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This is an **author produced version** of a paper published in:

European Journal of Protistology 81 (2021): 125839

**DOI:** <https://doi.org/10.1016/j.ejop.2021.125839>

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1 TITLE: Combining ultrastructural features of spore and capillitium as driver characters in the  
2 systematics of the order Trichiales (Myxomycetes, Amoebozoa).

3  
4 RUNNING TITLE: Ultrastructure in the order Trichiales.

5  
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16 ABSTRACT

17 The current Myxomycetes systematics relies on morphological features of the fruiting bodies. In the  
18 order Trichiales, the ornamental elements of spores and capillitium, i.e., a system of sterile  
19 filaments intermingled with the spores, drive its taxonomy, most usually approached with light  
20 microscopy. We here evaluate the broad diversity of spore and capillitium ultrastructural features in  
21 Trichiales with scanning and transmission electron microscopy (SEM and TEM, respectively). The  
22 spores of all the species analyzed with TEM show a two-layered wall, with the inner layer sub-  
23 divided into two sections. As seen with SEM, the spore ornamentation exhibits high diversity,  
24 including verrucae, bacula, pila, muri, and cristae elements. The muri and cristae are usually  
25 connected to create a reticulate pattern on the spore surface. The capillitium, here studied with  
26 SEM, also presents variable ornamentation, such as verrucae, spines, rings, reticula, spirals, or a  
27 combination of them. The introduction of molecular phylogenetic analyses in Myxomycetes  
28 systematics has challenged the morphology-based classifications and has pointed out distinct  
29 origins of several spore and capillitium ornamental elements. Thus, convergent evolutionary  
30 processes could be resulting in highly similar features. However, the combined consideration of  
31 spore and capillitium ornamentation may be advantageous in discerning emerging clades in the  
32 phylogeny.

33

34 KEY WORDS: microscopy, phylogeny, SEM, systematics, TEM.

## 35 1 INTRODUCTION

36 The Myxomycetes, or plasmodial slime molds, comprise one of the most diverse lineages within the  
37 supergroup Amoebozoa (Lara et al., 2020), with over 1,000 species described so far (Lado, 2005-  
38 2020). The general life cycle alternates microscopic, assimilative, and motile stages, myxamoeba,  
39 swarm cells, and plasmodia, and culminates with the development of usually macroscopic spore-  
40 bearing fruiting bodies (de Bary, 1859, Fiore-Donno et al., 2010). The spores are the reproductive  
41 units in myxomycetes, air-transported over long distances as the principal myxomycetes dispersion  
42 mechanism (Keller et al., 2017).

43

44 Trichiales is one of the most morphologically diverse orders within the Myxomycetes with an  
45 increasing taxa number described in the last decades, and currently comprises near 200 species  
46 (Lado, 2005-2020). In Trichiales taxonomy, the spore ornamentation is considered a valuable  
47 character for discrimination at the species level (Clark and Haskins, 2014) but questionable for  
48 higher taxonomic categories. As an example, species with reticulate-ornamented spores are present  
49 in different genera, even families, such as *Dianema* (family Dianemataceae), *Perichaena* (family  
50 Arcyriaceae), or *Hemitrichia* and *Trichia* (family Trichaiceae). Phylogenetic studies have suggested  
51 that the distinct spore ornamental elements originated several times independently in the Trichiales  
52 evolutionary history (Leontyev et al., 2019).

53

54 Spores in Trichiales species are usually globose, from 5-20  $\mu\text{m}$  in diameter, bright-colored, and  
55 present various elements that decorate their surfaces (Everhart and Keller, 2008, Lado and Eliasson  
56 2017). However, the spore ornamentation is at the resolution limit of light microscopy, and it is  
57 described vaguely as warted, spinulose, or reticulate (Martin and Alexopoulos, 1969). The first  
58 scanning electron microscopy (SEM) studies revealed that these ornamental elements were more  
59 diverse than traditionally recognized, and Rammeloo (1974) proposed a standardized terminology

60 based on palynology terms. While the description of the ornamentation with SEM has become used  
61 in specific articles, especially those describing new species, there are no other comparative studies  
62 considering the overall diversity of spore ornamental elements in Trichiales.

63

64 The ultrastructure and morphogenesis of the spores in myxomycetes appear barely represented in  
65 the literature. Most studies conducted with transmission electron microscopy (TEM) have detected  
66 spores with two-layered walls (Bechtel, 1977, Gaither, 1974, Scheetz and Alexopoulos, 1971,  
67 Schuster, 1964). The outer layer includes the ornamental elements, is formed first, and delimits the  
68 portion of protoplasm which will turn into a spore; the second layer develops internally, resulting in  
69 a thickening of the wall yet without increasing the global spore diameter (Mims, 1972). While this  
70 seems to be the case for most species, other reports have described different wall structures  
71 (Haskins and McGuinness, 1986), including three-layered spores in the trichiaceous *Minakatella*  
72 *longifila* (Keller et al., 1973). Nevertheless, the representation of species in the order Trichiales is  
73 quite limited in these studies.

74

75 The capillitium is a system of sterile filaments intermingled with the spores, and their features are  
76 also relevant characters in Trichiales systematics. These filaments present variable ornamental  
77 elements that have defined the different infra-order classifications (Lado and Pando, 1997, Martin  
78 and Alexopoulos, 1969). However, molecular phylogenies neither recovered these hypothesized  
79 relationships, pointing that the capillitium ornamentation, by itself, does not reflect the natural  
80 history of the group (Fiore-Donno et al., 2013). Moreover, ultrastructural approaches based on the  
81 capillitium study with TEM also hinted at different origins of these ornamental elements (Ellis et  
82 al., 1973, García-Cunchillos et al. 2021).

83

84 In this context, the main objectives of this study are: 1) to analyze comparatively the diversity of  
85 spore ornamental elements in Trichiales, as seen with SEM and TEM, 2) to detect whether the wall  
86 of the spores presents the usual two-layered structure or a different one, 3) to revise the previously  
87 defined spore ornamentation types and their descriptions, and 4) to explore the ability of a  
88 combined consideration of the spore and capillitium ornamentation to reflect the evolutionary  
89 history of the order in the current phylogenetic framework.

90

## 91 2 MATERIALS AND METHODS

### 92 2.1 SAMPLES

93 A total of 41 specimens from 28 species were studied with SEM and TEM, comprising 10 of the 14  
94 genera currently recognized in Trichiales (Supplementary Material). These species encompass the  
95 entire spectrum of ornamental elements observed in three of the four families in the order  
96 (Dianemataceae, Arcyriaceae, and Trichiaceae). Information on the remaining monospecific family  
97 (Minakatellaceae) is already available in the literature (Keller et al., 1973). All specimens proceed  
98 from the following herbaria (<http://sweetgum.nybg.org/science/ih/>): GB, Lado (private collection of  
99 C. Lado at the Real Jardín Botánico, CSIC), LSU, M, MA-Fungi, and PDD.

100

### 101 2.2 SCANNING ELECTRON MICROSCOPY

102 One completely developed fruiting body was collected from each specimen. Samples were  
103 dehydrated in an acetone series, 15 minutes in each step, and then subjected to a critical point  
104 drying (CPD 7501 Polaron Quorum Technologies). Later, they were gold-sputtered, with an SCD  
105 004 Sputter Coater, Blazers, Leica. Observations were done with a Hitachi S3000N scanning  
106 electron microscope, operating at 10-15 kV.

107

### 108 2.3 TRANSMISSION ELECTRON MICROSCOPY

109 From each specimen, one or two fruiting bodies were selected and collected in 1.5 safe-lock  
110 Eppendorf tubes. The preparation of samples follows the protocol of Ligrone and Duckett (1994),  
111 with minor modifications as previously published in García-Cunchillos et al. (2021). Briefly,  
112 samples were fixed in 3 % glutaraldehyde in a 0.1 M cacodylate buffer (pH=7.4) in two steps and  
113 post-fixed in 1 % osmium tetroxide for 2 hours at room temperature. Later, they were dehydrated  
114 in an ethanol series, transferred into propylene oxide (4° C) in three steps, and embedded in the  
115 Spurr low-viscosity embedding medium (Spurr, 1969) in seven steps, once every 24 hours, keeping  
116 the samples in darkness at 4° C during the embedding. Preparations were sectioned with a diamond  
117 knife (Leica Ultracut S ultramicrotome) and contrasted with lead citrate and uranyl acetate. Samples  
118 were studied with a JEOL JEM1010 transmission electron microscope, operating at 100kV and  
119 coupled to a Gatan Orius 200 SC camera. Observations included the highest number of sections and  
120 spores as possible, usually over 30 sections and ten spores.

