MARIA ISABEL MÜLLER

"Morfologia e Taxonomia Molecular de Myxosporea (Myxobolidae) e Monogenea (Dactylogyridae) de Brânquias de Pacu em Pisciculturas de São Paulo, Brasil."

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Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Maria Isabel Müller e aprovada pela Comissão Julgadora.

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Parasitologia.

Orientador(a): Prof(a). Dr(a). Marlene Tiduko Ueta

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Certificamos que o Protocolo nº **1476-1**, sobre **"Estudo dos principais parasitas que afetam brânquias de pacu (*Piaractus mesopotamicus*) em piscicultura"**, sob a responsabilidade de **Profa. Dra. Marlene Tiduko Ueta / Maria Isabel Müller**, está de acordo com os Principios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em **31 de março de 2008**.

CERTIFICATE

We certify that the protocol nº **1476-1**, entitled **"Study of main parasites of gills from cultivated pacu (*Piaractus mesopotamicus*)"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **March 31, 2008**.

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"Senhor, vós me perscrutais e me conheceis,
Sabeis tudo de mim, quando me sento ou me levanto.
De longe penetrais meus pensamentos.
Quando ando e quando repouso, vós me vedes,
observai todos os meus passos.
A palavra ainda não me chegou a língua,
e já, Senhor, a conheceis toda.
Vós me cercais por trás e pela frente,
e estendeis sobre mim vossa mão."
(Salmo 138)

"Não é o mais forte que sobrevive, nem o mais inteligente,
mas o que melhor se adapta as mudanças."
(C. Darwin)

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RESUMO

O pacu *Piaractus mesopotamicus* (Holmberg, 1887) é uma das principais espécies de importância econômica no Brasil. Peixe nativo da Bacia dos rios Paraná e Paraguai é uma das espécies mais comuns em pisciculturas. Mixosporídeos e monogêneas são parasitos de alta prevalência e causam danos em cultivos comerciais, tornando importante seu estudo detalhado. Neste estudo foram examinados 278 especimens de *P. mesopotamicus* de vários tamanhos, no período de Fevereiro de 2008 a Julho de 2010, coletados nos tanques do Centro de Pesquisa e Conservação de Peixes Continentais – CEPTA/ Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio, Pirassununga, SP e em pisciculturas particulares da mesma região. Os parasitos identificados foram os mixosporídeos *Henneguya piaractus* (Martins & Souza, 1997) e *Myxobolus colossomatis* (Molnár & Békési, 2007) e os monogêneas *Anacanthorus penilabiatus* (Boeger, Husak & Martins, 1995)*, Mymarothecium viatorum* (Boeger, Piasecki & Sobecka, 2002) e Dactylogyridae sp1 (não identificada). Análises ultraestruturais mostraram em *Henneguya piaractus* uma fina camada granular entre o plasmódio e as células do hospedeiro, uma única parede no plasmódio com numerosos canais de pinocitose. *Myxobolus colossomatis* apresentou uma camada plasmodial com numerosa atividade fagocítica em comunicação com as células do hospedeiro. A histopatologia apresentou hiperplasia nas lamelas branquiais em peixes altamente infectados e deformação das lamelas em relação a cistos de *H. piaractus*. A microscopia eletrônica de varredura mostrou para *A. penilabiatus* tegumento relativamente liso e microvilosidades nas regiões adesivas. *Mymarothecium viatorum* apresentou na superfície de seu tegumento, projeções similares a escamas com variações tipo microvilosidades. Análises moleculares utilizando marcadores ribossomais para mixosporídeos e monogeneas (porção18S) posicionaram as espécies em árvores filogenéticas. A descrição do genoma mitocondrial parcial de *A. penilabiatus* é apresentada.

Palavras-chave: *Piaractus mesopotamicus*, Myxosporea, Monogenea, parasitos branquiais, piscicultura, Brasil.

ABSTRACT

The freshwater fish *Piaractus mesopotamicus* (Holmberg, 1887), popularly known as "pacu", is one of the most economically important species of fish in Brazil. The fish is endemic to the Paraná-Paraguay river basin, and is one of the species that is most cultivated in fish farms. A detailed study of Myxosporea and Monogenea is important, due to their high prevalence and the damage they cause in commercial fish farms. A total of 278 specimens of *P. mesopotamicus* were examined from February 2008 to July 2010, collected in fish ponds at the Centro de Pesquisa e Conservação de Peixes Continentais (CEPTA), Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), in the city of Pirassununga, in the state of Sao Paulo, and in other commercial fish farms nearby. The main parasites found were the myxosporeans *Henneguya piaractus* (Martins & Souza, 1997) and *Myxobolus colossomatis* (Molnár & Békési, 2007) and the monogeneans *Anacanthorus penilabiatus* (Boeger, Husak & Martins, 1995), *Mymarothecium viatorum* (Boeger, Piasecki & Sobecka, 2002) and Dactylogyridae sp1 (not identified). Ultrastructural analysis of *H. piaractus* showed a thin layer of granular material between the plasmodial wall and the host cells. The single plasmodial wall was connected to a plasmodial ectoplasm zone through numerous and extensive pinocytotic canals. *Myxobolus colossomatis* showed a plasmodial ectoplasm zone with numerous and extensive phagocytic activity, and numerous projections in the direction of the host cells. Histopathological data showed hyperplasia in the lamellae in highly infected fish and deformation of the lamellae regarding cysts of *H. piaractus*. Scanning electron microscopy found that *A. penilabiatus* had a relatively smooth tegument and microvilli in the adhesive regions. However, the *M. viatorum* tegument showed that the top surface had variations similar to scales. Molecular analysis, using ribosomal markers for myxosporeans and monogeneans (18S rDNA) positioned the species in phylogenetic trees. The partial mitochondrial genome of *A. penilabiatus* is also recorded.

Keywords: *Piaractus mesopotamicus*, Myxosporea, Monogenea, gill parasites, fish farms, Brazil.

1- INTRODUÇÃO

A pesca teve sua origem na antiguidade em águas continentais, bem antes da população dar início a agricultura e agropecuária. Registros históricos datam do antigo Egito, contendo ilustrações que mostram cenas de pesca no rio Nilo e conservação de peixes em tanques, e antigos manuscritos asiáticos, onde peixes representam divindades (Feidi, 2001; Mekong River Commission, 2003).

Historicamente o cultivo controlado ou semi-controlado de animais teve seu início na China há aproximadamente 4 mil anos, com as macroalgas marinhas e posteriormente com o cultivo de carpas. Devido a sua história e grande experiência, a China é responsável por mais da metade da produção asiática, e por sua vez a Ásia representa 90% de toda produção mundial de alimentos provenientes de ambiente aquático (Camargo & Pouey, 2005). A partir da China, a cultura de peixes expande-se em países como Indonésia, Vietnã e Camboja, devido ao crescimento da população e modernização de métodos tradicionais de criação. Nesses países, o peixe mais cultivado é a carpa chinesa (Souza & Teixeira-Filho, 1985), mas existem, atualmente, esforços para o cultivo de peixes nativos. No Vietnã, há algumas décadas, a espécie *Pangasianodon hypophthalmus* nativa do rio Mekong, vem sendo intensamente cultivada nas regiões do rio Mekong e seus afluentes (Phuong & Oanh, 2010).

O consumo mundial de peixe como alimento aumenta gradualmente ano após ano. Segundo o relatório da FAO (2010), o consumo mundial de peixes subiu de 104,4 em 2004, para 117,8 milhões de toneladas em 2009, e o consumo per capita foi de 16,2kg em 2004 para 17,2kg em 2009.

Fatores como exploração indiscriminada do estoque pesqueiro natural e a crescente diferença entre a quantidade de pescado capturado e a demanda de consumo, tornaram a piscicultura uma das alternativas mais viáveis no mundo (FAO, 1997).

O Brasil, por possuir vasto potencial hídrico, diversidade de espécies de peixes nativas cultiváveis e clima favorável, tem a piscicultura como importante atividade econômica, e ainda, como uma alternativa a exploração indiscriminada

dos estoques pesqueiros costeiros. De acordo com o Ministério da Pesca e Aquicultura (MPA) (2009) a piscicultura cresceu cerca de 60,2% apenas entre os anos de 2007 a 2009 e juntamente com esse crescimento veio um aumento do consumo de peixe pelo brasileiro, que saltou de 6,46kg para 9,03kg por habitante/ano entre 2003 a 2009.

No Brasil, as espécies cultivadas em pisciculturas variam de acordo com a região: na região Norte, o pirarucu e o tambaqui, nas regiões Nordeste, Sudeste e Sul a tilapia e na região Centro-Oeste os destaques são o pacu, o tambaqui e os pintados (MPA, 2009).

1.1- *Piaractus mesopotamicus*

O pacu *Piaractus mesopotamicus* (Holmberg, 1887) é uma das principais espécies na pesca e piscicultura no Brasil. Espécie representante da superordem Ostariophysi na qual estão incluidos os peixes de maior valor comercial no Brasil. Pertencente aos Characiformes, Ordem dominante entre os peixes da América do Sul, com espécies herbívoras, carnívoras, onívoras e iliófagas. Esta ordem possui grande número de famílias sendo a Characidae a que possui o maior número de representantes em águas continentais brasileiras, alem de ser a mais complexa, com grande número de subfamílias (Urbinati & Gonçalves 2005).

Piaractus mesopotamicus (Characidae, Serrasalminae) é a espécie que apresenta mais informações técnicas para a piscicultura, possui ótimo valor de mercado e preferência popular nas regiões onde são abundantes (Chabalin & Ferraz de Lima, 1988). Sua alta capacidade reprodutiva, crescimento rápido e fácil aceitação comercial, faz desta espécie uma das mais cultivadas no território brasileiro. Em ambiente natural é encontrado nas Bacias dos rios Paraná, Paraguai e Uruguai e se distribui pelas planícies alagadas do Pantanal matogrossense (Godoy, 1975; Petrere, 1989). Popularmente conhecida como caranha, pacu-caranha ou pacu-guaçu é um dos peixes mais estudados nas regiões Sul, Sudeste e Centro-Oeste do Brasil (Urbinati & Gonçalves, 2005).

Alimenta-se basicamente de folhas e frutos, mas também caules, flores, e sementes, podendo ainda, ingerir insetos, aracnídeos, moluscos e restos de peixes (Ferraz de Lima et al., 1984; Dias - Koberstein et al., 2005), o que leva a avaliar que tenha habilidade especial na digestão e absorção de alimentos grosseiros e ricos em carboidratos (Dias - Koberstein et al., 2005). Em ambiente natural a alimentação varia com a época do ano, em período de seca, as folhas e frutos ingeridos são bastante triturados antes de chegar ao estômago, mas com a cheia dos rios, esses mesmos alimentos são parcialmente fracionados e digeridos, isso mostra que o peixe possui, além de diversas características positivas ao cultivo, a boa aceitação do arraçoamento, facilitando o sucesso da criação em sistemas de cultivos intensivos (Dias - Koberstein et al., 2005).

Em relação a reprodução apresenta desova total e fecundação externa. No ambiente natural, fazem longas migrações enquanto ocorre a maturação das gônadas nas estações secas (julho a outubro). A desova ocorre em novembro e dezembro, estação chuvosa, época em que os rios apresentam maior volume de água (Ferraz de Lima, et al., 1984). Em pisciculturas a reprodução é induzida através de estimulação hormonal, que permite que a liberação de gametas se complete. O período de reprodução em cativeiro ocorre nas épocas mais quentes, de outubro a março, com pico entre novembro e janeiro (Bernardino & Lima, 1999).

O tamanho médio da primeira maturação gonadal é de 34 cm, com idade média de 3 anos e a partir de 5 anos todas as fêmeas da população estão aptas a reprodução (Ferraz de Lima, et al., 1984). A desova está diretamente ligada a temperatura da água, (26 a 29°C), ao aumento do nível dos rios e das chuvas, o qual parece ser o fator ambiental mais influente na reprodução dessa espécie (Ferraz de Lima, 1981). Em pisciculturas as condições de desova são semelhantes.

O pacu é considerado um peixe promissor para o cultivo por resistir a concentrações de oxigênio de 3 mg/L e boa tolerância ao clima do Sudeste e Sul do País (Ferraz de Lima et al., 1988). A temperatura ótima para seu desenvolvimento é de 23 a 29°C e abaixo de 16°C pode ser letal (Garcia et al.,

2008). Em temperaturas inferiores a 22°C, o crescimento é prejudicado, embora alevinos possam resistir a oscilações térmicas médias de 5 a 6°C (Salaro et al., 1992).

Em relação ao parasitismo em pisciculturas, a alta densidade populacional facilita a transmissão de patógenos, determinando o desenvolvimento de parasitos e doenças (Bakke & Harris, 1998; Hudson et al., 2001) pelo maior contato entre indivíduos ou entre hospedeiros e patógenos, resultando em um aumento na transmissão e persistência da infecção (May & Anderson 1991; Hudson et al., 2001).

Em sistemas de cultivo, os principais parasitos encontrados em *P. mesopotamicus* são os monogêneas (*Anacanthorus penilabiatus*, *A. spathulatus, Mymarothecium viatorum* e *Urocleidoides* sp.), protozoários (*Trichodina* sp., *Ichthyophthirius multifilis*, *Piscinoodinium pillulare),* mixosporídeos (*Henneguya pellucida*, *H. piaractus, Myxoboulus cuneus, M. colossomatis*) e crustáceos (*Lernaea cyprinacea*, *Argulus* sp., *Dolops carvalhoi* e *Ergasilus* sp.) (Urbinati & Gonçalves, 2005). As espécies de *Henneguya*, *Myxobolus* e muitos monogêneas são reportados em peixes de água doce e as brânquias são os principais órgãos infectados (Barassa et al., 2003; Gioia & Cordeiro,1996).

1.2- Classe Myxosporea

Filo Myxozoa (mixosporídeos) é um grupo abundante e diverso que tem sido detectado em platelmintos (Overstreet, 1976; Siau et al., 1981; Freeman & Shinn, 2011), briozoários (Okamura, 1996), anelídeos (Lom et al., 1997), peixes (Lom & Arthur, 1989; Kaur & Singh, 2012), anfíbios (Hartigan et al., 2011), répteis (Johnson, 1969), aves (Bartholomew et al., 2008) e mamíferos (Friedrich et al., 2000).

A classificação do Filo possui uma longa história que se inicia com o descobrimento dos Myxosporea por Jurine (1825) e subsequentemente observações feitas por Müller (1841). Entretanto, os estudos sobre este grupo realmente começaram com Bütschli (1882) o qual classificou como Myxosporida

junto com Sarcosporida, subclasses da Classe Sporozoa (Lom & Diková, 2006). Por muito tempo foram considerados protistas, mas um grande número de características de metazoários como a multicelularidade e a presença de cápsulas com filamentos polares, foram importantes características para levantar a questão de que estes organismos não eram protozoários (Siddall et al., 1995; Kent et al., 2001; Canning & Okamura, 2004).

Análises filogenéticas, usando o DNA ribossomal 18S, determinaram sua origem entre os metazoários (Smothers et al., 1994). Embora confirmados como metazoários, estudos filogenéticos, utilizando diferentes marcadores moleculares, não foram ainda capazes de apontar a real posição dos Myxozoa dentro desse grupo, sendo que podem ser agrupados próximo aos Bilateria ou formam um clado altamente derivado dos cnidários (Evans et al., 2010).

O Filo Myxozoa está dividido em duas Classes, a Malacosporea e a Myxosporea. Esse Filo inclui 4 espécies malacosporea e mais de 2300 espécies de mixosporídeos os quais são distribuídos em diversos gêneros e a maioria é parasito de peixes de água doce (Lom & Diková, 2006; Sitjá-Bobadilla, 2008; Kent et al., 2001; Morris, 2010).

Os gêneros *Henneguya* e *Myxobolus* são os mais comuns em peixes (Adriano et al., 2002), ocorrem tanto em água doce como em ambiente marinho e causam sérios danos econômicos, pois sua presença pode provocar alta taxa de mortalidade em todo o mundo (Lom & Diková, 2006). A espécie mais conhecida é *Myxobolus cerebralis* Hojer, 1903 agente etiológico da doença do rodopio em salmonídeos. Essa doença causa deformidade da cartilagem da cabeça e da coluna vertebral provocando elevada mortalidade de peixes em diversas partes do mundo (Eiras, 1994).

Indivíduos do gênero *Henneguya* causam patogenias que podem ser responsáveis por grandes mortalidades de peixes em sistemas de cultivos intensivos e semintensivos (McCraren et al., 1975; Dykova & Lom, 1978). No Brasil, *H. piaractus* parasita de brânquias, causa altas mortalidades de *P. mesopotamicus* de sistemas de criação (Martins et al., 1997).

Os mixosporídeos formam plasmódios, de diferentes dimensões, repletos de esporos, em vários tecidos de seus hospedeiros. O desenvolvimento do plasmodio pode ser histozóico (intracelular ou intercelularmente) ou celozóico (localizados nas cavidades dos órgãos, soltos ou aderidos ao epitélio) (Lom, 1987; Eiras, 1994). Podem ser encontrados nas brânquias (mais comum), pele, cartilagens, músculos, fígado, baço, parede intestinal, vesícula biliar e bexiga natatória.

1.3- Classe Monogenea

Monogêneas são helmintos hermafroditos, monoxênicos, geralmente ectoparasitas de corpo e brânquias, mas algumas espécies podem ser endoparasitas de ureteres de peixes, estômago de tilápias (Jerônimo et al., 2010), bexigas urinárias de tartarugas, anfíbios e salamandras. Em mamíferos, é relatada uma única espécie, *Oculotrema hippopotami,* parasitando olhos de hipopótamos (Thurston & Laws, 1965).

Pertencente ao Filo Platyhelminthes, a Classe Monogenea, segundo estudos de filogenia utilizando dados morfológicos e moleculares feitos por Littlewood et al. (1999), é monofilética, com dois grandes grupos: Polyopisthocotylea e Monopisthocotylea, grupos irmãos de Cestoda e Monogenea e Cestoda grupos irmãos de Trematoda. Outra terminologia utilizada para a Classe é Monogenoidea, com as subclasses: Polyonchoinea (equivalente a Monopisthocotylea) e Heteronchoinea (= Polyopisthocotylea) (Boeger & Kritsky, 1997; 2001; Olson & Littlewood, 2002).

Monogêneas do grupo Polyopisthocotylea se alimentam de sangue e raramente causam mortalidade significativa entre seus hospedeiros. Os Monopisthocotylea que se alimentam de células epiteliais, são conhecidos por causar danos importantes em seus hospedeiros (Buchmann & Bresciani, 2006).

A grande maioria dos monogênea é ovípara, com exceção dos girodactilídeos que apresentam reprodução vivípara; possuem uma larva livre natante chamada oncomiracídio, que invade o hospedeiro e migra até o sítio de

fixação, comumente brânquias (Buchmann & Bresciani, 2006).

As infecções são responsáveis pelo aumento da mortalidade por ações diretas ou por favorecer outros processos infecciosos, como bacterioses e micoses (Ceccarelli et al*.,* 1990; Figueira & Ceccarelli, 1991).

As famílias que apresentam maior importância em relação a patologia em peixes são: Microbothriidae, Capsalidae, Dactylogyridae e Gyrodactylidae (Buchmann & Bresciani, 2006).

Dactylogyridae é aparentemente o táxon com maior número de representantes nas águas continentais da América do Sul, mas espécies de Gyrodactylidae são descritas, assim como poucos representantes de Diplectanidae, Monocotylidae e Hexabothriidae (Boeger & Vianna, 2006). Na região Neotropical são conhecidos 70 gêneros e cerca de 308 espécies de Monogenea de água doce parasitando 144 espécies de peixes (Boeger & Vianna, 2006).

Assim, os monogêneas e mixosporídeos, são os parasitos de maior incidência e os que causam os maiores danos em cultivos comerciais (Martins & Romero, 1996). As infecções por esses parasitos são responsáveis pelo aumento da mortalidade por ações diretas ou por favorecer outros processos infecciosos, como bacterioses e micoses (Ceccarelli et al., 1990; Figueira & Ceccarelli, 1991).

3- OBJETIVO GERAL

Caracterizar morfológica e molecularmente os parasitos da Classe Myxosporea e da Classe Monogenea encontrados em brânquias de *Piaractus mesopotamicus* oriundos de sistemas de criação do Estado de São Paulo.

4- OBJETIVOS ESPECÍFICOS

- Identificar, por microscopias de luz e eletrônicas de varredura e transmissão, as espécies de Myxosporea e Monogenea que ocorrem nas brânquias de pacu (*Piaractus mesopotamicus*) coletados em tanques de piscicultura.
- Observar, mediante análise histológica, a interação parasito-hospedeiro nos tecidos branquiais.

- Realizar o sequenciamento do gene 18S rDNA de mixosporídeos e 18S e 28S rDNA de monogêneas para a identificação das espécies.

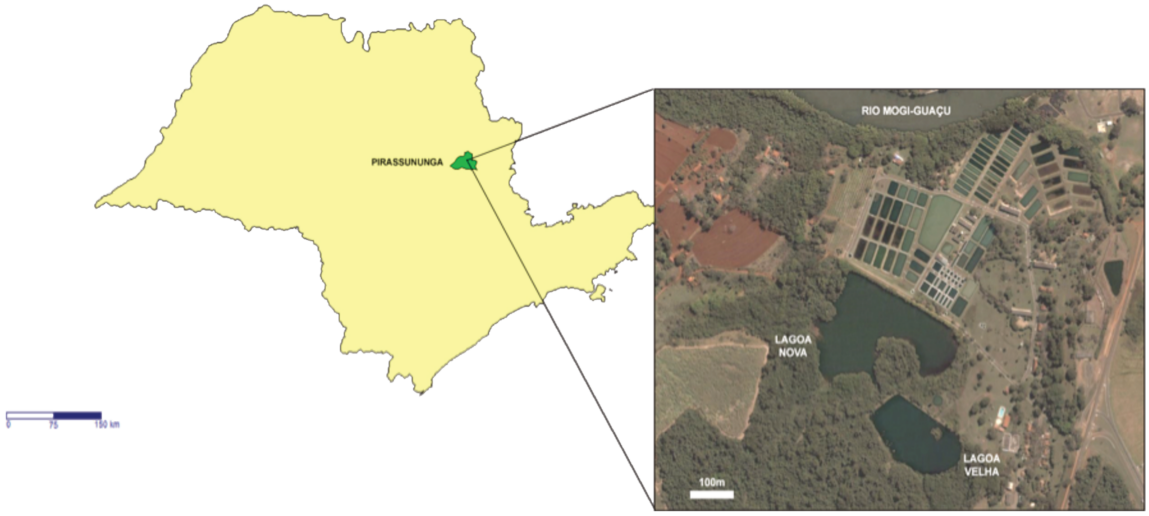
- Identificar as posições filogenéticas dos mixosporídeos e monogêneas encontrados neste estudo em relação as espécies de outros continentes.

- Realizar o sequenciamento do DNA mitocondrial de monogênea visando a descrição e identificação de marcadores moleculares para o estudo taxonômico.

5- MATERIAL E MÉTODOS

5.1- Área de Estudo

Os peixes utilizados no estudo são oriundos de reprodução induzida e foram obtidos em tanques da piscicultura do Centro Nacional de Pesquisa e Conservação de Peixes Continentais/ Instituto Chico Mendes de Conservação Da Biodiversidade– CEPTA/ ICMBio, município de Pirassununga, SP (Figura 1) e em pisciculturas particulares dos municípios de Mogi-Mirim e Itapira.



Fonte: Google

Figura 1: Centro Nacional de Pesquisa e Conservação de Peixes Continentais – CEPTA/ ICMBio, município de Pirassununga, localizado no Estado de São Paulo.

Este estudo foi realizado em parceria entre os Departamentos de Biologia Animal do Instituto de Biologia – UNICAMP, Campinas; Laboratório de Parasitologia do Departamento de Ciências Básicas – ZAB da Faculdade de Zootecnia e Engenharia de Alimentos – FZEA/USP, campus Pirassununga; Laboratório de Ictiopatologia do Centro Nacional de Pesquisa e Conservação de Peixes Continentais, também em Pirassununga e em colaboração com o Parasitic Worms Group, Wolfson – Wellcome Biomedical Laboratory, Department of Zoology – The Natural History Museum, cidade de Londres, Grã Bretanha.

5.2- Coletas e Microscopia

Coletas mensais foram realizadas com o auxílio de redes, tarrafa e anzóis. Os peixes foram transportados vivos para o laboratório, onde foram sacrificados por meio de transecção da coluna vertebral.

Foram coletados, em média, 9 especimens de pacu mês durante 30 meses, totalizando 278 peixes sendo 107 fêmeas, 111 machos e 60 de sexo indeterminado. Os peixes foram medidos, pesados e em seguida as brânquias retiradas e colocadas em placas de Petri com solução salina para pesquisa de parasitos.

Os cistos de mixosporídeos foram removidos das lamelas e arcos, armazenados em etanol 99% p.a. para estudos moleculares e em formalina 10% tamponada para as análises histológicas. Os esporos livres foram corados por Giemsa e montados em lâminas permanentes em Cytoseal^{TM} baixa viscosidade. As medidas foram feitas em um computador equipado com o programa Axivision 4.1 acoplado em um microscopio Axioplan 2 Zeiss. As espécies de mixosporídeos foram identificadas conforme os critérios estabelecidos por Lom & Arthur (1989).

Para estudo dos monogêneas, as brânquias foram colocadas em recipientes contendo água destilada quente (aproximadamente 60°C) e foram feitas agitações (40 - 60X) para que os monogêneas se desprendessem dos filamentos branquiais. Após esse período, o líquido contendo os monogêneas foi examinado ao estereomicroscópio, para coleta e quantificação (Boeger & Vianna, 2006). Os parasitas presentes foram fixados em etanol 99% p.a. para análise molecular e alguns foram fixados em formol a 5% para identificação morfólogica. Para o estudo das partes esclerotizadas, os monogêneas foram corados com Tricrômico de Gomori, montadas em meio de Hoyer e meio Grey & Wess (Humason, 1979; Putz & Hoffman, 1963; Kritsky et al., 1995; Eiras et al., 2000). As medidas dos órgãos internos para identificação, foram obtidas em microscópio de luz acoplado ao computador equipado com o programa Axioplan

2 de captura de imagem, situado no Departamento de Biologia Animal, Instituto de Biologia, UNICAMP.

A tese está dividida em 3 capítulos, portanto a metodologia específica para cada capítulo se encontra detalhada nos mesmos.

O projeto foi aprovado pelo Comitê de Ética no Uso de Animais do Instituto de Biologia da UNICAMP, protocolo número 1476-1.

6- RESULTADOS

Foram identificadas duas espécies de mixosporídeos e duas espécies de monogêneas. Os capítulos a seguir irão abordar aspectos morfológicos, ultraestruturais, histológicos, moleculares e dados parasitológicos dos seguintes parasitos: *Henneguya piaractus* e *Myxobolus colossomatis* (Myxosporea), *Anacanthorus penilabiatus* e *Mymarothecium viatorum* (Monogenea).

Capítulo 1: Diagnosis and molecular taxonomy of two myxosporeans species parasites of farmed *Piaractus mesopotamicus* (Characiformes: Characidae) in São Paulo State, Brazil.

Capítulo 2: Morphological and Molecular Analysis of *Anacanthorus penilabiatus* and *Mymarothecium viatorum* (Monogenea: Dactylogyridae) gill parasites from *Piaractus mesopotamicus* (Characidae: Serrasalminae) collected from fish farms in São Paulo State, Brazil.

Capítulo 3: Description of partial mitochondrial genome of *Anacanthorus penilabiatus* (Monogenea, Dactylogyridae) from *Piaractus mesopotamicus* (Characidae, Serrasalminae) and its use as possible molecular marker.

(Segundo padrões da Revista *Veterinary Parasitology*)

Capítulo 1

Diagnosis and molecular taxonomy of two myxosporean species parasites of farmed *Piaractus mesopotamicus* (Characiformes: Characidae) in the State of São Paulo, Brazil.

ABSTRACT

Among fish pathogens, Myxozoa parasites are an important group. The species *Myxobolus colossomatis* (Molnár & Békési, 2007) and *Henneguya piaractus* (Martins & Souza, 1997) are commonly found in the characid *Piaractus mesopotamicus* (Holmberg, 1887), an important species for fish farming in Brazil. The present study includes the morphological identification, histological, ultrastructural analyses, prevalence and mean intensity data and molecular taxonomy of *Henneguya piaractus* and *Myxobolus colossomatis* found to be infecting *Piaractus mesopotamicus* collected in fish farms in the State of São Paulo, Brazil. A total of 278 fish were collected between February 2008 and March 2010 from three fish farms. The prevalence and mean intensity varied throughout the study period and according to location. In Pirassununga, the prevalence and mean intensity of infection were 56.8%, and 24.5 cysts/fish for *H. piaractus* respectively and 60.5% and 6.0 cysts/fish for *M. colossomatis*. In Mogi Mirim, the values were 36% and 83.7 cysts/fish for *H. piaractus* and 40% and 40.6 cysts/fish for *M. colossomatis*. And in Itapira, findings were 100% and 47.1 cysts/fish for *H. piaractus* and 50% and 7.0 cysts/fish for *M. colossomatis*. Ultrastructural analysis for *Henneguya piaractus* showed a thin layer of granular material between the plasmodial wall and the host cells. The single plasmodial wall was connected to a plasmodial ectoplasm zone through numerous and extensive pinocytotic canals. *Myxobolus colossomatis* showed a plasmodial ectoplasm zone with numerous and extensive phagocytic activity and numerous projections in the direction of the host cells. Histopathological data showed hyperplasia in highly infected fish and deformation of lamellae caused by *H. piaractus* infection. Partial sequencing of the 18S rDNA gene resulted in 1.913 base pairs for *H. piaractus* and 1.341 base pairs for *M. colossomatis*. A phylogenetic tree, placing South American species in a global context, is provided.

Keywords: *Piaractus mesopotamicus*, Myxozoa, *Henneguya piaractus*, *Myxobolus colossomatis*, fish farm, Brazil.

1. INTRODUCTION

The Neotropical region has the most diversified fish fauna in the world, both of marine and freshwater fish species (Schaefer, 1998). Since advances in aquaculture, which has been growing throughout the world since the 1990s (Kent et al., 2001), fish disease has become an important area of investigation (Barassa et al., 2003a). The characid fish *Piaractus mesopotamicus* (Holmberg, 1887), a large riverine fish commonly known as "pacu", is considered an important species in Brazilian fish farming (Adriano et al., 2005). This species is naturally distributed in the Paraná, Paraguay and Uruguay river basin (Godoy, 1975) and is one of the most commonly studied fish in the south, southeast and center of Brazil (Urbinati & Gonçalves, 2005).

