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Intraspecific morphological variation in myxosporeans: high pleomorphic myxospores in the same plasmodium of *Myxobolus drjagini* (Akhmerov, 1954)

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Abstract: The taxonomy of myxosporeans was traditionally dependent solely upon the spore morphological and morphometric data. Intensive reports of intraspecific morphological variation, however, are increasingly challenging the taxonomic approaches for myxosporeans. In the present work, the morphological pleomorphism of myxospores of *Myxobolus drjagini* (Akhmerov, 1954) was observed. More interestingly, all of these pleomorphic myxospores occurred in the same plasmodium of *M. drjagini*, which refutes the previous hypothesis that morphological variation of *M. drjagini* was derived from its responses to differences in nutrition and immunological responses associated with different host tissues. Bearing the intraspecific morphometric and morphotype variation in mind, the combination of morphological, ecological and molecular data should be applied to the species identification and delimitation for myxosporeans. This is the first reported myxobolid species with high pleomorphic myxospores which are present in the same plasmodium.

Keywords: Morphology, pleomorphy, cranial cavity, silver carp, SSU rDNA

Myxosporeans are a group of morphologically diverse and widely distributed cnidarian endoparasites primarily infecting fish, with approximately 2,600 species reported over the world (Okamura et al. 2018). For the historical and technical limitations, most of the described myxosporeans were identified solely by spore morphology (Chen and Ma 1998). Recently, spore morphology, biological traits (host/organ specificity, tissue tropism) and molecular characteristics were suggested to be considered together for the myxosporeans' species identification (Atkinson et al. 2015), and a number of misidentification and confusions were corrected (e.g., Guo et al. 2018, Zhang et al. 2018). However, myxosporean taxonomists frequently run into a taxonomic dilemma when facing two or more following exceptional conditions which cover, but not limit to, extensive intraspecific morphological variation, several organ or tissue preference, multi-host with high genetic distance, high interspecific morphological similarity and ambiguous molecular sequence-based species boundaries or absent molecular sequence (Liu et al. 2010, Zhai et al. 2016, Guo et al. 2018). In a previous report, a strategy for the identification of species with two myxospore morphotypes was suggested when falling in the myxosporean's taxonomic dilemma (Guo et al. 2018).

Myxobolus drjagini (Akhmerov, 1954) Landsberg et Lom, 1991 was first reported from the head skin of silver carp *Hypophthalmichthys molitrix* (Valenciennes) from the Amur River in Russia by Akhmerov (1954). Subsequently, it was recorded to infect various organs of silver carp (blood, body cavity, brain, brain-lymph, eye socket, fins, gall bladder, gill arch, gills, heart, intestine, kidney, liver, nostrils, olfactory tract, optic nerve, semicircular canal, skin, spinal cord, swim bladder, trigeminus, vagus) in China (Wu et al. 1975, 1979, Chen and Ma 1998), although the nervous system was predominant site of infection (Wu et al. 1975). Due to the heavy pathological effect on the