121

## 122 2.4 TERMINOLOGY

123 The description of the spore ornamentation, as observed with SEM, follows the terminology  
124 proposed by Rammeloo (1974), who defined different ornamental elements according to their  
125 shapes (plural terms in parentheses).

126 *Verruca* (*verrucae*) is a rounded element, never pointed or constricted, in which its broader diameter  
127 is as large or larger than its height.

128 *Baculum* (*bacula*) is a cylindrical element in which height is higher than its broader diameter.

129 Bacula are usually straight and end abruptly in rounded or short-pointed tops.

130 *Pilum* (*pila*) refers to a compound element consisting of a straight cylindrical piece or baculum,  
131 which supports a *caput* (*caputs*), a spherical head-like structure at the top.

132 *Murus* (*muri*) is a strip-like element, wider than bacula, yet with also smooth tops. Muri may appear  
133 individually or connecting to create a *reticulum* (*reticula*). According to the reticulum pattern,

134 reticulate spores can be small-meshed, with the surface patched with numerous and small enclosed  
135 spaces, or broad-meshed, with few and large patches.

136 *Crista* (*cristae*) is an element similar to murus, although it is taller than them, and tops are  
137 irregularly shaped, analogous to caputs in pila. Unlike muri, cristae always appear connected,  
138 forming a reticulate or sub-reticulate (also named broken reticulum) pattern on the spore surface.  
139

140 For the capillitium ornamentation, we follow the terminology proposed by Rammeloo (1983,  
141 1984a, 1984b, 1986):

142 *Verruca* (*verrucae*), as described for spore ornamentation.

143 *Half-ring(s)* refers to an element that partially embraces a portion of the capillitium diameter.

144 *Spiral(s)* is a continuous band arranged helically along each capillitium filament.

145 *Irregular projection(s)* designate those ornamental elements without a definite shape.

146 *Reticulum* (*reticula*) is an ornamentation type in which a tridimensional network develops on the  
147 capillitium surface.

148 *Spine(s)* is a conical projection, tapering to a pointed tip.

149

## 150 3 RESULTS

### 151 3.1 TYPES OF SPORE ORNAMENTATION BY SEM

152 Spores of every specimen studied could be assigned to an ornamentation type already designated  
153 (Table 1). Names of these types are therefore conserved, with additions to their descriptions  
154 according to our observations. We further differentiate two new subtypes within the *cristate* one: the  
155 cristate reticulate and the cristate patched. None of the species showed the ornamentation with a  
156 suspended *tegillum*, as defined by Rammeloo (1974).

157



158 The **verrucate** type ornamentation consists of verrucae of two categorical sizes in every single  
159 spore, as seen with SEM. First, small verrucae, evenly distributed throughout the spore surface.  
160 Second, large verrucae, which seem to result from the joint of the smaller ones. These groups, or  
161 larger verrucae, are scarcer and distributed irregularly. Large verrucae can present a hole in their  
162 upper, central part (Figs. 2, 3, 5, 6, 41, 42). This hole is superficial and does not reach the spore  
163 surface, as seen with TEM (arrow in Fig. 64). Verrucae in species *Hemitrichia abietina* (not shown)  
164 do not present these two sizes and exhibit intermediate forms between verrucae and bacula. This  
165 ornamentation type shows the same characteristics when observed with TEM (arrow in Fig. 1).

166

167 The **baculate** type ornamentation consists of cylindrical elements with truncate or slightly rounded  
168 tops, as seen with SEM. These tops can sometimes present a lobulated appearance, as in *Perichaena*  
169 *corticalis*, *P. quadrata*, and *Prototrichia metallica* (Figs. 17, 18), but they are not that differentiated  
170 as caputs in pila. The bacula are taller in *Perichaena chrysosperma* (Figs. 13, 14) and *P. quadrata*  
171 and lower in *Dianema* species (Figs. 20, 21), *P. corticalis*, and *Prototrichia metallica*, which can  
172 present transitional forms to verrucae. Bacula in *Calonema foliicola* (Fig. 10) show comparatively  
173 broader diameters and exhibit longitudinal furrows (Fig. 9). Besides, they can join laterally, forming  
174 short muri-like elements. Bacula tops in this species vary from short-tipped to irregular, and tiny  
175 verrucae are present among the bacula. TEM observations highlight the cylindrical shape of the  
176 bacula (Figs. 8, 12, 16).

177

178 The **pilate** type differs from the baculate by the tops of the elements. As seen with SEM, the tops in  
179 pila are composed of several heads (caput) grouped upon a columnar structure. Caputs in these  
180 species (Table 1) are composed of three or more heads developing together, such as in *Metatrichia*  
181 *horrida* (Figs. 24, 25) and specimen GB 0146938 of *Hemitrichia leiocarpa* (Figs. 44, 45). TEM

182 observations reveal each pilum as composed of the same two pieces, i.e., the columnar structure and  
183 the caput, with no discontinuities separating them (Fig. 23).

184

185 Muri elements connect to form a continuous, small-meshed reticulum on the spore surface in the  
186 **simple reticulate** ornamentation (Figs. 28, 29). Muri in *Dianema depressum* are considerably taller  
187 and create a polygonal pattern (Figs. 31, 32). In species *Trichia decipiens*, the muri are short and  
188 form a sub-reticulate pattern (Fig. 47) rather than a continuous reticulum. In this species, it is  
189 possible to distinguish single bacula-like elements, connecting laterally to develop short rows (Fig.  
190 48). Although the ornamentation in *T. decipiens* differs slightly, observations with TEM (Fig. 59)  
191 revealed the same muri elements, distinct from cristae (see Discussion). The reticulate pattern is not  
192 evident when observed with TEM: in the spore section (arrow in Fig. 59), muri appear as radiating  
193 processes. Tops of muri sometimes bifurcate, acquiring a T- or Y-shape (Fig. 27).

194

195 The **cristate** type ornamentation consists of cristae elements connecting to form an overall  
196 reticulate pattern on the spore surface (Figs. 53, 54). In lateral view, cristae show smooth surfaces  
197 like muri, but they are usually taller and with roughly irregular tops, similar to the caputs in pila or  
198 the tops in bacula. Cristae connection creates small enclosed spaces, appearing as a secondary sub-  
199 reticulum (arrow in Fig. 38, Fig. 39). The overall pattern of the reticulum and the secondary sub-  
200 reticulum allow differentiating the reticulate and the patched subtypes.

201

202 The **cristate reticulate** subtype consists of cristae that create an overall small-meshed reticulum on  
203 the spore surface (Figs. 50, 51, 53). The secondary sub-reticulum in this subtype is barely  
204 developed (Figs. 35, 36). While most spores studied of *Hemitrichia serpula* showed very small  
205 verrucae on the spore surface, other spores from the same sporophore lacked them (Fig. 35). In  
206 species *Oligonema schweinitzii*, specimen MA-Fungi 40369 showed cristate reticulate ornamented

207 spores, unlike the two other representatives studied. Unfortunately, the TEM embedding protocol  
208 failed with species presenting this ornamentation subtype. Nonetheless, the TEM description of the  
209 patched subtype is expected to be highly similar to this one.

210

211 In the *cristate patched* subtype, cristae repeatedly bifurcate and anastomose, creating a highly  
212 developed sub-reticulum (arrow in Fig. 38, 57). The overall reticulum imprints a 'patched' broad-  
213 meshed pattern on the spore (Fig. 56) and, within these patches, there are other lower cristae  
214 connected to form small-meshed reticula (Figs. 39). As seen with TEM, cristae elements resemble  
215 muri, appearing as radiating processes that bifurcate, yet with a more complex pattern (Fig. 34).

216

### 217 3.2 STRUCTURE OF THE SPORE WALL BY TEM

218 The spore wall of all the species analyzed consists of two layers (Fig. 59), although there is a  
219 thickness variation in the whole wall and the individual layers (Figs. 59-64). The first outer layer  
220 (Fig. 59a) presents the highest electron density and is always very thin, never exceeding 0.1  $\mu\text{m}$ .  
221 This layer is continuous across the whole spore and forms the ornamental elements (Fig. 60). The  
222 second inner layer (Fig. 59b) consists of two sections with different electron densities, but their  
223 delimitation is no clear-cut (Fig. 61). The outer section of this second layer (Fig. 59c) presents an  
224 electron density intermediate between that of the outer layer (highest) and the electron translucent  
225 innermost section. In this outer section, the thickness is variable among species, although it is wider  
226 than the outermost layer. A low electron density characterizes the innermost section of the spore  
227 wall (Fig. 59d), in which the material becomes fibrillar or granular (Figs. 62, 63). This section  
228 shows the highest variability in extension and electron density among the species (Figs. 59-64).

