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Isolation and identification of different parasites from Indian major carps and exotic carps from South 24-Parganas, West Bengal

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ABSTRACT

The present study was conducted to isolate and identify different parasites from Indian major carps and exotic carps by morphological, histopathological study and by using molecular method from South 24 Parganas district, West Bengal, India. During the study period different types of parasites were observed such as *Thelohallenus* sp., *Trichodina* sp., *Argulus* sp., *Gregarine* sp., *Pisicola* sp., and *Myxobolus* sp. Most of the fishes were infested with different types of parasites in the gill and external body surface throughout the year. *Thelohallenus* sp., *Trichodina* sp. and *Argulus* sp. were identified from diseased *Labeo rohita.* Kidney plasmodia along with scattered cysts was seen with gill discolouration, focal gill necrosis, white spots on gills, sliming, visceral haemorrhages and swelled kidney. Diseased *Catla catla* with clinical signs of excess sliming and scale loss were infected with *Gregarine* sp., isolated from the intestine. *Pisicola* sp. and *Myxobolus* spp. were identified from diseased *Cirrhinus mrigala* with different clinical signs of cysts on the gill and excess sliming. *Argulus* sp. was observed in *Cyprinus carpio* with whitish gill and haemorrhages on the body. 1800 base pairs (bp) long fragment of 18S ribosomal RNA gene was obtained by PCR amplification with universal primers for parasitic isolates and approximately 1600 base pairs (bp) long fragment was obtained by PCR amplification for myxosporean isolates with specific primers for the family Myxobolidae. Among 5 parasitic strains, 1 was identified as *Myxobolus bhadrensis* and the other was *Thelohallenus bifurcate.* Sequence results were submitted to NCBI GenBank for accession number.

Key words: Carps, Histology, Morphology, Parasites, PCR

Carps are the major group of freshwater fish that have a global significance as a source of food and as experimental models for research. Modern fish farming with high stocking densities and intensive production units provide ideal conditions for the invasion and persistence of a range of pathogens (bacteria, viruses, protozoan and metazoan parasites). Infections by these disease-causing agents reduce the condition and survival of fish causing economical losses to farmers. However, treatments unavoidably cause problems related to environmental pollution, drug resistance and health issues, hence environmentally and ecologically sustainable solutions are now being sought in fish farming. Reaching this long-term objective will depend on the detailed information and knowledge on the ecology of main disease threats (Anssi *et al*. 2005). Ayyappan *et al*. (2006) reported that aquaculture in India is almost synonymous to carp culture and alone contributes to more than 75% of the total aquaculture production of the country. The carp culture mainly involves 2 groups, i.e. the 3 Indian major carps such as catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*), and 3 exotic carps such as silver carp (*Hypophthalmichthys molitr*ix), grass carp (*Ctenopharyngodon idella*) and common carp (*Cyprinus carpio*).

Carps are the mostly cultured fish species in West Bengal and infectious diseases of cultured freshwater carps are one of the major problems to successful aquaculture, which cause economic losses in aquaculture industry. To study fish diseases, it is important to acquire knowledge on different pathogens, their biology and life cycle. Cultured carps are susceptible to various kinds of diseases, viz. bacterial, fungal, viral, parasitic, environmental and nutritional. The dense populations of fish kept in particular environmental conditions may favour certain parasites to cause harm to a fish, but this condition varies considerably with the species and size of the host and its health status. Many parasites are host specific to at least some degree (Roberts 2001). Individual parasite species may also have widely differing effects on different host species. Carps are the most important freshwater species cultured in West Bengal but proper fish disease monitoring is still lacking, hence, suitable surveillance system is far from reality, so, protection of these fish against diseases is vital to the aquaculture industry.

So the objectives of the present study were to isolate and identify different parasites from Indian major carps and exotic carps by morphological, histopathological study and

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by using molecular method from South 24 Parganas district, West Bengal, India.

MATERIALS AND METHODS

Sampling: The diseased fish samples were collected from different fish farms located in different areas of South 24 Parganas district of West Bengal, viz. Chakgaria, Haripota, Katipota, Sonarpur, Bonhooghly, Canning II, Uttardanga, Bamanghata, Bhangar, Gangasagar.

