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# Evolutionary origin of *Ceratonova shasta* and phylogeny of the marine myxosporean lineage

Ivan Fiala [a,*], Marie Hlavničková [b], Alena Kodádková [a,b], Mark A. Freeman [c], Pavla Bartošová-Sojková [a], Stephen D. Atkinson [d]

[a] Institute of Parasitology, Biology Centre, The Czech Academy of Sciences, České Budějovice, Czech Republic
[b] Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic
[c] Institute of Ocean and Earth Sciences, University of Malaya, Kuala Lumpur, Malaysia
[d] Department of Microbiology, Oregon State University, Corvallis, USA

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ABSTRACT

In order to clarify the phylogenetic relationships among the main marine myxosporean clades including newly established *Ceratonova* clade and scrutinizing their evolutionary origins, we performed large-scale phylogenetic analysis of all myxosporean species from the marine myxosporean lineage based on three gene analyses and statistical topology tests. Furthermore, we obtained new molecular data for *Ceratonova shasta*, *C. gasterostea*, eight *Ceratomyxa* species and one *Myxodavisia* species. We described five new species: *Ceratomyxa ayami* n. sp., *C. leatherjacketi* n. sp., *C. synaphobranchi* n. sp., *C. verudaensis* n. sp. and *Myxodavisia bulani* n. sp.; two of these formed a new, basal *Ceratomyxa* subclade.

We identified that the *Ceratomyxa* clade is basal to all other marine myxosporean lineages, and *Kudoa* with *Enteromyxum* are the most recently branching clades. Topologies were least stable at the nodes connecting the marine urinary clade, the marine gall bladder clade and the *Ceratonova* clade. Bayesian inference analysis of SSU rDNA and the statistical tree topology tests suggested that *Ceratonova* is closely related to the *Enteromyxum* and *Kudoa* clades, which represent a large group of histozoic species. A close relationship between *Ceratomyxa* and *Ceratonova* was not supported, despite their similar myxospore morphologies. Overall, the site of sporulation in the vertebrate host is a more accurate predictor of phylogenetic relationships than the morphology of the myxospore.



## 1. Introduction

Myxosporeans (Cnidaria: Myxozoa) are microscopic parasites of vertebrates (fish, amphibians, rarely reptiles, birds and mammals) and invertebrates (polychaete and oligochaete worms). As myxosporean morphology is extremely simplified and unique, it took until the end of 20th century to demonstrate their taxonomic affinity to metazoan cnidarians (Siddall et al., 1995). The cnidarian origin was under discussion for more than 20 years utill multigene analyses (Jiménez-Guri et al., 2007; Nesnidal et al., 2013) and the identification of synapomorphic genes between the Cnidaria and Myxozoa (Holland et al., 2011) confirmed the myxozoan origin within Cnidaria. Myxosporeans evolved from a cnidarian ancestor closely related to the Medusozoa (Jiménez-Guri et al., 2007). They developed two novel forms, myxospores and actinospores, which are essential for their parasitic way of life. The only apparent synapomorphic morphological feature shared by both Cnidaria and Myxozoa are cells with extrudible filaments, referred to as nematocysts and polar capsules, respectively.

Taxonomy of the class Myxosporea has been based traditionally on morphology of myxospores, which develop in the intermediate vertebrate host. Myxospore morphology (structure, shape and size of spore, position of polar capsules etc.) has been the basis for assigning species to the ~60 myxosporean genera (Fiala and Bartošová, 2010), which contain ~2200 species (Lom and Dyková, 2006). Molecular studies have revealed discrepancies between this myxospore morphology-based taxonomy and phylogenetic relationships based on ribosomal DNA sequences. In many cases, morphologically distinct myxosporean species that had been assigned to different genera were shown by DNA sequencing to be related closely and some species with very similar spore morphologies are distantly related (Bartošová et al., 2009; Fiala, 2006; Holzer et al., 2004; Kent et al., 2001).

* Corresponding author at: Institute of Parasitology, Biology Centre of the CAS, Branišovská 31, 370 05 České Budějovice, Czech Republic. Fax: +42 0385310388.
*E-mail address:* fiala@paru.cas.cz (I. Fiala).

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Myxosporean evolutionary trends are indicated better by characters other than myxospore morphology. The primary division of myxosporean phylogenies is according to host habitat: either freshwater or marine (Fiala, 2006; Kent et al., 2001), with a third main grouping being the taxon-rich *Sphaerospora sensu stricto* clade (Bartošová et al., 2013; Jirků et al., 2007). However, significant exceptions (e.g. marine species clustering inside the freshwater lineage and *vice versa*) support the hypothesis that definitive host type (oligochaete vs. polychaete) might be the principle character that correlates with the main myxosporean lineages (Holzer et al., 2007).

Besides intermediate host environment and definitive host group, site of sporulation in the vertebrate host often correlates with subgrouping of species within both freshwater and marine myxosporean lineages (Bartošová et al., 2011; Fiala, 2006; Freeman et al., 2008; Holzer et al., 2004). The marine myxosporean lineage contains five clades sensu Fiala (2006) and Bartošová et al. (2011): the *Ceratomyxa* (1) and the marine gall bladder (2) clades (=gall bladder); the marine urinary clade (3) (=excretory system); *Enteromyxum* (4) and *Kudoa* (5) clades (=histozoic). Several of these clades contain species from multiple genera, i.e. multiple myxospore morphotypes: e.g. the marine urinary clade encompasses six genera (Bartošová et al., 2011; Kodádková et al., 2014); the marine gall bladder clade contains seven genera (Heiniger and Adlard, 2014).

One species within the marine myxosporean lineage with no firm relationship to any well-established clade is *Ceratonova shasta* (Noble, 1950) (syn. *Ceratomyxa shasta*). It is an economically important pathogen of salmonid fishes, in the Pacific Northwest of North America (Hoffmaster et al., 1988). The parasite sporulates typically in the fish intestine. *Ceratonova shasta* has a two host life cycle that alternates between salmonids and a freshwater polychaete, *Manayunkia* sp. (Bartholomew et al., 1997). Based on myxospore morphology, *C. shasta* was classified originally in the genus *Ceratomyxa*. However, its unique biological features and ambiguous phylogenetic position led to the transfer of *C. shasta* to the recently erected genus *Ceratonova* Atkinson, Foott, Bartholomew, 2014, which includes a newly discovered species *C. gasterostea* from the intestine of freshwater three-spined stickleback, *Gasterosteus aculeatus* Linnaeus, 1758 (Atkinson et al., in press). *Ceratomyxa* Thélohan, 1892 is a species-rich genus that includes 246 nominal species, which mostly are coelozoic in the gall bladder of marine fishes. Myxospore morphology of *Ceratomyxa* and *Ceratonova* species is similar, but *Ceratonova* differs from *Ceratomyxa* spp. by its site of sporulation (intestine vs. gall bladder), freshwater/brackish life cycle, and SSU rDNA sequence data (Atkinson et al., in press). Based on tissue tropism, *C. shasta* appears closely related to *Enteromyxum* spp. (Fiala and Bartošová, 2010), a relationship supported in the analyses of Freeman et al. (2008), though ambiguous in other analyses (e.g. Gunter et al., 2009).

In contrast to generally well-resolved clustering within the freshwater myxosporean lineage, the branching order of clades in the marine myxosporean lineage is not well-resolved and relationships of the main marine clades are unclear (Bartošová et al., 2009; Fiala, 2006). This is exemplified by the phylogenetic position of *C. shasta* in the SSU rDNA-based tree, which has only weak phylogenetic support for the nodes connecting this parasite with other myxosporeans. Resolution of the marine phylogeny has been improved by inclusion of data from other loci, primarily LSU rDNA (Bartošová et al., 2009) and the EF-2 gene (Fiala and Bartošová, 2010). Bootstrap analysis of LSU rDNA shows higher support for the main marine nodes, but subgroupings remained largely unresolved (Bartošová et al., 2009). *Ceratonova shasta* never clustered inside any of the five well-established clades, and its closest affinities vary depending on the analysis: e.g. sister to *Enteromyxum* spp. (Fiala, 2006; Fiala and Bartošová, 2010; Freeman et al., 2008), sister to *Parvicapsula* spp. (Gunter and Adlard, 2009; Heiniger et al., 2008; Køie et al., 2008), as a sister lineage to all marine groups except the marine gall bladder clade (Jirků et al., 2007) or as a sister to the marine gall bladder clade (Fiala and Dyková, 2004; Jirků et al., 2006). Analyses focused on all *Ceratomyxa* spp. available in GenBank placed *C. shasta* as a sister lineage to the *Ceratomyxa* clade (Gunter and Adlard, 2008; Gunter et al., 2009).

We sought to clarify the phylogenetic relationships between *C. shasta* and the main marine myxosporean clades, and draw conclusions about their evolutionary origins. We used comprehensive analyses of single and concatenated sequences of three molecular markers and statistical tests of topology. We provide new molecular data for *C. shasta*, *C. gasterostea*, and nine gall bladder-infecting myxosporeans (eight ceratomyxids and one *Myxodavisia* species, including novel species) to enlarge taxon sampling of our molecular dataset and be able to better reconstruct the phylogeny of the *Ceratonova*, *Ceratomyxa*, and the other main marine myxosporean clades.

## 2. Materials and methods

### 2.1. Myxospore samples and morphological analysis

*Ceratonova shasta* and *C. gasterostea* were obtained respectively from coho salmon (*Oncorhynchus kisutch*) and three-spined stickleback (*Gasterosteus aculeatus*), from the Klamath River, California, USA. Ceratomyxid samples were collected from the gall bladders of marine fishes in the North Sea, the Norwegian Sea, the Mediterranean Sea and the Andaman Sea (Table 1, Fig. 1). Myxospores were imaged under the Olympus BX53 microscope with Nomarski differential interference contrast equipped with an Olympus DP72 digital camera. Spore measurements given in the text as average (range) ± standard deviation followed the guidelines of Lom and Arthur (1989) and Heiniger et al. (2008). N specifies the number of spores measured.