229

### 230 3.3 CAPILLITIUM ORNAMENTATION BY SEM

231 The capillitium ornamentation is remarkably variable in the three families we studied:  
 232 Dianemataceae, Arcyriaceae, and Trichiaceae. Keller et al. (1973) examined, with TEM and SEM,  
 233 the only species of the family Minakatellaceae, *Minakatella longifila* (see Discussion).  
 234  
 235 Most species in Dianemataceae (here represented by the genus *Dianema*) show a smooth capillitium  
 236 surface, with occasional verrucae distributed irregularly. Verrucae are abundant in species *D.*  
 237 *succulenticola* (Fig. 22). Exceptionally, the capillitium in *D. depressum* (Fig. 33) presents reticulate  
 238 ornamentation, as well as the spores (Fig. 32).  
 239  
 240 In the family Arcyriaceae, the species show the most diverse capillitium ornamental elements within  
 241 Trichiales (Table 1). The species studied in the genus *Arcyria* present either half-rings (Fig. 4),  
 242 embracing variable tubule diameters, or verrucae connected by low ridges (Fig. 7). Other species in  
 243 this genus not studied here show different capillitium ornamentations (see Discussion). Capillitium  
 244 in *Arcyodes incarnata* also exhibits connected verrucae (not shown). In the genus *Perichaena*,  
 245 capillitium ornamentation includes long, tortuous spines in *P. chrysosperma* (Fig. 15) and irregular  
 246 projections in *P. corticalis* and *P. quadrata* (not shown).  
 247  
 248 In the family Trichiaceae, the capillitium ornamentation always consists of spiral elements (Table  
 249 1), yet the spirals show distinct features. They are loose, with separating spaces between them, in  
 250 specimen GB 0146938 of *Hemitrichia leiocarpa* (Fig. 46) and *Trichia decipiens* (Fig. 49), whereas  
 251 they are very tight in specimen PDD 74960 of *H. leiocarpa* (Fig. 43). In species *Oligonema*  
 252 *schweinitzii*, the spirals vary from very faint, almost undetectable (MA-Fungi 40369: Fig. 55), to  
 253 weak and very compact (MA-Fungi 86528: Fig. 58). Spirals in *Calonema foliiciola* are slightly  
 254 deviant, and sometimes they are arranged as sub-parallel longitudinal bands (Fig. 11).  
 255

Besides the spirals, some species in the family Trichiaceae exhibit secondary ornamental elements. These elements include long spines in *Hemitrichia serpula* (Fig. 37) or *Metatrichia horrida* (Fig. 26), short spines in *Trichia persimilis* (Fig. 40) and *T. scabra* (Fig. 52), or verrucae in *H. calyculata* (Fig. 30). Other species show no secondary ornamentation, such as *Prototrichia metallica* (Fig. 19) and *Trichia varia* (not shown). There are usually longitudinal striae between the spirals (Figs. 30, 37, 52) in this ornamentation type.

262

#### 4 DISCUSSION

##### 4.1 TYPES AND PATTERNS OF SPORE ORNAMENTATION IN TRICHIALES

Different spore ornamentation types concur in several genera of the order Trichiales (Table 1). The same results emerge when considering the distinction between reticulate and not-reticulate patterns of the ornamental elements.

268

The verrucate ornamentation is present in every species examined of the genera *Arcyodes* and *Arcyria* and in species *Hemitrichia abietina* and *H. leiocarpa* (Table 1). While Rammeloo (1974) did not discern between the two categorical sized verrucae, the only species that the author included within this type, *Arcyodes incarnata*, definitely showed them. Other studies based on SEM have also reported this same ornamentation in a wide range of *Arcyria* species (e.g., Lizárraga et al., 1998). Some authors consider the spore ornamental elements in *Minakatella longifila*, the only representative of the family Minakatellaceae, as flattened verrucae (Lister, 1921). However, they consist of truncate processes, as seen with SEM (Keller et al., 1973), ornamentation not detected in any other species, supporting its segregation from *Arcyria*.

278

The baculate ornamentation is present in four unrelated genera, *Dianema*, *Calonema*, *Perichaena*, and *Prototrichia*, comprising the three families studied. Other species of these genera, except in the

281 monospecific *Prototrichia*, show additional ornamentation types (Table 1), including simple  
 282 reticulate and pilate types (Keller and Reynolds, 1971, Lizárraga et al., 1999, Rammeloo, 1974).  
 283 The overall presence of this ornamentation type in distinct families and genera in the order seems to  
 284 compromise the taxonomic value of the baculate ornamentation.  
 285  
 286 The pilate ornamentation seems to be exclusive of the family Trichiaceae, chiefly in the genera  
 287 *Trichia* and *Metatrichia* (Rammeloo, 1974), but also in some species of *Hemitrichia* (Lizárraga et  
 288 al., 1999), *Calonema* (Bo and Li, 2015), and *Oligonema* (de Haan et al., 2004).  
 289  
 290 The simple reticulate ornamentation occurs in some species of the genera *Hemitrichia* and *Trichia*  
 291 (Table 1). Rammeloo (1974) designated the species *T. scabra* and *Oligonema schweinitzii* within  
 292 this type, while we regard them within the cristate one (see below). This author established a new  
 293 category, the *simple reticulate type with perforated muri*, represented by a single species, *H.*  
 294 *clavata*, based on perforations in some muri similar to those in *Stemonitis fusca* (order  
 295 Stemonitidales). Neither the specimen here studied nor the one analyzed by Lizárraga et al. (1999)  
 296 showed these perforations. Therefore, we do not consider this ornamentation in Trichiales.  
 297 Rammeloo (1974) regarded the spores of *T. decipiens* within the cristate type. Our observations,  
 298 particularly by TEM (Fig. 59), show that it better circumscribes within the simple reticulate one.  
 299 This ornamentation type is also present in some *Dianema* species (family Dianemataceae), as in *D.*  
 300 *aggregatum* (Moreno et al., 2004) and the here reported, *D. depressum*.  
 301  
 302 The cristate reticulate ornamentation is exclusively present in the family Trichiaceae, namely in  
 303 species *Hemitrichia serpula* and one specimen of *Oligonema schweinitzii* (Table 1). However, there  
 304 are other references in the bibliography to this subtype in the family Arcyriaceae, genus *Perichaena*  
 305 (Keller and Reynolds, 1971, Novozhilov and Stephenson, 2015). The ornamentation in *Trichia*

306 *scabra* is slightly deviant, intermediate with the simple reticulate type, as considered by Rammeloo  
307 (1974). The irregular tops of the ornamental elements, unlike the truncate ones of the muri  
308 elements, lead us to classify it within this subtype. The cristate patched subtype is also exclusively  
309 found in Trichiaceae, genera *Trichia* and *Oligonema* (Table 1).

310

#### 311 4.2 STRUCTURE OF THE SPORE WALL

312 Our study shows a very steady structure of the spore wall in all the species examined: two distinct  
313 layers, with the inner layer subdivided into two sections. Although this two-layered structure is in  
314 agreement with previous reports on a wide range of Myxomycetes species (Bechtel, 1977, Gaither,  
315 1974, 1976, Mims, 1972, Scheetz and Alexopoulos, 1971, Schuster, 1964), our observations allow a  
316 more accurate interpretation through the identification of two sections in the inner layer. Thus, most  
317 authors considered what we have defined as the outer section of the inner layer, as part of the  
318 outermost layer, probably due to insufficient contrast. Keller et al. (1973) did detect the same two  
319 sections of the inner layer, although they interpreted them as distinct layers in a three-layered wall.  
320 Our interpretation is similar to that reported by Randall and Lynch (1974). The wall structure here  
321 reported could be the basic pattern for most Myxomycetes. Slightly deviant forms in some species  
322 of the order Echinosteliales (Haskins and McGuinness, 1986) would merit a reexamination.

323

#### 324 4.3 COMBINATION OF SPORE AND CAPILLITIUM ORNAMENTATION AND

#### 325 PHYLOGENETIC IMPLICATIONS

326 In the order Trichiales, the taxonomic treatments consider the spore ornamentation as a character  
327 not able to reflect its systematics (e.g., Lado and Pando, 1997), as also reported by the available  
328 phylogenies (Fiore-Donno et al., 2013, Leontyev et al., 2019). The capillitium ornamentation  
329 neither seems to describe the evolutionary history (Ellis et al., 1973, García-Cunchillos et al., 2021).  
330 However, no studies have considered the combination of both characters.

