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A new microsporidium species, *Nucleospora braziliensis* n. Sp. infecting Nile tilapia (*Oreochromis niloticus*) from brazilian aquaculture

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Abstract
In November 2014 and March 2015, 360 *O. niloticus* were randomly sampled from six fish farms in Brazil and the organs were collected for histopathology, polymerase chain reaction, sequencing, and phylogeny tests. The histopathology revealed eosinophils, microsporidium, and inflammation in tissues of the majority of fish analyzed in this study. Microsporidia prevalence varied from 86.66% to 100%. Examination of wet mounts, revealed oval shaped microsporidian spores, their length ranging from 1.284 – 1.435 µm and their width 0.545 – 0.667 µm (n = 30). The nested PCR amplified a 407-bp product, which exhibited 97% sequence identity to *Nucleospora salmonis*. The phylogeny tests revealed that the microsporidia found in this study belonged to the *Nucleospora* genus. According to the clinical signs, histopathology findings, and molecular results, this study identifies an intranuclear microsporidium similar to *Nucleospora* infecting *O. niloticus*, which we named *Nucleospora braziliensis* sp. Nov.

Keywords: Micro-sporidia, Enterocytozoonidae, *Nucleospora*, aquaculture, fish disease

1. Introduction
Nile tilapia, *Oreochromis niloticus*, is distributed in some parts of the world. It occurs naturally in the coastal rivers of Israel [1], Nile Basin (including Lakes Albert, Edward, and Tana), Jebel Marra, Lake Kivu, Lake Tanganyika, Awash River, various Ethiopian lakes, the Omo River system, Lake Turkana, Suguta River, and Lake Baringo [2]. *Oreochromis niloticus* was introduced into Brazil in 1971 because it is a fish of easy to handle and adapt to water temperature, dissolved oxygen, and water quality [3].
Farmed fish are better for carrying out health observations than their wild counterparts because water parameters can be controlled and observation is easier in tanks. However, higher or lower water temperature, high density, and low oxygen in the water can cause immunosuppression leading to infections. Parasitic, bacterial, viral, and fungal infections have been reported in tilapia aquaculture, which can cause production loss and mortalities [4].
Nucleospora is an intranuclear microsporidium from the family Enterocytozoonidae [5]. Chilmonczyk *et al* [6]. described the first *Nucleospora* species infecting a fish host (*Oncorhynchus tshawystscha*), which was named as *Enterocytozoon salmonis*. Later, it was transferred to the genus *Nucleospora* [7]. There are two described species of this genus: *N. cyclopteri* infecting *Cyclopterus lumpus* [7] and *N. salmonis* infecting salmonids [8-10]. *Nucleospora* spp. primarily infect lymphoblast cells and contribute to chronic lymphoblastosis and a leukemia-like condition, leading to chronic mortality [6]. Clinical disease signs associated with *N. salmonis* are marked gill pallor associated with anemia, exophthalmia, enlarged spleen, kidney hypertrophy, and swelling of the large intestine [6, 11]. Confirmation of infection by conventional methods involves visualization of the spore or other developmental stage (e.g., meront stage) in host cell nuclei in either histological sections or stained kidney tissue imprints [6, 11]. However, the clinical signs are not pathognomonic, microscopic detection can be difficult because the small spores may not always be present, and the meront stage can be confused with intra-nuclear vacuoles [12]. Consequently, a number of molecular tests have been developed, including the ITS region [13], nested polymerase chain reaction (PCR) targeting the SSU gene [14], real-time quantitative PCR targeting the ITS region [15], loop-mediated isothermal amplification targeting the ITS region [16],

and *in-situ* hybridization using probes targeting the SSU gene [17]. In this study, we performed histological and molecular studies on *O. niloticus* tissues and describe a new microsporidium species.

2. Materials and Methods

2.1 Ethics statement

This study was carried out in strict accordance with the recommendations in the following laws: Law 11794/2008 and Decree 6899/2009. The protocol was approved by the Ethics Committee on Animal Use of Univ. Estadual Paulista (UNESP), and in accordance with established guidelines and policies at Ethics Committee on Animal Use of Univ. Estadual Paulista (UNESP) (Protocol Number: 724-CEUA). The owners of fish farms used in this study consent the use of their fish for detecting pathogens and other analysis, which is not present in this paper.

2.2 Nomenclatural acts

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in Zoo Bank, the online registration system for the ICZN. The LSID for this publication is: urn: lsid: zoobank.org: act: 92DAA172-4600-4880-B77E-CE79730024BD.

2.3 Sampling

Before starting this study, the project was sent to Ethics Committee on Animal Use of Universidade Estadual Paulista (UNESP), which was approved.

In November 2014, 180 *O. niloticus* specimens were randomly sampled at six fish farms in Brazil, and in March 2015 180 fishes were collected in the same places. The average of size and weight of fish samples were: Fish farm 1: 19.53 cm and 518.67 g in the first sampling, 21.64 cm and 441.10 g in the second sampling; Fish farm 2: 22.33 cm and 434.76 g in the first sampling, 22.00 cm and 518.99 g in the second sampling; Fish farm 3: 19.49 cm and 286.55 g in the first sampling, 22.02 cm and 537.56 g in the second sampling; Fish farm 4: 21.42 cm and 427.47 g in the first sampling, 20.73 cm and 430.86 g in the second sampling; Fish farm 5: 18.43 cm and 234.08 g in the first sampling, 22.77 cm and 396.82 g in the second sampling; Fish farm 6: 19.71 cm and 304.17 g in the first sampling, 21.49 cm and 424.77 g in the second sampling.

