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Parasites Causing Respiratory Manifestations in *Mullus surmulatus* Fish from Safaga at Red Sea Governorate

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ABSTRACT
Prosorhynchus spp., *Pseudohaliotrema* spp., and Paraniza larva formed a mixed parasitic infestation that was isolated from 120 red mullet fish (*Mullus surmuletus*) from the city of Safaga at the Red Sea governorate. *Prosorhynchus* spp. digenetic trematodes were found in a clear transparent sac in the buccal cavity; its prevalence was 65%. *Pseudohaliotrema* spp. Monogenetic trematodes were distributed in the gill fish with a heavy infestation in prevalence (84%). Paraniza larvae on the gills, fins, and tongue were recorded (32%). This mixed parasitic infestation caused suffering from respiratory manifestations with symptoms obvious in infested fish, such as congestion and erosion of gills in addition to excessive mucus secretion. For validation and identification of pathogenic *Prosorhynchus* spp., cox1gene was used by conventional PCR. The sequence was submitted to the Gene Bank (Accession No. OP103715). In the phylogenetic trees, our cox1 sequence had a close relationship and was clustered into one branch with the members of Family Bucephalidae.

INTRODUCTION

Commonly referred to as a "goatfish," is a fish with strikingly brilliant red skin. Red mullet can be found in tropical and warm waters including the Mediterranean; most digeneans are hermaphrodite and heteroxenous, as they need to complete their life cycle with more than one host. In this study, parasitic trematode *Prosorhynchus* spp. was isolated from a transparent cyst inside the gills of *Mullus surmuletus*; the weakening of the adductor muscle and castration are serious problems in mussels caused by infestation of *P*. spp. **(Cousteau *et al*., 1990; Cousteau *et al.*, 1993)**. Monogeneans are common parasites of both fresh and marine water fishes **(Eissa, 2002)**. Almost all monogeneans are obligatory ectoparasites on specific sites on the external body surface as gills, skin and fins. Some monogenean are characterized by temporary attachment to epithelial surfaces of fishes by secreation of adhesive materials **(Mahmoud *et al.*, 2020)**. Since their direct life cycle allows them to move from one host to another without stopping, these parasites cause indirect harm, increasing the risk of secondary infections in fish by destroying and rupturing the epithelium and mucous layer **(Ziarti *et al*., 2022)**. Most crustacean life cycles are spent by males, with the fertilized female acting as a parasite to

lay her eggs and maintain the rest of the life cycle and developmental stages in the water column **(Woo, 2006)**.

MATERIALS AND METHODS

1. Fish samples

A number of 120 barboni marine fish *Mullus surmulatus* with different lengths (18-28cm) and various body weights (70-305g.) were randomly collected life or moribunds from different areas of the Red Sea at Safaga City during the summer of 2022 where they were immediately examined.

2. Clinical picture and postmortem examination

Dead or recently deceased fish were subjected to examonation. Gross examinations were conducted on the fish samples to look for ectoparasites and other clinical anomalies, according to **Amlacker (1970)** and **Noga (2010)**. Additionally, for the the postmortem investigation, the techniques mentioned by **Lucky (1977)** were used. A stereoscopic dissecting microscope was used to thoroughly inspect the interior organs according to the procedures of **Conroy and Hermann (1981)**.

3. Parasitological examination

It was carried out using a magnified for the identification of external parasites on the samples' skin, fins and gills. Trematodes were stained and fixed according to **Kerrousha R. *et al*. (2022)**, and their gills were removed and observed under a stereomicroscope.

3. a: Digenetic trematodes

Before being stored in the refrigerator, the gathered worms were washed with physiological saline to eliminate mucus and debris and were left to completely rest. Once the dead samples relaxed, they were gently crushed between two glass slides, or a cover and glass slide according to the thickness of each specimen. The worms were preserved for 12–24 hours in 10% neutral buffered formalin. The fixed specimens were washed in distilled water many times for removing the excess fixative, then stained with acidified alum carmine, washed them in running water and destined in 1% acid alcohol before dehydrating them in ascending grades of ethyl alcohol. Specimens were then mounted in Canada balsam after being cleansed with clove oil and xylene.

