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FISH PARASITOLOGY - ORIGINAL PAPER

A new species *Myxodavisia jejuensis* n. sp. (Myxosporea: Sinuolineidae) isolated from cultured olive flounder *Paralichthys olivaceus* in South Korea

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Abstract

A new myxosporean parasite, *Myxodavisia jejuensis* n. sp. (Myxozoa; Bivalvulida) is described from the urinary bladder of olive flounder *Paralichthys olivaceus* cultured on Jeju Island, Korea. Two long lateral appendages with whip-like extensions were attached to mature spores of triangular to semi-circular shape. The spores were measured at 13.1 ± 1.1 μm in length, 17.2 ± 1.0 μm in thickness, and 13.1 ± 1.0 μm in width. Two spherical polar capsules, with a diameter of 5.0 ± 0.4 μm, were observed on opposite sides in the middle of the spore. The suture line was straight or slightly sinuous on the middle of spores. The 18S rDNA from *M. jejuensis* n. sp. was used in BLAST and molecular phylogenetic analysis. The results demonstrated that *M. jejuensis* n. sp. was closest to *Sinuolinea capsularis* and that the infection site tropism was correlated with the phylogeny of marine myxosporeans. In addition, we designed specific primers to detect the 18S rDNA gene of *M. jejuensis* n. sp.; the results showed specific amplification in *M. jejuensis* n. sp. among the myxosporeans isolated from the urinary bladder of the cultured olive flounder.

Keywords *Myxodavisia jejuensis* · Olive flounder · Infection site tropism · Polyphyly

Introduction

Over the past several decades, myxozoan parasites have been studied globally, and over 2000 species have been reported to date (Lom and Dykova 2006; Yang et al. 2014). In Korea, various species of myxosporeans have been reported in freshwater and marine fishes since the first identification of *Thelohanellus kitauei* in carp derived from Israel (Korean name: Hyang-eo or Israeli carp), *Cyprinus carpio*, in 1988 (Jun et al. 1988). The genus *Myxobolus*, including *M. miyairii*, *M. cheisini*, *M. koi*, *M. dispar*, *M. suturalis*, *M. episquamalis*, and *M. aeglefini*; genus *Thelohanellus*, including *T. kitauei* and *T. misguri*; and the genus *Henneguya*, including *H. tridentigeri* and *Henneguya* sp. were isolated from freshwater fish (Kim et al. 2002, 2013a, b; Kim and Kim 2005; Cho et al. 2006a; Choi et al. 2010; Kwon and Kim 2011; Shin et al. 2011; Seo et al. 2012; Jeon and Kim 2015). In marine fish, *Ceratomyxa protopsettae*, *C. oplegnathus*, *C. sparusaurati*, *Enteromyxum leei*, *Kudoa paralichthys*, *K. iwatai*, *K. septempunctata*, *Parvicapsula anisocaudata*, *P. curvatura*, and *Sphaerospora koreana* have been reported (Cho and Kim 2001, 2003, 2004, 2016; Cho et al. 2004, b; Song et al. 2013; Jeon et al. 2017; Shin et al. 2018b). In addition, parasites such as *K. septempunctata* and *E. leei* cause food poisoning and economic loss (Kim et al. 2018; Shin et al. 2018b).

The genus *Myxodavisia* is a replacement name for *Davisia*; 28 *Davisia* spp. were transferred to a new genus *Myxodavisia* in 2008 (Zhao et al. 2008). These parasites mainly infect the urinary system of various fishes (*M. amoena*, *M. anoplopoma*, *M. aurita*, *M. bidens*, *M. brachiophora*, *M. cella*, *M. cornuta*, *M. coryphaenoidia*, *M. diplocrepis*, *M. donecae*, *M. filiformis*, *M. galeiforme*, *M. hexagrammi*, *M. longibrachia*, *M. longifilus*, *M. narvi*, *M. newfoundlandia*, *M. nototheniae*, *M. opacita*, *M. ophidioni*, *M. pectoralis*, *M. reginae*, *M. sebastiscus*, *M. spectabilis*, and *M. spinosa*), but some of

