UNIVERSITY OF SÃO PAULO
FACULTY OF ANIMAL SCIENCE AND FOOD ENGINEERING

KASSIA ROBERTA HYGINO CAPODIFOGLIO

Systematics and molecular characterization of new myxosporean species parasites of *Colossoma macropomum* and *Piaractus brachypomus* from the Amazon basin and remarks on mitochondrial behavior in myxosporeans

Pirassununga
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Examination Board:

Prof. Dr.: Antonio Augusto Mendes Maia

Institution: Faculdade de Zootecnia e Engenharia de Alimentos – USP

President of the Examination Board

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“Estude e não esmoreça.
Pois traz erguida a cabeça,
quem sempre cumpre o dever.
A vida é luta e batalha e
nela só quem trabalha,
merece e deve vencer.”
(Avô de Rossandro Klinjey)

"Study and do not falters.
Because, it brings the head held high,
who always do your duty.
Life is struggle and battle,
and only those who work,
deserves and shall win. "
(Grandfather of Rossandro Klinjey)

*To **God**, I dedicate.*

BIOGRAPHY

Kassia Roberta Hygino Capodifoglio, daughter of Paulo Roberto Hygino and Filomena Aparecida da Silva Fujimura, was born in the city of Pirassununga, São Paulo on March 23, 1988. She is married to Eduardo Capodifoglio since January, 2012, and mother of two children, Theo (Three years) and Antonio (One year).

She is graduated in Biological Sciences by University Center Anhanguera in 2010.

At the beginning of 2012, Kassia began an internship in the Parasitology Laboratory of the Faculty of Animal Science and Food Engineering FZEA/USP and in June joined the FZEA/USP Master program in Animal Science, with a CAPES scholarship.

In 2014, Kassia was approved in the FZEA/USP Doctoral Program in Animal Science, with a FAPESP scholarship that linked with the BIOTA project supported by FAPESP under the support Prof. Dr. Edson Aparecido Adriano.

In 2016, Kassia collaborated in a scientific initiation project with undergraduate Caroline Munhoz Meira, also supported by FAPESP, which generated the third chapter of this thesis and received honorable mention in the 26th USP International Symposium of Undergraduate Research – SIICUSP (ATTACHMENT A) and which was presented at Ohio State University, in the United States.

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"We have to do what has to be done, including to honor of all those who came before us with much more difficulty, and who did much to make our way easier ..."
(Rossandro Klinjey)

I came from a creation from where it seemed impossible to reach this formation.
Today, I'm here and I don't know how I did it.
The only answer I have is that God has placed special people in my path to facilitate my journey. And without them this would not be possible.
And, now, I give thanks with great affection....

To **God**, first and always.

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"Whoever you are, whatever social position you have in life, the highest or the lowest, always have as goal a lot of strength, a lot of determination and always do everything with a lot of love and with a lot of faith in God, that someday you get there. Somehow you get there".

(Ayrton Senna)

ABSTRACT

CAPODIFOGLIO, K. R. H. **Systematics and molecular characterization of new myxosporean species parasites of *Colossoma macropomum* and *Piaractus brachypomus* from the Amazon basin and remarks on mitochondrial behavior in myxosporeans.** 2019. 150 f. Thesis (Doctorate) – Faculty of Animal Science and Food Engineering, University of São Paulo, Pirassununga, 2019.

The Amazon basin has about 7000 km^2 of extension and a vast biodiversity providing an ichthyofauna of approximately 5000 species of fish. Myxozoan parasites cause diseases in fish in both the natural environment and the breeding systemand they are responsible for high mortality rates. Myxozoans show a great diversity of species with some of them, highly pathogenic and, therefore, have been receiving attention of the researchers. In Brazil, due to the great diversity of fish species, the study of these parasites has been gaining the attention of the researchers, focusing on diversity, biology and parasite-host interaction. In this context, the objective of this work was to explore the parasite diversity of the Myxozoa subphylum, through morphological and molecular analyses of myxosporean parasites of Amazonian fish, important for the food market such as *Colossoma macropomum* (tambaqui) and *Piaractus brachypomus* (pirapitinga). For the taxonomic study, morphological analyses were performed using light microscopy and molecular analyses using ssrDNA. The fish were captured in the Amazon basin, in the Tapajós and Solimões Rivers, Santarém, PA and Manaus, AM, respectively. Two new myxosporean species, *Myxobolus* n. sp. 1 and *Myxobolus* n. sp. 2, were described infecting *C. macropomum,* based on morphological and molecular data. *Myxobolus colossomatis*, found infecting *C. macropomum*, was considered a distinct species of *Myxobolus* cf. *colossomatis* described in *Piaractus mesopotamicus*, through molecular and phylogenetic data. For *P. brachypomus* four new species were described. *Henneguya* n. sp. 1 and *Myxobolus* n. sp. 3, were described through of morphological, histological, ultra-structural and molecular analyses. *Henneguya* n. sp. 2 and *Myxobolus* n. sp. 4 were described based in morphological and molecular data. After observing the mitochondrial behavior of myxospores through electron microscopy studies, we performed an experiment of mitochondrial fluorescence microscopy and the results showed that few *Henneguya* sp. myxospores presented labeling for mitochondrial activity.

Key-words: Myxozoa, ssrDNA, tambaqui, pirapitinga, Amazon River, mitochondrial fluorescence microscopy.

RESUMO

CAPODIFOGLIO, K. R. H. **Sistemática e caracterização molecular de novas espécies de mixosporídeos parasitos de *Colossoma macropomum* e *Piaractus brachypomus* da bacia Amazônica e observações sobre o comportamento mitocondrial em mixosporídeos.** 2019. 150 f. Tese (Doutorado) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2019.

A bacia Amazônica tem cerca de 7000 km^2 de extensão e uma vasta biodiversidade abrigando uma ictiofauna de, aproximadamente, 5000 espécies de peixes. Parasitos mixozoários são causadores de doenças em peixes de ambiente natural e de sistemas de criação sendo responsáveis por altas taxas de mortalidade. Mixosporídeos apresentam uma grande diversidade de espécies com algumas destas, altamente patogênicas e, por isso, vêm recebendo atenção dos pesquisadores. No Brasil, devido à grande diversidade de espécies de peixes, o estudo destes parasitos vem ganhando cada vez mais destaque, com foco para a diversidade, biologia e a interação parasito-hospedeiro. Neste contexto, o objetivo deste trabalho foi explorar a diversidade de parasitos do subfilo Myxozoa, através de análises morfológicas e moleculares de mixosporídeos parasitos de peixes amazônicos, importantes para o mercado de alimentos como *Colossoma macropomum* (tambaqui) e *Piaractus brachypomus* (pirapitinga). Para o estudo taxonômico foram realizadas análises morfológicas, utilizando microscopia de luz, e análises moleculares utilizando o sequenciamento do ssrDNA. Os peixes foram capturados na bacia Amazônica, nos rios Tapajós e Solimões, Santarém, PA e Manaus, AM, respectivamente. Duas novas espécies de mixosporídeos, *Myxobolus* sp. n. 1 e *Myxobolus* sp. n. 2, foram descritas infectando *C. macropomum*, baseado em dados morfológicos e moleculares. *Myxobolus colossomatis* encontrada infectando *C. macropomum* foi considerada uma espécie distinta de *Myxobolus* cf. *colossomatis* descrita em *Piaractus mesopotamicus*, através de dados moleculares e filogenéticos. Para *P. brachypomus* quatro novas espécies foram descritas. *Henneguya* sp. n. 1 e *Myxobolus* sp. n. 3 foram descritas através de análises morfológicas, histológicas, ultra-estruturais e moleculares. *Henneguya* sp. n. 2 e *Myxobolus* sp. n. 4 foram descritas baseadas em dados morfológicos e moleculares. Após a observação do comportamento mitocondrial de mixosporídeos através de estudos de microscopia eletrônica, nós realizamos um experimento de microscopia de fluorescência mitocondrial e os resultados obtidos demonstraram que poucos mixosporos de *Henneguya* sp. apresentaram marcação para a atividade mitocondrial.

Palavras-chave: Myxozoa, ssrDNA, tambaqui, pirapitinga, Rio Amazonas, microscopia de fluorescência mitocondrial.

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ACRONYMS AND ABBREVIATIONS

α	alpha
µl	microliter
µm	micrometer
µM	micromolar
bp	base pairs
BLASTn	Basic Local Alignment Search Tool nucleotide
COI	Cytochrome C Oxidase I
CPL	Caudal Process Length
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine Tetraacetic Acid
FZEA	Faculty of Animal Science and Food Enginering
IB	Biology Institute
ITS-1	Intergenic Transcribed Spacer - 1
LED	Light Emitting Diode
LIP	Laboratory of Immunology of Parasite
$MgCl_2$	Magnesium Chloride
ML	Myxospore Length
mM	millimolar
MW	Myxospore width
NCBI	National Center for Biotechnology Information
NCF	Number of Coils of Polar Filament
ng	nanogram
nm	nanometer
PCL	Polar Capsule Length

PCW	Polar Capsule Width
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
rDNA	ribosomal Deoxyribonucleic Acid
rpm	rotations per minute
SDS	Sodium Dodecyl Sulfate
SMS	Smart Model Selection
ssrDNA	Small Subunit Ribosomal Deoxyribonucleic Acid
TAE	Tris-Acetate-EDTA
TL	Total Length
TH	Thickness
UNICAMP	State University of Campinas
UNIFESP	Federal University of São Paulo State
USP	University of São Paulo

LIST OF SYMBOLS

%	percentage
®	trademark
&	ampersand
×	multiplication
*	asterisk
°C	degree Celsius
'	minute
"	second
±	more or less
<	less-than
≈	almost equal to/ approximately

SUMMARY

1 INTRODUCTION

1.1 Basin of the Amazon River

The South American has potential for expansion of the aquaculture and a large number of native species, which represent 24% of the described species of the world (SCHAEFER, 1998). Furthermore, the continent has a large number of rivers and lakes that constitute the largest river basin in the world, the Amazon basin (TUNDISI, 2003).

Amazonian effluents are born in the Andes mountain range, in Peru (TRANCOSO, 2006), and it presents, in all its extension, a dense and complex network of streams, called igarapés (JUNK, 1983).

With about 6.500 km^2 of extension (GOULDING, 1996), Amazon basin is intersected by the Amazon River and thousands of others rivers (SIOLI, 1967) and is known for its vast water availability, with approximately 20% of all fresh water on the planet (TUNDISI, 2003).

In addition to having plenty of water, the Amazon basin has a vast biodiversity and is one of the planet's most productive and intact ecosystems (SANTOS; SANTOS, 2005), harboring an ichthyofauna of approximately 5000 species of fish (VAL; HONCZARYK, 1995).

According with Lundberg et al. (2000) it is believed that only 40% of the Amazonian ichthyofauna is known, indicating that this system is poorly understood and underestimated.

Brazil is home to a large part of this ichthyofauna, although the knowledge about its composition in the different Brazilian watersheds is deficient and irregular, such fact can be verified observing places with more species, with economic, ecological and social importance, and that several species threatened are not studied and preserved (MENEZES et al., 2007).

1.2 Amazonian fish of economic importance

Brazilian North, more specifically Amazonas and Pará states, is one of the main region producers of fish. In 2010, the region was responsible for than more two hundred thousand tons

of fish produced. Among the fish produced in the northern region the main targets in the extractive fishery are Characiformes fish. (MPA, 2012).

The Characiformes order is one of the largest groups of freshwater fish, with 2400 species divided in, approximately, 520 genera (NELSON, 2006). Serrasalmidae family, belonging to Characiformes order, shelters fish known popularly as “myleus”, “piranhas” and, with stand out, “pacus” (CALCAGNOTTO et al., 2005; MIRANDE, 2010).

The pacus group includes economically important species because they are considered high quality food, such as the species *Colossoma macropomum* Cuvier, 1816, commonly known as “tambaqui” and *Piractus brachypomus* Cuvier, 1818 known as “pirapitinga” (IBAMA, 2007).

Colossoma macropomum is the second largest scale fish in South America, weighing up to 30 kg and measuring approximately 90 cm in length, being a fish very appreciated by the riverside communities of the Amazon (LOPERA-BARRERO et al., 2011).

Tambaqui is a fish with solitary behavior and that survives in mineral-poor water (FROESE; PAULY, 2018).

It is a frugivorous fish in adulthood, making it one of the main seed dispersers for many species of plants in the Amazonian floodplains (ANDERSON et al., 2011).

The natural stocks of this species have been drastically reduced in recent years (SANTOS; SANTOS, 2005) and because this, the cultivation of this fish has been the solution for overexploitation. Tambaqui is the most cultivated species in the Brazilian Amazon and is widely used in breeding systems throughout the country, reaching 66% of all national production between 2007 and 2009 (LOPERA-BARRERO, 2011).

In 2011, Brazilian aquaculture produced about 628 thousand tons of fish. Of these, 544 thousand tons were produced by continental fish farming, with tambaqui being one of the main

species produced, making the country reach 12th place in the world ranking (BRAZIL, 2013a, BRAZIL, 2013b).

Piaractus brachypomus, commonly known as "pirapitinga", is a species that can to reach 80 cm in length and weigh up to 20 kg (SOBRINHO et al., 1984). It has an omnivorous food habit, with tendency to herbivory, consuming fruits and seeds in the period of the flood, in which its availability is greater. The pirapitinga makes two migrations during the year, one at the beginning of the flood, between November and February, for spawning and another at the beginning of the ebb, between June and October, when the species leaves the flooded forest to the headwaters of rivers (HERNÁNDEZ, 1994; SANTOS; FERREIRA; ZUANON, 2006).

The cultivation of *P. brachypomus* in Brazil reached a production of approximately 10 thousand tons among the pure native species (MPA, 2013a).

In addition to being widely used in fishing, pirapitinga has been used in fish farming at crossing with tambaqui, generating the hybrid tambatinga that has desirable characteristics for management (SANTOS; FERREIRA; ZUANON, 2006). In addition, the species presents great importance in the gastronomy and the commercial fishing as ornamental fish (SOBRINHO et al., 1984).

In the aquaculture production of freshwater, tambaqui and pirapitinga have aroused the interest of fish producers from several Brazilian states, where tambaqui production exceeded 54 thousand tons and pirapitinga reached 780 thousand tons in 2010 (MPA, 2012).

1.3 Myxozoans

Ichthyofauna may to host a diversity of organisms and, with this, affect the development of host species (EIRAS, 2004; FEIST; LONGSHAW, 2006). Studies involving parasitic infections in fish have intensified in recent years due to the morbidity and mortality that these pathogens can cause in fish from fish farms and the natural environment (FEIST;

LONGSHAW, 2006) and the aquatic environment facilitates the penetration of these pathogens (EIRAS, 1994). Fish breeding systems are more prone to infections due to the high density and handling where disease outbreaks are most likely to occur. Thus, the mass production of cultured fish is always accompanied by pathogens (RÜCKERT et al., 2008). Among the fish pathogens, stand out the parasites of the phylum Cnidaria, the myxozoans.

Myxozoans are common and occasionally highly problematic parasites of fish (OKAMURA; GRUHL; BARTHOLOMEW, 2015). These parasites are multicellular, spore forming and which have tissue tropism and host specificity (BÉKÉSI; SZÉKELY; MOLNÁR, 2002; ATKINSON, 2011). They are formed by the junction of two to seven valves in the form of myxospores, which are the resistant forms of the parasite. They present polar capsules, containing a polar filament in spiral form with function of fixation in the host. Inside the myxospore are also sporoplasm with amoeboid forms, which are infecting cells for the new host. These parasites develop in plasmodia that may or may not cause tissue compression and an inflammatory response of the host (EIRAS, 1994; FEIST; LONGSHAW, 2006).

Approximately 2,400 species are known, distributed in 63 genera (FIALA; BARTOŠOVÁ-SOJKOVÁ; WHIPPS, 2015), with the most prominent genera being *Henneguya* Thélohan, 1892 and *Myxobolus* Bütschli, 1882. The majority of species are not pathogenic, however, some cause significant damages to their hosts in the natural environment and/or breeding systems (FEIST; LONGSHAW, 2006).

Myxobolus cerebralis Hofer, 1903 is one of the most studied species, with great impact on mortality of salmonids and wide distribution, causing a disease called "whirling disease" (EL-WELL et al., 2009). The parasite develops cysts in the cartilaginous tissue, from which it feeds, causing inflammation and damage to the adjacent central nervous system causing the fish to have a rotating behavior (GILBERT; GRANATH, 2003). In the rocky Mountain region, in

the United States, *M. cerebralis* was identified as responsible for the decline in population of rainbow trout also in a natural environment (ALLEN; BERGERSEN, 2002).

In addition to *M. cerebralis*, several other species of myxosporeans are reported to cause significant damage to their hosts, such as *Kudoa thyrsites* Whipps and Kent, 2006, which cause, *postmortem*, muscle liquefaction in several species of marine fish, leading to significant economic losses to commercial fishing (WHIPPS; KENT, 2006). *Ceratonova shasta* (syn. *Ceratomyxa shasta*) causes enteronecrosis and is a common parasite in salmonids from North American responsible for high mortality rates in juvenile salmon in the states of California and Oregon (ATKINSON; BARTHOLOMEW, 2010). *Henneguya ictaluri* Pote, Hanson and Shivaji, 2000 causes proliferative disease of the gills in catfish from breeding systems in the South of the United States (GRIFFIN et al., 2008). *Henneguya pseudoplatystoma* cause stretching and deformation of the filament and lamella structure in catfish in the South American (NALDONI et al., 2009). The plasmodial development of *Myxobolus hilarii* in the renal tubules of *Brycon hilarii* from fish farm, causes compression and deformation of adjacent tissues and destruction of renal tubule cells (CAPODIFOGLIO et al., 2016).

1.4 Life cycle

For the first time, Wolf and Markiw, 1984, elucidated the life cycle of *M. cerebralis* in a revolutionary study that changed the understanding of the biology of the myxosporids, demonstrating that the species presented an indirect life cycle, involving a myxospore stage in vertebrate hosts and an actinospore stage that developed in invertebrate hosts.

The life cycle of the myxozoans, in general, involves two host which typically are invertebrates and vertebrates, as definitive and intermediate hosts, respectively. They utilise annelids as definitive hosts and, in addition to fish, amphibians, reptiles and homeotherms are

used as intermediate hosts of myxosporeans (OKAMURA; GRUHL; BARTHOLOMEW, 2015).

In the species in which the life cycle was studied, such as *M. cerebralis*, in the annelid phase it is initiated by the infection of these by myxospores. These myxospores are ingested by the annelid (usually an oligochaete), and when in contact with the intestinal epithelium, releases the polar filaments for anchorage of the parasite in the new host. At this time, the valves open and myxospores amoeboid sporoplasm is released, penetrating between the epithelial cells of the intestine. The binucleated sporoplasm undergoes several nuclear divisions and this process results in the formation of actinospores, which when released from the intestinal epithelial cells of annelids, which enter the aquatic environment together with the feces and serve as a source of infection for the fish. Free actinospores in water are infective to the intermediate host (usually fish), which in contact with the vertebrate host extrude the polar filaments, anchoring in the new host. Then, the sporoplasm is released from the actinospore and penetrates the host through the gills, skin, buccal cavity and also, intestinal. After penetration, the sporoplasm wall dissolves and the infecting cells invade adjacent tissues. The parasite reaches the target tissue, by circulation or intercellular migration, where occurs the development of the plasmodium and sporogony process, characteristic for each species, where are formed myxospores with distinct morphology of the actinospores (FEIST; LONGSHAW, 2006).

1.5 Taxonomy of myxosporean species

Due to importance of myxosporeans parasites, many studies have been developed by scientists in natural environments and aquacultures for the purpose of to describe new species, and to understand hosts–myxosporeans life cycles, morphology, hosts infection sites and phylogenetic position (ALAMA-BERMEJO; RAGA; HOLZER, 2011; AZEVEDO et al., 2014; ROCHA et al., 2015).

The tradicional taxonomic identification of the myxosporeans is based on morphological parameters and developmental stages of the myxospores and of the plasmodia, as well as the specificity to the host and the tissue that infect have been widely used. For a long time, studies of identification of news myxosporean species have used only morphologic methods (LOM; ARTHUR, 1989; OKAMURA; GRUHL; BARTHOLOMEW, 2015). Most myxozoan species have a specificity to single host species or family, being used as important character for distinguishing myxosporean species (MOLNÁR, 1994). In additional, other characters such as tissue specificity, traits of developmental stages data can be clear incorporated to improve classification (OKAMURA; GHRUL, 2015).

However, taxonomic classification based only in morphological analyses has its limitations due the high level of natural morphological and morphometric variation in spores and, sometimes, to present debatable results due the similarity between myxospores, hampering the taxonomic classification (ATKINSON et al., 2015).

Thus, Kent et al. (2001); Zhao et al. (2008); Fiala et al. (2015) proposed that the utilization of morphological and molecular techniques is fundamental and complementary for identification of news species and also to avoid taxonomic confusion in myxosporeans.

In South America, our group of the Laboratory of Immunology of Parasites (LIP), of the University of São Paulo was the first to associate morphological and molecular data for myxosporean species with the description of *Myxobolus cordeiroi* Adriano, Arana, Alves, Silva, Ceccarelli, Henrique-Silva and Maia, 2009 parasite of *Zungaro jahu* from Brazilian Pantanal. After this, many species have been described based on morphological and molecular data in economically important fish (AGUIAR; ADRIANO; MATHEWS, 2017; AZEVEDO et al., 2010; CAPODIFOGLIO et al., 2016; CARRIERO et al., 2013; MATHEWS; MAIA; ADRIANO, 2016; MILANIN et al., 2010; MOREIRA et al., 2014; NALDONI et al., 2009; 2011; ROCHA et al., 2014; VELASCO et al., 2016; ZATTI et al., 2015; 2018).

Approximately 120 myxosporean species were described in the South American freshwater and marine environments (ADRIANO; OLIVEIRA, 2019). And still there are many undescribed myxosporean species (ZATTI et al., 2018), as well diversity of other myxozoans is underestimated (ATKINSON et al., 2015).

1.6 Molecular markers for identification of myxosporeans

Recent molecular studies with myxosporeans have as main approach the amplification of the gene responsible for the coding of the small subunit Deoxyribonucleic Acid (ssrDNA) (MOLNÁR et al., 2002; FERGUSON et al., 2008; FIALA, 2006; MILANIN et al., 2010; NALDONI et al., 2011; CARRIERO, 2011). This approach has been shown to be efficient for identifying and separating phylogenetically distant species, but it is not indicated to evaluate the occurrence of intraspecific variations or between phylogenetically closely related species.

Through morphologic and morphometric data, and sites of infection was observed that species of *Myxobolus* and *Henneguya* genera that infect *Piaractus corruscans* from the Pantanal are also found infecting *Piaractus reticulatum* from the same environment (CARRIERO, 2011). These data were corroborated by the molecular analyses, from the partial sequence of the ssrDNA (CARRIERO, 2011; ADRIANO et al., 2012). Nevertheless, alignments of sequences showed variations, even if small, between samples of the same parasite species found in *P. corruscans* compared with samples from *P. reticulatum*.

In 2003, Herbet et al. proposed a system of molecular identification, a universal "bioidentifier", for the discrimination of all living species on the planet. This system was called DNA Barcodes and consists of the amplification of the mitochondrial Cytochrome C Oxidase I (COI) gene, has a greater number of phylogenetically informative sites in relation to other mitochondrial genes (KNOWLTON; WEIGT, 1998).

Takeuchi et al. (2015), sequenced by the first time the mitochondrial genome of myxosporeans and observed that *Kudoa* mitochondrial genome are divergent from other metazoans, suggesting that the mitochondrial of Myxozoa is, in general, potentially divergent.

1.7 Mitochondrial organelles of myxosporeans

Myxosporeans were first reported in 1825 infecting *Coregonus fera* from Lake Geneva as threats for fish due to cysts proliferation in the musculature of the host (JURINE,1825). Since then, these parasites have become aim of interest of researchers (MLADINEO et al., 2010) and the increasing knowledge of new species, as well other studies has allowed to understading better the morphology, parasite-host relationship, evolution and biology of the myxosporeans (ALAMA-BERMEJO et al., 2011; OKAMURA et al., 2015).

The technological advances of transmission electron microscopy allowed new insights on functional biology and cytological features of myxosporeans (OKAMURA et al., 2015). Some these studies documented developmental stages and while other focused on features of the constituents such as pinocytotic vesicles, microvilli, Golgi complexes and mitochondria (LOM; DYKOVÁ, 1992).

Mitochondria are cytoplasmatic organelles existing in practically all types of eukaryotic cells vegetables and animals (NICHOLLS; FERGUSON, 2002), in number proportional to the metabolic activity of each one, being then variable in the different cells, and, also, it is the primary source of hightly energetic compost in the cell (BEAL, 2005; BRAND; NICHOLLS, 2011).

In myxosporeans, mitochondria are presents in greater numbers in the periphery of plasmodia which high levels of pinocytotic activity (FEIST et al., 2015). Transformations of mitochondrial organelles are associated with not yet understood changes in metabolic activity and ion traffic at different stages of development of the myxosporeans and, in addition, there is

a great variability in the density of mitochondrial matrix and in the shape of cristae (LOM; DYKOVÁ, 1996). Just like the process sporogenesis also appears to follow a pattern observed in many myxosporean species, with the early stages of myxospore formation in the plasmodial periphery and mature myxospores located in the central part of the plasmodia (CURRENT et al., 1979; CASAL et al., 2002; ADRIANO et al., 2006).

Typically, myxospores are physiologically inactive, usually relatively immobile and rigid (FEIST et al., 2015) and are more resistant which malacospores and actinospores, being able to withstand extremes in temperature, desiccation and extended dormancy (HALLET; BARTHOLOMEW, 2012).

To understand the role of mitochondria in *Kudoa* myxospores, Takeuchi et al. (2015) observed *in vivo* the aerobic respiration, metabolism, and structure of the mitochondria, besides of the mitochondrial genome. The authors found eight proteins involved in the oxidative phosphorylation and, the aerobic respiration was observed, with fluorescent dye which detects proton gradients in the mitochondria, next to the polar capsules, suggesting that the aerobic respiration must to occur therein and is necessary to measure the acvitiy of each complex of oxidative phosphorylation for understading of the cellular respiration in myxosporeans.

HYPOTHESES

2 HYPOTHESES

- There is a diversity of myxosporean species infecting *Colossoma macropomum* and *Piaractus brachypomus* from the Amazon basin.

- COI gene can be a good molecular marker for the identification of new species.

OBJECTIVES

3 OBJECTIVES

3.1 General Objective

Identify and describe the species of myxosporeans found infecting tambaqui (*C. macropomum*) and pirapitinga (*P. brachypomus*) from the Amazon basin.

