



Phenotypic and genomic characterization of *Tylodelphys* sp. metacercaria (Diesing 1850) (Trematoda: Diplostomidae) recovered from *Lates niloticus* (Linna)

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Abstract:

Metacercaria of *Tylodelphys* sp. a member of the Diplostomidae, was isolated naturally from the gas bladder of *Lates niloticus* (Nile Perch), found in Lake Nasser (southern Egypt). The morphology, ultrastructure, and genomic characterization were investigated and described. The main characteristics were shown by the light microscope and these were confirmed by the electron microscope. The examined fish were found to be infected with *Tylodelphys* sp., with prevalence of 86%, with the highest prevalence shown to be in summer (26%) and the lowest in winter (16%). Morphologically, by light and scanning electron microscopy, the parasite was found to be linguiform, where the fore body comprised most of the worm and the hind body was reduced to a small, conical, pointed end. The tegument was unarmed and smooth. A small oral and a large ventral sucker were detected. The body contained two types of papillae; a fine anterior and a coarse posterior one. The genetic analysis of *Tylodelphys* sp. showed it was similar to those of the species of fish origin found in GenBank, and a description of a new Egyptian genotype (accession number MH367017) was provided. The morphotype detected was found to have about 99% genetic similarity in the 12S rRNA region with other helminthes. The obtained results support an evolutionary addition of the Egyptian species of *Tylodelphys* sp. aiding in its taxonomy and diagnosis.

Key words: *Tylodelphys* sp; genomic characterization; *Lates niloticus*; Lake Nasser ; phylogenetic tree.

INTRODUCTION

Tylodelphys spp. is trematodes of the family Diplostomatidae distributed worldwide, with a complicated life cycle. They need three hosts: lymnaeid snails / planorbid slugs as first intermediate host, a variety of fish species as second intermediate host, and the definitive hosts usually fish-eating birds and mammals (Dubois, 1970; Gibson et al., 2001; Niewiadomska and Laskowski, 2002).

The genus *Tylodelphys* was recorded firstly by Diesing (1850) in its metacercarial stages and later was proved by Dubois (1937). Morphologically there are a total of 17 adult species and 11 metacercarial stages of *Tylodelphys* spp. (Blasco-Costa et al., 2017).

The unencysted metacercarial stage of *Tylodelphys* spp. are causing serious damage and harmful effects to the host freshwater fish, where they inhabit the eyes, the cranial cavity of the brain, the pericardial sac, the body cavity and the gas bladder (Chibwana and

Nkwengulila, 2010; Chibwana et al., 2015; Otachi et al., 2015; García-Varela et al., 2016; Blasco-Costa et al., 2017).

Electron microscopy is one of the new aids used to confirm a diagnosis of helminth species infestation, and helps in the identification of more obscure structures (Bazh, 2012).

Polymerase Chain Reaction (PCR) is an excellent confirmative diagnostic method for helminthes diagnosis (El-Bahy et al., 2017).

Data on *Tylodelphys* spp. metacercariae from Egypt is limited. This may be due to the difficulty in identifying it. Few molecular data is available among the world. So, the aim of this study was to detect *Tylodelphys* sp. metacercaria in situ, its prevalence, describing it morphologically by light and scanning microscope and to distinguish it from related metacercarial stages and from other helminthes. In addition, the aim of the study was to provide the *Tylodelphys* sp. sequence using the genetic marker (12S rRNA) in order successfully to

characterize *Tylodelphys* genetically and molecularly from other trematodes and accurately to differentiate it from morphologically similar diplostomatids.

Material and Methods

Fish samples, clinical, postmortem and parasitological examinations

A total number of 100 *Lates niloticus* (Linnaeus, 1758) of different sizes (total length 25-53 cm) and weights (150-500 gm), (25 fish/ season) were collected alive from different localities of Lake Nasser, southern Egypt, from May 2017 till April 2018. The collected fish were transferred quickly to the Laboratory of Fish Diseases, Faculty of Fish and Fisheries Technology, Aswan University.

L. niloticus were clinically examined for any abnormalities, and then the fish were euthanized by the addition of 0.20 mL of clove oil to each 500 mL of water, according to the guidelines and policies of the local and national animal welfare laws (Javahery et al., 2012 and Fernandes et al., 2017). Then the external and internal gross lesions were recorded as

soon as possible according to the method described by Noga (2010).

Tylodelphys sp. metacercariae were recovered from *L. niloticus* by opening the gas bladder and leaving it in a Petri dish containing physiological saline for 30 minutes, at room temperature. Then the detached metacercariae in the saline were collected using a pipette. For morphological examination, the parasites were fixed in 70% ethanol, stained in acetic acid alum carmine and dehydrated through ascending grades of ethanol, then cleared in xyelene and mounted in DPX or Canada balsam for permanent slide preparation. The identification was according to keys of Gibson (1996) using nearly 30 samples.

