Description of new types of sphaeractinomyxon actinospores (Myxozoa: Myxosporea) from marine tubificid oligochaetes, with a discussion on the validity of the tetraspora and the endocapsa as actinospores collective group names

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Abstract Ten new types of sphaeractinomyxon actinospores are morphologically and molecularly described from the coelomic cavity of two marine oligochaete hosts, *Limnodriloides agnes* Hrabě, 1967 and *Tubificoides pseudogaster* (Dahl, 1960), from Aveiro

estuary, Portugal. The smallest sphaeractinomyxon type measured 17 µm (length) × 19 µm (width) × 19 µm (apical diameter), whereas the largest type measured 61 µm × 76 µm × 80 µm. While considering the ten types of sphaeractinomyxon, it was found that the number of spores developing inside pansporocysts varied between one, two, four and eight. The total prevalence of infection was of 19% for the two host species, with a maximum recorded for spring and summer (25-26%). While considering each type of sphaeractinomyxon individually, it was found that the prevalence values ranged between 0.3 and 1.7%. All described sphaeractinomyxons were most similar to mugilids infecting *Myxobolus* species. The validity of the tetraspora and endocapsa collective group names is discussed.

Keywords Sphaeractinomyxon; Oligochaeta; *Limnodriloides agnes*; *Tubificoides pseudogaster*; tetraspora; endocapsa

Introduction

The discovery and description, in 1899, of the first actinospore types – i.e. synactinomyxon, hexactinomyxon and triactinomyxon – is attributed to Antonin Štolc, who isolated them from specimens of oligochaetes collected in Vltava river in Czech Republic (Caullery and Mesnil 1905). A few years later, in 1904 the first sphaeractinomyxon actinospore, designated with a binomial name, i.e. *Sphaeractinomyxon stolci*, was described from marine oligochaetes (Caullery and Mesnil 1904, 1905). Since then, several other sphaeractinomyxons were described; *Sphaeractinomyxon gigas* in 1923, by Granata; *Sphaeractinomyxon danicae* in 192,3 by Georgevitch; *Sphaeractinomyxon ilyodrili* in 1940, by Jirovec (Marques 1984); *Sphaeractinomyxon amanieui* (Puytorac 1963); and *Sphaeractinomyxon rotundum* (Marques, 1984). Until this time, all actinospore types were considered to represent legitimate species. They were classified in a separate class, named Actinosporea, and named following the binomial nomenclature system. However, in 1984, Wolf and Markiw were able to demonstrate the alternation of the life cycle of *Myxobolus cerebralis* in an oligochaete, involving the formation of triactinomyxon actinospores. The class Actinosporea was then extinct and the genera of actinosporeans became collective group names (Kent et al. 1994). In the following, new types of sphaeractinomyxon were described. Sphaeractinomyxon types 1 and 2 (Hallett et al. 1997), *Sphaeractinomyxon ersei* (Hallett et al. 1998) and *Sphaeractinomyxon leptocapsula* (Hallett et al. 1999).

Two new actinospore collective group names, tetraspora and endocapsa, were erected to encompass the new types of sphaeractinomyxons exhibiting some variations in its characters (Hallett and Lester 1999; Hallett et al. 1999). Tetraspora actinospores differ from sphaeractinomyxon in the number of spores it develops inside pansporocysts, exclusively, i.e. four spores instead of the usual eight spores. Two types of tetraspora were described, *Tetraspora discoidea* and *Tetraspora rotundum* (Hallett e Lester, 1999). Endocapsa actinospores differ from sphaeractinomyxon in having small irregular valvular expansions and not protruding polar capsules. Four types of endocapsa were described, *Endocapsa rosulata* and *Endocapsa stepheni* (Hallett et al. 1999), endocapsa type 1 (Hallett et al., 2001), and endocapsa type of Székely et al. (2007).

Several life cycles have been demonstrated by experimental infection or inferred by molecular biology involving actinospore types from about half of the actinospore collective groups (Eszterbauer et al. 2015). Until now, sphaeractinomyxon actinospores were never associated with a life cycle of any identified myxosporean, but phylogenetically sphaeractinomyxons are associated with marine *Myxobolus* species (Kent et al. 2001).

The body of knowledge on myxosporeans and the group's taxonomy are still based in the myxosporean phase and myxospore morphology, despite the fact that molecular biology has demonstrated a real need for new ways to approach the myxosporean classification (Fiala 2006; Fiala et al. 2015). The study of actinospores is still very insipient, especially in what concern the marine species, and surely they will have a crucial role in the future classification of the Myxosporea Bütschli, 1881 class. This study is intended to represent a contribution for that goal, adding ten new marine sphaeractinomyxon actinospores, morphologically and molecularly described from two species of oligochaete hosts.

Material and methods

Actinospore sampling and morphological study

From 2013 to 2014, an actinospore survey was conducted in 651 oligochaete specimens from Aveiro estuary (40°40′N:8°45′W), Portugal. Oligochaetes were collected from the mud at low tide and kept individually in cell well plates containing salt water. A stereo microscope was used to examine the specimens for the release of actinospores during the following days. All specimens were posteriorly examined individually under a light microscope (200-400x of magnification) with a drop of salt water and gently pressured by a lamella, to detect the presence of actinospores in the coelomic cavity.

Developmental stages and free actinospores were examined and photographed using a Zeiss Axiophot microscope (Grupo Taper, Sintra, Portugal), equipped with a Zeiss AxioCam Icc3 digital camera. AxioVision 4.6.3 software (Grupo Taper) was used in image analysis. Morphology and morphometry were characterized using fresh material, in accordance to Lom et al. (1997). Measurements included the mean value±standard deviations (SDs), range of variation, and number of measured actinospores.

