1 *Txikispora philomaios* n. sp. n.g. & Parasitism in Filasterea

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3 *Txikispora philomaios* n. sp., n. g., a Micro-Eukaryotic Pathogen of 4 Amphipods, Reveals Parasitism and Hidden Diversity in Class Filasterea

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2 ## ABSTRACT

3 This study provides a morphological, ultrastructural, and phylogenetic characterization of a
4 novel micro-eukaryotic parasite (2.3-2.6 µm) infecting genera *Echinogammarus* and *Orchestia*.
5 Longitudinal studies across two years revealed that infection prevalence peaked in late April and
6 May, reaching 64% in *Echinogammarus* sp. and 15% in *Orchestia* sp., but was seldom detected
7 during the rest of the year. The parasite infected predominantly haemolymph, connective tissue,
8 tegument, and gonad, although hepatopancreas and nervous tissue were affected in heavier
9 infections, eliciting melanization and granuloma formation. Cell division occurred inside walled
10 parasitic cysts, often within host haemocytes, resulting in haemolymph congestion. Small subunit
11 (18S) rRNA gene phylogenies including related environmental sequences placed the novel
12 parasite as a highly divergent lineage within Class Filasterea, which together with
13 Choanoflagellatea represent the closest protistan relatives of Metazoa. We describe the new
14 parasite as *Txikispora philomaios* n. sp. n. g., the first confirmed parasitic filasterean lineage,
15 which otherwise comprises four free-living flagellates and a rarely observed endosymbiont of
16 snails. Lineage-specific PCR probing of other hosts and surrounding environments only detected
17 *T. philomaios* in the platyhelminth *Procerodes* sp. We expand the known diversity of Filasterea
18 by targeted searches of metagenomic datasets, resulting in 13 previously unknown lineages from
19 environmental samples.

20

21 **Keywords**

22 *Echinogammarus*; *Orchestia*; Holozoa; Histopathology, Intracellular parasite; Haemolymph
23 congestion; environmental DNA.

24

25 ## INTRODUCTION

26 The Class Filasterea Cavalier-Smith 2008 currently comprises five species (Shalchian-Tabrizi et
27 al. 2008; Hehenberger et al. 2017; Tikhonenkov et al. 2020a). Initially classified as a nucleariid,
28 *Capsaspora owczarzaki* was the first filasterean to be described (Stibbs et al. 1979; Owczarzak et
29 al. 1980; Amaral-Zettler et al. 2001; Hertel et al. 2002; Ruiz-Trillo et al. 2004). This filopodial
30 amoeba is a facultative endosymbiont (Harcet et al. 2016) isolated from explanted pericardial
31 sacs of laboratory-grown *Biomphalaria* sp. snails (Stibbs et al. 1979; Morgan et al. 2002), which
32 remains elusive in environmental samplings (Hertel et al. 2004; del Campo and Ruiz-Trillo 2013;
33 Shanan et al. 2015; Ferrer-Bonet and Ruiz-Trillo 2017; Arroyo et al. 2018). In contrast, the other
34 four species (*Ministeria vibrans*, *Ministeria marisola*, *Pigoraptor chileana*, and *Pigoraptor*
35 *vietnamica*) are free-living flagellates, sampled from marine and freshwater ecosystems
36 (Patterson et al. 1993; Tong et al. 1997; Hehenberger et al. 2017; Mylnikov et al. 2019).

37 Filasterea are also of interest (Ruiz-Trillo et al. 2008; Suga et al. 2013; Torruella et al.
38 2015; Hehenberger et al. 2017), as they branch phylogenetically close to the metazoan radiation,
39 being sister to Choanozoa (the Metazoa + Choanoflagellatea clade) (Shalchian-Tabrizi et al.
40 2008; Paps et al. 2013; Torruella et al. 2015; López-Escardó et al. 2019). Morphological (James-
41 Clark 1868), ultrastructural (Laval 1971; Hibberd 1975), and phylogenetic inference (Cavalier-

Smith 1993; Wainright et al. 1993; Snell et al. 2001; King 2004; Ruiz-Trillo et al. 2006) suggested a common evolutionary origin for Metazoa and Choanoflagellatea, which was later confirmed by phylogenomic analyses (King et al. 2005; Steenkamp et al. 2006; Ruiz-Trillo et al. 2008). Phylogenomic studies also revealed the relationship between genera *Capsaspora* and *Ministeria* and their sister-clade relationship to Choanozoa; shaping a novel class; Filasterea (Shalchian-Tabrizi et al. 2008 Torruella et al. 2012; Hehenberger et al. 2017). Since then, the genomes and transcriptomes of filasterean species have been thoroughly investigated to comprehend the evolutionary processes that drove the inception of animal multicellularity (Suga et al. 2013; Torruella et al. 2015; Sebé-Pedrós et al. 2017; Hehenberger et al. 2017; Grau-Bove et al. 2017).

For almost 40 years, our knowledge of filasterean ultrastructure came from a single paper (Owczarzak et al. 1980), describing *C. owczarzaki*. Recently, the ultrastructures of *M. vibrans* and *Pigoraptor* sp. have been investigated (Torruella et al. 2015; Mylnikov et al. 2019; Tikhonenkov et al. 2020a). Regarding the ecology and global distribution of the species within the Class, existing information is limited to the sampling locations of type species, and some feeding observations under culture conditions (Stibbs et al. 1979; Tong 1997; Hehenberger et al. 2017; Mylnikov et al. 2019; Tikhonenkov et al. 2020a). Given the low number of species described the influence of filastereans in the food web has been thought to be insignificant, at least in comparison to much bigger protistan clades, or notorious pathogenic taxa. However, recent environmental studies have suggested the relationship between an abundant clade of marine opisthokonts (MAOP-1) and Filasterea (del Campo et al. 2015; Hehenberger et al. 2017; Heger et al. 2018), challenging the idea of a small and scarce group. Excluding the facultative endosymbiont *C. owczarzaki,* all filastereans and choanoflagellates are free-living organisms, contrasting with the parasitic lifestyle of ichthyosporeans (mesomycetozoeans) (Mendoza et al. 2002; Glockling et al. 2013).

During a histopathological survey of invertebrates inhabiting the intertidal zone (Weymouth, UK), an unidentified protist was observed parasitizing two of the most common species of amphipods (*Echinogammarus* sp. and *Orchestia* sp.). Analysis by light microscopy of the structure and tissue tropism of the parasite did not allow a clear assignment of the organism to any of the pathogen groups commonly observed infecting amphipods or crustaceans. Similarly, examination of the ultrastructure by transmission electron microscopy (TEM), did not show any distinctive organelle suggesting taxonomic affiliation. Preliminary phylogenetic analyses of the 18S SSU rRNA gene (hereafter “18S”) strongly indicated that this lineage was a highly divergent novel genus within Holozoa. However, it did not consistently branch with any of the four established unicellular clades (Choanoflagellatea, Filasterea, Corallochytrea/Pluriformea, and Ichthyosporea/ Mesomycetozoea). When a greater diversity of environmental holozoan sequences were included in the analyses the parasite branched with Filasterea as the earliest diverging branch. This study comprises a complete histopathological, ultrastructural, and phylogenetic analysis based on the complete 18S of the novel parasite, described as *Txikispora philomaios* n. sp. n. g. We also present data on its prevalence, host range, biological cycle, and potential transmission routes. Additionally, we demonstrate novel filasterean diversity on the basis of sequences mined from environmental sequencing datasets. The description of *T. philomaios* and its parasitic lifestyle adds to a growing understanding of filasterean diversity, ecology, and lifestyle traits.

MATERIALS AND METHODS

Sample collection

Amphipods belonging to genera *Orchestia*, *Echinogammarus*, *Gammarus*, and *Melita* were collected in the Tamar estuary (Torpoint, Cornwall), Camel estuary (Padstow, Cornwall), Dart estuary (Dittisham, Devon) and Newton's Cove (Weymouth, Dorset, UK) between 2016 and 2018 (Table 1; Fig. 1). Individuals *of Echinogammarus* sp. and *Orchestia* sp. were sampled in the upper part of the intertidal zone, behind rocks and algae. Individuals of *Gammarus* sp. and *Melita* sp. were sampled in the lower part of the intertidal behind small stones and submerged algae. In addition to amphipods, other very abundant invertebrates sharing the same habitat in the upper part of the intertidal were also collected in Newton's Cove from May 2019 to September 2019 (Table 2). These organisms include *Capitella* sp. (Polychaeta, Annelida), *Procerodes* sp. (Turbellaria, Platyhelminthes) and harpacticoid copepods of the Ameiridae family (Crustacea, Arthropoda), all individually selected using a stereomicroscope.

