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Myxosporean phylogeny and evolution of myxospore morphotypes

Ph.D. thesis

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Annotation

Evolution of the Myxozoa, the group of extremely reduced metazoan parasites, has been studied on the molecular phylogenetic level for more than a decade. This thesis is focused on morphological and molecular characterization of myxozoan species with emphasis on genera with missing molecular data in GenBank as well as on revealing the hidden and cryptic species myxozoan diversity. Evolution of specific traits in myxospore morphotypes and estimation of the time of the myxozoan divergence is also investigated and further discussed.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

České Budějovice, 17. 10. 2014

RNDr. Alena Kodádková

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List of papers and author's contribution

The thesis is based on the following papers and manuscripts (listed chronologically):

Paper I:

Jirků M, Bartošová P, **Kodádková A**, Mutschmann F (2011) Another chloromyxid lineage: molecular phylogeny and redescription of *Chloromyxum careni* from amphibian. Journal of Eukaryotic Microbiology 58(1): 50–59, IF = 2.911

AK was responsible for processing the samples for the morphological documentation including ultrastructure.

Paper II:

Dyková I, **Kodádková A**, de Buron I, Fiala I, Roumillat WA (2013) *Sinuolinea* infections in the urinary system of *Cynoscion* species (Scianidae) and the search for the phylogenetic position of the type species of *Sinuolinea* Davis, 1917 (Myxozoa: Myxosporea) International Journal for Parasitology: Parasites and Wildlife 2:10-17, IF = not defined (new journal from 2012)

AK carried out the sequencing and phylogenetic analysis and participated on writing of the manuscript.

Paper III:

Kodádková A, Dyková I, Tyml T, Ditrich O, Fiala I (2014) Myxozoa in high Arctic: Survey on the central part of Svalbard archipelago International Journal for Parasitology: Parasites and Wildlife 3(1):41-56, IF = not defined (new journal from 2012)

AK conceived the study, participated in field sampling, morphological studies, conducted sequencing, phylogeny, and drafted the manuscript.

Paper IV:

Bartošová-Sojková P, Hrabcová M, Pecková H, Patra S, **Kodádková A**, Jurajda P, Tyml T, Holzer AS (2014) Hidden diversity and evotionary trends in malacosporean parasites (Cnidaria: Myxozoa) identified using molecular phylogenetics International Journal for Parasitology 44(8):565-577, IF = 3.404

AK participated in field sampling and contributed to the final draft of the manuscript.

Paper V:

Bartošová-Sojková P, **Kodádková A**, Pecková H, Kuchta R, Reed CC (2014) Morphology and phylogeny of two species of *Sphaeromyxa* Thélohan, 1892 (Cnidaria: Myxozoa) from marine fish (Clinidae and Trachichthyidae). Parasitology in press, IF = 2.350

AK participated in field sampling, morphological documentation including line drawings and prepared the figures, and contributed to the final draft of the manuscript.

Paper VI:

Kodádková A, Bartošová-Sojková P, Holzer AS, Fiala I *Bipteria vetusta* n. sp. - old parasite in an old host: tracing the origin of myxosporean parasitism in vertebrates. International Journal for Parasitology, submitted (International Journal for Parasitology)

AK carried out the phylogenetic analysis, drafted the manuscript, prepared the figures and participated on the molecular clocks analysis.

Paper VII:

Fiala I, Hlavničková M, **Kodádková A**, Freeman MA, Bartošová-Sojková P, Atkinson SD. Evolutionary origin of *Ceratonova shasta* and phylogeny of the marine myxosporean lineage, submitted (Molecular Phylogeny and Evolution)

AK participated on sequencing, prepared line drawings and some figures and contributed to the final draft of the manuscript.

Co-authors agreement

The senior and corresponding authors of the manuscripts included in this thesis, hereby confirm that AK contributed significantly to these publication, according to the statement above:

Ivan Fiala Iva Dyková Pavla Bartošová-Sojková

Miloslav Jirků

Myxosporean phylogeny and evolution of myxospore morphotypes

Contents

Chapter 1. General characteristic of the Myxozoa

1.1. History of myxozoan discovery

Myxozoa, a group of metazoan parasites, infect aquatic animals worldwide. This diverse group of parasitic animals has a long and rather complicated taxonomic classification history. The extremely reduced body structure due to a parasitic life strategy and the two-host life cycle puzzled pioneer researchers. French ichthyologist Jurine (1825) was the first one who noticed myxosporean infection and described it as "petite vérole des poisons" (small pox on fish) from the already extinct salmonid fish *Coregonus fera* from Lake Geneva. This first myxosporean observation is associated with *Henneguya zschokkei* (Gurley, 1893), which forms cysts in subcutaneous and superficial intermuscular tissue of salmonids. Myxosporeans were described under the term "psorosperms" by a German scientist Müller as early as 1841 (Müller 1841). Proper myxosporean taxonomy however, started several decades later when a German scientist Bütschli (1882) established the phylum Myxosporidia and placed it in the Sporozoa.

Myxosporidia together with another parasitic group Microsporidia Balbiani, 1882 were for a long time classified to Cnidospora Doflein, 1901 (Sporozoa), based on the typical morphological character of both parasitic groups – the polar filament of their spore. The mechanism of extrusion (simple evagination) of the polar filament is principally similar for both taxa. The differences are in the function of the polar filament. It serves as an anchor in the Myxosporea whereas it conveys the infection of host by injection of the host tissue with sporoplasm in Microsporidia. Auerbach (1910) included a new group of organisms into Cnidospora: Actinomyxidia (=later Actinosporea Noble, 1980), which was discovered in 1899 by a Czech scientist Štolc (1899). Both Myxosporidia Bütschli, 1881 and Actinomyxidia Štolc, 1899 shared the same nematocyst-like cells. During the 20th century, growing numbers of described species induced several major revisions of the taxonomic scheme. The systems proposed by Kudo (1933) and Tripathi (1948) can be considered among the first myxosporean classifications. Shulman (1959) built on these two classifications and proposed the main principles of the taxonomic system (Shulman 1966) which persists with some modifications, to the present. Since 1980, Myxosporea and Actinosporea began to be recognized as classes belonging to a separate phylum Myxozoa Grassé, 1970 (Levine et al. 1980). The last thorough revision of myxosporean taxonomy was conducted by Lom and Noble (1984) who adopted Shulman's classification (Shulman 1966) and arranged taxa within the class Myxosporea according to the re-evaluated characters important for classification.

The discovery of the myxosporean two-host life cycle by Wolf and Markiw (1984) can be considered as a taxonomic milestone. Members of Actinosporea were identified to be part of the life cycle of Myxosporea. Kent et al. (1994) thus suppressed the class Actinosporea, leaving a single class Myxosporea in the phylum Myxozoa and proposed that new myxosporean species should not be described based solely on actinosporean forms but on the myxosporean stages.

Another case enjoying the great scientific publicity was a re-discovery of an enigmatic worm *Buddenbrockia plumatellae* Schröder, 1910 as a primitive myxozoan (Monteiro et al. 2002, Okamura et al. 2002). Motile worms (= term myxoworm sensu Canning et al. 2008) filled with rounded bodies (spores) inside bryozoan body cavity were observed 60 years before (Dumortier & van Beneden 1850) the erection of the genus *Buddenbrockia* (Schröder, 1910). Class Malacosporea Canning, Curry, Feist, Longshaw et Okamura, 2000 was erected for *B. plumatellae* and *Tetracapsuloides bryosalmonae* and since that time the myxozoan classification has remained stable at the class level.

Currently the Myxozoa comprises more than 2300 nominal species (Morris 2010) and 60 genera classified to two classes, Myxosporea and Malacosporea. The latter species-poor class contains of only two genera *Tetracapsuloides* Canning, Tops, Curry, Wood et Okamura, 2002 and *Buddenbrockia* with only three nominal species. The vast majority of myxozoan genera are placed in the class Myxosporea (for the review see Lom & Dyková 2006).

1.2. Myxozoan morphology and classification

Myxozoan classification is based almost exclusively on spore morphology, which makes the system an arbitrary one. Lom and Arthur (1989) summarized the guidelines for species description and included the host and the vegetative stage as additional characteristics for classifying/describing myxosporeans. This system is suitable for Myxosporea but fishmalacospores of Malacosporea are rarely seen in fish hosts and do not possess sufficient diagnostic characters for their classification.

Therefore as well as from historical reasons (the fish stage described much later than the bryozoan-related stage), the class Malacosporea is characterised by the trophic worm- or sac-like stages that parasitize bryozoans (Canning & Okamura 2004). Recent molecular and morphological analyses revealed a high malacosporean species diversity and showed that the malacosporean clade includes parasites that develop as "myxoworms" (*B. plumatellae*, several undescribed species), sacs (*T. bryosalmonae, B. allmani*, *B. plumatellae*, several undescribed species), and elongate sacs with lobes (one undescribed malacosporean taxon) (Paper IV: Bartošová-Sojková et al. 2014a, Hartikainen et al. 2014). The sacs and "worm" forms occur across the phylogeny; both forms are present in the genus *Buddenbrockia* whilst within the genus *Tetracapsuloides* only the sac form was observed. Myxoworms possess four longitudinal muscle blocks sandwiched between inner and outer cell layers (Gruhl & Okamura 2012) but lack a gut and an apparent nervous system (Schröder 1910, Okamura et al. 2002). Morphologically uniform fishmalacospores, which are spherical with two unhardened valve cells enclosing two polar capsules

and one sporoplasm, substantially differ from homologous myxosporean spores (Fig. 1).

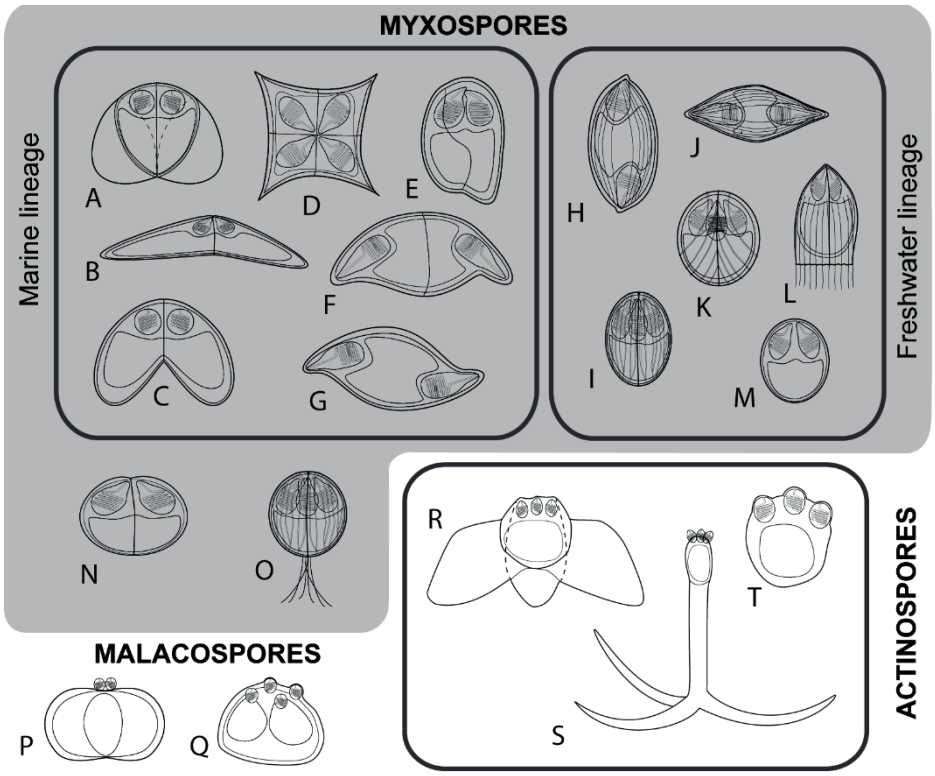

Fig. 1. Myxozoan morphotypes. (A-O) Myxospore morphotypes with one selected morphotype per each phylogenetic clade. (A) *Bipteria*, (B) *Ceratomyxa*, (C) *Ceratonova*, (D) *Kudoa*, (E) *Parvicapsula*, (F) *Enteromyxum*, (G) marine *Myxidium*, (H) freshwater *Myxidium*, (I) freshwater *Chloromyxum*, (J) *Myxidium lieberkuehni*, (K) *Chloromyxum careni*, (L) *Hoferellus,* (M) *Myxobolus*, (N) *Sphaerospora*, (O) marine *Chloromyxum*. (P-Q) Malacosporean morphotypes, (P) fishmalacospore of *Tetracapsuloides*, (Q) malacospore of *Tetracapsuloides* from bryozoan host (R-T) Common actinospore morphotypes, (R) aurantiactinomyxon, (S) triactinomyxon, (T) tetractinomyxon.

Myxosporean classification is based mostly on morphology of myxospores composed of valvogenic, capsulogenic and sporoplasmogenic cells. Each myxosporean genus includes species characterised by shared myxospore morphology ('morphotype') (Fiala & Bartošová 2010). The high variability of spore morphotypes (Fig. 1) provides many character features important for the classification, although all spores share certain features. Myxospores contain typically one to four polar capsules, exceptionally up to 13 and one or more sporoplasms, the actual infectious germs. The sporoplasm and polar capsules are enclosed in a hard shell composed of two to seven valves, which adhere together along one or more suture line(s). The classification at the order and suborder levels is mainly based on the number and configuration of shell valves and number and position of polar capsules to the suture plane. Thus, Myxosporea splits into two

orders: Bivalvulida Shulman, 1959 characterized by two shell valves and Multivalvulida Shulman, 1959 characterized by three to seven shell valves (Lom & Dyková 2006). Bivalvulida consists of two suborders: Variisporina Lom et Noble, 1984 with polar capsules in various positions in the spore and Platysporina Kudo, 1919 with bilaterally symmetrical spores as a rule flattened parallel to the suture line. Additional distinctive features such as character of polar filament, presence/lack of caudal appendages and shape of the suture line are used on the family and generic level. Finally, the classification at the species level is based, besides spore and polar capsule dimensions, on thorough details of the myxospore structure, e.g. the number of turns of polar filament, presence and number of ribs, ridges and striations on spore valves, character of appendages or presence of mucous envelope, mutual size relation of polar capsules, number of sporoplasms and their nuclei etc. Lom and Arthur (1989).

Despite the fact that myxospores possess several taxonomically informative morphological features, several morphotypes are overlapping in morphological characteristics, e.g. *Myxidium*, *Zschokkella*, *Cystodiscus*, *Sigmomyxa* and *Ellipsomyxa* or *Myxobolus* and *Cardimyxobolus*. These overlaps cause problems with correct assignment of new and existing taxa. Myxosporean phylogeny based on molecular markers can help to solve/prevent these problems.

1.3. Life cycles, habitats and tissue specificity

The discovery of the definitive host of *Myxobolus cerebralis* by Wolf and Markiw (1984) uncovered the complete myxosporean two-host life cycle with the definitive annelid and intermediate vertebrate host. The life cycle is different between Malacosporea and Myxosporea. Malacosporeans infect the excretory system of freshwater teleost fish as intermediate hosts, which results in the development of fishmalacospores. Released fishmalacospores infect freshwater bryozoans. Development in bryozoans results in the formation of spherical to ovoid sacs or elongate active worms. These large trophic proliferative stages (plasmodia) infect the basal lamina of the host body wall, attach to the peritoneum or float freely in host's metacoel. Plasmodia produce infectious spores (malacospores) in their internal cavity. To date, only one malacosporean life cycle has been fully resolved, i.e. *T. bryosalmonae* that infects salmonid fish and a bryozoan *Fredericella sultana* (Morris & Adams 2006). Interestingly no malacosporeans were found in the marine environment, despite a high diversity of bryozoans (Gordon 1999) and evolutionary origin of this host group in the marine environment. Furthermore, old records of vermiform stages reminiscent of malacosporean parasites in marine bryozoans are indicative of the existence of marine Malacosporea (Hastings 1943).

Myxosporean life cycles consist of myxospore and actinospore phases (Fig. 2). The cell-in-cell organisation (daughter cell enclosed in the mother cell) is typical for myxozoan development. The myxospore phase takes place in the intermediate hosts, which are mostly lower vertebrates, i.e. bony fish, rarely cartilaginous fish and amphibians. Myxosporeans are exceptionally found in reptiles, birds, mammals and

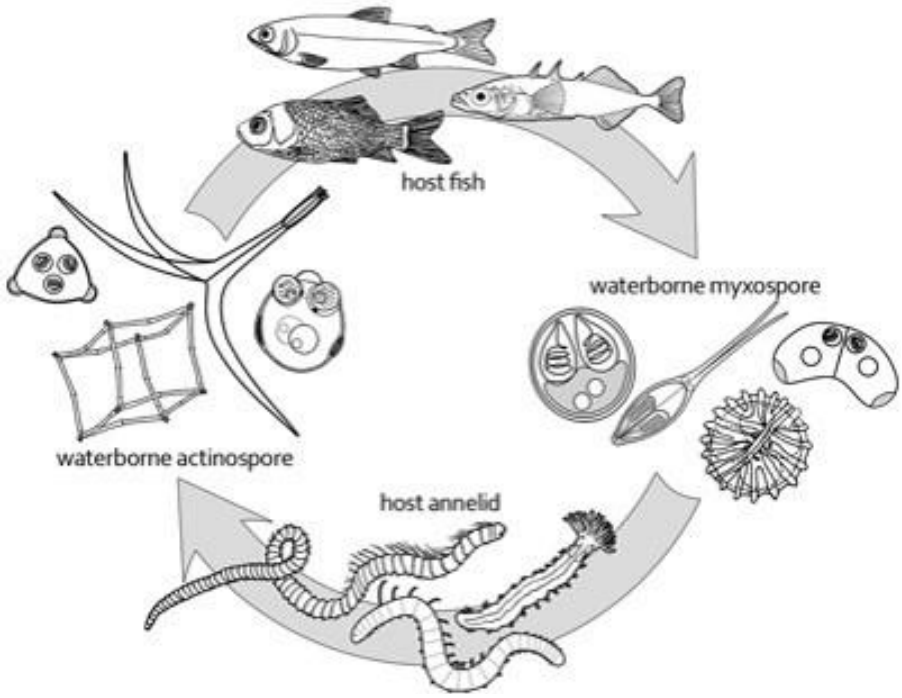

Fig. 2. Myxosporean life cycle © S. Atkinson

Agnatha; some curious findings are reported from Monogenea and Cephalopoda (Yokoyama & Masuda 2001, Lom & Dyková 2006, Freeman & Shinn 2011). Upon contact of the spore with the skin or gill epithelium, polar capsules discharge and polar filaments anchor to the host. In the case of terrestrial life cycle, the vertebrate host may be infected alimentary (Tyml 2010). Then the spore shell valves open and the sporoplasm enters the skin. The presporogonic stage develops and migrates to the sporogonic site of infection where plasmodium develops. Plasmodial stages may be histozoic, situated in tissues (liver, skin, kidney, testes etc.), or coelozoic, situated in the body cavities (mainly the excretory tract and biliary tract). The development in the sporogonic location results in myxospore formation. The actinospore phase, resulting in production of actinospores, involves a sexual phase, which takes place in definitive hosts from the superphylum Lophotrochozoa; typically annelids, rarely sipunculids. Actinospores are soft-shelled with typical triradiate symmetry with more than 30 described morphotypes (Lom & Dyková 2006). Most of the myxosporean life cycles are known from the freshwater environment (Kent et al. 2001). Marine myxosporean life cycles are poorly understood with only few described ones, i.e. *Ceratomyxa auerbachi*, *Gadimyxa atlantica*, *Sigmomyxa sphaerica*, two species of *Parvicapsula* and two species of *Ellipsomyxa.* All of them have a polychaete definitive host (Køie et al. 2004, Køie et al. 2007, Køie et al. 2008, Rangel et al. 2009, Karlsbakk & Køie 2012, Køie et al. 2013).

The exception to the two-host myxozoan life cycle is a direct fish to fish transmissions of *Enteromyxum* spp. vegetative stages (Diamant 1997, Redondo et al. 2002, Yasuda et al. 2002). *Enteromyxum leei* develops inside enterocytes of marine fish and developmental stages are dislodged and orally ingested by other fish, resulting in establishment of horizontal transmission (Diamant 1997, Yasuda et al. 2002, Sitjà-Bobadilla et al. 2007). This route of transmission may occur only in intensive culture system, where it facilitates rapid spread of the parasite. Another case of possible direct transmission was reported in *Kudoa ovivora* by ingestion of eggs in labrid fishes (Swearer & Robertson 1999). Therefore, myxospores of this species may also require development within an alternate host to complete the life cycle (Kent et al. 2001). Moreover, the epidemiological data on *Sphaerospora testicularis* suggest the possibility of the fish-to-fish transmission, but no successful experiments have yet confirmed this hypothesis (Sitjà-Bobadilla 2009).

1.4. Important diseases caused by myxozoans

Throughout the long history of interaction of Myxozoa and their hosts, the relation between these two became well balanced so that, relative to the number of species, only a minority of myxozoan species causes serious or fatal infections whereas the majority of them are harmless. However, this small minority causes the aquaculture industry large production and financial losses. Understanding of the biology of dangerous pathogens has high practical importance. *Tetracapsuloides bryosalmonae* is a malacosporean parasite of salmonid fishes, which causes Proliferative kidney disease (PKD). It is one of the most serious parasitic diseases causing high mortalities of salmonid populations and salmonid cultures in Europe and North America (Hedrick et al. 1993). Myxosporea include other numerous pathogenic species whose myxospore stages often cause considerable losses by fish deaths, retarding their growth, or rendering their meat unpalatable, e.g. *Ceratonova shasta* (syn. *Ceratomyxa shasta*), *Henneguya exilis, Myxobolus cerebralis* in freshwater fish cultures and *Enteromyxum leei, Kudoa thyrsites* or *Sphaerospora dykovae* (syn. *S. renicola*) in marine fish. A typical example of severe salmonid disease is the whirling disease caused by *M. cerebralis*, which damages fish cartilage and compromises its nervous system. With the huge expansion in marine fish aquaculture in the 1990s, particularly cage cultures of salmonids and sea bream, several myxozoans have been recognized as important pathogens. Increased efforts into the research of these marine myxozoans have advanced our understanding of their development and pathogenesis.

Despite the fact that Myxozoa are not human pathogens the consumption of raw fish meat with myxozoan infection is associated with diarrhoea and *Kudoa septempunctata* was identified as the etiological agent (Kawai et al. 2012). The pathogenicity of *K. septempunctata* was demonstrated on *in vitro* experiment on human intestinal cells, which were rapidly invaded by sporoplasm (Ohnishi et al. 2013).

Chapter 2. Phylogeny of Myxozoa

2.1. Myxozoan affinities with other groups

The discoveries of the multicellular nature of spores have impelled many debates about the myxozoan origin within the metazoans. Štolc (1899), Emery (1909) and Ikeda (1912) proposed that myxozoans were in fact Metazoa. Weill (1938) drew an intriguing parallel between myxosporeans and Cnidaria because of multicellularity (the cells of myxozoan spores joined by junctions) and the presence of polar capsules homologous to coelenterate nematocysts. Polar capsules with coiled eversible polar filament are the most distinctive organelles of Myxozoa. He also compared the development of myxosporean pansporoblasts, initiated by the union of pericyte and sporogonic cells, to the development of the larvae of *Polypodium hydriforme* (parasite of sturgeon eggs; Narcomedusae, Cnidaria) starting with a cell doublet consisting of an enveloping cell, a phorocyte, and an enclosed fertilized egg. However, these hypotheses about cnidarian origin of the Myxozoa, however, were frequently discounted by authoritative voices (Dogiel 1965, Desser et al. 1983, Lom 1990).

