First record of the genus *Hennegoides* Lom, Tonguthai and Dyková, 1991 from Punjab (India) infecting the catfish, *Sperata seenghala* (Sykes, 1839)

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**ABSTRACT**

*Sperata seenghala* (Sykes, 1839) the Giant river-catfish, is one of the largest freshwater catfish of Indian sub-continent and commonly called as singhara and seenghala. Catfish is a favourite food fish due to its palatability with high nutritional value. *S. seenghala* (25–27 cm in length) were procured from Chambkaur Sahib, a sub divisional town in the district of Rupnagar in the Indian State of Punjab. Prominent pale, thread-like plasmodia of *Hennegoides seenghalae* n. sp. were observed on the gills and histological examination located them in the epithelial lining of the gill filament (Intrafilamental epithelial type, FE). The prevalence was 32% (12 fish were infected out of 37 examined). The total myxospore length of *H. seenghalae* was 46.6 μm with myxospore body length 7.5 μm strongly vaulted from one side with caudal appendage length of 39.1 (25.5–45) μm. The myxospores were closely compared morphologically with five known species of the genus from Indo-Malayan region and another recently reported from the USA. Molecular analysis based on 18S rDNA sequence (1947bp) indicated 80%-91% sequence similarity with other myxozoan parasites (*Myxobolus*, *Henneguya*, *Hennegoides* and *Unicauda*). The most closely related species was *Hennegoides pangasii*, and was placed with the present species in the same subclade. The present study is the first report the genus *Hennegoides* from India.

1. Introduction

*Sperata seenghala* (Sykes, 1839) is a commercially important freshwater catfish and commonly known as singhara in Punjab (Gupta, 2014). The fish has a wide distribution throughout all the major rivers and reservoirs of India. Due to its high nutritional value and low number of intramuscular bones, this fish has large demand in the domestic markets. This fish species fetch higher prices than carps and the entire demand is fulfilled from natural sources, as aquaculture for catfishes is not yet developed in India (Acharya et al., 2019).

Myxozoa represents at least 20% of phylum Cnidaria, divided into 60 genera and more than 2300 species (Atkinson et al., 2018; Morris, 2010). Despite their diversity there are only a few descriptions of myxozoans from Indian fishes that include molecular data. For identification of myxosporans the information generated from molecular phylogeny in addition to morphological data greatly help in revealing the species complexes along with phylogeographic origin.

The type species of the genus, *Hennegoides longitudinalis* Lom et al. (1991) was reported from Thailand infecting intestinal mucosa of *Osphronemus gourami* (Lom et al., 1991). The new genus was created on the basis of asymmetrical myxosporides with non-axially attached caudal extensions, polar filaments arranged in elliptical turns lying in the longitudinal axis of the capsule. Another species, *Hennegoides obpyriformis* was originally described as *Henneguya obpyriformis* Ma et al. (1986) from China in the gills, spleen and liver of two species of stone loaches, *Nemacheilus jingiangensis* and *N. polytanu* (Order Cypriniformes). Three more species, *Hennegoides berlandi*, *Hennegoides malayensis* and *Hennegoides pangasii* Molnar et al. (2006), infecting gills of Cage-cultured suitchi catfish, *Pangasius hypophthalmus* (Order Siluriformes) from Malaysia were described (Molnar et al., 2006). The only *Hennegoides* species identified from outside of Asia is *Hennegoides flockae* Leis et al. (2019), the only description that includes molecular characterization for a species within the genus, although sequence are available for *He. pangasii* and *He. mekongensis*, the latter of which appears to be a species lacking a formal description.

Wagner (2016) compiled data and provided a guide to the identification of tailed myxobolids of the world and lists detailed information regarding five species of the genus *Hennegoides*. The present study provides a complete morphological description, histological evaluation, and phylogenetic analysis comparing a novel species identified from...
S. seenghala with other known species.

