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„Morphological convergence in distantly related myxozoans: Spores as ecotypes "

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Abstract

Myxozoans are miniaturized endoparasites belonging to the Cnidaria, with roughly 2200 species currently described. They are characterized by a two-host live cycle, including an invertebrate and a vertebrate host (mainly fish). Spores are formed as durable transmission stages between hosts and they represent pluricellular stages in a wide variety of shapes, sometimes with ornamentation and appendages. Myxozoan taxonomy has been based predominantly on these morphological features, however the incongruence of spore morphology and phylogenetic clustering of myxozoans is obvious. Different spore morphotypes, i.e. genera, can be extremely closely related while other morphotypes appear to have emerged more than once during the myxozoan evolution. The aim of the present study was to determine whether a relationship exists between the spore features and their habitats, both within the host and the environment. Spores of 10 species were collected, their SSU rDNA was sequenced and their surface structure characteristics studied by SEM. Furthermore, they were included in a large database of 258 taxa to perform statistical analyses in R. The most prominent correlation was found between spore characteristics and external habitat. Spores in freshwater habitats are usually characterized by some form of surface structure enlarging their surface area and increasing their buoyancy, whereas marine spores are predominantly smooth. Most exceptions can be explained by the habitat of the ancestor (as determined by SSU rDNA phylogeny). Despite significant differences between projections and ornamentation in slow flowing water, the water current is more likely to influence the shape of the spore than its ornamentation. Characteristic spore shapes exist also for each host organ system, while ornamentation is more variable. In summary, the present study shows that intrapiscine and external environment account for most of the variation in morphological spore features, demonstrating that spores are in fact ecotypes.

Keywords: Myxozoa, spore morphology, vertebrate host, ecology, infection habitat

Zusammenfassung

Myxozoa sind wenige Mikrometer große Parasiten, welche zu den Cnidaria gehören. Zurzeit sind ungefähr 2200 Arten innerhalb der Myxozoa beschrieben. Sie leben meistens als Endoparasiten innerhalb aquatischer Habitate und besitzen einen komplizierten Wirtswechsel zwischen Evertebraten und Wirbeltieren (am häufigsten Fischen). In den letzten Jahren zeichnete sich immer deutlicher ab, dass es einen Zusammenhang zwischen Form, Oberflächenstruktur (Rillen, Höhlen…) und Anhängen (Schwänze, Flügel, Lappen, Haare…) der Sporstadien und dem ökologischen Habitat, dem Wirtsgewebe und andern Faktoren geben muss. Somit müsste es auch möglich sein, Sporen anhand der beeinflussenden Faktoren als Ökotypen zu beschreiben. Diese Studie zeigt, dass das Infektionshabitat eine bedeutende Rolle in der Sporentwicklung einnimmt. Sporstadien aus dem Süßwasser zeigen immer eine Form von Oberflächenstrukturen (meistens Rillen), da sie ihre Oberfläche und so auch ihr Volumen vergrößern, um die im Wasser vorhandenen Strömungs- und Auftriebskräfte zu nutzen. Marine Arten sind in den meisten Fällen glatt, da im Salzwasser der Auftrieb aufgrund der höheren Dichte des Wassers größer ist. Ausnahmen lassen sich häufig durch die Verwandtschaftsbeziehungen erklären, da einige Arten ihr Habitat während ihrer Evolution wechselten. Weitere Unterschiede bei der Oberflächenstruktur und den Anhängen konnten nur in langsam fließenden Gewässern festgestellt werden, obwohl die Fließgeschwindigkeit am ehesten die Form der Sporen verändert. Die Form verändert sich jedoch wesentlicher in Abhängigkeit vom Organsystem innerhalb des Fisches, während die Oberflächenstruktur geringere Veränderungen aufweist. Zusammenfassend, konnte diese Studie jedoch bestätigen, dass sowohl Organsysteme als auch Faktoren des externen Habitats für die meisten morphologischen Veränderungen verantwortlich sind. Somit konnte bestätigt werden, dass die Sporen von Myxozoen tatsächlich Ökotypen darstellen.

Stichwörter: Myxozoa, Sporen, Wirbeltiere als Wirt, Parasiten, Infektionshabitat

1. Introduction

1.1. General introduction to the Myxozoa

Myxozoans are microscopic parasites belonging to the Cnidaria, with roughly 2200 species (Lom and Dyková 2006), currently described in 64 genera (Okamura, Gruhl and Bartholomew 2015). Myxozoans are extremely diverse and currently compose one fifth of all known cnidarians, however in hypothetical calculations as many as 16 000 myxozoan species are suggested for the Neotropics alone (Naldoni et al. 2011) and eDNA analyses predict the existence of many more taxa and phylogenetic lineages (Hartikainen et al. 2016), making the prospected number higher. Certain myxozoans cause severe diseases in their hosts, both in wild populations and aquaculture facilities, contributing to large ecological and economical losses. Especially in wild populations disease control is difficult. Young fish are often more susceptible to diseases and parasite infections causing extreme losses in wild stocks and in aquaculture.

Myxozoa were first described in 1880, however their classification changed from protozoans to the animal kingdom. Now it is clear that they are Cnidaria (Jimenez-Guri 2007; Jimenez-Guri, Okamura and Holland 2007, Holland et al. 2011), which diverged from a single ancestor. During this process myxozoans simplified their morphology and evolved an intricate parasitic life cycle. The two hosts live cycle is generally completed in aquatic habitats and usually includes an invertebrate and a vertebrate host. Fish serve as intermediate hosts (Okamura et al. 2015), whereas annelids or bryozoans suit as the final host. Though approximately 50 life cycles have been elucidated (Okamura et al. 2015), details of the development are only scarcely known in either host. However, a sophisticated host-parasite relationship and host manipulation has enabled them to spread from their teleost hosts to amphibians and reptiles (Jirků et al. 2011; Lom and Dyková 1993; Hartigan, Phalen and Slapeta 2013), waterfowl (Bartholomew et al. 2008) and even small mammals (Székely et al. 2015; Prunescu et al. 2007) in their host range.

1.2. Myxozoan taxonomy contradicts phylogeny

Myxozoan taxonomy has been and continues to be based predominantly on morphological features, mainly of the spore formed in the vertebrate host. Since their first discovery taxonomy was changed several times. Several changes were executed in the 1980s with the last revision by Lom and Noble (1984), however the important characters for classification stayed the same. Although spores vary in their appearance, they are all composed of a number of shell valves that are joined by a suture line, at least one polar capsule and one or more sporoplasms and their nuclei (Lom and Noble 1984). To characterize orders and suborders of myxosporeans two of those characteristics are important, either the number and configuration of the shell valves or the position of the polar capsules with regard to

the suture. Other characteristics for identification include details of the polar filaments, dimensions of the polar capsules, the presence of ridges or striations on the valve surface, the presence of appendages and the presence or absence of a mucous envelope. To determine a spore on species level Lom and Arthur (1989) proposed that characters, like host identification, host habitat, information about the vegetative stages and whether the sporogonic stages are mono-, di- or polysporic are important for a complete description.

Systematics is still mainly based on spore morphology, but since the 1990s, when first DNA sequences became available this system has become much debated, due to the incongruences between morphology and DNA based phylogenetic approaches. As myxozoan diversification started hundreds of millions of years ago, conservative markers are required to reconstruct their evolution. The 18S rDNA is found in all eukaryotic taxa and it has become the most commonly used phylogenetic marker of Myxozoans (Okamura et al. 2015). After phylogenetic analysis of molecular data for approx. 700 myxozoan taxa (National Centre of Biotechnology Information, NCBI, status April 2017) it is clear that most myxozoan genera are either para- or polyphyletic (Diamant et al. 2005; Diamant, Whipps and Kent 2004; Fiala 2006). While some spore morphotypes (genera) have emerged several times, occupying 5 or more different phylogenetic position (Heiniger, Gunter and Adlard 2011), molecularly monophyletic groups are artificially separated into different genera by minor differences such as presence/absence of caudal appendages, used to distinguish *Henneguya* from *Myxobolus* (Fiala 2006) or the number of sporoplasms which differentiated *Polysporoplasma* and *Sphaerospora* (Bartošová et al. 2013).

Although the SSU marker is very suitable for myxozoan phylogeny, there are certain problems with sticking to only one marker. The sequence length of myxosporeans varies a lot between marine and freshwater species. Marine species show the shortest SSU sequences (1500-1700 basepairs), whereas freshwater clades can be up to 2100 basepairs long. The Sphaerospora sensu stricto clade breaks ranks, as with up to 3700 basepairs they have one of the longest SSU rRNA sequences in all eukaryotes (Eszterbauer et al. 2013).

To produce the best outcome for myxozoan phylogeny Heiniger and Adlard (2013) propose a combination of morphological biological and molecular characters. Their data on ceratomyxids from cardinal fishes in Australian waters show significant differences between genetics and biological characters, but insignificant variances amongst spore characters.

1.3. Biological characteristics reflecting myxozoans phylogeny

Based on the controversy between spore morphology and molecular phylogeny, naturally one important question arises: Which characters if not morphological ones explain the phylogenetic clustering of the Myxozoa and play a key role in their evolution? Tissue localisation in the intermediate

host and host habitat (freshwater vs marine) was first identified as a factors explaining phylogenetic clustering of the morphologically paraphyletic genera (Holzer, Sommerville and Wootten 2004; Fiala 2006). Following further life cycle discoveries, it was suggested that the invertebrate host group mirrored large-scale myxozoan phylogeny and SSU rRNA secondary structure (Holzer, Wootten and Sommerville 2007). Most recently, Kodádková et al. (2015) determined that vertebrate host groups also mirror myxozoan phylogeny with cartilaginous fish representing an ancestral state for most myxozoan lineages.

An effect of geography on phylogenetic clustering was confirmed by Whipps and Kent (2006). Differences between four oceanic regions suggest significant barriers on a global scale, whereas the genetical exchange on a smaller geographic scale is not as meaningful, as morphological differences between different host species and locations could not be found. Therefore, this genetic study on *Kudoa thyrsites* suggests that the examined regions compromise endemic populations. The influence of geography on the 18S rDNA diversity of single species was also observed by other others as well (e.g. for *Zschokkella nova* (Fiala 2006) and for *Myxidium truttae* (Holzer et al. 2004)).

1.4. Spore morphotypes

If biological characteristics such as host tissue location, invertebrate host group or geography mirror phylogenetic clustering, another question arises: What influences spore shape, size and ornamentation? Apart from enduring unfavourable conditions outside the host, spore morphology should favour rapid/easy exit from the host, dispersion in the environment and should guarantee reaching the adequate invertebrate host. As such, spores likely represent ecotypes.

The common myxosporean ancestor had a smooth spore surface and was marine (Kodádková et al. 2014; Kodádková et al. 2015) Shell valves with ridges and striations appeared on entry into freshwater environments (Kodádková et al. 2014). Surface structures (ridges, pits…) and appendages of the spore appear to have the same main purpose, which is increasing the surface area of the spore. Based on the Archimedes' principle an upward buoyant force that is exerted on a body immersed in water. The density of the surrounding water is lower than the density of the spore, hence helping the spore to float. As any appendage or surface structure increases the surface area of the spore the surrounding buoyancy forces have more area to operate on and as such slow down the spore when sinking. Thus, spores have more time to float and can disperse further. As saltwater has a higher salinity as freshwater it is more viscous and spores sink slower than in freshwater, forcing freshwater spores to use mechanisms that counteract dispersal fast settlement rate. Hence one could speculate that spores in marine habitats can disperse even with a smooth surface, whereas freshwater spores should more likely have surface structures and appendages. In reality, this is not always the case as species descriptions show that most marine species have surface structures, whereas freshwater do not.

Bipteria vetusta spores enlarge their surface area with wing-like structures (Shul'man 1964). The deep-water genus *Palliatus* evolved a membranous veil (Kodádková et al. 2014). Both might enable better flotation in the high-pressure water column (Fiala and Bartošová 2010). Generally, appendages in myxozoans are thought to be adaptations to the out-of-host environment, and evolved many times independently e.g. in *Myxodavisia, Pseudalatospora* and *Henneguya* (Kodádková et al. 2014).

However, parasites can also adapt to the hosts' organ structure. Myxozoans live in restrained areas making morphological simplifications vital (Lom, Rohde and Dyková 1992). Therefore, absence or minimization of structures might represent an adaptation to the intrapiscine habitat. Most fish organs can be infested, myxozoans can be either coelozoic, inhabiting body cavities, or histozoic (inhabiting tissues). Myxospores with long appendages could be expected to be found in coelozoic environments, as release from the fish is otherwise difficult (Feist and Longshaw 2006). However, *Henneguya*, is known to have the longest appendages in myxozoans, lives in both marine and freshwater environments, and is found mainly in histozoic habitats, like gills (Barassa et al. 2003) or kidney (Wagner 2016). This hinders the spore from a rapid exit from the host organ. Most coelozoic spores do not have long appendages (Arndt et al. 2006, Landsberg and Lom 1991), but are covered with surfaces structures (Eiras et al. 2011; Eiras et al. 2012; Bartholomew et al. 2008; Hartigan et al. 2012). These surface structures might be useful to float in the e.g. bile of the host.

The water flow rate has direct and indirect effects on the spores. The temperature of the water body is highly dependent on the pace of the water flow. Low water flow promotes higher temperatures, as the water gets heated by the sun. Experimental studies also show that slow flowing water has a higher amount of temperature fluctuations, which may interfere with myxozoan development (Hallett and Bartholomew 2008). High flows might damage the spores and cause a higher dilution of the spores, suggested on actinospores (Kerans and Zale 2002). In a one-year experiment Hallett and Bartholomew (2008) demonstrated that habitats with lower water flow promote higher infection prevalence and greater dispersal in the invertebrate host. They also propose that the attachment of the actinospore to the fish is affected by the flow velocity, due to two observations, first more actinospores lacking sporoplasms could be found in slow flowing waters, second more fish became infected in the slow flowing experimental tanks. These are strong indications that myxozoan spores adapted their morphology to optimize host exit and transmission in the environment.

2. Aims

The aim of this thesis was to determine if a relationship exists between shape, cellular organisation and fine structure of myxozoan spores and the characteristics of their habitat, both within and outwith the fish host. Therefore, a comprehensive database of 258 taxa from all phylogenetic clades and known morphotypes (genera) was produced and a number of statistical models were tested to understand which morphological features can be related to which habitat. For example: Do the surface ridges and appendages of myxozoan spores vary in relation to a specific host habitat? Which spore structure represents which ecotype? Which myxozoan spore type is found in which host habitat? Additionally, morphological and ultrastructural data as well as 18S rDNA sequences were produced for new/previously undescribed taxa which were also added to the database.

3. Material and Methods

3.1. Collection of spores from fish

In each fish, the gallbladder, kidney (head and hind kidney), liver, gills, and skin (smears of lateral body surface of fish and from under pectoral fin) were checked for myxozoans by light microscopy (400x magnification). To take samples from the inner organs a ventral cut from the anus to the pelvic fins of the fish was performed. Then the body cavity was opened. During a 5-month-period, 125 fish belonging to 22 species and 13 families were checked for myxozoans (table 1). While myxozoan species were found in 16 fish species (table 1), only 10 were used for this study as for these species a sufficient number of spores for electron microscopy were found. If spores were detected in an organ they were isolated and observed at x400 magnification under an Olympus BX51 light microscope (Olympus Optical Co. (Europa) GmbH, Hamburg, Germany). Spores were immediately stored in TNES for further molecular examination as well as in water or saltwater (depending on origin of fish) for Scanning Electron Microscopy (SEM).

Table 1: Number of dissected and infected fish species and found myxozoan species. In the column of dissected fish, the number in parenthesis shows how many fish were infected with the myxozoan species in the corresponding column.