Metazoan features of Myxozoa along with the metazoan-like 18S ribosomal DNA sequences of myxozoans (Smothers et al. 1994) resulted in the general acceptance of metazoan origin of Myxozoa. However, the accurate placement of Myxozoa within Metazoa remained uncertain; it has been placed either at the base of Bilateria or within Cnidaria, depending on the genes analyzed and the phylogenetic analysis employed. The theory that Myxozoa are members of a primitive bilaterian lineage is based on an ultrastructural study, which revealed the triploblastic nature of the malacosporean *B*. *plumatellae*. This malacosporean species possesses four sets of longitudinal muscle blocks together with the presence of an outer and inner layer of cells in the body wall and thus indicated a close relation to the worm-like bilaterians (Okamura et al. 2002). The triploblastic origin (bilaterian animals) of Myxozoa found support in the molecular data as well. Anderson et al. (1998) published four myxozoan Hox genes that supported bilaterian origin. However, Jimenéz-Guri (2007) proved these genes were of host origin due to a host contamination. Pioneer phylogenetic analyses based on the myxozoan small subunit ribosomal RNA gene (SSU rDNA), showed the Myxozoa as either a sister group to Nematoda (with highest log-likelihood values) which was not significantly different from an alternative topology that placed Myxozoa sister to all bilaterian animals (Smothers et al. 1994). Subsequent phylogenetic analyses based on SSU rDNA have been supporting sister position of myxozoans to bilaterians (Zrzavý et al. 1998, Siddall & Whiting 1999, Zrzavý & Hypša 2003) or a triploblast affinities (Katayama et al. 1995, Hanelt et al. 1996, Schlegel et al. 1996, Kim et al. 1999).

On the other hand, SSU-based analyses including the sequence of narcomedusan *P. hydriforme* and combined analyses of molecular and morphological data, revealed that myxozoans are the sister group to narcomedusans and hence members of cnidarians (diploblast animals) (Siddall et al. 1995, Zrzavý et

al. 1998). It has been suggested that the conflicting results of phylogenetic analyses differing in the triploblastic vs. diploblastic myxozoan origin were caused by the long-branch attraction (LBA), which produces artifacts in phylogenetic analyses when taxa evolved at different evolutionary rates. Myxozoa and other metazoans (e.g. *P. hydridiforme* and Mesozoa) have unusually high divergence rates of their SSU rDNA (Siddall & Whiting 1999). Evans et al. (2010) demonstrated that there are conflicting signals in the phylogenomic data of *Buddenbrockia* and that a removal of only a few sites from their dataset changes the placement of Myxozoa from within cnidarians to an alternative position – at the base of Bilateria. The presumption of triploblastic nature of Myxozoa was based on a hypothesis about the endosymbiotic origin of polar capsules (Okamura & Canning 2003), despite the extraordinary similarity of myxozoan polar capsules and cnidarian nematocysts in their structure and function (e.g. Weill 1938).

Despite a strong triploblastic appurtenance, diploblasty (cnidarians affinity) continued to be favoured (Kent et al. 2001) and substantial evidence was later found. Although *Buddenbrockia* superficially resembles a bilaterian worm, another interpretation of the structure of *Buddenbrockia* vermiform body says its four blocks of muscles are radially distributed like in cnidarians (Jimenéz-Guri et al. 2007). Following ultrastructure study confirmed the latter interpretation of body organization by showing tetraradially arranged muscles in *B. plumatellle* (Gruhl & Okamura 2012). Molecularly, a homolog to minicollagens, which are cnidarian-specific nematocyst proteins (autapomorphy), was detected in the malacosporean *T. bryosalmonae* (Holland et al. 2011). Furthermore, phylogenetic analysis of multiple protein-coding genes placed Myxozoa as a highly derived Cnidaria, likely within their subgroup Medusozoa (Jimenéz-Guri et al. 2007, Nesnidal et al. 2013). However, a topology test of EST data did not reject a bilaterian origin of myxozoans (Jimenéz-Guri et al. 2007) and a Bayesian inference (CAT model) of genomic data from the myxosporean *M. cerebralis* did not strongly support the position of Myxozoa within the Cnidaria (Nesnidal et al. 2013).

Recently, a phylogenomic study of the myxosporean *Thelohanellus kitauei* strongly supported a sister position of Myxozoa to Medusozoa within Cnidaria. Several proto-mesodermal genes which are also present in the cnidarian *Hydra magnipapillata,* were found to robustly support the myxozoans being a derived cnidarian taxon with an affinity to Medusozoa (Feng et al. 2014). The battle of two possible hypotheses of myxozoan origin seems to be overly in favour of Cnidaria. Nevertheless, there are still secrets regarding the accurate phylogenetic relations of the Myxozoa with particular medusozoan groups, which will be hopefully resolved in future studies. Parallel to this scientific debate, our laboratory paradoxically still bares the historical name the Laboratory of Fish Protistology, even though we study parasitic cnidarians, which since their discovery have still been studied by protistologists.

2.2. Phylogeny within the Myxozoa

The methodology of molecular phylogenetics is essential to evaluate the evolutionary relationships among taxa, which also applies to the relationships within myxozoans. Phylogenetic reconstruction can also reveal the continuous process of diversification and speciation or history of character evolution. We can trace the first common ancestor of all extant myxozoan species that derived from an ancient cnidarian lineage. This first common myxozoan ancestor was most probably a malacosporean-like organism with myxoworm morphology that might have been similar to extant malacosporean *B. plumatellae* (Monteiro et al. 2002). After the split of Myxosporea from the malacosporean lineage, myxosporeans branched into three main lineages: sphaerosporids sensu stricto, the myxosporean freshwater and the

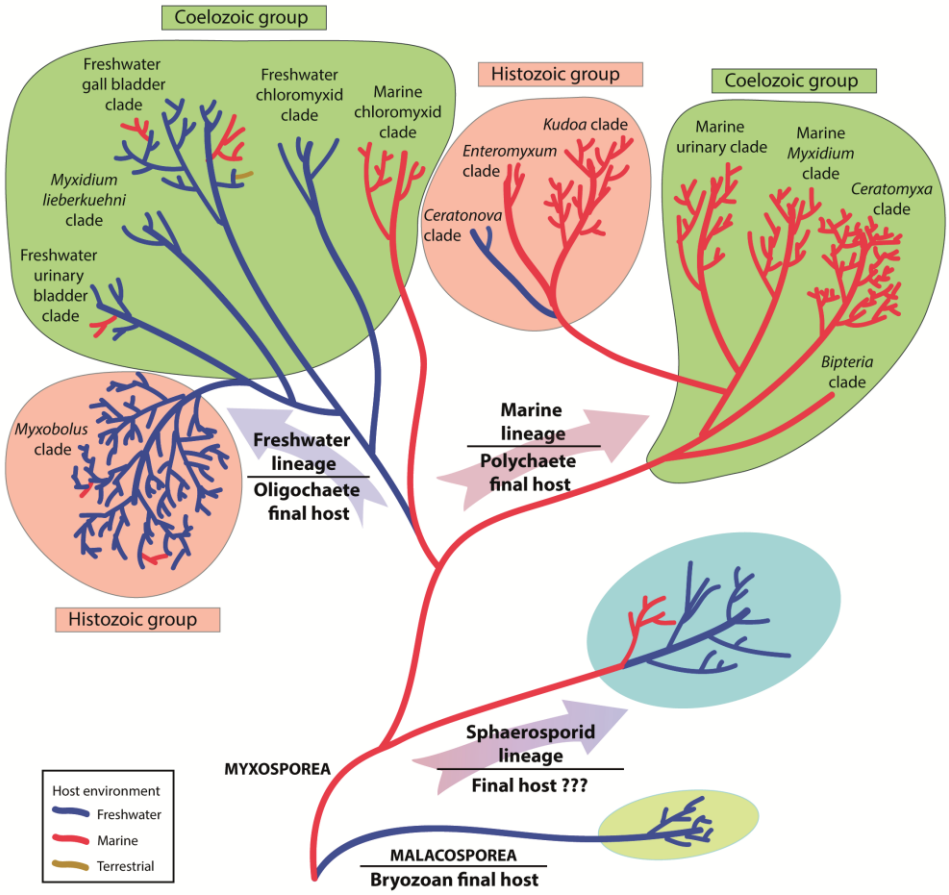

Fig. 3. Schematic evolution of the Myxozoa based on SSU rDNA data (Fiala & Bartošová-Sojková 2014).

marine myxosporean lineages (Fig. 3). These events happened deep in the myxozoan evolution presumably in the early Paleozoikum (Paper VI: Kodádková et al. submitted). Thus, for reconstruction of the molecular phylogeny of Myxozoa as an

organism, which underwent a deep ancient radiation, conservative phylogenetic markers are required.

SSU rDNA is a unique molecular marker that is widely used for phylogenetic reconstruction of many metazoan taxa at different taxonomic levels (Hillis & Dixon 1991) and has been proved to be sufficiently informative to estimate the phylogenetic relationships among myxozoan species (Kent et al. 2001). The popularity of this marker lies in its character of alternating variable and conservative DNA regions, which can be used at both deep-level and more recent phylogenetic relationships. Variable regions have frequent nucleotide substitutions and insertions/deletions, which are reflected in the length of the SSU rDNA. The length of SSU rDNA of myxozoan species conspicuously differs within the three main lineages: species clustering in the marine lineage have approx. 1800 bp, the members of freshwater lineage approx. 2000 bp and the species of the *Sphaerospora sensu stricto* lineage have up to 4000 bp long SSU rDNA (Fiala 2006, Bartošová et al. 2013). SSU rDNA has been successfully used to study both inter- and intra-specific relationships (e.g. Fiala 2006, Diamant et al. 2007, Gunter & Adlard 2008, Karlsbakk & Køie 2011), or to identify cryptic species (e.g. Whipps & Kent 2006, Bartošová et al. 2011, Paper II: Dyková et al. 2013). Multigene analyses are currently thought (e.g. Nosenko et al. 2013) to give more reliable results for tracing deep branching events in Metazoa but SSU rDNA is still extensively used in the phylogenetic analyses for above mentioned reasons. Moreover, it was found to be extremely problematic to amplify myxosporean protein-coding genes, which are essential to perform a large multigene phylogenetic study of Myxozoa (Fiala & Bartošová 2007).

Another frequently used phylogenetic marker is the large subunit ribosomal RNA gene (LSU rDNA) which has similar heterogeneity and informativeness as SSU rRNA gene. In Myxozoa, it has been mostly used as a marker supporting the SSU-based phylogeny and providing additional character information (Whipps et al. 2004b, Bartošová et al. 2009, Bartošová et al. 2013). As LSU rDNA has similar evolutionary history as SSU due to concerted evolution (Hillis & Davis 1988) and phylogenies based on LSU rDNA or concatenated SSU + LSU rDNAs can suffer the same phylogenetic artifact (LBA), as does SSU alone (Siddall & Whiting 1999). Therefore, other phylogenetic markers have been explored to better reconstruct myxosporean evolution, i.e. elongation factor 2 (EF2), heat shock protein 70 (HSP 70) and myosin heavy chain 2 (Fiala & Bartošová 2007). Unfortunately, only a single protein-coding gene – EF2 was successfully used for broader myxosporean phylogenetic studies (Fiala & Bartošová 2010, Bartošová et al. 2013, Paper IV: Bartošová-Sojková et al. 2014a, Paper VII: Fiala et al. submitted). Even though the phylogenetic signal of EF2 was congruent with the rDNA-based phylogeny (Fiala & Bartošová 2010), nevertheless, the informativeness of this marker was unfortunately revealed to be very low (Paper VII: Fiala et al. submitted).

Several studies have been performed to evaluate myxozoan intraspecies variability. The HSP 70 gene was used in phylogeographic study of *K. thyrsites*. However, due to its conservative nature, it was shown to be not appropriate for studies of myxozoan intra-specific relationships (Whipps & Kent 2006). On the other

hand, internal transcribed spacer region 1 (ITS 1), as rapidly evolving phylogenetic marker, was successfully used in several intra-specific studies. *Tetracapsuloides bryosalmonae* and *K. thyrsites* exhibited regional differentiation (Henderson & Okamura 2004, Whipps & Kent 2006), whereas *M. cerebralis* showed genetic homogeneity, supporting the recent anthropogenic spread of this parasite from Europe to North America (Whipps et al. 2004a). Moreover ITS 1 with ITS 2 revealed the existence of cryptic myxosporean species in amphibians (Hartigan et al. 2011).

The Malacosporean lineage with its *Buddenbrockia* myxoworm is supposed to be a primitive myxozoan clade derived from the myxozoan ancestor in the Cambrian era (Canning & Okamura 2004, Paper VI: Kodádková et al. submitted). Malacosporeans split into several sublineages recently uncovered independently by Hartikainen et al. (2014) and Bartošová-Sojková et al. (Paper IV: (2014a). These studies were focused on investigation into the malacosporean diversity in bryozoans and fish, respectively. No new malacosporean life cycles have been revealed after the comparison of SSU rDNA sequences of more than ten recently recognized malacosporean lineages (probably new species) from bryozoan and fish hosts (Fiala & Bartošová-Sojková 2014). These studies suggested the malacosporean diversity is much higher than previously expected and revealed high ubiquity of malacosporeans in both fish and bryozoans.

Phylogenetically, the Myxosporea are split into three main distinctive lineages – the marine, the freshwater, and the *Sphaerospora s.s.* clade (Fiala 2006, Jirků et al. 2007, Bartošová et al. 2013). The clustering of particular species in these lineages is well supported, however, certain instability was observed among the positions of these phylogenetic groups: *Sphaerospora s.s.* clade is either basal to the marine + freshwater lineage or sister to the marine lineage (Bartošová et al. 2013). The *Sphaerospora s.s.* clade groups mostly coelozoic myxosporeans infecting the urinary tract of marine and freshwater fish and amphibians. This clade encompasses morphologically similar species of the genus *Sphaerospora* who typically possess long inserts in their rRNA genes (Jirků et al. 2007, Gunter & Adlard 2010, Bartošová et al. 2013). The sphaerosporid clade splits in two lineages: lineage A including species from marine environment and lineage B having four subclades that correlate with host group and habitat e.g. closely-related sphaerosporids from freshwater – amphibian hosts (Bartošová et al. 2013). *Sphaerospora fugu* and *S. molnari* are the only histozoic species of the *Sphaerospora s.s.* clade, which cluster separately either in Lineage A or Lineage B, respectively. This indicates that histozoic way of life originated convergently at least two times in the sphaerosporid lineage from coelozoic species (Bartošová et al. 2013, Eszterbauer et al. 2013, Holzer et al. 2013).

The marine myxosporean lineage splits into six well-supported main clades: the *Ceratomyxa* and the marine *Myxidium* clades including species infecting the gall bladder; the marine urinary clade encompassing species from the host excretory system; the *Kudoa*, *Enteromyxum* and *Ceratonova* clades grouping histozoic species; and a separate lineage with only a single species *Bipteria vetusta* (Fiala 2006, Atkinson et al. 2014, Paper VI: Kodádková et al. submitted). The relationships between these clades are not entirely resolved. A number of published analyses have

provided mixed results showing variable/instable relationships among these clades that were always characterised by low nodal support (Fiala 2006, Bartošová et al. 2009). The clustering of species in particular clades follows tissue tropism criterion rather than myxospore morphology (Holzer et al. 2004, Fiala 2006). The marine urinary clade is typical in this respect: phylogenetically closely related myxosporeans of the genera *Parvicapsula*, *Gadimyxa*, *Sphaerospora*, *Sinuolinea*, *Latyspora*, and *Zschokkella* differ in spore morphology but have the same tissue tropism (Bartošová et al. 2011). Although, some species of the *Parvicapsula* subclade cause systemic infections and can be exceptionally found in epithelium of the gall bladder, the intestine, the pseudobranchs and testicles, most of the species infect the excretory system. The *Zschokkella* subclade contains species of the polyphyletic genus *Zschokkella* including its type species *Z. hildae* as well as type species of the polyphyletic genera *Latyspora* and *Sinuolinea* (Bartošová et al. 2011, Paper II: Dyková et al. 2013). The *Zschokkella* subclade is characterised by encompassing species of different genera with high variability in myxospore shape and position of PCs ranging from being set at opposite ends of the spore to be placed closely next to each other.

The freshwater lineage, with sister branching *Chloromyxum s.s* clade (= marine *Chloromyxum* clade) with species infecting elasmobranchs, is comprised of five clades: the hepatic biliary clade, the freshwater *Chloromyxum* clade, the *Myxidium lieberkuehni* clade, the *Myxobolus* clade, the urinary bladder clade and a single-species lineage of *Chloromyxum careni* (Fiala 2006, Paper I: Jirků et al. 2011, Kristmundsson & Freeman 2013). Similarly as in marine clades, tissue tropism is also the main criterion for species clustering in freshwater clades. Freshwater clades contain poly- or paraphyletic genera including species with different morphology but identical affinity to host organ or tissue. Despite the freshwater environment as the ancestral state for the species of the freshwater lineage, several species of this lineage invaded the marine (*Ortholinea orientalis*, sphaeromyxids, *Myxidium coryphaenoidium*, *Myxidium baueri*) and terrestrial environment (*Soricimyxum fegati*).

Most of the entries of myxozoan sequences in GenBank belong to their myxospore stages whereas significantly smaller portion of data is represented by their actinospore counterparts. The *Myxobolus* clade encompasses the majority of actinospore stages available in GenBank representing a wide variety of actinospore morphotypes from oligochaetes, e.g. hexactinomyxon, triactinomyxon, auractinomyxon, neoactinomyxum, synactinomyxon, echinactinomyxon, and endocapsa (Kent et al. 2001, Holzer et al. 2004, Eszterbauer et al. 2006). Within the *Myxobolus* clade, generally grouping freshwater taxa, a subclade of parasites from mixed environments occurs. Along with *Myxobolus* species from brackish and freshwater fish this subclade includes actinospore morphotypes from freshwater (endocapsa, triactinomyxon, neoactinomyxum, aurantiactinomyxon) as well as from marine oligochaetes (endocapsa, tetraspora, triactinomyxon, sphaeractinomyxon) (Hallett et al. 1999, Kent et al. 2001, Székely et al. 2007). The freshwater *Myxidium* clade includes the actinospore counterparts of *Zschokkella* and *Myxidium* spp. from freshwater oligochaetes, i.e. raabeia, guyenotia, and siedleckiella (Kent et al. 2001,

Eszterbauer et al. 2006). The freshwater *Chloromyxum* clade includes antonactinomyxon, aurantiactinomyxon actinospores of *Chloromyxum* spp. from freshwater oligochaetes (Holzer et al. 2004). The freshwater urinary bladder clade groups the actinospore counterparts of *Myxobilatus, Myxidium*, and *Chloromyxum* species from freshwater oligochaetes, i.e. triactinomyxon, aurantiactinomyxon, neoactinomyxum (Holzer et al. 2006). The marine myxosporean lineage is undersampled in regard to actinospore stages and so far includes only two actinospore morphotypes: i) tetractinomyxon of *Gadimyxa*, *Parvicapsula*, *Ceratonova*, *Ceratomyxa*, *Ellipsomyxa*, *Sigmomyxa*, and ii) unicapsulactinomyxon of *Enteromyxum* both from polychaetes (Køie et al. 2004, Rangel et al. 2011). As obvious from aforementioned trends and increasing number of exceptions of freshwater-marine separation, myxosporeans do not clearly cluster according to spore morphology and host environment but rather according to their definitive host groups, e.g. oligochaetes vs. polychaetes (Holzer et al. 2007, Paper VII: Fiala et al. submitted).

2.3. Discrepancies between taxonomy and phylogeny

First molecular data using mostly SSU rRNA gene sequences of Myxosporea has indicated that the classification based mainly on the structure and shape of the myxospore is not consistent with phylogenetic relationships (Smothers et al. 1994). This view was supported by Andree et al. (1999), who demonstrated that the genus *Myxobolus* is not monophyletic. Paraphyly of this genus was later confirmed by Kent (2001) in the first comprehensive phylogenetic analyses that deepened the incongruity of taxonomy and phylogeny of myxosporeans. Perhaps most surprising was the lack of monophyly of most genera examined. This analysis suggested that the only clearly monophyletic genus was *Kudoa,* which was later disrupted by positioning of *Sphaerospora dicentrarchi* within the *Kudoa* clade (Diamant et al. 2005). The other four genera evaluated, i.e. *Myxobolus*, *Henneguya*, *Sphaerospora* and *Myxidium*, were poly- or paraphyletic. Moreover, the polyphyletic genera *Henneguya*, *Sphaerospora*, *Myxidium*, *Zschokkella* and *Chloromyxum* were further confirmed in a comprehensive analysis based on more than 130 sequenced myxozoan species (Fiala 2006). In addition, paraphyly of genera *Myxobolus* and *Kudoa* was supported and paraphyly of the genus *Ceratomyxa* was revealed (Fiala 2006). Monophyletic groups were identified as additional DNA sequences became available for members of other genera, for example *Parvicapsula* (Nylund et al. 2005), *Enteromyxum* (Palenzuela et al. 2002) and *Sphaeromyxa* (Fiala 2006). However, recently erected genus *Gadimyxa* rendered the genus *Parvicapsula* paraphyletic (Køie et al. 2007).

Although myxosporean systematics is still based mainly on the spore morphology, results of molecular phylogeny have become to influence the classification of Myxosporea. For example, erection of the genus *Enteromyxum* and transfer of formerly described *Myxidium leei* to this genus was the first taxonomic revision based on molecular data (Palenzuela et al. 2002). In addition, replacement of *Bipteria formosa* as *Sphaerospora formosa* was driven by its affinity to

Sphaerospora s.s. clade (Bartošová et al. 2013). Later on, Whipps et al. (2004b) proposed taxonomic redescription of the Multivalvulidae. They synonymized three families and genera with the family Kudoidae and the genus *Kudoa*, respectively, as the only differential feature was the number of shell valves. The genus *Polysporoplasma* was suppressed due to the clustering of its type species within the *Sphaerospora s. s.* clade including members with very similar morphology and tissue tropism (Bartošová et al. 2013).

A combination of classic taxonomic approach and molecular data has led to incremental improvement of myxosporean classification. However, all the revisions driven by phylogeny should be carefully weighed up and preferably supplemented by other additional supporting characteristics, i.e. biological and bionomical traits.

Chapter 3. Ecology, diversity and evolution

3.1. Myxozoan host spectrum

Myxozoans are generally known as parasites of fish being their intermediate hosts. As dixenous parasites, myxozoans have a broad spectrum of intermediate and definitive hosts. Malacosporea infect the freshwater and anadromous bony fishes from orders Perciformes, Cypriniformes, Esociformes and Salmoniformes, however, their fish host spectrum may be larger due to cryptic species malacosporean diversity (Bartošová-Sojková et al. 2014a). The definitive host for Malacosporea are known from four families of freshwater Bryozoa: Cristatellidae, Fredericellidae, Lophopodidae and Plumatellidae. *Tetracapsuloides bryosalmonae* infects mostly salmonid fish and reports from nonsalmonid hosts were sporadic and likely caused by other myxosporean species (*Esox lucius* and *Rutilus rutilus*: Seagrave et al. 1981, Tops & Okamura 2005, Paper IV: Bartošová-Sojková et al. 2014a). Definitive hosts for *T. bryosalmonae* are members of three families of freshwater Bryozoa (Paper IV: Bartošová-Sojková et al. 2014a, Hartikainen et al. 2014). Additionally, intra-bryozoan cycles without involvement of a fish host might be possible for some malacosporeans (Hill & Okamura 2007).

Myxosporean intermediate hosts are mostly lower vertebrates, typically bony fish, rarely cartilaginous fish and amphibians, exceptionally reptiles and Agnatha. Higher vertebrates have been reported to be parasitized by myxosporeans only in a few cases; in birds (waterfowl, Bartholomew et al. 2008) and mammals (shrews, Prunescu et al. 2007). There are also some curious findings reported from Monogenea and Cephalopoda (Yokoyama & Masuda 2001, Lom & Dyková 2006, Freeman & Shinn 2011). The myxosporean definitive hosts are from the superphylum Lophotrochoza; typically annelids and rarely sipunculids (Lom & Dyková 2006).

3.2. Host specificity

Fish host specificity is an important research subject since these hosts are of great economic importance. While some of the pathogenic species seem to be strictly host specific (e.g. *Sphaerospora* spp. in the kidney of cyprinid fishes), others are highly polyxenous (e.g. *E. leei* and *K. thyrsites*) infecting many taxonomically different host species. It seems that host specificity varies in each genus (clade). A significant proportion of *Kudoa* species have relatively low host specificity, with a single species able to infect multiple host species representing various host families even from different host orders (Burger & Adlard 2011), while members of *Ceratomyxa* are generally restricted to a single host species. Moreover, it is common that a fish host harbours more than a single species of ceratomyxids (Gunter & Adlard 2009). Additionally, some myxosporean species such as *Myxobolus* and *Ceratomyxa* spp. branch according to the evolution of their hosts (Molnár et al. 2002, Gleeson & Adlard 2011).

