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

***Isabel Maria Vicente Guedes de Carvalho*[1], *Artur Trancoso Lopo de Queiroz*[2], *Rosiane Brito de Moraes*[4], *Helio Benites Gil*[3], *Rafael Alves*[5], *Andréa de Barros Pinto Viviani*[6], *James John Becnel*[7] *and Carlos José Pereira da Cunha de Araujo-Coutinho*[4]**

[1]. Laboratório de Parasitologia, Instituto Butantan, São Paulo, SP. [2]. Laboratório de Imunoparasitologia, Centro de Pesquisa Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, BA. [3]. Disciplina de Infectologia, Universidade Federal de São Paulo, São Paulo, SP. [4]. Laboratório de Entomologia Médica, Superintendência de Controle de Endemias, São Paulo, SP. [5]. Departamento de Medicina, Disciplina de Gastroenterologia, Universidade Federal de São Paulo, São Paulo, SP. [6]. Laboratório de Simulídeos, Superintendência de Controle de Endemias, Caraguatatuba, SP. [7]. United States Department of Agriculture, Mosquito and Fly Research Unit, Gainesville, Florida, USA.

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 ± 1.0 x 5.0 ± 0.7µm in persistent sporophorous vesicles. Similarly, *Amblyospora* spp. were characterized by the presence of eight uninucleate spores measuring 4.5 x 3.5µm 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 production[1-3]. Black fly control remains a major public health challenge. Microsporidia are unicellular, eukaryotic organisms that are obligate, intracellular parasites with public health relevance[4]. Several studies have suggested a new classification for microsporidia as fungi, but Ebersberger[5] 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 vigor[8].

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

Address to: Dr. Carlos José Pereira da Cunha de Araújo-Coutinho. Laboratório de Entomologia Médica/SUCEN. Rua Cardeal Arcoverde nº 2878, 05408-003 São Paulo, SP, Brasil.
Phone: 55 11 3032-2228
e-mail: cjpcacoutinho@gmail.com
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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 458,097km^2 and had a population at that time of 100,840[9]. 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 removed[10]. 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 overnight[11] 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 Sprague[12].

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 gene[13].

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 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 software[15].

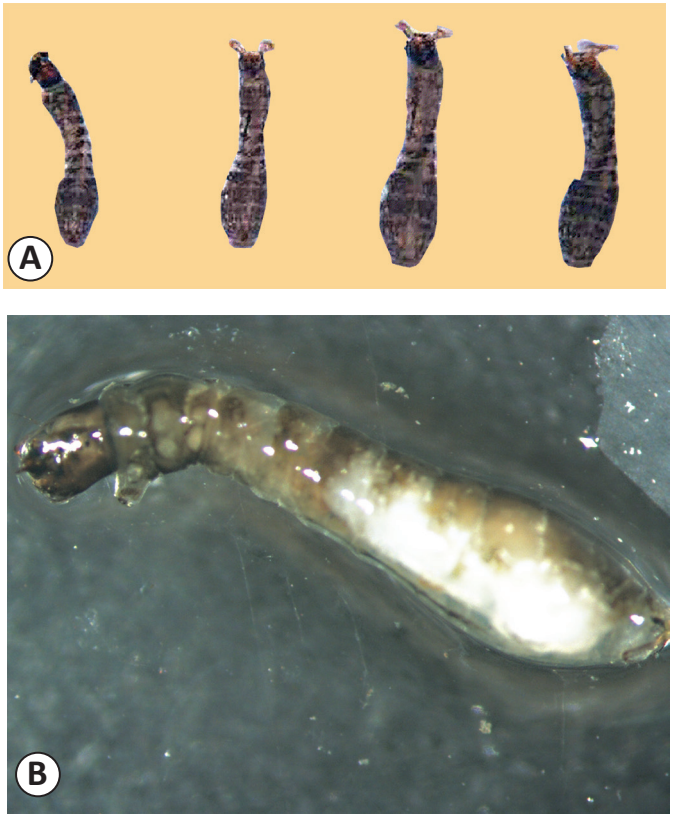

FIGURE 1 - ***Simulium pertinax*** **larvae. Healthy larvae with normal coloration of the integument (A). Larvae with symptoms of microsporidian infection in the fat bodies (B).**

