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Title:

A scanning electron microscopy study of early development in vitro of *Contracaecum multipapillatum s.l.* (Nematoda: Anisakidae) from a brown pelican (*Pelecanus occidentalis*) from the Gulf of California, Mexico.

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Running page head:

Early development of *C. multipapillatum s.l.* by SEM

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Abstract

Eggs obtained from the uteri of female nematodes, genetically identified as *Contracaecum multipapillatum s.l.*, found in a brown pelican (*Pelecanus occidentalis*) from Bahía de La Paz, Gulf of California, Mexico, were used to study the early developmental stages of this anisakid by scanning electron microscopy (SEM). Egg dimensions were approximately 54 x 45 µm measured by SEM. Observation of the eggs revealed an outer surface of fibrous appearance. The newly hatched larvae were ensheathed and highly motile. Observation with SEM showed that the sheaths of the larvae were striated and revealed an excretory pore and a cleft near the anterior end of the sheath, presumably to facilitate the opening of the sheath for the emergence of the larva. The hatched larvae were placed in nutritive culture medium, where they grew within their sheath, some exsheathing completely two weeks later. The surface patterns of the sheath and the cuticle of the exsheathed larvae were clearly different. Although they did not moult during culture, SEM revealed a morphology typical of third-stage larvae of *Contracaecum* from fish, as previously observed by optical microscopy. Thus, we suggest that newly hatched larvae from eggs of *C. multipapillatum* are third larval stage but with sheath of the second larval stage, as occurs in other anisakids.

Key words: Contracaecum multipapillatum; egg; larval early development; aquatic life cycle; morphology; scanning electron microscopy.

Introduction

The anisakids (family Anisakidae) are a group of nematodes which parasitize the digestive system of fish, fish-eating birds and marine mammals. After fertilization by the male, the female releases eggs which are passed into the water with the faeces of the definitive host. The eggs then develop at ambient temperature. It is still unclear which larval stage hatches from the egg: while some authors believe it to be the second larval stage, L2 (Huizinga 1966; Huizinga 1967; Measures and Hong 1995), others believe it to be L3 (Thomas 1937; Køie 1993; Køie and Fagerholm 1993; Køie et al. 1995; González 1998; Adroher et al. 2004; Moravec 2009). Furthermore, although in some species the larva appears to hatch in the water where it is ingested by the first intermediate host, usually an aquatic invertebrate (copepods, euphasiids,….), for other species it has been suggested that the larva hatches when the egg is broken by the mouthparts of these invertebrates during ingestion (Køie 1993; González 1998). The larvae grow in these intermediate hosts and become infective for the next intermediate hosts: fish and squid, although, in *Contracaecum rudolphii*, the direct experimental infection of fish with recently hatched larvae has been demonstrated (Thomas 1937; Huizinga 1966; Dziekońska-Rynko and Rokicki 2008; Moravec 2009). When L3 is sufficiently developed it is then able to infect the final host and complete the cycle. This life cycle is complex, involving one or more paratenic hosts (invertebrates, fish and squid).

In the genus *Contracaecum*, copepods seem to be the first intermediate hosts, fish the second intermediate/paratenic hosts and fish-eating birds or marine mammals the definitive hosts. In *C. multipapillatum s.l.* the fish are often mullet (several species) and the definitive hosts are birds, generally of the family Pelicanidae (Humphrey et al. 1978; Deardorff and Overstreet 1980; Iglesias et al. 1998; Valles-Ríos et al. 2000; Mattiucci et al. 2010; Iglesias et al. 2011; Valles-Vega et al. 2017). Humans can accidentally interfere with the anisakid life cycle if they ingest live L3 with raw fish or that which has not undergone suitable heating or freezing processes. These larvae can cause digestive problems resulting in anisakiasis. Although approximately 97% of these cases are due to *Anisakis* spp. and 3% to *Pseudoterranova* spp. (Rello-Yubero et al. 2004), at least five cases involving *Contracaecum* spp. have been reported (see Valles-Vega et

al. 2017 for references) and at least one for an immature female of *Hysterothylacium aduncum* (Yagi et al. 1996).

The present study describes for the first time the eggs from a female uterus of *C. multipapillatum s.l.*, the larval stages hatching from these eggs and early developmental stages in *in vitro* culture using scanning electron microscopy.

Materials and methods

Collection and culture of parasites

The uteri of the females of *C. multipapillatum s.l.* obtained from the digestive tract of a brown pelican (*Pelecanus occidentalis*) from Bahía de La Paz, B.C.S., Mexico, were dissected to extract the eggs. These eggs were processed for in vitro maintenance in physiological saline solution and the hatched larvae were cultured in nutritive modified Grace's medium supplemented with 20% (v/v) fetal bovine serum, as described by Valles-Vega et al. (2017). The larvae obtained after egg hatching and at different stages of development in culture were processed for SEM, as was a uterus with eggs.