The experimental fish for the present study include 3 species of Indian major and exotic carps, viz. *Labeo rohita*, *Catla catla, Cirrhinus mrigala* and *Hypophthalmichthys molitrix, Ctenopharyngodon idella, Cyprinus carpio* cultured in semi-intensive and intensive farms of the above mentioned areas.

Parasite isolation and phenotypic characterization: At the laboratory, external parasites from body surface, fin and gills region were removed by scrapping the slime with a sharp scalpel, it was mixed with a drop of physiological saline and was spread on a clean dry glass slide with cover slip on top of it.

Phenotypic characterization of all protozoans, monogenean and crustacean parasites were studied as described by Soulsby (1982). Photomicrographs were taken by using SCO-LUX camera, 16 MP attached with the microscope. The parasite identification was performed in the laboratory according to Lom and Arthur (1989). The myxosporean cysts infecting the gill and kidney of *L. rohita* were isolated carefully. The slides containing myxosporean spores were observed under oil immersion (100×) lens of Motic BA400 microscope with inbuilt digital camera. For endoparasites, fishes were dissected out ventrally by a sharp scalpel blade to observe parasites inside kidney, liver, spleen, stomach and intestine. Small worms were searched initially with the help of magnifying glass by scrapping out mucus (Akter *et al.* 2007). The morphometric measurements were done by Motic Image Plus Version2 software (Mondal *et al.* 2014). The results are presented in micrometer (µm) as mean±standard deviation (SD).

DNA extraction of myxosporean spore and PCR amplification of 18S rRNA gene: Genotypic characterization of selected myxosporean spores were done by 18S rRNA gene sequencing. The genomic DNA of myxospores were extracted by using genomic DNA isolation kit (Macherey-Nagel, Germany) as per the manufacturer's protocol. The 18S small subunit ribosomal RNA (18S rRNA) was amplified by PCR using a set of universal eukaryotic primers (UEP-F, 5′-ACCTGGTT-GATCCTGCCAG-3′ and UEP-R, 5′-CTTCCGCAGGTTCACCTACGG-3′) (Barta *et al.* 1997). These primers amplify approximately 1800 bp long fragment from the 18S rRNA gene.

Another set of species specific primers for the family Myxobolidae (Andree *et al*. 1999b) was (MX5-forward) 5'-CTGCGGACGGCTCAGTAAATCAGT-3'; the sequence of the reverse primer (MX3) was: 5'-CCAGGACATCTTAGG-GCATCACAGA-3'. These primers amplify approximately 1600 bp long fragment from the 18S rRNA gene.

The PCR products were analysed on 1.2% agarose (HiMedia, India) gels containing 0.5 µg/ml ethidium bromide in 1X Tris-acetate- EDTA (TAE) buffer. The PCR amplified products were sequenced at the Genomics Division, Xcelris Labs Ltd., Ahmedabad, India.

Histopathology: The different organs of diseased fish were fixed in alcoholic bouin's fixative for 48–72 h. After fixation the tissues were transferred to 70% ethyl alcohol and kept overnight. Histopathological analyses were made as described by Roberts (2001). The dried slides were stained by haematoxylin and eosin double staining (H&E) method described by Roberts (2012). Slides were permanently mounted using DPX (dibutyl phathalate xylene) mountant.

Microscopy and photomicrography: The sections were screened with the help of PC based microscope. Colour microphotographs were taken from the selected slides at different magnification with advanced Trinocular Research Microscope (Olympus, Japan, Model: BX51) by SCO-LUX camera, 16 MP attached with the microscope.

