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Spores of relictual bryophytes: Diverse adaptations to life on land



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ABSTRACT

Sporogenesis and the evolution of thick-walled meiospores were of paramount importance in the establishment of the first life on land nearly half a billion years ago. Indeed, the first evidence of plant life on land is the sudden appearance of spores (palynomorphs or cryptospores) about 470 million years ago, predating the first megafossils by some forty million years. Affinities of these fossil spores with living embryophytes are often difficult to assess due to significant gaps in knowledge of spore diversity in critical extant taxa. The bryophytes (liverworts, mosses and hornworts) are the most ancient living plant lineages. To fill in data and provide a framework in which to evaluate spore evolution, we conducted ultrastructural and developmental studies of eight early-divergent or relictual bryophytes, as identified by cladistic/molecular and morphological studies. Spore wall data are presented for the liverworts *Apotreubia*, *Haplomitrium*, and *Blasia*; the mosses *Takakia*, *Sphagnum*, *Andreaea*, and *Oedipodium*; and the hornwort *Leiosporoceros*. Wall ontogeny is based on tripartite lamellae (TPL) in all taxa except *Andreaea*, which has a unique spongy exine. While a single foundation TPL is typical of bryopsid mosses, *Takakia* has a multilamellate layer (MLL) composed of numerous appressed segments of TPL. Spores of *Sphagnum*, liverworts, and the hornwort *Leiosporoceros* have a MLL layer composed of continuous, not segmented, TPL. We conclude that TPL and multilamellate layers are plesiomorphic in sporoderm development in land plants. Proximal wall modifications evolved into distinct apertures in mosses and hornworts but not in liverworts. Analysis of relictual bryophyte species indicates that the ancestral spore types would be tetrahedral with trifacial proximal surfaces having less ornamentation than the distal surfaces. Identification of features specific to relictual liverwort, moss and hornwort spores provides a foundation for determining fossil spore affinities and interpreting spore evolution and diversification in the earliest land plants.

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1. Introduction

The innovation of sporogenesis at the algal/plant transition was critical to the establishment of life on land. Haploid meiospores enclosed in sporopollenin walls (sporoderm) provided secure storage of DNA during adverse periods, facilitated dispersal by air/wind, and allowed for dissemination of genetically different gametophytes. Spore fossils predate plant megafossils by 40 million years and afford the limited clues to the pioneering events in land colonization some 470 million years before present (Wellman, 2004; Edwards et al., 2014; Strother et al., 2015). The diverse, widespread and prolonged existence of only fossil spores supports the notion that spores evolved prior to the appearance of sporophytes (Brown and Lemmon, 2011). Affinities of the earliest fossil spores with living groups are often speculated in spite of significant

gaps in knowledge of spore diversity in phylogenetically important extant taxa (Wellman et al., 2003).

Spores are protected by some of the most elaborate and resistant walls produced by plants. All contain the highly durable substance sporopollenin (Wallace et al., 2011). In spite of the importance of spores in plant evolution and survival, the molecular biology and biochemistry of spore wall components and synthesis are little known (Quilichini et al., 2014). One outstanding developmental characteristic of spore walls obvious from comparative study of fossil spores as well as those of extant bryophytes and tracheophytes is the occurrence of distinct tripartite lamellae (TPL) in exospore development (Brown and Lemmon, 1990a; Wellman et al., 2003; Wellman, 2004; Taylor and Strother, 2008). As spore walls mature, the lamellae are cemented with sporopollenin and obscured, but a distinctive multilamellate layer (MLL) may remain as an inner exine, and in some liverworts, layers of MLL with sporopollenin form the entire outer wall.

The status of bryophytes (liverworts, mosses and hornworts) as the oldest living land plants is widely recognized (Mishler and Churchill,

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1984; Shaw and Renzaglia, 2004; Renzaglia et al., 2007). The diagnostic character of one sporophyte/one sporangium per fertilization event has led to thumb nailing these plants as monosporangiates. They differ from all other extant plants (polysporangiates) in the terminal position of a solitary sporangium on a sporophyte that is nourished by the gametophyte through a placenta. Since the gametophyte is the persistent vegetative generation that often reproduces asexually, sporophytes are not always obtainable in the right developmental stages to examine spore ultrastructure and development. Thus, spore characteristics in key, often elusive, early divergent bryophytes have not been described. To fill this scientific lacuna and to clarify misconceptions relating to variability in spore wall architecture in bryophytes, we present developmental summaries and ultrastructural data on eight early-divergent bryophyte genera, as identified by molecular and morphological phylogenies. We refer to these taxa as relictual bryophytes or living fossils because they are genetically isolated and they exhibit peculiar morphological features not seen in other plants within their major clades (i.e., liverworts, mosses or hornworts) (Renzaglia et al., 1994; Carafa et al., 2003; Renzaglia et al., 2007).

The premise of this study is that this basal grade of bryophytes has retained reproductive and structural traits that provide insight on early radiation into terrestrial habitats. Internal and external spore wall features, including development of wall ornamentation, number and nature of wall layers, and aperture existence and features, are documented for three liverworts – *Apotreubia* (Haplomitriopsida), *Haplomitrium* (Haplomitriopsida), and *Blasia* (Marchantiopsida) –, four mosses – *Takakia* (Takakiopsida), *Sphagnum* (Sphagnopsida), *Andreaea* (Andreaeopsida), and *Oedipodium* (Oedipodiopsida) –, and one hornwort – *Leiosporoceros* (Leiosporocerotopsida).

Although the spores of these relictual bryophytes are highly diverse when compared with each other, fundamental characters within each of the three bryophyte groups are evident. The information presented here extends the known characteristics in spore wall architecture in bryophytes and provides important comparisons with extinct and extant spores. We consider fossil spores and spores from more derived and better known bryophyte taxa to evaluate spore evolution within the three groups. Given that spores are the only evidence of the earliest land plants, this information is essential to assess more completely the initial stages of life on land.

1.1. Taxon selection

Generally viewed as sister to all other land plants (Qiu et al., 2006), the liverworts (Marchantiophyta) are key in assessing the hypothesis that sporogenesis and spores originated before the sporophyte through heterochrony and the transfer of specialized wall from zygotes in green alga to meiospores in plants (Brown and Lemmon, 2011). Therefore, the ephemeral liverwort sporophyte and spores contained within may be living evidence of the pioneering land plants that evolved following the “spores first” era of terrestrialization.

As a result of the Liverwort Tree of Life initiative and intensive molecular analyses over the past decade, phylogenetic relationships among Marchantiophyta largely have been solidified (Forrest et al., 2006). The phylum comprises three major lineages: Haplomitriopsida, Marchantiopsida (complex thalloids), and Jungermanniopsida (simple thalloids and leafy liverworts) with approximately 5000 species in some 400 genera (Crandall-Stotler et al., 2009a,b). Among these, the Haplomitriopsida, including Treubiales with two genera and the monotypic Haplomitriales, is sister to all other liverworts, while the Blasiales with two genera is sister to the remaining complex thalloids. As representative relictual taxa, spores of *Apotreubia*, and *Blasia* are described herein. Gametophytes of these three orders are morphologically distinct from other liverworts, including members of the diverse Jungermanniopsida that contains simple thalloid and leafy liverworts with over 90% of liverwort species. Because aspects of sporogenesis, especially patterns and behavior of plastids, microtubules and

centrosomes during meiosis, are exceptionally variable across liverworts (Brown and Lemmon, 2013), it is reasonable to expect similar diversity of spore wall architecture.

The mosses (Bryophyta) are the largest group of bryophytes consisting of some 10,000 species in three or four distinct clades usually recognized formally as classes (Goffinet et al., 2009; Shaw et al., 2011). The morphology of both life history phases is distinctive and features of the sporophyte and sporogenesis are remarkably uniform throughout most taxa in this specialized and successful group. All mosses investigated to date undergo monoplastic meiosis and most produce hilate spores (Brown and Lemmon, 1990b, 2013). Unlike liverwort spores that are inaperturate, spores of mosses may have well-developed apertures, which are localized modifications of the proximal pole involving all wall layers and functioning in germination. Although the order of diversification is controversial, it is clear that the Sphagnopsida with four genera and the monogeneric Takakiopsida are sister to all other mosses, followed by the Andreaeopsida with two genera and then the remainder of advanced orders (Renzaglia et al., 2007; Cox et al., 2010). Representatives from these three early divergent classes as described in this study exhibit peculiarities in patterns of spore wall deposition and organization. Together with the enigmatic *Oedipodium*, the taxon emerging as sister to peristomate mosses, *Takakia* and *Sphagnum* have distinct trilete apertures that distinguish them from the more derived hilate apertures of other mosses (Renzaglia et al., 1997; Shimamura and Deguchi, 2008; Brown and Lemmon, 2013).

Hornworts (Anthoceroophyta) are a small (about 200 species in 12 genera) but evolutionarily important group. Like the mosses, sporocyte quadripolarity is reflected consistently in cytoplasmic lobing and monoplastic meiosis. Most current phylogenies find hornworts to be the closest extant relatives of the tracheophytes (Qiu et al., 2006; Renzaglia et al., 2009; Chang and Graham, 2011), but this widely accepted hypothesis has been challenged recently by rigorous comprehensive analyses (Cox et al., 2014; Wickett et al., 2014). Within group classifications align the monospecific *Leiosporoceros* as sister to all other hornworts and in a separate class Leiosporocerotopsida (Renzaglia et al., 2009; Villarreal et al., 2010). A clade containing traditional members of *Anthoceros*, *Sphaerosporoceros* and *Folioceros* is the next to diverge. Here we describe spores of *Leiosporoceros*.

2. Materials and methods

2.1. Microscopy

Specimens were fixed in our laboratories over several decades using standard TEM and SEM procedures similar to those in Brown and Lemmon (1986) and Renzaglia et al. (1997). Developing capsules were cut in half and fixed in 4% glutaraldehyde pH 6.9–7.2 in 0.05 or 0.1 M phosphate or Pipes buffer for at least 30 min, postfixed in 1% osmium tetroxide for 15 min to 1 h, and washed overnight in d-H₂O (all at room temperatures). The materials were dehydrated in a graded acetone series and for SEM critical point dried, mounted on stubs and viewed. For light and transmission electron microscopy, acetone was replaced by propylene oxide and specimens were gradually infiltrated with Spurr's resin over several days. Semi-thin sections (0.5–1.0 μm) were collected on glass slides, stained with 1.0% toluidine blue containing sodium borate, monitored for mature or nearly mature spores and photographed on a light microscope. Ultrathin sections were stained sequentially with uranyl acetate and lead citrate, and viewed in a transmission electron microscope.