### 2.2. DNA isolation, PCR amplification, cloning and sequencing

DNA was extracted from fresh spores using a Jetquick Tissue DNA Spin Kit (Genomed, Germany) or by a standard phenol/chloroform method (Sambrook et al., 1989), after overnight digestion with proteinase K (50 μg/ml) at 55 °C. PCRs were performed in 25 μl reaction volumes, which comprised 10 pmol each primer, 250 μM each dNTPs, 2.5 μl 10 × PCR Buffer (Top-Bio, Czech Republic) and 1 U Taq-Purple polymerase (Top-Bio, Czech Republic). The SSU rRNA gene was amplified with universal eukaryotic primers ERIB1 and ERIB10 (Barta et al., 1997). If the PCR failed to amplify desired products, a second-round, nested PCR was done with novel *Ceratomyxa*-specific primers 18S-cerF and 18S-cerR (see Table 2 for nucleotide sequences of primers). The LSU rRNA gene was amplified as two sequentially overlapping products: the 5'end by nested PCR using *Ceratomyxa*-specific primers 28S-cer5-F1 and 28S-cer5-R1 (first round) and 28S-cer5-F2 and 28S-cer5-R2 or 28S-cer5-R3 (second round); the 3'end by nested PCR using primers 28S-cer3-F1 and 28S-cer3-R1 (first round) and 28S-cer3-F2 and 28S-cer3-R2 (second round). The EF-2 gene was amplified by nested PCR using primers EF2-F and EF2-R (Hashimoto et al., 1995) (first round) and EF2int2F and EF2int2R (Bartošová et al., 2013).

PCR amplification consisted of 10 min of initial denaturation at 95 °C, then 30 cycles of 95 °C for 1 min, 48 °C for 1 min and 72 °C for 2 min, followed by 10 min incubation at 72 °C. PCR products of expected size were purified with Gel Extraction Spin Kit (Genomed, Germany) and sequenced directly or cloned into pDrive Cloning vector from the Qiagen PCR Cloning Kit (Qiagen,

**Table 1**
Myxosporeans used in this study: host, site of infection, geographical area and GenBank accession numbers of available sequences (bold = new data). Int = intestine, Gb = gall bladder, Kid = kidney.

| Myxosporean | Host | Site | Geographical area | SSU | LSU | EF2 |
|---|---|---|---|---|---|---|
| *Ceratonova shasta* | *Oncorhynchus kisutch* | Int | Klamath River, California, USA | AF001579 | FJ981818 | **KM392431** |
| *Ceratonova gasterostei* | *Gasterosteus aculeatus* | Int | Klamath River, California, USA | KF751186 | **KM392422** | - |
| *Ceratomyxa longipes* | *Melanogrammus aeglefinus* | Gb | North sea, off Scotland | KJ419343 | KJ417061 | **KM392432** |
| *Ceratomyxa longipes* | *Merlangius merlangus* | Gb | North sea, off Scotland | **KM273021** | - | - |
| *Ceratomyxa informis* | *Merlangius merlangus* | Gb | North sea, off Scotland | **KM273022** | - | - |
| *Ceratomyxa arcuata* | *Lophius piscatorius* | Gb | North sea, off Scotland | KJ419344 | KJ419342 | **KM392433** |
| *Ceratomyxa arcuata* | *Merlangius merlangus* | Gb | North sea, off Scotland | **KM273023** | - | - |
| *Ceratomyxa arcuata* | *Callionymus lyra* | Gb | North sea, off Scotland | **KM273024** | - | - |
| *Ceratomyxa appendiculata* | *Lophius piscatorius* | Gb | Norwegian sea, off Norway | KJ439054 | **KM392423** | **KM392434** |
| *Ceratomyxa appendiculata* | *Lophius piscatorius* | Gb | North sea, off Scotland | **KM273025** | - | - |
| *Ceratomyxa synaphobranchi* | *Synaphobranchus kaupii* | Gb | North sea | **KM273026** | **KM392424** **KM392425** | **KM392435** |
| *Ceratomyxa verudaensis* | *Scorpaena porca* | Gb | Mediterraean sea, off Croatia | **KM273027** | **KM392426** | **KM392436** |
| *Ceratomyxa leatherjacketi* | *Aluterus monoceros* | Gb | Andaman Sea, off Malaysia | **KM273028** | **KM392427** **KM392428** | **KM392437** |
| *Ceratomyxa ayami* | *Aluterus monoceros* | Gb | Andaman Sea, off Malaysia | **KM273029** | **KM392429** | **KM392438** |
| *Myxodavisia bulani* | *Megalops cyprinoides* | Gb | Andaman Sea, off Malaysia | **KM273030** | **KM392430** | **KM392439** |
| *Parvicapsula minibicornis* | *Gasterosteus aculeatus* | Kid | Oregon, USA | AF201375 | KF874224 | **KM392440** |

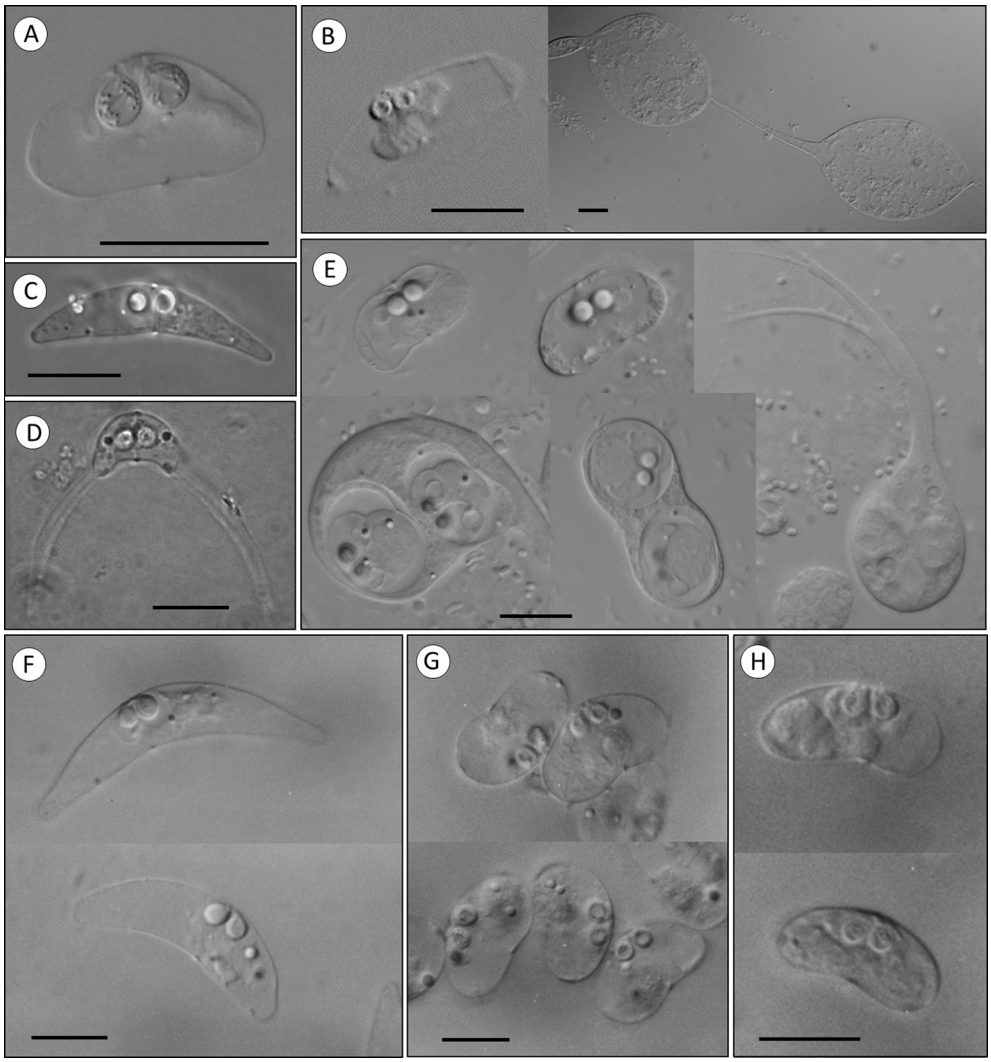


**Fig. 1.** Micrographs of mature myxospores and plasmodia. A – *Ceratomyxa verudaensis* n. sp.; B – *Ceratomyxa synaphobranchi* n. sp.; C – *Ceratomyxa leatherjacketi* n. sp.; D – *Myxodavisia bulani* n. sp.; E – *Ceratomyxa appendiculata*; F – *Ceratomyxa arcuata*; G – *Ceratomyxa informis*; H – *Ceratomyxa longipes*. Bars = 10 μm.

Germany) and transformed into competent *E. coli*-strain XL-1. Both strands of PCR products or their clones in plasmid vectors were sequenced on an ABI PRISM 3130XL automatic sequencer (Applied Biosystems, Czech Republic). Contigs were assembled using DNA Star SeqMan II v5.05 (DNASTAR Inc., Madison, Wisconsin).

**Table 2**
List of primeres used in the study with references.

| Primer name | Primer sequence (5́-3́) | References |
|---|---|---|
| ERIB1 | ACCTGGTTGATCCTGCCAG | Barta et al. (1997) |
| ERIB10 | CTTCCGCTGGTTCACCTACGG | Barta et al. (1997) |
| 18S-cerF | CTWGTTGGTADGGTAGTG | This study |
| 18S-cerR | GTACAAGAGGCAGAGACGTAT | This study |
| 28S-cer5-F1 | GTAACTGCGAGTGAAGCG | This study |
| 28S-cer5-F2 | CCGATAGCGAACAAGTAC | This study |
| 28S-cer5-R1 | TTTGCCKACTTCCCTTACCC | This study |
| 28S-cer5-R2 | TCACCTWGGAGACCTGMTGCGG | This study |
| 28S-cer5-R3 | AACCAGCTACTAGATGGTTCG | This study |
| 28S-cer3-F1 | GTGTCTAACAACTCACCTGCCG | This study |
| 28S-cer3-F2 | GTGCAGATCTTGGTGGTAGTAG | This study |
| 28S-cer3-R1 | GGGTGAACAATCCAACACTWTGGG | This study |
| 28S-cer3-R2 | TAGGAAGAGCCGACATCGAAGG | This study |
| A1 | GGNGCNGGNGARYTNCAYYTNGA | Hashimoto et al. (1995) |
| A2 | CCARTGRTCRAANACRCAYTGNGGRAA | Hashimoto et al. (1995) |
| EF2int2F | GATTTRGARGARGATCATGC | Bartošová et al. (2013) |
| EF2int2R | CAGTAAAACCRAAAGATTC | Bartošová et al. (2013) |

### 2.3. Alignments

Sequences were aligned using MAFFT v6.626b (Katoh et al., 2005) with the L-INS-i multiple alignment method and default parameters (gap opening penalty: 1.53 and gap extension penalty 0.0). Alignments were cross-checked using SEAVIEW v3.2 (Galtier et al., 1996). Alignments included our novel myxosporean sequences and all available GenBank sequences of representatives of the marine myxosporean lineage (see Table 1). Six sequences of freshwater taxa were set as outgroup. Five single-gene datasets were constructed: SSU-all = 158 myxosporean SSU rDNA sequences; LSU = 51 LSU rDNA sequences; SSU-as-lsu = 51 SSU rDNA sequences of identical myxosporean species as in the LSU dataset; EF2aa = 24 myxosporean EF-2 amino acid sequences, and EF2nt = 24 myxosporean EF-2 nucleotide sequences. Ambiguous characters were found using Gblocks v0.91b (Castresana, 2000) implemented in SEAVIEW with less stringent parameters and the beginning and end of the alignment trimmed manually. Two concatenated alignments were analysed: rDNA-con (=SSU-as-lsu + LSU datasets), and rDNA-EF2-con (=SSU-as-lsu + LSU + EF2aa). Three additional datasets were prepared: SSU-LBdel (=LSU excluding 11 taxa with long branches), SSU-ByEye (=SSU with manual exclusion of ambiguous characters), and SSU-untreated (=SSU with no characters excluded).

