

Remarks on the validity of *Myxobolus ampullicapsulatus* and *Myxobolus honghuensis* (Myxozoa: Myxosporea) based on SSU rDNA sequences

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Abstract In the present study, we isolated three populations of *Myxobolus ampullicapsulatus* from the gills of crucian carp, *Carassius auratus auratus*, two from Yongchuan, Chongqing area and one from Poyang Lake, Jiangxi area, China, sequenced their complete small subunit ribosome RNA gene, analyzed their genetic distance and gene similarity, and explored their relationship based on Bayesian inference and maximum likelihood analyses of their small subunit ribosomal DNA. The results combined with their morphological characteristics suggest that *M. ampullicapsulatus* infecting the gills and pharynx of allogynogenetic gibel carp, *Carassius auratus gibelio*, should be *Myxobolus honghuensis*. This study highlights the importance of DNA sequence comparisons for distinguishing *Myxobolus* species and indicates that the intra-species identification for the two *Myxobolus* species mentioned in the present research should be less than ten variation sites. In morphology, *M. honghuensis* Liu et al. (2012) parasitic on the gills of *C. auratus auratus* (goldfish) was collected from Chongqing area, and its mature spore was 16.5–19.5 × 8.5–10.0 μm in size, polar capsule was 7.0–10.0 × 2.5–4.0 μm in size, and polar filament had 9–10 coils. *M. honghuensis* Liu et al. (2012) isolated from the pharynx of *C. auratus gibelio* was sampled in Hubei area, and its mature spore was 15.1–19.5 × 9.0–11.3 μm in size, polar capsule was 7.9–8.1 × 3.0–4.5 μm in size, and polar filament had 7–8 coils.

Introduction

Myxobolus Bütschli, 1882 is a genus with the richest species in myxozoan. It has been reported to have more than 850 species (Eiras et al. 2005; Lom and Dyková 2006), most of which produce morphologically similar or identical spores from genetically related hosts. Therefore, its species identification merely based on their morphological characteristics is difficult and much more confusions will be caused. Fortunately, with the development of molecular biology, more and more new technologies have been widely applied in the classification and identification of *Myxobolus* species harbored from genetically closely related hosts and infection sites (Bartošová et al. 2009; Fiala 2006; Kent et al. 2001; Khelifa et al. 2012; Morsy et al. 2012; Whipps et al. 2004a, b; Zhao et al. 2008). To avoid mis-identification of myxosporean species, researches have recently suggested that morphology, host or organ specificity, tissue tropism, and molecular data should all be taken into consideration (Bartošová et al. 2009; Cone and Overstreet 1998; Dyková and Lom 2007; Lom and Dyková 2006; Molnár 1994; Morsy et al. 2012; Zhao et al. 2008).

Since Zhao et al. (2008) originally found *Myxobolus ampullicapsulatus* without causing any disease from the gills of *Carassius auratus* in Chongqing, China, described it in detail, and obtained its small subunit ribosomal DNA sequence, some researchers have studied this species and the related taxa from goldfish, crucian carp, and allogynogenetic gibel carp (Liu et al. 2012; Xi et al. 2011; Zhang et al. 2009, 2010). Among them, *Myxobolus wulii* (Wu and Li 1986), *M. ampullicapsulatus* Zhao et al. (2008) and *Myxobolus honghuensis* Liu et al. (2012) were found in *Carassius auratus gibelio* in China and *M. wulii* from the gills and hepatopancreas of goldfish in Japan (Liu et al. 2012; Xi et al. 2011; Zhang et al. 2010). Phylogenetic analyses of small

