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Biodiversity of Myxozoa based on extensive screening of fish and environmental samples

Ph.D. Thesis

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Annotation

Myxozoans represent a group of morphologically simplified endoparasites infecting mainly fish, annelid and bryozoan hosts including more than 2,600 nominal species. However, the diversity seems to be unexplored and undervalued. Host searching and invasive dissection is a commonly used method for the identification of myxozoan species diversity. Metabarcoding and amplicon analyses of environmental DNA from sediment, water and soil is developing approach for biodiversity assessment and have been performed in many biological areas to identify the diversity of organisms, communities and populations. Using classical morphological and molecular approaches, we explored the myxozoan diversity in selected areas and host groups. In addition, we propose a new methodological approach for the studying of myxozoan diversity based on environmental DNA from sediment and amplicon sequencing. Illumina Mi-Seq with metabarcoding and newly designed primer sets is suitable for analyzing myxozoan communities without the need to find and capture intermediate or final hosts.

Declaration

I hereby declare that I am the author of this dissertation and that I have used only those sources and literature detailed in the list of references.

České Budějovice, 4.7. 2022

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Martina Lisnerová

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List of papers and author's contribution

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

I. Bartošová-Sojková, P., Lövy, A., Reed, C.C., **Lisnerová, M.,** Tomková, T., Holzer, A.S., Fiala, I. (2018). Life in a rock pool: Radiation and population genetics of myxozoan parasites in hosts inhabiting restricted spaces. *PLoS ONE*, *13*, e0194042 (IF=2.776). DOI: 10.1371/journal.pone.0194042

ML participated in molecular analyses. Her contribution was 20%.

II. **Lisnerová, M.**, Blabolil, P., Holzer, A.S., Jurajda, P., Fiala, I. (2020). Myxozoan hidden diversity: The case of *Myxobolus pseudodispar*. *Folia Parasitologica*, 67 (IF=2.122). DOI: 10.14411/fp.2020.019

ML was responsible for molecular analyses. ML participated in data analyses and writing of the draft. Her contribution was 60%.

III. **Lisnerová, M.**, Fiala, I., Cantatore, D., Irigoitia, M., Timi, J., Pecková, H., Bartošová-Sojková, P., Sandoval, C., Luer, C., Morris, J., Holzer, A.S. (2020). Mechanisms and drivers for the establishment of life cycle complexity in Myxozoan parasites. *Biology*, *9*, 10 (IF=3.796). DOI: 10.3390/biology9010010

ML participated in sampling, molecular, phylogenetic and phylogeographic analyses. Her contribution was 40%.

IV. Cavaleiro, B., Serrão, J., Nogueira, S., Ribeiro, L., Hermida, M., Cruz, C., **Lisnerová, M.**, Fiala, I., Saraiva, A. (2021). Survey of *Kudoa* spp. (Myxozoa, Cnidaria) in fishes from the Madeira archipelago and the Portuguese mainland coast: Detection of *Kudoa thyrsites* in new hosts *Scomber colias* and *Micromesistius poutassou*. *Folia Parasitologica*, *68*, 1–7 (IF= 2.122). DOI: 10.14411/fp.2021.003

ML participated in molecular analyses. Her contribution was 10%.

V. **Lisnerová, M.**, Lisner, A., Cantatore, D.M., Schaeffner, B.C., Pecková, H., Tyml, T., Fiala, I., Bartošová-Sojková, P., Holzer, A.S. (2022). Correlated evolution of fish host length and parasite spore size: a tale from myxosporeans inhabiting elasmobranchs. *International Journal for Parasitology*, 52, 97–110

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ML was responsible for molecular and phylogenetic analyses. ML participated in statistical data analyses and writing of the draft. Her contribution was 60%.

VI. **Lisnerová, M.**, Martinek, I.N., Alama-Bermejo, G., Bouberlová, K., Schaeffner, B.C., Nkabi, N., Holzer, A.S., Bartošová-Sojková P. An ancient alliance: Matching evolutionary patterns of cartilaginous fishes (Elasmobranchii) and chloromyxid parasites (Myxozoa). Manuscript.

ML participated in molecular, phylogenetic, cophylogenetic and phylogeographic analyses and writing of the draft. Her contribution was 60%.

VII. **Lisnerová, M.**, Blabolil, P., Holzer, A.S., Fiala, I. Evaluation and optimization of eDNA metabarcoding assay for detection of freshwater myxozoan communities. Manuscript.

ML participated in sampling, molecular analyses and writing of the draft. Her contribution was 40%.

Co-author agreement

RNDr. Ivan Fiala, Ph.D., the supervisor of this Ph.D. thesis and co-author of papers I-V, VII, fully acknowledges the stated contribution of Mgr. Martina Lisnerová to these manuscripts.

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RNDr. Ivan Fiala, Ph.D.

RNDr. Pavla Sojková, Ph.D., first author of the paper I, last author of paper VI and the co-author of papers III and V, acknowledges the stated contribution of Mgr. Martina Lisnerová to these manuscripts.

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RNDr. Pavla Sojková, Ph.D.

Contents

Chapter I: General introduction

Myxozoa Grassé, 1970 are microscopic cnidarian endoparasites that live in various host groups, including amphibians, birds, elasmobranchs, mammals, reptiles, but mainly fish as intermediate hosts and annelids or bryozoans as final hosts (Dyková et al., 2007; Bartholomew et al., 2008; Fiala et al., 2015a; Espinoza et al., 2017, Cantatore et al., 2018). It has been 200 years since the first myxozoan parasite was found in the musculature of fish host (Jurine, 1825). Since then, more than 2,600 species have been described in various host tissues and organs (Okamura et al., 2018). These parasites have been detected in freshwater, marine, brackish water, and terrestrial ecosystems worldwide (e.g., Fiala 2006; Bartošová et al., 2011; Kodádková et al., 2014; 2015; Patra et al., 2018).

The coexistence of myxozoans in their hosts has long been adaptive, and interactions between parasites and their hosts appear to be in equilibrium (Holzer et al., 2021). Consequently, strong pathogenicity is not common among myxozoans, and only a minority of these parasites can cause severe disease in their fish hosts (Bjork and Bartholomew, 2010). However, some species are of global economic importance to fish aquaculture. *Tetracapsuloides bryosalmonae* Canning, Curry, Feist, Longshaw et Okamura, 1999, a parasite of salmon, causes proliferative kidney disease (PKD), one of the most serious parasitic diseases resulted in high losses in salmonid populations in Europe and North America (Hedrick et al., 1993). Other important myxozoan parasites are *Myxobolus cerebralis* (Hofer, 1903), which infects salmonids and causes whirling disease, and *Kudoa thyrsites* (Gilchrist, 1924), which parasite in marine fishes that devalues fish flesh. Infections by *Enteromyxum leei* (Diamant, Lom et Dyková, 1994) in the digestive tract and *Ceratonova shasta* (Noble, 1950) (syn. *Ceratomyxa shasta*) can lead to one hundred percent mortality in some fish stocks (Branson et al., 1999; Bjork and Bartholomew, 2010).

During the evolutionary development of myxozoans, parasites adapted to the parasitic life strategy by reducing their body structure and size to a few cells and a few micrometres (10-20 micrometres on average) (Fiala et al., 2015a), which is why they were incorrectly classified as protists (as Microsporidia Balbiani 1882 or Apicomplexa Levine, 1970; e.g. Bütchli, 1882; Dogiel, 1965). The first mention of multicellularity and concordance with metazoans was proposed by Štolc (1899). Recent molecular (18S rDNA) and ultrastructural findings (group-specific proteins) added the group to the metazoans, more specifically to the cnidarians (Siddall et al., 1995; Holland et al., 2011).

1.1 The life cycle and morphology of myxozoans

In the two-host life cycle of myxozoans, vertebrates are parasitized as intermediate hosts and invertebrates as final hosts (Wolf and Markiw, 1984). Of most myxozoan species, only the vertebrate part of the life cycle is known, as their invertebrate hosts, in particular, remain unknown. The life cycles of two myxozoan groups, Malacosporea Canning, Curry, Feist, Longshaw et Okamura, 2000 and Myxosporea Bütschli, 1882, are partially different. The complex life cycle of malacosporeans involves the development of fish malacospores that infect freshwater bryozoans. Subsequently, active sacs or elongated worms are formed in these invertebrate hosts (Figure 1A). Myxosporeans form actinospores that are produced in the final host (annelids - Oligochaeta or Polychaeta) and infect intermediate vertebrates where the myxospores are produced (Figure 1B) (e.g., Fiala et al., 2015a).

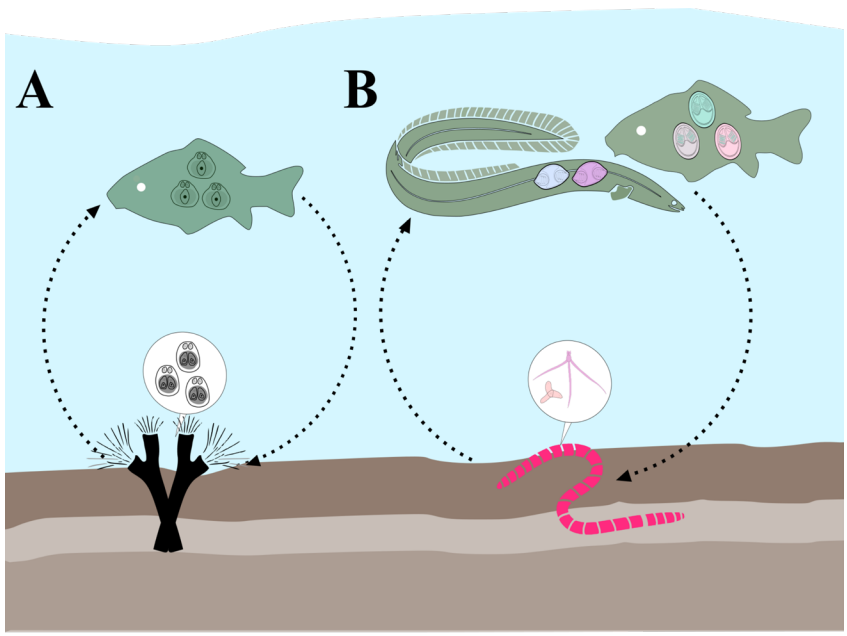

Figure 1: Simplified life cycle of myxozoans: A - malacosporean life cycle alternated with bryozoans and fish; B- myxosporean life cycle alternated with anellids and fish.

1.1.1 Myxosporean life cycle and morphology

Infection of vertebrates with myxospores begins with the penetration of an infective amoeboid sporoplasma of actinospores, which enters the body through the skin or gills. The sporoplasm invades the intercellular space and begins to form a "cell within a cell"

formation typical of Myxozoa, where secondary cells (which are formed endogenously) reside within primary cells (Alexander et al., 2015). Generative cells are formed around generative nuclei and plasmodia are differentiated. A multicellular plasmodium, often of macroscopic proportions, consists of multiple vegetative nuclei and generative cells. Plasmodium is an active form that even feeds by pinocytosis (Feist et al., 2015). Plasmodia form two different types: i) multinucleate plasmodia of large size (> 2 mm), whose cells form a large number of spores, ii) pseudoplasmodia, which are mononuclear and whose inner cells form only one or in some cases two spores (Alexander et al., 2015).

Myxospores are formed by proliferation, encapsulation and subsequent differentiation of the inner cells. Myxospores are multicellular structures consisting of one (or more) sporoplasm and one or more polar capsules that contain coiled polar filaments. The spores are released during host life in the urine or bile or after the death of intermediate hosts and infect the final host. Myxospores enter the intestine or coelomic cavity of the final invertebrate host. The sporoplasm contained in the spore is released and the associated actinospore phase begins with sexual reproduction and differentiation into actinospores (El-Matbouli and Hoffmann, 1998). These actinospores also contain an expulsive apparatus and sporoplasm with many amoeboid germs (Okamura et al., 2015). The first complex myxosporean life cycle was described by Wolf and Markiw in 1984 for *Myxobolus cerebralis*. To date, a complex myxosporean life cycle has been described with known vertebrate and invertebrate hosts in only 2% of known myxosporean diversity (Alexander et al., 2015).

1.1.2 Malacosporean life cycle and morphology

Malacosporeans form two different spores with soft valves that are not very resistant in the environment (Okamura et al., 2015). Malacospores developed in bryozoan hosts have eight shell valves, four polar capsules, and two sporoplasms, while malacospores produced in the excretory system of fish have two or four shell cells, two polar capsules, and one sporoplasm (Feist et al., 2015). The malacospores of fish are released into the water and give rise to sac-like or worm-like (myxoworm) stages (plasmodia) in the body cavity of their freshwater bryozoan hosts. The plasmodia produce infective malacospores in their internal cavity (Hartikainen et al., 2014). The full life cycle of malacospores is currently known only for a few spp. e.g., *Tetracapsuloides bryosalmonae*, the causative agent of proliferative kidney disease (PKD) in salmonids. The definitive host is the freshwater moss *Fredericella sultana* (Blumenbach, 1779) (Morris and Adams, 2006); or *Tetracapsuloides vermiformis* Patra, Hartigan, Morris, Kodádková et Holzer, 2017 parazited in common carp *Cyprinus carpio* L. and freshwater moss *Fredericella sultana* (Patra et al., 2017).

1.2. Classification, phylogeny and evolution of myxozoans

The current classification of Myxozoa is mainly based on spore morphology and does not reflect phylogenetic relationships within this group (Lom and Dyková, 2006; Fiala et al., 2015b), suggesting that inconsistencies exist between phylogenetic and morphological data within the myxozoan group. The shape of spores in Myxozoa is not an indicative character for the phylogenetic relationships; the same morphotypes of spores could have appeared independently multiple times during evolutionary history. Thus, many genera in the phylogenetic tree are polyphyletic or paraphyletic (e.g., the most widely distributed genus *Myxobolus* Bütschli, 1882, Liu et al., 2019a). Myxozoans are phylogenetically related based on other characteristics, such as the host environment in which they live (i.e., seawater vs. freshwater) or the tissue site of the parasite in the host (Fiala and Bartošová, 2010). The taxonomic division of fish species into groups (clades) of Myxosporea and Malacosporea is consistent with the results of phylogenetic analyses that also separate Myxosporea from Malacosporea (Canning et al., 2000), but in other respects, the molecular approach and subsequent phylogenetic analyses do not support the taxonomy (e.g., Fiala, 2006).

The taxonomy of myxozoans is based on the morphology and morphometrics of their spores and their developmental stages, host group/host species preference, and tissue specificity (Fiala et al., 2015b). Each spore consists of two or more valvae connected by a sutural line and enclosing one or more polar capsules and one or more sporoplasms. Information on the number of valvae, the number of polar capsules and their arrangement in the plane of the sutural line, and the type of twisting of the polar filament are used to distinguish families, genera, and orders, or suborders of Myxozoa. The most common morphotypes for genera are shown in Figure 2. Information on the size of spores/polar capsules, number of polar fibers, coils, surface features of spores (projections, grooves, ribs, fibers, tails), host species, geographical distribution, etc. are important for the identification of particular species (Lom and Dyková, 2006). However, some so-called cryptic species are not distinguishable by morphological and morphometric characters and can only be identified by molecular methods (Fiala and Bartošová, 2010).

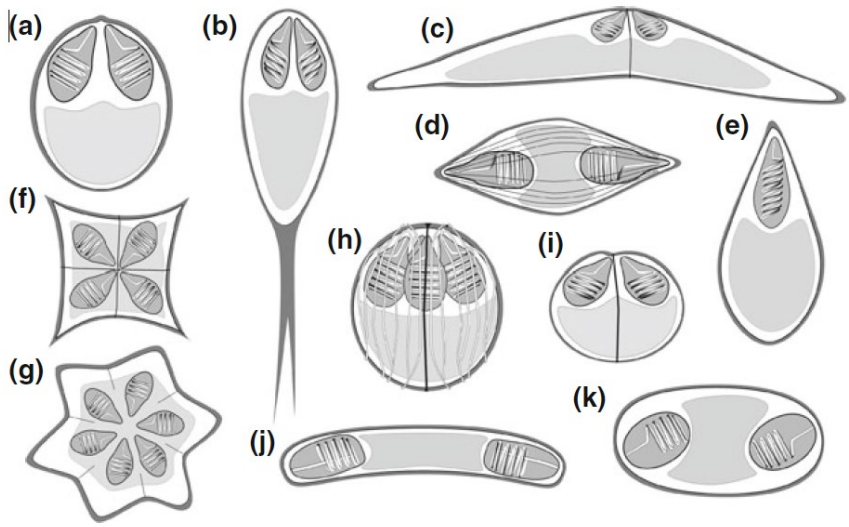

Figure 2: Ilustration of genera of major morphotypes. a *Myxobolus*, b *Henneguya*, c *Ceratomyxa*, d *Myxidium*, e *Thelohanellus*, f *Kudoa* (four valves), g *Kudoa* (six valves), h *Chloromyxum*, i *Sphaerospora*, j *Sphaeromyxa*, k *Zschokkella* (Fiala et al., 2015a).

The phylogenetic position of myxozoan species reflects the similarities of their habitats (e.g., Fiala, 2006). According to 18S rDNA analyses, the Myxozoa are divided into one malacosporean and three main myxosporean lineages: *Sphaerospora sensu stricto*, a lineage with mainly marine species parasitizing in polychaetes and a lineage with mainly freshwater species parasitizing in oligochaetes (Fiala et al., 2015b; Figure 3). For the reconstruction of myxozoan phylogeny, not only the 18S rDNA but also the LSU rDNA was used to observe the phylogenetic relationships (Bartošová et al., 2009). The results of the phylogeny based on this gene are similar to the topological relationships in the 18S rDNA-based phylogenetic tree. However, this gene is not widely used.

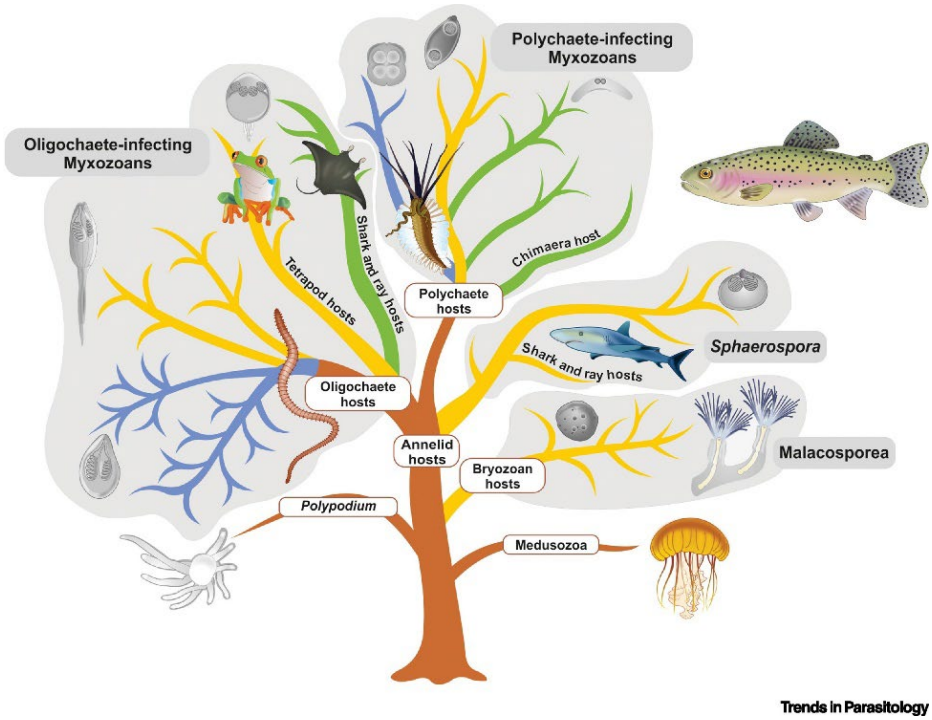

Figure 3: Illustration of phylogeny and evolutionary history of myxozoans with four major myxozoan lineages (malacosporeans, *Sphaerospora sensu stricto*, freshwater oligochaete-infecting group, marine polychaete-infecting group) and indicating hosts for every parasitic branch. The colours of the branches in the tree show a diversification in different host tissue and organ systems (yellow –urinary system, green – biliary system, blue – within tissues) (Alama-Bermejo and Holzer, 2021).

Myxozoan parasites evolved from free-living cnidarians and occurred probably in the late Cryogenian (651 million years ago). The last common ancestor of myxozoans dates back to the Ediakara period (588 million years) when Malacosporea parasiting in bryozoans and Myxosporea parasiting in primitive polychaetes (Haplodrili, Archiannelida) separated into two groups. The origin of sphaerosporids was estimated for the beginning of the Cambrian, 534 million years ago. The division of the two most diverse clades (oligochaeta-infecting myxosporean clade, polychaete-infecting myxosporean clade) took place in the late Cambrian and early Ordovician, 495 million years ago. The complex life cycle of studied parasites is a result of the incorporation of vertebrates into the simple life cycle including only aquatic invertebrates (Holzer et al., 2018). The first known vertebrate host is cartilaginous fish (sharks, rays, skates and chimaeras) (Kodádková et al., 2015; Cantatore et al., 2018; Holzer et al., 2018), which was incorporated into the life cycle later (about 450 million years ago) as secondary hosts

(Holzer et al., 2018). Sharks, rays, and chimaeras are the hosts for the oldest lineages of the two major myxosporean clades, particularly, *Bipteria vetusta* Kodádková, Bartošová-Sojková, Holzer et Fiala, 2015 found in *Chimaera monstrosa* L. clustered basally in polychaete-infecting myxosporean clade (Kodádková et al., 2015) and whole *Chloromyxum sensu stricto* clade found in different species of sharks and rays clustered basally in Oligochaeta infecting myxosporean clade (e.g. Cantatore et al., 2018; Figure 3). The inclusion of bony fish into the complex life cycles of Myxozoa took place several times independently and was followed by a huge species diversification of hosts and parasites (Holzer et al., 2018). Myxozoans show a high degree of coevolution with their hosts. The highest degree of agreement is estimated in the phylogeny of Myxozoa and their invertebrate hosts (Holzer et al., 2018). A significant cophylogenetic signal was also found in the myxozoan phylogeny and phylogeny of their vertebrate hosts (Patra et al., 2018; Holzer et al., 2018).

Chapter II: Myxozoan diversity

To date, more than 2,600 species of myxozoans have been formally described, and this number contributes significantly to the biodiversity of cnidarians with 14,000 described species (Okamura et al., 2018). Myxozoans are divided into two groups: Myxosporea (species-rich group: approx. 2600 nominal species) and Malacosporea (species-poor group: 5 nominal species) (Bartošová-Sojková et al., 2014; Patra et al., 2017; Okamura et al., 2018). The different patterns of myxozoan diversifications in malacosporean and myxosporean groups may be caused by a strong link with the species diversity of the final hosts. On the one hand, Annelida, the final hosts of Myxosporea, are relatively species-rich (about 11 500 oligochaete and polychaete species; Ruppert et al., 2004). In contrast, bryozoans, the hosts of Malacosporea, are very species-poor (69 species; Massard and Geimer, 2008). Therefore, the parasite species diversity is most likely correlated with host species diversity (Holzer et al., 2018; Okamura et al., 2018).

It is expected that the true species richness of myxozoans is considerably underestimated (Hartikainen et al., 2016; Okamura et al., 2018). Eiras et al. (2011) estimated 8,000 myxozoan species in Brazilian freshwater fishes, while Naldoni et al. (2011) even estimated the existence of about 16,000 myxozoan species for all fish hosts in the Neotropical region, which is related to their marked host specificity and the high diversity of freshwater fishes in the Amazon. Applying this estimation pattern to the whole world, there could be up to 70,000 myxozoan species (34,800 fish species, Froese and Pauly, 2022) and only about 3.7% of myxozoan diversity is known. According to other statistical calculation, it is estimated that there are something between 7,171 and

9,818 marine myxozoan species and only about 17% of the marine species in this group have been described (Appeltans et al., 2012).

2.1 Malacosporean diversification

Malacosporeans are considered as a species-poor group. To date, five nominal species (*Buddenbrockia almani* Canning, Curry, Hill et Okamura, 2007*, B. plumatellae* Schröder, 1910, *B. bryzoides, Tetracapsuloides bryosalmonae, T. vermiformis*) of malacosporeans have been described (Patra et al., 2017) and assigned to two genera (*Buddenbrockia* Schröder, 1910 and *Tetracapsuloides* Canning, Tops, Curry, Wood et Okamura, 2002). In addition, 13 undescribed species have been identified from bryozoan and fish hosts mainly based on molecular data (Hartikainen et al., 2014; Bartošová-Sojková et al., 2014; Patra et al., 2017; Naldoni et al., 2019; 2021). Based on sequencing of 18 rDNA, Hartikainen et al. (2014) detected five new malacosporean lineages (species) from bryozoan hosts, and Bartošová et al. (2014) found hidden malacosporean diversity of five new lineages (species) in freshwater fish. Interestingly, Naldoni et al., (2021) studied a wide range of fishes from the Neotropical region (Amazonia) and found one new malacosporean lineage with a broad fish host range.

Kidneys and renal tubules are typical infection sites for malacosporeans. Malacosporeans have been found in fishes from orders Cypriniformes, Esociformes, Perciformes, and Salmoniformes (Bartošová-Sojková et al. 2014; Hartikainen et al., 2014; Naldoni et al., 2019). Moreover, Malacosporea sp. (= *Buddenbrockia* sp. E in Naldoni et al., 2021), detected by PCR, was found in a variety of bony fishes, but also freshwater ray (Naldoni et al., 2021). The definitive hosts for Malacosporea are known from four families of freshwater Bryozoa: Cristatellidae, Fredericellidae, Lophopodidae and Plumatellidae (Patra et al., 2017).

All described and undescribed species were found in freshwater ecosystems in Europe, North and South America (Bartošová-Sojková et al., 2014, Hartikainen et al., 2014, Naldoni et al., 2019; 2021) and in Borneo (Hartikainen et al., 2014). Interestingly, no malacosporean species have been captured in the marine environment, despite the high diversification of bryozoans in the oceans. Moreover, the evolutionary origin of these final hosts is in the marine environment (Holzer et al., 2018) and an old record of worm-like stages reminiscent of malacosporeans in the marine bryozoans indicate possible existence of malacosporeans in the marine ecosystem (Hastings, 1943). It is therefore assumed that marine malacosporean species exist, but are not yet scientifically known.

Malacosporean group is likely more diverse than is currently known, mainly due to poor screening of hosts in tropical areas and relatively hidden infection that is difficult to find with classical methodological approaches (Hartikainen et al., 2014, Naldoni et al., 2021). With the new molecular approaches and their results, it is obvious that the species diversity is underestimated and new species for science are still waiting to be recorded and described.

2.2 Myxosporean diversification

Myxosporeans are considered a species-rich group of myxozoans. Until today, more than 2,600 nominal species (Okamura et al., 2018) in 64 genera and 19 families have been described (Lom and Dyková, 2006; Bartošová et al., 2011; Yang et al., 2017; Freeman and Kristmundsson, 2015; 2018) from a wide range of intermediate and definitive hosts (Fiala et al., 2015a). Diversity research has mainly focused on myxosporeans in freshwater and economically important fish (e. g., Liu et al., 2019b; Capodifoglio et al., 2020). Appeltans et al. (2012) estimated that more than 700 species of myxosporeans from marine are known, with the rest of the described diversity known from freshwater ecosystems. Most myxosporean species have been described from *Cyprinus carpio* (85 species, Liu et al., 2019b).

2.2.1 Myxosporean diversification in fish

Most of the myxosporean species (more than 978) belong to the genus *Myxobolus*, which infect various organs and tissues (mainly gills, kidneys, and muscles) of fish (Eiras et al., 2005a; 2014; 2021). This genus has a cosmopolitan distribution typical of freshwaters but also occurs in marine (around 30 species, Lom and Dyková, 2006) and brackish ecosystems (e.g., Liu et al., 2019a). The genus *Myxobolus* comprises more than 35% of the known myxosporean diversity (Eiras et al., 2005a). *Myxobolus* has from sutural view oval, round or ellipsoid spores with two often pyriform polar capsules of equal or unequal size (Lom and Dyková, 2006). The species of this genus cluster in Oligochaeta-infecting myxozoans with paraphyletic character together with the genera *Henneguya* Thélohan, 1892, *Thelohanellus* Kudo, 1933, *Hennegoides* Lom, Tonguthai et Dyková, 1991 and *Unicauda* Davis, 1944. The myxospores of *Henneguya* with more than 200 nominal species (third most common genus; Eiras, 2002; Eiras and Adriano, 2012) and *Unicauda* are very similar to *Myxobolus* spores. They differ only in the presence/absence of two caudal appendages (one caudal appendage in the case of *Unicauda*). These appendages have few independent evolutionary origins (Liu et al., 2019a). *Myxobolus, Henneguya, Thelohanellus, Hennegoides* and *Unicauda* cluster

polyphyletically in one clade (Figure 4). The evolutionary scenario seems to be host-associated in all parts of the *Myxobolus* clade (Liu et al., 2019a).

The most common myxosporean genus in the oceans in fish is *Ceratomyxa* Thelohan, 1892 (approximately 270 nominal species, Eiras, 2006; Eiras et al., 2018), which accounts for more than 10% of the known myxosporean diversity and typically infects the gallbladder; a few species parasitize in other organs (including urinary bladder and renal tubules of kidneys; Eiras, 2006). Species of the genus cluster paraphyletically in the *Ceratomyxa* clade in polychaete-infecting myxozoans (e.g., Fiala et al., 2015c). *Ceratomyxa* spp. have elongated or banana-shaped like spores with two round polar capsules near the sutural line at the anterior pole of the spore (Lom and Dyková, 2006). *Ceratomyxa* spp. have been found worldwide not only in seawater (Eiras, 2006; Eiras et al., 2018) but also in freshwater fish in Amazonia (Eiras, 2006; Zatti et al., 2017) and brackish water (Eiras, 2006).

Chloromyxum Mingazzini, 1890 is the fourth largest myxosporean genus counting more than 150 nominal species (Eiras et al., 2012). The myxospores of this genus are typically bivalved, smooth or ribbed, and contain four pear-shaped polar capsules and a binucleate, rarely uninucleate, sporoplasm (Lom and Dyková, 2006). *Chloromyxum*, originally established based on morphological characteristics, is a polyphyletic genus with species grouped into independent clades in the myxosporean phylogeny (Fiala and Bartošová, 2010; Gleeson and Adlard, 2012; Cantatore et al., 2018). All chloromyxids of elasmobranchs, including the type species *Chloromyxum leydigi* Mingazzini, 1890, fall into the *Chloromyxum sensu stricto* clade, while species that parasitize freshwater fishes and amphibians are grouped as *Chloromyxum sensu lato* (Gleeson and Adlard, 2012; Cantatore et al., 2018) within the oligochaete-infecting freshwater lineage.

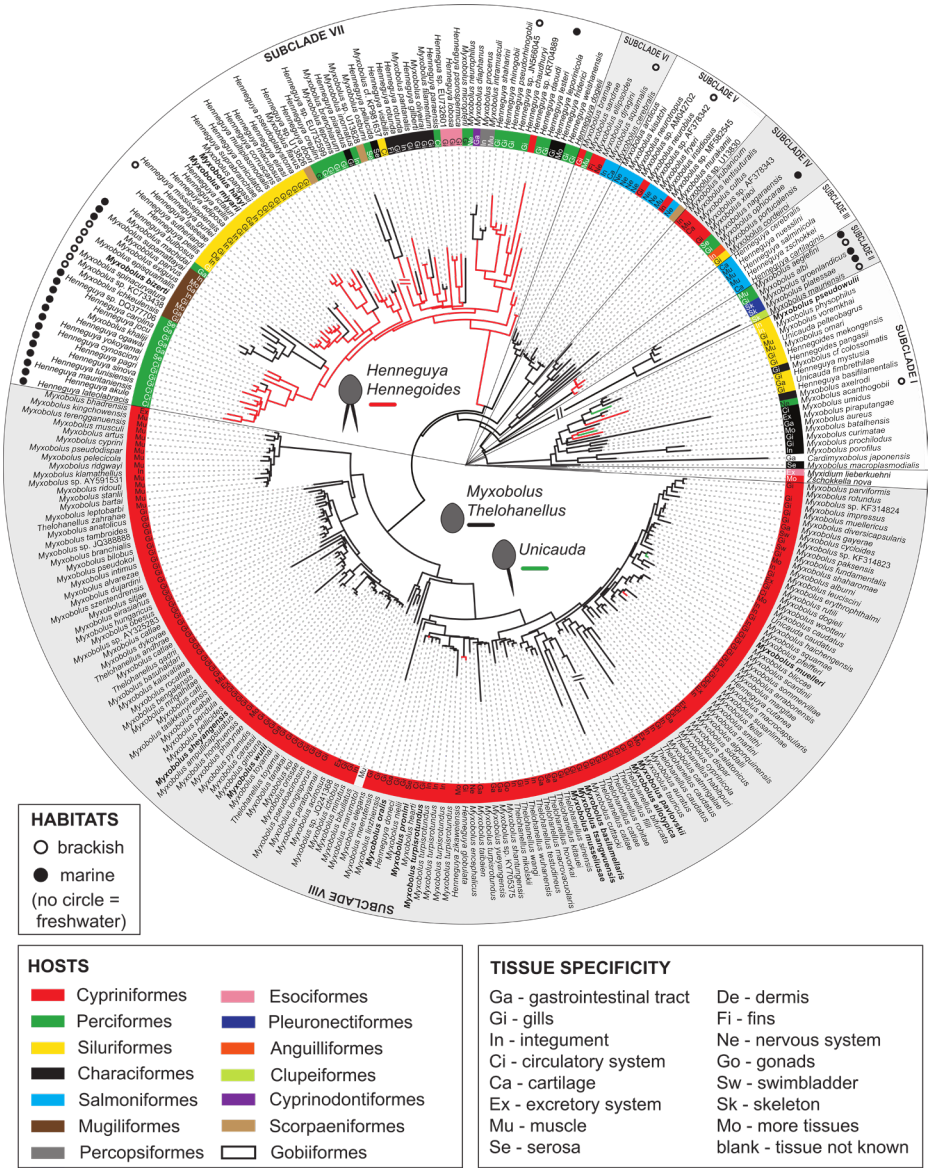

Figure 4: Maximum-likelihood phylogenetic tree of the Myxozoa (633 taxa), indicating different biological characteristics important for phylogenetic clustering: infection site in vertebrate host, aquatic habitat (freshwater vs. marine) as well as vertebrate and invertebrate host groups (Liu et al., 2019a).

Another common genus is *Sphaerospora* Thélohan, 1892. The polyphyletic genus *Sphaerospora* includes important fish pathogens and there is a clear phylogenetic trend toward clustering by organ specificity (e.g., Jirků et al., 2007). In total, this taxon has

103 described species (Patra, 2017) and they are mainly coelozoic species that parasitize in the kidneys of fish or amphibians (Lom and Dyková, 2006), apart from *S. molnari* Lom, Dyková, Pavlásková et Grupcheva, 1983 a histozoic species that parasitizes in gills (Lom et al., 1983).

Kudoa Meglitsch, 1947 is an economically important genus of myxosporeans with almost 100 nominal species that parasitise mainly in marine and estuarine fish (Eiras et al., 2014). This genus has atypical stellate, quadrate or rounded spores with four or more valves and pyriform polar capsules (Lom and Dyková, 2006) Some species can cause post-mortem liquefaction of the fish's muscle cells and lower the price of the fish, which has economic implications for the aquaculture industry (reviewed in Moran et al., 1999).

2.2.2 Diversification in other vertebrates

Myxosporeans are primarily typical fish parasites, but a few species parasite also in other vertebrate host groups: i) sharks, rays, chimaeras, ii) mammals, iii) birds, iv) amphibians and v) reptiles and vi) agnaths (e. g., Prunescu et al., 2007; Bartholomew et al., 2008; Roberts et al., 2008; Hartigan et al., 2013; Kodádková et al., 2015; Cantatore et al., 2018) (details in Figure 5).

i) To date, 53 myxosporean species have been described in about 110 species of chondrichthyan hosts (sharks, rays: elasmobranchs; chimaeras) from the genera *Bipteria* Kovaleva, Zubchenko et Krasin, 1983, *Ceratomyxa*, *Ellipsomyxa* Køie, 2003, *Chloromyxum*, *Kudoa*, *Myxidium* Bütschli, 1882, *Parvicapsula* Shulman, 1953, *Sinuolinea* Davis, 1917, and *Sphaerospora* Thelohan, 1892 (summarised in Paper III: Lisnerová et al., 2020b; Paper V: Lisnerová et al., 2022). Myxozoans found in chimaeras, sharks and rays parasite mainly in gall bladders (*Bipteria*, *Ceratomyxa*, *Ellipsomyxa*, *Chloromyxum*, *Myxidium*), kidneys (*Parvicapsula*, *Sinuolinea*, *Sphaerospora*) and less frequently in muscles (*Kudoa*). The most common genus in elasmobranchs (sharks and rays) is *Chloromyxum*, which has been described from hosts mainly in the Atlantic Ocean, Australian, and Tasmanian areas (Paper III: Lisnerová et al., 2020b, Paper VI; Lisnerová et al., submitted). The second most common genus in the sharks, rays and chimaeras is *Ceratomyxa* with 19 species described mainly from the group Carcharhiniformes from Australian waters (summarised in Paper III: Lisnerová et al., 2020b; Paper V: Lisnerová et al., 2022). Only two species are known in chimaeras, namely *Bipteria vetusta* Kodádková, Bartošová-Sojková, Holzer and Fiala, 2015 found in *Chimaera monstrosa* Linnaeus, 1758 (Kodádková et al., 2015) and *Ceratomyxa fisheri* Jameson, 1929 found in *Hydrolagus colliei* (Lay and Bennett, 1839) (Jameson, 1929).

ii) Only a few terrestrial species have been found as parasites of mammals. Two nominal species of the genus *Soricimyxum* Prunescu, Prunescu, Pucek et Lom, 2007 (*S. fegati* Prunescu, Prunescu, Pucek et Lom, 2007; *S. minuti* Székely, Cech, Atkinson, Molnár, Egyed et Gubányi, 2015) have been described from terrestrial shrews (Soricidae Fischer, 1814) in Central Europe (Prunescu et al., 2007; Székely et al., 2015). The spores are typical of ovoid shape with two polar capsules situated at opposite ends of the spore (Prunescu et al., 2007). There are also a few records of myxozoan spores from the human body, but none were viable infections with some development. These spores were identified in the faeces of patients with intestinal symptoms such as nausea or diarrhoea. The spores present were not associated with the symptoms, but with the consumption of fish (McClelland et al., 1997; Boreham et al., 1998; Lebbad and Willcox, 1998). The most recent detection in Japan is related to the occurrence of *Kudoa septempunctata* Matsukane, Sato, Tanaka, Kamata et Sugita-Konishi, 2010 as a causative agent of food poisoning by fish meat of *Paralichthys olivaceus* (Temminck & Schlegel, 1846) with more than 100 cases per year (Iijima et al., 2012). Moreover, allergic reactions are associated with the consumption of fish infected by *Kudoa* species (e. g., Martínez de Velasco et al., 2008). Overall, however, there is no known complex life cycle of myxosporeans involving humans as hosts.

iii) *Myxidium anatidum* Bartholomew, Atkinson, Hallett, Lowenstine, Garner, Gardiner, Rideout, Keel et Brown, 2008 is the first and single record of a myxozoan parasite infecting birds (specifically bile ducts in the liver of Pekin duck, *Anas platyrhynchos* L.) caught in different areas of the USA (Bartholomew et al., 2008), however, there are no records of this species or other myxozoans in birds from any other areas.

iv) Myxosporean infections have been reported also in amphibians worldwide (Eiras, 2005) in the orders Anura Merrem, 1820 (23 species infecting frogs and toads) and Caudata Scopoli, 1777 (four species infecting salamanders and newts) involving species of *Caudomyxum* Bauer, 1948, *Cystodiscus* Lutz, 1889, *Chloromyxum*, *Hoferellus*, *Myxobolus*, and *Sphaerospora* (Eiras, 2005; Hartigan et al., 2013; Patra, 2017; Vieira et al., 2021). The genus *Cystodiscus* exclusively contains parasites of amphibian hosts with 10 described species (Vieira et al., 2021). The genus was established mainly based on phylogenetic data rather than the usual morphological characters with ovoid spores and two polar capsules (Lom and Dyková, 2006).

v) All myxosporean infections reported in reptiles included the *Myxidium* spp. and all of these reports have occurred in turtles (e.g., Eiras et al., 2011; Aguilar et al., 2017).

vi) Agnaths represent an evolutionarily important group of hosts for many parasites (Holzer et al., 2018), but they are only marginally studied as potential hosts for myxozoans. There is only one record of the unspecified myxosporean species described from lamprey (Mori et al., 2000), although the finding of parasitic stages in the kidneys of the lamprey *Lethenteron camtschaticum* (Tilesius, 1811) is quite strikingly reminiscent of the developmental stages of malacosporeans (e.g., Canning et al., 2007). So far, three species of myxosporeans have been found in gall bladders of hagfish, all from the genus *Ceratomyxa* (Jameson 1929, Kondo and Yasumoto, 2020).

Myxosporeans seem to be more diverse than previously noted (Hartikainen et al., 2016; Patra et al., 2018). Even in areas where research on myxosporeans diversity has a long tradition, new species for science are very often found (e.g., Patra et al., 2018). The main reasons for the diversity underestimation are poor screening of vertebrate hosts and unavailability of hosts in hard-to-reach areas such as the tropics and artics. Furthermore, myxosporeans often manifest relatively hidden infections that are difficult to reveal using classical methodological approaches. With new molecular approaches (eDNA metabarcoding), it appears that species diversity is greatly underestimated and new species for science are still waiting to be found and formally described (Hartikainen et al., 2016).

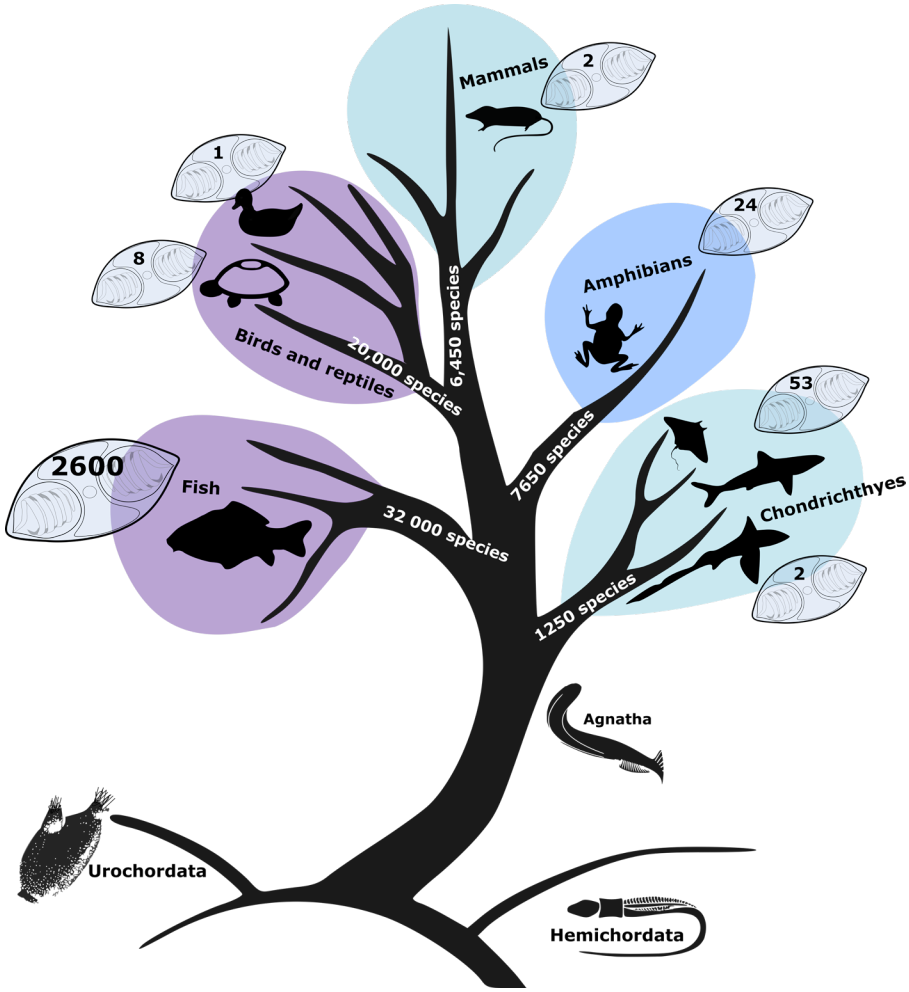

Figure 5: Illustrative tree of main vertebrate groups with the number of known potential host species (numbers in the tree branches) and with the number of described myxozoan species from individual host groups (numbers in illustrative myxozoan spores).

