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Endolimax piscium sp. nov. (Amoebozoa), causative agent of systemic granulomatous disease of cultured sole (*Solea senegalensis)*

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RUNNING TITLE: ~~Systemic granulomatous infection~~New species of ~~sole by~~ *Endolimax ~~piscium~~* ~~n. sp.~~

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Abstract.

A new amoeba species, pathogenic for Senegalese sole is described based on ultrastructural analysis and SSU rDNA phylogenetic inference. The parasite presents round to ovoid trophozoites (<5 µm) with a high degree of intracellular simplification. No mitochondria were observed but mitosome-like organelles were present. No cysts could be detected. Phylogenetic analysis confirmed the Senegalese sole parasite as an amitochondriate Archamoeba related to *Endolimax nana* and *Iodamoeba spp*., and we tentatively describe it as a new species in the genus *Endolimax*, *Endolimax piscium*. However, the genetic distance with *E. nana* is quite large, with only 60% pairwise identity between both SSU rDNA genotypes. Although the overall topology of the Archamoebae cladograms containing *E. piscium* was consistent, the support for the branching of *Endolimax spp.* relative to its closest neighbours was variable, being higher with distance or parsimony-based inference methods than with ML or Bayesian trees. The use of stringent alignment sampling masks also caused instability and reduced support for some branches, including the ~~monophily~~monophyly of *Endolimax* spp. in the most conservative datasets. The characterization of other Archamoebae parasitizing fish could help to clarify the status of *E. piscium* and to interpret the large genetic distance observed between *Endolimax* species.

Keywords: Archamoeba, Parasite, Solea senegalensis, Endolimax, Iodamoeba, Granulomatous disease

1. Introduction

Recently, systemic inflammatory lesions were described in cultured Senegalese sole, *Solea senegalensis* (Constenla and Padrós 2010). These lesions were characterised by lumps in the muscle, often noticeable at the skin surface, which make the fish unmarketable~~..~~. The disease was found to respond to the presence of large numbers of minute spherical, plasmodial protozoans at the periphery of granulomatous lesions ~~and abscesses~~, which were most evident in the skeletal muscle but also present in the digestive tract, liver, heart and kidney (Constenla and Padrós 2010). The differential diagnosis based on histopathological and preliminary TEM studies in this previous work initially pointed to a presumptive parasitosis due to amoeba, the so-called "X-cells" (Freeman, 2009), or stages from an unknown amitochondriate organism. Although no morphological unambiguous characters were found to confirm the ~~etiology~~aetiology of the disease, most of the ~~presumprive~~presumptive data strongly suggested that amoeba could be the causative agent.

Amoebic infections involving granulomatous inflammatory lesions and abscesses can affect different animal and human organs, especially the liver and the brain (Candreviotis 1977; Visvesvara, Schuster & Martinez 1993; Riestra-Castaneda, Riestra-Castaneda & Gonzalez-Garrido 1997). However, systemic ~~amoebiases~~amoebiasies have seldom being reported in fish and the amoebae involved have not been fully ~~characterized~~characterised (Nash, Nash & Schlotfeld 1988). This notwithstanding, a systemic granulomatous infection by a possibly related, amoeba-like organism was reported in goldfish, *Carassius auratus* L. (Voelker et al.

1977; Lom and Dyková 1992; Steinhagen, Jendrysek & Körting 1993) and recently also in Tench, *Tinca tinca* L. (Palíková et al. 2012).

Although a taxonomical hotchpotch for many years, recent studies have narrowed Amoebozoa to a diverse, but phylogenetically congruent clade grouping classical amoeboid Lobosa, slime moulds (Mycetozoa), and Archamoeba (Cavalier-Smith, Chao & Oates -2004; Nikolaev et al. 2006, Minge et al. 2009). Archamoebae includes amitochondriate, endocommensal or facultative parasitic organisms such as *Entamoeba* and *Endolimax*, reported from a wide variety of vertebrate hosts. Whereas *Entamoeba* spp. are better known due to their clinical importance in humans, *Endolimax* nana is the only species in its genus for which comprehensive data including genetic information are available, despite the existence of multiple reports of isolates and putative species descriptions in vertebrates and invertebrates (Table 1). This paucity of information maybe due to the relatively minor clinical importance of this taxon (Silberman et al. 1999), their pleomorphism and lack of distinct morphological characters, and the difficulties associated with their laboratory cultivation. In a recent study, *Iodamoeba,* considered the last genus of obligate parasitic human protists without proper phylogenetic ~~characterization~~characterisation, was placed as a sister taxa to *E. nana* although a striking intrageneric diversity was reported (Stensvold, Lebbad & Clark 2012).

The ~~aim~~aims of this study ~~was~~were the identification and description of the organism causing the systemic inflammatory disease in Senegalese sole. Molecular characterization and phylogenetic analyses, as well as an ultrastructural study of the organism were carried out. As a result, the organism was identified as a new archamoeba whose closest know relative is *E. nana*, and it is tentatively described as a new species in this genus.

2. Materials and methods

2.1. Source material.

In the course of parasitological surveys at different sole farms located in NW Spain, animals displaying obvious body bumps and inflammatory lesions in the muscle were selected. Affected regions were excised and preserved in 90% ethanol. Parallel subsamples were fixed in 10% neutral-buffered formalin and processed for paraffin-embedding and routine histopathological examination, in order to confirm the nature of the lesions prior to attempting further molecular work.

2.2. DNA isolation, cloning and sequencing.

Granulomatous lesions in ethanol-preserved skeletal muscle were excised under a binocular scope. Three fish from two sole farms, A and B, were used. From each fish, tissue from several lesions was sampled and pooled and Genomic DNA was extracted using a silica-based commercial kit (Roche Applied Science, Barcelona, Spain). Control DNA was also extracted from healthy juvenile sole. Different sets of primers targeting eukaryotic SSU rDNA were assayed (Table 2). All PCRs were carried out in 50 ul volumes containing 1x Taq buffer with 2.5mM $MgCl_2$, 0.2mM each deoxyribonucleotide triphosphate (dNTP), 1 U Taq DNA polymerase and 25 pmol of each primer. Cycling conditions consisted on an initial denaturation (2-3 min 94ºC) and 35x amplification cycles (94ºC / 1 min, 55ºC / 1 min, 72ºC / 1 min) followed by a final, 8min incubation at 72ºC. Reactions using primers 18S-EUK581-F and 18S-EUK1134-R (Bower et al., 2004) consisted of 40 cycles with a shorter (30s) annealing time.

