Benha Veterinary Medical Journal 46 (2024) 102-107

Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

Since 1990

Original Paper

Molecular characterization of *Glugea anomala* infecting Nile tilapia, *Oreochromis niloticus* and African Catfish, *Clarias gariepinus* and its associated pathological changes

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ARTICLE INFO

Keywords

African Catfish
Glugea anomala
Histopathology
Nile tilapia
18S ribosomal DNA

Received 03/02/2024
Accepted 14/03 /2024
Available On-Line 01/04/2024

ABSTRACT

The present study aimed to molecularly diagnose microsporidia in Nile tilapia and African catfish at the Qalyubia Governorate in Egypt and describe the histopathological alterations that are related to them. Seventy-nine out of 400 (19.75%) Nile tilapia and forty-eight out of 200 (24%) African catfish had white nodules in their liver and kidneys were collected from natural water resources. BLAST analysis showed that 18S rDNA of isolated *Glugea anomala* from Nile tilapia (acc. no. OR766288) and from African catfish (acc. no. OR766287) revealed 100% sequence similarity with *Glugea anomala* (AF044391, AB923879) and *Glugea arabica* (KT005391). The highest seasonal infection rates with *Glugea anomala* in Nile tilapia and African catfish were recorded in autumn (32%) and spring (36%) respectively. Histopathological examination revealed the presence of microsporidian spores in the cytoplasm of degenerated and necrotic hepatocytes and renal tubular epithelium. In conclusion, the study recorded *Glugea anomala* in naturally infected Nile tilapia and African catfish that negatively alter the tissue and hence fish health status.

1. INTRODUCTION

Nile tilapia (*Oreochromis niloticus*) (Linnaeus, 1758) is a freshwater species naturally distributed in the Nile River and its branches. In Egypt, Nile tilapia is the most important cultured fish species accounting for 80% of the total production and ranked third among the top global Nile tilapia producers in the world (El-sayed and Fitzsimmons, 2023). African catfish (*Clarias gariepinus*) (Burchell 1822) is a valuable freshwater species belonging to the family Clariidae. It is a widely distributed polyculture species in many African countries including Egypt, due to their fast growth rate and tolerance to poor water quality as well as cultured with Nile tilapia to control its overpopulation (Kaleem and Sabi, 2021). However, freshwater fishes are greatly affected by various parasites, which results in high fish mortality and reduced natural productivity resulting in a negative influence on the economy (El Asely et al., 2015; Shinn et al., 2023) .

Microsporidia are a group of obligatory intracellular fungi that can infect a wide range of invertebrate and vertebrate (including fish) hosts (Casal et al. 2016, Simakova et al. 2018). Within this phylum, about 1,700 microsporidia species have been described infecting a diverse range of hosts worldwide (Tournayre et al., 2024). About 220 Microsporidia genera are known to infect aquatic organisms, among which more than 160 species falling within 22 genera are known to infect fishes (Liu et al., 2019). Some of the most economically important microsporidia species that infect fish are Glugea, Loma, Pleistophora, Enterocytozoon and Nosema (Picard-Sánchez et al., 2021; Weng et al., 2023) .

The diagnosis of microsporidia dependent on the small subunit (SSU) rDNA sequencing molecular phylogeny beside spore morphological characters is becoming important for the classification and characterization of the microsporidia (Abdel-Baki et al., 2015 and Mansour et al., 2020). In addition, the histopathological imprints of microsporidian infection are significant in disease diagnosis, fish microsporidia are embedded in the cytoplasm of the host cell leading to cell hypertrophy and then forming xenoma which contain spores and other developmental stages of microsporidia (Lom and Nilsen, 2003).

In Egypt, microsporidian infection has been recorded among marine and freshwater fishes (Abdel-Ghaffar et al., 2009; Abd Rabo, 2017). The ongoing study was designed to survey, molecular diagnosis of microsporidia species affecting Nile tilapia and African Catfish inhabitants in natural watersheds (El-Riah El-Tawfiki, Qalyubia Governorate, Egypt) and describe histopathological changes associated with these species of microsporidia.

