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Molecular identification of a new myxozoan, *Myxobolus dermiscalis* n. sp. (Myxosporea) infecting scales of *Labeo rohita* Hamilton in Harike Wetland, Punjab (India)

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ABSTRACT

In the present study, a new species *Myxobolus dermiscalis* n. sp. infecting scales of *Labeo rohita*, an Indian major carp from Harike Wetland in Punjab, India has been described on the basis of spore morphology and amplification of a part of 18S rDNA gene. The pseudocysts of *M. dermiscalis* n. sp. are milky white with irregular outline, 0.5–3.6 mm in diameter embedded within the dermal scale in the form of a cavity. The spores 5.84–7.98 × 3.98–5.98 μm in size, having two equal polar capsules 3.98–5.98 × 1.85 –3.85 μm in size. The most differentiating feature from closely related species, *Myxobolus saugati* (Kaur and Singh, 2011) is the presence of two parietal folds at the posterior – lateral margins of the shell valves. The present species is regarded as host, organ and tissue specific in nature. The partial sequence of SSU gene of *M. dermiscalis* n. sp. clustered with other *Myxobolus* species infecting cyprinids available in the GenBank. Blast search revealed 98% homogeneity with *Myxobolus* sp (KM401439) infecting scales of *L. rohita* in Myanmar (unpubl. data). The present myxobolid parasite has been recorded to cause serious, highly symptomatic disease of the scales, causing their loosening from the skin of *L. rohita*. It rendered the host fish unsightly giving it cloudy appearance with white patches and mucoid body surface. Scale pseudocyst Index (SPI) has been provided to record the intensity of infection.

1. Introduction

Punjab (India) has 3 main wetlands, i.e. Harike, Kanjli and Ropar wetlands which are included in Ramsar list of International importance. Harike wetland (31° 17′ N latitude and 75° 12′ E longitude) is the largest freshwater wetland (in northern India) of 4100 ha area with Beas and Sutlej as primary inflows. It is a habitat for as many as 26 species of fishes which include *Catla*, *Cirrhinus*, *Channa*, *Mystus*, *Chitala chitala*, *Cyprinus* and *Ambassis ranga*. These wetlands are the major natural fisheries resource for food in whole of the Punjab state. The study indicates that large variety of fishes in these wetlands are infested with myxozoan parasites. Myxozoans include histozoic and coelozoic parasites infecting not only freshwater and marine fishes but have also been detected in molluscs, amphibians, reptiles, waterfowl and mammals (Moncada et al., 2001; Eiras, 2005). As demonstrated firstly by Wolf and Markiw (1984), it has been proven that myxozoan species require an alternate invertebrate host (usually an annelid) to complete the life cycle. Among myxosporeans, the genus *Myxobolus* includes the highest number of species. Eiras et al. (2005) reported 744 valid species, while Lom and Dykova (2006) counted 792 including 7 amphibian species. Several other reports on *Myxobolus* species are available from Punjab and West Bengal, India (Basu & Haldar, 2004-Basu et al., 2009; Bandyopadhyay et al., 2006/2007; Kaur and Singh, 2014). About 131 species of *Myxobolus* have been recorded in India (Kaur and Singh, 2012) and are mainly differentiated by morphological, morphometric characteristics of spores, besides host and organ or tissue specificity. Presently, the molecular analyses have been an important tool in the study of these parasites and this has expanded their taxonomy to the phylogenetic analyses. This has led to correct identification and differentiation of morphologically indistinguishable myxobolid species (Kent et al., 2001; Eszterbauer, 2002; Molnar et al., 2010; Cech et al., 2012; Bartosova et al., 2009). The most revealing aspects of analyses based on SSU rDNA sequences is the incongruence of phylogenetic trees with classification based 63on spore morphology alone (Bartosova et al., 2009). So far, there are 14 sequences i.e *caudatus* KC865607; *Myxobolus*

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cuttacki 465682; *M. orrissae* KF448527; *Myxobolus basuhaldari* KM029974, KM029975, KM029976; *M. kalavatiae* KM029973; *M. meerutensis* KM029977;*M. bhadrensis* KM029968, KM029969, KM029970, KM029971, KM029972 and *M. catlae* KM029967 (Mondal et al., 2014; Szekely et al., 2014; Rajesh et al., 2014; Abraham et al., 2015) from India are available in the Genbank. In this study, we present morphological and molecular characterization of *M. dermiscalis* n. sp. infecting the scales of *L. rohita* collected from Harike wetland, Punjab, India along with 18S rDNA based phylogenetic analysis with *Myxobolus* group and other related texa. In future, the molecular methods can be implied on the diagnostics of the economically important myxozoan parasites in this part of the world.

2. Material and methods

2.1. Collection

Fresh specimens of Indian major carp, rohu, *L. rohita* were collected from the fisher-man at Harike wetland during the period June 2013 to July 2014. The infected scales were removed with the help of forceps in a petridish containing 0.9% saline. The pseudocysts were visible with the naked eye and appeared as creamish white patches on the scale. The plasmodium were teased on a clean slide to liberate spores and were examined under the microscope. Fresh spores were treated with 8% KOH solution for the extrusion of polar filaments. For permanent preparation, air-dried smears were stained with Ziehl–Neelsen and Iron-haematoxylin. Spores were measured with the help of a calibrated ocular micrometre. All measurements were recorded in microns (μm).