Amplification products were analysed on TAE agarose gels and amplicons were cloned or used directly for automated sequencing. When necessary, bands of interests were excised from agarose gels, purified with a clean-up kit (PureLink, Quick gel Extraction and PCR Purification Combo Kit, Invitrogen, Paisley, UK) and sequenced. For cloning, fresh PCR products were ligated into a plasmid vector (PCR4-TOPO, Invitrogen), which was used to transform competent *E. coli.* Transformants were selected on LB-agar plates and plasmids were purified from overnight cultures in liquid media. The presence of the inserts of the expected size was

confirmed by restriction digestion analysis with *Eco*RI enzyme. Both strands of cloned products were sequenced using M13F and M13R primers, and additional walking primers s1, sx, r1 and r2 (table 2) designed for the purpose.

2.3. Phylogenetic analysis

DNA sequences were assembled and edited using MacVector software package (Rastogi 2000). Homologous positions presenting differences between contigs were detected and verified by eye inspection of the electropherograms. Consensus sequences were used as queries to the NCBI GenBank database using Blastn (Altschul et al. 1990) to identify the closest organisms. The final consensus sequence was inserted in an alignment of 2091 sequences available (November 2010) under the category "Amoebozoa" in the SSU_ r104 database release by SILVA (Pruesse et al. 2007: http://www.arb-silva.de). The alignment was refined by eye under ARB software (Ludwig et al. 2004) according to secondary structure criteria and the dataset was then pruned to the closest relevant taxa. Unambiguously aligned positions were sampled for phylogenetic inference using different methods and substitution models with MEGA v.5.0 software (Tamura et al. 2011). Bayesian phylogenetic inference was conducted with MrBayes (Huelsenbeck & Ronquist 2001; Ronquist & Hueslsenbeck 2003), under the EPoS framework (Griebel, Brinkmeyer & Böcker 2008).

2.4. Transmission electron microscopy (TEM)

Small pieces of muscular lesions were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), and postfixed in 1% osmium tetroxide. They were embedded in Eponate 12™ resin (Ted Pella Inc., Redding, CA, USA) and polymerized at 60°C for 48 h. Semi-thin sections (1 µm) were obtained with a Leica ultracut UCT microtome (Leica Microsystems GmbH, Wetzlar, Germany). Ultra-thin sections (70 nm) were mounted on copper grids and stained with uranyl acetate (30 min) and Reynolds' lead citrate (5 min) solutions. Some sections were mounted on gold grids and stained by the Thiéry reaction for carbohydrates (Thiéry 1967) and

OTO stain for lipids (Seligman, Wasserkr & Hanker 1966). Sections were observed with a Jeol 1400 transmission electron microscope (Jeol Ltd, Tokyo, Japan) equipped with a Gatan ES500W Erlangshen CCD camera. Parasites cells and subcellular structures were measured from micrographs and dimensions are given as the mean ± S.D (n=26 cells and 16 mitosomes).

3. Results

3.1. Obtaining of the parasites

Samples of *S. senegalensis* from different farms examined, which presented macroscopical lesions compatible with the systemic parasitic granulomatous disease, were chosen (Fig. 1). Histopathological examination of these samples demonstrated the presence of small (2-4 µm diameter), inconspicuous protozoans at the periphery of granulomatous lesions ~~and abscesses~~ (Figs. 2 & 3). The anatomopathological findings were identical to those described previously in detail (Constenla and Padrós 2010).

3.2. SSU rDNA sequence and phylogeny:

Universal primers 18SA and 18SB (Medlin et al. 1988) did not amplify any PCR product selectively from infected samples. The sequences obtained by this approach corresponded to the host's SSU rDNA (data not shown). Attempts with other primers sets, suggested for X-cell organisms, were also unsuccessful as no amplification was achieved. Reactions using universal eukaryotic primers described by Bower et al. (2004) yielded an amplicon that was differentially present in samples from parasitized ~~samples.~~fish, but absent in healthy (control) sole. This band was excised from agarose gels and sequenced. The 853 bps sequence obtained matched a short segment (191 bps) of *Mastigamoeba simplex* and *E. nana* as the closest organisms in Blast searches (85-91% ID respectively). The reactions with universal subterminal eukaryotic primers MM18Sf & MM18Sr (Palenzuela, Redondo & Álvarez-Pellitero 2002) also amplified

differentially a □3 kb product from ~~parasitized~~parasitised samples, from which ~~two clean,~~additional partial sequences could be obtained ~~(one with each amplification primer). These sequences also matched fragments~~. The sequence of ~~*E. nana* and other archeamoebae as~~ the ~~highest scoring hits in Blast searches.~~negative strand concatenated well with the product obtained with Bower's primers but the direct sequence of the positive strand failed repeatedly. With these segments of the organism SSU rDNA, new internal primers were designed ~~in combination with the subterminal mm18S primers, and the resulting~~and the entire 3 kb cloned product, as well as additional PCR products, were ~~cloned and~~ sequenced ~~entirely. No variability was detected in the sequences from several~~ . A final consensus sequence of 2971 bps comprising the coding region for helixes 1-48 of the 18S rRNA was resolved. Very minor heterogeneity (<0,1%) was found between sequences (PCR amplicons and cloned products~~, and the final consensus assembly comprised 2875 bps~~) obtained from the 3 fish sampled at two locations. Nevertheless, three different haplotypes were resolved presenting variations at three sites: a double consecutive nucleotide polymorphism located at a hypervariable region within V8 (positions 2769-70), and a SNP at position 147. Two of these variants were present in a cloned product from an individual fish (farm B) whereas a single haplotype was found in two fish from farm A. The most significant matches in Blastn searches in GenBank (lowest E-values) were SSU rDNA of *E. nana, Iodamoeba spp.,* and other Archamoebae. However, the matches were limited to 2 short segments, □350bps and □280 bps showing roughly 88% and 92% pairwise identity, respectively. Comparing the entire range of the sequences alignment, the most similar sequence, from *E. nana*, only reached 60% pairwise identity.

