Macroparasites of allis shad (*Alosa alosa*) and twaite shad (*Alosa fallax*) of the Western Iberian Peninsula Rivers: ecological, phylogenetic and zoonotic insights

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Abstract

Samples of anadromous *Alosa alosa* (Clupeidae) (n= 163), and *Alosa fallax* (Clupeidae) (n= 223), caught in Western Iberian Peninsula Rivers from 2008 to 2013, were examined for buccal, branchial and internal macroparasites, which were identified using morphological and molecular methods. *Alosa alosa* were infected with *Anisakis simplex* s.s., *Anisakis pegreffii*, *Hysterothylacium aduncum*, *Rhadinorhynchus pristis*, *Mazocraes alosae*, *Hemiurus appendiculatus*, *Ceratothoa italica* and an unidentified ergasilid copepod. *Ceratothoa italica* represents a new host record for *A. alosa*. *Alosa fallax* were infected with *A. simplex* s.s., *A. pegreffii*, *H. aduncum*, *H. appendiculatus*, *Clavellisa emarginata* and an unidentified cymothoid isopod. This is the first report of *C. italica*, *C. emarginata* and *M. alosae* in the Iberian Peninsula. The phylogenetic positions of *M. alosae*, *H. appendiculatus* and *C. emarginata* were assessed using 18S and 28S rRNA; our contributions provide a better understanding of the phylogenetic relationships within their groups. Qualitative and quantitative differences in the parasite faunas of these two shad species are consistent with different feeding strategies. The results provide information about host migration behaviour and transmission pathways through diet during the marine trophic phase of the shad's life cycle, and their roles as paratenic or final hosts and transporters of parasites between seawater and freshwater environments. The zoonotic parasites *A. simplex* s.s. and *A. pegreffii* pose a risk for consumers or riverine mammals (e.g. European otter). The use of parasites as biological tags for shad stocks in Western Iberian Rivers could be a useful approach in multidisciplinary studies concerning fish stock delimitation and characterization.

Key words

Alosa spp., macroparasites, *Anisakis* spp., phylogeny, freshwater, Iberian Rivers.

1. Introduction

The European shads, allis shad, *Alosa alosa* (Linnaeus, 1758), and twaite shad, *Alosa fallax* (Lacépède, 1803), are anadromous members of the family Clupeidae that have a pelagic behaviour at sea, especially in areas close to the shore, migrating into rivers to reproduce (Aprahamian et al. 2002; Baglinière et al. 2003; Acolas et al. 2004). Their original distribution extends from Iceland and Norway in the North to Morocco in the South (Aprahamian et al. 2002). *Alosa alosa* once occurred in the Mediterranean Sea, but is thought to have disappeared from these waters. *Alosa fallax*, however, still inhabits the Mediterranean, being especially abundant in the Aegean Sea; its occurrence is scarce in the Marmara and Black Seas (Baglinière 2000; Aprahamian et al. 2002; Ceyhan et al. 2012; La Mesa et al. 2015). Due to direct and indirect anthropogenic impacts (such as construction of dams on rivers that prevent spawning migrations, habitat loss, water pollution and overfishing) (Costa et al. 2001; Aprahamian et al. 2002; Baglinière et al. 2003; Doadrio et al. 2011; MIGRANET 2012), European shads have suffered significant reductions in their distribution and abundance in river basins, which led to their inclusion in Appendix III of the Bern Convention, Annexes II and V of the EU Habitats Directive. Moreover, in the case of *A. alosa*, as a result of its greater degree of anadromy and lower ecological plasticity (Baglinière 2000), marine catches and genetic loss appear to be accelerating population decline (OSPAR 2009; Jolly et al. 2012; Rougier et al. 2012). This decline was also observed in populations from Northwestern Iberian Peninsula (Galicia and North of Portugal) rivers (e.g. Minho and Ulla), so that both species are considered as endangered by some authors (Solórzano 2004; Cabral et al. 2006). Despite the fact that these populations are currently scarce, they still have great local importance from economic, recreational, cultural and ecological points of view both in Galician and north Portuguese river basin counties (XUNTA 2008; Mota and Antunes 2011; Pereira et al. 2013). Stable populations of both shads coexist in the River Minho (Mota et al. 2015). Formerly, *A. alosa* was reported

in the River Ulla, but this was probably a case of misidentification, since a monospecific population of *A. fallax* was recently discovered in this river (Cobo et al. 2010; Nachón et al. 2013; Silva et al. 2013). On the other hand, shads still migrate upstream in the River Mondego, despite the construction of a dam that restricted their spawning migration to the last 35 km of the river (Costa et al. 2001).

The spawning migration of adults usually starts in the late winter and continues throughout the spring. Shad upstream reproductive migration roughly occurs from March to July in the rivers referred to above. In addition, spawning takes place in fresh water during the night in both the tidal and non-tidal parts of the river (Aprahamian et al. 2002). The larvae and juveniles grow in fresh water and migrate to the ocean in their first year of life (Taverny et al. 2000; Aprahamian et al. 2002; Mota and Antunes 2012). After several years on marine feeding grounds (Taverny and Elie 2001a) they return to estuaries and begin their upstream spawning migration. During this upstream migration, most *A. alosa* do not feed (Mota et al. 2015), whereas at least part of the population of *A. fallax* may feed actively (Nachón et al. 2013).

There is a considerable lack of knowledge on the adult marine life stage of both *Alosa* species (Taverny et al. 2000; OSPAR 2009). Previous studies reported that both shads are coastal in their habit with different diet and depth preferences (Taverny et al. 2000; Taverny and Elie 2001a, 2001b; Trancart et al. 2014; La Mesa et al. 2015). Whilst *A. alosa* is mainly a zooplanktophagous species with small fish forming a less important part of the diet, *A. fallax* is predominantly ichthyophagous with small crustaceans as secondary prey (Assis et al. 1992; Taverny and Elie 2001b; Maitland and Lyle 2005; Ceyhan et al. 2012; Skóra et al. 2012). In the Bay of Biscay, both species remain in a range of about 30 miles away from the coast and above a depth of 115 m (Taverny and Elie 2001a). A recent study corroborated the presence of both species of shad at depths between 15 to115 m along the French coast (Trancart et al. 2014).

In NW Iberian waters this depth distribution might be even deeper for both species, with reports of their occurrence between 9 to 390 m (Bao et al. 2015).

By feeding on zooplankton during their marine phase or even in the estuaries during their upstream migration, anadromous fish acquire marine parasites, e.g. hemiurid digeneans, acanthocephalans and nematodes, which they accumulate and transport to freshwater ecosystems in their returning migration (MacKenzie 1987). Parasites can be used as indicators of trophic relationships and fish population structure (Williams et al. 1992). Thus, any information concerning the parasite ecology of both anadromous shad populations will be important to understand their natural life cycles.

The present paper provides new information about the macroparasite communities of spawning individuals' *A. alosa* and *A. fallax* caught in Western Iberian Peninsula Rivers; the specific aims were to: (1) identify the parasite species present, (2) obtain and compare parasite burdens between shad species and years, (3) assess the phylogenetic position of those parasites not present in the genetic database, (4) discuss the ecological implication of these findings within the life cycle of both parasites and shad species and (5) stress the zoonotic relevance due to the presence of *Anisakis* spp. in anadromous shads.

2. Materials and methods

2.1. Sampling

Two studies in relation to the ecology of *A. alosa* and *A. fallax* in the Rivers Minho and Ulla provided the opportunity to study the community richness and diversity of the macroparasite fauna (platyhelminthes, nematodes, acanthocephalans and crustaceans) of both shad species caught in estuaries and freshwater environments. In addition, individuals of *A. alosa*, caught by professional fishing in the River Mondego, made possible to broaden the study of the macroparasite fauna of shads of the Western Iberian Peninsula Rivers.

Shad were caught during their upstream spawning migration from 2008 to 2013 in the Rivers Minho, Ulla and Mondego by experimental, professional (i.e. trammel net) or sport fishing (i.e. rod and reel) (Table 1). For more detailed information on study area and sampling sites see Bao et al. (2015) and references therein.

2.2. Collection of parasites

Total length (measured in mm) was registered for each fish. Due to some fish having been previously processed, whole specimens were not always available for the location and identification of the parasites. Information and results from accessible shad samples are shown in Tables 1 and 2. When whole fish were available (Table 2), visual observation of the buccal cavity and gills for macroparasites was carried out. The head and branchial region was separated from the rest of the body, which was frozen for later visual inspection. The fish body was mostly available for internal parasites (Table 2). The stomach and internal organs were removed and conserved either frozen or in 70% ethanol. The branchial region was dissected and the gill arches extracted and examined under a stereomicroscope for macroparasites. The contents of the stomach were examined under a stereomicroscope and the surfaces of the other visceral organs were macroscopically examined for macroparasites. Later, the entire viscera and flesh of some shads were digested following artificial digestive methods in order to separate the remaining nematode larvae (Bao et al. 2015). All macroparasites found were removed and placed in 70% ethanol for further morphological and molecular diagnosis.

2.3. Parasite identification

2.3.1 Morphological identification

Parasites were identified morphologically under stereomicroscope using the following publications: Berland (1989) for nematodes, Horton (2000) for isopods, Kabata (1979, 1992)

for copepods, Yamaguti (1968), Dawes (1968) and Akmirza (2013) for monogeneans, Dawes (1968) and Gibson and Bray (1979, 1986) for digeneans, and Yamaguti (1963) and Gregori et al. (2013) for acanthocephalans.

2.3.2 Molecular identification

The following groups were selected for molecular analysis: 72 *Anisakis* spp. larvae 46 from *A. alosa* and 26 from *A. fallax*; 19 non-anisakid nematodes (*Hysterothylacium aduncum)* 15 from *A. alosa* and 4 from *A. fallax*; a single copepod (*Clavellisa emarginata*) from *A. fallax*; 18 digeneans (*Hemiurus appendiculatus*) 5 from *A. alosa* and 13 from *A. fallax*; 3 monogeneans (*Mazocraes alosae*) from *A. alosa*; and 13 acanthocephalans (*Rhadinorhynchus pristis*) from *A. alosa.*

Genomic DNA was isolated using Qiagen DNeasy™ Tissue Kit according to manufacturer's instructions. The identification of the parasites was performed amplifying different genes, depending on the group. For nematodes the entire ITS (ITS1, 5.8S rDNA gene and ITS2) was amplified using the forward primer NC5 (5´- GTA GGT GAA CCT GCG GAA GGA TCA TT-3´) and reverse primer NC2 (5´- TTA GTT TCT TTT CCT CCG CT-3´) (Zhu et al. 2000). Partial small subunit ribosomal RNA gene (18S rRNA or SSU) was selected to identify monogeneans, digeneans, acanthocephalans and the siphonostomatoid copepod, using the universal primers 18SU467F (5'- ATC CAA GGA AGG CAG CAG GC-3') and 18SL1310R (5'- CTC CAC CAA CTA AGA ACG GC-3') (Suzuki et al. 2008). Moreover, the large subunit ribosomal RNA gene (28S rRNA or LSU) was partially amplified on the digeneans with the primers LSU-5 (5'-TAG GTC GAC CCG CTG AAY TTA AGC A-3') and 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3') (Olson et al. 2003).

