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Comparative morpho-molecular identification of *Clinostomum phalacrocoracis* and *Clinostomum complanatum* metacercaria coinfecting Nile tilapia in Egypt

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ABSTRACT

Clinostomiasis is one of parasitic diseases infecting freshwater fish caused by digenetic trematodes that belong to family Clinostomatidae. During the course of the present study it was found that 392 out of 520 examined Nile tilapia Oreochromis niloticus were infected with EMC belonging to family: Clinostomatidae with overall prevalence in (mean ± SD) was 74.12 ± 5.19. The investigated *O. niloticus* were found to be simultaneously infected with two morphologically different encysted metacercariae (EMC); small cysts (Type 1) ranged from 1.0-3.0 mm in diameter and large cysts (Type 2) were measured 4.0-8.0 mm. Furthermore, an identification of the main morphological features (size of suckers, body length) in both excysted metacercariae was assessed to assist the morphological differentiation of these EMC; namely, *Clinostomum complanatum* and *C. phalacrocoracis*. The prevalence rates of *C. complanatum* and *C. phalacrocoracis* infections were 35.94± 2.24 and 60.93± 6.70, respectively. In the present study, the molecular identification was carried out by sequencing their COI mtDNA; BLAST analysis of C. complanatum (MT140101.1) showed 100% and 99.74% nucleotide similarity (MK501949.1; MF741769.1) in China, respectively. On the other hand, *C. phalacrocoracis* (MT140102.1) revealed 100% nucleotide identity (KY906238.1) in South Africa and 99.66% (KJ786967.1) in Israel.

INTRODUCTION

Clinostomiasis is one of parasitic diseases infecting freshwater fish caused by digenetic trematodes belonging to family *Clinostomatidae.* In Egypt, there are several species of the larval stages of *Clinostomatidae* (*Clinostomum complnatum* and *C. phalacrocoracis*) found as encysted progenetic metacercariae that infect the branchial cavity of Nile tilapia (*Oreochromis niloticus*) that act as the second intermediate host. *Clinostomum* sp. are well known as yellow grubs, marked with a bulge that contains whitish yellow and elliptical or diamond-shaped metacercariae in the branchial cavity of

fish. These metacercariae are zoonotic in nature that may cause Laryngo-pharyngitis disease in humans who eat improperly cooked fish, and may lead to death due to asphyxia **(Wang *et al*., 2017).** The digenetic trematodes *Clinostomidae*, including both vertebrates and invertebrates hosts, possess complicated life cycle that have recently stimulated reconsideration depending on both morphological and molecular analysis **(Caffara *et al*., 2020).**

Over the past few years, some researchers have revised the taxonomy of the *Clinostomum* species using morphological approaches **(Ukoli, 1966; Matthews & Cribb, 1998)** depending mainly on adult morphology. However, there are some difficulties in species morphological diagnosis due to the high similarity within them, while some relatively minor differences were diagnosed in their small/soft bodies like in some other digenean trematodes **(Graczyk, 1991).** In recent years, advanced molecular techniques have been successfully employed to identify trematode species and investigate the life cycles using genetic linkages among developmental periods. Therefore, significant taxonomic changes of *Clinostomum* species have been achieved using combined morphologic and molecular approaches to resolve the problem associated with the morphological intraspecific diversity and similarities. Propably, many *Clinostomum* species have not been identified or described precisely because of the lack of data in regard to the existing species in this genus **(Steenkiste *et al*., 2015; Simsek *et al*., 2018).**

Notably, no studies were conducted on molecular characterization of *C. complanatum* or *C. phalacrocoracis* from freshwater fishes in Egypt. In recent years, utilizing PCR and sequencing of partial mitochondrial cytochrome oxidase subunit I (mt-COI) gene regions, in addition morphological analyses have proved accurate giving precise identification of *Clinostomum* species that lead to better understanding their life cycles and development of treatment/control strategies against those parasites. This study was planned to determine the prevalence of *Clinostomum* metacercariae in Nile tilapia (*O. niloticus*) and molecular characterization of *C. complanatum* and *C. phalacrocoracis* based on the phylogenetic analysis and sequencing of mt-COI gene regions.

