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Phylogenetic analysis and characterization of a new parasitic cnidarian (Myxosporea: Myxobolidae) parasitizing skin of the giant mottled eel from the Solomon Islands

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ABSTRACT

Myxosporeans are microscopic cnidarians associated with severe diseases in aquaculture and wild fish populations. This group of parasitic cnidarians thus warrants close attention concerning its potential impact on susceptible fish stocks. At present, little is known about this group of parasites infecting anguillid eels. From myxospore specimens collected from a freshwater eel (*Anguilla marmorata*) in the Solomon Islands, we describe a new species belonging to the genus *Myxobolus* based on an integrative taxonomic analysis of morphological, biological traits and molecular data. Furthermore, we determined the phylogenetic position and relationships of this species among other platysporine myxosporeans. Molecular phylogenetic assessment of small subunit ribosomal DNA showed that the species clusters together with *Myxobolus portucalensis* and *Echinactinomyxon* type 5 Özer, Wootten and Shinn, 2002, in a well-

supported subclade. This is the first report of a myxosporean parasite infecting fish from the Solomon Islands

Keywords: Cnidaria, parasite, *Myxobolus choiseulae* n. sp., *Anguilla marmorata*, freshwater eel.

1. Introduction

Myxosporeans are globally distributed microscopic cnidarian endoparasites associated with severe diseases in aquaculture and wild fish populations (Alama-Bermejo and Holzer, 2021). They are biologically diverse with approximately 2,600 species described (Okamura et al., 2018), representing about one fifth of the cnidarian biodiversity known to date (Atkinson et al., 2018). Myxosporeans have a complex life cycle, including annelids as their definitive hosts and vertebrates such as fish (mainly), amphibians, reptiles, birds and mammals as intermediate hosts (Okamura et al., 2015, Liu et al., 2019). Within myxosporeans, *Myxobolus* Bütschli, 1882 is the richest genus with more than 900 described species, some of which are important fish pathogens in many regions around the world (Eiras et al., 2014). In the skin, some *Myxobolus* species have been described causing considerable pathological issues such as epithelial cell shedding and immunological cell infiltration, large skin nodules, erosion and attenuation of the epidermis and necroulcerative dermatitis (Rothwell et al., 1997; Zhang et al., 2017; Stilwell et al., 2020). Until this study only four *Myxobolus* species were reported from anguillid hosts: *Myxobolus kohlani* Molnár, Lom and Malik, 1985 and *Myxobolus portucalensis* Saraiva, Molnár, 1990 from *Anguilla anguilla*, *Myxobolus dermatobia* Ishi, 1915 and *Myxobolus fujitai* Fujita, 1929 from *Anguilla japonica*.

The eel genus *Anguilla* Garsault, 1764, includes predatory and migratory fish and consists of 19 species/subspecies widespread throughout the world from tropical,

subtropical to temperate geographical areas (Arai, 2016). The giant mottled eel *Anguilla marmorata* Quoy & Gaimard, 1824 is a tropical freshwater catadromous anguillid widely distributed in the southeast-central Pacific Ocean, Indian Ocean and adjacent freshwater habitats (Arai and Chino, 2018). It migrates between freshwater growth habitats and offshore spawning areas and can reach up to 2 meters in length and 21 kg in weight (Arai and Chino, 2018). *Anguilla marmorata* adults are highly valued commercial fish in some regions and their fingerlings are used as seed-stock for farming (Pike et al., 2020). At present, the conservation status of *A. marmorata* is Least Concern in the IUCN Red List of Threatened Species (Pike et al., 2020). Despite the ecological, and commercial importance of *A. marmorata*, little is known about its parasitic fauna, particularly the myxozoan parasites.

In the present study, based on an integrative taxonomic approach, a new cnidarian myxosporean parasitizing the skin of the giant mottled eel from the Solomon Islands is described. Furthermore, the phylogenetic position and relationships of this new species among other platysporine myxosporeans were determined.

