



Comparative spore wall architecture and development across hornwort diversity and across time

Karen S. Renzaglia¹ · Barbara Crandall-Stotler¹ · Wilson A. Taylor² · Amelia Merced³ · Scott Schuette⁴ · Juan Carlos Villarreal⁵ · D. Christine Cargill⁶

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Abstract

Hornworts are a small group of plants exclusively characterized by asynchronous sporogenesis and a sporangium that grows from its base. Among the most widely used and reliable features that delineate hornwort taxa are those related to spores, especially shape, color and spore wall ornamentation. The present study provides comparative data on the development and microanatomy of spores from all genera, representing the range of hornwort diversity. A key finding is that aspects of hornwort spores and sporogenesis are conserved across taxa (except the derived *Dendroceros*) and differentiate hornworts from other bryophytes. With isolated exceptions, spores are tetrahedral with a flattened proximal face and trilete mark or ridge that overlies an aperture with reduced sporopollenin. Monoplastidic meiosis is exhibited by all taxa and spore maturation occurs within the spore mother cell wall. The spore wall is typically three-layered (two exine layers with sporopollenin and one intine layer). A band of outer exine is laid down first, usually on tripartite lamellae, and this layer delineates the sculpto-derm, regardless of the external topography. An inner exine forms next and contains granular to globular sporopollenin that is progressively deposited as the spore matures. The intine varies in thickness and forms late in development, followed by deposition of a thin fourth layer of remnant spore mother cell wall on the spore surface. Ultrastructural and developmental deviations associated with modified hornwort spores are identified in taxa such as *Anthoceros fuciformis* (round spores) and *Dendroceros* (multicellular spores). Although sparse, the fossil record provides insight on the evolution of hornwort spore characteristics and the occurrence of hornwort spores in the Cretaceous and beyond.

Keywords Fossil spores · Hornworts · Spore wall · Sporogenesis · Sporopollenin

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✉ Karen S. Renzaglia
renzaglia@siu.edu

- ¹ School of Biological Sciences, Southern Illinois University Carbondale, Carbondale, IL 62901-6509, USA
- ² Department of Biology, University of Wisconsin-Eau Claire, Eau Claire, WI 54701, USA
- ³ Department of Biology, University of Puerto Rico Mayagüez, Call Box 9000, Mayagüez, Puerto Rico 00681-9000, USA
- ⁴ Western Pennsylvania Conservancy, Pennsylvania Natural Heritage Program, 800 Waterfront Drive, Pittsburgh, PA 15222, USA
- ⁵ Département de Biologie, Université Laval, 1030 Avenue de La Médecine, Québec, QC G1V 0A6, Canada
- ⁶ Centre for Australian National Biodiversity Research (a Joint Venture Between Parks Australia and CSIRO), Australian National Herbarium, GPO Box 1700, Canberra ACT 2601, Australia

Introduction

As a key innovation that was instrumental in the diversification of early land plants, spores are uniquely structured for efficient dispersal and for survival during adversity. These adaptive functions may be attributed to the fact that spores are surrounded by a specialized wall that is impregnated with sporopollenin, a chemically complex biopolymer that is found in the walls of all land plant spores and pollen (Suh and Ashton 2022). It has been suggested that the transmigration of green algae to land was not possible until a sporopollenin-containing wall evolved (Wellman 2004). As one of the most resistant organic substances (Grienenberger and Quilichini 2021), sporopollenin not only protects the spore from desiccation, ultraviolet radiation and mechanical damage, but this recalcitrant polymer has also persisted through millions of years of geological change. Not only do fossil spores provide the most complete and widespread evidence

of land plant colonization and subsequent conquest of the land surface, but they also are essential calibration points for inferring character transformation, biodiversification and historical biogeographic events (Wellman 2004; Blackmore 2007; Villarreal and Renner 2012; Peñaloza-Bojacá et al. 2025).

A consensus from modern phylogenetic studies is that bryophytes are monophyletic, with hornworts sister to a moss plus liverwort clade designated as setaphytes (Renzaglia and Garbary 2001; Renzaglia et al. 2018; Harris et al. 2022; Bechteler et al. 2023). Among hornworts, spore surface ornamentation, size and color are widely utilized in taxonomic delineation of species (Cargill et al. 2005, 2022; Cargill and Palsson 2021; Renzaglia and Crandall-Stotler 2026). As in other plants, the development of spore walls in the few hornwort taxa that have been examined involves the precise deposition of wall layers, including the ornate sporopollenin-containing exine (Renzaglia and Vaughn 2000; Renzaglia et al. 2009). Reviews on sporogenesis in bryophytes by Brown and Lemmon (1988, 1990a) provide general information on hornwort sporogenesis but highlight the limited data available for the group.

The present study was designed to clarify the ultrastructure, development and variability of spore wall features across hornwort diversity. The objectives of the study are to compare spore ontogeny and in particular the development and structure of the sculptoderm across hornworts and with other bryophytes, and to evaluate internal microstructure vis-à-vis surface ornamentation and spore variability. Perusal of the earliest fossil spores provides insight as to the origin and diversity of architectural features shared with hornwort spores.

Materials and methods

Species and Specimens Examined

Twenty-five species, representing the phylogenetic diversity of hornworts, were studied using a combination of light, fluorescence, differential interference contrast (DIC), scanning electron (SEM) and transmission electron (TEM) microscopy. Specimens examined include the following: *Leiosporoceros dussii*: PANAMA, Villarreal and Araújo 651, Araújo et al. 791, Villarreal 803a (PMA); *Anthoceros fuciformis* (= *Folioceros fuciformis*): AUSTRALIA, A. Cairns s.n. (CANB); *Anthoceros agrestis*: USA; ILLINOIS, Renzaglia 3004, 4146 (KSR), CALIFORNIA, Doyle 11342 (KSR); *Anthoceros fusiformis*: USA; OREGON, LONG 104 (SIUC), CALIFORNIA, Doyle s.n. (KSR); *Anthoceros macounii*: CANADA, Faubert s.n., (KSR); *Anthoceros punctatus*: PORTUGAL, Sérgio s.n. (CONN), USA; CALIFORNIA, Doyle s.n. (KSR); *Anthoceros laminifer*: NEW ZEALAND, Duckett

s.n. (KSR); *Anthoceros adscendens*: USA; MISSISSIPPI, Vaughn s.n. (KSR); *Paraphymatoceros pearsonii*: USA; CALIFORNIA, Doyle 11325 (KSR); OREGON, Long 107 (KSR); *Paraphymatoceros hallii*: USA; CALIFORNIA, Doyle 11363 (KSR); *Notothylas breutelii*: USA, LOUISIANA, Renzaglia 9902 (KSR); *Notothylas orbicularis*: USA; ILLINOIS, Long 101, 102, 105, Renzaglia 466 (KSR); *Notothylas javanicus*: JAPAN, Hasegawa s.n. (KSR); Villarreal 806 (PMA); *Phaeoceros laevis*: PORTUGAL, Sérgio s.n. (CONN); ITALY, Ligrone s.n. (KSR); *Phaeoceros carolinianus*: USA; ILLINOIS, Renzaglia 4139 (KSR); *Phymatoceros bulbiculosus*: PORTUGAL, Sérgio s.n. (CANB); *Phymatoceros phymatodes*: USA CALIFORNIA, Doyle 11338, 11353 (CANB); *Phaeomegaceros fimbriatus*: PANAMA, Villarreal 634, 799, Villarreal and Araújo 717 (PMA); *Phaeomegaceros hirticalyx*: NEW ZEALAND, Duckett s.n. (KSR); *Phaeomegaceros coriaceus*: NEW ZEALAND, Duckett s.n. (KSR); *Dendroceros crispatus*: AUSTRALIA, Cargill 28 (CANB); *Dendroceros granulatus*: NEW ZEALAND, Ligrone and Duckett s.n. (KSR); *Megaceros gracilis*: AUSTRALIA, NEW SOUTH WALES, Brown 338 (CANB); QUEENSLAND, Cargill 14 (CANB); *Nothoceros giganteus*: NEW ZEALAND, Duckett s.n. (KSR); *Nothoceros vincentianus*: PANAMA, Villarreal 207, 259 (PMA).

Microscopy

Scanning Electron Microscopy (SEM)

Sporophytes were excised and fixed in a 2.5% glutaraldehyde solution buffered with 0.05 M NaPO₄ pH 7.2 for 2 h at room temperature then refrigerated overnight. Specimens were rinsed in the buffer and post-fixed for 2 h in 2% OsO₄ buffered with 0.05 M NaPO₄ pH 7.2 followed by a graded ethanol dehydration series (25–50–75–85–100%). Following critical point drying, the specimens were mounted on stubs, gently opened to release spores and sputter-coated with gold palladium. Images were digitally captured on a Hitachi S570 SEM, with the exception of Fig. 1s, t whereby the spores were air dried, removed from the sporophyte, placed on double-sided sticky tape, gold-coated and images captured on a Joel JSM 6400 SEM.

Transmission Electron Microscopy (TEM)

Sporophytes were excised, cut into less than 1 cm pieces, fixed in 2.5–4% v/v glutaraldehyde in 0.05 M Pipes pH 7.4 or 0.05 M NaPO₄ pH 7.2 for 1–2 h at room temperature, stored overnight at 4 °C, and post-fixed in 2% (w/v) OsO₄ (1–2 h). Specimens were dehydrated 10–30 min each in a graded ethanol series (25–50–75–85–100%), followed by two rinses in 1:1 ethanol/propylene oxide solution and two rinses in 100% propylene oxide. Sporophytes were exchanged in increasing amounts of Spurr's resin over the

Fig. 1 Spore morphology across hornwort diversity. Phylogenetic tree based on comprehensive molecular analyses by Peñaloza-Bojacá et al. (2025, Fig. 1). SEM pairs show proximal face (left column) and distal face (right column) of spores. **a, b** *Leiosporoceros dussii*. Elongated smooth spore showing monolete mark and flattened lower side of (a). **c, d** *Anthoceros fuciformis* (= *Folioceros fuciformis*). Rounded spore with indistinct trilete mark. **e, f** *Anthoceros punctatus*. Distal face with coalescing spines, proximal face with fovea. **g, h** *Notothylas orbicularis*. Distal face with vermiculated ornamentation, punctuated by several irregular shaped tubercles, and flattened proximal face. **i, j** *Paraphymatoceros hallii*. Distal face with ellipsoid, rounded or ovoid protuberances, proximal face with a ring of verrucae in each facet. **k, l** *Phaeoceros carolinianus*. Distal face typically covered with short spines. **m, n** *Phymatoceros phymatodes*. Distal face partially covered by a thick SMC wall in (n). **o, p** *Phaeomegaceros fimbriatus*. A distinct triradiate mark on the proximal face, and six dimples on the distal face. **q, r** *Nothoceros vincentianus*. Distal face with a central mamilla surrounded by irregular verrucae and wart-like tubercles, and a thin trilete mark on the proximal face. **s, t** *Megaceros gracilis*. Central mamilla surrounded by irregular small verrucae and wart-like tubercles on the distal face, a thin trilete mark and verrucae on the proximal face. **u, v** *Dendroceros granulatus*. Distal surface finely papillate with a thin trilete mark on the proximal face. Bars = 10 mm

course of several days before embedding in Beem capsules and thermal cured in a 65 °C oven. Thick sections were cut, collected on slides and stained with 1% aqueous toluidine blue sprinkled with sodium borate for light microscope observations and photography. Thin Sects. (90–100 nm) were collected on 200 mesh nickel grids and post-stained with uranyl acetate (3 min) and Reynolds lead citrate (30 s). Samples were viewed, and micrographs were digitally captured on a Hitachi H7650 TEM.

