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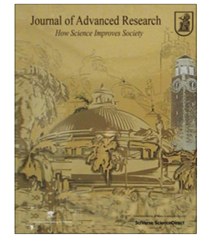

ORIGINAL ARTICLE

Morphological, molecular and pathological appraisal of *Callitetrarhynchus gracilis* plerocerci (Lacistorhynchidae) infecting Atlantic little tunny (*Euthynnus alletteratus*) in Southeastern Mediterranean

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ABSTRACT

The Atlantic little tunny, *Euthynnus alletteratus*, is widely distributed in temperate and tropical waters of the Atlantic Ocean, Black and Mediterranean Seas. In this study, wild-caught little tunny from Egypt, were found to be naturally infected with trypanorhyncha metacestodes, and the overall prevalence rate of infection was 38.7%. The blastocysts were either loosely attached to the mesentery of infected fish, or firmly attached and deeply embedded within the hepatic parenchyma. These encysted plerocerci are identified as *Callitetrarhynchus gracilis* (Trypanorhyncha, Lacistorhynchidae) based on its morphological and molecular characterization. The morphological characteristics of *C. gracilis* including scolex shape; the bothridia groove; the presence of frontal glands; the length of post-larval (appendix); metabasal armature;

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Trypanorhyncha
Callitetrarhynchus species
Histopathological studies

the existence of 'Chainette' and satellite hooks of different size were studied and described by Light and Scanning electron microscope. The phylogenetic analysis of lsrDNA gene of plerocerci confirmed the identification of the species to be deeply embedded in genus *Callitetrarhynchus*. The histopathological examination revealed severe pathological changes in the affected organs, including necrosis, inflammatory reactions, fibrosis and migratory tracts of the parasitic larvae together with marked visceral organs adhesions. To the best of our knowledge, this is the first report describing the detection of *C. gracilis* in little tunny collected from the Abu Qir landing site in Alexandria, Egypt.

Introduction

The Mediterranean Sea is considered as one of the main marine biodiversity hotspots on the earth [1], and fish parasites are a major component of such marine biodiversity [2]. Marine fish is commonly infected with a high diversity of parasites that could be a potential threat to fish abundance [3], and larval cestodes are some of the most damaging parasites to the viscera of infected fish. Order Trypanorhyncha [4] is a cosmopolitan group of marine cestodes, with more than 270 recorded species [5]. They use three or four intermediate hosts in their life cycles, before reaching the final host [6]. Larval trypanorhyncha encysted in visceral organs and musculature of marine teleosts, when being eaten by definitive hosts, and they excyst and form adult trypanorhyncha in the digestive tracts of elasmobranchs; sharks and rays [7].

The existence of larval trypanorhyncha in the fish flesh or body cavity reduces the market value of the fish by making them unappealing to consumers, thus causing economic losses [8]. Consumers may acquire this larval cestode through the consumption of infected raw, undercooked, or inadequately preserved fish [9]. There have been a few cases of accidental human infections by trypanorhyncha and they may also cause allergic reactions [10,11].

The migration of plerocercoid larvae of trypanorhyncha throughout visceral organs is typically associated with hepatic necrosis and extensive gonads and splenic damage [12]. This may reduce the reproductive capability and survival of affected fish [12]. Heavy tapeworm infections result in a mechanical obstruction of the gut and cause enteritis and degeneration of the intestinal wall [13]. Therefore, parasitological studies on fish accompanied with histopathological response are important when encapsulated metacestodes are found in commercially important species.

Atlantic little tunny *Euthynnus alletteratus* [14] is a member of family Scombridae that has wide distribution in the Mediterranean Sea [15]. Based on the available data, Atlantic little tunny *E. alletteratus* is the most abundant species among small tuna in Egypt and caught from the Southeastern coast of Mediterranean Sea [16]. In spite of its economic important, *E. alletteratus* is still poorly studied regarding its ichthyoparasitological problems.

In Egypt, most of the previous studies were carried out on trypanorhynchids from marine fish in the Red Sea [3,17,18], and there are no records for their existence in little tunny from the Egyptian Mediterranean Sea.

Therefore, this study reported the infection of little tunny collected from Egyptian Mediterranean coasts with a species of the trypanorhyncha cestode and provided information regarding its histopathological effects on the host. Morphological investigations of the recovered parasite species were carried out by light and scanning electron microscopy. In addition, the molecular analysis was also conducted for accurate identification of this parasite species.

Material and methods

Fish sample

During the period of October to December 2013, thirty-one specimens of Atlantic little tunny; *E. alletteratus* were collected by trap net method from the Coasts of Abu Qir landing site, Alexandria City, Egypt, located between longitude 29°47.1′–29°50.4′E and latitude 31°7.5′–31°09′N. The collected fish were preserved in isothermal boxes supplied with ice and transferred to the laboratories of the Biotechnology Center for Services and Researches, and fish diseases Department, Faculty of Veterinary Medicine, Cairo University, where specimens were identified, measured, and submitted for necropsy. Fish was medium-sized (19–28 cm long, and weighed 2025–3055 g).

