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Ceratomyxa gracillima n. sp. (Cnidaria: Myxosporea) provides evidence of panmixia and ceratomyxid radiation in the Amazon basin

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Abstract

We describe a new freshwater myxosporean species *Ceratomyxa gracillima* n. sp. from the gall bladder of the Amazonian catfish *Brachyplatystoma rousseauxii*; the first myxozoan recorded in this host. The new *Ceratomyxa* was described on the basis of its host, myxospore morphometry, ssrDNA and internal transcribed spacer region (ITS-1) sequences. Infected fish were sampled from geographically distant localities: the Tapajós River, Pará State, the Amazon River, Amapá State and the Solimões River, Amazonas State. Immature and mature plasmodia were slender, tapered at both ends, and exhibited vermiform motility. The ribosomal sequences from parasite isolates from the three localities were identical, and distinct from all other *Ceratomyxa* sequences. No population-level genetic variation was observed, even in the typically more variable ITS-1 region. This absence of genetic variation in widely separated parasite samples suggests high gene flow as a result of panmixia in the parasite populations. Maximum likelihood and maximum parsimony analyses placed *C. gracillima* n. sp. sister to *Ceratomyxa vermiformis* in a subclade together with *Ceratomyxa brasiliensis* and *Ceratomyxa amazonensis*, all of which have Amazonian hosts. This subclade, together with other *Ceratomyxa* from freshwater hosts, formed an apparently early diverging lineage. The Amazonian freshwater *Ceratomyxa* species may represent a radiation that originated during marine incursions into the Amazon basin that introduced an ancestral lineage in the late Oligocene or early Miocene.

Introduction

Myxozoans are a group of highly specialized endoparasites, characterized by morphologically reduced spores, and complex life cycles that involve both vertebrate (predominantly fish) and invertebrate (annelids or bryozoans) hosts (Kent *et al.* 2001; Lom and Dyková, 2006). With more than 2400 described species (Zhang, 2011) they have adapted to hosts in freshwater, marine and terrestrial habitats, and this adaptability may have been crucial for their persistence and diversity. There is strong morphological, molecular, phylogenetic and structural evidence for the relationships between myxozoans and free-living Cnidaria (Siddal *et al.* 1995; Jiménez-Guri *et al.* 2007; Holland *et al.* 2011; Nesnidal *et al.* 2013; Takeuchi *et al.* 2015); however, the mechanisms behind the transitions to parasitism, and subsequent radiation of these organisms remain obscure (Okamura *et al.* 2015).

Ceratomyxa Thélohan, 1892, is the second largest myxozoan genus, with over 240 described species, most of which infect the gall bladders of marine teleosts (Eiras, 2006; Gunter *et al.*, 2009; Gunter and Adlard, 2010; Fiala *et al.* 2015). *Ceratomyxa* myxospores are distinctly elongated, crescent-shaped or arcuate, with two shell valves and two subspherical polar capsules located at the spore apex, adjacent to a straight suture line (Lom and Dyková, 2006). Three other genera have morphologically similar myxospores: *Myxodavisia* (Zhao, Zhou, Kent and Whipps, 2008) myxospores are distinguished from *Ceratomyxa* on the basis of having valve cell appendages (Zhao *et al.* 2008); *Palliatus* Shulman Kovaleva and Dubina, 1979 myxospores have a membranous veil (Lom and Dyková, 2006), and *Meglitschia* Kovaleva, 1988 myxospores differ from other *Ceratomyxa* spp. by being highly arcuate, with large and elongated polar capsules (Lom and Dyková, 2006). Available molecular data for *Myxodavisia bulani* (Fiala, Kodadkova, Freeman, Bartošova-Sojkova and Atkinson, 2015), and *Palliatus indecorus* (Shulman Kovaleva and Dubina, 1979), demonstrate that these taxa cluster in a basal position within the '*Ceratomyxa*' clade of the marine lineage in ssrDNA-based phylogenetic studies (Fiala *et al.* 2015; Adriano and Okamura, 2017; Zatti *et al.* 2017). Molecular data from additional taxa, particularly from type species, are needed to better assess the validity of maintaining these groups as four distinct genera (Fiala *et al.* 2015; Adriano and Okamura, 2017).

Within the *Ceratomyxa*, only six species have been described from strictly freshwater environments, and five of these are from the Amazon basin: *C. mylei* (syn. *Meglitschia mylei*)

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(Azevedo, Ribeiro, Clemente, Casal, Lopes, Al-Quraishy and Matos, 2011), *C. microlepsis* (Azevedo, Rocha, Casal, Sao Clemente, Matos, Al-Quraishy and Matos, 2013), *C. amazonensis* (Mathews, Naldoni, Maia and Adriano, 2016), *C. vermiformis* Adriano and Okamura, 2017 and *C. brasiliensis* (Zatti, Atkinson, Bartholomew, Maia and Adriano, 2017). One of these Amazonian species, *C. vermiformis*, has elongated plasmodial stages that exhibit coordinated, undulatory locomotion, provided by plasmodial cytoskeleton elements, in the bile of its host (Adriano and Okamura, 2017). Superficially similar worm-like movement has been observed in myxoworm stages of taxa in class Malacosporea, where the movement is supported by four sets of longitudinal muscles (Gruhl and Okamura, 2012).

During a survey of Myxozoa of the Amazon basin in Brazil, we found a novel, freshwater *Ceratomyxa* species that parasitized the catfish *Brachyplatystoma rousseauxii* (Castelnau, 1855) (Siluriformes: Pimelodidae). Known as 'dourada' in Portuguese, this fish performs the longest strictly freshwater migration in the world, a journey of ~11 600 km (Barthem *et al.* 2017). Dourada spawn in Andean tributaries of the headwaters of the Amazon (Barthem and Goulding, 1997) and immature fish are transported downriver to the Amazon estuary region, where juveniles remain for 1·5–2 years. After this growth period, they then move upstream into the lower and middle Amazon, where they inhabit river channels and floodplains for another year (Batista and Alves-Gomes, 2006). Reproductive migration of this species back to the headwaters occurs June–November, when the river level starts to rise due to the rainy season (Batista and Alves-Gomes, 2006). Carvajal-Vallejos *et al.* (2014) observed some evidence of genetically distinct dourada populations in the Amazon basin, which have specific spawning areas.