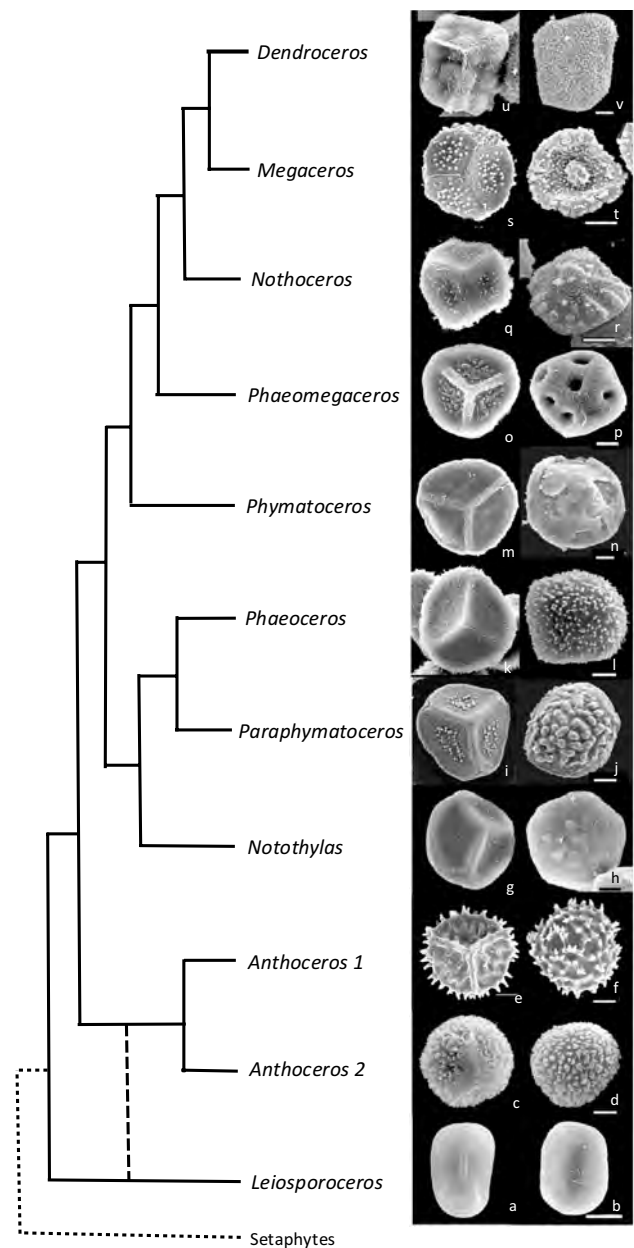
DIC and fluorescence microscopy

Sporophytes from live specimens were excised and sliced longitudinally to release spore mother cells and spores. Digital images were captured using autofluorescence, DIC and brightfield microscopy on a Leica DM5000 B compound microscope.

Results

Diversity of spore wall ornamentation

Evolution of spores in hornworts has involved modifications of the sporoderm architecture, with trends evident across diversity (Fig. 1). Most hornwort spores are highly polar with the distal surfaces typically more ornate than the proximal surfaces. Distal ornamentation is species-dependent and varies from smooth (Fig. 1b) to spinose/papillose (Figs. 1f, l, v), bacculate (Fig. 1d), tuberculate (Figs. 1r, t) and verrucate (Fig. 1j). The smooth spores of *Leiosporoceros* (Figs. 1a, b) are markedly different in shape because they develop in isobilateral tetrads with two spores appressed against each other



on opposite ends of the tetrad. Consequently, the spores are slightly elongated to bean-shaped with a proximal side distinguished by a monolete mark in the center and one flattened end (Fig. 1a). Considerable diversity in shape and surface ornamentation characterizes *Anthoceros* spores that are highly ornamented and bear coarse elements consisting of cones, spines, and warts (verrucae), some organized to form a loose reticulum. They lack a clear equatorial girdle and typically have prominent triradiate ridges across the proximal surfaces (Fig. 1e). The rounded spores of *A. adscendens* and *A. fuciformis* differ in that there are no flattened proximal surfaces and the trilete mark is poorly defined (Figs. 1c, d). The flattened proximal faces of many taxa in the Notothyladaceae [*Notothylas* (Fig. 1g), *Paraphymatoceros* (Fig. 1i),

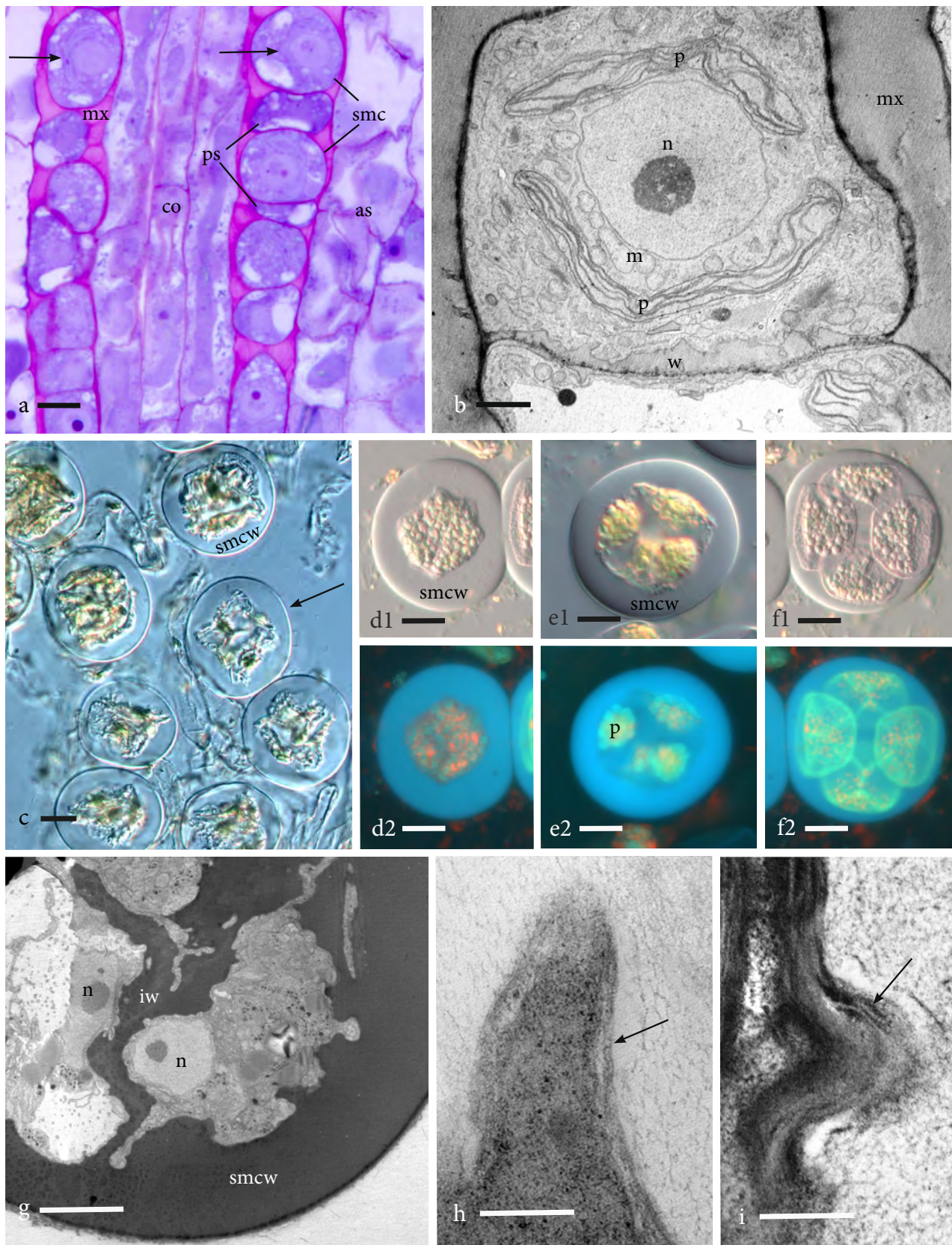


Fig. 2 Sporogenesis. **a–g** *Phaeoceros carolinianus*. **a** Longitudinal section of the sporophyte base directly above the meristem showing differentiation of spore mother cells (smc) and pseudoelaters (ps) in a matrix (mx) on either side of a central columella (co) and surrounded by assimilative tissue (as); smc with a single plastid at arrow. **b** TEM of a young spore mother cell in the matrix (mx) after the first plastid division, with thickening wall (w), a large central nucleus (n), numerous mitochondria (m) and two large plastids (p). **c** Differential interference contrast (DIC) image of rounded SMCs surrounded by a thick wall (smcw). Arrow points to an internally lobed SMC with quadripolar spindle. **d–f** Pairs of images of the same cells in DIC (1) and autofluorescence (2). **d** Four starch-filled plastids in an internally lobed SMC surrounded by a thick round wall (smcw). **e** SMC surrounded by thick round wall (smcw) with four large plastids (p) at the poles in anticipation of meiosis. **f** Newly formed tetrahedral spores with single starch-filled plastids in the round SMC wall. Spore wall ornamentation is beginning to form. **g** Irregularly outlined spores, immediately following meiosis, separated by an intersporal wall (iw) and surrounded by a thick and dense SMC wall (smcw). No wall has assembled yet in the nascent spores that contain prominent nuclei (n). **h** *Anthoceros agrestis*. Cytoplasmic projection in nascent spore with tripartite lamella (arrow) forming outside the plasmalemma; smcw, spore mother cell wall. **i** *Notothylas orbicularis*. Irregular tripartite lamellae (arrow) in early outer exine development; smcw, spore mother cell wall. Bars: **a, c, d–f**=20 µm; **b**=2.0 µm; **g, h**=5 µm; **i**=250 nm

and *Phaeoceros* (Fig. 1k)] and in *Phymatoceros* (Fig. 1m) have a finely vermiculate micro-ornamentation, with interspersed macro-ornamentation of variable size. These taxa have prominent trilete marks or projecting triradiate ridges (Figs. 1g, i, m) and well-defined equatorial girdles (also referred to as pseudosutures) (Figs. 1g–m). In comparison, *Phaeomegaceros* spores have larger vermiculae and prominent depressions (fovea) on the distal surface (Fig. 1p). A triradiate ridge and clear equatorial girdle are well developed on these spores (Fig. 1o). Spores of the tropical to subantarctic Dendrocerotaceae are green, and bear tubercles or papillae distally and vermiculae proximally with a clear trilete mark but no equatorial girdle nor triradiate ridge (Figs. 1q–v). *Megaceros* and *Nothoceros* spores are small (20–45 µm compared to > 40 µm for all other genera but *Leiosporoceros*) and morphologically similar, often with a prominent central mamilla on the distal surface surrounded by irregular verrucae (Figs. 1r, t). *Dendroceros* spores are irregularly shaped and contain numerous cells inside of a thin ornate sculptoderm (Figs. 1u, v).