3. Results

3.1. Developmental patterns

In *Haplomitrium* and *Apotreubia*, and retained in some core taxa, patterned precursors that define spore wall ornamentation are present on

the surfaces of the diploid sporocyte cytoplasm before meiosis (Plate I, 1–3). Distal walls are ornamented by exine laid down in these precursors after meiosis. No such precursors occur in the mosses or hornwort. Tripartite lamellae (TPL) play fundamental roles in the ontogeny of the outer spore wall (exospore or exine) in all the bryophytes examined, except *Andreaea* (Plate I, 4–9). TPL initiate exine formation where they form in association with the plasma membrane (Plate I, 5–9). In the three liverworts, they also appear in the precursors at some distance from the plasma membrane. Plates of TPL in mosses are associated with exine initiation (Plate I, 6) and a single continuous TPL (the so-called foundation layer) forms first (Plate I, 7), followed by exine development toward the outside (centrifugal) and then intine development toward the inside (centripetal). A foundation layer between exine and intine is visible in *Oedipodium* spores but development of wall layers has not been observed. Multilamellate layers (MLLs) comprised of compacted stacks of TPL, form a continuous layer in the initiation of exine in spores of *Sphagnum* (Plate I, 5), *Haplomitrium* and *Leiosporoceros* (Plate I, 8). Later exine development is centrifugal in *Sphagnum* but centripetal in *Leiosporoceros* and liverworts.

Sporocytes of all taxa except *Sphagnum* and *Takakia* are quadrilobed to varying degrees, with the three liverworts exhibiting the most pronounced lobing. Lobing is reflected in spore architecture. Specifically, distal surfaces that develop from unlobed to slightly lobed sporocytes such as *Takakia*, *Sphagnum* and *Andreaea* are restricted to the free spore surface. In sporocytes that are deeply lobed such as *Haplomitrium* and *Blasia* (Renzaglia et al., 1994), distal ornamentation curves around the lobed surface into the region where spores are juxtaposed and covers considerably more than half of the spore. Unlobed to slightly lobed sporocytes result in spores that have well-delineated proximal surfaces and are trifacial, often with trilete marks and aperture differentiation. In the mosses and hornworts, modifications of the proximal pole have evolved into germinal apertures of varying complexity. The descriptions and images below further illustrate these variations.

3.2. Spore descriptions

3.2.1. *Haplomitrium hookeri* (Smith) Nees

Spores of *Haplomitrium hookeri* (Plate II, 1–3) exhibit clavate ornamentation that covers the spore up to the small proximal pole (Plate II, 1, 3), where the cytoplasm of the quadrilobed polyplastidic sporocytes remains in common until meiosis/cytokinesis. The clavate projections occasionally fuse into ridges or clusters. These sculptural elements of the exine develop after meiosis within the sporocytic premeiotic precursors and consist of caps with scattered segments of TPL surrounded by sparse sporopollenin (Plate II, 2). In the mature spore, TPL are obscured by sporopollenin deposition. Subtending the caps is a middle spumous (frothy) layer forming a stalk, delimited by a single TPL, which in turn is underlain by an inner exine consisting of a continuous MLL (Plate II, 2). Between the MLL and the plasma membrane is a layer of intine. The proximal surface produced after simultaneous cytokinesis develops without precursors and is therefore poorly delimited and lacks ornamentation (Plate II, 3). No proximal aperture nor trilete mark is present.

3.2.2. *Apotreubia nana* (Sinski Hattori & Inoue) Sinski Hattori & Mizutani

In *Apotreubia nana* (Plate III, 1–3) there is pronounced definition of distal and proximal wall patterns, which are clearly demarcated by a low equatorial collar or cingulum (Plate III, 3). As in *Haplomitrium*, sporocytes are deeply lobed and the pattern of ornamentation is determined by exine precursors that develop in the sporocyte wall during meiotic prophase. A reticulate ornamentation covers the distal surface. The muri contain sporopollenin and slips of TPL that are more or less parallel in the radial elements and continue into the connecting ridges (Plate III, 1, 2). The outer ornamentation is underlain by a thin continuous MLL (Plate III, 2). The inner exine layer has a homogeneous texture (no discernible TPLs) at maturity and a medium opacity, distinct from

that of the sculptoderm. This smooth layer of exine is totally absent in *Haplomitrium hookeri*. The intine is approximately twice the thickness of the inner exine. The proximal surface adjacent to the cingulum exhibits a modified reticulate ornamentation, while the inner-most surface is rugulate, lacking an aperture and trilete mark (Plate III, 3).

3.2.3. *Blasia pusilla* L.

Sporocytes of *Blasia pusilla* are monoplastidic and lobed. Spores are round with a simple exospore structure (Plate IV, 1–3). The spore is covered with granulate ornamentation with a small ridge often delimiting distal and proximal surfaces (not illustrated). The baculae are made of loosely stacked TPL and are blunt at the apices (Plate IV, 2). Subtending the baculae are several loose wavy concentric layers of MLL and a relatively thick intine (Plate IV, 2). Granulate ornamentation extends in a modified, less pronounced form along the proximal spore surface (Plate IV, 1, 3). It is not known if the ornamentation is determined by exine precursors and if so how the process differs on the distal and proximal faces.

3.2.4. *Takakia ceratophylla* (Mitten) Grolle

Monoplastidic meiosis occurs in quadripolar but not quadrilobed sporocytes in *Takakia*. The distal spore wall consists of exine and a perine when nearly mature (Plate V, 1–3). Multiple segments of parallel TPL comprise the exine and are visible until spores are nearly mature (Plate V, 2). The proximal face is essentially smooth while the distal surface is covered with coarse regulate ornamentation (Plate V, 3). The spores are distinctively trilete with pronounced triradiate laesurae that overlie a well-developed aperture (Plate V, 1, 3). The commissure (slit) has a median projection surrounded by rounded margins (Plate V, 1). Until nearly mature, the aperture occupies half of the proximal surface and is protuberant with splayed TPL at the margins of the swollen exine (Plate V, 1). At maturity, the spore becomes more rounded and the outer exine is covered by globular perine on the distal and exposed surfaces (Plate V, 3). TPL in the exine are mostly obscured, and an intine develops internal to the exine late in development (not illustrated).

3.2.5. *Sphagnum lescurii* Sullivant

Quadripolarity is strongly expressed in monoplastidic meiosis and development of a quadripolar microtubule system (QMS), but sporocytes are unlobed and division planes marked only by small wall ingrowths at the surface of sporocytes. Spores of *Sphagnum* are distinctive and not likely to be confused with any others, fossil or living (Brown et al., 1982). The spore of *Sphagnum lescurii* (Plate VI, 1–4) has a smooth spore surface with raised trilaesurate proximal aperture, distal triradial ridges, and a pronounced cingulum (Plate VI, 1, 4). With five internal wall layers, the microanatomy of the sporoderm is the most complex found in bryophytes and is unique among bryophytes if not all plants. The wall (Plate VI, 2) consists of an inner intine, a pronounced and persistent MLL, a homogenous exine layer, a scaly layer of translucent material, and an outer coating of perine. The homogenous exine layer is much thickened at the equator and under the branched distal ornamentation (Plate VI, 1). All layers participate in the formation of the prominent trilaesurate aperture (Plate VI, 3). Perine-covered plates overarch the homogeneous exine, which is greatly thinned at the commissure. The aperture contains a much thickened inner granular exine and expanded intine (Plate VI, 1, 3).

3.2.6. *Andreaea rothii* F. Weber & D. Mohr

Monoplastidic meiosis occurs in slightly quadrilobed sporocytes in *Andreaea*. Spore walls of the *Andreaeopsida* are unusual with a flocculent appearance in the TEM (Plate VII, 1–3). Spore wall deposition occurs in the absence of TPL. Sporopollenin is deposited as loosely packed globules rather than in distinct layers (Plate VII, 1, 2). The mature wall consists of three layers, the inner intine, a loosely compacted spongy exine, and an outer globular exine covered by perine (Plate VII, 1, 3). The sporoderm is somewhat thinner on the proximal surface than the distal. The inaperturate spores are distinctly trifacial (Plate

VII, 3) with the angular faces of the proximal spore surface deposited while spores are contained in the long-lived sporocyte wall.

3.2.7. *Oedipodium griffithianum* (Dickson) Schwägrichen

Monoplastidic meiosis occurs in sporocytes that become lobed in conjunction with the tetrahedral positioning of the plastids. Spores of *Oedipodium* exhibit a marked polarity with clavate distal spore ornamentation and smooth proximal surface containing a trilaesurate aperture (Plate VIII, 1–6). The smooth proximal spore wall has a thickened intine, a narrow TPL-containing exine and a thin outer exine (Plate VIII, 1). The distal spore wall consists of a thin intine, a single exine and perine. Clavate distal elements are homogeneous projections of the exine covered by sporopollenin (Plate VIII, 2). A foundation layer is visible between exine and intine. The trilete area has a thick inner exine flanked by splayed TPL. At the equatorial region, the intine is thicker and the margin reinforced by separated segments of TPL in the inner exine (Plate VIII, 4). The mature wall is similar to bryopsid mosses except that TPL are more abundant and the trilaesurate aperture is more pronounced (Plate VIII, 5). Spores tend to remain in tetrads even after the persistent sporocyte wall is gone (Plate VIII, 6).

3.2.8. *Leiosporoceros dussii* (Stephani) Hässel

As is true in general for hornworts, *Leiosporoceros* is monoplastidic in both meiosis and vegetative tissue. The spherical sporocyte becomes lobed internally in conjunction with positioning of the four plastids in prophase. The elongated, smooth spores (Plate IX, 1–4) are the simplest of any bryophyte examined to date. Spore tetrads are isobilateral with spore pairs in contact at opposite ends of the tetrad (Plate IX, 1,4), leading to a monolete aperture and one slightly flattened end (Plate IX, 4). In development the wall begins with a pronounced MLL consisting of a stack of closely compressed TPL (Plate I, 8), much like that of *Sphagnum*, but the MLL forms the outer boundary of the spore, and sporopollenin is deposited within and to the inside of this layer. The simple mature wall consists of a two-layered exospore underlain by a thin fibrillar intine (Plate IX, 1). The simple monolete aperture is bordered by raised lips and consists of an intine, a thickened inner exine, and a thin outer exine at the commissure (Plate IX, 2).

3.3. Persistent tetrads and dyads

Variations in adherence of spores in tetrads are evident across the taxa in this study. These are not permanent connections as they are in *Sphaerocarpos* (Renzaglia et al., in press) but they are frequently encountered in nearly mature spores or when they are dispersed. In certain *Haplomitrium* species, spores remain in tetrads or dyads (Renzaglia and Crandall-Stotler, unpublished) even after maturity (Plate X, 1). These may be dispersed as such but often separate into monads. In several taxa, tetrads remain surrounded by the spore mother cell wall until late in development as exemplified by *Takakia* (Plate X, 2). Because of the unusual isobilateral tetrad, dyads may persist in *Leiosporoceros* (Plate X, 3).