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subunit ribosomal RNA gene sequences showed that *M. wulii* was closely related to *M. ampullicapsulatus* (Zhang et al. 2010). Meanwhile, Xi et al. (2011) isolated one *Myxobolus* species from the gills and pharynx of *C. auratus gibelio* (Bloch) reared in heavily infected pond in Jiangsu area, China and found that it could cause serious disease for host fish. After sequence comparison among the related *Myxobolus* species, they considered it as *M. ampullicapsulatus* based on the high similarity of SSU rDNA sequences. *M. honghuensis* from *C. auratus gibelio* with serious disease in Hubei area could be distinguished from *M. ampullicapsulatus* by its equal polar capsules and more polar filament turns and regarded as an independent species (Liu et al. 2012). In this study, we researched the differences and clarified the confusions among *M. wulii*, *M. ampullicapsulatus*, and *M. honghuensis* based on the genetic and molecular information.

Materials and methods

Sample collection and species identification

The host fish, *Carassius auratus auratus* were collected during July 2010 and July 2011 from Yongchuan, Chongqing area and Poyang Lake, Jiangxi area, China, necropsied, and examined under the binocular dissecting microscope at $\times 400$ to detect myxosporeans. Plasmodia of three populations of *M. ampullicapsulatus* were collected from the gills of the host fish, transferred on microscope slides, and ruptured to release spores. After rinsing three times with sterile distilled water, fresh spores were pelleted by centrifugation at $2,000\times g$, prepared as specimens as previously reported (Zhao et al. 2001), and observed and measured at $\times 1,000$ magnification using a Nikon E600 microscope. The illustrations based on fresh materials were drawn with the aid of the camera lucica and computer software. Photomicrographs were taken using a Nikon DXM1200 microscope at $\times 1,000$ magnification, and measurements based on 25 spores were given in microns (μm) as the arithmetic mean and standard deviation, followed by the range in parentheses. Species identification was conducted as previously reported (Zhao et al. 2001).

DNA extraction, cloning, and sequencing

Genomic DNA of myxosporean isolates from the fish gills were extracted using DNeasy Tissue Kit (QIAGEN). 18S rDNA was amplified using primers ERIB1: 5'-ACC TGG TTG ATC CTG CCA G-3' and ERIB10: 5'-CTT CCG CAG GTT CAC CTA CGG-3' at the following conditions: initial denaturation at 94 °C for 5 min followed by 35 cycles of 1 min at 94 °C, 1 min at 56 °C, and 2 min at 72 °C as well as a final elongation at 72 °C for 10 min. Products were purified using a Gel Extraction Kit, measured with the NanoDrop ND-1000 spectrophotometer, inserted into pMD19-T vector, and sequenced in both directions using the ABI PRISM® 3730 DNA sequencer.

Phylogenetic, sequence, and *P*-distance analyses

All sequences except those of the newly obtained SSU rRNA genes were obtained using the basic local alignment search tool (BLAST) from GenBank. Sequences selected include those of *Myxobolus* species which is morphologically similar to *M. ampullicapsulatus*. *Tetracapsuloides bryosalmonae* was used as an outgroup taxon. Twenty-two sequences were aligned using Clustal W. Maximum likelihood (ML) analyses were performed using PAUP*4.0b10 (Swofford 2003). Bootstrap confidence values were calculated with a heuristic search using simple sequence addition and 100 replicates. Bayesian analyses were conducted by MrBayes (Ronquist and Huelsenbeck 2003) with parameters setting to 1,000,000 generations and 10,000 trees. Sequences were assembled and manually edited in BioEdit (Hall 1999). In addition, the similarities of seven sequences were calculated by GenBank BLAST, and the *P*-distance was calculated using MEGA5 (Tamura et al. 2011).