2. Materials and methods

During field missions in the Solomon Islands in 2019, a fingerling specimen of *A. marmorata* measuring 9 cm in total length was caught using a DEKA 3000 electrofishing system (Gerätebau, Marsberg, Germany), in the Simbay Brook, near Bebelama Village, Province of Choiseul (7°21'503" S, 157°33'015" E). The specimen was euthanized using an overdose of clove essential oil (10%), stored and preserved in 95% ethanol. The procedures complied with the annex IV of the directive 2010/63/European Union animal welfare laws, guidelines and policies, which have been approved by the Solomon Islands authorities (EX2016/156). Fish sample was transported to the laboratory of Ichthyology of the Museum National d'Histoire

Naturelle, Paris, France, where complete parasitological examination was performed with visual examination of all organs to find the suspected myxosporean plasmodia by naked eye and further examined by preparing wet mounts of all inspected organs in a Nikon SMZ1000 stereomicroscope and an Olympus BX53 microscope equipped with an ocular micrometer.

Morphological and morphometric analyses were performed on mature myxospores isolated from plasmodia, which were post-fixed in 10% formalin and following the criteria outlined by Lom and Arthur (1989). Measurements and photographs were taken from 30 myxospores using a computer equipped with Axiovision 4.1 image capture software coupled to an Axioplan 2 Zeiss microscope (Carl Zeiss AG, Oberkochen, Germany). The dimensions of the myxospores included body length, width and thickness, polar capsule length and width, and are expressed as mean \pm standard deviation, followed by the range in brackets. Permanent slides were prepared with smears containing myxospores, which were air dried, fixed with methanol and stained with Giemsa solution and were deposited under MNHN-IK-2015-2692 in the cnidarian collection of the *Museum National d'Histoire Naturelle* - MNHN, Paris, France.

Molecular analysis was performed on plasmodia dissected from the host tissue and fixed in absolute ethanol ($\geq 99.5\%$). Extraction of genomic DNA was performed using QIAamp DNA Micro Kit (Qiagen, Valencia, USA), in accordance with the manufacturer's instructions. Small subunit ribosomal DNA (SSU rDNA) was amplified using the primer pairs ERIB1 (Barta et al. 1997) - ACT1r (Hallett and Diamant, 2001) and Myxgen4F (Diamant et al., 2004) - ERIB10 (Barta et al., 1997) (Table 1), which amplified two fragments containing approximately 1,000 and 1,200 base pairs (bp), respectively. Polymerase chain reactions (PCRs) were performed in accordance with Mathews et al. (2020a) and conducted in a final volume reaction of 20 μ L, which

comprised 1 μL of DNA extracted (10–50 ng), 0.3 μL of each primer (10 μM), and 0.625 U of Qiagen Taq DNA polymerase. PCRs were performed in a Thermocycler (Bio-rad T100) with initial denaturation at 94 $^{\circ}\text{C}$ for 5 min, followed by 39 cycles at 94 $^{\circ}\text{C}$ for 1 min, 58 $^{\circ}\text{C}$ for 1 min, 72 $^{\circ}\text{C}$ for 2 min, and then final elongation at 72 $^{\circ}\text{C}$ for 5 min. The amplicons were subjected to electrophoresis in 2% agarose gel in a TAE buffer (Tris–Acetate EDTA: Tris 40 mM, acetic acid 20 mM, EDTA 1 mM). The size of the amplicons was estimated by comparison with the 1 Kb Plus DNA Ladder (Invitrogen by Life Technologies). PCRs using the original primers and two additional primers, MC5 and MC3, (Molnár et al., 2002) (Table 1) were used in the sequencing to connect the overlapping fragments. Sequencing was performed using a BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems Inc., California, USA) in an ABI 3130 automatic DNA analyzer (Applied Biosystems Inc.™). The obtained sequence was visualized, assembled and edited using Geneious 7.5.1. A standard nucleotide BLAST search was conducted to verify the similarity of the newly sequence obtained with other sequences available in GenBank database (Altschul et al., 1997). Phylogenetic analysis was conducted using the 97 most closely related myxosporean sequences with BLAST similarity > 80% and these sequences were aligned with ClustalW within BioEdit version 7.1.3.0 (Hall, 1999). *Myxidium lieberkuehmi* (Accession number X76639) and *Chloromyxum leydigi* (Accession number AY604199) sequences were used as outgroups. Phylogenetic analysis was performed using Maximum Likelihood (ML) method with a Kimura 2-parameter (K2P) evolution sequence model in MEGA 6.0 (Tamura et al., 2013). Bootstrap analysis (1000 replicates) was employed to assess the relative robustness of the branches in ML tree. The pairwise method with the p-distance model in MEGA 6.0 was performed to

evaluate the genetic distance between the myxozoan species clustering together with the new sequence obtained.