A dataset containing SSU rDNA sequences of all *Ceratomyxa* spp. in GenBank was constructed for detailed analysis of the *Ceratomyxa* clade (72 ingroup taxa) and aligned using MAFFT. *Kudoa thyrsites*, *Parvicapsula minibicornis* and *Ellipsomyxa mugilis* were used as outgroup taxa. Ambiguous characters were found using Gblocks implemented in SEAVIEW, with less stringent parameters and manual trimming.

### 2.4. Phylogenetic analysis

Phylogenetic trees were calculated from the sequence alignments using maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI). ML analysis was done in RAxML v7.0.3 (Stamatakis, 2006) with a GTR + Γ model as recommended by author of the program. Concatenated genes were analysed using dataset partitioning. MP was done in PAUP* v4.0b10 (Swofford, 2001) with a heuristic search, random addition of taxa and Ts:Tv = 1:2. Bootstrap support was calculated from 500 replicates in ML and 1000 replicates in MP analysis. BI was done using MrBayes v3.0 (Ronquist and Huelsenbeck, 2003) with the GTR + Γ + I model of evolution (6 rates of substitution; gamma rate variation across sites; 8 categories used to approximate gamma distribution and covarion model) selected by ModelTest v3.1 (Posada and Crandall, 1998). Initially, MrBayes was run to estimate posterior probabilities over 1 million generations via 2 independent runs of 4 simultaneous Markov Chain Monte Carlo (MCMC) algorithms with every 100th tree saved. Then, the AWTY system (Nylander et al., 2008) was used to assess the length of each MCMC run. If the MCMC analysis had not run long enough to reach convergence and an effective sample size, MrBayes was set for another 1 million generations. Tracer v1.4.1 (Rambaut and Drummond, 2007) was used to ascertain the length of burn-in period.

### 2.5. Topology tests

TreeGraph v2.0.47-206 beta (Stöver and Müller, 2010) was used to generate constrained tree topologies from the phylogenetic analyses and alternate topologies. Topological tests were conducted on four datasets: SSU-all, SSU-as-lsu, LSU and concatenated rDNAs. ModelTest v3.1 and PAUP* were used to select the substitution model that best explained the data under the Akaike Information Criterion. The general time-reversible model with rate variation among sites, a gamma distribution and invariant sites category (GTR + Γ + I) was chosen for all datasets. Designed topologies in Newick format were specified in the assumption block. The data with selected ML parameters were executed in PAUP* to generate likelihood scores for each constrained tree. Resulted per-site log likelihood scores were analysed for significant differences in CONSEL v6.1 (Shimodaira and Hasegawa, 2001), using three likelihood-based tests: approximately unbiased (AU), Kishino-Hasegawa (KH), and Shimodaira-Hasegawa (SH).

### 2.6. Gene informativeness

We used PhyDesign (http://phydesign.townsend.yale.edu; Lopez-Giraldez and Townsend, 2011) to assess the gene informativeness of the SSU-as-lsu, LSU and EF2nt alignments. An ultrametric tree was generated in FigTree v1.3.1 (Rambaut, 2009) by transformation of branches to proportional ones. HyPhy v2.1.1 (Pond et al., 2005) was used to calculate phylogenetic informativeness of nucleotide-based data using empirical base frequencies and the time-reversible model of substitution.

## 3. Results

### 3.1. Taxonomic summary and new species descriptions

Phylum Cnidaria Hatschek, 1888

Unranked subphylum Myxozoa Grassé, 1970
Class Myxosporea Bütschli, 1881
Order Bivalvulida Shulman, 1959
Suborder Variisporina Lom and Noble, 1984
Family Ceratomyxidae Doflein, 1899
Genus *Ceratomyxa* Thélohan, 1892

*3.1.1. Ceratomyxa verudaensis n. sp. (Figs. 1A and 2A)*

**Type host**: *Scorpaena porcus* L., black scorpionfish (Scorpaeniformes: Scorpaenidae).

**Type locality**: Veruda Island, Mediterranean Sea off Pula, Croatia, N44°49′, E13°50′.

**Description of sporogonic stages**: Trophozoites floating freely in the bile, at different stages of maturation, disporous, ellipsoidal to sub-spherical. Size dependent on maturity, diameter usually 10–20 μm. Cytoplasmic extensions not observed.

**Description of myxospores**: Mature myxospores slightly crescent-shaped, length 6.4 (6.0–7.0) ± 0.5 μm and thickness 14.8 (13.0–16.0) ± 1.0 μm ($N$ = 10). Posterior spore angle slightly concave to straight 165–180°. Two smooth valves equal in size, or one slightly elongated, ovoid in lateral view. Sutural line straight. Spherical polar capsules, diameter 2.7 (2.5–3.0) ± 0.24 μm ($N$ = 20). 8–10 turns of polar filament in 2 rows.

**Site of sporogonic stages**: Gall bladder.

**Prevalence**: 20% (4/20).

**Materials deposited**: DNA sample (nr. 184) deposited at the Institute of Parasitology, BC CAS.

**Etymology:** The species name refers to the type locality.

**Molecular data**: 1897 nt SSU rDNA (GenBank Accession No. KM273027), 2567 nt LSU rDNA (GenBank Accession No. KM392426) and 711 nt EF-2 (GenBank Accession No. KM392436).

**Remarks:** *Ceratomyxa verudaensis* n. sp. has almost identical spore shape with *C. agilis* described by Thélohan (1892). *Ceratomyxa agilis* described from an elasmobranch *Trygon vulgaris* is slightly stumpy with lower thickness (11–12 μm) than *C. verudaensis* n. sp. Thélohan (1895) recorded *Scorpaena* sp. as the second host of *C. agilis*. However, this parasite identified as *C. agilis* from *Scorpaena* sp. might have been *C. verudaensis* n. sp. also found from the same fish genus. *Ceratomyxa verudaensis* n. sp. also resembles to *Ceratomyxa hepseti* (Thélohan, 1895) and *C. lubati* Gunter and Adlard, 2010 by its spore shape. They all have identical spore dimensions and are reported also from the Mediterranean Sea; however, *C. verudaensis* n. sp. has straight bottom side of the spore (frontal view) unlike the slightly curved shape of *C. hopseti* and *C. lubati*. *Ceratomyxa kovaljovae* has the same spore shape, parasitizes fish from the same order but has larger dimensions than *C. verudaensis*. Similarly, morphologically similar *C. elongata* Meglitsch, 1960 described from *Merluccius merluccius* in the Mediterranean Sea differs in spore dimensions. No DNA sequence data exist for these species for comparison. *Ceratomyxa arcuata* Thélohan, 1894, which we found in same fish host, has remarkably different spore morphology (*C. verudaensis* is more stumpy) and is genetically distant.

*3.1.2. Ceratomyxa synaphobranchi n. sp. (Figs. 1B and 2B)*

**Type host**: *Synaphobranchus kaupii* Johnson, 1862, Kaup's arrowtooth eel (Anguilliformes: Synaphobranchidae).

**Type locality**: North Atlantic, N56°49′, E9°40′, at 1800 m depth.

**Description of sporogonic stages**: Polysporous plasmodia floating freely in bile, oval, often narrowed in several regions to form bulb-like shapes, length up to 1 mm, width up to 50 μm.

**Description of myxospores**: Mature spores crescent shaped, length 6.3 (6.0–7.0) ± 0.94 μm and thickness 24.3 (22.0–32.0) ± 2.4 μm ($N$ = 10). Posterior spore angle slightly concave 155–165°. Valves slightly unequal, ovoid in lateral view. Sutural line straight. Polar capsules subspherical, length 3.4 (3.0–3.5) ± 0.12 μm and width 2.1 (2.0–2.5) ± 0.2 μm ($N$ = 10). 3–4 turns of polar filament.

**Site of sporogonic stages**: Gall bladder.

**Prevalence**: 9% (1/11).

**Materials deposited**: DNA sample (nr. 425) deposited at the Institute of Parasitology, BC CAS.

**Etymology:** The species name refers to the type host.

**Molecular data**: 1377 nt SSU rDNA (GenBank Accession No. KM273026), 1006 nt (5'end) and 1825 nt (3'end) LSU rDNA (GenBank Accession No. KM392424 and KM392425) and 712 nt EF-2 (GenBank Accession No. KM392435).

**Remarks**: *Ceratomyxa synaphobranchi* n. sp. myxospores resemble *C. allantoidea* Gaevskaya and Kovaleva, 1984, *C. crassa* Jameson, 1929, *C. hopkinsi* Jameson, 1929, *C. laxa* Meglitsch, 1960, *C. myoxocephala* Aseeva, 2002 and *C. platichthys* Aseeva, 2002 by spore shape. The former three species have greater spore lengths. The latter three species have similar spore sizes but are recorded from distant geographical localities – *C. laxa* (Pacific Ocean – New Zealand), *C. myoxocephala* and *C. platichthys* (Sea of Japan). Moreover, *C. laxa* has more pointed ends of the spore, spores of *C. myoxocephala* and *C. platichthys* have spherical PCs with greater dimensions (4–4.2 μm and 3.9–4.5 μm, respectively). All six species were found in different orders of fish hosts. No DNA sequence data exist for these species for comparison.

*3.1.3. Ceratomyxa leatherjacketi n. sp. (Figs. 1C and 2C)*

**Type host**: *Aluterus monoceros* L., 1758, unicorn leatherjacket (Tetraodontiformes: Monacanthidae).

**Type locality**: West coast of Peninsular Malaysia, N 3° 0', E 101°12'.

**Description of sporogonic stages**: Trophozoites floating in bile, at different stages of maturation, disporous, round, diameter 8–20 μm. Cytoplasmic extensions not observed.

