Acta Tropica 169 (2017) 100–106

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Amazonian waters harbour an ancient freshwater *Ceratomyxa* lineage (Cnidaria: Myxosporea)

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ARTICLE INFO

Article history:
Received 23 November 2016
Received in revised form 17 January 2017
Accepted 6 February 2017
Available online 7 February 2017

Keywords:
Myxozoa
Ceratomyxa
ssrDNA sequencing
Freshwater environment
Amazon

ABSTRACT

A new species of *Ceratomyxa* parasitizing the gall bladder of *Cichla monoculus*, an endemic cichlid fish from the Amazon basin in Brazil, is described using morphological and molecular data. In the bile, both immature and mature myxospores were found floating freely or inside elongated plasmodia: length 304 (196–402) μm and width 35.7 (18.3–55.1) μm. Mature spores were elongated and only slightly crescent-shaped in frontal view with a prominent sutural line between two valve cells, which had rounded ends. Measurements of formalin-fixed myxospores: length 6.3 ± 0.6 (5.1–7.5) μm, thickness 41.2 ± 2.9 (37.1–47.6) μm, posterior angle 147°. Lateral projections slightly asymmetric, with lengths 19.3 ± 1.4 μm and 20.5 ± 1.3 μm. Two ovoid, equal size polar capsules, length 2.6 ± 0.3 (2–3.3) μm, width 2.5 ± 0.4 (1.8–3.7) μm, located adjacent to the suture and containing polar filaments with 3–4 turns. The small subunit ribosomal DNA sequence of 1605 nt was no more than 97% similar to any other sequence in GenBank, and together with the host, locality and morphometric data, supports diagnosis of the parasite as a new species, *Ceratomyxa brasiliensis* n. sp. Maximum parsimony and maximum likelihood analyses showed that *C. brasiliensis* n. sp. clustered within the marine *Ceratomyxa* clade, but was in a basally divergent lineage with two other freshwater species from the Amazon basin. Our results are consistent with previous studies that show *Ceratomyxa* species can cluster according to both geography and host ecotype, and that the few known freshwater species diverged from marine cousins relatively early in evolution of the genus, possibly driven by marine incursions into riverine environments.

1. Introduction

Myxozoa is a diverse group of over 2400 species (Bartošova-Sojková et al., 2014). They are now regarded as endoparasitic Cnidaria that have complex life cycles, which typically require an intermediate vertebrate host (mainly fish and rarely amphibians, reptiles, birds and mammals) and a definitive invertebrate host (annelids or bryozoans) (Kent et al., 2001; Canning and Okamura, 2004; Lom and Dyková, 2006; Bartholomew et al., 2008). The evolutionary affinities of the Myxozoa with the Cnidaria has been supported by the presence of polar capsules, which are analogous to cnidarian nematocysts (Weill, 1934; Siddall et al., 1995), and by molecular and phylogenetic studies (Jiménez-Guri et al., 2007; Holland et al., 2011; Nesnidal et al., 2013; Chang et al., 2015; Takeuchi et al., 2015). Unlike the majority of free-living cnidarian species, which are marine, myxozoans have experienced massive radiations in both marine and freshwater habitats, however their exact origin relative to free-living Cnidaria is still obscure and controversial (Lom and Dyková, 2006; Okamura et al., 2015).

The genus *Ceratomyxa* Thélohan, 1892, is one of the most specious myxozoan groups, with more than 230 described taxa, representing about 8% of myxozoan biodiversity (Eiras, 2006; Gunter et al., 2009; Fiala et al., 2015a). *Ceratomyxa* species are found worldwide, and are mostly coelozoic in the gallbladders of a wide range of host fish; a few species are reported from other organs, including urinary bladder and renal tubules (Eiras, 2006). The overwhelming majority of known species are marine, and many of these from the North Atlantic (Gunter et al., 2009; Mackenzie

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http://dx.doi.org/10.1016/j.actatropica.2017.02.006
0001-706X/

and Kalavati, 2014; Fiala et al., 2015a). Nine species have been reported in anadromous and/or marine fishes that enter brackish-water lagoons and estuarine environments (Eiras, 2006; Froese and Pauly, 2009), and four species (three from the Amazon Basin) have been reported from freshwater fishes (Eiras, 2006; Azevedo et al., 2013; Mathews et al., 2016; Adriano and Okamura, 2017). Of these freshwater species, only *Ceratomyxa amazonensis* Mathews, Naldoni, Maia, Adrino, 2016, and *Ceratomyxa vermiformis* Adriano and Okamura, 2017; have ssrDNA sequence data available in the NCBI database (Mathews et al., 2016; Adriano and Okamura, 2016).

In the present study, we describe a new freshwater *Ceratomyxa* species found parasitizing the gall bladder of peacock bass *Cichla monoculus* Agassiz, 1831, known as *tucunaré* (in Portuguese), an economically important fish endemic to the Amazon basin (Batista and Petrere, 2003; Dos Santos et al., 2012). We used novel ssrDNA sequence data in combination with that of other *Ceratomyxa* spp. to explore the phylogenetic relationships between marine and freshwater species.