Development

Sporogenesis can be followed from the basal meristem upward toward the tip of the sporophyte. Early development is relatively uniform across hornworts and is illustrated in Figures 2 and 3. The first stages of sporogenesis occur directly above the basal meristem within the newly differentiated sporogenous tissue, which encircles a central columella and is surrounded by an outer zone of photosynthetic

assimilative tissue (Fig. 2a). Directly above the meristem, spore mother cells (SMCs) begin to round out and separate from alternating pseudoelaters, both of which are bathed in a matrix within which they differentiate (Figs. 2a, b). Typically, once separated and completely rounded, the SMCs deposit thickened walls (Figs. 2c–e); however, only modest wall thickenings are deposited in the SMCs of *Leiosporoceros* (Figs. 3a–c). Sporogenesis is monoplastidic and involves two rounds of divisions of the single large plastid (Figs. 2a, 3a) to produce two (Figs. 2b, 3b) then four prominent plastids that surround the central nucleus (Figs. 2d, e, 3c). Plastids are filled with thylakoids (and often starch) but lack a defined pyrenoid at this stage. A quadripolar meiotic spindle forms with the four plastids forming the focal points of the spindles (Figs. 2c–e, 3c). Lobing of the SMC is evident internally (Fig. 2c) in most taxa and externally as meiosis ensues in *Leiosporoceros* (Fig. 3c).

Newly formed spores remain in tetrads, which are enclosed in the round, typically thick SMC wall and separated by an irregular intersporal wall that forms during cytokinesis. At this stage, each spore of the tetrad is delineated by the plasmalemma (Fig. 2g) that lacks rigidity. Spore wall development begins with the deposition of tripartite lamellae just to the outside of the plasmalemma (Figs. 2f, h, i). In some taxa such as *Leiosporoceros*, the outermost spore wall layer consists of a multilamellate layer with neatly arranged layers of tripartite lamellae (Figs. 3d, e). In other taxa such as *Notothylas orbicularis*, this outer layer contains irregularly arranged slips of lamellae (Fig. 2i). In all cases that we have examined, except *Dendroceros*, this first-formed layer is a rigid exine layer (exine 1) that is built from tripartite lamellae in the nascent spore and is immediately impregnated with sporopollenin. Cytoplasmic projections are visible at this stage, which presumably are instrumental in producing the protrusions that characterize the ornamentation of the taxon as the outer exine is laid down (Figs. 2h, i).

Spore wall development occurs entirely within the confines of the round spore mother cell wall and spores fill the space as they develop (Fig. 4). Exine 1 is visible in early differentiation, forming a continuous band around the spore that determines the ornamentation of the spore wall (Figs. 4a–c). Wall development proceeds with the deposition of a second thick exine layer (exine 2) to the inside of exine 1 (Figs. 4b, c, e). As is evident in figure 4d, the spore wall ornamentation is fully developed on all spore surfaces while spores are still surrounded by the SMC wall; the distal ornamentation is imprinted in the SMC wall and the proximal ornamentation in the intersporal wall.

After thickening on both distal and proximal surfaces, the inner exine is progressively filled with granular sporopollenin in most taxa (Fig. 4e), or globular sporopollenin in *Anthoceros* (see below). In the mature exine, the exine

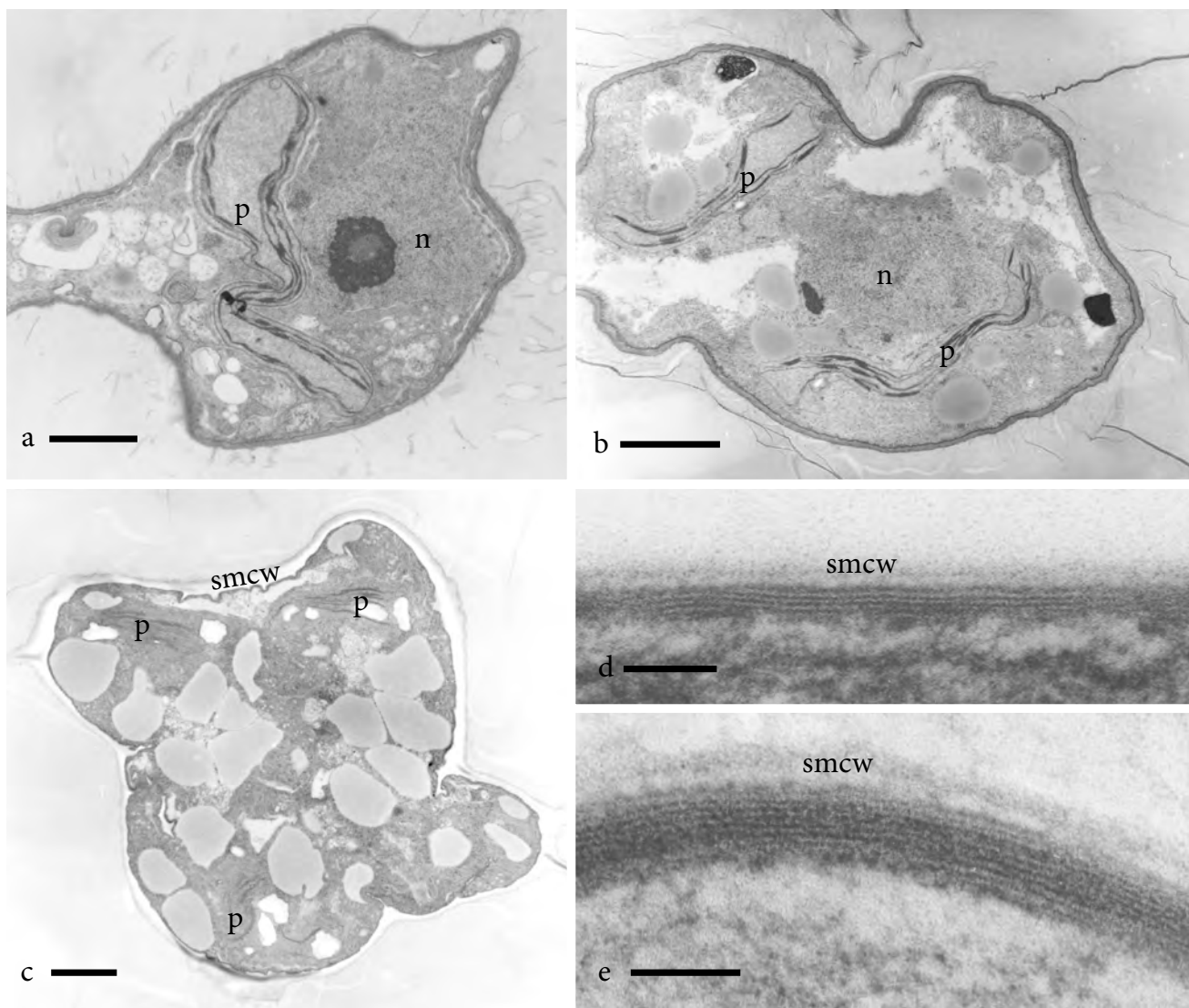


Fig. 3 TEM images of early sporogenesis in *Leiosporoceros dussii*. **a–c** Spore mother cells. **a** Single plastid (p) and large nucleus (n). **b** Two plastids (p) on either side of the nucleus (n). **c** Four plastids (p) (only three are visible) in each of four lobes of the SMC that has a slightly thickened wall (smcw). **d, e** Tripartite lamellae in newly

formed spore are progressively deposited from the plasmalemma into the SMC wall (smcw), forming a multilamellate band that comprises the outer exine layer. Subsequent sporopollenin deposition obscures the lamellae. Bars: **a–c** = 2.0 μm ; **d, e** = 0.5 μm

granules and globules become tightly packed and homogeneous, obscuring the substructure that is distinct during development. Brown and Lemmon (1990a) refer to the fully expanded inner exine before sporopollenin deposition as a primexine or spore special wall, terms that we feel complicate the process and suggest unrelated parallels with pollen development. An innermost intine or endospore is produced to the inside of the exine during sporopollenin deposition (Fig. 4e). In the final stages of spore maturation, toward the sporophyte apex where dehiscence occurs, the spore mother cell wall and intersporal walls dry down on the spore surface usually leaving a dense layer that we refer to as a pseudoperrine because it is not of spore or tapetal origin (Figs. 4f, g)

The development of *Dendoceros* spore walls differs from other hornworts in that the outer exine (exine 1) is produced directly from the plasmalemma as globules of sporopollenin without the involvement of TPL (Fig. 5a). The sporopollenin globules increase in size and number and coalesce to form an undulating exine 1 layer (Fig. 5b). The major papillose ornamentation is associated with cytoplasmic projections over which sporopollenin accumulates (Fig. 5b). The exine 2 layer forms and occupies the space between folds in exine 1 (Fig. 5c). Sparse sporopollenin is found in the exine 2 layer, making it difficult to distinguish from the intine. The three-layered spore wall is fully developed in the single-celled spore, prior to endosporic germination (Fig. 5c). Because

of its highly undulating contour, it is stretchable and can accommodate increases in spore size as mitosis produces more cells that remain enclosed in the spore wall (c.f., Figs. 5c and 6e).

Ultrastructural diversity

Because we examined *in situ* spores near the apex of dehiscing sporophytes, the spores illustrated in Figures 6, 7 and 8 were not dispersed and may not be fully mature. Spore release in hornworts is contingent upon the growing season with variations in temperature and moisture, and consequently spores can be released before the final stages of maturation. Spores in sporophytes range from containing numerous organelles (Figs. 6a, c) to being variously engorged with food reserves including oils, protein and starch in plastids (Figs. 6b, d, e). Plastid variability is immense with yellow-to black-spored taxa having obscure pleomorphic plastids that are nearly devoid of thylakoids (Figs. 6a–c). In green spores, the plastids are large, full of thylakoids (Figs. 6e, d) and as in *Nothoceros*, often starch-laden (Fig. 6d). Ultrastructural features that are associated with surface ornamentation of spores include similar substructure at the aperture and equatorial girdle in *Notothylas* (Fig. 6a), rounded and poorly defined proximal face of *A. fuciformis* (Fig. 6b), a prominent aperture along the triradiate ridge, equatorial girdle differentiation and large distal depressions or fovea in *Phaeomegaceros*, and thin walls with poorly developed aperture and no equatorial girdle in *Nothoceros* (Fig. 6d). The spores of *Dendroceros* undergo precocious, endosporic germination, and are multicellular when dispersed. Each multicellular spore is surrounded by a thin exine and an intine that is continuous with the primary walls of the internal cells (Fig. 6e). Each of the internal cells contains a large star-shaped plastid with a central pyrenoid (Fig. 6e).

