American Journal of

Botany

October 2015 ◆ Volume 102 ◆ Number 10

Official Publication of the Botanical Society of America, Inc.
www.amjbot.org

RESEARCH ARTICLE

AMERICAN JOURNAL OF BOTANY

Pollen structure and development in Nymphaeales: Insights into character evolution in an ancient angiosperm lineage[1]

Mackenzie L. Taylor[2,6], Ranessa L. Cooper[3], Edward L. Schneider[4], and Jeffrey M. Osborn[5]

PREMISE OF THE STUDY: A knowledge of pollen characters in early-diverging angiosperm lineages is essential for understanding pollen evolution and the role of pollen in angiosperm diversification. In this paper, we report and synthesize data on mature pollen and pollen ontogeny from all genera of Nymphaeales within a comparative, phylogenetic context and consider pollen evolution in this early-diverging angiosperm lineage. We describe mature pollen characters for *Euryale*, *Barclaya*, and *Nymphaea ondinea*, taxa for which little to no structural data exist.

METHODS: We studied mature pollen for all nymphaealean genera using light, scanning electron, and transmission electron microscopy. We reviewed published reports of nymphaealean pollen to provide a comprehensive discussion of pollen characters in water lilies.

KEY RESULTS: Nymphaeales exhibit diversity in key pollen characters, including dispersal unit size, ornamentation, aperture morphology, and tapetum type. All Nymphaeales pollen are tectate-columellate, exhibiting one of two distinct patterns of infratectal ultrastructure—a thick infratectal space with robust columellae or a thin infratectal space with thin columellae. All genera have pollen with a lamellate endexine that becomes compressed in the proximal, but not distal wall. This endexine ultrastructure supports the operculate hypothesis for aperture origin. Nymphaeaceae pollen exhibit a membranous granular layer, which is a synapomorphy of the family.

CONCLUSIONS: Variation in pollen characters indicates that significant potential for lability in pollen development was present in Nymphaeales at the time of its divergence from the rest of angiosperms. Structural and ontogenetic data are essential for interpreting pollen characters, such as infratectum and endexine ultrastructure in Nymphaeales.

KEY WORDS Cabombaceae; exine; Hydatellaceae; morphology; Nymphaeaceae; pollen; pollination; tapetum; ultrastructure; water lilies

Nymphaeales, or the water lilies, are a cosmopolitan order that comprises three families: Cabombaceae, Nymphaeaceae, and Hydatellaceae. Together, these families include eight to nine genera and less than 100 species (Borsch et al., 2008). Water lilies have long been considered to be among the oldest independent lineages of angiosperms (Walker, 1974a, b; Donoghue and Doyle, 1989; Hamby and Zimmer, 1992; Doyle, 1998), and the majority of molecular phylogenetic analyses indicate that Nymphaeales diverged from the second basalmost node of the extant angiosperm phylogenetic tree (Qiu et al., 1999, 2006; Löhne and Borsch, 2005; Saarela et al., 2007; APG III, 2009; Drew et al., 2014). Paleobotanical evidence also supports an early origin for water lilies. Nymphaeales are represented in the early angiosperm fossil record, and as many as three early Cretaceous fossils can be placed within the nymphaealean crown group (Friis et al., 2001, 2009, 2011; Mohr et al., 2008; D. W. Taylor et al., 2008; Doyle and Endress, 2014).

Despite a paucity of species, all restricted to aquatic habitats, water lilies exhibit considerable diversity in ecological and morphological traits in comparison to other early-divergent angiosperm lineages. This diversity includes variation in life history, carpel morphology, breeding system, and pollination biology (Osborn and Schneider, 1988; Schneider and Williamson, 1993; Endress and Igersheim, 2000;

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[1] Manuscript received 30 May 2015; revision accepted 25 August 2015.

[2] Department of Biology, Creighton University, 2500 California Plaza, Omaha, Nebraska 68178-0324 USA;

[3] Biology Department, Hillsdale College, 33 East College Street, Hillsdale, Michigan 49242 USA;

[4] The University of Minnesota Landscape Arboretum, 3675 Arboretum Drive, Chaska, Minnesota 55318 USA; and

[5] School of Science, The College of New Jersey, P.O. Box 7718, 2000 Pennington Road, Ewing, New Jersey 08628-0718 USA

[6] Author for correspondence (e-mail: MackenzieTaylor@creighton.edu)

doi:10.3732/ajb.1500249

Doyle and Endress, 2000; Endress, 2001, 2005; Rudall et al., 2007; Sokoloff et al., 2008; Thien et al., 2009; Taylor and Williams, 2009; Taylor et al., 2010). Because of this variation, Nymphaeales are an excellent system in which to investigate the evolution of reproductive traits in an early-diverging angiosperm lineage.

A knowledge of characters and character evolution in early-diverging angiosperm lineages, including Nymphaeales, is essential to understanding the evolutionary transitions that accompanied and facilitated the early diversification of flowering plants. Key reproductive characters, including several pollen structural and developmental characters, have been hypothesized to have played a critical role in flowering plant diversification (Stebbins, 1974; Doyle, 1978; Doyle and Donoghue, 1986; Williams, 2008, 2009, 2012). Pollen characters in Nymphaeales have historically figured prominently in discussions of the pollen of reputedly primitive angiosperms (e.g., Walker, 1974a, 1974b, 1976a, 1976b; Walker and Doyle, 1975) and have been important components of phylogenetic analyses of angiosperms and seed plants (e.g., Donoghue and Doyle, 1989; Doyle and Hotton, 1991; Doyle and Donoghue, 1993; Doyle, 1998; Doyle and Endress, 2000; Borsch et al., 2008). However, in general, there has been diminishing focus in recent years on exine structure and pollen developmental traits as critical evolutionary characters (but see Furness and Rudall, 2001, 2004; Doyle, 2005; Blackmore et al., 2007; Furness and Banks, 2010; Furness, 2011, 2013; Doyle and Le Thomas, 2012). Two key factors likely play a role in this decline. First, there is dearth of comparative analyses that have examined pollen characters at a high resolution. Second, there may be a misconception that pollen characters have essentially been documented, despite the lack of data for many taxa. Both have been true for Nymphaeales, a group in which key pollen data are lacking for multiple genera and for which a comprehensive, comparative evaluation of reported pollen characters is needed.

The objective of the current paper is to report and synthesize the available data on mature pollen and pollen ontogeny in Nymphaeales within a phylogenetic context and to consider the evolution of pollen characters in Nymphaeales, particularly as they may relate to transitions in life history and pollination biology. Whereas we have independently studied and summarized pollen characters for all genera, this paper also comprehensibly describes mature pollen characters for two water lily genera (*Barclaya*, *Euryale*) and one *Nymphaea* subgenus (*Anecypha*) for which there are no—or very limited—structural data available.

MATERIALS AND METHODS

Plant material—*Nymphaeaceae—*In all taxa, floral material was collected when anthers were mature and near the time of anther dehiscence (Figs. 1–6). *Victoria amazonica* (Poep.) Sowerby (Fig. 1) and *V. cruziana* D'Orbigny were collected from the private ponds of Ben and Kit Knotts in Cocoa Beach, Florida and from Lilypons aquatic nursery in Brookshire, Texas. *Euryale ferox* Salisbury (Fig. 2) was also collected at Lilypons aquatic nursery. *Nymphaea ondinea* Löhne, Wiersema & Borsch (Fig. 3) was collected in the Kimberley region of northern Western Australia. *Nymphaea odorata* Aiton (Fig. 4) was collected from Bastrop State Park in Bastrop, Texas. *Barclaya motleyi* Hooker f. was collected in Johore, Malaysia, and *Nuphar advena* (L.) Sm. (Fig. 6) was collected in Spring Lake, San Marcos, Texas. Voucher specimens for all species of Nymphaeaceae are stored in alcohol and housed in the liquid collection at the Santa Barbara Botanic Garden (SBBG).

*Cabombaceae—*Flowers of *Cabomba caroliniana* Gray (Fig. 7) were collected from Spring Lake in San Marcos, Texas, and flowers of *Brasenia schreberi* J.F.Gmel (Fig. 8) were collected in Toledo Bend Reservoir, East Texas on the Texas–Louisiana border. Voucher specimens for both species of Cabombaceae are stored in alcohol and housed in the liquid collection at the Santa Barbara Botanic Garden (SBBG). Additional floral material of *Brasenia* was collected from the Atlanta/Long Branch Conservation area near Macon, Missouri, and a voucher specimen is deposited in the Truman State University herbarium (NEMO; Appendix 1).

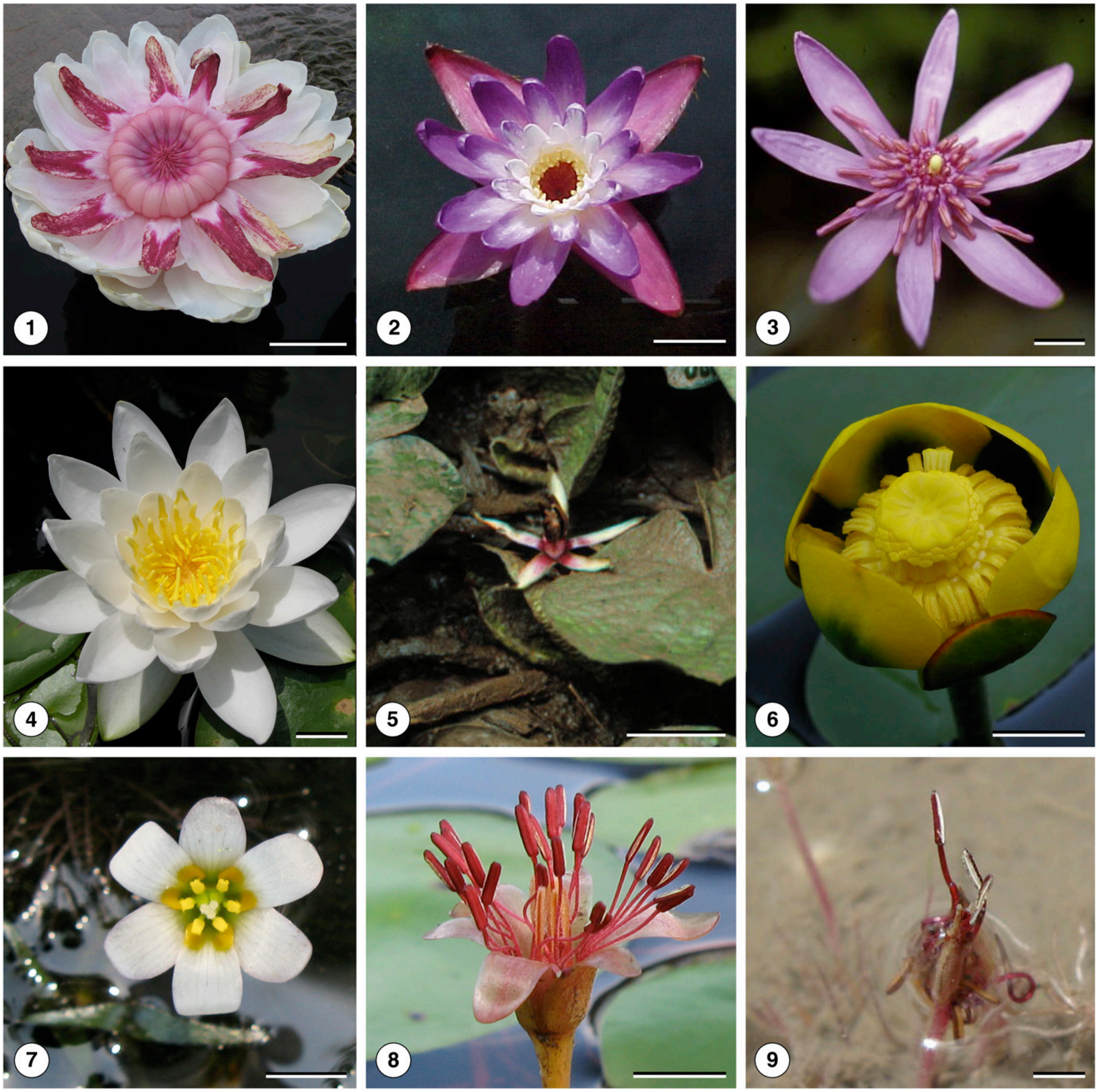
*Hydatellaceae—*Reproductive units of *Trithuria* were collected from southwest Western Australia (shire of Manjimup). *Trithuria submersa* Hook f. material was collected in Kulunilup Nature Reserve; *T. australis* (Diels) D.D.Sokoloff et al. material was collected in Frying Pan Swamp; and *T. austinensis* D.D.Sokoloff et al. (Fig. 9) material was collected in Branchinella Lake. Voucher specimens are deposited at the University of Tennessee herbarium (TENN; Appendix 1).

