Early Trait Evolution in Liverworts (Marchantiophyta), with an emphasis on Oil Bodies and Paleozoic Taxa

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Abstract

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Liverworts (Marchantiophyta) are a diverse and phylogenetically important group of land plants (Embryophytes). They diverged early on from the rest of the land plants, but few fossils exist from the Paleozoic or Mesozoic and those that do exist generally possess only a few vegetative characters. By the Cenozoic, when liverwort fossils become more common, modern groups had evolved and the fossils can be easily placed. Consequently little is known about early character evolution in liverworts. Recently, studies based on molecular data have resolved the liverwort phylogeny, and together with new fossil discoveries they offer an opportunity to reassess the early evolution of liverworts.

One of the most important liverwort characters are the unique organelles found in most of their cells called oil bodies. Considered a synapomorphy of liverworts, oil bodies are bound by a biological membrane and synthesize and sequester a wide variety of terpenoids and aromatic compounds, yet their exact role in the cell is not known. They can easily be observed through a light microscopy and are taxonomically informative. The first chapter of this thesis reviews what is known about oil bodies from a phylogenetic perspective. Structure and chemistry of oil bodies is reviewed, with special attention to assignment of oil body 'types' to groups based on the recent classification of liverworts.

Hypotheses of oil body evolution generally assume an ancestor with a single type of oil body, with the present diversity of oil bodies having evolved from the ancestral type. As documented in this review, similar variation in oil body types occurs in distantly related clades across the newly resolve phylogeny, including the earliest diverging groups, suggesting that the most recent common ancestor of the group may have been polymorphic for oil body types. Some liverwort taxa have secondarily lost oil bodies. Comparing these taxa to their sister groups could offer insight into oil body function. Some experimental evidence supports the anti-herbivory hypothesis of oil body function. Hypotheses of oil body evolution and oil body function need to be tested using both systematic and ecological methods.

In some groups oil bodies occur only in special, scattered cells that contain one large oil body. In my second chapter I test the hypothesized homology of the dark scattered cells of some Paleozoic liverwort fossils with the specialized oil body cells of some modern liverwort taxa. Fresh fossils of the oldest known liverwort, the recently discovered *Metzgeriothallus sharonae* Hernick, were collected and isolated from the matrix and their cells were compared to those of modern taxa using light microscopy, scanning electron microscopy, and fluorescence microscopy. Frequency of occurrence and area coverage of the dark cells were compared to several modern taxa. All results support the homology of the fossil dark cells and modern oil body cells, providing an important new character for use in assigning fossils to the liverworts and potentially to specific groups. It will also help elucidate the plesiomorphic state of liverwort oil bodies. The results showed that the dark cells of *M. sharonae* are more numerous near thallus margins providing circumstantial evidence for the anti-herbivory hypothesis of their function.

Metzgeriothallus sharonae is spectacularly preserved *in situ* and the acid maceration revealed complex branching previously unknown in Paleozoic liverworts. While *M. sharonae* was the dominant plant in the assemblage, similarly well-preserved organic remains of dozens of co-existing organisms were also recovered. In the third chapter of this thesis I describe these new features and the community these ancient liverworts were a dominant member of. The dimorphic structure of the plants, broad, dichotomizing thalli alternating with narrower, twisting thalli that have many intercalary branches suggests the plants were growing both above and below the surface of and along banks of shallow pools. *Metzgeriothallus sharonae* is the only Paleozoic thalloid liverwort to exhibit intercalary branching as well as ventral branching at the thallus apex. The Cairo Quarry liverwort lens represents a conservation lagerstatte, a miniature 'frozen' freshwater ecosystem that is the only known liverwort dominated community from the Paleozoic.

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Preface

Liverworts are, in a sense, the 'other' land plants. Like plants that evolved on a different, perhaps smaller planet, they charted their own evolutionary course. The crowning achievement of land plants is usually considered to be the diploid sporophyte with its stomata and vascular tissue that allowed embryophytes to reach toward the sky and transform terrestrial earth. Liverworts never developed their sporophytes -in all lineages of modern liverworts the sporophyte resembles a fungal fruiting body more than a plant, usually a simple circular or oval brown or black sporangium sitting atop a white stalk that is emerging from the gametophyte on which it grows parasitically. These tiny sporophytes are so ephemeral that they shrink and disappear within days after the spores are released, leaving no sign that they ever existed. Moss and hornwort sporophytes by contrast possess stomata and differentiated tissues and persist for months or more.

Liverwort gametophytes, on the other hand, evolved into a dazzling array of forms that flourish in every corner of the globe. Like mosses, some liverworts have stems and leaves (most extant species do in fact) but unlike moss leaves the tiny, one-cell thick liverwort leaves come in many forms. The elaborate and often bizarre complex thalloid taxa look especially alien, with their umbrella-like structures and reptilian textures, while the simple thalloids seem to exhibit every imaginable form between thalloid and leafy. Thus, liverworts are distinguished in simultaneously having both the simplest, most uniform sporophytes and the most complex, disparate gametophytes in the land plant kingdom.

I first became acquainted with the strange little plants while admiring the illustrations in a "Golden Book of Non-flowering Plants" many years ago. I was fascinated by the odd Marchantioid taxon pictured. The weirdly flattened, red and black leafy liverworts illustrated were distinctly odd yet beautiful also. I was captivated, but I was also a bit confused, especially when reading in the captions that these were common North American plants. How could that be? I'd never seen any of these plants. Then I read the fine print and realized I'd never seen (noticed?) them before because these were tiny plants and for a while, I forgot about them.

Years later I became interested in liverworts again through macrophotography. The more I observed these plants the more fascinated I became - there really is something different abut them. This time the fascination took root and I decided to study them. It was only after I began to study plant evolution that I was able to solve the mystery of why they seemed so strange; it's because they are strange; they've been going their own way since the Ordovician. It turns out liverworts are the sister group to the rest of the land plants, diverging from all other familiar land plants (mosses, ferns, and seed plants) sometime back in the Ordovician. The common ancestor of liverworts and other embryophytes was likely a tiny thalloid gametophyte and rudimentary sporophyte. Even though they have evolved many basic forms that often in some way mimic other plant groups, they have evolved all these forms convergently, hence the distinct alien quality.

In 2006, the year before I started grad school the Liverwort Tree of Life project had resolved many of the phylogenetic relationships in the group using molecular data.

A remarkable topology was revealed that mirrors that of the 'stomatophyte' sister group, with two species-rich leafy groups containing most species and a number of morphologically distinct 'early diverging' (asymmetric) lineages. At a scale one or two orders of magnitude smaller, liverworts evolved a disparity that mirrors it's sister group. I realized that the newly resolve phylogeny would provide an opportunity to learn more about the characteristics of the common ancestor of liverworts, and early liverwort trait evolution, and this became the subject of my dissertation. I decided to focus on liverwort oil bodies. Often the most conspicuous features of the liverwort cell when it is viewed through a light microscope, the function of these unique organelles is unknown. Liverwort oil bodies are as diverse as they are mysterious. They vary in size, color, texture and number per cell. I planned to do field experiments or genetic research to try to work out the function of liverwort oil bodies.

Then a serendipitous discovery in the paleobotanical world led to complete change of direction. Researchers in New York had discovered a treasure trove- the world's oldest liverwort, nearly 400 million years old, exquisitely preserved and present in great numbers, something unprecedented in a group with an especially depauperate fossil record. What was striking to me were the figures showing the dark, scattered cells throughout the thallus. Having been absorbed in study of oil bodies, I noticed right away that the dark cells were distributed in a way that was strikingly similar to the specialized oil body cells of some modern liverworts. I decided to collect more fossils, isolate them from the shale matrix by acid maceration, and compare the distribution and structure of the dark cells to modern oil body cells. This became my second thesis chapter.

Then came another extraordinary discovery; macerating the shales revealed two amazing things- a remarkably complex structure to the plants, with branching types never before seen in Paleozoic liverworts, and dozens of other co-occurring taxa - what the researchers in New York had actually discovered was an entire liverwort-dominated frozen ecosystem from the Devonian. Describing this remarkable plant and community for the first time became the subject of my third chapter. Of all the rewarding experiences I have had in graduate school, from the courses, the seminars with world class intellects, the travel to exotic field sites and museums, I don't think anything can compare to that moment when, after the long collection and maceration process, the isolates from a rock sample were finally placed under the stereo microscope and I could look through the eyepiece at the specimens swirling in the rinse water, never knowing in advance what I was going to see, what new feature of the plants, or weird never-before-seen arthropod or alga, would drift across my field of view. What a thrilling experience to be the first human being to set eyes on these miniature plants and animals that flourished and lived and died together in their little pond, only to be suddenly frozen in place for 400 million years until released from the shales to tell their story. Thus, I ended up where I wanted to be, illuminating the early days of terrestrial plant evolution but not in any way I could have predicted when I started out.

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Fieldwork in New Zealand could not have been done without Bill and Nancy Malcolm's hospitality and support in the field. I also appreciate Bill Malcolm's generous

willingness to share his bryo-photography tricks of the trade during my visit. I especially want to thank David Glenny for hospitality, fun times, and guidance in the field, and for his subsequent long-distance support and encouragement. Landcare Research and the Allen Herbarium generously provided me with support and a workspace during my visit to New Zealand. Field work in New York could not have happened without Linda Hernick. I thank her for teaching me how to be a field paleobotanist and taking me on as a partner in the exploration of the Catskill's liverwort localities. I'm also grateful for Ken Bartowski's practical support of our work in the field during my visits to New York.

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CHAPTER 1. Liverwort oil bodies: diverse organelles of unknown function

Abstract: Liverworts (Marchantiophyta) are a diverse and phylogenetically important group of land plants. Most taxa possess unique and morphologically diverse organelles called oil bodies. Considered a synapomorphy of liverworts, oil bodies are bound by a biological membrane and synthesize and contain a wide variety of terpenoids and aromatic compounds. Varying in characteristics such as size, number, color, and texture they are easily seen in the cell because of a highly refractive membrane, and are taxonomically informative. Most hypotheses of oil body function are anecdotal and usually easily disproven by counter examples. Few rigorous experiments have been made. Recent molecular phylogenies and new fossil evidence show that the traditionally described oil body types are dispersed broadly across the phylogeny, making ancestral state reconstruction difficult and raising the possibility that the most recent common ancestor of liverworts was polymorphic for oil body types. Hypotheses of oil body evolution and oil body function need to be tested using both systematic and ecological methods.

Introduction: Liverworts (Marchantiophyta) have been resolved as the earliest diverging group of extant embryophytes (Mishler & Churchill 1984; Graham 1993; Kenrick & Crane 1997; Qui et al. 1999; Qui et al. 2006). They represent a morphologically diverse group of 6000-8000 extant species that roughly fall into three morphological types: leafy, simple thalloid, and complex thalloid. They also include a considerable number of transitional and enigmatic forms, perhaps reflecting the deep divergences in the group (Heinrichs et al. 2007). Liverwort cells generally contain characteristic organelles called oil bodies that are found in no other organism (Schuster 1966). Unlike the lipid bodies found in the cells of many organisms (including liverworts), liverwort oil bodies are bound by a biological membrane and are considered to be 'true' organelles (Crandall-Stotler et al. 2009).

Schuster (1992) questioned whether oil bodies are truly a 'unique organelle' distinct from the oil droplets found in the cells of some liverworts that are thought to have lost oil bodies. He considered the matter unresolved because the point where the structures cease to be considered oil bodies and are instead referred to as oil droplets, is coincidentally the point at which light microscopy makes it difficult or impossible to discern the details of the structure, and adequate TEM studies of these taxa had not been carried out, a concern brought up more recently by He et al. (2013). Schuster (1992) also mentions early reports that the enigmatic, rare taxon *Takakia*, originally thought to be a liverwort but now placed with mosses, possessed oil bodies in marginal cells, but this is no longer believed to be the case (Renzaglia et al. 1997). Most researchers consider membrane-bound oil bodies to be a synapomorphy of the liverworts (Crandall-Stotler et al. 2009; Ligrone et al. 2012).

Oil bodies are present in approximately 90% of liverwort species across all major lineages, including the earliest diverging groups, although they were secondarily lost in a few derived lineages (Schuster 1966; Forrest et al. 2006; Crandall-Stotler et al. 2009). They vary in size, texture, and number per cell and serve as important taxonomic characters. In recent decades there has been increasing interest in the chemical contents of oil bodies (Asakawa 1995, 2004). Suire (2000a) has shown that all the major types of oil bodies develop in a similar manner, budding off from the ER soon after cell division. He has also found evidence of terpenoid biosynthesis within oil bodies (Suire 2000b). Despite being the subject of much taxonomic documentation, developmental and anatomical studies, and more recently, chemical analysis, the exact function of these unique organelles in the cell is not known (Schuster 1984; Duckett & Ligrone 1995).

Structure and development: Liverwort oil bodies are comprised of osmiophilic droplets containing a mixture of terpenoids and aromatic compounds dispersed in a granular protein-carbohydrate matrix surrounded by a biological membrane (Bartholomew-Began 1991; Pihakaski 1968). The membrane of oil bodies is underlined by a deposit of osmiophilic proteins early in their development (Suire 2000a). Because of the highly refractive nature of this membrane they are easily recognized through a light microscope, and in some cases large oil bodies can be seen with a strong magnifying glass (Bartholomew-Began 1991). Endoplasmic reticulum (ER) and rough ER are frequently seen encircling the oil bodies and they are surrounded by and suspended from cytoplasmic strands (Bartholomew-Began 1991; Schuster 1966) (Fig. 1.1). He et al. (2013) give an historical account of the discovery of liverwort oil bodies.

Oil bodies generally occur in all cells of the gametophyte and in the sporophyte in the Jungermanniopsida (Crandall-Stotler 2009). They can occur in the stems, slime cells, gametangial jacket cells, setae, and young capsule cell walls (Bartholomew-Began 1991) and have also been found in egg cells, spores, elaters, and gemmae (Schuster 1966; Stewart 1978). According to Suire (2000a), oil bodies in all the main liverwort lineages originate in the same way. Some earlier studies reached different conclusions, however this can be attributed to the fact that methods have improved since those studies occurred (He et al. 2013). Suire (2000a) looked at representatives of all the main lineages with light and transmission electron microscopy and concluded that in all cases oil bodies bud off from the endoplasmic reticulum (ER) early in ontogeny, after one or two cell divisions (Fig. 1.2). He found that the oil bodies in all liverwort lineages, including those with oil bodies in specialized cells, the process of development was the same and concluded that all the morphologically distinct types of oil bodies were homologous (Suire 2000a).

Chemistry: Many liverwort taxa have characteristic odors when crushed or dried, variously described as "earthy," "fruity," "carrot-like," or reminiscent of cedar oil and many others, presumably because of the release of volatile compounds that are sequestered in their oil bodies (Crum 2001). The amount of volatile oils in a liverwort species is correlated with the size and number of oil bodies found in their cells (Crum 2001). In recent decades there has been growing interest in the complex chemistry of liverworts. Over 700 terpenoids have been characterized (Asakawa 2004). Liverworts are especially rich in sesquiterpenes, including many structures unique to the group (Asakawa 2004; Lange et al. 2015). Other sesquiterpenoids are enantiomers of those found in flowering plants (Asakawa 1995). The second most common class of compounds found in oil bodies is bis-bibenzyls, aromatic compounds that are otherwise rare in nature (Asakawa 1995).

Asakawa (2004) has found that chemical analysis of the compounds found in liverwort oil bodies can be useful in identifying species, and that the larger clades of liverworts have characteristic oil body chemistry but his research suggests chemical composition has not proved especially useful for phylogenetic analysis as yet, because many of the terpenoid compounds appear to have evolved independently in different lineages (Ludwiczuk and Asakawa 2015).

Oil body characters & taxonomic systems: Oil bodies vary in a number of characteristics: size, color, texture, shape, and number per cell. Of these size is the most stable within a species and oil bodies can range from 1µm to as large as 60µm in diameter (Bartholomew-Began 1991; Schuster 1966; He et. al., 2013). The number of oil bodies present in each cell can vary from one to 100 or more (Schuster 1992; Engel & Glenny 2008). The number of oil bodies in a cell is generally consistent within a taxon but varies across tissues and can fluctuate greatly depending on the cell's location in the plant (Bartholomew-Began 1991;Suire 2000a). Oil bodies can be translucent and colorless or bluish, grayish, or brown, and rarely bright blue (Schuster 1966; Crandall-Stotler et al. 2009). Color can be affected by texture, which is determined by the number and size of the inner oil droplets along with the thickness of the stroma (Pihakaski 1968; Stewart 1978). The number and size of these lipophilic globules correlates to the granular appearance of the oil bodies at the light microscope level, so that those with a single, large inner droplet appear smooth and homogeneous, while those with numerous droplets appear granular and those with large globules that bulge out of the matrix appear botryoidal (Bartholomew-Began 1991). In a few lineages, single oil bodies that fill the entire cell are found in scattered, specialized cells while most cells contain no oil bodies.

These morphological characteristics of oil bodies, such as form, size, color, and texture, are highly variable across the liverworts but are consistent enough within closely related taxa to be important diagnostic characters for identification of liverwort species. (Schuster 1966; Asakawa 1985). The shape of oil bodies while also taxonomically useful, can be quite variable within a single taxon and even within an individual plant; and all oil body characters can change over time; for example, oil bodies of older cells in *Haplomitrium* tend to be slightly larger than those in younger cells, and tend to become more granular or botryoidal as they senesce (Bartholomew-Began 1991; He et al. 2013). Oil bodies tend to degrade quickly after collection and fresh material is necessary for taxonomic work because the oil bodies may change form and resemble other types when drying (Gradstein 1977).

Taxonomists have grouped oil body characteristics in various systems, sorting them into diagnostic "types," generally recognizing 4 or 5 types, although Schuster (1992) warned that these "types" are not really discrete (Crandall-Stotler et al. 2009) divided oil bodies into five general categories named after representative genera: two types of homogeneous, smooth, glistening types, *Massala* (small and many per cell) and *Bazzannia* type (larger and few per cell); *Calypogeia* type, which are botryoidal (usually medium-large and few per cell); and *Jungermannia* type which are granular (small and many/cell to large and 1-2 per cell). Those taxa that only possess single large oil bodies in some cells while other cells do not have oil bodies belong to a 5th type (Crandall-Stotler et al. 2009).

General distribution of oil body characteristics in the liverwort lineages: The taxonomic value of oil bodies occurs at different Linnaean hierarchal levels within each class of liverworts.

Haplomitiopsida:

Only 18 species in three genera, *Haplomitrium* (8 species)*,* *Treubia* (6 species), and *Apotreubia* (4 species), are extant in this sister group to the remaining liverworts (Marchantiopsida + Jungermanniopsida). *Haplomitrium* species have oil bodies in all cells of the gametophyte and possess several types of oil bodies, from small, numerous and shiny to few/cell and granular, often varying within a single species; while all *Apotreubia* and *Treubia* species are dimorphic having only specialized oil body cells filled with one large oil body and other cells that do not possess oil bodies (Fig. 1.3). No members of this *Apotreubia*-*Treubia*-*Haplomitrium* clade have secondary lost oil bodies.

Haplomitrium oil bodies are found in all cells in all parts of the plant. There is a great diversity of oil body shapes, size, color and texture in the clade (Bartholomew-Began 1991), from oil bodies distinct, small for cell size, from several to over 100 per cell, homogeneous (when minute) to granular, colorless to pale brownish (Engel & Glenny 2008). Schuster (1992) observed that *H. mnioides* oil bodies are minute, glistening, and homogeneous, but when highly magnified appear slightly granular. *Haplomitrium gibbsiae* oil bodies are numerous and highly polymorphous in shape and in size, with the smallest nearly homogeneous but under oil immersion "seen to be formed of small spherules" and not shiny and glistening like *H. hookeri* (Schuster 1992). They have been observed to change texture as they age, from finely granular in young leaves to coarsely granular in older leaves to botryoidal in dying leaves (Bartholomew-Began 1991). *H. intermedium* has the largest and most polymorphic oil bodies of any species in the genus (Bartholomew-Began 1991).

Treubia and *Apotreubia* have specialized oil body cells only, yet there is variation within the group in terms of number of oil body cells and size dimorphism of the oil body containing cells. *Apotreubia* has scattered oil body cells that are similar in size to the non-oil body containing cells (Inoue and Hattori 1954), while size dimorphism is marked in many *Treubia* species such as *T. lacunosa* (Fig. 1.3C). The number of oil body cells can vary within a species and can reach 100% in some populations of *T. pygmaea* (Glenny et al. 2015). In *T. pygmaea* some oil body cells are larger than other cells while others are the same size as the non-oil body cells (Fig. 1.3D,E).

Marchantiopsida:

All taxa either have only large, cell-filling oil bodies in specialized oil body cells, or appear to have secondarily lost oil bodies altogether. The two species that make up the isolated clade Blasiaceae, sister to the rest of the clade (Marchantiales), are without oil bodies. Nested well within the Marchantiales is the genus *Riccia* in the family Ricciaceae. This large clade contains about half of the species in the Marchantiopsida and none of the more than 200 species are known to have oil bodies. The exact placement of oil-body-free *Sphaerocarpos*, also in the Marchantiopsida (Forrest et al. 2006) is unresolved, but its sister taxon in the Sphaerocarpales, *Riella*, does have oil bodies in specialized oil-body cells. This suggests that there were at least three separate losses of oil-bodies in the Marchantiopsida.

The remaining taxa in the Marchantiopsida have scattered, specialized cells containing single, large granular to globular, brownish oil bodies that nearly fill the cell (Crandall-Stotler et al. 2009) and show little variation (Fig. 1.4). Often the oil body containing cells are smaller than surrounding cells (Figure 1.4 A-D). These cells were long thought to lack plastids; however, this was discovered to be incorrect (He et al. 2013). Oil bodies are not considered to be as taxonomically useful in the group beyond noting presence/absence of oil body cells, but the documentation that does exist and personal observation suggest variation in distribution of the oil body cells in the plants is fairly common. For example Schuster (1966) notes that a distinction between the closely related *Sauteria alpina* and *Athalamia hyalina* is that in the former oil body cells are found in the ventral tissues and scales and rarely in chlorenchyma or aerenchyma tissue; while in the latter the opposite situation is most common with oil body cells rarely found in ventral tissues and scales.

Jungermanniopsida:

This class is made up of four major lineages. The two main clades of leafy liverworts, Jungermanniales and Porellales, which comprise about 80% of liverwort species, form a monophyletic group sister to the Metzgeriales, a simple thalloid lineage. Sister to the Jungermanniales + Porellales + Metzgeriales clade is a second simple thalloid group, Pelliales. All oil body types are represented in the class, including specialized oil cells and secondary loss of oil bodies.

Pelliales: All taxa have oil bodies in all cells and all oil body types are represented: homogeneous, botryoidal, and granular (Fig. 1.5). In the sub order Fossombroniineae Schust. most taxa have few to many small homogeneous oil bodies, and in the suborder Pallaviciniineae Schust. few to many finely granulated or botryoidal to almost smooth, to one or two large oil bodies per cell (Schuster 1966). There are no known secondary losses of oil bodies in this group and no occurrences of specialized oil body cells.

Metzgeriales: Oil bodies found in this group are generally granular or botryoidal and medium to large in size. In one the two largest families, Metzgeriaceae, oil bodies are never found and assumed to have been secondarily lost (Schuster 1966). In the other large family, Aneuraceae, oil bodies are usually found in at least some cells, which contain a few larger ones, although some taxa have more oil bodies per cell. In the largest genus in the Aneuraceae, *Riccardia*, there is often great variation in presence/absence of oil bodies. Most species in the genus lack oil bodies in the marginal and epidermal cells, while most hypodermal cells have one or two large, grayish brown oil bodies. According to Schuster (1966) *R. latifrons* is normally oil body free *R. palmata* lacks oil bodies in epidermal cells and has single oil bodies in hypodermal cells, and *R. chamedryfolia* possesses a single large oil body in most epidermal cells which are especially apparent at the shoot tip (Fig. 1.5D). He also describes two rare taxa, *R. juata* and *R. stricta* as having

2-10 or sometimes more oil bodies in epidermal cells (Schuster 1966). There are no occurrences of specialized oil body cells in extant members of the group.

Porellales: All types of oil bodies are found in this order: homogeneous, granular and botryoidal (Fig. 1.6). There is often variation at the genus level; for example in the genus *Odontolejeunea* two types of oil bodies are found- homogeneous oil bodies in *O. lunulata* but segmented in *O. decemdentata* (Gradstein 2001)**.** Some taxa, primarily in the Lejuneaceae and Frullaniaceae, have oil bodies of one type in most cells and also large plate-like oil bodies filling special cells (called ocelli). Ocelli in this group have essentially the same structure as the oil body cells of the Marchantiales (Crandall-Stotler 2009). In the large genus *Lejeunea* oil bodies vary from very numerous (30-50 per cell) and minute and homogeneous, to several per cell (2-10) and finely segmented or papillose to finely botryoidal in appearance, rarely 1-2 per cell (Schuster 1992). Ocelli sometimes occur in a row but in some taxa they are scattered, with up to 200 per leaf in taxa such as *Stictolejeunea* (Crum 2001). Within the single species *Cyclolejeunea convexistipa* there is variation regarding ocelli, with some scattered, some basal, and some without any ocelli (Schuster 1992).

Jungermanniales:

All types of oil bodies are found in the order except specialized oil body cells (Fig. 1.7): homogeneous, botryoidal, granular, and at least two secondary losses in Lepidozeacea and Cephaloziaceae (Schuster 1966). Some large genera have more than one type, eg *Calypogeia*, *Lophozia*, and *Plagiochila*, that correlate with other morphologic characters and are taxonomically significant (Gradstein 1977). There is sometimes variation within a single taxon, for example in *Herbertus oldfieldanus* oil bodies in non-vitta cells occupy only a moderate portion of cell and there are only 3-6 per cell, while vitta cells are rather crowded with 13-15 globose oil bodies per cell (Engel & Glenny 2008). *Triandrophyllum* from the same family as *Herbertus* has very different, botryoidal, oil bodies (Gradstein 2001).

Oil body evolution:

Schuster concluded from his studies of over 500 liverwort species that complete secondary loss of oil bodies was a derived state (Schuster 1992); this has been confirmed by more recent phylogenetic analyses (Forest et al. 2006). Schuster hypothesized that the generalized condition, the one most often found i.e. cells bearing several, usually medium sized granular oil bodies, was plesiomorphic. He felt this was the most “rational” conclusion because this condition occurs most often in the in the most species-rich groups, the Metzgeriales and Jungermanniales (Schuster 1992). He also thought loss in only some cells, such as in taxa that have oil bodies only in scattered cells such as the Marchatiales, and the oil body free epidermal cells of Riccardia, which he also considered a form of cell dimorphism, was apomorphic in those groups (Schuster 1992). For that reason he considered *Treubia* to be "a true Jungermanniine genus" (Schuster 1992). More recently Crandall-Stotler et al (2005) mapped oil bodies onto a phylogeny and reconstructed oil bodies of the *Massalla* type (smooth, glistening, & colorless and many oil bodies per cell) as the pleisiomorphic state of oil bodies for liverworts.