Spore wall ultrastructure

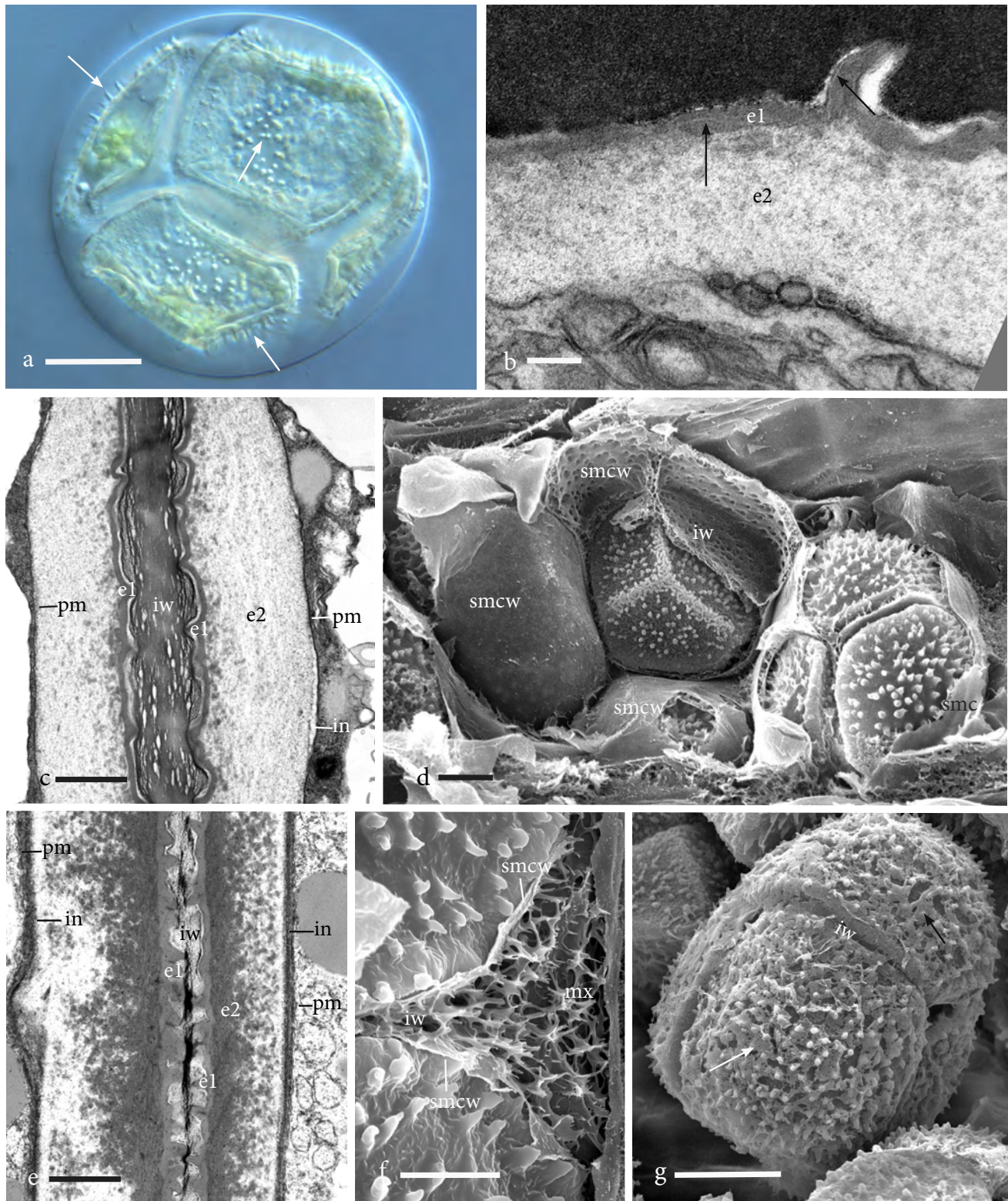
The diversity of spore wall ultrastructure in mature or nearly mature spores contained in sporophytes is illustrated in figure 7, showing distal wall substructure, and figure 8, showing proximal wall and aperture substructure. The majority of hornwort spores have a three-layered spore wall as described above (two exine layers and an intine) but some variability does exist. The outer exine (exine 1) is a continuous band of sporopollenin on the outside of the spore wall that forms a rigid and contoured surface lamina responsible for the taxon-specific ornamentation. This layer is variable in thickness, from comprising most of the spore wall in *Leiosporoceros* (Fig. 7a) to being a thin outer border as seen in spores of *Anthoceros* (Figs. 7b, c) and *Phaeoceros* (Fig. 7e). In *Anthoceros*, development of the inner exine is characterized by progressive deposition of rounded globules (Figs. 7b, 8a, b),

as opposed to the granular sporopollenin of other taxa, e.g. figures 7g and i. All hornwort spores are surrounded by the spore mother cell wall (Figs. 6a, 7a–e, g–i), that dries down on the exine forming a layer of dark material, the pseudoperine, late in development (Fig. 7f). There is no evident correspondence between the internal ultrastructure of the spore wall and its external ornamentation, as demonstrated by a comparison of the internal structure with the surface features seen in the SEM insets for each taxon.

Variations on this basic architecture are seen in *A. fuciformis* that has a spore wall that is essentially composed of an outer exine and intine separated by an obscure very thin inner exine (Fig. 7d). In the tropical or semi-aquatic *Leiosporoceros*, *Nothoceros* and *Megaceros* (Figs. 7a, j, k), the inner exine does not completely fill with sporopollenin and may even appear to be two-layered (Fig. 7j). The inner exine in spores of *Dendroceros* is thin, contains sparse sporopollenin and occupies spaces that project into the outer exine ornamentation (Figs. 5c, 7l). As these spores germinate and increase in cell number, they expand in size, stretching the undulating exine layer and filling spaces in the sporophyte. This process modifies the surface ornamentation by widening the space between papillae (cf., Figs. 5c and 7l).

Differences in density of layers is in part due to maturity level as seen in the more mature *A. macounii* spore that is full of lipids and has a homogeneous exine with obscured exine 1 and 2 layers, a dense intine and a clear pseudoperine forming (Fig. 7c). The spore of *Paraphymatoceros* is even further along in development as evidenced by the lack of differentiation between the two exine layers and the pseudoperine that is completely fused to the spore surface (Fig. 7f).

The same layering is found in the proximal spore surface (Fig. 8) with some variability in thickness compared with the distal wall (cf. Figures 7b and 8a in *Anthoceros*). Surface ornamentation is determined and bordered by the outer exine (Fig. 8a, b). Spores in tetrads are separated by the intersporal wall (Fig. 8a–c). Each spore typically has three flattened triangular proximal surfaces (Fig. 1) marked by a modified wall area or aperture (proximal suture) along the trilete mark where the four spores meet (Fig. 8d–i). The aperture is a weak area in the spore wall that is characterized by modified wall layering where sporopollenin deposition is highly reduced, especially in exine 2. The simplest aperture is found in *Leiosporoceros* along the monolete mark and it consists of expanded inner exine (e2) overarched by a thin outer exine (Fig. 8e). In taxa with trilete marks that are not swollen and ridge-like (= triradiate ridge) such as *Phaeoceros* (Fig. 1k, 8g), *Notothylas* (1 g) and *Nothoceros* (Fig. 1q, 8h), the aperture comprises a thin outer exine, often with a central linear beak, and thickened inner exine that is devoid of sporopollenin along its midline. In taxa with distinct triradiate ridges, the aperture projects from the surface and is pointed as in *Anthoceros* (Fig. 1e, 8f) or reinforced by a



thickened exine on either side of a sporopollenin-free zone in the inner exine as in *Phymatoceros* (Fig. 1m, 8i). Equatorial girdle ultrastructure is similar to that in the aperture in that it lacks sporopollenin in the usually expanded inner

exine (Figs. 6a, c, 8c). Both apertures and equatorial girdles are zones of weakness where the germinating sporeling ruptures the spore wall, often separating the wall into triangular valves. With lessened sporopollenin deposition, these areas

Fig. 4 Spore wall development. **a, b** *Phaeoceros carolinianus*. **a** DIC image of developing spores enclosed in the spore mother cell wall showing ornamentation of the outer exine (arrows) that is more developed than that in Fig. 2f1. The inner exine is not yet filled with sporopollenin. **b** TEM of tripartite lamellae (arrows) in outer exine (e1), filling with sporopollenin, while inner exine (e2) expansion occurs. **c** *Notothylas breutelli*. TEM of proximal walls of two adjacent spores in a tetrad. Outer exines (e1) that form the sculptured proximal surfaces are fully formed and abut the intersporal wall (iw). Inner exines (e2) are fully expanded before sporopollenin deposition is completed, while intine (in) deposition is initiated from the plasmalemma (pm). **d** *Phaeoceros carolinianus*. SEM of four tetrads of spores enclosed in rounded spore mother cell walls (smcw). Dissection of the tetrad in the center and removal of three spores reveals imprints of the distal wall ornamentation on the inside of the smcw wall and proximal wall ornamentation in the intersporal wall (iw). The tetrad on the left remains intact and the one on the bottom has a perforation in the smcw. Most of the smcw has been removed from the tetrad on the right but the four ornamented spores remain. **e** *Notothylas breutelli*. TEM of proximal walls of two adjacent spores in a tetrad as in (c), but further along in development. Sporopollenin has accumulated in the inner exines (e2) starting from the outer exines (e1) toward the compressed intersporal wall (iw). The intines (in) are continuous outside of the plasmalemma (pm). **f, g** *Phaeoceros carolinianus*. SEMs of nearly mature spores. **f** Adjacent spores in a tetrad with adhering SMC wall (smcw), separated by intersporal wall (iw) and embedded in a locular matrix (mx). **g** Tetrad with spores connected at intersporal wall (iw) and a partial cover of SMC wall (smcw) that is drying and adhering to the distal surface ornamentation (arrow). Bars: **a, d**, **g** = 20 μ m; **b** = 100 nm; **c, e** = 1.0 μ m; **f** = 5.0 μ m

also serve as hinges that allow the spore to change shape with maturation and drying as seen in the dehydrated spore in Fig. 8d where the equatorial girdle has collapsed.

Discussion

In contrast to the limited morphological diversity that makes sterile plants difficult to identify to species, spores of hornworts can show considerable species-specific variation that is essential in taxonomic delineations (Cargill et al. 2022; Peñaloza-Bojacá et al. 2022; Fig. 1). Consequently, the external spore features are well-documented and provide critical information in the assessment of hornwort systematics but have not been evaluated from a holistic perspective across hornworts and across plants (Renzaglia and Crandall-Stotler 2026). The present study fills the gap by comparing the development, microanatomy and evolution of spores in 25 species, representing all genera and a broad range of hornwort spore diversity.

The continuous production and development of spores from a sporangium that grows throughout the season place unique constraints on sporogenesis in hornworts. This asynchronous sporogenesis must be responsive to the fluctuations in temperature and moisture levels that occur during the season and from year to year. This is an important factor in the pattern of spore wall deposition and in coordinating

that process with continual and progressive dehiscence of the sporophyte (Renzaglia et al. 2017). Consequently, spore wall layering is simple and deposited rapidly following meiosis. Spores may be released and viable at various stages of maturation or may even germinate inside the sporophyte if conditions are right (Renzaglia 1978). Drying is a key element to sporogenesis and is in part facilitated and regulated by the sporophyte anatomy, including stomata (Renzaglia et al. 2017). The uniqueness of hornwort sporophytes must be considered in assessing sporogenesis and spore diversity across genera and with other plants.

Spore Development

With the exception of the derived multicellular spores of *Dendroceros* (see below), the basic steps in spore development are universal across the hornworts we examined, showing little variability, regardless of mature spore morphology. Spore mother cells (SMCs) round out as they separate from adjacent cells and become suspended in a matrix around the central columella. Prior to meiosis, the SMC wall thickens and cell polarity is accompanied by a meiotic spindle that emanates from four large plastids at each pole (Brown and Lemmon 1990b, 2013). Known as monoplastidic meiosis, this process involves a single plastid in the young sporocyte that undergoes two rounds of divisions followed by the migration of the resulting four plastids to the poles where microtubule nucleation occurs to form a quadripolar spindle (Fig. 2c). In cell division, monoplastidy establishes synchronization between plastid division and the host cell cycle (de Vries and Gould 2018). Monoplastidic meiosis is universal in hornworts and mosses but restricted in occurrence to a few liverworts and pteridophytes (Renzaglia et al. 1994; Brown and Lemmon 2001; Shimamura et al. 2003; Brown et al. 2010). Hornworts are the only group of land plants that have not escaped the so-called monoplastidic bottleneck and not only universally exhibit monoplastidic cell division (meiosis and mitosis) but also retain a monoplastidic phenotype in differentiated vegetative cells of several taxa (Renzaglia and Crandall-Stotler 2026). It is hypothesized that monoplastidic cell division was either inherited from green algal progenitors or derived through gene loss from polyplastidic ancestors (MacLeod et al. 2022). We suggest that the persistence of monoplastidy throughout the life cycle may be linked to the large size of hornwort chloroplasts, which is likely related to the occurrence of pyrenoids, a hornwort exclusive carbon-concentrating mechanism among land plants.