Phylogenetic trees constructed with different inference methods and models of substitution agreed on the clustering of *E. piscium with E. nana* (Fig.4a). *Endolimax* spp. were resolved as a sister group with *Iodamoeba* spp. genotypes and this clade grouping both parasitic amoebae lineages was always robustly supported (0.98-1.00). The *Endolimax* + *Iodamoeba* clade branched as sister to a clade of free-living Arcamoebae (*Mastigamoeba spp.* and *Mastigella*

208 *commutan*), although the later excluded *Mastigamoeba simplex,* whose position was somewhat
209 unstable. The overall topology of the Archamoebae cladograms was quite robust and consistent
210 using different inference methods, but these affected the bootstrap support for the branching of
211 *M. simplex* and *E. nana* relative to their closest neighbours. In most cases *M. simplex* branched
212 off basal to *Endolimax* + *Iodamoeba* clade with moderate (0.7-0.8) support, but the use of more
213 conservative alignment masks (i.e. disregarding more alignment positions of dubious homology
214 for some sequences), lowered this value. In addition, support for the monophyly of *Endolimax*
215 spp. was lower on ML-inferred trees than on Distance or Parsimony-based analyses, and it even
216 disappeared using the most stringent alignment sampling masks with ML and bayesian inference
217 methods. In these cases, *E. nana*, *E. piscium* and *Iodamoeba* spp. were resolved either as
218 independent branches from a multifurcating node (Fig. 4b) or as a weakly supported node
219 grouping *E. nana* plus *Iodamoeba* spp., from which *E. piscium* branched off at basal position
220 (Fig.4c).
221
222 *3.3. Ultrastructural observations*
223 Parasite stages were mostly round to ovoid in shape, measuring 3.33 μm ± 0.47 x 2.78 μm ±
224 0.42 (Fig. 5). They contained one vesicular nucleus (1.18 μm ± 0.07 μm in diameter) with a
225 large, central round nucleolus (diameter 0.54 μm ± 0.04) (Figs. 5 & 6) filling roughly half of the
226 nuclei surface. Small aggregates of heterochromatin associated with the nucleolus and peripheral
227 chromatin were usually observed (Fig. 6). A double-layered nuclear membrane, similar to that of
228 the plasma membrane, was noticed, which presented conspicuous pores (Fig. 6).
229 The parasites contained small glycogen granules, often forming aggregates (fig. 7), and a
230 variety of intracytoplasmatic structures such as some single-membrane bound vesicles, putative
231 digestive vacuoles containing particulate material, myelinic figures, or products of lysosomal
232 action (Fig. 5). No mitochondria were observed although double membrane-bounded, electron-
233 dense organelles with no apparent cristae were frequently observed within the cytoplasm (Fig.

8). These organelles were interpreted as mitosomes and they were present in variable numbers per cell, normally one to three on 70 nm-thick TEM sections. They were rounded to ovoid, 157.73 nm ± 46.77 x 107.71 nm ± 20.3. Elongated vesicles resembling dictyosomes of Golgi apparatus cisternae were sometimes observed (Fig. 9). Structures appearing as long rods or whirls of a bilayered membrane were commonly observed extending across the parasites or, sometimes, beside the nuclear membrane (Figs. 5 & 6). Vacuole-like structures surrounded by an electron-lucent, wide concentric aureole were sometimes observed within the parasites cytoplasm (Fig. 10). The parasites mostly presented a regular smooth surface but amoeboid stages presenting a more irregular shape with invaginations/evaginations could also be identified (Fig.7). Filopodia-like structures were detected in the surface of some ~~throphozoites~~trophozoites (Fig.11) and they were commonly observed sectioned transversally around the parasites (Figs. 7 & 10). No evident cysts could be detected in any of the samples from different tissues examined.

3.4. Description of the ~~Species~~

~~*Type*~~ *species~~:~~*

Endolimax piscium n. sp. (Amoebozoa)

Type host: *Solea senegalensis* Kaup, 1858

Locality: the parasite was detected in cultured Senegalese sole from fish farms located at different sites in NW Spain (Atlantic Ocean).

Location in the host: Systemic. Parasites were ~~localized~~localised as a compact layer at the periphery of granulomatous lesions in different tissues: muscle, liver, kidney, heart, intestine and ovary. Parasites were also found within the intestine epithelium.

Material deposited: Histological sections (sole tissues containing conspicuous granulomatous lesions ~~and abscesses~~ surrounded by *E. piscium* cells) have been deposited at the Museo Nacional de Ciencias Naturales (MNCN-CSIC), Madrid, Spain with accession numbers: MNCN 33.04/1 (Holotype) and MNCN 33.04/2 – MNCN 33.04/3 (paratypes). Partial SSU rDNA

sequences have been deposited in Genbank: *E. piscium* Clone MALEN3 (GenBank accession no. JX101953), *E. piscium* Clone PM1 (GenBank accession no. JX101944), and *E. piscium* Clone PM2 (GenBank accession no. JX101955).

4. Discussion

The combined results of the ultrastructural and genetic study of the parasite causing systemic granulomatous disease in sole (*E. piscium*) allowed its unambiguous identification as an amitochondriate Archamoeba related to *E. nana* and *Iodamoeba spp*., enteric commensals and parasitic species in humans and other mammals. The SSU rDNA sequence fragment obtained was ~~2875~~2,971 bps, and its total length, inferred from the alignments with other mastigamoebids, is estimated > 3,~~120~~107 bps. This is the second-longest known amoebozoan SSU rDNA, after that of the aberrant amoeboflagelate *Pelomyxa palustris* (~~3502~~3,502 bps) (Milyutina et al. 2001). Even though amoebozoans are characterised by SSU rDNAs longer than most eukaryotes and archamoebids represent an extreme to this tendence (Nikolaev et al. 2006), *E. piscium* sequence is 20% longer than its most similar, *E. nana*. The overall topology of the Archamoebae cladograms is quite robust and consistent using different phylogenetic inference methods, placing *Iodamoebae* and *Endolimax* spp. closer to Mastigamoebae than to Entamoebae and thus supporting different events of adaption to parasitic lifestyles or, alternatively, a re-adaption of mastigamoebae to free-living niches. This topology is congruous with previous molecular phylogeny studies of related amoebozoans (Cavalier-Smith, Chao & Oates 2004; Nikolaev et al. 2006; Tekle et al. 2008; Lahr et al. 2011; Stensvold, Lebbad M. & Clark 2012). Although *E. piscium* grouped together with *E. nana*, there is much variation between their rDNA sequences, with only 60% pairwise identity along the alignment of the available common segment. Interestingly, the recent molecular ~~characterization~~characterisation of *Iodamoeba* spp. showed two different rDNA lineages with 31% divergence among them and further substantial diversity within each lineage (Stensvold, Lebbad & Clark 2012). These values seem strikingly

