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Review Article

Molecular tools for identification and classification of Myxozoan parasites (Cnidaria: Myxosporea) in India: Current status

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Abstract
A substantial literature on myxosporea has been published to date using morphological characters and specificity of host tissue. Currently, there are some 2400 nominal species of myxosporea classified to 17 families and 64 genera. Approximately 300 species have been described from India and most of them have been described solely on the basis of morphological characteristics. Molecular markers like small subunit ribosomal (SSU) and large subunit ribosomal (LSU) DNA have been used worldwide for the identification and validation of fish myxosporeans. Maximum likelihood phylogenetic tree based on SSU rDNA sequences was used to study the phylogenetic relationship among myxosporeans infecting Indian fishes. Myxospore phylogenies disagree with traditional spore-based classification systems, probably due to extreme plasticity in myxospores morphologies that have resulted in extensive convergence. Morphological similarities exist among myxosporea that encounter several problems in categorizing them. That's why present-day research has shifted to molecular techniques for identification and correct systematics of myxosporeans. Molecular studies of myxozoans in India are still scarce and in infancy. To address persisting taxonomic and phylogenetic discrepancies, validation of these species by molecular tools is needed, because earlier species were reported only on the basis of morphological data. Therefore, the present study has summarized existing molecular data and current status of molecular taxonomy of myxosporeans parasitizing fresh and marine water fishes of India along with the approaches of myxozoan phylogenetics and information about the molecular markers, their interpretation in the identification of myxozoans parasitizing fishes.

Keywords: Myxosporea, Systematics, Fish parasites, Molecular marker, Phylogenetic analysis, Validation

INTRODUCTION

Myxosporea representing about 20% of described cnidarian species evolved as endoparasites and are radiated into a diverse range of morphologically different parasitic species (Fiala *et al.,* 2015). Myxosporean parasites are well known for infecting fishes, both freshwater as well as marine. For the first time in freshwater fish, myxozoan cysts were reported in the musculature of *Coregonus fera* from a lake in Geneva by Jurine in 1925, while in India the work on myxozoan was initiated by Bosanquet in 1910 (Abdel-Gaber *et al.,* 2017). A number of myxosporean species have also been described from annelids, amphibians, reptiles and birds.

More than 2400 species belonging to 64 genera have been reported worldwide (Lom and Dykova, 2006). Studies on myxosporean parasites of fishes in India have shown that among different genera, only *Myxobolus, Henneguya* and *Thelohanellus* have been frequently described (Kalavati and Nandi, 2007; Kaur and Singh, 2012; Singh and Kaur, 2012; Kaur, 2014). Approximately 300 species of myxobolids have been reported till date from India (Kaur and Singh, 2012). Myxobolids fauna has been investigated by a limited number of researchers in India (Gupta *et al.,* 2018; Chaudhary *et al.,* 2019). Many of the reported species have been identified and described solely on the basis of spore morphology. The end of the 20th century laid the foundation of the molecular era, and the controversies between the traditional spore-based taxonomy of myxosporeans and the rDNA-based phylogenies started.

It is true that myxospore morphology remains important, as it is the fundamental diagnostic feature for identification, but great discrepancies were found between the phylogenetic relationships of myxospores inferred from spore-based myxobolids taxonomy (Fiala and Bartosova, 2010). Morphological tools have failed to identify congeneric species, like those found in same infection site in different fishes with only minor differences in their morphology and structures of spores (Ye *et al.,* 2017). These problems have been resolved with the help of molecular studies (Smothers *et al.,* 1994). Smothers *et al.* (1994) used first time ribosomal DNA (rDNA) sequence to explore the phylogenetic position of the Myxozoa within Metazoa. After that, the use of molecular data started to explain the phylogenetic relationships within myxozoans. Different molecular markers, particularly small subunit ribosomal DNA (SSU rDNA), have been used most frequently to identify and study phylogeny of myxosporean parasites of fishes worldwide. Gradual enrichment of rDNA data on myxosporean species brought about the elimination of some genera and families. At present, a consolidated methodology consisting of spore morphology, site of sporulation, tissue as well as host explicitness and molecular characteristics has been generally suggested and acknowledged for taxonomic evaluation of myxosporean species and for segregation of species with morphological resemblance (Holzer *et al.,* 2004).