We sampled dourada from three widely separated rivers within the Amazon basin, and based on morphological, ssrDNA and internal transcribed spacer region (ITS-1) sequencing identified the same novel *Ceratomyxa* species in the gall bladder of fish from each locality. Lack of ITS-1 variation suggested that the parasite has a uniform population structure across broad spatial scales, a feature we suggest is evidence of panmixia in the parasite population as a consequence of the extensive migrations of the fish host.

Material and methods

Fish and parasite sampling

Thirty *B. rousseauxii* were collected by net from three localities (GPS coordinates given in Results section): fish were collected from the Tapajós River, Pará State, Brazil in October 2014 and March 2015 (average length 44 cm, range 34–59 cm, $N = 17$); from the Amazon River, Amapá State, Brazil, in October 2015 (length 52 cm, $N = 1$) and from the Solimões River, Amazonas State in December 2015 (average length 77 cm, range 33–103 cm, $N = 12$) (Fig. 1). The catches were authorized by the Brazilian Ministry of the Environment (SISBIO n° 44268-4), and the examination methodology was approved by the Ethics Committee on Animal Use, University of Campinas (CEUA/UNICAMP n° 3846). Fish were euthanized by overdose of benzocaine solution and necropsied. Gall bladders were removed and ruptured. Bile samples were examined by light microscope and those containing myxosporeans were subdivided and fixed in 10% neutral-buffered formalin for spore measurement (Lom and Arthur, 1989) and in 100% ethanol for DNA sequencing. Myxospores obtained from ten different plasmodia from two fish specimens (from Tapajós and Solimões rivers) were photographed using a Carl Zeiss Axio Imager A2 light microscope equipped with Axio Cam and AxioVision AxioVs 40V4.8.2 software, using differential interference contrast. Measurements of

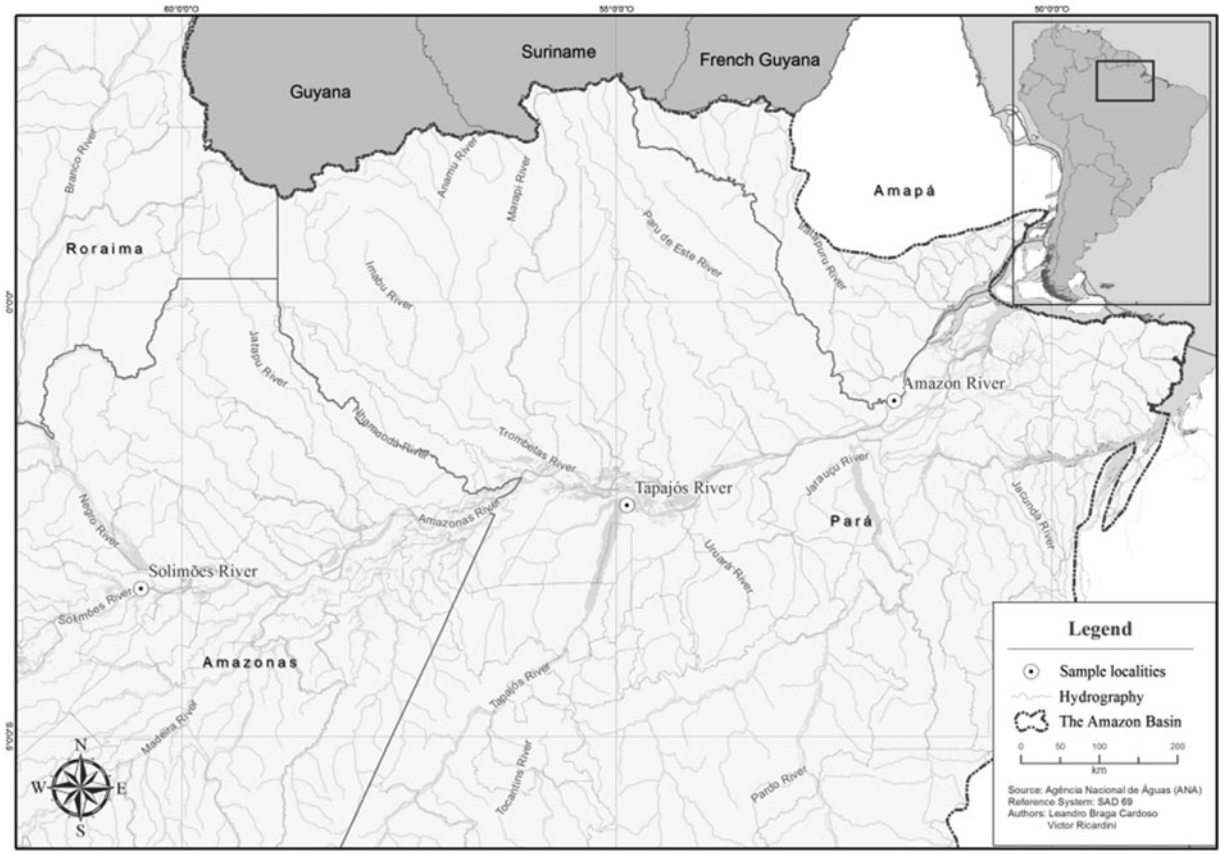

Fig. 1. Map of the myxozoan *Ceratomyxa gracillima* n. sp. collection localities in the Amazon basin: Solimões River (Amazonas State); Tapajós River (Pará State) and Amazon River (Amapá State).