3. b: Monogenetic trematodes

The discovered worms were fixed in 3% formalin; then a drop of glycerin alcohol was added at a ratio of 1:4, followed by a dehydration in increasing grades of ethyl alcohol (30, 50, 70, 80, 90, 100%), cleared with clove oil, then removed with xylene to remove the oil (each step takes 15 to 30 minutes), mounted in Canada balsam, and allowed to dry in a horizontal position in a hot air oven **(Ismail *et al* ., 2017)**.

Identification of the isolated parasites: - Monogenetic trematodes were identified according to **Yamaguti (1934)**, **Yamaguti (1958)** and **Tadros *et al.* (2020)**.

3. c: Paraniza larvae

Paraniza larvae were collected from the gills, fins and mouth of fish, then washed several times with physiological saline to be free from mucus. The larvae were preserved in 70% ethyl alcohol and then dissected and mounted in lactophenol as temporary slide preparations.

4. Molecular identification

4. a: DNA extraction

Following morphological identification, the samples were kept in 100% ethanol until DNA was extracted from them, using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH), with modifications to the manufacturer's instructions. Following a quick 1% SDS wash of the worms, 25 mg of the sample and 20µl of Qiagen protease were combined with 180µl of ATL buffer. To ensure sample homogeneity, tubes were inserted into the adapter sets, which are secured to the Qiagen tissue Lyser clamps. High-speed (30 Hz) shaking disruption was carried out for two minutes. Following that, samples were incubated at 56°C until lysis. A volume of 200µl of the lysate was added to 10µl of proteinase K and 200µl of lysis buffer, and they were incubated at 72°C for 10min. The lysate was then mixed with 200µl of 100% ethanol after incubation. Following the manufacturer's instructions, the sample was then cleaned and centrifuged. 100µl of the elution buffer included in the kit was used to elute the nucleic acid.

Utilizing a NanoDrop spectrophotometer, the amount of isolated DNA was determined and kept at a freezing temperature until usage.

PCR Amplification: To identify the isolated fluke, the mitochondrial cox 1 sequence was amplified using Dice1F (5´-TTAACCCTCACTAAATTWC NTTRGATCATAA-3´) and Dice11R (5´-AATACGACTCACTATAGCW GWACH AAATTTHCGATC-3´) [1]. The 25 L PCR reactions included 20 ng of template DNA, 1 L of each primer (50 pmol/L), 12.5 L of TaKARa Bio's Emerald Amp MAX Master Mix, and ddH2O up to 25 L.

4. b: Sequencing and sequence analysis

Homologous sequences were identified and downloaded by searching the Basic Local Alignment Search Tool (BLAST) on NCBI combined with Bioedit7.1 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The selected sequences were aligned b. According to **Van Steenkiste *et al*. (2015)**, an arrival PCR technique was applied. The 1.5% agarose gel/ethidium bromide electrophoresis used to analyze the PCR results was followed by UV transilluminator visualization. Big Dye Terminator (BDT) v3.1 (Applied Biosystems) was used to sequence the PCR product after purification using the QIAquick PCR Purification Kit (Qiagen, Germany). By Clustal W method and homology, percentage was calculated using the Megalign module of DNASTAR Lasergene.v7.1 software package.

4. c: Phylogenetic analysis

The phylogenetic trees were reconstructed from the aligned sequences by the neighbor-joining (NJ) method using MEGA5.0 software **(Nishimaki & Sato, 2021)**, based on the Akaike Information Criteria (AIC). A 1000-replicate bootstrap was used to measure nodal support and the dependability of the phylogenetic tree. Producing rooted trees required the use of the worm Onchocerca volvulus cox1 sequence (GenBank AM749284).

Ethics

This study was conducted following legal ethical guidelines of the Medical Ethical Committee of the Faculty of Veterinary Medicine, Suez Canal University, Egypt. (ethical approval no. 2023034).

RESULTS

Clinically: gills of infested red mullet *Mullus surmulatus* appear congested and eroded with excessive mucus on the external body surface as a defense mechanism due to external infestation.