RESULTS AND DISCUSSION

Prevalence of different parasites in diseased Indian major and exotic carps

Thelohallenus sp. and *Trichodina* sp. were identified from diseased *Labeo rohita* from Bonhooghly (Fig. 1a,c). Kidney plasmodia along with scattered cysts in the kidney was seen in *Labeo rohita* from Sonarpur with gill discolouration, focal gill necrosis, white spots on gills, sliming, visceral haemorrhages and swelled kidney. Diseased *Catla catla* from Canning II with clinical signs of excess sliming and scale loss were infected with *Gregarine* sp., isolated from the intestine (Fig. 1e). Leech (*Pisicola* sp.), *Myxobolus* spp., and *Trichodina* spp. were identified from diseased *Cirrhinus mrigala* from Haripota, Bhangar and Bonhooghly with different clinical signs of cysts on the gill and excess sliming (Fig. 1b,d,f,g). *Argulus* sp. was observed in *Cyprinus carpio* in Sonarpur with clinical signs of whitish gill and haemorrhages on the body (Fig. 1h).

Morphometric measurements

The parasitic strains (10) were randomly selected and

Table1. Description of the morphological features of myxosporean parasites observed

Name of parasite	Name of host	Organ	Length of spore (µm)	Width of spore (µm)
Myxobolus bhadrensis	*Labeo rohita*	Kidney	10.75±0.05	6.36±0.014
Myxobolus bhadrensis	*Labeo rohita*	Kidney	10.78±0.014	6.57±0.02
Thelohallenus bifurcata	*Labeo rohita*	Gill	16.16±0.014	4.18±0.014
Thelohallenus bifurcata	*Labeo rohita*	Gill	16.42±0.03	4.30±0.03
Thelohallenus bifurcata	*Labeo rohita*	Gill	16.19±0.014	4.46±0.02