Microscopy—Anthers of *Victoria*, *Cabomba*, *Brasenia*, and *Trithuria* were dissected from flowers, fixed in either Karnovky's fixative (50% v/v glutaraldehyde and 16% v/v paraformaldehyde in 0.2 M phosphate buffer; pH 7.4) or 3% v/v glutaraldehyde (in 0.2 M phosphate buffer; pH 7.4) for 24 h, and postfixed in 1–2% w/v osmium tetroxide (buffered in 0.2 M phosphate buffer; pH 7.4) for 2–6 h.

Flowers of *Euryale*, *Nymphaea odorata*, *N. ondinea*, *Barclaya*, and *Nuphar* were sectioned into pieces and fixed in FAA (40% formaldehyde, glacial acetic acid, and 95% ethanol) for 24 h. FAA fixation does not provide as much detail as glutaraldehyde-based fixatives for ultrastructural interpretation of developmental stages, but the key structural characters of the exine are adequately preserved to provide high-resolution data for mature pollen. Anthers were then dissected from flowers and postfixed in 1% osmium tetroxide (buffered in 0.2 M phosphate buffer; pH 7.4) for 3–6 h.

For light microscopy (LM) and transmission electron microscopy (TEM), chemically fixed anthers of all taxa were dehydrated in a graded ethanol series and embedded in Spurr's epoxy resin (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA). Thick sections (850 nm) were cut on an ultramicrotome using a diamond knife, collected on glass microscope slides, stained with either 10% toluidine blue O or Richardson's stain (methylene blue and azure II) and examined using an Olympus (Center Valley, Pennsylvania, USA) BHS compound light microscope. For investigation of pollen wall ultrastructure, thin sections (70–100 nm) were cut on an ultramicrotome using a diamond knife, collected on 1 × 2 mm copper slot grids and dried onto formvar support films (Rowley and Moran, 1975). Thin sections were stained with varying combinations of 1% w/v aqueous potassium permanganate (0–1 min), 1% w/v aqueous uranyl acetate (10–15 min), and 0.1% w/v aqueous lead citrate (8–10 min; Venable and Coggeshall, 1965) and examined using a JEOL (Peabody, Massachusetts, USA) JEM-100SX transmission electron microscope at 60–80 kV.

For studying pollen surface morphology with scanning electron microscopy (SEM), anthers were critical point dried, macerated using a syringe needle on a microscope slide, and mounted onto



FIGURES 1–9 Representative flowers of water lily genera. **1.** Male-phase flower of *Victoria amazonica* with dehiscing stamens and staminodes bent over the central syncarpous gynoecium. Bar = 5 cm. **2.** Female-phase flower of *Euryale ferox*. Bar = 1 cm. **3.** Male-phase flower of *Nymphaea ondinea* with dehiscing anthers reflexed from central gynoecium. Bar = 2 mm. **4.** Male-phase flower of *Nymphaea odorata* with dehiscing anthers surrounding and partially bent over central syncarpous gynoecium. Bar = 2 cm. **5.** Chasmogamous flower of *Barclaya rotundifolia*. Bar = 5 cm. **6.** Male-phase flower of *Nuphar advena* with dehiscing anthers reflexed from the central syncarpous gynoecium. Bar = 1 cm. **7.** Male-phase flower of *Cabomba caroliniana* with six dehiscent anthers and three carpels aggregated in the floral center. Bar = 5 mm. **8.** Male-phase flower of *Brasenia schreberi* with many dehiscing anthers supported by long, slender filaments. Bar = 5 mm. **9.** Male reproductive unit of *Trithuria austinensis* with three dehiscing anthers and at least one immature anther supported above the water surface on long filaments. Bar = 2 mm.

aluminum stubs to which double-sided adhesive tape had been adhered. Stubs were sputter-coated with gold-palladium, and the pollen was examined using a JEOL JSM-6100 scanning electron microscope at 5 kV. *Trithuria* specimens were sputter-coated with gold and examined using a LEO (Thornwood, New York, USA) 1525 scanning electron microscope at 5 kV.

RESULTS

In this section, we present new data on the structure of mature pollen from *Euryale ferox*, *Nymphaea ondinea*, and *Barclaya motleyi*. Pollen characters for the other six water lily genera that we studied, and for which varying quantities of structural data exist in the literature, are summarized in Tables 1 and 2.

Pollen terminology used in this paper follows Hesse et al. (2009). Unless otherwise noted, measurements of pollen grain size are given as the length of the major equatorial axis by the length of the polar axis. All reported pollen grain size measurements are based on scanning electron micrographs, but are consistent with measurements from light micrographs. We also present relevant size and shape data from the literature, but it should be noted that slight variation in size and shape is expected across studies due to different preservation/preparation techniques (Doyle and Endress, 2014).

Euryale*—Euryale* is a monotypic genus (*Euryale ferox* Salisbury) native to Asia (Kadono and Schneider, 1987; Ajaib et al., 2010). *Euryale* is sister to *Victoria*, and this clade is hypothesized to be either nested within *Nymphaea* (Borsch et al., 2008) or sister to *Nymphaea* (Borsch et al., 2007, 2011). The present paper is the first high-resolution investigation of *Euryale* pollen. Select pollen characters have previously been described by Erdtman (1943, 1952), Khanna (1964), and Meyer-Melikian and Diamandopulu (1996), with the latter illustrating the pollen wall, but these studies were accompanied by only line drawings that display characters at low resolution. Walker (1976b) and Hesse and Zetter (2005) described surface features of *Euryale* pollen with accompanying scanning electron micrographs.

Mature pollen—*Euryale* pollen grains are dispersed as monads (Fig. 10). Although Erdtman (1943) reported grains to be more ellipsoidal (51 × 37 μm), pollen grains in our material were approximately spheroidal (35 μm in diameter). Pollen grains exhibit a ring-like aperture that is slightly displaced toward the distal pole (Table 1). The apertural membrane consists of only the intine and lacks ornamentation (Fig. 10; Table 2). The nonapertural pollen surface is microechinate, with spines measuring up to 1 μm in height (Figs. 11–13; Hesse and Zetter, 2005). The exine is tectate-columellate, comprising a distinct tectum, an extremely thin foot layer, and short, indistinct columellae that span a narrow infratectal space (Figs. 13, 14; Table 2). Small, spherical elements are also present in the infratectum, particularly near the infratectum–tectum interface (Fig. 13). Developmental studies of *Brasenia schreberi* (Taylor and Osborn, 2006) revealed that similar elements in that species were, in fact, part of the tectum and not the infratectum. We hypothesize that the same is true for *Euryale,* although this needs to be investigated with developmental studies. A thick, lamellate endexine is present, as well as a membranous granular layer composed of small, spherical bodies (Fig. 14). Both the sculptural microechinae and tectum are dissected with microchannels (Figs. 13, 14). Exine ultrastructure is similar in the distal and proximal walls (Fig. 12).

Pollen development—The only observation of pollen developmental characters in *Euryale* is by Khanna (1964), who reported on the formation of tetrahedral and isobilateral tetrads and a secretory tapetum (Table 1). The resolution of characters in that investigation is low, and the pollen ontogeny in *Euryale* is in need of re-evaluation.

Nymphaea ondinea*—Nymphaea ondinea* Löhne, Wiersema & Borsch is endemic to nonperennial sandstone streams of the northern Kimberley District in Western Australia (Schneider, 1983). Historically considered to comprise the monotypic genus *Ondinea*, this species has long been thought to be closely related to *Nymphaea*

TABLE 1. Summary of select pollen structural and developmental characters in Nymphaeales. Dispersal unit size is given as either the average diameter or the average length of the equatorial axis by polar axis. Data from this study are reported and, when applicable, representative variation that has been reported in the literature is included in parentheses. Variation for *Nymphaea* is listed with *N. odorata*.

	Dispersal unit			Surface ornamentation		Aperture		
Taxon	Type	Grain shape	Dispersal unit size (μm)	Pattern	Stage initiated	Type	Surface ornamentation	Tapetum type
Nymphaeaceae								
Victoria	tetrad	oblate to spheroidal	78 (63–86)[a, b]	psilate	—	ring-like	microgemmate	secretory[m]
Euryale	monad	spheroidal (oblate)	35 (51 × 37)[b]	microechinate	?	ring-like	psilate	secretory[n]
N. ondinea	monad	spheroidal (oblate)	44 (37.1 × 25.7)[c]	psilate to verrucate	—	ring-like	psilate	?
N. odorata	monad	oblate to spheroidal	24 × 19 (22–59)[b, d–f]	Microgemmate (psilate–baculate)[e, l]	free microspore[l]	ring-like	microgemmate	secretory[o, p] cyclic invasive[l, q]
Barclaya	monad	oblate	19 × 10 (41 × 24)[b]	verrucate	—	ring-like	psilate	?
Nuphar	monad	oblate to spheroidal	41 × 30 (40 × 34–58 × 46)[g]	echinate	early tetrad[g]	monosulcate	psilate	secretory[r]
Cabombaceae								
Cabomba	monad	elongate	81 x 61[h] (47 × 33–91 × 58)[i]	Striate (verrucate in *C. palaeformis*)[i]	early free microspore[h]	monosulcate	scabrate	secretory[s] invasive[h]
Brasenia	monad	elongate	50 × 35[j]	scabrate	middle free microspore[j]	monosulcate	psilate	secretory[j]
Hydatellaceae								
Trithuria	monad	spherical (oblong)	19 (17 × 12–27 × 22)[k]	perforate	—	monosulcate	psilate	?