3.4. Summary of characters

For ease of comparison, the major features in spore wall development of taxa discussed herein are provided in Table 1.

4. Discussion

Sporogenesis has long been thought to be a conservative process, especially when viewed from the perspective of angiosperms, but the overall complex program is extremely variable in bryophytes (Brown and Lemmon, 2013). Variation is seen in the precocious cytoplasmic preparation for cytokinesis, organization of the cytoskeleton, origin of meiotic spindles, determination of spore ornamentation pattern, ontogeny of wall layers, and complexity of proximal apertures, if they occur. Spore wall ontogeny in bryophytes has been described generally and the degree of variability in spore-related features recognized (Brown and Lemmon, 1988, 1990a), but no attempt had been made to critically examine the taxa occupying basal positions in the phylogenies of the major groups. The diversity of characters revealed in this study corroborates the concept that the establishment of spore bearing plants on land was accompanied by a sweeping radiation into open and abundant niches. The early fossil record supports this notion and reveals a spore flora that was diverse and unique long before megafossils appeared (Wellman and Gray, 2000; Edwards et al., 2014).

Plate I. Basic patterns of exine initiation in bryophyte spores. All illustrations are oriented with the sporocyte wall uppermost. Magnification is based on the single bar in 1. 1–3. Wall pattern is determined by distinct precursors (P) associated with cytoplasmic stalks in the 2n sporocyte wall (SW) in certain liverworts. PM, plasma membrane. 1. TEM of capitulate precursors in *Haplomitrium*. Bar = 0.2 µm. 2. Aniline blue stain showing callosic nature of reticulate exine precursors in the deeply lobed sporocyte of *Pallavicinia*. Bar = 6.1 µm. 3. TEM of exine precursors of *Pallavicinia* showing the elongate cytoplasmic stalks capped by exine precursors. Bar = 0.33 µm. 4. *Andreaea*. The spore wall is deposited by the young spore within the sporocyte wall (SW) as globules of initial exine (E) adjacent to the plasma membrane (PM). Bar = 0.09 µm. 5. *Sphagnum*. The first layer of exine in the young spore is a continuous multilamellate layer (MLL) that develops in the sporocyte wall (SW). The plasma membrane (PM) is underlain by microtubules (MT). Bar = 0.12 µm. 6. *Takakia*. Segments of tripartite lamellae (TPL) develop outside the plasma membrane in the sporocyte wall (SW) that surrounds the young spore. Bar = 0.1 µm. 7. In bryopsid mosses, a single continuous layer of tripartite lamella (TPL) establishes the foundation layer in the spore wall. SW, sporocyte wall. Bar = 0.1 µm. 8. *Leiosporoceros*. Following meiosis, a distinct multilamellate layer (MLL) is deposited outside the plasma membrane in the sporocyte wall (SW). Bar = 0.12 µm.

Plate II. *Haplomitrium hookeri*. Magnification is based on the single bar in 1. 1. Cross section through a nearly mature spore. Capitulate ornamentation on circumference of spore except for the narrow proximal pole (PX). A mass of central amyloplasts is adjacent to the nucleus (N) and lipid droplets (L) are scattered peripherally. Bar = 1.5 µm. 2. Detail of wall layers. The cap (C) of short TPL tops the spongy stalk (S). The outer layers are subtended by a continuous MLL and intine (I). Bar = 0.25 µm. 3. SEM of three spores of the tetrahedral tetrad. The circumference of the spores is uniformly capitulate except for the narrow region surrounding the proximal poles. Bar = 5.0 µm. (See on page 6)

Plate III. *Apotreubia nana*. Magnification is based on the single bar in 1. 1. Cross section through a nearly mature spore. Amylochloroplasts (A) surround the central nucleus (N). Bar = 1.9 µm. 2. The mature wall from plasma membrane (PM) out consists of a prominent intine (I), inner exine (IE) that includes a multilamellate layer (MLL) impregnated with sporopollenin, and an outer exine (OE) that constitutes the ornamentation. Bar = 0.3 µm. 3. SEM of three spores of the tetrahedral tetrad showing distinct proximal and distal faces. Bar = 3.3 µm. (See on page 7)

Plate IV. *Blasia pusilla*. Magnification is based on the single bar in 1. 1. Cross section through a nearly mature spore. Amylochloroplasts (A) with extensive thylakoid systems surround nucleus (N) and protein bodies are abundant. Papillate ornamentation surrounds the spore with less regular and lower papillae on the proximal face (PX). Bar = 1.0 µm. 2. Detail of wall layers. Papillate ornamentation composed of successive layers of curving TPL grade into an irregular subtending layer of MLL. Plasma membrane, P; Intine, I. Bar = 0.8 µm. 3. Thinner, less ornate proximal walls of adjacent spores in a tetrad with prominent electron-lucent intines. Bar = 0.5 µm. (See on page 8)

Plate V. *Takakia ceratophylla*. Magnification is based on the single bar in 1. 1. Cross section through a portion of a nearly mature tetrad prior to spore rounding showing the large apertures of expanded exine at the inner contact faces. A thin intine is present only at the aperture. Bar = 1.4 µm. 2. Detail of wall layers showing amorphous perine (P), TPL throughout the exine (E), and no intine. Bar = 0.18 µm. 3. SEM of spores. The uppermost shows the proximal trilaesurate aperture (arrow) on a smooth proximal face. Bar = 7.2 µm. (See on page 9)

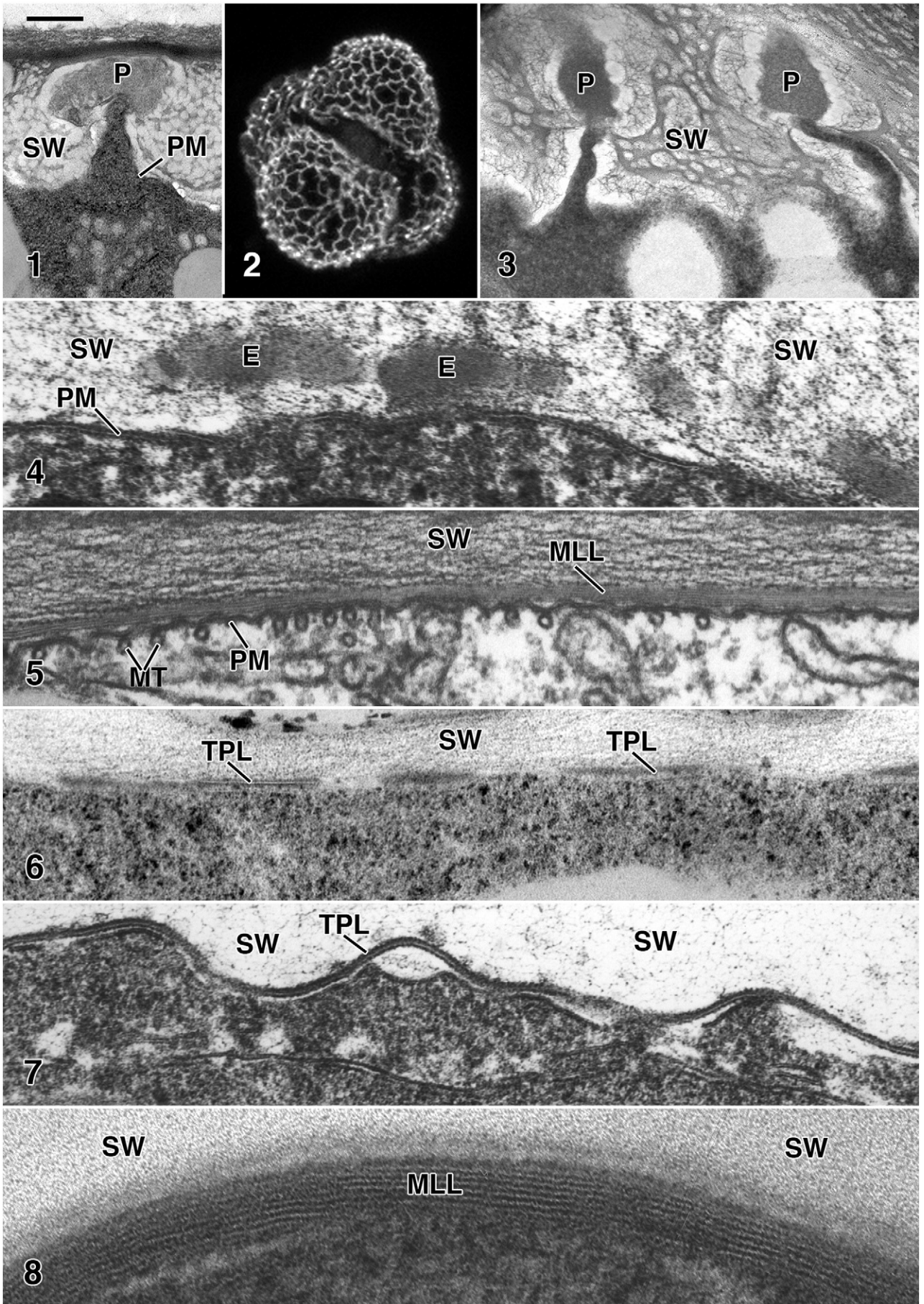


Plate I.