PCR reactions were performed in a total volume of 25 µl, containing 1 µl of genomic DNA (20-40 ng), PCR buffer at 1x concentration, 1.5 mM $MgCl_2$, 0.2 mM nucleotides (Roche

Applied Science), 0.3 µM primers and 0.025 U/µl Taq DNA polymerase (Roche Applied Science). The cycling protocol used to amplify the ITS1, 5.8S and ITS2 genes from anisakids was as follows: 2 min at 94 ºC, then 35 cycles of 30 s at 94 ºC, 30 s at 55 ºC and 75 s at 72 ºC, followed by a final elongation of 7 min at 72 ºC. The cycling protocol for 18S and 28S rRNA genes was 3 min at 94°C, 40 cycles of 30 s at 94°C, 45 s at 56°C and 2 min at 72°C, followed by 7 min extension hold at 72°C. All PCRs were carried out in a Tgradient thermocycler (Biometra) and a negative control (no DNA) was included for each set of PCR reactions. Positive PCR products were purified for sequencing using ExoSAP-IT© (USB corporation). Sequencing was performed in a specialized service (Secugen, Madrid). All sequences were subjected to an identity search using Basic Local Alignment Search Tool (BLASTn) through web servers of the National Center for Biotechnology Information (NCBI) database. Sequences obtained in this study were deposited in GenBank under accession numbers (Table 3).

2.4. Phylogenetic analysis

Since the sequences of the digenean *H. appendiculatus*, the monogenean *M. alosae* and the copepod *C. emarginata* were not present in the genetic database, phylogenetic trees were built to assess their phylogenetic position. Sequences that displayed highest matches on BLASTn were downloaded to infer the phylogeny of the monogenean polyopisthocotyleans (n= 37 for SSU), the higher digenean plagiorchiideans (n= 46 for SSU and n= 49 for LSU) and the siphonostomatoid copepods (n= 41 for SSU). The digenean *Petasiger phalacrocoracis* (accession number AY245709) was used as an outgroup for the monogenean tree; the diplostomid *Schistosoma haematobium* (accession number Z46521) was used as an outgroup for the digenean trees; and the calanoid copepod *Calanus finmarchicus* (accession number AF367719) was used as the outgroup for the siphonostomatoids.

The different subsets of sequences were aligned using Mafft implemented in Geneious (version 7.1.7). GBlocks (Castresana 2000) was then used to identify and remove highly divergent regions and poorly aligned positions, which are common features in these ribosomal genes. Afterwards, the best model of nucleotide substitution was selected under the corrected Akaike information criterion (Akaike 1974) in Modeltest2 (Darriba et al. 2012). In all the subsets the general time reversible model with estimates of invariant sites and gamma-distributed model (GTR+I+G) was chosen. For the digenean *H. appendiculatus*, datasets of partial SSU sequences (n= 46), partial LSU sequences (n= 53), and concatenated SSU and LSU sequences (n= 31) were analysed. Maximum likelihood (ML) and Bayesian Inference (BI) analysis were run in Geneious 7.1.7 implemented with PhyML and MrBayes. For ML phylogenetic trees, nodal support was estimated with a bootstrap procedure with 1000 replicates (Felsenstein 1985). Bayesian analysis was run twice and Log-likelihoods were estimated over 1.100.000 generations using 4 Markov Chain Monte Carlo (MCMC) chains, with every 200th tree saved. Nodal support for BI trees was given by posterior probabilities.

2.5. Quantitative descriptors

The quantitative descriptors of parasite infections, such as prevalence, mean abundance and mean intensity, were calculated as described in Bush et al. (1997). Prevalences of infection were compared using the chi-squared test or Fisher's exact test, and abundances using the non-parametric Mann-Whitney *U*-test or the Kruskal-Wallis test. Statistical analyses were carried out using GraphPad Prism 6. The level of statistical significance was set at 95% ($p < 0.05$).

3. Results

3.1 Parasite identification

Ten parasite taxa were found, eight of which were identified to species using morphology and genetics (Figures 1 and 2). Five of these had perfect matches against the genetic database (100% homology), and three were not present in the genetic database (see section 3.3, and Table 3 for accession numbers). Two parasites – the unidentified copepod from *A. alosa* and the unidentified isopod from *A. fallax* – failed to amplify the selected genes and thus morphological identification was not confirmed.

The digenean *H. appendiculatus* was common to both species from all sampling rivers, as were the three nematode species. The larval anisakid nematodes were found to represent two species: *A. simplex* s.s. and *A. pegreffii*. In addition, the acanthocephalan *R. pristis* was identified in *A. alosa* from the Rivers Minho and Mondego. The monogenean *M. alosae*, the cymothoid isopod *C. italica* and an unidentified ergasilid copepod (genus *Ergasilus*) were found in *A. alosa* from the River Minho. The lernaeopodid copepod *C. emarginata* and an unidentified mouth-dwelling isopod (family Cymothoidae) were found in *A. fallax* samples from the River Minho.

3.2 Comparative infection data.

The quantitative descriptors of parasitic infections in the two shad species (all samples combined) using visual inspection methods are shown in Table 2. *Anisakis* spp. larvae and *M. alosae* were the most prevalent parasites of *A. alosa*, whereas *H. appendiculatus* and *H. aduncum* were the most common species infecting *A. fallax*. A single specimen each of the isopod *C. italica* and an unidentified parasitic copepod were found on *A. alosa*, and a single specimen of an unidentified isopod was found on *A. fallax*.

A comparative analysis of the three common parasites of *A. alosa* and *A. fallax* (all samples combined) was performed (Table 4). Mean abundance was significantly higher for *H. appendiculatus*, *H. aduncum* and *Anisakis* spp. in *A. alosa,* while prevalence was significantly

higher for *H. appendiculatus* and *Anisakis* spp. in *A. alosa,* but the difference was not statistically significant for *H. aduncum*.

A similar comparison was carried out with infection values of the same parasite species found in both shad species from the River Minho in 2011 (Table 5). Prevalence and mean abundance were significantly higher for *H. appendiculatus* and significantly lower for *Anisakis* spp. in *A. fallax* than in *A. alosa*. Differences in prevalence and mean abundance of *H. aduncum* between host species were not statistically significant.

Infection levels of the three most common parasites were compared for *A. alosa* of the River Minho for the years 2009, 2010 and 2011 (Table 6). Mean abundance decreased significantly during the sampling years for all parasites but the only significant decrease in prevalence was for *H. aduncum*. Differences in mean abundance were statistically significant for all three parasites.

3.3. Molecular phylogeny

A total of 500 bp were aligned for partial SSU gene (37 species) for the monogenean *M. alosae*. Of these, 396 were unambiguously alignable (77%). Phylogenetic trees constructed with ML and BI displayed similar topologies for the basal groups, but differed in the resolution of the most divergent order Mazocraeidea (Figure 3). The monogenean identified on the gills of *A. alosa* belonged to the Mazocraeidae family with high ML bootstrap values 84.8 and BI posterior probability of 0.99. The topology obtained is in agreement with previous works, despite only a small region of the SSU gene being analysed. Polystomatids (parasites of tetrapods) are the sister group of the oligonchoinea (parasites of fishes). Among the latter group *Pseudohexabothrium taeniurae* (parasite of sharks) displays a basal position in relation to the parasites of teleost fishes (Order Mazocraeidea). Within this group the family Mazocraeidae, where the parasite found in the shad *A. alosa* is located, is the sister group of all the others.

For the digenean *H. appendiculatus*, a total of 349 sites were aligned for partial SSU gene (46 species), though only 229 were unambiguously aligned and informative (65%), while 450 sites were aligned for partial LSU gene (53 species), but only 233 were unambiguous (51%). The interrelationships found with the SSU sequences were better resolved than in the monogenean tree, showing that this region is more variable for digeneans. The phylogenetic trees constructed showed similar topologies for both genes when analysed individually (Figure 4 and Figure 5), although deeper nodes were less resolved on LSU tree (Figure 5). The combined analysis of LSU and SSU (Figure 6) shows a robust phylogeny where most of the relationships are well supported. The basal Plagiorchiida (named after Olson et al. 2003) are grouped together, with the monophyletic suborders Bivesiculata and Transversotremata being the basal lineages, and the monophyletic suborder Hemiurata as the sister clade. Within it, the superfamily Azygioidea (represented by *Otodistomum cestoides*) is basal to the superfamily Hemiuroidea. The relationships found within the latter lineage show two main clades, as shown on the individual LSU and SSU trees: the first clade comprises the families Derogenidae (polyphyletic), Hirudinellidae, Syncoelidae, Accacoelidae, Sclerodistomidae and the monophyletic Didymozoidae; while the second comprises the polyphyletic families Lecithasteridae and Hemiuridae. Independently of the gene analysed, the digenean found in the digestive tract of *Alosa* falls within a supported Hemiuroidea lineage constituted by the subfamilies Lecithochirinae, which is monophyletic and sister clade to: Plerurinae (polyphyletic), Dinurinae (polyphyletic), Elytrophallinae (monophyletic) and Hemiurinae (monophyletic). Within the latter hemiurid clade *H. appendiculatus* consistently appears basal to the subfamilies Dinurinae and Elythrophallinae. In the SSU tree, this digenean is basally located in the Hemiurinae subfamily with *Hemiurus communis* (95% bootstrap, Figure 4) in a supported clade (65.6% and 0.91 posterior probability) that included other Hemiuridae subfamilies (Dinurinae and Elytrophallinae).

For the copepod *C. emarginata* a total of 804 bp were aligned (41 species) and 788 bp were unambiguously aligned (98 %) (Figure 7). The topologies obtained by ML and BI were equivalent and therefore we added the Bayesian posterior probabilities to the ML tree. The phylogenetic relationships obtained show a low bootstrap support and posterior probabilities on the deeper nodes. Nonetheless, the more divergent relationships are well resolved and locate the copepod *C. emarginata* within a highly supported clade (100% bootstrap and posterior probability of 1) that includes the family Sphyriidae (represented by *Paeon elongatus*) basal to the members of the family Lernaeopodidae. *Clavellisa emarginata* falls within the Lernaeopodidae family with high bootstrap value (93.6%) and posterior probabilities (1).

4. Discussion

4.1 Metazoan parasite fauna

To date, parasitological studies on European shads have been done mainly on adult specimens from rivers or estuaries flowing into the Bay of Biscay, Celtic Sea, North Sea and Baltic Sea for *A. alosa*, and from the same geographical areas, plus Mediterranean Sea, for *A. fallax* (Aprahamian et al. 2002 and references therein; Doherty et al. 2004; Rokicki et al. 2009). Recently, Mota et al. (2015) cited the presence of *A. pegreffii*, *H. aduncum* and *R. pristis* in *A. alosa* from the River Minho. In addition, Bao et al. (2015) reported the presence of mixed infections of *A. simplex* s.s. and *A. pegreffii* for the first time in both shad species from Western Iberian Peninsula Rivers. Notwithstanding the foregoing, this is the first report of the quantitative descriptors and ecology aspects of the metazoan parasite fauna of both anadromous shad species in Western Iberian Peninsula Rivers. In addition, to the best of our knowledge this is the first time that *C. italica* has been reported on *A. alosa* and, additionally, in a clupeid host. Moreover, the presence of *C. italica*, *C. emarginata* and *M. alosae* is reported for the first time in the Iberian Peninsula. In general, the parasite fauna of *A. alosa* and *A. fallax* was

characterized by anisakid and rhapidascarid nematodes and hemiurid digeneans, whilst acanthocephalans, monogeneans, copepods and isopods were less important.

