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

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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).

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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., 2014). *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., 2014). 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,