Among fish pathogens, the Myxozoa are an important group. The Phylum Myxozoa is composed by highly specialized metazoan parasites, mainly of aquatic hosts with a wide host range (Feist & Longshaw, 2006). Interest in the group has intensified along with the development of aquaculture, since many species can lead to serious outbreaks in farmed fish species (Feist & Longshaw, 2006). The myxosporeans, especially species belonging to the genera *Myxobolus* and *Henneguya*, are among the most common and remarkable of fish parasites (Thatcher, 2006). These parasites form spore filled cysts in host tissues that vary in size from less than a millimeter to more than a centimeter. Such cysts can be found in several locations on the host, such as the gills and skin, muscles, liver, spleen and intestinal wall (Thatcher, 2006).

The *Myxobolus* (Butschli, 1882) is the most common genus, with the greatest number of species (790 valid species approximately) (Eiras et al., 2005) infecting both marine and freshwater fishes. Many of these are recognized as pathogenic, such as *Myxobolus cerebralis*, which causes Whirling Disease (Kent et al., 2001; Feist & Longshaw, 2006). In South America 30 *Myxobolus* species have been reported (Azevedo et al., 2010; Eiras et al., 2010), although this number will certainly increase.

The *Henneguya* (Thélohan, 1892) is the second largest genus of the Myxobolidae (Lom & Diková, 2006) and many species have been reported

parasitizing freshwater fishes throughout the world (Casal et al., 2003). It is the most abundant genus in South America, and its importance as a pathogen is described by several authors (Lom & Diková, 1995; Martins & Souza, 1997; Martins et al., 1999). The species *Myxobolus colossomatis* and *Henneguya piaractus* have been reported as important pathogens of the characid *Piaractus mesopotamicus* in fish farms in Brazil (Martins et al., 1997, Adriano et al., 2005).

The majority of studies related to these parasites are of morphological nature, or are ultrastructural and histopathological studies. Recently, a number of molecular analyses have been carried out. The development of specific diagnosis methods, including the use of molecular phylogenetic techniques, has improved pathogenesis studies, providing fundamental advances in taxonomical studies of Myxozoa (Feist & Longshaw, 2006). The 18S rDNA has been the most common molecular marker used for the detection, identification and phylogenetic analysis for myxozoans (Holzer et al., 2006). Molecular data using 18S rDNA gene sequences demonstrated that classification based mainly on morphological characteristics was not consistent with phylogenetic relationships (Smothers et al., 1994; Andree et al., 1999).

The aim of the present study was to evaluate the myxosporeans species found in farmed *Piaractus mesopotamicus*, with emphasis on morphological identification, ultrastructural study, parasitological data, histological analysis and elucidation of the relationship between the species, using a large set of molecular data available in GenBank, to establish their phylogenetic position, using the 18S rDNA gene molecular marker.

2. MATERIAL AND METHODS

2.1- Sampling and Morphological Study

A total of 278 specimens of *Piaractus mesopotamicus* (25.8 cm ±10.36 in length and 458.0 kg ± 543.1 in weight) were collected monthly from February 2008 to July 2010 from three different fish farms located in the state of São

Paulo: 218 fish from Pirassununga (21°55'51.68"S, 47°22'29.01"W), 50 fish from Mogi Mirim (22°28'16.63"S, 47°00'40.47"W) and 10 fish from Itapira (22°26'10.54"S, 46°49'19.52"W).

Fish were collected using nets and fishing rods and transported alive to the laboratory, where they were killed, measured and necropsied. The gills were immediately screened for myxozoans using a stereomicroscope and measurement of the spores was performed based on studies by Lom & Arthur (1989). Measurements were performed using a computer equipped with Axivision 4.1 software linked to an Axioplan 2 Zeiss microscope.

Rubbings containing free spores were fixed in methanol, stained with Giemsa solution (pH 7.2), and a low viscosity mounting medium Cytoseal^{TM}, was used to create permanent slides. Identifications were done following Martins & Souza (1997), Azevedo et al. (2010) and Molnár & Békési (2007). Fragments of the gills containing cysts obtained from *P. mesopotamicus* were fixed in buffered 10% formalin for 24h and placed in paraffin for histological analysis. Cross sections (µm) stained with hematoxylin-eosin and permanent slides were made and analyzed using the light microscope (Adriano et al., 2002).

Prevalence and mean intensity of cysts were calculated annually according to Bush et al. (1997). One way variance analysis was conducted using PROC GLM SAS 9.1 software (SAS Institute Inc., Carry, NC, USA), and Duncan's Multiple Range Test was applied to compare the prevalence and mean intensity by location, year, and species ($p \leq 0.05$ significance level).

2.2- Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM).

Free spores were deposited on a cover slip coated with poly-L-lysine and fixed for 2 h at room temperature with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for observation using scanning electron microscopy observations. After washing in the same buffer, the preparations were

dehydrated in ethanol, critical-point dried with CO_2, coated with metallic gold and examined using a JEOL JSM 35 microscope operating at 15 kV (Adriano et al., 2002).

Plasmodia were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 12 h, washed in a glucose-saline solution for 2 h and post-fixed in OsO_4, performed at 4 ºC, for transmission electron microscopy observation. After dehydration in an acetone series, the material was embedded in EMbed 812 resin. Ultrathin sections double stained with uranyl acetate and lead citrate were examined with a LEO 906 electron microscope operating at 60 kV (Adriano et al., 2005).

2.3- Molecular Study

The content of the plasmodium was collected in a 1.5 ml microcentrifuge tube and fixed in 99% ethanol for molecular study. DNA was extracted using the Qiagen Dneasy® Blood and Tissue kit, following manufacturer's instructions, with final volume of 100µl.

DNA content was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific) at 260 nm. Polymerase chain reaction (PCR) was carried out in a final volume of 25 µl, containing 10 to 50 ng of extracted DNA, 1x Taq DNA Polymerase buffer (Invitrogen), 0.2 mmol of dNTP (Invitrogen), 1.5 mmol of MgCl2, 0.2 pmol of each primer (Invitrogen), 0.25 µl (1.25 U) of Taq DNA polymerase (Invitrogen), and ultrapure (MilliQ) water. The Eppendorf AG 22331 Hamburg Thermocycler was used.

Table 1: Primers used to amplify and sequence 18S fragments of *Henneguya piaractus* and *Myxobolus colossomatis.*

Primers Name		**Sequence (5' to 3') F - foward, R - reverse**	**Source**
gene18S rDNA			
ERIB1	F	ACCTGGTTGATCCTGCCAG	Barta et al. (1997)
ACT1R	R	AATTTCACCTCTCGCTGCCA	Hallet & Diamant (2001)
MYXGEN4f	F	GTGCCTTGAATAAATCAGAG	Diamant et al. (2004)
ERIB10	R	CTTCCGCAGGTTCACCTACGG	Barta et al. (1997)
MC5	F	CCTGAGAAACGGCTACCACATCCA	Molnár et al. (2002)
MC3	R	GATTAGCCTGACAGATCACTCCACGA	Molnár et al. (2002)

Fragments of the SSU rDNA gene were amplified using the primer set ERIB1 (Forward) and ACT1R (Reverse), MYXGEN4f (Forward) and ERIB10 (reverse) (Barta et al., 1997; Andree et al., 1999; Hallett & Diamant, 2001; Diamant et al., 2004) (Table 1). Initial denaturation step was carried out at 95º C for 5 min, followed by 35 cycles of denaturation (95º C for 60 s), annealing (62º C for 60 s) and extension (72º C for 120 s) and a final extended elongation step at 72º C for 5 min. PCR products were electrophoresed in 1.0% agarose gel (BioAmerica) in a TBE buffer (0.045 MTris-borate, 0.001 M EDTA, pH 8.0), stained with ethidium bromide and analyzed in a FLA-3000 (Fugi) scanner. The size of the amplified fragments was estimated by comparisons with the 1 kb DNA Ladder (Invitrogen). Amplicons were purified using the QIAquick® Spin Handbook, PCR Purification Kit, Qiagen. Sequencing was prepared using also the primer pair MC5-MC3 (Table 1) (Molnár et al., 2002).

Sequencing was performed with the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc., Foster City, California) in an ABI 3730 DNA sequencing analyzer (Applied Biosystems).

A standard nucleotide-nucleotide BLAST search was conducted (Altschul et al., 1997). The Bioedit (Hall, 1999) program was used to align the comparison sequence with those found in the GenBank. To ensure the accuracy of the analysis, GenBank sequences smaller than those obtained in this study were not

used. Phylogenetic analyses using *Ceratomyxa sparusaurati* as the outgroup were conducted with the MEGA 5.0 program (Tamura et al., 2011), employing the maximum likelihood (ML) phylogenetic method. The Kimura two-parameter (K2P) evolution sequence model was used in the analysis with gaps treated with complete deletion. Bootstrap analysis (500 replicates ML) was employed to assess the relative robustness of the tree branches.

3. RESULTS

The species of myxosporean identified in all three locations were *H. piaractus* and *M. colossomatis* based on original descriptions. *Henneguya piaractus* plasmodia, located in the lamella, were polysporic, white, round to ellipsoidal (majority) 84.3 µm (± 24.8) in diameter and 88.9 µm (± 22.6) in length. *Myxobolus colossomatis* plasmodia, located in the arches tissue, were polysporic, round, white, 81.1 µm (± 10.4) diameters and 127.1 µm (± 9.4) in length.

During the study period, the prevalence for both species was high (> 50%) or moderate (50%) for fish gathered from Pirassununga and Itapira. The exception was for fish captured from the Mogi Mirim farm, in 2010. Mogi Mirim fish presented significant results concerning to prevalence and mean intensity by year. In 2009 the highest values were found ($p < 0.05$) and decreased in 2010 (Table 2).

Annual prevalence values for Pirassununga fish farm were high or moderate during the study period. The highest prevalence for *Henneguya piaractus* was in 2008 (P=71.2%). During the following years the prevalence fell, and did not vary (Table 2), but not significantly different. In terms of data for *M. colossomatis* in fish from Pirassununga, high prevalence in 2010 (P=73.5%), with the lowest percentage in 2008 (P=53.1%) was observed. *Myxobolus colossomatis* was the most prevalent parasites in Pirassununga compared to

other fish farms ($p<0.05$) (Duncan values of 63.3 Pirassununga, compared to 21.3 Mogi and 16.6 Itapira).

At the Mogi Mirim fish farm, the prevalence rate in 2009 was 65% for *H. piaractus* and 55% for *M. colossomatis*, decreasing in 2010 to 16.6% and 9.0%, respectively (Table 2). Comparing prevalence and mean intesity by year for Mogi Mirim, significantly different results showed that these parameters had the highest results in 2009, followed by 2010. At the Itapira fish farm only one sample was collected, during an outbreak in spring 2009. During this period prevalence was high for *H. piaractus* and moderate for *M. colossomatis* (Table 2).

The ANOVA test was used for statistical analysis and presented significant results regarding prevalence versus location and year, and also mean intensity versus year ($p<0.05$). The Duncan test showed that Pirassununga had a higher prevalence and mean intensity compared with the other fish farms. For Duncan analysis, 2009 showed significant overall data for prevalence (76.2 for 2009; 30.6 for 2010 and 23.2 for 2008) and mean intensity (56.1 for 2009; 16.4 for 2010 and 8.7 for 2008), compared to 2010 and 2008 (Table 2).

Annual mean intensity data presented a different scenario. For *H. piaractus* (Pirassununga) mean intensity was 39.8 cysts/fish in 2008, although it has decreased considerably in the subsequent years (Table 2). At Mogi Mirim and Itapira the mean intensity was high throughout the study period (Table 2). For *M. colossomatis* in the Pirassununga and Itapira fish farms, mean intensity was low, while in Mogi Mirim the intensity has been found high in 2009, but declined in 2010.

Histological studies showed that plasmodia of *H. piaractus* intralamellar type were located between the gill lamellar epithelium and the capillary (Fig. 1). The parasite caused accentuated dilatation and deformation of infected gill lamellae, leading to displacement, deformation and fusion of neighboring lamellae. Intense epithelial hyperplasia was observed, but no inflammatory

infiltration (Fig. 1B, 1C and 1D). Plasmodia were observed in all regions of the gill lamella.

The plasmodia of *M. colossomatis* were distributed throughout the tissue of the gill arches, rakers and basifilamental portion. The parasite formed plasmodia among the gill arch and gill rakers and basifilamental position between the osseous and cartilaginous tissue (Fig. 2D). Mature spores were encountered on the plasmodia. A thin layer of the plasmodium was in direct contact with layer of the host cells (Fig. 2D). No tissue damage was observed.

Scanning electron microscopy for *H. piaractus* showed ruptured intralamelar plasmodia with some external spores (Figs. 3A and 3B). Ellipsoidal tailed spores were observed along the external features such as suture lines and two equally demarked polar capsules with polar filaments in the spore wall (Figs. 3C to 3F). Transmission electron microscopy showed a thin layer of granular material between the plasmodial wall and the host cells. The thick single plasmodial wall was connected to a plasmodial ectoplasm zone through numerous and extensive pinocytotic canals (Figs. 4A, 4B and 4C). Mitochondria and the earliest stages of sporogenesis were observed at the periphery of the endoplasm (Figs. 4A, 4B and 4C), whereas mature spores were found in the central region (Fig. 4D).

Scanning electron microscopy for *M. colossomatis* showed mature spores pear-shaped to sub-spherical with sutural folds (Figs. 5A and 5B). Ultrastructural analysis revealed the plasmodia in direct contact with the host cells. The plasmodial wall consisted of a single layer separating the host environment from the parasite (Figs. 5C, 5D and 5E). A plasmodial ectoplasm zone was formed with numerous and extensive phagocytic activity and numerous projections in the direction of the host cells. No pinocytotic canal was observed (Figs. 5C and 5D). Numerous mitochondria were found in the periphery of the endoplasm, and there were signs of the earliest stages of sporogenesis and mature spores (Figs. 5C, 5D and 5E).

Regarding molecular analyses, the primer pairs ERIB1–ACT1R and MYXGEN4f–ERIB10 successfully amplified partial fragments of the 18S rDNA

gene for *H. piaractus* and *M. colossomatis* found in the gills of *P. mesopotamicus*.

Sequencing of the 18S rDNA gene resulted in sequences of 1.913 bp for *H. piaractus* and 1.341 bp for *M. colossomatis*. These sequences did match with Myxozoa species sequences available in GenBank. Sequences will be deposited in Genbank after the acceptance of the article.

Phylogenetic analysis of 37 species of *Myxobolus* and *Henneguya* sequences available from GenBank were aligned using Maximum Likelihood analyses.

The phylogenetic tree shows Myxozoa species forming two distinct clades, with the Cypriniform *Myxobolus* spp. and *Henneguya* spp. parasites forming the largest clade, and the other clade being composed by Siluriform and Characiform parasites. *Myxobolus colossomatis* appears as a basal species of the clade composed by Siluriform/ Characiform parasites. *Henneguya piaractus,* clustered together with *M. oliveirai,* forming sister groups in the clade composed of *Henneguya* spp. parasites of North American siluriforms (Fig. 6).

Table 2: Mean values of *Henneguya piaractus* and *Myxobolus colossomatis* parasites of the gills of *Piaractus mesopotamicus* from three fish farms during February 2008 to July 2010. Different letters indicate significant difference among samples along the years, for Pirassununga and Mogi Mirim fish farms ($p<0.05$).

Parameters	**Pirassununga**				**Mogi Mirim**			**Itapira**
Host's Total Number	2008 n= 94	2009 n= 90	2010 n=34	total n=218	2009 n=20	2010 n=30	total n=50	2009 n=10
Prevalence %								
H. piaractus	71.2 a	54.5 b	50 b	56.8	65 a	16.6 b	36	100
M. colossomatis	53.1 b	63.3 b	73.5 a	60.5	55 a	9 b	40	50
Mean Intensity								
H. piaractus	39.8 a	5.7 b	5.4 b	24.5	64.3 a	51.6 a	83.7	47.1
Range	1 to 1183	1 to 33	1 to 20		2 to 389	1 to 606	5 to 115	
M. colossomatis	5.4 b	7.3 b	6.6 b	6.0	57.5 a	19.8 b	40.6	7.0
Range	1 to 37	1 to 30	1 to 27		5 to 340	2 to 85	1 to 16	

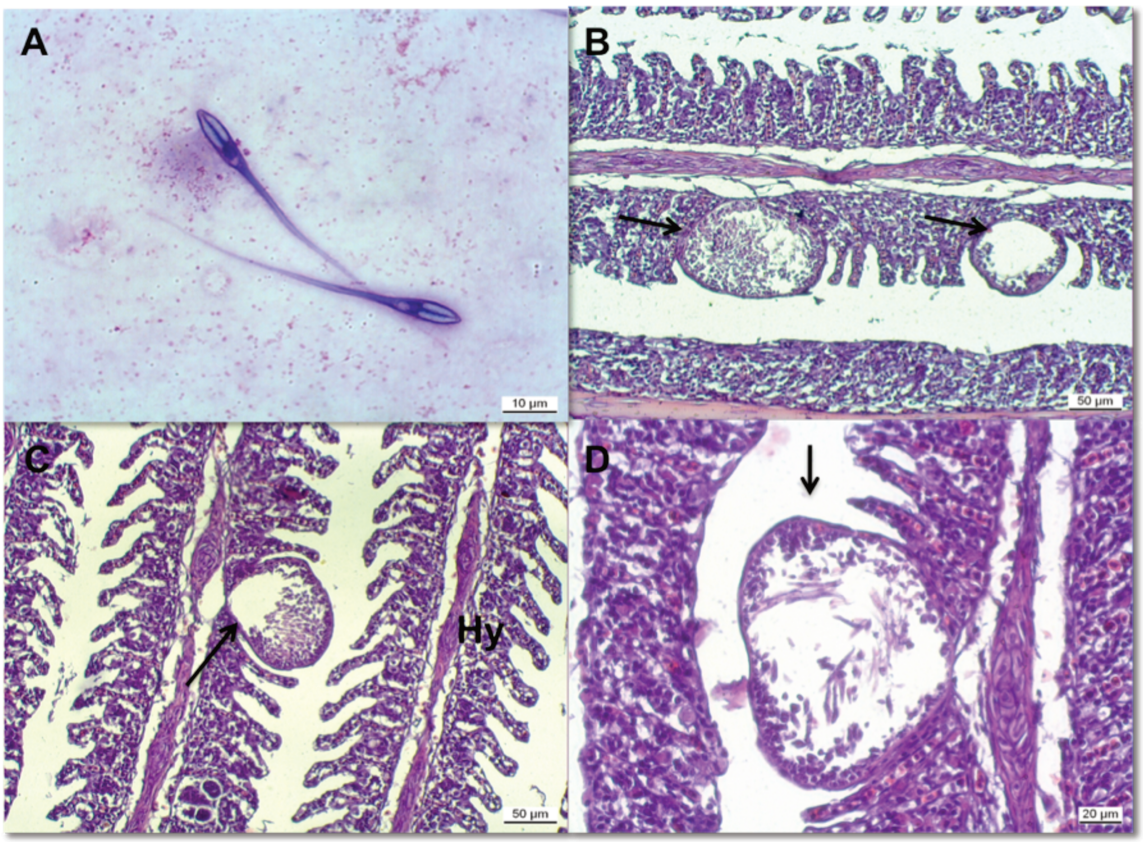


Figure 1: Light photomicrographs of spores of (A) *Henneguya piaractus* stained with Giemsa; (B, C and D) histological longitudinal sections of the lamellae showing *H. piaractus* cysts, black arrows and hyperplasia (Hy) at the base of secondary lamellae, stained with HE.

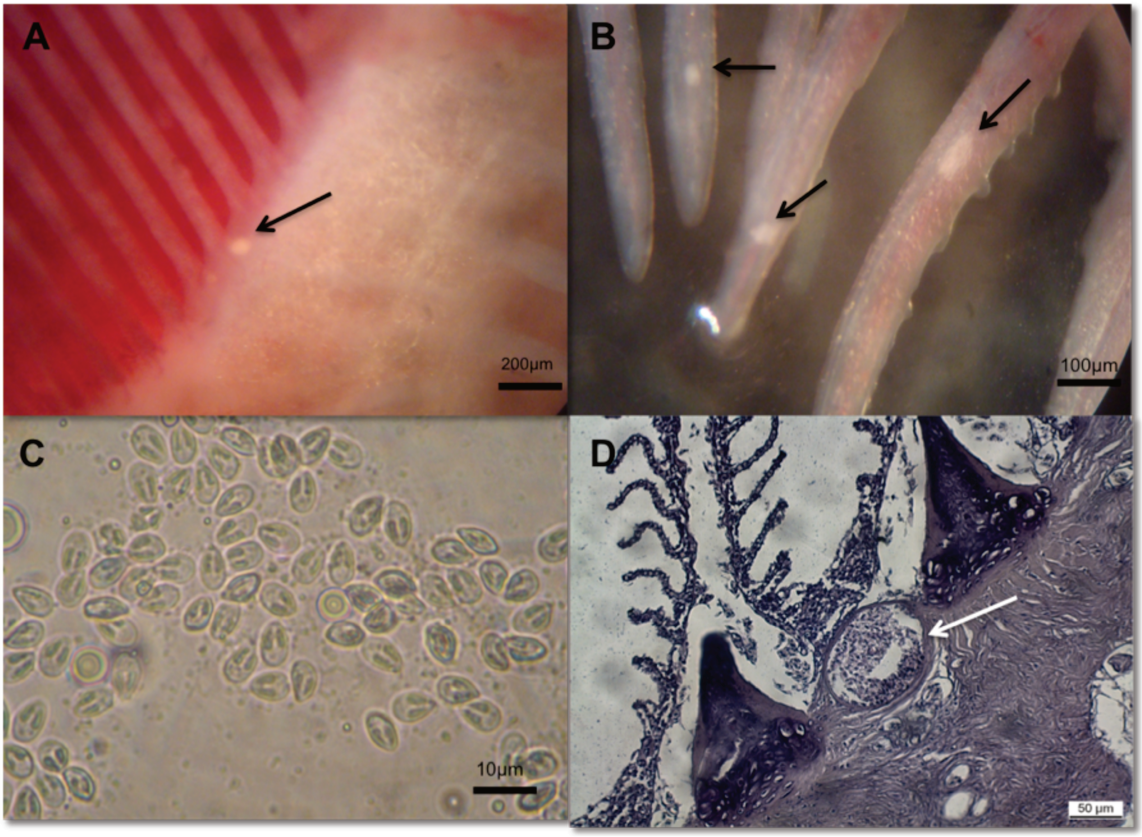


Figure 2: Light photomicrographs of gills of *Piaractus mesopotamicus* with the cysts (A- B) of *Myxobolus colossomatis* on the arches, (A) base of the filament, (B) and in the gill rakers (black arrows). (C) *Myxobolus colossomatis* mature fresh spores. (D) histological longitudinal section showing the cyst in the gill arch (white arrow), stained with HE.

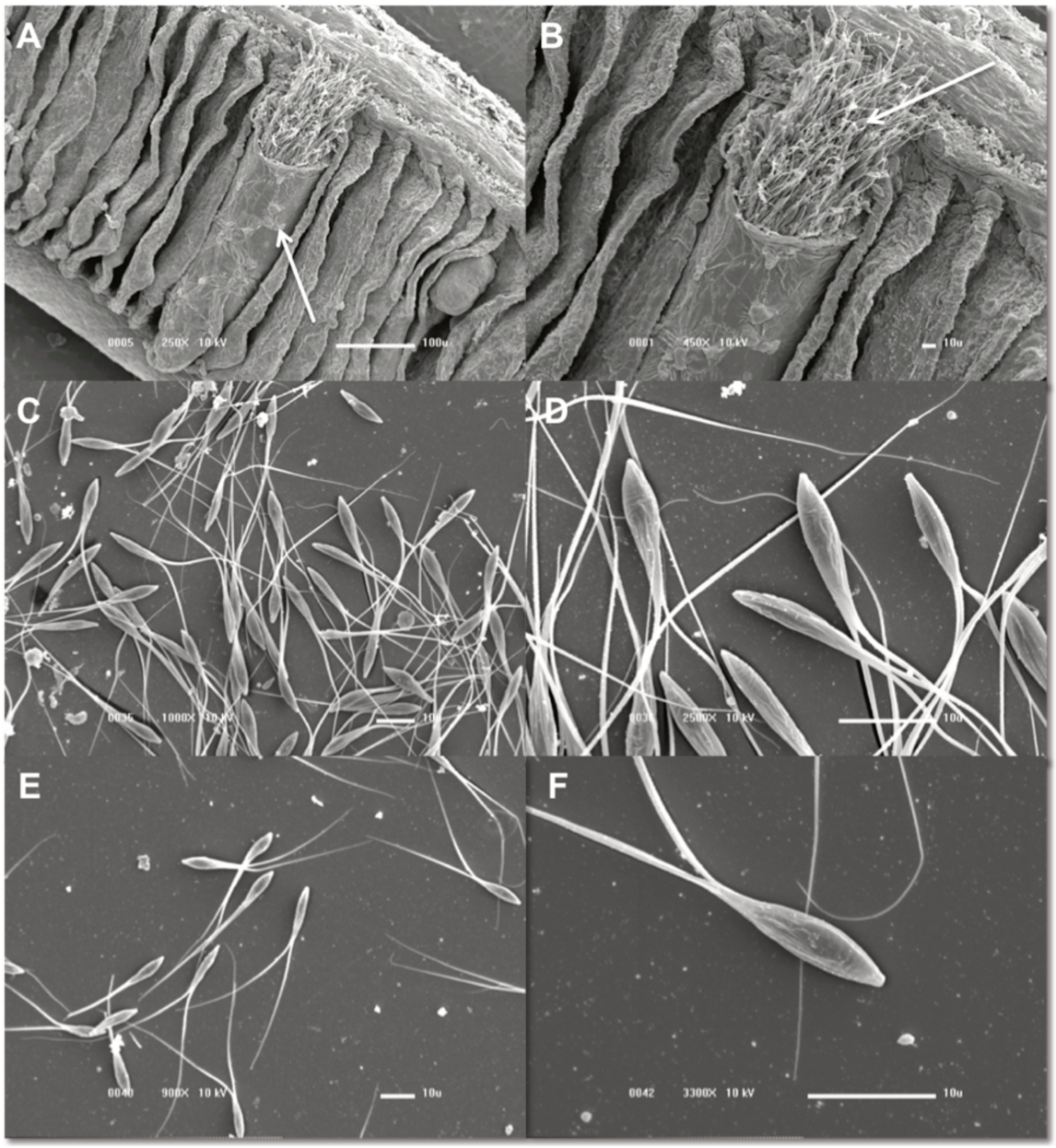


Figure 3: Scanning electron micrographs of *Henneguya piaractus*. (A and B) Secondary lamellae with cyst (white arrow). (B) A closer view of the cyst showing *Henneguya piaractus* liberated spores (white arrow). (C -F) Spore morphology: spindle-shaped spores with a long bifurcate tail.

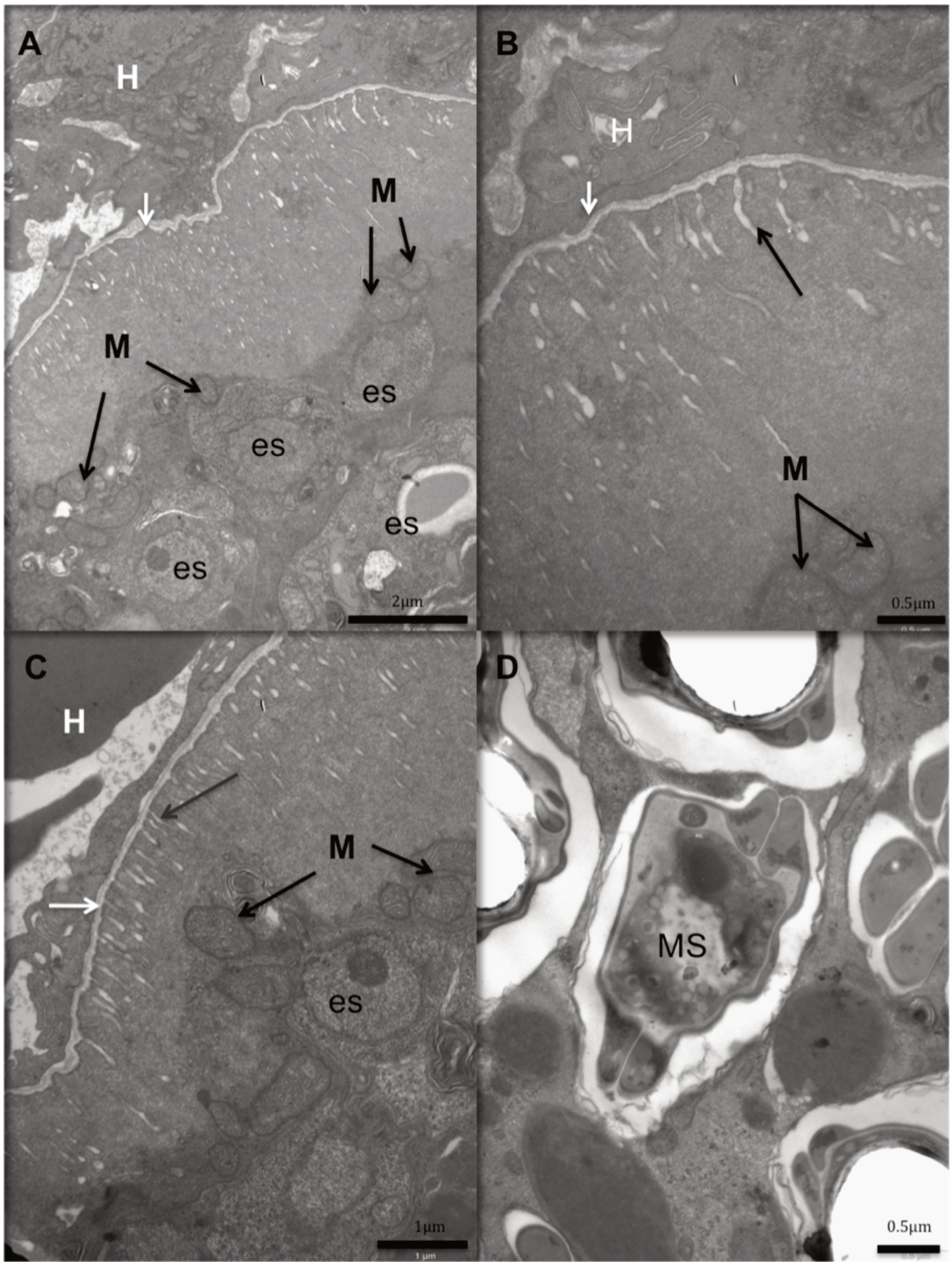


Figure 4: Electron micrography of the gills of *Piaractus mesopotamicus* (A-D)infected with *Henneguya piaractus*. Note a thin layer of granular material between the plasmodial wall and the host (white arrows); a single plasmodial wall connected to a plasmodial ectoplasm zone through numerous and extensive pinocytic canals (black arrows). Host (H). Mitochondria (M). Earliest stages of sporogenesis (es) and mature spore (MS).