Results

Morphology remarks

Infection sites and plasmodia morphology for three populations of *M. ampullicapsulatus* collected from Chongqing area



Fig. 1 Comparison of spores among the populations of *M. ampullicapsulatus* and *M. honghuensis* morphologically. **a** *M. ampullicapsulatus* CQLg (from Zhao et al. 2008). **b** *M. ampullicapsulatus* CQ collected in the present study.

c *M. ampullicapsulatus* JXa.g collected in the present study. **d** *M. ampullicapsulatus* JSa.g (from Xi et al. 2011). **e** *M. honghuensis* HBa.g (from Liu et al. 2012). Bar = 10 μm

and Poyang Lake, Jiangxi area, China show resemblance to the original population of *M. ampullicapsulatus* (Zhao et al. 2008). Mature spores were pyriform with bluntly pointed apex and rounded posterior in valvular view (Fig. 1 and Table 1). Generally, the population of *M. ampullicapsulatus* from Poyang Lake possesses narrower and slightly elongated spores compared with that of *M. ampullicapsulatus* from Chongqing. In addition, the population of *M. ampullicapsulatus* isolated from goldfish and crucian carp can be distinguished from *M. ampullicapsulatus* isolated from the pharynx and gills of *C. auratus gibelio* from Jiangsu by having a lower ratio of length to width and more bluntly pointed apex (Xi et al. 2011), but displays obvious similarity in both spore morphology and host type to *M. honghuensis* from the pharynx and gills of *C. auratus gibelio* in Honghu Lake (Liu et al. 2012). Based on spore morphology and host specificity, *M. ampullicapsulatus* was mainly parasitic on the gills of goldfish and crucian carp. Thus, *M. ampullicapsulatus* collected from the pharynx and gills of *C. auratus gibelio* should be classified as *M. honghuensis*.

Sequence analyses

The three new sequences of *M. ampullicapsulatus* were obtained and submitted to GenBank (Fig. 2), and their lengths are shown in Table 1. Based on GenBank BLAST searches, the three new sequences were most similar to that of *M. ampullicapsulatus* CQLg (DQ339482) with similarities of 99, 100, and 100 %, respectively, and to that of *M. ampullicapsulatus* HBa.a (JQ690373) with similarity of 99 % for all (Table 2). Furthermore, Clustal W multiple alignment shows that the coverage length of seven sequences was 1,554 nucleotides, and sequence analyses show that 18S rDNA sequences had little variability among the former five populations of *M. ampullicapsulatus* (Fig. 3). A total of ten variation sites was scattered in the five sequences. However, 24 variation sites were found in both populations of *M. ampullicapsulatus* JS and *M. ampullicapsulatus* CQLg (Zhao et al. 2008; Xi et al. 2011) and only two variation sites in both populations of *M. ampullicapsulatus* JS and *M. honghuensis* (Liu et al. 2012). The similarities of the seven sequences are listed in Table 2 and Fig. 3.

P-distance analyses

P-distance inferred from 18S rDNA data for the seven sequences are shown in Table 2. Their P-distances to the former five *M. ampullicapsulatus* (<0.005) are far smaller than their distances to *M. ampullicapsulatus* JS and *M. honghuensis* (0.013–0.016). In addition, the P-distances between the latter two is only 0.001.

Table 1 Morphometrical comparison between populations of *M. ampullicapsulatus* and *M. honghuensis* and its synonyms from different sources