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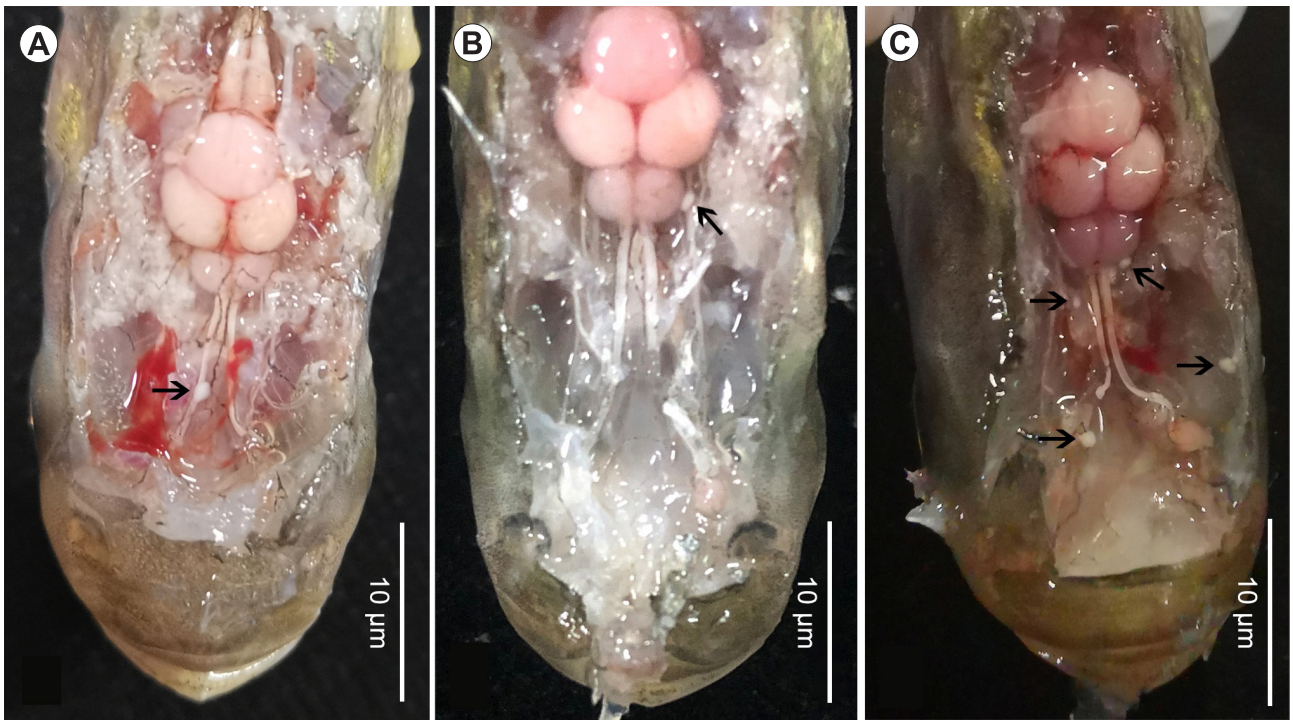

Fig. 1. The plasmodia of *Myxobolus drjagini* (Akhmerov, 1954) (arrows) in the brain-lymph of silver carp *Hypophthalmichthys molitrix* (Valenciennes).