2. Material and methods

2.1. Sampling and morphological analysis

Twenty-nine adult *C. monoculus*, length 33.3 (26–51) cm, were collected from the Tapajós River, Pará State, Brazil (02°20′214″S, 54°52′ 890″ W) in October 2014 and March 2015. The catches were authorized by Brazilian Ministry of the Environment (SISBIO n° 44268-4) and our methodology was approved by Ethics Committee on Animal Use of University of Campinas (CEUA/UNICAMP n° 3846-1). The fish were euthanized in a benzocaine solution (70 mgL^{-1}), then necropsied and examined for myxosporeans. Bile samples containing parasites were fixed in formalin (10%) for measurements (Lom and Arthur, 1989) and in 100% ethanol for DNA sequencing. Thirty spores from one host fish were photographed with differential interference contrast using a Carl Zeiss Axio Imager A2 light microscope equipped with Axio Cam and AxioVision AxioVs 40V4.8.2 software. Measurements were taken following the guidelines of Lom and Arthur (1989) and expressed as mean with standard deviation and range. Descriptions of plasmodia use terminology of Adriano and Okamura (2017).

2.2. DNA extraction, amplification, and sequencing

About 20 μl of ethanol-preserved bile was pelleted at 17,900 × *g* for 5 min and the ethanol removed. DNA was extracted from the pellet using a DNeasy® Blood & Tissue Kit (animal tissue protocol) (Qiagen Inc., California, USA) following the manufacturer's instructions. The product was eluted in 60 μl buffer AE.

ssrDNA was amplified using a semi-nested PCR. The first round amplification targeted nearly the entire ssrDNA with primers 18E (CTGGTTGATTCTGCCAGT; Hillis and Dixon, 1991) and 18R (CTACGCAAACCTTGTTACG; Whipps et al., 2003), followed by a second round with 18E and ACT1R (AATTTCACCTCTCGCTGCCA; Hallett and Diamant, 2001) and Myxigen4f (GTGCCTTGAATAAATCAGAG; Diamant et al., 2004) or novel primer MXATK2f (ACGCTTGCGAAGYGTGCCTT) with 18R. PCR was conducted in a 20 μl reaction volume, comprising: 1 μl template DNA (10–50 ng/μl), 1.25 U GoTaq Flexi polymerase (Promega, San Luis Obispo, California, USA), 0.2 μl mM each dNTPs, 0.50 μl each primer (10 pmol), 4 μl 5 × GoTaq Flexi clear buffer, 2.4 μl $MgCl_2$ (1.5 mM), 0.50 μl BSA, 0.4 μl Rediload dye (Invitrogen, Carisbad, California, USA) and 10.05 μl ultrapure water.

PCR was performed on a PTC-200 Thermocycler (MJ Research Inc., Watertown, Massachusetts, USA) with initial denaturation at 95 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 120 s with first-round primers (or 60 s when using sec-

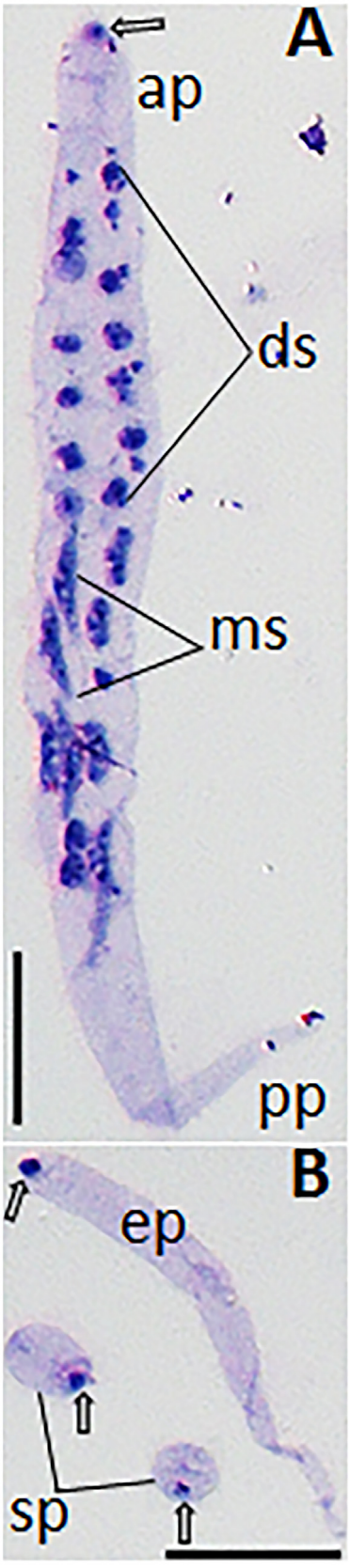

Fig. 1. Light photomicrographs of plasmodia of *Ceratomyxa brasiliensis* n. sp. parasite of the gallbladder of *Cichla monoculus*. A: young plasmodia. Note spherical early stages (sp) with nuclei (thin arrows) and elongated plasmodium (ep) with nucleus (large arrow) in the growth centre at the anterior pole (ap); no sporogonic stages are present. B: elongated plasmodium with nucleus (large arrow) in the growth centre at the anterior pole (ap), early sporogonic stages (es) and mature myxospores (ms), and a narrow posterior pole (pp). Bar = 25 μm. Stain = Giemsa.