For the correct host identification, the mitochondrial DNA gene cytochrome c oxidase subunit I (CO1) was sequenced. Thus, DNA was extracted from a fragment of dorsal fin, using a DNeasy® Blood & Tissue Kit (Qiagen, California, USA), in accordance with the

manufacturer's instructions. The CO1 gene was amplified using the primer pair FishF1 and FishR1 (Ward et al., 2005) (Table 1), which amplified a sequence of approximately 655 bp. PCR was conducted according to Ward et al. (2005) with a final reaction volume of 20 µL, which included 1.5 µL of DNA extracted, 2 µL of 10 × PCR buffer, 0.3 µL of each primer (10 µM), 1.25 µL of MgCl₂ (25 mM), 0.2 µL of each dNTP (6.6mM), and 0.625 U of Qiagen Taq DNA polymerase. The amplification was performed with an initial denaturation step at 94 °C for 4min, followed by 39 cycles at 94 °C for 40s, 49 °C for 30s, 72 °C for 1 min and final elongation at 72 °C for 10 min. Sanger sequencing was performed in both directions by a commercial company (Genoscreen, Lille, France). Basic Local Alignment Search Tool (BLAST) was conducted to verify the similarity of the sequence obtained with other fish sequences available in GenBank database (Altschul et al., 1997).

3. Results

The epidermis layer of the skin of the wild fingerling specimen of *A. marmorata* harboured macroscopically visible plasmodia (Fig. 1A) containing large quantities of myxospores belonging to the genus *Myxobolus*. Plasmodia were not found in any other organ and no other metazoan parasites were observed.

Taxonomic summary

Phylum: Cnidaria Hatschek, 1888

Subphylum: Endocnidozoa Schuchert, 1996

Class: Myxozoa Grassé, 1970

Subclass: Myxosporea Bütschli, 1881

Order: Bivalvulida Shulman, 1959

Family: Myxobolidae Thélohan, 1892

Genus: *Myxobolus* Bütschli, 1882

Species: *Myxobolus choiseulae* n. sp.

Type host: *Anguilla marmorata* (Anguilliformes: Anguillidae)

Site of infection: Epidermis of the skin

Type locality: Simbay Brook, near Bebelama Village, Province of Choiseul (7°21'503" S, 157°33'015" E), Solomon Islands.

Type of material: Syntypes-Air-dried slide stained with Giemsa (MNHN-IK-2015-2692) deposited in the cnidarian collection of the Museum national d'Histoire naturelle-MNHN, Paris, France. The SSU rDNA sequence was deposited in GenBank under accession number MW794256.

Etymology: The specific name (*M. choiseulae*) is based on the geographic area (Province of Choiseul, Solomon Islands), where the eel specimen was collected.

Morphological characterization

Plasmodia were whitish, measuring 3-4 mm in diameter and localized in the epidermis layer of the skin (Fig. 1A). Mature myxospores featured a subspherical shape from the

frontal view and measured $13.8 \pm 0.3 \mu\text{m}$ ($13.7\text{--}13.9 \mu\text{m}$) in length, 10.8 ± 0.4 ($9.8\text{--}11 \mu\text{m}$) in width and $7.5 \pm 0.2 \mu\text{m}$ ($7.2\text{--}7.5 \mu\text{m}$) in thickness (Fig. 1B-C). Two pyriform equally-sized polar capsules located side by side at the myxospore anterior pole, measuring $5.6 \pm 0.2 \mu\text{m}$ ($4.8\text{--}5.8 \mu\text{m}$) in length and $2.8 \pm 0.18 \mu\text{m}$ ($1.9\text{--}2.8 \mu\text{m}$) in width (Fig. 1B-C). The polar tubules presented four (rarely five) turns (Fig. 1C).