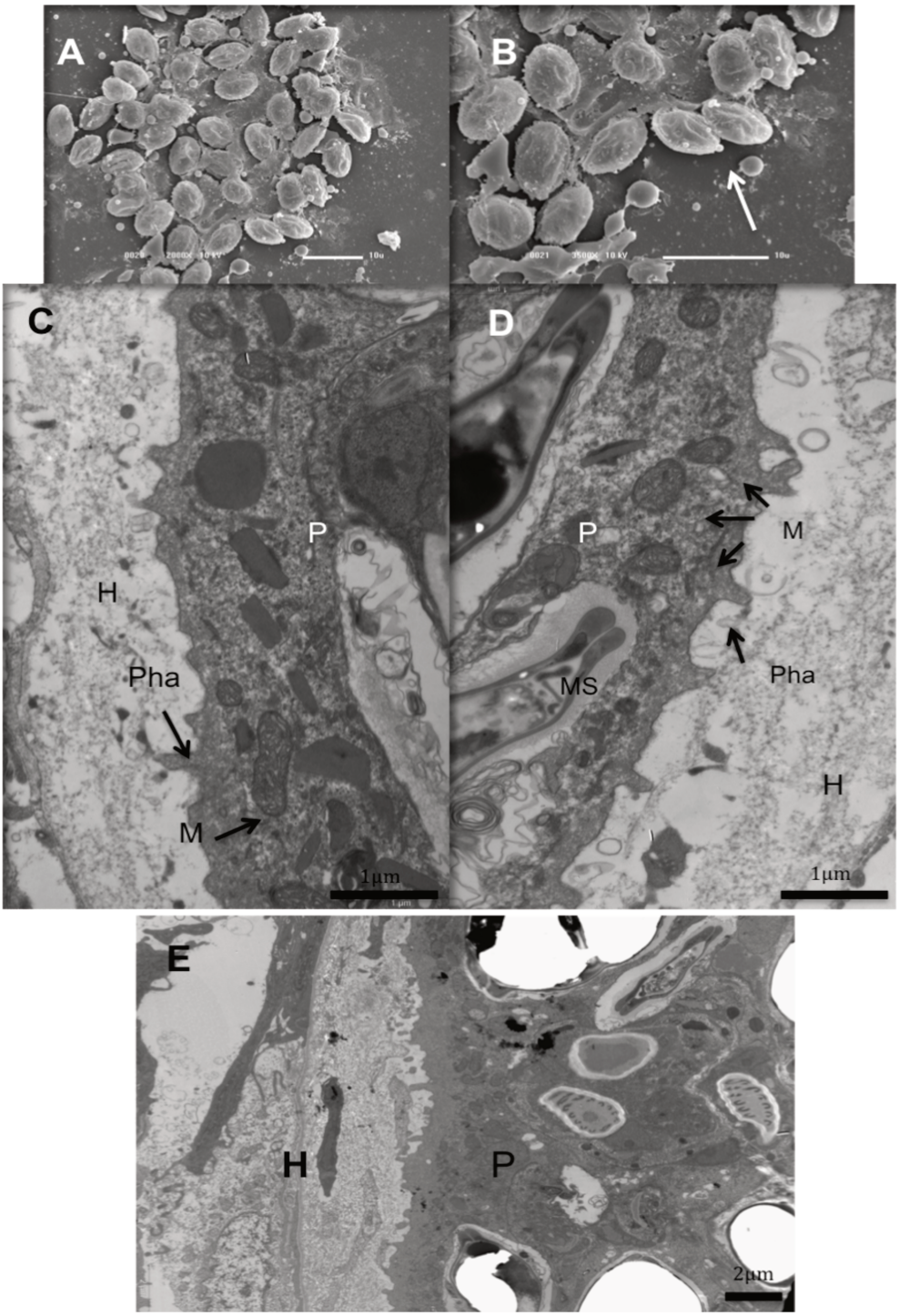


Figure 5: Electron micrographs of *Myxobolus colossomatis*, gill arches parasite of *Piaractus mesopotamicus*. (A - B) Scanning electron micrographs of *M. colossomatis.* (C - E). Transmission electron micrographs of *M. colossomatis*, (C - D) showing structures from host tissue (H). Parasite (P). Phagocytosis activity (Pha). Mitochondria (M) and mature spore (MS). (E) A panoramic view of the structure of the cyst wall.

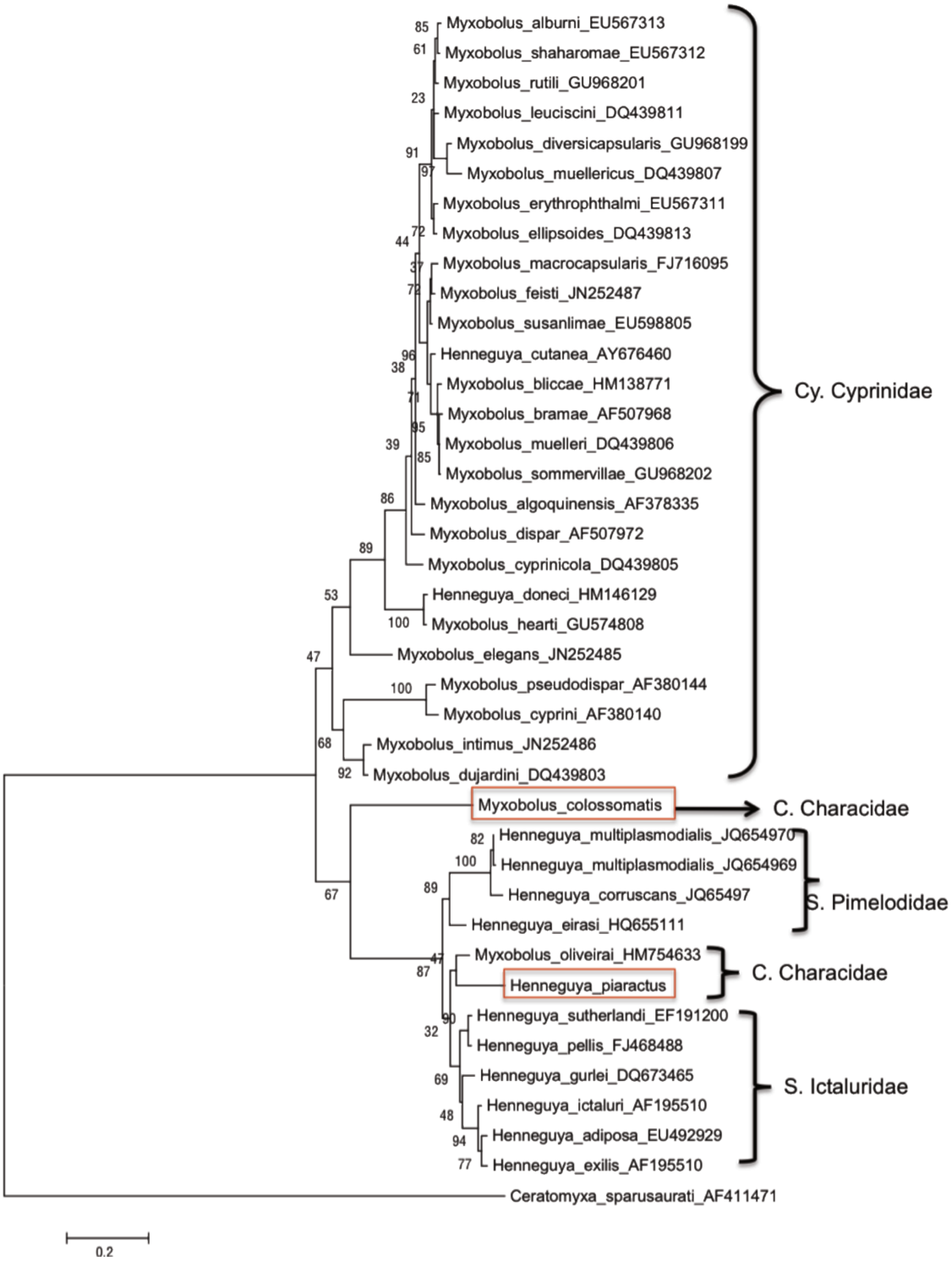


Figure 6: Phylogenetic tree of Myxobolidae species based on Maximum Likelihood analysis of the SSU rDNA data showing the relationship of the South American species *Henneguya piaractus* and *Myxobolus colossomatis*. Values above nodes indicate bootstrap confidence levels. Cy - Cyprinifoms, C - Characiforms and S - Siluriforms.

3- DISCUSSION

The prevalence values were moderate to high for both species of myxosporean species evaluated, except for the sample gathered at the Mogi Mirim fish farm in 2010. The significantly smaller prevalence observed at Mogi Mirim in 2010 is probably related to the chemical treatment carried out by the owner during that year. The high parasitological numbers at the Itapira fish farm are related to an outbreak during the spring of 2009, when fish were heavily infected, especially with protozoans (*Ichthyophthirius multifiliis* and *Trichodina* sp.) and monogeneans (*Anacanthorus penilabiatus* and *Mymarothecium viatorum*). Countant (1998) says that outbreaks during changing seasons are often reported at fish farms, especially if environmental conditions, such as changes in the aquatic parameters, climate stress, and introduction of pathogens, are not favorable, as these are factors that can increase the susceptibility of the host to parasites, and provoke an imbalance of the host/parasite/environment system.

Little information is known about myxozoan prevalence in collected freshwater fish in aquaculture or in nature in Brazil (Martins & Onaka, 2006). Parasites in Pirassununga had the highest prevalence and mean intensity of infection values among the other locations. This may be due to the period of study, which was carried out over a three-year period, as all months were sampled equally, and a large number of fish studied.

Infections in the gills can compromise the respiratory organ if parasites are present in high numbers, and the disruption of the gill epithelium through the release of spores can provide an entry route for secondary infections. The most common pathologies associated with myxozoan infections in the gills, include fusion of lamellae, inflammation, hyperplasia, pressure atrophy and cellular necrosis (Feist & Longshaw 2006; Reimschuessel, 2008). *Henneguya piaractus* is an important pathogen that causes pathological alterations in the gills of cultivated pacu (Martins et al., 1997; Adriano et al., 2005), including hemorrhage and severe inflammatory foci in the gill epithelium (Martins et al., 1997). Adriano

et al. (2005) reported marked dilatation of the infected lamellae, compression of the capillary and adjacent tissues, displacement of neighboring lamellae and discreet epithelial hyperplasia, but infiltrated inflammation was not observed. The results presented here corroborate those reported by Adriano et al. (2005), and, in part, those recorded by Martins et al. (1997) regarding infections of *H. piaractus* in the gills of fish farm *P. mesopotamicus*. Similar histological damages have been observed for other myxosporeans such as for *Henneguya curvata* (see Barassa et al., 2003a), *Henneguya chydadea* (see Barassa et al., 2003b) and *Henneguya creplini* (see Mólnar, 1998).

Gill infecting species are frequently considered pathogens of serious effect, as pathological responses to their presence can affect respiratory function. However, in many cases, the tissue response is rather localized, especially for cyst-forming species (Feist, 2008). *Myxobolus colossomatis* infects the gill arches and sometimes the basifilamental but no major pathology was found. For myxozoans, the importance of the pathology depends on the locality of the infection, the organ or tissue parasitized. Regarding gills infection, the formation of cysts in other sites, other than the gills lamellae, cannot be pathogenic (Feist & Longshaw, 2006). However, for the myxosporean *M. basilamellaris,* infections can cause a localized pathological response to the parasite cysts within the gills of the host (Kovács-Gayer & Molnár, 1983). The cysts develop in a basifilamental position in the gill, and the growth of the cyst lifts the basal part of the gill filament and deforms the adjoining lamellae, reducing the respiratory surface. Those cysts developing in the gill arch restrict nerves and blood vessels passing through the gill arch, leading to local blockages (Feist & Longshaw, 2006). These features were not observed in this study of *M. colossomatis*, but pathology associated with these parasites is thought to occur in the latter stages, mainly in high parasite loads, when the parasites release the spores. In this case, the foci were the parasite present, which can open doors for secondary infections.

Ultrastructural analysis showed the way that this parasite communicates and feeds on the host. It differs between the two species. The single plasmodial wall of *H. piaractus* was separated from the wall of the host cell by a thin layer of

granular material with pinocytic channels through the cyst wall showing how communications between host and parasite occur. The presence of pinocytic activity is also reported in several other *Henneguya* species (Hallet & Diamant, 2001; Azevedo & Matos, 2002; 2003; El-Mansy & Bashtar, 2002). Adriano et al. (2005) reported, in addition to pinocytic activity, phagocytic activity in the wall of the plasmodia of *H. piaractus*. In contrast, in the present study no evidence of this activity was found. The plasmodium wall is seen as the organelle responsible for feeding the entire plasmodium and knowledge of the structure of this organelle is necessary to understand the stress placed on the host by the parasite (El-Mansy & Bashtar, 2002).

Myxobolus colossomatis also presented a single wall, but in this case, the parasite wall has direct contact with the host cells, and phagocytic activity can be observed in the interface between the host and the parasite, through the plasmodia wall, a different system to that observed for *H. piaractus*. Phagocytosis as a form of obtaining nutrients has been reported in other myxosporeans. Phagocytosis followed by intracellular digestion within a food vacuole was observed in *Kudoa quadratum* (Uspenskaya, 1982). Phagocytosed chondrocytes were observed in *M. cerebralis*, and phagocytosis of the erythrocytes was also found in the *Sphaerospora* sp. (Lom & Diková, 1995). Pinocytosis and phagocytosis are important nutrient transport systems and are necessary for the growth of parasites (Hallet & Diamant, 2001; El-Mansy & Bashtar, 2002).

The sporogenesis in *H. piaractus* is fully described in prior studies (Adriano et al., 2005; Azevedo et al., 2010), and the results here obtained follows the pattern of reports previously described before, as well as of other *Henneguya* species (Azevedo & Matos, 2002; 2003; El-Mansy & Bashtar, 2002; Vita et al., 2003). The earliest stages of sporogenesis were observed in the periphery of endoplasm and of the mature spores in the central region. For *M. colossomatis* the sporogenesis also follows the pattern of other *Myxobolus* species (Current et al., 1979, Casal et al., 1996, 2002, Adriano et al., 2006) with early stages in the outer periphery and mature spores located centrally.

Ultrastructural investigations using sectioned material are rarely able to identify species, but scanning electron microscopy provides valuable diagnostic data on the surface morphology of spore stages (Lom & Dyková, 1993). Scanning electron microscopy for this study provided morphological features to help diagnosis of the gill myxosporeans species from *P. mesopotamicus*.

Diagnosis of myxozoans is still mainly reliant on morphological characteristics of mature spore stages, using established criteria (Lom & Arthur, 1989). However, taxonomic classification of Myxosporea based on morphology alone has been refined through the application of molecular methods (Molnár et al., 2007, 2008; Andree et al., 1999, Adriano et al., 2012). Molecular tools became necessary when several authors came across difficult questions regarding this interesting metazoan group. Questions regarding specificity, location on/or in the host and differences and similarities to morphology itself, have been answered by previous studies. Adriano et al. (2009), studying a new species infecting *Zungaro jahu*, *Myxobolus cordeiroi*, found that the parasite can be located in the gill arch, skin, serosa of the body cavity, urinary bladder and eye. Using molecular tools these authors confirmed that the same myxosporean species could be found at all these sites of infection.

The most used marker for Myxozoa is the 18S rDNA gene. Molecular studies have indicated that classification based mainly on the structure and shape of the myxospore is not consistent with phylogenetic relationships (Smothers et al., 1994). Molecular phylogenetic analysis found that the *Myxobolus* genus is not monophyletic, and also showed the polyphyly of the genera *Henneguya* (Fiala, 2006) and separated freshwater from marine myxozoans in two major branches (Kent et al., 2001), with the exception of some species that do not follow this trend (Fiala, 2006).

The phylogenetic position of *H. piaractus* and *M. colossomatis* among the myxosporeans available in Genbank are divided into two major clades. To enhance the accuracy of the analysis, smaller specie sequences were not used, the majority being approximately 1500bp or more. The results showed *Myxobolus* and *Henneguya* species clustering into two clades. Only freshwater

and some brackish species were chosen for analysis, and the results clearly show the tendency to cluster according to fish families, corroborating Ferguson et al. (2008) Naldoni et al. (2011) and Adriano et al. (2012).

The most numerous clade grouped *Henneguya* and *Myxobolus* species parasites in Cypriniformes. The smallest was composed predominantly by Siluriforme parasite species, but also three parasites species of Characiformes, where *M. colossomatis* appears as a basal species. This clade was further divided into two clades. One composed only by *Henneguya* spp. parasites of South American pimelodidae and another that further divided into a clade composed by *Henneguya* spp. parasites from North American ictalurids, which clustered with *H. piaractus* and *Myxobolus oliveirai*, parasites of characids, forming a sister clade.

Among the myxosporean species parasites from South American freshwater fish, just a few species have been sequenced and deposited in the GenBank. South American species of the *Myxobolus* genus contained in GenBank are *M. cordeiroi* (Adriano et al., 2009), which was the first to be sequenced and deposited, consisting of around 500bp, although it was not used in this study, and *M. oliveirai*, parasite of a characid host (Milanin et al., 2010). Within the *Henneguya* genus, South American species that are sequenced and deposited in the GenBank are *H. eirasi*, *H. plasmodialis* and *H. corruscans*, all parasites of pimelodids hosts. *H. piaractus* is the first species of a characid host to be 18S rDNA gene sequenced.

Other than *M. colossomatis*, *M. oliveirai* and *H. piaractus* were parasites of characid hosts that clustered together*. Myxobolus. colossomatis* does not appear in clustering in a clade, in contrast to the tendency to cluster according to fish families found by Ferguson et al. (2008), Naldoni et al. (2011) and Adriano et al. (2012). However, given the lack of data concerning sequencing of myxosporens parasites of characiform fish available in the GenBank, this result may reflect the small number (three) of species involved in phylogenetic analysis. In the future, a larger sample group from the same geographic region should be used, to properly define the evolutionary history of the South American myxosporean

species from characid hosts, in relation to parasite species from other orders/families hosts.

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6- REFERENCES

Adriano, E.A., Arana, S., Ceccarelli, P.S., Cordeiro, N.S., 2002. Light and scanning microscopy of *Myxobolus porofilus* sp. n. (Myxosporea: Myxobolidae) infecting the visceral cavity of *Prochilodus lineatus* (Pisces: Characiformes: Prochilodontidae) cultivated in Brazil. Folia Parasitol. 49, 259-262.

Adriano, E.A., Arana, S., Cordeiro, N.S., 2005. Histology, ultrastructure and prevalence of *Henneguya piaractus* (Myxosporea) infecting the gills of *Piaractus mesopotamicus* (Characidae) cultivated in Brazil. Dis. Aquatic Organ. 64, 229-235.

Adriano, E.A., Arana, S., Cordeiro, N.S., 2006. *Myxobolus cuneus* n.sp. (Myxosporea) infecting the connective tissue of *Piaractus mesopotamicus* (Pisces: Characidae) in Brazil: histopathology and ultrastructure. Parasite. 13, 137-142.

Adriano, E.A., Arana, S., Alves, A.L., Silva, M.R.M., Ceccarelli, P.S., Henrique-Silva, F., Maia, A.A.M., 2009. *Myxobolus cordeiroi* n. sp., a parasite of *Zungaro jahu* (Siluriformes: Pimelodidae) from brazilian Pantanal: morphology, phylogeny and histopathology. Vet. Parasitol. 162, 221-229.

Adriano, E.A., Carriero, M.M., Maia, A.A.M., Silva, M.R.M., Naldoni, J., Ceccarelli, P.S., Arana, S., 2012. Phylogenetic and host-parasite relationship analysis of *Henneguya multiplasmodialis* n. sp. infecting *Pseudoplatystoma* spp. in Brazilian Pantanal wetland. Vet. Parasitol. 185, 110-120.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl. Acids Res. 25(17), 3389-3402.

Andree K.B., Székely C., Molnár K., Gresoviac S.J., Hedrick R.P., 1999. Relationships among members of the genus *Myxobolus* (Myxozoa: Bivalvidae) based on small subunit ribosomal DNA sequences. J. Parasitol. 85 (1), 68-74.

Azevedo, C., Matos, E., 2002. Fine structure of the myxosporean, *Henneguya curimata* n. sp., parasite of the Amazonian fish, *Curimata inormata* (Teleostei, Curimatidae). J. Euk. Microbiol. 49, 197-200.

Azevedo, C., Matos, E., 2003. Fine structure of *Henneguya pilosa* n. sp. (Myxozoa: Myxosporea), parasite of *Serrasalmus altuvei* (Characidae) in Brazil. Folia Parasitol. 50, 37-42.

Azevedo, C., Casal, G., Mendonça, I., Carvalho, E, Matos, P., Matos, E., 2010. Light and eletron microscopy of *Myxobolus sciades* n. sp. (Myxozoa), a parasite of the gills of the brazilian fish *Sciades herzbergii* (Block, 1794) (Teleostei: Ariidae). Mem. do Inst. Oswaldo Cruz. 105, 203-207.

Barassa, B., Cordeiro, N.S., Arana, S., 2003b. A new species of *Henneguya*, a gill parasite of *Astianax altiparanae* (Pisces: Characidae) from Brazil, with comments on histopathology and seasonality. Mem. do Inst. Oswaldo Cruz. 98(6), 761-765.

Barassa, B., Adriano, E.A., Arana, S., Cordeiro, N.S., 2003b. *Henneguya curvata* sp. n. (Myxosporea: Myxobolidae) parasitizing the gills of the *Serrasalmus spilopleura* (Characidae: Serrasalminae), a South American freshwater fish. Folia Parasitol. 50, 151-153.

Barta, J.R., Martin, D.S., Liberator, P.A., Dashkevicz, M., Anderson, J.W., Feighner, S.D., Elbrecht, A., Perkins-Barrow, A., Jenkins, M.C., Danforth, H.D., Ruff, M.D., Profous-Juchelka, H., 1997. Phylogenetic relationships among eight *Eimeria* species infecting domestic fowl inferred using complete small subunit ribossomal DNA sequences. J. Parasitol. 83, 262-271.

Bush, A.O., Lafferty, K.D., Lotz, J.M., Shostak, A.W., 1997. Parasitology meet ecology on its own terms: Margolis *et al*. revisited. J. Parasitol. 83 (4), 575-583.

Casal, G., Matos, E., Azevedo, C., 1996. Ultrastructural data on the life cycle stages of Myxobolus braziliensis n. sp., parasite of an Amazonian fish. Eur. J. Protistol. 32, 132-127.

Casal, G., Matos, E., Azevedo, C., 2002. Ultrastructural data on the spore of *Myxobolus maculatus* n. sp. (phylum Myxozoa), parasite from the Amazonian fish *Metynnis maculatus* (Teleostei). *Dis. Aquatic Organ.* 51, 107-112.

Casal, G., Matos, E., Azevedo, C., 2003. Light and eletron microscopic study of the myxosporean, *Henneguya friderici* n. sp. from the Amazonian teleostean fish, *Leporinus friderici*. Parasitol. 126, 313-319.

Coutant, C.C., 1998. What is normative for fish pathogens? A perspective on the controversy over interactions between wild and cultured fish. J. Aquatic Ani. Health, Bethesda. 10, 101-106.

Current, W.L., Janovy, Jr.J., Knight, S.A., 1979. *Myxosoma funduli* Kudo (Myxosporida) in *Fundulus kansae*: ultrastructure of the plasmodium wall and sporogenesis. J. Protozool. 26, 574-583.

Diamant, A., Whipps, C.M., Kent, M.L., 2004. A new species of *Sphaeromyxa* (Myxosporea: Sphaeromyxina: Sphaeromyxidae) in devil firefish, *Pterois miles* (Scorpaenidae), from the northern red sea: morphology, ultrastructure and phylogeny. J. Parasitol. 90(6), 1434-1442.

Eiras, J.C., Molnár, K., Lu, Y.S., 2005. Synopsis of the species of *Myxobolus* Butschli, 1882 (Myxozoa: Myxosporea: Myxobolidae). Syst. Parasitol. 61, 1-46.

Eiras, J.C., Monteiro, C.M, Brasil - Sato, M.C., 2010. *Myxobolus franciscoi* sp. nov. (Myxozoa; Myxosporea: Myxobolidae), a parasite of *Prochilodus argenteus* (Actinopterygii: Prochilodontidae) from the upper São Francisco River, Brazil, with a revision of the *Myxobolus* spp from South America. Zoologia. 27, 131-137.

El-Mansy, A., Bashtar, A.R., 2002. Histopathological and ultrastructural studies of *Henneguya suprabranchiae* Landsberg, 1987 (Myxosporea: Myxobolidae) parasitazing the suprabranchial organ of the freshwater catfish *Clarias gariepinus* Burchell, 1822 in Egypt. Parasitol. Res. 88, 617-626.

Feist, S., Longshaw, M., 2006. Phylum Myxozoa. In: P.T.K. Woo. Fish Diseases and Disorders. Volume 1, Second Edition. CAB International, University of Guelph, Canada. pp. 230 -296.

Feist, S., 2008. Myxozoan Diseases. In: Eiras, J.; Segner, H.; Wahli, T, Kapoor, B.G. (eds.) Fish Diseases, Volume 2. Science Publishers, Enfield (NH), Jersey, Plymouth. 1312p.

Ferguson, J.A., Atkinson, S.D., Whipps, C.M., Kent, M.L., 2008. Molecular and morphological analysis of *Myxobolus* spp of salmonid fishes with the description of a new *Myxobolus* species. J. Parasitol. 94, 1322-1334.

Fiala, I., 2006. The phylogeny of Myxosporea (Myxozoa) based on small subunit ribossomal RNA gene analysis. Int. J. Parasitol. 36, 1521-1534.

Godoy, M.P., 1975. Peixes do Brasil: subordem Characoidei: bacia do rio Mogi-Guassu. Piracicaba: Franciscana. 1-4, 216pp.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acid. Symp. Ser. 41, 95-98.

Hallet, S.L., Diamant, A., 2001. Ultrastructure and small-subunit ribossomal DNA sequence of *Henneguya lesteri* n. sp. (Myxosporea), a parasite of sand whiting *Sillago analis* (Sillaginidae) from the coast of Queensland, Australia. Dis. Aquat. Organ. 46, 197-212.

Holzer, A. S., Sommerville, C., Wooten, R., 2006. Molecular studies on the seasonal occurrence and development of five myxozoans in farmed *Salmo trutta* L. Parasitol. 132, 193-205.

Kent, M. L., Andree, K.B., Bartholomew, J.L., El-Matbouli, M., Desses, S.S., Delvin, R.H., Feist, S.W., Hedrick, R.P., Hoffmann, R.W., Khattra, J., Hallet, S.L., Lester, R.J.G., Longshaw, M., Palenzeula, O., Siddall, M.E., Xiao, C.X., 2001. Recent Advances in our knowledge of the Myxozoa. J. Euk. Microbiol. 48, 395-413.

Kovács-Gayer, É.,, Molnár, K. 1983. Studies on the biology and pathology of the common carp parasite *Myxobolus basilamellaris* Lom *et* Molnár, 1983 (Myxozoa: Myxosporea). Acta Vet. Hung. 31, 91–102.

Lom, J., Arthur, J.R., 1989. A guideline for the preparation of species description in Myxosporea. J. Fish Dis. 12, 151-156.

Lom, J., Dyková, I., 1993. Scanning eletron microscopic revision of common species of the genus *Chloromyxum* (Myxozoa: Myxosporea) infecting European freshwater fishes. Folia Parasitol. 40, 161-174.

Lom, J., Dyková, L., 1995. Myxosporea (Phylum Myxozoa). In: PTK, Woo. Fish Diseases and Disorders - Protozoan and Metazoan Infections. Vol 1, CAB International. pp. 87-147.

Lom, J., Dyková, I., 2006. Myxozoan genera; definition and notes on taxonomy, life-cycle terminology and pathogenic species. Folia Parasitol. 53, 1-36.

Martins, M.L., Souza, V.N., 1997. *Henneguya piaractus* n. sp. (Myxozoa: Myxobolidae), a gill parasite of *Piaractus mesopotamicus* Holmberg, 1887 (Osteichthyes: Characidae), in Brazil. Rev. Bras. Biol. 57, 239-245.

Martins, M.L., Souza, V.N., Moraes, F.R., Moraes, J.R.E., Costa, A.J., 1997. Pathology and behavioral effects associated with *Henneguya* sp. (Mixozoa: Mixobolidae) infections of captive pacu *Piaractus mesopotamicus* in Brazil. J. World Aquatic Soci. 28, 297-300.

Martins, M.L., Souza, V.N., Moraes, J.R.E., Moraes, F.R., Costa, A.J., 1999. Comparative evaluation of the suscetibility of cultivated fishes to the natural infection with myxosporean parasites and tissue changes in the host. Rev. Bras. Biol. 59, 263-269.

Martins, M.L., Onaka, E.M., 2006. *Henneguya garavelli* n. sp. and *Myxobolus peculiaris* n. sp. (Myxozoa: Myxobolidae) in the gills of *Cyphocharax nagelli* (Osteichthyes: Curimatidae) from Rio do Peixe Reservoir, São José do Rio Pardo, São Paulo, Brazil. Vet. Parasitol. 137, 253-261.

Milanin, T., Eiras, J.C., Arana, S., Maia, A.A.M., Alves, A.L., Monteiro, M.R.M., Carriero, M.M., Ceccarelli, P.S., Adriano, E.A., 2010. Phylogeny, ultrastructure, histopathology and prevalence of *Myxobolus oliveirai* sp. nov., a parasite of *Brycon hilarii* (Characidae) in the Pantanal wetland, Brazil. Mem. do Inst. Oswaldo Cruz, Rio de Janeiro. 105(6), 762-769.

Molnár, K., Békési, L., 1993. Description of a new *Myxobolus* species, *M. colossomatis* n. sp. from the teleost *Colossoma macropomum* of the Amazon River basin. J. Appl. Ichthyol. 9(1), 57‑63.

Mólnar, K., 1998. Taxonomic problems, seasonality and histopathology of *Henneguya creplini* (Myxosporea) infection of the pikeperch *Stizostedion lucioperca* in Lake Balaton. Folia Parasitol. 45, 261-269.