Parasitic investigation

Fish samples were dissected for recovery of the prevailing parasites. Body cavity and viscera were examined using a stereoscopic dissecting microscope and the capsulated plerocerci were removed from the infected organs. Walls of parasite blastocysts were opened to remove the juvenile scoleces. The isolated worms were washed with saline solution and fixed in 10% buffered formalin. The fixed specimens were stained with acetic carmine, dehydrated and then mounted in Canada balsam and morphologically identified following the guidelines of Carvajal and Rego [19] and Palm [10]. Drawing of specimens was done by using the microscope tube (Nikon, Japan) Faculty of Veterinary Medicine, Cairo University, Egypt. Measurements were taken in millimeters.

Scanning electron microscope

For scanning electron microscopy, larvae were fixed in 4% glutaraldehyde, washed in cacodylate buffer, dehydrated in ascending alcohol series, processed in a critical point drier "Bomer-900" with Freon 13 and sputter coated with gold–palladium in a Technics Hummer V and then examined under an Etec AutoScan at 20 kV JEOL scanning electron microscope (Etec, USA) in the Electron Microscope unit at Ain Shams University, Egypt. Measurements were taken in millimeters.

Fig. 1 Photomicrographs of the larval cestode *Callitetrarhynchus gracilis* stained with acetic carmine and infecting *Euthynnus alletteratus*. (a) Whole mounts of larval cestode showing the bothridia (BO), followed by tentacles within tentacle sheaths (TS) coiled till the bulb base (BU), four bulbs (BU), and post-bulbosa area (PB). (b–g) High magnifications of: (b and c) The anterior part is showing the four bothridia (BO), tentacles (T) within tentacle sheaths (TS). (d) One of tentacles (T) within tentacle sheaths (TS). (e) Four tentacle sheaths (TS) coiled till the bulb base (BU). (f) The cephalic glands (frontal glands) do not extend to the par bulbosa (BU). (g) Long Post-bulbosa area (appendix) (PB).

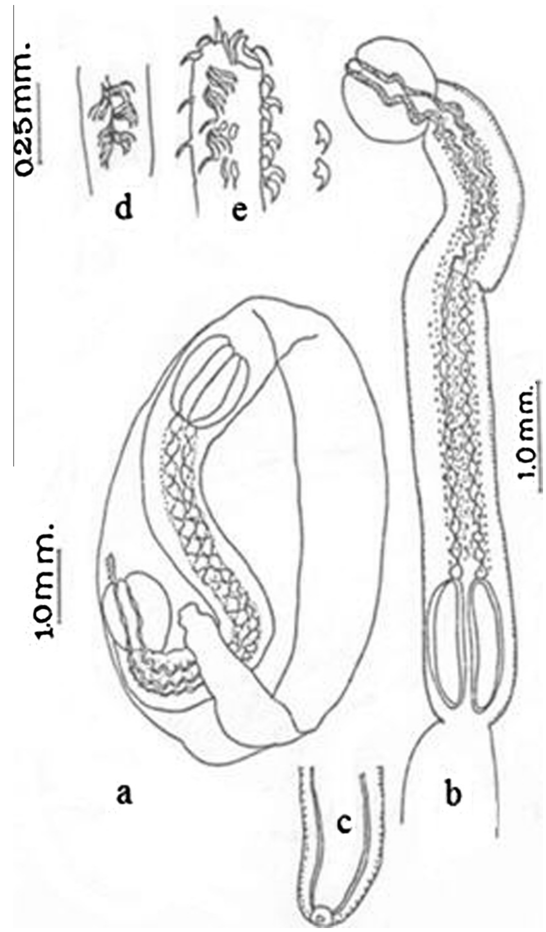

Fig. 2 Drawing of stained *C. gracilis*. (a) The host capsule is bladder-like to elongated. (b) Scolex showing distribution of cephalic glands do not extend to the par bulbosa. (c) Posterior end of appendix. (d–e) Metabasal tentacle armature showing external face, note the chainette (The figure adopted from Mahdy et al. [17]).

