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Morphological and molecular-genetic characterization of *Chloromyxum trilineatum* n. sp. (Myxosporea: Bivalvulida) in the gall bladder of pale chub (*Zacco platypus*) in Japan

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Abstract

The genus *Chloromyxum* (Myxozoa: Myxosporea: Bivalvulida) is defined as having ridged or smooth bivalvular myxospores containing four polar capsules, with/without caudal filaments. Currently containing more than 140 nominal species, this genus is reasonably speciose with myxospores of unique but heterogeneous morphology. Recent phylogenetic studies have demonstrated its polyphyletic nature. During our myxosporean survey of freshwater fish, a new coelozoic myxosporean species, *Chloromyxum trilineatum* n. sp., was detected in the gall bladder of the pale chub, *Zacco platypus* (Cypriniformes: Cyprinidae), which originated from central Japan. Spores were subspherical, measuring 8.5–9.1 (8.8) μm in length, 7.6–8.2 (8.0) μm in width, and 6.8–7.8 (7.4) μm in thickness ($n = 20$). The valvular surface was smooth and three or four distinct ridges ran parallel to the suture line. Four almost equal polar capsules, 2.9–3.8 (3.3) μm in length and 1.6–2.4 (2.0) μm in width, assembled at the apical part of the spores. The partial nucleotide sequence of the 18S ribosomal RNA gene, 2014 bp in length, was closest to that of morphologically distinct *Chloromyxum ellipticum*, infecting the gall bladder of grass carp (*Ctenopharyngodon idella*) in China with 96.99% (1673/1725) identity and three insertion/deletion (indel) sites, followed by *Chloromyxum legeri*, infecting the gall bladder of common carp (*Cyprinus carpio*) in the Czech Republic with 89.97% (1803/2004) identity and 14 indel sites. Other myxosporean species, including *Chloromyxum* spp. from the gall bladder or urinary system of freshwater and marine fish, were phylogenetically distant from the present species.

Keywords Myxozoa · *Chloromyxum* · *Zacco platypus* · Morphology · SEM · 18S rDNA · Phylogeny

Introduction

Myxospores of the genus *Chloromyxum* Mingazzini, 1890 (Myxozoa: Myxosporea: Bivalvulida) are heterogeneous in morphology but invariably characterized by ridged or smooth bivalvular myxospores with four polar capsules and a uni or binucleated sporoplasm (Lom and Dyková 2006). Currently containing more than 140 nominal species (Bartošová and Fiala 2011; Eiras et al. 2012; Gleeson and Adlard 2012; Rocha et al. 2013; Sanders et al. 2015; Liu et al. 2017; Cantatore et al. 2018; Chandran et al. 2018), this genus is reasonably speciose. Despite a certain degree of morphological consistency among *Chloromyxum* myxospores, phylogenetic studies have demonstrated their polyphyletic nature with multiple phylogenetic lineages (Fiala and Dyková 2004; Gleeson and Adlard 2012; Rocha et al. 2013; Liu et al. 2017; Zhang et al. 2017; Chandran et al. 2018). As a

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consequence of the fairly limited availability of the nucleotide sequences of *Chloromyxum* spp.—currently comprising the 18S ribosomal RNA gene (rDNA) sequences of approximately 19% of the nominal species—the molecular-genetic background of the genus is not yet sufficiently understood.

During our myxosporean survey of freshwater fish, a coelozoic myxosporean species of the genus *Chloromyxum* was detected in the gall bladder of the pale chub, *Zacco platypus* (Temminck et Schlegel, 1846) (Cypriniformes: Cyprinidae), which originated from the central part of Japan. Its subspherical spores were clearly unique in morphology under light microscopy. In the present study, we morphologically and molecular-genetically characterize this specimen and erect a new species, namely *Chloromyxum trilineatum* n. sp.

Materials and methods

Fish samples and parasitological examination

The Gifu World Fresh Water Aquarium (Aqua Totto Gifu) in Japan exhibits approximately 28,500 freshwater fish of 260 species. Surveillance checks for dead fish are carried out on a daily basis, where the specimens are subsequently frozen in order to investigate the cause of death for disease monitoring and other purposes. For this study, the bodies of 92 dead fish of 34 species (13 families) were examined between October 10, 2017 and September 22, 2018 (Supplementary Table 1). The frozen fish bodies were thawed, and the body weights and total and standard body lengths were recorded. The external and internal organs were then individually examined by the naked eye and under a dissection microscope. To detect myxosporeans microscopically, the contents of the luminal organs, such as the gall bladder, urinary bladder, and gastrointestinal tract, were smeared on glass slides and treated with Diff-Quik™ stain (Sysmex Co., Kobe, Japan). A coelozoic myxosporean species of the genus *Chloromyxum* was detected in the gall bladder of two of the five examined pale chub individuals.