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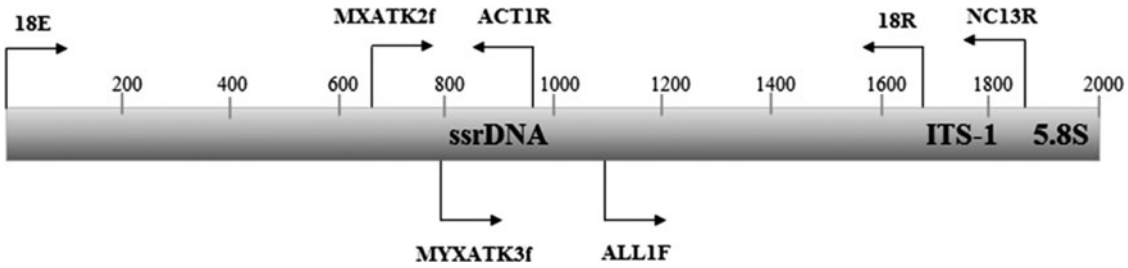

Fig. 2. Schematic representation of part of the ribosomal DNA of *Ceratomyxa gracillima* n. sp., which shows locations of gene regions and PCR primers.

formalin-fixed myxospores followed the general guidelines of Lom and Arthur (1989), and Gunter *et al.* (2009) for *Ceratomyxa* spp., with modifications suggested by Adriano and Okamura (2017) for strongly arcuate myxospores. We use the more structurally accurate term 'polar tubule' instead of 'polar filament' (Ben-David *et al.* 2016). Type myxospores were also air-dried onto glass slides, stained with Giemsa, and deposited in the Museum of Zoology 'Adão José Cardoso' University of Campinas (UNICAMP), São Paulo State, Brazil.

Molecular studies

DNA was extracted from 50 μL of infected bile preserved in ethanol. The sample was pelleted at 15 700 G for 10 min and the ethanol removed. DNA was extracted from the pellet using a DNeasy® Blood & Tissue Kit (animal tissue protocol) (Qiagen Inc., Redwood City, California, USA).

The ssrDNA was amplified using a semi-nested PCR: first round amplification targeted the entire ssrDNA with universal primers 18E (CTGGTTGATTCTGCCAGT; Hillis and Dixon, 1991) and 18R (CTACGCAAACCTTGTTACG; Whipps *et al.* 2003) (Fig. 2). PCR was conducted in 20 μL reaction volumes, comprising: 1 μL template DNA (10–50 ng μL^{-1}), 0·25 μL GoTaq Flexi polymerase (Promega, San Luis Obispo, California, USA), 0·2 μL mM each dNTPs, 0·50 μL each primer (10 pL), 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, 62 °C for 30 s (or 58 °C and 60 s with second-round primers) and 72 °C for 120 s, followed by a terminal extension at 72 °C for 7 min. Semi-nested second-round PCR generated overlapping fragments using primers 18E and ACT1R (AATTTCACCTCTCGCTGCCA; Hallett and Diamant, 2001) and MXATK2f (ACGCTTGCGAAGYGTGCCTT, Zatti *et al.* 2017) with 18R (Fig. 2).

For the ITS-1 amplification, the first round was performed with the novel primer MYXATK3f (CATTTGAGGGCGTTAGT ACTTG) paired with NC13R (GCTGCGTTCTTCATCGAT, Gasser *et al.* 1993) followed by a second round with ALL1F (GCGGCTTAATTTGACTCAACACGGG, Hallett *et al.* 2002) and NC13R (Fig. 2). These primers produced fragments that extended from the ssrDNA through the ITS-1 and terminated in the 5·8S. The PCR protocol was the same as above, but with an annealing temperature of 60 °C. All amplicons were electrophoresed in 1·5% agarose gels with Tris-borate-EDTA buffer (0·045 M Tris-borate, 0·001 M EDTA, pH 8·0) stained with SYBRsafe (Invitrogen By Life Technologies, Maryland, USA) alongside a 1 kb Plus DNA Ladder (Invitrogen By Life Technologies, Maryland, USA), then analysed on a Compact Digimage System transilluminator (Major Science, Saratoga, California, USA). Products from PCRs that produced a single bright band were purified with QIAquick PCR

