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Ph.D. Thesis

Phylogenetic analyses of myxosporeans based on the molecular data

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ANNOTATION

This thesis is focused on the assessment of the phylogenetic position of the Myxozoa within the Metazoa, study of the evolutionary relationships within myxosporeans and investigation into the cryptic species assemblages of several myxosporeans based on the ribosomal and protein-coding data. The major part of this work was to confirm the evolutionary trends within myxosporeans based on a single gene by other molecular markers in order to find out if the reconstructed relationships correspond to the real organismal phylogeny. This has been a crucial step for future actions in solving the discrepancies between the myxosporean phylogeny and taxonomy.

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DECLARATION

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

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České Budějovice, 26th July, 2010 .....

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I declare that Pavla Bartošová substantially contributed to the results in this thesis:

RNDr. Ivan Fiala, Ph.D. .....

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Miloslav Jirků, Ph.D. .....

CONTENTS

PREFACE

This thesis is based on following papers that will be referred to in the text by their Roman numerals:

I. **Bartošová P.**, Fiala I., Hypša V. (2009). Concatenated SSU and LSU rDNA data confirm the main evolutionary trends within myxosporeans (Myxozoa: Myxosporea) and provide an effective tool for their molecular phylogenetics. *Molecular Phylogenetics and Evolution* **53**, 81-93.

II. Fiala I., **Bartošová P.** (2010). History of myxozoan character evolution on the basis of rDNA and EF-2 data. *BMC Evolutionary Biology* **10**, 228.

III. **Bartošová P.**, Freeman M. A., Yokoyama H., Caffara M., Fiala I. Phylogenetic position of *Sphaerospora testicularis* and *Latyspora scomberomori* n. gen., n. sp. (Myxozoa) within the marine urinary clade and the evolution of the nature of the sutural line in the marine lineage of Myxosporea (submitted to *Parasitology*).

IV. Jirků M., **Bartošová P.**, Kodádková A., Mutschman F. Another chloromyxid lineage: molecular phylogeny and redescription of *Chloromyxum careni* from amphibian (submitted to *Journal of Eukaryotic Microbiology*).

V. **Bartošová P.,** Fiala I. Molecular evidence for the existence of cryptic species assemblages of several myxosporeans (Myxozoa) (submitted to *Diseases of Aquatic organisms*).

ABBREVIATIONS

SSU rDNA – small subunit ribosomal DNA

LSU rDNA – large subunit ribosomal DNA

EF1, EF2 – elongation factor 1, 2

SEM – scanning electron microscopy

TEM – transmission electron microscopy

LBA – long branch attraction

LogDet – log determinant

MP – maximum parsimony

ML – maximum likelihood

BI – Bayesian inference

HSP – heat shock protein

ITS – internal transcribed spacer

OBJECTIVES OF RESEARCH

- investigation into the evolution within myxosporeans based on the single LSU rDNA data and combined SSU + LSU rDNA data
- comparison of the LSU- vs. the known SSU-based myxosporean phylogenies
- selection of the most informative rDNA regions useful for future reconstructions of the myxosporean relationships
- comparison of the rDNA-based phylogenetic trends within myxosporeans with the phylogenies reconstructed on the protein-coding data
- utilization of the new LSU rDNA and protein-coding data to investigate the myxozoan relationships with metazoan groups
- study of the cryptic species assemblages of *Chloromyxum* and *Zschokkella* spp. based on rDNA data

CHAPTER 1. GENERAL INTRODUCTION

It is almost over 200 years since the first myxozoan has been reported in the musculature of its fish host (Jurine 1825). Since then, more than 2300 representatives of the phylum Myxozoa Grassé 1970 have been described in various tissues and organs of the fish or other vertebrate and invertebrate hosts (Morris 2010). These microscopic metazoan endoparasites have always attracted extensive attention not only as important pathogens of fish, especially in fisheries and aquacultures, but also for their fascinating life cycle, morphology, putative protozoan nature, and their enigmatic relationships with metazoan groups. Moreover, after the beginning of the molecular era at the end of the 20th century, the controversies between the traditional spore-based taxonomy of myxosporeans and the findings of the rDNA-based phylogenies became the subject of never-ending discussions.

1.1. Multicellular origin of the Myxozoa

Myxozoans are characterized by transmission via multicellular spores that possess nematocyst-like polar capsules with a coiled polar filament and an infective germ (sporoplasm) serving for the attachment to the host and its invasion. The multicellular nature of myxozoan spores has firstly been suggested by Štolc (1899) and later confirmed not only by TEM but also by molecular data (Smothers et al. 1994, Katayama et al. 1995, Siddall et al. 1995, Schlegel et al. 1996). The metazoan affinities of the Myxozoa are manifested by the multicellular spores consisting of several differentiated cells and by cell-to-cell junctions (including gap juntions). Moreover, the myxozoan relationships to the Metazoa are indicated by the similarities in detailed structure of the myxozoan polar capsules and the nematocysts of non-anthozoan cnidarians (Medusozoa) (Weill 1938, Siddall et al. 1995). However, myxozoan polar capsules differ from the typical nematocysts of cnidarians in lacking chemo- and/or mechanosensory structures and neural connections that modulate discharge (Westfall 2004). Before the findings that Myxozoa represent highly degenerate metazoans, they were grouped with protistan taxa (Microsporidia and Apicomplexa) within the former groups Sporozoa and Cnidospora (Bütschli 1881, Dogiel 1965).

1.2. Myxozoan classification and taxonomy

The phylum Myxozoa is composed of two classes: the class Malacosporea Canning, Curry, Feist, Longshaw and Okamura, 2000 with three described species and the class Myxosporea Bütschli, 1881 with the overwhelming majority of myxozoan species.

Members of the class Myxosporea form vegetative stages (plasmodia; coelozoic in the body or organ cavities and histozoic in the tissues) and two types of spores (myxospores – Fig. 1 and actinospores). The current classification of myxosporeans is based on the morphology of their myxospores since their vegetative stages usually do not possess sufficient features for the classification. Important characters are the size and shape of the myxospore and of the polar capsules, number of shell valves, polar capsules and sporoplasms, position of the polar capsules to a plane of the suture and their location in the spore, the presence of the surface ridges, projections and envelopes of spore, character of the polar filament etc. Besides the spore characteristics, information about the vegetative stages, development of the spore (with/without pansporoblast), the host species, host environment and final site of infection in the host are important for proper determination of the parasite or description of a new species (Shulman 1966, Lom and Noble 1984, Lom and Dyková 1992, 2006).

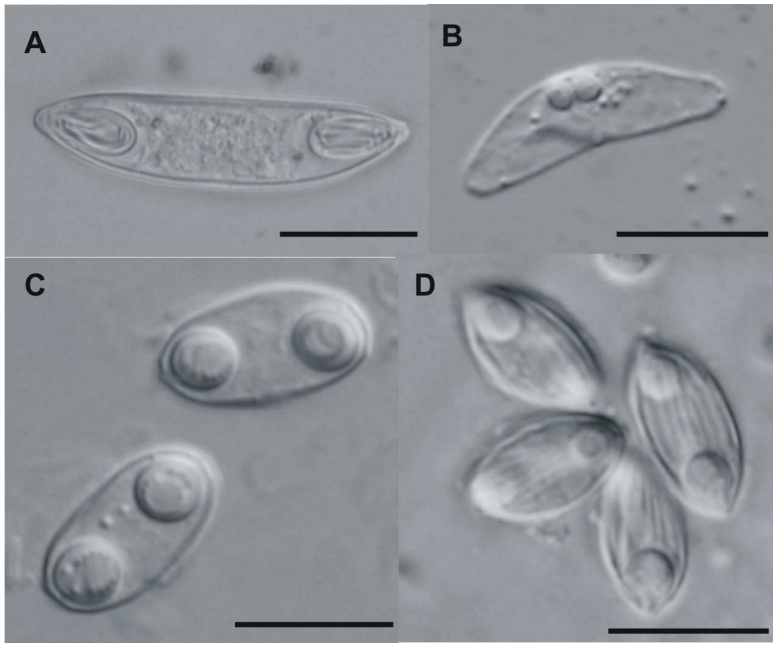

Figure 1. The myxospores of (A) *Sphaeromyxa* sp. from *Gephyroberyx darwinii*, Java, Indonesia; (B) *Ceratomyxa* sp. from *Scorpaena porcus*, off Croatia; (C) *Zschokkella nova* from *Ctenopharyngodon idella*, Czech Republic; (D) *Myxidium polymorphum* from *Rhinogobius giurinus*, China. Scale bar: 10 μm.