The contents of the gall bladder containing myxospores were diluted with physiological saline. These were then observed using a light microscope equipped with differential interference contrast imaging, photographed at a magnification of × 800, then transformed into digital images using Adobe® Photoshop® ver. 11.0 (Adobe Systems, San Jose, CA, USA). Then, the photographs were printed at a high magnification. Measurements were conducted on multiple printed photographs following the guidelines of Lom and Arthur (1989). All measurements are expressed in micrometers (μm) unless otherwise stated. Ranges with the means in parentheses are presented. Following the removal of a portion of the spores for DNA extraction, the parasite was fixed in 10% neutral-buffered formalin solution and 70% ethanol solution. The specimens collected in the present work were deposited in the Meguro Parasitological Museum, Tokyo, Japan, under collection no. 21458.

For scanning electron microscopy (SEM), a portion of the formalin-fixed spores was washed three times in 0.2 M Na_2HPO_4–NaH_2PO_4 solution (PB) at pH 7.8 and immersed in 2.5% glutaraldehyde in PB overnight. Following three washes in PB, the sample was post-fixed in 1% (*w*/*v*) osmium tetroxide in PB for 1 h. After washing three times in PB, the sample was dehydrated through a graded ethanol series, immersed in warmed *t*-butyl-alcohol, and cooled at 4 °C for 2 h. All processing was conducted using SEMpore® with 0.6-μm pore size (JEOL, Akishima, Tokyo, Japan) to reduce spore loss. The polycarbonate membrane of SEMpore® was then freeze-dried (model JFD-300; JEOL), mounted on stubs, and sputter-coated with gold-palladium at 200 Å (model JFC-1500; JEOL). The samples were examined using a scanning electron microscope (model JSM-6100; JEOL) at an accelerating voltage of 15 kV.

DNA extraction, polymerase chain reaction, and sequencing

Parasite DNA was extracted from collected myxospores using an Illustra™ tissue and cells genomicPrep Mini Spin Kit (GE Healthcare UK, Buckinghamshire, UK) according to the manufacturer's instructions. PCR amplification of two overlapping fragments of the 18S rDNA was performed in a 20-μl volume containing a DNA polymerase, Blend Taq-Plus-(TOYOBO, Dojima Hama, Osaka, Japan), and two primer pairs (MyxospecF [5′-TTCTGCCCTATCAACTWGTTG-3′] and Act1R [5′-AATTTCACCTCTCGCTGCCA-3′], and Myxo18S_575F [5′-CGCGGTAATTCCAGCTCCAG-3′] and SSU18R [5′-TGATCCTTCYGGAGGTTCAC-3′]) with reference to Hallett and Diamant (2001), Fiala (2006), and Kato et al. (2017). Alternatively, a combination of universal eukaryotic primers, Eurib1 (5′-ACCTGGTTGATCCT GCCAG-3′) and reverse Eurib2 (5′-CTTCCGCTGGTTCA CCTACGG-3′), was used to amplify almost the whole length of the 18S rDNA at once (Kopečná et al. 2006; Li et al. 2012). When direct sequencing was not satisfactory, the purified PCR products were cloned into the plasmid vector pTA2 (TArget Clone™; TOYOBO) and transformed into *Escherichia coli* JM109 cells (TOYOBO) according to the manufacturer's instructions. Following propagation, the plasmid DNA was extracted using a FastGene Plasmid Mini Kit (NIPPON Genetics Co., Tokyo, Japan), and inserts from multiple independent clones, at least three, were sequenced using universal M13 forward and reverse primers.

The nucleotide sequence obtained in the present study is available from the DDBJ/EMBL/GenBank databases under accession no. LC417364.