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them (*M. bulani*, *M. cynoglossi*, *M. filiformis*, *M. haldarae*, *M. murtii*, and *M. sauridae*) are isolated from the gall bladder (Zhao et al. 2008; Sarkar 2010; Fiala et al. 2015). To date, *Myxodavisia* spp. have not been reported in Korea and their pathogenicity is poorly understood. The Fish Vaccine Research Center of Jeju National University has been monitoring parasitic infections in cultured olive flounder *Paralichthys olivaceus*, starry flounder *Platichthys stellatus*, and rock bream *Oplegnathus fasciatus*. In our previous studies, we have reported infections of *E. leei*, *P. anisocaudata*, and *P. curvatura* in olive flounder (Shin et al. 2018a, b). In the present study, we isolated a *Myxodavisia* species in the urinary bladder of cultured olive flounder. Morphological and phylogenetic analyses of the specimens suggested that this parasite represented a new species, which is described here as *Myxodavisia jejuensis* n. sp.

Materials and methods

Parasite samples and partial purification

Olive flounder samples ($n = 18$; total length, 26.0 ± 2.5 cm) were obtained dead from two olive flounder farms located on Jeju island. Several fish exhibited urinary bladder enlargement. Extracts were aseptically obtained using a syringe and the urine suspensions were filtered through a 40-μm cell strainer, followed by centrifugation at 10,000×*g* (3 min at 20 °C). The pellets were resuspended in lysis buffer (RIPA, Merck, Germany) for 5 min, and the suspensions were subsequently centrifuged at 10,000×*g* (1 min at 20 °C). The supernatant was discarded, and the pellet resuspended in phosphate buffered saline (PBS). The sample was collected and preserved at 4 °C until further use.

Morphological identification

The urine suspensions and partially purified parasites were wet-mounted and observed under a light microscope and photographed at 400 or × 1000 magnification. Myxospore measurements were taken from 20 spores using the image processing program ImageJ, (available at http://rsb.info.nih.gov/ij/) according to the criteria of Lom and Arthur (Lom and Arthur 1989).

Molecular identification and phylogenetic analysis

DNA was extracted from partially purified parasites using QIAmp DNA Mini Kit (QIAGEN, Germany), following the manufacturer's instructions. Portions of 18S rDNA were amplified by PCR using a combination of primers designed by us (MyxodaF: 5′-ACTATGTTTAATACAGCTTGGTTG-3′ and MyxodaR: 5′-TCATTATTCAACTTGGTTCCC-3′) and other groups (SSU_F04: 5′-GCTTGTCTCAAAGATTAAGCC-3′, MYXGEN4f: 5′-GTGCCTTGAATAAATCAGAG-3′, ACT1R: 5′-AATTTCACCTCTCGCTGCCA-3′, and ERIB10: 5′-CTTCCGCAGGTTCACCTA-3′) (Barta et al. 1997; Hallett and Diamant 2001; Diamant et al. 2004; Fonseca et al. 2010). PCR was conducted with the following cycling program: initial denaturation at 95 °C for 5 min followed by 40 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. PCR products were treated with AccuPrep Genomic PCR Purification Kit (BIONEER, Korea) to remove excess primers and dNTPs, and directly sequenced with BigDyeTM Terminator v3.1 in an ABI 3730xl Sequencer.

Multiple alignments of 18S rDNA sequence were performed by Clustal X 2.0 (Larkin et al. 2007) with the homologous sequences of *Myxodavisia bulani* available on the GenBank database, and the similarities of the present isolate and *M. bulani* based on 18S rDNA were calculated in MEGA 7.0 (Kumar et al. 2016) and Clustal Omega (Sievers et al. 2011). Bayesian inference (BI) was used to reconstruct the phylogenetic tree from datasets containing 67 sequences of the 18S rDNA from the marine myxosporeans with the malacosporeans, namely *Tetracapsuloides bryosalmonae* (KF731712), which was used as an outgroup. Ambiguously aligned regions in 18S rDNA datasets were removed using Gblocks v0.91b (Castresana 2000) under default parameters, allowing up to half the taxa to have gaps. For BI analysis, nucleotide substitution models were selected using the Akaike information criterion (AIC) and the Bayesian information criterion (BIC), implemented in jModeltest 2.1.7 (Guindon and Gascuel 2003; Darriba et al. 2012) and GTR + I + G was chosen as the best-fit nucleotide substitution model for the 18S rDNA data sets. The metropolis-coupled Markov chain Monte Carlo (MCMC) algorithm implemented in MrBayes 3.2.4 (Ronquist et al. 2012) was run for a sufficient number of generations until the average standard deviation of the split frequencies was < 0.05. The sampling frequency was set at every 100 generations for 1000,000 generations. The first 100,000 generations from each run were discarded as burn-in, and the remaining was analyzed using the "sumt" command in MrBayes. Gaps were treated as missing data. A consensus tree was created using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Diagnostic PCR for detecting *M. jejuensis* n. sp.

