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Morphology and molecular phylogeny of trypanorhynchid metacestodes infecting commercial fish of the Mediterranean Sea

[*Morfologia e filogenia molecular das metacestodes de tripanorhynchid que infectam os peixes comerciais do Mar Mediterrâneo*]

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ABSTRACT

Members of the order Trypanorhyncha are cestode parasites that are frequently found infecting the muscles of several marine fish species, affecting fish health, and resulting in consumers' rejection of fish. Fifty-two specimens of marine fish were freshly caught throughout the year 2020 from boat landing sites at the Alexandria coast along the Mediterranean Sea in Egypt, including the grey trigger fish *Balistes carolinensis* (F: Balistidae); the mottled grouper *Mycteroperca rubra* (F: Serranidae) and the common sole *Solea vulgaris* (F: Soleidae). Blastocysts were isolated and ruptured; the generated pleurocerci were described morphologically and morphometrically by light and scanning electron microscopy. Also, multiple–sequence alignment was performed, and a phylogenetic tree was constructed following maximum likelihood analysis of the 18s and 28s ribosomal RNA sequences of the recovered worms. Thirty fish were infected; the infection was recorded as blastocysts embedded in fish flesh. Three different parasitic species were recovered and classified morphologically as *Gymnorhynchus isuri*, *Pseudotobothrium dipsacum* and *Heteronybelinia estigmena*. The taxonomic position of these parasites was justified by molecular analysis of their 18s and 28s rRNAs, which revealed high percentages of homology with species recovered from the GenBank. The accession numbers ON157059, ON139663 and ON139662 were respectively assigned to the recovered parasites after their deposition in GenBank. The results obtained from the molecular analyses confirmed the morphological records of the recovered parasites. Since metacestodes are found in the musculature of infected fish specimens, it is necessary to remove these areas in the commercialization of fish.

Keywords: Cestoda, Trypanorhyncha, Taxonomy, morphology, molecular analysis

RESUMO

Os membros da ordem Trypanorhyncha são parasitas de cestóides que são freqüentemente encontrados infectando os músculos de várias espécies de peixes marinhos, afetando a saúde dos peixes e resultando na rejeição do peixe por parte dos consumidores. Cinqüenta e dois espécimes de peixes marinhos foram capturados recentemente durante todo o ano de 2020 nos locais de desembarque de barcos na costa de Alexandria ao longo do Mar Mediterrâneo, no Egito, incluindo o peixe de gatilho cinzento Balistes

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carolinensis (F: Balistidae); a garoupa mosqueada Mycteroperca rubra (F: Serranidae) e o linguado comum Solea vulgaris (F: Soleidae). Os blastocistos foram isolados e rompidos; os pleurocistos gerados foram descritos morfologicamente e morfometricamente por microscopia eletrônica de luz e varredura. Além disso, foi realizado o alinhamento de sequências múltiplas e uma árvore filogenética foi construída seguindo a análise de máxima probabilidade das sequências de RNA ribossômico de 18s e 28s dos vermes recuperados. Trinta peixes foram infectados; a infecção foi registrada como blastocistos embutidos na carne do peixe. Três espécies diferentes de parasitas foram recuperadas e classificadas morfologicamente como Gymnorhynchus isuri, Pseudotobothrium dipsacum e Heteronybelinia estigmena. A posição taxonômica desses parasitas foi justificada pela análise molecular de seus rRNAs de 18 e 28 anos, que revelou altas porcentagens de homologia com espécies recuperadas do GenBank. Os números de acesso ON157059, ON139663 e ON139662 foram respectivamente atribuídos aos parasitas recuperados após sua deposição no GenBank. Os resultados obtidos a partir das análises moleculares confirmaram os registros morfológicos dos parasitas recuperados. Como as metacestodes são encontradas na musculatura dos espécimes de peixes infectados, é necessário remover estas áreas na comercialização dos peixes.

Palavras-chave: Cestoda, Trypanorhyncha, Taxonomia, morfologia, análise molecular

INTRODUCTION

Members of the order Trypanorhyncha Diesing (1863) represent parasitic cestodes of fish and sea invertebrates; adults infect the stomach and intestines of sharks and rays as definitive hosts, while the larval stages are found in the musculature and coelomatic cavity of teleosteans as intermediate hosts (Campbell and Beveridge, 1994; Palm, 2004; Morsy *et al.,* 2013; Santoro *et al.,* 2020). Detection of these parasites among infected fish poses marketing problems (Morsy *et al.,* 2013). Humans can be accidentally infected by larvae of Trypanorhyncha after ingesting raw fish meat which, in most cases, leads to allergic reactions. Further, the presence of larvae in the fish musculature may release toxins that affect humans (Deadorff *et al.,* 1984; Caira and Jensen, 2017). Previous reports have concluded that experimental inoculation of Trypanorhyncha species extracts is responsible for immune responses in mice, indicating the possibility of allergic reactions in humans (Vásquez–López *et al.,* 2001; Gòmez–Morales *et al.,* 2008; Al Quraishy *et al.,* 2019). Despite the worldwide distribution of these parasites in commercial fishes, and the great diversity of their species, trypanorhynchids are still a relatively poorly studied group (Palm, 2004; Menezes *et al.,* 2018). Only a few life cycles are completely known, but those that involve several intermediate hosts before the final infestation of sharks are still missed. Few reports have been published on these parasites, likely due to the challenges associated with classification (Menezes *et al.,* 2018). Trypanorhynchid cestodes are characterized by the presence of two or four bothria and a tentacular apparatus, which consists of tentachular sheaths with tentacles that bear numerous hooks. The hooks originate at the anterior extremity of bulbs and extend in a spiral anteriorly toward the scolex (Dollfus, 1942; Richmond and Caira, 1991; Campbell and Beveridge, 1994; Palm, 1995, 1997). Taxonomists originally identified the species of a larva in an invertebrate or teleost intermediate host based on the shape of the scolex, number of bothridia, tentacular armature (Palm and Caira, 2008), zoogeographical distribution (Palm, 2004, Palm *et al.,* 2007), and parasite evolution (Palm and Klimpel, 2007; Palm *et al.,* 2009) as the most important morphological features of the trypanorhynchid taxonomy. During a recent parasitological survey of marine fish of the Mediterranean Sea at the Alexandria coasts in Egypt, three species of trypanorhynchid metacestodes were captured from the musculature and coelomatic cavities of three examined fish species belonging to families Balistidae, Serranidae and Soleidae. The taxonomic status of the parasites was determined based on both morphological characterization and the molecular analysis of the parasites' 18s and 28s rRNA.