Table 1 *Chloromyxum* spp. reported in Japan

Species name	Host	Location	Locality	Reference
	Cypriniformes			
C. cristatum Léger, 1906 (syn. *C. koi* Fujita, 1913; *C. cyprini* Fujita, 1927)	*Cyprinus carpio*	Gall bladder	Hokkaido; Biwako Lake, Shiga Prefecture	Fujita 1913, 1927; Lom and Dyková 1993; Bartošová and Fiala 2011
C. fujitai Kudo, 1916	*Misgurnus anguillicaudatus*	Gall bladder	Tokyo	Kudo 1916
C. misgurni Kudo, 1916	*Misgurnus anguillicaudatus*	Gall bladder	Tokyo	Kudo 1916
C. oviforme Fujita, 1925	*Noemacheilus barbatulus toni*	Gall bladder	Hokkaido	Fujita 1925
C. richardsonii Fujita, 1925	*Tribolodon hakonensis*	Gall bladder	Toyohira River, Hokkaido	Fujita 1925
C. trilineatum n. sp.	*Zacco platypus*	Gall bladder	Gifu Prefecture	Present study
	Siluriformes			
C. parasiluri Fujita, 1927	*Silurus asotus*	Gall bladder	Biwako Lake, Shiga Prefecture	Fujita 1927
C. sphericum Fujita, 1927	*Silurus asotus*, *Zacco platypus*[a]	Gall bladder	Biwako Lake, Shiga Prefecture	Fujita 1927
	Salmoniformes			
C. wardi Kudo, 1929 (syn. *C. chitosense* Fujita, 1923)	*Oncorhynchus keta*	Gall bladder	Hokkaido	Fujita 1923; Urawa and Hiroi 1986
C. giganteum Fujita, 1923	*Oncorhynchus gorbuscha*	Gall bladder	Hokkaido	Fujita 1923
C. oncorhynchi Fujita, 1936	*Oncorhynchus masou*	Gall bladder	Hokkaido	Fujita 1936
C. quadriforme Fujita, 1923	*Oncorhynchus keta*, *O. masou*, *O. gorbuscha*	Gall bladder	Hokkaido	Fujita 1923, 1936
C. salvelini Fujita, 1923	*Salvelinus leucomaenis leucomaenis*	Gall bladder	Hokkaido	Fujita 1923; Urawa and Hiroi 1986
C. tanakai Fujita, 1936	*Oncorhynchus keta*	Gall bladder	Hokkaido	Fujita 1936

[a] An additional host for *C. sphericum*, *Z. platypus*, belongs to Cypriniformes

Phylogenetic analysis

For phylogenetic analysis, the newly obtained 18S rDNA nucleotide sequence in the present study and related bivalvulid myxosporean sequences retrieved from the DDBJ/EMBL/GenBank databases were aligned using the CLUSTAL W multiple alignment program (Thompson et al. 1994), with subsequent manual adjustment. The accession numbers of the sequences analyzed in the present study are provided in the figure showing a phylogenetic tree. Regions judged to be poorly aligned and characters with a gap in any sequence were excluded from subsequent analyses; 812 characters, of which 435 were variable, were retained for subsequent analysis. Maximum likelihood (ML) analysis was performed using the PhyML program (Guindon and Gascuel 2003; Dereeper et al. 2008) provided on the "phylogeny.fr" website (http://www.phylogeny.fr/) as conducted previously (Matsukane et al. 2010; Li et al. 2012, 2013). The probability of inferred branch was assessed by the approximate likelihood-ratio test (aLRT), an alternative to the non-parametric bootstrap estimation of branch support (Anisimova and Gascuel 2006). Marine bivalvulid species of genera *Auerbachia* and *Coccomyxa* were used as an outgroup for the construction of the ML phylogenetic tree (Gleeson and Adlard 2012).

Results

Numerous subspherical spores were detected in the contents of the gall bladder of two of the five dead pale chub individuals examined. The infected fishes were 11.5 cm in standard body length and 46.8 g in body weight, and 11.2 cm in standard body length and 28.7 g in body weight, and no macroscopic changes related to their death were observed. Microscopic examination of isolated bivalvular spores revealed four polar capsules and three sutural ridges parallel to the suture line. Fine striations on the shell valvular surface were not observed. The morphology of the present bivalvular spores was well coincident with the definition of the genus *Chloromyxum* by Lom and Dyková (2006), but was distinct from any of the known species of the genus.