2.2. Prevalence

The prevalence (in percentage) of *M. dermiscalis* infecting scales of *L. rohita* was calculated according to Bush et al. (1997). The intensity of infection was determined by following the index proposed by Kaur and Attri (2015) for infection on the scales. 0 = no infection; 1 = one pseudocyst per scale in 10% of total scales (indicating light infection); 2 = two pseudocysts per scale in 15–20% of total scales (moderate infection); 3 = three to four pseudocysts per scale in 50% of total scales (heavy infection); 4 = four to five pseudocysts per scale in 100% of scales (severe infection).

2.3. Molecular analysis

2.3.1. DNA extraction, polymerase chain reaction

The pseudocysts (50 in numbers) present on ethanol fixed scales were ruptured with the help of a sharp needle in a watch glass having double distilled water. The contents in the watch glass containing spores were collected in 1.5 ml micro centrifuge tubes. The DNA was Scientific, Wilmington, USA) spectrophotometer at 175 ng/μl. Polymerase chain reaction (PCR) was carried out according to the Andree et al. (1999) at the final volume of 25 μl using the primers MX5-MX3 which amplified the fragments of 1597 bp respectively of the 18S rDNA gene. The amplification reactions were conducted with 30–75 ng of genomic DNA, 12.5 μl of 1 × reaction buffer (Hi media), 1.0 μl of each primer, 1.0 μl of total DNA and 10.5 μl of double purified water. Amplification was done by initial denaturation at 95 °C for 3 min, followed by 33 cycles of denaturation at 95 °C for 30 s, annealing of primers at 58 °C for 30 s, extension at 72 °C for 1 s. The final extension was at 72 °C for 10 s. The PCRproducts were analysed agarose gel electrophoresis, and size was estimated by comparison with the1Kb Plus DNA ladder (Fig. 1).

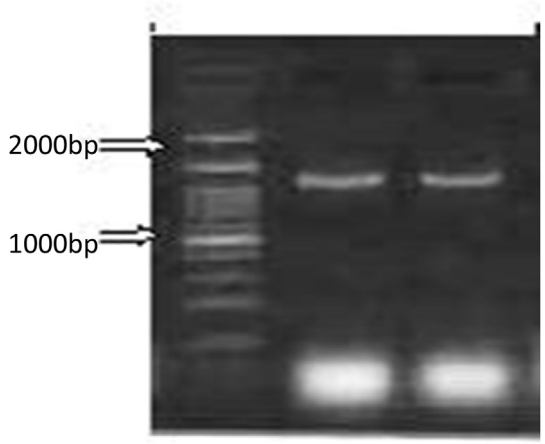

Fig. 1. Agarose gel (1.8%) showing amplified 18S rDNA gene of *M. dermiscalis* n. sp. infecting scales of *Labeo rohita*.
Lane 1: 1kb DNA Ladder
Lane 2, 3: M. dermiscalis n. sp. (1597bp)

2.3.2. DNA sequencing, sequence alignment and phylogenetic analysis

Extracted from spores using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instructions. The product was then quantified in a Nanodrop (Thermocycler). In the present study, the PCR amplified products were sequenced at the Molecular Diagnostics & Research Laboratories (MDRL) Pvt. Ltd. Chandigarh, India. 1597 bp of 18S rDNA sequences of *M. dermiscalis* n. sp. were deposited in GenBank with accession number KM092529. Phylogenetic analysis involving 23 nucleotide sequences was performed using the Bayesian, Maximum likelihood (ML), Maximum parsimony (MP) and Neighbour joining (NJ) methods. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). The evolutionary distances were computed using the Kimura 2-parameter method Kimura (1980) and units of the number of base substitutions per site. The rate variation among sites was modelled with a gamma distribution. The best fit model for analysis for the current data was GTR + G with lowest BIS (Bayesian Information Criterion) was estimated by using model test tool in Mega 6.06.

3. Results

3.1. Morphological characteristics of Myxobolus dermiscalis n. sp.

3.1.1. Pseudocyst (Fig. 2)

Round to irregular, white, 4-5pseudocysts per scale, histozoic, present within a cavity and measure 0.5–3.6 mm in diameter. 400–500 of spores were present per pseudocyst.

3.1.2. Spore description (Fig. 3)

(Measurements based on 10 spores in frontal view).

The spores measure 5.84–7.84 × 3.98–5.98 μm, oval to spherical in frontal view having rounded anterior and posterior ends. Both the shell valves are thick, symmetrical and 0.5 μm in thickness. Parietal folds two, present on the posterio-lateral margins of the shell valves. Polar capsules are two, equal, measure 3.98–5.98 × 1.85–3.85 μm and are pyriform with distinct neck at the anterior end. They converge anteriorly and are placed at a distance posteriorly. Polar filaments form 5–6 coils and are arranged obliquely to the polar capsule axis, 7.2 μm in length when extruded. An intercapsular process is absent. Two capsulogenic