

Description of microsporidia in simuliids: molecular and morphological characterization of microsporidia in the larvae of *Simulium pertinax* Kollar (Diptera: Simuliidae)

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ABSTRACT

Introduction: Microsporidia constitute the most common black fly pathogens, although the species' diversity, seasonal occurrence and transmission mechanisms remain poorly understood. Infections by this agent are often chronic and non-lethal, but they can cause reduced fecundity and decreased longevity. The objective of this study was to identify microsporidia infecting *Simulium* (Chirostilbia) *pertinax* (Kollar, 1832) larvae from Caraguatatuba, State of São Paulo, Brazil, by molecular and morphological characterization. **Methods:** Larvae were collected at a single point in a stream in a rural area of the city and were kept under artificial aeration until analysis. *Polydispyrenia* spp. infection was characterized by the presence of at least 32 mononuclear spores measuring $6.9 \pm 1.0 \times 5.0 \pm 0.7 \mu\text{m}$ in persistent sporophorous vesicles. Similarly, *Amblyospora* spp. were characterized by the presence of eight uninucleate spores measuring $4.5 \times 3.5 \mu\text{m}$ in sporophorous vesicles. **Results:** The molecular analysis confirmed the presence of microsporidian DNA in the 8 samples (prevalence of 0.51%). Six samples (Brazilian larvae) were related to *Polydispyrenia simulii* and *Caudospora palustris* reference sequences but in separate clusters. One sample was clustered with *Amblyospora* spp. *Edhazardia aedis* was the positive control taxon. **Conclusions:** Samples identified as *Polydispyrenia* spp. and *Amblyospora* spp. were grouped with *P. simulii* and *Amblyospora* spp., respectively, corroborating previous results. However, the 16S gene tree showed a considerable distance between the black fly-infecting *Amblyospora* spp. and the mosquito-infecting spp. This distance suggests that these two groups are not congeneric. Additional genomic region evaluation is necessary to obtain a coherent phylogeny for this group.

Keywords: Microsporidae. *Amblyospora* spp. *Polydispyrenia* spp. Phylogenetic analysis.

INTRODUCTION

Black flies (Diptera: Simuliidae) cause severe medical and veterinary problems worldwide. Simuliidae species are able to transmit parasites that can result in severe disease in humans and animals. In addition, their bites can cause allergic reactions and dermatitis in sensitized individuals, resulting in severe economic losses to tourism centers and negatively impacting animal production¹⁻³. Black fly control remains a

major public health challenge. Microsporidia are unicellular, eukaryotic organisms that are obligate, intracellular parasites with public health relevance⁴. Several studies have suggested a new classification for microsporidia as fungi, but Ebersberger⁵ stated that phylogenetic analysis did not support fungal characterization for this group.

Microsporidia are the most common black fly pathogens, although the species' diversity, seasonal occurrence and transmission mechanisms remain poorly understood^{6,7}. Infections caused by this agent are often chronic and non-lethal, but they can cause sub-lethal host effects, such as reduced fecundity, decreased life span and general loss of vigor⁸.

The objective of this study was to identify microsporidian species infecting *Simulium* (Chirostilbia) *pertinax* (Kollar, 1832) larvae from Caraguatatuba City, on the north coast of State of São Paulo, by molecular and morphological characterization.

The city's economy greatly depends on tourism. Thus, the Simuliidae population plays an important role because black

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fly bites annoy visitors and have deleterious effects on the local economy. Monitoring and controlling black flies are essential to avoiding seasonal population outbreaks.

METHODS

Sampling and biological material processing

The sampling period was from May to August 2013, and the samples were collected from a stream in Caraguatatuba City, located on the north coast of the State of São Paulo, Brazil, which has a total area of 458,097km² and had a population at that time of 100,840⁹. All of the larvae were held in aerated containers with water from the breeding site until examination. Tissues showing evidence of infection (whitish abdomens or whitish digestive tracts) were dissected in NaCl 0.9% solution, and fat bodies and adjacent tissues were removed¹⁰. Processed samples were frozen in 1.5ml tubes with 30µl of diethylpyrocarbonate (DEPC) (Invitrogen® Life Technologies, Carlsbad, CA, USA). Fresh smears of fat bodies were made, fixed with methanol for 5min and stained with 10% Giemsa in 7.4 pH buffer for 20min. The slides were washed in water and dried at 25°C overnight¹¹ for further morphological analysis of spores.