4.1.1. *Mazocraes alosae* (Hermann, 1782).

The mazocraeid monogenean *Mazocraes alosae* is a specific parasite of the gills of *Alosa* spp. It was reported from *A. alosa* and *A. fallax* by Dawes (1968). Its occurrence in European shads was recorded in the Gironde system (France), River Rhine (Germany and Switzerland), River Barrow and Waterford estuary (Ireland), and in the Irish Sea, River Severn and estuary and at Plymouth and Aberdeen (Britain) (Dawes 1968; Aprahamian et al. 2002 and references therein), Caspian, Black and Azov Seas (Mamaev 1982; Özer et al. 2013) and the NE Aegean Sea (Akmirza 2013). Monogeneans are parasites which may cause serious damage in both wild and farmed fish (Dezfuli et al. 2007; Lia et al. 2007; Akmirza 2013 and references therein).

4.1.2. *Hemiurus appendiculatus* (Rudolphi, 1802).

Digenetic trematodes of the family Hemiuridae usually occur in the stomachs of marine and freshwater teleosts and the lungs of piscivorous sea-snakes. They have a unique organ - a "tail" or *ecsoma* at the posterior region of the body - which has the ability to be retracted within the body or *soma*. This structure is thought to be a feeding organ extruded only when conditions in the stomach are favourable (Gibson and Bray 1979, 1986). According to Gibson and Bray (1986), *H. appendiculatus* appears to be restricted to *A. alosa* and *A. fallax* (especially in the latter) in Mediterranean and European Atlantic waters as far north as southern Norway (Moravec 2004). In addition, it was previously reported in *A. fallax* of the Atlantic coast of Africa and the Portuguese Coast (Rodrigues et al. 1972 cited in Aprahamian et al. 2002).

H. appendiculatus showed higher values of prevalence and abundance in *A. alosa* (Table 4), even though it was found to be the most prevalent and abundant parasite species of *A. fallax* following visual methodologies (Table 2).

4.1.3. *Anisakis* spp. (*Anisakis simplex* sensu stricto (Rudolphi, 1809); *Anisakis pegreffii* (Campana-Rouget & Biocca, 1955)).

The presence of mixed infections of *A. simplex* s.s. and *A. pegreffii* in both *Alosa* spp. was previously reported and discussed in Bao et al. (2015). Herein, we report the quantitative descriptors of *Anisakis* spp. infection using visual methodologies in order to compare infection values with other parasite data obtained using visual methods. Thus, *Anisakis* spp. was clearly the most common and abundant parasite of *A. alosa* (Table 2) (Bao et al. 2015), whilst this predominance was not so evident for *A. fallax*, which showed low prevalence and abundance values following visual methods (Table 2), but can reach values of up to 83% prevalence and 44.17 mean abundance using a combination of visual and digestive methods (see Bao et al. 2015).

4.1.4. *Hysterothylacium aduncum* (Rudolphi, 1802).

Many teleost fish species have been shown to be definitive hosts of *H. aduncum*, while crustaceans (copepods, amphipods, isopods, euphausiids and mysids) act as obligate intermediate hosts. In addition, non-crustacean invertebrates (ctenophores, chaetognaths, polychaetes and ophiuroids), as well as fish, may act as obligate second intermediate or transport hosts (Smith 1983; Køie 1993; Shih and Jeng 2002). The presence of both the fourth larval stage and adults of *H. aduncum* in both *Alosa* species confirms their role as definitive hosts in the life cycle of this rhapidascarid nematode. Additionally, *H. aduncum* was shown as a component parasite of both shad species, but was more abundant in *A. alosa* (Table 4).

4.1.5. *Rhadinorhynchus pristis* (Rudolphi, 1802).

The presence of *R. pristis* in *A. alosa* was previously recorded in one specimen caught in the River Rhine by Golvan (1969) and also by Mota et al. (2015) in the River Minho. This acanthocephalan has a complex life cycle, using euphausiids (e.g. *Nyctiphanes couchii*) as

intermediate hosts and marine fish as definitive hosts in the NW Atlantic area (Gregori et al. 2013). This parasite was only found in *A. alosa*, which is consistent with its feeding habits.

4.1.6. *Ceratothoa italica* (Schioedte & Meinert, 1883).

The mouth-dwelling isopod *C. italica* has a direct life cycle which involves fish of the family Sparidae as final hosts. It was previously reported in Mediterranean and North-West African waters (Horton 2000; Sala-Bozano, 2012). European shads can migrate long distances from the rivers where they born and reproduce to their feeding grounds at sea (Sabatié 1993). Our results might suggest a similar migrating behaviour; a movement from the Western Iberian Rivers to southern productive sea areas of the Coast of Portugal, which is the northern limit of distribution of this parasite and where shads may have better chances of infection. This hypothesis was previously suggested by Bao et al. (2015).

4.1.7. *Clavellisa emarginata* (Krøyer, 1837).

The lernaeopodid copepod *C. emarginata* is a specialist parasite of the gills of clupeid fish belonging to the genera *Alosa*, *Caspialosa* and *Clupeonella* (Kabata 1992). Its occurrence includes the North, Irish, Mediterranean, Black and Azov Seas, the lower reaches of the Danube, Southern Bug, Dnieper and Don and also in the Gironde system (France), River Rhine (Germany), River Barrow and Waterford estuary (Ireland), River Severn and estuary and at Plymouth (Britain), and is probably even more widespread (Kabata 1992; Aprahamian and references therein, 2002).

4.2 Phylogenetic position of the parasites

4.2.1. *Mazocraes alosae* (Hermann, 1782).

Monogeneans are primarily ectoparasites of fishes, with few exceptions, and their mode of feeding splits the two major clades: the Monopisthocotylea (that erode the epidermis) and the Polyopisthocotylea (specialized to feed on blood) (Olson and Tkach 2005). Large and small ribosomal RNA (LSU and SSU) data shows that polyopisthocotylean parasites (also known as

Heteronchoinea) are monophyletic with a basal split between radiations in tetrapods (Polystomatoinea) and fishes (Oligonchoinea) (Boeger and Kritsky 1997; Littlewood et al. 1999; Mollaret et al. 2000; Jovelin and Justine 2001; Olson and Littlewood 2002; Olson and Tkach 2005). The latter clade shows a split of lineages with the oligonchoinean parasites of chondrichthyans (chimaeras: Chimaerocolidae, sharks: Hexabothriidae) as a sister group of the oligonchoinean parasites of teleosts (Mazocraeidea) (Jovelin and Justine 2001). The more derived oligonchoineans, the mazocraeids, are a monophyletic clade with poorly supported interrelationships, as observed herein, either using morphology or phylogeny (Jovelin and Justine 2001; Olson and Littlewood 2002). Nonetheless, within this group of parasites the basal position of the Mazocraeidae is consistent, being mainly parasites of clupeid fishes, as well as scombrids (Mollaret et al. 2000; Jovelin and Justine 2001).

Even though only a small region of the SSU gene was analysed herein, the phylogeny obtained is in agreement with previous studies (Mollaret et al. 2000; Jovelin and Justine 2001, Olson and Littlewood 2002), placing *Mazocraes alosae* consistently as a polyopisthocotylean monogenean (Order Mazocraeidea, Family Mazocraeidae). The low bootstrap values or posterior probabilities as well as the small length of the terminal branches in the sister clade of Mazocraeidae (Figure 3) are noteworthy. These features are the result of low divergent rates in this lineage - four times lower than those measured for the Polyonchoinea lineage in analyses of the complete SSU sequences (Olson and Littlewood 2002) - and the main reason for the lack of support for higher interrelationships in mazocraeids. Fortunately, the basal relationships of the mazocraeids are well resolved and the monogenean found in the gills of *A. alosa* undoubtedly belongs to the basal Mazocraeidae.

This is the first time that a sequence of 18S rRNA from a mazocraeid obtained from its clupeid host has been deposited on GenBank, since the other mazocraeid available on GenBank for this region, *Kuhnia scombri,* was isolated from the scombrid *Scomber scombrus* (see Olson and

Littlewood 2002). This genetic information is valuable since it allows testing of the hypothesis suggested by Bychowsky (1961) and reassessed with molecular data by Mollaret et al. (2000). These authors suggested that the family Mazocraeidae is parasitic in the relatively early divergent teleost fish family Clupeidae, with later host-switching to the scombrids, represented by the species *Kuhnia* and *Grubea* (Mollaret et al. 2000; Jovelin and Justine 2001). Our results support this hypothesis, since the mazocraeids *M. alosae* and *K. scombrii* constitute a monophyletic group basal to all other polyopisthocotyleans, suggesting that they diverged earlier. Moreover, it is also in agreement with the systematic work of Boeger and Kritsky (1997) on the coevolution of the monogeneans with their fish hosts. Within the mazocraeids, *M. alosae* is basal to *K. scombrii,* confirming that the parasitic relationship first evolved in clupeids, with a later host-switching to the most derived scombrids that share the coastal pelagic ecosystems with clupeids (Rosen 1982). There is another study on the mazocraeid *Mazocreaoides gonialosae* found in the gizzard shad, *Konosirus punctatus*, but it is focused on the variability in the mitochondrial cytochrome *c* oxidase subunit I gene (COI) to study the phylogeographical patterns along the coast of China (Li et al. 2011). Although these authors did not ask phylogenetic questions about the position of *M. gonialosae*, according to our results we hypothesize that this species will be placed basal to the other mazocraeids *Kuhnia* or *Grubea*, close to *M. alosae*, since both share a clupeid host. Unfortunately, since we were not able to amplify the 28S region of *M. alosae*, we cannot test this hypothesis with molecular data.

4.2.2. *Hemiurus appendiculatus* (Rudolphi, 1802).

The digenean phylogenies of the basal Plagiorchiida obtained analysing either individual regions of SSU and LSU and combined, show similar topologies to previous studies in the basal relationships with minor modifications on the most divergent groups. The relationships found within the superfamily Hemiuroidea, with two main clades separating the [Accacoelidae + Derogenidae + Syncoeliidae + Sclerodistomidae + Didymozoidae + Isoparorchiidae] and the

[Hemiuridae + Lecithasteridae], are in agreement with previous studies (Blair et al. 1998; Cribb et al. 2003; Olson et al. 2003; Pankov et al. 2006). Nonetheless, our results differ slightly from those found by Blair et al. (1998), since we found the Hemiuridae and Lecithasteridae to be polyphyletic, since more genetic data was included in the present analysis. The combined analysis of both ribosomal regions showed a topology very similar to that obtained by Pankov et al. (2006), but in our analysis the basal topology of the clade that includes the families Hemiuridae and Lecithasteridae was better resolved. The lecithasterid *Machidatrema chilostoma,* the hemiurid *Opisthadena dimidia* and the monophyletic hemiurid subfamily Bunocotylinae constitute a consistent clade that is basal in the hemiurid/lecithasterid clade. The basal position of the monophyletic subfamily Bunocotylinae in the hemiurid/lecithasterid clade does not change according to the region studied, but their closest relatives do when the different regions are analysed separately. The SSU region shows the bunocotylinids strongly supported with the lecithasterids *Hysterolecitha* and *Thulinia* (as in Pankov et al. 2006, Figure 5), while the LSU region shows a poorly resolved relationship with the lecithasterid *Machidatrema chilostoma,* the hemiurid *Opisthadena dimidia* and the monophyletic hemiurid subfamily Quadrifoliovariinae as a sister clade. When analysing the LSU region considering the gaps, the latter relationship is strongly supported both by bootstrap 97% and posterior probabilities 1 (data not shown). These inconsistencies may be the result of multiple factors: i) the phylogenetic signal found in both regions is not very strong, ii) a consequence of including different taxa to the different regions, like the hemiurid subfamily Quadrifoliovariinae that is represented in the database with LSU data but is absent on SSU, and also iii) the inclusion of an outgroup, which is not present in the combined analysis of partial LSU and complete SSU carried out by Pankov et al. (2006).