Parasitological: This study isolated parasitic trematode ***Prosorhynchus*** **spp. metacercariae** removed from a cyst in gills. There was a clear transparent cyst under the surface of the chin (Fig. 1a) or under the right or left gill cover (Fig. 1b) of infected fish containing a huge number of metacercariae of *Prosorhyncus* spp. in 65 out of 100 (65%)

Classification:
Class: Trematoda
Subclass: Digenea
Order: Strigeata
Family: Bucephlidae
Subfamily: Bucephaline (Poche, 1907)
Genus: Prosorhynchinae (Nicol, 1914)
Species: Prosorhynchus spp.
Location: Gills

The body is plump to elongate (Fig. 2C) in an average of 1041 x 566mm. Rhynchus is funnel-shaped with an average of 0.35 ± 0.08mm and up to 0.56mm in the most developed cercariae, without tentacular appendages (Fig. 2A). The mouth is opening usually in the middle third of the body. The intestine is short. Gonads are near midbody anterior to cirrus sac. Testes are tandem in the middle third of the body with an average of 104 x89 mm. Cirrus pouch contains tubular vesicular semiannually and well-developed prostatic complex. The genital pore is in the posterior part (Fig. 2B). The ovary is in front of the anterior testis occasionally intertesticular, with an average of 107 x 90 mm.

Pseudohaliotrema **spp.** Attaching individual hollow branch gills of *M. surmulatus* in 84 out of 100 (84%), with large numbers that may reach 16/ field. (Fig. 3)

Classification:
Class: Trematoda
Subclass: Monogenea
Order: Monopithocotylea
Family: Dactylogyridae
Subfamily: Ancyrocephalinae
Genus: Pseudohaliotrema

The body is small and elongated. The haptor is well-marked off, with two pairs of anchors and 12 – 14 marginal hooklets; each pair of anchors is supported by a transverse bar, (Fig. 4a, d). Head with one pair or two of the lateral lobes containing head organs. Neck more or less distinctly marked out. Mouth subterminal; pharynx well developed; esophagus moderately long; intestinal crura simple, united posteriorly. Testis post equatorial, vas deferens not looped around the intestinal limb. The seminal vesicle turned back on itself behind intestinal bifurcation. Copulatory organs are not simple. Ovary anterior or anterolateral to testis (Fig. 4b). Vagina opening ventrally or laterally. Vitelline follicles are small, extending from behind the pharynx to the posterior end of the body (Fig. 4c).

Praniza larva: showed on the gills, fins and tongue (Fig. 5). The fusion of the gill lamellae, mechanical harm to the gill epithelium, and necrosis are all negative effects of the praniza larva on the fish host. Due to complete loss of gill function in cases of severe infestation, it may result in hypoxia **(Hassan, 2018)**. When you consider that a single parasite can consume up to 1.89 mg of blood 1/20 of a fish's weight, you can see why this is a significant quantity. It was found in 32 out of 100 cases (about 32%). As a result, severe infestations can result in severe anemia, which can be fatal, especially in small fish **(Muller-Karanassos *et al.*, 2021)**. Hemogregarine bigeminal, a protozoan blood parasite of fish, can be transmitted by praniza larva **(Hassan, 2018)**

Classification:

Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Crustacea Brünnich, 1772
Class: Malacostraca Latreille, 1802
Order: Isopoda Latreille, 1781
Family: Gnathiidae Leach, 1814
Genus: Gnathia Leach, 1814

It has a long body consisting of head, thorax and abdomen. The head (Cephalon) has two pairs of eyes on its lateral sides and one pair of antennae; thorax (Pereon) is the second part of the body which is swollen, round and longer than Cephalon; it has five pairs of thoracic legs. The abdomen (Pleon), the last part of a larva, is the narrowest part of the larva and has a pair of legs.

