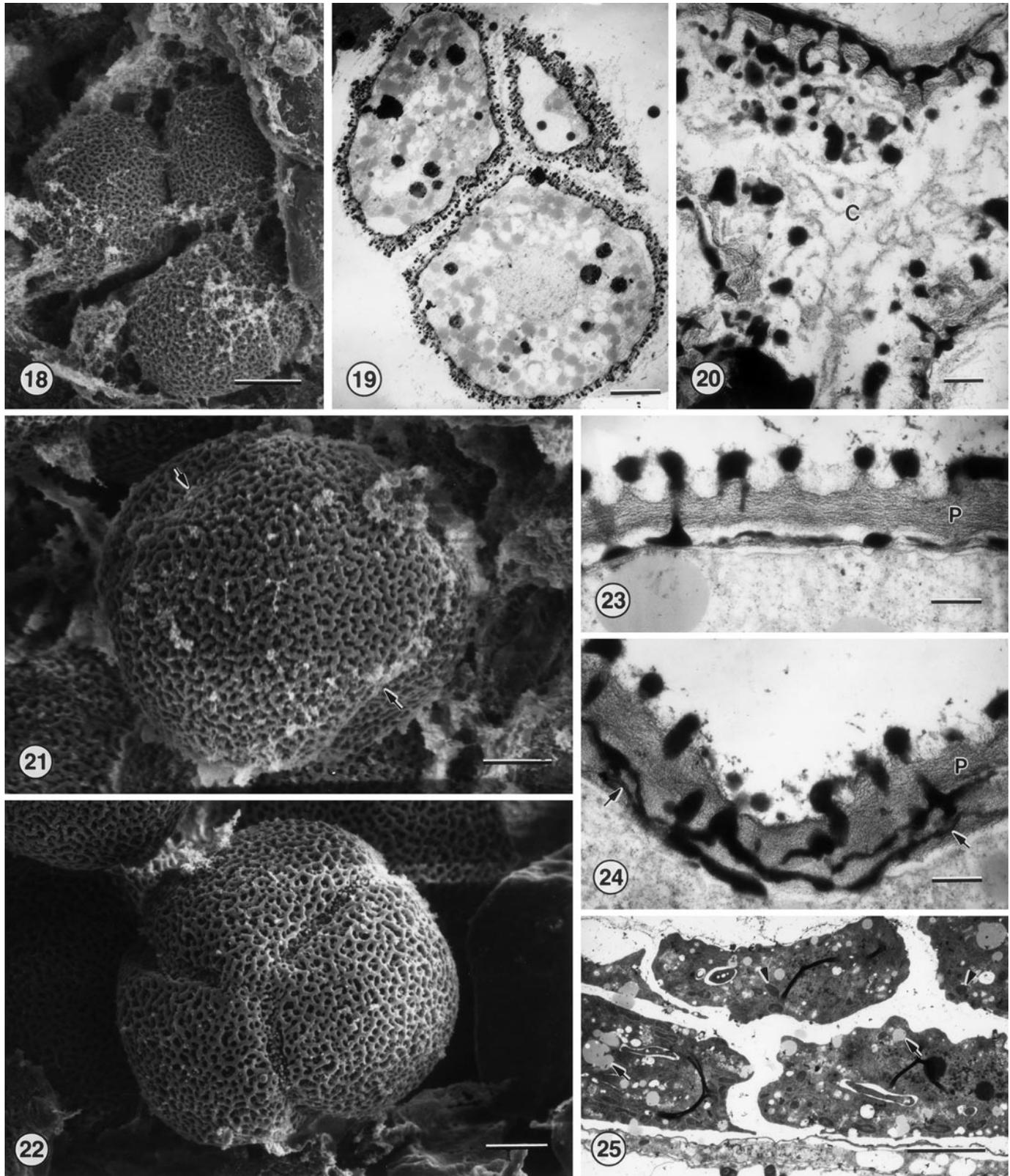
Anthers containing mature pollen range in length from 34 to 38 mm. At this stage, secondary cell wall thickenings form in the endothelial cells. The U-shaped thickenings form along the anticlinal and inner periclinal walls of the cells and are distinctly fibrous (Figs. 44–46). These thickenings form only after the tapetum has degraded. The thickenings cause the anthers to dehisce longitudinally (Fig. 44). Although the tapetum degrades in the latest free spore stage, remnant orbicules, lipid globules, and tapetal membranes are still present in the periphery of the locules (Figs. 47–48).