331

332 Species with verrucate spores, with verrucae of two categorical sizes, only occur in a monophyletic  
333 clade comprising the closely related genera *Arcyria* and *Arcyodes* (Fig. 65). The species in these  
334 genera show diverse capillitium ornamental elements, such as spines, cogs, and reticula, besides the  
335 half-rings and connected verrucae here reported, but they never consist of spirals. The species  
336 *Hemitrichia abietina* also present verrucate spores, although verrucae do not show the two sizes.  
337 Moreover, capillitium ornamentation in this species is also different from that in *Arcyria* and  
338 *Arcyodes*, consisting of spirals. Phylogenetic relationships also reflected these differences, with *H.*  
339 *abietina* (*Hemitrichia* 01 in Fig. 65) showing higher affinities with other species with spiral  
340 capillitium ornamentation yet reticulate spores.

341

342 Remarkably, we have found wide diversity in the spores of *Hemitrichia leiocarpa*, with verrucate  
343 and pilate ornamentations depending on the specimen (Table 1). The verrucate ones better fit the  
344 definition of the genus *Arcyria*, although with spiral capillitium. Moreover, *Arcyria leiocarpa* exists  
345 as a synonym of the currently accepted *H. leiocarpa* (Lado, 2005-2020). Walker et al. (2015)  
346 reported phylogenetic affinities of this species to the *Arcyria* clade, pointing out a possible  
347 independent origin of the spirals in Trichiales, while the spore ornamentation of that specimen is  
348 unknown. The specimen showing pilate spores better fits the definition of *Hemitrichia*. Thus, this  
349 ‘*leiocarpa*’ complex could comprise a group of unrelated species that would merit further studies.

350

351 There are three phylogenetic clades with species presenting exclusively baculate spores (Table 1,  
352 Fig. 65). First, this ornamentation type occurs in the clade comprising the genera *Dianema* and  
353 *Prototrichia*, differing in the capillitium ornamentation (see Results). Exceptionally, species *D.*  
354 *depressum* possesses simple reticulate spores (Fig. 32), although there is no molecular data  
355 available on this species. Besides, species of the genus *Calomyxa* (not studied), closely related to



356 *Dianema* and *Prototrichia* (Fiore-Donno et al., 2013), also present these spore ornamental elements  
357 (Lado et al., 2014). Second, it appears in the two clades that include *Perichaena* species (Table 1,  
358 Fig. 65), here represented by *P. corticalis* (*Perichaena* 1) and *P. chrysosperma* (*Perichaena* 2), with  
359 an unknown position of *P. quadrata*. The capillitium ornamentation within this paraphyletic genus  
360 is highly diverse (Lado et al., 2009), and a broader phylogenetic sampling is necessary to elucidate  
361 its systematics. Third, baculate spores also occur in species *Calonema foliicola*, with spirally  
362 arranged smooth bands, which also lacks molecular information.

363

364 The species here reported presenting pilate spores always show spiral capillitium. These spirals  
365 usually exhibit spines as secondary elements (Table 1, Fig. 65), except in *Trichia varia*, with  
366 uncertain phylogenetic affinities (Fiore-Donno et al., 2013). Phylogenies of the order have  
367 recovered a close relationship between these species and those presenting cristate spores (Fiore-  
368 Donno et al., 2013, Leontyev et al., 2019). However, these results are not conclusive to discern  
369 whether the pilate ornamentation originated once or several times in Trichiales.

370

371 Simple reticulate spores occur in some *Hemitrichia* and *Trichia* species, represented in this study by  
372 *H. calyculata* and *T. decipiens* (*Hemitrichia* 2 and *Trichia* 1 in Fig. 65, respectively), with an  
373 unknown position of *H. clavata*. These species and *H. abietina* (verrucate spores, see above)  
374 comprised a new clade in the Trichiales phylogeny (Fiore-Donno et al., 2013), all of them  
375 presenting spiral capillitium ornamental elements. However, this association between capillitium  
376 and spore ornamentations does not apply in species *Dianema depressum*, with simple reticulate  
377 spores but reticulate capillitium, although, as previously stated, its phylogenetic affinities are  
378 unknown.

379

Species with cristate spores occur in a single clade in the Trichiales phylogeny (Fig. 65), which also encompasses species with pilate spores (e.g., *Trichia varia*) or with verrucae-like elements, like *Hemitrichia intorta* (*Hemitrichia* 3), not studied here but see Ronikier et al. (2020). Species with cristate spores include *Trichia* species (*Trichia* 2 in Fig. 65, represented by *T. scabra* and *T. persimilis*) and *Oligonema schweinitzii*. However, we can not ascertain a single origin of cristate spores in Trichiales since relationships within this clade remained uncertain (Fiore-Donno et al., 2013, Ronikier et al., 2020). The same applies when considering the distinction between the subtypes cristate reticulate and patched. Most of these species in this clade exhibit spines as secondary elements in the capillitium, except *O. schweinitzii* and *T. varia*, with smooth spirals.

The diversity of spore ornamentation in Trichiales encompasses verrucae, truncate processes (not studied), bacula, pila, muri, and cristae elements. Muri and cristae connect to form a pattern from sub-reticulate to reticulate on the spore surface. Most of these elements occur in different genera and families, restricting their use as taxonomical characters in the formulation of systematic classifications. The spore wall structure in Trichiales is constant among the species and consists of two layers: an outer one, including the ornamental elements, and an inner one sub-divided into two sections. The capillitium ornamentation is even more variable, including cogs, half-rings, rings, irregular projections, reticula, spines, spirals, verrucae, and combinations of them.

Despite the long-standing tradition in the study of Myxomycetes, the introduction of molecular phylogenetic analyses has challenged its systematics. In the order Trichiales, the capillitium ornamentation does not reflect the evolutionary history, and some ornamental elements seem to be the result of convergent evolution into highly similar features. As shown here, the elements that decorate the spore surface could also be affected by these processes. However, considering both

404 characters may help discriminate among the emerging clades in the still cryptic and incomplete  
405 phylogenetic scenario.

406

#### 407 ACKNOWLEDGMENTS

408 We acknowledge to Yolanda Ruiz-León from the Real Jardín Botánico (CSIC), for her technical  
409 support with the scanning electron microscopy, and to Francisco R. Urbano Olmos and Covadonga  
410 Aguado Ballano from the Universidad Autónoma de Madrid for their help with the transmission  
411 electron microscopy. We thank Carlos de Mier Ruiz for his invaluable technical support with the  
412 image processing. We also thank the assistance of the staff from the different herbaria: Claes  
413 Persson (University of Gothenburg), Laura Lagomarsino and Jennifer Kluse (Louisiana State  
414 University), Dagmar Triebel (Staatliche Naturwissenschaftliche Sammlungen Bayerns), Margarita  
415 Dueñas (Real Jardín Botánico, CSIC), and Mahajabeen Padamsee (Manaaki Whenua – Landcare  
416 Research). This study was supported by the Spanish Government (project PGC2018-094660-B-  
417 I00). The first author thanks the Spanish National PhD grant (BES-2015-072763).

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419 TABLES

420 **Table 1.** Spore and capillitium ornamentation in the species studied of the order Trichiales. Names  
 421 in bold: type species of a genus. Shaded in grey: species for which molecular phylogenetic data are  
 422 available. <sup>1</sup>Vouchers are only specified when distinct specimens of the same species showed  
 423 different ornamental elements.