3.2 Specifics Objectives

3.2.1 Carry out taxonomic studies of myxosporean parasites of tambaqui (*C. macropomum*) and pirapitinga (*P. brachypomus*), through morphological and molecular, by sequencing the ssrDNA, data;

3.2.2 Evaluate the phylogenetic relationship of parasites involved in this study in relation to other myxosporeans described in Brazil and in the world;

3.2.3 Sequence the mitochondrial gene Cytochrome C Oxidase I of myxosporeans in *C. macropomum* and *P. brachypomus*.

4 MATERIALS AND METHODS

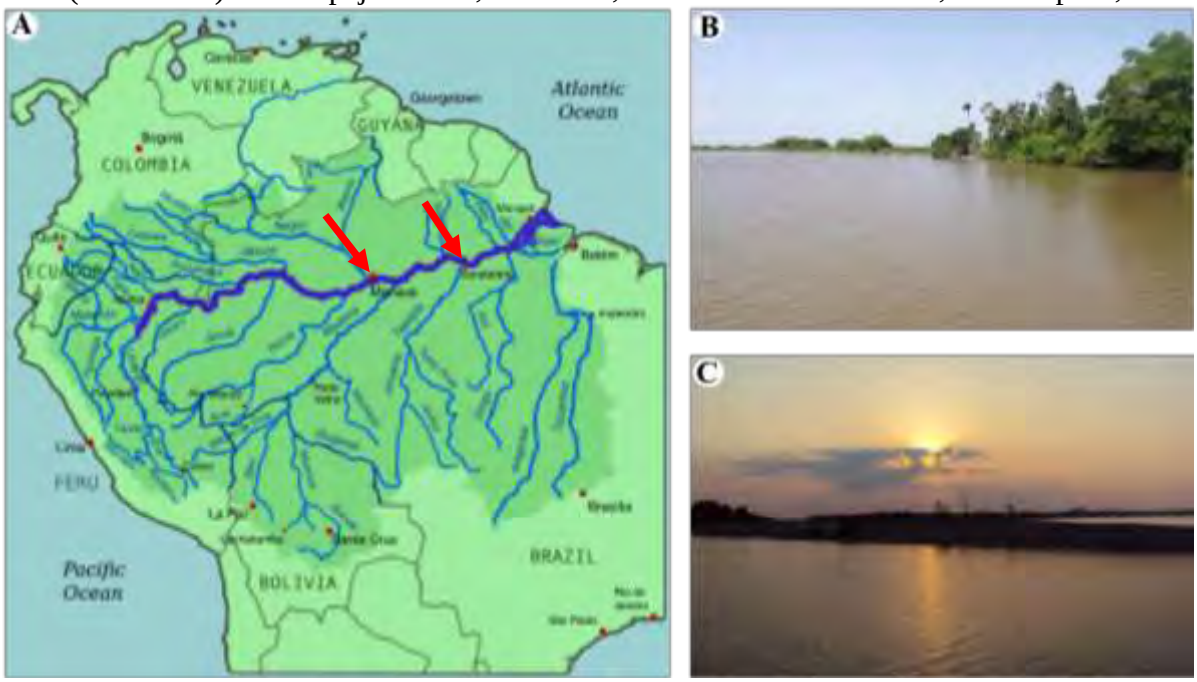
4.1 General Methodology

This project was linked to the project "Systematics and parasite-host interaction of parasites of Myxozoa subphylum in fish of economic importance of the Amazon Basin" of the Prof. Dr. Edson Aparecido Adriano, process FAPESP: 2013/21374-6 and was developed in the laboratory of Immunology of Parasites (LIP), of the Department of Veterinary Medicine, of the Faculty of Animal Science and Food Engineering, University of São Paulo, FZEA/USP, Pirassununga – SP, in partnership with Department of Ecology and Evolution Biology, Federal University of São Paulo – UNIFESP, Campus Diadema – SP; Department of Biochemistry and Cell Morphology of the Institute of Biology IB/UNICAMP, Campinas, SP.

4.1.1 Samples collection

The collections were performed in the Amazon basin, in the Tapajós and Solimões rivers, Santarém, PA and Manacapuru, AM, respectively (Figures 1 A – B).

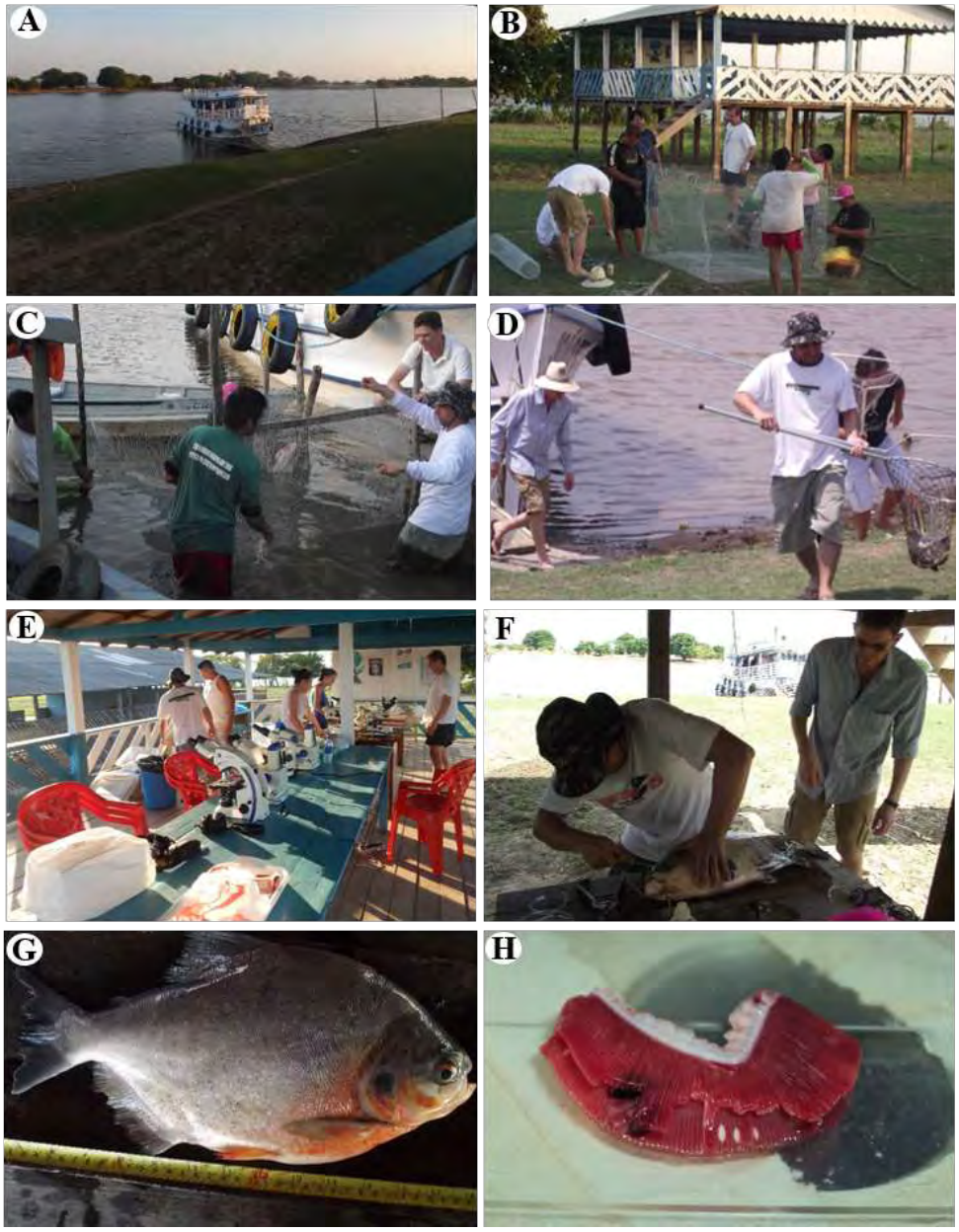
Figure 1. Collections local. **A** – Map showing the Amazon basin with highlight for Amazon river (red arrows). **B** – Tapajós River, Santarém, PA. **C** – Solimões River, Manacapuru, AM.



Source: A – By KMusser - https://commons.wikimedia.org/w/index.php?curid=24862395. B and C – personal archive.

Boats were used to reach the site collection (Figure 2A). Fish were caught (SISBIO nº 44268-4 and SisGen nº A33CB83) using boats and nets and kept in captivity (Figure 2B and C) until they were transported alive (Figure 2D) to the field laboratory (Figure 2E), mounted in the proximity of the collection point. After euthanasia, by transection of the spinal cord (Figure 2F), methodology approved by the Ethics Committee of the Faculty of Animal Science and Food Engineering, FZEA/USP, Process USP: 14.1.391.74-9 (ATTACHMENT B), the fish were weighed and measured (Figure 2G) and, subsequently, a necropsy was performed with exposure of the gills and visceral cavity, in order to detect the presence of parasites or cysts (Figure 2H). Fragments of tissues of the host fish containing plasmodia were collected and fixed in formaldehyde and absolute ethanol, respectively, for morphological and molecular analyses.

Figure 2. Field collection. **A** - Boat used for fishing. **B** - Assembly of tanks. **C** - Tank network where the fish caught were kept until the necropsy. **D** - Fish transport. **E** - Field laboratory. **F** - Euthanasia by transection of the spinal cord. **G** - Biometry and necropsy of fish. **H** – Plasmodia of myxosporeans.



Source: personal archive.

4.1.2 Molecular characterization of host fish

The correct identification of the host fish was performed through the amplification of the α-tropomyosin gene. The tissue of the fish infected with plasmodium was macerated in a slide and collected in 1,5 mL microtubes for DNA extraction.

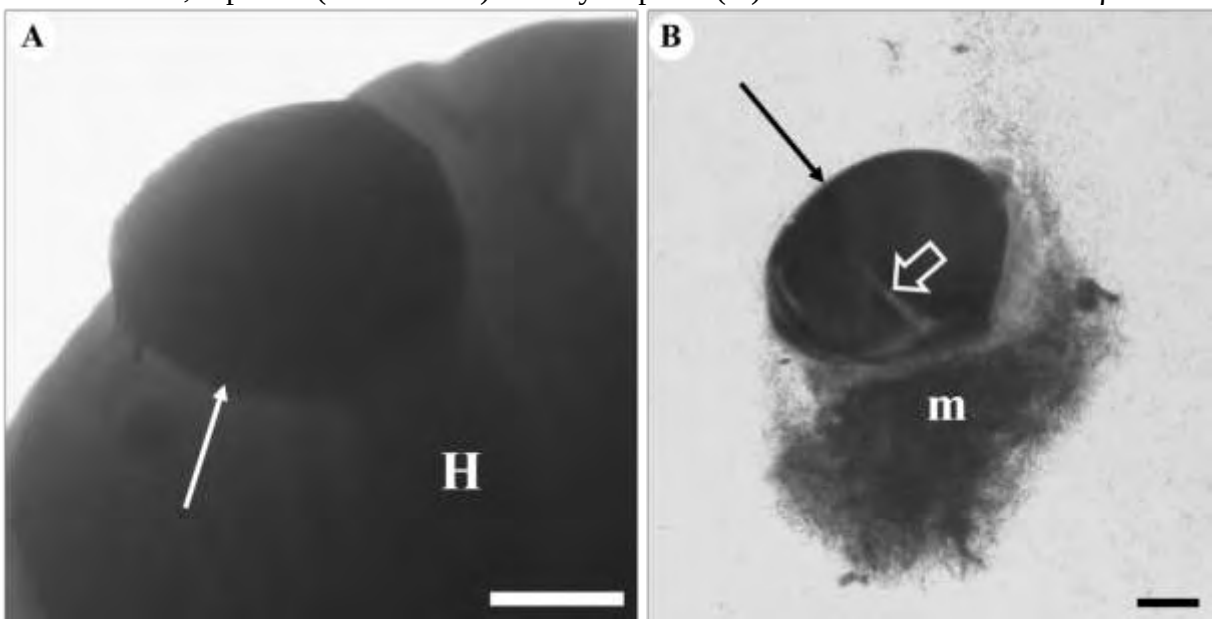
DNA from the tissues infected of the collected fish was extracted with the DNeasy® Blood & Tissue kit (Qiagen Inc., California, USA), according to the manufacturer's instructions. DNA extracted was quantified in NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA) at 260 nm.

The amplification and sequencing of the α-tropomyosin gene of the specimens of fish infected was performed according to Hashimoto et al. (2011), using the primer pair TROPF and TROPR (Table 1) and the product obtained was sent for sequencing. The obtained sequences were submitted to Basic Local Alignment Search Tool nucleotide (BLASTn) analysis of the GenBank database (ALTSCHUL et al.,1990).

4.1.3 Morphologic analyses

Myxosporean cysts (Figure 3A) fized in formaldehyde were carefully separated from the host tissue and individually ruptured (Figure 3B) in glass slides. The released myxospores (Figure 3B) were analyzed under light microscopy and then photographed. The myxosporean species were identified and measured according to the criteria established by Lom and Arthur (1989). The slides were then fixed with methanol and stained with Giemsa to be deposited in the collection of the Adão José Cardoso Museum of Zoology, of the State University of Campinas (UNICAMP), Brazil.

Figure 3. Plasmodium of myxosporeans. **A** – Tissue of the host (H) infected with plasmodium (white arrow). Scale bar = 400 µm. **B** – Plasmodium (black arrow) separated individually of the host tissue, ruptured (white arrow) and myxospores (m) released. Scale bar = 100 µm.



Source: personal archive.

To characterize the species, the morphological characteristics of mature myxospores were analyzed, such as: myxospore shape and structure, polar capsule form, myxospore projections or caudal appendages, size relation between polar capsules, relative position of the anterior end of the polar capsules, position of sporoplasm in the spore cavity, dimensions of myxospores, variability of shape and size of myxospores. These data were obtained by light microscope coupled to a computer equipped with the Axivision 4.1 image capture program.

4.1.4 Molecular analyses

After capturing images, the slides containing myxospores were washed with PBS buffer and the myxospores were collected in sterile 1.5 mL microtubes for DNA extraction.

DNA extraction

DNA from the isolated samples was extracted with the DNeasy® Blood & Tissue kit (Qiagen Inc., California, USA), according to the manufacturer's instructions and was quantified in NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA) at 260 nm.

Polymerase Chain Reaction (PCR)

All PCR were performed with final volume of 25 µL, containing 10-50 ng genomic DNA, 12.5 µL of 2 × DreamTaq PCR Master Mix (Thermo Scientific – Carlsbad, California, USA), 0.2 µM of each primer and free water of nucleases (Thermo Scientific – Carlsbad, California, USA). For genes amplification, two specific pairs of primers were used, some them designed for this study (Table 1, Figure 4). A ProFlex PCR Systems (Applied Biosystems™ Inc., California, USA) thermocycler with specific program were used to each gene (Table 2).

Table 1. Primers used in the amplification and sequencing of the ssrDNA, α-tropomyosin and Cytochrome C oxidase (COI) genes.

Gene	Primer	Sequence 5' – 3'	Reference
ssrDNA	TEDF	AATTACCCAATCCAGACAAT	Capodifoglio et al. (2015)
	ACT1R	AATTTCACCTCTCGCTGCCA	Hallett and Diamant (2001)
	ERIB1 F	ACCTGGTTGATCCTGCCAG	Barta et al. (1997)
	ERIB10 R	CTTCCGCAGGTTCACCTACGG	Barta et al. (1997)
	MC5 F*	CCTGAGAAACGGCTACCACATCCA	Molnár et al. (2002)
	MC3 R*	GATTAGCCTGACAGATCACTCCACGA	Molnár et al. (2002)
	BOBF	GTTACAGCATGGAACGAAC	Present study
α-tropomyosin	TROPF	CGGTCAGCCTCTTCAGCAATGTGCTT	Friesen et al. (1999)
	TROPR	GAGTTGGATCGGGCTCAGGAGCG	Friesen et al. (1999)
COI	LCO F	GGTCAACAAATCATAAAGATATTG	Folmer et al. (1994)
	HCO R	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
	F14 F	CCTTAATTGGTGATTTCCTG	Takeuchi et al. (2015)
	R19 R	TCTAGGGATTCCACAAAGAC	Takeuchi et al. (2015)
	TITIF	ACTATGTTGGGGGACGACCATC	Present study
	GABIR	GTTATAGCCCCGGCCAATACGG	Present study
	XISF	AGTAGCCATAGGHTAYTGAAG	Present study
	XISR	ACAGCCTAAATAGCCTG	Present study
	COIF	AGGCTGAACCATGTATCC	Present study

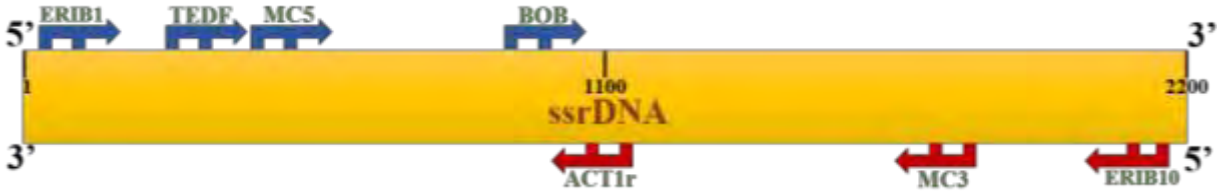
*Primers used only for sequencing.
Source: own authorship.

Table 2. Programs used in the thermocycler for amplification reactions. SsrDNA – small subunit ribosomal DNA; COI – Cytochrome C Oxidase I.

Molecular Marker	Program				
	Initial Step	35 cycles			Final Step
		Denaturation	Hybridization	Extension	
ssrDNA	95°C 5'	95°C 60"	60°C 60"	72°C 90"	72°C 5'
α-tropomyosin	-	95°C 30"	64°C 30"	72°C 10"	_
COI*	-	95°C 60"	40°C 60"	72°C 90"	72°C 7'

*preliminary conditions described by Folmer et al. (1994).
Source: own authorship.

Figure 4. Schematic representation of the localization of primers for amplification and sequencing of ssrDNA.



Source: own authorship.

All PCR products were subjected to 1.5% agarose gel electrophoresis in TAE buffer (EDTA Tris-Acetate: Tris 40 mM, Acetic Acid 20 mM, EDTA 1 mM), stained with Sybr Safe DNA gel stain (Thermo Scientific – Carlsbad, California, USA) and analyzed in Light Emitting Diode (LED) transilluminator (Kasvi, Paraná, Brazil). The size of the amplified fragments was estimated by comparison to the standard 1 Kb Plus DNA Ladder (Thermo Scientific – Carlsbad, California, USA).

The PCR products were then purified using the QIAquick PCR kit (Qiagen Inc., California, USA), according to the manufacturer's specifications. The purified product was quantified in 2% agarose gel electrophoresis and compared to Low DNA Mass (Thermo Scientific – Carlsbad, California, USA).

Sequencing

The sequencing of the purified PCR products was performed with primers specific for each gene (Table 1), in the ABI 3730 DNA sequencer (Applied Biosystems™ Inc., California,

USA), using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems™ Inc., California, USA).

The obtained sequences were then visualized, edited and aligned in the program BioEdit 7.1.3.0 (HALL, 1999) using the algorithm ClustalW (THOMPSON, 1994), forming a consensus sequence for each sample. Each consensus sequence was submitted to BLASTn (ALTSCHUL et al., 1990) analysis for the search for similarity in the GenBank database National Center for Biotechnology Information (NCBI).

4.1.5 Phylogenetic analyses

For the phylogenetic analyses, alignments were performed with sequences of myxosporeans deposited in GenBank database by the algorithm ClustalW (THOMPSON et al., 1994) coupled to the program BioEdit 7.1.3.0 (HALL, 1999).

The maximum likelihood method was performed by the PhyML 3.0 program (GUINDON et al., 2010), with automatic model selection by Smart Model Selection (SMS) and bootstrap value of 1000 repetitions. The resulting trees were visualized in the FigTree 1.3.1 program (RAMBAUT, 2008) and edited with Adobe Illustrator (Adobe Systems Inc., San Jose, USA).

The p-distance analysis was performed by the MEGA 7.0 software (KUMAR; STECHER; TAMURA, 2016) to compare the genetic differences among the species studied with other myxosporeans species of the GenBank database.

Host fish data, such as family and/or order, were obtained from the FishBase website (FROESE; PAULY, 2018).

4.2 Supplementary Methodology

Due to the availability of material and the willingness to enrich the data of this study, histological and electron microscopy analyses were performed for some samples. Microscopy fluorescence was perfomed to observe the behavior *in vivo* of mitochondria in *Henneguya* spp.

4.2.1 Histologic analyses

For histologic analyses, fragments containing plasmodia were fixed in buffered 10% formalin for 24h and placed in paraffin. Sections of 4 µm thickness were then stained with hematoxylin-eosin and analyzed in Leica DM 1000 Microscope coupled with Leica EC3 camera and LAS EZ image capture software.

4.2.2 Transmission Electron Microscopy analyses

For transmission electron microscopy, small fragments of infected tissue were fixed in 2.5% glutaraldehyde diluted in 0.1 M sodium cacodylate (pH 7.4) for 12h, washed in glucose-saline solution for 2h and fixed in OsO_4 for 4h. After, these samples were dehydrated in increasing concentrations of acetone and embedded in Embed 812 resin. Uranyl acetate citrate was used to treat ultrathin sections that, posteriorly, were examined in a LEO 906 transmission electron microscope operating at 60 kV (UNICAMP).

4.2.3 Fluorescence Microscopy analyses

Live myxospores were collected of fins of *Astyanax altiparanae* in a fish farm and kept in microtube containing PBS. Spores were centrifuged and stained with MitoTracker Orange® (Thermo Scientific – Carlsbad, California, USA) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Thermo Scientific – Carlsbad, California, USA) and visualized in inverted microscope Zeiss AxioVert A1 coupled with photographic camera Axio Can 503.

The results of this work are divided in four chapters, which generated two articles. The first one already published and the second sent for publication. The third chapter refers to results

obtained by undergraduated Caroline Munhoz Meira. The fourth chapter refers to studies of the COI gene and fluorescence microscopy in myxosporeans.

Specific methodologies are described in each chapter.

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5 RESULTS

CHAPTER 1. THE RESOLUTION OF THE TAXONOMIC DILEMMA OF *Myxobolus colossomatis* AND DESCRIPTION OF TWO NOVEL MYXOSPOREANS SPECIES OF *Colossoma macropomum* FROM THE AMAZON BASIN

(PAPER PUBLISHED IN ACTA TROPICA – ATTACHMENT C)

CHAPTER 1. THE RESOLUTION OF THE TAXONOMIC DILEMMA OF *Myxobolus colossomatis* AND DESCRIPTION OF TWO NOVEL MYXOSPOREANS SPECIES OF *Colossoma macropomum* FROM THE AMAZON BASIN

ABSTRACT

This study presents morphologic, molecular and phylogenetic data about two new species of the genus *Myxobolus* and of the previously described *Myxobolus colossomatis,* all which are found infecting the *Colossoma macropomum*, a fish whose natural habitat is the Amazon Basin of Brazil, from where the specimens for this study were caught. A total of 51 *C. macropomum* specimens were examined between October of 2014 and January of 2016. Plasmodia of the myxosporeans were found infecting several organs: *Myxobolus* n. sp. 1 and *Myxobolus* n. sp. 2 were respectively found in the inner face of the operculum and in the wall external surface of the stomach, intestine and gill arch. *Myxobolus* n. sp. 1 were 9.6 ± 0.4 µm in length, 7.0 ± 0.3 µm in width and 5.0±0.3 µm in thickness of the myxospore. *Myxobolus* n. sp. 2 measured 19.1 ± 0.4 µm in length, 9.4 ± 0.3 µm in width and 8.3±0.4 µm in thickness. The polar capsules, which were elongated, showed 4.3 ± 0.4 µm in length and 1.9 ± 0.1 µm in width for *Myxobolus* n. sp. 1 and 10.5 ± 0.2 µm in length and 2.5 ± 0.1 µm in width for *Myxobolus* n. sp. 2. *Myxobolus colossomatis* had two myxospores morphotypes: 1) Ellipsoidal myxospores measuring 11.6 ± 0.4 µm in length and 7.6 ± 0.2 µm in width. Their elongated polar capsules measured 5.6 ± 0.2 µm in length and 2.5 ± 0.2 µm in width; 2) Oval myxospores measuring 10.4 ± 0.5 µm in length and 7.7 ± 0.3 µm in width. Their polar capsules were 5.4 ± 0.2 µm in length and 2.4 ± 0.0 µm in width. The number of coils of the polar filament was 7-8 coils. The molecular comparison of ssrDNA showed a genetic divergence of 10.3% between *Myxobolus* n. sp. 1 and *M. colossomatis,* 22.4% between *Myxobolus* n. sp. 1 and *Myxobolus* n. sp. 2, and 23.2% between *Myxobolus* n. sp. 2 and *M. colossomatis*. *Myxobolus* cf. *colossomatis,* a parasite of *Piaractus*

mesopotamicus, showed 11.1% of genetic divergence to *M. colossomatis*, demonstrating them to be a distinct species. Phylogenetic analysis, based on sequences of the ssrDNA, showed the *Myxobolus* n. sp. 1 to be a sister species of *M. colossomatis,* and it also showed *Myxobolus* n. sp. 2 to be a sister branch in the lineage composed by *Myxobolus* cf. *cuneus* and *Henneguya pellucida*.

Keywords: *Myxobolus*; *Myxobolus colossomatis;* ssrDNA sequencing; phylogeny; prevalence.

INTRODUCTION

Colossoma macropomum Cuvier, 1816, is a serrasalmid fish commonly known in Brazil as *tambaqui*. It is considered the second largest scaled fish from Amazon River Basin, and in its natural environment it may reach up to 100 cm long and weigh 30 kg (NAKATANI et al., 2001). *Tambaqui* is the most cultivated species in the Brazilian Amazon (GOMES et al., 2010; LOPERA-BARRERO et al., 2011) and one of the most important species in Brazilian aquaculture. Almost 60.000t were marketed in 2010, with 4.200t caught by fishing and 54.300t harvested from fish farms (MPA, 2012). C*olossoma macropomum* is also important when it is crossbred in fish farms with other serrasalmid~~s~~ species, such as the *Piaractus mesopotamicus* Holmberg, 1887, which results in a hybrid called *tambacu*, or with the *Piaractus brachypomus* Cuvier, 1818, which results in the hybrid *tambatinga*. These produced around 21.600t and 5.000t respectively in 2010 (MPA, 2012).

Due to its economic importance as one of the most important species of the amazonic icthyofauna (GOULDING; CARVALHO, 1982), *C. macropomum* has been targeted for the study of several parasitic infections, including those of the myxosporeans. Myxosporea is a morphologically and biologically diverse group of parasite cnidarians that cause diseases and threaten fish in their natural habitats and in fish farms in various parts of the world. They are

also responsible for high mortality rates in some species and are associated with ecological and economic impacts (LOM; DYKOVÁ, 2006; ELWELL et al., 2009; FIALA; BARTOŠOVÁ, 2010). Despite the importance of *C. macropomum*, only *Myxobolus colossomatis* Molnár and Békési, 1993 and *Ceratomyxa vermiformis* Adriano and Okamura, 2017 have so far been described in this iconic Amazon fish. In this study, however, morphologic and small subunit ribosomal DNA (ssrDNA) sequencing data were obtained from *M. colossomatis* and of two new *Myxobolus* Bütschli, 1882 species found parasitizing *tambaqui* in the Amazon River Basin, Brazil, and a phylogenetic analysis is also provided.

MATERIAL AND METHODS

Specimens of *C. macropomum* were caught (SISBIO nº 44268-4 and SisGen nº A33CB83) in the Tapajós River in the municipality of Santarém, Pará State (N = 35) and also in the Solimões River in the municipality of Manacapuru, Amazônia State (N = 16), both in the Brazilian Amazon. The fish were caught using nets and/or hooks and taken alive to a field laboratory near their point of collection. Before the examination, the fish were euthanized with spinal transection methodology approved by the ethics research committee of the University de São Paulo – Process USP 14.1.391.74.9 (ATTACHMENT B) measured, necropsied and examined for myxosporeans.

