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The occurrence of *Contracaecum* sp. larvae (Nematoda: Anisakidae) in four teleostean species from Lake Nasser, Egypt: morphological and molecular studies

Abuelhassan Elshazly Younis[1*], Atef Ibrahim Saad[1*] and Jihan Moharam Rabei[2]

Abstract

Background: Nematodes of the family Anisakidae are parasites of many fishes and aquatic invertebrates which act as intermediate or paratenic hosts, while mammals and fish-eating birds are definitive hosts. Infective L3 larvae may be incidentally taken by human through eating raw or undercooked fish meat, causing anisakidosis. The main purpose of this study is to provide a basis for the future investigations to discover the genetic diversity of this widely distributed parasite nematodes of fishes and fish eating animals and their effect on fisheries and public health in Egypt and worldwide.

Results: One thousand, one hundred and fifteen specimens belong to nine fish species were collected from Lake Nasser, Egypt, and examined for infection with Anisakid larvae. Four fish species (*Oreochromis niloticus*, *Tilapia galilaea, Lates niloticus*, and *Hydrocynus forskahlii*) were found infected with third stage larvae of *Contracaecum* spp. No other Anisakid nematodes were detected. Larvae were found in the body cavity adhering to mesenteries by a thin membrane, except in *Oreochromis niloticus* and *Tilapia galilaea* were found free in branchial chambers. The highest prevalence was recorded in *L. niloticus* (100%) and *H. forskahlii* (82%). The mean intensity of infections were 0.17–4.12 and 5.1–10.3 in *L. niloticus* and *H. forskahlii* respectively. For further identification, the internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA from isolated larvae ($n = 54$) were amplified by PCR, followed by single-strand conformation polymorphism (SSCP) analysis which revealed five possible profiles.

Conclusion: Light and scanning electron microscope studies revealed that all anisakid larvae in the present study showed the most typical features of the genus *Contracaecum*. The sequencing ($n = 28$) and sequence and phylogenetic analyses showed that the present nematode larvae are likely belonging to *C. multipapillatum*.

Keywords: *Contracaecum*, Lake Nasser, Fish, Internal transcribed spacers

Background

Parasites usually influence the quality and marketing of commercially produced fish and may contribute to high fish mortalities and economic losses or threaten the abundance and diversity of fish species, accordingly, raising a lot of public health concerns, particularly in regions where raw or smoked fish are eaten (Adams, Murrell, & Cross, 1997; Paperna, 1996; Barson, 2004).

Nematodes of the family Anisakidae are parasites of many fishes and aquatic invertebrates which acting as intermediate or paratenic hosts, while mammals and fish-eating birds are definitive hosts. The most widespread genera are *Anisakis*, *Pseudoterranova*, and *Contracaecum* which have similar life cycles. Third stage larvae (L3) of *Contracaecum* (Railliet et Henry, 1912) were found usually in the body cavity, branchial chambers, and mesenteries of the fish while the adults were found in the gut of the fish-eating birds as mentioned by Whitfield and Heeg (1977). It was reported in Cichlids and Catfish in different African countries, such as Egypt

* Correspondence: abssan2000@aswu.edu.eg; atefsaad2002@yahoo.com
[1]Zoology Department, Faculty of Science, Aswan University, Aswan 81528, Egypt
Full list of author information is available at the end of the article

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(Amin, 1978), East Africa (Aloo, 2001) and South Africa (Boomker, 1982, 1994; Van As & Basson, 1984). These larvae were the most prevalent nematode parasite identified from the mesenteries of *Clarias gariepinus*, *Barbus acutirostris*, *B. tsanensis*, and *B. brevicephalus* and from the pericardial cavity of Nile Tilapia (*Oreochromis niloticus*) captured from Lake Tana Ethiopia (Yimer & Enyew, 2003). Kaddumukasa, Kaddu, and Makanga (2006) investigated the positive correlation between intensity of infection and length of Nile tilapia, *O. niloticus*, in Lake Wamala. The high spread of *Contracaecum* in fish might influence their health; subsequently, it can affect the commercial value of fish, particularly when it was found in the musculature, and thus represent some economic loss for fisheries industry (Angot & Brasseur, 1995). At the point when in the stomach of the definitive host, L3 molts to become L4 and both the larvae and the adults may influence the host health negatively (Martins, Onaka, & Fenerick Jr., 2005; Girişgin, Alasonyalilar-Demirer, & Girişgin, 2012). In case of the larvae are incidentally taken by human through eating raw or undercooked fish meat, they may cause anisakidosis, a zoonotic infection causing stomach pains, fever, diarrhea, and vomiting (Sakanari & McKerrow, 1989; Kaneko, 1991; Arslan, Dinçoğlu, & Güven, 1995; Audicana, Ansotegui, de Corres, & Kennedy, 2002; Palm, 2004; Shamsi & Butcher, 2011).

The identification of *Contracaecum* spp. from various host groups attracted attention of scientists in different geographical parts of the world using both light and scanning electron microscopy for morphological examination, as well as the evaluation of genetic markers as the ribosomal internal transcribed spacers (ITS-1 and ITS-2) for the molecular characterization (Nadler, D'Amelio, Dailey, Paggi, Siu, et al., 2005; D'Amelio, Barros, Ingrosso, Fauquier, Russo, et al., 2007; Shamsi, Gasser, Beveridge, & Shabani, 2008; Shamsi, Norman, Gasser, & Beveridge, 2009; Shamsi, Gasser, & Beveridge, 2011; Garbin, Mattiucci, Paoletti, González-Acuña, & Nascetti, 2011; Garbin, Mattiucci, Paoletti, Diaz, Nascetti, et al., 2013; Jabbar, Fong, Kok, Lopata, Gasser, et al., 2013; Borges, Santos, Brandã, Santos, Miranda, et al., 2014).

The Fishes are an important source of protein for humans and other animals. The fish industry likewise provides livelihood chances to numerous individuals, as well as income at household and national levels (FAO, 1996).

Lake Nasser is a reservoir constructed by the High Dam in southern part in Egypt and extends to the second cataract in Sudan. It is about 480 km long, 300 km in Egypt (Lake Nasser), and 180 km in Sudan (Lake Nubia). Lake Nasser is considered as important source of fish and of considerable economic potential in Egypt. Its estimated commercial value of the fishery (including both fresh and salted fish) is around US $17 million (Béné, Bandi, & Durville, 2008).

Fifty-seven species of fish belonging to 16 families were recorded in Lake Nasser. *Tilapia nilotica* (*Oreochromis niloticus*), Nile perch (*Lates niloticus*), Tiger fish (*Hydrocynus forskahlii*), and *Alestes spp.* are the most common species and important for the fishery (Latif, 1974; Rashid, 1995). While *Tilapia* and Nile perch are landed fresh, Tiger fish and *Alestes* are usually processed before being sold as salted fish (Bishai, Abdel-Malek, & Khalil, 2000).