Partial sequencing of lsrDNA and phylogenetic analyses

For molecular analysis, DNA from the preserved worm samples was extracted according to the protocol of tissue Gene Jet TM Genomic DNA purification Kit (Fermentas life sciences, Lithuania). The D2 variable region (~600 bp) of the nuclear large subunit ribosomal DNA (lsrDNA) gene was sequenced to identify the plerocercoid. This region of the lsrDNA has been found to be informative for both diagnostic and phylogenetic work in tetraphyllidean and related taxa [20,21]. Polymerase chain reaction (PCR) was carried out to amplify the target D2 variable region of lsrDNA using the following primers: 300F (5-CAA GTA CCG TGA GGG AAA GTT-3) and ECD2 (5-CTT GGT CCG TGT TTC AAG ACG GG-3), as described by Aznar et al. [21], in a 25-μl reaction mixture comprising 1 μl of extracted genomic DNA, 5 μl of 1 mM deoxyribonucleotide triphosphates (dNTPs, MBI Fermentase), 0.25 μl of each primer (50 pmol μl^{-1}), 2.5 μl of 10× Taq polymerase buffer (MBI Fermentase), 2 μl of 25 mM Mgc l, 1 μl Taq DNA polymerase (2 U) (MBI Fermentase), and 13 μl of distilled water. The PCR cycle consisted of an initial denaturation step of 94 °C for 4 min, followed by 40 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 60 s, was finished with terminal extension at 72 °C for 7 min, and then rested at 4 °C. The PCR products were electrophoresed in 1.0% agarose gel in Tris–acetate–EDTA-buffered gel stained with 1% ethidium bromide and visualized with a UV transilluminator. PCR products were purified using standard techniques (Qiaquick PCR Purification Kit, Qiagen Company, CA) and run against a standard mass ladder (100 bp) on an agarose gel to estimate the concentration of DNA. The PCR product was directly sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with 310 Automated DNA Sequencer (Applied Biosystems, USA) using the same primers used in PCR. The sequence obtained was edited manually using BioEdit version 7.0 [22], and aligned with other lsrDNA sequences available in GenBank using Mega 5 [23]. The phylogenetic analysis was based on Kimura's 2-parameter model for the neighbor-joining method (substitutions included transversions and transitions, pattern among lineages assumed