Populations	Host	Location	Site of infection	Length of sequence (nt)	Spore length × width (μm)	Polar capsule length × width (μm)	Polar filament coils	Source
<i>M. ampullicapsulatus</i> CQLg	<i>Carassius auratus auratus</i> (goldfish)	Dazu, Chongqing	Gills	1,576	16.5–19.5 × 8.5–10.0	7.0–10.0 × 2.5–4.0	9–10	Zhao et al. (2008)
<i>M. ampullicapsulatus</i> CQYg	<i>C. auratus auratus</i> (goldfish)	Yongchuan, Chongqing	Gills	2,005	16.5–22.7 × 8.2–14.0	6.2–11.8 × 1.8–4.1	9–10	Present study
<i>M. ampullicapsulatus</i> CQYa.a	<i>C. auratus auratus</i>	Yongchuan, Chongqing	Gills	2,008	–	–	–	Present study
<i>M. ampullicapsulatus</i> JXa.a	<i>C. auratus auratus</i>	Poyang Lake, Jiangxi	Gills	1,990	18.4–20.4 × 8.0–11.6	8.8–14.4 × 3.2–4.4	9–10	Present study
<i>M. ampullicapsulatus</i> HBa.a	<i>C. auratus auratus</i>	Wuhan, Hubei	Gills	1,962	–	–	–	–
<i>M. ampullicapsulatus</i> JSa.g	<i>C. auratus gibelio</i>	Yancheng, Jiangsu	Pharynx, gills	1,996	16.7–18.5 × 8.4–9.6	8.4–9.6 × 3.0–3.6	8–10	Xi et al. (2011)
<i>M. honghuensis</i> HBa.g	<i>C. auratus gibelio</i>	Honghu Lake, Hubei	Pharynx	1,555	15.1–19.5 × 9.0–11.3	7.9–8.1 × 3.0–4.5	7–8	Liu et al. (2012)

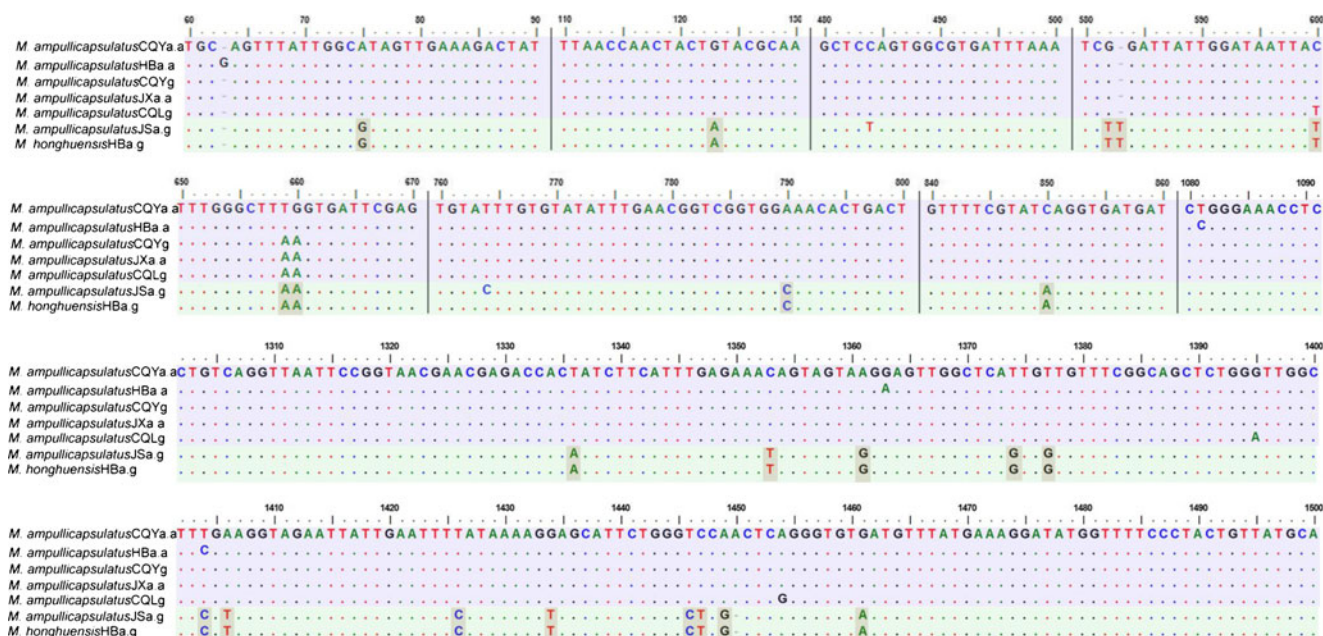


Fig. 2 Regions of alignment of SSU rDNA gene sequences of populations of *M. ampullicapsulatus* and *M. honghuensis*. Regions are separated by vertical lines and the reference sequence. Insertions and deletions

were compensated by introducing alignment gaps (dash). Matched sites are represented by dots. Distinct sequence signatures of each clade are shaded