heterogeneous for congeneric species, although amoebozoans are known examples of high rDNA heterogeneity and evolution rate. Thus, some instability on the branching of *E. piscium* relative to *E. nana* and *Iodamoeba* spp. was noticed that was particularly patent when bayesian or maximum likelihood inference methods and, specifically, when stringent alignment sampling masks were used. A very similar instability was reported to affect the placement and monophyly of *Iodamoeba* genotypes with these reconstruction methods, even though they are firmly resolved by distance or MP-based methods and despite the fact that multiple synapomorphic motifs are apparent by eye inspection of the alignments, as previously pointed out (Stensvold, Lebbad & Clark 2012). Since the placement and relative support for *E. piscium* branching in phylogenetic analyses is influenced by the inference method, we conservatively describe the new archamoeba in the genus *Endolimax* but the significant genetic distance with the closest *Endolimax* and I*odamoeba* genotypes, as well as the putative discovery of additional piscine genotypes, could support a higher-level taxon corresponding to a distinct lineage of parasitic archamoebae.

Cavalier-Smith (1998) in his "Revised six kingdom system of life" grouped entamoebids, *Endolimax*, and *Mastigamoeba spp.* in the subphylum Conosa (infraphylum Archamoebae), in spite of their great phenotypic diversity. Members of these lineages share features such as absence of mitochondria, and an apparently simplified intracellular ~~organization~~organisation (Martinez-Palomo 1986; Simpson et al. 1997). Whereas mastigamoebae are generally free-living and have flagellated cells, known *Endolimax* and *Entamoeba* are commensals or parasites with amoeboid trophozoites displaying locomotion by pseudopodia (Silberman et al. 1999). *E. nana* and *Iodamoeba* develop cyst stages, which are also present in most *Entamoeba* spp. Even though these archamoebae are regarded as marginally pathogenic to vertebrates, the role of different *Entamoeba* spp, in gastrointestinal disorders of humans is well known (Jetter et al. 1997; Heredia, Fonseca & López 2012). In 90% of cases these amoebic infections are asymptomatic and self-limited (Haque et al. 2003), with trophozoites remaining in intestine lumen as

commensals and some encysting for the perpetuation of the cycle through fecal-oral spread (Mortimer and Chadee 2010). However, *E. histolytica* is able to play a pathogenic phenotype disrupting the mucosal barrier, entering the portal circulation and dispersing to soft organs, generally producing abscesses (Espinosa-Cantellano & Martinez-Palomo 2000; Mortimer & Chadee 2010). *E. nana* and *Iodamoeba* spp. have also been ~~characterized~~characterised as occasional causes of gastrointestinal disorders (Stauffer et al. 1974) cutaneous processes (Veraldi, Schianchi-Veraldi & Gasparini 1991), reumathoid arthritis (Burnstein & Liakos 1983) and brain granuloma (Arava et al. 2010). *Endolimax piscium* is usually found as causative agent of systemic granulomas ~~and abscesses~~ in Senegalese sole viscera, but it can also be detected in the intestinal epithelium of asymptomatic fish (Constenla and Padrós 2011). However, even in these cases, the amoebae appear always in intraepithelial locations and occasionally with some degree of host response associated (author's unpublished data). Although the whole transmission and developmental cycle of *E. piscium* is yet unknown, the data available so far suggests a primary parasitic, rather than commensal ~~behavior~~behaviour in this piscine host. Contaminated water sources like swimming pools, freshwater ponds, lakes, or drinking supplies are recognized sources of human and animal amoebiases, but the occurrence of amoebae in the fish that inhabit aquatic environments has been neglected for a long period of time (Dyková and Lom 2004).

Species of *Endolimax* have been classically described as intestinal amoebas with eruptive pseudopodia, lacking cilia, centrioles, contractile vacuoles or intracellular crystals (Cavalier-Smith, Chao & Oates 2004). They have been reported from multiple vertebrate hosts (Wenyon and O'Connor 1917; Brug 1920; Chian 1925; McFall 1926; Hegner 1926; Lucas 1927; Gutierrez–Ballesteros & Wenrich 1950), and even from invertebrates (e.g., Kirby 1927). However, a single isolate of *E. nana* from monkey was till now the only species in this genus with some genetic data available (Silberman et al. 1999). Furthermore, no species of *Endolimax* has been described from fish to date. Comparison of *E. piscium* with species from these dated and incomplete descriptions, on morphological grounds, is quite difficult due to the lack of

characters. In any case, the ~~throphozoites~~trophozoites of *Endolimax* spp. have been reported to measure between 5 µm to 14 µm depending on the species, and to contain one vesicular nucleus (1.5 µm - 6 µm) with a large karyosome. Thus, *E. piscium* appears to be the smallest species in the genus, with ~~throphozoites~~trophozoites smaller than 5 µm. Our ultrastructural analysis of *E. piscium* revealed some similarities with *Entamoeba* spp. stages, such as the absence of mitochondria, Golgi apparatus and rough endoplasmic reticulum (Ludvik & Shipstone 1970; Martinez-Palomo 1986). However, structures resembling isolated dictyosomes were observed in some *E. piscium* cells. Ultrastructural data of *E. nana* or *Iodamoeba* are quite scarce (Zaman, Howe & Ng 1998; Zaman et al. 2000). In *E. nana* cysts, Zaman et al. (2000) pointed out the existence of tubular structures made up of a double row of ribosome-like particles with a single membrane running between them. Since these had not been described from any other intestinal amoeba, they were suggested to be species-specific characters. Our material from Senegalese sole did not include any cyst, but we observed similar, conspicuous tubular rods within some trophozoites. However, in *E. piscium* they appeared as a continuous double membranous layer, not associated with ribosomes and resembling nuclear membrane. Since they were not observed in all the stages and they frequently extended across the entire parasite, they could be structures related with the cell cycle or proliferation.