Vertebrate host specificity has been a topic for many studies; however research on invertebrate host specificity is scarce. A crucial problem is very low prevalence of myxosporeans in annelid hosts, which makes the assessment of the host specificity problematic. According to recent studies, the annelid *Tubibex tubifex* get infected by more than ten species of the genus *Myxobolus* (list of complete life cycles by Kent et al. 2001). On the other hand, *Myxobolus preudodispar* is known from four annelid hosts (Marton & Eszterbauer 2012). In the marine environment myxosporeans display some degree of specificity, e.g. *Ellipsomyxa gobii* uses two species of *Nereis* as invertebrate hosts (Køie et al. 2004), whereas *Sigmomyxa sphaerica* apparently use only one species of *Nereis* (Karlsbakk & Køie 2012). However, much data about host specificity have to be taken with caution since the determination of the myxosporean species, especially in the older literature, may not be reliable. In addition, myxozoan species reported from a wide range of hosts might in fact represent a complex of cryptic species necessary to be identified by molecular analysis.

3.3. Tissue specificity and character of developmental stages

Myxosporea has been found in a variety of tissues and organs. Coelozoic species infect body and organ cavities, such as the gall bladder and the urinary tract up to renal corpuscles, and attach to the tissue walls or float freely in the fluid within the cavity. Exceptionally, they occupy other sites, e.g. the pericardium or blood vessels or are attached to intestinal epithelium (Cuadrado et al. 2007). Coelozoic plasmodia can be very small (10–20 m) and are mostly disporic (producing two spores) or occasionally monosporic (producing one spore) as typical for example for sphaerosporids s. s. (Lom & Dyková 1992, Bartošová et al. 2013). Plasmodia of intermediate size (e.g. tens to hundreds of micrometres) may be mono-, di- or polysporic and are produced by myxosporeans of both marine and freshwater lineages e.g. *Ceratomyxa*, *Chloromyxum* and *Parvicapsula*. Some coelozoic plasmodia can be large (up to several millimetres), e.g. *Sphaeromyxa*. Histozoic

species live intercellularly in various tissues. Intracellular parasitism is also *Sphaeromyxa* (Kristmundsson & Freeman 2013). Intracellular parasitism is also common as many species invade cells in the initial proliferative phase of their life cycle. Species of the order Multivalvulida live intracellularly, mostly in muscle cells. Plasmodia of histozoic myxosporeans often grow to enormous size, up to several millimetres. These large plasmodia can be encased within a fibroblast envelope and are visible as large cysts in infected tissues, e.g. in *Myxobolus*, *Henneguya* and *Kudoa*. The histozoic parasitism is often associated with some pathology (Molnár & Kovacsgayer 1985).

Tracing the character history of infection site revealed the common myxosporean ancestor was coelozoic and evolved into histozoic forms two times independently. It occurred within the marine lineage in the common ancestor of the *Ceratonova* and *Enteromyxum* + *Kudoa* clades and as well as in the common ancestor of the most robust myxosporean clade, the *Myxobolus* clade, in the freshwater lineage (Fiala & Bartošová 2010, Paper VI: Kodádková et al. submitted).

3.4. Evolutionary history of myxospore morphotypes

Although every myxosporean species has typically actinospore and myxospore morphotypes, the former is mostly unknown for the majority of described species. However it is now becoming clear that several myxospore morphotypes share the same actinospore morphotype, e.g. *Ceratomyxa auerbachi*, *Ceratonova shasta*, *Gadimyxa atlantica*, *Parvicapsula minibicornis* and *Ellipsomyxa gobii,* and *E. mugilis* have tetractinomyxon actinospore morphotype. This suggests that myxospores may have undergone greater morphological differentiation than actinospores although further sampling of actinospores is required to confirm this assumption. We can also speculate that myxosporeans first invaded invertebrate host during evolution with the actinosporean morphotype of spores since the sexual phase occurs in annelids. Myxospore morphotypes developed later in the evolution when the secondary hosts were involved in the life cycle. The presence of morphologically distinct actinospores and myxospores within the same life cycle demonstrates considerable plasticity in spore design and may be related to maximising the efficiency of transmission to another host. Furthermore, it may be inappropriate to equate actinospores and myxospores as homologous stages that are reiterated within a life cycle.

There are several main myxospore morphotypes (Fig. 1) that are associated with the majority of myxosporean diversity (i.e. those produced in species of *Myxobolus*, *Henneguya*, *Ceratomyxa*, *Myxidium*, *Zschokkella*, *Chloromyxum*, *Sphaerospora*, *Kudoa*, *Thelohanellus* and *Sphaeromyxa*) and thus can be considered as the most evolutionarily successful ones. About 50 remaining morphotypes (50 genera) (according to Lom & Dyková 2006) represent only a small fraction of all described myxosporeans but they illustrate the broad range of myxospore morphologies that have evolved. The most common myxospore morphotype is that of *Myxobolus* – the most species rich myxosporean genus which harbours more than 800 species (Liu et al. 2013). Eight myxospore morphotypes are very similar to the

Myxobolus morphotype varying in only minor ways (e.g. loss of one polar capsule, unequal size of polar capsules, development of spore caudal appendages) and their appurtenance to be different are sometimes speculative, e.g. *Henneguya* and *Hennegoides*. They all probably evolved from the *Myxobolus* morphotype as resulting from phylogenetic analyses placing *Henneguya*, *Thelohanellus* and *Unicauda* in the *Myxobolus* clade. The *Myxobolus* morphotype with its morphotype variations altogether are associated with about half of the myxosporean species described to date.

The phylogenetic analyses revealed many cases when unrelated species bear the same myxospore morphotype. These species are typical examples of convergent evolution due to, for example parasitism in the same tissue or organ. The genera *Myxidium* and *Zschokkella*, which are characterised by polar capsules situated at opposite ends of an elongate myxospore are sometimes extremely difficult to distinguish (Diamant & Palenzuela 2005). Based on the phylogeny these very similar morphotypes evolved several times independently. The *Chloromyxum* morphotype represents another case of remarkable convergent evolution with evolutionary reconstruction suggesting multiple origins of this morphotype (Fiala & Bartošová 2010). In many cases, a single myxosporean clade includes a number of different morphotypes. For example, each of the closely related species in the freshwater urinary clade (*Acauda*, *Chloromyxum*, *Hoferellus*, *Myxidium*, *Myxobilatus*, *Ortholinea* and *Zschokkella*) possess highly distinct spore morphotypes (Fiala 2006, Karlsbakk & Køie 2011, Whipps 2011). Similar levels of variation amongst myxospore morphologies characterise species in the marine urinary clade (Bartošová et al. 2011, Paper III: Kodádková et al. 2014). This is in contrast to rather morphologically uniform myxospores produced in the *Ceratomyxa*, *Kudoa* and *Sphaerospora* clades.

Tracing the character history of spore morphology (Fiala & Bartošová 2010) revealed that the common myxozoan ancestor had similar spore morphology to the current species of the genus *Sphaerospora* infecting the renal tubules of freshwater fishes. This supports the hypothesis of Jirků et al. (2007) that current myxozoans are derived from the *Sphaerospora* morphotype. A hypothetical ancestor of a stem lineage leading to the marine lineage has *Ceratomyxa* morphotype as deduced from the firm position of the *Ceratomyxa* clade as the basal taxon of the marine myxosporean lineage (Paper VII: Fiala et al. submitted). The ancestor of the freshwater lineage is supposed to evolve from the *Chloromyxum* morphotype after the separation of elasmobranch-infecting *Chloromyxum* species from the marine lineage. *Chloromyxum* morphotype probably evolved from sphaerosporid ancestor by the duplication of polar capsules (Fiala & Bartošová 2010).

Chapter 4. Objectives of the research

- Characterisation of selected myxosporean species using morphology and molecular approach
- Study of the cryptic species from closely related fish host
- Investigation into the malacosporean diversity
- Investigation into the myxosporean evolutionary trends
- Tracing the myxosporean character evolution and estimation of the time of myxozoan divergence

Chapter 5. Summary of results and discussion

Highlights of the core findings of the present thesis:
(i) Discovery of a novel myxosporean lineage within the freshwater clade and a description of two myxosporean species of the *Sphaeromyxa* clade (Paper I, V)

(ii) Detection of high prevalence and diversity of malacosporeans and discovery of cryptic myxosporean species (Paper II, III, IV)

(iii) Discovery of phylogenetic position of new myxospore morphotypes within the marine urinary clade with the revision of the marine lineage (Paper II, III, VI, VII)

(iv) Estimation of dating of the myxozoan evolution and tracing the history of evolution of the selected morphological and bionomical characters (Paper VII)

5.1. New myxosporean lineages

Myxosporean parasites have been identified in amphibians around the world but very little is known about their diversity, biology and pathological impact on host. Our phylogenetic analysis of SSU rDNA of *Chloromyxum careni* from Malayan horned frog, *Megophrys nasuta,* revealed that this myxosporean represents an evolutionary distinct myxosporean branch within the freshwater lineage. It is the first amphibian-infecting *Chloromyxum* sequenced to date (Paper I: Jirků et al. 2011) out of four so far described ones. Altogether, there are seven amphibian-infecting species clustering in 3 distinct clades (Paper I: Jirků et al. 2011, Hartigan et al. 2012, Bartošová et al. 2013). Thus, it is clear that amhibians were infected by myxosporeans several times independently. Furthermore, in our Paper I we introduced the marine *Chloromyxum* clade to include *Chloromyxum* species from elasmobranchs. This clade, also called *Chloromyxum s.s.* clade as it contains the type species *Chloromyxum leydigi*, was later enriched by adding several new chloromyxids (Gleeson & Adlard 2012).

Unlike many myxosporean genera (including the above mentioned genus *Chloromyxum*), sphaeromyxids represent a monophyletic group of marine species

clustering within the hepatic biliary clade of the freshwater lineage (Kristmundsson & Freeman 2013). Sphaeromyxids share a unique autapomorphy among myxosporeans, as they have a ribbon-like zig-zag folded polar filaments. *Sphaeromyxa* species have probably evolved by a change in the character of polar filament from the spindle-shaped *Myxidium* ancestor, which then returned to the marine environment (Fiala & Bartošová 2010, Kristmundsson & Freeman 2013). We described new *Sphaeromyxa* species, *S. limocapitis* and *S. clini* (Paper V: Bartošová-Sojková et al. 2014b), which cluster within the newly recognized '*limocapitis*' lineage and within the '*incurvata*' group, respectively. *Sphaeromyxa clini* was found to be most similar to *Sphaeromyxa noblei* also described from clinid hosts but from different geographical realms. *Sphaeromyxa limocapitis* appears to represent a missing link in the evolution of sphaeromyxids. This is reasoned by similarity in its overall morphology with *Myxidium* morphotype represented by *Myxidium coryphaenoideum* and *Myxidium baueri* with spindle shape spores, which are sisterly related to sphaerosporids.

5.2. Myxozoan diversity and cryptic species phenomenon

The diversity of myxosporean species and genera is much higher than the diversity of malacosporeans. Our PCR screening of fish hosts from different fish hosts and habitats revealed an unexpectedly high prevalence and hidden diversity of malacosporeans in several fish hosts in European freshwater habitats. We discovered five new phylogenetic groups representing very likely new species of *Buddenbrockia* and *Tetracapsuloides* from cyprinid and perciform fishes (Paper IV: Bartošová-Sojková et al. 2014). We also reported co-infections of up to three malacosporean species in individual fish. We suggested that hidden biodiversity in malacosporeans is most probably due to the cryptic nature of malacosporean sporogonic and presporogonic stages and mostly asymptomatic infections in the fish hosts.

It was not only the hidden diversity of malacosporeans that surprised us during the research within the scope of this thesis. The molecular phylogeny approach along with morphological data helped us to reveal also the presence of cryptic species in myxosporeans (Paper II: Dyková et al. 2013). Sequencing of the type species of the genus *Sinuolinea*, *S. dimorpha*, from the type fish host *Cynoscion regalis* and its closely related *Cynoscion nebulosus* uncovered the existence of two morphologically undistinguishable species. The first species was found to infect both fish hosts and the second species was found in *C. nebulosus* only. The cluster containing exclusively sequences of *Sinuolinea* from the type species has been designated as representing *S. dimorpha* because of its origin from the type host (Paper II: Dyková et al. 2013). Further study revealed *Zschokkella siegfriedi* as a cryptic species of *Zschokkella hildae* known from numerous gadid fish (Paper III: Kodádková et al. 2014). Moreover, apart from the genetic difference, localization of *Z. siegfriedi* in the upper excretory system (*Z. hildae* in the collecting duct) and different but very closely related host species support *Z. siegfriedi* as a distinct species from *Z. hildae*.

These findings contribute to acknowledging myxozoan cryptic species diversity, an important topic that emphasizes the general necessity of species elimination and of continued effort to improve our knowledge of Myxozoa based both on morphology of spores and molecular data.

5.3. Phylogenetic position of new morphotypes and evolution of the myxospore characters

Molecular taxonomy helped us to detect otherwise morphologically indistinguishable new myxozoan species. Nevertheless, it was mainly a great tool to reveal the relationship of species with myxospore morphotypes (genera) with unknown sequence data in GenBank (NCBI database) as well as to reveal the evolution of specific myxospore traits.

We revealed the phylogenetic position of *Schulmania aenigmatosa* that is the only known species of its genus with the molecular data available in GenBank (Paper III: Kodádková et al. 2014). In that particular paper, we also found *Latyspora*-like organism that morphologically resembles *Latyspora scomberomori*, the only so far described member of this genus. We described new species *Bipteria vetusta* and also provided the first molecular data for this genus. This species turned up to be a representative of a new basal branch within the marine myxosporean lineage (Paper VI: Kodádková et al. submitted). Finally, we described *Myxodavisia bulani*, which is also the only representative of its genus in the phylogenetic tree. We revealed that *Myxodavisia* is closely related to *Ceratomyxa* with broad range of described and phylogenetically characterised species (Paper VII: Fiala et al. submitted).

The myxospore shape of species from the marine urinary clade is significantly variable in comparison with morphotypes shared among species from other e.g. *Ceratomyxa* and *Kudoa* clades. Variability of the myxospore morphology can be determined by the position of PCs, twisting of the suture line around the valves and by alterations of the overall spore shape e.g. prolongation and broadening of the spore.

Bartošová et al. (2011) investigated the evolution of the suture line in the marine urinary clade and found the character of the suture line to be a typical homoplastic feature. Our phylogenetic analysis of the myxosporeans reported from Svalbard represented by the genera *Zschokkella*, *Parvicapsula*, *Sinuolinea*, *Latyspora* (all with curved or sinuous suture line) and *Schulmania* (straight suture line) clustering within the marine urinary clade, supported the homoplasy of this feature. Moreover, Bartošová et al. (2011) traced the evolutionary character of the suture line i.e. sinuous or curved vs. straight on the SSU rDNA-based phylogeny. They found that an ancestor of the marine urinary clade possessed the curved suture line. *Latyspora*-like organism as the basal species of the *Zschokkella* subclade, has a remarkably straight suture line (Paper III: Kodádková et al. 2014). Therefore, the evolutionary history of this feature would be different if we again trace this character on the tree, which is in congruence with the statement of Bartošová et al. (2011), that poor taxon sampling influences the tracing of character evolution.

Another distinctive morphotype's character is the presence of appendages or wing-like projections, e.g. in *Bipteria vetusta*, *Myxodavisia bulani*. We assume that wing-like appendages of morphotypes from the marine lineage have evolved numerous times independently as an adaptation of Myxosporea infecting deep-water fish for better floating in the water column (Fiala & Bartošová 2010, Paper VI: Kodádková et al. submitted). The morphological adaptions of the basal marine myxosporean *B. vetusta* spore could have originated from its ancestor with supposed *Ceratomyxa*-like morphotype by evolution of wing-like appendages (Paper VI: Kodádková et al. submitted). Similarly, deep-water myxosporeans of *Palliatus indecorus* may have evolved a membranaceous veil as a morphological adaptation with similar functional significance (Paper VII: Fiala et al. submitted).

Variability of the myxospore morphology was also studied at the level of a single species e.g. *Zschokkella pleomorpha* and *Sphaerospora formosa* during spore development. It was documented that the maturation process changes the shape and dimensions of the myxospore (Lom & Dyková 1995) as well as it affects formation of lateral wings (Karlsbakk & Køie 2009). We assume that the lateral wings of *Schulmania aenigmatosa* undergo similar maturation changes as described for *S. formosa*. Therefore, from these reasons it is important to provide morphometric data from the completely mature spores to avoid obtaining of misleading spore dimensions (Paper II: Dyková et al. 2013).

Our revision of the phylogenetic relationships among the main clades of the marine lineage based on recent phylogenetic analysis and topology tests with all available sequences suggested that the *Ceratomyxa* clade is the first basal branch of the marine lineage. There is a large polytomy of unresolved or weakly supported relationships in the middle of the tree among the marine urinary clade, the marine *Myxidium* clade and the newly designated *Ceratonova* clade. Nevertheless, topological tests suggested *Ceratonova* to be closely related to *Enteromyxum* and *Kudoa* thus constituting a large histozoic group (Paper VII: Fiala et al. submitted). Therefore, *Ceratonova* clade was proved to be distantly related from the *Ceratomyxa* clade containing species with similar spore morphology.

5.4. History of myxozoan evolution

We morphologically and phylogenetically described a new myxosporean *Bipteria vetusta* from an evolutionary old holocephalan cartilaginous fish. This is the first myxosporean with available molecular data from such an evolutionary ancient fish host group, which is reflected by the fact that it represents a basal lineage of all marine myxosporeans. This finding provoked us to implement the first molecular dating of myxozoan evolution (Paper VI: Kodádková et al. submitted). We used SSU rDNA for the molecular dating of myxozoan evolution, which was justified by the aforementioned popularity and accessibility of this molecular marker in myxozoans.

Pioneer researcher Schulman (1966) considered that primitive myxosporeans were probably initially coelozoic parasites of the gall and urinary bladders of marine teleost of the Actinopterygii (superclass of bony fishes) in the Cretaceous period (approx. 100 Ma). His estimation was challenging when we

consider the state of knowledge at that time. Our estimation of the time of divergence of the Myxosporea revealed that the first myxosporeans evolved at the time of the origin of cartilaginous fish, which can be assumed as the first vertebrate hosts of myxosporeans (Paper VI: Kodádková et al. submitted). The split of *Sphaerospora s.s.* clade and a lineage leading to both marine and freshwater lineages was dated to about 430 Ma in the Silurian era, which correlates with the origin of Chondrichthyes (Inoue et al. 2010). This supports our character evolution analysis that suggested Chondrichthyes as hosts for primitive Myxosporea (Paper VI: Kodádková et al. submitted). Subsequent species radiation within the freshwater and marine myxosporean lineages is in congruence with radiation of teleost hosts.

The diversity and especially phylogenetic position of myxosporeans infecting Chondrichthyes is scarce. The few molecular records of myxosporeans from sharks and rays (Elasmobranchii) revealed these parasites to be ones of the most basal representatives in the myxosporean phylogenetic tree suggesting their old evolutionary history (*Bipteria,* chloromyxids and ceratomyxids infecting elasmobranchs (Gleeson & Adlard 2011, Paper VII: Fiala et al. submitted). Their basal phylogenetic position suggested that the common ancestor of both marine and freshwater myxosporean lineages was a parasite of Chondrichthyes. Our analysis also suggested that recently sequenced *Kudoa* spp. (Gleeson et al. 2010) from sharks skipped from parasitism in teleosts back to Chondrichthyes.

More intense exploration of myxosporean species from other evolutionary old hosts (Agnatha, lampreys, etc.) may help to better understand the diversity and early evolution of the Myxozoa. Moreover, a focused search for possible phylogenetic links between the primitive and extant myxosporean lineages can pave the way towards a better resolution of phylogenetic relationships of the myxozoans. This may facilitate the resolution of the persisting discrepancy between the phylogeny and current taxonomic scheme of the Myxozoa.

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Chapter 6. List of papers

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Paper I

I

Another chloromyxid lineage: molecular phylogeny and redescription of *Chloromyxum careni* from the Asian Horned frog *Megophrys nasuta*

Another Chloromyxid Lineage: Molecular Phylogeny and Redescription of *Chloromyxum careni* from the Asian Horned frog *Megophrys nasuta*

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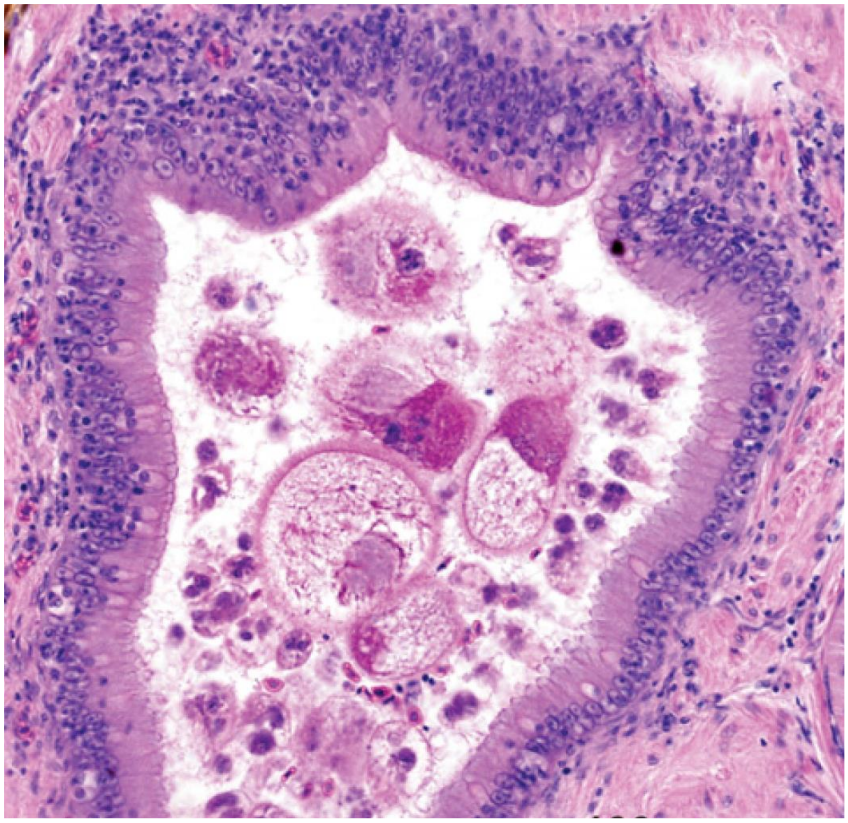
Abstract

Infection with *Chloromyxum careni* Mutschmann, 1999 was found in the Asian horned frog *Megophrys nasuta* from Malaysia and Indonesia. Kidney was the only organ infected. Coelozoic plasmodia up to 300 mm were localized in Bowman's space, embracing the glomerulus from all sides, or rarely in lumina of renal tubules. Plasmodia are polysporic, containing disporic pansporoblasts. Myxospores observed by light microscopy are colorless, variable in shape and size, measuring 6.0-8.5 x 5.0-6.5 mu m, composed of two symmetrical valves joined by a meridian suture, containing four pyriform polar capsules 3.0-4.0 x 2.5-3.0 mu m and a single sporoplasm. Each valve possesses 14-24 (median 21) fine longitudinal ridges clearly visible only in scanning electron microscopy. Rarely, atypical spores with a markedly pointed posterior pole and only 6-10 surface ridges are present in plasmodia together with typical spores. Both small subunit (SSU) and large subunit (LSU) rRNA gene sequences possess extremely long GU-rich inserts. In all SSU and LSU rDNA-based phylogenetic analyses, *C. careni* clustered as a distinct basal branch to the *Myxobolus+Myxidium lieberkuehni* clade, out of the marine *Chloromyxum* clade containing *Chloromyxum leydigi*, the type species of the genus. These morphological and phylogenetic data suggest erection of a new genus for the *C. careni* lineage, but we conservatively treat it as a *Chloromyxum sensu lato* until more information is available.