424

Spores ornamentation	Capillitium ornamentation	Species (voucher <sup>1</sup> )	Family
Verrucate	Joint verrucae	<i>Arcyodes incarnata</i>	Arcyriaceae
		<i>Arcyria globosa</i>	
		<i>Arcyria nigella</i>	
		<i>Arcyria versicolor</i>	
	Half-rings	<i>Arcyria denudata</i>	Trichiaceae
		<i>Arcyria ferruginea</i>	
		<i>Arcyria insignis</i>	
	Spirals	<i>Hemitrichia abietina</i>	Trichiaceae
<i>Hemitrichia leiocarpa</i> (MA 80548, PDD 74960)			
Baculate	Spines	<i>Perichaena chrysosperma</i>	Arcyriaceae
	Irregular projections	<i>Perichaena corticalis</i>	
		<i>Perichaena quadrata</i>	
	Single verrucae	<i>Dianema corticatum</i>	Dianemataceae
		<i>Dianema harveyi</i>	
		<i>Dianema succulenticola</i>	
	Spirals	<i>Calonema foliicola</i>	Trichiaceae
		<i>Prototrichia metallica</i>	
Pilate	Spirals	<i>Hemitrichia leiocarpa</i> (GB 0146938)	Trichiaceae
		<i>Metatrichia horrida</i>	
		<i>Trichia varia</i>	
Simple reticulate	Reticulate	<i>Dianema depressum</i>	Dianemataceae
	Spirals	<i>Hemitrichia calyculata</i>	Trichiaceae
		<i>Hemitrichia clavata</i>	
		<i>Trichia decipiens</i>	
Cristate reticulate	Spirals	<i>Hemitrichia serpula</i>	Trichiaceae
		<i>Oligonema schweinitzii</i> (MA 40369)	
		<i>Trichia scabra</i>	
Cristate patched	Spirals	<i>Oligonema schweinitzii</i> (MA 86528, MA 85559)	Trichiaceae
		<i>Trichia affinis</i>	





426 FIGURES LEGENDS

427 **Figures 1-7. Verrucate ornamentation. 1-4. *Arcyria denudata*. 1.** Cross-section of the spore wall  
428 and the ornamental elements (arrow) (TEM). **2.** Detail of the spore ornamentation (SEM). **3.** Spore  
429 (SEM). **4.** Capillitial filament (SEM). **5-7. *Arcyria nigella*. 5.** Detail of the spore ornamentation  
430 (SEM). **6.** Spore (SEM). **7.** Capillitial filament (SEM). **Figures 8-22. Baculate ornamentation. 8-**  
431 **11. *Calonema foliicola*. 8.** Cross-section of the spore wall and the ornamental elements (TEM). **9.**  
432 Detail of the spore ornamentation (SEM). **10.** Spore (SEM). **11.** Capillitial filament (SEM). **12-15.**  
433 ***Perichaena chrysosperma*. 12.** Cross-section of the spore wall and the ornamental elements (TEM).  
434 **13.** Detail of the spore ornamentation (SEM). **14.** Spore (SEM). **15.** Capillitial filament (SEM). **16-**  
435 **19. *Prototrichia metallica*. 16.** Cross-section of the spore wall and the ornamental elements (TEM).  
436 **17.** Detail of the spore ornamentation (SEM). **18.** Spore (SEM). **19.** Capillitial filament (SEM). **20-**  
437 **22: *Dianema succulenticola*. 20.** Detail of the spore ornamentation (SEM). **21.** Spore (SEM). **22.**  
438 Capillitial filament (SEM). Scale bars: 0.2  $\mu\text{m}$  (1), 1  $\mu\text{m}$  (2, 5, 9, 13, 17, 20), 5  $\mu\text{m}$  (3, 4, 6, 7, 10,  
439 11, 14, 15, 18, 19, 22), 2  $\mu\text{m}$  (22).

440

441 **Figures 23-26. Pilate ornamentation. *Metatrichia horrida*. 23.** Cross-section of the spore wall and  
442 the ornamental elements (TEM). **24.** Detail of the spore ornamentation (SEM). **25.** Spore (SEM).  
443 **26.** Capillitial filament (SEM). **Figures 27-33. Simple reticulate ornamentation. 27-30.**  
444 ***Hemitrichia calyculata*. 27.** Cross-section of the spore wall and the ornamental elements (TEM). **28.**  
445 Detail of the spore ornamentation (SEM). **29.** Spore (SEM). **30.** Capillitial filament (SEM). **31-33.**  
446 ***Dianema depressum*. 31.** Detail of the spore ornamentation (SEM). **32.** Spore (SEM). **33.** Capillitial  
447 filament (SEM). **Figures 34-40. Cristate ornamentation. 34. *Oligonema schweinitzii*. Cross-**  
448 **section of the spore wall and the ornamental elements (arrow) (TEM). 35-37. *Hemitrichia serpula*.**  
449 **35.** Detail of the spore ornamentation (SEM). **36.** Spore (SEM). **37.** Capillitial filament (SEM). **38-**  
450 **40. *Trichia persimilis*. 38.** Detail of the spore ornamentation and secondary sub-reticulum (arrow)

451 (SEM). **39.** Spore (SEM). **40.** Capillitial filament (SEM). Scale bars: 0.5  $\mu\text{m}$  (23, 27), 1  $\mu\text{m}$  (24, 28,  
452 31, 33, 34, 35, 36, 38), 5  $\mu\text{m}$  (25, 26, 29, 30, 32, 37, 39, 40).

453

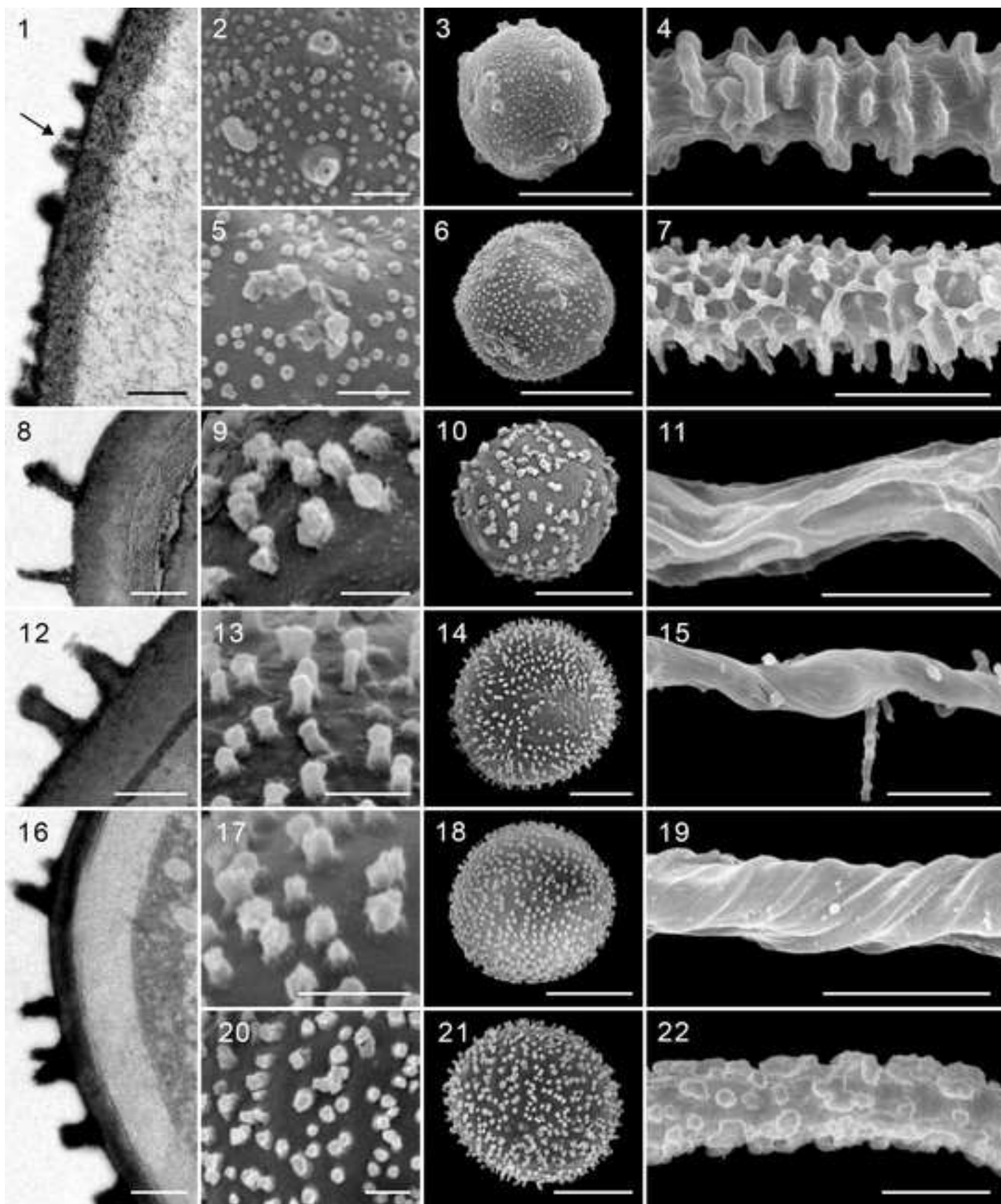
454 **Figures 41-58. Spores and capillitium of selected species (SEM). 41-43. *Hemitrichia leiocarpa***  
455 **(PDD 74960). 41.** Spore. **42.** Detail of the spore ornamentation. **43.** Capillitial filament. **44-46.**  
456 ***Hemitrichia leiocarpa* (GB 0146938). 44.** Spore. **45.** Detail of the spore ornamentation (SEM). **46.**  
457 **Capillitial filament. 47-49. *Trichia decipiens*. 47.** Spore. **48.** Detail of the spore ornamentation. **49.**  
458 **Capillitial filament. 50-52. *Trichia scabra*. 50.** Spore. **51.** Detail of the spore ornamentation. **52.**  
459 **Capillitial filament. 53-55. *Oligonema schweinitzii* (MA-Fungi 40369). 53.** Spore. **54.** Detail of the  
460 **spore ornamentation. 55.** Capillitial filament. **56-58. *Oligonema schweinitzii* (MA-Fungi 86528).**  
461 **56.** Spore. **57.** Detail of the spore ornamentation. **58.** Capillitial filament. Scale bars: 2  $\mu\text{m}$  (41, 44,  
462 47, 50, 53, 56), 1  $\mu\text{m}$  (42, 45, 48, 51, 54, 57), 5  $\mu\text{m}$  (43, 46, 49, 52, 55, 58).

