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ANISAKIASIS OF *PSEUDOTOLITHUS ELONGATUS* (BOBO CROAKER) AND *BOSTRYCHUS AFRICANUS* (SLEEPER GOBY) BY MOLECULAR IDENTIFICATION TECHNIQUES, IN NIGERIA

By

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Abstract

Anisakis species species are identified by using molecular biology techniques due to the absence larval diagnostic features in the fish host. This study identified anisakids by molecular techniques in *Pseudotolithus elongatus* and *Bostrychus africanus* caught from Rivers State. Parasite identification was accomplished at the Regional Center for Biotechnology and Bioresource Center, University of Port Harcourt, Nigeria. NCBI blast confirmed *Anisakis simplex, Anisakis brevispiculata* from *Pseudotolithus elongatus* and *Goezia spinulosa*, and *Raphidascaroides brasiliensis* from *B. africanus*.

Key words: Nigeria, Abonnema, Rivers State, Fish, Anisakids, Molecular identification.

Introduction

Anisakidosis is the commonest fish-borne nematodiasis with about 20,000 reported cases worldwide of which >90% were from Japan (Eiras *et al*, 2018), acquired by consmption of raw and/or under-cooked fish (Shamsi *et al*, 2023). Ogbeibu *et al*. (2014) reported *Ichthyanisakis* sp., *Pseudanisakis* sp., *Contracaecum* sp., and *Terranova* sp. from *Pseudotolithus elongatus* in Buguma Creek in Nigeria's Niger Delta. Odum *et al*. (2021) identified Anisakid larvae from *Caranx hippos*, and *Sardinella maderensis* from Okrika, Rivers State, and Bamidele (2021) identified *Raphi dascaroides* sp. from *Rhinogobius ocellatus* from Lekki Lagoon, based on the larval morphological diagnosis.

Aibinu *et al*. (2019) in Africa listed the fish-borne *Anisakis* spp. as *Merluccius merluccius, Trachurus trachurus, Boops boops, Scorpaena porcus,* and *S. japonicas* from Algeria, Egypt, Libya, Mauritania, Morocco and Tunisia.

The study aimed to evaluate *Anisakis* species infecting edible fish, *Pseudotolithus elongatus* and *Bostrychus africanus* caught from Abonnema, Rivers State, Nigeria.

Materials and Methods

Study area and fish collection: *Pseudotolithus elongatus* and *Bostrychus africanus* were harvested from Abonnema River, Rivers State in the Niger Delta of Nigeria. The-location contained brackish water and man grove vegetation, and connected to the Sombriero River, at 4.7231°N & 6.7788°E (Ideriah *et al*, 2012). Abonnema is a fishing area, and fishing is the main occupation identified after Sentongo *et al*. (1986).

Fish examination: Sixty samples each of *Pseudotolithus elongates* and *Bostrychus africanus* were collected from May and October, 2022 and immediately transported to the Entomology and Parasitology Laboratory. Each fish was dissected out by an incision via the anal pore to expose the gastrointestinal organs, which were excised into separate Petri-dishes with 0.9% saline solution. A longitudinal cut through each organ was made to expose any detached parasites. They were examined microscopically for endo-parasites, which were removed by Pasteur pipettes and fixed in absolute ethanol. Before fixation, each parasite was placed on a clean slide with few drops of normal saline solution, covered with a suitable cover slip, and examined. The recovered parasites were carefully submitted to the Regional Center for Biotechnology and Bioresource Center, University of Port Harcourt, for molecular identification.

DNA was extracted from the parasites by using the ZR Fecal DNA MiniPrep™ Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. NGS

primers were designed to generate DNA reference libraries for each genetic locus used and libraries were prepared for sequencing on the Illumined platform. Contamination was avoided, and both the DNA extraction and initial library preparation steps were completed in a laboratory designated for the pre-PCR protocols conducted on low copy, and highly degraded DNA. Filtered barrier tips were used, and plastics and reagents (if appropriate) were all UV-treated prior the use. DNA extracts were amplified by PCR in triplicates for the target locus (28S rDNA). The positive and negative controls were included side by side for the locus.

PCR primers: The primer sets 28S_F_Ces (GAGTAAACAGTACGTGAAGC), & 28S _R_Ces (CCACCGGTCGTGGTGTTC) targ eting the 28S rDNA gene was used based on the high helminthic yielded (Greiman *et al*, 2018).