MATERIALS AND METHODS

A total of 52 specimens of marine fish were freshly caught throughout the year 2020 from boat landing sites at the Alexandria coasts along the Mediterranean Sea, Egypt. These were the grey trigger fish *Balistes carolinensis* (F:

Balistidae, no. 15); the mottled grouper *Mycteroperca rubra* (F: Serranidae, no. 20); the common sole *Solea vulgaris* (F: Soleidae, no. 17). Fish specimens were transported to the laboratory and were morphologically identified according to the methods of Kvach *et al.* (2018).

Morphology: After fish dissection, blastocysts were isolated in an isotonic saline solution (7%) in a Petri dish, where they were ruptured to release the coiled larvae that were left to relax between two slides within hot 10% formalin as a fixative. The fixed worms were washed with distilled water to remove the excess fixative. The worms were stained using acetic acid alum carmine (Carleton, 1976). Dehydration was achieved using an ascending series of ethyl alcohol, cleared in clove oil and xylene, and then the worms were permanently mounted in Canada balsam (Ergens, 1969). The worms were subsequently examined and photographed using a BX53 microscope (Olympus Corporation, Toyko, Japan) and drawn using a camera lucida. The nomenclature of the different body parts followed the convention published by) for trypanorhynchids. Measurements were given in millimeters (mm) and were reported as means and ranges in parentheses. To study the surface ultrastructure of worms by scanning electron microscopy (SEM), the worms were fixed in buffered glutaraldehyde (3%, pH 7.3, 3 hours), washed in the same buffer, and post–fixed in osmium tetroxide (4 hours) according to the instructions detailed by Madden and Tromba (1976). The worms were dehydrated in acetone solution, dried in a BOMER–900 drier (Leica Microsystems, Wetzlar, Germany), Jones *et al*. (2004mounted on an aluminum stub, coated with gold palladium in a JEOL JEC– *DNA Extraction, PCR, and Sequencing*: Genomic DNA (gDNA) was extracted from the preserved samples in 70% ethanol using a DNeasy tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of partial 18s ribosomal RNA sequences was carried out on an MJ Research PTC–150 thermocycler (Marshall Scientific, Hampton, NH, USA) using the universal primers 1F 5′–AACCTGGTTGATCCTGCCAG–3′ and 1528R 5′–TGATCCTTCTGCAGGTTCACCTAC–3′. The PCR was conducted using a final volume of 25µL containing 3.5mM of $MgCl_2$, 0.5 mM of each primer, 0.2 mM of dNTPs, 0.6 units (U) of *Thermus aquaticus* (Taq) polymerase in 1× PCR buffer, 0.1µg of extracted parasite genomic DNA, and nuclease–free sterile double–distilled water up to 25µL. The thermocycling conditions were as follows: 94°C for 2 minutes; 3 cycles of 94°C for 40 seconds, 51°C for 40 seconds, 72°C for 1 minute; 5 'touchdown' cycles of 94°C for 40 seconds, 50°C–46°C for 40 seconds (dropping 1°C per cycle), 72°C for 1 minute; 35 cycles of 94°C for 40 seconds, 45°C for 40 seconds, 72°C for 1 minute; and a final extension at 72°C for 5 minutes. DNA gel electrophoresis (1.5% agarose gel) was used to confirm the amplified product (10–15 µL). The DNA bands were stained with ethidium bromide (0.5 µg/mL) against the GeneRuler 100 bp Plus ready–to–use DNA ladder (Fermentas, Waltham, MA, USA) as a molecular weight marker. A DNA gel purification kit (Abgene, Portsmouth, NH, USA) was used to purify the appropriate–sized PCR amplicons from the gel. The sequencing reactions were carried out with 10 µL and contained 1 µL BigDye Terminator (BDT) v3.1 (Applied Biosystems, Waltham, MA, USA), 2 µL of BDT buffer, 0.16 µM of primer, and 1–2 µL of PCR product. Sequencing products were purified with the DyeEx® 2.0 Spin Kit (Qiagen) and run on a 3130*xl*Genetic Analyzer (Applied Biosystems). The sequences were aligned and compared with different trypanorhychid species previously accessed in GenBank.

Phylogeny: Phylogenetic analysis and evolutionary history for the isolated parasites were carried out using the Maximum Likelihood method and Tamura 3–parameter model. The recovered sequences were aligned and compared against Trypanorhyncha species previously accessible in the GeneBank. Sequence identity for the recovered data was checked using the Basic Local Alignment Search Tool (BLAST, available at http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence trimming for the congeneric species recovered was carried out by BIOEDIT v7.5.3; sequence alignment was done by CLUSTAL W v2 while the phylogenetic tree was constructed using MEGA 7 programme.

RESULTS

Three species of trypanorhynch metacestodes were isolated from the peritoneal cavity and mesenteries of the examined fish as blastocysts.

All the included species represent the first locality records in the investigated area. These include: *Gymnorhynchus isuri* (Figure 1a) isolated from the grey trigger fish *Balistes carolinensis* (46.7%, 7/15), *Pseudotobothrium dipsacum* (Figure 1b) from the mottled grouper *Mycteroperca rubra* (50.0%, 10/20), *Heteronybelinia estigmena* (Figure 1c) from the common sole *Solea vulgaris* (76.5%, 13/17). Worms were encapsulated within blastocysts; after rupture, each blastocyst generated a post larva called pleurocercus (Figure 1d; *plural* pleurocerci).