Molnár, K., Eszterbauer, E., Székely, C., Dán, Á. and Harrach, B., 2002. Morphological and molecular biological studies of intramuscular *Myxobolus* spp. of cyprinid fish. J. Fish Dis. 25, 643–652.

Molnár, K., Marton, S.Z., Eszterbauer, E., Székely, C., 2007. Description of *Myxobolus gayerae* sp. n. and redescription of *Myxobolus leuciscini* infecting europeanchub from the Hungarian stretch of the river Danube. Dis. Aquatic Organ. 78, 147-153.

Molnár, K., Cech, G., Székely, C., 2008. Infection of the heart of the common bream, *Abramis brama* (L), with *Myxobolus s.l. dogieli* (Myxozoa, Myxobolidae). J. Fish Dis. 31, 613-620.

Naldoni, J., Arana, S., Maia, A.A.M., Silva, M.R.M., Carriero, M.M., Ceccarelli, P.S., Tavares, E.L.R., Adriano, E.A., 2011. Host-parasite environment relationship, morphology and molecular analyses of *Henneguya eirasi* n. sp. parasite of two wild *Pseudoplatystoma* spp. in Pantanal wetland, Brazil. Vet. Parasitol. 177, 247-255.

Reimschuessel, R., 2008. General Fish Histopathology. In: Eiras, J.; Segner, H.; Wahli, T & Kapoor, B.G., (eds.). Fish Diseases, Volume 1. Science Publishers, Enfield (NH), Jersey, Plymouth. 612pp.

SAS Institute Incorporation, 2002-2003. Statistical Analysis System. Release 9.1. (Software). Cary. USA.

Schaefer, S.A., 1998. Conflict and resolution: impact of new taxa on phylogenetic studies of the neotropical cascudinhos (Siluroidea: Loricariidae). In: L.R. Malabarba, R.E. Reis, R.P. Vari, Z.M.S. Lucena, C.A.S. Lucena (eds). Phylogeny and Classification of Neotropical Fishes. EDIPUCRS, Porto Alegre. pp. 375-400.

Smothers, J.F., Von Dohlen, C.D. Smith Jr, L.H., Spall, R.D., 1994. Molecular evidence that the myxozoan protists are metazoans. Science. 265, 1719-1721.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. Mega5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance and Parsimony methods. Mol. Biol. Evo. 28(10), 2731-2739.

Thatcher, V. 2006. Amazon Fish Parasites. Vol. 1. Second Edition. Pensoft ed, Sofia, Moscow. 508pp.

Uspenskaya, A.V., 1982. New data on the life cycle and biology of myxosporida. Archiv. Protist. 126, 309-338.

Urbinati, E.C., Gonçalves, F.D., 2005. Pacu (*Piaractus mesopotamicus*). In: B. Baldisserotto, L.C. Gomes (eds.). Espécies nativas para a piscicultura no Brasil. UFSM, Santa Maria. pp.470.

Vita, P., Corral, L., Matos, E., Azevedo, C., 2003. Ultrastructural aspects of the myxosporean *Henneguya astyanax* n. sp. (Myxozoa: Myxobolidae), a parasite of the Amazonian teleost *Astyanax keithi* (Characidae). Dis. Aquatic Organ. 53, 55-60.

(Segundo padrão da Revista *Veterinary Parasitology*)

Capítulo 2

Morphological and Molecular Analysis of *Anacanthorus penilabiatus* and *Mymarothecium viatorum* (Monogenea: Dactylogyridae) gill parasites from *Piaractus mesopotamicus* (Characidae: Serrasalminae) collected from fish farms in São Paulo State, Brazil.

ABSTRACT

The dactylogyrid monogeneans *Anacanthorus penilabiatus* (Boeger, Husak & Martins, 1995) and *Mymarothecium viatorum* (Boeger, Piasecki & Sobecka, 2002), commonly found in large quantities in farmed *Piaractus mesopotamicus* (Holmberg, 1887) in Brazil, were collected from three fish farms located in the State of São Paulo. Morphological identification, scanning electron microscopy, histopathological data, prevalence and mean intesity of infection values and molecular approaches were performed. A total of 278 specimens of *P. mesopotamicus* were gathered from February 2008 and July 2010. Prevalence (P) and mean intensity of infection (MI) data was taken at each location for *A. penilabiatus*, *M. viatorum* and a Dactylogyridae sp1 (not identified). The data, counted all species together, showed prevalence and mean intensity annually, with numbers such as P= 91.2% and MI= 50 worms/fish (Pirassununga), P= 60% and MI= 39.2 worms/fish (Mogi Mirim), and P= 100% and MI= 204.8 worms/fish (Itapira). Histopathological results showed hyperplasia of the secondary lamellae, and hypersecretion of mucus was also observed. Scanning electron microscopy found that *Anacanthorus penilabiatus* presented a relatively smooth tegument and microvilli in the adhesive regions. *Mymarothecium viatorum* tegument showed the top of surface with variations in the form of projections similar to scales, with microvillus-like projections. The SEM method showed closely the morphology of anchors and the way the sclerotized material was disposed to the formation of the anchor. A phylogenetic position was inferred using complete SSU (18S) ribosomal rDNA, giving a position to *Anacanthorus penilabiatus* and *Mymarothecium viatorum* among other Monopisthocotyleans available in Genbank. A PCR-RFLP analysis using the LSU (28S) rDNA, was successfully performed to provide a rapid diagnostic evaluation of these species.

Keyword: Monogenea, *Anacanthorus penilabiatus*, *Mymarothecium viatorum*, phylogenetic position, PCR - RFLP.

1. INTRODUCTION

With the decline of stocks of ocean fisheries, aquaculture has been expanding in almost all regions of the globe (Nowak, 2007). Aquaculture in Brazil has shown rapid development over the past years, especially with an increase in intensive fish farming (Martins et al., 2002). The Brazilian government is investing heavily in this activity and the country has increased its consumption of fish from 6.46kg to 9.03kg per year (MPA, 2009).

The freshwater fish *Piaractus mepotamicus*, popularly known as "pacu", is an important species for aquaculture, and popular among consumers. Diseases caused by parasites in fish farms are a common problem. These diseases can result in considerable economic losses, so it is necessary to know which pathogens inhabit the environment, to perform a reliable diagnostic to prevent and control (Souza et al., 2000).

Identification of parasite pathogens at species level can be a complex and time-consuming task. In the face of a potential outbreak of a disease, the need for a fast and proper diagnostic process is essential (Shinn et al., 2010).

Monogenea (Platyhelminthes) are mainly fish parasites, mostly ectoparasites, and are hermaphroditic, with direct life-cycles (Boeger & Vianna, 2006). They can be found in fish in marine, brackish water and freshwater habitats (Buchmann & Bresciani, 2006). In South America the most abundant taxon is probably the Dactylogyridae family (Monopisthocotylea), however species belonging to the Gyrodactylidae family are also recorded, along with a few examples of species from the Diplectanidae, Monocotylidae and Hexabothriidae families (Boeger & Vianna, 2006).

Monogenean species present considerable variety between species, and some species present small variations, which require accurate diagnosis. Sometimes, morphology does not easily lend itself to routine use in a diagnostic laboratory (Huyse et al., 2007). Thus, molecular identification methods have mainly used the nuclear DNA encoding ribosomal RNA from both large and small subunits of monogeneans ribosomes (Mollaret et al., 2000; Olson & Littlewood,

2002; Matejusová & Cunningham, 2004) and also internal transcribed spacers (ITS) (Zietara et al., 2002; Huyse et al., 2003; Matejusová et al., 2003).

Molecular biological methods have become important diagnostic and taxonomic tools (Liu et al., 2011). Sequencing entire PCR products elucidates differences and variations between species and ribosomal DNA genes 18S and 28S and spacers have been found to be effective indicators due to intraspecific variation, which is very low (Buchmann & Bresciani, 2006). A fast molecular diagnostic tool that has been used is PCR-RFLP (Cunningham, 1997; Shinn et al., 2010), which uses restriction enzymes to cut fragments from the 28S and 18S PCR products. This technique can distinguish species in a short period of time.

The dactylogyrid monogeneans *Anacanthorus penilabiatus* and *Mymarothecium viatorum*, commonly found in high quantities in farmed *Piaractus mesopotamicus* fish in Brazil, represent a risk to productivity and can cause high fish mortality. In the present study a morphological, histopathological and molecular approach of the species *A. penilabiatus* and *M. viatorum* was carried out. Morphological identification, histopathology, scanning electron microscopy methods, parasitological data, phylogenetic position and PCR-RFLP were used.

2. MATERIAL AND METHODS

2.1- Sampling and Morphological Study

A total of 278 specimens of *P. mesopotamicus* (25.8 cm ±10.36 in length and 458.0 kg ± 543.1 in weight) were monthly collected between February 2008 and July 2010 from three different fish farms in the state of São Paulo. A total of 218 fish were collected from the Pirassununga fish farm (21°55'51.68"S, 47°22'29.01"W), 50 fish from Mogi Mirim (22°28'16.63"S, 47°00'40.47"W) and 10 fish from Itapira (22°26'10.54"S, 46°49'19.52"W). Fish were collected using nets and fishing rods, and following killing, the gills were immediately screened for

monogenean infections, using a stereomicroscope, following the hot water method described by Boeger & Vianna (2006).

For morphological identification, monogenean specimens were kept in diluted formaldehyde (5%) and mounted in Hoyer medium and Grey & Wess and stained in Gomori's Trichrome (Putz & Hoffman, 1963; Kritsky et al., 1995; Eiras et al., 2000). The keys for identification used were Boeger et al., (1995), Cohen & Kohn (2005), Kritsky et al., (1979,1992,1996). The measurements of the internal and sclerotized organs for identification were taken using a light microscope linked to a computer equipped with an Axioplan2 image program.

Prevalence and mean intensity data was calculated following Bush et al. (1997). These data was calculated annually. Statistical analysis was performed using SAS 9.1 software (SAS Institute Inc, Carry, NC, EUA). ANOVA (Analysis of Variance) and Duncan's Multiple Range Test were applied to compare the prevalence and mean intensity according to location, year with a $p \leq 0.05$ significance level. The prevalence and mean intensity from both species were not counted separately, so the numbers are from both species together.

For histological analysis, gill arches were stored in buffered formaldehyde 10% and later transferred to alcohol 70%. The material was subsequently dehydrated in crescent solutions of alcohol (70, 80, 90 and 100%) and clarified with xylol. The material was embedded in paraffin, cut on microtome (4μm) and stained in Hematoxylin and Eosin (Adriano et al., 2002). Permanent slides were made and analyzed using the light microscope.

2.2 - Scanning Electron Microscope (SEM)

Two procedures were used for the monogeneans. The first procedure was performed at the Helminthology Laboratory, Department of Animal Biology, UNICAMP and the second procedure was performed at the University of Hamburg, *Biozentrum Grindel und Zoologisches Museum Hamburg*.

In the first procedure specimens were fixed in glutaraldehyde 3% buffered Sodium cacodylate 0.1M buffered in pH 7.4. After fixation the material was washed several times in buffer and fixed in 1% osmium for one hour. The material was then dehydrated, using ethanol, to critical point, and following the preparation routine was scanned using an electron microscope (Jeol JMS 35 operated at 10 kV) (Adriano et al., 2002) from the Department of Microscopy, Biology Institute, UNICAMP.

The second procedure was performed with specimens already fixed in 100% ethanol, which were transferred, several times, to different amyl acetate dilutions in ethanol for 15 minutes each time, until the last phase, at amyl acetate at 100%, also for 15 minutes. The samples were transferred in amyl acetate into the critical point drying device where the amyl acetate at 5°C against CO^2 was exchanged. By heating the sample chamber to 40°C, the critical values were reached, and the CO^2 was then released slowly. After coating with carbon or gold in an Leybold – Heraeus PD170AZ evaporation unit the critical point-dried specimens were observed and photographed in a LEO 1525 scanning electron microscope (Carl Zeiss SMT) (Brandão, 2010).

2.3 - Molecular Study

The samples were collected according to Thatcher (2006) and kept in 96% (v/v) ethanol at 5°C. For morphological identification, specimens were cut in half, with one part used as a voucher specimen for morphological identification (*Anacanthorus penilabiatus* the anterior part and *Mymarothecium viatorum* the posterior part) using the Hoyer medium, and the other part for DNA extraction.

For genomic DNA extraction, a total of 15 parasite parts were briefly air dried with a vacuum centrifuge to remove the ethanol, and the total genome was extracted using DNeasy® Blood & Tissue Kit (Qiagen) according to manufacturer's protocol, with final volume of 100µl.

Conventional PCR was performed with 25µl primary PCR amplifications, with 2µl DNA extract, 1.0µl each primer and Ready-to-Go PCR beads (Pure

Taq™Ready-to-Go™PCR beads, GE Healthcare), the solution consisted of stabilizers, BSA, dATP, dCTP, dGTP, dTTP, ~2.5 units of puReTaq DNA polymerase and reaction buffer. With the reconstituted bead to a final volume of 25 µl final, the concentration of each dNTP was 200 µM in 10 mM Tris-HCl, (pH 9.0 at room temperature), 50 mM KCl and 1.5 mM MgCl2. Cycling conditions were as follows: initial denaturation for 3 min at 94°C followed by 40 cycles of 30 sec at 94°C, 30 sec at 40° to 63°C, 2 min at 72°C and final extension at 10 min at 72°C.

The primers used for phylogenetic position analysis were ribosomal large subunit 28S and ribosomal small subunit 18S. For PCR - RFLP 28S rDNA was used as digestion fragment. The set of primers used are expressed in Table 1.

Table1: Primers used to amplify and sequence 28S and 18S fragments of *Anacanthorus penilabiatus* and *Mymarothecium viatorum*.

Primers Name		**Sequence (5' to 3') F - foward, R - reverse**	**Source**
gene18S rDNA			
wormA	F	GCGAATGGCTCATTAAATCAG	Littlewood & Olson (2001)
worm B	R	CTTGTTACGACTTTTACTTCC	Littlewood & Olson (2001)
300F	F	AGGGTTCGATTCCGGAG	Littlewood et al. (2008)
600R	R	ACCGCGGCKGGCTGGCACC	Littlewood et al. (2008)
930F	F	GCATGGAATAATGGAATAGG	Littlewood et al. (2008)
1270R	R	CCGTCAATTCCTTTAAGT	Littlewood & Olson (2001)
1200F	F	CAGGTCTGTGATGCCC	Littlewood & Olson (2001)
1200R	R	GGGCATCACAGACTTG	Littlewood & Olson (2001)
gene 28S rDNA			
ZX-1*	F	ACCCGCTGAATTTAAGCATAT	van der Auwera et al. (1994)
1500R	R	GCTATCCTGAGGGAAACTTCG	Littlewood et al. (2008)
ECD2	R	CCTTGGTCCGTGTTTCAAGACGGG	Littlewood *et al.* (2000)
900F	F	CCGTCTTGAAACACGGACCAAG	Littlewood *et al.* (2000)
1200F	F	CCCGAAAGATGGTGAACTATGC	Littlewood *et al.* (2000)
1200R	R	GCATAGTTCACCATCTTTCGG	Littlewood *et al.* (2000)

*Original ZX-1 (ACCCGCTGAAYTTAAGCATAT) Y was replaced with T.

PCR products were run on agarose gel using gel red and loading buffer, and purified using QIAquick PCR Purification Kit (Qiagen). Automated sequencing was performed directly on purified PCR products using ABI Big Dye chemistry following the manufacturer's protocols for cycle sequencing. Sequences were alcohol precipitated and run on an ABI prism 377 automated

sequencer. The sequence identity was verified using the Basic Local Alignment Search Tool (BLAST) and sequences were assembled and edited using Sequencher v. 4.10.1 (Gene Codes, Ann Arbor, MI).

Sequences were aligned using Clustal X program (Larkin et al. 2007) and adjustments were made by eye using MacClade (Maddison & Maddison, 2000).

For phylogenetic analyses, was conducted the SSU 18S rDNA data set in an analysis containing 37 ingroup taxa from the Dactylogyridae family and two outgroup taxa belonging to the Protogyrodactylidae family, available from Genbank. The method used was Maximum Likelihood (GTR+I+G; PhyML) using Geneious v5.4 (Drummond et al. 2011).

PCR - RFLP (Restriction Fragment Length Polymorfism) analysis was carried out using LSU 28S fragment following (Shinn et al. 2010). Both sequences, *A. penilabiatus* and *M. viatorum*, were used for enzyme cut analysis on MacVector with Assembler 12.0.6 (MacVector Inc.). Five microliter aliquots of PCR product were digested with restriction enzyme HindiIII (Invitrogen) in the incubator at 37°C for 2 to 3 hours. For the digestion reaction the following was used: 2 µl of reaction buffer (Invitrogen), 2 µl of HindiIII, 5 µl of PCR product and 11 µl of H2O, total volume 20 µl. The digested fragments were analyzed in electrophoresis with 2 µl loading buffer and gel red, 1 µl laedder and 20 µl RFLP product.

3. RESULTS

Two dactylogyrid species (*A. penilabiatus* and *M. viatorum*) were identified in all three locations. In Pirassununga, a dactylogyrid species was also observed and counted together with the others species, but not yet identified. Morphology and measurements of the specimens of *Anacanthorus penilabiatus* and *Mymarothecium viatorum* from *Piaractus mesopotamicus* are in agreement with original description from each species. *Mymarothecium viatorum* was identified by having a posteromedial projection on the bars and the structure of the copulatory complex agreed with the original description. *Anacanthorus*

penilabiatus was identified by the copulatory organ morphology, such as, the longer "lip" on the distal aperture of the copulatory organ, by a median longitudinal flap on the copulatory organ and a flattened spathulated distal portion of the acessory piece.

As regards annual prevalence, in the Pirassununga fish farm, prevalence was high during the study period. The highest prevalence was found in 2009, followed by 2010, with the lowest percentage in 2008 (Table 2). Mean intensity per year at Pirassununga was the highest in 2009, (68.2 worms/fish), followed by 2010 (41.5 worms/fish), and finally in 2008 (31.8 worms/fish). ANOVA statistical analysis showed significant results regarding prevalence versus location and year, and also mean intensity versus year ($p<0.05$). The Duncan test also showed that Pirassununga had the highest prevalence and mean intensity by the monogeneans compared to the other fish farms. Also, the year 2009 showed significant overall data for prevalence (Duncan values - 76.2 for 2009; 30.6 for 2010 and 23.2 in 2008) and mean intensity (Duncan values - 56.1 for 2009; 16.4 for 2010 and 8.7 in 2008) in relation to the years between 2008 and 2010.

At Mogi Mirim fish farm, prevalence was 100% in 2009, declining to 33.4% in 2010. Mogi Mirim had 44.0 worms/fish in 2009, which decreased to 28.4 worms/fish in 2010 (Table 2). Significantly different results were found for prevalence/mean intensity for this fish farm in 2009, and higher numbers were found for this year. The sample gathering at Itapira fish farm was carried out just once during spring 2009. Prevalence was 100%, and the mean intensity was 204.8 worms/fish, varying from 35.0 to 546.0 worms/fish.

Several gills were analyzed for histopathology, therefore the following results are from fish with high level of infections. Histological analysis indicated the haptor penetrating into the basement membrane and connective tissue of the lamella (Fig. 1A, 1B). The attachment of the worm to the host's gill epithelium is

ensured by the anchors (*M. viatorum*) and hooks (*A. penilabiatus*). A depression on the gill filament caused by the attachment was observed, showing the attachment zone of the worm (Fig. 1E). Also observed was hyperplasia of secondary lamellae (Fig. 1C, 1D e 1E) and hypersecretion of mucus in the gills (not shown). Hyperplasia results in cellular proliferation, causing fusion of the lamellae.

The scanning electron microscopy tool (SEM) for external morphological study of monogeneans presented some obstacles. The procedures used apparently need some modification to achieve better results, and this was not possible for this study. Some problems appeared during the process and some specimens were lost during the technique. However, positive results were ultimately achieved.

This technique easily differentiated between the two genera, *A. penilabiatus* and *M. viatorum*, with both having different external morphology.

Anacanthorus penilabiatus has a symmetrical elongated body with a posterior haptor (Fig. 2A). The tegument has slight folding along the body. The bucal cavity and the opening of the genital atrium lie in the anterior part of the ventral surface. Adesive glands could be clearly observed at the anterior end, six in total, three on each side of the anterior part, containing several surrounding microvilli structures and three unequal openings (Fig. 2B and 2C). The genital atrium presents a tegument with ripples characterizing a proper aperture (Fig. 2D). The haptor apparently shows distended muscles at the peduncle portion, followed by the posterior part visibly bilobed, symmetrical and with possible retractile hooks (Fig. 2E and 2F).

Mymarothecium viatorum is smaller than *A. penilabiatus*, and also has an elongated body (Fig. 3A). The tegument showed interesting scale annulation projections all over the body (Fig. 3D) and some specimens showed smooth parts and no visible scales. Also in the tegument some pores could be observed.

The haptor, symmetrical, showed hooks and similar anchors. The SEM method showed closely the morphology of the anchors and the way the sclerotized material was disposed by the formation of the anchor (Fig. 3E and 3F). The hooks and anchors possess a tegument like a membrane surrounding these structures and this characteristic suggests that it might be retractile. The attachment is between the secondary lamellae (Fig. 3A and 3B). Figure 3C shows the exact site of the attachment and where the haptor was found.

The fragments amplified by complete SSU rDNA (18S) and partial LSU rDNA (28S) molecular markers for both species, *A. penilabiatus* accession number (which will be deposited after article submission) and *M. viatorum* accession number (which will be deposited after article submission), could successfully diagnose the species, and distinguish then. The sequences for partial 28S *A. penilabiatus* presented a length of approximately 1235bp and *M. viatorum* a length of 1212bp. Complete 18S sequences had a length of approximately 2240bp for *A. penilabiatus* and 2000bp for *M. viatorum*.

For phylogenetic analysis, Genbank holds various sequences for dactylogyrids, but few freshwater, or South American representatives, have been characterized. With the sequencing of complete 18S rRNA gene of *A. penilabiatus* and *M. viatorum,* it was possible to establish the phylogenetic relationship of these two species with other sequences available in GenBank.

Complete SSU rDNA (18S), were aligned across all 37 dactylogyrids sequences available on GenBank. Maximum likelihood analysis (GTR+I+G; PhyML; using Geneious) (Drummond et al. 2011) yielded a tree with reasonable nodal support, with *A. penilabiatus* and *M. viatorum* grouping together, as a sister taxon of the clade composed by the other Dactylogyridae spp. (Fig. 4).

The PCR-RFLP (Restriction Fragment Length Polymorfism) method with 28S gene successfully distinguished *A. penilabiatus* and *M. viatorum.* Eletrophoresis separated the digested fragments in 2 bands (one cut) for *A.*

penilabiatus with sizes approximately 380bp and 820bp and fragments and 3 bands (two cuts) for *M. viatorum* with sizes approximately 226bp, 394bp and 580bp. (Fig. 5).

Table 2: Prevalence and mean intensity of monogeneans *Anacanthorus penilabiatus* and *Mymarothecium viatorum* from the fish farms studied during February 2008 to July 2010. Different letters indicate significant difference among samples along the years, for Pirassununga and Mogi Mirim fish farms ($p<0.05$).

Parameters	**Pirassununga**				**Mogi Mirim**			**Itapira**
Host's Total Number	2008 n= 94	2009 n= 90	2010 n=34	total n=218	2009 n=20	2010 n=30	total n=50	2009 n=10
Prevalence %	85.1 b	98.8 a	94.1 b	91.2	100 a	33.4 b	60	100
Mean Intensity	31.8 b	68.2 a	41.5 b	50.0	44.0 a	28.4 b	39.2	204.8
Range	1 to 172	1 to 309	2 to 153		5 to 207	3 to 92		35 to 546

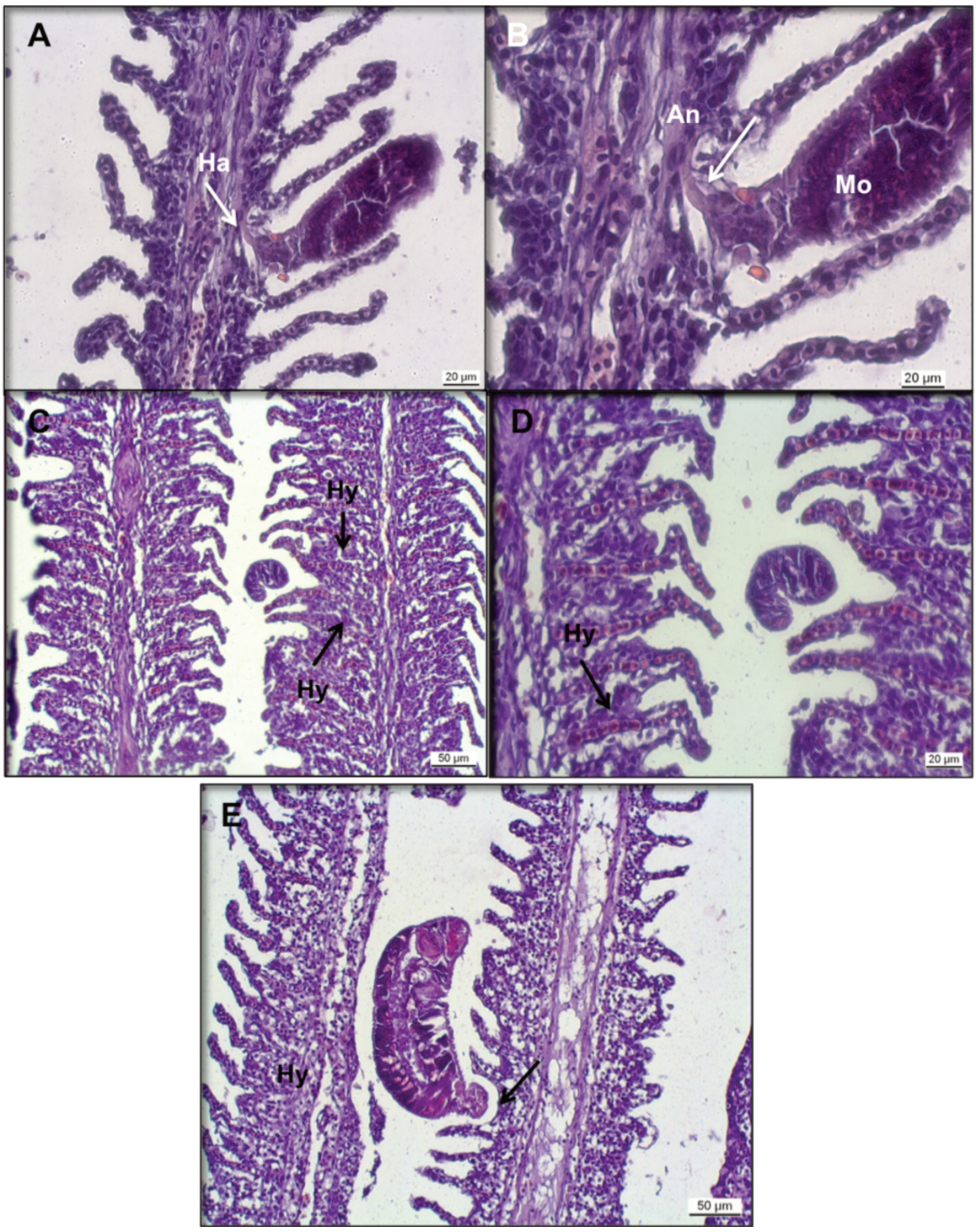

Figure 1: Histological longitudinal sections of monogeneans parasitizing gills of *Piaractus mesopotamicus*. (A and B) *Mymarothecium viatorum* haptor's attachment in the epithelium of gill lamellae (Ha) haptor (Ha), anchor (An), monogenean (Mo); (C and D) Highly infection causing hyperplasia (Hy); (E) *Anacanthorus penilabiatus* causing depression at the worm's attachment zone (black arrow).

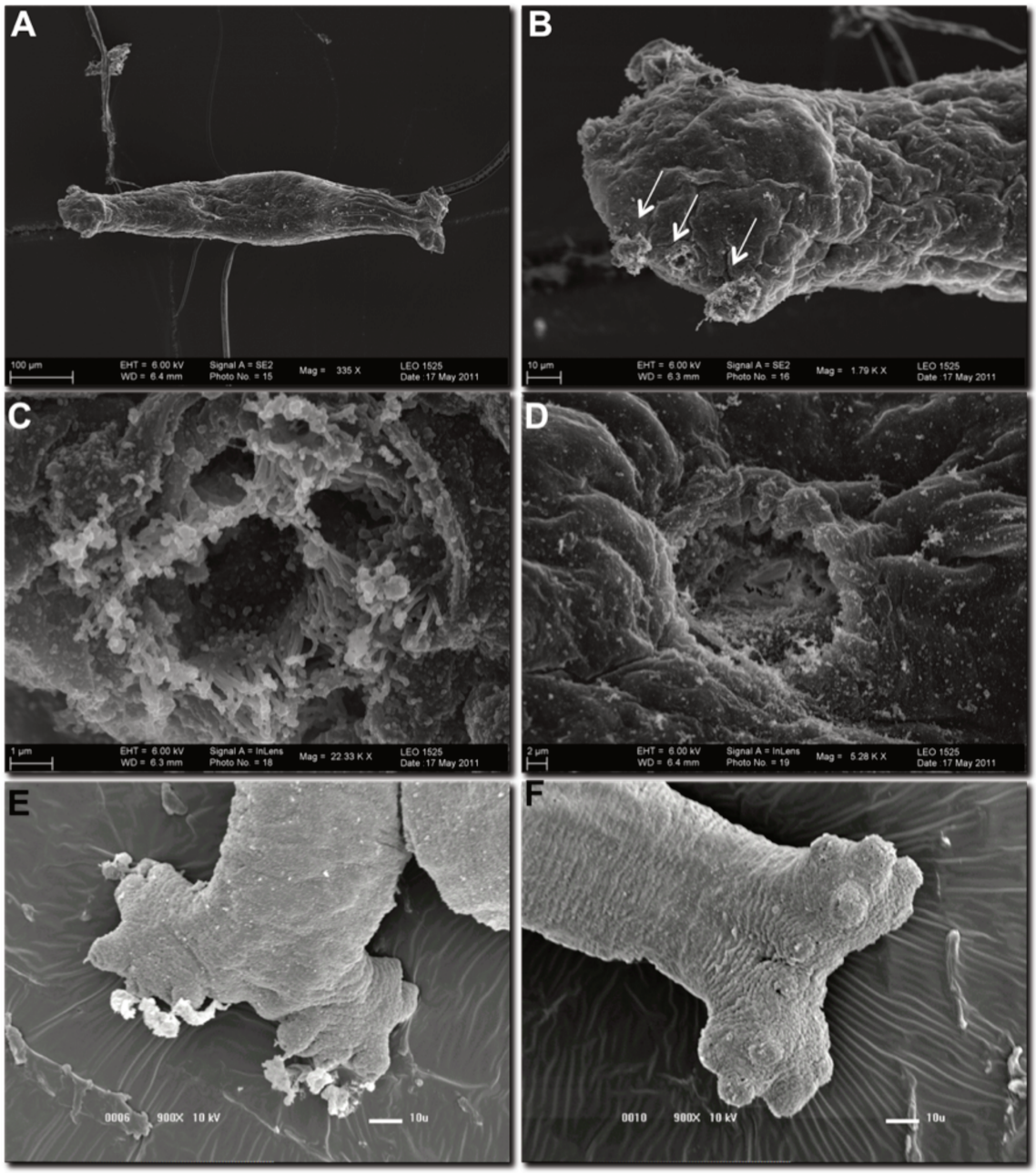

Figure 2: *Anacanthorus penilabiatus* electron micrographs showing external morphological features. The micrographs A, B, C and D are from the second protocol and E and F pictures are from the first used protocol. (A) *Anacanthorus penilabiatus* body morphology. (B) closer view at the anterior region, highlighting the 3 adhesive glands (white arrow). (C) the adhesive gland, closer magnification, showing the microvillus surrounding the 3 openings. (D) genital pore, (E and F) the haptor morphology, (E) dorsal view and (F) ventral view.