Molecular characterization and phylogeny

The sequencing of the SSU rDNA from the myxospores of *M. choiseulae* n. sp. obtained from the *A. marmorata* specimen resulted in a sequence of 1672 bp with a guanine-cytosine-GC content of 49.7%. A basic local alignment search (BLASTn) revealed that this sequence did not match any myxosporeans available in the GenBank database. The comparison of the new sequence obtained by BLASTn revealed the highest sequence similarity to *Myxobolus portucalensis* Saraiva and Molnar, 1990 (GenBank accession number AF085182.1, query coverage 92%, maximum identities 90.4%), recovered from pectoral and caudal fins of the European eel *Anguilla anguilla* Linnaeus, 1758 from North of Portugal. Phylogenetic analysis inferred by the Maximum Likelihood method placed *M. choiseulae* n. sp. together with *M. portucalensis* and Echinactinomyxon type 5 Özer, Wootten, Shinn, 2002, in a well-supported subclade with 100% bootstrap support (Fig. 3). Pairwise analysis of the SSU rDNA sequences of the myxosporean species that cluster in the same clade with the *M. choiseulae* n. sp. sequence revealed an important genetic divergence among these species, with a difference of 8.8% to *M. portucalensis*, 9.5% to Echinactinomyxon type 5 Özer, Wootten, Shinn, 2002, 17.5% to *Hungactinomyxon* sp. Rácz, Eszterbauer, Molnár, 2005, 18.3% to *Myxobolus lentisuturalis* Dyková, Fiala, Nie, 2002, 18.9% to *Myxobolus nagaraensis* Yokoyama, Kageyama, Ohara, Yanagida, 2007 and 19.7% to *Myxobolus cultus* Yokoyama, Ogawa, Wakabayashi, 1995.

4. Discussion

Although a number of myxosporean cnidarians, particularly *Myxobolus* spp., have been described infecting different fish species in many regions around the world (Eiras et al. 2014; Mathews et al., 2020b, c; Gupta and Kaur 2020; Batueva, 2020; Milanin et al., 2021), there are geographical areas for which there is a gap in the knowledge of myxosporean diversity (Okamura et al., 2018). Nothing is known about this group of parasites infecting vertebrates from the Solomon Islands. To the best of our knowledge, this is the first report of a *Myxobolus* species parasitizing a fish from the Solomon Islands and the first to describe this parasite infecting *A. marmorata*. In this study, based on an integrative taxonomic approach that included morphology, biological traits, molecular data, and phylogeny we have described a new myxobolid species, *M. choiseulae* n. sp., thus increasing our knowledge of cnidarian myxosporean diversity.

In the literature available, most *Myxobolus* species have been described solely based on myxospore morphology (Eiras et al., 2005; Eiras et al., 2014). However, molecular methods involving genetic markers have revealed discrepancies between traditional myxospore-based classification and phylogenies based on gene sequences (Fiala et al., 2015). Yet, myxospore morphology-based comparisons remain necessary to differentiate the numerous *Myxobolus* spp. that lack molecular data (Rocha et al., 2019). In our study, considering that *Myxobolus* species display a strong tendency to cluster, based on host phylogenies (Ferguson et al., 2008; Carriero et al., 2013; Mathews et al., 2018), morphological and morphometric comparisons of mature myxospores of *M. choiseulae* n. sp. were conducted only with the four *Myxobolus* species previously reported from closely related anguillid eels (Table 2). Compared to these congeners, *M. choiseulae* n. sp. myxospores were overall bigger, further differing in the number of coils of the polar tubules (Table 2). Other differences include the presence of a

membranous appendage around the posterior border of *M. fujitai* myxospores, and 10 sutural markings around the edge of the myxospores of *M. portucalensis* (Saraiva and Molnár, 1990; Eiras et al., 2005). Another important difference is related to the site of infection as the organ and/or tissue specificity has been considered a taxonomic key for the identification of freshwater histozoic platysporines, particularly for *Myxobolus* species (Molnár and Eszterbauer, 2015). Accordingly, differences are observed as the plasmodia of *M. choiseulae* n. sp. were located in the epidermis of skin, whereas *M. kotlani* plasmodia are found in the subcutaneous and intermuscular connective tissue of the head, *M. dermatobia* plasmodia in circular gastric, intestinal muscle layer and tegument, *M. fujitai* plasmodia in the subcutaneous tissue of fins, and *M. portucalensis* plasmodia in the subepithelial connective tissue of the fins (Table 2). Currently, molecular data is highly recommended for *Myxobolus* species-level classification (Atkinson et al., 2015). However, of the four anguillid *Myxobolus* species previously described, molecular data is available for only *M. portucalensis* in the NCBI Nucleotide database. Comparison between *M. choiseulae* n. sp. and *M. portucalensis* showed 91.2% genetic similarity in the BLAST search. This genetic distance is reinforced by the pairwise similarity analysis that revealed a high level of genetic divergence (8.8%) in the SSU rDNA of these two species.