Electron microscopy

Ten freshly collected *Tylodelphys* sp. were washed several times in isotonic saline solution, then washed in phosphate buffer solution (PBS) and fixed in 3% glutraldehyde (pH 7.4) at 4 °C in 0.1 M phosphate buffer, then washed three times in PBS (for 30 minutes). Specimens

were post fixed in 1% osmium tetra oxide in 0.1 M phosphate buffer solution for 1-2 hours, and then washed three times in the same buffer. Fixed specimens were dehydrated through series grades of ethanol. Complete dehydration was performed in 2 changes of absolute ethyl alcohol and dried in the critical point drying apparatus, and then mounted on stubs with double adhesive tape, coated with gold and examined with a JEOL 5300 JSM electron microscope at an accelerating voltage of 25 K.V and magnification power ranged from X 150 to X 500 (Bazh, 2012).

DNA extraction, PCR reaction and phylogenetic analysis

Genomic DNA of 200 *Tylodelphys* sp. metacercariae was extracted. The extraction process was performed by using a QIAamp tissue kit (Qiagen Inc., Valencia, CA, USA).

The used primers were presented in table 1. PCR reactions volume was 50 µl according to (Lee et al., 2013). A

negative control was used with every PCR reaction to detect contamination.

Purification process of the excellent PCR product was done by using the GeneJET PCR Purification Kit (Thermoscientific kits, Germany) on GATC Company by using a ABI 3730xl DNA sequencer (Applied Biosystems, Foster City, CA, USA).

The obtained nucleotide sequence was aligned using (BLAST) program (BLAST <http://www.ncbi.nlm.nih.gov/blast>) in the NCBI GenBank. Thus to obtain the first tree view for the nucleotide sequence by joining it to its neighbor.

The obtained nucleotide sequence of *Tylodelphys* sp. was submitted to GenBank for an accession number. The accession number then was analyzed and compared with the gene sequence of different helminthes as described in table (2). The MEGA7 software was used to evaluate the identity and diversity of the sequences (Kumar et al., 2015). The tools of the phylogenetic tree construction

were considered as the model for the interpretation and the explanations of the results. The sequences were obtained only from specific small subunit ribosomal RNA (12S rRNA) genes, which were about 747bp in length (**Lee et al., 2013**). The phylogenetic tree was obtained by using the evolutionary neighbor-joinin distances, and a maximum parsimony distance.

Fig 1: A. *Tylodelphys* sp. attached to the congested inner wall of gas bladder of *Lates niloticus* (arrows) and aggregates of them inside the circle. B. Gas bladder of *Lates niloticus* infected with *Tylodelphys* sp. Showing the metacercariae attached to the congested inner wall (arrows) and several detached ones in the Petri dish

