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A new *Myxidium* species (Myxozoa: Myxosporea) infecting the gallbladder of the turtle *Podocnemis unifilis* (Testudines: Podocnemididae) from Peruvian Amazon

Luis L. Espinoza[a], Omar Mertins[b], Gabriella S. Gama[b], Ana C.M. Fernandes Patta[b,c], Patrick D. Mathews[d,*]

[a] Laboratory of Biology and Molecular Genetics, Faculty of Veterinary Medicine, National University of San Marcos, 2800 San Borja, Lima, Peru
[b] Department of Biophysics, Paulista School of Medicine, Federal University of São Paulo, 04023-062 São Paulo, Brazil
[c] Faculty of Biological Sciences, Federal Institute of Education, Science and Technology, 01109-010 São Paulo, Brazil
[d] Department of Animal Biology, Institute of Biology, University of Campinas, Avenida Bertrand Russel, s/n, Cx. 6109, 13083-970 Campinas, SP, Brazil

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ABSTRACT

A new myxosporean species, *Myxidium peruviensis* n. sp., is described parasitizing the gall bladder of the yellow-spotted river turtle *Podocnemis unifilis* kept in captivity in an Amazonian Peruvian turtle rescue unit in the city of Iquitos, State of Loreto, Peru. The parasite was found in four of ten (40%) *P. unifilis* examined. The new species was characterized based on morphological and molecular phylogeny analyses. SSU rDNA sequence of the spores of M. peruviensis n. sp. resulted in 1876 nucleotides and this sequence did no match any of the Myxozoa available in the GenBank. Phylogenetic analysis identified the new species as a sister species of *Myxidium turturibus*, the unique *Myxidium* species described in a Neotropical turtle. Nevertheless, the SSU rDNA sequences of the new species and *M. turturibus* have only a 91.5% similarity. This is the first description and molecular study of a Myxozoa in a reptile from Peru. Considering the status of *P. unifilis* as vulnerable species, the infection by *Myxidium* parasites is emphasized as possible disease impeller, representing menace to the turtle conservation.

1. Introduction

The yellow-spotted river turtle *Podocnemis unifilis* Troschel, 1848, is a semi-aquatic turtle widely distributed in South America. This species inhabits in aquatic environment of the Amazon and Orinoco basins (Arraes and Tavares-Dias, 2014) In the last decades, the natural populations of *P. unifilis* has declined due to the excessive exploitation by Amazonian communities as a food source, as well as the increasing destruction of their natural nesting habitat (Conway-Gomez, 2007). Currently, *P. unifilis* is classified a vulnerable species in the Red List of Threatened Species (IUCN, 2015). In this context, several strategies towards conservation have been introduced by local authorities in order to minimize impacts on wild populations of *P. unifilis*, for example, the establishment of illegality of hunting, creation of conservation areas, breeding and educational programs (Alves et al., 2012; Alcântara, 2014).

Myxozoans are microscopic metazoan endoparasites of worldwide distribution (Okamura et al., 2015). Annelids and bryozoans are the definitive hosts, releasing actinospores to water. Primarily fish and rarely other vertebrates as amphibians, reptiles, birds and small mammal can be intermediate hosts (Okamura et al., 2015) and may become infected with actinospores by ingestion or upon contact with the skin or gill epithelium (Canning and Okamura, 2004; Okamura et al., 2015). Among the myxosporean, species of the genus *Myxidium* Bütschli, 1882, are typically coelozoic parasites virtually infecting all taxon of vertebrates and within a wide geographical range (Lom and Dykova, 2006; Bartholomew et al., 2008; Eiras et al., 2011; Székely et al., 2015). However, information about infection on reptiles is still scarce and, to our knowledge, nothing is known about occurrence in reptiles in Peru.

This study describes a new freshwater *Myxidium* species from the gallbladder of *P. unifilis* kept in captivity in the Amazon region, Peru. The new species was characterized based on morphological and molecular phylogeny analyses and stress the finding concerning its implication as a possible threat to the yellow-spotted turtle survival.

