Parasitology Research (2019) 118:1061–1066
https://doi.org/10.1007/s00436-019-06234-9

FISH PARASITOLOGY - SHORT COMMUNICATION

The impacts of geographic and host species isolation on population divergence of *Myxobolus lentisuturalis*

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Received: 12 January 2018 / Accepted: 24 January 2019 / Published online: 1 February 2019

Abstract

Samples of *Myxobolus lentisuturalis* were found in the gallbladder of *Carassius auratus* in Chongqing, China, without obvious disease symptoms, which were different from samples reported in Hubei, China, and Italy which were described as highly pathogenic muscle-infecting species. In order to improve our understanding of the relationships between these different samples, we analyzed geography, DNA sequence data, and site specificity. The results indicated that (1) the genetic relationship between Chongqing and Italy samples of *M. lentisuturalis* was much closer than relationship between each of them and the Hubei samples; (2) host species isolation was more important than the geographic isolation in divergence of *M. lentisuturalis* samples, and the species might be specialized among its different host species; and (3) geographic isolation and infection-site variation played a limited impact in genetic differentiation among different samples of *M. lentisuturalis* infecting the same host species.

Keywords *Myxobolus lentisuturalis* · Geography · Host species · Isolation · Divergence · Infection site

Introduction

Myxozoans are the most abundant, diversified, and economically important group of microscopic metazoan parasites (Kent et al. 2001; Lodh et al. 2012). They are characterized by multicellular spores, with polar capsules containing an extrusible polar filament (Feist and Longshaw 2006; Zhang et al. 2010; Abdel-Ghaffar et al. 2012). These myxosporean parasites are common parasites of ectothermic vertebrates, particularly fish (Székely and Molnár 1999; Abdel-Ghaffar et al. 2005; Ali et al. 2006; Zhao et al. 2015). To date, more than 2200 myxosporean species have been identified worldwide (Okamura et al. 2015). However, few cases about their population divergence have been reported. An extensive investigation of *Myxobolus cerebralis* (Hofer 1903) from Europe and America shows that sequences of ITS-1 are identical among samples of *M. cerebralis* from different localities, which suggests current distribution of *M. cerebralis* in North America is a result of recent introductions followed by dispersal via anthropogenic means, largely through the stocking of infected trout for sport fishing (Whipps et al. 2004). Another study revealed that *Tetracapsuloides bryosalmonae* (Canning et al. 1999) in North America and Europe have been isolated for a long enough time to result in differences in ITS-1 sequences between the majority of isolates from the two continents (Henderson and Okamura 2004). Based on 18S rDNA, population of *Parvicapsula minibicornis* (Kent et al.1997) has strong signals of structuring by both geography and salmonid host species in the Pacific Northwest of North America (Atkinson et al. 2011). A study of population divergence on *Kudoa thyrsites* (Gilchrist 1924) was conducted with samples collected around the world and found genetic types correlated to four broad oceanic regions: Japan, Australia, eastern Pacific, and eastern Atlantic (Whipps and Kent 2006). Genetic structure exists in inter-regions suggesting there are significant barriers to gene flow of *K. thyrsites* between regions. While little genetic differentiation within-regions suggests high gene flow within individual sampling areas (Whipps and Kent 2006). *Myxobolus lentisuturalis* was first described by Dyková et al. (2002) as a highly pathogenic myxozoan species (hereafter called the Hubei samples of *M. lentisuturalis*) infecting the muscles of *Carassius gibelio*

Section Editor: Christopher Whipps

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in Hubei, China. Then, a report from an Italian group showed a case of muscle infection on *Carassius auratus* caused by *M. lentisuturalis* (hereafter called the Italy samples of *M. lentisuturalis*) (Caffara et al. 2009). Otherwise, no other infection cases of *M. lentisuturalis* have been recorded. In the present study, we reported a new locality and infection site of *M. lentisuturalis* (hereafter called Chongqing samples of *M. lentisuturalis*). In order to better understand the population evolution of this species, we investigated the morphological and genetic divergence of the three geographic samples of *M. lentisuturalis*.