**Description of myxospores**: Mature myxospores crescent-shaped and elongated transversely, length 6.3 (5.8–6.5) ± 0.19 μm and thickness 27.5 (24.5–31.0) ± 1.85 μm ($N$ = 20). Posterior spore angle slightly concave 155–165°. Two smooth valves, equal in size or one elongated slightly. Sutural line straight, sometimes causing a slight constriction at the spore centre. Polar capsules spherical, diameter 2.7 (2.5–3) ± 0.18 μm ($N$ = 20). Polar filament turns not visible.

**Site of sporogonic stages**: Gall bladder.

**Prevalence**: 50% (13/26).

**Materials deposited**: DNA sample (nr. 1616) deposited at the Institute of Parasitology, BC CAS.

**Etymology**: The species name refers to the common name of the fish host.

**Molecular data**: 1695 nt SSU rDNA (GenBank Accession No. KM273028), 1104 nt (5'end) and 1451 nt (3'end) LSU rDNA (GenBank Accession No. KM392427 and KM392428) and 673 nt EF-2 (GenBank Accession No. KM392437).

**Remarks**: Polar capsules not always in a stable position, spores often seen with both polar capsules occupying one valve. Myxospore size and morphology of *C. leatherjacketi* n. sp. is similar to that of *C. elegans* Jameson, 1929. However, *C. leatherjacketi* n. sp. spore is narrower and PCs fill the half of the spore thickness in contrast to *C. elegans* whose PCs fill one third of the spore. *Ceratomyxa elegans* is found in a different order of fish host, the toadfishes Batrachoidiformes, from the species *Porichthys notatus* that is exclusively found on the Pacific coast of America. *Ceratomyxa leatherjacketi* n. sp. has also similar spore shape with *C. fistulariae* Kpatcha, Diebakate, Faye and Toguebaye, 1996, but with spore dimensions much larger than *C. leatherjacketi* n. sp. No DNA sequence data exist for these species for comparison.

*3.1.4. Ceratomyxa ayami n. sp. (Fig. 2E)*

**Type host**: *Aluterus monoceros* L., unicorn leatherjacket (Tetraodontiformes: Monacanthidae).

**Type locality**: Pangkor Island, Peninsular Malaysia, N4°12′, E100°33′.

Description of sporogonic stages: Not observed.

**Description of myxospores**: Mature myxospores ovoid, with a constriction at the centre, length 10.6 (9.0–11.5) ± 1.8 μm and thickness 17.1 (15.0–18.5) ± 1.2 μm ($N$ = 18). Posterior angle slightly concave to straight 170–180°. Two smooth valves of different size with rounded distal ends, oval in lateral view. Sutural line straight. Spherical polar capsules, diameter 4.2 (4.0–4.5) ± 0.35 μm ($N$ = 18). 4 turns of polar filament. Extruded polar filament length 47.3–50.6 μm.

**Site of sporogonic stages**: Gall bladder.

**Prevalence**: 31% (8/26).

**Materials deposited**: DNA sample (nr. 1617) deposited at the Institute of Parasitology, BC CAS.

**Etymology**: The species name refers to the local Malay name of the fish host Ikan Ayam (chicken fish).

**Molecular data**: 2004 nt SSU rDNA (GenBank Accession No. KM273029), 2415 nt LSU rDNA (GenBank Accession No. KM392429) and 675 nt EF-2 (GenBank Accession No. KM392438).

**Remarks**: *Ceratomyxa ayami* n. sp. differs from most *Ceratomyxa* spp. by its myxospore central constriction. *Ceratomyxa constricta* Meglitsch, 1960 has similar anterior constriction but the thickness of spore is much greater (25 μm) than in *Ceratomyxa ayami* n. sp. Similar morphology and size as *Ceratomyxa ayami* n. sp. also have *C. vepallida* Meglitsch, 1960 and *Ceratomyxa* sp. ex *Zebrasoma veliferum* (Gunter et al., 2010). However, both of them are parasites of fishes from different orders, with the former described from a different biogeographic realm (Temperate Australasia). *Ceratomyxa vepallida* has subspherical PCs with smaller dimensions and *Ceratomyxa* sp. ex *Zebrasoma veliferum* is phylogenetically distantly related. *Ceratomyxa ayami* n. sp. is morphologically different from *C. leatherjacketi* n. sp. found in the same host and geographic region.

Micrographs of mature spores of species with newly obtained molecular data are shown on Fig. 1E–H, i.e. *Ceratomyxa appendiculata* Thélohan, 1892, *C. arcuata* Thélohan, 1892, *C. informis* (Auerbach, 1910) and *C. longipes* (Auerbach, 1910).

Family Sinuolineidae Schulman, 1959

Genus *Myxodavisia* Zhao, Zhou, Kent, and Whipps, 2008

*3.1.5. Myxodavisia bulani n. sp. (Figs. 1D and 2D)*

**Type host**: *Megalops cyprinoides* (Broussonet, 1782), Indo-Pacific tarpon (Elopiformes: Megalopidae).

**Type locality**: Langkawi, Peninsular Malaysia, N6°25′, E99°52′.

Description of sporogonic stages: Not observed.

**Description of myxospores**: Mature myxospores crescent-shaped with long, hollow lateral appendages, length 7.0 (6.8–7.8) ± 0.38 μm and thickness 37.0 (31.0–43.0) ± 3.72 μm including lateral appendages, spore body without appendages, thickness 13.3 (12.0–13.5) ± 0.5 μm ($N$ = 10). Posterior spore angle slightly concave 130–140°. Two smooth, equal size valves. Sutural line straight. Subspherical polar capsules, diameter 2.8 (2.5–3.3) ± 0.28 μm ($N$ = 10). Polar filament with 3–4 turns.

**Site of sporogonic stages**: Gall bladder.

**Prevalence**: 2% (1/50).

**Materials deposited**: DNA sample (nr. 1618) deposited at the Institute of Parasitology, BC CAS.

**Etymology**: The species name refers to the local Malay name of the fish host Ikan Bulan (moon fish).

**Molecular data**: 1798 nt SSU rDNA (GenBank Accession No. KM273030), 1924 nt LSU rDNA (GenBank Accession No. KM392430) and 712 nt EF-2 (GenBank Accession No. KM392438).

**Remarks**: *Myxodavisia bulani* n. sp. differs from most of existing species of its genus by smaller spore dimensions. Only *M. murtii* has the size in the range of *M. bulani* n. sp. *Myxodavisia murtii* (Padma Dorothy and Kalavati, 1994) is characterised by sinuous sutural line (straight in *M. bulani* n. sp.) and by shorter spore appendages than *M. bulani* n. sp.

*3.2. Phylogenetic relationships among Ceratomyxa spp.*

We obtained novel SSU rDNA, LSU rDNA and EF-2 sequence data for eight *Ceratomyxa* and one *Myxodavisia* gall bladder-infecting species and for two intestine-infecting species – *Ceratonova shasta* and *C. gasterostea*. Phylogenetic analyses showed that all nine gall bladder-infecting species branched inside the *Ceratomyxa* clade (Fig. 3). *Ceratomyxa leatherjacketi* n. sp. and *Myxodavisia bulani* n. sp. clustered together as the most basal subclade A with high bootstrap support and BI posterior probability. *Ceratomyxa informis* from codfish was placed in subclade B as a sister taxon to four *Ceratomyxa* species, which infect sharks. *Ceratomyxa synaphobranchi* n. sp. clustered with *Palliatus indecorus* and newly sequenced *Ceratomyxa* sp. ex *Notacanthus bonapartei* in the small, well-resolved subclade C. *Ceratomyxa arcuata* was closely related to *C. cretensis* and *C. filamentosi*, and all clustered with long-branching *Ceratomyxa* species, which include the newly sequenced *Ceratomyxa ayami* n. sp., which has the longest branch in the *Ceratomyxa* tree. *Ceratomyxa longipes* and *C. appendiculata* clustered in the clade that contains long-branching ceratomyxids *C. aegyptiaca*, *C. vikrami*, *C. pantherini* and *C. anko*. *Ceratomyxa verudaensis* n. sp. was a close relative of *C. nowakae*.

The intraspecific sequence similarity of *C. arcuata* from the North Atlantic fish *Lophius piscatorius*, *Merlangius merlangus* and *Callionymus lyra* ranged from 98.41% to 99.27% over 1442 bp of SSU. The intraspecific sequence similarity of *C. longipes* from two gadid fish hosts *M. merlangus* and *Melanogrammus aeglefinus* off Scotland coast was 98.90% over 1445 bp of SSU. The sequence similarity of two SSU rDNA sequences of *C. appendiculata* from *L. piscatorius* from two geographic regions (off Scotland coast and off Norway coast) was 98.67% over 1510 bp of SSU.

*3.3. Phylogenetic relationships of Ceratonova and main marine subclades*

All phylogenetic analyses revealed a close relationship between *Ceratonova shasta* and *C. gasterostea*, which constitute the well-supported *Ceratonova* clade within the marine myxosporean lineage (Fig. 4). The position of the *Ceratonova* clade within the marine lineage was not stable and depended on the alignment and phylogenetic analysis used.

Ten different tree topologies were obtained from the phylogenetic analyses of five single rDNA, one EF-2 and two concatenated rDNA datasets using three phylogenetic methods (Figs. 4 and 5). In most of the topologies, the *Ceratomyxa* clade was the first branching clade (the most basal group) and *Enteromyxum* and *Kudoa* were the most derived groups. Topological instability was evident in the central part of the trees with unstable relationships of the marine urinary clade, the marine gall bladder clade and the *Ceratonova* clade. In two topologies, the *Ceratonova* clade branched at the base of the tree (Fig. 5, Topologies 6 and 9). The *Ceratonova* and *Ceratomyxa* clades did not cluster together in any analysis.