Since the analyses of Schuster (1992) and Crandall-Stotler (2005), the topology of the liverwort phylogeny has been better resolved and many important changes make the question of the plesiomorphic state worth revisiting. Most importantly, *Treubia* + *Apotreubia* clade, which has specialized oil cells only, has been placed with *Haplomitrium* in the Haplomitriopsida, now considered sister to the remaining liverwort lineages (Forrest et al. 2006). Additionally the dark, scattered cells found in the oldest known fossil liverworts have been shown to be homologous to the oil body containing cells of modern taxa (Chapter 2 of this dissertation). The two Devonian taxa, *Metzgeriothallus sharonae* Hernick and *Pallavicinites devonicus* Hueber (Schust)., that possess these dark, scattered cells, have been systematically placed, based on superficial similarity, in the Metzgeriales and Pelliales respectively. These two orders do not have representative taxa with specialized oil body cells today (Schuster 1992). If they have been placed correctly, this indicates that along with the newly resolved placement of *Treubia* in the Haplomitriopsida (Forrest et al 2006) specialized oil body cells are more widespread across the phylogeny than previously considered. Phylogenetic analyses are needed to incorporate fossils into recent phylogenies while mapping oil body types onto the phylogeny to correctly ascertain the ancestral state of these organelles.

Are oil body types homologous?

Based on the observations of Suire (2000a) there is little doubt that the common ancestor of extant liverworts had oil bodies and that the organelles found across the liverwort clade are homologous structures at a basic developmental level, developing in the ER soon after cell division. But what about the specialized oil-body cells filled with a single large oil body found today in the Treubiaceae, the Marchantiales and the Porellales; or the small glistening oil bodies found in *Haplomitrium hookeri* and distantly related *Fossombronia*?

All the hypotheses of oil body evolution discussed so far assume a single ancestral condition for oil bodies that presumably evolved into the other oil body types. But with the resolved liverwort phylogeny, and the undisputable fact that the various types of oil bodies are widely distributed across the liverwort tree and appear in multiple, distantly related lineages suggests that there two possible scenarios of oil body evolution. One, that these are rapidly evolving organelles with one of the types being plesiomorphic and all other types evolving from it, or that more than one type existed in the most recent common ancestor, and they have sorted out in the extant groups. Careful reconstructions need to be made after adding the fossils to the phylogeny, and considerations of function need to be taken into account to try to understand the likelihood of parallel evolution.

Oil body function: A number of hypotheses of oil body function have been proposed, and include: energy storage, defense against herbivores and/or microbes, freezing tolerance, and desiccation tolerance (Schuster 1966: Duckett & Ligrone 1995; Pressel et al. 2009). However, none of these hypotheses have been rigorously tested. The most frequently proposed function is anti-herbivory and/or anti-microbial. The reasoning is that liverworts appear to be resistant to herbivores and many of the compounds found in the oil bodies, particularly the terpenoids and aromatic compounds, are thought to fulfill this function in seed plants (Lange et al. 2015). This is countered by the fact that nearly all bryophytes, including those liverworts that do not contain oil bodies, appear to be similarly resistant to herbivores. Generally, all these “armchair hypotheses" are countered with examples of liverwort taxa that endure the same environmental stress but lack oil bodies. For example Hieronymous suggested that they provided protection from light intensity and Schuster countered that *Anthelia* and *Riccia* species grow in intense light but do not have oil bodies (Schuster 1966).

Some tentative support for the anti-desiccation hypothesis was noted by Pressel et al (2009) in their observational studies of desiccation tolerance in liverworts. They noted a number of active changes in the oil bodies that seemed to track with important events in the desiccation and rehydration in desiccation tolerant liverwort taxa (Pressel et al. 2009). However, cause and effect is not clear; the oil body changes could be an *effect* of desiccation rather than any kind of mechanism. There is some evidence for liverworts and their terpenoids and other secondary compounds as anti-herbivory or anti-microbial. For example, Wurzel et al (1990) recorded molluscicidal activity from constituents from the liverwort *Ricciocarpos natans*, and Millar et al. (2004) tested the antimicrobial properties of three liverworts and found antimicrobial activity to be more pronounced in taxa with oil bodies. Some researchers have pointed out that the membrane bound oil bodies appear to be a type of specialized compartment that shields the plant's own tissues from the potentially toxic effects of the secondary compounds within that is analogous to the secretory glands of vascular plants (Lange 2015).

It is also possible that oil bodies have multiple functions and that these functions may have diverged during the long evolution of the group. The null hypothesis of course is that they have no particular function; this needs to be rejected before accepting a putative function, but rigorous tests have been lacking to date. This will require both systematic and ecological studies. Hypotheses of oil body function need to be tested experimentally; the function can then be mapped on the cladogram to find out whether the proposed function arose first, making the trait an adaptation, or the trait arose first and the proposed function later, which would make the function an exaptation (Mishler 1988).

Future directions: In order to elucidate the evolution of these unique organelles and understand their function in the liverwort cell, more study is needed. The numerous examples of sister groups with different oil body characteristics, or even presence/absence, offer many possibilities. At the largest scale, it would be ideal to compare the two sides of the liverwort phylogeny, Haplomitriopsida vs Marchntiopsida+Jungermanniopsida. Haplomitiropsida is particularly interesting because of the small number of species (18) yet great variation that almost mirrors the 6000-8000 species on the other side of the liverwort phylogeny. Taxa such as *Treubia pygmaea* that have populations with large oil bodies in larger cells as in typical in the genus, but also have large single oil bodies in nearly 100% of the other normal sized cells (Figure 1.3E) appear to be "transitional"- although they are considered to have only special oil body cells, they have oil bodies in all cells in some populations- and may be worthy of more study.

An important question that needs to be asked is: what do phylogenetic changes in oil body type correlate with? Correlation of oil body types with ecological characteristics may produce hypotheses that could be tested with field experiments, or by eventually by knocking out genes involved in oil body development. Transcriptomes of closely related taxa such as the *Riccardia* species where one taxon has oil bodies and a close relative does not, or in taxa such as *H. gibbsiae* where oil body expression changes over the course of the life cycle, could help to identify genes involved in oil body expression. Gene knockout experiments could then test fitness under different conditions.

The observations of Pressel et al. (2009) in their desiccation tolerance study suggest that complementary approach may be simple observational study of live cells. With differential interference contrast (DIC) microscopy subcellular structures such as organelles, cytoplasmic strands, and vesicles of live cells can be observed over time, as cells react to light or other stimuli, perhaps providing further clues. Finally, it is important to incorporate fossils into the liverwort phylogeny so that they can be placed correctly. Continued study and refinement of hypotheses of oil body type homology and mapping onto the resolved phylogeny can eventually elucidate the ancestral type (or types) of liverwort oil bodies.

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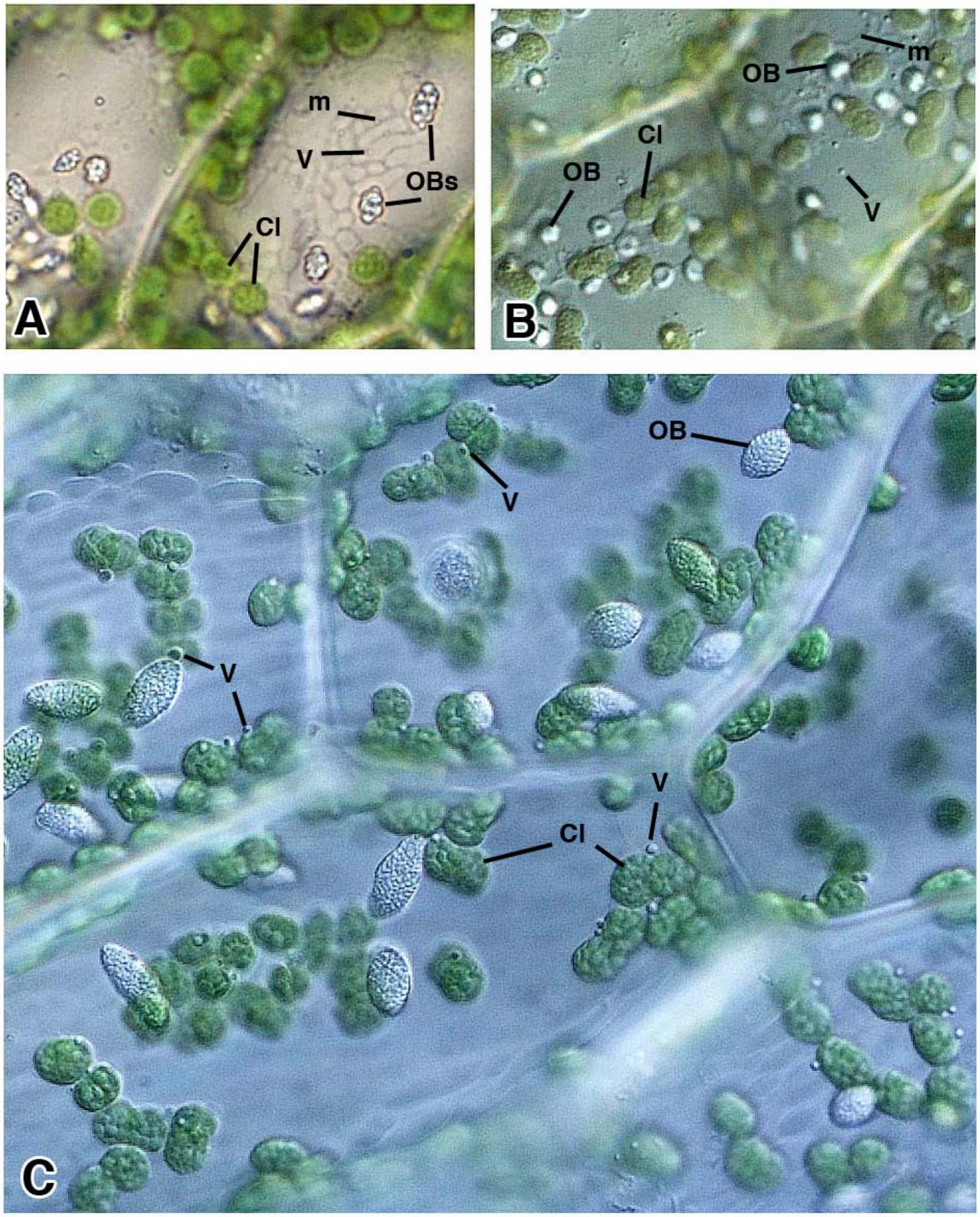

Figure 1.1*. Oil bodies and other cell elements: chloroplasts, vesicles, and cytoplasmic strands. A, Cytoskeleton lined with vesicles surrounds the botryoidal oil bodies of Pallavicinia; B, Microfilaments, vesicles, chloroplasts and small, glistening oil bodies (in this case smaller than the chloroplasts) of Fossombronia; C, Gyrothyra underwoodiana* M. Howe granular oil bodies, chloroplasts and vesicles. B,C Differential Interference Contrast (DIC) microscopy.OB=oil body, V=vesicle, Cl=chloroplast, M=microtubule

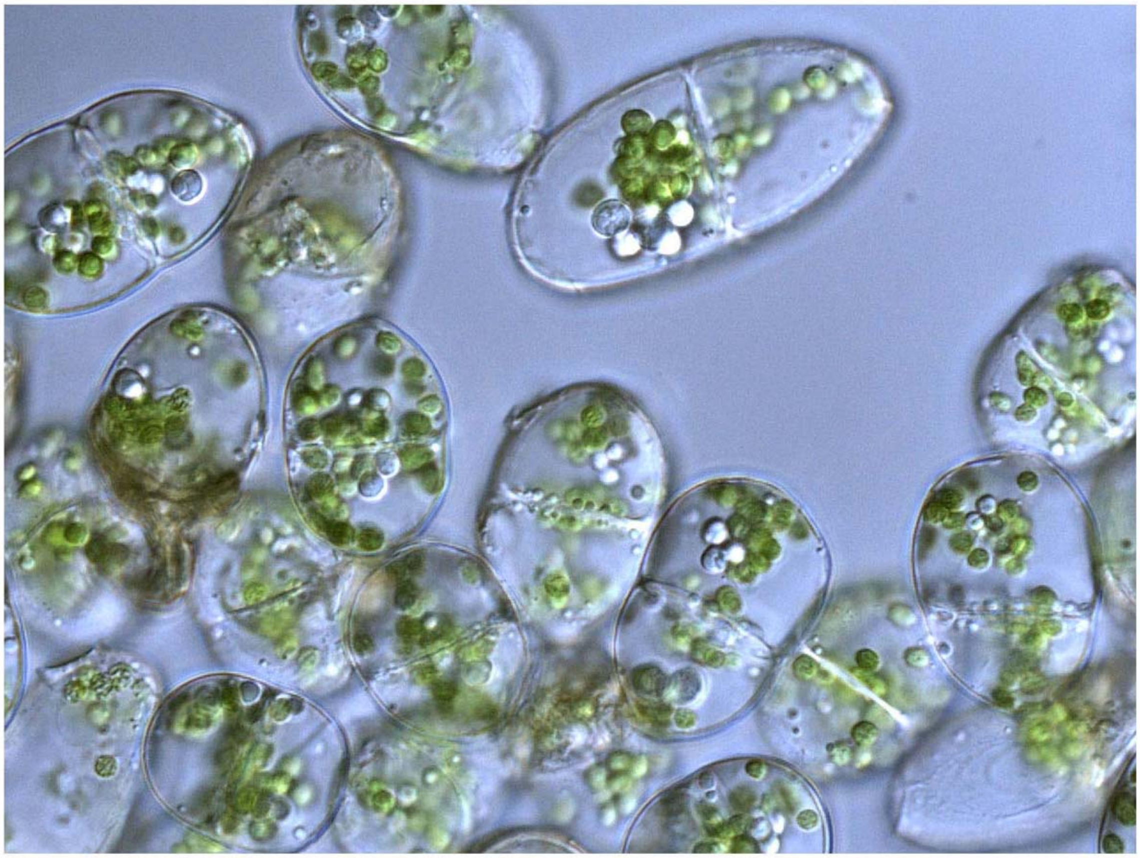

Figure 1.2. Oil bodies already forming in 2-celled asexual propagules of *Calypogeia.* In some gemmae small oil bodies appear to be forming near the point of cell division. Some structure can be seen in the larger oil bodies. Mature plants of *Calypogeia* have botryoidal oil bodies.

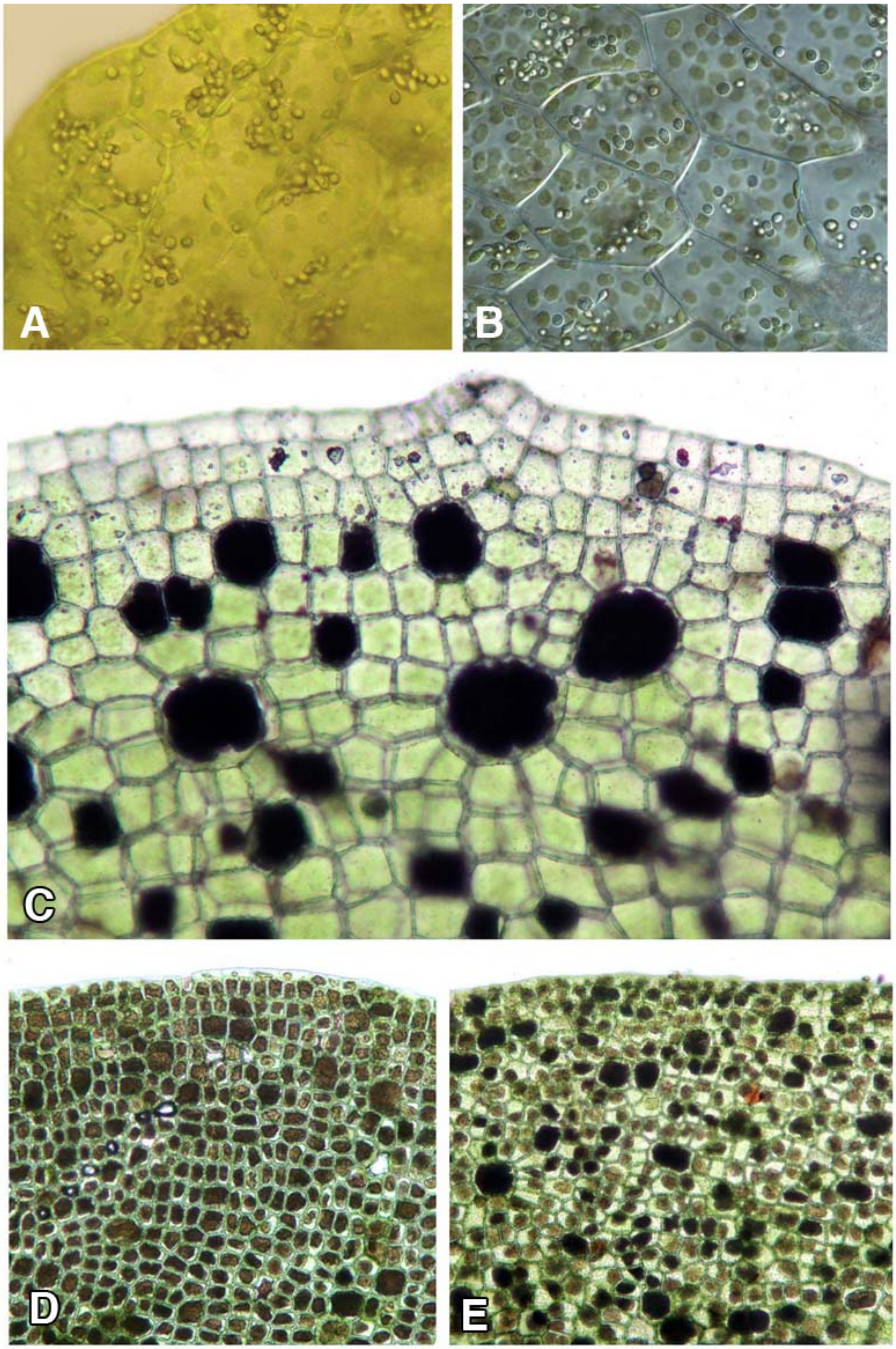

Figure 1.3. Diversity of oil bodies in the class Haplomitriopsida. A,B, *Haplomitrium hookeri* (100x) light microscopy (A) and differential interference contrast microscopy (B); C, Oil body cells in the unistratose 'leaves' of *Treubia lacunosa [Cobb]*; D,E, Oil bodies fill nearly 100% of the cells in these samples from two different populations of *T. pygmaea*

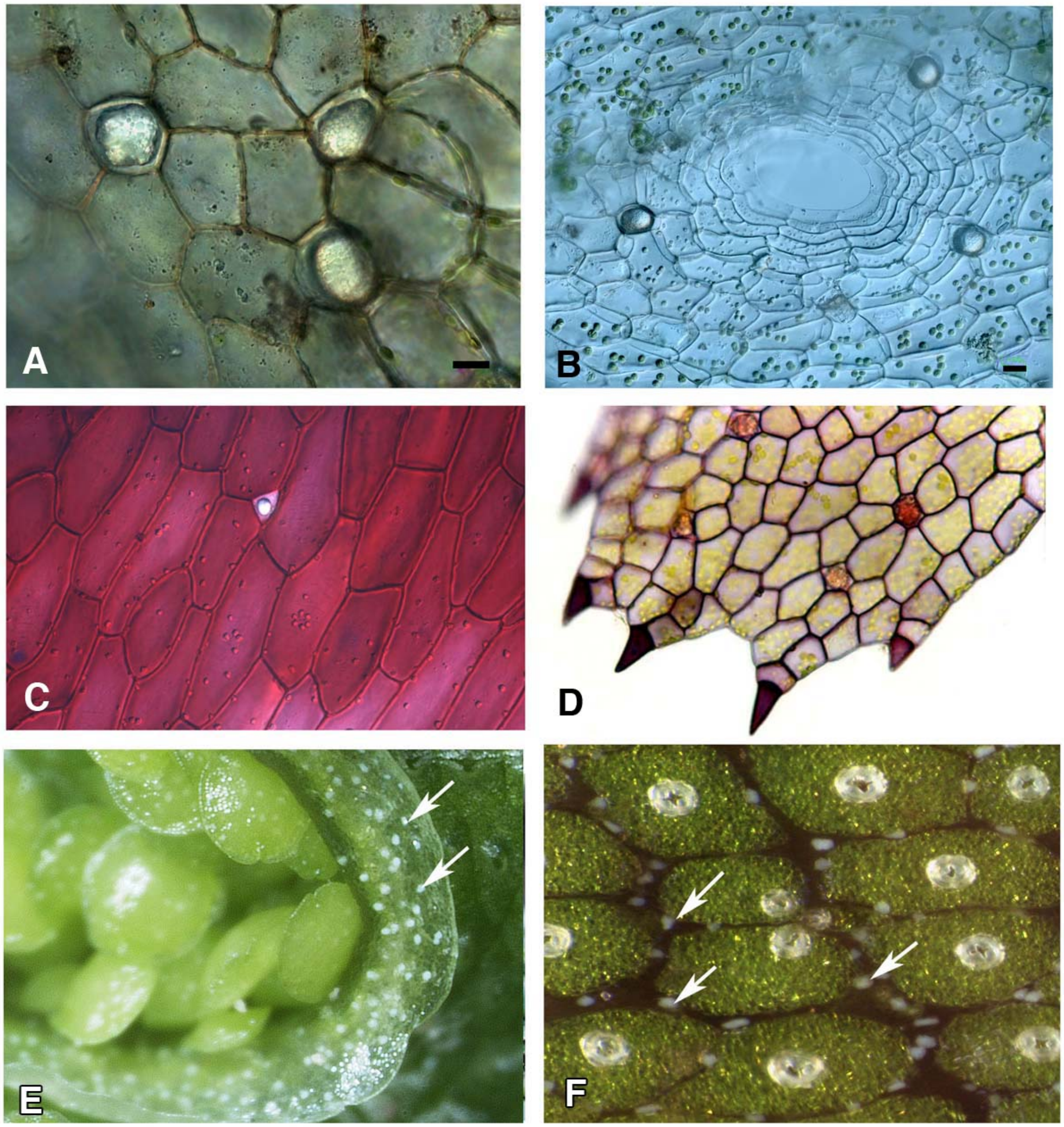

Figure 1.4. Diversity scattered, specialized oil body cells of in the class Marchantiopsida. A,B, Oil bodies sequestered in scattered cells in the ventral epidermis (A) and surrounding a dorsal pore of *Conocephalum conicum*; C,D, Oil cells in the unistratose scales of complex thalloid liverworts *Reboulia hemisphaerica* (C) and *Ricciocarpos natans* (D); E,F, The large oil bodies of *Lunularia cruciata* (E) and *Marchantia* (F) appear white in reflected light (arrows), here seen under a stereomicroscope. A,B Scale bars=10µm

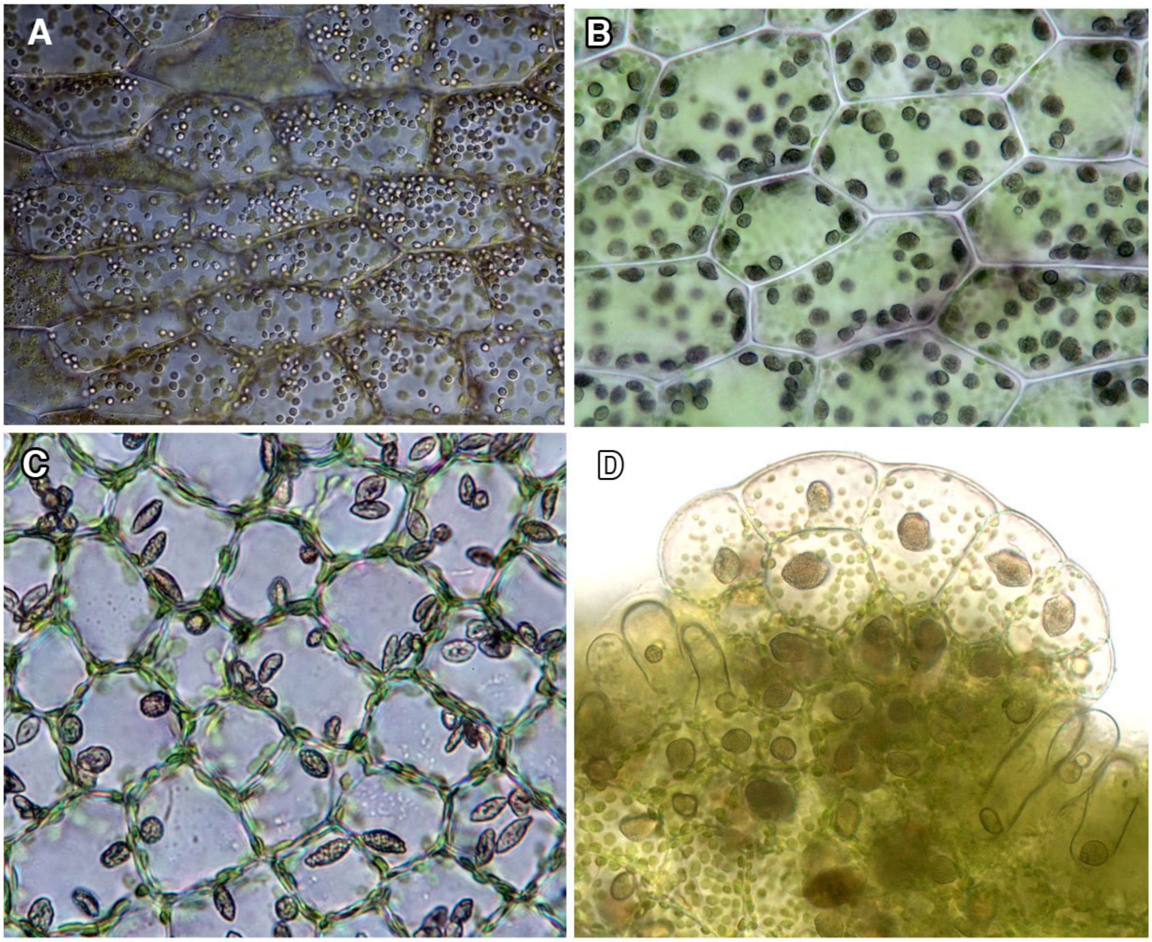

Figure 1.5. Diversity of oil bodies in the Pelliales and of Metzgeriales. A, Small, glistening, *Masalla* type oil bodies in *Fossombronia* (63x)here photographed with DIC microscopy. B, Granular oil bodies of *Hymenophyton flabellatum;* Oil bodies of *Pallavacinia;* D, Single, large oil bodies in growing tip of *Riccardia chamedryfolia*.

