Molecular characterization of *Myxobolus catmrigalae* (Myxosporea: Myxobolidae) infecting the gill lamellae of carp *Cirrhinus mrigala* (Hamilton)

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Abstract The present study attempted sequencing the 18S rRNA gene of *Myxobolus catmrigalae* infecting the gill lamellae of carp, *Cirrhinus mrigala* and compared its genetic homology and phylogenetic characteristics with 18S rRNA genes of other *Myxobolus* spp. The infected fish had up to 3 small, creamy white plasmodia per gill filament with 30–50 spores each. The spore size was $17.90 \pm 0.70 \times 7.40 \pm 0.40 \mu\text{m}$. The sporoplasm contained two large nuclei of size $0.57 \pm 0.09 \mu\text{m}$ and no iodophilous vacuole. The DNA sequence of *M. catmrigalae* was clustered phylogenetically with other *Myxobolus* spp. infecting the gills of cyprinids available in GenBank, which showed 77–87 % homogeneity. On the phylogenetic tree, *M. catmrigalae* (KC933944) was clustered with *M. pavlovskii* (HM991164) infecting the gill lamellae of silver carp, *Hypophthalmichthys molitrix*. The species most closely related to *M. catmrigalae* in GenBank was *M. pavlovskii* (AF507973) infecting the gill lamellae of big head carp, *Aristichthys nobilis* with 87 % homogeneity. This is the first report on molecular characterization of gill lamellae infecting *M. catmrigalae*.

Keywords Aquaculture · Indian major carps · Myxosporea · *Myxobolus catmrigalae* · Molecular characterization · Phylogenetic analysis

Introduction

Aquaculture is one of the fastest growing animal food producing sectors. The world aquaculture production of food fish is increased by 6.2 % from 59 million tonnes in 2010 to 62.7 million tonnes in 2011. India is the second largest producer of fish in the world contributing to about 5.43 % of global fish production. Scientific culture of carps has become a common practice in many parts of India. The aquaculture production of India reached 4.573 million tonnes in 2011 from 3.786 million tonnes in 2010 (FAO 2013). The state West Bengal, India is one of the leading producers of aquacultured freshwater fish. Total fish production in the State has increased from 12.15 lakh tonnes in 2004–2005 to 14.72 lakh tonnes in the year 2011–2012 (DAHDF 2013). Diseases of bacterial, parasitic and fungal origin are one of the major constraints on aquaculture production. Ulcerative bacterial diseases, myxosporeans, monogeneans, digeneans, larval cestodes and ectoparasitic crustaceans have been regularly reported in India, but mortalities associated with these pathogens are few (Mohan and Bhatta 2002). Among the various groups of fish pathogens, parasitic infestations play a major role, which contribute to 78 % of the disease occurrences in India (Lakra et al. 2006). Some of these pathogens are reportedly responsible for significant chronic mortalities and poor growth. Annual losses of US\$ 1.0 million due to disease induced mortality and impaired growth have been reported in carp culture in Andhra Pradesh, India (Mohan and Bhatta 2002).

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The myxosporeans are economically important group of microscopic parasites, best known for the diseases they cause in commercially important fish (Kent et al. 2001; Feist and Longshaw 2006; Xi et al. 2011). These parasitic protozoans are getting more and more attention as new myxosporeans are continually emerging and threatening the aquaculture development. Among myxosporeans, the genus *Myxobolus* includes the highest number of species. In a synopsis of this genus, Eiras et al. (2005) reported 744 valid species, while Lom and Dyková (2006) counted 792 including 7 amphibian species. Several reports on *Myxobolus* spp. are available from Punjab and West Bengal, India (Basu and Haldar 2004; Bandyopadhyay et al. 2006; Basu et al. 2009; Kaur and Singh 2012; Singh and Kaur 2012). About 131 species of *Myxobolus* have been observed in India (Kaur and Singh 2012). *Myxobolus* is characterized by ellipsoidal, ovoid or rounded spores in valvular view, which look biconvex in sutural view. Spores always have smooth shell valves and two pyriform, sometimes unequal polar capsules. Sporoplasms are binucleate, often with an iodophilous vacuole (Lom and Dyková 2006). *Myxobolus* spp. can be differentiated by the morphological characteristics of their spores and the location of plasmodia. Until recently, only spore morphology served as a means of identification of the myxosporean species. As molecular methods have become increasingly important in parasitological studies, the taxonomic classification of myxosporeans has been expanded via phylogenetic analyses. Molecular biological methods offer great scope for the correct identification and differentiation of morphologically indistinguishable *Myxobolus* spp. (Kent et al. 2001; Eszterbauer 2002; Molnár et al. 2010; Cech et al. 2012). The present study was aimed at sequencing the 18S rRNA gene of *Myxobolus catmrigalae* infecting the gill lamellae of carp, *Cirrhinus mrigala* and comparing its genetic homology and phylogenetic characteristics with 18S rRNA genes of other *Myxobolus* spp.