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2 . MATERIAL AND METHODS

2.1 .Samples collection

Four hundred Nile tilapia (100 fish/season) and two hundred African catfish (50 fish/ season) were collected from El-Riah El-Tawfiki and its branches at Qalyubia Governorate, Egypt during the period from December 2020 to September 2021. Fishes were transported to the diagnostic Lab of Aquatic Animal Medicine, Faculty of Veterinary Medicine, Benha University, Egypt for clinical and parasitological examination .

This research was conducted according to the guidelines of the Scientific Research Ethics Committee of the Faculty of Veterinary Medicine, Benha University, Egypt (BUFVTM: 13-10-22).

2.2 .Clinical and postmortem inspection

The collected fishes were examined for the presence of external nodules (skin and underlying musculature, gills, and dendritic organ) and internal (liver, spleen, kidneys, intestine, heart, and gonads) according to Noga (2010). The total and seasonal prevalence of isolated microsporidia species were calculated according to Margolis et al.(1982) .

2.3 .Microscopical examination

Squash preparation was performed from nodules observed in the kidney and liver and carefully compressed between two clean glass slides with a drop of physiological saline. The positive slides were then allowed to dry at room temperature before being fixed by methanol and stained by Geimsa stain according to Abd Rabo (2017). Tissue specimens containing nodules from the kidneys and liver of Nile tilapia and African catfish were incised and fixed in 10% neutral buffered formalin for histopathological diagnosis. In addition, another sample from infected tissue was preserved at -80oC for molecular analysis .

2.4 .Pathological investigations

Specimens from the infected fish tissues (kidney and liver) were collected and fixed in 10% neutral buffered formalin. These specimens were dehydrated using a series of graded ethyl alcohols cleared in xylol then embedded in paraffin wax. Using a rotatory microtome, tissue paraffin sections of 5 µm thicknesses were cut and then stained with Meyer's hematoxylin and eosin according to Bancroft and Gamble (2007). In addition, special staining techniques using Periodic acid-Schiff (PAS) stain was carried out to demonstration and identification the variety of the spore-forming parasites (Bruno et al., 2006). These sections were examined under a light microscope (Nikon, Eclipse, E800), and representative microphotographs were captured with an Olympus DP 72 digital camera.

2.5 .Molecular characterization, sequencing, and BLAST analysis

The genomic DNA from frozen infected kidney and liver tissues was extracted after following the manufacturer's instructions of the GF-1® Tissue DNA extraction Kit (Vivantis, Malaysia). The quality and concentration of the extracted DNA were measured with a Nano Drop™ ND-1000 Spectrophotometer (Thermo Scientific, Germany). A set of primers 18F (CACCAGGTTGATTCTGCC) and 1492R (GGTTACCTTGTTACGACTT) was selected to allow amplification of the 18S ribosomal DNA (18S rDNA) gene from the retrieved Microsporidia spp according to Vossbrinck and Andreadis (2007). A 25µl reaction mixture comprising 12.5 µl of 2x MyTaq™ Red Mix (Cat.no. BIO-25043, Meridian Life Science Inc., USA), 0.5 µl of each primer (10 Mmol), and 2 µl of target DNA were used for PCR reaction. The amplification reactions were carried out for 35 cycles after an initial denaturation for 4 min at 94 °C. Each PCR cycle comprised three steps: denaturation at 94 °C for 30s, annealing at 50 °C for 45s, and extension at 72 °C for 80 s with a final extension of 7 min at 72 °C then rested at 4 °C. Gel electrophoresis was performed with an expected product of 1300bp.

The Gene JET Gel Extraction Kit (K0691, Thermo Fisher, USA) was used to clean two of the positive PCR products targeting 18S rDNA genes from Glugea spp in Nile tilapia and African catfish. The sequencing was performed in Macrogen Company (Korea). Two-way sequencing using the specific primers used in PCR served as a confirmation of the data's accuracy. The programs Bioedit 7.0.4.1 and MUSCLE were used to examine the nucleotide sequences acquired in this work. Using a neighbour-joining technique of the aligned sequences implemented in the application CLC 6, the obtained sequences were aligned with reference sequences of 18S rDNA genes of Microsporidia spp (Table 1) .

Table. 1. *Microsporidia Spp*. 18 S rDNA gene sequences from GenBank used for phylogenetic tree construction.