However, the parasitic fauna of fishes from different inland water in Egypt were studied by several investigators; only few published data based on morphological characteristics were performed on helminth infections in Lake Nasser (Saoud & Wannas, 1984; Al-Bassel, 1992; Saad, 2007; Ezzat, ElKorashey, & Sherif, 2012). Thus, our knowledge about the prevalence, abundance, and the genetic diversity of nematodes of fishes is still limited. The present investigation is the first study to use an integrated molecular and morphological approach toward identification and detailed description of socioeconomically important anisakid nematodes from four infected out of nine examined species of fishes in Lake Nasser, Egypt.

Methods

Parasite

Guidelines and rules of Aswan University were followed in terms of dealing with and use of animals. Approvals from the committee of Graduate Studies and from the College Council were taken before starting the study.

During 2 years, from November 2013 to October 2015,1115 fish specimens were collected from Lake Nasser, Egypt, and examined for infection with anisakid larvae. These fishes were *Oreochromis niloticus* ($n = 73$); *Tilapia galilaea* ($n = 712$); *Lates niloticus* ($n = 85$); *Synodontis frontosus* ($n = 40$); *Mormyrus caschive* ($n = 53$); *Chrysichthys auratus* ($n = 43$); *Hydrocynus forskahlii* ($n = 35$); *Alestes baremose* ($n = 44$); and *Lebeo niloticus* ($n = 30$). They were caught from 6 different localities (Khors) of the Lake, Allaqi, Dehmet, Khor Ghazal, Khor Maria, Khor Galal, and Wady abyad. Then, they were immediately transported to the laboratory for parasite examinations. Subsequently, they were dissected and the alimentary tract, liver, and gills were taken out and examined in physiological saline. Also, the body cavity was examined for investigation of nematode infection. The worms were collected and washed extensively with physiological saline. The prevalence and intensity were calculated for each fish species.

Morphological identification

Each individual nematode was divided into three pieces. Small pieces of the middle parts were kept in − 20 in

70% ethanol for molecular studies, while the anterior and posterior parts fixed in hot 70% ethanol (60–70 °C) for relaxing their bodies, and then preserved in 70% ethanol with few drops of glycerin to avoid distortion of the outer layer of the cuticle. Before light microscopic study, worms were cleared in Lactophenol for 24 h. All measurements were made specifically with an eyepiece micrometer and given in millimeters, unless mentioned otherwise. The photomicrographs were taken for the obtained nematodes using Olympus light microscope attached with a camera model HBS (HB2) and digital microscope model Olympus CX41. Identifications were based on key features and descriptions (Yamaguti, 1961; Nadler & Hudspeth, 1998; Anderson, 2000; Martins et al., 2005; Olivero-Verbel, Baldiris-Avila, Guette-Fernandez, Benavides-Alvarez, Mercado-Camargo, et al., 2006). For the scanning electron microscope (SEM) examinations, specimens were fixed in 5% glutaraldehyde solution for 24 h and then washed five times in Sodium cacodylate buffer solution (15 min each). Post-fixation, specimens were rinsed in 1% of osmic acid for 2 h. After washing, three changes in Sodium cacodylate buffer solution (15 min each) took place, and then the specimens were dehydrated in ascending grade of alcohols; 30, 50, 70, 90, and 100% alcohol for 30 min each. Then, the specimens were dried to acritical point as a result of exposure to 25 °C. Subsequently, samples were placed on holder and were coated by a very thin layer of gold (thickness of 150–200 Angstroms). Finally, the specimens were examined by scanning the electron microscope type JSM 5400 LV: Jeol.

Molecular characterization

Genomic DNA isolation, PCR, and sequencing

Genomic DNA was isolated from individual larvae ($n = 54$), as described before (Younis, Geisinger, Ajonina-Ekoti, Soblik, Steen, et al., 2011) with slight modifications. In brief, the individual parasite materials were digested for 3–4 h at 56 °C with proteinase K in ALT buffer (Dneasy kit, Qiagen) under a constant agitation. gDNA extracted with the standard phenol/chloroform method and treated with RNase A was then precipitated with 5.2 M ammonium acetate and dissolved in 20–30 μl HPLC water. Concentrations were determined by spectrophotometry.

The specific primer sets SS1/NC13R and SS2/NC2 were used to amplify the two nuclear ribosomal markers ITS-1 and ITS-2 respectively, under the same conditions as described previously (Zhang, Hu, Shamsi, Beveridge, Li, et al., 2007). The negative control (gDNA from intact fish muscles and non-DNA) samples were used. The PCR products were analyzed by 1% *w*/*v* agarose gel, stained with ethidium bromide and photographed using a gel documentation system, were then purified using QIAquick® PCR purification kit (Qiagen), according to the manufacturer's protocols. The resulted products were sequenced by a dideoxy termination method using an applied Biosystems 373 DNA sequencer (LGC Genomics GmbH, Germany) in the two directions (forward and reverse) by the same PCR-used primers.

Single-strand conformation polymorphism (SSCP)

All amplicons (ITS-1 and ITS-2) were subjected to SSCP analysis (Gasser, Hu, Chilton, Campbell, Jex, et al., 2006) to screen nucleotide sequence variation among amplicons. In details, 5 μl of individual amplicons were mixed with 10 μl of loading buffer (75% formamide, 2.5 mM Tris, 0.0075 bromophenol blue, 0.0075% xylene cyanol, 15% glycerol, 15 mM EDTA), denaturated at 96 °C for 15 min and snapped cooling on a freeze block (– 20 °C) for 5 min. Individual samples (15 μl) were loaded into the wells of a 10% acrylamide/bis gel (29:1), containing 7% glycerol, and 1X TBE buffer and casted in mini protein tetra vertical chamber (Bio-Rad). Gels were subjected to electrophoresis for 7 h at constant power (70 V) and temperature 8 °C in a pre-cooled 1XTBE. The gels were then stained with ethidium bromide (1 μg/ml in 1XTBE buffer for 15 min), de-stained in 1XTBE for 10 min and photographed by an ultraviolet transilluminator.

Computer-based sequence analysis

For each sequence, the NCBI Blast program was used for homology search (http://www.ncbi.nlm.nih.gov/). After that, each sequence (forward) was compared to its complement (reverse) and then adjusted manually to get one sequence. Subsequently, the resulted sequences were aligned to each other and to the most homologous sequences in the database using CLUSTALW multiple sequence alignment program. The phylogenetic trees were constructed using online tool software Phylogeny.fr program (Dereeper, Guignon, Blanc, Audic, Buffet, et al., 2008).

Results

Morphological identification

Morphological examinations and measurements showed that all the nematodes larvae examined in the present study showed the most typical features of L3 of the genus *Contracaecum* with convergent shapes and morphology. Interestingly, there were no significant morphological differences of larvae among the fish species, with exception that there were two forms of L3 infected *L. niloticus* and *H. forskahlii*, short form (SF) and long form (LF).