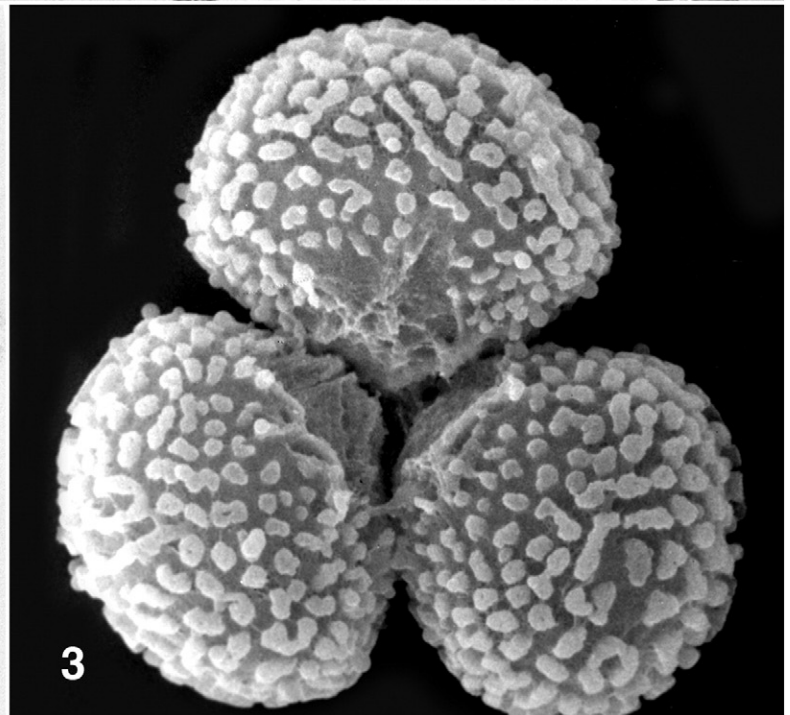
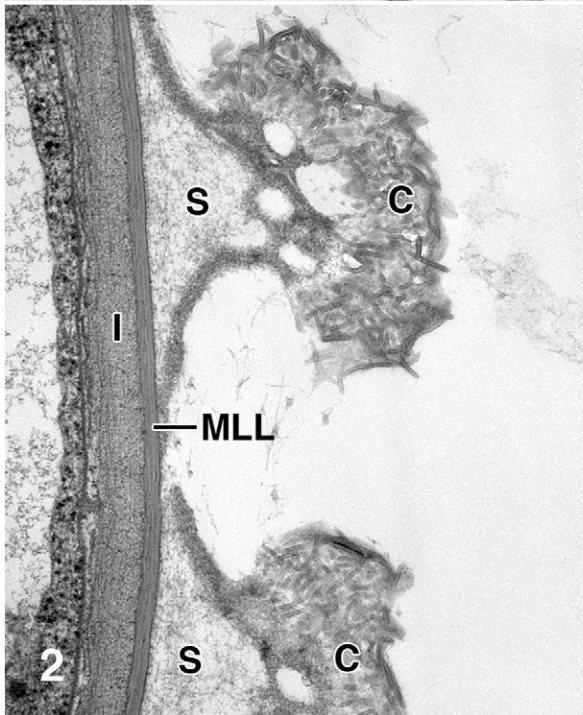
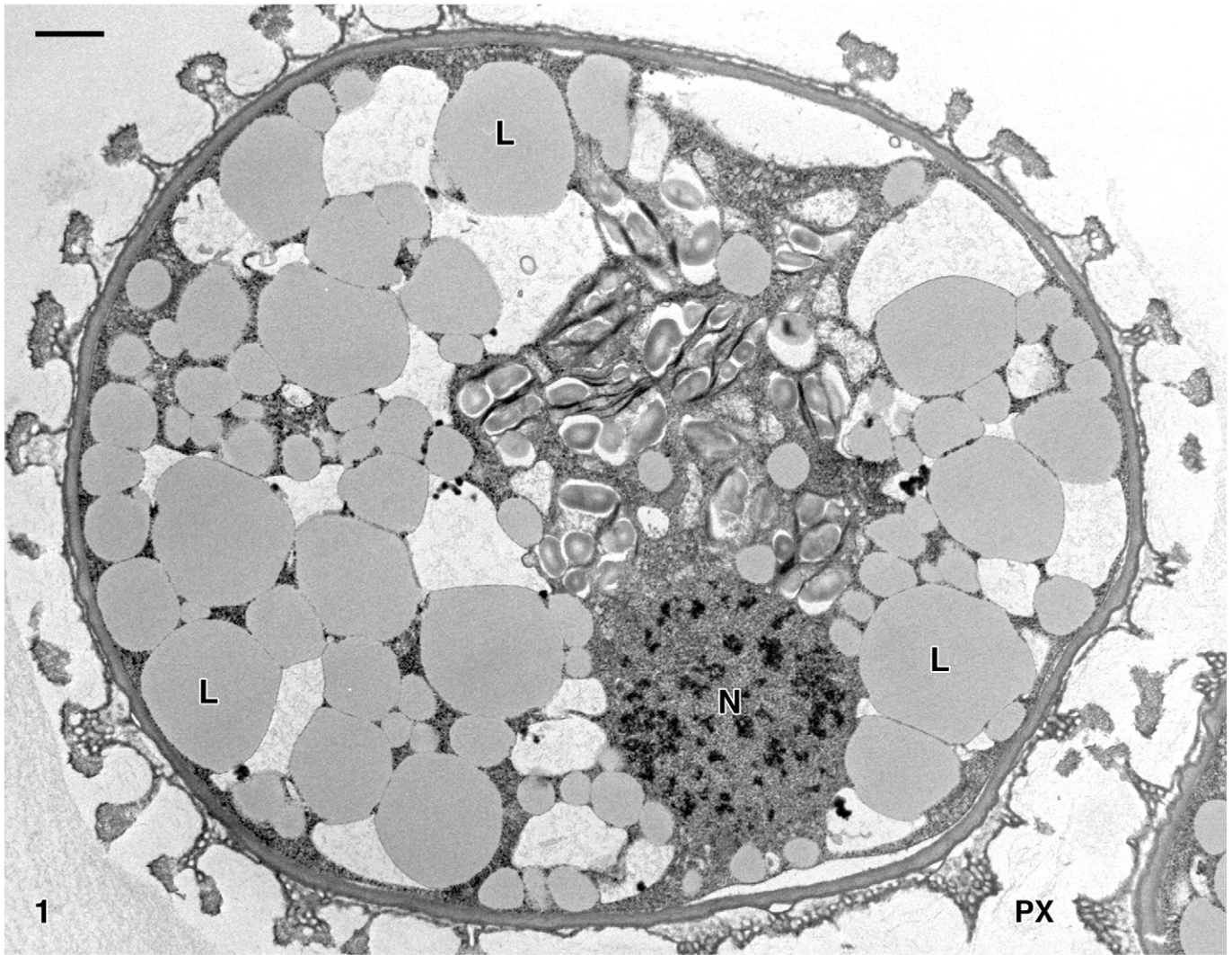


Plate II. (caption on page 4).

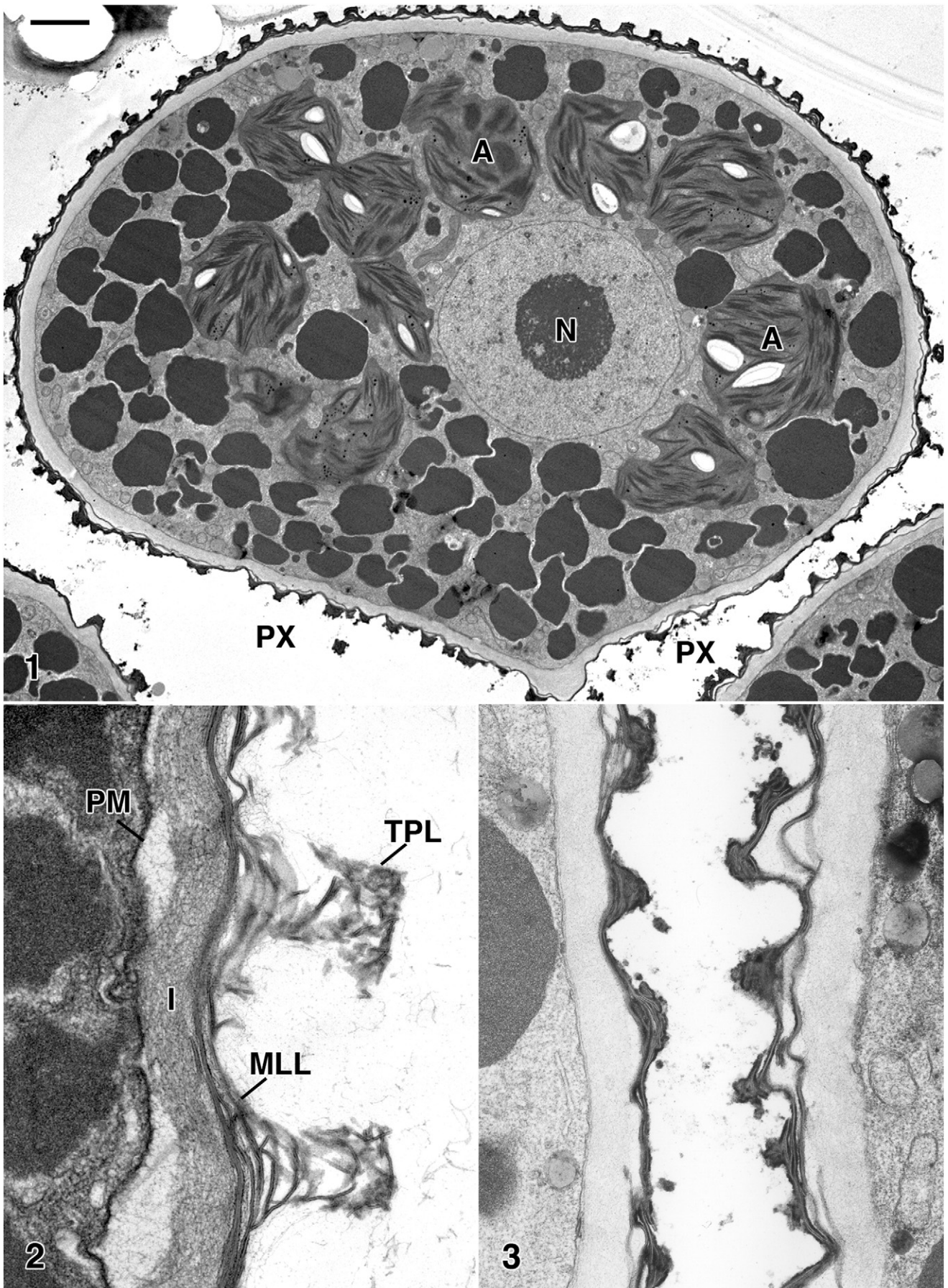


Plate IV. (caption on page 4).