Only two genera, *Tetracapsuloides* and *Buddenbrockia,* are included within the class Malacosporea. They differ from the myxosporean taxa by the production of sac-like or vermiform stages in bryozoans and by having spores with eight unhardened shell valves (malacospores, Fig. 2). Their spores are topped with four polar capsules and include two sporoplasms with secondary cells and dense bodies (sporoplasmosomes). The malacosporean

spores developing in the fish host (fishmalacospores) possess four valve cells, two polar capsules and one sporoplasm. The sporoplasm contains sporoplasmosomes but lacks a secondary cell (Hedrick et al. 2004, Grabner and El-Matbouli 2010). Shulman´s system (1966) did not include criteria for the classification of Malacosporea since this class has been established many years later (Canning et al. 2000). The morphological differences between both malacosporean genera rest on the distinct shape and inner structure of their sac-like stages, spore formation, structure of polar capsules and pathogenicity (Canning and Okamura 2004).

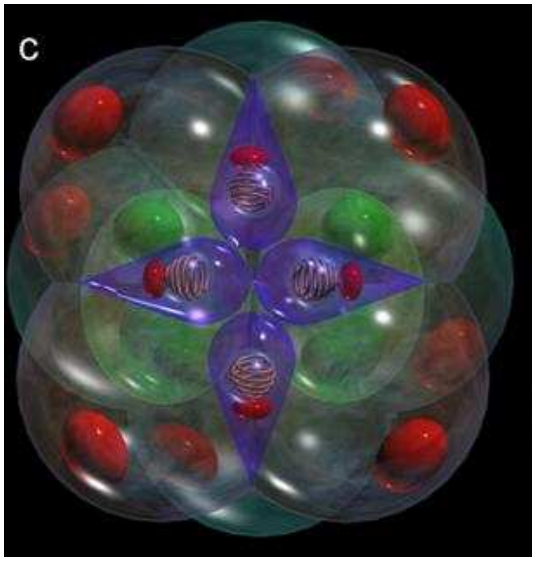

Figure 2. The scheme of the *Tetracapsuloides bryosalmonae* malacospore containing four capsulogenic cells (with spherical polar capsules and internal coiled polar filaments), eight valve cells and two sporoplasms each comprising nucleated primary and secondary cell (McGurk et al. 2005).

The classification proposed by Shulman (1966) was the base for the myxosporean taxonomy (Lom and Noble 1984) that predominantly persists until now. This system has been enriched with the class Malacosporea in the most recent taxonomic revision of the Myxozoa (Lom and Dyková 2006). The class Malacosporea encompasses only one order Malacovalvulida Canning, Curry, Feist, Longshaw and Okamura, 2000 with two already mentioned genera. The much larger class Myxosporea is divided into the orders Bivalvulida Shulman, 1959 and Multivalvulida Shulman, 1959 which distinction is based on the number of shell valves (two vs. three to seven) (Lom and Dyková 2006). Bivalvulids split into three suborders Sphaeromyxina Lom et Noble, 1984, Variisporina Lom et Noble, 1984 and Platysporina Kudo, 1919 mainly differing by the character of the polar filament and the position of polar capsules in the spore. Coelozoic representatives of Sphaeromyxina possess two polar capsules at the opposite ends of spore and polar filament is flat, zig-zag folded and tapering from its base to the tip. Mostly coelozoic variisporinids have two (rarely one or four) polar capsules placed either at the opposite ends of the spore or at one pole. In the latter case, they do not lie solely in the sutural plane or they lie perpendicularly to it. Generally two polar capsules of histozoic platysporinids are positioned concurrently with the sutural plane (Lom and Dyková 2006).

The former class Actinosporea was suppressed (Kent et al. 1994) after the groundbreaking discovery of Wolf and Markiw (1984) who proved that the representatives of this class are

actually the sexual developmental stages of the complex myxosporean life cycle. Recently, former genera of the class Actinosporea are named only in the vernacular using the collective group names (Kent and Lom 1999). Nevertheless, even the actinospores represent the definitive stages of the myxosporean life cycle, the International Code for Zoological Nomenlature does not state that sexual stages must be used for the taxonomic and nomenclature purposes (Kent and Lom 1999).

Increasing number of the rDNA data on myxosporean species induced the suppression of some genera or even the families. Families Pentacapsulidae, Hexacapsulidae and Septemcapsulidae, that include multivalvulids with more than four valves and polar capsules, have been synonymized with Kudoidae and their species have been transferred to the genus *Kudoa*. The reason was to avoid the paraphyletic status of the genus *Kudoa* (Whipps et al. 2004b). The recent demise of the genus *Leptotheca* has been impelled by the vague boundaries among this genus and the genera *Ceratomyxa* and *Sphaerospora*. *Leptotheca* species infecting the host gall bladder have been transferred to the genus *Ceratomyxa* and urinary system-inhabiting *Leptotheca* species were moved to the genus *Sphaerospora*. Only two representatives of the genus *Leptotheca* were transferred to the genera *Ellipsomyxa* and *Myxobolus* (Gunter and Adlard 2010).

1.3. Hosts and life cycle of the Myxozoa

1.3.1. Malacosporean hosts and life cycle

The most common bryozoan (Bryozoa: Phylactolaemata) hosts of malacosporeans are *Plumatella fungosa, P. rugosa, P. repens, Cristatella mucedo, Pectinatella magnifica,* and *Fredericella sultana*. The development begins with the infection of the bryozoan epithelium by the cryptic stages that give rise to the sac- or worm-like trophic stages developing in the bryozoan body cavity into the malacospores.

Tetracapsuloides bryosalmonae is known to form sac-like stages only. Despite there are some clues on the existence of the vermiform stages of *T. bryosalmonae* in the bryozoan hosts (Taticchi et al. 2004), more convincing data are still lacking. The sacs of *T. bryosalmonae* in the bryozoan are composed of an outer layer of mural cells and incomplete inner layer. The inner layer contains B cells and stellate cells both giving rise to malacospores. The fish hosts are *Salmo, Oncorhynchus* and *Thymallus* fingerlings in North America and Europe but the *T. bryosamonae* infection was also observed in a non-salmonid host (*Esox*). This parasite is an agent of the proliferative kidney disease (PKD): its cell-in-cell stages are the main replicative

phase of the life cycle, proliferate in the kidney interstitium and provoke a vigorous defense reaction. The onset of sporogony is indicated by an engulfment of one secondary cell by another to form a secondary-tertiary doublet and by the parasite migration to the lumen of kidney tubules. Then, the pseudoplasmodium is formed in which a single fishmalacospore develops (Morris and Adams 2008). Mature spores were found in the urine of rainbow trout *Oncorhynchus mykiss* (Hedrick et al. 2004) and within the pseudoplasmodia in the lumen of kidney tubules of brown trout *Salmo trutta* (Morris and Adams 2008). The complete life cycle has been demonstrated by the development of PKD in fish exposed to infected bryozoans (Feist et al. 2001) and also by the successful experimental transmission of *T. bryosalmonae* from infected brown trout to naïve *Fredericella sultana* colonies (Morris and Adams 2006). It has been proved that this parasite is not transmitted through fisheries activities (Henderson and Okamura 2004).

Buddenbrockia plumatellae forms both sac- and worm-like stages in the same bryozoan host (Canning et al. 2002, Okamura and Canning 2003). Its vermiform proliferative stage has an outer and inner layer of cells, a basal lamina and four longitudinal muscle blocks. Special B cells of the inner wall proliferate and give rise to the pre-spore cells later becoming typical spherical malacospores (Canning and Okamura 2004). The successful experimental transmission of *B. plumatellae* bryozoan stages to the cyprinid fishes *Cyprinus carpio* and *Phoxinus phoxinus*, where sporogonic stages in the kidney tubules similar to that of *T. bryosalmonae* were present, have shown that *Buddenbrockia* also possess a life cycle involving a fish host (Grabner and El-Matbouli 2010).

1.3.2. Myxosporean hosts and life cycle

The myxosporean life cycle includes the alternation of two hosts. The myxospore phase takes place in the vertebrate whereas the stages of the actinospore phase undergo their development in the invertebrate. The typical vertebrate host is the teleost fish, but several myxosporean species have been found in the cartilaginous fishes (Lom and Dyková 1992). Other hosts from the water environment are amphibians, reptiles and ducks (Eiras 2005, Jirků et al. 2006, Bartholomew et al. 2008). The recent findings of a myxosporean species in the shrews demonstate that myxozoans can also infect terrestrial homeothermic vertebrates (Prunescu et al. 2007, Dyková et al. 2007). A very few species have been reported to occur in their myxosporean phase in invertebrates (Weidner and Overstreet 1979, Yokoyama and Masuda 2001). The invertebrate hosts of the actinosporean phase of the freshwater myxosporeans are annelids (mainly oligochaetes and rarely polychaetes). As for the marine

myxosporeans, polychaetes and rarely sipunculids (Ikeda 1912) act as hosts of the actinosporen phase.