Immediately after sampling, *O. niloticus* was put into a bottle to be euthanized by an overdose of MS-222 (1g/L; Sigma-Aldrich). Euthanasia was achieved within 5 to 10 minutes according to fish body size.

2.4 Microscopy

Necropsy was performed according to Noga[4]. The organs sampled were the gill, gut, heart, kidney, liver, muscle, spleen, and stomach for histopathology and molecular analysis. According to Noga [4], a 1-cm³ portion of each tissue was fixed in 10% neutral buffered formalin followed by processing using standard histological techniques and embedded in paraffin. Hematoxylin and eosin were used for staining. The slides were stained with Ziehl–Neelsen for microsporidium visualization. After this procedure, the size and shape of microsporidium were compared with others measures of *Nucleospora* species.

2.5 DNA extraction, PCR, and sequencing

The organ tissues collected from each fish were pooled and 20 mg of each sample was used for molecular analysis. The DNA extraction was performed using the Wizard® SV Genomic DNA Purification System kit (Promega Corporation®) according to the manufacturer's instructions. The DNA was eluted in elution buffer (nuclease-free water) and kept at −20°C until use. Purity and quantification of extracted DNA was measured using a 260/280 absorbance rate in a Nanodrop 2000c (Thermo Fisher Scientific®). Only DNA with a ratio of >1.7 (260/280 rate) was used in this study.

Because we observed structures similar to microsporidium in the histology, we performed a PCR that amplifies the small subunit ribosomal RNA (srRNA) gene for all microsporidia. This PCR used the primers 18f (5′ CAC-CAG-GTT-GAT-TCT-GCC 3′) and 1492r (5′ GGT-TAC-CTT-GTT-ACG-ACT-T 3′) according to Baker *et al* [18]. After sequencing the amplified product, we identified the *Nucleospora salmonis* sequence. We then performed a second PCR followed by nested PCR specific for this pathogen, using two pairs of oligonucleotides (PCR: ES-1a 5′ CTT-TGT-GAA-CCC-AGA-CGG-G 3′ and ES-2a 5′ TGC-CTT-AGT-GAG-ACA-CTG-TTA-C 3′; nested-PCR: ES-3a 5′ GAC-ATT-CTC-TGT-CCA-GCG-G 3′ and Es-4a 5′GAG-CTA-ATC-CTG-CTC-ATC-C 3′) described by Barlough *et al* [14], which amplify a portion of the 16S small subunit ribosomal RNA (srRNA) gene of *N.* (*Enterocytozoon*) *salmonis*.

Both PCR reactions were performed in a 20-µL reaction that comprised 3 µL of extracted genomic DNA, 10 µL GoTaq Green Master Mix 2X (Promega), 0.3 µM each primer, 0.5 µL DMSO, and nuclease-free water (q.s.p.). The nested-PCR used 1 µL of extracted genomic DNA. The polymerase chain reaction cycle profile was performed in a Veriti thermocycler (Thermo Fisher Scientific®). The first reaction consisted of an initial denaturation step of 95°C for 5 min, followed by 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, finishing with terminal extension at 72°C for 5 min and a hold at 22°C. The PCR and nested PCR for *N. salmonis* consisted of an initial denaturation step of 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 45°C for 2 min, and 72°C for 90 s, finishing with terminal extension at 72°C for 7 min and a hold at 22°C. The PCR products were electrophoresed on a 1.5% agarose gel stained with 1% SYBR safe (Thermo Fisher Scientific®), alongside a High Ranger 100-bp DNA ladder (Norgen Biotek Corp.®). The 1270-bp amplicons produced with the primers 18f and 1492r were excised from the gel and purified with an Ilustra Microspin™ S-400 HR Columns Kit (GE Healthcare®) according to the manufacturer's instructions.

After purification of the amplified products, Sanger sequencing was performed. For this purpose, the purified amplicon was sequenced in both directions using Big Dye™ Terminator Cycle Sequencing Kit (Applied Biosystems) on an Applied Bio systems capillary 3500 Genetic Analyzer. The quality of the electropherograms was assessed in Sequencing Analysis version 5.4 (Applied Bio systems).

Phylogenetic analysis of the data sets was performed in MEGA version 6.0 [19]. The microsporidia sequences (genbank data) used to build the phylogenetic tree were selected based on reports in fish and the lesions caused. The *N. salmonis* sequence used was U78176 (host: *Oncorhynchus*

tshawyscha). We also used: *Nucleospora* sp. (AF186007, host: *Pleuronectes vetulus*), *N. cyclopteri* (KC203457, host: *Cyclopterus lumpus*), *Enterocytozoon salmonis* (U10883, host: *Oncorhynchus tschawytscha*), *Enterocytozoon bieneusi* (AY257180 and AF024657), *Microsporidium* sp. (AF394528, KR871371, and FJ794873), *Endoreticulatus* sp. (EU260046), *Cystosporogenes* sp. (GU299511 and GQ379704), *C. legeri* (AY233131), *Vittaforma corneae* (KP099422), *Glugoides intestinalis* (AF394525), and *Orthosomella operoptherae* (AJ302316 and AJ302317). The *Ordospora colligata* (AF394529) sequence was used as an out group.