Materials and methods

Morphometric analyses of *Myxobolus*

A *Myxobolus* species infecting the gill lamellae of carp, *Cirrhinus mrigala* collected from Nabagram (Lat. 22°27'48"N; Long. 88°24'31"E), South 24 Parganas District, West Bengal, India during the routine survey work on parasitic infection of carps in winter season, i.e., January 2013 was characterized by morphometric and molecular techniques. The myxosporean identification was performed according to Lom and Arthur (1989). The myxosporean plasmodia attached to the gill lamellae of *C. mrigala* of

weight 155 g were isolated carefully. For detailed study, fresh plasmodia were first taken on clean grease free glass slides and slightly ruptured on one end with a sharp needle. The spores released from the plasmodia were smeared on clean grease free slides with few drops of distilled water, covered with cover slips and sealed with DPX for examination of fresh spores under the oil immersion (100×) lens. Some of the fresh smears were treated with various concentrations of KOH (2–10 % w/v) for the extrusion of polar filament. The Indian ink method (Lom and Vavrá 1963) was employed for observing the mucous membrane around the spores. For the detection of iodophilic vacuoles in the sporoplasm, fresh spores sealed with cover slips were treated with Lugol's iodine solution and examined under oil immersion lens. Permanent mounting of myxosporeans were done by staining with giemsa. Air dried smears on grease free clean slides were treated with acetone free absolute methyl alcohol for about 8 min to fix the myxosporeans and again dried. The stock solution of giemsa was diluted with phosphate buffer (pH 7.2) in the ratio of 1:2. The slides were then placed on a staining rack and covered with dilute giemsa stain for 40 min. The slides were finally washed by pouring neutral distilled water until the colour did not turn to a noticeable extent and the slides were air dried. The slides containing myxosporean spores were observed under the oil immersion (100×) lens of Motic BA400 microscope with inbuilt digital camera. The morphometric measurements were done by Motic Image Plus Version-2 software. The results are presented in micrometer (µm) as mean ± standard deviation (SD).

Scanning electron microscopy

For scanning electron microscopy (SEM), myxosporean plasmodia attached to the gill lamellae of *C. mrigala* (Hamilton) were isolated with the help of a sterile forcep and placed on to thin cover slips pre-coated with poly-L-lysine. These were then ruptured and the envelopes removed with the tip of a needle. Utmost care was taken to remove entire remnants of the host tissue so as to free the samples from any foreign particles. The spores were then fixed with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at 30 ± 2 °C. Following dehydration in a standard ethanol gradient, the samples were transferred to a series of absolute alcohol and amyl acetate mixture (3:1, 1:1 and 1:3) for 30 min each before finally reaching to amyl acetate (Adriano et al. 2002). The samples were then critical point dried using CO₂ in a HCP-2 Critical Point Dryer (Hitachi), coated with metallic gold in an IB-2 ion coater and examined using Hitachi S-530 Scanning Electron Microscope at accelerating voltages of 15 and 20 kV.

Molecular analyses

The plasmodia filled with mature spores were ruptured by a sharp needle and the contents collected carefully in 1.5 ml microfuge tubes. The spores were then centrifuged at $1000\times g$ for 10 min. The DNA was extracted by suspending the spores in 500 μ l 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 μ l of phenol: chloroform (1:1) was added to the digested spores, mixed gently, and centrifuged at $5200\times g$ for 10 min. The upper phase was transferred to a new tube. The extraction step was repeated, if necessary and followed by phenol: chloroform (1:1) treatment. After the last centrifugation, the upper phase was transferred to a fresh tube and mixed with 1/10th volume of sodium acetate (3 M, pH 5.2) with 2 times the volume of 96 % ethanol (Amersco, USA). The DNA was precipitated at –20 °C for overnight and pelleted by centrifugation at $10000\times g$ for 30 min. The pellet was washed once with 70 % ethanol, air-dried for several minutes and resuspended in molecular biology grade water (30 μ l).