Histology and transmission electron microscopy

Amphipods were kept alive in bottles containing moist algae and dissected within 3-4 hours post collection. The head and two first thoracic segments were fixed in 100% molecular grade ethanol. The following proximate segments of the thorax of about 2 mm in size, were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for TEM. The remainder of the body, which included the last 4-5 segments of the pereon and the pleon, were fixed in Davidson's seawater fixative (Hopwood 1969) for 24 hours, and then transferred to 70% ethanol. Fresh smears were produced by cutting the distal part of the antennae or uropods before fixation; after a preliminary analysis, slides were left to air-dry. Once dry, slides were stained for 1 minute with Toluidine Blue (1%) and washed with distilled water before being cover-slipped.

For histology, Davidson's fixed samples were processed from ethanol to wax in a vacuum infiltration processor using established laboratory protocols (Stentiford et al. 2013). Tissue sections (2.5-3 µm) were cut on a Finnese® microtome, left to dry for 24 hours, mounted on VWR™ microscope slides, and stained with H&E (Bancroft and Cook 1994). Cover-slipped sections were examined for general histopathology by light microscopy (Nikon Eclipse E800). Digital images and measurements were obtained using the Lucia™ Screen Measurement software system (Nikon, UK).

Specimens observed by light microscopy to be infected with *T. philomaios* (one *Echinogammarus* sp. and one *Orchestia* sp.), were selected for TEM analysis. Glutaraldehyde-fixed samples were rinsed in 0.1 M sodium cacodylate buffer (pH 7.4) and post-fixed for 1 hour in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Samples were washed in three changes of 0.1 M sodium cacodylate buffer before dehydration through a graded acetone series. Then, they were embedded in epoxy resin 812 (Agar Scientific pre-Mix Kit 812, Agar Scientific, UK) and polymerised overnight at 60 °C. Semi-thin sections (1 µm) were stained with 1% Toluidine Blue and analysed by light microscope, to identify target areas containing sufficient parasites. Ultrathin sections (70-90 nm) were framed on uncoated copper grids and stained with uranyl acetate and Reynold's lead citrate (Reynolds 1963). Grids were examined using a JEOL JEM 1400 transmission electron microscope and digital images captured using a GATAN Erlangshen ES500W camera and Gatan Digital Micrograph™ software.

1 **Cell cultures**

2 Concomitant to the production of fresh smears described in the above section, the antenna of
3 infected amphipods was suspended to "bleed" over the following culturing media: Marine Broth
4 (MB), Brain-Heart Infusion Broth (BHI), and modified PYNFH, habitually used for filastereans
5 (Hertel et al. 2002, Torruella et al. 2015). All media contained 10 µg/ ml of gentamycin,
6 streptomycin, and penicillin. The process was conducted in a local exhaust ventilation system
7 (post UV for 15 mins). The media, containing a drop of the haemolymph of infected amphipods,
8 were incubated at 22-23 ºC (Tikhonenkov et al, 2020a, Parra-Acero et al. 2020)

9 **DNA extraction, polymerase chain reaction, cloning and sequencing**

10 The head and anterior part of the thorax (preserved in 100% molecular grade ethanol) of 23
11 amphipods found to be infected via histology (pereon, pleon, and uropods fixed in Davidson's
12 seawater fixative) were selected for DNA extraction. Infected tissues were disrupted and
13 digested overnight (12 hours) using Fast Prep® Lysing Matrix tubes containing 0.2 mg (6 U)
14 Proteinase K (Sigma-Aldrich®) diluted 1/40 in Lifton's Buffer (100 mM EDTA, 25 mM Tris-
15 HCl, 1% (v/v) SDS, pH 7.6). Next, a 1/10 (v/v) of 5 M potassium acetate was added to each of
16 the 23 tubes containing digested sample, Proteinase K, and Lifton's buffer. The solution was
17 mixed and incubated on ice for 1 hour. From here DNA was extracted using the phenol-
18 chloroform method described in (Sambrook et al. 1989). The resulting pellet was diluted in 50 µl
19 of molecular grade water and DNA concentration quantified using NanoDrop™ (Thermo Fisher
20 Scientific). *T. philomaios*' 18S was amplified by PCR using primers targeting different
21 overlapping regions (Table 3), and the following PCR conditions: A total reaction volume of 20
22 µl included 10 µl molecular water, 5 µL GoTaq® Flexi Buffer, 2.0 mM $MgCl_2$, 0.2 mM of each
23 deoxyribonucleotide, 40 pM of each primer, 0.5 U GoTaq® Polymerase (Promega), and 200 ng
24 of the extracted DNA. The PCR cycling parameters for primer pair (SA1nF + 631R; Bass et al.
25 2012, and in-house design respectively; Table 3) included denaturation for 5 minutes at 95 °C,
26 followed by 35 cycles alternating: 95 °C (30 s), 57 °C (30 s), and 72 °C (90 s); before a final
27 extension and incubation of the amplicons at 72 °C for 10 minutes. Same conditions were used
28 for primer combinations (S47-152F + S47-617R and S47-472F + S47-1027R; Table 3) except
29 for the annealing temperature which was 67 °C (30 s). Amplicons were cleaned using 20%
30 polyethylene glycol 8000 (Sigma-Aldrich®) followed by ethanol precipitation, and a-tailed to
31 improve cloning efficiency before another PEG 8000 clean. Clone libraries were created using
32 Strategene's cloning kit (Agilent Technologies, Santa Clara, CA, USA) as per manufacturer's
33 protocol. Bacterial colonies were picked from LB/ampicillin plates and suspended in 20 µl PCR
34 water and lysed at 95 °C for 5 minutes. Eight clones from each library were amplified with 1 µl
35 lysed culture DNA and M13F/M13R primers (Invitrogen™ – Thermo Fisher Scientific) using
36 the mastermix concentrations described previously, and the manufacturers program. PCR
37 products were bead-cleaned and a total volume of 15 µl was mixed with 2 µl of the M13F
38 forward primer, before being single-read Sanger sequenced (Eurofins®Genomics).

39 ***In-situ* hybridization**

40 Tissue sections (4 µm) from the individuals of interest were recovered from the 42 °C water bath
41 (without Sta-On tissue-adhesive) using Polysine® Slides (Thermo Fisher Scientific) and left to
42 dry for 24 hours. The forward S47-152F and reverse S47-617R primers were used to amplify
43 part of the 18S extracted from an infected individual of *Orchestia* sp. DNA amplification and
44 purification were carried out using the same concentrations and conditions explained in previous

section. Purified DNA was digoxigenin (DIG)-labelled using same primers and PCR conditions above, but changing the concentration of reagents, say: 10 µl 5X Colorless GoTaq® Reaction Buffer, 5 µl $MgCl_2$ solution (Promega), 5 µl of PCR DIG labelling mix (Roche), 3 µl template DNA, 1 µl of forward and reverse primers, 0.5 µl of GoTaq Polymerase, and 24.5 µl molecular grade water. The control slide was produced amplifying the same 18S region using non-labelled standard DNTPs. Products generated via PCR were purified as described in previous section, total DNA quantified (NanoDrop 1000 Spectrophotometer® Thermo Scientific) and diluted to 1 ng/µl for a total volume of 50 µl.

Dry tissue sections were dewaxed and rehydrated: Clearene for 5 minutes (2 times), followed by 100% IDA (industrial denatured alcohol) for 5 minutes and 70% IDA another 5 minutes. Slides were rinsed in 0.1M TRIS buffer (0.1 M TRIS base, 0.15 M NaCl, adjust the pH to 7.5 adding HCl) and placed in a humid chamber. Each slide was covered with 300 µl of 0.3% Triton-X diluted in 0.1M TRIS buffer (pH 7.5) for 20 minutes and rinsed with 0.1M TRIS buffer (pH 7.5). Tissue was covered with Proteinase K diluted to 25 µg/ml in prewarmed (37 °C) 0.1M TRIS buffer (pH 7.5) and kept for 20 minutes at 37 °C within the humid chamber to prevent evaporation. Slides were washed in 70% IDA for 3 minutes and 100% IDA for another 3 minutes before rinsing them in SSC 2X for 1 minute while gently agitating (SSC 1X is 0.15 M sodium chloride and 0.015 M sodium citrate). Slides were kept in 0.1 M TRIS buffer (pH 7.5) until the *in-situ* hybridization frame seals (BIO-RAD) were glued to the slide around the sample. Then, the DIG-labelled probe and the non-labelled probe (control), both 50 µl in volume, were diluted by adding 50 µl of hybridization buffer and added to the cavity created by the gel frames in the slide, with the sample in the middle. After DNA denaturation at 94 °C for 6 minutes, slides were hybridized overnight (16 h) at 44 °C.