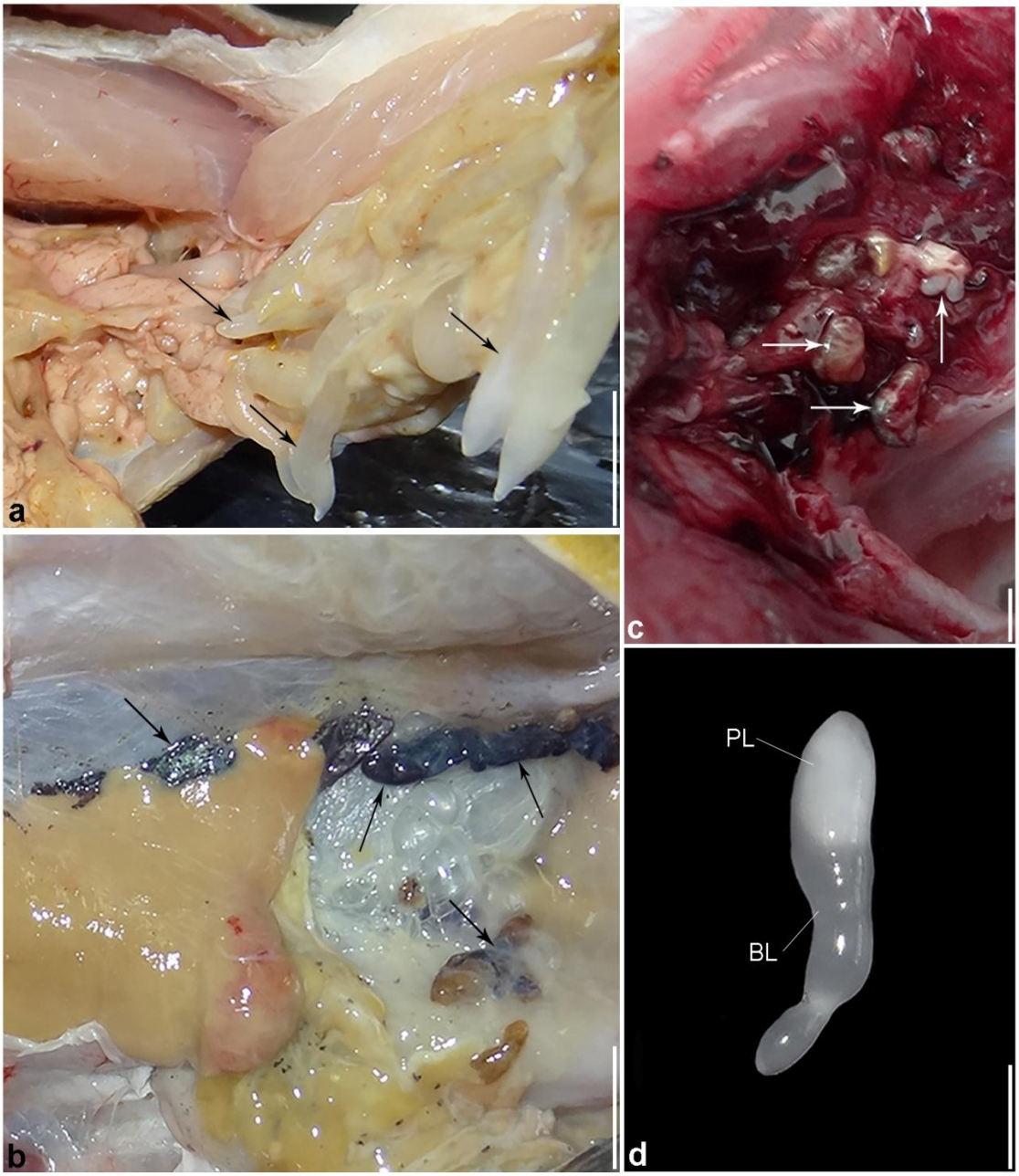

Figure 1. (a–d) Photographs showing encapsulated blastocysts of trypanorhynch metacestodes (arrows) in the peritoneal cavity of: (**a)** *Balistes carolinensis*; **(b)** *Mycteroperca rubra*; (**c)** *Solea vulgaris*; (**g)** Blastocyst sheathed a post larvae (plerocercus), Bars: a-d 1 cm; d 0.2 cm.

***Morphology*:**
Super family: Gymnorhynchoidea Dollfus (1935)
Family: Gymnorhynchidae Dollfus (1935)
Genus: *Gymnorhynchus* Rudolphi (1819)
***Gymnorhynchus isuri* Robinson (1959)**

Description (based on 8 pleurocerci): The capsule ranged from bladder–like to elongate and was usually white; the blastocyst was 1500–2800 (2630) µm long. The post larva had an elongated acraspedote and cylindrical scolex (Figure 2a) that was 740–880 (800) µm long × 87–145 (102) µm wide and featuring two short, auriculate, bothridia 211–243 (237) µm long x 278 –386 (329) µm wide in lateral view with rounded edges (Figure 3a, b). It is divided into four parts; the anterior pars bothridialis, the middle pars vaginalis, and the posterior pars bulbosa and pars post bulbosa. The length of the pars vaginalis was 351–483 (442) µm long, that of the pars bulbosa was 244–392 (363) µm, and that of the pars post bulbosa was 56–97 (72) µm. The tentacles (Figure 2b, 3c) were relatively short and tapered with corona of long falciform hooks around base of tentacles, and spiral rows of hooks, ascending from internal to external part of tentacle. The tentacle sheaths were spiral and tightly coiled. The tentacle bulbs (Figure 2c) reached the end of the scolex, but they did not occupy its entire width; they were about three times longer than their width. The metabasal armature was poeciloacanthous, with hollow hooks began on the internal surface. The longest hooks are in the middle row, 9 hooks/ row, 56–188 long, 12–68 wide, hooks 4(4') 60–195 long, 16–76 wide, hooks 5(5') 64–196 long, 20–76 wide, hooks 6(6') 40–192 long, 12–68 wide. Hooks 7(7')–9(9'), pointed, spiniform, becoming smaller in size; hooks 7(7') 36–168 long, 12–40 wide, hooks 8(8') 32–124 long, 12–40 wide, hooks 9(9') 28–76 long, 8–28 wide. Figure 4 (a, b), showing line diagrams for the recorded *G. isuri.*

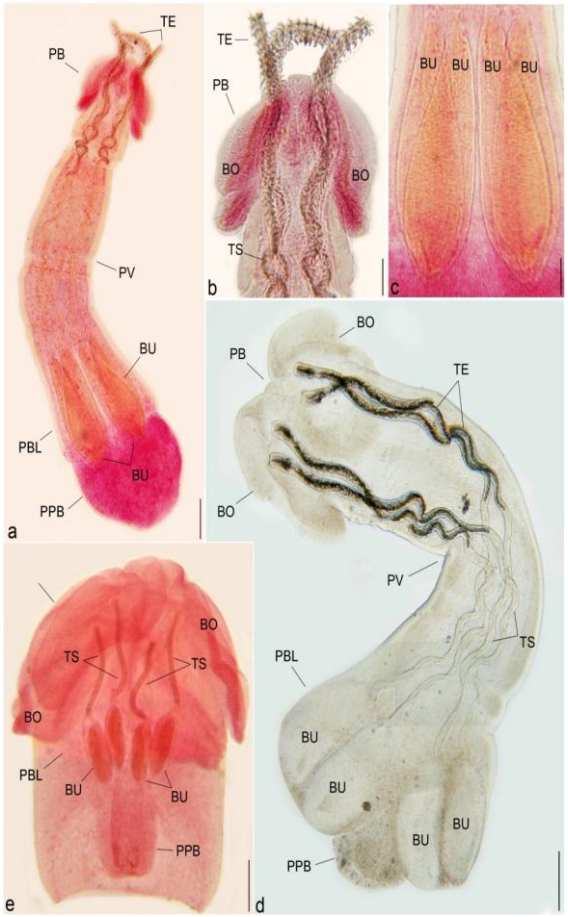

Figure 2. Photomicrographs of trypanorhynch metacestodes, isolated from the examined fish showing: (**a–c)** *Gymnorhynchus isuri*, **(a)** Entire worm, lateral view, PB pars bothridialis, PV pars vaginalis, PBL pars bulbulosa, PPB pars post bulbulosa, BU bulbs, Bar 500 µm; (**b)** The anterior part, BO bothridia, TS tentacle sheaths, TE tentacles, Bar 200 µm; (**c)** Four bulbs (BU), Bar 200 µm; (**d)**. *Pseudotobothrium dipsacum*, Entire worm, lateral view, Bar 500 µm; **(e)** *Heteronybelinia estigmena*, Entire worm, lateral view, Bar 200 µm.