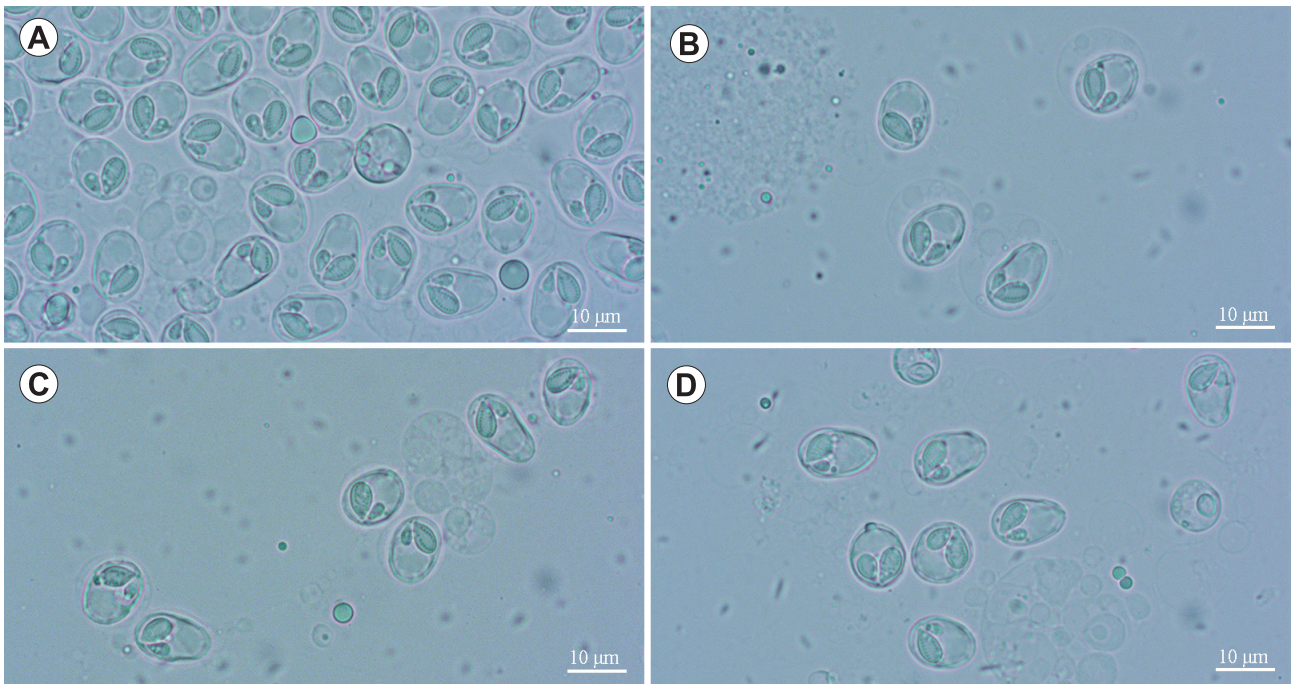

Fig. 2. Myxospores of *Myxobolus drjagini* (Akhmerov, 1954) from the same plasmodium.

nervous system, *M. drjagini* could cause the so-called twist disease and finally the massive mortality of silver carp, which was reported in China (Wu et al. 1975). With the colonisation of silver carp, *M. drjagini* was introduced into Hungary where it was reported to infect the head skin, cornea, operculum and buccal cavity of host (Molnár 1971, El-Mansy and Molnár 1997). Recently, two myxospore morphotypes (elliptical vs. obovate) of *M. drjagini* in different sites of infection (head skin vs. cranial cavity) were reported (Xi et al. 2019). The possible causes of the presence of two morphotypes were suggested, based on the differential responses of *M. drjagini* to the nutrition and immunological differences associated with different host organs (Xi et al. 2019). In the present study, a mixture of high pleomorphic myxospores were found to be present in the same plasmodium located in the cranial cavity of silver