CLUSTAL_W (MEGA version 5.1) was used for the initial sequence alignments of the SSU rRNA gene. For MEGA, the item "Find Best DNA/Protein Model" recommended the General Time-Reversible Model, maximum likelihood for our sequence as the most parameter-rich evolutionary model. Therefore, the settings used for analysis were nst=5, with the gamma-distributed rate variation across sites, a proportion of invariable sites (rates – invgamma), and bootstraps with 1000 replicates.

2.6 Statistical analysis

The prevalence of microsporidium was calculated for each fish farm sampled. The occurrence of lesions observed and positive PCR and nested-PCR results were also analyzed. All statistical analyses were performed in R software version 3.2.3 [20] and visualized in Graph Pad Prism version 5.00 for Windows, Graph Pad Software, San Diego California USA, www.graphpad.com.

3. Results

3.1 Macroscopic and microscopic lesions

During necropsy we observed many lesions as described in Table 1, the microscopic lesions are shown in Figures 1, 2, and 3. The most prevalent were hepatomegaly, melanisation of the skin and splenomegaly. In some cases, it is observed increase depending of the alteration analyzed during the two sampling periods.

The prevalence of microsporidium by histopathology in fish farms vary between 86.66% to 100% (Table 2). Fish farming 1 presented decrease of microsporidium in the second sampling, but fish farmings 4 and 5 have an increase of the occurrence of this pathogen in the second moment. Fish farming 2, 3, and 6 had the same prevalence of 100% in both samplings. The most prevalent lesions were inflammatory response, xenome, and eosinophils presence. Examination of wet mounts, revealed oval shaped microsporidium spores, their length ranging from 1.284 – 1.435 µm (mean ± stdev = 1.34 ± 0.08) and their width 0.545 – 0.667 µm (mean ± stdev = 0.61 ± 0.06) (n = 30). The size of this microsporidium is different from others described until now (Table 3). The material (tissues naturally infected and slides fixed) was deposited in Biotechnology Institute, situated in Botucatu, São Paulo, Brazil, under number IBTEC 0003/2016.

3.2 Molecular results

The PCR performed with universal primers (18f and 1942r) amplified a product of 1270-bp, which 1071 were sequenced and presented 96% identity with *Nucleospora salmonis* (Genbank: AF185991). The nested PCR specific for this microsporidium amplified a 407-bp product. The sequence obtained exhibited 95% identity with *Nucleospora salmonis* (Genbank: AF185991). The sequence has been submitted to GenBank under the accession number: KT777455. Therefore, the positive identification of *Nucleospora* by molecular techniques was lower when compared with histopathology (Table 2). The fish farmings with the highest detection was 2, 4, 5, and 6. Between samplings, fish farming 1 presented decrease in the second moment of collection as number 3, 4, and 6. Others (2 and 5) presented an increase of this detection by molecular techniques.

The sequence obtained in this study (Genbank: KT777455) had a well clade support with other *Nucleospora* species, indicating that the microsporidium found in this study belongs to the genus *Nucleospora* (Fig. 4). Based on the differences observed (Fig. 5) and the phylogeny results, we infer that this could be a new species, named as *Nucleospora braziliensis* sp. nov. Urn: lsid: zoobank.org: act: 92DAA172-4600-4880-B77E-CE79730024BD.