Fish species	dissected(infected) fish	Fish family	Found myxozoan species
Albramis brama	17 (5)	Cyprinidae	*Zschokella nova/Myxobolus sp.*
Anguilla anguilla	1 (1)	Anguillidae	*Myxidium giardi*
Centropyge acanthops	5	Pomacanthidae	-
Carassius auratus	1(1)	Cyprinidae	*Sphaerospora sp.*
Crassius gibelio	14(3)	Cyprinidae	*Myxobulus sp.*
Ctenopharyngodon idella	3 (2)	Cyprinidae	*Zschokella nova, Chloromyxum sp.*
Cyprius carpio	4(2)	Cyprinidae	*Thelohanellus wuhanensis/Myxobolus sp.*
Datnioides microlepsus	3	Datnioididae	Plasmodium
Esox lucius	8	Esocidae	-
Gobiodor okinawae	3(1)	Gobiidae	*Ceratomyxa sp.*
Merluccius productus	1(1)	Merlucciidae	*Kudoa sp.*
Microsynodontis batesii	5	Mochokidae	-
Oligoancistrus sp.	1	Loricariidae	-
Otocincius affinis	5	Loricariidae	-
Paracanthurus hepatus	5(1)	Acanthuridae	-
Pseudanthias squamipinnis	5(5)	Serranidae	*Ceratomyxa cardinalis*
Rhodeus amarus	1(1)	Cyprinidae	*Myxidium sp.*
Rutilus rutilus	16(14)	Cyprinidae	*Zschokella nova, Myxobolus sp.*
Scathophagus argus	5(2)	Scatophagidae	*Myxobolus sp.*
Squalius cephalus	1(1)	Cyprinidae	*Myxobolus muelleri*
Tetradon nigrovirigis	18(11)	Tetraodontidae	*Ortholinea aurata, Ceratomyxa arcuata*
Tinca tinca	3(1)	Cyprinidae	*Chloromyxum cyprini*

3.2. Spore morphology

Digital photos of isolated spores were taken at x1000 magnification with an Olympus DP70 camera (Olympus Optical Co. (Europa) GmbH, Hamburg, Germany) mounted on the Nomarski interference microscope Olympus BX51. Measurements of 10 spores of each myxozoan species were taken on digital images with the program ImageJ, Version: 64-bit Java 1.6.0_20 for Windows (National Institutes of Health, Maryland, US). Measurements of the spores follow the guidelines of Lom & Arthur (1989) and include spore length, spore width, spore thickness as well as polar capsule length and polar capsule width. The definitions and how to measure was incurred from Lom and Dyková (1992). Spore length is defined by the distance between the apex and the posterior end. The width is measured perpendicular to the length from one end of the suture to the other. The thickness is measured perpendicular to the suture plane, from the most distant point of one valve to the other. Polar capsule length and width are measured like the rest of the spore (figure 1).

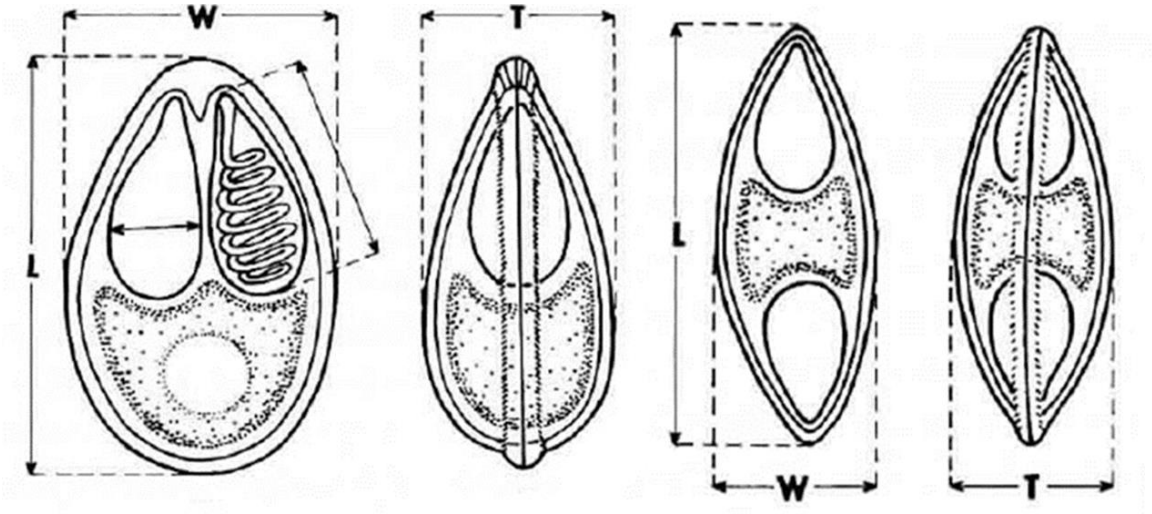

Figure 1: How to measure length (L), width (W) and thickness (T) of a myxozoaon spore. (Lom and Dyková, 1992)

Ultrastructure of myxozoan spores

For SEM sample preparation, a protocol of Alama-Bermejo (2009) was followed. Round cover slips were washed in 96% ethanol (EtOH). The dried slides were coated with 0,1% poly-D-lysine on the surface of which spores were left to settle for 30 min in their preferred medium (water or seawater, depending on origin of fish). Afterwards the spores were fixed for 30 min on the coverslip using 2,5% glutaraldehyde in 0,1M Cacodylate buffer. Then the spores were rinsed twice in the same buffer for 15 minutes each. Post-fixation was performed with 1% osmium tetroxide in 0,1M sodium cacodylate buffer, for 30 minutes. The coverslips where washed with distilled water for 15 minutes and dehydrated in an ascending alcohol series from 30% to 100% EtOH, for 5 minutes each. Thereafter, the samples were critical point dried (PELCO CPD2, Ted Pella Inc, Redding, USA), mounted on stubs and gold-sputtered with a BAL-TEC, SCD 050 sputter coater (BAL-TEC, Leica Biosystems Nussloch GmbH,

Nussloch, Germany). The spores were examined with a JEOL JSM-7401F scanning electron microscope (JEOL, Akishima Tokyo, Japan).

3.3. Molecular analyses

DNA analysis

To extract DNA from the spores they were stored in 400µl TNES urea buffer (10mM Tris-HCl, 125mM NaCl, 10mM ethylenediaminetetraacetic acid, 0,5% sodium dodecyl sulphate, 4M urea) directly after fish dissection. The spores were digested with Proteinase K (20 µg/ml) at 55°C overnight. For DNA extraction, 400µl phenol (pH=8 buffered) and 400µl chloroform were added. After mixing, the different phases were separated by centrifugation (15 000g for 5 min). Thereafter, 330µl of the aqueous top layer including the DNA was removed into a fresh tube. The DNA was then precipitated mixing the sample with triple amount of ice-cold 92% EtOH. After pelleting the DNA by centrifugation (13 000 g, 20 min) and washing the sample with 70% EtOH, the sample was dried on a heating plate at 55°C until all alcohol had completely evaporated. The dried DNA was then resuspended in 50µl of RNAsa/DNAsa-free water and left in the fridge to dissolve overnight.

Table 2: Annealing temperatures of all primer pairs

Primer Pairs	Annealing temperature
18e, 18R	62°C
MyxGP2F, Act1R	58°C
18e, 18g	62°C
MyxGEN4F, 18g	60°C
Myx1F, Mx3	66°C
SSU CER-For, SSU CER-Rew	57 °C

Polymerase Chain Reaction was performed with a programmable thermal cycler (Thermal Cycler-Life Pro, BIOER Technology, Hangzhou, China or TPersonal, Biometra GmbH, Göttingen, Germany) in a final volume of 10µl. Each tube contained 1µl dissolved DNA (50-100 ng), 7.35 µl nanopure water, 1 µl titanium buffer (containing 1,5Mm $MgCl_2$, ABgene, Epsom, UK), 0.2 mM dNTPs each, 0.5 µM forward and 0.5 µM reverse Primer, as well as 0,25 U Titanium Taq Polymerase. In the first PCR a universal set of primers targeting the 18S rDNA was used (18E, 18R). For the nested PCR different primer pairs were used depending on the screened myxozoan species. The volume and composition of the nested PCR were the same, with 1µl of the first PCR product used in the nested assays. The PCR protocol consisted of initial DNA denaturation at 95°C for 3min, and 30 cycles of DNA separation at 94° for 50 seconds, annealing for 50 sec at different temperatures depending on the Primers (table 2; table 3), 2 minutes elongation at 68°C, as well as a final elongation step at 68°C for one minute and a final hold at 10°C. After checking if DNA was present in a 1% agarose gel in TRIS-borate-EDTA buffer, the eluted DNA was purified for sequencing with a Kit (PCR Cleanup Kit Gel/PCR DNA Fragments Kit (DF300), Geneaid Biotech Ltd., Taiwan) and sent off for commercial sequencing by SEQme (SEQme s.r.o., Czech Republic).

Table 3: Primer sequences. The table shows all used primer sequences and the corresponding basepairs

Primers	Sequence	paired with	Primers	Sequence	paired with
18e	TGGTTGATCCTGCCAGT	18R, 18g	**Myx1F**	GTGAGACTGCGGACGGCTCAG	Mx3
18g	GGTAGTAGCGACGGGCGGTGT	18e, MyxGEN4F	**Mx3**	CCAGGACATCTTAGGGCATCACAG	Myx1F
18R	CTACGGAAACCTTGTTACG	18e	**SSU CER-For**	CTWGTTGGTADGGTAGTG	SSU CER-Rew
MyxGP2F	GGATAACCGTGGGAAATCTA	Act1R	**SSU CER-Rew**	GTACAAGAGGCAGAGACGTAT	SSU CER-For
Act1R	AATTTCACCTCTCGCTGCCA	MyxGP2F	**MyxGEN4F**	GTGCCTTGAATAAATCAGAG	18g

Cloning

When the obtained sequences had double peaks (contamination or mixed infection), the nested PCR product was cloned. Therefore 2.5 µl of ligation master mix, 2.0 µl of the eluted PCR product and 0.5 µl cloning vector were mixed and incubated for 2 hours at 14°C in a thermal cycling block (ligation). After placing the ligation reaction mix on ice, 50µl of competent bacterial cells (*Escherichia coli*- strain DH5α) were added. The mixture was then incubated for 5-8 minutes on ice. The tubes were heated in a 42°C water bath for 30 seconds (vector inclusion) and then incubated on ice again for 2 minutes. Then 200µl of room temperature SOC medium was added and the tubes were shaken horizontally at 37°C for an hour. Meanwhile 40µl of X-gal was spread on each LB agar plate and the plates were incubated until ready for use at 37°C. After spreading the transformation mixture on the prepared agar plates, they were incubated overnight at 37°C.

The next day white and blue colonies were visible on the agar plates. White colonies, containing the PCR product, were taken up with a pipette tip and placed into a tube containing 30µl of nanopure water. The tubes where then shaken at 37°C for 10 minutes. After that a new PCR was performed with a total amount of 10µl per tube. Each tube contained 2µl of cell suspension, 1µl of 10x Taq buffer, 0.2 mM of dNTP, 10µM of M13 forward primer and 10µM M13 reverse Primer, 0.24 U of polymerase and 6µl of PCR water. The PCR protocol consisted of an initial denaturation step at 95°C for 10min, and 20 cycles of DNA denaturation at 95° for 30 seconds, annealing at 54°C for 1 minute, 1 minute elongation at 72°C and a final extension at 72°C, for 10 minutes. Finally, the plasmid PCR products were visualized by agarose gel electrophoresis. If the electrophoresis showed a band of the expected size, the corresponding bacterial colonies were put into tubes with 12µl ampicillin and 3ml culture solution and shaken overnight at 37 °C. To isolate the plasmids from the competent cells, the High Pure Plasmid Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used. DNA was then sent for commercial sequencing to SEQme.

The obtained sequences were submitted to the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI) to screen for matches or close relatives. For the alignment of 18S rDNA sequences Geneious (Biomatters Ltd., Auckland, New Zealand) was used.

3.4. Database of morphological and habitat features

To determine whether a relationship exists between the morphological features of myxozoan spores (size, shape, surface structure, appendages etc.) and their habitats, both within the host and the environment a database of 258 species was complied, including all currently recognised myxozoan genera. For statistical analyses the following characteristics were picked: spore length, spore width, spore thickness (and ratios of the latter three), the occurrence, size and structure of appendages, the occurrence of surface structures, the type of surface structure and general information about the habitat of the spore/host (marine, freshwater, brackish), as well as the organ location of the spore inside the vertebrate host (table 4).

Table 4: For statistical analysis, all characteristics were changed into categories (represented by numbers). For all spore measurements the mean was calculated. The infection habitat of the spore was grouped into freshwater, marine and all habitats (FW, M, FWMB). Ancestral habitat is represented by four categories (FW, M, FW to M, M to FW). Categories were ascribed for each organ system and for the general location within the host (coelozoic, histozoic, ectoparasitic). Water current was categorized from standing water (1) to very fast flowing water (5). Surface structures and appendages were put into subjective categories from none at all (as the lowest number) to a lot of them (the highest number). The category "others" in appendages includes appendages that only occur in one myxozoan species. The shape of the spore was also put into categories based on descriptions in the literature. The shape category "others" includes shapes that occur only in one myxozoan species.

	CATEGORIES						
CHARACTERS	**1**	**2**	**3**	**4**	**5**	**6**	**7**
ancestral habitat	marine	freshwater	marine to freshwater	freshwater to marine			
infection habitat	freshwater	marine	freshwater, marine, brackish				
water current	1	2	3	4	5		
host organ tissue	urinary system	gills and skin	biliary system	reproductive and digestive system	muscles and skeleton	brain and nerves	
inhost habitat	histozoic	coelozoic	ectoparasitic				
ornamentation Differences	smooth	fine/thin ridges	thick ridges/ furcated	circular ridges	pockets and pits		
appendages	mucuos envelope	hairs	short to long tail	wings	others	no appendages	
shape	disc	banana to horseshoe	spherical	subspherical (-like)	club to drop	shamrock	others

3.5. Statistical analyses

The ratio between length and thickness, thickness and width, and width and length was calculated by dividing one by the other. Normality of the measurements and ratios was tested with the Shapiro-Wilkinson-Test in the R-package “nortest” version 2.0-4 (Gross and Ligger 2015) and Q-Q plots version 7.3-47 (Wickham 2009). Thereafter a Kruskal-Wallis-Test (R-package rcompanion, version 1.5.6, Mangiafica 2017) was performed. If a significant relationship between a measurement and habitat could be detected a Dunn-Test, R-package “FSA” version 0.8.13 (Ogle 2017) was performed to show significance between the different habitat categories. To test significance of the association between two kinds of nominal categories a Fisher's exact test of contingency tables was performed, including multiple comparisons for each combination with the R-package rcompanion, e.g. differences between ornamentation types in the marine habitat or differences between appendages in the biliary system. All statistical analysis was conducted in R (R Core Team, version 3.3.3, 2017, Vienna, Austria), plots were done with ggplot2 version 2.2.1 (Wickham 2009).

3.6. Phylogeny

To draw an exemplary phylogenetic tree including all SSU rDNA sequences obatained in this study as well as selected sequences from GenBank, sequences were assembled and aligned with Geneious (Biomatters Ltd., Auckland, New Zealand) using MAFFT v7.017 (Katoh et al. 2002) with default parameters. Phylogeny was reconstructed using the maximum likelihood method in RAxML (Stamatakis 2006) using the GTR+G model. The malacosporean *Buddenbrockia plumatellae* was set as an outgroup. The tree was visualized in Fig-Tree v 1.4.3. (Rambaut 2007).

4. Results

4.1. Ultrastructure of myxozoan spores

Examples presented in figure 2 show that most marine myxozoan spores have a smooth surface and no ornamental structures of any kind. *Kudoa sp. ex Merluccius productus* (Figure 2C) has four valve cells, whereas the three-other species have two valve cells. Both, *Ceratomyxa sp. ex Paracanthurus hepatus* (Figure 2A) and *Ceratomyxa cardinalis* (Figure 2B) have elongated, banana shaped spores with a straight suture. *Myxobolus portucalensis from Anguilla anguilla* (Figure 2D) is disc shaped with a straight suture line.