Molecular characterization

Genomic DNA from actinospores and oligochaete hosts was extracted using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich), following the manufacturer's instructions. The 18S ribosomal RNA (rRNA) gene of actinospores was PCR amplified with the universal eukaryotic primers ERIB1 and ERIB10 (Table 1). PCR was carried out in a 25 µl reaction volume, using 2 µl of extracted genomic DNA, 0.5 µl of 10 mM deoxyribonucleotide triphosphates (dNTPs; nzyTech), 0.25 µl of 10 pmol of each primer,

2.5 µl of 10× Taq DNA polymerase buffer, 1.25 µl of 50 mM $MgCl_2$, 1.25 U of Taq DNA polymerase (nzyTech), and 18 µl of water. The reactions were run on a Bio-Rad - MJ Mini Gradient Thermal Cycler, with initial denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The final elongation step was performed at 72 °C for 7 min. This was followed by a nested PCR, using as a template, 1 µl of the initial PCR and specific myxosporeans primers (Table 1). The PCR mixture reaction was the same as for the first PCR, except for the 0.5 µl of 10 pmol of each primer. The nested PCR cycle had an initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 53 °C for 45s, and 72 °C for 1:30 min, and a final elongation at 72 °C for 7 min. Concerning the oligochaete hosts, the 16S rRNA gene was PCR amplified using the universal primers 16sar-L and 16sbr-H (Table 1). The PCR was carried out in a single reaction in the same conditions as for the actinospores DNA nested PCR, but using 2 µl of extracted genomic DNA. All PCR products were electrophoresed through a 1 % agarose 1× tris-acetate-EDTA buffer (TAE) gel stained with GreenSafe Premium (nzyTech). The PCR amplification products were purified and sequenced by STABVida (Portugal).

Staden Package software (pregap4 and gap4) version 2.0.0 (Staden et al. 2000) was used to assist the 16S rDNA and 18S rDNA consensus sequences assembling. Similarities between sequences (pairwise p-distance) were calculated using MEGA 5 software (Tamura et al. 2011). Consensus sequences were submitted to a standard nucleotide BLAST search for close relatives from NCBI (http://blast.ncbi.nlm.nih.gov).

Results

During the survey, 651 oligochaetes were examined for parasites. From these, 122 (18.7%) were found infected with actinospores identified as representatives of sphaeractinomyxon collective group.

All examined oligochaete hosts looked alike, with similar dimensions, bifid setae and a body tegument devoid of papillae. However, molecular biology has demonstrated the existence of two distinct oligochaete species. After submitting the 16S rRNA gene sequence from both oligochaete species to a BLAST search, one consensus sequence with 537 bp, obtained from nine specimens (GenBank acceccion number ########), was found to be 99.8% similar to *Limnodriloides agnes* Hrabě, 1967 (KR025871), while the other, with 527 bp, obtained from five specimens (########), was 98.8% similar to *Tubificoides pseudogaster* (Dahl, 1960) (HM459968), specifically, *T. pseudogaster* from the lineage II, as

reported by Kvist et al. (2010). Accordingly, the prevalence of infection was calculated considering the total sample of both oligochaetes.

Sphaeractinomyxons were found in oligochaetes throughout the year. Seasonal prevalence was 8.1% for winter (January–March; *n*= 211), 26.3% for spring (April–June; *n*=137), 19.5% for summer (July–September; *n*=123), and 25.0% for autumn (October–December; *n*=180). From the total number of infected oligochaetes, 30.3% had only initial developmental stages and/or immature spores, especially in the autumn season, with a maximum of 40.0%; 5.7% had co-infections with another type of sphaeractinomyxon, and, in one case, with a triactinomyxon type. The individual prevalence of infection for each type of actinospore was very low, ranging from 0.3 to 1.7%, even considering that it must be underestimated, because they were calculated comprising two different hosts.

Taxonomy and morphology of studied actinospores

Sphaeractinomyxon type 1 (new type)

Figs. 1a-c, Table 2

Description: Mature spores spherical in apical and lateral view. Spores (*n*=36) 17.2±1.5 (15.2–20.5) µm in length, 18.8±1.3 (16.7–21.7) µm in width and 18.8±0.9 (16.9–21.0) µm in diameter; three pyriform polar capsules 4.7±0.4 (3.8–5.8) µm in length and 3.4±0.3 (2.7–3.8) µm in width. Polar filaments exhibiting 3 to 4 longitudinal coils. Sporoplasm having many secondary cells. Spores developing in number of 8 inside pansporocysts.

Type host: *Limnodriloides agnes* Hrabě, 1967

Type locality: Aveiro Estuary, Portugal

Site of infection: Coelomic cavity

Prevalence of infection: 1.7% (11 out of 651)

GenBank accession no.: ########

Remarks: The sequences in GenBank most similar to this type were Triactinomyxon sp. SH-2006 (DQ473515) and Endocapsa sp. SH-2006 (DQ473516), with 91% of similarity, followed by *Myxobolus exiguus* Thélohan, 1895 (AY129317), *Myxobolus muelleri* Bütschli, 1882 (AY129314) and *Myxobolus episquamalis* Egusa et al., 1990 (JF810537), with 90% of similarity.

Sphaeractinomyxon type 2 (new type)

Fig. 1d, Table 2

Description: Mature spores spherical to angular in apical and lateral view. Spores (*n*=10) 23.3±0.8 (22.1–24.8) µm in length, 28.4±1.0 (26.2–29.8) µm in width and 28.6±1.2 (26.6–31.1) µm in diameter; three pyriform polar capsules 5.9±0.2 (5.7–6.0) µm in length and 4.1±0.2 (4.0–4.3) µm in width. Polar filaments exhibiting 3 to 4 longitudinal coils. Sporoplasm having many secondary cells. Spores developing in number of 8 inside pansporocysts.

Type host: *Limnodriloides agnes* Hrabě, 1967

Type locality: Aveiro Estuary, Portugal

Site of infection: Coelomic cavity

Prevalence of infection: 0.5% (3 out of 651)

GenBank accession no.: ########

Remarks: The sequences in GenBank most similar to this type were *Myxobolus* sp. WSK-2013 (KC733438) and *Sphaeractinomyxon ersei* (AF306790), with 94% of similarity, and *Myxobolus ichkeulensis* Bahri and Marques, 1996 (AF378337), with 93% of similarity.