Species	Host	Site of infection	Country	Accession. no
Glugea anomala	*Oreochromis niloticus*	Liver	Egypt	OR766288 (our study)
Glugea anomala	*Clarias gariepinus*	Kidney	Egypt	OR766287 (our study)
Nucleospora braziliensis	*Oreochromis niloticus*	Gill, gut, heart, kidney, liver, muscle, spleen, and stomach	Brazil	MW491352
Nucleospora sp.	*Oreochromis niloticus*	Gill, gut, heart, kidney, liver, muscle, spleen, and stomach	Brazil	KT777455
Pleistophora sp.2	*Zeugopterus punctatus*	Skeletal muscles	Norway	AF044389
Pleistophora sp.3	*Taurulus bubalis*	Skeletal muscles	Norway	AF044390
Glugea anomala	*Gasterosteus aculeatus*	Migratory mesenchyma cells	Norway	AF044391
Loma sp.	*Encelyopus cimbrius*	……..	……..	AF104081
Pleistophora mirandellae	*Rutilus rutilus*	……	…….	AF104085
Pleistophora typicalis	*Myoxocephalus scorpius*	muscles	Norway	AF044387
Spraguea lophii	*Lophius piscatorius*	……	……..	AF104086
Heterosporis lessepsianus	*Saurida lessepsianus*	Abdominal cavity, skeletal muscle and mesenteric tissues	Egypt	MF769371
Microsporidia sp.	*Saurida undosquamis*	abdominal cavity, mesenteric tissues	Persian Gulf (Iran)	MT192526
Pleistophora pagri	*Pagrus pagrus*	Epithelial cell and peritoneal cavity	Egypt	JF797622
Glugea anomala	*Gasterosteus aculeatus*	…… .	France	AF056016
Microsporidium aurata	*Sparus aurata*	muscles, connective tissues, and the intestinal epithelium	Egypt	KF022044
Glugea arabica	*Epinephelus polyphekadion*	Intestinal wall	Saudi Arabia	KT005391
Glugea anomala	…… ...	……	India	AB923879

3 . RESULTS

3.1 .Gross lesions

The most common gross lesions were detected only in the liver and kidney of infected Nile tilapia and African catfish such as enlarged congested liver and kidneys with the presence of macroscopic whitish nodules of variable size (Fig 1). Some cases recorded an enlarged pale liver with a distended abdomen.

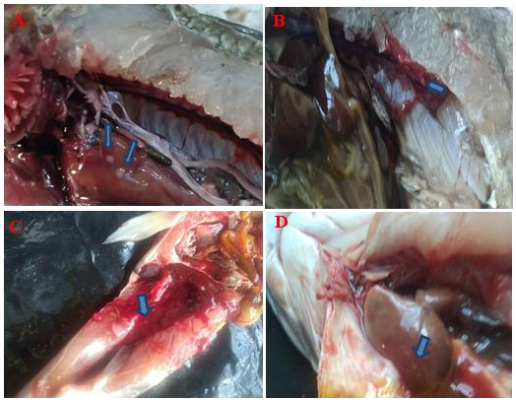

Fig 1: Nile tilapia infected with *G. anomale.* showing whitish nodules in (**A**) liver, and (**B**) kidneys. (**C-D):** African catfish infected with *G. anomale* showing whitish nodules in the kidneys (**C**), whitish nodules and enlargement of pale liver (**D**).

3.2 .Microscopical examination

The squash preparation from infected liver and kidneys of Nile tilapia and African catfish showed multi stages of *G. anomala* division (sporophorous, uninucleated, multinucleated, sporoblast and mature form) (Fig 2).