Superfamily: Otobothrioidea Dollfus (1942)
Family: Otobothriidae Dollfus (1942)
Genus: *Pseudotobothrium* Linton (1897)
***Pseudotobothrium dipsacum* Linton (1897)**

Description (based on 10 pleurocerci): Plerocerci were isolated from club–shaped blastocysts with a broad anterior and blunt posterior ends, blastocysts were 1362–1857 (1557) µm long x 340–621 (566) µm wide at the broad end. The post larva was found attached to one end of the cyst by their heads (Figure 2d). Scolex craspedote, 462–856 (738) µm long; the maximum width was at mid–level of pars bulbosa, 436–568 (511) µm; pars bothrialis 361–495 (482) µm. Two patelliform notched bothria were observed, 234–315 (291) µm long. The length of the pars bothridialis was 163–195 µm, that of the pars vaginalis was 75–96 µm, that of the pars bulbosa was 22–35 µm. Pars post bulbosa reduced. The four tentacle sheaths were highly coiled and started from bothridial region till the anterior end of each bulb without tentacular swelling. The tentacular armature was heteroacanthous, heteromorphous; at the distal and metabasal regions consisted of longitudinal rows of slender hooks (Figure 3d) which were identical within each longitudinal row. Four ovoid bulbs were observed, three times longer than wide, 46–80 µm long x 15.1–19.6 µm wide. Seven hooks were observed per row with prominent space between in between. Hooks 1(1') uncinate, 13–17 (15) µm long; hooks 2(2') smaller, uncinate, 10–14 (13) µm long, hooks 3(3') uncinate, 11–15 (13) µm long; hooks 4(4') and 5(5') uncinate, smaller, 10–13 (12) and 8–10 (9) µm long respectively; hooks 6(6'), 7(7') smaller, falcate, 2–6 (3) mm long, 1–5 (4) µm long respectively. Figure 4 (c, d) showing line diagrams for the recorded *P. dipsacum.*

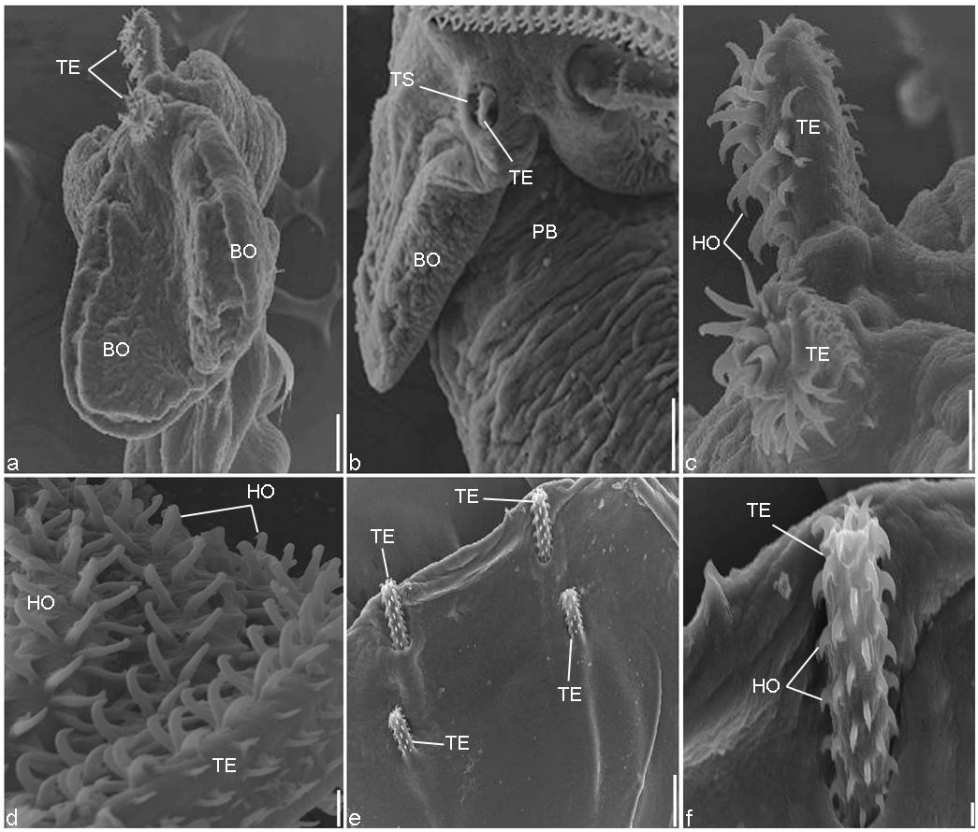

Figure 3. Scanning electron micrographs showing: **(a–c)** *Gymnorhynchus isuri*, **(a, b)** Pars bothridialis (PB), bothridia (BO), tentacles (TE), and tentacle sheaths (TS), Bar 100 µm, 50µm, **(c)** Tentacles and hooks (HO), Bar 50µm**; (d)** *Pseudotobothrium dipsacum*, tentacles and hooks, Bar 20µm; **(e, f)** *Heteronybelinia estigmena*, **(e)** Pars bothridialis and the four tentacles, Bar 100µm, **(f)** One tentacle and their hooks, Bar 10µm.