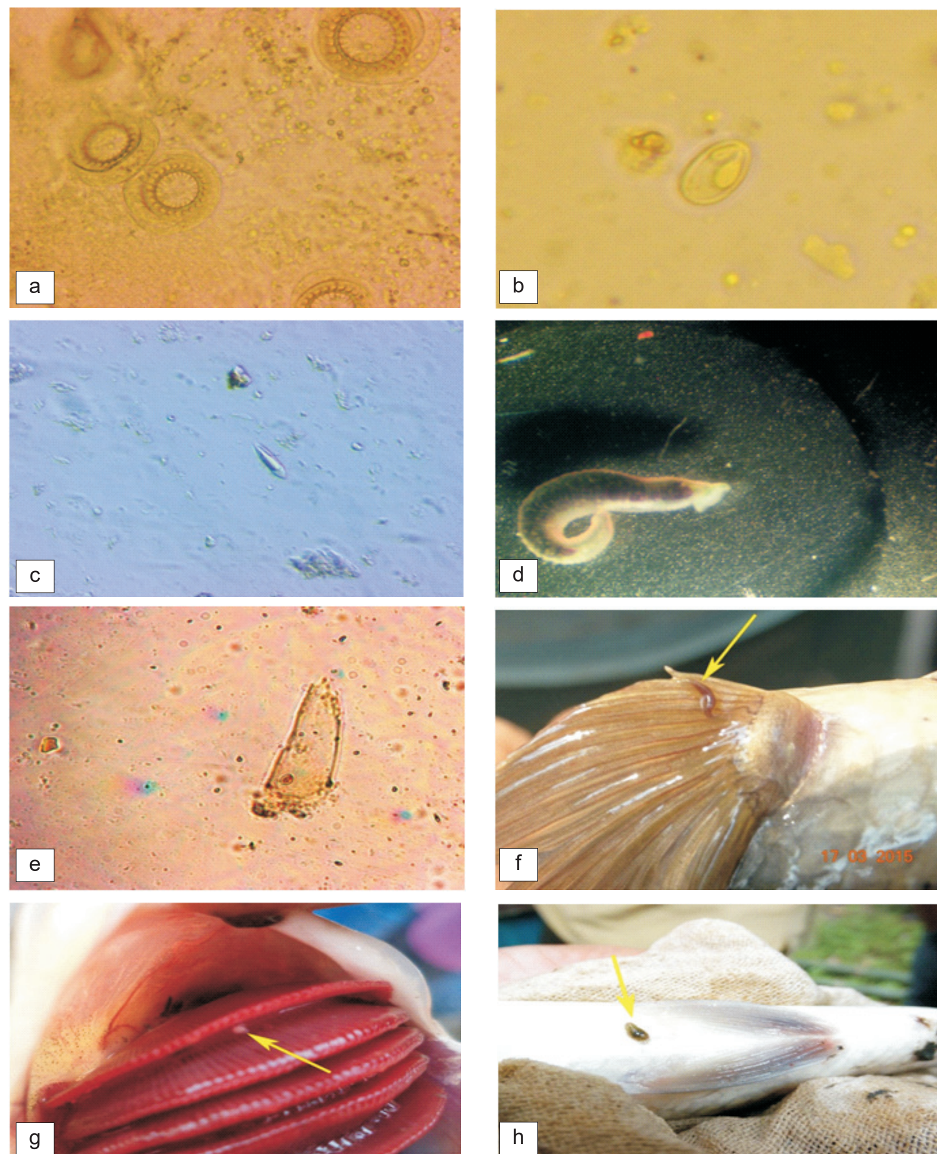

Fig. 1(a-h). (**a**) Microscopic observation of *Trichodina* sp. present on *Hypophthalmichthys molitrix* (10×) (wet mount). (**b**) *Myxobolus bhadrensis* isolated from kidney of *Labeo rohita* (40×) (wet mount). (**c**) *Thelohanellus* sp. isolated from gill of *Labeo rohita* (20×) (wet mount). (**d**) *Piscicola* sp. from tail region of *Catla catla* (wet mount). (**e**) *Gregarine* sp. isolated from intestine of *Catla catla* (20×) (wet mount). (**f**) *Piscicola* sp. attached at the tail region in *Catla catla.* (**g**) *Myxobolus* sp. cyst attached in gill. (**h**) *Argulus* sp. attached on body of *Labeo rohita.*

observed under microscope by wet mount method (Table 1). One of the strains was isolated from the plasmodia in the kidney of *Labeo rohita*. After microscopic observation, morphometric measurements were taken. The length of the spores was 10.75±0.05µm and the width 6.36±0.014 µm. Polar capsules were pear shaped, filling most of the spore cavity. Larger capsule was 6.5±0.014 µm, while the smaller capsule was 3.695±0.02 µm. The morphometric measurements of the spores were close to the measurements reported for *Myxobolus bhadrensis*. Other than *Myxobolus bhadrensis* strain, other 4 strains were also isolated from gills and kidney of *Labeo rohita*. The morphometric measurements of the other *Myxobolus bhadrensis* spore, length was 10.78±0.014 µm and width 6.57±0.02 µm. Polar capsules were pear shaped, filling most of the spore cavity. Larger capsule was 6.275±0.02 µm, while the smaller capsule was 3.71±0.02 µm. The morphometric measurements of the other 3 strains from gills of *Labeo rohita* were, length 16.16±0.014 µm, 16.42±0.03 µm, 16.19±0.014 µm and width 4.18±0.014 µm, 4.3±0.03 µm, 4.455±0.02 µm, respectively. Length of polar capsule was 10.635±0.02 µm, 10.49±0.014 µm, 10.405±0.02 µm, respectively. The length/width ratio of the spore was 3.86, 3.76, 3.63 and 3 strains were identified as *Thelohallenus bifurcata*.

18S rRNA gene analysis

Randomly selected 5 eukaryotic parasitic strains were further characterized and identified through 18S rRNA gene analysis. In 1.2% agarose gel electrophoresis, approximately

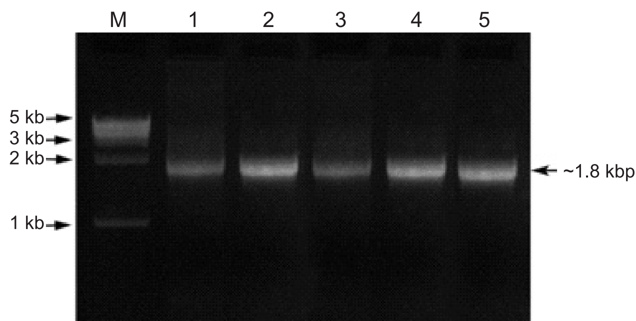

Fig. 2. Agarose gel (1.2%) showing 18S rRNA gene amplicons of myxosporean strains of diseased Indian major and exotic carps. Lane M, 1 Kb molecular weight DNA marker; lane 1, *Myxobolus bhadrensis;* lane 2, *Myxobolus* sp.; lane 3, *Thelohanellus* sp.; lane 4, *Thelohanellus* sp.; lane 5, *Thelohanellus* sp.