Sphaeractinomyxon type 3 (new type)

Figs. 1e-f, Table 2

Description: Mature spores angular in apical view and spherical to ellipsoidal in lateral view. Spores (*n*=16) 30.5±1.8 (27.9–33.4) µm in length, 33.6±2.2 (28.4–38.0) µm in width and 33.9±0.8 (32.5–35.3) µm in diameter; three pyriform polar capsules 7.5±0.7 (6.5–8.3) µm in length and 6.2±0.4 (5.8–7.0) µm in width. Polar filaments exhibiting 3 longitudinal coils. Sporoplasm having many secondary cells. Spores developing in number of 4 inside pansporocysts.

Type host: *Limnodriloides agnes* Hrabě, 1967

Type locality: Aveiro Estuary, Portugal

Site of infection: Coelomic cavity

Prevalence of infection: 0.5% (3 out of 651)

GenBank accession no.: ########

Remarks: The sequences in GenBank most similar to this type were *Endocapsa rosulata* (AF306791), with 91% of similarity, and *M. exiguus* (AY129317) and *M. muelleri* (AY129314), with 90% of similarity.

Sphaeractinomyxon type 4 (new type)

Fig. 1g, Table 2

Description: Mature spores spherical to angular in apical view and spherical to slightly ellipsoidal in lateral view. Spores (*n*=19) 33.2±2.8 (28.1–36.7) µm in length, 36.5±2.3 (32.4–42.6) µm in width and 37.9±2.2 (34.1–40.3) µm in diameter; three pyriform polar capsules 7.8±0.4 (7.0–8.7) µm in length and 6.6±0.4 (5.8–7.1) µm in width. Polar filaments exhibiting 2 to 3 longitudinal coils. Sporoplasm having many secondary cells. Spores developing in number of 4 inside pansporocysts.

Type host: *Limnodriloides agnes* Hrabě, 1967

Type locality: Aveiro Estuary, Portugal

Site of infection: Coelomic cavity

Prevalence of infection: 0.8% (5 out of 651)

GenBank accession no.: ########

Remarks: The sequences in GenBank most similar to this type were Endocapsa sp. SH-2006 (DQ473516), with 89% of similarity, and *M. episquamalis* (JF810537), *M. exiguus* (AY129317) and *M. muelleri* (AY129314), with 91% of similarity.

Sphaeractinomyxon type 5 (new type)

Figs. 1h-i, Table 2

Description: Mature spores angular in apical view and ellipsoidal in lateral view. Spores (*n*=10) 42.5±2.1 (38.8–45.6) µm in length, 54.8±2.7 (50.0–59.5) µm in width and 56.0±2.4 (52.9–58.8) µm in diameter; three pyriform polar capsules 9.1±0.3 (8.8–9.6) µm in length and 7.6±0.3 (7.0–7.9) µm in width. Polar filaments exhibiting 2 to 3 longitudinal coils. Sporoplasm having many secondary cells. Spores developing in number of 4 inside pansporocysts.

Type host: *Limnodriloides agnes* Hrabě, 1967

Type locality: Aveiro Estuary, Portugal

Site of infection: Coelomic cavity

Prevalence of infection: 0.6% (4 out of 651)

GenBank accession no.: ########

Remarks: The sequences in GenBank most similar to this type were *M. exiguus* (AY129317) and *Endocapsa rosulata* (AF306791), with 90% of similarity, and *M. episquamalis* (JF810537), with 89% of similarity.

Sphaeractinomyxon type 6 (new type)

Figs. 1j-k, Table 2

Description: Mature spores angular in apical view and ellipsoidal in lateral view. Spores (*n*=20) 51.5±5.2 (46.0–56.9) µm in length, 64.5±3.3 (61.6–69.2) µm in width and 62.1±2.8 (56.7–68.4) µm in diameter; three pyriform polar capsules 10.2±0.4 (9.9–10.9) µm in length and 8.9±0.8 (7.5–9.7) µm in width. Polar filaments exhibiting 2 to 3 longitudinal coils. Sporoplasm having many secondary cells. Spores developing in number of 2 to 4 inside pansporocysts.

Type host: *Limnodriloides agnes* Hrabě, 1967

Type locality: Aveiro Estuary, Portugal

Site of infection: Coelomic cavity

Prevalence of infection: 0.8% (5 out of 651)

GenBank accession no.: ########

Remarks: The sequences in GenBank most similar to this type were *M. episquamalis* (JF810537), *M. exiguus* (AY129317) and *M. muelleri* (AY129314), with 90% of similarity, and *Endocapsa rosulata* (AF306791), with 87% of similarity.

Sphaeractinomyxon type 7 (new type)

Figs. 1l-n, Table 2

Description: Mature spores angular in apical view and ellipsoidal in lateral view. Spores (*n*=10) 60.9±5.6 (55.0–68.7) µm in length, 75.6±6.2 (64.7–85.8) µm in width and 80.3±5.1 (71.8–91.4) µm in diameter; three pyriform polar capsules 10.6±0.9 (9.4–11.9) µm in length and 9.7±0.4 (9.2–10.1) µm in width. Polar filaments exhibiting 2 to 3 longitudinal coils. The sporoplasm having many secondary cells. Spores developing in number of 1 to 4 inside pansporocysts.

Type host: *Limnodriloides agnes* Hrabě, 1967

Type locality: Aveiro Estuary, Portugal

Site of infection: Coelomic cavity

Prevalence of infection: 1.2% (8 out of 651)

GenBank accession no.: ########

Remarks: The sequences in GenBank most similar to this type were *Endocapsa rosulata* (AF306791), *Myxobolus bizerti* Bahri and Marques 1996 (AY129318), *M. exiguus* (AY129317) and *M. muelleri* (AY129314), all with 90% of similarity.

Sphaeractinomyxon type 8 (new type)

Fig. 1o, Table 2

Description: Mature spores spherical in apical and lateral view. Spores (*n*=21) 8.1±0.9 (17.0–21.3) µm in length, 18.6±0.8 (17.7–21.1) µm in width and 18.1±1.0 (17.1–20.0) µm in diameter; three pyriform polar capsules 4.7±0.2 (4.3–5.2) µm in length and 3.3±0.2 (3.0–3.5) µm in width. Polar filaments exhibiting 2 to 3 longitudinal coils. The sporoplasm having many secondary cells. Spores developing in number of 8 inside pansporocysts.