Paper II

II

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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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 + Γ + 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

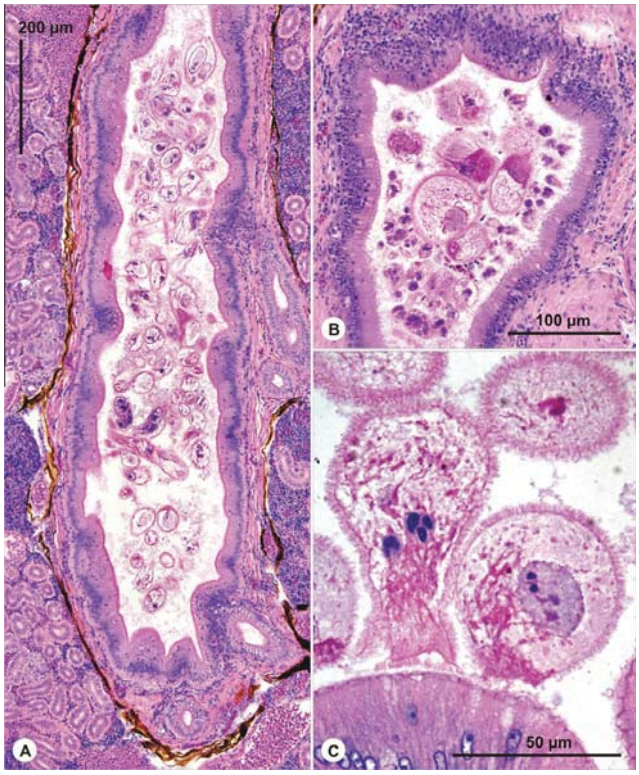

Fig. 1. *Sinuolinea dimorpha* infection in urinary tract of *Cynoscion nebulosus*. Histological sections stained with haematoxylin and eosin. (A) Plasmodial stages within the lumen of collecting duct. (B) Plasmodial stages in terminal part of ureter, small ones seen attached to epithelium, giant ones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

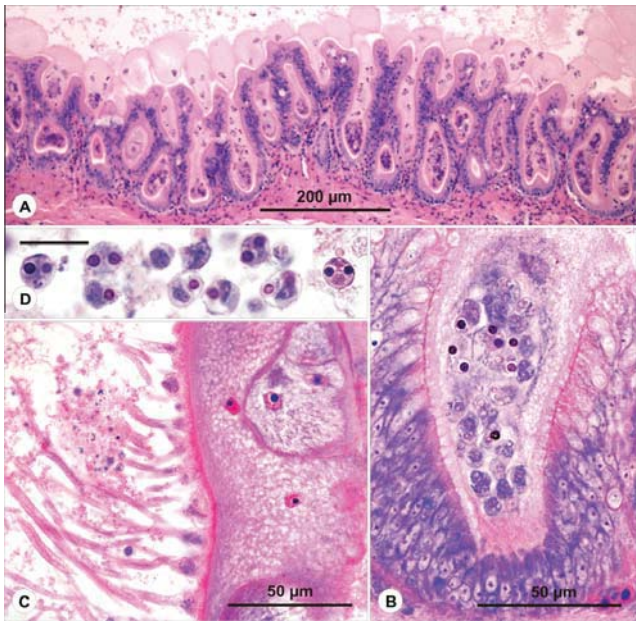

Fig. 2. *Sinuolinea dimorpha* infection in urinary bladder of *Cynoscion nebulosus*. Histological sections stained with haematoxylin and eosin. (A) Plasmodial stages with developing spores filling spaces between folds of urinary bladder wall. Longitudinal section. (B) Detail of plasmodium from urinary 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%.

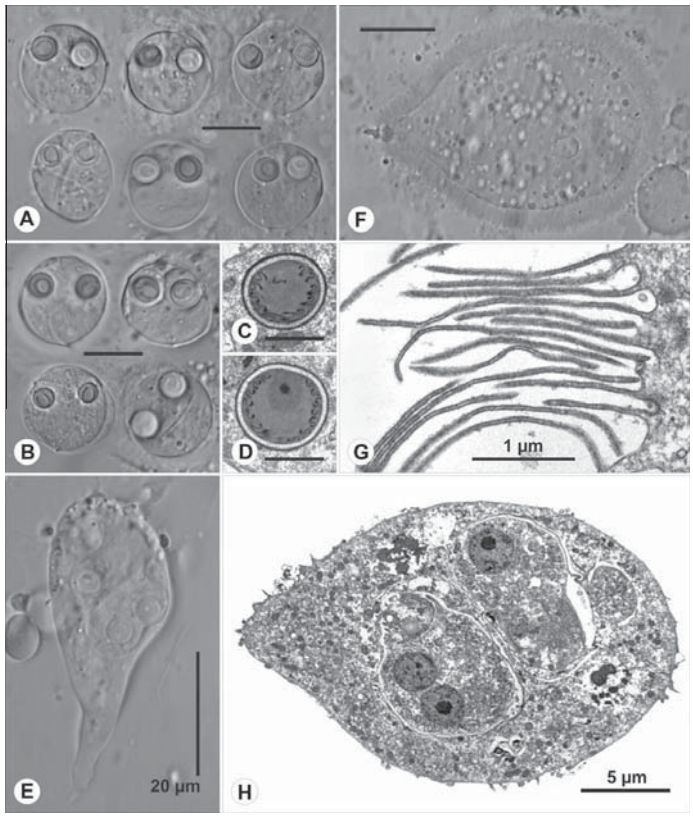

Fig. 3. *Sinuolinea dimorpha* spores documented from fresh material. Scale bars = 10 µm. (A) Spores from urinary bladder of *Cynoscion regalis*. Note left bottom spore with sinuous suture line seen in its entire course. (B) Spores from urinary bladder of *C. nebulosus*. (C and D) Polar capsules with 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%.

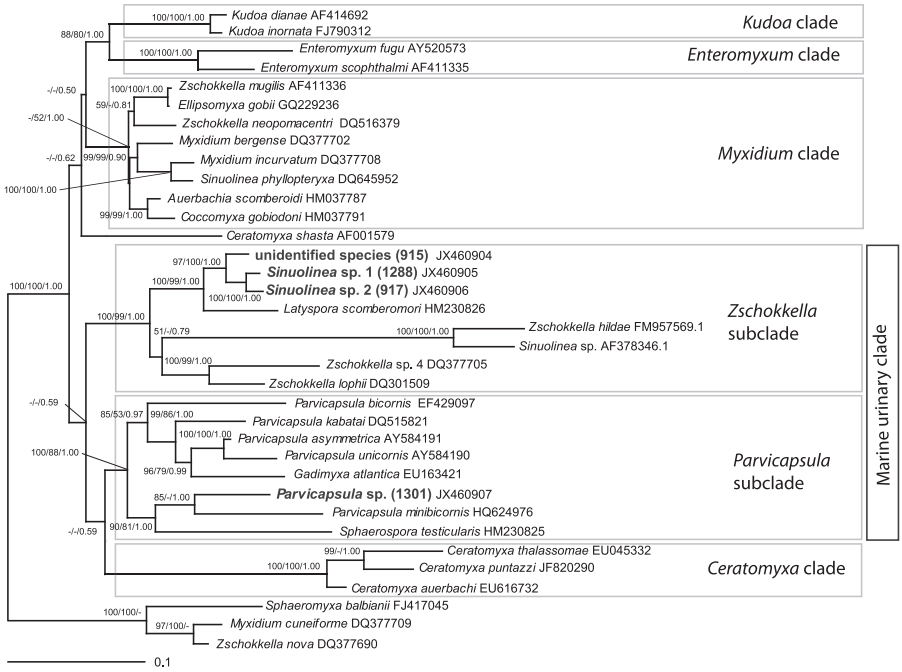

Fig. 4. Maximum likelihood tree (−ln = 2829.6205) based on the SSU rDNA of the selected sequences of the marine myxosporean clades. Representatives of newly obtained sequences clustering in the *Zschokkella* subclade as well as *Parvicapsula* sp. in the *Parvicapsula* subclade are in bold. *Sphaeromyxa 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.

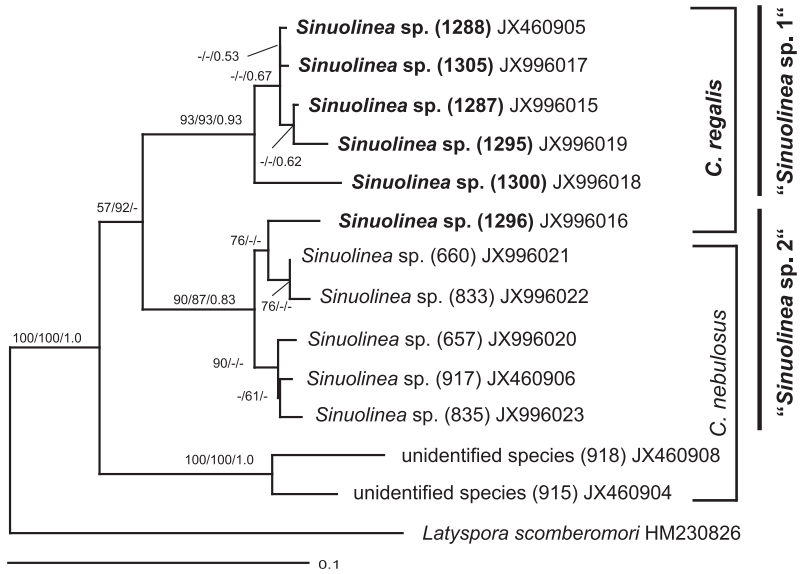

Fig. 5. Maximum likelihood tree (−ln = 13803.0789) based on the SSU rDNA of all newly obtained sequences which cluster within the *Zschokkella* subclade. *Latyspora scomberomori* was set as outgroup. Numbers at the nodes represent the bootstrap values and Bayesian posterior probability (ML/MP/BI) gaining 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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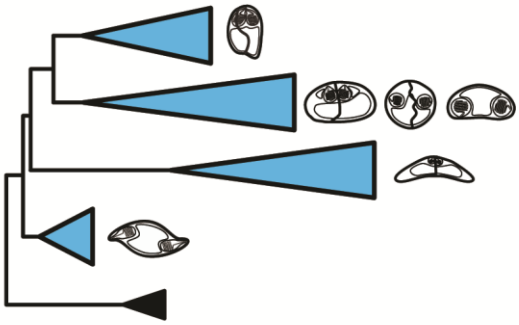
Paper III

III

Myxozoa in high Arctic: Survey on the central part of Svalbard archipelago



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Myxozoa in high Arctic: Survey on the central part of Svalbard archipelago

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ABSTRACT

Myxosporeans (Myxozoa), microscopic metazoan parasitic organisms, are poorly studied in the Arctic region. Our survey of benthic and pelagic fish ($n = 234$) collected in Isfjorden (Svalbard, Norway) together with detailed morphological and molecular examination revealed the presence of nine myxosporean species. We compared observed myxosporean diversity with diversity documented in regions close to the Arctic and revealed that water depth rather than geographic distribution is an important factor influencing myxosporean fauna.

We describe three new myxosporean species: *Zschokkella siegfriedi* n. sp. from kidney of *Boreogadus saida*, *Parvicapsula petuniae* n. sp. from the urinary bladder of *Gymnocanthus tricuspis*, and *Sinuolinea arctica* n. sp. from the urinary bladder of *Myoxocephalus scorpius*. We characterise *Latyspora*-like organism from kidney of *Clupea harengus*. We provide new data for *Ceratomyxa porrecta*, *Myxidium gadi*, *Myxidium finnmarchicum*, *Schulmania aenigmatosa*, and *Parvicapsula irregularis* comb. nov. The phylogenetic analyses including the newly obtained SSU and LSU rDNA data revealed that most of the species studied cluster in the marine urinary clade within the marine myxosporean lineage. Newly obtained sequences including the first molecular data for the member of the genus *Schulmania*, substantially enriched the *Zschokkella* subclade. *C. porrecta* and the two *Myxidium* species cluster within the *Ceratomyxa* and marine *Myxidium* clade, respectively.

Newly described species, *Z. siegfriedi* n. sp., was revealed to be morphologically indistinguishable but genetically diverse from *Zschokkella hildae* known from numerous gadid fish. Therefore, we consider *Z. siegfriedi* to be a cryptic myxosporean species that might be misidentified with *Z. hildae*. A *Latyspora*-like organism was found to be taxonomically problematic due to its suture line and its distant phylogenetic position from the type species *Latyspora scomberomori* did not allow us to assign it to the genus *Latyspora*. Based on an increased taxon sampling and SSU + LSU rDNA-based phylogeny, evolutionary trends within the marine urinary clade are investigated.

1. Introduction

Arctic ecosystems draw our attention due to their rapid responses to climate change (Post et al., 2009). The Arctic region can be defined as north of the Arctic Circle, and consists the Arctic Ocean, northern part of Eurasia and North America, Greenland, Iceland, Svalbard archipelago etc. The Arctic can be divided into the Low Arctic and High Arctic, according to various environmental and biological characteristics. The Svalbard archipelago is located in the High Arctic. The Arctic Ocean is the most extreme ocean in regard to the seasonality of light and its seasonally fluctuating ice cover. In general, species richness is lower in the Arctic than at lower latitudes and is to some degree constrained by biotic and abiotic mechanisms that define species occurrences and associations (Hoberg and Kutz, 2013). Furthermore, species richness tends to decline from low to high Arctic (Payer et al., 2013). Low numbers of host species is usually correlated to low numbers of parasites. Moreover, water temperature may influence transmission dynamics and parasite development (e.g. Kerans et al., 2005). Arctic fjords in the west coast of the Svalbard archipelago region are exceptional in terms of significantly higher temperatures caused by the Gulf Stream. Variations in the number of parasites were found in morphotypes of threespine sticklebacks living in different temperatures; higher numbers of parasites were found in the morphotype from the deep-cold water habitat compared to

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two warmer water dwelling morphotypes in the same Iceland lake (Karvonen et al., 2013). The enriching effect of warmer temperatures on higher abundance and species richness of ectoparasites was demonstrated in more than 100 fish hosts. This effect is not an artefact, but rather an indication of the importance of temperature in the diversification of fish parasites in the tropics (Poulin and Rohde, 1997).

Myxosporean fauna has been poorly studied in the Arctic region. One of the most parasitologically and ecologically studied marine fish with high economical importance occurring in sub-Arctic and Arctic waters is the Atlantic cod *Gadus morhua* (Hemmingsen and MacKenzie, 2001; Perdiguero-Alonso et al., 2008). Apart from a number of protozoan and metazoan parasites (mostly helminths), 11 myxosporean species have been found in Atlantic cod (Hemmingsen and MacKenzie, 2001; Køie et al., 2007a; Holzer et al., 2010). A survey of parasite fauna of Atlantic cod revealed relatively rich and abundant regional macroparasite fauna dominated mostly by generalist parasites with Arctic-Boreal distribution in six localities in the North East Atlantic (Perdiguero-Alonso et al., 2008). These high-level fauna comparisons suggest that differences in the feeding behaviour of cod amongst localities which could affect the prevalence and abundance of parasite species. Kerans et al. (2005) found that water temperature influenced parasite development rates and was a primary determinant for the release of actinospores of the myxozoan *Myxobolus cerebralis* in strains of its definitive host *Tubifex tubifex*. In addition to latitudinal temperature gradients, sea depth is an important factor for parasite ecology. Low parasite richness was reported in different meso- and bathypelagic fishes in comparison to benthopelagic species in the Arctic Ocean (Klimpel et al., 2006).

This study is focused on the Myxozoa, microscopic metazoan parasites characterised by simplified bodies. Evolutionary history of the Myxozoa has been questioned until recent molecular evidence proved the cnidarian origin (Jiménez-Guri et al., 2007; Holland et al., 2011). Myxozoans infect various organs in the vertebrate, mainly fish, hosts: coelozoic species multiply in the cavities of body organs (gall bladder, urinary tract, renal corpuscles etc.) whereas histozoic species are intercellular in various tissues (liver, skin, kidney, testes etc.). The phylum Myxozoa is divided into two classes: Malacosporea with only three described species and Myxosporea with the overwhelming majority of the myxozoan species. Until now, approximately 2310 myxosporean species assigned to 60 genera have been described (Morris, 2010). Myxosporean genera are characterised by the morphology of the spore: spore shape, number of spore valves and polar capsules (PCs), and position of suture lines towards the PCs are considered the main taxonomic features. However, many myxospore morphological features are not synapomorphic since great discrepancies were found between the classic taxonomic approach and the phylogenetic relationships (Holzer et al., 2004; Fiala and Bartošová, 2010).

Myxosporeans form two main phylogenetic lineages according to host habitat, i.e. marine and freshwater (Fiala, 2006), plus a recently revised third basal sphaerosporid lineage (Bartošová et al., 2013). The marine lineage exclusively consists of marine species with the exception of *Ceratomyxa shasta*. There are five clades within the marine lineage: the marine *Myxidium* clade, the *Ceratomyxa* clade, the *Enteromyxum* clade, the *Kudoa* clade and the marine urinary clade divided into the *Parvicapsula* and *Zschokkella* subclade (Fiala, 2006; Bartošová et al., 2011). With the exception of the *Enteromyxum* clade, the remaining clades include non-monophyletic genera. The clustering of species in particular clades follows tissue tropism criterion rather than myxospore morphology (Holzer et al., 2004; Fiala, 2006). The marine urinary clade is typical in this respect: phylogenetically closely related myxosporeans of the genera *Parvicapsula*, *Gadimyxa*, *Sphaerospora*, *Sinuolinea*, *Latyspora*, and *Zschokkella* differ in spore morphology but predominately infect the excretory tract (Bartošová et al., 2011). However, some species of the *Parvicapsula* subclade also infect other sites such as the epithelium of the gall bladder, the intestine, the pseudobranchs and testicles. The monophyly of the genus *Parvicapsula* was disrupted by clustering of *Gadimyxa* spp. with parvicapsulids as well as by the sister relationship of *P. minibocornis* and *Sphaerospora testicularis* (Køie et al., 2007a; Bartošová et al., 2011). The *Zschokkella* subclade contains species of the polyphyletic genus *Zschokkella* including its type species *Z. hildae* as well as type species of the genera *Latyspora* and *Sinuolinea* (Bartošová et al., 2011; Dyková et al., 2013). The *Zschokkella* subclade is characterised by species with high variability in myxospore shape with the position of PCs ranging from set at opposite ends of the spore, to directly next to each other.

This paper attempts to characterise myxosporean fauna on the Svalbard archipelago: (i) detailed morphological and molecular characterization of myxosporean species; (ii) phylogeny and evolutionary trends; (iii) comparison of parasite diversity from the Arctic with other regions.

2. Material and methods

2.1. Fish hosts

Eight species of teleost fish were collected in part of the Billefjorden, Isfjorden, Petunia Bay (78° 69′ N, 16° 53′ E) in the central part of Svalbard archipelago during the summer season (July and August 2011). A total of 234 individuals of 8 fish species from 7 families were dissected. Families, namely Cottidae: *Myoxocephalus scorpius* (Linnaeus, 1758) (n = 98), *Gymnocanthus tricuspis* (Reinhardt, 1830) (n = 22); Clupeidae *Clupea harengus* Linnaeus, 1758 (n = 66); Osmeridae: *Mallotus villosus* (Müller, 1776) (n = 16); Gadidae: *Boreogadus saida* (Lepechin, 1774) (n = 14); Pleuronectidae: *Hippoglossoides platessoides* (Fabricius, 1780) (n = 9); Myctophidae: *Lumpenus lampretaeformis* (Walbaum, 1792) (n = 8); and Salmonidae: *Salmo salar* Linnaeus, 1758 (n = 1). Fish were caught using gillnets in littoral habitat (maximum depth of gillnets was 40 m). After euthanasia all organs were checked for the presence of the Myxozoa in squash preparations by light microscopy (Olympus BX 53). Contents of gall and urinary bladders were examined fresh, under cover slips, on slides covered with a thin layer of 1% agar. In some cases we failed to obtain samples of gall and urinary bladders and the missing data are considered in prevalence records within species descriptions. A DNA sample of *Parvicapsula minibicornis* was obtained from the kidney of *Oncorhynchus nerka* (Walbaum, 1792) in Cultus Lake (British Columbia, Canada).

2.2. Myxosporean collection and documentation

Pictures of fresh spores were made using an Olympus BX 53 microscope with Nomarski differential interference contrast equipped with an Olympus DP72 digital camera. Measurements of spores were analysed in ImageJ v.1_44p (Wayne Rasband, http://imagej.nih.gov/ij). Measurements are presented in micrometres. Means, standard deviation (SD) and range in the parentheses were calculated for each spore dimension. Range of plasmodia size is followed by mean and median in parentheses. For examination of fine structure of myxosporean spores and plasmodia by transmission electron microscopy (TEM), whole urinary bladders as well as samples of their contents and kidney tissue were fixed in cacodylate buffered 3% glutaraldehyde at 4 °C, rinsed in 0.1 M cacodylate buffer and postfixed in 1% osmium tetroxide. After graded acetone dehydration, the samples were embedded in Spurr's 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 paging system using analySIS software.

For histological examination, organs were fixed for 24 h in Davidson fixative, stored in 70% ethanol; samples were routinely dehydrated and embedded into paraffin. Sections were stained by haematoxylin and eosin (HE) and Giemsa. Positive samples were preserved in TNES buffer (10 mM Tris–HCl pH 8, 125 mM NaCl, 10 mM EDTA, 0.5% SDS, 4 M urea) for DNA isolation and selected samples were molecularly characterised by sequencing of rRNA genes.

2.3. DNA isolation and PCR

Total DNA was extracted by standard phenol–chloroform method 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 or by the combination of MyxospecF-ERIB10 and ERIB1-MyxospecR primers (Barta et al., 1997; Fiala, 2006). If the primary PCR failed, the reaction with ERIB primers was followed by nested PCR with combinations of MyxospecF-ERIB10, ERIB1-MyxospecR, and/or MyxospecF-MyxospecR primers. PCRs of the SSU rDNA were carried out in a 25 μl reactions using 1 × Taq buffer, 250 μM of each dNTPs, 10 pmol of each primer, 1 U of Taq-Purple polymerase (Top-Bio, Czech Republic), 1 μl of DNA and sterile dd H_2O. Cycling parameters for the primary/nested PCR were as follows: denaturation at 95 °C for 3 min, then 30 cycles of amplification at 95 °C for 1 min, 48 °C/50 °C for 1 min, 72 °C for 2/1 min and followed by a 10 min of extension at 72 °C. If above mentioned PCR combinations failed to amplify the desired product TITANIUM Taq DNA polymerase (BD Biosciences Clontech) was used instead of Taq-Purple polymerase. PCRs were conducted in 10 μl reactions with 0.025 U μl^{-1} TITANIUM Taq DNA polymerase, 10 × buffer containing 5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.5 mM of each primer, and 0.5 μl DNA. Cycling parameters for the primary/nested PCR were as follows: denaturation at 95 °C for 2 min, then 30 cycles of amplification at 95 °C for 40 s, 52 °C/56 °C for 40 s, 68 °C for 1 min 40 s/1 min and followed by a 8 min of extension at 68 °C. The 3′ end of the LSU rDNA was obtained using the NLF1050-NLR3284 primer set (Bartošová et al., 2009; Van der Auwera et al., 1994). When these PCRs failed to amplify the desired products, a nested PCR approach with NLF1260-NLR3113 (Bartošová et al., 2009; Van der Auwera et al., 1994) primers was used. The LSU rDNA of *P. minibicornis* failed to amplify with primers listed above but was amplified by nested PCR using primers 28Scer5F1-28Scer5R1 (first PCR) and 28Scer5F2-28Scer5R2 (second PCR) according to Fiala et al. (in prep.). PCRs of the LSU rDNA were carried out in a 25 μl reactions using 1 × LA buffer, 0.5 μl DMSO, 250 μM of each dNTPs, 10 pmol of each primer, LA DNA polymerase (Top-Bio, Czech Republic), 1 μl of DNA, and sterile dd H_2O. Cycling parameters of LSU rDNA samples in the primary/nested PCR were denaturation at 95 °C for 3 min, then 30 cycles of amplification at 95 °C for 1 min, 50 °C/54 °C for 1 min, 68 °C for 2 min/1 min 40 s and followed by 8 min of extension at 68 °C.