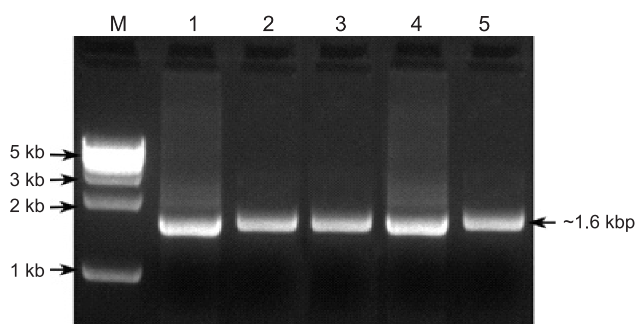

Fig. 3. Agarose gel (1.2%) showing Myxobolidae family specific 18S rRNA gene fragment amplicons of myxosporean strains of diseased Indian major and exotic carps. Lane M, 1 Kb molecular weight DNA marker; lane 1, *Myxobolus bhadrensis;* lane 2, *Myxobolus* sp.; lane 3, *Thelohanellus* sp.; lane 4, *Thelohanellus* sp.; lane 5, *Thelohanellus* sp.

1.8 kbp bands were obtained by PCR amplification for universal primers for parasitic isolates and approximately 1.6kbp band was obtained by PCR amplification for myxosporean isolates with specific primers for the family Myxobolidae (Figs 2, 3). Among 5 parasitic strains, 1 was identified as *Myxobolus bhadrensis*. The sequence results of other parasitic isolates are awaited.

Histopathology

Labeo rohita from Sonarpur, South 24 Parganas with clinical signs of focal gill necrosis, sliming, haemorrhage and cysts in kidney was diagnosed as mixed infection (kidney myxoboliasis with bacterial infection). Histopathological observations on kidney were depicted in Fig. 4 a, b. The study depicted alterations like plasmodia with spores of *Myxobolus bhadrensis* developing in the kidney, extensive necrosis of haematopoietic tissue, inflammed nephric tubule, constricted nephric tubule with vacuolated surrounding (Fig. 4a), and plasmodium in the kidney and congestion of inflamed nephric tubules with vacuolated surrounding (Fig. 4b).

Labeo rohita from Sonarpur, South 24 Parganas district with clinical signs of gill necrosis, gill fouling, haemorrhage, white spots/cysts in the gill and swelling of kidney were diagnosed to have mixed infection. Histopathological observations on kidney were depicted in Fig. 4c, d. Histopathological alterations like thickening around the inflamed nephric tubule, inflamed nephric tubule, widen lumen, congestion of nephric tubules with vacuolated surrounding, necrosis and hyperplastic haematopoietic tissue were observed (Fig.4c). Histologically, the kidney section depicted alterations like highly inflamed nephric tubule, thickening around highly inflamed nephric tubule, highly constricted nephric tubules, haemorrhage and necrosis (Fig. 4d). *Labeo rohita* in 3 cases from Sonarpur, South 24 Parganas district with clinical signs of focal gill necrosis, high sliming, haemorrhage and cysts in kidney; gill fouling, haemorrhages and swelling of kidney; and white spots/cysts in the gill were diagnosed as mixed infection. Histopathological observations on the gills of diseased *Labeo rohita* are depicted in Fig. 4e, f. Histologically, the gill section had alterations like loss of inter-lamellar space due to hyperplasia of respiratory cells, fusion of gill lamellae, inter-lamellar epithelium type large plasmodium deforming several gill lamellae, destruction of gill lamellae and extensive necrosis (Fig. 4e).