Samples were washed for 10 minutes with room temperature washing buffer (25 ml of SSC 20X, 6M Urea, 2 mg/l BSA), before being washed twice with preheated (38 °C) washing buffer for 10 minutes each. Slides were rinsed with preheated (38 °C) SSC 1X for 5 minutes (2 times) and with 0.1M TRIS buffer (pH 7.5) another 2 times. The blocking step was carried out with a solution of 6% dried skimmed milk diluted in 0.1M TRIS buffer (pH 7.5) for 1 hour at room temperature and washed with 0.1M TRIS buffer (pH 7.5) for 5 minutes twice.

Slides were incubated with 1.5 U/ml of anti-DIG-AP Fab fragments (Roche) diluted in 0.1M TRIS buffer (pH 7.5) for 1 hour at room temperature in darkness. The excess of Anti-DIG-AP was removed by 4 successive washes in 0.1M TRIS buffer (pH 7.5) for 10 minutes each. Slides were transferred to 0.1M TRIS buffer (pH 9.5) which is (0.1M TRIS base, 0.1M NaCl, adjust pH to 9.5 adding HCl) for 2 minutes and then tissue was covered with NBT/BCIP stock solution (Roche) diluted in 0.1M TRIS buffer (pH 9.5) at 20 µl/ml, and incubated in darkness and room temperature until the first clear signs of blue staining appeared (about 30 minutes). Slides were washed in 0.1M TRIS buffer (pH 9.5) for 1 minute twice and stained with 1% Bismark Brown for 6 minutes. Finally, slides were dehydrated by immersing them for 30 seconds in 70% IDA, 45 seconds in 100% IDA and 2 washes in clearene for 1 minute each. Slides were air dried for 30 minutes and permanently cover-slipped with DPX mounting medium (Sigma-Aldrich) .

Sequence alignment and phylogenetic analysis

The PCR-amplified 18S was BlastN-searched (Zhang et al. 2000) against the GenBank nucleotide (nt) database. Holozoan 18S gene sequences, as well as sequences from those

uncultured organisms showing highest similarity, were downloaded and aligned with the consensus 18S rRNA gene sequence from *T. philomaios* in MAFFT v.7 (Katoh et al. 2017) using the accurate option L-INS-i. The alignment was trimmed by trimAl v.1.4.rev22 (Capella-Gutiérrez et al. 2009) using the (-gt 0.1) option, and manually curated in SeaView v.4 (Gouy et al. 2010). In turn, the best-fitting model (GTR + F + G) for the alignment was selected using ModelFinder (Kalyaanamoorthy et al. 2017) as implemented in IQ-TREE v.1.6.10 (Nguyen et al. 2015) and used to generate a ML tree in IQ-TREE. Branch support was obtained from 1,000 ultrafast bootstrap values (Minh et al 2013). A second maximum likelihood phylogenetic tree was constructed using RAxML v8.2.12 (Stamatakis 2014); support values calculated using 1,000 bootstrap replicates were mapped onto the tree with the highest likelihood value (evaluated under GTRGAMMA model). A Bayesian inference consensus tree was built using MrBayes v.3.2 (Ronquist et al. 2012) under default parameters except for the following: the number of substitution types was mixed; the model for among-site rate variation, Invgamma; the use of covarion like model, activated. The MCMC parameters changed were: 5 million generations; sampling frequency set to every 1,000 generations; burnin fraction value = 0.25; starting tree set to random, and all compatible groups consensus tree. A final consensus tree figure was created using FigTree v1.4.3 (Rambaut 2017) and based on the Bayesian topology.

A second 18S phylogenetic tree was constructed including environmental and unclassified sequences branching with or within Filasterea, by mining different databases. The 18S of *T. philomaios* was used as a bait to fish highly-similar sequences, by blastn searching against the following GenBank archives: nt, whole genome shotgun contigs (WGS), sequence read archive (SRA), and high throughput genomic sequence archive (HTGS). The same approach was followed for SILVA (www.arb-silva.de), ENA (www.ebi.ac.uk) and DDBJ (www.ddbj.nig.ac.jp) databases. All environmental sequences branching within Filasterea or sister to it in a preliminary tree were retained for subsequent analyses (Table 4), as were as a selection of highly divergent uncultured mesomycetozoean and choanoflagellate sequences. Sequences belonging to uncultured organisms that branched robustly to existing species in Ichthyosporea, Choanoflagellatea or Metazoa were excluded from the final alignment (the selected sequences were realigned). The alignment and subsequent phylogenetic analysis were constructed as described above.

RESULTS

Clinical signs and prevalence

Two amphipod genera, *Echinogammarus* and *Orchestia*, were found infected by *T. philomaios*. The genera *Gammarus* (n = 279) and *Melita* (n = 101) were also investigated, but no signs of infection were observed histologically. However, the number of individuals examined was considerably lower (Table 1). Infection by *T. philomaios* was suggested macroscopically in heavily infected individuals by a yellowish and opaque tegument (Fig. 2). The carapace thickened and lost rigidity (Fig. 2B), impeding to discern internal organs, especially the intestine, which is evident in young healthy individuals. Besides, gross examination of the most translucent appendages (antennae, uropods, and gills) using a stereomicroscope permitted detection of the parasite in haemolymph (Fig. 3). Infected individuals often appeared lethargic and unresponsive to stimuli (during dissection) while habitually displaying reduced jumping ability (during collection) in the case of the sandhopper (*Orchestia* sp.).

1 Discrimination between haemolymph cells (8-10 µm) and *T. philomaios* cells (2-4 µm)
2 was possible on the basis of the cell diameter and nuclear size (Fig. 3A, 3B). Haemolymph
3 smears (Fig. 3C) evidenced the difference between the spherical and peripheral nucleus of *T.*
4 *philomaios* (~1 µm) and the central and irregular one in haemocytes (6-8 µm) (Fig. 3C).
5 Additionally, fresh preparations allowed to notice the occurrence of up to 10 parasite cells inside
6 hosts haemocytes. Toluidine staining of the dry smears emphasised the structures, allowing the
7 observation of cell aggregates (Fig. 3D). The occurrence of *T. philomaios* infection was
8 consistent throughout the years of study (2016-2018) showing a distinct prevalence peak
9 between late April and early June; at least for the regularly sampled *Echinogammarus* sp.
10 population present in Newton's Cove. These outbreaks of *T. philomaios* infection were usually
11 short-lived, lasting no more than three weeks. However, the prevalence of infection was high,
12 varying between 24% (2018) and 64% (2016) in the coastal location of Weymouth. Although the
13 limited data from the other sampling sites precluded direct comparison, the parasite was present
14 in the Dart, Tamar and Camel estuaries at low levels in both spring and autumn (Fig. 4A).
15 *Orchestia* sp. was less frequently and abundantly sampled, but in Newton's Cove, infection also
16 seemed to peak during May and early June (Fig. 4B). While in *Orchestia* sp. sampled in
17 Newton's Cove the prevalence was lower (10%), the parasite was more frequently detected in
18 the Dart and Tamar estuaries. The prevalence of infections in *Echinogammarus* sp. during the
19 rest of the year (from June to early April) was low (1.9%, n = 1136), and infection was never
20 systemic. The few parasitic cells observable during these months were almost exclusively
21 associated with the testis.