2. Material and methods

2.1. Fish collection and morphological characterization of myxospores

Bagrid catfishes, *Sperata seenghala* (n = 37; 25–27 cm in total length) were procured from the fish market (Chamkaur sahib, Punjab, India). Fresh, dead specimens were examined for the presence of plasmodia on the external body surfaces (i.e., gills, skin, fins, and scales). After external examination, the fishes were dissected and various organs such as gills, eye, buccal cavity, muscle, intestine, liver, gall bladder and kidney were examined under a stereomicroscope for the presence of plasmodia. The
only infection observed was found in the gills in the form of elongated, thread-like, pale coloured plasmodia (Fig. 1 a,b,c). Each plasmodium was gently removed from the gill tissue, teased onto a clean slide to liberate the myxospores which were later treated with 8% KOH for the extrusion of polar filaments. Photography of fresh myxospores was completed with phase contrast microscopy (Leica DM3000LAD) in the Sophisticated Instrumentation Laboratory of Department of Zoology, Panjab University, Chandigarh. Stains such as Iron-haematoxylin, Ziehl-Neelsen and Giemsa were used to stain the myxospores (Fig. 2 a,b,c) and measurements were done with the aid of a calibrated ocular micrometer. Line diagrams (frontal view and sutural view) of stained slides were made with the aid of camera lucida (Fig. 3 a,b). Taxonomic classification was determined according to the keys provided in Okamura et al. (2015).

The parasite abundance was calculated as GPI (Gill Plasmodium Index) on the basis of number of plasmodia present per gill on one side (Kaur and Attri, 2015). Plasmodial size was categorized according to Kaur and Katoch (2016). The prevalence was calculated as described in Bush et al. (1997).

Histological evaluation was completed on gills preserved in Bouin’s fixative. Following tissue preservation, the tissue was washed and dehydrated in ascending series of ethanol, cleared in xylene, embedded in paraffin wax and sectioned at 5–8 µm thin sections and then stained with Luna’s stain (Katoch and Kaur, 2016). The tissue location was also determined according to the guidelines in Molnár (2002a).

2.2. Molecular and phylogenetic characterization of myxospores

A subsample of ethanol-fixed plasmodia was removed from gills, gently placed on microscope slide. After checking the morphology, the myxospore were taken into 1.5 ml microcentrifuge tube, pelleted by centrifugation at 10,000 × g for 5 min. DNA extraction was completed using according to the protocol specified in DNeasy Blood and Tissue Kit (Qiagen) and DNA was stored at –20 °C. Quantification of the DNA product was done using Nanodrop spectrophotometer. PCR amplification of 18S rDNA gene was done by universal primers 18e, 5-CTGTTGTATTCTGCCAGT-3 (Hillis and Dixon, 1991) and 18R, 5-CTACGGAAACCTTGTTACG-3 (Whipps et al., 2003). A 25 µl of reaction mixture was made using 2.5 µl PCR buffer, 1 µl of MgCl₂, 2 µl each primer, 2 µl DNA template, 0.6 µl taq polymerase and 13 µl of molecular grade water. Amplification was completed with an initial denaturation at 95 °C for 30s, annealing of primers at 56 °C for 30s, an extension at 72 °C for 1 min 20s. The final extension was at 72 °C for 10min. The amplified PCR product was analysed on a 1.7% agarose gel and a 100 bp DNA ladder was used to estimate the size of amplified product. The amplified product was sent for sequencing by Chromus BIOTECH, Karnataka (India) using the Dideoxy sequencer (Applied Biosystem, USA). The sequence was submitted to NCBI under accession MN322671. Ceratomyxa shasta from USA was used to root the phylogenetic position of the present species with other tailed myxosporeans.

Phylogenetic and molecular evolutionary analyses were conducted by selection of 18S rDNA sequence that comprise the new species (MN322671) and 21 additional sequence from closely related species using MEGA version X (Kumar et al., 2018) with 1000 bootstrap replicates. Sequence alignments were performed by MUSCLE (Multiple Sequence Comparison by Log-Expectation). A Maximum likelihood (ML) phylogenetic tree was generated by using the present sequence (MN322671) along with closely related sequence from NCBI GenBank database. Substitution pattern and rates were estimated under the (Kimura, 1980) 2-parameter model (+G).