Aquaculture is one of the most economically important applied strategies allover the world and fishes are one of the most beneficial and nutritional resources for human beings. India has witnessed an overwhelming growth in the aquaculture sector for the past 2 decades. It is presently ranked second in aquaculture production. Among the various culture fisheries, carp culture plays an indispensable role in freshwater aquaculture production of India (FAO 2010). West Bengal is the only state in India, where fishes have been cultivated in every kind of water bodies, i.e. brackish water, freshwater, sewage water and marine water as well. In West Bengal different types of freshwater fish are cultured commercially and captured live from wild regions for commercial purposes. Among these, Indian major carps contribute 70–75%, followed by exotic carps and catfishes contributing 25–30% of the total freshwater fish production in India (FAO 2014). In West Bengal, 1.2% of the total human population is involved in fisheries and related activity (Korakandy 2008). Mostly cultured freshwater fish in West Bengal include 3 species of Indian major carps, viz. *Labeo rohita, Catla catla* and *Cirrhinus mrigala* and 3 species of exotic carps *Cyprinus carpio, Hypophthalmichthys molitrix* and *Ctenopharyngodon idella.*

Apart from the bacterial diseases, mixed infection, parasitic infestation and non-infectious diseases were also noticed in the present study. Next to bacterial diseases, parasites are the most dominating pathogen affecting the carp production. Therefore, correct identification of parasites is also necessary. The identification by wet mount as well as morphometric characterization is not enough for the diagnosis of the parasitic diseases. The 18S rRNA gene sequence method is used for the identification of parasites. Parasites being eukaryotic cells and sequence data from these genes are widely used in molecular analysis to

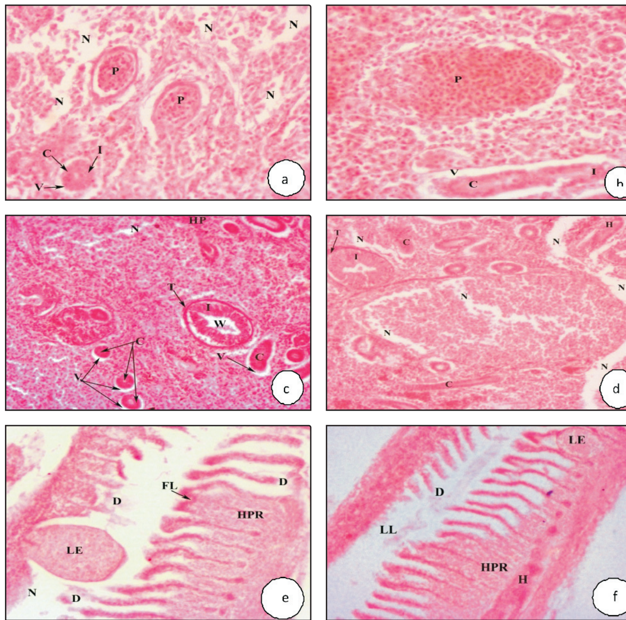

Fig.4(a-f). (**a**). The kidney of diseased *Labeo rohita* showing: plasmodia with spores of *Myxobolus bhadrensis* developing in the kidney (P), extensive necrosis of haematopoietic tissue (N), inflammed nephric tubule (I), constricted nephric tubule (C) with vacuolated surrounding (V). H&E, ×200. (**b**). The kidney of diseased *Labeo rohita* showing: plasmodium in the kidney (P) and congestion (C) of inflamed nephric tubules (I) with vacuolated surrounding (V). H&E, ×200. (**c**). The kidney of diseased *Labeo rohita* showing: thickening around the inflamed nephric tubule (T), inflamed nephric tubule (I), widen lumen (W), congestion of nephric tubules (C) with vacuolated surrounding (V), necrosis (N), hyperplastic haematopoietic tissue (HP). H&E, ×100. (**d**). The kidney of diseased *Labeo rohita* showing: highly inflamed nephric tubule (I), thickening around highly inflamed nephric tubule (T), highly constricted nephric tubules (C), haemorrhage (H), necrosis (N), congestion of nephric tubules (C). H&E, ×100. (**e**). Histopathological changes in the gill of diseased *Labeo rohita* showing: loss of inter-lamellar space due to hyperplasia of respiratory cells (HPR), fusion of gill lamellae (FL), inter-lamellar epithelium type large plasmodium deforming several gill lamellae (LE), destruction of gill lamellae (D), extensive necrosis (N). H&E, ×100. (**f**). The gill of diseased *Labeo rohita* showing: loss of inter-lamellar space due to hyperplasia of respiratory cells (HPR), inter-lamellar epithelium type large plasmodium deforming several gill lamellae (LE), loss of gill lamellar structure (LL), destruction of gill lamellae (D), haemorrhage (H) along with haemorrhagic pockets at the tips of gill lamellae (H). H&E, ×100.

reconstruct the evolutionary history of organisms, especially in vertebrates, as its slow evolutionary rate makes it suitable to reconstruct ancient divergences.

