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Supplementary studies on *Anacanthorus penilabiatus* and *Mymarothecium viatorum* (Monogenea: Dactylogyridae) from *Piaractus mesopotamicus* (Characiformes: Serrasalmidae) in Brazil

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Abstract

The dactylogyrid monogeneans *Anacanthorus penilabiatus* (Boeger, Husak and Martins, 1995) and *Mymarothecium viatorum* (Boeger, Piasecki and 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, Brazil. Morphological and molecular identification, scanning electron microscopy, histopathological and descriptive parameters data 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 (MII) data was taken at each location. The data showed prevalence and mean intensity of infection annually, with numbers such as P = 91.2% and MII = 50 worms/fish (Pirassununga), P = 60% and MII = 39.2 worms/fish (Mogi Mirim), and P = 100% and MII = 204.8 worms/fish (Itapira). Histopathological results showed hyperplasia of the secondary lamellae, and hypersecretion of mucus was also observed. A phylogenetic topology was inferred using complete SSU (18S) ribosomal rDNA, positioning *Anacanthorus penilabiatus* and *Mymarothecium viatorum* among other monopisthocotyleans available in GenBank.

Keywords

Freshwater fish, pacu, monogenean, ribosomal DNA

Introduction

The freshwater fish *Piaractus mepotamicus*, popularly known as "pacu", is an important species for aquaculture and popular among consumers. Because of its high reproductive capacity, rapid growth and commercial value, it is one of the most cultivated freshwater fish in Brazil. Diseases caused by parasites in fish farms are a common problem.

Monogenea (Platyhelminthes) are mainly fish parasites, mostly ectoparasites, and are hermaphroditic, with direct life-cycles (Boeger and Vianna 2006). They can be found in fish in marine, brackish water and freshwater habitats (Buchmann and Bresciani 2006). In South America the most abundant taxon is probably the Dactylogyridae family (Monopisthocotylea), although species belonging to the species-rich family Gyrodactylidae are also recorded (Boeger and 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 and Littlewood 2002; Matejusová and Cunningham 2004) and also internal transcribed spacers (ITS) (Cunningham 1997; 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 suitable PCR products elucidates differences and variations between species, and ribosomal DNA genes 18S and 28S and spacers have been found to be effective indicators (e.g. Buchmann and Bresciani 2006).The dactylogyrid monogeneans

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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. The aim of the present study was to collect and calculate descriptive parameters data, such as prevalence and mean intensity of infection, from the monogeneans found in *P. mesopotamicus* in different fish farms, also access the external morphological characteristics from the worms and relate it, with histopathological data to study the disease. In addition, the complete ribosomal gene SSU (18S) of the species *A. penilabiatus* and *M. viatorum* was characterized to evaluate its use for species diagnostic and phylogenetic purposes.

Materials and Methods

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 and Vianna (2006). This study is in agreement with the Ethical Principles for Animal Research established by the Brazillian College for Animal Experimentation (COBEA) protocol number 1476-1 and appproved by the institutional Commitee for Ethics in Animal Research of the State University of Campinas – Unicamp.

For morphological identification, monogenean specimens were kept in diluted formaldehyde (5%) and mounted in Hoyer medium and Grey and Wess and stained in Gomori's Trichrome (Putz and Hoffman 1963; Kritsky *et al.* 1995; Eiras *et al.* 2000). The keys for identification used were Boeger *et al.* (1995, 2002), Cohen and 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 of infection data were calculated annually according to Bush *et al.* (1997). Intensity was evaluated by counting the total monogenea in each infected organ (gills). 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 averages of prevalence and mean intensity of infection for location, year and species ($p < 0.05$ significance level).

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.

For SEM 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 tetroxide 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).