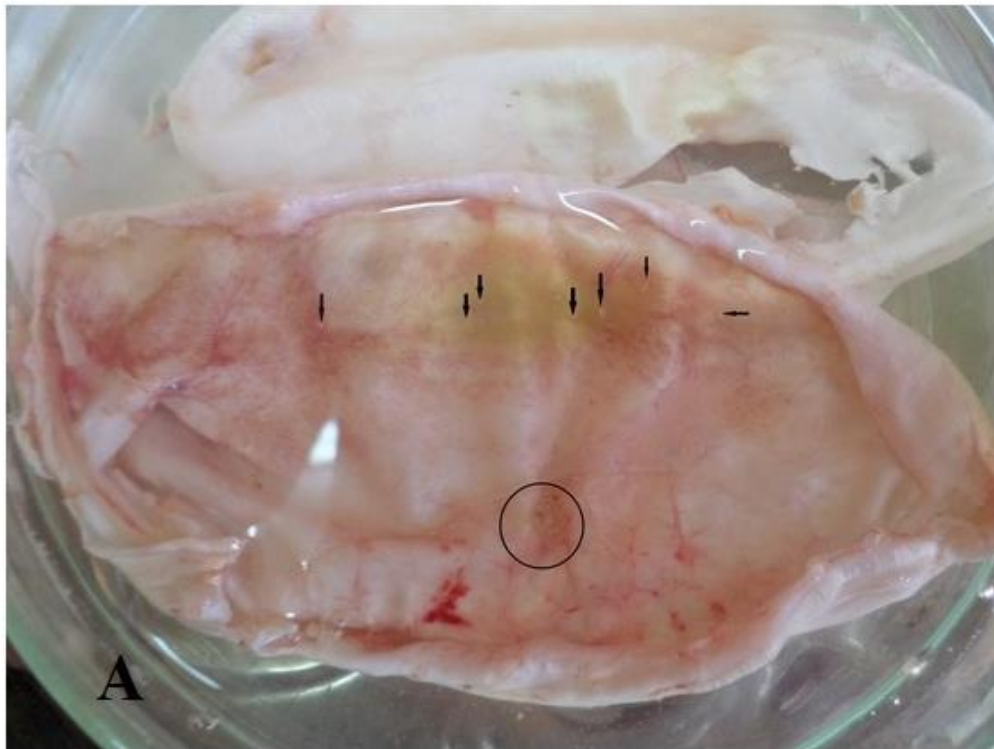


Fig 2: Prevalence of *Tyloodelphys* sp. metacercariae in *Lates niloticus* from Nasser Lake during the period of May 2017 to April 2018 (examined number= 100).

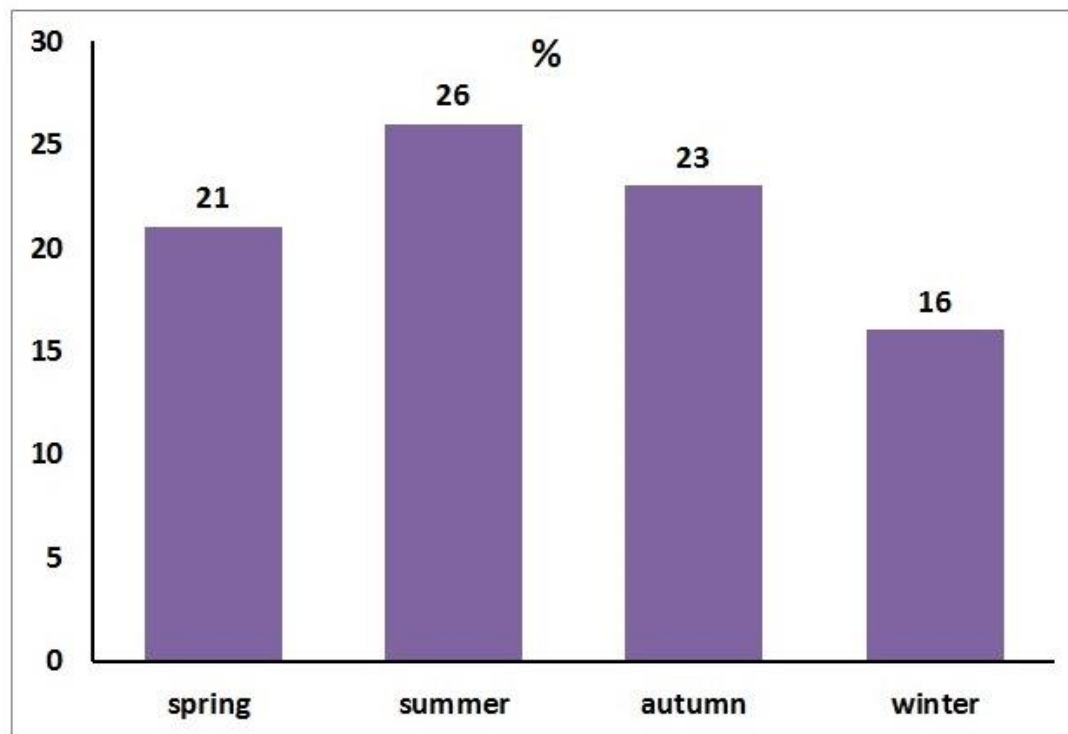


Fig. 3: *Tylodelphys* sp. metacercariae. Stained specimen (Carmin stain) x132. Scale bar= 100µm. OS=oral sucker, PS= pseudosucker, VS ventral sucker, Osp= oeshagus, Int= intestine, HiB= hind body, G= genital groove, EP= excretory pore.