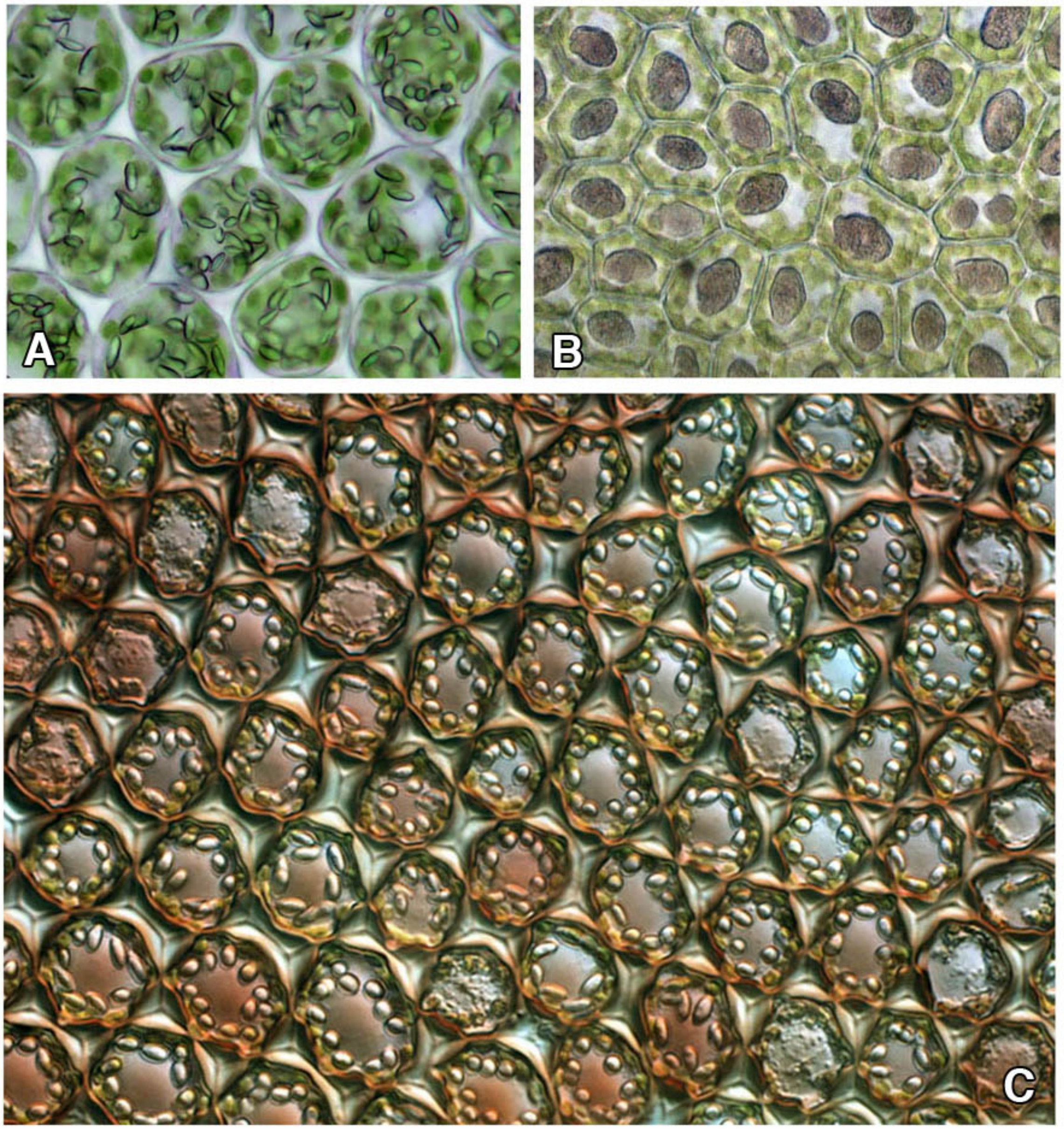

Figure 1.6. Diversity of oil bodies in the order Porrelales. A, Smooth, glistening oil bodies of *Porella*; B, Large, single, granular oil bodies of *Radula*; C, Oil bodies of *Lepidolaena taylorii.*

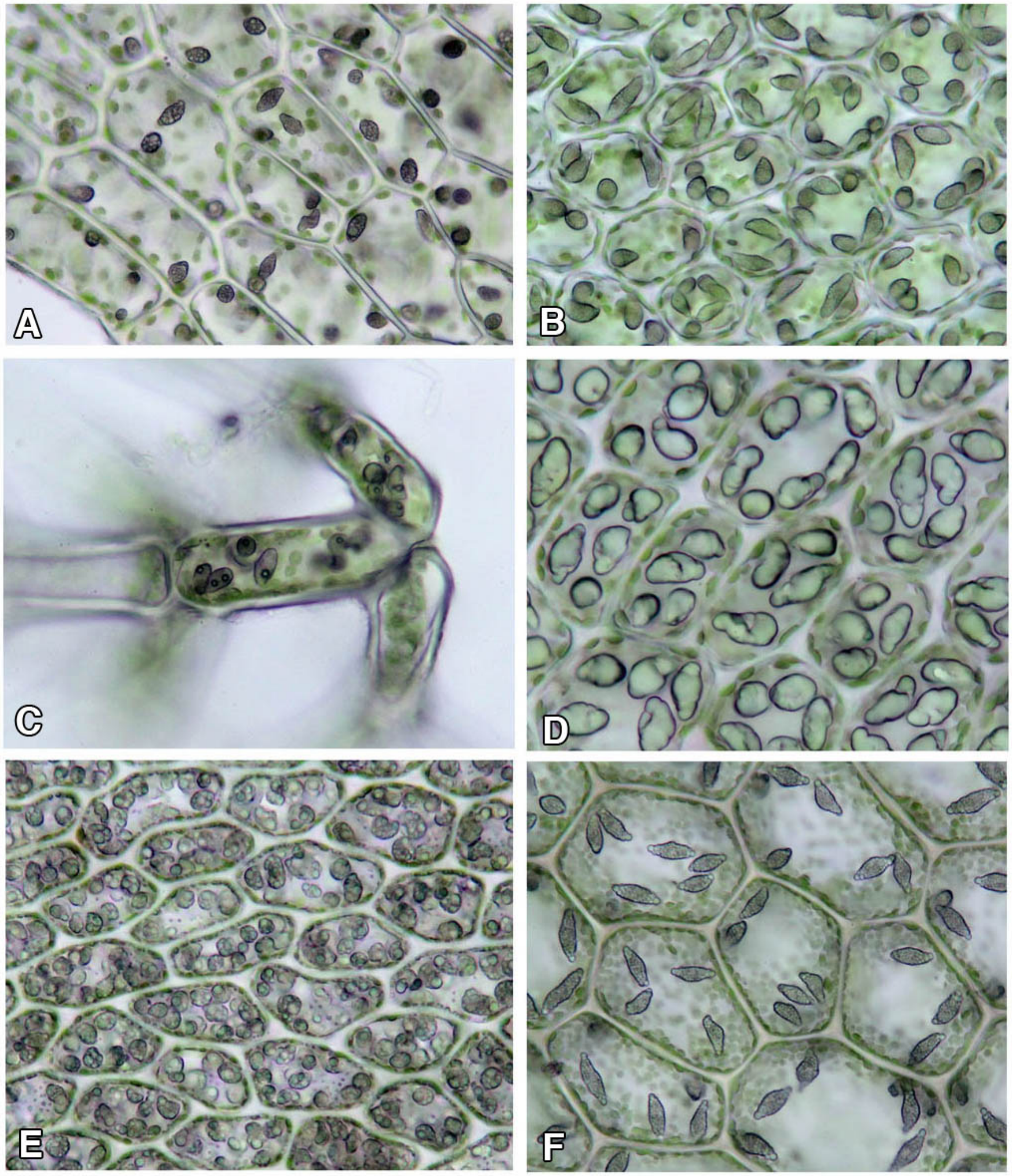

Figure 1.7 Diversity of oil bodies in the order Jungermanniales. A, Coarsely-granular, nearly botryoidal oil bodies of *Ballantiopsis*; B, Large, granular oil bodies of *Plagiochila*; C, Oil bodies with large internal oil broplets in leaves of *Trichocolea mollisima;* D, Large homogeneous *Bazzania* type oil bodies in a *Bazzania* species; E, Botryoidal oil bodies in *Schistochila*; F, Coarsely granular oil bodies of *Chilocyphus.*

CHAPTER 2. An investigation into the homology of the dark, scattered cells of Paleozoic liverworts (Marchantiophyta) with the specialized oil body cells of modern liverworts

Abstract

Liverworts (Marchantiophyta) are an early-diverging, morphologically disparate group of land plants with a sparse fossil record. Several of the earliest known Paleozoic liverworts possess dark, scattered cells that have been compared to the specialized oil body containing cells of some extant taxa. Oil bodies are unique, lipid containing, membrane bound organelles of unknown function that are considered an important synapomorphy of the liverwort clade. In 2007 a Middle Devonian shale lens was discovered in New York State, that contained hundreds of specimens of a new fossil liverwort, *Metzgeriothallus sharonae.* The spectacular preservation and large number of specimens provide a unique opportunity for direct comparison with modern taxa that possess specialized oil body cells in order to test this homology. Shale fragments were collected from four locations along the exposure and the carbonaceous fossils were isolated from the matrix by hydrofluoric acid maceration. Museum shale specimens of the slightly more recent taxon, *Pallaviciniites devonicus*, were also obtained using acid maceration. The presence and position of the dark cells in specimens of different apparent stages of development and in various structures and were compared with extant representatives in the Marchantiales. The dark cells of *M. sharonae* were also characterized by light, fluorescence, and scanning electron microscopy. A quantitative comparison was made between the frequency and area coverage of the dark cells of the unistratose wings of *M.sharonae* and the oil body cells of the unistratose tips of the 'leaves' of *Treubia lacunosa.* Finally, the anecdotal observation that the distribution of the dark cells in *M. sharonae* appear relatively even, but with a higher occurrence near thallus edges, was tested by measuring the distribution of all dark cells in relation to the mibrib and thallus edges in a set of randomly selected specimens. The relative frequency and area taken up by both the oil body cells of extant taxa and the fossil dark cells was fairly constant both within and across taxa. The dark cells of *M. sharonae* show a very prominent clumping near the edge of the thallus and an even distribution elsewhere. The morphological, chemical, and quantitative analyses all support the hypothesis that the dark, scattered cells of *M. sharonae* and other Paleozoic liverworts are homologous to the specialized oil body cells found in some extant liverwort lineages. These results provide an important new character that can be used in assigning fossils and to help elucidate the evolution of oil bodies.

Introduction

Liverworts (Marchantiophyta) are a morphologically and ecologically diverse group of land plants. Most phylogenetic analyses resolve them as sister to the remaining embryophytes (Mishler & Churchill 1984; Kenrick and Crane 1997; Qui et al. 2006; for alternative resolutions see Cox et al. 2014 and Wickett et al 2014). The fossil record supports the liverworts as diverging early from the remainder of the extant land plants, as much of the earliest evidence of embryophytes, mainly enigmatic spores and cellular fragments, is thought to have liverwort affinities (Graham1993; Strother 2000), and the earliest mesofossils with bryophytic affinities have been placed in the liverwort lineage (Krassilov and Schuster 1984; Oostendorp 1987; Lacey 1969; Hueber 1961; Hernick 2007). Unfortunately, until recently the only Paleozoic mesofossils that have been placed unequivocally in the liverwort clade were represented by only a small number of specimens from a single locality which exhibited only vegetative characters (Krassilov and Schuster 1984; Hernick 2007), which are, as in all embryophytes, prone to homoplasy. Because of this sparse fossil record and rampant homoplasy in the group, early character evolution in liverworts remains poorly understood. One roadblock has been assigning fossils to liverworts with so few characters available. Until now, one of the most important synapomorphies of the group, their unique organelles called oil bodies, has never been used to assign fossils. The ability to do so would be especially advantageous as oil bodies vary in a number of characters that are taxonomically informative within the liverwort clade (Schuster 1966; Crandall-Stotler et al. 2005).

Oil bodies are true, membrane-bound organelles found only in liverworts. Present in 90% of liverwort species across all main lineages, they have been secondarily lost in several groups (Schuster 1966). They are known to sequester lipophilic compounds (Asakawa 1995) and Suire (2000) has shown evidence of terpenoid synthesis in the oil bodies of *Marchantia polymorpha* L. Varying in size, texture, and color, they often impart characteristic odors to a particular species. The ultrastructure, chemical composition, and taxonomic significance of liverwort oil bodies have been extensively investigated (Schuster 1966; Asakawa 2004) yet their exact function in the cell is not known (Xe 2013). They are usually found in all cells of the plant, and depending on the taxon, an individual liverwort cell can contain one to as many as fifty or more oil bodies. In Marchantiopsida and Treubiales (Haplomitriopsida), however, they are only found in scattered, specialized cells that are nearly filled with a single large oil body.

The four major lineages of extant land plants, the liverworts, mosses, hornworts, and vascular plants, had diverged by the Middle Silurian (Graham, 1993; Heinrichs et al., 2007). All six major lineages of liverworts are thought to have diverged by the end of the Paleozoic (Heinrichs, 2007). The sparse liverwort fossil record improves very little in the Mesozoic, with the most notable discovery being *Naiadita lanceolata* Brodie, an aquatic liverwort found at the Triassic-Jurassic border across Britain that was originally described as a vascular plant. What is exceptional about *Naiaditia* is that the reproductive structures were eventually described by Harris (1938, 1939) in great detail. Fossil liverworts become more numerous in the Cenozoic. The majority of these are leafy liverworts found in amber, or complex thalloid liverworts, with fossils from both groups practically indistinguishable from modern taxa by that point in time and easily placed in modern families, even genera (Lacey 1969; Krassilov & Schuster 1984; Oostendorp 1987; Edwards 2000).

The first Paleozoic fossil liverworts were described by Walton, who named five taxa and a potential sixth taxon, from the Middle and Upper Carboniferous (Walton1925; Walton 1928). Most of these were described as having characteristics of the Metzgeriales, or "simple thalloid" liverworts (now understood to be two paraphyletic lineages (Forrest et al. 2006). *Hepaticites metzgerioides* Walton had a midrib and unistratose wings very similar in appearance to those of modern Metzgeriaceae, while *H.langi* Walton had a more generalized simple thalloid structure, one-several cells thick and a dichotomizing thallus. One taxon, *Treubiites kidstonii* (Walton) Schuster, is thought to resemble the extant genus *Treubia*, a taxon that was long thought to belong to one of the simple thalloid lineages but turns out to be part of a small sister group to the rest of the liverworts (Forrest et al. 2006). The first Devonian liverwort, *Palaviciniites devonicus* (Hueber) Schuster, was described by Hueber in 1961. Although it had very similar midrib and unistratose wings to *H. metzgerioides*, Schuster (1966) thought the fossil bore a closer resemblance to *Pallavicinia* with it's rhizomes and its toothed thallus edges. *Palaviciniites devonicus* was accepted as the oldest fossil liverwort, in fact the oldest known bryophyte of any kind, until 2007 when the even older, spectacularly well-preserved *Metzgeriothallus sharonae* Hernick was discovered occurring in large numbers in a lens of dark gray shales in a Middle Devonian outcrop in New York State.

Pallaviniites devonicus, *M. sharonae*, and Walton's Carboniferous taxa were all discovered in fine shales and mudstones, and despite their relative rarity are extremely well-preserved, exhibiting fine cellular detail (Walton; 1925, 1928; Hernick 2007; Hueber 1961). A curious feature of these fossils is the presence of dark, scattered cells that appear denser and more opaque than the surrounding cells. These cells have been described as possible storage cells (Hernick 2007) or the site of waste accumulation or secretory cells (Walton, 1925). Schuster (1966) compared them to the oil body cells found in some extant lineages, an explanation that seems most likely if a homologous structure in modern groups is to be proposed. Other types of specialized cells/structures can occur in liverworts, such as endogenous gemmae (asexual propagules) in the Fossombroniacaeae but these are found only on occasion in some individual plants and are confined to specific parts of the plant (Crandall-Stotler pers. comm.). As far as specialized cells that are ubiquitous and occur on all parts of the plant, the only example in extant liverworts are the specialized oil body cells found in the Treubiales, the Marchantiales, and some leafy liverworts. It was partly based on the presence of scattered dark cells that Schuster placed the Carboniferous taxon *Treubiites kidstonii* in the modern Treubiaceae (Schuster 1984).

The recent discovery of unprecedented numbers of well-preserved *M. sharonae* fossils presents an opportunity to test this hypothesis of homology. Even when viewed on the shale surface the large number of well-preserved thallus fragments clearly exhibit the scattered dark cells, making apparent their striking resemblance in frequency and distribution to the oil body cells of modern liverworts. Recent molecular phylogenies have resolved the evolutionary relationships of the main extant lineages and placed previously enigmatic groups, yet many questions remain about the timing of divergences and character evolution in the clade (Crandall-Stotler 2005). There is an urgent need for comparative study of morphological characters in order to incorporate the fossils into modern phylogenies to understand the relationships between the major plant groups and to understand character trait evolution in the liverwort clade itself (Wiens 2004). As *M. sharonae* is the oldest known liverwort, occurring at a time when the main extant lineages may not have yet diverged (Heinrichs et al. 2007) it could provide insights into the evolution of traits such as oil bodies.

The modern definition of homology is "similarity by descent" (Roth 1991). Owen (1848) outlined three criteria for postulating homology that still apply today; positional, structural, and transitional. Homologous structures should be in the same relative position, should be made of the same materials, and develop in the same way. Initial hypotheses of homology are then testing by congruency on a phylogeny (Mishler 2005). In the case of fossils we can't follow development very precisely, but the large numbers of fossil specimens of *M. sharonae* available afford an opportunity to more rigorously compare the position and frequency of the cells to some modern taxa. The preservation of the fossils also means we can take a closer look at the structure and composition of the dark cells in the fossils as compared to surrounding cells. These results can provide insight into the as yet unknown function of these unique organelles. Early investigations of the *M. sharonae* fossils indicated that the dark cells appear to be more numerous near the edge of the unistratose thallus wings, suggesting that oil bodies may have an anti-herbivory function as the edge of a foliar structure would be more likely to be attacked by predators (Labandeira et al. 2013). This study used the large numbers of available fossils to quantify the distribution of these putative oil body cells as well. The goals were: (1) to test the homology hypothesis by examining the relative position of the dark cells and compare them to oil body cells of modern taxa; (2) to characterize the cells themselves with microscopy techniques to see if these results are consistent with the dark cells having originally contained oil bodies, and (3) to test quantitatively the observation that the fossil dark cells are more concentrated near thallus edges.

Methods

Sample collection and processing

Fossils: *Metzgeriothallus sharonae* material originates from the Cairo Quarry, New York State, in August 2011 and June 2012. This locality is an old quarry located just south of New York State Route 145 at the Cairo Highway department headquarters, 3.22 km NW of Cairo, Greene County, New York (42.32˚ N and 74.04˚ W, NAD 83).The material was collected from three locations approximately 3m apart along the 25m wide exposed lens of dark gray shales and mudstones where the liverworts were discovered. Unmacerated material (still in the shale matrix) collected from the type locality by Fran Hueber, and from which he isolated *Pallaviciniites devonicus* was obtained from NMNH (Accession nr 348094) for the purposes of this study. Shale fragments were immersed in 58% hydrofluoric acid in order to dissolve the shale matrix. The isolated carbonaceous fossils were rinsed in water until a neutral pH was obtained, and then rinsed two more times. Thousands of carbonaceous *M. sharonae* fossils, mostly fragmentary, were isolated. Selected specimens were dehydrated to a 50% glycerine solution, then the fossil fragments were mounted on glass slides and fixed with permanent slide mounting medium (Glycerin Gelatin). Over six hundred permanent slides were made. Specimens were photographed through a Nikon D90 digital camera mounted on a Leica DMRB compound microscope. High-resolution images of the mounted specimens were taken with a Leica DM2500 microscope using Differential Interference Contrast, a Plan Apo 63x Oil objective, and a Nikon DS-Fi1 Digital Camera. Extended depth of field images were generated using Adobe Photoshop's stacking feature.

Fossil specimen selection for quantitative analyses: One hundred and twenty *Metzgeriothallus sharonae* samples were selected for the quantitative analyses based on the following criteria: unistratose thallus fragments larger than 1mm x 1mm that showed good preservation and included a portion of the midrib. Specimens were chosen from a variety of rocks from the three different locations along the layer in roughly equal measure. The selected specimens were permanently mounted on glass slides and imaged at 100 magnification and saved as TIF files, all at the same orientation, with the midrib partially visible along the lower edge. Closer examination of the photographs showed that many specimens had other dark types of structures in their cells, and many had variably fading dark cells in multiple shades of gray. Also many specimen images included damaged areas. The original 120 photographs were eventually narrowed down to those specimens that had little damage, clearly defined dark cells, and little evidence of other cellular structures, leaving 40 specimen photographs to use for quantitative analyses.

Extant taxa: *Targionia hypophylla* L. was collected in Sonoma County and *Marchantia polymorpha* gemmae were collected from the teaching collection housed at the University greenhouse. Fresh material of *Treubia lacunosa* (Colenso) Prosk. was collected from four localities in New Zealand's South Island and *Monoclea forsteri* Hook. from one Stewart Island locality. They were imaged while the material was still fresh at 100x magnification using a Leica compound microscope fitted with a digital camera. Voucher specimens of *T. lacunosa* and *M. forsteri* will be deposited in the University Herbarium, UC Berkeley, (Tremblay specimen numbers 504, 547, 548, 549, 559, 560, 563, 565, and 664). While not endangered, *T. lacunosa* is relatively rare, growing in isolated colonies, so collection is limited. A small number of individual plants was collected from several areas at each locality. Two-six 'leaf' tips per plant were used, from the 'middle' leaves only from each individual plant, mainly the unistratose region. Only those that were undamaged were used for the analyses. Hand sections of *M. forsteri* were made along the plane of the upper and lower epidermis of the multistratose thalloid plants where most of the oil body cells occur using a single edged razor blade. The sections were hand cut as thinly as possible, generally 2-3 cells thick.

SEM and stereo microscopy:

Specimens still on the matrix were initially examined with a Leica Wild MZ8 stereo microscope using polarized fiber optic light source and a rotatable analyzer in order to increase contrast (Bengston, 2000; VanAller Hernick, 2007). Water or cedarwood oil was applied to rock surfaces with a fine brush before photographing the compressions to help resolve details (Hernick, 2007). For higher magnifications a Nikon D90 digital camera mounted on a Leica DMRB compound microscope was used. Scanning electron microscopy (SEM) was done with the Hitachi TM-1000 SEM on uncoated specimens. Add file format.

Fluorescence microscopy:

Mounted specimens were photographed with a Leica DM2000 epifluorescence microscope equipped with violet and UV excitation filter sets and a phototube, which fits a Nikon DS-Fi1 Digital Camera.

Cell counts for fossil taxa:

For the frequency analysis a subset of 10 images was selected using a random number generator (RNG). A vector box was drawn on the first selected image in the middle of the sample to avoid the edges and midrib and the image was cropped to the size of the vector. This vector was saved and applied to all other samples so that the area being examined was consistent. The cleaned images were saved as JPEGs for analysis in imageJ (NIH, Bethesda, MD). The analyses method cellcounter (File→analyze→Cell counter) was used, with the dark cells marked with one tag and all empty cells were marked with a second counter. Cells that were less than 25% visible inside the box were not counted. The counts were totaled and recorded in an excel file. Because only three specimens of *Pallaviciniites devonicus* were well-preserved enough for analysis all three were used and all cells (excluding the midrib) of each specimen were counted.

Cell counts for extant taxa:

The same vector box from the *Metzgeriothallus sharonae* series was transferred and applied to the photographs of the 'leaf' tips of *T. lacunosa* in order to use a similar area for the extant and extinct taxa, however this meant that portions of the 'leaf' that were more than one cell thick were included. Care was taken when imaging and counting to focus on the nearest layer of cells in those cases. The images were analyzed in imageJ (https://imagej.nih.gov/ij/) using the cell counter and the data were recorded, with oil body containing cells tagged one way and cells without oil bodies tagged another and the frequency was calculated for each population.

Surface area analysis for fossil and extant specimens:

The same slides/images used for *M. sharonae* and *T. lacunosa* frequency counts were used for the area analysis. The original vector box used for the other taxa was applied to the 100x *Monoclea forsteri* photographs. First a cleanup step was performed with the “magic wand” tool in Photoshop to select everything similar in shade to the dark cells. This, however, selected the cell walls in the *M. sharonae* photographs and other material in the extant taxa in addition to the oil bodies so an additional clean up step was performed. The image was inverted to allow for the use of analyze→analyze particles command in imageJ to give the total area of the oil bodies and entire image, as well as the area fraction.

Spatial distribution:

To assess the spatial distribution of the dark cells in *M. sharonae* and to test the hypothesis of increased frequency near the edges of the thallus wings, the 40 *M. sharonae* images were narrowed down to those that included on entire thallus margin opposite the midrib. Eight images fit this criterion. For each specimen the distance of each oil body from the costa relative to the edge was measured. Because the edge is curved and the fossil thalli were not all the same size, a ratio was used, ranging from 0% directly on the costa to 100% directly on the outer edge of the wing. For each dark cell two points were recorded; the distance from the costa to the midpoint of each dark cell and the distance from the costa to the edge of the thallus, yielding a ratio of how far from the costa the oil body is. This was done on for every oil body on all eight separate fossil samples. It should be noted that the first row of cells adjacent to the costa are generally of a transitional shape and are very thin-walled, and sometimes a few cells were missing because the thalli tend to pull away from the costa. For these reasons we measured from the first row of 'normal-shaped' cells. All 432 data points from the eight specimens were combined and to reduce noise the points were clumped into 10 blocks and an absolute frequency graph was made.

Results

General observations of distribution of dark cells in fossil liverworts:

The vast majority of *M. sharonae* specimens that were isolated from the shale fragments possess the scattered, dark cells in all tissues of the plant in both the midrib (comprised of 2-3 layers of elongate cells) and in the unistratose wings of the thallus (Fig. 2.1). The dark cells appear more numerous in younger tissues such as new branches budding off the main thallus (Fig. 2.2B and 2.2D), the base of more mature ventral branches (Fig. 2.3C) and branch apices (Fig. 2.2A). Very occasionally, specimens appear to have no dark cells and many specimens have similarly distributed cells that are faded to varying shades of gray (Fig. 2.2A except for apex), sometimes very pale, so it was difficult to determine if specimens that appeared to lack the cells had originally possessed them. Occasionally, *M. sharonae* specimens exhibited an uncharacteristically large number of dark cells, often in combination with other unusual features. For example, part of a thallus fragment might have more than 50% dark cells, while other areas might have fewer dark cells, separated by an irregular border. (Fig. 2.2C,E). Few general observations could be made about the distribution of the dark cells in *Pallaviciniites devonicus* as the newly macerated material obtained from the shale fragments was generally in poor condition. Only three thallus fragments were usable for the frequency analysis (see below) because preservation was very uneven even within a single fragment of cells. However the cells of both midrib and thallus wing were nearly indistinguishable between the two taxa (Fig. 2.5A, C).

Characterization of dark cells of M. sharonae (microscopy):

The dark cells of both costa and wing are partially to completely opaque in transmitted light. In some specimens there are round holes in the fossils that occur within the dark cells, while in some cases portions of the dark cells are partially separated but still remained attached. The holes or dis-attached portions form nearly perfect circles, even when they occur in the greatly elongated cells of the costa (Fig. 2.4A,B). Conversely when studying the shale surface with polarized light, black dots are sometimes visible in a similar arrangement as the dark cells of nearby fossil thalli (Fig. 2.4C).

Scanning Electron Microscopy:

When the uncoated fossils were imaged with SEM, the dark cells appeared consistently darker than other cells even though there was clear evidence that there was little variation in surface height (Fig. 2.4E). Figures 2.4F-H shows increasingly close views of a portion of one of the dark cells that is breaking away from the rest of the fossil.

Fluorescence Microscopy:

The dark cells did not autofluoresce at all, while the non-dark cells autofluoresced only very faintly in the yellow-orange part of the spectrum (Figure 4D; For comparison the much more brightly autofluorescing spots in Figure 2.4D likely represent dust and modern contaminants of organic origin).

Frequency and area of dark cells (fossils) and oil body cells (extant taxa):

The frequency of dark cells of the two fossil taxa *M. sharonae* and *P. devonicus* (Fig. 2.5A-D) and oil body cells of extant taxa *T. lacunosa* and *M. forsteri* (Fig. 2.5E-H) shows a high degree of conservation both within various *T. lacunosa* populations and between all taxa (Fig. 2.6). All *T. lacunosa* populations showed an average frequency of oil bodies within a range of 15-20%. *M. sharonae* and *P. devonicus* are also very close in both mean and standard deviation although the small sample size of *P. devonicus* was only three specimens. All values for both fossil species fell within the range of values of the *T. lacunosa* populations. The percent area values for all taxa were also consistent between taxa, with the percent area coverage consistently slightly smaller than the frequency and the range in area slightly larger than in the frequency (Fig.2.7). Despite how visually different and occasionally dimorphic the oil body cells/dark cells are in various taxa the frequency of the oil bodies and the area taken up by oil bodies remains quite consistent, with all samples showing a mean of 9-18% area covered by oil cells/dark cells (Fig. 2.7).