In the phylogenetic analysis performed in this study, myxosporean species showed a general tendency to cluster based on phylogenetic affinity of the fish hosts, even if the fishes are from different geographical areas, which has been reported previously (Ferguson et al., 2008; Carriero et al., 2013; Rocha et al., 2019). In the phylogenetic tree, *M. choiseulae* n. sp. is in the same subclade as *M. portucalensis* and the actinospore Echinactinomyxon type 5 Özer, Wootten, Shinn, 2002, with 100% bootstrap support. The presence of this actinosporean type in this subclade is in

accordance with Holzer et al. (2004) who described Echinactinomyxon type 5 Özer, Wootten, Shinn, 2002, as a closely related sister species of *M. portucalensis*. In our study, the genetic divergence analysis shows that Echinactinomyxon type 5 Özer, Wootten, Shinn, 2002 shares 90.5% and 93.9% sequence similarity with *M. choiseulae* n. sp. and *M. portucalensis* respectively, suggesting that this actinospore might represent the alternate stage of an undescribed freshwater myxosporean species that infects anguillid fish. However, it is important to highlight that this is only the second phylogenetic study of a myxobolid parasite of anguillid eels and there is little SSU rDNA data available from other myxosporeans infecting eels of the genus *Anguilla*. Thus, a comprehensive data set including molecular data and phylogenetic analysis of the many yet-to-be-discovered *Myxobolus* species from these underrepresented hosts should help to elucidate the evolutionary context of *M. choiseulae* n. sp. as well of the anguillid myxosporeans as a whole.

Declaration of Competing Interest

The authors declare that they have no conflict of interest regarding the publication of this article.