The 18S small subunit ribosomal RNA (18S rRNA) was amplified by Gradient PCR system (Eppendorf Master cycler Pro S) using a set of universal eukaryotic primers-UEP-F, 5'-ACCTGGTTGATCCTGCCAG-3' and UEP-R, 5'-CTTCCGCAGGTTACCTACGG-3' (Barta et al. 1997). The PCR was run using a mixture containing 50 ng of genomic DNA, 10 μ M of each primer, 2 $\times$ PCR TaqMixture (HiMedia, Mumbai). Amplification was done by initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing of primers at 51 °C for 30 s and extension at 72 °C for 60 s. The final extension was at 72 °C for 5 min. The PCR products were analysed on a 1.5 % agarose (HiMedia, Mumbai) gel containing 0.5 μ g/ml ethidium bromide in 1 $\times$ Tris-acetate-EDTA (TAE) buffer.

Sequence and phylogenetic analyses

The PCR amplified products were sequenced at the Genomics Division, Xcelris Labs Ltd, Ahmedabad, India. Amplified PCR product was purified using EXO-SAP treatment. The concentration of the purified DNA was determined and subjected to automated DNA sequencing on ABI 3730xl Genetic Analyzer (Applied Biosystems, USA). Sequencing was carried out using BigDye[®] Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) following manufacturers' instructions. Electrophoresis and data analysis was carried out on the ABI 3730xl Genetic Analyzer. The nucleotide sequences of 18S rRNA gene of 19 gill infecting *Myxobolus* spp. such as *M. pavlovskii* (AF507973, HM991164), *M. macrocapsularis*

(AF507969), *M. dispar* (AF507972), *M. bramae* (AF507968), *M. impressus* (AF507970), *M. bliccae* (HM138772), *M. muelleri* (AY325284), *M. basillamellaris* (AF507971), *M. leuciscini* (DQ439811), *M. margitae* (EU598803), *M. bilobus* (DQ008579), *M. intimus* (JX390690), *Myxobolus* spp. (FJ716098, FJ716097, FJ716096), *M. intimus* (JX390691), *M. tambroides* (JX028236), *M. longisporus* (AY364637), three muscle infecting *Myxobolus* spp. such as *M. musculi* (AF380141), *M. cyprini* (AF380140), *M. pseudodispar* (AF380145), one fin infecting *Thelohanellus nikolskii* (DQ231156) of cyprinids, two gill infecting *Henneguya* spp. such as *H. exilis* (AF021881), *H. ictaluri* (AF195510) and one fin infecting *H. gurlei* (DQ673465) of ictalurids from NCBI GenBank were subjected to phylogenetic analysis. *Ceratomyxa shasta* (AF001579) infecting the intestinal tissue of rainbow trout (*Oncorhynchus mykiss*) was selected as an out-group. The sequences were compared by standard nucleotide BLAST (www.ncbi.nlm.gov/BLAST/). Data analysis and multiple alignments were performed by ClustalX (Thompson et al. 1997) and MEGA5 (Tamura et al. 2011) softwares, respectively. The evolutionary history was inferred using the maximum parsimony method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). The nucleotide sequence generated in the present study has been deposited in the Genbank database under accession number KC933944.