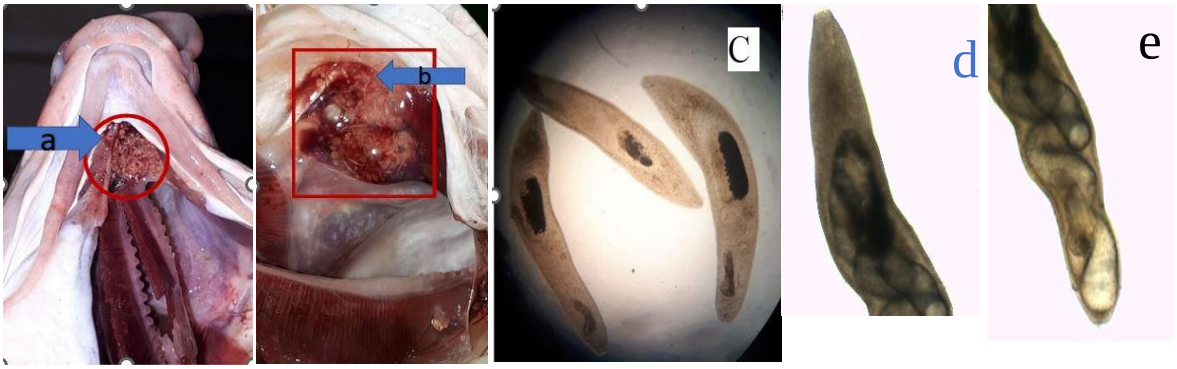

Fig. 1. A photo showing ectoparasitic infestation in red mullet, **(a)** *Prosorhynchus* spp. metacercariae in a clear transparent cyst under the surface of the chin of *M. surmulatus* or **(b)** Under the medial surface of the operculum in a branchial chamber, **(c)** Light photomicrograph of *M. surmulatus* under the field of the microscope. **(d), (e)** Ant. and post. ends of *M. surmulatus* under the stereo microscope.

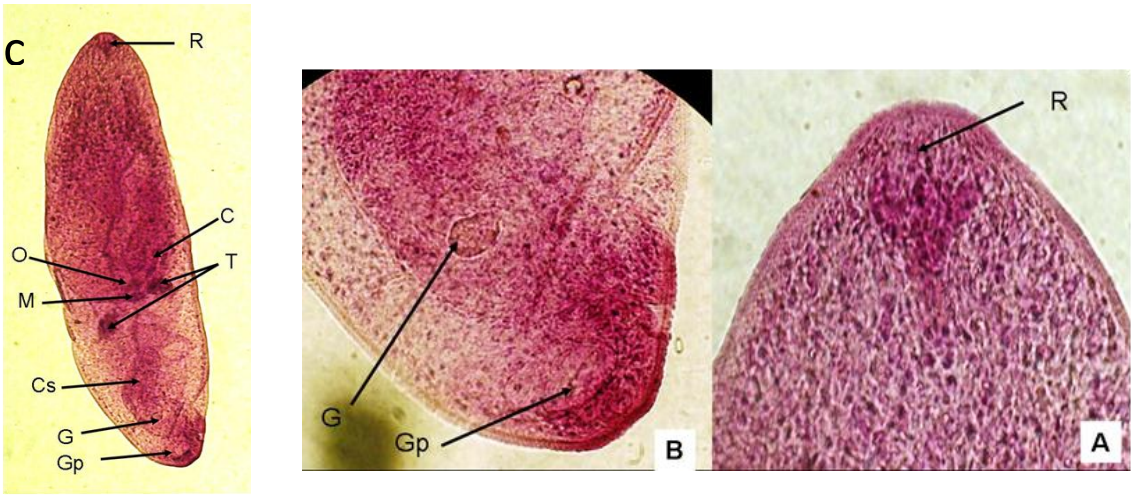

Fig. 2. *Prosorhynchus* spp.: R= Rhynchus, C= Cecum, O= Ovary, T= Testes, M: Mouth; Cs: Cirrus sac; G= Genital lobe, Gp= Genital pore. **(A)** Ant. part, **(B)** Post. part, R= Rhynchus; G= Genital lobe, Gp= Genital pore.