BI analysis of SSU-all dataset resulted in a topology with a polytomy, which included the marine urinary, the marine gall bladder and the lineage leading to the *Ceratonova* clade, *Kudoa* clade + *Enteromyxum* clade (Fig. 4). ML analyses of the taxon-rich SSU-all dataset placed the *Ceratonova* clade between the marine urinary clade and the marine gall bladder clade (Fig. 4, Fig. 5 – Topology 1). MP analysis gave a topology with *Ceratonova* as the

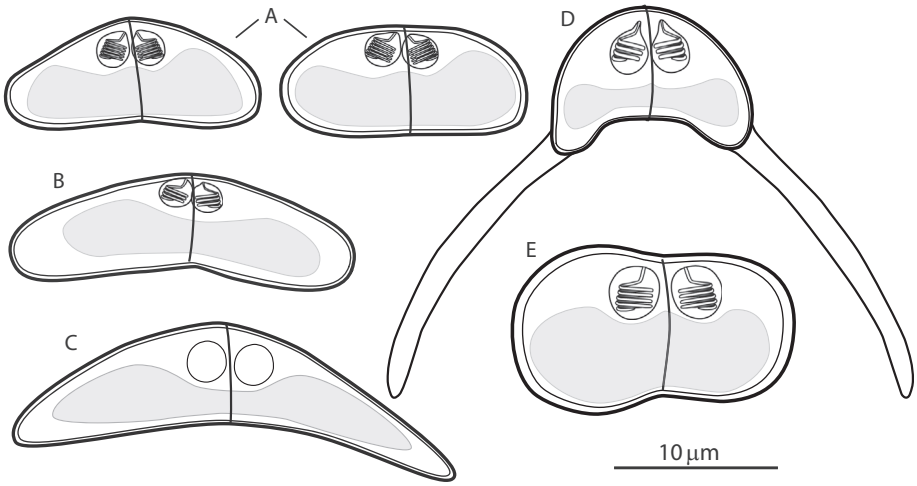


**Fig. 2.** Line drawings of myxospores. A – *Ceratomyxa verudaensis* n. sp.; B – *Ceratomyxa synaphobranchi* n. sp.; C – *Ceratomyxa leatherjacketi* n. sp.; D – *Myxodavisia bulani* n. sp.; E – *Ceratomyxa ayami* n. sp.

second clade branching ancestrally to the marine urinary clade + the marine gall bladder clade + *Kudoa* clade + *Enteromyxum* clade (Fig. 5 – Topology 2). The ML and BI analyses of the LSU dataset suggested the *Ceratonova* clade to be closely related to the *Enteromyxum* + *Kudoa* clades (Fig. 5 – Topology 5). The MP analysis of the LSU dataset resulted in the exceptional position of the *Ceratonova* as the most basal clade of the marine myxosporean lineage (Fig. 5 – Topology 6). The ML and BI analyses of the concatenated rDNA genes resulted in similar tree (Fig. 5 – Topology 7) as in the analyses of data providing Topology 3 (Fig. 5). The only difference was the sister relation of the marine urinary and the marine gall bladder clades. The MP analysis of the concatenated rDNA data revealed the close relationship of the *Ceratonova* with the *Enteromyxum* clade (Fig. 5 – Topology 8). The ML and BI concatenated analyses of all three genes showed the identical topology (Fig. 5 – Topology 7) as obtained by the concatenated rDNAs analysis. Single analyses of the EF-2 gene datasets gave ambiguous results with non-monophyletic main clades of the marine lineage. The only exception was the ML of the EF2-aa dataset that was almost identical (Fig. 5 – Topology 9) with the results of SSU-all ML/BI analyses differing in the *Ceratonova* clade position (Fig. 5 – Topology 1).

### 3.4. Topology tests

We performed statistical tests to (i) construct a confidence set of 9 topologies derived from the phylogenetic analyses based on four sequence datasets (Fig. 5), (ii) confirm branching of *Ceratonova* spp. out of the *Ceratomyxa* clade, (iii) evaluate the sister relation of the *Ceratonova* clade to the *Ceratomyxa* clade and (iv) assess the *Ceratomyxa* clade status as sister to the rest of the ingroup (Supplementary File 1). Seven topologies that resulted from the phylogenetic analyses were not included in the statistical testing since the relationships of the main clades were not resolved (polytomies occurred) and/or the main clades were not monophyletic.

The best topology determined by the all topological tests from all the possible 945 rooted topologies (out of six ingroups) based on the SSU-all dataset was the topology with the *Ceratomyxa* as the most basal clade of the tree and the *Ceratonova* clade as a sister lineage to the *Enteromyxum* + *Kudoa* clades (monophyly of the histozoic species) (Supplementary File 1). This topology is almost identical with the topology obtained from the BI analysis of SSU-all dataset (Fig. 4) and similar to topology 5 (Fig. 5), which resulted from ML and BI analyses of the LSU. Topology derived from the ML analyses of SSU-all dataset, was the fourth best scoring topology selected by the AU test (Fig. 5 – Topology 1, Supplementary File 1). However, this topology was rejected by the AU test of LSU data. All hypothetical topologies with constrained *Ceratonova* species within the *Ceratomyxa* clade were rejected by all statistical tests under all sequence datasets (Supplementary File 1). Majority of topologies that constrained *Ceratomyxa* + *Ceratonova* as sister clades were rejected (Table 3), e.g. topology tests based on LSU and rDNA-con rejected 96 and 86 out of 105 topologies, respectively (Supplementary File 1, yellow boxes). The *Ceratomyxa* clade as sister to the rest of the ingroup was always as the best scoring topology in all performed tests. Seven topologies with this *Ceratomyxa* phylogenetic position were ranked amongst the ten topologies with the highest *p*-value in AU tests derived from SSU and rDNA-con datasets (Supplementary File 1, green boxes).

### 3.5. Gene informativeness

The phylogenetic informativeness of the SSU per nucleotide was higher than the LSU for relationships highest in the tree (i.e. most recent evolution) but was lower in the middle part of the tree, during the diversification of the main marine clades (Fig. 6). LSU informativeness peaked approximately at the time of splitting of the *Enteromyxum* and *Kudoa* clades and remained higher than the SSU. The phylogenetic informativeness of both rDNAs was relatively high at the time of branching of the most unstable clades (*Ceratonova*, marine gall bladder and marine urinary). EF-2 phylogenetic informativeness was considerably lower than both rDNA loci at all times.

## 4. Discussion

### 4.1. Limits of phylogenetic reconstructions in the Myxozoa

The issue of whether adding taxa or characters into a dataset is the most important factor for phylogenetic reconstruction, has been discussed often in molecular taxonomy in last decade (e.g. DeBry, 2005; Heath et al., 2008). Although some authors do not consider incomplete taxon sampling as a problem for phylogeny inference (e.g. Rosenberg and Kumar, 2001), taxon sampling is one of the most important criteria for correct phylogenetic reconstruction (e.g. Hedtke et al., 2006; Zwickl and Hillis, 2002). In the present study, we added novel ceratomyxid taxa and analysed

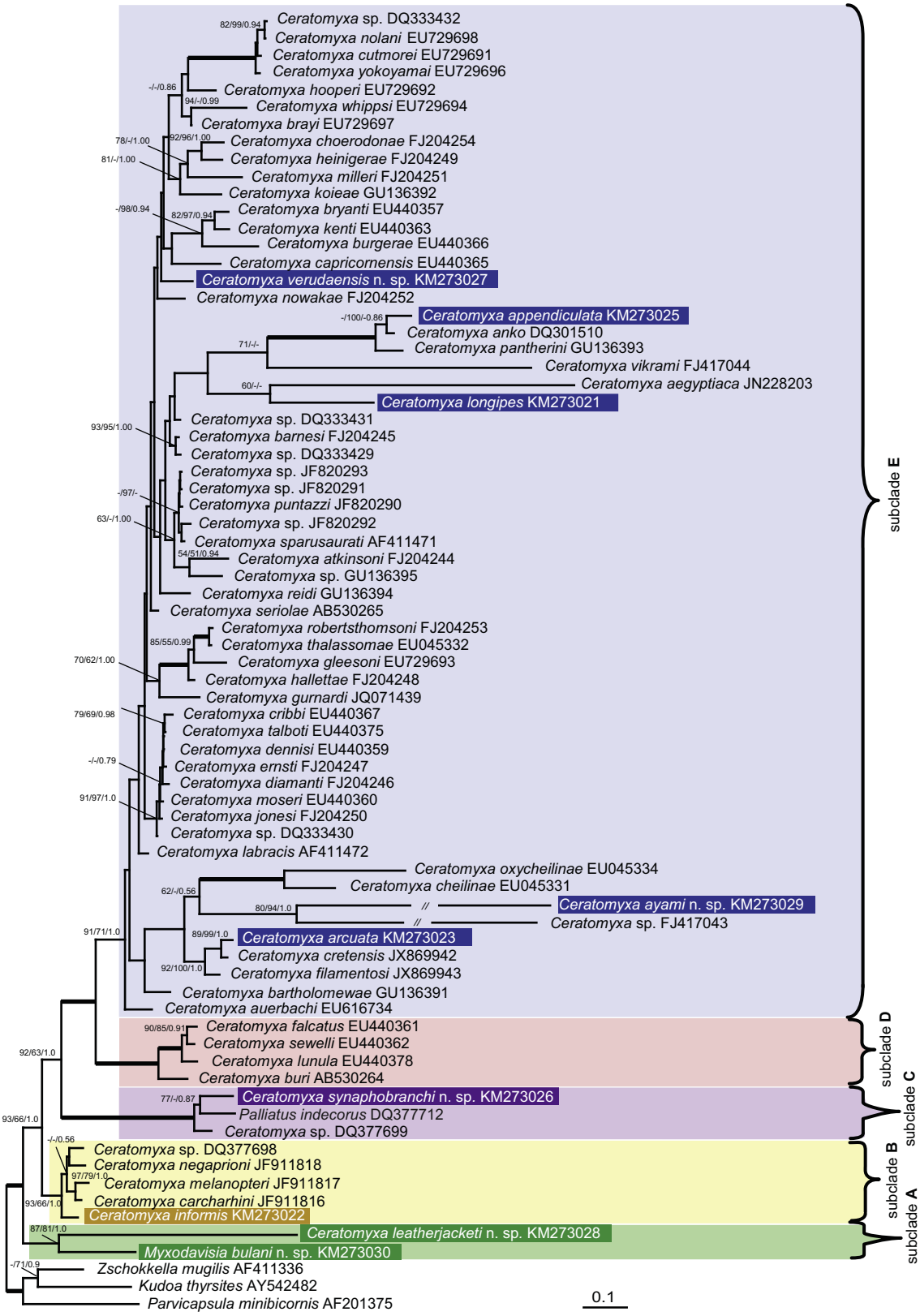


**Fig. 3.** SSU rDNA maximum likelihood tree topology of the *Ceratomyxa* clade. GenBank accession numbers are beside taxon names; newly sequenced taxa are boxed. Numbers at nodes = maximum likelihood/maximum parsimony bootstrap support, and Bayesian posterior probabilities. Bold branches designate nodes with highest bootstrap support and Bayesian posterior probability. Dashes indicate nodes with support <50% (0.5), or not present in the maximum parsimony or Bayesian trees.

these and existing taxa at multiple genetic loci, then compared the results of the different approaches.