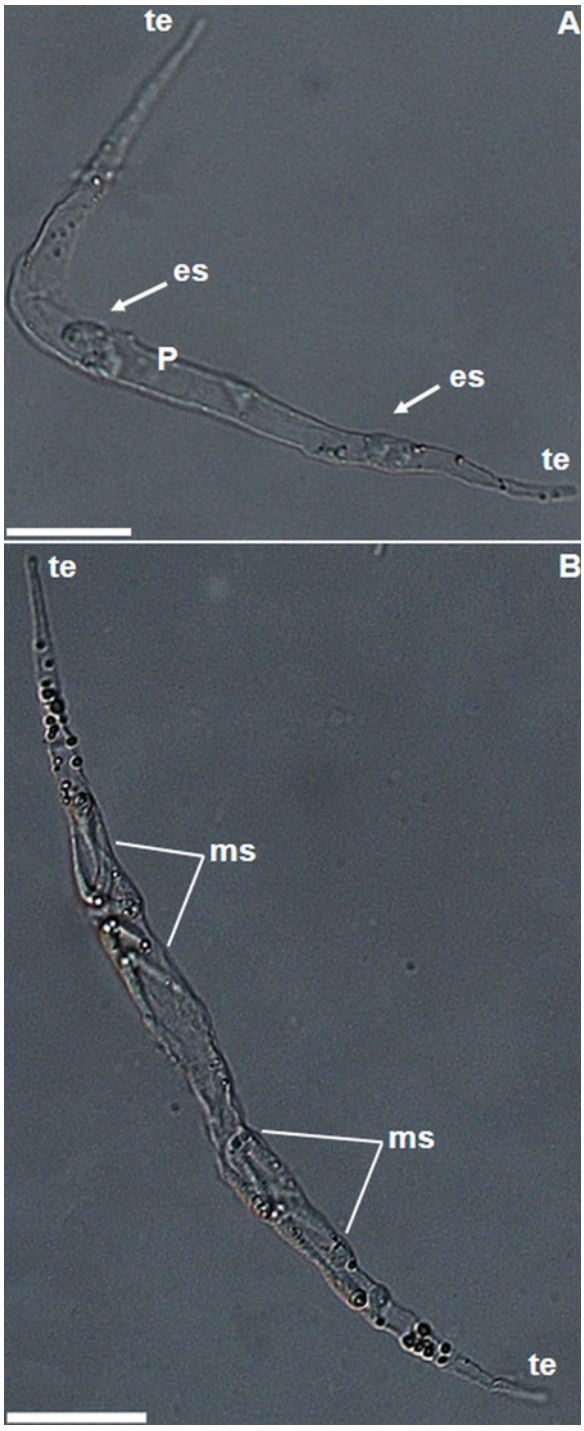

Fig. 3. Light photomicrographs of plasmodia of *Ceratomyxa gracillima* n. sp. from the gallbladder of *Brachyplatystoma rousseauxii*. (A) Immature plasmodium. Note the tapered ends (te) at both poles, and early sporogonic stages (es) in the medial region of the plasmodium (P). (B) Mature plasmodium showing mature myxospores (ms). Bars: 20 μm.

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Purification Kit (Qiagen) according to the manufacturer's instructions, then sequenced in both directions. Sequencing reactions were performed using a BigDye 102 Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, California, USA) in an ABI 3730 DNA 103 Analyzer (Applied Biosystems) at the Oregon State University Center for Genome Research and Biocomputing.

Sequence assembly, alignment and phylogenetic analyses

Forward and reverse sequences were aligned by eye and assembled into consensus sequences using BioEdit (Hall, 1999). Chromatograms were examined for polymorphic loci, indicated by coincident peaks. We used a visual threshold for recording secondary peaks only if they were at least 50% of the height of the primary peak. Consensus sequences were searched using BLASTn (Altschul *et al.* 1997) with the GenBank database to identify the most closely related taxa.

Phylogenetic analyses were performed on an alignment of 81 ssrRNA sequences from related species, which included all available *Ceratomyxa* spp., *P. indecorus* and *M. bulani*, retrieved from the NCBI database (accession numbers are indicated in the phylogenetic tree). Sequences were aligned with ClustalW (Thompson *et al.* 1997) using default parameters. Gaps were treated as missing data. Phylogenetic trees were calculated using maximum likelihood (ML) and maximum parsimony (MP) methods. The optimum evolutionary model for the dataset was obtained by the Akaike information criterion using jModelTest 0.1.1 (Posada, 2008), which identified GTR + I + G as the best-fit model. ML analysis was made using PhyML version 3.0 (Guindon and Gascuel, 2003) implemented *via* the web server (http://www.atgc-montpellier.fr/phyml/) (Guindon *et al.* 2010). MP was computed in PAUP* v.4.0b10 (Swofford, 2002) employing a heuristic search with ten repetitions of random sequence addition, the ACCTRAN-option, with tree bisection and reconnection branch swapping. Basal myxosporean taxa *Chloromyxum auratum* (AY971521) and *Chloromyxum clavatum* (JQ793641) were used as outgroups. Bootstrap support was calculated using 1000 replicates for both methods. Trees were visualized using Figtree 1.3.1 (Rambaut, 2008) and annotated in Adobe Photoshop (Adobe Systems Inc., California, USA). Sequence divergence among all *Ceratomyxa* from Amazon were estimated using *p*-distance and distance (base pairs differences) methods in MEGA 7.0 (Kumar *et al.* 2016) using default parameters.

Results

Morphological, molecular and host data supported description of a new *Ceratomyxa* species. We found the parasite in gall bladders of *B. rousseauxii* at each of the three sample locations: 82% (14/17) from the Tapajós River, 100% (1/1) from the Amazon River and 50% (6/12) from the Solimões River. The average prevalence was 70% (21/30). Parasite plasmodia were elongated, and exhibited undulatory, worm-like locomotion, swimming freely in the bile.

Morphological description and taxonomic summary

Phylum: Cnidaria Verrill, 1865
Unranked sub-phylum: Myxozoa Grassé, 1970
Class: Myxosporea Bütschli, 1881
Order: Bivalvulida Shulman, 1959
Family: Ceratomyxiidae Doflein, 1899
Genus: *Ceratomyxa* Thélohan, 1892
Ceratomyxa gracillima n. sp. (Figs 3–5).

Vegetative stages

Motile, elongated plasmodia at different stages of development were found swimming freely in the bile of *B. rousseauxii*. Immature plasmodia contained few sporogonic stages in medial regions and no mature myxospores (Fig. 3A), and showed an average length of 112 μm (range 72–134 μm, $N = 7$); and average width of 8·9 (range 4·6–11·8 μm, $N = 7$). Mature plasmodia contained mature myxospores in their medial portion (Fig. 3B). Average plasmodia length 181 μm (range 173–246 μm, $N = 10$) and average width 11·4 μm (range 7·2–16·2 μm, $N = 10$).