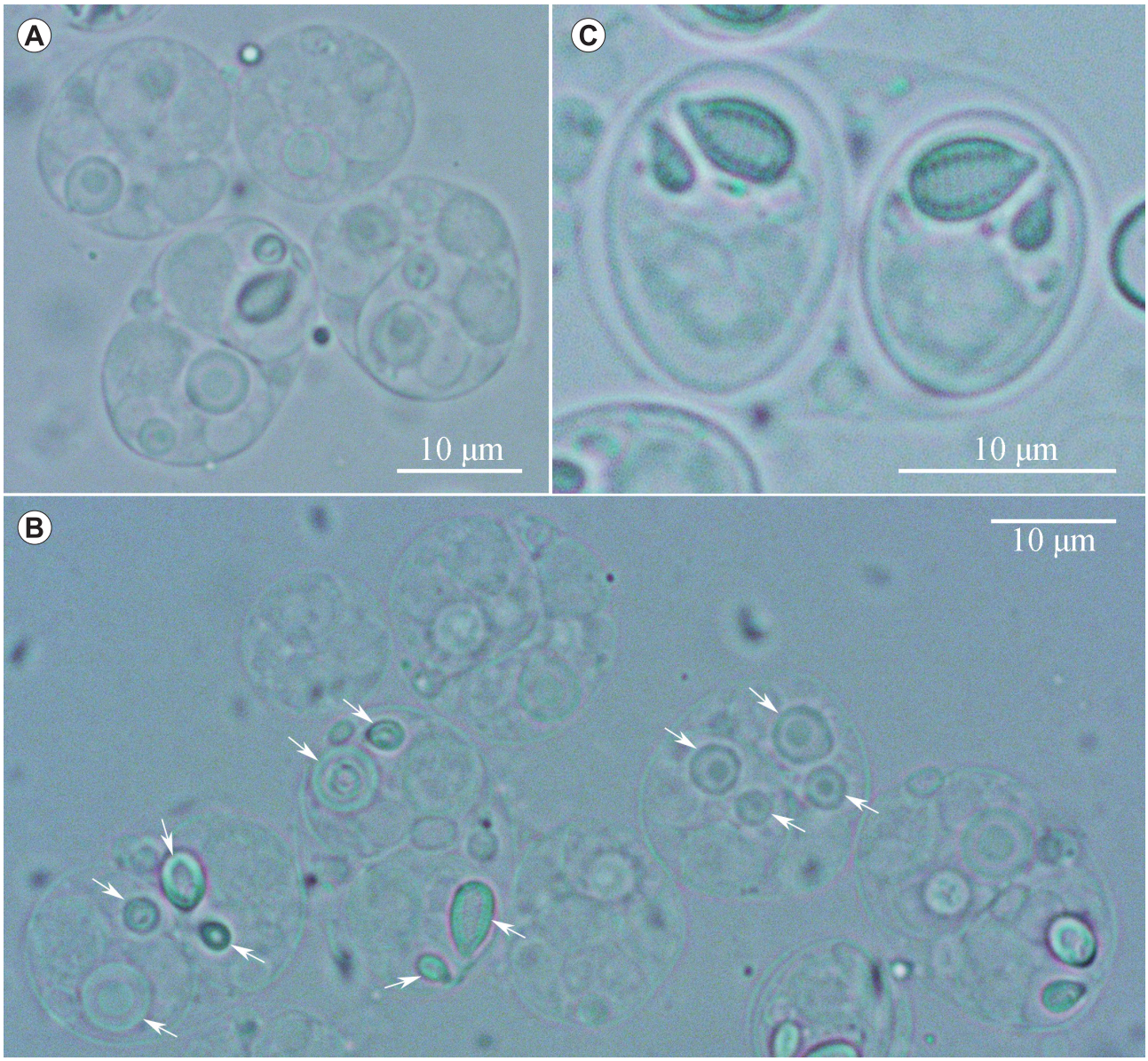

Fig. 3. Some developmental stages of *Myxobolus drjagini* (Akhmerov, 1954). **A** – the early pansporoblast; **B** – the advanced pansporoblast; arrows show capsulogenic cells; **C** – two spores produced by the pansporoblast.

carp which refuted the previous suggestion for its morphological variation.

Twenty-four silver carp (16.1–19.9 cm in total length) from Weishan Lake in Jining City, Shandong Province in China were transported alive to the Laboratory of Aquatic Animal Parasitology in School of Marine Science and Engineering of the Qingdao Agricultural University in Qingdao City, Shandong Province and held temporarily in aquaria, where they were anesthetised with MS222 (SIGMA, Louis, USA) prior to the dissection. Subsequently, all external and internal organs were examined for myxospores by the naked eye and preparing tissue smear under the light microscopy.

The plasmodia containing myxospores were separately singled out from brain-lymph with a pipette, and immediately fixed in absolute alcohol for molecular analysis. The fresh plasmodia from ten out of 24 infected silver carp were ruptured on slides to release the covering presporogonic stages and mature spores which were examined and photographed with ZEISS Lab.A1 microscope equipped with the ZEISS Axiocam 105 colour camera (Zeiss, Oberkochen, Germany). Morphological and morphometric analysis of fresh spores was performed according to Lom and Arthur (1989). Measurements are given in micrometres (µm) unless stated otherwise. The detailed spore morphological comparisons between the present reported strain and all previously recorded data for *M. drjagini* were conducted to uncover how much morphological variation was present in this species.