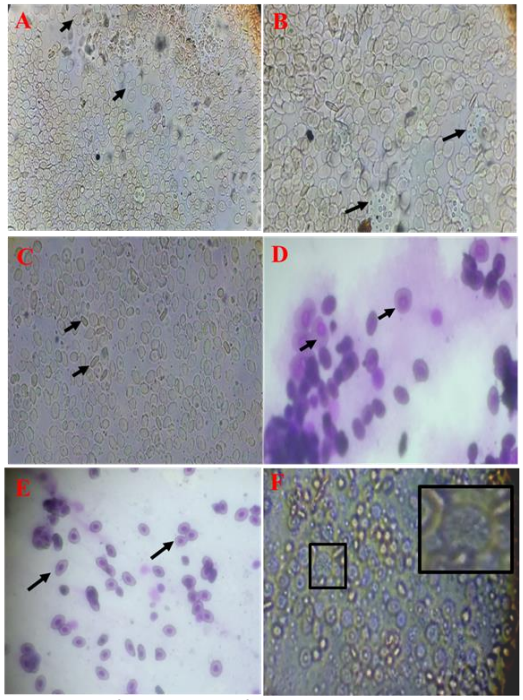

Fig 2: Wet mount from liver nodules of Nile tilapia. (**A**) *G. anomala* spores with a polar tube (arrows) (X40); (**B**) Multinucleate merogonal plasmodium (arrows) (X40); (**C**) Rod-shaped mature spore (arrows) (X40); (**D-E**) Giemsa-stained smear from kidney nodules showing large number of enlarged uninucleated meront (arrows) (X40); (**F**) sporophorous vesicules of *G. anomala* (X40).

3.3 .Total and seasonal prevalence

The total prevalence of *G. anomala* in Nile tilapia was 19.75 % (79/400) with the highest seasonal infection rate in autumn (32%), followed by winter (20%), summer (18%) and spring (9%) (Fig 3). African catfish showed a total infection rate of 24% (48/200) with the highest seasonal prevalence in spring (36%), then autumn (22%), summer (20%), and winter (18%) (Fig 4).

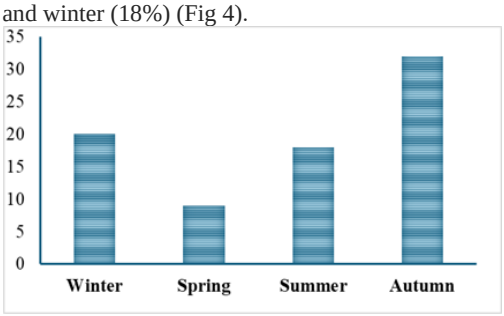

Fig 3. Seasonal prevalence of *G. anomala* in Nile tilapia

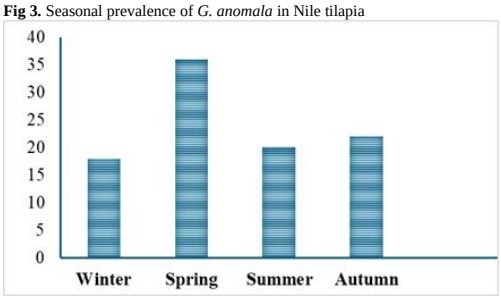

Fig 4. Seasonal prevalence of *G. anomala* in African catfish**.**

3.4 .Histopathological alterations

The histopathological examination of the liver sections of African catfish revealed aggregation of numerous PAS-positive, pink to magenta spherical or oval spores in the cytoplasm of hepatocytes (Fig. 5A). Severe fatty degeneration and necrosis of the hepatocytes were prevalent, particularly in the vicinity (Fig. 5B). Most infected hepatocytes had pyknotic, karyorrhectic, or karyolitic nuclei. An inflammatory cellular reaction was prominent, as seen by the aggregation of eosinophilic granular cells, lymphocytes, and macrophages near the infected hepatic cells with the parasitic spores (Fig. 5C). Many spores markedly distended some of these macrophages. In addition, some of these microsporidian spores were found in the infected cells within blood vessels. Furthermore, these microsporidia spores were also seen within the pancreatic acini in Nile tilapia (Fig.5D) associated with congestion of central veins and hepatoportal veins with bile duct hyperplasia (Fig. 5E) .

The microscopic examination of the kidney sections in the African catfish and Nile tilapia showed the presence of microsporidia spores inside the cytoplasm of the renal tubular epithelium with widespread renal haemorrhages as well as tubular epithelial degeneration and necrosis (Fig. 5F). Renal tubular epithelial apoptosis was also frequently observed (Fig.5G). Furthermore, melanomacrophage centre activation and severe renal congestion, with glomerular haemorrhage were occasionally seen (Fig. 5H) .