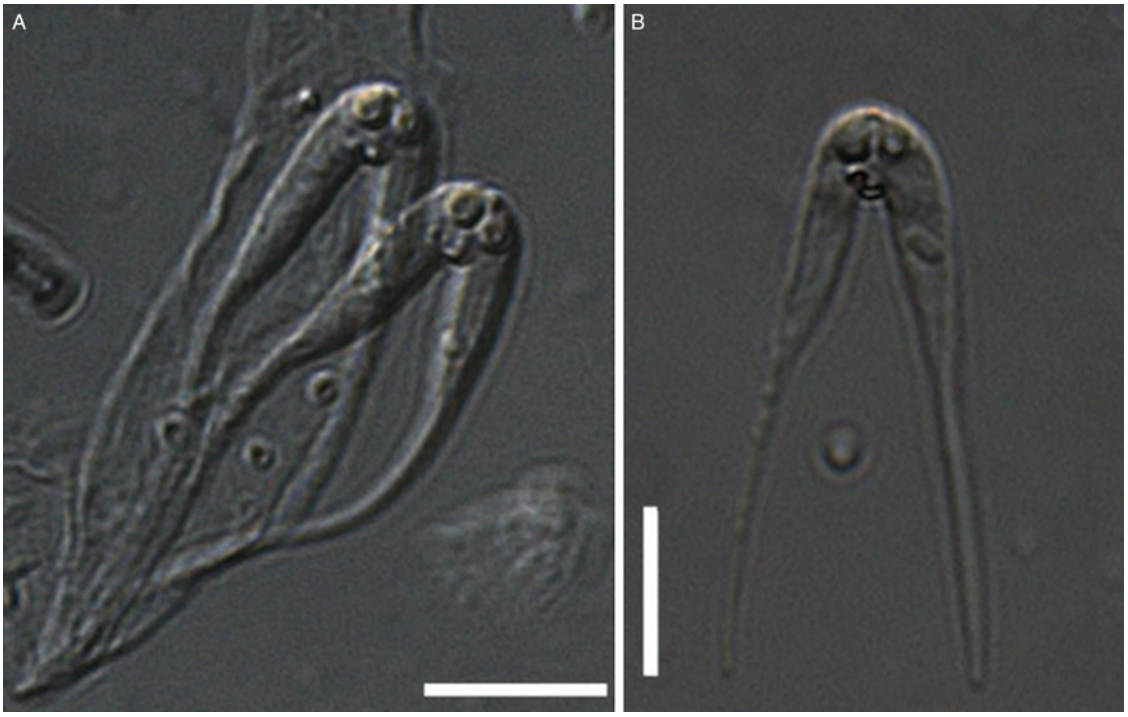

Fig. 4. Light photomicrographs of *Ceratomyxa gracillima* n. sp. A and B: mature myxospores. Bars: 10 μm.

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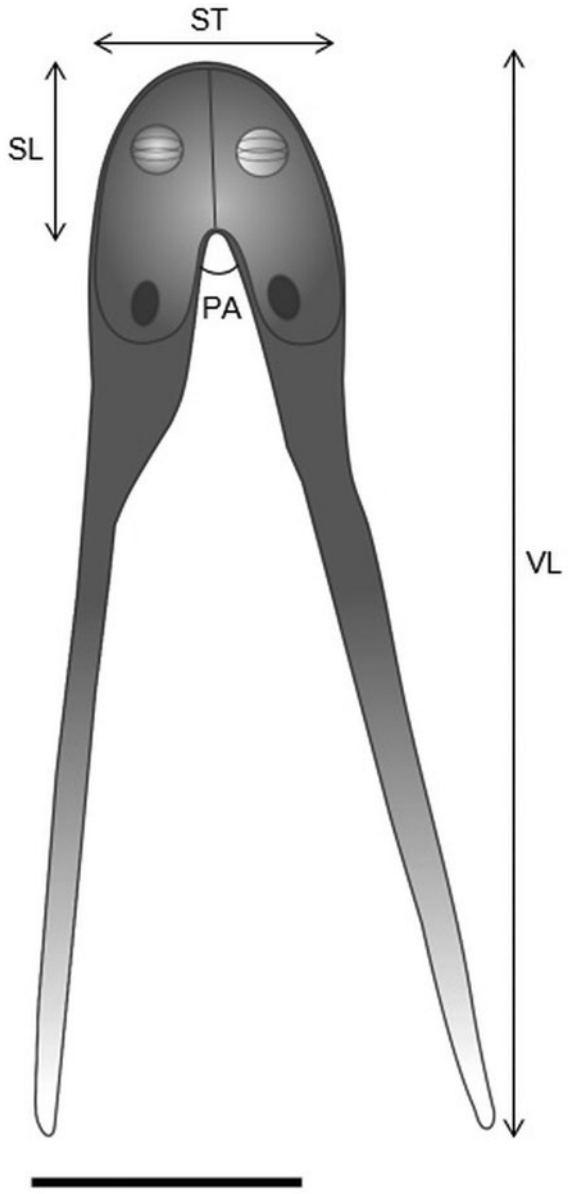

Fig. 5. Schematic drawing of *Ceratomyxa gracillima* n. sp. showing details of the principal measured components. SL, spore length; ST, spore thickness; VL, valves length; PA, posterior angle. Bar: 10 μm.

Mature myxospores

Spores were strongly arcuate in side view, average total length 28 μm (range 23·3–35·3 μm, $N = 33$); average spore length of 4·4 μm (range 3·0–5·7 μm, $N = 33$); average spore thickness 7·0 μm (range 6·0–8·2 μm, $N = 33$). Two spherical, equal-sized polar capsules, diameter 1·9 μm (range 1·5–2·5 μm, $N = 30$), located anteriorly, adjacent to the straight suture. Each polar capsule containing a polar tubule with 2–3 turns around the longitudinal axis. Posterior angle averaged 36·6° (range 35°–40°, $N = 10$) (Figs 4A, B and 5).

Type host: *Brachyplatystoma rousseauxii* (Siluriformes: Pimelodidae)

Prevalence across the three sites: 21 of 30 (70%).

Type locality: Tapajós River, municipality of Santarém, Pará State, Brazil, (02°20′28″S, 54°52′56″W). Other localities: Amazon River, municipality of Vitoria do Jari, Amapá State (01°08′17″S, 51°48′31″W) and Solimões River, municipality of Manacapuru, Amazonas State (03°18′08″S, 60°28′00″W).

Site of infection: Lumen of gall bladder.

Type material: A glass slide with fixed, stained myxospores (syntype) was deposited in the Museum of Zoology 'Adão José Cardoso' University of Campinas (UNICAMP), São Paulo, Brazil (Zuec MYX 65). Concatenated ssrDNA, ITS-1 and partial 5·8S sequences were deposited in the NCBI GenBank database (accession numbers: KY934182 from the Tapajós River, KY934183 from the Solimões River and KY934184 from the Amazon River).

Etymology: The specific name *gracillima* (in English, *gracile* = slender, thin, slim) refers to the features of the plasmodia.