Description

Chloromyxum trilineatum n. sp. (Myxosporea: Bivalvulida)

Plasmodia unknown. Coelozoic in the gall bladder. Bivalvular myxospores subspherical, measuring 8.5–9.1 (8.8) μm in length, 7.6–8.2 (8.0) μm in width, and 6.8–7.8 (7.4) μm in thickness ($n = 20$). The valvular surface was smooth with three or four distinct ridges parallel to the suture line. Four almost equal polar capsules, pyriform, 2.9–3.8 (3.3) μm in length and 1.6–2.4 (2.0) μm in width, assembled at the apical portion of spores.

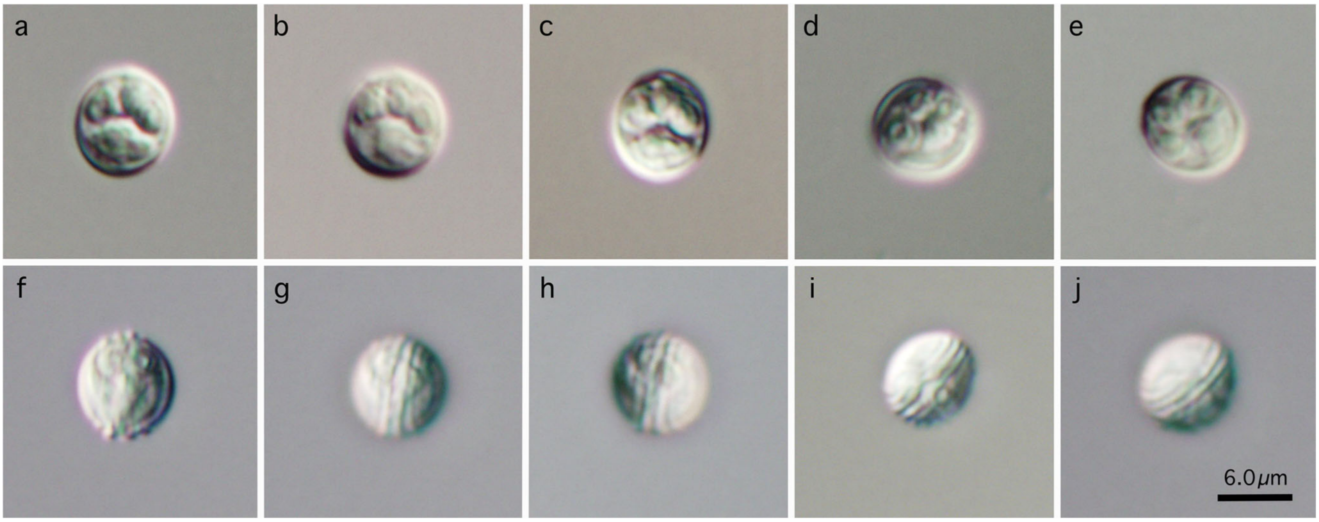


Fig. 1 Microscopic view of myxospores of *Chloromyxum trilineatum* n. sp. Valvular view (**a**–**c**), apical view (**d**, **e**), sutural view nearly at midline of sagittal plane (**f**), and sutural view nearly at surface (**g**–**j**) at the same magnification. Scale bar is shown in **j**

Binucleated sporoplasm in the remaining space. The turns of polar filaments were invisible.

Taxonomic summary

Host: *Zacco platypus* (Temminck et Schlegel, 1846), pale chub (Actinopterygii: Cypriniformes: Cyprinidae: Xenocypridinae).

Locality: Collected from Gifu World Fresh Water Aquarium (Aqua Totto Gifu), Gifu Prefecture, Japan, following its relocation from one of the rivers running through Gifu Prefecture, Japan. Circumstantially, the infection might originate from the water in the natural habitat and not in the aquarium.