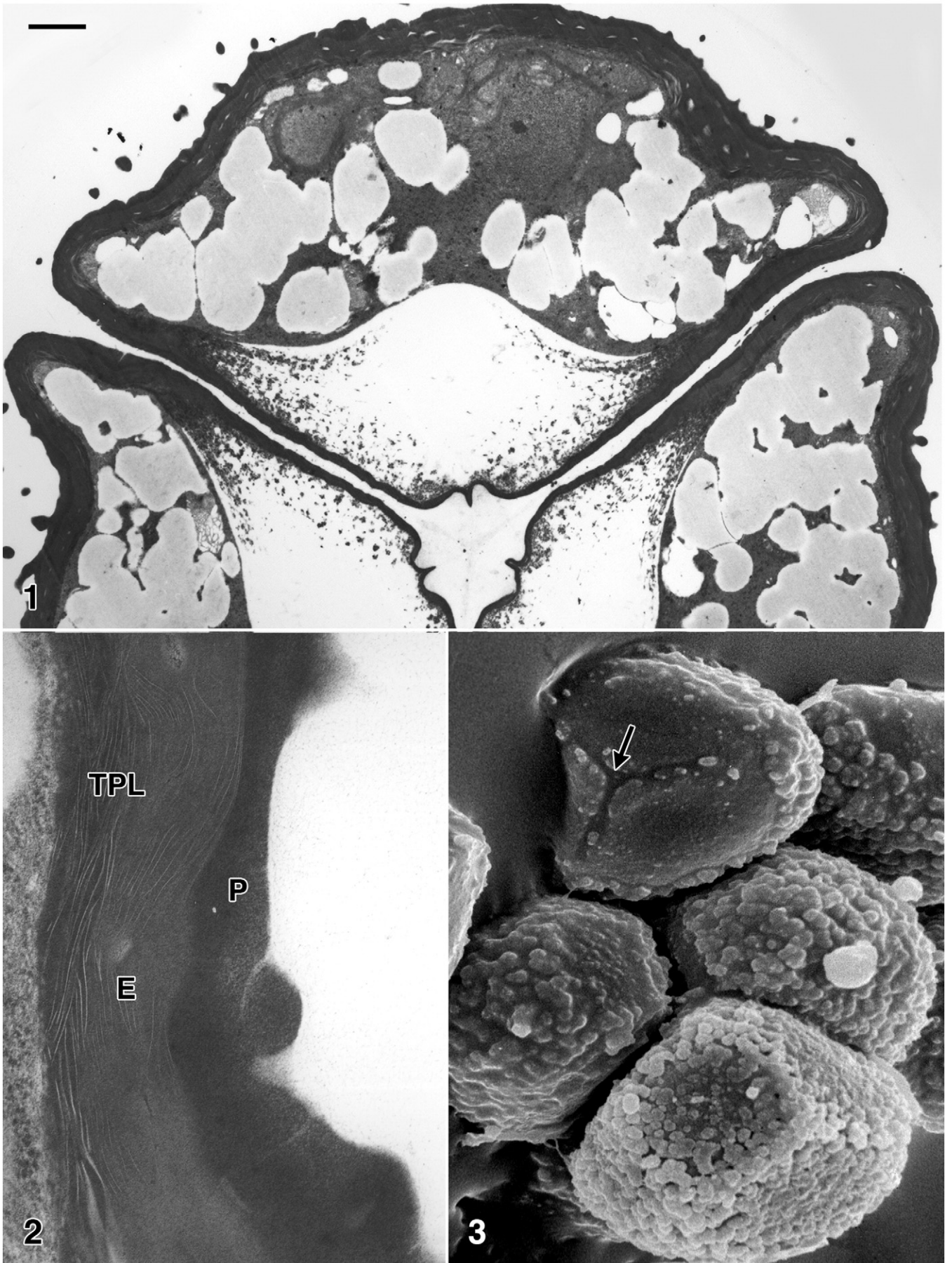


Plate V. (caption on page 4).

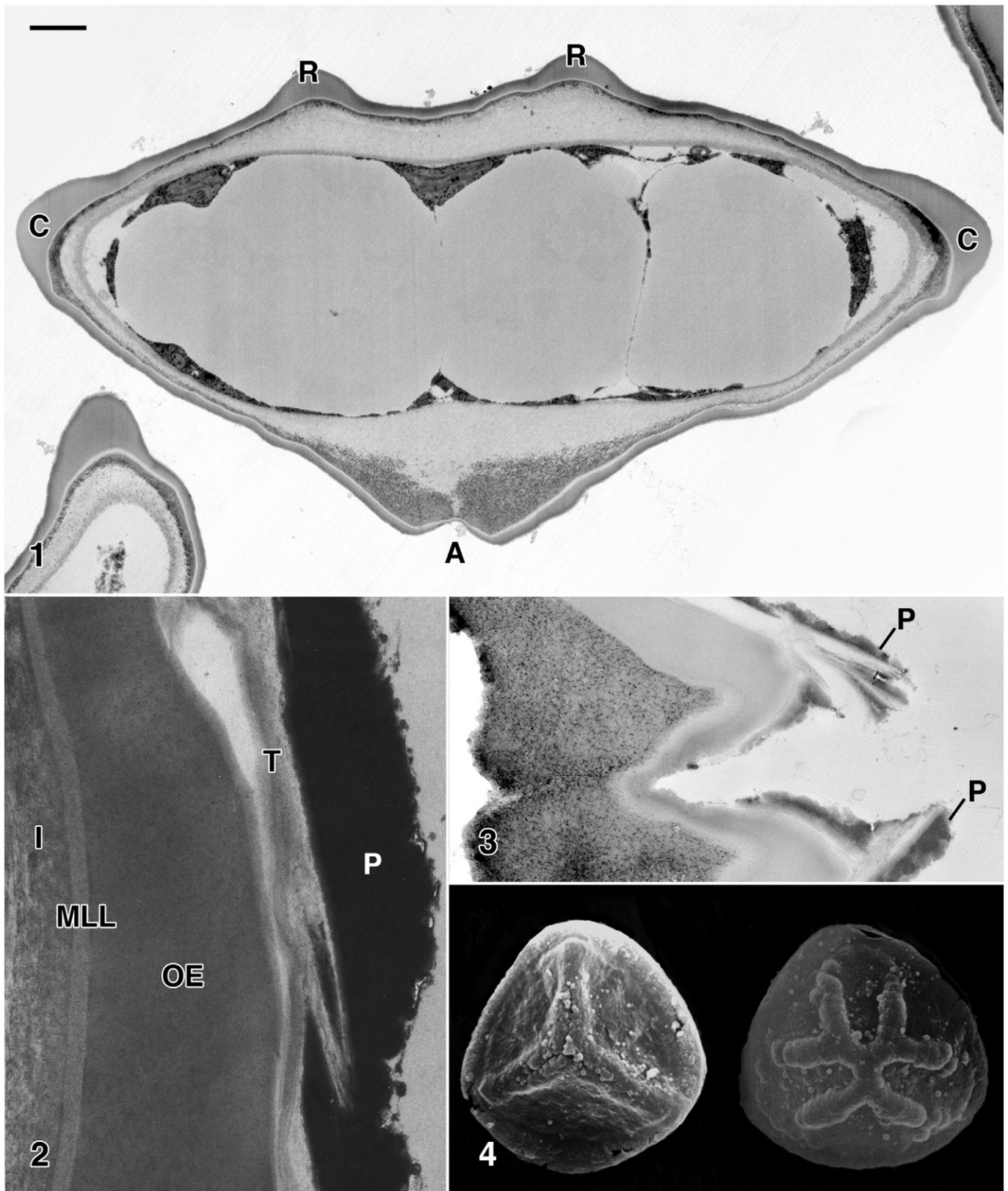


Plate VI. *Sphagnum lescurii*. Magnification is based on the single bar in 1. 1. Cross section through a nearly mature spore filled with lipids. Ornamental thickenings clearly revealed. The exine is thicker at the cingulum (C) and less so over distal ridges (R). An arm of the trilaesurate aperture (A) is seen on the proximal face. Bar = 1.8 μm . 2. Detail of five layered spore wall that consists of intine (I), foundational multilamellate layer (MLL), outer exine (OE), translucent layer with scales (T), and perine (P). Bar = 0.2 μm . 3. Section through portion of an aperture showing thickened intine, deep cleft in the exine, and perine (P) covered translucent plates cover the aperture opening. Bar = 0.5 μm . 4. SEM of spores. Proximal face with conspicuous trilaesurate aperture (left) and distal surface with triradial markings (right). Bar = 7.2 μm .

A central feature of bryophyte sporogenesis is the extensive preparation for division polarity and cytokinesis that is clearly manifested in quadrilobing (Brown and Lemmon, 1988, 2013). The sporocytes of all of the relictual bryophytes studied are quadrilobed with the exception of *Sphagnum* and *Takakia*, which have spherical sporocytes with slight wall invaginations that mark division planes. The developmental pattern of tetrahedral bryophyte spores stems from quadripolarity of the sporocyte established early in meiosis (see Brown and Lemmon, 2013). Quadrilobing frequently involves monoplastidy, the presence of a single plastid in the emergent sporocyte. The single plastid undergoes two rounds of division and the resultant four plastids are distributed to the tetrad poles where they are involved in the nucleation of microtubule arrays throughout the process of meiosis (Brown and Lemmon, 1990b, 2013).

Monoplastidic meiosis occurs in all mosses and hornworts and although uncommon in liverworts, in two of the early divergent taxa considered here, i.e., *Haplomitrium* and *Blasia*. The report of both monoplastidic and polyplastidic meiosis in lobed sporocytes of different species of *Haplomitrium* (Renzaglia et al., 1994) suggests that additional studies focused on the small Haplomitriopsida may shed light on the origin of polyplastidic meiosis. Some complex thalloid taxa exhibit monoplastidic meiosis without quadrilobing of the sporocyte (Brown and Lemmon, 2013; Shimamura et al., 2001, 2003a). The Blasiales (*Blasia* and *Cavicularia*) are unusual among the complex thalloid clade in that they undergo monoplastidic meiosis in deeply quadrilobed sporocytes (Renzaglia et al., 1994; Shimamura et al., 2005). An inference based on these observations is that monoplastidy and quadrilobing are plesiomorphic in bryophytes.

A fundamental component of exine development in land plants is the participation of TPL that serve as sites for sporopollenin deposition. TPL, also known as white line lamellae, each consist of a translucent layer sandwiched between two osmiophilic layers to produce a trilayered structure about 10 nm thick. TPL have the cross sectional ultrastructure of a unit membrane. The production of TPL is poorly understood and perhaps best illustrated in *Fossombronina*, where TPL first appear as isolated discoid platelets outside the plasma membrane that stack up to form the initial spore wall (Brown and Lemmon, 1986, 1993). Similar TPL and derived multilamellate layers (MLLs) made of many TPL are seen in the oldest fossil palynomorphs found in the Ordovician (ca. 470 million years before present) and are widely distributed in extant pollen and spores from bryophytes to seed plants (Brown and Lemmon, 1990a; Lugardon, 1990; Wellman, 2004). TPL are described in all but one of the relictual bryophytes describe herein, and among all bryophytes are absent from spore wall development solely in the Andreaeopsida (Brown and Lemmon, 1984). MLLs are most prominent in spores of many liverworts, especially complex thalloid taxa, where very thick, ornate walls are made up exclusively of layer upon layer of sporopollenin covered MLLs (Brown and Lemmon, 1990a). Development of these complex sporoderms is essentially an elaboration of the liverwort type wall described in this paper, which consists of outer sculpted elements made up of TPLs subtended by an irregularly thickened inner exine consisting of a band of MLLs.