Molecular characterization has been broadly accepted as a significant criterion for taxonomy and discrimination of congeneric myxosporean species (Bartosova *et al.,* 2013). There are certain ambiguities in the systematics of these parasites that need to be resolved for future taxonomists working in this field. Therefore, the aim of the present review is to summarize existing molecular data of myxosporea parasitizing the fishes of India.

USE OF MOLECULAR MARKERS IN MYXOZOAN SYSTEMATICS

In cnidarian evolution, myxosporea derived from a common ancestor and exhibited an ancient cnidarian lineage (Kent *et al.,* 2001). Conservative phylogenetic markers, like small and large subunit ribosomal DNA gene, are required to reconstruct myxosporean diversification. Nowadays, SSU rDNA sequences are most important in the systematics of myxosporea and have been used frequently to understand their phylogeny (Fiala, 2006; Evans *et al.,* 2010; Zhao *et al.,* 2013; Naldoni *et al.,* 2018), due to heterogeneous nature of its conserved as well as variable regions that facilitate accurate differentiation of myxosporean parasites at different taxon levels. The conserved regions of the molecular marker are helpful in DNA sequences multiple alignments and also facilitate the development of primers for amplification purpose while variable regions give information about the diversification process. Studies proved that this gene evolved very rapidly because SSU rDNA molecular marker of myxosporean fauna shows high substitution rates in their variable regions (Saulnier *et al.,* 1999; Hartikainen *et al.,* 2014).

Large subunit ribosomal DNA (LSU rDNA) sequences have been used for myxozoan phylogenetics due to their conserved and variable regions. LSU rDNA molecular marker has also been used in congruence with SSU rDNA based phylogenetic analysis and sometimes used in combination for particular inferences (Whipps and Kent, 2006). According to several workers (Fiala 2006; Bartošová *et al.,* 2009; Fiala and Bartosova, 2010), the LSU rDNA sequences are more reliable than SSU rDNA for myxobolids phylogeny with which we also agree. However, myxosporean SSU rDNA sequences are more in numbers than LSU rDNA in National Centre of Biotechnology Information (NCBI) database, being the first choice of marker for those working in the analysis of myxozoan phylogenetics. At intraspecies level, the internal transcribed spacer region 1 (ITS 1) is found to be more reliable than SSU rDNA to find the similarity or dissimilarity and relatedness between congeneric myxosporean species (Andree *et al.,* 1999). Phylogenetic analysis of myxosporean species on the basis of elongation factor (EF2) genes was also found reliable with those based on ribosomal DNA (Fiala and Bartosova, 2010). Molecular markers such as HSP70, mitochondrial gene 12S, COX -1 and some protein-coding genes like rad51 and rpl23a have also been used for molecular identification of myxosporean parasites (Whipps *et al.,* 2004).

Molecular data of Myxozoan parasites from India

According to Kalavati and Nandi (2007), approximately 300 species have been described from the Indian subcontinent and most of them have been described solely on the basis of morphological characteristics. Myx-

obolids parasites possess several morphological features that can be used as a distinguishing feature among them, but several genera demonstrate similar morphological characteristics. Therefore, in myxobolids taxonomy, the molecular analysis should be in congruence with morphological characteristics that can verify and offer great independent evidence relevant for phylogenetic based systematics. Several studies show that phylogenetic analysis with some molecular markers more reliable and consistent over morphological taxonomic features (Palenzuela *et al.,* 2002; Gupta *et al.,* 2018; Kosakyan *et al.,* 2019). In India, identification of myxosporean parasites through molecular tools is at its initial stage as several workers have reported these parasites using morphological classical taxonomic tools. GeneBank database of NCBI for Indian myxobolids molecular data comprises of only 66 sequences of 18S rDNA belonging to 40 species of myxobolids (Table 1).

