Molecular data infers the involvement of a marine aurantiactinomyxon in the life cycle of the myxosporean parasite *Paramyxidium giardi* (Cnidaria, Myxozoa)

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Abstract

An aurantiactinomyxon type is described from the marine naidid *Tubificoides pseudogaster* (Dahl, 1960), collected from the lower estuary of a Northern Portuguese River. This type constitutes the first of its collective group to be reported from Portugal, and only the fourth described from a marine oligochaete worldwide. Extensive morphological comparisons of new aurantiactinomyxon isolates to all known types without available molecular data are proposed to be unnecessary, given the artificiality of the usage of morphological criteria for actinosporean differentiation and the apparent strict host specificity of the group. Recognition of naidid oligochaetes as the hosts of choice for marine types of aurantiactinomyxon and other collective groups, suggests that the family Naididae played a preponderant role in the myxosporean colonization of estuarine communities. Molecular analyses of the type in study further infer its involvement in the life cycle of *Paramyxidium giardi* (Cépède, 1906) Freeman and Kristmundsson, 2018, a species that infects the kidney of European eel *Anguilla anguilla* (Linnaeus, 1758) and that has been reported globally, including from Portuguese waters. The low intraspecific difference registered in relation to Icelandic isolates of *P. giardi* (0.6%) is hypothesized to result from the emergence of genotypically different subspecies due to geographic isolation.

Introduction

Myxosporeans are parasitic cnidarians that mainly infect aquatic vertebrates and invertebrates. The involvement of annelids in the life cycle of myxosporeans was first revealed by Wolf and Markiw (1984), upon their discovery that the salmonid-infecting species *Myxobolus cerebralis* Hofer, 1903 has a life cycle stage that develops in the freshwater oligochaete *Tubifex tubifex* (Müller, 1774). Since then, several studies have provided direct and indirect evidence for a two-stage life cycle of these parasites, in which a myxosporean stage that develops in a fish alternates with an actinosporean stage that develops in an oligochaete or polychaete (see Eszterbauer *et al.*, 2015). Both life cycle stages are characterized by the production of spores necessary to achieve dissemination and transmission between vertebrate and invertebrate hosts. Spores are multicellular, comprised by two to several external valve cells that form a wall surrounding one to many polar capsules, and one to many sporoplasms. Despite sharing these main morphological features, the myxospores produced in the fish host and the actinospores produced in the annelid host exhibit highly distinct morphotypes (Lom and Dyková, 2006).

Currently, actinospore morphotypes are divided into ca. 20 collective groups; the differentiation and classification of distinct types within these groups being traditionally based on morphological criteria. The latter include the shape and size of the actinospores' body and valvular processes, shape and position of the polar capsules, and number of secondary cells in the sporoplasm (Lom *et al.*, 1997; Lom and Dyková, 2006). Actinospores of the aurantiactinomyxon collective group, specifically, are defined as having three stout, semicircularly curved, leaf-like valvular processes attached to an ellipsoidal body with protruding polar capsules at the apex and containing a sporoplasm with many secondary cells (Lom and Dyková, 2006). In the past few decades, however, the implementation of molecular tools to the study of actinosporeans has revealed that morphological criteria are unreliable for performing differential diagnoses between types (e.g. Hallett *et al.*, 2002, 2004; Eszterbauer *et al.*, 2006; Xi *et al.*, 2015; Rangel *et al.*, 2016a; Zhao *et al.*, 2016). Congruently, actinospores descriptions are currently based on the combined analysis of morphological and molecular data.

Initially, life cycle studies relied solely on data acquired through means of experimental transmission between vertebrate and invertebrate hosts. The complicated logistics and time-consuming nature of this methodology, however, hinders the achievement of results.

Moreover, its reliability has been discredited by the usage of molecular tools, which show that mixed and non-spore forming infections are common and may lead to erroneous associations (Holzer et al., 2004; Atkinson and Bartholomew, 2009; Marton and Eszterbauer, 2011; Székely et al., 2014). Consequently, life cycle studies now mainly rely on sequencing of the SSU rDNA gene for inferring correspondence between myxosporean and actinosporean stages (see Eszterbauer et al., 2015).

