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Morphological and ssrDNA sequence based molecular characterization of a novel *Thelohanellus* species (Myxosporea: Myxobolidae) infecting the fins of Goldfish, *Carassius auratus* L. with special reference to its histopathological alteration

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ABSTRACT

A new species of myxozoan, *Thelohanellus goldi* n. sp. is described using morphological and molecular data, parasitizing the fin filaments from 18 of 25 host specimens (72.5%) of *Carassius auratus* collected from different ornamental fish farms of India. Mature spore of the new species were oval to spherical in frontal view having rounded posterior ends and tapering anterior end measures 8.7–10.26 (9.50) × 4.10–7.89 (5.84) µm. The single large polar capsule, round to oval in shape but slightly pointed at the anterior end measuring 4.91–7.63 (5.60) × 2.3–3.1 (2.96) µm and located just below the anterior end of the spore. Polar filament only at distal end with 5–6 loose coils. The most differentiating feature from closely related species was carried out by morphotaxonomic affinities with previously described species which are tremendously supported by molecular taxonomy by partial sequencing of the 18S rDNA gene resulted in a total of 2124 bp fragment of newly obtained small subunit ribosomal RNA gene sequence of the new species which Exhibit 93–95% homogeneity with other closely related species available in GenBank. The BLAST search and high genetic diversity of distance matrix of *Myxobolus* sp. did not properly match with any available sequences in GenBank and make sister clade with *Thelohanellus caudatus* and *Thelohanellus habibpuri* in the *Thelohanellus* clade including most of *Thelohanellus* spp. The study of evolutionary history enables us to understand the evolution of modern species and supports some uncertain topologies which are being presented regarding the morphometric analysis. The severity of myxozoan infection has been assessed in this article by observing the histopathological changes of fins of the *C. auratus* along with the diversity, distribution and taxonomic description of the new *Thelohanellus* species with their new host and locality records.

1. Introduction

Ornamental fish farming represents a consolidated market over the world and rapidly gaining an important economic activity for the diet of different countries especially in the tropics and subtropics. In recent times, there has been tremendous increase in the development of fish farming and culture attributable to the increased need for affordable animal protein especially in the tropics (Mathews et al., 2017). Parasitic infection and diseases are some of the factors hindering high productivity in fish farming. In the last years, several studies have shown widespread distribution of myxosporeans in many ornamental fish which may act as main cause of hindrance in these profit markets of ornamental aquaculture industry (Saha and Bandyopadhyay, 2017a). Myxozoa is a diverse group of over 2400 species (Zatti et al., 2017). They are now regarded as cnidarians 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). Although the evolutionary origin of myxozoans has been elusive, which show close relationship with cnidarians, a group that include coral, sea anemone, jellyfish, and hydroid, is supported by some phylogenetic studies and the observation that the distinctive myxozoan structure, the polar capsule, is remarkably similar to the stinging structures known as nematocysts in cnidarians (Chang et al., 2015). Now a day, spore-based taxonomic key has been taken into account the

Abbreviations: PC, polar capsule; PF, polar filament; IV, iodinophilous vacuole; NU, nucleus; SL, suture line

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recent recognition of the cnidarians origin of myxozoans as well as important revisions at generic, family and suborder levels over the last decade (Fiala, 2006).

The genus *Thelohanellus* is one of the most specious myxozoan groups, with more than 108 described taxa, representing of myxozoan biodiversity (Zhang et al., 2013). Owing to the technical limitations of the past, most of species were identified solely based on spore morphology (Chen and Ma, 1998; Liu et al., 2016; Saha and Bandyopadhyay, 2017b,c). Given the incredible diversity coupled with the simplicity of the diagnostic stage of these species, it is often difficult to determine the validity of morphologically similar species using spore morphology alone. As a result, large number of misidentified and cryptic species exists in the morphologically identified species and the present species diversity of myxosporeans is not reliable. Thus, the taxonomic classification of Myxosporea based on morphology alone (Lom and Arthur, 1989) has been refined with the application of molecular biological methods (Molnar et al., 2002, 2006, 2008; Mathews et al., 2017; Zatti et al., 2017) become a sharp blade in myxosporean classification and a number of misidentification and confusions are resolved (Atkinson et al., 2015; Liu et al., 2016).

In the present study, we describe a new species of *Thelohanellus* found parasitizing the fin of *Carassius auratus*, an economically important ornamental fish on the basis of their light and scanning electron microscopic data. We also used novel ssrDNA sequence data in combination with that of other Myxozoan spp. to explore the phylogenetic relationships between the current and closely related species. Furthermore, this parasite may result in large scale histopathological features according to the infectious stage and host susceptibility which has also been presented in this paper.