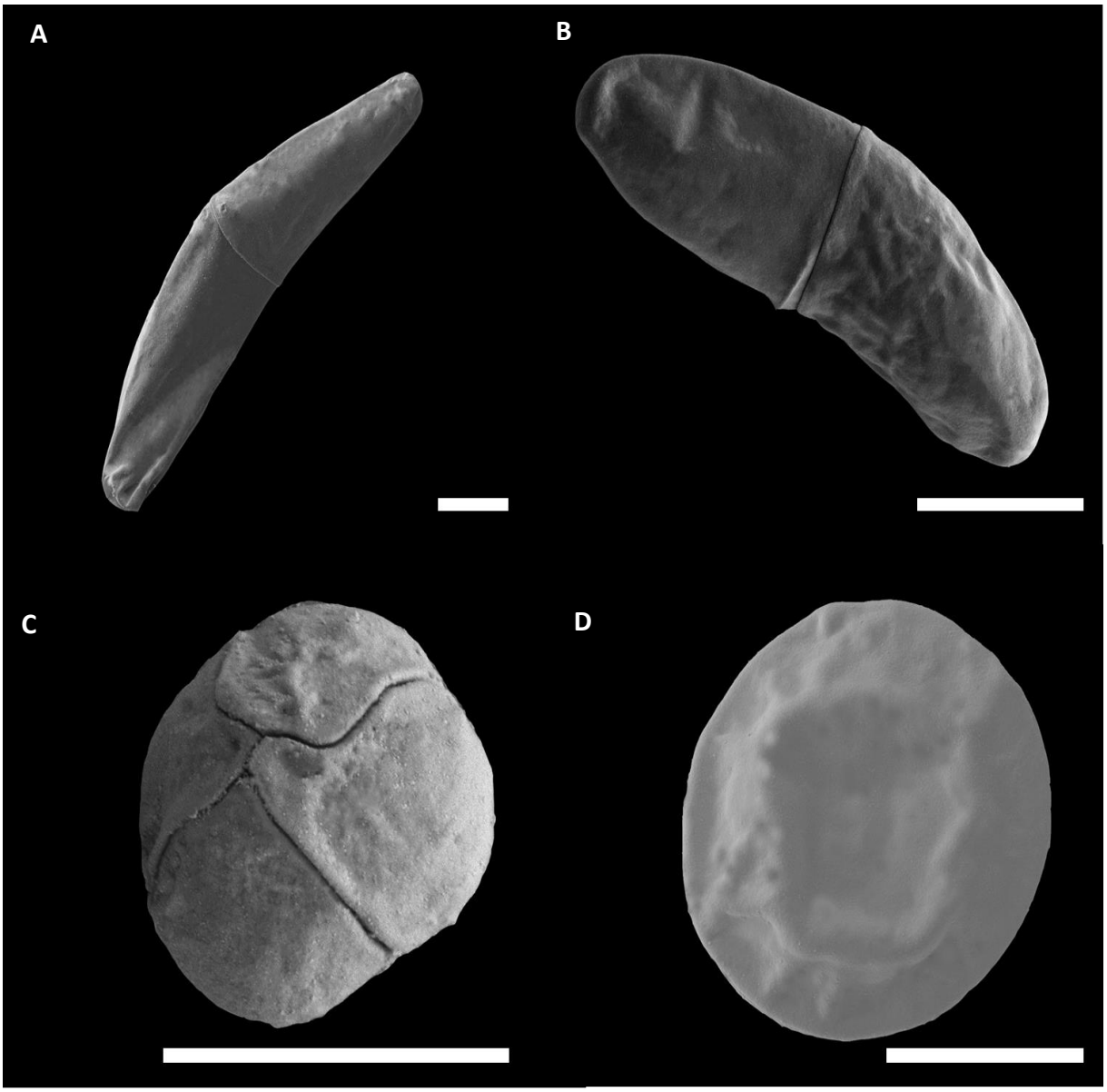

Figure 2: Examples of myxospores with a smooth spore surface. A: *Ceratomyxa sp.* from the gallbladder *of Paracanthurus hepatus*, marine B: *Ceratomyxa cardinalis* from the gallbladder of *Pseudanthias squamipinnis*, marine C: *Kudoa sp.* from the muscle tissue *of Merluccius productus*, marine D: *Myxobolus portucalensis from the gills of Anguilla anguilla*, freshwater scale bar= 5µm

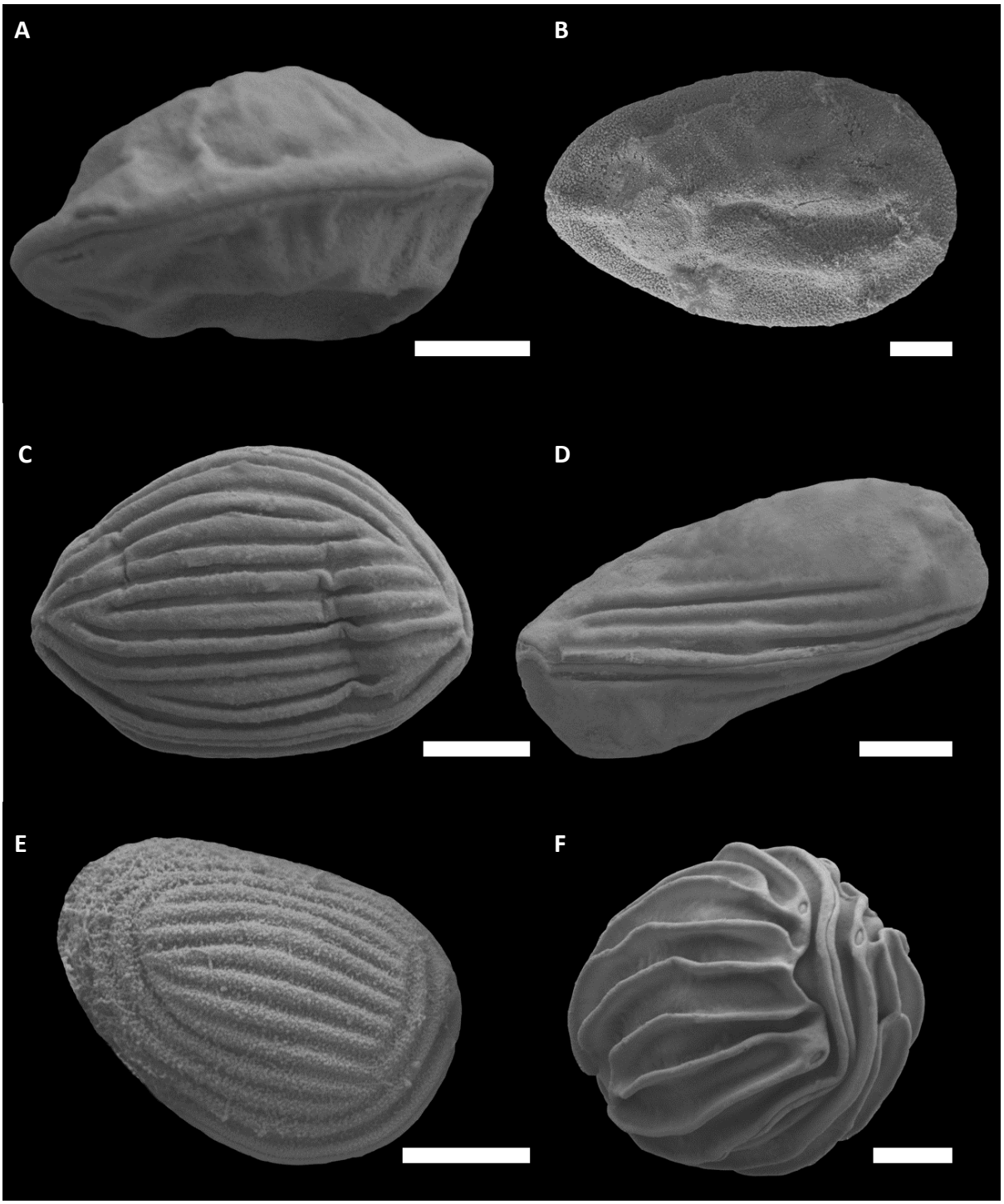

Figure 3: Examples myxospores with an ornamented spore surface and from freshwater habitats. A: *Myxobolus dispar* from the muscle of *Cyprinis carpio, B: Thelohanellus sp.* from the fin rays of *Cyprius carpio,* C: *Myxidium rhodei from the kidney of Rhodeus amarus*, D: *Zschokkella sp.* from the gallbladder of *Ctenopharyngodon idella*, E: *Ortholinea sp.* from the kidney tubules of *Tetradon nigrovirigis*, F: *Chloromyxum cristatum* from the gall bladder of *Tinca tinca,* scale bar= 2µm

As seen in figure 3 most freshwater species have surface structures. However, the species-rich histozoic genera *Myxobolus spp.* (figure 3A and 2B) and *Thelohanellus spp.* (figure 3B) have disc-like spores that have smooth valves and straight sutures. *Myxidium rhodei from Rhodeus amarus* has a straight suture and thin longitudinal ridges (figure 3C). The pictured *Zschokkella spp.* from gallbladder of *Ctenopharyngodon idella* shows only three longitudinal ridges on either side of the straight suture line, which appears to run diagonal due to the position of the polar capsules (figure 3D). Otherwise the spores are smooth. Parallel to the suture of the spores of *Ortholinea spp.* three circular ridges are visible (figure 3E). In the middle of the circle thin longitudinal parallel ridges are formed. *Chloromyxum cristatum* from the gallbladder of *Tinca tinca* shows a sinuous suture and 8-10 tall ridges on either spore valve (figure 3F).

4.2. Phylogenetic relationship between myxozoan taxa based on 18S rDNA sequences

Ten new 18S rDNA sequences were obtained in the present study which clustered in 8 different subclades (figure 4). *Henneguya*, *Thellohanellus* and *Myxobolus* cluster together in several subclades of the oligochaete-infecting freshwater clade of myxozoans. As previously stated, most myxozoan genera are poly- or paraphyletic. *Myxidium spp.* serve as an extreme example for polyphyly whereas other genera like *Ceratomyxa* and *Kudoa* are almost monophyletic.

While all belonging to a single genus, *Myxidium* species with smooth valves cluster together in one subclade. All other species have ridges somewhat parallel to the suture. Differences in spore length, thickness and width as well as variations of the polar capsules have no visible effect on the phylogenetic position. Yet, one clade including *Myxidium amazonense, Myxidium rhodei* and *Myxidium cuneiforme* seems to have slightly elongated spores. This subclade also is an exception to all other *Myxidium* species as those three species have a suture perpendicular to the polar capsule plane and striations, whereas a perpendicular suture is normally found in smooth spores while polar capsules and suture are in the same plane in spores with striations. As fine, longitudinal ridges are present in all but one subclade, and the shape of all subclades with striations is very similar it is difficult to differentiate these subclades, even by morphology supported by ultrastructure.

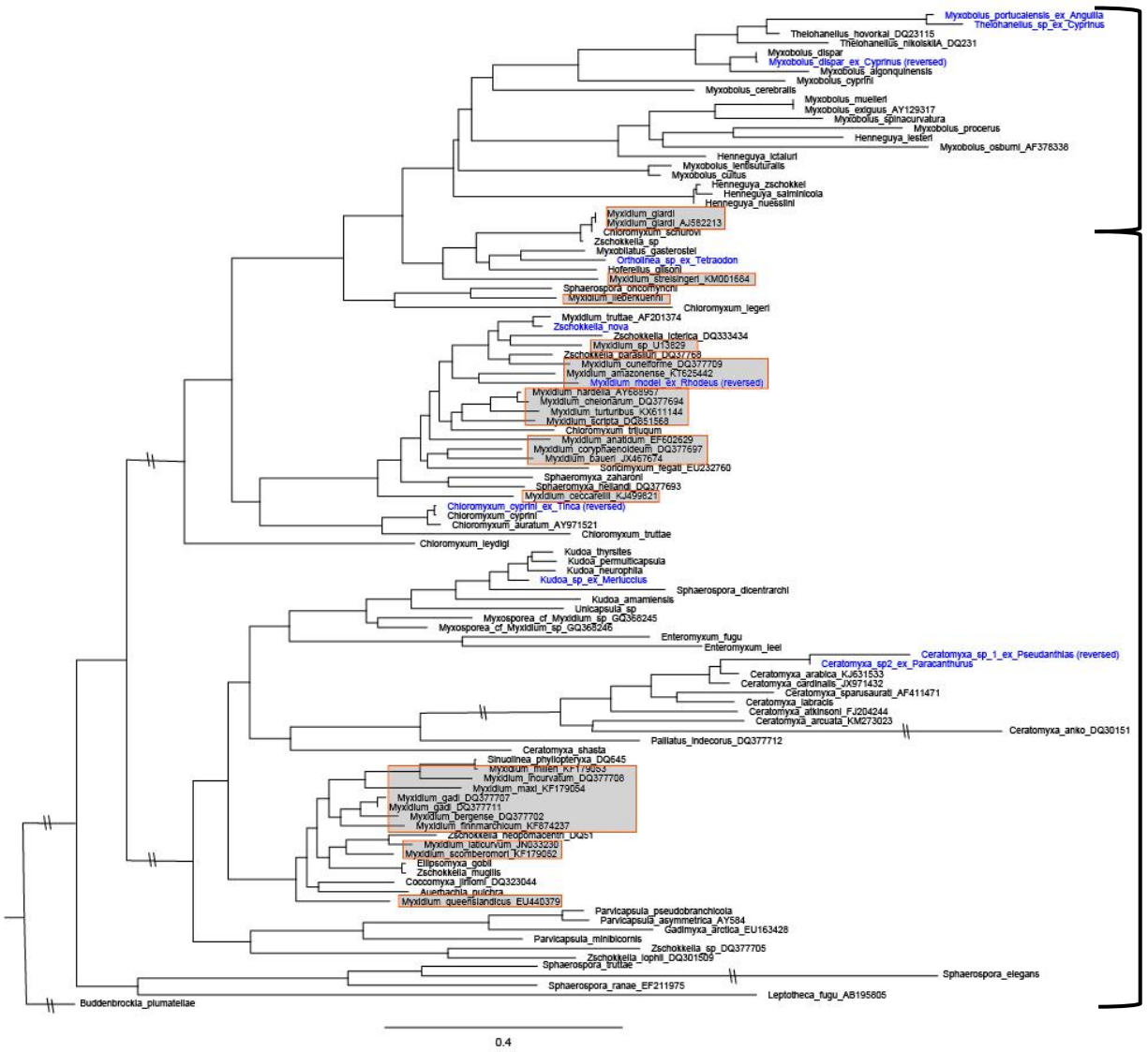

Figure 4: 18S rDNA-based phylogenetic tree indicating the polyphyletic nature of myxozoan genera as demonstrated for *Myxidium* (in orange boxes), a genus (morphotype) which evolved multiple times in both, the oligochaete- (freshwater) and polychaete-infecting (marine) clades of myxozoans. Sequences obtained in the present study are highlighted in blue ‖ indicates branch shortening by 90% in basal branches (left part of image) and by 50% for *S. elegans* and *C. anko*; *B plumatellae* was used as an outgroup.

4.3. Spores as ecotypes

4.3.1 Relation between spore sizes/shapes and their habitats

Most myxozoans are between 10-29µm long, 15-20µm thick and around 10µm wide. In marine habitats, they appear to be slightly longer and wider than in freshwater habitats (figure 5A). In the biliary system spore length, thickness and width show a much higher variation than in all other organ systems. However only thickness is significantly different from all other categories in the biliary system and the external epithelia. Spores in the muscle (support, mostly marine species belonging to the genera *Kudoa* or *Unicauda*) are the smallest.