Superfamily Tentacularioidea Poche (1926)
Family Tentaculariidae Poche (1926)
Genus *Heteronybelinia* Palm (1999)
***Heteronybelinia estigmena* Dollfus (1960)**

Description (based on 8 pleurocerci): Plerocerci were isolated as encapsulated larvae from blastocysts which had broad anterior and tapered posterior ends (Figure 2e), they were 423–644 (451) µm long x 122–203 (140) µm wide at the broad end. Scolex short, with broad anterior and posterior ends, 122–253 (189) µm long x 61–84 (066) µm wide at the bothridial region. The total length/ bulbs ratio was 2.1–3.6 length of pars bothridialis 44–68 (57) µm long, pars vaginalis 49–73 (55) µm long, pars bulbosa 35–46 (0.38) µm long, pars post bulbosa reduced. The tentacle sheaths 29–35 (32) µm long, tentacles reached the apical end of the bulbs with no tentacular swelling (Figure 3e). Bulbs 146–210 (177) µm long x 7.5–18.1 (13.2) µm wide. The tentacular armature was homeoacanthous and heteromorphous, the hooks (Figure 3f) were rose–thorn shaped and diminished in size towards the basal part of the tentacle. Figure 4 (e, f), showing line diagrams for the recorded *H. estigmena.*

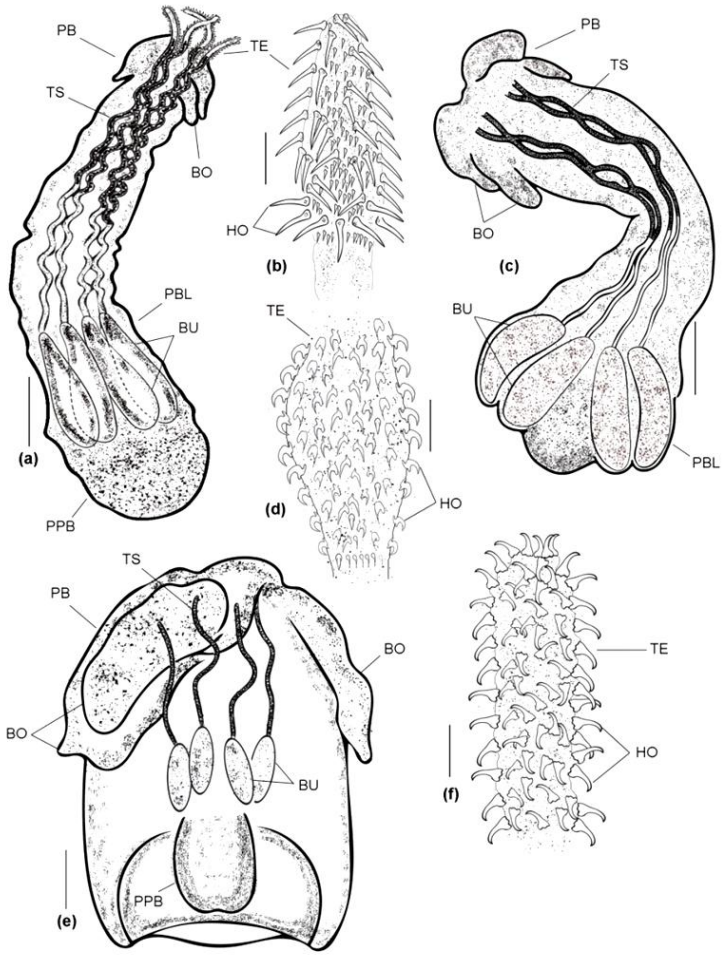

Figure 4. Line diagrams of trypanorhynch metacestodes isolated in the present study: Gymnorhynchus isuri, (a) Entire worm, Bar 500µm; (b) Enlarged tentacle, Bar 20µm; Pseudotobothrium dipsacum, (c) Entire worm, Bar 500µm; (d) Enlarged tentacle, Bar 20µm; Heteronybelinia estigmena, (e) Entire worm, Bar 200µm; (f) Enlarged tentacle, Bar 300 µm. PB pars bothridialis, PV pars vaginalis, PBL pars bulbulosa, PPB pars post bulbulosa, BO bothridia, TS tentacle sheaths, TE tentacles, HO hooks, BU bulbs.

Molecular Study: A phylogenetic tree was constructed from the sequences of the partial 18s and 28s ribosomal RNA isolated from the three species of trypanorhynchid larvae (Figure 5). Calculating the estimates of evolutionary divergence between sequences, the recovered data were compared to the available sequences of some Trypanorhyncha members recovered from GenBank. According to the phylogenetic analyses, there are four major lineages within the order Trypanorhyncha: the first clade includes the superfamilies "Lacistorhynchoidea", "Otobothrioidea ", "Gymnorhynchoidea" and "Tentacularioidea". Members of the first major lineage include monophyletic trypanorhych cestodes of family Lacistorhynchidae and consist of the genera *Grillotia*, *Grillotiella*, *Paragrillotia*, *Pseudogilquinia*, *Floriceps*, *Lacistorhynchus*, and *Callitetrarhynchus*. Families Pseudotobothriidae , Otobothriidae are the sister groups to this clade. The monophyletic clade of Tentacularioidea has a sister group that includes members of the family Tentaculariidae and Sphyriocephalidae. Members of Gymnorhynchidae and Phyllobothriidae are sister groups to Gilquiniidae. The constructed tree was polyphyletic and included the three queued species in different clades. The query sequences of the cestode parasite isolated from *B. carolinensis* showed high similarity (identity percentages of 98.91%, 96.58) with the previously deposited sequences of *G. isuri* in GenBank (DQ642909.1, MT667257.1). The recovered sequences were deposited in GenBank under accession number ON157059. The BLAST results also indicated that the 18s rRNA sequences of the cestode isolated from *Mycteroperca rubra* showed different identities from *Pseudotobothrium* species, which was identified in GenBank. The maximum identity was 98.40% with *P. dipsacum* (Acc. No. AF286972.1), followed by 96.59% with *P. balli* (FJ572959.1), and 94.20% with *P. arii* (Acc. No. DQ642910); it was deposited in GenBank under accession number ON139663. The 28s RNA sequences of the parasite isolated from *Solea vulgaris* yielded identity percentages with *Heteronybelinia* species, 90.73% with 28s ribosomal RNA sequences of *H. yamagutii* (Acc. No. FJ572932.1), with a maximum identity was 95.25% with *H. estigmena* (Acc. No. FJ572931.1) recovered from GenBank. The recovered sequences were deposited in GenBank under accession number ON139662.

DISCUSSION

There are 277 species of marine cestodes within Trypanorhyncha Diesing (1863) that use elasmobranches as their final hosts (Palm, 2004; Palm *et al.,* 2009). The present study provides the first data on the spectrum of trypanorhynch infestations among commercially important teleost fishes from the Mediterranean Sea, as illustrated through morphological and molecular analyses. The three recovered metacestodes in the present study possess most of the characteristic features of the order Trypanorhyncha, which include the following: the presence of two or four bothria and a tentacular apparatus with 4 eversible tentacles at its apex; and tentacles that generally bear a complex array of diverse hooks used to attach to the mucosa of the gastrointestinal tract (Dollfus, 1942; Richmond and Caira, 1991; Campbell and Beveridge, 1994; Palm, 1995, 1997; Morsy *et al.,* 2013). Cestodes in this group are unique because a specialist can often identify the larval species, usually in an invertebrate or teleost intermediate host, simply by observing the morphology of the scolex. The cestodes serve as a model group for understanding the patterns of host specificity (Palm and Caira, 2008), zoogeographic distribution, and parasite evolution within the marine ecosystem (Palm, 2004, Palm and Klimpel, 2007, Palm *et al.,* 2007, Palm *et al.,* 2009, Palm and Caira, 2008). The genus *Gymnorhynchus* includes two species, namely *G. gigas* and *G. isuri* (Knoff *et al.,* 2007; Santoro *et al.,* 2020). The number of hooks in the basal armature is the most conspicuous point of differentiation; *G. gigas* has approximately a ring of 18 large hooks in its basal armature, whereas *G. isuri* has only a ring of 8 or 9 of widely varying size (Caira and Bardos, 1996).