MATERIALS AND METHODS

2.1. Collection of samples

Five hundred and twenty *O. niloticus* were collected from different fish markets in Giza Governorate, Egypt at weekly intervals during different seasons of 2018-2019. The fish samples were collected, stored in ice bags and transported to the Faculty of Veterinary Medicine, Cairo University laboratories. Fresh fish specimens were examined for parasitological investigation. Fish examinations were undertaken to detect weight, total length, any macroscopically lesions and visible parasites **(Eissa *et al*., 2015, 2020).**

2.2. Parasitological examination

Fish samples were thoroughly examined with the naked eye and by hand-held magnifying glass to detect the large sized metacercarial cysts in skin, gills, fins and buccal cavities **(Abdelsalam *et al.*, 2016, 2020).** The dissected fish were carefully investigated internally for observing the changes in internal organs as liver, heart, kidneys and gonads **(Mahdy *et al.*, 2020).**

2.2.1. Isolation and identification of encysted metacercariae

The isolated EMC from different body parts were morphologically identified and measured using a light microscope, and all measurements expressed as mean ± Standard error (SE). For both morphological and molecular analysis, all parasites were kept in ethanol 70%.

2.2.2. Morphological analysis

Ten metacercariae (five for each metacercaria species) were included in the Morphological analysis. The measurements of the parasites were given in micrometers unless specified otherwise and taken afterwards **(Matthews & Cribb, 1998).** Parasites were identified based on the international keys of the family *Clinostomidae* **(Caffara *et al.*, 2014, 2017).**

2.3. Molecular characterization

2.3.1. PCR amplifications

For molecular analysis, 10 metacercariae (five from each metacercaria sp.) were thoroughly washed with sterile distilled water and preserved in sterile Eppendorf tubes. Extraction of the total DNA was performed using the manufacturer's protocol of GeneJET Genomic DNA Purification Kit (Thermo Scientific #K0721). Amplification of a fragment of *cytochrome oxidase* I (COI mtDNA) was performed as followed; the total volume of all PCRs was 25 µL and involved 1× PCR buffer (20 mM Tris HCl pH 8.4, 50 mM Cl), 2.5 mM $MgCl_2$, 1.25 pmol of each primer (MplatCOX1dF- 5′ - TGTAAAACGACGGCCAGTTTWCITTRGATCATAAG-3′; MplatCOX1dR-5′ - AGGAAACAGCTATGACTGAAAYAAYAGGATCCCAC-3′) according to **Moszczynska *et al.* (2009)** and **Caffara *et al.* (2014),** 50 µM of each dNTP, 0.6 U of Platinum *Taq* Polymerase (Invitrogen) and approximately 50 ng (COI PCR) of DNA template. The conditions of PCR were started with initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. 1% agarose gel (Sigma) stained with SYBR Safe DNA Gel Stain in 0.5× TBE (Molecular Probes-Invitrogen) was used to electrophoresis the PCR products.

2.3.2. DNA Sequencing

The amplicons of PCR were purified using QIAGEN Extraction Kit (Hilden, Germany) followed the manufacturer's protocol. The purified amplicons were sent to Animal Health Research institute in Egypt for sequencing, using Big Dye Terminator v3.1 cycle sequencing kit chemistry. The DNA sequencing reactions were electrophoresed on ABI's Prism 3700 DNA Analyzers. The obtained sequences were checked and edited using Bio Edit **(Hall, 1999).** The *cytochrome oxidase* I (COI mtDNA) assembled sequences of *C. complanatum* and *C. phalacrocoracis* that were aligned with other COI region of trematodes available in the GenBank by nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and then, deposited into the database of the GenBank with accession numbers MT140101 and MT140102, respectively. The phylogenetic analysis of assembled sequences was created by neighbor-joining model using MEGA X **(Kumar *et al.*, 2018).** The following parameters were employed: substitutions involved transversions and transitions, rate of variation among sites was uniform, and the pattern among lineages was homogeneous with 1000 bootstrap replicates.