Results

Morphological characteristics of *Myxobolus catmrigalae*

The polysporic plasmodia of *Myxobolus* species isolated from the gill lamellae of *C. mrigala* were small, 0.80–0.90 mm in size and creamy white. The infected fish had up to 3 plasmodia per gill filament and each plasmodium contained about 30–50 spores. The morphometric measurements of the mature spores and spore index of the *Myxobolus* species are presented in Table 1. The mature spores were elongated and pyriform in valvular view and lenticular in sutural view. They were bluntly pointed anteriorly with a rounded posterior end. The size of the spore was 17.90 ± 0.70 (17.20–19.30) μ m $\times$ 7.40 ± 0.40 (6.80–8.50) μ m. The polar capsules were elongated and pyriform in shape. They occupied most of the sporoplasm and ran parallel to each other, but sometimes converged

Table 1 Morphological observations of *Myxobolus catmrigalae* spores isolated from the gill lamellae of *Cirrhinus mrigala*

Measurement	Range	Mean	SD	SE	CV (%)
Length of the spore (LS) (μm)	17.2–19.3	17.9	0.707	0.158	3.95
Width of the spore (WS) (μm)	6.8–8.5	7.4	0.468	0.104	6.33
Length of the larger polar capsule (LLPC) (μm)	9.9–13.2	11.3	1.014	0.226	8.97
Width of the larger polar capsule (WLPC) (μm)	2.7–3.4	3.0	0.221	0.049	7.37
Length of the smaller polar capsule (LSPC) (μm)	9.2–12.4	10.6	0.977	0.218	9.21
Width of the smaller polar capsule (WSPC) (μm)	2.5–3.2	2.8	0.165	0.037	5.90
No. of nucleus	2	–	–	–	–
No. of coils in larger polar capsule	20–24	–	–	–	–
No. of coils in smaller polar capsule	19–22	–	–	–	–
Spore index					
LS:WS	1:0.413	–	–	–	–
LLPC:WLPC	1:0.265	–	–	–	–
LSPC:WSPC	1:0.264	–	–	–	–
LLPC:LSPC	1:0.938	–	–	–	–
WLPC:WSPC	1:0.933	–	–	–	–

– No data, *SD* standard deviation, *SE* standard error, *CV* co-efficient of variation
 Values are on the basis of 20 observations

Fig. 1 Mature spores of giemsa stained *Myxobolus catmrigalae* (bar 1 μm). Note that there is no iodophilous vacuole. *MS* Mature spore, *FC* polar filament coiling

slightly towards the anterior end (Fig. 1). One polar capsule was slightly larger [11.30 ± 1.02 (9.90–13.20) $\mu\text{m} \times 3.00 \pm 0.22$ (2.70–3.40) μm] than the other [10.60 ± 0.98 (9.20–12.40) $\mu\text{m} \times 2.80 \pm 0.16$ (2.50–3.20) μm]. Polar filaments formed 20–24 coils in the larger and 19–22 coils in the smaller polar capsules, respectively (Fig. 2). The polar filaments were coiled perpendicularly to the axis of the capsules in both cases. The sporoplasm contained two large sporogenic nuclei of size $0.57 \pm 0.09 \mu\text{m}$. No glycogen containing iodophilous vacuole in the sporoplasm and mucus envelope around the spore was found (Fig. 2). The spores were also devoid of any intercapsular ridge or parietal folds. The spore length

Fig. 2 Mature spores of giemsa stained *Myxobolus catmrigalae* (bar 1 μm). *LPC* Larger polar capsule, *SPC* smaller polar capsule

(LS) and spore width (WS) ratio of the *Myxobolus* was 1:0.413 (Table 1). On the basis of the morphometric measurements and their comparison with previous descriptions (Kaur and Singh 2012), the *Myxobolus* infecting the gill lamellae of *C. mrigala* was identified as *M. catmrigalae*. The SEM study of the mature spores of *M. catmrigalae* revealed that spore surface was smooth and

Fig. 3 Scanning electron micrograph of *Myxobolus catmrigalae* mature spores (bar 10 μm). PC Polar capsule, PFP polar filament pore

Fig. 4 Scanning electron micrograph of *Myxobolus catmrigalae* mature spores (bar 10 μm). SL Suture line, SV smooth valve

devoid of mucous (Fig. 3). The sutural line, formed by the fused thick edges of each valve, was slightly curved (Fig. 4). A slit like aperture was found above the lower middle part of the sutural line.