Type host: *Tubificoides pseudogaster* (Dahl, 1960)

Type locality: Aveiro Estuary, Portugal

Site of infection: Coelomic cavity

Prevalence of infection: 0.3% (2 out of 651)

GenBank accession no.: ########

Remarks: The sequences in GenBank most similar to this type were Triactinomyxon sp. SH-2006 (DQ473515) and Endocapsa sp. SH-2006 (DQ473516), with 94% of similarity, and *M. exiguus* (AY129317) and *M. muelleri* (AY129314), with 93% of similarity.

Sphaeractinomyxon type 9 (new type)

Fig. 1p, Table 2

Description: Mature spores spherical in apical and lateral view. Spores (*n*=21) 20.9±1.0 (19.0–23.4) µm in length, 22.0±1.5 (20.4–26.9) µm in width and 22.6±1.2 (20.7–26.5) µm in diameter; three pyriform polar capsules 5.6±0.4 (4.8–6.0) µm in length and 4.3±0.3 (3.9–4.8) µm in width. Polar filaments exhibiting 2 to 3 longitudinal coils. The sporoplasm having many secondary cells. Spores developing in number of 8 inside pansporocysts.

Type host: *Tubificoides pseudogaster* (Dahl, 1960)

Type locality: Aveiro Estuary, Portugal

Site of infection: Coelomic cavity

Prevalence of infection: 0.9% (6 out of 651)

GenBank accession no.: ########

Remarks: The sequences in GenBank most similar to this type were Triactinomyxon sp. SH-2006 (DQ473515), with 96% of similarity, Endocapsa sp. SH-2006 (DQ473516), with 94% of similarity, and *M. exiguus* (AY129317) and *M. muelleri* (AY129314), with 93% of similarity.

Sphaeractinomyxon type 10 (new type)

Figs. 1q-r, Table 2

Description: Mature spores spherical in apical view and spherical to slightly ellipsoidal in lateral view. Spores (*n*=47) 22.3±1.1 (19.3–24.9) µm in length, 24.0±1.7 (19.6–27.4) µm in width and 24.3±1.6 (21.2–27.5) µm in diameter; three pyriform polar capsules 5.2±0.3 (4.5–6.2) µm in length and 4.0±0.2 (3.4–4.5) µm in width. Polar filaments exhibiting 3 longitudinal coils. Sporoplasm having many secondary cells. Spores developing in number of 8 inside pansporocysts.

Type host: *Tubificoides pseudogaster* (Dahl, 1960)

Type locality: Aveiro Estuary, Portugal

Site of infection: Coelomic cavity

Prevalence of infection: 0.8% (5 out of 651)

GenBank accession no.: ########

Remarks: The sequences in GenBank most similar to this type were Triactinomyxon sp. SH-2006 (DQ473515), with 93% of similarity, and Endocapsa sp. SH-2006 (DQ473516), *M. exiguus* (AY129317) and *M. muelleri* (AY129314), with 92% of similarity.

The spores and corresponding developmental stages were always found in the coelomic cavity, for all described actinospore types (Fig. 1a); in some cases, they were also found inside reproductive structures cavities (Fig. 1k). Spore development was asynchronous, as all different developmental stages were observed simultaneously in a same individual. Nevertheless, spore development inside each individual pansporocyst was usually synchronous, even though, asynchronous development was also observed in some rare cases.

After observing the developmental stages of the several new sphaeractinomyxon types, it is possible to infer the successive steps in spores development (Fig. 2). The first identifiable stage corresponded to binucleated cells (Fig. 2a). These cells divide twice, forming a set of four cells (Figs. 2b-c), which then create the initial pansporocyst with two somatic cells forming the pansporocysts walls and another two generative cells inside the pansporocyst (Fig. 2d). The two internal cells start to divide in three (Fig. 2e) and four internal cells (Fig. 2f). Two such internal cells then divide further two times, resulting in stages with six (Fig. 2g) and then 10 smaller cells (Fig. 2h), while the other two larger cells only then start to divide (Fig. 2i). In the end of the gametogamy, it is found a pansporocyst with 16 morphologically indistinct cells (Fig. 2j). The gametogamy ends with the formation

of zygotes (Fig. 2k). The gametogamy stage had similar developmental stages in all different types of described actinospore; however, the sporogony stage was found to differ to a little extent among the different types, according to the final number of spores formed in each pansporocyst. In the beginning of the sporogony, for the actinospore types with the development of eight spores, it were found pansporocysts with a set of eight cells centrally located and eight sporoplasmic cells leaning against the pansporocyst wall (Fig. 2l). The centrally located cells divide, forming eight involucres formed by three valvogenic cells surrounding three capsulogenic cells (Fig. 2o). As the spores develop, the sporoplasmic cells migrate inside the spore involucre (Fig. 2p). When immature (Figs. 2q, r), the spores look larger compared to mature spores, and their appearance can give the false idea of spores with expansions (Fig. 2r). For the actinospore types with the development of two or four spores, it were found pansporocysts with two (Fig. 2m) or four (Fig. 2n) involucres and the same amount of sporoplasmic cells leaning against the pansporocyst wall. In these stages, it is also observed some elongated cells, whose function or purpose can only be speculated, and which seem to correspond to vegetative cells that do not develop to form spores. In the end of the sporogony it is possible to observe pansporocysts with different number of mature spores, according to the different actinospore types (Figs. 2s, t, u). In pansporocysts with less than eight mature spores, it was possible to observe some small vegetative cells, as the example in Fig. 2t, where four vegetative cells are easily seen between the four mature spores. These vegetative cells were also observed in mature pansporocysts of the sphaeractinomyxon type 1 in oligochaetes heavily infected. This type of actinospore usually develops eight spores per pansporocyst, but several pansporocysts had a smaller number of spores and the missing spores were compensated by the presence of these vegetative cells.

The mature spores were never seen exiting the worm body, not by the intestine either by the gonophores or the nephridiopores, even in heavily infected worms. In some cases, when the worm host body was excessively pressured by a cover slip, the spores free in the coelom could break through the intestine epithelium and invade its lumen, but this was not a common occurrence.