Out of total 66 sequences submitted to NCBI, 32 sequences belong to genus *Myxobolus,* followed by genus *Thelohanellus* with 23 sequences, 04 sequences belonging to *Ceratomyxa,* 03 sequences belonging to *Henneguya*, 02 sequences belonging to *Chloromyxum* and one each from genus *Ortholinea* and *Zschokkella* respectively. In India, studies on these groups of cnidarian parasites on the basis of molecular taxonomy has been done by a very limited number of researchers mainly from West Bengal, Uttar Pradesh, Kerala and Punjab (Singh and Kaur, 2012; Kaur, 2014; Rajesh *et al.,* 2014; Abraham *et al.,* 2015; Szekely *et al.,* 2015). Due to very few rDNA sequences of Indian myxosporean, it puts a question mark on the taxonomy of these parasites in India. Szekely *et al.* (2015) analyzed 18S rDNA gene sequences of 05 different *Myxobolus* species infecting carp fishes from Meerut, Uttar Pradesh. Chaudhary *et al.* (2017, 2018, 2019) reported 04 myxozoan species from catfishes belonging to genus *Henneguya* and *Myxobolus* based on molecular taxonomy. Similarly from Meerut, (Uttar Pradesh) Gupta *et al.* (2018) also analyzed three isolates of *Thelohanellus wallagoi* infecting different organs of freshwater fish *Wallago attu* viz; gills, kidney and intestine, and found that all the sequences were similar to each other. All these researchers validated the status of the reported parasites after many years of its original description. Kaur and Attri (2015), Kaur and Gupta (2017a, b), Gupta and Kaur (2017) and Kaur *et al.* (2014) investigated molecular characterization of 04 species of *Thelohanellus* and 01 species of *Henneguya* based on 18S rDNA gene in Punjab. Abraham *et al.* (2015) from West Bengal and Banerjee *et al.* (2015a, b, 2017) analyzed 18S rDNA gene sequences of 04 myxozoan species belonging to genus *Myxobolus* and *Thelohanellus*. Sanil *et al.* (2017) and Chandran *et al.* (2018, 2020) from Kerala analyzed 18S rDNA gene sequences of 03 myxozoan species belonging to genus *Chloromyxum* and *Ortholinea* (Table 1).

Myxosporea systematics demands, extensive work on molecular taxonomy of these parasites. Revisions of myxobolids taxonomy are also needed in accordance with existing paraphylectic and polyphylectic genera (Anjan *et al.,* 2014; Abidi *et al.,* 2015; Fariya *et al.,* 2020). Molecular markers like SSU and LSU have a tremendous role in the systematics of myxobolids parasites because of many congeneric species' presence, having highest morphological similarities.

Phylogenetic analysis of Myxozoans sequenced from India

The phylogenetic analysis based on SSU rDNA sequences conducted with MEGA X (Kumar *et al.,* 2016) in the present study revealed discrepancies between spore-based classification system and phylogeny-based schemes. The phylogenetic analysis supported a split of myxosporeans into two main lineages separating freshwater species (freshwater clade) and marine species (marine clade) (Fig. 1). The freshwater clade split into two distinct lineages (*Myxobolus* clade and *Thelohanellus* clade). Marine clade consisted of *Ceratomyxa* spp. Six of seven genera studied (*Myxobolus, Henneguya, Thelohanellus, Chloromyxum, Ortholinea, Zschokkella*) are paraphyletic. *Ceratomyxa* is the only monophyletic genera and molecular phylogeny based on SSU data of such groups belongs to myxosporea showed consistency with traditional, morphology-based classification. Representatives of genus *Myxobolus* were intermixed with morphologically dissimilar *Henneguya* spp. The species of genus *Henneguya* probably arose from *Myxobolus* ancestors several times in myxosporean evolution. The caudal appendages do not represent a valid character for the distinction of genera *Myxobolus* and *Henneguya.* The freshwater clade is composed of myxosporean infecting freshwater fishes but includes two exceptional species, *Chloromyxum argusi* and *Ortholinea scatophagi* from *Scatophagus argus*. Both species are found to infect coastal fish in a marine environment that clusters with freshwater myxosporeans. It seems that the common ancestor of marine *Chloromyxum* spp. and *Ortholinea* spp. was the freshwater myxosporean. The position of such myxosporean species from coastal fish among the clade of freshwater species seems logical since hosts of these species often enter estuaries and rivers. It is unexpected the phylogenetic position of *Zschokkella auritis* within *Thelohanellus* clade clustered with *Myxobolous cuttacki* was supported with low bootstrap value. These *Zschokkella* spp. may be sister taxa to *Myxobolus* spp. or they may be *Myxobolus* species.