Phylogenetic analysis

The analyses were performed using the Seaview software[16]. A phylogenetic tree was constructed, with reference sequences[32-46] 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 3.0.6[18], 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
Amblyospora bracteata	*Odagamia ornata*	Czech Republic	AY090068[32]
Antonospora scoticae	*Andrena scotica*	USA	AF024655*
Paranosema grylli	*Gryllus bimaculatus*	St. Petersburg, FL, USA	AY305325[33]
Polydispyrenia simulii	*Odagamia ornata*	Czech Republic	AY090069[32]
Weiseria palustris	*Cnephia ornithophilia*	USA	AF132544*
Nosema algerae	*Anopheles stephensi*	Illinois, USA	AF069063[34]
Thelohania solenopsae	*Solenopsis invicta*	USA	AF031538[28]
Janacekia debaisieuxi	*Odagamia ornata*	USA	AY090070[35]
Hamiltosporidium magnivora	*Daphnia magna*	Russia	AJ302318.1*
Ichthyosporidium sp.	*Leiostomus xanthurus*	Not Informed	L39110[31]
Glugea anomala	*Gasterosteus aculeatus*	Norway	AF044391.1[36]
Vavraia oncoperae	*Wiseana* spp.	New Zealand	X74112[37]
Vavraia culicis	*Aedes albopictus*	USA	AJ252961[29]
Endoreticulatus schubergi	*Lymantria dispar*	Switzerland	L39109[31]
Vittaforma corneum	*Homo sapiens*	USA	L39112[31]
Nucleospora salmonis	*Oncorhynchus tshawytscha*	Canada	U78176[38]
Enterocytozoon bieneusi	*Homo sapiens*	USA	AF024657[39]
Encephalitozoon cuniculi	*Oryctolagus cuniculus*	USA	Z19563.1[40]
Encephalitozoon intestinalis	*Homo sapiens*	USA	U09929[41]
Encephalitozoon hellem	*Homo sapiens*	USA	L19070[42]
Nosema bombycis	*Bombyx mori*	Switzerland	L39111[31]
Vairimorpha necatrix	*Malacosoma americanum*	Not Informed	Y00266[4]
Nosema vespula	*Species Unknown*	USA	U11047*
Nosema apis	*Apis mellifera*	New Zealand	U97150.1[43]
Amblyospora ferocious	*Psorophora ferox*	Argentina	AY090062[32]
Amblyospora criniferis	*Aedes cernifera*	Argentina	AY090061[32]
Amblyospora stimuli	*Diacyclops bicuspidatus*	USA	AY090050[32]
Amblyospora canadensis	*Ochlerotatus canadensis*	USA	AY090056[32]
Amblyospora cinerei	*Aedes cinereus*	USA	AY090057[32]
Amblyospora cinerei	*Acanthacyclops vernalis*	USA	AY090059[32]
Amblyospora cinerei	*Acanthacyclops vernalis*	USA	AY090058[32]
Amblyospora cinerei	*Cyclops venustoides*	USA	AY090060[32]
Amblyospora connecticus	*Ochlerotatus cantator*	USA	AF025685*
Amblyospora excrucii	*Ochlerotatus excrucians*	USA	AY090043[32]
Amblyospora stimuli	*Aedes stimulans*	USA	AF027685[27]
Amblyospora excrucii	*Acanthocyclops vernalis*	USA	AY090044[32]
Amblyospora khaliulini	*Ochlerotatus communis*	USA	AY090045[32]
Amblyospora khaliulini	*Acanthocyclops vernalis*	USA	AY090046[32]
Amblyospora khaliulini	*Acanthocyclops vernalis*	USA	AY090047[32]

Table 1 - continues....

TABLE 1 - Continuation.