Genetic distances between the 10 sphaeractinomyxon new types were calculated and are presented in Table 3. The lowest genetic differences found were between sphaeractinomyxon type 4 and type 7 (5.2%), while the highest respected sphaeractinomyxon type 1 and type 2 (14.3%). A BLAST search for the 10 sphaeractinomyxon new types found no match in the GenBank. The most similar species were *Myxobolus* species (89–94%), especially *M. exiguus* (AY129317), *M. muelleri* (AY129314), *Myxobolus* sp. (KC733438), *M. ichkeulensis* (AF378337), *Myxobolus spinacurvatura* Maeno, Sorimachi, Ogawa and Egusa,

1990 (AF378341), *M. episquamalis* (JF810537) and *M. bizerti* (AY129318). They also had similarities with some actinospore types in GenBank (86–96%): *Sphaeractinomyxon ersei* (AF306790), Triactinomyxon sp. SH-2006 (DQ473515), *Endocapsa rosulata* (AF306791), Endocapsa sp. SH-2006 (DQ473516), *Tetraspora discoidea* (AF306793) and Raabeia TGR-2014 (KF263539).

Discussion

Oligochaete worms in Aveiro estuary were found infected with 10 new types of sphaeractinomyxon actinospore (species), duplicating the number of the strictly described sphaeractinomyxons (Marques, 1984; Hallett et al. 2001). The new types of sphaeractinomyxon can be distinguished in morphological terms by the size of the spores and polar capsules, within each oligochaete species host.

One obvious conclusion that can be drawn from the results of this study is the strict specificity of the different sphaeractinomyxon types to the oligochaete host species. Each specific sphaeractinomyxon type was never found in both species of oligochaetes *L. agnes* and *T. pseudogaster*. This great specificity turns useless the morphological comparison between sphaeractinomyxons infecting different host species. From the 16 sphaeractinomyxon types (10 sphaeractinomyxon, two tetraspora and four endocapsa) described in the literature, none was reported to infect *L. agnes* or *T. pseudogaster*. Another conclusion is the need to use molecular biology in future descriptions of these types of spores from different species of oligochaete hosts, since spore measurements can overlap in many different sphaeractinomyxon types. Therefore, in ecological studies, besides spore's measures, the host oligochaete species is also an important character to identify the sphaeractinomyxon type without the use of molecular biology, and eventually locality can also play an important role in species separation.

The developmental stages observed in this study follow the same path described for the sphaeractinomyxon types, since the first development description made by Caullery and Mesnil (1905). The major differences between these actinospores and the others types, is the fact that sphaeractinomyxons can develop a different number of spores (from one to eight) inside pansporocysts, while all other described actinospore types have always eight spores.

Main differentiating characteristics between the different sphaeractinomyxon types, besides the number of spores developing inside the pansporocysts, are the size and form of the spores. The form and number of the developing spores seem to be related with the size of the spores. The spore form changes from round in apical and lateral view, in the smaller

sphaeractinomyxon types, to more angular in apical view and ellipsoidal in lateral view, in the larger sphaeractinomyxon types. The spore shape change is not only a species specific character but also an environmental influenced character. For instance, spores observed under the pressure of a cover slip, tend to become more angular as the pressure increases; accordingly, researchers should have some caution when observing these types of spores in a microscope slide. Spore size is also related with the number of spores developing inside the pansporocyst. The smaller sphaeractinomyxon types develop in number of eight inside pansporocysts, while the median size sphaeractinomyxon types, develop in number of two to four spores inside pansporocysts, and the larger sphaeractinomyxon types, develop in number of one to two inside pansporocysts. This character seems to be not only typical for each species, but also, an environmentally influenced character. For instance, in heavily infected worms, the number of mature spores inside the pansporocysts was smaller than expected; in these cases, the missing spores were 'replaced' by small vegetative cells, suggesting that the lack of space or excessive pressure exerted in the worm coelomic cavity had some kind of inhibiting effect during the spore's development.

None of the infected oligochaetes was found to release sphaeractinomyxons from the coelomic cavity. There is some literature reporting the presence of this type of spores in the intestinal lumen of their hosts (Hallett et al. 1998, 1999, 2001), but, in this study, the spores where only observed in the lumen of the intestine when excessive pressure was applied to the cover slip over the oligochaete under microscopy observation. This suggests that the presence of spores inside the intestine lumen is likely accidental, and not a normal exiting route.

Overall prevalence of infection of all new types of sphaeractinomyxon, for the two hosts species, was of 19%, and could reach a maximum of 26% in spring, with a minimum in winter. Parasite infection was found throughout the year but, the spring and summer months seem to be more appropriate for the dissemination of these type of actinospores since, in autumn, there is a great number of worms with only developmental stages and immature spores. Nevertheless, individually the prevalence of infection was low, which is consistent with the prevalence values reported in the literature (Yokoyama et al. 2012).

Molecular biology could demonstrate a close proximity of the 10 new types of sphaeratinomyxon to *Myxobolus* species, in particular, to species infecting mugilid fishes. The same is true for the other sphaeractinomyxon, tetraspora and endocapsa types in GenBank. Myxosporean species infecting mullets worldwide amount to 64, according to the last counting, and, from these, 32 are *Myxobolus* species (Yurakhno and Ovcharenko 2014). Only 31% of the mullets infecting *Myxobolus* have DNA sequences published in GenBank, and surely, many new species are yet to be discovered. Nevertheless, it is premature to associate

the sphaeractinomyxon actinospores as the invertebrate stage of mugilid infecting *Myxobolus* species.

The number of spores developing inside pansporocysts was used as argument for the erection of tetraspora as a new actinospore collective group (Hallett and Lester 1999); nevertheless, these authors considered that, in morphological terms these spores represented typical sphaeractinomyxons, only developing in a set of four spores. In face of the results obtained in this study, no reason is found to justify the existence or creation of new collective groups for sphaeractinomyxons based in this character.