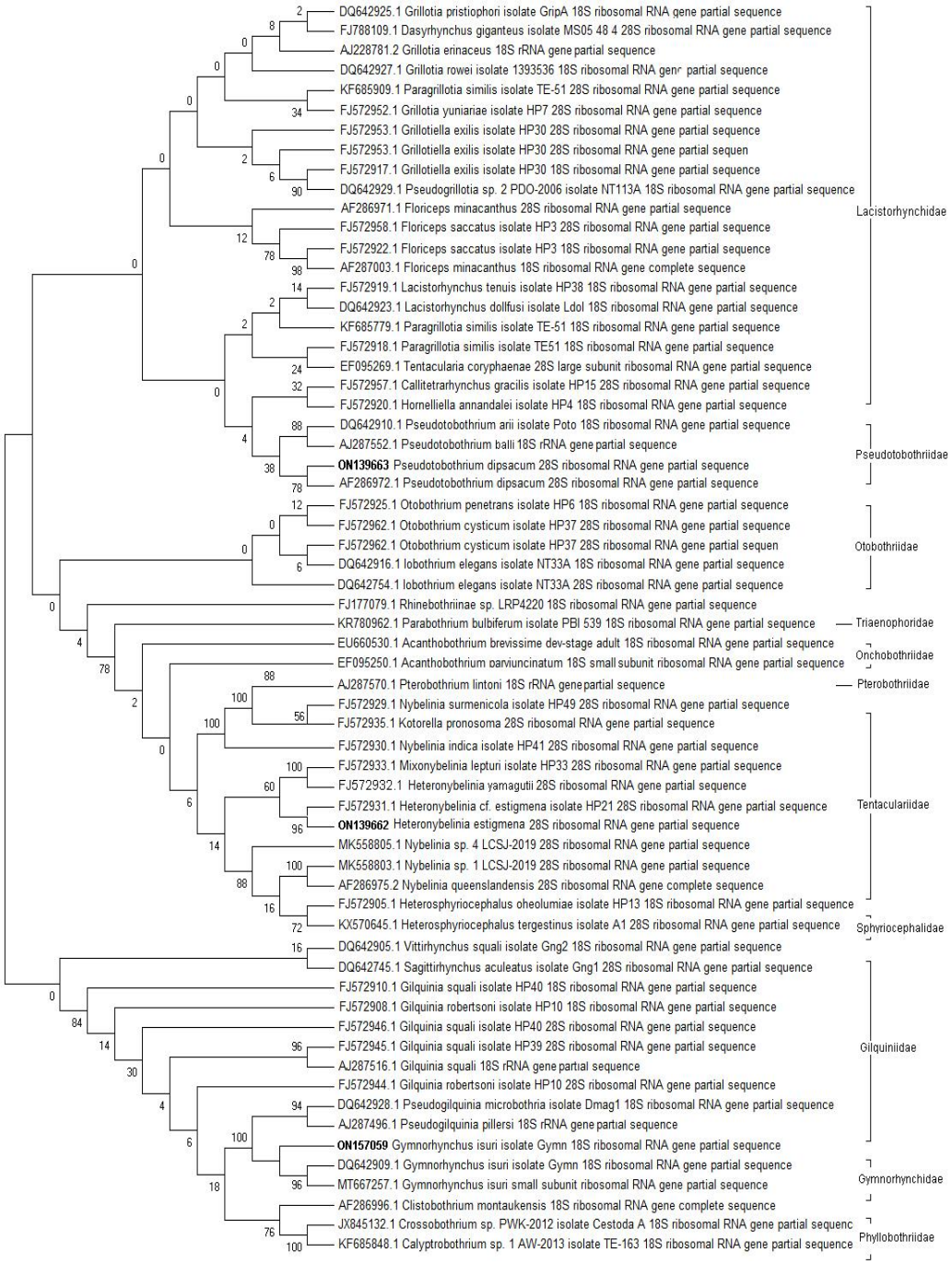

Figure 5. Phylogenetic analysis and evolutionary history using the Maximum Likelihood method and Tamura 3-parameter model according to the parasites 18s and 28s rRNA sequence analyses, the percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree (s) for the heuristic search was obtained by applying the BioNJ method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 60 nucleotide sequences. There were a total of 1000 positions in the final dataset.