22 **Histopathology and ultrastructure**

23 Cells of *T. philomaios* were virtually spherical (width = 1.94 ± 0.21 µm; length = 2.36 ± 0.23
24 µm; n = 50) when fixed in Davidson's seawater fixative, and 2.26 ± 0.34 µm by 2.60 ± 0.41 µm;
25 n = 50) when preserved in glutaraldehyde. By light microscopy, a nucleus in the periphery of the
26 cell was distinguished in a very translucent cytoplasm. Parasites were present in the haemolymph
27 and frequently intracellularly within haemocytes (Fig. 5A). Infected haemocytes (containing up
28 to 10 *T. philomaios* cells) were often necrotic, with a clear loss of cellular integrity. In contrast,
29 the parasites inside them appeared to be intact. Aggregates of *T. philomaios* cells occurred free
30 or within haemocytes, where similar sized stages were contained within a membrane. However,
31 it was not possible to discern by light microscopy if aggregation was the result of single cells
32 actively joining, or clusters of cells remaining together after the rupture of the haemocyte
33 containing them. Proliferation of *T. philomaios* cells was associated with congestion of haemal
34 sinuses of the tegumental gland and the connective tissue associated with the cuticular
35 epithelium (Fig. 5B). In such systemic infections (with haemolymph, connective and tegument
36 affected), *T. philomaios* was frequently observed infecting the hepatopancreas (Fig. 5C), and
37 seldom in nervous tissue. In the hepatopancreas, the parasite was associated with structural
38 damage with significant inflammation and granuloma formation, often encapsulating *T.*
39 *philomaios* cells (Fig. 5C). The testis and ovary (Fig. 5D, 5E) also became infected, notably in
40 early-stage infections that did not show evidence of the parasite in other organs and tissues.
41 However, intracellular infections in oocytes and spermatozoids were not observed.

42 At the TEM, *T. philomaios* was found more often as single cells, but also forming
43 clusters containing 3-4 cells (Fig. 6A, 6B). Single cells, often coated by a cell wall, contained a
44 pale staining nucleus with a peripheral compact nucleolus, small mitochondria with lamellar
45 cristae and lipoid structures of varying electron-density (Fig. 6A). These lipoid inclusions

1 displayed morphologic plasticity and variable staining characteristics between *T. philomaios*
2 cells of different size. (Fig. 6C, 6D). Electron-lucent granules appeared integrated within the
3 cytoplasm, while darker granules were often membrane bound and associated to evaginations of
4 the cell wall (Fig. 6G, 6H). The multi-layered cell wall varied in thickness (Fig. 6G, 6H, 6I) and
5 in approximately 30% of the cells examined, appeared detached from the plasma membrane (Fig.
6 6A, 6G). In few cases, a matrix was observable between cell wall and the detached plasma
7 membrane (Fig. 6C).

8 A multicellular stage of *T. philomaios* was also frequently prominent (Fig. 6B); tricellular
9 in appearance a hidden fourth cell was occasionally observed (Fig. 7D). In several multicellular
10 clusters (Fig. 6B, 6E, 6F) the cells were indistinguishable from the unicellular stages present in
11 the haemolymph (Fig. 6A, 6C, 6D). Occasionally, one or more individual cells contained within
12 the walled parent cell were necrotic (Fig. 6B). Numerous peripheral mitochondria were observed
13 in cells with a thickened wall (Fig. 6A, 6I). The thickening of the electron-dense wall of the inner
14 cells was concurrent with a diminishing wall of the receptacle (Fig. 6D, 6F). The presence of
15 unicellular and divisional forms of *T. philomaios* inside host haemocytes and tegumental gland
16 hinted by light microscopy was corroborated by TEM analysis (Fig. 7A, 7B, 7C). Parasite cells
17 appeared healthy in contrast to the compromised integrity of the infected host cell (Fig. 7C). The
18 multicellular form appeared more often within haemocytes (Fig. 7A, 7B), while unicellular
19 stages were more commonly observed free in the haemolymph or inside cells of the host
20 tegument (Fig. 7C)

21 The majority of *T. philomaios* cells examined corresponded to one of the two main cell
22 cycle stages described above. The occasional occurrence of intermediate forms and structures
23 (Fig. 7E, 7F) suggested how unicellular cells were released from multicellular stages. The wall
24 of the receptacle became reduced until it fractured, allowing dispersal of the walled inner cells.
25 Just before being released, or immediately after (Fig. 7E, 7F), some of the released cells became
26 less electron-dense, with a fine matrix between wall and plasma membrane. In later stages, the
27 cell wall thickened and separated from the plasma membrane, possibly aided by co-occurring
28 cellular projections (Fig. 7F). At this stage, some of the electron-dense lipid vesicles (Fig. 7G,
29 7H), seemingly enclosed by a double membrane, were absorbed, or excreted. Occurrence of non-
30 walled unicellular forms of *T. philomaios* constituted the only stages in which the presence of
31 microvilli (Fig. 7I) and maybe a flagellum (Fig. 7J) were noticeable. Inside haemocytes non-
32 walled parasitic cells were loosely enclosed by a membrane of unknown origin (Fig. 7K).
33 Coinfection of *T. philomaios* with *Haplosporidium* sp. (Urrutia et al. 2020) was not uncommon
34 (Fig. 7L), but only *T. philomaios* cells were observed inside haemocytes. *In-situ* hybridization
35 confirmed that the ultrastructure and histopathology of the amphipod infecting microeukaryotes
36 matched with the 18S identified as *T. philomaios* (Fig. 8). The size and distribution of the DIG-
37 NTB stained structures coincided with their immediate histological H&E stained sections. Round
38 blue stains (2-4 μm) appeared concentrated in tegument, connective tissue, (Fig. 8A, 8B), gills,
39 haemolymph (Fig. 8C, 8D), and inside haemocytes (Fig. 8E, 8F).

40 **Life cycle and potential vectors**

41 The occurrence of a multicellular stage provided strong evidence that *T. philomaios* was
42 proliferating inside amphipod hosts. Two different amphipod genera were found to be
43 susceptible to infection by *T. philomaios*, raising questions about host specificity. Therefore,
44 some common invertebrates cohabiting with *Echinogammarus* sp. and *Orchestia* sp. were

analysed histologically and by PCR. In Newton's Cove, co-occurring polychaetes of genus *Capitella*, the turbellarian *Procerodes* sp., and harpacticoid copepods were sampled (Table 2). While evident systemic *T. philomaios* infection in amphipods is limited to late April and May, we recognized the possibility that the parasite might be present in other hosts during a different time of the year. Thus, abundantly co-occurring invertebrates were sampled during May, June, July, August, and September. No clear evidence of *T. philomaios* cells were observed in the histopathological survey of *Procerodes* sp., *Capitella* sp. or harpacticoid copepods. However, PCR analysis carried out using sets of individuals representing these taxa indicated the presence of DNA of *T. philomaios* in a batch sample comprising *Procerodes* individuals, collected during May 2019.

Phylogenetic analyses

Initially, a partial SSU sequence (ca. 705 bp long, including variable regions V5, V7, V8, and partial V9) was coincidentally amplified by haplosporidian-specific primers (Hartikainen et al 2014) from an *Echinogammarus* sp. sample later shown to be infected by *T. philomaios*. The top Blastn match for this sequence was the ichthyosporean *Dermocystidium salmonis* (91.5% similarity; 92% coverage; e-value = 0). Phylogenetic analysis of this 705 bp sequence (not shown) placed *T. philomaios* within clade Holozoa, with low branch support for any particular position, but often grouping with Ichthyosporea or Filasterea. A longer, equivalent 18S region of 1679 bp generated from an infected *Echinogammarus sp*. individual, resulted in a Blastn match of 87.90% similarity (99% coverage) to the free living filasterean *Pigoraptor chileana*. Phylogenetic analysis of the 1679 bp region (Fig. 9) was consistent with that using the shorter fragment, and robustly placed *T. philomaios* as an holozoan, but very weakly branching as the earliest diverging lineage in Holozoa.

Several databases were mined for environmental sequences (process specified in section 2.5) related to *T. philomaios* (Table 4). The resulting phylogenetic tree (Fig. 10) showed some interesting differences when compared to the tree without environmental sequences (Fig. 9). In particular, in Fig. 10 *T. philomaios* branched within Filasterea, in a clade mostly comprising environmental sequences, but also Ministeria. The filasterean clade was more strongly supported with the inclusion of the environmental sequences, with supports of (0.98, 21, 72; posterior probability, ML bootstrap, and ML ultrafast bootstrap, respectively) compared to (0.9, -, -) in Fig. 10. The metazoan, choanoflagellate, and fungal clades were again fully/strongly supported, although the ML bootstrap support for the ichthyosporean clade was lower: 1, 34, 68 in Fig. 10 to 0.99, 73, 82 in Fig. 9. The phylogenetic position of the two pluriformean species as basal to choanoflagellates was maintained, but the support for *C. limacisporum* in that position increased from (0.32, -, 46) to (0.91, 19, 64).