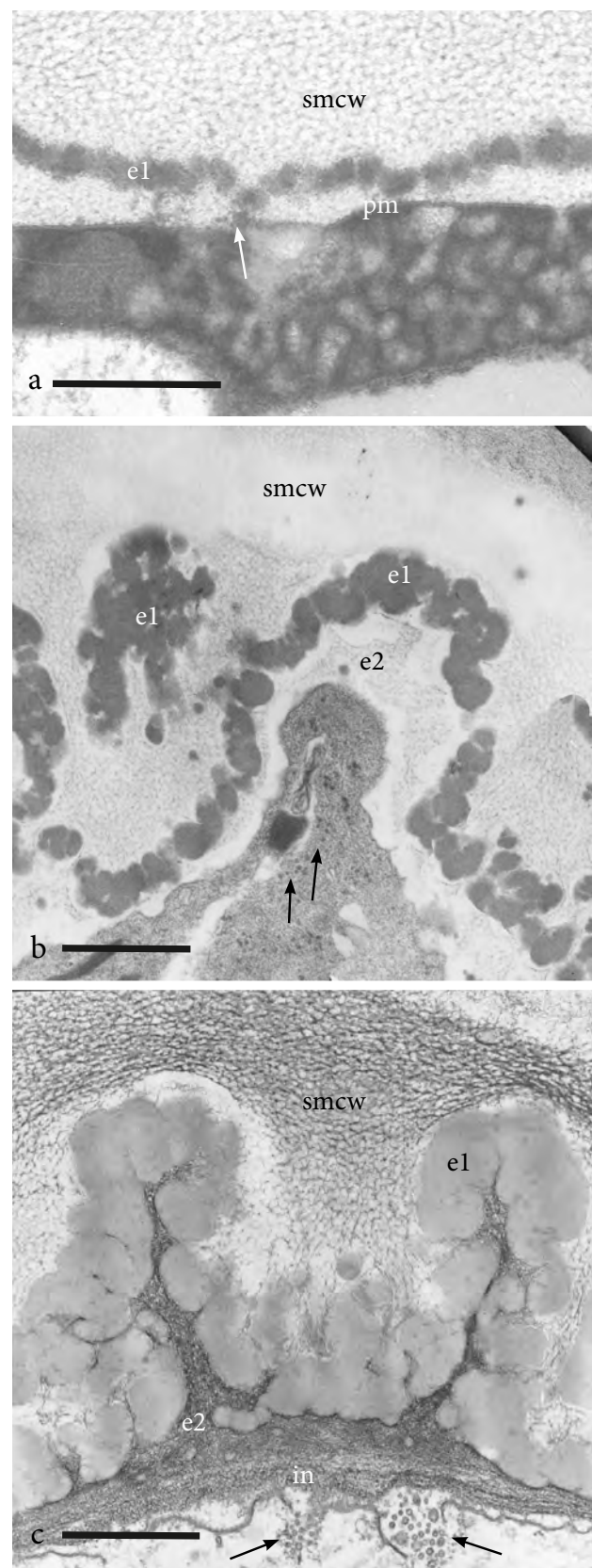
In bryophytes, lobing of the SMC cytoplasm is widespread, with the exception of most complex thalloid liverworts, and may occur in both monoplastidic and polyplastidic plants (Brown and Lemmon 2013). Deeply lobed sporocytes (SMCs) are characteristic of jungermanniopsis liverworts and many mosses, while lobing in hornwort

Fig. 5 TEMs of spore wall development in *Dendroceros*. **a** *Dendroceros granulatus*. The outer exine (e1) originates from small globules of sporopollenin deposited (arrow) directly from the plasmalemma (pm) into the spore mother cell wall (smcw). **b, c** *Dendroceros crispatus*. **b** Cytoplasmic projection containing small electron dense material (arrows) overarched by inner exine (e2) and coalescing globules of sporopollenin in the outer exine (e1) contained within the spore mother cell wall (smcw). **c** Spore wall of a single-celled spore in the SMC (smcw) wall. The thickened and convoluted outer exine (e1) forms the ornamentation and consists of fused sporopollenin globules. The inner exine (e2) is thin, lacks sporopollenin and fills the space between outer exine projections. Intine (in) deposition is ongoing presumably via the numerous small vesicles (arrows) at the plasmalemma. Bars = 1.0 μ m

sporocytes is more subtle, being evident only internally or late in meiosis as in *Leiosporoceros*. Both monoplastidic meiosis and lobing of sporocytes define the four future spore domains in the diploid cell prior to meiosis.

The early stages of hornwort sporogenesis are in line with those in the moss *Physcomitrium* (Renzaglia et al. 2023) but postmeiotic development tracks a separate pathway in the two groups. Unlike mosses where spores expand during differentiation, in hornworts there is little size increase in spores following meiosis. The nascent spores of hornworts remain within the SMC wall until spore wall development is complete and the spores are mature. In comparison, in mosses only the newly formed spores are contained in the SMC wall; as the spores enlarge, they separate from each other and are released from the SMC into the locule of the capsule where they continue to expand (Renzaglia et al. 2023). Although development in both mosses and hornworts begins in a round SMC that undergoes monoplastidic meiosis, mature spores in most hornworts are decidedly tetrahedral and polar, whereas in bryopsid mosses, they are typically spheroidal with less differentiated proximal surfaces. Early separation and expansion of moss spores outside the confines of the SMC wall may in part allow for their spherical shape.

In liverworts, the early stages of sporogenesis are more variable than in hornworts and mosses, as is spore morphology that ranges from apolar to strongly polar (Brown and Lemmon 1990a, 2013). This diversity is especially extreme in the complex thalloid lineage, ranging from monoplastidic cell division in lobed sporocytes of *Blasia* to derived spherical sporocytes that lack quadripolarity in *Conocephalum*, resembling those of tracheophytes (Brown et al. 1988, 2010). Unknown from any other plant group is the origin of callosic exine precursors in the diploid sporocyte that predict spore wall patterning in certain, seemingly disparate, liverworts (e.g., *Haplomitrium*, *Pallavicinia* and *Cephalozia*) (Brown et al. 1986). These precursors are associated with projections from the cytoplasm similar to those described here in hornworts.



Spore wall development and the origin of the sculptoderm

Development of a thickened spore wall and its signature ornamentation is a key part of sporogenesis. In hornworts and liverworts, the spore wall develops centripetally and the sculptoderm is laid down first. In mosses, the characteristic spore ornamentation is laid down in the final stages of spore maturation via an externally produced sporopollenin-containing perine layer, typically over an undecorated exine (Renzaglia et al. 2023).

Exine development in most bryophytes involves the production of tripartite lamellae (TPL), which are white line centered sheets or slips of unit membrane thickness. The TPL of hornworts are elusive and visible only in the newly formed spore because they are rapidly impregnated with sporopollenin, which obscures them (Brown et al. 2015). In all but *Dendroceros*, the lamellae serve as the framework for the outer exine which is an outer, often thin, lamina that contorts and shapes to form the external sculpturing. In hornworts and liverworts, spore surface sculpturing is determined early and laid down by the spore cytoplasm. In liverworts, TPL are important constituents of the exine and are deposited as slips, single lines or multilaminate bands. They are typically visible in mature spore walls and are partially coated by sporopollenin (Brown et al. 1986, 2015; Renzaglia et al. 2015a).

As noted above, moss spores undergo dramatic increases in size during development and certain spore wall constituents, especially sporopollenin, are contributed externally from the locular matrix and tapetum (Renzaglia et al. 2023). TPL serve as a foundation layer between exine and intine. Among the sister taxa to true mosses, *Takakia* and *Sphagnum* spores contain abundant TPL that are visible in the mature spore, while the mosses *Andreaea*, *Andreaebryum*, and the hornwort *Dendroceros* are the only bryophytes that are reported to lack TPL during all stages of spore wall development (Brown and Lemmon 1984; Renzaglia et al. 1997; Brown et al. 2015; Polevova et al. 2022). The aperture of mosses may be reinforced by multiple TPL, as in *Physoomitrium patens* (Renzaglia et al. 2023).

The involvement of callose is universal in plant sporogenesis but the precise role of this transient cell wall polysaccharide is highly variable (Schuette et al. 2009; Wallace et al. 2011; Ünal et al. 2013; Brown et al. 2015; Renzaglia et al. 2020). Among bryophytes, liverworts widely utilize callose in the early stages of sporogenesis as it commonly appears in the cell wall as a template or a matrix in which the exine is systematically and progressively constructed (Brown et al. 1986; Renzaglia et al. 2015b). In mosses, the occurrence of this polymer has been verified via aniline blue staining and immunolabelling in the aperture during its formation (Schuette et al. 2009). In hornworts, there is no trace of callose during spore development until it appears in the

intine of the mature spore, clearly distinguishing these plants from all other embryophytes (Renzaglia et al. 2020).

Spore morphological diversity and evolution

The majority of hornwort spores are polar, tetrahedral and trilete with a well-defined aperture with or without a defined equatorial girdle. These spores are found in most *Anthoceros* species, *Phymatoceros*, *Phaeomegaceros* and members of the Notothyladaceae, including *Notothylas*, *Phaeoceros* and *Paraphymatoceros*. Spore wall sculpturing in these plants is more pronounced on the distal surface than on the flattened proximal surfaces. However, significant deviations in spore morphology are seen in *Leiosporoceros* and some species of *Anthoceros*, the sister taxa to other hornworts (Brown et al. 2015; Cargill et al. 2022), and in the derived tropical members of the Dendrocerotaceae.

Small, smooth and elongated spores are diagnostic of *Leiosporoceros*. The unique isobilateral arrangement is responsible for the monolete mark that functions as an aperture. Comparable isobilateral spores that are devoid of ornamentation are unknown in any other living plant, although spores with smooth, unornamented walls have scattered occurrences in a few species of the liverwort *Riccia*, e.g., *R. albida*, *R. crustata* and *R. singularis* (Jovet-Ast 1973, 1996). Laevigate spores are widespread in the early plant fossil record, including some of the oldest known fossil plant spores (Wellman et al. 1998, 2003; Taylor et al. 2017; Taylor and Strother 2024), but they are tetrahedral in contrast to the elongate spores of *Leiosporoceros*.

In *Anthoceros adscendens* and *A. fuciformis*, spore polarity is unclear as demarcation between distal and proximal surfaces is obscured and the trilete mark is reduced to absent (Cargill et al. 2022). The distinctly rounded shape of these spores may be related to the virtual lack of an inner exine (Fig. 7d), and the less substantial SMC wall as evident in our micrographs (Figs. 6b, 7d). Both features can impart flexibility in shape. Equally defined are the derived spores in the mostly tropical crown group Dendrocerotaceae. In *Nothoceros* and *Megaceros*, spores are small and green and lack differentiation of the equatorial girdle. Large, irregularly-shaped, multicellular spores characterize all species of *Dendroceros* and one species of *Nothoceros*, a condition that is likely associated with the epiphytic habit as it is in other bryophytes (Schuette and Renzaglia 2010).