Molecular identification

The females of the nematode were processed for genetic identification using the sequence of ITS1-5.8-ITS2 from nuclear ribosomal DNA. This procedure has been described previously (Valles-Vega et al. 2017). Briefly, DNA extracted from the nematode specimens was amplified with primers described by Zhu et al. (2000) and then purified and sequenced by MacroGen (South Korea). Sequences were aligned with the Clustal X software. Neighbour Joining analysis, based on Kimura-2-parameters (K2P) distance (Kimura 1980) values, was used to construct phenetic trees, using the MEGA 5.05 software (Tamura et al. 2011) and reliability of the measure of stability of the branches was tested by 10000 bootstrap replications. The comparison of the ITS1 sequence with another 15 from *Contracaecum* from fish-eating birds, and the sequence of *Ascaris suum* as an outgroup, deposited in the GeneBank was carried out (Fig. 1).

Results

The females of *Contracaecum* from brown pelican were genetically identified as *C. multipapillatum s.l.* (K2P<0.07 and Fig. 1) and markedly separated from other species of *Contracaecum* from fish-eating birds (K2P>0.30 and Fig. 1). The eggs from the uteri of these females (Fig. 2), examined using SEM, were ovoid, measuring ± standard deviation 54.0 ± 2.9 x 44.8 ± 2.0 µm (n = 13). Using SEM, the external layer of these eggs appeared smooth (Fig. 3a), but, at higher magnification, the surface was seen to consist of a fibrous structure (Fig. 3b). This was laid out in plateaux surrounded by incipient ridges (Fig. 3c), although some eggs also exhibited eggshell areas with more or less parallel ridges (Fig. 3d). Furthermore, a rounded area, differentiated from the rest of the surface, was observed on some eggs. This may be an "opercular region" (Fig. 4).

Using SEM the recently hatched larvae in saline solution measured 209 ± 21 x 14.7 ± 0.9 µm (n = 5) excluding the sheath. They showed a striated sheath (Fig. 5) with annuli occasionally subdivided into two (Fig. 6), the sheath corresponding to the cuticle of the previous larval stage. An oval excretory pore of ca. 0.14 x 0.24 µm diameter (Fig. 5b, inset) can be seen at its anterior end. This pore is ~0.7 µm from the first striation of the sheath and 1.4-1.7 µm from a cleft situated between the first and second annulus (Fig. 5b). The posterior end gradually narrow, finishing in a blunt point (Fig. 5c) with adhesive properties (Fig. 5d). During preparation of these larvae for SEM, the sheath contracted, emphasizing the body of the larva within it. Although the sheath appears to have a tooth (Fig. 5b), the sheath is actually covering the boring tooth of the L3 below. Consequently, we do not relate the position of the excretory pore to the position of the tooth, as is usually the case in descriptions of L3. These ensheathed larvae were placed in nutritive culture medium and allowed to grow to fill their sheaths. After two weeks, some larvae started to free themselves from the sheath (Fig. 7). This seemed to take place as a result of the sheath breaking at the above-mentioned cleft, near (~3 µm) the anterior end (Fig. 5b), when the larvae measured on average 273 ± 31 µm (n = 5) by SEM. The

larvae freed themselves from the sheath at different times using rapid movements. The freed larvae also adhered to substrates by the tail, although less frequently than ensheathed larvae. No new moulting in culture medium was observed during the duration of the experiments (2 months).

Observed with SEM, the exsheathed larvae exhibited a striated cuticle (Fig. 6b), except at the anterior end, where it was smooth (Fig. 7b). The first striation was observed some 3-4 µm from the anterior end (the width of the larva at this point ~9-10 µm). The mean measurement of the annuli was 0.64 µm (range 0.46-0.85 µm) with non-uniform parallel vertical bands (Fig. 6b). These larvae had a boring tooth (height ~1.5 µm) with an excretory pore with an apparently oval-shaped opening at its base (Fig. 7b inset), between the ventrolateral lips. Delimiting the mouth, which appears as a groove, are the lips, relatively undeveloped, with papillae (2 dorso-lateral papillae on the dorsal lip and 1 papilla on each of the ventrolaterals, Fig. 7b), incipient interlabium, mouth, lateral cuticular suture, starting with the first cephalic striation (Fig. 7b) and running along the body (Fig. 7c) on both sides, almost as far as the tail of the larva, which finishes in a slightly thickened, unornamented, blunt point (Fig. 7d). No cephalic collar, described by Chandler (1935) thus: "*Just behind head cuticle conspicuously marked with annulations, which are very close together and end rather abruptly*"; was observed, nor was a spine at the posterior end, described by some authors in the most well-developed L3 (~2-3 cm) found in host fish (Fernández-Bargiela 1987; Valles-Vega 2011; Valles Vega et al. 2014), although Chandler (1935) described it as a "*demarcated conical lobe*" rather than a spine.