All larvae were found to share most of morphological characters with slight differences (Table 1; Figs. 1, 2, and 3).

Table 1 Comparison of morphological descriptions and measurements, between various *Contracaecum* larval types found in Lake Nasser fishes

	Contracaecum A	*Contracaecum* B	*Contracaecum* C (SF)	*Contracaecum* C (LF)	*Contracaecum* D (SF)	*Contracaecum* D (LF)
Number of larvae examined	10	4	10	10	10	10
Host	*Oreochromis niloticus*	*Tilapia galilaea*	*Lates niloticus*	*Lates niloticus*	*Hydrocynus forskalii*	*Hydrocynus forskalii*
Localization in host	Free in pericardial cavity and gills	Free in pericardial cavity and gills	Body cavity adhering to the alimentary canal	Body cavity adhering to the alimentary canal	Body cavity adhering to the alimentary canal	Body cavity adhering to the alimentary canal
Larva description	• Mouth opening slit-like shape, surrounded by three small lips, one dorsal, two ventrolateral with ill boring tooth between them. • Four cephalic papillae were present • Cuticle striated, forming circular ridges. Cuticular ridges very closely anteriorly, covered with heavy host mucus.	Same previous	Same previous except: • A smooth transparent membrane covering it, head end rounded without host mucus and posterior end pointed. • A triangular mouth opening • Four cephalic papillae were present • cuticle striated with fine transversely lines.	• Triangular mouth opening • Four cephalic papillae were present, two on dorsal lip and the two others were one on each ventral lip. • Tail provided with terminal process which appear as a projection and covered by thin membrane. • cuticle was transversely striated with fine longitudinal lines	• Body covered by transparent sheath. • anterior rounded, while posterior pointed. • Four cephalic papillae were present • The cuticle was transversely striated.	• Mouth opening triangular • excretory pore was anteriorly • Four cephalic papillae were present • tail conical provided with rounded tapered process. • The cuticle was striated longitudinally and transversely forming net – like shape
L	40–52 (47)	39–50 (45)	29–39 (34)	39–50 (46)	23–35 (30)	32–50 (42)
W	1.3–1.64 (1.45)	1.32–1.76 (1.48)	0.78–1.22 (1.05)	1–1.26 (1.16)	0.7–1.13 (0.96)	0.94–1.4 (1.15)
B.T.	0.007–0.009 (0.008)	0.007–0.0084 (0.008)	0.02–0.025 (0.023)	0.02–0.024 (0.022)	0.018–0.02 (0.019)	0.01–0.015 (0.013)
Oes.	4.4–6.2 (5.18)	4.2–5.9 (4.9)	2.46–3.72 (3.07)	3.6–4.6 (4.13)	3.2–5 (4.28)	2.9–4.4 (3.62)
Int.c.	3.4–5 (4.1)	3.2–4.8 (3.9)	1.91—3.12 (2.42)	2.8–3.8 (3.22)	2–4 (3.35)	2–3.6 (2.75)
VA	1.66–3.2 (2.43)	1.5–2.8 (2.15)	0.78–0.84 (0.81)	0.84–1.3 (1.11)	0.56–0.66 (0.6)	0.84–1.2 (0.98)
t.	0.16–0.38 (0.23)	0.16–0.35 (0.2)	0.1–0.24 (0.16)	0.1–0.2 (0.16)	0.06–0.24 (0.22)	0.2–0.22 (0.2)
t.p.	0.01–0.015 (0.012)	0.01–0.013 (0.012)	0.05–0.06 (0.055)	0.07–0.075 (0.072)	0.073–0.075 (0.07)	0.032–0.038 (0.035)
Oes./L	10–12% (11%)	10–12% (11%)	8.4–9.5% (9%)	9.2–9.6% (9.43%)	13.9–14.3% (14%)	9.1–10% (9.6%)
Int.c./Oes.	77–81% (79%)	76–81% (80%)	77–84% (78.8%)	78–83% (80%)	63–78% (76.2%)	69–80% (77%)
VA/Oes.	37.7–51.6% (46.9%)	35.7–47.4% (43.8%)	22–32% (26.4%)	23–30.6% (26.8%)	13–17.5% (14%)	23–29% (27%)
VA/Int.c.	48.8–64% (59%)	46.8–58% (55%)	27–41% (33.8%)	30–39% (34.5%)	16.5–28% (18%)	33.3–42% (35.6%)
t./L	0.4–0.7% (0.55%)	0.4–0.66% (0.53%)	0.34–0.6% (0.47%)	0.3–0.5% (0.35%)	0.3–0.6% (0.58%)	0.47–0.6% (0.5%)

Measurements are in millimeters

Abbreviations: *LF* long form, *SF* short form, *L* body length, *W* body width, *B.T.* boring tooth, *Oes.* length of esophagus, *Int.c.* length of intestinal caecum, *VA* length of ventricular appendix, *t.* tail length, *t.p.* tapered process