3. Results

3.1. Description of Hennegoides seenghalae n. sp.

**Plasmodia:** Plasmodia thread-like 3–5 mm in length, elongated, pale coloured, visible with naked eye, present on the gill filament. 1 to 5 plasmodia present per gill and gills laden with thick mucus.

**Myxospore (n = 50):** Mature myxospores asymmetrical in frontal view, inverted pyriform, rounded anterior end and tapering posterior end leading into a long bifurcated caudal prolongation. Myxospore body strongly vaulted from one side and other side straight. The total body...
Comparative description of
form 3
lamellae. and enlargement of the plasmodial site lead to atrophy of the gill
and were present along the epithelium bounding the gill filament. plasmodia were histozoics in nature, elongated measuring 3
epithelial type (FE) of plasmodia as discussed in (Molnár et al., 2006).

Famil Design name-seenghala/seenghara, Family- Bagridae

3.2. Taxonomic summary of Hennegoides seenghalae n sp.

Host: Sperata seenghala (Sykes, 1839), vernacular name-seenghala/seeenghara, Family- Bagridae
Location: Chamkaur sahib Punjab, India.
Site of infection: Gills (Intrafilamental epithelial type, FE).
Syntypes: Myxospores stained in Giemsa, Ziehl-Neelsen and Iron-haematoxylin, deposited in Parasitology Laboratory in the Department of Zoology, Panjab University, Chandigarh, India (Catalogue No: H/G/April 12, 2019; H/ZN/April 12, 2019 and H/IH/April 12, 2019).

Prevalence of infection: 32% (12/37).
Gill Plasmodial Index (GPI): Light infection (1–5) plasmodia per gill.

Type of Plasmodia: Type C - (size 3–5 mm).
Etymology: “seenghalae” has been named after specific name of the fish host, Sperata seenghala.

3.3. Histological location of plasmodia

Histological studies revealed the presence of long intrafilamental epithelial type (FE) of plasmodia as discussed in (Molnár, 2002a). The plasmodia were histozaics in nature, elongated measuring 3–5 mm long, and were present along the epithelium bounding the gill filament. Numerous myxospores were present within each plasmodium (Fig. 4). The long intrafilamental plasmodia distorted the gill filament structure and enlargement of the plasmoidal site lead to atrophy of the gill lamellae.

4. Remarks

4.1. Morphological comparison

A comparison of the present species, He. seenghalae n. sp has been made with morphologically similar myxospores belonging to the genus Hennegoides viz. He. longitudinalis, He. obpyriformis, He. berlandi, He. malayensis He. pangasi and He. flocake. It has been characterized in having an inverted pyriform shape with broad anterior end and tapering posterior end measuring 7.5 μm and caudal extension having length of 39.1 μm. The polar capsules were perpendicular to the body axis therefore differed from He. longitudinalis in which the polar capsules were longitudinally arranged to the body axis. Therefore, the species under study differed from all the already known species in the size of the myxospore and length of the caudal appendage (Table 1). However, in this respect, the present species was closest to He. berlandi in having same body ratio 1.9 (myxospore length and length of caudal appendage) but differed from it in having smaller polar capsules (1.5 × 0.75 μm in He. seenghalae n. sp vs. 2.8 × 1.8 in He. berlandi).

4.2. Phylogenetic analysis

The 18S universal primers 18e (5-CTGGTTGATTCGGCAGT-3) and 18R (5-CTACGGAAAACTCGTTACG-3) successfully amplified 1947 bp fragments of the 18S rDNA gene from the present sample of Hennegoides infecting gills of Sperata seenghala from Chamkaur Sahib, Punjab, India. The edited nucleotide sequences obtained from the myxospores of Hennegoides seenghalae n. sp. were deposited in the GenBank under the accession number MN322671.