Morphological analysis

The Nis Elements F 3.0 NIKON H550S software, with phase III objective scale 100X settings, was used for spore measurement. Morphological characterization was performed according to Sprague¹².

Molecular assay

Molecular assays were performed with frozen tissues from infected larvae, and *Aedes aegypti* larvae infected with *Edhazardia aedis* were used as positive controls.

DNA extraction

Larvae exhibiting symptoms of infection had deoxyribonucleic acid (DNA) extracted using a viral DNA kit (QIAamp® viral RNA, Qiagen, Inc, Hilden, Germany). Healthy larvae (**Figure 1A**) were discarded. Tissue samples were processed with a proteinase K kit, incubated at 56°C for 2h and mixed every 20min. The supernatants were used to amplify the r16S ribosomal gene¹³.

Small subunit ribosomal gene (SSUrDNA) PCR (r16S)

Polymerase chain reaction (PCR) amplification was performed with 18f (CAC CAG GTT GAT TCT GCC) and 1492r (GGT TAC CTT GTT ACG ACT T), according to Vossbrinck et al.¹⁴.

The amplification products were visualized on 2% agarose gels, with positive and negative controls and a 100 bps ladder (Invitrogen® Life Technologies, Carlsbad, CA, USA), following electrophoresis.

Nucleotide sequencing

PCR products were purified with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Limited, Little Chalfont, Buckinghamshire, UK) and were quantified with 2% agarose gel ethidium bromide staining, according to the Low



FIGURE 1 - *Simulium pertinax* larvae. Healthy larvae with normal coloration of the integument (A). Larvae with symptoms of microsporidian infection in the fat bodies (B).

DNA Mass Ladder (Invitrogen®) protocol. The products were sequenced using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems), following the standard manufacturer protocols. The data were analyzed with the phred/phrap software, and the contigs were assembled with the cap3 software¹⁵.

Phylogenetic analysis

The analyses were performed using the Seaview software¹⁶. A phylogenetic tree was constructed, with reference sequences³²⁻⁴⁶ from **Table 1** (supplementary file), using the maximum likelihood method with the general time reversible (GTR) model of nucleotide substitution and gamma distribution (G) (GTR + G)¹⁷. The model was selected by the Modeltest software, version 3.0.6¹⁸, and was optimized by the Seaview software. We calculated the bootstrap values with 1,000 replications to support the verification of branches in the topologies of the trees obtained, and bootstrap values greater than 70 were considered significant.

Nucleotide sequences and accession numbers

The nucleotide sequences obtained in this work were submitted to the GenBank nucleotide sequences databank under the following accession numbers GenBank: KC855552-KC855557 (L1_L6); and GenBank: KC855558 (L2).

TABLE 1 - Sequences and accession numbers used for phylogenetic analysis.