In order to detect *M. jejuensis* n. sp. in infected olive flounder, diagnostic PCR was carried out with the primers designed for this study. Each 20 μL reaction contained 0.2 μM MJdiaF (5′-ACATTAAGCGAGAAACTATTGAAG-3′) and MJdiaR (5′-

CAACCAAGCTGTATTAAACATAGT-3′), 1 × EmeraldAmp PCR Master Mix (TaKaRa, Japan), and 1 μL of sample DNA. Cycling conditions were initial denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 7 min. To test the sensitivity and specificity of the developed diagnostic PCR, different dilutions of *M. jejuensis* n. sp. myxospores and other myxosporeans infecting urinary bladder of olive flounder (such as *P. anisocaudata*, *P. curvatura*, *Sinuolinea capsularis*, and *Ortholinea* sp.) were tested.

Results and discussion

Description of disporogonic plasmodia and mature spores

Mature spores, triangular to semi-circular in shape, with two long lateral appendages with whip-like extensions (Fig. 1a). The spores measure 13.1 ± 1.1 μm in length, 17.2 ± 1.0 μm in thickness, and 13.1 ± 1.0 μm in width. Two spherical polar capsules, with a diameter of 5.0 ± 0.4 μm and a coiled polar filament with four to five turns, in a plane perpendicular to the sutural plane. The suture line straight or slightly sinous in the middle of the spores. The sporoplasm fills spore cavity almost or entirely with granular globules (Fig. 1b–e). Disporogonic plasmodia, with 25–28 μm in length, had two immature spores with the posterior ends of both spores facing each other. In addition, two mature spores with appendages, released from the disporogonic plasmodia (Fig. 1f, g).

Taxonomic summary

Myxodavisia jejuensis n. sp.

Host: *Paralichthys olivaceus*, Olive flounder (Pleuronectiformes; Paralichthyidae).

Locality: Olive flounder culture farm, Jeju Self-Governing Province, Republic of Korea (33° 33′ N, 126° 41′ E)

Site of infection: Urinary bladder

Date of sampling: March 2017

Host size: 24–30 cm

Prevalence: Seven of the18 fish examined were infected (38.8%)