Organism	Host	Geographic locale	Accession number
Amblyospora weiseri	*Ochlerotatus cantans*	USA	AY090048[32]
Amblyospora stictici	*Ochlerotatus sticticus*	USA	AY090049[32]
Edhazardia aedis	*Aedes aegypti*	Thailand	AF027684[27]
Amblyospora sp.	*Cyclops strenuus*	Czech Republic	AY090055[32]
Amblyospora californica	*Culex tarsulis*	USA	U68473[44]
Amblyospora sp.	*Culex nigripalpus*	USA	AY090053[32]
Amblyospora sp.	*Culex salinarius*	USA	U68474[44]
Amblyospora salinaria	*Culex salinarius*	USA	AY326270[32]
Culicospora magna	*Culex restuans*	USA	AY090054[32]
Culicospora magna	*Culex restuans*	USA	AY326269[32]
Intrapredatorus barri	*Culex fuscanus*	Norway	AY013359[45]
Amblyospora indicola	*Culex sitiens*	India	AY090051[32]
Amblyospora opacita	*Culex territans*	USA	AY090052[32]
Hyalinocysta chapmani	*Culiseta melanura*	USA	AF483837[46]
Hyalinocysta chapmani	*Orthocyclops modestus*	USA	AF483838[46]
Culicosporella lunata	*Culex pilosus*	USA	AF027683[27]
Parathelohania anophelis	*Anopheles quadrimaculatus*	USA	AF027682[27]
Parathelohania obesa	*Anopheles crucians*	USA	AY090065[32]
Trichotuzetia guttata	*Cyclops vicinus*	Czech Republic	AY326268[32]
Hazardia milleri	*Culex quinquefasciatus*	Argentina	AY090067[32]
Hazardia sp.	*Anopheles crucians*	USA	AY090066[32]
Marsoniella elegans	*Cyclops vicinus*	Czech Republic	AY090041[32]
Gurleya vavrai	*Daphnia longispina*	Finland	AF394526[30]
Gurleya daphniae	*Daphnia pulex*	Austria	AF439320[30]
Larssonia obtusa	*Daphnia pulex*	Sweden	AF394527[30]
Berwaldia schaefernai	*Daphnia galeata*	Czech Republic	AY090042[32]
Varimorpha sp.	*Solenopsis richteri*	USA	AF031539[28]
Amblyospora sp.	*Simulium* 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 ± 1.0 x 5.0 ± 0.7µm (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 x 3.5µm (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.

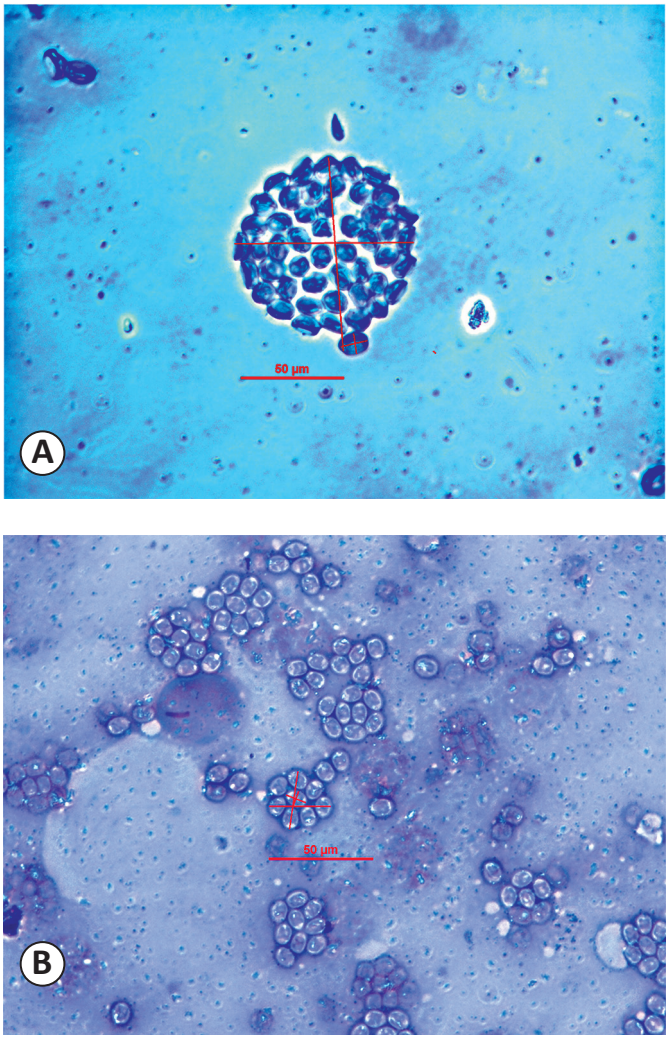

FIGURE 2 - Phase-contrast microscopy of smear slides of *Simulium pertinax* infected by microsporidia. Sporophorous vesicle of *Polydispyrenia* sp. containing 32 mononuclear spores (A). Octospores of *Amblyospora* spp. containing 8 uninucleate spores each (B).