463

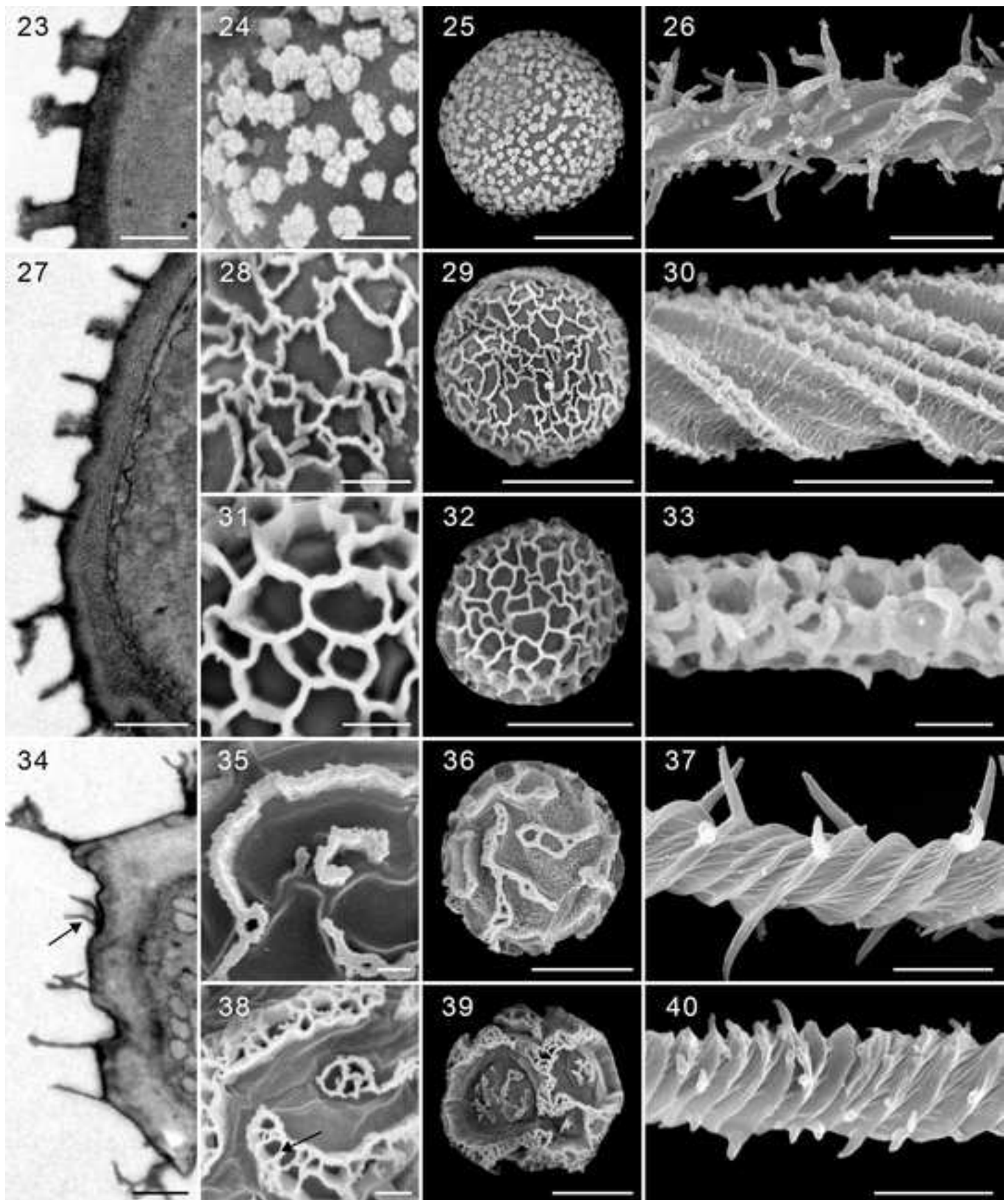
464 **Figures 59-64. Cross-section of the spore walls and the ornamental elements (TEM). 59.**  
465 ***Trichia decipiens*. Outer layer (a), inner layer (b), outer section (c), inner section (d). 60.**  
466 ***Hemitrichia clavata*. Ornamental elements (arrow). 61. *Dianema harveyi*. 62. *Perichaena***  
467 ***chrysosperma*. 63. *Calonema foliicola*. 64. *Arcyria insignis*. Arrow points to the upper hole of the**  
468 **large verrucae. Scale bars: 0.5  $\mu\text{m}$  (59, 60, 61, 62), 1  $\mu\text{m}$  (63, 64).**