4.2.3. *Clavellisa emarginata* (Krøyer, 1837).

The recovered phylogeny of the available sequences of the order Siphonostomatoida shows the same family groupings as those obtained in previous works (Huys et al. 2006, 2007), but the relative position of these families is slightly different. This difference may rely on the phylogenetic signal of the SSU rDNA, the species included in the analysis, and the analysis used (maximum likelihood versus maximum parsimony). Previous works used the whole sequence of the SSU rDNA from 16 (Huys et al. 2006) and 20 siphonostomatoid taxa (Huys et al. 2007), while we used less than half to build our alignment file (804 bp) with up to 40 siphonostomatoid taxa. Accordingly, they obtained a better resolution on the deeper nodes (indicated by the higher bootstrap values and posterior probabilities), since they have more informative positions. Nonetheless, as more taxa are added into our phylogenetic tree, the relative position of the families may change and new relationships appear. Apart from that, we have enough signal in our database to assign consistently the parasitic copepod *C. emarginata* within the family Lernaeopodidae, which includes parasites of marine and anadromous fishes. Our analysis reveal a consistent sister taxa relationship between the families Lernaeopodidae and Sphyriidae (parasites of sharks). These two families are the sister group of a clade that includes the families Entomolepidae, parasites of sponges (as shown in Huys et al. 2006) and Nicothoidae (represented by *Choniosphaera maenadis*, a parasite of crabs). It is important to note that the relative position of *C. maenadis* differed between ML and BI analysis, with the BI representation placing it within the Dirivultidae family as in Huys et al. (2007).

It is interesting to point out the parallelism found between host evolution in monogeneans and siphonostomatoid copepods. With the available data, the phylogenetic trees suggest that the parasites present in clupeiform fishes (monogenean and copepods) evolved from those present in elasmobranchs. This is clearly shown in the monogeneans, where the family Hexabothriidae is basal to the Mazocraeidae (Olson et al. 2003), and supported by the systematic work carried out by Boeger and Kritsky (1997). Surprisingly, this host switching seems to have occurred in

a similar way in the siphonostomatoid copepods where the family Lernaeopodidae (parasites of clupeiforms and other marine fishes) evolved from the Sphyriidae (parasites of elasmobranchs and deep see fishes). This parallelism suggests a coevolution of two different groups of parasites in their hosts and deserves further study.

4.3 Feeding and transmission pathways

According to Williams et al. (1992) and Arthur (1997), the parasite fauna of a host species reflects its diet and characterizes the feeding ecology of the host.

Alosa alosa is mainly zooplanktophagous, with euphausiids (e.g. *Nyctiphanes couchii*), copepods (*Calanus* spp.), and mysids as favourite preys and small clupeids (e.g. *Engraulis encrasicolus* and *Sprattus sprattus*) as less important parts of the diet at sea (Taverny and Elie 2001b; Maitland and Lyle 2005). *Alosa fallax* is mainly ichthyophagous, with small pelagic fish (e.g. *Engraulis encrasicolus*, *Sprattus sprattus*, *Atherina boyeri*, *Sardina pilchardus*, *Pomatoschistus minutus*, *P. microps*) as preferred preys, followed in importance by euphausiids (e.g. *Nyctiphanes couchii*) and mysids (*Neomysis* spp.). Other crustaceans, such as decapods (*Crangon crangon*), amphipods, isopods, ostracods and insects are of less importance (Assis et al. 1992; Taverny and Elie 2001b; Maitland and Lyle 2005; Ceyhan et al. 2012; Skóra et al. 2012). Thus, copepods do not seem to be an important part of the diet of adults at sea, even though calanoid and harpacticoid copepods may be important prey for 0+ juveniles during their estuarine phase (Aprahamian 1989; Nunn et al. 2008). Moreover, *Alosa fallax nilotica* feed on fish and crustaceans (Decapoda and Mysidacea) at the sea bottom (160 m.) during the winter months, and on fish (*S. sprattus*, *S. pilchardus*, *E. encrasicolus*, *Atherina* spp.) close to the surface during the summer months (Ceyhan et al. 2012 and references therein). Furthermore, *A. alosa* do not feed during their spawning migration (Mota et al. 2015), whereas

A. fallax might feed actively, especially on aquatic and terrestrial invertebrates, but also on fish (*Atherina boyeri*, *Pseudochondrostoma duriense*) (Nachón et al. 2013).

The euphausiid *N. couchii* was found to be an intermediate host of the acanthocephalans *Bolbosoma balaenae* and *Rhadinorhynchus* sp., and of the *Anisakis simplex* complex (*A. simplex* s.s. and *A. pegreffii*) in NW Iberian Peninsula waters (Gregori et al. 2012, 2013, 2015). It was additionally reported as intermediate host of *A. simplex* on the Scottish East Coast, and of *Hysterothylacium* sp. on the Scottish East Coast and Portuguese Coast (Smith 1983). Furthermore, it is the main component of the marine diet of *A. alosa* and the secondary diet of *A. fallax* (Taverny et al. 2001a). It is also the main euphausiid in the European continental shelf, with high concentrations present near the Spanish coast (Roura et al. 2013). Hence, *N. couchii* may represent an important transport host for *A. simplex*, *A. pegreffii*, *H. aduncum* and *R. pristis* to both *Alosa* spp. off Western Iberian Marine waters.

The high infection values of these anisakid and rhapidascarid nematodes found in *A. alosa* and the comparatively low ones found in *A. fallax* (Table 4) appear to be consistent with their feeding habits, linking zooplankton as intermediate hosts and main transmission vectors through shads (especially *A. alosa*) and small pelagic fish as paratenic hosts and secondary transmission vectors through shads (especially *A. fallax*). In relation to this, small pelagic fish usually carry low anisakid burdens, or at least lower than bigger specimens of the same species, since accumulation of parasites during the host lifetime has been previously reported in numerous studies (Mladineo and Poljak 2014 and references therein), which may explain the relatively low anisakid infection values of *A. fallax*. When shad samples from the same river (Minho) and sampling year (2011) were compared, *Anisakis* spp. also showed higher infection values in *A. alosa* than *A. fallax* (Table 5). Moreover, the presence of *R. pristis* in *A. alosa* but not in *A. fallax* also supports the previously suggested "parasite-host" transmission pathway by feeding routes.

The life cycle of *H. appendiculatus* is not known but may be assumed to follow a similar pattern to those of other hemiurids, having a marine mollusc as first intermediate host, metacercariae in second intermediate hosts (crustaceans, especially copepods and chaetognaths) and adults in the stomach of *Alosa* spp. (Gibson and Bray 1986; Moravec 2004). In relation to this, cercariae of the hemiurid digenean *Hemiurus communis* were found in the opisthobranch snail *Retusa truncatula* and calanoid copepods were found to be second intermediate hosts (Køie 1995). Likewise, cercariae of *Hemiurus luehei* were found in the opisthobranch *Philine denticulata*, while the chaetognath *Sagitta* sp. was found to be naturally infected by metacercariae, probably by feeding on its second intermediate hosts, calanoid copepods (*Temora longicornis*, *Acartia tonsa*, unidentified copepod), which were shown to be susceptible to infection by metacercaria of *H. luehei* (Køie 1990). Thus, the high hemiurid infection found in *A. alosa* (Table 2, 4) is in accordance with their zooplanktophagous diet. However the high values of *H. appendiculatus* found in *A. fallax* (Table 2, 4) suggest that zooplankton (especially calanoid copepods) could also be an important part of their diet in the marine environment, which may have been underestimated previously. Moreover, comparison of hemiurid infection values of both shads in the River Minho in 2011 showed even higher values of *H. appendiculatus* in *A. fallax* than in *A. alosa* which is consistent with this hypothesis (Table 5).

Mazocraes alosae, *C. italica* and *C. emarginata* are ectoparasites with direct life cycles, so reinfection or parasite transmission from infected to uninfected specimens will occur directly.

4.4 Shad parasites as biological tags

Parasites can be used effectively as biological tags or indicators in population studies of their hosts. This method is particularly useful for anadromous fish species which acquire different parasites during their stay in freshwater, brackish and marine environments in the course of their migrations (MacKenzie and Hemmingsen 2014). Bao et al. (2015) found that levels of

infection with *A. pegreffii* were higher than those of *A. simplex* in all Western Iberian shad samples. *Anisakis pegreffii* is more common further south off the coast of Portugal, which suggests that these shads became infected during feeding migrations to these southern areas. This hypothesis is supported by the occurrence in our samples of the isopod *C. italica*, which was previously reported as a parasite of sparid fishes in the Mediterranean and off the northwest coast of Africa (Horton 2000). The marine cestode *Eubothrium fragile* is a specific parasite of shads (*Alosa* spp.), but appears to be restricted to northern Europe (Kennedy 1981; Aprahamian et al. 2002; Kuchta et al. 2005) and has not been reported from shad south of the Bay of Biscay. We did not find *E. fragile* in our samples, which suggests that Western Iberian shads do not migrate to more northern feeding grounds. In this regard, Martin et al. (2015), based on otolith microchemistry and microsatellite genetic analyses, reported that migrations of *A. alosa* of hundreds of kilometres might occur, either south or northward from natal to spawning rivers, even though such long distance straying was not frequent. Therefore, migration of some Western Iberian shads to northern feeding grounds cannot be completely discounted.

4.5. General comments

Finally, we report the first presence of *M. alosae* and *C. emarginata* in NW Iberia. This finding is not unexpected since both are specific parasites of *Alosa* spp. and this is the first time that the macroparasite community of shads has been described in this area. In this regard, *M. alosae* and *C. emarginata* were found only in *A. alosa* and *A. fallax* respectively, from River Minho, whereas infections of both gill parasites in both *Alosa* spp. might be expected. Nonetheless, further assumptions cannot be made since a comparison is not possible because gill samples of *A. alosa* were only available in 2013, but no *A. fallax* samples were available that year.

Significantly decreasing abundances were found for *H. appendiculatus*, *H. aduncum* and *Anisakis* spp. in *A. alosa* during 2009, 2010 and 2011 (Table 6), but this decrease in infection was not always confirmed for prevalence of infection. This variation of annual infection values

is not easily explained since parasite burdens can be influenced by many biotic and abiotic variables (Kleinertz et al. 2012; Mladineo and Poljak 2014). Further research will be needed to confirm or refute this trend.