We sequenced 9 new gall bladder-infecting *Ceratomyxa* spp., and provided new molecular data for the two known *Ceratonova* species *C. shasta* and *C. gasterostea*. Addition of *C. gasterostea*, which is closely related to relatively long-branching *C. shasta*, allowed us to break the long-branch; this is a method used for improving phylogenetic reconstruction (Poe, 2003). Inclusion of new basal

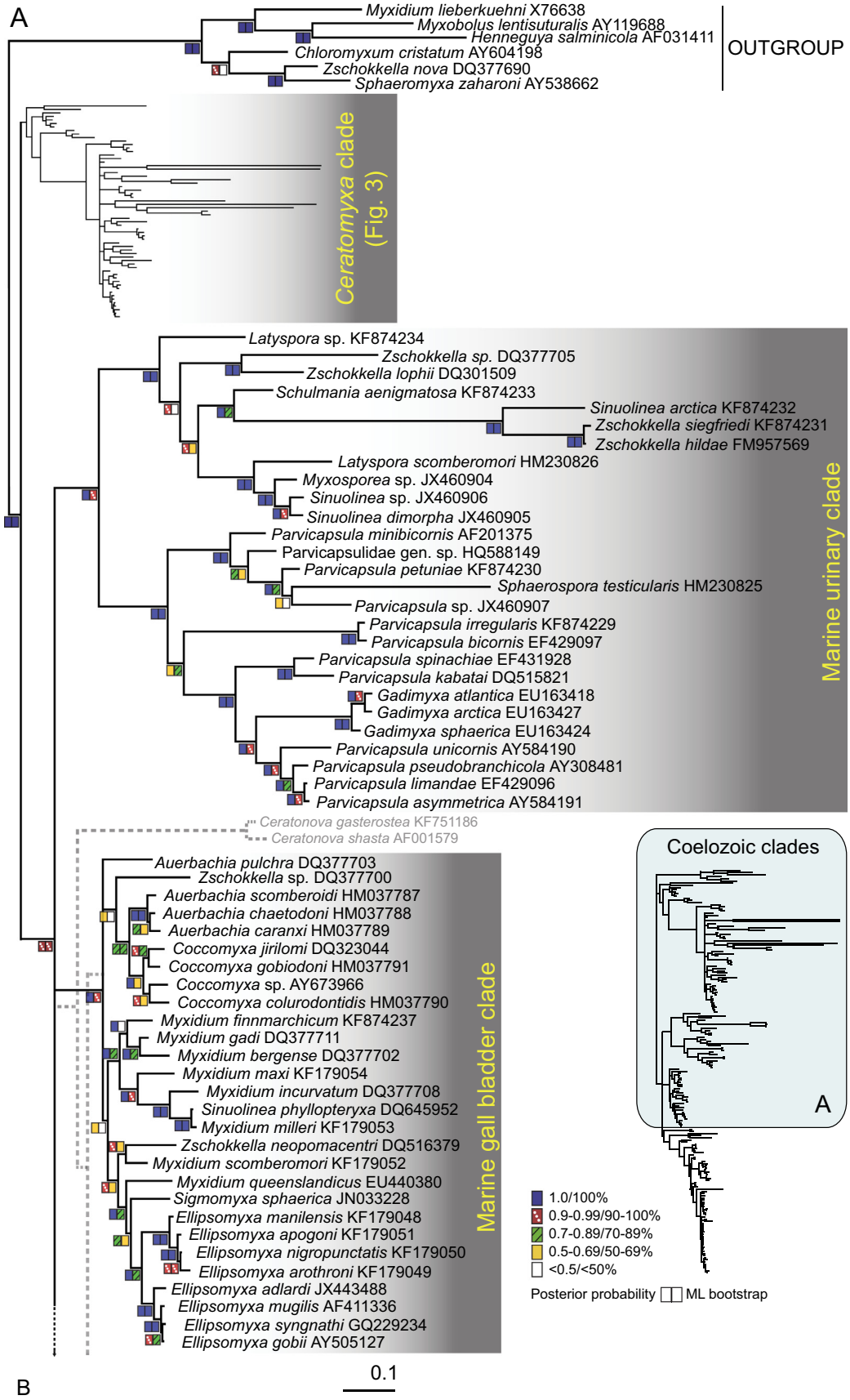


**Fig. 4.** Bayesian inference tree topology based on all available SSU rDNA sequences of myxosporeans that branch in the marine myxosporean lineage. Bayesian posterior probabilities and maximum likelihood bootstrap supports are displayed as coloured boxes. Alternative maximum likelihood topology of the *Ceratonova* clade is displayed by dashed lines. GenBank accession numbers are in parentheses. Scale bar is under the tree.

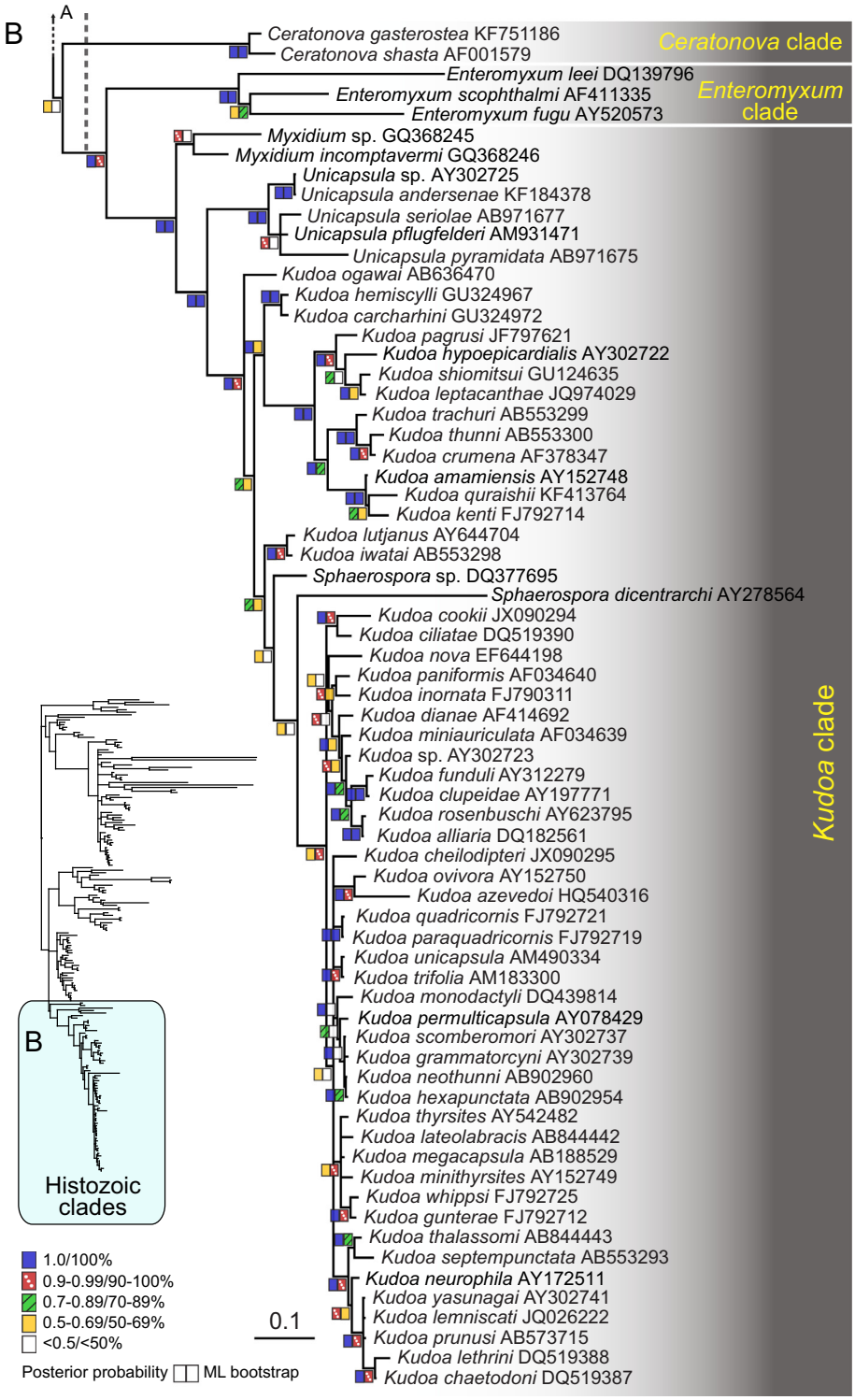


**Fig. 4** (*continued*)

*Ceratomyxa* sequences, *C. leatherjacketi* n. sp. and *M. bulani* n. sp., helped to better assess the relationships of *Ceratomyxa* with other marine clades.