Discussion

The eggs of *C. multipapillatum s.l.* collected in the present study measured approximately 54 x 45 µm by SEM. These values are similar to those obtained by optical microscopy by Vidal-Martínez et al. (1994) 53 x 38 µm, and Valles-Vega et al. (2017) who reported 53 x 43 µm for the eggs of the females, molecularly identified, from the same brown pelican host. Other authors reported higher values such as 60 x 50 µm (Lucker 1941) and 65 x 58 µm (Huizinga 1967). These differences may be due to intraspecific variability or that they correspond to different species from the same *sibling* species complex.

According to Huizinga (1967), the eggshell of *C. multipapillatum* had a lightly mamillated outer surface, although Valles-Vega et al. (2017) reported eggs with a smooth or slightly rough surface by optical microscopy. Observed with SEM, the outer layer of the eggs was smooth (Figs. 2, 3a, 4a). However, at higher magnification, it was seen to be generally formed of plateaux surrounded by incipient ridges with a fibrous appearance (Fig. 3b,c), although, in some eggs, the outer surface appeared uniform (Fig. 4a). The plateaux may be due to the pressure exerted on the eggs in the uterus, forcing them close together (Fig. 2), which could create these plateaux and their corresponding ridges. This gives these areas the appearance of a crater with wilted fibres oriented towards the outside of the plateaux (Fig. 3c). The greater or lesser development of these ridges may determine whether the outer surface appears slightly rough or smooth (Fig. 3), as described previously in *C. multipapillatum* (Huizinga 1967; Valles-Vega et al. 2017) and other anisakids (see Anderson 2000). The fibrous material of the eggshells (Figs. 3, 4) has been described previously in the uterine layer (the outermost layer) of the eggs of other ascaridoids and may be composed of proteins and mucopolysaccharides (Cruthers et al. 1974; see Wharton 1980 for review). Moreover, in some eggs we observed a rounded zone, differentiated from the rest of the surface, which could serve as an "exit" during hatching (Fig. 4). This is also described as an "opercular region" in some ascaridoids (Ubelaker and Allison 1975).

The SEM photos show hatched larvae in which the sheath covering them is seen to be striated, ending in a blunt point with adhesive properties (Fig. 5). In some parts of the sheath the annuli can be seen to subdivide in two, which may be a growth process of the cuticle (Fig. 6a). Near the anterior end of the sheath is an excretory pore and a cleft (Fig. 5b) where it can be supposed that the sheath breaks, thus allowing the larva to emerge from the sheath at the anterior end. Huizinga (1967) described it thus: *"the anterior end of the sheath became swollen and a cap separated from the remainder of the sheath, permitting the larva to escape"*, which is consistent with the cleft detected in the present study (Fig. 5b). According to this author, exsheathment takes place in the intestine of the copepod, the first host of the parasite, prior to the larva entering the haemocoel, as has also been observed in other species of *Contracaecum* both those parasitizing birds and those parasitizing seals (Huizinga 1966; Davey 1969; Moravec 2009). It is still not known whether this exsheathment is simply a physical action resulting from the pressure of the larva on the sheath or whether substances secreted by the larvae are involved. The role of nematode proteases, not only in the nutrition and

development of the parasite, but also in hatching, exsheathment and moulting has recently been examined (Malagón et al. 2013). Live larvae observed by optical microscopy measured a mean of 260 µm (Valles-Vega et al. 2017) while those measured by SEM were only 210 µm. The same occurred with larvae measured at the start of exsheathment (315 and 270 µm, respectively). This discrepancy of 15-20%. may be a consequence of the method used to prepare the larvae for observation by SEM. In any case, the increase in size from hatching to exsheathment is still ~55-60 µm. It is likely that this increase in size of the larva is involved in the breaking of the sheath at the cleft (Fig. 5b), although the release of substances by the larva to aid escape from the sheath should not be discounted.

The characteristics observed in the exsheathed larva (Fig. 7) coincide with those of a typical L3 of *Contracaecum* from a fish host, as described previously with SEM (Valter et al. 1982; Weerasooriya et al. 1986; Fernández-Bargiela 1987; Valles-Vega et al. 2014). Thus, the anterior end exhibits three labia, one dorsal with two dorsolateral papillae and two ventrolateral labia with one papillum each and a boring tooth, with an excretory pore in its base (Fig. 7b). At its anterior end the cuticle is first smooth and then transversely striated, although without a cephalic collar (present in L3 from fish). However, the annuli exhibit longitudinal bands all along the striated cuticle (Fig. 6b). The cuticle does not show subdivided annuli like the sheath, probably because they are recently formed. Different superficial patterns have been observed in the cuticle of other anisakids, depending on species and developmental stage (Valter et al. 1982; Weerasooriya et al. 1986). The posterior end finishes in a blunt point (Fig. 7d) without the spine present in L3 of *C. multipapillatum s.l.* from fish (Fernández-Bargiela 1987; Valles-Vega 2011; Valles-Vega et al. 2014).