DISCUSSION

Herein, we reported microsporidia parasitizing *S. pertinax* larvae in the State of São Paulo, with a prevalence of 0.51%. Araújo-Coutinho[6] 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 Crosskey[19] 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 Janeiro[6]. 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 ± 1.0µm in length x 5.0 ± 0.7µm in width. Araújo-Coutinho[6] reported spores of a similar size for a *Polydispyrenia* sp. from *S. pertinax* that was ovocylindrical and measured 7.0 ± 0.6 x 4.9 ± 0.8µm. However, Castello-Branco and Andrade[20] reported larger-sized spores measuring 8.3µm in length x 6.3µm in width for *P. simulii* from *S. pertinax* collected in State of São Paulo, Brazil. Sprague[12] stated that the spore dimensions were 4.5 to 5.5µm x 2.5 to 3.5µm 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 4.5µm in length x 3.5µm in width, similar to that found by Araújo-Coutinho[6] 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 Europe[21]. According to Sprague[12] 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 results[22-26] 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 aquatics hosts[27-30].

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),* simulids *(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 Paleartic[29]; 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 simulids,

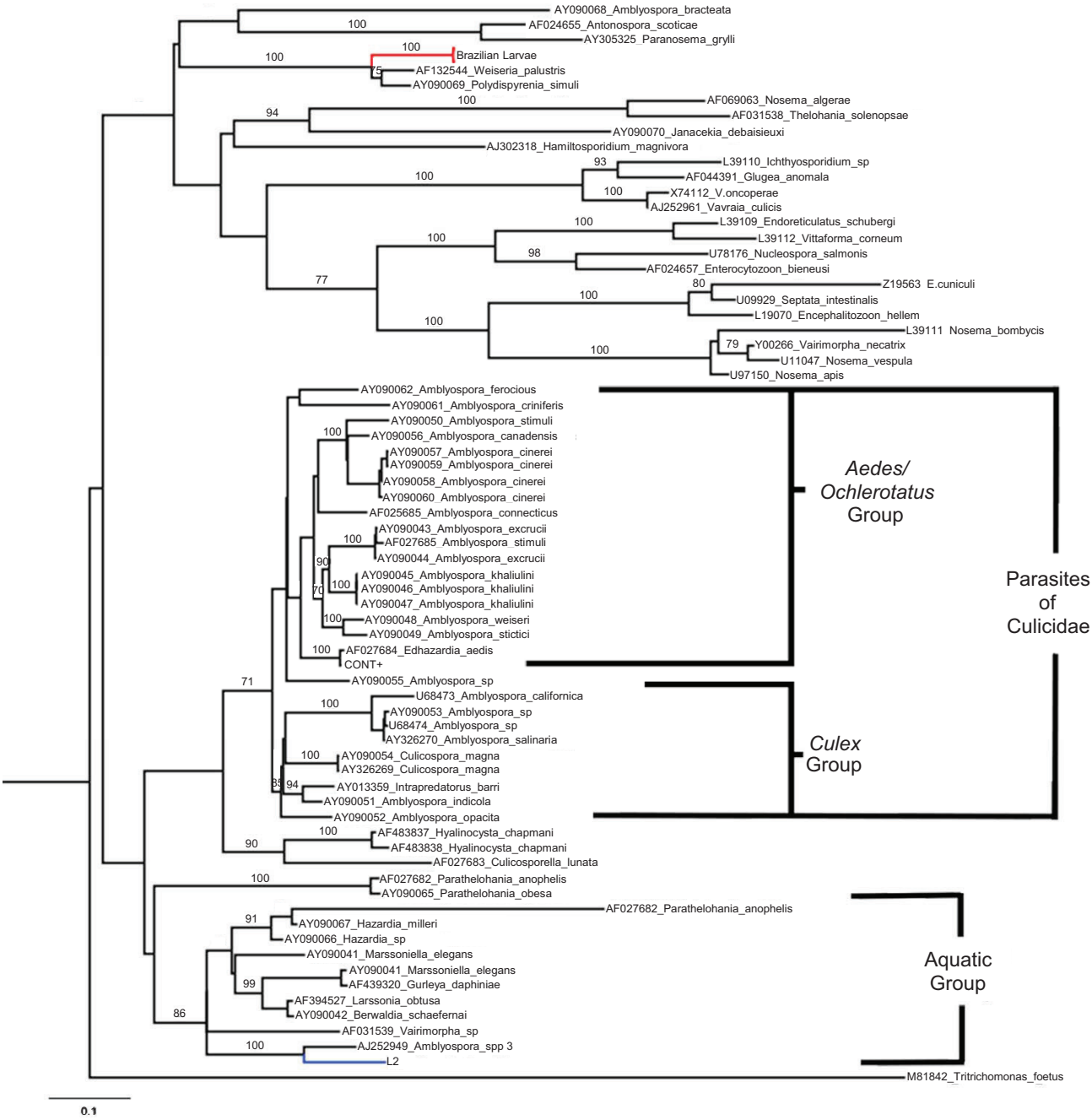

FIGURE 3 - Phylogenetic tree generated for microsporidia. Unrooted tree constructed with the maximum likelihood method using the general time reversible model of nucleotide substitution and gamma distribution (GTR + G), using Seaview software. The robustness of the phylogenetic groups was evaluated using 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 origins[31]. 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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REFERENCES