The lack of mitochondria in *E. piscium* is consistent with its phylogenetic affinities. In Archamoebae, this absence is a derived condition, either by loss of mitochondria or by conversion of these organelles in mitosomes (Tovar, Fischer & Clark 1999) as adaptation to anaerobic environments (Cavalier-Smith 2002; Cavalier-Smith, Chao & Oates 2004). Mitosomes are mitochondrion-related organelles found in a range of unicellular eukaryotic organisms that inhabit oxygen-poor environments, usually parasites invading digestive tract and other tissues, and including Archamoebae (Cavalier-Smith 1991; Tachezy & Smíd 2008). Mitosomes in *Entamoeba histolytica* and in *Mastigamoeba balamuthi* are described as ovoid to elongate double-membrane organelles, with electron-dense material (Tachezy & Smíd 2008). These bear

a strong resemblance to the structures found in *E. piscium* cells, with two tightly opposing membranes without intermembrane space. The size and number of mitosomes in different organisms is variable (Tachezy & Smíd 2008). Based on TEM studies, they measure about 0.5 µm to 2.0 µm in *E. hystolytica* (Tovar, Fischer & Clark 1999; Ghosh et al. 2000), but more precise studies by confocal microscopy revealed estimated sizes of 0.5 µm (León-Avila and Tovar, 2004), and of 0.1 to 0.2 µm in *M. balamuthi* (Gill et al. 2007). In TEM sections, *E. piscium* mitosome-like organelles appear smaller than those ~~decribed~~described in *Entamoeba*, which can be due to the minute size of this parasite or simply reflect the methodological inaccuracy. It must be stressed that even though the morphological evidence strongly suggests the presence of mitosomes in *E. piscium*, further biochemical and genetic studies would be required to confirm the nature of these double-membrane organelles.

The vacuole-like structures surrounded by a bright concentric aureole are very similar to those described in the ~~parasitic~~parasite causing systemic granulomatosis affecting goldfish (Paperna & Kim 1996; Dyková et al. 1996) and interpreted as endocytotic channels. Unfortunately, no invagination of the organism's body wall was observed in this work to verify this possibility, since *E. piscium* cells appeared rather smooth and only presented some slender filopodia-like structures in the sectioned material. From these reports, the parasite from goldfish seems quite similar to this new *E. piscium* from Senegalese sole. Further ~~characterization~~characterisation of that parasite, and eventually of other piscine parasitic archamoebae, might contribute to clarify their relationships with *E. nana* and to interpret the large genetic distance observed between *Endolimax* species.

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571

Table 1: Species of the genus *Endolimax* described to date and their respective hosts. (*) The genotype available in Genbank was isolated from mangabey (*Cercocebus albigena*), but assimilated to *Endolimax nana* (Clark and Diamond 1997; Silberman et al. 1999) and deposited as such in the American Type Culture Collection (ATCC #50293).

Species	**Host**	**References**
E. nana	Human (*)	Weynon and O'Connor 1917
E. kueneni	Monkey	Brug 1920
E. reynoldsi	Common swift	McFall 1926
E. caviae	Guinea pig	Hegner 1926
E. janisae	Domestic fowl	Hegner 1926
E. termitis	Termite	Kirby 1927
E. blattae	Cockroaches	Lucas 1927
E. gildemeisteri	Human	Momcilo 1936
E. leptocoridis	Hemiptera	Kay 1940
E. suggrandis	Termite	Henderson 1941
E. clevelandi	Turtle	Gutierrez-Ballesteros and Wenrich 1950
E. tayassusi	Pig	Mello et al. 1951

Table 2: List of primers used for primary amplification and sequencing of *Endolimax piscium* SSU rDNA gene. The positions of the binding region (relative to the sense strand of *E. piscium*) are noted when available. Primers flagged with an asterisk are reverse primers. References: [1] Medlin et al. 1988; [2] Palenzuela, Redondo & Álvarez-Pellitero 2002; [3] Bower et al. 2004; [4] Freeman 2009.

Primer	**Sequence (5'- 3')**	**References**	**Target**
18A	CCGAATTCGTCGACAACCTG GTTGATGCTG	[1]	(-17) → 13
18B(*)	CCCGGGATCCAAGCTTGATC CTTCTGCAGGTTCACCTAC	[1]	n/a
MM18Sr(*)	CGGTACTAGCGACGGGCG	[2]	2954 ← 2971
MM18Sf	CTGGTTGATTCTGCCAGTGG TC	[2]	1 → 22
18S-EUK581-F	GTGCCAGCAGCCGCG	[3]	923 → 938
18S-EUK1134-R(*)	TTTAAGTTTCAGCCTTGCG	[3]	2095 ← 2113
280f	ATCCATCAGCCATCGACGC	[4]	n/a
1300r(*)	TCGTCCGATCCTCAGTCGG	[4]	n/a
sx	ACAGAGCCAAGGTTCAGTG A	This study	1477 → 1496
s1	GCCCATTCTAACAGACACAA C	This study	273 → 293
r1(*)	GAAGAGGTTGTTGCCGAGA AG	This study	1629 ← 1649
r2(*)	TCCCACACTTGTTCGTATG	This study	1168 ← 1186