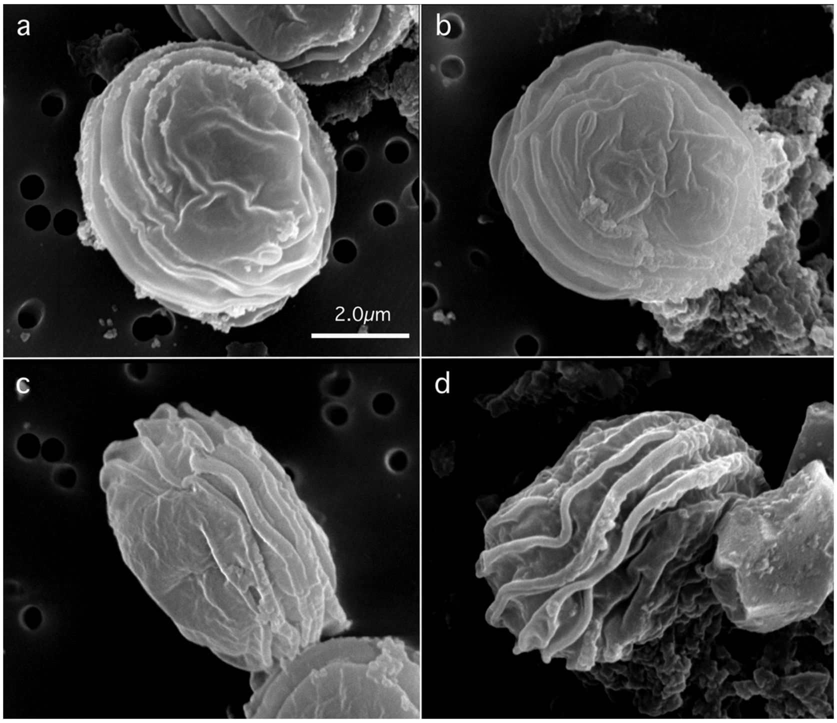
Site of infection: Gall bladder (coelozoic).

Materials deposited: Hapantotype no. 21458, Meguro Parasitological Museum, Tokyo, Japan.

Prevalence: Two of the five pale chubs examined.

Etymology: The species name refers to the characteristic morphology of spores, i.e., predominantly three distinct ridges parallel to the suture line.

Fig. 2 Scanning electron microscopy of myxospores of *Chloromyxum trilineatum* n. sp. Valvular view (**a**, **b**) and sutural view (**c**, **d**) at the same magnification. Scale bar is shown in **a**. Wrinkles at valvular surfaces are artifacts due to dehydration. Please note that originally subspherical spores became simple hamburger-shaped structures due to shrinkage of convex shell valves



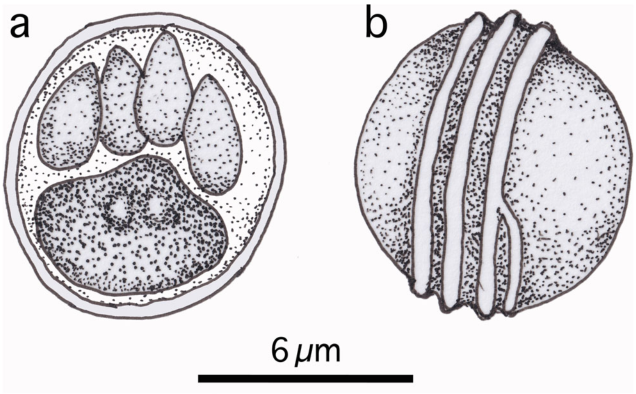


Fig. 3 Stylized illustrations of *Chloromyxum trilineatum* n. sp. Valvular view (**a**) and sutural view (**b**) at the same magnification

Remarks

In Japan, 13 *Chloromyxum* spp. have been described from freshwater and anadromous fish (Table 1). In 1927, *C. sphericum* was recorded from the same host species, i.e., pale chub, and Amur catfish (*Silurus asotus* L.) in Lake Biwa, Shiga Prefecture (Fujita 1927). Although the almost spherical spore shape and dimensions of *C. sphericum* are similar to the present species, the former species has only a single sutural ridge (elevation), but with nine or 10 extrasutural ridges on the shell valve, distinct from the present new species. With the exception of *Chloromyxum colchicum* (Chernova, 1969) in the gall bladder of *Rutilus rutilus* (L.) from Russia and *Chloromyxum dubium* (Auerbach, 1908) in the gall bladder of *Lota lota* (L.) from Karelia and the northwestern margin of Russia, other recorded species in Japan and other locations are distinct from the present species in having a variety of extrasutural edges on shell valves. *Chloromyxum colchicum*, however, has more edges parallel to the suture line, i.e., a sutural elevation and two thin extrasutural edges on each valve, and smaller spherical spores with a diameter of 6.6–7.3 μm (Chernova 1969). *Chloromyxum dubium* also has more edges parallel to the suture line, i.e. six extrasutural edges on each valve, and larger spherical spores with a diameter of 10.0–11.0 μm (Auerbach 1908). Another species, *Chloromyxum trijugum* (Kudo, 1920), in the gall bladder of *Lepomis megalotis* (Rafinesque, 1820) from North America has three distinct ridges encircling the spore, i.e., a thick sutural elevation and a thick straight, sometimes slightly zig-zag, ridge running parallel to the suture line in each valve (Kudo 1920). Although this feature is fairly similar to the phenotype of spores of the present new species, *C. trijugum* spores are somewhat flattened (8–10 μm in diameter from the frontal view and 5–7 μm in thickness), having eight to 12 short ridges from the prominent ridge towards the center of each valve (Kudo 1920). Thus, the present new species, *C. trilineatum* n. sp., is morphologically distinct from any known *Chloromyxum* spp.