2.1 Morphological analyses

Myxosporean plasmodia were carefully separated from the host tissue and individually ruptured in glass slides. A total of 30 mature myxospores were analyzed in a light microscope, photographed and measured according Lom and Arthur (1989). The dimensions of the myxospores are expressed as mean ± standard deviation in µm. These slides were then fixed

with methanol and stained with Giemsa to be deposited in the collection of the Adão José Cardoso Museum of Zoology, of the State University of Campinas (UNICAMP), Brazil.

2.2 Molecular analyses

SsrDNA analyses were performed in the samples of myxospores and taxonomic and phylogenetic studies were initiated. The α-tropomyosin gene of the host were sequenced from host tissue aiming the correct host identification of the fish.

The DNA of each sample was extracted separately with DNeasy® Blood & Tissue kit (Qiagen Inc., California, USA) according to manufacturer's instructions. PCR reactions were performed with a final volume of 25 µL containing 10 - 50 ng of genomic DNA, DreamTaq 12.5 µL of 2 × PCR Master Mix (Thermo Scientific – Carlsbad, California, USA), 0.2 µM of each specific primer and nuclease free water (Thermo Scientific – Carlsbad, California, USA). For ssrDNA amplification, primer pairs ERIB1 (BARTA et al., 1997) with ACT1R (HALLET; DIAMANT, 2001) and TEDF (CAPODIFOGLIO et al., 2015) with ERIB10 (BARTA et al., 1997) were used. The amplifications of the α-tropomyosin gene were performed using the primer pair TROPF and TROPR (FRIESEN et al., 1999) in accordance with Hashimoto et al. (2011), followed by modifications. A ProFlex PCR Systems thermocycler (Applied Biosystems™ Inc., California, USA) was utilized for amplification of the genes with a program as follows: ssrDNA (initial denaturation at 95°C for 5 minutes; 35 cycles: 95°C for 60 s, 60°C for 60 s and 72°C for 90 s; final extension at 72°C for 5 minutes), α-tropomyosin (35 cycles: 95°C for 30 s, 64°C for 30 s and 72°C for 10 s). All PCR products were analyzed by 1.5% agarose gel electrophoresis in a TAE buffer (Tris–Acetate EDTA, Tris 40 mM, Acetic Acid 20 mM, EDTA 1 mM), stained with Sybr Safe DNA gel stain (Thermo Scientific – Carlsbad, California, USA), and analyzed in a LED transilluminator (Kasvi, Paraná, Brazil). The sizes of the amplified fragments were estimated by comparison with the 1 Kb Plus DNA Ladder

(Thermo Scientific – Carlsbad, California, USA). The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc., California, USA) and sequenced using the same primer pairs used in the reaction, as well as the additional primers for sequencing of ssrDNA: BOBF 5'-TGTTACAGCATGGAACGAAC-3' (Present study); MC5 and MC3 (MOLNÁR et al., 2002), in order to overlap the fragments obtained. Sequencing of all samples was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™ Inc., California, USA) and an ABI 3730 DNA sequencing analyzer (Applied Biosystems™ Inc., California, USA).

The DNA sequences obtained were visualized and assembled using BioEdit 7.1.3.0 (HALL, 1999) software and compared with other myxozoans sequences available in the NCBI database using BLASTn (ALTSCHUL et al., 1997) for confirmation of myxozoans and *C. macropomum* DNA.

2.3 Phylogenetic analysis

The alignment was performed using the closest myxozoans sequences to each one of the species here studied, based on GenBank data by BLASTn. The sequences of the ssrDNA of myxosporeans were obtained from the NCBI database, and a total of 31 sequences of *Henneguya* species, 45 of *Myxobolus* species and one of *Thelohanellus* Kudo, 1933 species were inserted in the analysis. *Ceratomyxa amazonensis* Mathews, Naldoni, Maia and Adriano, 2016 and *Ceratomyxa vermiformis* Adriano and Okamura, 2017 were chosen as the outgroup. All sequences were aligned using the ClustalW algorithm (THOMPSON et al., 1994) by software BioEdit 7.1.3.0 (HALL, 1999).

Phylogenetic analysis was conducted using maximum likelihood method in PhyML software (GUINDON et al. 2010), with NNI search, automatic model selection by SMS, under a GTR + G + I substitution model (with four categories), equilibrium frequencies optimized,

transition/transversion ratio estimated, proportion of in-variable sites estimated (0.246) and gamma shape parameter estimated (0.533). Bootstrap analysis (1000 replicates) was employed to assess the relative robustness of the tree branches.

The phylogenetic tree was viewed using FigTree v1.3.1 (RAMBAUT, 2008) and edited with Adobe Illustrator (Adobe Systems Inc., San Jose, USA). The pairwise method, with the p-distance model (KUMAR; STECHER; TAMURA, 2016), was carried out in the MEGA 7.0 software to compare the genetic differences of the ssrDNA sequences among the focus species of this study with *Myxobolus* spp. of other serrasalmids fish. The data of the host fishes, as family and/or order, were obtained from FishBase web site (FROESE; PAULY, 2017).

2.4. Statistical analysis

The G test (with Williams correction) was performed with the BioEstat 5.0 (AYRES et al., 2000); it had a level of significance set at $p < 0.05$ and was used to compare if there were any significant differences of the prevalence among the *Myxobolus* species and the places of study.

RESULTS

Epidemiologic data

In addition to the morphological identification of all the hosts, this study also provided the identification of three *tambaqui* specimens by the sequencing of α-tropomyosin and comparison with GenBank data by BLASTn.

Three *Myxobolus* species were found infecting *C. macropomum* specimens in this study. Of the total of 51 *tambaqui* sampled, 35 (68.6%) had infections by at least one of these *Myxobolus* species. *Myxobolus colossomatis* was observed in 6.2% (1/16) of the fish examined

in Amazonas State and in 17.1% (6/35) in the Pará State. These prevalence did not differ significantly (G test = 1.14, df = 1). The parasite was found in several organs, such as the fin, pyloric cecum, gill arch and abdominal cavity. Two of the three *Myxobolus* species were unknown myxosporeans, and are below described: *Myxobolus* n. sp. 1 was found infecting the inner surface of the operculum, with prevalence of 6.2% (1/16) in Amazonas State and 5.7% (2/35) in Pará State, and these prevalence did not differ significantly between (G test = 0.004, df = 1); *Myxobolus* n. sp. 2 was observed parasitizing the external wall of the stomach, intestine and epithelium of the gill arch. The prevalence was of 8.5% (3/35) in Pará State and this species was not found in Amazonas State. The comparison of the total prevalence among each one of the three *Myxobolus* species studied showed that there were not significant differences (G test = 2.76, df = 2).

Taxonomic data

***Myxobolus* n. sp. 1** (Figures 5-6, A)

Description: plasmodia white and ~ 0.1 mm were found in the inner surface of the operculum of *C. macropomum.* Mature myxospores were ellipsoid in frontal view (Figure 5A), measuring 9.6 ± 0.4 (9.1 to 10.8) µm in length, 7.0 ± 0.3 (6.5 to 7.6) µm in width and biconvex in lateral view, with 5.0 ± 0.3 (4.6 to 5.5) µm in thickness and valves symmetric. The polar capsules elongated and were equal in size, measuring 4.3 ± 0.4 (3.3 to 5.0) µm in length and 1.9 ± 0.1 (1.6 to 2.2) µm in width, and occupying half of the spore body length (Figure 5A). It was not possible to count the number of coils of the polar tubules by light microscopy (Table 3).

Figure 5. Photomicrographs of mature myxospores of *Myxobolus* spp. parasites of *Colossoma macropomum* from the Amazon basin. **A** – Frontal view of *Myxobolus* n. sp. 1 infecting the inner face of the operculum. **B** – Frontal and sutural view of *Myxobolus* n. sp. 2 found infecting the external surface of the stomach. **C** – Frontal and sutural view of *Myxobolus colossomatis* found infecting the pyloric cecum. Note the sutural line (black arrow). Scale bars = 10 µm.

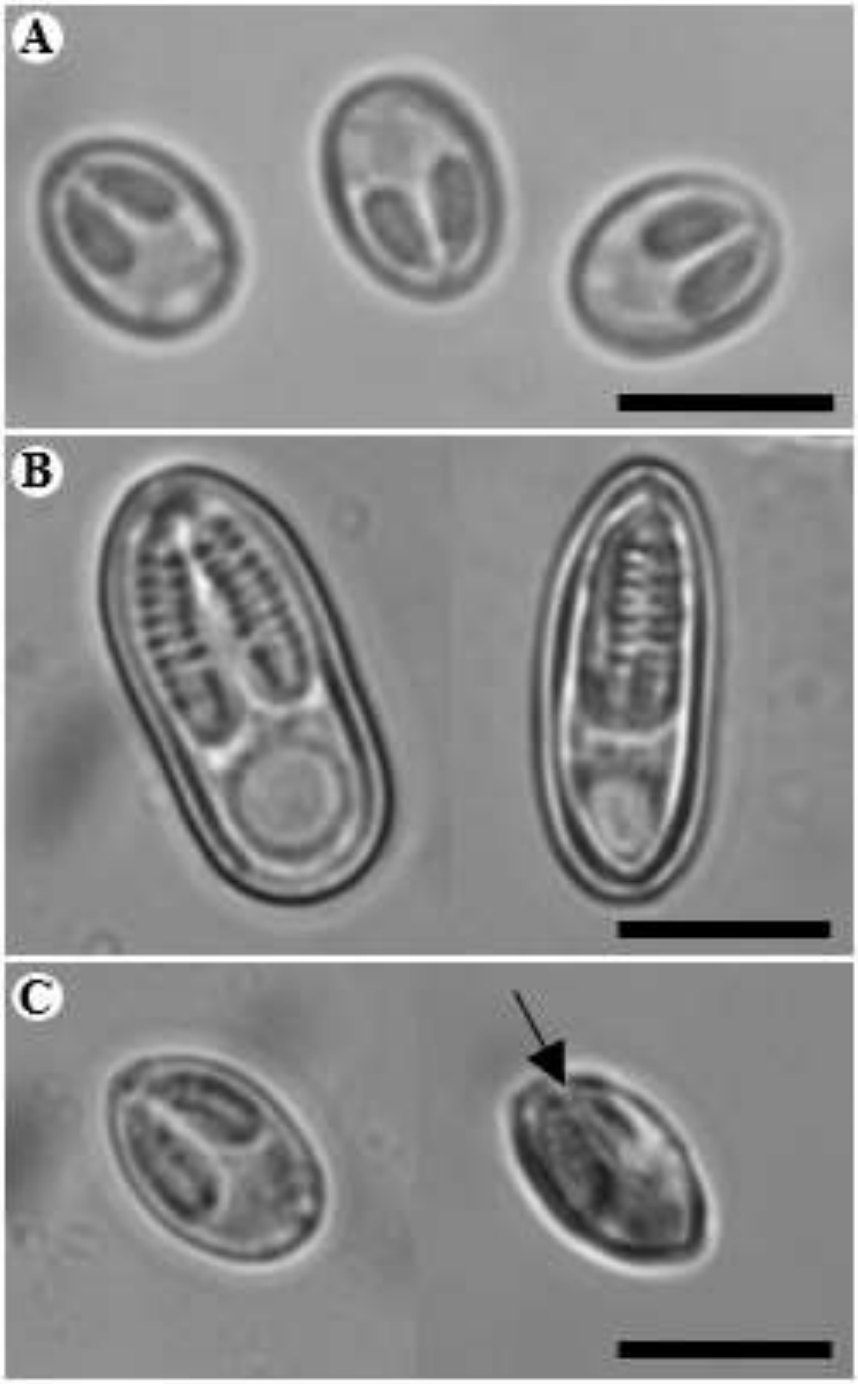
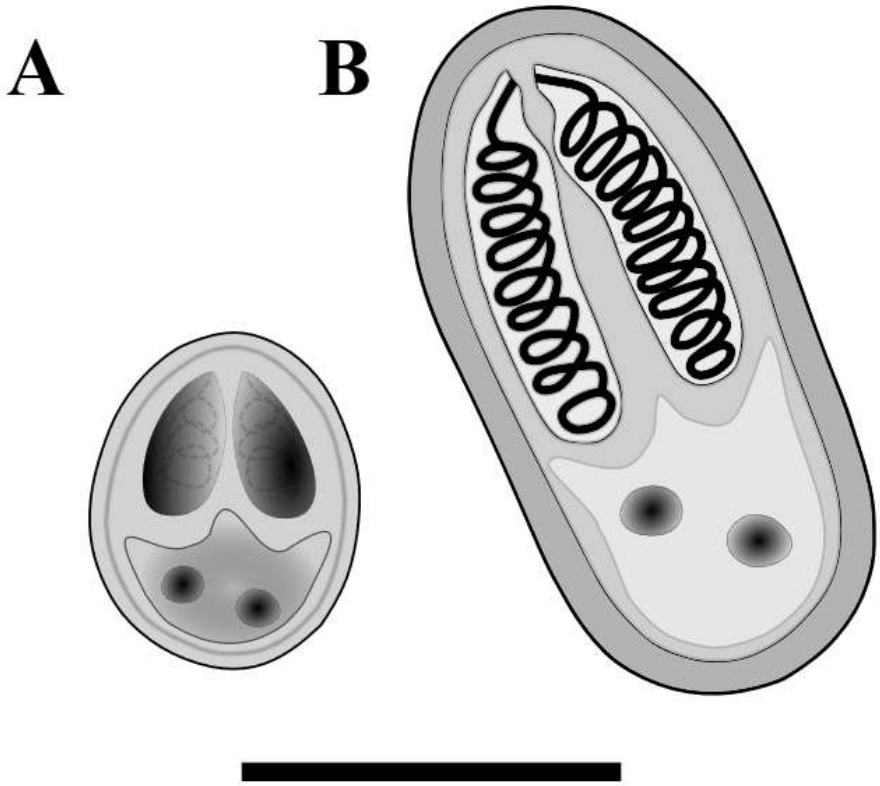


Figure 6. Schematic representations. **A** – *Myxobolus* n. sp. 1. **B** – *Myxobolus* n. sp. 2. Scale bars = 10 µm.



Type host: *Colossoma macropomum* Cuvier, 1816, Characiformes: Serrasalmidae.

Localities of collection: Solimões and Tapajós Rivers, municipality of Manacapuru, Amazonas State and Santarém, Pará State, respectively, Brazil.

Prevalence: 3 of 51 (5.9%).

Site of infection: Inner surface of the operculum.

Type of material: Slides stained with myxospores were deposited in the collections of the Adão José Cardoso Museum of Zoology, of the State University of Campinas (UNICAMP), Brazil (accession number ZUEC – MYX89).

Molecular data: Partial sequence, with 1956 bp of the ssrDNA were deposited in GenBank under the accession number MK032219.

Remarks: *Myxobolus* n. sp. 1 was compared with all species of *Myxobolus* known to infect freshwater fish from South America and other continents (EIRAS et al., 2005, 2014), and also with the other new species described in this study. Initially, the comparison was done with parasite species of *C. macropomum. Myxobolus* n. sp. 1 differs in some morphological and morphometric aspect as length and width of the body, length and width of the polar capsules and thickness in comparison to *Myxobolus* n. sp. 2 and *M. colossomatis* (Table 3). Of the species found infecting other fishes of the Serrasalmidae family, *Myxobolus* n. sp. 1 presented measurements closest with *M.* cf. *colossomatis,* a parasite of *Piaractus mesopotamicus,* to which this new species was similar in length and width of the polar capsules (respectively: 4.3/1.9 for *Myxobolus* n. sp. 1 and 4.4/1.8 for *M.* cf. *colossomatis*), but the body shape differed in the anterior extremity, once *Myxobolus* n. sp. 1 presented rounded anterior end and *M.* cf. *colossomatis* exhibited tapered anterior end. In comparing the *Myxobolus* with parasite species of other hosts, *Myxobolus* n. sp. 1 differed in morphometric/morphological features in at least one of the following properties: the size and/or shape of the plasmodia and myxospores, the number of polar tubules coils, the site of infection, and region of occurrence (EIRAS 2002; EIRAS; ADRIANO 2012).

The ssrDNA sequencing of *Myxobolus* n. sp. 1 resulted in a partial sequence of 1956 bp, which did not match with any myxosporeans sequences available in GenBank database. Analysis of the genetic similarity of the myxosporean parasites of serrassalmid fish showed that the closest species to *Myxobolus* n. sp. 1 was *Myxobolus colossomatis* (Present study) with a 10.3% of difference (Table 4). Thus, based on morphological and molecular data, *Myxobolus* n. sp. 1 is proposed to be a new taxon.

***Myxobolus* n. sp. 2** (Figures 5-6, B)

Description: White plasmodia measuring ~ 0.2 mm were found in the external wall of the stomach, intestine and gill arch of *C. macropomum.* Mature myxospores were elongated (Figure 5B), measuring 19.1 ± 0.4 (18.0 to 20.0) µm in length, 9.4±0.3 (8.6 to 10.4) µm in width, biconvex in lateral view, with 8.3 ± 0.4 (7.6 to 8.9) µm in thickness. Polar capsules elongated with equal size, measuring 10.5 ± 0.2 (9.7 to 11.1) µm in length and 2.5 ± 0.1 (2.1 to 2.8) µm in width, occupied a little more than half the body of the myxospores. Polar tubules with 9 to 10 coils were arranged perpendicularly to the longitudinal axis of the polar capsule (Figure 5B, Table 3).

Type host: *Colossoma macropomum* Cuvier, 1816, Characiformes: Serrasalmidae.

Locality: Tapajós River, municipality of Santarém, Pará State, Brazil.

Prevalence: 3 of 51 (5.9%).

Sites of infection: The external wall of the stomach, intestine and gill arch.

Type of material: Slides with stained myxospores were deposited in the collections of the Adão José Cardoso Museum of Zoology, of the State University of Campinas (UNICAMP), Brazil (accession number ZUEC – MYX90).

Molecular data: Partial sequence with 1945 bp of the ssrDNA were deposited in GenBank under the accession number MK032221.

Remarks: *Myxobolus* n. sp. 2 was also compared with all species of *Myxobolus* that infect freshwater fish of the South America and other continents (EIRAS et al., 2005, 2014). *Myxobolus myleus* Azevedo, Clemente, Casal, Matos, Alves, Al-Quraishy and Matos, 2012, a parasite also of the Amazon serrasalmid *Myleus rubripinnis*, presented similar dimensions of the myxospores body length (19.3 for *M. myleus* and 19.1 for *Myxobolus* n. sp. 2), but differed in the body shape when viewed frontally; *M. myleus* exhibited a lightly-tapered anterior end and pear-shaped myxospores. Unfortunately, it was not possible to perform the molecular

comparison between these two species, since the *M. myleus* description was based on only morphological data. *Myxobolus* cf. *cuneus* (MILANIN et al., 2015), a parasite of hybrid serrasalmids, presented the same number of coils of the polar filaments [9-10] (Table 3), but these two species differed in the morphology, since *M.* cf. *cuneus* presented a pyriform shape, while *Myxobolus* n. sp. 2 presented an ellipsoidal shape. Comparing *Myxobolus* species parasites of other hosts, this new species differed in morphometric/morphological features at least in one of the properties, such as size and/or shape of the plasmodia and myxospores, number of polar tubules coils, site of infection and region of occurrence (EIRAS 2002; EIRAS; ADRIANO 2012). The ssrDNA sequencing of *Myxobolus* n. sp. 2 resulted in a partial sequence of 1945 bp, which did not match to any myxosporean sequences available in GenBank database. In the genetic similarity analysis of the myxosporeans parasites of serralmids fish *Myxobolus* n. sp. 2, the closest species was *M.* cf. *cuneus* with a 14.8% of difference (Table 4). The morphological and molecular data support the creating of the new taxon.

Myxobolus colossomatis Molnár and Békési, 1993 (Figures 5-6, C)

Morphological characteristics: White plasmodia, measuring ~ 0.1 mm and containing innumerous mature and immature myxospores were found in different organs, such as pyloric cecum, skin and gill arch. The majority of the mature myxospores were ellipsoidal in frontal view (Figure 5C), measuring 11.6 ± 0.4 (10.8 to 12.3) µm in length and 7.6 ± 0.2 (7.2 to 8.1) µm in width. Polar capsules elongated and were of equal size, measuring 5.6 ± 0.2 (5.0 to 5.9) µm of length and 2.5 ± 0.2 (2.2 to 3.4) µm of width and occupying little more than half of the spore. A few myxospores had oval shape (with slightly narrower anterior region), measuring 10.4 ± 0.5 (9.6 to 10.8) µm in length, 7.7 ± 0.3 (7.3 to 8.0) µm in width. The polar capsules were elongated and with equal size, measuring 5.4 ± 0.2 (5.1 to 5.7) µm in length and 2.4 ± 0.0 (2.3 to 2.4) µm in width (Table 3). The polar tubules had 7 to 8 coils.

Host: *Colossoma macropomum* Cuvier, 1816, Characiformes: Serrasalmidae.

Localities of collection: Solimões and Tapajós Rivers, municipality of Manacapuru, Amazonas State and Santarém, Pará State, respectively, Brazil.

Prevalence: 7 of 51 (13.7%).

Sites of infection: Liver, spleen**,** pyloric cecum, fin, wall intestinal, digestive tract and gill arch.

Material deposited: Slides with stained myxospores were deposited in the collections of the Adão José Cardoso Museum of Zoology, of the State University of Campinas (UNICAMP), Brazil (accession number ZUEC – MYX 91).

Molecular data: Partial sequence with 1936 bp of the ssrDNA were deposited in GenBank under the accession number MK032220.

Remarks: The morphological and morphometric data of the *Myxobolus* species were found parasitizing the pyloric cecum, skin and gill arch of *C. colossomatis* from the Amazon and concurred to those of the *M. colossomatis* originally described by Molnár and Békési (1993) (Table 3). The ssrDNA sequencing of *M. colossomatis* performed in this study resulted in a partial sequence of 1936 bp, which did not match to any myxosporean sequences available in GenBank database or in the genetic similarity analysis. Considering parasite species of serrasalmids, *Myxobolus* n. sp. 1 was the closest related species to *M. colossomatis*, with 10.3% of difference (Table 4).

Table 3. Comparative data of myxospore dimensions, host, sites of infection and locality of *Myxobolus* spp. obtained in this study and others *Myxobolus* spp. parasites of serrasalmids fish. **ML –** myxospore length; **MW –** myxospore width; **TH** – Thickness; **PCL –** polar capsules length; **PCW –** polar capsules width; **NCF –** number of coils of polar filaments; Dashes – no data.

Species	Shape	ML	MW	TH	PCL	PCW	NCF	Host	Site of infection	Locality	Reference
***Myxobolus* n. sp. 1**		**9.6±0.4**	**7.0±0.3**	**5.0±0.3**	**4.3±0.4**	**1.9±0.1**	-	***Colossoma macropomum***	**Inner surface of the operculum**	**Amazon Basin**	**Present study**
***Myxobolus* n. sp. 2**		**19.1±0.4**	**9.4±0.3**	**8.3±0.4**	**10.5±0.2**	**2.5±0.1**	**9-10**	***Colossoma macropomum***	**External wall of stomach**	**Amazon Basin**	**Present study**
M. colossomatis	**Ellipsoidal**	**11.6±0.4**	**7.6±0.2**	-	**5.6±0.2**	**2.5±0.2**	**7-8**	***Colossoma macropomum***	**Pyloric cecum**	**Amazon Basin**	**Present study**
	Oval	**10.4±0.5**	**7.7±0.3**	-	**5.4±0.2**	**2.4±0.0**					
M. colossomatis	Ellipsoidal	11.8	6.9	3.7	6.0	2.1	7-8	*Colossoma macropomum*	Several organs	Amazon River	Molnár; Békési (1993)
	Oval	10.5	8.0	-	-	-					
M. cf. *colossomatis*		10.3±0.7	6.4±0.8	-	4.4±0.4	1.8±0.2	7-8	*Piaractus mesopotamicus*	Gills	Fish Farm	Müller et al. (2013)
M. cuneus		10.0±0.6	5.1±0.3	-	5.7±0.3	1.7±0.2	8-9	*Piaractus mesopotamicus*	Several organs	Fish Farm	Adriano et al. (2006)
M. cf. *cuneus*		10.5±0.3	5.4±0.2	4.3±0.3	6.0±0.3	2.0±0.3	9-10	Hybrid patinga*****	Spleen	Fish Farm	Milanin et al. (2015)
M. cunhai		9-11	4-6	-	-	-	-	*Pygocentrus piraya*	Intestinal content	Brazil	Penido (1927)
M. noguchi		13.6	8.5	-	6.8	2.2	-	*Serrasalmus spilopleura*	Gills	Brazil	Pinto (1928)
M. serrasalmus		14.8	8.6	-	7.7	3.1	-	*Serrasalmus rhombeus*	Kidney, Spleen, Liver	Brazil	Walliker (1969)
M. maculatus		21.0	8.9	7.5	12.7	3.2	14-15	*Metynnis maculatus*	Kidney	Amazon River	Casal et al. (2002)
M. metynnis		13.1	7.8	3.9	5.2	3.2	8-9	*Metynnis argenteus*	Orbicular region	Amazon River	Casal et al. (2006)
M. myleus		19.3±0.5	8.3±0.5	4.0±0.3	13.2±0.4	3.0±0.3	19-21	*Myleus rubripinnis*	Gall bladder	Sapuruá Lagoon	Azevedo et al. (2012)

*****Result of the crossing between *Piaractus mesopotamicus* X *Piaractus brachypomus.*
Source: own authorship.

Table 4. Comparative analysis of the genetic identity of ssrDNA of *Myxobolus* spp. parasites of the serrasalmids fish. The area above the diagonal shows % pairwise distance identity. The area below the diagonal shows nucleotide differences.