Genomic DNA of an individual plasmodium was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR amplification of the small subunit ribosomal DNA (SSU rDNA) sequence was performed using universal eukaryotic primer pairs ERIB1/ERIB10 (Barta et al. 1997) in a 50 µl reaction mixture, comprising 2 µl extract-

Table 1. Comparison of *Myxobolus drjagini* (Akhmerov, 1954) described in the present study and in the literature.

Infection site	SS	SL	SW	ST	LPCL	LPCW	SPCL	SPCW	Reference
Skin	obovate	14.0	9–10	6.0	6.0	3.0	4.0	2.0–2.5	Akhmerov 1954
Head skin, cornea	-	-	-	-	-	-	-	-	Molnár 1971
Skin, nostrils, blood, gills, brain, brain-lymph, semicircular canal, heart, spinal cord, olfactory tract, optic nerve, trigeminus, vagus	-	-	-	-	-	-	-	-	Wu et al. 1975
Head skin, operculum, buccal cavity	-	-	-	-	-	-	-	-	El-Mansy and Molnár 1997
Gill arch, skin, eye socket, heart, brain-lymph, brain, kidney, swim bladder, intestine, gall bladder, body cavity, fins, liver	obpyriform, elliptical	13.2 (10.8–14.4)	7.4 (7.2–7.8)	6.0 (6.1–6.2)	5.8 (5.5–6.0)	3.7 (3.6–3.8)	3.4 (2.6–3.6)	2.5 (2.4–2.6)	Chen and Ma 1998
Head skin	obovate	13.9 (12.6–14.7)	8.4 (7.5–9.9)	6.5	5.2 (4.7–5.8)	3.7 (3.1–4.2)	3.1 (2.3–3.5)	1.9 (1.2–2.6)	Xi et al. 2019
Cranial cavity	elliptical	12.8 (11.8–14.0)	8.9 (8.3–9.6)	7.1	5.8 (5.2–6.5)	3.3 (2.9–3.7)	3.0 (2.3–3.7)	1.7 (1.4–2.0)	Xi et al. 2019
Cranial cavity	elliptical	12.4 (11.7–13.3)	8.9 (8.4–9.9)	6.3	5.5 (4.9–6.2)	3.3 (2.8–3.8)	3.2 (2.6–4.4)	1.9 (1.4–3.0)	Xi et al. 2019
Cranial cavity	pleomorphic	12.5 ± 0.8 (10.8–14.2)	9.0 ± 0.5 (7.9–11.4)	-	6.6 ± 0.3 (5.9–7.4)	3.8 ± 0.2 (3.3–4.1)	4.0 ± 0.4 (3.1–5.4)	2.3 ± 0.3 (1.8–3.6)	Present study

SS – spore shape; SL – spore length; SW – spore width; ST – spore thickness; LPCL – large polar capsule length; LPCW – large polar capsule width; SPCL – small polar capsule length; SPCW – small polar capsule width; (−) data not given.

ed genomic DNA, 25 µl 2× Es Taq MasterMix (CWBIO, Beijing, China), 1 µl (10 µM) of each primer, 21 µl double distilled water. The PCR reaction was performed with initial denaturation for 4 min at 94 °C, followed by 35 cycles of the denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, and a final elongation step at 72 °C for 10 min. PCR products were separated using a 1.0% agarose gel, purified using a PCR purification kit (CWBiotech, Beijing, China) and sequenced directly with an ABI PRISM® 3730XL DNA sequencer (Applied Biosystems Inc., Foster, USA). The contiguous sequences were assembled according to the corresponding chromatograms with the SeqMan™ utility of the Lasergene software package and submitted to the National Center for Biotechnology Information (NCBI) nucleotide database.

One to five plasmodia of *M. drjagini* were observed in the brain-lymph of 24 out of 24 (100%) silver carp. The plasmodia were white, round or elliptical, measuring 0.5–1.5 mm in diameter (Fig. 1). The myxospores from each of examined plasmodia of *M. drjagini* presented as high pleomorphic (Fig. 2). The spores were 10.8–14.2 (12.5 ± 0.8) in length, 7.9–11.4 (9.0 ± 0.5) in width. Two unequal polar capsules were pyriform, measuring 5.9–7.4 (6.6 ± 0.3) × 3.3–4.1 (3.8 ± 0.2) and 3.1–5.4 (4.0 ± 0.4) × 1.8–3.6 (2.3 ± 0.3), respectively. Polar filament was seven to eight turns in the larger polar capsules and four to six turns in the smaller polar capsules.