DISCUSSION

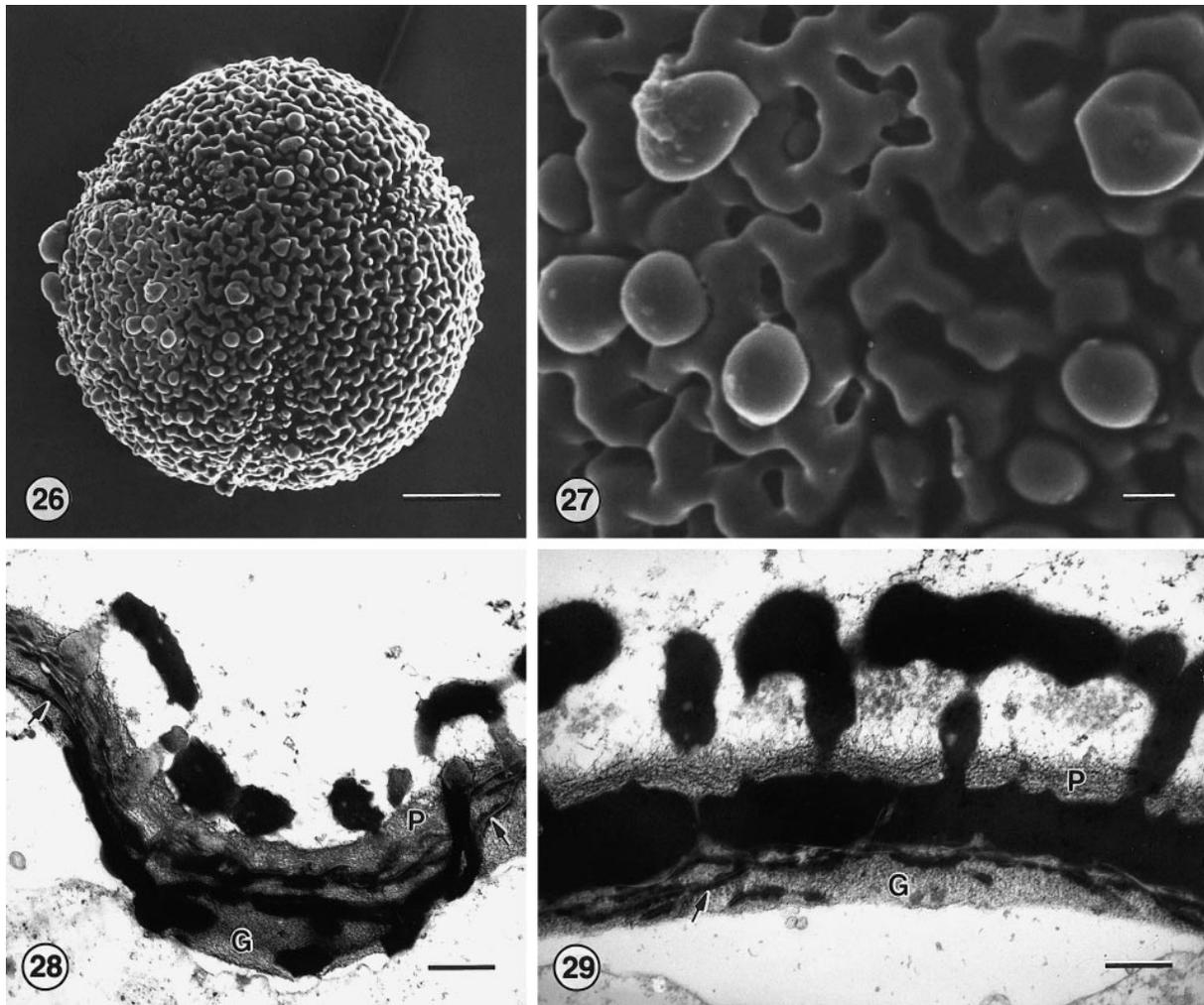
This is the first study to comprehensively examine pollen and anther ontogeny in *Nelumbo*, as well as investigate any aspect of pollen development in *Nelumbo lutea* using electron microscopy. The major events of sporoderm and tapetal ontogeny are summarized in Fig. 49 and specific developmental characters are discussed below. Furthermore, new data on pollen and anther development are interpreted regarding their phylogenetic implications.

Aperture ontogeny—Aperture formation in *Nelumbo lutea* appears to occur at a later stage of development than in many other angiosperms, where apertures are initially established during the tetrad stage (e.g., Blackmore and Barnes, 1990; Schmid, Eberwein, and Hesse, 1996). At the tetrad stage of *N. lutea*, the primexine is distributed completely and uniformly around the individual microspores, and there does not appear to be accumulation of endoplasmic reticulum at potential aperture sites. This coincides with Flynn and Rowley's (1971a) report that the primexine at the tetrad stage did not indicate future aperture positions in *Nelumbo nucifera*. The first evidence of aperture formation detected in *N. lutea* was in the earliest free spores, where apertures appear as indentations at the equatorial regions and are the sites of the first identifiable endexine lamellae (Fig. 49). The current study provides confirmation of the post tetrad establishment of apertures in *Nelumbo*, as early reports of this in *N. nucifera* (Flynn and Rowley, 1971a; see also Rowley, 1975) have not been widely recognized.

Observations of the two tetrad configurations and co-occurring monoaperturate and triaperturate pollen in *Nelumbo lutea* complement those of Kuprianova (1979), who documented the same in *Nelumbo nucifera*. Blackmore, Stafford, and Persson (1995) also reported boat-shaped monoaperturate grains in *N. nucifera*, however,



Figs. 18–25. Early free spore stage. **18.** Surface view of three free spores still in a tetrad configuration following callose dissociation. Bar = 10 μm . **19.** Section through three, young free spores still in a tetrahedral tetrad arrangement. Bar = 5 μm . **20.** Detail of the contact region between the three free spores seen in Fig. 19. The dissociating callose (C), fibrillar primexines, and developing electron-dense exines are visible. Bar = 0.5 μm . **21.** Single free spore with two developing apertures (arrows) visible as indentations at the equatorial regions of the grain. Bar = 5 μm . **22.** Polar view of a single free spore in a later stage of development than seen in Fig. 21 showing three, immature apertures that have extended to the poles of the grain. Bar = 5 μm . **23.** Nonapertural wall of a single free spore in transverse section showing the early, electron-dense ectexine. Widely



Figs. 26–29. Middle free spore stage. **26.** Polar view of a triaperturate grain. The apertures have widened and lipid globules are visible on the surface of the pollen grain. Bar = 10 μm . **27.** Detail of the nonapertural wall of the same grain seen in Fig. 26 showing variation in tectum morphology. Note that the tectum at left has formed a more dense ornament and may be further developed than that at right. Bar = 1 μm . **28.** Transverse section of an apertural region. The degrading primexine (P) is visible with the marginal tectum and columellae distinct. Note also the abundance of white-line-centered endexine lamellae (arrows) and an underlying layer of minute granules (G). Bar = 0.5 μm . **29.** Transverse section of a nonapertural wall showing a partially degraded primexine (P). The reticulate tectum, wide columellae, and thick foot layer are distinct. Multiple white-line-centered endexine lamellae (arrow) are also visible, underlaid and intermixed with a layer of minute granules (G). Bar = 0.5 μm .