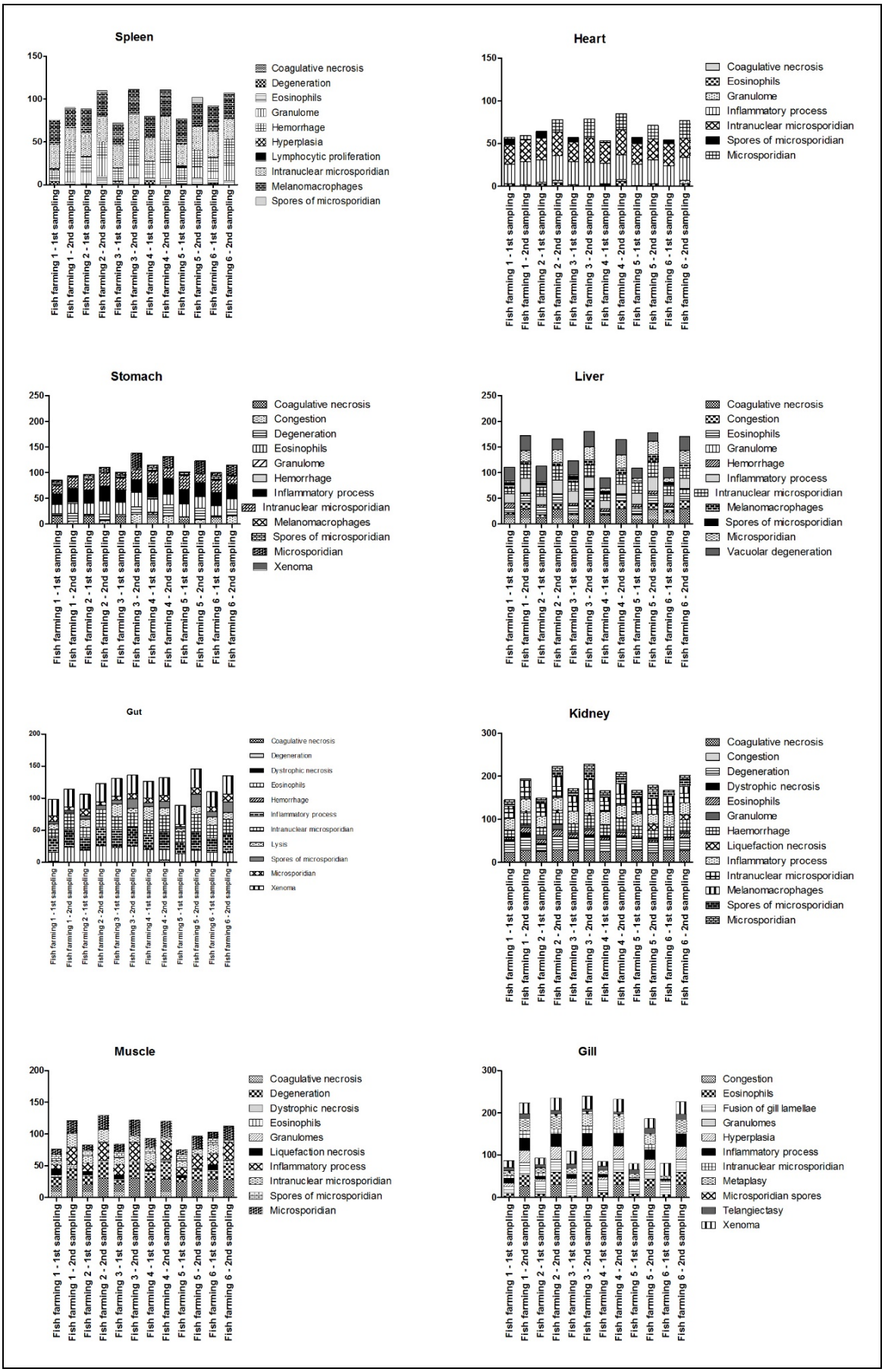

Fig 1: Occurrence of lesions observed in histopathology of the gills, gut, heart, kidney, liver, muscle, spleen, and stomach at fish farms 1, 2, 3, 4, 5, and 6 in the first and second sampling.