Organism	Host	Geographic locale	Accession number
<i>Amblyospora bracteata</i>	<i>Odagamia ornata</i>	Czech Republic	AY090068 ³²
<i>Antonosporea scoticæ</i>	<i>Andrena scotica</i>	USA	AF024655*
<i>Paranosema grylli</i>	<i>Gryllus bimaculatus</i>	St. Petersburg, FL, USA	AY305325 ³³
<i>Polydispyrenia simulii</i>	<i>Odagamia ornata</i>	Czech Republic	AY090069 ³²
<i>Weiseria palustris</i>	<i>Cnephia ornithophilia</i>	USA	AF132544*
<i>Nosema algerae</i>	<i>Anopheles stephensi</i>	Illinois, USA	AF069063 ³⁴
<i>Thelohania solenopsae</i>	<i>Solenopsis invicta</i>	USA	AF031538 ²⁸
<i>Janacekia debaisieuxi</i>	<i>Odagamia ornata</i>	USA	AY090070 ³⁵
<i>Hamiltosporidium magnivora</i>	<i>Daphnia magna</i>	Russia	AJ302318.1*
<i>Ichthyosporidium</i> sp.	<i>Leiostomus xanthurus</i>	Not Informed	L39110 ³¹
<i>Glugea anomala</i>	<i>Gasterosteus aculeatus</i>	Norway	AF044391.1 ³⁶
<i>Vavraia oncoperæ</i>	<i>Wiseana</i> spp.	New Zealand	X74112 ³⁷
<i>Vavraia culicis</i>	<i>Aedes albopictus</i>	USA	AJ252961 ²⁹
<i>Endoreticulatus schubergi</i>	<i>Lymantria dispar</i>	Switzerland	L39109 ³¹
<i>Vittaforma corneum</i>	<i>Homo sapiens</i>	USA	L39112 ³¹
<i>Nucleospora salmonis</i>	<i>Oncorhynchus tshawytscha</i>	Canada	U78176 ³⁸
<i>Enterocytozoon bieneusi</i>	<i>Homo sapiens</i>	USA	AF024657 ³⁹
<i>Encephalitozoon cuniculi</i>	<i>Oryctolagus cuniculus</i>	USA	Z19563.1 ⁴⁰
<i>Encephalitozoon intestinalis</i>	<i>Homo sapiens</i>	USA	U09929 ⁴¹
<i>Encephalitozoon hellem</i>	<i>Homo sapiens</i>	USA	L19070 ⁴²
<i>Nosema bombycis</i>	<i>Bombyx mori</i>	Switzerland	L39111 ³¹
<i>Vairimorpha necatrix</i>	<i>Malacosoma americanum</i>	Not Informed	Y00266 ⁴
<i>Nosema vespula</i>	<i>Species Unknown</i>	USA	U11047*
<i>Nosema apis</i>	<i>Apis mellifera</i>	New Zealand	U97150.1 ⁴³
<i>Amblyospora ferocious</i>	<i>Psorophora ferox</i>	Argentina	AY090062 ³²
<i>Amblyospora criniferis</i>	<i>Aedes cernifera</i>	Argentina	AY090061 ³²
<i>Amblyospora stimuli</i>	<i>Diaxyclops bicuspidatus</i>	USA	AY090050 ³²
<i>Amblyospora canadensis</i>	<i>Ochlerotatus canadensis</i>	USA	AY090056 ³²
<i>Amblyospora cinerei</i>	<i>Aedes cinereus</i>	USA	AY090057 ³²
<i>Amblyospora cinerei</i>	<i>Acanthacyclops vernalis</i>	USA	AY090059 ³²
<i>Amblyospora cinerei</i>	<i>Acanthacyclops vernalis</i>	USA	AY090058 ³²
<i>Amblyospora cinerei</i>	<i>Cyclops venustoides</i>	USA	AY090060 ³²
<i>Amblyospora connecticus</i>	<i>Ochlerotatus cantator</i>	USA	AF025685*
<i>Amblyospora excrucii</i>	<i>Ochlerotatus excrucians</i>	USA	AY090043 ³²
<i>Amblyospora stimuli</i>	<i>Aedes stimulans</i>	USA	AF027685 ²⁷
<i>Amblyospora excrucii</i>	<i>Acanthocyclops vernalis</i>	USA	AY090044 ³²
<i>Amblyospora khaliulini</i>	<i>Ochlerotatus communis</i>	USA	AY090045 ³²
<i>Amblyospora khaliulini</i>	<i>Acanthocyclops vernalis</i>	USA	AY090046 ³²
<i>Amblyospora khaliulini</i>	<i>Acanthocyclops vernalis</i>	USA	AY090047 ³²

Table 1 - continues....

TABLE 1 - Continuation.