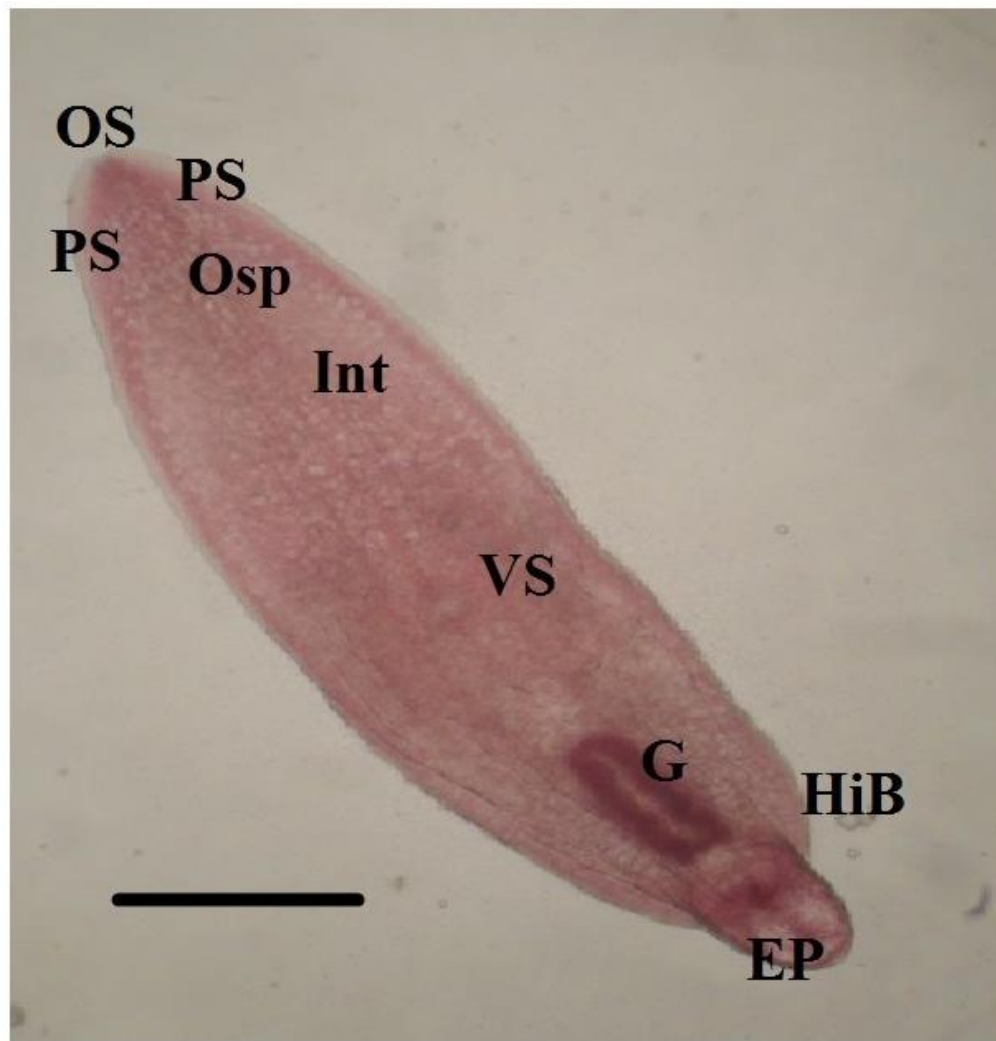


Fig. 4 (a & b) scanning electron microscopy of *Tylodelphys* sp. metacercariae showing AP= anterior papillae, PP= posterior papillae.