Finally, Moravec (2009), in *C. rudolphii*, which parasitizes cormorants, observed that the development of the oesophageal appendix and the appearance of the boring tooth did not take place until moulting to L3. Smith et al. (1990) did not observe moulting during the development of newly hatched larvae of *C. osculatum* until they had attained a length of more than 13 mm in the visceral cavity of an experimental host fish, identifying them as L3 both morphologically and morphometrically. The SEM description of L3 of *Contracaecum* collected from host fish (Valter et al. 1982 and references herein; Weerasooriya et al. 1986) concur with the data from the present study, except, of course, in the size of the larvae. Consideration of these

previous studies, those of Køie with different anisakids including *C. osculatum* (Køie 1993; Køie and Fagerholm 1993; Køie et al. 1995) and our own results (Valles-Vega et al. 2017; and present report), leads the present authors to suggest that the larva hatching from the egg in *C. multipapillatum s.l.* is the third stage larva since development of the boring tooth, the oesophageal appendix and other structures typical of L3 of *Contracaecum* was observed without any moulting. As development is still not complete, the characteristic collar and spine of L3 of *C. multipapillatum s.l.* are still not observed.

Further work is required in order to achieve in vitro culture of these anisakids which would then enable us to study their development and identify the differences between stages and species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Legends to Figures

Fig. 1 Neighbor Joining reconstruction between sequences of females of *Contracaecum multipapillatum* collected from brown pelican (in bold, indicating the host) to this study and sequences of *Contracaecum* species of birds from the NCBI database, with the tree inferred from the ITS1 data set. The numbers on the tree branches represent the percentage of bootstrap resampling (with 10000 replicates). *Ascaris suum* was used as an outgroup. The GenBank accession nos. are in front of species names.

Fig. 2 Uterus with eggs of a gravid female of *Contracaecum multipapillatum s.l.* viewed with SEM

Fig. 3 Eggs of *Contracaecum multipapillatum s.l.* (SEM). (a) Egg with smooth covering with plateaux and ridges. b) Detail of fibrous surface. (c) Detail of ridges around plateaux. (d) Egg surface ridges more or less parallel

Fig. 4 Eggs of *Contracaecum multipapillatum s.l.* with putative opercular region. (a) Egg with smooth covering, without ridges, and putative opercular (*) region (SEM). (b) Detail of this opercular region (SEM). (c) Hatching eggs (optical microscopy) with exit hole (*) visible (right), slightly (*) visible (left) and not visible (middle)

Fig. 5 Ensheathed larvae of *Contracaecum multipapillatum s.l.* (a) Larva. (b) Anterior end with boring tooth under the sheath; note the cleft in the sheath (arrow) where it could break and allow the larva to emerge. Inset (b_i): the excretory pore of the L2 on the sheath (arrowhead). (c, d) End of tail of larvae with adhesive blunt point; note material adhered in (d)

Fig. 6 Cuticle of *Contracaecum multipapillatum s.l.* (a) Sheath from ensheathed larva, note division of some annuli. (b) Cuticle of exsheathed larva, note the vertical bands and the thickening of the lower edge of the annuli. As shown in Fig. 6b, it appears that when the larva is curved, the upper part of an annulus is introduced into the lower part of the previous one on the inner side of the curve, while on the outer side, the annuli are fully expanded

Fig. 7 Exsheathing (a) and exsheathed (b, c, d) larvae of *Contracaecum multipapillatum s.l.* (a) Note the point of strangulation caused by the sheath during larval exsheathment. (b) Cephalic end, note mouth as a slit and, in inset (b_i), excretory pore in the base of the boring tooth. (c) Lateral suture in mid body. (d) Tail ending in blunt point. *Abbreviations*: a, annulus; bt, boring tooth; dl, dorsal labium; ep, excretory pore, il, incipient interlabium; ls, lateral suture; m, mouth; p, papillae; s, striations of cuticle; vl, ventrolateral labium