2.2.3 Diversification in invertebrates

Invertebrates are the final hosts of myxosporeans, in which sexual reproduction takes place and actinospores evolved. Invertebrates were the first hosts of these parasites in their evolution, initially infecting primitive polychaetes (Haplodrili, Archiannelida). The complex life cycle of myxosporeans evolved as a result of vertebrate host incorporation into the simple life cycle including only aquatic invertebrates (Holzer et al., 2018).

Historically, actinosporean stages were described separately as a different group of organisms called Actinosporea. New insights into the myxozoan life cycle (Wolf and Markiw, 1984) resulted in the demise of this class (Kent et al., 1994). More than 200 actinosporean records have been currently comprised within 21 collective groups (Lom and Dyková, 2006; Rocha et al., 2019), however, this number seems low and should be increased given the discrepancy with much larger number of known myxospores and myxosporeans (2,600 species; Okamura et al., 2018). Today, only around 50 complex life cycles with two hosts are described with 25 species of polychaetes and oligochaetes as definitive hosts with the most common host *Tubifex tubifex* (Alexander et al., 2015). Other groups of invertebrates (whether as intermediate or final hosts) have also been found as hosts for myxosporeans, including Monogenea (Aguilar et al., 2004), Cephalopoda (Yokoyama and Masuda, 2001), Trematoda (Overstreet, 1976), or Sipunculida (Ikeda, 1912).

2.3 Methods for identification of myxozoan diversity

Research on myxozoan biodiversity is largely focused on the screening of fish hosts, for which endoparasite infection has been reported worldwide (e.g., Kodádková et al., 2014; Bartošová-Sojková et al., 2018: Paper I; Patra et al., 2018). The usual methodology for determining myxozoan diversity is to capture and dissect host species (mainly fish), light microscopical investigation of host tissues and organs, myxozoan identification and documentation. This methodological strategy that leads to the taxonomic characterisation of individual myxosporeans, can present many pitfalls, especially for the following reasons: (i) availability of intermediate fish hosts - demanding trapping techniques; less common host species; endangered host species; unusual or unexpected host groups (ii) frequent low prevalence; (iii) life cycle and seasonality (fish parasites may not be present in fish when examined); (iv) limited number of fish samples (prevalence of Myxozoa may be low and only detectable when small sizes of fish are examined) (v) hidden infections.

The alternative method, that can overcome most of the problems associated with the host availability, is the use of environmental DNA (eDNA) sequencing. In recent years, the use of eDNA has been shown to effectively determine microbial, protist, fungal, and eukaryotic diversity in many areas of biological and ecological research (Bass et al., 2015; Hartikainen et al., 2016; Boussarie et al., 2018). In addition, eDNA has also been successfully used to detect low-density populations in freshwater environments (Balasingham et al., 2018; Furlan et al., 2019; Priestley et al., 2021). Myxozoan parasites are transmitted between intermediate and definitive hosts by mature spores, which are released into the environment (water, sediment) (Køie et al., 2007; Caffara et al., 2009).

Therefore, obtaining and screening eDNA by next-generation sequencing from water filtrates or sediment samples containing myxozoan spores is feasible to discover the diversity of the group under study (Hartikainen et al., 2016; Richey et al., 2020; Marshall et al., 2022), although the spore density contained in the environment is unknown.

Next generation sequencing (NGS) enables the detection of species diversity based on molecular data on a large scale. The increasing availability of myxozoan molecular data (especially SSU rDNA) can serve as a measure of known diversity and as a framework into which novel sequence types can be placed in the absence of morphological data. Using NGS, Hartikainen et al., (2016) discovered great myxosporean diversity, particularly in a relatively large clade called the environmental clade. Most sequences falling into this clade were not assigned to intermediate hosts, let alone morphotypes. Furthermore, the authors discovered only 7% of the myxozoan operational taxonomic units (OTUs) that match previously known sequences. This is a unique and, to date, only study focusing on large-scale eDNA metabarcoding of myxozoans. Myxozoan species or OTUs are rarely detected in large-scale eDNA studies that are generally interested in eukaryotic diversity using universal SSU rDNA primers (e.g., Ortiz-Alvarez et al., 2018; Briscoe et al., 2022).

Hartikainen et al. (2016) used in their study water filtration of large volumes (more than 100 litres) as a source of myxozoan DNA. However, in the freshwater eutrophic ecosystems in Central Europe (e.g., ponds with a large number of algae), the filtration of large quantities of water is not possible because the filters become clogged after filtration of smaller quantities. Furthermore, the eDNA contains a mixture of DNA from different groups of organisms and PCR requires specific primers to amplify target organisms and does not allow amplification of different taxonomic groups (e.g., algae, ciliates, diatoms).

Expanding knowledge about the biodiversity of myxozoans can help us to find answers to questions about their evolutionary trajectories, the parasite-host spectrum and parasite adaptations, their prevalence in fish, and parasitism in general. Detection of biodiversity can help not only to detect hidden species but also help to monitor the expansion of these parasites along with invasive fish species.

Chapter III: Aims of the study

The aims of this Ph. D. project were:

1. To elucidate myxozoan species diversity (including morphological descriptions and phylogenetic analyses) in selected localities by host screening.

2. To optimize the methodology of myxozoan environmental screening: i) find the most efficient method of spore capturing from the water environment (free water and sediment) in freshwater ecosystems in Central Europe, ii) determine the optimal method of DNA extraction from environmental samples containing spores of Myxozoa, iii) optimize PCR reaction.

3. Using amplicon sequencing, to evaluate the biodiversity of Myxozoa in selected localities from water and sediment samples and compare it with the diversity found by classical examination of fish hosts.

Chapter IV: Summary of the PhD. thesis

The main highlights of the present thesis:

I. Revealing the hidden species diversity and host range, cryptic characteristics, and phylogenetic relationships of specific groups/species of the highly diverse *Ceratomyxa* clade, *Kudoa thyrsites* and *Myxobolus pseudodispar* complex at selected localities (Papers I, II, IV).

II. Conducting a comprehensive search for myxozoans in evolutionarily ancient fishes (sharks, rays, and chimaeras) with cophylogenetic, phylogenetic, and phylogeographic analyses (Papers III, V, VI).

III. Study of the evolution of spore size within three phylogenetic groups, *Ceratomyxa, Sphaerospora sensu stricto* and *Parvicapsula*, correlating spore size with phylogeny of parasite and size of host (Paper V).

IV. Introduction of the new methodological approach to study myxozoan diversity in sediments with group-specific primers (Paper VII).

V. First preliminary data comparing myxozoan diversity in fish and in the environment using the eDNA approach (Paper VII).

4.1. Hidden diversity of myxosporeans in fish

The diversity of myxozoans seems to be greatly underestimated even in fish hosts and places where the study of this parasite group has a long tradition (Hartikainen et al., 2016; Paper II: Lisnerová et al., 2020a). Europe is a region where myxozoan diversity has been studied not only by classical microscopy but also by molecular approaches (e.g., Fiala, 2006; Bartošová et al., 2014; Patra et al., 2018). New species for science have been

found even in commonly studied hosts such as *Cyprinus carpio* L., *Rutilus rutilus* (L.) or *Phoxinus phoxinus* L. (Batueva, 2020; Paper II: Lisnerová et al., 2020a; Chen et al., 2021). Moreover, large undiscovered diversity still lies in rare and endangered host species and discrete localities (Reed et al., 2007; Paper I: Bartošová-Sojková et al., 2018).

We found a high species diversity represented by i) **seven *Ceratomyxa* species, including the previously described *Ceratomyxa dehoopi* Reed, Basson, Van As et Dyková, 2008 and *C. cottoidii* Reed, Basson, Van As et Dyková, 2008 in clinids in South Africa** (Paper I: Bartošová-Sojková et al., 2018) and by ii) **at least 10 new well-recognised clades in the complex of the species *Myxobolus pseudodispar* Gorbunova, 1936 in Europe** (Paper II: Lisnerová et al., 2020a). *Ceratomyxa* species caught in clinids in South Africa are a diverse group of closely related species that have evolved rapidly. They have high prevalence, low host specificity, and high levels of co-infection in their hosts. Developmental stages of the genus *Ceratomyxa* were found in 40% of the clinids examined, mainly in gall bladders. *C. cottoidii* was the most common ceratomyxid parasite in clinids (Paper I: Bartošová-Sojková et al., 2018). Further, in our comprehensive screening of various freshwater fish species (over 450 specimens, 36 species) from the Czech Republic and Bulgaria, we found that the well-known *Myxobolus pseudodispar* has cryptic species and paraphyletic character, with a clear phylogenetic grouping (13 well-recognised clades) according to the fish host. At least ten new species of *Myxobolus* can be recognised based on molecular data in the *M. pseudodispar* sequence group (Paper II: Lisnerová et al., 2020a). We also focused on the myxozoan parasite iii) ***Kudoa thyrsites***, which is an economically important parasite with a wide range of marine intermediate hosts, including commercial tunas, mackerels, salmonids, and flatfishes. **Two new hosts, *Micromesistius poutassou* (A. Risso, 1827) and *Scomber colias* Gmelin, 1789, were found for the studied species, which was assigned to the eastern Atlantic *K. thyrsites* genotype** (Paper IV: Cavairo et al., 2021).

4.2 Myxozoans in evolutionarily ancient fishes (sharks, rays and chimaeras)

The known myxozoan species diversity in ancestral elasmobranch hosts represents only 2% of the total myxozoan species diversity (Holzer et al., 2018; Paper III: Lisnerová et al., 2020b; Paper V: Lisnerová et al., 2022; Paper VI: Lisnerová et al., submitted). We screened different orders of elasmobranch hosts obtained around the world (mainly the coast of Argentina and the coast of South Africa) and the species of the genera *Ceratomyxa, Chloromyxum, Kudoa, Ortholinea* Shulman, 1962*, Parvicapsula* and

Sphaerospora were observed (Paper III: Lisnerová et al., 2020b; Paper V: Lisnerová et al., 2022; Paper VI: Lisnerová et al., submitted). Based on myxozoan phylogeny, the most basal clades in polychaete-infecting myxozans and oligochaete-infecting myxozoans parasite in sharks, rays and chimaeras (Kodádková et al., 2015; Cantatore et al., 2018). For this reason, they play a key role in the evolution of myxozoans.

We more than doubled the existing SSU rDNA sequences and provided 11 formal descriptions of myxozoans from sharks and rays. We determined three new lineages of myxozoans in Chondrichthyes (*Ortholinea*, *Parvicapsula* and *Sphaerospora*) (Paper III: Lisnerová et al., 2020b; Paper V: Lisnerová et al., 2022; Paper VI: Lisnerová et al., submitted). Obtained species create clearly defined elasmobranch-associated groups at the base or within the teleost-infecting myxozoan clades similarly as reported previously (Fiala and Dyková, 2004; Fiala, 2006; Azevedo et al., 2009; Gleesen et al., 2010; Gleeson and Adlard, 2011; 2012). On the one hand, newly found *Parvicapsula* sp. clusters in the basal position, on the other hand, *Ortholinea* sp. cluster within teleost-infecting group. Surprisingly, sphaerosporids from sharks do not occupy the most basal position in the *Sphaerospora sensu stricto* clade, however, they fall basal to species infecting frogs indicating that they probably settled higher vertebrates by host switch from Chondrichthyes (Paper III: Lisnerová et al., 2020b; Paper V: Lisnerová et al., 2022). Last but not least, our new data influenced the phylogenetic tree of the Myxozoa and thus changed our view on the myxozoan evolution. For example, a large number of newly provided evolutionary distinct sequences of *Chloromyxum* clustering in basal positions changed the relationship between the major clades of the myxozoan tree (Paper III: Lisnerová et al., 2020b; Paper V: Lisnerová et al., 2022).

In the Paper III (Lisnerová et al., 2020b) and Paper VI (Lisnerová et al., submitted), we demonstrated, that the phylogeny of *Chloromyxum sensu stricto*, the most diverse myxozoan clade from elasmobranchs, correlates with the phylogeny of its hosts (Holzer et al., 2018; Patra et al., 2018). The ancestral state reconstruction of geographic origin in this largest monophyletic dataset in elasmobranchs did not demonstrate strict patterns (Paper III: Lisnerová et al., 2020b; Paper VI: Lisnerová et al., submitted), in other words, it seems that the phylogeny of elasmobranch-infecting myxozoans is not affected by host geographical origin but is defined by other ecological or biological conditions (Paper III: Lisnerová et al., 2020b; Paper VI: Lisnerová et al., submitted). However, for stronger conclusions, we need records mainly from areas of the Indian and Pacific Oceans.

4.3 The evolution of spore size within *Ceratomyxa*, *Sphaerospora sensu stricto* and *Parvicapsula* clades

The morphological aspects of myxozoan developmental stages linked with phylogeny are poorly studied. We found that the elasmobranch-infecting myxozoans from *Ceratomyxa*, *Parvicapsula* and *Sphaerospora* clades were overall larger in myxospore size than their teleost-infecting relatives and this difference was highly significant for the *Ceratomyxa* species (Paper V: Lisnerová et al., 2022).

Investigation of phylogenetic signal expressed by the lambda method in myxospore size (myxospore section area) differences determined that the spore size is significantly influenced by phylogenetic position in all studied groups. Further, we focused on size of spores deeper in the most diverse *Ceratomyxa* group (data for other groups are limited) and we found that mature spores of *Ceratomyxa* spp. from larger hosts tended to be significantly larger in size as revealed by a strong positive correlation of myxospore size and body length of both elasmobranchs and teleost fishes. The positive correlation of larger-bodied parasites in larger hosts, named Harrison's rule (Harrison, 1915), can be associated with more efficient usage of available niche space by parasite individuals in larger hosts (Sasal et al., 1999). Furthermore, the correlation between the depth where hosts live and size of ceratomyxids is also strongly positively correlated (Paper V: Lisnerová et al., 2022). The dominance of myxospores with large size and their host in the deep sea has been found with clearly defined bigger-deeper phenomenon which was also found in various groups of marine organisms such as crustaceans (Ardizzone et al.,1990).

4.4 The methodological approach to study myxozoan diversity in sediment

The metabarcoding analyses of the environmental DNA have been used for the identification of biodiversity (Bass et al., 2015; Hartikainen et al., 2016) or detection of rare (Furlan et al., 2019) and endangered animal populations (Ikeda, 2016) in many ecological and phylogenetic studies. It has been proven that the eDNA methodology for myxozoan diversity assessment by next-generation sequencing of samples from water filtrates or sediment samples containing myxozoan spores is feasible (Hartikainen et al., 2016; Richey et al., 2020; Marshall et al., 2022). However, the eDNA approach is not an easy task with many obstacles during sampling, laboratory processing and bioinformatic processing (Alama-Bermejo and Holzer, 2021).

We proposed a new methodological approach for studying myxozoan diversity based on environmental DNA from sediments in freshwater eutrophic ecosystems using amplicon sequencing which provides a new view on the identification of myxozoan diversity. We found Illumina metabarcoding with newly designed primer sets for the V4 variable region of approximately 350 bp long region of SSU rDNA gene suitable for analyzing myxozoan communities and hidden diversity in freshwater ecosystems in Central Europe. We tested our methodology on the sediment samples from the river, pond, dam and brook. Our metabarcoding approach with newly designed primer sets is suitable for non-invasive analyzing of freshwater myxozoan communities without the need of obtaining, killing and dissection of intermediate or final hosts and for detection of myxozoan parasites in habitats with a large number of different eukaryotic organisms (Paper VII: Lisnerová et al., in advanced preparation).

4.5 Comparison of myxozoan diversity in fish and in the environment using the eDNA approach: a pilot study

We demonstrated the application of our methodology on the samples of sediment from four different ecosystems: river, pond, dam and brook. We compared our results obtained from sediment samples with data obtained from fish from the same locality. Our pilot eDNA study based on small volumes of sediment samples successfully determined a relatively high number of myxozoan OTUs. In the case of Švihov reservoir and Rájský pond, we detected 14 myxozoan OTUs in each locality and Hostačovka brook we determined 7 OTUs. In addition, single sample from the Douro river (Porto, Portugal) revealed 17 myxobolid OTUs. Although it was considerable smaller number of detected OTUs in the environment than detected by metabarcoding in fish tissues in the case of the Czech localities, we proved eDNA as a very promising methodology for assessing myxozoan biodiversity (Paper VII: Lisnerová et al., in advanced preparation).

For overall detection of myxozoan diversity in selected localities, it is necessary to sample more sediment samples than we showed in the pilot study. The amount of sediment to be sampled to determine myxosporean diversity should correlate with the type and the size of the habitat and the size of the fish spectrum.

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Chapter V: The identification of myxozoan diversity in fish in selected localities

5.1 Paper I

Life in a rock pool: Radiation and population genetics of myxozoan parasites in hosts inhabiting restricted spaces

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Life in a rock pool: Radiation and population genetics of myxozoan parasites in hosts inhabiting restricted spaces

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Abstract

Introduction

Intertidal rock pools where fish and invertebrates are in constant close contact due to limited space and water level fluctuations represent ideal conditions to promote life cycles in parasites using these two alternate hosts and to study speciation processes that could contribute to understanding the roles of parasitic species in such ecosystems.

Material and methods

Gall bladder and liver samples from five clinid fish species (Blenniiformes: Clinidae) were morphologically and molecularly examined to determine the diversity, prevalence, distribution and host specificity of *Ceratomyxa* parasites (Cnidaria: Myxozoa) in intertidal habitats along the coast of South Africa. Phylogenetic relationships of clinid ceratomyxids based on the SSU rDNA, LSU rDNA and ITS regions were assessed additionally to the investigation of population genetic structure of *Ceratomyxa cottoidii* and subsequent comparison with the data known from type fish host *Clinus cottoides*.

Results and discussion

Seven *Ceratomyxa* species including previously described *Ceratomyxa dehoopi* and *C. cottoidii* were recognized in clinids. They represent a diverse group of rapidly evolving,

closely related species with a remarkably high prevalence in their hosts, little host specificity and frequent concurrent infections, most probably as a result of parasite radiation after multiple speciation events triggered by limited host dispersal within restricted spaces. *C. cottoidii* represents the most common clinid parasite with a population structure characterized by young expanding populations in the south west and south east coast and by older populations in equilibrium on the west coast of its distribution. Parasite and fish host population structures show overlapping patterns and are very likely affected by similar oceanographic barriers possibly due to reduced host dispersal enhancing parasite community differentiation. While fish host specificity had little impact on parasite population structure, the habitat preference of the alternate invertebrate host as well as tidal water exchange may be additional crucial variables affecting the dispersal and associated population structure of *C. cottoidii*.

Introduction

Rock pools are unique and complex habitats in intertidal areas. They harbor large numbers of fishes and invertebrates that are well adapted to life in harsh conditions of strong fluctuations in water temperature, oxygen levels and salinity [1]. Fishes of the family Clinidae (Blenniiformes: Blennioidei), locally known as klipfish, are well adapted to such turbulent and unstable conditions [2, 3]. They are prominent inhabitants of these intertidal and shallow subtidal habitats and represent up to 98% of the total number of fish species in rock pool communities of South Africa [2, 3]. Distribution and genetic structure of South African clinids are strongly affected by oceanic circulation, paleoclimate changes, contemporary environmental variables, specific biological features and habitat preferences [4–6]. Significant biogeographic boundaries that affect South African marine fauna are created by two very distinct ocean currents that flank the west and south coasts. The Benguela Current, on the west coast, brings cold nutrient-rich water to the surface as it moves northwards along the west coast of the country. The Agulhas Current carries warm, nutrient-poor surface waters southwards along the east and south coasts of the country. A biogeographical barrier is created over the Agulhas Bank where these two currents meet (Fig 1) [4]. The latest glaciation events also affected the distribution of intertidal marine organisms in South Africa as a result of changes in sea levels and associated availability of rocky shore habitats [4–7].

The South African clinid genera *Clinus*, *Muraenoclinus*, *Blennophis*, and *Pavoclinus* as well as their sister-related Australian genera *Heteroclinus* and *Cristiceps* [8] are viviparous species giving birth to well-developed postflexion larvae [9]. This is in contrast to North and South American clinids which are oviparous [9]. Thus, the potential for free dispersal in South African live-bearing clinid fishes is highly reduced

[10], creating more pronounced population genetic structures [6, 11, 12]. *Clinus cottoides* Valenciennes, 1836 (bluntnose klipfish) is a viviparous fish species with restricted dispersal capacity [10], which is endemic to South Africa and has a clear population structure with discontinuities along the oceanographic barriers that influence gene flow patterns. The first main genetic break lies on the southern South African coast (Gaansbai–Cape Agulhas; HB2 in Fig 1) while the second break is located between the east coast and other locations on the south (Knysna–Port Alfred; HB3 in Fig 1) [11]. Studies have also revealed additional pronounced genetic differentiation of *C. cottoides* populations between Jacobs Bay and Sea Point (HB1 in Fig 1) [12]. Similar isolation of populations on the east coast has been documented for the super klipfish *Clinus superciliosus* (L.) whereas a recent colonization event of the eastern range distribution has been suggested for the nosestripe klipfish *Muraenoclinus dorsalis* (Bleeker, 1860) [6, 12]. Both of these fish species represent species complexes showing evidence of cryptic speciation [6, 13].

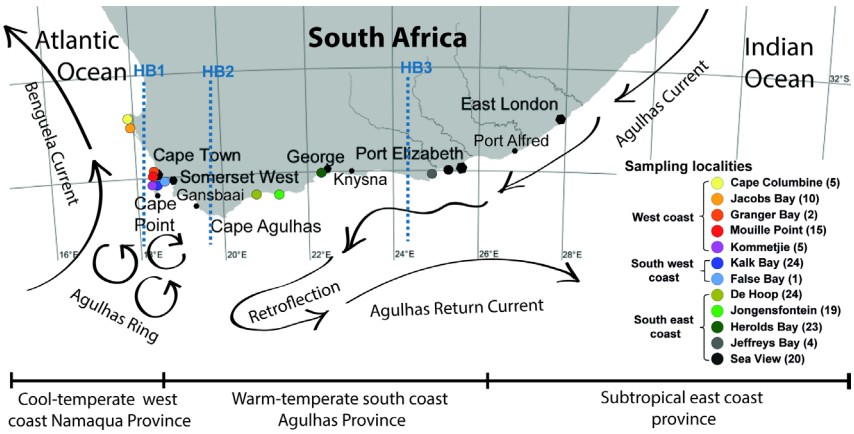

Fig 1. Map showing the sampling area of clinid fish in this study.

The twelve sampling localities, grouped into three geographical regions, are labeled by round colored circles. The number of samples obtained at each locality is shown in parentheses. Moreover, the major oceanographic features around the South African coastline, the Benguela and Agulhas Currents, that influence population structuring of *Clinus cottoides* are shown along with the barriers to gene flow (blue vertical lines) as HB1: Jacobs Bay–Sea Point (at Cape Town), HB2: Gaansbai–Cape Agulhas, HB3: Knysna–Port Alfred [11, 12]. Agulhas ring eddies and the Agulhas counter-current are also highlighted.

Rock pools are important habitats in intertidal environments as they support a diverse assemblage of organisms [14, 15]. Moreover, these habitats represent ideal conditions for parasite life cycles as, due to the limited space, different host groups are in constant close contact [16]. The Myxozoa, a group of microscopic cnidarian parasites alternating

mainly between the fish intermediate and annelid definitive hosts, are very common in marine habitats [17]. Representatives of five myxozoan genera *Henneguya*, *Ceratomyxa*, *Myxidium*, *Ortholinea* and *Sphaeromyxa* have so far been described from South African clinids [18–23]. *Ceratomyxa* is a species-rich genus characterized by crescent shaped spores with two polar capsules. Individual species mostly infect gall bladders and bile ducts of marine fishes and generally differ by spore measurements, host preference and SSU rRNA gene sequences [17, 24]. Ceratomyxids are highly host specific [25–30] and very few taxa infect more than one host species [25, 30]. Therefore, host as an informative diagnostic character has become particularly valuable for morphologically and genetically closely related species of *Ceratomyxa* [25–30]. Even though ceratomyxids in general cause little harm to their hosts, some species have been documented to induce severe histopathological damage [31–33] and obstruction and distension of bile ducts due to the presence of masses of spores and plasmodial stages [20]. The ceratomyxid life cycle has so far been elucidated only for *Ceratomyxa auerbachii* Kabata, 1962 which alternates between a clupeid fish and a marine polychaete [34]. Though globally distributed, the knowledge of *Ceratomyxa* species diversity in South African clinids is very limited. *Ceratomyxa obovalis* (Fantham, 1930) was found to parasitize blennioids *C. cottoides*, *C. superciliosus*, bull klipfish *Clinus taurus* Gilchrist & Thompson, 1908 and horned blenny *Parablennius cornutus* (L.) from False Bay and Kalk Bay [18, 19]. *Ceratomyxa cottoidii* Reed, Basson, Van As et Dyková, 2007 and *Ceratomyxa dehoopi* Reed, Basson, Van As et Dyková, 2007 were described from *C. cottoides* and *C. superciliosus*, respectively, from De Hoop [20], one of South Africa's largest marine reserves. This area is located just east of Cape Agulhas, a dominant genetic break for marine intertidal organisms [6, 11, 35, 36]. A further undescribed *Ceratomyxa* species, morphologically similar to *C. cottoidii*, was reported from *M. dorsalis* from Kommetjie, Granger Bay and Jacobs Bay [37]. Interestingly, this species [37] and other ceratomyxids [26, 31, 38] display a high degree of morphological spore plasticity. This raises questions whether the observed variation is determined by deformations of their presumably thin-walled shell valves, a feature previously documented for some ceratomyxids [39], or alternatively is due to the concurrent infections of several *Ceratomyxa* species of different morphology, as mixed myxozoan infections [17], including those of *Ceratomyxa* spp. [40], are a common phenomenon.

Myxozoans are generally recognized as parasites with an accelerated rate of molecular evolution [41, 42] explained by an extraordinary level of radiation [43, 44]. Such rapid molecular evolution and associated long-branch attraction (LBA) can greatly obscure the results of phylogenetic studies of the Myxozoa [41]. Substitution rates significantly differ among myxozoan lineages. For example, the *Ceratomyxa* clade, placed within the marine myxosporean lineage [45, 46] includes several long-branching taxa sometimes

clustering together, most probably due to LBA [46]. *Ceratomyxa* spp. from the same fish host family (and sometimes from the same geographic location) tend to cluster together, however, this is not always the rule [27, 29].

Though population structure of both, host and parasite, is most relevant to co-evolutionary processes [47–49] and myxozoan evolutionary history has been significantly linked to host-parasite co-evolution [50, 51], unfortunately, to date, investigations determining the population structure of myxozoan parasites in relation to that of their hosts are still missing. For this purpose, we selected the host-parasite model system of *Clinus cottoides*, rendering the detailed knowledge of its biology, distributional range, and population structure [10–12], and *Ceratomyxa cottoidii* for which information on its fish host species and distribution was collected during the previous [20] and these studies. Moreover, an ecological system with geographically limited host dispersion as seen in *C. cottoides* is ideal for study.

Our aims were to i) examine diversity, prevalence, distribution and host species spectra of ceratomyxids from South African clinids, ii) reconstruct the phylogenetic relationships among clinid ceratomyxids and to other members of the *Ceratomyxa* clade, and iii) reveal the patterns of population structuring of *C. cottoidii* and to investigate if they are linked to geographical or host-driven isolation of parasite populations. Based on previous knowledge, we hypothesized that ceratomyxids species in South African clinids are strictly host-specific, closely related and occur in mixed infections. Finally, we hypothesized that *C. cottoidii* as a specialist parasite follows the population structure of its fish host *C. cottoides*.

Material and methods

Sample collection, study area and time schedule

In total, 143 fish specimens of the family Clinidae (Perciformes) belonging to *Clinus acuminatus* (Bloch & Schneider), *Clinus brevicristatus* Gilchrist et Thompson, *C. cottoides*, *C. superciliosus* and *M. dorsalis* were collected from intertidal rock pools using small hand nets, at several localities in South Africa in March–April 2008, November 2009 and October–November 2012 (Fig 1, S1 Table). Fish were captured and treated under the terms and conditions of the MCM permit number RES2008/15, 2008/V8/LT and 2009/V1/LT of the Science Faculty Animal Ethics Committee (SFAEC) of University of Cape town (UCT) that approved this study. Captured fish were identified using Branch et al., [52].

Processing of samples

Fish livers and gall bladders (in total 152 samples; S1 Table) were examined directly in the field or fixed in 70% ethanol and examined subsequently in the laboratory for the presence of myxosporean infections by light microscopy (LM) on Leica DM750 and Olympus BX51 microscopes. Plasmodia and myxospore morphology were documented with an Olympus DP70 digital camera using differential interference contrast. Samples were stored in 400 µL of TNES urea buffer (10 mM Tris-HCl with pH 8, 125 mM NaCl, 10 mM EDTA, 0.5% SDS and 4 M urea) or in 90% ethanol for subsequent DNA extraction.

DNA extraction, PCR amplification, cloning and sequencing

Total DNA was extracted from all collected samples using a standard phenol-chloroform protocol, after an overnight digestion with proteinase K (50 µg mL^{-1}; Serva, Germany), at 55°C. DNA was re-suspended in 50–100 μL^{-1} of DNAse-free water and left to dissolve overnight at 4°C.

Different regions of molecular markers within the nuclear ribosomal operon (SSU rDNA, LSU rDNA, ITS region) were amplified in this study with primer combinations as listed in Table 1. At the beginning we aimed at obtaining the sequence data of all three markers for *Ceratomyxa* parasites using the general *Ceratomyxa* (SSU rDNA) or general clinid *Ceratomyxa* (LSU rDNA, longer ITS region) primers from a random selection of several samples to compare the phylogenetic clustering patterns. Later on, we selected the longer ITS region as the most suitable marker for species detection based on the highest amount of data informativeness and best tree resolution and continued in PCRs of additional samples only for this region. Using these approaches, seven *Ceratomyxa* spp. were uncovered (*C. cottoidii*, *C. dehoopi*, further undescribed species *Ceratomyxa* sp. 1, *Ceratomyxa* sp. 2, *Ceratomyxa* sp. 3, *Ceratomyxa* sp. 4 and *Ceratomyxa* sp. 5). However, amplification of all three loci from samples chosen for amplification of all markers was not successful in all cases. This situation was further complicated by identification of concurrent infections of multiple *Ceratomyxa* spp. from clones placed in different clinid *Ceratomyxa* subclades (representing species) originating from single PCR products. To overcome these obstacles, which would bias the exact assessment of diversity and prevalence of *Ceratomyxa* spp., we designed seven sets of species-(lineage) specific primers targeting the shorter ITS region of each *Ceratomyxa* species (Table 1). This region was nested within the previously amplified longer ITS region and provides enough interspecific sequence variation and sufficient number of sequences for primer design. We subsequently PCR-screened all 152 samples.

PCRs of the first three regions were carried out in a total volume of 25 μl consisting of 1x Taq Buffer, 250 μM of each dNTP, 10 pmol of each primer, 1 U of Taq-Purple polymerase (Top-Bio, Czech Republic), 1 μl of DNA (50 to 150 ng) and sterile distilled H2O. For the PCR screening based on the shorter ITS region, an AmpOne HS-Taq premix (GeneAll Biotechnology, South Korea) was used to prepare PCR reactions containing 10 μl of HS-Taq premix, 0.5 μl of each primer (25 pmol), 8 μl of sterile distilled H2O and 1 μl of DNA (50 to 150 ng). The following cycling parameters were used for these primary/nested PCRs i) SSU rDNA: 95°C 3 min, 30x (95°C 1 min, 52°C/55°C 1 min, 72°C 1 min 40s/1 min 15s), 72°C 7 min, ii) LSU rDNA: 95°C 3 min, 30x (95°C 30 s, 50°C/55°C 30s, 72°C 1 min), 72°C 7 min, iii) longer ITS region: 95°C 3 min, 30x (95°C 30 s, 50°C/56°C 30s, 72°C 1 min/40 s), 72°C 7 min, and iv) shorter ITS region: 95°C 3 min, 30x (95°C 30 s, 56°C/55°C 30s, 72°C 40 s/30s), 72°C 7 min.

Table 1. Primers used to amplify target regions of the nuclear ribosomal operon of clinid* Ceratomyxa *spp.

Primer name	Primer sequence	Target group/species	PCR type/ Amplicon length (bp)	Reference
SSU rDNA				
ERIB1	ACCTGGTTGATCCTGCCAG	Universal eukaryotic	Primary PCR ≈ 1900–2050	[53]
ERIB10	CTTCCGCAGGTTCACCTACGG	Universal eukaryotic		[53]
18S-cerF	CTWGTTGGTADGGTAGTG	All *Ceratomyxa*	Nested PCR ≈ 1300–1450	[46]
18S-cerR	GTACAAGAGGCAGAGACGTAT	All *Ceratomyxa*		[46]
LSU rDNA				
NLF-CerCot-out	ACCGTGATTGTCCCAGTAACTGCG	Clinid *Ceratomyxa*	Primary PCR ≈ 1050–1150	This study
NLR-CerCot-out	AAAGTGGCCCACTTGGAGCGC	Clinid *Ceratomyxa*		This study
NLF-CerCot-in	ACAAGAGCCCGTAGTCGAATCGC	Clinid *Ceratomyxa*	Nested PCR ≈ 950–1050	This study
NLR-CerCot-in	TTAGGAGCCTGCTCGCGGTTGGG	Clinid *Ceratomyxa*		This study
Longer ITS region (last 50 bp of SSU rDNA, full ITS1, full 5.8S rDNA, full ITS2, first 85 bp of LSU rDNA)				
CerCot_ITS_for	CACGGCGACGGTGTGAAAACG	Clinid *Ceratomyxa*	Primary PCR ≈ 700–1050	This study
C_cot-ITS-R-out	GCGATTCGACTACGGGCTCTTGT	Clinid *Ceratomyxa*		This study
C_Cot-ITS-F-in	GAAGTAAAAGTCGTAACAAGG	Clinid *Ceratomyxa*	Nested PCR ≈ 500–850	This study
C_Cot-ITS-R-in	CGCAGTTACTGGGACAATCACGGT	Clinid *Ceratomyxa*		This study
Shorter ITS region (cca 60–300 bp of ITS1 + 5.8S rDNA + cca 60–100 bp of ITS2)				
C_Cot-ITS-F-in	GAAGTAAAAGTCGTAACAAGG	Clinid *Ceratomyxa*	Primary PCR ≈ 500–850	This study
C_Cot-ITS-R-in	CGCAGTTACTGGGACAATCACGGT	Clinid *Ceratomyxa*		This study
CCotITS162-for	TACGRKWRVARGTACTKTGAGGATG	*C. cottoidii*	Nested PCR ≈ 300	This study
CCotITS730-rev	TTTTMCGGCAGAGTAACC	*C. cottoidii*		This study
CDehITS154-for	AGAGTRCGTWTGGTGCGTTGAAG	*C. dehoopi*	Nested PCR ≈ 300	This study
CDehITS789-rev	TCGAGTCGGGTCAGTTGTTGTTC	*C. dehoopi*		This study
Csp1ITS223-for	AGTGTAAATATATGSCTACMTG	*Ceratomyxa* sp. 1	Nested PCR ≈ 300	This study
Csp1ITS801-rev	TTCATCAAGGGCAAATGTCGTC	*Ceratomyxa* sp. 1		This study
Csp2ITS315-for	AAGAGTAAAGATGAATGCCTAC	*Ceratomyxa* sp. 2	Nested PCR ≈ 350	This study
Csp2ITS824-rev	TTGTAATTTACAGGGGTCTAGG	*Ceratomyxa* sp. 2		This study
Csp3ITS198-for	TGAACGAAAGATGAAWTWAAGC	*Ceratomyxa* sp. 3	Nested PCR ≈ 350–550	This study
Csp3ITS785-rev	TGTTACTTGGTTGCCAGTCAG	*Ceratomyxa* sp. 3		This study
Csp4ITS199-for	AKGTRGACTGTGCTGCTTGCAG	*Ceratomyxa* sp. 4	Nested PCR ≈ 350	This study
Csp4ITS798-rev	TTCTAGTTGTCGGGYTTGTACCG	*Ceratomyxa* sp. 4		This study
Csp5ITS151-for	AGGTGAAATGGTGCAGTCTATC	*Ceratomyxa* sp. 5	Nested PCR ≈ 350	This study
Csp5ITS782-rev	TTCACCGTAACCATCATTCAC	*Ceratomyxa* sp. 5		This study

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PCR products were purified using a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd, USA) and sequenced directly. In most cases, the amplified fragments were cloned into the pDrive Vector with a PCR Cloning Kit (Qiagen, Germany) and transformed into TOP10 chemically competent *Escherichia coli* cells (Life

Technologies, Czech Republic). Plasmid DNA was extracted using a High Pure Plasmid Isolation Kit (Roche Applied Science, Germany) and up to 1–8 (mostly three) colonies (clones) were sequenced (SeqMe, Czech Republic).

A table with detailed information on sample localities and fish species, DNA extracted, numbers of PCR products and clones sequenced for each locus of a particular *Ceratomyxa* species is supplemented (S1 Table).

Sequence alignments

Five datasets were created for the phylogenetic analyses and the haplotype network: i) Ceratomyxa-SSU rDNA dataset (1176 bp) composed of 70 sequences representing seven newly sequenced *Ceratomyxa* spp. from clinids (2 sequences for each species, in total 14 sequences), other selected members of the *Ceratomyxa* clade retrieved from the GenBank (54 sequences) and two outgroup sequences (basal ceratomyxids *Ceratomyxa leatherjacketi* and *Myxodavisia bulani*) ii) clinid-*Ceratomyxa*-SSU rDNA dataset (1465 bp) including 106 SSU rDNA sequences of seven newly sequenced *Ceratomyxa* spp. from clinids (104 sequences) and two outgroup sequences (*Ceratomyxa longipes* and *Ceratomyxa barnesi*), iii) clinid-*Ceratomyxa*-LSU rDNA dataset (1068 bp) including 122 LSU rDNA sequences of five newly sequenced *Ceratomyxa* spp. from clinids (118 sequences) and four outgroup sequences (*Ceratomyxa appendiculata*, *Ceratomyxa cardinalis, Ceratomyxa vikrami, Ceratomyxa verudaensis*), iv) clinid-*Ceratomyxa*-ITS dataset (908 bp) including 173 ITS sequences of seven newly sequenced *Ceratomyxa* spp. from clinids (170 sequences) and three outgroup sequences (*Ceratonova shasta*, *Cystodiscus australis, Parvicapsula* sp.), and v) Ceratomyxa cottoidii-ITS haplotype dataset (264 bp) including 76 new ITS sequences of C. cottoidii. This dataset, prepared for the haplotype networks and population genetics analyses, contained sequences from both the longer and the shorter ITS region that were trimmed to the length of the shortest ones (see Table 1). Nucleotide sequences in each dataset were aligned in MAFFT v7.017 algorithm [54] implemented in Geneious v8.0.5 [55] using the E-INS-i multiple alignment method, with a gap opening penalty (–op) of 1.53 and gap extension penalty (–ep) of 0.123. The alignments were manually edited and highly variable sections were removed manually in Geneious.

Phylogenetic analyses

Maximum parsimony (MP) analysis was performed in PAUP* v4.b10 [56], using a heuristic search with random taxa addition, the ACCTRAN option, TBR swapping algorithm, all characters treated as unordered and gaps treated as missing data. Maximum

likelihood (ML) analysis was performed in RAxML v7·0·3 [57] using the GTR + Γ model of nucleotide substitution. Bootstraps were based on 1000 replicates for both analyses. Bayesian inference (BI) analysis was performed in MrBayes v3.0 [58], using the GTR + Γ + I model of evolution. Analyses were initiated with random starting trees, four simultaneous MCMC chains sampled at intervals of 100 trees and posterior probabilities estimated from 1 million generations for the Ceratomyxa-SSU rDNA dataset (burn-in 100,000), 3 million generations for the clinid-Ceratomyxa-SSU rDNA dataset (burn-in 600,000), 3.5 million generations for the clinid-Ceratomyxa-LSU rDNA dataset (burn-in 350,000) and the clinid-Ceratomyxa-ITS dataset (burn-in 710,000). Suitable burn-in levels were chosen in Tracer v1.4.1 [59].

Distances

The intraspecific and interspecific divergences were determined from proportional distances (in %) which were calculated in Geneious from the datasets previously used for the phylogenetic analyses. These datasets were additionally adjusted by excluding the very short sequences and by trimming the 5' and 3' ends of the remaining sequences to achieve their same length. The intraspecific cut-offs for the studied markers in the group of long branching ceratomyxids from clinid hosts were as follows: SSU rDNA–7.5%, LSU rDNA–10%, ITS region–20%.

Haplotype networks, AMOVA and population genetic statistics

The ITS region (Ceratomyxa cottoidii-ITS haplotype dataset, 76 sequences) was selected for haplotype networks, analysis of molecular variance (AMOVA) and population genetic statistics, as it provides enough sequence variability to distinguish the population structure patterns in myxozoans [60].

To examine the evolutionary relationships among haplotypes in populations of *C. cottoidii* from different localities and clinid fish species statistical parsimony networks (TCS) based on pairwise differences were constructed using PopART v1.7 [61]. Sampling localities were set as traits for the haplotype network which grouped the sequences (number of sequences = n) with regard to geography (Cape Columbine n = 6, Jacobs Bay n = 2, Granger Bay n = 1, Mouille Point n = 4, Kommetjie n = 1, Kalk Bay n = 24, False Bay n = 1, De Hoop n = 14, Jongensfontein n = 2, Herolds Bay n = 19, Jeffreys Bay n = 2). Fish host species were set as traits for the haplotype network which grouped the sequences with regard to host specificity (*C. cottoides* n = 61, *C. superciliosus* n = 9, *C. acuminatus* n = 1, *C. brevicristatus* n = 1, *M. dorsalis* n = 4).

Population structure was estimated by AMOVA in PopART. Individuals represented by ITS region sequences were grouped by sampling locality to populations and then nested within groups delimited by putative oceanographic barriers and host-driven isolation. We performed AMOVAs at three different levels to quantify how much variation is partitioned: (i) among individuals within populations, (ii) among populations within groups, and (iii) among groups of populations. A priori groupings were as follows: i) "Geographic groups" formed according to the geographic separation of the South African coastline [11]: "West coast group" (n = 14): localities Cape Columbine, Jacobs Bay, Granger Bay, Mouille Point, Kommetjie; "South west coast group" (n = 25): Kalk Bay, False Bay; "South east coast group" (n = 37): De Hoop, Jongensfontein, Herolds Bay, Jeffreys Bay, ii) "Barrier groups" formed in accordance with the main barriers to gene flow recognized for the populations of the type fish host Clinus cottoides (HB1, HB2 and HB3 in Fig 1) [11, 12] with an exception that the most eastern locality, Jeffreys Bay, was not analyzed as a separate group due to low sample size (n = 2), so it was merged into a single group with the south coast localities De Hoop, Jongensfontein and Herolds Bay: "B1 group" (n = 8): Cape Columbine, Jacobs Bay; "B2 group" (n = 31): Kalk Bay, False Bay, Granger Bay, Mouille Point, Kommetjie; "B3 group" (n = 37): De Hoop, Jongensfontein, Herolds Bay, Jeffreys Bay, and iii) "Host groups" formed according to fish host species: "Clinus cottoides" (n = 61): C. cottoides from Kalk Bay, De Hoop, Jongensfontein, Herolds Bay, Jeffreys Bay; "Clinus superciliosus" (n = 9): C. superciliosus from Cape Columbine, Mouille Point, Kommetjie; and "Muraenoclinus dorsalis" (n = 4): *M. dorsalis* from Jacobs Bay, Granger Bay and Mouille Point. The variation was not quantified for *C. acuminatus* and *C. brevicristatus* due to small sample size (1 sequence for each host species).