2. Materials and methods

2.1. Sampling

During the period of March 2014–March 2017, around thirty fish farms, of different districts of West Bengal, India, namely, Nadia (23.4710° N, 88.5565° E), Hooghly (22.8963° N, 88.2461° E), Howrah (22.5958° N, 88.2636° E), South 24-parganas (22.1352° N, 88.4016° E) and North 24-parganas (22.6168° N, 88.4029° E) have been surveyed and checked for myxozoan cyst-like plasmodia. Goldfish, *C. auratus* along with myxozoan cyst-like plasmodia are brought alive at the Parasitology laboratory, University of Kalyani, Kalyani for examination. Water quality parameters of all the fish farms have been checked from where the fishes have been collected. (26.6 ± 2.3 °C, pH 6.9 ± 0.1 with dissolved oxygen 6.0–7.8 mg/L).

2.2. Isolation of pathogens

Length, weight, (weighting 4.38 ± 0.28 gm, length 3-9 ± 0.89 cm) external symptoms and general health conditions of each fish have been recorded immediately at the Parasitology laboratory. Forty six number of whitish cyst-like plasmodia of similar morphology were found in the fins of goldfish. The majority of collected cyst-like plasmodia was deep frozen in 1.5-mL centrifuge tubes in 70% ethanol and stored for molecular studies. Three cyst-like plasmodia are taken for phase contrast microscopic preparation and one for scanning electron microscopic studies. Infected organ along with the cyst were fixed in Bouin's solution for histopathological observation. Large numbers of samples of myxospores collected from different specimens of the same fish host species and subjected for light microscopic studies which indicating the same species infecting all the goldfishes. Remaining fishes were kept into the aquaria for further study and they were fed once at 1% body weight daily with commercially available fish pellet.

2.3. Light microscopic study

The cyst-like plasmodia has been kept on clean glass slides with a drop of 0.9% NaCl solution, covered with cover slips, ruptured and examined for the presence of myxospores spore. The methodology for permanent specimens has been made following the guidelines of Saha and Bandyopadhyay (2017b). Photomicrographs of fresh and stained spores were taken at 1000× magnification. Drawings were made from stained materials with the aid of camera lucida. Measurements based on thirty five fresh spores were done with a calibrated ocular micrometer according to Lom and Arthur (1989) and Lom and Dykova (1992). The myxosporean identification has been done as per previous descriptions (Lom and Arthur, 1989; Kaur and Singh, 2012; Zhang et al., 2013).

2.4. Scanning electron microscopic study

The myxozoans were fixed in 2.5% glutaraldehyde solution for two hours at 4 °C for SEM studies, following the methodology of Saha and Bandyopadhyay (2017b). The cyst-like plasmodia attached tissue of the host fish examined and fixed in 2.5% glutaraldehyde solution for two hours at 4 °C followed by dehydration with ethanol, and washing with absolute acetone and amyl acetate mixture in 3:1, 2:2 and 1:3 ratios respectively and finally with 100% amyl acetate following the guideline of Saha and Bandyopadhyay (2017a).

2.5. DNA extraction

After morphometric confirmation, the plasmodia filled with mature spores were ruptured by a sharp needle and subjected for DNA isolation following the methods of Mondal et al. (2014) and the contents were collected carefully in 1.5 mL microfuge tubes. The spores were then centrifuged at 1000*g* for 10 min. The DNA was extracted by suspending the spores in 500 µL lysis buffer (100 mM NaCl, 10 mM Tris, 10 mM EDTA, 0.2% SDS, 0.4 mg/mL Proteinase K) and incubating overnight at 55 °C. Then, 500 µL of phenol: chloroform (1:1) was added to the digested spores, mixed gently, and centrifuged at 5200*g* for 10 min. The upper phase was transferred to a new tube and mixed with 1/10th volume of sodium acetate (3 M, pH 5.2) and 2 vols of 96% ethanol (Amresco, USA). If necessary, the extraction step and phenol-chloroform treatment were repeated. The DNA was precipitated at −20 °C for whole night and pelleted by centrifugation at 10000*g* for 30 min. The pellet was washed once with 70% ethyl alcohol, airdried for few minutes and resuspended in 30 µL of molecular biology grade water. Quality was then evaluated on 0.8% Agarose Gel (Fig. 1).

2.6. Polymerase chain reaction amplification

The polymerase chain reaction (PCR) was carried out as described by Molnar et al. (2002). Isolated DNA was amplified with protozoa specific 18S rRNA Primers (MC5 and MC3) using Veriti® 99 well Thermal Cycler (Model No. 9902) which amplified the fragment of

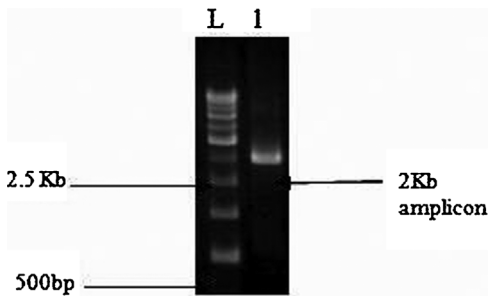

Fig. 1. Agarose gel (1.8%) showing amplified 18S rDNA gene of infecting *Thelohanellus goldi* n. sp. isolated from the fin and gill of the *Carassius auratus*. Lane 1: 1 kb DNA Ladder Lane 2: *Thelohanellus goldi* n. sp. (1794 bp).