Stacks of TPLs resulting in MLLs are a key feature in the construction of spore walls in land plants and are absent in algal spores. Based on the occurrence of lamellae (MLLs) in several Ordovician and Silurian fossils and their existence in most of the relictual bryophyte spores presented herein it can be inferred that TPL and MLL are plesiomorphic in embryophyte spores as concluded by Blackmore and Barnes (1987) based on morphologic character assessment.

The exceptional layering of multiple segments of TPL not associated with the aperture in *Takakia*, and to a lesser extent *Oedipodium*, is unique among bryophytes and significantly extends TPL involvement in exine development in mosses. The so-called lamellae illustrated in spores of *Cymbosporites* from the Silurian (Johnson and Taylor, 2005) are remarkably similar to those in *Takakia* and suggest moss affinity. *Sphagnum* has an exine with a concentric MLL layer and in most

bryopsid mosses there is only a single TPL upon which the exine is built. Slips of TPL may also occur at the margin of apertures (Brown and Lemmon, 1988, 1990a) as in *Takakia* and *Oedipodium*. TPL are more elusive in hornworts and consequently escaped detection in early developmental studies (Brown and Lemmon, 1990a). However, an MLL is a conspicuous element of exine initiation in *Leiosporoceros* where the role of the TPL differs somewhat from that in mosses and liverworts. In *Leiosporoceros*, the MLL is displaced to the outside of the exine, where it is bathed in sporopollenin. Folding of this outer rigid exine forms the ornamentation in hornwort spores. Loose sporopollenin is deposited centripetally from this outer layer inward to form the bulk of the thickness of the simple wall. An intine forms late in development and is not always evident in spores.

Liverwort spores do not have apertures. There is a difference in proximal and distal ornamentation that largely reflects cytokinesis and the delimitation and arrangement of spores in the tetrad. Liverwort spore formation is typified by extensive remodeling of the 2n sporocytes in anticipation of meiosis and cytokinesis. The sporocytes of the Haplomitriopsida are deeply quadrilobed and the pattern of ornamentation is determined by the formation of exine precursors at the surface of the 2n sporocytes (Brown et al., 1986). The spore domains are defined by quadrilobing and remain attached only by a narrow common isthmus. This isthmus region is never covered by exine precursors and is not separated until cytokinesis; a low sculpture pattern develops in this region marking the proximal polar region. There is no discernible structure associated with germination and there is no evidence of a thickened or otherwise modified central proximal wall. This is in contrast to the mosses and hornwort *Leiosporoceros*, all of which develop a distinct aperture on the proximal surface. All of the relictual mosses, with the exception of *Andreaea*, have trilaesurate apertures that develop at the common contact faces of the tetrahedral spore tetrads. The apertures exhibit modifications in all wall layers such as a thinned outer exine and thickened inner exine and intine. The aperture in hornworts is simple in design with a thin outer exine and widened inner exine, but this region is devoid of sporopollenin at the commissure, a condition that is also often evident at the equatorial region (cingulum). Consequently, when many hornwort spores germinate, the proximal wall dissociates from the distal wall and separates into three triangular units (Renzaglia, 1978). This spore wall structure is evident in the fossil *Ephanisporites*, indicating hornwort-like spores had diversified by the early Devonian (Taylor et al., 2010).

The presence of trilete apertures in relictual moss and hornwort spores is in contrast to the inaperturate spores of liverworts and the specialized hilate aperture that consists of a localized disclike thickening of the intine in the more derived peristomate mosses (Brown and Lemmon, 1980). Spores of *Leiosporoceros* exhibit an unusual monolaesurate proximal aperture, which reflects an unusual isobilateral arrangement (Plate X, 3) (Hässel de Menéndez, 1986). Hornwort spores typically occur in tetrahedral tetrads and exhibit trifacial proximal surfaces or well-developed trilaesurate apertures (Renzaglia et al., 2009). The preponderance of trilete apertures and trifacial spores in relictual bryophytes is important in the interpretation of fossil spores because trilete spores abound in the Silurian and Devonian (Wellman and Gray, 2000). It should be noted that in the paleobotanical literature trilete refers to spores that are both trifacial and trilaesurate, while here we restrict the term trilete to those spores with trilete apertures.

Based on the existence of quadripolarity and quadrilobing in spores of relictual bryophytes, early bryophyte spores would have proximal faces with trilete impression marks (Y-shaped), which would remain distinct or become rounded as the spores enlarge and mature. The tetrahedral arrangement of spores that results from precocious delineation of spore domains and the QMS is considered typical of the first spores (Brown and Lemmon, 2011). It is possible that in the earliest stages of embryophyte evolution the distal spore surfaces were adequately walled with sporopollenin while the proximal surfaces had little or no outer wall covering (exospore). The simple laevigate wall of

Leiosporoceros is reminiscent of the abundant early fossil spores that entirely lack ornamentation (Wellman et al., 1998; Wellman and Gray, 2000). Smooth walls without any sculpturing (cf., *Sphagnum*) are unknown from any other bryophyte and their existence in a relictual hornwort is significant. The wall is initiated by TPL, which are compressed into a single continuous MLL. The MLL is displaced to the surface where it forms the spore boundary and the remainder of the wall develops to the inside. There is little differentiation of the proximal/distal surfaces except for the proximal monolete aperture. Although more elaborate sculptoderms are found in hornworts, all can be derived from this basic developmental pattern. The sole occurrence of isobilateral tetrads in *Leiosporoceros*, in contrast, identifies the monolete spores of this hornwort as a derived condition, not ancestral.

A basic pattern of wall development for liverworts could stem from that of *Blasia* where the entire exine is comprised of repeated stacks of TPL. The walls of many liverworts, some with highly ornate exines, are built from repeated stacks of MLL, which are impregnated with sporopollenin to comprise the mature wall (Brown and Lemmon, 1988, 1990a). Some authors (e.g. Taylor and Strother, 2008) posit that this pattern (multilamellate sporoderms built from fusion of multiple lamellae) is the “primitive plant sporoderm type.” Also found in early fossil spores (Taylor, 2000), these lamellae are reminiscent of more derived extant liverworts such as *Sphaerocarpos* and *Petalophyllum* with more elaborate spore walls and abundant food reserve for periods of dormancy. In addition to these basic patterns, there is evidence of early divergence in developmental patterns. Both *Apotreubia* and *Haplomitrium* possess pre-meiotic templates suggesting that diploid determination of wall patterning is plesiomorphic in liverworts and perhaps in land plants. This is consistent with the hypothesis that spores and their elaborate walls evolved directly from an algal zygote wall prior to sporophyte evolution (Brown and Lemmon, 2011). Accelerated spore production is interpreted as highly advantageous in early terrestrial habitats where resources for survival were limited and sporadic. A recent study of permanent tetrads in *Sphaerocarpos* points to the role of a diploid-derived callose layer in establishing layers of MLL that cross spore boundaries and provides a simple mechanism for the development of permanent dyads and tetrads that are abundant in the early fossil record (Renzaglia et al., in press).

The diversity of bryophyte spores indicates multiple lines of evolution into diverse habitats. It is noteworthy that the three relictual liverworts have spores that appear ready to germinate, i.e., they are green with little food reserve, thin exospores and no perine. Perhaps many of the earliest fossil spores were also green with sufficient walls for protection just until germination but not during long periods of adversity. Because such spores would not preserve well in the fossil record, this supposition is difficult to evaluate. However, the relictual spores described in mosses and *Leiosporoceros* contain abundant lipid reserve and thicker exospores than the liverworts, indicating greater desiccation tolerance and longevity.

Colonization of land was possible in large part because of the evolution of spores with thick durable walls capable of withstanding adversities such as desiccation, UV radiation, abrasion, and pathogens (Wellman, 2004; Brown and Lemmon, 2011). We can envision that meiosis was accelerated with precocious determination of division planes, and multiple forms of MTOCs responsible for nucleating the complex microtubule systems associated with meiosis, cytokinesis, and spore wall initiation (see Shimamura et al., 2003a, 2003b; Brown and Lemmon, 2011, 2013). We can also assume that it was advantageous for meiospores to be enclosed in a multilayered wall with thinner areas for germination, and eventually specific germinal apertures. Adherence of spores to each other either within a persistent spore mother

cell wall or by connections between adjacent spores is commonplace among relicts of each of the three bryophyte clades. Comparisons between the spores described herein and fossil spores provide evidence that the so-called envelope-enclosed early fossil tetrads may well be tetrads with persistent spore mother cell walls. Naked tetrads are known from a few living bryophytes such as *Haplomitrium hookeri* and naked dyads do occur naturally as seen in *Leiosporoceros*. No envelope-enclosed dyads occur in extant taxa and these are more problematic to explain, but may be related to successive cytokinesis that is indicated in the cryptospore fossil record prior to the Darriwilian (Taylor and Strother, 2009). Furthermore, variability in the number of meiotic spores produced per zygote in early cryptospores suggests that the control of meiosis was actively being selected for early in bryophyte evolution, presumably in response to survival in subaerial habitats. Variation in bryophytic meiosis (Brown and Lemmon, 2013) and sporogenesis today supports this hypothesis, while the widespread occurrence of simultaneous cytokinesis and tetrads in extant bryophytes indicates that these features were established early in bryophyte evolution.

5. Conclusions

In this paper we compare data from sporogenesis in basal liverworts, mosses, and a hornwort in an attempt to determine plesiomorphy and variability in the critical process of sporogenesis in bryophytes. Starting with liverworts, our observations point to the likelihood that the ancestral spore wall was simple and consisted of a prominent intine and slips of TPL and MLLs forming a thin ornamented exine. TPL and MLLs may or may not be evident in fully mature spore walls. The spores were trifacial or the proximal wall may have been indistinct with irregular projections reflecting a deeply lobed sporocyte. There are no germinal apertures and consequently no trilete spores in liverworts. Relictual mosses demonstrate considerable variability. TPL are abundant and evident in mature spore walls of *Takakia* and *Oedipodium* and an MLL occurs in *Sphagnum*. No TPL are found in *Andreaea* spores. Elaborate apertures characterize most moss spores but are lacking, presumably lost, in *Andreaea*. Perine is unique among bryophytes to moss spores. *Leiosporoceros* produces monolete, elongated spores that are smooth and may adhere in dyads. The peculiar shape of these spores is a function of the orientation in isobilateral tetrads.