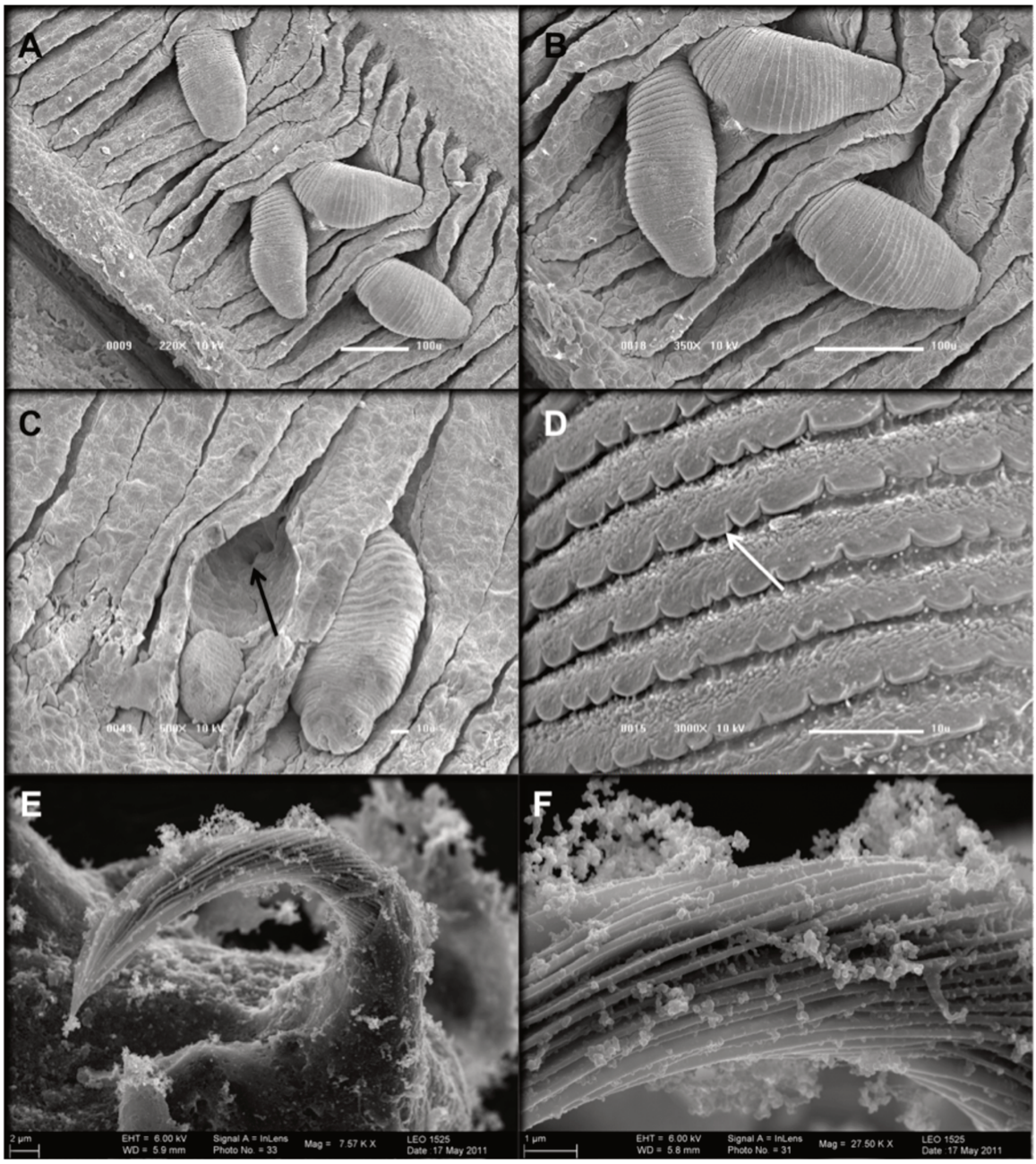

Figure 3: *Mymarothecium viatorum* electron micrographs showing external morphological features. The micrographs A, B, C and D are from the first protocol and E and F pictures are from the second protocol used. (A and B) shows the way monogeneans are inserted in gill filament. (C) foci where the parasite used to be placed in and the damage caused by the hooks (black arrow). (D) specialized tegument of the *Mymarothecium viatorum*. (E and F) the anchor morphology with a closer magnification showing the structure of the sclerotized material.

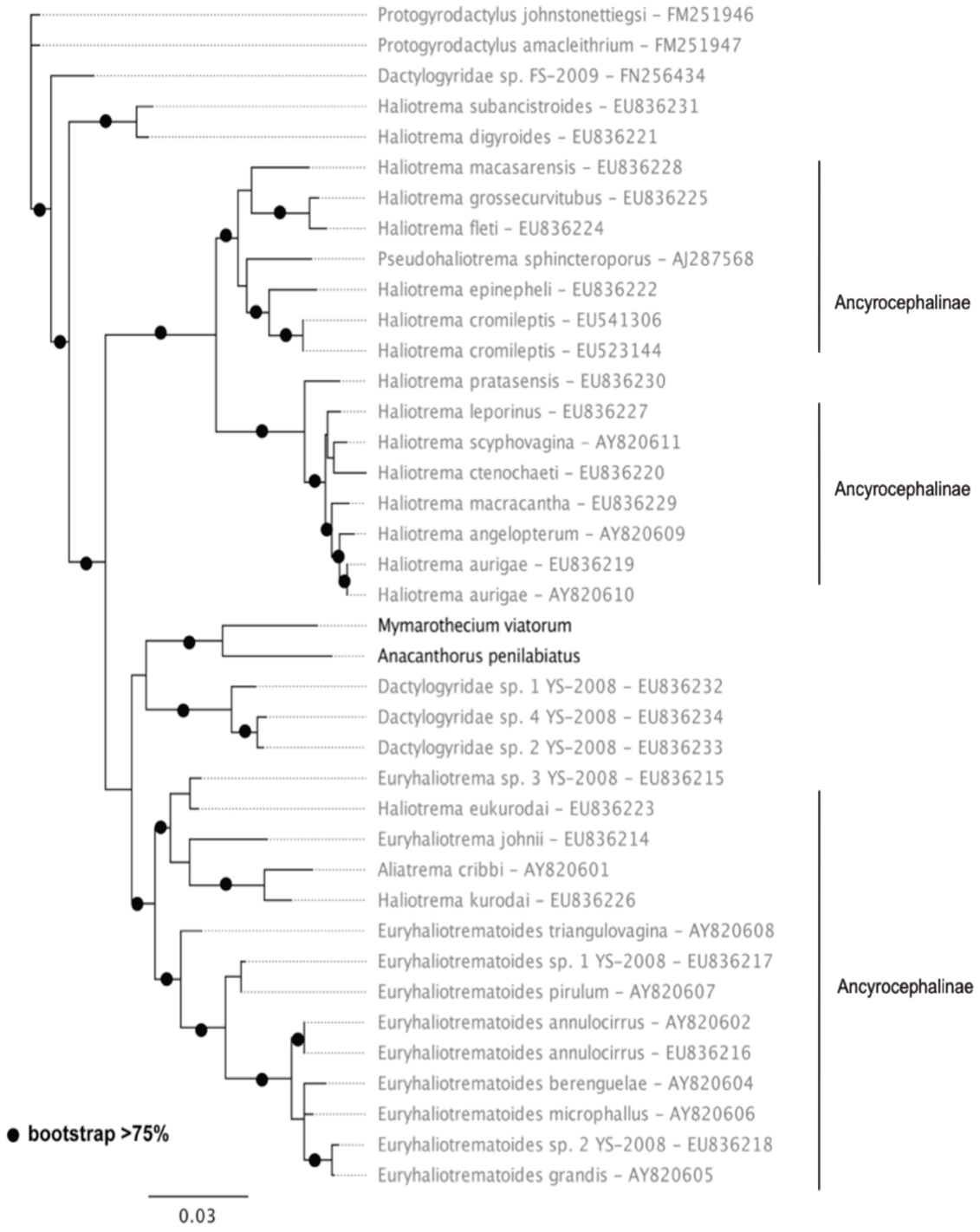

Figure 4: Phylogenetic position of *Anacanthorus penilabiatus* and *Mymarothecium viatorum* among available dactylogyrids 18S rDNA sequences, using Maximum Likelihood.

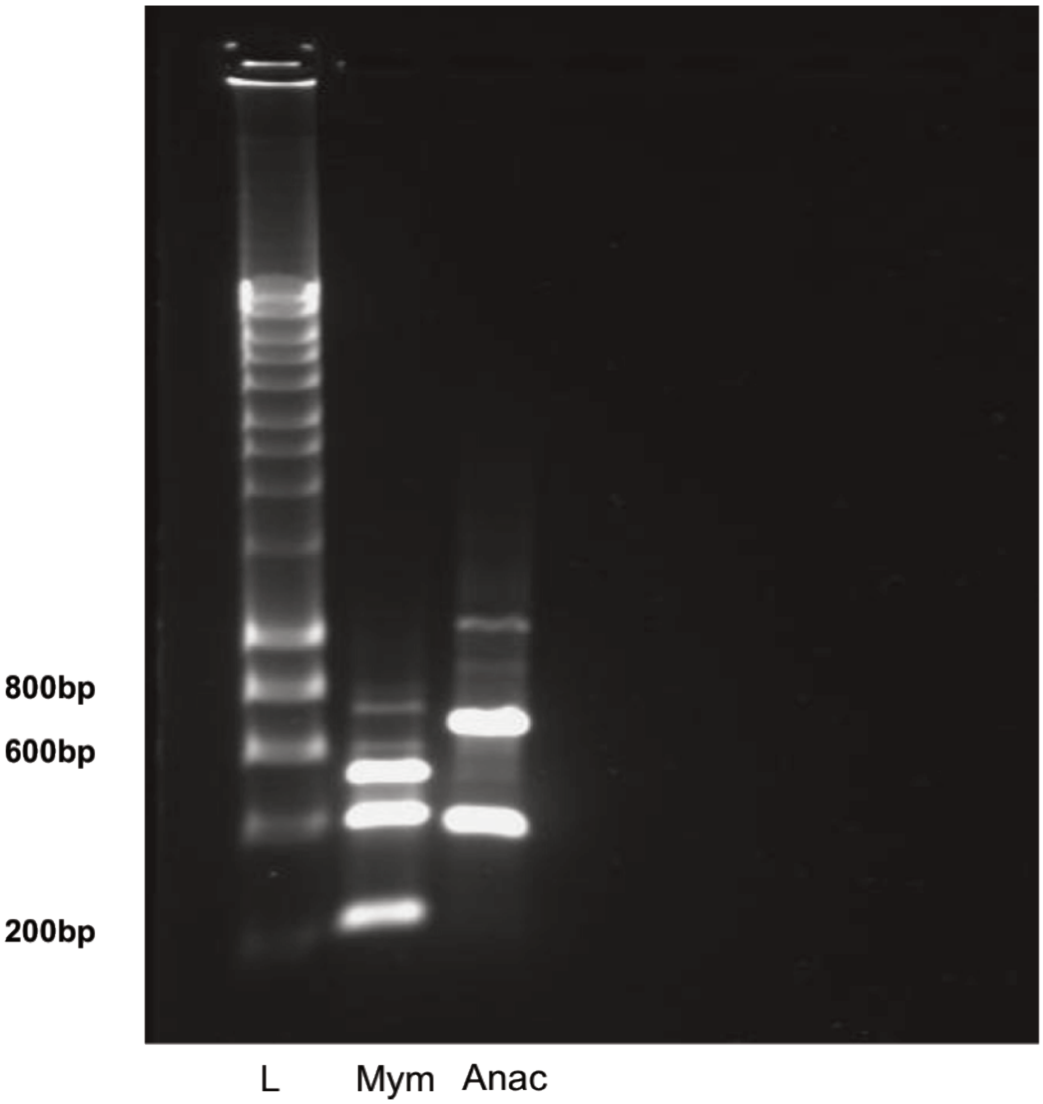

Figure 5: PCR-RFLP method from the 28S partial fragment from *Anacanthorus penilabiatus* (2 bands) (Anac) and *Mymarothecium viatorum* (3 bands) (Mym) digested with HindIII enzyme on agarose gel stained in gel red, (L) Ladder. Secondary bands are non-digested PCRproduct.

4. DISCUSSION

The parameters of prevalence and intensity, traditionally used to quantify parasite populations or severity of infection, are also a subject of variation (Poulin, 2006). The majority of the results presented a small degree of variation regarding prevalence and mean intensity during the study group. At the Mogi Mirim fish farm, two years were sampled. In the first year, 2009, prevalence was 100% and mean intensity was 44.0 worms/fish. The considerable decrease in prevalence and mean intensity in 2010 is probably related to the treatment performed by the owner of this fish farm, who used medication to treat fish disease.

In Itapira the high numbers were related to an outbreak during spring 2009, when fish were heavily infected and died. Outbreaks during changing seasons are often reported at fish farms, especially if the environmental conditions, such as changes in the aquatic parameters, climate stress, introduction of pathogens, are not favorable, as all are factors that can increase the host´s susceptibility to parasites and provoke an imbalance of the host/parasite/environment system (Coutant, 1998).

Branchial lesions are particularly important, as these organs react to the presence of parasites by hyperplasia from the epithelium cells and with an increase of mucous production, which causes damage among gas and ionic change functions (Thatcher & Brites-Neto, 1994; Martins et al., 1999; Tavares-Dias et al., 2001).

Hyperplasia response can be observed due to poor water quality, toxicant exposures, nutritional deficiencies and parasitic infections (Reimschuessel, 2008). As observed in this study, the occurrence of severe epithelial hyperplasia of gills was also detected during the attachment of the dactylogyrids in *Cyprinus carpio* (Paperna, 1996) and the cultured Australian dhufish, *Glaucosoma hebraicum* (Pironet & Jones, 2000; Kritsky & Stephens, 2001).

This study showed hypersecretion of mucus in the gills, presumably a defense response to the parasites. Haaparanta et al. (1997) also observed an increase in mucus formation on secondary lamellae in perch caused by monogeneans. Buchmann (1999) states that infection by monogeneans can stimulate mucus production in the host as a protective mechanism in response to parasitism. However, the production of excess mucus can cause hypoxia and induce gill dysfunction in fish (Monteiro et al., 2004; Chavez et al., 2006). This may lead to death in severe infestations. Damaged areas caused by parasites can facilitate the entry or establishment of secondary infections by fungi, viruses and bacteria (Martins & Romero, 1996).

The scanning electron microscope photographs showed that *A. penilabiatus* and *M. viatorum* were distinguished, and external morphology were observed.

The tegument of the monogeneans is an outer lining of a syncytial tegument composed by an outer nucleated layer and a basal nucleated layer in the parenchyma (Lyons, 1970; El-Naggar & Kearn, 1983b). The outer tegumental plasma membrane is covered by a glycocalyx showing positive reactions to various carbohydrates (Buchmann & Bresciani, 2006). Although the tegument of many species is smooth, some possess microvilli in adhesive regions such as cephalic openings, as in *Gyrodactylus* spp. (Buchmann and Bresciani, 1999). *Anacanthorus penilabiatus* presented a relatively smooth tegument and microvilli in the adhesive regions, similar to the *Gyrodactylus* species.

Mymarothecium viatorum tegument showed the top of surface with some variations such as projections similar to scales, with microvillus-like projections. Some monogenean species present projections on the surface area of the tegument, such as *Paranaella luquei* (Cohen et al., 2001), *Metamicrocotyla macracantha*, and also Microcotylidae (Kohn et al., 1994) and *Gyrodactylus* spp. (Buchmann & Bresciani, 1999). The ridges and microvillus-like projections present in the tegument increase the surface area, suggesting metabolic exchange and absorption of micro molecular nutrients from the surrounding environment (Olivier, 1981; Ramasamy & Hanna, 1986).

The tegument seems to be quite active and may possess osmoregulatory and excretory functions (Buchmann & Bresciani, 2006). Some authors as Halton (1978) and Cohen et al. (2001) suggest that the occurrence of microvilli and the absorptive function of the tegument provide evidence of a close phylogenetic relationship to the Cestoda group.

The anterior glands observed in *A. penilabiatus*, may participate in the attachment activity of the worm in the host gills. The forward part of the monogeneans plays an important role in attachment, movement, feeding and reproduction (Whittington et al., 2000). Although a limited number of studies regarding this subject have been conducted, the gland cells function in the cephalic region is assumed to play a crucial role in attachment and communication with the host (El-Naggar & Kearn, 1980; Whittington et al., 2000).

Many studies on the posterior attachment organ (haptor) of monogeneans from fishes are available, but on the other hand little information regarding methods of temporary attachment by the anterior end of monogeneans is available. There are some studies about the ultrastructure of anterior secretions, such as those by Kritsky (1978) and El-Naggar & Kearn (1980) for *Gyrodactylus eucaliae* (Gyrodactylidae) and 2 species of *Dactylogyrus* (Dactylogyridae), El-Naggar & Kearn (1983a) and Rees & Kearn (1984) for *Entobdella soleae* (Capsalidae) and *Acanthocotyle lobianchi* (Acanthocotylidae) respectively, and Cribb et al. (1997) which studied the anterior adhesive glands, and other glands in *Monocotyle spiremae* (Monocotylidae).

In respect of molecular studies, the genes18S and 28S ribosomal markers could successfully diagnose between these two genera by looking at interspecific genetic variation. These genes have been shown to provide excellent tools for phylogenetic studies and for diagnostic purposes (Buchmann & Bresciani, 2006). Other studies have been reported using the ribosomal genes 18S and 28S for diagnostics and molecular phylogeny from monogeneans (Lockyer et al., 2003; Mendlová et al., 2010; Shinn et al., 2010; Simková et al., 2007; Wu et al., 2007).

The RFLP method successfully differentiated both species, agreeing with Shinn et al. (2010), where ITS RFLP was used to diagnose *Gyrodactylus*

species. Also this method showed applicability, and the results are done based on a brief time period. However the chosen amplified sequence has to be previously known. Several enzymes can be used. The MacVector program gave a list of more than 70 enzyme possibilities and the selection criteria was the most common and cheapest enzyme.

The enzyme Hindi III differentiated between *A. penilabiatus* and *M. viatorum*, however it might not work as well for other similar species, so the choice of enzyme and molecular marker choice is important, and should be tested first.

The 18S complete sequences provided a phylogenetic position of the South American monogeneans *A. penilabiatus* and *M. viatorum* among dactylogyrids sequences available in Genbank. Both species are sister taxa with a bootstrap bigger than 75% and form a clade of South American species. However, the data is combined with species from other continents, such as Asia and Europe, and no species from South America. Little molecular data is available for monogeneans in South America. The Dactylogyridae is overwhelmingly the most abundant taxon in the continental waters of South America (Boeger & Vianna, 2006), but no molecular studies are currently available.

The Dactylogyridae is constituted of 9 subfamilies, i.e. the Dactylogyrinae, Ancyrocephalinae, Linguadactylinae, Linguadactyloidinae, Haerocephalinae, Heterotesiinae, Ancylodiscoidinae, Pseudodactylogyrinae and Anacanthorinae (Kritsky & Boeger, 1989). Phylogenetic relationships among families and subfamilies within Dactylogyrinea Bychowsky, 1937 remain unresolved (Kritsky & Boeger, 1989; Lim, 1998; Simková et al., 2003). For example, the unresolved Ancyrocephalidae terminology *sensu* Bychowsky & Nagibina (1978) and Ancyrocephalinae *sensu* Kritsky & Boeger (1989) have been examined in several published phylogenetic studies such as Klassen (1994), Lim (1998), Mollaret et al., (2000), Simková et al., (2003), Plaisance et al., (2004, 2005). The phylogenetic position of the Brazilian dactylogyrid species *M. viatorum* (Ancyrocephalinae) and *A. penilabiatus* (Anacanthorinae) are placed between

Ancyrocephalinae clades. These clades are most represented by *Haliotrema* genera and *Euryhaliotrematoides* genera. The clade taxa represented by Dactylogyridae (1, 2, 3 and 4) YS 2008, are unpublished and unresolved taxa.

Haliotrema is a taxonomic group including more than 100 species, which exhibit different morphologies and parasitize a large number of hosts with a wide range of ecology and morphology (Plaisance et al., 2004). The *Haliotrema* group is suggested to be a polyphyletic taxon, however the validity of each species group or genus and their relationships remains unresolved (Wu et al., 2006). The *Euryhaliotrematoides* genus is only found on fish from the Chaetodontidae family and is suggested as a monophyletic genus (Plaisence et al., 2005).

In the context of Dactylogyridae phylogenetic relationships, clearly several questions must be answered, and morphology, together with molecular tools will be beneficial to assess relationships within and between subfamilies (Wu et al., 2006). In the case of *A. penilabiatus*, an Anacanthorinae species, and *M. viatorum* an Ancyrocephalinae species, both are placed together within Ancyrocephalinae clades in which most of the species belong to other continents. Probably these species (*A. penilabiatus* and *M. viatorum*) would be apart, placed with other more closely related species, and/or other clades. Therefore, a wider range of taxa from South America should be sampled and more DNA markers displaying various evolutionary rates should be tested to study the phylogenetic relationships between species, and within subfamilies of the South American Dactylogyridae.

In conclusion this work presents a external morphological and histopathological analysis of the dactylogyrids *A. penilabiatus* and *M. viatorum*, from *P. mesopotamicus*, an important fish for aquaculture and also a useful molecular tool for diagnostics of monogeneans in South America. With the lack of molecular information regarding monogeneans in South America, hopefully this work will encourage others to solve phylogenetic questions about the Dactylogyridae.

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6. REFERENCES

Adriano, E.A., Arana, S., Ceccarelli, P.S., Cordeiro, N.S., 2002. Light and scanning microscopy of *Myxobolus porofilus* sp. n. (Myxosporea: Myxobolidae) infecting the visceral cavity of *Prochilodus lineatus* (Pisces: Characiformes: Prochilodontidae) cultivated in Brazil. Folia Parasitol. 49, 259-262.

Boeger, W., Husak, W.S., Martins, M.L., 1995. Neotropical Monogenoidea. 25. *Anacanthorus penilabiatus* n. sp. (Dactylogyridae, Anacanthorinae) from *Piaractus mesopotamicus* (Osteichthyes, Serrasalmidae), cultivated in the State of São Paulo, Brazil. Mem. do Inst. Oswaldo Cruz, Rio de Janeiro. 90(6), 699-701.

Boeger, W.A., Piasecki, W., Sobecka, E., 2002. Neotropical Monogenoidea. 44. *Mymarothecium viatorum* sp. n. (Ancyrocephalinae) from the gills of *Piaractus brachypomus* (Serrasalmidae, Teleostei) captured in a warm-water canal of a power plant in Szczecin, Poland. Acta Ichthyol. et Pisc. 32, 157-162.

Boeger, W.A., Vianna, R.T., 2006. Monogenoidea. In: Thatcher, V.E. *Amazon* Fish Parasites. Vol.1. Second Edition. Pensoft ed. pp.508.

Brandão, S., 2010. Macrocyprididae (Ostracoda) from the Southern Ocean: taxonomic revision, macroecological patterns, and biogeographical implications. Zool. J. Linn. Soci. 159, 567-672.

Buchmann, K., 1999. Immune mechanisms in fish skin against monogeneans - a model. Folia Parasitol. 46, 1-9.

Buchmann, K., Bresciani, J., 1999. Rainbow trout leukocyte activity: influence on the ectoparasitic monogenean *Gyrodactylus derjavini*. Dis. Aquatic Organ. 35, 13–22.

Buchmann, K., Bresciani, J., 2006. Monogenea (Filo Platyhelminthes). In: Woo, P.T.K. Fish Diseases and Disorders, Volume 1: Protozoa and Metazoan Infections Second Edition. University of Guelph, Canada. CABI. 801pp.

Bush, A.O., Lafferty, K.D., Lotz, J.M., Shostak, A.W., 1997. Parasitology meet ecology on its own terms: Margolis *et al*. revisited. J. Parasitol. 83 (4), 575-583.

Bychowsky, B.E., Nagibina, L.F., 1978. Revision of Ancyrocephalinae Bychowsky, 1937. Parazitol. Sbornik. 28, 5-15.

Chavez, L.S., Luvizzotto-Santos, R., Sampaio, L.A.N., Bianchini, A., Martinez, P.E., 2006. Immune adaptive response induced by *Bicotylophora trachinoti* (Monogenea: Diclidophoridae) infestation in pompano *Trachinotus marginatus* (Perciformes: Carangidae). Fish Shelf. Immunol. 21, 242-250.

Cohen, S.C., Kohn, A., Baptista-Farias, M.F.D., 2001. Scanning and transmission eletron microscopy of the tegument of *Paranaella luquei* Kohn, Baptista-Farias & Cohen, 2000 (Microcotylidae, Monogenea), parasite of a brazilian catfish, *Hypostomus regani*. Mem. Inst. Oswaldo Cruz*,* Rio de Janeiro. 96(4), 555-560.

Cohen, S. C., Kohn, A., 2005. A new species of *Mymarothecium* and new host and geographical records for *M. viatorum* (Monogenea: Dactylogyridae), parasites of freshwater fishes in Brazil. Folia Parasitol. 52, 307-310.

Coutant, C.C., 1998. What is normative for fish pathogens? A perspective on the controversy over interactions between wild and cultured fish. J. Aquatic Ani. Health*,* Bethesda. 10, 101-106.

Cribb, B.W., Whittington I.D., Chisholm, L.A., 1997. Observations on ultrastructure of the anterior glands in the monogenean, *Monocotyle spiremae* (Monocotylidae), from the gills of *Himantura fai* (Dasyatididae). Int. J. Parasitol. 27, 907–917.

Cunningham, C.O., 1997. Species variation within the internal transcribed spacer (ITS) region of *Gyrodactylus* (Monogenea, Gyrodactylidae) ribosomal RNA genes. J. Parasitol. 83, 215–219.

Drummond, A.J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Duran, C., Field, M., Heled, J., Kearse, M., Markowitz, S., Moir, R., Stones-Havas, S.,

Sturrock, S., Thierer, T., Wilson, A., 2011. Geneious v5.4, Available from http://www.geneious.com/

Eiras, J. C., Takemoto, R. M., Pavanelli, G. C., 2000. Métodos de estudo e técnicas laboratoriais em parasitologia de peixes. Editora Universidade Estadual de Maringá, Maringá. 173p.

El-Naggar, M.M., Kearn, G.C., 1980. Ultrastructural observations on the anterior adhesive apparatus in the monogenean *Dactylogyrus amphibothrium* Wagener, 1857 and *D. hemiamphibothrium* Ergens, 1956. Zeits. Parasiten. 61, 223–241.

El-Naggar, M.M., Kearn, G.C., 1983a. Glands associated with the anterior adhesive areas and body margins in the skin-parasitic monogenean *Entobdella soleae.* Int. J. Parasitol. 13, 67–81.

El-Naggar, M.M., Kearn, G.C., 1983b. The tegument of the monogenean gill parasites *Dactylogyrus amphibothrium* and *D. hemiamphibothrium.* Int. J. Parasitol. 13, 579–592.

Haaparanta, A., Valtonen, E.T., Hoffmann, R.W., 1997. Gill anomalies of perch and roach from four lakes differinf in water quality. J. Fish Biol. 50, 575-591.

Halton, D.W., 1978. Trans-tegumental absorption of alfa-alanine and alfa-leucine by a monogenean *Diclidiophora merlangi*. Parasitol. 76, 29-37.

Huyse, T., Audenaert, V., Volckaert, F.A.M., 2003. Speciation and host-parasite relationships in the parasite genus *Gyrodactylus* (Monogenea, Platyhelminthes) infecting gobies of the genus *Pomatoschistus* (Gobiidae, Teleostei). Int. J. Parasitol. 33, 1679-1689.

Huyse, T., Plaisance, L., Webster, B.L., Mo, T.A., Bakke, T.A., Bachmann, L., Littlewood, D.T.J., 2007. The mitochondrial genome of *Gyrodactylus salaris* (Platyhelminthes: Monogenea), a pathogen of Atlantic salmon (*Salmo salar*). Parasitol. 134, 739-747.

Klassen, G.J., 1994. Phylogeny of *Haliotrema* species (Monogenea, Ancyrocephalidae) from boxfishes (Tetraodontiformes, Ostraciidae) - are

Haliotrema species from boxfishes monophyletic? J. Parasitol. 80, 596-610.

Kohn, A., Cohen, S.C., Baptista-Farias, M.F.D., 1994. A redescription of the morphology of *Metamicrocotyla macracantha* (Alexander, 1954) Koratha, 1955 (Monogenea, Microcotylidae) from *Mugil liza* in Brazil. Syst. Parasitol. 27, 127-132.

Kritsky, D.C., 1978. The cephalic glands and associated structures in *Gyrodactylus eucaliae* Ikezaki and Hoffman 1957 (Monogenea: Gyrodactylidae). Proc. Helminthol. Soc. Wash. 45, 37–49.

Kritsky, D.C., Thatcher, V.E., Kayton, R.J., 1979. Neotropical Monogenoidea 2. The Anacanthorinae Price, 1967, with the proposal of four new species of *Anacanthorus* Mizelle & Price, 1965, from Amazonian fishes. Acta Amazon. 9(2), 355-361.

Kritsky, D.C., Boeger, W.A., 1989. The phylogenetic status of the Ancyrocephalinae Bychowsky 1937 (Monogenea: Dactylogyridae). J. Parasitol. 75, 207-211.