Organism	Host	Geographic locale	Accession number
<i>Amblyospora weiseri</i>	<i>Ochlerotatus cantans</i>	USA	AY090048 ³²
<i>Amblyospora stictici</i>	<i>Ochlerotatus sticticus</i>	USA	AY090049 ³²
<i>Edhazardia aedis</i>	<i>Aedes aegypti</i>	Thailand	AF027684 ²⁷
<i>Amblyospora</i> sp.	<i>Cyclops strenuus</i>	Czech Republic	AY090055 ³²
<i>Amblyospora californica</i>	<i>Culex tarsulis</i>	USA	U68473 ⁴⁴
<i>Amblyospora</i> sp.	<i>Culex nigripalpus</i>	USA	AY090053 ³²
<i>Amblyospora</i> sp.	<i>Culex salinarius</i>	USA	U68474 ⁴⁴
<i>Amblyospora salinaria</i>	<i>Culex salinarius</i>	USA	AY326270 ³²
<i>Culicospora magna</i>	<i>Culex restuans</i>	USA	AY090054 ³²
<i>Culicospora magna</i>	<i>Culex restuans</i>	USA	AY326269 ³²
<i>Intrapredatorus barri</i>	<i>Culex fuscanus</i>	Norway	AY013359 ⁴⁵
<i>Amblyospora indicola</i>	<i>Culex sitiens</i>	India	AY090051 ³²
<i>Amblyospora opacita</i>	<i>Culex territans</i>	USA	AY090052 ³²
<i>Hyalinocysta chapmani</i>	<i>Culiseta melanura</i>	USA	AF483837 ⁴⁶
<i>Hyalinocysta chapmani</i>	<i>Orthocyclops modestus</i>	USA	AF483838 ⁴⁶
<i>Culicosporella lunata</i>	<i>Culex pilosus</i>	USA	AF027683 ²⁷
<i>Parathelohania anophelis</i>	<i>Anopheles quadrimaculatus</i>	USA	AF027682 ²⁷
<i>Parathelohania obesa</i>	<i>Anopheles crucians</i>	USA	AY090065 ³²
<i>Trichotuzetia guttata</i>	<i>Cyclops vicinus</i>	Czech Republic	AY326268 ³²
<i>Hazardia milleri</i>	<i>Culex quinquefasciatus</i>	Argentina	AY090067 ³²
<i>Hazardia</i> sp.	<i>Anopheles crucians</i>	USA	AY090066 ³²
<i>Marsoniella elegans</i>	<i>Cyclops vicinus</i>	Czech Republic	AY090041 ³²
<i>Gurleya vavrai</i>	<i>Daphnia longispina</i>	Finland	AF394526 ³⁰
<i>Gurleya daphniae</i>	<i>Daphnia pulex</i>	Austria	AF439320 ³⁰
<i>Larssonsonia obtusa</i>	<i>Daphnia pulex</i>	Sweden	AF394527 ³⁰
<i>Berwaldia schaefernai</i>	<i>Daphnia galeata</i>	Czech Republic	AY090042 ³²
<i>Varimorpha</i> sp.	<i>Solenopsis richteri</i>	USA	AF031539 ²⁸
<i>Amblyospora</i> sp.	<i>Simulium</i> sp.	UK	AJ252949 ²⁹

USA: United States of America; FL: Florida; UK: United Kingdom.*Unpublished.

RESULTS

A total of 1,574 *S. pertinax* larvae were examined. Eight larvae exhibited symptoms of microsporidian infection localized to the fat body (**Figure 1B**).

Morphological characterization indicated *Polydispyrenia* spp. infections in 7 larvae (**Figure 2A**), representing 87.5% of the infected larvae. *Amblyospora* sp. infection was observed in one larva (12.5% of the infected larvae) (**Figure 2B**). The prevalence of microsporidia parasitizing larvae of *S. pertinax* was 0.51%.

Polydispyrenia spp. infections were characterized by the presence of at least 32 mononuclear spores contained within a persistent sporophorous vesicle, with the spores measuring $6.9 \pm 1.0 \times 5.0 \pm 0.7 \mu\text{m}$ ($n = 23$). Similarly, *Amblyospora* spp. were characterized by the presence of eight uninucleate spores contained within a sporophorous vesicle, with the spores measuring $4.5 \times 3.5 \mu\text{m}$ ($n = 12$).