Notes: Literature reports are from the following papers: [a] Ikuse, 1956; [b] Erdtman, 1943; [c] Muller, 1970; [d] Chaturvedi, 1974; [e] Volkova and Shipunov, 2007; [f] Coiro and Lumaga, 2013; [g] Takahashi, 1992; [h] Taylor et al., 2008; [i] Ørgaard, 1991; [j] Taylor and Osborn, 2006; [k] Remizowa et al., 2008; [l] Gabarayeva and El Ghazaly, 1997; [m] Taylor et al., 2012; [n] Khanna et al., 1964; [o] Schnarf, 1931; [p] Gabarayeva at al., 2001; [q] Rowley et al., 1992; [r] Zhou and Fu, 2008; [s] Gabarayeva at al., 2003.

TABLE 2. Comparative exine ultrastructure in Nymphaeales. Absolute values (µm) are provided for the total thickness of the exine (ektexine + endexine, excluding the membranous granular layer) in each representative taxon measured in this study. The proportion of the total proximal ektexine that is comprised by each layer (tectum, infratectum, and foot layer) is indicated, with the thickest layer indicated by an underline. In the case of *Nymphaea ondinea*, in which the tectum and foot layer undulate, the average thickness of each of those layers was calculated from measurements taken in both the thickest and thinnest regions of that layer. The presence or absence of a membranous granular layer (MGL) and the composition of the wall directly over the aperture are also indicated.

			Layer composition of the Ektexine (%)				
Taxon	**Overall exine type**	**Total exine thickness (µm)**	**Tectum**	**Infratectum**	**Foot layer**	**MGL**	**Composition of apertural membrane**
Nymphaeaceae							
Victoria	tectate-columellate	1.4	50	8	41	present	globular sporopollenin elements, foot layer, endexine, MGL, intine
Euryale	tectate-columellate	0.9	50	33	16	present	intine
N. ondinea	tectate-columellate	1.6	49	5	46	present	intine
N. odorata	tectate-columellate	1.4	12	8	80	present	large sculptural elements, reduced ektexine, endexine, MGL, intine
Barclaya	tectate-columellate	0.6	41	43	17	present	reduced ektexine, endexine, MGL, intine
Nuphar	tectate-columellate	0.7	31	24	46	present	sculptural spines, reduced ektexine, endexine, MGL, intine
Cabombaceae							
Cabomba	tectate-columellate	1.1	36	51	13	absent	reduced ektexine, endexine, intine
Brasenia	tectate-columellate	0.5	33	43	24	absent	intine
Hydatellaceae							
Trithuria	tectate-columellate	1.0	27	43	30	absent	intine

(den Hartog, 1970; Muller, 1970; Williamson and Moseley, 1989). Recent molecular analyses have indicated that the former *Ondinea purpurea* den Hartog is nested within *Nymphaea* subgenus *Anecypha* (Borsch et al., 2007, 2011; Löhne et al., 2008, 2009). Löhne et al. (2009) published a taxonomic revision, proposing the new name *Nymphaea ondinea* Löhne, Wiersema & Borsch.

The current paper is the first investigation of pollen in this taxon, other than a brief description by Muller (1970), who used only light microscopy. Previous investigations of pollen in subgenus *Anecypha* are limited to brief observations of *N. gigantea* (Meyer-Melikian and Diamandopulu, 1996; Coiro and Lumaga, 2013).

Mature pollen—Nymphaea ondinea pollen grains are dispersed as monads with a ring-like aperture displaced toward the distal pole (Figs. 15–17; Table 1). Muller (1970) described grains as ellipsoidal to spheroidal-suboblate (37.1 × 25.7 µm), whereas in our material, pollen grains were nearly spheroidal (44 µm in diameter).

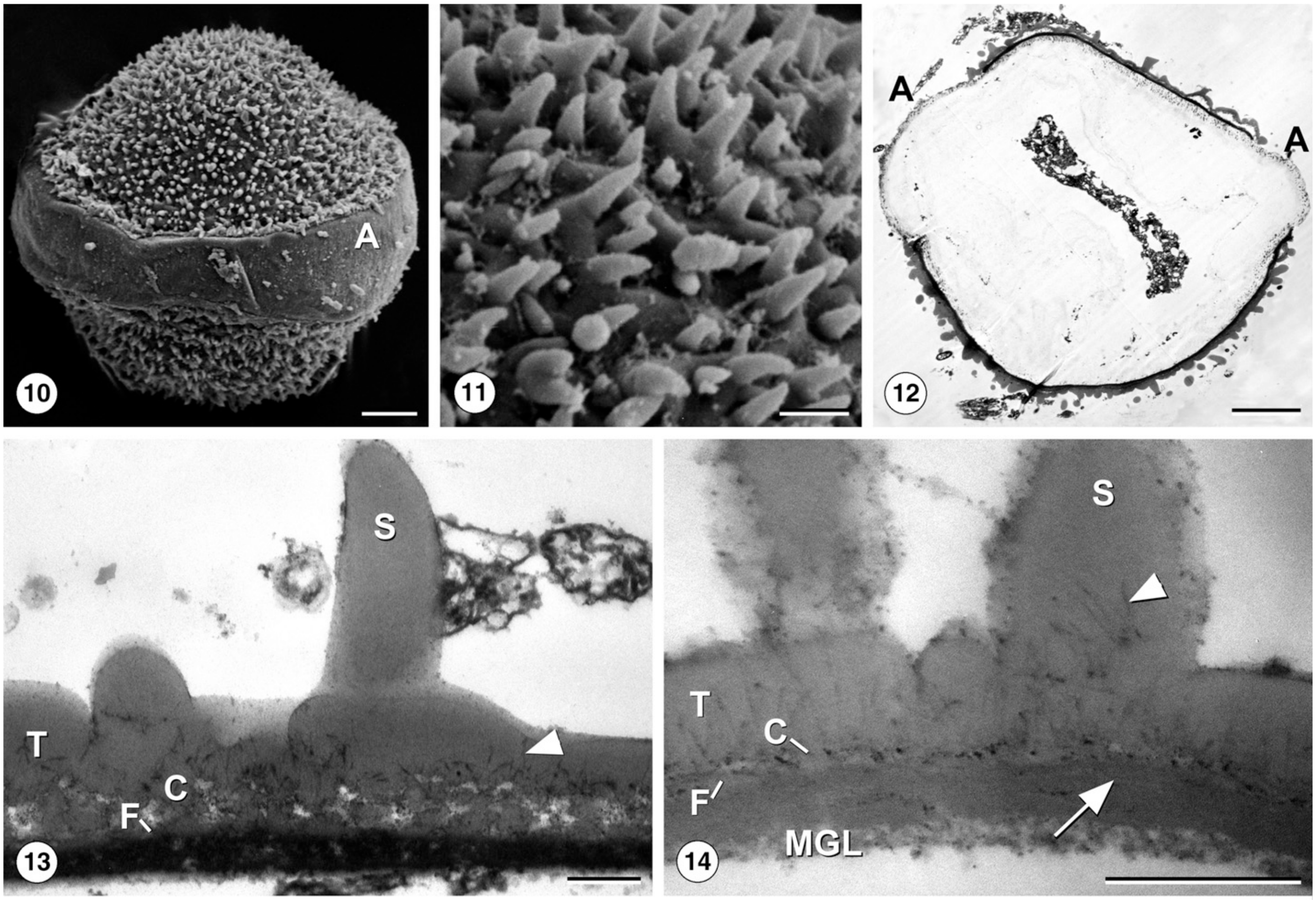
The surface of the nonapertural wall is psilate to verrucate, whereas the apertural surface is psilate, with the apertural membrane consisting of only intine (Figs. 18–21). We interpret the exine of *N. ondinea* to be tectate-columellate. The distal and proximal walls differ considerably. In the proximal wall, the tectum and foot layer are robust and vary in thickness such that they appear undulated (Fig. 20; Table 2). The infratectum is highly compressed; however, distinct infratectal columellae are present. These elements are short, measuring less than 0.1 µm in height, but span the entire infratectal space (Fig. 20). In the distal wall, tectum thickness is more consistent, and the foot layer is much thinner than in the proximal wall (Fig. 21). The infratectal space is thicker in overall height (0.30 µm), but columellae are less organized. Minute spherical elements are also visible within the infratectal space among the columellae in the distal wall. In both the proximal and distal walls, the endexine is distinctly lamellate, but the endexine is twice as thick in the distal wall. The identification of this layer as endexine, rather than foot layer, is supported by its staining darker than adjacent layers. A membranous granular layer is present in both the proximal and distal walls, as well (Figs. 20, 21).

Barclaya—The genus *Barclaya* is endemic to Southeast Asia and comprises four species: *Barclaya motleyi* Hooker f., *Barclaya longifolia* Wallich, *Barclaya kunstleri* (King) Ridley, and *Barclaya rotundifolia* Hotta. Previous reports of *Barclaya* pollen are limited in scope (Erdtman, 1943; Batygina and Shamrov, 1983; Meyer-Melikian and Diamandopulu, 1996). The current paper is the first study to provide high-resolution data on pollen structure in *Barclaya*.

Mature pollen—Pollen of *Barclaya rotundifolia* is oblate (19 × 10 µm) with a ring-like aperture displaced toward the distal pole (Figs. 22, 23). Erdtman (1943, 1952) described the grains of *Barclaya* sp. as "bean shaped" and much larger (30 × 48 × 33 µm; 41 × 24 µm) than the grains in our material. The nonapertural surface is verrucate, whereas the apertural surface is psilate (Fig. 22; Table 1). The apertural membrane primarily consists of endexine and a reduced tectal layer. *Barclaya* pollen is tectate-columellate, with the columellae much more robust in the proximal wall (Fig. 24; Table 2). In the distal wall, the columellae are less distinct, and the foot layer is nearly absent (Fig. 25). The tectum is discontinuous, with narrow gaps between sections of the tectum, much like that observed in *Brasenia* (Figs. 24, 25). An endexine—with distinct white-line centered lamellae—is present, with very robust lamellae in the distal wall (Fig. 25). A membranous granular layer, consisting of minute spherical bodies, is present beneath the endexine.