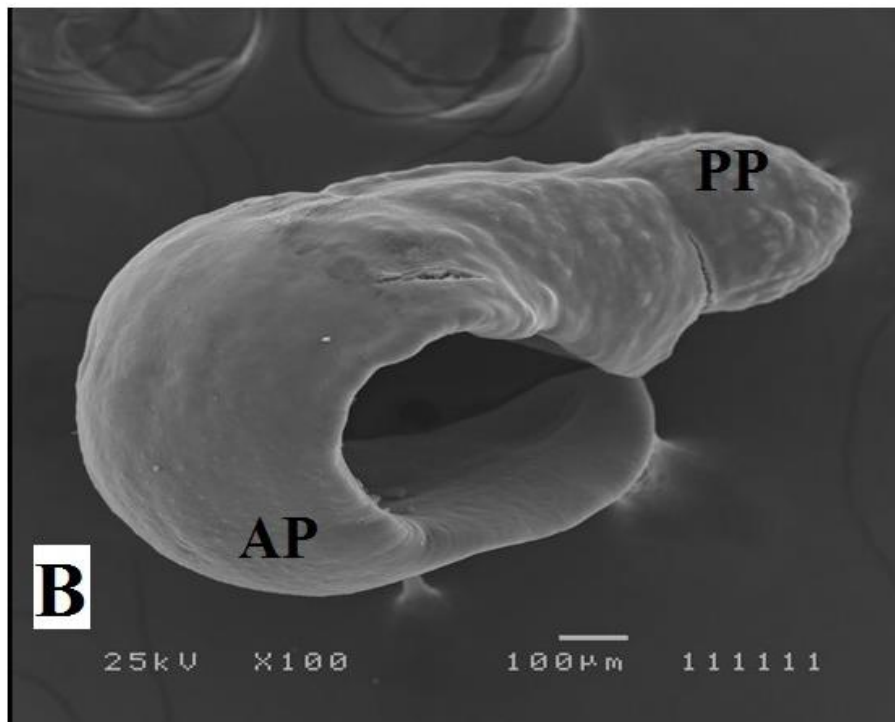
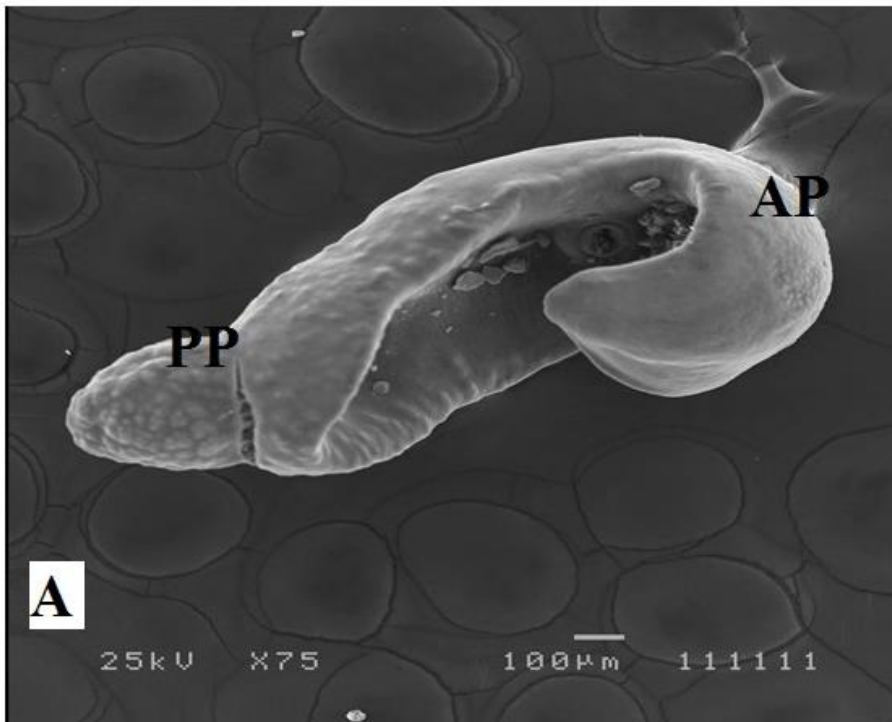


Fig. 5 (a, b& c) scanning electron microscopy of *Tylodelphys* sp. metacercariae showing OS=oral sucker, PS= pseudosucker, VS ventral sucker, HiB= hind body, G= genital groove.