Distribution of dark cells in the unistratose 'wings' of Metzgeriothallus sharonae:

The dark cells in *Metzgeriothallus sharonae* are present in a much higher frequency along the edge of the fossil thalli than in the middle of each wing. Furthermore, the distribution in the remainder of the thallus is relatively even, at least in terms of 'lateral' spread of dark cells between costa and edge (Fig. 2.8); the 'longitudinal' spread along the thallus was not measured, though it appears similarly evenly distributed (Fig. 2.1). There is relatively little difference in frequency in any region from 0-70% away from the midrib. However the last 1/10 of the graph shows a very large peak indicating a much higher concentration of oil bodies in that portion than the rest of the fossil. There are 2-3 times more oil bodies near the edge of the thallus than at any other point (Fig. 2.8). Calculating the results separately for the eight samples and placing all the graphs side-by-side (Fig. 2.9) confirms that there is a very limited window of variance. In every sample the last 1/10 had the highest frequency of cells and no sample had any recorded values below 3% or above 15% for the first 9/10.

Discussion

The concentration of dark cells in younger, growing areas of *M. sharonae* such as shoot apices and new branch buds is consistent with the hypothesis of their homology with the specialized oil body cells of modern liverworts, and with oil bodies in general. For example, oil bodies are known to disappear in older tissues and in the leafy liverwort *Lepicolea attenuata* they are only visible in young leaves (Stewart 1978). The distribution of specialized oil body cells in gametophytes of Marchantiales and other groups that possess these cells has not been extensively described in the literature; oil bodies are chiefly used taxonomically in the Jungermanniales. However, comparisons were made with several complex thalloid taxa that were investigated in this study. The concentration of dark cells around branch bases and edges of laminar plant structures can be compared to similar observations in modern taxa such as the ventral branch bases of the modern taxon, *Targionia hypophylla* (Fig. 2.3C,D) and the gemmae (asexual propagules) of *Marchantia polymorpha* (Fig.12.0C).

The observations of increased frequency of the dark cells in growing areas and young thalli also may support the anti-herbivory hypothesis of oil body function, although indirectly, as these young tissues are less protected and potentially more attractive to herbivores. The anti-herbivory hypothesis has some experimental support in the modern liverworts (Millar 2004) and is supported indirectly by results that show similar terpenoid compounds to those found in liverwort oil bodies having anti-herbivore effects in vascular plants (Lange 2015). While a clear analog to the increased frequency at edges of wings in modern taxa isn't available for comparison it is worth noting that both *Treubia lacunosa* and *Monoclea forsteri* have most oil body cells in the cell layers near the upper and lower epidermis in the multistratose parts of the plants (Fig. 2.3A,B). The vast majority of the hundreds of specimens isolated through acid maceration appear to have a similar frequency of dark cells, although there were some exceptions. While the "dark cells" that are in fact found in varying shades of gray can be explained by taphonomic processes, it is curious that some specimens, including one of the young thallus buds, had no trace of these cells at all. One possible explanation is that these tissues had died shortly before deposition. Oil bodies generally disintegrate after a plant is collected and begins to dry out (Schuster 1966). The specimens with unusually dense concentrations of dark cells are more difficult to explain. It is possible that these represent some natural variation. In some species of *Treubia* today there is a great deal of variation in oil body cell frequency (Glenny et al. 2015).

The SEM, light, stereo, and autofluoresence microscopy results all confirm the very different nature of the dark cells and non-dark cells of the *Metzgeriothallu*s *sharonae* fossils. Additionally, the SEM, light, and stereo microscopy results are consistent with a physical similarity between the fossil dark cells and modern liverwort oil body cells. Liverwort cells, like the cells of most land plants, are largely comprised of water. Specialized oil body cells, in contrast, are nearly filled with one large oil body. The terpenoids, bisbibenzyls and other organic compounds in oil bodies are carbon rich (Asakawa 2004). The often total opaqueness seen in transmitted light suggests that more organic material has been preserved in those cells, which is consistent with the content of the carbon-rich lipids of extant liverwort oil bodies. Furthermore, the breaking away of some material from the dark cells in a regular circular fashion (Fig. 4A,B,F-H) suggest that these areas had a denser concentration of material, that over time, because of taphonomic processes, shrank and pulled away from the surrounding material. The SEM backscatter indicates an area of greater density with a chemical composition with low atomic number, or both, in the dark cells as compared to the non-dark cells which also supports the hypothesis of carbon-rich contents of the cells before fossilization occurred.

The autoflurescence results were somewhat surprising and initially appeared to conflict with the light and SEM results. Autofluorescence, or primary fluorescence, is based on the faculty of organic compounds to transpose UV light into an emission of light in the visible part of the spectrum. The faint fluorescence and the yellow orange color are both consistent with the great age of the fossils (van Gijzel 1966). Where there is a greater concentration of organic material there would be an expectation of greater fluorescence in those areas, yet we see the opposite in these images. The non-dark, ordinary cells that were presumably mostly water and appear nearly translucent under a compound microscope have a faint, unevenly distributed fluorescence, and the dark cells do not appear to autofluoresce at all. An explanation for this could be that the material that is fluorescing is not within the cells at all, but from remains of the waxy covering on the surface of the thallus. Cutins are long-lived compounds as evidenced by the long preservation of vascular plant cuticles (Taylor et al. 2009). Because the Cairo Quarry liverworts are likely semi-aquatic, and appear to have been growing in compact layers, or tiers (refer to chapter 3), much as modern thalloid liverworts often do today, they may have had only a thin cuticle on the upper surface. If the specimens were oriented under the microscope with the waxy side down, the opaque material of the dark cells may have blocked the faint fluorescence completely. Autofluorescence is also indicative of the rank of coal, so that in fossils in the final stages of coalification fluorescence is completely extinguished (van Gijzel 1966). This may also indicate that the fossils, aside from the cuticle, may be completely coalified.

The frequency of oil body distribution is highly conserved between and within the four *Treubia lacunosa* populations and the two Devonian fossil taxa and may imply a conserved function. Although *T. lacunosa* cells were di-morphic, with many oil cells significantly larger than non-oil cells, both the frequency counts and the area coverage ratios were similar to those of the fossil taxa. It should be mentioned that other *Treubia* species vary considerably in oil cell frequency (Glenny et al. 2015) so these results are not representative of all liverwort taxa with specialized oil body cells. Interestingly, *Apotreubia nana* (S. Hatt. & Inoue) S. Hatt. & Mizut. is different enough from *Treubia* that it placed in its own genus (Stech et al. 2002). *Apotreubia* closely resembles the fossil liverworts in oil cell distribution, especially in the lack of size dimorphism and greater unistratose area in 'leaf' tips (Inoue and Hattori 1954). Unfortunately it is rare and it was not possible to obtain specimens for this study. Figure 11 illustrates the differences among species. The *T. lacunosa* counts were slightly higher than those found by Glenny et al. 2015). This could be a result of the different methods used. Rather than count a specific number cells, I counted all the cells in a squared-off area. Furthermore, although I counted into the multistratose region in order the use a similar area of tissue for all taxa, I were careful to count only one layer, something that turned out to be difficult in practice. Adding area coverage of specialized oil body cells to the comparison (Fig. 2.7) provided an opportunity to add an example from another major lineage, the multistratose Marchantiales, and provides further evidence of how oil body cell distribution is conserved among and within liverwort taxa.

The results of the distribution analysis confirm the observation of the non-random, even distribution of the dark cells of the fossil taxa, and of their increased frequency near the edge of the vegetative thalli (Labandeira et al. 2013). In addition, they confirm the suggestion by Labandeira et al. (2013) of a possible early oil body anti-herbivory function, if the distribution is in fact functional and not an artifact of development. The eight thalli that were used for the distribution analysis were by necessity on the small end of the distribution, as only those specimens that showed both a portion of midrib and an entire edge in the 100x magnification photographs were used in the analysis.

Interestingly, neither of the two modern lineages that the Devonian fossil taxa have been systematically placed in have specialized oil body cells today (Forest et al 2006; Crandall-Stotler 2005). Modern liverworts in the Metzgeriaceae have secondarily lost oil bodies; most of their sister taxa have oil bodies in all or most cells. And in the family Pallavicinaceae, oil bodies are found in most cells of the plant, with each cell usually containing several oil bodies rather than one large oil body (Schuster 1966). Yet the results presented here strongly suggest that the dark cells are indeed homologous to modern oil body cells. This puts specialized oil body cells in five of the six main lineages, including in extant taxa in the two earliest diverging lineages, suggesting that specialized oil cells may be the plesiomorphic character state for liverworts (Fig. 2.12). If the fossil taxa are placed correctly many nodes may have to be pushed back from current estimates (e.g., Heinrichs 2007) by 100 million years or more.

It is also possible the fossils may not have been placed correctly in the modern groups. They may represent ancestral morphologies, or possibly convergent morphologies. The fossil record itself is biased toward taxa that occur in wet, basinal areas, where fossilization is more likely to occur (Taylor et al. 2009). Modern liverworts in the "simple thalloid" lineages such as *Metzgeria* and *Pallavicinia* are restricted to moist places even today (Schuster 1966) so it is perhaps not surprising that these lineages would be over represented, and those more xerophytic under-represented. It is possible that the Devonian taxa are related to early representatives of the lineage that evolved into modern, dry-adapted Marchantiales. This possibility would also support the hypothesis of specialized oil body cells as ancestral to other states (see Fig. 2.11 for a possible evolutionary transition).

The extant enigmatic *Treubia* was long placed in the simple thalloids as well. Recent molecular studies have resolved the Metzgeriales as two paraphyletic lineages, or polyphyletic if *Treubia* is included in the simple thalloids, as *Treubia* has been resolved, along with its sister group *Haplomitrium*, as sister to all other liverworts. After publication Schuster (1984) doubted his placement of *Treubiites* in Treubiaceae and considered Blasiales a more likely placement. This, however, would not affect the fact that the specialized oil cells can now be considered a character state of five of the six main lineages, as Treubiales and Marchantiales both have specialized cells today, and they occur in some taxa in one of the main leafy clades (Fig. 2.12). This means that the early Paleozoic fossils have actually been placed in three of the six main lineages, and in all three of the lineages that were hypothesized to exist in the Middle Devonian (Frey & Stechs 2005; Heinrichs et al. 2007). A formal phylogenetic analysis including fossil and modern taxa is needed to determine the most likely placement of these important fossil taxa.

The morphological, chemical, and quantitative analyses overwhelmingly support the hypothesis that the dark, scattered cells of *Metzgeriothallus sharonae* and *Pallavicinites devonicus,* and presumably other Paleozoic liverworts that possess these cells, are homologous to the specialized oil body cells of extant liverworts in the Marchantiales and Treubiales and in a few leafy liverworts. Fossil liverworts, like all fossils, are relatively depauperate in characters that can be used for incorporation in phylogenies and for use in the calibration of phylogenies. Since oil bodies are an important synapomorphy of the liverwort clade these results provide an important new character that can be used in assigning fossils, and that may even be used to interpret some of the earliest evidence of plants on land, the so-called 'waifs and strays' or scraps of cellular material (Graham 2004) that along with spores comprise the only fossil evidence of the first 50 million years of plant life on land.

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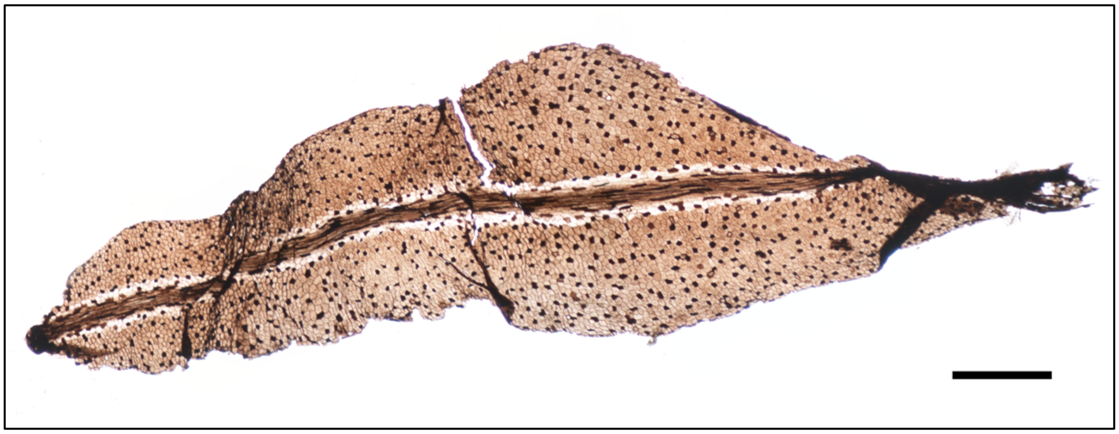

Figure 2.1. The oldest fossil liverwort *Metzgeriothallus sharonae* showing thallus comprised of midrib and unistratose wings with scattered dark cells. Light micrograph. Scale bar=1mm.

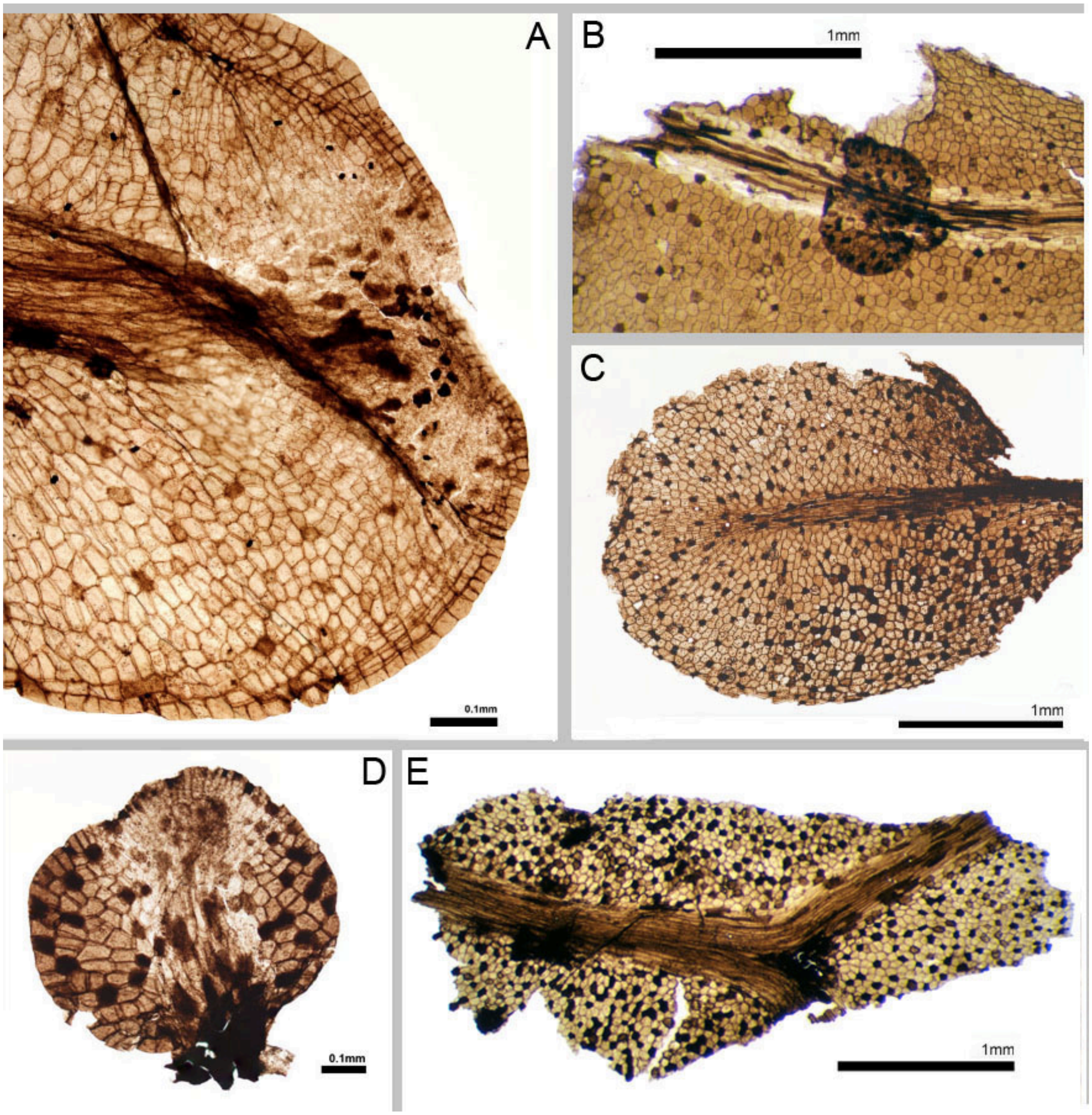

Figure 2.2. Variation in distribution of dark cells of fossil taxon *Metzgeriothallus sharonae* A. Shoot apex with more concentrated and opaque dark cells in growing region; B. Young thallus growing from midrib has higher concentration of dark cells than the parent thallus; C. Clearly defined region in lower right part of thallus has more dark cells; D. New thallus branch E. Fragment with many dark cells and other dark material in additional cells.

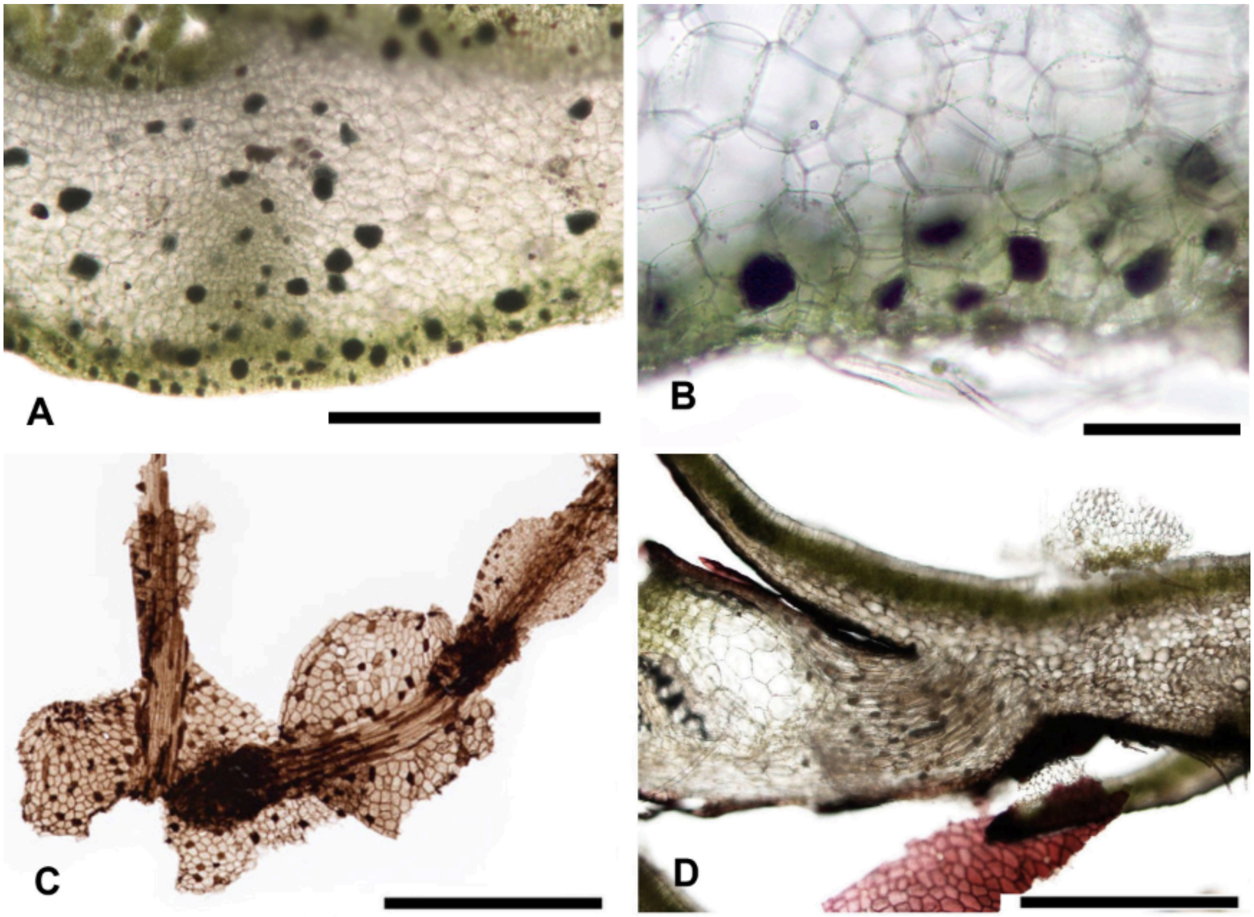

Figure 2.3. Concentration of oil body cells (extant liverworts) and dark cells (fossils) inepidermal issues and branching points. A, Cross section of *Treubia lacunosa* axis showing oil body cells concentrated near surface and around central region near concentration of fungal endophytes. B, Lower surface of *Monoclea forsteri*. C, Fossil taxon *Metzgeriothallus sharonae* with two ventral branches with concentration of dark cells at branch bases. D, Section of *Targionia hypophylla* showing concentration of oil body cells at base of ventral branch point. Scale bars A, C, D, 1mm; B, 0.1mm

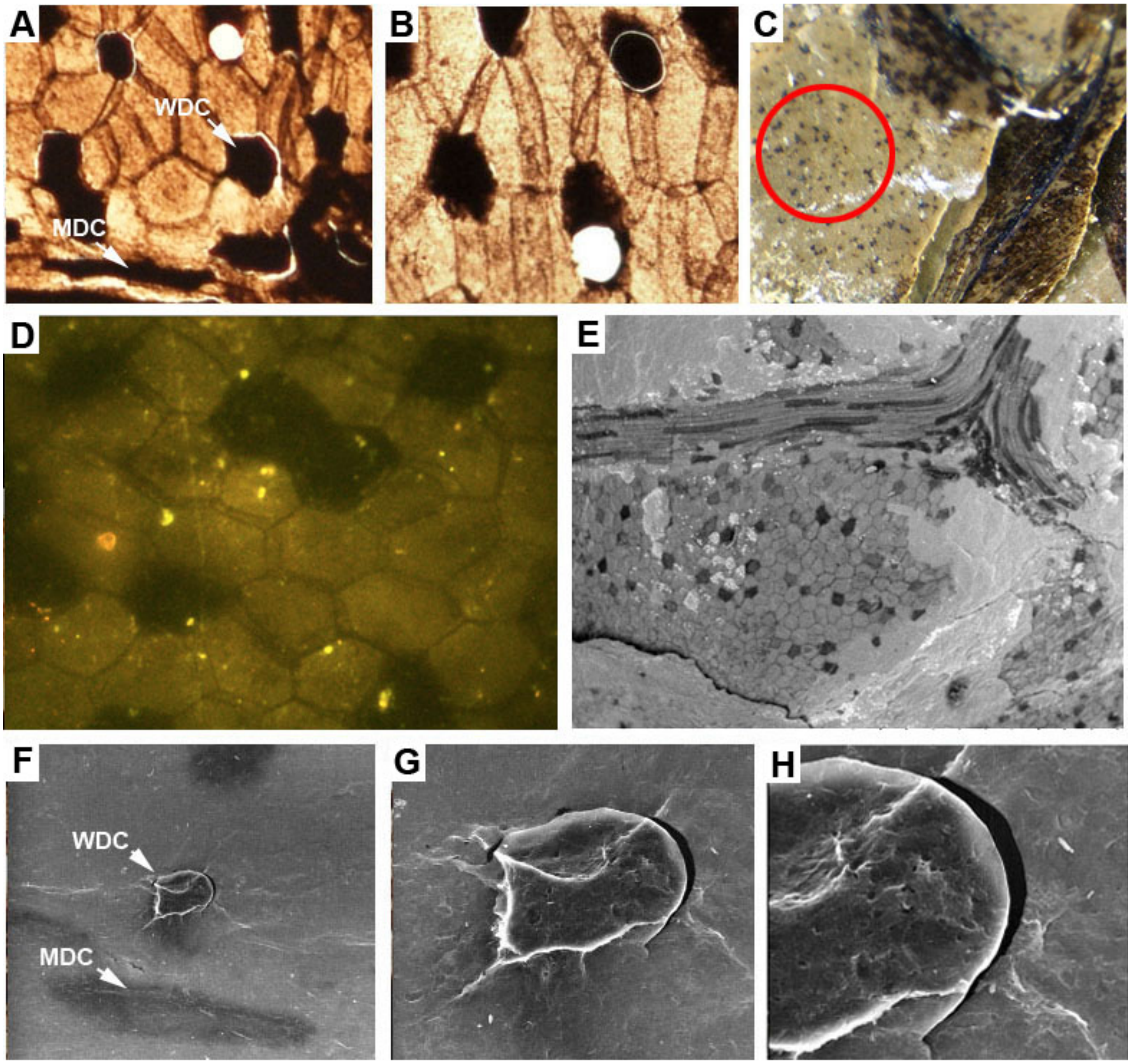

Figure 2.4. Dark cells of *Metzgeriothallus sharonae* A, B. Circular center of dark cells partially or completely separated from fossil visible in light microscopy. C. Surface of shale matrix under polarized light showing thallus on right, on left are remains of dark cells only (circled). D. Weak, yellowish autofluorescence is confined to non-dark cells. E. Scanning electron micrograph of uncoated surface of shale fragment near the region of a branch dichotomy. F, G & H. SEM of a dark cell in the thallus wing that is beginning to separate WDC=wing dark cell, MDC=midrib dark cell.

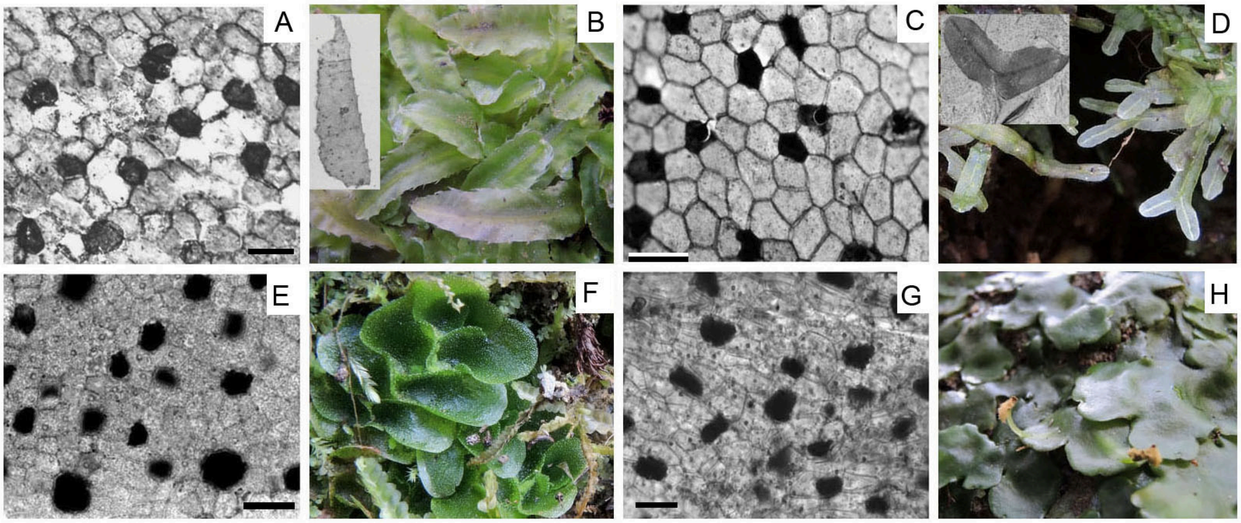

Figure 2.5. Extant oil body cells and fossil 'dark cells' with respective habit photographs of taxa used in the quantitative analyses. Top row: Cells and thallus of fossil taxa *Pallaviciniites devonicus* (Hueber) Schuster, (A and inset of B) and *Metzgeriothallus sharonae* (C and inset of D) with representatives of the modern families they have been assigned to: *Pallavicinia xiphoides* (B) and *Metzgeria furcata* (L.) Dumort. (D). Bottom row: Cells and habit photos of extant liverworts *Treubia lacunosa* (Colenso) Prosk. (E, F) and *Monoclea forsteri* Hook. (G, H). Because cell size tends to vary in liverworts, in order to facilitate comparison the four cell photos have been slightly adjusted for scale to show a similar number of cells. Scale=0.1mm.