This study suggested that morphological characters are significant for the taxonomy of myxosporea as the

Table 1. List of Indian species of Myxosporean parasites with their host, location and accession numbers.

Myxosporean species	Authors and Year	Host	Location	Accession number
Myxobolus bengalensis	Abraham *et al.* 2014*	*Catla catla*	West Bengal	KJ476883, MK412934
Myxobolus rocatlae	Abraham *et al.* 2014*	*Catla catla*	West Bengal	KJ476878
Myxobolus ticto	Abraham *et al.* 2014*	*Puntius ticto*	West Bengal	KJ476887
Thelohanellus catlae	Abraham *et al.* 2014*	*Catla catla*	West Bengal	KJ476881, KJ476882
Thelohanellus rohita	Abraham *et al.* 2014*	*Labeo rohita*	West Bengal	KF170927, KF170926 MK412940
Thelohanellus habibpuri	Abraham *et al.* 2014*	*Labeo rohita*	West Bengal	KM252683, KM252681
Thelohanellus jiroveci	Abraham *et al.* 2014*	*Labeo rohita*	West Bengal	KJ476885
Thelohanellus seni	Abraham *et al.* 2014*	*Labeo rohita*	West Bengal	KJ476884
Myxobolus cuttacki	Rajesh *et al.* 2014	*Labeo bata*	West Bengal	KF465682
Thelohanellus filli	Kaur *et al.* 2014	*Labeo rohita*	Punjab	KR340464
Thelohanellus caudatus	Anjan *et al.* 2014	*Labeo rohita*	West Bengal	KJ476877, MK412941
Myxobolus auratus	Banerjee *et al.* 2015*	*Carassius auratus*	West Bengal	KX399851
Henneguya bicaudi	Kaur and Attri 2015	*Cirrhinus mrigal*	Punjab	KP099967
Myxobolus arcticus	Abidi *et al.* 2015	*Clarius batachus*	Uttar Pradesh	KF662475
Myxobolus basuhaldari	Szekely *et al.* 2015	*Catla catla/Labeo rohita*	Uttar Pradesh	KM029976, KM029974, KM029975
Myxobolus bhadrensis	Szekely *et al.* 2015	*Labeo rohita / Catla catla*	Uttar Pradesh	KM029970, KM029971, KM029968, KM029969, KM029972
Thelohanellus qadrii	Banerjee *et al.* 2015a	*Amblypharyngodon mola*	West Bengal	KF170928, MK412939
Myxobolus carnaticus	Banerjee *et al.* 2015b	*Cirrhinus mrigala*	West Bengal	KF796620
Myxobolus catlae	Szekely *et al.* 2015	*Cirrhinus cirrhosis*	Uttar Pradesh	KM029967, KR819276
Myxobolus cerebralis	Abidi *et al.* 2015	*Cyprinus carpio*	Uttar Pradesh	KM671790, KJ701267
Myxobolus kalavatiae	Szekely *et al.* 2015	*Cirrhinus cirrhosis*	Uttar Pradesh	KM029973
Myxobolus meerutensis	Szekely *et al.* 2015	*Labeo rohita*	Uttar Pradesh	KM029977
Myxobolus orissae	Abraham *et al.* 2015	*Cirrhinus mrigala*	West Bengal	KF448527
Myxobolus catli	Abraham *et al.* 2016*	*Catla catla*	West Bengal	KR819272
Myxobolus mrigalhitae	Abraham *et al.* 2016*	*Catla catla*	West Bengal	KJ476880, KJ476879 KR819275
Thelohanellus andhrae	Abraham *et al.* 2016*	*Labeo rohita*	West Bengal	KR819270
Thelohanellus bifurcate	Abraham *et al.* 2016*	*Labeo rohita*	West Bengal	KJ476886, KR819274
Ceratomyxa collarae	Sanil *et al.* 2017	*Chaetodon collare*	Kerala	KU726595, KU726593, KU726592
Ceratomyxa leucoster-noni	Sanil *et al.* 2017	*Acanthurus leu-costernon*	Kerala	KU726596
Henneguya chaudhuryi	Chaudhary *et al.* 2017	*Channa punctata*	Uttar Pradesh	KT279402
Myxobolus catmrigalae	Banerjee *et al.* 2017	*Cirhinus mrigal*	West Bengal	KC933944
Thelohanellus boggoti	Kaur and Gupta 2017a	*Labeo dero*	Punjab	KU884967
Thelohanellus muscularis	Kaur and Gupta 2017b	*Catla catla*	Punjab	KT387308
Thelohanellus theinensis	Gupta and Kaur 2017	*Labeo bata*	Punjab	KP792568
Zschokkella auratis	Paul *et al.* 2017*	*Channa striata*	Odisha	MF978273
Chloromyxum argusi	Chandran *et al.* 2018	*Scatophagus argus*	Kerala	MG029441, MG029440
Myxobolus ompok	Chaudhary *et al.* 2018	*Ompok pabda*	Uttar Pradesh	MG760574, MG760575
Thelohanellus wallagoi	Gupta *et al.* 2018	*Wallago attu*	Uttar Pradesh	MG099779, MG099781, MG099782
Henneguya mystasi	Chaudhary *et al.* 2019	*Mystus vittatus*	Uttar Pradesh	MH300136
Myxobolus cylindricus	Chaudhary *et al.* 2019	*Channa gachua*	Uttar Pradesh	MH424126
Myxobolus toyamai	Fariya *et al.* 2020	*Cyprinus carpio*	Uttar Pradesh	MG800830
Ortholinea scatophagi	Chandran *et al.* 2020	*Scatophagus argus*	Kerala	MN310514