Phylogenetic analysis

Since the topology of ML tree is similar to that of Bayesian inference (BI) tree, they were combined into one (Fig. 3). Twenty-one myxosporeans were grouped into two main phylogenetic lineages: freshwater lineage and marine lineage. The result of topologies of the two trees strongly supports that *M. ampullicapsulatus* and *M. honghuensis* should be placed into two clades. However, *M. ampullicapsulatus* JSa.g from the pharynx and gills of *C. auratus gibelio* in Yancheng, Jiangsu area (Xi et al. 2011) is clustered with *M. honghuensis* from the same host and infection site. The sister clade of *M. honghuensis* contains five

populations of *M. ampullicapsulatus* from infected gills of *C. auratus auratus*. Bayesian analyses strongly support a large clade including a sister group formed by *M. wulii* with another one consisting of *Myxobolus koi* and *Myxobolus longisporus*.

Discussion

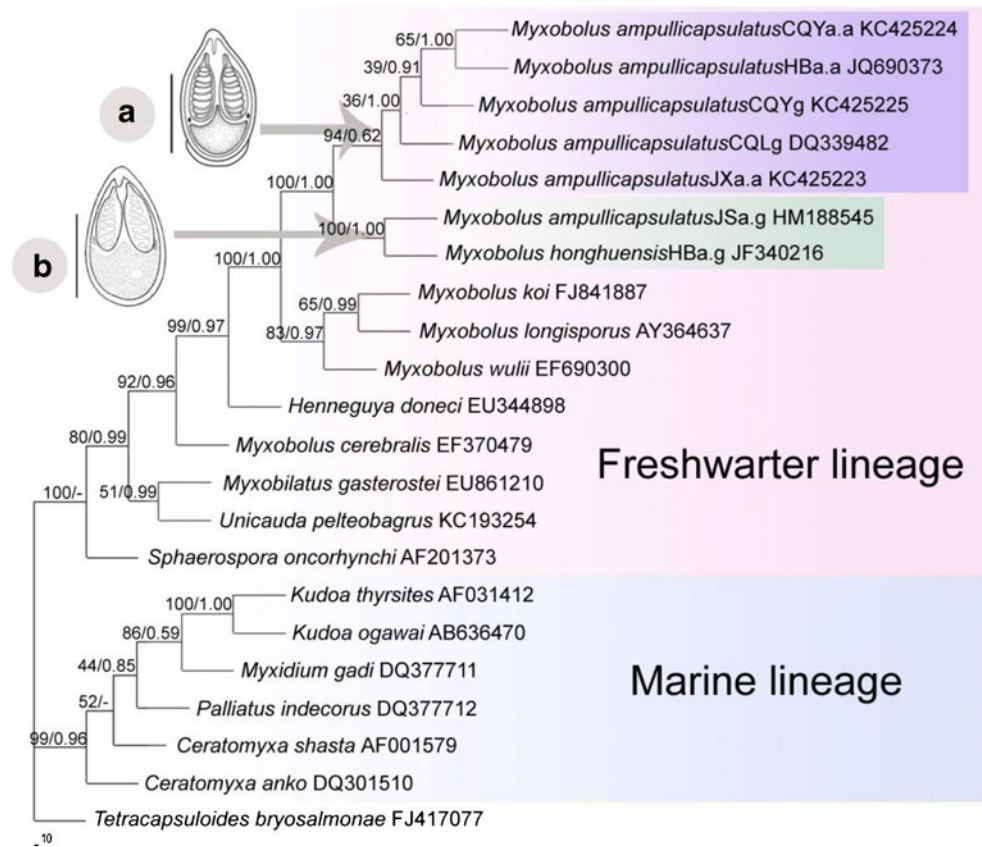
Morphological comparison for four populations of *M. ampullicapsulatus* and *M. honghuensis* indicates that the former three populations of *M. ampullicapsulatus* have longer pointed apex and ampullaceous polar capsules with longer neck region