Kritsky, D.C., Boeger, W.A., Van Every, L.R., 1992. Neotropical Monogenoidea. 17. *Anacanthorus* Mizelle & Price, 1965 (Dactylogyridae, Anacanthorinae) from Characoid Fishes of the Central Amazon. J. Helminthol. Soc. Wash. 59(1), 25-51.

Kritsky, D.C., Boeger, W.A., Popazoglo, F., 1995. Neotropical Monogenoidea. 22. Variation in *Scleroductus* species (Gyrodactylidea, Gyrodactylidae) from Siluriform fishes of southeastern Brazil. *Proc. Helminthol Soc. Wash.* 62(1), 53-56.

Kritsky, D.C., Boeger, W.A., Jégu, M., 1996. Neotropical Monogenoidea. 28. Ancyrocephalinae (Dactylogyridae) of piranha and their relatives (Telostei, Serrasalmidae) from Brazil and French Guiana: species of *Notozothecium* Boeger & Kritsky, 1988, and *Mymarothecium* gen.n. J. Helminthol. Soc. Wash. 63(2), 153-175.

Kritsky, D.S., Stephens, F., 2001. *Haliotrema abaddon* sp. n. (Monogenoidea: Dactylogyridae) from the gills of wild and maricultured West Australian

dhufish, *Glaucosoma hebraicum* (Teleostei: Glaucosomatidae), in Australia. J. Parasitol. 87, 749-754.

Larkin, M.A., Balckshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., MvWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Bioinformatics (Oxford, England). 23(21).

Lim, L.H.S., 1998. Diversity of monogeneans in Southeast Asia. Int. J. Parasitol. 28, 1495-1515.

Littlewood, D.T.J., Curini-Galletti, M., Herniou, E.A., 2000. The interrelationships of Proseriata (Platyhelminthes: Seriata) tested with molecules and morphology. Mol. Phylo. Evol. 16 449-466.

Littlewood, D.T.J., Olson, P.D., 2001. Small subunit rDNA and the phylum Platyhelminthes: signal, noise, conflict and compromise. In: D.T.J. Littlewood and R.A. Bray (eds.). Interrelationships of the Platyhelminthes. Taylor & Francis, London. pp. 262-278.

Littlewood, D.T.J., Waeschenbach, A., Nikolov, P.N., 2008. In search of mitochondrial markers for resolving the phylogeny of cyclophyllidean tapeworms (Platyhelminthes: Cestoda) – a test study with Davaineidae Parasitologica. 53**,** 133-144 .

Liu, Z., Gu, Z., Zhang, Y, Zeng, L., 2011. Redescription and molecular analysis of *Myxobolus shantungensis* Hu, 1965 (Myxozoa: Myxosporea) infecting common carp *Cyprinus carpio haematopterus.* Parasitol. Res. 109, 1619-1623.

Lockyer, A.E., Olson, P.D., Littlewood, D.T.J., 2003. Utility of complete large and small subunit rRNA genes in resolving the phylogeny of the Neodermata (Platyhelminthes): implications and a review of the cercomer theory. Biol. J. Linn. Soci. 78, 156-171.

Lyons, K.M., 1970. Fine structure of the outer epidermis of the viviparous monogenean *Gyrodactylus* sp. from the skin of *Gasterosteus aculeatus*. J. Parasitol. 56, 1110–1117.

Maddison, W.P., Maddison, D.R., 2000. MacClade. Sunderland, MA: Sinauer Associates.

Martins, M.L., Romero, N.G., 1996. Efectos del parasitismo sobre el tejido branquial en peces cultivados: estudio parasitologico e histologico. Rev. Bras. de Zool. 13(2), 489-500.

Martins, M.L., Souza, V.N., Moraes, J.R.E., Moraes, F.R., 1999. Gill infection of *Leporinus macrocephalus* Garavello e Britski, 1988 (Osteichthyes: Anostomidae) by *Henneguya leporinicola* n. sp. (Myxozoa: Myxobolidae). Description, histopathology and treatment. Rev. Bras. de Biol., São Carlos. 59(3), 527-534.

Martins, M.L., Onaka, E.M., Moraes, F. R., Bozzo, F.R., Paiva, A.M.F.C., Gonçalves, A., 2002. Recent studies on parasitic infections of freshwater cultivated fish in the state of São Paulo, Brazil. Acta Sci., Maringá. 24(4), 981-985.

Matejusová, I., Gelnar, M., Verneau, O., Cunnigham, C.O., Littlewood, D.T.J., 2003. Molecular phylogenetic analysis of the genus *Gyrodactylus* (Platyhelminthes: Monogenea) inferred from rDNA ITS region: subgenera versus species groups. Parasitol. 127, 603-611.

Matejusová, I., Cunningham, C.O., 2004. The first complete monogenean ribossomal RNA gene operon: sequence and secondary structure of the *Gyrodactylus salaris* Malmberg, 1957, large subunit ribosomal RNA gene. J. Parasitol. 90 (1), 146-151.

Mendlová, M., Pariselle, A., Vyshocilová, M., Simková, A., 2010. Molecular phylogeny of monogeneans parasitizing african freshwater Cichlidae inferred from LSU rDNA sequences. Parasitol. Res. 107, 1405-1413.

Ministério da Pesca e Aquicultura - MPA, 2009. *Boletim* Estatístico da Pesca e Aquicultura, Brasil 2008-2009. Ministério da Pesca e Aquicultura, Governo Federal. Brasília. 99p. Disponível em: http://www.mpa.gov.br/images/Docs/Publicidade/anuário%20da%20pesca%20completo2.pdf. Acesso em 21/12/2011.

Mollaret, I., Jamieson, B.G.M., Justine, J.-L., 2000. Phylogeny of the Monopisthocotylea and Polyopisthocotylea (Platyhelminthes) inferred from 28S rDNA sequences. Int. J. Parasitol. 30, 171–185.

Monteiro, F.E., Crespo, S., Padrós, F., De La Gándara F., Garcia, A., Raga, J.A., 2004. Effects of the gill parasite *Zeuxapta seriolae* (Monogenea: Heteraxinidae) on the amberjack *Seriola dumerili* Risso (Teleostei: Carangidae). Aquaculture. 232, 153-163.

Nowak, B.F., 2007. Parasitic diseases in marine cage culture. An example of experimental evolution of parasites? Int. J. Parasitol. 37, 581-588.

Olivier, G., 1981. Etude de *Microcotyle labracis* Van Beneden et Hesse, 1863 (Monogenea, Polyopisthocotylea, Microcotylidae) au microscope életronique á balayage. *Zeits. Parasiten*. 65, 235-240.

Olson, P.D., Littlewood, D.T.J., 2002. Phylogenetics of the monogenea – evidence from a medley of molecules. Int. J. Parasitol. 32, 233–244.

Paperna, L., 1996. Parasites, infections and diseases of fishes in Africa - An update CIFA, Technical Paper. N. 31, FAO, Rome, Italy. p. 220.

Pironet, F., Jones, J., 2000. Treatments for ectoparasites and diseases in captive Western Australia dhufish. Aquacul. Int. 8, 349-361.

Plaisance, L., Bouamer,S., Morand,S., 2004. Description and redescritption of *Haliotrema* species (Monogenoidea: Polyonchoinea: Dactylogyridae) parasitizing butterfly fishes (Teleostei: Chaetodontidae) in the Indo-West Pacific Ocean. Parasitol. Res. 93, 72-78.

Plaisance, L., Littlewood, D.T.J., Olson, P.D., Morand, S., 2005. Molecular phylogeny of gill monogeneans (Platyhelminthes, Monogenea, Dactylogyridae) and the colonization of Indo-West Pacific butterflyfish hosts (Perciformes, Chaetodontidae). Zool. Scripta. 34, 425-436.

Poulin, R., 2006. Variation in infection parameters among populations within parasite species: Intrinsic properties versus local factors. Int. J. Parasitol. 36, 877-885.

Putz, R. E., Hoffman, G. L., 1963. Two new *Gyrodactylus* (Trematoda, Monogenea) from cyprinid fishes with synopsis of those found on North American fishes. J. Parasitol. 49, 559-566.

Ramasamy, P., Hanna, R.E.B., 1986. The surface topography of a monogenean *Heterapta chorinemi* from the gills of *Scomberoides commersonianus*. Int. J. Parasitol. 16, 595-600.

Rees, J.A., Kearn, G.C., 1984. The anterior adhesive apparatus and an associated compound sense organ in the skin-parasitic monogenean *Acanthocotyle lobianchi*. Zeits. Parasiten. 70, 609–625.

Reimschuessel, R., 2008. General Fish Histopathology. In: Eiras, J.; Segner, H.; Wahli, T & Kapoor, B.G. Fish Diseases, Volume 1. Science Publishers, Enfield (NH), Jersey, Plymouth. 612pp.

SAS Institute Incorporation, 2002-2003. Statistical Analysis System. Release 9.1. (Software). Cary. USA.

Shinn, A.P., Collins, C., Garcia-Vásquez, A., Snow, M., Matejusová, I., Paladini, G., Longshaw, M., Lidenstrom, T., Stone, D.M., Turnbull, J.F., Picon-Camacho, P.M., Vásquez-Rivera, C., Duguid, R.A., Mo, T.A., Hansen, H., Olstad, K., Cable, J. Harris, P.D., Kerr, R., Graham, D., Monagham, S.J., Yoon, G.H., Buchmann, K., Taylor, N.G.H., Bakke, T.A., Raynard, R., Irving, S., Bron, J.E., 2010. Multi-centre testing validation of current protocols for the identification of *Gyrodactylus salaris* (Monogenea). Int. J. Parasitol. 40,1455-1467.

Simková, A., Plaisence, L., Matejusová, I., Morand, S., Verneau, O., 2003. Phylogenetic relationships of the Dactylogyridae Bychowsky, 1933 (Monogenea: Dactylogyridae): the need for the systematic revision of the Ancyrocephalinae Bychowsky, 1937. Syst. Parasitol. 54, 1-11.

Simková, A., Pecinková, M., Rehulková, E., Vyskocilová, M., Ondracková, M., 2007. *Dactylogyrus* species parasitizing european *Barbus* species: morphometric and molecular variability. Parasitol. 134, 1751-1765.

Souza, M.L.R., Martins, M.L., Santos, J.M., 2000. Microscopia eletrônica de varredura de parasitos branquiais de *Piaractus mesopotamicus*

Holmeberg, 1887 cultivados no Estado de São Paulo, Brasil. *Acta Scient.* 22(2), 527-531.

Tavares-Dias, M., Moraes, F.R., Martins, M.L., Kronka, S.N., 2001. Fauna parasitária de peixes oriundos de "pesque-pague" do município de Franca, São Paulo, Brasil. II. Metazoários. Rev. Bras. de Zool., Curitiba. 18(1), 81-95.

Thatcher, V.E., Brites-Neto, J., 1994. Diagnóstico, prevenção e tratamento das enfermidades de peixes neotropicais de água doce. Rev. Bras. de Med. Vet., Rio de Janeiro. 16(3), 111-128.

Thatcher, V. 2006. Amazon Fish Parasites. Vol.1. Second Edition. Pensoft ed. 508pp.

van der Auwera, G., Chapelle, S., de Wachter, R., 1994. Structure of the large ribosomal subunit RNA of *Phytophthora megasperma*, and phylogeny of the oomycetes. FEBS Letters. 338, 133–136.

Whittington, I.D., Cribb, B.W., Hamwood, T.E., Halliday, J.A., 2000. Host-specificity of monogenean (platyhelminth) parasites: a role for anterior adhesive areas? Int. J. Parasitol. 30, 305–320.

Wu, X.Y., Zhu, X.Q., Xie, M.Q., Li, A.X., 2006. The radiation of *Haliotrema* (Monogenea: Dactylogyridae: Ancyrocephalinae): molecular evidence and explanation inferred from LSU rDNA sequences. Parasitol. 132, 659-668.

Wu, X.Y., Zhu, X.Q., Xie, M.Q., Li, A.X., 2007. The evaluation for generic-level monophyly of Ancyrocephalinae (Monogenea, Dactylogyridae) using ribossomal DNA sequence data. Mol. Phylo. Evol. 44, 530-544.

Zietara, M.S., Huyse, T., Lumme, J., Volckaert, F.A., 2002. Deep divergence among subgenera of *Gyrodactylus* inferred from rDNA ITS region. Parasitol. 124, 39-52.

(Segundo padrão da Revista *Parasitology*)

Capítulo 3

Description of partial mitochondrial genome of *Anacanthorus penilabiatus* (Monogenea, Dactylogyridae) from *Piaractus mesopotamicus* (Characidae, Serrasalminae) and its use as molecular markers.

ABSTRACT

The objective of the present study was to describe the mitochondrial genome from the dactylogyrid *Anacanthorus penilabiatus* parasite of the freshwater characid *Piaractus mesopotamicus*, an important species for aquaculture in Brazil. A partial genome with length of 6547bp has been described, and gene order is similar to other monogeneans available in Genbank. The fragment comprises 4 complete protein-coding genes (cox2, nad6, nad5 and cox3), 1 partial protein-coding gene (cytb), 1 non-coding region with 3 tandem repeats, partial rrnL, complete rrnS and 9 tRNAs. There are differences in gene rearrangement compared to *Gyrodactylus* spp. and *Benedenia seriolae*. The overall nucleotide composition of all coding site is A (27.3%), C (10.9%), G (23.1%) and T (38.6%). The partial genome has an overall A+T content of 65.9%. Partial genome of *A. penilabiatus* presented a non-coding region of 357bp followed by 3 identical repeat regions of 186bp long. Among the monopisthocotyleans genomes available, *A. penilabiatus* is the first to show non-coding region with repetitive sequences. Two ribosomal genes have been sequenced for *A. penilabiatus*, partial sequence of 16S, rrnL, the large subunit and complete 12S, rrnS, small subunit. The total length of this fragment is 1262bp and between those two ribosomal genes a $tRNA^{CYS}$ has been annotated. Other two species successfully sequenced the same fragment, *Mymarothecium viatorum* and Dactylogyridae sp. (not yet identified). Sliding window analysis was performed for the *A. penilabiatus* genes and successfully displayed conserved and highly variable regions for possible molecular markers design to solve future diagnostics, phylogenetics and population studies for dactylogyrids. This is the first study of the dactylogyrid mitochondrial genome.

Keywords: *Piaractus mesopotamicus*, Monogenea, *Anacanthorus penilabiatus*, Dactylogyridae, mitochondrial genome.

1. INTRODUCTION

Piaractus mesopotamicus (Characidae, Serrasalminae) is a freshwater and migratory fish species from the Paraná - Paraguay River Basin, Brazil. Popularly known as "pacu", a considerable amount of information exists about the technical farming, market value and consumer popularity of the fish in regions where it is abundant (Chabalin et al., 1988). Because of its high reproductive capacity, rapid growth and commercial value, it is one of the most cultivated freshwater fish in Brazil.

Parasites cause important losses in industrial aquaculture, with greater relevance in Neotropical regions due to climate characteristics, which results in faster disease propagation (Thatcher and Brites-Neto, 1994). Among fish parasites, monogeneans are a frequent threat, which, due to their high pathogenicity value, affects fish stock health.

The Class Monogenea (Platyhelminthes), also known as Class Monogenoidea (Boeger and Kritsky, 1993), is a group of obligatory and monoxenic parasites that infest a number of invertebrates such as squids and copepods, vertebrates such as amphibia, reptiles, mammals, and especially fish.

Anacanthorus (Monogenea, Dactylogyridae) is a diverse taxon of parasites from the gills of Characiform fishes (Boeger et al., 1995). The genus *Anacanthorus* is represented by 63 species, all from the Amazon River Basin (Pamplona - Basilio et al., 2001), with the exception of *Anacanthorus penilabiatus* (Boeger et al., 1995), which was first described in *P. mesopotamicus* (Holmberg, 1887) in fish farms in São Paulo State, Brazil. The species *A. penilabiatus* is recorded in high quantities in artificial environments and is considered a risk to productivity as it is the main cause of mortality and economical loss for the farmers in the north east of São Paulo State (Boeger et al., 1995, Martins et al., 2002).

In the Neotropical region 70 genera of freshwater Monogenea are known, distributed across approximately 308 species, parasitizing 144 fish species

(Boeger and Vianna, 2006). The Dactylogyridae is, apparently, the major taxon in continental waters in South America, although species from the family Gyrodactylidae have also been described, as well as a number of species from the Diplectanidae, Monocotylidae and Hexabothriidae families (Boeger and Vianna, 2006).

According to Simková et al. (2003; 2004) the Dactylogyridae family is extremely diverse and studying its phylogenetic position and speciation, it can be observed that the group derived recently from Monopisthocotylea.

A few molecular studies of dactylogyrids are available, while most of the molecular studies of monogeneans are concerned with gyrodactylids, where 50% is related to the genus *Gyrodactylus* (Olson and Vasyl, 2005). This is of relevance as *Gyrodactylus* are major parasites in Europe, especially in Scotland, Norway and others Nordic countries, where a great number of salmonids are lost in the wild, and in fish farms (Huyse et al., 2007, 2008; Bakke et al., 2007).

Most references regarding monogeneans refer to morphological taxonomy, descriptions, ecology and treatment. Monogenea taxonomy is usually based on morphological features, mainly on the sclerotized parts and reproductive organs, which are extremely similar in many species and difficult to visualize, therefore requiring accurate study. The determination of genus, species and phylogeny study based on traditional methods, has been questioned in molecular analysis by ribosomal DNA and mitochondrial genome (Pouyaud et al., 2006; Huyse, et al., 2007).

The molecular study of monogeneans has aroused considerable interest and a number of studies have been carried out in recent years (Huyse et al., 2007; Wu et al., 2007; Hansen et al., 2007; Matejusová and Cunningham 2004). Molecular methods such as ribosomal DNA (rDNA) have been used for genus identification, but they are not sufficiently precise to differentiate the small variations that characterize species (Matejusová et al., 2001, 2003; Huyse et al., 2003; Zietara et al., 2002; Hansen et al., 2007).

Nowadays, the interest in several aspects of the mitochondrial genome is growing exponentially (Gasiev and Shaikhaev, 2008). The mitochondrial genome

is commonly used to infer a metazoan phylogeny, haploid status, and genes that are conserved and of small size, all of which are important factors for solving systematic and evolutionary questions in a wider taxonomic context (Sinniger, et al., 2007; Waeschenbach et al., 2006).

The monogenean mitochondrial DNA displays great efficiency. Due to its maternal inheritance, the haploid mitochondrial genome should provide useful markers for diagnostic and population studies (Huyse et al., 2007, 2008; Bueno-Silva et al. 2011). The mitochondrial genome should also overcome problems with heterozygosis presence in nuclear markers (Huyse et al., 2008). Huyse et al. (2007) and Plaisance et al. (2007) were the first to sequence the complete mitochondrial genome from the monogenean species, *Gyrodactylus salaris* and *Gyrodactylus thymalli*, species that are morphologically similar and of great commercial importance.

Mitochondrial genes are useful as molecular markers for diagnostics, phylogenetics analysis, speciation and epidemiological studies (Escalante et al., 1998; Blouin et al., 1998).

In Brazil, at the moment, there are few studies of the diagnostics, phylogeny, population patterns or evolutionary development of the Dactylogyridae, which makes studies of this diverse group necessary. In the present study the partial mitochondrial genome was sequenced from *A. penilabiatus*, an important species for fish farms in Brazil, and some mitochondrial genes were characterized, such as gene order, codon usage, tRNA features, gene boundaries compared with other monopisthocotyleans and polyopisthocotyleans available. Sliding window analysis was also performed, to reach variable and conserved regions for possible primer design. Mitochondrial sequence data of rrnL and rrnS genes was also included from other dactylogyrids *Mymarothecium viatorum* and Dactylogyridae sp., collected from *P. mesopotamicus* in the same locality. This is the first study on the mitochondrial genome from the Dactylogyridae, and that is from South America.

2. MATERIAL AND METHODS

2.1. Sample collection and DNA extraction

The monogenean *A. penilabiatus*, *M. viatorum* and a Dactylogyridae sp1 (in identification process) from *P. mesopotamicus* were collected in fish farms from the state of São Paulo, Brazil from September 2008 to July 2010. The samples were collected in accordance with Thatcher (2006) and kept in 96% (v/v) ethanol at 5°C. For morphological identification, specimens were cut in half, with one part used as a voucher specimen for morphological identification using the Hoyer's medium, and the other part used for DNA extraction.

For genomic DNA extraction, a total of 15 parasites were briefly air dried with a vacuum centrifuge to remove ethanol, and the total genome was extracted using DNeasy® Blood & Tissue Kit (Qiagen) according to manufacturer's protocol with a final volume of 100 µl.

2.2. Conventional and long polymerase chain reaction (PCR)

The map of the complete mitochondrial genome of *Gyrodactylus derjavini* was used to delineate the strategy for PCR amplification.

Five mitochondrial fragments were amplified and sequenced: cytb, 16Sa, 12Sa, nad1 and cox1, using specific Forward and Reverse primers. Primers were especially designed for the specimens using sequences from other helminthes available from Genbank (Table 1). Some primers were used from previous studies and the majority was specially designed for this study (Table 2). The conventional PCR was performed with a 25 µl primary PCR amplifications, with 2 µl DNA extract, 1.0 µl each primer and Ready-to-Go PCR beads (Pure Taq™Ready-to-Go™PCR beads, GE Healthcare), the solution consisted stabilizers, BSA, dATP, dCTP, dGTP, dTTP, ~2.5 units of puReTaq DNA polymerase and reaction buffer. With the reconstituted bead to a 25 µl final volume, the concentration of each dNTP was 200 µM in 10 mM Tris-HCl, (pH 9.0

at room temperature), 50 mM KCl and 1.5 mM MgCl2. Cycling conditions were as follows: initial denaturation for 3 min at 94°C follow by 40 cycles of 30 sec at 94°C, 30 sec at 40° to 63°C, 2 min at 72°C and final extension at 10 min at 72°C.

Table 1: List of complete mt sequences from Genbank used to design monogenean primers for this study.

Species	Class	Accession Number
Microcotyle sebastis	Monogenea	DQ412044
Gyrodactylus derjavini	Monogenea	EF524575
Gyrodactylus salaris	Monogenea	DQ988931
Dictyocotyle coeliaca	Monogenea	AY157171
Diphyllobothrium nihonkaiense	Cestoda	AB544064
Diphyllobothrium latum	Cestoda	GU997615
Echinococcus vogeli	Cestoda	NC009462
Echinococcus granulosus	Cestoda	FJ645851
Echinococcus shiquicus	Cestoda	HM064449
Echinococcus multilocularis	Cestoda	NC000928
Taenia solium	Cestoda	NC004022
Taenia asiatica	Cestoda	GQ202130
Taenia crassiceps	Cestoda	NC002547
Hymenolepis diminuta	Cestoda	NC002767
Fasciola hepatica	Trematoda	NC002546
Paragonimus westermani	Trematoda	NC002354
Schistosoma haematobium	Trematoda	DQ677664
Schistosoma spindale	Trematoda	DQ157223
Schistosoma mansoni	Trematoda	NS000200

The mitochondrial genome was amplified by Long PCRs using Expand 20 Kb Plus PCR System, dNTPack (Roche Applied Science) were carried out using all possible primer combinations. Three fragments were amplified, Anac_rrnF + cytbR (≈7kb), Anac_cytbF + Anac_rrnR (≈7kb) and Mym_cytbF + rrnR (≈7kb). Cycling conditions were: initial denaturation for 2 min at 92°C following with 40 cycles of 15 sec at 92°C, 30 sec at 57°C and 7min at 68°C, adding 30 sec at each cycle and final extension of 10 min at 68°C. To increase the quantity of Long PCR product, nested primers were designed using Sequencher 4.10.1, Mac Vector, BBEdit Lite 6.1 for OS X and NetPrimer (available at www.premierbiosoft.com/netprimer/index.html). All the PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and some were extracted

directly from the agarose gel using Zymoclean ™ Gel DNA Recovery Kit (ZymoResearch).

2.3. Nested primers and Sequencing

Nested primers (Table 2) were designed to increase the quantity of Long PCR products. Automated sequencing was performed directly on purified PCR products using ABI Big Dye chemistry following the manufacturer's protocols for cycle sequencing. Sequences were alcohol precipitated and run on an ABI prism 377 automated sequencer. The sequence identity was verified using the Basic Local Alignment Search Tool (BLAST) and sequences were assembled and edited using Sequencher v. 4.10.1 (Gene Codes, Ann Arbor, MI). The fragment Anac_rrnF + Anac_cytbR were sequenced by primer walking (Table 2).

Table 2: List of primers designed to amplify monogeneans' fragments, *Anacanthorus penilabiatus* and *Mymarothecium viatorum*.

Gene/Primer Conventional PCR	**Sequence 5'- 3'**	**Source**
mit DNA		
Cytb (Boore&Brown)	cytbF GGWTAYGTWYTWCCWTGRGGWCARAT cytbR GCRTAWGCRAAWARRAARTAYCAYTCWGG	Boore & Brown (2000)
Cox1 (Folmer)	LCO GGTCAACAAATCATAAAGATATTG HCO TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
mMon_cytbF	TATTATTGGTGTAGTGG	Designed from aligned helminth sequences
mMon_cytbR	CCTARRAAHGCTTCHACCATCAT	Designed from aligned helminth sequences
mMon_Nad1F	TGTTTRTTYGCWTGTGA	Designed from aligned helminth sequences
mMon_Cox1R	ACWGGRTCHCCTCCNCCC	Designed from aligned helminth sequences
mMon_Cox1F	TTTACTATMGGDGGKGTRACVGGG	Designed from aligned helminth sequences
mMon_16SR	ACTGGCTTACACCGGTYTTAACTCAACTC	Designed from aligned helminth sequences
mMon_16SFa	TGACTGTGCTAAGGTAGCAT	Designed from aligned helminth sequences
mMon_12SaR	AYCGAGATTGACGGGCGGTATGT	Designed from aligned helminth sequences
Long PCR		
Anac_cytbR	CAAATCAAGGGAATAATGCG	Designed from *Anacanthorus* sequences
Anac_rrnF	CAGCCAACTAATCCTGGTAGG	Designed from *Anacanthorus* sequences
Nested Primers		
Anac_cytbRn	ACATCTGCAACGTACAATAGGG	Designed from *Anacanthorus* sequences
Anac_rrnFn	GCTGCTAACATAAAGGGGAGAG	Designed from *Anacanthorus* sequences
Sequencing Primers		
Anac_seqF1	TGTTCTTTTGGGAGGAGC	Designed from *Anacanthorus* sequences
Anac_seqR1	TGGTATGCTGTGGCTGTAAC	Designed from *Anacanthorus* sequences
Anac_seqF2	AAAGAGGTGGACAAGCCTG	Designed from *Anacanthorus* sequences
Anac_seqR2	ACTGGCTCCCCAAAGGTAAC	Designed from *Anacanthorus* sequences
Anac_seqF3	ATTCTCTCATTGGAGGGAGCGAGG	Designed from *Anacanthorus* sequences
Anac_seqR3	ATCATCTCAACAACCCCTCGCTCC	Designed from *Anacanthorus* sequences
Anac_seqF4	TTTTCCAAAGCAAAGACC	Designed from *Anacanthorus* sequences
Anac_seq F5	GTTACAGCCACAGCATACCA	Designed from *Anacanthorus* sequences
Anac_seqF6	TTTTGTAGTGGTGCTGAGGGGG	Designed from *Anacanthorus* sequences
Anac_seqR6	AGATAGGTAACCCCCTCAG	Designed from *Anacanthorus* sequences
Anac_seqR7	ATTAGGTCTTTGCTTTGG	Designed from *Anacanthorus* sequences
Anac_seqR8	CCTTTTACTCAGTTACATTTTC	Designed from *Anacanthorus* sequences
Anac_seqR9	ATCATCTCAACAACCCCTCGCTCC	Designed from *Anacanthorus* sequences
Anac_seqF10	GGACGAGTTTATGGGAGTG	Designed from *Anacanthorus* sequences
Anac_seqF11	CGTGAGACTGTAAATCTTGC	Designed from *Anacanthorus* sequences
Anac_seqF12	TCGTTTTGGGGATGTAGG	Designed from *Anacanthorus* sequences
Anac_seqR10	CATCCCCAAAACGAGAAG	Designed from *Anacanthorus* sequences
Anac_intF1	TAAAGATGGATGCCGTCCCAGG	Designed from *Anacanthorus* sequences
Anac_intR1_3X	GTCCTATCATCTCAACAACCCCTC	Designed from *Anacanthorus* sequences
Anac_intF2	CCGTGATTGTTATGCTTATTGCTC	Designed from *Anacanthorus* sequences
Anac_intR2	AAACCTTGTCAACCTACTTCCAGAC	Designed from *Anacanthorus* sequences
Anac_intF3	GGCTTGTTTAGGACGAGTTTATGG	Designed from *Anacanthorus* sequences
Anac_intR3	ATGGTGGTATGCTGTGGCTG	Designed from *Anacanthorus* sequences
Anac_seqF13	TATCTTGCTTGCGGTAGGC	Designed from *Anacanthorus* sequences
Anac_seqF14	GGGAGGACTTTTGGTTATTG	Designed from *Anacanthorus* sequences
Anac_seqR11	CAATAACCAAAAGTCCTCCC	Designed from *Anacanthorus* sequences

2.4. Annotation of sequences

Contiguous sequence fragments were assembled using Sequencher v. 4.10.1 (Gene Codes, Ann Arbor, MI). Genome annotation was performed using Mac Vector with Assembler 12.0.6 (MacVector Inc.). The open reading frames (ORFS) were verified using BLAST and the start and stop codons were verified aligning sequences from other monogeneans (Monopisthocotylea) found in Genbank. The monogeneans were *Benedenia hoshinai* (NC 014591), *Benedenia seriolae* (NC 014291), *Gyrodactylus derjavinoides* (NC 010976), *Gyrodactylus thymalli* (NC 988931). Protein coding regions were translated with the echinoderm and flatworm mitochondrial code (Table 9, NCBI). The program Arwen tRNA scan (available at http://130.235.46.10/ARWEN/) was used to identify the tRNAs. The non-coding region was scanned for repeat elements using the program Tandem Repeats Finder, Benson, 1999 and the two rRNAs genes were identified using BLAST searches.

2.5. Sliding Window Analyses

To study the distribution of variation along the genome and to find conserved regions for primer design, the program DnaSP was performed using the sliding window tool (Librado & Rozas, 2009). Sliding window analysis of 100bp was carried out to indicate the most variable regions, whereas a window size of 25bp was used to identify the relatively conserved regions.