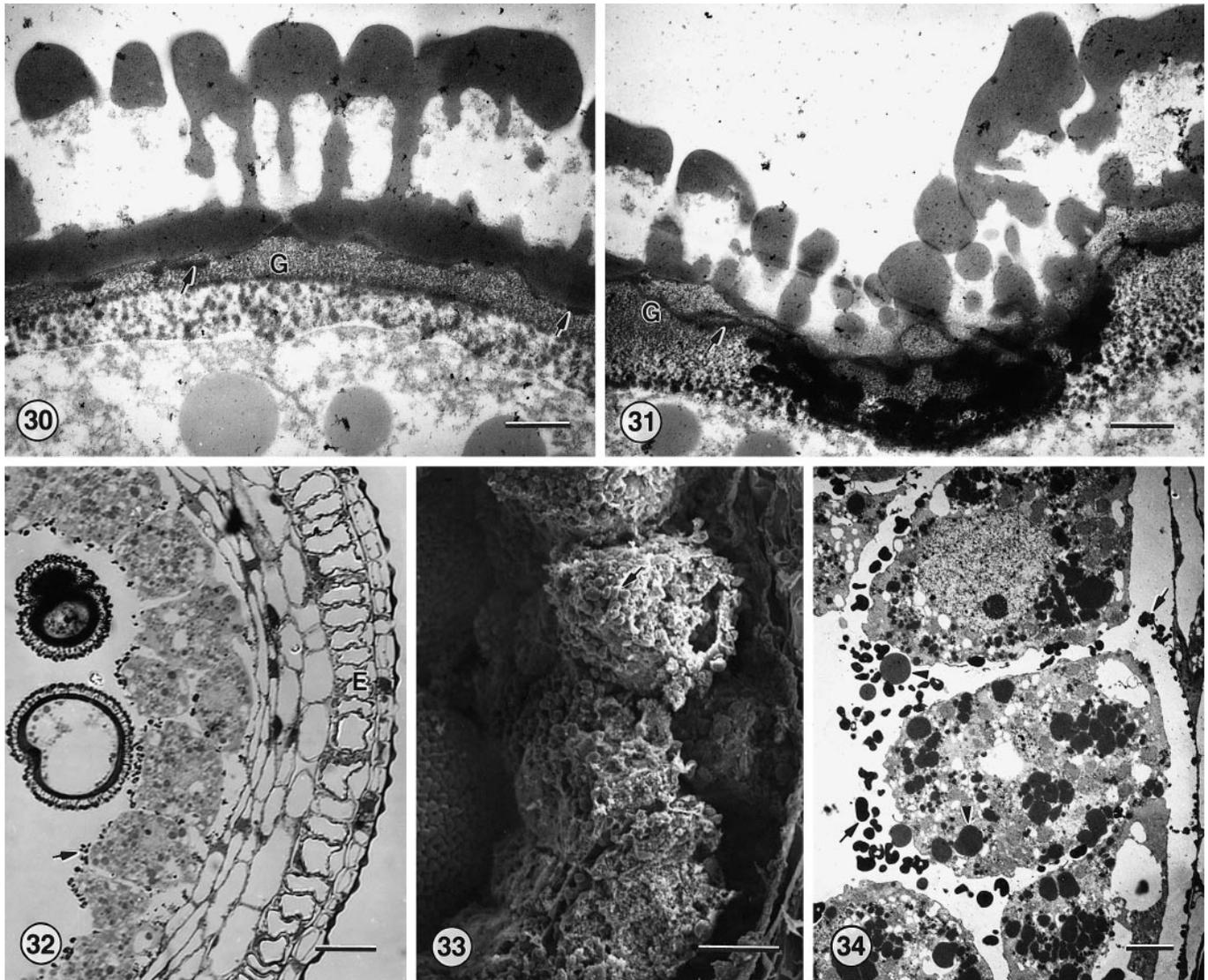
the monoaperturate grains of *N. lutea* found in the present study were spheroidal, as are the common triaperturate grains. Kuprianova (1979) has suggested that the co-occurring aperture types in *Nelumbo* could be an important transition in angiosperms, from monoaperturate to triaperturate pollen. However, as demonstrated in this study, several other aperture types also exist in *N. lutea* and have also recently been reported in *N. nucifera* (Borsch and Wilde, 1999).

An alternative explanation to Kuprianova's (1979)

transitional hypothesis is that aperture variability in *Nelumbo* may be correlated with the lateness of aperture ontogeny in the genus. Borsch and Wilde (1999) have also suggested that aperture variation in *Nelumbo* may be due to weak control of the developmental processes that govern aperture formation. These authors further suggest that such plasticity in aperture control mechanisms may be a primitive condition and that this plesiomorphic state may be present in *Nelumbo* (Borsch and Wilde, 1999). Although this postulate complements the developmental hy-

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spaced tectal elements, a single forming columella, and a thin foot layer have formed on the primexine (P). Bar = 0.5 μm . **24.** Section through an early apertural wall showing widely spaced and nonconnected tectal elements at the apertural margins, a foot layer thicker than that in nonapertural regions, endexine lamellae (arrows), and primexine (P). Bar = 0.5 μm . **25.** Section through the tapetum showing partially degraded cell walls. Note that the tapetal cytoplasm remains relatively dense and contains electron-lucent lipid globules (arrows) and more electron-dense pre-orbicular bodies (arrowheads). Bar = 5 μm .