Phylogenetic analysis of the rDNA

The partial nucleotide sequences of the 18S rDNA of two *Chloromyxum* isolates from different pale chubs in the present study were absolutely identical, and the 2.014-bp long sequence (DDBJ/EMBL/GenBank accession no. LC417364) was closest to that of morphologically distinct *Chloromyxum ellipticum* Li et Nie, 1973, infecting the gall bladder of grass carp (*Ctenopharyngodon idella* (Valenciennes, 1844)) in China (KX424575). This species and *C. trilineatum* n. sp. had 96.99% (1673/1725) identity of the 18S rDNA with three insertion/deletion (indel) sites, followed by *Chloromyxum legeri* Tourraine, 1931, infecting the gall bladder of common carp (*Cyprinus carpio* L.) in the Czech Republic (AY604197) with 89.97% (1803/2004) identity and 14 indel sites. Other myxosporean species, including *Chloromyxum* spp. from the gall bladder or urinary system of freshwater, estuarine, and marine fish as well as amphibians, were phylogenetically distant from the present new species, *C. ellipticum*, and *C. legeri* (Fig. 4).

Figure 4 depicts the phylogenetic relationships of *Chloromyxum* spp. and other representative myxosporean taxa with deposited 18S rDNA nucleotide sequences at the DNA databases, demonstrating the distinct polyphyletic nature of *Chloromyxum* spp. with multiple phylogenetic lineages. Marine (elasmobranch-infecting) *Chloromyxum* clade that presently included at least 11 taxa (Fiala et al. 2015; Cantatore et al. 2018) was independent from other five lineages as follows: (1) species infecting the gall bladder of cyprinids and salmonids (*C. auratum*, *C. cyprini*, *C. fluviatile*, *C. peleci*, *C. thymalli*, and *C. truttae*); (2) species infecting the gall bladder of cyprinids (*C. ellipticum*, *C. legeri*, *and C. trilineatum* n. sp.); (3) species infecting the gall bladder of North American freshwater fish (*C. trijugum*); (4) species infecting the urinary system of estuarine fish (*C. argusi* and *C. kurisi*); and (5) species infecting the urinary system of amphibians (*C. careni*).

Discussion

As stated above, the genus *Chloromyxum* is speciose and comprises approximately 140 nominal species, according to Eiras et al. (2012). Lom and Dyková (2006) previously cataloged 112 species from marine and freshwater fish and three species from amphibians in this genus. The difference between these two reports can be partially ascribed to the addition of at least 13 new species to the genus since the publication of Lom and Dyková's report (Hallett et al. 2006; Abdel-Baki 2007; Sarkar 2007; Azevedo et al. 2009; Casal et al. 2009; Reda 2010; Gleeson and Adlard 2012), as well as to the different perspectives on synonyms (Shul'man 1966; Lom and Dyková 1993; Fiala and Dyková 2004). For