Figure Legends

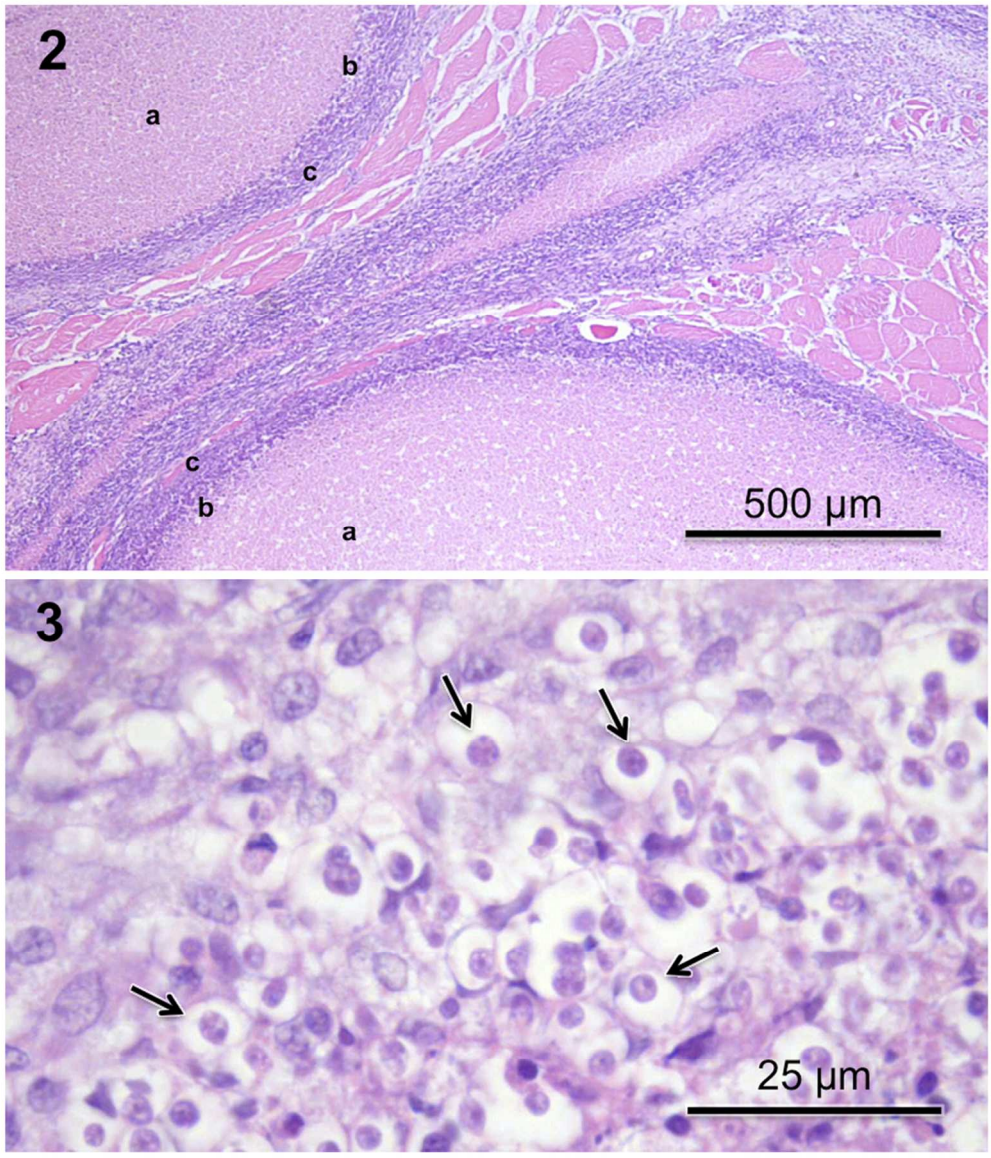
Fig. 1: Senegalese sole clinically infected by *Endolimax piscium*, with nodules in muscle and conspicuous lumps on the skin surface.

Figs. 2- 3: Paraffin-embedded histological sections of Senegalese sole skeletal muscle showing the typical inflammatory reaction to *Endolimax piscium*. Fig. 2: Granulomatous inflammatory reactions in which different layers are differenced: a necrotic core (a); a peripheral band containing macrophages and parasites (b); and an external layer with inflammatory cells and fibroblasts (c). Fig. 3: Throphozoites of *E. piscium* are observed as very small, round uninucleate cells (arrows), mostly within macrophages in the periphery of a granulomatous lesion. Stain: H&E.

Fig. 4: SSU rDNA gene-based phylogeny of *Endolimax piscium* and its closest amoebozoan relatives. The topology was inferred using three alignment sampling masks disregarding variable numbers of positions of dubious homology, and multiple reconstruction methods and models of nucleotide substitution. (a) “Relaxed” dataset (1,493 sites). Numbers at nodes represent bootstrap values after 500 resamplings, determined by Distance methods (Tajima-Nei model with a gamma distribution parameter G=0.36, determined from the dataset) / Maximum Parsimony (with Close-Neighbor Interchange on Random Trees) / and Maximum Likelihood (GTR Model with 4 gamma categories). (b) Very stringent alignment sampling mask (1,350 sites) using the Maximum Likelihood method with the General Time-Reversible (GTR) model and 4 gamma categories. (c) Stringent alignment sampling mask (1,410 sites): numbers at nodes represent posterior probabilities resulting from a Bayesian analysis using the GTR model. Only the relevant subtrees are represented in (b) and (c).

Figs. 5- 11: Transmission electron micrographs of *Endolimax piscium* trophozoites. Fig 5: Amoeba cell displaying a nucleus (N) with a single nucleolus (n), myelinic figures (*), whirls of bilayered single membrane extending across the parasites (arrows) and mitosomes (arrowheads); Fig. 6: Detail of the nucleus with a large central karyosome. Note the presence of nuclear pores (arrows) and peripheral chromatin (*); Fig. 7: Presence of carbohydrates as small glycogen granules, often forming aggregates over the whole surface of the trophozoite stage, demonstrated by the Thiéry stain; Fig. 8: Detail of various mitosomes: note the electron-dense, homogeneous matrix without cristae and the double membrane around them; Fig. 9: Elongated vesicles resembling dictyosomes close to the nucleus; Fig. 10: Vacuole-like structure within the parasite cytoplasm (*), containing a withered matrix and an electron-lucent concentric aureole. Note several filopodia seccioned transversally at the periphery of the amoeba (arrows); Fig. 11: Detail of one of these filopodia-like structures emerging at the surface of a trophozoite.