To characterize the diversity of populations and their demographic history we performed several population genetic statistics for C. cottoidii groups that showed significant variation among the groups tested in the previous AMOVA (i.e. above defined geographic and barrier groups). DNA polymorphism statistics (number of haplotypes, number of segregating sites, nucleotide genetic diversity, haplotype diversity) and statistical tests aiming at detection of population size changes (Tajima's D, Fu & Li's F and D and Ramos-Onsins and Rozas R2) were calculated using DNAsp v6.10.04 [62]. The significance of all tests was determined by 10,000 coalescent simulations implemented in DNAsp. Highly significant negative values of Fs and small positive values of R2 indicate population growth whereas positive Fs values can result from balancing selection, population bottlenecks or the presence of population structure [63, 64]. Significantly negative values of Tajima's D indicate recent range expansion (excess of low frequency polymorphisms), whereas significantly positive values are a signature

of bottleneck or selection. A non-significant D is consistent with a population at drift-mutation equilibrium [64, 65].

Results

Ceratomyxa *diversity as observed by light microscopy*

Myxospores (Fig 2A–2E) and/or plasmodia (Fig 2F) of the genus *Ceratomyxa* were found in 40% of examined samples with the majority represented by infected gall bladders (58/152), and with few of them (3/152) found in bile ducts (liver). High morphological plasticity of *Ceratomyxa* spores was commonly observed in our samples with spore morphotypes similar to previously described *C. cottoidii* (Fig 2A) and *C. dehoopi* (Fig 2B) typical for their slender spores, however, oval round spores suggestive of novel species were also frequently seen (Fig 2C and 2D). Spore shape and size (length and thickness) varied even within single fresh samples. Aberrant *Ceratomyxa* spores with three polar capsules and three valves were occasionally observed (Fig 2E). Concurrent infections of *Ceratomyxa* with other myxozoan genera, *Sphaeromyxa* and *Kudoa* were also detected.

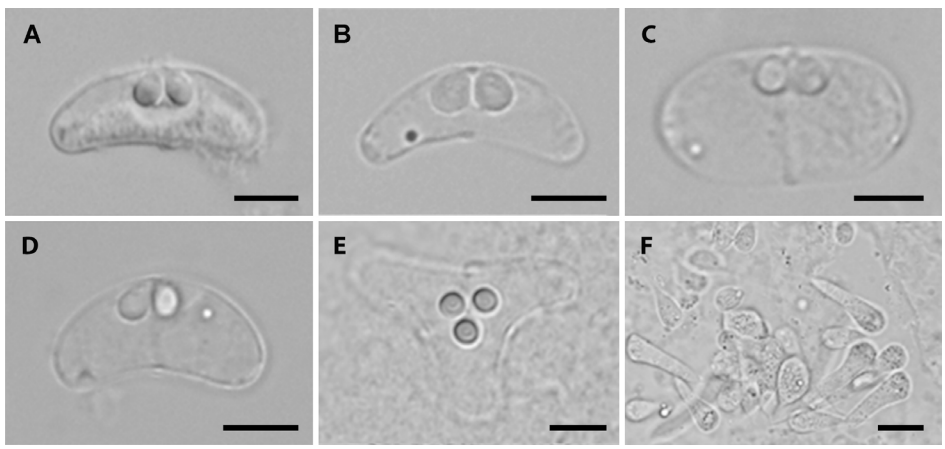

Fig 2. Light microscopy pictures of* Ceratomyxa *spp. spores of different morphotypes and plasmodia from clinid fish as seen in nomarski differential interference contrast. (A) *Ceratomyxa cottoidii* from *Clinus cottoides* from Herolds Bay. (B) *Ceratomyxa dehoopi* from *Clinus superciliosus* from Jacobs Bay. (C) *Ceratomyxa* sp. from *Clinus cottoides* from Jongensfontein. (D) *Ceratomyxa* sp. from *Muraenoclinus dorsalis* from Mouille Point. (E) Aberrant spore of *Ceratomyxa* sp. from *Clinus cottoides* from De Hoop. (F) Plasmodia of *Ceratomyxa* sp. from *Clinus cottoides* from Jongensfontein. Scale bar 5 μm (A–E), 20 μm (F).

Phylogenetic positioning of clinid ceratomyxids within the Ceratomyxa clade

The *Ceratomyxa* clade is an assemblage of diverse groups that evolved initially at different substitution rates and some of them have undergone subsequent radiation events [46]. This pattern is evident in ceratomyxids from South African clinids that clustered into a single well-supported long-branched lineage ("*Ceratomyxa* group from clinid fish") which further split into several short-branched species (Fig 3). The whole lineage grouped with other extremely long-branching taxa with various scenarios in tree topology, most probably due to LBA artifacts. In ML and BI analyses (Fig 3), clinid ceratomyxids grouped with *Ceratomyxa aegyptiaca* from soleid fish from a Mediterranean coastal lagoon in northern Africa and *Ceratomyxa longipes* from a gadid fish sampled in the North Sea. The whole group then clustered either with a group including *Ceratomyxa ayami* and *Ceratomyxa* sp. M0304 (ML analysis, Fig 3) or formed a polytomy with two groups (*Ceratomyxa ayami* and *Ceratomyxa* sp. M0304; *C. anko*, *C. appendiculata*, *C. pantherini*, *C. vikrami* and *Pseudalataspora kovalevae*, BI analysis, BI posterior probability = 0.81). Another alternative in tree topology was observed in MP analysis, where clinid ceratomyxids grouped with *C. longipes* and then with the group formed by *C. anko*, *C. appendiculata*, *C. pantherini*, *C. vikrami* and *Pseudalataspora kovalevae* (MP bootstrap support <50%).

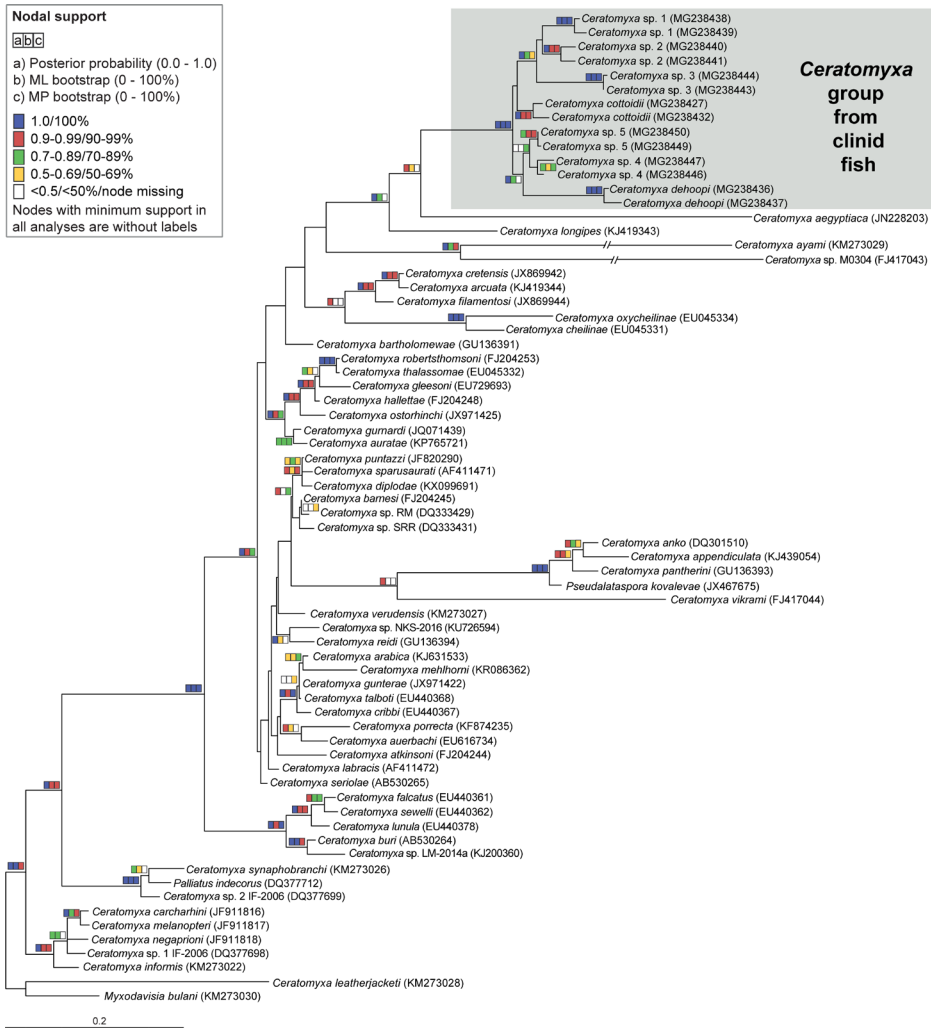

Fig 3. SSU rDNA-based maximum likelihood phylogenetic tree showing the position of clinid ceratomyxids within the* Ceratomyxa *clade.

Nodal supports are shown for maximum likelihood and maximum parsimony bootstraps and Bayesian inference posterior probabilities. Branches of Ceratomyxa ayami and Ceratomyxa sp. M0304 were shortened to 50% of their original length. Ceratomyxa leatherjacketi and Myxodavisia bulani were used as outgroups.

Phylogenetic relationships within the clinid Ceratomyxa *group*

In this study, we amplified almost 400 clinid *Ceratomyxa* sequences belonging to different molecular markers of the nuclear ribosomal operon (SSU rDNA: n = 104; LSU rDNA: n = 118; Longer and shorter ITS region: n = 170, S1 Table) that were all used for subsequent phylogenetic analyses. After performing the analyses, unique sequence data

representing each *Ceratomyxa* species from different fish hosts and localities were selected for GenBank submission (74 sequences, S2 Table). All other sequences are available in the S1 Dataset containing the untrimmed and trimmed fasta datasets of all molecular markers analyzed in this study. Blasting of newly obtained sequences against GenBank entries has shown that our data represent the first publicly available sequences of *Ceratomyxa* spp. from clinids. Phylogenetic analyses of SSU rDNA (S1 Fig) and ITS data (Fig 4) recognized seven well-supported novel lineages of *Ceratomyxa*, likely species, from South African clinids, whereas only five lineages were recovered in the LSU rDNA-based tree (S2 Fig) due to missing data from the two remaining lineages (*Ceratomyxa* sp. 2 and *Ceratomyxa* sp. 3). Based on the consensus of biological data (type host species and type locality) and, wherever possible, morphological data two of the lineages are considered to represent the previously described species *C. cottoidii* and *C. dehoopi*. The remainder of the sequences likely represents novel species (see paragraph "PCR screening, prevalence, distribution, co-infections and morphological remarks"). In the ITS-based tree, *C. cottoidii* clustered with a well-supported clade of *Ceratomyxa* sp. 1, *Ceratomyxa* sp. 2 and *Ceratomyxa* sp. 3 to create a common sister group to *C. dehoopi* (Fig 4). However, positioning of *C. cottoidii* and *C. dehoopi* varied depending on the marker used (S1 and S2 Figs). Another stable clade in all single gene analyses was formed by *Ceratomyxa* sp. 4 and *Ceratomyxa* sp. 5. Some members, i.e. *Ceratomyxa dehoopi* and *Ceratomyxa* sp. 3, created long branches to their sister taxa within the clinid *Ceratomyxa* group (Figs 3 and 4, S1 and S2 Figs).

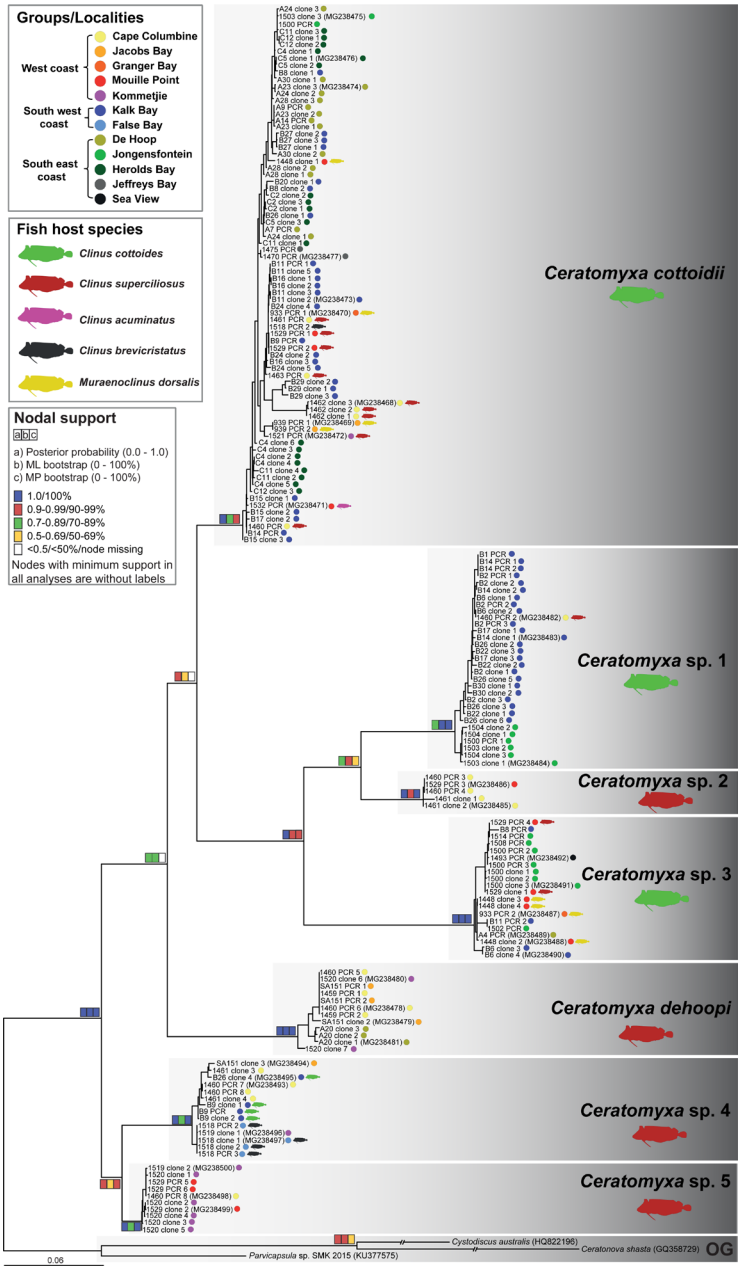

Fig 4. Maximum likelihood phylogenetic tree of the ITS region showing the relationships of* Ceratomyxa *spp. from South African clinids.

Nodal supports are shown for maximum likelihood and maximum parsimony bootstraps and Bayesian inference posterior probability. Cystodiscus australis, Ceratonova shasta *and* Parvicapsula *sp. were used as outgroups; branches of the first two outgroup taxa were shortened to 50% of their original length. Host species and localities are shown with colored symbols.*

Species distances

SSU rDNA sequence variability among clinid ceratomyxids ranged from 7.8 to 15.7% whereas intraspecific divergence ranged from 0.4% in *Ceratomyxa* sp. 5 to the significantly higher level (7%) in *C. cottoidii* (Fig 5A). A similar trend was observed for the LSU rRNA gene with interspecific variation of 18.2–28.3% and intraspecific variation ranging from 4.7 to 9% (Fig 5B). The highest variation was calculated for the ITS region showing 20.9–33.6% interspecific divergence and 1.7–18.4% intraspecific distance (Fig 5C).

PCR screening, prevalence, distribution, co-infections and morphological remarks

Species-specific PCR screening revealed 100% of samples positive at least for one *Ceratomyxa* species compared to only 40% parasite prevalence determined based on morphological observations (see paragraph "*Ceratomyxa* diversity as observed by light microscopy"). By species-specific PCR, *C. cottoidii* was recognized to be a true generalist with a high prevalence in all fish host species including its type host *C. cottoides*, while general clinid *Ceratomyxa* PCR revealed its presence in four out of five fish host species (Table 2). It is widely distributed from the west (Cape Columbine) to the east coast (Sea View) of South Africa with almost 100% prevalence at all studied localities. The only exception is its lower prevalence in Jacobs Bay (west coast) (Fig 6) where it was missing in all *C. superciliosus* and present in 80% of *M. dorsalis* samples. However, *C. superciliosus* was a common host of *C. cottoidii* at other localities (Table 2). On the other side, *C. dehoopi* is a parasite with a narrower host range (three out of five clinid species) and with the highest prevalence in *C. superciliosus* (Table 2). Its distributional range spans from Cape Columbine (west coast) to Herolds Bay (south coast). It was not detected at any of the two east coast localities (Fig 6). The remainder of *Ceratomyxa* spp. is represented by generalists, present in 4–5 examined fish species and distributed from the west to the east coast of South Africa with variable prevalence at each locality (Table 2, Fig 6). Importantly, the application of species-specific PCR was especially crucial for the assessment of the fish host species spectra of *C. dehoopi*, *Ceratomyxa* sp. 1 and *Ceratomyxa* sp. 5 for which general clinid *Ceratomyxa* PCR amplification was suggestive of their strict host specificity only in one fish species (Table 2).

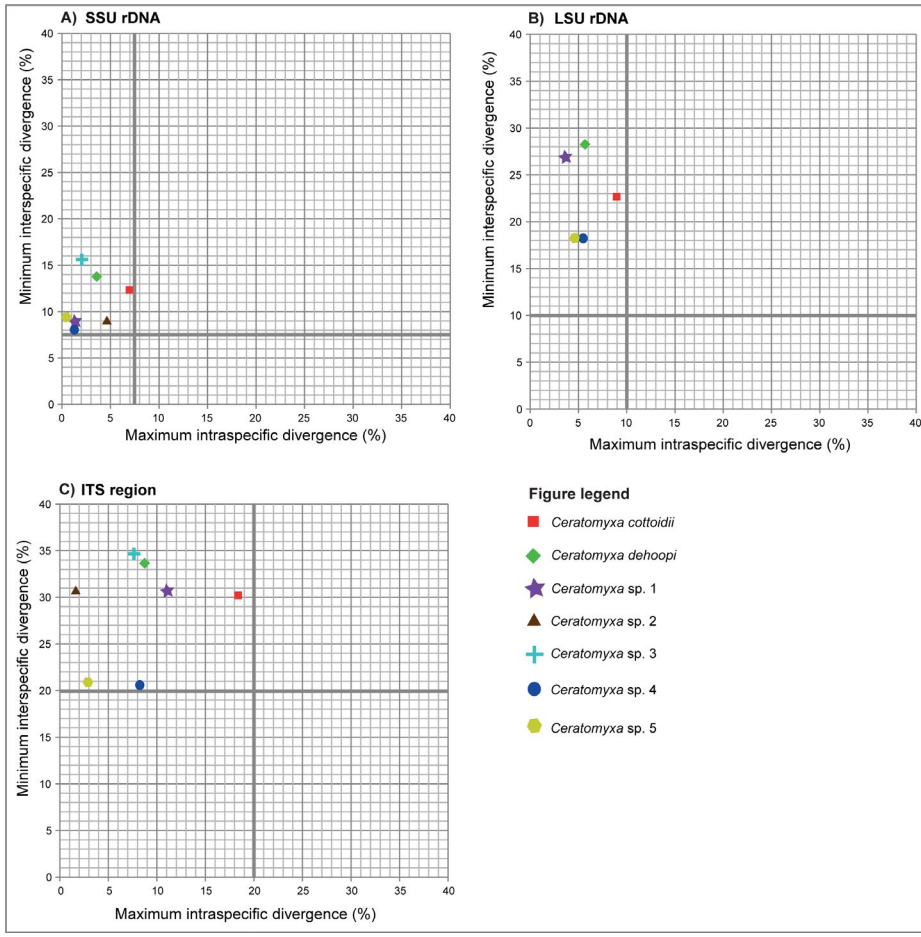

Fig 5. Graphic interpretation of maximum intraspecific distances plotted against minimum interspecific distances (dissimilarities) among the sequences of (A) SSU rDNA, (B) LSU rDNA and (C) ITS region of clinid* Ceratomyxa *spp. Intraspecific cut-off values (thresholds) are shown with thick grey-colored line.

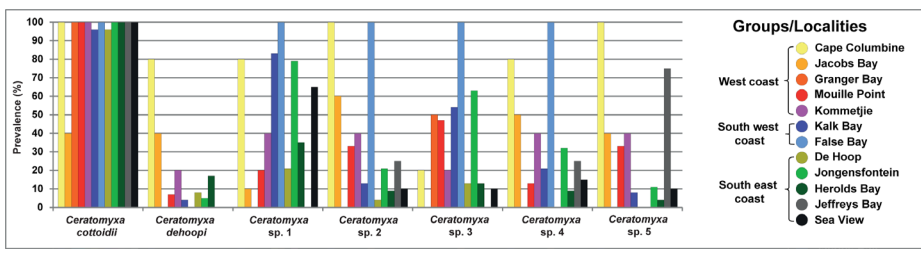

Fig 6. Prevalence of clinid* Ceratomyxa *spp. at each locality based on the PCR-screening of the shorter ITS region of all samples.

Co-infections of single fish host samples by several *Ceratomyxa* species were extremely common and occurred in 71% of all samples. One sample from *C. superciliosus* from Cape Columbine even contained all *Ceratomyxa* spp. which was confirmed by variable shapes and dimensions of myxospores observed in this sample. On the other hand, many samples with single spore morphotype observed by LM showed multiple species infections. Single infections (29% of all samples) were mainly represented by *C. cottoidii* (42 samples) and only two samples exclusively contained *C. dehoopi* or *Ceratomyxa* sp. 5.

We were not able to draw any conclusions regarding the measurements of *C. cottoidii* spores from the original species description (Reed et al., 2007) and from our samples with single infections, for which LM measurements were taken, as the comparison would be biased by potential spore shrinkage of our ethanol-fixed material (details on spore measurements in S3 Table). Unfortunately, the lack of any samples with single infections of *C. dehoopi* and *Ceratomyxa* sp. 1–5 accompanied by spore measurements prevented analysis of spore morphometry for species comparison/descriptions. Therefore, *Ceratomyxa* sp. 1–5 currently represent phylogenetic lineages and remain undescribed species.

Table 2. Prevalence of studied* Ceratomyxa *spp. in the fish host samples identified by PCR with general* Ceratomyxa *SSU rDNA, LSU rDNA and longer ITS region primers and species-specific shorter ITS-based PCR screening.

Myxozoan species	Marker	*Clinus cottoides*	*Clinus superciliosus*	*Clinus acuminatus*	*Clinus brevicristatus*	*Muraenoclinus dorsalis*
Ceratomyxa cottoidii	SSU rDNA	80% (31/39)	22% (2/9)	100% (4/4)	0% (0/1)	67% (2/3)
	LSU rDNA	86% (18/21)	14% (1/7)	100% (2/2)	0% (0/1)	100% (1/1)
	ITS region	71% (20/28)	14% (1/7)	N/A	0% (0/1)	100% (1/1)
	Screening	98% (110/112)	67% (12/18)	100% (6/6)	100% (1/1)	93% (14/15)
Ceratomyxa dehoopi	SSU rDNA	0% (0/39)	33% (3/9)	0% (0/4)	0% (0/1)	0% (0/3)
	LSU rDNA	0% (0/21)	29% (2/7)	0% (0/2)	0% (0/1)	0% (0/1)
	ITS region	0% (0/28)	43% (3/7)	N/A	0% (0/1)	0% (0/1)
	Screening	6% (7/112)	56% (10/18)	17% (1/6)	0% (0/1)	0% (0/15)
Ceratomyxa sp. 1	SSU rDNA	21% (8/39)	0% (0/9)	0% (0/4)	0% (0/1)	0% (0/3)
	LSU rDNA	38% (8/21)	0% (0/7)	0% (0/2)	0% (0/1)	0% (0/1)
	ITS region	32% (9/28)	0% (0/7)	N/A	0% (0/1)	0% (0/1)
	Screening	55% (61/112)	39% (7/18)	17% (1/6)	100% (1/1)	13% (2/15)
Ceratomyxa sp. 2	SSU rDNA	3% (1/39)	33% (3/9)	0% (0/4)	0% (0/1)	0% (0/3)
	LSU rDNA	0% (0/21)	0% (0/7)	0% (0/2)	0% (0/1)	0% (0/1)
	ITS region	0% (0/28)	14% (1/7)	N/A	0% (0/1)	0% (0/1)
	Screening	12% (13/112)	83% (15/18)	17% (1/6)	100% (1/1)	13% (2/15)
Ceratomyxa sp. 3	SSU rDNA	10% (4/39)	0% (0/9)	0% (0/4)	0% (0/1)	33% (1/3)
	LSU rDNA	0% (0/21)	0% (0/7)	0% (0/2)	0% (0/1)	0% (0/1)
	ITS region	7% (2/28)	14% (1/7)	N/A	0% (0/1)	100% (1/1)
	Screening	29% (32/112)	22% (4/18)	0% (0/6)	100% (1/1)	47% (7/15)
Ceratomyxa sp. 4	SSU rDNA	3% (1/39)	11% (1/9)	0% (0/4)	100% (1/1)	0% (0/3)
	LSU rDNA	10% (2/21)	29% (2/7)	0% (0/2)	100% (1/1)	0% (0/1)
	ITS region	7% (2/28)	43% (3/7)	N/A	100% (1/1)	0% (0/1)
	Screening	14% (16/112)	67% (12/18)	17% (1/6)	100% (1/1)	7% (1/15)
Ceratomyxa sp. 5	SSU rDNA	0% (0/39)	22% (2/9)	0% (0/4)	0% (0/1)	0% (0/3)
	LSU rDNA	0% (0/21)	43% (3/7)	0% (0/2)	0% (0/1)	0% (0/1)
	ITS region	0% (0/28)	43% (3/7)	N/A	0% (0/1)	0% (0/1)
	Screening	9% (10/112)	61% (11/18)	33% (2/6)	0% (0/1)	20% (3/15)

N/A: PCR not done.

https://doi.org/10.1371/journal.pone.0194042.t002

Haplotype networks of Ceratomyxa cottoidii *populations*

The haplotype network of *C. cottoidii* ITS region sequences with traits defined as geographic localities was represented by 39 haplotypes and showed a considerable complexity of haplotype grouping (Fig 7A). Isolates of *C. cottoidii* fell into six main haplotype groups. Haplotype group A, separated from the rest of the network by 11 mutations (nucleotide changes), was represented by samples from Cape Columbine, the most western locality of our sampling range. Haplotype groups B and C were mainly created by samples from Kalk Bay (south west coast) mixed with samples from the western localities. Haplotype group D was mainly represented by samples from De Hoop and Herolds Bay (south east coast) mixed with samples from the south-western locality Kalk Bay. Haplotype group E contained exclusively samples from the south-eastern coast. Two haplotypes from the south-eastern coast locality Jeffreys Bay formed haplotype group F separated from haplotype group E by two mutations. Haplotype groups B–E showed a radial branching pattern with the main haplotype surrounded by satellite low frequency haplotypes which is typical for young expanding populations. Due to small sample size (4 and 2 sequences, respectively), existence of a similar burst pattern cannot be excluded for haplotype groups A and F (Fig 7A).

The separation of haplotypes according to the fish host species was not evident in our analyses. Haplotypes from different fish species mixed with each other and the only two unmixed groups of parasite haplotypes were biased by their exclusive origin from a single host species (*C. cottoides*) sampled in Kalk Bay, De Hoop, Jongensfontein, Herolds Bay, and Jeffreys Bay, localities from which data from other host species were not available (Fig 7B).

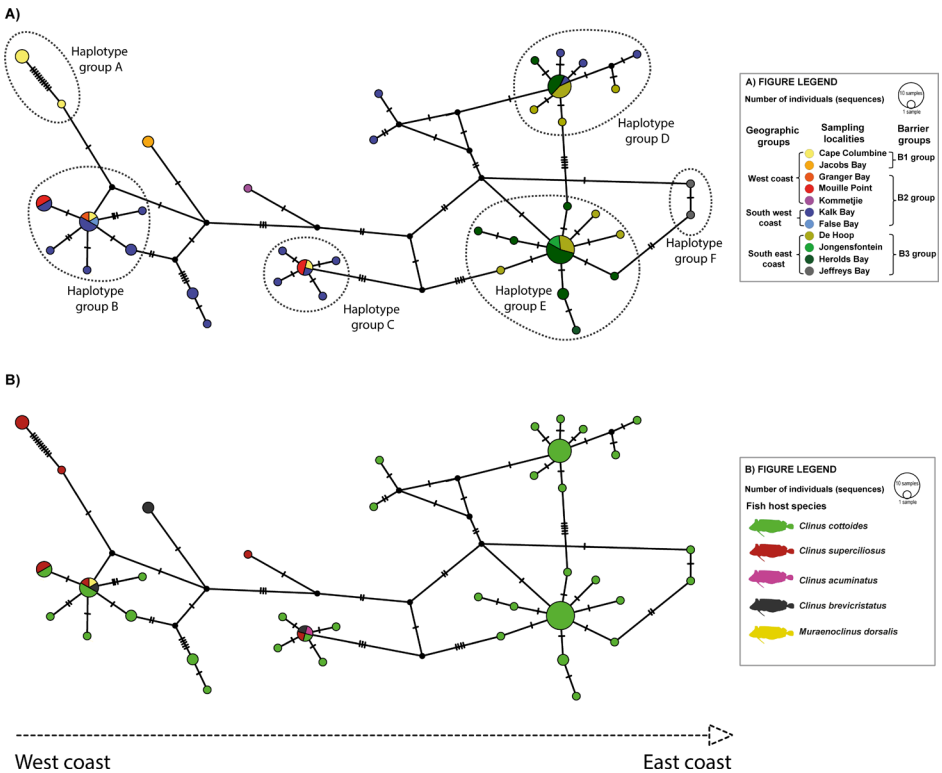

Fig 7. Haplotype network of* Ceratomyxa cottoidii *ITS dataset calculated using statistical parsimony networks (TCS) in PopArt v1.7.

Haplotype network with (A) sampling localities and (B) host species used as traits. The sizes of haplotypic nodes are relative to the sample size. Each line represents one mutational step and dots represent alternative one-step mutations.

Population genetic structure of Ceratomyxa cottoidii

Approximately half (55.4%) of the variation in *C. cottoidii* was attributable to highly significant differences within populations whereas about 42.6% was associated with significant differences among geographic groups. On the other hand, very low variation

was observed among populations (2.6%). The second AMOVA showed slightly higher variation among the barrier groups (50.0%) with highly significant covariance components. The within population variation was comparable with the one from the previous analysis. The lowest level of among group variation was found for AMOVA of host groups (26.0%), the analysis reaching highly significant levels only within and among populations (Table 3). In summary, our *a priori* AMOVA settings dividing *C. cottoidii* populations into groups delimited by major oceanographic barriers, especially if followed by the separation according to host gene flow barriers, explained significant amount of variation, however, additional factors except of fish host species, seem to impact population structuring of *C. cottoidii*. As revealed by AMOVA and haplotype networks, calculations of population genetics statistics for *C. cottoidii* using the dataset of parasite sequences from different fish species and their subsequent comparison with the population data from the type host *C. cottoides* is not a problem due to the lack of significant structuring of *C. cottoidii* populations according to fish host species.

***Table 3. AMOVA partitioning of genetic variance for shorter ITS region within populations and groups of* Ceratomyxa cottoidii.** *A priori groupings followed the separation of* C. cottoidii *sequences according to the i) geography of South African coastline (geographic groups), ii) main barriers to gene flow recognized for the populations of the fish host* Clinus cottoides *(barrier groups) and iii) fish host species (host groups).*

% variation	Geographic groups	Barrier groups	Host groups
Among groups	42.58*	50.04**	26.02
Among populations	2.55	-3.06	25.35**
Within populations	55.36**	53.02**	48.63**

The covariance components are indicated as

** for highly significant ($P < 0.01$) and

* for significant ($P < 0.05$).

https://doi.org/10.1371/journal.pone.0194042.t003

Sequence diversity indices and demographic history of Ceratomyxa cottoidii *populations*

The differences between *C. cottoidii* populations based on geographic separation observed in haplotype networks and AMOVA were also evident in their population genetics statistics (Table 4). Results of DNA polymorphism statistical analysis showed generally high values of haplotype diversity with small differences between the analyzed groups. From the total of 39 haplotypes, the highest number of haplotypes was found in the south west (n = 20) and south east coast (n = 20) for the geography-related groups and in B2 (n = 22) for barrier groups. The nucleotide diversity was the lowest for the south east coast (resp. B3 group). Tajima's test showed significant negative values only for south east and B3 populations whereas Fu and Li's tests revealed significant negative values for the south west, south east coast, B2 and B3 populations. The R2 test was significant only for the south east coast and B3 group populations (Table 4). The results

indicate that the south west, south east coast, B2 and B3 populations are expanding with the evidence of recent expansion in south east coast and B3 populations. In contrast, equilibrium and no population growth are suggested for the west and B1 populations at the edge of the distributional range of *C. cottoidii*.

***Table 4. Results of population genetics statistics of ITS region for* Ceratomyxa cottoidii.**

Statistic tests	Geographic groups			Barrier groups		
	West coast	South west coast	South east coast	B1	B2	B3
Number of sequences (n)	14	25	37	8	31	37
Number of haplotypes (h)	7	20	20	5	22	20
Number of segregating sites (S)	27	38	20	23	38	20
Haplotype diversity (Hd)	0.901	0.983	0.908	0.857	0.972	0.908
Nucleotide diversity (π)	0.043	0.042	0.015	0.045	0.038	0.015
Tajima's D (D)	1.233	0.259	-0.688*	1.470	0.040	-0.688*
Fu and Li's D* (D*)	0.968	-1.030**	-1.512**	0.260	-1.743**	-1.512**
Fu and Li's F* (F*)	0.893	-0.785*	-1.453**	0.363	-1.408**	-1.453**
Ramos-Onsins & Rozas' (R2)	0.197	0.125	0.086*	0.227	0.112	0.086*

The total number of unique haplotypes for all groups is 39; some haplotypes are shared by the groups. The statistical significance of covariance components is indicated as

** highly significant ($P < 0.05$) and

* significant ($P < 0.1$)

https://doi.org/10.1371/journal.pone.0194042.t004

Discussion

This is the first study to determine myxozoan diversity, distribution, prevalence and host specificity in a variety of clinid fish species, typical inhabitants of intertidal environments in South Africa. Moreover, the evolutionary history of the rapidly evolving clinid ceratomyxids has been assessed within this group as well as within the *Ceratomyxa* clade. Lastly, the most common clinid parasite in South Africa, *Ceratomyxa cottoidii*, has been subjected to population genetics analyses and its population structure has been compared with the data known for its type fish host, *Clinus cottoides*. This is a novel approach in myxozoan research as none of the previous studies compared the host-parasite genetic structure as they were solely focused on parasite population structuring [60, 66–69] and phylogeography [70–72].

We have revealed a high degree of species diversity represented by seven closely related *Ceratomyxa* spp. from the intertidal fishes Clinidae along the coast of South Africa. Such parasite radiation is probably the result of multiple speciation events triggered by limited host dispersal within this endemic area. Future application of a holistic approach in species descriptions based on detailed LM examination, proper photographic documentation and in-depth molecular identification is a good direction in accounting myxozoan species diversity, which currently seems to be underestimated [30, 73–76].

PCR screening with species-specific primers was crucial for the discovery of the remarkably high prevalence of ceratomyxids in clinid fish, with all examined samples

infected with at least one parasite species and with some ceratomyxids even reaching 100% prevalence in certain hosts and at certain sites. Our findings underpin the rock pools as ideal habitats for promoting myxozoan life cycles and for the establishment of a diverse assemblage of organisms as reported previously for other animal groups [14–16]. Specific PCR assays were also essential for the exact assessment of *Ceratomyxa* spp. diversity in clinids, as frequent mixed infections, indeed common in myxozoans [17, 40], complicated such evaluation. Present evidence of multiple *Ceratomyxa* spp. infections indicates that the extreme spore variability observed in our samples, most likely resulted from concurrent infections of closely related species of different morphology. This may also be a case for *Ceratomyxa* sp. from *M. dorsalis* [37]. Moreover, high rates of mixed *Ceratomyxa* spp. infections encountered in such a restricted organ space are suggestive of a low or even lacking within-host competition in this niche. Nevertheless, a temporal and spatial separation of the infections during the host's development may still occur similarly as reported for two competing marine myxozoan species [77].

In this study, we have unexpectedly revealed that *Ceratomyxa* spp. from South African clinids are not strictly host-specific parasites as a general rule for other ceratomyxids [25–30]. This may be caused by the close evolutionary relationships of South African clinids [8] and associated likely similar immune system traits of these fish as well as by generally low host immune response to coelozoic myxozoan parasites [78, 79]. We assume that several clinid fish species are true hosts for individual *Ceratomyxa* spp. as spores have been observed in the site of sporogony (gall bladder) at least in several samples in which the parasite has been molecularly detected.

The phylogenetic relationships of clinid *Ceratomyxa* spp. with other ceratomyxids were provided in this study. However, their accurate assessment was difficult due to their unstable clustering and weak nodal supports within the *Ceratomyxa* clade most probably due to LBA artifact. Nevertheless, increased taxon sampling with some closely related species, possibly from other blenniiform fishes, would facilitate to break the long-branch [46, 80] and thus improve inferring the exact positioning of this fast-evolving parasite group.

There is no universal criterion regarding what constitutes a sufficient level of sequence variation to represent distinct species in the genus *Ceratomyxa*. The intraspecific threshold values used for clinid ceratomyxids in present study are higher than commonly reported in other myxozoan clades [17] but they have been carefully evaluated with regard to the fast evolution of the studied molecular markers in this long-branched parasite group and we recommend using these cut-offs for this specific group of

ceratomyxids in future studies. For example, relatively low values of maximum SSU rDNA intraspecific divergences were reported for ceratomyxids from Indian (up to 0.8%) [81], Australian (up to 1.3%) [26, 27, 30] and North Atlantic fishes (up to 1.6%) [46], whereas wide ranges up to 7% in *C. cottoidii* were encountered for South African clinid ceratomyxids (present study). Such large intraspecific variability of *C. cottoidii* may be a result of fast evolution of its genes concordant with increased substitution rates and associated tree branch lengths [82]. Therefore, settings of the intraspecific cut-offs and the following assessment of intraspecific and interspecific variation of a molecular marker have to be taken into account in relation to the substitution rate in a particular *Ceratomyxa* group. As evolutionary rates also vary for other myxozoan lineages, investigations into the sequence variation for a particular phylogenetic group are crucial for the assessment of the species concept in the Myxozoa. Moreover, it would be desirable to assess the intragenomic variation of ribosomal sequences for each myxozoan species from one individual (spore), however, low DNA yields associated with single spore extractions are limiting factors for such approach. Information about the intragenomic variation would be of interest also in the present population genetics analysis of *C. cottoidii*, however, distinguishing of multiple haplotype infections from the real intragenomic variability was not possible due to the fact that DNA was extracted from hundreds of spores from each infected fish individual. Moreover, spores in each host specimen may have been a genetic mixture that developed after an infection from a mixed pool of invertebrate alternate hosts harboring different *C. cottoidii* haplotypes. It is highly probable that intragenomic variation exists in *C. cottoidii* but this would only have a significant effect on "within individual level of variation" that was not assessed in our analyses. An alternative how to get around the intragenomic variability issue would be the use of mitochondrial (maternally inherited) genes, for example cytochrome c oxidase I or cytochrome b, but this approach was unfortunately not feasible in our case as such genes are difficult to amplify in myxosporeans in general and lead to several misinterpretations due to occurrence of pseudogenes (numts) [73].

C. cottoidii was shown to represent the most common parasite of clinids with a widespread distribution along the South African coastline. Investigation of a potential host-driven isolation of *C. cottoidii* populations, impelled by the newly discovered generalist life history of this parasite, showed no evidence for separation of parasite haplotypes by fish hosts. However, a certain bias may exist as the largest haplotype groups were characterized by a single host species. On the other hand, population structuring based on geography-related groupings (geographic and barrier groups) showed important overlapping patterns with fish host population structure [11, 12] consistent with the hypothesis that reduced host dispersal, as typical for *C. cottoides* [10], enhances parasite community differentiation. *C. cottoidii* population structure is formed

by young expanding populations as well as by older populations in equilibrium. The older populations are represented by haplotypes from B1 group, which includes the most western localities, most probably at the edge of the distributional range of *C. cottoidii*. In these peripheral populations, no haplotype mixture is evident in the associated haplotype group A, likely representing the populations under recent speciation, which further supports the clustering of Cape Columbine sample (nr. 1462) as a separate lineage within (Fig 4) or at the base of the *Ceratomyxa* clade (S1 and S2 Figs). The young expanding populations of *C. cottoidii* are mainly represented by haplotypes from groups B2 and B3 covering western, south-western and south-eastern South African localities. Though some of the western localities are present in geographically mixed haplotype groups B and C (west + south west coast) and D (south west + south east coast), the west coast haplotypes never mixed with south east coast haplotypes. This pattern of population mixing does imply existence of some gene flow barriers on one side along with still ongoing migration between south west coast and south east coast locations on the other side, which is most probably facilitated by Agulhas ring eddies between Cape of Good Hope and Cape Agulhas in the zone of contact of Agulhas and Benguela current (Fig 1). Haplotype network suggests that some additional barrier to gene flow may exist within the south east coast populations (separation of haplotype group F), however, analysis of this hypothesis by splitting the south east group into south coast (De Hoop, Jongensfontein, Herolds Bay) and east coast (Jeffreys Bay) groups was not feasible due to low sample size from the eastern locality. Therefore, a more efficient fish taxon sampling, especially from the peripheral eastern and western localities, and following haplotype sequencing are necessary to support our conclusions which would additionally provide more data for detailed gene flow and migration analyses.

Our data suggest that oceanographic barriers around Cape Point, Cape Agulhas and east of Jeffreys Bay (east of Algoa Bay) region play important roles in the distribution and population structuring of the parasite as they do for the fish host *Clinus cottoides* [6, 11, 83]. This may be caused by closely linked host-parasite co-evolution in these space-limited habitats as genetic structure and co-divergence of host-parasite populations is higher in parasites infecting hosts with limited dispersal abilities [84]. However, only 43–50% of molecular variation in *C. cottoidii* sequences was ascribed to differences among groups associated with geography (Table 3) while a higher percentage of variation among groups (localities) was encountered for *C. cottoides* populations exhibiting significant oceanographic separation [11]. Such difference in variation may be linked to the complex myxozoan life cycle involving a vertebrate (intermediate) and an invertebrate (definitive) host, which adds another variable to the dispersal and associated population structure of *C. cottoidii*, a myxozoan with a presumably two-host life cycle [34]. *C. cottoides* is most commonly associated with mid-shore areas [2, 12],

and while this fish host shows strong site fidelity, its ceratomyxid parasites may have a high dispersal capacity by distributing spores to other rock pools via tidal water exchange or wave splashes. An invertebrate host can be present in these rock pools or alternatively, it can inhabit other terrestrial or planktonic habitats and thus contributing further to the gene flow in *C. cottoidii* populations. The high infection prevalence in fish, however, suggests that the invertebrate host habitat overlaps with that of *C. cottoides*. Excellent candidates that are common in the intertidal rocky shore habitats are sedentary polychaetes (Annelida: Polychaeta) from the families Cirratulidae, Spionidae, Orbiniidae, Arenicolidae, Flabelligeridae, Sabellariidae, Terebellidae, Sabellidae, Serpulidae and Spirorbidae [52, 85]. A systematic approach specifically targeting the species present in rock pools in order to find myxozoan parasites within these is needed.