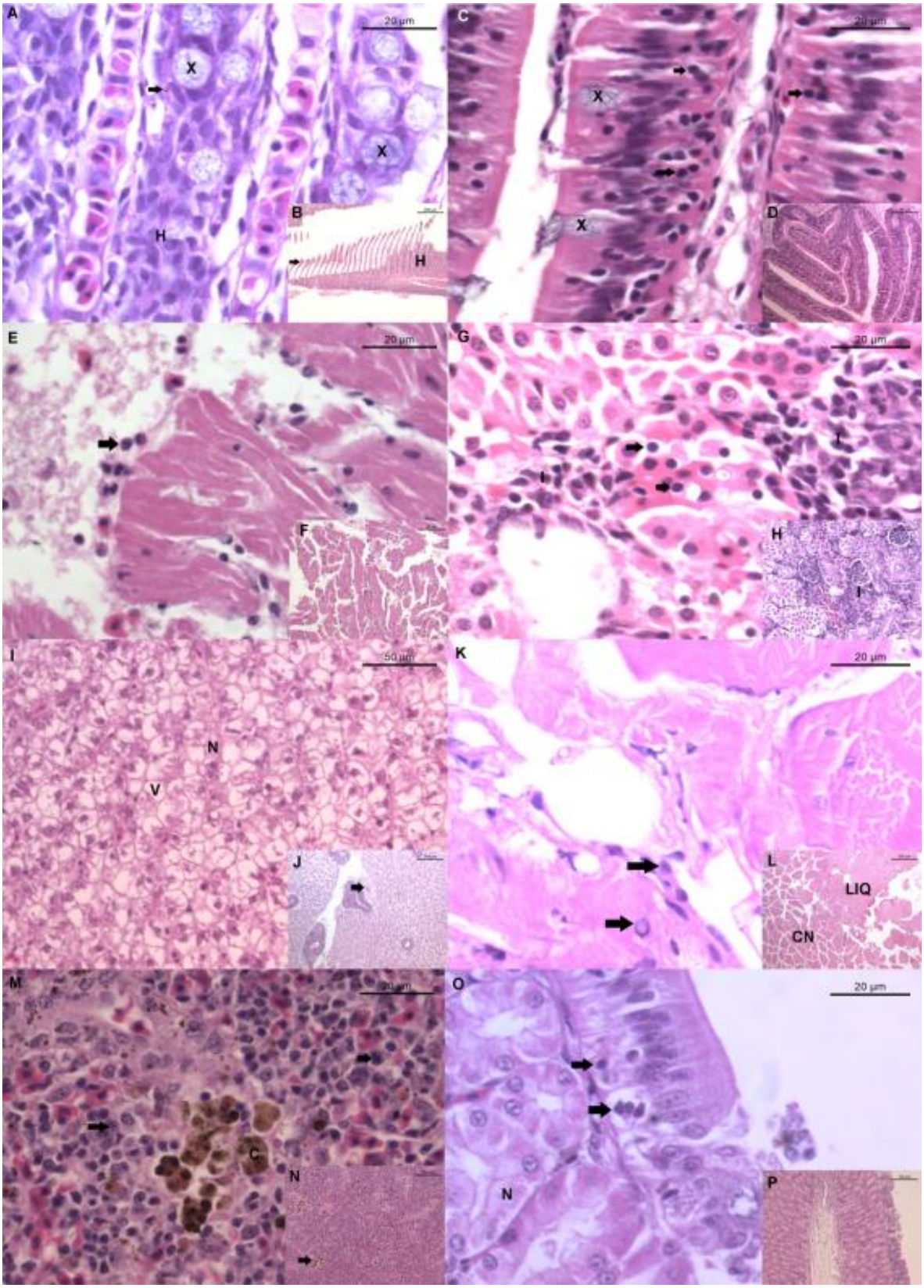

Fig 2: Microscopic lesions observed in *Oreochromis niloticus*. A: Hyperplasia (H), structures similar to microsporidium (arrow), and xenoma in the gill. B: Hyperplasia (H) and telangiectasia (arrow) in the gills. C: Xenomas (X) and inflammatory cells (arrow) in the gut. D: gut. E: Intranuclear structures similar to microsporidium (arrow) in the heart. F: heart. G: Inflammatory cells (I) and intranuclear structures similar to microsporidium (arrow) in the kidney. H: Inflammatory process (I). I: Vacuolar degeneration (V) and coagulative necrosis (N) in the liver. J: Hemorrhage (arrow) in the liver. K: Structures similar to microsporidium spores (arrow) in the muscle. L: Coagulative necrosis (CN) and liquefaction necrosis (LIQ) in the muscle. M: Intranuclear structures similar to microsporidium (arrow) and melanomacrophages centers (C) in the spleen. N: Melanomacrophages centers (arrow) in the spleen. O: Coagulative necrosis (N) and intranuclear structures similar to microsporidium (arrow) in the stomach. P: Stomach. Hematoxylin and eosin (HE).

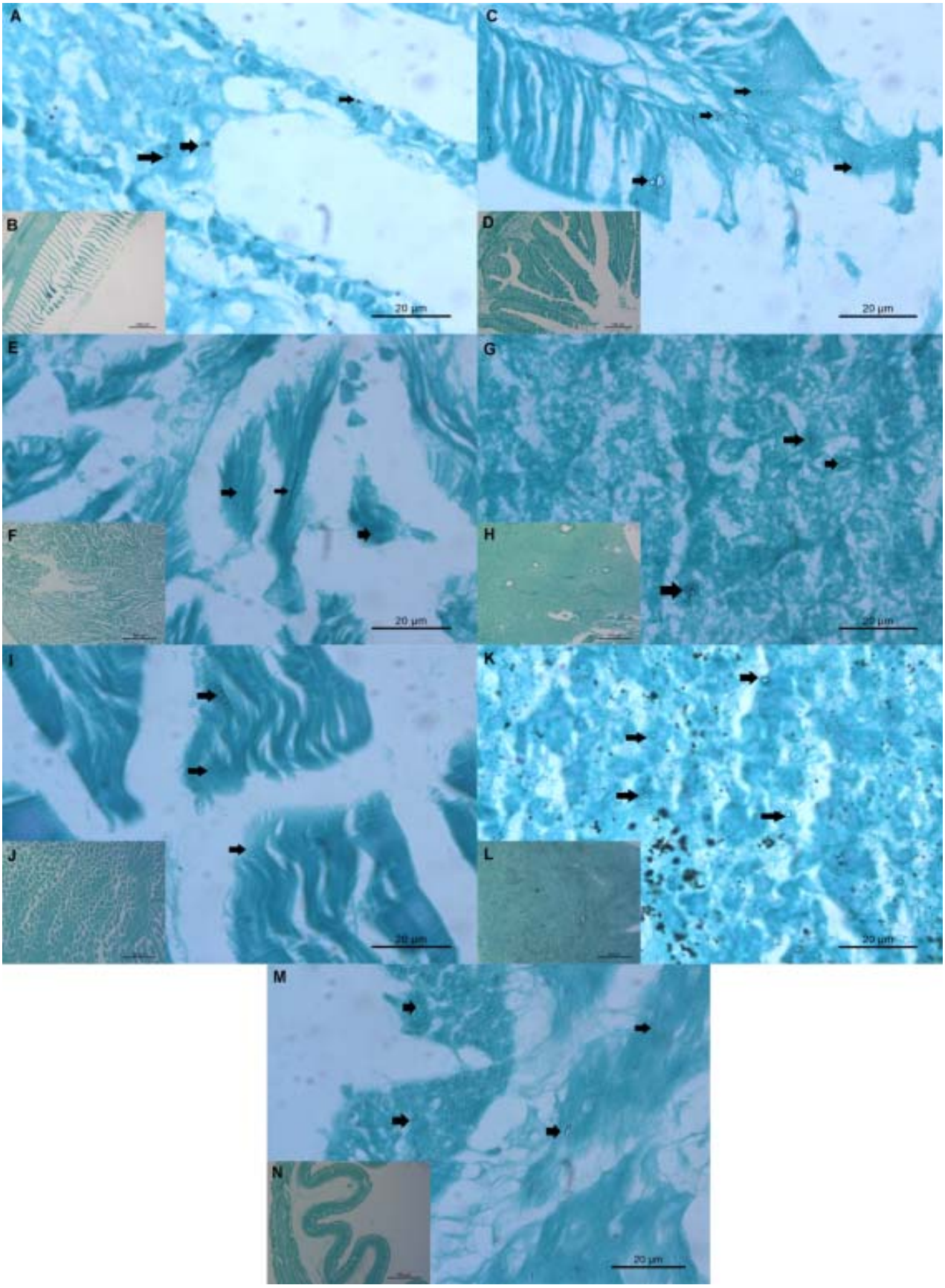

Fig 3: Observation of microsporidium (arrow) stained with Ziehl–Neelsen. A and B: gills, C and D: gut, E and F: heart, G and H: liver, I and J: muscle, K and L: spleen, M and N: stomach.