Endocapsa collective group, erected by Hallett et al. (1999), is another group of actinospores similar to the sphaeractinomyxons and, in the same way, these authors state that, by default, the endocapsa can be considered as sphaeractinomyxons. The major differences described are the 'submerged' polar capsules and the presence of irregular 'processes in the form of swellings'. In the work of Hallett et al. (1999), several forms of different swellings are drawn, and an important detail is described by these authors, the swellings do not change when in contact with water, contrary to what happens to the other actinospores types with typical processes. Besides the later record, there is another more recent record for the endocapsa type in Lake Assad (Euphrates River), Syria (Székely et al. 2007), which is the only freshwater endocapsa isolate until now. Unfortunately, however, spores illustrations in that work do not help to support the endocapsa actinospores, as depicted spores have the aspect of being degraded. We cannot dispute this collective group without observing the described spores, but, in the course of this study, it was possible to observe more than one hundred infected oligochaetes and thousands of mature spores ranging from 15 µm to 91 µm in size, and their developmental stages, and had never found spores with swellings or processes. However, many immature spores, very similar to the description made for the endocapsa actinospores, were observed with 'submerged' polar capsules and a kind of swellings but, side by side, we also observed mature spores with its typical sphaeractinomyxon form within the same host (for instance, see Fig. 2r). Another important detail were the modifications suffered by spores, especially when they started to dehydrate, in which the sporoplasm concentrates more in the centre of the spores, and the corners of the more angular spores start to appear as swellings.

In conclusion, the endocapsa collective group needs a validation by new records that can indisputably confirm them as a legitimated collective group. On the other hand, it is mandatory to considerer if the number of spores developing inside pansporocysts is reason enough to divide the sphaeractinomyxon actinospores in new and different collective groups

(for instance, tetraspora for four spores, dispora for two spores, unispora for one spore, etc.). In our opinion, that there is no need for such separation.

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Table captions

Table 1 Primers used in actinospores and oligochaete DNA amplification and sequencing

Table 2 Morphology of sphaeractinomyxon types, isolated from the marine oligochaetes *Limnodriloides agnes* and *Tubificoides pseudogaster* (the mean±SD with the range in parentheses; in µm)

Table 3 Pairwise genetic differences between the 18S rDNA sequences of the ten new sphaeractinomyxon types

Figure legends

Fig. 1 Spores of sphaeractinomyxon types found in the coelomic cavity of *Limnodriloides agnes* (a-n) and *Tubificoides pseudogaster* (o-r). **a** Sphaeractinomyxon type 1 filling the coelomic cavity of *L. agnes*. **b** Sphaeractinomyxon type 1 in lateral view. **c** Sphaeractinomyxon type 1 in apical view. **d** Sphaeractinomyxon type 2 in apical and lateral view. **e** Sphaeractinomyxon type 3 in lateral view. **f** Sphaeractinomyxon type 3 in apical view. **g** Sphaeractinomyxon type 4 in lateral view. **h** Sphaeractinomyxon type 5 in apical view. **i** Sphaeractinomyxon type 5 in lateral view. **j** Sphaeractinomyxon type 6 in apical and lateral view inside a bisporous pansporocyst. **k** Developmental stages of sphaeractinomyxon type 6 inside a gonad cavity. **l** Sphaeractinomyxon type 7 in apical view. **m** Sphaeractinomyxon type 7 in lateral view. **n** Polar capsules of a sphaeractinomyxon type 7 smashed spore exhibiting the polar filaments coiled longitudinally. **o** Sphaeractinomyxon type 8 in apical and lateral view. **p** Sphaeractinomyxon type 9 in apical and lateral view inside a pansporocyst. **q** Sphaeractinomyxon type 10 in lateral view. **r** Sphaeractinomyxon type 1o in apical view. Scale bars=20 µm.

Fig. 2 Developmental stages of the sphaeractinomyxons in the coelomic cavity of *Limnodriloides agnes* and *Tubificoides pseudogaster*. **a** A binucleated cell. **b-c** Binucleated cells dividing into two and four cells. **d** Initial pansporocyst with two enveloping cells and

two internal cells. **e-f** Pansporocysts with three and four internal cells. **g-h** Pansporocysts in which two internal cells divided successively two times originating a final set of 8 smaller cells while two other larger cells rest undivided. **i** Pansporocysts in which the lager undivided cells from the anterior stages started their division. **j** Final stage of gametogamy with a pansporocysts with 16 inner cells. **k** Pansporocyst in the beginning of the sporogony showing 8 zygotes. **l** Pansporocyst with 8 set of cells in a more central area and sporoplasmic cells (*double asterisks*) leaning against the pansporocyst wall. **m** Pansporocyst with a more advanced stage of sporogony that will produce two final spores. It is visible two sporoplasmic cells (*double asterisks*) against the pansporocyst wall, two involucres formed by three valvogenic cells surrounding three inner capsulogenic cells (*asterisks*) and some elongated cells with unknown function. **n** Pansporocyst in the same sporogony stage as the one in the previous figure but in a type of sphaeractinmyxon that will produce four final spores. **o** Pansporocysts in a slightly more advanced sporogony stage than the previous two figures, but producing eight final spores. The capsulogenic cells are already forming the polar capsules. **p** Pansporocyst in a more advanced sporogony stage, were the majority of the spores have already the sporoplasmic cells inside the spore involucre, but two sporoplasmic cells (*double asterisks*) are still outside their spore involucre (*asterisks*). **q** Pansporocyst with eight immature spores. **r** Some immature spores outside a busted pansporocyct accompanied by a solitaire mature spore on the right side. **s-u** Pansporocysts with mature spores from three different types of sphaeractinomyxon. One type developing two spores (*s*), another type developing four spores (*t*) and, finally a type developing eight spores (*u*). Note the presence of four vegetative cells (*asterisks*) around the four mature spores in figure *t*. Scale bars=20 µm.