To date, near 60 myxosporean life cycles have been elucidated (see Eszterbauer et al., 2015). Most refer to myxobolid species (Myxobolus, Henneguya and Thelohanellus) that infect fish and oligochaetes in freshwater environments (e.g. Styer et al., 1991; Kent et al., 1993; Lin et al., 1999; Eszterbauer et al., 2000, 2006, 2015; Kallert et al., 2005; Marton and Eszterbauer, 2011; Székely et al., 2014), with fewer providing information pertaining to the life cycles of myxosporean genera such as Ceratomyxa, Ceratonova, Chloromyxum, Ellipsomyxa, Gadimyxa, Hofferellus, Myxidium, Myxobilatus, Ortholinea, Paramyxidium, Parvicapsula, Sigmomyxa, Sphaerospora and Zschokkella (Grossheider and Körting, 1992; Benajiba and Marques, 1993; Yokoyama et al., 1993; Bartholomew et al., 1997, 2006; Holzer et al., 2004, 2006; Køie et al., 2004, 2007, 2008; Atkinson and Bartholomew, 2009; Rangel et al., 2009, 2017; Karlsbakk and Køie, 2012). In marine environments, polychaetes appear to be the hosts of choice (Køie et al., 2004, 2007, 2008; Rangel et al., 2009, 2011, 2016b), with a few known exceptions occurring in estuarine waters, including Portuguese Rivers (Bartholomew et al., 1997, 2006; Rangel et al., 2015, 2017). Overall, few studies have aimed to investigate myxosporean biodiversity in invertebrate communities of this geographic region, with a total of 20 actinosporean types described from the raabeia (1 type), triactinomyxon (2 types), tetractinomyxon (2 types), sphaeractinomyxon (13 types), synactinomyxon (1 type) and unicapsulaticnomyxon (1 type) collective groups (Székely et al., 2005; Rangel et al., 2009, 2011, 2015, 2016a, 2016b, 2017; Rocha et al., 2019a, b). Apart from the Raabeia type of Rocha et al. (2019a) and the Synactinomyxon type of Székely et al. (2005), which were described from the freshwater oligochaetes Ilyodrilus templetoni (Southern, 1909) and Tubifex tubifex, respectively, all the other above-mentioned types were found infecting marine oligochaetes or polychaetes in downstream estuaries. Both the triactinomyxon and tetractinomyxon types have been paired with their respective myxosporean counterparts, through the positive match of SSU rDNA sequences. The triactinomyxon types, one reported from the oligochaete Limnodriloides agnes Habrě, 1967 and the other from an undetermined species of the genus Tectidrilus Erséus, 1982, were molecularly identified as the species Ortholinea auratae Rangel et al., 2015 and O. labracis Rangel et al., 2017, which parasitize the urinary bladder of gilthead seabream Sparus aurata Linnaeus, 1758 and European seabass Dicentrarchus labrax (Linnaeus, 1758), respectively (Rangel et al., 2015, 2017). In turn, both tetractinomyxon types were described from marine polychaetes, Hediste diversicolor (Müller, 1776) and an undetermined species of Capitella Blainville, 1828, and molecularly shown to be involved in the life cycle of Ellipsomyxa mugilis (Sitjà-Bobadilla and Alvarez-Pellitero, 1993) from the gall bladder of grey mullets and Sphaerospora dicentrarchi Sitjà-Bobadilla and Alvarez-Pellitero, 1992 from systemic infections in D. labrax, respectively (Rangel et al., 2009, 2016b). To our best knowledge, further 17 myxosporean species have been reported from fishes inhabiting Portuguese rivers and estuaries, including Paramyxidium giardi (Cépède, 1906) Freeman and Kristmundsson, 2018 (see Eiras, 2016).

The myxosporean species Paramyxidium giardi was originally described as a member of the genus Myxidium, from infections in the kidney of European eel Anguilla anguilla (Linnaeus, 1758) in France (Cépède, 1906). Over time, numerous reports of this species were performed from infections occurring in multiple organs of anguillid eels worldwide (Ishii, 1915; Fujita, 1927; Hine, 1975, 1978, 1980; Copland, 1981; Treasurer and Cox, 1997), including specimens of A. anguilla from Portuguese estuaries (Ventura and Paperna, 1984; Azevedo et al., 1989; Saraiva and Chubb, 1989; Saraiva and Eiras, 1996; Hermida et al., 2008). In 1993, Benajiba and Marques Rocha et al., 2019a identified the actinosporean counterpart of P. giardi as belonging to the aurantiactinomyxon collective group. The authors used cysts from the kidney of infected A. anguilla to experiment infection on samples of Tubifex that were reported to include non-parasitized specimens, having been collected from a previously studied area (presumably in France). Recent phylogenetic analyses corroborate the potential involvement of aurantiactinomyxon, as well as of raabeia and synactinomyxon types, in the life cycle of Paramyxidium spp. (Freeman and Kristmundsson, 2018; unpublished results). Nonetheless, molecular data has not been acquired that confirms the association between this genus and any of the above-mentioned collective groups.

In this study, microscopic and molecular tools are used to describe an aurantiactinomyxon type found infecting the intestinal epithelium of the marine oligochaete Tubificoides pseudogaster (Dahl, 1960) in the lower estuary of the River Minho, Portugal. This type constitutes the first of its collective group to be reported from Portuguese waters, and only the fourth described from a marine oligochaete. Its involvement in the life cycle of Paramyxidium giardi is molecularly inferred by comparison of the newly obtained SSU rDNA sequence to that available in GenBank from Icelandic isolates used in the species re-description.

Materials and methods

Sampling sites and morphological characterization

As part of a two-year myxozoan survey conducted in the River Minho between 2015 and 2016, mud was collected at low tide from the lower estuary, near the village of "Caminha" (41° 52′ N, 08° 50′W). In the laboratory, oligochaetes were collected from the mud and placed individually in 12-well plates containing brackish water (15‰ salinity) and kept at 4 °C. Salinity was chosen according to the value range (15 to 40‰) generally registered for this parameter in the lower estuary throughout the year (see Dias et al., 2016). All oligochaetes were individually examined using the light microscope for the detection of actinosporean stages in internal tissues and cavities. Developmental stages and free actinospores were examined and photographed using an Olympus BX50 light microscope (Olympus, Japan). Morphometry was determined from fresh material, in accordance to Lom et al. (1997). Measurements include the mean value ± standard deviation (S.D.), range of variation, and number of measured actinospores (n).