Molecular and phylogenetic analyses

We obtained partial rDNA sequences (18S-ITS-1-5.8S) for *C. gracillima* n. sp. from each of the three localities (1839 bp from the Tapajós River, 1855 bp from the Solimões River and 1849 bp from the Amazon River). Apart from differences in length, the sequences were identical. The BLASTn search revealed no other sequence with more than 94% similarity to *C. gracillima* n. sp. The ITS-1 region began approximately 1635 bp from the start of primer 18E and was about 165 bp long. At four positions – 1725, 1747, 1763 and 1809 bp – we observed ~50/50 mixtures of alleles as single nucleotide polymorphisms (SNPs) at the same positions in the *C. gracillima* n. sp. sequences from the three rivers.

Phylogenetic analysis revealed two main clades, A and B (Fig. 6). The large clade A contains a subclade A1 of exclusively *Ceratomyxa* marine species, plus two small marine subclades. Subclade A2 comprised *Ceratomyxa* sp. (DQ377699), *Ceratomyxa synaphobranchi* (Fiala, Hlavnickova, Kodadkova, Freeman, Bartošova-Sojkova and Atkinson, 2015), and *P. indecorus*, all of which are parasites of deep water marine fish. Subclade A3 comprised parasites of sharks and cod.

Ceratomyxa gracillima n. sp. clustered in the smaller clade B with *C. vermiformis*, *C. amazonensis* and *C. brasiliensis* to form a subclade of species with exclusively freshwater Amazonian fish hosts. This subclade was sister to *Ceratomyxa leatherjacketi* (Fiala, Hlavnickova, Kodadkova, Freeman, Bartošova-Sojkova and Atkinson, 2015) and *Ceratomyxa tunisiensis* (Thabet, Mansoura, Omar and Zouaria, 2015). Together with *M. bulani* these isolates collectively formed an early-diverging clade within the marine myxosporean lineage (Fig. 6).

Discussion

We found a novel *Ceratomyxa* species, *C. gracillima* n. sp. in the bile of an economically important Amazonian catfish, *B. rousseauxii*. The parasite developed in the gall bladder and the myxospores are typical of *Ceratomyxa* spp., apart from being arcuate in frontal view and having extended valve cells characteristic of *Meglitschia*. There are, however, limited morphological characters to distinguish *Meglitschia* from *Ceratomyxa*, and a distinction of the two genera is not supported by molecular data (Zhao *et al.* 2008; Adriano and Okamura, 2017). Accordingly, we compared the morphology of *C. gracillima* n. sp. primarily with other freshwater *Ceratomyxa* spp. and then, with other congeners from marine/brackish hosts, guided by the species checklist of Eiras (2006) and other recent taxonomic studies (Gunter *et al.* 2009; Azevedo *et al.* 2011; Azevedo *et al.* 2013; Fiala *et al.* 2015; Mathews *et al.* 2016; Adriano and Okamura, 2017; Zatti *et al.* 2017).

The strongly arcuate myxospores of *C. gracillima* n. sp. resemble those of both *C. vermiformis*, a parasite of *Colossoma macropomum*, and *C. mylei*, a parasite of *Myleus rubripinnis*. Both of these hosts are serrasalmid fishes from the Amazon basin. *Ceratomyxa gracillima* n. sp. myxospores are longer, narrower and have smaller polar capsules with fewer turns of the polar tubules than those of *C. vermiformis* and *C. mylei* (Table 1).

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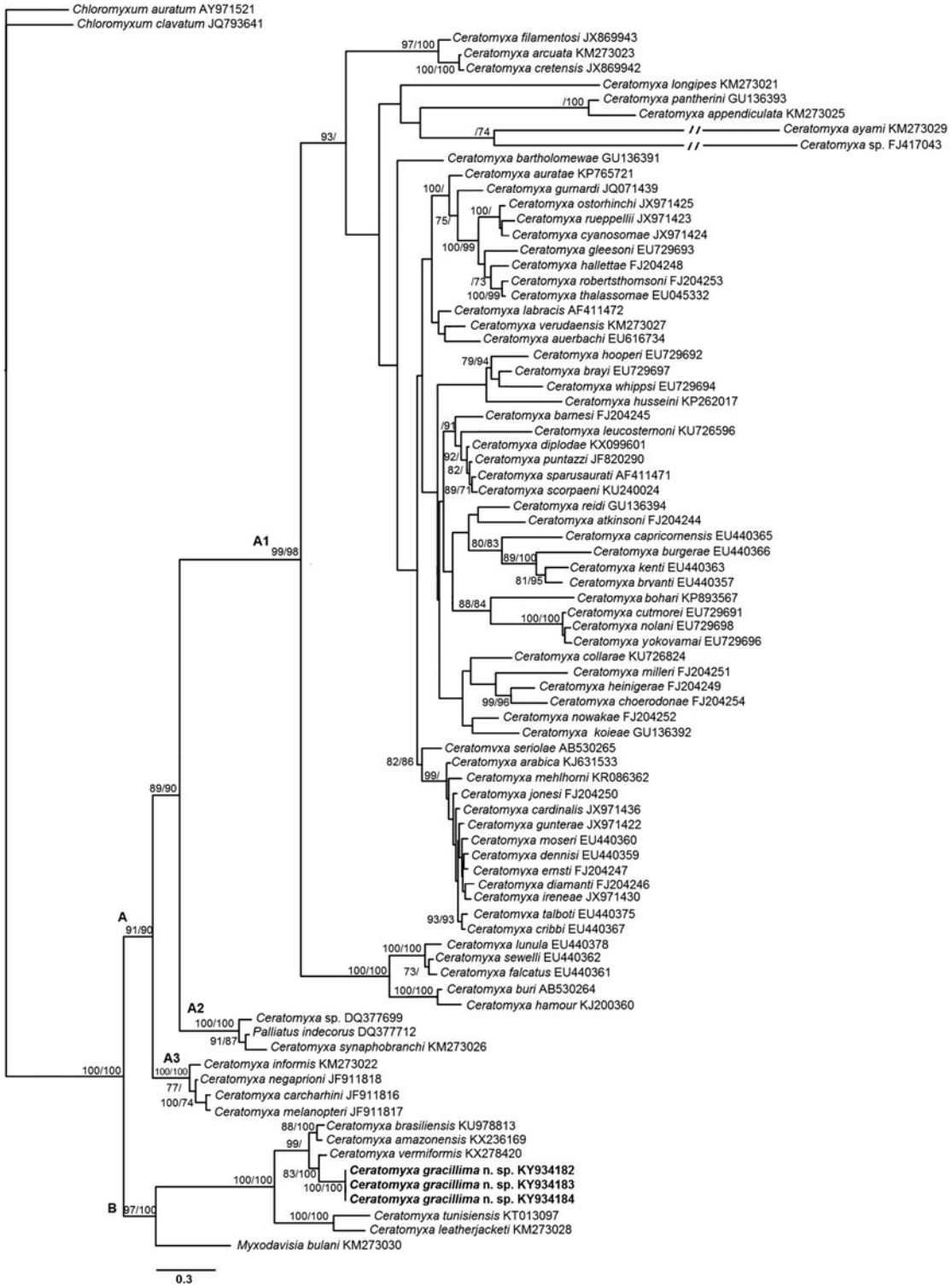