Spores were of paramount importance to early survival on land where there was no soil and no shade/protection from severe ultraviolet radiation. The first plants on earth could have survived as spores for long periods until intermittent favorable conditions permitted a round of growth and reproduction. As such, spore characteristics were subject to heavy selection resulting in the diversity of spore types seen in the fossil record and radiation of the sporoderm preceded evolution of the plant body (Taylor and Strother, 2008; Brown and Lemmon, 2011; Strother et al., 2015). Some of this variation in sporoderm is found today in living bryophytes. Further studies of spore wall development in more representatives within each of the three bryophyte groups will be valuable in interpreting the fossil record, and will contribute to knowledge of the evolution and development of a terrestrial land flora from green algal ancestors.

Acknowledgments

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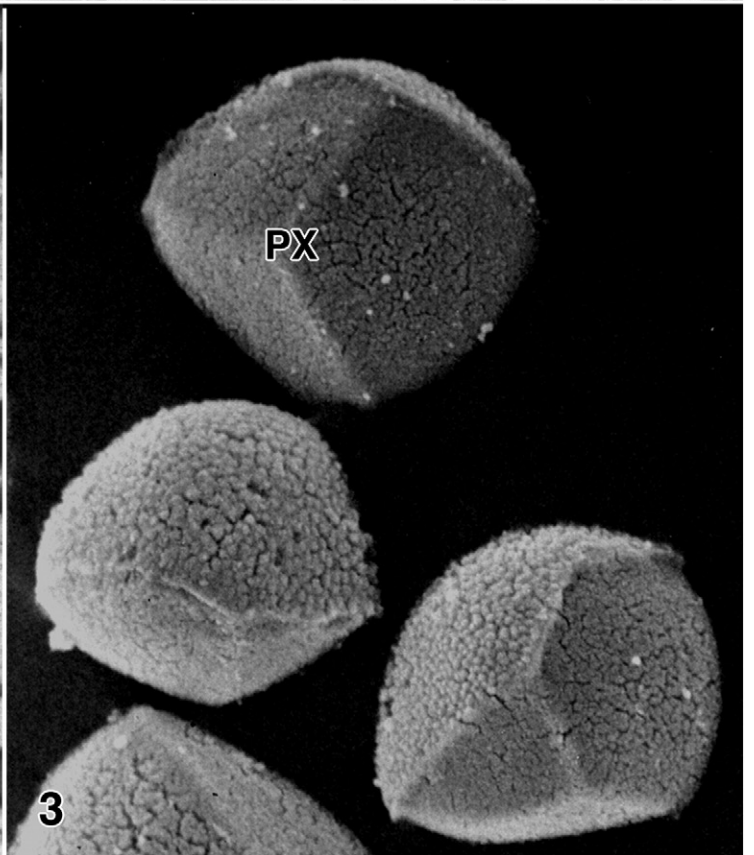
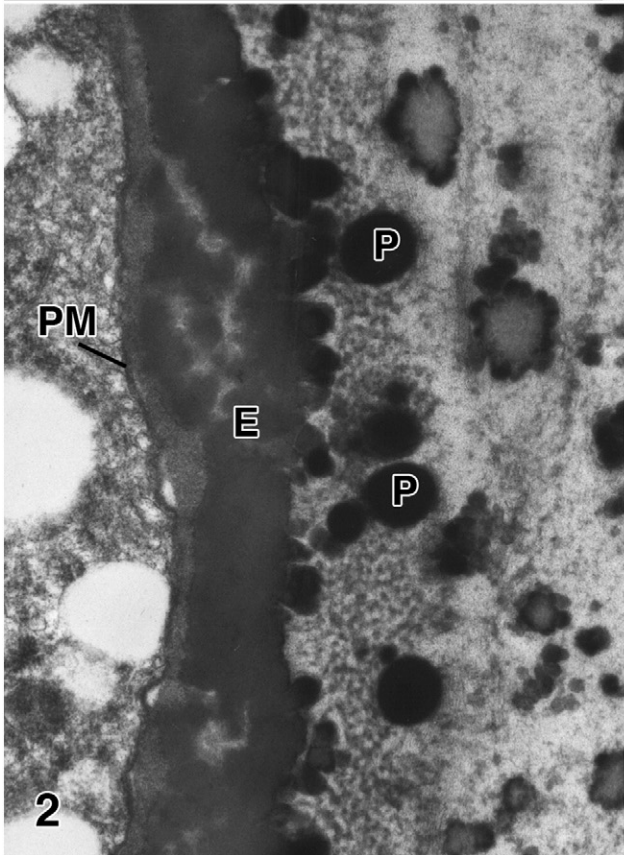
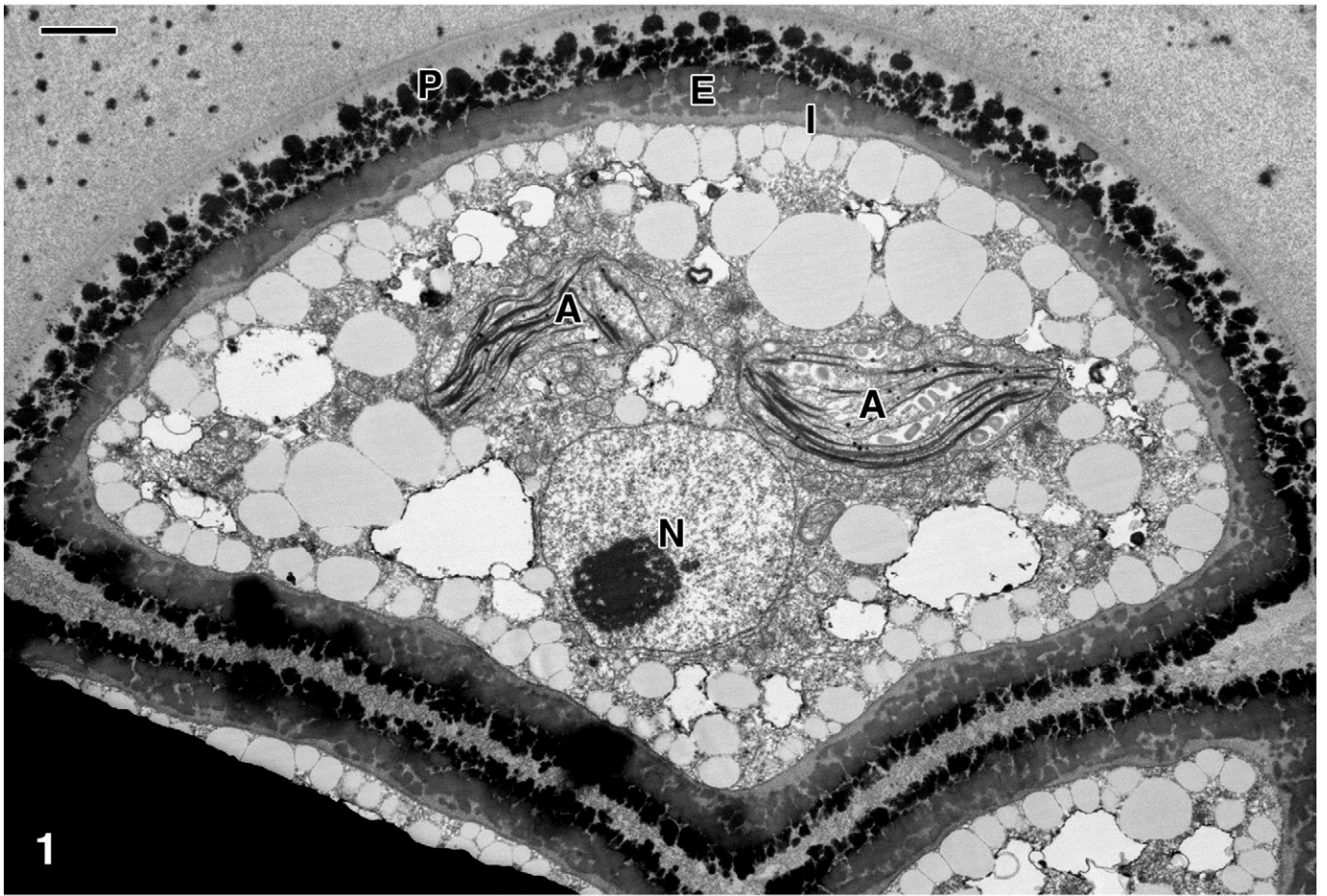


Plate VII.