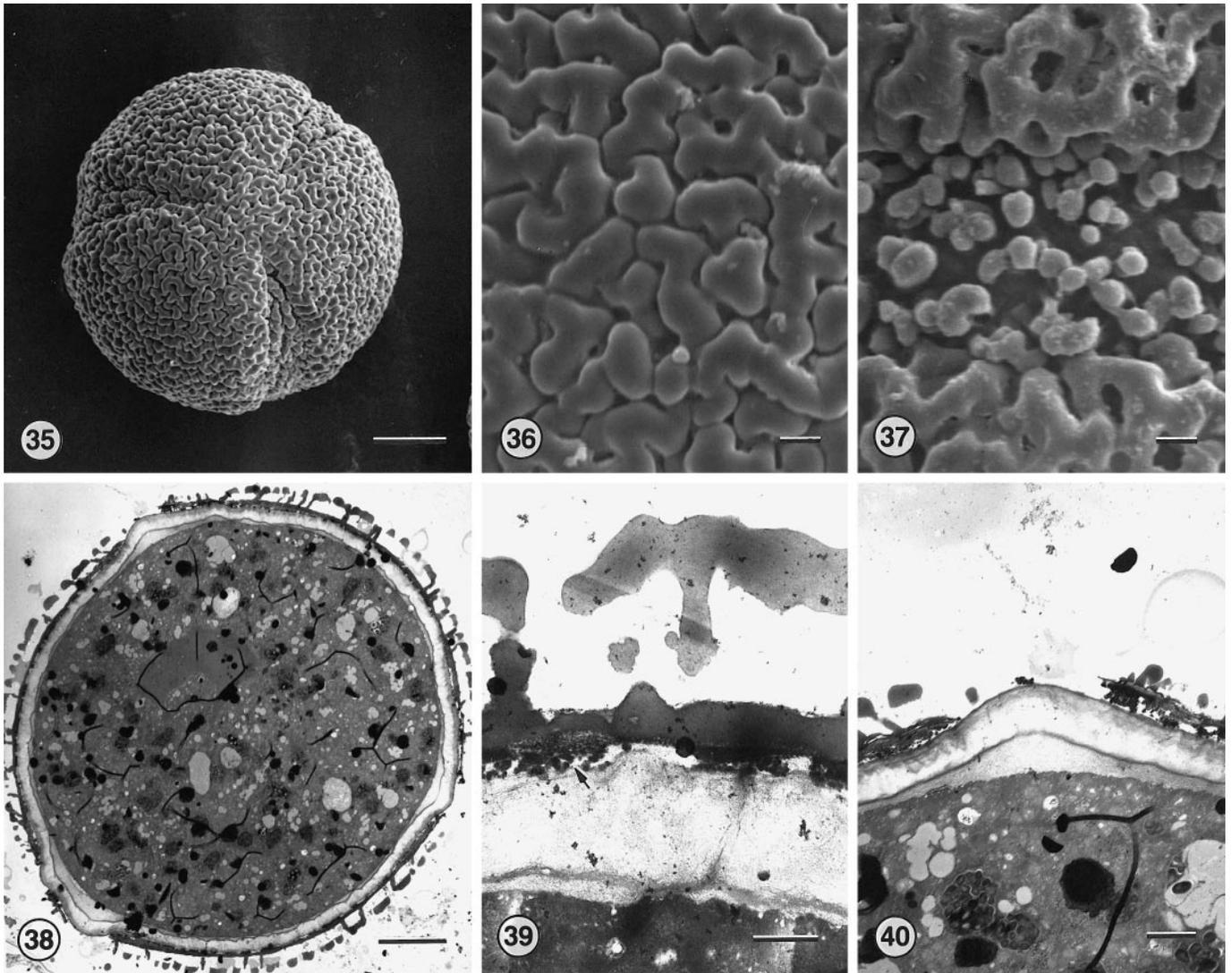
Figs. 30–34. Late free spore stage. **30.** Transverse section of the nonapertural wall showing complete ectexine layers (tectum, columellae, and foot layer). The endexine lamellae (arrows) have been compressed between the foot layer and the granular layer (G), which has thickened and developed an inner layer of larger granules. Bar = 1 μm . **31.** Transverse section of an apertural region showing complete ectexine and marginal endexine lamellae (arrow), which thicken and anastomose at the apertural membrane. The granular (G) layer is present below and intermixed with the endexine lamellae, but is reduced at the apertural membrane. Bar = 1 μm . **32.** Section through a portion of the anther wall and locule. Orbicules (arrow) are visible on all sides of the tapetal cells. Note the 4–5 middle layers and that the endothecium (E) lacks cell wall thickenings. Bar = 60 μm . **33.** Detail of tapetal cells showing bulbous shape and numerous orbicules (arrow) on the cell surfaces. Bar = 10 μm . **34.** Detail of several tapetal cells showing complete cell wall degradation and numerous intracellular and extracellular lipid globules (arrowheads). Orbicules (arrows) are abundant on the locule-facing surfaces at left, but also present both between individual cells and between the tapetum and the middle layers. Bar = 5 μm .

pothesis posed in the present paper, the degree to which aperture variation in *Nelumbo* can be attributed to phylogeny vs. ontogeny requires additional study.

Exine ontogeny—The primexine is the location for sporopollenin deposition of the exine. Although procolumellae form during the late tetrad stage, exine development does not occur until the free spore stage. As the free spore stage progresses, the ectexine layers mature more or less simultaneously; the tectal elements thicken, the columellae widen, and the foot layer thickens (Fig. 49). Some sporopollenin of the tectum may be deposited

late in the free spore stage from tapetal orbicules, thereby contributing to the reticulate morphology. This may not occur uniformly across the surface of the pollen, as isolated patches of varying tectum density are found in some free spores.