DNA extraction, amplification and sequencing

Genomic DNA from infected oligochaetes was extracted using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St Louis, USA), following the manufacturer's instructions. The DNA was stored in 50 μL of TE buffer at −20 °C. The SSU rDNA gene of the actinospores was amplified using both universal and myxosporean-specific primers: the 5′-end by pairing the primer 18E (5′-CTG GTT GAT CCT GCC AGT-3′) (Hillis and Dixon, 1991) with the primers MyxospecR (5′-CAA CAA GTT GAT AGG GCA GAA-3′) (Fiala, 2006) and MYX4R (5′-CTG ACA GAT CAC TCC ACG AAC-3′) (Hallett and Diamant, 2001), and the 3′-end by pairing

the primers MyxospecF (5′-TTC TGC CCT ATC AAC TTG TTG-3′) (Fiala, 2006) and MYX4F (5′-GTT CGT GGA GTG ATC TGT CAG-3′) (Rocha *et al.*, 2015) with the primer 18R (5′-CTA CGG AAA CCT TGT TAC G-3′) (Whipps *et al.*, 2003). In turn, the 16S mitochondrial DNA (mtDNA) gene of the oligochaete host was amplified using the universal primers 16sar-L (5′-CGC CTG TTT ATC AAA AAC AT-3′) and 16sbr-H (5′-CCG GTC TGA ACT CAG ATC ACG T-3′) (Palumbi *et al.*, 2002). All PCRs were performed in 50 μL reactions using 10 pmol of each primer, 10 nmol of each dNTP, 2.0 mM MgCl$_2$, 5 μL 10× *Taq* polymerase buffer, 1.5 units *Taq* DNA polymerase (NZYTech, Lisbon, Portugal), and 3 μL (approximately 100–150 ng) of genomic DNA. The reactions were run on a Hybaid PxE Thermocycler (Thermo Electron Corporation, Milford, Massachusetts), with initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 53 °C for 45 s, and 72 °C for 90 s. The final elongation step was performed at 72 °C for 7 min. Five-μL aliquots of the PCR products were electrophoresed through a 1% agarose 1× tris-acetate-EDTA buffer (TAE) gel stained with ethidium bromide. PCR products were purified using Puramag™ magnetic beads coated with carboxylic acid groups (MCLAB, San Francisco, California, USA). Sequencing reactions were performed with the same primers used for amplification, using a BigDye Terminator v3.1 Cycle Sequencing Kit from AppliedBiosystems (Thermo Fisher Scientific, Waltham, Massachusetts, EUA), and were run on an ABI3700 DNA analyzer from AppliedBiosystems (Thermo Fisher Scientific, Waltham, Massachusetts, EUA).

Sequence assembly and distance estimation analysis

The SSU rDNA sequence of the parasite was assembled in MEGA7 (Kumar *et al.*, 2016), by aligning the partial sequences obtained for each PCR fragment. A dataset was generated according to the highest similarity scores obtained using BLAST search, and included all known *Paramyxidium* spp., as well as closely related sequences of the aurantiactinomyxon, raabeia and synactinomyxon collective groups. All SSU rDNA sequences available for more distantly related aurantiactinomyxon types were also included in the dataset. Sequences were aligned using MAFFT version 7 available online, and distance estimation was calculated in MEGA7, with the p-distance model and all ambiguous positions removed for each sequence pair.

Results

Myxozoan survey, prevalence of infection and host identification

During this study, 577 oligochaetes were isolated from the mud collected in the lower estuary of the River Minho. Myxozoan infections were determined in a total of 9 specimens, in all of which it was possible to observe mature actinospores alongside younger developmental stages. Five specimens displayed infection in the coelomic cavity by members of the sphaeractinomyxon collective group. The remaining 4 specimens displayed actinosporean development in the intestinal epithelium: two by a type of the aurantiactinomyxon collective group, and the other two by a type of the synactinomyxon collective group. The description of the aurantiactinomyxon type is presented here; descriptions of the remaining types will be published elsewhere.

All 9 oligochaete specimens displaying myxozoan infection were identified as belonging to the marine species *Tubificoides pseudogaster* (Dahl, 1960). Identification was based on combined morphological features and molecular data of the 16S mitochondrial DNA (mtDNA). Infected oligochaetes looked alike, having

similar dimensions, bifid setae and a body tegument devoid of papillae. Sequences of the 16S mtDNA were 98–100% similar to those available for the lineage II of this species, as reported by Kvist *et al.* (2010).

Characterization of the novel Aurantiactinomyxon type (Cnidaria, Myxozoa)