All PCR products were purified using Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., 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 3130XL automatic sequencer (Applied Biosystems, Czech Republic).

2.4. Phylogenetic analyses

The overlapping partial sequences of both SSU and LSU rDNA markers were assembled into the contigs in the SeqMan II program v5.05 (DNASTAR Inc., Madison, Wisconsin). The SSU and LSU rDNA alignments were created in program MAFFT v6.864 (Katoh et al., 2002) using L-INS-i strategy and default parameters. Alignments contain newly obtained sequences and sequences retrieved from GenBank. Highly variable parts of the alignments were determined and excluded in SeaView v4 (Gouy et al., 2010) by Gblocks (Castresana, 2000) using less stringent parameters and slightly adjusted by eye mainly at the beginning and at the end of the alignment.

Five alignments were assembled: SSU rDNA-muc alignment with all newly sequenced myxosporeans and all sequences of taxa within the marine urinary clade available in GenBank (1491 characters) plus the representatives of the other marine clades; LSU rDNA alignment (1987 characters) with all newly sequenced LSU rDNA and those ones available in GenBank; concatenated SSU rDNA-muc + LSU rDNA alignment (3487 characters); SSU rDNA-mar-myxid alignment focused on the marine *Myxidium* clade (1549 characters); and SSU rDNA-cer alignment focused on the *Ceratomyxa* clade (1389 characters). Three myxosporean species from the freshwater lineage were selected as outgroup in the analyses of SSU rDNA-muc alignment and SSU rDNA + LSU rDNA alignment. Outgroups for the SSU rDNA-mar-myxid alignment, SSU rDNA-cer alignment, and LSU rDNA alignment were selected as follows: two species from *Zschokkella* subclade, three ceratomyxids from elasmobranchs and two species from the freshwater lineage, respectively.

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 + Γ + 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. Tracer v1.4.1 (Rambaut and Drummond, 2007) was used to set the length of burn-in period. For ML and MP, the bootstrap supports were calculated from 500 replicates. Genetic distances (converted to similarities in %) were computed in PAUP* v4.0b10 with default P parameter from the SSU rDNA-muc and SSU rDNA-mar-myxid alignments.

3. Results

3.1. Findings of myxosporean infections

A total of five (i.e. *M. scorpius*, *G. tricuspis*, *C. harengus*, *B. saida*, *H. platessoides*) out of eight fish species were positive for the presence of Myxozoa (Table 1). 18% out of all dissected fishes were infected by Myxosporea. Two fish were infected with more than one myxosporean: *M. scorpius* with four myxosporeans and *H. platessoides* with two myxosporeans. In this fish species, several concomitant infections occurred (stated in the species descriptions below). The highest prevalence of myxosporean infection was observed in *H. platessoides* and *B. saida* (Table 1).

We obtained 9 SSU rDNA sequences of *Parvicapsula irregularis*, *P. petuniae*, *Zschokkella siegfriedi*, *Sinuolinea arctica*, *Schulmania aenigmatosa*, *Latyspora*-like organism, *Ceratomyxa porrecta*, *Myxidium gadi*, and *M. finnmarchicum*. We obtained 6 LSU rDNA sequences of *P. petuniae*, *P. minibicornis* (from *Gasterosteus aculeatus*; Oregon, USA), *Latyspora*-like organism, *P. irregularis*, *S. arctica*, and *S. aenigmatosa*.

Table 1
List of myxosporeans, hosts, site of infection (gb = gall bladder, ub = urinary bladder), prevalence (number of infected organs/number of analysed organs) and measurements of species found on Svalbard. Spore: Length (*L*), Width (*W*), Thickness (*T*), Polar capsules (PCs). All measurements in μm.

Myxosporean species	Host	Site of infection	Prevalence	Spores			PCs	Plasmodia	References
				L	*W*	*T*			
Ceratomyxa porrecta	*Myoxocephalus scorpius*	gb	4% (3/79)	2.9 ± 0.4 (2.3–3.2)	29.1 ± 4.8 (25.5–34.4)	–	1.9 ± 0.2 × 1.7 ± 0.2	3.9 ± 0.5 × 10.2 ± 2.2 (3.5–13.8)	This study
				4–5	23–34	–	3 × 3	–	Dogiel (1948)
Myxidium gadi		gb	6% (5/79)	11.3 ± 0.1 (11.2–11.4)	5.3 ± 2.0 (3.9–6.7)	–	3.4 ± 0.5 × 2.8 ± 0.7	–	This study
				8.5–14	4–7.5	–	4.5–4.7	–	Géorgevitch (1916) (quoted from Shulman (1966))
Myxidium finnmarchicum		gb	7% (6/79)	15.3 ± 1.6 (13.1–17.8)	9.2 ± 1.3 (7.2–10.1)	9 ± 0.5 (8.4–9.7)	4.8–6.4 × 3.2–4.8	24.0 ± 3.2 (22.2–27.7) × 27.5 ± 3.6 (25.0–31.6)	This study
				17.6–22.4	6.4–6.9	–	4.8–6.4 × 3.2–4.8	30–36 × 40–50	MacKenzie et al. (2010)
Sinuolinea arctica		ub	10% (5/48)	15.7 ± 0.9 (14.7–16.6)	15.4 ± 0.8 (14.2–16.6)	16.1 ± 2.9 (14.0–18.1)	5.1 ± 0.3 × 5.1 ± 0.3	21.8 ± 4.7 (16.6–31.3) × 27.4 ± 7.0 (18.0–39.5)	This study
Parvicapsula petuniae	*Gymnocanthus tricuspis*	ub, kidney	9% (2/22)	11.0 ± 0.7 (9.9–12.3)	7.9 ± 0.6 (7.4–8.3)	8.7 ± 2.1 (6.7–11.9)	3.6 ± 0.2 × 2.8 ± 0.3	–	This study
Zschokkella siegfriedi	*Boreogadus saida*	kidney	43% (6/14)	17.4 ± 0.7 (16.7–18.2)	10.5 ± 1.2 (9.2–11.6)	9.8 ± 0.7 (8.5–11.0)	5.2 ± 0.3 × 5.1 ± 0.3	16.6 ± 4.2(11.5–24.0) × 20.8 ± 5.3 (14.0–25.1)	This study
Parvicapsula irregularis	*Hippoglossoides platessoides*	ub, kidney	44% (4/9)	11.0 ± 0.7 (11.0–15.1)	7.9 ± 0.6 (6.1–10.4)	8.7 ± 2.1 (7.4–9.0)	3.6 ± 0.2 × 2.8 ± 0.3	10.6 ± 0.7 (10.1–11.1) × 13.3 ± 1.5 (12.3–4.3)	This study
				8.0–11.0 (mean 10.6)	6.0–9.0 (mean 7.1)	–	2.2	15–20 × 20–25	Kabata (1962)
Schulmania aenigmatosa		ub	22% (2/9)	20.3 ± 1.6 (17.2–22.9)	16. 9 ± 1.6 (15.0–19.5), with wings 17.4 ± 1.5 (16.0–19.6)	16.4 ± 6.3 (14.9–18.0)	6.7 ± 0.6 × 6.3 ± 0.5	29.3 ± 3.3 (27.2–31.2) × 30.8 ± 3.4 (28.0–37.4)	This study
				19.9–23.1	11.9–13.3	11.9–16.0	5.9–6.7	26.6–42.0 × 31.9–55.9	Kovaleva et al. (1983)
Latyspora-like organism	*Clupea harengus*	kidney	14% (9/66)	10.7 ± 0.7 (9.2–11.2)	22.6 ± 1.6 (21.2–26.2)	20.7 ± 2 (17.7–23.3)	7.8 ± 0.4 × 4.7 ± 0.4	28.4 ± 4.1 (23.1–33.7) × 29.8 ± 3.2 (25.3–32.8)	This study

3.2. Myxosporean species

3.2.1. Additional data on described species

Ceratomyxa porrecta Dogiel, 1948 (Fig. 1A).

Type host: Gymnocanthus herzensteini Jordan and Starks, 1904.

Other hosts: *M. scorpius* (Linnaeus, 1758), shorthorn sculpin, average standard length 18.9 cm; *Bero elegans* (Steindachner, 1881); *Myoxocephalus brandtii* (Steindachner, 1867).

Type locality: Peter the Great Bay, Japan Sea.

Other locality: Greenland Sea, part of the Billefjorden, Isfjorden, Petunia Bay in the central part of Svalbard archipelago (78° 69′ N, 16° 53′ E).

Description of sporogonic stages: disporic plasmodia with filopodia; for dimensions see Table 1.

Description of myxospores: crescent shape with markedly elongated shell valves; PCs with a straight central shaft of the filament, located close to the suture line in a plane perpendicular to it; posterior angle 220°; for dimensions see Table 1.

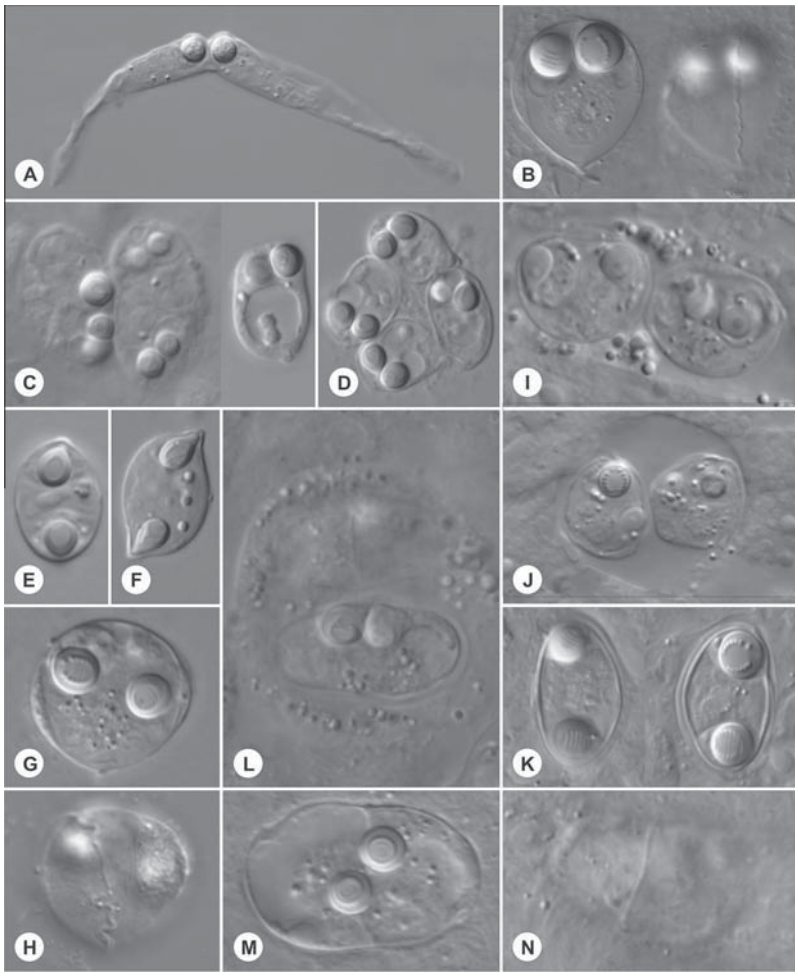

Fig. 1. Mature spores and plasmodia. (A-N) Myxospores and myxosporean plasmodial stages as seen in Nomarski differential interference contrast. Measurements are listed in Table 1. (A) Mature spore of *Ceratomyxa porrecta*. (B) Spores of *Schulmania aenigmatosa* with focus on polar capsules (left) and sinuous valve suture (right). (C) Plasmodial stages (left) and mature spore of *Parvicapsula irregularis* (right). (D) Mature spores of *Parvicapsula petuniae*. (E) Mature spore of *Myxidium gadi*. (F) Mature spore of *Myxidium finnmarchicum*. (G, H) Spores of *Sinuolinea arctica* in frontal (G) and sutural (H) view. (I, J) Plasmodial stages of *Zschokkella siegfriedi*. (K) Mature spores of *Zschokkella siegfriedi*. (L) Plasmodial stage of *Latyspora*-like organism. (M, N) *Latyspora*-like organism spores with focus on polar capsules and part of valve suture, respectively.

Localization of sporogonic stages: coelozoic, gall bladder.

Prevalence: 4% (3 of 79 gall bladders; 1 sample co-infected with *Myxidium finnmarchicum*).

Pathology: no material available for evaluation the species pathogenicity.

Materials deposited: DNA sample (nr. 1373) stored in −80 °C in the Laboratory of Fish Protistology, Institute of Parasitology, BC ASCR; SSU (GenBank accession No. KF874235) rDNA sequence.

Remarks: Ceratomyxa porrecta has identical spore shape with original description of *C. porrecta* (Dogiel, 1948). Measurements differences (see Table 1): PCs are remarkably larger (3 × 3 μm), and spore length is longer (4.5 μm) in Dogiel's description of *C. porrecta* contrary to our measurements of *C. porrecta* from this study (PC 1.9 × 1.7 μm; spore length 2.9 μm). The type host of *C. porrecta* is *G. herzensteini* Jordan and Starks, 1904. We suggest that *M. scorpius* is another host for *C. porrecta*. Although our material does not originate from the type host of *C. porrecta* and there is no sequence data for *C. porrecta*. We assign our molecular and morphological findings to *C. porrecta* based on identical spore morphology and on the close genetic relationship of hosts *M. scorpius* and *M. brandtii* (Knope, 2013).

Myxidium gadi Georgévitch, 1916 (Fig. 1E).

Type host: Pollachius pollachius (Linnaeus, 1758), pollack.

Other hosts: *M. scorpius* (Linnaeus, 1758), shorthorn sculpin, average standard length 18.5 cm; *G. morhua* Linnaeus, 1758, Atlantic cod; *Pollachius virens* (Linnaeus, 1758), saithe; *Merlangius merlangius* (Linnaeus, 1758), whiting; *Melanogrammus aeglefinus* (Linnaeus, 1758), haddock; *Pleuronectes flesus* Linnaeus, 1758, European flounder; *Solea solea* (Linnaeus, 1758), common sole.

Type locality: Roscoff, off France coast.

Other localities: Barents Sea, White Sea, Atlantic Ocean: off Canada coast, Greenland Sea, part of the Billefjorden, Isfjorden, Petunia Bay in the central part of Svalbard archipelago (78° 69′ N, 16° 53′ E).

Description of sporogonic stages: plasmodia not observed in our material.

Description of myxospores: fusiform shape with pointed ends; pyriform PCs at each end of the spore; for dimensions see Table 1.

Localization of sporogonic stages: coelozoic, gall bladder.

Prevalence: 6% (5 of 79 gall bladders).

Pathology: no material was available for evaluation the species pathogenicity.

Materials deposited: DNA sample (nr. 1320) stored in −80 °C in the Laboratory of Fish Protistology, Institute of Parasitology, BC ASCR; SSU (GenBank accession No. KF874236) rDNA sequence.

Remarks: Myxidium gadi has a wide host species spectrum and has been reported from five gadids and two flatfish (MacKenzie et al., 2010; Shulman, 1966). *M. scorpius* is a new host for *M. gadi*, broadening its host spectrum in the family Cottidae, as spore measurements of our material from shorthorn sculpin basically correspond to the original description of *M. gadi* from *P. pollachius* (Table 1). Moreover, *M. gadi* from shorthorn sculpin and *M. gadi* from haddock are genetically highly similar (98.8%; Supplementary Fig. 2A, Table 2). Moreover intraspecific variability based on partial SSU rDNA sequence of *M. gadi* is high, on the edge of the border resolving two species. This variability can be driven by wide host spectrum with intermixing infrapopulations of *M. gadi*. Infrapopulation of *M. gadi* on the edge of distribution area can be for some period of time without any gene flow with the rest of infrapopulations. Four *Myxidium* species were found in cottids: *M. scorpii* Schulman-Albowa, 1950, described from atypical infection site in the urinary bladder of *M. scorpius*; *M. arcticum* Zhukov, 1962 described from *Myoxocephalus axillaris*; *M. japonicum* Dogiel, 1948 described from *Myoxocephalus brandtii;* and *M. myoxocephali* Fantham, Porter and Richardson, 1940 described from *Myoxocephalus octodecemspinosus*. *Myxidium myoxocephali* appears to be identical with *M. incurvatum* based on their similar morphology, morphometrics and distribution area, although Fantham et al. (1940) noted that the parasite was larger than *M. incurvatum* (Khan et al., 1986) and both myxosporeans also differ in host species and molecularly. Generally, marine *Myxidium* species clustering within the marine *Myxidium* clade have fusiform or S-shape spores. The spore measurements and shape of *M. gadi* correspond to those of *M. scorpii* described from the same host but they differ in tissue tropism and PCs size. *Myxidium scorpii* described from an atypical infection site in the urinary bladder has slightly smaller PCs (1.8–2.0 μm) than *M. gadi* (3.4 × 2.8 μm) originating from gall bladder of *M. scorpius* (Table 1).

Myxidium finnmarchicum MacKenzie et al., 2010 (Fig. 1F).

Type host: *Merlangius merlangus* (Linnaeus, 1758), Whiting.

Other host: *M. scorpius* (Linnaeus, 1758), Shorthorn sculpin, average standard length 16.5 cm;

Type locality: off Sørøya, North Norway (70° 47′ N, 22° 58′ E).

Other localities: Greenland Sea, part of the Billefjorden, Isfjorden, Petunia Bay in the central part of Svalbard archipelago (78° 69′ N, 16° 53′ E).

Description of sporogonic stages: spherical disporic plasmodia; for dimensions see Table 1.

Description of myxospores: sigmoid shape with pointed ends; fine transverse ridges; pyriform PCs at each end of the spore, for dimensions see Table 1.

Localization of sporogonic stages: coelozoic, gall bladder.

Prevalence: 7% (6 of 79 gall bladders; 2 samples co-infected with *Ceratomyxa porrecta*).

Pathology: no material available for evaluation the species pathogenicity.

Materials deposited: DNA sample (nr. 1610) stored in −80 °C in the Laboratory of Fish Protistology, Institute of Parasitology, BC ASCR; SSU rDNA sequence (GenBank accession No. KF874237).

Remarks: M. scorpius is a new host for *M. finnmarchicum*, broadening its host species spectrum in the family Cottidae. *Myxidium finnmarchicum* was described with 4–6 fine longitudinal striations (MacKenzie et al., 2010) which were not observed in this study. On the other side, we observed fine transverse ridges on *M. finnmarchicum* spores under the light microscope. However, scanning electron micrographs are required for re-evaluation of the spore surface. SSU rDNA sequences of the myxosporean from our three samples were almost identical with SSU rDNA data of *M. finnmarchicum*. *Myxidium finnmarchicum* has a similar spore shape as *M. gadi* but differs in larger spore size and genetic similarity is 94.4% (Supplementary Table 2).

Schulmania aenigmatosa (Kovaleva et al., 1983) (Fig. 1B, Figs. 2 and 3).

Type host: *H. platessoides* (Fabricius, 1780) American plaice, average standard length 10.5 cm.

Other hosts: *Hippoglossoides robustus* Gill and Townsend, 1897, Bering flounder; *Hippoglossoides elassodon* Jordan and Gilbert, 1881, Flathead sole.

Type locality: south off the Labrador.

Other localities: Sea of Okhotsk, Chukchi Sea, Bering Sea, Greenland Sea, part of the Billefjorden, Isfjorden, Petunia Bay in the central part of Svalbard archipelago (78° 69′ N, 16° 53′ E).

Description of sporogonic stages: spheric or plane (oval in cross section) disporic plasmodia; ectoplasm separated from endoplasm; for dimensions see Table 1.

Description of myxospores: spores notably large, inversely pyramidal in sutural view with pointed posterior pole; suture line waved; lateral wings partially visible in the light microscope and clearly in TEM, lateral wings on spore present as pocket-like extensions separated from the spore body by membrane; PCs in posterior pole apposed closely to each other and discharging forward in the direction slightly toward the axis of the spore, 7 coils of polar filament; for dimensions see Table 1.

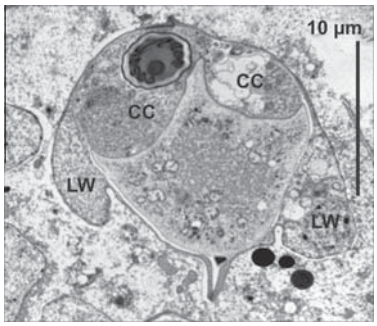

Fig. 2. Ultrathin section of almost mature spore of *Schulmania aenigmatosa* with lateral wings (LW) typical for the genus. CC capsulogenic cell, CC with polar capsule (left).

Localization of sporogonic stages: coelozoic, urinary bladder.

Prevalence: 22% (2 of 9 urinary bladders; 2 samples co-infected with *P. irregularis*).

Pathology: high numbers of rodlet cells observed in epithelium of heavily infected segments of renal tubules (Fig. 3); even seen in early infections but not present in epithelium of collecting ducts and urinary bladder.

Materials deposited: DNA sample (nr. 1415) stored in −80 °C, paraffin blocks nrs. 786/10, 819/10, 900/10 and blocks in resin nr. 550i stored in the Laboratory of Fish Protistology, Institute of Parasitology, BC ASCR; SSU (GenBank accession No. KF874233) and LSU (GenBank accession No. KF874228) rDNA sequences.

Remarks: The spores of *S. aenigmatosa* from our material were wider in frontal view compared to the original description of Kovaleva et al. (1983) (12 µm vs. 17 µm). *Schulmania aenigmatosa* is very similar by its spore size with *S. ovale*; the type species of the genus *Schulmania*. Mainly four longitudinal keel-like stiff membranes and wing shaped extensions were partially visible by light microscopy and clearly visible in TEM (Fig. 2). The lateral wings are one of the most characteristic features of the genus *Schulmania*. However, due to our rare observation of this feature one can deduce that this structure changes during its maturation as observed in other myxosporeans e.g. immature spores of *Bipteria formosa* have empty-looking pockets at each spore side which detach posteriorly in maturing spores and later open thus releasing their content (Karlsbakk and Køie, 2009). *Schulmania aenigmatosa* is the first sequenced member of the genus *Schulmania*.

***Parvicapsula irregularis* comb. nov.** (Kabata, 1962) (Fig. 1C).

Synonyms: Sphaerospora irregularis Kabata, 1962; *Myxoproteus irregularis* (Kabata, 1962); *Ortholinea irregularis* (Kabata, 1962).

Type host: *H. platessoides* (Fabricius, 1780), American plaice (syn. *Drepanopsetta platessoides* Fabricius, 1780); average standard length 10.6 cm.

Other hosts: unknown.

Type locality: Northern North Sea.

Other localities: Greenland Sea, part of the Billefjorden, Isfjorden, Petunia Bay in the central part of Svalbard archipelago (78° 69′ N, 16° 53′ E).

Description of sporogonic stages: disporic plasmodia of various shapes i.e. round, oval or irregular with protrusions; pseudopodia with rounded ends; for dimensions see Table 1.

Description of myxospores: spore shape roughly pyriform with considerable degree of irregularity, widest diameter of spore about middle of the long axis, narrowing somewhat towards the poles, particularly towards the anterior pole; slightly flattened in the sutural plane; spherical PCs close together located anteriorly; single sporoplasm occupying more than three quarters of the spore, sporoplasm with two nuclei; for dimensions see Table 1.

Localization of sporogonic stages: coelozoic, renal tubules, urinary bladder.

Prevalence: 44% (4 of 9 urinary bladders; 2 samples co-infected with *Schulmania aenigmatosa*).

Pathology: no material available for evaluation the species pathogenicity.

Materials deposited: DNA sample (nr. 1376) stored in −80 °C in the Laboratory of Fish Protistology, Institute of Parasitology, BC ASCR; SSU (GenBank accession No. KF874229) and LSU (GenBank accession No. KF874226) rDNA sequences.