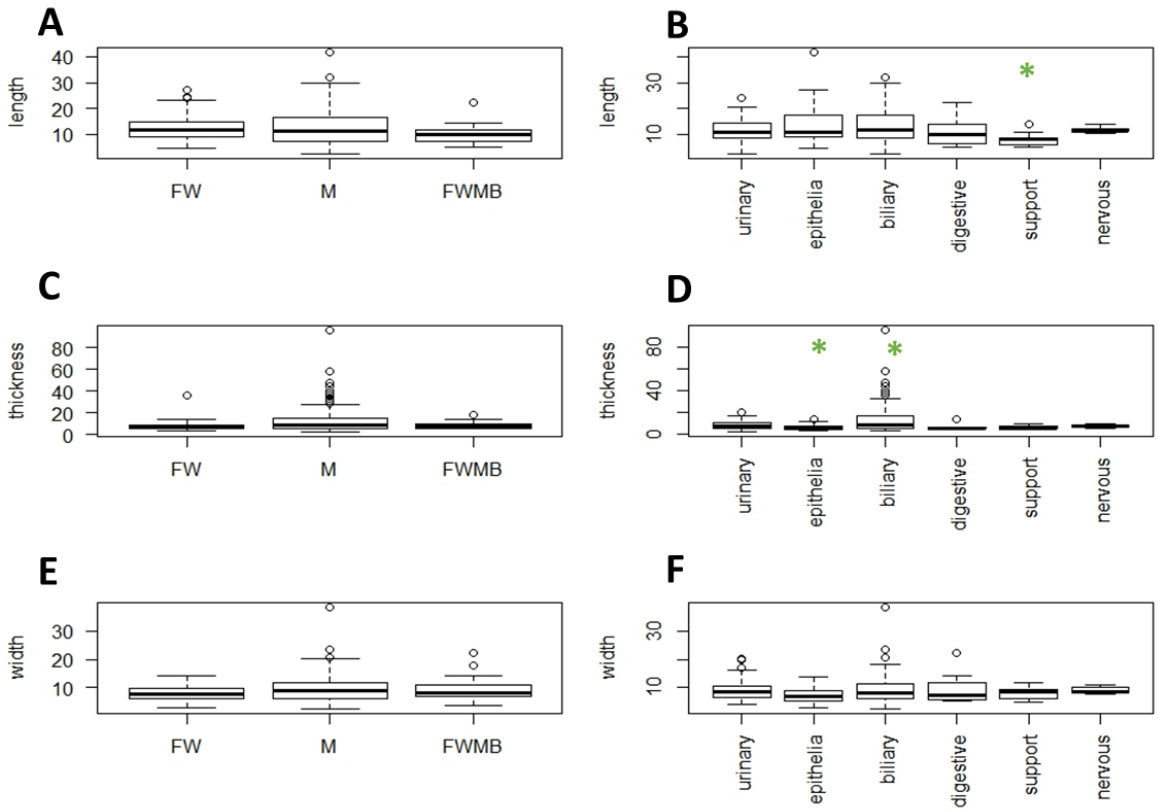

Figure 5: Relation between absolute spore measurements (length, thickness and width) and their external as well as host organ habitat. A: spore length - external habitat, B: spore length - infected organ system, C: thickness - external habitat, D: thickness - infected organ system, E: width - infection habitat, F: spore thickness - infected organ. FW-freshwater, M-marine, FWMB-all aquatic habitats (freshwater, marine and brackish), * shows categories significantly different from all other categories

When comparing the ratios between different external habitats, the variety in the marine habitat is still the highest. However, the ratio length:thickness shows that the highest values are obtained in the freshwater habitat, meaning spores in freshwater are as long as in the marine habitat (figure 6A) but are significantly thinner and hence disc shaped rather than spherical. This is mostly the case for myxozoans producing spores in external epithelia (genera *Myxobolus*, *Henneguya*, *Thelohanellus*). The ratio thickness:width only shows differences in the biliary system, whereas the ratio width: length is very variable, but shows significance in four organ systems.

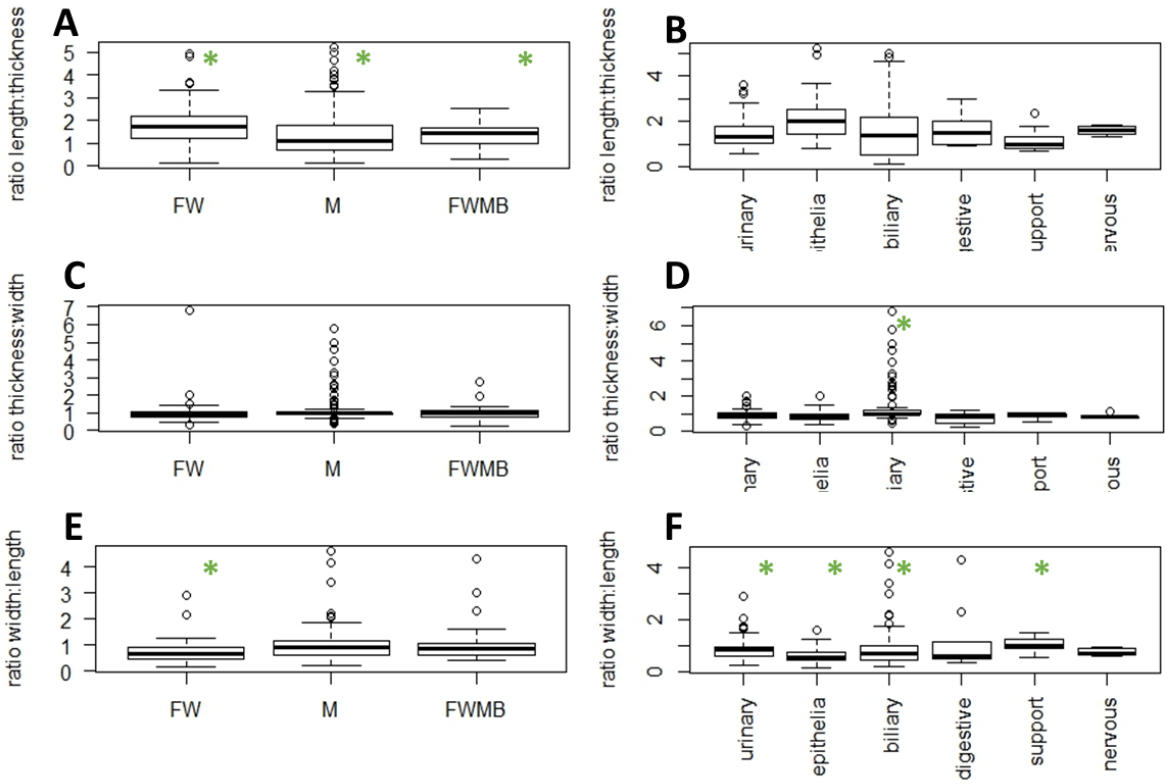

Figure 6: Relation between spore measurement ratios (length: thickness, thickness: width, width: length) and their external as well as host organ habitat. A: ratio length:thickness - external habitat, B: ratio length:thickness - infected organ system, C: ratio thickness:width - external habitat, D: ratio thickness:width - infected organ system, E: ratio width:length - external habitat, F: ratio width:length - infected organ system. FW-freshwater, M-marine, FWMB-all aquatic habitats (freshwater, marine and brackish), * shows categories significantly different from all other categories

4.3.2 Relation between spore shape, appendages and ornamentation

When testing the correlation between appendages and their spore-shape, tails are significantly more common in club-shaped spores than in any other spore shape. Wings were mainly found in the shape category “others”. As expected appendages were absent in all categories except the club shaped and “others” spore category. All other appendages (mucous envelope, hairs and other special structures) were not significantly related to any specific spore shape. Similarly, ornamentations (thin ridges, thick ridges, circular structures as well as pockets and pits) showed no statistically significant relation to a specific spore shape, however, a smooth spore surface can be found in all shape categories (Figure 7).

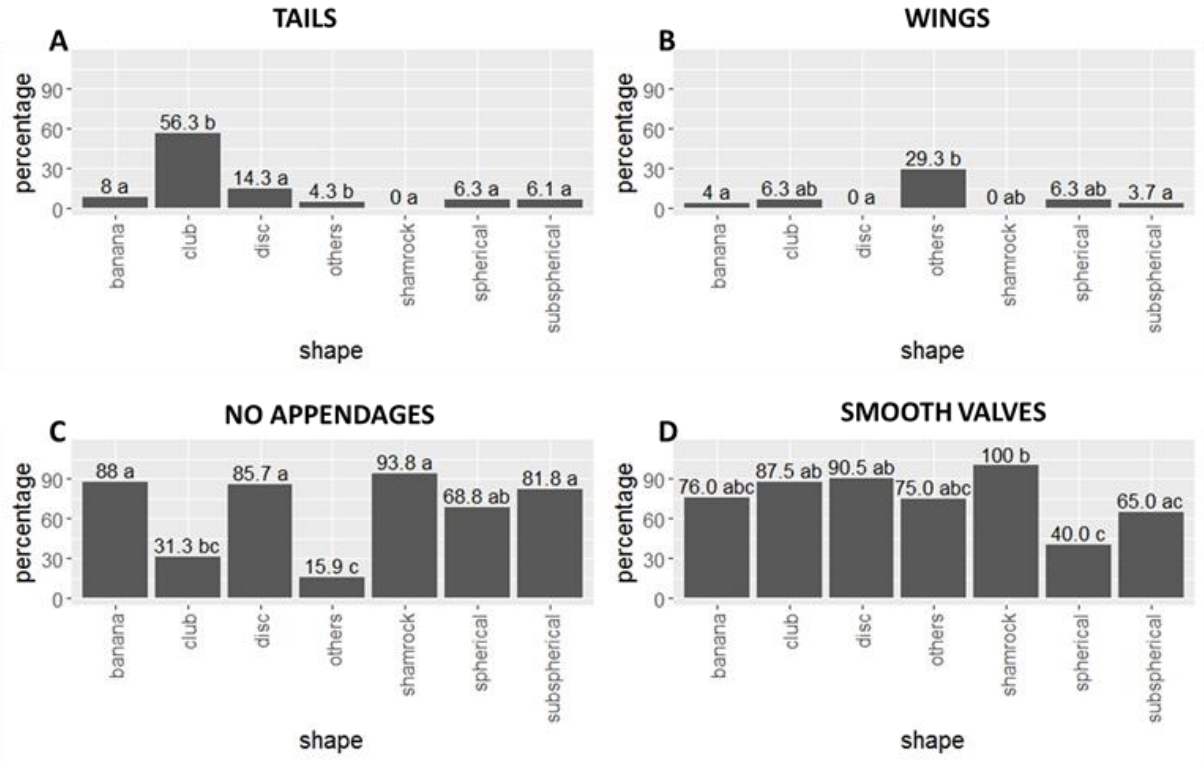

Figure 7: Relation between spore shapes and appendages/ornamentation. A: presence of tails in different shapes, B: presence of wings in different shapes, C: lack of appendages in different shapes, D: presence of a smooth valve surface in different spore shapes. Significance was tested with a Fisher's exact test of contingency tables.

4.3.3 Relation between water current and spore surface/appendages

In slow-flowing water ecosystems significantly more myxozoans have tails than any other appendage structure. All other water currents did not show a significant correlation with a specific appendage or ornamentation. However, any form of ridges (thin and thick ridges as well as pockets and pits) on the spore surface is significantly correlated with slow flowing water (figure 8).

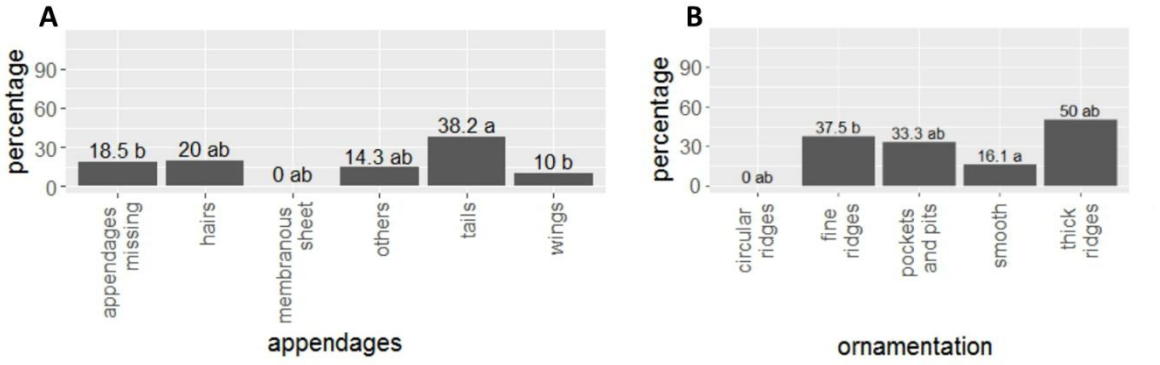

Figure 8: Relation between water flow and A. appendages or B. ornamentation of the spores. Significance was tested with a Fisher's exact test of contingency tables.

4.3.4 Relation between spore ornamentation and ancestral habitat

Marine spores, whose ancestors also occurred in marine habitats as of their phylogenetic position, are smooth rather than ridged. Spores with pockets and pits show no significant difference to smooth spores, when comparing spore ornamentation in different ancestral habitats (figure 9B). In marine species with an ancestor occurring in freshwater, ridges are most prominent. In freshwater spores with an ancestor in freshwater, ridges are more likely to occur than a smooth spore surface. However, smooth spores also occur in freshwater. A significant difference between circular and fine ridges was also observed (figure 9A). In marine species with an ancestor occurring in freshwater, ridges are most prominent. Thick ridges occur in 100% of the spores that switched from freshwater habitats to marine habitats. However, the proportion between thin and thick ridges is not significant, whereas circular ridges show a significant difference in comparison with thick and thin ridges (figure 9C).

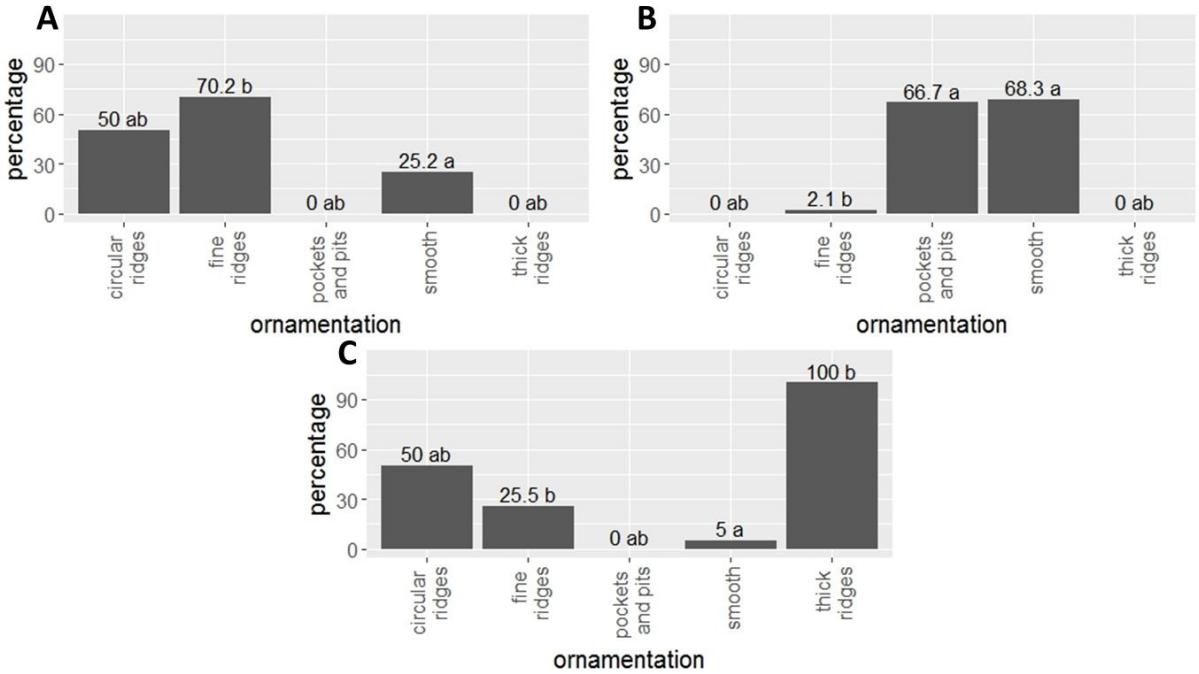

Figure 9: Relation between different ancestral habitats and ornamentation of the spore. A: ancestor and successor occur in freshwater habitats, B: ancestor and successor occur in marine habitats, C: ancestor occurred in freshwater and successor is living in marine habitats

4.3.5. Relation between spore appendages, ornamentation, shape and external habitat

Significant differences between appendages, ornamentation and shape of spores could only be observed in freshwater and marine habitats. The most common appendage in freshwater habitats are tails. Wings could not be found in any myxozoan species from freshwater. A significant difference between all appendages and freshwater habitat can be observed, except for the categories “appendages missing” and “others”. Most species with wings can be found in marine habitats. Significant differences were found between all appendages, except for the categories “hairs” and “others”, and the external habitat they occur in (figure 10 A and B).