Description: Developmental stages of the gametogamy and sporogony phases developing in the intestinal epithelium (Fig. 1). Youngest developmental stages observed were binucleated cells (Fig. 2A). The latter divided twice to produce a set of four cells that form the initial pansporocyst, comprised by two somatic cells surrounding two generative inner cells (Fig. 2B and C). Three successive mitotic divisions and one meiotic division of the generative cells produced a final set of 16 haploid cells within the pansporocysts (Fig. 2D–F). At the end of gametogamy, these cells united two by two to form 8 diploid zygotes (Fig. 2G). Subsequent mitotic divisions of the zygotes originated 8 immature actinospores, each comprised by a sporoplasmic cell connected to three valvogenic cells and three capsulogenic cells. The valvogenic cells surrounded the capsulogenic cells and appeared positioned centrally, while corresponding sporoplasmic cells leaned against the pansporocyst's wall. Sporoplasmic cells increased in size and multiplied the number of secondary cells prior to migrating into the involucre formed by the valvogenic cells (Fig. 2H–I). At the end of sporogony, 8 mature actinospores were observed within each pansporocyst. Mature actinospores subspherical, 14.4 ± 0.6 (13.6–15.9) μm long ($n = 25$) and 12.7 ± 0.7 (11.3–13.3) μm wide ($n = 25$), entirely embraced by 3 equally sized valvular processes, leaf-like with rounded tips, 22.4 ± 2.4 (18.1–27.6) μm long ($n = 47$) and 15.5 ± 0.9 (13.3–17.0) μm wide at the base ($n = 25$). Three equally sized spherical polar capsules protruding from the anterior end of the actinospores, 2.6 ± 0.3 (1.9–3.5) μm in diameter ($n = 53$), each containing a polar tubule exhibiting 4 to 5 coils (Figs 2J–L and 3). Sporoplasm containing an undetermined number of secondary cells.

Type host: *Tubificoides pseudogaster* (Dahl, 1960) (Annelida, Oligochaeta).

Type locality: The lower estuary of the River Minho, near "Caminha" (41° 52′ N, 08° 50′ W), Portugal.
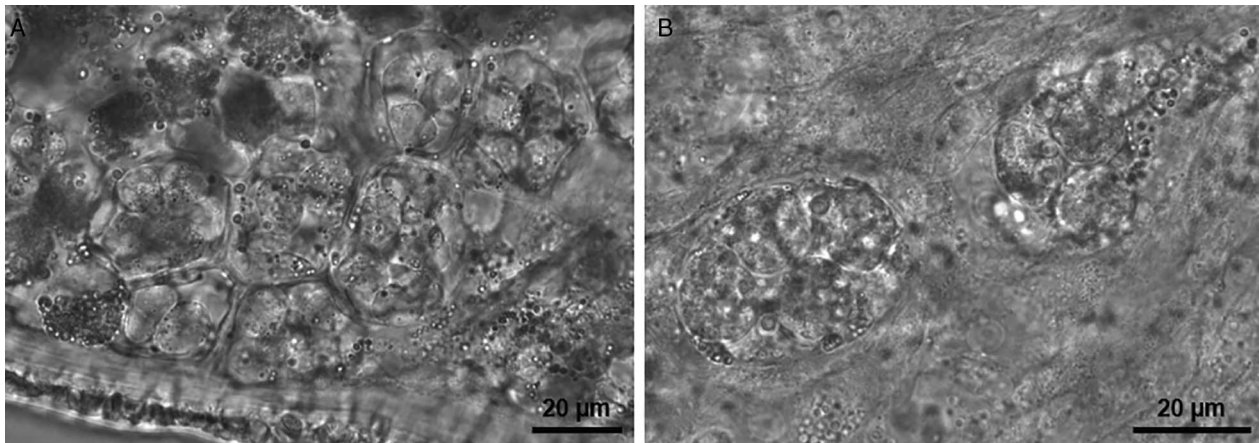
Site of infection: The intestinal epithelium.

Prevalence: 0.3% (2 infected in a total of 577 oligochaetes examined).

Deposition of material: Series of phototypes deposited together with a representative DNA sample in the Type Material Collection of the Laboratory of Animal Pathology, Interdisciplinary Centre of Marine and Environmental Research, Porto, Portugal, reference CIIMAR 2019.44.

Molecular data: One SSU rDNA gene sequence with a total of 2027 bp, deposited in GenBank with the accession no. MK635346. The latter is representative of two identical sequences that were separately obtained from the actinosporean developmental stages in the intestinal epithelium of two infected oligochaetes.

Remarks: Morphometry was determined from mature actinospores observed in both infected hosts. Morphometric variation between individual measurements was not recorded. Comparison to the ca. 39 known aurantiactinomyxon types without available molecular data revealed some morphometric similarity to the actinospores of the Aurantiactinomyxon type of McGeorge *et al.* (1997), Aurantiactinomyxon type of Xiao and Desser (1998) and Aurantiactinomyxon type 2 of Özer *et al.* (2002). Nonetheless, these types parasitize different oligochaete hosts in freshwater habitats of distant geographic locations (Canada and Scotland), further differing from the one in study in specific morphological aspects of the actinospores. Both the