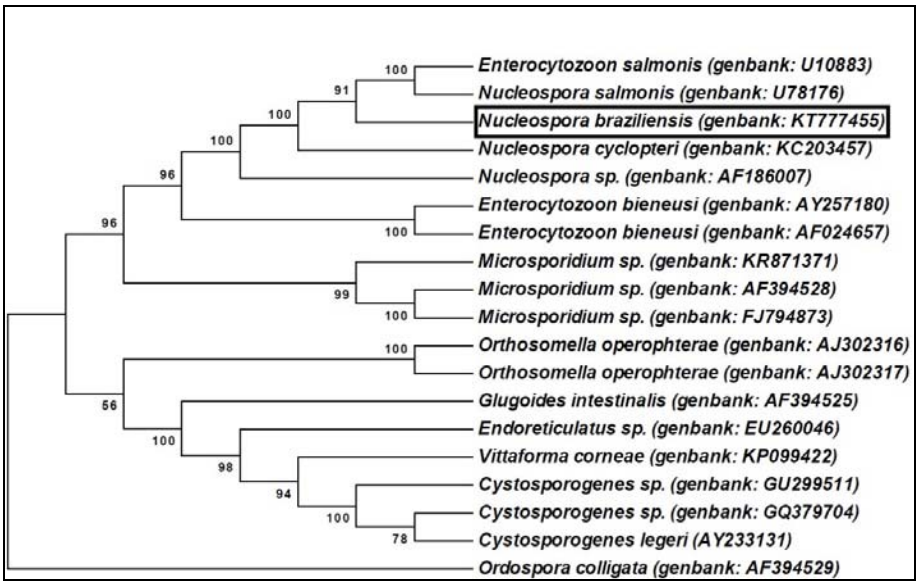

Fig 4: Maximum likelihood tree (General Time-Reversible Model) for 16S small subunit ribosomal gene (16S SSU rDNA) for the sequence obtained in this study (marked with rectangle), *Nucleospora* species, and other selected microsporidia species. The numbers of the branches are bootstrap confidence levels on 1000 replicates.

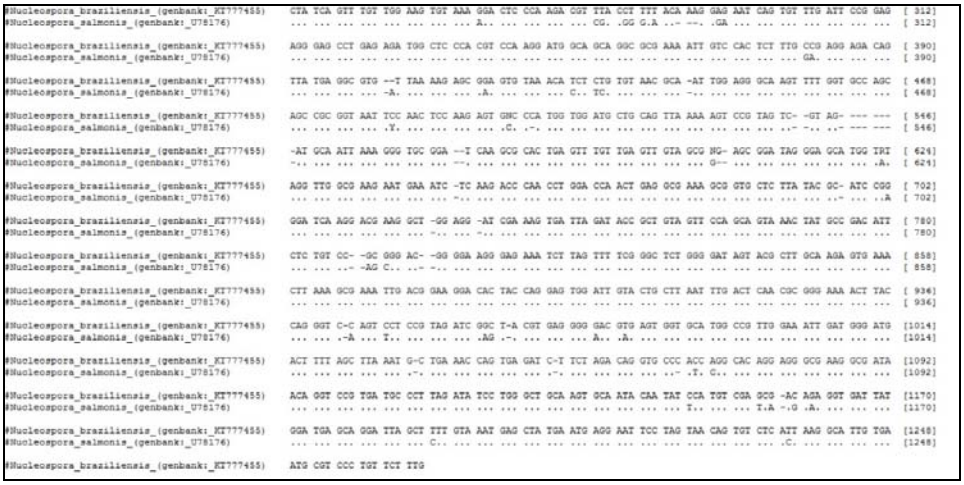

Fig 5: Visualization of the differences in *Nucleospora braziliensis* n. sp. and *Nucleospora salmonis* (Genbank: U78176). Letters (A, C, T, and G) demonstrate the differences and (-) gap between the sequences analyzed.

4. Discussion

The microsporidia are a diverse parasitic phylum infecting host groups from all major *taxa* in all environments [21, 22]. The vast field of research into the Microsporidia has focused on terrestrial hosts, ranging from those infecting pest and beneficial insects to important human parasites. However, despite the fact that almost half of the known microsporidium genera infect aquatic hosts, the emergence of this discipline is more recent. Recent research demonstrating the potential for extreme morphological plasticity and an ability to infect all known organ and tissue systems is important not only for its effects at the individual level. It also highlights the urgency with which we need to enhance our understanding of the role of Microsporidia in ecological communities and their potential to cause emergent diseases in aquatic animals and humans [23]. In the present study, we detected and identified a new microsporidium species infecting *O. niloticus* that caused severe lesions.

Infections caused by *N. salmonis* have been reported in Chinook salmon (*Oncorhynchus tshawytscha*) in the western United States and Canada, steelhead (*O. mykiss*) in Idaho, United States, rainbow trout (*O. mykiss*) in France, Atlantic salmon (*Salmo salar*) and coho salmon (*O. kisutch*) in Chile, and Atlantic salmon in British Columbia, Canada [6, 8-11, 24]. At present, there is no report in either *O. niloticus* or in other Brazilian fishes, so this study is the first report of *Nucleospora* species in this country and in this host species.