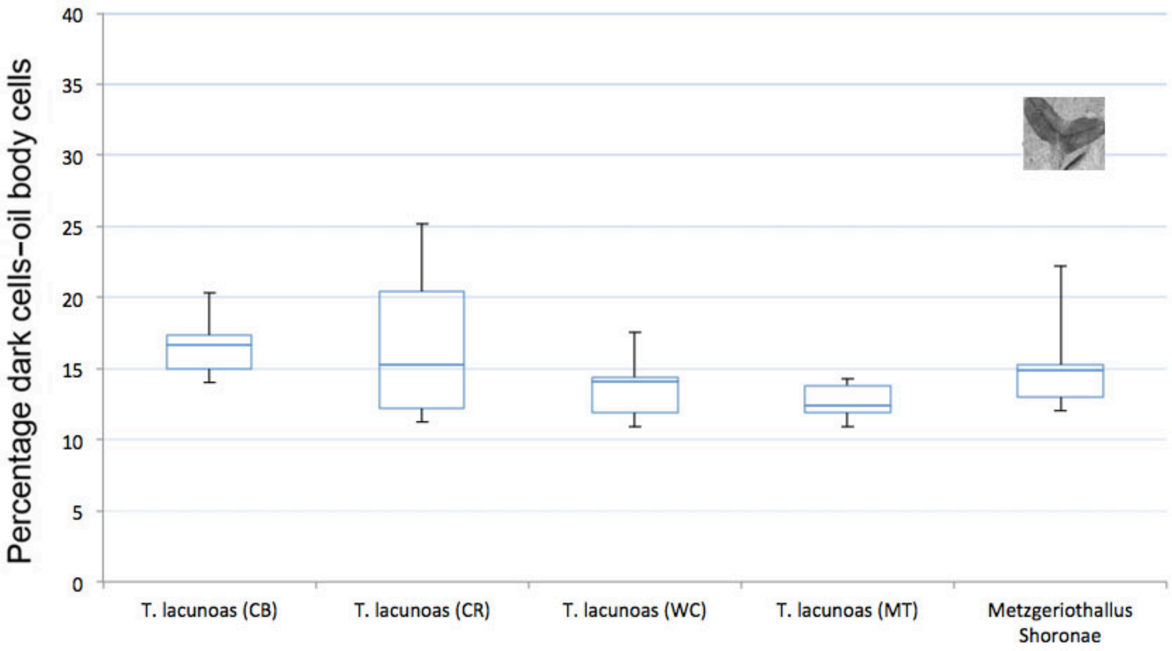

Figure 2.6. The percentage of oil body cells (dark cells in fossil taxa) versus total number of cells for four *Treubia lacunosa* populations and the two fossil taxa *Metzgeriothallus sharonae* and *Pallaviciniites devonicus*. *T. lacunosa* localities: Constant Bay (CB); Cobb River (CR); Waterfall Creek (WC); Maori Track (MT).

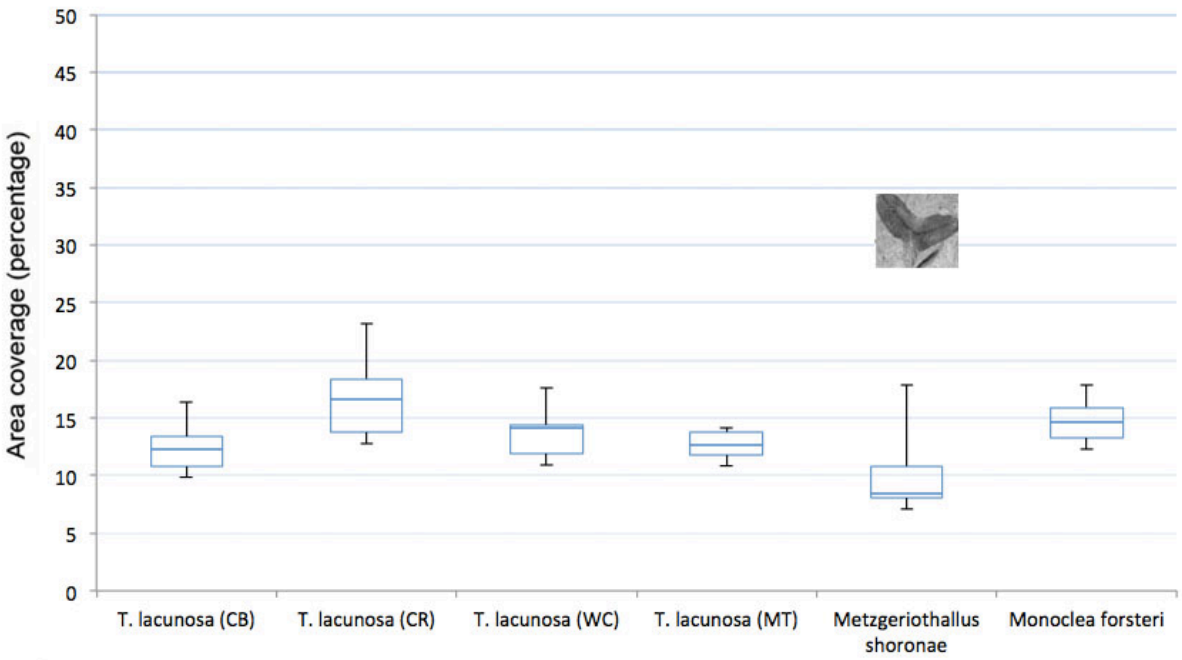

Figure 2.7. Percentage of surface area taken up by oil body cells (extant taxa) or dark cells (fossils) vs the total area for the four *Treubia lacunosa* populations and the fossil taxon *Metzgeriothallus sharonae* and the extant taxon *Monoclea forsteri. Treubia lacunosa.* localities: Constant Bay (CB); Cobb River (CR); Waterfall Creek (WC); Maori Track (MT).

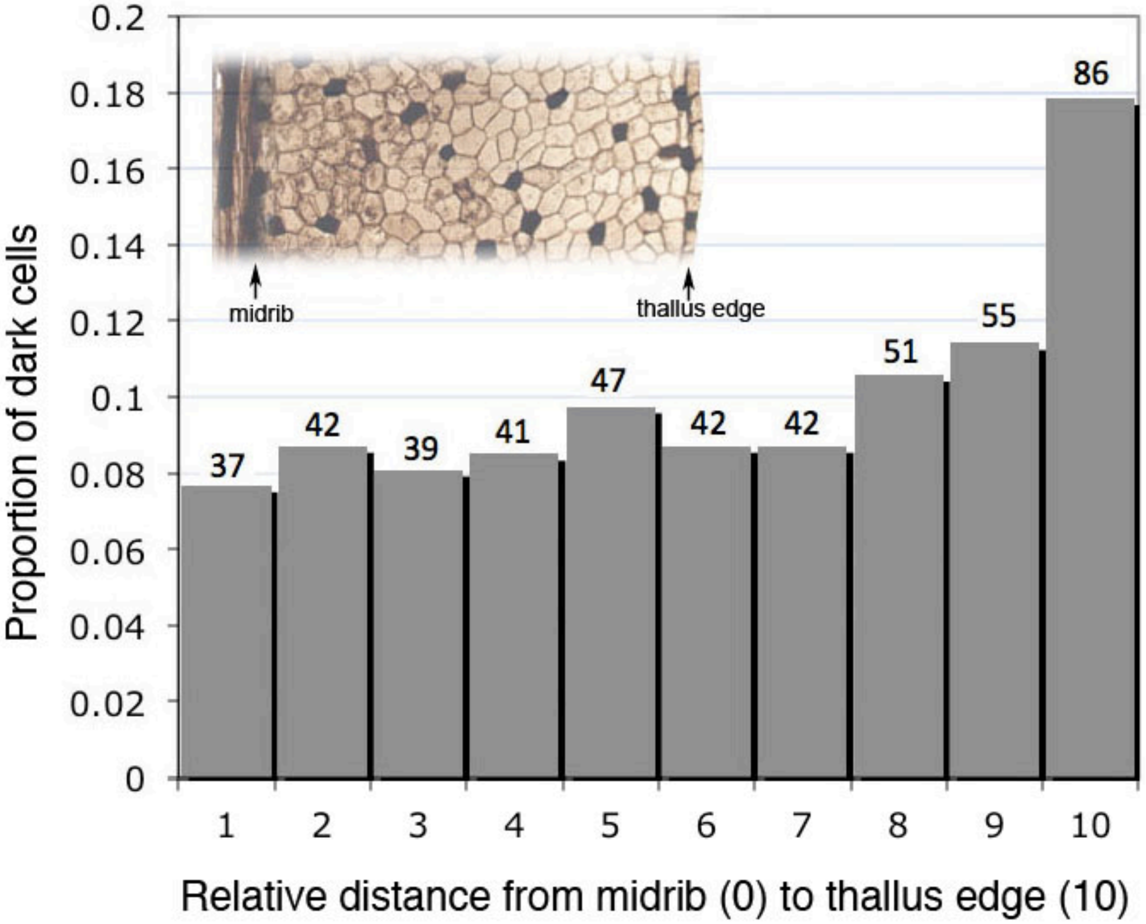

Figure 2.8. Absolute frequency distribution of the relative distance of dark cells from the costa to the edge of the thallus wings of *M. sharonae*. Proportion of dark cells combining all data points for all eight specimens (432 dark cells total) and assigning them to ten bins from (left to right) midrib to thallus edge. Actual numbers in each bin at top of bars.

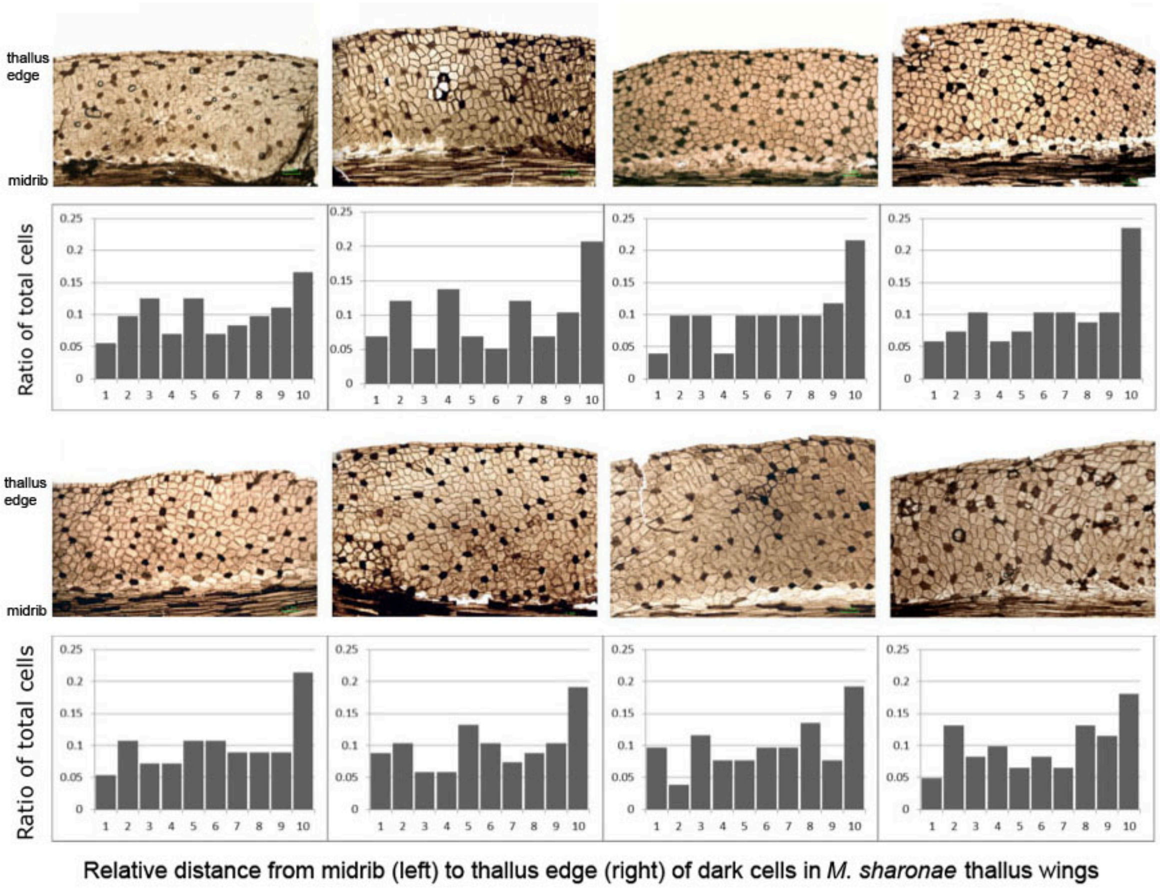

Figure 2.9. Absolute frequency of dark cells spatial position as relative distance away from the midrib (0) to thallus edge (10) for each of the eight specimen photographs of *M. sharonae* used for the distribution analysis. Bottom: Percentage of dark cells in each specimen in each of ten bins from (left to right) midrib to thallus edge for each specimen.

Figure 2.10. Distribution of fossil dark cells and extant oil body cells. Light micrographs showing a young thallus branch (A) and a portion of unistratose wing (B) of *Metzgeriothallus sharonae* with apparent concentration of dark cells near the thallus edge. C, Oil body cells are concentrated near the edges of asexual propagules in the extant liverwort *Marchantia polymorpha*. The oil bodies of *M. polymorpha* appear white in reflected light. Scale bars A,1mm; B,C, 0.1m

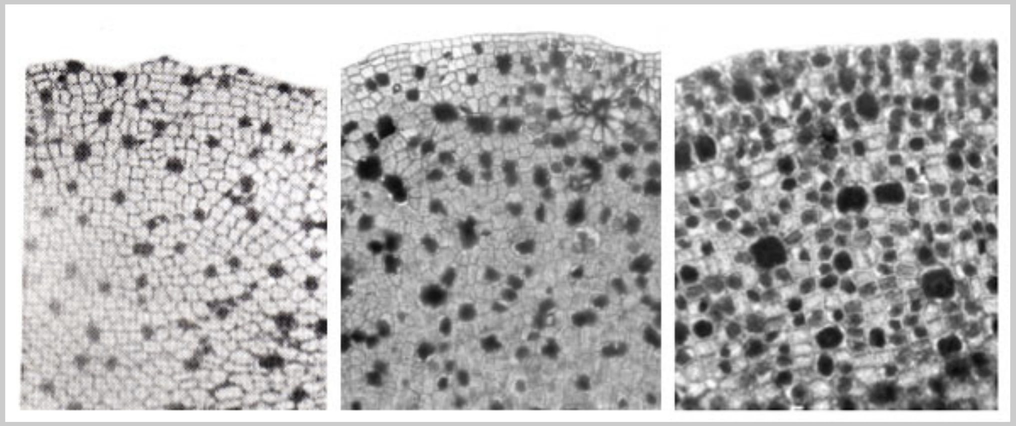

Figure 2.11. Diversity of oil body cells in 'leaves' of extant Treubiales. Left, *Apotreubia nana* (from Inoue and Hattori 1955); Middle, *Treubia lacunosa* (UC 2036933); Right, *Treubia pygmaea* (UC2036944).

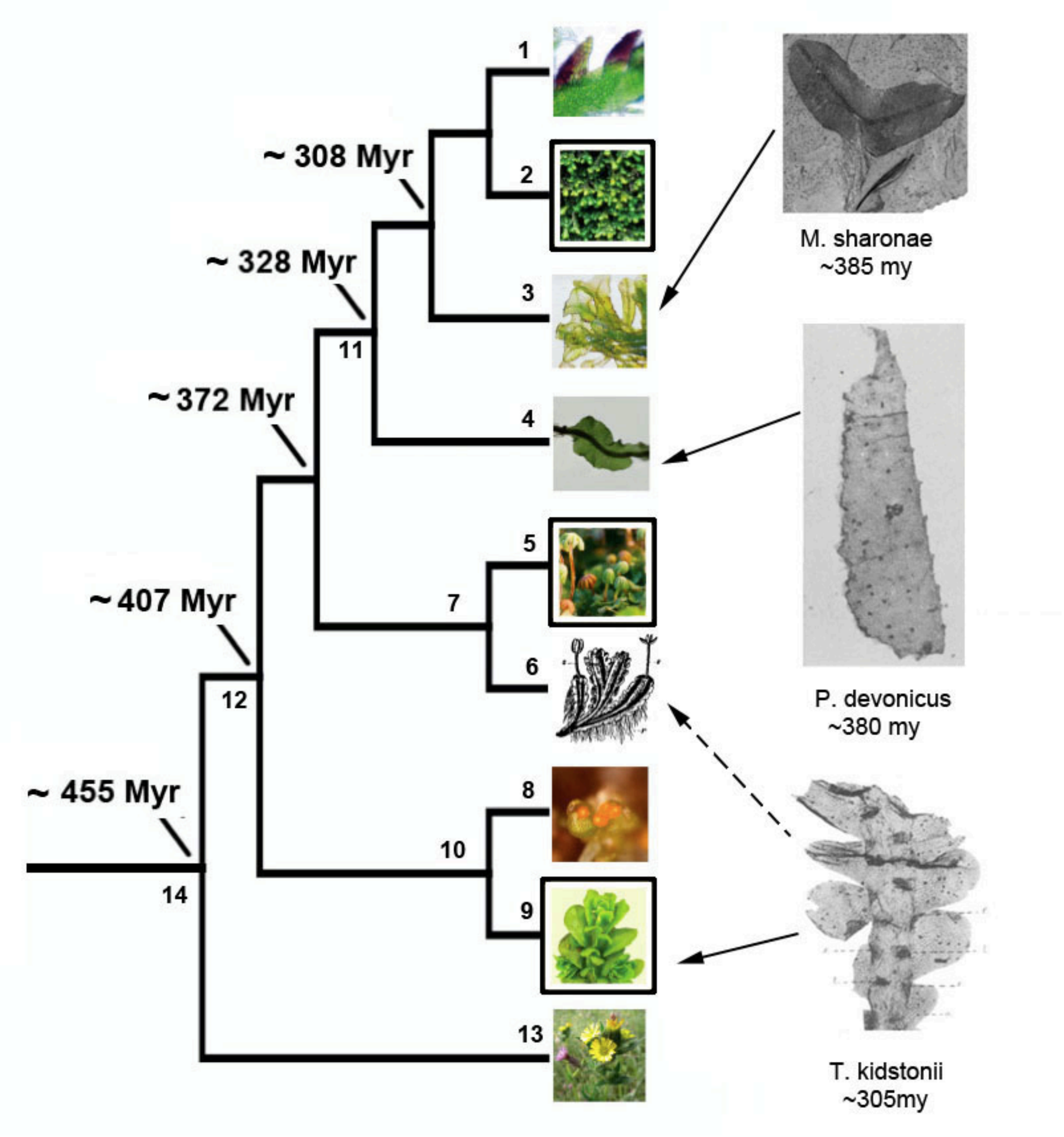

Figure 2.12. The main lineages of extant liverworts and the current systematic placement of three Paleozoic fossils that possess the dark scattered cells: *Metzgeriothallus sharonae*, *Pallaviciniites devonicus*, and *Treubiites kidstoni* (right). Extant taxa that have specialized oil body cells are outlined in black. Divergence estimates from Heinrich et al. (2007). Taxa:1, Jungermanniales; 2,Porellales; 3, Metzgeriales; 4, Pelliales; 5 Marchantiales; 6, Blasiales; 7, Marchantiopsida; 8, Calybriales; 9, Treubiales; 10, Haplomitriopsida; 11, Jungermaniopsida; 12, liverworts (Marchantiophyta);13, Mosses, hornworts and vascular plants; 14, Land plants (Embryophyta)

CHAPTER 3: The complex structure of a Middle Devonian liverwort *Metzgeriothallus sharonae*, the dominant species in a newly discovered lagerstatte

Abstract

Non-vascular plants dominated the earliest terrestrial ecosystems; however, they left little fossil record because of their small size and delicate tissues. Liverworts (Marchantiophyta) represent an early-diverging group of land plants (Embryophytes) whose early evolution is poorly understood. Recently, remarkably well-preserved fossil liverworts were described in a Middle Devonian (~388mya) bed of dark gray shales at the Cairo Quarry in Eastern New York State. *Metzgeriothallus sharonae* Hernick is now widely accepted as the oldest confirmed liverwort. For the present study, approximately 60 shale fragments were collected from four locations along the lens exposure where *M. sharonae* was discovered. The organic fossil remains were isolated from the shale matrix using hydrofluoric acid maceration. Morphological analyses showed that *M. sharonae* has a more complex structure and branching architecture than originally thought. It is the only Paleozoic thalloid liverwort to exhibit intercalary branching as well as ventral branching at the thallus apex. Rhizoids and sporophytes were not observed and the plants appear to be mainly reproducing asexually. Their dimorphic architecture suggests that they may have grown both below and above the surface of shallow pools and on the surrounding shore banks. While *M. sharonae* was the dominant plant in the assemblage, similarly well-preserved organic remains of dozens of co-existing organisms were also recovered. Species interactions are preserved in fine detail, including filamentous epiphytes, fungal hyphae decomposing liverwort thalli, and stalked egg/spore cases of unknown origin attached to the liverwort thalli. Most of these delicate structures are disarticulated and fragmented; yet their exquisite preservation and even distribution suggests rapid *in situ* burial and fossilization in an anoxic environment, with the disarticulation having a secondary origin. The Cairo Quarry liverwort lens represents a conservation lagerstatte, a miniature 'frozen' freshwater ecosystem, and the only known liverwort dominated ecosystem from the Paleozoic. Continued study of the bed will likely lead to discovery of more reproductive structures and evidence of ecological relationships of *Metzgeriothallus sharonae,* providing a uniquely comprehensive view of a non-vascular plant dominated ecosystem early in the history of terrestrial life.

Introduction

The colonization of land by plants (Embryophyta) occurred around 470 million years ago (Wellman 2003; Graham and Gray 2001). It was one of the most important events in the evolution of terrestrial life on earth, yet the only fossil remains from this critical period are enigmatic cellular fragments and cryptospores (Edwards 2014; Wellman 2003). Morphological and molecular analyses suggest that the bryophytes (liverworts, mosses, and hornworts) diverged paraphyletically relative to the vascular plants (Mishler and Churchill 1984; Qui et al. 2006) with all four major extant lineages of land plants: the liverworts, mosses, hornworts, and vascular plants, having diverged by the Middle Silurian, ~425 mya (Graham 1993; Heinrichs et al. 2007). Liverworts (Marchantiophyta) have been resolved as the sister group of all other extant embryophytes by most phylogenetic analyses (Mishler and Churchill 1984; Graham, 1993; Kenrick and Crane 1997; Qui et al. 1999; Forrest et al. 2006). They have morphologically diverse and often complex gametophytes, ranging from leafy, to simple thalloid, complex thalloid forms, and uniformly simple, ephemeral sporophytes. Leaves appear to have evolved independently in more than one lineage (Crandall-Stotler 2005). Despite their reputation as a relictual lineage, liverworts are a successful, diverse clade of 6000-8000 extant species in six major, long-diverged lineages. Within the group are many isolated and enigmatic taxa as well as a few lineages undergoing current radiations, most notably in the epiphyllic Lejeuneaceae (Heinrichs et al. 2007).

Based on fossil and molecular evidence, it is estimated that the major lineages of liverworts had diverged by the end of the Permian (252 mya; Stech 2002; Heinrichs 2007). Paleozoic evidence of liverworts, is, however, extremely rare. Only a handful of fossil taxa that are accepted as having liverwort affinities have been described, and most of those are represented by a small number of specimens from single localities. This is repeated, with a few exceptions, throughout the Mesozoic as well (Lacey, 1969; Krassilov & Schuster 1984; Oostendorp 1987; Edwards, 2000). Fossil liverworts do not become more numerous until the Cenozoic. The majority of the Cenozoic specimens are leafy liverworts found in amber, or mats of complex thalloid liverworts, with fossils from both groups practically indistinguishable from modern taxa and easily placed in modern families (Lacey 1969; Krassilov and Schuster 1984; Oostendorp 1987; Edwards 2000). The depauperate fossil record of this group contributed to long term questions about liverwort evolution, the relationships between groups, and the many isolated, enigmatic taxa. Comparisons of fossil and extant taxa have helped elucidate key events in plant evolution, as well as the timing of events (Crane et al. 2004). Fossils can be more informative than extant groups in revealing relationships among major lineages, as they often possess unique combinations of characters (Donoghue et al. 1989). Even when fossils do not have a significant effect on phylogenies, they can offer insight into the sequence of events of character evolution. Hennig stressed the usefulness of fossils in challenging ideas of homology and the importance of the stratigraphic order of appearance of character states as evidence of polarity (Doyle and Donoghue 1989). Recent molecular phylogenies have resolved the evolutionary relationships of the main lineages and enigmatic groups, yet many questions remain about character evolution in the clade (Crandall-Stotler 2005).

The first Paleozoic fossil liverworts were described by Walton in the early 1900s (Walton 1925, 1928). He named five taxa, and a potential sixth taxon, dated from the Middle to Upper Carboniferous. The plant remains were carbonaceous in nature, and isolated from fine shales or mudstones by macerating the rocks with acid. The delicate, often unistratose plants were remarkably well-preserved with clearly defined cells; however, these were fragmentary fossils exhibiting a limited number of vegetative characters and no reproductive characters. All taxa were described as having characteristics of the Metzgeriales, or simple thalloid liverworts, a "bin" grouping now understood to be paraphyletic (or polyphyletic if the Treubiaceae are included; Forrest et al. 2006). One of Walton's discoveries, *Hepaticites kidstoni*, was initially thought by

Schuster (1966) to resemble the extant genus *Treubia.* At a later stage he later speculated that it may be closer to *Blasia* based on his interpretation of the orientation of the shoots, *Treubiites kidstoni*, however, remains the accepted name. The first widely accepted Devonian liverwort was discovered in the 1960s, isolated from the fine shales of New York State's Catskill Mountains using Walton's maceration technique (Hueber 1961). *Hepaticites devonicus* Hueber was composed of a multicellular midrib of elongated cells with unistratose wings with a serrated edge along with a basal portion of the plants that consisted of a rhizome bearing unicellular rhizoids. The remains were fragmentary and the thalli and rhizomes were not found in organic connection. Based on the similarity of midrib cells, and frequent co-occurrence, Hueber concluded the fossils belonged to the same plant. Because of the gross morphological similarity to several modern liverwort taxa, particularly the species *Pallavicinia xioides*, Schuster (1984) placed the fossil in the Pallaviciniaceae. Although *P. xioides* has a similar unistratose wing with a serrated edge, the midribs of modern taxa in the Pallaviciniaceae are more complex and include a central strand of conducting tissue. The 375 million year old *Pallavicinites devonicus* (Hueber) Schuster was accepted as the oldest fossil liverwort, in fact the oldest known bryophyte of any kind, for over four decades.