Initiation of the endexine occurs after initial formation of the ectexine in the early free spore stage, as in many angiosperms (e.g., Blackmore and Barnes, 1990). However, distinguishing between endexine and foot layer is often difficult, especially at apertural regions, where these layers are thickened, noncontinuous, and often anastomose. Furthermore, the endexine and foot layer com-



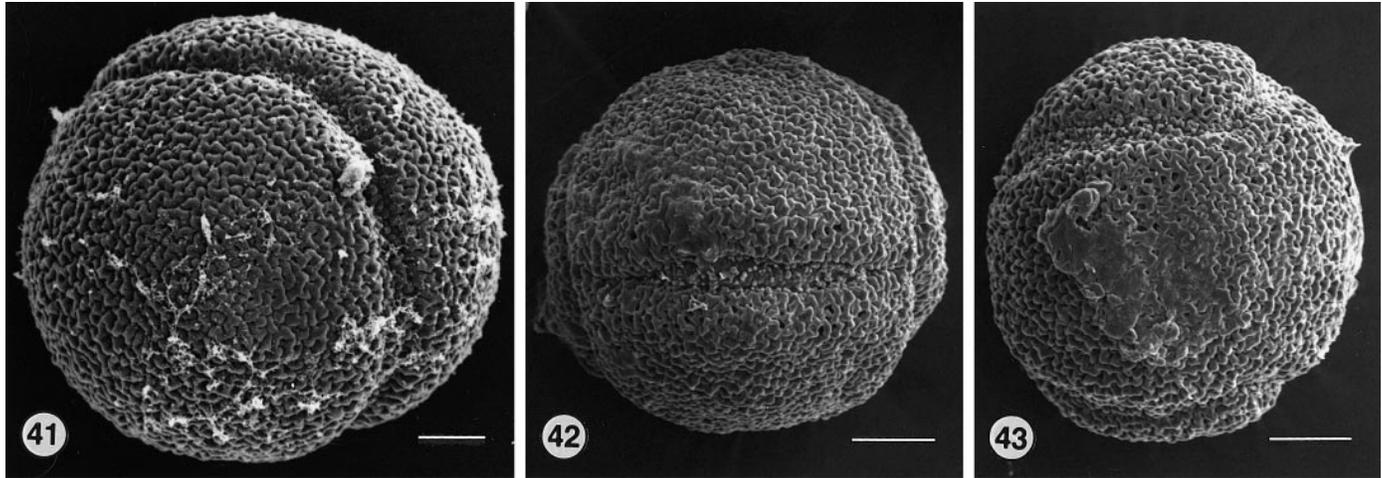
Figs. 35–40. Mature pollen grain stage. **35.** Polar view of a triaperturate pollen grain. Portions of the three apertures are visible. Bar = 10 μm . **36.** Detail of the nonapertural pollen surface. Note that the tectum forms a densely reticulate ornament. Bar = 1 μm . **37.** Detail of the apertural pollen surface. Note that the aperture is intectate and that short, ectexine elements project from the underlying foot layer. Bar = 1 μm . **38.** Equatorial section through a pollen grain showing complete sporoderm and three slightly bulging apertures. Bar = 10 μm . **39.** Detail of the nonapertural wall in transverse section showing a complete ectexine (tectum, columellae, and foot layer). The endexine lamellae and granular layer (arrow) have been compressed between the thick foot layer and the two-layered intine. Bar = 1 μm . **40.** Transverse section through the apertural wall showing the thickened endintine, that causes the entire intine to bulge. Note that minimal exine is visible at the apertural membrane in this ultrathin section. Bar = 2 μm .

monly have similar electron densities, an occurrence related to staining properties that has been noted in other taxa as well (e.g., Weber, 1998; El-Ghazaly, Swedish Museum of Natural History, personal communication). When the two layers exhibited similar staining densities in *N. lutea* pollen, the endexine lamellae were identified by the presence of white lines.

At the middle free spore stage, a two-zoned granular layer forms below the endexine lamellae. This layer appears to compress the lamellae against the foot layer. The granules persist into the mature pollen stage, but the layer is reduced in thickness (Fig. 49). It is possible that the granular layer is another component of the endexine. Similar granules have been identified in other angiosperms and found to be acetolysis resistant, although not

definitively described as endexine (e.g., Huysmans, El-Ghazaly, and Smets, 1998). Alternatively, such a granular layer has been suggested to be an intine precursor (see below).

Intine ontogeny—The only stage in which an intine layer was detected was in fully mature pollen grains, located within dehiscent or near dehiscent anthers. Furthermore, the intines documented were well developed and two layered (Fig. 49). Consequently, it is presumed that the events of intine development occur rapidly. As discussed above, it is possible that the granular layer below the endexine lamellae, first detectable in the free spore stage, could be a precursor to the intine. A layer of similar appearance has been described as an intine pre-



Figs. 41–43. Mature pollen grain stage—aperture variation. **41.** View of a monoaperturate grain with a single, encircling aperture. Bar = 5 μm . **42.** Equatorial view of a diaperturate grain showing one, typical colpus and one spirally encircling aperture. Bar = 10 μm . **43.** View of a monoaperturate grain showing a single, spirally encircling aperture. Bar = 10 μm .

cursor in *Liriodendron chinense* (Hemsl.) Sarg (Gabarayeva, 1996).

Locule and anther wall ontogeny—The locular matrix present at the microspore mother cell and tetrad stages can be abundant and often fills the locular space. This matrix material surrounds the microspore mother cells or tetrads and appears to suspend the cells within the anther locule. It is not clear whether or not the more dense matrix present between adjacent cells is a different substance or whether it is the same matrix material found throughout the locules, but has a different ultrastructure as a result of fixation influences. The abundant matrix material is presumed to be secreted by the tapetum, as tapetal cells have large vacuoles containing a substance that is similar in ultrastructure and electron density. A locular matrix described as “colloidal” was identified by Farr (1922) in *Nelumbo lutea*, but it was reported to occur only in the microspore mother cell stage. Following the tetrad stage, individual free spores and pollen grains occupy the entire locular space.