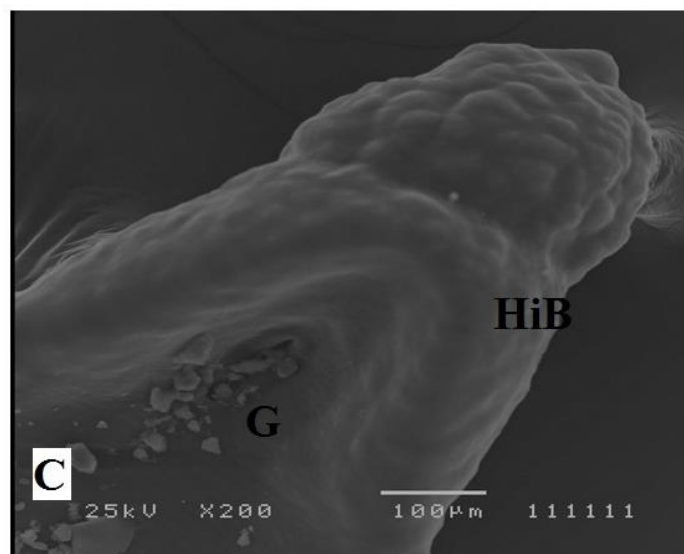
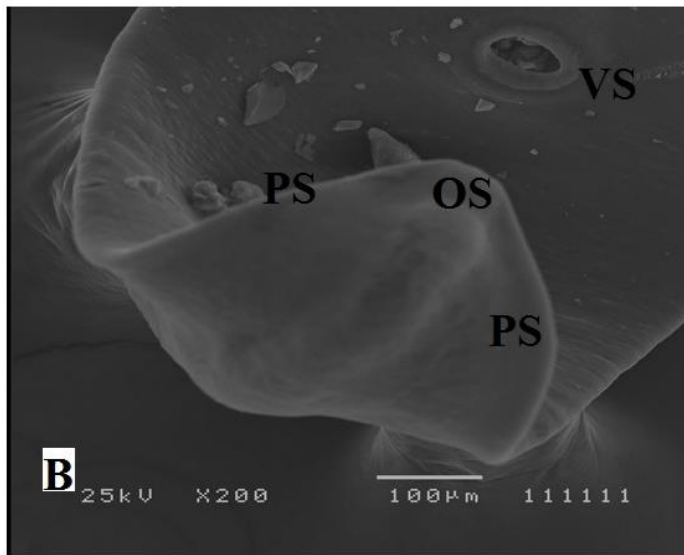
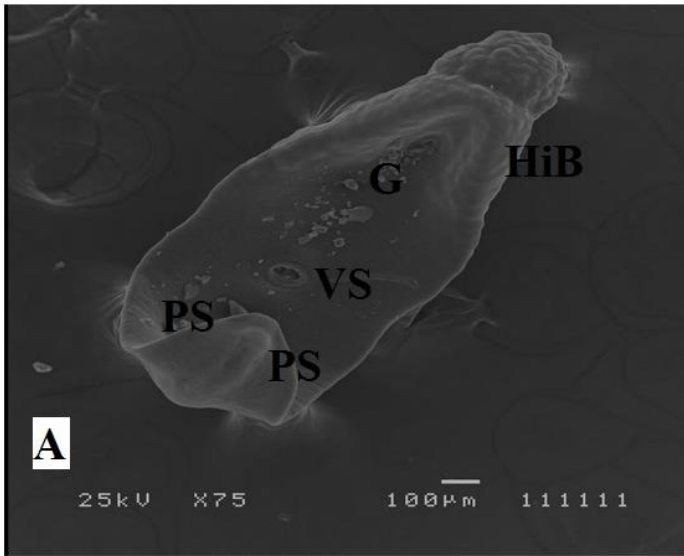
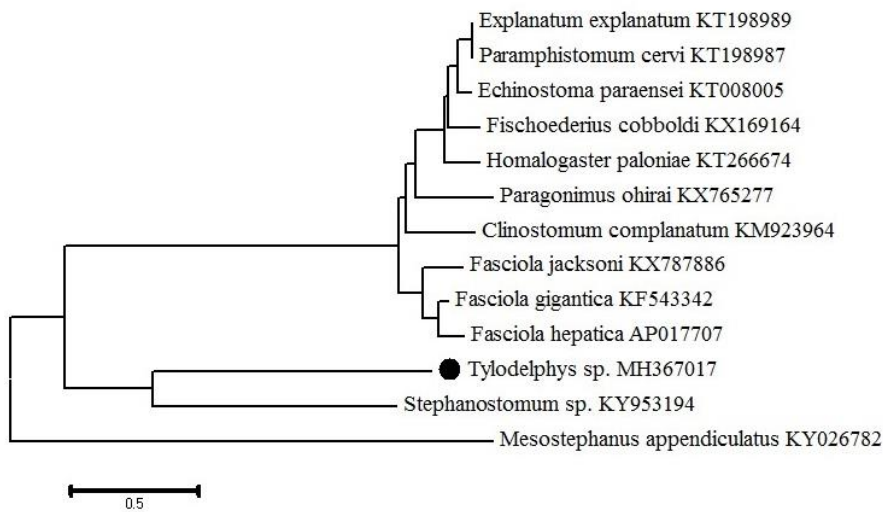


Fig. 6: Phylogenetic analysis of *Tylodelphys* sp. metacercariae. (MH367017) with different trematode families by the neighbor-joining method using the BLAST program and MEGA7.



Results

Clinical, postmortem examinations, seasonal prevalence and Parasitological examinations. Parasitological examinations.

The clinical examination of infected *L. niloticus* showed no pathognomonic abnormalities but the postmortem examination revealed the presence of several *Tylodelphys* sp. attached to the inner wall of the gas bladder, in addition to hemorrhagic patches scattered along the inner wall of the bladder. Detached, visible, unencysted and highly motile metacercariae could be seen by naked the eye in the plate (Fig. 1).

By examining 100 *L. niloticus*, eighty-six (86%) of them harbored *Tylodelphys* sp. metacercariae. The highest prevalence was in summer (26%), then autumn (23%), spring (21%) and the lowest prevalence was in winter (16%), (Fig. 2).

The detected metacercaria is related to:

Superfamily Diplostomoidea Poirier, 1886

Syn. Strigeoidea Railliet, 1919

Family Diplostomidae Poirier, 1886

Subfamily Diplostominae Poirier, 1886

Genus: *Tylodelphys* Diesing, 1850

Tylodelphys sp. metacercaria was recovered from the inner wall of the gas bladder of *L. niloticus*. It was linguiform in shape, free, unencysted, highly active and white in color. The body was fusiform, measuring a few millimeters, not clearly divided into two regions, with a rounded anterior end and a bluntly conical, pointed posterior end. The ventral concavity in the anterior body was very indistinct. The tegument was devoid of spines. The oral sucker was sub-terminal, well-developed and smaller than the ventral one. Pseudosuckers (lappets) were positioned on either side of the oral sucker, barely differentiated (indistinct) and only discernible on three specimens. The esophagus was long and the intestinal caeca was terminated posteriorly. The hind body was small, not

well differentiated and about 13% of the total body length. Excretory bladder had a pore at the posterior tip of the body (Fig. 3).