Spore wall ultrastructure

Despite considerable variability in external ornamentation across hornwort diversity, there is a common internal structure of the spore wall. At its essence, the hornwort spore wall is three-layered (Fig. 7). The outer exine, or exine 1 layer, is derived from TPL and forms the outer sculpturing wall. The

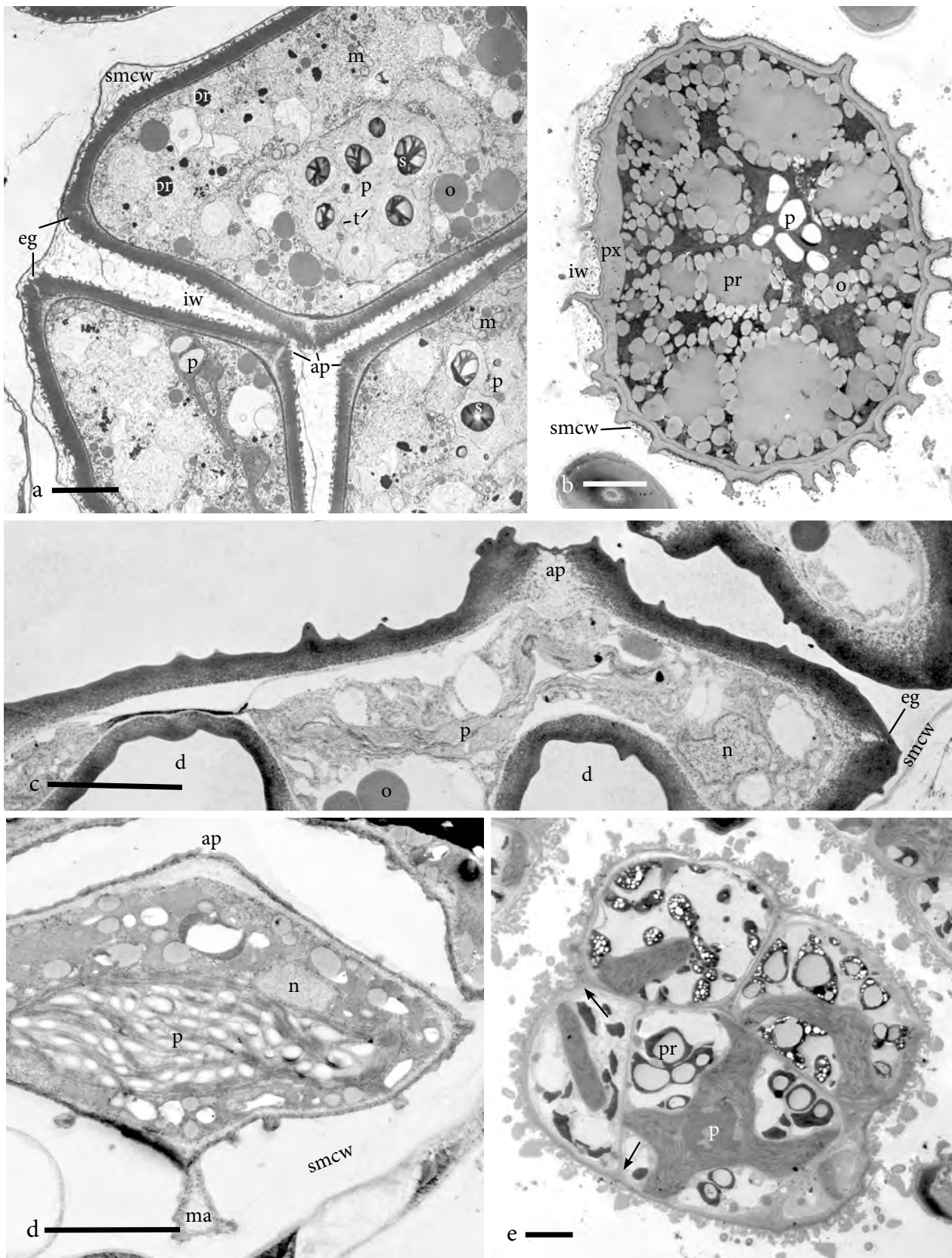


Fig. 6 TEMs of mature or nearly mature spores retained in sporophytes. **a** *Notothylas breutellii*. Nearly mature tetrad enclosed in the SMC wall (smcw). Spores are separated by an intersporal wall (iw) and contain abundant organelles including a large pleomorphic plastid (p) with scattered starch (s) and few thylakoids (t), mitochondria (m), oil droplets (o) and protein (pr). The spore walls are three layered and an aperture (ap) subtends the trilete mark on the proximal face of each spore. A morphologically similar wall extends around each spore of the tetrad forming an equatorial girdle (eg). **b** *Anthoceros fuciformis*. A single mature spore engorged with oils (o) and protein (pr), obscuring organelles except the starch-filled plastid (p). The inner spore wall, intine, is expanded along the proximal face (px). The spore mother cell wall (smcw) and intersporal wall (iw) adhere to the spore surface. **c** *Phaeomegaceros fimbriatus*. Part of a tetrad showing a nearly mature spore with sparse cytoplasm containing plastid (p), lateral nucleus (n) and scattered oils (o). The spore wall contains distal fovea (d), a well-developed proximal aperture (ap), and a “closed” equatorial girdle (eg). The tetrad is surrounded by the spore mother cell wall (smcw). **d** *Nothoceros vincentianus*. A single spore in the tetrad stage, with one large starch-filled plastid (p) and lateral nucleus (n). The spore wall is thin all around except for the expanded intine at the proximal aperture (ap) and mamilla (ma) distally. The tetrad is surrounded by the spore mother cell wall (smcw). **e** *Dendroceros*. Five cells are visible in this multicellular spore, each with a single plastid containing thylakoids and a pyrenoid (p), and protein bodies (pr). The thin spore wall is composed of an undulating outer exine, thin inner exine and intine that is continuous with the primary walls between cells (arrows). Bars = 5.0 μ m

inner exine, or exine 2 layer, is usually thicker and initially contains sporopollenin particles or globules that compact, potentially obscuring demarcation between the two exine layers. Both layers are acetolysis resistant, supporting the presence of sporopollenin (Long 2003; Grienemberger and Quilichini 2021). The innermost spore wall layer is a late deposited intine that contains primary cell wall polymers (cellulose, hemicellulose and pectin) as well as callose (Renzaglia et al. 2020).

Because hornwort spores are contained in the SMC wall throughout sporogenesis, the final process leading to release of single spores is the drying down of the SMC wall on the spore surface, leading to the deposition of a fourth layer, referred to as a pseudoperine because it is not of tapetal origin (Crandall-Stotler et al. 2006, 2008; Renzaglia et al. 2009). This layer can be thick and dense as in *Phymatoceros* or thin and variable as in most *Dendrocerataceae*.

Aperture

Spore apertures are structurally distinct, usually proximal, regions that are designed to facilitate water uptake and serve as a suture area for spore wall rupture during germination (Olesen and Mogensen 1978). Among bryophytes, apertures occur only in hornworts and mosses and are likely of independent origin (Renzaglia et al. 2023). The hornwort aperture is a modified spore wall region subtending the trilete or monolete mark that contains sparse sporopollenin (Renzaglia and Crandall-Stotler 2026). The apertures of

Leiosporoceros, *Notothylas* and *Phaeoceros* exhibit a basic structure of thin outer exine and expanded inner exine with rarified sporopollenin (Brown et al. 2015). In taxa with highly developed trilete ridges such as *Phymatoceros*, the outer exine may be thickened adjacent to the inner exine region forming reinforcement that flanks the suture (Fig. 8i). Based on this design, it is easy to envision water uptake and spore wall rupture through the aperture. Less obvious is the likely role of the hornwort aperture in desiccation tolerance, especially in temperate hornworts. The equatorial girdle that defines many hornwort spores, e.g., *Notothylas* and *Phaeoceros*, is a zone of weakness that encircles the spore where proximal and distal faces meet. It is a structural mimic of the proximal aperture in that it contains sparse sporopollenin. At germination, the spore wall often separates along the trilete mark and equatorial girdle in these hornworts, leaving three triangular valves at the base of the germinating spore or sporeling (Renzaglia 1978). Both aperture and equatorial girdle also allow for partial spore collapse during drying and are consequential in the drying process of dispersed spores, enabling spores to stay viable by losing water without losing contact between the plasmalemma and spore wall.

The proximal aperture of mosses is much more elaborate in design than that of hornworts (Brown and Lemmon 1980, 1981; Renzaglia et al. 2023). In contrast to hornworts in which the aperture subtends only the trilete mark or ridge, the aperture of mosses is a complex modification of spore wall layers across the entire proximal surface. The moss spore aperture consists of a thickened intine, an annulus, and a central pad (pore) with radiating fibers. This region of the spore is the first to develop during spore wall ontogeny and it is accompanied by the production of an elaborate system of microtubules that guide the construction of the germinal pore region. In *Physcomitrium* spores, the aperture region is elastic and collapses when dry, forming a concave surface, and expands when wet.

Dendroceros as an outlier

The unique microanatomy of *Dendroceros* spores is unparalleled in land plants and appears to be related to the multicellular condition of released spores in these epiphytic taxa (Schuette and Renzaglia 2010). Compared with those of other hornworts, the spores exhibit a suite of developmental and structural features that include deposition of sporopollenin micro-globules devoid of TPL that coalesce to form a highly convoluted and stretchable outer exine layer. This manner of sporopollenin deposition via micro-globules is identical to that illustrated by Gabarayeva (1995, 2000) as one of several modes of initial sporopollenin deposition in microspores of angiosperms (c.f., Fig. 5c with Fig. 8 in Gabarayeva 2000). In *Dendroceros*, the exine 2 layer is highly reduced and occupies the space between folds of