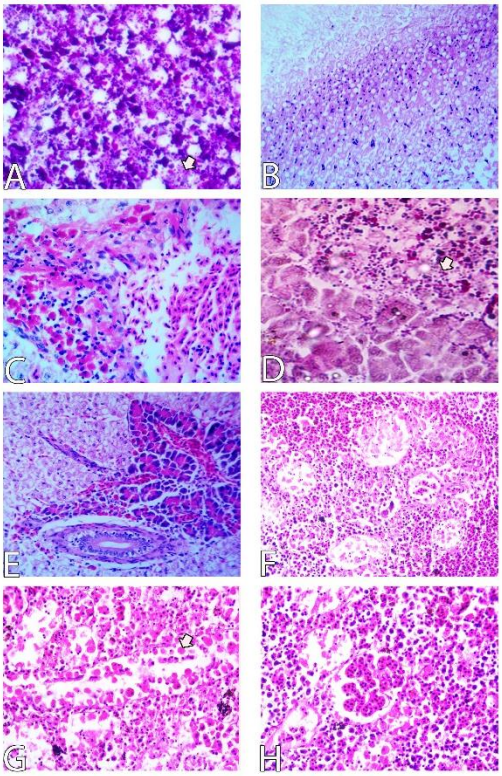

Fig 5: Photomicrograph of liver sections of African catfish infected with *G. anomala* (A-C) A- PAS-positive tiny, spherical to ellipsoidal spores within the cytoplasm of hepatocytes (arrow) PAS stainX400; B-extensive necrosis and degeneration of hepatocytes X200 (C) congestion of central vein with perivascular eosinophilic granular cells aggregation X400). Liver sections of Nile tilapia infected with *G. anomala* (D-E) D-PAS-positive small, spherical to oval spores within the cytoplasm of pancreatic acini PAS stain X400; E-congestion of hepatoportal vein and bile ductal hyperplasia H&E stain X200. of kidney sections of Nile tilapia infected with *G. anomala* (F-H) F-interstitial haemorrhage and tubular epithelial necrosis H&E stain X200; G- Renal tubular apoptosis (arrow) H&E stain X200; H- Glomerular haemorrhage. H&E stain X200.

3.5 .Molecular and phylogenetic analysis

PCR amplification of the 18S rDNA for Glugea spp isolated from the liver of Nile tilapia and the kidney of African catfish revealed 1600bp (Fig 6). The assembled sequence of the 18S rDNA fragment from the isolated *G. anomala* from Nile tilapia and African catfish in the current study were deposited in the GenBank under the accession number OR766288 and OR766287 respectively. The percentage of similarity between these sequences and other microsporidia isolates from other hosts demonstrated 100% identity to the sequence of *G. anomala* (AF044391 and AB923879) and 100% similarity to that of G. arabica (KT005391) that were isolated from intestinal wall samples of Epinephelus polyphekadion in Saudi Arabia. The derived phylogenetic tree of the 18S rDNA gene of *G. anomala* exhibited strong nodal support for one major lineage (Fig 7) .

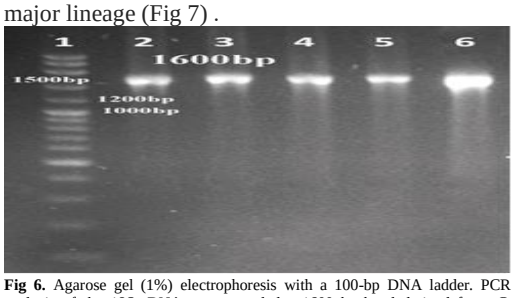

Fig 6. Agarose gel (1%) electrophoresis with a 100-bp DNA ladder. PCR analysis of the 18S rDNA gene revealed a 1600 bp band derived from *G. anomala*.

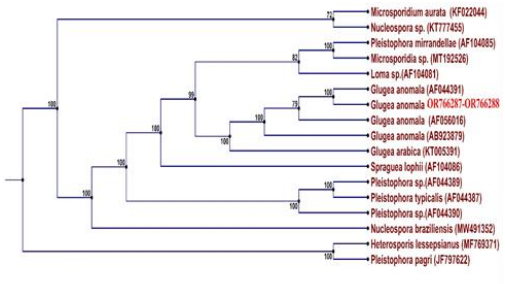

Fig 7. Phylogenetic tree constructed based on the 18S rDNA sequences of *G. anomala* (OR766288 and OR766287) and its closest *Microsporidia spp.* using the neighbor-joining method.