During necropsy, the main lesions observed in *O. niloticus* from all of the fish farms sampled included congestion of the stomach, hepatomegaly, and splenomegaly (Table 1). However, other symptoms were also highly prevalent, e.g., congestion of the liver, exophthalmy, hemorrhaging in the liver, skin, stomach, and spleen, liquefaction of the kidney, and melanization of the skin. These findings are similar to those reported in other studies [6, 9-11, 14, 17], which observed anemia characterized by pale gills, darkened body color, exophthalmia, kidney hypertrophy, renomegaly, splenomegaly, ascites, and swelling of the large intestine. Nylund *et al*. [25] have also reported that some fish can have skin hemorrhaging, loss of scales, pale gills with a high

production of mucus, and strong pigmentation at the base of the primary lamellae, as observed in this study (Table 1).

In the histopathology, the most prevalent symptoms observed were: xenoma in the gills and gut, fusion of gill lamellae, hemorrhaging in the kidney and spleen, intra-nuclear structures similar to microsporidium and inflammatory cells in all of the tissues analyzed, coagulative necrosis in the liver, kidney, muscle, and stomach, presence of eosinophils in the liver, gut, and stomach, vacuolar degeneration in the liver, lysis of the villus gut, and degeneration of microsporidium spores in the muscle (Fig. 1). In the second sampling, fish analyzed exhibited more lesions when compared with the first collection, which demonstrate that the microsporidium is causing these alterations in tissue observed. These findings have also been reported by others researchers, Barlough *et al*. [14] found severe lymphoblastosis with leukemia-like features, i.e., intra-nuclear-stage microsporidium in lymphocytic cells. According to Nylund *et al*. [25], hypertrophy and hyperplasia with necrosis and invasion of leukocytes were observed in the gill epithelium, as was inflammation in the kidney, heart, spleen, gut, and exocrine pancreas.

The xenoma is a complex cyst-like structure comprising a hypertrophic host cell harboring microsporidia in multiple stages of development [26]; it is considered a completely separate entity from the rest of the host and has a dual function: 1) it protects the parasite of the host's immune response and 2) it confines the parasite in one site, preventing its dissemination throughout the host body [27]. Rupture of the xenomas and spore release cause noticeable muscular degeneration, necrosis, and obvious infiltration by mononuclear cells, such as macrophages and lymphocytes [28]. The inflammatory tissue reaction associated with fish microsporidium has been studied at the ultrastructural level, identifying many of the inflammatory cells that actively participate in spore elimination, e.g., macrophages, lymphocytes, neutrophils, fibroblasts, and eosinophilic granular cells [29, 30]. This report explains why the histopathology in this study revealed many fishes with inflammatory cells (Figs. 1 and 2), suggesting that this response might be involved in the elimination of the parasite. Matthews and Matthews [31] reported that the microsporidium *Tetramicra brevifilum*, which infects turbot (*Scophthalmus maximus*), causes several xenomas in the muscle fiber connective tissue. Spore release was followed by leukocyte infiltration, uptake of spores by macrophages, and severe local necrosis. Lovy *et al*. [30] also reported that infection by microsporidia can cause granuloma formation, also observed in higher quantity in the second sampling, demonstrating that the permanence of this microsporidia in the aquaculture. In agreement with Matthews and Matthews [31] and Lovy *et al*. [30], this study also observed that the most frequent lesions were xenoma, leukocyte infiltration, macrophages, and necrosis (Fig. 1).

One of the most recognized mechanisms for the elimination of microsporidia spores from the host tissue is the phagocytic process [32]. The role that neutrophils and macrophages play in spore destruction is well documented. Because spore release corresponds to the final stage of parasite development, it is clear that spore phagocytosis represents an important defense mechanism for microsporidia tissue clearance. After phagocytes arrive at the xenoma site, they actively ingest and destroy spores avoiding dissemination [33].

Initial experimental studies with *N. salmonis* have shown that two natural routes (cohabitation with infected fish and the feeding of infected tissues) result in transmission of the parasite [34]. Oral ingestion of spores is presumed to be the route of entry of the infective stage. The parasite then spreads from the initial site to the major cellular targets in hematopoietic tissues, mainly the spleen and kidney [9, 10]. Barlough *et al*. [14] also detected this microsporidium species in the feces of infected fish by PCR, supporting an intestinal role in excretion and shedding of the parasite, which presumably is a major route for fish-to-fish transmission.

According to Canning *et al*. [35] the prevalence is particularly elevated when the fish population density is extremely high, increasing the morbidity and mortality rates in young fish, which explains the high prevalence observed in this study because *O. niloticus* are cultured in high densities in Brazil. Although we analyzed samples from different reservoirs in the São Paulo state, the prevalence observed was similar in all of the fish farms investigated in this study.

Historically, the ability to detect microsporidia infections was based on external clinical signs [36]. However, the recent application of pathological and molecular diagnostic approaches has allowed for the detection of cryptic and subclinical infections [37]. However, this study detected fewer positive animals by PCR when compared with the histology results. This low detection by PCR could be related to the stage of infection (chronic), which suggests that the animals were infected but the fungus was either inactive or being eliminated. However, the sequencing and phylogeny following detection was crucial in identifying the presence of the pathogen in *O. niloticus*. Furthermore, we did not use a positive control, which demonstrates that the microsporidium was present in the samples and it was not due to contamination.