Vertebrates are considered as the intermediate hosts since the sexual process (gametogony) has been observed during the actinospore phase in the invertebrates (definitive host). Up to date, the life cycles including the actinospore stages have been described in a total number of 34 myxosporean species mainly infecting freshwater (eventually anadromous or catadromous) fish (Holzer et al. 2006b, Lom and Dyková 2006, Caffara et al. 2009, Figs. 3A, B). Only four marine life cycles have been elucidated up to date (Køie et al. 2004, 2007, 2008, Rangel et al. 2009, Fig. 3C). However, a direct fish-to-fish transmission without the requirement of the alternate actinosporean development has been proved in enteric *Enteromyxum* species (Fig. 3D). In this case, not spores, but the proliferative stages are responsible for the transmission of the disease (Diamant 1997, Redondo et al. 2002). However, it has been hypothesized that a heteroxenous life cycle involving an actinosporean phase may exist for *E. scophthalmi*. Its fish host, *Scophthalmus maximus*, may be an accidental host of this parasite (Redondo et al. 2004). Moreover, the epidemiological data on *Sphaerospora testicularis* suggest the possibility of the fish-to-fish transmission, but no successful experiments have yet confirmed this hypothesis (Sitjà-Bobadilla 2009).

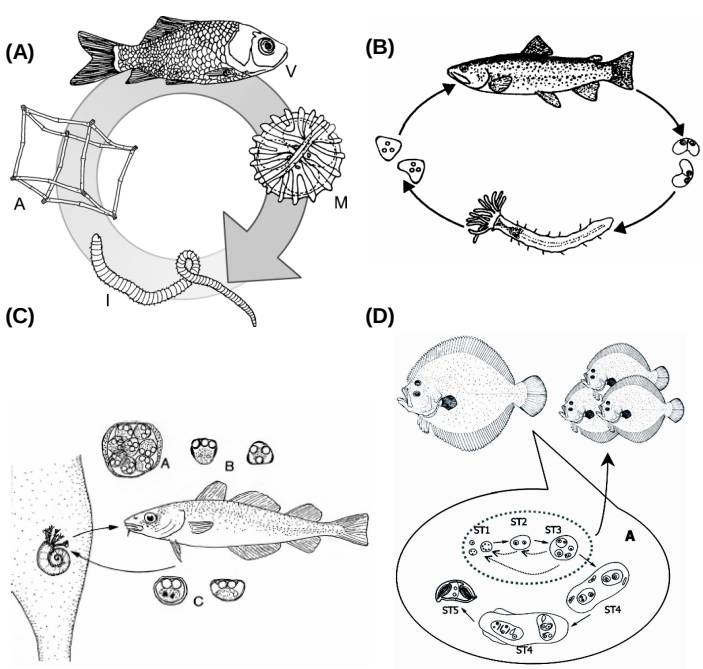

Figure 3. The life cycles of the Myxozoa. (A) freshhwater life cycle of *Chloromyxum auratum* with the antonactinomyxon actinospore stage in the freshwater oligochaete and the myxospore stage in common goldfish, *Carassius auratus* (Atkinson et al. 2007); (B) freshwater life cycle of *Ceratomyxa shasta* with the tetractinomyxon actinospore stage in the freshwater polychaete and the myxospore stage in North American salmonids (Bartholomew et al. 1997); (C) marine life cycle of *Gadimyxa atlantica* with an actinospore stage in the polychaete *Spirorbis* sp. and the thick-walled and thin-walled myxospore stages in Atlantic cod, *Gadus morhua* (Køie et al. 2007); (D) direct fish-to-fish transmission of *Enteromyxum scophthalmi* in turbot, *Scophthalmus maximus*, with ST2 to ST3 stages responsible for transmission to other fish through the faeces (Redondo et al. 2004).

Upon contact with the skin or gills of the vertebrate host the actinospore coming out of the annelid into the water discharges its polar capsules fastening itself to the host and releasing its sporoplasm. In the case of the terrestrial life cycle, the vertebrate host may be infected alimentary with the actinospore stages released from the digested terrestrial annelid (earthworm) (Tyml 2010). Then, the presporogonic development based on the unique myxosporean stage, cell-in-cell organization, follows. It is characterized by the endogenously produced secondary cells that persist inside the primary cell. Sometimes the tertiary or quaternary cells develop. The internal cells were firstly suggested to form within a surrounding cell by the endogenous budding (Thélohan 1890). However, the recent TEM study of the formation of the actinospore sporoplasm has demonstrated that endogenous budding does not occur and the myxozoan internal cells arise from cells surrounding one another (Morris 2010). Presporogonic stages ensure the multiplication of the parasite to a sufficient number and spread the infection throughout the host organs. Sometimes, a massive presporogonic replication in tissues and organs different from the final site of infection (extrasporogonic development) occur e.g. the extrasporogonic stages of *Sphaerospora dykovae* (syn. *S. renicola*), *S. ictaluri* and *S. elegans* in the blood and other organs (swimbladder, rete mirabile of the eye). They are exclusively proliferative phase of the cycle serving for an additional parasite proliferation and spreading the parasite throughout the fish organs (Dyková and Lom 1988, Lom and Dyková 1992).

When the presporogonic stages reach the final site of infection, they enter the sporogonic phase of the cycle and transform in the plasmodia (trophozoites) in which the myxospores develop. There are many types of plasmodia differing by their size, number of nuclei, number of the generative cells and by the localization in the host (coelozoic or histozoic; mono-, disporic pseudoplasmodia or polysporic plasmodia). Histozoic plasmodia of some members of genera *Myxobolus*, *Henneguya* and *Kudoa* may be encased with host connective tissue and appear as whitish “cysts”. The assignment of myxosporeans as coelozoic or histozoic is sometimes difficult to assess as many species develop both within the body cavities and in various tissues e.g. *Sphaerospora dykovae* and *Myxidium lieberkuehni*. Actinospores, the final stages of the myxosporean life cycle in the invertebrate, develop within the pansporocyst. Actinospore stages have typical triradiate symmetry, sometimes possess the caudal projections and may form a group of eight spores as a result of the development within the pansporocyst (Lom and Dyková 1992, 2006). Interestingly, a single actinosporean genotype may display two different phenotypes in the same oligochaete host (Hallett et al. 2002,

Eszterbauer et al. 2006). These phenotypes are possibly designed for different fish hosts (Holzer et al. 2004).

1.4. Importance of the Myxozoa

Among the large number of described myxozoan species, only a fraction is known for the diseases they cause to their fish hosts. Many of these pathogenic species infect feral or trash fishes, but there are also numerous serious pathogens of commercially important fishes. The parasites may impair the growth of the fish, damage its tissues and organs or even cause the death of the host. The pathogenic effect depends on the myxosporean species, life cycle stage, host immune reaction, temperature and intensity of infection.

One of the best studied pathogenic myxosporean species is *Myxobolus cerebralis* causing the whirling disease of freshwater salmonids. Disease symptoms are the destruction of cartilage and associated tissues in juvenile fish. Other myxozoan species infect the renal system of the fish such as the malacosporean *Tetracapsuloides bryosalmonae* and many myxosporeans e. g. *Sphaerospora dykovae, S. ictaluri*, *Polysporoplasma sparis* and *Parvicapsula minibicornis*. The digestive tract of sparid or salmonid fishes may be seriously damaged by histozoic myxosporeans *Enteromyxum leei* and *Ceratomyxa shasta*, respectively. The gall bladder of cultured *Sparus aurata* may be infected by *Ceratomyxa sparusaurati*. Certain myxosporean species parasitize the reproductive tract of the fish such as *Sphaerospora testicularis*, *Henneguya testicularis* (testes) and *Kudoa ovivora* and *Wardia ovinocua* (ovaries). Muscle-invading multivalvulid myxosporeans of the genus *Kudoa* cause dramatic changes in the flesh (myoliquifaction) after the death of the fish. This is caused by the effect of powerful proteolytic enzymes released by the parasites after the disintegration of the pseudocyst. Also some bivalvulid species like *Henneguya zschokkei* and *Myxobolus cyprini* may form cysts in the muscles. The fish gills may be impaired by cyst-forming species e. g. *Henneguya exilis*, *H. psorospermica*, *Thelohanellus pyriformis* and *Myxobolus muelleri*. There are also several myxosporeans causing systemic infections of the host such as *Sphaerospora dicentrarchi* and *Kudoa lutjanus* (Lom and Dyková 1992, 2006).

CHAPTER 2. RELATIONSHIPS OF THE MYXOZOA

Knowledge into the myxozoan phylogeny and identification of the myxozoan affinities to metazoan groups is important not only for the study of an early metazoan evolution but also

from the economic point of view as it could help to understand myxozoan life-history patterns and design efficient intervention strategies (Siddall and Whiting 1999). Smothers et al. (1994) were the first to use the ribosomal DNA (rDNA) sequence analysis to investigate the phylogenetic position of the Myxozoa within Metazoa. Since then, molecular data have been employed to reveal the phylogenetic relationships within myxozoans, to study their life cycles and to develop PCR diagnostic tests for the pathogenic myxozoan species.

2.1. Relationships of the Myxozoa with the metazoan groups

The discoveries of myxozoan cellular complexities have impelled many debates about affinities of the Myxozoa to the metazoan taxa. Structural similarities of myxozoan polar capsules and cnidarian nematocysts and the parallels between the development of myxozoan sporoblasts and parasitic stages of *P. hydriforme* Ussov 1885 suggested that the Myxozoa and the Cnidaria share a common ancestor (Weill 1938).