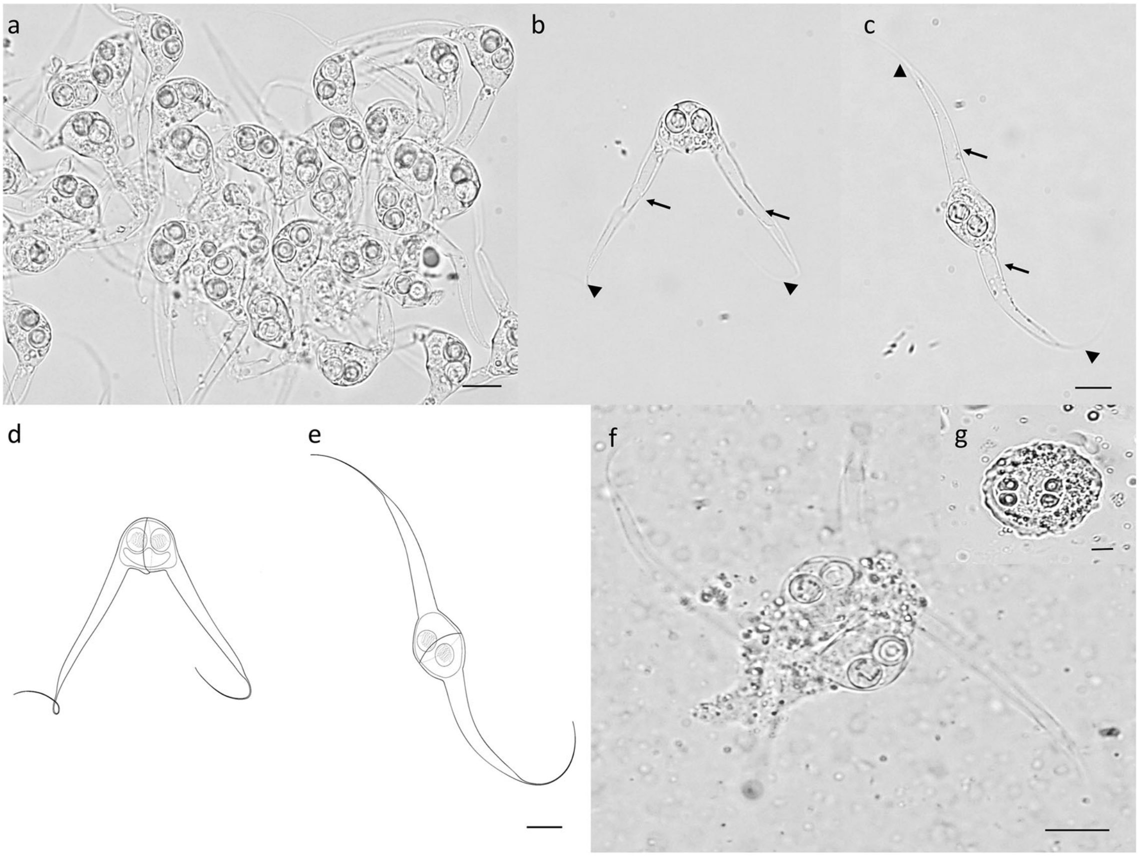

Fig. 1 *Myxodavisia jejuensis* n. sp. from the urinary bladder of *Paralichthys olivaceus*. **a** Myxospores of *M. jejuensis* after partial purification. **b**, **c** Mature spores of *M. jejuensis* with appendages (black arrow) and whip-like extensions (arrow heads) in the sutural view **b** and the apical view **c**. **d–e** Line drawing of spores of *M. jejuensis* in sutural and top view. **f–g** Disporic plasmodium of *M. jejuensis* with/without appendages. Scale bar = 10 μm

Type material: Diff-Quik–stained smears were deposited in the parasitological collection of the Fish Vaccine Research Center, Jeju National University, under accession number PCFVRC20170301A.

Etymology: specific name refers to the locality Jeju island.

Morphological comparison

Zhao et al. (2008) suggested a new genus name *Myxodavisia* and summarized the biological characters of 29 *Myxodavisia* spp. Based on the biological characters (such as host, infection site, and morphological similarity), we compared the species isolated in the present study with thirteen similar *Myxodavisia* spp. (Table 1). *M. newfoundlandia* and *M. reginae* showed five similarities to the present isolate in infection site, spore length, spore width, polar capsule diameter or appendages, and whip-like extension. However, they have different hosts from the isolate: *M. newfoundlandia* has a longer appendage (123–248 μm), which was speculated to be a whip-like extension, and *M. reginae* has a smaller polar capsule (4 μm). *M. donecae*, *M. galeiforme*, *M. longibrachia*, and *M. sebastisca* have four similarities to the isolate: infection site, spore length, polar capsule diameter, and whip-like extension. However, they have longer (*M. longibrachia*, and *M. sebastisca*) or shorter (*M. donecae* and *M. galeiforme*) appendage lengths than the isolates. *M. galeiforme* has the most similar morphology with the isolate, however the parasite has found in different host (*Lycodapus australis*) which inhabit in South America (Falkland-Patagonian area). Although the three *Myxodavisia* spp. (*M. branchiophora*, *M. opacita*, and *M. spinosa*) have similar hosts (Genus level: *Paralichthys*) to the isolates, they have comparatively different morphology. *M. branchiophora* and *M. opacita* have rounded spore chambers, and *M. spinosa* has an oval spore chamber; whereas the isolate displayed a semi-circular spore chamber. *M. hexagrammi* and *M. narvi* have smaller spore chambers and different hosts (Order level). *M. filiformis* has a different host (order level), infection site (gall bladder), and an oval spore chamber. Morphologically *M. amoena* has a smaller polar capsule, different host (family level), and longer appendage (150–167 μm). Based on morphological and biological differences of *M. jejuensis* n. sp. to published records of *Myxodavisia* spp., we justify the erection of a new species.