When testing the relation between the external habitats and ornamentation, a clear difference between the freshwater habitat and the marine habitat can be seen. Any form of ridges occur mainly in freshwater habitats, with circular ridges only occurring in freshwater habitats. A smooth surface and pockets and pits are more popular in marine habitats. However, all different ornamentation categories, except circular ridges, can be found in both habitats (figure 10 C and D).

The shape of spores varies a lot between those of freshwater habitats and those of marine habitats. All different shapes can be found in marine habitats, and the “shamrock” shape does not occur in freshwater habitats. In freshwater, no significant difference between club, spherical and other shapes could be found. Disc-shaped myxozoans are most common in freshwater habitats, however there is only a 9% difference to the next popular shape (spherical). In marine habitats “shamrock” shape and “banana” shape are the most common ones, disc-shaped spores are hardly found amongst marine species (figure 10 E and F).

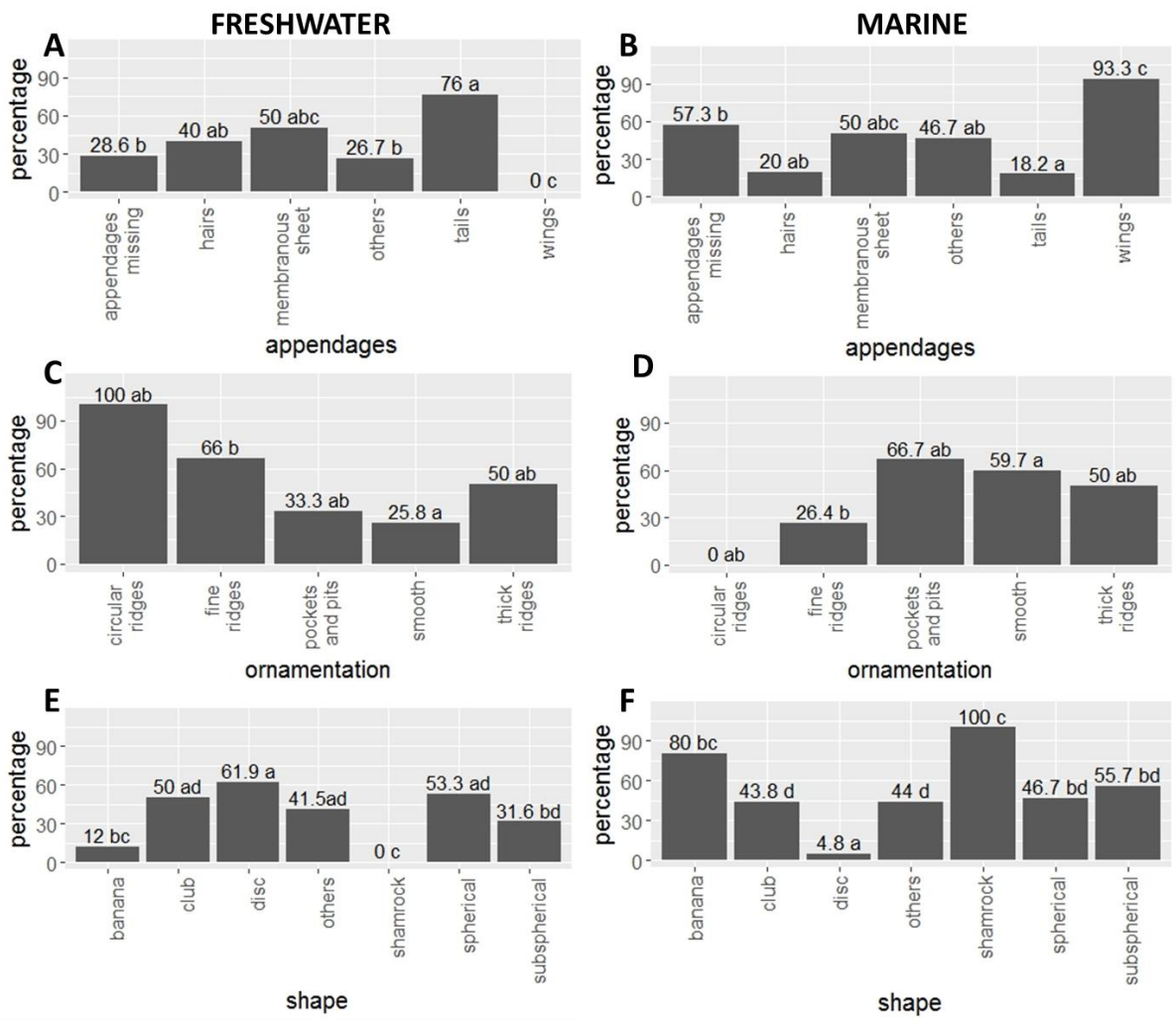

Figure 10: Relation between external habitat and appendages, spore ornamentation and shape. A: appendages - freshwater habitats, B: appendages - marine habitats, C: ornamentation - freshwater habitats, D: ornamentation - marine habitats, E: shape- freshwater habitats, F: shape - marine habitat

4.3.6. Relation between spore shape and host target organ system

In the external epithelia of the host, disc-shaped myxozoas are most common, club-shaped and others are also significantly more prevalent in external epithelia though less common than disc-shaped ones. In the urinary system, spherical, subspherical spores as well as “others” shapes are significantly more common, with little difference between each other. In the biliary system, banana-shaped myxozoans are most common. Despite spherical and subspherical spores showing a similar high prevalence in the biliary system their morphology differs significantly from each other. In the muscle “shamrock” shaped myxozoans are most common with 76.9% of the tested myxozoans in the muscle having this shape. In the nervous system, the only shape present are disk shaped myxozoans belonging to the genus *Myxobolus* (figure 11).

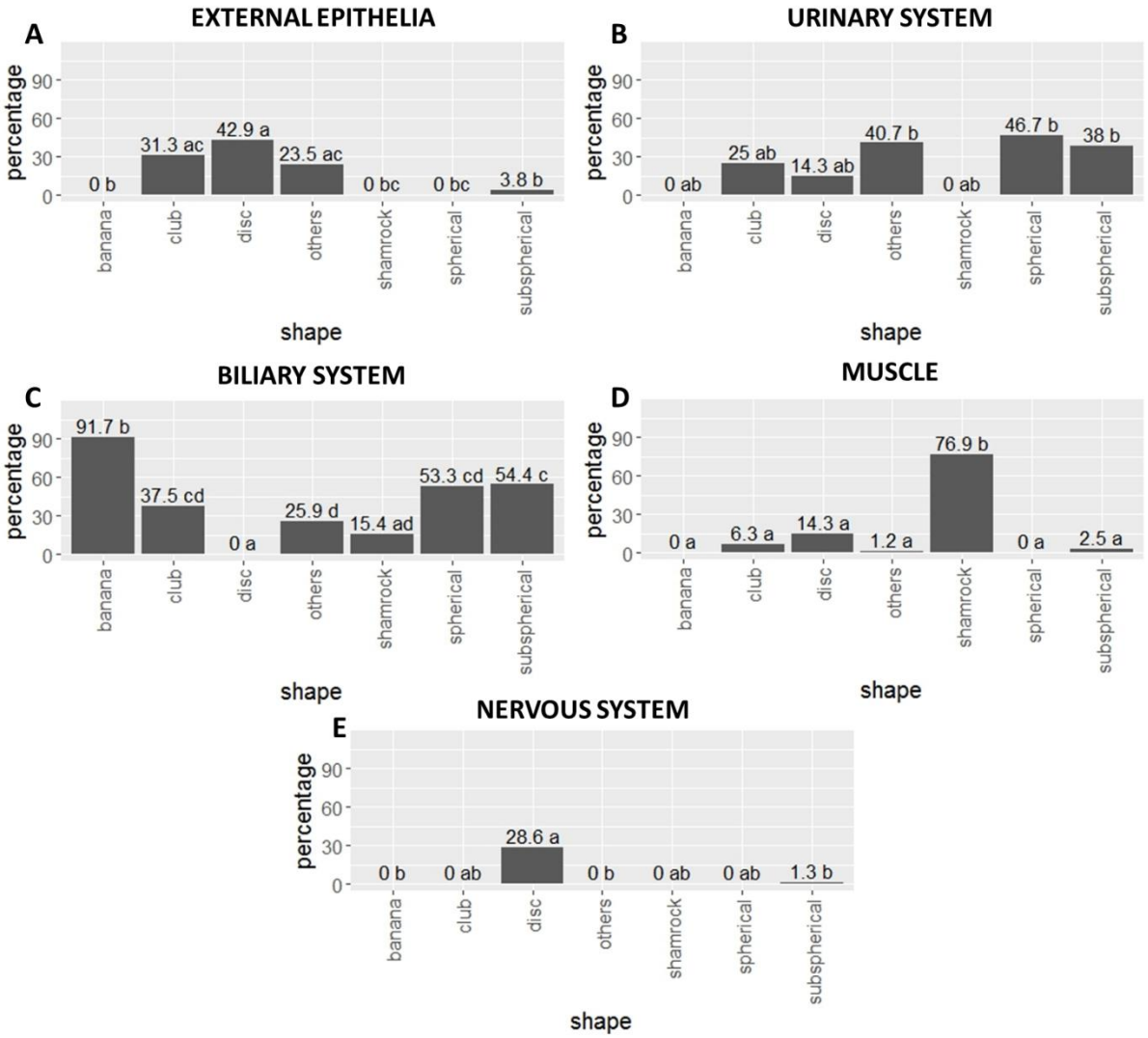

Figure 11: Relation between spore shape and host target organ systems. A: external epithelia, B: urinary system, C: biliary system, D: muscle, E: nervous system

In the hosts' urinary system spores with pockets and pits are most common, however, all different forms of ridges are present in the urinary system. In the external epithelia, spores are most likely smooth (figure 12 C and D). A significant difference between appendage types was found in the biliary system and the external epithelia. Within the biliary system no significant differences were found in the prevalence of "appendages missing", "others" and "wings", the most common appendages. In the external epithelia tails are the most common feature, a shape that is significantly more common than all other appendages in the epithelia (figure 12 A and B).

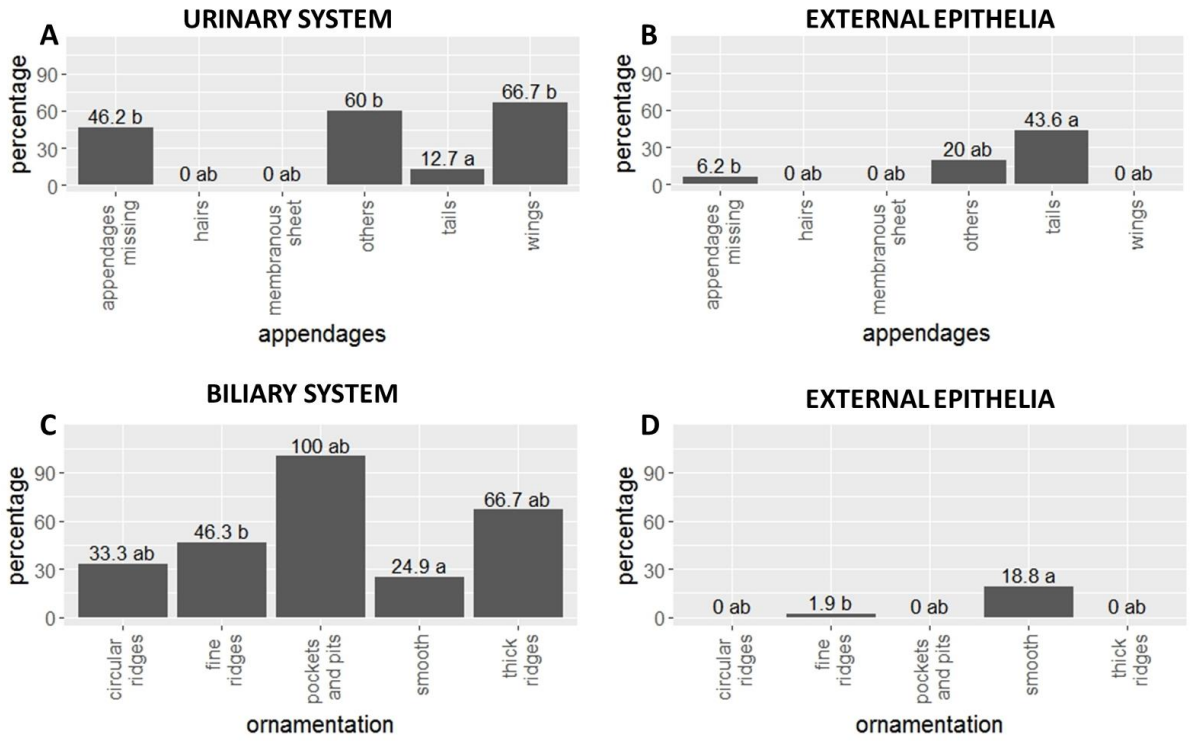

Figure 12: Relation between spore ornamentation/appendages and different host organ systems. A: appendages - biliary system, B: appendages – external epithelia, C: ornamentation-urinary system, D: ornamentation – external epithelia

When comparing histozoic with coelozoic myxozoans it is imminent that species with a membranous sheet are only histozoic, whereas species with wings are mainly coelozoic. However, all other appendages occur in both, histozoic and coelozoic myxozoans (figure 13 A and B). Differences between the different ornamentation categories are not significant. When comparing the different shapes, disc-like spores are strictly histozoic and shamrock-shaped myxozoans are mainly histozoic. Coelozoic spores are mainly banana shaped, but also frequently spherical and subsperical (figure 13C and D).