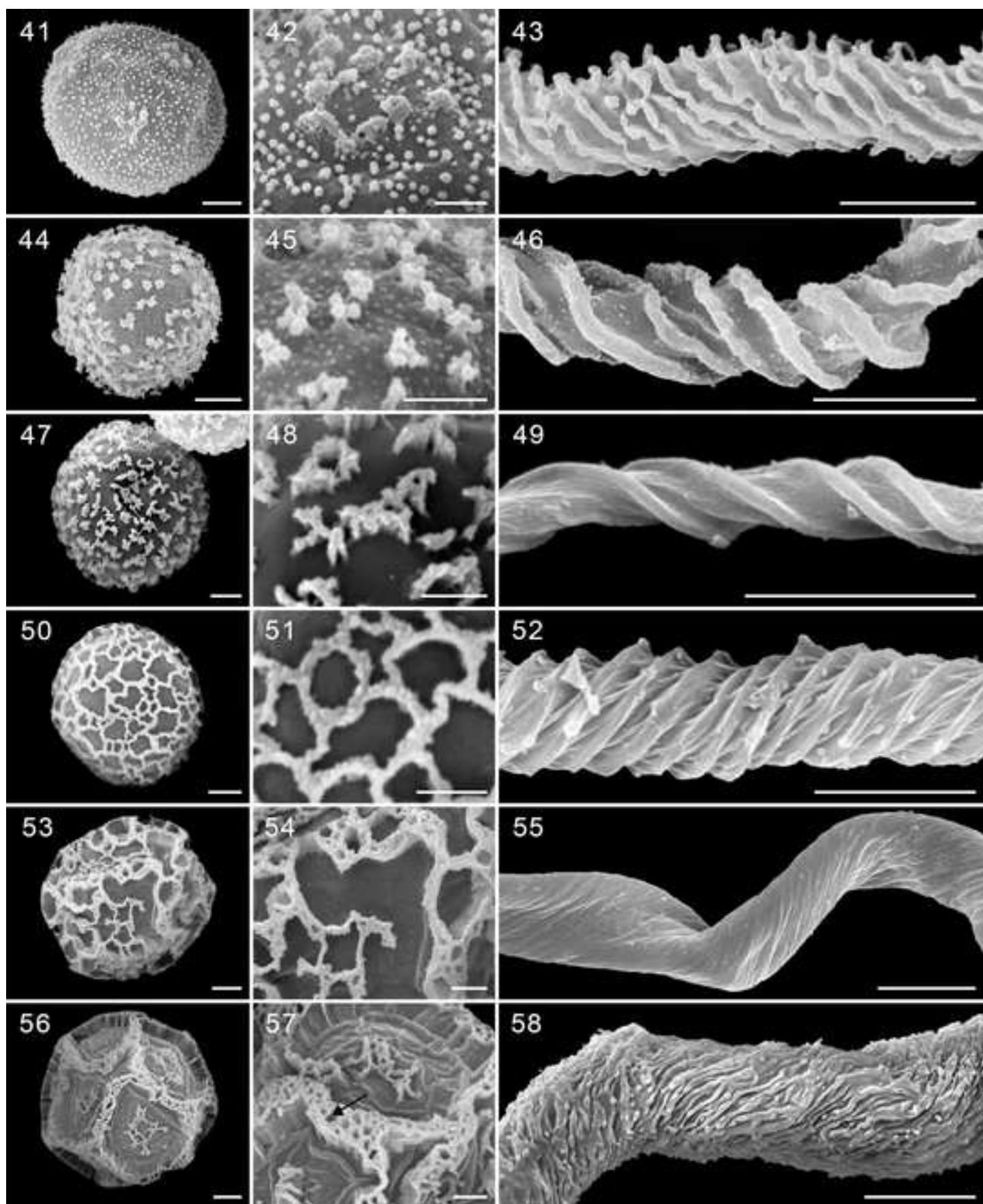
469

470 **Figure 65.** Mapping capillitium ornamentation, spore ornamental elements, and patterns of spore  
471 ornamentation on a schematic representation of the phylogenetic relationships among the principal  
472 clades in the order Trichiales. Branch lengths indicate neither genetic distance nor time. <sup>1</sup>Not  
473 studied here, but see Lado et al. (2014).

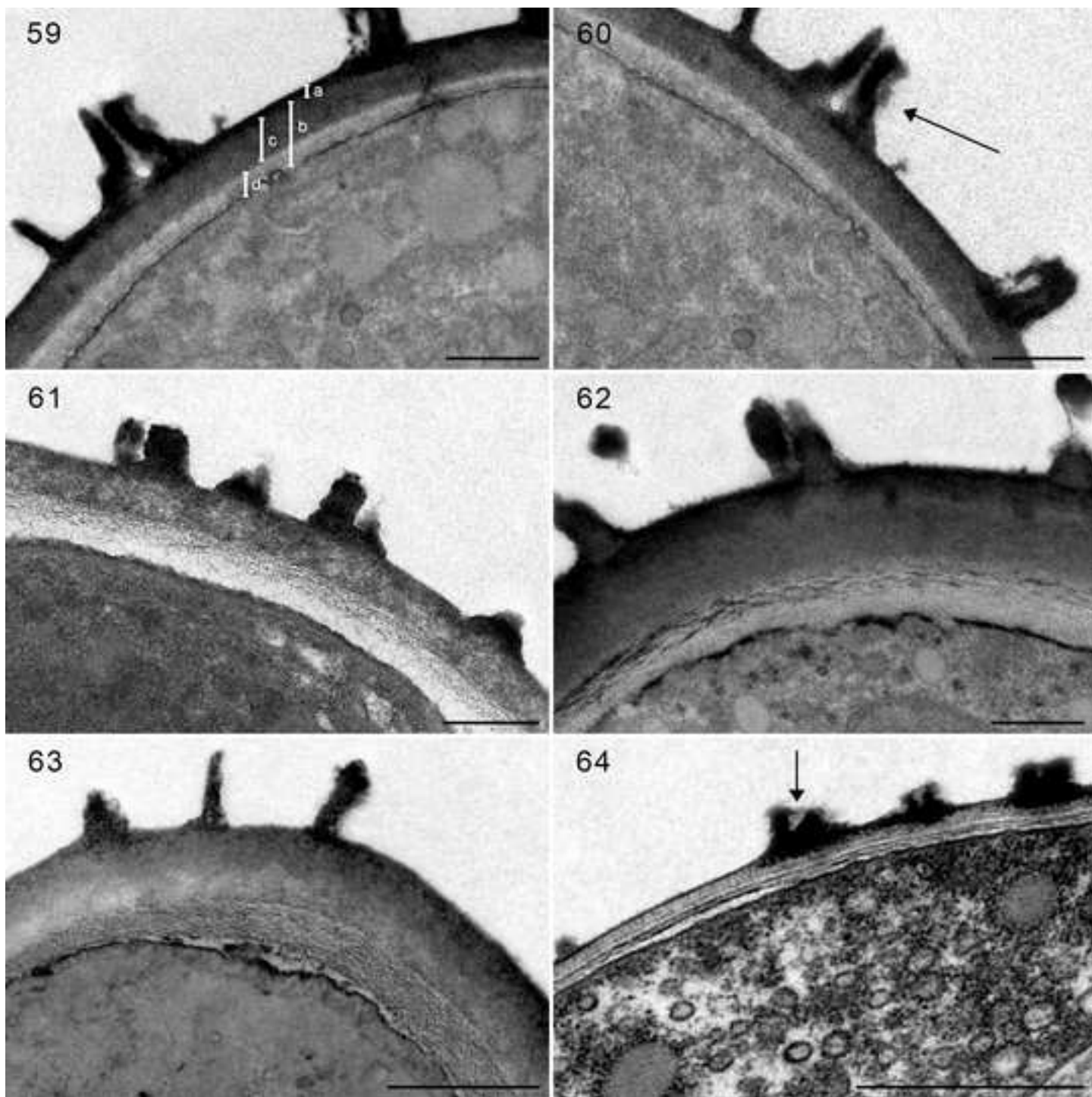


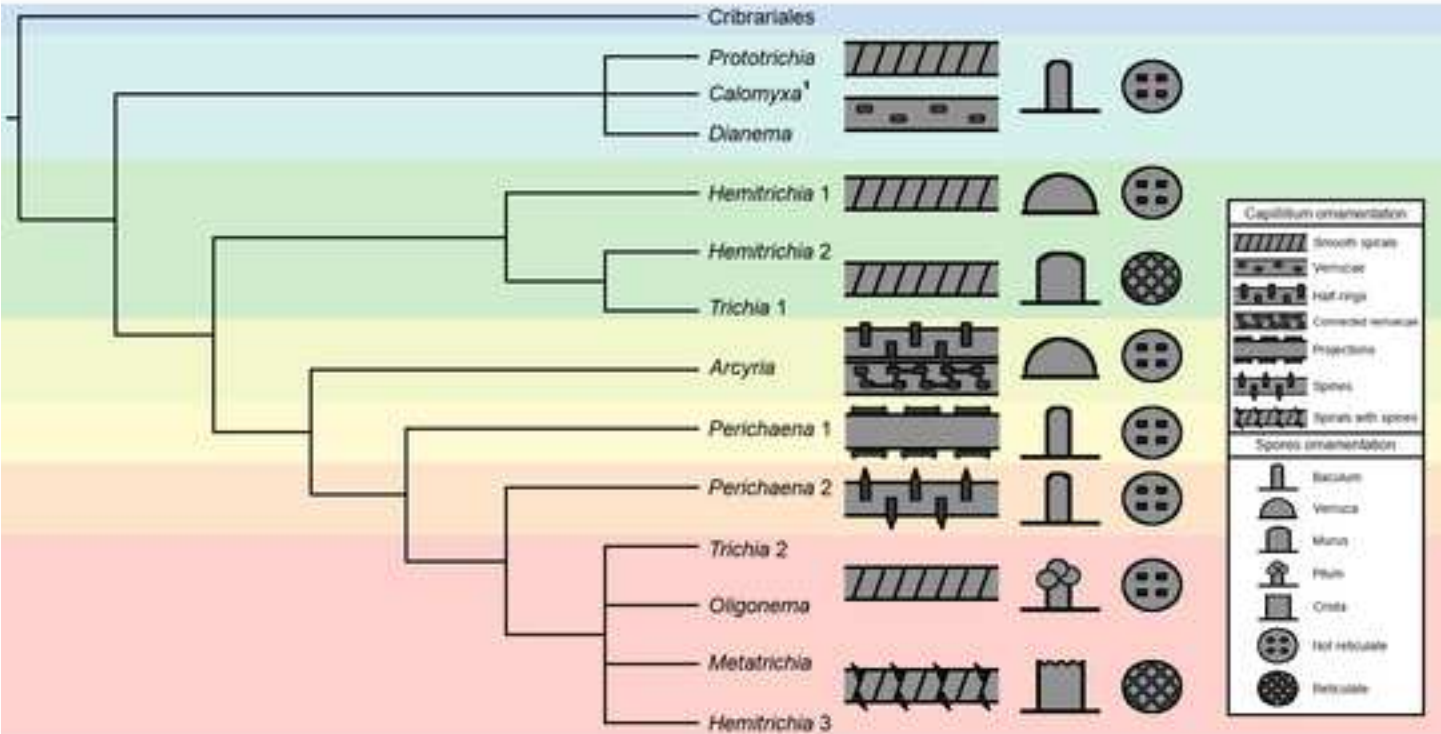














**Supplementary Material.** List of the specimens included in the study with additional information including voucher, country, province and locality, collectors, and collection date. See herbaria codes in <http://sweetgum.nybg.org/science/ih/>, except the private collection of Carlos Lado (Lado).