Conclusions

We show that ceratomyxid species from South African clinids are a diverse group of fast evolving closely related parasites with high prevalence in their fish hosts, little host specificity and frequent concurrent infections, most probably as a result of radiation and no competition within the space limited host niche. *Ceratomyxa cottoidii* shows overlapping population structure with its type fish host, *C. cottoides*, however, data on the definitive host is required to unravel the complex network.

Several genetic studies of marine organisms, including *C. cottoides*, have shown that the sections of South African coastline coined as marine protected areas, representing hotspots of species richness and endemism and including a high diversity of habitats [86], require more protection [11, 83]. As both parasite diversity and distribution are closely linked to that of the host, any change in the conservation status and distribution of the host directly impacts that of the parasite and *vice versa* [87]. As *Ceratomyxa* parasites of clinid fishes are well represented in the South African marine fauna (present study), they may significantly impact their fish host populations. Investigations into the aquatic parasite biodiversity and distribution are of highest priority, as global climate change can shift the balance in healthy parasite-rich ecosystems where parasites represent one of the most susceptible groups to environmental change [87]. Besides the implications of global climate change for fish populations, a more detailed research of myxozoan life cycles as well as the roles of these parasites in food webs and trophic transfers and their impact on the health of fish hosts are desirable not only from a South African [23] but also from a global perspective.

Information on Electronic Supplementary Material

S1 Table. List of clinid fish samples molecularly examined on the presence of Ceratomyxa spp.
A detailed information on the host species and organ, locality, result of species-specific ITS-based PCR screening (+/-) and number of SSU rDNA, LSU rDNA and ITS PCR amplicons and/or clone sequences are available for each *Ceratomyxa* species along with the GenBank accession numbers (corresponding to those present in the phylogenetic trees). gb = gall bladder.
https://doi.org/10.1371/journal.pone.0194042.s001 (XLSX)

S2 Table. GenBank accession numbers of newly amplified Ceratomyxa sequences.
https://doi.org/10.1371/journal.pone.0194042.s002 (DOCX)

S3 Table. List of spore measurements of Ceratomyxa cottoidii from the present study and from the original species description.
https://doi.org/10.1371/journal.pone.0194042.s003 (DOCX)

S1 Dataset. The fasta datasets of untrimmed and trimmed molecular markers used for the phylogenetic analyses. https://doi.org/10.1371/journal.pone.0194042.s004 (TXT)

S1 Fig. Maximum likelihood phylogenetic tree of the SSU rDNA sequences showing the relationships of Ceratomyxa spp. from South African clinids.
Nodal supports are mapped for maximum likelihood and maximum parsimony bootstraps and Bayesian inference posterior probabilities. *Ceratomyxa barnesi* and *Ceratomyxa longipes*, for which the branches were shortened to 50% of their original length, were used as outgroups. Host species and localities are shown with colored symbols.
https://doi.org/10.1371/journal.pone.0194042.s005 (EPS)

S2 Fig. Maximum likelihood phylogenetic tree of the LSU rDNA sequences showing the relationships of Ceratomyxa spp. from South African clinids.
Nodal supports are mapped for maximum likelihood and maximum parsimony bootstraps and Bayesian inference posterior probabilities. *Ceratomyxa appendiculata*, *Ceratomyxa vikrami*, *Ceratomyxa cardinalis* and *Ceratomyxa verudaensis*, for which the branches were shortened to 50% of their original length, were used as outgroups. Host species and localities are shown with colored symbols.
https://doi.org/10.1371/journal.pone.0194042.s006 (EPS)

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5.2 Paper IV

Survey of *Kudoa* spp. (Myxozoa, Cnidaria) in fishes from the Madeira Archipelago and the Portuguese mainland coast: detection of *Kudoa thyrsites* in new hosts *Scomber colias* and *Micromesistius poutassou*

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Survey of *Kudoa* spp. (Myxozoa, Cnidaria) in fishes from the Madeira Archipelago and the Portuguese mainland coast: detection of *Kudoa thyrsites* in new hosts *Scomber colias* and *Micromesistius poutassou*

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Abstract

Myxozoan parasites of the genus *Kudoa* Meglitsch, 1947 are associated with *post-mortem* tissue degradation that causes great financial losses to commercial fisheries. *Kudoa thyrsites* (Gilchrist, 1924) is a species with a very wide host range including commercial tunas, mackerels, salmonids and flatfishes. A sample of 190 fishes of 18 species from the Madeira Archipelago and 30 Atlantic chub mackerel, *Scomber colias* Gmelin, and 30 blue whiting, *Micromesistius poutassou* (Risso), from the Portuguese mainland coast were examined for the presence of species of *Kudoa*. The prevalence of *Kudoa* spp. was 80% in *M. poutassou* and 60% in *S. colias*. No spore was detected in *S. colias* from Madeira, which was confirmed by specific PCR screening of the muscle from all individuals of *S. colias*. SSU rDNA analysis revealed that *M. poutassou* and *S. colias* from the Portuguese mainland coast were infected with *K. thyrsites*, an economically

important myxozoan parasite. Both sequences were identical with sequences of the eastern Atlantic *K. thyrsites* genotype, including that from the type host of this parasite. This is the first report of *K. thyrsites* from *M. poutassou* and *S. colias*. The fact that spores of species of *Kudoa* were not detected in fishes screened in the Madeira Archipelago may be explained by various ecological factors, such as the absence of a continental shelf, a short insular shelf, and oceanic waters with low productivity, all resulting in reduced abundance of benthic organisms. Consequently, it is possible that as yet unknown annelid definitive hosts of *Kudoa* spp. are absent or very rare near Madeiran coasts.

Keywords: Atlantic, food safety, parasites, Portugal, scombrid, tuna, Atlantic chub mackerel, blue whiting.

The study of fish parasites has grown over the last decades mainly due to their economic implications in fisheries, aquaculture and, in some cases, risk to human health. Fish parasites that occur in edible parts of the fish are the most relevant for human health. Multivalvulida (Cnidaria: Myxozoa) are widespread and can be associated with mortality or poor flesh quality of commercially important marine fishes (Whipps and Diggles 2006, Kristmundsson and Freeman 2014, Kasai et al., 2016) and, in some cases, foodborne diseases (Suzuki et al., 2015, Takeuchi et al., 2016). *Kudoa* Meglitsch, 1947 is one of the most studied myxozoan genera whose species infect a wide range of marine fishes from different geographic areas. Around 100 species of *Kudoa* have been described from wild fishes in different regions of the world (Eiras et al., 2014).

In the Eastern Atlantic, they have been reported as parasites of several small pelagic fish species such as *Sardina pilchardus* (Walbaum), *Scomber scombrus* Linnaeus, *Trachurus trachurus* (Linnaeus) and *Trachurus picturatus* (Bowdich) (Gaevskaya and Kovaleva 1985, Cruz et al., 2003, Levsen et al., 2008, Cruz et al., 2011). Spores of species of *Kudoa* have already been detected in the western Mediterranean, and more southwards alongside the African coast to the Gulf of Guinea in some large pelagic fish species, such as *Thunnus obesus* (Lowe) (Yurakhno and Gorchanok 2011). Wild fishes are not the only suitable intermediate hosts of *Kudoa* spp., because there are also reports of infections in various species from aquaculture. Atlantic salmon, *Salmo salar* Linnaeus, is one of the most affected species, with considerable commercial impact (Goater et al., 2014, Lafferty et al., 2015).

The life cycles of marine myxozoans are poorly known (Eszterbauer et al., 2015), with no data available for species of *Kudoa*. Generally, myxosporean life cycle has two different phases: an actinospore-phase taking place in invertebrate definitive hosts

(annelids), where actinospores are produced, and a myxospore-phase that typically occurs in fishes, which are intermediate hosts. The actinospore is released into the water by the annelid and infects fish through direct contact with the gill epithelium or skin (Lom and Dyková 2006, Rangel et al., 2015, Rangel et al., 2016a,b, Atkinson et al., 2019).

Commercial fishes infected by *Kudoa* spp. can be rejected by consumers due to the presence of whitish or black hypertrophic muscular cells, repugnant appearance, or soft texture (*post-mortem* myoliquefaction) induced by these parasites, causing important commercial losses. However, the impact of some species of *Kudoa* on human health is more serious. Several cases of gastrointestinal problems, such as vomiting and diarrhea, were observed after ingestion of raw portions of fish infected with *Kudoa septempunctata* Matsukane, Sato, Tanaka, Kamata et Sugita-Konishi, 2010 (see Kawai et al., 2012, Iwashita et al., 2013). Since 2003 there has been an increase in raw fish consumption and consequently cases of food poisoning caused by *Kudoa* spp. (Kawai et al., 2012).

Myxozoans are generally specific parasites with a few exceptions, which include *Kudoa thyrsites* (Gilchrist, 1924) reported thus far from 40 fish species representing 18 families (Whipps and Kent 2006). This species is associated with *post-mortem* myoliquefaction in a wide range of host species. The analysis of genetic structure of *K. thyrsites* suggested a correlation of genetic samples with their geography and the existence of significant barriers in gene flow at a global scale that may lead to geographic isolation of regionally endemic populations or even emergence of cryptic species (Whipps and Kent 2006).

Scombrids are very important for fisheries in the Northeast Atlantic, with tunas and Atlantic mackerels in the ranking of the most landed species in the Portuguese mainland coast and the Madeira archipelago (INE 2018). However, to the best of our knowledge, studies on the occurrence of *Kudoa* in marine fishes have been carried out only in the Portuguese mainland coast in *T. trachurus* and *S. pilchardus* (Cruz et al., 2003, 2011). There is also a report of the presence of *Kudoa* sp. in *Scomber colias* Gmelin from the Portuguese mainland coast (Alves 2016).

Considering gaps in the knowledge of the occurrence of *Kudoa* spp. in the Northeast Atlantic, the aim of this study was to evaluate the presence and infection levels of these myxozoans in marine fishes from the Portuguese mainland coast and, in particular, from the Madeira Archipelago. We focus mainly on scombrid fish because of their importance to local fisheries (Hermida and Delgado 2016), as well as on a variety of other fish species to provide a better picture of the occurrence of species of *Kudoa* in this region.

Materials and methods

Sampling

A total of 250 fishes from 19 species were sampled for detection of spores of *Kudoa* spp., between May 2016 and October 2017. The main focus of the study were scombrids from the Madeira Archipelago in the North Atlantic Ocean: 22 albacore *Thunnus alalunga* (Bonnaterre), 33 Atlantic chub mackerel *Scomber colias*, 30 bigeye tuna *Thunnus obesus*, and 30 skipjack tuna *Katsuwonus pelamis* (Linnaeus) (Table 1).

Furthermore, a mixed sample of different species of fishes were included in this study to carry out a comprehensive survey of *Kudoa* spp. in important commercial species from the Madeira Archipelago: three grey triggerfish *Balistes capriscus* Gmelin, one barred hogfish *Bodianus scrofa* (Valenciennes), five leafscale gulper shark *Centrophorus squamosus* (Bonaterre), three pink dentex *Dentex gibbosus* (Rafinesque), one sharktooth moray *Gymnothorax maderensis* (Johnson), one island grouper *Mycteroperca fusca* (Lowe), one black gemfish *Nesiarchus nasutus* Johnson, two forkbeard *Phycis phycis* (Linnaeus), one wreckfish *Polyprion americanus* (Bloch et Schneider), 16 greater amberjack *Seriola dumerili* (Risso), 30 longfin yellowtail *Seriola rivoliana* Valenciennes, eight blacktail comber *Serranus atricauda* Günther, two red scorpionfish *Scorpaena scrofa* Linnaeus, and one yellowmouth barracuda *Sphyraena viridensis* Cuvier. In addition, 30 Atlantic chub mackerel *S. colias* and 30 blue whiting *Micromesistius poutassou* (Risso) from the northern part of the Portuguese mainland coast (Matosinhos) were also examined. Total length (TL), fork length (FL) and weight (W) were determined, and muscle samples were collected from each fish.

Parasitological examination

The method used to detect spores of *Kudoa* spp. was the one proposed by Saraiva et al., (2017). One gram of frozen dorsal muscle collected just posterior to the head of each fish was used, except for specimens of *S. colias* and *M. poutassou*, from which one gram of frozen muscle from each region of the fish (anterior, middle and posterior) was used. The muscle was placed on a lip of a Petri dish, moistened with 5 ml of phosphate buffered saline (PBS), macerated with a scalpel and squashed with the base of the Petri dish. The squashed muscle was initially observed under a stereomicroscope for detection of infected hypertrophic muscle cells. Afterwards, the liquid was squeezed out into a centrifuge tube, allowing the spore suspension to settle for 30 minutes. Three drops of liquid (~ 25 µl) were then pipetted from the bottom of the tube to a microscope slide and covered with a coverslip.

Slides were observed under a Differential Interference Contrast (DIC) microscope at 400× magnification. Detected kudoid spores were photographed with a camera integrated into the microscope and measured according to Burger and Adlard (2010). Spore width and thickness and polar capsules width and length were measured in spores in apical view and spore length was measured in spores in lateral view.

Data analysis

Mean and standard deviation were calculated for host parameters (TL, FL, and W). Prevalence, mean intensity and mean abundance of spores were determined based on definitions proposed by Bush et al., (1997), except that the quantification was done not on the host but on a sample of host muscle obtained by the methodology described above.

Statistical analyses were carried out using IBM SPSS 25 statistics software (IBM 2017). Differences in parasite abundance among the three regions of *M. poutassou* and *S. colias* were compared using non-parametric tests for related samples. The Cochran test was used for occurrence comparisons and Friedman's analysis of variance by ranks for abundance comparisons. For all tests, statistical significance was accepted when $p < 0.05$.

DNA extraction, PCR amplification and sequencing

Total DNA was extracted from samples using a standard phenol-chloroform protocol, after an overnight digestion with proteinase K (50 µg ml-1; Serva, Heidelberg, Germany), at 55 °C (Holzer et al., 2004). DNA was resuspended in 50–100 µl−1 of DNAse-free water and left to dissolve overnight at 4 °C. PCRs were performed using an AccuPower® PCR PreMix (Bioneer, Daejeon, South Korea) with 25 pmol of each primer, 18 µl of water and 1 µl (approx. 100 ng) of extracted DNA.

Initially, we used a primer pair MyxospecF+18R (Whipps et al., 2003, Fiala 2006). This approach failed to give a positive PCR result for the sample from *M. poutassou* as well as for samples from *S. colias* from Madeira. Therefore, nested PCR was performed with initial primer pair 18e + 18g (Hillis and Dixon 1991) followed by PCR with specific *Kudoa* primer pair KUD1f + KUD2r (Hervio et al., 1997) or semispecific KUD1f + 18g.

Cycling parameters were set up as follows: initial PCR run: denaturation 95 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min s, 72 °C for 2 min and after cycles a terminal extension at 72 °C for 10 min; II run: denaturation 95 °C for 3 min, followed by 35 cycles of 94 °C for 40 s, 55 °C for 50 s, 72 °C for 1 min 40 s with terminal extension at 72 °C for 10 min. PCR products were purified using a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan) and

sequenced directly (SeqMe, Dobříš, Czech Republic). Sequences were deposited in GenBank under the accession numbers MT991409 and MT991410.

Phylogenetic analysis

Based on an initial BLAST search of SSU rDNA, sequences from *M. poutassou* and *S. colias* showed a high match score with *K. thyrsites*. Therefore, we retrieved nucleotide sequences of *K. thyrsites* and its related species from GenBank and aligned them with the newly obtained sequence using MAFFT v7.450 algorithm (Katoh et al., 2013) implemented in Geneious v8.0.5 (Kearse et al., 2012) using the E-INS-i multiple alignment meth od, with a gap opening penalty (–op) of 1.5 and gap extension penalty (–ep) of 0.123. Maximum likelihood (ML) analysis was performed in RAxML v7.0.3 (Stamatakis 2006) using the GTR + Γ model of nucleotide substitution. *Kudoa lateolabracis* Yokoyama, Whipps, Kent, Mizuno et Kawakami, 2004 was set as an outgroup. Bootstraps were based on 1,000 replicates. The intraspecific differences were determined from proportional distances of SSU rDNA, which were calculated in Geneious (p-dist) from the dataset previously used for the phylogenetic analysis.

Results

Taxonomic and biometric data of all 250 examined fishes are detailed in Table 1. From all examined specimens from the Madeira Archipelago, no hypertrophic muscle cells were evident macroscopically. Only a single spore of a species of *Kudoa* was found in one specimen of *Thunnus obesus* by light microscopy. The spore was stellate in apical view and had four pyriform capsules of unequal size (Fig. 1). We found 18 out of 30 *Scomber colias* and 24 out of 30 *Micromesistius poutassou* individuals positive for infection with species of *Kudoa* (60% and 80% prevalence, respectively). In both cases, spores were observed in the muscle from all three sampled body regions (anterior, middle and posterior), and infection levels varied between them (Table 2). However, in *S. colias*, no significant differences were observed either in the occurrence or in the abundance (measured as the number of spores in the three drops of liquid examined) among the three regions. Likewise, no significant differences were detected in the occurrence of parasites in *M. poutassou*, but significant differences in abundance were detected among regions ($p = 0.040$). Spores of *Kudoa* sp. found in *S. colias* (Fig. 2) and *M. poutassou* (Fig. 3) were stellate in apical view and had four pyriform polar capsules of three different sizes: one large, two intermediate and one small. Myxospore measurements from spores detected in *T. obesus* and *M. poutassou* are shown in Table 3.

Table 1. Fish species examine from the Madeira archipelago and the Portuguese mainland coast, number of specimens (n) and their biological parameters: total length (TL), fork length (FL) and weight (W). Values of measured biological parameters are listed as a mean ± standard deviation.

Species	n	TL (cm)	FL (cm)	W (g)
Madeira Archipelago				
Balistes capriscus	3	48.5 ± 1.6	41.8 ± 3.9	1,822 ± 346
Bodianus scrofa	1	51.0	N/A	2362
Centrophorus squamosus	5	107.0 ± 4.4	N/A	6,205 ± 918
Dentex gibbosus	3	88.4 ± 11.0	79.4 ± 9.7	9,211 ± 2749
Gymnothorax maderensis	1	90.0	a	1589
Katsuwonus pelamis	30	53.6 ± 3.3	50.6 ± 3.1	2,869 ± 642
Mycteroperca fusca	1	69.0	65.7	4533
Nesiarchus nasutus	1	127.0	N/A	5385
Phycis phycis	2	51.9 ± 3.0	N/A	1,576 ± 117
Polyprion americanus	1	64.7	N/A	4565
Scomber colias	33	24.1 ± 4.5	21.9 ± 4.0	128 ± 110
Scorpaena scrofa	2	44.0 ± 3.7	N/A	1,655 ± 208
Seriola dumerili	16	110.5 ± 33.7	99.1 ± 29.6	17,505 ± 9417
Seriola rivoliana	30	52.2 ± 13.6	46.4 ± 11.8	2,344 ± 3842
Serranus atricauda	8	30.6 ± 3.2	N/A	385 ± 178
Sphyraena viridensis	1	44.0	38.4	313
Thunnus alalunga	22	95.1 ± 4.7	87.9 ± 3.8	14,992 ± 2282
Thunnus obesus	30	85.3 ± 8.9	77.6 ± 9.5	10,654 ± 3399
Portuguese Continental Coast				
Micromesistius poutassou	30	19.4 ± 1.0	17.7 ± 0.9	41 ± 9.6
Scomber colias	30	28.4 ± 3.1	26.2 ± 3.0	178 ± 64

N/A - not available

Fig. 1. Spore of* Kudoa *sp. from* Thunnus obesus *(Lowe) from Madeira Archipelago.

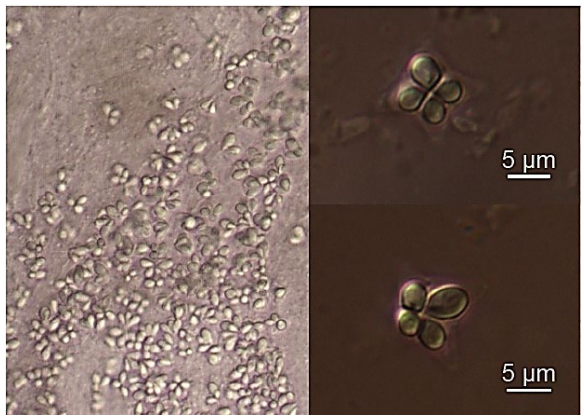

Fig. 2. Spore of* Kudoa *sp. from* Scomber colias *Gmelin from Portuguese mainland coast.

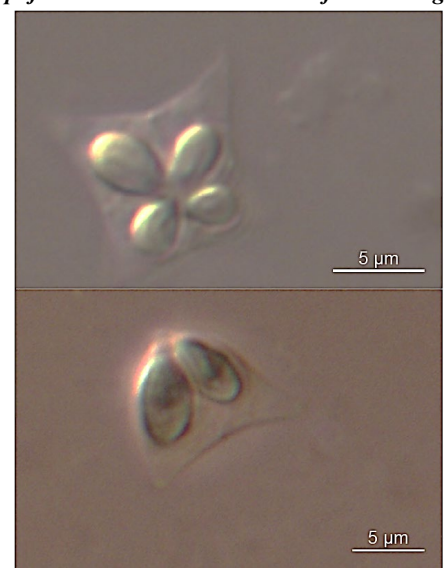

Fig. 3. Spore of* Kudoa *sp. from* Micromesistius poutassou *(Risso) from Portuguese mainland coast.

Both SSU rDNA sequences of *Kudoa* from *S. colias* and *M. poutassou* were identical and revealed 100% identity with *Kudoa thyrsites* (AY078430) from its type species *Thyrsites atun* (Euphrasen) from the coast of South Africa as well as with *K. thyrsites* from *Merluccius capensis* Castelnau from the coast of South Africa (AY941819) and *S. scombrus* from Northern North Sea (EU154349) and the South coast of England, North Sea (AY542482). The comparison was done based on a 1,096 bp and 824 bp long sequence of *Kudoa* sp. from *S. colias* and *M. poutassou*, respectively. Phylogenetic analysis revealed four lineages of *K. thyrsites* genotypes (Fig. 4). *Kudoa* sp. from *S. colias* and *M. poutassou* clustered with sequences of *K. thyrsites* (AY078430,

AY941819, EU154349, AY542482, AY542481) with 91% bootstrap support (Fig. 4). Molecular screening of 33 specimens of *S. colias* from the Madeira Archipelago was negative for infection with *Kudoa* spp.

Table 2. Infection levels of* Kudoa *Meglitsch, 1947 in three muscle regions of Atlantic chub mackerel, Scomber colias Gmelin (n = 30) and blue whiting,* Micromesistius poutassou *(Risso) (n = 30) from the Portuguese mainland coast. P (%), CI – 95% confidence interval, SD (range).

Muscle region	Prevalence (CI) %	Mean intensity (±SD)	Mean abundance (±SD)
S. colias			
Anterior	50 (33–70)	88 ± 219 (1–831)	45 ± 159 (0–831)
Middle	43 (27–63)	137 ± 438 (1–1650)	597 ± 301 (0–1,650)
Posterior	33 (17–50)	140 ± 247 (1–734)	47 ± 153 (0–734)
M. poutassou			
Anterior	60 (43–77)	29 ± 43 (1–144)	18 ± 35 (0–144)
Middle	70 (53–87)	41 ± 63 (1–232)	29 ± 56 (0–232)
Posterior	73 (57–87)	63 ± 94 (1–306)	46 ± 85 (0–306)

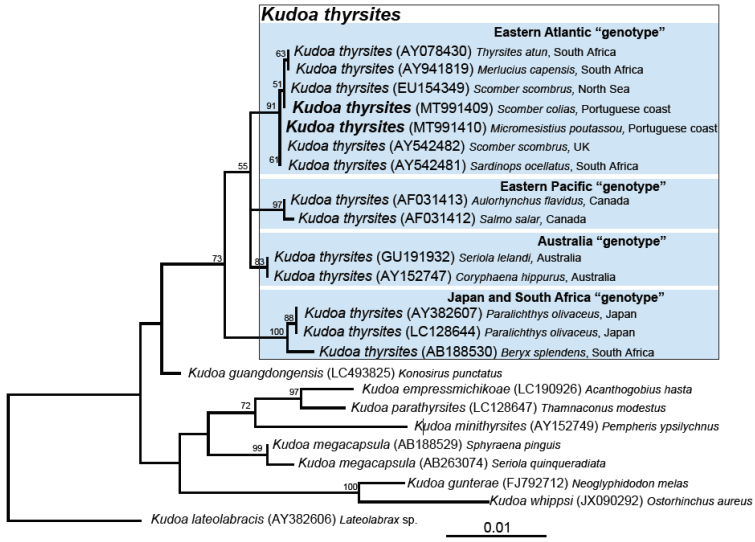

Fig. 4. Maximum likelihood tree of SSU rDNA sequences of* Kudoa thyrsites *(Gilchrist, 1924) and its closely related species. Newly identified sequences are in bold. Maximum likelihood bootstrap nodal supports are shown at every node. GenBank acc. numbers are given after the species names and scale is given under the tree.

Discussion

Only around ten species of *Kudoa* have been described from the northeast Atlantic Ocean out of approximately 100 nominal species (Eiras et al., 2014). In the Portuguese mainland coast, their occurrence was reported in greater amberjack *Seriola dumerili* (*Kudoa insolita* Kovaleva, Shulman et Yakovlev, 1979), Atlantic horse mackerel *Trachurus trachurus* (*Kudoa* sp.) (Cruz et al., 2003) and European pilchard *Sardina pilchardus* (*Kudoa* sp. with morphology and morphometry consistent with *Kudoa thyrsites*) (Cruz et al., 2011). However, there are no studies on the occurrence of *Kudoa* spp. in the Madeira Archipelago. Our survey of 190 fishes of 18 different species from this archipelago seems to indicate the absence/very rare occurrence of parasites of the genus *Kudoa* in this region.

This is the first reference to the occurrence of the genus *Kudoa* in the blue whiting *Micromesistius poutassou*. Based on spore morphology and molecular analysis, this species was also identified as *K. thyrsites*, confirming the euryxenous host specificity of this parasite at the level of intermediate fish hosts. This *Kudoa* sp. as well as *Kudoa* sp. from *Scomber colias* from the Portuguese mainland coast were morphologically identical with *K. thyrsites*, which was confirmed by molecular analysis that showed

100% identity of both sequences with *K. thyrsites* from the type host. SSU rDNA sequences of our samples clustered with sequences of *K. thyrsites* from the eastern Atlantic genotype *sensu* Whipps and Kent (2006), which includes isolates from fish in the Eastern Atlantic.

Table 3. Measurements (W – width; T – thickness; L – length) of the spores detected in the muscle of *Micromesistius poutassou* (Risso) and *Thunnus obesus* (Lowe) in µm.

				Apical view				Side view			
	Spore			Large polar capsule		Small polar capsule		Large polar capsule		Small polar capsule	
	W	T	L	L	W	L	W	L	W	L	W
M. poutoussou											
Mean	15.2	8.3	8.4	4.7	3.2	3.7	2.5	6.1	3.2	5.0	2.6
Sd	0.90	0.67	0.53	0.48	0.25	0.51	0.30	0.61	0.30	0.41	0.23
Minimum	14.0	7.5	7.4	3.6	2.5	3.1	1.3	5.3	2.5	4.0	2.4
Maximum	17.8	10.8	9.0	6.3	3.6	5.9	3.0	6.9	3.7	5.6	3.1
n	30	30	15	35	35	35	35	13	13	12	12
T. obesus	-	7.4	-	4.9	2.7	3.0	2.5	-	-	-	-
n		1		1	1	1	1				

Our phylogenetic analysis including the newly identified *K. thyrsites* from *M. poutassou* and *S. colias* from the Portuguese mainland coast supported the idea that the phylogeny of *K. thyrsites* genotypes correlates with geographic distribution. The host range of *K. thyrsites* is thus broadened to include two economically important fishes, namely Atlantic chub mackerel and blue whiting.

A high prevalence (60%) of *K. thyrsites* detected in *S. colias* from the mainland coast contrasts greatly with no detection of *K. thyrsites* in *S. colias* from the Madeira Archipelago. This divergence is heightened by the almost complete absence of any species of *Kudoa* in fishes from Madeira, which is in accordance with the study of Shukhgalter (2004) who reported *Kudoa* sp. in chub mackerel from Mauritania (a coastal zone) and its absence in specimens from the Azores, where environmental conditions are similar to those in Madeira, which lacks a continental shelf, and where the insular shelf is quite narrow. Results of the present study may also provide additional information about the stock structure of *S. colias* in the Northeastern Atlantic. The presence of *K. thyrsites* in Atlantic chub mackerel from the Portuguese mainland coast and no detection in fish from the Madeira archipelago suggests that the specimens observed may belong to two different populations.

To the best of our knowledge, no life cycle of any species of *Kudoa* has yet been described (Eszterbauer et al., 2015). However, the life cycle is likely similar to that of other genera of the Myxosporea, with the presence of two hosts, annelids (probably a marine polychaete) and fishes. In the Madeira Archipelago, the ocean depth increases steeply at a short distance from the coast; there is no continental shelf, and the insular

shelf is quite narrow. In addition, oceanic waters in this region have low productivity (Hermida and Delgado 2016). These conditions may explain the reduced abundance of benthic organisms, and consequently, it is possible that the occurrence of annelids near the Madeiran coasts is very low, or even that the specific hosts of *Kudoa* spp. might not be present in this region.

Thunnus spp. have been reported as hosts of several species of parasites of the genus *Kudoa* in wild and cultured fishes from Japan (Zhang et al., 2010, Meng et al., 2011, Abe and Maehara 2013). Although Japan is also an archipelago, it has an extended continental shelf, which can provide the ideal environmental conditions for the occurrence of benthic annelids, allowing *Kudoa* spp. to complete their life cycle in their waters. This could explain the difference between the high infection rates by *Kudoa* spp. in fishes from Japan and the extremely low prevalence of *Kudoa* spp. in this study.

Furthermore, the presence of *Kudoa* sp. in *T. obesus* does not indicate that this parasite can complete its life cycle in Madeira, since bigeye tuna is a highly migratory species, and thus could have acquired the infection elsewhere. In the Eastern Atlantic, bigeye tuna could acquire the infection, for example, in the African continental shelf, where this parasite was previously reported by Henning et al., (2013).

The results of this study indicate that the occurrence of *Kudoa* spp. in Madeiran waters appears to be uncommon. This seems to indicate that fishes from the Madeira Archipelago present a limited risk in terms of the danger of *Kudoa* food poisoning. In any case, European Union recommendations of the adequate preparation of fish products should be followed (EFSA 2010).

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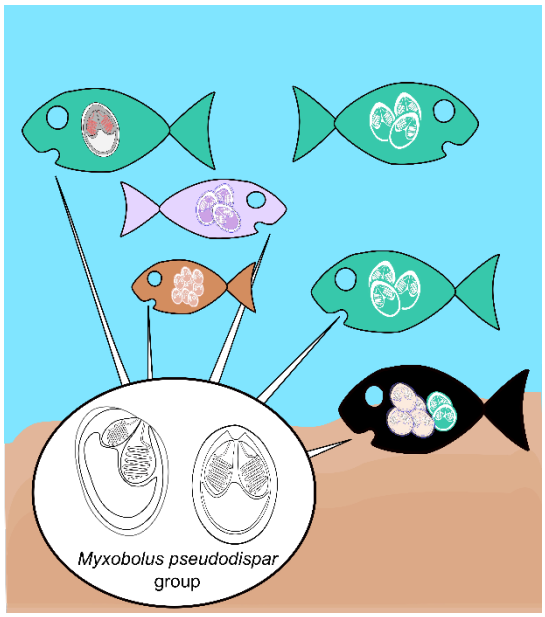
5.3 Paper II

Myxozoan hidden diversity: the case of *Myxobolus pseudodispar* Gorbunova, 1936



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Myxozoan hidden diversity: the case of *Myxobolus pseudodispar* Gorbunova, 1936

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Abstract

Myxobolus pseudodispar Gorbunova, 1936 (Myxozoa) was originally described as a parasite of common roach, *Rutilus rutilus* (Linnaeus), with developing stages in muscles and spores disseminated in macrophage centres of different organs and tissues. Later, this parasite was described from several other cyprinids, but with relatively large intraspecific differences based on SSU rDNA gene sequences. Within our long-term study on myxozoan biodiversity, we performed a broad microscopic and molecular screening of various freshwater fish species (over 450 specimens, 36 species) from different localities. We investigated the cryptic species status of *M. pseudodispar*. Our analysis revealed four new unique SSU rDNA sequences of *M. pseudodispar* as well as an infection in new fish host species. *Myxobolus pseudodispar* sequence analysis showed clear phylogenetic grouping according to fish host criterion forming 13 well-recognised clades. Using 1% SSU rDNA-based genetic distance criterion, at least ten new species of *Myxobolus* Bütschli, 1882 may be recognised in the group of *M. pseudodispar* sequences. Our analysis showed the paraphyletic character of *M. pseudodispar* sequences and the statistical tests rejected hypothetical tree topology with the monophyletic status of the *M. pseudodispar* group. *Myxobolus pseudodispar* represents a species complex and it is a typical example of myxozoan hidden diversity phenomenon confirming myxozoans as an evolutionary very successful group of parasites with a great ability to adapt to new hosts with subsequent speciation events.

Keywords: Myxozoa, phylogeny, PCR screening, cryptic species, host specificity.

Introduction

The Myxozoa is a diverse group of endoparasites with a worldwide distribution numbering more than 2,600 de-scribed species, which represents approx. 20% of cnidarians (Okamura et al., 2015, 2018). However, much of myxo-zoan biodiversity appears to be hidden (Bartošová-Sojková et al., 2014, Hartikainen et al., 2016). Their complex life cycle involves two hosts: invertebrates (Oligochaeta, Polychaeta and Bryozoa) as a definitive host, and vertebrates (mainly fish, rarely amphibians, reptiles, birds or mammals) as an intermediate host (Okamura et al., 2015). Myxozoan infections in fish are frequently inconspicuous; however, several myxozoans are highly pathogenic for their hosts causing whirling disease or proliferative kidney disease in salmonid fish (Hedrick et al., 1993, Gilbert and Granath, 2003).

Myxobolus Bütschli, 1882 is the largest genus of the Myxozoa with more than 850 described species (Eiras et al., 2005, 2014). Species of *Myxobolus* have been reported from fish worldwide (e.g., Carriero et al., 2013, Lövy et al., 2018, Folefack et al., 2019). They infect mostly freshwater fish but they have been found also in brackish and marine hosts (e.g., Liu et al., 2019, Rocha et al., 2019). The diversity success of the *Myxobolus* is assumed to be caused by the shape of spores with lateral flattening that enabled eas-ier invasion of tissues from precursors that lived in body liquids and cavities. Therefore, they could colonise many types of tissues (Fiala et al., 2015). Species of *Myxobolus* constitute the largest clade in the phylogenetic tree branch-ing within the oligochaete-infecting lineage of myxozoans (Holzer et al., 2018). However, it is a paraphyletic taxon with *Henneguya* Thélohan, 1892; *Thelohanellus* Kudo, 1933; *Unicauda* Davis, 1944; *Cardimyxobolus* Ma, Dong et Wang, 1982 and species of *Hennegoides* Lom, Tonguthai et Dyková, 1991 clustering with species of *Myxobolus* (see Fiala 2006, Liu et al., 2019).

The genus *Myxobolus* is morphologically characterised by ovoid or rounded spores with smooth shell valves and with two mostly pyriform, sometimes unequal polar capsules (Lom and Dyková 2006). Myxospore valve projec-tions (appendages) typical for species of *Henneguya*, *Unicauda* and *Hennegoides* appeared or have been lost several times independently in myxobolid evolution (Liu et al., 2019) similarly as a loss of one of the polar capsules, the trait characteristic for species of the genus *Thelohanellus* (see Zhang et al., 2019).

It has been proved many times by phylogenetic analyses based on molecular data that molecular taxonomy of the genus *Myxobolus* and other myxozoan genera does not

entirely reflect morphological similarities of myxozoan spores. Phylogenetic relationships rather correlate with host environ-ment, host and host tissues preferences (Andree et al., 1999, Kent et al., 2001, Eszterbauer 2004, Fiala 2006, Fiala and Bartošová 2010, Holzer et al., 2018). Although a study by Salim and Desser (2000) demonstrated clustering of species of *Myxobolus* according to the morphology of the spores, analysis by Andree et al., (1999) and Eszterbauer (2004) reported relationships based on the site of infection. Host-as-sociated phylogenetic pattern of Myxobolus spp. has been suggested by Carriero et al., (2013) as well as by Liu et al., (2019), who made a comprehensive analysis of all available sequences of SSU rDNA of *Myxobolus* spp.

Myxobolus pseudodispar Gorbunova, 1936 was origi-nally described as a common myxozoan parasite of com-mon roach (*Rutilus rutilus* [Linnaeus]) with developing stages in muscles and spores disseminated in macrophage centres of different organs and tissues. Since the original description, *M. pseudodispar* has been documented from different organs (gills, kidney, liver, muscle, skin and spleen) of many cyprinids e.g., *Abramis brama* (Linneaus); *Alburnus alburnus* (Linnaeus); *Blicca bjoerkna* (Linnaeus); *Pseudochondrostoma polylepis* (Steindachner); *Squalius squalus* (Bonaparte); *Achondrostoma arcasii* (Steindachner); *Scardinius erythrophthalmus* (Linnaeus) (Gorbunova 1936, Gonzalez-Lanza and Alvarez-Pellitero 1985, Baska 1987, Eszterbauer et al., 2001, Székely et al., 2001, Molnár et al., 2002).

Spores of *M. pseudodispar* are ovoid or rounded with an irregular shape. Unequal polar capsules are located laterally from the longitudinal axis of the spore, which is the character distinguishing *M. pseudodispar* from morphologically very similar *Myxobolus dispar* (Gorbunova 1936). *Myxobolus pseudodispar* was considered as a junior synonym of *Myxobolus cyprini* Doflein, 1898 because of great morpho-logical variability of their spores (Lom and Dyková 1992). However, Molnár et al., (2002), based on SSU rDNA analysis, suggested that *M. pseudodispar* is a valid species. This work also indicated that muscle-dwelling parasites cluster according to fish host species. Forró and Eszterbauer (2016) confirmed this and defined five different clades of *M. pseudodispar* correlating with fish host species.

The SSU rDNA sequence differences among these clades suggested cryptic species status of *M. pseudodispar.* Cryptic species phenomenon was also reported for other *Myxobolus* spp. (e.g., Rocha et al., 2019) and for other myxozoan genera e.g., *Chloromyxum* Mingazzini, 1890; *Sphaerospora* Thélohan, 1892; *Zschokkela* Auerbach, 1910 (see Bartošová and Fiala 2011, Holzer et al., 2013). Moreover, the study of Molnár et al., (2002) indicated that morphologically similar species of *Myxobolus* from different

fishes seem to be different species. In contrast, Guo et al., (2018) suggested that SSU rDNA difference as low as 2.4% does not necessarily define conspecificity.

In the present study, we aimed to analyse data from a large-scale screening of freshwater fishes to resolve the cryptic species status of *M. pseudodispar*. We assess the host specificity of each *M. pseudodispar* clade and trace the myxospore morphology of *M. pseudodispar* and relat-ed species on the evolutionary tree.

Material and methods

Sample collection and light microscopy

During our large-scale research on the biodiversity of myxozoan parasites of freshwater fish in Europe, overall, 452 fish (36 species from different fish orders) were collected in ponds, dams and rivers in the Czech Republic (408) and in the Danube River (44) in Bulgaria during the years 2016–2018. Host species numbers are summarised in Supplementary Table 1.

Fish from the Czech Republic were parasitologically examined using light microscopy (Olympus BX51 microscope) at 400× magnification to detect the presence of spores, plasmodia or other development stages of Myxozoa in gills, kidney and gall bladder. Other host tissues were not included in the examination, and therefore myxosporean plasmodia in muscles or skin could not be formed. Digital photos of fresh spores were taken at 1000× magnification using an Olympus DP70 camera. Spores were measured from digital images using ImageJ 1-48q (Wayne Ras-band, http://imagej.nih.gov/ij). Gills, kidneys and gall bladders of fish from Bulgaria were fixed only for molecular processing.

DNA isolation, PCR, cloning and sequencing

All obtained fish tissue samples were kept in 400 µl of TNES urea buffer (10 mM Tris-HCl with pH 8; 125 mM NaCl; 10 mM EDTA; 0.5% SDS and 4 M urea). Genomic DNA extraction was performed by standard phenol-chloroform extraction method with four-hour digestion with proteinase K (50 µg ml-1 180; Serva, Heidelberg, Germany) at 55 °C and final elution in 100 µl of DNAse-free water (Holzer et al., 2004).

We screened all obtained DNA samples for myxozoan infection using nested PCR by general myxozoan primer combination sets for partial SSU rDNA: 18e+18g (Hillis and Dixon 1991) in the first run; MyxGP2 F (Kent et al., 1998) + Act1R (Hallett and Diamant 2001) in the second run. The PCR cycling parameters were set up for first run:

denaturation 95 °C for three min, followed by 35 cycles of three steps: 95 °C for one min, 62 °C for one min, 72 °C for two min, and after cycles a final extension 72 °C for ten min; second run: denaturation 95 °C for three min, followed by 35 cycles of three steps: 95 °C for 40 s, 56 °C for 50 s, 72 °C for one min 40 s with a final incubation 72 °C for ten min (details in Table 1).

Table 1. List of primers used for PCR screening including sequence of primers.

Primer name	Sequence (5'→3')	Annealing temperature, ˚C	References
18e	TGGTTGATCCTGCCAGT	64	Hillis and Dixon (1991)
18g	GGTAGTAGCGACGGGCGG TGTG		
MyxGP2 F	WTGGATAACCGTGGGAA A	58	Kent et al., (1998)
Act1R	AATTTCACCTCTCGCTGCC A		Hallett and Diamant (2001)
CobF424	GGWTAYGTWYTWCCWTG RGGWCARAT	50	Boore and Brown (2000)
CobR876	GCRTAWGCRAAWARRAA RTAYCAYTCWGG		

Determination of the host species was verified by PCR and sequencing if the host identification was doubtful. We used CobF424 + CobR876 primers (Boore and Brown 2000) for amplification of partial mitochondrial cytochrome b gene. The PCR cycling parameters were set up as follows: denaturation 95 °C for three min, followed by 30 cycles of three steps: 95 °C for one min, 50 °C for one min, 72 °C for one min with a final incubation 72 °C for five min.PCRs of the partial SSU rDNA of the parasite and cytochrome b gene of the host were performed using an AmpOne HS-Taq premix (GeneAll Biotechnology Co., Ltd., Seoul, South Korea) with 10 µl of AmpOne HS-Taq premix, 0.5 µl of each primer (25 pmol), 8.5 µl of water and 0.5 µl of extracted DNA (100–300 ng/µl). Obtained PCR products were purified by Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., New Taipei, Taiwan) and sequenced directly by Sanger sequencing (Seqme, Dobříš, Czech Republic). PCR products were cloned if the direct sequencing revealed myxozoan coinfections. PCR fragments were cloned into the pDrive vector using a PCR Cloning Kit (Qiagen, Hilden, Germany) and then transformed into competent *Escherichia coli* cells (Life Technologies, Prague, Czech Republic). Cloned DNA plasmids were extracted and purified by the High Pure Plasmid Isolation Kit (Roche Applied Science, Penzberg, Germany) and three colonies of each plasmid were sequenced by Sanger sequencing (Seqme).