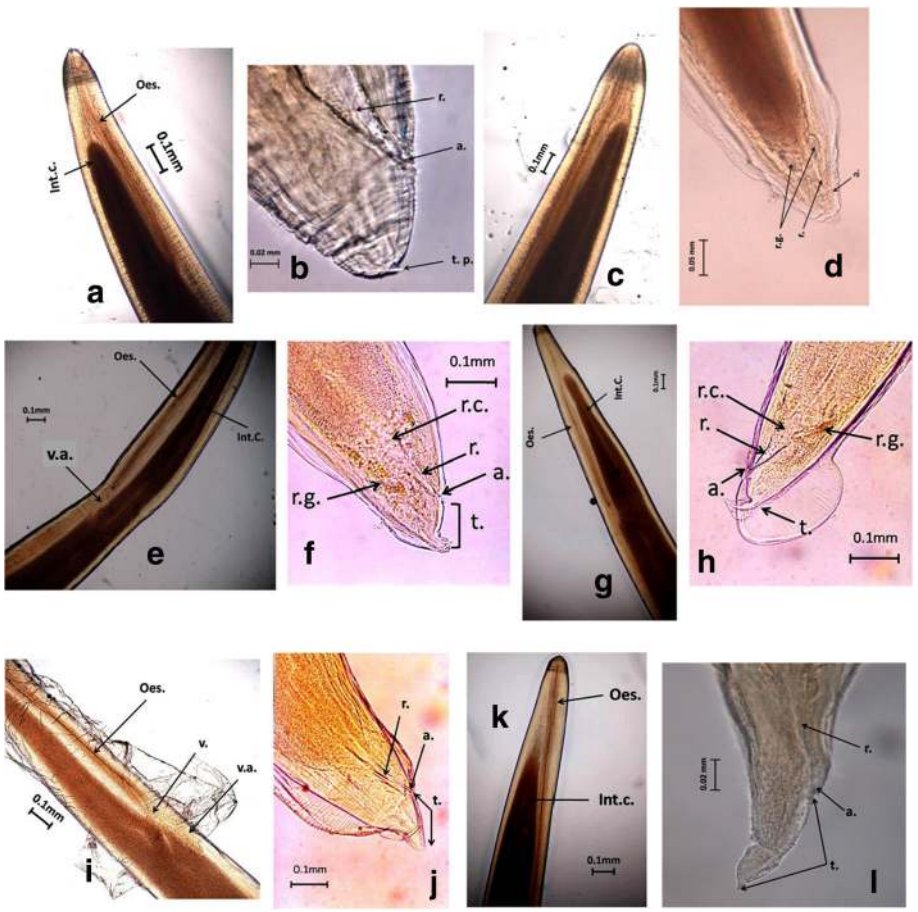

Fig. 1 Light microscopic study. **a**, **b** Anterior and posterior end of larva collected from *Oreochromis niloticus*; **c**, **d** anterior and posterior end of larva collected from *Tilapia galilae*; **e**, **f** anterior and posterior end of short form larva collected from *Lates niloticus*; **g**, **h** anterior and posterior end of long form larva collected from *Lates niloticus*; **i**, **j** anterior and posterior end of short form larva collected from *Hydrocynus forskahlii*; and **k**, **l** anterior and posterior end of long form larva collected from *Hydrocynus forskahlii*. Magnifications: **a**, **c**, **e**, **g**, **i** and **k** (×4); **b** and **l** (×40); **d** (×20); **f**, **h** and **j** (×10)

Living larvae were reddish-yellow in color. The body was long, cylindrical with rounded anterior end and tapered posterior one. Light and scanning electron microscopes data can be summarized as follows: the mouth is triangular or transverse in shape and surrounded by three lips, one dorsal and two ventrolateral; lips are provided with four papillae, two on the dorsal lip and one on each ventrolateral lip; well-defined boring tooth is located in between the ventrolateral lips; excretory pore is located anteriorly just below to the boring tooth; length of esophagus, ventricular appendix, intestinal caecum, and the ratios between each other are not the same; anal opening is located near the posterior end; the length of tail is also not equal between larvae collected from different fish species; and tail is blunt in larvae collected from *Oreochromis niloticus* and *T. galilae*, while being conical in larvae collected from other fishes and tail ending with a tapered process like spine.

Regarding SEM study, the cuticle is striated with regular and irregular rings, which are narrower anteriorly and become wider as extended posteriorly and showing different ornamentations between larvae where cuticle of collected larvae from *Oreochromis niloticus* and *T. galilae* is striated transversely and longitudinally and forming well-defined circular ridges, which are very close anteriorly and covered with heavy mucus derived from the host (Fig. 2a, c). Larvae collected from *Lates niloticus* present in two sizes: short form (SF) and long form (LF). They are decorated with wavy striations in SF and net shaped in LF as shown in Fig. 4(b, c). Larvae (SF and LF) found in *Hydrocynus forskahlii* were striated transversely and longitudinally with overlapping strips (dendritic shape) respectively as shown in Fig. 4(d, e).

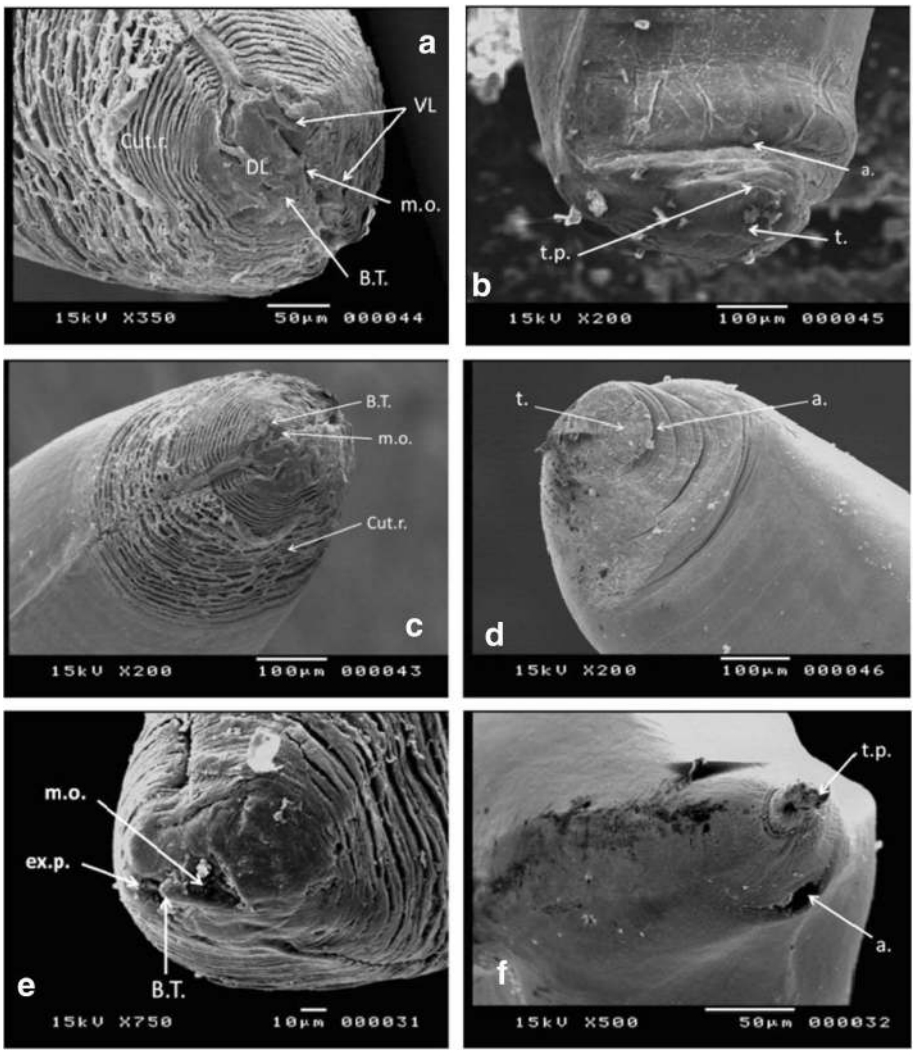

Fig. 2 Scanning electron micrographs: **a**, **b** anterior and posterior end of larva collected from *Oreochromis niloticus*; **c**, **d** anterior and posterior end of larva collected from *Tilapia galilae*; **e**, **f** anterior and posterior end of short form larva collected from *Lates niloticus*

Prevalence and infection intensity

Nine species of Lake Nasser fishes were examined for infection with *Contracaecum* larvae. Four fish species (*Oreochromis niloticus*, *Tilapia galilaea*, *Lates niloticus*, and *Hydrocynus forskahlii*) were found infected with L3 of *Contracaecum*, and the other five fish species (*Synodontis frontosus*, *Mormyrus caschive*, *Chrysichthys auratus*, *Alestes baremose*, and *Lebeo niloticus*) were found free in infection. *Contraceacum* L3 was found free in pericardial cavity and branchial chambers (*O. niloticus* and *T. galilaea*) and in the body cavity adhering to the alimentary canal (*L. niloticus* and *H. forskahlii*). The worm burden, prevalence and mean intensities per infected fish varied among different fishes (Table 2), with 100% prevalence in *L. niloticus* and the highest intensity in *H. forskahlii*. Interestingly, there were no significant differences in the data collected from different Khors of the Lake Nasser.