* Corresponding author. Department of Animal Biology, Institute of Biology, University of Campinas, Avenida Bertrand Russel, s/n, Cx. 6109, 13083-970 Campinas, SP, Brazil. Tel.: +55 19 35 21 62 75; fax: +55 19 35 21 63 74.
E-mail address: patrickmathews83@gmail.com (P.D. Mathews).

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2. Materials and methods

In April 2015, gallbladders of ten hatchlings yellow-spotted river turtles (ranging from 7.9 to 8.3 cm in length) infected with spores of a *Myxidium* species were obtained from a turtle rehabilitation unit in the city of Iquitos (3°44′ S, 73°15′ W), Loreto State, Peru. In the unit, the turtles are kept in a density of about two turtles/m^2 in earthen ponds of 18 m^2 approximately. The turtles were seized from illegal hunters and transported to this rescue unit. The turtles examined in this study were collected as a result of a mortality event of turtle hatchlings, occurred in the rescue unit above mentioned. After necropsy, gallbladders infected with myxosporeans were removed and examined using light microscopy. A drop of bile was placed on a microscope slide, covered with a cover slip and examined by light microscopy in the Laboratory of Biology and Molecular Genetics at the National University of San Marcos. Morphological and morphometric analyses were performed on mature spores based on the criteria outlined by Lom and Arthur (1989). Measurements were taken from 30 spores using an optical microscope equipped with Nomarski differential interference contrast (DIC), at Department of Biophysics, Federal University of São Paulo. Smears containing free spores were air-dried and stained with Giemsa solution and mounted in a low-viscosity mounting medium (Cytosealtm) on permanent slides.

For molecular analysis, samples were preserved in absolute ethanol (Merck). DNA was extracted using a DNeasy® Blood & Tissue Kit (Qiagen, USA), in accordance with the manufacturer's instructions. The concentration of the DNA was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA). Polymerase chain reactions (PCRs) were conducted in accordance with Mathews et al. (2015). The final reaction volume was 25 µl, which comprised 10–50 ng of extracted DNA, 1× Taq DNA Polymerase buffer (Invitrogen by Life Technologies, MD, USA), 0.2 mmol dNTPs, 1.5 mmol $MgCl_2$, 0.2 pmol for each primer, 0.25 µl (1.25 U) Taq DNA polymerase (Invitrogen by Life Technologies, MD, USA), and ultrapure water. The amplification of fragments containing approximately 1.000 and 1.200 bp was performed with the primer pairs ERIB1-ACT1R and MYXGEN4f-ERIB10, respectively (Barta et al., 1997; Kent et al., 2000; Hallett and Diamant, 2001). PCRs were performed in an iCycler (Bio-Rad, Hercules, CA, USA) with initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 60 s, 64 °C (ERIB1-ACT1R) or 58 °C (MYXGEN4f-ERIB10) for 60 s, 72 °C for 120 s, and then final elongation at 72 °C for 5 min. PCR products were subjected to electrophoresis in 1.0% agarose gel (BioAmerica, Miami, FL, USA) in a TAE buffer (Tris–Acetate EDTA, Tris 40 mM, Acetic Acid 20 mM, EDTA 1 mM), stained with Sybr Safe DNA gel stain (Invitrogen by Life Technologies, CA, USA), and then analysed with a Stratagene 2020E transilluminator. PCR products were purified using USB® ExoSap-IT® (OH Cleveland, USA) in accordance with the manufacturer's instructions and sequenced using the same PCR primers and additionally MC5 and MC3 primers (Molnár et al., 2002). Sequencing was performed at Paulista School of Medicine, Federal University of São Paulo, using a BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems Inc., CA, USA) in an ABI 3130 automatic DNA analyzer (Applied Biosystems™).

To verify the similarity of the sequence obtained in this study with other sequences available in the GenBank database, a standard nucleotide BLAST (blastn) search was conducted (Altschul et al., 1997). In order to visually assemble sequence fragments and to compare the coding sequence obtained with the 40 most closely related myxozoan taxa, as determined by the BLAST search, a Bioedit was used (Hall, 2011).