Molecular identification and phylogenetic analysis

Partial sequence of the 18S rDNA (1861 bp) was obtained from *M. jejuensis* n. sp. and deposited in GenBank (accession number MK614162). The analysis of Max Score by BLAST indicated that the 18S rDNA gene of *Sinuolinea capsularis*

Table 1 Comparison of spore measurements of *Myxodavisia jejuensis* n. sp. with the other related species. *UB* urinary bladder; *GB* gall bladder

Species	Host	Infection site	Spore length	Spore thickness	Spore width	Polar capsule diameter	Appendage length	Whip-like extension	References
Myxodavisia jejuensis	*Paralichthys olivaceus*	UB	13.1 ± 1.1	17.2 ± 1.0	13.1 ± 1.0	5.0 ± 0.4	59.2 ± 5.0	+	Present study
M. amoena	*Hippoglossoides platessoides*	UB	10.6–13.9	–	10.7–13.9	3.4–4.0	150–167	+	Gayevskaya et al. (1980)
M. brachiophora	*Paralichthys albigutta*	UB	9.0–11.0	–	9	3.5	18–22	–	Davis (1917)
M. donecae	*Trachurus capensis*	UB	10.0–12.0	10.6	9.6–12.0	4.2–4.7	45.2–49.2	+	Gayevskaya and Kovaleva (1979)
M. filiformis	*Thryssa setirostris*	GB	12.0–14.0	–	16.0–20.0	8.0–10.0	60–88	+	Padma Dorothy et al. (1998)
M. galeiforme	*Lycodapus australis*	UB	12.0–17.3	–	–	5.3–6.0	25.3–46.0	+	Kovaleva and Rodjuk (1991)
M. hexagrammi	*Hexagrammos otakii*	UB	9.5–10.5	10.0–12.0	–	3.5–4.0	44–59	+	Zhao et al. (2002)
M. longibrachia	*Callionymus lyra*	UB	12.0–15.0	12	–	5	100	+	Kabata (1962)
M. narvi	*Myxocephalus brandtii*	UB	10.0–11.5	–	11.5–14.4	4.0–4.5	20–27	+	Aseeva (2002)
M. newfoundlandia	*Macrourus holotrachys*	UB	9.6–12.0	6.4	10.7–12.8	2.7–4.3	123–248	+	Gaevskaya et al. (1980)
M. opacita	*Paralichthys albigutta*	UB	12.0–13.0	–	–	4	68	–	Davis (1917)
M. reginae	*Sebastes serranoides*	UB	11.0–15.0	11.5	12.0–20.0	2.5–4.5	28–85	+	Love and Moser (1976)
M. sebastisca	*Sebastiscus marmoratus*	UB	12.7–13.6	10.9–13.5	–	3.2–4.6	119.4–335.2	+	Zhao et al. (2008)
M. spinosa	*Paralichthys albigutta*	UB	13	–	7	4	70	+	Davis (1917)

(MK072735, 84.5%, 1601/1873) was the most similar sequence. To date, *M. bulani* (KM273030) is the only *Myxodavisia* species available in GenBank, to which *M. jejuensis* n. sp. showed a genetic similarity of 67% (1891 nt alignment). *M. bulani* was isolated from the gall bladder of an Indo-Pacific tarpon *Megalops cyprinoides* (Fiala et al. 2015) and it has a distinct morphology from *M. jejuensis* n. sp. *M. bulani* has a crescent-shaped spore chamber, and the sizes of the spore chamber and polar capsule are smaller than *M. jejuensis* n. sp. In addition, the parasites have a different host (Order level: Elopiformes vs Pleuronectiformes) and locality (approximately 4000 km distance). We consider that low genetic similarity between *M. jejuensis* n. sp. and *M. bulani* results from the difference in biological traits.

Phylogenetic analysis of myxozoa in previous studies revealed that marine myxosporeans were divided by characteristics of the infection site, such as histozoic and coelozoic groups, and the coelozoic group was further divided by the infection site (i.e., urinary system and gall bladder) (Fiala et al. 2015). In addition, the marine urinary clade consists of *Parvicapsula* and *Zschokkella* subclades (Bartosova et al. 2011). The phylogenetic tree constructed by this study also has similar division of groups, and the obtained sequence of *M. jejuensis* n. sp. clustered with the *Zschokkella* subclade in the marine urinary clade (Fig. 2). Site of infection has a strong effect on phylogenetic clustering of myxsporeans (Holzer et al. 2004; Fiala 2006; Shin et al. 2014, 2016). The 18S rDNA similarity of *M. jejuensis* n. sp. was the closest to *S. capsularis* isolated from cultured olive flounder (Shin et al. 2019), and was also present in the urinary bladder infection clade. Interestingly, *M. jejuensis* n. sp. clustered with the urinary system infection group, while *M. bulani* clustered with *C. leatheriacketi* isolated from the gall bladder of unicorn leatherjacket *Aluterus monoceros* (Fiala et al. 2015). In addition, Fiala et al. (2015) also predicted that other gall bladder-infecting *Myxodavisia* species will cluster within the *Ceratomyxa* clade, and that the species that infect the urinary bladder will either branch inside the marine urinary clade or form a separate clade within the marine lineage. Phylogenetic analysis in the present study supported the suggestion of previous study and the polyphyly of genus *Myxodavisia*.