*Shows as unpublished sequences on NCBI.

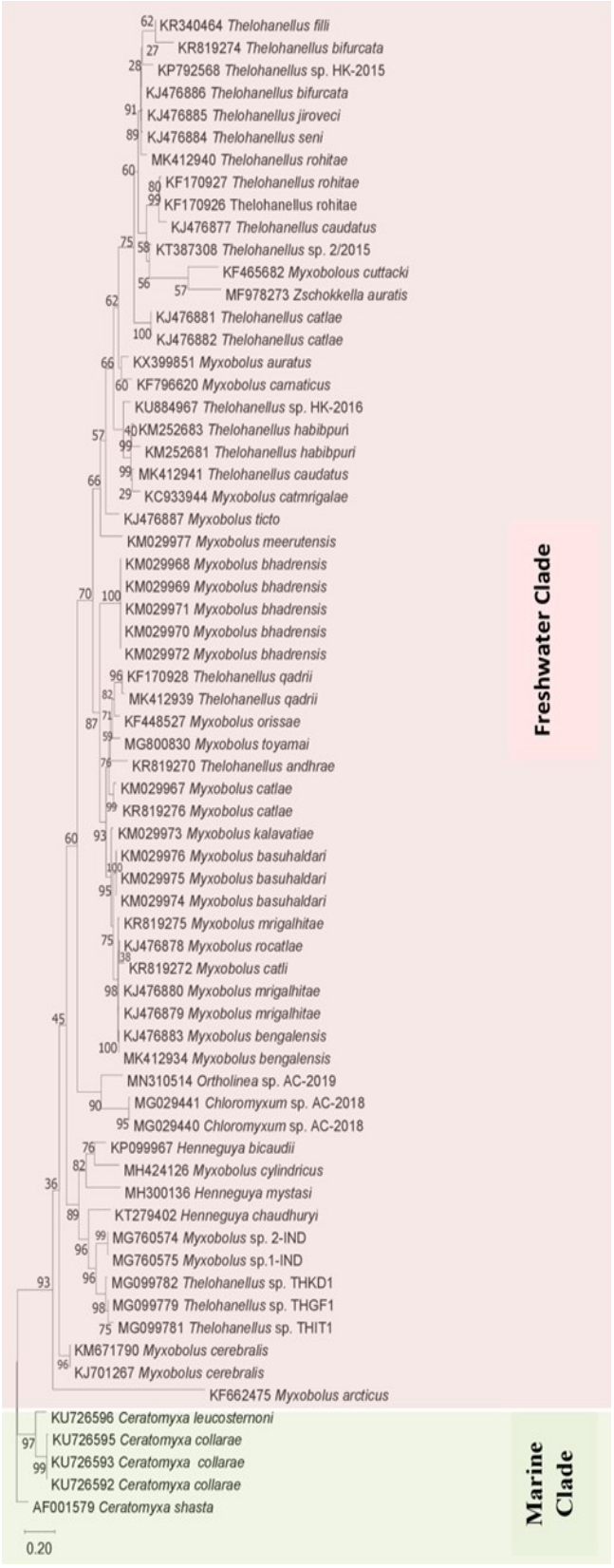

Fig. 1. *Maximum likelihood phylogenetic tree based on SSU rDNA sequences of all myxosporean species available in NCBI database from India. Ceratomyxa shasta is as outgroup species. Numbers at nodes indicate ML bootstrap values (1000 replications). The scale bar indicates the number of substitution per site.*

clades showed the affinity to tissue tropism and present findings corroborate with previous studies that tissue tropism is an important factor that should be considered for myxozoan phylogenetic studies (Molnár, 1994). Several studies were conducted on the basis of phylogenetic analysis and many revisions of myxozoan taxonomy were done (Smothers *et al.,* 1994; Palenzuela *et al.,* 2002; Whipps *et al.,* 2004; Jirku *et al.,* 2007; Heiniger *et al.,* 2011). Molecular data have largely driven and showed incremental improvement of myxozoan classification system. As the sequencing become more common, resulting more sequences available, more revisions can be proposed with the help of more precise studies of phylogenies of myxozoan. The genus *Myxobolus* is paraphyletic and difficult to distinguish from *Zschokkella*, in such conditions molecular data along with the distinctive morphological characters might be used as guiding tool. The polyphyly of *Choloromyxum* is now well recognized and several clarifications have been made between this and other genera using molecular phylogenies reassigning other species too and from this group (Gunter and Adlard, 2010). Therefore, it is demonstrated that molecular data are likely to be necessary for myxozoan classification and many species descriptions should be obtained for discriminating or describing myxozoans related to these problematic taxa. Regardless of the increasing number of available SSU rDNA sequences, the proportion of sequenced species is low and many species are still unknown to science. The finding of the present