The filasterean clade in Fig. 10 was moderately well supported by Bayesian Inference (0.98, 21, 72) but contained a large proportion of partial environmental sequences yielding disparity between ML methods. *Txikispora* was a robustly placed sister to Metagenome seq. OBEP010137028) sampled from sandy/muddy sediments associated with algae in Ulvedybet in Limfjorden (northern Denmark) (Karst et al. 2018). These, together with *Ministeria*, formed a clade with other environmental sequences from fresh groundwater systems in Denmark (OBEP010275669, OBEP010278239, OBEP010275324, OBEP010275456, OBEP010276073) and New York State (ORJL011316691) (Karst et al. 2018; Wilhelm et al. 2018), with the exception of OBEP010162136, which also came from the coastal location of Ulvedybet in

Limfjorden (Karst et al. 2018). The other characterised filasterean taxa, *Capsaspora* and *Pigoraptor*, grouped separately within the filasterean clade, and potentially more closely to each other than to *Ministeria* and *Txikispora* (Fig. 10)

Several environmental sequences branched close to *Capsaspora* and *Pigoraptor*. Metagenomic sequence OBEP01433235, collected from the sediments in a freshwater lake (Denmark) was very closely related to *Pigoraptor.* Additionally, two almost identical sequences (FPLL01002905 and FPLS01019718) collected from soil samples in Denmark (Karst et al. 2016) were robustly sister to *C. owczarzaki* (100, 100, 100). Two further clades of environmental sequences branched within the filasterean clade as shown on Fig. 10. One was an abundant group of uncultured marine organisms named "MAOP1-Marine Opisthokonts", which was weakly sister to *Pigoraptor* in Hehenberger et al. (2017). The other was a clade formed by short sequences (indicated on Fig. 10 as "LN******") collected from a subterranean colony of ants adjacent to Chagres river, Panama (Scott et al. 2010).

DISCUSSION

Phylogeny and diversity

Until the recent addition of *Pigoraptor* by Hehenberger et al. (2017), Class Filasterea comprised only two genera: *Capsaspora* (*C. owczarzaki*) and *Ministeria* (*M. vibrans* + *M. marisola*). Hehenberger et al. (2017), also suggested the inclusion of an abundant group of marine opisthokonts "MAOP-1" (del Campo and Trillo 2013) into Filasterea. The ecology of these clade formed by uncultured organisms remains entirely undetermined except for an apparent inclination for the low oxygen fraction of the water column in coastal waters of the Indian, Atlantic and Pacific Oceans. Our results (Fig. 10) further support the inclusion of MAOP-1 in Filasterea. However, none of the ML analyses are conclusive, and the relative phylogenetic position of the group among existing filasterean species varies. In Hehenberger et al. (2017), MAOP-1 appeared as sister to *Pigoraptor* sp. (ML Bootstrap = 52%), but our analysis showed it as weakly sister to *Pigoraptor* spp, plus *C. owczarzaki* (in both cases with related environmental sequences) plus the "LN******" environmental sequences. Our 18S phylogenetic analysis without environmental sequences (Fig. 9) also supported the inclusion of *T. philomaios* into Holozoa, but not its association with Filasterea. Ongoing phylogenomic analyses seek to place *T. philomaios* using a much larger number of genes.

It is well established that single-gene trees are unable to resolve deep eukaryotic phylogenetic relationships. This is particularly evident for holozoan relationships, as shown by Simion et al. (2017) among others. Our results suggest that the use of uncharacterized environmental sequences in phylogenetic studies based on 18S provide additional phylogenetic information that may assist in resolving evolutionary relationships of novel holozoan organisms, as has previously been demonstrated for other eukaryotic groups and eukaryotes as a whole (e.g. Berney et al 2004; Cavalier-Smith 2004; Bass et al. 2018; Hartikainen et al. 2016).

Several environmental sequences were closely related to existing filasterean species (Fig. 10). The uncultured sequence Metagenome seq. OBEP011433235 most likely belongs to a novel *Pigoraptor* sp. species, which evidences the preference of the genus for the sediments of stagnant freshwater systems, and a global distribution (Denmark, Chile, Vietnam). However, the

1 environmental sequences FPLL01002905 and FPLS01019718, although sister to *C. owczarzaki*,
2 are too distantly related to sensibly infer any lifestyle or other phenotypic similarity between
3 them and *Capsaspora*. Interestingly, its occurrence in a Danish grassland (Karst et al. 2016),
4 contrasts with the rest of environmental sequences associated to Filasterea, which were sampled
5 from aquatic ecosystems. Although it is not possible to determine whether other filasterean
6 environmental sequences are parasites, other symbionts, or free-living, our discovery of a true
7 filasterean parasite means that this is now a realistic working hypothesis.

8 At some point in the evolutionary history of their lineages, *C. owczarzaki* and *T.*
9 *philomaios* evolved endosymbiotic and parasitic behaviours closely associated with host
10 haemolymph and haemocytes, highly uncommon target cells/tissues in the related clade
11 Ichthyosporea (Glockling et al. 2013). Whether filasterean radiation preceded that of early
12 metazoans 650-833 million years ago (Paps 2018) remains unresolved. Nonetheless, a common
13 tissue trophism could suggest certain predisposition in the early ancestors of filastereans to
14 colonize the haemolymph (or precursor cells) of other organisms, that could be shared by related
15 uncultured filastereans.

16 **Clinical signs and histopathology**

17 *T. philomaios* cells congest the host's haemolymph and tegument, making heavily infected
18 amphipods present a light-yellow colouration and reduced carapace transparency (internal organs
19 are not easily visible though the carapace). Definite colour alterations of the host's carapace have
20 been documented for other parasitic infections, such as those produced by acanthocephalans,
21 cestodes, and trematodes (Lagrue et al. 2016; Johnson and Heard 2017). Other microeukaryotic
22 cells targeting tegument and haemolymph in amphipods (*Haplosporidium* sp.) have also been
23 associated with a pallid carapace and opacity. However, amphipods with heavy haplosporidiosis
24 look whitish rather than yellowish, at least in *Echinogammarus* sp. and *Orchestia* sp. (Urrutia et
25 al. 2019). The formation of cell aggregates, very evident in fresh haemolymph smears, are
26 characteristic among filastereans (Sebé-Pedrós et al. 2013) and facilitates the differentiation
27 between *T. philomaios* and other protistan parasites with similar size. We have also observed
28 infected hosts to be more sessile and unresponsive to stimuli, but this is the case for other protist
29 parasite infections as well, not only in amphipods (Feist et al. 2009; Lefèvre et al. 2009).

30

31 **Morphology and ultrastructure**

32 Measuring less than 3 µm in diameter *T. philomaios* is one of the smallest known holozoans. In
33 clade Filasterea only the bacterivorous *M. vibrans* would have a similar size, with its round cells
34 being 2.1-3.6 µm in diameter (Mylnikov et al. 2019). The highly motile predators *P. vietnamica*
35 *and P. chileana* tend to be considerably bigger (5-12 µm), in the size range of most
36 choanoflagellates and corallochytreans (Raghu-kumar 1987; Dayel and King 2014; Tikhonenkov
37 et al. 2020a). Only the zoospores of few species of ichthyosporean parasites such as
38 *Sphaerothecum destruens* or *Dermocystidium percae* have been reported to have a similar or
39 even smaller size than *T. philomaios* (Pekkarinen and Lotman 2003, Andreou et al. 2011).
40 *Tunicaraptor*, a free-living predatory holozoan protist of unresolved phylogenetic affinity
41 described during the production of the present study, is slightly bigger (3-5.1 µm) than *T.*
42 *philomaios* (Tikhonenkov et al. 2020b), overlapping in size with most filastereans. Reduced
43 body and genome size have been linked to parasitism in other protistan (Keeling and Fast 2002;

Keeling 2004; Holzer et al. 2018), but not in unicellular holozoans, possibly due to the absence of parasites among choanoflagellates, and rarity of free-living forms in Ichthyosporea (Mendoza et al. 2002; Glockling et al. 2013; Hassett et al. 2015).