Alignments and phylogenetic analyses

Two alignments were constructed to reveal phylogenetic relationships of myxobolid SSU rDNA sequences. The first alignment composed of 117 myxosporean partial SSU rDNA, from which 81 sequences were newly obtained in this study and 36 sequences were retrieved from GenBank. The second alignment consisted of the selected sequences representing the groups of very closely related sequences with identical or almost identical sequences. Sequences in both datasets were aligned in MAFFT v7.017 (Katoh et al., 2005) using E-INS-i multiple alignment method implemented in Geneious v11.0.3 (Kearse et al., 2012). The alignments were manually edited and ambiguously aligned regions were removed.

Phylogenetic analysis was done by Bayesian inference (BI) and maximum likelihood (ML) methods. ML analysis was performed with RAxML v7.2.8 (Stamatakis 2006) with a GTR + Γ model selected by jModelTest2 (Posada 2008). Bootstrap supports were calculated from 1,000 replications. BI was performed in MrBayes v3.0 (Ronquist and Huelsenbeck 2003) with the GTR + Γ model of evolution. MrBayes was run to estimate posterior probabilities over one million generations via two independent runs of four simultaneous Markov Chain Monte Carlo (MCMC) algorithms with every 100th tree saved. Species-specific divergences were identified from proportional distances (in %) which were calculated in program Geneious based on the SSU rDNA dataset of all sequences under study.

Topology test

We used TreeGraph v2.0.47-206 beta (Stöver and Müller 2010) to generate constrained alternate tree topology from the phylogenetic analysis. Designed alternate topology in Newick format was specified in the assumption block of the PAUP* program (Swofford 2001). The dataset with selected ML parameters was executed in PAUP* to generate likelihood scores of the best topology and the constrained tree. Resulted per-site log likelihood scores were analysed for significant differences in CONSEL v6.1 (Shimodaira and Hasegawa 2001), using three likelihood-based tests: approximately unbiased (AU), Kishino-Hasegawa (KH), and Shimodaira-Hasegawa (SH).

Results

Morphology of Myxobolus pseudodispar

Myxozoan spores corresponding to the morphological diagnosis of *Myxobolus pseudodispar* were observed microscopically in fresh tissue preparations of four cypriniform fish: *Abramis brama*, *Gobio gobio* (Linnaeus), *Phoxinus phoxinus* (Linnaeus) and *Rutilus rutilus* (Fig. 1). Plasmodia of the *Myxobolus* origin were observed in gills of *A. brama*, in kidney and gills of *R. rutilus* and in kidney of *P. phoxinus* and in gills of *Scardinius erythrophthalmus* (see Table 2).

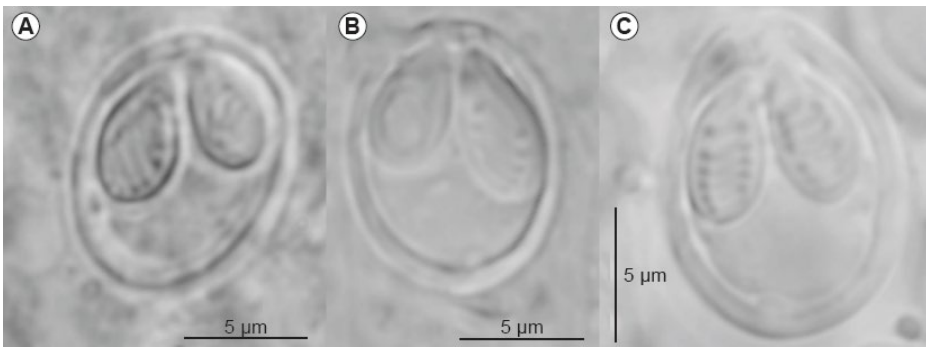

Fig. 1 Myxobolid spores obtained from the kidneys of different fish hosts. **A** – spore of *Myxobolus* sp. from *Rutilus rutilus* (Linnaeus) from Rájský pond, CZ; **B** – spore of *Myxobolus* sp. from *Phoxinus phoxinus* (Linnaeus) from Hostačovka brook, CZ; **C** – spore of *Myxobolus* sp. from *Gobio gobio* (Linnaeus) from Hostačovka brook, CZ.

Spore dimensions mean with standard deviation were measured for myxobolid spores from (i) *R. rutilus* from Švihov Reservoir (RRZE1b, n = 9) with length 10.34 µm (± 0.61 SD), width 6.95 (± 0.32 µm), polar capsules length 4.91 µm (± 0.67 SD) and width 3.12 µm (± 0.86 SD); (ii) *R. rutilus* from Rájský pond (RRRA3 + RRRA9, n=5) with length 10.06 µm (±0.62 SD), width 7.59 µm (± 0.96 SD) and polar capsules length 4.77 µm (± 0.67 SD) and width 2.89 µm (± 0.31 SD); (iii) *P. phoxinus* from Hostačovka brook (PPHV1, n = 5) with length 10.85 µm (±0.92 SD), width 8.93 µm (± 0.14 SD) and polar capsules length 5.14 µm (± 1.1 SD) and width 3.09 µm (± 0.38 SD); iv) *G. gobio* from Hostačovka brook (GGHV3, n=7) with length 11.7 µm (±0.2 SD), width 8.3 µm (± 0.8 SD) and polar capsules length 5.5 µm (± 0.2 SD) and width 2.9 µm (± 0.3 SD) (see Table 3 for spore dimension details of all myxobolids included in the phylogenetic analysis).

Table 2. Prevalence of *Myxobolus* spp. from *Myxobolus pseudodispar* group in individual host species screened in present study including localities and their coordinates.

Host fish	Nr of fish	Locality	Coordinates	Positive in total	PCR screening	Prevalence in total	Spores/ Plasmodia observed	Clade
***Abramis brama* (Linnaeus)**	3	Obecník pond, CZ	49°48'46.440"N; 15°28'28.920"E	0% (0/3)	gills=0/3; kidney=0/3	36.1% (13/36)	*NA*	*Abramis* clade
	3	Římov Reservoir, CZ	48°49'58.440"N; 14°29'0.960"E	0% (0/3)	gills=0/3; kidney=0/3		*NA*	
	29	Švihov Reservoir, CZ	49°40'27.480"N; 15°9'48.600"E	44.8% (13/29)	bile=0/3; gills=3/29; kidney=12/29		Spores in kidney	
	1	Horusický pond, CZ	49°9'22.320"N; 14°40'28.200"E	0% (0/1)	gills=0/1		*NA*	
***Blicca bjoerkna* (Linnaeus)**	6	Švihov Reservoir, CZ	49°40'27.480"N; 15°9'48.600"E	0% (0/6)	gills=0/6; kidney=0/6	27.3% (3/11)	*NA*	*Blicca/Vimba* clade
	5	Novo Selo, Danube river, BG	44°09´46"N; 22°47´17"E	60% (3/5)	gills=3/5; kidney=3/5		*NA*	
	6	Hostačovka brook, CZ	49°48'56.707"N; 15°31'48.786"E	33.3% (2/6)	gills=0/6; kidney=2/6	16.7% (2/12)	Spores in kidney	*Gobio* clade

Host fish	Nr of fish	Locality	Coordinates	Positive in total	PCR screening	Prevalence in total	Spores/ Plasmodia observed	Clade
***Gobio gobio* (Linnaeus)**	3	Švihov Reservoir, CZ	49°40'27.480"N; 15°9'48.600"E	0% (0/3)	gills=0/3; kidney=0/3		*NA*	
	2	Klíčava Reservoir, CZ	50°4'11.640"N; 13°55'48.000"E	0% (0/2)	gills=0/2; kidney=0/2		*NA*	
	1	Jihlava river, CZ	49°12'45.714"N; 15°56'44.319"E	0% (0/1)	gilss=0/1; kidney=0/1		*NA*	
***Leuciscus idus* (Linnaeus)**	10	Hostačov pond, CZ	49°50'51.720"N; 15°29'44.160"E	0% (0/10)	gills=0/9; kidney=0/10	6.3% (1/16)	*NA*	*Leuciscus idus* clade
	2	Švihov Reservoir, CZ	49°40'27.480"N; 15°9'48.600"E	0% (0/2)	gills=0/2; kidney=0/2		*NA*	
	1	Lužnice river, CZ	49°3'14.760"N; 14°45'46.800"E	0% (0/1)	gills=0/1; kidney=0/1		*NA*	
	1	Novo Selo, Danube river, BG	44°09´46"N; 22°47´17"E	100% (1/1)	gills=1/1; kidney=1/1		*NA*	
	1	Topolovec, Danube river, BG	43°56'27.395"N; 22°50'21.820"E	0% (0/1)	gills=0/1; kidney=0/1		*NA*	

Host fish	Nr of fish	Locality	Coordinates	Positive in total	PCR screening	Prevalence in total	Spores/ Plasmodia observed	Clade
***Leuciscus leuciscus* (Linnaeus)**	2	Jihlava river, CZ	49°12'45.714"N; 15°56'44.319"E	0% (0/2)	gills=0/2; kidney=0/2	20% (1/5)	*NA*	*Leuciscus leuciscus* clade
	3	Římov Reservoir, CZ	48°49'58.440"N; 14°29'0.960"E	33.3% (1/3)	gills=1/3; kidney=1/2		*NA*	
***Perca fluviatilis* Linnaeus**	3	Hostačov pond, CZ	49°50'51.720"N; 15°29'44.160"E	0% (0/3)	gills=0/3; kidney=0/3	5.3% (2/38)	*NA*	*Abramis* clade
	10	Milada Lake, CZ	50°39'14.040"N; 13°56'52.440"E	0% (0/10)	gills=0/10; kidney=0/10		*NA*	
	4	Obecník pond, CZ	49°48'46.440"N; 15°28'28.920"E	0% (0/4)	gills=0/4; kidney=0/4		*NA*	
	4	Rájský pond, CZ	49°49'45.840"N; 15°28'5.880"E	0% (0/4)	gills=0/4; kidney=0/4		*NA*	
	3	Římov Reservoir, CZ	48°49'58.440"N; 14°29'0.960"E	0% (0/3)	gills=0/3; kidney=0/1		*NA*	
	7	Švihov Reservoir, CZ	49°40'27.480"N; 15°9'48.600"E	14.3% (1/7)	gills=0/7; kidney=1/7		*NA*	
	6	Zlatý brook, CZ	49°7'46.188"N; 13°34'43.040"E	16.7% (1/6)	gills=1/6; kidney=1/6		Plasmodia in gills	

Host fish	Nr of fish	Locality	Coordinates	Positive in total	PCR screening	Prevalence in total	Spores/ Plasmodia observed	Clade
	1	Horusický pond, CZ	49°9'22.320"N; 14°40'28.200"E	0% (0/1)	gills=0/1		*NA*	
***Phoxinus phoxinus* (Linnaeus)**	14	Hostačovka brook, CZ	49°48'56.707"N; 15°31'48.786"E	71.4% (10/14)	gills=4/14; kidney=7/14; muscle=1/1		Spores in kidney and gills; plasmodia in kidney	
	2	Černá river, CZ	48°43.44528'N; 14°35.60745'E	50% (1/2)	gills=0/2; kidney=0/2; skin=1/1	64.7% (11/17)	Spores on skin	*Phoxinus* clade
	1	Hostačov pond, CZ	49°50'51.720"N; 15°29'44.160"E	0% (0/1)	gills=0/1; kidney=0/1		*NA*	
***Rutilus rutilus* (Linnaeus)**	15	Švihov Reservoir, CZ	49°40'27.480"N; 15°9'48.600"E	81.3% (13/16)	gills=6/16; kidney=6/16 (*Rutilus* clade 2); gills=2/16; kidney=2/16 (*Rutilus* clade 1)	21.4% (9/42)	Spores in gills and in kidney; plasmodia in gills (*Rutilus* clade 2); Spores in kidney (*Rutilus* clade 1)	*Rutilus* clade 1/2
	5	Hostačovka brook, CZ	49°48'56.707"N; 15°31'48.786"E	0% (0/5)	gills=0/5; kidney=0/5		*NA*	
	12	Rájský pond, CZ	49°49'45.840"N; 15°28'5.880"E	83.3% (10/12)	gills=7/12; kidney=5/8		Spores in gills and in kidney;	

Host fish	Nr of fish	Locality	Coordinates	Positive in total	PCR screening	Prevalence in total	Spores/ Plasmodia observed	Clade
					(*Rutilus* clade 2)		plasmodia in gills (*Rutilus* clade 2)	
	3	Římov Reservoir, CZ	48°49'58.440"N; 14°29'0.960"E	0% (0/3)	gills=0/3; kidney=0/3		*NA*	
	2	Zlatý brook, CZ	49°7'46.188"N; 13°34'43.040"E	100% (2/2)	gills=0/2; kidney=2/2 (*Rutilus* clade 1)		Spores in kidney (*Rutilus* clade 1)	
	2	Obecník pond, CZ	49°48'46.440"N; 15°28'28.920"E	100% (3/3)	gills=0/3; kidney=1/3 (*Rutilus* clade 2); gills=1/3; kidney=1/3 (*Rutilus* clade 1)	45.2% (19/42)	*NA*	
	1	Horusický pond, CZ	49°9'22.320"N; 14°40'28.200"E	0% (0/1)	gills=0/1		*NA*	
	16	Obecník pond, CZ	49°48'46.440"N; 15°28'28.920"E	31.3% (5/16)	gills=3/16; kidney=2/16	29.4% (5/17)	*NA*	*Rutilus* clade 1

Host fish	Nr of fish	Locality	Coordinates	Positive in total	PCR screening	Prevalence in total	Spores/ Plasmodia observed	Clade
***Sander lucioperca* (Linnaeus)**	1	Švihov Reservoir, CZ	49°40'27.480"N; 15°9'48.600"E	0% (0/1)	gills=0/1; kidney=0/1		*NA*	
***Scardinius erythrophthalmus* (Linnaeus)**	2	Bolevecký pond, CZ	49°46'25.680"N; 13°23'54.240"E	50% (1/2)	gills=1/2; kidney=0/2	3.8% (1/26)	Plasmodia in gills	*Rutilus* clade 1
	5	Obecník pond, CZ	49°48'46.440"N; 15°28'28.920"E	0% (0/5)	gills=0/5; kidney=0/5		*NA*	
	8	Rájský pond, CZ	49°49'45.840"N; 15°28'5.880"E	0% (0/8)	gills=0/8; kidney=0/8		*NA*	
	6	Švihov Reservoir, CZ	49°40'27.480"N; 15°9'48.600"E	0% (0/6)	gills=0/6; kidney=0/6		*NA*	
	4	Nežárka river	49°9'24.480"N; 14°46'5.520"E	0% (0/4)	gills=0/4; kidney=0/4		*NA*	
***Sabanejewia bulgarica* (Drensky)**	5	Novoselo, Danube river, BG	44°09´46"N; 22°47´17"E	20% (1/5)	gills=1/5; kidney=0/5	20% (1/5)	*NA*	*Abramis* clade
	2	Vodňany pond, CZ	49°9'17.458"N; 14°9'39.708"E	50% (1/2)	gills=0/2; kidney=0/1	85.7% (6/7)	*NA*	*Blicca/Vimba* clade

Host fish	Nr of fish	Locality	Coordinates	Positive in total	PCR screening	Prevalence in total	Spores/ Plasmodia observed	Clade
***Vimba vimba* (Linnaeus)**	5	Novoselo, Danube river, BG	44°09′46"N; 22°47′17"E	100% (5/5)	gills=4/5; kidney=5/5		*NA*	

Host species spectrum and coinfections

We screened using PCR 452 fish from several localities in the Czech Republic and two localities in Bulgaria and subsequently sequenced positive PCR products (Supplementary file 1). The highest prevalence of *M. pseudodispar* was detected in vimba bream, *Vimba vimba* (Linnaeus) (86%; 6/7), common roach, *R. rutilus* (67%; 28/42) and common minnow, *P. phoxinus* (65%; 11/17). Lower prevalence has been found in other cyprinids: freshwater bream *A. brama* (36%; 13/36) white bream, *Blicca bjoerkna* (27%; 3/11) gudgeon, *G. gobio* (17%; 2/12) ide, *Leuciscus idus* (Linnaeus) (6%; 1/16) common dace, *Leuciscus leuciscus* (Linnaeus) (20%; 1/5), *Sabanejewia bulgarica* (Drensky) (20%; 1/5), and common rudd, *S. erythrophthalmus* (3.8%; 1/26). Myxozoans were detected in two perciform fish: Eu-ropean perch, *Perca fluviatilis* Linnaeus (5%; 2/38) and pikeperch, *Sander lucioperca* (Linnaeus) (29%; 5/17) (Table 2). One sample of the gall bladder from *P. phoxinus* was PCR positive for *M. pseudodispar,* which may represent detection of pre-sporogonic stages of this parasite since we did not observe any spores and plasmodia in the bile. *Myxobolus diversicapsularis* Slukhai in Shulman, 1966; *M. elegans* Kashkovski in Shulman, 1966; *M. parviformis* Kallert, Eszterbauer, Erseus, El-Matbouli et Haas, 2005; *Myxidium* spp.; *Henneguya* sp. and *Chloromyxum* sp. were morphologically and molecularly identified in coinfections with *M. pseudodispar.*

Table 3 Details of all myxobolids included in the phylogenetic analyses inclusive name of the host, fish organ or tissue, GenBank accession numbers for parasite taxa and spore dimensions (length x width).

Myxozoan species	Host	Fish organ or tissue	GenBank Acc No	Spore dimensions (length x width) ± standart deviation	Reference
***M. artus* Akhmerov, 1960**	*Cyprinus carpio*	Skeletal muscle	FJ710799	*NA*	Zhang et al., (2010)
***M. bartai* Salim et Desser, 2000**	*Notropis cornutus*	Body wall musculature	AF186835	11.0 (10.3–11.4) × 10.8 (10.0–11.3)	Salim and Desser (2000)
***M. bhadrensis* Seenappa et Manohar, 1981**	*Labeo rohita*	Kidney	KX832046	10.0 ± 0.41 (9.2–10.4) x 6.6 ± 0.37 (6.0–7.2)	Zhang et al., (2018)
***M. cyprini* Doflein, 1898**	*Cyprinus carpio*	Muscle	AF380140	*NA*	Molnár et al., (2002)
***M. kingchowensis* Chen et Ma, 1998**	*Carassius gibelio*	Muscle	KP400624	11.21 ± 0.64 (9.63–12.20) × 8.43 ± 0.32 (7.83–9.14)	Zhang et al., (2018)
***M. klamathellus* Atkinson et Banner, 2017**	*Gila coerulea*	Subcutaneous and kidney	KX261616	14.3 ± 0.4 (13–15) x 9.7 ± 0.4 (9–10)	Atkinson and Banner (2017)
***M. ladogensis* Rumyantsev et Shulman, 1997**	-	Muscle	KU160629	*NA*	Liu et al., (2015), unpublish

Myxozoan species	Host	Fish organ or tissue	GenBank Acc No	Spore dimensions (length x width) ± standart deviation	Reference
***M. musculi* Keisselitz, 1908**	*Luciobarbus bocagei*	Muscle	JQ388891	*NA*	Molnár et al., (2002)
***M. pseudodispar* Gorbunova, 1936**	*Abramis brama*	Muscle, kidney, gills, skin	AF380144 AF466648–9 KU340984–6	*NA*	*present study*, Molnár et al., (2002)
***M. pseudodispar* Gorbunova, 1936**	*Alburnus alburnus*	Muscle	KU340981	*NA*	Forró and Eszterbauer (2016)
***M. pseudodispar* Gorbunova, 1936**	*Rutilus rutilus, Abrasmis brama*	Gills, kidney	EF466088 KU340983	10.34 ± 0.61 (9.4–11.1) x 6.95 ± 0.32 (6.4–7.3)	*present study*
***M. pseudodispar* Gorbunova, 1936**	*Rutilus rutilus, Sander lucioperca, Scardinius erythrophthalmus*	Gills, kidney	KU340991 MK100343	10.06 ± 0.62 (9.3–10.8) x 7.59 ± 0.96 (6.3–8.6)	*present study*
M. pseudodispar	*Rutilus rutilus*	Muscle	AF380145 AF466651	*NA*	Molnár et al., (2002)

Myxozoan species	Host	Fish organ or tissue	GenBank Acc No	Spore dimensions (length x width) ± standart deviation	Reference
Gorbunova, 1936					
***M. pseudodispar* Gorbunova, 1936**	*Rutilus rutilus*	Muscle	KU340988-90	*NA*	Forró and Eszterbauer (2016)
***M. pseudodispar* Gorbunova, 1936**	*Scardinius erythrophthalmus*	Muscle	KU340979	*NA*	Forró and Eszterbauer (2016)
***M. pseudodispar* Gorbunova, 1936**	*Scardinius erythrophthalmus*	Muscle	KU340976–8 KU340980 AF466650 AF380142	*NA*	Forró and Eszterbauer (2016)
***M. ridouti* Easy et Cone, 2009**	*Pimephales notatus*	Muscle	GQ292745	9..9 ± 0.3 (9.5–10.5) x 10.1 ± 0.4 (9.4–10.9)	Easy and Cone (2009)
***Myxobolus* sp.**	*Ptychocheilus oregonensis*	Muscle	AY591531	*NA*	Kent et al., (2004)
***M. stanlii* Iwanowicz et Iwanowicz, 2013**	*Campostoma oligolepis*	Muscle	DQ779995	10.03 ± 0.7 (7.5–11.0) x 8.8 ± 1.5 (6.3–11.3)	Iwanowicz et al., (2013)

Myxozoan species	Host	Fish organ or tissue	GenBank Acc No	Spore dimensions (length x width) ± standart deviation	Reference
***M. terengganuensis* Székely, Shaharom-Harrison, Cech, Ostoros et Molnár, 2009**	*Osteochilus vittatus*	Muscle	EU643629	12.7 ± 0.51 (12–13.4) x 7.4 ± 0.53 (6.7–8.3)	Székely et al., (2009)
***Myxobolus* sp.**	*Gobio gobio*	Kidney	MN401328	11.75 ± 0.24 (11.5–12.1) x 8.28 ± 0.79 (7.3–9.5)	*present study*
***Myxobolus* sp.**	*Leuciscus leuciscus*	Gills, kidney	MN401336	*NA*	*present study*
***Myxobolus* sp.**	*Leuciscus idus*	Gills, kidney	MN401333	*NA*	*present study*
***Myxobolus* sp.**	*Perca fluviatilis*	Gills	MN401331	*NA*	*present study*
***Myxobolus* sp.**	*Phoxinus phoxinus*	Gills, kidney, skin, bile, muscle	MN401332	10.85 ± 0.92 (9.2–11.86) x 8.93 ± 0.14 (8.8–9.2)	*present study*
***Myxobolus* sp.**	*Sabanejewia bulgarica*	Gills	MN401330	*NA*	*present study*
***Myxobolus* sp.**	*Sander lucioperca*	Gills, kidney	MN401335	*NA*	*present study*
***Myxobolus* sp.**	*Scardinius erythrophtalmus*	Gills	MN401334	*NA*	*present study*
***Myxobolus* sp.**	*Vimba vimba*	Gills, kidney	MN401329	*NA*	*present study*

Phylogenetic analyses

The phylogenetic tree based on SSU rDNA revealed that all newly obtained sequences clustered in close relation to *M. pseudodispar* sequences from GenBank (Fig. 2). They clearly grouped according to fish host species preferences forming 13 well-recognised clades. Part of the newly obtained sequences are identical or almost identical to already known sequences that clustered in clades named according to their predominant hosts: *Abramis*, *Blicca*/*Vimba*, *Rutilus* I and *Rutilus* II. Four new clades were explored by our new sequence data: *Gobio*, *L. leuciscus*, *L. idus* and *Phoxinus*. None of our newly obtained sequences clustered to five already identified *M. pseudodispar* sequences forming clades *Alburnus*, *Rutilus* III and IV, *Scardinius* I and II (Fig. 2). Sequence similarities among most of the clades in the *M. pseudodispar* group ranged from 90.1% to 99.8%, whereas only three clades exceeded 99% of sequence similarity between their members (Fig. 3).

Our analysis suggested that not all phylogenetic clades of *M. pseudodispar* shared a common ancestor. The paraphyletic character of *M. pseudodispar* group is caused by the clustering of *Myxobolus bartai* Salim et Desser, 2000 (AF186835), *M. klamathellus* Atkinson et Banner, 2017 (KX261616), *M. ladogensis* Rumyantsev et Shulman, 1997 (KU160629), *M. ridouti* Easy et Cone, 2009 (GQ292745), *M. stanlii* Iwanowicz et Iwanowicz, 2013 (DQ779995) and *Myxobolus* sp. (AY591531) within this group. Particularly, *Alburnus* and *Gobio* clades are sister related to the above-mentioned species of *Myxobolus* except for *M. ladogensis*, which is a sister taxon to *Rutilus* III, *Rutilus* IV and *Scardinius* I clades. We performed statistical tests to assess the possible monophyletic status of *M. pseudodispar* sequences. The best topology resulted from the ML analysis with paraphyletic character tested against the constrained topology with monophyletic *M. pseudodispar* sequences. All statistical tests (AU, SH and KH) rejected the monophyly of *M. pseudodispar* at the significance level 0.05 (Supplementary Table 2).

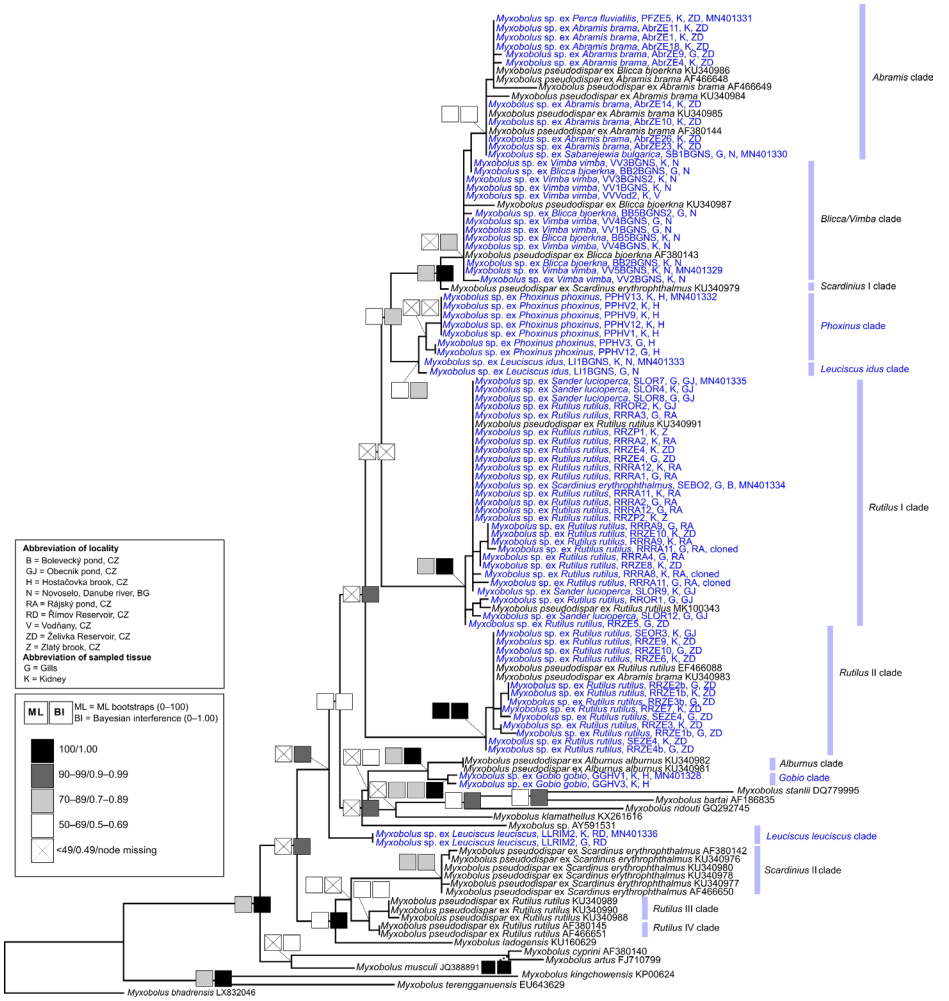

Fig. 2. The phylogenetic tree based on SSU rDNA including all sequences of the *Myxobolus pseudodispar* Gorbunova, 1936 group and closely related *Myxobolus* spp. with highlighted individual clades. *Myxobolus bhadrensis* Seenappa et Manohar, 1981 was used as outgroup. Newly identified sequences are in blue and bold. Maximum likelihood/Bayesian inference nodal supports are shown at every node by colour square according to the appropriate scale shown in the legend.

Although *M. pseudodispar* SSU rDNA sequence clades reflect host species affinity, there are few exceptions to this trend. *Myxobolus pseudodispar* from *R. rutilus* (*Rutilus* I clade) was also identified in five specimens of *S. lucioperca* and in one individual of *S. erythrophthalmus*. This clade showed high nodal support in both ML and BI analyses (87/1.0). The *Rutilus* II clade with ML/BI nodal support (100/1.0) includes the sequence from one specimen of *A. brama* (KU340983). The *Abramis* clade with predominant

sequences from *A. brama* also includes sequences from *B. bjoerkna*, *S. bulgarica* and the perciform fish *P. fluviatilis* (Fig. 3).

No phylogenetic trend according to the site of infection was observed except the *Phoxinus* clade that showed a clear pattern of kidney and gills associated sequence clusters. Similarly, there was no tendency of clustering of *M. pseudodispar* sequences from the same geographic region.

In order to assess the influence of the taxon sampling on phylogenetic distribution, nodal support and cryptic status of *Myxobolus pseudodispar*, we created a less comprehensive dataset for the phylogenetic analysis, in which a representative sequence of each *M. pseudodispar* clade was included (Fig. 3). The resulting tree was additionally supplied by information about the systematic classification of the hosts and the comparison of the morphology of myxozoan spores (if available). This restricted analysis suggested almost the
same topology as the comprehensive phylogenetic analysis of all obtained sequences. The only difference is the switch of the branching of the *Rutilus* I and *Rutilus* II clades. Nodal support remained generally low, however, several nodes were better supported in the restricted dataset. The exclusion of the repetitive identical sequences in the restricted dataset caused the different topological arrangements of the taxa at the tree and in connection with that, the tree is more explicit with the clear paraphyletic character of the sequences assigned to *M. pseudodispar*. SSU rDNA sequences of the *M. pseudodispar* group have so far been obtained only from cypriniform hosts, from the Leuciscinae (*A. brama*, *B. bjoerkna*, *R. rutilus* and *S. erythrophthalmus*) and Alburninae (*Alburnus alburnus*). We revealed identical sequences of *M. pseudodispar* recorded in GenBank from four additional hosts: *S. bulgarica*, *V. vimba* (both Cypriniformes: Leuciscinae), *P. fluviatilis* and *S. lucioperca* (both Perciformes) (Fig. 3). Four new unique sequences very similar to *M. pseudodispar* sequences from GenBank and clustering within the *M. pseudodispar* group were acquired from tissues of *L. leuciscus*, *L. idus*, *P. phoxinus* (Cypriniformes: Leuciscinae) and *G. gobio* (Cypriniformes: Gobioninae).

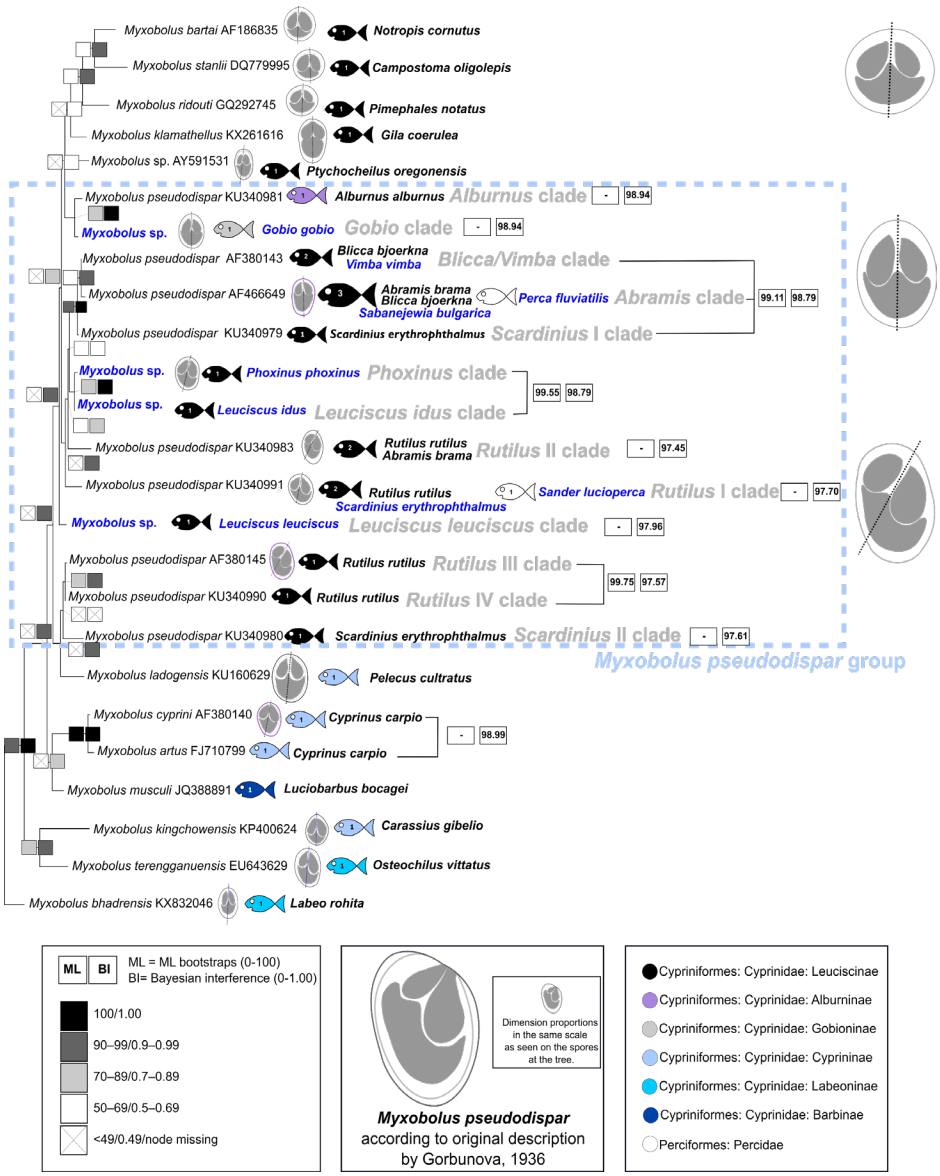

Fig. 3. The phylogenetic tree based on the restricted alignment, in which a representative sequence of each individual clade was included. Each taxon is provided with fish host species the names and comparison of the morphology of myxozoan spores (if available) schematically drawn in the same scale including spore of original description of *Myxobolus pseudodispar* Gorbunova, 1936 (morphometric data are not available for *Leuciscus* and *Blicca*/*Vimba* clades). *Myxobolus bhadrensis* Seenappa et Manohar, 1981 was used as outgroup. Newly identified clades and host species are in blue and bold. Maximum likelihood/Bayesian inference nodal supports are shown at every node by colour square according to the appropriate scale shown in the legend. Clamps connect taxa with the sequence similarities higher than 99% - indicated by the number in the left

rectangle next to clade name. The number in the right rectangle shows the sequence similarity of the *M. pseudodispar* clade (or clade assemblages with similarities higher than 99%) with the closest related clade. GenBank acc. numbers are given behind the species names.

The comparison of myxospore morphologies of *M. pseudodispar* and closely related *Myxobolus* spp. revealed that from an evolutionary point of view, there is a slight trend of change of myxospore morphology from an irregulary oval shape to oval and finally to round spores (Fig. 3). The typical irregular shape was seen in three *Rutilus* clades (for the fourth *Rutilus* clade no morphology data are available) as documented in the original description of *M. pseudodispar*. Oval symmetrical spores of *Myxobolus* spp. were obsewed in *G. gobio*. Sequences of this species, forming the above-mentioned *Gobio* clade, is very closely related to *M. pseudodispar* (KU340981) from *A. alburnus*, for which no morphology data are available.

Discussion

During a large host screening of various freshwater fish species, we focused on species identity of detected *Myxobolus* spp. morphologically resembling *Myxobolus pseudodispar*, a common myxosporean parasite of cyprinid fish with pleomorphic myxospore morphology and wide host spectrum. The large diversity of species of *Myxobolus* together with a very simple myxospore morphology as the main taxonomic criterion is the cause of difficulties in the identification species of *Myxobolus*. Some myxosporeans may display a certain degree of myxospore polymorphism in the shape and size (Guo et al., 2018), which makes the myxosporean taxonomy even more problematic.

Myxobolus pseudodispar and its closely related species represent a typical example of this problem. Pleomorphic character and irregular shape of spores make difficulties in distinguishing species according to morphology and it has been suggested that *M. pseudodispar* may be a cryptic species assemblage with affinities of individual species to specific hosts (Forró and Eszterbauer 2016). Therefore, molecular taxonomy based on DNA sequences is indispensable for species determination in these cases. However, there are no exact rules for determining whether particular differences in morphology and molecular data can be used to discriminate intra- or interspecific variation in the Myxozoa.

Our SSU rDNA-based phylogenetic analysis showed differences in sequences of *M. pseudodispar* from different fish hosts and thus supported the cryptic character of different species of *Myxobolus* in the investigated group rather than congruence with a single species. Records of *M. pseudodispar* from different fish hosts represent very likely

individual species of the genus *Myxobolus* with very similar myxospore morphology parasitising specific hosts or a specific group of fish. The SSU rDNA similarities within sequences of individual clades were more than 99%, whereas interclade similarities ranged from 90.1 to 99.8%, similarly as reported by Forró and Eszterbauer (2016). To find the boundary for species delimitation in the percentage of sequence similarities is often problematic. The similarity of SSU rDNA of *M. cyprini* and *M. artus*, the most closely related species in our analysis, is 99%. Therefore, we assume that dissimilarity of the sequences around 1% may be the boundary to delimitate the species of *Myxobolus*. However, several clades have similarity higher than 99% with another clade (e.g., similarity between the *Blicca*/*Vimba* and *Abramis* clade), suggesting rather single species existence than existence of two independent species. Nevertheless, host specificity is still clearly evident in these clades. Using only the genetic distance criterion, at least ten new species of *Myxobolus* may be described in the future from the *M. pseudodispar* group.

Myxobolus pseudodispar was originally described as an endoparasite with developing stages in the muscle and spores scattered in different tissues of the common roach, *Rutilus rutilus* (see Gorbunova 1936). Phylogenetically, several different sequences identified as *M. pseudodispar* were documented from the common roach in previous studies (Molnár et al., 2002, Kallert et al., 2007, Forró and Eszterbauer 2016). We supported these sequence differences in the present study and suggested four different phylogenetic clades of *M. pseudodispar* in *R. rutilus*. Morphology and dimensions of myxobolid spores of three phylogenetic clades from the common roach correspond to the original description of *M. pseudodispar* by Gorbunova (1936). No morphology data are available for the fourth *Rutilus* clade, but its SSU rDNA shows very high similarity with the sequence of the *Rutilus* clade III. thus, we may predict similar morphology of myxospores of these two clades. We assume that *M. pseudodispar* firstly seen in *R. rutilus* by Gorbunova (1936) belongs to one of these four *Rutilus* clades, but because of the *M. pseudodispar* (and related species) myxospore pleomorphy and lack of additional morphological diagnostic features, it is not possible to decide which clade corresponds to the species in the original description.

Myxobolus pseudodispar has been supposed to have a relatively wide host spectrum. At least, five closely related fish species from the Cyprinidae were reported to be infected by this parasite (e.g., Gorbunova 1936, Baska 1987, Molnár et al., 2002, Forró and Eszterbauer 2016). Our fish revealed another six cyprinid and two perciform fishes to be infected by *Myxobolus* spp. with similar morphology to *M. pseudodispar* and phylogenetically closely related to *M. pseudodispar* sequences retrieved from GenBank. However, with the knowledge of the phylogenetic clade host specificity of these

Myxobolus specimens and their genetic distances, it is obvious that the host spectrum of the originally described *M. pseudodispar* is rather narrow. As described above, we assume that the sequence of this species fits in one of the four clades according to the type host. Only three other hosts, *Abramis brama*, *Sander lucioperca* and *Scardinius erythropthalmus*, were recorded as hosts for myxobolids in these four respective clades. Moreover, this myxozoan sequence was detected only once in *A. brama* (Baska 1987, Forró and Eszterbauer 2016) and once in *Scardinius erythrophthalmus*. Most of other *M. pseudodispar* related clades studied in the present work have also a narrow host spectrum similarly as many other species of *Myxobolus*, e.g., *M. bejeranoi* Lövy, Smirnov, Brekhman, Ofek et Lotan, 2018 from hybrid tilapia of *Oreochromis aureus* (Steindachner) male × *Oreochromis niloticus* (Linnaeus) female, or *M. pseudowulii* Zhang, Zhai, Liu et Gu, 2017 from the yellow catfish *Pylodictis olivaris* (Rafinesque) (Zhang et al., 2017, Lövy et al., 2018). The *Abramis* clade is the exception of the narrow host spectrum with four hosts (*Abramis brama*, *Blicca bjoerkna*, *Perca fluviatilis* and *Sabanejewia bulgarica*) detected to be infected by this myxobolid.

SSU rDNA sequences of *Myxobolus pseudodispar* and its closely related *Myxobolus* spp. (e.g., *Myxobolus bartai* and *M. musculi* Keisselitz, 1908) were obtained previously only from cyprinid hosts (Salim and Desser 2000, Molnár et al., 2002, Kent et al., 2004, Easy and Cone 2009, Iwanowicz et al., 2013, Forró and Eszterbauer 2016, Atkinson and Banner 2017, Zhang et al., 2018), specifically, from the subfamily Leuciscinae (*A. brama*; *B. bjoerkna*; *Campostoma oligolepis* Hubbs et Greene; *Gila coerulea* [Girard]; *Luxilus cornutus* [Mitchill]; *Ptychocheilus oregonensis* [Richardson]; *R. rutilus* and *S. erythrophthalmus*), Cyprininae (*Carassius gibelio* [Bloch]; *Cyprinus carpio* Linnaeus); Labeoninae (*Labeo rohita* [Hamilton]; *Osteochilus vittatus* [Valenciennes]), Barbinae (*Luciobarbus bocagei* [Steindachner]) and Alburninae (*Alburnus alburnus*).

These myxobolids form a diverse group of muscle-infecting *Myxobolus* spp. within a large subclade VIII according to Liu et al., (2019), which is specific for the Cypriniformes. Among newly identified hosts for *M. pseudodispar*, there are mostly cyprinids from the subfamily Leuciscinae (*S. bulgarica*; *Vimba vimba*; *Leuciscus leuciscus*; *Leuciscus idus*; *Phoxinus phoxinus*) and newly from the subfamily Gobioninae (*Gobio gobio*). Interestingly, we detected, by PCR and identified by sequencing, these myxobolids from two perciform fish *P. fluviatilis* and *S. lucioperca*. *Myxobolus pseudodispar* identified in *P. fluviatilis* (identical with *M. pseudodispar* GenBank AccNo. AF466649) was also detected in other three cyprinid fish species suggesting a wide host spectrum for the *M. pseudodispar* group which was broadened by host switching and adaptation even to a phylogenetically more distant host from Perciformes. However, no spores or development stages of *Myxobolus* were

microscopically determined in these two perciform hosts. Hence, the presence of *M. pseudodispar* in *P. fluviatilis* and *S. lucioperca* may be also explained by the occurrence of these species in accidental hosts and a blind at by of the parasite development. The prevalence of *M. pseudodispar* was higher (67%; 28/40) in the type host (*R. rutilus*) in the present work in comparison with 41% recorded in Molnár et al., (2010) and much higher than 17%, 0.4% and 19%, respectively, which were recorded by Athanassopoulou and Sommerville (1993). At very high prevalence (94%) was documented by Forró and Eszterbauer (2016) after experimental exposure, which likely does not correlate with levels of natural infection. Discrepancies in different levels of prevalence can be explained by different localities under study, different age of screened fish or different sampling time of the year. The screening methodology is also very important for the prevalence analysis. PCR is very sensitive and detects also microscopically undetectable infections (Grossel et al., 2005). In our study, we derived the prevalence from combination of light microscopy, PCR and sequencing, which is likely the reason for higher prevalence values we observed for compared to these based only on the microscopical examination. The highest prevalence, interestingly, was detected in vimba bream *V. vimba* (86%; 6/7) and in common minnow *P. phoxinus* (65%; 11/17), the hosts in which *M. pseudodispar* has never been observed.