Fig. 1. Light micrographs showing pansporocysts of the new type of aurantiactinomyxon developing in the intestinal epithelium of the marine oligochaete *Tubificoides pseudogaster* in the lower estuary of the River Minho.
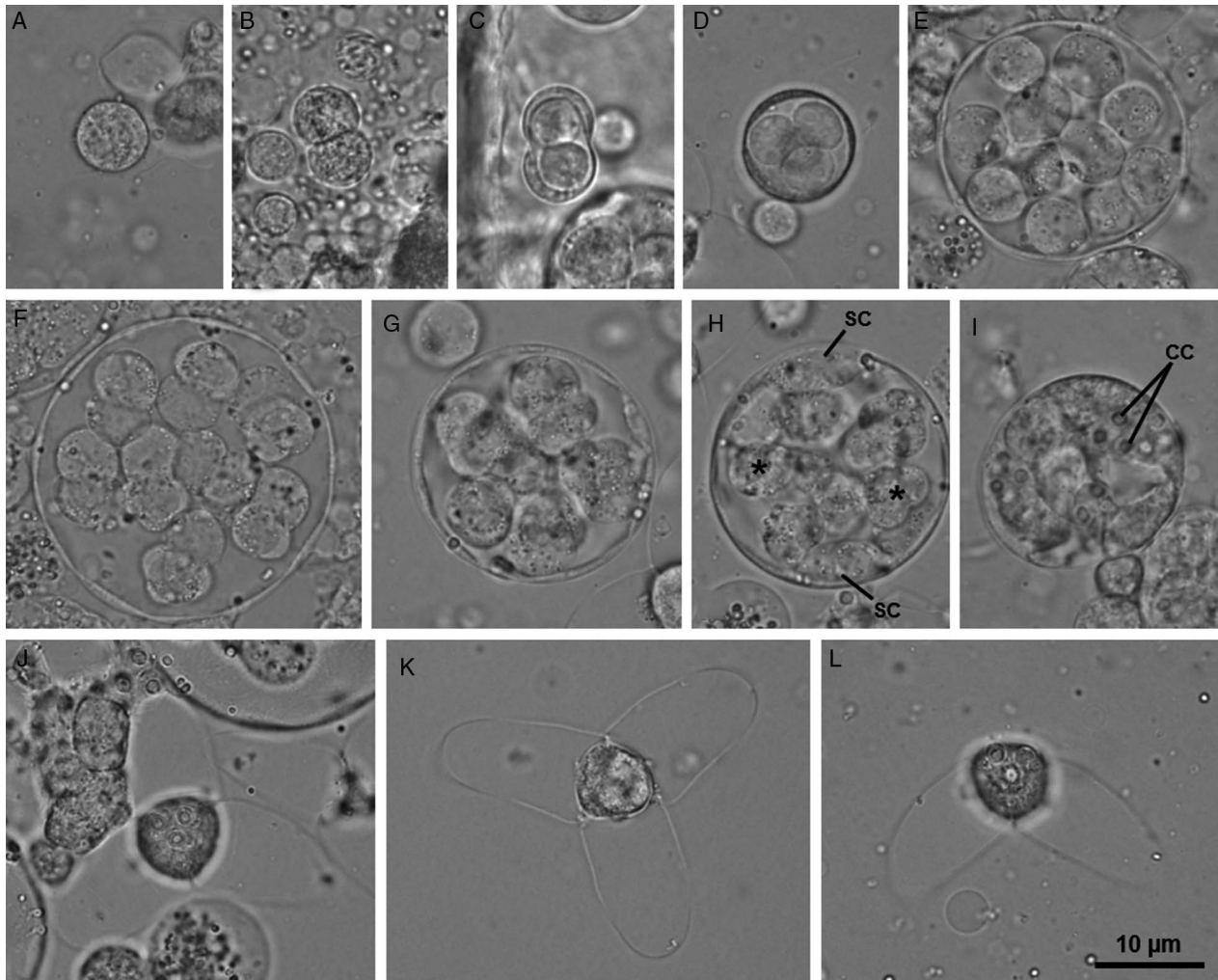
actinospores of the Aurantiactinomyxon type of McGeorge *et al.* (1997) and of the Aurantiactinomyxon type 2 of Özer *et al.* (2002) are spherical instead of subspherical, with the first further having thinner valvular processes, and the second being generally bigger. In turn, the Aurantiactinomyxon type of Xiao and Desser (1998) has pyriform polar capsules instead of spherical, and a slightly smaller actinospore body. It should be noted that no gross similarity was determined in relation to the three marine aurantiactinomyxon types reported in the literature (Hallett *et al.*, 1997), all of which are without available molecular data. In turn, morphological comparison to the freshwater Aurantiactinomyxon type of Benajiba and Marques (1993), previously reported to be the life cycle counterpart of *P. giardi*, was strongly hampered by the paucity of morphometric data given in its original description. Molecular comparison could be performed only in relation to 20 freshwater types with SSU rDNA sequences available from either their original descriptions, follow-up studies, or from the myxosporean species determined to be their life cycle counterparts through experimental transmission. The results showed significant genetic differences of the SSU rDNA sequences in all cases (ranging from 1.4% to as high as 24.8%). In turn, a high similarity value (99.4%) was obtained in relation to the sequence of *Paramyxidium giardi* available in GenBank (MH414925) from infections in the kidney of European eel *A. anguilla*. These two sequences differ in a total of 9 nucleotide positions over 2020 bp. Overall, the parasite was more related to other members of the genus *Paramyxidium*, as well as to actinosporean types known to be phylogenetically associated with the latter, namely raabeia, synactinomyxon and aurantiactinomyxon (similarity values ranged from 98.6% to as low as 81.6%). All other myxosporean sequences available in GenBank shared less than 80% similarity with the new Aurantiactinomyxon type.