PCR of ITS-1 and ITS-2 and mutation scanning analysis

Following the morphological identification, the ITS-1 and ITS-2 regions were amplified by PCR from genomic DNA samples ($n = 54$) from individual larvae. Agarose gels analyses revealed the same size for each ITS region. Amplicons were ~ 530 bp and ~ 430 bp for the ITS-1 and ITS-2, respectively (Fig. 5a), confirming that all sequences are of the same genus. Following, all ITS-1 and ITS-2 amplicons were analyzed using SSCP analysis to scan for sequence variability within and among individual specimens. For each ITS region, five possible profiles were observed on the SSCP gels for all 54 samples

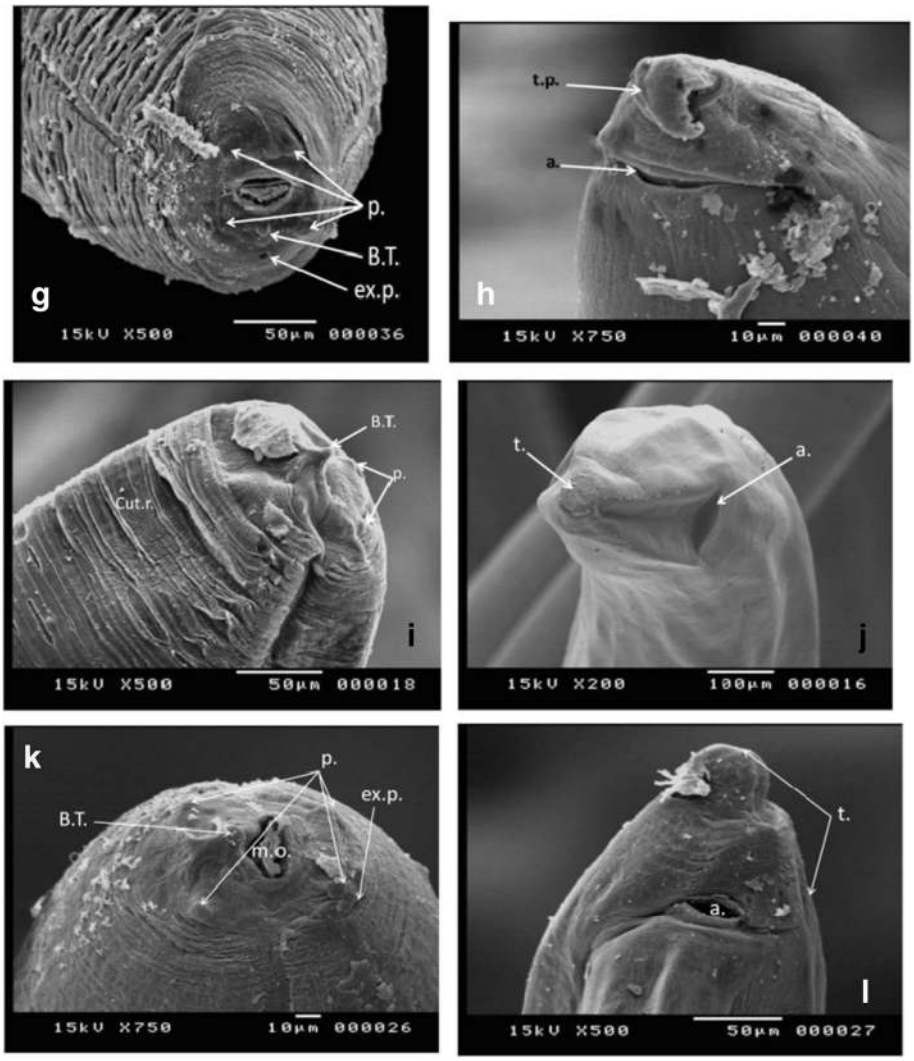

Fig. 3 Scanning electron micrographs: **g**, **h** anterior and posterior end of long form larva collected from *Lates niloticus*; **i**, **j** anterior and posterior end of short form larva collected from *Hydrocynus forskahlii*; **k**, **l** anterior and posterior end of long form larva collected from *Hydrocynus forskahlii*

(Fig. 5b, c). Selected amplicons ($n = 28$) representing each possible genotype, taking fish species into consideration, were subjected to sequencing. No polymorphism was detected in any sequences that have the same SSCP profile.

Sequence and phylogenetic analyses

Alignment of resulted sequences revealed considerable variation of each ITS region, which may indicate the presence of more than one type of larvae. Scores of identities between ITS-1 sequences were ranging from 65% (between LF larvae collected from *L. niloticus* and *H. forskahlii*) to more than 99.3% (between SF larvae collected from *L. niloticus* and *H. forskahlii*), while scores of identities between ITS-2 sequences were ranging from 42% (between LF larvae collected from *H. forskahlii* and larvae from *T. galilaea*) to more than 98.7% (between larvae collected from *O. niloticus* and larvae from *T. galilaea*) (Fig. 6).

The Blast analyses revealed that all sequences of both ITS-1 and ITS-2 in the present study were different from all sequences deposited in GenBank databases. ITS-1 sequences of *Contracaecum* larvae obtained were deposited in GenBank under accession numbers KX580602-KX580607, and ITS-2 sequences from the corresponding larvae were deposited in GenBank under accession No. KX580607-KX5806013. ITS-1 sequences obtained from larvae-infected *T. galilaea* showed the highest similarity (89%) to ITS-1 sequence of L3 of *Contracaecum* sp. 1 (Accession numbers KF990491), isolated from the body cavity and the intestines of *H. forskahlii* and *T. zillii* in Lake Turkana, Kenya (Otachi, Szostakowska, Jirsa, & Fellner-Frank, 2015). The last in turn draw an analogy (89%) to the *Contracaecum*