3. RESULTS AND DISCUSSION

3.1. Genome organization and base composition.

One fragment of *A. penilabiatus* mitochondrial genome were amplified, the portion Anac_rrnF + Anac_cytbR. Partial 16S and 12S genes of *M. viatorum* and another Dactylogyridae sp1 (not yet identified) were sequenced with success. Thus, this study will focus mostly on the partial genome fragment Anac_rrnF +

Anac_cytbR and its use for possible molecular marker design. *M. viatorum* and Dactylogyridae sp1 will be discussed in Paragraph 3.5, Ribosomal genes.

The partial mitochondrial genome of *A. penilabiatus* sequenced is 6547bp. The fragment has been annotated and deposited in Genbank under the accession number (will be deposited after article submission) and is depicted in figure1. Gene order is similar to other monogeneans, with some differences. The partial genome comprises 4 complete protein coding genes (cox2, nad6, nad5 and cox3), 1 partial protein coding gene (cytb), 1 non-coding region with 3 tandem repeat regions, partial rrnL, complete rrnS and 9 tRNAs. Between the genes tRNAs are represented. As for all Platyhelminthes, all genes are described in the same strand. Metazoan mitochondrial genomes are usually unicircular molecules with a size of approximately 16kb that encode a set of 37 genes, 2 rRNAs (rrnL and rrnS), 22 tRNAs and 13 protein coding genes (cox1, cox2, cox3, nad1, nad2, nad3, nad4, nad4L, nad5, nad6, cytb, atp6, atp8) (Boore & Brown, 2000).

Comparing the gene rearrangement to *Gyrodactylus salaris* and *Gyrodactylus derjavinoides* genomes (Huyse et al., 2007, 2008) the difference is the conserved NC2 region, between the nad6 and nad5 protein coding genes, and, for *A. penilabiatus* the non-coding region, is located after nad5, between nad5 and cox3. *Benedenia seriolae* on the other hand, have no non-coding region, and in this case *B. seriolae* has only one tandem repeat region (TA_{12}) between ND4L and ND4 (Perkins et al., 2010). The tRNA rearrangements are similar, as *Gyrodactylus* spp. and *B. seriolae* (Huyse et al., 2007, 2008; Perkins et al., 2010).

As for *Gyrodactylus* spp. and *B. seriolae*, *A. penilabiatus* presents $tRNA^{CYS}$ between rrnL and rrnS, $tRNA^{GLU}$ between cox2 and nad6, $tRNA^{GLY}$ between nad5 and cox3, and $tRNA^{HIS}$ between cox 3 and cytb. In *Gyrodactylus* spp. there are 7 tRNAs between nad6 and nad5, $tRNA^{TYR}$, $tRNA^{LEU1}$, $tRNA^{GLN}$, $tRNA^{MET}$, a non-coding region, $tRNA^{SER2}$, $tRNA^{LEU2}$ and $tRNA^{ARG}$ (Huyse et al., 2007, 2008). By contrast, *A. penilabiatus*, and *B. seriolae* has 5 between nad6 and nad5, but in different positions. *Benedenia seriolae* tRNA between nad6 and

nad5 are $tRNA^{TYR}$, $tRNA^{LEU1}$, $tRNA^{SER2}$ and $tRNA^{ARG}$ and this gene order follows exactly the same tRNA rearrangement seen between nad6 and nad5 in the polyopisthocotylean *Microcotyle sebastis* (Park et al., 2007a; Perkins et al., 2010). For *A. penilabiatus* the tRNAs between nad6 and nad5 are: $tRNA^{LEU1}$, $tRNA^{SER}$, $tRNA^{TYR}$, $tRNA^{LEU2}$ and $tRNA^{ARG}$ (Fig.1).

Genome rearrangements were shown to serve as a possible source of phylogenetic information (Hypsa, 2006). Mitochondrial genomes have the advantage of lying on a limited area which allows determination of homologies and hence evaluation of gene rearrangements (Wolstenholme, 1992; Boore & Brown, 1998). The basis of using mitochondrial gene order as phylogenetic information started with the assumption that rearrangements are relatively infrequent events (Boore & Brown, 1998). However, the frequency and distribution of gene rearrangements may constrain their phylogenetic applicability to only some taxa (Hypsa, 2006). Gene order was similar for all monogeneans compared above.

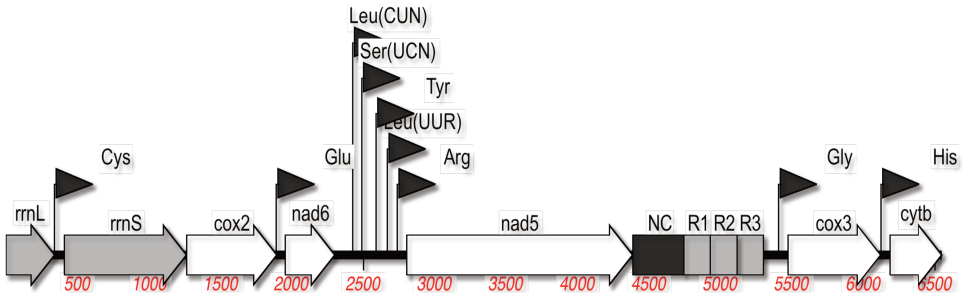

Figure 1: Partial mitochondrial genome map from *Anacanthorus penilabiatus*. Partial ribosomal genes 16S (rrnL) and complete 12S (rrnS), cytochrome oxidase (cox2 and cox3), subunits of nicotinamide adenine dinucleotide dehydrogenase (nad6 and nad5), non-coding region (NC), tandem repeats (R1, R2 and R3), partial cytochrome b (cytb) and tRNAs between genes.

The overall nucleotide composition of all coding sites is: A (27.3%), C (10.9%), G (23.1%) and T (38.6%). The partial genome has an overall A+T content of 65.9%. Gene boundaries are described in the table 3, where the position is described as annotated, although not as gene order should be, as the genome is partial. Comparing these genes with other mitochondrial genes from

monogeneans closely related to *Gyrodactylus* spp., there are not many differences. This information is expressed in table 4.

Table 3: Data from the partial mitochondrial genome from *Anacanthorus penilabiatus*, with the start and stop codon positions for individual protein coding genes and other gene characteristics. Base pairs (Bp), and amino acids (aa).
* Position as the annotated partial genome.

	Length		**Codon**				**Length**	
Genes	**Bp**	**aa**	**Start**	**Stop**	*** Position 5' to 3'**	**Genes**	**bp**	***Position 5' to 3'**
Protein						RNAs		
cox2	633	211	ATG	TAG	1263: 1895	*rrnL(partial)*	338	1: 338
nad6	345	115	ATG	TAA	1955: 2299	*rrnS*	854	409: 1262
nad5	1581	527	ATG	TAA	2805: 4385	*trnC*	70	339: 408
cox3	648	216	ATG	TAG	5472: 6119	*trnG*	67	1891: 1957
cytb (partial)	363	121	ATG	------	6185: 6547	*trnL*	65	2426: 2490
						trnS	62	2490: 2551
Non-coding						*trnT*	63	2588: 2650
NC	357				4386: 4742	*trnL*	69	2668: 2736
R1	186				4743: 4928	*trnA*	68	2736: 2803
R2	186				4929: 5114	*trnG*	68	5405: 5472
R3	186				5115: 5300	*trnH*	63	6122: 6184

Table 4: Mitochondrial genes comparison, *Anacanthorus penilabiatus* genes with *Gyrodactylus* spp. genes available in Genbank.

Gene	***Gyrodactylus salaris***			***Gyrodactylus derjavioides***			***Anacanthorus penilabiatus***		
	Length	Start/stop	%A+T	Length	Start/stop	%A+T	Length	Start/stop	%A+T
cox2	582	ATG/TAA	62.7	582	ATG/TAA	65.5	633	ATG/TAG	60.5
nad6	483	ATG/TAA	67.0	483	ATG/TAA	73.5	345	ATG/TAA	65.0
nad5	1551	ATG/TAG	58.8	1551	ATG/TAA	66.8	1581	ATG/TAA	66.5
cox3	639	ATG/TAA	61.3	639	ATG/TAA	68.0	648	ATG/TAG	66.3
cytb	1074	ATG/TAG	59.7	1074	ATG/TAA	63.9	363	ATG/------	66.1

3.2. Protein-coding genes and codon usage

All protein coding genes have the initiation codon ATG, as predicted, TAG for cox2 and cox3 and TAA for nad6 and nad5 as termination codons. The cytb gene is partial so only the start codon ATG was detected. Comparing the monopisthocotyleans available, all initiations are the same and differ in terminations codons. Cox2 are the same as *B. seriolae,* but different in *Gyrodactylus* spp. and cox3 are different from all (Table 4). Other invertebrate initiations codons, such as ATC and GTG, were not found. Regarding the length, all are quite similar except nad6, which is approximately 130bp shorter (Table 4).

The most frequent codons were TTT(155), TTA(128), TAG(83), GTT(78) and ATT(76), and the least frequent codons were CGC(2), GCC(3), TCG(6), CCC(7), CAC(7), CGT(7), TCC(8), CGG(8) and ACC(8) (Table 5). As base composition, T is the most frequent base, G is less frequent, A and C are intermediate. This observation is also commonly found for other invertebrates (Waeschenbach et al., 2006). This result shows considerable A + T richness consistent with other mt genomes from monogeneans (Huyse et al., 2007,2008, Plaisance et al., 2007; Park et al., 2007a; Perkins et al., 2010; Zhang et al., 2011).

Table 5: *Anacanthorus penilabiatus* partial genome, codon usage table. Amino acids (aa) and number of nucleotides (N).

aa	Codon	N	Percent	aa	Codon	N	Percent
Phe	TTT	155	7.1	Ile	ATT	76	3.5
	TTC	33	1.5		ATC	20	0.9
Leu	TTA	128	5.9		ATA	54	2.5
	TTG	72	3.3	Met i	ATG	32	1.5
Ser	TCT	26	1.2	The	ACT	32	1.5
	TCC	8	0.4		ACC	8	0.4
	TCA	22	1.0		ACA	13	0.6
	TCG	6	0.3		ACG	8	0.4
Tyr	TAT	65	3.0	Asn	AAT	53	2.4
	TAC	29	1.3		AAC	18	0.8
ter*	TAA	74	3.4		AAA	50	2.3
ter*	TAG	83	3.8	Lys	AAG	52	2.4
Cys	TGT	68	3.1	Ser	AGT	46	2.1
	TGC	26	1.2		AGC	14	0.6
Trp	TGA	33	1.5		AGA	30	1.4
	TGG	47	2.2		AGG	54	2.5
Leu	CTT	42	1.9	Val	GTT	78	3.6
	CTC	12	0.5		GTC	15	0.7
	CTA	26	1.2		GTA	54	2.5
	CTG	26	1.2		GTG	29	1.3
Pro	CCT	22	1.0	Ala	GTC	19	0.9
	CCC	7	0.3		GCC	3	0.1
	CCA	11	0.5		GCA	15	0.7
	CCG	9	0.4		GCG	10	0.5
His	CAT	25	1.1	Asp	GAT	37	1.7
	CAC	7	0.3		GAC	13	0.6
Gln	CAA	17	0.8	Glu	GAA	39	1.8
	CAG	25	1.1		GAG	35	1.6
Arg	CGT	7	0.3	Gly	GGT	49	2.2
	CGC	2	0.1		GGC	24	1.1
	CGA	12	0.5		GGA	25	1.1
	CGG	8	0.4		GGG	42	

3.3. Non-coding region (NC)

Gyrodactylus spp. have two large non-coding regions located between $tRNA^{MET}$ and $tRNA^{SER2}$ and other between ND4 and ATP6. No repetitive regions were found for *Gyrodactylus* spp. and the alignment of these two regions shows great conservation. Therefore, this region warrants characterization for additional strains and species of gyrodactylids (Huyse et al., 2007, 2008). These large non-coding regions are absent in *B. seriolae*, which is more compact (Perkins et al., 2010).

In contrast, the partial genome of *A. penilabiatus* presents a non-coding region with 357bp long, followed by three identical repeat regions with length of 186bp and A + T content of 67.8%. While PCR was difficult for these three tandem repeat fragments, it was achieved. Repeat regions are often associated with the origin of replication, or control region, as it is known (Wolstenholme, 1992). These regions have been found in many different mt genomes, but are considered uncommon and hotspots for replication slippage errors and translocation (Le et al., 2002). The complete mitochondrial genome of *Trichobilharzia regenti* (Platyhelminthes, Digenea) has also three repeat region fragments of 184bp each, which suggests that this region may include the control region of origin of replication for the mitochondrial genome, because it folds readily into stable stem loop structures (Webster et al., 2007).

Directly repeated segments are found in mtDNA molecules of a number of metazoan species. The most common type comprises sequences repeated in tandem in the putative control region (Wolstenholme, 1992). Additionally, some mitochondrial genomes, such as *Caenorhabditis elegans* and, *Meloidogyne* sp. (Nematoda), contain DNA repeats in non-coding control region (Feagin, 2000). Typical control regions have proved difficult to locate in parasitic platyhelminths (Perkins et al., 2010). However, the origin of replication may be located following the same determined features as the control region, but this designation remains putative (Huyse et al., 2008).

Among available monopisthocotyleans genomes, *A. penilabiatus* is the first to show non-coding region with repetitive sequences. The poliopisthocotylean *M. sebastis* possesses a highly repetitive region between cox3 and nad6 (Park et al., 2007a), and also *Polylabris halichoeres* possesses a highly repetitive region located between $tRNA^{S(UCN)}$ and nad6, including 17 repeats units of a 94bp sequence (Zhang et al., 2011). According to Zhang et al. (2011) *P. halichoeres* mitochondrial genome is the largest monogenean genome sequenced so far and NC regions from polyopisthocotylean species (*P. halichoeres* and *M. sebastis*), much larger in length (53-107bp) with more clusters and repeated motifs. In accordance with general consideration, the findings described above show that polyopisthocotyleans mt genomes have a typically long repetitive region in the NC area compared to monopisthocotyleans (Zhang et al., 2011). In contrast to these statements, the partial mt genome of the monopisthocotylean *A. penilabiatus* shows a NC region with a length of 915bp with 3 repetitive regions, not as large as *P. halichoeres,* but so far the first NC region with repetitive regions for monopisthocotyleans. Additionally, more taxa regarding mt genomes from polyopisthocotyleans and monopisthocotyleans must be sequenced for a more effective conclusion.

Small non-coding regions have been found separating genes and tRNAs, those located between gene nad6 and $tRNA^{Leu}$ (CUN), 127 nucleotides, between $tRNAs^{Ser}$ (UCN) and $tRNA^{Tyr}$, 37 nucleotides, $tRNA^{Tyr}$ and $tRNA^{Leu}$ (UUR), 18 nucleotides, a non-coding region between the last repetitive region R3 and $tRNA^{Gly}$, 105 nucleotides and a non-coding region between the gene cox3 and $tRNA^{His}$, 3 nucleotides.

3.4. tRNA secondary structures and overlap between adjacent genes

Nine typical putative tRNAs were identified using tRNAscan (Laslett & Canback, 2008). All nine possessed the DHU stem and loop arm and T¥C stem and loop arm (Figure 2). Starting with $tRNA^{Cys}$ features, anticodon GCA, 70 bases and GC content 38.6%, looks to possess a variable loop before the T¥C

arm; $tRNA^{Glu}$ anticodon TTC, 67 bases and GC content 25.4%; $tRNA^{Leu}$ (CUN) anticodon TAG, 65 bases, GC content 41.5%; $tRNA^{Ser}$ (UCN) anticodon TGA, 62 bases, GC content 35.5%; $tRNA^{Tyr}$ anticodon GTA, 63 bases, GC content 28.6%; $tRNA^{Leu}$ (UUR) anticodon TAA, 69 bases, GC content 30.4%; $tRNA^{Arg}$ anticodon TCG, 68 bases, GC content 38.2%; $tRNA^{Gly}$ anticodon TCC, 68 bases, GC content 22.1% and $tRNA^{His}$ anticodon GTG, 63 bases, GC content 39.1%. No TV replacements or D replacements were found.

Unlike *B. seriolae* (Perkins et al., 2010) overlaps between protein coding genes and tRNAs and between tRNAs with each other were found and ranged from 1 to 4 nucleotides. There was overlap between 3' end of cox2 protein coding gene with 5' end of $tRNA^{Glu}$ by 4 nucleotides, 3' end of $tRNA^{Glu}$ overlaps with 5' end of protein coding gene nad6 by 2 nucleotides, 3' end of $tRNA^{Gly}$ with 5' end of protein coding gene cox3 by 1 nucleotide, 3' end of $tRNA^{Leu}$ (CUN) overlaps with 5' end of $tRNA^{Ser}$(UCN) by one nucleotide and 3' end of $tRNA^{Leu}$(UUR) with 5' end of $tRNA^{Arg}$ by one nucleotide also.

The overlaps between protein coding genes and tRNAs, and between tRNAs with each other are not unusual (Waeschenbach et al., 2006) and other taxons have been reported with similar features (Anderson et al., 1981; Boore & Brown, 1994, 1995; Clary and Wolstenholme, 1985; Huyse et al., 2007 and Waeschenbach et al., 2006).

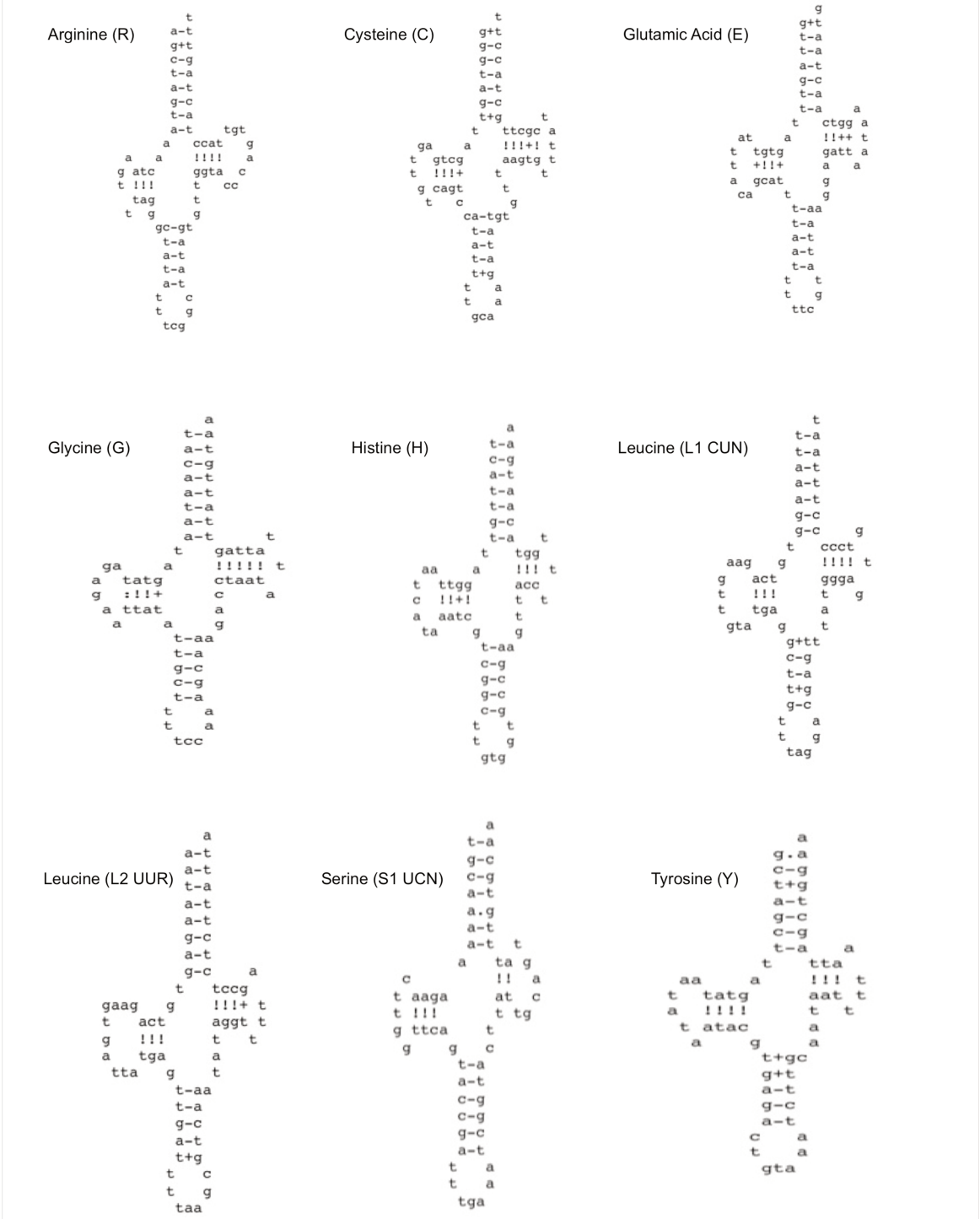

Figure 2: Inferred secondary structures of nine mitochondrial tRNAs from *Anacanthorus penilabiatus* partial mitochondrial genome, in alphabetical order. For the order in the genome see Figure 1.

3.5. Ribosomal genes

Two ribosomal genes were sequenced in this study for *A. penilabiatus*, partial sequence of 16S, rrnL, the ribosomal large subunit and 12S, rrnS, ribosomal small subunit. The total length of this fragment is 1262bp and between those two ribosomal genes a $tRNA^{CYS}$ has been annotated.

Other two species had successfully the same fragment sequenced, *M. viatorum* and Dactylogyridae sp1 (not yet identified) and annotated using MacVector. Like *A. penilabiatus*, have the same structure, partial rrnL followed by the $tRNA^{CYS}$ and rrnS, with lengths as 1511bp for *M. viatorum* and 1103 for Dactylogyridae sp1. The overall nucleotide composition for these genes is: A (31.7%), C (10.5%), G (18.1%) and T (39.7%) for *M. viatorum* and A (33.2%), C (11.0%), G (17.9%) and T (37.9%) for Dactylogyridae sp1. The fragments cited above, have been annotated and deposited in Genbank under the accession number (which will be deposited after article submission) and number (which will be deposited after article submission) respectively. The primer set for all the ribosomal genes amplification were mMom_16SFa and mMom_12SaR expressed at Table 2.

Alignments from these fragments were performed with Clustal X 2.1 and results were well conserved and possessed variable regions, and the species were successfully differentiated by the alignment. A study by Greig et al. (2005) of shark species discrimination with mitochondrial genes 16S and 12S, found that these genes have regions containing sufficient signals to generate reasonable phylogenetic reconstructions and as such are useful markers for species identification.

Regarding the molecular phylogeny of gill monogeneans, dactylogyrids from the Indo-West Pacific butterfly fish, Plaisance et al. (2005) used a combination of nuclear 18S (small subunit) and 28S (large subunit) rDNA ribosomal primers, mitochondrial gene16S rDNA ribosomal primers. The data combining the three set of primers revealed congruent results with those

obtained from each data set analyzed separately for relationships between main groups, whereas minor differences were found within the same clades.

Studying molecular markers for resolving the phylogeny of cyclophyllidean tapeworms, Littlewood et al. (2008), tested molecular markers as ssrDNA, lsrDNA genes, and the mitochondrial markers rrnL (16S), cox1 and nad1. The results showed that the mitochondrial genes provided almost twice as many variables and twice as many parsimony informative sites. According to the present study, genes with the most informative sites were partial rrnL (38.6%), nad1 (37.0%), partial cox1 (33.6%), against lsrDNA (23.7%) and ssrDNA (15.0%).

For the present study, mitochondrial ribosomal markers, the fragment partial rrnL(16S), tRNACYS and rrnS(12S), brought positive results for the species previously mentioned, and can possibly be useful markers for diagnostics, and a potential tool for future phylogenetic studies of dactylogyrids. However, in order to confirm its usefulness more taxa must be sampled, and further study is required.

3.6. Sliding window analysis

Sliding window analysis was performed for the *A. penilabiatus* genes: partial rrnL (16S), complete rrnS (12S), followed by the complete protein coding genes cox2, nad6, nad5, cox3 and partial cytb. The other taxon chosen for the analysis was *G. salaris* and the gene arrangement was the same as described for *A. penilabiatus* with homologous partial genes (16S and cytb). This analysis had the purpose of showing variable and conserved regions useful for possible primer design.

Nucleotide diversity (average number of nucleotide differences per site between two sequences,π) showed regions with high and low variability (Figure 3). The most conserved regions are located in the gene rrnL (16S) following rrnS (12S) and two highly conserved regions in nad5 gene. The most variable regions

were located in the protein coding genes nad6, nad5, cox3, cox2 and a portion located in the rrnS (12S) gene (Figure 3).

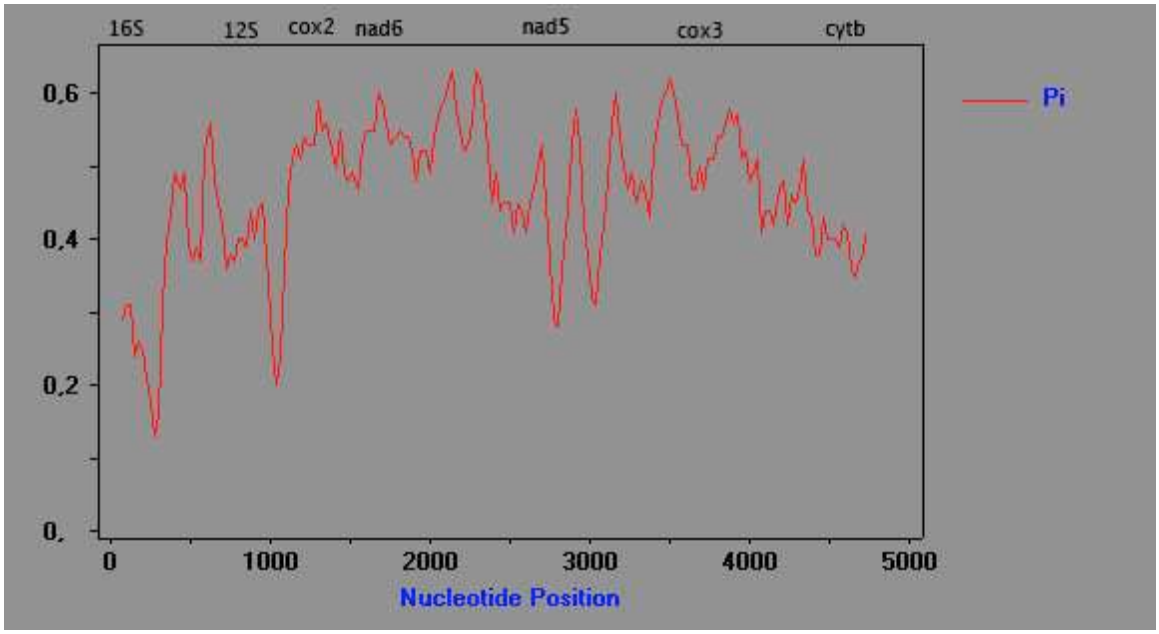

Figure 3: Sliding window analysis of the alignment of *Anacanthorus penilabiatus* and *Gyrodactylus salaris*. The line shows the value of nucleotide diversity (π) in a sliding window analysis of window size 500bp with step size 25. Gene boundaries are indicated above. The peaks are highly variable and the descendent decreases means highly conserved regions.

Sliding window analysis for partial *A. penilabiatus* as for *G. salaris* and *Gyrodactylus derjavinoides* (Huyse et al., 2008), showed nucleotide diversity distributed along the mitochondrial genes, with alternating regions of high and low variability, the majority of which were high variability regions.

The nucleotide diversity tool showed a potential region of 4818bp to design possible molecular markers for monopisthocotyleans capturing high variable and conserved regions. According to Huyse et al. (2008), the choice of molecular markers usually depends primarily on the availability of suitable PCR primers and there is a certain degree of conservatism in the approach. Researchers tend to re-use PCR primers from authors who have worked on the same or similar taxa, and these primers can become widely used, or are sometimes chosen for convenience, while not representing the most suitable marker (Huyse et al., 2008). So, primer design can be a preferable choice to maximize specific DNA fragments than adopting markers with unknown scope

and potential (Huyse et al., 2008). The information about the variable and conserved regions provided in this study can be useful for developing new molecular markers for monogeneans, especially for South American species.

Mitochondrial genomes of various parasitic helminthes have been sequenced, usually to provide access to the full suite of potential molecular markers within the genome (Webster et al., 2007). The number of complete mitochondrial genomes, or nearly complete, of the platyhelminthes species, is increasing (Le et al., 2001; Park et al., 2007*a*,*b*; Nickisch-Rosenegk et al., 2001; Blair et al., 1999; Littlewood et al., 2006; Jeon et al., 2007; Shekhovtsov et al., 2010).

There are a total of seven complete mitochondrial genomes for monogeneans available in Genbank, five of which are monopisthocotyleans as *Gyrodactylus salaris*, *G. thymalli*, *G. derjavinoides*, *Benedenia hoshinai*, *B. seriolae*, and two of which are polyopisthocotyleans *M. sebastis* and *P. halichoeres* (Huyse et al., 2007,2008, Plaisance et al., 2007; Perkins et al., 2010; Park et al., 2007a; Zhang et al., 2011). Studies of mitochondrial genomes or even other molecular tools for Monogenea are of considerable interest to researchers, to solve diagnostic and phylogenetic questions.

In conclusion, the partial mitochondrial genome of *A. penilabiatus* from *P. mesopotamicus* has been sequenced and annotated, the first time this has been performed for a Dactylogyridae, and the first such study in South America. Partial and complete genes sequenced showed highly variable and conserved features that enable the design of molecular markers to allow future diagnostic, phylogenetic and population studies of dactylogyrids. To confirm these statements, more taxa should be sampled, and tested further with these markers.

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5. REFERENCES

Anderson, S., Bankier, A.T., Barrell, B.G., De Bruijn, M.H.L. and Coulson, A.R. (1981). Sequence and organization of the human mitochondrial genome. *Nature* 290, 457-465.

Bakke, T.A., Cable, J. and Harris, P.D. (2007). The biology of gyrodactylid monogeneans: the "Russian-doll killers". *Advances for Parasitology* 64,161-376.

Benson, G. (1999). Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Research* 27 (2), 573-580.

Blair, D., Le, T.H., Després, L. and McManus, D.P. (1999). Mitochondrial genes of *Schistosoma mansoni*. *Parasitology* 119, 303-313.

Blouin, M.S., Yowell, C.A., Courtney, C.H. and Dame, J.B. (1998). Substitution bias, rapid saturation, and the use of mtDNA for nematode systematics. *Molecular Biology and Evolution* 15, 1719-1727.

Boeger, W.A. and Kritsky, D.C. (1993). Phylogeny and a revised classification of the Monogenoidea Bychowsky, 1837 (Platyhelminths). *Systematic Parasitology* 26, 1-32.