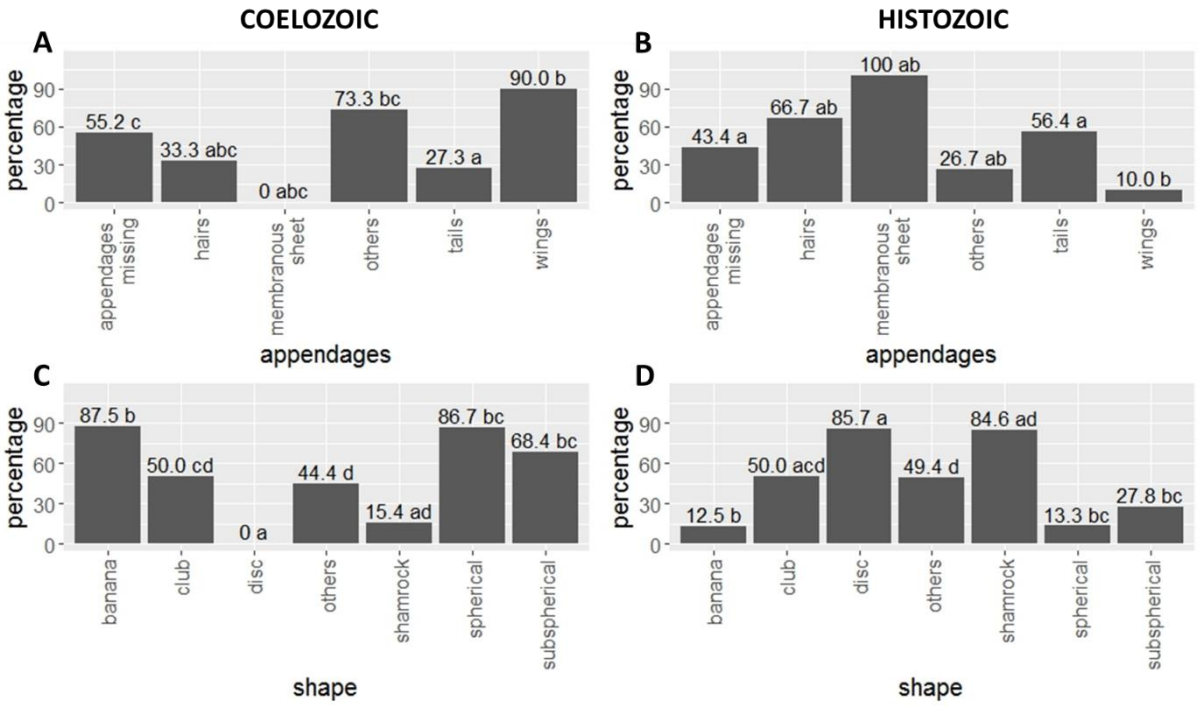

Figure 13: Relation between spore appendages/shape and coelozoic or histozoic location in the host. A: distribution of appendages in coelozoic myxozoans, B: distribution of appendages in histozoic myxozoans, C: different shapes of coelozoic myxozoans, D: different shapes of coelozoic myxozoans

5. Discussion

Possible correlations between spore morphology and spore environment were speculated long time ago (Shulman 1966), and later in relation to contradicting morphology- and SSU- based phylogenetic trees (Fiala and Bartošová, 2010), however, past studies never focused on evaluating this correlation based on statistical models applied to a large database, making this study the first of its kind. The statistical evaluation of spore morphology and ultrastructure in relation to the intrapiscine and aquatic spore environment has proven a valuable tool for understanding that the large variety of different spore morphologies that evolved in myxozoans represent a response to environmental and functional pressures, and explain much of their evolution into different, highly specialized morphotypes in different habitats.

5.1. Phylogenetic relationship between species

Myxozoans were collected from a number of different organs and habitats, hence resulting in a variety of phylogenetic origins in the oligochaete- (freshwater) and polychaete-infecting (marine) clades of myxozoans. In this tree, *Myxidium* was chosen as a representative to demonstrate the general polyphyletic distribution of most myxozoan genera, with 4 different origins within all urinary and biliary tract parasite clades. The different clustering of *Myxidium* species can be partially explained by different spore morphotypes. One *Myxidium* clade tends to be smooth, whereas all others are with

ridges, however their shape differs between all subclades. Summarizing, there are morphological/ultrastructural features that allow to differentiate some *Myxidium* spp. subclades that cluster separately based on their molecular phylogeny. As *Myxidium* is not an isolated case, I would suggest combining DNA sequences with specific morphological characters for designing a new systematic scheme of the Myxozoa that will be able to correctly accommodate newly described species. While this combination was also recommended in previous studies (Fiala and Bartošová, 2010) the present study points out which morphological features are statistically significantly ascribed to a certain phylogenetic genotype. It would make sense to merge subclades together when morphological differences are minor, and hence reduce the number of genera. The position of the suture to the polar capsules as well as the ornamentation of the spore would be suitable to differentiate *Myxidum* species of different phylogenetic origin.

5.2. Spore measurements and ratios

In general, myxozoan spores are microscopic, showing very little size variation between species, likely due to the enormous size reduction as an adaptation to parasitism. Most myxozoans are between 10-29µm long, 15-20 µm thick and around 10µm wide. In marine habitats, they appear to be slightly longer and wider than in freshwater habitats, which was already recognized by Shulman (1966), who stated that the genera *Chloromyxum, Unicapsula* and *Sphaerospora* are the smallest, whereas *Thellohanellus, Henneguya* and partly *Myxobolus* and Ceratomyxa are the largest. In the biliary system, length, thickness and width have a considerably higher variability than in all other organ systems.

When comparing the ratios with the infection habitat, the variation in the marine habitat is still the highest. However, the ratio length:thickness shows that the highest ratio can be found in the freshwater habitat. This high ratio might be an intent to increase surface in relation to weight. Previous studies already showed that spore dimension is a shared character within phylogenetic groups and suggested that their ancestor had a spore thicker or of the same thickness as width, i.e. a spherical shape (Fiala and Bartošová, 2010). The definitions for spore measurements (length, width, thickness) are based on the orientation of the polar capsules relative to the spore and the orientation of the suture relative to the polar capsules, and while important for taxonomy, as individual measurements they fail to represent the spore shape as a whole. While ratios are more likely to address shape and proved to improve statistical significance for the analyses relating spore shapes and their intrapiscine or external habitat characteristics in the present analyses, they still do not fully represent the three dimensions of different spore shapes. A mathematical model is required to define this relationship and simple models have been developed e.g. for plant pollen (Reponen et al., 2010), however, this model was not complex enough to reflect all different shapes found in myxozoan spores. In the future, it would be useful to develop a mathematic model for all myxozoan spore shapes as empirical evaluation

as well as the superior significance of relationships from ratios rather than raw measurements indicate that spore shape is essential for myxozoans in their specific habitats.

Most spore measurements are performed following clear definitions and guidelines by Lom and Arthur (1989). However, one cannot be completely sure that thickness, length and width are always measured correctly in original descriptions. Especially if descriptions predate the guidelines for species descriptions by Lom & Arthur (1989) one of the three measurements is frequently missing, or if all spores always lie in a certain way in the microscope, so that one measurement cannot be taken (e.g. thickness in *Ceratomyxa* spp.). Furthermore, some species do not comply to the standard form of myxozoans. The thickness of *Ceratomyxa* is a lot larger than in all other genera, whereas in other species the suture is sinuous, and measurements become somewhat subjective, depending on the angle at which they were taken. Moreover, some species do not have the standard spore features of two valves, two polar capsules, one suture. *Kudoa* has four valves and four polar capsules, causing the suture-lines to "cross". Other species of this genus can have up to thirteen valves and polar capsules (*Kudoa permulticapsula*). *Unicapsula* has three valves, two smaller and one larger one. Others i.e. *Auerbachia* only has one polar capsule but two valves. In summary, measurements are difficult to compare between different myxozoan genera and a shape-based mathematical model would eliminate most of these problems.

5.3. Relationships between spore morphology and intrapiscine/external habitats

Using a database of 258 species and 64 taxa it was determined that most marine myxozoans have a smooth spore surface, whereas a large number of freshwater species have some form of surface ridges. As saline waters are more viscous spores sink slower to the ground anyway and therefore a surface enlargement as in freshwater spores is not needed. Ridges of any form are more present in freshwater habitats than in marine habitats. Due to the Archimedes principle this surface enlargement results in a higher surface area that the surrounding buoyancy forces can interact with and hence the spores have more time in the water column and therefore get dispersed further from their infection site. Fine ridges create the highest surface area, which may be why circular ridges are very prominent as well. Circular ridges are only found in freshwater habitats. However, thick ridges were as popular in marine habitats as in freshwater habitats. As thick ridges produce a smaller surface area than thin ridges on the valve, thick ridges can be an evolutionary adaptation to a new habitat, derived from thin ridges, which is also shown by the fact that all spores with thick ridges in the database have a freshwater ancestor and are now marine.

The main function of appendages is the same as for surface ornamentation, however the increase in surface area might not be the only reason to prefer appendages. The most popular projection in freshwater habitats, as well as slow flowing water current, are tails. This might be to hook the spore

on the ground/vegetation and stay there over a longer period of time after dispersal. Correlations between spore shape and type of appendage showed a clear significance regarding club and drop-like shaped spores developing a tail as appendage. This tail is always located on the narrower end of the spore, which might result in a more streamlined appearance, presenting little resistance to water flow in their environment. Most species with wings were found in marine habitats. Most wings have a similar form and function as in seeds of a maple tree or in veils on sailing boats. However, wings are rare and were hence designated to the category "others", which included spore shapes that did not fit in any other category. Difficulties when describing the spores within the "others" category may occur, as not all spore descriptions are strictly after Lom and Arthur, 1989.

The theoretical ancestor of all myxozoans had a spherical spore (Kodadkova et al 2014). New shapes occurred in both freshwater and marine species (Fiala and Bartošová, 2010). The present study shows that, shape variations between freshwater and marine infection habitat complement each other. The shamrock shape is only present in marine habitat, within the genera *Kudoa* and *Unicapsula*, with more than two valves. Banana-shaped spores were mainly found in marine environments as well. Disc-shaped spores are more popular in freshwater than in marine habitats. The disc-like spore shape might have developed to impede the opening of the spore in stress situations or to get a higher surface to volume ratio to prevent a rapid descent in the water. In stress situations, a spore will break along the suture and the disc-like spore shape will work as a lever breaking the valves from each other. Spherical spores have a lever-arm as large as the radius of the spore. Disc shaped spores with a suture lining the edge of the disc have a reduced lever length and are therefore least likely to open in a stress situation (Shulman, 1966). Cigar-shaped, banana- or horseshoe-shaped spores are more likely to break due to the increased leverage (Noble, 1950). With this regard, surface ridges may not only increase surface area but may also serve as an enforcement to the valve (Shulman, 1966). Another way of staying longer in the water habitat, regardless of marine or freshwater habitat, is to decrease the volume to surface ratio. Spheres have a small surface area, which makes them sink to the bottom quicker, which also explains the size reduction of spherical species. Discs have an intermediate ratio, whereas cigar-shaped spores have a large surface to volume ratio. However, *Kudoa* spp. slow down their descent by their shamrock shape, which functions as a parachute (Shulman, 1966) and allows them to stay in the water column as long as necessary. Although a significant correlation between shape and water current was not found in our data. Spherical spores sink slowly, disc-shaped with an intermediate speed and fusiform spores very quickly (Wittenburg et al 1989). However, banana-shaped spores and elongated subspherical spores tend to rotate when sinking, which slows the sinking speed down (Leger, 1931). This might also be a possible explanation for the formation of pockets and pits in spherical spores, as this specific ornamentation even intensifies the tumbling process.

When testing the correlation between organ systems of the host and different morphological spore characters it becomes clear that most myxozoans adapt a specific shape for each organ system. In nervous tissue only disc-shaped spores can be found and external epithelia is preferred by disc-shaped spores as well. However, the disc-shaped category is the only one present in all organ systems. Epithelia-inhabiting myxozoans are mainly represented by the *Myxobolus* clades, which includes all disc-shaped freshwater myxozoans which lack ornamentation. The fact that disc-shaped spores are prevalent in every organ system suggests that this is a highly practical shape and likely one of the best shape solutions for transmission in the environment. Observations by light microscopy show that disc shaped spores are located very close to each other and they can be packed better than spherical spores into a plasmodium. This enlarges the number of spores leaving the intermediate host and, more importantly, infecting the final host even greater. The urinary system has spherical and subspherical genera which is also mirrored in the biliary system, likely because of the similar pressure coming from each all sides in liquid-filled spaces such as the gallbladder (Akhmerov et al., 1958). Banana-shaped spores are most common in the biliary system. This shape might be used for flotation in the bile. However, not all shapes are organ-dependent (Leger, 1931), but likely represent an adaptation to the external habitat, since the time spores spend in the fish is limited (Shulman, 1966). Significant organ-specific ornamentation was found in the urinary system. Most popular are spores with pockets and pits, followed by any kind of ridges. Therefore, the pockets and pits might be a spore feature for better grasping in the host tissue, since most of these spores are in close contact with the microvilli of the excretory canals. Tails are the most common appendages in epithelia. These tails can entangle with the host tissue for a better hold, but could also be used for a better release from the host tissue. The high variability of appendages in different organs indicates that they are not so much an adaptation to the in-host environment, which is supported by our statistical analyses and by different descriptions over the years (Feist and Longshaw, 2006; Barassa et al, 2003).

Regardless of explanatory incongruences for the function of different ornamentations, appendages and shapes of myxozoans are strongly correlated with their habitat. This study shows the vast differences between myxozoan species and genera and tries to explain ecological adjustments of their spore features, for the first time based on statistical analyses, making it a fundamental starting point for in-depth future studies into the topic of functional myxozoan spore morphology.

6. Conclusion

This is the first time that correlations between myxozoan spore morphology/ultrastructure and habitat within the fish host and the environment were analysed statistically. The results clearly demonstrate that myxozoan spores represent ecotypes rather than morphotypes mirroring the phylogenetic tree based on SSU rDNA sequence data. Some genera, that are strongly polyphyletic (e.g. *Myxidium*) in reality represent multiple groups of similar spore morphotypes with specific and differentiable spore dimensions and surface structures. The results of this study identify characteristics of myxozoan spore morphology and ultrastructure that are useful for myxozoan taxonomy as they mirror phylogenetic clustering. At the same time, it unveils some presently used taxonomic features as redundant as they clearly represent ecological adaptations. While it has been repeatedly stated that a better taxonomic scheme for the Myxozoa is absolutely required it can be concluded from the present study that such a system would clearly involve not only spore features which are strongly biased by ecological requirements but more details on earlier stages of parasite development in the host as well as phylogenetic information on the origin and ancestry of a taxon to be described. Follow-on studies concentrating on ecological adaptations of myxozoan spores should focus on developing a mathematic model to better describe spore shape than simple measurements and optimizing/narrowing down the artificially designed morphological categories for statistical analysis to join functionally identical features and better reflect their correlation with habitat features. However, this study only gives a first insight into spore ecology and changes in the spore because of influencing factors.