Fig. 6. Consensus maximum likelihood phylogenetic tree based on ssrDNA sequences of *Ceratomyxa gracillima* n. sp., other *Ceratomyxa* spp. plus *Palliatus indecorus* and *Myxodavisia bulani*. Nodal supports are indicated for maximum likelihood (ML) and maximum parsimony (MP), respectively, with a bootstrap of 1000 replicates. Branches with bootstrap ⩽70% support are blank. GenBank accession numbers are shown after taxon names. Clade A: Marine *Ceratomyxa* spp., including *P. indecorus*. Clade B: Amazonian *Ceratomyxa* spp. plus *C. leatherjacketi*, *C. tunisiensis* and *M. bulani*.

The elongate plasmodia of *C. vermiformis* have blunt ends and a gradient in maturation of developmental stages demonstrates that one of these blunt ends (the anterior end) serves as a centre of growth (a growth pole) (Adriano and Okamura, 2017). The elongate plasmodia of *C. gracillima* n. sp. have thin, tapering ends (Fig. 3A, B), and we did not observe a distinct growth pole in this species (although early developmental stages were present in medial regions). Another Amazonian myxozoan, *C. brasiliensis*, has elongated plasmodia but these were not observed to be motile (Zatti *et al.* 2017). *Ceratomyxa gracillima* n. sp. was distinct from most *Ceratomyxa* species on the basis of its strongly arcuate spores and elongated valve cells. The myxospores of other ceratomyxids with arcuate spores (*C. insolita*, *C. inconstans* Jameson, 1929, and *C. sphairophora* Davis, 1917) differ in valve lengths and spore thickness relative to *C. gracillima* n. sp., *C. vermiformis* and *C. mylei* myxospores (Meglitsch, 1960; Eiras, 2006; Azevedo *et al.* 2011; Adriano and Okamura, 2017).

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Table 1. Comparison of morphometry of *Ceratomyxa gracillima* n. sp. with Amazonian *Ceratomyxa* spp. with arcuate myxospores

Species	*Ceratomyxa gracillima* n. sp. (This study)	*Ceratomyxa vermiformis* (Adriano and Okamura, 2017)	*Ceratomyxa mylei* (syn. *Meglitschia mylei*) (Azevedo *et al.* 2011)
Measurements for comparison with *M. mylei* (Azevedo *et al.* 2011)			
STL	28 ± 3 (23–35)	26 ± 2 (22–29)	24·6 ± 0·8
LA	24 ± 2 (19–29)	18·8 ± 0·7 (17·6–19·3)	20·1 ± 0·7
		17·5 ± 0·8 (16·4–18·3)	
SW	7·0 ± 0·5 (6·0–8·2)	8·4 ± 0·4 (7·9–9·3)	8·7 ± 0·4
ST	4·4 ± 0·4 (3·3–5·7)	5·4 ± 03 (4·9–5·7)	5·1 ± 0·3
PC	1·9 ± 0·3 (1·5–2·5)	2·7 ± 0·1	2·1 ± 0·3
PT	2–3	3–4	5–6
Measurements based on Gunter *et al.* (2009) for comparison with *Ceratomyxa* spp.			
SL	4·4 ± 0·4 (3·3–5·7)	4·5 ± 0·2 (4·2–4·8)	
VL	28 ± 3 (23·3–35·3)	23·7 ± 0·7 (22·1–24·3)	
		21·9 ± 08 (20·6–23)	
SW	—	5·4 ± 03 (4·9–5·7)	
ST	7 ± 0·5 (6–8·2)	8·4 ± 0·4 (7·9–9·3)	
PA	36·6° ± 2·9° (35°–40°)	30·2° ± 6·6° (22°–43°)	
Type host	*Brachyplatystoma rousseauxii*	*Colossoma macropomum*	*Myleus rubripinnis*
Locality	Tapajós River, PA, Amazon River, AP and Solimões River, AM	Tapajós River, PA, and Amazon River AP	Lagoon of Sapuruá, AM

Dimensions are given in micrometres expressed as the mean ± standard deviation followed by the range in parentheses. STL, spore total length; LA, length of appendices; SW, spore width; ST, spore thickness; PC, polar capsule diameter; PT, polar tubule turns; SL, spore length; VL, valve length; PA, posterior angle; PA, Pará State; AP, Amapá State; AM, Amazonas State.