Materials and methods

Sample collection and species identification

A total of 20 specimens of *C. auratus* were collected from a river in Bishan District, Chongqing, China, on 15 March 2017. *Myxobolus lentisuturalis* was observed from gallbladders of three fish. Fresh spores were rinsed three times with sterile distilled water, followed by centrifugation at 2000*g*. Species identification and specimen treatment were performed using a previously described method (Zhao et al. 2001). All specimens were observed and photographed at × 1000 magnification using a Leica DM6000B microscope (Leica Microsystems, CMS GmbH, Germany). The illustrations, based on fresh materials, were drawn with the aid of a camera lucida and computer software. Measurements, based on 30 spores, were given in microns (μm) and expressed as the arithmetic mean and standard deviation, followed by the range in parentheses.

DNA isolation, cloning, and sequencing

Genomic DNA of the myxosporea isolated from the fish gallbladder was extracted using the DNeasy Blood & Tissue Kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany). Samples were analyzed by polymerase chain reaction (PCR) using different primer pairs, and the 18S rDNA was amplified in first round by ERIB1 (ACCTGGTTGATCCTGCCAG; Barta et al. 1997) and ERIB10 (CTTCCGCAGGTTCACCTACGG; Barta et al. 1997) and second round by 18E (CTGGTTGATCCTGCCAGT; Hillis and Dixon 1991) and 18R (CTACGGAAACCTTGTTACG; Whipps et al. 2003). PCR was performed in a 25-μL reaction system containing 3 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.5 μM of each primer, 15 ng of genomic DNA, and 1.5 U of Taq Ex DNA polymerase (TaKaRa, Otsu, Japan). Briefly, after an initial denaturation step at 95 °C for 5 min, amplifications were carried out with 35 cycles at a melting temperature of 95 °C for 30 s, an annealing temperature of 54 °C for 55 s, and an extension temperature of 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR products were subjected to electrophoresis on a 1.5% agarose gel and purified with the DNA Agarose Gel Extraction Kit (Omega Bio-Tek, Norcross City, GA). The purified PCR products were inserted into a pMD18-T vector (TaKaRa, Otsu, Japan), and two clones were sequenced on an ABI Prism 377 DNA Sequencer (Applied Biosystems Inc., Foster City, California).

Results and discussion

The mature spores collected from the gallbladder were ellipsoidal in outline with symmetrical shell valves, and the sporoplasm occupied the posterior half of the spore body (Fig. 1a). The length of the fresh spores was 11.9 (10.4–12.9) μm, and the width was 7.3 (6.4–7.9) μm ($n = 30$). Two equal anteriorly-tapered pyriform polar capsules were located at the anterior part of the spore. The anterior ends of the polar capsules were separated from each other, and the polar filaments were coiled 4–5 times. The length of the polar capsule