DISCUSSION

Nymphaeales are an early-divergent angiosperm lineage with a long, independent evolutionary history. The age of the lineage combined with diversification and specialization in flower morphology, pollination biology, and other reproductive traits, makes Nymphaeales an



FIGURES 10–14 *Euryale*. **10.** Equatorial view of a grain with microechinate surface ornamentation on the nonapertural surface and a ring-like aperture with no ornamentation. SEM, bar = 5 μm. **11.** Detail of pollen grain surface with microspine sculptural elements. SEM, bar = 1 μm. **12.** Section through an entire pollen grain showing similar proximal and distal pollen walls. TEM, bar = 5 μm. **13.** Detail of the pollen wall showing a microspine continuous with the substantial tectum; the tectum is dissected by microchannels (arrowhead). Infratectal columellae are present, but not robust and the foot layer is very thin. The endexine is relatively thick and compact. TEM, bar = 0.5 μm. **14.** Detail of the pollen wall showing microchannels (arrowhead) dissecting the tectum and microspines. The infratectal space is narrow with very short columellae. Individual lamellae are visible in the thick endexine layer (arrow). TEM, bar = 0.5 μm. A, aperture; C, columella, F, foot layer; MGL, membranous granular layer; S, sculptural microspine; T, tectum.

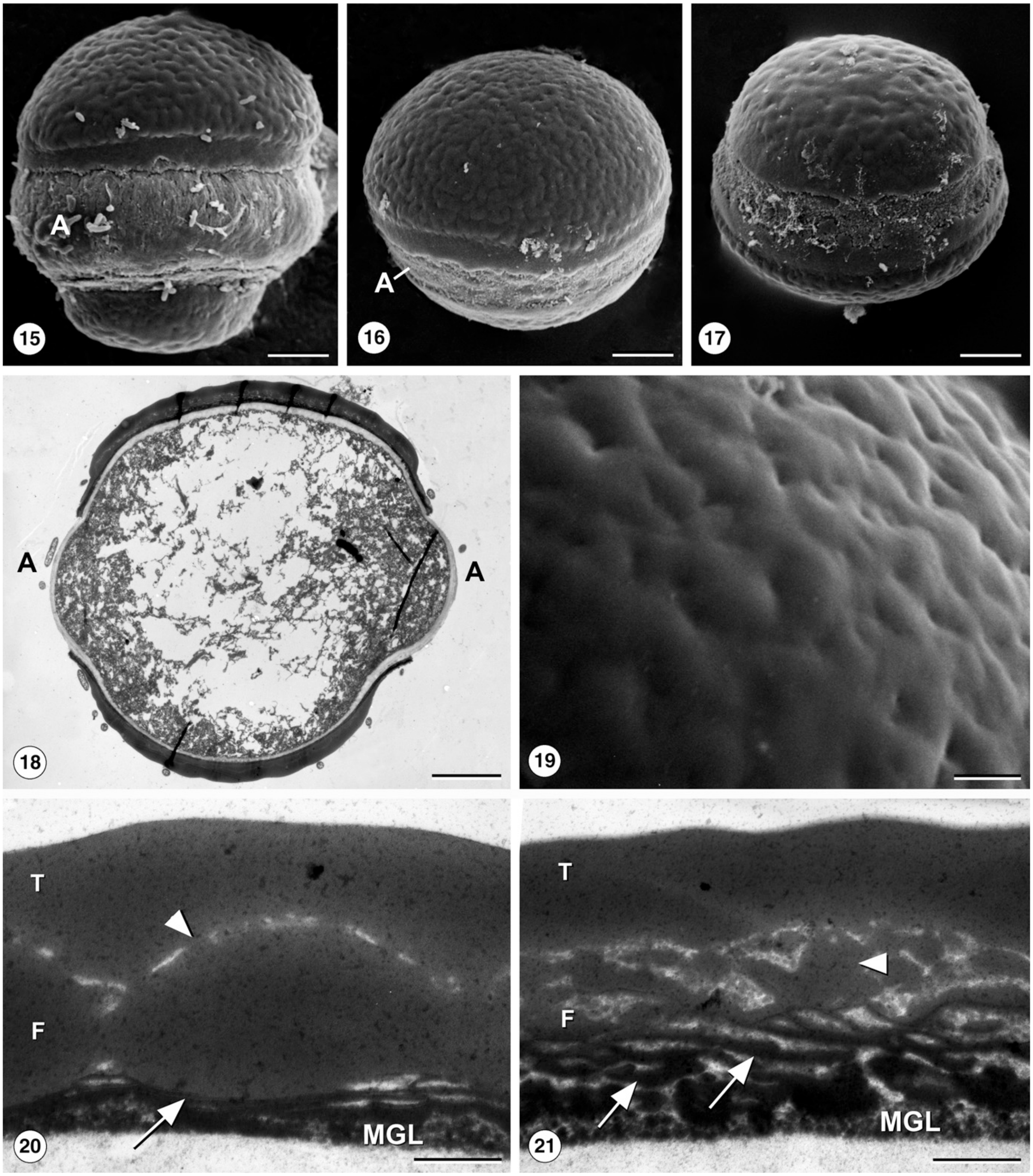
excellent system in which to investigate pollen evolution and the lability of the pollen developmental program. In this section, we discuss selected pollen characters in Nymphaeales in a comparative context.

Pollen dispersal unit—*Dispersal unit size* —The size of the dispersal unit varies both within and among species in Nymphaeales, with most taxa exhibiting pollen grains measuring between 25 and 50 μm (Fig. 26; Table 1). This range is consistent with the size range for pollen in ANITA lineages, and such medium-sized pollen grains have been hypothesized to be ancestral in angiosperms and Nymphaeales (Doyle and Endress, 2000, 2014; Doyle, 2005).

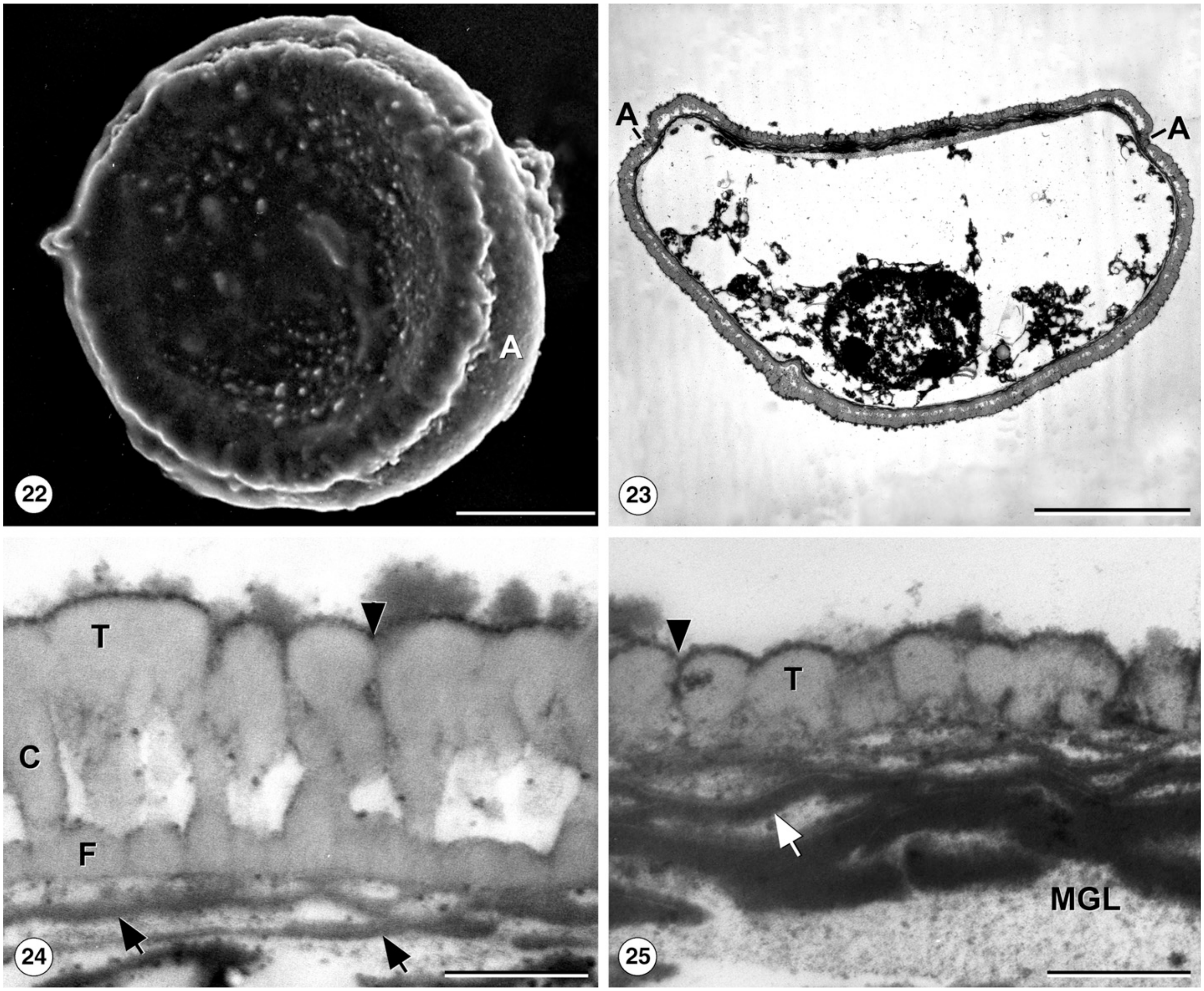
Barclaya and *Trithuria* exhibit pollen that is typically smaller than other water lilies. In *Barclaya*, this small size is potentially correlated with breeding system. *Barclaya motleyi*, the species studied here, likely exhibits a high proportion of selfing (Williamson and Schneider, 1994). Plants with high levels of selfing are expected to exhibit less investment in male function, which may result in smaller pollen (Charnov, 1982; Barrett et al., 1996). Erdtman (1943) reported the size of *Barclaya* pollen to be considerably larger than that in our study, but it was not noted which species he examined. It is possible that pollen size varies according to breeding system in *Barclaya*, with insect-pollinated species producing larger pollen than selfing species.

It is unlikely that small pollen size in *Trithuria* is correlated with breeding system because pollen grains of predominantly selfing species and obligately outcrossing species are similar in size (Remizowa et al., 2008). Small pollen in *Trithuria* is, instead, likely due to an overall reduction in plant size, including anther size, which occurred with the transition to ephemeral pools (Taylor et al., 2010).