To conclude, both shad species are shown to represent suitable final hosts for a number of ecto- and endo-parasite species. They also form strong connections between intermediate hosts (zooplankton) and larger transport or definitive hosts (larger fish and marine mammals), in both freshwater and marine environments of Western Iberia. Monitoring of parasite diversity introduced from the marine environment to the freshwater ecosystem is thus desirable to confirm these connections and also to control parasite and/or allergen risk to human consumers and riverine mammals due to zoonotic *A. pegreffii* and *A. simplex* s.s. (Bao et al. 2015). In relation to this, it is noteworthy to mention that an Inuk woman was previously diagnosed with gastric anisakiasis after the ingestion of raw anadromous fish (arctic char, *Salvelinus alpinus alpinus*) caught in a local river of northern Quebec (Canada) (Bhat and Cleland 2010). Our results provide new information regarding the life cycle and ecology of these macroparasites and also suggest host feeding habits during the marine trophic phase and migration patterns of *A. alosa* and *A. fallax* in the Western Iberian Peninsula. Overall, this study contributes to a better understanding of the phylogenetic relationships within monogenean and digenean platyhelminthes as well as to the diverse order of the Siphonostomatoid copepods. Further research integrating the use of parasites as biological tags in a multidisciplinary approach (i.e. molecular genetics, biometrics, life histories, modelling, otolith microchemistry, artificial tags) (Catalano et al. 2014) with appropriate statistical methods would be desirable to confirm feeding behaviour and migration routes and also to determine recruitment and aggregation patterns at sea and to differentiate stocks of these vulnerable shad species in Western Iberia.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MB and SP conceived and designed the study; MB, MM, DJN, CA and FC collected the data and collaborated in designing the study; MB performed the morphological identification studies; MB and AR performed the molecular genetic and phylogenetic studies; MB and KM performed the statistical analysis; MB, AR and KM analysed the data and drafted the manuscript. All authors critically reviewed the manuscript and gave the final approval of the version to be published.

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TABLES

Table 1. Sampling details obtained by *Alosa* species, river, year (period), method, number of fish analysed (*n*) and total length mean and standard deviation (TL ± SD (min-max) (cm)). *Samples obtained by sport fishing.

Host species	River	Sampling dates	*n*	TL ± SD (min – max)
Alosa alosa	Minho	2009 (23/03 – 21/07)	41	62.7 ± 4.30 (55.5 – 74.0)
		2010 (21/03 – 2/08)	90	65.0 ± 3.09 (57.0 – 73.0)
		2011 (3/04 – 9/07)	13	66.0 ± 5.88 (56.0 – 73.0)
		2013 (6/05)	9	63.9 ± 5.92 (53.5 – 71.6)
	Mondego	2012 (14/05)	5	56.6 ± 2.38 (54.5 – 60.0)
		2013 (15/05)	5	54.0 ± 2.57 (49.5 – 56.0)
Alosa fallax	Ulla	2008 (01/05 – 08/06)*	6	39.6 ± 5.39 (31.0 – 44.7)
		2011 (15/04 – 06/06)	89	45.4 ± 5.72 (33.7 – 56.2)
		2012 (30/03 – 27/07)	59	44.8 ± 4.45 (32.8 – 55.0)
	Minho	2011 (29/04)	27	38.16 ± 3.37 (34.7 – 50.0)
		2012 (14/05)	42	36.7 ± 2.58 (28.0 – 42.6)

Table 2. Quantitative descriptors of parasitic infections in both shad species examined by visual methods, all samples per *Alosa* species combined. Area inspected for macroparasites (organ); *n*, number of fish examined; I, number of fish infected; prevalence (P), mean abundance and standard deviation (mA ± SD); mean intensity, standard deviation and range (mI ± SD (range)).

Alosa alosa						
Parasite	**Organ**	***n***	**I**	**P(%)**	**mA ± SD**	**mI ± SD (range)**
M. alosae	Gill	9	8	88.9	7.3 ± 15.0	8.2 ± 15.7 (1-47)
H. appendiculatus	Stomach and viscera	163	118	72.4	24.1 ± 47.9	33.3 ± 53.6 (1-314)
H. aduncum	Stomach and viscera	162	101	62.3	10.6 ± 15.3	17.0 ± 16.3 (1-99)
Anisakis spp.	Stomach and viscera	162	156	96.3	171.8 ± 205.5	178.5 ± 206.6 (1-1206)
R. pristis	Stomach and viscera	162	24	14.8	0.4 ± 1.3	2.92 ± 2.19 (1-8)
C. italica	Buccal cavity	9	1	11.1	0.1 ± 0.3	1
Unidentified copepod	Gill	9	1	11.1	0.1 ± 0.3	1
Alosa fallax						
Parasite	**Organ**	**n**	**I**	**P(%)**	**mA ± SD**	**mI ± SD (range)**
H. appendiculatus	Stomach and viscera	220	123	55.9	6.5 ± 14.8	11.6 ± 18.2(1-100)
H. aduncum	Stomach and viscera	74	36	48.6	1.6 ± 3.3	3.3 ± 4.1 (1-25)
Anisakis spp.	Stomach and viscera	214	25	11.7	1.8 ± 8.8	15.7 ± 21.9 (1-89)
C. emarginata	Gill	49	14	28.6	1.1 ± 3.0	4.0 ± 4.7 (1-17)
Unidentified isopod	Buccal cavity	49	1	2.0	0.02 ± 0.1	1

Table 3. Accession numbers and related information of parasite sequences available at GenBank.

Fish host	Parasite species	River	Year	Location	Accession number
A. alosa	*H. aduncum*	Minho	2013	Stomach	KR349114
		Mondego	2013	Stomach	KR349115
	R. pristis	Minho	2009	Visceral cavity	KR349116
		Mondego	2013	Visceral cavity	KR349117
	H. appendiculatus	Minho	2013	Stomach	KR349118
	M. alosae	Minho	2013	Gill	KR349119
A. fallax	*H. aduncum*	Minho	2012	Stomach	KR349120
	H. appendiculatus	Ulla	2012	Stomach	KR349121
		Minho	2009	Stomach	KR349123
	C. emarginata	Minho	2012	Gill	KR349122

Table 4. Comparative prevalences and mean abundances of parasite taxa common to the two shad species, all samples combined, with results of tests of statistical significance. NS = not significant, * = significant, ** and *** = highly significant.

Parasite	Prevalence (%)			Mean abundance		
	A. alosa	*A. fallax*	P	*A. alosa*	*A. fallax*	P
H. appendiculatus	72.4	55.9	0.0013**	24.12	6.48	<0.0001***
H. aduncum	62.3	48.6	0.064 NS	10.59	1.61	<0.0001***
Anisakis spp.	96.3	11.7	<0.0001***	171.84	1.83	<0.0001***

Table 5. Comparative prevalences and mean abundances of parasite taxa common to the two shad species caught in the river Minho in the same year (2011), with results of tests of statistical significance. NS = not significant, * = significant, ** and *** = highly significant.

Parasite	Prevalence (%)			Mean abundance		
	A. alosa	*A. fallax*	P	*A. alosa*	*A. fallax*	P
H. appendiculatus	69.2	100	<0.0001***	15.8	32.9	0.002*
H. aduncum	38.5	61.5	0.196 NS	5.2	2.4	0.692 NS
Anisakis spp.	76.9	23.1	0.002*	23.3	1.3	0.0014**

Table 6. Comparative prevalences and mean abundances of the most common parasite taxa in samples of *A. alosa* caught in River Minho in different sampling years. NS = not significant, * = significant, ** and *** = highly significant, NV = test not valid because of zeros and numbers <5 in some cells. The 2013 sample was excluded because of the small number of fish examined.

Parasite	Year	Prevalence (%)	P	Mean abundance	P
H. appendiculatus	2009	88		58.0	
	2010	69		14.7	
	2011	69	0.072 NS	15.8	<0.0001***
H. aduncum	2009	78		12.6	
	2010	57		7.6	
	2011	38	<0.017*	5.2	<0.0001***
Anisakis spp.	2009	100		313.2	
	2010	97		84.1	
	2011	77	NV	23.3	<0.0001***

FIGURE LEGENDS

Figure 1. Parasite taxa recorded, with sites of infection in *Alosa alosa*. **A.** The posterior (tail) region of an adult male of the rhapidascarid nematode *Hysterothylacium aduncum*. **B.** Two specimens of the monogenean *Mazocraes alosae* in situ on the gill filaments. **C.** The digenean *Hemiurus appendiculatus* with ecsoma everted. **D.** Ultraviolet photograph of an opened *A. alosa* stomach showing the intensity of the infection and the different colours and fluorescence brightness of the anisakid and rhapidascarid nematode larvae. Several parasite larvae were molecularly identified, thus larvae nematodes number 1, 2, 3 and 4 belong to adult *H. aduncum*; number 5 and 7 to *A. simplex* s.s. and 6 to *A. pegreffii* third larval stage. **E.** The acanthocephalan *Rhadinorhynchus pristis* showing the body spination and proboscis. **F.** The mouth-dwelling isopod *Ceratothoa italica* in situ. **G.** Female unidentified ergasilid copepod (probably *Ergasilus* spp.) attached to the gill filaments.

Figure 2. Parasite taxa recorded, with sites of infection in *Alosa fallax*. *Anisakis* spp. usually appeared in the visceral cavity but one larva was observed in the stomach of one fish. **A.** The nematode *Hysterothylacium aduncum* and the digenean *Hemiurus appendiculatus*. **B.** The parasitic copepod *Clavellisa emarginata* in situ on the gill filaments. **C.** An unidentified mouth-dwelling isopod in situ in the buccal cavity.

Figure 3. Maximum likelihood phylogenetic tree using GTR+I+G model based on partial small subunit ribosomal RNA gene (18S rRNA or SSU) to infer the phylogenetic position of the monogenean platyhelminth morphologically identified as *Mazocraes alosae.* Nodal support is given by bootstrap percentages after 1000 replicates above the node and Bayesian posterior probability values ≥ 0.7 below the node.

Figure 4. Maximum likelihood phylogenetic tree using GTR+I+G model based on partial SSU gene to infer the phylogenetic position of the digenean platyhelminth morphologically

identified as *Hemiurus appendiculatus.* Nodal support is given by bootstrap percentages after 1000 replicates above the node and Bayesian posterior probability values ≥ 0.7 below the node.

Figure 5. Maximum likelihood phylogenetic tree using GTR+I+G model based on partial large subunit ribosomal RNA gene (28S rRNA or LSU) to infer the phylogenetic position of the digenean platyhelminth morphologically identified as *Hemiurus appendiculatus.* Nodal support is given by bootstrap percentages after 1000 replicates above the node and Bayesian posterior probability values ≥ 0.7 below the node.

Figure 6. Maximum likelihood phylogenetic tree using GTR+I+G model based on combined partial SSU and LSU to explore the phylogenetic position of the digenean platyhelminth morphologically identified as *Hemiurus appendiculatus.* Nodal support is given by bootstrap percentages after 1000 replicates above the node and Bayesian posterior probability values ≥ 0.7 below the node.

Figure 7. Maximum likelihood phylogenetic tree using GTR+I+G model based on partial SSU gene to infer the phylogenetic position of the siphonostomatoid copepod morphologically identified as *Clavellisa emarginata.* Nodal support is given by bootstrap percentages after 1000 replicates above the node and Bayesian posterior probability values ≥ 0.7 below the node.