In 2007 a remarkable discovery was made (Hernick et al.2007) in a Middle Devonian (~388 mya) lens of dark gray and black shales at the Cairo Quarry, Eastern New York State, not far from the *P. devonicus* site. While studying shale fragments containing freshwater arthropods and using polarized light to increase contrast, paleontologists discovered that the surface was covered with exquisitely preserved liverworts with midrib and thallus wings. The specimens were very similar to *P. devonicus* but with a entire thallus border. Further distinguishing the new taxon were the absence of rhizomes. A few unattached rhizoids as well as a single sporophyte with a four-valved sporangium (also not in direct organic connection) were, however, observed on the shale surface (Hernick et al. 2007). The liverwort fossils were preserved in a 5 cm thick layer of fine shales and mudstones and measuring approximately 13 meters across. *Metzgeriothallus sharonae* Hernick is presently accepted to be the oldest confirmed liverwort. The association with freshwater arthropods and charophyte algae suggested the plants were growing in a shallow pool, and that the fossils were preserved in place (Feist and Hernick 2014).

Not only are the *M. sharonae* fossils preserved *in situ*, the upper and lower surfaces of the thin shale layers are distinct, with well preserved liverwort thalli covering most of the upper surface and amorphous material and masses of ostracods typically found on the lower surface. Together these indicate that the thin shale layers likely represent individual burial events. The goal of this study was to take advantage of the unprecedented abundance of the plant fossils in their original location by sampling across the exposed bed, using acid maceration to dissolve the shale matrix. More specifically, aims were to: (1) learn more about the structure of this important early land plant than can be deduced by studying the shale surface alone; (2) reconstruct the paleocommunity to collect data about other co-existing organisms to add to our understanding of the original community; and (3) use the results of these investigations to build a more complete ecological picture of this early bryophyte.

Methods

Collection:

Metzgeriothallus sharonae material was collected in August 2011 and June 2012 from an exposed lens of dark gray and black shales and siltstones, approximately 3-5 cm thick and 25m across, at the Cairo Quarry, where the fossil liverworts were originally discovered (Figure 1A). This locality is an old quarry located just south of New York State Route 145 at the Cairo Highway department headquarters, 3.22 km NW of Cairo, Greene County, New York (42.32˚ N and 74.04˚ W, NAD 83). For this study shales were collected from four roughly equidistant localities 3-5 meters apart (C0-C3, C=Cairo) along the lens exposure; including the location where the original specimens were collected (for this study referred to as C2). Through trial and error three other areas along the same liverwort-rich layer (itself comprised of many thinner laminae) were found where the shale could be readily accessed. Although an effort was made to collect equally from all four sites, the original locality (C2) was slightly undersampled in this study, and C0 is slightly over-represented in these results. With some practice it was possible to discern the liverwort fossils on the shale surface while in the field, and further winnowing of the samples under polarized light was done back in the lab. Only samples that included liverwort fossils were used, which resulted in approximately 60 shale fragments used for this study.

HF maceration and slide preparation:

Each of the collected shale fragments was carefully split until it was no longer possible to split any further, leaving fragments approximately 3-6 cm per side and several millimeters thick (Figure 1B,C). While the top of the shale surface showed nicely preserved thalli, the bottom was more often less distinct, with a jumble of coalified material with the only distinct feature being clusters of 3-dimensionally preserved ostracodes that are rarely seen on the upper surface. Each fragment was then washed gently with deionized water to remove contaminants and air-dried. The rocks were individually immersed in 58% hydrofluoric acid in custom-made polypropylene containers with a built-in polypropylene sieve until the silicates dissolved and the organic fossils released from the matrix. The isolated fossils were then immersed in a series of rinses in deionized water until a neutral pH was reached, and then rinsed twice more leaving the fossils immersed in the rinse bath for at least 24 hours each step. Initially a 250 µm sieve mesh was used, but changed to 125 µm mesh in order to capture smaller structures. The rinse water of a few samples was reprocessed through a 35µm sieve to capture more mesofossil structures. The fossil remains were sorted under a dissecting scope).

Well-preserved specimens and ones with informative morphological structures were mounted for further study. The isolated specimens were gradually dehydrated to 50% glycerine; only 50% because the fossils were too delicate to handle in a higher viscous medium. Exposing the thin fossil thalli to the air-water interface caused damage including rapid deformation that was often irreversible. Therefore the delicate fossils were carefully transferred using plastic pipettes with enlarged tips, cut to various opening sizes as needed. After transferring the selected specimen on a glass slide excess glycerin solution was wicked away with blotting paper. After a short time air-drying on a slide warmer a drop of warmed Glycerin jelly was added, a cover slip placed over the specimen, and the slide placed on the warmer for a brief period. Add sealing info. Using this method 642 specimens were made into permanent slides, 497+ of which were *M. sharonae* specimens. Prepared slides and extra material, including unmounted specimens and unmacerated rocks, will be deposited in the University of California Museum of Paleontology (UCMP).

Microscopy and microphotography:

Specimens still on the matrix were initially examined with a Leica Wild MZ8 stereo microscope using polarized fiber optic light source and a rotatable analyzer in order to increase contrast (Hernick, 2007). Water or cedarwood oil was applied to rock surfaces with a fine brush before photographing the compressions to help resolve details (Rowe, 1999; Hernick, 2007). For higher magnifications a Nikon D90 digital camera mounted on a Leica DMRB compound microscope was used. High-resolution images of the mounted specimens were taken with a Leica DM2500 microscope using Differential Interference Contrast, a Plan Apo 63x Oil objective, and a Nikon DS-Fi1 Digital Camera. Extended depth of field images were generated using add program (and website). Specimens for scanning electron microscope (SEM) were rinsed with demineralized water, mounted on SEM stubs covered with adhered processed black-and-white film, and examined under low vacuum conditions with a Hitachi TM-1000 SEM. Illustrated specimens will be stored in the UCMP Type and Illustrated Collections.

Results

Fossil yield:

Typically, hundreds of organic meso-fossils were isolated from each rock. The remains were fragmented, with the largest specimens a few centimeters long and less than one centimeter wide. Most were much smaller. Some rocks contained mainly fragments of thallus wings that had fallen off the midrib, and fragments of midribs. Samples with poorly preserved specimens or ones that appeared to have modern contaminants were discarded. Specimens selected for study originate from 41 of the 60 rocks that were macerated. The fossil yield and distribution of preserved characters in the samples was uneven. Some rocks contained far more varied, morphologically complex and well-preserved specimens than others. Two samples from locality C0 (C0R08 and C0R10) accounted for nearly a third of the selected specimens.

Basic structure and branching:

Most of the isolated fossil remains still retained some degree of 3-dimensionality. Many thalli (53 in total) exhibited a 'twisted' morphology, where the thallus twists at regular length intervals resulting in a flipped the orientation of the shoot (Fig. 3.1 D,G). Branches and structures attached to the thalli separate out in different directions in an apparently natural way when observed floating in water (Fig. 3.1E-G; also see 12D and 13G). While *M. sharonae* was originally described as having only simple dichotomous branching, maceration revealed far more complexity in branching structure. Three main types of "ventral" branching were observed: (1) branches emerging from the main midrib of the thallus (intercalary), (2) branches emerging from the apex of the thallus (tip branching), and (3) branches emerging from the origin of a dichotomy (see Fig. 3.2 for general examples). Out of the 497 specimens of *M. sharonae* used in this study 97 displayed dichotomous branching (Fig. 3.3A-I) and 105 ventral branching (Fig. 3.4A-G). These branch types were observed at varying stages of maturity, and were found either separated or still attached to the main thallus. The bases of the branches emerging along a midrib were generally very clearly preserved and showed the branches forming through a twisted, pleating type of growth (Fig. 3.5). Twenty two specimens possessed both ventral and dichotomous branching (Fig. 3.2A), with thirteen of those involving branching at the point of division of a dichotomy (Fig.3.6). There was at least one example of tertiary ventral branching (Fig. 3.4D) and many specimens had multiple ventral branches (Figures 3.1E, 3.2D, 3.4E). Many specimens were fragmented while handling and this was disproportionately true for the long thalli and those thalli with multiple branches. Despite the vast number of thalli sequestered in each shale specimen, except for one or two equivocal examples, plant bases were not found and, and no rhizomes or rhizoids were discerned.

Shoot apices and enigmatic, possibly reproductive structures on midrib and thallus wings:

Of the 497 selected specimens, 114 had complete or partial shoot apices (Fig. 3.7). Many of the partial apices were damaged in a characteristic way that included evidence of reaction tissue, mainly thickened cells walls around the damaged area (Fig. 3.7C). Besides the clearly identifiable ventral branches, many additional structures were observed along midribs or at shoot apices (Fig. 3.8). Some are likely new branches while others remain enigmatic. In total 68 specimens have some type of structure, ranging from clearly discernible budding branches to opaque "bumps" that are found in the same relative position as the other structures along the midrib of the thallus, at the point where the midrib transitions into the thallus wings (Fig. 3.8A). Commonly these structures are found at roughly 1 mm intervals. Seventy-eight specimens possessed oblong smaller-celled structures scattered on the surface of thallus wings (Fig. 3.9). While irregularly placed and seemingly randomly oriented, they were located about midway between the midrib and the thallus edge, becoming more closely spaced near thallus apices (Fig. 3.9D). In a few cases they were found clustered on what appear to be smaller, specialized thalli (Fig.3.9A). In some well-preserved examples very small structures can be observed within the small-celled oblong structures (Fig.3.9B). In many instances these structures appear to have fallen out of the thallus leaving a hole behind (Fig. 3.9C).

Damaged areas with reaction tissue, oil body cells, sub-cellular details:

Nearly all *M. sharonae* specimens exhibited the dark, opaque, scattered cells that are homologous to the specialized oil body cells of modern liverworts (Chapter 2 of this dissertation). Many *M. sharonae* specimens contain various types of opaque structures inside their cells, possibly fungi, other microbial symbionts, or pathogens (Fig. 3.10), and 126 specimens show evidence of damaged areas with thick reaction tissue, (Figures 3.2D, 3.3A,G, 3.5F,G, 3.6C, 3.7C) which indicates that the damage occurred while the plant was still living.

Interspecies relationships: algal epiphytes, saprobic fungi, attached egg cases:

Some *M. sharonae* specimens preserve evidence of a direct relationship with other organisms in fine detail (Figures 3.11 and 3.12). Twenty-one well-preserved specimens were covered to varying degrees by fine filaments, probably an algal epiphyte (Fig. 3.11B), often completely cover the thalli like a dense coat of fur. The same structures cover some of the charophyte algae specimens from the same rocks (Fig. 3.11A). In a number of rocks the fungal hyphae decomposing the fossil thalli appear to be clearly of fossil origin (Fig. 3.11C) however this is still under investigation. Finally, a number of specimens of stalked egg/spore cases of unknown origin were recovered, most floating freely in rinse water after isolation from the rocks (Figure 3.12A-E) but in two cases still attached at the base of the stalk to *M. sharonae* thalli (Figures 3.11D and 3.12 F-G) and a third with a few *M. sharonae* cells still adhering to the base of the stalk (Fig. 3.12D,E). In several of the free-floating specimens the egg/spore contents were still inside (Fig. 12A,B). Although all recovered specimens were very similar in size, averaging about 0.7mm long from base of stalk to tip, there was some variation in shape and in how the structures appeared to dehisce (Fig. 3.12C).

The broader freshwater community:

Similarly well-preserved organic remains of dozens of co-existing organisms were recovered, including other non-vascular plants and charophyte algae, disarticulated fragments of aquatic and terrestrial arthropods, many types of resting structures, egg cases, and large spores, and a number of other enigmatic structures. Trilete spores were common in the samples although they were only found on the few occasions when smaller sieve sizes were used. However, even at the larger sieve sizes, the surfaces of both the liverworts (48 of the *M. sharonae* specimens) and charophyte algae were sometimes covered with small, alete, smooth, and oblong spores 10-15 microns long.

Discussion

Description of *M. sharonae* and its local environment:

Isolating the fossils from the shale matrix revealed that *Metzgeriothallus sharonae* had a considerably more complex structure than the flat, ribbon-like, dichotomously branching thalli described in the original publication. That description was based on the portions of the fossils that were visible on the shale matrix (Hernick 2007). In the current study relatively flat, meandering *M. sharonae* thalli that exhibited simple dichotomous branching were frequently observed both on the surface of the shale fragments and floating in the rinse water after maceration of the rocks. Furthermore, hydrofluoric acid maceration showed that the *M. sharonae* remains were distributed throughout the matrix and that many thalli were somewhat narrower than those seen on the surface, had a twisting morphology, and were producing new intercalary branches. In many specimens, new thallus branches emerge from the midrib of mature thalli, from thallus apices, and from the origin of a dichotomy. In terms of gross morphology at least, they did so in ways that were remarkably similar to some modern liverworts. Until this study, thalloid liverworts with anything but simple dichotomous branching were unknown from the Paleozoic. *M. sharonae* is therefore not only the oldest known liverwort taxon, it is the only Paleozoic thalloid liverwort known to exhibit ventral branching. The broad bifurcating thalli on the upper surface of the shale layers and narrower, twisting ventral branching within the rocks suggests a dimorphic morphology as is typically found in semi-aquatic thalloid liverworts alive today, such as *Ricciocarpus natans* and *Riccia fluitans*, both in the complex thalloid Marchantiales.

Considering the nearly ubiquitous presence of the freshwater arthropods such as ostracodes and conchastratons, and the many algae specimens found in most samples, there is little doubt that the liverworts were growing in a freshwater environment. Whether they were growing submerged or on the surface and/or along banks is still unclear. The concentration of specimens in each rock is very high, even though the layers are very thin. Evidence of this can sometimes be seen at the edges of the shale fragments even before the rocks are macerated (Fig. 3.13). This suggests that the liverworts often grew densely layered in the water, or on the surface or on banks, with newer thalli growing directly over older thalli as liverworts commonly do today (Fig. 3.13). It is possible that the flooding events that led to the plants being buried and fossilized on a repeated basis occasionally reached higher levels than normal, so that plants growing on the bank were buried by sediments. This could explain why in some rocks fungi are decomposing the liverwort thalli, while specimens from most rocks, even in the case of seemingly older thalli, do not ever show fungal hyphae. Yet some of these other specimens, from rocks that produce no evidence of fungi, exhibit the "furry" phenotype, where the surface is covered with fine epiphytic growth. These very fine hyphae or algal filaments would have been extremely delicate and surely growing in water or very moist environment at least. As these filaments do not appear to have crosswalls they could be of cyanobacterial origin. That far more specimens were found covered in fine filaments is consistent with the hypothesis that the plants were growing in a shallow pool. A broader ecological study of the locality with sampling at a much finer scale could provide additional information about the microenvironment (shallow pool, banks) that could be linked with individual fossil specimens.

Evidence that the plants have been preserved *in situ*, and that each shale fragment that no longer breaks apart easily represents a single burial event, is that there appears to be a distinct 'top' and 'bottom' to each fragment. This would explain why there were no rhizomes or rhizoids found in the rocks despite high fossil concentration and the presumably complete capture of the ecosystem. If the deposition was relatively steady, for example if this was a shallow pond that was filling in slowly with fine sediments over a number of years, after each burial event (seasonal storms perhaps) most of the plants might be buried, but the few whose shoot tips left emerging could be the ones that branched out again to create a new colony. If a colony is regularly being buried spore release may not be as reliable a method for reproduction as spores may be washed away or buried too deeply; therefore, asexual reproduction may have been more reliable. The plant material is poorly preserved at the lower surface. This could be where many of the poorly preserved fragments in the rocks are coming from, and their poor preservation could reflect their state at the time of fossilization; in other words they may have already been in a state of deterioration, perhaps the wings of older thalli. These wings seem to have readily fallen away from the midribs. The cell walls in the area between the midrib and wing are very thin, and this appears to form a sort of abscission zone. As the older thalli produced ventral branches that continued to branch, the older thallus wings may have fallen off and collected at the bottom of the shallow pond. The older midribs could then form "branches" anchoring the plants even after a flooding and burial event (Fig. 3.14).

The *M. sharonae* thalli, with their delicate unistratose wings are some of the earliest foliose organs showing evidence of both abiotic and biotic interactions. A large number of *M. sharonae* thalli show evidence of damage and reaction tissue and many had structures inside cells, possibly fungi, although usually associated hyphae are not found. These structures could be unicellular fungi or other types of microbes either symbiotic or pathological. Sometimes the structures occurred in particular areas surrounded by thickened cell walls but often not. The reaction tissue commonly seen may have both biotic and abiotic causes. Several types of herbivory on *M. sharonae* were identified previously (Labandeira et al. 2013) including the significant discovery of the earliest example of foliage feeding. Arthropod herbivory, other microbial infections and intermittent drying may all have been factors leading to the creation of reaction tissue. All three direct relationships that were observed: the epiphytic algae, the attached egg cases, and saprobic fungi decomposing liverwort thalli were likely facultative. The epiphytes were also growing on the charophyte algae, and one stalked egg case was found attached to a clam shrimp 'shell'.

Systematic placement of *M. sharonae*:

Metzgeriothallus sharonae has an unusual suite of vegetative characters, and without a phylogenetic analysis, it is difficult to place unequivocally in a particular liverwort clade. Placement in the liverworts itself is more assured. Characteristics typically used to differentiate liverworts from the other bryophytes are unicellular rhizoids, slime papillae, and their unique organelles called oil bodies (Crandall-Stotler et al. 2005). Rhizoids were not found in this study. Slime papillae are single-celled structures that often surround growing areas and other parts of the plant, sometimes exuding mucilage (Schuster 1984). One specimen (Ms174) appears to show similar structures to slime papillae (Figure 3.7D), and occasionally cells at the thallus margins of the *M. sharonae* thalli appear to be broken off even though the specimen may be otherwise well-preserved with an intact edge. The broken off structures may have been slime papillae since these are especially fragile cells. Oil bodies are considered an important synapomorphy of the group (Schuster 1984). In some lineages, including the early diverging *Treubia* and Marchantiales (complex thalloid liverworts) a single large oil body fills specialized cells, and the remaining cells do not contain oil bodies (ref). In the previous chapter, I showed strong support for the hypothesis that the scattered dark cells of M. sharonae are homologous to the specialized oil body cells of modern liverworts (Chapter 2 of this dissertation).

In plants, vegetative characters are often convergent (Donoghue and Doyle 1989). Sexual characters are generally preferred for identification, yet all potential reproductive structures found in this study are to a degree equivocal. Another liverwort apomorphy is the presence of simple, ephemeral sporophytes. A possible four-valved sporangium, the most common dehiscence type in liverworts, was discovered on the shale surface described in the original publication (Hernick et al. 2007). Unfortunately, no sporophytes were found in this larger study, so the presence of four-valved sporophytes was not confirmed. However, the original described structure was well preserved and can be accepted, especially considering the rarity and ephemeral nature of liverwort sporophytes. Four-valved sporophytes are typically found in many lineages of the Jungermanniopsida, which includes both major simple thalloid lineages as well as the leafy liverworts, but they are never found in the Marchantiopsida (complex thalloids and their close relatives). Mosses and liverworts grow by a single apical cell. In liverworts, the shape of the cells is diagnostic of major lineage and influences the gametophyte's structure. There is some question about the existence of growth by apical cells in the fossil material, as the expected resulting pattern of merophytes (Ligrone et al. 2012) was not clearly observed. While determination of the shape of the apical cells of *M. sharonae* is as yet unclear, additional study, perhaps using techniques such as transmission electron microscopy may be able to answer that question.

Damaged areas and structures on the liverwort thalli have been previously diagnosed as arthropod damage (Labandeira et al 2013). At least one of those, the oblong, multi-celled structures scattered on the thalli that were though to be insect galls, may merit new analysis. It is possible that these are antheridia, the male reproductive structures. These 100-200 µm long structures are fairly common, seem to be forming at the thallus apex, and are approximate the size and shape of liverwort antheridia (refer to figure). Additionally, nothing that resembles any arthropod larval stage or structure has been found in any specimen. Two objections to this hypothesis are that extant simple thalloid liverworts generally produce antheridia on the midrib rather than the thallus wings (of those that have this midrib and wings structure). Also there is not clear evidence of a supporting neck, or stalk, which is present in all liverwort antheridia (Ligrone et al. 2012)). Interestingly, *Apotreubia* and *Haplomitrium,* both in the small clade Haplomitriopsida, which is sister to the remaining liverworts (Marchantiopsida+jungermanniopsida), have a very short, one cell stalk (Engel 1981). A similar base could be present in the *M. sharonae* antheridia, but just very short and obscured. A few specimens do exhibit a possible stalk-like structure (Fig. 3.9E), but others seem to exhibit organic connection along an entire side, which would not be consistent with an antheridial interpretation. The oblong structures definitely deserve more study, particularly of a few specimens that may show earlier developmental stages.

Based on the site of occurrence of reproductive structures in many modern liverworts, a number of other structures could very well be reproductive. These structures are either positioned along midribs, often alternating from one side of the costa to the other, or found at the tips of thallus branches. Some of the structures could be archegonia, including one specimen that bears a striking resemblance to specialized female reproductive branches of *Metzgeria* species (Fig. 3.8C). However, the *M. sharonae* specimen does not appear to have the characteristic 'collar' at the base of the branches. All these structures need further study and their (occasional) presence in the results of this study, along with the vast number of still sequestered, easily accessible fossils, gives reasonable hope that an understanding of the sexual lifecycle of *M. sharonae* may eventually be understood.

While the ventral branching appears superficially similar to that of some *Metzgeria* species today, the branch bases are unique. The twisted branch bases of the many intercalary branches produced along the midribs of the larger mature thalli and possible female branch are unlike those of modern taxa. So while the overall structure is even more similar to modern taxa than originally described (Hernick et al. 2007), some evidence suggests that this may be a result of homoplasy. Further complicating the placement in modern lineages, some *M. sharonae* characters are more similar to the Marchantiales (complex thalloids), particularly the specialized oil body cells that are found in that clade, as well as in the Treubiales. These specialized cells are not found in any simple thalloid liverworts today, although they are occasionally found in one of the two main leafy liverwort clades (ref). Another example that evokes the complex thalloids is the repeated short ventral branches from the apex of a shoot that resembles those some genera (Fig. 3.15).

Liverworts in the Devonian and Carboniferous:

It is not surprising that a small sampling would include only vegetative characters because of the ephemeral nature of plant reproductive structures (including the entire sporophyte itself in the case of liverworts). However, with this larger study that examined thousands of specimens and found scant, equivocal evidence of reproductive structures and no sporophytes, we can conclude that *M. sharonae* thalli were mainly reproducing asexually. Additionally no rhizoids or unambiguously individual plants were found. While a number of very tiny "thallus primordia" were found, these were always attached to a mature plant or show clear evidence that they were originally attached. No examples of newly germinating thalli were seen. This predominance of asexual reproduction is consistent with what we know of vascular plants from the Devonian from the Rhynie Chert (several of which do not have a vascular bundle). The only other known Devonian simple thalloid liverwort, the slightly younger *Pallaviciniites devonicus,* was discovered in similar shale deposits in the same geographic area. *Pallaviciniites devonicus* has a very similar dichotomously branching midrib and unistratose wings, yet it also has a rhizomatous portion with rhizoids, which was not found in *M. sharonae*. Despite the lack of rhizomes *M. sharonae* appears to be branching freely and spreading asexually through ventral branching.

It is interesting that *P. devonicus* and *M. sharonae* have strong morphological similarities, and both have been placed in two of the six main, long-diverged lineages. Besides having a similar thallus morphology and size, the cells in both the thallus and midribs are practically indistinguishable between the two taxa. Have we discovered representatives of the two lineages not long after divergences, or have these taxa retained similar primitive characters even though they are no longer closely related? Many of the Devonian and Carboniferous taxa were growing in both spatial and temporal proximity, especially during the Devonian (Wellman 2003). These two Devonian taxa were found in the Catskill Mountains within a few miles of each other in localities separated by less than 10 million years, suggesting that they or closely related precursers, were likely to have grown sympatrically. If so, perhaps *P. devonicus* flourished on the banks around the pools *M. sharonae* was growing in. Both share some characteristics with the Carboniferous liverworts described by Walton (1925, 1929) from England and Wales. While New York State and the United Kingdom are widely separated today and have very different climates, in the Devonian the two regions were much closer together. Both were part of the equatorial Euramerican floral realm. This implies that the localities of the Carboniferous taxa from the UK had diverged from a similar latitude as the New York Devonian taxa.

It would be hasty to jump to too many conclusions regarding the similarity of the 'simple thalloid' forms that are most commonly found in sediments from these early periods for two reasons: the overall rarity of Paleozoic liverwort species discovered to date and bias of the fossil record. Both the particular types of localities these early liverwort taxa are found in and their simple thalloid morphology may be artifacts of bias in the fossil record. These were basinal areas or areas where delicate liverworts were able to fossilize because of deposition of fine silt, forming the shales and mudstones that virtually all these taxa have been found in. Phylogenetic analysis will be needed to test the placement of these earliest fossil liverworts. According to current molecular dating estimates, the two families that the Devonian taxa have been placed in, Metzgeriaceae and Pallavicinaceae, had not even diverged by the end of the Devonian (Heinrichs et al., 2007). If the fossil placement is found to be correct, some nodes will indeed need to be pushed back considerably. Alternatively they may represent earlier stem groups, or possibly ancestral forms that existed before the split.

The Cairo Quarry lens represents an *in situ* conservation lagerstatte, a miniature 'frozen' freshwater ecosystem. *Metzgeriothallus sharonae* fossils remain sequestered in large numbers in the lens and likely at other locations in the quarry. *Metzgeriothallus sharonae* has already been found at the nearby Bates Hollow quarry but less well preserved (Hernick 2007). Further extraction and maceration of these fossil remains will likely reveal important structures such as sporophytes. *Metzgeriothallus sharonae* can thus serve as a Paleozoic liverwort fossil model system, one informative for understanding liverwort and land plant evolution as well as a model system for understanding interrelationships between plants and other freshwater organisms. As the only known non-vascular plant-dominated ecosystem from this Era, this unique cache of spectacularly well-preserved *in situ* fossils has the potential to be a complement to the Rhynie Chert, as a slightly younger version of a frozen freshwater ecosystem and one that is not a hot springs locality but a normal pond.