Remarks: Kabata (1962) described *Sphaerospora irregularis* from American plaice in northern North Sea. This species was later assigned to other genera: *Myxoproteus* (Gaevskaya and Kovaleva, 1984), and *Ortholinea* (Arthur and Lom, 1985). After re-examination, Køie et al. (2007b) suggested *S. irregularis* may belong to *Parvicapsula*. Despite *S. irregularis* was reported from another host, *Pleuronectes platessa* (MacKenzie et al., 1976), this report most probably corresponds to *Parvicapsula bicornis* later described from this host (Køie et al., 2007b). Unfortunately, the report of "*S. irregularis*" by MacKenzie et al. (1976) lacked sufficient morphological documentation and comparison with similar species. Therefore, *P. bicornis* from *P. platessa* was regarded as syn. part. of *S. irregularis* (Køie et al., 2007b). Since this species is now re-examined and molecularly characterised we claim that *P. bicornis* and the re-described *P. irregularis* are two morphological and molecularly different species. *S. testicularis* as the closest relative of *P. irregularis* has a wider and thicker spore.

3.2.2. Description of new taxa

***Zschokkella siegfriedi* n. sp.** (Fig. 1I–K, Figs. 4–6).

Family Myxidiidae Thélohan, 1892.

Genus *Zschokkella* Auerbach, 1910.

Type host: *B. saida* (Lepechin, 1774), Polar cod (officially accepted common name; commonly used name Arctic cod for *B. saida* is valid for *Arctogadus glacialis* (Peters, 1872) (Froese and Pauly, 2013)); average standard length 14.4 cm.

Other host: unknown.

Type locality: Greenland Sea, part of the Billefjorden, Isfjorden, Petunia Bay in the central part of Svalbard archipelago (78° 69′ N, 16° 53′ E).

Other localities: none.

Description of sporogonic stages: plasmodia mostly di-, rarely polysporic; round to oval in shape; clear differentiation between smooth ectoplasm and granular endoplasm; for dimensions see Table 1.

Description of myxospores: shape of spores considerably variable, from spores with one side vaulted appearing almost rounded triangular to spores of ellipsoidal shape; suture line irregularly oblique, two shell valves completely asymmetrical; subspherical to spherical PCs located in the spore ends and discharging to opposite sides parallel with axis of the spore from the apical view, 7 coils of polar filament; for dimensions see Table 1.

Localization of sporogonic stages: coelozoic, renal tubules.

Prevalence: 43% (6 of 14 kidney samples).

Pathology: regressive changes of importance developed in the epithelial cells of infected renal tubules manifested as pronounced changes of staining properties of individual cells in semithin sections; mitochondria with various degrees of mitochondrial electron-density suggestive of necrotic changes revealed in ultrathin sections (Fig. 5, Fig. 6).

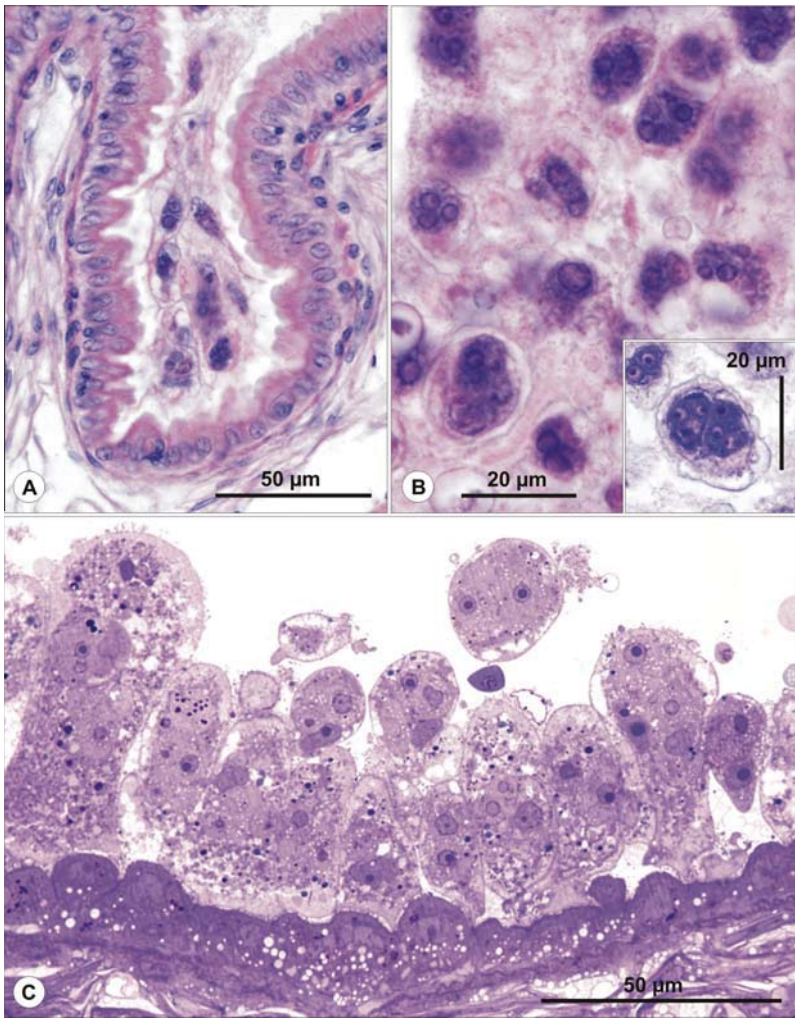

Fig. 3. Histology of *Schulmania aenigmatosa* infection. (A-C) *Schulmania aenigmatosa* infection in excretory system of *Hippoglossoides platessoides*. (A) Early plasmodial stages localised in ureter as seen in histological section stained with HE. (B) Advanced plasmodial stages filling urinary bladder. Giemsa stained stage (inserted). (C) Semithin section stained with toluidine blue documents numerous plasmodial stages attached to the wall of urinary bladder. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Materials deposited: DNA sample (nr. 1608) stored in −80 °C and blocks in resin nrs. 541a and 543a in the Institute of Parasitology, Laboratory of Fish Protistology, BC ASCR; SSU rDNA sequence (GenBank accession No. KF874231).

Etymology: The species name of *Z. hildae*, type species of the genus *Zschokkella*, refers to Hilda (a shorten version of the German name) used by author Auerbach (1910) in honour of his wife. We name *Z. siegfriedi* n. sp. according to the German heroic poem "The Song of Nibelungs" with the lovers Siegfried and Kriemhilda (Hilda) reflecting the close phylogenetic relationship between *Z. hildae* and our new species.

Remarks: We found *Zschokkella siegfriedi* from the kidney of polar cod to be genetically distinct (2.8% of dissimilarity) (Supplementary Table 1) from *Z. hildae* SSU rDNA sequence from *G.*

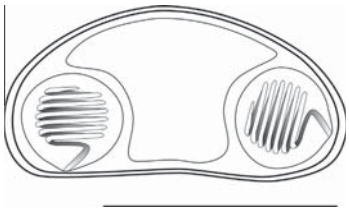

Fig. 4. Line drawing of *Zschokkella siegfriedi*, sutural view. Scale bar = 10 μm.

morhua. *Zschokkella hildae*, the type species of the genus *Zschokkella*, typically infects fish from the family Gadidae and was previously reported from *B. saida* without providing any molecular data (Aseeva, 2002; Køie, 2009). In the light of the new data, we suggest *B. saida* was most likely either infected with *Z. siegfriedi* in the report of Aseeva (2002) or this host is susceptible for both *Z. hildae* and *Z. siegfriedi* species. The spores of *Z. hildae* possess some degree of pleiomorphy during maturation; morphologically, *Z. hildae* and *Z. siegfriedi* are indistinguishable. However, *Z. hildae* was found to infect the host's urinary bladder and collecting duct of the kidney, unlike *Z. siegfriedi* which develops in the upper excretory system and the renal tubules. Nevertheless, we expect *Z. siegfriedi* to infect also urinary bladder as reported for *Z. hildae* since we were not able to cheque the urinary bladder of *B. saida*. We determined that *Z. siegfriedi* is a distinct species based on biology and genetics; biologically, *Z. siegfriedi* has (i) significant genetic difference based on SSU rDNA; (ii) localization of sporogonic stages in renal tubules vs collecting duct; (iii) different but very closely related host species to that of *Z. hildae*.

***Parvicapsula petuniae* n. sp.** (Fig. 1D, Fig. 7).

Family Parvicapsulidae Shulman, 1953.

Genus *Parvicapsula* Shulman, 1953.

Type host: *G. tricuspis* (Reinhardt, 1830), Arctic staghorn sculpin; average standard length 13.9 cm.

Other hosts: unknown.

Type locality: Greenland Sea, part of the Billefjorden, Isfjorden, Petunia Bay in the central part of Svalbard archipelago (78° 69′ N, 16° 53′ E).

Other localities: none.

Description of sporogonic stages: disporic plasmodia, early plasmodia subspherical to oval, sometimes with filopodial projections; plasmodia located in renal tubules; for dimensions see Table 1.

Description of myxospores: spores asymmetrical with somewhat curved and wavy suture line, ellipsoidal in frontal view; two pyriform PCs of equal size; closely apposed, discharging in the same apical direction, 8 coils of polar filament; single distinct binucleate sporoplasm; measurements see Table 1.

Localization of sporogonic stages: coelozoic, renal tubules, urinary bladder.

Prevalence: 9% (2 of 22 kidney samples and of 17 urinary bladders).

Pathology: No material was available for evaluation the species pathogenicity.

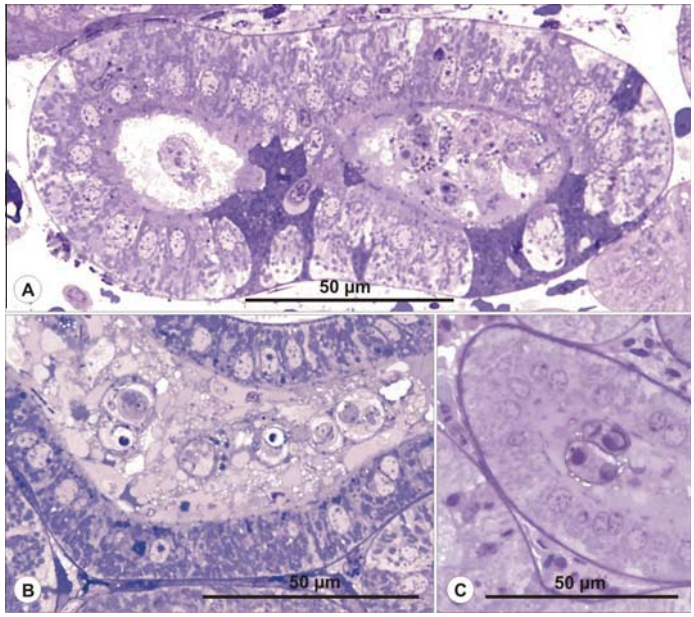

Fig. 5. Histology of *Zschokkella siegfriedi* infection. (A–C) *Zschokkella siegfriedi* infection in renal tubules of *Boreogadus saida* as seen in semithin sections stained with toluidine blue. (A) Infected segment of renal tubule with plasmodial stages in its lumen and densely stained cells in its epithelial lining. (B) Advanced plasmodial stages and amorphous material completely filling the lumen of renal tubule. All epithelial cells are densely stained. (C) Almost mature spores localised in the lumen of renal tubule. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

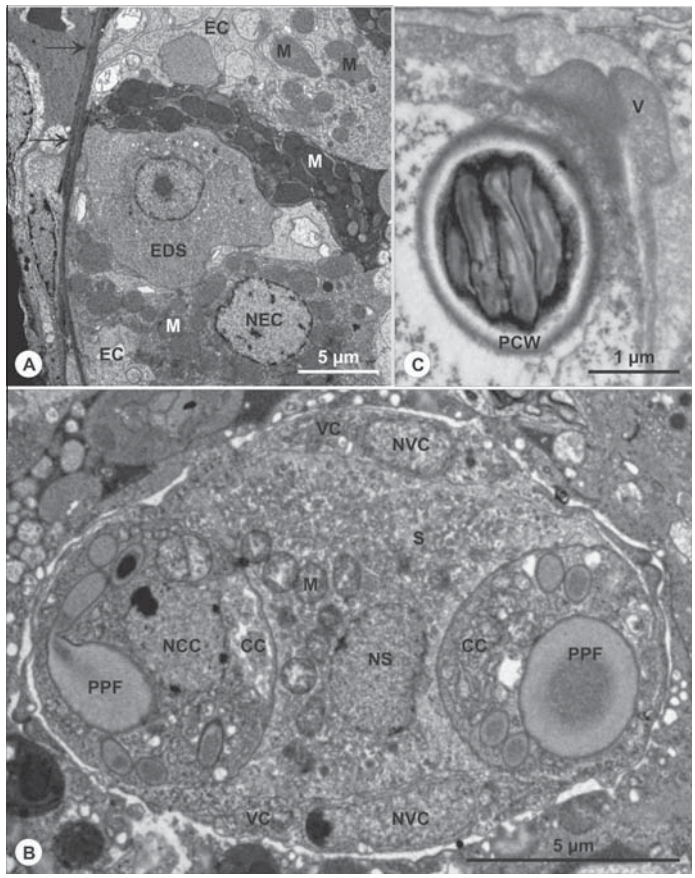

Fig. 6. Ultrastructure of *Zschokkella siegfriedi* infection. (A–C) Details of ultrastructure of *Zschokkella siegfriedi* as seen in transmission electron microscope. (A) Early developmental stage (EDS) localised within epithelium of renal tubule. Epithelial cells (EC) differ substantially in electron-density due to differences in density of mitochondria. Arrows mark basal membrane of renal tubule, NEC nucleus of epithelial cells, M mitochondria. (B) Almost mature spore in longitudinal section. VC valvogenic cell, NVC nucleus of VC, CC capsulogenic cells, NCC nucleus of CC, S sporoplasm, NS nucleus of S, PPF primordium of polar filament, M mitochondria. C. Valves (V), the polar capsule wall (PCW), and some sections of the polar filament coils.

Materials deposited: DNA sample (nr. 1423) stored in −80 °C in the Laboratory of Fish Protistology, Institute of Parasitology, BC ASCR; SSU (GenBank accession No. KF874230) and LSU (GenBank accession No. KF874223) rDNA sequences.

Etymology: specific name refers to the type locality Petunia Bay.

Remarks: This is the first report of a *Parvicapsula* species from *G. tricuspis*. The shape and size of *P. petuniae* spores are similar to the asymmetrical spores of *P. hoffmani* infecting the intestinal epithelium of mullet (India) (Dorothy and Kalavati, 1993) and even more similar to *P. karenii* infecting the urinary bladder of a flatfish from the Yellow Sea (Zhao et al., 2000). Nevertheless, the above mentioned morphologically similar *Parvicapsula* species differ in their host species preference and with different distributions from *P. petuniae* thus considering it to be a distinct species.

***Sinuolinea arctica* n. sp.** (Fig. 1G, H, Fig. 8).

Family Sinuolineidae.

Genus *Sinuolinea* Davis, 1917.

Type host: *M. scorpius* (Linnaeus, 1758), Shorthorn sculpin; average standard length 18.7 cm.

Other hosts: unknown.

Type locality: Greenland Sea, part of the Billefjorden, Isfjorden, Petunia Bay in the central part of Svalbard archipelago (78° 69′ N, 16° 53′ E).

Other localities: none.

Description of sporogonic stages: mostly disporic, rarely polysporic spherical plasmodia; freely floating in urine; spores maturing either inside the plasmodium or in pansporoblasts which are as a whole separated from the plasmodium and where spores

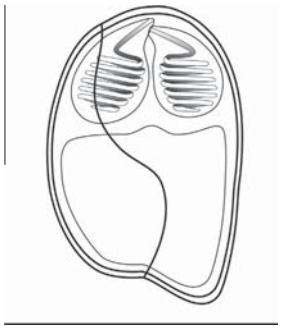

Fig. 7. Line drawing of *Parvicapsula petuniae*, sutural view. Scale bar = 10 μm.

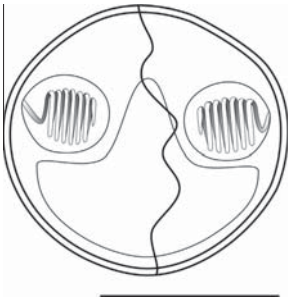

Fig. 8. Line drawing of *Sinuolinea arctica*, sutural view. Scale bar = 10 μm.

subsequently undergo complete maturation; for dimensions see Table 1.

Description of myxospores: spores spherical with protrusive sinuous suture line twisted in its axis; valves with smooth surface; two spherical PCs of equal size, separated from one another and discharging sideways, 7 coils of polar filament; spores with a single distinct sporoplasm; for dimensions see Table 1.

Localization of sporogonic stages: coelozoic; urinary bladder.

Prevalence: 10% (5 of 48 urinary bladders).

Pathology: unknown.

Materials deposited: DNA sample (nr. 1317) stored in −80 °C in the Laboratory of Fish Protistology, Institute of Parasitology, BC ASCR; SSU (GenBank accession No. KF874232) and LSU (GenBank accession No. KF874227) rDNA sequences.

Etymology: species name refers to the geographic origin in Arctic.

Remarks: Sinuolinea arctica is the first *Sinuolinea* species described from *M. scorpius*. Its size and morphology are similar to those of the type species *S. dimorpha*, but the spore of *S. arctica* is slightly bigger (14.8–15 μm vs. 15.4–16 μm) (Dyková et al., 2013). *Sinuolinea* sp. from urine of *M. scorpius* was previously reported by Lom (1984) and has identical morphology to *S. arctica*. However, dimensions of *Sinuolinea* sp. are significantly larger (L 22.9 μm and W 20.7 μm) than of *S. arctica* (L 15.7 ± 0.9 and W 15.4 ± 0.8). Assigning taxonomic status of *Sinuolinea* sp. would require molecular characterisation. *Myxoproteus myoxocephali* Fantham, 1940 (family Sinuolineidae) was described from gall bladder of *M. scorpius*. However, infecting gall bladder, which is not a typical site of infection of sinuolineid species, and poor morphological description of *M. myoxocephali* puts doubt on correct systematic position of this species.

3.2.3. Characterization of new organism

***Latyspora*-like organism** (Fig. 1L–N, Figs. 9 and 10).

Family Sinuolineidae Shulman, 1959.

Genus *Latyspora* Bartošová, Freeman, Yokoyama, Caffara and Fiala, 2010.

Type host: *C. harengus* Linnaeus, 1758, Atlantic herring; average standard length 20.9 cm.

Other hosts: unknown.

Type locality: Greenland Sea, part of the Billefjorden, Isfjorden, Petunia Bay in the central part of Svalbard archipelago (78° 69′ N, 16° 53′ E).

Other localities: none.

Description of sporogonic stages: disporic plasmodia globular in shape containing numerous refractile granules; plasmodia developing in renal tubules (attached to the epithelium and sometimes invading into epithelium); for dimensions see Table 1.

Description of myxospores: spores bean-shaped or trapezoidal from frontal view, oval from the apical view; both valves smooth with rounded shape; spore folds formed by the shell valve at its posterior pole; straight sutural line running perpendicularly between two spherical PCs of equal size, PCs located close together at anterior pole and oriented in the same direction, discharging sideways, PCs with a straight central shaft of the filament, 6–7 coils of polar filament; single sporoplasm with two nuclei; for dimensions see Table 1.

Localization of sporogonic stages: coelozoic; renal tubules.

Prevalence: 14% (9 of 66 kidney samples).

Pathology: advanced infection associated with alteration to the epithelium of renal tubules either by atrophy of epithelial cells and pyknosis of cell nuclei or complete loss of integrity of epithelium due to necrotic changes; hypertrophy of renal corpuscles caused by foreign material accumulated in dilated Bowman's spaces not possible to unambiguously associate with infection (Fig. 10).

Materials deposited: DNA sample (nr. 1365) stored at −80 °C and paraffin blocks nrs. 695/09, 700/09, 704/09 stored in the Laboratory of Fish Protistology, Institute of Parasitology, BC ASCR; SSU (GenBank accession No. KF874234) and LSU (GenBank accession No. KF874225) rDNAs sequences.

Remarks: Classification of this species near the genus *Latyspora* is based on the current state of *Latyspora* taxonomy (Bartošová et al., 2011). The taxonomic status of *Latyspora*-like organism will be emended in the future when myxozoan taxonomy and in particular the genus *Latyspora* is revised. *Latyspora*-like organism differs morphologically from the genus *Latyspora* in one morphological characteristic: the sutural line is straight in *Latyspora*-like

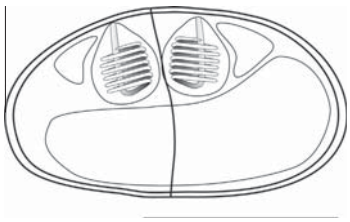

Fig. 9. Line drawing of *Latyspora*-like organism, sutural view. Scale bar = 10 μm.

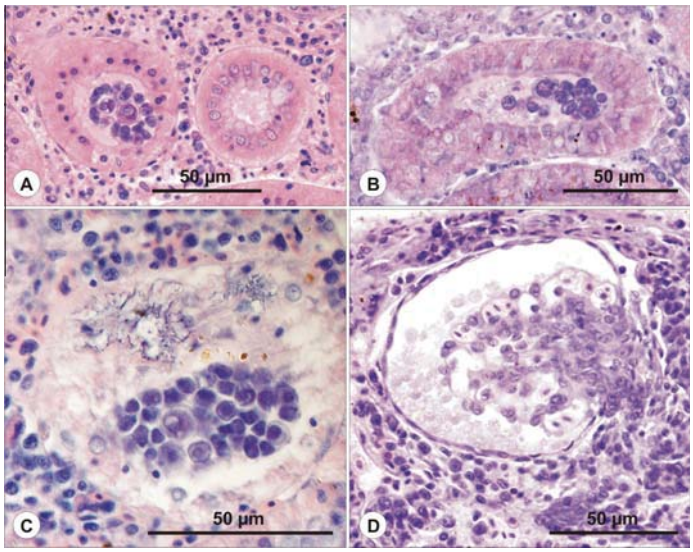

Fig. 10. The histology of kidney infected with *Latyspora*-like organism. (A–B) Advanced stage of *Latyspora*-like organism infection in renal tubules of *Clupea harengus*. (A) Epithelium in infected segments of renal tubules consisting of cells with pyknotic nuclei suggestive of cellular necrosis. (B) Early stage of epithelial disintegration. (C) Loss of integrity of epithelium due to advanced necrotic changes. Basophilic remnants seen in necrotic epithelium indicate hypertrophy of some nuclei. (D) Hypertrophy of renal corpuscles containing foreign material in Bowman's spaces was observed but cannot be solely associated with *Latyspora*-like organism infection.

organism vs. sinuous in the type species *Latyspora scomberomori*. Other morphological and biological characteristics e.g. localization in the fish host fully correspond to *Latyspora* and phylogenetically, the genus type species *L. scomberomori* and *Latyspora*-like organism are distantly related.

3.3. Phylogenetic analyses

Seven newly molecularly characterised myxosporeans clustered within the marine myxosporean lineage in the rDNA-based phylogenies (Fig. 11, Supplementary Fig. 1A). Phylogenetic tree based on five new LSU rDNA and concatenated analysis based on SSU + LSU rDNA shows the marine urinary clade monophyletic, however subclades are not well resolved (Supplementary Fig. 1A, B).

All species, except *Ceratomyxa porrecta*, clustered within the clade of marine myxosporeans mainly infecting the urinary bladder of fish i.e. the marine urinary clade according to Bartošová et al. (2011). *Ceratomyxa porrecta* branched in the *Ceratomyxa* clade (Supplementary Fig. 2B) with a close relationship to *C. auerbachi* described from the North Sea and Norwegian Sea region. SSU rDNA sequences of *M. gadi* and *M. finnmarchicum*, obtained in this study, clustered with sequences of these species currently available in GenBank (Supplementary Fig. 2A). Their sequence similarities within the species were higher than 98% (Supplementary Table 2). The analysis of the marine *Myxidium* clade confirmed the sister relationship of *M. gadi* and *M. bergense* and revealed the position of *M. finnmarchicum* as an early branching species closely related to *S. phyllopteryxa* and *M. incurvatum* (Supplementary Fig. 2A).