RESULTS

3.1.Epidemiological Study

During the course of the present study, it was noticed that 392 from520 investigated *O. niloticus* were found to be infected with EMC belonging to family *Clinostomatidae* with overall mean ± SE 74.12 ± 5.19 **(Table 1).** Furthermore, two morphologically different types of EMC (type1and type2) were recognized in this survey and were found inhabiting the branchial cavity of infected fish. The overall prevalence of EMC in mean intensity ± SE of EMC; type1 and type2 were 35.94^{B} ± 2.24 and 60.93^{A} ± 6.70, respectively. The aggregation index of all recorded EMC showed that type 2 was the most prevalent cyst reported in this study followed by type 1. Regarding the prevalence of infection in different seasons the findings revealed it was significantly different (P = <.0001). The most infected season was summer (85.45 ± 7.27 and 40.91 ± 4.55), while the lowest infection was recorded in winter (40.32± 4.02 and 28.90 ± 4.50) in *C. complanatum* and *C. phalacrocoracis* **(Table 2).** With regard to the effect of weight and length of the examined fishes, statistically, significant differences were found in mean intensity of the examined fishes according to host weight and length (P = <.0001). In addition, the prevalence was higher in the lower host weight and length. The prevalence of EMC was higher in weight (50-100 gm) and length (15-20cm), while the lowest one was in fish more than 150 gm and 20 cm in weight and length, respectively **(Table 3).**

Table 1. Seasonal prevalence of *Clinostomaum EMC* from examined fishes

Season	No. of Samples (Ex. fish)		Infected Fish		
			Number (PR%)	PR% per sample	
				mean ± *SE*	*p* value
Autumn	3	(162)	123 (75.93)	75.88^{bc} ± 3.93	
Spring	2	(110)	93 (84.55)	84.54 ± 8.19	
Summer	2	(110)	100 (90.91)	90.91 ± 9.09	*P*= 0.0023
Winter	3	(138)	76 (55.07)	54.24 ± 2.98	
All	10	**(520)**	392 (75.83)	74.12 ± 5.19	

Table 2. Seasonal prevalence of *EMC* of two *species* of *Clinostomatidae*

Season	*Encysted metacercaria spp.*		
	Mean ± *SE* PR% per sample (no. infected)		*p* value
	C.phalacrocoracis	*C.complanatum*	
Autumn	51.85 ± 6.82 (84)	35.74 ± 1.61 (58)	*P*<0.0001
Spring	80.90 ± 4.54 (89)	41.82 ± 1.82 (46)	
Summer	85.45 ± 7.27 (94)	40.91 ± 4.55 (45)	
Winter	40.32± 4.02 (54)	28.90 ± 4.50 (39)	
All	60.93^{A} ± 6.70 (321)	35.94^{BC} ± 2.24 (188)	*P*<0.0001

Table 3. Seasonal prevalence of *EMC* regarding to weight and length groups

Season	Mean PR% per sample ± *SE* (number of infected fish)							
	Weight groups (g)			*p* value	Length groups (cm)			*p* value
	50-100	100-150	>150		10-15	15-20	>20	
Autumn	93.48 ± 6.52	54.80 ± 11.49	41.07 ± 22.28		66.40 ± 23.38	82.93 ± 17.08	50.00 ± 24.53	
Spring	100.00 ± 0.00	89.59 ± 10.42	41.67 ± 8.34		93.75 ± 6.25	100.00 ± 0.00	36.11 ± 13.89	
Summer	96.55 ± 3.45	81.67 ± 1.67	0.00 ± 0.00		83.34 ± 16.67	87.12 ± 3.79	41.03 ± 25.65	
Winter	83.79 ± 10.29	64.41 ± 25.79	14.29 ± 14.29		40.00 ± 30.55	84.07 ± 10.33	11.11 ± 11.11	
All	92.38^{A} ± 3.94	70.01^{A} ± 8.71	26.41^{B} ± 9.76	*P* = <.0001	67.34^{A} ± 12.32	88.03^{AB} ± 4.77	35.24^{B} ± 10.60	*P* = <.0081

3.2. Identification of the obtained metacercaria

Morphological analysis

The observed EMC was classified according to its morphological characteristics such as size, shape, cyst thickness, presence and degree of pigmentation in the excretory bladder. Based on those features, EMCs have belonged to two different species of family: *Clinostomatidae* according to the described keys of **Caffara *et al*. (2014, 2020).**

