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Sinuolinea infections in the urinary system of *Cynoscion* species (Sciaenidae) and phylogenetic position of the type species of *Sinuolinea* Davis, 1917 (Myxozoa: Myxosporea)

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ABSTRACT

Myxosporean infections that we diagnosed frequently in the urinary tract of *Cynoscion nebulosus* (Cuvier, 1830) and *Cynoscion regalis* (Bloch and Schneider, 1801) (Sciaenidae) collected in the estuarine systems of SC, USA, are described together with their etiological agent. Based on the morphology of spores and plasmodial stages, we identified the agent, in both fish host species, as *Sinuolinea dimorpha* (Davis, 1916), which is the type species of the genus. Based on sequences of SSU rDNA generated in this study from type host material, this species of *Sinuolinea* Davis, 1917 has found its place in the current phylogenetic reconstruction of Myxozoa and enlarged the limited number of myxosporean genera represented in phylogenetic analyses by sequences of type species. Sequences of SSU rDNA of *S. dimorpha* from *Cynoscion* host species formed two clusters, irrespective of their host species, and also revealed differences within each cluster. These findings contribute to the acknowledgement of myxosporean cryptic species diversity, an important topic that emphasizes the general necessity of species delimitation and of continued effort to improve our knowledge of Myxosporea based on both morphology of spores and molecular data.

1. Introduction

As part of an investigation of parasite fauna of *Cynoscion nebulosus* (Cuvier, 1830), an important food and sport fish of the family Sciaenidae, internal organs were screened histologically for the presence of myxosporean infections. *Kudoa* infections in muscles and *Henneguya* infections in hearts of this host have been described previously. Along with these infections, myxosporean plasmodial stages containing spherical spores were found in ureters and urinary bladders of over 40% of *C. nebulosus* examined (Dyková et al., 2009, 2011).

These latter findings turned our attention to known myxosporean infections of the urinary system of a phylogenetically closely related host, *Cynoscion regalis* (Bloch and Schneider, 1801; Vergara-Chen et al., 2009). Myxosporean infections of this latter host were studied by Herbert Spencer Davis, Professor of zoology at the University of Florida, USA, 100 years ago. He described a new myxosporean species, *Sphaerospora dimorpha* Davis, 1916 from the urinary bladder of *C. regalis* collected in the Beaufort region, North Carolina (Davis, 1916). A year later, he erected a new genus *Sinuolinea* Davis, 1917 and *Sphaerospora dimorpha* became its type species as *Sinuolinea dimorpha* (Davis, 1916). The study containing this taxonomic decision was published as document No. 855 of the Bulletin of the United States Bureau of Fisheries, issued on December 17, 1917 (Davis, 1917), which became part of the Bulletin of the United States Bureau of Fisheries vol. 35 published in 1918 although in an unchanged form (Davis, 1918).

The goal of the current study was to obtain comparative data to identify the myxosporean with spherical spores from *C. nebulosus*. This was accomplished by the examination of fresh material from urinary bladders of both *C. nebulosus* and *C. regalis*. The specimens collected were characterised on the basis of morphology and by molecular analyses, and a phylogenetic analysis was performed, with the aim of developing a dataset of type species sequences for *Sinuolinea* Davis, 1917. These data are not currently available for *Sinuolinea* or for most other myxosporean genera.

2. Materials and methods

2.1. Fish hosts

As in previous papers (Dyková et al., 2009, 2011), and in agreement with other authors (see review by Bortone, 2003), we follow

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Nelson et al. (2004) and use the officially accepted common names "spotted seatrout" for *C. nebulosus* (Cuvier, 1830) and "weakfish" for *C. regalis* (Bloch and Schneider, 1801).

A total of 227 spotted seatrout was collected for histopathological survey of myxosporean infections of this host in four estuaries (Winyah Bay, Bull's Bay, Charleston Harbor, Ashepoo-Combahee-Edisto Basin) of SC from April 2009 through May 2010 (Dyková et al., 2009, 2011). Total length of spotted seatrout specimens examined histologically ranged from 276 to 663 mm. In June, July and the beginning of August 2011, the sample was supplemented with 36 specimens whose total length ranged from 263 to 496 mm. A sample of 43 weakfish with total lengths ranging from 55 to 120 mm was collected from crustacean trawls in the Charleston Harbor, SC in June and July 2011. The fish collected in 2011 were used for examination of fresh myxosporeans, for the study of their ultrastructure, and for molecular studies. DNA samples from all *C. nebulosus* collected and from a sub-sample of *C. regalis* were archived at the Charleston Department of Natural Resources, SC, USA.

2.2. Myxosporea

Contents of 15 *C. nebulosus* and 15 *C. regalis* urinary bladders were examined fresh, under cover slips, on slides covered with a thin layer of 1.5% agar, and also in "chambers" (Davis, 1916). An Olympus BX40 microscope was used for observation and a digital camera was used for documentation.

For examination of fine structure of myxosporeans, whole urinary bladders as well as samples of their contents were fixed in cacodylate buffered 2.5% glutaraldehyde at 4 °C, stored in cacodylate buffer with sucrose, rinsed in 0.1 M cacodylate buffer and postfixed in 1% osmium tetroxide. After graded acetone dehydration, the samples were embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEOL JEM 1010 electron microscope operating at 80 kV. Images were collected with Megaview II soft imaging system using analySIS software.