7. References

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Appendix

Species Name	Vertebrate Host	ancestral habitat	infection Habitat	water current	Organ Tissue	in host habitat	SlengthMean	SwidthMean	SthicknessMean	shape	ornamentation	Ornamentation Differences	appendages	appendagesBin
Sphaerospora ranae	Rana dalmatina	3	1	1	1	1	10.1	5.7	11.6	7	1	5	6	0
Sphaerospora motemarini	Lutjanus griseus	1	2	2	1	1	11.6	19.85	20.41	7	1	5	6	0
Sphaerospora molnari	Cyprinus carpio	3	1	2	2	1	9.9		10.0	7			5	1
Sphaerospora dykovae (renicola)	Cyprinus carpio	3	1	2	1	1	7.0	7.02	7.18	7	1	2	6	0
Sphaerospora epinepheli	Epinephelus coioides, E. malabaricus, E. fmcoguttatm E. stictus E. bleekeri	1	2	3	1	1	8.8	13.4	8.3	7	0	1	6	0
Sphaerospora poljanskii	Rutilus rutilus	3	1	1	1	1	9.75	9.5		7	0	1	3	1
Wardia lucii	Esox lucius		1	1	1	1	8.5	8.25	5.5	7	1	2	6	0
Wardia ovinocua	Lepomis humilis		1	2	4	1	9.5	11	6	7	1	2	3	1
Ceratomyxa cretensis	Synodus saurus	1	2	1	3	2	6.7	6.7	30.7	2	0	1	6	0
Ceratomyxa filamentosi	Aulopus filamentosus	1	2	5	3	2	8.2	8.2	40.5	2	0	1	6	0
Ceratomyxa carcharhini	Carcharhinus melanopterus	1	2	2	3	2	10.0	10.0	58.1	2	0	1	6	0
Ceratomyxa melanopteri	Carcharhinus melanopterus	1	2	2	3	2	11.3	11.3	44.5	2	0	1	6	0
Ceratomyxa negaprioni	Negaprion acutidens	1	2	3	3	2	11.1	11.1	27.7	2	0	1	6	0
Ceratomyxa puntazzi	Diplodus puntazzo	1	2	2	3	2	9.2	9.2	29	2	0	1	6	0
Ceratomyxa tenuispora	Aphanopus carbo	1	2	3	3	2	11.0	12.1	28.5	2	0	1	3	1
Ceratomyxa microlepsis	H.microlepis		1	5	3	2	5.2	5.2	35.5	2	0	1	6	0
Ceratomyxa cardinalis	Cheilodipterus artus	1	2	2	3	2	5.3	5.3	13.8	2	0	1	6	0
Ceratomyxa cardinalis	Pseudanthias squamipinnis	1	2	2	3	2	8.83	8.83	17.53	2	0	1	6	0
Ceratomyxa cyanosomae	Ostorhinchus cyanosoma	1	2	2	3	2	6.1	6.1	20	2	0	1	6	0
Ceratomyxa sp. ex Paracanthurus hepatus	Paracanthurus hepatus	1	2	2	3	2	2.44	2.44	11.17	2	0	1	6	0
Myxodavisia bulani	Megalops cyprinoides	1	3	2	3	2	7.0	7.0	13.3	7	0	1	5	1
Myxodavisia haldarae	Sardinella longiceps	1	2	2	3	2	13.2		8.0	7	0	1	3	1
Myxodavisia cornuta	Neogobius fluviatilis	1	3	1	1	2	8.8		10.65	7	0	1	3	1
Myxodavisia longifilius	Hippoglossoides dubius	1	2	2	1	2	10.5	12.35	12.35	7	0	1	3	1
Pseudalataspora kovalevae	Macruronus magellanicus	1	2	2	3	2	9.1	15.7	10.2	4	0	1	4	1
Pseudalataspora umbraculiformis	Gaidropsarus mediterraneus	1	2	2	3	2	8.65	15.95	7.35	4	0	1	4	1
Pseudalataspora pontica	Liza aurata	1	3	3	3	2	6	6.5	17.95	4	0	1	6	0
Pseudalataspora scombri	Scomber japonicus	1	2	2	3	2	5.32		9.28	4	0	1	6	0
Zschokkella lophii	Lophius litulon	1	2	2	1	2	20.1	14.9		4	0	1	6	0
Zschokkella sigfriedi	Boreogadus saida	1	2	2	1	1	17.4	10.5	9.8	4	0	1	6	0

Zschokkella hildae	Gadus morhua	1	2	3	1	1	16.2	10.4	9.6	4	0	1	6	0
Sinuolinea dimorpha	Cynoscion regalis, C. nebulosus	1	2	2	1	2	14.9	14.8	14.9	3	0	1	6	0
Sinuolinea phyllopteryxa, Sinuolinea sp. 2. Dykova 2012	Phyllopteryx taeniolatus	1	2	2	1	1	17.1	16.4	15.6	3	0	1	6	0
Sinuolinea tetraodontoni	Tetraodon palembangensis (Pao palembangensis)	3	1	3	1	2	9.56	10.11		3	0	1	6	0
Sinuolinea arctica	Myoxocephalus scorpius	1	2	2	1	2	15.7	15.4	16.1	3	0	1	6	0
Parvicapsula minibicornis	Oncorhynchus spp.	1	3	2	1	2	11.0	7.5	6.8	7	0	1	5	1
Parvicapsula pseudobranchicola	Salmo salar, S. truttae	1	3	5	2	1	12.4		6.2	7	0	1	6	0
Parvicapsula irregularis	Hippoglossoides platessoides	1	2	3	1	2	11.0	8.9	8.7	7	0	1	6	0
Parvicapsula bicornis	Pleuronectes platessa	1	2	3	1	1	6.8	5.3	3.44	7	0	1	3	1
Parvicapsula spinachiae	Spinachia spinachia	1	2	2	1	1	10	5	3.98	7	1	2	5	1
Gadimyxa atlantica	Gadus morhua	1	2	3	1	1	5.3	7.5	5.3	4	0	1	6	0
Gadimyxa sphaerica	Gadus morhua	1	2	3	1	2	4.8	10.0	4.1	4	0	1	6	0
Gadimyxa arctica	Arctogadus glacialis	1	2	3	1	2	5.7	10.0	5.6	4	0	1	6	0
Gadimyxa ex Sprattus sprattus (Karlsbakk)	Sprattus sprattus	1	2	2	1	1	2.55	4.2	2.45	4	0	1	6	0
Gadimyxa ex Clupea harengus (Karlsbakk)	Clupea harengus	1	2	2	1	1	2.55	4.2	2.45	4	0	1	6	0
Auerbachia pulchra	Coryphaenoides rupestris	1	2	2	3	2	30	11	11	5	0	1	6	0
Auerbachia maamouni	Gnathanodon speciosus	1	2	2	3	2	6.2	7.9	7.9	5	0	1	6	0
Auerbachia scomeroidi	Scomberoides lysan	1	2	2	3	2	21.4	7.5	7.5	5	0	1	6	0
Auerbachia chaetodonti	Chaetodon unimaculatus	1	2	2	3	2	32.2	9.1	9.1	5	0	1	6	0
Auerbachia caranxi	Caranx papuensis	1	2	2	3	2	16.8	6.7	6.7	5	0	1	6	0
Coccomyxa gobiodoni	Gobiodon citrinus	1	2	2	3	2	10.6	6.5	6.5	4	0	1	6	0
Coccomyxa colurodontis	Colurodontis paxmani	1	2	2	3	2	12.1	6	6	4	0	1	6	0
Coccomyxa jirilomi	Bathygobius cyclopterus	1	2	2	3	2	10.1	6	6.1	4	0	1	6	0
Coccomyxa baleswarensis	Hilsa ilisha	1	3	5	3	2	11.36	5.17	5.17	4	0	1	6	0
Coccomyxa morovi	Sardina pilchardus	1	3	3	3	2	14	5.5	5.5	4	0	1	6	0
Myxidium gadi	Merlangius merlangus, Pollachius virens	1	2	1	3	2	11.3	5.3	5.3	4	0	1	6	0
Myxidium bergense	Pollachius virens	1	2	2	3	2	18.54	6.87	6.25	4	0	1	6	0
Myxidium finnmarchicum	Merlangius merlangus	1	2	1	3	2	15.3	9.2	9	4	0	1	6	0
Myxidium milleri	Corythoichthys schultzi	1	2	2	3	2	9.2	5.2	5.97	4	0	1	6	0
Myxidium incurvatum	Callionymus lyra	1	2	2	3	2	11.62	4.92	4.92	4	0	1	6	0
Myxidium amazonense	Corydoras melini	2	1	1	3	2	17.0	3.7		2	1	2	6	0
Myxidium sp. Ex Rhodeus amarus	Rhodeus amarus	2	1	1	1	2	4.9	14.3	4.9	4	1	2	6	0
Zschokkella sp1 IF2006	Eugerres plumieri	4	3	1	3	2	11.5	13.4		4			6	0
Sigmomyxa sphaerica	Belone belone	1	2	3	3	2	18.0	11.7	8.2	7			6	0
Ellipsomyxa gobii	Pomatoschistus microps	1	3	1	3	2	7.0	8.7	11.6	4	0	1	6	0
Ellipsomyxa syngnathi	Syngnathus rostellatus	1	2	2	3	2	6.8	8.1	10	4	0	1	6	0

Ellipsomyxa mugilis	Liza saliens	1	2	1	3	2	7.25	11.75	6.8	4	0	1	6	0
Ceratonova gasterostea	Gasterosteus aculeatus	1	3	2	4	1	5.2	22.4	5.2	2	0	1	6	0
Ceratonova shasta (before Ceratomyxa shasta)	Gasterosteus aculeatus	1	3	3	4	1	6	14	6	2	0	1	6	0
Enteromyxum leei	Takifugu rubripes	1	3	4	4	1	14.1	7.75		7	0	1	6	0
Enteromyxum scophthalmi	Scophthalmus maximus	1	3	1	4	1	22.2	11.7	14	7	0	1	6	0
Gastromyxum bulani	Elops machnata	1	2	2	4	1	10.29	5.64	4.15	7	0	1	6	0
Gastromyxum rafii	Elops machnata	1	2	3	4	1	7.92	5.42	5.35	7	0	1	6	0
Unicapsula pflugfelderi	Spicara smaris	1	2	2	5	1	5.16	6.02	6.02	6	0	1	6	0
Unicapsula fatimae	Siganus canaliculatus	1	2	2	4	1	6.23	6.8	6.8	6	0	1	6	0
Unicapsula pyramidata	Scolopsis monogramma	1	2	2	5	1	5.1	7	7	6	0	1	6	0
Unicapsula seriolae	Seriola dumerili	1	2	3	5	1	6.1	6.1	6.1	6	0	1	6	0
Unicapsula andersenae	Acanthopagrus australis	1	2	2	5	1	5.8	5.8	5.8	6	0	1	6	0
Kudoa sp. ex Merluccius productus	Merluccius productus	1	2	2	5	1	6.23	5.59	5.59	6	0	1	6	0
Kudoa carcharini	Carcharhinus cautus	1	2	2	5	1	8.16	10.14	9.79	6	0	1	6	0
Kudoa thyrsites	Paralichthys olivaceus	1	2	2			7.31	15.26	7.57	6	0	1	6	0
Kudoa iwatai	Acanthopagrus schlegelii	1	2	2			6.0	6.8	6.0	6	0	1	6	0
Kudoa septempunctata	Paralichthys olivaceus	1	2	2	5	1	8.5	11.8	9.4	6	0	1	1	1
Kudoa gunterae	Neoglyphidodon melas	1	2	2			5.42	10	5.62	6	0	1	6	
Kudoa azoni	Pleurogrammus azonus, Hexagrammos octogrammus	1	2	2	5	1	5.5	6.7	5.6	6	0	1	6	0
Kudoa permulticapsula	Scomberomorus commerson,	1	2	2	5	1	6	9.15	7.7	6	0	1	6	0
Chloromyxum clavatum	Raja clavata	4	2	1	3	2	14.4	11.9	9.4	3	0	1	5	1
Chloromyxum menticirrhi	Squalus acanthias	4	2	3	1	2	10.5	9.8	10.1	3	1	3	6	0
Chloromyxum riorajum	Rioraja agassizii	4	2	1			11.41	8.48	5.92	3	1	3	2	1
Sphaeromyxa artedielli	Triglops murrayi	4	2	1	3	2	17.5	5.6	5.6	2	1	2	6	0
Sphaeromyxa balbianii	Gaidropsarus vulgaris	4	2	1	3	2	15	5	5	2	1	2	6	0
Sphaeromyxa longa	Trisopterus minutus	4	2	1	3	2	20	5	5	2	0	1	6	0
Sphaeromyxa cannolii	Hippocampus erectus	4	2	2	3	2	17.6	5.5	5.5	2	0	1	6	0
Sphaeromyxa schulmani	Salilota australis	4	2	3	3	2	19.3	5	5	2	0	1	6	0
Sphaeromyxa kenti	Gobiosoma bosc	4	2	3	3	2	18.5	4.4	4.4	2	1	2	6	0
Sphaeromyxa zaharoni	Pterois miles	4	2	2			13.7	4.8	4.2	2	1	2	6	0
Sphaeromyxa lycodi	Lycodes sp.	4	2	2	3	1	22.4	5.7	4.5	2	0	1	6	0
Soricimyxum fegati	Sorex minutus			2	3	1	7.0	5.4	3.5	4	1	2	6	0
Soricimyxum minuti	Sorex minutus			2	3	1	12.6	9.2	8.0	4	1	2	6	0
Myxidium baueri	Macruronus magellanicus	4	2	2	3	2	17.55	4.65	4.65	4	1	2	6	0
Myxidium coryphaenoideum	Coryphaenoides rupestris	4	2	2	3	2	23.3	5	5.0	4	1	2	6	0
Cystodiscus melleni	Pseudacris triseriata triseriata			1	3	2	12.3	7.6	4.82	4	1	2	6	0
Cystodiscus axonis	Typhlonectes compressicauda	2	1	1	3	2	13.66	7.83	4.84	4	1	2	3	1

Cystodiscus australis	Limnodynastes peronii	2	1	1	3	2	16.0	8.7	10.34	4	1	2	6	0
Cystodiscus immersus	Rhinella marina	2	1	1	3	2	12.55	8.05		4	1	2	6	0
Myxidium anatidum	Anas platyrhynchos	2	1	1	3	1	23.1	10.8	11.2	4	1	2	6	0
Myxidium scripta	Trachemys scripta elegans (turtle)	2	1	1	1	1	18.5	5.1	5.1	4	1	2	6	0
Myxidium chelonarum	Kachuga smithii (turtle)	2	1	1	3	2	14.5	4.5	5.5	4	1	2	6	0
Myxidium truttae	Oncorhynchus kisutch	2	3	3	3	1	11.5	7.2	7.15	4	1	2	6	0
Myxidium ceccarellii	Leporinus elongatus	2	1	1	3	2	17.7	10.4	10.1	4	1	2	6	0
Zschokkella jaimeae	Tylosurus gavialoides	4	2	2	3	2	11.4	7.1		4	1	2	6	0
Zschokkella balistoidi	Balistoides viridescens	4	2	2	3	2	11.1	6.3	5.86	4	1	2	6	0
Zschokkella auratis	Sparus aurata	4	2	2	3	2	9.5	7.1	6.55	4			6	0
Zschokkella icterica	Siganus luridus	4	2	2	3	2	12.4	7.1	4.4	4	1	2	6	0
Zschokkella nova	Carassius carassius, Carassius auratus gibelio	2	1	1	3	2	10	6	6	4	1	2	6	0
Zschokkella sp. ex Ctenopharyngodon idella	Ctenopharyngodon idella	2	1	1	3	2	4.88	10.46	4.88	4	1	2	6	0
Chloromyxum auratum	Carassius auratus	2	1	2	3	2	18.8	9.8	9.8	3			3	1
Chloromyxum cristatum	Hypophthalmichthys molitrix	2	1	2	3	2	13.6	12.6	13.1	3	1	2	6	0
Chloromyxum cyprini	Tinca tinca	2	1	1	3	2	13.24	12	13.34	3	1	2	6	0
Chloromyxum fluviatile	Hypophthalmichthys molitrix, Rutilus rutilus, leuciscus cephalus	2	1	2	3	2	7.8	8.0	7.45	3	1	4	6	0
Chloromyxum truttae	Salmo salar	4	1	2	3	2	9.4	7.4	9.15	3	1	4	6	0
Chloromyxum thymalli	Thymallus nigrescens	2	1	2	3	2	9.3	6.65	8.9	3	1	2	5	1
Chloromyxum careni	Megophrys nasuta			1	1	1	7.25	5.75	5.76	3	1	2	6	0
Myxidium lieberkuehni	Esox lucius	2	1	3	1	1	20	6	6	4	1	2	6	0
Chloromyxum legeri	Cyprinus carpio and other cyprinids	2	1	2	3	2	7.5	7.5	7.5	3	1	2	6	0
Myxobolus arcticus	Oncorhynchus masou masou	2	3	1	6	1	14.1	8.7	9.76	1	0	1	6	0
Myxobolus neurobius	Salmo trutta	2	3	2	6	1	11	8.0	6	1	0	1	6	0
Myxobolus fryeri	Oncorhynchus clarki	2	3	3	6	1	12.9	8.6	7.2	1	0	1	6	0
Myxobolus neurotropus	Oncorhynchus mykiss	2	3	3	6	1	11.8	10.8	8.8	1	0	1	6	0
Myxobolus murakamii	Oncorhynchus masou ishikawae	2	3	3	6	1	11.15	10.05	7.4	1	0	1	6	0
Ortholinea auratae	Sparus aurata	4	2	2	1	2	9.0	8.3	7.2	4	1	2	6	0
Ortholinea orientalis	Clupea harengus, Sprattus sprattus	4	2	3	1	2	9.0	7.9	5.6	4	1	2	6	0
Ortholinea labracis	Dicentrarchus labrax	4	3	2			7.6	7.2	6.5	4	1	2	6	0
Ortholinea aurata	Tetradon nigrovirigis	2	1	5	1	1	6.97		8.97	4	1	2	6	0
Hoferellus alosae	Alosa alosa	2	3	2	1	1	9.7	8.4	7.7	7	1	2	2	1
Hoferellus cyprini	Cyprinus carpio	2	1	3	1	1	8.5	6.7	5.15	7	1	2	2	1
Hoferellus gnathonemi	Gnathonemus petersii	2	1	2	1	1	11.9	11		7	1	2	3	1
Hoferellus anurae	Hyperolius kivuensis	2	1	1	1	1	8	7	3.9	7	1	2	3	1
Hoferellus wuchangensis	Crassius auratus auratus	2	1	2	1	2	9.5	7.5	6.6	7	1	2	5	1
Hoferellus liocasis	Tachysurus brashnikowi = Liocassis brashnikowi	2	1		2	1	8.5	7.2	7.3	7	1	2	5	1
Hoferellus gilsoni	Anguilla anguilla	2	3	3			7.8	7.6	8.19	7	1	2	2	1