Type of locality and geographic origin of the samples do not seem to have an influence on the phylogenetic relationships within the species of *M. pseudodispar* complex. Samples in the present study were obtained from three different locality types (pond, reservoir and river/brook) and from two different countries: Czech Republic (ponds, reservoirs, rivers) and Bulgaria (Danube River). Several sequences from identical hosts but different localities were found to be identical, e.g., *Myxobolus* sp. SSU rDNA sequences from *V. vimba* from the pond in Vodňany (CZ) and from the Danube River in Bulgaria revealed 100% sequence identity. In addition to that, several sequences from the samples from the same geographic locality and the same host, e.g.: *M. pseudodispar* from *R. rutilus* from Švihov Reservoir (CZ), clustered in different lineages at the phylogenetic tree with similarity around 96%. This may be due to the ability of M. pseudodispar to infect several oligochaete host species (Marton and Eszterbauer 2012) and thus the observed diversity of *Myxobolus* may be the result of co-evolution of a definitive host and its parasite.

The evolutionary success of the species of *Myxobolus* diversity is obvious and has been documented many times (recently reviewed in Liu et al., 2019). We proved that *M. pseudodispar* is a complex of closely related species that parasitise not only in the different hosts but also in different host tissues as the spores were observed frequently out of muscles, where the development of plasmodia was documented (e.g., Gorbunova

1936, Gonzalez-Lanza and Alvarez-Pellitero 1985, Baska 1987). *Myxobolus pseudodispar* is a typical example of a great ability of myxozoans to speciate after adapting to new hosts either by host switching or by host-parasite coevolutionary processes.

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Information on Electronic Supplementary Material

Supplementary Table 1. Number of fish species used in the present study.

Supplementary File 1. List of fasta sequences

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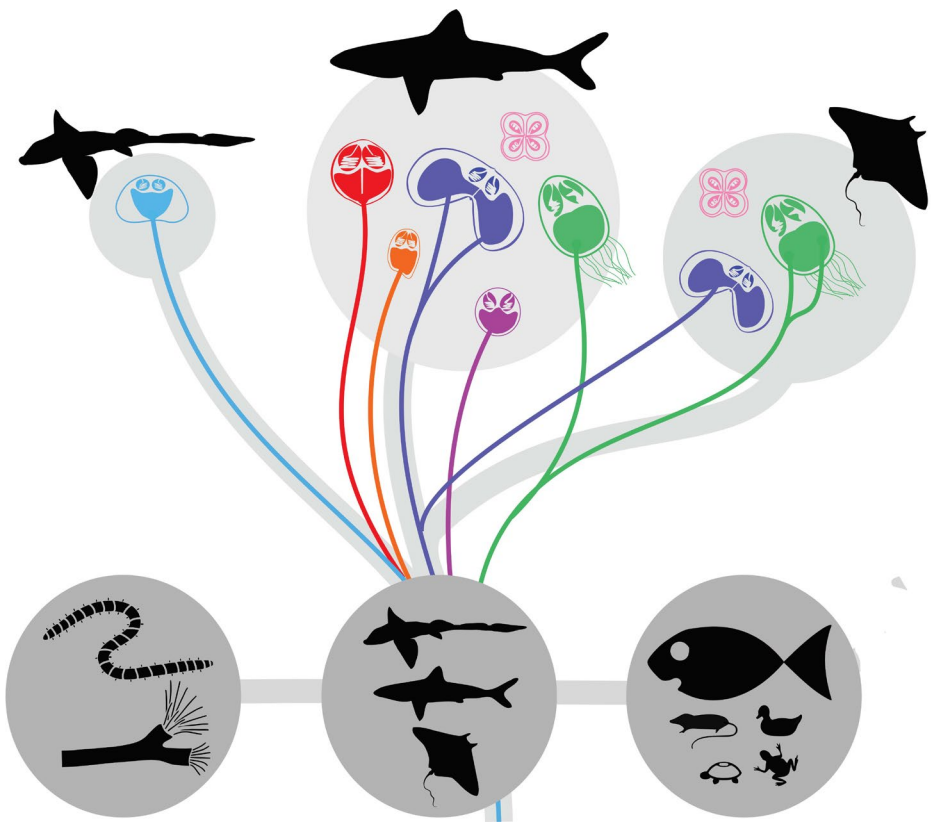
Chapter VI: The identification of myxozoan diversity in elasmobranchs

6.1 Paper III

Mechanisms and Drivers for the Establishment of Life Cycle Complexity in Myxozoan Parasites



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Mechanisms and Drivers for the Establishment of Life Cycle Complexity in Myxozoan Parasites

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Abstract

It is assumed that complex life cycles in cnidarian parasites belonging to the Myxozoa result from incorporation of vertebrates into simple life cycles exploiting aquatic invertebrates. However, nothing is known about the driving forces and implementation of this event, though it fostered massive diversification. We performed a comprehensive search for myxozoans in evolutionary ancient fishes (Chondrichthyes), and more than doubled existing 18S rDNA sequence data, discovering seven independent phylogenetic lineages. We performed cophylogenetic and character mapping methods in the largest monophyletic dataset and demonstrate that host and parasite phylogenies are strongly correlated, and that tectonic changes may explain phylogeographic clustering in recent skates and softnose skates, in the Atlantic. The most basal lineages of myxozoans inhabit the bile of chondrichthyans, an immunologically privileged site and protective niche, easily accessible from the gut via the bile duct. We hypothesize that feed-integration is a likely mechanism of host acquisition, an idea supported by feeding habits of chimaeras and ancient sharks and by multiple entries of different parasite lineages from invertebrates into the new host group. We provide exciting first insights into the early

evolutionary history of ancient metazoan parasites in a host group that embodies more evolutionary distinctiveness than most other vertebrates.

Keywords: Chondrichthyes; myxozoa; cnidaria; co-phylogeny; co-diversification; phylogeography; migration; feed-integration

1. Introduction

Parasite life cycle changes generally have adaptive components and are not purely accidental [1]. Changes in life cycle complexity, i.e., the acquisition of a new host group can either be a necessary response to changes in external environmental conditions or the outcome of the differential success of alternative transmission strategies under stable external conditions, and is usually associated with evolutionary advantages [2].

Myxozoans are a group of cnidarians that are estimated to have emerged approximately 601–700 million years ago (mya), and there is compelling evidence from phylogeny and life history, that they first settled as parasites of aquatic invertebrate hosts [3]. Following the occurrence of aquatic vertebrates on Earth, myxozoans implemented fish, which emerged 410–447 mya [4,5], as secondary hosts in their life cycles. This event fostered massive host-associated biodiversification [3] and likely presents the main reason for the distinct success of the Myxozoa, when compared with other parasitic cnidarians. Myxozoans are extremely reduced in size, and only their polar capsules, homologues of stinging cells of free-living cnidarians, give away their affiliation with the phylum and support their inclusion into the Cnidaria (e.g., [6,7,8]). Though predominantly known as pathogens of fishes, myxozoans can infect a wide variety of vertebrate groups, including amphibians, reptiles, birds, and even terrestrial mammals ([9] and references therein), and they appear to be extraordinarily flexible with regard to host switches from fish to completely unrelated groups such as mollusks or platyhelminths [10,11,12,13]. Not surprisingly, it appears that fish were acquired as secondary hosts multiple times during myxozoan evolution [3,14].

Myxozoans are highly diverse, representing about one fifth of the cnidarian biodiversity known to date [15]. Since their diversification is strongly linked with vertebrate host speciation [3], the evolutionary event of secondary host acquisition is of particular importance, yet it is unknown which vertebrate hosts were settled first and by what mechanism the successful life cycle expansion event was implemented in this group of parasites, as well as why complex life cycles did not evolve in other lineages of parasitic cnidarians. While agnaths are poorly explored as hosts for myxozoans (single record from hagfish [16], no DNA sequences), the oldest vertebrate group known to harbor an important number of myxozoans is cartilaginous fish, i.e., sharks, rays, skates, and

chimaeras e.g., [14,16,17,18,19,20]. To date, 46 species belonging to seven genera have been formally described from Chondrichthyes (Table S1), with phylogenetic positions of 19 taxa determined, using 18S rDNA sequences. The most basal branches in two out of the four major clades of myxozoans are represented by species from cartilaginous fishes [3], while the remaining clades are devoid of lineages from Chondrichthyes, potentially due to missing data.

Worldwide, over 1250 species of Chondrichthyes are known [21], and they are by far the most evolutionarily distinct radiation of all jawed vertebrates, with the average species embodying 26 million years of unique evolutionary history [22]. With the exception of the living fossil lineages Coelacanthiformes and Dipnoi, only Agnatha embody more evolutionary history per species than the average chondrichthyan [22]. Hence, this class of ancient vertebrates is of special importance to reconstruct the early evolutionary history of myxozoans in vertebrates. However, Chondrichthyes are among the most imperiled marine organisms, with up to a quarter facing increased risk of extinction [23], together with the potential for their evolutionarily distinct parasite fauna.

Considering the paucity of myxozoan sequence data from cartilaginous fishes, and the great potential of such data to unveil ancient evolutionary events that shaped this parasite group, we screened sharks and rays from seven different orders for myxozoan infections. We more than doubled existing 18S rDNA sequences of myxozoans from these hosts, and used phylogenetic, cophylogenetic, and character mapping methods to unveil the early history of myxozoan settlement in evolutionarily ancient fishes. We aimed at identifying phylogenetic origins and diversity hotspots with regard to geography as well as host groups and in relation to host migratory behavior. Finally, we discuss the potential mechanisms for establishing a complex life cycle in predominantly large, predatory fishes.

2. Materials and Methods

2.1. Isolation and Characterization of Myxozoans from Chondrichthyes

Twenty one species of sharks and rays, belonging to 7 different orders (Table S1) were obtained from 3 geographical areas: the Gulf of Mexico (coast of Florida, USA), the Atlantic off South Carolina (USA), and the Atlantic off Mar del Plata (Argentina). Gulf of Mexico specimens Rostroraja eglanteria, Sphyrna tiburo, and Carcharhinus limbatus were collected using trotline or gill nets in local waters off Sarasota, FL, under Special Activities License (SAL) research collecting permits issued by the Florida Fish and Wildlife Conservation Commission. All other specimens were commercial catches landed in Charlston Harbor (Rhizoprionodon terraenovae) and at the port of Mar del

Plata (all other species), after transport on ice by the fishing vessels, and were dissected at the fish processing plant.

Fish tissues were analyzed fresh or post freezing (storage). Microscopic preparations of bile/liver and kidney of all species as well as muscle of some specimens were screened microscopically at 400× magnification and the same tissues/liquids were stored in TNES urea for DNA extraction using a conventional phenol-chloroform protocol [24]. Depending on access and time, samples from several other organs (gills, skin, heart, stomach, intestine, gonads) were also screened visually and by PCR, in 31% of fishes. Digital images of unfixed myxozoan spores were obtained whenever visible and their morphology was compared with published records to determine unique morphological characteristics. As 18S rDNA is the most taxa-rich sequence collection of myxozoan DNA sequences presently available, extracted DNA was subjected to PCR amplification of myxozoan 18S rDNA, using an optimized methodology based on a combination of universal and more specific primers [24,25]. PCR products were separated by electrophoresis on 1% agarose gels. Bands were cut, purified (Gel/PCR DNA Fragments Extraction Kit, Geneaid Biotech Ltd., New Taipei City, Taiwan) and commercially Sanger sequenced (www.seqme.eu).

2.2. Novel Lineages, Their Phylogeny, and Diversity

To determine phylogenetic relationships between new unique 18S rDNA sequences and published ones, comprehensive alignments were prepared that included all available sequences of myxozoan isolates from cartilaginous hosts (21 new and 18 published taxa) as well as selected myxozoan representatives from all previously defined large clades (total alignment of 137 taxa, 1746 bp; Table S2). Nucleotide sequences were aligned applying Mafft version 7.017 [26] implemented in Geneious Prime v 11.1 [27], using the E-INS-i algorithm, with a gap opening penalty of 2.0. Alignments were edited to remove highly variable sections. Maximum likelihood (ML) analyses were performed using RAxML v7.2.8 [28] with the GTR + Γ model of nucleotide substitution. Maximum parsimony (MP) analyses were done in PAUP* v4.0b10 [29], using heuristic search with random taxa addition, Ts:Tv = 1:2 and random addition of taxa. Bootstrap support was calculated from 1000 replicas.

In order to determine whether a correlation exists between parasite diversity and host migratory behavior, we quantified the number of parasite and host species in known infected chondrichthyan orders and defined the migration status of each species into categories (Table S1). Movement patterns are frequently categorized as ‘migratory, nomadic, or resident’, based on definitions in Mueller and Fagan [30], however, by

definition this approach would have left us with a too small dataset (n = 3) for statistical analyses in the 'nomadic' category. Furthermore, some species such as Squalius acanthias can cover either extremely large distances or migrate smaller distances seasonally while others even reside as local population. We hence created 3 categories based on ranges of distances travelled per year (resident/movements up to 500 m radius (1), >500 m but <1000 km movements (2), and >1000 km migrations), taking into account that some migratory species can travel the same distance as nomads, potentially encountering the same number of parasites. Migratory behavior was categorized based on the information available in the IUCN Red List of Threatened Species https://www.iucnredlist.org or by using data from peer-reviewed studies using transponders and tracking the movements of individuals. Subsequently, we analyzed diversity and migration data using generalized linear models [31] with a Poisson distribution (logarithmic link function) and tested the significance of correlation by the χ^2 criterion. All analyses were performed in R [32].

2.3. Host-Parasite Cophylogeny and Geographic Character Correlation in Chloromyxum *spp.*

The largest number of parasite 18S rDNA sequences obtained from a single lineage (23 taxa) and a diverse spectrum of Chondrichthyes (9 orders) is represented by Chloromyxum spp. This lineage likely represents a single entry into secondary hosts followed by radiation predominantly in cartilaginous fishes (see Section 3.2). We analyzed this clade in more detail with regard to potential host-related and geographic radiation. For cophylogenetic studies, parasite trees of Chloromyxum spp. were produced as stated above, with all nucleotide positions included in phylogenetic analyses (1901 bp). To obtain a reliable host phylogeny, a DNA supermatrix of 15 coding and non-coding regions (60,121 bp; [22]) was used for the related 21 host species, according to availability of these genes on GenBank (March 2019). Phylogeny estimates were performed as described above. To compare host and parasite phylogenies, we used an event-based tree reconciliation method, CoRe-PA v 0.5 [33], with data-based cost estimation. Reliable trees are a prerequisite for meaningful tree-based cophylogenetic analyses. We used the best ML host and parasite trees (RAxML), acknowledging low support for several branches in the Chloromyxum spp. tree (Figure S3) and as a consequence also analyzed partially unresolved trees (Figure S4). To further support codivergence of host and parasite lineages, we applied a global fit estimate of cophylogeny based on a matrix of raw patristic distances and hence a method that is independent from tree topologies, using PARAFIT [34], implemented in the APE package, version 3.4 in R. We additionally performed historical biogeographical reconstructions in order to illuminate the evolutionary history of chloromyxids in sharks

and rays, in space and time. We used dispersal-vicariance analyses (DIVA) [35,36] and tested for geographic-phylogenetic character correlations using S-DIVA [37], thereby reconstructing ancestral states.

3. Results

3.1. Preferred Cartilaginous Hosts of Myxozoans

All major chondrichthyan lineages, the subclass Holocephali (chimaeras) and all three superoders within the subclass Elasmobranchii, i.e., Batoidea (rays, skates, and sawfish), Galeomorphii (modern sharks) and Squalomorphii (ancient sharks) were found to harbor myxozoan parasites (Figure 1), with 70 taxa known to date (Table S1) and at least seven independent historic entries into cartilaginous hosts (Figure 2), based on limited molecular data available to date (41 distinct 18S rDNA sequences). We discovered three new lineages of myxozoans in elasmobranchs (*Sphaerospora*, *Ortholinea*, and *Parvicapsula*), amounting to a total of 6 lineages in this group (Figure 2). *Chloromyxum* spp. occur in 14 families of elasmobranchs (Figure 1) and represent the most diversified lineage of myxozoans in these hosts, accounting for 38 species, exclusively found in elasmobranchs. The second most diverse genus is *Ceratomyxa*, with 20 species from sharks, rays, and skates, which cluster in a mixed clade with *Ceratomyxa* spp. from teleosts. In contrast to elasmobranchs, in chimaeras, only two species are known, *Bipteria vetusta* which occupies a unique basal lineage (Figure 2) and *Ceratomyxa fisheri* [16] (no DNA sequence data available). Kidneys and gall bladders/liver were commonly found infected with myxozoans, some muscle samples also harbored parasites, however, no other organs tested positive by microscopy or PCR-based DNA analyses.

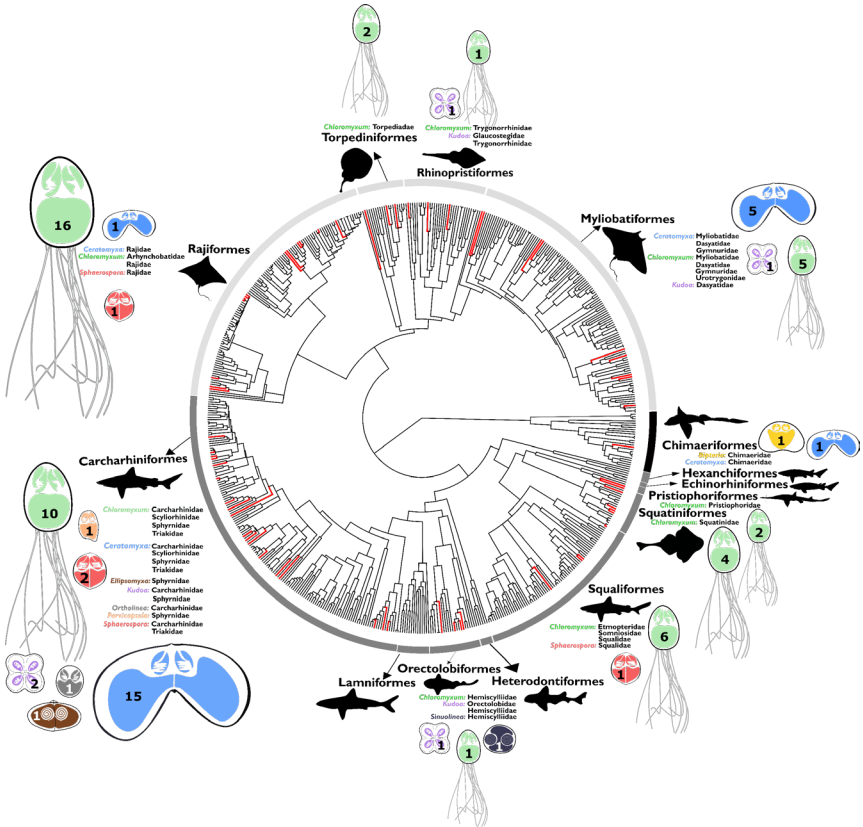


Figure 1. Representative taxon-complete tree of Chondrichthyes based on a DNA supermatrix of 15 coding and non-coding DNA regions (60,121 bp; [22]), indicating fish species with myxozoan infection (red lineages) and distribution of myxozoan genera in different families of chimaeras, sharks, rays, and skates. Number within myxozoan spores and their size is indicative of parasite species found in the relevant lineage.

The family Carcharhiniformes (ground sharks) as one of the most diverse families of elasmobranchs (280 species; [22]) presently exhibits the highest diversity and number of myxozoans (7 genera, 32 species; Table S1) and we observed a clear correlation between the number of myxozoans described and the host diversity in a given order of Chondrichthyes ($\chi^2 = 21.855$, df = 12, $p < 0.001$; Figure S1). Larger numbers of parasite species were observed in migratory host species than in species with a low geographic range or non-migratory behavior (Figure S2), however, the correlation was not significant ($\chi^2 = 4.36$, df = 2, $p = 0.11$).

3.2. Origin of Lineages and Comparison with Phylogenetic Position of Teleost Congeners

The addition of 22 new and unique 18S rDNA sequences to the existing 19 led to a change in the arrangement of the major myxozoan clades (Figure 2), due to the addition of distinct basal lineages. The phylogenetic tree of myxzoans is characterized by four well-supported main lineages, the bryozoan-infecting malacosporeans, the Sphaerospora sensu stricto clade as well as a polychaete- and and oligochaete-infecting clade of myxozoans (recently reviewed in [3]). Here, myxozoans belonging to the *Sphaerospora sensu stricto* clade were sequenced for the first time from elasmobranchs. This clade is normally consistently positioned basal to oligochaete- and polychaete-infecting lineages [3,38,39] while it was reconciliated as sister to the polychaete-infecting clade, with 82% bootstrap support in both, maximum likelihood and maximum parsimony analyses, in the present study.

Bipteria vetusta from *Chimaera monstrosa* represents the most basal lineage in polychaete-infecting myxozoans, similar to *Chloromyxum* spp. from elasmobranchs, which form the most basal branch of all oligochaete-infecting myxozoans. *B*. *vetusta* and all *Chloromyxum* spp. are parasites of the biliary system of their cartilaginous hosts and hence share a common host tissue location, despite being phylogenetically independent lineages. At present, members of the *Bipteria* and the *Chloromyxum* sublineage from Chondrichthyes represent parasites exclusively from cartilaginous hosts and they differ in phylogenetic origin from the relevant generic representatives in bony fishes (Figure 2).

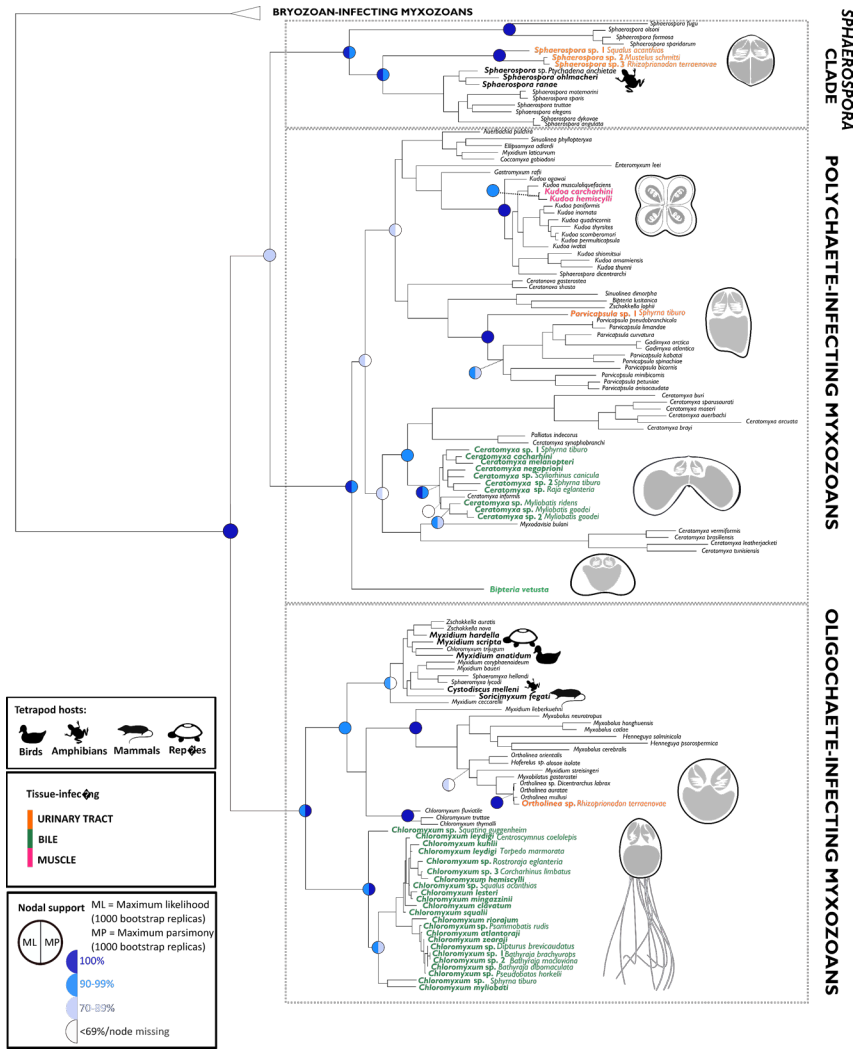


Figure 2. Phylogenetic tree of myxozoans based on 18S rDNA sequences, indicating the four main lineages (Malacosporea = bryozoan-infecting myxozoans, *Sphaerospora sensu stricto*, polychaete-infecting myxozoans, and oligochaete-infecting myxozoans) and the origin of myxozoan lineages in sharks, rays and skates (seven independent lineages, colored species names). Note *Bipteria vetusta* and *Chloromyxum* spp. represent the most basal lineages in their respective clades. These inhabit the bile and, to the present knowledge, occur only in Chondrichthyes.

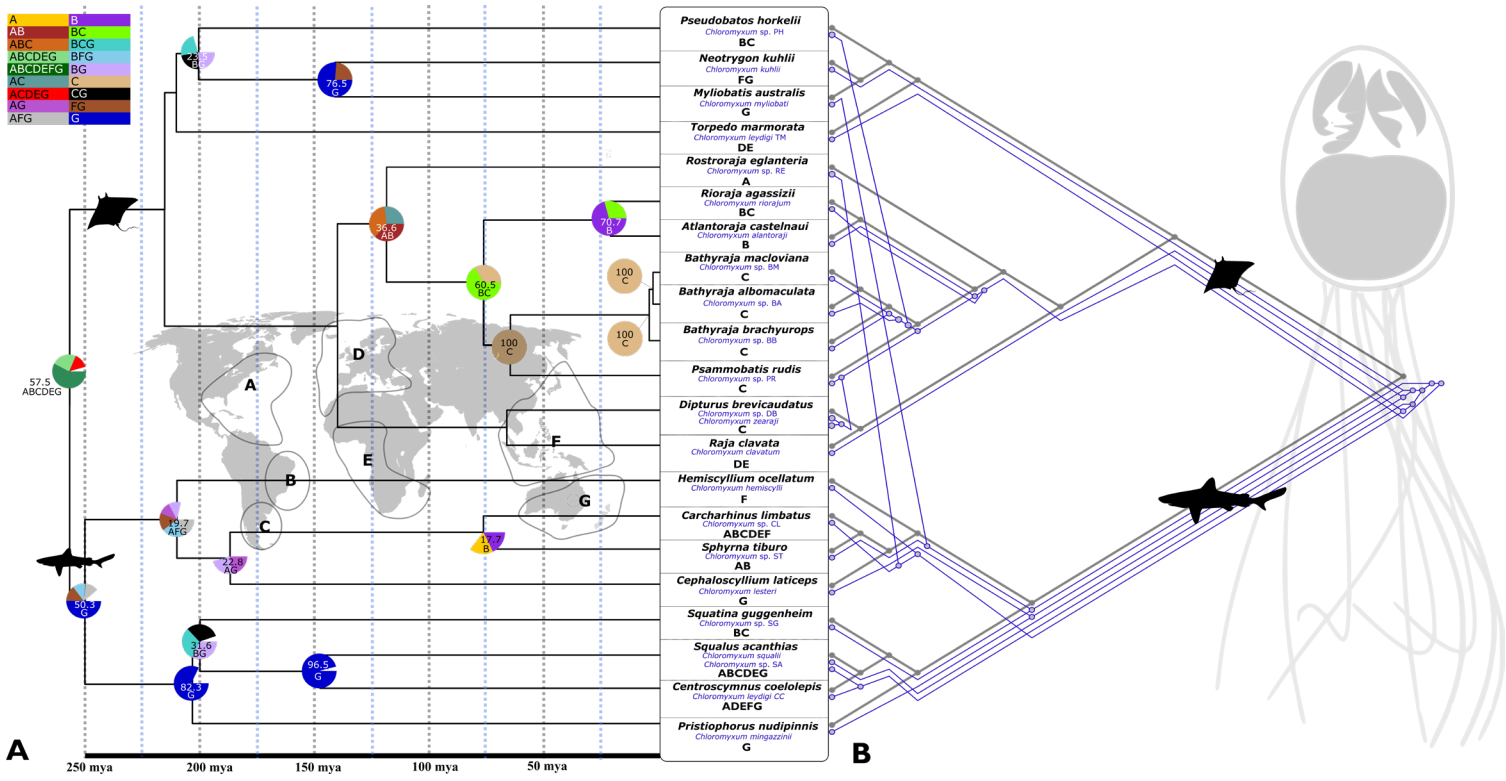
In contrast to the polychaete- and oligochaete-infecting phylogenetic lineages, members of *Sphaerospora sensu stricto* from sharks cluster together but do not branch basal to all other species in this clade, however, they are positioned basal to representatives infecting

amphibians and teleosts (Figure 2). *Parvicapsula* sp. from the bonnethead, *Sphyrna tiburo*, represents the first branch in the subtree harboring all members of the family Parvicapsulidae, which were without exception sequenced from teleosts. The other myxozoan genera from Chondrichthyes (*Ceratomyxa* spp. and *Ortholinea* sp.) cluster in two lineages that are characterized by mixed assemblages of parasites from cartilaginous and teleost hosts (Figure 2). These lineages are derived, and within them, species from elasmobranchs do not cluster basal.

3.3. Evolution of Myxozoans in Cartilaginous Fish Hosts: Host-Parasite Co-Diversification and Phylogeography

We used the largest monophyletic dataset of myxozoans from elasmobranchs (23 species of *Chloromyxum*) to better understand the evolutionary history of myxozoans after initial conquest of the new host group and establishment of a two-host life cycle. We investigated whether chloromyxids and their cartilaginous hosts co-diversified and whether geographic barriers impacted on the radiation of *Chloromyxum* spp. in sharks and bathoids.

Analyses in CoRe-PA determined that parasite and host phylogenies are strongly correlated (Table S3; Figure 3b). The best scenarios based on fully resolved trees estimated 10 cospeciation events (Table S3). Partially collapsed parasite trees with polytomies that accounted for the low support of some branches still correlated significantly with host phylogenies, with eight cospeciation events determined (Table S3) (21 hosts, 23 parasites, in all analyses). All scenarios further indicated that several lineages of *Chloromyxum* invaded elasmobranchs (Figure 3b—basal node), indicating multiple successful establishments. Global fit analyses also supported a cophylogenetic scenario in 14 out of 19 host-parasite pairs, however, with a significant but marginal p-value = 0.044, likely due to a limited dataset (19 parasites, 18 hosts; dataset reduced due to missing full-length sequences for several species (excluded)).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Chloromyxum sp. PH
BC
Neotrygon kuhlii
Chloromyxum kuhlii
FG
Myliobatis australis
Chloromyxum myliobati
G
Torpedo marmorata
Chloromyxum leydigi TM
DE
Rostroraja eglanteria
Chloromyxum sp. RE
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Rioraja agassizii
Chloromyxum riorajum
BC
Atlantoraja castelnaui
Chloromyxum alantoraji
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Bathyraja macloviana
Chloromyxum sp. BM
C
Bathyraja albomaculata
Chloromyxum sp. BA
C
Bathyraja brachyurops
Chloromyxum sp. BB
C
Psammobatis rudis
Chloromyxum sp. PR
C
Dipturus brevicaudatus
Chloromyxum sp. DB
Chloromyxum zearaji
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Raja clavata
Chloromyxum clavatum
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Cephaloscyllium laticeps
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Squatina guggenheim
Chloromyxum sp. SG
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Squalus acanthias
Chloromyxum squalli
Chloromyxum sp. SA
ABCDEG
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Chloromyxum leydigi CC
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Pristiophorus nudipinnis
Chloromyxum mingazzinii
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22.8
AG
17.7
B
50.3
G
31.6
BG
96.5
G
82.3
G
A
B
C
D
E
F
G
250 mya
200 mya
150 mya
100 mya
50 mya

Figure 3. Phylogenetic trees of *Chloromyxum* spp. and their respective cartilaginous fish hosts: (**a**) timed tree of chondrichthyans with dated species emergence, based on multi-gene phylogenetic analysis and molecular clock analyses [22], indicating ancestral states of geographic–phylogenetic character correlations determined by dispersal-vicariance analyses (S-DIVA). Geographic areas defined by letters A-G in coastal zones around the world (background of Figure 3a), numbers of nodes show highest support (%) for an origin in a certain area. (**b**) Result of co-phylogeny analyses of chondrichthyans (black cladogram; same tree as in Figure 3a) and their *Chloromyxum* spp. (blue cladogram, mapped to host; based on 18S rDNA data), using CoRe-PA, showing significant overlap of host and parasite phylogenies, with only three estimated host switches.

Historical biogeographical reconstructions in S-DIVA showed that *Chloromyxum* spp. in softnose skates (Arhynchobatidae) belonging to the genera *Rioraja*, *Atlantoraja*, *Bathyraja*, and *Psammobatis* evolved from a common ancestor in a restricted coastal area of South America, i.e., along the coastline of southern Chile, Argentina, Uruguay, and south to central Brazil (zones B and C, Figure 3a). Amongst these, the genera *Bathyraja* and *Psammobatis* speciated most recently with 100% support for a common ancestor in the most southern part of Argentina and Chile (zone C, Figure 3a). Significant agreement regarding phylogenetic and geographic clustering and relevant support for a common ancestor was only detected in one other section of the tree, i.e., in one of the two major lineages of shark hosts, including Squatiniformes (*Squatina*), Squaliformes (*Squalus*, *Centroscymnus*), and Pristiophoriformes (*Pristiophorus*) (Figure 3a). S-DIVA indicates a birth zone for these species and their parasites in the Pacific around Southern Australia (support of 96.5% and 82.3%). However, the present dataset is extremely limited and two of the four species in this group are actually highly migrant, circumglobal species (*Squalus acanthias* and *Centroscymnus coelolepis*). Otherwise, there was no clear correlation between phylogeny and biogeography (Figure 3a).

Discussion

The nature of the deepest branches in the phylogenetic tree as well as the last common ancestor and host are key questions in myxozoan biology, having wide ramifications for understanding diversity, evolutionary history, and adaptive strategies in this parasite group. Myxozoans are intriguing group of obligate parasites that evolved at the base of metazoans, showing extremely derived genomes [8] and complex life histories (summarized in [3]). Given that it is likely that the vast majority of myxozoan diversity has not yet been sampled and molecular data are only available for approximately 30% of described species [40], our approach represents only a first step for strengthening the

base of the tree by adding evolutionary ancient parasite sequence data from some of the first fish hosts available on Earth for secondary host acquisition. While broader sampling is certainly required, we hoped to characterize at least some isolates that pre-date the epoch of within teleost mass-diversification.

It is likely that ancient chondrichthyan lineages are represented by two subclades, which constitute new host acquisitions in chimaeras (*B. vetusta*) and elasmobranchs (*Chloromyxum* spp.). We hypothesize that both lineages originated and diversified only in cartilaginous hosts, potentially largely prior to the occurrence of teleosts on Earth. This idea is supported by molecular dating of the basal divergence of polychaete- and oligochaete-infecting lineages of myxozoans which was estimated to have occurred 537–447 mya [3] while Chondrichthyes emerged 447–410 mya [4,5], and ray-finned fishes (Actinopterygii) and teleosts 379–340 mya [41]. Furthermore, in contrast to all other chondrichthyan lineages of myxozoans, both *Bipteria* and *Chloromyxum* differ in morphotype and phylogenetic origin from the relevant generic representatives in bony fishes (Figure 1; [14,42,43]), with para- or polyphyletic-lineages being a common problem in the Myxozoa. As an example, *Chloromyxum* spp. known from freshwater teleosts and amphibians are spherical to ovoid with abundant surface ridges while species belonging to the monophyletic group from Chondrichthyes are ovoid to pyriform, with only partial ridges and long posterior appendages in the shape of filaments (reviewed in [43]).

As a surprising result from the present study, myxozoans from elasmobranchs in the Sphaerospora sensu stricto clade do not occupy the most basal position in the respective clade, as in the other two annelid-infecting clades. However, they cluster basal to species infecting amphibians and teleosts (Figure 2), indicating that they likely settled in higher vertebrates and teleosts by host switch from Chondrichthyes, thereby reflecting the evolutionary history of these hosts ([3]; see also below). This raises the question if other, evolutionarily even more ancient fish (Agnatha: cyclostomes or the extinct conodonts and ostracoderms) served as initial vertebrate hosts for the first subclade within sphaerosporids. Since this first subclade harbors exclusively marine species [38], hagfish could be a feasible ancestral host group. A single record of a myxozoan in hagfish exists [16], however DNA sequence data is missing. On the other hand, it may well be that this first subclade distinguishes itself from the remainder of sphaerosporids due to an independent historic event of invertebrate host acquisition in an annelid stem group, such as Haplodrilii (basal polychaetes) or Sipuncula (known hosts of myxozoans without DNA sequence records [44]), much earlier in the evolution of sphaerosporids [38], when they first invaded invertebrates. Future investigations into these potential host groups likely bear exciting outcomes. Another surprising finding regarding this particular

parasite clade is that, despite screening large numbers of Batoidea, we did not detect sphaerosporids in these hosts, though a single record from rays exists ([45]; no DNA sequence data).

The phylogenetic position of ceratomyxids and of *Ortholinea* sp. from Chondrichthyes indicates that these chondrichthyan host acquisitions are likely the result of host switches from teleost ancestors. They occur in derived clades with a mixed teleost and chondrichthyan host composition. Furthermore, ceratomyxids in sharks occur in relatively recent shark and ray species (Carcharhiniformes and Myliobatiformes), which diverged from other groups 193–181 mya and diversified 163–130 mya [22], when teleosts were already roaming the Earth [41]. This shows that, despite bony fishes being predominant in aquatic habitats (at present, teleosts outnumber cartilaginous fish approx. 22 times) cartilaginous fishes can yet be preferred as hosts by some myxozoan species.

Considering that chondrichthyans represent some of the first vertebrate hosts of myxozoans and data originates from limited host screenings when compared with teleosts, the diversity in this group is remarkable, with 70 taxa known to date. In contrast to elasmobranchs, only two species are known from chimaeras and it is unsure if this is due to a bias in sampling, or if limited diversification of the host group (49 species of Holocephali vs. 1143 species of Elasmobranchii; [22]) could be linked to a lower potential for parasite encounter and diversification [46,47]. The latter would suggest that most of the chondrichthyan host acquisitions happened after the emergence of elasmobranchs. Supportive for this hypothesis is that one of the two species known from chimaeras, B. vetusta occupies a unique basal lineage (Figure 2) and morphotype [14] when compared with myxozoans from elasmobranchs, however, more parasite sequence data from chimaeras is required to confirm the proposed independent host entries and evolutionary trajectories. In contrast, the second myxozoan parasite species described from chimaerids is of a morphotype that is common in teleosts which suggests that it was likely acquired by host switch from bony fishes, as stated above.

The addition of 22 new unique 18S rDNA sequences of myxozoans from cartilaginous hosts allowed for new insights into the origins of myxozoan parasites in sharks, skates, and rays. Most importantly, adding a large number of evolutionarily distinct sequences in basal positions to the phylogenetic tree changed the relationship between the major clades. Of the four major clades known, three are characterized by different invertebrate host groups, Malacosporeans in phylactolaemate bryozoans, polychaete-infecting myxozoans and oligochaete-infecting myxozoans. The life history of members of the fourth clade, Sphaerospora sensu stricto, is yet to be confirmed, as a single experimental study exists that identified an oligochaete as a sphaerosporid invertebrate host [48]. The

reconciliation of Sphaerospora sensu stricto as a sister clade to the polychaete-infecting myxozoans is uncommon but has occasionally occurred previously [49,50] and may suggest that sphaerosporids, like members of polychaete- and oligochaete-infecting clades, emerged as parasites of annelid stem groups [3,38], rather than representing an isolated group parasitizing a different invertebrate group, such as the Malacosporeans. Malacosporeans invade freshwater bryozoans belonging to the Phylactolaemata, and though known to parasitize cyprinid and salmonid fishes, have never been found in Chondrichthyes. It has been hypothesized that the last common ancestor of today's Phylactolaemata, the radix group of all bryozoans, first evolved in marine environments and only secondarily occupied freshwaters habitats [51]. However, it is uncertain if malacosporeans and their phylactolaemate hosts ever coincided with cartilaginous fishes in marine habitats. To the present knowledge, it is hence unclear if malacosporeans initially parasitized ancient cartilaginous fishes and if extant species lost infections due to the absence of Phylactolaemata in marine habitats. About 5% of all chondrichthyans occur in freshwater habitats, with different evolutionary entries into these environments and some species being completely euryhaline [52]. These may be an interesting target group in the search for ancient malacosporeans in Chondrichthyes.

Our diversity data clearly supports a correlation between high parasite speciation rates and high diversification rates of elasmobranch host groups, which had recently been observed in teleosts [3]. Our analyses spotlight the Carcharhiniformes (ground sharks) as a species-rich shark family, from which 32 out of the total of 70 parasite taxa have been reported. Myxozoan-infected ground sharks are to a large extent migratory species. Migration is commonly assumed to enhance the geographical spread of parasites and can expose animals to a higher diversity of infective stages as they move between breeding and wintering or feeding grounds [53,54]. Resident bird species, for example harbor lower parasite richness of nematodes and helminths in general compared to migratory species [55,56]. Apart from being one of the most diverse families of elasmobranchs, migratory behavior may serve as an additional reason for ground sharks being attractive hosts for myxozoans. Higher numbers of myxozoans were present in highly migratory elasmobranchs such as groud sharks, though additional data is required to proof significant correlations.

Historical biogeographical reconstructions of the evolutionary history of myxzoans and their elasmobranch hosts in the monophyletic group of chloromyxids identified the Atlantic as the birthplace of softnose skates (Arhynchobatidae), skates (Rajidae), and their parasites. This is however based on a dataset of species (genera *Rioraja*, *Atlantoraja*, *Bathyraja*, *Psammobatis*, *Dipturus*, *Rostroraja* and *Beringraja*) inhabiting the coasts of the Atlantic (zones A–E in Figure 3a) and clustering together in

phylogenetic analyses. In remains unclear whether their phylogeny mirrors the speciation pattern of their hosts or in fact the biogeographic history of settlement of these species in the Atlantic. Rifting in the central Atlantic occurred 220–198 mya, with seafloor spreading beginning ca. 200 mya in Georgia/North and South Carolina and 180 mya in Massachusetts/Nova Scotia, while the South Atlantic began to open 120 mya [57,58]. These dates are in agreement with the emergence of the chondrichthyan hosts relevant to this analyses (Figure 3a; [22]) and likely that of their chloromyxids (Figure 3b). However, it would be of particular interest to enrich the *Chloromyxum* spp. dataset with closely related ray species from outside the Atlantic to elucidate these relationships in more detail and challenge the present biogeographic observations. The birth zone of Squatiniformes (*Squatina*), Squaliformes (*Squalus*, *Centroscymnus*), and Pristiophoriformes (*Pristiophorus*) and their parasites, in the Pacific around Southern Australia, estimated by S-DIVA, is likely artificial as two of the four species are highly migrant and we believe that additional species from these shark orders are required to confirm or (likely) refute this pattern. The general lack of correlation between phylogeny and biogeography in older lineages and the estimated panglobal ancestor of chloromyxids in chondrichthyans may well be explained by the fact that many chondrichthyan hosts and their myxozoans emerged in Panthalassa, before the present tectonic arrangement of landmasses [58].