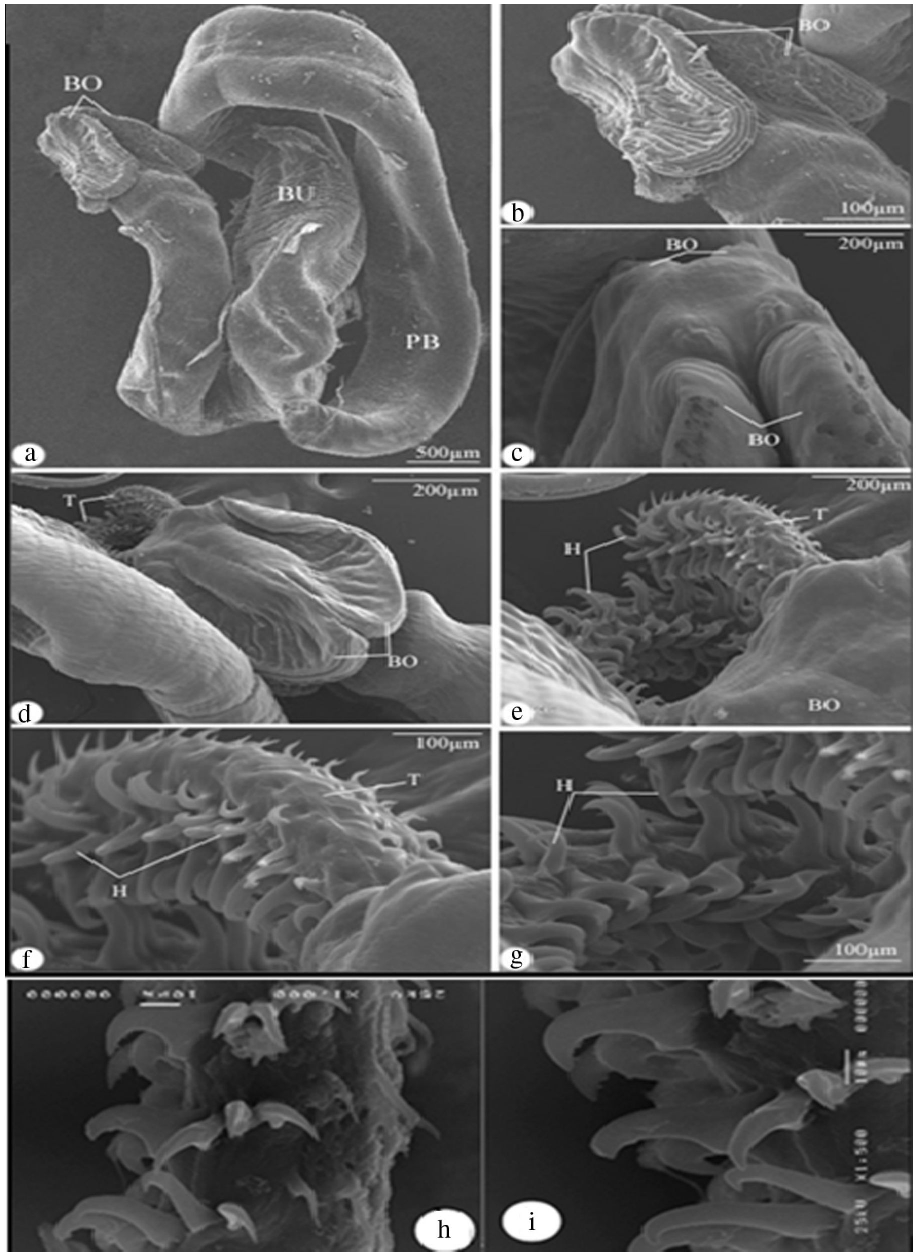

Fig. 3 Scanning electron micrographs of *C. gracilis* showing: (a) the whole body with 2 bothridia (BO), bulb base (BU) and parbulbosa area (PB). (b–d) Scolex note the distinct bothridial groove near the border (BO). (d) Scolex with heart shaped bothridia, and armed tentacle. (f and g). Tentacle with continuous spiral rows of hooks. (h and i) Metabasal armature showing external face, note the 'chainette' and satellite hooks of different size.

homogeneous, and the rate variation among sites uniform) with 1000 bootstrap replicates. The nucleotide sequences obtained were submitted to the GenBank under the accession number KP300037.

Histopathological examination

Tissue specimens from liver and tissue masses showing adhesion between visceral organs and peritoneum were fixed in 10% neutral buffered formalin for routine histopathological examinations. The fixed samples were washed in tap water overnight and exposed to ascend concentrations of ethanol (70%, 80%, 90% and 100%), cleared in xylene and embedded in paraffin. Tissue slides of 5 μm thick sections were prepared and stained with hematoxylin and eosin (H&E). The histopathological preparation was performed according to Roberts [24].

Results

Clinical investigation

Naturally infected fish showed slight abdominal distension. The gross lesion revealed the presence of encysted trypanorhyncha larvae in mesentery, liver and other internal organs within the peritoneal cavity. The encysted larvae were slender or bladder-like in shape with white color. The older

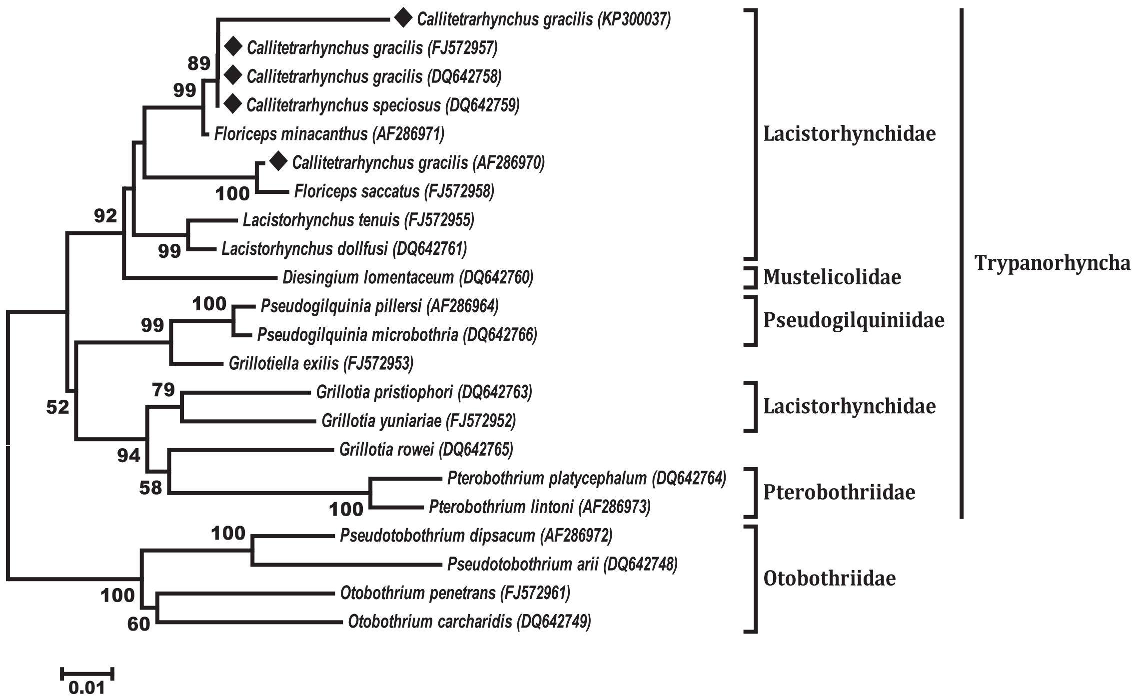

Fig. 4 Dendrogram showing the relationship between the present *C. gracilis* with other Lacistorhynchoidea species recovered from GenBank.

larval capsules, however, were brown to blue black and slightly iridescent. The overall prevalence of 31 examined fish was 38.7% (12/31).