4 . DISCUSSION

Infection of aquatic animals with microsporidia has received great attention due to their effect on fish productivity as well as it has zoonotic importance (Chen et al., 2023; Moratal et al., 2023). The examined Nile tilapia and African catfish infected with *G. anomala* in the current study revealed the presence of whitish nodules of variable size in the liver and kidneys. Similarly, lesions caused by Microsporidia spp were observed in African catfish (Abd Rabo., 2017; Abd El-Lateif and Torra., 2020) and Nile tilapia (Rodrigues et al., 2017; Nur et al., 2020) .

In the present study, microscopic findings of *G. anomala* from the liver and kidneys revealed different developmental stages (merogonal plasmodia, sporonts, sporoblasts, and mature spores). Abd El-Lateif and Torra (2020) revealed the presence of mature ovoid to pyriform Microsporidia spp spores as well as Abd Rabo (2017) observed different division stages of Glugea sp including sporophorous vesicle, uninucleated meront, and mature egg-shape spores in kidneys of naturally infected African catfish .

The total prevalence of *G. anomala* in the current study was 19.75% and 24% in Nile tilapia and African catfish respectively. This result was nearly similar to the findings reported by Abdel Mawla and Mohamed (2010); and Abd El-Lateif and Torra (2020). However, our findings were higher than that of Abd Rabo (2017) who reported a total infection rate with Glugea spp (7%) in African catfish, and lower than that reported by Peyghan et al. (2009) who observed total infection (44%) in lizardfish, Saurida undosquamis. This difference could be due to the difference in fish species, water quality, fish immunity, fish size, and intensity of parasites.

The highest seasonal infection rate among the examined Nile tilapia in this study was in autumn (32%), and the lowest infection rate (9%) was in spring. In this respect, Shehab El-Din (2008) showed that the highest seasonal prevalence of microsporidia infection in Hack fish was in autumn. In our study, the highest seasonal infection rate of African catfish with *G. anomala* was 36% in spring, and lowest in winter (18%). This result comes in agreement with Abd Rabo (2017) who recorded Glugea spp in African catfish during the spring season at an infection rate of 28%. Moreover, Abd El-Lateif and Torra (2020) reported that the low infection rate in African catfish infected with Microsporidia spp was in winter (6%). Our finding could be attributed to the

water temperature which is considered an important factor in development of microsporidia in different species of fish (Sveen et al., 2012). Additionally, it could explain the increase in the microsporidian infection rate in spring that fish may be eat excess infected zooplankton (Marzouk et al., 2010).

Histopathological studies are one of principal tools for the diagnosis of microsporidiosis (Sanders et al., 2020). In the current study, histopathological examination of infected liver and kidneys with *G. anomala* in Nile tilapia and African catfish showed aggregation of numerous PAS-positive, pink to magenta spherical or oval spores in the cytoplasm of hepatocytes and pancreatic acini as well as the renal tubular epithelium with severe hepatorenal damage. Also, many of the spores were observed in the cytoplasm of macrophages and infected leucocytes within blood vessels. The infected macrophage could be responsible for spreading the infection within the host. Winters et al., (2016) and Abd Rabo (2017) observed nearly similar microscopic changes in which xenomas lodged in tissues were surrounded by mononuclear leucocytes and tissue degeneration. Severe inflammation and myocyte rupture in skeletal muscles with proliferative phases of spores were recorded (Phelps et al., 2015; Abd El-Lateif and Torra, 2020).

In the present study, molecular analysis of 18S rDNA gene sequences of *G. anomala* isolated from the liver of Nile tilapia and kidneys of African catfish revealed 1600bp in length and deposited in the GenBank under the accession number OR766288 and OR766287 respectively. Azevedo et al. (2016) recorded that PCR amplification SSU rRNA gene sequence of *Glugea arabica* isolated from camouflage grouper Epinephelus polyphekadion resulted in 1763 bp in length. The blast analysis of the isolated *G. anomala* in our study exhibited 100% sequence similarity to that of *G. anomala* (AF044391), *G. anomala* (AB923879) and G. arabica (KT005391) .

5 .CONCLUSIONS

The current study revealed that the high infection rate with *G. anomala* was during autumn in Nile tilapia and in spring in African catfish. Additionally, severe pathological changes were recorded in infected tissue with Glugea spp. Based on phylogenetic analysis of the 18S rDNA sequence gene of *G. anomala* showed high similarity with *G. anomala* and G. arabica.

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