Fresh smears of *T. philomaios* showed the presence of cell-projections comparable to the flagellar structures described by light microscopy and TEM in *M. vibrans* and *Pigoraptor* sp. (Torruella et al. 2015; Hehenberger et al. 2017; Mylnikov et al. 2019). However, no evidence of a flagellum was observed in the histopathological analysis, and we only have limited ultrastructural evidence of its occurrence by TEM (Fig. 7J). While inconclusive, we must note that in fresh smears *T. philomaios* cells were exposed to a substrate and marine water, but histology and TEM analysed them fixed in tissues and haemolymph. A non-flagellated *T. philomaios* would imply a secondary loss of the structure (based in our phylogeny, Fig. 10), the second one within Filasterea after *C. owczarzaki*. Two losses are less parsimonious but could strengthen the idea of a parasitic/endosymbiotic lifestyle driving them, which has also been suggested for non-flagellated ichthyosporean parasites in order Ichthyophonida (Marshall and Berbee 2011, Torruella et al. 2015).

Microvilli are actin-based filopodial structures present in filozoans (Karpov et al. 2016; Sebé-Pedrós et al. 2017; Mylnikov et al. 2019). Contrasting with choanoflagellates, they are evenly distributed around the cell in all filastereans and pluriformeans (Mylnikov et al. 2019; Tikhonenkov et al. 2020a), clades in which they can be up to three or four times the length of the cell However, they are not present in cystic and dividing stages, what could explain the reduced evidence for them in *T. philomaios* (Fig. 7I). Moreover, their occurrence was not noticed in the original descriptions of *C. owczarzaki* done on explanted pericardial sacs of snails (Owczarzak et al. 1980), but they are evident when the facultative symbiont is in axenic culture (Sebé-Pedrós et al. 2013), where they have been shown to facilitate movement, cell-cell adhesion, and food particle capture (Parra-Acero et al. 2020). It is possible that microvilli are not desirable in the haemolymph of a host, where the current impedes movement and there is no substrate surface other than target haemolymph cells.

Opisthokonts are characterized by flat non-discoid cristae (Cavalier-Smith and Chao 1995). Mitochondria in *T. philomaios* follows the norm and possesses lamellar cristae (Fig. 7G, 7I). The radial distribution of numerous mitochondria in the periphery of non-cystic stages (Fig. 6A) could indicate a close in time cell division between daughter cells, as observed in the ichthyosporean parasite *Sphaerothecum* sp. (Borteiro et al. 2018). In contrast, the absence of mitochondria in stages with a thicker wall suggests a resistant spore-like stage, as it is the case in the ichthyosporean *Amphibiocystidium* sp. (González-Hernández et al. 2010). However, the structure and activity of mitochondria in parasites has been observed to be extremely flexible (Zíková et al. 2016), as they would be able to use mitochondrial metabolites of the host (de Melo and Souza 1992).

Numerous electron-dense bodies comparable to those observed in other filasterean species (Owczarzak et al. 1980, Tikhonenkov et al. 2020a) are scattered in the cytoplasm of *T. philomaios* (Fig. 6A, 6C, 6D, 7G, 7H, 7K). However, their occurrence is not characteristic of filastereans or even holozoan protists, as they have been observed in distantly related clades such as apicomplexans, ascetosporeans or dinoflagellates (Speer et al. 1999; Stentiford and Shields 2005; Feist et al. 2009). Nevertheless, their size and number has been suggested to be of taxonomic value in Mesomycetozoea (Pereira et al. 2005), and indicative of the function of

certain life stages and their maturation (Vilela and Mendoza 2012; Fagotti et al. 2020). These bodies have been described as lipid globules in *M. vibrans* (Mylnikov et al. 2019) and reserve substances (most likely glycoprotein) in genera *Pigoraptor* and *Syssomonas* (Tikhonenkov et al. 2020a). In contrast, the occurrence of a double lipidic layer around them in *C. owczarzaki* made Owczarzak et al. (1980) suggest that these "lipid filled vacuoles" were excreted. In *T. philomaios* we observe two main forms; the first is a smaller and electron-lucent body similar to those observed in genera *Ministeria*, *Pigoraptor*, and *Syssomonas*. The second form is a larger and electron-dense body surrounded by a double lipid layer (Fig. 6H, 7G) that appears to be excreted (Fig. 7H) as proposed for *C. owczarzaki*. However, its implication in the formation of the cell wall should be considered, as it is not clear how the ejected material could trespass the outer membrane (Fig. 7G).

Life cycle and transmission strategies

So far, all filastereans have been culturable (Stibbs et al. 1979; Cavalier-Smith and Chao 2003; Hehenberger et al. 2017; Mylnikov et al. 2019), allowing a detailed description of their life cycle in culture conditions. In contrast, *T. philomaios*, like most parasites in the clade Ichthyosporea remains uncultured (Cafaro 2005; Glockling et al. 2013). According to the diagnostic description of Class Filasterea Cavalier-Smith 2008, trophic stages in this lineage do not possess a cell wall (Shalchian-Tabrizi et al. 2008). In free-living genera *Pigoraptor* and *Ministeria*, this non-walled stage corresponds to a flagellated amoeba which uses its retractile microvilli to capture preys and attract food particles (Hehenberger et al. 2017; Mylnikov et al. 2019). In turn, trophocytes of the endosymbiont *C. owczarzaki* lack a flagellum, and even microvilli if cultured in explanted tissues of *B. glabrata* (Owczarzak et al. 1980; Sebé-Pedrós et al. 2013). Although morphologically different, the behaviour of trophocytes is the same in all known filasterean species; they can either divide by binary fission or encyst when the food source is depleted (Hertel et al. 2002; Tikhonenkov et al. 2020a). The binary fission observed by light microscopy in few walled cells of *P. vietnamica* (Tikhonenkov et al. 2020a) represents the only known exception of cellular division occurring outside the trophic stage. Interestingly, our TEM analysis indicates that quite the contrary occurs for *T. philomaios*, in which cell division appears to occur exclusively inside walled cells (Fig. 6B, 6I, 7D) as in Corallochytrea and Ichcthyosporea (Raghu-Kumar 1987; Lotman et al. 2000; Pekkarinen et al. 2003; Glockling et al. 2013). If flagella and/or microvilli occur in *T. philomaios* trophocytes (Fig. 7I, 7J), these structures are lost when parasitic cells either penetrate or are engulfed by host haemocytes (Fig. 7K).

A single host haemocyte can contain up to ten *T. philomaios* cells, in which four walled endospores arise inside walled parent cells (Fig. 7D). Comparable cellular structures containing 16-32 endospores are the result of a palintomic division in corallochytrean cystic stages (Raghu-Kumar et al. 1987; Tikhonenkov et al. 2020a). Once mature, *T. philomaios* endospores would leave the parent cells through an opening formed in its wall, by which time its thickness is much reduced, as in Corallochytrea and Ichthyosporea (Mendoza et al. 2002; Marshall and Berbee 2011; Tikhonenkov et al. 2020a). The wall thickness, electron-density and amount of reserve material vary greatly among endospores. Some cells appear active even before exiting the ruptured parent cell (Fig. 7E), presumably ready to re-infect other haemocytes and tissues in the same host, as it has been shown for several ichthyosporeans (Arkush et al. 2003; Marshall et al. 2008; Kocan 2019). Other cysts seem to be resistant (Fig. 6D, 6F), perhaps capable of infecting other amphipods or even remaining viable in the environment for months (Marshall and Berbee 2010; Gozlan et al. 2014; LaPatra and Kocan 2016).

The transmission method for *T. philomaios* cells is unknown, as for *C. owczarzaki* (Harcet et al. 2016), and most parasites in Ichthyosporea (Glockling et al. 2013). A direct cycle by consumption of infected prey has been demonstrated in the ichthyophonids (Kramer-Schadt et al. 2010), and could be possible for *T. philomaios*, given the high levels of interspecific predation (Dick et al. 1999), cannibalism (Kinzler and Maier 2003), and scavenging of conspecifics (Agnew and Moore 1986) observed in amphipods. The thicker ameboid endospores observed in *T. philomaios* are also remindful of the infective waterborne cells observed in ichthyophonid parasites too (Olson et al. 1991, Andreou et al. 2009, Kocan 2019), which unlike those in order Dermocystida, lack a flagellum (Mendoza et al. 2002). Additionally, cysts of the so called "TMS" ichthyosporean infecting *Tenebrio molitor*, persist in the connective tissues associated to the gonads, and are transmitted with sperm during copulation (Lord et al. 2012). The presence of few *T. philomaios* cells infecting amphipod gonads throughout the year (although with low prevalence = 1.9%) leaves open the possibility of a similar "nuptial transmission" for the novel parasite.