Victoria exhibits the largest dispersal unit in Nymphaeales (78 μm), followed by *Cabomba* (81 × 61 μm; M. L. Taylor et al., 2008, 2013). We hypothesize that pollen in these two genera has increased in size, relative to the ancestral condition. This increase occurred independently and through different developmental mechanisms. In *Cabomba*, increase in dispersal size is due only to enlargement of the pollen grain, whereas in both species of *Victoria*, the large dispersal unit is a consequence of both larger individual pollen grains,



FIGURES 15–21 *Nymphaea ondinea.* **15.** Equatorial view of a pollen grain showing the aperture displaced toward the distal pole. SEM, bar = 5 µm. **16.** Pollen grain in oblique proximal view showing the verrucate pollen surface. SEM, bar = 5 µm. **17.** Pollen grain in oblique distal view. SEM, bar = 5 µm. **18.** Slightly oblique section through a whole pollen grain. TEM, bar = 5 µm. **19.** Detail of pollen surface. SEM, bar = 1 µm. **20.** Detail of proximal wall in section showing substantial tectum, very narrow infratectal space with distinct columellae (arrowhead), and very thick foot layer. The endexine lamellae are apparent (arrow), as is the membranous granular layer. TEM, bar = 0.5 µm. **21.** Detail of distal wall in section showing tectum similar to the proximal wall, but a much thicker infratectal space with less-organized columellae (arrowhead) and a much thinner foot layer. The endexine is also thicker, with distinct lamellae (arrows); a membranous granular layer is present. TEM, bar = 0.5 µm. A, aperture; F, foot layer; MGL, membranous granular layer; T, tectum.



FIGURES 22–25 *Barclaya.* **22.** Pollen grain in distal polar view showing smooth pollen surface and ring-like aperture. SEM, bar = 5 µm. **23.** Longitudinal section through a pollen grain, with ring-like aperture nearer to the distal pole. TEM, bar = 5 µm. **24.** Detail of the proximal exine in section showing a robust tectum with discontinuities (arrowhead), a thick infratectal space with distinct columellae, a thick foot layer, and endexine lamellae (arrows). TEM, bar = 0.25 µm. **25.** Detail of a distal exine in section showing a tectum with discontinuities (arrowhead), a highly compressed infratectal space, reduced foot layer, thick endexine lamellae (arrow), and thin membranous granular layer. TEM, bar = 0.25 µm. A, aperture; C, columella; F, foot layer; MGL, membranous granular layer; T, tectum.

relative to other water lilies, and fusion of microspores into permanent tetrads (Taylor et al., 2013).

Dispersal unit type —Most water lily pollen grains are dispersed as monads. However, pollen grains of both *Victoria* species are dispersed as permanent, calymmate tetrads (Roland, 1965; Taylor et al., 2013). Permanent tetrads have also been reported in *Nymphaea tetragona* (Ueno, 1962) and *Trithuria inconspicua*; however, in the case of *T. inconspicua*, these tetrads appear to be collapsed and sterile (Furness and Rudall, 1999; Remizowa et al., 2008). The production of permanent tetrads in these taxa indicates that there was more than one origin of compound pollen in Nymphaeales. The developmental mechanism of microspore cohesion that occurs in *Victoria* is unique and has not been described in other compound pollen-producing angiosperm taxa (Taylor et al., 2013). It is not known whether *N. tetragona* exhibits the same processes or if variation in microspore cohesion exists in Nymphaeales.

Compound pollen production is not associated with pollination syndrome in Nymphaeales (Taylor et al., 2013). *Victoria* exhibits beetle entrapment, whereas *Nymphaea tetragona* is reported to be pollinated by flies (Hill, 1988). Furthermore, *Nymphaea lotus* and *Nymphaea rudgeana*, which exhibit pollination via beetle entrapment like *Victoria,* do not produce permanent tetrads (Cramer et al., 1975; Ervik and Knudsen, 2003; Löhne et al., 2008). Compound pollen in *Victoria,* instead, likely evolved as part of a suite of reproductive characters that accompanied an increase in flower size. Taylor et al. (2013) hypothesized that larger pollen grains in *Victoria* may adhere more readily to beetles or may provide three-dimensionality in the same way that sculptural elements do in other species. Permanent tetrads ultimately result in four potential pollen tubes—and, therefore, four potential fertilization events—with every transfer of a dispersal unit (Taylor et al., 2013).

Surface ornamentation—The surface ornamentation of water lily pollen is variable (Table 1). *Barclaya, Nymphaea ondinea,* and *Victoria* (Taylor et al., 2013) each exhibit a psilate to verrucate surface. *Brasenia* (Taylor and Osborn, 2006), *Euryale*, *Trithuria*, and other species of *Nymphaea* (Gabarayeva and El Ghazaly, 1997; Volkova and Shipunov, 2007) exhibit small ornamental elements, but of varying types.

Cabomba and *Nuphar* exhibit large sculptural elements, but these are markedly different (Fig. 26). *Nuphar* pollen has large, vertically oriented spines (Takahashi, 1992), whereas the pollen of *Cabomba* has longitudinally oriented striae (M. L. Taylor et al., 2008). The large sculptural elements in these two genera have different developmental origins. In *Cabomba*, the sculptural elements are supratectal and ontogenetically first appear during the early free-microspore stage, after tectum initiation (M. L. Taylor et al., 2008). In contrast, the spines of *Nuphar* are the first pollen wall element to form, developing along the plasmalemma during the early tetrad stage and accumulating significant sporopollenin before the rest of the exine is initiated (Takahashi, 1992). These spines extend through the entirety of the exine and above the tectum to a height of over 6 μm.

In both *Cabomba* and *Nuphar*, we hypothesize that the large sculptural elements are adaptive for insect pollination. In *Cabomba*, large amounts of pollenkitt are deposited on the surface of the pollen grain, and the pollenkitt accumulates between the grooves formed between the supratectal striae and within the tectal microchannels. This pollenkitt serves to adhere pollen grains to the bristles of pollinating flies (Osborn et al., 1991; M. L. Taylor et al., 2008). In *Nuphar*, the spines may act to increase surface area and three-dimensionality and, therefore, the likelihood that a pollen grain will adhere to a visiting insect.

Surface ornamentation in Nymphaeales differs from that found in other early-divergent angiosperm lineages. Pollen of *Illicium*, *Schisandra*, and *Kadsura* is reticulate (Wang et al., 2010), and *Trimenia* pollen has been characterized as reticulate to rugulose (Sampson and Endress, 1984; Sampson, 1987). Pollen of *Austrobaileya* exhibits a perforate tectum (Zavada, 1984), whereas pollen of *Amborella* exhibits a tectum comprised of cupule elements that have not been recognized elsewhere in angiosperms (Sampson, 1993; Hesse, 2001). Because there is building evidence for the role of genomic factors, as well as self-assembly in the patterning of exine ultrastructure and sporopollenin deposition (Gabarayeva, 2014), understanding the evolutionary pressures that drive both modification and conservation of surface ornamentation may yield valuable data regarding this biophysical process.

Pollen apertures—There are two types of aperture morphology in Nymphaeales, monosulcate apertures and ring-like apertures (Table 1; Fig. 26).

Monosulcate apertures—Pollen grains of *Brasenia*, *Cabomba*, *Nuphar*, and *Trithuria* are monosulcate, with elongated apertures situated distally and extending the full length of the grain. Having monosulcate apertures is the plesiomorphic condition in Nymphaeales (Fig. 26; Borsch et al., 2008). The exine is completely absent from the apertural membrane in *Brasenia* and is typically lacking in *Trithuria*; however, some species of *Trithuria* exhibit small exine fragments over the aperture (Remizowa et al., 2008). In contrast, pollen grains of both *Cabomba* (Osborn et al., 1991; Gabarayeva et al., 2003; M. L. Taylor et al., 2008; Coiro and Lumaga, 2013) and *Nuphar* (Erdtman, 1943; Jones and Clarke, 1981; Zhou and Fu, 2008; Coiro and Lumaga, 2013) exhibit a coherent exine over the aperture (Table 2).

Ring-like apertures—The pollen grains of all other nymphaealean genera exhibit a ring-like aperture, and this character is a synapomorphy of Nymphaeaceae excluding *Nuphar* (Fig. 26). There has previously been uncertainty regarding aperture type in *Barclaya*. Pollen of *Barclaya* has been characterized as possibly nonaperturate (Erdtman, 1952), as monocolpate (Erdtman, 1943; Meyer, 1966), and as having a circular distal aperture (Batygina and Shamrov, 1983). Doyle (2005) coded the aperture in *Barclaya* as "sulculate," whereas Borsch et al. (2008) coded it as "uncertain." Our data unequivocally demonstrate that *Barclaya* exhibits a ring-like aperture.

The ring-like apertures in Nymphaeaceae have also been termed zonasulculate. Apertures in *Victoria amazonica*, *V. cruziana*, *Euryale ferox*, *Nymphaea odorata*, *N. ondinea*, and *Barclaya motley* are displaced toward the distal pole and have been characterized as anazonasulculate (Fig. 26), as have those of *Nymphaea candida* (Walker, 1974a) and *N. mexicana* (Gabarayeva and El-Ghazaly, 1997). Other water lily taxa, such as *Nymphaea lotus*, have been characterized as having an equatorial, or zonizonasulculate aperture (Walker, 1974a). In nymphaealean taxa with ring-like apertures, aperture position occurs along a continuum from strongly distal (*Nymphaea odorata*) to nearly equatorial (*Euryale ferox*) to equatorial (*Nymphaea lotus*). We feel that emphasizing a distinction between "anazonasulculate" and "zonizonasulculate" artificially exaggerates differences among taxa for a character state with little phylogenetic signal, and we therefore prefer to use the more general term "ring-like" (Hesse et al., 2009) for Nymphaeales.

Evolution of aperture type—There has been much debate regarding the origin of the ring-like aperture in Nymphaeales, with two hypotheses emerging. In the first hypothesis, the ring-like aperture resulted from a transformation from the monsulcate condition of *Nuphar* (Meyer, 1964; see Coiro and Lumaga, 2013). In the second scenario, the operculate hypothesis, pollen grains are monoporate, with the entire distal hemisphere of the grain comprising a modified aperture and the distal exine comprising an operculum (Wodehouse, 1935; Erdtman, 1943; Walker, 1974a; Faegri and Iversen, 1975; Jones and Clarke, 1981; Coiro and Lumaga, 2013).