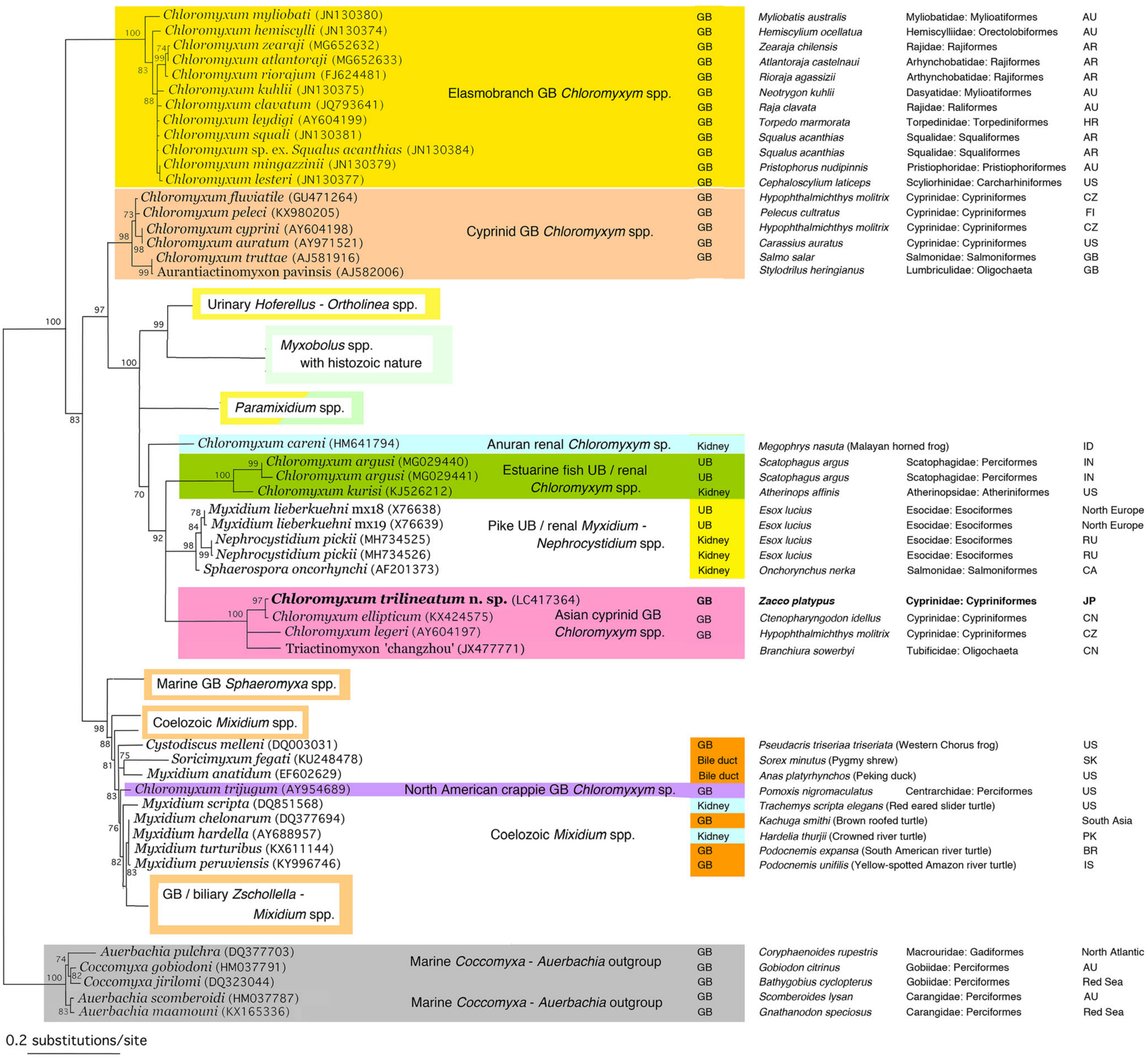


Fig. 4 ML phylogenetic trees based on the 18S rDNA sequence of *Chloromyxuum* spp. and related bivalvulid myxosporeans. A majority of non-*Chloromyxum* spp. were omitted from this figure as individuals, but shown as groups. In the right half of the figure, tissue preference, host fish species with its family and order in classification, and country name as the type locality are shown. Abbreviations of tissue preference: GB, gall bladder; and UB, urinary bladder. Abbreviations of country names: AR, Argentin; AU, Australia; BR, Brazil; CA, Canada; CN, China; CZ, Czech Republic; FI, Finland; GB, United Kingdom; HR, Croatia; ID, Indonesia; IN, India; IS, Iceland; JP, Japan; PK, Pakistan; RU, Russian Federation; SK, Slovakia; and US, United States