Table 1 Primers used in actinospores and oligochaete DNA amplification and sequencing

Name	Sequence (5'-3')	Pared with	Source
ERIB1	ACC TGG TTG ATC CTG CCA G	ERIB10	Barta et al. 1997
ERIB10	CTT CCG CAG GTT CAC CTA CGG	ERIB1	Barta et al. 1997
18e	CTG GTT GAT CCT GCC AGT	ACT3r, Myx4r, ACT1r	Hillis and Dixon 1991
18r	CTA CGG AAA CCT TGT TAC G	MyxospecF, ACT3f	Whipps et al. 2003
MyxospecF	TTC TGC CCT ATC AAC TTG TTG	ACT1r, 18r	Fiala 2006
ACT3f	CAT GGA ACG AAC AAT	ACT1r, 18r	Hallett and Diamant 2001
ACT3r	ATT GTT CGT TCC ATG	18e	Hallett and Diamant 2001
Myx4r	CTG ACA GAT CAC TCC ACG AAC	18e	Hallett and Diamant 2001
ACT1r	AAT TTC ACC TCT CGC TGC CA	18e, MyxospecF	Hallett and Diamant, 2001
16sar-L	CGC CTG TTT ATC AAA AAC AT	16sbr-H	Palumbi et al. 2002
16sbr-H	CCG GTC TGA ACT CAG ATC ACG T	16sar-L	Palumbi et al. 2002

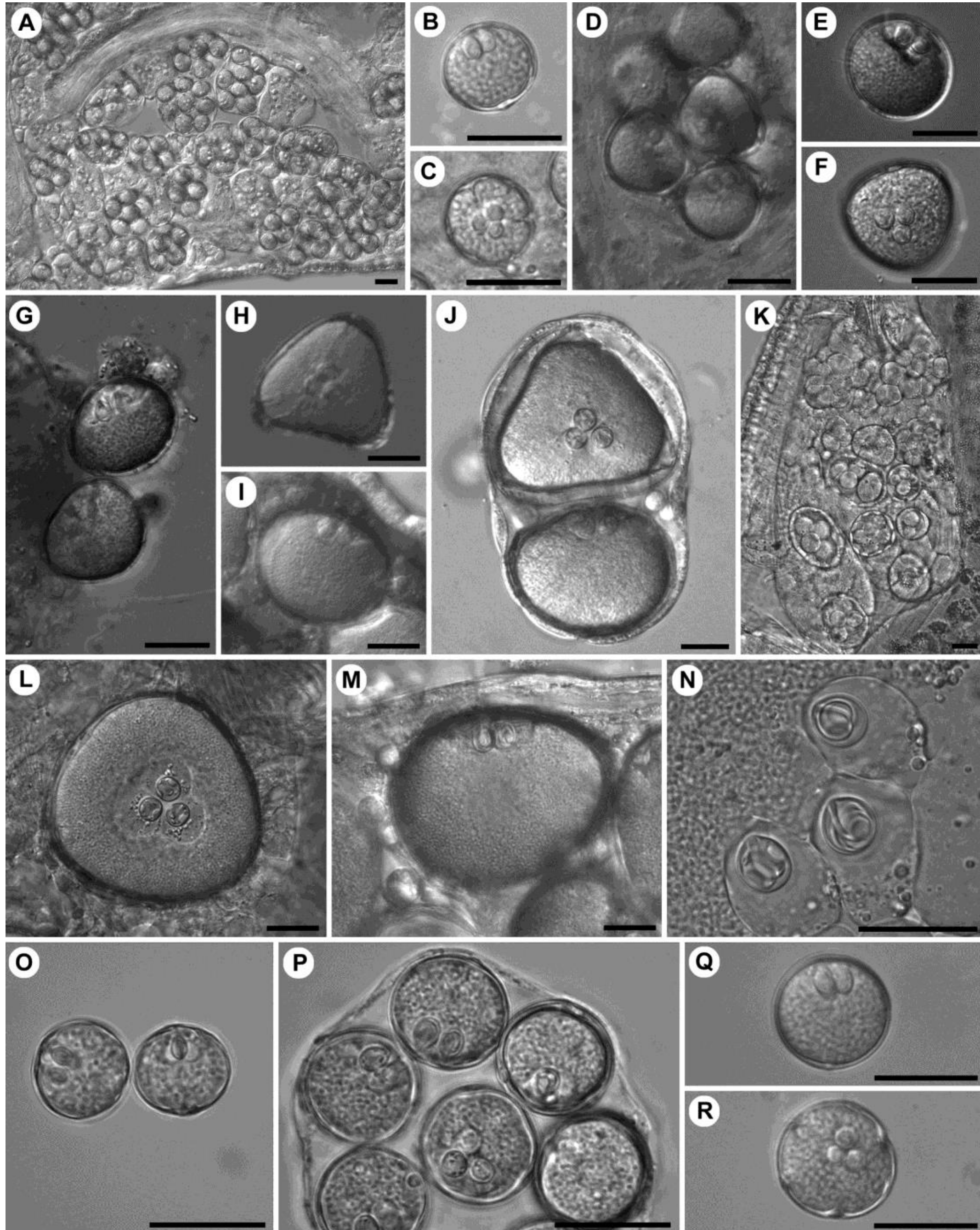
Table 2 Spore morphology of sphaeractinomyxon types from *Limnodriloides agnes* (types 1–7) and *Tubificoides pseudogaster* (types 8–10).

Spore type	Spore length	Spore width	Spore diameter	Polar capsule length	Polar capsule width
1	17.2±1.5 (15.2–20.5)	18.8±1.3 (16.7–21.7)	18.8±0.9 (16.9–21.0)	4.7±0.4 (3.8–5.8)	3.4±0.3 (2.7–3.8)
2	23.3±0.8 (22.1–24.8)	28.4±1.0 (26.2–29.8)	28.6±1.2 (26.6–31.1)	5.9±0.2 (5.7–6.0)	4.1±0.2 (4.0–4.3)
3	30.5±1.8 (27.9–33.4)	33.6±2.2 (28.4–38.0)	33.9±0.8 (32.5–35.3)	7.5±0.7 (6.5–8.3)	6.2±0.4 (5.8–7.0)
4	33.2±2.8 (28.1–36.7)	36.5±2.3 (32.4–42.6)	37.9±2.2 (34.1–40.3)	7.8±0.4 (7.0–8.7)	6.6±0.4 (5.8–7.1)
5	42.5±2.1 (38.8–45.6)	54.8±2.7 (50.0–59.5)	56.0±2.4 (52.9–58.8)	9.1±0.3 (8.8–9.6)	7.6±0.3 (7.0–7.9)