Morphological description (based on 10 specimens)

The plerocercoid body was elongated and measured 9.1 ± 0.1 (8.7–11.5) mm in length and 1.1 ± 0.2 (1.0–1.5) mm in width at the level of bulbs. The scolex supplied with four long cylindrical and sheathed tentacles measuring 6.3 ± 0.2 (5.8–6.9) mm in length (Figs. 1a–c and 3a–d). The scolex was measured 6.9 ± 0.2 (5.4–6.3) mm in length with two short, heart-shaped bothridia (Fig. 3b–d). The bothridia were 1.1 ± 0.2 (0.91–1.4) mm in length and 0.43 ± 0.2 (0.27–0.69) mm in width. It has a clear distinct bothridial groove near the border (Fig. 3d). The anterior body has a frontal glands do not extend to the par bulbosa (Fig. 2a–b). Metabasal tentacular armature poeciloacanthus atypical external surface with chainette elements and intercalary hooks (Fig. 3f–g). A principal hooks from continuous half spiral rows of seven hooks beginning on the internal surface; hooks 1 (1′) their points are convergent and uncinate (supplement 2-B); hooks 2 (2′) are uncinate; hooks 3–5 (3′–5′) are falciform (Fig. 3h–i); hooks 6–7 (6′–7′) are spiniform and situated near external surface; principle hook 7 (7′) and intercalary hooks a (a′) in satellite position to chainette elements, the intercalary hook is smaller than the principle hook (7) (supplement 2-A). The tentacle sheaths were regularly coiled until the base of the bulb and supplied with hooks in continuous spirals (Fig. 1d and e). Four symmetrically arranged bulbs were present at the end of a scolex. Each one measured 1.1 ± 0.02 (0.87–1.05) mm in length and 0.29 ± 0.02 (0.26–0.33) mm in width (Figs. 1a, f and 3a, b). The post-bulbosa (appendix) area was long with no granules, and measured 0.29 ± 0.02 (0.26–0.31) mm in length and 0.14 ± 0.002 (0.11–0.20) mm in width (Fig. 1a and g).

Taxonomic summary

Parasites name: *Callitetrarhynchus gracilis* [25]
Family: Lacistorhynchidae [26]
Host: Little Tunny *E. alletteratus* [14] (Family: Scombridae)
Locality: Coasts of Abu Qir landing site, Alexandria City, Egypt
Site of infection: Plerocerci larvae were found in the coelomic cavity of infected fish
Prevalence of infection: 12 out of 31 examined fish were infected (38.7%).
Material deposition: Voucher specimens were deposited in the Parasitology Laboratory, Zoology Department, Faculty of Science, Cairo University, Egypt.

Phylogenetic analysis

An approximately 560 bp fragment of the D2 variable region of lsrDNA gene of the studied species was obtained. Comparison of the nucleotide sequences and divergence showed that the present trypanorhynchid cestode is deeply embedded in the genus *Callitetrarhynchus*, with 97% identities for (FJ572957, AF286970, DQ642758) of *C. gracilis*, 96% for (DQ642759) of *C. speciosus*, 95% for (AF286971) of *Floriceps minacanthus*, (DQ642761) of *Lacistorhynchus dollfusi* and