***Clinostomatidae* encysted metacercariae**

The metacercarial cyst was found heavily distributed in the branchial cavity of fish. Either the cysts encysted or excysted metacercaria, they were reddish rather than yellowish. The measurements of the metacercariae main morphological characters were min-max (mean ± SD) mm. The cysts were found in two different sizes; large cysts with measures 4.0-8.0 mm, whereas small cysts ranged from 1.0-3.0 mm in diameter. The excysted small metacercaria were measured 3.798 – 6.34 (5.182 ± 0.44) mm long, and 0.988-2.05 (1.483 ± 0.207) mm wide**. (Fig.1a-c)**

- ***Clinostomum phalacrocoracis* excysted metacercaria Dubois, 1930**

The body of excysted metacercaria was stout, slightly wider in gonadic region; 9.20-16.00 (12.35 ± 1.374) mm long, 1.72-3.567 (2.606 ± 0.346) mm wide. Oral sucker, 0.38-0.53 (0.447 ± 0.031) mm long and 0.476-0.722 (0.614 ± 0.049) mm wide, smaller than oral collar, 0.73-1.20 (1.022 ± 0.087) mm wide; intestinal caeca extended laterally to ventral sucker, testes were arranged in side by side in the posterior third of body. Anterior testis is fan-shaped with measures 0.67-1.376 (1.073 ± 0.155) mm long × 0.741-1.296 (1.027 ± 0.112) mm wide. Anterior testis consists of several lobes with blunt end, these lobes ranged from four to eight, some of them were divided into sub-lobed; sometimes, the right lobe was located at left side of uterus. Posterior testis is fan-shaped measured 0.570-1.08 (0.807 ± 0.096) mm long × 0.646-1.420 (0.965 ± 0.155) mm wide. It is placed anteriorly in the posterior third of body, composed mainly of three lobes, two are lateral lobes and one is posterior lobe, each lobe is subdivided into sub-lobes. Ovary is 0.095–0.386 (0.201± 0.057) mm long × 0.115–0.382 (0.214 ± 0.052) mm wide, irregular, round, located in the dextral space between testicles. The uterus extends directly from the ventral sucker to the anterior testicle. **(Fig.1b-d, white arrow)**

- ***Clinostomum complanatum* excysted metacercaria Rudolphi, 1814**

The body of metacercariae is slightly narrow at level of ventral sucker region and wide at the level of the gonads;3,798 – 6,340 (5,182 ± 0.44) mm length and 0.988-2.050 (1.483 ± 0.207) mm width. Oral collar is well developed and visible, 0.622-0.867 (0.750 ± 0.052) wide. The oral sucker, 0.232-0.310 (0.274 ± 0.016) mm length and 0.265-0.457 (0.36 ± 0.04) mm wide, smaller than the ventral sucker, 0.664-0.842 (0.754 ± 0.038) mm length and 0.56-0.79 (0.668 ± 0.043) mm width. The intestinal ceca were run to the posterior part of the body and lateral to the ventral sucker until genital organs. Testes

resemble a triangular with a lobed structure. Anterior testis, 0.27-0.65 (0.466 ± 0.075) mm length and 0.195-0.484 (0.33 ± 0.054) width, is placed at the end of middle third of the body, while the posterior testis, 0.205-0.38 (0.301 ± 0.032) mm length and 0.323-0.554 (0.443 ± 0.043) mm width, is placed at anteriorly in posterior third of the body. The genital pore is in front of the anterior testis, located nearly at the midline of the body. The ovary, 0.11-0.16 (0.133± 0.010) mm length and 0.093-0.143 (0.116 ± 0.009) mm width with an irregular shape was smaller in size than the cirrus sac and it is placed between two testes. **(Fig.1b-d, black arrow)**