Agarose gel electrophoresis: After PCR reaction, its products were separated on a 1.5 % agarose gel, and one hundred base pair (100bp) DNA ladder (Solis Biodyne), and used as the DNA molecular weight marker. Electrophoresis was done at 80 V for one hour, and gel was viewed under UV light after ethidium bromide staining, and determined the nucleotide order. Sequences generated by the sequencer was visualized using Bioformatic Algorithms such as Chromaslite for base calling. Bio-Edit was used for the sequence editing, before performing a Basic Local Alignment Search Tool) using NCBI (National Centre for Biotechnology Information) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Similar sequences were downloaded and aligned with ClusterW and phylogenetic tree drawn with the MEGA 6 software.

Phylogenetic tree: The evolutionary history was inferred using Neighbor-Joining method (Saitou *et al*, 1987). Replicate trees percentage associated with taxa clustered together in the bootstrap test (1500) was shown next to the branches (Felsenstein, 1985).

Tree was drawn to scale, with branch lengths in same units as the evolutionary distances used to infer the phylogenetic tree computed using the Jukes-Cantor method and were in the units of the number of base substitutions per site. Analysis involved ten nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*, 2013).

Ethical approval: This study was approved by the Academic Board, Department of Animal and Environmental Biology, Rivers State University, Port Harcourt, Nigeria.

Statistical analysis: Data were computerized and statistically analyzed (Bush *et al*, 1997).

Results

Fish; *Pseudotolithus elongatus* and *Bostrychus africanus* showed (total length & wet body weight of 23.94cm±1.67x117.6g ±25.8 and 111.85cm±0.76x19.42g±4.15), respectively. Molecular identification identified three nematodes from *Pseudotolithus elongatus* and one from *B. africanus*.

Genomic DNA quantification by the nanoDrop spectrophotometer, and agarose gel electrophoresis showed that DNAs extracted from the nematodes were pure, with an index ranged between 1.79 & 1.88 with concentration between 97.4 & 391.1ng/µl. *Cox*1 primer was efficient in amplifying the cytochrome c oxidase subunit I (*cox1*) gene of interest. Six isolates showed amplification with amplicon size of about 700bp.

Sequences of *P. elongatus* and *B. Africanus* were deposited at GenBank with accession numbers: OQ509666-OQ509671. *Anisakis simplex* six isolates were RCBBR _15, and RCBBR_16, *A, brevispiculata* isolate RCBBR_17, & RCBBR_18, *Goezia spinulosa* RCBBR_19 was isolated from *P. elongates*, & *R. brasiliensis* RCBBR _20 was isolated from *B. africanus*.

The details were given in tables (1, 2 & 3), figures (1, 2, 3, 4, 5, & 6) and plates (1 & 2).

Table 1: Genomic DNA quantification result using NanoDrop spectrophotometer

Lab. Isolate	DNA Conc. (ng/µl)	Absorbance (nm)		
		260	280	260/280
RCBBR_N15	97.4	1.948	1.047	1.86
RCBBR_N16	115.6	2.311	1.234	1.88
RCBBR_N17	135.1	2.703	1.453	1.86
RCBBR_N18	391.1	5.821	3.130	1.86
RCBBR_N19	371.7	5.434	3.036	1.79
RCBBR_N20	346.6	4.184	2.286	1.83

Table 2: GenBank characteristics of the partially sequenced *cox1* genes of the isolates

SN	Closest GenBank Match	Accession No	LAB	Strain	%similarity
1	*Anisakis simplex* TA02-10	OQ509666	*Anisakis simplex*	RCBBR_15	99.28
2	*Anisakis simplex* isolate Jumunjin	OQ509667	*Anisakis simplex*	RCBBR_16	99.28
3	*Anisakis brevispiculata* isolate 7	OQ509668	*Anisakis brevispiculata*	RCBBR_17	99.55
4	*Anisakis brevispiculata* isolate 3	OQ509669	*Anisakis brevispiculata*	RCBBR_18	100
5	*Goezia spinulosa* isolate E472	OQ509670	*Goezia spinulosa*	RCBBR_19	100
6	*Raphidascaroides brasiliensis* PAX11	OQ509671	*Raphidascaroides brasiliensis*	RCBBR_20	100

Table 3: Parasitic infection in *Pseudotolithus elongatus* and *Bostrychus africanus*, Rivers State (n=60)

Parasite	*Pseudotolithus elongatus*		*Bostrychus africanus*	
	Infected hosts	Percentage	Infected hosts	Percentage
Anisakis simplex	3/60	5.0%	-	-
Anisakis brevispiculata	8/60	13.3%	-	-
Goezia spinulosa	2/60	3.3%	-	-
Rhaphidascaroides brasiliensis	-	-	44/60	73.3%
Total	13/60	21.7	44/60	73.3