Figure 1

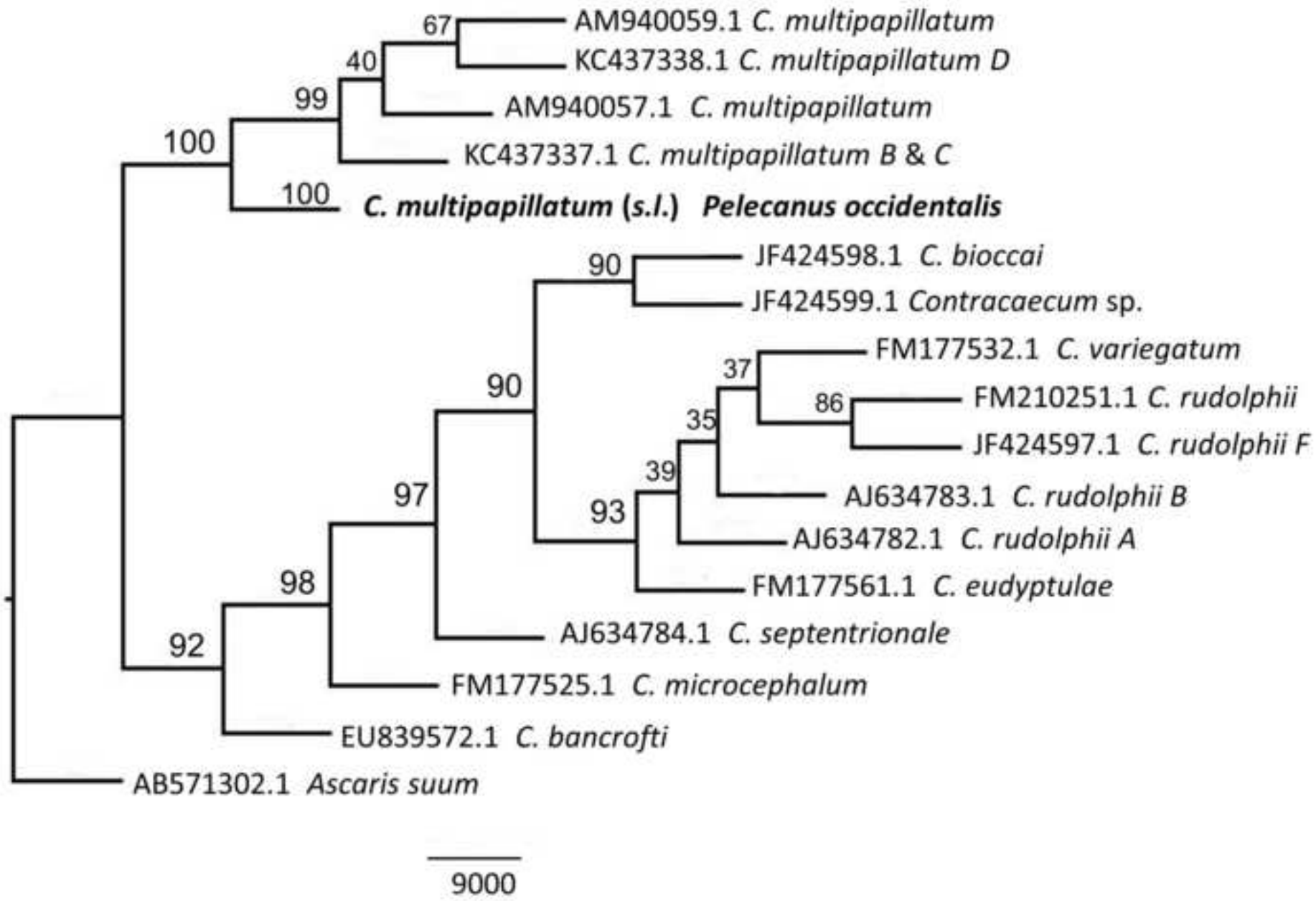


Figure 2