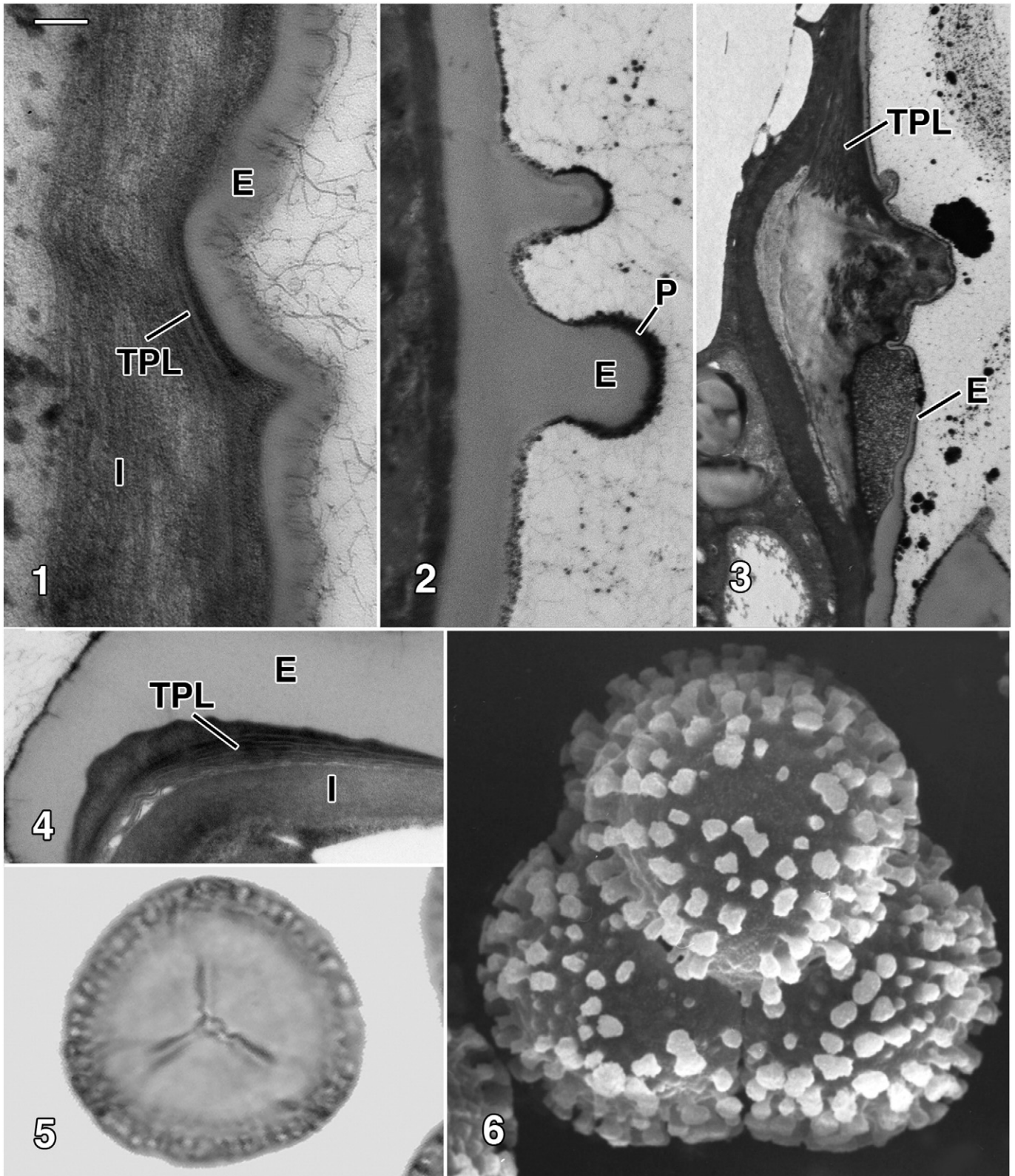


Plate VIII. *Oedipodium griffithianum*. Magnification is based on the single bar in 1. 1. TEM detail of the proximal wall of a nearly mature spore with extensive intine (I), homogeneous outer exine (E) and thin inner exine with TPL. Bar = 0.1 μm . 2. TEM of distal ornamentation in nearly mature spore showing clavate projections of outer exine (E) with sporopollenin deposition to form perine (P). Bar = 0.5 μm . 3. TEM of proximal wall at aperture consisting of thin outer exine and expanded inner exine flanked by TPL and nearly devoid of sporopollenin. Bar = 1.5 μm . 4. Thick outer exine (E) and inner exine prominent with TPL over intine (I) at the equatorial spore region. Bar = 0.25 μm . 5. Light micrograph of proximal face showing distinct trilaesurate aperture. Bar = 4.5 μm . 6. SEM of persistent tetrahedral tetrad. Bar = 4.2 μm .

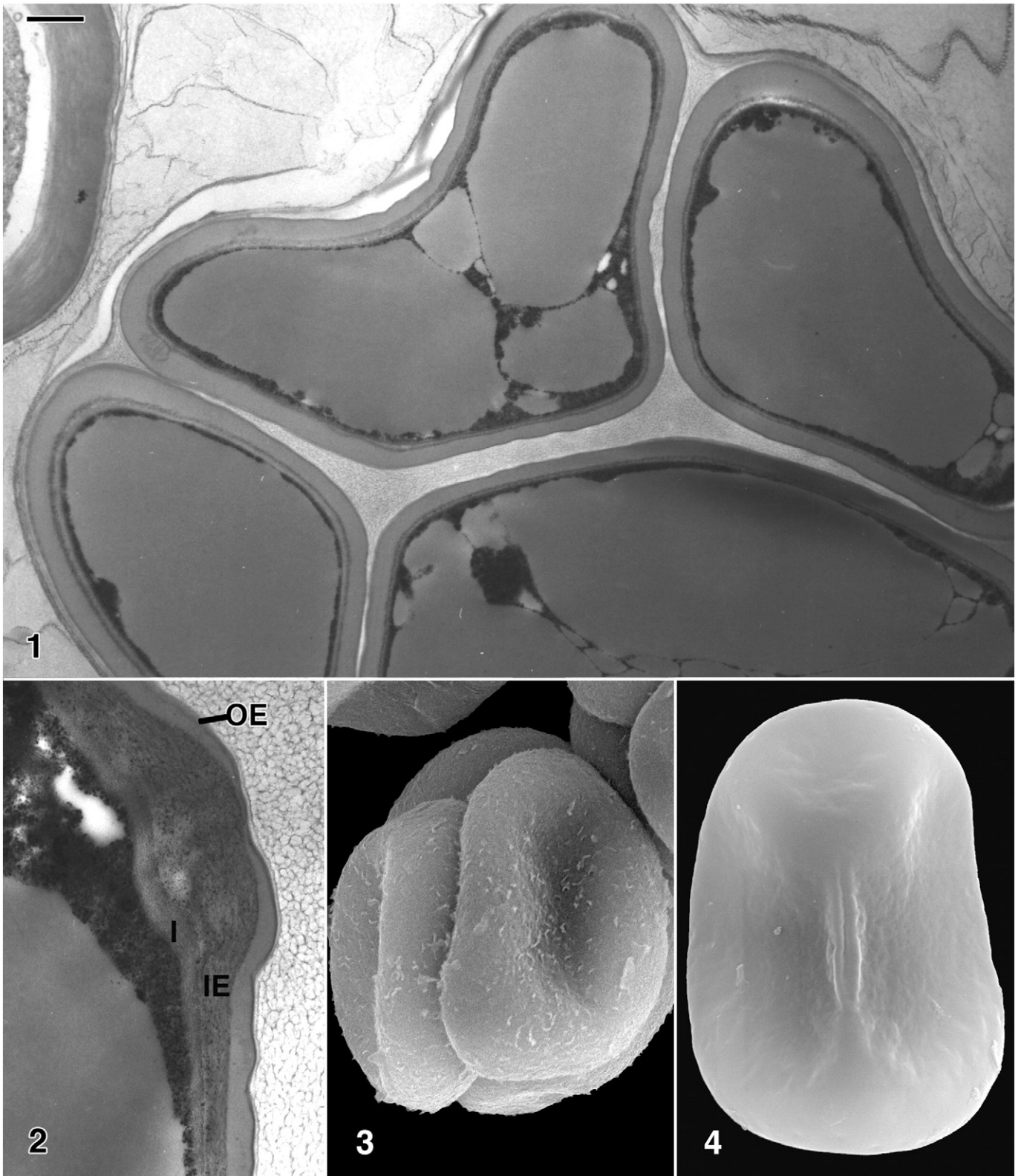


Plate IX. *Leiosporoceros dussii*. Magnification is based on the single bar in 1. 1. Cross section through a nearly mature tetrad. Spores filled with lipid reserves. Bar = 0.9 μm . 2. TEM detail of the wall flanking the aperture that consists of intine (I), expanded inner exine (IE), and thin outer exine (OE). Bar = 0.4 μm . 3. SEM of isobilateral tetrad with smooth spores speckled with spore mother cell wall remnants. Bar = 3.0 μm . 4. SEM of the smooth proximal spore surface showing short monolete aperture. Bar = 2.3 μm .

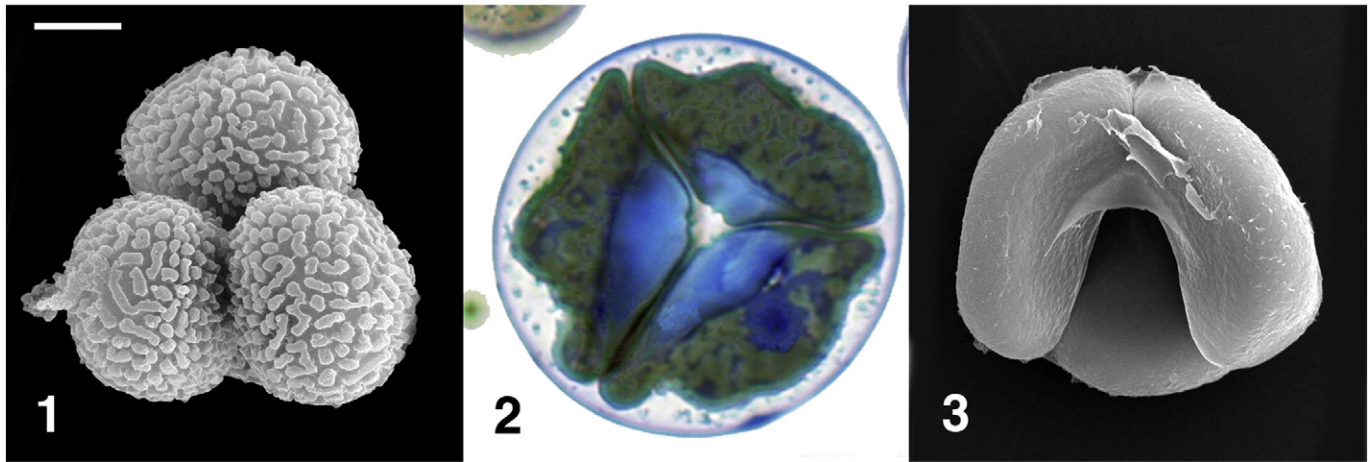


Plate X. Post-cytokinetic spore arrangements. Magnification is based on the single bar in 1. 1. Adherent spore tetrad in *Haplomitrium hookeri*. Bar = 10 μm . 2. Tetrad enclosed within long-lived sporocyte wall in *Takakia*. Apertures are prominent until the spore mother cell wall dissociates, the spores round out and perine is deposited. Bar = 5.0 μm . 3. Three spores of isobilateral tetrad. Two spores of *Leiosporoceros* are connected along one end and often adhere as dyads. The other two spores, of which only one remains, are in contact at the opposite side of the tetrad. The monolete mark is a result of this arrangement. Bar = 5.8 μm .

Table 1
Summary of the major developmental and spore wall features of relictual bryophytes.

	Monoplastid	Quadrilobing	Exine precursors	Trilete with aperture	Trifacial	TPL	MLL	Homogeneous exine	Perine	Prominent	SMC wall persistent
<i>Haplomitrium</i>	+/- ^a	+	+	–	–	+	+	–	–	+	–
<i>Apotreubia</i>	–	+	+	–	+	+	+	+	–	+	–
<i>Blasia</i>	+	+	?	–	–	+	+	+	–	+	–
<i>Takakia</i>	+	^b	–	+	+	+	+	+	+	–	+
<i>Andreaea</i>	+	+	–	–	+	–	–	+	+	–	+
<i>Sphagnum</i>	+	^b	–	+	+	+	+	+	+	–	+
<i>Oedipodium</i>	+	+	–	+	+	+	–	+	+	–	+
<i>Leiosporoceros</i>	+	+	–	Monolete ^d	–	+	+	+	–	–	+

^a Both monoplastidic and polyplastidic species in the genus.

^b Precise peripheral wall ingrowth.

^c Texture similar but with interstices.

^d With a single laesura.

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