66x14mm (300 x 300 DPI)



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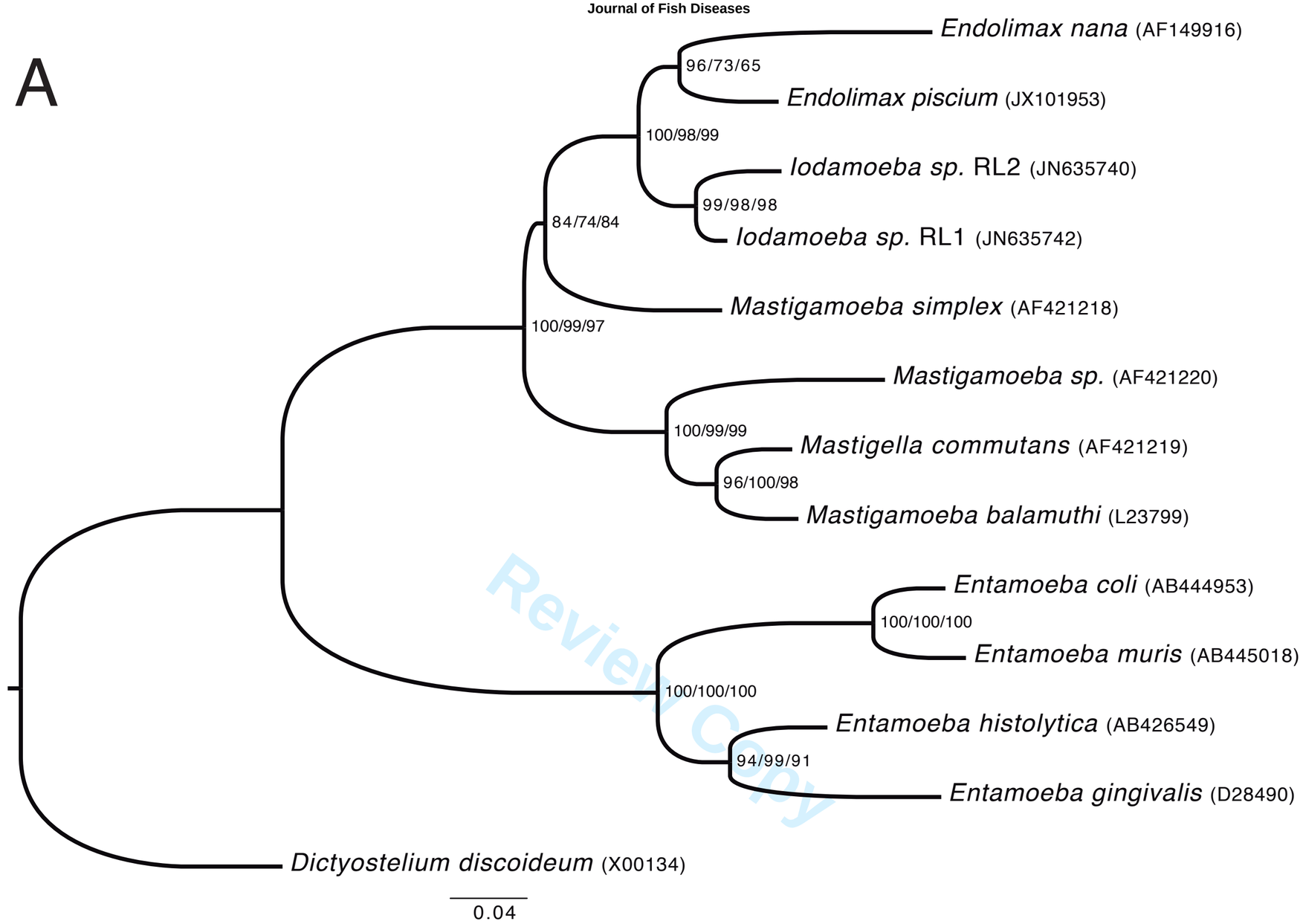
A



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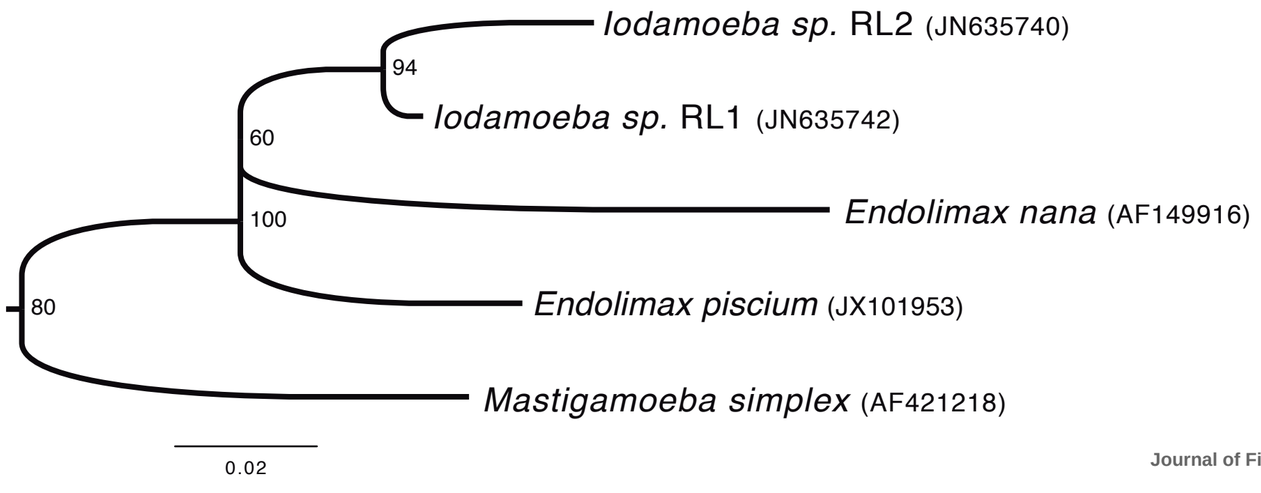

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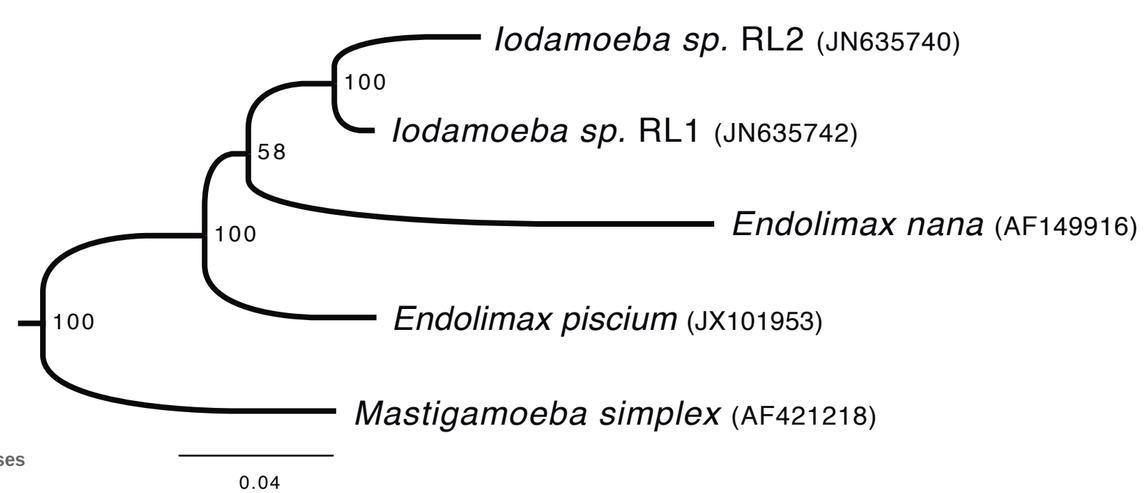