The marine urinary clade was enlarged by the addition of six newly sequenced species (Fig. 11). *Parvicapsula irregularis* was very closely related to pathogenic *P. bicornis* in both SSU and SSU + LSU rDNA-based trees with maximum bootstrap support (Fig. 11 and Supplementary Fig. 1A) and with a high sequence similarity of 98.1% (Supplementary Table 1). *Parvicapsula petuniae* clustered with *S. testicularis* with high nodal support in the SSU rDNA-based ML and BI (MP bootstrap support was low; Fig. 11) and their sequence similarity was 85.9% (Supplementary Table 1). Both newly obtained *Parvicapsula* sequences branched within the *Parvicapsula* subclade of the marine urinary clade with high nodal support in the SSU rDNA-based tree (Fig. 11). The *Zschokkella* subclade is enriched almost 1.5 times. *Latyspora*-like organism was the most basal species of the *Zschokkella* subclade and did not cluster with the type species *L. scomberomori* in any analyses (Fig. 11, Supplementary Fig. 1A). *Sinuolinea arctica* and *Zschokkella siegfriedi* clustered together with both *Z. hildae* and *Sinuolinea* sp. with high nodal support in the SSU and SSU + LSU rDNA-based phylogenies (Fig. 11 and Supplementary Fig. 1A). The clade of the four aforementioned species was characterised by the long branch in the phylogenetic trees (Fig. 11, Supplementary Fig. 1A). *Zschokkella siegfriedi* and *Z. hildae* were closely related (Fig. 11, Supplementary Fig. 1A) with relatively high sequence similarity of 97.2% (Supplementary Table 1). *Schulmania aenigmatosa* was revealed as the sister taxon to the abovementioned long-branching group with low nodal support in the SSU rDNA tree (Fig. 11). Concatenated analysis of SSU + LSU rDNA data supported the relationship of *S. aenigmatosa* with the group containing *L. scomberomori* and *S. dimorpha* (99% bootstrap support in ML) and revealed the long-branching group of two *Zschokkella* spp. and two *Sinuolinea* spp. inside the *Parvicapsula* subclade (Supplementary Fig. 1A). In addition, the topology within the marine urinary clade was identical after changing the outgroup (three *Ceratomyxa* species instead of three freshwater myxosporeans) in the analysis testing the influence of the selected outgroup on the resulting topology (tree not shown). Based on the three main alignments focused on the Myxosporea infecting urinary

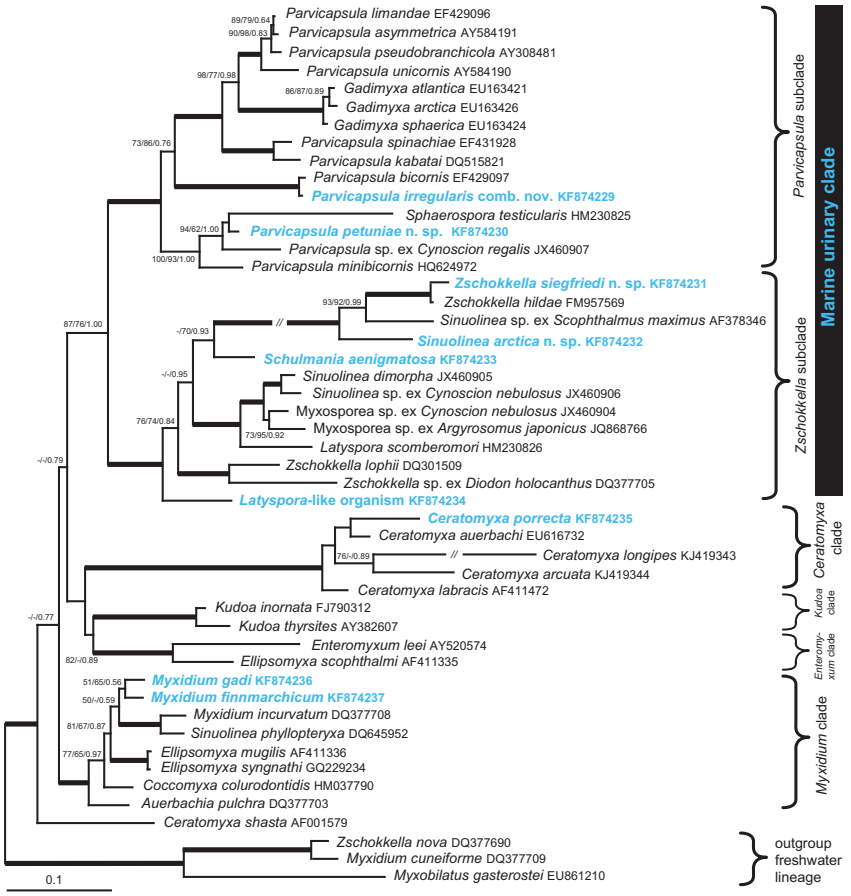

Fig. 11. Maximum likelihood tree (-ln = 18360.2248) based on 52 SSU rDNA myxosporean sequences available in GenBank and newly obtained data (in bold blue colour) belonging to the marine urinary clade. Numbers at the nodes represent the bootstrap values and the Bayesian posterior probability (ML/MP/BI) gaining more than 50% support (ML and MP) and 0.5 posterior probability (BI), respectively. Bold branches lead to a node with a bootstrap support of ⩾95 and a Bayesian posterior probability of ⩾0.97. Scale bar is given under the tree. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bladder, the marine urinary clade was a well resolved group with nodal supports (ML/MP/BI) of 87/76/1.00 in the SSU rDNA-based tree, 60/80/1.00 in the LSU rDNA-based tree and 100/99/0.92 in the SSU + LSU rDNA-based tree, respectively (Fig. 11, Supplementary Fig. 1A, B).

4. Discussion

All our findings of myxosporean species in this study are the most Northern records of the Myxosporea. *Myxidium gadi* has an extended geographic distribution around the north part of the Northern hemisphere. Polar cod, host of *Zschokkella siegfriedi*, is one of the most northerly distributed gadid fish, has a circumpolar distribution (Froese and Pauly, 2013). We can assume that most of the myxosporeans described from Svalbard have a wide distribution not limited by the Svalbard archipelago but not exceeding the range of their intermediate fish hosts.

Differences in prevalence and infection intensity of Myxosporea were detected in the fish hosts. The highest myxosporean prevalence and infection intensity was observed in benthopelagic fish *B. saida* infected with *Zschokkella siegfriedi* (43%) and in benthic fish *H. platessoides* infected with *P. irregularis* (44%). Generally, the prevalence of all four myxosporeans found in benthic fish *M. scorpius* was low, however, this host was infected with the highest number of myxosporean species (Table 1). The rich myxosporean fauna of *M. scorpius* was revealed during sequencing of selected microscopically myxosporean positive samples. Hidden mixed infections of *Myxidium finnmarchicum* and *M. gadi* revealed by

PCR suggest the presence of presporogonic or sporogonic stages with low infection intensity, which can be easily overlooked or misidentified with stages of belonging to myxosporean species with high prevalence. The differences in infection intensity and parasite abundance between hosts may be explained by competition or other negative interactions among parasites in the fish host (Seppala et al., 2009).

Myxosporeans have not been reported on Svalbard or the surrounding marine environment so far except the finding of *Z. hildae* from *B. saida* (Køie, 2009). Therefore, we can only provide a comparison of myxosporean parasitofauna with geographic regions close to the Arctic. We chose the ratio of total number of myxosporean species found/number of dissected fish species as a measure to determine and compare the biodiversity among the regions. The ratio in our study (1.3) was very similar to the ratio (1.5) obtained in the study of gadid fish in the North Sea and Norwegian waters (Kalavati and MacKenzie, 1999). However, a much lower ratio (0.5) was recorded in 28 meso- and bathypelagic fish species from the continental shelf of Newfoundland and Labrador (water depth from 200 to 1000 m) (Khan et al., 1986). On the other side, one parasite per fish species in average (ratio 1.0) was revealed in mesopelagic fish in the North Atlantic (Yoshino and Noble, 1973). Therefore, it seems sea water depth rather than geographic distribution is an important factor influencing myxosporean fauna. Deep water fish (except benthopelagic) had the lowest ratio of myxosporeans per fish, which corresponds to observation of low parasite richness by Klimpel et al. (2006) in different meso- and bathypelagic fish. In contrast to high ratio of myxosporean infections observed in epi- and mesopelagic gadid fish (Kalavati and MacKenzie, 1999) which is one of the most dominant Arctic fish families. Although we did not dissect any Atlantic cod, whose parasitofauna has been well studied and includes a total 11 myxosporean species, we did examine fishes from the same depth range with similar parasite/host ratios. Heteroxenous parasite expansion is dependent also on the other host involved in the life cycle. It means that the myxosporean distribution area is restricted not only by fish abundance but also by the particular definitive host.

The morphologically simplified body organisation of the Myxozoa together with ancestral polymorphism and convergent evolution limit the number of characteristic features important for the classification of myxozoan genera (Avise, 2004). Moreover, myxosporeans often possess a certain degree of spore plasticity within evolutionary closely related species, especially within species clustering in the marine urinary clade (Fiala and Bartošová, 2010). Phylogenetic positions of myxosporeans obtained in this study strengthened the typical myxosporean discrepancies between taxonomy based on the morphological similarities and the observed phylogenetic relationships. This is evident in the close relationship of *P. petuniae* with *S. testicularis* and unrelated phylogenetic positions of the *Latyspora*-like organism and *L. scomberomori* as well as *Sinuolinea arctica* and *S. dimorpha*.

The myxospore shape of species from the marine urinary clade is very variable in comparison with shapes shared among species in other e.g. *Ceratomyxa* and *Kudoa* clades. Variability of the myxospore morphology can be seen in the position of PCs, twisting of the suture line around the valves and by alterations of the overall spore shape e.g. prolongation and broadening of the spore. Bartošová et al. (2011) investigated the evolution of the suture line in the marine urinary clade. They found the character of the suture line to be a typical homoplastic feature. Phylogenetic positions of the myxosporeans reported from Svalbard represented by the genera *Zschokkella*, *Parvicapsula*, *Sinuolinea*, *Latyspora* (all with curved or sinuous suture line) and *Schulmania* (straight suture line) supported the homoplasy of this feature. Moreover, Bartošová et al. (2011) traced the evolutionary character of the suture line i.e. sinuous or curved vs. straight on the SSU rDNA-based phylogeny. They found that an ancestor of the marine urinary clade possessed the curved suture line. *Latyspora*-like organism as the basal species of the *Zschokkella* subclade, has a remarkably straight suture line. Therefore, the evolutionary history of this feature would be different if we again trace this character on the tree which is in congruence with the statement of Bartošová et al. (2011) that poor taxon sampling influences the tracing of character evolution.

Latyspora-like organism is a problematic species, a taxonomic "hard nut to crack", detailed in the description above. It has the straight suture line and differences in PC discharge and its phylogenetic position from the type species thus not allowing us to assign it to the genus *Latyspora*. The genera *Latyspora* and *Ceratomyxa* have very similar types of spores, nevertheless characters of suture line and position of PCs distinguish these two genera (Bartošová et al., 2011). The appropriate focus plane is crucial for the correct characterisation of the suture line as seen in the picture of *Latyspora*-like organism in Fig. 1N. We assume that the documentation of sinuous suture line of *L. scomberomori* (Bartošová et al., 2011) is questionable in that halo effect around the PCs, may have resulted in misinterpretation of the character of suture line. In any case, these two species are not phylogenetically closely related and thus *Latyspora*-like organism should not be assigned to the genus *Latyspora* which would make this genus polyphyletic. However, *Latyspora*-like organism may be representative of another so far undescribed genus.

Variability of the myxospore morphology was also studied at the level of a single species e.g. *Zschokkella pleomorpha* and *Bipteria formosa* during spore development. It was documented that the maturation process changes the shape and dimensions of the myxospore (Lom and Dyková, 1995) or formation of lateral wings (Karlsbakk and Køie, 2009). We assume that the lateral wings of *Schulmania aenigmatosa* undergo similar maturation changes as those in *B. formosa*. In these cases, it is important to provide morphometric data from the completely mature spores to avoid obtaining of misleading spore dimensions.

Speciation is not always accompanied by morphological change and many species remain undescribed (Bickford et al., 2007). Research on cryptic species has increased since molecular tools helped to distinguish closely related and morphologically similar or identical species. In our study, two species of the genus *Myxidium*, *M. finnmarchicum* and *M. gadi*, were hard to distinguish based on the morphology of the spores, which is a tool of classic myxosporean taxonomy. Both species occurred in the same host species and were present in low prevalence. The presence of these two different species was uncovered based on SSU rDNA screening of the sample and supported by a detailed morphometric analysis.

Another example is a cryptic myxosporean species found in Polar cod kidney tubules. Aseeva (2002) observed this myxosporean in Polar cod and classified it as *Zschokkella hildae* based on identical morphological and biological features. However, we revealed this myxosporean to be a cryptic species based on the genetic differences in the SSU rDNA and we named it as *Zschokkella siegfriedi*. *Zschokkella hildae* has been recorded in nine gadid fish including Arctic cod from Arctic region of Greenland (Køie et al., 2008b). Up to now SSU rDNA data of *Z. hildae* are available from the Atlantic cod only (Holzer et al., 2010). Hypothetically, more species can be revealed from the family Gadidae by molecular characterisation and they can represent hidden or misidentified species as in the case of *Z. siegfriedi* from Polar cod. The type host of *Z. hildae*, the Greater forkbeard *Phycis blennoides*, phylogenetically clusters apart from the other reported hosts of *Z. hildae* (Møller et al., 2002; Teletchea et al., 2006; Roa-Varon and Orti, 2009). This may suggest that *Z. hildae* from the type host may not correspond to the myxosporean described (and sequenced) from Atlantic cod. More information about the *Zschokkella* subclade including increased taxon sampling effort together with providing biological characters

from life cycles, development, ecology of definitive host etc. may lead to the radical taxonomic changes. Pleomorphic myxospores resembling *Zschokkella* morphotype and presence of the *Zschokkella* type species in the *Zschokkella* subclade may provoke assignment of all members of this subclade to the genus *Zschokkella*.

Discovery of *Z. siegfriedi*, morphologically identical species with *Z. hildae*, based on SSU rDNA sequence divergence underlines the importance of molecular data for species description and for parasite new host records. However, the level of myxosporean genetic interspecific dissimilarity is fluctuating, which do not allow simple use of arbitrary chosen level of genetic dissimilarity to discriminate between species. For example, members of the genus *Ceratomyxa* have much lower sequence difference up to 0.4% (Gunter and Adlard, 2009), which is in contrast to *Chloromyxum leydigi* with 1.8% intraspecific variation (Glesoon and Adlard, 2012). Similarly in this study, *Myxidium gadi* a generalist parasite of gadid fish has 1.2% of intraspecific variation and, on the other hand, sequence dissimilarity between *Parvicapsula limandae* and *P. assymetrica* is 0.9%, and among *Ellipsomyxa* spp. is even about 0.5%. As already discussed in Gunter and Adlard (2009), the level of DNA sequence difference must be assessed on a case to case basis using a whole evidence approach.

Marine myxosporean life cycles are poorly resolved with only few described ones for specifically *Ceratomyxa auerbachi*, *Gadimyxa atlantica*, *Sigmomyxa sphaerica*, two species of *Parvicapsula* and two species of *Ellipsomyxa*. All of them have a polychaete definitive host in their life cycle (Køie et al., 2004; Køie et al., 2007a; Køie et al., 2008a; Rangel et al., 2009; Karlsbakk and Køie, 2012; Køie et al., 2013). Lower levels of species richness may give polar regions an advantage for studying myxozoan life cycles compared to species-rich subtropical or tropical regions. Therefore, the Svalbard coast may be a suitable area for life cycle studies, supported by preliminary data on the life cycle of *Gadimyxa sphaerica* (results will be published elsewhere). We may hypothesise a polychaete worm as a host for *P. petuniae*, since the closely related *P. minibicornis* uses a freshwater polychaete, *Manayunkia speciosa* as a host (Bartholomew et al., 2006). Nevertheless, the elucidation of the life cycles of myxosporeans from Svalbard region is a task for future studies.

Except of the universal SSU rDNA marker, we also sequenced LSU rDNA of Myxosporea in order to add more molecular data to our analyses. Nevertheless, the single LSU rDNA analysis of the marine urinary clade contained significantly less taxa compared with the SSU rDNA analysis. This discrepancy in amount of the characters for particular taxa may cause the different topological pattern of SSU vs SSU + LSU rDNA analyses. Moreover, LSU rDNA has higher phylogenetic signal and may suppress the signal of SSU rDNA leading to different topology (Bartošová et al., 2009).

Our research indicates that increased taxon sampling effort is needed to elucidate myxosporean relationships, mainly of species from the urinary system clustering in the marine urinary clade. This clade accommodates many diverse myxosporean morphotypes and therefore, new molecular data for species from urinary systems of marine fish, especially those classified to genera with missing molecular data, are needed. There is also an obvious importance of studying parasites from the Arctic as a region most influenced by climate change (Post et al., 2009) in order to monitor its changing parasitofauna. New phylogenetic data from species infecting urinary tract contribute to the knowledge of evolution of the marine myxosporeans.

5. Conclusions

Our focus on myxosporeans of benthic and pelagic fish collected in the central part of Svalbard revealed the presence of several new myxosporean species. Results of the present study increase the species richness of myxosporeans in a polar region as well broaden the spectrum of their hosts and their distribution in the studied area. We mostly found myxosporean species infecting the urinary tract that are distinguished by the morphologically variable spores and classified to five myxosporean genera. These species clustered together based on shared tissue tropism rather than their myxospore morphology. Based on adequate taxon sampling and SSU and LSU rDNA-based phylogeny, we discussed evolutionary trends within the marine urinary clade.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijppaw.2014.02.001.

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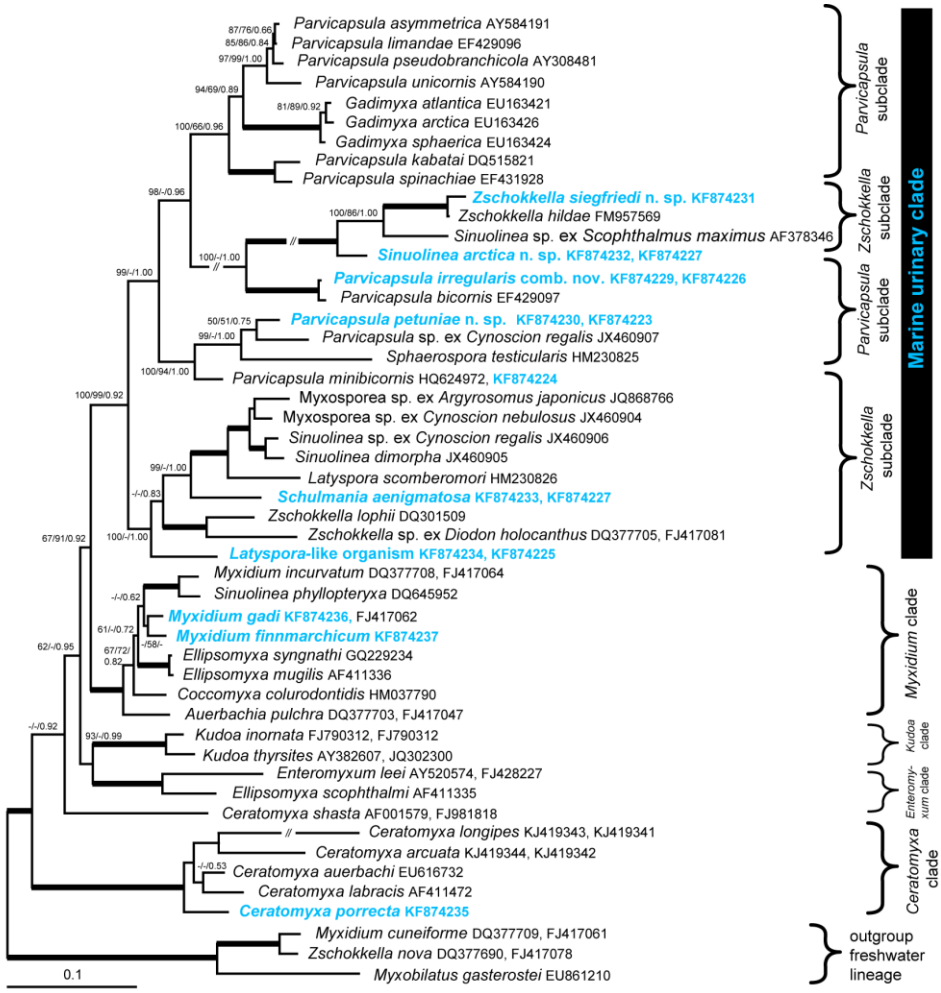

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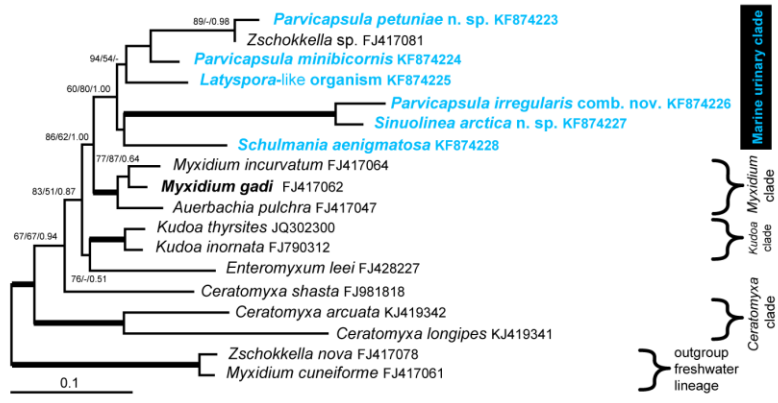
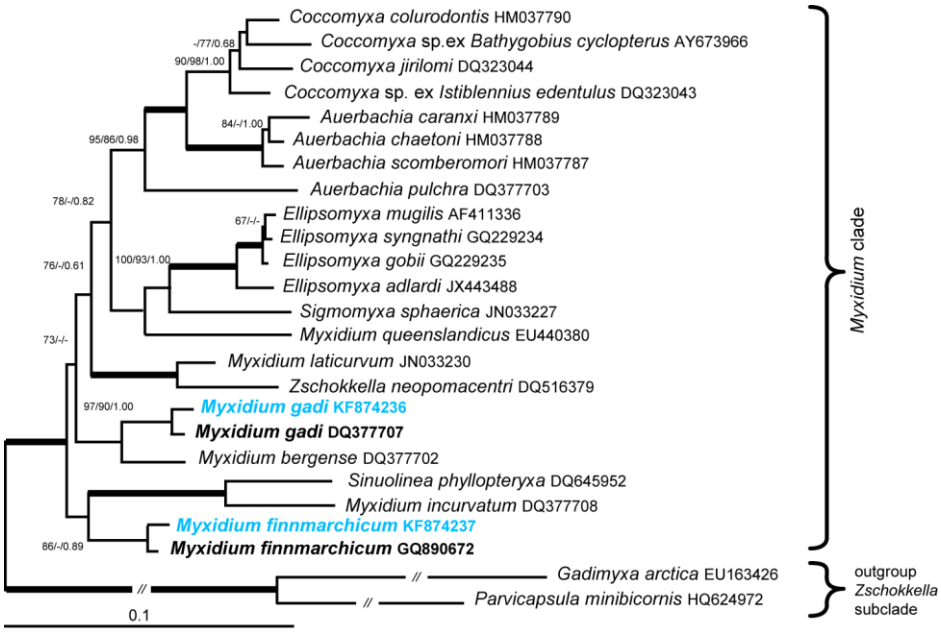


B.

Supplementary Fig. 1.