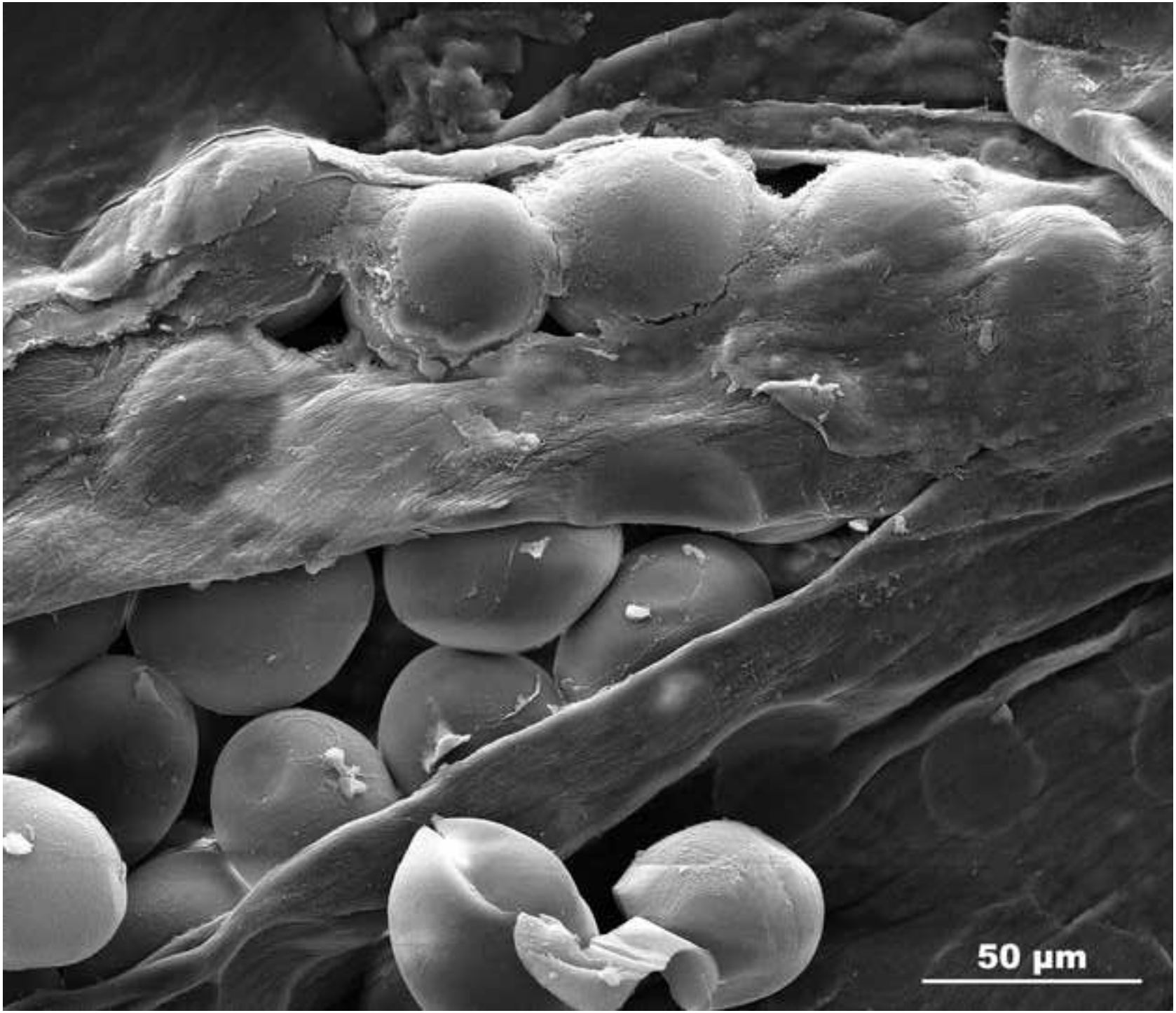
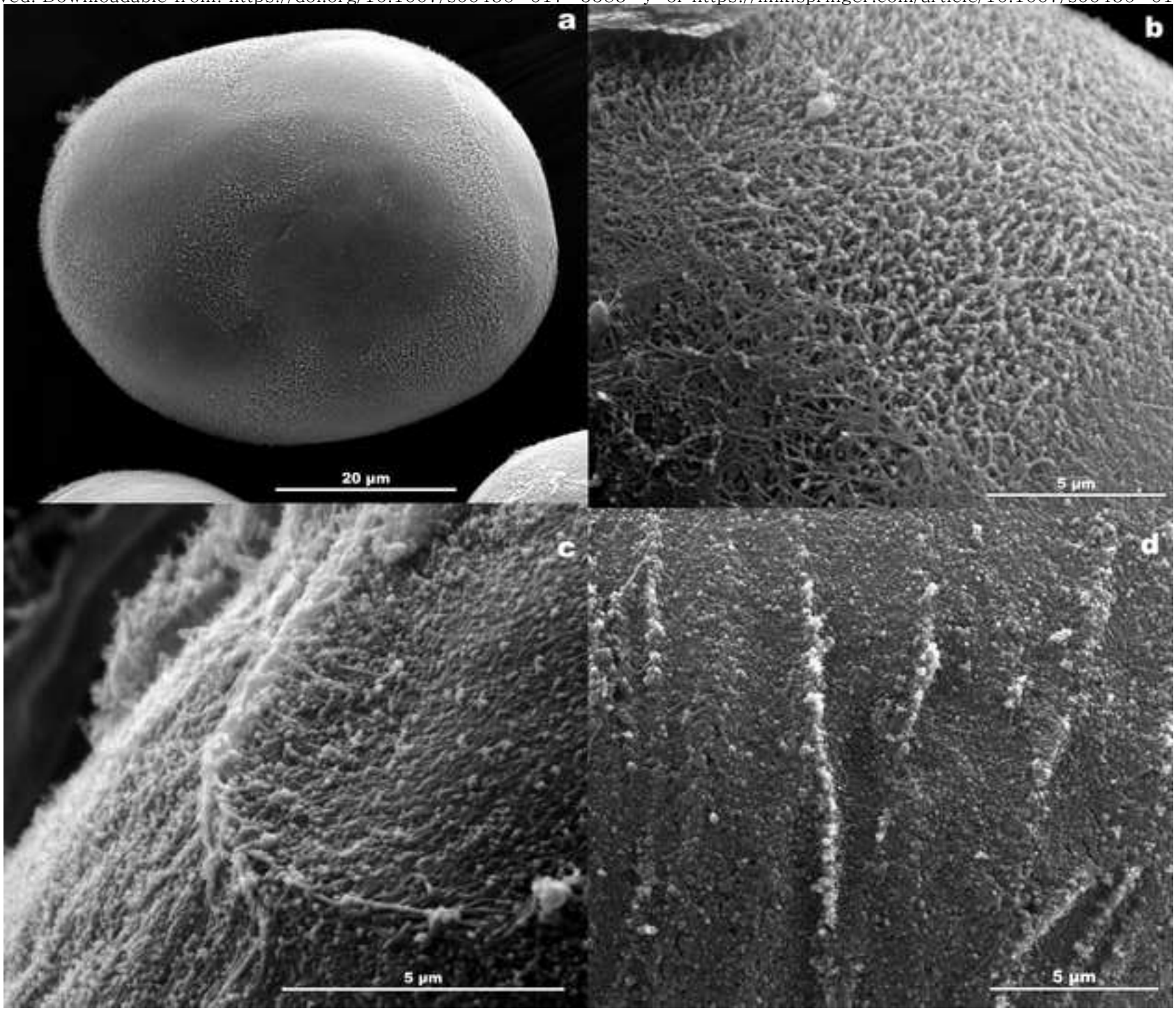