The PCR products targeting the 16S region and electrophoresis agarose gel analysis confirmed the presence of microsporidian DNA in 8 samples.

Six samples (Brazilian larvae) were found to be related to, but in a separate cluster (**Figure 3**) than, the *Polydispyrenia simulii*

[GenBank: AY090069] and *Caudospora palustris* [GenBank: AF132544] reference sequences (with 100% bootstrapping). One sample (L2) was clustered with *Amblyospora* spp. [GenBank: AJ252949] with 100% bootstrapping. The *Edhazardia aedis* positive control (CONT+) taxon was clustered with *Edhazardia aedis* [GenBank: AF027684] with 100% bootstrapping.

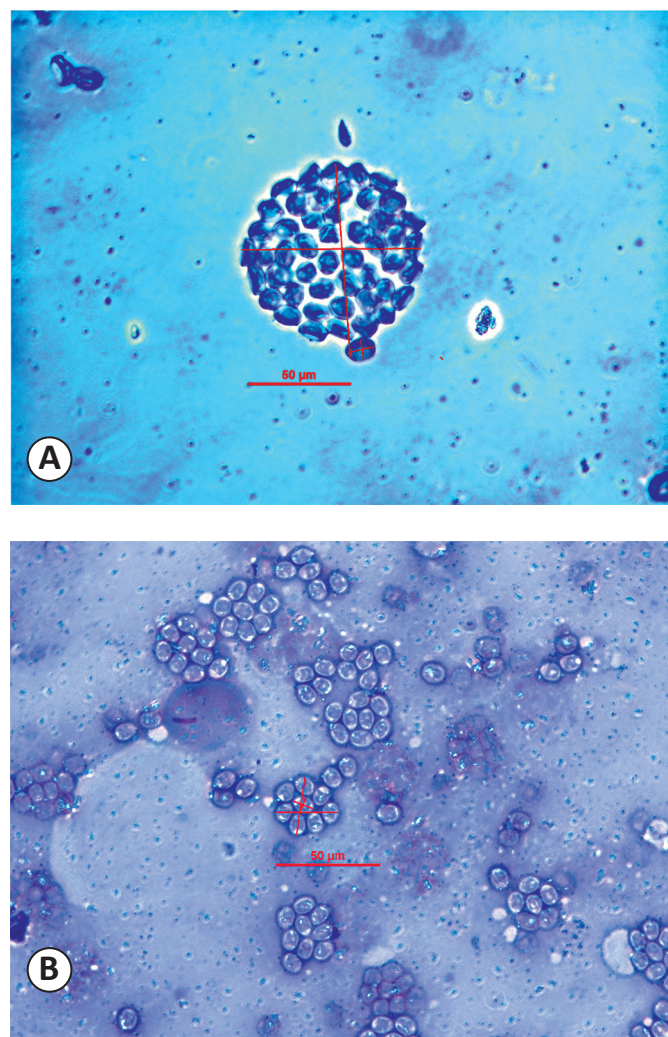


FIGURE 2 - Phase-contrast microscopy of smear slides of *Simulium pertinax* infected by microsporidia. Sporophorous vesicle of *Polydispyrenia* sp. containing 32 mononuclear spores (A). Octospores of *Amblyospora* spp. containing 8 uninucleate spores each (B).

DISCUSSION

Herein, we reported microsporidia parasitizing *S. pertinax* larvae in the State of São Paulo, with a prevalence of 0.51%. Araújo-Coutinho⁶ previously reported a 0.5-2.0% prevalence of microsporidia in *S. pertinax* in State of Rio de Janeiro. Our study showed a similar prevalence to that previously reported by Crosskey¹⁹ in other populations of black flies, with rates

of up to 1%. *Polydispyrenia* spp. were the most prevalent parasitic species in *S. pertinax* from Caraguatatuba/SP in this study, while *Amblyospora* spp. showed a higher prevalence in Rio de Janeiro⁶. This difference could be explained by the small sample size, which prevented further analysis of the species population dynamics between *S. pertinax* from Rio de Janeiro and Caraguatatuba.