Molecular comparison

The universal primer sets UEP-F and UEP-R successfully amplified approximately 2048 bp fragments of the 18S rRNA gene from *M. catmrigalae* (Fig. 5). The edited nucleotide sequence was 1000 pb, which was deposited in GenBank under the accession number KC933944. It revealed 77–87 % sequence homology between the 18S rRNA gene of *M. catmrigalae* and most of the gill infecting *Myxobolus* spp. (Table 2). The DNA sequence of the morphologically identified *M. catmrigalae* infecting the gill lamellae of *C. mrigala* was clustered phylogenetically with *M. pavlovskii* (HM991164) infecting the gill lamellae of silver carp, *Hypophthalmichthys molitrix* from Hungary

Fig. 5 Agarose gel (1.5 %) showing 18S rRNA gene amplification of *Myxobolus catmrigalae* from the gill lamellae of *Cirrhinus mrigala*. Lane M: 1 kb DNA Ladder (Takara Bio Inc., Japan); Lane 1: *Myxobolus catmrigalae*

(Fig. 6). Among the 18S rRNA gene sequence of selected gill infecting *Myxobolus* spp. from GenBank, the species most closely related to *M. catmrigalae* was another *M. pavlovskii* (AF507973) infecting the gill lamellae of big head carp, *Aristichthys nobilis* with 87 % homogeneity. Among the selected gill infecting *Myxobolus* spp., *M. longisporus* (AY364637) infecting the gill lamellae of *Cyprinus carpio haematopterus* from China exhibited the least (77 %) homogeneity with *M. catmrigalae* (Table 2). The out-group species *C. shasta* (AF001579) infecting the intestinal tissue of rainbow trout (*O. mykiss*) was found to be very different and not clustered with gill infecting *Myxobolus* spp. (Figure 6).

Discussion

Gill myxosporeans are common on aquacultured and wild fish of India and vast array of literatures are available on the taxonomy and host ranges (Basu and Halder 2004; Bandyopadhyay et al. 2006; Basu et al. 2009; Kaur and Singh 2012; Singh and Kaur 2012). Spore morphology has been used as a basic feature to identify myxosporean species. Myxosporeans are characterized as host, organ and tissue specific organisms (Molnár 1994), who opined that host and infection site are important characters for specific assignment. On the basis of morphometric data, *Myxobolus* species of the present study bear close resemblance with

Table 2 Homogeneity of 18S rRNA gene sequences of selected myxosporean species available in GenBank and *Myxobolus catmrigalae* (Accession number KC933944)