Figure 3



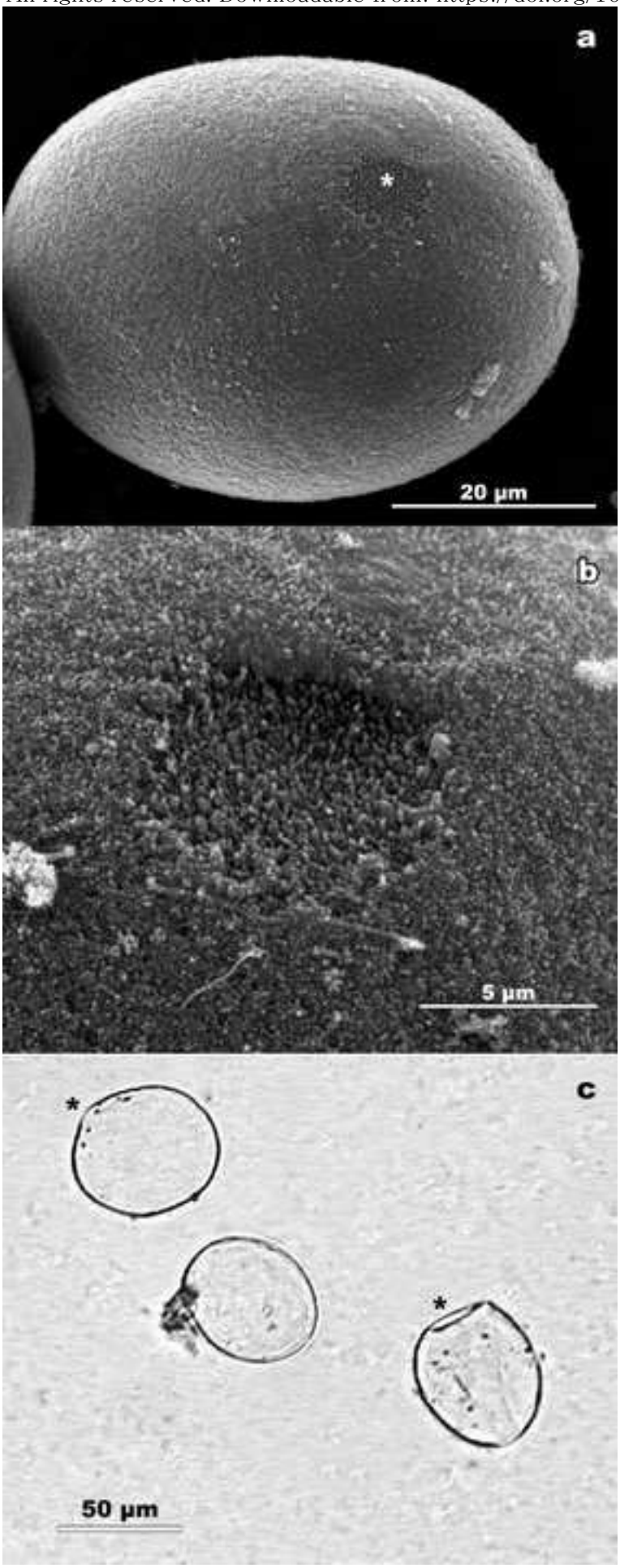
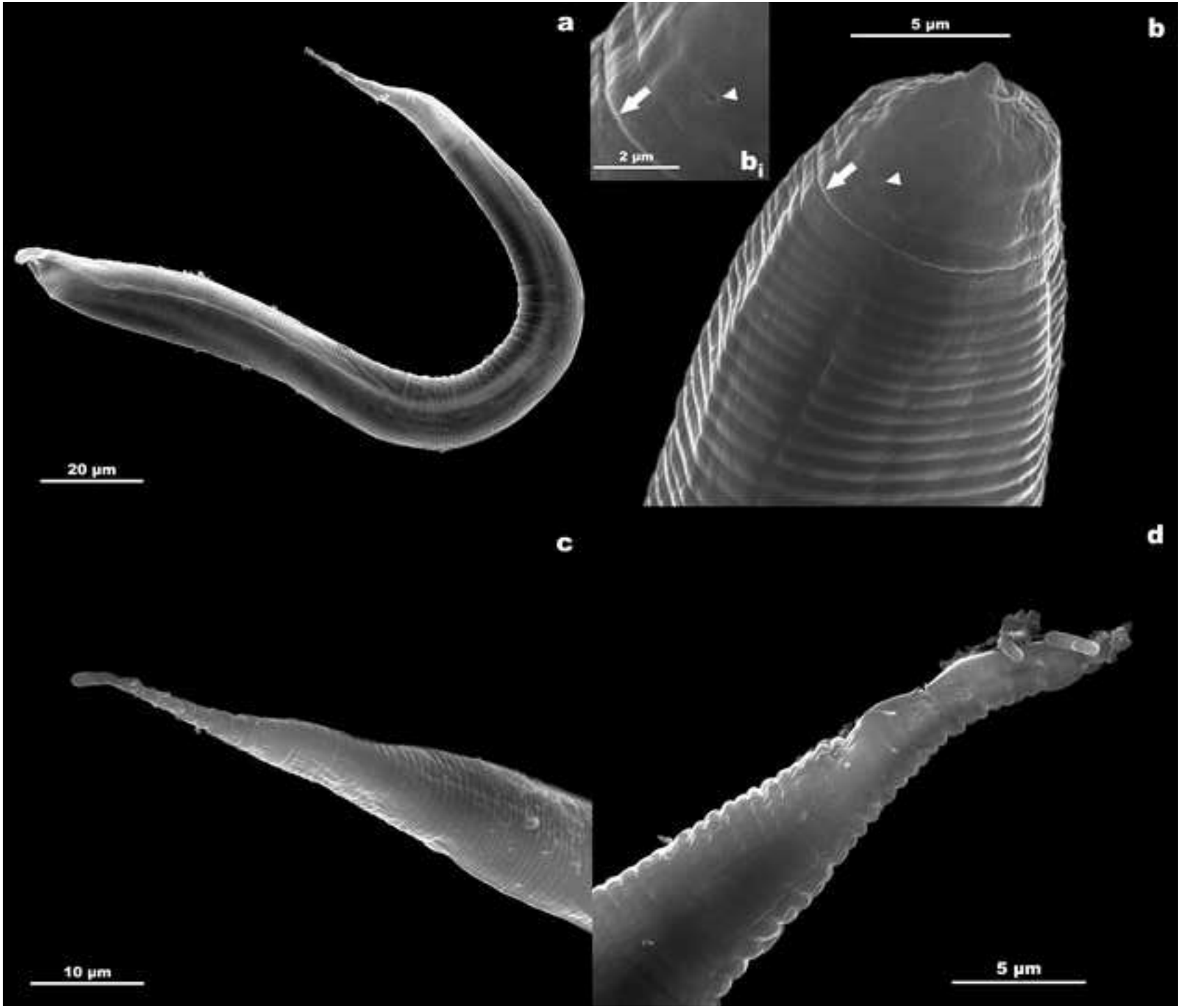