Electron microscopy

Electron microscopy revealed that the tegument was apparently smooth. The fore body included most of the worm, whereas the hind body was reduced to a small conical posterior end. The oral sucker was smaller than the ventral one. Pseudosuckers were present. The ventral surface of the fore body was flat or slightly concave, and the dorsal surface was somewhat convex. Two types of papillae (oval granular inclusions) were present; small delicate papillae were found on the fore body and coarse ones were identified on the region from the posterior end of the body to the ventral sucker (Fig. 4a, b). The ventral sucker was oval, well developed and located in the middle third of the body. The holdfast organ was a strong, muscular, deep, longitudinal oval slit and was located midway between ventral sucker and the

posterior end of the body. Directly posterior to the holdfast organ, primordia of gonads were found within the hind body (Fig. 5 a, b& c).

Molecular analysis

The PCR amplification of the 12S rRNA region of the rDNA from *Tylodelphys* sp. metacercariae resulted in a single product of identical size, i.e. (700 bp) long. After the analysis of the PCR product, this product was fit for sequencing. The sequence obtained was submitted to the GenBank which provided an accession number of gb (MH367017).

A nucleotide BLAST of the novel 12S rRNA sequence of *Tylodelphys* sp. (metacercariae) on (BLASTn) revealed the existence in GenBank of several high similarity sequences, all belonging to species of different trematodes.

To evaluate the sequence from the *Tylodelphys* sp. obtained in this study, partial 12S rRNA sequences for named Pairwise alignments were performed using the same partial 12S rRNA,

Tylodelphys sp. was found to be closely related to most digenetic trematodes.

Phylogenetic tree analyses were illustrated, based on the alignment of partial sequences of the 12S region of the rRNA, using the Neighbor Joining method and the maximum parsimony tree. The trees obtained presented bootstrap consensus that the topologies of the trees so obtained were similar (Fig. 6).

Discussion

There are different types of metacercariae inhabit fish as second intermediate host; one of them is *Tylodelphys* sp., which is an unencysted larval stage trematode previously called *Diplostomulum*.

Tylodelphys sp. (Diesing, 1850) was detected in different sites; the eyes (vitreous humour), body cavity, gas bladder and the brain of freshwater teleost and in the spinal cord of amphibians. Otachi (2009) detected *Tylodelphys* sp. from *Oreochromis nilotica*. Moema et al. (2013) and Chibwana et al. (2015) collected it from the cranial cavity of *Clarias gariepinus*.

Garcia-Varela et al. (2016) isolated it from the body cavity of freshwater fishes (Goodeidae and Cyprinidae). In (Blasco-Costa et al., 2017; Chaudhary et al., 2017) recovered it from the vitreous humour of different freshwater fish species. Hamouda et al. (2018) found it in the gas bladder of *L. niloticus*.

The adults were detected in piscivorous birds (Gibson, 1996). In Egypt, El-Naffar et al. (1980) and Tadros et al. (2013) isolated the adult *Tylodelphys aegyptius* from the giant heron (*Ardea goliath*) and from herring gulls (*Larus argentatus*) respectively. Chibwana et al. (2015) recovered adult *Tylodelphys mashonense* from the grey heron (*Ardea cinerea*) and the white egret (*Egretta alba*) from Tanzania.

Tylodelphys sp. was detected with a prevalence of 86%, showing a high prevalence in summer (26%), followed by autumn (23%), spring (21%) and then winter (16%). It showed nearly uniform frequency during the different seasons. This may be attributed to the ecological

factors for this parasite, as the snails (the first intermediate host), and aquatic birds (the definitive host) are exist in and around Lake Nasser all over the year. This finding was nearly similar to that recorded by **Hamouda et al. (2018)** which examined the same fish species from Lake Nasser. Lower prevalence was recorded by **Öztürk (2017)**, who isolated *Tylodelphys* sp. from some cyprinid fish from Turkey, *Chondrostoma nasus*, *Capoeta tinca* and *Barbus plebejus*, where the prevalence was found to be 5.1%, 10.6% and 12.5% respectively. **Öztürk (2017)** recorded the infection of *C. tinca* in all seasons but *B. plebejus* and *C. nasus*, only infected in winter. This may be due to the difference in the species of the fish examined and to the collection area.