2.3. DNA data collection

To sample material for DNA extraction, we collected either the contents of urinary bladders using sterile Pasteur pipettes, or in the case of the smallest *C. regalis*, the whole urinary bladders. A total of 23 samples were collected from *C. nebulosus* and 21 from *C. regalis*. Samples were fixed in 95% ethanol or in 400 µl of TNES urea buffer (10 mM Tris–HCl pH 8, 125 mM NaCl, 10 mM EDTA, 0.5% SDS, 4 M urea) and kept in the refrigerator. Samples in ethanol were centrifuged for 3 min at 2500*g*. Ethanol was removed by pipetting and by evaporation at 50 °C. TNES urea buffer (400 µl) was added into the dried sample. DNA was extracted by standard phenol–chloroform protocol after digestion with proteinase K (100 µg ml^{-1}) overnight at 55 °C. The extracted DNA was resuspended in 100 µl of sterile dd H_2O and kept at 4 °C. SSU rDNA sequences were obtained by PCR using universal eukaryotic ERIB1–ERIB10 primers (Barta et al., 1997). If the PCR failed, a nested secondary PCR was performed using a set of two primers ERIB1–Act1R (Hallett and Diamant, 2001) and MyxGen4F (Diamant et al., 2004)–ERIB10 to obtain a complete SSU rDNA sequence from two overlapping fragments. Alternative nested PCR with MyxospecF–MyxospecR (Fiala, 2006) was performed to obtain a partial sequence in the case of negative results of the nested PCR. All PCRs were carried out in a 25 µl reaction using 1× Taq buffer, 250 µM of each dNTPs, 10 pmol of each primer, Taq-Purple polymerase (Top-Bio, Czech Republic) and sterile dd H_2O. Cycling parameters for the primary PCR were 95 °C for 3 min, then 30 cycles at 95 °C for 1 min, 48 °C for 1 min, 72 °C for 1 min and 45 s and followed by a 10 min incubation at 72 °C. Amplification of nested PCR consisted of 95 °C for 3 min, then 30 cycles at 95 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min and followed by a 10 min incubation at 72 °C.

PCR products were separated by agarose gel electrophoresis, cut out of the gel and purified using Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech, USA). PCR products were sequenced directly or cloned into pDrive Cloning vector (Qiagen, Germany) and transformed into the competent *Escherichia coli* strains XL-1. PCR products or plasmid were sequenced on an ABI PRISM 3130x1 automatic sequencer (Applied Biosystems, Czech Republic).

In order to verify the host species for one *Sinuolinea* sample (No. 1296), cytochrome *c* oxidase (CO1) of the host was amplified. PCR was carried out using HCO2198 and LCO1490 primers (Folmer et al., 1994) and with the same PCR reagents as those used for the amplification of the SSU rDNA. PCR cycling parameters were 94 °C for 3 min, then 30 cycles 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min and incubated for 10 min at 72 °C.

2.4. Phylogenetic analysis

The contigs were assembled in the SeqMan II program v5.05 (DNASTAR Inc., Madison, Wisconsin). The SSU rDNA alignments were created in programme MAFFT v6.864 using L-INS-i strategy and default parameters (Katoh and Hiroyuki, 2008). Alignments were performed on newly obtained sequences and data retrieved from the GenBank. For one sequence of *Sinuolinea* sp. (Acc. No. AF378346), 450 bp were excluded because they were identical to the fish host *Psetta maxima* as previously reported by Holzer et al. (2010). Highly variable parts of the SSU rDNA sequences were determined and excluded by Gblocks (Castresana, 2000) using less stringent parameters. The alignments were visualised and checked in the Clustal X v2.1 (Larkin et al., 2007).

Phylogenetic analyses were performed using Maximum Likelihood (ML), Maximum Parsimony (MP) and Bayesian Inference (BI). ML was done in the RAxML v7.0.3. (Stamatakis, 2006) with GTR GAMMA model of evolution. MP was performed in the PAUP* v4.0b10 (Swofford, 2003) with heuristic search with random taxa addition and the TBR swapping algorithm. All characters were treated as unordered, Ts:Tv ratio was set to 1:2, and gaps were treated as missing data. BI was computed in the MrBayes v3.0 (Ronquist and Huelsenbeck, 2003) with the $GTR + \Gamma + I$ model of evolution. Posterior probabilities were calculated over 1,000,000 generations via two independent runs of four simultaneous Markov chain Monte Carlo chains with every 100th tree saved. The length of burn-in period was set to 10% (100,000 generations). For ML and MP, the bootstrap supports were calculated from 500 replicates. Trees were visualised in the TreeViewX v0.5.0 (Page, 2005). The distance matrix of the sequences listed in Table 1 was calculated in PAUP *v4.0b10 using uncorrected *p*-distances.

Table 1
Genetic similarities (in %) among two representative sequences of each three lineages ("*Sinuolinea* sp. 1", "*Sinuolinea* sp. 2" and unidentified species) with closest relative *Latyspora scomberomori*.

		1	2	3	4	5	6	7
1	*Sinuolinea* sp. 1 (1288)	–						
2	*Sinuolinea* sp. 1 (1295)	98.2	–					
3	*Sinuolinea* sp. 2 (833)	92.4	91.1	–				
4	*Sinuolinea* sp. 2 (917)	92.7	92.0	97.2	–			
5	Unidentified species (915)	90.1	88.9	90.6	90.8	–		
6	Unidentified species (918)	89.4	88.6	89.6	90.2	95.1	–	
7	*Latyspora scomberomori*	85.0	82.0	84.1	84.1	83.6	83.6	–