Species	Author(s)	Voucher	Country	Province: locality	Collector(s)	Collection date
<i>Arcyodes incarnata</i>	(Alb. & Schwein.) O.F. Cook	Lado 25434	Peru	Junín: Huancayo	A.Estrada-Torres, I.García-Cunchillos, C.Lado, I.Treviño	27 Apr 2017
		Lado 25437	Peru	Junín: Huancayo	A.Estrada-Torres, I.García-Cunchillos, C.Lado, I.Treviño	27 Apr 2017
<i>Arcyria denudata</i>	(L.) Wettst.	MA-Fungi 80405	Argentina	Catamarca: Santa María	C.Lado, A.Estrada-Torres, D.Wrigley de Basanta	26 Nov 2006
		MA-Fungi 83327	Argentina	Chubut: Alto Río Senguer	A.Estrada-Torres, E.García, C.Lado, A.Ronikier, D.Wrigley de Basanta	22 Apr 2010
<i>Arcyria ferruginea</i>	Saut.	MA-Fungi 62942	Spain	Valencia: Bocairente	F.García, F.P.Martínez, M. Oltra	16 Feb 2000
		MA-Fungi 86476	Argentina	Chubut: Cushamen	A.Estrada-Torres, E.García, C.Lado, A.Ronikier, D.Wrigley de Basanta	24 Nov 2009
<i>Arcyria globosa</i>	Schwein.	MA-Fungi 52762	France	Auvergne-Rhône-Alpes: Belley	M.Meyer	08 May 1993
<i>Arcyria insignis</i>	Kalchbr. & Cooke	MA-Fungi 87857	Peru	Arequipa: Islay	A.Estrada-Torres, C.Lado, I.Treviño, D.Wrigley de Basanta	04 Oct 2012
		MA-Fungi 87859	Peru	Arequipa: Islay	A.Estrada-Torres, C.Lado, I.Treviño, D.Wrigley de Basanta	04 Oct 2012
<i>Arcyria nigella</i>	Emoto	LSU 00169415	United States	Lousiana: New Orleans	A.L.Weden	14 Apr 2011
<i>Arcyria versicolor</i>	W. Phillips	MA-Fungi 85279	Spain	Lleida: Espot	M.A.Ferrández, C.Lado	05 Aug 1981
<i>Calonema foliicola</i>	Estrada, J.M.Ramírez & Lado	MA-Fungi 50729	Mexico	Chihuahua: Bocoyna	C.Lado, A.Estrada-Torres	30 Jul 2001
		MA-Fungi 50730	Mexico	Chihuahua: Bocoyna	C.Lado, A.Estrada-Torres	30 Jul 2001
<i>Dianema corticatum</i>	Lister	MA-Fungi 73204	Spain	Lleida: Salardú	C.Lado, S.Santamaría	29 Jun 1994
<i>Dianema depressum</i>	(Lister) Lister	MA-Fungi 80673	Chile	Valparaíso: Quillota	C.Lado, A.Estrada-Torres, D.Wrigley de Basanta	02 Apr 2006
<i>Dianema harveyi</i>	Rex	M 0114483	Germany	Baden-Wurtemberg: Sinzheim	H.Neubert	29 Jan 1977
<i>Dianema succulenticola</i>	Lado, Estrada & D. Wrigley	MA-Fungi 81389	Chile	Coquimbo: Vicuña	C.Lado, A.Estrada-Torres, D.Wrigley de Basanta	05 Apr 2006
		MA-Fungi 81391	Chile	Coquimbo: Vicuña	C.Lado, A.Estrada-Torres, D.Wrigley de Basanta	28 Jan 2004
		MA-Fungi 81394	Chile	Coquimbo: Vicuña	C.Lado, A.Estrada-Torres, D.Wrigley de Basanta	28 Jan 2004
<i>Hemitrichia abietina</i>	(Wigand) G. Lister	MA-Fungi 52877	France	Auvergne-Rhône-Alpes: Rognaix	M.Meyer	12 Nov 1991
<i>Hemitrichia calyculata</i>	(Speg.) M.L. Farr	MA-Fungi 80629	Chile	Valparaíso: Peñuelas	C.Lado, A.Estrada-Torres, D.Wrigley de Basanta	30 Mar 2006
<i>Hemitrichia clavata</i>	(Pers.) Rostaf.	MA-Fungi 68961	Spain	Valencia: Bocairente	A.Conca, F.García	-
		GB 00146938	Lithuania	Klaipeda County: Juodkrantė	G.Adamonyte	01 Sep 2010
<i>Hemitrichia leiocarpa</i>	(Cooke) Lister	MA-Fungi 80548	Chile	Araucania: Curacautin	C.Lado, A.Estrada-Torres, D.Wrigley de Basanta	25 Mar 2006
		PDD 74960	New Zeland	Campbell Island: -	S.L.Stephenson	-
<i>Hemitrichia serpula</i>	(Scop.) Rostaf. ex Lister	MA-Fungi 64141	Mexico	Veracruz: Misantla	C.Lado, A.Estrada-Torres, M.Ramírez, L.Hernández	06 Oct 2000
<i>Metatrichia horrida</i>	Ing	MA-Fungi 51490	Mexico	Guerrero: Lázaro Cárdenas	A.Estrada-Torres, C.Lado	25 Nov 1999

		MA-Fungi 51493	Mexico	Veracruz: Catemaco	A.Estrada-Torres, C.Lado	01 Dec 1999
		MA-Fungi 40369	Spain	Huelva: Castaño del Robledo	L.Romero de la Osa	25 May 1996
<i>Oligonema schweinitzii</i>	(Berk.) G.W. Martin	MA-Fungi 86528	Argentina	Futaleufú: Esquel	A.Estrada-Torres, E.García, C.Lado, A.Ronikier, D.Wrigley de Basanta	19 Nov 2009
		MA-Fungi 85559	Portugal	Algarve: Aljezur	C.Lado	20 Feb 1991
<i>Perichaena chrysosperma</i>	(Curr.) Lister	MA-Fungi 64131	Mexico	Veracruz: Totalco	C.Lado, A.Estrada-Torres, M.Ramírez, L.Hernández	05 Oct 2000
<i>Perichaena corticalis</i>	(Batsch) Rostaf.	MA-Fungi 68788	Spain	Valencia: Alfarrasí	M.Oltra	18 Feb 2006
<i>Perichaena quadrata</i>	T. Macbr.	MA-Fungi 83632	Argentina	Río Negro: Ministro Ramos Mexía	A.Estrada-Torres, E.García, C.Lado, A.Ronikier, D.Wrigley de Basanta	-
<i>Prototrichia metallica</i>	(Berk.) Massee	MA-Fungi 73209	Spain	Lleida: Arties	C.Lado, S.Santamaría	05 Jun 1996
		MA-Fungi 80049	France	Hautes-Alpes: Gap	François	24 Apr 2004
<i>Trichia affinis</i>	de Bary	MA-Fungi 78912	Argentina	Santa Cruz: El Calafate	A.Estrada-Torres, C.Lado, D.Wrigley de Basanta	25 Jan 2005
<i>Trichia decipiens</i>	(Pers.) T. Macbr.	MA-Fungi 78951	Argentina	Río Negro: San Carlos de Bariloche	A.Estrada-Torres, C.Lado, D.Wrigley de Basanta	29 Jan 2005
<i>Trichia persimilis</i>	P. Karst.	MA-Fungi 81444	Madagascar	Fianarantsoa: Fianarantsoa	A.Estrada-Torres, C.Lado, M.Schnittler, D.Wrigley de Basanta	10 May 2009
<i>Trichia scabra</i>	Rostaf.	MA-Fungi 81001	Poland	Lower Silesian Voivodeship: Klento	W.Stojanowska	19 Sep 1991
<i>Trichia varia</i>	(Pers. ex J.F. Gmel.) Pers.	MA-Fungi 64440	Spain	Asturias: Pola de Somiedo	C.Lado, D.Wrigley de Basanta	04 Oct 2005

## AUTHORSHIP STATEMENT

Manuscript title: Combining ultrastructural features of spore and capillitium as driver characters in the systematics of the order Trichiales (Myxomycetes, Amoebozoa).

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