In our opinion, the optimal temperature which required for the maintenance of snails and aquatic birds in and around Lake Nasser all over the year may be the reason for the high infection rate of *L. niloticus* with *Tylodelphys* sp.

Electron microscopy findings showed the *Tylodelphys* sp. was to some extent similar to the genus *Diplostomum*, especially the tegument and the presence of the papillae, with some noted differences (**Moema et al., 2013; Garcia-Varela et al., 2016; Blasco-Costa et al., 2017**).

In Egypt, the taxonomy of *Tylodelphys* sp. depends mainly on the conventional diagnostic methods, such as microscopic examination; no data on its genotypic diversity is currently available.

The PCR and DNA sequencing methods now possible are recommended to confirm the findings as these are the most sensitive tools for the detection and diagnosis of parasitic diseases (**Bazh, 2013a&b; Bazh et al., 2016**). Such molecular techniques have facilitated the accurate identification of the phylogenetic relationships among the different parasite species in the family Diplostomidae (**Moema et al., 2013; Garcia-Varela et al., 2016; Chibwana et al., 2015;**

Blasco-Costa et al., 2017; Sereno-Uribe et al 2019; Sokolov et al 2022).

The sequence analysis of *Tylodelphys* sp., depending on the nuclear ribosomal gene (12S rRNA), revealed its relation with most of the Digenean helminthes (Fasciolidae, and Echinostomatidae). It is also closely related to other *Tylodelphys* spp. That has been recovered from different localities, all over the world. The obtained trees presented bootstrap consensus that the topologies of the resulting trees were similar. That similarity was close to 100% with the other samples of *Tylodelphys* sp., from different countries in the world. Bootstrap represents (99%) similarity with other trematodes (**Blasco-Costa et al., 2017; Chaudhary et al., 2017**). However, other findings by use of cox1 gene; ITS1–5.8S–ITS2 rDNA (Sereno-Uribe et al 2019, Sokolov et al 2022) revealed mostly the same phylogenetic relationship with other diplostomatid trematodes

The results of sequences and phylogenetic analysis proved that modern

molecular technology is a confirmative tool to detect the relations among the Platyhelminthes.

In conclusion, *Tylodelphys* sp. is a metacercaria digenetic trematode found in fish as the second intermediate host. The infective stages in birds and mammals come from the free metacercariae in the fish gas bladder of *L. niloticus* they consume. The present work concerned with the seasonal prevalence, the morphology (using light and electron microscopy) and the genomic sequencing of *Tylodelphys* sp. using the 12S rRNA gene. This provided a GenBank accession number and produced a new Egyptian genotype. Phylogenetic analysis provided a sister relationship between *Tylodelphys* sp. and other digenetic trematodes, and also demonstrated the remarkably close relation with other *Tylodelphys* spp., all over the world.

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الملخص العربي

التوصيف الظاهري والجينومي لطفيل **Tylodelphys** ميتاسركاريا الموجودة في أسماك البياض النيلي

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تعتبر **Tylodelphys** ميتاسركاريا واحدة من عائلة الديبلوستوميدي وتم عزلها من عدوى طبيعية في حويصلة الغاز لأسماك قشر البياض النيلي ببحيرة ناصر جنوب مصر وتم تحديد الوصف المورفولوجي (الشكل) وباستخدام المجهر الإلكتروني وكذلك الفحص الجيني لها. الصفات الأساسية ظهرت بوضوح باستخدام الميكروسكوب الضوئي كانت نسبة الإصابة ٨٦% وكانت أعلى نسبة إصابة في الصيف (26%) وأقلها في الشتاء (16%).

الفحص باستخدام الميكروسكوب الضوئي و الإلكتروني كان الطفيل لساني الشكل؛ الجزء الأمامي منه يكون معظم الحسم بينما الجزء الخلفي يمثل جزء صغير مخروطي الشكل وله نهاية مدببة. الغشاء الخارجي للجسم ناعما لا يحتوي على أي أشواك؛ الممص الفمي صغير بينما الممص البطني كبير. الجسم يحتوي على نوعين من الزوائد ملساء في المقدمة وأخرى خشنة (غليظة) في الخلفية. التحليل الجيني لها كان مقارب ومشابه لتلك الأنواع التي لها أصل من الأسماك ومدرجة في بنك الجينات وتم وصف النوع المصري (MH367017). وبفحص الشكل النهائي وجد أنها تشكل 99% تشابه جيني في الجزء (SrRNA12) مع الديدان الأخرى.

النتائج التي تم الحصول عليها تدعم إضافة **Tylodelphys** ميتاسركاريا النوع المصري مما يساهم في التصنيف والتشخيص لها.