The transition to the ring-like aperture under the operculate hypothesis requires only the expansion of the distal aperture into a large pore and the concomitant broadening of an existing thin operculum (Walker, 1974a), such as that observed in *Cabomba* and *Nuphar*. If the distal exine represents a modified operculum, the ultrastructure of that region is expected to differ from that of the proximal exine (Furness and Rudall, 2003). In water lily pollen, differences between proximal and distal ornamentation and/or exine ultrastructure have been observed in *Nymphaea alba* (Jones and Clarke, 1981; Coiro and Lumaga, 2013), *N. candida* (Walker, 1974a; Jones and Clarke, 1981), *N. mexicana* (Gabarayeva and El Ghazaly, 1997), and *N. colorata* (Gabarayeva and Rowley, 1994). In contrast, variation between the proximal and distal ektexine was not observed in *Nymphaea caerulea*, nor in the sculpturing pattern in *N. gigantea* (Coiro and Lumaga, 2013).

In the current study, we found considerable structural variation in the ektexine and endexine between the proximal and distal walls in three taxa of Nymphaeaceae: *Barclaya motleyi*, *Nymphaea odorata*, and *N. ondinea.* In contrast, we did not observe major differences in the proximal and distal ektexines of *Euryale* or *Victoria* pollen, other than those caused by microspore cohesion in *Victoria* (Fig. 26). However, these taxa, as well as *Nymphaea caerulea* (Coiro and Lumaga, 2013), do exhibit differences between the proximal and distal endexines. In the proximal wall, the endexine is narrow, with highly compressed lamellae in the proximal wall, whereas the endexine is thick, with loosely arranged lamellae in the distal wall. The loosely arranged distal endexine in all five genera of Nymphaeales with a ring-like aperture closely resembles the apertural endexine of the four genera with monosulcate apertures. These data support the hypothesis that the entire distal hemisphere of the grain in these taxa represents a modified aperture (i.e., the operculate hypothesis).

Phylogenetic implications —We support the hypothesis that structural similarity in the ektexine in the proximal and distal wall is the derived state in Nymphaeaceae, and that structural differentiation between the proximal and distal exines is plesiomorphic and has been lost in more-derived taxa (Fig. 26). The loss of this structural variation would have occurred either through co-option of the proximal ontogenetic program in the distal hemisphere or from similar selection pressure on both proximal and distal wall development (Coiro and Lumaga, 2013). The strong exine differentiation observed in *Barclaya*, the earliest-diverging lineage in Nymphaeaceae other than *Nuphar*, supports this hypothesis. Similarly, as expected under this hypothesis, taxa of *Nymphaea* subgenus *Nymphaea* (*N. alba*, *N. candida*, *N. mexicana*, *N. odorata*) also continue to exhibit exine differentiation (Walker, 1974a; Jones and Clarke, 1981; Gabarayeva and El Ghazaly, 1997; Coiro and Lumaga, 2013).

For the most part, taxa of Nymphaeaceae that are thought to be more deeply nested (Borsch et al., 2008), such as *Nymphaea caerulea* (subg. *Brachyceras*), *N. gigantea* (subg. *Anecypha*), *Victoria,* and *Euryale*, exhibit loss of ektexine differentiation. However, they have retained endexine differentiation. The similarity between *Euryale* and *Victoria* further supports the hypothesis that these taxa form a clade (Fig. 26).

However, there are exceptions; we observed significant exine differentiation in *Nymphaea ondinea* (*Nymphaea* subg. *Anecypha*), and Gabarayeva and Rowley (1994) observed exine differentiation in *N. colorata* (subg. *Brachyceras*). To explain this latter exception, Coiro and Lumaga (2013) suggested (1) that Gabarayeva and Rowley (1994) observed immature pollen grains of *N. colorata* in which the intine was not yet fully developed and (2) that the differences Gabarayeva and Rowley (1994) observed might be due only to differences in developmental timing of the distal and proximal walls and that variation in exine structure might not be observed in fully mature grains. We consider this an unlikely scenario, because significant exine modification would need to occur quite late in development, but ontogenetic data from other water lilies indicate that little modification in exine ultrastructure occurs after initiation of the intine (Taylor and Osborn, 2006; M. L. Taylor et al., 2008, 2013). Furthermore, our observations of *Nymphaea ondinea* are of fully mature grains. It is more likely that exine differentiation has been lost more than once, and to varying degrees, in the core Nymphaeaceae. Better resolution of relationships among these genera, combined with additional pollen ultrastructural data, may reveal this character to have a phylogenetic signal.

Infratectum ultrastructure—Infratectum ultrastructure is a critical character for reconstructing pollen evolution and determining relationships among both extant and fossil seed plants (e.g., Walker, 1974b; Osborn, 2000; Doyle, 2005; Doores et al., 2007; Doyle and Endress, 2014). Accurately characterizing the infratectum in Nymphaeales is essential for determining the ancestral angiosperm condition and the evolution of infratectum ultrastructure in flowering plants.

The majority of early papers described or depicted water lilies, including taxa in both Nymphaeaceae and Cabombaceae, as having an infratectal structure that would now be described as granular (Snigerevskaya, 1955; Ueno and Kitaguchi, 1961; Ueno, 1962; Meyer, 1966; Walker, 1976b). Due, in part, to this characterization, a granular infratectum was commonly hypothesized to be plesiomorphic in angiosperms (Walker, 1976b; Donoghue and Doyle, 1989; Doyle and Donoghue, 1993; Doyle et al., 1994). A granular infratectum was also used to unite Nymphaeales with gymnospermous anthophytes (e.g., Osborn and Taylor, 1995; Osborn, 2000).

Characterization of the infratectum in water lilies as granular, however, was not consistent even in the early literature (e.g., Ueno and Kitaguchi, 1961; Roland, 1965), and more recent investigations have consistently revealed the infratectum in Nymphaeales to be columellate (e.g., Osborn et al., 1991; Gabarayeva and Rowley, 1994; Gabarayeva and El Ghazaly, 1997; Gabarayeva et al., 2001, 2003; Taylor and Osborn, 2006; Remizowa et al., 2008; M. L. Taylor et al., 2008, 2013). Revised interpretation of this character supports the more recent hypothesis that a columellate infratectum is ancestral in angiosperms (Doyle, 2005; Borsch et al., 2008).

Despite the broad reevaluation of this character in early-angiosperm lineages along with multiple developmental studies characterizing the infratectum in core Nymphaeaceae as columellate (Rowley et al., 1992; Gabarayeva and Rowley, 1994; Gabarayeva and El Ghazaly, 1997; Gabarayeva et al., 2001; Taylor et al., 2013), the infratectum continues to be considered "intermediate" or "granular-intermediate" in phylogenetic analyses (Doyle and Endress, 2000, 2014; Borsch et al., 2008), likely due to its enigmatic appearance in mature pollen. Comparative examination of the infratectum in Nymphaeales reveals two distinct types of mature infratectal ultrastructure in mature water lily pollen, and ontogenetic data clearly indicate that both are columellate.

Robust columellae and thick infratectal spaces—Pollen grains of Hydatellaceae, Cabombaceae, and *Barclaya* (Nymphaeaceae) exhibit a thick infratectal space and robust columellae. The infratectal space accounts for nearly half of the total ektexine thickness and have robust columellae (Table 2; Figs. 26, 27), and these taxa are unquestionably columellate.

Misinterpretation of the infratectal elements in Cabombaceae as "granular" by Ueno and Kitaguchi (1961) and Walker (1976b) was likely due to observations of the exine in oblique section, which is apparent in the published images referenced by these authors. In addition, there are ontogenetic characteristics of the exine that may lead to misinterpretation in these genera. In the case of *Brasenia*, developmental studies revealed the presence of an inner, discontinuous layer of the tectum (Taylor and Osborn, 2006) that could be misinterpreted as a partially granular or intermediate infratectum. In *Cabomba aquatica*, Gabarayeva et al. (2003) found that the infratectum became laterally stretched during development, thereby fragmenting the columellae. Such stretching does not occur in *C. caroliniana*, but in this species the columellae do become

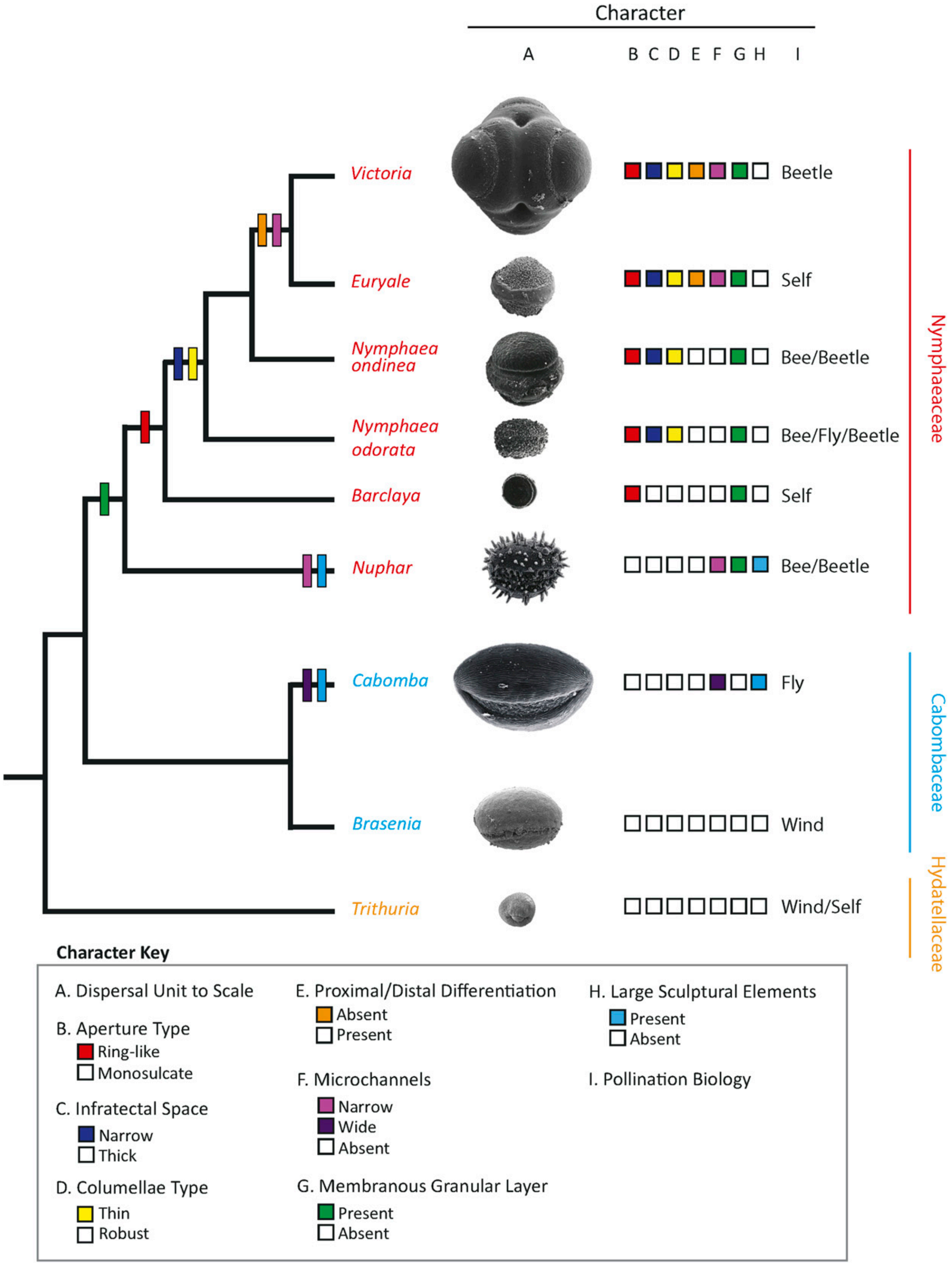


FIGURE 26 Phylogeny of Nymphaeales with pollen structural characters and pollination characters mapped onto the tree. Scanning electron micrographs of whole pollen grains are shown to scale. Phylogeny simplified from Borsch et al. (2008).