3. Results

3.1. Histological findings in the urinary system of C. nebulosus

Myxosporean plasmodial stages that differed in size and structure within the same host were found in urinary bladders and collecting ducts of 45.8% (104 out of 227) *C. nebulosus* examined (Figs. 1 and 2). In numerous cases these stages almost completely filled the ureters (Fig. 1A and B) and formed a continuous layer covering the inner surface of urinary bladders (Fig. 2A). They were never found in the convoluted tubules of the kidney. Small (Fig. 1B), as well as giant plasmodial stages (Fig. 1C), could be seen attached to the urinary bladder epithelium in some sections. The giant plasmodia frequently exceeded 200 μm in length, and contained fine granular or fine reticulate cytoplasm with a small number of developing pansporoblasts that appeared to be hollow. Plasmodia had a pronounced brush border-like layer on their surface (Fig. 1C). Spores in various stages of development were detected in plasmodial stages but in only 10% of fish (Fig. 2B). Spores were mostly immature and had an irregular shape whereas mature, rounded spores with well-defined shells were rare. Those mature spores had two rounded polar capsules (visualised with Giemsa staining) and some showed two nuclei of sporoplasm (Fig. 2D). Other rare findings included elongated, ribbon-like plasmodial stages attached to the surface of giant plasmodia but that did not contain either developing or mature spores (Fig. 2C).

3.2. Fresh material

Observation of fresh contents of urinary bladders of *C. nebulosus* and *C. regalis* showed that myxosporeans were very similar. Examination of this material confirmed the observations from histological sections of *C. nebulosus* and added information on plasmodial stages and spores (Fig. 3). Both giant plasmodial stages (Fig. 3F) and much smaller disporic stages (with the length ranging from 30 to 60 μm) (Fig. 3E) were observed in both fish hosts.

In each fish host, mature spores displayed features of the genus *Sinuolinea* Davis, 1917 (Fig. 3A–D): shape spherical (rounded in all views); diameter range 14.8–15.0 μm; shell valves thin, well defined in mature spores only; sutural line sinuous, difficult to observe over its entire course; two rounded polar capsules of equal size, set wide apart; 5–7 coils of polar filament. Under slight

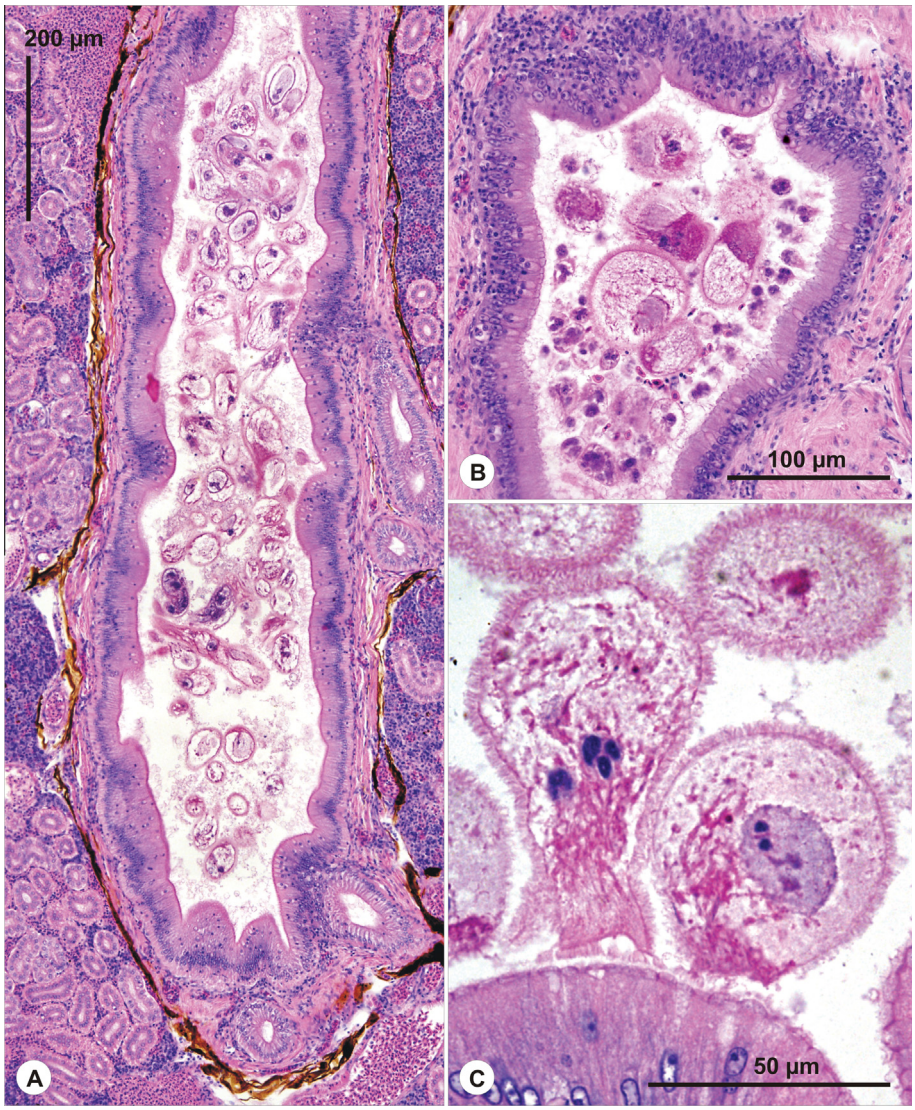

Fig. 1. *Sinuolinea dimorpha* infection in urinary tract of *Cynoscion nebulosus*. Histological sections stained with haematoxylin and eosin. (A) Plasmodial stages within the lumen of collecting duct. (B) Plasmodial stages in terminal part of ureter, small ones seen attached to epithelium, giant ones with brush border-like periphery. (C) Detail of giant plasmodial stages with hair-like processes on their entire free surface.