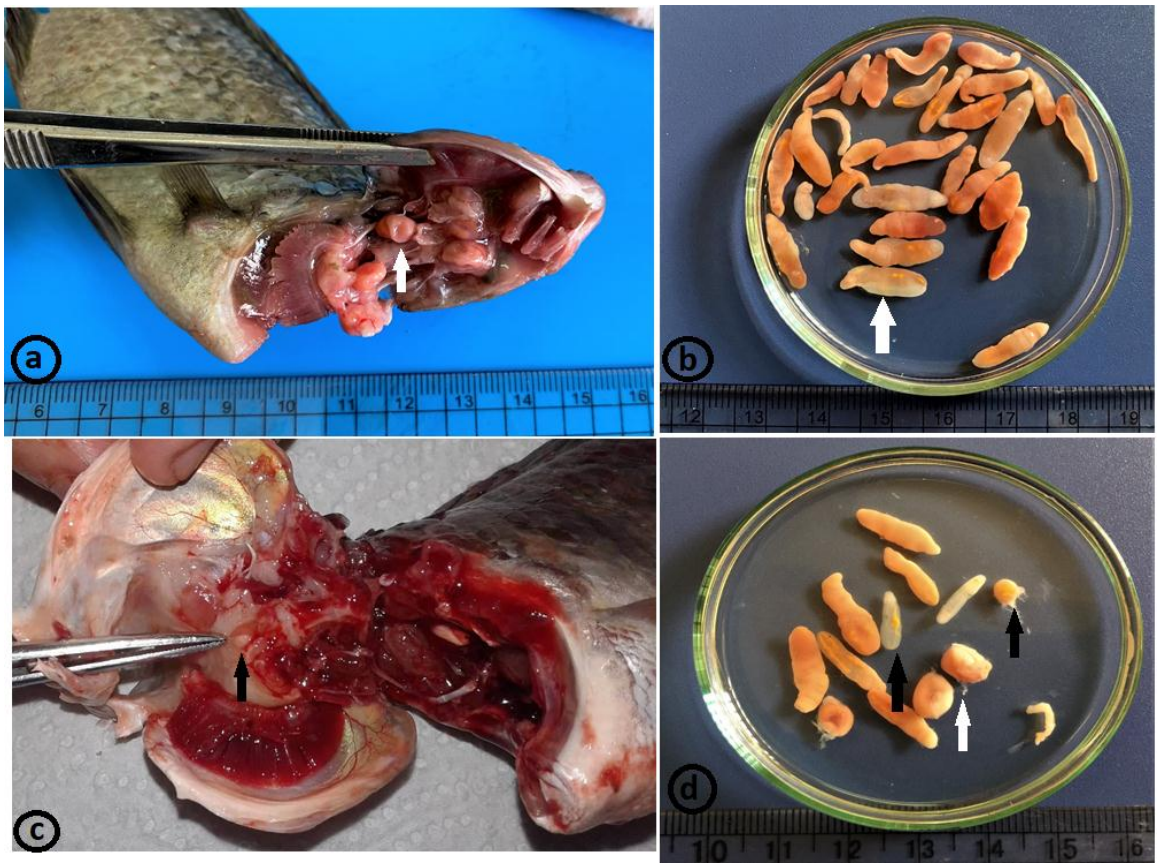

Fig. 1: Infected *Oreochromis niloticus* with *Clinostomum* species a) *O.niloticus* infected with large encysted metacercaria of *C. phalacrocoracis* in the buccal cavity (white arrows). b) large sized excysted metacercariae of *C. phalacrocoracis*. c) *O. niloticus* infected with small-encysted metacercariae of *C. complanatum*in the buccal cavity (black arrows). d) Two types of clinostomatid; encysted and excysted metacercariae for *C. phalacrocoracis* (white arrows) and *C. complanatum* (black arrows).

3.3. Molecular analysis

The COI mtDNA region of the two excysted metacercaria morphologically identified as *clinostomatidae* were successfully amplified using the primers mentioned above in this study. Two purified PCR products from the two different excysted

metacercaria were directly sequenced and yielded 593and 591bp, respectively. In GenBank, these two sequences were deposited under the following accession numbers MT140101.1 and MT140102.1,respectively. Comparing these DNA sequences fragments with other, the nucleotide sequences and divergence of trematodes in GenBank showed that those two excysted metacercaria were identified as *C. complanatum* and *C. phalacrocoracis*.