Finally, an indirect transmission cycle has been contemplated as well, given the generalist infectivity observed in *T. philomaios* and ichthyosporean parasites (Andreou et al. 2012; Rowley et al. 2013; Combe and Gozlan 2018). Copepods have been proposed as the missing intermediate host for ichthyosporean parasites (Hershberger et al. 2002; Gregg et al. 2012). Interestingly, harpacticoid copepods are some of the most common invertebrates co-occurring with amphipods in the upper part of the intertidal in Newton's Cove, Camel, Dart and Tamar estuaries (personal observation; Hicks and Coull 1983). However, our PCR based search for *T. philomaios* in copepods (n = 1300 individuals) was negative, just like the histopathological analysis. In turn, the results for the turbellarian *Procerodes* sp. were PCR positive during May. The platyhelminth, which is very common in the north Atlantic, appears to predate on diseased *Echinogammarus* sp. preys and carcasses (Den Hartog 1968; Taylor 1986), showing a link and a possible role as intermediate host. A more extensive histopathological analysis of *Procerodes* sp. will be necessary to substantiate its possible role as intermediate host of *T. philomaios*. If uninfected the turbellarian could still be a vector helping the dispersal of viable *T. philomaios* cysts.

Distribution, prevalence, and ecological significance

The low number of filasterean species and their rare appearance in environmental samplings have prevented any previous estimation of their temporal prevalence, as it has been assayed for larger holozoan clades Ichthyosporea and Choanoflagellatea (Marchant and Perrin 1990; Kasesalu et al. 2000; Pekkarinen and Lotman 2003). The prevalence of *C. owczarzaki* in *Biomphalaria glabrata* has been observed to vary from 1% to 45% depending on the strain (Hertel et al. 2002), but the measurement, done on cultured snails, does not estimate occurrence on a time period. Our study is the first one to reveal a temporal pattern in the abundance of a filasterean species. The quickly vanishing peak in prevalence observed for *T. philomaios* during May, exposes seasonality as an until now unaccounted bias for the scarcity of filasterean sequences in environmental samplings (del Campo et al. 2015; Hehenberger et al. 2017; Mylnikov et al. 2019). A similar short temporary window in the transmission of *C. owczarzaki* between snails, could explain, at least partially how it has eluded sampling efforts to find it in the wild (Ferrer-Bonet and Ruiz-Trillo 2017). Additionally, our failed efforts to amplify the 18S of *T. philomaios* from filtered water collected in Newton's Cove during May, reinforces the hypothesis of a reduced detection capability of eDNA for parasites/endosymbionts (Dumonteil et al. 2018).

So far, it has been observed that *T. philomaios* is able to infect at least two different amphipod genera, indicating certain range of hosts specificity that could expand notably if infection in the turbellarian *Procerodes* sp. is substantiated by histology. In this study, the prevalence of *T. philomaios* was as high as 64% (May 2016), with about a third of the infected individuals presenting heavy infections associated to tissue disruption and haemolymph congestion by parasitic cells. From the point of view of pathology, other protistan parasites that tend to multiply and congest the haemolymph of crustacean hosts, such as the dinoflagellate *Hematodinium* sp. have been associated with a reduced oxygenation capability and diminished overall fitness (Taylor et al. 1996; Stentiford et al. 2001). The observed unresponsiveness to stimuli in infected amphipods, is consistent with the systemic damage observed in the tegument, which functions as the sensorial system (Steele and Oshel 1987). Collectively, numerous protistan parasites have been found to profoundly alter the populations of amphipods and other crustaceans (Morado 2011; Ironside and Alexander 2015). Considering that several ichthyosporean parasites are responsible for important mortality (Raffel et al. 2008; Kirkbright et al. 2016) it would be interesting to monitor the influence of *T. philomaios* in the amphipod population; *Echinogammarus* and *Orchestia* are amongst the most common and abundant crustaceans in coastal ecosystems of Northern Europe (Marques and Nogueira 1991; Mantzouki et al. 2012), and important invasive species outside the continent (Van Overdijk et al. 2003; Herkül et al. 2006).

TAXONOMIC SUMMARY

Eukaryota Chatton, 1925 / Eukarya Margulis and Chapman, 2009: Opisthokonta Adl, 2005: Holozoa Adl, 2012: Filasterea Shalchian-Tabrizi, 2008: Ministerida Cavalier-Smith, 1997

Family Txikisporidae Urrutia, Feist & Bass n. fam.
Diagnosis. Naked unicellular and uninucleated protists morphologically similar to individuals in family Ministeriidae Cavalier-Smith 2008, but with a parasitic lifestyle.
Type genus. Txikispora n. g. (see below)
Genus *Txikispora* Urrutia, Feist & Bass n. g.
Etymology. 'txiki': small and 'spora': a seed (Basque). The name has been chosen to reflect relatedness with the filasterean endosymbiont *Capsaspora* Hertel, 2002 ("the quick eating seed") and its small size, while putting a distance with other small spore forming parasitic lineages with Latin stems.
Diagnosis. As for species (see below)
Type species. Txikispora philomaios (see below)
***Txikispora philomaios* Urrutia, Feist & Bass n. sp.**
Etymology. Txiki-: small, spora: spore, philo-: lover, maios: the month of May. "The little May-loving spore", referring to its predominant detection (as a parasite of amphipods) in that month.
Diagnosis. Virtually spherical monokaryotic stages, with a length of 2.6 ± 0.41 µm and a width of 2.26 ± 0.34 µm. The round and walled multinucleated stage contains four walled cells inside, which resemble a lot the monokaryotic stages. The size of this divisional stage is slightly bigger (3.17 µm ± 0.24 in diameter). Infection develops principally inside host haemocytes and connective tissues, especially those associated to the tegument. Infection in amphipods in the southwest of UK occurs consistently during late April and May, the prevalence of the parasite during the rest of the year is anecdotical (< 2%). The parasite has been also linked to the gonads,

being the only organ that appears to be infected during the rest of the year. There is host reaction to the parasite in form of melanization and granuloma formation, especially when the parasite affects the hepatopancreas.

Type host. Amphipods *Echinogammarus* sp. and *Orchestia* sp**.**

Type location. Coastal waters in Newton's Cove (UK)

Type material. Original slides used for this paper are stored together with biological material embedded in wax and epoxy resin in Cefas Weymouth Lab. Type material is stored as RA16020 (specimen no. 19) and RA17028 (specimen no. 53) and (specimen no. 287). The 18S SSU rRNA gene sequence is deposited in GenBank under accession number (OK181898).

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FIGURE LEGENDS

Fig. 1. Map showing the coastal locations in which amphipods of the genera *Echinogammarus*, *Orchestia*, *Melita* and *Gammarus* were collected. A. Western Europe, the black rectangle showing the area of UK sampled. B. Area contained within the black rectangle (A). The blue lines show the rivers and estuaries; arrows indicate the sampling locations. Precise coordinates of the locations (Table 1).

Fig. 2. Stereo-microscopical images of live *Echinogammarus* sp. amphipods collected in Newton's Cove. A. Uninfected individual. Antennae, pereopods and uropods (arrowheads), internal organs (arrow) B. Individual heavily infected by *Txikispora philomaios*. The tegument of the infected individual appears more opaque, the gut (arrow) is not evident, especially in the posterior fraction of the body (pleon). Scale bars = 100 µm for (A & B).

Fig. 3. Light microscopic images of antennae (A, B), and haemolymph (C, D) from healthy (A) and infected (B, C, D) amphipods of genus *Echinogammarus*. A. Stereo microscope image of the antennae (inset) of a healthy amphipod individual, showing (≈ 10 µm) haemocytes (arrowhead) flowing in the open circulatory system between the antennal gland and the tegument (asterisk). B. Cells of *Txikispora philomaios* (empty arrow) can be differentiated from haemocytes (filled arrowhead) by their smaller size and small nucleus. C. Composed microscope image of an unstained fresh preparation of the haemolymph showing *T. philomaios* cells free in the haemolymph (empty arrowhead) and within haemocytes (filled arrowhead). D. Toluidine blue-stained preparation of haemolymph from an infected amphipod showing *T. philomaios* single cells (empty arrowhead), parasitic cells inside haemocytes (filled arrowhead), and parasite cells forming multicellular groups (arrow). Scale bars = 10 µm for (A, B, C, D), and 20 µm for inset in (A).