In this study, spores of the *Polydispyrenia* spp. measured $6.9 \pm 1.0 \mu\text{m}$ in length x $5.0 \pm 0.7 \mu\text{m}$ in width. Araújo-Coutinho⁶ reported spores of a similar size for a *Polydispyrenia* sp. from *S. pertinax* that was ovocylindrical and measured $7.0 \pm 0.6 \times 4.9 \pm 0.8 \mu\text{m}$. However, Castello-Branco and Andrade²⁰ reported larger-sized spores measuring $8.3 \mu\text{m}$ in length x $6.3 \mu\text{m}$ in width for *P. simulii* from *S. pertinax* collected in State of São Paulo, Brazil. Sprague¹² stated that the spore dimensions were 4.5 to $5.5 \mu\text{m}$ x 2.5 to $3.5 \mu\text{m}$ for *P. simulii* with the hosts listed as *S. pertinax* and *S. perflavum* from Brazil.

In this study, for *Amblyospora* spp. from Caraguatatuba, the spore measurement was $4.5 \mu\text{m}$ in length x $3.5 \mu\text{m}$ in width, similar to that found by Araújo-Coutinho⁶ for *Amblyospora* spp. infecting *S. pertinax* in the State of Rio de Janeiro. Both of these results were similar to those from *Amblyospora bracteata* and *Amblyospora varians*, described in black flies in North America and Europe²¹. According to Sprague¹² the morphological similarity between species of microsporidia, particularly the spore measurements, makes identification difficult, and other methods are needed for identification. Such evidence indicates that spore dimension diversity is too variable; thus, molecular analysis could help in species identification.

Our sample, identified morphologically as *Polydispyrenia* spp., was grouped with the *P. simulii* and *C. palustris* clusters. This identification corroborated previous results²²⁻²⁶ regarding the phylogeny of these parasites.

The genera *Parathelohania*, *Hazardia*, *Marsoniella*, *Gurleya*, *Larssonia*, *Berwaldia*, *Varimorpha*, *Amblyospora* and the *Amblyospora* sp. from *S. pertinax* in this study form a separate group from the main *Amblyospora* cluster (**Figure 3**). Excluding the *Varimorpha* sp., which was characterized in an ant species, *Solenopsis richteri* (Forel, 1909), all genera in this group are parasites of aquatic hosts²⁷⁻³⁰.

Because the *Amblyospora* group is divided into two clades, corresponding to the hosts (*Culex* or *Aedes/Ochlerotatus*)²⁸, the aquatic group also demonstrated distinct phylogenetic characteristics according to the host. The genera that infect both *Culex quinquefasciatus* (SAY, 1823) and crustaceans (*Hazardia*, *Marsoniella*, *Gurleya*, *Larssonia* and *Berwaldia*) are the main members of this clade. The genera that infect anopheline mosquitoes (*Parathelohania*), simuliids (*Amblyospora* spp. 3 in this study) and a species of ant (*Varimorpha* sp.), are more closely related to the aquatic group than to the main *Amblyospora* group. The *Amblyospora* spp. in this study were clustered with *Amblyospora* sp. (AJ252949) from *Simulium* spp. from the Palearctic²⁹; confirming the morphological and molecular similarities between these 2 species.

Phylogenetic analysis with the 16S gene showed considerable distance between the *Amblyospora* spp., which infect simuliids,