Species	Host fish	Site of infection (G: gill; F: fin; M: muscle; I: intestine)	Accession number	Country	DNA sequence homogeneity (%) to <i>Myxobolus catmrigalae</i> (KC933944)
<i>Myxobolus pavlovskii</i>	<i>Aristichthys nobilis</i>	G, Interlamellar/epithelium	AF507973	Hungary	1054/1054 (87)
<i>Myxobolus pavlovskii</i>	<i>Hypophthalmichthys molitrix</i>	G, Interlamellar/epithelium	HM991164	Hungary	1104/1104 (86)
<i>Myxobolus macrocapsularis</i>	<i>Abramis brama</i>	G, Intralamellar/vascular	AF507969	Hungary	1012/1012 (86)
<i>Myxobolus dispar</i>	<i>Cyprinus carpio</i>	G, Intralamellar/vascular	AF507972	Hungary	1045/1045 (86)
<i>Myxobolus bramae</i>	<i>Abramis brama</i>	G, Intralamellar/vascular	AF507968	Hungary	1029/1029 (86)
<i>Myxobolus impressus</i>	<i>Abramis brama</i>	G, Interlamellar/epithelium	AF507970	Hungary	1021/1021 (86)
<i>Myxobolus bliccae</i>	<i>Vimba vimba</i>	G, Intrafilamental/vascular	HM138772	Hungary	930/930 (86)
<i>Myxobolus muelleri</i>	<i>Leuciscus cephalus</i>	G, Intralamellar/vascular	AY325284	Hungary	1000/1000 (85)
<i>Myxobolus basilamellaris</i>	<i>Cyprinus carpio</i>	G, Basifilamental type	AF507971	Hungary	1009/1009 (85)
<i>Myxobolus leuciscini</i>	<i>Leuciscus cephalus</i>	G, Intrafilamental/vascular	DQ439811	Hungary	982/982 (85)
<i>Myxobolus margitae</i>	<i>Alburnus alburnus</i>	G, Intrafilamental/vascular	EU598803	Hungary	883/883 (84)
<i>Myxobolus bilobus</i>	<i>Notemigonus crysoleucas</i>	G, Interfilamental/epithelium	DQ008579	Canada	729/729 (79)
<i>Myxobolus intimus</i>	<i>Leuciscus idus</i>	G, Intralamellar/vascular	JX390690	Hungary	740/740 (79)
<i>Myxobolus</i> sp.	<i>Leuciscus idus</i>	G, Intralamellar/vascular	FJ716098	Hungary	731/731 (79)
<i>Myxobolus intimus</i>	<i>Leuciscus idus</i>	G, Intralamellar/vascular	JX390691	Hungary	737/737 (79)
<i>Myxobolus</i> sp.	<i>Aspius aspius</i>	G, Intralamellar/vascular	FJ716097	Hungary	720/720 (79)
<i>Myxobolus</i> sp.	<i>Leuciscus idus</i>	G, Interfilamental/epithelium	FJ716096	Hungary	715/715 (79)
<i>Myxobolus tambroides</i>	<i>Tor tramboides</i>	G, Intralamellar/vascular	JX028236	Malaysia	682/682 (78)
<i>Myxobolus longisporus</i>	<i>Cyprinus carpio haematopterus</i>	G, Interlamellar/epithelium	AY364637	China	717/717 (77)
<i>Myxobolus musculi</i>	<i>Barbus barbus</i>	M, Intramuscular	AF380141	Hungary	580/685 (84)
<i>Myxobolus cyprinid</i>	<i>Cyprinus carpio</i>	M, Intramuscular	AF380140	Hungary	572/699 (84)
<i>Myxobolus pseudodispar</i>	<i>Rutilus rutilus</i>	M, Intramuscular	AF380145	Hungary	565/586 (78)
<i>Henneguya gurlei</i>	<i>Ameiurus nebulosus</i>	F, Caudal fin	DQ673465	USA	405/514 (79)
<i>Henneguya exilis</i>	<i>Ictalurus punctatus</i>	G, Intralamellar and Interlamellar	AF021881	USA	425/534 (78)
<i>Henneguya ictaluri</i>	<i>Ictalurus punctatus</i>	G, Interlamellar-epithelial	AF195510	USA	430/451 (73)
<i>Thelohanellus nikolskii</i>	<i>Cyprinus carpio</i>	F, Caudal fin	DQ231156	Hungary	902/902 (83)
<i>Ceratomyxa shasta</i>	<i>Oncorhynchus mykiss</i>	I, Intestinal tissue	AF001579	USA	Out group

that of *M. catmrigalae*, a species described by Basu and Haldar (2004) from a hybrid carp *C. catla* Ham. × *C. mrigala* Ham. in West Bengal, India. In both cases, *M. catmrigalae* infected the gill lamellae, exhibited tissue and host specificity but differed slightly in its spore size. The minor discrepancies in morphometric data and prevalence of myxosporeans are influenced by several factors perhaps by differences in location, ecology, ecological condition and age of the fish (Moshu and Molnár 1997). They recorded spores of larger size more frequently in older fish than from fingerlings. Nevertheless, as shown in Table 1, the spore length (LS) and spore width (WS) ratio (1:0.413) as well as the polar capsules length (LLPC and LSPC) and polar capsules width (WLPC and WSPC) ratios (1:0.265 and 1:0.264) are in conformity with original descriptions of *M. catmrigalae*, LS:WS = 1:0.314; LLPC:WLPC = 1:0.210; and LSPC:WSPC = 1:0.209 (Basu and Haldar

2004). Several gill infecting *Myxobolus* spp. with unequal polar capsules other than *M. catmrigalae* are known from Indian cyprinid and non-cyprinid fishes (Kaur and Singh 2012). Comparison of the morphometric data of *M. catmrigalae* of the present study with representatives of *Myxobolus* spp. infecting the gills of cyprinids and non-cyprinids (Kaur and Singh 2012) revealed that it differed from all of them by morphometric characters and spore morphology. These observations, thus, confirm that the *Myxobolus* species found on the gill lamellae of *C. mrigala* corresponds to *M. catmrigalae* in its morphology, host specificity and tissue tropism. Perhaps the slight differences observed in size and shapes are within the limits of natural variations typical of population or species.