For molecular techniques, 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 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 and 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

Table I. Primers used to amplify and sequence 18S fragments of *Anacanthorus penilabiatus* and *Mymarothecium viatorum*

Primers Name		Sequence (5' to 3') F – foward, R – reverse	Source
gene18S rDNA			
worm A	F	GCGAATGGCTCATTAAATCAGL	Littlewood and Olson (2001)
worm B	R	CTTGTTACGACTTTTACTTCC	Littlewood and 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 and Olson (2001)
1200F	F	CAGGTCTGTGATGCCC	Littlewood and Olson (2001)
1200R	R	GGGCATCACAGACTTG	Littlewood and Olson (2001)

Table II. Prevalence, mean intensity of infection and range in parenthesis 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)

Locality	Year	Hosts (n)	Prevalence (%)	Mean intensity
Pirassununga	2008	94	85.1b	31.8b (1–172)
	2009	90	98.8a	68.2a (1–309)
	2010	34	94.1b	41.5b (2–153)
Mogi Mirim	2009	20	100a	44.0a (5–207)
	2010	30	33.4b	28.4b (3–92)
Itapira	2009	10	100	204.8 (35–546)

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 analysis were ribosomal small subunit 18S. The set of primers used are expressed in Table I.

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 and Maddison 2000).

The phylogenetic analyses, was conducted using 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).
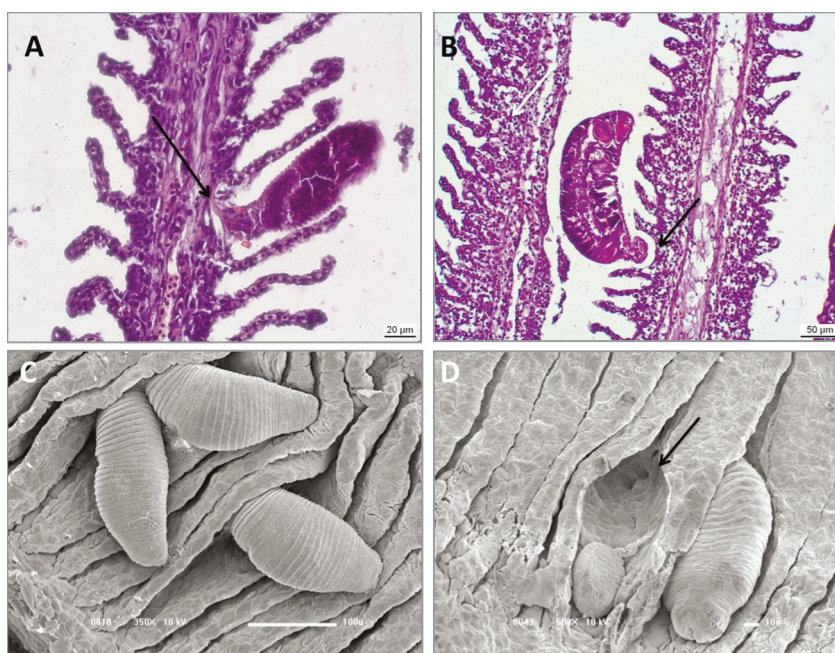
Fig. 1. Histological longitudinal sections of monogeneans parasitizing gills of *Piaractus mesopotamicus*. **A** – *Mymarothecium viatorum* haptor's attachment in the epithelium of gill lamellae (black arrow), **B** – *Anacanthorus penilabiatus* haptor's attachment in the epithelium of the gill (black arrow) with highly infection causing hyperplasia (white arrow); (**C** and **D**) Scanning electron micrographs of *Mymarothecium viatorum* infection, **D** – Empty space caused by the worm, showing a depression at the worm's attachment zone (black arrow)
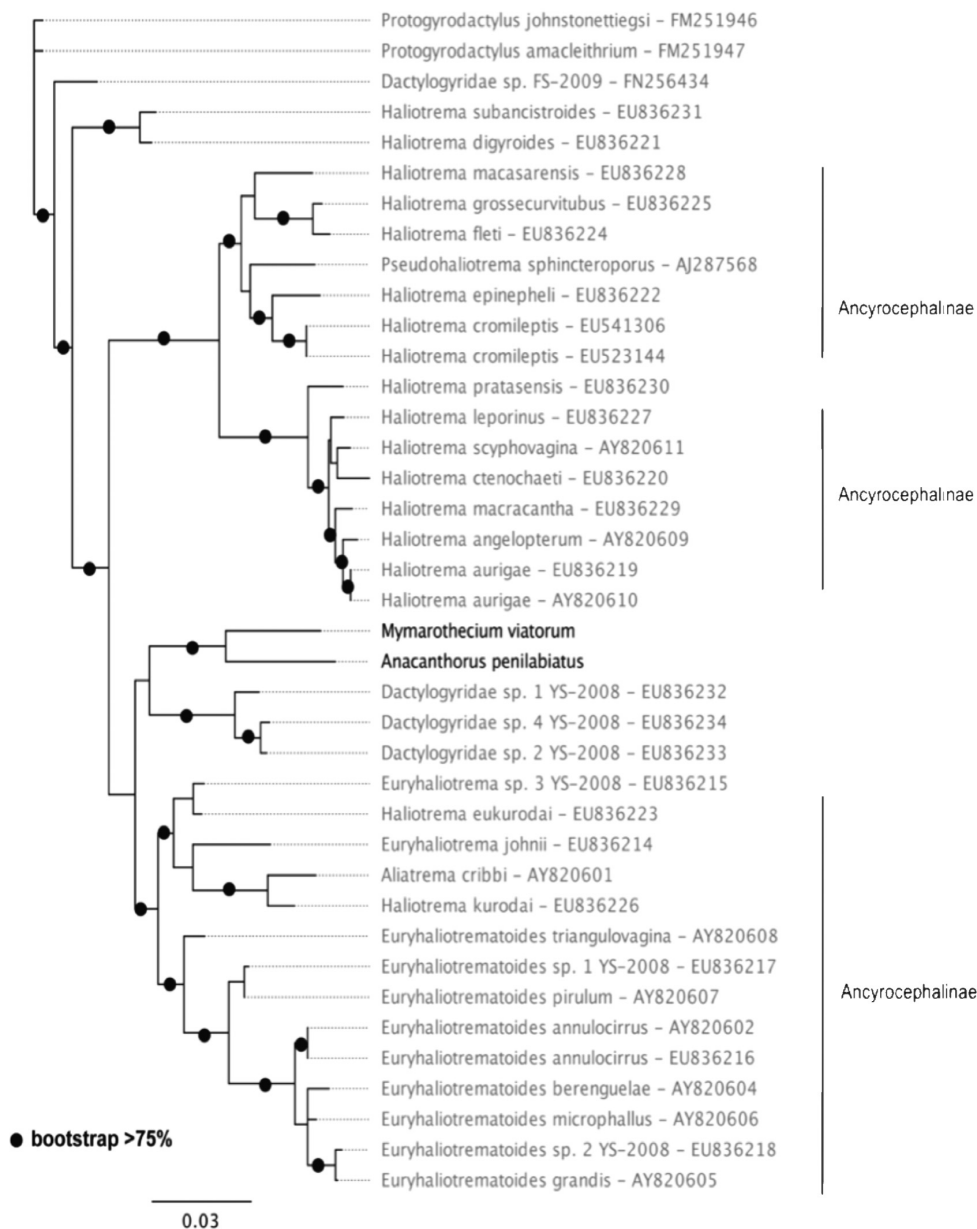
Results