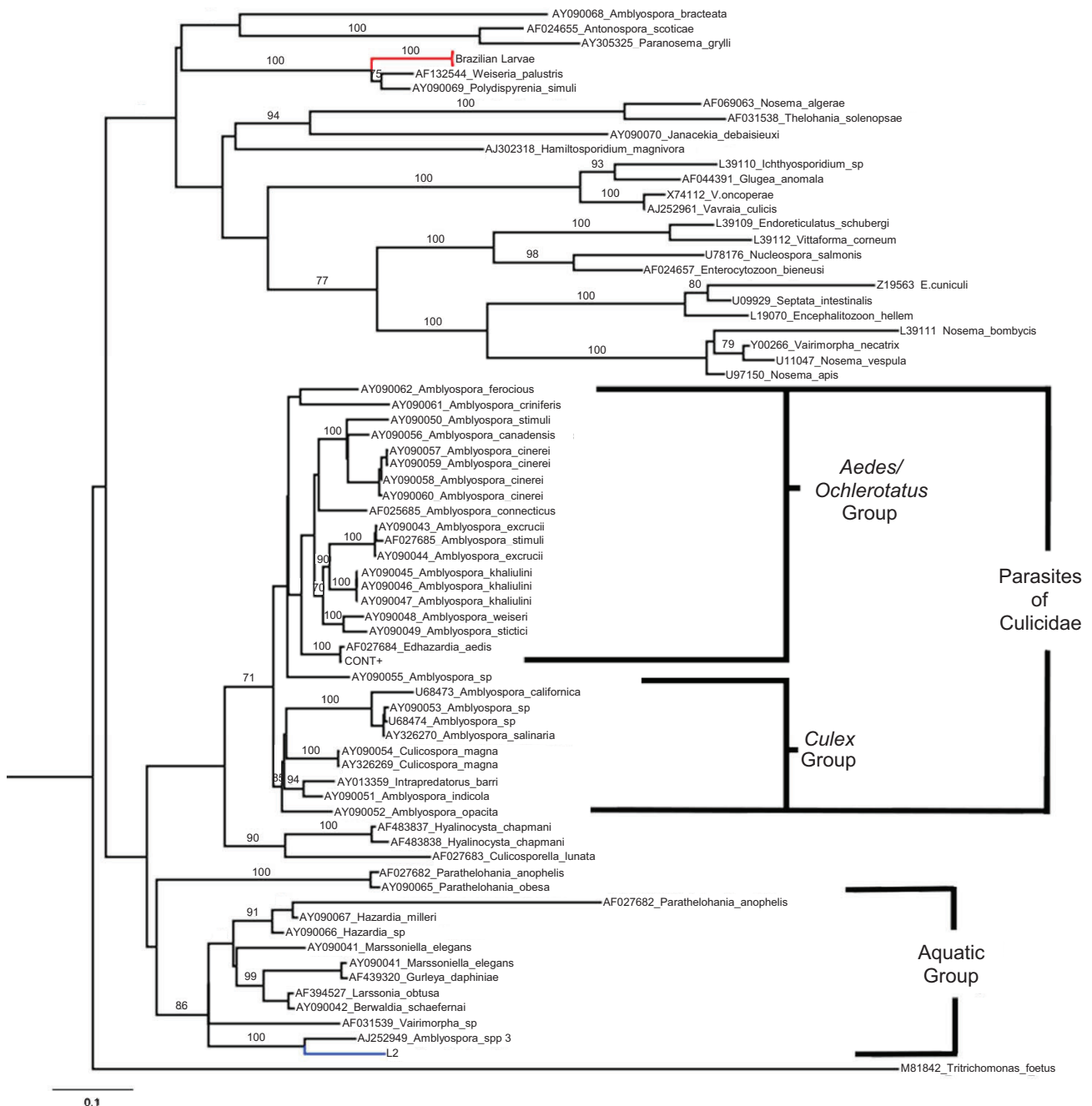


FIGURE 3 - Phylogenetic tree generated for microsporidia. Unrooted tree constructed with the maximum likelihood method using the general time reversible model of nucleotide substitution and gamma distribution (GTR + G), using Seaview software. The robustness of the phylogenetic groups was evaluated using 1,000 bootstrap replicates, and bootstrap values greater than 70 were considered significant.

and the main group of *Amblyospora* spp., which infects mosquitoes, indicating that these groups are not congeneric. The differences between taxonomic relationships, based on phylogenetic placement and classical morphological characteristics, could probably be explained by the possibility that some of these characteristics (diplokaryon, sporophorous vesicles, and meiosis) appear to have multiple origins³¹. Thus, molecular analysis of other genomic regions could improve the phylogenetic understanding of microsporidia. This work

contributes to the phylogenetic analysis of microsporidia because it provides two genus sequences from these parasites.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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