Fig. 3. Original photo of *pseudohalotrima* spp. in the gills of red mullet fish

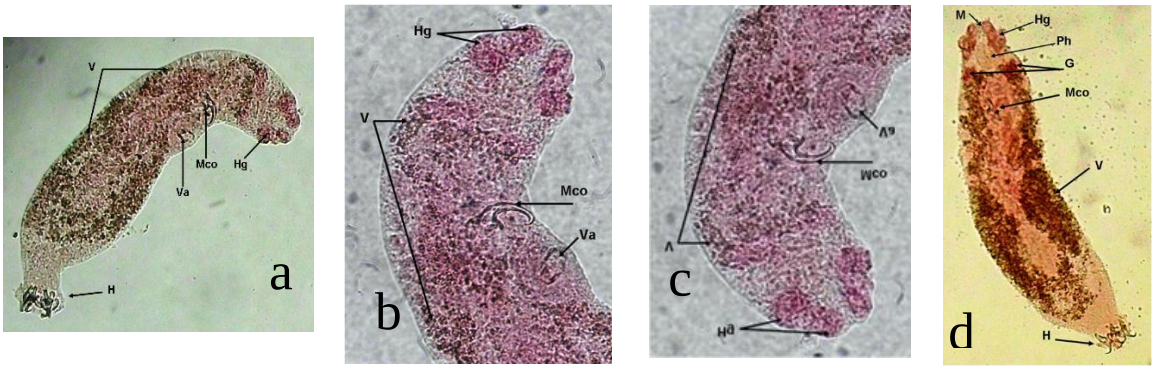

Fig. 4. (a, d) *Pseudohaliotrema* sp.: M= mouth, Hg= Head gland, Ph= Pharenx, Mco= Male copulatory organ, V= Vitelline follicle, Va= Vagina, H=Haptor. G= gland. **(b)** Ant. Part, **(c)** Post. part, S= Spine; An= Pair of Anchor, Tb= Transverse bar.