BLAST analysis of *C. complanatum* (MT140101.1) of this study showed 100% similarity with *C. complanatum* (MK501949.1) in China, 99.74% with *C. complanatum* (MF741769.1) in China, 97.62% with *C. complanatum* (MF928769.1) in Turkey, 97.09%, with *C. sinensis*(MK801719.1) in Italy, 96.83% with *C. complanatum* (MT602068.1) in Turkey, 96.56% with *C. complanatum* (MK814187.1) in USA, 93.12% with *Clinostomum* sp. morphotype 4 (KY865661.1) in Italy and 89.69%with *C. phalacrocoracis* (KY906227.1) in South Africa. On the other hand, *C. phalacrocoracis* (MT140102.1) in this study revealed 100% similarity with *C. phalacrocoracis* (KY906238.1) in South Africa, 99.83% similarity with *C. phalacrocoracis* (KY906229.1) in South Africa, 99.66% with *C. phalacrocoracis* (KJ786967.1) in Israel, 99.66% with *C. phalacrocoracis* (KP110522.1) in Canada,90.68% *with C. cutaneum* (KP110515.1) in Canada,89.66% with *C. tilapiae* (KY649357.1) in Italy,89.49% with *C. tilapiae* (MN709048.1) in Egypt,89.49% with *C. tilapiae* (KY649358.1) in Nigeria, 88.81% with *C. sinensis* (KP110535.1) in China, 88.64% with *C. sinensis* (MK801715.1) in Taiwan, 88.47% with *C. complanatum* (KM923964.1) in China, 87.99% with *C. complanatum* (KU236382.1) in Italy, 87.65% with *C. complanatum* (MK814187.1) in , USA, 86.13% with *C. brieni* (MH253048.1) in Italy and85.45% with *C. detruncatum* (KP110519.1) in China.

The phylogenetic tree was constructed using the neighbor joining model of the COI mtDNA region of C. complanatum and C. phalacrocoracis showing strong nodal support of two major clades. The first one comprised two subclades. The first subclade included C. complanatum of Egypt that embedded among other C. complanatum, and grouped together with C. sinensis. While the second subclade included C. Phalacrocoracis of Egypt and was classified with other C. phalacrocoracis and grouped together with C. tilapiae and C. cutaneum **(Fig.2).** On the other hand, the second clade grouped as C. brieni was separate from other groups.