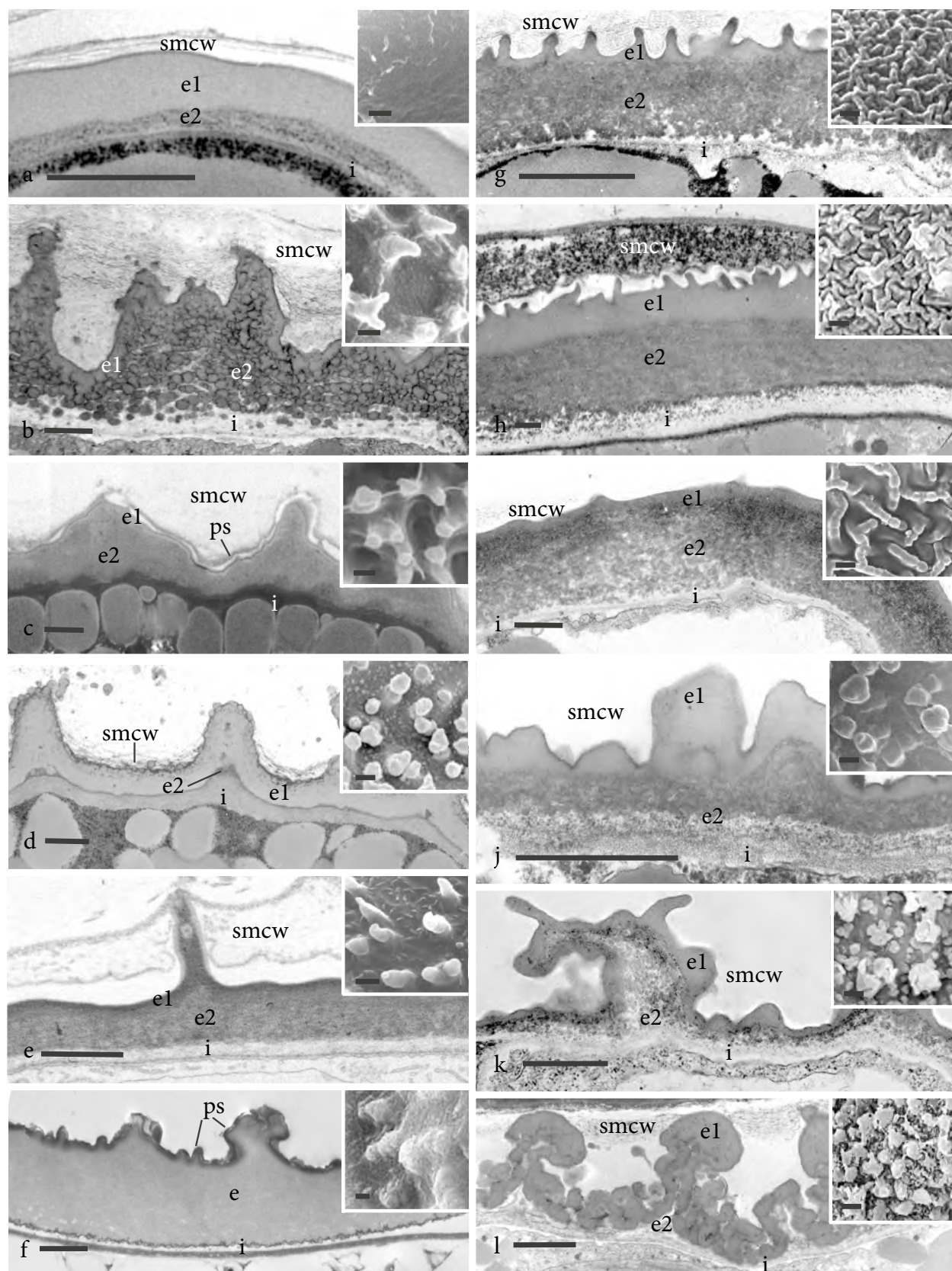


Fig. 7 TEM of distal wall architecture from mature or nearly mature spores inside of the sporophyte with SEM insets showing surface ornamentation. Deposition of the pseudoperine (ps) from the spore mother cell wall (smcw) has not been completed, except in (f). Spore wall layering from outside to inside is outer exine (e1) that forms the sculptured layer, inner exine (e2) and intine (i). **a** *Leiosporoceros dussii*. Three-layered sporoderm with thick outer exine (e1) and minimal deposition of sporopollenin in the inner exine (e2). **b** *Anthoceros laminifer*. Three-layered sporoderm with irregular outer exine (e1) and globular sporopollenin in the inner exine (e2). **c** *Anthoceros macounii*. Highly compacted three-layered sporoderm with thin outer exine (e1) and pseudoperine (ps) beginning to accumulate. **d** *Anthoceros fuciformis*. Three-layered sporoderm with minimal inner exine (e2) filling spaces between folds in the thick outer exine (e1) and thick intine (i). **e** *Phaeoceros carolinianus*. Three-layered spore wall with distinction between outer (e1) and inner exine (e2) obscured. **f** *Paraphymatoceros hallii*. Compacted sporoderm in fully mature spore with lack of distinction between outer and inner exine (e) and thin layer of pseudoperine (p) from drying down of spore mother cell wall. **g** *Notothylas orbicularis*. Three-layered sporoderm. **h** *Phymatoceros bulbiculosus*. Prominent spore mother cell wall (smcw) (c.f., Fig. 1n) covering the thick outer exine (e1), dense inner exine (e2), and intine (i). **i** *Phaeomegaceros fimbriatus*. Three-layered sporoderm. **j** *Nothoceros vincentianus*. The inner exine (e2) in this spore wall is only partially filled with sporopollenin. **k** *Megaceros gracilis*. This spore wall with minimal sporopollenin in the inner exine (e2). **l** *Dendroceros granulatus*. Spore wall in spore at multicellular stage; the prominent but irregular outer exine (e1) undulates over a thin inner exine (e2). Bars = 1.0 μ m

exine 1. The intine is extensive when compared with that found in other hornworts and it is continuous with the cell walls that separate the multiple cells within each spore.

Spore color

Spore color has been shown to be related to ecological niches in hornworts (Peñaloza-Bojacá et al. 2023). Here we document anatomical features associated with spore color. In general, spore walls that are thicker than 1 μ m (as evidenced by the 1 μ m bars on each image in Figure 7) are dark colored and those that are yellow or green at maturity have a thinner spore wall that is less than 1 μ m thick, excluding spines and projections. Other factors also contribute to spore color. *Anthoceros* sensu lato is the only hornwort clade in which spore color is consistently dark brown to black, a condition that is likely related to the deposition of large globular sporopollenin in highly thickened inner exines. For several species, spore color has been noted to darken during maturation, a phenomenon that is consistent with an increase in sporopollenin in the inner exine that occurs during spore development and the adherence of SMC wall in the final stages of drying. This is particularly exaggerated in *Phymatoceros* and some species of *Paraphymatoceros* and *Notothylas* where spore color has been noted to darken from yellow to brown during the final stages of maturation, concomitant with the deposition of a particularly thick SMC wall onto the spore wall. The pale yellow and green spores of

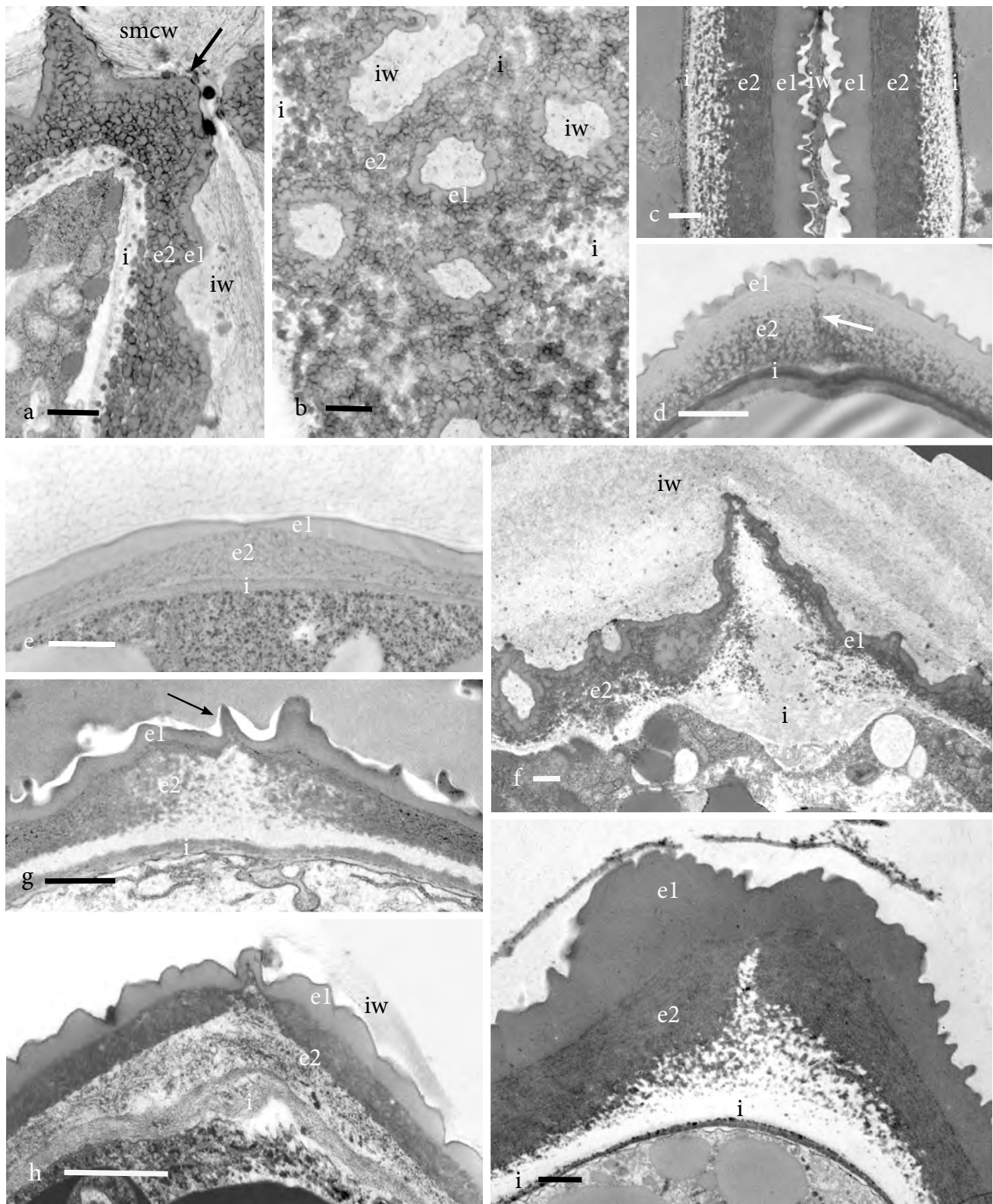
Nothoceros, *Megaceros* and *Dendroceros* have significantly reduced inner exine layers, in thickness and sporopollenin deposition, and thick intines, making them somewhat translucent and allowing the green color of their large plastids to be seen. In the green spores of *Dendroceros*, the wavy outer exine stretches as spores divide by mitosis and enlarge, becoming multicellular, with single plastids in each cell, in anticipation of immediate germination following release onto leaf and branch surfaces of host plants (Schuette et al. 2010).

Fossil studies

In recent fossil-constrained molecular divergence time analyses, crown-hornworts are estimated to have originated in the Carboniferous, approximately 322.5 Ma to 337.6 Ma, nearly 100 million years later than the presumed origin of mosses and liverworts (Villarreal and Renner 2012; Peñaloza-Bojacá et al. 2025; Xu et al. 2026). According to these estimates, radiation within hornworts began in the Permian with the divergence of the *Leiosporoceros/Anthoceros* stem and the subsequent divergence of the *Notothyladales* and *Dendrocerotales* stem in the Jurassic and the *Phymatocerotales* stem in the Cretaceous and Palaeogene. Despite a presumed origin in the Carboniferous, fossil spores that can be reliably identified as hornwort spores are first found in the Cretaceous.

Although the earliest fossil land plant spores are not directly attributed to hornworts, they do provide clues as to the origin of the following diagnostic characters of hornwort spores: 1) spore wall development occurring entirely within the tetrad while still contained within the spore mother cell wall; 2) dispersed spores with trilete geometry having differentiated proximal surfaces and a well-developed suture system; 3) exine development in which transient tripartite lamellae are obscured early by sporopollenin, forming a homogeneous non-lamellar layer; and 4) a spore wall that is 3- or 4-layered, comprised of a 2-layered exine, single layered intine, and an inconsistent outer pseudoperine produced from the adherent spore mother cell wall.

Many organisms in the Ordovician and Silurian produced envelope-enclosed tetrads and dyads (Gray 1985; Taylor 1995, 1996; Wellman et al. 2003; Wellman 2004; Edwards et al. 2014, Edwards et al. 2022). These envelopes may be loose or tightly adherent to the spore walls, or even sculptured as in *Velatitetras* (Edwards et al. 2014). They have been variously interpreted, including the view that some may represent a persistent spore mother cell wall (Taylor and Strother 2008), as occurs in all hornworts. Among bryophytes, permanent dyads and tetrads are produced by only a few liverworts but, unlike hornworts, the spores are united by a spore wall that develops after meiosis during callose-mediated spore wall maturation (Renzaglia et al. 2015a).