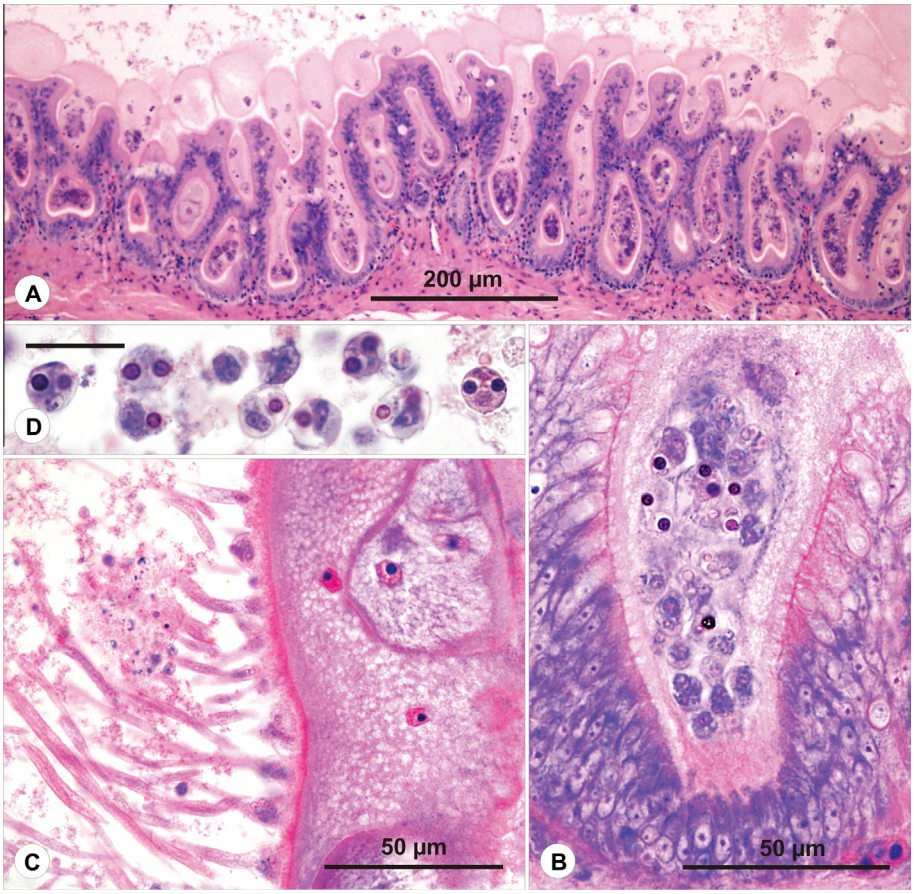

Fig. 2. *Sinuolinea dimorpha* infection in urinary bladder of *Cynoscion nebulosus*. Histological sections stained with haematoxylin and eosin. (A) Plasmodial stages with developing spores filling spaces between folds of urinary bladder wall. Longitudinal section. (B) Detail of plasmodium from urinary bladder containing developing spores. (C) Enigmatic thin ribbon-like plasmodia (left) attached to the surface of giant plasmodium (right). (D) Spores of *S. dimorpha* as seen in histological sections. Among developing spores only one (right-most) seems to be mature. Scale bar = 20 µm.

coverslip pressure, the entire course of the suture line could be observed and was identical to that documented in the original description (Davis, 1916), but the shape of the spore slightly changed under the pressure (Fig. 3A lower left). Also, part of the material collected during one trip was observed repeatedly over 48 h, during which time spores that were not fully mature changed quickly, increasing in diameter/volume. All features observed in fresh specimens indicated that the myxosporean species studied in *C. nebulosus* and *C. regalis* was morphologically consistent with *Sphaerospora dimorpha* as described by Davis in 1916 (and 1917 when he erected the new genus *Sinuolinea*).

In addition to being infected with *Sinuolinea*, urinary bladders of two *C. nebulosus* specimens contained several spores resembling those determined by Davis (1918) as *Leptotheca* sp. Mixed myxosporean infections of *C. regalis* specimens could not be ruled out, because urinary bladders were too small for their contents to be checked before sampling for DNA extraction.

3.3. Ultrastructure of spores and plasmodial stages

The presence of disporic and giant plasmodial stages in the urinary bladders was confirmed in *C. nebulosus* and *C. regalis*. Details of spore and plasmodial structures that were difficult to observe in fresh material and in histological sections were visualised on ultrathin sections. Although only developing spores were found in ultrathin sections (Fig. 3H), the structure of polar capsules, including the number of polar filament coils (Fig. 3C and D), was identical in both fish hosts. Also, the brush border-like layer (the microvillous surface) of giant plasmodial stages (Fig. 3G) could be observed in detail in both fish hosts.

3.4. SSU rDNA sequence data

PCR analysis of DNA samples of from the urinary bladders of *C. regalis* revealed 8 myxosporean-positive samples out of 21. Cloning of PCR products and subsequent sequencing determined 10 different sequences. Comparison of the sequences obtained to those available in the GenBank using the BLAST search revealed the highest sequence similarities of six sequences to *Latyspora scomberomori* Bartošová et al., 2011 (with 97% query coverage and 85% maximum identities) and of four sequences to *Parvicapsula minibicornis* Kent, Whitaker and Dawe, 1997 (76% query coverage, 96% maximum identities and 98.6–99.3% sequence similarity). Two DNA samples contained mixed infections of these two myxosporeans.

PCR analysis of DNA samples from the urinary bladders of 23 *C. nebulosus* revealed 5 myxosporean-positive samples. Five different sequences were obtained, which were most similar to *L. scomberomori* according to the BLAST search (98% mean query coverage and 85% mean maximum identities).

The sequence similarity suggests that the myxosporean sequences revealed by the BLAST analysis to be closely related to *L. scomberomori* may be of different species origin (Table 1). The similarity of *Sinuolinea* sequences to *L. scomberomori* is 82.0–85.0%. The variability within the two *Sinuolinea* clades is low, and the sequence similarity is 91.1–98.2%.