Two dactylogyrid species (*A. penilabiatus* and *M. viatorum*) were identified in all three locations. Morphology and measurements of the specimens of *Anacanthorus penilabiatus* and *Mymarothecium viatorum* from *P. mesopotamicus* are in agreement with original description from each species. As regards annual prevalence, in the Pirassununga fish farm, prevalence was high during the studied period. The highest prevalence was found in 2009, followed by 2010, with the lowest percentage in 2008 (Table II). Mean intensity of infection 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 analyses 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

Fig. 2. Phylogenetic position of *Anacanthorus penilabiatus* and *Mymarothecium viatorum* among available dactylogyrids 18S rDNA sequences, using Maximum Likelihood

II). 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 on spring 2009, during an outbreak. 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). Scanning electron micrographs showed the worms in the filament (1C) and a depression on the gill filament caused by the attachment, showing the attachment zone of the worm (Fig. 1D). Also observed was cellular proliferation (hyperplasia) and fusion of the lamellae (Fig. 1B) and hypersecretion of mucus in the gills (not shown).

The fragments amplified by complete SSU rDNA (18S) molecular markers for both species, *A. penilabiatus* accession number KU941837 and *M. viatorum* accession number KU941838, could successfully diagnose the species, and distinguish then. Complete 18S sequences had a length of approximately 1890bp for *A. penilabiatus* and 1855bp 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 (>75%) nodal support, with *A. penilabiatus* and *M. viatorum* grouping together, as a sister taxon of the clade composed by the other Dactylogyridae spp. (Fig. 2).