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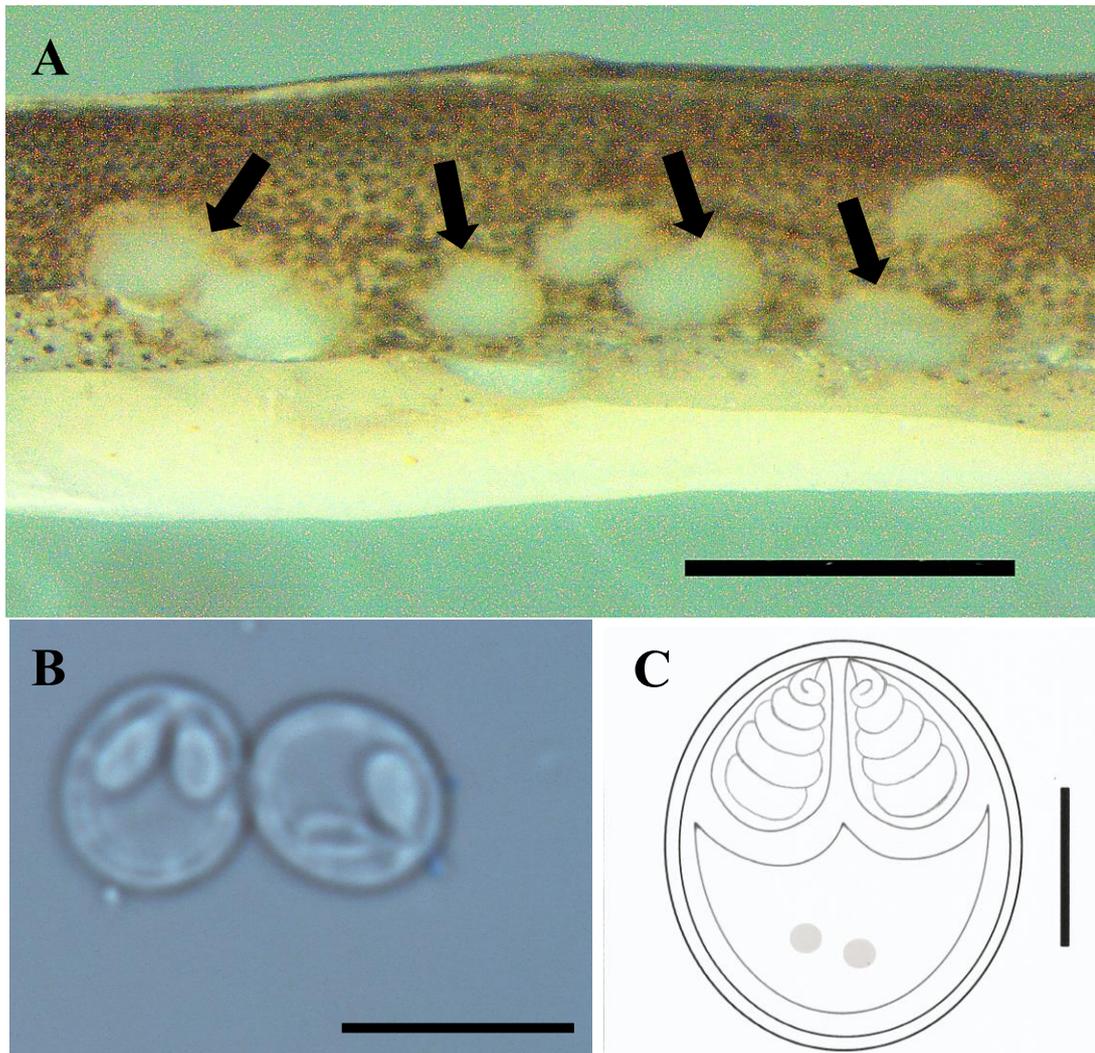
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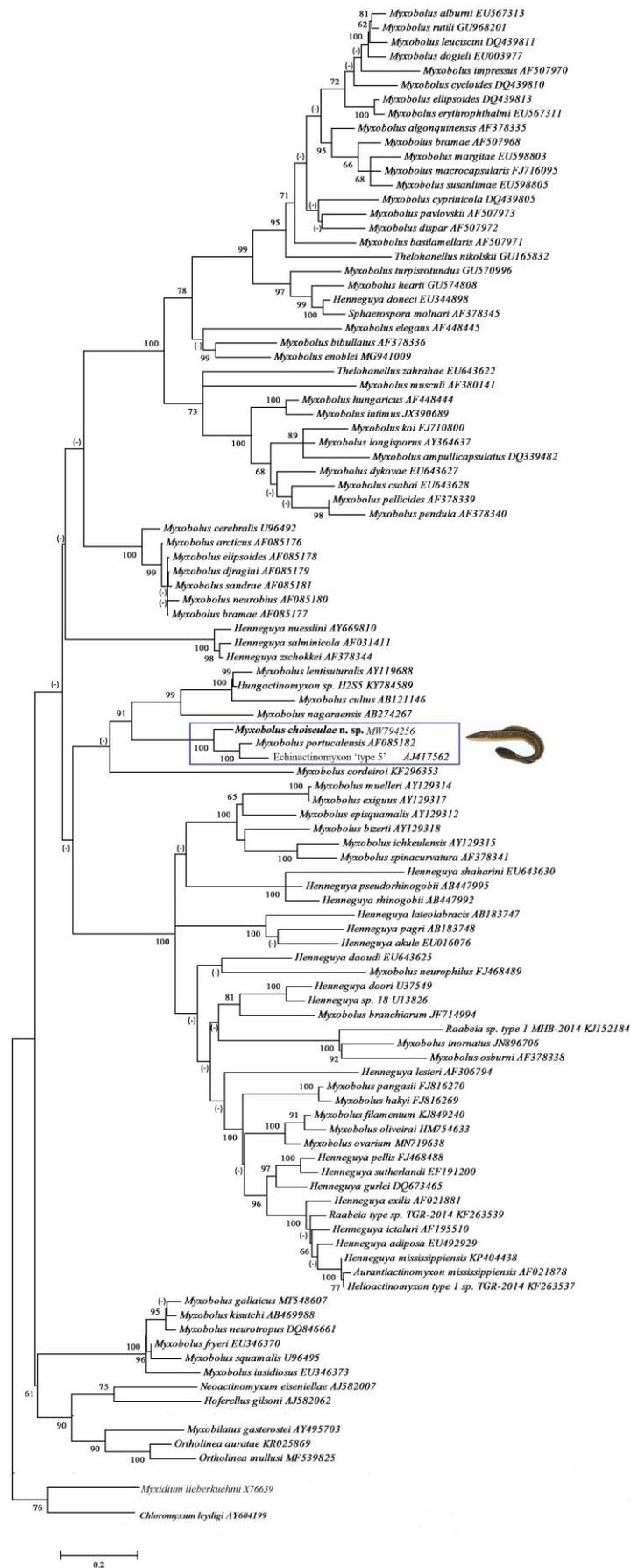
Legends