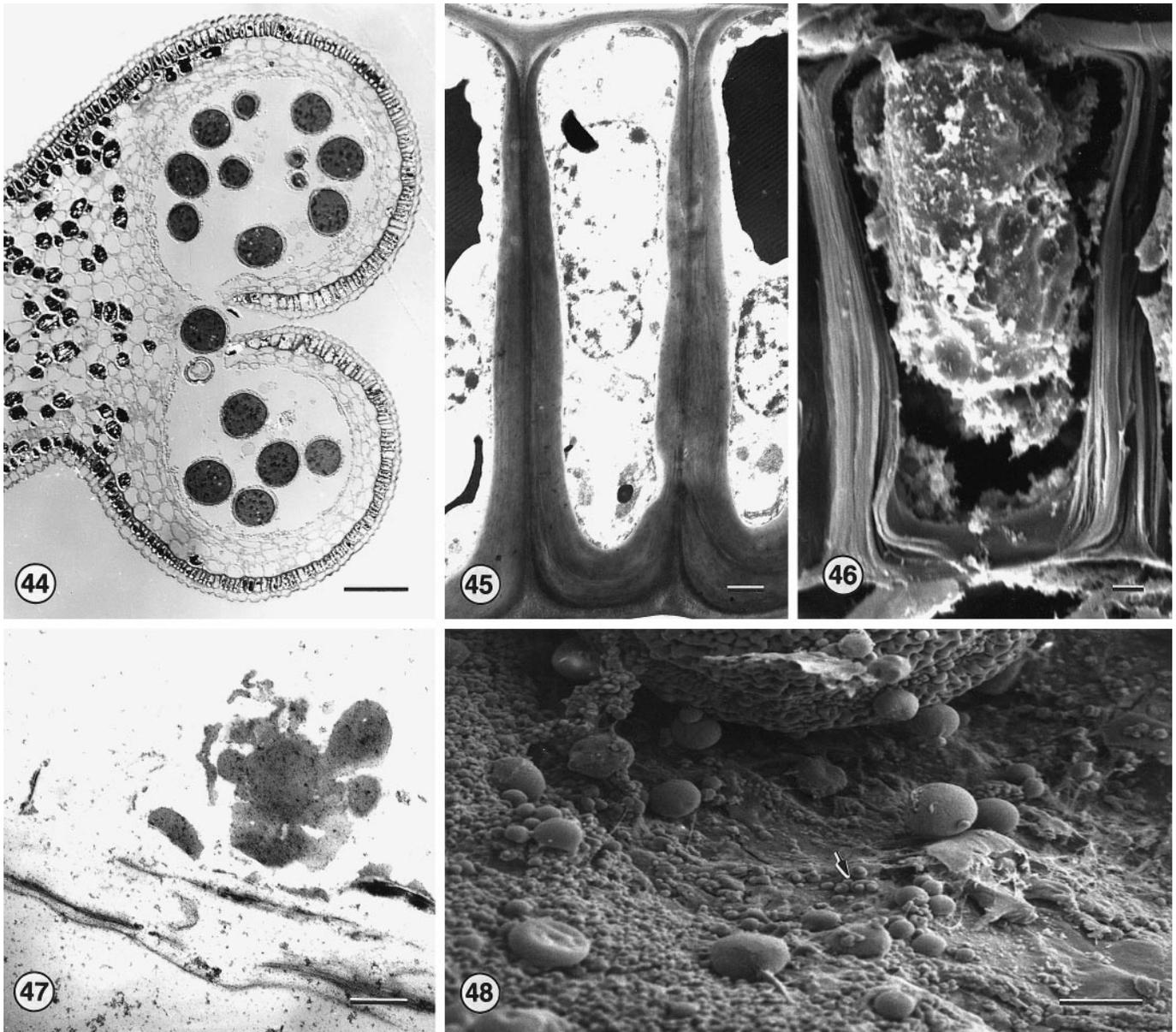
The tapetum is first distinguishable at the microspore mother cell stage and is of the secretory type. Tapetal cells retain a similar morphology throughout the microspore mother cell and tetrad stages. Tapetal degradation occurs at the free spore stage, during which cell walls break down and numerous orbicules are synthesized and secreted (Fig. 49). A secretory tapetum has also been described in *N. nucifera* based on studies using LM (Batygina et al., 1980; Batygina and Shamrov, 1983).

Endothelial thickenings are clearly U-shaped in *Nelumbo lutea* and were observed only in locules containing mature pollen grains (i.e., with complete intines). Endothelial cells with early to intermediate secondary wall thickenings were not detected at earlier ontogenetic stages. Consequently, it is presumed that the cell walls thicken rapidly during the latest free spore or earliest mature pollen grain stages. Previously, only Moseley (1958) has described U-shaped endothelial wall thickenings in *Nelumbo nucifera*. Other studies have described the endothelial thickenings of *N. nucifera* as both “feeble” (Khan-

na, 1965) and “fibrous,” but masked by tannins (Gupta and Ahluwalia, 1979); however, it was not clear from these studies whether the thickenings were U-shaped. The abundant tannins described by Gupta and Ahluwalia (1979) in *N. nucifera* may be related to the fixatives used (formalin-acetic acid-alcohol and Nawaschin’s fluid); tannins were not detected in the current study of *N. lutea*.

Phylogenetic implications—As discussed above, *Nelumbo* has been phylogenetically allied with both Nymphaeales and more recently with lower eudicots, especially *Platanus*. Therefore, comparisons of pollen ontogeny among *Nelumbo* and these groups may provide new insight into phylogenetic relationships. Six published studies have examined ultrastructural aspects of pollen development in Nymphaeales (water lilies). These include investigations of two genera of Nymphaeaceae: *Nymphaea* (Gabarayeva, 1991; Rowley, Gabarayeva, and Walles, 1992; Gabarayeva and Rowley, 1994; Gabarayeva and El-Ghazaly, 1997) and *Nuphar* (Flynn and Rowley, 1971b; Takahashi, 1992), although only limited developmental stages and characters have been reported for *Nuphar*. Only one published study has focused on pollen ontogeny in *Platanus* (Suarez-Cervera, Marquez, and Seoane-Camba, 1995).