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. Pirassununga had higher prevalence and mean intensity values than Mogi Mirim fish farm. This fact may be associated with the characteristics of Pirassununga fish farm, which belongs to a Brazilian government research institution (CEPTA-ICMBio) and the main goal is research and not commercial production. Sampling at the Mogi Mirim fish farm was undertaken over two years. 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 could be related to the

handling method performed by the owner of this fish farm, who reported using salt to kill parasites before releasing the fishes to the ponds.

The main goal of the Mogi Mirim fish farm is as a hatchery, and at each production cycle, the ponds are emptied and dried. Meanwhile, Pirassununga fish farm is a non-profitable research facility, and its principle aim is not to optimize the productivity, thus the techniques of pond emptying and drying are not routinely used. Pirassununga fish farm is an old fish farm, which was founded in 1939, and has a long history of development study of handling techniques from Neotropical fishes, mainly *P. mesopotamicus*, which is the focus of this study.

In Itapira the high parasite 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, such as temperature and oxygen, 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 problematic, 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 and Brites-Neto 1994; Martins *et al.* 1999; Tavares-Dias *et al.* 2001).

Hyperplasia response can be observed as a result of 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 and Jones 2000; Kritsky and 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 and Romero 1996).

In respect of molecular studies, the gene 18S ribosomal marker 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 and Bresciani 2006). Other studies have been reported using the ribosomal

gene 18S 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; Gilmore *et al.* 2012; Prikrylová *et al.* 2013).

The 18S complete sequences provided a phylogenetic topology of the South American monogeneans *A. penilabiatus* and *M. viatorum* among dactylogyrids sequences available in GenBank. The species are resolved as 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 and Vianna 2006), and although little molecular studies are currently available, results here suggest the 18S rRNA gene may be of use in providing some phylogenetic structure to these taxa.

The Dactylogyridae represent a highly diversified group and is constituted of 9 subfamilies, i.e. the Dactylogyrinae, Ancyrocephalinae, Linguadactylinae, Linguadactyloidinae, Haerocephalinae, Heterotesiinae, Ancylodiscoidinae, Pseudodactylogyrinae and Anacanthorinae (Gibson *et al.* 1996; Kritsky and Boeger, 1989). Phylogenetic relationships among families and subfamilies within Dactylogyrinea Bychowsky 1937 remain unresolved (Kritsky and Boeger 1989; Lim 1998; Simková *et al.* 2003). For example, the unresolved Ancyrocephalidae terminology *sensu* Bychowsky and Nagibina (1978) and Ancyrocephalinae *sensu* Kritsky and Boeger (1989) have been examined in several published phylogenetic studies such as Klassen (1994), Lim (1998), Mollaret *et al.* (2000), Simková *et al.* (2003, 2006), Plaisance *et al.* (2004, 2005).

Kritsky and Boeger (1989) used morphological characters to resolve the phylogenetic relationships among families and subfamilies of Dactylogyridea and they revealed the non-monophyly of the Ancyrocephalidae. Simková *et al.* (2003) tested the status Ancyrocephalidae terminology *sensu* Bychowsky and Nagibina (1978) and Ancyrocephalinae *sensu* Kritsky and Boeger (1989) using molecular data with SSU marker and confirmed the polyphyletic relationships. Later studies confirmed the non-monophyly of the Ancyrocephalinae such as Simková *et al.* (2006), Plaisance *et al.* (2005).

The phylogenetic position of the Brazilian dactylogyrid species *M. viatorum* (Ancyrocephalinae) and *A. penilabiatus* (Anacanthorinae) are well supported and placed between Ancyrocephalinae clades which also confirm the non-monophyly relationships between these subfamilies. The other 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 include 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 mor-

phology (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 (Plaisance *et al.* 2005).

In the context of dactylogyrid 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 prevalence and mean intensity of infection data and histopathological analysis of the dactylogyrids *A. penilabiatus* and *M. viatorum*, from *P. mesopotamicus* in fish farms from Sao Paulo state, an important fish for aquaculture and also, useful molecular tool for diagnostics of monogeneans in South America. Although molecular information regarding monogeneans in South America is generally lacking, and there remains a need to resolve the interrelationships of the Dactylogyridae, 18S rDNA has been shown to be of utility in broad scale phylogenetic studies.

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