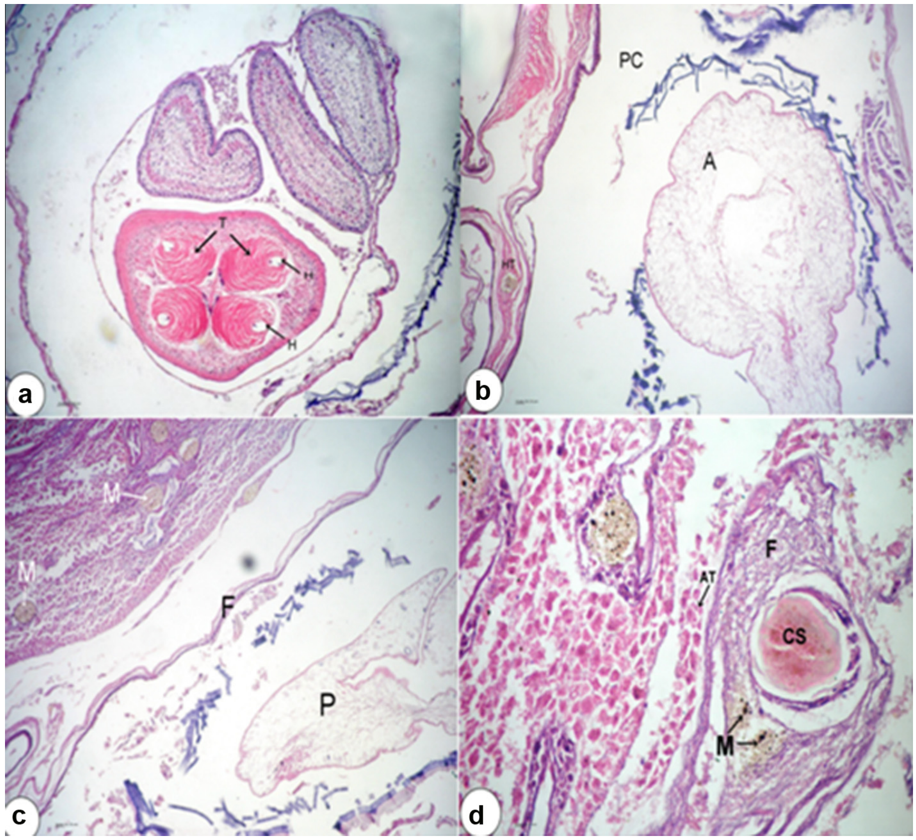

Fig. 5 (a) Histopathological section of parasitic nodules showing anterior part of the parasite with tentacle (T) and hooks (H). (b–d) Histopathological section of fish viscera showing: (b) Parasitic larvae attached to intestinal serosa. Notice, the anterior part of the parasite (A) in the peritoneal cavity (PC) near the intestinal and hepatic tissue (HT). (c) The anterior part of the parasite (P) in the abdominal cavity surrounded with a thin layer of fibrous connective tissue (F), notice, the melanophores aggregation (M) in the hepatic tissue. (d) Fish liver, with a cross section of parasitic larvae (CS) surrounded with a thin layer of fibrous connective tissue proliferation (F), melanophores aggregation (M) and atrophied hepatocytes (AT), (H&E stain).

(FJ572955) of *Lacistorhynchus tenuis*, 94% for (DQ642760) of *Diesingium lomentaceum*, 93% for (DQ642765) of *Grillotia rowei*, (AF286967) of *Grillotia erinaceus*, and (DQ642763) of *Grillotia pristiophori*. The present trypanorhynchid cestode revealed sequence identities under family Lacistorhynchidae ($\geqslant$91%). The phylogenetic analysis revealed strong nodal support for two major lineages (Fig. 4). The first major clade represents Lacistorhynchoidea species and consisted of two larger subclades, in which Pseudogilquiniidae, Mustelicolidae, and Petrobothriidae are sister to Lacistorhynchoidae with weak nodal support. The other major clade stands for a monophyletic origin for Otobothrioidea species.

Pathological findings

The Gross examination of the affected fish showed the presence of the parasites' nodules in the abdominal cavity and it evoked adhesion between the different visceral compartments causing difficulties in separating of individual organs. The parasitic nodules were noticed only in the liver, intestinal serosa and peritoneum causing adhesion of such parts.

The histopathological examination revealed the presence of multiple parasitic larvae attached to intestinal serosa. The histopathological examination of the parasitic nodules revealed the characteristic shape of the anterior part of the cestodal larvae in tissue section (Fig. 5a). A thin layer of fibrous connective tissue surrounds the parasites and holds fast them to the intestinal tissue (Fig. 5b). Remnants of the larvae were also noticed in the hepatic tissue with prominent melanophores aggregation (Fig. 5c). Some cases reported characteristic passage tracts formed of necrotic tissue with marked hepatocytes destruction. The parasitic larvae were wrapped with active thin layer of proliferative fibrous tissue, melanophores aggregation and atrophied hepatocytes (Fig. 5d). In such tracts, areas of hemorrhage were frequently noticed along with melanophores aggregates and mild to marked fibrosis (Fig. 6a). Mononuclear inflammatory cell infiltration (Fig. 6b) and hepatocytes necrosis were common findings with multiple pyknotic nuclei in the affected hepatic tissue (Fig. 6c). The examination of the spleen revealed marked activation of melanomacrophage centers while the homeopathic tissue showed depletion (Fig. 6d).

Discussion

This study reported the prevalence of infection with one of the trypanorhyncha metacestodes in *E. alletteratus* which is a common pelagic species in Mediterranean fisheries. Larvae of