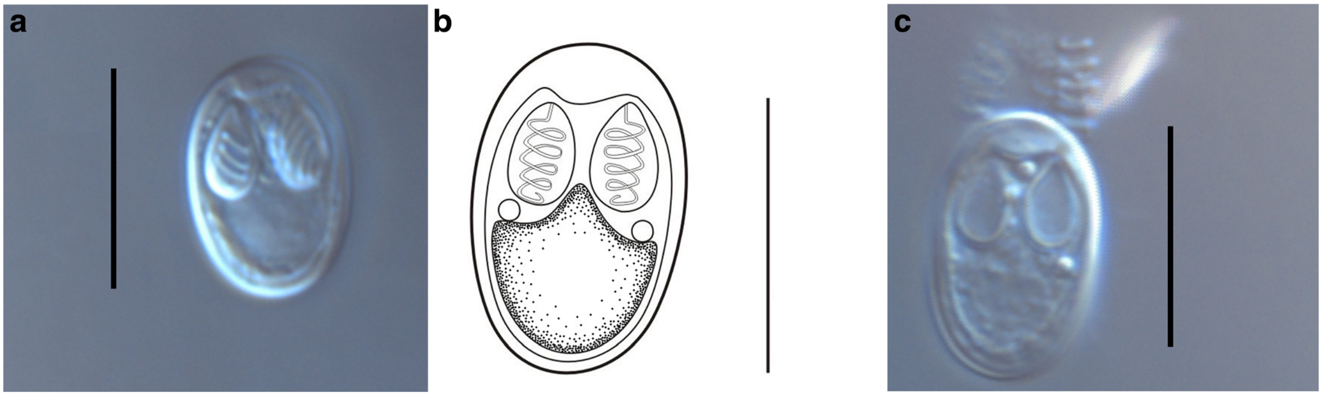

Fig. 1 Mature spores of *Myxobolus lentisuturalis.* **a**, **c** Light micrographs of *M. lentisuturalis*; **c** polar filament released from spores of *M. lentisuturalis*; **b** line drawing of *M. lentisuturalis.* Scale bars = 10 μm

was 4.1 (3.4–5.0) μm, and the width was 2.4 (1.8–2.8) μm ($n = 45$).

Spores of *M. lentisuturalis* from Chongqing samples morphologically resembled those of the Hubei and Italy samples of *M. lentisuturalis* (Dyková et al. 2002; Caffara et al. 2009), *Myxobolus cultus* (Yokoyama et al. 1995), *Myxobolus gayerae* (Molnár et al. 2007), *Myxobolus cycloides* (Molnár et al. 2006), *Myxobolus fundamentalis* (Molnár et al. 2010), and *Myxobolus rutili* (Molnár et al. 2010). Overall, spores from the Chongqing samples of *M. lentisuturalis* were most morphologically consistent with those of the Hubei and Italy samples of *M. lentisuturalis*, although the spores of Italy samples were slightly smaller than those of the other two samples (Table 1). Additionally, *M. cultus* could be distinguished from *M. lentisuturalis* based on a lower ratio of length to width of spores. Compared with *M. lentisuturalis*, the spore sizes of *M. gayerae*, *M. cycloides*, *M. fundamentalis*, and *M. rutili* were bigger, and their shapes were more elliptical.

A 1822-bp fragment of 18S rDNA was successfully sequenced for the present species, and such sequence was deposited in GenBank with an accession number of MF150547. The genetic differentiation on samples of *M. lentisuturalis* from three localities was compared, and the results showed that the sequence of the Chongqing samples was completely identical to the Italy samples. Only two mutation sites were detected between the Chongqing and Hubei samples, which had a similarity of 99.9%.

The samples of *M. lentisuturalis* from Hubei and Italy were reported as highly pathogenic, infecting the muscles of *C. gibelio* and *C. auratus*, respectively, but the Chongqing samples were found in gallbladder of *C. auratus* without obvious pathology. This suggests that virulence might be related to infection site and/or host species, or that a certain level of divergence has happened in the three geographic samples. Generally, for free-living animals, geographic isolation plays an important role in population divergence, and the longer geographic distance may cause stronger divergence between populations. In the present study, Chongqing and Hubei are geographically very close in China with about 1140 km between them and the places where the fish were collected belong to the same watershed of Yangtze River, while Italy is much farther from each of them. However, the genetic relationship between the samples of *M. lentisuturalis* from Chongqing and Italy was closer than that between each of them and the samples from Hubei. It seemed that the close geographic distance did not guarantee the close genetic relationship between different samples of *M. lentisuturalis*. The samples of *M. lentisuturalis* from Chongqing and Italy infected the same host species (*C. auratus*), while the host of Hubei samples was different (*C. gibelio*), which might explain why the Hubei samples were genetically distinct from other two samples. This finding suggested that host species isolation was more important than geographic isolation in population

Table 1 Morphological characteristics and measurements of *Myxobolus lentisuturalis* and similar species (all measurements are provided in μm)