The phylogenetic tree based on the final edited alignment (1947) with Maximum-Likelihood showed maximum homogeneity of 87.07% with Unicauda pelteobagrus (KC193254) infecting muscles of Pelteobagrus fulvidraco followed by 85.83% homogeneity with Hennegoides pangasi (EU732605) infecting the gills of Pangasius hypophthalmus from Malaysia. The phylogenetic tree comprised of 21 myxosporean species of genus Henneguya, Unicauda, Myxobolus, Myxobilatus and Hennegoides.
from different regions including countries such as Malaysia, Nigeria, Vietnam, Brazil, Mexico and USA (Fig. 5). *Ceratomyxa shasta* (AF001579) infecting intestine of *Oncorhynchus mykiss* from USA was used as an out-group (Fig. 5).

Minimum evolutionary divergence among the sequence of *Henne-goides seenghalae* n. sp. and other myxosporea was estimated using Bayesian Information Criterion (BIC scores) using K2+G as the best substitution model with lowest BIC score of 2077.869. It showed 0.36 divergence with *Hennegoides flockae*, 0.31 with *Unicauda pelteobaguars*, 0.19 with *Henneguya* sp, 0.22 with *Henne-goides pangasi*, 0.18 with *Uni-cauda fimbrethilae* and 0.32 with *Ceratomyxa shasta* (Outgroup). Rates of different nucleotides substitution (r) from one base (transitional) and to another base (transversional) were estimated. The rates of transition were more than transversion for all base substitutions. Maximum transition was C→T 16.80 probability value followed by T→C 12.94, A→G 12.37 and G→A 11.84. The analysis involved 21 nucleotide sequences. All positions containing gaps and missing data were deleted. The D value in the Tajima’s Neutrality Test was found to be −1.187662 indicating recent sweep and increase in the population of the myxozoans.

4.3. Discussion

It is for the first time, we investigated a new species belonging to the genus *Hennegoides* infecting a new host *Sperata seenghala*. The phylogenetic tree using maximum likelihood based on final edited alignment formed two major clades with a moderate bootstrap value including the present species (*Hennegoides seenghalae*) and the related species in one clade and *Myxobilatus gaterostei* in the other clade. *Ceratomyxoa shasta* infecting *Oncorhynchus mykiss* was taken as the out-group. The present species formed a separate subclade including *He. pangasi*, *M. omari*, *He. mekongensis*, *Myxobolus pseudowulii* and *U. pelteobargus* with a high bootstrap value of 81. Similar studies have been made by Molnár (2002b) where the genus *Thelohanellus* was characterized by strict tissue specificity and geographical location. Later, it was found that clustering of species also occurs on the basis of tissue location, host and geographical location of the host (Zhao et al., 2008). The other subclade consisted species such as *Hennegoides flockae*, *Henneguya laseeae*, *H. bulbous*, *H. exilis* and *H. adiposa* from USA with a high bootstrap value of 100. The topmost clade comprised species such as *Henneguya fusi-formis, Unicauda fimbrethilae, H. mystusia, H. gnathonemus, H. sp. and
H. basifilamentalis with a moderate bootstrap value of 50 in which H. nyongensis and Henneguya sp. formed a sister clade with a high bootstrap value of 100. Another important aspect of the present study is the presence of Myxobolus, Henneguya and Unicauda species along with Hennegoides in the BLAST results.

He. flockae formed a separate clade in the phylogenetic tree along with some Henneguya species having same geographical area. Leis et al. (2019) found that the genus Henneguya was polyphyletic however a majority of its species has a common ancestor and groups in the second largest clade of the Myxobolus clade. The BLAST results from the current study revealed a significant number of species belonging to different genera such as Unicauda, Myxobolus, Henneguya, Myxobilatus and Hennegoides. This situation highlights the importance of using both molecular and morphological methods to identify and study of inter-relationships associated with these enigmatic cnidarians (Leis et al., 2019; Liu et al., 2019).

Based on molecular and morphological data, phylogenetic tree and evolutionary data, Hennegoides scenghalae is considered a novel species and represents the first record of the genus Hennegoides from the Indian subcontinent.

Declaration of competing interest

The authors declare that they have no conflict of interest.

5. Ethical clearance

Not required.

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