Discussion

The majority of known actinosporean stages have been described based solely on morphological criteria. The implementation of molecular tools to the study of myxosporeans, however, has revealed that species having morphologically similar actinospores may be only distantly related based on SSU rDNA sequence data. In the other way around, species having actinospores with divergent morphology may be closely related (Hallett *et al.*, 2002, 2004; Eszterbauer *et al.*, 2006; Xi *et al.*, 2015; Rangel *et al.*, 2016*a*; Zhao *et al.*, 2016). In the case of aurantiactinomyxon, for instance, previous studies showed that distinct morphotypes can, in fact, share the same genotype (see Hallett *et al.*, 2002; Eszterbauer *et al.*, 2006; Zhao *et al.*, 2016). Consequently, the morphological

characterization of actinospores is important but not enough for the description of new types, making the acquisition of molecular data indispensable for the identification of actinosporean stages. Our study strengthens this contention, given that without the use of molecular data, the Aurantiactinomyxon type described here could easily have been misidentified with the Aurantiactinomyxon type 1 of Negredo and Mulcahy (2001), as these types share a strong morphological resemblance (Negredo and Mulcahy, 2001; Negredo *et al.*, 2003). Despite morphological comparison to types without molecular data having revealed significant similarity to the Aurantiactinomyxon type of McGeorge *et al.* (1997), Aurantiactinomyxon type of Xiao and Desser (1998) and Aurantiactinomyxon type 2 of Özer *et al.* (2002), correspondence to any of these types could never be established with confidence, given their different hosts, habitats and geographical origins. In addition to the known unreliability of the usage of morphological criteria for type differentiation (see Hallett *et al.*, 2002; Eszterbauer *et al.*, 2006; Zhao *et al.*, 2016), aurantiactinomyxon appear to have strict host specificity, considering that no type has been molecularly proven to infect more than a single annelid species. Thus, we suggest that extensive morphological comparisons to all types that are without molecular data and have distinct oligochaete hosts are unnecessary. Actinospores of new isolates should only be identified either by DNA match or through a comprehensive morphological and biological comparison to known types sharing the same annelid host, regardless of there being high morphological similarity to other types.

Morphological features and genetic data identified the host species of the new Aurantiactinomyxon type as being *Tubificoides pseudogaster*. This annelid species is known to host several types of sphaeractinomyxon in Portuguese estuaries (this study; Rangel *et al.*, 2016*a*) and is further reported here to host infection by a member of the synactinomyxon collective group. Despite this significant biodiversity, concomitant infections were never observed in individual specimens. This is congruent with the very low infection rates commonly reported in wild oligochaete populations (e.g. Hallett *et al.*, 2001; Negredo and Mulcahy, 2001; Atkinson and Bartholomew, 2009). In the same manner, the low value of individual prevalence of infection determined in this study (0.3%) agrees with those previously reported for aurantiactinomyxon types (e.g. Xiao and Desser, 1998; Xi *et al.*, 2015; Zhao *et al.*, 2016; Milanin *et al.*, 2017). Actinospore development was further demonstrated to follow the pattern typically reported for members of the aurantiactinomyxon collective group; development within the host was asynchronous, but sporogony within the pansporocysts was synchronous. Multiple layers of pansporocysts were present in the intestinal epithelium,
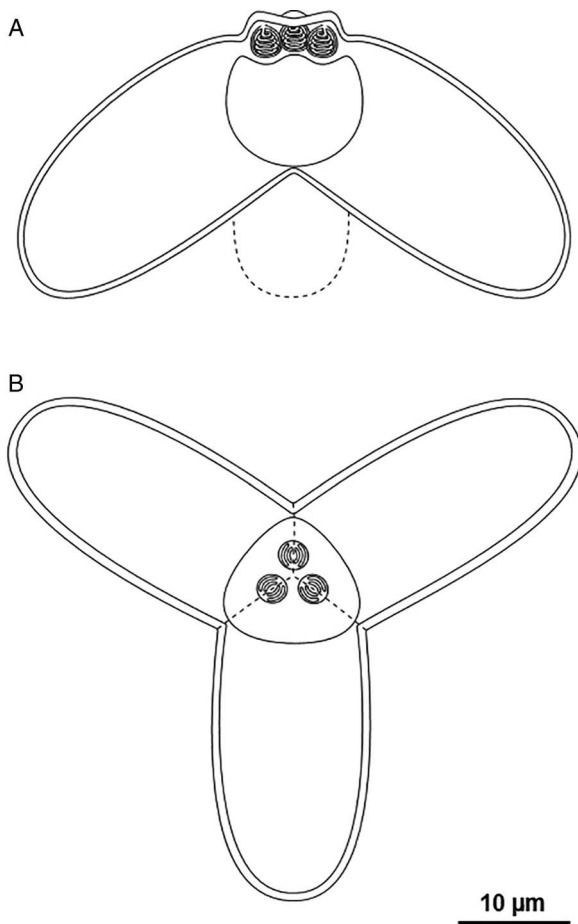
Fig. 2. Light micrographs of some developmental stages of the new type of aurantiactinomyxon found infecting the marine oligochaete *Tubificoides pseudogaster* in the lower estuary of the River Minho. (A–F) Gametogamy phase: (A) Binucleated cell; (B) Binucleated cell in cellular division; (C) Initial pansporocyst with two involucre cells and two inner cells; (D–F) Pansporocysts with four, 14 and 16 inner cells, respectively. (G–I) Sporogony phase: (G) Pansporocyst with 8 zygotes undergoing cellular divisions; (H) Pansporocyst showing the valvogenic and capsulogenic cells located centrally (*), while the sporoplasmic cells (SC) lean against the pansporocyst wall; (I) Pansporocyst containing 8 immature actinospores. Notice the capsulogenic cells; (J, K) Mature actinospores in apical view, evidencing the 3 spherical polar capsules protruding from the anterior end, and the rounded tips of the 3 equally sized leaf-like valvular processes; (L) Mature actinospore as observed in lateral view.

partially protruding into the coelomic cavity (e.g. Marcucci *et al.*, 2009; Zhao *et al.*, 2017).