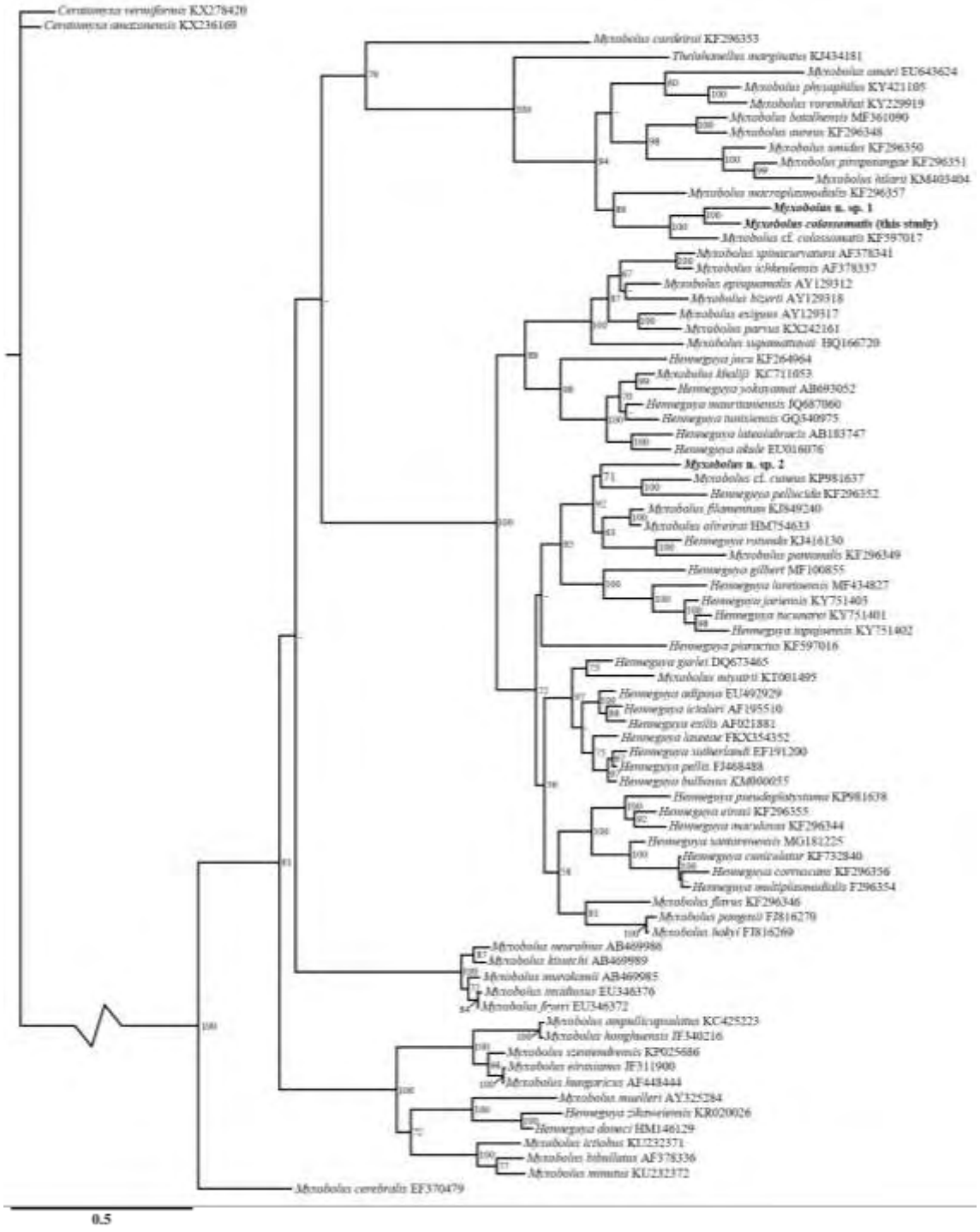
Species	**1**	**2**	**3**	4	5
1 *Myxobolus* n. sp. 1	-	22.4	10.3	12.7	22.1
2 *Myxobolus* n. sp. 2	419	-	23.2	21.1	14.8
3 *Myxobolus colossomatis* (Present study)	198	435	-	11.1	22.1
4 *Myxobolus* cf. *colossomatis*	170	280	148	-	21.3
5 *Myxobolus* cf. *cuneus*	395	274	396	283	-

Source: own authorship.

Phylogenetic analysis

Maximum likelihood analyses, based on the ssrDNA sequences of the myxosporeans species most closely related to each of the species studied here and, according to BLASTn, with well-supported bootstrap, showed that *Myxobolus* n. sp. 1 appears as sister species to *M. colossomatis,* in a subclade that also are present *M.* cf. *colossomatis,* parasite of *P. mesopotamicus* and *Myxobolus macroplasmodialis* Molnár, Ranzani-Paiva, Eiras and Rodrigues (1998), parasite of the bryconid *Salminus brasiliensis*. *Myxobolus* n. sp. 2 appears, with low bootstrap support, in a lineage composed by three species parasites of South American serrasalmids fish (Figure 7).

Figure 7. Maximum likelihood showing relationship among *Myxobolus* spp. and other myxobolids based on ssrDNA. The numbers above the nodes indicate bootstrap confidence levels. Dashes are shown for values under 60%.



DISCUSSION

Based on morphological/morphometric and sequencing of the ssrDNA, this study described two novel species of genus *Myxobolus* and provided molecular data for *M.*

colossomatis. All these were found parasitizing *C. macropomum* in its natural habitat of the Amazon Basin.

The BLASTn search was conducted for each ssrDNA sequences obtained in this study to confirm amplification of myxozoan DNA, and no close matches to any myxosporeans sequences available in GenBank. The p-distance analysis displayed genetic divergences of 10.3% between *Myxobolus* n. sp. 1 and *M. colossomatis,* 22.4% between *Myxobolus* n. sp. 1 and *Myxobolus* n. sp. 2, and 23.2% between *Myxobolus* n. sp. 2 and *M. colossomatis.* This demonstrates that, in its natural habitat, *C. macropomum* harbors at least three distinct *Myxobolus* species, two of which were hitherto unknown. The third parasite, *M. colossomatis,* had already been described by Molnár and Békési (1993) to infect gills, fins, liver, heart, muscle and intestines of farmed specimens from Ceará State, in the Brazil's Northeast, although molecular data were not provided. Later, in a search for myxosporeans in farmed fish in São Paulo State in Brazil's Southeast, Martins et al. (1999) reported the occurrence *M. colossomatis* in the kidney of *C. macropomum*, but these authors also identified the occurrence of this same parasite in kidney, liver, spleen and gallbladder of *P. mesopotamicus*, another serrasalmid fish popularly known as "pacu", but whose the natural distribution is restricted to La Plata River Basin (LIMA et al. 2003). In studies carried out in the natural environment of Brazil's Pantanal wetlands (a part of the La Plata Basin) Adriano et al. (2002) and Campos et al. (2008) also referred to a *Myxobolus* species and reported specimens of *P. mesopotamicus* as *M. colossomatis*; however, these authors, (MARTINS et al.,1999), did not display morphometric and molecular data, thus leaving the real taxonomic position of the studied parasites inconclusive. Müller et al. (2013), studying myxosporeans of farmed *P. mesopotamicus,* in São Paulo State, found gills infected by a *Myxobolus* sp. whose spores resembled that of the *M. colossomatis* described by Molnár and Békési (1993), but

in addition to morphometric data, the authors also performed ultrastructure and ssrDNA sequencing. Because the myxospores morphologic/morphometric data had very few differences to those of the original description of *M. colossomatis* (Table 1), and as there was no molecular data for comparison, Müller et al. (2013) preferred to call it *Myxobolus* cf. *colossomatis* and wait for the availability of ssrDNA sequences of *M. colossomatis* obtained from the host type before defining it as the same species. Notably, this study now reveals a *Myxobolus* species found infecting pyloric cecum, skin and gill arch of specimens of *C. macropomum* caught in its natural habitat (Amazon Basin), and in which morphologic/morphometric data and biological traits have matched those reported by Molnár and Békési (1993), in their original descriptions. Hence, this parasite has been identified as *M. colossomatis*, and this has finally provided the ssrDNA sequence of this parasite from the host type, settling the taxonomic dilemma. The ssrDNA sequence of *M. colossomatis* showed 11.1% of genetic divergence to that of *M.* cf. *colossomatis* found by Müller et al. (2013). It was discovered parasitizing farmed *P. mesopotamicus* in São Paulo State and thus demonstrates that this is an unnamed species waiting for a name.

However, despite the fact that naturally *C. macropomum* and *P. mesopotamicus* inhabit distinct watersheds and fish farms, these two species are farmed throughout Brazil, and in some regions, they are crossed bred to obtain a hybrid fish called "tambacu," which is largely accepted by consumers. It is therefore not possible to ensure that in these conditions the *Myxobolus* species do not circulate between *C. macropomum* and *P. mesopotamicus*, or even between their hybrids.

In the phylogenetic analysis, the three *Myxobolus* parasite species of *C. macropomum* were not monophyletic, with the strong likelihood that *Myxobolus* n. sp. 1 and *M. colossomatis* appear to be sister species in a lineage. Also, the *M.* cf. *colossomatis*, provided by Muller et al. (2013). *Myxobolus* n. sp. 2 (albeit with low bootstrap support),

appears in a sister branch of the lineage composed by *Henneguya pellucida* Adriano, Arana and Cordeiro (2005) and *Myxobolus* cf. *cuneus*; both parasites are also of the serrassalmid *P. mesopotamicus*.

In addition to the taxonomic and phylogenetic data above provided, this study also introduces and provides data about the prevalence of the *Myxobolus* species. Considering the two regions that were sampled, it was possible to observe that in cases of *M. colossomatis* and *Myxobolus* n. sp. 1, which were reported in both places, the prevalence did not differ significantly. In the same way, no significant difference was observed when compared to the prevalence among the three *Myxobolus* species found. Additionally, these data show, that despite being low, the prevalence was similar to the different regions and species, except with the *Myxobolus* n. sp. 2, which was absent in Amazonas state. This absence may, however, be related to the lower sampling in this region.

In summary, this study provided data on the prevalence, morphology, molecular and phylogeny of two new species of *Myxobolus* and of *M. colossomatis*, and it clarified the taxonomic context of the latter. Further to this, our data reinforce the suggestions of Kent et al. (2001), Lom and Dyková (2006) and Fiala et al. (2015), that the association of morphological and molecular data are fundamental to avoid taxonomic confusion in myxosporeans.

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CHAPTER 2. NOVEL MYXOSPOREAN SPECIES PARASITES OF *Piaractus brachypomus*, AN ECONOMICALLY IMPORTANT FISH FROM THE AMAZON BASIN

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ABSTRACT

Myxosporeans are a group of parasitic cnidarians, with severe pathogeny of some species. This paper describes two new species of myxobolid found infecting specimens of *Piaractus brachypomus* arising from the Amazon basin, Brazil, based on morphology, histology, ultrastructural and phylogeny analysis. The fish were caught in the Tapajós river Santarém, PA, Brazil. The plasmodial development of *Henneguya* n. sp. 1 was intralamellar and of *Myxobolus* n. sp. 3 was in the pyloric cecum according with histological analysis. For both species was observed no inflammatory infiltrate. For *Henneguya* n. sp. 1 mature myxospores were ellipsoidal measuring: 13.07 ± 0.5 µm in length, 4.3 ± 0.2 µm in width, 3.8 ± 0.2 µm in thickness. The polar capsules were elongated measuring 6.3 ± 0.5 µm in length and 1.6 ± 0.2 µm in width, each containing polar filament with 4 coils. The caudal process was greater than the length of the body, measuring 44.7 ± 3.0 µm and the total length of the myxospore was 57.5 ± 3.5 µm. *Myxobolus* n. sp. 3 showed rounded mature myxospores measuring: 10.4 ± 0.3 µm in length, 7.2 ± 0.1 µm in width and 5.8 ± 0.2 µm of the thickness. The polar capsules were equal size and occupied less than half the myxospore, measuring: 3.8 ± 0.1 µm in length and 2.6 ± 0.1 µm in width, each containing polar filament with 6-7 coils. Phylogenetic analysis, based on partial ssrDNA sequences and using the closest myxosporean sequences to each one of the species here studied, showed that *Henneguya* n. sp. 1. clustered in a well-supported smaller subclade composed by *Henneguya* species that

infect gills of the serrasalmid hosts, *Myxobolus* n. sp. 3 clustered in a subclade composed only for *Myxobolus* species parasite of the different organs.

Key-words: *Myxobolus*, *Henneguya*, Tapajós river, pirapitinga, ssrDNA.

INTRODUCTION

Myxozoans are widespread cnidarian endoparasites and diverse and important components for ecosystems. They have a complex life-cycle which involves annelids and bryozoans as definitive invertebrate hosts and mainly fish, but also other vertebrates, as intermediate hosts (OKAMURA et al., 2015). Phylogenetic DNA studies have shown that the *Myxobolus* lineage (*Myxobolus/Henneguya*) is the largest within the myxozoan class (FIALA et al., 2015, Chapter 5), with around 850 *Myxobolus* and 200 *Henneguya* species described, representing almost half the diversity of these cnidaria subphyla. However, only 100 species (around 10%) have been reported infecting South American fish species (EIRAS; ADRIANO, 2012; EIRAS et al., 2014; ADRIANO; OLIVEIRA, 2019).

The Amazon basin is approximately 6.500 km^2 in size, and is one of the most productive and intact ecosystems on the planet. Due to its abundant water, it harbors vast fish diversity (VAL; HONCZARYK, 1995; GOULDING, 1996; SANTOS; SANTOS, 2005). The native fish *Piaractus brachypomus* Cuvier, 1818, popularly known in Brazil as “pirapitinga”, which occurs in the Amazon and Orinoco basins and can reach a weight of up to 20 kg and a length of up to 80 cm (SOBRINHO et al., 1984; MPA, 2013; FROESE; PAULY, 2018), is economically important for the region. Due to the high quality of its meat and rapid growth, the species is widely farmed in South America, and has also been used in fish farming in Asia (FRESNEDA et al., 2004; HONGLANG, 2007; FLORES NOVA, 2007; LIN et al., 2015). In Brazil, *P. brachypomus* is among the largest

native fish species farmed, and is also important for crossing with other native species to produce hybrid fish such as tambatinga (female *Colossoma macropomum* × male *P. brachypomus*) and patinga (female *Piaractus mesopotamicus* × male *P. brachypomus*), which are equally important in fish farm production (MPA, 2013; IBGE, 2016).

Despite the commercial importance of this species in the context of fishing and fish farming in South America and in other regions where it has been introduced, nothing is so far known about the myxozoan fauna parasites of *P. brachypomus*. The present study, which is part of ongoing research into South American myxozoans, describes two new myxosporean species found parasitizing *pirapitinga* specimens caught in a natural environment in the Amazon basin. The study was carried out using morphologic, histopathologic, ultrastructural and small subunit rDNA (ssrDNA) based phylogenetic analyses.

MATERIAL AND METHODS

Twenty-five *P. brachypomus* specimens were caught between October 2014 and January 2016 in the Tapajós River (2°20’03.92”S 54°52’33.44”W), in the municipal district of Santarém, in the state of Pará, Brazil. Authorization for the fish capture and the genetic heritage access report were obtained from the Brazilian Ministry of the Environment under processes SISBIO nº 44268-4 and SisGen nº A33CB83, respectively. The fish were transported live to a field laboratory and euthanized by spinal column transection. This methodology was approved by the ethics research committee of the Faculty of Animal Science and Food Engineering of the University of São Paulo (Process USP: 14.1.391.74.9) in accordance with Brazilian law (Federal Law nº 11794, dated 8 October 2008 and Federal Decree No. 6899 dated July 15, 2009).

Morphological analysis

Myxosporean plasmodia were carefully separated from the host tissue and individually ruptured in glass slides. Mature myxospores were measured using a Leica DM 1000 Microscope coupled with Leica EC3 camera and LAS EZ image capture software. The dimensions of mature myxospores (N = 25) were obtained in according Lom and Arthur (1989) and expressed as mean ± standard deviation (SD) and range, in µm. The slides were then fixed with methanol and stained with Giemsa to be deposited in the collection of the Adão José Cardoso Museum of Zoology, of the State University of Campinas (UNICAMP), Brazil.

Histological analysis

For histological analysis tissue fragments containing the plasmodia were fixed in buffered 10% formalin for 24h and placed in paraffin. Sections with a thickness of 4 µm were then stained with hematoxylin-eosin and analyzed and photographed in a Leica DM 1000 Microscope coupled with a Leica EC3 camera and LAS EZ image capture software.

Transmission electron microscopy

For transmission electron microscopy, fragments of infected tissue were fixed, for at least 12h, in 2.5% glutaraldehyde diluted in the cacodylate buffer at 0.1 M sodium containing 3% of glucose at pH 7.4. The sample were then washed in the same buffer per 2h and fixed in OsO_4 for 4h. After dehydration in increasing concentrations of acetone the samples were embedded in Embed 812 resin. Uranyl acetate and lead citrate were used to treat ultrathin sections that, posteriorly, were examined in a LEO 906 transmission electron microscope operating at 60 kV.

Molecular analysis

DNA extraction, PCRs and Sequence assembly

Host tissue was used for correct host identification by sequencing the α-tropomyosin gene. Ethanol-fixed myxospores were washed with Phosphate Buffered Saline (PBS) for DNA extraction and separate in microtubes containing the myxospores and host tissue were centrifuged at 13.000 rpm for three minutes, before the PBS buffer was removed.

The DNA was extracted with DNeasy® Blood & Tissue kit (Qiagen Inc., California, USA) according to manufacturer's instructions. For PCR reactions were used 10 - 50 ng of genomic DNA, DreamTaq 12.5 µL of 2 × PCR Master Mix (Thermo Scientific – Carlsbad, California, USA), 0.2 µM of each specific primer and nuclease free water (Thermo Scientific – Carlsbad, California, USA) with final volume of 25 µL. In the ssrDNA amplification of the myxosporeans were used the primers ERIB1 (BARTA et al., 1997), ACT1R (HALLET; DIAMANT, 2001), TEDF (CAPODIFOGLIO et al., 2015) and ERIB10 (BARTA et al., 1997). The TROPF and TROPR (FRIESEN et al., 1999) primers were used to amplifications of the α-tropomyosin gene in accordance with Hashimoto et al. (2011), followed by modifications. The PCRs were carried out in a ProFlex PCR Systems thermocycler (Applied Biosystems™ Inc., California, USA) with a program of 35 cycles for α-tropomyosin gene at 95°C for 30 s, 64°C for 30 s and 72°C for 10 s. For ssrDNA was used an initial denaturation at 95°C for 5 minutes; 35 cycles: 95°C for 60 s, 60°C for 60 s and 72°C for 90 s; and a final extension at 72°C for 5 minutes.

All the PCR products were analyzed by 1.5% agarose gel electrophoresis in a TAE buffer (Tris–Acetate EDTA, Tris 40 mM, Acetic Acid 20 Mm and EDTA 1 mM), stained with Sybr Safe DNA gel stain (Thermo Scientific – Carlsbad, California, USA), and analyzed in a LED transilluminator (Kasvi, Paraná, Brazil). The sizes of the amplified

fragments were estimated by comparison with the 1 Kb Plus DNA Ladder (Thermo Scientific – Carlsbad, California, USA). The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen Inc., California, USA) and sequenced with the same primer pairs used in the reaction, as well as the additional primers MC5f, MC3r (MOLNÁR et al., 2002) and BOBf (Present study, Chapter 1) for sequencing of ssrDNA. The sequencing of all samples was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™ Inc., California, USA) and an ABI 3730 DNA sequencing analyzer (Applied Biosystems™ Inc., California, USA).

The DNA sequences obtained were visualized and assembled using BioEdit 7.1.3.0 (HALL, 1999) software. A BLASTn search (ALTSCHUL et al., 1997), available in the NCBI database, was performed to confirm the myxozoan and *P. brachypomus* host.

Phylogenetic analysis

Phylogenetic analysis was performed using the sequences obtained plus ssrDNA sequences of the closest myxozoans to each one of the new species, based on the BLASTn searches carried out in the NCBI database. Four species of the genus *Ceratomyxa* Thélohan, 1892 were used as outgroup. All the sequences were aligned using the ClustalW algorithm (THOMPSON et al., 1994) by software BioEdit 7.1.3.0 (HALL, 1999).

The Maximum likelihood (ML) method was applied using the PhyML3.0 (GUINDON et al., 2010) software, with a bootstrap value of 1000 replications. The FigTree v1.3.1 (RAMBAUT, 2008) and Adobe Illustrator (Adobe Systems Inc.) software programs were used to view and to edit, respectively, the tree generated. Only bootstrap values above 50 were displayed, and data related to the host fish, such as order and family, were obtained from FishBase (FROESE; PAULY, 2018).

To evaluate the genetic distance between the ssrDNA sequences of the new species with other myxosporean parasites of serrasalmids fish, a new alignment was performed using the species focus and the pairwise method was applied using the p-distance model in the MEGA 7.0 software program (KUMAR; STECHER; TAMURA, 2016).

RESULTS

Occurrence

Of 25 *P. brachypomus* specimens collected, 22 (88%) contained myxosporean plasmodia. Seventeen (68%), had plasmodia containing myxospores typical of the *Henneguya* genus in the gill filament, while four (16%) had plasmodia harboring myxospores with characteristics of the *Myxobolus* genus in the pyloric cecum. The morphological and ssrDNA sequence analyses revealed that both are new myxosporean species, and their descriptions are provided below.

Taxonomic data

***Henneguya* n. sp. 1** (Figures 8A, 9 – 11)

Type host: *Piaractus brachypomus* Cuvier, 1818, Characiformes: Serrasalmidae.

Locality of collection: Tapajós River, municipal district of Santarém, state of Pará, Brazil (2°23'07.5"S 54°47'05.9"W).

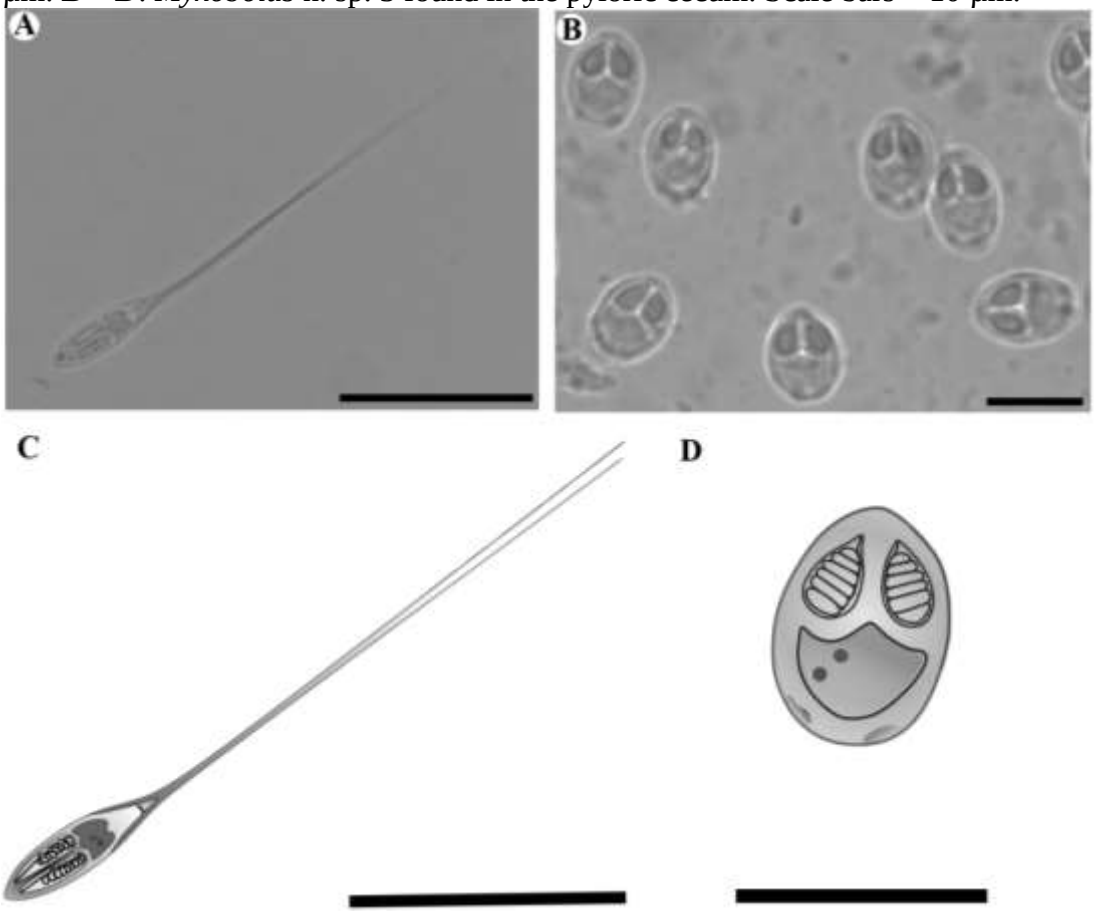
Prevalence: 17 of 25 (68%).

Site of infection: Gill lamellae.

Type of material: Slides with stained myxospores were deposited in the collections of the Adão José Cardoso Museum of Zoology, of the State University of Campinas (UNICAMP), São Paulo state, Brazil (ZUEC –MYX 86).

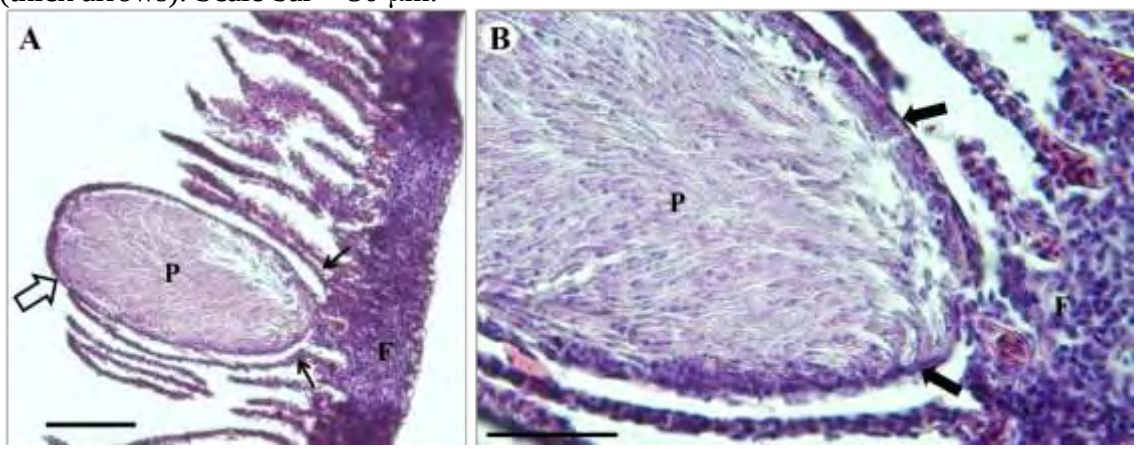
Description and host-parasite relationship: plasmodia white and elongated measuring up to 0.3 mm in length were found in the gill filament of *P. brachypomus*. The mature myxospores had fusiform shape in the frontal view and measured 13.0 ± 0.5 (11.7 to 13.8) µm in length, 4.3 ± 0.2 (4.0 to 4.6) µm in width, with caudal processes bigger than the myxospore body, measuring 44.7 ± 3.0 (40.5 to 48.1) µm and a total length of 57.5 ± 3.5 (52.4 to 61.6) (Figure 8A). In lateral view the myxospores were 3.8 ± 0.2 (3.5 to 4.3) µm in thickness. Polar capsules elongated, occupying more than half of the myxospore and measuring 6.3 ± 0.5 (5.6 to 7.3) µm in length and 1.6 ± 0.2 (1.3 to 2.0) µm in width, each containing a polar filament with 8-9 coils (Figures 8A, 10B and 11A) (Table 1).

Figure 8. Photomicrographs of mature myxospores (A – B) and respective schematic representations (C – D) of new myxobolid species parasites of *Piaractus brachypomus* from the Tapajós River. **A** – **C**: *Henneguya* n. sp. 1 found in the lamella. Scale bars = 20 µm. **B** – **D**: *Myxobolus* n. sp. 3 found in the pyloric cecum. Scale bars = 10 µm.



Histological analysis showed that *Henneguya* n. sp. 1 developed in the gill lamellae (Figures 9A – B). The parasite development caused stretching of the epithelium and compression of the capillary and adjacent tissues. The hypertrophy of the infected lamellae produced displacement and deformation of the neighboring lamellae (Figure 9). No inflammatory infiltrate was observed.

Figure 9. Photomicroscopy of histological section of *Piaractus brachypomus* filament (F) infected by *Henneguya* n. sp. 1. **A** – Development of the plasmodium (P) in the gill lamellae (large unfilled arrow). Note that the growth of the plasmodium pushes the neighboring uninfected lamellae (thin arrows). Scale bar = 100 μm. **B -** Magnification of A showing the lamellar epithelium stretching due to the development of the plasmodium (thick arrows). Scale bar = 50 μm.