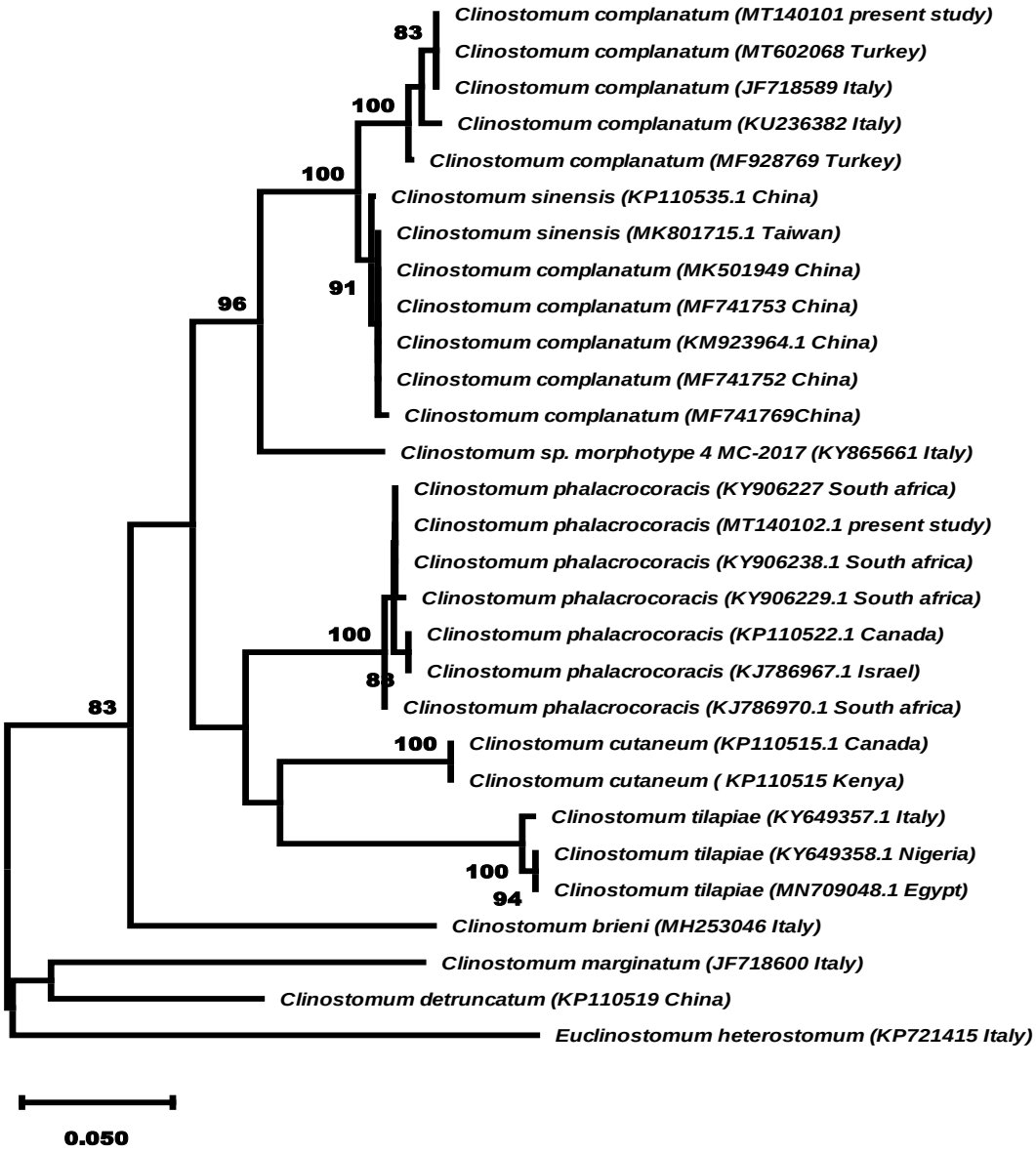

Fig. 2: Phylogeny of *C. complanatum* and *C. phalacrocoracis* based on neighbor-joining model using COI mtDNA

DISCUSSION

The metacercarial infection in warm freshwater fish possesses a superior position of parasitic infection. Away from its drastic effect on fish health, it usually has a negative drawback on fish marketing as well as a prominent health hazards on the human fish consumers **(Aly *et al*., 2005).** The present investigation detected that the overall infection rate of Clinostomatidae EMC among the investigated *O. niloticus* in Egyptian water was 74.12 ± 5.19. This prevalence is inconsistent with that found by **(Khattab, 1990)** who

reported a higher prevalence (87.06 %) of *Clinostomum* metacercariae from *Tilapia niloticus* in Egypt. While, current result was higher than that prevalence reported by **Taher (2009),** who revealed that (62.25%) of *O. niloticus* were infected with different species of Clinostomatidae metacercaria. Such variation in prevalence may be related to differences in habitat, food supply, the abundance of aquatic snails (the intermediate hosts), and the abundance of aquatic piscivorous birds, which play the main role in completing the life cycles of some digenetic trematodes **(Mutengu & Mhlanga, 2018).** Regarding to the current result of *C. phalacrocoracis* and *C. complanatum* cysts of infected *O. niloticus* the prevalence was detected as 60.93 ± 6.70 and 35.94 ±2.24, respectively. The present prevalence of *C. phalacrocoracis* metaceracriae is considered higher than that determined by **Ebraheem (1992)** and **Taher (2009)** who recorded that infection was 35.17% and 30.75% in the Sohag and Assiut governorates of Upper Egypt, respectively. Furthermore, **Mohamadin (1989)** and **Thabit (2004)** noted relatively low prevalence values of 5.26% in Quena and 10.0% in Assiut Governorates in Egypt. Furthermore, the current result of prevalence of *C. complanatum* cysts of the infected *O. niloticus* under investigation from Giza Egypt was higher than that recorded by **EL-Shahawy *et al.* (2017)** who revealed that (6.9%) from *O.niloticus* was collected from the River Nile at Qena Governorate, southern Egypt

Furthermore, the highest number of Clinostomatidae EMC was detected in branchial cavity (66.93± 4.91) in investigated *O. niloticus.* The present result is in agreement with that of *Clinostomum* sp. recorded by **Taher (2009)** who found that prevalence of infection was 47.5% in the buccal cavities of *O. niloticus* ,and lower than that recorded by **Caffara *et al.* (2014)** from *T.zilli* (23.4%). The difference in prevalence was based on the types of EMC that has a preference site. This may be related to several factors, including species of the host, geographical distribution, and genetic variation of each type of metacercariae.