A cnidarian *Polypodium hydriforme* (phylogenetic position based on SSU rDNA data; Evans et al. 2009) has a life cycle including both intracellular stages parasitizing oocytes of acipenseriform fishes (Fig. 4A) and free-living medusoid stages (Fig. 4B). Besides a single type of polar capsule/nematocyst (atrichous isorhizae) and the the early cell-in-cell development, other characteristic shared by *Polypodium* and the *Myxozoa* are fish parasitism, loss of epidermal ciliation and mode of attachment of the infective stages to the host tissue by using nematocysts (Raikova 1994).

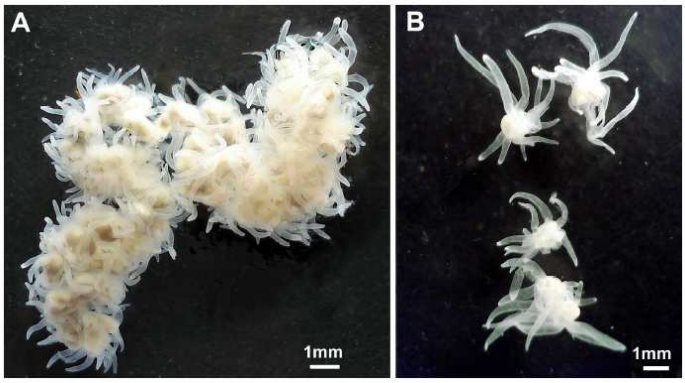

Figure 4. *Polypodium hydriforme*. (A) Stolon stage just after emerging from the host oocyte; (B) Four specimens of free-living *Polypodium* with 12 tentacles (Evans et al. 2008).

Unlike myxozoan affinities to cnidarians, another hypothesis considers Myxozoa to be the members of a primitive bilaterian animal lineage. This theory is supported by the lack of gut and central nervous system and the triploblast organization of the vermiform body of the rediscovered enigmatic malacosporean *Buddenbrockia plumatellae* Schröder, 1910 (Fig. 5). It has been suggested that *Buddenbrockia* represents a missing link in the evolution of myxozoans from a bilaterian ancestor to the degenerate plasmodia characterizing most of myxosporean species (Canning et al. 2002, Okamura and Canning 2003). Contrary to this, another interpretation of the structure of *Buddenbrockia´*s vermiform body was provided by Jiménez-Guri et al. (2007). They claimed that its four blocks of muscles are radially

distributed like in cnidarians. Therefore, *Buddenbrockia* is a tetraradial worm with two axes of symmetry across a transverse section. A motile worm form evolved independently within cnidarians by the loss of the opening to the gastrovascular cavity and subsequent acquisition of hydrostatic skeleton (Jiménez-Guri et al. 2007).

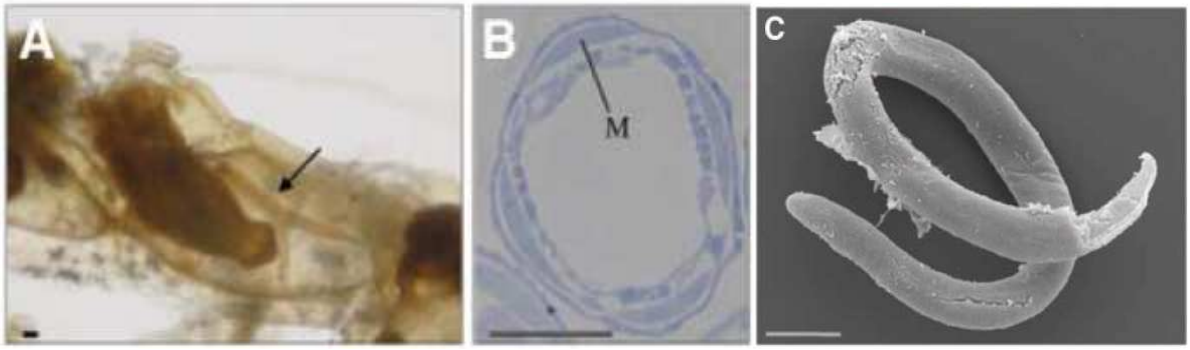

Figure 5. (A) A zooid of the bryozoan *Plumatella* with *Buddenbrockia* worms (arrow) in the body cavity. Scale bar, 40 μm; (B) Cross section of an immature *Buddenbrockia plumatellae* worm. Note the presence of four longitudinal muscle blocks (M) and absence of gut. Scale bar, 20 μm; (C) Scanning electron microscopy image of a *Buddenbrockia plumatellae* worm. Scale bar: 100 μm (Jiménez-Guri et al. 2007).

Further evidence for a cnidarian (diploblast) vs. bilaterian (triploblast) origin of myxozoans came from the molecular studies. Pioneer phylogenetic analyses based on the SSU rDNA data revealed the Myxozoa as a sister group to Nematoda/all Bilateria or a member of the Bilateria (Smothers et al. 1994). Triploblast affinities of the Myxozoa have later been supported by other authors (Katayama et al. 1995, Hanelt et al. 1996, Schlegel et al. 1996, Kim et al. 1999). However, other SSU-based analyses including the sequence of *P. hydriforme* showed that Myxozoa and *Polypodium* group in one clade (Endocnidozoa). This clade forms a basal lineage to all bilaterians (Zrzavý et al. 1998, Siddall and Whiting 1999, Zrzavý and Hypša 2003) or it clusters within cnidarians (Siddall et al. 1995, Siddall and Whiting 1999). It has been suggested that the conflicting results of the molecular analyses were caused by the long-branch nature of the myxozoan and many metazoan (Acoela, Mesozoa, *Polypodium* and a nematode *Caenorhabditis elegans*) SSU rDNA sequences. These taxa have unusually high divergence rates of their SSU rDNA sequences that can cause grouping of the unrelated taxa (LBA; Siddall and Whiting 1999). Moreover, selection of too distant outgroups and a poor taxonomic sampling were proposed as other reasons for the discrepancy between results of the analyses (Siddall et al. 1995, Kim et al. 1999).

Therefore, certain approaches were used to resolve this problem. Application of different tree-building methods (Siddall and Whiting 1999), a combination of SSU rDNA data with morphological characters (Siddall et al. 1995, Zrzavý et al. 1998) as well the use of the long-branch extraction method (Siddall and Whiting 1999) have shown that the grouping of the

Myxozoa and *Polypodium* is not an artifact caused by LBA. Inclusion of more cnidarian taxa (Siddall and Whiting 1999) confirmed the previously suggested placement of the Endocnidozoa within Cnidaria (Siddall et al. 1995). On the other side, application of different tree building methods excluded the long-branch and distant outgroup artifacts as a source of the basal clustering of the Endocnidozoa to the Bilateria (Zrzavý and Hypša 2003). Performing single analyses of another ribosomal gene (LSU rRNA) sequences as well as its concatenated analyses with the SSU rDNA data showed that Myxozoa cluster as a sister taxon of bilaterians inside the Metazoa (Fiala and Bartošová 2007, appendix I). This placement of Myxozoa has been supported by the recent comprehensive analyses of myxozoan, cnidarian and other metazoan SSU and LSU rDNA sequences, where Myxozoa clustered as an early diverging and well supported clade of the Bilateria (Evans et al. 2010). In contrast, the phylogenetic analyses of the protein-coding data (EF1, EF2, α-tubulin) placed Myxozoa as a sister group to all Metazoa (Fiala and Bartošová 2007, appendix II). The re-investigation of the four myxozoan Hox genes, supporting the bilaterian affinities of Myxozoa, has revealed that these sequences were derived from the host DNA (Jiménez-Guri et al. 2007).

The relatedness of myxozoans to the Bilateria or to all Metazoa has been contradicted by the comprehensive multi-gene analyses of 50 different protein-coding gene sequences that placed a malacosporean, *B. plumatellae*, within Cnidaria forming a clade with Medusozoa (Fig. 6). This has been extrapolated to all Myxozoa rendering them members of a cnidarian lineage (Jiménez-Guri et al. 2007). This finding supports the previously mentioned myxozoan affinities to cnidarians indicated by morphological data (Weill 1938, Jiménez-Guri et al. 2007).