Table 2 Estimates of evolutionary divergence between sequences and highly similar sequence. SSU rDNA gene sequence genetic distances (lower triangle) and similarities (upper triangle) among seven sequences

	1	2	3	4	5	6	7
<i>M. ampullicapsulatus</i> CQLg	–	100 %	100 %	99 %	99 %	98 %	99 %
<i>M. ampullicapsulatus</i> JXa.a	0.002	–	100 %	99 %	100 %	98 %	99 %
<i>M. ampullicapsulatus</i> CQYa.a	0.003	0.001	–	99 %	99 %	98 %	98 %
<i>M. ampullicapsulatus</i> HBa.a	0.005	0.003	0.002	–	99 %	98 %	98 %
<i>M. ampullicapsulatus</i> CQYg	0.002	0.000	0.001	0.003	–	98 %	99 %
<i>M. ampullicapsulatus</i> JSa.g	0.015	0.014	0.016	0.016	0.014	–	99 %
<i>M. honghuensis</i> HBa.g	0.014	0.013	0.014	0.015	0.013	0.001	–

1 *M. ampullicapsulatus* CQLg, 2 *M. ampullicapsulatus* JXa.a, 3 *M. ampullicapsulatus* CQYa.a, 4 *M. ampullicapsulatus* HBa.a, 5 *M. ampullicapsulatus* CQYg, 6 *M. ampullicapsulatus* JSa.g, 7 *M. honghuensis* HBa.g

Fig. 3 Phylogenetic tree generated by maximum likelihood analysis of the aligned small subunit ribosomal RNA gene sequences. Nodal supports are indicated for bootstrap confidence values resulting from ML and posterior probability of Bayesian analysis. “-” reflects minor differences between maximum likelihood and other methods. GenBank accession numbers for each taxon are listed. The distance scale is shown under the tree. Schematic of (a) *M. ampullicapsulatus* and (b) *M. honghuensis*. Bar = 10 m



(Fig. 1), while *M. ampullicapsulatus* from Jiangsu area and *M. honghuensis* possess ampullaceous polar capsules with shorter neck region. Moreover, the spores of *M. ampullicapsulatus* from Jiangsu area can be distinguished from those of the former *M. ampullicapsulatus* by having a lower ratio of length to width and more bluntly pointed apex and displaying a similarity to *M. honghuensis*. Liu et al. (2012) have mentioned that *M. honghuensis* is differed from *M. ampullicapsulatus* by subtle morphological differences. As for the host range and tissue tropism, *M. ampullicapsulatus* from Jiangsu and *M. honghuensis* were both from the pharynx and gills of *C. auratus gibelio* (Liu et al. 2012; Xi et al. 2011), whereas *M. ampullicapsulatus* from Chongqing, Jiangxi, and Hubei were all obtained from the gills of *C. auratus auratus* (Zhao et al. 2008). All the abovementioned morphological characteristics, host specificity, and tissue tropism play important roles in the classification of the complicated myxosporean species (Li et al. 2012; U-taynapun et al. 2011). Therefore, the different host and parasite locations can be used as good references for taxonomy prior to recognizing the importance of the minor differences in the spore shape and size.