Phylogenetic analysis was conducted using maximum likelihood (ML) methods with a Kimura 2-parameter (K2P) evolution sequence model in the MEGA 6.0 program (Tamura et al., 2013). Bootstrap analysis (1000 replicates) was employed to assess the relative robustness of the tree branches. *Tetracapsuloides bryosalmonae* KF731712 sequence was used as out-group. Additionally, a second alignment of the SSU rDNA sequences clustering together with the new parasite here described was performed to evaluate the genetic distance between them, using the pairwise method with the p-distance model in MEGA 6.0 software (Tamura et al., 2013).

3. Results

Mature myxospores floating free in the bile of an unknown *Myxidium* species were found in the gallbladder of hatchlings specimens of *P. unifilis*. The vegetative stages were not observed. The prevalence of the parasite was 40% (4/10) and they were not found in any other organs.

3.1. Morphological description and taxonomic placement

Mature myxospores were fusiform, lightly arcuate and ending in pointed extremities, measuring 11.3 ± 0.1 (11.2–11.5) µm in length and 3.5 ± 0.2 (3.4–3.6) µm in width. The two polar capsules were pyriform in shape, equal in size and situated at both ends of the spore, with 2.7 ± 0.3 (2.6–2.9) µm in length and 1.9 ± 0.2 (1.8–2.1) µm in width. They occupied only the anterior third of the myxospore body and had a polar filament with four to five coils. The valval surface with five grooves was longitudinal (Fig. 1A–C).

Phylum Cnidaria Verrill, 1865
Class Myxosporea Bütschli, 1881
Order Bivalvulida Shulman, 1959
Suborder Variisporina Lom and Noble, 1984
Family Myxidiidae Thélodan, 1892
Genus *Myxidium* Bütschli, 1882
Myxidium peruviensis n. sp.

Type host: *Podocnemis unifilis* Troschel, 1848 (Testudines: Podocnemididae).

Site of infection: Gall bladder (spores floating free in the bile).

Type locality: CREA rescue unit, city of Iquitos, Loreto State, Peru.

Prevalence: From ten examined turtles, four were infected (40%).

Type of material: glass slides with stained spores (syntype) deposited in the collection of the Museum of Zoology "Adão José Cardoso" State University of Campinas (UNICAMP), State of São Paulo, Brazil (accession numbers Zuec Myx 61). The SSU rDNA sequence was deposited in GenBank (accession number KY996746).

Etymology: The specific name is derived from the geographic region of origin of the host species.

3.2. Phylogenetic analysis

The SSU rDNA sequence of the mature spores of *M. peruviensis* n. sp. obtained from the gall bladder of *P. unifilis* resulted in 1876 nucleotides with a CG content of 46.2%. BLAST search revealed no identical sequences in the database. The comparison of the obtained sequence with other Myxosporean sequences available in GenBank showed that the highest percentage of identity was with *Myxidium turturibus* Aguiar et al. (2016) (query coverage 95%, maximum identities 95%). Phylogenetic analysis, showed the formation of two clades (Fig. 2). The smaller clade was composed of four marine myxosporean (three *Myxidium* and one *Sphaerospora* species). The larger clade comprised marine and freshwater taxa from the Myxidiidae as well as Sphaeromyxidae, Sphaerosporidae, Chloromyxidae and Ceratomyxidae families (Fig. 2). In this phylogenetic tree, *M. peruviensis* n. sp. cluster together in a same sub-clade with *Myxidium hardella* Garner et al. (2005), *Myxidium chelonarum* Johnson, 1969 and *M. turturibus*, which are coelozoic parasites of turtles. In this same clade, *M. peruviensis* n. sp. appears as a sister species of *M. turturibus*, the unique *Myxidium* species described from a South American turtle. The analysis of the genetic distance among the SSU rDNA sequences of the *Myxidium* species parasites of turtles that cluster together in the same clade with the new parasite herein described resulted in 89.5% to *M. chelonarum*, 89.9% to