1. Shelley A, Hernandez L, Maia-Herzog M, Luna Dias A, Garritano D. The blackflies (Diptera: Simuliidae) of Brazil. *In:* Arias JR, Golovatch S, Wantzen KM, Dominguez E, editors. Aquatic biodiversity in Latin America (ABLA). Vol. 6. Pensoft:Sofia-Moscow; 2010. p. 821.
2. Coscarón S, Coscarón-Arias CL. Neotropical Simuliidae (Diptera: Insecta). *In:* Adis J, Arias JR, Rueda-Delgado G, Wantzen KM, editors. Aquatic biodiversity in Latim America (ABLA). Vol. 3. Pensoft: Sofia-Moscow; 2007. p. 685.
3. Ribeiro do Amaral-Calvão AM, Maia-Herzog M. Adolpho Lutz's collection of black flies (Diptera: Simuliidae), its history and importance. Hist Cienc Saude Manguinhos 2003; 10:259-271.
4. Vossbrinck CR, Maddox JB, Friedman S, Debrunner-Vossbrink BA, Woese CR. Ribosomal RNA sequence suggests microsporidia are extremely ancient eukaryotes. Nature 1987; 326: 411-414.
5. Ebersberger I, Simões RM, Kupczok A, Gube M, Kothe E, Voigt K, et al. A consistent phylogenetic backbone for the fungi. Mol Biol Evol 2012; 29:1319-1334.
6. Araújo-Coutinho CJ, Nascimento ES, Figueiro R, Becnel JJ. Seasonality and prevalence rates of microsporidia in *Simulium pertinax* (Diptera: Simuliidae) larvae in the region of Serra dos Órgãos, Rio de Janeiro, Brasil. J Invertebr Pathol 2004; 85:188-191.
7. Ginarte CA, Andrade CFS, Gaona JC. Larvas de simulídeos (Diptera, Simuliidae) do Centro Oeste, Sudeste e Sul do Brasil, parasitadas por microsporídeos (Protozoa) e mermitídeos (Nematoda). Ser Zool 2003; 93:325-334.
8. Castello-Branco A. Effects of *Polydispyrenia simulii* (Microspora; Duboscqiidae) on Development of the Gonads of *Simulium pertinax* (Diptera; Simuliidae). Mem Inst Oswaldo Cruz 1997; 94:421-424.
9. Online Archive of Instituto Brasileiro de Geografia e Estatística [Internet][cited 2013 May]. Available at: http://cod.ibge.gov.br/2335A.
10. Undeen A H, Vavra J. Research Methods for Entomopathogenic Protozoa *In:* Lacey LA. Manual of Techniques in Insect Pathology. Chapter 4. 1st ed. Great Britain: Academic Press; 1997. p. 115-151.
11. Becnel J J. Preparations of Entomopathogens. *In:* Lacey LA. Manual of Techniques in Insect Pathology. Chapter VIII-1. 1st ed. Great Britain: Academic Press; 1997; p. 337-353.
12. Sprague V, Becnel JJ, Hazard EI. Taxonomy of Phylum Microspora. Crit Rev Microbiol 1992; 18:285-395.
13. Adler PH, Becnel JJ, Moser B. Molecular charaterization and taxonomy of a new species of Caudosporidae (Microsporidia) from black flies (Diptera: Simuliidae), with host-derived relationships of the North American caudosporids. J Invertebr Pathol 2000; 75:133-143.
14. Vossbrinck CR, Andreadis TG, Vavra J, Debrunner-Vossbrink BA. Verification of intermediate hosts in the life cycles of Microsporidia by small subunit rDNA sequencing. J Eukaryot Microbiol 1998; 45:290-292.
15. Huang X, Madan A. CAP3: A DNA sequence assembly program. Genome Res 1999; 9:868-877.
16. Gouy M, Guindon S, Gascuel O. SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol Biol Evol 2010; 27:221-224.
17. Waddell PJ, Steel MA. General time-reversible distances with unequal rates across sites: mixing gamma and inverse Gaussian distributions with invariant sites. Mol Phylogenet Evol 1997; 8:398-414.
18. Posada D, Crandall KA. MODELTEST: testing the model of DNA substitution. Bioinformatics 1998; 14:817-818.
19. Crosskey RW. The Natural History of Black flies. 1st ed. West Sussex, England: Wiley; 1990.
20. Castello-Branco A, Andrade CFS. Studies on *Polydispyrenia simulii* (Microspora; Pleistophoridae) in *Simulium pertinax* (Diptera; Simulidae) in Brazil. Mem Inst Oswaldo Cruz 1993; 88:167.