Almost all aurantiactinomyxon types reported in the literature infect oligochaete species belonging to the family Naididae Ehrenberg, 1828 [currently includes the members of the former Tubificidae (Erséus *et al.*, 2008)], be it in freshwater or marine environments. Aurantiactinomyxon pavinsis constitutes the only exception, having been widely reported to infect the freshwater lumbriculid *Stylodrilus heringianus* Claparède, 1862 (see Marques, 1984; Oumouna *et al.*, 2003; Holzer *et al.*, 2004; Marcucci *et al.*, 2009). A few types have their oligochaete hosts identified only up to the genus- or family-level, but all infect naidids (see Marques, 1984; Grossheider and Körting, 1992; Benajiba and Marques, 1993), while the Aurantiactinomyxon types 7 and 11 of El-Mansy *et al.* (1998), Aurantiactinomyxon type 1 of Oumouna *et al.* (2003) and Aurantiactinomyxon type 1 of Hallett *et al.* (2006) constitute the only members of the collective group that lack host information (El-Mansy *et al.*, 1998; Oumouna *et al.*, 2003; Hallett *et al.*, 2006). Up until this study, only the three aurantiactinomyxon types described by Hallett *et al.* (1997) were known to occur in the marine environment, parasitizing the naidids *Limnodriloides toloensis* Erséus, 1984, *Pacifidrilus vanus* (Erséus, 1984) and *P. darvelli* (Erséus, 1984).

The species *Tubificoides pseudogaster* is, therefore, identified here as another member of the family Naididae serving as host for the aurantiactinomyxon collective group in marine environments.

To date, few other actinosporean types have been described from marine oligochaetes and all to parasitize naidids. These marine oligochaete-infecting types mostly belong to the sphaeractinomyxon collective group, with few representatives belonging to endocapsa and triactinomyxon. The 13 distinct types of sphaeractinomyxon recently reported from Portuguese estuaries are hosted by *T. pseudogaster*, its congener *T. insularis* (Stephenson, 1923), and *Limnodriloides agnes* Hrabě, 1967 (see Rangel *et al.*, 2016b; Rocha *et al.*, 2019b). Worldwide, the remaining known marine sphaeractinomyxon types were also reported from naidids belonging to the genera *Ainudrilus* Finogenova, 1982, *Aktedrilus* Knöllner, 1935, *Bathydrilus* Cook, 1970, *Doliodrilus* Erséus, 1984, *Heronidrilus* Erséus and Jamieson, 1981, *Limnodriloides* Pierantoni, 1903 and *Thalassodrilides* Brinkhurst and Baker, 1979 (Hallett *et al.*, 1997, 1998, 2001; Hallett and Lester, 1999). Similarly, the three marine types included within the endocapsa collective group were reported from naidids of the genera *Heronidrilus*, *Heterodrilus* Pierantoni, 1902 and *Thalassodrilides* (Hallett *et al.*, 1999, 2001). Lastly, to our best knowledge there

Fig. 3. Schematic drawing depicting a mature actinospore of the new type of aurantiactinomyxon found infecting the marine oligochaete *Tubificoides pseudogaster* in the lower estuary of the River Minho, as observed in lateral (A) and apical (B) view.

are 5 marine triactinomyxon types described in the literature from naidids of the subfamily Limnodriloidinae Erséus, 1982 (including *Limnodriloides* and *Tectidrilus* spp.) (see Roubal *et al.*, 1997; Hallett *et al.*, 2001; Rangel *et al.*, 2015, 2017). Overall, this suggests that the family Naididae played a preponderant role in the establishment and evolution of myxosporeans in invertebrate communities of estuarine and marine habitats.

Recently, a comprehensive co-phylogenetic study by Holzer *et al.* (2018) evidenced that oligochaetes and polychaetes are the most ancient hosts of myxosporeans. As such, it can be assumed that the composition, ecology, spatial distribution and susceptibility of these hosts is crucial for myxosporean evolution and diversification. The Naididae is probably the most diverse and cosmopolitan oligochaete family, being present in all biogeographic regions, including sub-Antarctic islands (Timm and Martin, 2015). Its involvement in myxosporean settlement and evolution, therefore, most likely relates to the high availability of its species in different aquatic habitats worldwide. Conjectures to other potential factors possibly influencing the reported range of myxosporean infections in naidids are difficult to be performed based on the limited information currently available for annelid-myxosporean interactions, especially in marine habitats. Future research should, therefore, aim to provide insight into the host-, parasite-, and environmental-related factors that influence myxosporean infection in annelid hosts. The acquisition of further information on this subject will, most certainly, prove to be relevant in improving our knowledge of myxosporean evolution.