Discussion

Generally speaking, *Anisakis* species, *Contracaecum* and *Pseudoterranova* spp., cause severe gastrointestinal anisakidoses disturbance, if accidentally consumed by humans (Nieuwenhunzien *et al*, 2013). Villazanakretzer *et al*. (2016) reported abdominal pain, nausea, and vomiting as the most common symptoms of the infection. Marine mammals are their definitive hosts, and several species of fish and crustaceans served as intermediate hosts (Audicana *et al*, 2008), as well as fresh water snails (Abo-Madyan *et al*, 2003). The main definitive host of *Anisakis brevispiculata* was sperm whale; *Kogia breviceps* (Mattiuci *et al*, 2001). Together with *A. physeteris* and *A. paggiae*, *A. brevispiculata* is in Clade 3 of *Anisakis* species (Valentini *et al*, 2006). *Anisakis* species in the Clade 1 are members of *A. simplex* complex, and referred to as *A. simplex sensu lato*, which included *A. berlandi, A. pegreffii* and *A. simplex* (s.s). Clade 2 *Anisakis* species are *A. ziphidarum* and *A. nascettii* based on their genetic relatedness (Mattiuci *et al*, 2011). Besides, Mattiucci *et al*. (2018) used molecular techniques to identify the larval stages of *A. brevispiculata* from *Xiphias gladius* (the swordfish) from waters of the Central Atlantic Ocean and Tropical Equatorial Atlantic. Species was identified from fish species in Japan (Quiazon *et al*, 2011), Taiwan (Chen *et al*, 2015), and from the lantern fishes (myctophids) in the Arabian Sea (Cabrera-Gil *et al*, 2018).

In Africa, *A. brevispiculata* was reported from *Scomber japonicus*, *Trachyrincus scabrus, Sardina pilchardus, Merluccius polli, Trachurus trachurus*, and *Hoplostethus cadenati* caught from the sea coasts of Mauritania and Morocco (Kijewska *et al*, 2009). Also, Ogbeibu *et al*. (2014) reported *Ichthyanisakis* sp. and *Pseudanisakis* sp. with *Contracaecum* sp. and *Terranova* sp. from *Pseudotolithus elongatus* from Buguma Creek, Rivers State, Nigeria. Moreover, Farjallah *et al*. (2008) in North African coasts reported *A. simplex* from *Scomber scombrus* and *Merluccius merluccius*

In the present study, both *A. brevispiculata* and *A. simplex* from *Pseudotolithus elongatus* caught from Abonnema, Rivers State represented new host and geographical recorded species. *Raphidascaroides* (family Ani-

sakidae) are characterized by a thick cuticle, and mouth with three lips (Bamidele, 2021). *Raphidascaroides brasiliensis* was isolated from intestine of the African knife fish *Gymnarchus niloticus* in Lekki Lagoon caused polycyclic aromatic hydrocarbons in them (Isibor *et al*, 2020). *R. Africanus* was reported from Rivers State gobies, Nigeria (Khalil *et al*, 1988; Robert *et al*, 2022; Ezenwaka *et al* (2024). But, *R. brasiliensis* from gobiid fish hosts in Nigeria was lacking. As such, the *R. brasiliensis* in the present elicits the molecular identification need of more *Raphidascaroides* parasites from their fish hosts in Nigeria to prove their distribution.

Also, *Goezia spinulosa* is an anisakid nematode closely related to *Raphidascaroides* and is commonly isolated from the arapaima *Arapaima gigas* and other fish species that had been fed with plankton from arapaima farms (Santos *et al*, 2009; Menezes *et al*, 2011). It is high pathogenic due to its spiny body, causing ulcers in the stomach or intestine of the fish hosts (Silva *et al*, 2017). Ogbeibu *et al*. (2014) reported *Goezia sigalasi*, and *G. spinulosa* from *Pseudotolithus senegalensis* and *P. elongatus* from the Niger Delta Tidal Creek.