21. Vávra J, Undeen AH. Microsporidia (Microspora: Microsporida) from Newfoundland blakflies (Diptera: Simuliidae). Can J Zool 1981; 59:1431-1446.
22. Lom J, Nilsen F, Dykova I. Thelohania contejeani Henneguy, 1892: dimorphic life cycle and taxonomic affinities, as indicated by ultrastrutural and molecular study. Parasitol Res 2001; 87:860-872.
23. Terry RS, Smith JE, Sharpe RG, Rigaud T, Littlewood TDJ, Ironside JE, et al. Widespread vertical transmission and associated host sex-ratio distortion within eukaryotic Phylum Microspora. Proc R Soc Lond 2004; 271:1783-1789.
24. Vossbrinck CR, Debrunner-Vossbrinck BA. Molecular Phylogeny of the Microsporidia: ecological, ultrastructural and taxonomic considerations. Folia Parasitol 2005; 52:131-142.
25. Smith JE. The ecology and evolution of microsporidian parasites. Parasitol 2009; 136:1901-1914.
26. Dong S, Shen Z, Xu L, Zhu F. Sequence and phylogenetic analisys of SSU rRNA gene of five Microsporidia. Curr Microbiol 2010; 60:30-37.
27. Baker MD, Vossbrinck CR, Becnel JJ, Andreadis TG. Phylogeny of *Amblyospora* (Microsporida: Amblyosporidae) and related genera based on small subunit ribosomal DNA data: A possible example of host parasite cospeciation. J Invertebr Pathol 1998; 71:199-206.
28. Moser BA, Becnel JJ, Maruniak J, Patterson RS. Analysis of the ribosomal DNA sequences of the microsporidia *Thelohania* and *Vairimorpha* of fire ants. J Invertebr Pathol 1998; 72:154-159.
29. Cheney SA, Lafranchi-Tristem NJ, Canning EU. Phylogenetic relationships of *Pleistophora*-like microsporidia based on small subunit ribosomal DNA sequences and implications for the source of *Trachipleistophora hominis* infections. J Eukaryot Microbiol 2000; 47:280-287.
30. Refardt D, Canning EU, Mathis A, Cheney SA, Lafranchi-Tristem NJ, Ebert D. Small subunit ribosomal DNA phylogeny of microsporidia that infect *Daphnia* (Crustacea: Cladocera). Parasitol 2002; 124:381-389.
31. Baker MD, Vossbrinck CR, Didier ES, Maddox JV, Shadduck JA. Small subunit ribosomal DNA phylogeny of various microsporidia with emphasis on AIDS related forms. J Eukaryot Microbiol 1995; 42:564-570.
32. Vossbrinck CR, Andreadis TG, Vavra J, Becnel JJ. Molecular phylogeny and evolution of mosquito parasitic Microsporidia (Microsporidia: Amblyosporidae). J Eukaryot Microbiol 2004; 51:88-95.
33. Sokolova YY, Dolgikh VV, Morzhina EV, Nassonova ES, Issi IV, Terry RS, et al. Establishment of the new genus *Paranosema* based on the ultrastructure and molecular phylogeny of the type species *Paranosema grylli* Gen. Nov., Comb. Nov. (Sokolova, Selezniov, Dolgikh, Issi 1994), from the cricket *Gryllus bimaculatus*. Deg J Invertebr Pathol 2003; 84:159-172.
34. Muller A, Trammer T, Chioralia G, Seitz HM, Diehl V, Franzen C. Ribosomal RNA of *Nosema algerae* and phylogenetic relationship to other microsporidia. Parasitol Res 2000; 86:18-23.
35. Vossbrinck CR, Andreadis TG, Vavra J, Becnel JJ. Molecular phylogeny and evolution of mosquito parasitic Microsporidia (Microsporidia: Amblyosporidae). J Eukaryot Microbiol 2004; 51:88-95.
36. Nilsen F, Endresen C, Hordvik I. Molecular phylogeny of microsporidians with particular reference to species that infect the muscles of fish. J Eukaryot Microbiol 1998; 45:535-543.
37. Malone LA, Broadwell AH, Lindridge ET, McIvor CA, Ninham J. Ribosomal RNA genes of two microsporidia, *Nosema apis* and *Vavraia oncoperae* are very variable. J Invertebr Pathol 1994; 64:151-152.
38. Docker MF, Kent ML, Hervio DL, Khattra JS, Weiss LM, Cali A, et al. Ribosomal DNA sequence of *Nucleospora salmonis* Hedrick, Groff and Baxa, 1991 (Microsporea: Enterocytozoonidae): implications for Phylogeny and Nomenclature. J Eukaryot Microbiol 1997; 44:55-60.