6	51.5±5.2 (46.0–56.9)	64.5±3.3 (61.6–69.2)	62.1±2.8 (56.7–68.4)	10.2±0.4 (9.9–10.9)	8.9±0.8 (7.5–9.7)
7	60.9±5.6 (55.0–68.7)	75.6±6.2 (64.7–85.8)	80.3±5.1 (71.8–91.4)	10.6±0.9 (9.4–11.9)	9.7±0.4 (9.2–10.1)
8	18.1±0.9 (17.0–21.3)	18.6±0.8 (17.7–21.1)	18.1±1.0 (17.1–20.0)	4.7±0.2 (4.3–5.2)	3.3±0.2 (3.0–3.5)
9	20.9±1.0 (19.0–23.4)	22.0±1.5 (20.4–26.9)	22.6±1.2 (20.7–26.5)	5.6±0.4 (4.8–6.0)	4.3±0.3 (3.9–4.8)
10	22.3±1.1 (19.3–24.9)	24.0±1.7 (19.6–27.4)	24.3±1.6 (21.2–27.5)	5.2±0.3 (4.5–6.2)	4.0±0.2 (3.4–4.5)

Table 3 Pairwise genetic differences between the 18S rDNA sequences of the ten new sphaeractinomyxon types

	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6	Type 7	Type 8	Type 9	Type 10
Type 1 (########)										
Type 2 (########)	0.143									
Type 3 (########)	0.112	0.130								
Type 4 (########)	0.109	0.125	0.057							
Type 5 (########)	0.112	0.138	0.098	0.098						
Type 6 (########)	0.109	0.131	0.099	0.100	0.080					
Type 7 (########)	0.109	0.131	0.072	0.052	0.099	0.110				
Type 8 (########)	0.076	0.131	0.107	0.104	0.115	0.109	0.114			
Type 9 (########)	0.079	0.134	0.106	0.102	0.110	0.109	0.112	0.057		
Type 10 (########)	0.082	0.133	0.113	0.104	0.116	0.120	0.118	0.058	0.061	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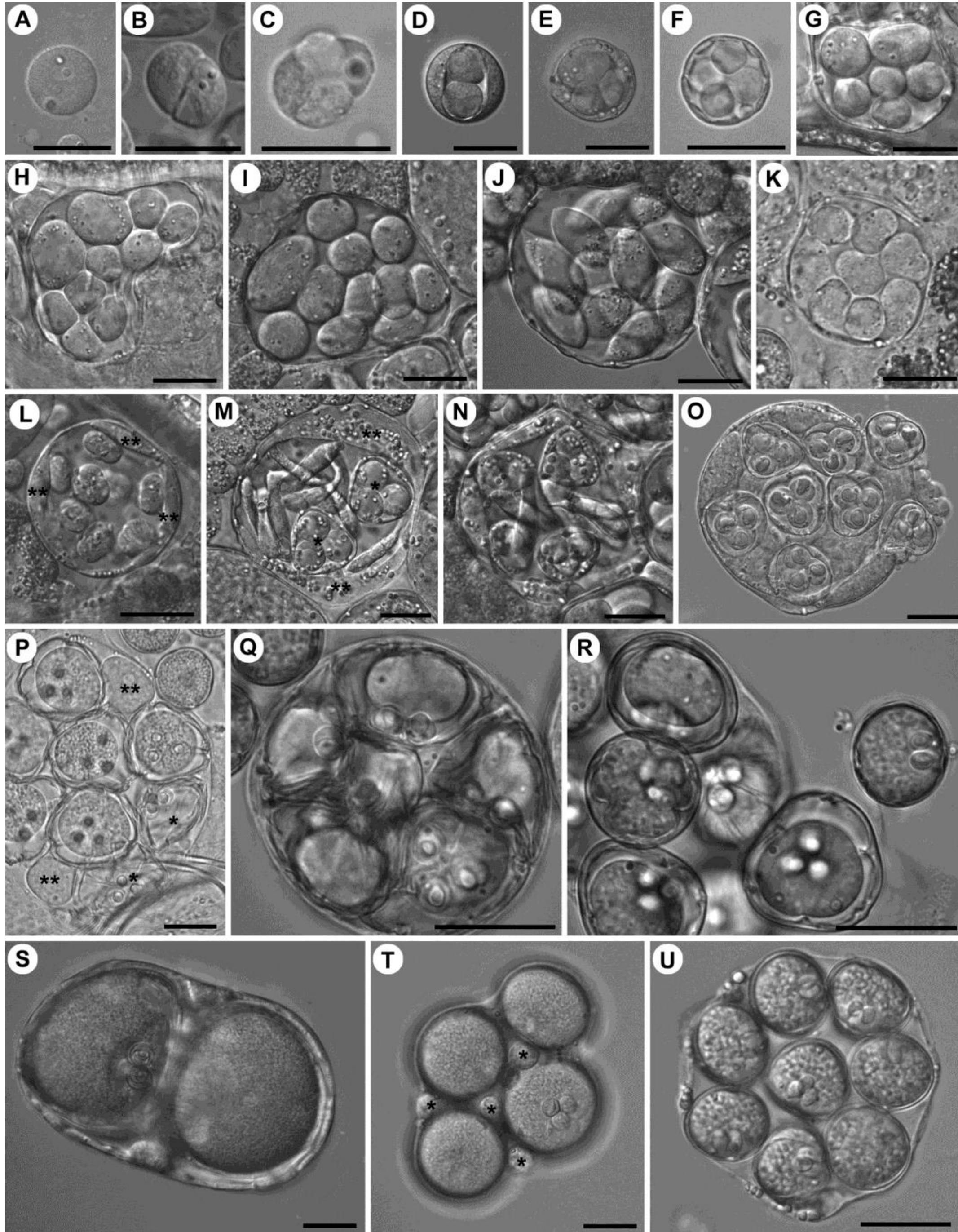
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