The sequence of the amplified 18S rRNA gene of *M. catmrigalae* isolated from the gill lamellae of *C. mrigala* was compared with those of other gill infecting *Myxobolus*

Fig. 6 Phylogenetic tree generated by maximum parsimony of the 18S rRNA gene sequences of *Myxobolus* spp. infecting the gill, fin and muscle of cyprinids with *Myxobolus catmrigalae*/KC933944/India and related taxa. Bootstrap confidence values are shown at nodes (1000 replications); G, gill; M, muscle; CF, caudal fin; C, *Ceratomyxa*; H, *Henneguya*; M, *Myxobolus*; T, *Thelohanellus*

spp. sequences available in GenBank. Phylogenetic cluster was established on the basis of edited alignment, which was 1000 bp in length. The similarity data of DNA sequences indicated that the myxosporeans originating from different fish species are closely related to each other (77–87 %), and suggested that these myxosporeans could represent populations of the same species. The 18S rRNA gene sequence of *M. catmrigalae* was clustered phylogenetically with other cyprinids gill infecting *Myxobolus* species. The overall topology of the phylogenetic analyses (Fig. 6) corresponded well with the *Myxobolidae* clade described by Fiala (2006). The sequences of muscle infecting *M. musculi*, *M. cyprini* and *M. pseudodispar*, fin infecting *Thelohanellus nikolskii* of cyprinids, and gill and fin infecting *Henneguya* spp. of non-cyprinids were also clustered with *M. catmrigalae*, but with a low bootstrap value. The phylogeny of *C. shasta* (AF001579) infecting the intestinal tissue of salmonid was found to be very different from those of gill infecting *Myxobolus* spp. It is important to mention here that no detailed information is currently available regarding the life cycle and pathogenicity of *M. catmrigalae* except the prevalence rate

of 65.70 % on the gill lamellae of catla–mrigal hybrid carp (Basu and Haldar 2004). On the other hand, the present study recorded a myxosporean prevalence rate of 85.71 %, i.e., 18 out of 21 numbers of *C. mrigala* in January. The 18S rRNA gene sequence of *M. catmrigalae* phylogenetically clustered with *M. pavlovskii* (HM991164). *Myxobolus pavlovskii* was found to infect the gills and develop in the stratified epithelium between gill lamellae, where the developing plasmodia can fill the interlamellar space (Molnár 2002) and cause pathological changes (Marton and Eszterbauer 2011). Histologically, a marked hypertrophy at the secondary lamellar tip of infected *C. mrigala* gill was observed (data not shown) in the present study, indicating the pathogenic potential of *M. catmrigalae*. The evolutionary tree revealed by this study demonstrated that tissue tropism may play an important role in genetic relationships among myxozoan species. According to Eszterbauer (2004) tissue specificity is an important factor in myxozoan speciation. It is important to mention here that all of the available DNA gene sequences of *Myxobolus* spp. in the GenBank are mostly from Hungary, Germany, Czech Republic, Portugal, Norway, Greenland, Italy, UK, China,

South Korea, Japan, Thailand, Malaysia, Canada, USA, Brazil and Tunisia, and this study is the first to report the molecular and phylogenetic characterization of gill infecting *Myxobolus* sp. particularly *M. catmrigalae* from Indian subcontinent.

The need for a more accurate diagnosis for myxozoa has increased recently because of insufficient morphological characteristics of many *Myxobolus* species. Kent et al. (2001) recommended that SSU rRNA gene sequence data should be provided when describing a new species. As the list of Indian species of the genus *Myxobolus* has increased over the years (Basu and Haldar 2004; Bandyopadhyay et al. 2006; Singh and Kaur 2012), the future studies on Indian *Myxobolus* species and other myxosporean group must focus the morphological, molecular and phylogenetic characterization together with host, organ and tissue specificity of the given myxosporea for the correct identification of species. Molecular characterizations of Indian myxosporeans of fish are currently in progress in our laboratory, which would throw more lights on their phylogenetic relationships, geographical distribution, genetic diversity in different ecological niches, host susceptibility to infections and correlation between hosts and clinical manifestations besides molecular taxonomy.

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