The development of these permanent tetrads may be similar to those producing the widespread naked (not envelope-enclosed) permanent tetrads of ancient fossils (Wellman

and Gray 2000). In hornworts, although the spores remain in tetrads during spore wall formation, they are dispersed

Fig. 8 TEM of proximal wall and suture architecture, including aperture variability and equatorial girdle. **a, b** *Anthoceros laminifer*. **a** Junction of distal spore wall (overlain by SMC wall (smcw)) and proximal wall (adjacent to the intersporal wall (iw)) showing similar layering of the outer exine (e1), globular inner exine (e2) and intine (i), and the absence of a differentiated equatorial girdle (arrow). **b** Surface section of proximal wall showing outer exine (e1) surrounding proximal dimples filled with intersporal wall (iw), globular sporopollenin in the inner exine (e2) and intine (i). **c** *Phymatoceros bulbiculosus*. Proximal surfaces of two adjacent spores in a nearly mature tetrad separated by the intersporal wall (iw) showing wall layering similar to the distal wall with thick outer exine (e1), compacting inner exine (e2) and intine (i). **d** *Notothylas orbicularis*. Equatorial girdle in a 'closed' state following spore shrinkage. The zone of sparse sporopollenin serves as a hinge that allows the spore to fold inward slightly (arrow) and decrease in volume; (e1) outer exine, (e2) inner exine, (i) intine. **e-i** Spore aperture variability. **e** *Leiosporoceros dussii*. Simple aperture with thin outer exine (e1) and expanded inner exine (e2) subtending the monolete mark. **f** *Anthoceros laminifer*. Protruding aperture at the trilete ridge consisting of thin outer exine (e1) and expanded inner exine (e2) with rarefied sporopollenin and inner core of intine (i); iw, intersporal wall. **g** *Phaeoceros carolinianus*. Outer exine (e1) with a pointed central ridge (arrow) and modified inner exine (e2) with sparse sporopollenin in the middle subtended by intine (i). **h** *Nothoceros vincentianus*. Thin outer exine (e1) with a pointed central ridge penetrated by a thicker region of rarefied sporopollenin in inner exine (e2), subtended by intine (i). Remnant intersporal wall (iw). **i** *Phymatoceros bulbiculosus*. Aperture along the triradiate ridge is reinforced by thickened outer exine (e1) flanking expanded inner exine (e2) that lacks sporopollenin in the middle and is subtended by intine (i). Bars: **a-d, f, i** = 1.0 μm ; **e, g, h** = 0.5 μm

as singlets following drying down of the spore mother cell wall (Fig. 4g).

Trilete spores and dispersed tetrads of spores, which are considered proxies for embryophytes, first occur in the lower Silurian, e.g. *Ambitisporites avitus* (Wellman et al. 2013; Strother and Taylor 2024). Spores with homogeneous walls that lack lamellar construction at maturity, as occur in hornworts, appear by the Middle Ordovician, e.g., *Cryptotetras erugata* (Taylor et al. 2017), although this spore wall type does have a single basal lamina at the innermost extent of the wall. Taylor et al. (2017) interpret this as the earliest example of a spore produced in a sporangium and suggest that the original lamellar construction was obscured by the addition of sporopollenin from a surrounding source, perhaps a sporangial tapetum. Among all extant bryophytes sterile tissues within the sporangium contribute to the developing sporocytes (Crandall-Stotler et al. 2009), but the addition of sporopollenin polymers to the spore wall from a tapetum has been verified only in mosses (Renzaglia et al. 2023) and is absent in both hornworts and liverworts (Wallace et al. 2011; Brown et al. 2015; Filin and Platonova 2025a).

By the Silurian/Devonian boundary, the ultrastructural diversity of spore walls was considerable, including lamellate, granular, and lacunate walls, some of which consist of several layers (Edwards et al. 2014; Wellman 2024). Dispersed monolete spores with single laesurae, a character also

expressed in *Leiosporoceros*, first appear in the fossil record in the late Devonian (Breuer and Steemans 2013). Some of the oldest known fossil plant spores have laevigate or smooth walls, a character also seen in *Leiosporoceros* but are trilete (with three laesurae), not monolete (Wellman et al. 1998, 2003). Based on ultrastructural features, Taylor et al. (2011) proposed a possible hornwort affinity for the dispersed spore taxa *Emphanisporites schultzei* and *E. rotatus*. This assignment was based on the presence of an equatorial girdle with reduced sporopollenin (pseudosuture), and a single wall lamina at the spore surface. The argument was supported by the occurrence of *Emphanisporites* (*E. decoratus*) in situ in the sporangia of the Rhynie chert plant *Horneophyton lignieri* (Wellman et al. 2004; Cascales-Miñana et al. 2022). However, several other very simple plants described from Wales by Dianne Edwards and her colleagues (Edwards and Richardson 2000) also contain *Emphanisporites* in their sporangia, and several other spore types, including *Retusotriletes* have been found in the sporangia of *Horneophyton* (Eggert 1974; Cascales-Miñana et al. 2022). Germinating spores identified from *Horneophyton* (Bhutta 1973) do resemble those of bryophytes in that the spore wall ruptures at the trilete mark on the proximal face, but the sporeling morphology is not like that of modern hornworts (Renzaglia 1978). Of course, comparisons between the spores of *Horneophyton* and those of hornworts are intriguing since both taxa have sporangia with a cylindrical shape and a central columella of sterile tissue. However, unlike monosporangiate bryophytes, the sporangia of *Horneophyton* terminate a dichotomously branched cylindrical sporophyte and are themselves dichotomously branched twice (Eggert 1974). Within a single *Horneophyton* sporangium, spore maturation appears to be acropetal, with some asynchrony possibly present, a feature found only in hornworts among extant plants (Taylor et al. 2009, p. 238).

Developmental comparisons between hornworts and fossils may also be assessed based on in situ spores preserved in sporangia. For example, developing SMCs in hornworts (Fig. 2b) are strikingly similar to those of *Aglaophyton major* from the Rhynie Chert (illustrated in Wellman et al. 2006, Plate II, Figs. 1 and 2). Moreover, in *A. major* the tetrads are enclosed in a round matrix speculated to be a 'callosic wall' (without verification), which may represent the sporocyte wall, as in hornworts. This organization also implies that the spore wall ornamentation is formed inside the SMC wall without the involvement of a tapetum. Indeed, similarities in spore wall development and structure between hornworts and these Devonian fossils lends support to the hypothesized ancestral link between hornworts and the rhyniophytes as proposed by Haskell (1949), as well as to the current view that bryophyte and tracheophyte lineages originated independently from a group of early land plants

and have diverged both genetically and morphologically since that time (Harris et al. 2022).

The first fossil spores that are widely accepted to have hornwort affinity appeared in the Mesozoic during the Upper Jurassic and lower Cretaceous some 151 Ma and 118 Ma, respectively (Archangelsky and Villar de Seone 1996; Villarreal and Renner 2012). With warts on the proximal faces, these fossils resemble spores of two extant Neotropical *Anthoceros* species (Peñaloza-Bojacá et al. 2025). Dispersed spores of the Jurassic *Foraminisporis dailyi* are speculated to be related to *Phymatoceros* or *Phaeoceros* due to a small number of distal protuberances (Dettman 1963), while Jarzen (1979) ascribed three forms of North American fossil spores from the upper Cretaceous to the genus *Phaeoceros* s. l. A later dispersed spore fossil from the Lower Miocene (15–23 Ma) of Costa Rica closely resembles extant *Phaeomegaceros fimbriatus* in size and ornamentation, with six depressions on its distal face (Villarreal and Renner 2012). It is curious that although there are many reports of dispersed hornwort-like fossil spores since the early Cretaceous, pseudoelaters have not been described (Jarzen 1979; Warny et al. 2012), and can be explained by either their absence or a lack of decay-resistant compounds in their walls (Kremer et al. 2004). In this regard, a mixture of sterile cells (pseudoelaters) and spores are present inside the petrified sporangium of *Notothylites nirulai* from Upper Cretaceous deposits in India (Chitaley and Yawale 1980), confirming that pseudoelaters were present in Cretaceous-aged hornworts.

Data on the structure of in situ fossil hornwort spores is extremely limited due to the paucity of unequivocal hornwort sporangia preserved as macrofossils (Blanco-Moreno et al. 2025; Filin and Platonova 2025b). In *Notothylites nirulae* spores are round, 33–47 µm, smooth-walled, and somewhat transparent, surrounded by many 1- (rarely 2-celled), rectangular, thick-walled, pseudoelaters, 55–64 µm × 33–48 µm (Chitaley and Yawale 1980). In the original publication describing the impression macrofossil *Notothylacites filiformis* from Upper Cretaceous deposits in the Czech Republic, only spores were recovered from the sporangia studied, with a mix of tetrads and single spores (Němejc and Pacltová 1974). The spore wall was described as consisting of four layers, a 2-layered "perine," which dissolves and peels off during maceration, and a 2-layered exine that is acetolysis resistant. Our studies of spore wall structure in extant hornworts suggest that the "perine" layers of this fossil likely represent the spore mother cell wall, which does peel off with acetolysis (see Filin and Platonova 2025b, Fig. 7). Recently, Veselá et al. (2025) reinterpreted *Notothylacites filiformis* as a conglomerate of a hornwort sporangium and *Riccia*-like liverwort thalli found with it, fused by taphonomic effects. The original mesofossil of the hornwort sporangium was lost and is illustrated only by a line drawing in the original publication (Němejc and

Pacltová 1974). In SEM views, the extracted spores resemble dispersed spores of Jarzen's *Phaeoceros* form B (1979), being triradiate with a slightly thickened equatorial girdle and thin laesurae, granulate proximal surfaces and a vermiculate distal surface. While this designation is plausible, assigning such simple spores with few diagnostic characters to specific taxonomic categories should be approached with caution. Nonetheless, the presence of both tetrads and single spores within the single sporangial sample provide evidence of nonsynchronous sporogenesis in the Upper Cretaceous, a diagnostic character of hornworts.

Conclusion

Prior to this study, the ultrastructural features of hornwort spores were known from a limited number of descriptions, mostly scattered in more comprehensive studies of morphology and bryophyte sporogenesis (Brown and Lemmon 1988, 1990a; Renzaglia et al. 2009; Brown et al. 2015). With examination of 25 species, representing the range of hornwort diversity, our study provides critical developmental information related to ultrastructural and morphological diversity that is pertinent to interpretations of phenotypic variability and evolution of spores, which were essential adaptations to life on land. Through a comparative approach, we identify a central process in the development of spores in hornworts and a common spore wall architecture across hornwort genera except *Dendroceros*. Hornwort exclusive features of sporogenesis include the retention of spores in the spore mother cell wall throughout development and the adherence of this wall to the spore surface in the final stages of spore maturation forming an inconsistent pseudoperine. In addition to this outermost pseudoperine, mature spore walls are characterized by a 2-layered homogeneous exine and inner intine, and a simple aperture with rarified sporopollenin. The characters of the hornwort spores and their pattern of development described herein are well represented in the early fossil record and may represent plesiomorphic features of land plant spores. The findings of this study serve as the basis for continued evo-devo studies, and for structural and developmental comparisons with other plants and with fossil spores across geological history.

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Author contribution KSR and WAT conceived and designed the study. Material collection, acquisition and preparation were carried out by KSR, JCV, SS, AM and DCC. Data collection and interpretation were performed by KSR, BCS, JCV, SS, and AM. The manuscript was written by KSR and BCS, with DCC providing critical revisions. All authors read and approved the final manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

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