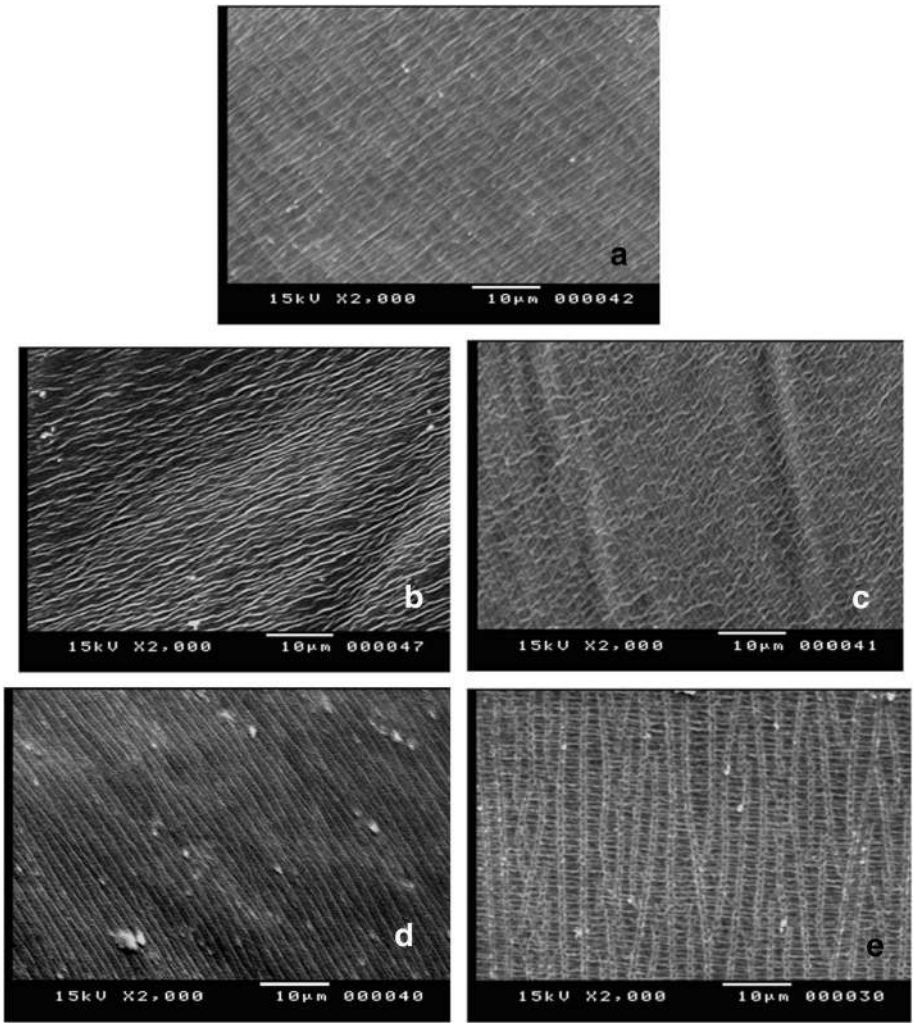

Fig. 4 Scanning electron micrographs showing the pattern of cuticular striations in: **a** larva collected from *Oreochromis niloticus* and *Tilapia galilae*; **b** short form larva collected from *Lates niloticus*; **c** long form larva collected from *Lates niloticus*; **d** short form larva collected from *Hydrocynus forskahlii*; **e** long form larva collected from *Hydrocynus forskahlii*

multipapillatum (von Drasche, 1882) from the Australian pelican *Pelecanus conspicillatus* (Accession numbers AM940056) (Shamsi et al., 2008). In addition, ITS-2 from the same larvae showed the highest similarity (86%) to three ITS-2 sequences of *Contracaecum* L3 (Accession numbers FM210437- FM210438- FM210439) from Iranian fish (recorded by Shamsi in 2008 to the GenBank). Similarly, sequences of ITS-1 and ITS-2 of L3 from *O. niloticus* showed 99 and 86% similarity to *Contracaecum multipapillatum* (Accession numbers KF990491) and to *Contracaecum* L3 (Accession numbers FM210437-FM210439) respectively. In *H. forskahlii*, two forms (differ in size) of larvae were found, SF and LF. The last is morphologically similar to all other larvae found in other fishes. ITS-1 of SF larvae collected from *H. forskahlii* showed the highest similarity (97%) to three ITS-1 sequences (Accession numbers FM210433-FM210434- FM210435) from *C. multipapillatum* L3

Table 2 The prevalence and mean intensity of *Contracaecum* larvae relative to fish species

Fish species	No. of examined fish	No. of infected fish	Prevalence %	No. parasite in each fish	Mean intensity
O. niloticus	73	26	35.6	15–50	0.6–1.9
T. galilaea	712	1	0.14	4	4
H. forskhalii	35	29	82	150–300	5.1–10.3
L. niloticus	85	85	100	15–350	0.17–4.12

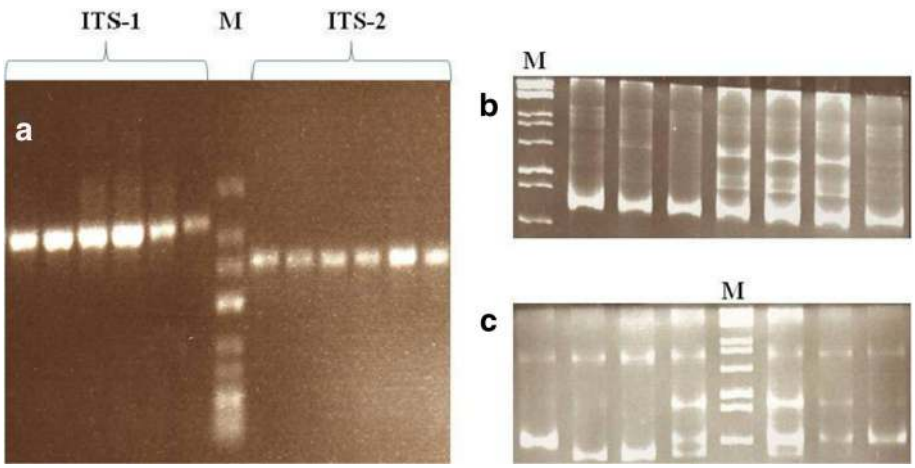

Fig. 5 Ethidium bromide-stained gel analysis of gDNA of *Contracaecum* L3 types. **a** Image of PCR products of ITS-1 and ITS-2 sequences of *Contracaecum* larvae in an 1.5% agarose gel under UV light. **b** SSCP patterns of ITS-1 sequences of *Contracaecum* larvae. **c** SSCP patterns of ITS-2 sequences of *Contracaecum* larvae. M: DNA ladder