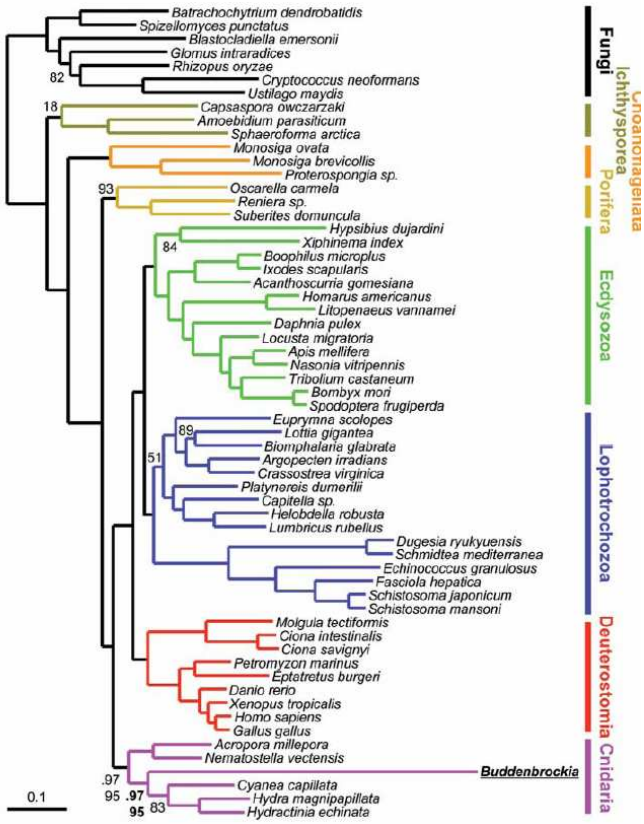

Figure 6. Phylogenetic analysis of the genomic data showing the clustering of *Buddenbrockia* within Cnidaria (Jiménez-Guri et al. 2007).

Despite the conclusions of the phylogenomic study of Jiménez-Guri et al. (2007) seem to be based on the convincing results, certain issues like the missing data, model choice and inference methods were found to have a substantial effect on the placement of the Myxozoa within the Metazoa. For example, the performed analyses were only based on 50 of a total number of 129 genes resulting in the lack of about 74% aminoacid positions in *Buddenbrockia* compared to other taxa (Fiala 2008). Moreover, this study has a relatively limited sampling of cnidarians and does not include *Polypodium* (Evans et al. 2008). Other aspects like the long branch of *Buddenbrockia´s* sequence in the analyses and its clustering with the Bilateria in MP analysis should be also taken into account (Fiala 2008). In-depth re-investigation of the phylogenomic dataset by the selection of different models has changed the position of *Buddenbrockia* from within Cnidaria to the alternative hypothesis at the base of Bilateria. This demonstrates that conflicting signals exist within the phylogenomic dataset and careful data exploration and a model selection are important when investigating the phylogenetic placement of the highly divergent taxa (Evans et al. 2010).

New approaches such as the sequencing of the whole mitochondrial genome of the myxozoan representatives can show to be essential to resolve this long-time discussed topic. The first step has begun by the most recent successful amplification of *P. hydriforme* and seven myxosporean mitochondrial 16S sequences. These data may be useful for the design of the probe targeting the myxosporean mitochondrial genome. The ML phylogenetic analysis of these sequences with the available cnidarian 16S data (Cinková 2010) has confirmed the close relationships of the Myxozoa and the Hydrozoa (Cnidaria) as proposed previously (Jiménez-Guri et al. 2007) and revealed the position of *P. hydriforme* within anthozoans.

2.2. Phylogenetic relationships within the Myxozoa

The first considerations about the evolution of myxosporeans but with no molecular support were suggested by Shulman (1966). He proposed that coelozoic urinary bladder myxosporeans infecting the marine teleost fishes evolved from the myxozoans inhabiting the gall bladder. Histozoic forms arose later from the coelozoic ones. Shulman (1966) has also suggested that ancestral myxozoans were variisporinids. These myxosporeans gave rise to platysporinids in the fresh water. The evolution of the coelozoic marine bivalvulid genus *Ceratomyxa* went to the marine histozoic multivalvulids through *Trilospora* with three valves and polar capsules and to *Kudoa* with four up to seven valves and polar capsules (Shulman 1966). This could have happened by a genetically fixed change or disorder in cell division producing supernumerary cell valves and polar capsules and by their stabilization by selective

environmental pressures (Lom and Noble 1984). The contrary process (suppression of one capsulogenic cell) may have led to the evolutionary line from *Myxobolus* to *Thelohanellus* (Lom and Noble 1984).

As for the malacosporeans, *Tetracapsuloides bryosalmonae* has been linked to the myxosporean genera *Sphaerospora* and *Parvicapsula* based on the similarities in the morphology of tetracapsuloid sporoblasts and spores of these myxosporeans (Kent and Hedrick 1986). Later, the SSU rDNA data on *T. bryosalmonae* have rejected its relationships to both myxosporean species since it clustered basally to all myxosporeans (Kent et al. 1998). Moreover, *T. bryosalmonae* grouped with another malacosporean, *Tetracapsula bryozoides*, and they still created a basal lineage to all Myxosporea in the SSU-based analysis (Kent et al. 2000). Latter SSU rDNA studies have shown that the sac-like *T. bryozoides* is co-specific with the worm-like organism *Buddenbrockia plumatellae* and *T. bryozoides* became a junior synonym of the firstly described *B. plumatellae* (Monteiro et al. 2002). It has been suggested that malacosporeans diverged from the myxozoan evolutionary line before the radiation of the numerous myxosporean genera (Kent et al. 1998, 2000).

Many of Shulman's hypotheses (1966) on the evolution of Myxosporea have later been confirmed by the phylogenetic analyses based on the SSU rDNA data (Kent et al. 2000, 2001, Fiala and Dyková 2004, Fiala 2006). It has been a well known fact that the classification of Myxosporea does not reflect many biological features such as the life cycle characteristics with the alternation of hosts, morphology of actinospores and host and tissue preferences. Moreover, already the very first molecular SSU-based analysis has revealed an important finding that the spore-based myxosporean taxonomy does also not reflect phylogenetic relationships retrieved from the molecular data (Smothers et al. 1994). This fact has later been stressed by many authors (Andree et al. 1999b, Kent et al. 2001, Holzer et al. 2004, Fiala 2006). The SSU-based phylogenetic studies have shown many genera to be paraphyletic (e.g. *Kudoa*, *Myxobolus*, *Parvicapsula*) or polyphyletic (e.g. *Henneguya*, *Sphaerospora*, *Zschokkella*, *Chloromyxum*) (Smothers et al. 1994, Andree et al. 1999b, Kent et al. 2001, Fiala 2006). Recently, the only monophyletic myxosporean genera are *Enteromyxum*, *Gadimyxa* and *Sphaeromyxa*. Despite the existence of significant discrepancies between the myxosporean taxonomy and phylogeny, there have been found at least some bionomical characters (the host environment and site of infection) that are congruent with myxozoan evolution (Andree et al. 1999b, Holzer et al. 2004, Whipps et al. 2004b, Eszterbauer et al. 2006, Fiala 2006). These non-morphological attributes are also very important for the species description.

Myxosporeans split into two main lineages, the freshwater and the marine one (Fig. 7), according to the environment of their hosts as firstly reported by Kent et al. (2001). This trend is mostly still valid (paper II) but some exceptions exits like the clustering of the freshwater species *Ceratomyxa shasta* and *Parvicapsula minibicornis* within the marine lineage and marine *Sphaeromyxa* spp. within the freshwater lineage (Fiala 2006). There is an interesting correlation between the length of the sequences and the host environment. The species of the marine lineage have of about 250 bp shorter SSU rDNA sequences than the freshwater ones (Fiala and Dyková 2004, Fiala 2006). These differences are due to the insertions within the variable regions of the freshwater myxosporean SSU rDNA sequences. For example, the E43 subhelix of V7 SSU region of the marine species retains its basic condition as in the malacosporean *Tetracapsuloides bryosalmonae*. Freshwater species extend this subhelix by forming various bifurcations. Only *Parvicapsula minibicornis* infecting the freshwater and anadromous fishes and clustering within the marine urinary clade has significantly longer SSU rDNA sequence than other marine myxosporeans. This is caused by the bifurcation of *P. minibicornis* E43 subhelix (Holzer et al. 2007).

Besides the two mentioned lineages there is also one minor group (Fiala 2006, Fig. 7) termed as the sphaerosporid clade (Jirků et al. 2007). This minor clade is composed of two subclades. The first well supported *Sphaerospora* sensu stricto subclade encompasses only freshwater sphaerosporids, including the type species *S. elegans* (Fiala 2006, Holzer et al. 2007, paper III). The second subclade includes the marine species, *Bipteria formosa* and *Sphaerospora fugu* (Fiala 2006, Karlsbakk and Køie 2009, paper III). Besides the shared site specificity (coelozoic in the urinary system, Jirků et al. 2007) and similar sporogonic development (Morris and Adams 2008), the feature uniting the representatives of the *Sphaerospora* sensu stricto subclade is the presence of extensive insertions in the V4 and V7 regions of their SSU rDNA sequences (Fiala 2006, Holzer et al. 2007, Jirků et al. 2007). *Sphaerospora elegans* possesses the longest V4 region among all myxosporeans (Holzer et al. 2007). Contrary to this, *B. formosa* and *S. fugu* do not have such long insertions as the representatives of the first subclade (Holzer et al. 2007, paper III). Moreover, *S. fugu* is a histozoic parasite of the fish digestive tract (Tun et al. 2000). Interestingly, there is also one representative of the freshwater lineage, frog kidney-infecting myxosporean *Chloromyxum careni,* which possesses extensive inserts in its variable SSU rDNA regions. Its V7 region contains extraordinary long insertions (paper IV) that are even longer than in the members of the sphaerosporid clade (Holzer et al. 2007).