39. Silva AJ, Schwartz DA, Visvesvara GS, Moura H, Slemenda SB, Pieniazek NJ. Sensitive PCR diagnosis of Infections by *Enterocytozoon bieneusi* (microsporidia) using primers based on the region coding for small-subunit rRNA. J Clin Microbiol 1996; 34:986-987.

40. Zhu X, Wittner M, Tanowitz HB, Cali A, Weiss LM. Nucleotide sequence of the small ribosomal RNA of *Encephalitozoon Cuniculi*. Nucleic Acids Res 1993; 21:1315.

41. Visvesvara GS, Silva AJ, Croppo GP, Pieniazek NJ, Leitch GJ, Ferguson D, et al. *In vitro* culture and serologic and molecular identification of *Septata intestinalis* isolated from urine of a patient with AIDS. J Clin Microbiol 1995; 33:930-936.

42. Visvesvara GS, Leitch GJ, Silva AJ, Croppo GP, Moura H, Wallace S, et al. Polyclonal and monoclonal antibody and PCR-amplified small-subunit rRNA identification of a microsporidian, *Encephalitozoon hellem*, isolated from an AIDS patient with disseminated infection. J Clin Microbiol 1994; 32:2760-2768.

43. Gatehouse HS, Malone LA. The ribosomal RNA gene region of *Nosema apis* (Microspora): DNA sequence for small and large subunit rRNA genes and evidence of a large tandem repeat unit size. J Invertebr Pathol 1998; 71:97-105.

44. Baker MD, Vossbrinck CR, Becnel JJ, Maddox JV. Phylogenetic position of *Amblyospora* Hazard & Oldacre (Microspora: Amblyosporidae) based on small subunit rRNA data and its implication for the evolution of the microsporidia. J Eukaryot Microbiol 1997; 44:220-225.

45. Nilse F, Che WJ. rDNA phylogeny of *Intrapredatorus barri* (Microsporida: Amblyosporidae) parasitic to *Culex fuscanus* Wiedemann (Diptera: Culicidae) Parasitology 2001; 122:617-623.

46. Andreadis TG, Vossbrinck CR. Life cycle, ultrastructure and molecular phylogeny of *Hyalinocysta chapmani* (Microsporidia: Thelohaniidae), a parasite of *Culiseta melanura* (Diptera: Culicidae) and *Orthocyclops modestus* (Copepoda: Cyclopidae). J Eukaryot Microbiol 2002; 49:350-364.