which were collected from intestine and body cavity of barboid fishes in Parishan Lake, Iran (Shamsi & Aghazadeh-Meshgi, 2011). Further, ITS-2 of the same larvae (SF L3 from *H. forskahlii*) showed the highest similarity (98%) to the previously abovementioned three ITS-2 sequences of *Contracaecum* L3 (Accession numbers FM210437-FM210438-FM210439). Also, ITS-1 of LF larvae collected from *H. forskahlii* showed the highest similarity (78%) to the three abovementioned ITS-1 sequences of *C. multipapillatum* L3 (Accession numbers FM210433- FM210434- FM210435). Differently, ITS-2 sequence of the same larvae (LF L3 from *H. forskahlii*) showed the highest similarity (92%) to 18S- 5,8S-, 28S-rDNA-cluster of *Contracaecum osculatum* (Rudolphi, 1802) larvae (Accession numbers KM273050) in cod (*Gadus morhua*) from the Baltic Sea (Mehrdana, Bahlool, Skov, Marana, Sindberg, et al., 2014). Moreover, *L. niloticus* had SF and LF larvae. ITS-1 of SF larvae collected from *L. niloticus* showed the highest similarity (98%) to the three abovementioned ITS-1 sequences of *C. multipapillatum* L3 (Accession numbers FM210433-FM210434- FM210435). Also, ITS-2 of the same larvae (SF L3 from *L. niloticus*) showed the highest similarity (98%) to the abovementioned three ITS-2 sequences of *Contracaecum* L3 (Accession numbers FM210437-FM210438-FM210439). Finally, ITS-1 of LF larvae collected from *L. niloticus* showed the highest similarity (92%) to the three abovementioned ITS-1 sequences of *C. multipapillatum* L3 (Accession numbers FM210433-FM210434- FM210435). In addition, ITS-2 of SF L3 from *L. niloticus* showed the highest similarity to the abovementioned three ITS-2 sequences of *Contracaecum* L3 (Accession numbers FM210437-FM210438-FM210439).

Phylogenetic analysis for both ITS-1 and ITS-2 sequences produced nearly similar results, of which the first is displayed in Fig. 7. The identities and phylogenetic distances of the selected and most related ITS sequences showed nematode larvae investigated in this study are closely related to *C. multipapillatum* and likely are of different sub-species.

Discussion

The present investigation is the first study to use integrated molecular and morphological approaches toward characterization of larval anisakid nematodes from four infected out of nine examined species of fishes in Lake Nasser, Egypt.

Based on morphological characteristics, all anisakid larvae collected from *O. niloticus*, *Tilapia galilaea, Lates niloticus*, and *Hydrocynus forskahlii* fishes in present study are belonging to the genus *Contracaecum*. They were found in the body cavity adhering to the mesentery by a thin membrane, except in *O. niloticus* and *Tilapia galilaea* were found free in pericardial cavity and gills. It was noticed that the parasitic fauna of Lake Nasser is changeable and there was a peak of infection by this larvae in fish species. Al-Bassel (1990) found the juveniles of *Contracaecum* sp. in the abdominal and branchial cavities of a variety of Lake Nasser fish species, such as *Clarias gariepinus, Barbus bynni, Lates niloticus, Synodontis schall, Sarotherodonl galilaeus*, and *Mormyrus kannume*. Garo (1993) reported that such juveniles were found in the branchial cavity and mainly in the sinus venosus of 51.6% of *Serotherodon galilaeus* fish from Lake Nasser. This fluctuation in the parasitic fauna of Lake Nasser may be a result of the change of water level, temperature degrees of the Lake, and the intensity of

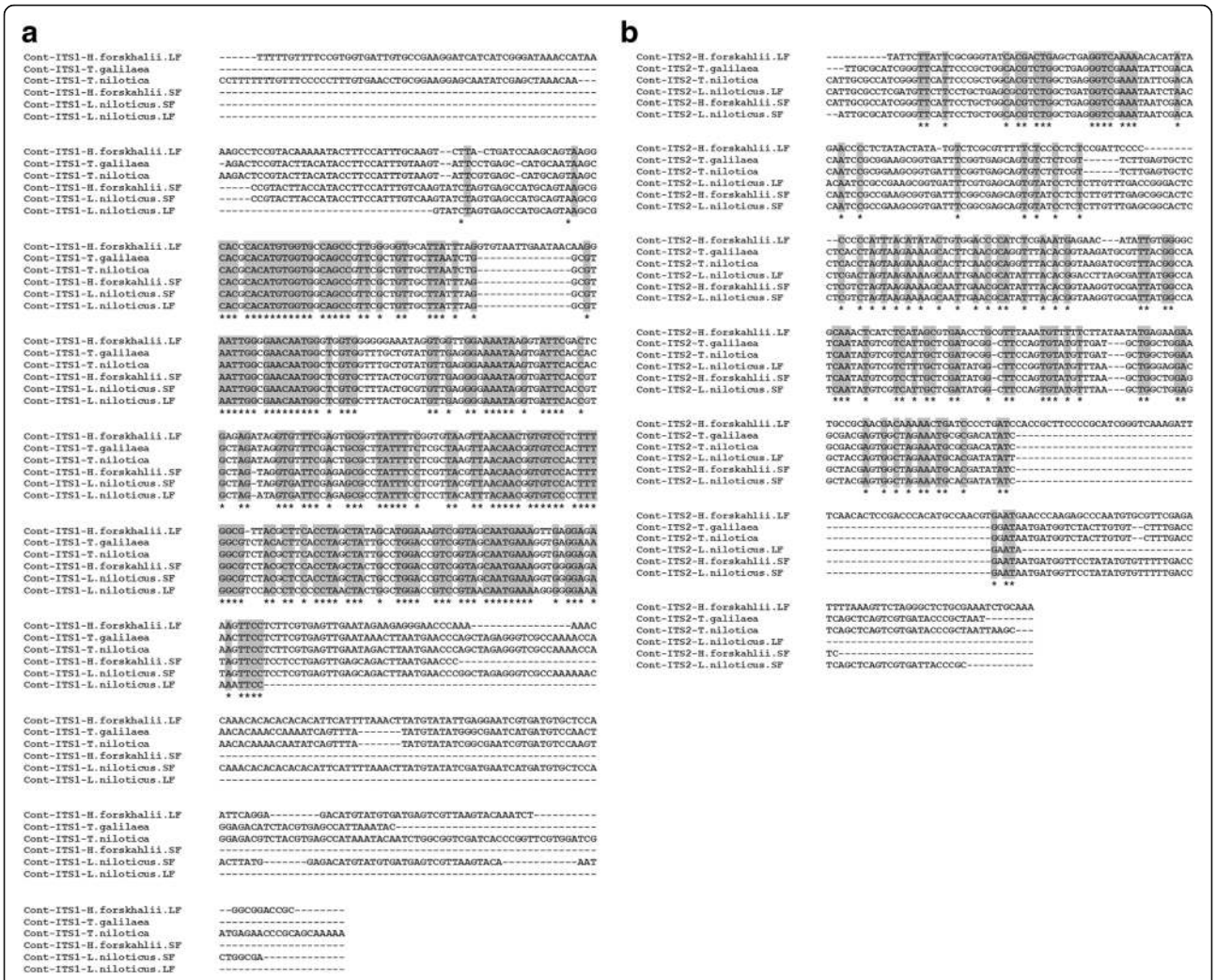

Fig. 6 Nucleotide sequences alignment: **a** ITS-1 region alignment of *Contracaecum* larvae (sequences were deposited to the GenBank database under the accession numbers (KX580602-KX580607). **b** ITS-2 region alignment of *Contracaecum* larvae (sequences were deposited to the GenBank database under accession numbers (KX580607- KX5806013) .Samples are labeled according to host fish. Symbols: identical (asterisks; gray highlighted), no nucleotide (dashes)