Fig. 4. Prevalence of *Txikispora philomaios* infection in *Echinogammarus* sp. (A.), and *Orchestia* sp. (B.) from April 2016 to August 2018. Dates on the x-axis correspond to sampling information in (Table 1, 2). Y-axis: *T. philomaios* infection prevalence (%). Blue spheres refer to amphipods collected in Newton's Cove; red triangles = Tamar estuary; green diamonds = Dart estuary; yellow spheres = Camel estuary.

Fig. 5. Histological appearance of *Txikispora philomaios* infecting different tissues in *Orchestia* sp. A. Parasite cells were observed free in the haemolymph (empty arrowhead) and inside haemocytes (arrows). Non-infected haemocytes (filled arrowhead), tegument (t) and connective tissue (co), in the pereopods of the amphipod. B. Masses of parasitic cells (*) in the haemolymph and tegumental gland (t) associated to the cuticle of the carapace. C. Parasite cells (*) infiltrating the hepatopancreas (h). Granulomas and melanization (empty arrowhead) and muscle fibres (m). D. *T. philomaios* cells infiltrated between muscle fibres (filled arrowhead) and inner connective layers (empty arrowhead) of male gonads (mg). E. Disrupted female gonadal tissue (fg) associated to parasitic cells (empty arrowhead). Unaffected intestine (i) and its lumen (lu). Scale bars = 20 µm for (A, B, C, D, E).

Fig. 6. Transmission Electron Microscope (TEM) micrographs of *Txikispora philomaios* cells infecting *Orchestia* sp. A. Unicellular stages of the parasite show a single amorphous nucleus (n)

with a peripheral nucleolus (nu), peripheric mitochondria (m) electron-dense lipidic vesicles (*), and electron-lucent vesicles (i). The cell wall (filled arrowhead) appears detached from the plasma membrane (arrow). B. Dividing form of the parasite, with outer cell wall (arrow) and walled inner cells (filled arrowhead). One of the inner cells appears necrotic (*). C. Unicellular stage attached to host cell (h); amorphous material between wall and plasma membrane (filled arrowhead); (i) electron-lucent vesicles (reserve material). D. Unicellular stage full of electron-dense vesicles (x) with disrupted cell wall around (filled arrowhead). E. Dividing form, with inner cells (r) partially sharing the same matrixial material (me) with the outer walled cell. F. Electron-dense tricellular stage still within an indistinct walled outer cell. G. Detail of the thin wall (filled arrowhead) of a unicellular parasite cell inside a host haemocyte. Electron-lucent vesicles (i) and granular cytoplasm (*). H. Detail of a unicellular parasite cell with a thickening and evaginating cell wall (arrowhead). I. Detail of outer (empty arrowhead) and inner (filled arrowhead) cell walls, plasma membrane (arrow), and mitochondria (m). Scale bars = 500 nm for (A, B, C, D, E, F, G) and 100 nm for (H, I).

Fig. 7. Transmission Electron Microscope (TEM) micrographs of *Txikispora philomaios* cells infecting *Orchestia* sp. A. Intracellular stage of *T. philomaios* with a fine and closely attached cell wall (filled arrowhead). The host (h), its nucleus (hn), and nucleolus (nu) are shown. B. Five unicellular and a single multicellular stage (empty arrowhead) inside a host haemocyte (h), with a presumed parasite cell wall (*) attached to it. The inset shows the presence of a more electron-dense dividing form of *T. philomaios* (filled arrowhead) inside a host cell (h). C. Necrotic haemocyte containing three intact *T. philomaios* cells, with one vacuole containing a necrotic *T. philomaios* cell (*). The infected host cell is unable to maintain its normal structure, also true for its nucleus (hn). D. Divisional stage of *T. philomaios*. Four electron-dense daughter cells increase in size inside the wall of the parent cell, which still contains an evident cytoplasmatic matrix (*). E. Three daughter cells inside a parent cell without matrix and a very reduced cell wall (arrowhead). One of the daughter cells is more translucent (empty arrowhead) than its sister cyst-like cells. F. Two unicellular stages, one of them with an open thin wall (filled arrowhead) similar to the one marked with an asterisk in figure 7B. The other with short projections of the outer cell wall (empty arrowhead). Detail of the inner structure of the projection in the inset. G. Detail of two electron-dense vesicles surrounded by a double lipidic membrane (empty arrowhead) in the immediate periphery of the cell. Mitochondria (m). H. Unicellular stage showing detachment of the outer cell-wall. The wall presents several subtle evaginations (filled arrowhead). An electron-dense vesicle (empty arrowhead) is excreted to the space between plasma membrane and cell wall. I. Surface projections on a free *T. philomaios* cell (arrowheads). J. Parasite cell with mitochondria (m) and nucleus (n) in contact with a host cell (h). At least two flagellar structures (black arrows) have been observed flanking *T. philomaios* cells K. Intracellular stage of *T. philomaios* inside a host haemocyte with a thin detached wall (filled arrowhead) L. Coinfection of *T. philomaios* (empty arrowhead) and the ascetosporean parasite *Haplosporidium orchestiae* (filled arrowheads) in *Orchestia* sp. Only developing *T. philomaios* cells (empty arrowhead) are visible inside host haemocytes (h). Scale bars = 2 µm for (A, B, C, L), and 500 nm for (D, E, F, G, H, I, J, K). Inset in (B) is 2 µm; inset in (F) is 100 nm.

1 **Fig. 8.** Histological sections of *Orchestia* sp. tissues following *In-Situ* Hybridization (ISH) using
2 a DIG-labelled probe (A, C, E) and the respective consecutive histological H&E-stained section
3 obtained from the same host (B, D, F). A. & B. *Txikispora philomaios* cells can be observed
4 infecting the tegument (filled arrowhead) the cuticle (c) and haemocytes present in the cardiac
5 tissues. Female gonads (fg) appear uninfected in this individual. C. & D. Infected gill cells
6 (arrowhead) usually have ciliates attached (arrow), which are not infected in this occasion.
7 Haemolymph circulating through the gills is heavily infected with *T. philomaios* cells. E. & F.
8 Uninfected muscle (m) forming the cardiac tissue, pumps infected haemocytes (h) to other
9 tissues. Scale bars = 100 µm for (A, B) and 25 µm for (C, D, E, F).

10 **Fig. 9.** Bayesian phylogenetic analysis of 18S and 28S rRNA genes places the novel amphipod
11 parasite *Txikispora philomaios* (1679 bp) within Holozoa. The alignment included the 1679 bp
12 18S rRNA gene sequence of *T. philomaios* and the 18S of the rest of species (28S sequences
13 were included where available in Genbank). The tree includes a selection of the main
14 opisthokont groups and unicellular holozoan lineages. Branch support values are shown in
15 clusters of three, representing Bayesian posterior probability (pp) run on MrBayes, maximum
16 likelihood bootstrap support (bs) generated using RAxML with 1,000 replicates, and ML
17 ultrafast 1,000 replicates bootstrap support (UF) from IQ-TREE, respectively. Branches with
18 values (> 0.95 pp, > 95% bs, > 95% UF) are represented by a black dot on the branch. Species
19 belonging to clades Protostomia, Vertebrata, Basydiomycota, Glomeromycota, Mucuromycota,
20 Chytridiomycota, and Rozellida were collapsed.

21 **Fig. 10.** Bayesian phylogenetic analysis of 18S and 28S rRNA genes, including environmental
22 sequences, places the novel amphipod parasite *Txikispora philomaios* (1679 bp) within
23 Filasterea. 28S sequences were included where available in Genbank. Branch support values are
24 shown in clusters of three, representing Bayesian posterior probability (pp) run on MrBayes,
25 maximum likelihood bootstrap support (bs) generated using RAxML, and ML ultrafast bootstrap
26 support (UF) from IQ-TREE, respectively. Branches with values (> 0.95 pp, > 95% bs, > 95%
27 UF) are represented by a black dot on the branch. Species belonging to Metazoa and Fungi were
28 collapsed, as were Eccrinales and Dermocystida (Ichthyosporea). Environmental sequences are
29 indicated by their GenBank accession numbers.