Table 2. Pairwise identities of ssrDNA sequences from *Ceratomyxa* species described from Amazon basin fish hosts, adjusted for missing data. The upper triangular matrix shows the number of nucleotide differences; the lower triangular matrix shows the percentage nucleotide difference

Species	1	2	3	4
1. *Ceratomyxa gracillima* n. sp.	–	92	109	109
2. *Ceratomyxa vermiformis*	5·2	–	82	74
3. *Ceratomyxa brasiliensis*	6·8	5·1	–	38
4. *Ceratomyxa amazonensis*	6·9	4·7	2·4	–

The ssrDNA sequence data that are available clearly distinguish *C. gracillima* n. sp. from its congeners. Thus, it was at least 5·2% different from its nearest relative, *C. vermiformis* (Table 2), but we note that there are no molecular data for the Amazonian species *C. mylei* for comparison. Levels of interspecific genetic variation amongst myxosporeans vary (Ferguson *et al.* 2008; Carriero *et al.* 2013), and *Ceratomyxa* species have been shown to differ by 2% to more than 20% (Gunter *et al.* 2009). The interspecific differences between available ssrDNA sequences for Amazonian *Ceratomyxa* spp. range from 2·4% (*C. brasiliensis vs C. amazonensis*) to 6·9% (*C. gracillima* n. sp. *vs C. amazonensis*) (see Table 2).

Despite the lack of ssrDNA sequence data for one of the Amazonian ceratomyxids (*C. mylei*), we consider that the considerable morphological differences in plasmodia and spores described above and the exploitation of the different fish hosts provide evidence consistent with species-level differences and hence the designation of *C. gracillima* as a new species.

As ssrDNA is typically conserved within isolates of the same species, the neighbouring, more variable ITS-1 was explored to identify intraspecific differences (strains/genotypes) (Whipps and Kent, 2006; Atkinson and Bartholomew, 2010*a*, *b*). Accordingly, we identified a series of polymorphic loci (SNPs) in the ITS-1 which were evident as two mixed peaks at the same four locations in all sequence chromatograms. The absence of variability in the sequences in this genome region was surprising and is evidence that the parasite population is genetically uniform for this highly variable region despite wide geographic separation of the collection localities. In a straight line, the Solimões River site is ~660 km from the Tapajós River site and around 1000 km from the Amazon River site, while the distance between Tapajós and the Amazon sites is ~370 km. The similarity of ITS-1 sequences amongst parasites collected from such widely separated localities leads us to hypothesize that this uniformity is evidence of high gene flow across broad spatial scales, and is evidence of panmixia. We consider that this mixing is a result of the fish host's exceptionally long-distance migration from tributaries in the foothills of the Andes to the coastal estuary of Brazil, and eventually back again (Barthem and Goulding, 1997; Barthem *et al.* 2017). Pathogens and parasites are potentially carried with fish along this migration route and their populations may therefore be mixed over great distances.

The addition of *C. gracillima* n. sp. to ML and MP molecular phylogenetic analyses of *Ceratomyxa* species provides further support for a distinct Amazonian *Ceratomyxa* lineage (Mathews *et al.* 2016; Adriano and Okamura, 2017; Zatti *et al.* 2017). Our analysis revealed *C. gracillima* n. sp. as sister to the morphologically similar *C. vermiformis* and both are placed in a subclade that also contains *C. brasiliensis* and *C. amazonensis*. Those species, together with *C. tunisiensis*, *C. leatherjacketi* and *M. bulani*, in turn form a well-supported and apparently early-diverging ceratomyxid clade (clade B; Fig. 6). This apparent early-diverging position for the Amazonian freshwater lineage relative to the larger, fully marine *Ceratomyxa* clade may be a genetic signal of historical marine incursions into the Amazon basin (Zatti *et al.* 2017).

Although the Amazonian *Ceratomyxa* spp. studied so far have been reported only in hosts belonging to evolutionary lineages of freshwaters species (i.e. Serrasalmidae, Cichlidae and Pimelodidae) (Nelson *et al.* 2016; Bloom and Lovejoy, 2017), several vertebrate and invertebrate freshwater organisms from the Amazon region are closely related to extant marine lineages, including stingrays, croakers, sponges, mollusks, parasitic monogenoids (Boeger and Kritsky, 2003; Lovejoy *et al.* 2006; Cooke *et al.* 2011; Bloom and Lovejoy, 2017). Several hypotheses have been proposed to explain the origin of these marine-derived lineages, including opportunistic invasions *via* estuaries, vicariance events and marine incursions (Lovejoy *et al.* 2006; Hoorn *et al.* 2010; Bloom and Lovejoy, 2017). There is evidence, based on fossil records, geomorphological history, distributional data and phylogenetic results, that marine incursions into South America occurred between late Oligocene and early Miocene were involved in the adaptation of marine fish to the freshwater environment in the Amazon (Cooke *et al.* 2011; Bloom and Lovejoy, 2017). Apart from South America, the occurrence of *Ceratomyxa* spp. in hosts inhabiting exclusively freshwater (or estuarine) environments is exceptional and restricted to *Ceratomyxa hongtzensis* Hsieh and Chen, 1984, *Ceratomyxa anguillae* Tuzet and Ormières,1957 and *Ceratomyxa hungarica* Molnár, 1992 (Eiras, 2006; Froese and Pauly, 2013). In South America, there is still no record of *Ceratomyxa* species infecting freshwater fish outside the Amazon basin (Eiras, 2006; Adriano and Oliveira, 2017). In this context, the contrast between the uncommon presence of *Ceratomyxa* spp. infecting freshwater hosts from other continents and watersheds and the recent finding of five species related to marine taxa parasitizing Amazonian freshwater fish (Azevedo *et al.* 2011, 2013; Mathews *et al.* 2016; Adriano and Okamura, 2017; Zatti *et al.* 2017) suggest that this freshwater diversity may have arisen from ancestral marine ceratomyxids that invaded the Amazon during marine incursions in the late Oligocene and early Miocene, and subsequently adapted to freshwater environments. Subsequent adoption of freshwater hosts possibly leads to the radiation of Amazonian ceratomyxids shown by phylogenetic analyses. If this hypothesis is valid, then dating of the transition of ancestral *Ceratomyxa* species, from marine to freshwater, could provide an indication of the rate of molecular evolution for myxozoans, which is potentially valuable and unique in the understanding of the evolution of these enigmatic parasites.

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