Previous studies have suggested the possibility that spore morphology of the myxosporeans (such as genus *Parvicapsula* and *Kudoa*) correlates with genetic relatedness (Shin et al. 2016, 2018a). However, we could not reveal the relatedness in this study because there were only two 18S rDNA sequences of *Myxodavisia* spp. available for analysis. In addition, *M. jejuensis* n. sp. has a morphological feature (appendage) that is distinct from other myxosporeans included in the *Zschokkella* subclade. Host specificity is an important

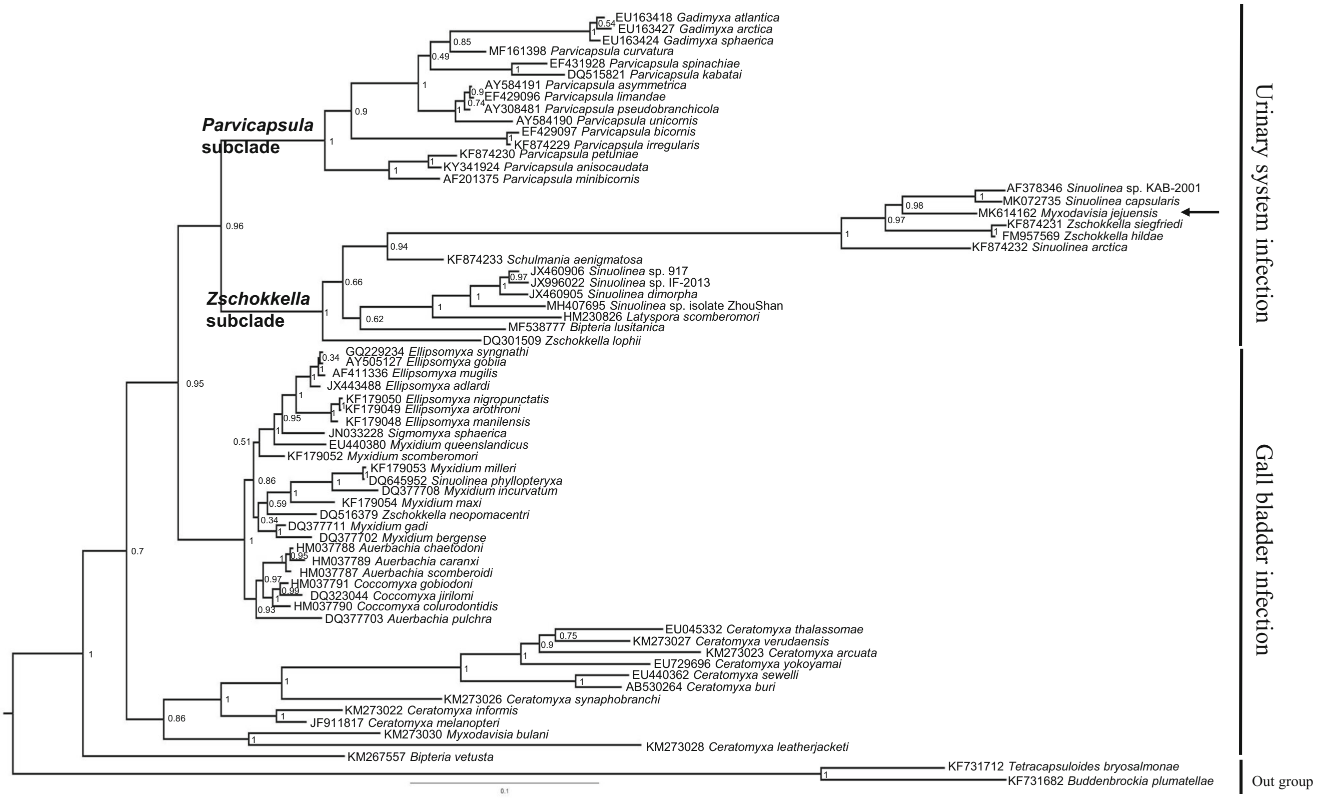

Fig. 2 Phylogenetic tree generated by Bayesian inference (BI) of the aligned partial 18S rDNA sequences of *M. jejuensis* obtained from the present study and related marine myxosporeans. The present species, indicated by an arrow, was shown to cluster within the *Zschokkella* subclade. *Tetracapsuloides bryosalmonae* was set as the out group and posterior probabilities were listed on the branches

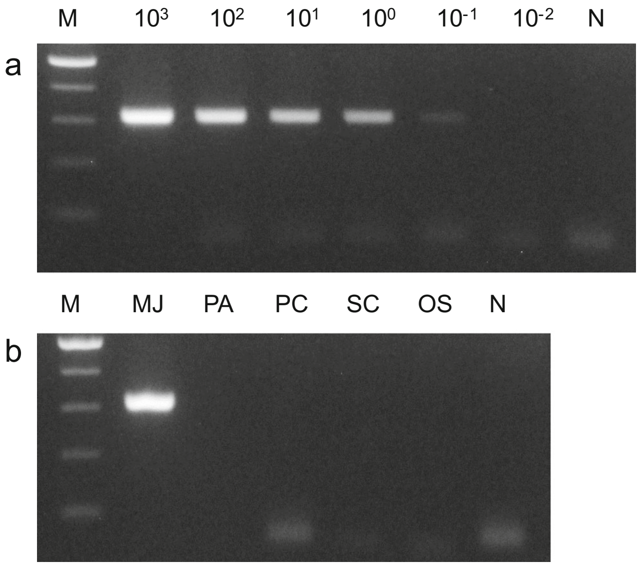

Fig. 3 Detection of *M. jejuensis* by diagnostic PCR: **a** Sensitivity test of diagnostic PCR: numbers of *M. jejuensis* myxospores are given at each lane. **b** Specificity of diagnostic PCR: M = 100 bp marker, MJ = *M. jejuensis*, PA = *P. anisocaudata*, PC = *P. curvatura*, SC = *S. capsularis*, OS = *Ortholinea* sp., N = negative control

factor used to characterize myxozoans and *M. jejuensis* n. sp. showed close genetic similarity to two of the *Sinuolinea* spp. isolated from the same fish host and a taxonomically close fish host, *Paralichthys olivaceus* (*S. capsularis*; Pleuronectiformes) and *Scophthalmus maximus* (*Sinuolinea* sp. KAB-2001; Pleuronectiformes), respectively. However, we cannot claim that there is a specific relationship between