Figure 5



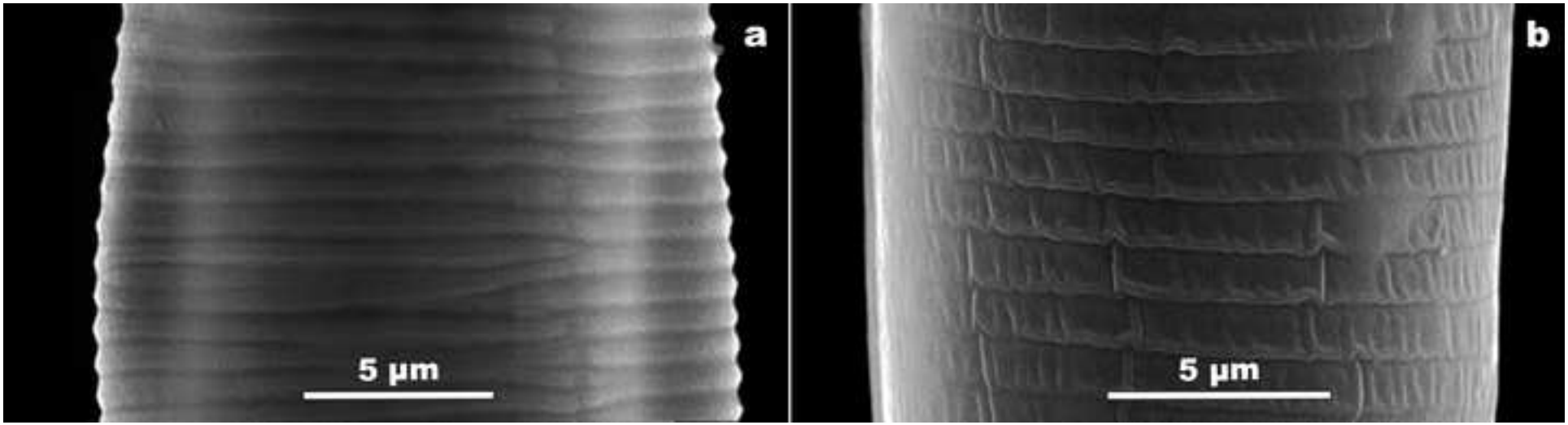


Figure 7