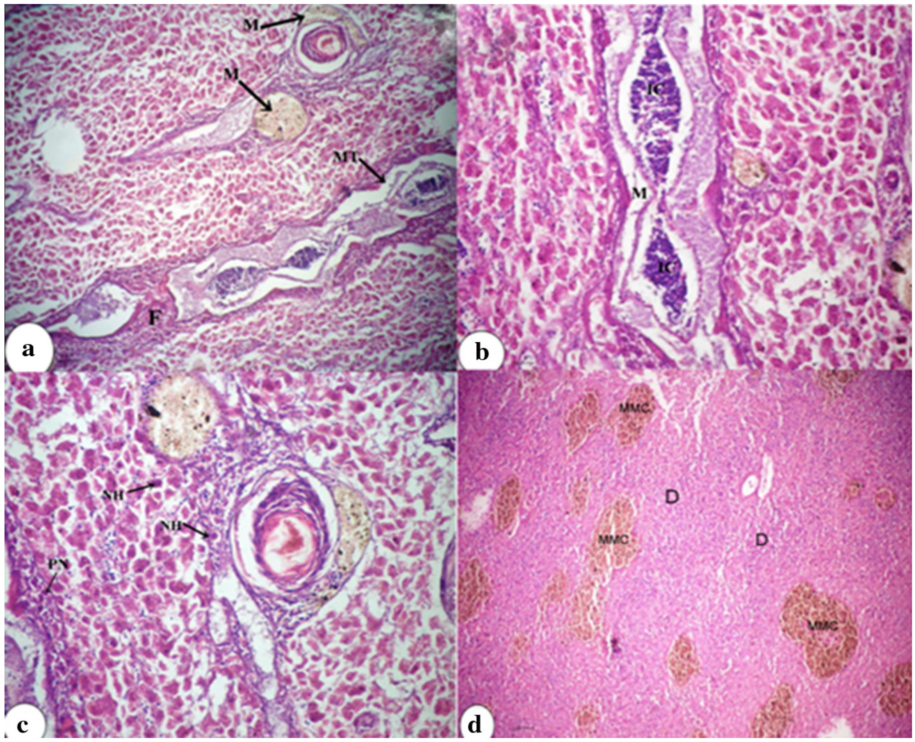

Fig. 6 (a) Histopathological section of fish liver showing migratory tracts (MT), melanophores aggregation (M), fibrosis (F) and atrophied cells (AC) of hepatocytes. (b) Histopathological section of fish liver showing migratory tract of the parasite in hepatic tissue with mononuclear inflammatory cells (IC) infiltration. (c) Histopathological section of fish liver showing necrosis of the hepatocytes (NH) and pyknosis of the nuclei (PN). (d) Histopathological section of fish spleen showing the activation of melanomacrophage centers (MMC) and depletion (D) of the hemopoietic tissue, (H&E stain).

trypanorhyncha were found to be encysted in mesentery, liver and other internal organs within the peritoneal cavity of *E. alletteratus*. Trypanorhyncha use crustaceans and invertebrate animals as the first intermediate hosts [27,28], some of which constitute food items for *E. alletteratus*, which act as the second intermediate host.

The recovered plerocercoid was identified as *C. gracilis* [25]. To our knowledge, the present finding of *C. gracilis* in Alexandria coasts represents its new geographical record in the Southeastern Mediterranean Sea. This parasite was also recorded from *E. alletteratus* in Turkey and the prevalence rate was 91.3% [29], while it was 38.7% in this study. The present finding of *C. gracilis* in Egypt and Turkey indicates its common occurrence in *E. alletteratus* of the Mediterranean Sea. The existence of variation in *C. gracilis* prevalence in *E. alletteratus* from two different countries in the Mediterranean Sea may indicate the presence of an uneven distribution in density of first intermediate hosts.

C. gracilis was also isolated from more than 150 fish species worldwide such as California [30], Brazil [27], Arabian Gulf [28,31], and Red Sea in Egypt [3]. In Arab Gulf, Bates [32] and Abdou and Palm [3] recorded *Callitetrarhynchus* parasite from *Scomberoides cammmersoniaus*. The occurrence of *Callitetrarhynchus* species in many species of teleosts suggested a wide distribution of this parasite, and the existence of certain un-specificity of this parasite to its fish hosts.

Taxonomists are considered scolex shape, bothrial groove, spread of cephalic glands, and the length of post-bulbosa. Tentacular armature was the most important characters for trypanorhyncha taxonomy [19,33–36]. The morphological characters of *C. gracilis* found in the present study were similar to other *Callitetrarhynchus* species described previously. Such similarity was represented by the presence of pars postbulbosa, heterocanthus, homeomorphous, and unicate hooks that were arranged in continuous spirals. *C. gracilis* revealed specific characteristic morphological features that distinguish it from *C. speciosus*. These morphological differences include the presence of a clear distinct bothridial groove near the border in *C. gracilis*, while it is weakly developed in *C. speciosus*; the frontal glands do not extend to the par bulbosa in *C. gracilis*, while it extends to par bulbosa in *C. speciosus*; hooks 1 (1′) in *C. gracilis* have their points convergent, while in *C. speciosus* they are arranged in a parallel pattern; in *C. gracilis* the intercalary hook is smaller than the principle hook no. 7 (7′), while in C. *speciosus* they are almost equal in size. By morphometrical comparison (Table 1), the parasite from the present study mostly resembles to *C. gracilis* in *Lethrinus nebulosus* and in *Carangoides malabaricus*y [18,19,36,37], but showing minor variation in the dimensions of the different body parts.