FIGURES

Fig. 1

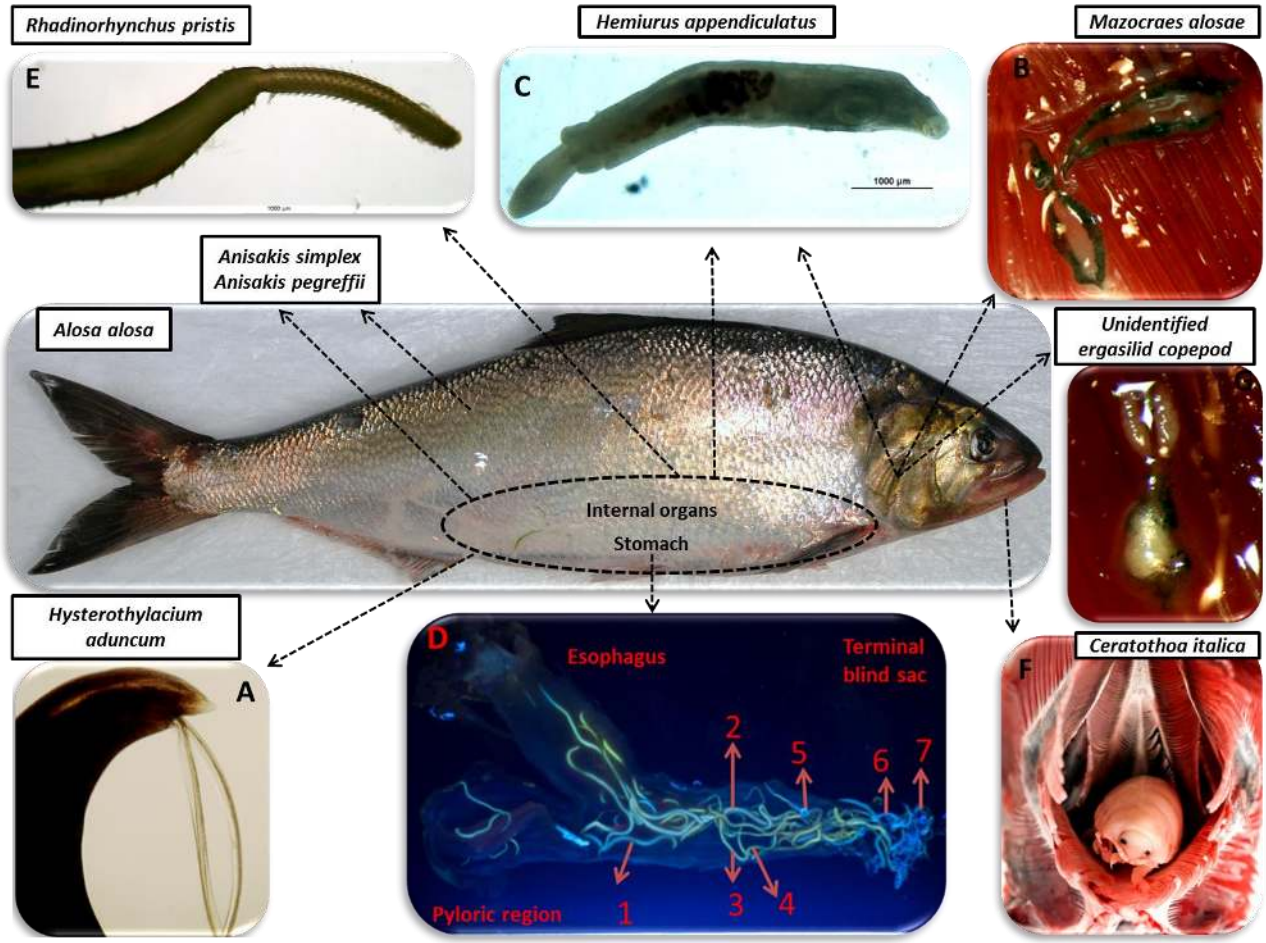
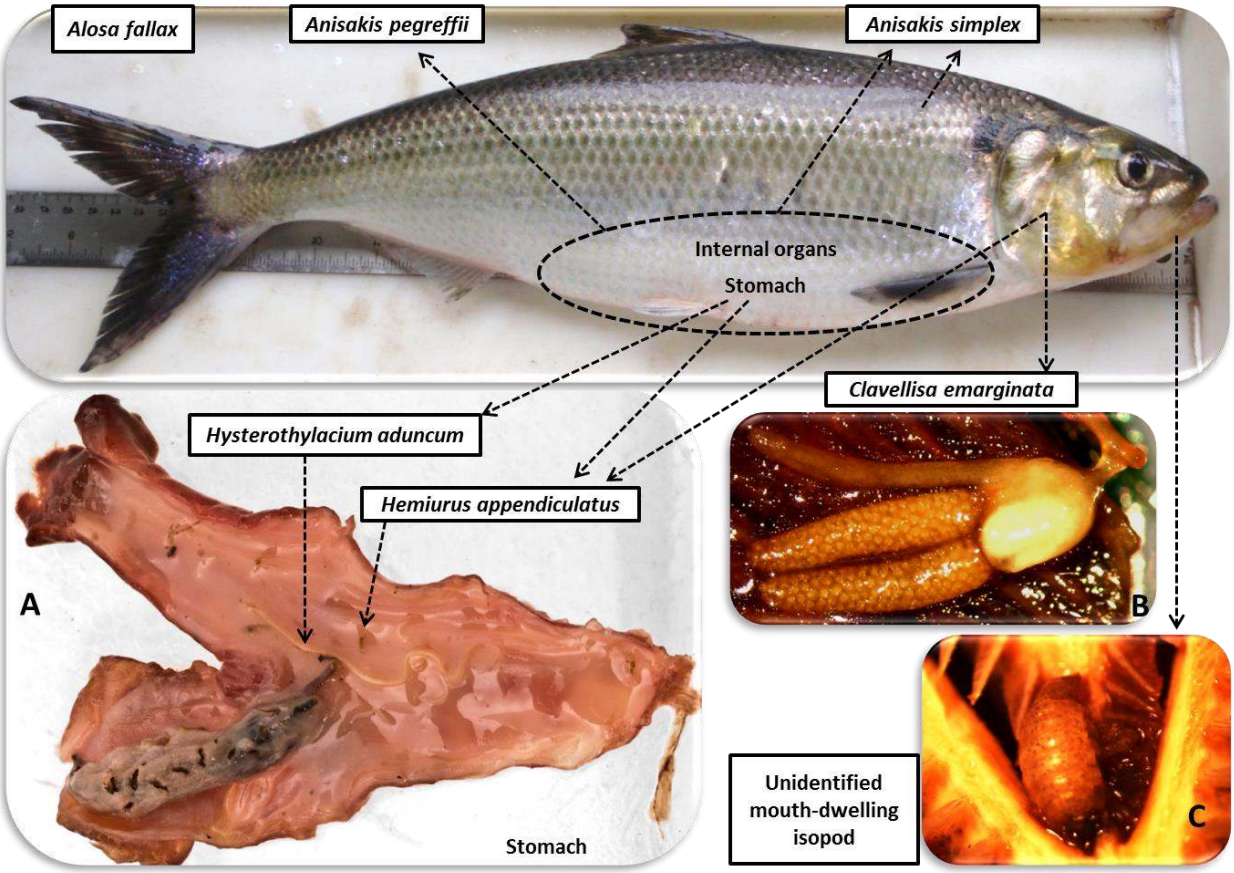


Fig. 2


Alosa fallax
Anisakis pegreffii
Anisakis simplex
Internal organs
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Hysterothylacium aduncum
Hemiurus appendiculatus
A
B
C
Stomach
Unidentified mouth-dwelling isopod