Ultrastructural analysis of gill lamellae parasitized by *Henneguya* n. sp. 1 revealed thick peripheral ectoplasm, with extensive and numerous pinocytic channels connecting the external of the plasmodium to the interior and numerous mitochondria. Generative cells and young and mature myxospores were observed inside the plasmodium revealing an asynchronous sporogonic process, germinal nucleus, young spores and mature spores observed inside the plasmodium reveal an asynchronous sporogonic process (Figure 10). Young myxospores presented a polar capsule with a polar tubule, and a sporoplasm with sporoplasmosomes and some mitochondria (Figures 10B, 11A).

Figure 10. Electron micrograph of branchial filament of *Piaractus brachypomus* infected by *Henneguya* n. sp. 1 showing the host-parasite interface: lamella (L); plasmodium (P) with plasmodial wall (broad arrow); ectoplasm zone (ec); generative cell (gc); young myxospores (ym) with polar capsules (pc) and polar tubule in uninternalized formation (white arrow), sporoplasmosomes (thin arrow); and mature spore (mm). Scale bar = 5 μm.

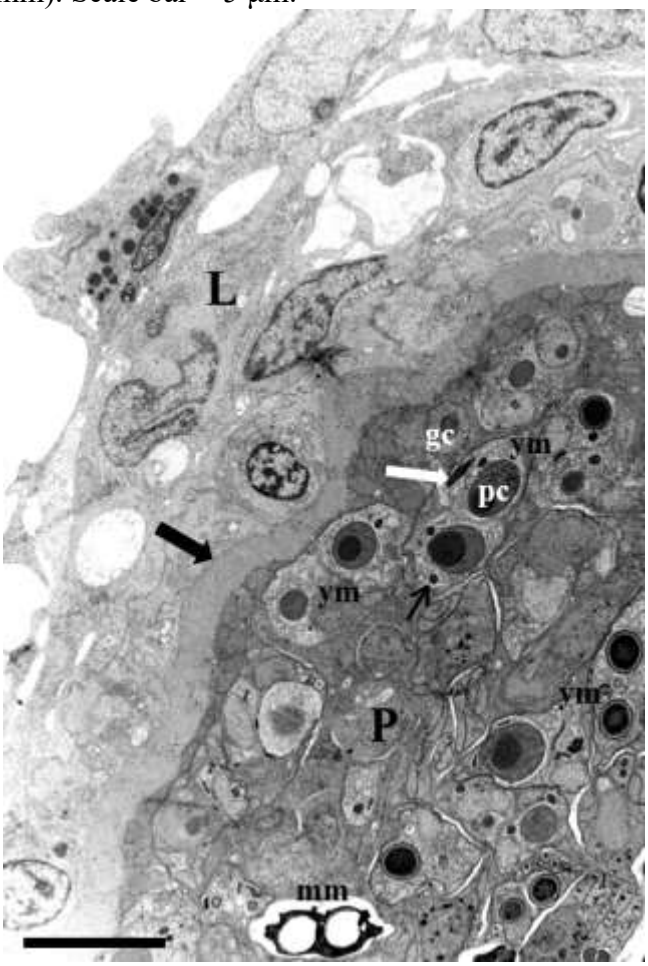
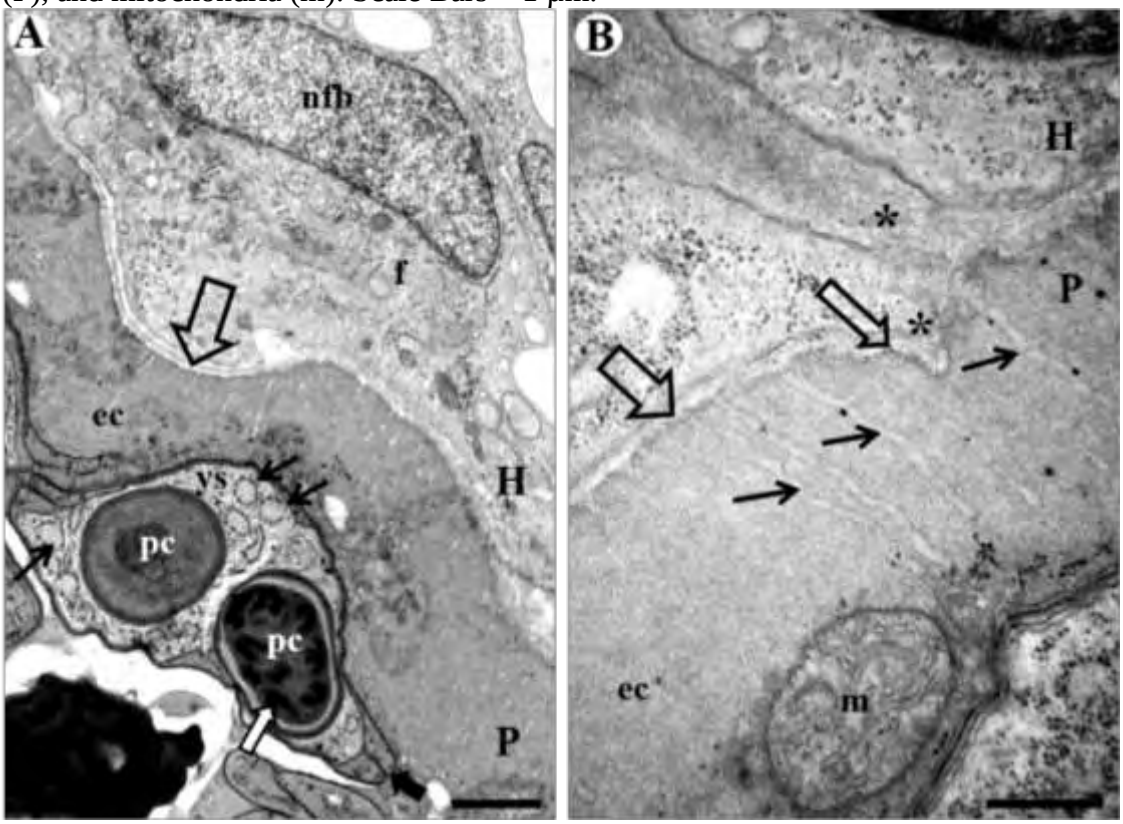


Figure 11. Electron micrograph of branchial filament of *Piaractus brachypomus* infected by *Henneguya* n. sp. 1. **A** – Host-parasite interface showing the plasmodial wall (large empty arrow), ectoplasm zone (dotted line), young spores (ym) and mature myxospores (mm). Note the junction point of valves (thin white arrows), polar capsule (pc) with internalized polar tubules (large white arrow). Nucleus of respiratory epithelial cell (ren); Host (H); Plasmodium (P). **B -** Detail of parasite-host interaction showing host epithelium (H), plasmodial wall (large empty arrow); numerous and extensive pinocytic channels (black arrows) connecting the external medium to the ectoplasm (ec) of the plasmodium (P), and mitochondria (m). Scale Bars = 1 µm.



Genetics: The partial ssrDNA sequence with a fragment of 1971 bp, obtained from a single plasmodium of *Henneguya* n. sp. 1, was deposited in GenBank under accession number MK491272. BLASTn search revealed no match with any other myxosporeans ssr DNA available in genBank. Pairwise comparison considering parasites of serrasalmid hosts revealed *Henneguya piaractus* Martins and Souza, 1997 as the closest species to *Henneguya* n. sp. 1, with 5.1% of genetic divergence (Table 6).

Remarks: the new *Henneguya* species was morphologically and genetically compared with all species reported infecting freshwater fishes (EIRAS et al., 2005, 2014; MADDEN, 2002). The morphologically closest species to *Henneguya* n. sp. 1 was *H.*

piaractus, which is a parasite of *P. mesopotamicus*, a congeneric fish to *P. brachypomus*, but endemic to the La Plata Basin. These two species are so similar that morphological distinction is an impossible task (Fig 8A and Table 5). In a typical sign of cryptical speciation ssrDNA sequencing revealed 5.1% of genetic divergence between the two. In terms of *Henneguya* spp. parasites of Amazonian fishes, the myxospores of *Henneguya* n. sp. 1 resembled those of *Henneguya amazonica* Rocha, Matos and Azevedo, 1992, a parasite of *Crenlcichla lepldota* Heckel, 1840. They were similar in myxospore length, polar capsule width, caudal process length, total myxospore length, and infection site. They differed in myxospore width (4.3 µm for *Henneguya* n. sp. 1 and 5.7 µm for *H. amazonica*), polar capsule length (6.3 µm for *Henneguya* n. sp. 1 and 3.3 µm for *H. amazonica*), number of polar tubule coils (8-9 for *Henneguya* n. sp. 1 and 6 for *H. amazonica*), and host order/family (Characiformes: Serrasalmidae for *Henneguya* n. sp. 1 and Perciformes: Cichlidae for *H. amazonica*). In the comparison with *Henneguya* spp. parasites of fish species from other regions, *Henneguya* n. sp. 1 exhibited morphometric/morphological differences at least one of the following properties: size and/or shape of the plasmodia and myxospores, number of polar tubule coils, site of infection and region of occurrence (EIRAS et al., 2005; 2014). It also differed in genetic identity comparison (Table 6 and BLASTn analysis). The creation of a new taxon is therefore proposed.

***Myxobolus* n. sp. 3** (Figures 8B, 12 – 14)

Type host: *Piaractus brachypomus* Cuvier, 1818 Characiformes: Serrasalmidae.

Type locality: Tapajós River, municipal district of Santarém, state of Pará, Brazil.

Prevalence: 4 of 25 (16%).

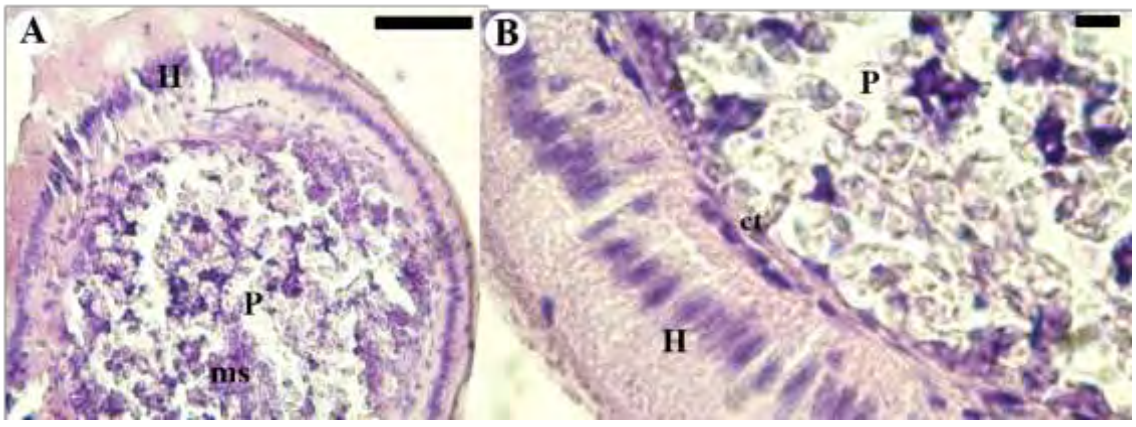
Site of infection: Pyloric cecum.

Type of material: Slides with stained myxospores were deposited in the collections of the Adão José Cardoso Museum of Zoology, of the State University of Campinas (UNICAMP), state of São Paulo, Brazil (ZUEC – MYX 85).

Description and host-parasite relationship: plasmodia white and rounded measuring up to 0.2 mm in diameter were found in the pyloric cecum of *P. brachypomus*. Mature myxospores with an oval shape in the frontal view, measuring 10.4 ± 0.3 (10.0 to 11.1) µm in length, 7.2 ± 0.1 (7.0 to 7.6) µm in width, and measuring 5.8 ± 0.2 (5.4 to 6.3) µm in thickness in the lateral view (Figure 8B). The pyriform polar capsules occupied less than half of the myxospore, measuring 3.8 ± 0.1 (3.5 to 4.0) µm in length and 2.3 ± 0.1 (2.0 to 2.6) µm in width (Figure 8B), each containing a polar filament with 6-7 coils (Figure 14A) (Table 5).

The parasite developed in the pyloric cecum, and the plasmodia were surrounded by a thin layer of connective tissue, but no inflammatory infiltrate was observed (Figures 12A – B).

Figure 12. A – B Photomicrograph of histological section of pyloric cecum of *Piaractus brachypomus* parasitized by *Myxobolus* n. sp. 3. **A** – Plasmodium (P) filled with mature myxospores (ms). Scale bar = 50 µm. **B** – Magnification of A showing the connective tissue capsule (ct) surrounding the plasmodium (P). Host (H). Scale bar = 10 µm.



Ultrastructure analysis displayed a thin ectoplasm zone, with few pinocytic channels, small vesicles and numerous mitochondria, which in the majority had a degraded internal membrane (Figures 13A – B). The sporogenesis process was

asynchronous and the polar capsules of the myxospores had a filament with 6-7 coils (Figures 13 and 14).

Figure 13. Electron micrographs of pyloric cecum of *Piaractus brachypomus* infected by *Myxobolus* n. sp. 3. **A** – Host-parasite interface showing connective tissue layer (ct) with conspicuous fibroblasts (fb) and its nucleus (n) separating the plasmodial wall (large empty arrow) from the host tissue (H). Inside the plasmodium (P) distinct sporogonic developmental stages can be seen, with the sporoblast (sb) containing young myxospores (ym) with a polar capsule (pc) and internalized (black arrow) or free (white arrow) polar tubules, and some mature myxospores (mm). Scale bar = 5 μm. **B** – Magnification of the host-parasite interface showing connective tissue layer (dotted line) composed of fibroblast (fb) and collagenic fibers (cf). Note the plasmodial wall (large empty arrow), ectoplasms (ec) with pinocytic channels (white arrows), the fragment of a sporoblast with immature myxospores exhibiting binucleate sporoplasm cells (sp), their numerous sporoplasmosomes (black arrows) and mitochondria (m). Nucleus (n); Host (H). Scale bar = 1 μm.

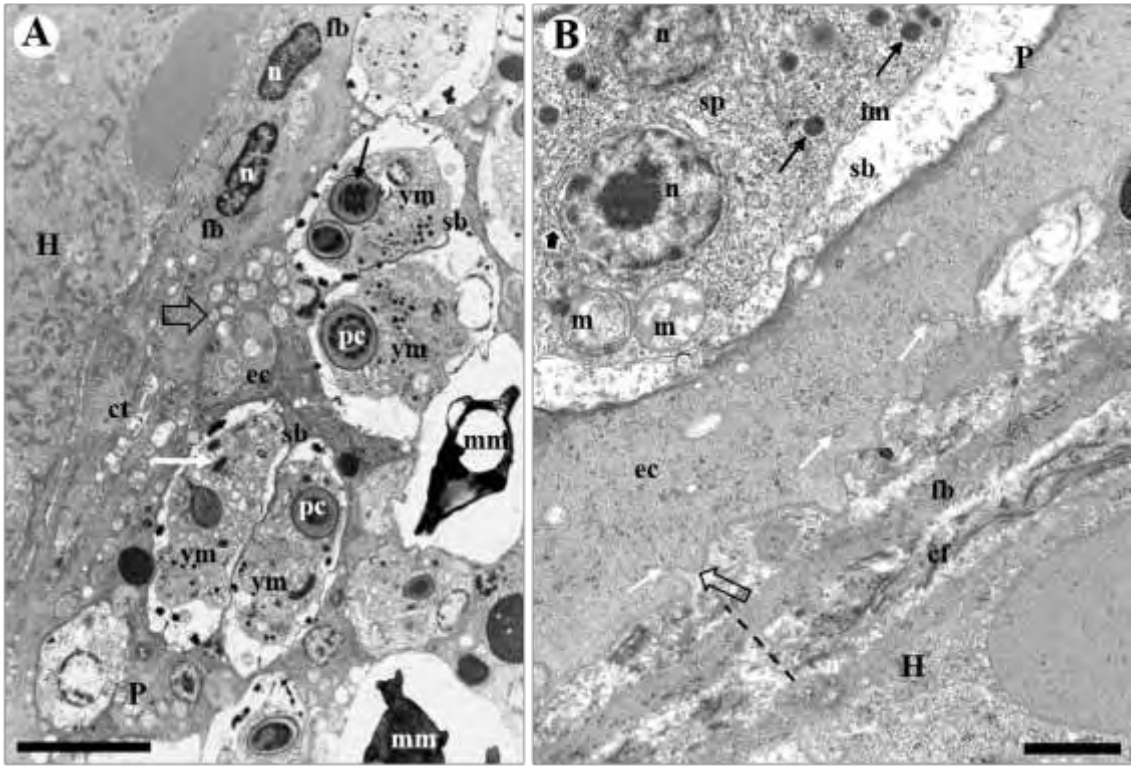
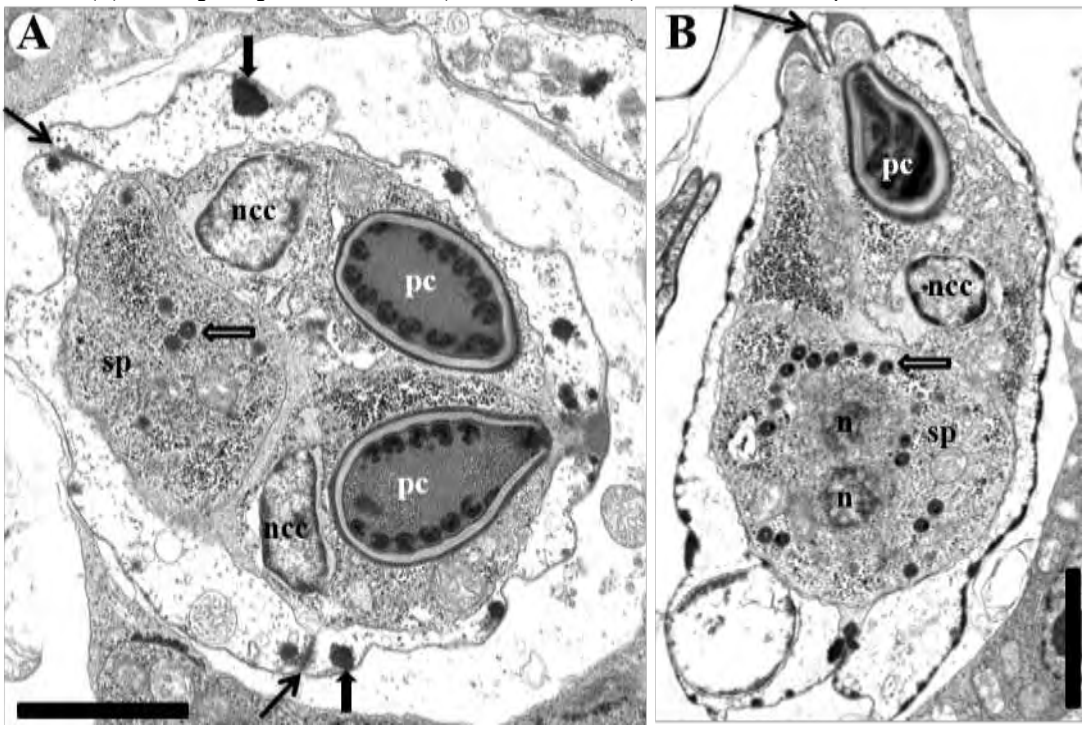


Figure 14. Electron micrograph of immature myxospores of *Myxobolus* n. sp. 3 parasite of pyloric cecum. **A**- Immature myxospore in longitudinal section showing the junction points of the valves (thin black arrows), valve-forming material (large black arrows), sporoplasm (sp) with sporoplasmosomes (unfilled arrow), polar capsule (pc) and capsulogenic cell nucleus (ncc). Scale bar = 2 μm. **B** - Side longitudinal view of immature myxospore showing points of valve junction (black arrow), polar capsule (pc), capsulogenic cell nucleus (ncc), sporoplasm (sp) with two nuclei (n) and sporoplasmosomes (unfilled arrow). Scale bar = 2 μm.



Genetics: A ssrDNA fragment with 1891 bp, obtained from a single plasmodium of *Myxobolus* n. sp. 3, was deposited in GenBank under accession number MK492647. In the BLASTn search, there was no match with the other available myxosporean ssrDNAs. *Myxobolus colossomatis* Molnár and Békési 1993 and *Myxobolus* cf. *colossomatis* were the closest species to *M. pirapitingae* n. sp., with a similarity of 89%.

Remarks: The new *Myxobolus* species was morphologically and genetically compared with all the species reported infecting freshwater fish (EIRAS et al., 2005; 2014; MADDEN, 2002). Of the parasites of serrasalmid fish, *M. colossomatis*, which also is a parasite of the Amazonian *C. macropomum*, was the morphologically closest species to *M. pirapitingae* n. sp. Similarities were observed in the length and width of the myxospores, the width of the polar capsules and the number of polar filament coils. But

significant differences were noted in the length of the polar capsules (3.8 µm for *Myxobolus* n. sp. 3 and 5.6 µm for *M. colossomatis*), and the existence of myxospores with elongated-ellipsoidal or oval shapes in *M. colossomatis,* compared with only oval shapes in the new species. In addition to the differences in size, the polar capsules of *Myxobolus* n. sp. 3 were pear-shaped and occupied less than half the myxospore, while *M. colossomatis* exhibited elongated polar capsules which occupied half the myxospore. P-distance analysis revealed large genetic divergences of 10.7% between these species. In relation to other South American *Myxobolus* species, *Myxobolus* n. sp. 3 myxospores resembled those of *Myxobolus cordeiroi* Adriano, Arana, Alves, Silva, Ceccarelli, Henrique-Silva and Maia, 2009. The resemblances were related to myxospore length, width, and thickness, while significant differences were observed in polar capsule length (3.8 µm for *Myxobolus* n. sp. 3 and 5.2 µm for *M. cordeiroi*) and width (2.6 µm for *Myxobolus* n. sp. 3 and 1.4 µm for *M. cordeiroi*), number of polar filament coils (6-7 for *Myxobolus* n. sp. 3 and 5-6 for *M. cordeiroi*), and order/family host (Characiformes: Serrasalmidae for *Myxobolus* n. sp. 3 and Siluriformes: Pimelodidae for *M. cordeiroi*). The genetic analysis corroborated the distinctions between these species, showing them in different evolutionary lineages in the phylogenetic tree (Figure 15). In comparison with parasites of fish species from other regions, *Myxobolus* n. sp. 3 exhibited morphometric/morphological differences in at least one of the following properties: the size and/or shape of the plasmodia and the myxospores, the number of polar tubule coils, the site of infection and the region of occurrence (EIRAS et al., 2005; 2014); it also differed in genetic identity comparison (Table 6 and BLASTn analysis). Based on these data, the creation of a new taxon is proposed.

Table 5. Morphometric comparison of *Myxobolus* n. sp. 3 and *Henneguya* n. sp. 1 with other species of *Myxobolus/Henneguya* belonging to Serrasalmidae. **ML** – myxospore length; **MW** – myxospore width; **TH** – Thickness; **PCL** – polar capsule length; **PCW** – polar capsule width; **CPL** – caudal process length; **TL** – total length; **NCF** – numbers of coils of the polar filament; Dashes – no data. All measurements are given as mean ± standard deviation in μm.

Species	ML	MW	TH	PCL	PCW	CPL	TL	NCF	Host	Site of infection	Locality	Reference
***Henneguya* n. sp. 1**	**13.0±0.5**	**4.3±0.2**	**3.8±0.2**	**6.3±0.5**	**1.6±0.2**	**44.7±3.0**	**57.5±3.5**	**8-9**	***Piaractus brachypomus***	**Gill lamellae**	**Brazil**	**Present study**
H. piaractus	12.7	3.6	-	6.7	1.2	41.2	52.5	-	*Piaractus mesopotamicus*	Gill filament	Brazil	Martins; Souza, (1997a); Adriano et al. (2005); Muller et al. (2013)
H. curvata	16.4±0.8	4.7±0.2	-	7.8±0.3	1.4±0.2	23.3±2.3	41.7±2.7	10-11	*Serrasalmus spilopleura*	Interlamellar epithelium	Brazil	Barassa et al. (2003)
H. pellucida	11.4±0.3	4.1±0.4	-	4.0±0.4	1.6±0.2	24.1±1.5	33.3±1.5	6-7	*Piaractus mesopotamicus*	Serous membrane of visceral cavity	Fish farm	Adriano; Arana; Cordeiro (2005)
H. pilosa	21.1	5.9	-	7.4	1.2	31.1	54.2	11-12	*Serrasalmus altuvei*	Gill filament	Brazil	Azevedo; Matos (2003)
***Myxobolus* n. sp. 3**	**10.4±0.3**	**7.2±0.1**	**5.8±0.2**	**3.8±0.1**	**2.6±0.1**	-	-	**6-7**	***Piaractus brachypomus***	**Pyloric cecum**	**Amazon basin**	**Present study**
Myxobolus n. sp. 1	9.6±0.4	7.0±0.3	5.0±0.3	4.3±0.4	1.9±0.1	-	-	-	*Colossoma macropomum*	Inner surface of the operculum	Amazon basin	Present study
Myxobolus n. sp. 2	19.1±0.4	9.4±0.3	8.3±0.4	10.5±0.2	2.5±0.1	-	-	9-10	*Colossoma macropomum*	External wall of the stomach	Amazon basin	Present study
M. colossomatis	11.8	6.9	3.7	6.0	2.1	-	-	7-8	*Colossoma macropomum*	Several organs	Amazon river basin	Molnár; Békési (1993)
M. cuneus	10.0±0.6	5.1±0.3	-	5.7±0.3	1.7±0.2	-	-	8-9	*Piaractus mesopotamicus*	Several organs	Fish farm	Adriano et al. (2006)
M. cf. cuneus	10.5±0.3	5.4±0.2	4.3±0.3	6.0±0.3	2.0±0.3	-	-	9-10	*Hybrid patinga******	Spleen	Fish farm	Milanin et al. (2015)
M. cf. colossomatis	10.3±0.7	6.4±0.8	-	4.4±0.4	1.8±0.2	-	-	7-8	*Piaractus mesopotamicus*	Gills	Fish farm	Müller et al. (2013)
M. maculatus	21.0	8.9	7.5	12.7	3.2	-	-	14-15	*Metynnis maculatus*	Kidney	Amazon river	Casal et al. (2002)
M. serrasalmus	14.8	8.6	-	7.7	3.1	-	-	-	*Serrasalmus rhombeus*	Kidney, Liver	Brazil	Walliker (1969)
M. myleus	19.3±0.5	8.3±0.5	4.0±0.3	13.2±0.4	3.0±0.3	-	-	19-21	*Myleus rubripinnis*	Gall bladder	Sapuruá lagoon	Azevedo et al. (2012)
M. metynnis	13.1	7.8	3.9	5.2	3.2	-	-	8-9	*Metynnis argenteus*	Orbicular region	Amazon river	Casal et al. (2006)
M. noguchi	13.6	8.5	-	6.8	2.2	-	-	-	*Serrasalmus spilopleura*	Gills	Brazil	Pinto (1928)
M. cunhai	9-11	4-6	-	-	-	-	-	-	*Pygocentrus piraya*	Intestinal content	Brazil	Penido (1927)

*****Result of the crossing between *Piaractus mesopotamicus* X *Piaractus brachypomus.*
Source: own authorship.

Table 6. Comparison of the genetic identity of ssrDNA of *Myxobolus* spp. and *Henneguya* spp. parasites of serrasalmid fish. The area above the diagonal shows % pairwise distance identity. The area below the diagonal shows nucleotide differences.