host specificity and phylogeny of *M. jejuensis* n. sp. in this study because other myxosporeans (*S. arctica*, *Z. siegfriedi*, and *Z. hildae*) reported from other fish (order level) were also clustered with *M. jejuensis* n. sp. in the *Zschokkella* subclade (Fig. 2).

Diagnostic PCR

The diagnostic PCR primers successfully amplified 344 bp of the 18S rDNA from *M. jejuensis* n. sp. while it did not amplify in *P. anisocaudata*, *P. curvatura*, *S. capsularis*, and *Ortholinea* sp. The fragments from *M. jejuensis* n. sp. were clearly visible for 1000 to 1 myxospores, but faint for the dilution equivalent of 0.1 myxospores (Fig. 3). Based on the present diagnostic PCR result, we suggest the PCR assay is specific for *M. jejuensis* n. sp.

Recently, we found diseased olive flounders with enlarged urinary bladders that contained *M. jejuensis* n. sp. but also other myxosporeans in coinfections. Since it is still unclear which species is responsible for the pathological enlargement, this requires further investigation. We have a plan to design specific primers for the myxosporenas infected with urinary system of olive flounder, and the primers containing designed primers in the present study will be applied to reveal an etiological agent causing the extension of the urinary bladder; however, further study is needed to identify the sequences of other myxosporeans. Based on morphological molecular analysis, the present isolate is identified as a new species, *M. jejuensis* n. sp. More myxozoans including the genus *Myxodavisia* spp. need to be sequenced to reveal the relationship between biological traits and phylogeny as well as the evolution of the parasite.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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