Fig. 3

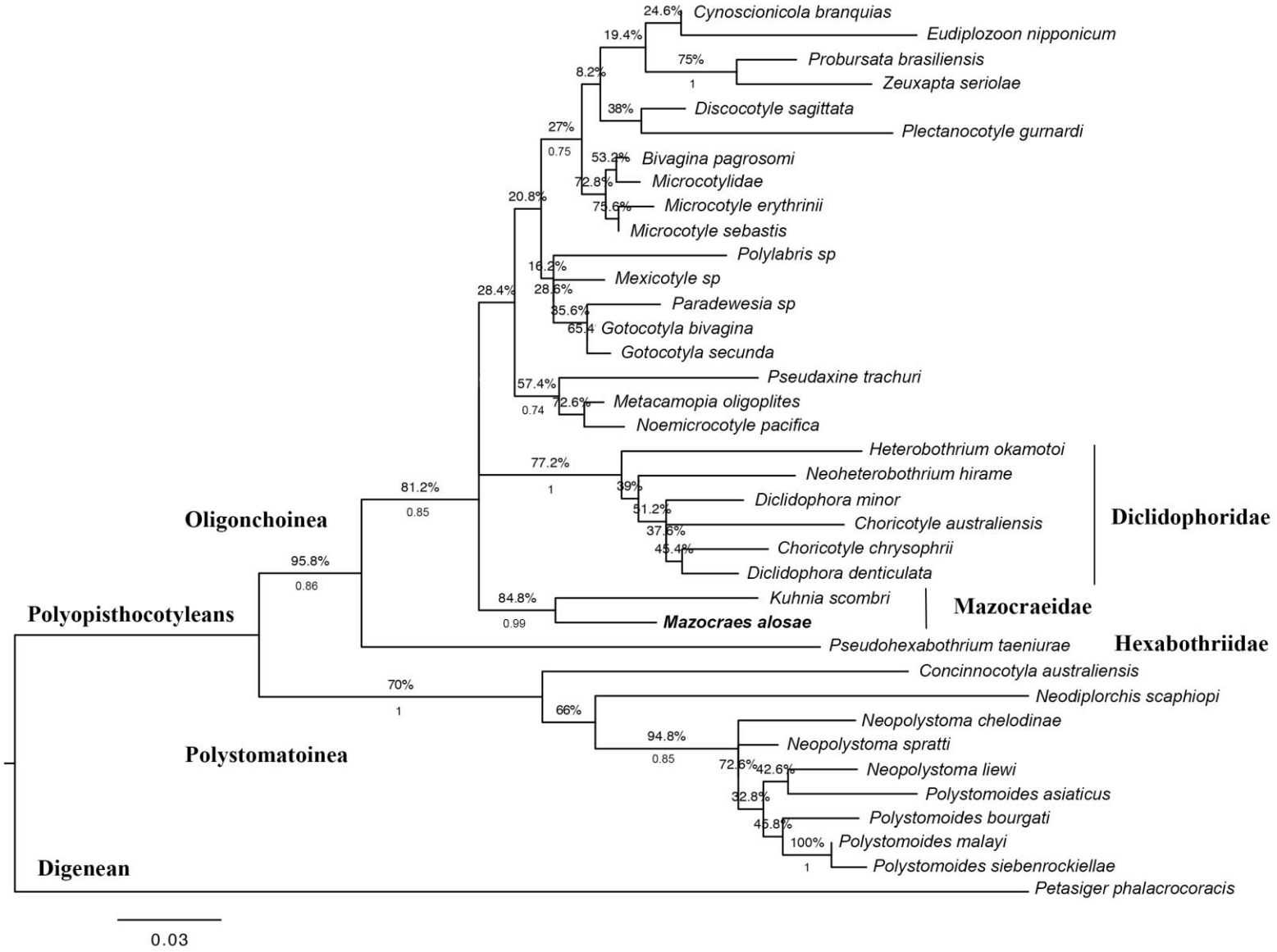
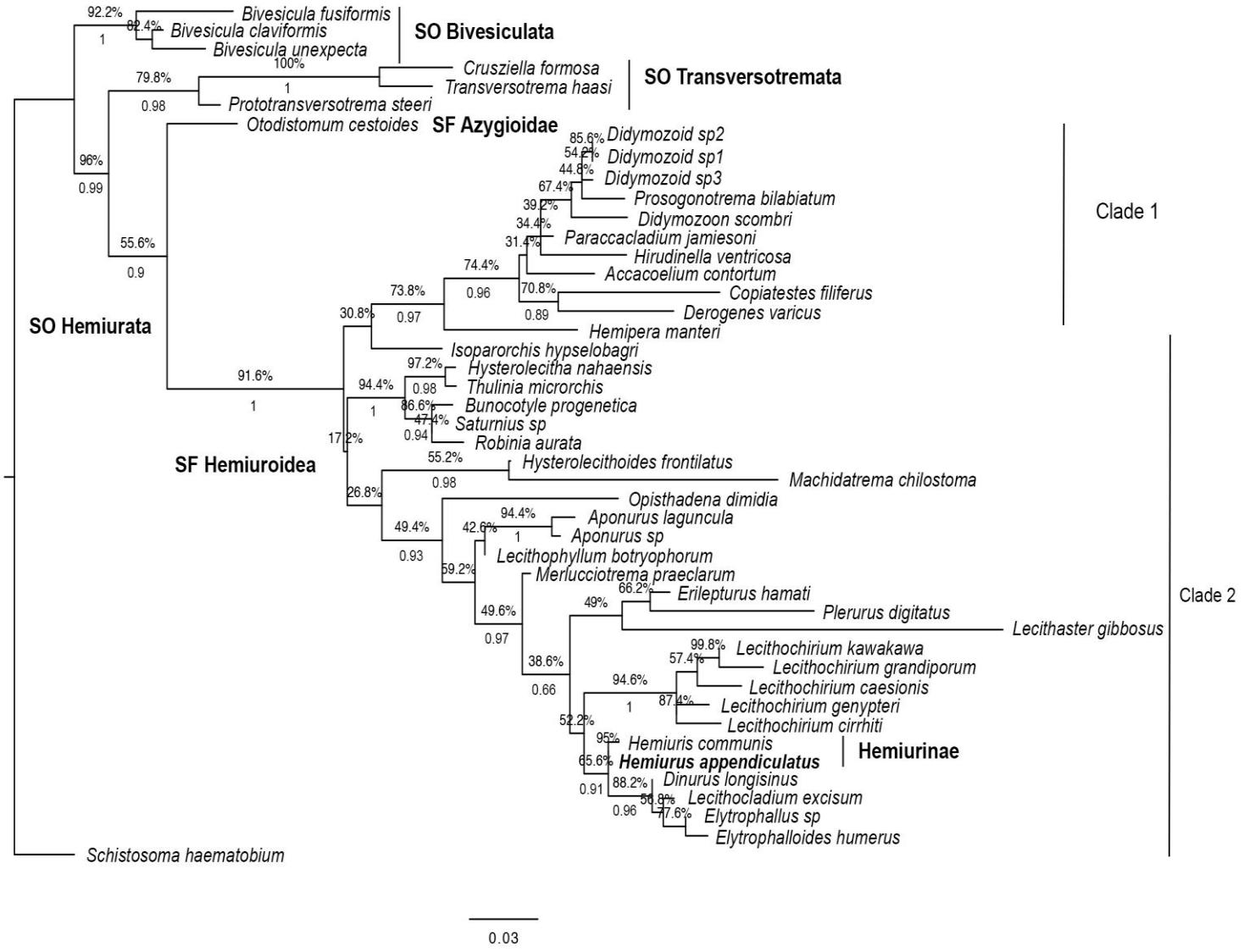

Cynoscionicola branquias
Eudiplozoon nipponicum
Probursata brasiliensis
Zeuxapta seriolae
Discocotyle sagittata
Plectanocotyle gurnardi
Bivagina pagrosomi
Microcotylidae
Microcotyle erythrinii
Microcotyle sebastis
Polylabris sp
Mexicotyle sp
Paradewesia sp
Gotocotyla bivagina
Gotocotyla secunda
Pseudaxine trachuri
Metacamopia oligoplites
Noemicrocotyle pacifica
Heterobothrium okamotoi
Neoheterobothrium hirame
Diclidophora minor
Choricotyle australiensis
Choricotyle chrysophrii
Diclidophora denticulata
Kuhnia scombri
Mazocraes alosae
Pseudohexabothrium taeniurae
Concinnocotyla australiensis
Neodiplorchis scaphiopi
Neopolystoma chelodinae
Neopolystoma spratti
Neopolystoma liewi
Polystomoides asiaticus
Polystomoides bourgati
Polystomoides malayi
Polystomoides siebenrockiellae
Petasiger phalacrocoracis
Oligonchoinea
Polyopisthocotyleans
Polystomatoinea
Digenean
Diclidophoridae
Mazocraeidae
Hexabothriidae
0.03

Fig. 4


Bivesicula fusiformis
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SO Bivesiculata
Crusziella formosa
Transversotrema haasi
SO Transversotremata
Prototransversotrema steeri
Otodistomum cestoides
SF Azygioidae
Didymozoid sp2
Didymozoid sp1
Didymozoid sp3
Prosogonotrema bilabiatum
Didymozoon scombri
Paraccacladium jamiesoni
Hirudinella ventricosa
Accacoelium contortum
Copiatestes filiferus
Derogenes varicus
Hemipera manteri
Clade 1
SO Hemiurata
Isoparorchis hypselobagri
Hysterolecitha nahaensis
Thulinia microrchis
Bunocotyle progenetica
Saturnius sp
Robinia aurata
SF Hemiuroidea
Hysterolecithoides frontilatus
Machidatrema chilostoma
Opisthadena dimidia
Aponurus laguncula
Aponurus sp
Lecithophyllum botryophorum
Merlucciotrema praeclarum
Erilepturus hamati
Plerurus digitatus
Lecithaster gibbosus
Clade 2
Lecithochirium kawakawa
Lecithochirium grandiporum
Lecithochirium caesionis
Lecithochirium genypteri
Lecithochirium cirrhiti
Hemiuris communis
Hemiurus appendiculatus
Hemiurinae
Dinurus longisinus
Lecithocladium excisum
Elytrophallus sp
Elytrophalloides humerus
Schistosoma haematobium
0.03

Fig. 5

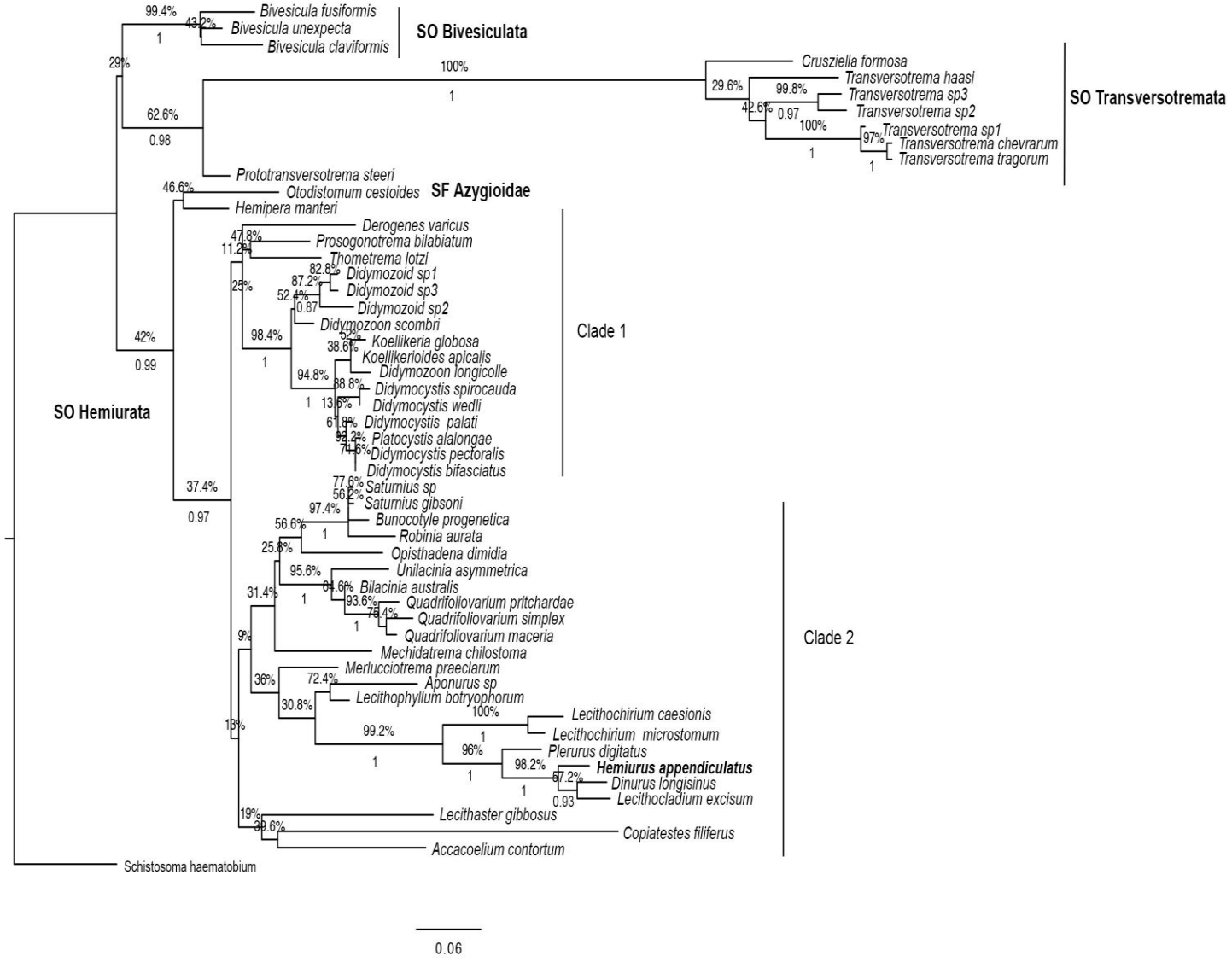

Bivesicula fusiformis
Bivesicula unexpecta
Bivesicula claviformis
SO Bivesiculata
Crusziella formosa
Transversotrema haasi
Transversotrema sp3
Transversotrema sp2
Transversotrema sp1
Transversotrema chevrarum
Transversotrema tragorum
SO Transversotremata
Prototransversotrema steeri
Otodistomum cestoides
SF Azygioidae
Hemipera manteri
Derogenes varicus
Prosogonotrema bilabiatum
Thometrema lotzi
Didymozoid sp1
Didymozoid sp3
Didymozoid sp2
Didymozoon scombri
Koellikeria globosa
Koellikerioides apicalis
Didymozoon longicolle
Didymocystis spirocauda
Didymocystis wedli
Didymocystis palati
Platocystis alalongae
Didymocystis pectoralis
Didymocystis bifasciatus
Clade 1
SO Hemiurata
Saturnius sp
Saturnius gibsoni
Bunocotyle progenetica
Robinia aurata
Opisthadena dimidia
Unilacinia asymmetrica
Bilacinia australis
Quadrifoliovarium pritchardae
Quadrifoliovarium simplex
Quadrifoliovarium maceria
Mechidatrema chilostoma
Merlucciotrema praeclarum
Aponurus sp
Lecithophyllum botryophorum
Lecithochirium caesionis
Lecithochirium microstomum
Plerurus digitatus
Hemiurus appendiculatus
Dinurus longisinus
Lecithocladium excisum
Clade 2
Lecithaster gibbosus
Copiatestes filiferus
Accacoelium contortum
Schistosoma haematobium
0.06