We increased the number of molecular characters in our analyses to improve the phylogenetic signal strength (Huelsenbeck et al., 1996). We combined single gene SSU rDNA datasets with available LSU rDNA data. Although taxa with poor sequence coverage may not influence the phylogenetic accuracy (Wiens and Tiu, 2012), considerable differences in availability of SSU data compared with LSU, led us to include in combined analyses only taxa with both rDNA markers available. Comparison of rDNA-based single vs. concatenated SSU + LSU rDNA analyses showed significantly higher bootstrap support for separation of the *Ceratomyxa* clade from the rest of the marine myxosporeans in the concatenated analysis. This topology pattern was also supported by our topology test analysis in which the concatenated dataset revealed the

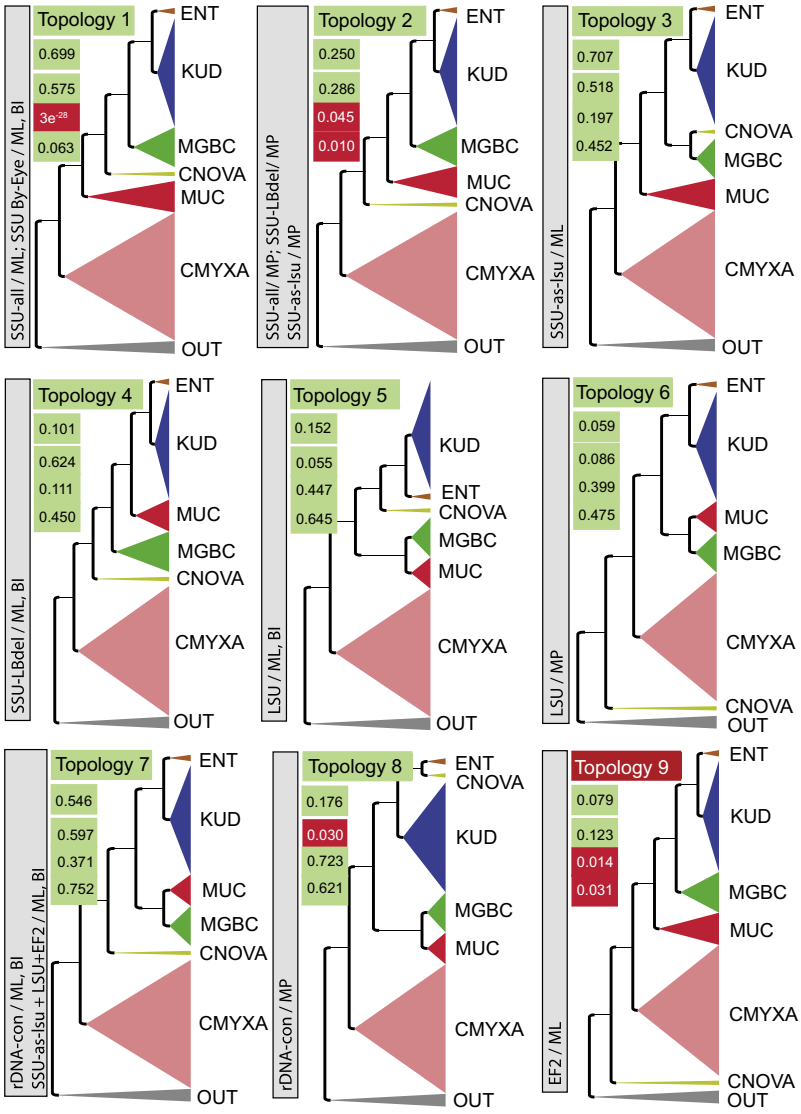


**Fig. 5.** Tree topologies. Schematic drawings of the tree topologies obtained from phylogenetic analyses (analysis type stated in the vertical box) with the results of topology tests. Numbers in boxes represent the *p*-values of the approximately unbiased (AU) test. Test datasets from top to bottom: SSU-all, SSU-as-lsu, LSU, rDNA-con. The green boxes with black writing show topologies that were not rejected and the red boxes with white writing mark the rejected topologies at the significance level 0.05. OUT = outgroup, CMYXA = *Ceratomyxa* clade, CNOVA = *Ceratonova* clade, MUC = marine urinary clade, MGBC = marine gall bladder clade, KUD = *Kudoa* clade, ENT = *Enteromyxum* clade. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 3**
Number of rejected topologies to all possible topologies with specified criteria.

| Dataset | *Ceratonova* within *Ceratomyxa* | *Ceratomyxa* sister to *Ceratonova* | *Ceratomyxa* sister to all ingroups |
|---|---|---|---|
| SSU | 105/105 | 79/105 | 65/105 |
| LSU | 105/105 | 96/105 | 61/105 |
| rDNA-con | 105/105 | 86/105 | 77/105 |
| SSU-as-lsu | 105/105 | 36/105 | 47/105 |

highest *p*-values for the topologies with the *Ceratomyxa* as the first branching clade of the marine myxosporean lineage. This suggests that a combination of the two markers increased the phylogenetic signal at this node. Combined analyses did not increase resolution of the particular position of the *Ceratonova* clade, whose phylogenetic signal was weak in all analyses.

To assess effects of bias from different alignment methods (Lemmon et al., 2009), we removed ambiguous characters in rDNA alignments both automatically (Gblocks) and manually. We found no difference in the resultant topologies: branching order of the main clades was the same. This indicated that the strength of the phylogenetic signal in untreated data exceeded any bias from ambiguous characters, which could affect the principal topology.

One of the most common phylogenetic artefacts that can mislead investigators attempting to estimate evolutionary trees is the phenomenon of long-branch attraction (LBA) (e.g. Anderson and Swofford, 2004). Although associated commonly with MP, LBA can also affect ML and distance methods (Swofford et al., 2001). In our comprehensive SSU rDNA tree, there are several long-branching species, mainly in the *Ceratomyxa* and marine urinary clades, which may affect the overall topology. Analyses excluding the long-branching taxa showed that the position of the *Ceratomyxa* clade was stable, but the position of the marine urinary clade changed in ML and BI analyses. The urinary clade shifted to have a closer relationship to histozoic *Enteromyxum* and *Kudoa* species, which may indicate the effect of LBA in the complete dataset, due to attraction of long-branches in the *Ceratomyxa* and the

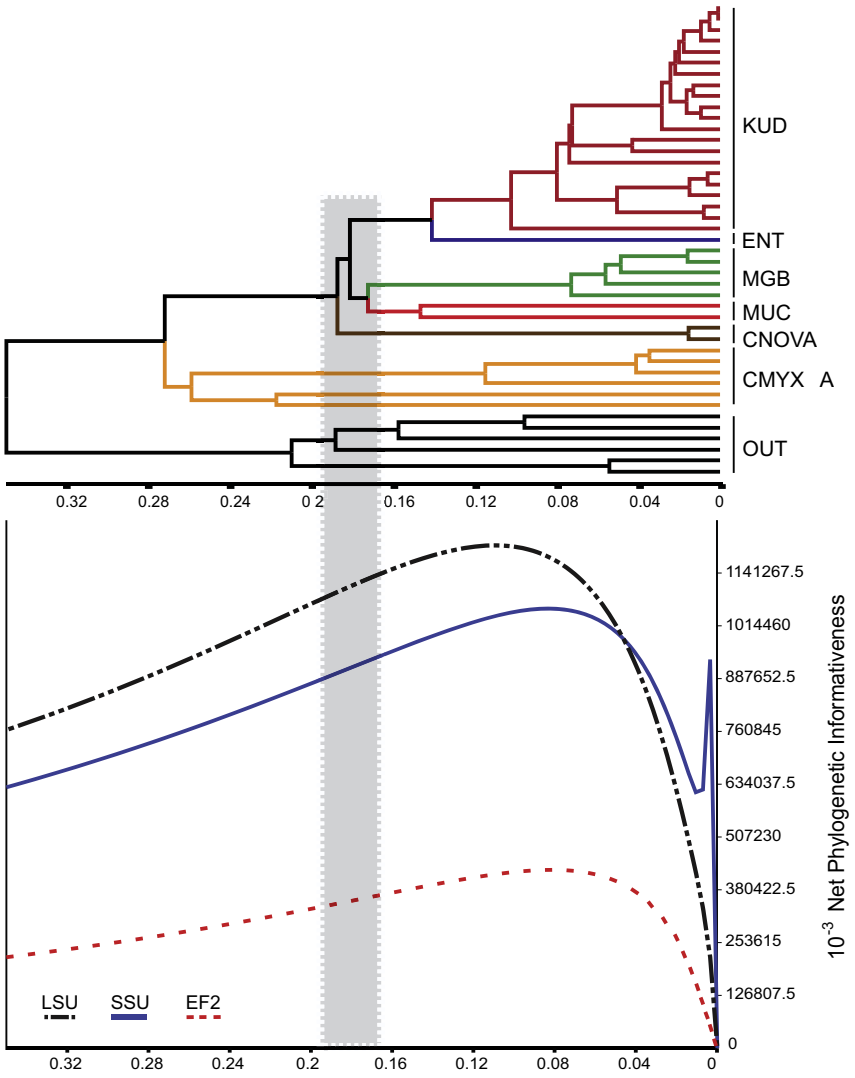


**Fig. 6.** Graphical representation of phylogenetic informativeness of each dataset, relative to principal phylogenetic tree topology. LSU = large subunit rRNA gene, SSU = small subunit rRNA gene, EF2 = Elongation Factor 2 gene. OUT = outgroup, CMYXA = *Ceratomyxa* clade, CNOVA = *Ceratonova* clade, MUC = marine urinary clade, MGB = marine gall bladder clade, KUD = *Kudoa* clade, ENT = *Enteromyxum* clade.

marine urinary clade. As *C. shasta* itself formed a relatively long branch, the basal position (MP analysis of LSU rDNA) of the *Ceratonova* clade could have been affected by LBA as well. Clustering of LB taxa close to the outgroup taxa is a typical consequence of LBA (Bergsten, 2005). Our ML and BI analyses of the same dataset revealed the most reliable position of the *Ceratonova* clade was close to the histozoic species.

To overcome the insufficiency of rDNA markers in resolving particular nodes of the marine myxosporean lineage, we included EF-2 gene sequences, as recommended previously (Fiala and Bartošová, 2010). Trees inferred from the EF-2 gene generally supported the grouping of myxosporean species into the main marine clades; however, concatenated analyses including EF-2 did not give a better resolution of the main clades. Our PhyDesign comparison of phylogenetic informativeness of the rDNAs and EF-2 showed approximately a three times lower informative value for the EF-2 than both rDNAs (Fig. 6). This can be explained by lower information content of the short EF-2 alignment (only 370 amino-acid positions), which contained many conserved regions. Nevertheless, the EF-2 single gene-based ML analysis reconstructed the three main marine clades, based only on nine ceratomyxids, three kudoids and *Sphaerospora dicentrarchi* and two members of the marine urinary clade. Moreover, the relationships among *Ceratomyxa* species within the *Ceratomyxa* clade agreed with the rDNA phylogeny, which supports congruence of the rDNA and EF-2 as phylogenetic markers.

### 4.2. Phylogeny within the Ceratomyxa clade

Our analyses showed that the *Ceratomyxa* clade was consistently the first branching clade of the marine myxosporean lineage, irrespective of the marker, taxon sampling, character treatment and phylogenetic method used. This agrees with previous analyses (Fiala and Bartošová, 2010) and suggests that a *Ceratomyxa*-like myxospore was the ancestral morphotype of marine myxosporeans. Retention or reappearance of a *Ceratomyxa*-like morphotype occurred in the *Ceratonova* clade.