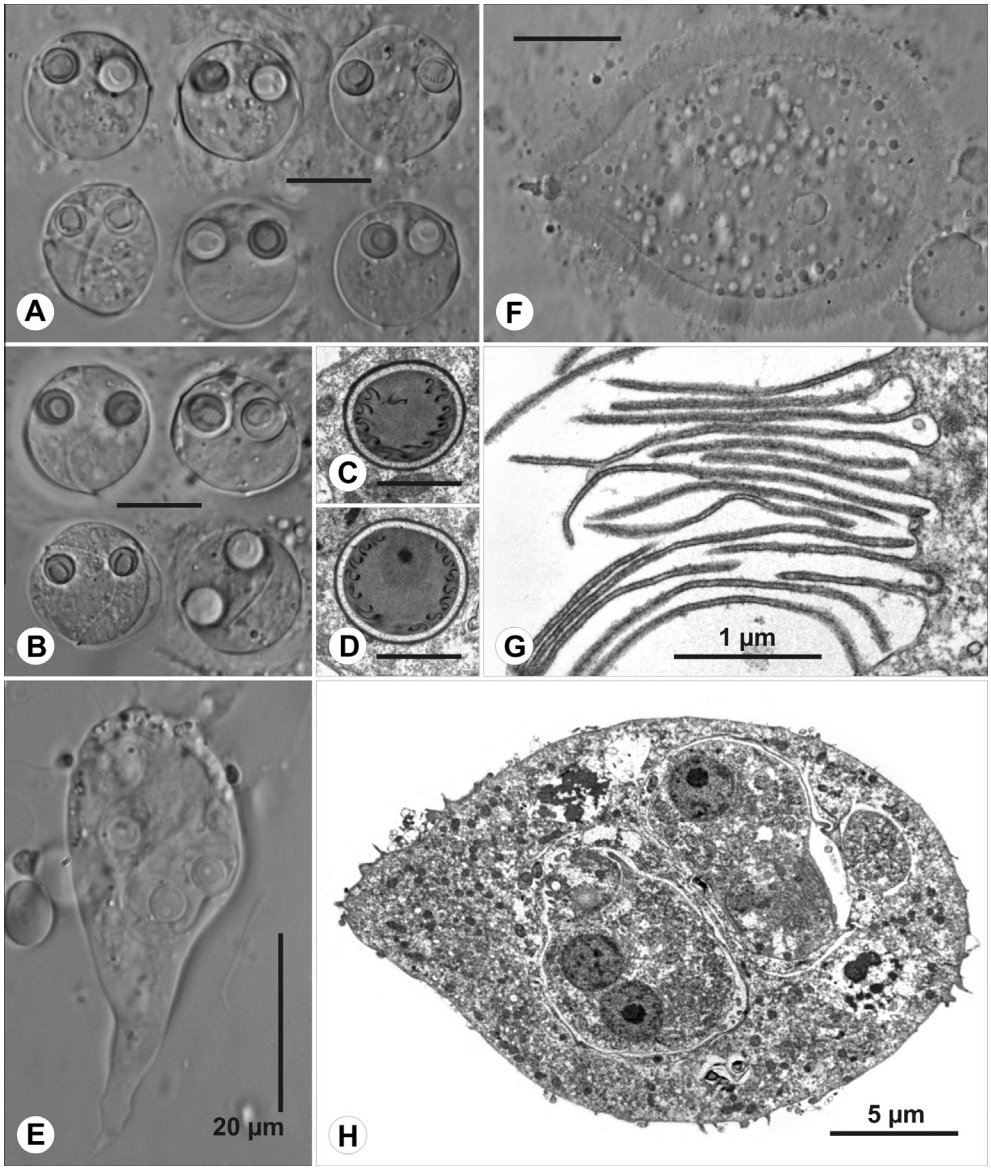

Fig. 3. *Sinuolinea dimorpha* spores documented from fresh material. Scale bars = 10 µm. (A) Spores from urinary bladder of *Cynoscion regalis*. Note left bottom spore with sinuous suture line seen in its entire course. (B) Spores from urinary bladder of *C. nebulosus*. (C and D) Polar capsules with polar filaments sectioned at the level of their turns. Ultrathin sections, scale bars = 2 µm. (E) Small disporic plasmodium from urinary bladder of *C. regalis*, seen in fresh. (F) Giant plasmodium of *S. dimorpha* from urinary bladder of *C. regalis* with long hair-like projections on its periphery, seen in fresh material. Scale bar = 100 µm. (G) Ultrastructure of brush border-like surface of giant plasmodium from urinary bladder of *C. regalis*. (H) Disporic plasmodium of *S. dimorpha* from *C. regalis*. Ultrathin section.

3.5. Phylogenetic analysis

All newly obtained sequences clustered together within the marine urinary clade (MUC) as defined in Bartošová et al. (2011) (Fig. 4). Altogether, 13 newly obtained sequences from three distinct groups (Fig. 5) clustered together within the *Zschokkella* subclade. Based on sequence data currently available, the closest relative of these three groups is *L. scomberomori*. This relationship is stable in all performed analyses and is supported with high bootstrap values and posterior probability. Five of six sequences from the *C. regalis* samples clustered together in one group that comprised species designated as "*Sinuolinea* sp. 1" because the differences in sequences represent intragenomic variability of a single species. The sequence similarity is 98.2% (Table 1). The second group consists of five sequences obtained from *C. nebulosus* and one sequence (*Sinuolinea* sp. 1296) from *C. regalis*. The determination of the host species of *Sinuolinea* sp. 1296 was verified by molecular analysis (the host's CO1 gene sequence obtained was 100% identical with CO1 of *C. regalis* available in the GenBank). The latter group of *Sinuolinea* sequences represents the second phylogenetically distinct *Sinuolinea* species ("*Sinuolinea* sp. 2") (the sequence similarity is 97.2%). The third group is composed of two sequences of unidentified myxosporean species from the urinary bladder of *C. nebulosus* and is basal to both these *Sinuolinea* spp. (the sequence similarity is 95.1%).