The sphaerosporid clade forms a basal branch to all myxosporean species in the MP analysis where malacosporeans are used as outgroups (Fiala 2006). Such position may be affected by LBA since the long branches of the sphaerosporid clade members could be attracted by the outgroup sequences. Nevertheless, the strong support for the basal position of this clade has also been achieved by BI and distance Logdet methods (Fiala 2006). The latter method is theoretically able to recover the correct tree topology by correction of base composition inequalities (Huelsenbeck 1997). The basal position of the *Sphaerospora* sensu stricto clade to all myxosporeans also support certain unique secondary structure characteristics of the representatives of this clade (the presence of subhelix E23_3) shared with malacosporeans but absent in other myxosporeans (Holzer et al. 2007). Despite many studies supported these findings (Holzer et al. 2007, 2010, Jirků et al. 2007), other analyses revealed the clustering of this clade within or at the base of the marine lineage (Holzer et al. 2004, Karlsbakk and Køie 2009). However, the nodal support for the sphaerosporid clade has always been weak in the latter analyses.

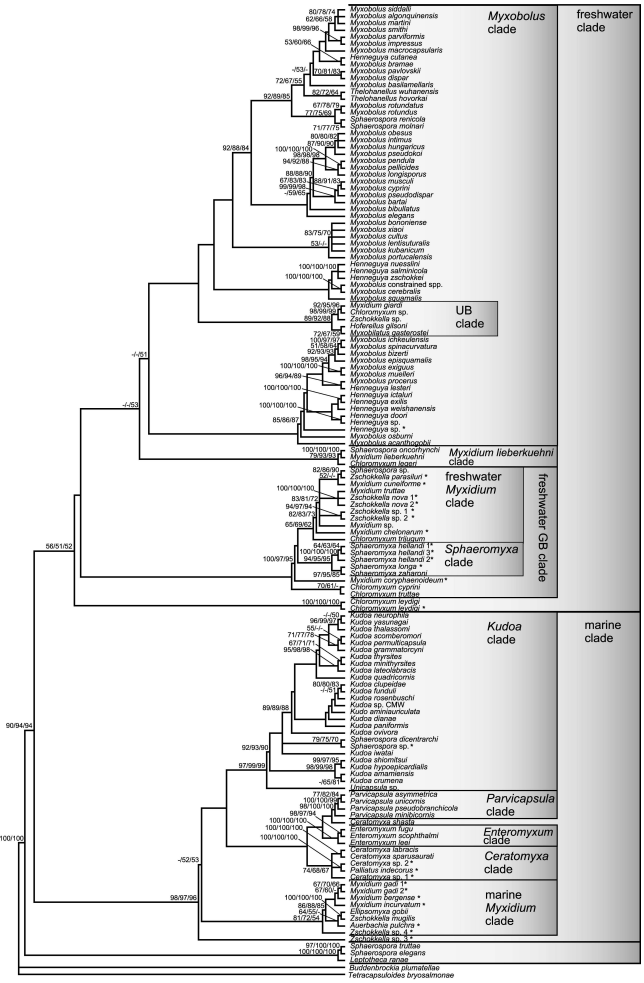

Figure 7. The phylogeny of Myxosporea based on the SSU rDNA data (Fiala 2006).

The freshwater and marine lineages divide into several clades that follow the site specificity (tissue tropism) of the parasites within the hosts to a great extent (Andree et al. 1999b, Eszterbauer 2004, Holzer et al. 2004, 2010, Fiala 2006, paper III, IV). Histozoic myxosporeans cluster within both the marine (*Myxobolus* clade) and freshwater lineage (*Kudoa* clade). Similarly, gall bladder-infecting parasites can be found within the freshwater *Myxidium* and *Sphaeromyxa* clades and within the marine *Myxidium* and *Ceratomyxa* clades. Myxosporeans infecting the urinary system of their hosts mainly cluster within the marine urinary clade composed of the *Parvicapsula* and *Zschokkella* subclades and within the freshwater *Myxidium lieberkuehni* clade. *Enteromyxum* clade includes the parasites of the intestine (Fiala 2006, Holzer et al. 2010, paper III). The site and host specificity have been shown to be a less important factor of evolution than spore morphology in the phylogenetic study of the *Myxobolus* species (Salim and Desser 2000). However, better sampling has shown that *Myxobolus* representatives cluster according to their site specificity rather than to their spore morphology (Andree et al. 1999b, Bahri et al. 2003, Eszterbauer 2004).

Similarly, despite geography has been found to play a role in the evolution of five *Kudoa* species (Hervio et al. 1997), larger dataset of the SSU rDNA kudoid sequences did not confirm this statement (Whipps et al. 2004b, Yokoyama and Itoh 2005). No correlation of the myxosporean geographic origin with their phylogeny has also been shown in other studies (Eszterbauer and Székely 2004, Fiala 2006, Holzer et al. 2006a). However, geographic origin may be important at the population level when the differences among the various geographic isolates of one species are assessed. Such phylogeographic analyses based on the SSU, LSU rDNA, HSP70 and ITS sequences were performed to investigate the intra- and intergenomic variability of *Myxobolus cerebralis*, *Kudoa thyrsites*, and *Tetracapsuloides bryosalmonae* (Andree et al. 1999a, Henderson and Okamura 2004, Whipps et al. 2004a, Whipps and Kent 2006).

The host specificity has also been proposed to be an important factor affecting the myxozoan evolution (Hervio et al. 1997, Molnár et al. 2002, Bahri et al. 2003). However, most of the authors do not agree with this opinion and claim that there is no or only a little correlation with the myxozoan phylogeny (Salim a Desser 2000, Holzer et al. 2004, 2006a, Eszterbauer 2004, Eszterbauer and Székely 2004, Fiala 2006).

The type of the host (fish, amphibian, reptile, bird and mammal) does not seem to be congruent with the myxosporean phylogeny. For example, myxosporeans infecting the turtles can be found among the fish-inhabiting species and only the turtle parasites *Myxidium chelonarum* and *M. hardella* cluster together. The frog parasites, *Chloromyxum careni* and

Myxidium melleni, are found at the different positions in the SSU-based phylogenetic tree. Myxosporeans infecting the homoeothermic animals (water birds and shrews), *Myxidium anatidum* and *Soricimyxum fegati*, do not group together. However, their relationships within the clade, to which these myxosporeans belong, are weakly supported. One can be more confident about the correlation between the type of host and clustering of myxosporeans, if more myxozoan parasites of amphibians, reptiles, birds and mammals are sequenced. Recent phylogenetic trees show, that all of the myxosporeans rather group according to their site specificity than to the type of host (Fig. 31 in paper IV).

Despite many evolutionary trends in myxosporeans have been revealed by the analyses of the SSU rDNA data, certain relationships among myxosporean groups have still not been fully resolved. Under question were e.g. the interrelationships of the main clades within the marine lineage, the position of the sphaerosporid clade to other myxosporeans etc. The insufficient resolution of the single-gene analyses is often ascribed to the limited number of alignable nucleotides (Nei et al. 1998). Moreover, the discrepancies between the myxozoan phylogeny and taxonomy have introduced a question if the phylogeny based on the single SSU rRNA gene corresponds to the real organismal evolution.

Alternative sets of data have been shown to be very helpful to resolve these problems. The LSU rRNA gene has a great potential as a phylogenetic marker for the assessment of phylogenetic relationships of various animal groups. It is especially advantageous in the combined analyses with the SSU rDNA data (Medina et al. 2001, Winchell et al. 2004, Moreira et al. 2007). Unlike some protein-coding genes, the mechanism of concerted evolution in the ribosomal genes eliminates the problems linked with the presence of orthologs (Hillis and Davis 1986). Moreover, the LSU rDNA is much larger than SSU rDNA and contains a greater number of variable regions (Hassouna et al. 1984).