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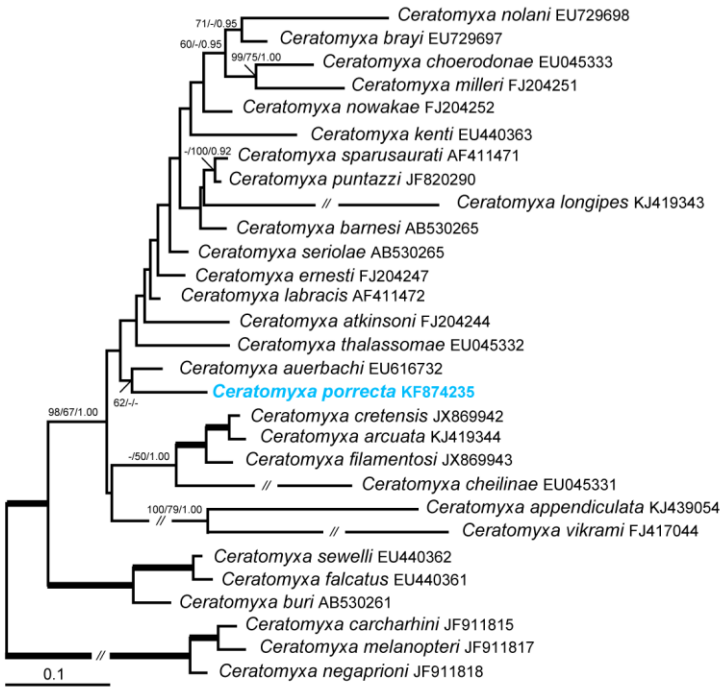


B.

Supplementary Fig. 2.

Supplementary Table 1.

	Species from the Marine urinary clade	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	Zschokkella siegfriedi																											
2	Zschokkella hildae	97.2																										
3	Sinuolineasp. ex Scophthalmus maximus	84.9	82.2																									
4	Sinuolinea arctica	83.5	81.1	81.8																								
5	Schulmania aenigmatosa	75.4	73.6	70.8	74.4																							
6	Sinuolinea dimorpha	72.4	69.6	69.8	70.9	82.5																						
7	Sinuolineasp. ex Cynoscion nebulosus	73.2	70.2	69.9	71.5	82.5	92.2																					
8	Myxosporea sp. ex Cynoscion nebulosus	71.7	70.6	69.5	70.5	81.5	89.8	89.8																				
9	Myxosporea sp. Argyrosomus japonicus	72.3	68.9	64.8	68.8	79.7	89.5	89.7	90.3																			
10	Latyspora scomberomori	72.3	69.8	70.9	72.7	80.7	83.1	83.6	83.2	82.6																		
11	Zschokkella lophii	75.2	74.1	73.3	75.9	85.6	84.2	84.1	85.8	83.7	82.4																	
12	Zschokkellasp. ex Diodonholocanthus	70.9	68.5	68.7	71.2	79.1	77.5	78.2	78.2	77.6	76.0	84.9																
13	Latyspora-like organism	76.4	74.2	72.8	76.3	86.1	83.6	84.1	85.3	82.5	83.6	84.8	81.6															
14	Parvicapsula limandae	76.0	69.4	69.7	69.4	78.2	77.4	78.1	77.6	72.7	76.7	79.8	76.5	82.4														
15	Parvicapsula asymmetrica	75.4	69.5	69.2	70.9	79.4	76.5	76.9	79.0	76.3	76.0	78.3	75.6	80.1	99.1													
16	Parvicapsula pseudobranchicola	75.9	70.3	68.9	71.0	79.3	76.1	76.0	79.3	76.0	75.4	77.7	75.5	79.7	94.5	94.5												
17	Parvicapsula unicornis	74.4	69.6	69.1	71.8	78.9	75.9	76.3	78.7	75.5	75.2	76.0	74.8	79.7	90.5	91.1	90.4											
18	Gadimyxa atlantica	74.4	72.1	72.7	73.8	81.6	80.6	80.5	79.4	79.4	79.4	79.9	78.2	81.7	86.6	87.1	87.2	87.0										
19	Gadimyxa arctica	74.2	72.1	72.9	74.1	81.8	80.8	80.5	79.7	79.4	79.5	80.3	78.4	82.3	86.7	87.3	87.3	87.5	98.8									
20	Gadimyxa sphaerica	74.3	72.4	72.6	73.7	82.2	80.6	80.2	79.6	78.7	79.5	80.2	77.7	82.0	86.7	87.3	87.1	87.0	98.3	97.9								
21	Parvicapsula spinachiae	75.7	69.5	69.3	70.6	79.0	79.4	79.7	79.6	74.9	77.5	80.4	77.6	82.7	84.3	84.7	85.0	86.6	84.1	84.1	84.0							
22	Parvicapsula kabatai	75.1	69.9	68.1	69.6	77.6	78.2	78.8	78.5	74.9	77.3	80.3	78.0	81.9	82.9	84.7	85.0	85.8	83.1	83.2	82.3	88.0						
23	Parvicapsula bicornis	73.5	69.7	67.7	69.7	78.0	77.5	78.3	78.3	74.8	75.6	78.0	76.7	80.5	82.0	82.8	83.0	82.0	80.9	80.5	80.4	82.3	82.2					
24	Parvicapsula irregularis	73.8	69.7	68.4	71.9	78.9	77.4	78.0	79.3	76.6	75.4	77.8	76.3	78.9	82.7	81.4	81.0	80.7	82.0	81.8	81.5	82.6	83.0	98.1				
25	Sphaerospora testicularis	73.2	69.9	68.4	71.0	79.1	76.7	77.4	78.1	75.4	75.4	77.3	75.4	78.4	81.9	81.2	80.4	80.4	82.2	82.2	82.4	82.1	81.9	80.8	80.1			
26	Parvicapsula petuniae	70.7	67.8	65.4	67.1	74.8	74.5	74.8	74.5	72.4	73.7	79.5	74.1	81.9	81.8	83.3	83.6	83.3	81.3	81.0	81.0	81.1	81.1	82.5	84.2	85.9		
27	Parvicapsula sp. ex Cynoscion regalis	72.6	66.2	57.8	64.8	71.2	72.0	71.5	71.8	69.2	71.6	78.4	75.8	78.5	75.3	80.7	81.2	80.8	77.6	78.1	77.3	72.6	73.6	77.1	79.4	78.7	83.8	
28	Parvicapsula minibicornis	72.4	66.7	67.4	69.5	75.8	77.1	77.1	77.8	75.6	75.0	78.7	74.6	80.1	82.0	80.4	79.6	81.2	83.2	83.4	82.9	82.9	83.0	82.1	81.7	83.6	85.3	80.6

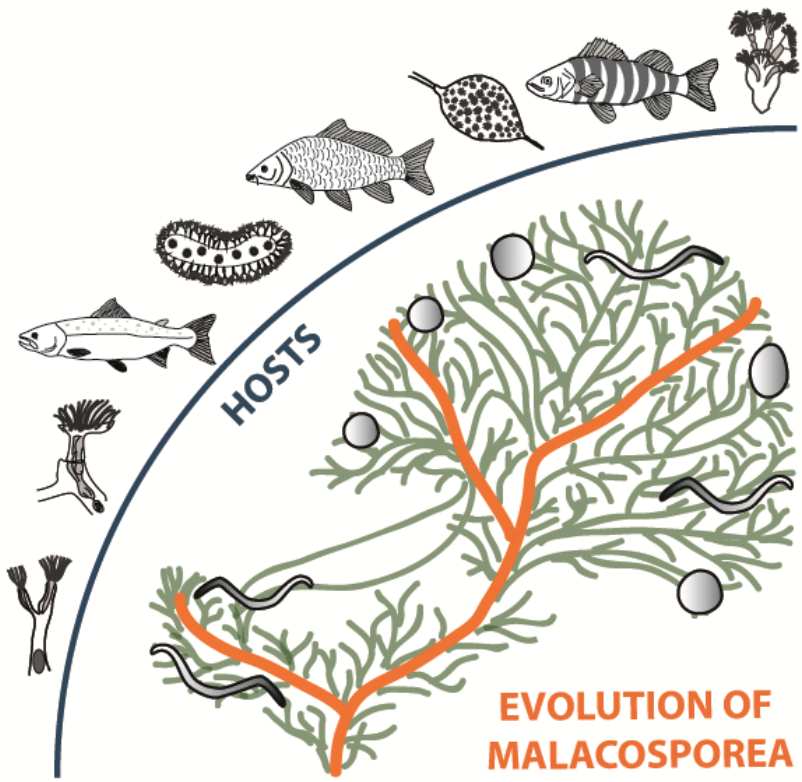
Supplementary Table 2.

	Species from the Myxidium clade	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	Myxidium gadi																								
2	Myxidium gadiex Myoxocephalus scorpius	98.8																							
3	Myxidium finnmarchicum	95.6	94.1																						
4	Myxidium finnmarchicumex Myoxocephalus scorpius	95.5	94.4	98.4																					
5	Myxidium bergense	96.8	95.0	93.3	92.2																				
6	Myxidium laticurvum	94.6	91.6	92.3	91.2	92.7																			
7	Myxidium queenslandicus	93.0	92.4	91.6	91.3	92.6	93.1																		
8	Mixidium incurvatum	91.2	90.4	91.3	90.2	90.7	90.3	90.3																	
9	Sinuolinea phyllopteryxa	91.2	89.2	90.5	89.1	90.6	90.1	90.5	93.8																
10	Auerbachia pulchra	93.3	90.3	91.5	90.0	92.1	91.4	91.8	89.3	88.6															
11	Auerbachia caranxi	93.2	93.3	93.1	92.0	93.0	92.7	92.7	90.3	90.9	92.9														
12	Auerbachia scomberoidi	93.8	94.0	93.1	92.0	93.3	92.8	93.3	90.6	91.1	93.2	98.0													
13	Auerbachia chaetodoni	94.2	94.2	93.6	92.6	93.4	93.0	92.9	90.7	91.5	93.5	98.2	98.8												
14	Coccomyxa colurodontidis	93.8	93.5	93.4	92.4	93.4	93.0	92.9	90.5	91.0	93.1	95.4	96.0	96.0											
15	Ellipsomyxa syngnathi	93.9	91.3	91.4	89.9	91.6	91.5	93.5	89.5	90.5	91.3	93.0	93.5	93.8	93.2										
16	Ellipsomyxa gobii	93.9	91.2	91.3	89.7	91.6	91.5	93.6	89.6	90.4	91.3	92.8	93.2	93.5	93.1	99.5									
17	Ellipsomyxa mugilis	94.5	91.6	91.4	90.0	91.9	92.0	93.5	90.1	90.5	91.7	93.0	93.4	93.9	93.6	99.6	99.5								
18	Ellipsomyxa adlardi	93.7	91.5	91.7	90.6	91.9	91.3	93.6	89.9	90.2	90.9	93.2	93.8	93.9	93.1	97.4	97.5	97.8							
19	Zschokkella neopomacentri	94.1	90.4	90.5	89.3	91.8	96.0	91.8	90.2	90.0	91.1	92.1	92.4	92.5	92.4	91.5	91.4	91.5	91.5						
20	Coccomya jirilomi	93.6	90.5	91.3	89.6	92.3	91.4	92.3	89.8	89.6	92.2	95.6	95.9	96.0	97.6	91.8	91.7	91.8	92.1	91.0					
21	Coccomyxasp. ex Bathygobius cyclopterus	93.8	91.0	91.2	90.2	91.7	91.6	92.0	89.3	89.8	91.8	95.8	95.8	96.4	97.6	92.1	92.0	92.0	91.9	91.1	96.1				
22	Coccomyxasp. ex Istiblennius edentulus	93.7	91.9	91.8	90.9	92.6	92.3	92.2	89.9	90.2	92.3	95.6	95.6	96.0	97.1	92.3	92.3	92.2	92.3	91.3	96.7	96.6			
23	Sigmomyxa sphaerica	93.0	90.5	91.0	90.2	90.6	91.7	93.5	89.4	89.0	91.4	92.3	92.5	92.8	92.5	93.4	93.3	93.6	93.4	91.8	91.1	90.7	91.3		
24	Gadimyxa arctica	83.4	82.5	82.4	81.7	82.3	82.5	82.0	81.4	80.9	83.5	81.7	82.0	82.2	82.4	82.7	82.7	82.6	82.4	81.9	81.7	81.8	82.4	83.4	
25	Parvicapsula minibicornis	84.5	82.8	83.1	81.7	83.0	82.3	81.5	82.0	82.4	82.8	84.0	84.0	83.9	83.8	82.1	82.1	83.7	82.6	82.5	83.5	83.7	84.3	82.0	83.4

Paper IV

IV

Hidden diversity and evolutionary trends in malacosporean parasites (Cnidaria: Myxozoa) identified using molecular phylogenetics



Hidden diversity and evolutionary trends in malacosporean parasites (Cnidaria: Myxozoa) identified using molecular phylogenetic

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Abstract

Malacosporeans represent a small fraction of myxozoan biodiversity with only two genera and three species described. They cycle between bryozoans and freshwater fish. In this study, we (i) microscopically examine and screen different freshwater/marine fish species from various geographic locations and habitats for the presence of malacosporeans using PCR; (ii) study the morphology, prevalence, host species/habitat preference and distribution of malacosporeans; (iii) perform small subunit/large subunit rDNA and Elongation factor 2 based phylogenetic analyses of newly gathered data, together with all available malacosporean data in GenBank; and (iv) investigate the evolutionary trends of malacosporeans by mapping the morphology of bryozoan-related stages, host species, habitat and geographic data on the small subunit rDNA-based phylogenetic tree. We reveal a high prevalence and diversity of malacosporeans in several fish hosts in European freshwater habitats by adding five new species of *Buddenbrockia* and *Tetracapsuloides* from cyprinid and perciform fishes. Comprehensive phylogenetic analyses revealed that, apart from *Buddenbrockia* and *Tetracapsuloides* clades, a novel malacosporean lineage (likely a new genus) exists. The fish host species spectrum was extended for *Buddenbrockia plumatellae* and *Buddenbrockia* sp. 2. Co-infections of up to three malacosporean species were found in individual fish. The significant increase in malacosporean species richness revealed in the present study points to a hidden biodiversity in this parasite group. This is most probably due to the cryptic nature of malacosporean sporogonic and presporogonic stages and mostly asymptomatic infections in the fish hosts. The potential existence of malacosporean life cycles in the marine environment as well as the evolution of worm- and sac-like morphology is discussed. This study improves the understanding of the biodiversity, prevalence, distribution, habitat and host preference of malacosporeans and unveils their evolutionary trends.

Paper V

V

Morphology and phylogeny of two new species of *Sphaeromyxa* Thélohan, 1892 (Cnidaria: Myxozoa) from marine fish (Clinidae and Trachichthyidae) (in press)

Morphology and phylogeny of two new species of *Sphaeromyxa* Thélohan, 1892 (Cnidaria: Myxozoa) from marine fish (Clinidae and Trachichthyidae)

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Running title: *Sphaeromyxa* spp. from clinids and *Gephyroberyx darwinii*

Summary
Our survey of fish from South Africa and Indonesia revealed the presence of two new myxosporean species of the genus *Sphaeromyxa* for which we provide morphological and sequence data. *Sphaeromyxa clini* n. sp. detected in three *Clinus* spp. and *Muraenoclinus dorsalis* from South Africa is morphologically similar to *Sphaeromyxa noblei* previously described from *Heteroclinus whiteleggii* from Australia and to several other sphaeromyxids with arcuate spores and rounded ends. This similarity is reflected by phylogenetic positioning of *S. clini* n. sp. which clusters within the '*incurvata*' group of the *Sphaeromyxa* clade. It differs from morphologically similar species by spore and polar capsule dimensions, host specificity and geographic distribution. *Sphaeromyxa limocapitis* n. sp., described from *Gephyroberyx darwinii* from Java, is morphologically similar to sphaeromyxids with straight spores and to marine *Myxidium* species with spindle shaped spores but differs from them by spore and polar capsule dimensions, host specificity and geographic distribution. *Sphaeromyxa limocapitis* n. sp. represents a separate lineage of the *Sphaeromyxa* clade and appears to be a missing link in the evolution of sphaeromyxids.

Key words
Myxosporea; Clinidae; *Gephyroberyx*; ultrastructure; evolution; *Sphaeromyxa* clade; SSU rDNA.

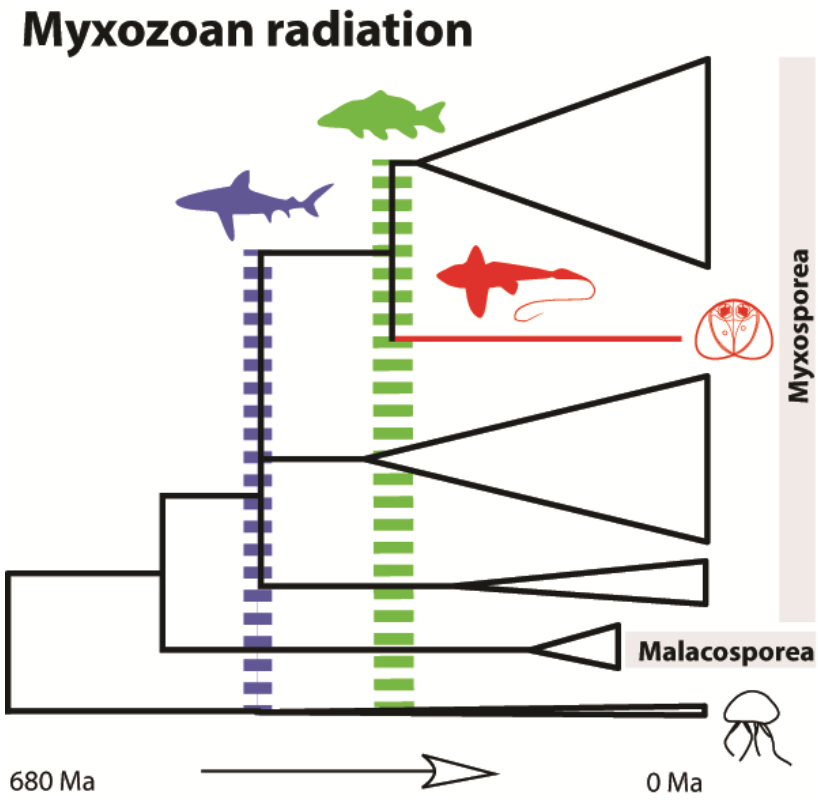
Key findings

- South African and Indonesian fish were examined for the presence of *Sphaeromyxa*.
- Light microscopical, ultrastructural, histological and molecular data are provided for *S. clini* n. sp.
- *Sphaeromyxa limocapitis* n. sp. is described from *Gephyroberyx darwinii*.
- Evolution of the *Sphaeromyxa* clade is discussed.

Paper VI

VI

Bipteria vetusta n. sp. - old parasite in an old host: tracing the origin of myxosporea parasitism in vertebrates (submitted)



Bipteria vetusta n. sp. – old parasite in an old host: tracing the origin of myxosporean parasitism in vertebrates

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Abstract

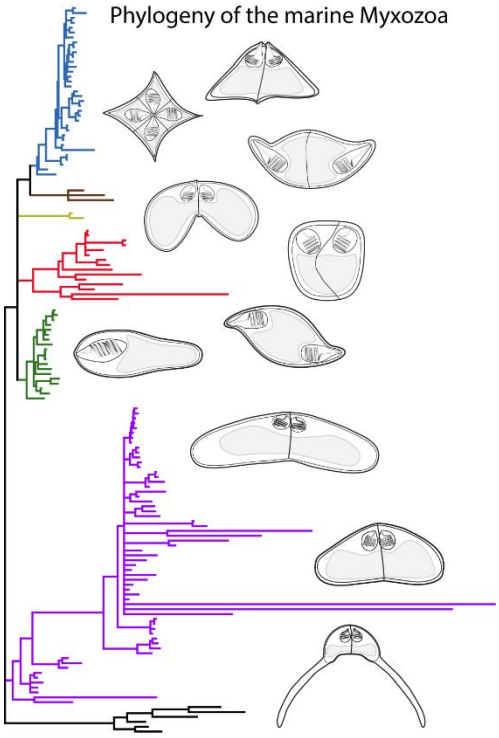
Myxosporea (Myxozoa), a group of parasitic Cnidaria, use mostly bony fish (Teleostei) as intermediate hosts; however, they can also parasitize other vertebrates such as cartilaginous fish (Chondrichthyes). Scarce molecular data of myxosporeans from sharks and rays (Elasmobranchii) revealed these parasites to be one of the most basal representatives in the myxosporean phylogenetic tree suggesting their old evolutionary history. A new myxosporean species *Bipteria vetusta* n. sp. was found in the gall bladder of rabbit fish *Chimaera monstrosa* (Holocephali; Chondrichthyes) and SSU rDNA-based phylogeny revealed its basal position within the marine myxosporean lineage. Molecular dating suggested the origin of a stem lineage leading to the marine myxosporean lineage at the time of the origin of Chondrichthyes in the Silurian era. The two common lineages of Myxozoa, Myxosporea and Malacosporea, were estimated to have split from their common ancestor in the Cambrian era. Tracing the history of evolution of the "vertebrate host type" character in context of molecular dating showed that cartilaginous fish represented an ancestral state for all myxosporeans. Teleosts were very likely subsequently parasitized by myxozoans, four times independently. Myxosporean radiation and diversification seem to correlate with intermediate host evolution. The first intermediate hosts of myxosporeans were cartilaginous fish. When bony fish evolved and radiated, myxosporeans switched and adapted to bony fish and subsequently greatly diversified in this new host niche. The present study is the first attempt of molecular dating of myxozoan evolution based on an old myxosporean species - a living myxosporean fossil.

Keywords: Myxosporean evolution; molecular clock; *Bipteria*; Holocephali; vertebrate host; cartilaginous fish; phylogeny; co-evolution.

Paper VII

VII

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



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

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Running title: *Ceratonova* and the 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*, *C. leatherjacketi*, *C. synaphobranchi*, *C. verudaensis* and *Myxodavisia bulani*; 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 phylogenetic affinities of myxosporeans were in congruence with site of sporulation in the vertebrate hosts rather than myxospore morphology.

Keywords: Myxozoa, *Ceratomyxa*, topology test, evolutionary trends, taxonomy

Appendix

Curriculum vitae

Alena Kodádková

Personal details

Born: 8 November 1984, Prachatice, Czech Republic
email: alena.kodadkova@gmail.com

Current position

Research assistant at the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic

Education

2009 until now PhD student at the Department of parasitology, Faculty of Science, University of South Bohemia in České Budějovice, Thesis*: Myxosporean phylogeny and evolution of myxospore morphotypes*

2011 RNDr. Parasitology, Department of parasitology, Faculty of Science, University of South Bohemia in České Budějovice, Thesis*: Activated CD8+ T cells contribute to clearance of gastric Cryptosporidium muris infections*

2007-2009 MSc. Parasitology, Faculty of Science, University pf South Bohemia in České Budějovice, Thesis: *Roles of CD4+ and CD8+ T-lymphocytes in immune response to the gastric cryptosporidiosis*

2007-2005 BSc. Biology, Faculty of Science, University of South Bohemia in České Budějovice, Thesis: *Occurrence of cryptosporidia in exotic mammals kept in zoological gardens*

Research interest

Taxonomy, biology and phylogeny of Myxozoa with the focus of morphotypes without any molecular data

Interships and stay abroad

2011 September- November Department of Zoology, University of Cape Town, South Africa, Population study of *Ceratomyxa cottoidi* from *Clinus cottoides*

2010 July - August Polar ecology course on Svalbard archipelago, study of myxosporeans in the arctic fishes

Teaching

Teaching assistant of the practical course “Medical parasitology and diagnostics methods“ and “Field parasitology“(Faculty of Science, University of South Bohemia in České Budějovice).

Awards

Student award for the conference of 16th European Association of Fish Parasitology

Publications

Bartošová-Sojková P, **Kodádková A**, Pecková H, Kuchta R, Reed CC (2014) Morphology and phylogeny of two species of *Sphaeromyxa* Thélohan, 1892 (Cnidaria: Myxozoa) from marine fish (Clinidae and Trachichthyidae). Parasitology in press

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Kodádková A, Tyml T, Ditrich O, Kašparová E, Mašová Š, Tušer M, Fiala I (2012) Fish parasites beyond the Arctic Circle: Myxozoa (poster). Polar Ecology conference, České Budějovice, Czech Republic

Kodádková A, Dyková I, Fiala I (2012) On the phylogenetic position of the type species of the genus *Sinuolinea* (Myxozoa) and myxosporean cryptic species diversity talk). 41th Jírovec's Protozoological days, Kletečná, Czech Republic

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Kodádková A, Ditrich O, Kváč M, Xiao L (2008) *Cryptosporidium* sp. in mammals kept in four zoological gardens in the Czech Republic (poster) 10th European Multicolloqium of Parasitology, Paris, France

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