Palm et al. [7] accepted Otobothrioidea characterized by bothrial pits as a superfamily, despite its derived placement among the Lacistorhynchoidea. In our analyses, the Lacistorhynchoidea grouped as sister to the Mustelicolidae species and thus we recognize both taxa as monophyletic superfamilies, which coincided with Olson et al's study [38]. The present trypanorhynchoid showed the highest percentage of identity with other species within Lacistorhynchoidea. The

Table 1 Comparative measurements (in millimeters) of the present *Callitetrarhynchus gracilis* and those described previously.

Species	Host fish	Dimensions of			
		Total body L	Pars bothridialis L.	Pars bulbosa L.	Postbulbosa L.
Callitetrarhynchus speciosus [33]	*Pomatomus saltatrix*	13–17 (15)	1.70–2.0 (1.92)	1.8–2.2 (2)	1.6–3.4 (2.0)
Callitetrarhynchus gracilis [34]	*Lethrinus nebulosus*	8.5–14.8	0.96–1.3 (1.13)	0.85–0.99 (0.92)	(0.22–0.29) 0.25
Callitetrarhynchus speciosus [19]	*Pagrus pagrus*	10.30–13.8 (12.97 ± 2)	0.8–1.2 (1.00 ± 0.2)	1.22–1.35 (1.32 ± 0.02)	2.6–3.30 (3.00 ± 0.2)
Callitetrarhynchus gracilis [35]	*Carangoides malabaricus*	25	0.27	0.12	–
Callitetrarhynchus gracilis (The present study)	*Euthynnus alletteratus*	8.7–11.5 (9.1 ± 0.1)	0.91–1.4 (1.1 ± 0.2)	0.87–1.05 (1.1 ± 0.02)	0.26–0.31 (0.29 ± 0.02)

phylogenetic analyses supported its taxonomic position within the genus of *Callitetrarhynchus* with indistinguishable relationship to other *C. gracilis* and *C. speciosus*. This finding was in agreement with the previous reports of Olson et al. [38]. Therefore, according to data from morphological and molecular analyses, the present parasite belongs to family Lacistorhynchoidea and classified as, *C. gracilis* with new locality records in *E. alletteratus* from the Egyptian water.

Relatively few studies have investigated the effects of trypanorhyncha on their hosts [39]. Paperna [40] reported that encysted larvae of cestodes might not interfere with fish physiological functions and homeostasis, even when numerous in the mesenteries. However, Adjei et al. [41] attributed an increased mortality of *Saurida tumbil* due to the pressure of *C. gracilis* blastocysts on the ventral aorta.

In this study, the gross examination of infected fish showed severe adhesion in the internal viscera typically associated with the presence of the encapsulated blastocysts. In some fish, adhesion with internal organs looks like a big mass of tissue. This adhesion could be attributed mainly to the development and migration of plerocercoids within the host. In this scenario, the parasitic cestodes with penetrative type scoleces [42] elicited mechanical tissue damages that end up with chronic inflammatory lesions with adhesive nature. Here the plerocercoids were noticed to be attached to the surface of the internal organs or frequently found loose in the abdominal cavity. This finding agrees with the results of Al-Niaeem et al. [39].

The observed plerocercoids were either migrating under the wall of the intestine or dug inside the hepatic and splenic tissues causing their destruction. The inflamed sites in affected tissues were recognized by aggregation of mononuclear cells and melanophores. Such histological damage and inflammatory response were previously addressed by Bahram et al. [43]. Interestingly, no plerocercoids were found in the musculature of infected fish. These results revealed that these plerocercoids mainly harbor the internal organs and, therefore, do not comprise the edible portion of the fish. Since the cestode parasites (*C. speciosus*) were previously reported in musculature of two different edible fish species *of Cephalopholis hemistiktos* and *L. nebulosus*, in the Arabian Gulf [44], further studies should be required, however, to exclude the possibility of musculature infestation in *E. alletteratus* fish species.

There is still a considerable shortage in knowledge of tapeworms from Scombridae fish, and considering existing information about parasites from tunas, the number of species known to parasitize these tunas is proportionally lesser than that of other fish, perhaps due to shortage of surveys of their helminth fauna.

Conclusions

This is the first record documents the infection of little tunny fish by *C. gracilis* with 38.7% prevalence rate, and represents its new geographical record in Southeastern Mediterranean Egyptian coast. The infection was confirmed by both morphological and molecular tools. The infected fish showed encapsulated blastocysts-related visceral adhesion that attributed to the mechanical damage induced by plerocercoids development and migration.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jare.2015.07.004.

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