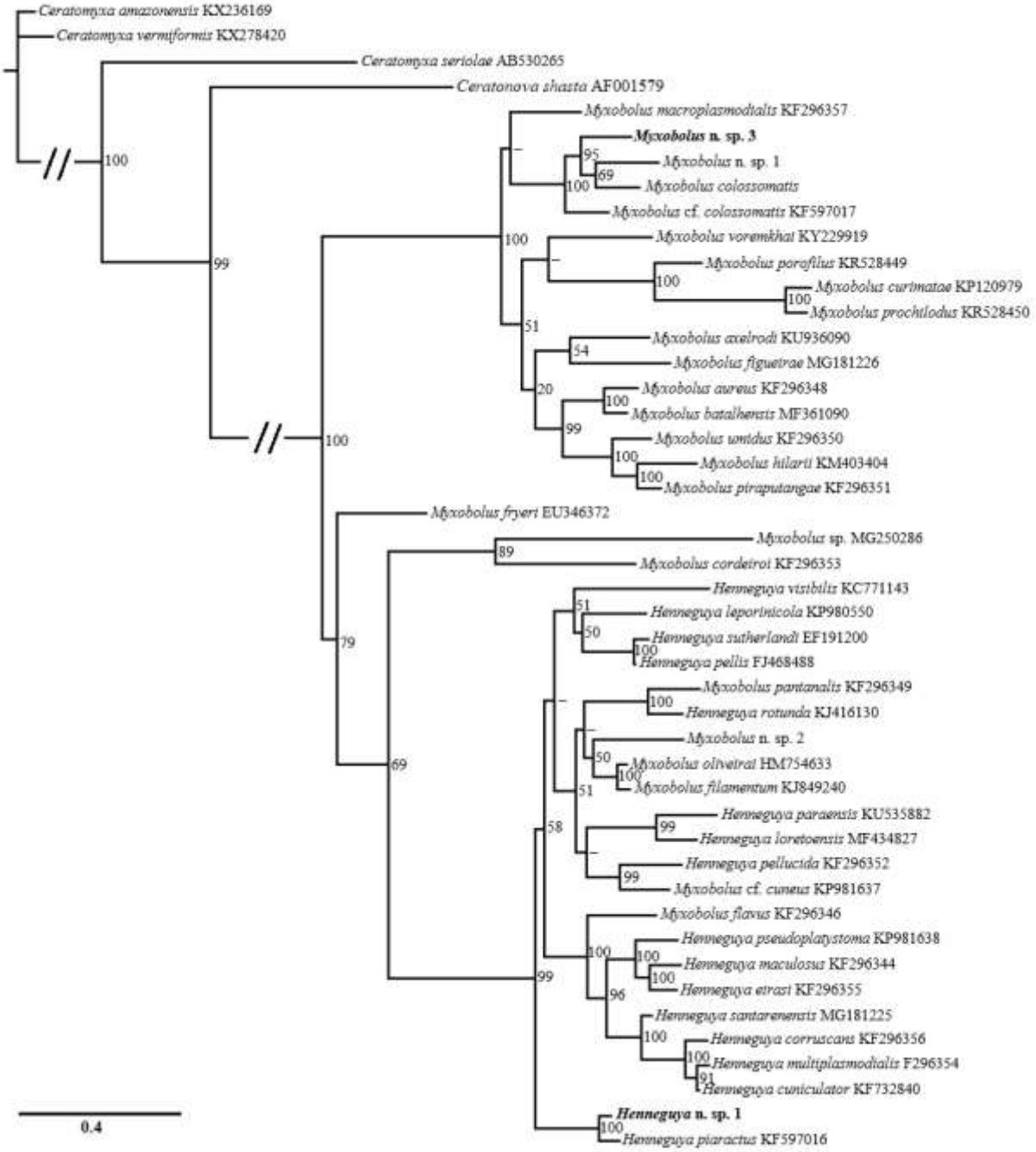
Species	**1**	2	3	**4**	5	6	7	8	9
1 *Henneguya* n. sp. 1	-	17.9	5.1	20.9	20.3	17.0	22.1	19.8	15.7
2 *Henneguya pellucida*	279	-	17.7	26.5	29	17.3	27.8	26.9	15.1
3 *Henneguya piaractus*	97	276	-	22.3	20.6	16.4	21.5	21.1	14.6
4 *Myxobolus* n. sp. 3	388	203	407	-	10.9	23.1	10.7	11.5	21.6
5 *Myxobolus* n. sp. 1	385	223	379	205	-	22.4	10.3	12.7	22.1
6 *Myxobolus* n. sp. 2	327	132	311	428	419	-	23.3	21.1	14.8
7 *Myxobolus colossomatis*	413	213	396	201	198	435	-	11.1	22.1
8 *Myxobolus* cf. *colossomatis*	263	165	280	152	170	280	148	-	21.3
9 *Myxobolus* cf. *cuneus*	291	115	269	384	395	274	396	283	-

Source: own authorship

Phylogenetic analysis

Phylogenetic analysis using the ssrDNA sequences of the closest myxosporeans to each of the new species based on BLAST searches placed the grouping in two main lineages: the smaller clustered only *Myxobolus* species, while the larger grouped the *Myxobolus*/*Henneguya* species. *Myxobolus* n. sp. 3 appears in the sister branch of *Myxobolus* n. sp. 1 (Present study, Chapter 1) and *M. colossomatis*. *Henneguya* n. sp. 1 appears as a sister species of *H. piaractus*, in a sister branch of the larger lineage composed of *Myxobolus*/*Henneguya* species (Figure 15).

Figure 15. Maximum likelihood tree showing the relationship between *Henneguya* n. sp. 1 and *Myxobolus* n. sp. 3 and other myxobolids based on ssrDNA. The numbers above the nodes indicate bootstrap confidence levels. Dashes are shown for values under 50%.



Genetic host identification

The host species were confirmed as pure though the sequencing of the α-tropomyosin gene of approximately 300 bp, demonstrating 100% of similarity with *P. brachypomus* species using BLASTn of the GenBank database.

DISCUSSION

Henneguya n. sp. 1 and *Myxobolus* n. sp. 3 are the first myxozoans described infecting pirapitinga, an iconic species of fishing and fish farming in the Amazon. Histological analysis of both species revealed the absence of inflammatory infiltrate, which is a common feature in myxosporean infections (SITJA-BOBADILLA, 2008). However, in *Henneguya* n. sp. 1 the growth of the plasmodia induced stretching of the epithelium and compression of the capillaries of the lamellae. In addition, there was displacement and deformation of the neighboring lamellae, demonstrating the pathogenic potential of this species in high intensities in farmed fish. Similar tissue damage has been observed in infections by *H. piaractus*, commonly found parasitizing the gill lamellae of the congeneric fish *P. mesopotamicus* in Brazilian fish farms (MARTINS et al., 1997; ADRIANO et al., 2005; MULLER et al., 2013). Under the conditions of the present study, *Myxobolus* n. sp. 3 did not demonstrate pathogenic potential, largely due to the site of infection and the changes observed therein.

Ultrastructural analysis of the host-parasite interaction revealed pinocytic channels in both the species here described, a common strategy by which myxosporeans obtain nutrients to support sporogonic development inside the plasmodia (HALLETT; DIAMANT, 2001; EL-MANSY; BASHTAR, 2002; AZEVEDO; MATOS, 2003; OVCHARENKO et al., 2017). Generative cells and young developmental spore stages occurred in the periphery of the plasmodium, while advanced sporogonic stages and mature myxospores were found in the central section, following a common sporogenesis pattern of *Myxobolus/Henneguya* plasmodia (ADRIANO et al., 2005; NALDONI et al., 2018).

Phylogenetic analysis based on ssrDNA sequences revealed *Henneguya* n. sp. 1 clustering in a basal branch as a sister species to *H. piaractus,* which parasites the gill lamellae of the congeneric host *P. mesopotamicus*, but which is endemic to the La Plata basin (MARTINS; SOUZA; 1997; ADRIANO et al., 2005). *Myxobolus* n. sp. 3 clustered in a sub-

clade composed of *Myxobolus* species which also parasite other South American serrasalmid fish. These results showed *Henneguya* n. sp. 1 and *Myxobolus* n. sp. 3 clustering in distinct lineages in the tree, corroborating the absence of monophyly in the lineage of *Myxobolus/Henneguya* parasites of South American serrasalmids, as stated in the present study (Chapter 1).

The present study expands our knowledge of the diversity of myxosporean parasites of Amazonian Serrasalmidae fish, describing two new species, and offering new insights into the evolution and host-parasite relationship of myxosporean infections in these important native fish for fishing and fish farming.

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CHAPTER 3. MORPHOLOGY AND PHYLOGENY OF TWO MYXOSPOREAN SPECIES PARASITIZING *Piaractus brachypomus* FROM THE AMAZON BASIN

CHAPTER 3. MORPHOLOGY AND PHYLOGENY OF TWO MYXOSPOREAN SPECIES PARASITIZING *Piaractus brachypomus* FROM THE AMAZON BASIN

ABSTRACT

Mixosporids, belonging to the Cnidaria phylum, are obligate endoparasites, which mainly infect fish and which comprise more than 2,400 described species. They have a complex life cycle, involving an invertebrate and vertebrate host. Among the species of fish that harbor these parasites, the species *Piaractus brachypomus*, popularly known as pirapitinga, has great economic importance, especially in the region of the Amazon basin, where it is widely found. The objective of this study was to characterize, through morphological, molecular and phylogentic analyses, the new species of myxosporids found infecting *P. brachypomus*. In this study, 25 specimens of *P. brachypomus* were collected, of which 16 (64%) were infected in the gill filament with a *Henneguya* species and 3 (12%) with a *Myxobolus* species in intestine. The morphological and morphometric analysis of *Henneguya* n. sp. 2 revealed myxospores with ellipsoid shaped and prolongation of the valves were lager than the length of the body. The polar capsules were elongated and occupied less than half of the body. Molecular analysis generated a partial sequence of 1946 pb of the ssrDNA. Phylogenetic analysis revealed a cluster according to the order/family of the host, with *Henneguya piaractus* being the closest related species. *Myxobolus* n. sp. 4 in morphological analysis revealed myxospores with an oval shaped body and polar capsules of the same size occupying less than half of the body. Molecular analysis generated a 1950 bp partial sequence of the ssrDNA and phylogenetic analysis revealed a cluster as a sister species of *Myxobolus colossomatis.* Sequencing of the *α-tropomyosin* gene confirmed that the collected fish belonged to the species *P. brachypomus*. The results obtained for *Henneguya* n. sp. 2 and *Myxobolus* n. sp. 4 confirmed that both isolates are from species not yet described in the literature.

Key-words: *Henneguya*, *Myxobolus*, Tapajós River, pirapitinga, ssrDNA.

INTRODUCTION

Myxozoa subphylum is composed of microscopic cnidarians that have adaptations to parasitism (OKAMURA et al., 2015). There are some 2400 described species which the great majority are myxosporeans (FIALA et al., 2015). These parasites infect mainly fish, in the natural environment and fish farms, but also birds, amphibians, and reptiles (Bartholomew et al., 2008). With the development of aquaculture, the interest in the group has intensified, since many species cause serious disease outbreaks that can generate significant economic impacts, especially where prevalence rates are high. (FEIST; LONGSHAW, 2006).

The myxosporeans are highly specialized metazoan parasites characterized by multicellular spores, with polar capsules containing an extrusible polar filament (FEIST; LONGSHAW, 2006). These organisms can be found infecting several organs and have high specificity both for the host and for the tissues they infect (BÉKÉSI et al., 2002). In addition, parasites develop in plasmodia that may or may not cause tissue compression and an inflammatory host response (EIRAS, 1994; FEIST; LONGSHAW, 2006). Most species are not pathogenic, but several of them cause significant damage to their hosts (FEIST; LONGSHAW, 2006).

Among the Myxosporea class, the genera *Henneguya* Thélohan, 1892 and *Myxobolus* Bütschli, 1882 are the most specious, with approximately 192 species described of *Henneguya* and 850 of *Myxobolus* (EIRAS; ADRIANO, 2012; EIRAS et al. 2014).

The present study is part of a research that involves morphological and molecular analyses for the identification of new species of myxosporeans found infecting the species *Piaractus brachypomus* Cuvier, 1818. Using light microscopy and amplification of the ssrDNA, we described new species of *Henneguya* and *Myxobolus.*

MATERIALS AND METHODS

Sample collection

Twenty-five specimens of *P. brachypomus* were caught (SISBIO nº 44268-4 and SisGen nº A33CB83) between October 2014 and January 2016 from Tapajós River, municipality of Santarém, Pará State, Brazil. In total, twenty specimens (80%) were infected with myxozoan plasmodia. All the fish handling was approved by the ethics committee for animal welfare of the Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo – USP Process USP: 14.1.391.74-9 (ATTACHMENT B).

Immediately after capture, the fish were transported alive to the field laboratory where they were euthanized by transection of the spinal cord. After euthanasia, then were weighed, measured and an external analysis was performed in search of parasite plasmodia. Subsequently, the necropsy was performed to expose of the gills and the visceral cavity, in order to detect possible alterations in the characteristics of the organs and the presence of parasites. Fragments of host tissue infected with Myxozoa plasmodia were also collected for molecular confirmation of the host. All material was fixed in absolute ethanol for molecular analyses and in formaldehyde for morphological characterization of the myxospores.

Morphological analysis

For morphological analysis, plasmodia fixed in formaldehyde were separated from the host tissue, placed on a slide and ruptured. The measures of the myxospores was performed in accordance with Lom and Arthur (1989), using a computer equipped with Axivision 4.1 software, linked to Axioplan 2 Zeiss microscope. Thirty-six mature myxospores of *Henneguya* n. sp. 2 and thirty-one of *Myxobolus* n. sp. 4 were photographed. The dimensions were expressed as the mean ± standard deviation (SD). The slides were then fixed with methanol and

stained with Giemsa to be deposited in the collection of the Adão José Cardoso Museum of Zoology, of the State University of Campinas (UNICAMP), Brazil.

Molecular analysis and sequencing

In molecular analysis, after capturing images, the slides containing myxospores were washed with PBS buffer and collected in a microtube. After centrifugation, one more washing was done with PBS buffer to remove remnants of the ethanol. DNA was extracted using the DNeasy® Blood & Tissue Kit (Qiagen Inc., California, USA), according to the manufacturer's instructions. DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA) at 260 nm. The ssrDNA was amplified by PCR and the reactions were performed with a final volume of 25 µL containing 10-50 ng of genomic DNA, 12.5 µL of 2 × DreamTaq PCR Master Mix (Thermo Scientific – Carlsbad, California, USA), 0.2 µM of each primer and nuclease-free water (Thermo Scientific – Carlsbad, California, USA). The PCR amplifications were performed in a ProFlex PCR Systems (Applied Biosystems™ Inc., California, USA) thermocycler with a program consisting of initial denaturation carried out at 95 °C for 5 minutes, followed by 35 cycles of denaturation (95 °C for 60 s), hybridization (60 °C for 60 s) and extension (72 °C for 90 s) and a final extended elongation step at 72 °C for 5 minutes.

Partial sequence of the ssrDNA was amplified using two primers pairs: ERIB1 (BARTA et al., 1997) with ACT1R (HALLET; DIAMANT, 2001) and TEDF (CAPODIFOGLIO et al., 2015) with ERIB10 (BARTA et al., 1997). PCR products were analyzed by 1.5% agarose gel electrophoresis in a TAE buffer (Tris–Acetate EDTA, Tris 40 mM, Acetic Acid 20 mM, EDTA 1 mM), stained with Sybr Safe DNA gel stain (Thermo Scientific – Carlsbad, California, USA) and analyzed with a LED transilluminator (Kasvi, Paraná, Brazil). The sizes of the amplified fragments were estimated by comparison using the 1 Kb Plus DNA Ladder (Thermo Scientific

– Carlsbad, California, USA). Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen Inc., California, USA) according to the manufacturer's specifications, quantified in 2% agarose gel electrophoresis and compared to Low DNA Mass (Thermo Scientific – Carlsbad, California, USA).

Sequencing of the purified PCR products was performed with specific primers on the ABI 3730 DNA sequencer (Applied Biosystems™ Inc., California, USA), using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems™ Inc., California, USA). Afterwards, the obtained sequences were visualized, edited and aligned in the program BioEdit 7.1.3.0 (HALL, 1999) using the ClustalW algorithm (THOMPSON, 1994), and a consensus sequence was obtained for each sample. The consensus sequences were submitted to BLASTn analysis for the search for similarity in the GenBank database (NCBI). The p-distance analysis was performed in the MEGA 7.0 (KUMAR; STECHER; TAMURA, 2016) to verify the genetic distance between the species of this study and other myxozoan sequences available in the NCBI.

For the molecular characterization of *P. brachypomus* species, the DNA extraction from the fish tissue was carried out in the same way as for myxosporeans. The amplification and sequencing of the α-tropomyosin gene from the two specimens of fish infected by *Henneguya* and *Myxobolus* were performed according to Hashimoto et al. (2011) using the primer pair TROPF and TROPR (FRIESEN et al., 1999). The amplification product was obtained and then sent for sequencing. The sequences obtained were subjected to BLASTn analysis of the GenBank database to search for genetic similarity and confirmation of the host species.

Phylogenetic analysis

For phylogenetic analysis the partial ssrDNA sequences of *Henneguya* n. sp. 2 and *Myxobolus* n. sp. 4 and closest DNA sequences from the NCBI database of genera *Heneguya* and *Myxobolus* were used to phylogenetic analysis. The analysis was performed using

maximum likelihood in PhyML software (GUINDON et al., 2010) and a bootstrap analysis of 1000 replicates. *Ceratomyxa vermiformis* Adriano and Okamura, 2017 and *Ceratomyxa amazonensis* Mathews, Naldoni, Maia and Adriano, 2016 were used as the outgroup.

RESULTS

Identification of the host collected

The amplification of the α-tropomyosin gene, resulted in a fragment of approximately 300 pb. The sequences obtained were aligned with two other *P. brachypomus* sequences deposited on GenBank (accession numbers: HQ420087 and AY817271), demonstrating 100% similarity.

Taxonomic data

Two new species found infecting *P. brachypomus* from Tapajós River were characterized through of the morphological, molecular and phylogenetic analyses.

***Henneguya* n. sp. 2** (Figures 16A, 17A)

Description: Plasmodia were rounded and yellow. Mature myxospores of *Henneguya* n. sp. had ellipsoid shape in frontal view, measuring 13.6 ± 0.5 μm length and 3.6 ± 0.2 μm width. In lateral view, the body spores were fusiform and symmetrical with 2.5 ± 0.4 μm thickness. Prolongation of the valves were lager than the length of the body, measuring 40.3 ± 2.7 μm, with a total spore length of 53.4 ± 2.9 μm. The polar capsules were elongated and occupied less than half of the body, measuring 5.0 ± 0.5 μm in length and 1.4 ± 0.2 μm in width (Table 7). It was not possible to count the number of coils of the polar filament by light microscopic (Figure 16A).

Figure 16. Photomicrograph of mature myxospore species parasites of *Piaractus brachypomus* from the Tapajós River. **A** – *Henneguya* n. sp. 2 infecting the gill filament. **B** - *Myxobolus* n. sp. 4 found infecting the intestine. Scale bars = 10 µm.

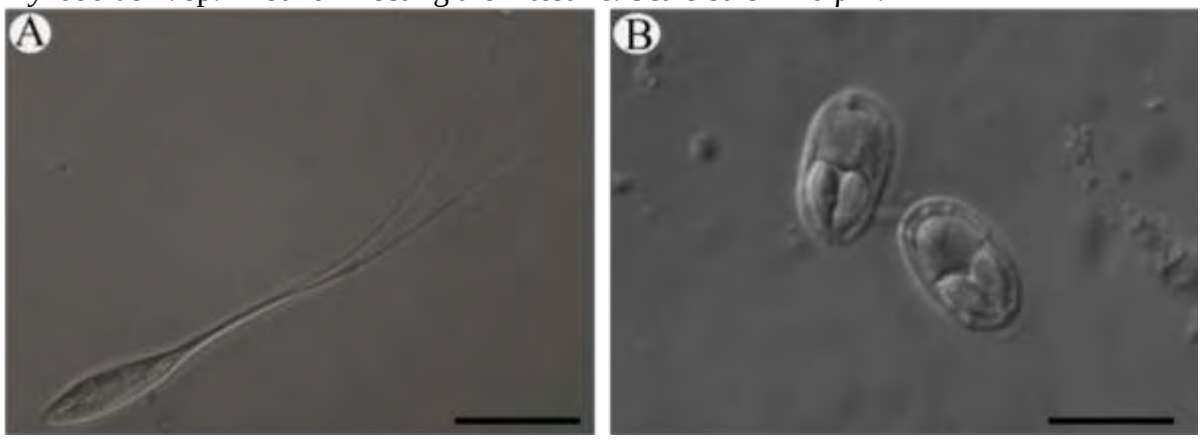
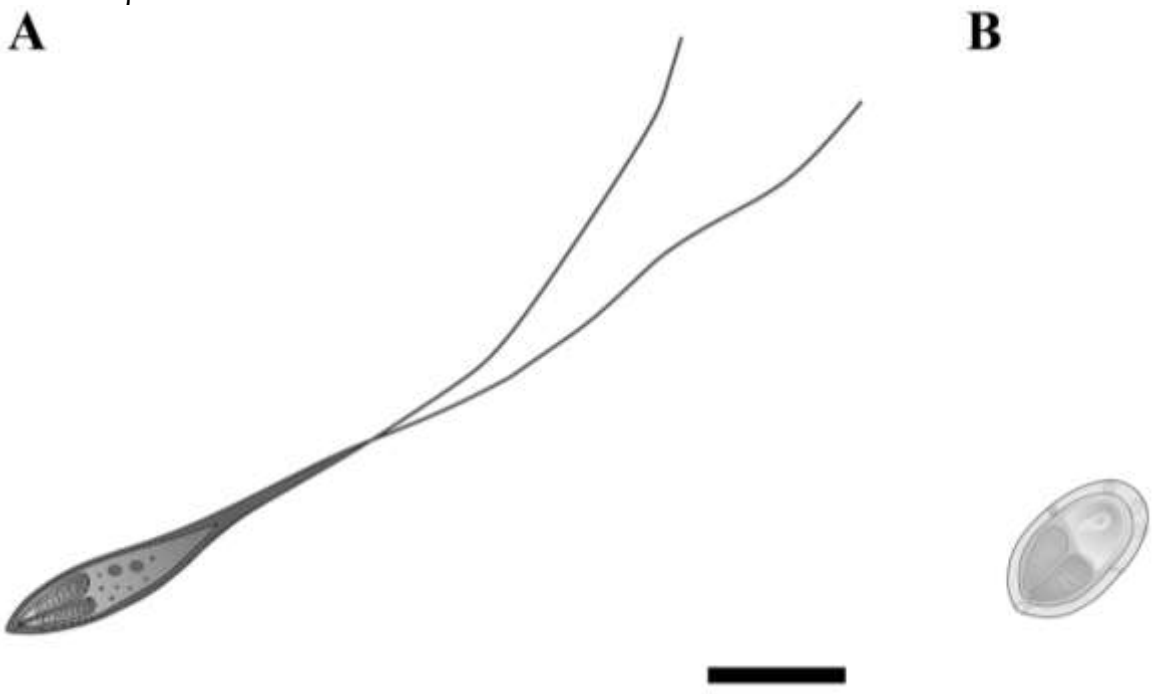


Figure 17. Schematic representations. **A** – *Henneguya* n. sp. 2. **B** – *Myxobolus* n. sp. 4. Scale bars = 10 µm



Type locality: Tapajós River, municipal district of Santarém, state of Pará, Brazil (2°23'07.5"S 54°47'05.9"W).

Occurrence: 16 of 25 (64%).

Site of infection: Gill filament.

Type of material: Slides stained with myxospores was deposited in the collections of the Adão José Cardoso Museum of Zoology, of the State University of Campinas (UNICAMP), São Paulo state, Brazil (accession number ZUEC –MYX88).

Molecular data: Sequencing of the ssrDNA resulted in a partial sequence of 1946 pb.

Remarks: According to the BLASTn analysis, based on the ssrDNA sequence, the closest species of *Henneguya* n. sp. 2 was *Henneguya pellucida* Adriano, Arana and Cordeiro, 2006, showing 89% similarity, which was described as infecting *Piaractus mesopotamicus* from fish farming in the state of São Paulo. The comparison between these two species, through morphological and morphometric analysis, showed some differences. With respect to the morphology, *Henneguya* n. sp. 2 showed elongated body when compared to *H. pellucida* and the caudal extension of *Henneguya* n. sp. 2 was longer. The morphometric comparison revealed differences with respect to: body lengh (13.6 μm for *Henneguya* n. sp. 2 and 11.4 μm for *H. pellucida*), polar capsule length (5.0 μm for *Henneguya* n. sp. 2 and 4.0 μm for *H. pellucida*), total length (53.4 μm for *Henneguya* n. sp. 2 and 33.3 μm for *H. pellucida*) and length of the caudal extension (40.3 μm for *Henneguya* n. sp. 2 and 24.1 μm for *H. pellucida*). In addition to the morphological and morphometric differences, there are differences in relation to the host (*P. brachypomus* for *Henneguya* n. sp. 2 and *P. mesopotamicus* for *H. pellucida*) and the locality (the Amazon basin for *Henneguya* n. sp. 2 and fish farms in the state of São Paulo for *H. pellucida*) (Table 7). The p-distance analysis, based in parasites species of serrasalmids fish, showed *Henneguya* n. sp. 2 as closest species of *Henneguya* sp. 1 (Present study, Chapter 2) with 16.3% of genetic difference (Table 8).

***Myxobolus* n. sp. 4** (Figures 16B, 17B)

Description: Plasmodia were rounded and yellow. Mature myxospores of *Myxobolus* n. sp. 4 had an oval body shape, measuring 12.9 ± 0.5 μm in length, 7.5 ± 0.3 μm in width and

5.4 ± 0.2 μm in thickness. The polar capsules were of equal size, presenting 5.4 ± 0.3 μm in length and 2.2 ± 0.1 μm in width (Table 7). It was not possible to count the number of coils of the polar filament (Figure 16B).

Type locality: Tapajós River, municipal district of Santarém, state of Pará, Brazil (2°23'07.5"S 54°47'05.9"W).

Occurrence: 3 of 25 (12%).

Site of infection: Intestine.

Type of material: Slides stained with myxospores was deposited in the Adão José Cardoso Museum of Zoology, of the State University of Campinas (UNICAMP), São Paulo state, Brazil (accession number ZUEC – MYX87).

Molecular data: Sequencing of the ssrDNA resulted in a partial sequence of 1950 pb.