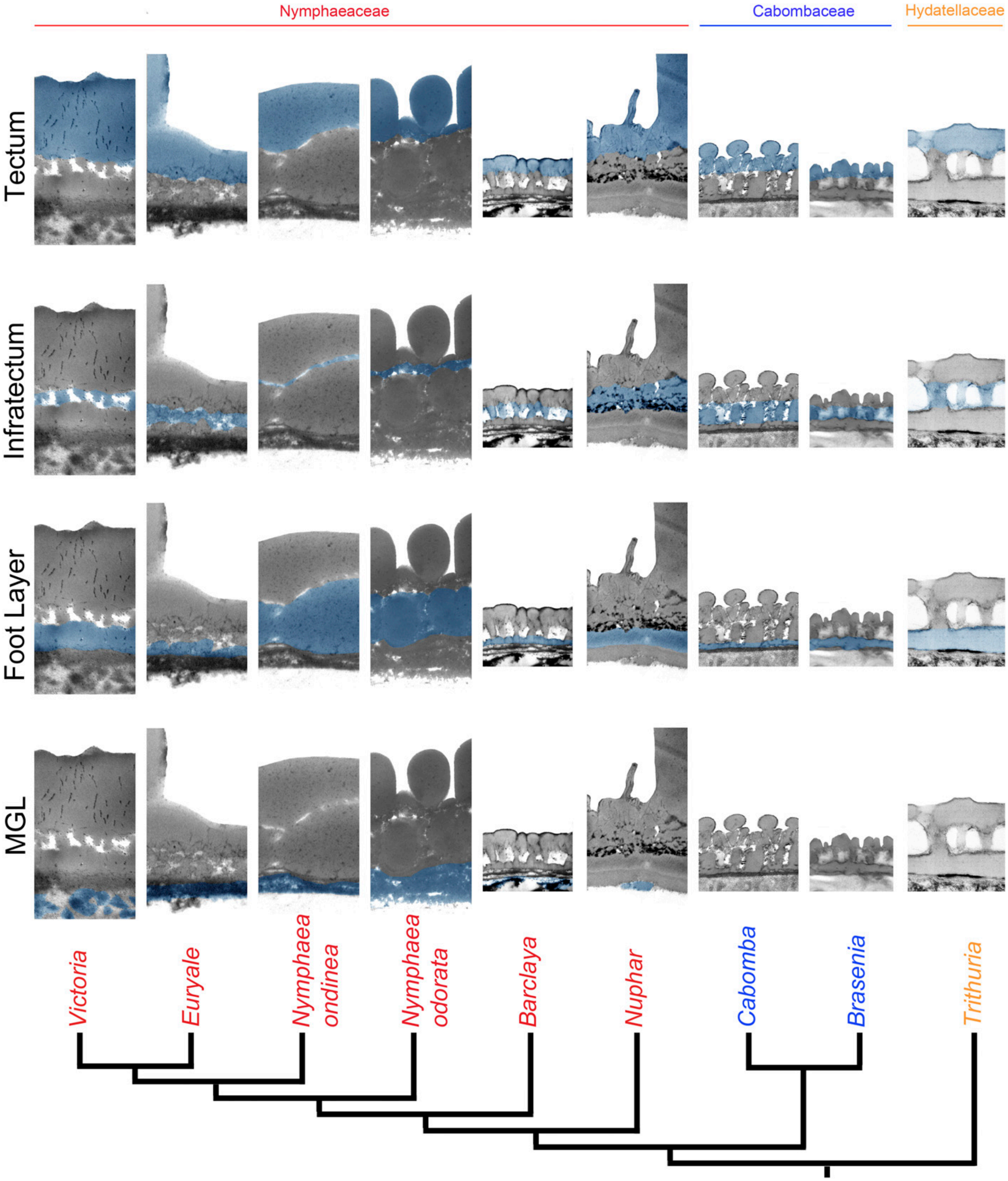


FIGURE 27 Comparative pollen wall ultrastructure in representative taxa for each genus of Nymphaeales. Proximal exine fragments are shown to scale in transmission electron micrographs, with the indicated pollen wall layer (tectum; infratectum; foot layer; MGL, membranous granular layer) colorized in blue and arranged in horizontal rows. Phylogeny simplified from Borsch et al. (2008).

branched and tapered during development (M. L. Taylor et al., 2008), which may also lead to the infratectum appearing to be granular or intermediate.

The infratectum of *Nuphar* is less distinctly columellate, but ontogenetic data support this characterization. The infratectum comprises nearly a quarter of the ektexine thickness in *Nuphar* (Table 2), and the infratectal elements are large, globular, and fill the infratectal space. Developmental investigations reveal the presence of slender, clearly defined columellae early in ontogeny (Takahashi, 1992). Takahashi's (1992) study focused primarily on development of the spines in *Nuphar* and, unfortunately, the published work does not elucidate the developmental steps leading from slender to globose columellae. However, developmental studies of *Victoria,* in which a similar phenomenon occurs, indicate that more significant thickening of the tectum and a narrowing of the infratectal space during sporopollenin deposition leads to short, globular columellae that fill the infratectal space (Taylor et al., 2013).

Thin columellae and narrow infratectal spaces—Several nymphaealean taxa exhibit thin columellae and a narrow infratectal space. Pollen of these taxa has been most commonly described as having a granular or intermediate infratectum. *Victoria* and *Nymphaea* exhibit a narrow infratectal space that comprises less than 10% of the total ektexine thickness (Figs. 26, 27; Table 2). These taxa also exhibit infratectal columellae that are considerably thinner than those of other water lilies.

In *Euryale*, the infratectum accounts for approximately one-third of the ektexine thickness, a larger proportion than in the other taxa with thin columellae and is more similar to *Nuphar* in relative thickness. However, columellae in *Euryale* are thin in overall morphology and are similar to those of *Victoria* and *Nymphaea* (Fig. 27).

The importance of ontogenetic characters in determining the nature of the infratectum in Nymphaeales cannot be overstated. Distinct columellae are apparent during the tetrad stage in every genus for which the exine has been documented at this ontogenetic stage, including both taxa with robust mature columellae (*Brasenia*, *Cabomba*, *Nuphar*, *Trithuria*) and taxa with thin, mature columellae (*Nymphaea*, *Victoria*). In *Nymphaea* and *Victoria*, thickening of the exine (primarily the tectum and/or foot layer; see below) during the free-microspore stage results in a narrowing of the infratectal space, which restricts columellae development and results in mature columellae that are much less robust than those found in other water lilies. There is no evidence for a fundamental change in infratectal element development in water lilies (i.e., from columellae to granules). Instead, elaboration of the tectum and/or foot layer during later developmental stages results in an infratectal ultrastructure in mature pollen grains that is different in appearance.

Ontogenetic data are key to accurately characterizing mature character states. Practically, using ontogenetic data to inform the characterization of mature character states may make this characterization more difficult, but ontogenetic data reveal fundamental similarities that may be masked by modifications that occur late in development, such as the thickening of the tectum in the pollen wall. Differences in the mature state can, and should, be recognized, but as variations of a fundamental type.

Mature exine ultrastructure is consistently and recognizably different in core Nymphaeaceae (Fig. 27), and we understand the inclination to distinguish it from the robust columellate infratectum found in other water lilies. However, we do not agree with interpreting it as "granular," "granular-intermediate," or "intermediate" because infratectal ultrastructure in this group is a variation on the typical columellate type. Moreover, there is no evidence, as of yet, that it is an intermediate step in the evolution of a granular infratectum. If anything, this "thin columellae and narrow infratectal spaces" type of infratectal ultrastructure should be considered to be a novel, derived character state that is potentially unique to Nymphaeales.

Tectum and foot layer ultrastructure—All Nymphaeales exhibit a thick tectum (Fig. 27). In *Victoria* and *Euryale*, the tectum is the thickest layer of the ektexine (Table 2), with the mature tectum in *Victoria* homogenous, without clear discontinuities. In contrast, *Trithuria*, *Brasenia*, and *Barclaya* exhibit a mature tectum comprising discrete, appressed segments. Tectal homogeneity varies in *Nymphaea* (Gabarayeva and Rowley, 1994; Gabarayeva and El Ghazaly, 1997; Gabarayeva et al., 2001; Coiro and Lumaga, 2013; this study).

The foot layer in Nymphaeales is much more variable than the tectum (Fig. 27). This layer is quite thin in *Cabomba*, *Barclaya*, and *Euryale*, whereas in *Nymphaea odorata* it comprises 80% of the total ektexine thickness (Table 2). In *Trithuria*, *Brasenia*, *Nuphar*, *Nymphaea mexicana* (Gabarayeva and El Ghazaly, 1997), *N. capensis* (Gabarayeva et al., 2001), *N. colorata* (Gabarayeva and Rowley, 1994), *N. caerulea* (Coiro and Lumaga, 2013), and *N. ondinea*, the foot layer and the tectum are similar in thickness, both accounting for about half of the total exine thickness (Table 2). Both the tectum and the foot layer undulate at regular intervals in *N. ondinea*, a condition that has not yet been observed in other *Nymphaea* species.

Exine microchannels—Exine microchannels have been shown to facilitate the transport of sporophytic material from the tapetum to the cytoplasm of the developing microspores (Rowley et al., 1987, 2003; Abadie et al., 1988; Carretero and Rodriguez-Garcia, 1995). In *Victoria*, the ontogenetic appearance of microchannels coincides with tapetal degradation, supporting the hypothesis that these channels function in the transport of tapetally derived material. In taxa without microchannels (*Brasenia*, *Barclaya*, *Trithuria*), mature pollen grains exhibit grooves between discontinuous tectal elements, which may serve the same function in the transport of substances through the exine. There are two distinct types of microchannels found in Nymphaeales (Fig. 26).