Pollen development in *Nelumbo* shares some similarities with that of both water lilies and *Platanus*; however, the ontogenetic sequence of the genus is different in many ways. Aperture formation occurs in the free spore stage in *Nelumbo*, which is later than that in both *Nymphaea mexicana* A. Gray (Gabarayeva and El-Ghazaly, 1997) and *Platanus acerifolia* (Aiton) Willdenow (Suarez-Cervera, Marquez, and Seoane-Camba, 1995). In the pollen of both *N. mexicana* and *P. acerifolia*, initiation of the apertures is apparent at the tetrad stage. Aperture number and position are also characters that do not clearly link *Nelumbo* to either water lilies or *Platanus*. *Platanus* pollen is triaperturate, with three equatorially positioned colpi, as are the majority of *Nelumbo* pollen grains. In contrast, the occurrence of monoaperturate pollen in *Nelumbo*, in which the aperture encircles the grain,



Figs. 44–48. Mature pollen grain stage. **44.** Section through two, now-continuous anther locules showing ruptured stomium and mature grains. Note the absence of a tapetum and the mature endothecium with thickened cell walls. Bar = 80 μm . **45.** Transverse section of three endothelial cells. Note that the secondary cell wall is thickened in a U-shaped pattern along the two anticlinal walls and the lower periclinal wall. Bar = 1 μm . **46.** Detail of a single endothelial cell showing fibrous secondary cell wall thickenings. Bar = 1 μm . **47.** Oblique section through an orbicula and several resistant tapetal membranes. Bar = 0.5 μm . **48.** Inner surface of an anther locule following tapetal dissociation. A portion of one pollen grain is shown (at top); remnant orbicules (arrow), tapetal membranes, and larger, more spherical lipid globules are also visible. Bar = 10 μm .

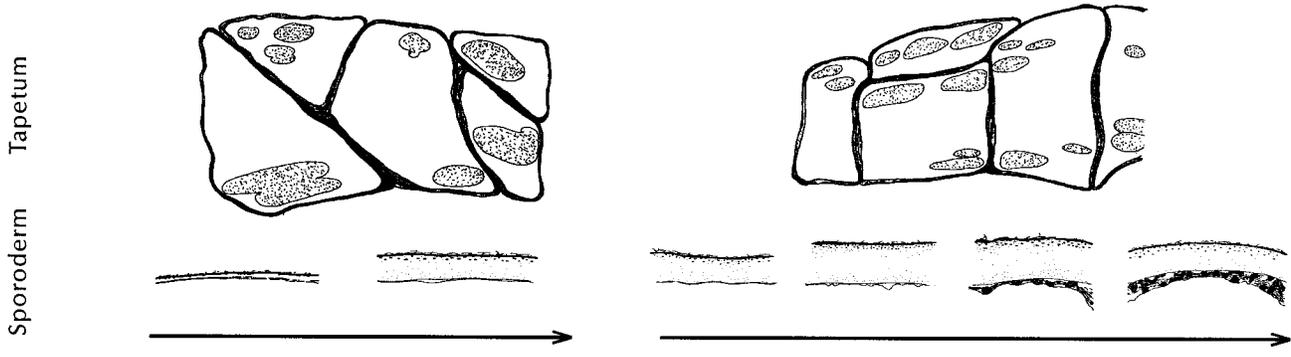
correlates with the pollen of *Nymphaea*, *Ondinea*, *Barclaya*, and *Euryale* (Nymphaeaceae; e.g., Walker, 1976; Osborn, unpublished data). However, as discussed above, aperture variation in *Nelumbo* may be related to the late ontogenetic timing of aperture establishment and not necessarily informative in a strict phylogenetic context. Therefore, this character alone may be difficult to compare with that of other taxa until more information is available about aperture development.

Comparisons of exine morphology and architecture are also relevant. The tectum of *Nelumbo* pollen is reticulate, like that of *Platanus* and many other lower eudicot taxa

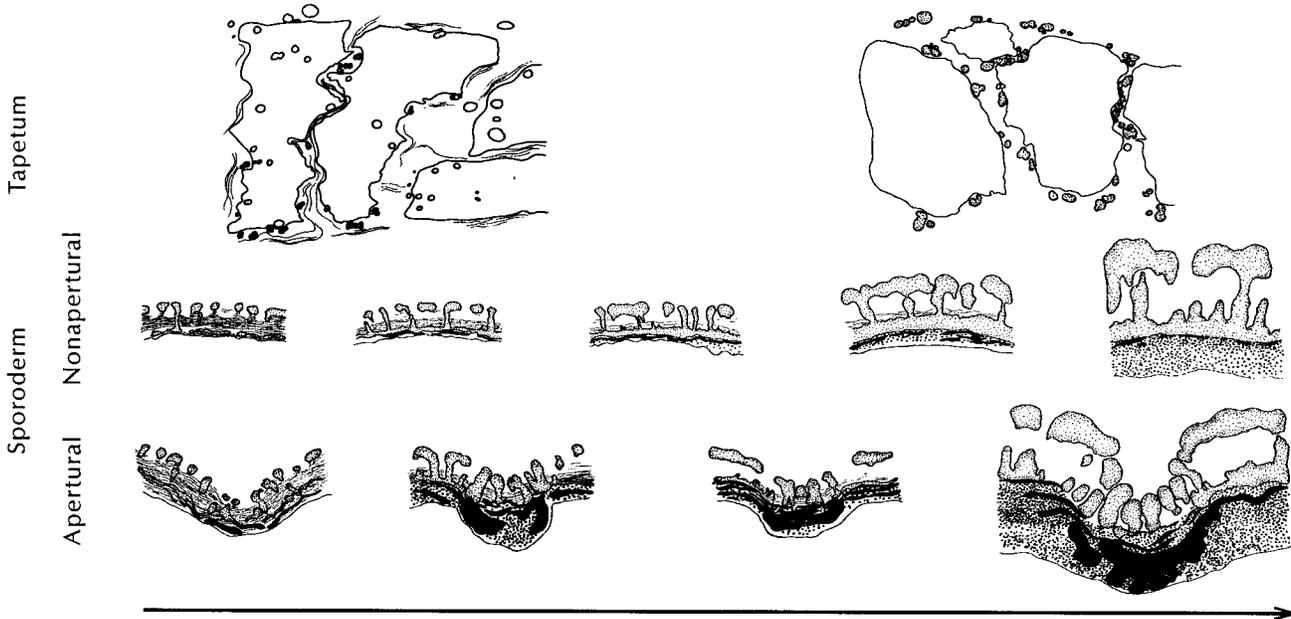
(Suarez-Cervera, Marquez, and Seoane-Camba, 1995; Blackmore, Stafford, and Persson, 1995), whereas the pollen of water lilies has a variety of nonreticulate ornamentation patterns (e.g., verrucate, scabrate, psilate, spinose, and striate; Walker, 1976; Osborn, Taylor, and Schneider, 1991; Takahashi, 1992; Gabarayeva and El-Ghazaly, 1997). Regarding the infratectal layer, the columellate infratectum of *Nelumbo* has been considered more derived than the historically described granular infratectum of water lilies (e.g., Walker, 1976) and thereby more similar to that of *Platanus* and other lower eudicots. More recent studies, however, have documented a colu-