The major finding of this study is that a strong correlation exists between host and parasite phylogenies in chloromyxids from elasmobranchs, even after collapsing the branches of the parasite tree that showed low bootstrap support, and by support from non-tree-based methods. All co-phylogenetic scenarios further suggested multiple establishments of different *Chloromyxum* spp. in the new host group. Repeated transmissions would require accessibility or frequent physical contact of parasites and elasmobranchs. The two ancient lineages of chondrichthyan myxozoans (*B. vetusta* and *Chloromyxum* spp.) form spores exclusively in the gall bladders of their hosts, indicating a potential route of entry and mechanism of new host acquisition. Chimaeroids and ancient sharks are predatory, eating primarily hard foods that they crush with their tooth plates. Their diet consists primarily of benthic invertebrates including bivalves, gastropods, various crustaceans, polychaetes, and echinoderms [59,60]. It appears that trophic transmission followed by migration of the parasites to the gall bladder via the common bile duct represents a likely pathway of acquisition of the new host. The bile is considered an immunologically privileged site where the hosts' immune system has reduced surveillance capability [61] and hence represents a close-by protective niche on ingestion, where the parasite was able to survive despite the advanced immune system capabilities of vertebrates which can mount parasite-specific adaptive responses [62]. Thereby survival in a new host group is considered favorable [63,64] and compensates

the initial costs related to generalism [65]. The feed-integration hypothesis receives further support from myxozoan infection trials in teleosts. Gills and skin were identified as common portals of myxozoan entry into fish, however, the buccal cavity and the digestive tract are other evidenced invasion sites [66]. The myxozoan Thelohanellus hovorkai is not only able to use digestive epithelia for entry into common carp, but even develops higher infection levels after intubation than following bath exposure [67], despite its target organ being the skin.

On acquisition of the chondrichthyan host, myxozoans successfully colonized a host of a higher trophic level. Johnson et al., (2010) [68] aptly termed the incorporation of predators as hosts into parasite life cycles 'ghosts of predators past'. In such an 'upward incorporation' [65], parasite life cycles lengthen by adding a new host higher up the food chain, and usually this host subsequently becomes the definitive host, while the original definitive host becomes an intermediate host, with a prolonged larval stage [69]. This succession is explained by host size, as larger hosts can accommodate larger adult parasites and produce more offspring. Hence, a priori it might be expected that complex life cycles should be observed in derived taxa. Blaxter (2003) [70], however, reviewed evidence from different parasite groups such as gnathostomes or ascarid nematodes that convincingly demonstrates that there appears to be no greater barriers to moving from vertebrate to nonvertebrate hosts than vice versa. This pattern of host usage suggests that their recruitment is based on what is adaptive to the parasite, and not restricted by phylogenetic history [70]. Myxozoans likely maintained invertebrates as their definitive host as they remain microscopic parasites throughout their whole life cycle, even though they sometimes produce large plasmodia in fish (e.g., *Kudoa thyrsites*, *Thelohanellus kitauei*) or in invertebrates (e.g., *Buddenbrockia plumatellae*), which harbor millions of individual spores. However, myxozoans never show cell differentiation, organ formation or development into larger individuals, and sexually reproducing stages still appear to be restricted to the original invertebrate hosts {reviewed by [71]). This likely allowed them to switch between completely different host groups several times, acquiring e.g., platyhelminth [10,11,13] or mollusk [12] invertebrate hosts by switches from secondary fish hosts [3]. It is likely that chondrichthyan or teleost host acquisitions were not exploited with regard to an advantage for parasite growth in large vertebrates compared with smaller invertebrate hosts, but rather because they facilitated alternative transmission and dispersion strategies and provided diverse new niches in different host organs, leading to massive diversification of myxozoans in these hosts and their organs [3,24,39].

5. Conclusions

In order to reconstruct the evolution of life cycle complexity in the Myxozoa and study the patterns of diversification in the newly acquired vertebrate host, we more than doubled existing 18S rDNA sequence data of myxozoan parasites from evolutionarily ancient fishes belonging to the Chondrichthyes. Our results demonstrate that the oldest lineages of oligochaete- and polychaete-infecting myxozoans evolved in cartilaginous fishes. Furthermore, we provide the first evidence that they speciated as a function of host diversification, as well as biogeographic changes, with phylogeny clearly mirroring the formation and settlement of host species in the Atlantic Ocean. We propose feed-integration of infected invertebrates as a likely mechanism for the establishment of life cycle complexity, and migration and initial development in the gall bladder/bile of chondrichthyans as an immunologically privileged site, confirmed in two independent ancient lineages. The addition of new sequences in basal positions of the myxozoan tree resulted in a change in the organization of the major myxozoan phylogenetic lineages and shows that data from ancient vertebrates embodying large periods of unique evolutionary history are extremely useful for investigating the initial settlement and diversification of myxozoans in vertebrates. Thereby, agnaths offer promising future perspectives as they represent the oldest vertebrate group and are evolutionarily even more distinct than cartilaginous fishes, while they are almost unexplored to date as hosts for this enigmatic group of cnidarian parasites.

Supplementary Materials

The following are available online at https://www.mdpi.com/2079-7737/9/1/10/s1.

Table S1: Complete data of myxozoan species described in chondrichthyan hosts.

Table S2: BenBank Accession numbers of myxozoan SSU rDNA sequences using in phylogenetic analyses.

Table S3: Outcome of co-phylogenetic analyses using CoRe-PA.

Figure S1: Correlation between number of chondrichthyan species and number of myxozoan parasites found in each host order.

Figure S2: Number of myxozoan species in relation to the migration status of their hosts.

Figure S3: Fully resolved ML phylogenetic tree of *Chloromyxum* spp. SSU rDNA sequences; Figure S4: Partially resolved ML phylogenetic tree of *Chloromyxum* spp. SSU rDNA sequences, with branches showing bootstrap support <50% collapsed.

Author Contributions

Conceptualization, A.S.H.; data curation, M.L., I.F., D.C., M.I., J.T., and A.S.H.; formal analysis, M.L., I.F., and A.S.H.; funding acquisition, D.C. and A.S.H.; investigation, M.L., H.P., P.B.-S., and C.M.S.; project administration, A.S.H.; resources, D.C., J.T., C.L., and J.M.; validation, A.S.H.; visualization, M.L.; writing—original draft, A.S.H.; writing—review and editing, M.L., I.F., D.C., M.I., J.T., H.P., P.B.-S., C.M.S., C.L., J.M., and A.S.H. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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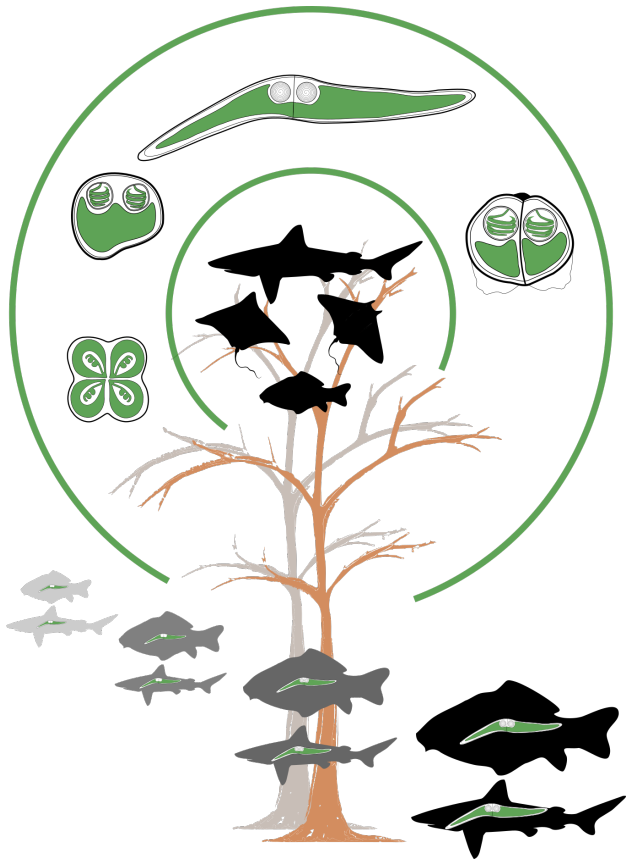
6.2 Paper V

Correlated evolution of fish host length and parasite spore size: a tale from myxosporeans inhabiting elasmobranchs



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Correlated evolution of fish host length and parasite spore size: a tale from myxosporeans inhabiting elasmobranchs

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Highlights

• Eight myxozoans were found in five of nine examined elasmobranch species.

• Five new parasite species are formally described.

• Myxozoans from elasmobranchs cluster in host group-defined phylogenetic clades.

• *Ceratomyxa* spores from elasmobranchs are approximately 5 times larger than those from teleosts.

• *Ceratomyxa* spore size strongly correlates with host body length and habitat depth.

Abstract

Myxozoa represent a diverse group of microscopic cnidarian endoparasites alternating between invertebrate and vertebrate hosts. Of the approximately 2,600 species described predominantly from teleost fish, only 1.8% have been reported from cartilaginous fishes (Elasmobranchii). As ancestral vertebrate hosts of myxozoans, elasmobranchs may have played an important role in myxozoan evolution, however, they are also some of the largest vertebrate hosts known for this group of parasites. We screened 50 elasmobranchs belonging to nine species and seven families, from various geographical areas, for myxozoan infection. We found a 22% overall prevalence of myxozoans in elasmobranchs and describe five species new to science. We investigated, for the first known time, the evolution of spore size within three phylogenetic clades, *Ceratomyxa*, *Sphaerospora* sensu stricto and *Parvicapsula*. We found that spores from elasmobranch-infecting myxozoans were on average 4.8× (*Ceratomyxa*), 2.2× (*Parvicapsula* clade) and 1.8× (*Sphaerospora* sensu stricto except polysporoplasmic *Sphaerospora* spp.) larger than those from teleosts. In all analysed clades, spore size was correlated with phylogenetic position. In ceratomyxids, it was further strongly positively correlated with fish body size and habitat depth, independent of cellular composition of the spores and phylogenetic position in the tree. While in macroparasites a host size-correlated increase in parasite size occurs on a large scale and is often related to improved exploitation of host resources, in microscopic parasites size ranges vary at the scale of a few micrometres, disproportionate to the available additional space in a large host. We discuss the ecological role of these changes with regard to transmission under high pressure and an invertebrate fauna that is adapted to deeper marine habitats.

Keywords

Body size, *Ceratomyxa*, Chondrichthyes, Depth, Harrison's rule, Parasites

1. Introduction

Elasmobranchs tend to be much larger than teleost fishes. The most massive teleost, the bump-head sunfish (*Mola alexandrini*) at 2.3 tonnes is almost an order of magnitude smaller than the largest elasmobranch fish, the whale shark (*Rhincodon typus*) of approximately 20.6 tonnes and 20 m long (Freedman and Noakes, 2002). Maximum length is available for tens of thousands of taxa (Froese and Pauly, 2020, www.fishbase.org, accessed 5 June 2020) with 90% of teleosts measuring only between 3 and 83 cm while 90% of elasmobranchs (sharks, rays and skates) grow from 36 to 403 cm (Fig. 1). Apart from their large size, elasmobranchs are interesting from an

evolutionary perspective as they are phylogenetically distinct, ancient vertebrate groups, with almost 1,200 species (Stein et al., 2018) which are mostly marine and distributed all over the world.

Although elasmobranchs are parasitised by a multitude of protist and metazoan parasites, relatively little information is available on myxozoans (Cnidaria: Myxozoa). This diverse group of cnidarian endoparasites has a complex life cycle, which likely arose by the incorporation of a vertebrate (mainly fish) into simple life cycles involving only aquatic invertebrate hosts (annelids or bryozoans) (Holzer et al., 2018, Lisnerová et al., 2020). Currently, there are some 2,600 myxozoan species distributed into two classes: Myxosporea, including the majority of known species; and Malacosporea, with fewer than 20 known species. Diversity of myxozoans seems to be strongly underestimated (Hartikainen et al., 2016, Okamura et al., 2018). This appears to be true also for myxozoans from chimaeras and elasmobranchs (Gleeson and Adlard, 2012, Kodádková et al., 2015, Lisnerová et al., 2020) as only 1.8% of all myxozoan species have so far been reported from elasmobranchs (Lisnerová et al., 2020), with 45 myxozoan species belonging to seven genera (*Ceratomyxa*, *Chloromyxum*, *Ellipsomyxa*, *Kudoa*, *Myxidium, Sinuolinea,* and *Sphaerospora*) formally described to date. In elasmobranchs, myxozoans show a worldwide geographic distribution, although the majority of studies are from Australian waters (Gleeson et al., 2010, Gambi and Giangrande, 1986, Gleeson and Adlard, 2011), the Atlantic coast of Europe (Fiala, 2006, Rocha et al., 2014) and the Atlantic coast of the Americas (Azevedo et al., 2009, Cantatore et al., 2018, Lisnerová et al., 2020).

The study of elasmobranch myxosporeans can give us a better understanding of the ancient evolutionary history of this parasite group and host-parasite co-evolutionary patterns (Fiala, 2006, Kodádková et al., 2015, Lisnerová et al., 2020). Myxozoans from elasmobranchs cluster in six independent lineages, which are frequently placed at the base of teleost-infecting clades (Fiala and Dyková, 2004, Fiala, 2006, Azevedo et al., 2009, Gambi and Giangrande, 1986, Gleeson and Adlard, 2011, Rocha et al., 2014, Cantatore et al., 2018, Lisnerová et al., 2020). *Ceratomyxa* represents one of the most comprehensively sampled marine genera and includes 278 described species (Eiras, 2006, Eiras et al., 2018 DNA GenBank records at 12 May 2020) with parasite ssrDNA sequences available from both, teleost and elasmobranch hosts encompassing a variety of host total lengths, ranging from 100 to 380 cm in elasmobranchs and from 4.2 to 200 cm in teleosts (included in Fig. 1). Moreover, ceratomyxids from elasmobranchs do not cluster basally within ceratomyxids (Lisnerová et al., 2020).

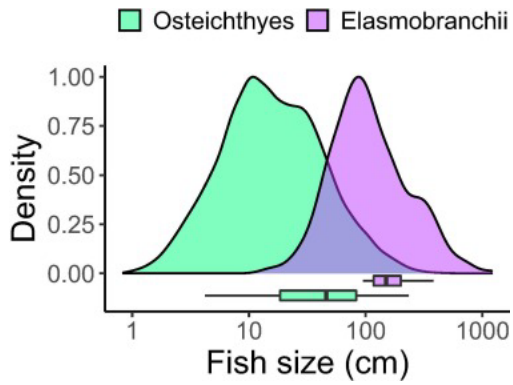

Fig. 1. Distribution of fish host body size of Elasmobranchii (purple) and Osteichthyes (green). Elasmobranchs measure around 145 cm on average while bony fish grow to around 26 cm on average. Variability in size of host species with myxozoan infections (*Ceratomyxa*, *Parvicapsula* and *Sphaerospora* sensu stricto clades) recorded in the present study is represented by boxplots.

Host body size is considered one of the most important factors determining parasite abundance, parasite body size and species richness (Poulin, 2007, Kamiya et al., 2014), but such character correlations have not previously been studied for myxozoans. Spores, which are responsible for parasite dispersal and host invasion (Alexander et al., 2015), contain most characters used in myxozoan taxonomy, with spore size and shape being key for systematics (Lom and Dyková, 2006). Myxospores harbour polar capsules, which are homologous to nematocysts of free-living cnidarians (Americus et al., 2020) and essential for host attachment and invasion. Myxozoan spore morphology frequently contradicts phylogenetic clustering of species (Fiala, 2006, Liu et al., 2019), rendering the majority of genera paraphyletic/polyphyletic, and suggesting a host or environmental impact on spore morphology.

During our recent investigations into myxozoans from chondrichthyan hosts (Lisnerová et al., 2020), we noticed that several parasite species in elasmobranchs develop unusually large spores, encouraging investigations into the evolution of size-related patterns. To shine a light on characters prone to morphological change, and physical and ecological factors associated with modifications in myxozoan spore size and morphology throughout evolution, the present study aimed at describing myxospore morphology in large-bodied fish groups (≥1 m), as well as undertaking comparative analyses to determine if morphological and morphometric differences of myxozoans from the same phylogenetic clade correlate with phylogeny, host body size or host habitat depth. We address whether the spore/body size correlation between myxozoans and their hosts is

adaptive and discuss the physical and biological challenges for transmission in meso- and bathypelagic zones of the oceans versus shallower water habitats, in relation to myxospore morphological characters, for the first time in this group of parasites.

2. Materials and methods

2.1. Sample collection and parasite morphology

In total, 50 elasmobranch specimens belonging to nine species of seven families were collected off South Africa (n = 15), off Argentina (n = 28), in the Gulf of Mexico (n = 6), and off South Carolina, USA (n = 1), between 2011 and 2018 (Table 1). Argentinian elasmobranchs were obtained as by-catch by commercial trawlers operating off the coast of Buenos Aires Province. *Rhizoprionodon terraenovae* was captured in the Charleston Harbor region, South Carolina, USA. At both sites, animals were captured following the terms and conditions of local or national regulations. South African elasmobranch specimens were collected from various sampling sites off the coast of Hermanus, Western Cape, South Africa, by longline, handline or by handfishing. Permits for the collection of sharks were issued by the South African Department of Agriculture, Forestry and Fisheries (Permit nos.: RES2018-58 and RES2019-61 issued to the South African Shark Conservancy, and RES2019-105 issued to BCS (Bjoern C. Schaeffner). Gulf of Mexico bonnetheads were collected using trotline in local waters off Sarasota, Florida, USA, under Special Activities License (SAL) research collecting permits issued by the Florida Fish and Wildlife Conservation Commission, USA. Valid names of host species follow the Catalog of Fishes (Eschmeyer et al., 2021, http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp, accessed 30 January 2021).

Table 1. Elasmobranch hosts sampled during the present study with information on host species, number of individuals and locality.

Host species	Number of individuals	Date of collection	Locality
Haploblepharus edwardsii	1	7 October 2018	Hermanus, SA
Haploblepharus fuscus	2	5 October 2018	Hermanus, SA
Haploblepharus pictus	4	4–11 October 2018	Hermanus, SA
Mustelus mustelus	6	8–11 October 2018	Hermanus, SA
Myliobatis goodei	5	15 December 2014	off the coast of Buenos Aires Province, ARG
Rhizoprionodon terraenovae	1	20 May 2011	Cape Romain Harbor, South Carolina, USA
Rostoraja alba	2	2019	Hermanus, SA
Sphyrna tiburo	6		Gulf of Mexico, USA
Squalus acanthias	23	2018	Off the coast of Buenos Aires Province, ARG
Total number	**50**		

SA, South Africa; ARG, Argentina; USA, United States of America.

Based on previous experience demonstrating the presence of myxozoans in the biliary and urinary systems of elasmobranchs (Gleeson and Adlard, 2012, Cantatore et al., 2018, Lisnerová et al., 2020), kidneys and gall bladders ($n = 50$) were primarily screened, while liver samples ($n = 21$), muscle ($n = 7$) and gills ($n = 6$) were examined only in a few specimens. Light microscopy was used to detect myxozoan infections. Digital pictures of myxozoan spores and plasmodia from fresh tissues were documented at 400× and 1,000× magnification using an Olympus BX51 microscope equipped with an Olympus DP70 camera (Olympus, Japan). Measurements (in micrometres), which followed the guidelines of Lom and Arthur, 1989, Heiniger et al., 2008 were obtained from digital images of 20–30 (in one case only seven) spores using ImageJ 1.53e (Schneider et al., 2012) and are presented in species descriptions as the mean ± S.D., with the range in parentheses.

2.2. ssrDNA amplification

All obtained elasmobranch tissue samples (microscopically positive and negative) were stored in 400 µL of TNES urea buffer (10 mM Tris-HCl, pH 8, 125 mM NaCl, 10 mM EDTA, 0.5% SDS and 4 M urea) or in 96% ethanol. DNA was extracted using a phenol–chloroform protocol (Holzer et al., 2004) with overnight digestion using proteinase K (50 µg mL^{-1}, Serva, Germany) at 55 °C.

All (even microscopically negative) samples were PCR-screened for myxozoan infections. Initial PCRs were carried out with primer combinations 18e + 18R (first run) and MyxGP2F + Act1R (second run) using the PCR composition given by Holzer et al., (2004) and PCR cycling parameters following the TITANIUM polymerase protocol of Patra et al., (2018). Partial ssrDNA sequences of myxozoans from positive samples were afterwards obtained by various combinations of general eukaryotic/myxozoan clade-specific primer sets (see Supplementary Table S1).

PCR amplicons were extracted from 1% agarose gels using the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., USA) and sequenced directly (Seqme, Czech Republic). PCR products with low DNA concentrations or unclear chromatograms were cloned into the pDrive Vector with a Qiagen PCR Cloning Kit (Qiagen, Germany) and transformed into *Escherichia coli* competent cells. Plasmid DNA was extracted using a High Pure Plasmid Isolation Kit (Roche Applied Science, Germany) and 2–5 colonies were sequenced (Seqme, Czech Republic). All sequences obtained in this study were submitted to GenBank at the National Center for Biotechnology Information (NCBI, USA). Four ssrDNA sequences were obtained in a previous study (Lisnerová et al., 2020), where morphological descriptions were, however, missing. These corresponding records were cross-referenced.

2.3. Phylogenetic analyses

Four datasets were created for phylogenetic analyses: (i) a full myxozoan dataset showing the position of all lineages from elasmobranchs, i.e. ssrDNA dataset of 138 sequences; (ii) a *Ceratomyxa*-ssrDNA dataset composed of 111 sequences of *Ceratomyxa* spp.; (iii) a *Sphaerospora* ssrDNA dataset composed of 34 sequences of *Sphaerospora* sensu stricto spp. (including polysporoplasmic sphaerosporids as they form part of this monophyletic clade) and (iv) a *Parvicapsula* ssrDNA dataset composed of 17 sequences of *Parvicapsula* spp., *Gadimyxa* spp. and *Sphaerospora testicularis*. The sequences were aligned in MAFFT v6.864b (Katoh et al., 2005) using the E-INS-i

algorithm implemented in Geneious Prime 2019.0.4 (Kearse et al., 2012) and manually edited by removal of ambiguously aligned regions.

Maximum likelihood (ML) analyses were performed in RAxML v7.0.3 (Stamatakis, 2006) implemented in Geneious Prime 2019.0.4 using the GTR + Γ model which was selected as the best-fitting model of evolution in jModelTest (Posada, 2008). Bootstraps were calculated from 1,000 replicates. Bayesian inference (BI) analysis was performed in MrBayes v3.0 (Ronquist and Huelsenbeck, 2003) implemented in Geneious Prime 2019.0.4 with the GTR + Γ model of evolution. Posterior probabilities were calculated over 1 million generations via two independent runs of four simultaneous Markov Chain Monte Carlo algorithms with every 100th tree saved and burn-in set to 10% (100,000 generations).

2.4. Relationships between spore characteristics and host/environmental parameters

Data for the maximum body length of Elasmobranchii and Osteichthyes species were retrieved from FishBase (Froese and Pauly, 2020, www.fishbase.org, accessed 5 June 2020) using the function popchar in the rfishbase package (Boettiger et al., 2012) and visualized by means of kernel density distribution function. Variability in size of host species with myxozoan infections recorded in the present study (*Ceratomyxa*, *Parvicapsula* and *Sphaerospora* sensu stricto clades) was visualized by boxplots in Fig. 1.

For parasite morphometry associations, the following parameters were considered: myxospore length, myxospore width or thickness, section area (two-dimensional (2D) representation of spore), polar capsule length, polar capsule width, and cellular composition of sporoplasms. The myxopore section area was estimated in ImageJ 1.53e (Schneider et al., 2012) from proportional 2D line drawings of spores, which were depicted according to myxospore dimensions given in original species descriptions (Supplementary Tables S2–S4). Based on the significant correlation of myxospore length, thickness, and section area in *Ceratomyxa* (Supplementary Fig. S1), for downstream analyses investigating the relationship between spore size and host/ecological features, myxospore section area was used as a proxy for size.

With regard to host parameters, body sizes of the fish host species were represented by maximum total lengths of each species while host habitat depths were represented by the maximum depths recorded, both according to FishBase (Froese and Pauly, 2020, www.fishbase.org, accessed 5 June 2020). Information on maximum fish host body size,

maximum host habitat depth and the myxospore size of each species is listed in Supplementary Tables S2–S4.

We used three different datasets encompassing the members of (i) the *Sphaerospora* sensu stricto clade (34 records, 30 species, myxospore section area 23.6–246.8 μm^2, host size 6.2–234.0 cm, host habitat depth 12–1,460 m, 1–12 sporoplasms), (ii) the *Parvicapsula* clade (17 records, 16 species, myxospore section area 31.6–145.5 μm^2, host size 22.0–203.0 cm and host habitat depth 80–3,000 m) and (iii) the *Ceratomyxa* clade (112 records, 98 species, myxospore section area 33.8–979.8 μm^2, host size 4.2–380.0 cm and host habitat depth 1–4,800 m). The latter dataset represents the most comprehensive one and is characterised by a large range of host sizes and habitat depths in both teleost and elasmobranch hosts. It was thus used for all analyses, while the remainder of the datasets were only analysed where statistical analyses were possible (see Section 3 for details).

To determine if morphological character changes in myxozoan spores can be associated with phylogenetic position and/or host (host size) and ecological features (host habitat depth), different approaches were used. To determine if spore size is a proxy for the evolutionary age of a species and if phylogeny has an influence on myxospore size, we calculated phylogenetic signal using Pagel's λ in the phytools package (Revell, 2012). Thereafter, we performed ancestral spore size reconstruction and mapped host and parasite traits to the tree using the fastAnc and dotTree functions in phytools. To characterise the relationship between polar capsule length and myxospore size characteristics (length and thickness of the spore, myxospore section area) and to estimate if host size and host habitat depth are good predictors of myxospore section area, we used phylogenetic generalized linear models (pGLS) in the caper package (Orme et al., 2013, https://cran.r-project.org/web/packages/caper/index.html). We used pGLS models to eliminate the influence of phylogenetic signal on the studied relationships. The analyses were performed separately for all studied parasite groups. In the case of *Sphaerospora,* spores can have one to several sporoplasms, potentially occupying more space and hence influencing spore size. Thus, we also included sporoplasm composition in the analysis of this group. Furthermore, linear regression was used to analyse the relationship between fish host body size and fish host habitat depth (to observe if the results of the two previous analyses may be affected by the fact that fish size depended on fish habitat depth). For all size-related analyses, response (myxospore size attributes) and quantitative explanatory variables (host body size, host habitat depth, sporoplasm number) were logarithmically transformed prior to the analyses to avoid highly positively skewed distribution of the data. All analyses were conducted using the R software (R Core Team, 2020, https://www.R-project.org) and

visualised using phytools and ggplot2 (Wickham, 2011) packages. The level of statistical significance is expressed as *P* value. The *P* value threshold for statistical significance was 0.05.

2.5. Data accessibility

R code, alignments and tables with details about hosts and parasites used in analyses are available at MendeleyData via the link: https://doi.org/10.17632/xjv5ymk8zs.1#file-39bcedbd-cf40-4700-ba41-b60c48a97816.

3. Results

3.1. Uncharted diversity of myxosporeans in elasmobranch fishes

Spore-forming plasmodia or mature spores were found in 56% (5/9) of elasmobranch species, representing 22% of examined specimens (11/50). Infections were encountered in gall bladders (10%; 5/50), kidneys (12%; 6/50) and muscle (14%; 1/7), while livers (0/21) and gills (0/6) did not contain any myxozoan parasites, even when screened by PCR. Mature myxospores (and rarely also plasmodia) of coelozoic myxosporeans belonging to *Ceratomyxa* and *Sphaerospora,* which are formally described in this study (Table 2), were observed in three sharks (*Mustelus mustelus, Rhizoprionodon terraenovae, Squalus acanthias)* and one ray (*Myliobatis goodei*) (Fig. 2A–N). Spores of *Ceratomyxa* sp. (Fig. 2T) and *Parvicapsula* sp. (Fig. 2O–Q) were observed in the bile of *Sphyrna tiburo* and in the kidney of *S. tiburo*, respectively, but we do not describe them as new species due to an insufficient amount of morphological data. Spores of a histozoic *Kudoa* sp. (Fig. 2R, S) matching the original morphological description and published ssrDNA sequence data of *K. hemiscylli* were found in the muscle of *S. tiburo* (Table 2).

Table 2. Comparison of newly described species with related myxosporeans.

Myxozoan species	Fish host	ssrDNA GenBank Acc. No.	Spore dimensions (length × width/thickness in µm)	Reference
***Ceratomyxa sigmoidea* n. sp.**	*Mustelus mustelus*	**MW396732**	SP 11.1 ± 0.6 (10.3–12.6) × 84.4 ± 5.4 (77.2–96.8); PC 4.8 ± 0.5 (4.0–5.9) × 4.8 ± 0.5 (4.1–6.0)	**Present study**
***Ceratomyxa barbini* n. sp.**	*Myliobatis goodei*	**MK937839**	SP 11.7 ± 1.3 (9.5–13.8) × 32.9 ± 8.1 (20.0–44.9); PC 3.7 ± 0.4 (3.0–4.4) × 3.7 ± 0.3 (3.0–4.2)	Lisnerová et al., (2020); **Present study**
Ceratomyxa* sp. 2 ex *Sphyrna tiburo	*Sphyrna tiburo*	**MK937838**	SP 6.9 ± 0.0 (6.9–6.9) × 48.9 ± 2.7 (46.2–51.5); PC 2.7 ± 0.1 (2.6–2.8) × 2.8 ± 0.1 (2.7–3)	Lisnerová et al., (2020); **Present study**
Ceratomyxa abbreviata Davis, 1917	*Rhizoprionodon terraenovae*	NA	SP 14 × 17; PC 4.5 × 4.5	Davis (1917)
Ceratomyxa agilis Thélohan, 1892	*Dasyatis pastinaca*	NA	SP 6–7 × 11–12	Thélohan (1892)
Ceratomyxa attenuata Davis, 1917	*Rhizoprionodon terraenovae*	NA	SP 9 × 115; PC 4.5 in diameter	Davis (1917)
Ceratomyxa carcharhini Gleeson et Adlard, 2011	*Carcharhinus melanopterus*	**JF911815–16**	SP 10.0 ± 0.7 (9–11.5) × 58.1 ± 7.1 (46–77.5); PC 3.8 ± 0.2 (3.5–4) × 3.7 ± 0.2 (3.5–4)	Gleeson and Adlard (2011)
Ceratomyxa flagellifera Davis, 1917	*Carcharhinus* sp.	NA	SP 12 × 118; PC 6 in diameter	Davis (1917)
Ceratomyxa jamesoni Kudo, 1933 (*C. taenia* Jameson, 1931)	*Triakis semifasciata*	NA	SP 7.5–9.5 × 95–117	Jameson, 1931, Kudo, 1933
Ceratomyxa lunata Davis, 1917	*Galeocerdo cuvier*	NA	SP 7–9 × 15–30; PC 4 in diameter.	Davis (1917)
Ceratomyxa melanopteri Gleeson et Adlard, 2011	*Carcharhinus melanopterus*	**JF911817**	SP 11.3 ± 1.2 (10–13.5) × 44.5 ± 9.3 (28–58.5); PC 3.9 ± 0.1 (3.5–4) × 4.0 ± 0.2 (3.5–4.5)	Gleeson and Adlard (2011)

Myxozoan species	Fish host	ssrDNA GenBank Acc. No.	Spore dimensions (length × width/thickness in µm)	Reference
Ceratomyxa mesospora Thélohan, 1892	*Sphyrna zygaena; Sphyrna tiburo*	NA	SP 8 × 50–60; PC 4.5 in diameter	Thélohan (1892)
Ceratomyxa negaprioni Gleeson et Adlard, 2011	*Negaprion acutidens*	**JF911816**	SP 11.1 ± 0.7 (10–12.5) × 27.7 ± 2.7 (20.5–33); PC 5 ± 0.4 (3.5–5.5) × 4.9 ± 0.3 (3.5–5.5)	Gleeson and Adlard (2011)
Ceratomyxa recurvata Davis, 1917	*Sphyrna zygaena*	NA	SP 8–9 × 16; PC 4.5 in diameter	Davis (1917)
Ceratomyxa sphaerulosa Thélohan, 1892	*Mustelus canis*; *Galeorhinus galeus*	NA	SP 10–12 × 90–100; PC 6–7 × 5	Thélohan (1892)
Ceratomyxa sphairophora Davis, 1917	*Rhizoprionodon terraenovae*	NA	SP 12 × 115–149; PC 6 in diameter	Davis (1917)
Ceratomyxa taenia Davis, 1917	*Rhizoprionodon terraenovae*	NA	SP 6 × 140–150; PC 3 in diameter	Davis (1917)
Ceratomyxa scissura (*Leptotheca scissura*) Davis, 1917	*Dasyatis hastata; Gymnura altavela*	NA	SP 11 × 22	Davis (1917)
Kudoa hemiscylli Gleeson, Bennett et Adlard, 2010	*Sphyrna tiburo*	NA	SP 11.0 ± 0.3 (10.6–11.6) (spore apical width) × 9.3 ± 0.4 (8.5–9.6) (spore apical thickness) × 10.6 ± 0.4 (10.1–11.0) (spore lateral width) × 7.8 ± 0.1 (7.8–7.9) (spore length)	Lisnerová et al., (2020); **Present study**
Kudoa hemiscylli Gleeson, Bennett et Adlard, 2010	*Hemiscyllium ocellatum*	**GU324949**	SP 9.9 ± 0.43 (9.1–10.8) (spore apical width) × 9.5 ± 0.38 (8.7–10.1) (spore apical thickness) × 9.6 ± 0.36 (9.1–10.5) (spore lateral width) × 7.6 ± 0.35 (7–8.2) (spore length)	Gleeson et al., (2010)
Kudoa carcharhini Gleeson, Bennett et Adlard, 2010	*Carcharhinus cautus*	**GU324968**	SP 10.1 ± 0.49 (9.5–11.2) (spore apical width) × 9.8 ± 0.6 (7.8–10.6) (spore apical thickness) × 9.8 ± 0.49 (8.5–10.6) (spore lateral width) × 8.2 ± 0.56 (7.2–9.5) (spore length)	Gleeson et al., (2010)

Myxozoan species	Fish host	ssrDNA GenBank Acc. No.	Spore dimensions (length × width/thickness in µm)	Reference
Parvicapsula* sp. ex *Sphyrna tiburo	*Sphyrna tiburo*	**MK937852**	SP 11.3 ± 0.4 (10.7–11.6) × 13.3 ± 0.0 (13.3–13.3) × 11.9 (thickness); PC 4.0 ± 0.2 (3.8–4.2) × 3.9 ± 0.2 (3.8–4.1)	Lisnerová et al., (2020); **Present study**
***Sphaerospora alata* n. sp.**	*Mustelus mustelus*	**MW396733**	SP 10.4 ± 0.7 (9.0–11.9) × 11.5 ± 0.8 (10.2–12.9); PC 3.8 ± 0.4 (2.9–4.5) × 3.7 ± 0.4 (2.9–4.2)	**Present study**
***Sphaerospora terraenovae* n. sp.**	*Rhizoprionodon terraenovae*	**MK937855**	SP 11.0 ± 0.8 (10.0–12.2) × 13.6 ± 0.8 (13.0–15.2); PC 4.0 ± 0.2 (3.6–4.3) × 3.8 ± 0.2 (3.8–4.2)	Lisnerová et al., (2020); **Present study**
***Sphaerospora argentinensis* n. sp.**	*Squalus acanthias*	**MK937853**	SP 11.7 ± 1.1 (10.3–13.7) × 13.4 ± 2 (7.2–16.3); PC 4.5 ± 0.3 (4.1–5.1) × 4.4 ± 0.3 (3.6–4.8)	Lisnerová et al., (2020); **Present study**
Sphaerospora araii Arthur et Lom, 1985	*Beringraja rhina* as *Raja rhina*	NA	SP 14.3–18.4 × 14.8–18.5	Arthur and Lom (1985)

SP, spore; PC, polar capsule; NA, data not available

3.2. Taxonomic descriptions

In this study, we provide formal species descriptions of five myxosporean parasites of elasmobranch hosts based on the morphological and molecular (ssrDNA) sequence differences from published species.

3.2.1. Taxonomic description of Ceratomyxa *spp.*

Phylum Cnidaria Hatschek, 1888
Subphylum Myxozoa Grassé, 1970
Class Myxosporea Bütschli, 1881
Order Bivalvulida Schulman, 1959
Family Ceratomyxidae Doflein, 1899
Genus *Ceratomyxa* Thélohan, 1892

***Ceratomyxa sigmoidea* Lisnerová n. sp. (**Fig. 2**A–C)**

Type host: *Mustelus mustelus* (L.), common smooth-hound (Carcharhiniformes: Triakidae), adult specimen collected in October 2018.

Type locality: Rietfontein, Hermanus, South Africa (34° 26′ 332″ S, 19° 13′ 486″ E).

Other localities: Vermont, Hermanus, South Africa (34° 26′ 210″ S, 19° 13′ 663″ E).

Site of infection: Coelozoic in gallbladder (myxospores floating in the bile).

Prevalence of infection: Overall: 67% (4/6); Vermont, Hermanus, South Africa: 100% (1/1); Rietfontein, Hermanus, South Africa: 60% (3/5).

Etymology: Refers to the Greek word sigmoidis, which means S-shaped, with one spore valve curving upwards and the other one curving downwards

ZooBank registration: urn:lsid:zoobank.org:act:9A1481B1-D2F0-430D-B424-86FC6E8B1C17.

Molecular data (two host specimens): Identical partial ssrDNA sequences (1,384 bp and 1,093 bp; expected total gene length around 2,000 bp) obtained from two host specimens.

Material deposited: DNA material stored at the Protistological Collection of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice (Acc. number IPCAS Prot 62), ssrDNA sequence (1,384 bp) deposited under the GenBank Acc. Number MW396732.

Description of sporogonic stages: Plasmodia not observed.

Description of myxospores (three host specimens)**:** Mature spores of arcuate shape, typically with one valve curved upwards and other one curved downwards. Spore length 11.1 ± 0.6 (10.3–12.6) µm and thickness 84.4 ± 5.4 (77.2–96.8) µm (n = 26; type host); spherical polar capsules, length 4.8 ± 0.5 (4.0–5.9) µm and width 4.8 ± 0.5 (4.1–6.0) µm (n = 26; type host), 6–7 polar filament coils (n = 2, type host). Length of suture position 42.1 ± 4.7 (35.0–56.0) µm (n = 26; type host). Sutural line straight. Posterior angle convex 123.4–173.2° (n = 20, type host). Non-significantly different measurements of spores and polar capsules from other two host specimens given in Supplementary Table S5. One binucleate sporoplasm.

Remarks: In total, 15 *Ceratomyxa* species have so far been described from elasmobranchs and most of them seem to be strictly host-specific (Lisnerová et al., 2020) (Table 2). *Ceratomyxa sigmoidea* n. sp. is distinguishable from its shark- and ray-inhabiting congeners by the unique spore shape (one end curved upwards, one downwards), larger dimensions (generally longer and thicker spores, except for *C. sphaerulosa*, *C. sphairophora* and *C. taenia*, which are thicker), and by host species (shark *M. mustelus* vs. teleost fishes) (Table 2).

***Ceratomyxa barbini* Cantatore et Lisnerová n. sp. (Fig. 2D–F)**

Type host: *Myliobatis goodei* (Garman, 1885), Southern eagle ray (Myliobatiformes: Myliobatidae), adult specimen collected in December 2014.

Type locality: Off the coast of Buenos Aires Province, Argentina (34°−42° S, 53°−62° W).

Site of sporogonic development: Coelozoic in gallbladder (plasmodia and myxospores floating in the bile).

Prevalence of infection: 20% (1/5).

Etymology: In honor of the Argentinian ichthyologist Santiago Barbini who has made many contributions to the knowledge of the biology, ecology and diversity of elasmobranchs in the Argentine Sea.

ZooBank registration: urn:lsid:zoobank.org:act:43D91159-5A66-43B6-8B4C-10DEB050AD08.

Molecular data (single host specimen): Partial ssrDNA sequence (1,647 bp; expected total gene length around 2,000 bp) obtained from a single host specimen refers to *Ceratomyxa* sp. 1 MG6 in Lisnerová et al., (2020).

Material deposited: DNA material and slides with spores stored at the Protistological Collection of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice (Acc. number IPCAS Prot 61), ssrDNA sequence deposited under the GenBank Acc. Number MK937839.

Description of sporogonic stages (single host specimen)**:** Disporic plasmodia, highly polymorphic of spherical, oval or irregular shape with variable dimensions, 19.1 ± 8.9 (6.6–36.5) µm maximum length (n = 10) (Fig. 2F).

Description of myxospores (single host specimen): Mature spores of arcuate shape with pointed valves; spore length 11.7 ± 1.3 (9.5–13.8) µm and thickness 32.9 ± 8.1 (20.0–44.9) µm (n = 20; type host); polar capsules spherical, equal in size, length 3.7 ± 0.4 (3.0–4.4) µm and width 3.7 ± 0.3 (3.0–4.2) µm (n = 20); 4–5 coils in each polar filament (n = 2). Valves roughly equal. Length of suture position 16.8 ± 3.9 (10.0–22.5) µm. Sutural line straight. Posterior angle convex (72.6–166.4°). One binucleate sporoplasm.

Remarks: *Ceratomyxa barbini* n. sp. overlapps by spore dimensions with the elasmobranch-infecting species *C. negaprioni* and *C. melanopteri*. However, they differ by valve shape (pointed vs. round) and ssrDNA sequence data in which *C. barbini* n. sp. matches the sequence of *C. negaprioni* by 90.5% (131 nt differences across 1350 bp) and *C. melanopteri* by 90.5% (131 nt differences across 1337 bp). The newly described species can be distinguished from other *Ceratomyxa* spp. reported from elasmobranchs by unique spore shape/dimensions, ssrDNA sequences and host species (Table 2).

3.2.2. Taxonomic description of Sphaerospora *spp.*

Phylum Cnidaria Hatschek, 1888
Subphylum Myxozoa Grassé, 1970
Class Myxosporea Bütschli, 1881
Order Bivalvulida Schulman, 1959
Family Sphaerosporidae Davis, 1917
Genus *Sphaerospora* Thélohan, 1892

***Sphaerospora alata* Lisnerová n. sp. (Fig. 2G–I)**

Type host: *Mustelus mustelus* (L.), common smooth-hound (Carcharhiniformes: Triakidae), adult specimen collected in October 2018.

Type locality: Vermont, Hermanus, South Africa (34° 26′ 210″ S, 19° 13′ 663″ E).

Other locality: Rietfontein, Hermanus, South Africa (34° 26′ 332″ S, 19° 13′ 486″ E).

Site of infection: Coelozoic in kidney renal tubules.

Prevalence of infection: Overall: 50% (3/6); Vermont, Hermanus, South Africa: 100% (1/1); Rietfontein, Hermanus, South Africa: 40% (2/5).

Etymology: Refers to the Latin word "ala" which means wing and corresponds to small wing-like postero-lateral protuberances on the mature spore.

ZooBank registration: urn:lsid:zoobank.org:act:59C73DDB-9F8B-40E0-B5F6-02D285811E52.

Molecular data (single host specimen): Partial ssrDNA sequence (1,058 bp; expected total gene length around 2,600 bp) obtained from a single host individual.

Material deposited: DNA material stored at the Protistological Collection of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice (Acc. number IPCAS Prot 63), ssrDNA sequence deposited under the GenBank Acc. Number MW396733.

Description of sporogonic stages (single host specimen): Disporic pseudoplasmodia of oval shape ranging 25.4–28.6 µm (n = 1).

Description of myxospores (three host specimens): Mature spores wider than long, pointed apically in frontal view; length 10.4 ± 0.7 (9.0–11.9) µm and thickness 11.5 ± 0.8 (10.2–12.9) µm; polar capsules equally-sized, almost spherical, length 3.8 ± 0.4 (2.9–4.5) µm and width 3.7 ± 0.4 (2.9–4.2) µm (n = 30; type host); each polar filament with 4–5 coils (n = 12; type host). Non-significantly different measurements of spores and polar capsules for other two host specimens given in Supplementary Table S5. Spore surface without striations but containing small postero-lateral protuberances. Two uninucleate sporoplasms.