example, Lom and Dyková (1993) conducted an intensive SEM investigation on *Chloromyxum* spores of multiple origins. They concluded that *C. koi* Fujita, 1913, *C. cyprini* Fujita, 1927, and *C. barbi* Dogiel, 1934, may be junior synonyms of *C. cristatum* Léger, 1906, with the additional possible synonyms of *C. ctenopharyngodonis* Akhmerov, 1960, *C. hypophthalmichtydis* Akhmerov, 1960, and *C. orbiculatum* Akhmerov, 1960. Interestingly, the majority of *Chloromyxum* spp. were rarely detected again. This is partially due to the coelozoic nature of myxosporeans, which makes it difficult to detect parasitism with the naked eye, as well as the limited availability of spores. Consequently, this parasitic mode undoubtedly hampers the taxonomic revision of *Chloromyxum* spp.

Ultrastructural observation of *Chloromyxum* myxospores appears to be critical to characterize individual species. It allows for taxonomic characterization based on suture elevation and the running condition of the extrasutural edges on the shell valves (Lom and Dyková 1993). At the same time, we should take into account the recent achievements regarding *Myxobolus* spp., the most speciose genus of Myxosporea, which suggest that morphologically similar spores of *Myxobolus* spp. from different localities do not prove their conspecificity (Salim and Desser 2000; Bahri et al. 2003; Ferguson et al. 2008; Liu et al. 2010, 2013; Zhang et al. 2010; Zhao et al. 2013; Liu et al. 2016). In this respect, the contribution of molecular techniques is required, particularly rDNA nucleotide sequencing, for the reliable specific differentiation of myxozoans. Furthermore, this technique allows the phylogenetic relationships of different species to be inferred. As mentioned above, the currently recognized *Chloromyxum* spp. are heterogeneous in both morphology and phylogeny (Fiala and Dyková 2004; Holzer et al. 2006; Lom and Dyková 2006; Jirků et al. 2011; Gleeson and Adlard 2012; Rocha et al. 2013; Liu et al. 2017; Zhang et al. 2017; Chandran et al. 2018).

Since only a limited number of rDNA nucleotide sequences of *Chloromyxum* spp. is currently available (27 taxa; ca. 19%), the phylogenetic relationships of only some representative members of the genus are disclosed at present, as shown in Fig. 4. *Chloromyxum* spp. form at least two major distant clades: a marine (elasmobranch-infecting) *Chloromyxum* clade (currently 11 taxa) and a cyprinid and salmonid fish-infecting *Chloromyxum* clade (currently six taxa). By adding the present new species, *C. trilineatum* n. sp., to the phylogenetic analysis, a second cyprinid fish-infecting *Chloromyxum* clade (three taxa) becomes evident. All members of these three major clades, currently only 20 taxa, are coelozoic in the gall bladder of fish hosts. Regarding the three species in the second freshwater fish-infecting *Chloromyxum* clade, the myxospores of *C. legeri* from *Hypophthalmichthys molitrix* and *C. ellipticum* from *Ctenopharyngodon idella* have a relatively similar morphology with fine extrasutural edges on the shell valves (Fiala and Dyková 2004; Zhang et al. 2017); however, *C. trilineatum* n. sp. from *Zacco platypus* is distinct from them, having three sutural ridges and a smooth valvular surface (Figs. 1, 2, and 3). Additional nucleotide sequence data of more *Chloromyxum* spp., particularly that related to species demonstrating a solitary position on a phylogenetic tree based on the 18S rDNA, such as *C. careni* from the urinary system of frogs (Mutschmann 1999), *C. argusi* and *C. kurisi* from the urinary system of estuarine fish (Sanders et al. 2015; Chandran et al. 2018), and *C. trijugum* from the gall bladder of centrarchid fish (Mitchell et al. 1980; Hallett et al. 2006), would help to resolve the taxonomic complications and determine the evolutional pathways of species currently classified under the *Chloromyxum* genus (Fiala and Dyková 2004; Hallett et al. 2006; Jirků et al. 2011; Gleeson and Adlard 2012; Rocha et al. 2014; Liu et al. 2017). Furthermore, as is evident in Fig. 4, the enrichment of available nucleotide sequences, at least those of the rDNA of not only different homoplasious groups of the genus *Chloromyxum* but also other genera, is fundamental to fully understand the evolution of Myxosporea (Fiala 2006; Fiala et al. 2015).

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Compliance with ethical standards

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

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