Nowadays, the recognition of myxosporean life cycles is mainly based on DNA match between myxosporean and actinosporean counterparts. This molecular approach for establishing life cycle connections relies on the typically significant interspecific variability and limited intraspecific variability of the SSU rDNA gene of myxosporeans (Køie *et al.*, 2004; Atkinson and Bartholomew, 2009). In some cases, however, it can be difficult to distinguish between different species and different geographic genotypes of one single species. Molecularly inferred life cycles range from 99.4% to 100% similarity between the SSU rDNA sequences of myxosporean and actinosporean counterparts (see Eszterbauer *et al.*, 2015). These values agree with the intraspecific variability generally accepted for myxosporeans, which can range between 0–3.6%, but is usually lower than 1% (Atkinson *et al.*, 2015 and references therein). In this study, comparative molecular analysis of the SSU rDNA gene revealed the Aurantiactinomyxon type infecting *T. pseudogaster* sharing 99.4% of similarity with *P. giardi*, thus inferring its involvement in the life cycle of this myxosporean pathogen. The low genetic difference obtained (0.6%) is most likely representative of intraspecific variability due to the different geographic origin of the biological material, as previously suggested in the life cycle studies of *Myxidium truttae* Léger, 1930, *Myxobolus cultus* Yokoyama *et al.*, 1995 and *Thelohanellus kitauei* Egusa and Nakajima, 1981 (see Holzer *et al.*, 2004; Eszterbauer *et al.*, 2006; Xi *et al.*, 2013; Zhao *et al.*, 2016). In these cases, the emergence of genotypically different subspecies was hypothesized to be the outcome of geographic isolation, with subsequent evolutionary adaptation to distinct environmental factors, and possibly different invertebrate hosts. In order to confirm our assumption of intraspecific variability between geographic isolates of *P. giardi*, a myxozoan survey on *A. anguilla* from the River Minho is being prepared and will aim to acquire molecular information from myxospores. This will allow to verify if genetic variation also occurs within a given geographic location and/or between life cycle counterparts. In this context, acquiring information from molecular markers other than the SSU rDNA gene may prove necessary in order to either confirm or refute the observations of relatedness performed here. Similarly, it would be interesting to sample marine oligochaetes from Icelandic Rivers where infections by *P. giardi* are known to occur.

The information thus far available for myxosporean life cycles demonstrates that there is no obvious correspondence between actinosporean and myxosporean morphotypes. Accordingly, phylogenetic analyses show most collective groups spanning distinct myxosporean suborders, as is the case of aurantiactinomyxon (e.g. Rocha *et al.*, 2019b). Members of aurantiactinomyxon have been implicated in the life cycles of species belonging to the genera *Chloromyxum*, *Henneguya*, *Hoferellus*, *Myxobolus*, *Myxidium* and *Thelohanellus* (Styer *et al.*, 1991; Grossheider and Körting, 1992; Benajiba and Marques, 1993; Lin *et al.*, 1999; Holzer *et al.*, 2004; Eszterbauer *et al.*, 2006; Molnár *et al.*, 2010). Our results further broaden the array of genera associated with the aurantiactinomyxon collective group, by molecularly inferring the involvement of the type described here in the life cycle of *Paramyxidium giardi*. This finding is corroborated by the experimental transmission study performed by Benajiba and Marques (1993), who also identified the actinosporean counterpart of *P. giardi* as an aurantiactinomyxon type, but developing in a freshwater tubificid. Accordingly, Freeman and Kristmundsson (2018) suggested freshwater oligochaetes as the most probable annelid hosts of *Paramyxidium* spp., considering that known fish hosts [European eel and Indo-Pacific tarpon *Megalops cyprinoides* (Broussonet, 1782)], were sampled from freshwater habitats. Nonetheless, both these species are migratory fish that spawn at sea producing leptocephalic larvae that migrate into inland waters to grow. Taking into account that a significant portion of their lives is spent in fresh- and brackish waters, it is possible that

infection is acquired from marine oligochaetes inhabiting estuarine habitats, as our results suggest in the case of *P. giardi*. During our myxozoan survey, molecular evidence was also found for the development of a single type of sphaeractinomyxon in both a freshwater oligochaete species of the genus *Potamothrix* Vejdovský and Mrázek, 1903 and *T. pseudogaster*, suggesting that the myxosporean counterpart of this type uses a fish host that is not entirely restricted to freshwater, most probably a grey mullet (unpublished results). Congruently, the possibility of *P. giardi* having acquired both marine and freshwater oligochaetes as hosts cannot be disregarded, considering that the ecology of the European eel allows it to contact with intrinsically distinct invertebrate communities throughout its life.

The genus *Paramyxidium* Freeman and Kristmundsson, 2018 encompasses species that, whilst being morphologically similar to *Myxidium*, differ by having histozoic development in various tissues of fish from Elopomorpha (see Freeman and Kristmundsson, 2018). The European eel is the sole representative of Elopomorpha in Portuguese waters. Thus far, it has been recognized to host four myxosporean species in this geographic location, including *P. giardi* from infections that occur in multiple organs (Ventura and Paperna, 1984; Azevedo *et al.*, 1989; Saraiva and Chubb, 1989; Saraiva and Eiras, 1996; Hermida *et al.*, 2008). The study by Freeman and Kristmundsson (2018), however, showed that *P. giardi* is not systemic; while myxospore morphology is conserved and, therefore, unreliable for identification at the species-level, the site of infection appears to be an important diagnostic feature for the group. As such, it is expected that the myxozoan survey of *A. anguilla* in Portuguese Rivers will lead to the discovery of other *Paramyxidium* spp.

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