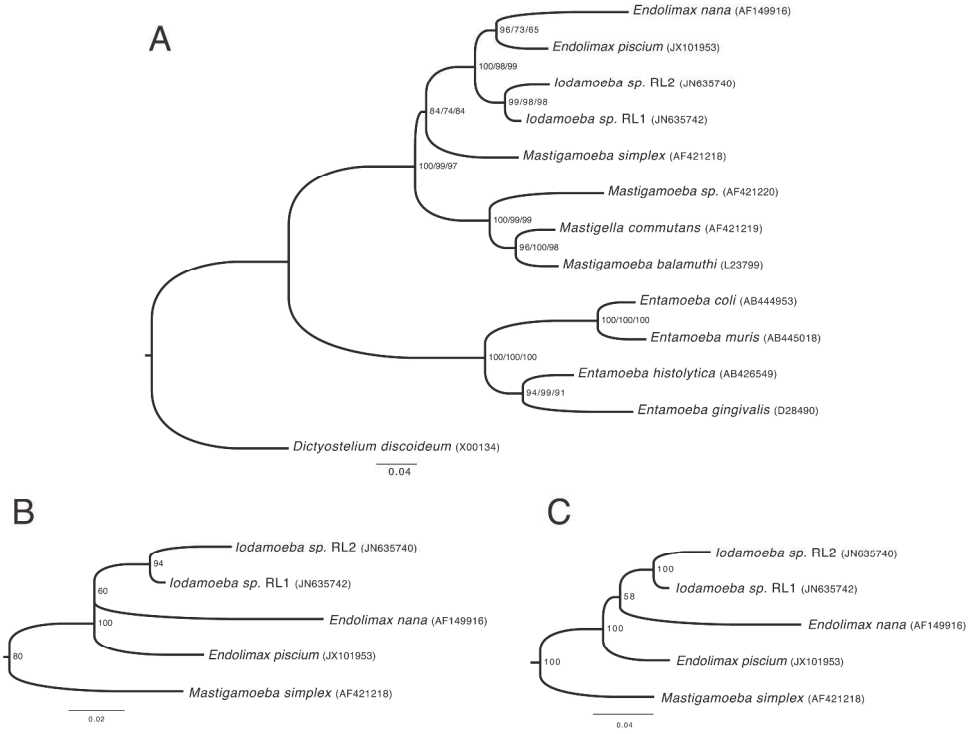

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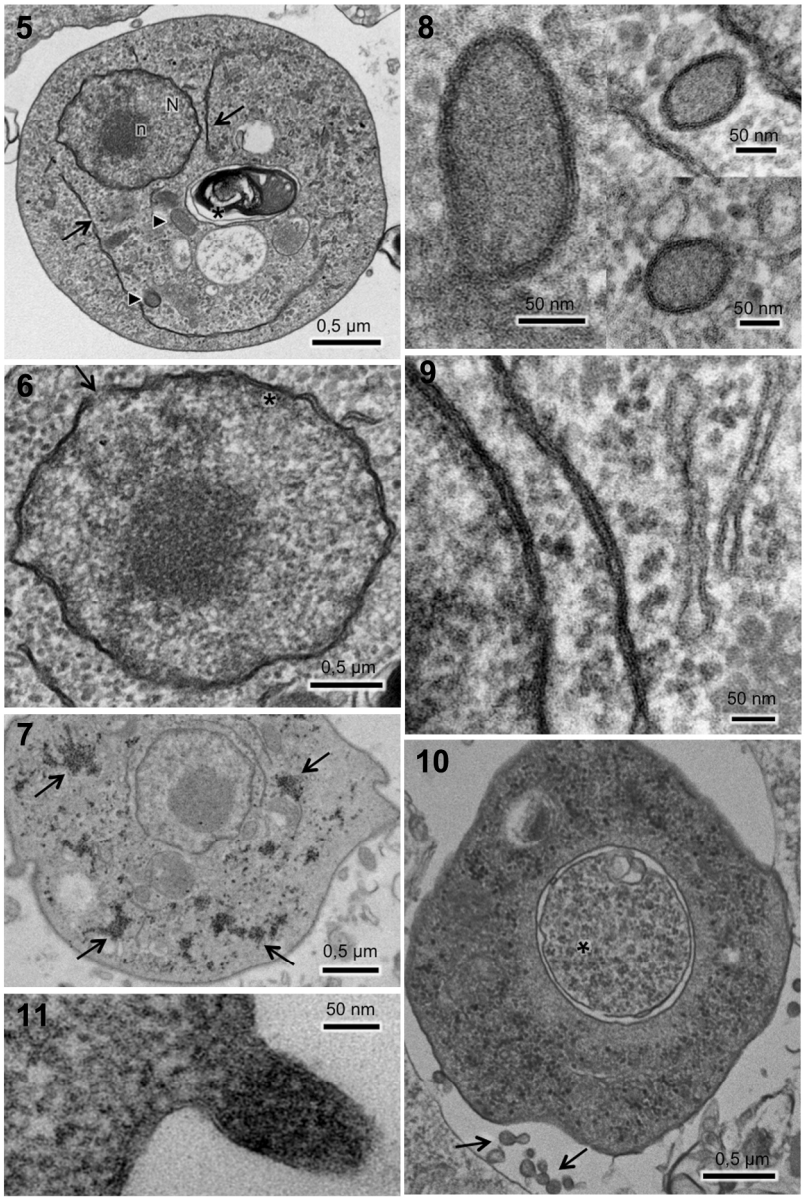

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