In the present study, *P. elongatus* prevalence was lower (21.7%) than in *B. Africanus* (73.3%). However, three *Anisakis simplex*, *Anisakis brevispiculata* and *Goezia spinulosa* were isolated from *P. elongatus*, and only one from *B. africanus*. The differences could be due to differences in the feeding habits of both fish hosts; gobiids are omnivorous (Bamidele, 2021) whereas the croaker, *P. elongatus* has crustaceans and juvenile fish as major dietary components (Akpan *et al*, 2004; Isangedighi *et al*, 2016). *P. elongatus* showed more chances of acquiring parasites for crustaceans and juvenile fish serve as intermediate and the paratenic hosts. Bamidele (2021) reported only one *Raphidascaroides* sp. from the gobiid fish, *Rhinogobius ocellatus* from Lekki Lagoon. Robert *et al*. (2022) also reported one *Raphidascaroides Africanus* from *Bostrychus africanus* obtained from two locations. This could mean that gobiid fish generally didn't harbor a large diversity of parasitic helminth species.

In the present study, *A. simplex* (5%) was in *P. elongatus*. Abattouy *et al*. (2011) in Morocco reported *A. simplex* (67.9%) from *Scomber japonicus* and 57.0% in the Mediterranean Sea. Goffredo *et al*. (2019) in Italy reported that *Anisakis* spp. ranged from 0.04 (~4%) in *Sardina pilchardus* to 0.67 (67%) in *S. japonicus*. Also, the present *A. brevispiculata* was 13.3%. Chen *et al*. (2015) in Taiwan reported 0.5% in *Scomber australasicus*, Cabrera-Gil *et al*. (2018) in Arabian Sea reported >50% of *A. brevispiculata* in myctophid samples. Ogbeibu *et al*. (2014) reported *Ichthyanisakis* sp. (0.3%), *Pseudanisakis* sp. (5.7%), *Contracaecum* sp. (0.1%), and *Terranova* sp. (0.7%). This could be due to ecological and climatic differences (Martin-Carrillo *et al*, 2022).

In the present study, *Goezia spinulosa* was 3.3%. Ogbeibu *et al* (2014) reported 0.3% & 4.2% in *Pseudotolithus elongatus* and *P. senegalensis* respectively. Silva *et al* (2017) reported 29.69% in arapaimas from the northwestern Brazil.

In the present study, *R. brasiliensis* was 73.3% in *Bostrychus africanus*. This was more or less identical to 70% by Bamidele (2021). Robert *et al*. (2022) in Nigeria recorded *R. africanus* of 6.7%, and 100%, respectively. Isibor *et al* (2020) in Nigeria reported *R. brasiliensis* from *Gymnarchus niloticus* as a new host and a new location.

No doubted, sterilization of plankton was highlighted as a control measure against the fish parasites (Santos *et al*, 2009). Abiotic factors and the presence of zoonotic parasites are also of notable importance (Kuhn *et al*, 2016). Fish and fishing are very indicated in Nigeria (Aibinu *et al*, 2019), and many national and international countries Safonova *et al*, 2021).

Conclusion

Anisakis simplex (3.3%), *A. brevispiculata* (5.0%), and *Goezia spinulosa* (13.3%) were isolated from *Pseudotolithus elongates*. *Rap-*

hidascaroides brasiliensis (73.3%) was isolated from *Bostrychus africanus* collected from Abonnema, Rivers State.

Authors' contributions: The authors equally shared in the field and practical study, wrote, reviewed the manuscript and approved its publication.

Recommendations

Molecular identification methods and bioinformatics should be included in parasitological research to aid the accurate identification of species.

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Explanation of figures

Plate 1: Gel electrophoresis image of genomic DNA from the parasitic nematodes (Lane 1= DNA ladder; Lanes 2 to 7= genomic DNAs of isolates RCBBR_N15 to RCBBR_N20, respectively). DNA ladder used 1kb.

Plate 2: Gel electrophoresis image of amplified *cox1* gene from the parasitic nematodes (Lane 1= DNA ladder; Lanes 2 to 7 = *cox1* genes of isolates RCBBR_N15 to RCBBR_N20, respectively). DNA ladder used 100bp.

Fig. 1: Neighbor-joining phylogenetic tree of isolate RCBBR_N15 (Accession Number: OQ509666).
Fig. 2: Neighbor-joining phylogenetic tree of isolate RCBBR_N16 (Accession Number: OQ509667).
Fig. 3: Neighbor-joining phylogenetic tree of isolate RCBBR_N17 (Accession Number: OQ509668).
Fig. 4: Neighbor-joining phylogenetic tree of isolate RCBBR_N18 (Accession Number: OQ509669),
Fig.5: Neighbor-joining phylogenetic tree of isolate RCBBR_N19 (Accession Number: OQ509670).
Fig. 6: Neighbor-joining phylogenetic tree of isolate RCBBR_N21 (Accession Number: OQ509671).