Fig. 1. *Myxobolus choiseulae* n. sp. infecting skin of the giant mottled eel *Anguilla marmorata* from the Solomon Islands. (A) Whitish plasmodia macroscopically visible in the skin (arrows). Scale bar= 10 mm. (B) Two mature myxospores in frontal view, showing two pyriform equally-sized polar capsules located side by side at the anterior pole. Scale bar= 15 μ m. (C) Schematic representation of a mature myxospore. Scale bar= 5 μ m.

Fig. 2. Phylogenetic tree generated by maximum likelihood analysis of SSU rDNA sequences of *Myxobolus* species and other closely related myxosporidian species based on

BLAST. GenBank accession numbers are given in front of species. Bootstrap values below 60 are indicated in brackets.





Primers	Sequences (5'-3')	References
ACT1r	AATTTACCTCTCGCTGCCA	Hallett and Diamant 2001
ERIB1	ACCTGGTTGATCCTGCCAG	Barta et al. 1997
ERIB10	CTTCCGCAGGTTACCTACGG	Barta et al. 1997
Myxgen4F	GTGCCTTGAATAAATCAGAG	Diamant et al. 2004
MC5	CCTGAGAAACGGCTACCACATCCA	Molnár et al. 2002
MC3	GATTAGCCTGACAGATCACTCCACGA	Molnár et al. 2002
FishF1	TCAACCAACCACAAAGACATTGGCAC	Ward et al. 2005
FishR1	TAGACTTCTGGGTGGCCAAAGAATCA	Ward et al. 2005

Table 1. Primers used for the amplification and sequencing of the SSU rDNA gene of *Myxobolus choiseulae* n. sp. and the partial *COI* gene of host fish *Anguilla marmorata*.

Species	SL	SW	PCL	PCW	NCT	Host	Site of infection	Type locality	Source
<i>Myxobolus choiseulae</i> n. sp.	13.8±0.3	10.8±0.4	8.7±0.43 (6.9-9.3)	3±0.2 (3-3.6)	4-5	<i>Anguilla marmorata</i>	Epidermis of skin	Solomon Islands	This study
<i>Myxobolus kotlani</i>	10.3 (8.7-11.7)	7.6 (6.9-8.5)	5.6 (3.9-6.1)	2.7 (2.2-3.4)	7-8 Rarely 6-9	<i>Anguilla anguilla</i>	Subcutaneous, intramuscular tissue of head	Hungary	Molnár, Lom and Malik, 1985
<i>Myxobolus portucalensis</i>	12.6 (11.2-15.0)	8.9 (7.5-10.0)	5.8 (3.7-7.5)	3.0 (2.5-3.7)	9-11	<i>Anguilla anguilla</i>	Subepithelial connective tissue of fins	Portugal	Saraiva, Molnár, 1990
<i>Myxobolus dermatobia</i>	6.3-7	4.2-4.9	2.8-3.5	-	6-7	<i>Anguilla japonica</i>	Integument	Japan	Eiras, Molnár, Lu, 2005
<i>Myxobolus fujitai</i>	9.2	8.4	4.9	-	-	<i>Anguilla japonica</i>	Fins, dorsal side of body	Japan	Eiras, Molnár, Lu, 2005

Table 2. Comparison of *Myxobolus choiseulae* n. sp. with other congeners described parasitizing freshwater anguillid eels. Myxospore dimensions, hosts, infection sites are given. SL: spore length, SW: spore width, PCL: polar capsule length, PCW: polar capsule width, NCT: number of coils in polar tubule, dashes: no data. All measurements are in μm .