We consider the type species *S. dimorpha* to be defined by the sequences of "*Sinuolinea* sp. 1" (Fig. 5). The length of the representative, complete SSU rDNA, sequence of *S. dimorpha* (Acc. No. JX460905) is 1991 bp with a GC content of 44%. The length of the complete SSU rDNA sequence of "*Sinuolinea* sp. 2" (Acc. No. JX460906) is 1990 bp with a CG content of 45%. The partial sequence of the unidentified species UB 915 (Acc. No. JX460904) is 1694 bp long with a CG content of 47%, and the partial sequence of another unidentified species UB 918 (Acc. No. JX460908) is 1691 bp long with a CG content of 47%.

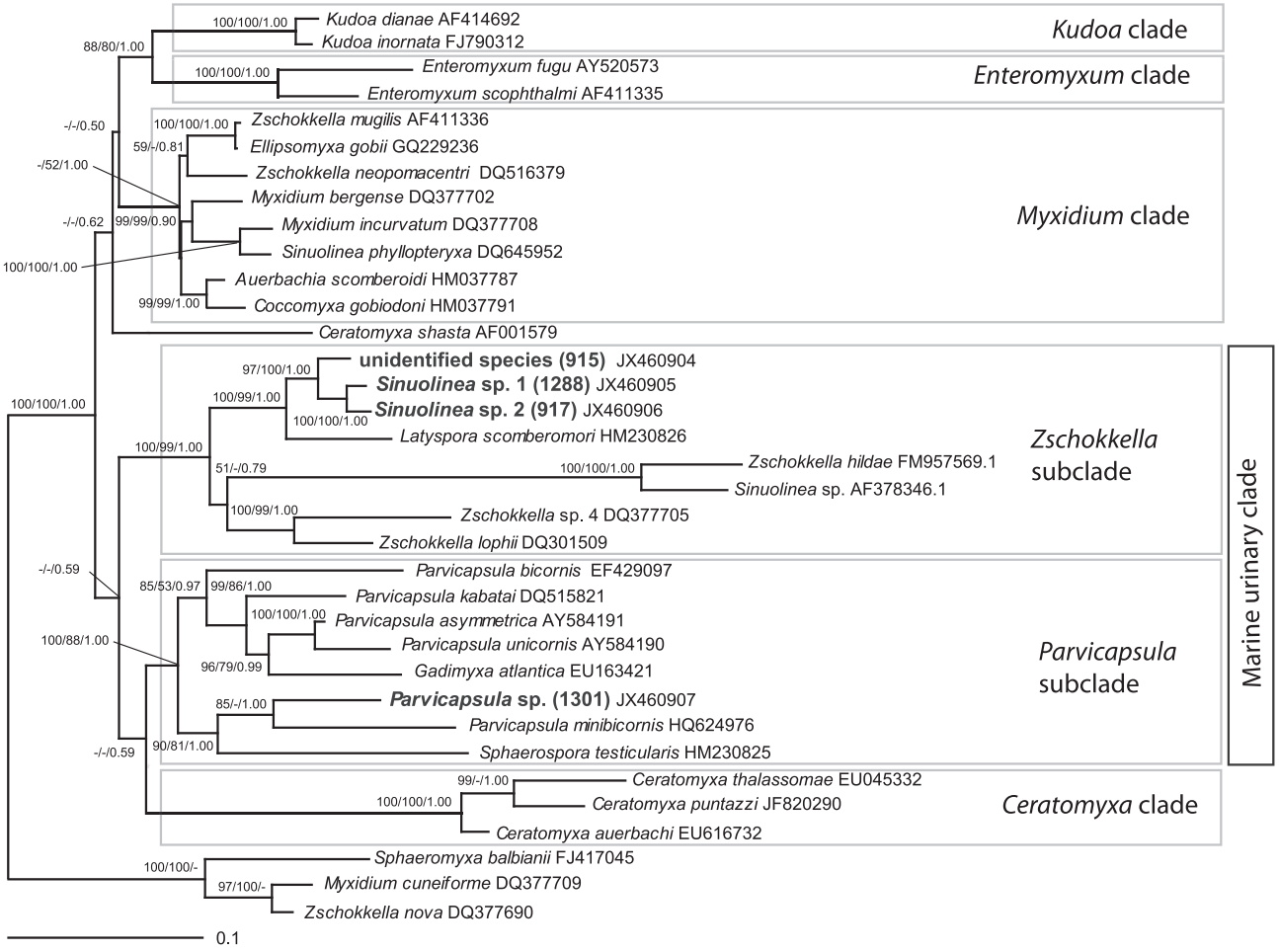

Fig. 4. Maximum likelihood tree (−ln = 2829.6205) based on the SSU rDNA of the selected sequences of the marine myxosporean clades. Representatives of newly obtained sequences clustering in the *Zschokkella* subclade as well as *Parvicapsula* sp. in the *Parvicapsula* subclade are in bold. *Sphaeromyxa balbianii*, *Myxidium cuneiforme* and *Zschokkella nova* were set as outgroup. Numbers at the nodes represent the bootstrap values and the Bayesian posterior probability (ML/MP/BI) gaining more than 50% support (ML and MP) and 0.5 posterior probability (BI), respectively. The scale bar is provided under the tree.