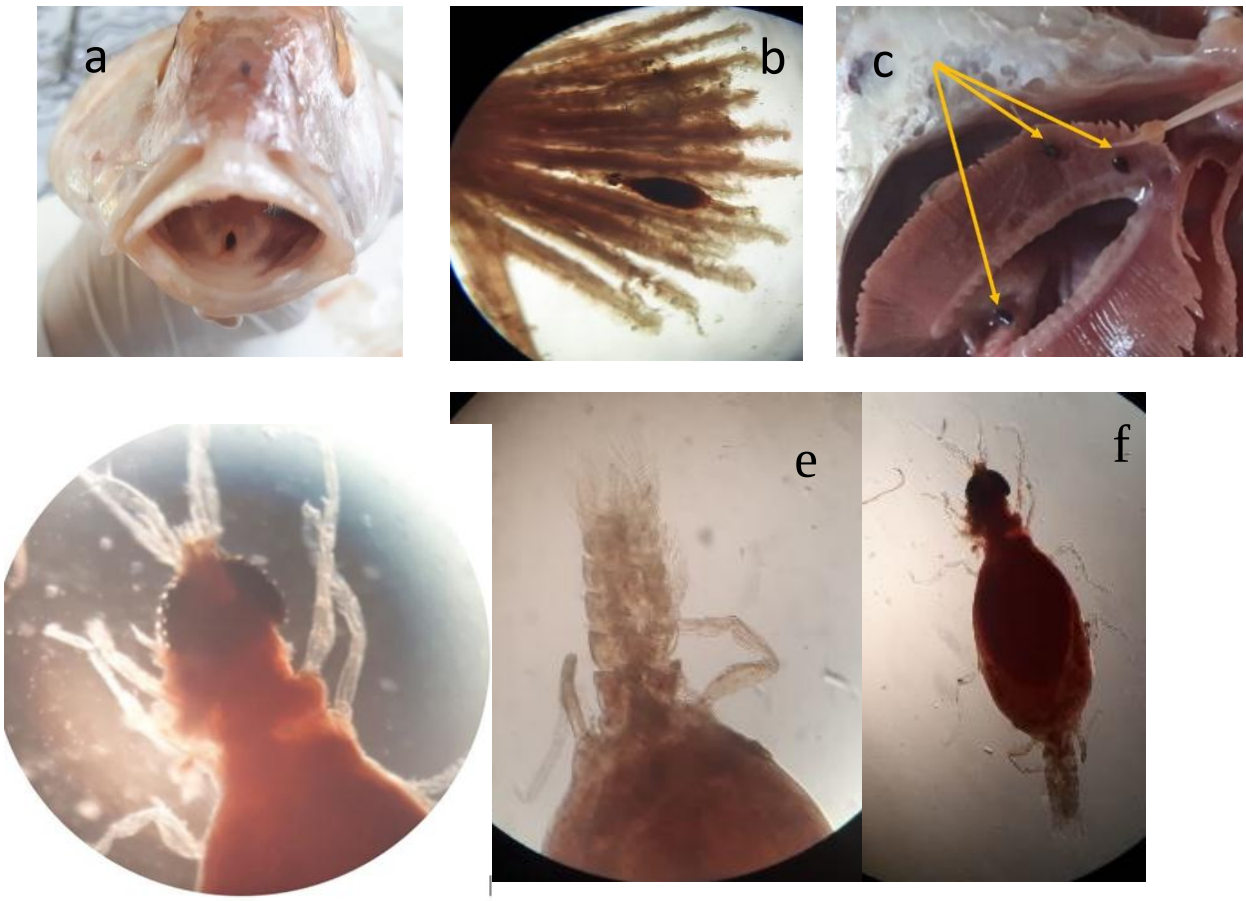

Fig. 5. Paraniza larva in mouth (**a**), fins (**b**), gills (**c**) of *M. surmulatus*.
(**d**), (**e**), Ant. and post. of paraniza larva.

The DNA sequences PCR assay used for identification of *Prosorhynchus* spp. digenitic trematodes samples. It was based on primers for the cox1 gene. It revealed that the amplification products of expected molecular size at cox1 (cytochrome oxidase subunit 1), which is specific for genus, *Prosorhynchus* spp. were queried in GenBank and identified (Accession No. OP103715).

Bioinformatic analysis

Homology analysis showed that the cox1 gene from our sample had the highest similarity (81.3%) to Rhipidocotyle spp. (KM538111.1) specimen voucher from Richelieu River, Quebec, Canada, followed by *Aenigmatrema grandiovum* (MT815812.1) isolated from obtuse *barracuda marine* fish (Sphyraena obtusata) in Australia with 80.4% homology (Fig. 6).

Phylogenetic analysis

In the phylogenetic tree (fig. G), our cox1 sequence had a close relationship and clustered into one branch with members of Family Bucephalidae, including Rhipidocotyle spp. (KM538111.1), *Aenigmatrema grandiovum* (MT815812.1), and *Bucephalus minimus* (KF880466.1).

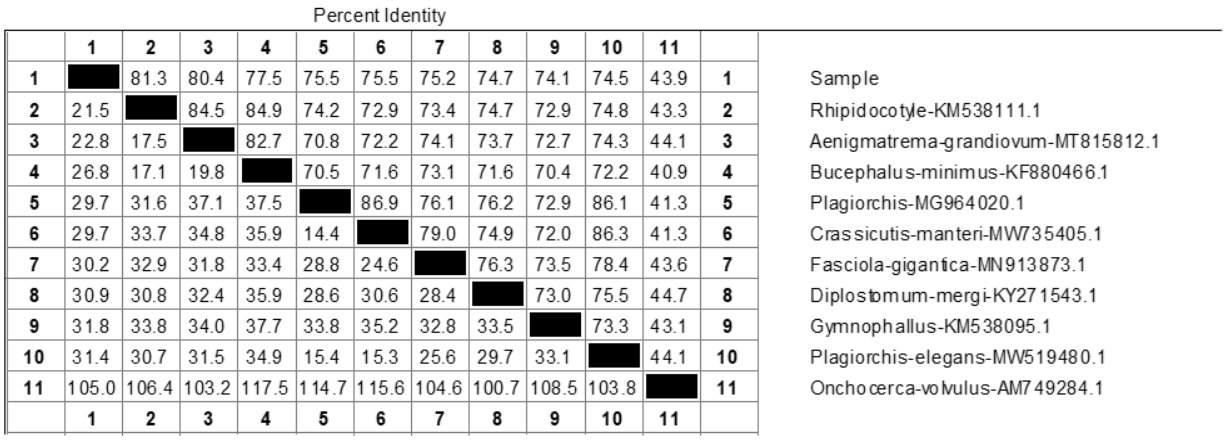

Fig. 6. Sequence distance analysis of Prosorhynchus spp. COX1.