The first twelve myxosporean LSU rDNA data have been published in the phylogenetic study investigating the relationships within multivalvulids. The single SSU/LSU rDNA-based analyses yielded similar results as the combined ones (Whipps et al. 2004b). However, the representatives of only two genera (*Kudoa* and *Unicapsula*) were included as ingroups and only partial LSU rDNA data have been used in these analyses. Similarly, despite the increased number of the new LSU rDNA sequences in the most recet work of Burger and Adlard (2010), only multivalvulid relationships were investigated. The comprehensive phylogenetic study including 35 myxosporean species representing all the main clades has confirmed the same evolutionary trends within myxosporeans based on both complete SSU and complete LSU rDNA data (paper I). Myxosporean species that have been shown to possess extensive

inserts within the SSU rDNA sequences contained significant insertions in their LSU divergent (D) regions (papers I, IV). The most variable LSU parts were D2, D8 and D10 domains (paper I). Similar length differences among the D domains have been observed in cnidarians (Chen et al. 2000, paper I). The relative branch lengths of myxosporean sequences were identical in the SSU- vs. LSU-based trees. The SSU-based evolutionary trends were confirmed by the split of myxosporeans into the freshwater/marine lineages and clustering of myxosporeans within the same clades in the LSU- and SSU-based trees. These findings suggested that SSU-based phylogeny corresponds to the organismal phylogeny (paper I). The only differences were revealed in the clustering of *Chloromyxum cristatum* and *Sphaerospora ranae*. Surprisingly, *S. ranae*, the representative of the sphaerosporid clade, clustered basally to the members of the freshwater lineage in the LSU rDNA based analyses. The informative superiority of the LSU rDNA data has been manifested by an increase in the resolution, nodal supports and tree indexes in single LSU and combined LSU+SSU rDNA analyses. The D5-3´end has been shown as the most informative region of the LSU rRNA gene. Since the first half of the LSU rRNA gene mostly includes ambiguous characters that are usually excluded from the phylogenetic analyses the combination of the complete SSU rDNA data with the most informative D5-3´end LSU part has been recommended as the most effective strategy for inferring phylogenetic relationships within myxosporeans (paper I). However, the variable character of the first half of LSU rDNA can be useful at the population level. For example the variable D2 region of LSU rDNA has been shown to be a useful molecular marker for the discrimination of cryptic species assemblages of *Chloromyxum fluviatile* and *Zschokkella nova* (paper V).

The failure of the ribosomal genes to resolve several branches in the phylogenetic tree or to be subject to LBA may arise from mutational saturation due to multiple substitutions, poorly aligned regions or base frequency heterogeneity among taxa (Hasegawa and Hashimoto 1993, Galtier and Gouy 1995). The long-branch nature of many myxosporean species was observed in the rDNA-based phylogenetic studies (Siddall et al. 1995, Kim et al. 1999, Fiala 2006, paper I). Several appproaches such as the use of model-based methods (ML or BI) instead of MP and additional taxon sampling have been proposed as efficient tools to avoid potential LBA problems and to resolve some nodes (Anderson and Swofford 2004). Generating additional LSU rDNA sequences for the diverse myxosporean species should be a next future step as the number of the LSU rDNA myxosporean sequences is still low compared to SSU rDNA data. The addition of information contained in other genes may also improve the accuracy of the phylogenetic trees (Rokas and Carroll 2005). The protein-coding

genes may be especially advantageous compared to the ribosomal genes since they are considered to be less affected by mutational saturation (Hasegawa and Hashimoto 1993). Moreover, another advantage of the protein-coding genes rests on their non-linked evolution unlike rDNA repeats which undergo homogenization through concerted evolution (Hillis and Davis 1986, Rokas et al. 2003).

Protein-coding genes have widely been used to assess the phylogenetic relationships of myxozoans with the metazoans (Fiala and Bartošová 2007, Jiménez-Guri et al. 2007, Fiala 2008, Cinková 2010) or at the population level (Whipps and Kent 2006). Recently, the protein-coding gene sequences have been exploited to infer the phylogeny within myxosporeans. The attempts to obtain a representative number of the HSP70 myxosporean sequences and subsequently infer myxosporean relationships faced the difficulties with the successful amplification. In the positive case, several different paralogs of this gene from cytoplasm and endoplasmic reticulum were obtained. The low number of obtained cytoplasmic HSP70 paralogs was not sufficient to recontruct the phylogenetic tree. Unlike the cytoplasmic sequences, the analyses of endoplasmic reticulum paralogs have shown similar clustering of myxosporeans as in the rDNA-based trees. However, more HSP70 genes should be obtained to confirm these results (Bartošová and Fiala 2007, appendix III). On the other side, the analyses based on twelve myxosporean EF2 sequences have verified the evolutionary trends within myxosporeans based on rDNA data. Congruence of ribosomal and protein-coding data supported the relevance of SSU rDNA as a useful marker for the assessment of myxosporean relationships (paper II).

The confirmation of the SSU-based phylogeny by other two genes provided the strong support for the statement that the phylogenetic relationships among myxosporeans correspond to the organismal evolution. The even more supported discrepancies between the myxosporean phylogeny and taxonomy introduced a question how did the morphological characters in the Myxozoa evolve. New light has been shed by the mapping of morphological and bionomical character evolution on the SSU-based tree (paper II). The performed analyses have shown that the only synapomorphic character corresponding to both myxozoan taxonomy and phylogeny is the character of polar filament. This structure is flat and zig-zag folded only in the *Sphaeromyxa* species contrary to other myxosporeans with spirally wound polar filament. Other morphological characters mainly relating to the spore characteristics were homoplastic. However, certain morphological characters like the number of shell valves, the relative position of polar capsules to the sutural plane and the position of sporoplasm, the ratio of spore width to thickness and the presence of surface ridges partially corresponded to

the phylogeny. The bionomical characters such as the site of infection and host environment highly correlated with the phylogeny. Tracing the character evolution revealed that the ancestor of all myxozoans inhabited the freshwater environment and infected the excretory system. Then, the evolution led to the ancestor of myxosporeans infecting the gall bladder of marine hosts from which arose the lineage of freshwater myxosporeans (paper II). Such trend in myxosporeans has also been previously proposed by other studies (Shulman 1966, Kent et al. 2001, Holzer et al. 2004). The spore morphology of the ancestor of all Myxozoa was similar to the current species of the genus *Sphaerospora* and this ancestor infected the renal tubules (paper II). This discovery supports not only the previously suggested hypothesis about the ancestral sphaerosporid morphotype (Jirků et al. 2007) but the similar morphology of the myxozoan ancestor with the *Sphaerospora* species clustering in the basal sphaerosporid clade also supports the basal position of this clade to all other myxosporeans. The evolution of the myxozoan ancestor with a sphaerosporid morphotype went to the *Ceratomyxa* morphotype in the marine lineage and to the *Chloromyxum* morphotype in the freshwater lineage and marine *C. leydigi*. Other morphotypes evolved by the changes in the spore shape, position, number of polar capsules etc. (paper II). The sphaerosporid morphotype probably also occurred in the ancestor of the marine urinary clade. This organization has been retained in a group of *Parvicapsula minibicornis* and *Sphaerospora testicularis* that resemble to current sphaerosporids but are different to some extent (paper II, III). *Parvicapsula minibicornis* has an elongated shape of spore compared to the spherical, subspherical or ovoid shape of all sphaerosporids. *Sphaerospora testicularis* is an atypical *Sphaerospora* not only for its phylogenetic position (paper III) but also for its unusual morphology (Sitjà-Bobadilla and Alvarez-Pellitero 1993). Many other sphaerosporids are spread throughout the whole myxosporean phylogeny. This supports the idea that the sphaerosporid morphotype might be a successful spore design arisen by a convergent evolution (Holzer et al. 2004).

2.3. Future steps for the myxozoan taxonomy

The knowledge into the phylogenetic relationships within the Myxozoa and evolution of their morphological and bionomical characters provides the basic information for future taxonomic revisions that are essential to solve the persisting taxonomic and phylogenetic discrepancies. Since only one synapomorphic morphological character has been found, the combination of the bionomical characters with the characters that at least partially correspond to the myxozoan phylogeny could be used for future taxonomic changes (paper II).

The available sequence data on myxosporeans suspect of their mis-identification with other myxosporean species caused by their co-infection in the samples should be re-sequenced (Lom and Dyková 2006, paper III). Taxon sampling of certain genera for which only a small fraction of species has been sequenced (*Sphaerospora*, *Myxobolus*, *Henneguya*, *Myxidium*, *Ceratomyxa*, *Thelohanellus* etc.), should increase (Fig. 5 in paper II). Moreover, the representatives of many genera, for which no molecular data are available (*Polysporoplasma*, *Kentmoseria*, *Triangula*, *Davisia*, *Myxoproteus*, *Fabespora*, *Unicauda*, *Dicauda* etc.), should have their rDNA characterized. Especially, molecular data of the type species of each genus should be available before any taxonomic changes are made (Fiala 2006, Lom and Dyková 2006, Holzer et al. 2010). Up to date, only 15 of a total number of 59 myxozoan type species have been sequenced. The sequence characterization of the type species of the genus *Sphaerospora* and the finding of the unique features within the rDNA sequences of *S. elegans* and its closely related sphaerosporid species has led to the first definition of the myxozoan genus based not only on the morphological but also on the molecular data (Jirků et al. 2007). Recently, many species are known to cluster out of the clades containing the type species of their correspoding genus. Therefore, there is an urgent need for future revision of the existing polyphyletic and paraphyletic genera. A whole evidence approach based on combining biological, morphological (host tissue location, morphology of sporogonic and other developmental stages) and molecular characteristics should be applied for species description (Lom and Dyková 2006, Holzer et al. 2010).

Moreover, other morphological and bionomical characters should be investigated to find more features corresponding to the myxozoan phylogeny. The incongruent results of the cladistic analyses based on the morphological characters of the myxospore and actinospore life cycle stages have shown that the systematics of myxosporeans solely based on one of their stages (myxospore) may result in the incorrect supraspecific level groupings. It was suggested that a more stable and predictive classification of myxozoans should be based on a combined total evidence of both life-cycle stages (Xiao and Desser 2000). Another feature of taxonomic value is the information about the myxozoan development in the host. It has been shown that five distinct sporogonial sequences that are congruent with five myxozoan phylogenetic clades, can be identified for the Myxozoa (Morris and Adams 2008).