Myxobolus spp.	Host(s)	Tissue	Locality	Length	Width	Thickness	PCL	PCW	References
M. lentisuturalis (MF150547)	*Carassius auratus*	Gallbladder	Chongqing (China)	11.9 (10.4–12.9)	7.3 (6.4–7.9)	–	4.1 (3.3–5.0)	2.4 (1.8–2.8)	The present study
M. lentisuturalis (AY278563)	*Carassius auratus*	Muscle	Bologna (Italy)	10.5 (9.0–11.0)	6.6 (6.0–7.0)	3.9 (3.0–5.0)	3.7 (3.0–4.5)	2.2 (2.0–3.0)	Caffara et al. (2009)
M. lentisuturalis (AY119688)	*Carassius gibelio*	Muscle	Hubei (China)	11.8 (11.2–12.4)	7.6 (7.2–8.4)	5.2	4.2 (4.0–4.4)	2.5 (2.0–2.8)	Dyková et al. (2002)
M. cultus (HQ613409)	*Carassius auratus gibelio*	Gill filament	Jiangsu (China)	11.9 (11.5–12.7)	8.6 (8.4–9.1)	6.3 (5.6–7.5)	5.0 (4.5–5.6)	3.0 (2.7–3.4)	Xi et al. (2013)
M. gayerae (DQ439809)	*Leuciscus cephalus*	Intestinal wall	Hungary	15.41 (15–16.5)	13.16 (12.5–14)	7.9 (7.3–8.7)	5.79 (5–6.5)	4 (3.5–4.5)	Molnár et al. (2006)
M. cycloides (DQ439810)	*Leuciscus cephalus*	Swim bladder	Hungary	14.1 (13–15.2)	10.43 (9.8–11.1)	7.61 (7.2–8.5)	5.2 (4.1–6.5)	3.36 (3–4)	Molnár et al. (2006)
M. fundamentalis (GU968200)	*Rutilus rutilus*	Gill arch	Hungary	15.5 (14.4–17.0)	11.8 (11.2–13.2)	9.2 (9.0–9.6)	6.7 (6.5–7.2)	4.0 (3.7–4.3)	Molnár et al. (2010)
M. rutili (GU968201)	*Rutilus rutilus*	Gill filament	Hungary	13.1 (11–15.7)	9.9 (8–10.8)	6.8 (6.0–9.0)	5.9 (4.0–7.2)	3.4 (3.0–3.7)	Molnár et al. (2010)