Remarks: The partial sequence of ssrDNA of *Myxobolus* n. sp. 4 showed, according to BLASTn analysis, to be a distinct species from others species with sequences deposited in GenBank. The closest species was *Myxobolus* c*olossomatis* Molnár and Békési (1993) with 94% similarity, described infecting several organs of *Colossoma macropomum* collected in the Amazon basin. Considering parasite species of serrasalmids, *Myxobolus* n. sp. 4 was the closest species to *M. colossomatis*. The comparison between these two species, through morphological and morphometric analysis, showed differences. With respect to morphology, *Myxobolus* n. sp. 4 had an ellipsoidal shape and the polar capsules occupied half of the myxospore body, whereas *M. colossomatis* had an oval shape, an anterior end, and polar capsules occupying half of the myxospore body. The morphometric comparison revealed similarities with respect to body length and polar capsules width. The differences were with respect to: body width (7.5 μm for *Myxobolus* n. sp. 4 and 6.9 μm for *M. colossomatis*), polar capsule length (5.4 μm for *Myxobolus* n. sp. 4 and 6.0 μm for *M. colossomatis*). In addition, there are differences in relation to the host (*P. brachypomus* for *Myxobolus* n. sp. 4 and *C. macropomum* for *M. colossomatis*) (Table 7).

In additional, the p-distance analysis, based in parasites species of serrasalmids fish, showed *Myxobolus* n. sp. 4 as closest species of *M. colossomatis* (Present study, Chapter 1) with 6.3% of genetic difference (Table 8).

Table 7. Comparative data of *Henneguya* n. sp. 2 and *Myxobolus* n. sp. 4 with other species of *Henneguya/Myxobolus* described in fish of the Serrasalmidae family from South America. **ML –** body length; **MW –** body width; **PCL –** polar capsule length; **PCW –** polar capsule width; **CPL –** caudal process length; **TL –** total length; **TH** – Thickness and **NCF –** numbers of coils of the polar filament; Dashes – no data. All measurements are given as mean ± standard deviation in μm.

Species	ML	MW	PCL	PCW	CPL	TL	TH	NCF	Host	Site of infection	Locality	Reference
***Henneguya* n. sp. 2**	**13.6±0.5**	**3.6±0.2**	**5.0±0.5**	**1.4±0.2**	**40.3±2.7**	**53.4±2.9**	**2.5±0.4**	-	***Piaractus brachypomus***	**Gill filament**	**Amazon basin**	**Present study**
H. piaractus	12.8±0.7	4.1±0.2	6.5±0.4	1.2±0.2	46.4±2.1	59.6±2.3	-		*Piaractus mesopotamicus*	Gill filament	Brazil	Martins; Souza (1997)
H. pellucida	11.4±0.3	4.1±0.4	4.0±0.4	1.6±0.2	24.1±1.5	33.3±1.5	-	6-7	*Piaractus mesopotamicus*	Visceral cavity	Fish farm	Adriano; Arana; Cordeiro (2005)
H. curvata	16.4±0.8	4.7±0.2	7.8±0.3	1.4±0.2	23.3±2.3	41.7±2.7	-	10-11	*Serrasalmus spilopleura*	Interlamellar epithelium	Brazil	Barassa et al. (2003)
H. pilosa	21.1	5.9	7.4	1.2	31.1	54.2	-	11-12	*Sserrasalmus altuvei*	Gill filament	Brazil	Azevedo; Matos (2003)
Henneguya n. sp. 1	13.0±0.5	4.3±0.2	6.3±0.5	1.6±0.2	44.7±3.0	57.5±3.5	3.8±0.2	8-9	*Piaractus brachypomus*	Gill filament	Amazon basin	Present study
***Myxobolus* n. sp. 4**	**12.9±0.5**	**7.5±0.3**	**5.4±0.2**	**2.2±0.1**	-	-	**5.4±0.2**	-	***Piaractus brachypomus***	**Intestine**	**Amazon basin**	**Present study**
Myxobolus n. sp. 3	10.4±0.3	7.2±0.1	3.8±0.1	2.6±0.1	-	-	5.8±0.2	6-7	*Piaractus brachypomus*	Pyloric cecum	Amazon basin	Present study
M. colossomatis	11.8	6.9	6.0	2.1	-	-	3.7	7-8	*Colossoma macropomum*	Several organs	Amazon river	Molnár; Békési (1993)
M. cf. *colossomatis*	10.3±0.7	6.4±0.8	4.4±0.4	1.8±0.2	-	-	-	7-8	*Piaractus mesopotamicus*	Gills	Fish farm	Müller et al. (2013)
M. cuneus	10.0±0.6	5.1±0.3	5.7±0.3	1.7±0.2	-	-	-	8-9	*Piaractus mesopotamicus*	Several organs	Fish farm	Adriano et al. (2006)
M. cf. *cuneus*	10.5±0.3	5.4±0.2	6.0±0.3	2.0±0.3	-	-	4.3±0.3	9-10	Hybrid patinga*****	Spleen	Fish farm	Milanin et al. (2015)
M. cunhai	9-11	4-6	-	-			-	-	*Pygocentrus piraya*	Intestinal content	Brazil	Penido (1927)
Myxobolus n. sp.2	19.1±0.4	9.4±0.3	4.3±0.4	2.5±0.1	-	-	8.3±0.4	9-10	*Colossoma macropomum*	Wall of the stomach	Tapajós river	Present study
M. maculatus	21.0	8.9	12.7	3.2	-	-	7.5	14-15	*Metynnis maculatus*	Kidney	Amazon river	Casal et al. (2002)
Myxobolus n. sp. 1	9.6±0.4	7.0±0.3	4.3±0.4	1.9±0.1	-	-	5.0±0.3	-	*Colossoma macropomum*	Surface of the operculum	Amazon basin	Present study
M. metynnis	13.1	7.8	5.2	3.2	-	-	3.9	8-9	*Metynnis argenteus*	Orbicular region	Amazon river	Casal et al. (2006)
M. myleus	19.3±0.5	8.3±0.5	13.2±0.4	3.0±0.3	-	-	4.0±0.3	19-21	*Myleus rubripinnis*	Gall bladder	Sapuruá lagoon	Azevedo et al. (2012)
M. noguchi	13.6	8.5	6.8	2.2	-	-	-	-	*Serrasalmus. spilopleura*	Gills	Brazil	Pinto (1928)
M. serrasalmus	14.8	8.6	7.7	3.1	-	-	-	-	*Serrasalmus rhombeus*	Kidney, Liver	Brazil	Walliker (1969)

*****Result of the crossing between *Piaractus mesopotamicus* X *Piaractus brachypomus.*

Source: own authorship.

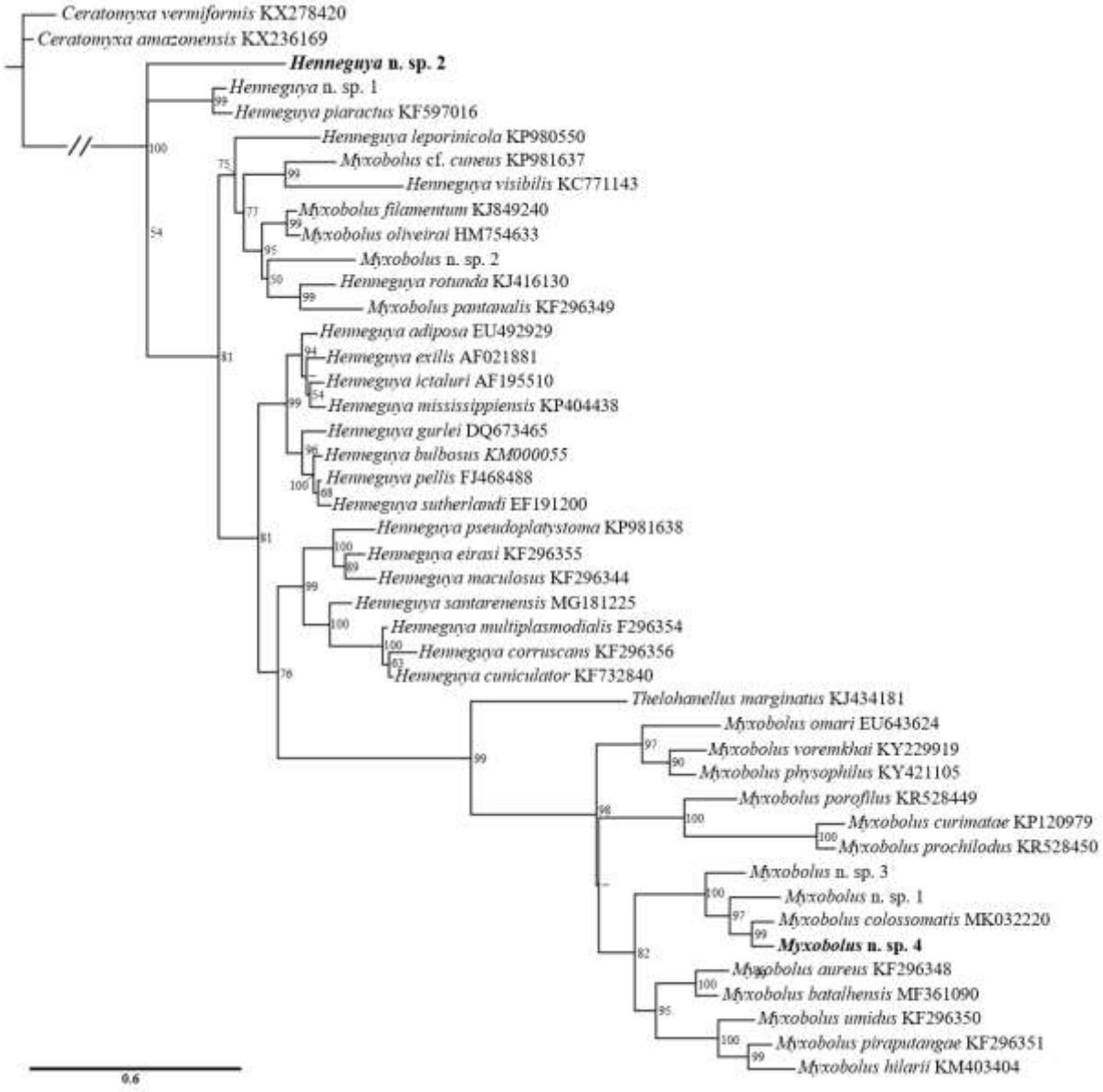
Table 8. P-distance comparison of ssrDNA of *Henneguya* spp. and *Myxobolus* spp. parasites of the fish belonging Serrasalmidae family. The area above the diagonal shows % pairwise distance identity. The area below the diagonal shows nucleotide differences.

Species	1	2	3	4	5	6	7	8	9	10	11
1 *Henneguya* n. sp. 1	-	17.9	5.1	**16.3**	20.9	20.3	17.0	22.1	19.8	15.7	**22.3**
2 *Henneguya pellucida*	279	-	17.7	**20.3**	26.5	29	17.3	27.8	26.9	15.1	**26.3**
3 *Henneguya piaractus*	97	276	-	**16.9**	22.3	20.6	16.4	21.5	21.1	14.6	**22.7**
4 *Henneguya* n. sp. 2	**316**	**316**	**319**	-	**22.4**	**23.2**	**18**	**22.1**	**20.8**	**18.6**	**23.1**
5 *Myxobolus* n. sp. 3	388	203	407	**415**	-	10.9	23.1	10.7	11.5	21.6	**11**
6 *Myxobolus* n. sp. 1	385	223	379	**436**	205	-	22.4	10.3	12.7	22.1	**10.9**
7 *Myxobolus* n. sp 2	327	132	311	**345**	428	419	-	23.3	21.1	14.8	**23.2**
8 *Myxobolus colossomatis*	413	213	396	**413**	201	198	435	-	11.1	22.1	**6.3**
9 *Myxobolus* cf. *colossomatis*	263	165	280	**275**	152	170	280	148	-	21.3	**10.8**
10 *Myxobolus* cf. *cuneus*	291	115	269	**343**	384	395	274	396	283	-	**22.4**
11 *Myxobolus* n. sp. 4	**419**	**413**	**418**	**434**	**208**	**211**	**434**	**121**	**144**	**401**	-

Phylogenetic analysis

Henneguya n. sp. 2 and *Myxobolus* n. sp. 4 aggrouped in according with the family and order of the host as observed by Fiala et al. (2006). *Henneguya* n. sp. 2 aggrouped alone in a clade. *Myxobolus* n. sp. 4 aggrouped as a sister specie of *M. colossomatis* described infecting gills of *C. macropomum* in a subclade composed only *Myxobolus* species that infecting Characiformes fish (Figure 18).

Figure 18. Maximum likelihood showing relationship among *Henneguya* n. sp. 2 and *Myxobolus* n. sp. 4 with others myxobolids based on ssrDNA. The numbers above the nodes indicate bootstrap confidence levels. Dashes are shown for values under 60%.



DISCUSSION

Henneguya sp. 2 and *Myxobolus* sp. 4 were compared, morphological/morphometrically, with others myxosporean species around of the world and with all species of *Henneguya* and *Myxobolus* described in the South America freshwater fish (EIRAS et al., 2014), indicating that the species described here differs of others species (EIRAS, MOLNÁR; LU, 2005), besides the host and locality of collection.

For myxosporean species that infecting fish of the Serrasalmidae family 14 myxobolids were described. *Henneguya pilosa* Azevedo; Matos, 2003, *Henneguya pellucida* Adriano, Arana and Cordeiro, 2005, *Henneguya curvata* Barassa et al. (2003), *Henneguya piaractus* Martins and Souza, 1997, *Henneguya* sp. 1; *Myxobolus colossomatis* Molnár and Békési, 1993, *Myxobolus cuneus* Adriano et al., 2006, *Myxobolus maculatus* Casal et al., 2002, *Myxobolus metynnis* Casal et al., 2006, *Myxobolus myleus* Azevedo et al., 2012, *Myxobolus* cf. *colossomatis* Muller et al., 2013, *Myxobolus* sp. 1 (Present study) and *Myxobolus* sp. 2 (Present study). In *P. brachypomus* two myxosporean species have been related previously, with *Henneguya* n. sp. 1 (Present study) parasitizing the gill filament and *Myxobolus* n. sp. 3 (Present study) parasitizing the intestine.

Based on ssrDNA sequences of myxosporean parasites of serrasalmids fish, *Henneguya* n. sp. 2 was the closest to *Henneguya* n. sp. 1, both parasites of *P. brachypomus* from the Amazon basin, with 16.3% of difference, demonstrating that this host specie harbors two distinct *Henneguya* species which infect the gill filament. For *Myxobolus* n. sp. 4, *M. colossomatis* was closest species with 6.3% of difference, but this species showed differences morphologic and host, being sufficient to consider these two distinct species.

Our phylogenetic analysis demonstrated the tendency of the myxosporeans to group according to the host order as previous studies (FIALA, 2006; FERGUSON et al., 2008; CARRIERO et al., 2013; MOREIRA et al., 2014), as well as the influence of the infected tissue in which clustering occurred primarily by host affinity and after by tissue or organ tropism, positioning *Henneguya* n. sp. 2, as well *Henneguya* n. sp. 1 and *Henneguya piaractus*, as basal species of the major clade separated from the others myxosporids. *Myxobolus* n. sp. 4 aggrouped as a sister specie of *M. colossomatis* in a well-supported subclade composed only with *Myxobolus* species parasites of Characiformes fish from the Amazon basin and which

was closely related to *Myxobolus* species that infecting South American characiforms as observed by Zatti et al. (2018).

We provide here morphological and molecular data of two news species of myxosporeans that infecting *P. brachypomus* from the Tapajós River, contribuiting for the knowledgement of the biodiversity of myxosporean species.

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CHAPTER 4. REMARKS ON MITOCHONDRIAL BEHAVIOR IN MYXOSPOREANS

CHAPTER 4. REMARKS ON MITOCHONDRIAL BEHAVIOR IN MYXOSPOREANS

ABSTRACT

Myxozoa are parasites that mainly infect fish, but also reptiles, amphibians, birds and mammals and has a complex life cycle. The initial aim of this study was to amplify the mitochondrial gene Citochrome C Oxidase I of myxosporean parasites of *Colossoma macropomum* and *Piaractus brachypomus* from Amazon basin. Primers and protocols described in the literature as well as others primers were used for amplification, however, no amplification was obtained. Thus, the present study consists in to report remarks about mitochondrial behavior in myxosporeans of *Henneguya* sp. found infecting *Astyanax altiparanae* from CEPTA fish farm, of Pirassununga municipality, São Paulo State, Brazil. A total of 17 specimens of *A. altiparanae* were collected and 9 presented *Henneguya* sp. plasmodia in the fins. Plasmodia was ruptured and myxospores were stained with MitoTracker® Orange and 4′,6-diamidino-2-phenylindole (DAPI). The results showed that only some *Henneguya* sp. myxospores presented labeling for mitochondrial activity and all myxospores presented labeling for nucleuos.

Key-words: *Henneguya* sp., Myxozoa, mitochondrial fluorescence microscopy and COI.

INTRODUCTION

As important model organisms, cnidarians are fundamentals for research, where the *Hydra* genus is the model organism used for understanding processes of morphogenesis and regeneration (CHAPMAN et al., 2010) and, also, as model of non-senescence (BOEHM et al. 2013).

Belonging to Cnidaria phylum, myxozoans are parasites that infect a wide range of invertebrate and vertebrate hosts in worldwide (LOM; DYKOVÁ, 2006). With more than 2400 nominal species (ZHANG, 2011), they are distributed in 63 genera, and classified mainly by myxospore morphology (FIALA et al., 2015). The genus *Henneguya* Thélohan, 1892, encompasses approximately 200 species and are most commonly found in fresh water fishes (EIRAS; ADRIANO, 2012). Although most species do not cause disease, some are highly pathogenic causing severe disease in economically important fish and give rise to serious ecological on their host populations (KENT et al. 2001; FEIST; LONGSHAW, 2006; FEIST, 2008; OKAMURA; GRUHL; BARTHOLOMEW, 2015).

With a complex life cycle, myxosporeans have been identified in fish, repteils, amphibians and birds (BARTHOLOMEW et al., 2008), around the world, but little is known about their diversity, pathological impact on host, as well the biology these parasites (KODÁDKOVÁ, 2014). Regarding the biology of myxosporeans, myxospores are formed within plasmodia/pseudoplasmodia and they are characterized by multicellular myxospores, comprised of external valve cells that enclose polar capsules containing extrudable polar filaments and one or two sporoplasm cells (FEIST; LONGSHAW, 2006; OKAMURA, GRUHL; BARTHOLOMEW, 2015).

Takeuchi et al. (2015) sequenced for the first time the mitochondrial genome of *Kudoa* species and suggested that the genome of Myxozoa is in general extremely divergent from others metazoans.

The initial objective of this study was to amplify the mitochondrial gene of myxosporeans found in Amazonian fish as *Colossoma macropomum* and *Piaractus brachypomus*. In additional, was performed fluorescence microscopy to observe the mitochondrial activity of *Henneguya* sp. found infecting *Astyanax altiparanae*.

MATERIAL AND METHODS

COI amplification in myxosporeans found in Amazonian fish

Samples collection

A total of 61 fish (25 of *P. brachypomus* and 36 of *C. macropomum*) were caught (SISBIO nº 44268-4 and SisGen nº A33CB83) in October 2014, March 2015 and January 2016 in the Amazon basin, in Santarém, Pará State and in Manaus, Amazonas State, Brazil. The fish were transported live to a field laboratory and euthanized by transection spinal column. The methodology of the present study was approved by the ethics research committee of the Faculty of Animal Science and Food Engineering, University of São Paulo – Process USP: 14.1.391.74.9 (ATTACHMENT B) in accordance with Brazilian law (Federal Law nº 11794, dated 8 October 2008 and Federal Decree No. 6899 dated July 15, 2009).

DNA extraction

DNA from the isolated myxosporeans samples was extracted with the DNeasy® Blood & Tissue kit (Qiagen Inc., California, USA), according to the manufacturer's instructions and was quantified in NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA) at 260 nm.

Polymerase Chain Reaction (PCR)

PCR for amplification mitochondrial gene were performed with final volume of 25 µl, containing 10-50 ng genomic DNA, 12.5 µl of 2 × DreamTaq PCR Master Mix (Thermo Scientific – Carlsbad, California, USA), 0.2 µM of each primer and free water of nucleases (Thermo Scientific – Carlsbad, California, USA). For gene amplification, were used two pairs of primers (Table 9).

For PCR amplifications were used a ProFlex PCR Systems (Applied Biosystems™ Inc., California, USA) thermocycler. Initially, the reactions were performed in according to Folmer

et al. (1994) (Table 10). After this, primers were designed and others techniques for amplification of gene were tested (Tables 9,10).

Table 9. Primers used in the amplification Cytochrome C Oxidase I (COI) gene.

Gene	Primer	Sequence 5' – 3'	Reference
COI	LCO F	GGTCAACAAATCATAAAGATATTG	FOLMER et al. (1994)
	HCO R	TAAACTTCAGGGTGACCAAAAAATCA	FOLMER et al. (1994)
	14 F	CCTTAATTGGTGATTTCCTG	TAKEUCHI et al. (2015)
	19 R	TCTAGGGATTCCACAAAGAC	TAKEUCHI et al. (2015)
	TITI F	ACTATGTTGGGGGACGACCATC	Present study
	GABI R	GTTATAGCCCCGGCCAATACGG	Present study
	XIS F	AGTAGCCATAGGHTAYTGAAG	Present study
	XIS R	ACAGCCTAAATAGCCTG	Present study
	COI F	AGGCTGAACCATGTATCC	Present study
	COI R	TAGGTGGAGGTTTAACAGGAA	Present study
	16S F	CGCCTGTTTATCAAAAACAT	PALUMBI et al. (1991)
	16S R	CCGGTCTGAACTCAGATCACGT	PALUMBI et al. (1991)

Source: own authorship.

Table 10. Thermocycler programs used for Cytochrome C Oxidase I (COI) amplification in Conventional PCR.

Pair Primer (Forward/Reverse)	Technique	Initial Step	35 cycles			Final Step
			Denaturation	Hybridization	Extension	
LCO/HCO*	Conventional PCR	-	95°C 60"	40°C 60"	72°C 90"	72°C 7'
LCO/HCO	Gradient PCR	-	95°C 60"	(50 – 70)°C 60"	72°C 90"	72°C 7'
LCO/HCO	Conventional PCR	95°C 5'	95°C 60"	60°C 60"	72°C 60"	72°C 7'
LCO/TITI	Semi-nested PCR	-	95°C 60"	60°C 60"	72°C 90"	72°C 7'
LCO/GABI	Semi-nested PCR	-	95°C 60"	58°C 60"	72°C 90"	72°C 7'
LCO/HCO	Touchdown PCR	94°C 5'	94°C 60"	10 × 60°C 60"	72°C 90"	72°C 5'
				25 × 68°C 60"		
14/19**	Conventional PCR	95°C 3'	95°C 30"	55°C 30"	68°C 60"	68°C 5'
COI/COI	Conventional PCR	95°C 5'	95°C 60"	60°C 60"	72°C 60"	72°C 5'
16S/16S	Conventional PCR	95°C 5'	95°C 60"	60°C 60"	72°C 60"	72°C 5'
16S/16S	Nested PCR	95°C 5'	95°C 60"	60°C 60"	72°C 60"	72°C 5'
XIS/XIS	Conventional PCR	95°C 5'	95°C 60"	60°C 60"	72°C 60"	72°C 5'

*Primers and reaction performed in according to Folmer et al. (1994).
**Primers and reaction performed in according to Takeuchi et al. (2015).
Source: own authorship.

Transmission electron microscopy in myxosporeans found in Amazonian fish

For transmission electron microscopy, plasmodia were fixed in 2.5% glutaraldehyde diluted in 0.1 M sodium cacodylate (pH 7.4) for 12h, washed in glucose-saline solution for 2h and fixed in OsO_4. After, these samples were dehydrated in increasing concentrations of acetone and embedded in Embed 812 resin. Uranyl acetate citrate was used to treat ultrathin sections that, posteriorly, were examined in a LEO 906 transmission electron microscope operating at 60 kV.

Fluorescence microscopy in myxosporeans found in *Astyanax altiparanae*

Sample collection

Seventeen specimens of *A. altiparanae* were collected in May 2018 from Center National Research and Conservation Continental Aquatic Biodiversity (CEPTA) - ICMBio, municipality of Pirassununga, São Paulo State, Brazil. The fish were caught using nets and taken alive to laboratory. Before the examination, the fish were euthanized by lidocaine overdose. All the fish handling was approved by the ethics committee for animal welfare of the Faculty of Animal Science and Food Engineering, University of São Paulo Process USP: 14.1.391.74-9 (ATTACHMENT B).

After euthanasia, an external analysis was performed in search of parasite plasmodia. Subsequently, the necropsy was performed with exposure of the gills and the visceral cavity, in order to detect possible alterations in the characteristics of the organs and the presence of plasmodia. Myxosporean plasmodia were carefully separated from the host tissue and kept in microtubes containing PBS.

Staining with MitoTracker® Orange

Plasmodium containing live myxospores was ruptured and myxospores released were washed with PBS and centrifuged. After this, the myxospores were stained with MitoTracker®

Orange (Thermo Scientific – Carlsbad, California, USA) for 30 minutes at concentration of 250 nM at room temperature. After stain, myxospores were washed three times with PBS and visualized in inverted microscope Zeiss AxioVert A1 coupled with photographic camera Axio Can 503.

Permeabilization and Staining with DAPI and MitoTracker® Orange

Plasmodium containing live myxospores was ruptured and myxospores released were washed with PBS and centrifuged. After this, the myxospores were permeabilized with PBS containing 1% Triton X-100 for 1 h. Myxospores permeabilized were washed three times with PBS, centrifuged, and stained with 4′,6-diamidino-2-phenylindole dihydrochloride - DAPI (Thermo Scientific – Carlsbad, California, USA) 0.004 mg mL^{-1} for 10 minutes. The samples were rinsed with PBS and centrifuged to remove supernadant. Myxospores were then stained with MitoTracker Orange® (Thermo Scientific – Carlsbad, California, USA) for 30 minutes at concentration of 250 nM at room temperature. After stain, myxospores were washed three times with PBS and visualized in inverted microscope Zeiss AxioVert A1 coupled with photographic camera Axio Can 503.

RESULTS

COI amplification in myxosporeans found in Amazonian fish

Despite our efforts to obtain a COI gene sequence of myxosporids, no amplification was obtained. 16S F and 16S R are universal primers and were used for PCR walking method. However, the fragment of approximately 550 bp obtained was submitted BLASTn analysis (ALTSCHUL et al., 1997) which showed 100% of similarity with *C. macropomum* fish.

Transmission electron microscopy in myxosporeans found in Amazonian fish

Ultrastructural analysis of *Myxobolus* n. sp. 3 found infecting pyloric cecum of *P. brachypomus* showed several mitochondria in the periphery of the plasmodium and a reduced number of mitochondria in the central part of plasmodium (Figure 19A). In ectoplasm was observed a misshapen mitochondrion with the crests clearly evident and spherical mitochondria inside of secondary cells, with the mitochondrial crests less evident (Figure 19B). *Henneguya* n. sp. 1 found infecting gill filament of *P. brachypomus* showed large mitochondria near of ectoplasm and smaller mitochondria within of the myxospore at an advanced stage of development (Figures 20A – B).

Figure 19. Ultrastructure of *Piaractus brachypomus* cecum pyloric infected by *Myxobolus* n. sp. 3. **A** – Host – parasite interface showing the wall (leaked arrow) of the plasmodium (P) with numerous mitochondria (white arrow) in the periphery of the plasmodium and a smaller number of mitochondria (black arrow) in the central part of the plasmodium. Host (H). Scale bar = 5 μm. **B** – Magnification of parasite-host interface showing the wall (black arrow leaked) of the plasmodium (P). In ectoplasm (ec) were observed pinocytosis channels (white arrow leaked), a large shapeless mitochondria (m1) near of the sporoblast (white arrow), spherical mitochondria (m2) inside of the sporoblast and sporoplasmosomes (black arrow). Scale bar = 1 μm.