study does not correspond between SSU rDNA phylogeny and current taxonomy. The present study revealed paraphyletic and polyphyletic genera within myxosporeans. Other genes should also be incorporated whether SSU rDNA phylogeny corresponds to organismal phylogeny. The combined analysis of appropriate genes could also increase the resolution of myxosporean phylogeny.

Conclusion

Parasites substantially play fundamental ecological and evolutionary roles and also contribute to biodiversity. Existing knowledge on the myxosporean on molecular taxonomy is incomplete in India because most of the taxa have been described on the basis of traditional spore-based taxonomy. Molecular data helps in testing the morphological results and provide independent evidence relevant for a classification system based on phylogenetic analysis. Careful consideration is needed in selecting more useful molecular marker for the systematics of myxosporeans. The increasing number of rDNA data on myxosporean species demonstrated the existence of conflicting signals within the phylogenomic dataset. The use of molecular studies in addition to

morphological characters, will be very useful for subsequent workers to understand the gaps in myxozoan diversity and their taxonomy. Additional taxon sequencing is needed for certain genera e,g, *Zschokkella, Chloromyxum, Ortholinea* from Indian subcontinent for which few of species have been sequenced. Therefore, re-investigation of the traditional spore-based myxozoan taxonomy with the help of markers like SSU and LSU rDNA to address persisting taxonomic and phylogenetic discrepancies of this highly divergent taxa and also to facilitate the development of novel strategies to effectively prevent and control disease outbreaks and their economic impacts.

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Conflict of interest

The authors declare that they have no conflict of interest.

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