In the present study, there were significant variations in the intensity and prevalence rate of EMC among different seasons which were high in summer due to the increase of the releasing rate of the temperature dependent cercariae from the snail host and the successfully transmitted to the fish as previously described by **Elsheikha and Elshazly (2008)** and **Ibrahim and Soliman (2010).** On the other hand, the decreased prevalence and intensity of EMC during the cold seasons may be related to the death of the temperature dependent cercariae/metacercariae **(Taher, 2009).**

Regarding to the morphological analysis, description of *C.phalacrocoracis* coordinates with **Kabunda and Sommerville (1984)** and **Thabit (2004),** while description of *C. complanatum* coincides with **Taher (2009).** Precise identification of parasite species is more valuable especially in those concerns with the treatment and control of the parasitic infections demonstrated by **Moszczynska *et al*. (2009).** Due to the difficulties in morphological identification, molecular methods have become useful and popular to distinguish morphologically similar species, and identify species at any

developmental stage of parasites demonstrated by **Simsek *et al.* (2018).** In recent years, mt-COI sequence analyses combined with morphological definition have been successfully utilized to identify and redefine trematode species as previously described by **Gustinelli *et al.* (2010), Caffara *et al.* (2011, 2017)** and **Wang *et al.* (2017).** COI gene region has also been used as DNA barcoding region in many studies as demonsterated by **Moszczynska *et al.* (2009)** and **Steenkiste *et al.* (2015)** due to its ability to identify the specimens in a wide taxonomic range. Furthermore, it has been shown that barcode COI region exhibits better resolution than ITS especially for exploring the intraspecific genetic divergence and revealing cryptic species as previously described by **Moszczynska *et al.* (2009)** and **Steenkiste *et al.* (2015).** Furthermore, in Africa, many papers were published without any morphological and/or molecular identification reporting unidentified metacercariae of *Clinostomum* in different species of tilapia (**Yimer & Enyew, 2003).**

The molecular identification was carried out by sequencing their COI mtDNA; BLAST analysis of *C. complanatum* (MT140101.1) of this study showed 100% and 99.74% similarity with *C. complanatum* (MK501949.1; MF741769.1) in China, respectively. On the other hand, *C. phalacrocoracis* (MT140102.1) revealed 100% with *C. phalacrocoracis* (KY906238.1) in South Africa and 99.66% with *C. phalacrocoracis* (KJ786967.1) in Israel. The results of this study provide the first data on the C. c*omplanatum* and *C. phalacrocoracis* lineages infecting freshwater fishes in Egypt using the combination of molecular and traditional morphological techniques. Many important changes have been made in the taxonomy of species belonging to genus of *Clinostomum* due to the morphological similarities between the species and the lack of description of valid species as previously described by **Ukoli (1966)** and **Dzikowski *et al*. (2004).** The results of the morphological features of the obtained metacercariae agreed with the results of *C. complanatum* as described by **Caffra *et al.* (2011)** and **Wang *et al.* (2017).** Moreover, the results concerning *C. phalacrocoracis* agree with that of **Gustinelli *et al*. (2010)** and **Caffara *et al.* (2014)** in tilapias and herons from Kenya and Israel, respectively. The morphological characterization knowledge of *C. phalacrocoracis* in Africa is few **(Ukoli, 1966).**

In this study, we are coupling the traditional morphological identification and the newly molecular characterization of *C. phalacrocoracis* and *C. complnatum* metacercariae from *O. niloticus* in Egypt.

CONCLUSION

This interesting study defined two types of Clinostomum spp.; *C. Complanatum* and *C. phalacrocoracis. C. Complanatum*, the first species with its zoonotic importance could present a potential health risk for eating undercooked fish. The current study is a leading investigation for the first comparative results performed using the morphological and molecular characteristics of two clinostomides infection of freshwater fish

(*O.niloticus*) from northern Nile River Egypt. Detailed studies with a large number of samples in a wide geographical area are recommended to explore the true picture of population genetic structure within the genus *Clinostomum* in freshwater fish based on the principle of an integrative taxonomic approach that combines morphological and molecular characteristics.

Compliance with ethical standards

All guidelines of institutional, ethical and animal welfare were conducted in accordance with the Laboratory Animal Care and Use Gide (Vet-CU-10102019100).

Conflict of interest

The authors state that they have no conflict of interest.

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