migratory birds. It may also be owing to the increasing numbers of intermediate and paratenic hosts and the change of the habitat of these hosts. The reports of Skov, Kania, Olsen, Lauridsen, and Buchmann (2009) and Mathenge (2010) proved that the infection in wild fish was higher than farmed fish due to the high abundance of definitive piscivorous birds in the wild regions; also, the prevalence and intensity in Catfish were higher than in *Tilapia*; this may be according to the feeding habits of *Tilapia*, which is herbivores and is less likely to get infected directly, while Catfish are omnivorous that will feed on all intermediate hosts (Malvestuto & Ogambo-Ongoma, 1978). These are compatible with the present study in that the highest prevalence and intensity were recorded in *L. niloticus* which represented 100%, followed by *H. forskahlii*, which have a prevalence of 82%, and the lowest were in *O. niloticus and T. galilaea* that recorded prevalence of 35.6 and 0.14% respectively.

Human anisakidosis (previously known as anisakiasis) is a disease that became of major health and economic importance. Humans were considered accidental hosts in the life cycle, as a result of consumption of raw fish, and these nematodes never develop inside the alimentary canal of human and may penetrate the tract and associated organs with severe pathological consequences. This disease was recorded in Egypt by Cocheton, Cabout, and Lecomte (1991). Early evisceration is recommended to avoid anisakidosis disease, due to the movement of the larvae from the digestive tract into muscles within a few days (Declerck, 1988). Also, freezing at – 20 °C for 3 days or heating to 70 °C because the larvae can survive at 50 °C. Sakanari and McKerrow

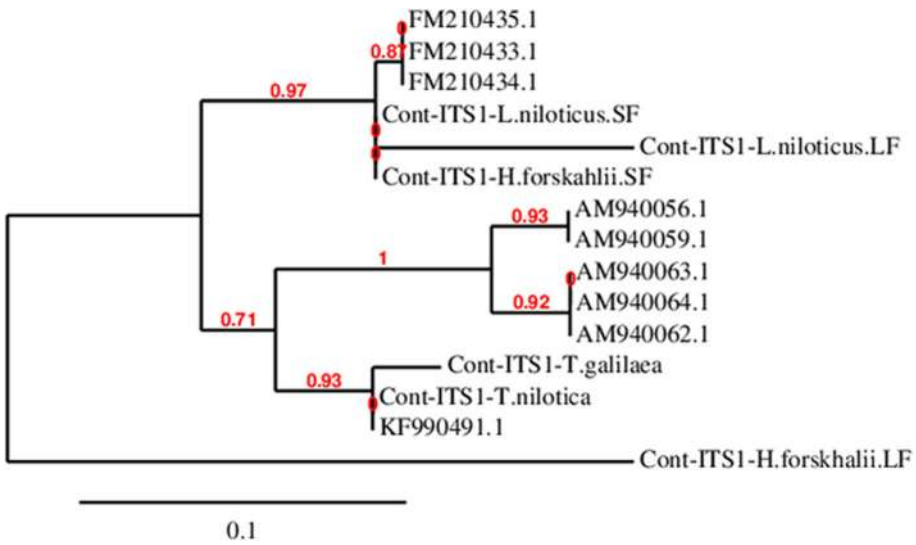

Fig. 7 Phylogenetic tree of the aligned 15 ITS-1 sequences from *Contracaecum* larvae including 6 ITS-1 sequences, identified in this study, using the Phylogeny.fr program (http://www.phylogeny.fr/). Bootstrap support values are shown on the branches. The compared sequences are represented by accession numbers

(1989) indicated that larvae resist salting, smoking, and 51 days in vinegar. According to the US Food and Drugs Administration (FDA) agency, all fish products not intended for cooking or processing at temperature > 60 °C should be deep frozen at − 35 °C for > 15 h, or at − 23 °C for a minimum period of 7 days (Deardorff, Kayes, & Fukumura, 1991). Interestingly, *C. magnipapillatum* was redescribed from cormorants in Wadi Al-Raiyan lake area, Fayoum, Egypt, by Al-Bassel, 2006. In the present study, *Hydrocynus forskahlii* was the most Egyptian traditional salted fish (local name is Kalb-El Samak). This fish was exposed to direct sun in open air for about 24 h. The larger sized fish were gutted before being salted while small fishes were salted as a whole. *Contracaecum* larvae infected this fish by a prevalence of 82% and this may affect the human health in Egypt.

The SSCP analysis of ITS-1 and ITS-2 revealed five possible profiles for each. The sequence and phylogenetic analyses showed that the nematode larvae investigated in this study are closely related to *C. multipapillatum,* which is a species complex and it was shown that it consists of at least 4 distinct species found in the USA, Europe, and Australia (Nadler, D'Amelio, Fagerholm, Berland, & Paggi, 2000; Mattiucci, Turchetto, Bragantini, & Nascetti, 2002; D'Amelio et al., 2007; Shamsi et al., 2008).

Conclusion

It seems that *Contracaecum* larvae in Lake Nasser are belonging to *C. multipapillatum* complex and differ from each other in the sub-species level, with the possibility of the presence of at least one new species. It is necessary to complete the life cycles of different larvae of *Contracaecum* in the laboratory (running experiments) and examine adult worms using detailed morphological examination and molecular biology with complete ITS sequences and additional genetic markers.

Abbreviations

a.: Anus; B.T: Boring tooth; Cut.r.: Cuticle ridges; DL: Dorsal lip; ex.p.: Excretory pore; Int. c.: Intestinal caecum; ITS: Internal transcribed spacer; LF: Long form; m.o.: Mouth opening; Oes.: Esophagus; p.: Papillae; r.: Rectum; SF: Short form; t.: Tail; t.p.: Tapered process; v.: Ventriculus; v.a.: Ventricular appendages; VL: Ventral lip

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Authors' contributions

This work is part of the doctoral thesis of JR. She has done most of the experiments. AY is assistant supervisor who has done the practical part of molecular biology, data analyses and subsequent interpretations. AS is the main supervisor who prepared the research plan and contributed to the explanation of the morphological part. All coauthors contributed in the writing and revision of this manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Author details
[1]Zoology Department, Faculty of Science, Aswan University, Aswan 81528, Egypt. [2]Fishery Management Center, High Dam Lake Development Authority, Aswan, Egypt.

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