The lack of microtriches in several regions of the scolex is in agreement with the observations of Caira and Bardos (1996), who only detected densely packed, slender, filiform microtriches on distal bothrial surfaces. The recovered *P. dipsacum* possessed a characteristic morphology similar to the species previously described trypanorynch cestodes of the genus *Pseudotobothrium* (Dollfus, 1942; Carvajal and Rego, 1985; Palm, 2004). It is one of the five most common trypanorhynch species found in teleosts on coral reefs by Beveridge *et al.* (2014). *Pseudotobothrium* sp. showed remarkably low specificity, it has been recorded previously from three orders and seven families, it was previously recorded from perciform fish teleosts of the families Apogonidae, Carangidae, Labridae, Lutjanidae, Serranidae; from Tetraodontiformes fish of the family Balistidae and from Scorpaeniformes fish of the family Platycephalidae (Beveridge *et al.,* 2014). The absence of any detectable specificity in these species leads to the prediction that further sampling will lead to even larger host ranges for these species. The morphological description of Plerocercoids of *P. dipsacum* agrees with that isolated from from *Lutjanus argentimaculatus*, *Pomadasys argenteus* given by Al–Zubaidy and Mhaisen (2011) from the Yemeni coasts of the Red Sea and those of Beveridge *et al*. (2000) from teloest fishes off Australian coasts. The prevalence of infection reported in the present study (50%) is higher than that reported from *P. maculates* (22%) from the Northeast Brazilian coastal waters (Palm, 1997) and from *P. argenteus* (24%) and *Thunnus tonggol* (36.7%) from the Red Sea (Al–Zubaidy and Mhaisen, 2011). Species of the genera *Gymnorhynchus* occur as adults in pelagic fish, with the larval stages infecting a wide range of teleosts and sharks (Campbell and Beveridge, 1994; Palm, 2004). Our present description of the trypanorhnch cestode isolated from *Solea vulgaris* and the morphology of its heteromorphous tentacular hooks assigns it to the genus *Heteronybelinia* as described by Palm (1999). *Heteronybelinia estigmena* is a typical tentacullariid trypanorhynch, its scolex consisting of a pedunculus scolecis bearing four anterior bothria with free margins and four apical tentacles. By comparison, the current specimen is similar in morphology to *H. alloiotica*, *H. punctatissima* and *H. dakari*, all are similar in having a heteromorphous tentacular armature with hooks diminishing in size towards the basal part of the tentacle, with no characteristic basal armature. Also, all these species have a very similar scolex and hook morphology, mainly differing from each other by a different bulb ratio and different scolex proportions. *H. alloiotica* differs from *H. estigmena* in having a bulb ratio of about 4, *H. punctatissima* have a slightly different bulb ratio and different scolex dimensions, while the bulb ratio of *H. dakari* was small (about 2.5:1). The parasite recorded in the present study is very similar to *H. estigmena* described by Dollfus (1960), both have similar morphology as well as bulb ratios and the presence of four tentacles equipped with solid, homeomorphous hooks arranged in a homeoacanthous pattern in accordance with (Palm, 1995; Palm and Walter, 1999). The phylogenetic analysis used 18s and 28s small ribosomal RNA for the recovered metacestodes, which led to the construction of multiple alignments that supported the taxonomic position of these parasites representing three genera: *Gymnorhynchus*, *Pseudotobothrium* and *Heteronybelinia*. These genera are sister taxons to *Pseudogilquinia pillersi*, *Otobothrium penetrans*, and *Nybelinia* sp., respectively, in accordance with Olson *et al*. (2010). The molecular evidence shows that Trypanorhncha consists of four well–supported lineages, and important morphological cross–linking has been mapped, where the highly variable armature pattern represents the main morphological diagnostic tool. The molecular phylogeny and tree topology in the present study are similar to the cladistic analysis of trypanorhynch cestodes reported by Palm (2004), where, there are four lineages within Trypanorhyncha, the branch including lacistorhynchoids consists of two main paraphyletic clades: poeciloacanthous multiatypical (*Dasyrhynchus*, *Protogrillotia*, and *Grillotia*) and poeciloacanthous atypical (*Pseudotobothrium* and *Gymnorhynchus*) with heteroacanthous, heteromorphous tentacular armature with a monophyletic sister taxon, *Otobothrium* and *pseudogilquinia* species (Palm and Overstreet, 2000, Palm *et al.*, 2009). Also, the monophyletic clade of superfamily Tentacularioidea includes metacestodes of family Tentaculariidae (*Heteronybelinia*) with homeoacanthous and heteromorphous armature with *Nybelinia* species of as sister taxons, this in accordance with the study of Palm and Overstreet (2000).

CONCLUSION

Both the molecular analysis and morphological characterization performed in the present study support the taxonomic identification of four parasitic metacestodes: *Gymnorhynchus isuri*, *Pseudotobothrium dipsacum* and *Heteronybelinia estigmena*. To ensure good food hygiene, trypanorhynch cestodes should be removed from infected fish, as parasitized fish are generally rejected by consumers due to their repulsive appearance, and humans are at greater risk for accidental infection and allergic reactions following the ingestion of raw infected fish meat.

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REFERENCES

AL QURAISHY, S.; DKHIL, M.A.M.; ABDEL–GABER, R. *et al.* Morphological and molecular insights of a new species of trypanorhynchid cestode parasite, *Nybelinia exostigmi*, in the Narrowstripe cardinal fish *Apogon exostigma*. *Rev. Bras. Parasitol. Vet.*, v.28, p.266-282, 2019.

AL–ZUBAIDY, A.B.; MHAISEN, F.T. First record of *Derogenes varicus* and *Dinurus scombri* (Digenea: Hemiurata) from Red Sea fishes, Yemen. Mesopot. *J. Mar. Sci.*, v.26, p.122-133, 2011.

BEVERIDGE, I.; BRAY, R.A.; CRIBB, T.H.; JUSTINE, J.L. Diversity of trypanorhynch metacestodes in teleost fishes from coral reefs off eastern Australia and New Caledonia. *Parasite*, v.21, p.60, 2014.

BEVERIDGE, I.; CAMPBELL, R.A.; JONES, M.K. New records of the cestode genus *Pseudotobothrium* (Trypanorhyncha: Otobothriidae) from Australian fishes. *Trans. R. Soc. S. Aust.*, v.124, p.151-162, 2000.

CAIRA, J.N.; BARDOS, T. Further information on *Gymnorhynchus isuri* (Trypanorhyncha: Gymnorhynchidae) from the shortfin mako shark. *J. Helminth. Soc. Wash.*, v.63, p.188-192, 1996.

CAIRA, J.N.; JENSEN, K. *Tapeworms from vertebrate bowels of the earth, Planetary Biodiversity Inventory (2008–2017)*: University of Kansas, Natural History Museum, Lawrence: [University of Kansas], 2017. (Special Publication No. 25).

CAMPBELL, R.A.; BEVERIDGE, I. Order Trypanorhyncha Diesing, 1863. In: KHALIL, L.F.; JONES, A.; BRAY, R.A. (Eds.). *Keys to the cestode parasites of vertebrates*. Wallingford: CAB International, 1994. p.51-148.

CARLETON, H. *CARLETON'S histopathological technique.* 4.ed. New York: Oxi. Univ. Press, 1976.

CARVAJAL, J.; REGO, A.A. Critical studies on the genus *Callitetrarhynchus* (Cestoda: Trypanorhyncha) with recognition of *Rhynchobothrium speciosum* Linton, 1897 as a valid species of the genus *Callitetrarhynchus*. *Syst. Parasitol.*, v.57, p.161-167, 1985.

DEARDORFF, R.B.; RAYBOURNE, R.B.; MATTIS, T.E. Infections with trypanorhynch plerocerci (Cestoda) in Hawaiiasn fishes of commercial importance. *Sea Grant. Quart.*, v.6, p.1-6, 1984.

DIESING, K.M. Sitzungsberichte der Kaiserlichen Akademie der Wissenschaften, Mathematisch–Naturwissenschaftliche Classe, Austria. *Akad. Wiss. Math. Naturwiss. Classe*, v.48, p.200345, 1863.

DOLLFUS, R.P. *Études critiques sur les tetrarhynchiques du Muséum de Paris*. Paris: Archives du Muséum D'Histoire Naturelle, 1942.

DOLLFUS, R.P. Sur quelques parasites de poissons recoltes a Castiglione (Algerie). *Bull. Trav.*, v.2, p.199-279, 1935.