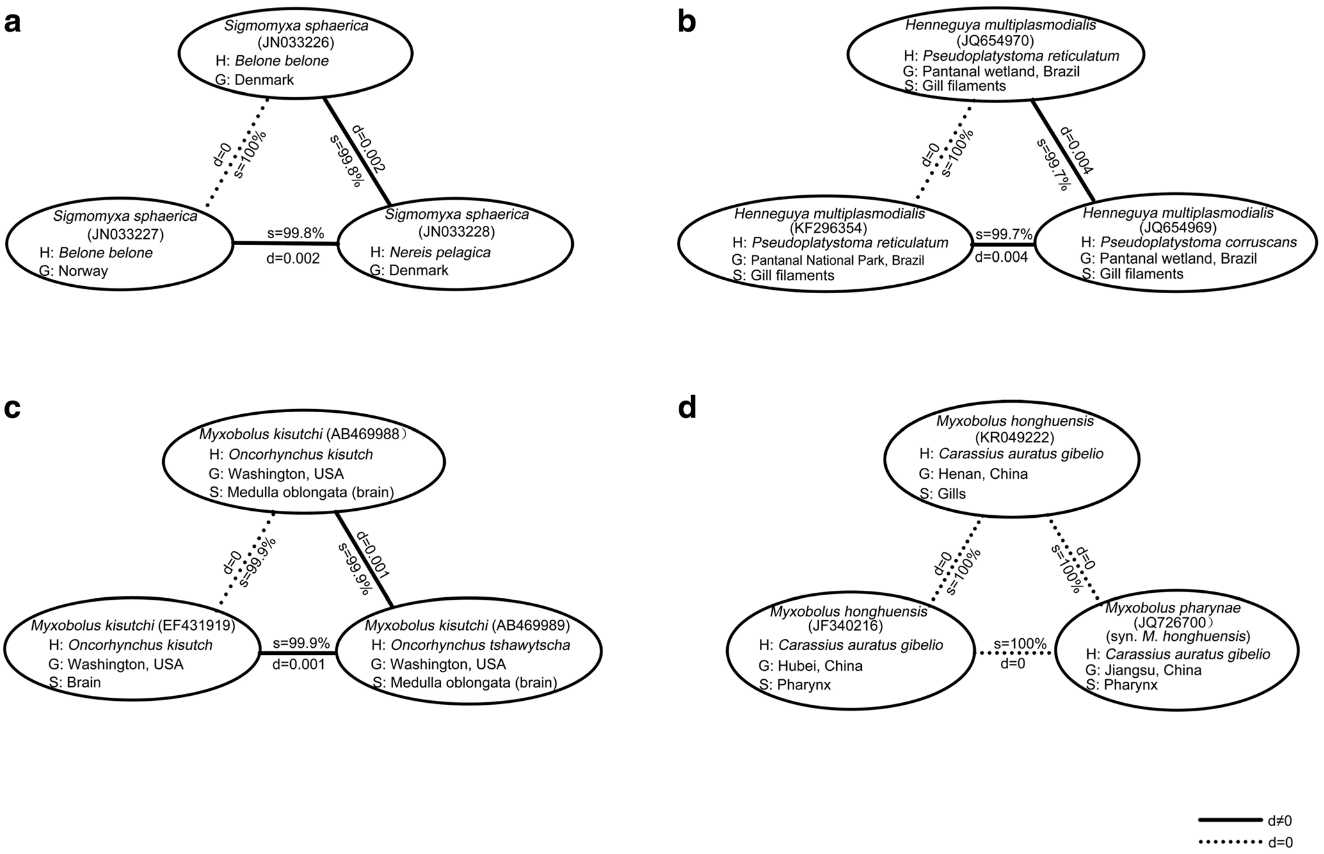

Fig. 2 Genetic relationship among samples of different myxozoan species. H: host species; G: geography; S: site; d: genetic distance; s: similarity

divergence of *M. lentisuturalis*, and the species might be specialized among its different host species. Two geographic samples of *Sigmomyxa sphaerica* (Thélohan 1895) from Denmark and Norway, infecting the same host species *Belone belone*, had closer genetic relationship than that between each of them and other samples from Denmark infecting different host species *Nereis pelagic* (Karlsbakk and Køie 2012) (Fig. 2a). Similarly, among three samples of *Henneguya multiplasmodialis* (Adriano et al. 2012), two samples infecting the same host species had closer genetic relationship than that between each of them and the different host-infecting samples (Adriano et al. 2012; Carriero et al. 2013) (Fig. 2b). Sample relationships of *Myxobolus kisutchi* (Yasutake and Wood 1957) also fitted the pattern well (Hogge et al. 2008; Urawa et al. 2009) (Fig. 2c).

Many studies have also shown that some parasites may be specialized among its different host species, and stronger genetic differentiation has been found between populations with different host species than between geographic isolated populations in the same host species (McCoy et al. 2003; Criscione et al. 2005; Dietrich et al. 2011).

In the present study, the samples of *M. lentisuturalis* from Chongqing and Italy infected *C. auratus* at different sites (gall bladder vs. muscle). However, the two geographic samples exhibited no genetic difference. Moreover, we also compared the three samples of *Myxobolus honghuensis* (syn. *Myxobolus pharynae*) (Liu et al. 2012), which infect the same host species *Carassius auratus gibelio* at different sites (gills vs. pharynx) (Liu et al. 2012; Lu et al. 2012; Liu et al. 2016) (Fig. 2d). The results showed that there was no genetic difference among them ($d = 0$, $s = 100\%$) (Fig. 2d). Even though we did not find much evidence for a relationship between population divergence and infection-site, and the sample size was small, our data suggest that the infection-site variation did not affect the genetic diversity of *M. lentisuturalis*. We also realized that 18S rDNA may have not enough information for illuminating the intraspecific divergence of a species. Hence, in order to better access the genetic variation at a population level of *M. lentisuturalis*, a more variable genetic marker such as ITS-1 could be used in future studies.

Funding information This work was supported by grants from the National Natural Science Foundation of China (nos. 31471980, 31501845, and 31672280), project of Chongqing Science & Technology Commission (no. cstc2017jcyjAX0165).

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