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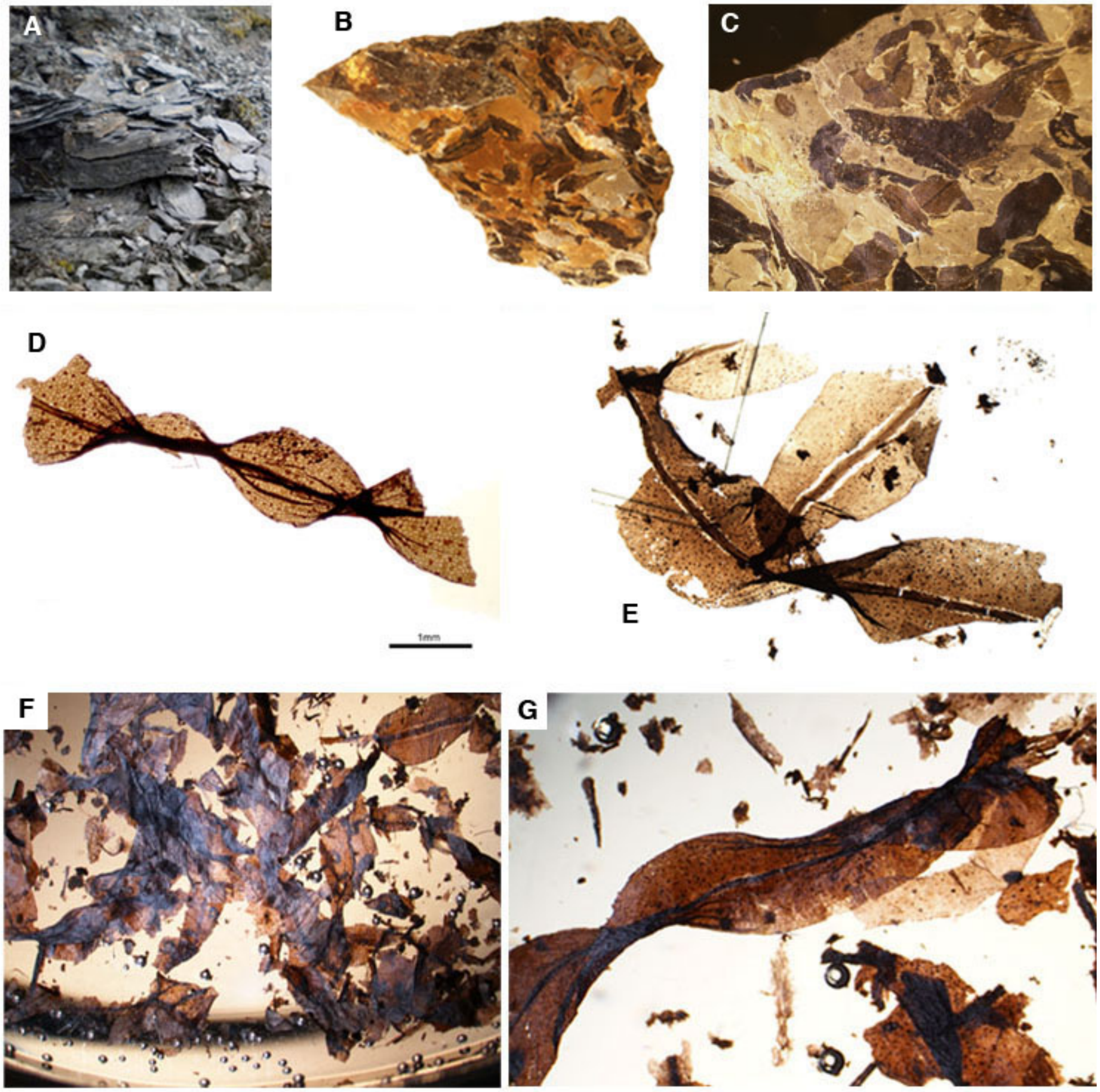

Figure 3.1. Collection and processing of *Metzgeriothallus sharonae* fossils. A, Liverwort-rich layer at Cairo Quarry in Eastern New York; B,C, Shale fragment under polarized light revealing a surface covered with liverwort thalli; D-G, Specimens isolated from the matrix showing filamentous and 3-dimensional structure; E-G, Specimens still floating in rinse water; D is a permanent slide.

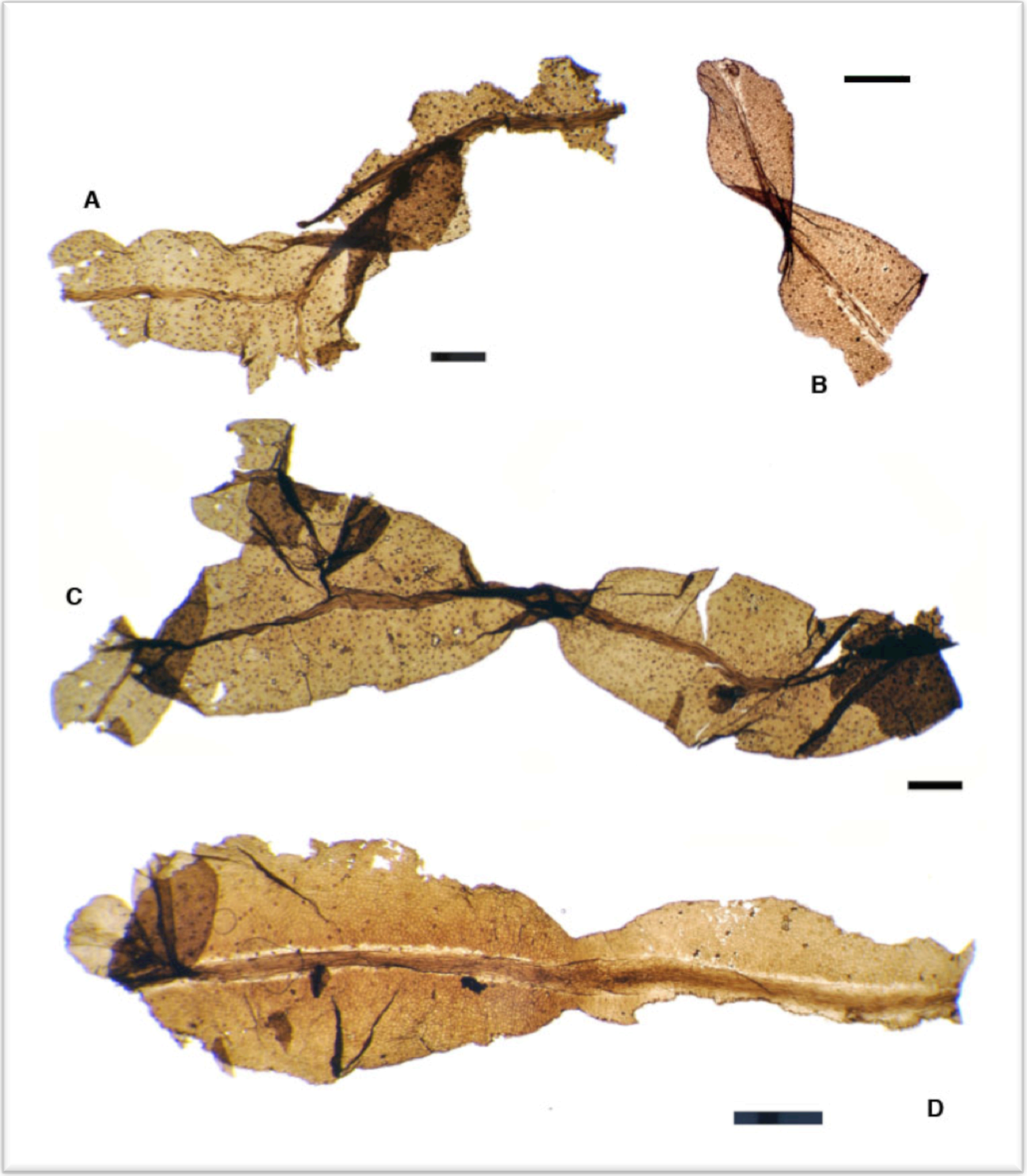

Figure 3.2. Examples of variation in *M. sharonae* thalli. A, Dichotomizing, intercalary branch emerging from the midrib of the main thallus. Only a fragment of the main thallus remains; B, Thallus twisting to flip orientation. A structure, possibly a new branch, is developing at apex; C, Multiple examples of dichotomous branches; D, This specimen possesses two intercalary ventral emerging from nearly the same spot. It also exhibits the narrow small-celled regions of some specimens, and some damaged areas with reaction tissues at lower right. Scale bars=1mm.

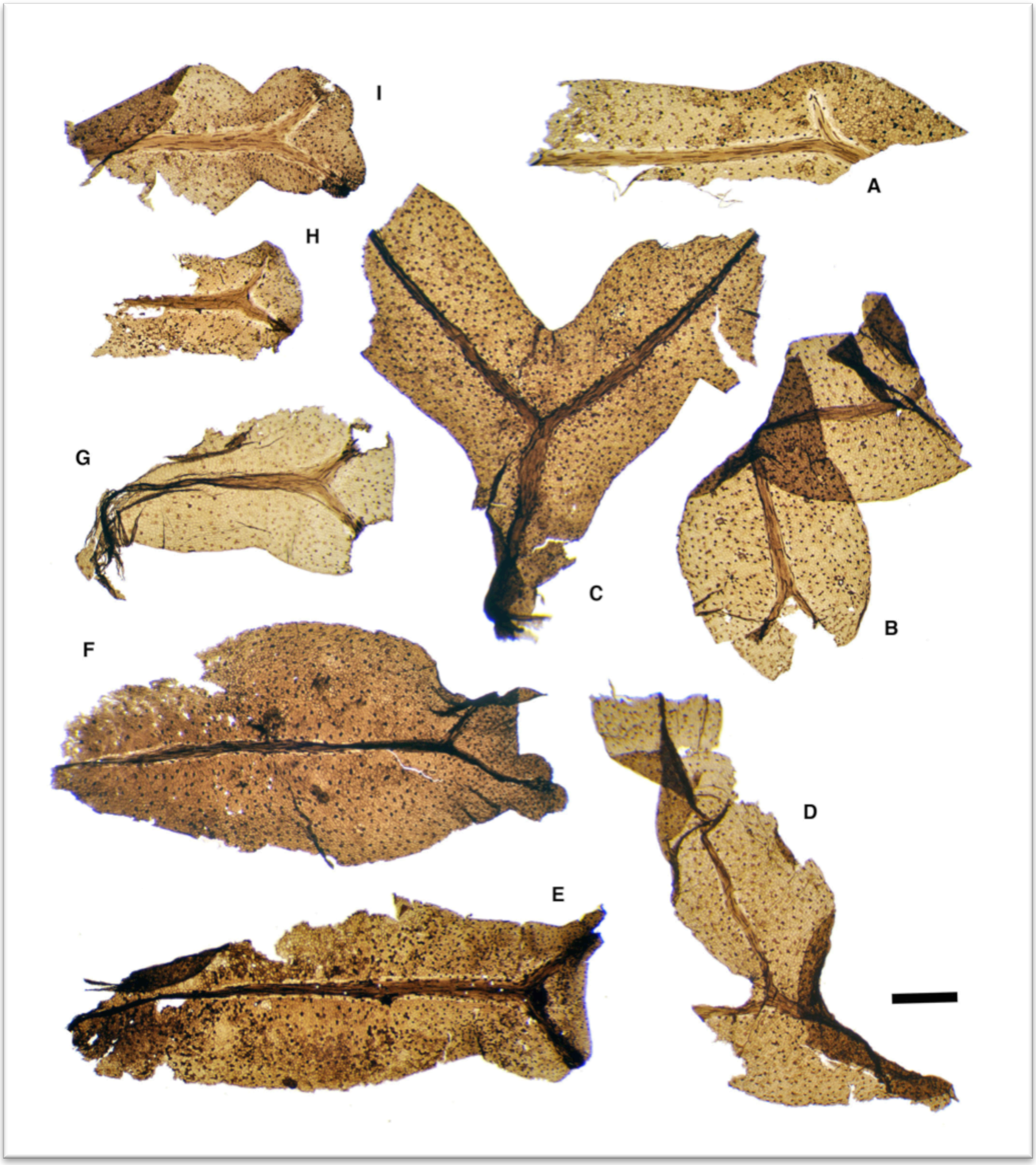

Figure 3.3. Examples of dichotamous branching in *M. sharonae.* Scale bar=1mm.

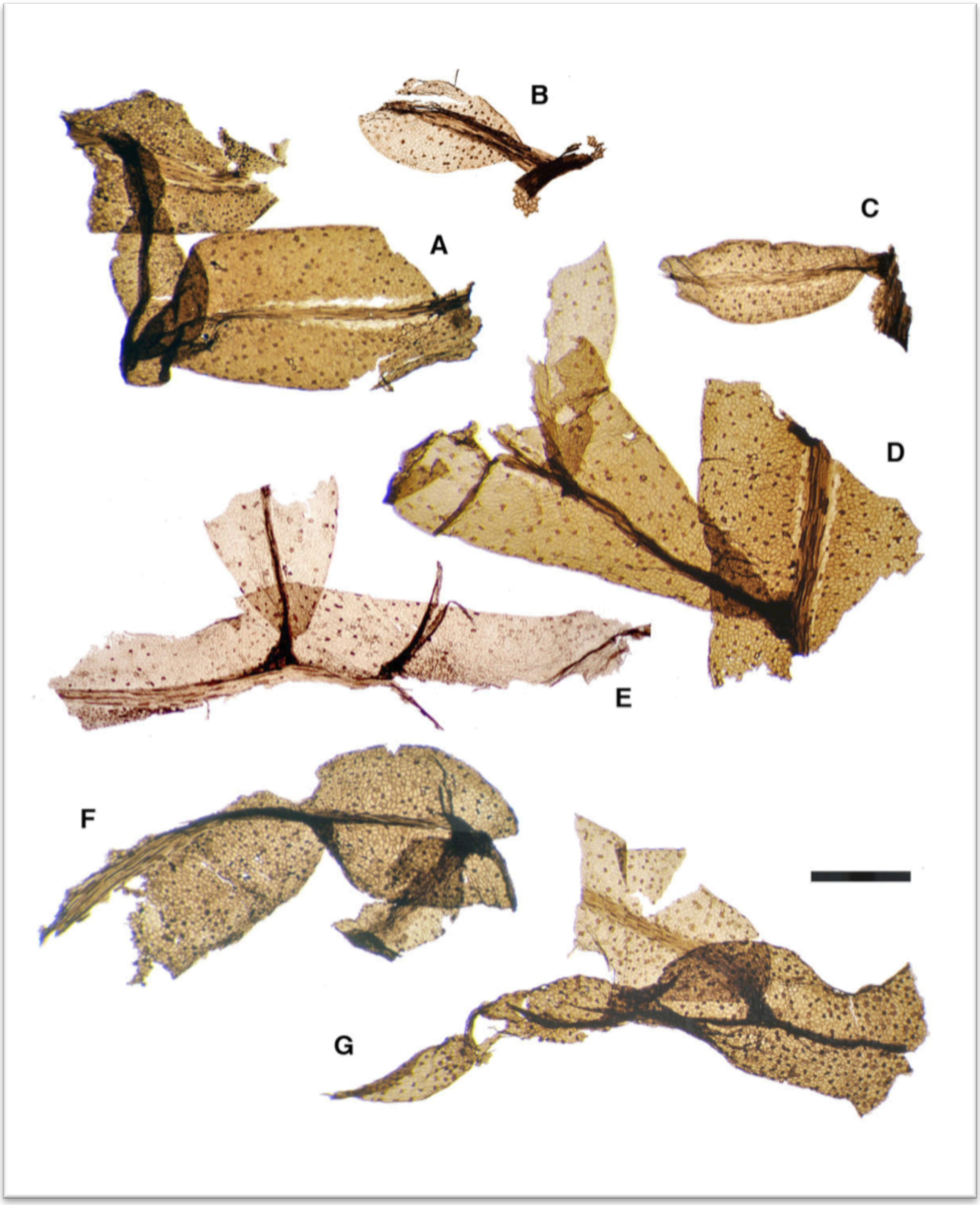

Figure 3.4. Examples of ventral branching in *M. sharonae*. D, The intercalary thallus branch has a tertiary branch. E.Two intercalary ventral branches approximately one millimeter apart. Scale bar=1mm.

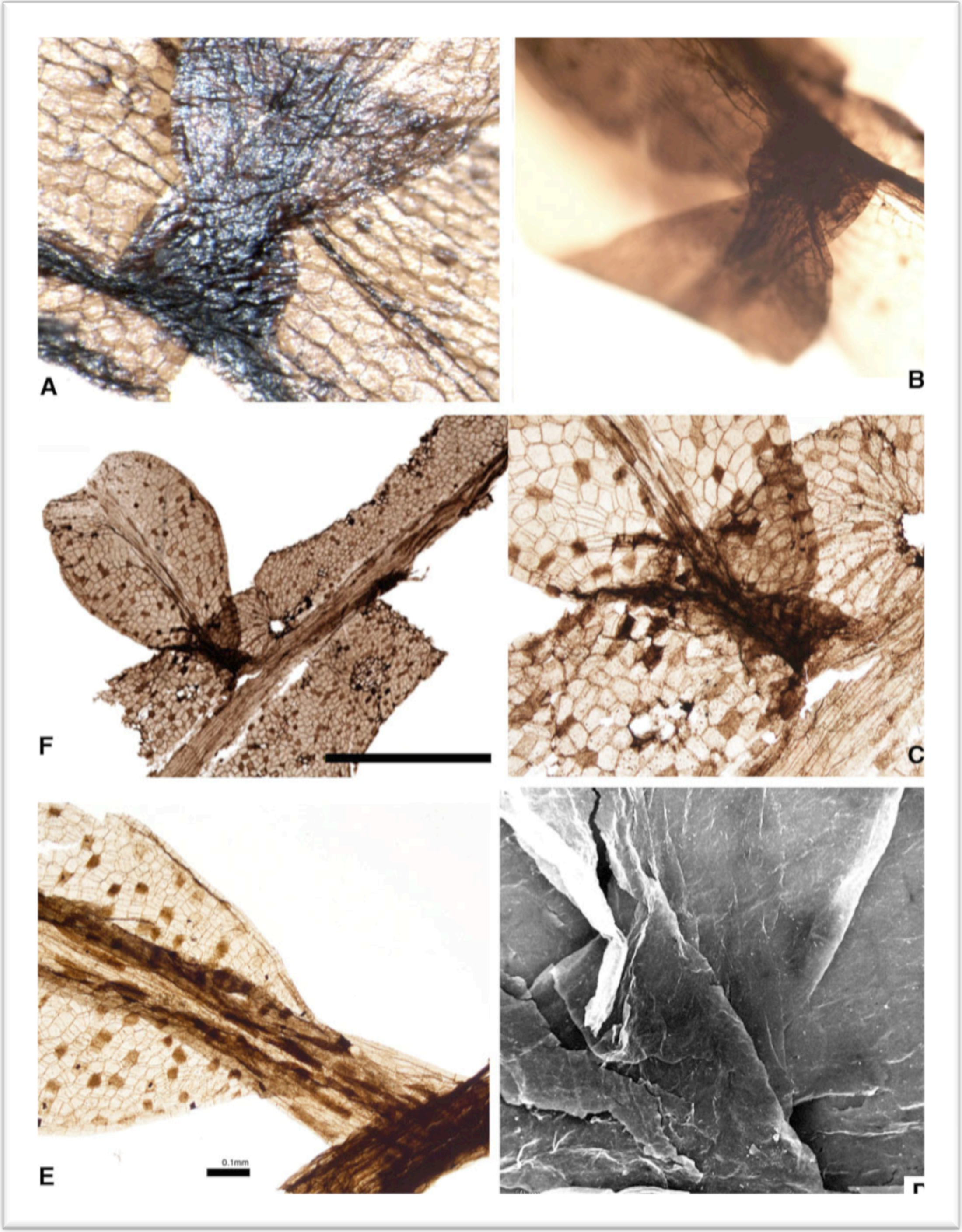

Figure 3.5. Close ups of ventral branch points in *M. sharonae*; C,F are same specimen D. SEM of branch base; Scale bar F, 1mm; E.0.1mm

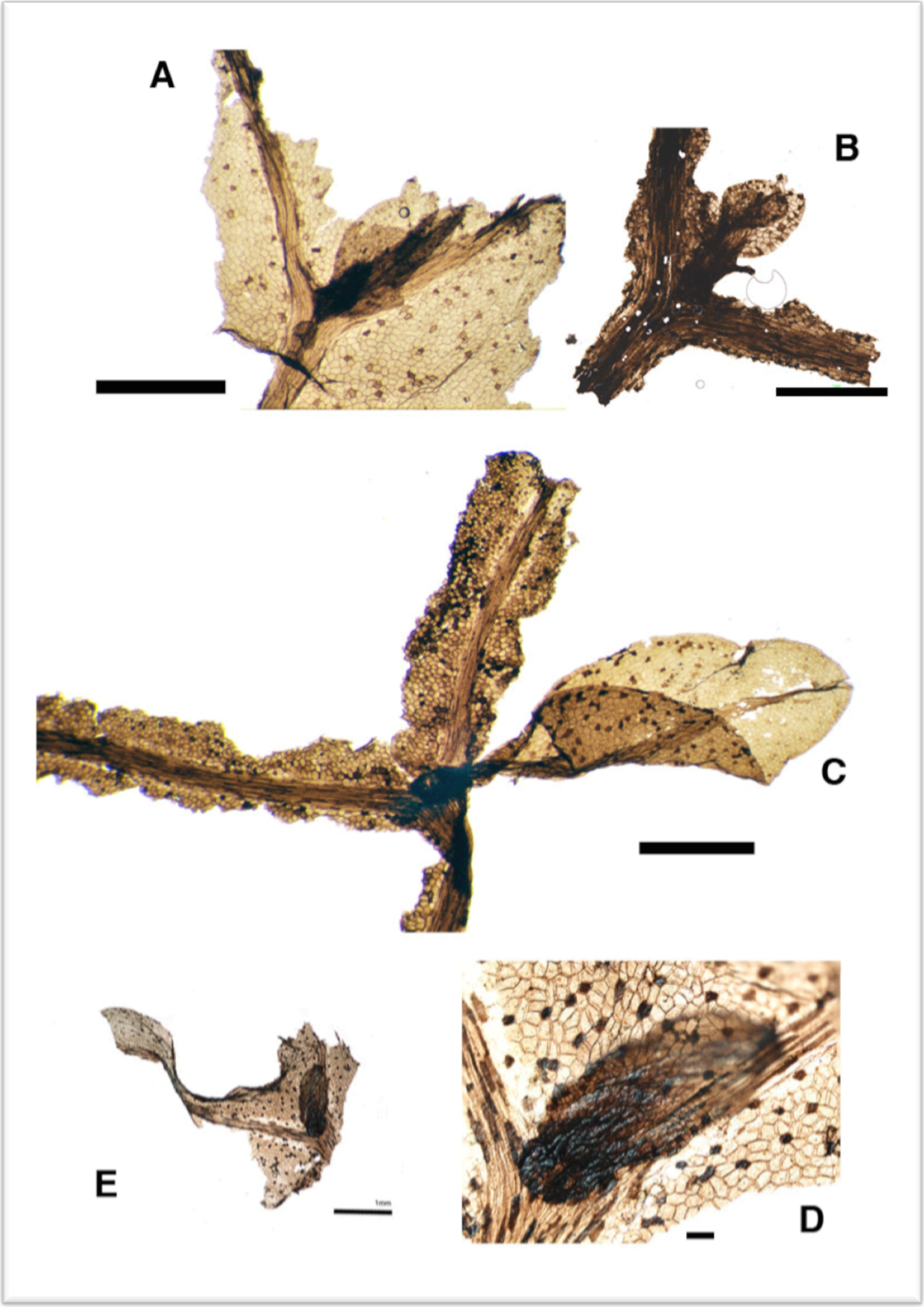

Figure 3.6. Ventral branches from origination of dichotomy in *M. sharonae*.

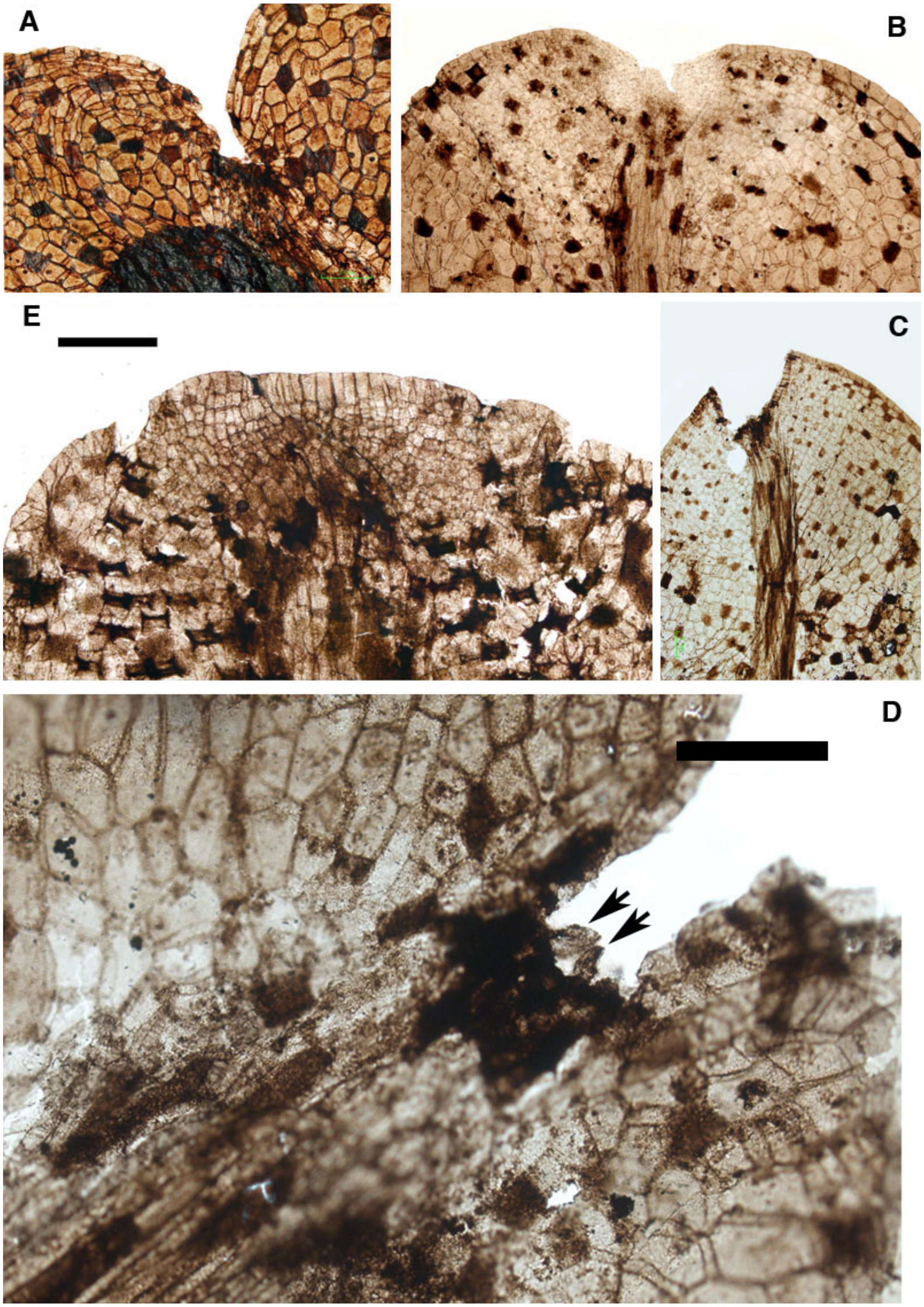

Figure 3.7. Variation in Shoot apices in *M. sharonae*; C. Damaged apex is likely evidence of arthropod tip feeding. F, Two structures at apical region resemble modern slime papillae (arrows) Specimen no. Ms174. Scale bars 100 µm