Hoferellus carassii	Carassius gibelio	2	1	2	1	2	13.1	9.6	7.25	5	1	2	2	1
Myxobolus muelleri	Leuciscus cephalus	2	1	2	2	1	8.3	7	4.7	1	0	1	6	0
Myxobolus buckei	Leuciscus cephalus	2	1	3	5	1	14.0	11.5		1	0	1	6	0
Myxobolus muellericus	Leuciscus cephalus	2	1	3	2	1	9.7	8.1	5.05	1	0	1	6	0
Myxobolus cerebralis	Oncorhynchus mykiss	2	1	3	5	1	8.55	8.5	6.8	1	0	1	6	0
Myxobolus tambroides	Tor tambroides	2	1	2	2	1	9.9	7.4	7.2	1	0	1	6	0
Myxobolus sp ex C. carpio	Cyprinus carpio	2	1	2	2	1	10.39	5.75	8.38	1	0	1	6	0
Myxobolus sp. ex Anguilla anguilla	Anguilla anguilla	2	3	3	2	1	9.66	5.21		1	0	1	6	0
Henneguya zschokkei	Prosopium williamsoni	2	1	4	5	1	11	9		5	0	1	3	1
Henneguya chydadea	Astyanax altiparanae	2	1	1	2	1	18.8	4.4	3.8	5	0	1	3	1
Henneguya tunisiensis	Symphodus tinca	4	2	2	2	1	41.8	9.1	8	5	0	1	3	1
Henneguya curimata	Curimata inormata	2	1	2	1	1	16.6	6.2		5	0	1	3	1
Thelohanellus kitauei	Cyprinus carpio	2	1	2	1	2	23.9	10.0	8.6	4	0	1	6	0
Thelohanellus hovorkai	Cyprinus carpio	2	1	2			21.25	12.5		4	0	1	1	1
Thelohanellus nikolskii	Cyprinus carpio	2	1	2	2	3	18.9	9.6	8	4	0	1	6	0
Thelohanellus wuhanensis	Carassius auratus gibelio	2	1	2	2	3	22.9	13.3	10.6	4	0	1	3	1
Thelohanellus wuhanensis	Cyprius carpio	2	1	2	2	3	20.22	8.68		4	0	1	3	1
Cardimyxobolus japonensis	Odontobutis obscura	2	1	2			9.4	11.9	5.8	4	0	1	6	0
Acauda hoffmani	Lepomis macrochirus	2	1	2	1	1	19.85	8.95	9.12	4	1	2	6	0
Acauda elongata	Lepomis cyanellus	2	1	2	1	1	16	5.5	5	4	1	2	6	0
Agarella gracilis	Lepidosiren paradoxa, Anura	2	1	1	4	1	17.7	6.6	5.9	7	0	1	3	1
Alataspora samaroidea	Chlorophthalmus atlanticus	1	2	3	3	2	10.15		39.75	7	0	1	4	1
Alataspora budegassai	Lophius budegassa	1	2	3	3	2	13.2		35.8	7	0	1	6	0
Alataspora africana	Callanthias ruber	1	2	1	3	2	12.8		33.1	7	0	1	4	1
Alataspora longialata	Hippoglossoides dubius	1	2	1	3	2	13.0		96	7	0	1	4	1
Alataspora adelia	Glyptocephalus stelleri	1	2	1	3	2	7.5		48	7	0	1	4	1
Alataspora merluccii	Merluccius australis	1	2	2	3	2	13.51	9.89		7	0	1	4	1
Biptera formosa	Merlangius merlangus	1	2	2	1	1	6.6	6.6	11	7	1	5	4	1
Bipteria vetusta	Chimaera monstrosa	1	2	3	3	2	9.2	12	9.1	7	0	1	4	1
Bipteria admiranda	Pagellus acarne	1	2	2	1	2	12.3	10.82	11.31	7	0	1	4	1
Bipteria nototheniae	Patagonotothen ramsayi	1	2	2	1	1	11.35	12.35	12.7	7	0	1	4	1
Bipteria magna	Coryphaenoides pectoralis	1	2	2	1	2	17.29	13.3	14.63	7	0	1	4	1
Bipteria minima	Coryphaenoides pectoralis	1	2	2	1	2	11.68	11.82	16.95	7	0	1	4	1
Caudomyxum nanum	Lota lota		1	1	1	1	5.8	5.25	5.25	7	0	1	3	1
Dicauda atherinodi	Notropis atherinoides		1	2	5	3	10.3	9.3	7.3	1	0	1	3	1
Fabespora nana	Scorpaena porcus, Proterorhinus marmoratus		2	2	3	2	3.25	7.15		4	0	1	3	1
Fabespora vermicola	Archosargus probatocephalus, Crassicutis archosargi,		2	2	5	1	8.4	4.7	4.7	4	1	2	6	0
Hennegoides longitudinalis	Osphronemus gourami	2	1	2	4	1	11.5	5.4		7	0	1	3	1
Hennegoides obpyriformis	Noemacheilus yingjangensis, Plectorhinchus polytaenia	4	2	2	2	1	10.2	6.5	5.0	7	0	1	3	1

Hennegoides berlandi	Pangasius hypophthalmus	2	1	2	2	1	8.5	2.8	3.0	7	0	1	3	1
Hennegoides malayensis	Pangasius hypophthalmus	2	1	2	2	1	13.7	6.8	5.1	7	0	1	3	1
Hennegoides pangasii	Pangasius hypophthalmus	2	1	2	2	1	27.3	12.6	13.6	7	0	1	3	1
Kentmoseria alata	Chaetodon rainfordi		2	2	1	1	12.6	9.6	9.9	4	0	1	4	1
Kentmoseria fluviatilis	Dichotomyctere fluviatilis		1	2	1	1	8.3	7.8	6.8	4	1	4	6	0
Kentmoseria indica	Macrospinosa cuja		3	2	1	1	7.38	6.17		4	0	1	6	0
Laterocaudata mastacembala	Mastacembelus aculeatus		1	2	2	3	8.4	5.0	4.1	1	0	1	3	1
Laterocaudata armati	Mastacembelus armatus		1	2	2	1	8.4	5.2		1	0	1	3	1
Latyspora scomberomori	Scomberomorus guttatus	1	2	2	1	1	9.2	9.6	16.1	1	0	1	6	0
Meglitschia mylei	Myleus rubripinnis		1	1	3	2	24.6	8.7	5.1	2	1	3	3	1
Myxobilatus anguillaris	Taenioides anguillaris		3	1	2	1	6.2	3.7		7	0	1	3	1
Myxobilatus cheni	Schizothorax davidi		1	1	1	2	11.9	5.1	6.3	7	1	2	3	1
Myxobilatus gasterostei	Gasterosteus aculeatus	2	1	1	1	1	12.9	5.8		7	1	2	3	1
Myxobilatus gobii	Gobio gobio	2	1	2	1	1	11.85	7.25	6.1	7	1	2	3	1
Myxobilatus hemiculteri	Hemiculter leucisculus	2	1	5	1	1	12.2	6.7	6.25	7	1	2	3	1
Myxobilatus mictosporus	Micropterus salmoides	2	1	1	1	2	14.25	8.5	6.75	7	1	2	3	1
Myxobilatus noturi	Noturus gyrinus	2	1	3	1	2	8.9	6.6	6.3	7	1	2	3	1
Myxobilatus sichuanensis	Carassius auratus auratus	2	1	1	1	2	12.9	4.8	4.6	7	0	1	3	1
Myxoproteus cheni	Thamnaconus septentrionalis		2	1	1	2	14.0	12.8	12.5	4	0	1	6	0
Myxoproteus elongatus	Anarhichas lupus, Licichthys deuyiculatus		2	2	1	2	11.75	6.5	7.75	4	0	1	6	0
Myxoproteus meridionalis	Merluccius hubbsi		2	2	1	2	10.5	8.65		4	0	1	6	0
Myxoproteus cujaeus	Macrospinosa cuja		3	2	1	2	10.5	9.2		4	0	1	6	0
Myxoproteus hubbsi	Coelorinchus coelorhincus carminatus		2	2	1	2	5.8	5.2	3.9	4	0	1	6	0
Myxoproteus abyssus	Bathygadus antrodes, Coryphaenoides ariommus, C. armatus		2	2	1	2	11.8	10.2	7.2	4	0	1	6	0
Myxoproteus rosenblatti	Coryphaenoides acrolepis		2	2	1	2	17.0	10.5	9.5	4	0	1	6	0
Neobipteria macrouri	Coryphaenoides acrolepis		2	2	1	2	13.8	16		7	0	1	4	1
Neohenneguya tetraradiata	Odontamblyopus rubicundus		2	2	2	3	18.9	5.4	5.4	7	0	1	3	1
Neomyxobolus ophiocephalus	Channa argus		1	2	1	1	9.05	10		1	1	3	6	0
Noblea admiranda	Urophycis chuss		2	2	1	2	17.3	13.3		3	0	1	4	1
Neothelohanellus catlae	Catla catla		1	2	1	1	8.5	7.7		4	0	1	6	0
Neothelohanellus krishnagarensis	Labeo calbasu		1	2	6	1	11.2	7.7	6.5	4	0	1	6	0
Neoparvicapsula ovalis	Gobionotothen gibberifrons		2	2	1	2	14.6	8.2	8.2	5	1	2	3	1
Neoparvicapsula monoalata	Microspinosa cuja		3	2	1	2	14.25	7.0	8.4	5	0	1	4	1
Octospina tongrensis	Ctenopharyngodon idella		1	1	3	1	12.9	9.0	9.0	5	0	1	3	1
Palliatus mirabilis	Xenodermichthys copei,(old name Xenodermichthys socialis)	1	2	2	3	2	19.45	23.5		4	0	1	5	1
Palliatus grandis	Alepocephalus australis	1	2	3	3	2	26	17	25	4	0	1	5	1

Palliatus indecorus	Alepocephalus rostratus	1	2	3	3	2	23.5		21	4	0	1	5	1
Palliatus indicus	Liza macrolepis	1	3	3	3	2	9.8	7.35		4	0	1	5	1
Palliatus binus	Hippoglossoides dubius	1	2	1	3	2	18.5	18.25		4	0	1	5	1
Palliatus magellanicus	Macruronus magellanicus	1	2	3	3	2	14.4	16.3	17	4	0	1	5	1
Paramyxoproteus reinhardti	Reinhardtius hippoglossoides		2	3	1	2	20.4	20.12	11.5	4	0	1	3	1
Phlogospora mysti	Mystus bleekeri		1	1	2	1	16.0	4.25		5	0	1	3	1
Phlogospora oculatus	Mystus oculatus		1	3	2	1	18.47	3.07		5	0	1	3	1
Phlogospora gulio	Mystus gulio		1	3	2	1	19.8	5.9		5	0	1	3	1
Pseudalataspora lophii	Lophius piscatorius		2	2	3	2	9.8	18.2	17.5	7	0	1	4	1
Pseudalataspora atlantica	Chlorophthalmus agassizi		2	2	3	2	8.35	38.55	38.55	7	0	1	4	1
Pseudalataspora indecora	Dentex angolensis		2	2	3	2	4	16.7	16.7	7	0	1	4	1
Pseudalataspora pontica	Liza aurata		3	2	3	2	6	17.95	17.95	7	0	1	4	1
Pseudalataspora scombri	Scomber japonicus		2	2	3	2	5.32	9.26	9.28	7	0	1	4	1
Pseudalataspora squamiformis	Lepidonotothen squamifrons		2	3	3	2	6	20.6	20.6	7	0	1	4	1
Pseudalataspora umbraculiformis	Gaidropsarus mediterraneus		2	2	3	2	8.65	15.95	15.95	7	0	1	4	1
Renispora simae	Patagonotothen sima		2	2	3	2	24.4	10.8		2	0	1	4	1
Spirosuturia hypophthalmichthydis	Hypophthalmichthys molitrix		1	2	5	1	8.4	8.4	5.5	4	0	1	6	0
Spirosuturia carassi	Carassius auratus		1	2	1	1	13.8	8.6	8.6	4	0	1	6	0
Schulmania aenigmatosa	Hippoglossoides platessoides	1	2	2	1	2	20.3	16.9	16.4	7	0	1	4	1
Schulmania pacifica	Coryphaenoides longifilis	1	2	3	3	2	19	11.35	11.35	7	0	1	4	1
Schulmania ovale	Lycodes esmarkii	1	2	2	3	2	19	13.95	13.95	7	0	1	4	1
Schulmania albinae	Sebastolobus macrochir, Sebastes helvomaculatus	1	2	3	3	2	14.65	8.95	8.95	7	0	1	4	1
Schulmania japonica	Dasycottus setiger	1	2	2	3	2	17.5	11.3	11.3	7	0	1	4	1
Trigonosporus acanthogobii	Acanthogobius flavimanus		3	3	2	1	8.7	13.9	5.5	7	0	1	3	1
Tetrauronema macropodus	Macropodus opercularis		1	1	2	1	11.9	6.04	5.45	7	0	1	3	1
Tetrauronema desaequalis	Hoplias malabaricus		1	1	2	3	13.6	6.5	3.7	7	0	1	3	1
Trilospora californica	Gibbonsia elegans, Typhlogobius californiensis		2	1	3	2	7.2	16	16	6	0	1	6	0
Trilospora muscularis	Molva dypterygia		2	2	5	1	5.9	8.25	8.25	6	0	1	6	0
Trilosporoides platessae	Pleuronectes platessa L.,		2	2	3	2	24.4	9.4	9.4	6	0	1	6	0
Trigonosporus acanthogobii	Acanthogobius flavimanus		3	2	2	1	8.7	13.9	5.5	7	0	1	5	1
Triangula egyptica	Oreochromis niloticus		1	2	1	1	12.85	11.22	7.32	1	0	2	6	0
Triangula illinoisensis	Ictiobus bubalus		1	2	2	1	10.2	12.8		1	0	1	6	0
Triangula yangkiangensis	Rhinogobius giurinus, Rhodeus lightii		3	2	2	3	9.6	9.1		1	0	1	6	0
Triangula percae	Perca fluviatilis		1	2	6	1	10.6	10.4		1	0	1	6	0
Unicauda wuhanensis	Arabibarbus grypus	2	1	3	2	3	4.85	3.05	6.1	7	0	1	3	1
Unicauda pelteobragus	Hybognathus nuchalis	2	1	1	2	1	13.58	7.38	5.4	7	0	1	3	1

Unicauda magna	Pimephales promelas	2	1	1	2	3	15.5	12.1		7	0	1	3	1
Unicauda lumae	Carassius auratus gibelio	2	1	1	2	3	11.0	8.5	11.75	7	0	1	3	1
Unicauda crassicauda	Campostoma anomalum	2	1	2	2	1	9.5	6.5	6.5	7	0	1	3	1
Unicauda clavicauda	Notropis blennius, N. hudsonius	2	1	2	2	1	12.3	10.1	6	7	0	1	3	1
Unicauda brachyura	Notropis anogenus	2	1	1	5	1	10.75	8.4	4.5	7	0	1	3	1
Unicauda aristichthyalis	Hypophthalmichthys nobilis	2	1	2	1	2	9.53	5.08	4.6	7	0	1	3	1