DOLLFUS, R.P. Sur une collection de tetrarhynques homeacanthes de la famille des tentaculariidae recotes principalement dans la region de Dakar. *Bull. Ins. Fr. Afr. Noire*, v.22, p.788-852, 1960.

ERGENS, R. The suitability of ammonium picrate-glycerin in preparing slides of lower Monogenoidea. *Folia Parasitol.*, v. 16, p. 320, 1969.

GÒMEZ–MORALES, M.A.; LUDOVISI, A.; GIUFFRA, E. *et al*. Allergenic activity of *Molicola horridus* (Cestoda, Trypanorhyncha), a cosmopolitan fish parasite, in a mouse model. *Vet. Parasitol.*, v.157, p.314-320, 2008.

JONES, M.K.; BEVERIDGE, I.; CAMPBELL, R.A.; PALM, H.W. Terminology of the sucker-like organs of the scolex of trypanorhynch cestodes. *Syst Parasitol*., v. 59, p.121-126, 2004

KNOFF, M.; CLEMENTE, S.C.; PINTO, R.M.; LANFREDI, R.M.; GOMES, D.C. Redescription of *Gymnorhynchus isuri* (Cestoda: Trypanorhyncha) from Isurus oxyrinchus (Elasmobranchii: Lamnidae). *Folia Parasitol.*, v.54, p.208-214, 2007.

KVACH, Y.; ONDRAČKOVÁ, M.; JANÁČ, M.; JURAJDA, P. Methodological issues affecting the study of fish parasites. III. Effect of fish preservation method. *Dis. Aquat. Organ.*, v.12, p.213-224, 2018.

MADDEN, P.A.; TROMBA, F.G. Scanning electron microscopy of the lip denticles of *Ascaris suum* adults of known ages. *J. Parasitol.*, v.62, p.265-271, 1976.

MENEZES, P.Q.F.; KNOFF, M.; FELIZARDO, N.N. *et al*. *Callitetrarhynchus gracilis* (Rudolphi, 1819) Pintner, 1931 (Cestoda: Trypanorhyncha) parasitizing the musculature of *Sardinella brasiliensis* (Steindachner, 1879) (Actinopterygii) off the coast of the state of Rio de Janeiro, Brazil. *PLoS One*, v.14, p.e0206377, 2018.

MORSY, K.; BASHTAR, A.R.; ABDEL–GHAFFAR, F. *et al*. First identification of four trypanorhynchid cestodes: *Callitetrarhynchus speciouses*, *Pseudogrillotia* sp. (Lacistorhynchidae), *Kotorella pronosoma* and *Nybelinia bisulcata* (Tentaculariidae) from Sparidae and Mullidae fish. *Parasitol. Res.*, v.112, p.2523-2532, 2013.

OLSON, P.D.; CAIRA, J.N.; JENSEN, K. *et al*. Evolution of the trypanorhynch tapeworms: parasite phylogeny supports independent lineages of sharks and rays. *Int. J. Parasitol.*, v.40, p.223-242, 2010.

PALM H. W.; WALTER T. *Nybelinia southwelli* sp. nov. (Cestoda: Trypanorhyncha) with the re–description of *N. perideraeus* (Shipley & Hornell, 1906) and synonymy of N. herdmani (Shipley & Hornell, 1906) with Kotorella pronosoma (Stossich, 1901). *Bull. Br. Mus. Nat. His. Zool.*, v.65, p.123-131, 1999.

PALM, H.W. *Nybelinia* Poche, 1926, *Heteronybelinia* gen. nov. and *Mixonybelinia* gen. nov. (Cestoda, Trypanorhyncha) in the collections of the Natural History Museum, London. *Bull. Nat. Hist. Mus. London Zoolog*., v.65, p.133-153, 1999.

PALM, H.W. *The Trypanorhyncha Diesing, 1863*. Borgo: PKSPL, IPB Press, 2004.

PALM, H.W. Trypanorhynch cestodes of commercial fishes from Northeast Brazilian coastal waters. *Mem. Inst. Oswaldo Cruz.*, v.92, p.69-79, 1997.

PALM, H.W. Untersuchungen zur Systematik von Rüsselbandwürmern (Cestoda: Trypanorhyncha) aus atlantischen Fischen Christian–Albrechts–Universität Kiel, Kiel, Germany, 238 pp. *Ber. Inst. Meereskd. Christian Albrechts Univ. Kiel*, v.275, p.1-238, 1995.

PALM, H.W.; CAIRA, J.N. Host specificity of adult versus larval cestodes of the elasmobranch tapeworm order Trypanorhyncha. *Int. J. Parasitol.*, v.38, p.381-388, 2008.

PALM, H.W.; KLIMPEL, S. Evolution of parasitic life in the ocean. *Trends Parasitol.*, v.23, p.10-12, 2007.

PALM, H.W.; OVERSTREET, R.M. New records of trypanorhynch cestodes from the Gulf of Mexico, including *Kotorella pronosoma* (Stossich, 1901) and *Heteronybelinia palliata* (Linton, 1924) comb. n. *Folia Parasitol.*, v.47, p.293-302, 2000.

PALM, H.W.; WAESCHENBACH, A.; LITTLEWOOD, D.T. Genetic diversity in the trypanorhynch cestode *Tentacularia coryphaenae* Bosc, 1797: evidence for a cosmopolitan distribution and low host specificity in the teleost intermediate host. *Parasitol. Res.*, v.101, p.153-159, 2007.

PALM, H.W.; WAESCHENBACH, A.; OLSON, P.D.; LITTLEWOOD, D.T. Molecular phylogeny and evolution of the Trypanorhyncha Diesing, 1863 (Platyhelminthes: Cestoda). *Mol. Phylogenet. Evol.*, v.52, p.351-367, 2009.

RICHMOND, C.; CAIRA, J.N. Morphological investigations into *Floriceps minacanthus* (Trypanorhyncha: Lacistorhynchidae) with analysis of the systematic utility of scolex microtriches. *Syst. Parasitol.*, v.19, p.25-32, 1991.

ROBINSON, E.S. Some new cestodes from New Zealand marine fishes. *Trans. R. Soc. N. Z.*, v.86, p.381-392, 1959.

SANTORO, M.; IACCARI`NO, D.; BELLISARIO, B. Host biological factors and geographic locality influence predictors of parasite communities in sympatric sparid fishes off the southern Italian coast. *Sci. Rep.*, v.10, p.13283, 2020.

VÁZQUEZ–LÓPEZ, C.; ARMAS–SERRA, C.; BERNARDINA, W.; RODRÍGUEZ–CAABEIRO, F. Oral inoculation with *Gymnorhynchus gigas* induces anti–parasite anaphylatic antibody production in both mice and rats and adverse reactions in challenge mice. *Int. J. Food Microbiol.*, v.64, p.307-315, 2001.