Boeger, W.A., Husak, W.S. and Martins, M.L. (1995). Neotropical Monogenoidea. 25. *Anacanthorus penilabiatus* n. sp. (Dactylogyridae, Anacanthorinae) from *Piaractus mesopotamicus* (Osteichthyes, Serrasalmidae), cultivated in the State of São Paulo, Brazil. *Memórias do Instituto Oswaldo Cruz* 90(6), 699-701.

Boeger, W.A. and Vianna, R.T (2006). Monogenoidea. In: *Aquatic Biodiversity in Latin America. V. 1. Amazon fish parasites.* (Second edition), Vernon E.

Thatcher. Eds. Joachim Adis, Jorge R. Arias, Guillermo Rueda-Delgado and Karl Matthias Wantzen. Pensoft Series Faunísticas. Sofia-Moscow.

Boore, J.L. and Brown, W.M. (1994). Complete DNA sequence of the mitochondrial genome of the black chiton, *Katharina tunicata*. *Genetics* 138, 423-443.

Boore, J.L. and Brown, W.M. (1995). Complete sequence of the mitochondrial DNA of the annelid worm *Lumbricus terrestris*. *Genetics* 141, 305-319.

Boore, J.L. and Brown, W.M. (1998). Big trees from little genomes: mitochondrial gene order as a phylogenetic tool. *Current Opinion in Genetics and Development* 8, 668-674.

Boore, J.L. and Brown, W.M. (2000). Mitochondrial genomes of *Galathealinum*, *Helobdella* and *Platynereis*: sequence and gene arrangement comparisons indicate that Pogonophora is not a Phylum and Annelida and Arthropoda are not a sister taxa. *Molecular Biology and Evolution* 17 (1), 87-106.

Bueno- Silva, M., Boeger, W.A. and Pie, M.R. (2011). Choice matters: Incipient speciation in Gyrodactylus corydori (Monogenoidea: Gyrodactylidae). *International Journal for Parasitology* 41, 657-667.

Chabalin, E., Ferraz de Lima, J.A. and Alves, L.B.O.A. (1988). Análise comparativa entre o valor comercial do pacu (*Colossoma mitrei*) capturado e cultivado - Mercado de peixes de Cuiabá. *Boletim Técnico do CEPTA*, Pirassununga 1 (1), 49-60.

Clary, D.O. and Wolstenholme, D.R. (1985). The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization and genetic code. *Journal of Molecular Evolution* 22, 252-271.

Escalante, A.A., Freeland, D.E., Colins, W.E. and Lal, A.A. (1998). The evolution of primate malaria parasites based on the gene encoding cytochrome b from the linear mitochondrial genome. *Proceedings of National Academy of Sciences* USA 95, 8124-8129.

Feagin, J. E. (2000). Mitochondrial genome diversity in parasites. *International Journal for Parasitology* 30, 371-390.

Folmer, O., Black, M., Hoeck, W., Lutz, R. and Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cythocrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3 (5), 294-299.

Gasiev, A.I. and Shaikhaev, G.O. (2008). Lesions on mitochondrial genome and ways of its preservation. *Russian Journal of Genetics* 44 (4), 373-388.

Greig, T.W., Moore, M.K. and Woodley, C.M. (2005). Mitochondrial gene sequences useful for species identification of western North Atlantic Ocean sharks. *Fishery Bulletin* 103, 516-523.

Hansen, H, Bakke, T.A. and Bachmann, L. (2007). DNA taxonomy and barcoding of monogenean parasites: lessons from *Gyrodactylus*. *Trends in Parasitology* 23 (8), 363-367.

Huyse, T., Audenaert, V. and Volckaert, F.A.M. (2003). Speciation and host-parasite relationships in the parasite genus *Gyrodactylus* (Monogenea, Platyhelminthes) infecting gobies of the genus *Pomatoschistus* (Gobiidae, Teleostei). *International Journal for Parasitology* 33,1679-1689.

Huyse, T., Plaisance, L., Webster, B.L., Mo, T.A., Bakke, T.A., Bachmann, L. and Littlewood, D.T.J. (2007). The mitochondrial genome of *Gyrodactylus salaris* (Platyhelminthes: Monogenea), a pathogen of Atlantic salmon (*Salmo salar*). *Parasitology* 134, 739-747.

Huyse, T., Buchmann, K. and Littlewood, D.T.J. (2008). The mitochondrial genome of *Gyrodactylus derjavinoides* (Platyhelminthes: Monogenea) - A mitogenomic approach for *Gyrodactylus* species and strain identification. *Gene* 417, 27-34.

Hypsa, V. (2006). Parasite histories and novel phylogenetic tools: Alternative approaches to inferring parasite evolution from molecular markers. *International Journal for Parasitology* 36, 141-155.

Jeon, H.K., Kim, K.H. and Eom, K.S. (2007). Complete sequence of the mitochondrial genome of *Taenia saginata*: comparison with *T. solium* and *T. asiatica*. *Parasitology International* 56, 243-246.

Laslett, D. and Canbäck, B. (2008). ARWEN, a program to detect tRNA genes in metazoan mitochondrial nucleotide sequences. *Bioinformatics* 24, 172-175.

Le, T.H., Blair, D. and McManus, D.P. (2001). Complete DNA sequence and gene organization of the mitochondrial genome of the liverfluke, *Fasciola hepatica* L. (Platyhelminthes: Trematoda). *Parasitology* 123, 609-621.

Le, T.H., Blair, D. and McManus, D.P. (2002). Mitochondrial genomes of parasitic flatworms. *Trends in Parasitology* 18, 206-213.

Librado, P. and Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25, 1451-1452 doi:10.1093/bioinformatics/btp187.

Littlewood, D.T.J., Lockyer, A.E., Webster, B.L., Johnston, D.A. and Le, T.H. (2006). The complete mitochondrial genomes of *Schistosoma haematobium* and *Schistosoma spindale* and the evolutionary history of mitochondrial genome changes among parasitic flatworms. *Molecular Phylogenetics and Evolution* 39, 452-467.

Littlewood, D.T.J., Waeschenbach, A. and Nikolov, P.N. (2008). In search of mitochondrial markers resolving the phylogeny of cyclophyllidean tapeworms (Platyhelminthes, Cestoda) - a test study with Davaineidae. *Acta Parasitologica* 53 (2), 133-144.

Martins, M.L., Onaka, E.M., Moraes, F.R., Bozzo, F.R., Paiva, A.M.F.C. and Gonçalves, A. (2002). Recent studies on parasitic infections of freshwater cultivated fish in the State of São Paulo, Brazil. *Acta Scientarium* 24 (4), 981-985.

Matejusová, I., Gelnar, M., McBeath, A.J.A., Collins, C.M. and Cunningham, C.O. (2001). Molecular markers for gyrodactylids (Gyrodactylidae: Monogenea) from five fish families (Teleostei). *International Journal for Parasitology* 31, 738-745.

Matejusová, I., Gelnar, M., Verneau, O., Cunnigham, C.O. and Littlewood, D.T.J. (2003). Molecular phylogenetic analysis of the genus *Gyrodactylus* (Platyhelminthes: Monogenea) inferred from rDNA ITS region: subgenera versus species groups. *Parasitology* 127, 603-611.

Matejusová, I. and Cunningham, C.O. (2004). The first complete monogenean ribossomal RNA gene operon: sequence and secondary structure of the *Gyrodactylus salaris* Malmberg, 1957, large subunit ribosomal RNA gene. *The Journal of Parasitology* 90 (1), 146-151.

Nickisch-Rosenegk, M.von, Brown, W.M. and Boore, J.L. (2001). Complete sequence of the mitochondrial genome of the tapeworm *Hymenolepis diminuta*: Gene arrangements indicate that Platyhelminthes are Eutrochozoans. *Molecular Biology and Evolution* 18 (5), 721-730.

Olson, P.D. and Vasyl, V.T. (2005). Advances and Trends in the Molecular Systematics of the Parasitic Platyhelminthes. *Advances in Parasitology* 60, 1-79.

Pamplona-Basilio, M.C., Kohn, A. and Feitosa, V.A. (2001). New host records and description of the egg of *Anacanthorus penilabiatus* (Monogenea, Dactylogyridae). *Memórias do Instituto Oswaldo Cruz* 96 (5), 667-668.

Park, J.K., Kim, K.H., Kang, S., Kim.W., Eom, K.S. and Littlewood, D.T.J. (2007a). A common origin of complex life cycles in parasitic flatworms: evidence from the complete mitochondrial genome of *Microcotyle sebastis* (Monogenea: Platyheminthes). *BMC Evolutionary Biology* 7, 11. doi:10.1186/1471-2148-7-11.

Park, J.K., Kim, K.H., Kang, S., Jeon, H.K., Kim, J.H., Littlewood, D.T.J and Eom, K.S. (2007b). Characterization of the mitochondrial genome of *Diphyllobothrium latum* (Cestoda: Pseudophyllidea) - implications for the phylogeny of eucestodes. *Parasitology* 134, 749-759.

Perkins, E.M., Donnellan, S.C., Bertozzi, T. and Whitttington, I.D. (2010). Closing the mitochondrial circle on paraphyly of the Monogenea (Platyhelminthes) infers evolution in the diet of parasitic flatworms. *International Journal for Parasitology* 40, 1237-1245.

Plaisance, L., Littlewood, D.T.J., Olson, P.D. and Morand, S. (2005). Molecular phylogeny of gill monogeneans (Platyhelminthes, Monogenea, Dactylogyridae) and colonization of Indo-West Pacific butterflyfish hosts (Perciformes, Chaetodontidae). Zoological Science 34, 425-436.

Plaisance, L., Huyse, T., Littlewood, D.T.J., Bakke, T.A. and Bachmann, L. (2007). The complete mitochondrial DNA sequence of the monogenean *Gyrodactylus thymalli*, a parasite of grayling (*Thymallus thymallus*). *Molecular Biochemical Parasitology* 154, 190-194.

Pouyaud, L., Desmarais, E., Deveney, M. and Pariselle, A. (2006). Phylogenetic relationships among monogenean gill parasites (Dactylogyridae, Ancyrocephalidae) infesting tilapiine hosts (Cichlidae): systematic and evolutionary implications. *Molecular Phylogenetic Evotution* 38, 241-249.

Shekhovtsov, S.V., Katokhin, A.V., Kolchanov, N.A. and Mordvinov, V.A. (2010). The complete mitochondrial genomes of the liver flukes *Opisthorchis felineus* and *Clonorchis sinensis* (Trematoda). *Parasitology International* 59, 100-103.

Simková , A., Plaisance, L., Matejusová , I., Morand, S. and Verneau, O. (2003). Phylogenetic relationships of the Dactylogyridae Bychowsky, 1933 (Monogenea: Dactylogyridea): the need for the systematic revision of the Ancyrocephalinae Bychowsky, 1937. *Systematic Parasitology* 54, 1–11.

Simková , A., Morand, S., Jobet, E., Gelnar, M. and Verneau, O. (2004). Molecular phylogeny of congeneric monogenean parasites (*Dactylogyrus*): a case of intrahost speciation. *Evolution* 58, 1001–1018.

Sinniger, F., Chevaldoné, P. and Pawlowski, J. (2007). Mitochondrial genome of *Savalia savaglia* (Cnidaria, Hexacorallia) and early metazoan phylogeny. *Journal of Molecular Evolution* 64, 196-203.

Thatcher, V.E. and Brites-Neto, J. (1994). Diagnóstico, prevenção e tratamento das enfermidades de peixes neotropicais de água doce. *Revista Brasileira de Medicina Veterinária*, Rio de Janeiro 16 (3), 111-128.

Waeschenbach, A., Telford, M.J., Porter, J.S. and Littlewood, D.T.J. (2006). The complete mitochondrial genome of *Flustrellidra hispida* and the phylogenetic position of Bryozoa among Metazoa. *Molecular Phylogenetics and Evolution* 40, 195-207.

Webster, B.L., Rudolfová, J, Horák, P. and Littlewood, D.T.J. (2007). The complete mitochondrial genome of the bird schistosome *Trichobilharzia regenti* (Platyhelminthes: Digenea), causative agent of cercarial dermatitis. *Journal of Parasitology* 93 (3), 553-561.

Wolstenholme, D.R. (1992). Genetic novelties in mitochondrial genomes of multicellular animals. *Current Opinions in Genetics and Development* 2, 918-925.

Wu, X.Y., Zhu, X.Q., Xie, M.Q., Wang, J.Q. and Li, A.X. (2007). The radiation of *Thaparocleidus* (Monogenoidea: Dactylogyridae: Ancylodiscoidinae): phylogentic analyses and taxonomic implications inferred from ribosomal DNA sequences. *Parasitology Research* Springer.

Zhang, J., Wu, X., Xie, M. and Li, A. (2011). The mitochondrial genome of *Polylabris halichoeres* (Monogenea: Microcotylidae). *Mitochondrial DNA* 22 (1-2), 3-5.

Zietara, M.S., Huyse, T., Lumme, J. and Volckaert, F.A. (2002). Deep divergence among subgenera of *Gyrodactylus* inferred from rDNA ITS region. *Parasitology* 124, 39-52.

CONCLUSÕES

Os resultados obtidos neste estudo permitiram concluir que:

1) *H. piaractus* e *M. colossomatis* possuem diferentes sistemas de interação parasito-hospedeiro, como pinocitose para *H. piaractus* e fagocitose para *M. colossomatis*.

2) Posicionando filogeneticamente as espécies de mixosporídeos, *H. piaractus* e *M. colossomatis* agrupam no clado sul-americano.

3) Marcadores moleculares ribossomais, SSU e LSU identificaram e posicionaram filogeneticamente os monogeneas *A. penilabiatus* e *M. viatorum*, como táxons irmãos e agrupam com espécies da subfamília Ancyrocephalinae de outros continentes.

4) O genoma mitocondrial parcial de *A. penilabiatus*, possui rearranjos similares aos representantes disponíveis do grupo Monopisthocotylea e uma região não codificada com repetições em "tandem", se diferenciando de qualquer genoma disponível do mesmo grupo.

5) Os genes mitocondriais 16S (parcial) e 12S (completo) sequenciados para os monogeneas possuem características conservadas e variáveis, podendo ser usados como marcadores moleculares para dactylogyrídeos.

7- REFERÊNCIAS BIBLIOGRÁFICAS

Adriano, E. A.; Arana, S.; Ceccarelli, P. S. & Cordeiro, N. S. (2002). Light and scanning electron microscopy of *Myxobolus porofilus* sp. n. (Myxosporea: Myxobolidae) infecting the visceral cavity of *Prochilodus lineatus* (Pisces: Characiformes: Prochilodontidae) cultivated in Brazil. *Folia Parasitologica*. Vol. 49, p. 259-262.

Barassa, B.; Adriano, E. A.; Arana, S. C. & Cordeiro, N. C. (2003). *Henneguya curvata* sp. n. (Mixosporea: Myxobolidae) parasiting the gills of *Serrasalmus spilopleura* (Characidae: Serrasalmidae), a South American freshwater fish. *Folia Parasitologica*. Vol. 50, p. 151-153.

Bakke, T. A. & Harris, P. D. (1998). Diseases and parasites in wild Atlantic salmon (*Salmo salar*) populations. *Canadian Journal of Fisheries and Aquatic Sciences*. Vol. 55 (suppl. 1).

Bartholomew, J. L.; Atkinson, S. D.; Hallett, S. L.; Lowenstine, L. J.; Garner, M. M.; Gardiner, C. H.; Rideout, B. A.; Keel, M. K. & Brown, J. D. (2008). Myxozoan parasitism in waterfowl. *International Journal for Parasitology*. Vol. 38, p. 1199-1207.

Bernardino, G. & Lima, R. V. (1999). Situação da criação de *Colossoma* e *Piaractus* no sudeste do Brasil (1988-1991). In: *Criação de Colossoma e Piaractus no Brasil.* Brasília: IBAMA, p. 262-266.

Boeger, W. A. & Kritsky, D. C. (1997). Coevolution of the Monogenoidea (Platyhelminthes) based on a revised hypothesis of the parasite phylogeny. *International Journal for Parasitology*. Vol. 27, p. 1495-1511.

Boeger, W. A. & Kritsky, D. C. (2001). Phylogenetic relationships of the Monogenoidea. In: Littlewood, D.T.J. and Bray, R.A. (eds.). *Interrelationships of the Platyhelminthes*. Taylor & Francis. London. p.92-102.

Boeger, W. A. & Vianna, R.T. (2006). *Monogenoidea*. *In*: Aquatic Biodiversity in Latin America. V. 1. Amazon fish parasites. (Second edition), Vernon E.

Thatcher. Eds. Joachim Adis, Jorge R. Arias, Guillermo Rueda-Delgado and Karl Matthias Wantzen. Pensoft Series Faunísticas. Sofia-Moscow.

Buchmann, K. & Bresciani, J. (2006). Monogenea (Filo Platyhelminthes). In: Woo, P.T.K. Fish Diseases and Disorders, Volume 1: Protozoa and Metazoan Infections Second Edition. University of Guelph, Canada. CABI. 801p.

Bütschli, O. (1882). Myxosporidia. *In:* Bronn's Klassen und Ordnungen des Tierreichs. 1. *Protozoa*. Second Edition. C.F. Winter, Leipizig. p. 590-603.

Camargo, S. G. O. & Pouey, J. L. O. F. (2005). Aquicultura - Um mercado em expansão. *Revista Brasileira de Agrociências,* Pelotas. Vol. 11(4), p. 393-396.

Canning, E. U. & Okamura, B. (2004). Biodiversity and evolution of the Myxozoa. *Advances in Parasitology*. Vol. 56, p. 43-141.

Chabalin, E. & Ferraz de Lima, J. A. (1988). Análise econômica de um cultivo intensivo de pacu (*Colossoma mitrei*) no Centro-Oeste do Brasil. *Boletim Técnico do CEPTA*, Pirassununga, Vol.1(1), p. 61-68.

Ceccarelli, P. S.; Figueira, L. B.; Ferraz de Lima, C. L. B. & Oliveira, C. A. (1990). Observações sobre a ocorrência de parasitos no CEPTA entre 1983 e 1990. *Boletim Técnico do CEPTA.* Vol.3, p. 43-54.

Dias-Koberstein, T. C. R.; Carneiro, D. J. & Urbinati, E. C. (2005). Tempo de trânsito gastrintestinal e esvaziamento gástrico do pacu (*Piaractus mesopotamicus*) em diferentes temperaturas de cultivo. *Acta Scientarium of Animal Sciences.* Maringá, Vol. 27 (3), p. 413- 417.

Dyková, I. & Lom, J. (1978). Histopathological changes in fish gills infected with myxosporidian parasites of the genus *Henneguya*. *Journal of Fish Disease*. Vol.12, p. 197-202.

Eiras, J. C. (1994). *Elementos de Ictioparasitologia*. Porto, Portugal: Fundação Eng. Antônio de Almeida, 339p.

Eiras, J. C.; Takemoto, R. M. & Pavanelli, G. C. (2000). *Métodos de estudo e técnicas laboratoriais em parasitologia de peixes.* Editora Universidade Estadual de Maringá, Maringá. 173p.

Evans, N. M.; Holder, M. T.; Barbeiros, M. S.; Okamura, B. & Cartwright, P. (2010). The phylogenetic position of Myxozoa: exploring conflicting signals in phylogenomic and ribossomal data sets. *Molecular Biology and Evolution*. Vol. 27 (2), p. 2733-2746.

FAO. (1997). Aquaculture development. FAO Technical Guidelines for Responsible Fisheries. N. 5 Rome, b 40p. Disponível em: ftp//.fao.org/docrep/fao/003/W4493e/W4493e00.pdf acesso em 22/12/2011.

FAO. (2010). The State of World Fisheries and Aquaculture. FAO Fisheries and Aquaculture Department. Food and Agriculture Organization od the United Nations. Rome. 218p. Disponível em: http://www.fao.org/docrep/013/i1820e/i1820e00.htm acesso em 22/12/2011.

Ferraz de Lima, J. A. (1981). A pesca no Pantanal de Mato Grosso (rio Cuiabá: biologia e ecologia pesqueira). In: II Congresso Brasileiro de Engenharia de Pesca, Recife. Anais. p. 503-515.

Ferraz de Lima, J. A.; Barbieri, G. & Verani, Jr. (1984). Período de reprodução, tamanho e idade de primeira maturação gonadal do pacu, *Colossoma mitrei*, em ambiente natural (Rio Cuiabá, Pantanal do Mato Grosso). In: *Simpósio Brasileiro de Aqüicultura ,* Anais, São Carlos, p. 477-497.

Ferraz de Lima, J. A.; Ferrari, V. A.; Colares de Melo, J. S. et al. (1988). Comportamento do pacu, *Colossoma mitrei*, em cultivo experimental, no Centro-Oeste do Brasil. *Boletim Técnico do CEPTA*, Vol.1, p.15-28.

Feidi. (2001). Gift of the Nile. *Samudra*. Vol. 28, p. 3–7.

Figueira, L. B. & Ceccarelli, P. S. (1991). Observações sobre a presença de ectoparasitos em pisciculturas tropicais do interior (CEPTA e região). *Boletim Técnico do CEPTA.* Vol. 4, p. 57-65.

Freeman, M. A. & Shinn, A. P. (2011). Myxosporean hyperparasites of gill monogeneans are basal to the Multivalvulida. Parasites & Vectors Vol. 4,p. 220. http://www.parasitesandvectors.com/content/4/1/220

Friedrich, C.; Ingolic, E.; Freitag, B.; Kastberger, G.; Hohmann, V.; Skofitsch, G.; Neumeister, U. & Kepka, O. (2000). A myxozoan-like parasite causing

xenomas in the brain of the mole, *Talpa europea* L., 1758 (Vertebrata, Mammalia). *Parasitology*. Vol. 121, p. 483-492.

Garcia, L. O.; Copatti, C. E.; Wachholz, F.; Pereira-Filho, W. & Baldisserotto, B. (2008). Freshwater temperature in the state of Rio Grande do Sul, Southern Brazil, and its implication for fish culture. *Neotropical Ichthyology*. Vol. 6 (2), p. 275-281.

Gioia, I. & Cordeiro, N. C. (1996). Brazilian Mixosporidian checklist (Myxozoa). *Acta Protozoologica.* Vol. 35, p. 137-149.

Godoy, M. P. (1975). *Peixes do Brasil: subordem Characoidei: bacia do rio Mogi Guassu.* Piracicaba: Franciscana, Vol. 1-4, 216p.

Hartigan, A.; Fiala, I.; Dyková, I.; Jirku, M.; Okimoto, B.; Rose, K; Phalen, D. N. & Slapeta, J. (2011). A suspected parasite spill-back of two novel *Myxidium* spp. (Myxosporea) causing disease in australian endemic frogs found in two invasive cane toad. *Plos One*. Vol. 6 (4), p. 1-12.

Hudson, P. J.; Rizzoli, A.; Grenfell, B. T.; Heesterbeek, H. & Dobson, A. P. (2001). The ecology of wildlife diseases. Oxford University Press, Oxford. 197p.

Humason, G. L. (1979). Animal Tissue Techniques. Forth ed. W.H. Freeman and Co., San Francisco.

Jerônimo, G. T., Speck, G. M. & Martins, M. L. (2010). First report of *Enterogyrus cichlidarum* Paperna 1963 (Monogenoidea: Ancyrocephalidae) on Nile Tilapia *Oreochromis niloticus* cultured in Brazil. *Neotropical Helminthology*. Vol. 4 (1), p. 75-80.

Johnson, C. A. (1969). A redescription of *Myxidium chelonorum* Johnson, 1969 (Cnidospora: Myxidiidae) from various north american turtles. *Journal of Protozoology.* Vol. 16, p. 700-702.

Jurine, L.L. (1825). Histoire des poissons du Lac Léman. *Mémoires de la Société de Physique et d'Histoire Naturelle de Genève.* Vol. 3.

Kaur, H. & Singh, R. (2012). A synopsis of the species of *Myxobolus* Bürschli, 1882 (Myxozoa: Bivalvulida) parasitising Indian fishes and a revised

dichotomous key to myxosporean genera. *Systematic Parasitology*. Vol. 81, p. 17-37.

Kent, M. L.; Andree, K. B.; Bartholomeu, J. L.; El-Matbouli, M.; Desser, S. S.; Delvin, R. H.; Feist, S. W.; Hedrick, R. P.; Hoffmann, R. W.; Khattra, J.; Hallet, S. L.; Lester, R. J. G.; Longshae, M.; Palenzeula, O.; Siddall, M. E. & Xiao, C.X. (2001). Recent advances in our knowledge of the Myxozoa. *Journal Eukariotic Microbiolog*y. Vol. 48, p. 395-413.

Kritsky, D. C.; Boeger, W. A. & Popazoglo, F. (1995). Neotropical Monogenoidea. 22. Variation in *Scleroductus* species (Gyrodactylidea, Gyrodactylidae) from Siluriform fishes of southeastern Brazil. *Proceedings of Helminthological Society of Washington* Vol. 62 (1), p. 53-56.

Littlewood, D. T. J.; Rhode, K. & Clough. (1999). Interrelationships of all major groups of Platyhelminths: phylogenetic evidence from morphology and molecules. *Biological Journal of the Linnean Society.* Vol. 66, p. 75-114.

Lom, J. (1987). Myxosporea: a new look at longa t long - Known parasites of fish. *Parasitology Today*. Vol. 3, p. 327-332.

Lom J, & Arthur J.R. (1989). A guideline for the preparation of species description in Myxosporea. *Journal of Fish Diseases*. Vol. 12, p. 151-156.

Lom, J.; Yokohama, H. & Diková, I. (1997). Comparative ultrastructure of *Aurantiactinomyxon* and *Raabeia*, actinosporean stages of myxozoan life cycles. *Archiv Protistenkunde.* Vol. 148, 173–189.

Lom, J. & Diková, I. (2006). Myxozoan genera; definition and notes on taxonomy, life-cycle terminology and pathogenic species. *Folia Parasitologica*. Vol. 53, p. 1-36.

Martins, M. M. & Romero, N. G. (1996). Efectos del parasitismo sobre el tecido branquial en peces cultivados: estudo parasitologico histopatologico. *Revista Brasileira de Zoologia*. Vol. 13 (2), p. 489-500.

Martins, M. L.; Souza, V. N.; Moraes, F. R.; Moraes, J. R. E & Costa, A. J. (1997). Pathology and behavioral effects associated with *Henneguya* sp. (Mixozoa: Mixobolidae) infections of captive pacu *Piaractus mesopotamicus* in Brazil. *Journal of World Aquaculture Society*. Vol. 28, p. 297-300.

May, R. M. & Anderson, R. M. (1991). Infectious diseases of humans. Oxford University Press, Oxford. 757p.

Mekong River Commission. (2003). Lao legends. Catch and culture. Vol. 9 (1): 11.

McCraren, J. P.; Landolt, M. L. & Hoffman, G. L. (1975). Variation in response of channel catfish to *Henneguya* sp. Infections (Protozoa: Myxosporidea). *Journal of Wildlife Diseases.* Vol. 11, p. 2-7.

Morris, D. J. (2010). Cell formation by myxozoan species is not explained by dogma. *Proceedings of the Royal Society Biological Sciences*. Vol. 277, p. 2565-2570.

Ministéria da Pesca e Aquicultura - MPA (2009). *Boletim Estatístico da Pesca e Aquicultura, Brasil 2008-2009.* Ministério da Pesca e Aquicultura, Governo Federal. Brasília. 99p. Disponível em: http://www.mpa.gov.br/images/Docs/Publicidade/anuário%20da%20pesca%20completo2.pdf. Acesso em 21/12/2011.

Müller, J. (1841). Über Psorospermien. *Archiv für Anatomie, Physiologie und wissenschaftliche Medicin.* Vol. 5, p. 477-496.

Overstreet, R. M. (1976). *Fabespora vermicola* sp. n., the first Myxosporidan from a Platyhelminth. *Journal of Parasitology*. Vol. 62, p. 680–684.

Okamura, B. (1996). Occurrence, prevalence, and effects of the myxozoan *Tetracapsula bryozoides* parasitic in the freshwater bryozoan *Cristatella mucedo* (Bryozoa: Phylactolaemata). *Folia Parasitologica*. Vol. 43, p. 262–266.

Olson, P. D. & Littlewood, D. T. J. (2002). Phylogenetics of the Monogenea - evidence from a medley of molecules. *International Journal for Parasitology*. Vol. 32, p. 233-244.

Petrere, Jr. M. (1989). River fisheries in Brazil: a review. *Regulated Rivers: Research and Management.* Vol. 4, p. 1-16.

Phuong, N. T.& Oanh, D. T. (2010). Striped catfish aquaculture in Vietnam: A decade of unprecendented development. In: Sena S. De Silva & F.Bryan Davy (editors). *Success Stories in Asian aquaculture*. Springer. 214p.

Putz, R. E. & Hoffman, G. L. (1963). Two new *Gyrodactylus* (Trematoda, Monogenea) from cyprinid fishes with synopsis of those found on North American fishes. *Journal of Parasitology.* Vol. 49, p. 559-566.

Salaro, A. L.; Pezzato, L. E.; Barros, M. M.; Del Carratore, C. R. & Rosa, G. J. M. (1992). Influência da oscilação térmica sobre o ganho de peso de alevinos de pacu, tambacu, carpa comum durante o inverno de Botucatu. In: *Simpósio Brasileiro de Aquicultura. Anais*, Peruíbe. Vol. 7, p. 117-121.

Siau, Y.; Gasc, C. & Maillard, C. (1981). Premiéres observations ultrastructurales d'une myxosporidie appartenant au genre *Fabespora*, parasite de trématode. *Protistologica*. Vol. 17, p. 131–137.

Siddal, M. E.; Martin, D. S.; Bridge, D.; Cone, D. M. & Desser, S. S. (1995). The demise of a phylum of protists. Phylogeny of Myxozoa and other parasitic cnidaria. *Journal of Parasitology*. Vol. 81, p. 961-967.

Sitjá-Bobadilla, A. (2008). Fish immune response to myxozoan parasites. *Parasite*. Vol. 15, p. 420-425.

Smothers, J. F.; von Dohlen, C. D.; Smith, L. H. & Spall, R. D. (1994). Molecular evidence that the myxozoan protists are metazoans. *Science*. Vol. 265, p. 1719-1721.

Souza, E. C. P. M. & Teixeira-Filho, A. R. (1985). *Piscicultura Fundamental*. USP, Nobel. 88p.

Thurston, J. P. & Laws, R. M. (1965). *Oculotrema hippopotami* (Trematoda: Monogenea) in Uganda. *Nature*. Vol. 205, p. 1127.

Urbinati, E. C. & Gonçalves, F. D. (2005). Pacu (*Piaractus mesopotamicus*) in: Baldisserotto, B. & Gomes, L.C. *Espécies Nativas para piscicultura no Brasil.* Editora UFSM. Santa Maria. 470p.