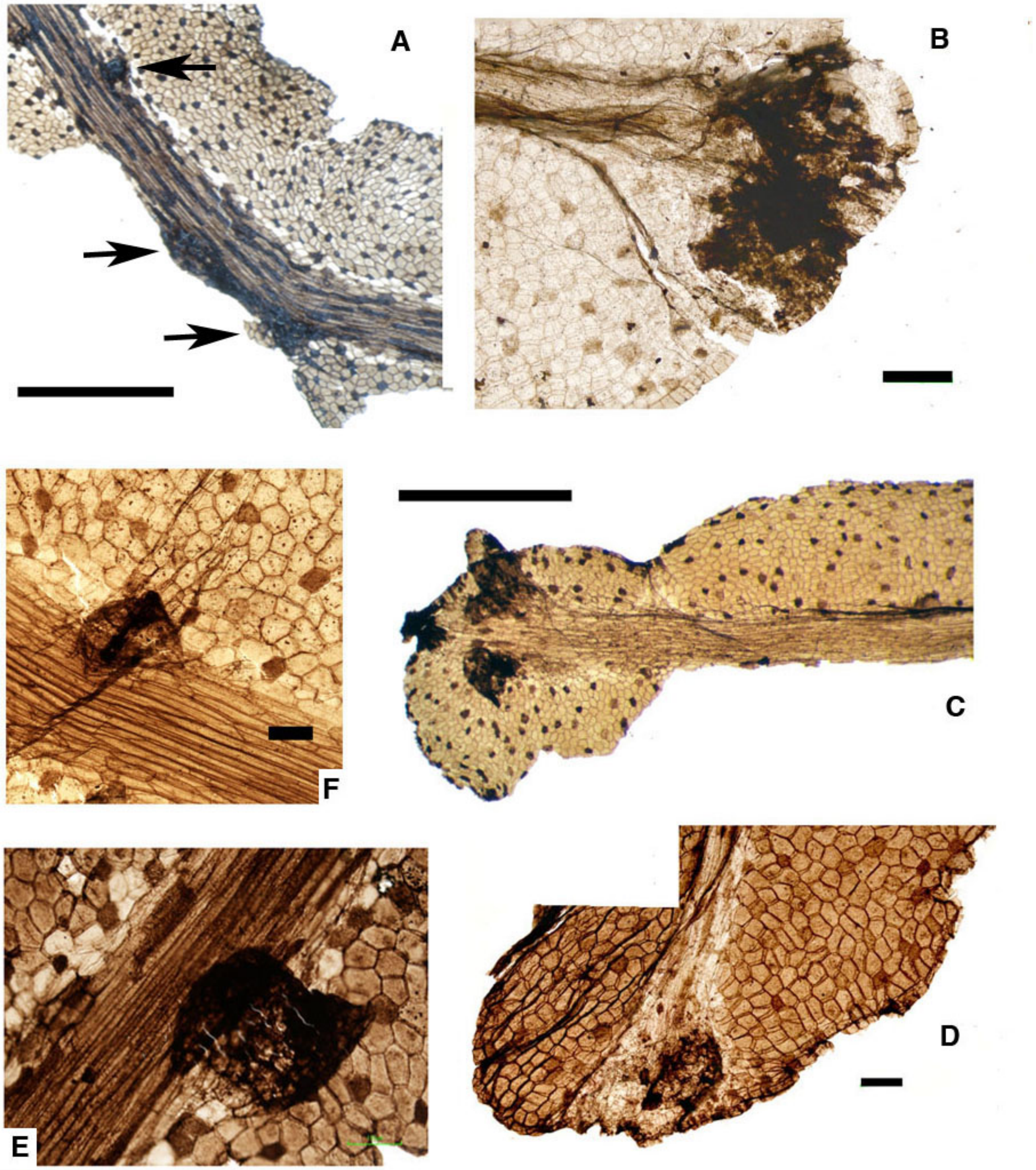

Figure 3.8. Enigmatic, possibly reproductive structures at midribs and apices of *M. sharonae* specimens. A, Example of thickened areas at often found at alternating intervals; B,D, Dense area of poorly defined, smaller cells at thallus apex; C, Two structures that superficially resemble female reproductive structures of some liverworts; E,F, Enigmatic developing structures. Scale bars A,C,1mm; B,D,F, 100 µm

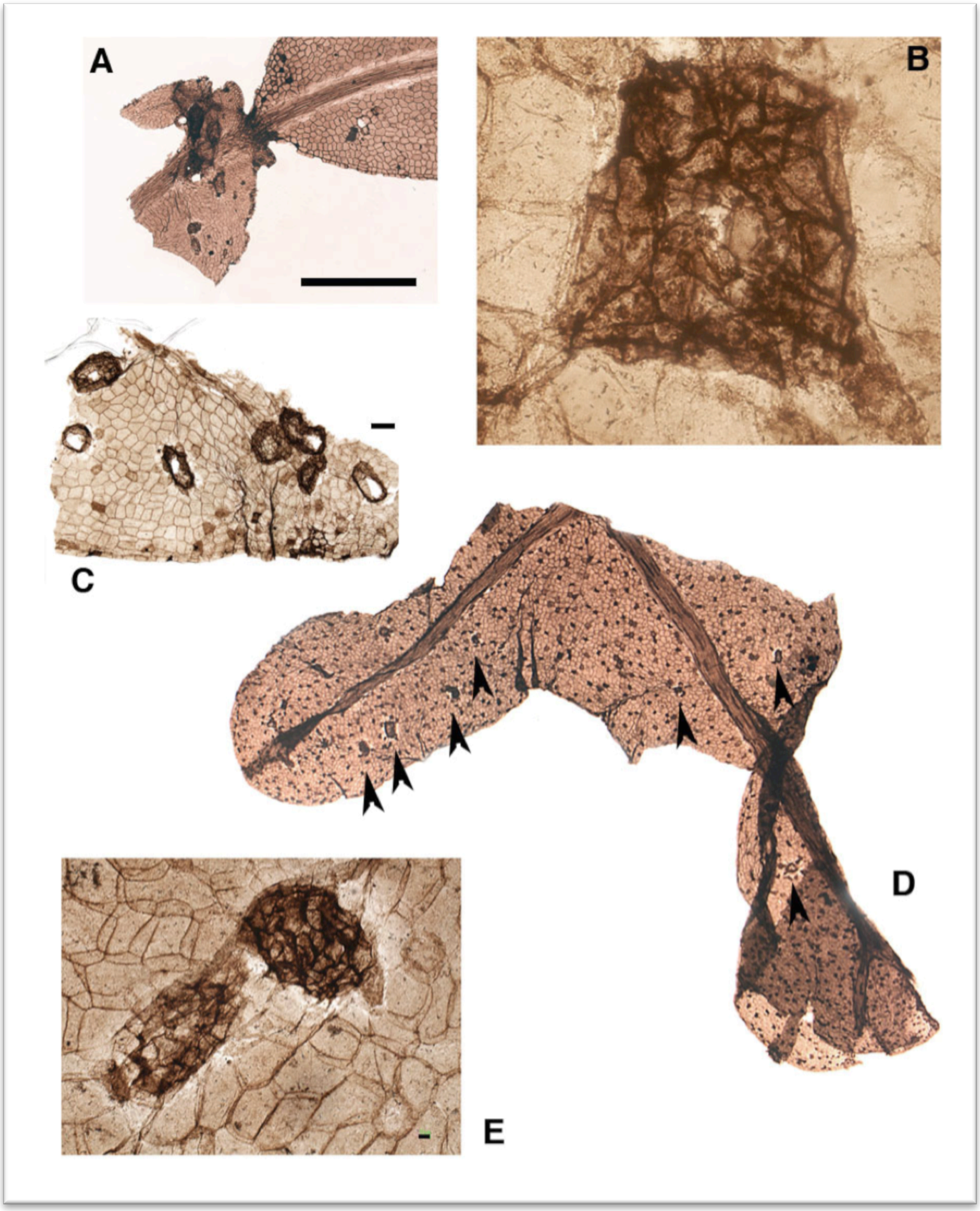

Figure 3.9. Enigmatic, antheridia-like structures on some *M. sharonae* thalli. A, Unusual, perhaps specialized branch has clusters of the oblong structures; B, At a high magnification small round objects can be seen inside some of the thallus structures; C, Many of the oblong structures have disappeared leaving characteristic holes in the thallus; D, The structures more often appear irregularly space mid-thallus(arrows) in flat, relatively wide dichotomizing thalli; E, Two structures that show hints of a possible short stalk. Scale bars A, 1mm; C, 100 µm

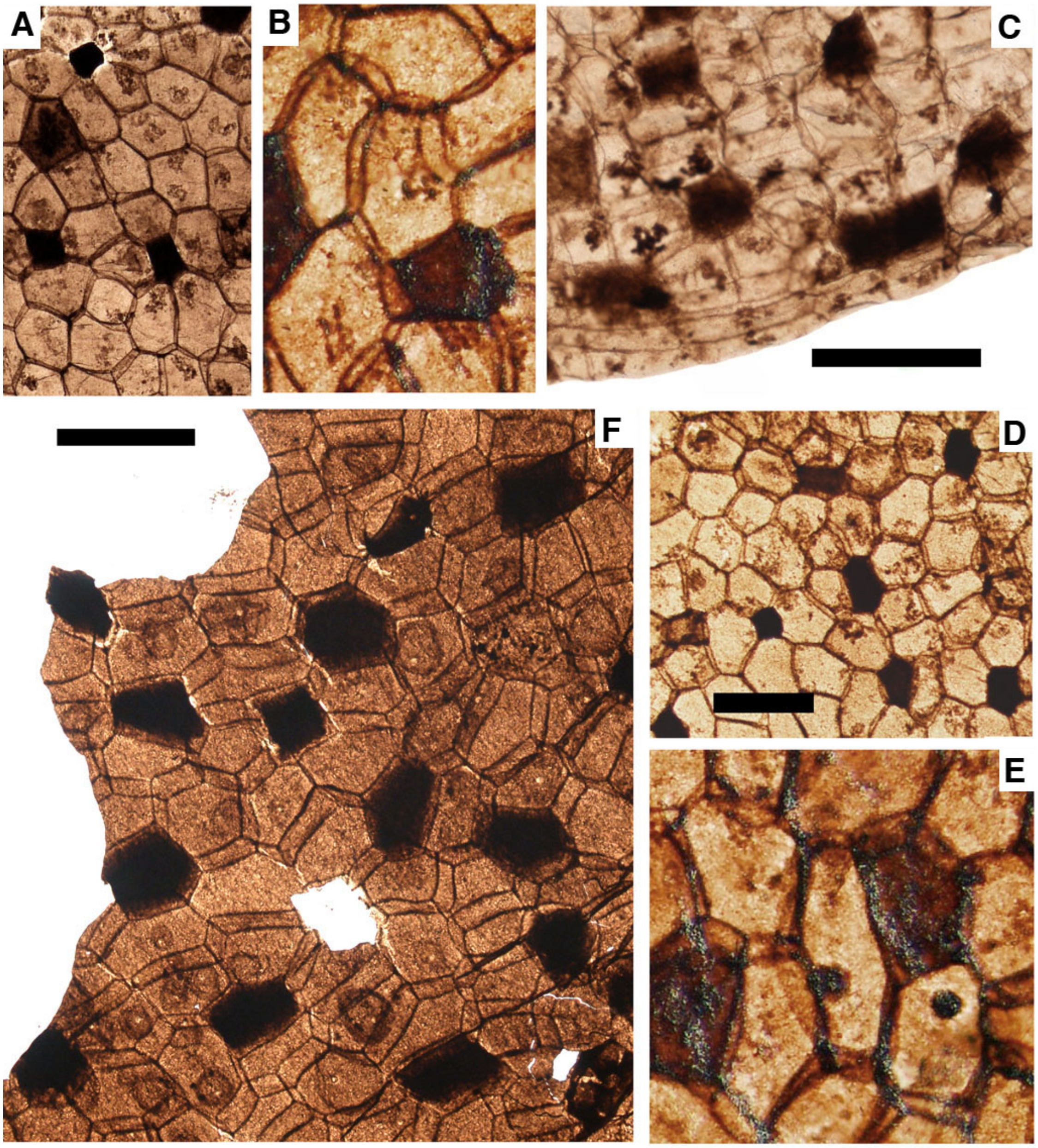

Figure 3.10. Examples showing the wide variety of enigmatic structures preserved within *M. sharonae* cells. Scale bars 100 μm

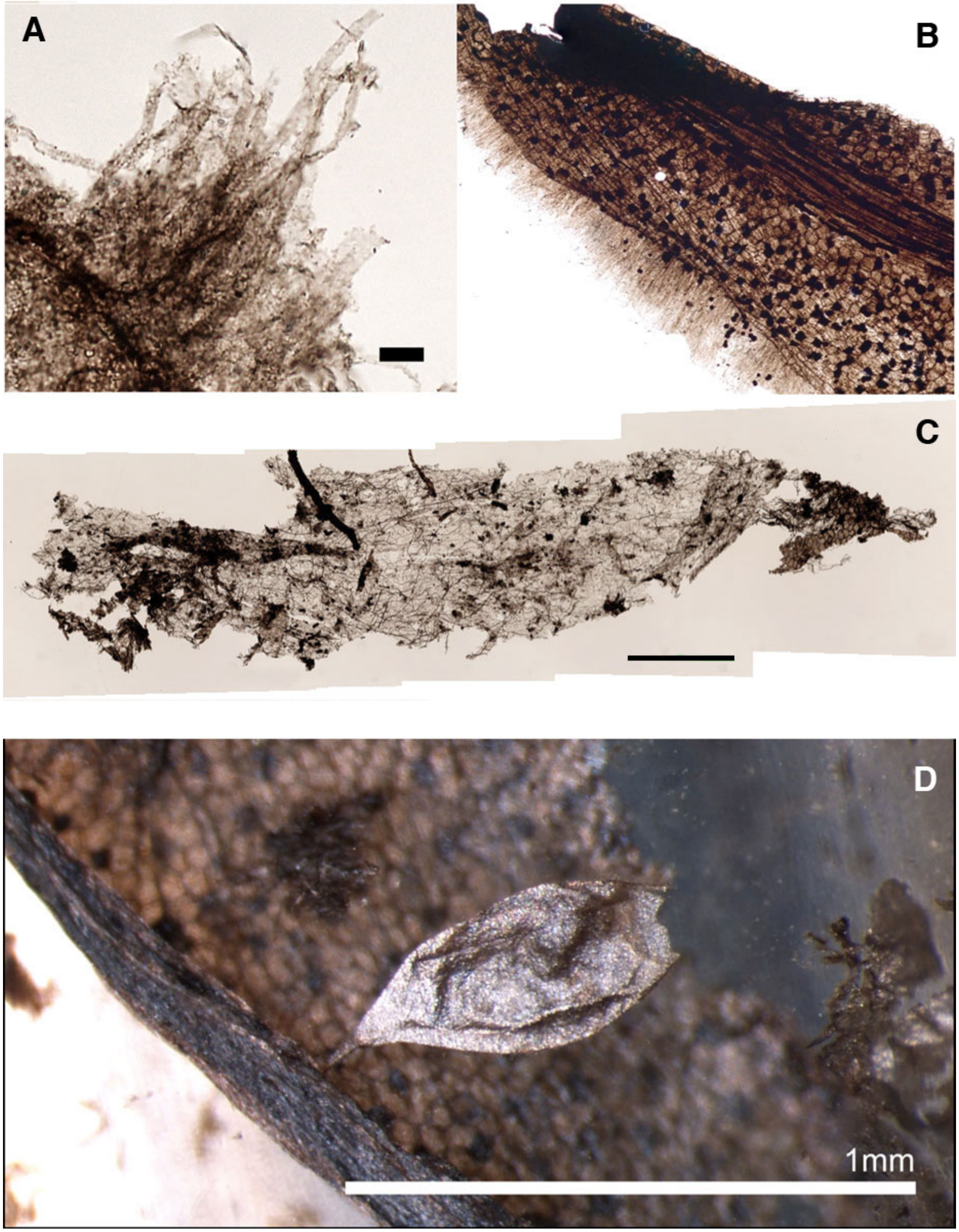

Figure 3.11. Species interactions between *M. sharonae* and co-occurring taxa. A,B, filamentous epiphyte growing on both Charophyte algae (A) and *M. sharonae* (B) thalli; C, Liverwort thallus filled with hyphae of decomposing fungal species; D. Liverwort thallus with putative egg case attached at base of stalk but otherwise floating free in rinse water, photographed with reflected light (see also 13G). Scale bars A, 10 µm, C,D 1mm

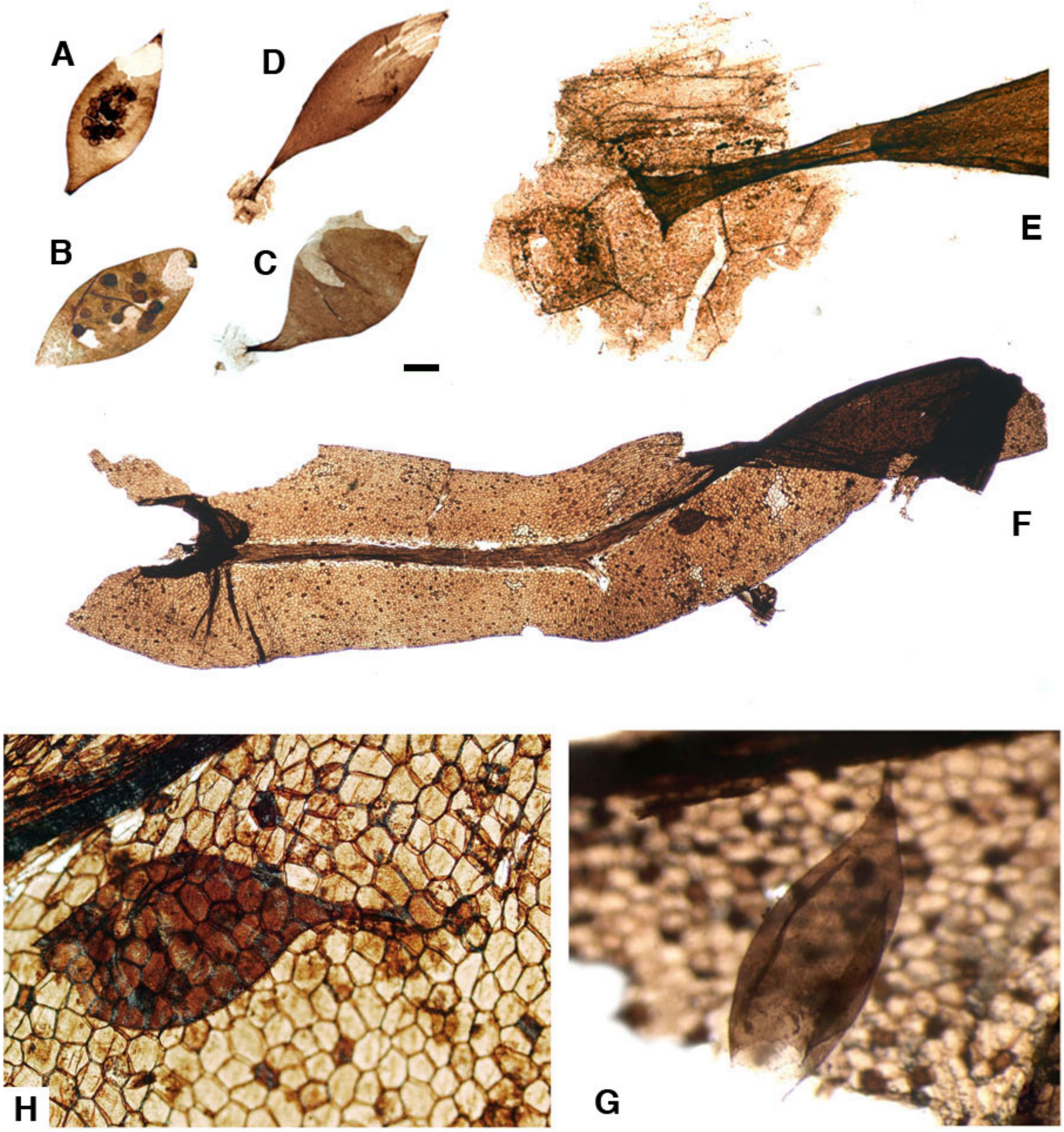

Figure 3.12. Examples of egg cases found with and at times attached to *M. sharonae* liverwort thalli. A,B, Dis-attached specimens similar to those found attached to liverwort thalli, filled with round structures. C, A similar type of strcture was attached to the 'shell' of a clam shrimp. D,E, Dis-attached stalked egg case with a few *M. sharonae* cells still adhering to base of stalk; F,G, Egg case attached to a liverwort thallus (preserved slide); G. Same specimen as 12D with only transmitted light. Scale bars 100 µm

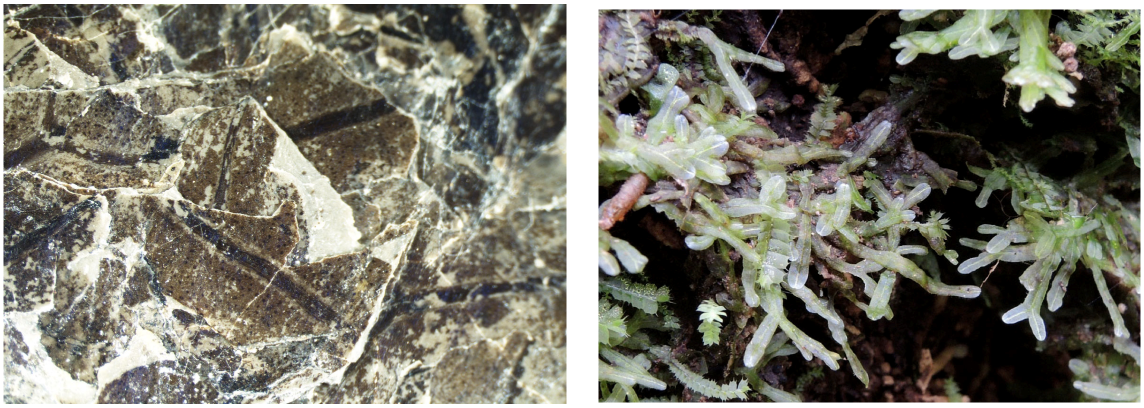

Figure 3.13. The densely packed *M. sharonae* thalli (left, as seen on edge of shale fragment, photographed with polarized light) may have been growing in a layered fashion similar to modern taxa such as *Metzgeria furcata* (right).

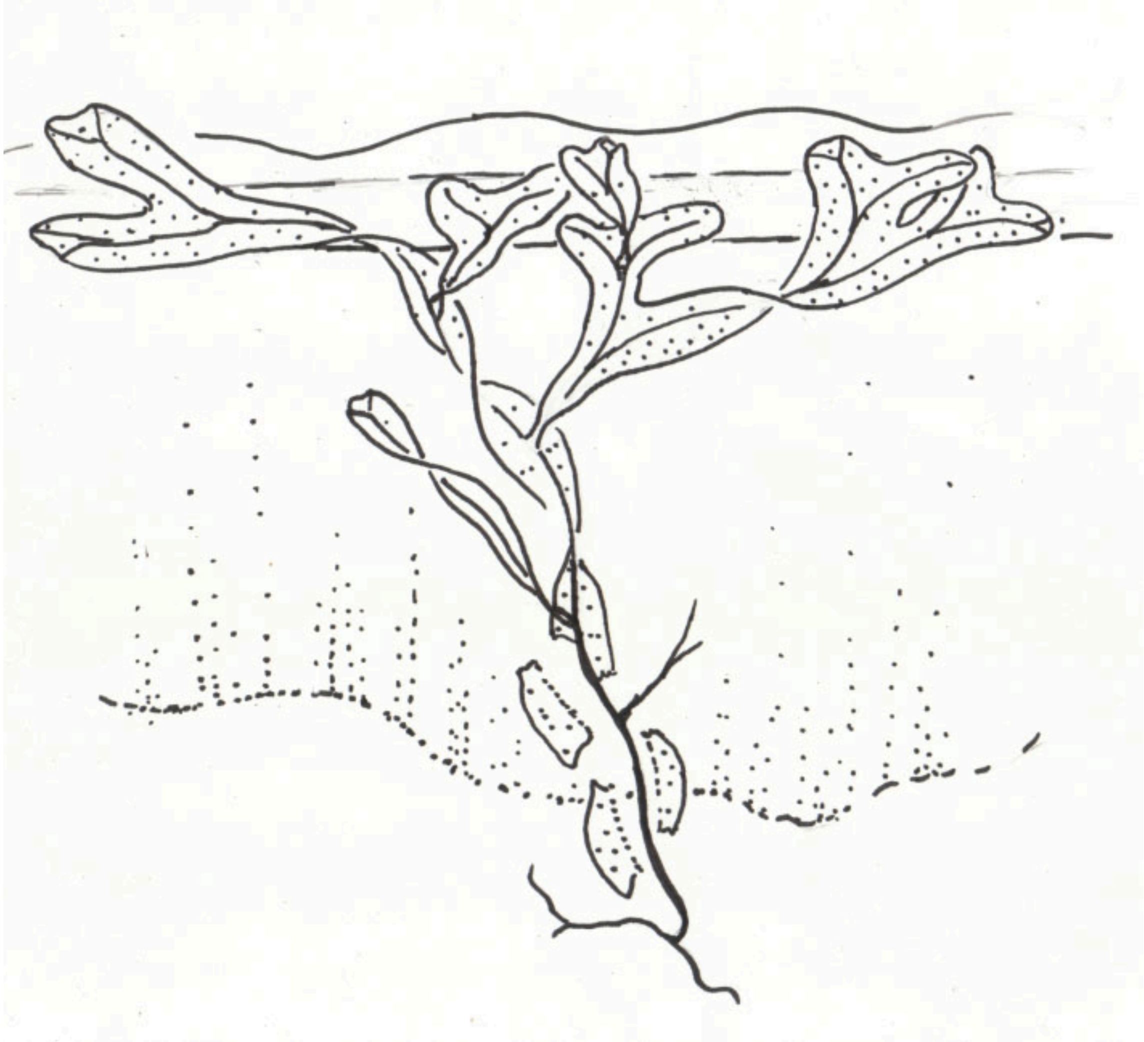

Figure 3.14. Reconstruction of *M. sharonae*. The results of this study suggest that the plants may have been growing both below and above the surface of shallow pools. The twisting, narrower thalli with intercalary branches may have predominated below the surface, and the flat broad dichotomizing thalli above the surface, and perhaps on surrounding banks. The thallus wings likely dropped from older parts of the thalli, with the thickened midribs serving as 'stems' that also served to anchor the plants in the silt. After periodic flooding events, the thalli still visible above the new layer of silt would have repeated the process.

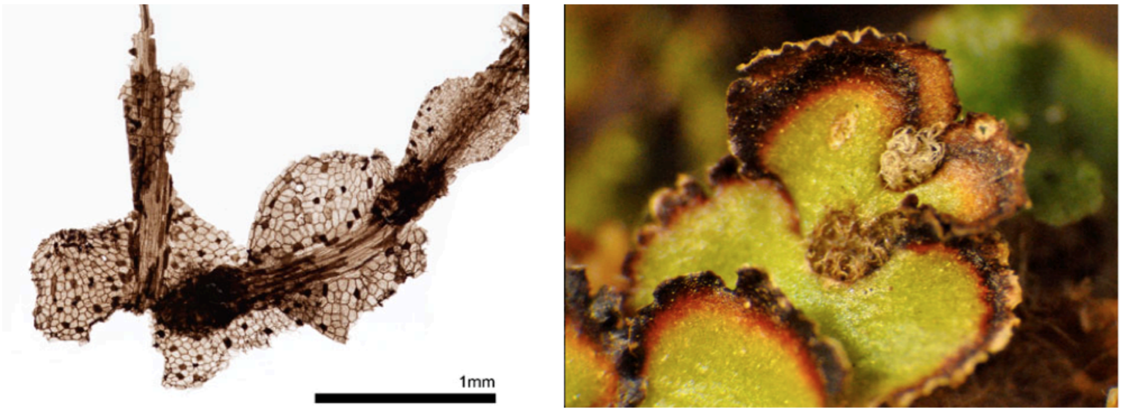

Figure 3.15. Branching comparison between *M. sharonae* and modern taxa. *Metzgeriothallus sharonae* intercalary branch branches again at apex to form two short branches, which is reminiscent of some taxa in the Marchantiales, such as the short branches of *Plagiochasma rupestre* that produce reproductive structures. Scale bar 1mm