Analysis of variation sites based on the alignment of SSU rRNA gene sequences also revealed that *M. ampullicapsulatus* obtained previously from Jiangsu area is distinct from the five populations of *M. ampullicapsulatus* in the study. The sequences of the five *M. ampullicapsulatus* isolated from *C.*

auratus auratus have high identity (99–100 %) to each other but slightly lower identity (98–99 %) to the sequences of two *M. ampullicapsulatus* isolated from *C. auratus gibelio* (Table 2). Moreover, the sequence of *M. ampullicapsulatus* from *C. auratus gibelio* is nearly identical to that of *M. honghuensis* from *C. auratus gibelio* (99 % over 1,996 nt), with only two variation sites. Genetically, these two sequences are intra-specific variations among those reported species of myxozoans (Ferguson et al. 2008; Molnár et al. 2006; Whipps et al. 2004a, b; Whipps and Diggles 2006; Whipps and Kent 2006; Suo et al. 2010). Therefore, defining the population of *M. ampullicapsulatus* from *C. auratus gibelio* as *M. ampullicapsulatus* is debatable, and *M. ampullicapsulatus* and *M. honghuensis* from *C. auratus gibelio* should be regarded as the same species. Blast analyses indicate that *M. ampullicapsulatus* JSa.a is distinguishable from the other *M. ampullicapsulatus* sequenced to date. In conclusion, the result indicates that the intra-species identification for the two *Myxobolus* species mentioned in the present research should be less than ten variation sites.

Depending on estimates of evolutionary divergence among the seven sequences (Table 2) except *M. ampullicapsulatus* JSa.a, the genetic distances inferred from 18S rDNA sequences for five populations of *M. ampullicapsulatus* are very close (<0.005). However, their genetic distances to *M. ampullicapsulatus* JSa.a and *M. honghuensis* are greater

(>0.013). The population of *M. ampullicapsulatus* JSa.a is genetically closer to *M. honghuensis* (0.001), indicating that they are in the same species. The study suggests that *M. ampullicapsulatus* from *C. auratus gibelio* was mis-identified and should be *M. honghuensis*.

ML and BI phylogenetic trees strongly suggest that *M. ampullicapsulatus* and *M. honghuensis* should be grouped into two different clades: one contains all the populations for *M. ampullicapsulatus* except *M. ampullicapsulatus* JSa.a isolated from the pharynx and gills of *C. auratus gibelio* in Yancheng, Jiangsu area and the other only includes both *M. ampullicapsulatus* JSa.a and *M. honghuensis* Liu et al. (2012) from the pharynx of *C. auratus gibelio* in Honghu Lake, Hubei area. *M. ampullicapsulatus* was originally described from the gills of goldfish. The two populations of *M. ampullicapsulatus* in the present research were also from the gills of goldfish or crucian carp, indicating that tissue specificity is an important factor in the evolution of myxozoans (Blaylock et al. 2004; Burger et al. 2007; Cone et al. 2005; Easy et al. 2005; Eszterbauer 2004; Fiala 2006; Kent et al. 2001; Molnár et al. 2006; Whipps et al. 2004a, b; Zhao et al. 2008).

Based on the discussion above, *M. ampullicapsulatus* seems to be a gill-specific parasite of *C. auratus auratus*, whereas *M. honghuensis* is a pharynx or gill parasite, which has a shift in tissue microhabitat. *M. ampullicapsulatus* and *M. honghuensis* can be distinguished with careful scrutiny, as the latter has shorter bottleneck, a lower ratio of length to width, and less polar filament turns. Furthermore, the study infers that *M. ampullicapsulatus* and *M. honghuensis* are two different species, and the population of *M. ampullicapsulatus* from Jiangsu area should be regarded as the same species as *M. honghuensis*. Taken together, this study highlights the importance of DNA sequence comparisons for distinguishing *Myxobolus* species and also indicates that morphologically similar or identical spores collected from genetically close hosts are important for species classification. The intra-species identification for the two *Myxobolus* species mentioned in the present research should be less than ten variation sites and more than ten variation sites for inter-species identification of *Myxobolus* can be inferred. However, some molecular criterion for the intra-species or inter-species identification based on the comprehensive factors, including sequence length, coverage, similarity, variation sites and genetic distances, should be extracted and proved by much more research data.

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