Highly similar SSU rDNA sequences that branched in the *Parvicapsula* subclade and are represented in Fig. 4 by *Parvicapsula* sp. (Acc. No. JX460907) apparently belong to an undetermined parvicapsulid species that was not observed in material examined with light microscopy.

3.6. Taxonomic implications

The identical morphology of *Sinuolinea* spores and plasmodial stages under study allowed us to assign them as a single species, *S. dimorpha* (Davis, 1916). Molecular analyses revealed the existence of genetically diverse cryptic species, however, with SSU rDNA sequences that grouped into two well supported clades. The concept of cryptic species within the *S. dimorpha* complex is supported by the fact that *S. dimorpha* sequences from *C. regalis*, the type host of this species, form branches in both clades.

4. Discussion

Examination of fresh and fixed material from urinary bladders of *C. nebulosus* and *C. regalis* confirmed the original description of *Sinuolinea dimorpha* (Davis, 1916). To the best of our knowledge there are only a few myxosporean species for which spore and plasmodial-stage morphology was described as meticulously as for *S. dimorpha*, and thus the original description does not require emendation. Also, the line drawing documentation provided by Davis (1916) is exhaustive and can only be improved by supplementation with micrographs. Ultrastructural details obtained herein supported the original light microscope observations. Unfortunately, despite all efforts and because of collection conditions and the time required for transport of fish to the laboratory, the material designated for ultrastructural study could not be fixed as quickly as classical methods require. The best results were obtained when urinary bladders from small specimens of *C. regalis* were fixed on the boat immediately after they were extracted from the trawl net.

To the best of our knowledge, no voucher nor topotype specimens of *S. dimorpha* exist for comparison with the current material. Based on our experience, we stress the necessity to observe and document spores as fresh as possible, since they lose their typical form due to rapid post-mortem change in the urinary bladder contents. This detail is of importance because even spores with well-developed polar capsules and coils of filaments (seen in light microscopy) may still have thin shell valves when collected and thus, may change size after the death of the fish. Our investigations were restricted to the same months (June, July and August) as Davis (1916) dedicated to his original study of *S. dimorpha*, although the localities were about 400 km away from his collecting site. The rarity of mature spores in the fresh material examined in the current study supports the observation by Davis (1916) that these spores are voided very rapidly after release from the plasmodia.

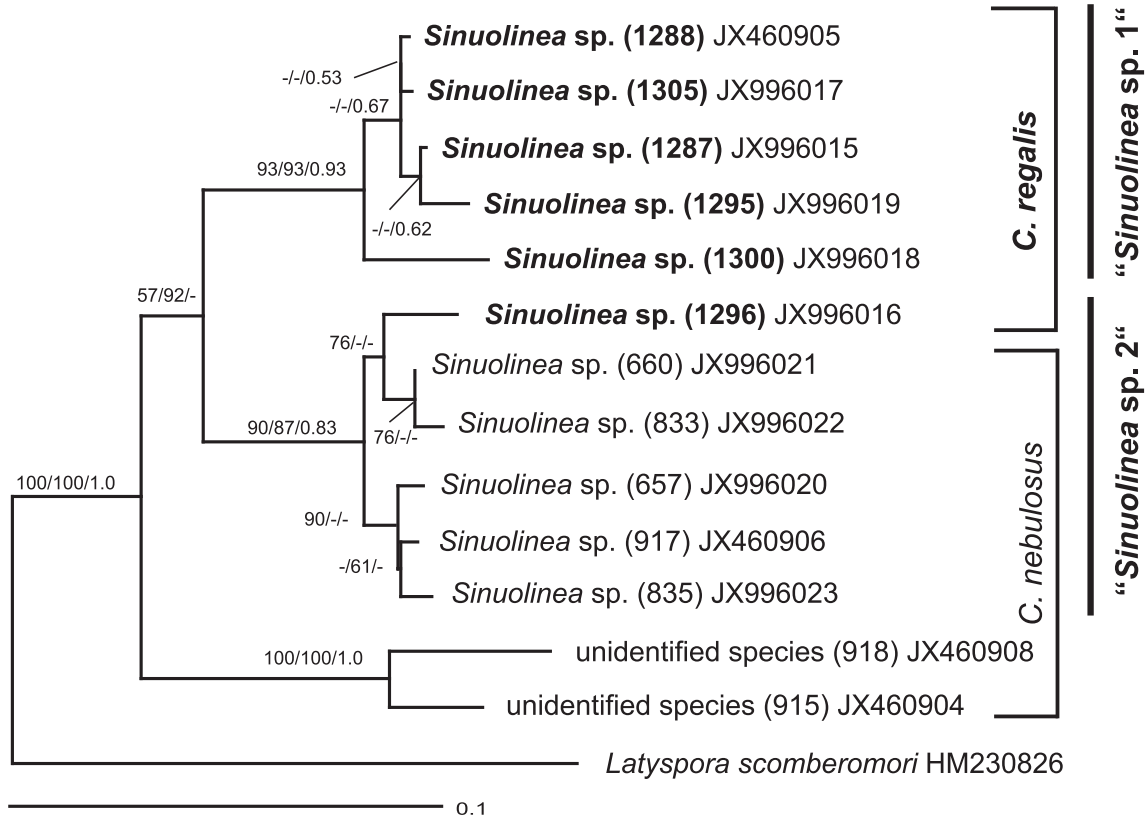

Fig. 5. Maximum likelihood tree (−ln = 13803.0789) based on the SSU rDNA of all newly obtained sequences which cluster within the *Zschokkella* subclade. *Latyspora scomberomori* was set as outgroup. Numbers at the nodes represent the bootstrap values and Bayesian posterior probability (ML/MP/BI) gaining more than 50% support (ML and MP) and 0.5 posterior probability (BI), respectively. The scale bar is provided under the tree.