In myxozoan phylogenetics, *Ceratomyxa* (marine) and *Myxobolus* (freshwater) have been the most intensively studied groups. Here, we analysed 70 *Ceratomyxa* species, which represent about 30% of all described species. We added nine newly sequenced myxosporeans to the *Ceratomyxa* clade, from which two constituted a new basal subclade (subclade A, Fig. 3). In total, our phylogenetic reconstructions resolved five-subclades within *Ceratomyxa*. The majority of *Ceratomyxa* spp. clustered within the most recently branching taxon-rich subclade (subclade E) with

unresolved deeper nodes, a result that agrees with the polytomic BI topology of Gunter et al. (2009). Apart from a large polytomy, subclade E is characterised by the presence of two groups of extremely long-branching taxa, though their propensity to cluster together, especially in the MP analysis, is most probably due to LBA and thus their exact relationships are uncertain.

The majority of sequenced species have been found in fish from Australian waters (Pacific Ocean; Gunter and Adlard, 2009). We added data from three species from Malaysian waters (Indian Ocean) and six species from the Atlantic Ocean, to broaden phylogeographic sampling. The evolutionary study of Gunter et al. (2009) revealed, intriguingly, that ceratomyxids from different geographic localities grouped together. Our data support this trend: species from the Atlantic Ocean (*Ceratomyxa* spp. ex *Gadus morhua*, *C. informis*, *C. appendiculata*) cluster with species from the Indian Ocean (*C. ayami* n. sp.), Japan (*C. anko*) and Australia (*C. pantherini*). However, the genetically most similar species came from the same geographic area: e.g. *C. arcuata*, *C. cretensis*, *C. filamentosi* and *C. synaphobranchi* n. sp.

Moreover, *Myxodavisia bulani* n. sp. and *C. leatherjacketi* n. sp., both from the Indian Ocean, clustered together in the basal ceratomyxid branch, and thus represent an evolutionary old ceratomyxid lineage. The genus *Myxodavisia* includes both gall bladder and urinary bladder-infecting species. Given the correlation of phylogenetic grouping with vertebrate host tissue tropism, we were not surprised to find that the gall bladder-infecting *M. bulani* n. sp. clustered with *Ceratomyxa* species. Moreover, *Myxodavisia* myxospore morphology highly resembles *Ceratomyxa* morphology, with addition of valve cell appendages (Zhao et al., 2008). We predict that other gall bladder-infecting *Myxodavisia* species will cluster within the *Ceratomyxa* clade, and that species that infect the urinary bladder will either branch inside the marine urinary clade or form a separate clade within the marine lineage.

Phylogenetic analyses place *Palliatus indecorus* inside the *Ceratomyxa* clade (Fiala, 2006; Gunter et al., 2009; this study). The newly discovered species, *C. synaphobranchi* n. sp., from deep-sea fish is closely related to *Palliatus indecorus* and together with another deep-sea fish-infecting *Ceratomyxa* sp. (DQ377699; ex *Notacanthus bonapartei*) they form the well-resolved subclade C (Fig. 3). This implies ceratomyxids from deep-sea fish have a common evolutionary history. The only morphological feature that differentiates *Palliatus* from *Ceratomyxa* is the presence of a membranaceous veil (Lom and Dyková, 2006). All six described *Palliatus* spp. are parasites of deep-sea fishes from various fish orders and geographic regions. We hypothesise that the membranaceous veil evolved in an ancestral ceratomyxid, which lived in the deep-sea, and possibly enabled its myxospores to float better in the cold, high pressure environment, and thus be more widely distributed to invertebrate hosts. If the feature evolved only once with the radiation of *Ceratomyxa* spp., its use to define *Palliatus* and *Ceratomyxa* as separate genera is invalid. However, since we do not know the phylogenetic position of *P. mirabilis*, the type species, we do not propose suppression of genus *Palliatus* at this time.

We identified three *Ceratomyxa* species each from different fish hosts and/or geographic regions. They were characterised by identical myxospore morphology; however their SSU sequence difference reached in some cases more than 1% but never exceeded 2%. We classified these myxosporeans as single species; however further research is required to exclude the possibility of presence of cryptic species (Bartošová and Fiala, 2011).

### 4.3. Origin and phylogeny of genus Ceratonova

Although having morphologically very similar myxospores, a close relationship between *Ceratonova* and *Ceratomyxa* spp. is not supported as they differ in their tissue tropism, host environment and phylogenetic positioning. *Ceratonova* spp. never clustered within the *Ceratomyxa* clade and when constrained with all available *Ceratomyxa* spp., the topology was rejected with very high confidence levels in all tests. However, when *Ceratonova* spp. were constrained to be sister to the *Ceratomyxa* clade the set of these topologies was not completely rejected, and thus there is a remaining possibility that all *Ceratomyxa* and *Ceratonova* species may have had a close common ancestor, as proposed previously (Gunter et al., 2009). Nevertheless, this hypothesis is not supported by our phylogenetic analyses, which never showed a close (sister) relationship of the *Ceratomyxa* and *Ceratonova* clades. Instead, the *Ceratonova* clade had different positions depending on the dataset and analysis performed.

The stable position of the freshwater *Ceratonova* clade inside the marine lineage clearly supports a marine evolutionary history of this taxon (e.g. Fiala, 2006; Gunter et al., 2009; Kent et al., 2001; this study). The finding of *C. gasterostea* parasitizing freshwater stickleback (Atkinson et al., in press) informs about the puzzling evolution of this genus. The three-spined stickleback is originally a marine fish that recently adapted to freshwater (Jones et al., 2012). Adaptation of the originally marine parasite to the freshwater environment may have happened when their hosts entered the freshwaters. The presumed ancestors of *Ceratonova* spp. are either salmonid fish migrating between marine and freshwater environment or marine sticklebacks that were trapped in freshwater lakes in North America during the last ice age (Jones et al., 2012). *Ceratonova* parasites introduced from the sea to the freshwater found a suitable invertebrate host, likely freshwater polychaetes, and were able to survive in the new environment. Our phylogenetic analyses showed that both *C. gasterostea* and *C. shasta* are closely related and have very short branch lengths, which suggest a relatively recent split of the two species.

### 4.4. Evolution of the marine myxosporean lineage

The deep evolutionary origin of the main clades within the marine lineage causes difficulties in phylogenetic reconstruction. Our analyses supported that the first branching clade of the marine lineage was *Ceratomyxa*. Then there is a large polytomy of unresolved or weakly supported relationships among the marine urinary clade, the marine gall bladder clade and the newly designated *Ceratonova* clade, and the more recent lineage of the *Enteromyxum* and *Kudoa* clades. The relationships among these clades were unstable in all phylogenetic trees and were highly dependent on the phylogenetic method and marker used, taxon sampling and on the treatment of ambiguously aligned characters in the datasets (Fig. 5). There are insufficient informative characters to resolve the basal nodes in the middle part of the tree, which may reflect rapid radiation into marine subgroups, early in the evolution. These evolutionary deep divergences have very little phylogenetic signal in extant taxa to establish branching order (Townsend et al., 2012). During a short evolutionary time, nucleotide synapomorphies of closely related taxa cannot be fixed. Furthermore, if the diversification happened earlier in their evolution, the nucleotide synapomorphies of closely related clades can be suppressed by a subsequent nucleotide substitution in the DNA marker during the evolution. Therefore, the stability and high bootstrap support of the *Enteromyxum* + *Kudoa* clade can be explained by the recent split of these two clades and from a relatively long lasting common ancestor, resulting in synapomorphies in their molecular markers becoming fixed. Furthermore, we showed that highest phylogenetic informativeness of both rDNA markers correlates with separation of these two clades.

The SSU rDNA gene is the most widely used locus for molecular phylogenetic analyses, especially for evaluation of deep-level relationships (Van de Peer et al., 2000) and it has been used

extensively for myxozoan phylogenetics. While LSU rDNA may be more informative than SSU for myxozoan phylogenies (Bartošová et al., 2009; this study), LSU alone or in combination with SSU was unable to resolve the unstable nodes in the middle part of the myxosporean marine lineage. Despite that, ML analyses of the LSU and topology tests based on both single SSU and LSU support grouping of all marine histozoic species including *Ceratonova* spp., and suggest that they evolved from a common coelozoic ancestor (Fiala and Bartošová, 2010; Shulman, 1966). Although LSU suffers from lower taxon sampling than SSU, our best LSU tree topology was identical to that of the taxon-rich SSU, which showed close relationships of all histozoic myxosporeans and supported a common origin of histozoic *Ceratonova*, *Enteromyxum* and *Kudoa* from a coelozoic ancestor.

Fiala and Bartošová (2010) suggested that the marine myxosporean lineage had a sphaerosporid-like ancestor, which infected the gall bladder of marine fish. This split occurred after the separation of the progenitor of the freshwater lineage, which may have been a parasite of a marine elasmobranch host still inhabiting the marine environment, since extant basal species of the freshwater lineage are *Chloromyxum* species that infect rays and sharks (Gleeson and Adlard, 2012; Kristmundsson and Freeman, 2013; Rocha et al., 2014). The first myxospore morphotype of the marine myxosporean clade was probably *Ceratomyxa*-like (Fiala and Bartošová, 2010), a hypothesis supported by our phylogenetic evidence, which shows that the *Ceratomyxa* clade is the first branch of the marine lineage. Within the *Ceratomyxa* clade, the most basal taxa were found in sharks (Fiala, 2006; Gleeson and Adlard, 2011). Thus, the ancestors of both the marine and freshwater myxosporean lineages very likely parasitized elasmobranch vertebrate hosts and had polychaete definitive host. We revealed two ceratomyxids from bony fish at the base of the *Ceratomyxa* clade in our analysis. Thus ancestor of ceratomyxids could be equally likely parasite of elasmobranch fish as well as bony fish.

In spite of the ever increasing number of myxosporean sequences in the NCBI database, phylogenetic reconstructions of myxozoan evolution still suffer from low taxon sampling, as about half of the described myxosporean genera (i.e. myxospore morphotypes) lack molecular data for any of their representatives. Future research of myxosporean phylogeny should be focused on sequencing representatives from these 'missing' genera, and thus help to find the missing links between the main marine subclades.

## 5. Conclusions

We have reviewed the phylogenetic relationships among all species of the main clades of the marine myxosporean lineage. Histozoic *Ceratonova shasta* and *C. gasterostea* are not closely related to the *Ceratomyxa* clade, which includes gall bladder infecting myxosporeans with similar spore morphology. SSU rDNA, LSU rDNA and EF2 molecular markers show low phylogenetic informativeness in the middle part of the phylogenetic tree of the marine myxosporean lineage causing instability of the clade relationships in this part of the tree. However, phylogenetic analysis and statistical topology tests suggest histozoic *Ceratonova*, *Enteromyxum* and *Kudoa* species to be the most derived marine myxosporeans with a common ancestor and *Ceratomyxa* as the most basal marine clade.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.03.004.

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