MICROSPORE MOTHER CELL STAGE

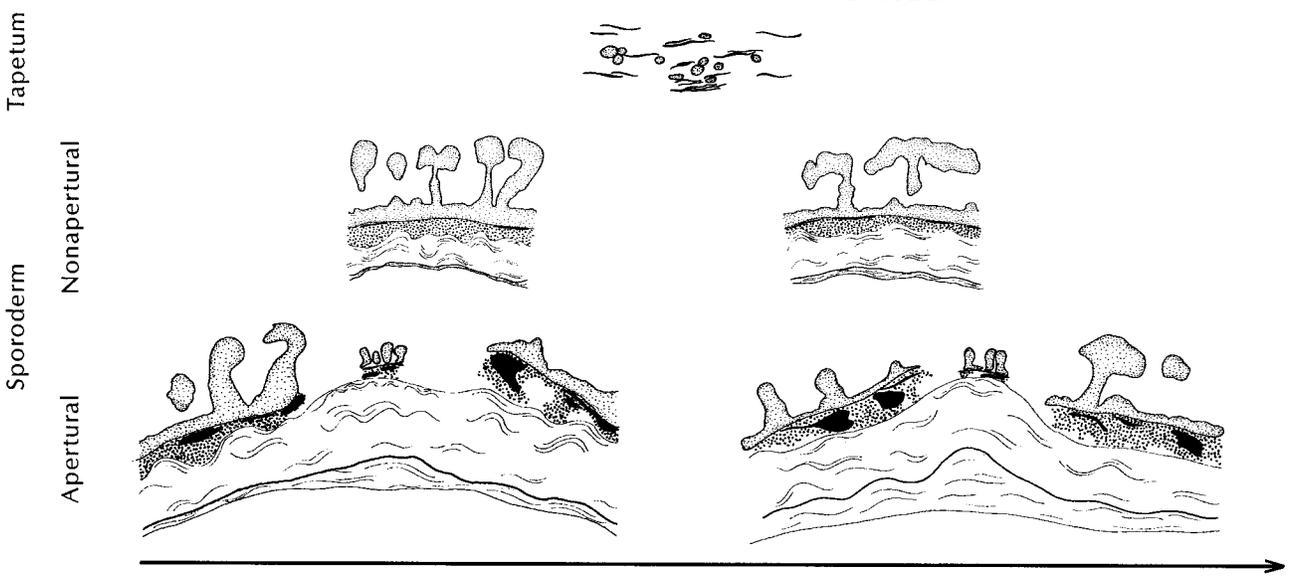
TETRAD STAGE



FREE SPORE STAGE



MATURE POLLEN GRAIN STAGE



mellate infratectum in the pollen of *Brasenia* and *Cabomba* (Cabombaceae; Osborn, Taylor, and Schneider, 1991), as well as in *Nymphaea* pollen (Nymphaeaceae; Gabarayeva and Rowley, 1994; Gabarayeva and El-Ghazaly, 1997). The columellae of these three water lily taxa are not as distinct as those of *Nelumbo*; in particular, the columellae of *Nymphaea* are somewhat irregularly shaped. The synchronous timing of ectexine development and the subsequent formation and ultrastructure of endexine lamellae in *Nelumbo* pollen are similar to that in both *Nymphaea* and *Platanus* pollen. The granular layer that forms beneath the lamellae in *Nelumbo* also occurs during pollen ontogeny in *Nymphaea* (Gabarayeva and El-Ghazaly, 1997), but not in *Platanus* (Suarez-Cervera, Marquez, and Seoane-Camba, 1995).

Most recent research concerning the systematic position of *Nelumbo* has been based on molecular characters and has placed *Nelumbo* among lower eudicots (e.g., Chase et al., 1993; Soltis et al., 1997; Nandi, Chase, and Endress, 1998; Qiu et al., 1998). However, the historical association of *Nelumbo* with Nymphaeales has also recently been supported based on analyses using character compatibility (Meacham, 1994). Unfortunately, results from the present study of *Nelumbo* do not clearly link the genus to either lower eudicots or to Nymphaeales. The new data on pollen and anther ontogeny in *Nelumbo*, however, do fill an important gap in understanding this interesting plant, and they provide the opportunity for additional, comprehensive comparisons. Further clarification of the systematic position of *Nelumbo* will be aided by more investigations of pollen development in a greater number of other lower eudicot and nymphaean taxa.

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Fig. 49. Summary of the major ontogenetic events during sporoderm and tapetum development in *Nelumbo lutea*. Sporoderm characters illustrated include callose (lightly stippled), primexine (horizontal long dashes), ectexine (densely stippled), endexine (solid black), granular layer (large, dark dots), and intine (discontinuous, wavy lines). Tapetal characters illustrated include integrity of cell walls, vacuoles containing a locular matrix-like material (short hashes), lipid droplets (empty circles), pre-orbicular bodies (black circles), and orbicules (densely stippled). All sporoderm images are depicted at the same scale, as are all tapetal cell drawings; however, illustrations of sporoderm and tapetum are not depicted to scale relative to one another.

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