Although the morphology of *S. dimorpha* was described in detail by Davis (1916, 1917), no data were included for size of hosts (*C. regalis*), which is partly a reflection of host age, or for the number of specimens examined for *Sphaerospora dimorpha* Davis, 1916 and *Sinuolinea dimorpha* (Davis, 1917). Davis (1916), however, recorded that "A large number of fish were examined and in no case was the bladder found to be entirely free from infection although there was great variation in the abundance of the parasites". His description of the handling of urinary bladders to collect spores and plasmodial stages suggests that he examined larger specimens of *C. regalis* than in the current study.

Dimorphism of plasmodial stages, a characteristic of *S. dimorpha*, is known in other myxosporeans, e.g., *Myxidium lieberkuehni* Bütschli, 1882 (see Lom et al., 1989). However, giant polysporic plasmodia of *S. dimorpha* are unique in having a brush border-like layer on their periphery whereas small disporic plasmodia do not exhibit this feature. In three newly described *Sinuolinea* species, Zhao and Song (2003) mentioned ecto- and endoplasm and pseudopodia of various shapes without providing any information about villosity on the surface of the plasmodia. Plasmodial stages of *Sinuolinea tetraodoni* El-Matbouli and Hoffmann, 1994 also lack this feature (El-Matbouli and Hoffmann, 1994).

Phylogenetic analysis revealed that all myxosporean sequences obtained from the samples of urinary bladders of both *C. nebulosus* and *C. regalis* clustered in the marine urinary clade. This branching pattern supports the previous observation that there is a general pattern, albeit with some inconsistencies, of myxozoan species clustering by tissue tropism (Holzer et al., 2004; Fiala, 2006). Of all the *Sinuolinea* species whose morphology has been described, the SSU rDNA sequence is only available for *Sinuolinea phyllopteryxa* from weedy sea dragons (Garner et al., 2008). The only other sequence deposited in GenBank (Acc. No. AF378346.1 under the misspelled name '*Sinuoelinea*') is not accompanied by a morphological description. The *Sinuolinea* sequences obtained in the current study did not show close relationships with either of these two sequences (*S. phyllopteryxa* clusters within the *Myxidium* clade, and *Sinuolinea* sp. with Acc. No AF378346.1 clusters in the *Zschokkella* subclade). Therefore, members of the genus *Sinuolinea* do not constitute one monophyletic group, which accentuates the incongruence of their taxonomy with their phylogeny.

Morphological similarity between *Sinuolinea* spp. and phylogenetically "closely" related *L. scomberomori* is limited to the sinuous suture line of the spore valves. Sequence similarity of *Sinuolinea* and *Latyspora* (currently the closest relative) is relatively low (85%), which can be explained by the lack of sequence data of more closely related species, an early evolutionary separation of these taxa, or fast evolution of their SSU rDNA.

The phylogenetic analysis and comparison of sequence similarity revealed that there are more than one species of *Sinuolinea* in the urinary bladder of both *Cynoscion* hosts. "*Sinuolinea* sp. 1" is the most abundant one in specimens of *C. regalis*; it also appears to be host specific for *C. regalis* whereas "*Sinuolinea* sp. 2" was found in both fish species but predominantly in *C. nebulosus*. The type species of *Sinuolinea*, *S. dimorpha*, was described from *C. regalis* but we do not know which one of the two *Sinuolinea* spp. we detected in *C. regalis* via molecular analysis was described by Davis (1916). We can assume, however, that he observed either "*Sinuolinea* sp. 1" only or both *Sinuolinea* sp. 1 and sp. 2, as we did, because we collected specimens from this type host during the same period of the year as Davis did, and within the area of distribution of *C. regalis*, although at a distance of about 400 km from the type locality. The cluster containing exclusively sequences of

Sinuolinea from *C. regalis* has been designated as representing *S. dimorpha* because of its origin from the type host.

The identification of cryptic species can potentially be a serious problem if the morphological description of a new species is not supplemented with proper molecular characterisation or if molecular data for a previously described species with well described morphology are limited to sequencing a single sample of DNA. The taxonomic challenge posed by cryptic species was recognized years ago (see Bickford et al., 2007 for review) and was also reported for myxosporeans, e.g., *Kudoa thyrsites* (see Whipps and Kent, 2006), *Chloromyxum* spp. (Bartošová and Fiala, 2011), *Cystodiscus* spp. (see Hartigan et al., 2012). On the other hand, some species can display a high morphological variability of myxospores during development (Lom and Hoffman, 1971; Lom and Dyková, 1995) and there may be differences in morphotypes of spores with identical SSU rDNA sequences as reported for *Kudoa hypoepicardialis* by Blaylock et al. (2004), although these were interpreted by Whipps and Kent (2006) as caused by genetic differences not resolved by the genes chosen for analyses. According to recent studies (e.g., Rissler and Apodaca, 2007; de Buron et al., 2011), patterns of genetic divergence may be strongly associated with divergence in ecological niche and environmental data may be important for species delimitation. This is of importance especially in myxosporeans, who have life cycle phases alternating between vertebrate and invertebrate hosts. The evolutionary origin of myxosporean cryptic species may be explained, therefore, by switching of vertebrate or invertebrate hosts.

Note

Nucleotide sequence data reported in this paper are available in the GenBank™ under the Acc. No. JX460904, JX460905, JX460906, JX460907, JX460908, JX96015, JX996016, JX996017, JX996018, JX996019, JX996020, JX996021, JX996022, JX996023, JX996024, JX996025

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