The phylogenetic analysis and measures (Table 3) revealed that *Nucleospora braziliensis* sp. nov. Was most closely related to the *N. salmonis* isolates, but some differences were observed, which supports the conclusion that this is a new species of the *Nucleospora* genus.

Table 1: Prevalence (%) of macroscopic lesions observed in *Oreochromis niloticus* sampled in six different fish farms in the first and second sampling.

	Fish farm											
Lesions	**1[a]**	**1[b]**	**2[a]**	**2[b]**	**3[a]**	**3[b]**	**4[a]**	**4[b]**	**5[a]**	**5[b]**	**6[a]**	**6[b]**
Congestion												
Gut	0.00	0.00	10.00	6.66	3.33	0.00	3.33	0.00	3.33	3.33	10.00	0.00
Heart	0.00	0.00	6.66	10.00	0.00	0.00	0.00	10.00	0.00	0.00	0.00	0.00
Liver	10.00	43.33	40.00	90.00	50.00	70.00	16.66	56.66	46.66	36.66	46.66	40.00
Stomach	66.66	10.00	30.00	30.00	70.00	33.33	66.66	53.33	43.33	43.33	23.33	13.33
Endophthalmy	3.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Exophthalmy	6.66	6.66	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	26.66	0.00
Haemorrhage												
Kidney	0.00	30.00	0.00	23.33	0.00	20.00	0.00	10.00	0.00	3.33	0.00	3.33

Liver	0.00	13.33	13.33	6.66	10.00	0.00	33.33	0.00	0.00	16.66	40.00	3.33
Skin	66.66	26.66	0.00	23.33	26.66	0.00	20.00	0.00	3.33	3.33	20.00	0.00
Spleen	100	0.00	26.66	0.00	13.33	0.00	16.66	0.00	10.00	3.33	0.00	0.00
Stomach	0.00	0.00	0.00	3.33	3.33	0.00	23.33	0.00	0.00	0.00	46.66	0.00
Hepatomegaly	90.00	100.00	90.00	96.66	93.33	100.00	100.00	90.00	90.00	100.00	100.00	100.00
Hyperemia of the gut	0.00	6.66	0.00	6.66	0.00	6.66	13.33	3.33	6.66	0.00	0.00	3.33
Hyperplasia												
Kidney	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.33	3.33	0.00	3.33
White pulp	0.00	3.33	3.33	3.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Hypertrophy												
Liver	0.00	0.00	3.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Spleen	0.00	46.66	0.00	10.00	6.66	10.00	3.33	6.66	3.33	10.00	3.33	13.33
Ischemia of the liver	16.66	0.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00	3.33	0.00	10.00
Liquefaction												
Heart	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.66	0.00
Kidney	6.66	0.00	16.66	23.33	0.00	23.33	6.66	30.00	0.00	40.00	36.66	30.00
Melanisation of the skin	3.33	96.66	6.66	70.00	20.00	46.66	30.00	40.00	93.33	46.66	80.00	3.33
Necrosis												
Gills	0.00	0.00	0.00	3.33	0.00	0.00	0.00	0.00	0.00	0.00	3.33	0.00
Liver	20.00	0.00	13.33	0.00	3.33	0.00	6.66	0.00	0.00	0.00	3.33	6.66
Splenomegaly	90.00	63.33	73.33	100.00	73.33	90.00	100.00	63.33	30.00	70.00	63.33	66.66

[a] First sampling, [b] Second sampling.

Table 2: Prevalence (%) of microsporidia by histopathology, PCR and nested-PCR in *Oreochromis niloticus* sampled in six different fish farms in the first and second sampling.

	Fish Farming											
Test	**1[a]**	**1[b]**	**2[a]**	**2[b]**	**3[a]**	**3[b]**	**4[a]**	**4[b]**	**5[a]**	**5[b]**	**6[a]**	**6[b]**
Histopathology	100.00	96.66	100.00	100.00	100.00	100.00	86.66	100.00	90.00	100.00	100.00	100.00
PCR	3.33	0.00	3.33	73.33	0.00	6.66	0.00	16.66	0.00	36.66	6.66	20.00
Nested-PCR	16.66	6.66	33.33	73.33	23.33	10.00	63.33	33.33	26.66	70.00	63.33	60.00

[a] First sampling, [b] Second sampling.

Table 3: Host, size, width, target, and shape described of *Nucleospora* species.

Microsporidia	Host	Size	Width	Target	Shape	Reference
Nucleospora braziliensis n. sp.	*Oreochromis niloticus*	1.2-1.4 µm	0.5-0.6 µm	Gill, gut, heart, kidney, liver, muscle, spleen, and stomach	Ovoid	This study
Nucleospora cyclopteri	*Cyclopterus lumpus*	2.9-3.5 µm	1.1-1.5 µm	Kidney	Ovoid	Freeman *et al*., 2013
Nucleospora salmonis	*Oncorhynchus tshawyscha* and others salmonids	1 µm	2 µm	Spleen, kidney, and blood leukocytes	Ovoid	Chilmonczyk *et al*., 1991

5. Conclusion

Using clinical signs, histopathology findings, and molecular results, this study identified an intra-nuclear microsporidium, similar to *Nucleospora*, infecting *O. niloticus*. This is the first report in both *O. niloticus* and in Brazil.

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