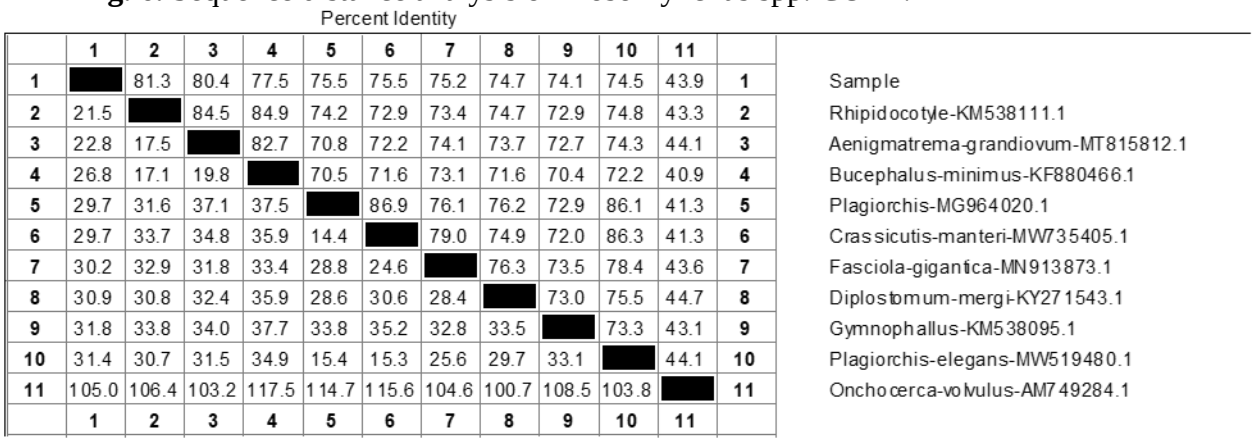

Fig. 7: Phylogenetic tree based on cytochrome oxidase subunit 1 (COX1) sequences from 11 parasite species including sequence obtained in this study (Sample: *Prosorhynchus* spp).

DISCUSSION

Digeneans, monogeneans, crustaceans and nematodes are considered as the major groups of fish parasites causing harmful effects to their hosts (**Woo, 2006; Luque & Poulin, 2008)**.

***Prosorhyncus* spp**. This genus is characterized by the presence of rhyncus, which acts as the muscular attachment organ at its anterior end instead of the oral pharynx. There are metacercariae under the chin in a translucent, clear sac or at a pseudobranch on the medial surface of the operculum in the branchial chamber. thus due to the migration of digenetic trematodes which is supported by **Eissa *et al.* (2020a)** were found *Tangenarchopsis chinensis* of alongside the tips of the gill filaments and on the operculum's internal surface *Diplectanum labrax* and in the branchial cavity of *Diplectanum punctatus*, and **Klimpel *et al.* (2019**) who recorded that digenean parasites are primarily found in fish guts, but they can also be found in fishes swim bladders, body cavities, urinary bladders, gall bladders, skin, ovaries, and circulatory systems. Additionally, the present finding agrees with **Hassanine (2002)** who mentioned that

individuals of *Prosorhynchoides arcuatus* were only discovered in the pyloric caeca or clustered there, however, they were discovered in the anterior intestine of recently captured fish. Due to feeding quietness in the field, the movement of food in the intestine was linked to the short-term migration of the worms (reverse migration) **(MacKenzie & Gibson, 1970)**. Based on an experimental investigation of a similar situation discovered that when malnourished flounders were force-fed, the trematodes migrated into the anterior intestine instead of remaining primarily in the rectum as was the case in the starved flounder *Plaiichthys jksus*. Attaching individual hollow branch gills of *M. surmuletus* in 84 out of 100 (84%) is higher than the value recorded by **Laffargue *et al*, (2004)**, with 65% in *Solea solea*. Moreover, **Akmirza (2013)** recorded that *Prosorhynchus crucibulum* in *Conger conger* was 14.29% and 33.33% in *Muraena helena* fish. This difference may be attributed to the locality from which fish samples were obtained and the type of fish. The description of *Prosorhynchus epinepheli* coincides with that reported in the study of **Yamaguti (1958)** for *Epinephelus akaara* marine fish collected from Iinland Sea. In addition, it was reported in the Arabian Gulf, by **mohameh *et al.* (1988)** from *E. chlorostigma*. Moreover, it was isolated from *E. areolatus* by **Nahhas *et al.* (2006)**. Moreover, the isolated *Prosorhynchus* spp. in this work is an extremely similar species to *P. longisaccatus* described by **Bray and Justine (2013)** but is probably a wider and shorter rhynchus.