Narrow microchannels—*Victoria*, *Euryale*, and *Nuphar* each exhibit narrow microchannels that are readily apparent as electron-dense structures dissecting the mature exine (Fig. 26). In *Victoria*, these first become apparent during the early free-microspore stage as electron-dense elements running perpendicular to the microspore plasmalemma. These microchannels are close to each other and clearly run through distinct subunits of the tectum. As the exine thickens, the microchannels become less regularly organized (Taylor et al., 2012).

Although not clearly defined exine microchannels, discontinuities in the tectum were also observed during pollen development in *Nymphaea colorata* and termed microchannels by Gabarayeva and Rowley (1994). Similar tectal discontinuities can be observed in images of the developing exine of *N. capensis* (Gabarayeva et al., 2001), as well as in *Brasenia* (Taylor and Osborn, 2006), *Cabomba aquatica* (Gabarayeva et al., 2003), and *Nuphar* (Takahashi, 1992). In mature grains of *N. colorata* and *N. capensis*, these discontinuities more closely resemble the electron-translucent gaps found between distinct tectal subunits observed in *Brasenia* and *Trithuria*, than the needle-like, electron-dense microchannels of *Victoria*, *Euryale*, and *Nuphar*. In *Nymphaea mexicana* (Gabarayeva and El-Ghazaly, 1997), tectal discontinuities are present early in development, but disappear as the tectum thickens. It is not known whether tectal discontinuities are similarly present in the developing exine of *N. ondinea* because pollen ontogeny in this taxon has not been characterized.

Additional developmental studies in Nymphaeaceae are necessary to provide more information about the distribution of this character and the potential developmental homology of the tectal discontinuities and narrow microchannels. We think it is likely that these structures have different developmental origins, but serve the same function, transporting material through the exine.

Wide microchannels—Cabomba exhibits microchannels that are much wider than the narrow microchannels found in Nymphaeaceae. In *Cabomba caroliniana,* microchannels develop in the exine during the early free-microspore stage (M. L. Taylor et al., 2008) and are filled at maturity with electron-dense material. These channels have been hypothesized to store the copious amounts of pollenkitt, which is produced by the tapetum, and helps to facilitate successful fly pollination in *Cabomba* (Osborn et al., 1991; M. L. Taylor et al., 2008).

Membranous granular layer (MGL)—All members of Nymphaeaceae exhibit an MGL, and the presence of this enigmatic layer is a key pollen synapomorphy for the family (Figs. 26, 27). The MGL is initiated after endexine deposition is complete, and it develops fully before deposition of the intine. The MGL is characterized by small sporopollenin granules that often coalesce into a more homogenous layer as ontogeny proceeds (El-Ghazaly and Huysmans, 2001). This layer exhibits a different mode of deposition and chemical composition than the endexine lamellae, and the MGL is distinguished from the intine by its staining properties and resistance to acetolysis (El-Ghazaly and Huysmans, 2001). The MGL has been observed in a variety of taxa from across the angiosperm phylogeny (see El-Ghazaly and Huysmans, 2001).

Osborn (2000) hypothesized that the MGL is the result of a loss of substructural white-line units of the inner endexine. Subsequently, late-formed sporopollenin, originally of endexinous origin, is unable to aggregate onto the white-line units that have been lost and, instead, forms the granules that make up the MGL (Osborn, 2000). The presence of the MGL in Nymphaeaceae indicates a loss of these white-line units, without concomitant loss of extra endexine material, before the diversification of the Nymphaeaceae crown group (Fig. 26).

This hypothesis may have mechanistic support from the recent explanation for the appearance of white lines in the center of each endexine lamella. The white line has been suggested to represent the gap between bilayers in laminate micelles that form during self-assembly of the exine (Hemsley and Gabarayeva, 2007; Gabarayeva, 2014; Gabarayeva and Grigorjeva, 2014). The absence of white lines indicates that the endexine is instead formed on the base of a different type of self-assembly structure, such as spherical micelles (Gabarayeva et al., 2011). Developmental changes in the chemical composition of the periplasmic space, where exine development occurs, result in the formation of these different micellar mesophases (Gabarayeva, 2014). The appearance of the MGL following lamellar endexine formation may signal such a developmental change that results in the deposition of endexinous material onto a different type of substructure.

The presence of an MGL is likely a derived trait that has arisen once in Nymphaeaceae and multiple times in angiosperms. Taxa from across the angiosperm phylogeny exhibit an MGL-like layer, including *Betula* (El-Ghazaly and Huysmans, 2001), *Cucurbita* (Ciampolini et al., 1993), *Nelumbo* (Kreunen and Osborn, 1999), and *Zea* (Skvarla and Larson, 1966). However, there are few data regarding the phylogenetic distribution of the MGL or variation in its ultrastructure. Consistent recognition of the MGL will be necessary to elucidate both its developmental and evolutionary origins.

Tapetum type and orbicules—Tapetum type is labile in Nymphaeales (Table 1). *Brasenia schreberi* (Taylor and Osborn, 2006), *Cabomba aquatica* (Gabarayeva et al., 2003), *Nuphar pumila* DC. (Zhou and Fu, 2008), *Nymphaea capensis* (Gabarayeva et al., 2001), and *Nymphaea alba* (as *Castalia alba* Schnarf, 1931) each exhibit a secretory tapetum. *Victoria* also exhibits a secretory tapetum, with tapetal cells that never detach from the anther wall (Taylor et al., 2012). However, tapetal cells in *Victoria* exhibit a high degree of separation along their radial walls and become elongated, extending into the locule farther than the tapetal cells in other water lily taxa with secretory tapeta (Taylor et al., 2012). These modifications would be expected to occur in the transition to an invasive tapetum, which, in Nymphaeaceae, is exhibited by at least two species of *Nymphaea, N. colorata* and *N. mexicana.*

Both *Nymphaea colorata* and *N. mexicana* exhibit a "cyclic" invasive tapetum, in which tapetal cells physically migrate into the locular space and then later retreat back to the anther wall multiples times during ontogeny (Rowley et al., 1992; Gabarayeva and El-Ghazaly, 1997). This tapetum type has, itself, been hypothesized to be an intermediate step between the secretory and the noncyclic invasive tapetum types (Tiwari and Gunning, 1986; Furness and Rudall, 2001). In Cabombaceae, *Cabomba caroliniana* also exhibits tapetal cells that migrate into the locule, but retain many characteristics of a secretory tapetum (M. L. Taylor et al., 2008). Invasive tapeta likely evolved multiple times in Nymphaeales: once in *Cabomba*, once in *Nymphaea* subg. *Nymphaea*, and once in *Nymphaea* subg. *Brachyceras*.

The majority of taxa in Nymphaeales for which the tapetum has been studied exhibit orbicules, including *Brasenia schreberi* (Taylor and Osborn, 2006), *Cabomba caroliniana* (M. L. Taylor et al., 2008), *Victoria* (Taylor et al., 2012, 2013), *Nymphaea colorata* (Rowley et al., 1992), and *Nymphaea mexicana* (Gabarayeva and El-Ghazaly, 1997). The presence or absence of orbicules is not reported in *Cabomba aquatica*, *Nuphar pumila*, or *Nymphaea alba*. Orbicules are almost exclusively associated with secretory tapeta (Furness and Rudall, 2001), although exceptions do exist (Lombardo and Carraro, 1976; Galati et al., 2007). The presence of orbicules in water lilies with invasive tapeta may indicate that the secretory function is not completely lost in those species.

Tapetum diversity in early-divergent lineages of angiosperms is potentially greatly underestimated. Many of the reports of tapetum type in these taxa date back to large surveys that only used light microscopy (e.g., Clausen, 1927; Schnarf, 1931; Wunderlich, 1954; see Furness and Rudall, 2001 for additional references). Even in more recent studies, tapetum type is typically categorized into one of a few "types," with ontogeny rarely described in detail or documented in published images, making it difficult to identify intermediate or transitional types of tapetum development. Moreover, the tapetum is challenging to characterize, due to the difficulty of quantifying the timing of ontogenetic events or the degree of cell separation and locular intrusion. Greater attention to this critical tissue layer may yield informative data regarding the evolution of anther development and its interplay with pollen ontogeny.

CONCLUSION

Nymphaeales exhibit diversity in key pollen characters, including dispersal unit size, ornamentation, aperture morphology, and tapetum type. Some of this diversity, such as in dispersal unit size and the presence of large sculptural elements, is associated with pollination biology or breeding system. In contrast, the source, or potential functionality, of variation in traits such as microsurface ornamentation is not understood. Variation in pollen characters within Nymphaeales indicates that significant potential for lability in pollen development was present in the water lily lineage at the time of its divergence from the rest of angiosperms. Furthermore, the apparent rarity of traits such as a cyclic-invasive tapetum and the ring-like aperture in more derived angiosperm lineages indicates that these may have been the result of "evolutionary experimentation" that occurred relatively early in angiosperm history but did not underlie significant diversification, as is hypothesized for the transition to tricolpate pollen in eudicots (Furness and Rudall, 2004).

Comparative structural and developmental data are essential for interpreting pollen characters in Nymphaeales and, thus, for understanding the evolutionary transitions that have occurred in water lily pollen. For example, such a comparative approach that integrates ontogenetic data is essential in unequivocally supporting the interpretation that all water lilies have a columellate infratectum and exhibit endexine lamellae. The importance of high-resolution structural and ontogenetic data underscores the need for continued investigation of mature pollen and pollen developmental traits using modern microscopical methods and other tools.

ACKNOWLEDGEMENTS

We thank Ben and Kit Knotts (Cocoa Beach, Florida, USA) for access to their private ponds, assistance with field collection, and sharing unpublished information about *Euryale* pollen. We also thank J. Dunlap and the University of Tennessee (Knoxville) Advanced Microscopy and Imaging Center. We extend special thanks to James A. Doyle and an anonymous reviewer for helpful comments on this manuscript. This study was supported in part by the National Science Foundation (grants IBN-0212521 and MRI-0216391 to J.M.O. and Doctoral Dissertation Improvement Grant DEB-0910171 to M.L.T.), Creighton University, and The College of New Jersey.

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APPENDIX 1 Voucher specimen information.

Taxon, locality, *Voucher specimen*, (Herbarium).

Barclaya motleyi Hook.f, Johore, Malaysia, *Schneider 803* (SBBG); ***Brasenia schreberi*** J.F. Gmel, Missouri, *M. Taylor 57* (NEMO), ***Trithuria austinensis*** D.D.Sokoloff, Remizowa, T.D.Macfarl. & Rudall, Western Australia, *M. Taylor 64* (TENN); ***Trithuria australis*** (Diels) D.D.Sokoloff, Remizowa, T.D.Macfarl. & Rudall, Western Australia, *M. Taylor 62* (TENN); ***Trithuria submersa*** Hook.f., Western Australia, *M. Taylor 61* (TENN).