Remarks: Only one sphaerosporid species has so far been described from an elasmobranch host (*Sphaerospora araii* Lom et Arthur, 1985) which has much larger spores and infects a different host species (sphaerosporids are usually strictly host-specific; Patra et al., 2018) than *S. alata* n. sp. Unfortunately, no ssrDNA sequence data is available for *S. araii* for comparison.

***Sphaerospora terraenovae* Lisnerová n. sp. (Fig. 2J–K)**

Type host: *Rhizoprionodon terraenovae* (Richardson, 1836), Atlantic sharpnose shark (Carcharhiniformes: Carcharhinidae), adult specimen collected in May 2011.

Type locality: Charleston Harbor region, South Carolina, United States of America (33° 03′ 528″ N, 79° 21′ 268″ W).

Site of sporogonic development: Coelozoic in kidney renal tubules.

Prevalence of infection: 100% (1/1).

Etymology: Refers to the type-host species *Rhizoprionodon terraenovae*.

ZooBank registration: urn:lsid:zoobank.org:act:8A69A053-F40D-4773-BC5C-76BA6165D881.

Molecular data (single host specimen): Partial ssDNA sequence (1,214 bp; expected total gene around 2,600 bp) refers to *Sphaerospora* sp. 3 RT_2 of Lisnerová et al., (2020).

Material deposited: DNA material stored at the Protistological Collection of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České

Budějovice (Acc. number IPCAS Prot 65), rDNA sequence deposited under the GenBank Acc. Number MK937855.

Description of sporogonic stages (single host specimen): Disporic pseudoplasmodia of round, oval or irregular shape ranging 20.6–27.0 µm in diameter (n = 4).

Description of myxospores (single host specimen): Mature spores wider than long, with pointed apical end in frontal view, length 10.0–12.2 µm and thickness 13.0–15.2 µm; polar capsules subspherical, equally sized, length 3.6–4.2 µm and width 3.8–4.2 µm (n = 7); 4–5 polar filament coils in each polar capsule (n = 2). Spore without any striations, filaments or protuberances. Two uninucleate sporoplasms.

Remarks: The only sphaerosporid species that has so far been described from an elasmobranch host (*S. araii*) has much longer spores and a different host than the newly described *S. terraenovae* n. sp. *Sphaerospora terranovae* n. sp. can be distinguished from *S. alata* n. sp. by the lack of postero-lateral protuberances on the mature spore, the larger size of mature spores, by a different host species, and by ssrDNA sequences (similarity 98.2%; 20 nt differences across 1058 bp).

***Sphaerospora argentinensis* Cantatore et Lisnerová n. sp. (Fig. 2L–N)**

Type host: *Squalus acanthias* (L.), Picked dogfish (Squaliformes: Squalidae), adult specimen collected in 2018.

Type locality: Off the coast of Buenos Aires Province, Argentina (34°–42° S, 53°–62° W).

Site of sporogonic development: Coelozoic in kidney renal tubules.

Prevalence of infection: 8.7% (2/23).

Etymology: Refers to the country of origin, Argentina.

ZooBank registration: urn:lsid:zoobank.org:pub:FC429562-47E1-44D6-8B4A-473D9AE682EE.

Molecular data (two host specimens): Identical partial ssDNA sequences (2,213 and 2,526 bp; expected total gene length around 2,600 bp) obtained from two host specimens refers to *Sphaerospora* sp. 1 SA in Lisnerová et al., (2020).

Material deposited: DNA material and slides with spores stored at the Protistological Collection of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice (Acc. number IPCAS Prot 64), ssrDNA sequence (2,526 bp) deposited under the GenBank Acc. Number MK937853**.**

Description of sporogonic stages (type host): Disporic pseudoplasmodia of round to oval shape ranging 21.7–27.5 µm (*n* = 3).

Description of myxospores (type host): Mature spores subspherical, length 11.7 ± 1.1 (10.3–13.7) µm and thickness 13.4 ± 2.0 (7.2–16.3) µm. Two equally-sized spherical polar capsules, length 4.5 ± 0.3 (4.1–5.1) µm and width 4.4 ± 0.3 (3.6–4.8) µm (*n* = 20). Polar filaments with 4 coils (*n* = 1). Valves with 4 longitudinal striations and postero-lateral bulges on each valve. Sutural line straight and prominent. Two uninucleate sporoplasms.

Remarks: *Sphaerospora argentinensis* n. sp. can be distinguished from *S. araii* which has much longer spores (Table 2). *Sphaerospora argentinensis* n. sp. can be distinguished from *S. terranovae* n. sp. and *S. alata* n. sp. by different spore sizes, smaller postero-lateral protuberances than those of *S. alata* n. sp. and by host species (Table 2).

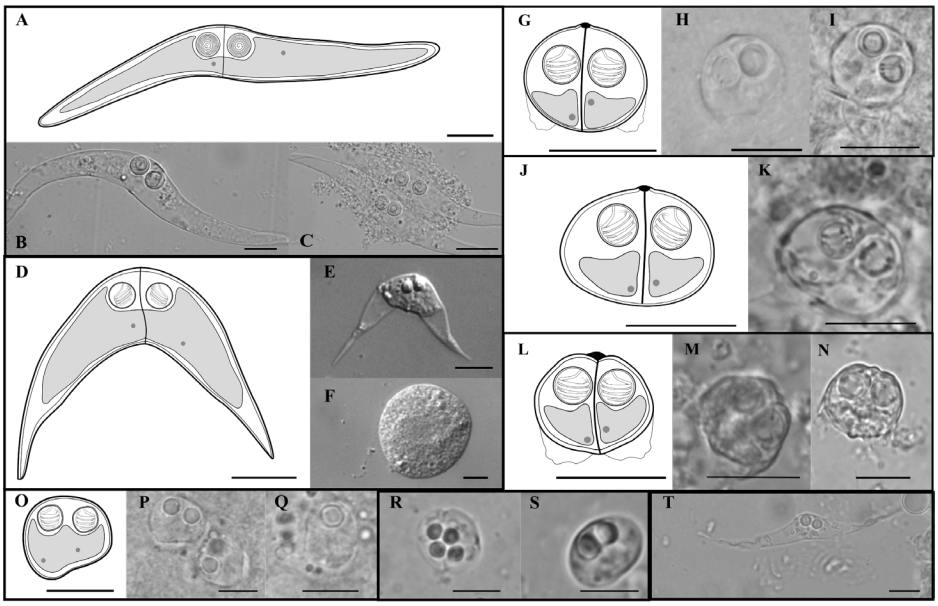

Fig. 2. Drawings and light microscopy pictures of newly described species in this study. *Ceratomyxa sigmoidea* n. sp. (A) Line drawing, (B) mature spore in frontal view and (C) spore doublet after the rupture of disporoblast; *Ceratomyxa barbini* n. sp. (D) Line drawing, (E) mature spore in frontal view, (F) round-shaped plasmodium; *Sphaerospora alata* n. sp. (G) Line drawing, (H) mature spore in frontal view with posterior protuberances and (I) mature spore with focus on polar filament turns; *Sphaerospora terraenovae* n. sp. (J) Line drawing, (K) mature spore in frontal view; *Sphaerospora argentinensis* n. sp. (L) Line drawing, (M and N) mature spores in frontal view with posterior protuberances; *Parvicapsula* sp. ex *Sphyrna tiburo.* (O) Line drawing, (P) mature spores in frontal view, (Q) mature spore in side view; *Kudoa hemiscylli.* (R) Mature spore in apical view and (S) in side view; mature spore of *Ceratomyxa* sp. ex *Sphyrna tiburo* (T). Scale bars = 10 µm.

3.2.3. Details about Parvicapsula sp. (Fig. 2O–Q)

Type host: *Sphyrna tiburo* (L.), Bonnethead (Carcharhiniformes: Sphyrnidae).

Type locality: off coast of Florida, USA, Gulf of Mexico, United States of America.

Site of infection: Coelozoic in kidney renal tubules.

Prevalence of infection: 17% (1/6).

Molecular data: Partial ssDNA sequence (1,433 bp; MK937852; expected total gene length around 2,000 bp) obtained from single host individual refers to *Parvicapsula* sp. 1 ST_4 of Lisnerová et al., (2020).

Description of sporogonic stages: Plasmodia not observed.

Description of myxospores (single host specimen): Mature spores asymmetric, wider than long, length 12.6 (11.6–13.5) µm, width 13.4 (13.2–13.5) µm and thickness 8.6 µm; relatively large equally-sized polar capsules, almost spherical in shape, length 4.0 (3.8–4.2) µm and width 3.9 (3.8–4.1) µm (n = 2); number of polar filament coils not determined. Spore surface smooth. Single distinct binucleate sporoplasm.

3.3. Elasmobranch-infecting myxozoans create unique host group-defined phylogenetic branches

The species described in the present study clustered in two out of four major myxozoan lineages, i) the polychaete-infecting lineage harbouring the members of the *Ceratomyxa* and *Parvicapsula* clades, and ii) the *Sphaerospora* sensu stricto clade accommodating most sphaerosporids (Fig. 3). Ceratomyxids from elasmobranchs formed a strongly supported subclade of the *Ceratomyxa* clade (ML/BI = 99/1; Fig. 3) containing only one species, *C. informis,* from a teleost host. This subclade emerged more recently than some of the teleost-infecting lineages, and its branches are comparatively short, indicating limited divergence of ssrDNA sequences. In contrast to *Ceratomyxa*, the shark-infecting *Parvicapsula* sp. ex *Sphyrna tiburo* created the most basal lineage of the whole *Parvicapsula* clade with maximum nodal support in our analyses (ML/BI = 100/1; Fig. 3). All three newly described sphaerosporids, *S. argentinensi*s n. sp., *S. alata* n. sp. and *S. terraenovae* n. sp. clustered together with another shark-infecting *Sphaerospora* sp. ex *Mustelus schmitti* (ML/BI = 100/1; Fig. 3), as a separate elasmobranch-infecting group within the *Sphaerospora* sensu stricto clade. This subclade was positioned at the base of the two subclades, one composing species from freshwater + marine fish and the other one species from tetrapods. Another subclade of sphaerosporids infecting marine teleosts clustered more basal to the elasmobranch-infecting species. These findings are in line with previous studies (most recently Cantatore et al., 2018, Lisnerová et al., 2020).

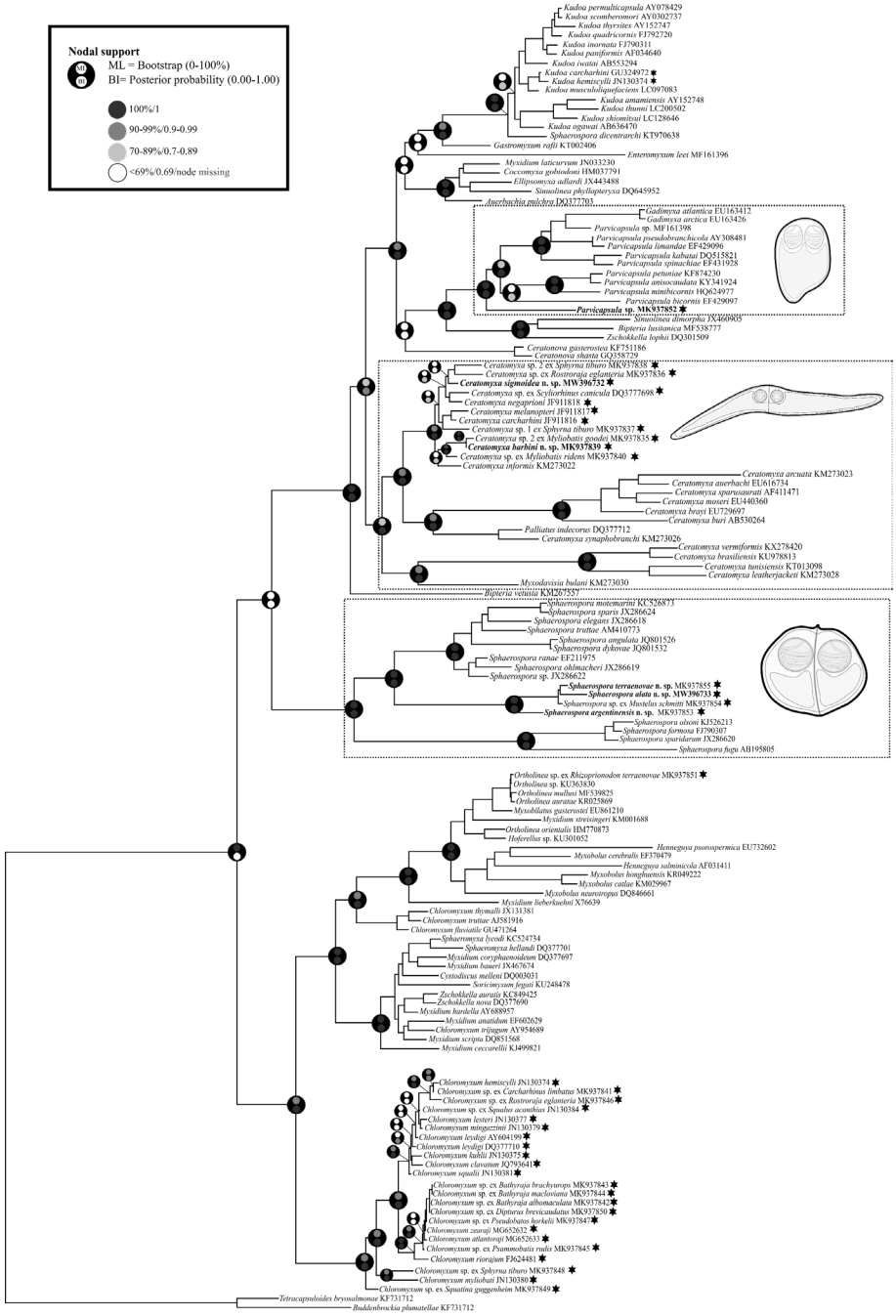

Fig. 3. The ssrDNA-based maximum likelihood phylogenetic tree including all elasmobranch-infecting (asterisk) and selected teleost-infecting myxosporeans. Malacosporeans *Tetracapsuloides bryosalmonae* and *Buddenbrockia plumatellae* were used as the outgroup. Newly described species in this study are in bold. Maximum

likelihood/Bayesian inference nodal supports are shown at each node by the shaded circle according to the scaling scheme shown in the legend.

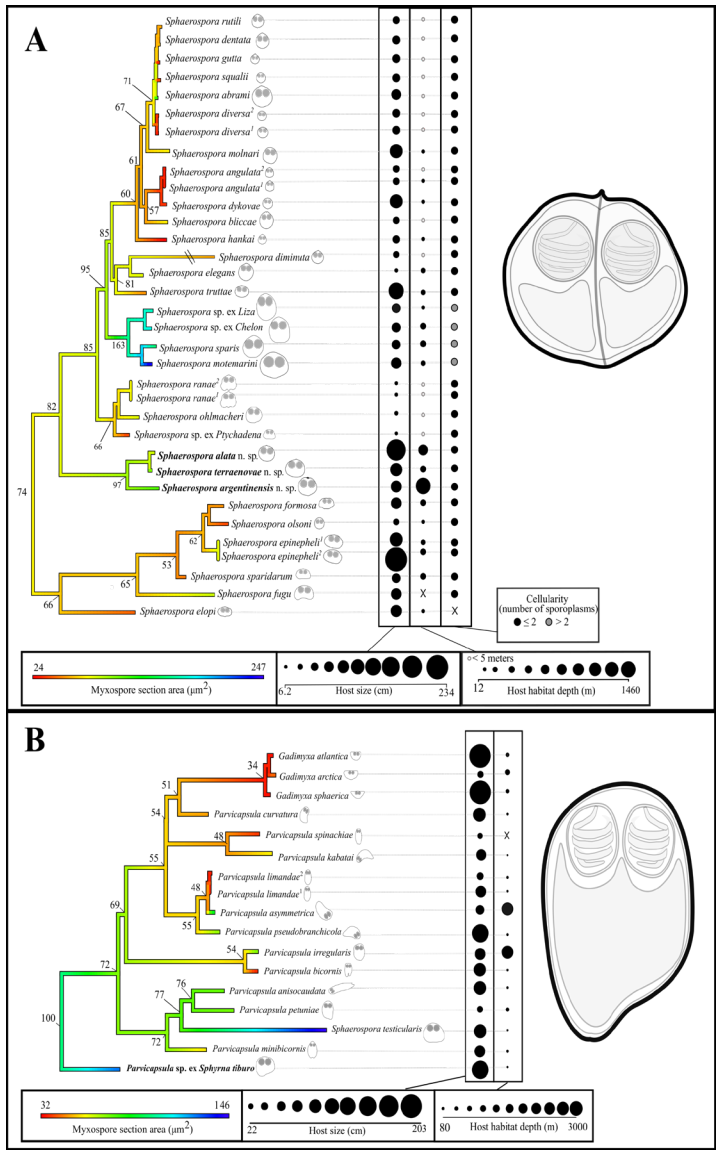

Fig. 4. The ssrDNA-based maximum likelihood phylogenetic trees with myxospore shape, host habitat depth, host size, cellularity (in the case of *Sphaerospora* sensu stricto) and estimation of ancestral myxospore section area for important nodes, mapped to the tree. The tree heat map refers to spore size (myxospore section area). Elasmobranch-

infecting species are marked in bold. Data not available for a given species are marked with X. (A) *Sphaerospora* sensu stricto clade; (B) *Parvicapsula* clade.

3.4. Spore size is dependent on phylogeny and elasmobranch myxozoans have larger spores than those from teleosts

Spore section area was related to phylogeny in all studied groups (*Sphaerospora* sensu stricto*:* $\lambda = 0.808$, $P < 0.001$; *Parvicapsula*: $\lambda = 0.741$, $P = 0.010$; *Ceratomyxa:* $\lambda = 0.999$; $P < 0.001$) indicating that spore size does not change sporadically but that an increase/decrease in spore size is characteristic to particular subclades. Myxozoan spores from elasmobranchs were generally larger in size than those of teleosts (Figs. 4A, B, 5).

3.5. The largest ceratomyxids inhabit large-bodied fishes from mesial and bathyal habitats

We tested the influence of host size and the environmental factor of habitat depth on spore size (myxospore section area), performing phylogenetic correction, and showed that only the spore size of *Ceratomyxa* is influenced by host size ($F_{1,104} = 335.07$; $P < 0.001$) and habitat depth ($F_{1,103} = 108.06$; $P < 0.001$) (Fig. 6B), while the sizes of *Parvicapsula* and *Sphaerospora* sensu stricto clade members were not significantly influenced by host size ($F_{1,14} = 1.01$; $P = 0.33$*;* $F_{1,30} = 0.23$, $P = 0.64$), habitat depth ($F_{1,13} = 0.07$; $P = 0.79$*;* $F_{1,28} = 3.01$, $P = 0.09$) or the number of sporoplasms ($F_{1,29} = 0.06$; $P = 0.81$; *Sphaerospora*). We further found that larger fish in our database inhabit deeper waters ($t_{104} = 7.426$; $P < 0.001$; Fig. 6C).

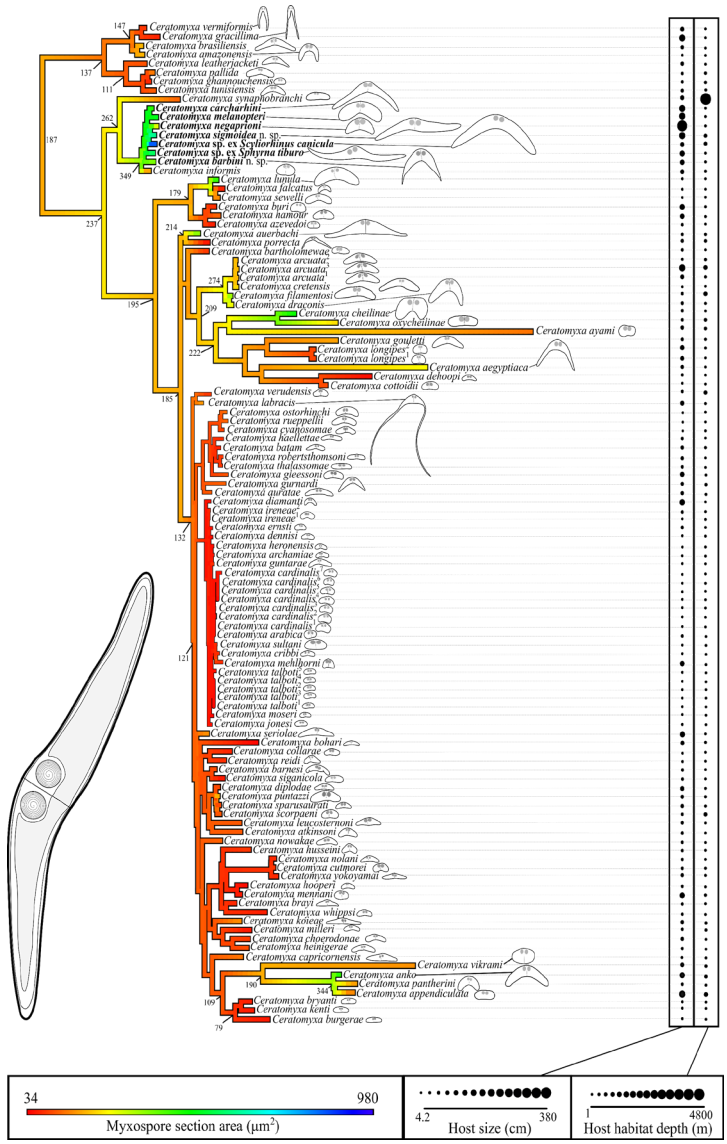

Fig. 5. The ssrDNA-based maximum likelihood phylogenetic tree of the *Ceratomyxa* clade with myxospore shape, host habitat depth, host size, and estimation of ancestral myxospore section area for important nodes, mapped to the tree. Tree heat map refers to spore size (myxospore section area). Elasmobranch-infecting species are marked in bold.

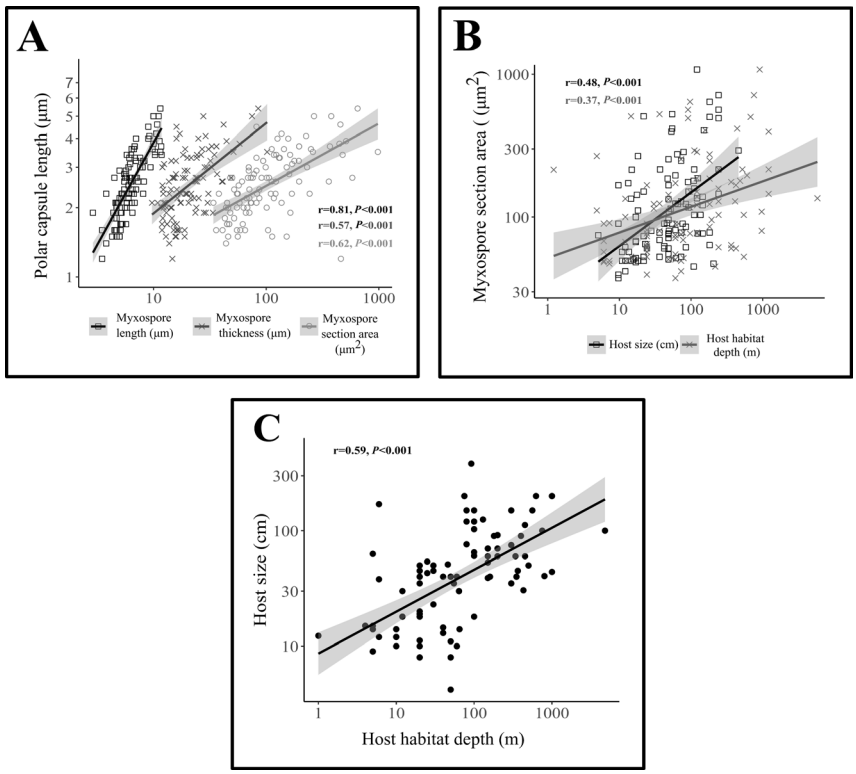

Fig. 6. Relationship between host-defined parameters and myxospores of *Ceratomyxa* spp. Generalized linear models with phylogenetic correction of (A) average length of polar capsules and average myxospore length/thickness/myxospore section area; (B) myxospore section area and maximum fish body size/host habitat depth; (C) maximum fish body size and maximum fish habitat depth. The *P* value threshold for statistical significance was 0.05.

4. Discussion

This study provides insights into the diversity, morphology and phylogeny of myxozoan parasites encountered in a large variety of elasmobranch hosts. Elasmobranchs are an ancestral host group for the Myxozoa (Kodádková et al., 2015, Holzer et al., 2018), however, they have so far been poorly explored as myxozoan hosts. We provide morphological descriptions and ssrDNA sequences for new species of myxozoans from large-bodied fish, thereby providing unique datasets for phylogenetic and morphometric analyses. We noticed that myxozoans from sharks and rays occupy separate phylogenetic

clades and that their spores are considerably larger than those obtained from most of their teleost-inhabiting congeners. Comprehensive statistical methods and evolutionary models were used to analyse spore size-related patterns in three phylogenetic clades, with particular focus on the species-rich *Ceratomyxa* clade.

Although cellularity and basic spore shapes of myxozoans in small and large fish hosts are generally the same (with the exception some of *Sphaerospora* spp.), remarkable differences in myxospore section area were observed in different clades, especially in *Ceratomyxa* spp., where size incrementation was almost five-fold. Size-related correlations in parvicapsulids and *Sphaerospora* sensu stricto showed limited significance, likely due to small sample size. However, in the comprehensive *Ceratomyxa* dataset, myxospore section area was strongly correlated with both, host body size and habitat depth. In contrast to parvicapsulids and ceratomyxids, sphaerosporids are not largest in elasmobranchs. Indeed, the largest spores are found among the marine teleost-infecting *Sphaerospora* spp. harbouring multiple sporoplasms (i.e., polysporoplasmic *Sphaerospora* spp., Sitjà-Bobadilla and Alvarez-Pellitero, 1995), with cellularity having a more significant impact on myxospore section area than host size. This can be explained by the extreme reduction of these parasites to only a few cells, with every additional cell causing a significant size change.

As described previously (Gleeson and Adlard, 2011, Lisnerová et al., 2020), *Ceratomyxa* spp. from ancient elasmobranch hosts do not cluster in the most basal position. Their positioning may be explained by an initial invasion of teleost intermediate hosts and a later switch to elasmobranchs (Lisnerová et al., 2020), or an origin in ancient Chondrichthyes and a switch by movement of teleosts into freshwater habitats (Zatti et al., 2017) by the first ceratomyxids. However, it allows us to conclude that spore size changes are adaptive and large size is not an ancestral condition.

Various biological factors have so far been recognized to act on parasite size with host body size being one of the most obvious host-related factors (Poulin, 2007). The positive correlation of large parasites in large-bodied hosts, coined as Harrison's rule (Harrison, 1915), may be associated with more efficient usage of available niche space by parasite individuals in larger hosts (Sasal et al., 1999). In fact, larger hosts have larger organs which represent larger habitats that provide more space and nutrients for parasites to exploit (Kuris et al., 1980, Poulin, 1995). Examples of Harrison's rule have been documented for a wide spectrum of other parasites and their hosts, e. g. wing lice and birds (Johnson et al., 2005), different insect species and their hosts (Kirk, 1991), digenean flukes and their fish hosts (Sasal et al., 1999) and rhizocephalan parasites and crustaceans (Poulin and Hamilton, 1997). However, as myxozoans are truly microscopic

parasites and spore size changes are effective at some tens of micrometres it is unclear whether the size of the host body, which varies at a much larger scale, plays a crucial role in the evolution of spore size variations, when compared with large-bodied metazoan parasites (Kirk, 1991, Poulin and Hamilton, 1997, Sasal et al., 1999, Johnson et al., 2005).

It is important to relate changes in spore morphology not only to the host in which these spores are produced but also to the habitat for their dispersion and to their target hosts. The size of parasite developmental stages in an intermediate host can be associated with larger body size in the final host (Fredensborg and Poulin, 2005, Benesh and Hafer, 2012). Ceratomyxid myxospores infect polychaete invertebrates as final hosts (Køie et al., 2008), in which a different morphotype of spore is produced that re-infects the fish host, whereby the typical dixenous myxozoan life cycle is completed. Polychaetes represent a major component of macrobenthic assemblages, in terms of abundance, diversity and biomass, at every bathymetric level (Gambi and Giangrande, 1986, Abbiati et al., 1987, Baldrighi et al., 2013), with many large species, e. g. the sulfid worm *Paralvinella sulfincola* (up to 7 cm) or the giant tube worm (*Riftia pachyptila*, up to 3 m), occurring in deep sea habitats. Whether larger myxospores are required to infect large polychaetes is unclear, since very few life cycles have been elucidated in polychaetes (only nine are known to date; Atkinson et al., 2019) and the data are too scarce to discover any trends.

Interestingly, large elasmobranch-infecting *Ceratomyxa* spp. inhabit fishes that grow to greater sizes and inhabit deeper oceanic habitats (75–750 m, Smith and Brown, 2002; this study), likely due to selective pressures exerted by environmental conditions (Poulin and Hamilton, 1995). The predominance of large-sized organisms at greater depth, defined as the 'bigger-deeper phenomenon', has been described in different groups of marine organisms such as e.g., crustaceans (Ardizzone et al., 1990), cephalopods (Roper and Boss, 1982), free-living cnidarians (Matsumoto et al., 2003, Daly, 2006) or fish (Massutí et al., 1996). The deep sea is the largest ecosystem on Earth but organisms living there must contend with high pressure, low temperature, decreased dissolved oxygen levels, food scarcity and scarcely available or irregularly distributed hosts. However, hardly anything is known about myxozoans inhabiting the bathyal (>1,000 m) or abyssal (>5,000 m) zones (Noble, 1966, Yoshino and Noble, 1973, Lom et al., 1975, Threlfall and Khan, 1990). In these habitats, environmental parameters themselves may be responsible for a larger spore size as higher pressure causes increased water density and hence spores may have to be larger (heavier relative to surface area) to sink and reach benthic polychaete hosts at all. Our parasite size-depth correlations are based on the maximum depth reported for the relevant host species and for more meaningful

outcomes, future analysis of spore sizes along depth gradients should focus on single species in non-migratory hosts occurring at a variety of depths, such as *C. arcuata* or *C. appendiculata* in the angler, *Lophius piscatorius*, which can be found between 20 and 1,000 m.

In parasitic crustaceans, body size tends to increase with both water depth and latitude, environmental correlates of temperature (Poulin, 1995, Poulin and Hamilton, 1995). Comparing sister taxa, those inhabiting higher latitudes or deeper water tend to be larger-bodied than their relatives in warmer waters, independent of other variables such as host type or size. This complies with Bergmann's rule that states that within a broadly distributed taxonomic clade, populations of species of larger size are found in colder environments and offers a further interpretation of our data from myxozoans. Similar patterns can be found in monogeneans (Poulin, 1996) and sea lice (Samsing et al., 2016), while opposite trends are often observed in non-aquatic hosts (e.g. Dallas et al., 2019). These examples illustrate that responses of parasite body size to selective environmental pressures such as temperature or depth can vary and, in myxozoans, spore size may further be influenced by infection requirements in the invertebrate host. However, few studies failed to find an association between host size and parasite size (Poulin, 2007), and the present data strongly indicates a size correlation for several myxozoan genera and their fish hosts. The fact that spores of larger size appear in isolated clades characterized only by large hosts may additionally suggest that adaptive constraints limit the range that myxozoans can use, as has been demonstrated in lice (Clayton et al., 2003). Size may thus reinforce cospeciation by preventing parasites from switching to hosts of smaller size, despite the generally extraordinary capacity for host switching in this parasite group, which is associated with an extremely high evolutionary diversification rate (Holzer et al., 2018).

This is the first time that size-related models of evolution and host-associated changes were elucidated in myxozoans. We believe that we have confirmed Harrison's rule for myxozoans by a positive correlation between fish host size and myxozoan spore size, however, other effects such as depth, water pressure and invertebrate target host can at present not be excluded as factors contributing to the observed size variation. The adaptive basis of the observed phenomenon remains poorly understood and requires further investigation. Future research on myxozoans from large elasmobranch and teleost hosts inhabiting a range of different depths as well as on infections and spore types in deep-water invertebrate hosts could further contribute to an understanding of adaptive morphotypes and their distribution in different aquatic habitats, as well as to the evolutionary history of the Myxozoa.

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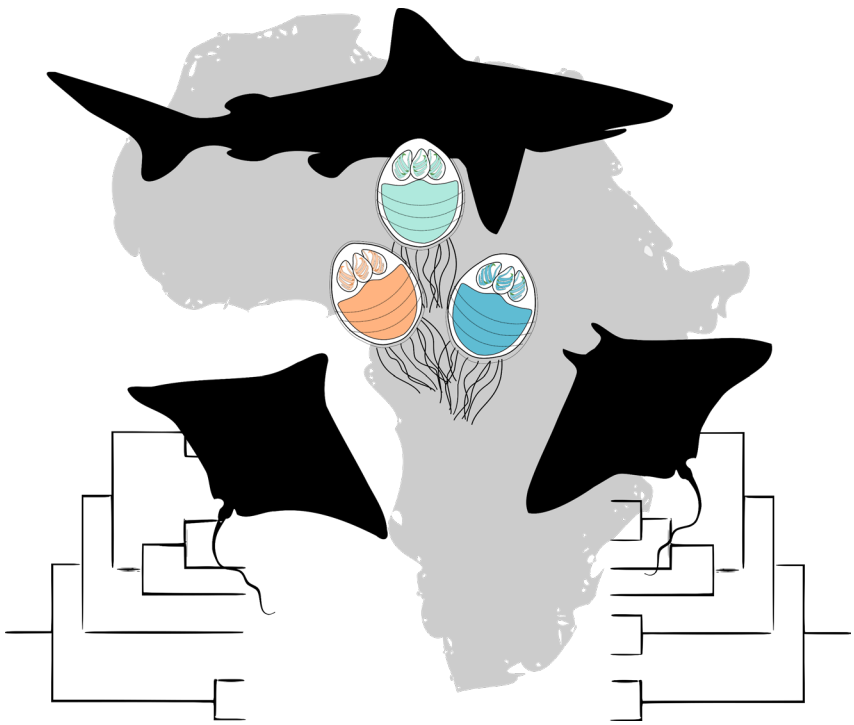
6.3 Paper VI

An ancient alliance: Matching evolutionary patterns of cartilaginous fishes (Elasmobranchii) and chloromyxid parasites (Myxozoa)



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This part is comprised of unpublished data, which is present in the original thesis deposited at the Faculty of Science, University of South Bohemia.

An ancient alliance: Matching evolutionary patterns of cartilaginous fishes (Elasmobranchii) and chloromyxid parasites (Myxozoa)

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Abstract

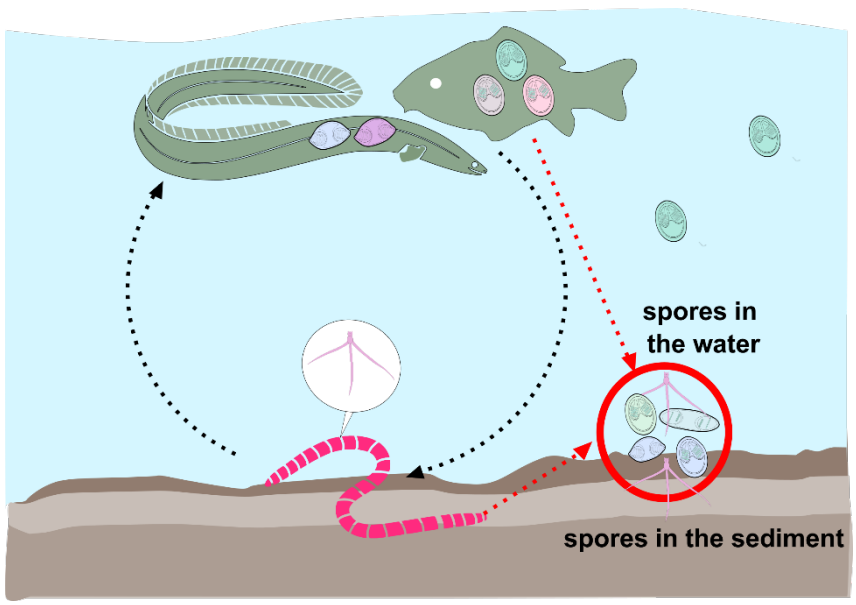
Myxozoa is a group of endoparasitic cnidarians covering almost 2,600 species but merely 53 species, mostly from the genus *Chloromyxum*, have been reported from sharks, rays, and skates (Elasmobranchii). Elasmobranchs play a key role in the study of evolutionary trajectories of myxozoans as they represent ancestral vertebrate hosts. Our study provides new data on *Chloromyxum* spp. from 57 elasmobranchs, covering 20 species from geographical regions and host groups not previously investigated, such as Lamniformes and Hexanchiformes, the most basal phylogenetic shark lineage. In total, 28% of elasmobranchs were infected with *Chloromyxum* spp., indicating high diversity. Of the seven distinguished species, six are formally described based on morphological, morphometric, and genetic (18S rDNA) data. Comprehensive co-phylogenetic analyses and ancestral state reconstruction revealed that parasite and host phylogenies are clearly

correlated, resulting in a distinct phylogenetic separation of chloromyxids from selachid (shark) vs. batoid (ray and skate) hosts. Species infecting the most ancient elasmobranchs formed a sublineage, branching off in the middle of the *Chloromyxum sensu stricto* clade. Our findings indicate that chloromyxids likely invaded an ancestral elasmobranch prior the time of divergence of shark and batoid lineages. Our analyses did not show a clear phylogeographic pattern of *Chloromyxum* parasites, probably due to the cosmopolitan distribution and migratory behaviour of many elasmobranch hosts, but geographical sampling must be extended to confirm or refute this observation. This study provides a complex view on species diversity, phylogeny, evolution, host-parasite co-phylogeny, and the phylogeographic origin of *Chloromyxum* species from elasmobranchs. Our results highlight the importance of adding missing data from previously un- or undersampled geographical regions and host species which results in a more accurate estimate of myxozoan biodiversity and a better understanding of the evolution of this parasite group in their hosts and in the different oceans of our planet.

Chapter VII: The identification of myxozoan diversity in environment

7.1 Paper VII

Evaluation and optimization of eDNA metabarcoding assay for detection of freshwater myxozoan communities



Manuscript in advanced preparation.

This part is comprised of unpublished data, which is present in the original thesis deposited at the Faculty of Science, University of South Bohemia.

Evaluation and optimization of eDNA metabarcoding assay for detection of freshwater myxozoan communities

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Abstract

1. The environmental DNA (eDNA) metabarcoding approach has become a useful tool for detecting the diversity of many species, including parasites. Myxozoa represent a unique group of morphologically simplified endoparasites that mainly infest fish and whose diversity remains largely unexplored. The parasitological study of host tissues is an invasive technique that requires the acquisition of a fish host and encounters difficulties such as access to rare, threatened, or endangered host species or the seasonal occurrence of myxozoans in the host. eDNA metabarcoding of infectious myxozoan spore stages occurring in the aquatic environment for transmission is a promising non-invasive method to assess myxozoan biodiversity at a given site. In addition, assessing the distribution and diversity of fish parasites is essential for fish disease control and for describing parasite communities.

2. Using alignment of 330 sequences, we employed In-silico PCR to score primer pairs, designed to amplify the V4 region of the SSU rDNA of different sublineages, aiming at amplification of the entire diversity of the oligochaete-infecting freshwater myxozoans. Eight clade-specific primer sets were selected for metabarcoding, avoiding amplification of the enormous diversity in eutrophic freshwaters.

3. Metabarcoding analysis of selected fish tissue samples revealed 89 different myxozoan OTUs, more than double that of Sanger sequencing with general myxozoan primers and almost seven times higher detection than microscopic examination.

Moreover, new approach used in sediment samples of small volume demonstrated more than one third of the diversity observed in a large number of fish (35 vs 90 OTUs). An important overlap between OTUs from the two sources was only found in a size-restricted habitat (small pond, no flow), thus providing valuable insights into quantitative sampling requirements for realistic future diversity estimates. Moreover, a pilot project of Douro sediment revealed high myxozoan abundance in small amounts of the sampled material. Using specific primer sets allowed for a high rate of detection of myxozoan reads (63.1-100%), in all datasets.

4. Our results show that the new metabarcoding approach is an excellent tool for non-invasive and sensitive detection of myxozoan biodiversity in aquatic sediments. The assay can also be utilised to monitor myxozoan diseases that threaten economically important fish in aquaculture.

Curriculum Vitae

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Conferences:

Lisnerová, M., Fiala, I., Cantatore, D., Irigoitia, M., Timi, J., Pecková, H., Bartošová-Sojková, P., & Holzer, A. The initiation of life cycle complexity in the Myxozoa: evolutionary events and potential mechanism. 19th Internation conference on Diseases of fish and shellfish (EAFP), 9th - 12th September 2019, Porto, Portugal (oral).

Fiala, I., **Lisnerová, M.,** Baiko, D., Blabolil, P., Lövy, A., & Holzer, A. The initiation of life cycle complexity in the Myxozoa: evolutionary events and potential mechanism. 19th Internation conference on Diseases of fish and shellfish (EAFP), 9th - 12th September 2019, Porto, Portugal (oral).

Lisnerová, M., & Fiala, I. Myxozoa Wherever You Look: Uncovering Myxozoan Species Diversity. 48th Jírovec's Protozoological Days, 30th April -4th May 2018, Kunčice pod Ondřejníkem, Czech Republic.

Grants:

GAJU (The Grant Agency of the University of South Bohemia in České Budějovice) one-year 2020 (027/2020/P): Detection of myxozoan species diversity by metabarcoding analysis.

Grant Programme Biology Centre of the Czech Academy of Science (Ibera) 2020: participation in a foreign stay*:* stay cancalled due to covid situation

Grant Programme Biology Centre of the Czech Academy of Science (Ibera) 2019: participation at an international scientific conference

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October 2019, University of Bologna (Italy), Maria-Letizia Fioravanti

Teaching and supervision:

Field practice „Terenní parazitologie“. Fish dissection, demonstration of hosts and parasites. Summer 2018.

Biology of parasitic protists. Teachig assistant, 2018-2019.

Monika Bürgerová: Myxozoa parasites in fish from dams in the river Rhine: morphological and molecular characterization. Supervision of the Bachelor Thesis, 2019-2021

Kateřina Bouberlová: Diversity and evolution of the Myxozoa in ancestral hosts: A retrospective look at the evolution of cnidarians. Co-supervision of the Bachelor Thesis, 2019-2021

Bc. Monika Bürgerová: Biodiversity of Myxozoa in Římov reservoir based on environmental DNA analysis. Co-supervision of the Master Thesis, 2021-current

Bc. Veronika Žánová: Monitoring the variability of myxozoan diversity in the Malše river by environmental DNA analysis Co-supervision of the Master Thesis, 2021-current

List of publications (non-included in Ph.D. thesis):

Tyml, T., **Lisnerová, M**., Kostka, M., & Dyková, I. (2018). Current view on phylogeny within the genus *Flabellula* Schaeffer, 1926 (Amoebozoa: Leptomyxida). *European Journal of Protistology*, *64*, 40-53.

Korytář, T., Wiegertjes, G. F., Zusková, E., Tomanová, A., **Lisnerová, M**., Patra, S., Sieranski, V., Šíma, R., Born-Torrijos, A., Wentzel, A. S., Blasco-Monleon, S., Yanes-Roca, C., Policar, T., & Holzer, A. S. (2019). The kinetics of cellular and humoral immune responses of common carp to presporogonic development of the myxozoan *Sphaerospora molnari*. *Parasites & Vectors*, *12*, 1-16

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