Fig. 6

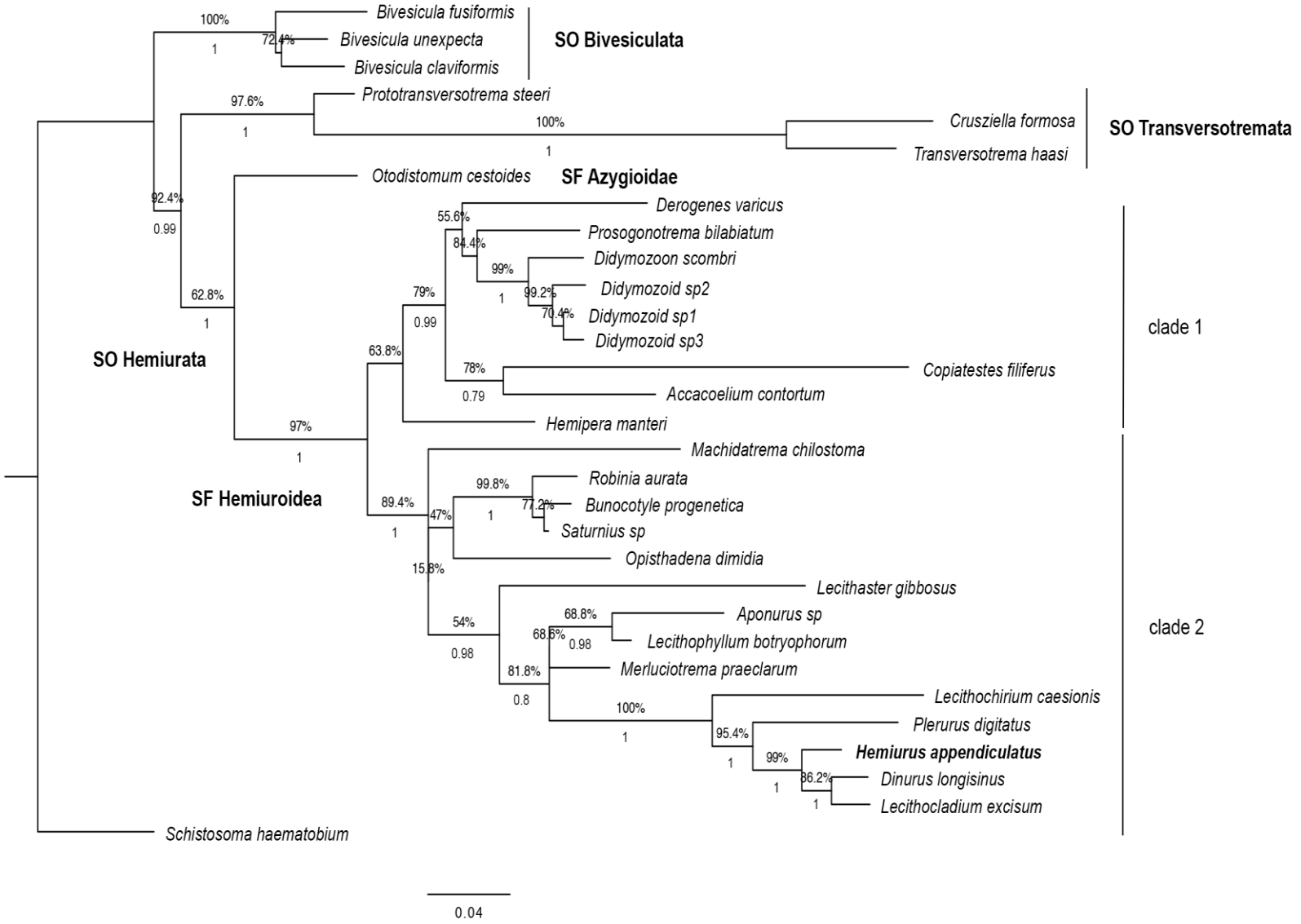
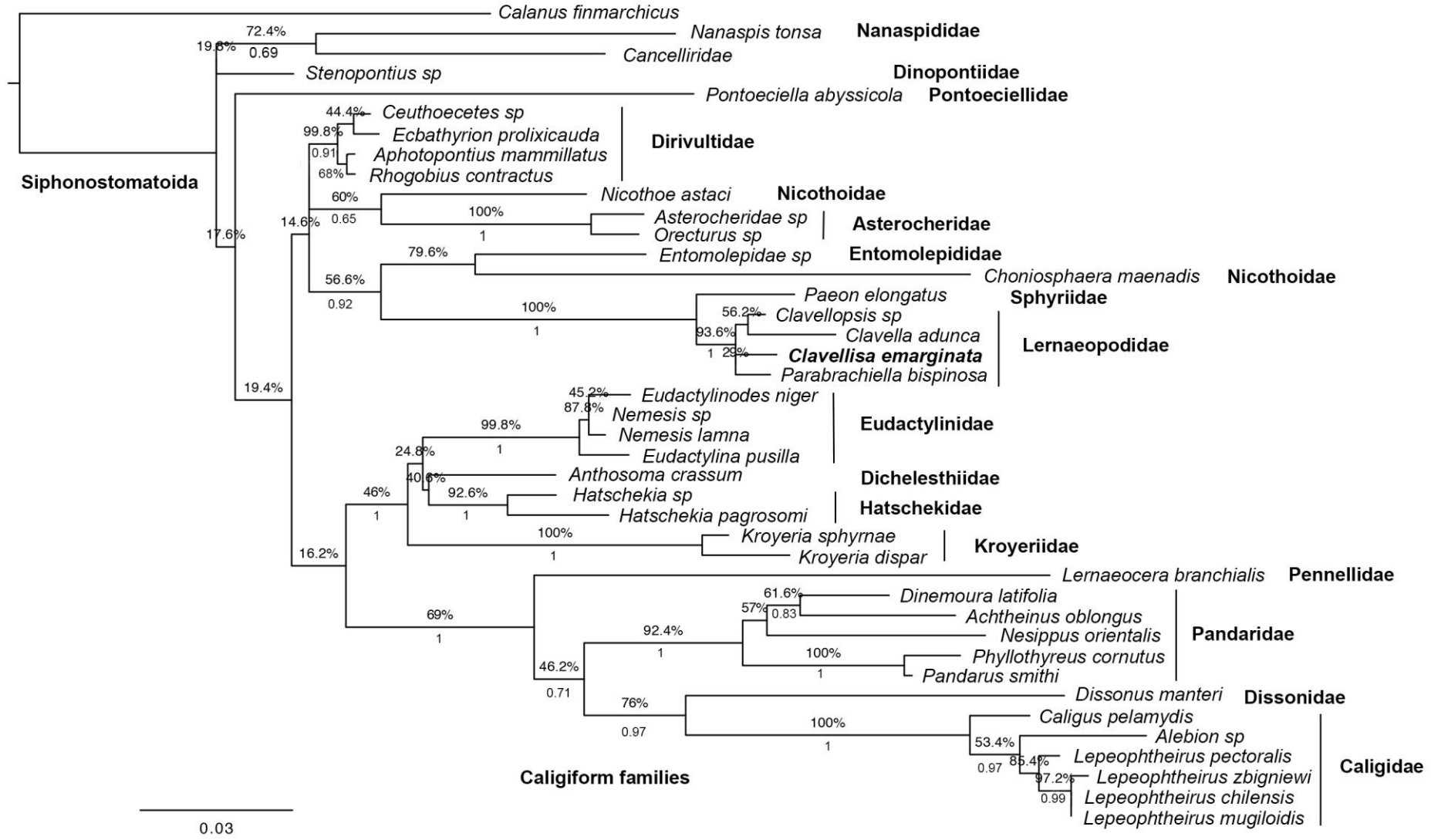

Bivesicula fusiformis
Bivesicula unexpecta
Bivesicula claviformis
SO Bivesiculata
Prototransversotrema steeri
Crusziella formosa
Transversotrema haasi
SO Transversotremata
Otodistomum cestoides
SF Azygioidae
Derogenes varicus
Prosogonotrema bilabiatum
Didymozoon scombri
Didymozoid sp2
Didymozoid sp1
Didymozoid sp3
SO Hemiurata
Copiatestes filiferus
Accacoelium contortum
Hemipera manteri
clade 1
Machidatrema chilostoma
Robinia aurata
Bunocotyle progenetica
Saturnius sp
SF Hemiuroidea
Opisthadena dimidia
Lecithaster gibbosus
Aponurus sp
Lecithophyllum botryophorum
Merluciotrema praeclarum
Lecithochirium caesionis
Plerurus digitatus
Hemiurus appendiculatus
Dinurus longisinus
Lecithocladium excisum
clade 2
Schistosoma haematobium
0.04

Fig. 7


Calanus finmarchicus
Nanaspis tonsa
Nanaspididae
Cancelliridae
Stenopontius sp
Dinopontiidae
Pontoeciella abyssicola
Pontoeciellidae
Ceuthoecetes sp
Ecbathyrion prolixicauda
Aphotopontius mammillatus
Rhogobius contractus
Dirivultidae
Siphonostomatoida
Nicothoe astaci
Nicothoidae
Asterocheridae sp
Orecturus sp
Asterocheridae
Entomolepidae sp
Entomolepididae
Choniosphaera maenadis
Nicothoidae
Paeon elongatus
Sphyriidae
Clavellopsis sp
Clavella adunca
Clavellisa emarginata
Parabrachiella bispinosa
Lernaeopodidae
Eudactylinodes niger
Nemesis sp
Nemesis lamna
Eudactylina pusilla
Eudactylinidae
Anthosoma crassum
Dichelesthiidae
Hatschekia sp
Hatschekia pagrosomi
Hatschekidae
Kroyeria sphyrnae
Kroyeria dispar
Kroyeriidae
Lernaeocera branchialis
Pennellidae
Dinemoura latifolia
Achtheinus oblongus
Nesippus orientalis
Phyllothyreus cornutus
Pandarus smithi
Pandaridae
Dissonus manteri
Dissonidae
Caligus pelamydis
Alebion sp
Lepeophtheirus pectoralis
Lepeophtheirus zbigniewi
Lepeophtheirus chilensis
Lepeophtheirus mugiloidis
Caligidae
Caligiform families
0.03