The early pansporoblast stage of *M. drjagini* was observed in the plasmodia (Fig. 3A). In the advanced pansporoblast, four capsulogenic cells were recognisable, but the development of capsulogenic cells was asynchronous (Fig. 3B). Finally, the pansporoblast produced two spores (Fig. 3C).

A SSU rDNA sequence 1,920 bp long was obtained in the present study (accession number MW577455). A BLAST search indicated that the present SSU rDNA sequence was most similar to the sequences of *M. drjagini* (MH119079, 99.95%; MH119078, 99.90%) and *Myxobolus* sp. [*Hypophthalmichthys molitrix*, China] (MF543859, 99.95%; MF543857, 99.95%; MF543858, 99.85%).

In the present study, a myxosporean species was observed in the brain-lymph of silver carp. According to the host, infection site, character of plasmodia, measurements of spores (Table 1) and molecular sequence, the present species could be identified as *M. drjagini*. However, the spores from the individual plasmodium did not show a single morphotype and high pleomorphic myxospores were observed in the same plasmodium, which is different from the previous reports (e.g., Chen and Ma 1998, Xi et al. 2019).

Most species of *Myxobolus* Bütschli, 1882 show relatively strictly host specificity, organ specificity and tissue tropism (e.g., Molnár 1994, Atkinson et al. 2015). However, *M. drjagini* was reported to infect 25 organs (Table 1) of host (Molnár 1971, Wu et al. 1975, 1979, El-Mansy and Molnár 1997, Chen and Ma 1998, Xi et al. 2019), even though it was first described from the head skin of silver carp (Akhmerov 1954). So high variation of infection sites for this species allows us to cast doubts upon identification of previously reported myxospores. Actual site of infection should be identified based on the location where the sporogony occurs. According to the previous reports (Akhmerov 1954, Molnár 1971, Wu et al. 1975, 1979, El-Mansy and Molnár 1997, Chen and Ma 1998, Xi et al. 2019) and the present study, the plasmodia of *M. drjagini* were observed in 15 organs of silver carp, which could be divided into the skin system (head skin, fin, operculum, buccal cavity, nostrils), nervous system (brain, brain-lymph, spinal cord, optic nerve, trigeminus, vagus, olfactory tract), sensory system (cornea, semicircular canal), and visceral organs (swimbladder). In some organs of silver carp, however, only trophozoites or mature spores of *M. drjagini* were observed (Wu et al. 1975, 1979). It can be assumed that these organs may be just the passageway through which the trophozoite moves to the target organ or mature spores released from the target organs are excreted to the outside of fish (Wu et al. 1979).

Recently, two sites of infection (head skin and cranial cavity) were validated for *M. drjagini* by molecular evidence (Xi et al. 2019). Meanwhile, the myxospore morphotypes of *M. drjagini* from these two different infection sites were found to be distinct, and the authors suspected that various environmental pressure from different host organs resulted in the morphological variation (Xi et al. 2019). However, high pleomorphic myxospores of *M. drjagini* observed here in the same plasmodium refutes this hypothesis. So far, distinct morphological variation has been reported for several myofiber-infecting species of *Kudoa* Meglitsch, 1947 in the same plasmodium (e.g., Heiniger et al. 2013, Sakai et al. 2019). Mechanisms driving these morphological variations of myxosporeans in the same plasmodium remain unknown.

To conclude, it is obvious that *M. drjagini* is a species with high pleomorphic myxospores in the same or different plasmodia and it may be a good model material to explore the genetic mechanism underlying the myxosporean intraspecific morphological variation.

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