Monogenean. Gill lamellae, which represent the main location of gas exchange, are preferred for attachment. Water and gases are transferred between the blood flowing through the lamellae and the channels made by the interdigitation lamellar tissues through the thin epithelial layer of the lamellae. They were distributed in the gill fish with a heavy infestation, with a prevalence of 84%, presented in large numbers that may reach 16/ field. This result is closer to that obtained by **Hussein (2017)** who reported *Pseudohaliotrema sphincteroporus* in 23 out of 28 samples (82%) collected from *Sengas luridus* (Siganidae) and lower than that obtained by **Putri *et al.* (2022)**, the prevalence of *Pseudohaliotrema* sp. reached 100% from cultured *Siganus guttatus*. The high incidence is caused by stress from the culture tank's high stocking density, lower water exchange systems and high stocking densities in fish cultured allow quick growing of the presence parasites to grow quickly. The description of the genus *Pseudohaliotrema* coincides with that described by **Yamaguti (1963)** from the gill of marine fish, and **Lim (2002)** in Singapore, where three species of *Pseudohaliotrema* were isolated from two *Siganus* species,

Praniza larvae were detected in 32 out of 100 (32%), a quantity which is higher than that detected by **Hassan (2018)**, who recorded a percentage value of 4.4% in *Pomacanthus maculosus* and 3.4% in *Acanthopagrus bifaciates* fish. While, **El-Lamie and Abdel-Mawla (2015)** registered a different value (10%) among *Siganus revulatus.* On the other hand, **Diniz *et al.* (2008)** recorded a value of 9.1% in *Conodon nobilis*., which is lower than that recorded by **Diniz *et al.* (2008)** (42.3%) in *Anableps anableps*. The aforementioned values are also less than those recorded in the study of **Bayoumy *et al.* (2013)** (58.33%) in *Epinephelus tauvina* fish and in the work of **Bakhraibah (2018)** which was 40%. Biotic factors such as pathogen, size, age and species are responsible for the differences in parasitic infestation as well as abiotic factors like salinity, pH, and other temperature environmental factors. The red mullet's condition appeared unchanged,

but the larvae's clever placement, particularly those in the gills, struck. This suggests that this type of infection could impair some biological functions such as adaptation, disease resistance and growth rates.

Homology analysis showed that the cox1 gene from our sample had the highest similarity compared to **Van steenkiste *et al.* (2015)** who isolated and identified *Rhipidocotyle* sp. (KM538111.1) specimen voucher from Richelieu River, Quebec, Canada, through mitochondrion *Rhipidocotyle* spp., followed by **Corner *et al.* (2020)** whoisolated and identified *Aenigmatrema grandiovum* (MT815812.1), isolated from *Obtuse barracuda* marine fish (*Sphyraena obtusata*) in Australia, with 80.4% homology. Whereas, **Feis *et al.* (2013)** isolated and identified (*Sphyraena obtusata*) from mitochondrion *Bucephalus minimus*, isolated from cockles *Cerastoderma edule* in Australia, with 80.4% homology.

CONCLUSION

The distribution of *Prosorhynchus* spp., *Pseudohaliotrema* spp. and Paraniza larva was a mixed parasitic infestation isolated from red mullet fish. The highest infestation was *Pseudohaliotrema* spp. monogenetic trematodes (84%) with large numbers that may reach 16/ field. Followed by Prosorhynchus digenetic trematodes (65%), found in a clear transparent cyst under the surface of the chin or under the right or left gill cover of infected fish containing a huge number of cercariae, and the lowest was Praniza larva (32%). Gill of red mullet *Mullus surmulatus* appears congested and eroded with excessive mucus on the external body surface. *Prosorhynchus* spp. were queried in GenBank and identified based (Accession No. OP103715). Our cox1 sequence had a close relationship and was clustered into one branch with the members of Family Bucephalidae, Therefore, the molecular techniques were developed providing useful tools for this study on the geographical origins of *Prosorhynchus* spp.

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