Few parasitic strains were selected and observed under microscope by wet mount method. One of the strains were isolated from the plasmodia in the kidney of *Labeo rohita*. After microscopic observation, morphometric measurements were taken. The morphometric measurements of the spore were close to the measurements reported for *Myxobolus bhadrensis* by Szekely *et al.* (2015). The strain was further confirmed as *Myxobolus bhadrensis* by using specific primer for the family Myxobolidae. In 1.2% agarose gel electrophoresis, approximately 1.6 kbp bands were obtained by PCR amplification (Figs 2, 3). Likewise, Szekely *et al.* (2015) reported *Myxobolus bhadrensis* from *Labeo rohita* kidney following characterization by 18S rRNA gene sequence. The morphometric measurements of spores from gill of *Labeo rohita*, length of the spores was 16.16±0.014 µm, 16.21±0.03 µm, 16.19±0.014 µm and the width was 4.18±0.014 µm, 4.3±0.03 µm, 4.455±0.02 µm, respectively. Length of polar capsule was 10.635±0.02 µm, 10.49±0.014 µm, 10.405±0.02 µm, respectively. The length/width ratio of the spore was 3.86, 3.76 and 3.63 respectively. The results corroborate closely with the result of Kaur and Katoch (2014) and 3 strains were identified as *Thelohallenus bifurcata* isolated from *Labeo rohita* gill where the length/width ratio of the spore was 3.74. The results of molecular characterizations are awaited.

In the present study, parasitic infestation was documented with 10% incidence rate. Thelohanellosis and argulosis were documented in *Labeo rohita.* Mondal *et al.* (2014) reported that *Thelohanellus caudatus* infecting the caudal fin of carp *Labeo rohita* was characterized morphologically and by 18S rRNA gene sequence analysis in West Bengal. Similarly, Kaur *et al.* (2014) reported that *Thelohanellus dykovi* was a pathogenic gill parasite in *Labeo rohita* in Punjab. Dash *et al.* (2014) also reported both *Thelohanellus* sp. and *Argulus* sp. in minor carps, *Labeo bata* in West Bengal. Gregarine infection was observed in *Catla catla* in Canning II. However, no reports are available on the incidence of gregarine infection in major craps. The culture system was polyculture system and the area being under brackishwater and freshwater zone, along with Indian major carps, shrimp, brackishwater species were cultured. As stocking density was not maintained properly, improper feed and lack of scientific culture practices might led to the infection. Argulosis was documented in *Cyprinus carpio* in Sonarpur block. Steckler and Yanong (2012) reported *Argulus* infection in the cyprinidae family. Rahman (1996) documented *Argulus* sp. in *Cyprinus carpio.* Gill myxoboliasis was observed in *Cirrhinus mrigala*. Similarly, heavy carp mortality associated with gill myxoboliasis has raised concern among fish farmers (Chandra *et al.* 1996). The present results corroborate the study of Banerjee *et al.* (2015), who characterized a myxozoan parasite, *Myxobolus carnaticus* infecting the gill lamellae of mrigal carp, *Cirrhinus mrigala* by the 18S rRNA gene sequence.

The parasitic infestation was mostly affected by climate change in aquatic systems and most organisms and their functional roles in the ecosystem. The parasites in aquatic systems depend on both the abiotic conditions of the environment and the distribution and abundance of their hosts for transmission, reproduction, survival, and other basic life history functions. Likewise, the surveyed aquaculture system was based on sewage-fed fisheries, industrial effluents and household sewage water, which are loaded with organic matters, thereby expediting the parasitic infestation.

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