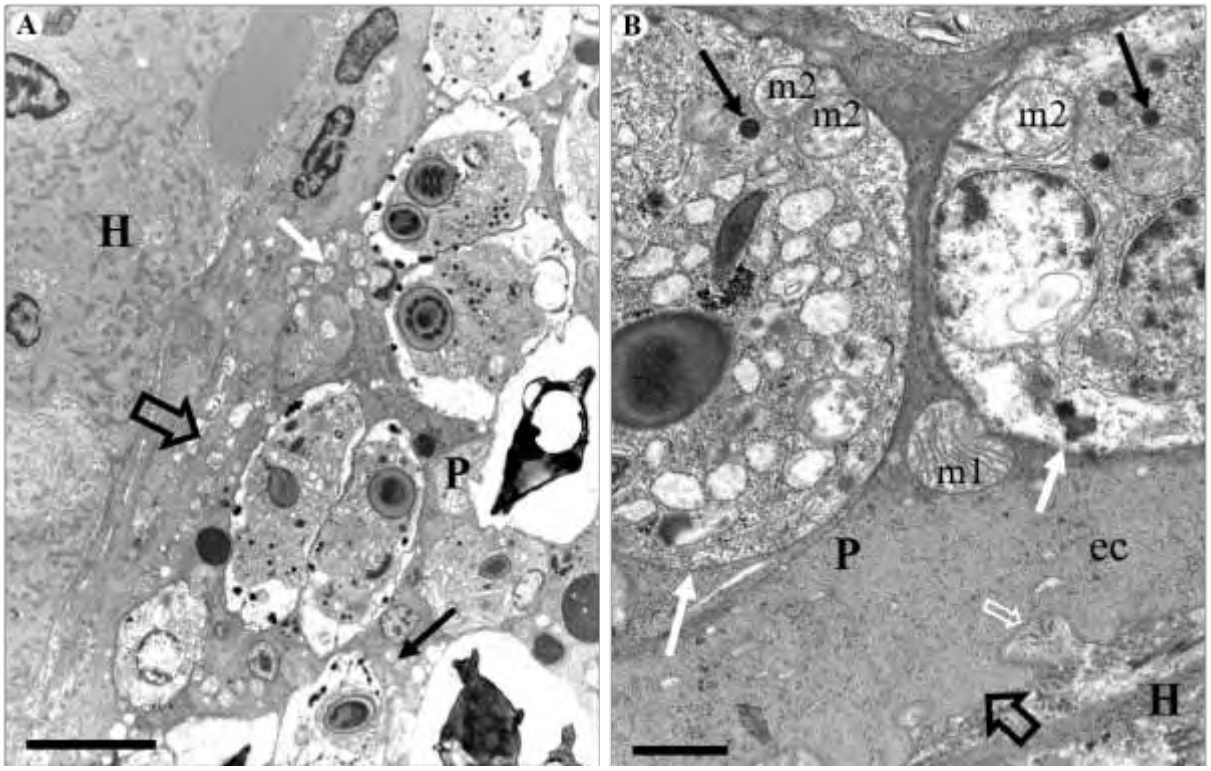
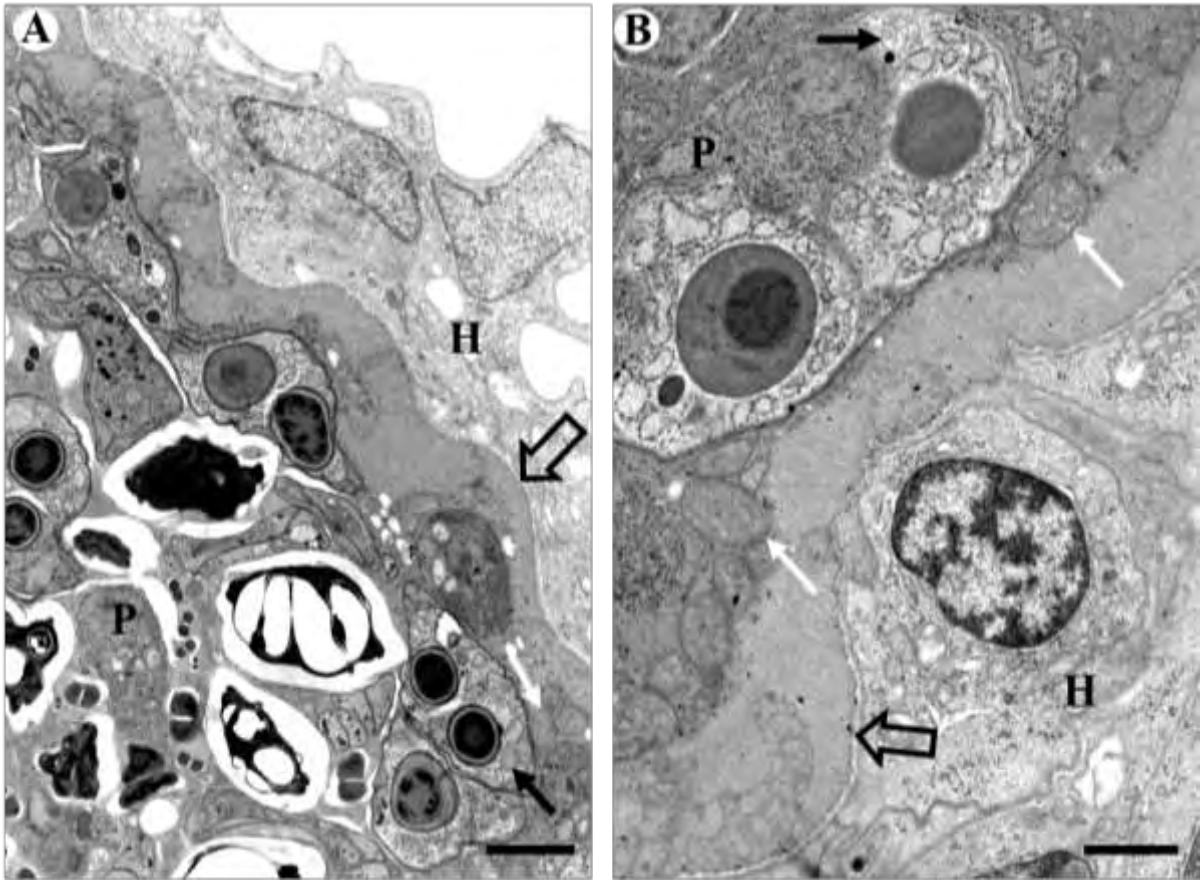


Figure 20. Ultrastructure of *Piaractus brachypomus* gill filament infected by *Henneguya* n. sp. 1. **A** and **B** – Host – parasite interface showing the wall (leaked arrow) of the plasmodium (P). Larger mitochondria (white arrow) in the periphery of the plasmodium "Pre-sporogony". Small mitochondria within the myxospore "Sporogony" (black arrow). Scale Bars = 2 μm and 5 μm, respectively.



Fluorescence microscopy in myxosporeans found in *Astyanax altiparanae*

Sample collection

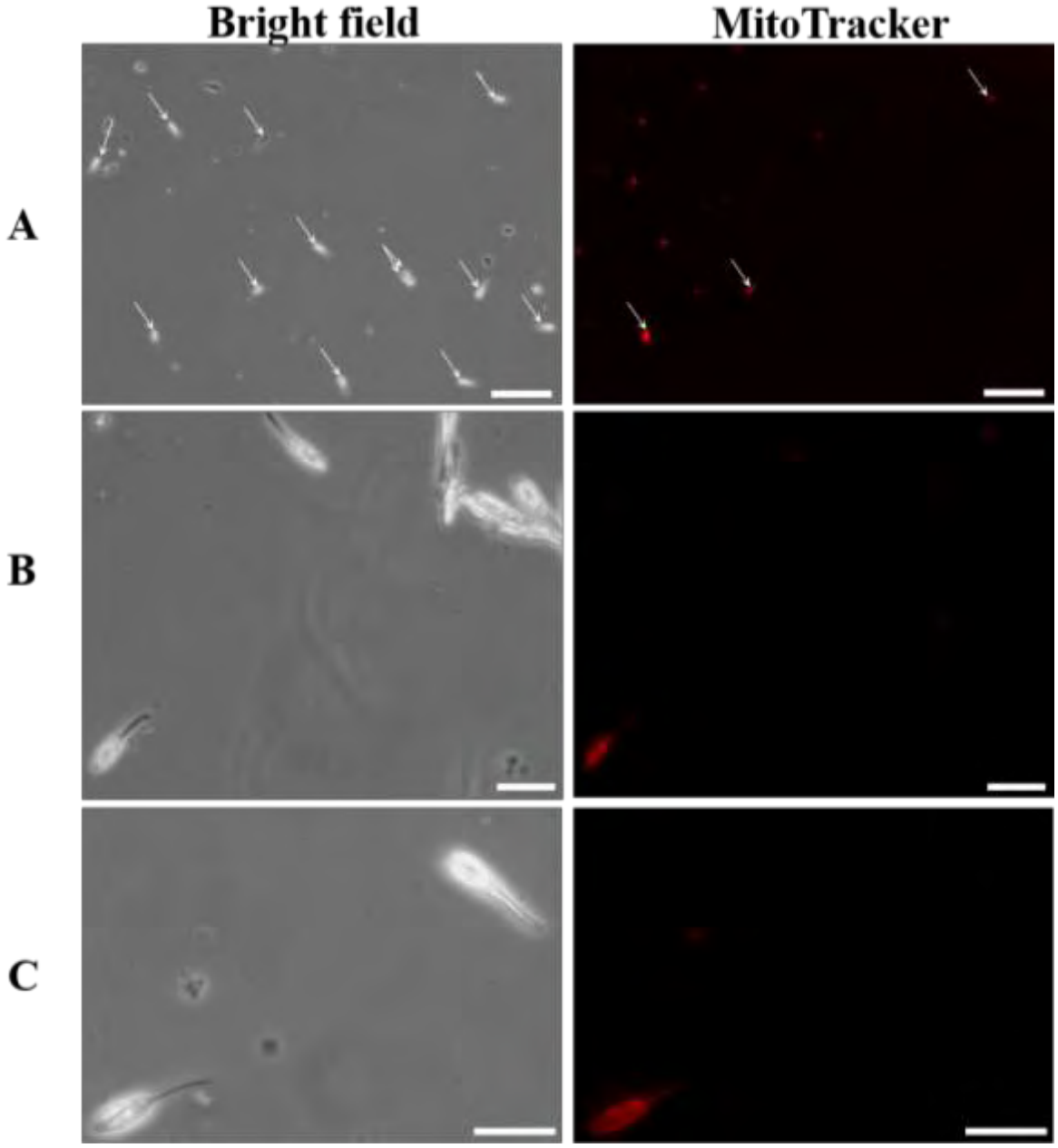
A total of 17 specimens of *A. altiparanae* were collected in CEPTA fish farm, 9 (53%) had plasmodia harboring *Henneguya* sp. infecting the fins.

Staining with MitoTracker® Orange

Henneguya sp. was stained with MitoTracker Orange, which is an orange-fluorescent dye able to stain mitochondria in live cells and its accumulation is dependent upon membrane potential. For this reason, staining was performed with fresh myxospores.

The results showed that only a few myxospores presented labeling for mitochondrial activity (Figure 21).

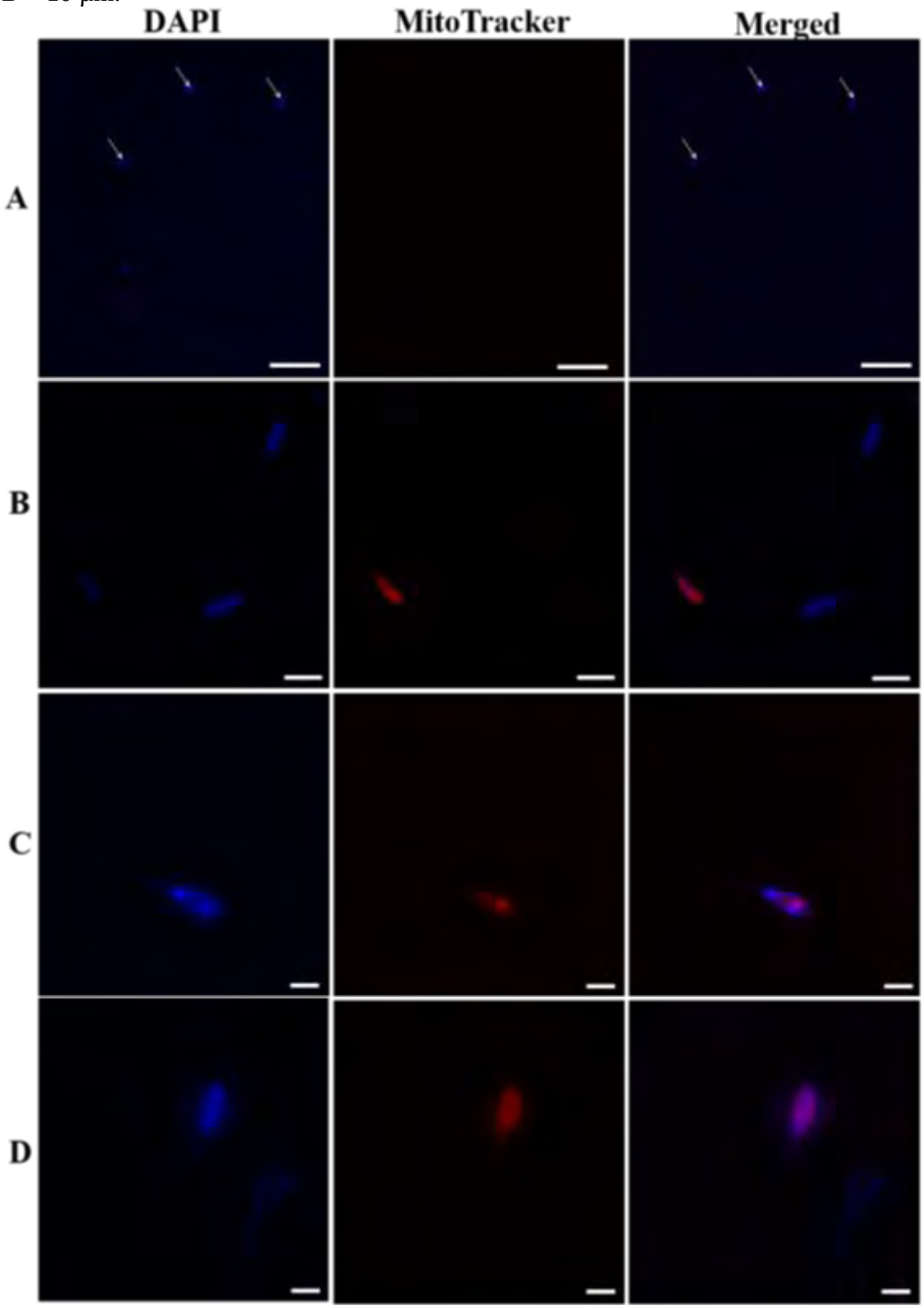
Figure 21. Photomicroscopy and fluorescence microscopy in the same view field of *Henneguya* sp. myxospores stained with MitoTracker® Orange in three different magnifications. White arrows indicate *Henneguya* sp. myxospores. Scale bars = A – 200 µm; B – 20 µm; C – 10 µm.



Permeabilization and Staining with DAPI and MitoTracker® Orange

The results showed that all myxospores presented labeling for nucleus, but only some myxospores presented reading for mitochondrial activity (Figure 22).

Figure 22. Fluorescence microscopy of *Henneguya* sp. myxospores stained with DAPI and MitoTracker® Orange in the same view field, in three different magnifications. White arrows indicate *Henneguya* sp. myxospores. Scale bars = A – 100 µm; B – 20 µm; C and D – 10 µm.



DISCUSSION

For COI mitochondrial amplification were initially used protocols and universal primers described by Folmer et al. (1994), unsuccessfully. Then, primers (Table 9) were designed based on COI gene sequences of species phylogenetically closer to the myxosporids deposited in GenBank. PCR conditions such as primer hybridization temperature (Table 10), extension time, DNA concentration and $MgCl_2$ were evaluated for standardization for each primer studied. However, no amplification was obtained.

Takeuchi et al. (2015) sequenced the mitochondrial genome of three species of the genus *Kudoa* and observed that the genomes are not conserved among these species, suggesting that the mitochondrial genes of myxosporids are, in general, potentially divergent. This may be one of the reasons why we can not achieve the amplification of the mitochondrial COI gene, since there may not be a conserved region for the design of specific primers for amplification mitochondrial gene of myxosporeans.

In view of the difficulty found in the amplification of the COI, we begin to investigate the mitochondria of the parasite analyzing the ultrastructural images of *Henneguya* n. sp. 1 and *Myxobolus* n. sp. 3, (Figures 19, 20), as well as other images of myxosporeans already published in the literature. And we observed, as well as Alama-Bermejo et al. (2012), that mitochondria are smaller in the sporogony phase than in the pre-sporogony phase. In addition to a reduced number of these organelles in the central part of the plasmodium where mature myxospores become agglomerated.

Wood et al. (2002) attempted to amplify mitochondrial genes of myxosporids without success and thus stated that these parasites are amitocondrials. This statement is contradictory when compared to ultrastructural studies, where it is possible to identify many mitochondria in the periphery of the plasmodium (OKAMURA et al., 2015).

Based on these studies, we assume that the mitochondrial activity could be related to the development phase of the parasite, being less active in mature myxospores.

For first experiment was only used MitoTracker® dye as an initial study and the myxospores no marked suggested a possible waterproofing of the chitin wall of the mature myxospore leaflets and as a consequence the non-penetration of the reagent.

For this reason, for the second experiment was performed the permeabilization of the myxospore as also the labeling for nucleus with DAPI dye as described by Adriano and Okamura (2017) for the nucleus is a structure present in mature or immature myxospores. After this, the same samples were stained with MitoTracker® Orange to the labeling for mitochondrial activity. The results showed that all myxospores observed were able to emit fluorescence for labeling of nucleus, but only some showed labeling for mitochondrial activity.

Accordding to Jirků et al. (2007) in mature phase, the myxospore leaflets harden causing a dormancy stage. Thus, the energy expenditure of mature myxospores can be reduced during this period.

In myxosporeans is evident that the mitochondrial cristae can assume different shapes (GRIPARIC; VANDER BLIEK, 2001) and sometimes this can reflect in differences in biochemistry within the same tissues (RIVA et al., 2005).

In a study of the ultrastructural features of *Ceratomyxa* sp. Diamant and Paperna (1989) reported mitochondria elongated and tubular in the plasmodium ectoplasm, while those inside the sporoblast and developing myxospores were typically spherical and contained considerably more matrix. Elmansy and Bashtar (2002) observed that numerous mitochondria, containing more cristae were usually located close to the nucleus of the sporoplasm in myxospore maturation of *Henneguya suprabranchiae* Landsberg (1987). Furthermore, glycogen-like material were more common in the sporoplasm of the mature myxospores of *H. suprabranchiae,* can represent an energy supply necessary for subsequent developmental stage

of life cycle and may be similar to sporoplasmosomes of other myxosporeans (EL-MATBOULI et al. 1990).

Sporoplasmosomes are intracellular structures present in the sporoplasm of the parasite and its function is still unknown (LOM et al., 1989), suggesting for us a possible energy storage through these structures so that the parasite becomes viable during the period of dormancy that, according to Yokoyama et al. (1993), can last for months or years until the myxospore is ingested by the invertebrate host.

Thus, according to Feist et al. (2015), cellular processes involved in development have been highly modified in myxosporeans and many these modifications require further research and clarification.

KEY QUESTIONS FOR FUTURE STUDY

- Why only some myxospores presented mitochondrial activity?
- Does mature myxospores really have inactive mitochondria?
- Is there any structure inside mature myxospores able of storing energy?

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6 FINAL CONSIDERATIONS

- *Myxobolus* n. sp. 1 and *Myxobolus* n. sp. 2 are new species infecting *C. macropomum;*

- *Henneguya* n. sp. 1, *Henneguya* n. sp. 2, *Myxobolus* n. sp. 3 and *Myxobolus* n. sp. 4 are new species infecting *P. brachypomus*;

- *Myxobolus colossomatis* and *Myxobolus* cf. *colossomatis* are distinct species;

- In natural environment both *C. macropomum* and *P. brachypomus* can harbor more than one species of *Myxobolus* and/or *Henneguya;*

- *Henneguya* myxospores apparently do not present mitochondrial activity in some development stages.

ATTACHMENTS

A

Universidade de São Paulo

26º SIICUSP - Simpósio Internacional de Iniciação Científica e Tecnológica da Universidade de São Paulo

MENÇÃO HONROSA

A Pró-Reitoria de Pesquisa da Universidade de São Paulo atribui Menção Honrosa a **Caroline Munhoz Meira** pela apresentação do trabalho **CARACTERIZAÇÃO MORFOLÓGICA, MOLECULAR E FILOGENÉTICA DE HENNEGUYA SP. E MYXOBOLUS SP. INFECTANDO PIARACTUS BRACHYPOMUS DA BACIA AMAZÔNICA**, na área de **Ciências Agrárias** na Etapa Internacional do **26º** Simpósio Internacional de Iniciação Científica e Tecnológica da USP - SIICUSP, sob a orientação de **Antonio Augusto Mendes Maia**, com a colaboração de **Kassia Roberta Hygino Capodifoglio**, em **2018**.

Prof. Dr. Sylvio Roberto Accioly Canuto
Pró Reitor de Pesquisa
Universidade de São Paulo

Documento emitido às **16:10:49** horas do dia **05/12/2018** (hora e data de Brasília).
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B

UNIVERSIDADE DE SÃO PAULO
Faculdade de Zootecnia e Engenharia de Alimentos
Comitê de Ética em Pesquisa (CEP)

CONCLUSÃO DE PARECER ÉTICO

Processo USP: 14.1.391.74.9

Projeto: Taxonomia, Morfologia, Biologia e Patogenia de Parasitas e bactérias que infectam peixes capturados em pisciculturas e rios brasileiros.

Esta Comissão solicita e compromete-se a manter total sigilo do conteúdo sobre a solicitação enviada e parecer gerado.

Recomendação do Comitê de Ética:

☒ **Aprovação.**

☐ **Não aprovado.**

☐ **Considerado em PENDÊNCIA até esclarecimentos dos pontos levantados.**

☐ **Retirado de pauta.**

Pirassununga, 28 de maio de 2014.

Notifique-se o interessado.

Profa. Dra. Daniele dos Santos Martins - Presidente do CEP/FZEA

Via do Parecer retirada pelo interessado:

Nome/Assinatura: _____

Data:

C

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The resolution of the taxonomic dilemma of *Myxobolus colossomatis* and description of two novel myxosporeans species of *Colossoma macropomum* from Amazon basin

Kassia R.H. Capodifoglio[a,*], Edson A. Adriano[b], Marcia R.M. Silva[a], Antonio A.M. Maia[a]

[a] Laboratory of Parasitology, Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering, University of São Paulo (USP), Pirassununga, SP, Brazil
[b] Department of Ecology and Evolutionary Biology, Federal University of São Paulo (UNIFESP), Diadema, SP, Brazil

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ABSTRACT

This study presents morphologic, molecular and phylogenetic data about two new species of the genus *Myxobolus* and of the previously described *Myxobolus colossomatis*, all which are found infecting the *Colossoma macropomum*, a fish whose natural habitat is the Amazon Basin of Brazil, from where the specimens for this study were caught. A total of 51 *C. macropomum* specimens were examined between October of 2014 and January of 2016. Plasmodia of the myxosporeans were found infecting several organs: *Myxobolus matosi* n. sp. and *Myxobolus longissimus* n. sp. were respectively found in the inner face of the operculum and in the wall external surface of the stomach and gill arch. *M. matosi* n. sp. were 9.6 ± 0.4 μm in length, 7.0 ± 0.3 μm in width and 5.0 ± 0.3 μm in thickness of the myxospore. *M. longissimus* n. sp. measured 19.1 ± 0.4 μm in length, 9.4 ± 0.3 μm in width and 8.3 ± 0.4 μm in thickness. The polar capsules, which were elongated, showed 4.3 ± 0.4 μm in length and 1.9 ± 0.1 μm in width for *M. matosi* n. sp. and 10.5 ± 0.2 μm in length and 2.5 ± 0.1 μm in width for *M. longissimus* n. sp. The *Myxobolus colossomatis* had two myxospore morphotypes: 1) Ellipsoidal myxospores measuring 11.6 ± 0.4 μm in length and 7.6 ± 0.2 μm in width. Their elongated polar capsules measured 5.6 ± 0.2 μm in length and 2.5 ± 0.2 μm in width; 2) Oval myxospores measuring 10.4 ± 0.5 μm in length and 7.7 ± 0.3 μm in width. Their polar capsules were 5.4 ± 0.2 μm in length and 2.4 ± 0.0 μm in width. The number of turns of the polar filament was 7–8 coils. The molecular comparison of the small subunit ribosomal DNA (ssrDNA) showed a genetic divergence of 10.3% between *M. matosi* n. sp. and *M. colossomatis*, 22.4% between *M. matosi* n. sp. and *M. longissimus* n. sp., and 23.2% between *M. longissimus* n. sp. and *M. colossomatis*. *Myxobolus* cf. *colossomatis*, a parasite of *Piaractus mesopotamicus*, showed 11.1% of genetic divergence to *M. colossomatis*, demonstrating them to be distinct species. Phylogenetic analysis, based on sequences of the ssrDNA, showed the *M. matosi* n. sp. to be a sister species of *M. colossomatis*, and it also showed *M. longissimus* n. sp. to be a sister branch in the lineage composed by *Myxobolus* cf. *cuneus* and *Henneguya pellucida*.

1. Introduction

Colossoma macropomum (Cuvier, 1816), is a serrasalmid fish commonly known in Brazil as *Tambaqui*. It is considered the second largest scaled fish from Amazon River Basin, and in its natural environment it may reach up to 100 cm long and weigh 30 kg (Nakatani et al., 2001). The *Tambaqui* is the most cultivated species in the Brazilian Amazon (Gomes et al., 2010; Lopera-Barrero et al., 2011) and one of the most important species in Brazilian aquaculture. Almost 60.000 t were marketed in 2010, with 4.200 t caught by fishing and 54.300 t harvested from fish farms (Brasil. Ministério da Pesca e Aquicultura - MPA, 2012). The *Colossoma macropomum* is also important when it is crossbred in fish farms with other serrasalmid species, such as the *Piaractus mesopotamicus* (Holmberg, 1887), which results in a hybrid called *tambacu*, or with the *Piaractus brachypomus* (Cuvier, 1818), which results in the hybrid *tambatinga*. These produced around 21.600 t and 5.000 t respectively in 2010 (Brasil. Ministério da Pesca e Aquicultura - MPA, 2012).

Due to its economic importance as one of the most important species of the amazonic icthyofauna (Goulding and Carvalho, 1982), *C.*

* Corresponding author at: Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering, University of São Paulo (USP), Av. Duque de Caxias Norte, 225, Jardim Elite, CEP 13635-900, Pirassununga, SP, Brazil.
E-mail address: kacapo@usp.br (K.R.H. Capodifoglio).

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