It may happen that genera established on small differences like *Myxidium* and *Zschokkella* or *Myxobolus* and *Henneguya* will fuse or be suppressed while some existing genera may be split in the future e.g. the aforementioned demise of the genus *Leptotheca* (Gunter and Adlard 2010).

CHAPTER 3.

RESEARCH PAPERS

3.1. **PAPER I.**

Concatenated SSU and LSU rDNA data confirm the main evolutionary trends within myxosporeans (Myxozoa: Myxosporea) and provide an effective tool for their molecular phylogenetics

Bartošová P., Fiala I., Hypša V.

Molecular Phylogenetics and Evolution (2009) **53**, 81-93

Views on myxosporean phylogeny and systematics have recently undergone substantial changes resulting from analyses of SSU rDNA. Here, we further investigate the evolutionary trends within myxosporean lineages by using 35 new sequences of the LSU rDNA. We show a good agreement between the two rRNA genes and confirm the main phylogenetic split between the freshwater and marine lineages. The informative superiority of the LSU data is shown by an increase of the resolution, nodal supports and tree indexes in the LSU rDNA and combined analyses. We determine the most suitable part of LSU for the myxosporean phylogeny by comparing informative content in various regions of the LSU sequences. Based on this comparison, we propose the D5–30-end part of the LSU rRNA gene as the most informative region which provides in concatenation with the complete SSU a well resolved and robust tree. To allow for simple amplification of the marker, we design specific primer set for this part of LSU rDNA.

2. **PAPER II.**

History of myxozoan character evolution on the basis of rDNA and EF-2 data

Fiala I., **Bartošová P.**

BMC Evolutionary Biology (2010) **10,** 228

Background

Phylogenetic relationships among myxosporeans based on ribosomal DNA data disagree with traditional taxonomic classification: a number of myxosporeans with very similar spore morphology are assigned to the same genera even though they are phylogenetically distantly related. The credibility of rDNA as a suitable marker for Myxozoa is uncertain and needs to be proved. Furthermore, we need to know the history of myxospore evolution to understand the great diversity of modern species.

Results

Phylogenetic analysis of elongation factor 2 supports the ribosomal DNA-based reconstruction of myxozoan evolution. We propose that SSU rDNA is a reliable marker for inferring myxozoan relationships, even though SSU rDNA analysis markedly disagrees with the current taxonomy. The analyses of character evolution of 15 morphological and 5 bionomical characters show the evolution of individual characters and uncover the main evolutionary changes in the myxosporean spore morphology and bionomy. Most bionomical and several morphological characters were found to be congruent with the phylogeny. The summary of character analyses leads to the simulation of myxozoan ancestral morphotypes and their evolution to the current species. As such, the ancestor of all myxozoans appears to have infected the renal tubules of freshwater fish, was sphaerosporid in shape, and had a spore with polar capsules that discharged slightly sideways. After the separation of Malacosporea, the spore of the common myxosporean ancestor then changed to the typical sphaerosporid morphotype. This species inhabited the marine environment as a parasite of the gall bladder of

marine fish and ultimately separated into the three main myxosporean lineages evident today. Two of these lineages re-entered the freshwater environment, one as a myxosporean with

Chloromyxum and another with a primitive sphaerosporid morphotype. The common ancestor of all marine myxosporeans had a ceratomyxid shape of spore.

Conclusions

We support rDNA based myxozoan phylogeny by the analysis of a protein coding gene and demonstrate the reliability of rDNA as a marker explaining myxozoan relationships. Our tracing the history of myxozoan character evolution discloses ancestral morphotypes and shows their development over the course of evolution. We point out several myxozoan characters that are to a certain extent congruent with the phylogeny and determined that the discrepancy between phylogeny and current taxonomy based on spore morphology is due to an extreme myxospore plasticity occurring during myxozoan evolution.

3.3. **PAPER III.**

Phylogenetic position of *Sphaerospora testicularis* and *Latyspora scomberomori* n. gen., n. sp. (Myxozoa) within the marine urinary clade and the evolution of the nature of the sutural line in the marine lineage of Myxosporea

Bartošová P., Freeman M. A., Yokoyama H., Caffara M., Fiala I.

Unpublished

An amendment of the family Sinuolineidae (Myxozoa: Myxosporea) is proposed in order to include a newly described genus *Latyspora* n. gen. The type species *Latyspora scomberomori* n. sp. is coelozoic in the kidney tubules of *Scomberomorus guttatus*. *Latyspora lobosa* n. comb. previously assigned within the genus *Sphaerospora* has been transferred to *Latyspora*. In addition to the molecular characterization of *L. scomberomori*, we also present SSU rDNA data on *Sphaerospora testicularis*, a serious parasite of *Dicentrarchus labrax*. The phylogenetic analyses revealed the clustering of both species within the marine urinary clade. *Sphaerospora testicularis* is the closest relative to *Parvicapsula minibicornis* and *L. scomberomori* is the basal species of the *Zschokkella* subclade. Long inserts within the variable SSU rDNA regions were observed in *L. scomberomori* similarly as in other members of the *Zschokkella* subclade. Tracing the evolution of the spores' sutural line revealed several evolutionary trends for this character within the marine urinary clade. The similarities of *P. minibicornis* with the characteristics of the genus *Sphaerospora* are emphasized. The sequence data provided on *S. testicularis* can help in future revisions of the strongly polyphyletic genus *Sphaerospora*. We recommend re-sequencing of several sphaerosporids as an essential step before such taxonomic changes are accomplished.

3.4. **PAPER IV.**

Another chloromyxid lineage: molecular phylogeny and redescription of *Chloromyxum careni* from amphibian

Jirků M., **Bartošová P.**, Kodádková A., Mutschman F.

Unpublished

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

3.5. **PAPER V.**

Molecular evidence for the existence of cryptic species assemblages of several myxosporeans (Myxozoa)

Bartošová P., Fiala I.

Unpublished

Myxosporeans *Chloromyxum cristatum, C. fluviatile* and *Zschokkella nova* (Myxozoa) are common gall bladder parasites of the cyprinid fishes frequently persisting as co-infections. Despite they are believed to be innocuous endocommensals, *C. cristatum* clearly displays the potential of a serious pathogen since it may pervade fish liver parenchyma and cause its necrosis. Employing the comparison of genetic distances among the myxosporean rDNA sequences and performing phylogenetic analyses we demonstrate that cryptic species assemblages exist in *C. fluviatile* and *Z. nova*. Sequence comparison revealed that *Chloromyxum legeri,* previously assigned as a junior synonym of *C. fluviatile,* is a valid species. The same method is used to display the distinction of *Z. nova* isolates from China and the Czech Republic. We show that *C. cristatum* is not an assemblage of more species and our results support the synonymy of *Chloromyxum cyprini* with *C. cristatum*. We have developed a multiplex PCR as an effective tool for the detection and discrimination of *Z. nova*, *C. cristatum* and *C. fluviatile*. It is especially advantageous for the distinction of the non-mature plasmodia of both *Chloromyxum* species. This method also helped to assess the exact prevalence of these parasites in examined samples and enabled to select single infected host samples for the intended population studies.

SUMMARY OF RESULTS

In this Ph.D. thesis, phylogenetic analyses of the ribosomal and protein-coding gene sequences were performed to study the phylogeny of the Myxozoa at three evolutionary levels. The higher-level phylogenetic relationships of metazoan groups were investigated to clarify the widely discussed position of the Myxozoa within the Metazoa. The study of the lower-level phylogeny within the Myxosporea aimed to confirm the evolutionary trends based on a single gene by other molecular markers in order to find out if the reconstructed relationships correspond to the real organismal phylogeny. This has been a crucial step for future attempts to solve the discrepancies between the myxosporean phylogeny and taxonomy. At last, the relationships of myxosporeans at the population level were assessed to reveal the presumed existence of cryptic species assemblages of several myxosporean species.

Study of the myxozoan relationships among the Metazoa has revealed the existence of conflicting phylogenetic signals between the ribosomal and protein-coding genes. The SSU rDNA dataset of available GenBank myxozoan sequences was enlarged with the complete nuclear LSU rDNA data. The concatenated analyses of rDNA sequences placed Myxozoa as a sister taxon to the Bilateria (appendix I). This supported one of the two most prevailing hypotheses about the position of the Myxozoa within the Metazoa (Siddall and Whiting 1999, Evans et al. 2010). Since these rDNA-based analyses could have possibly been influenced by LBA, we obtained protein-coding gene sequences of EF1, EF2 and α-tubulin and analyzed them as concatenated data. Surprisingly, these analyses showed Myxosporea as an early-branching basal metazoan lineage (appendix II) that disagreed with the results of the rDNA-based phylogenies. Searching for other protein-coding genes was terminated after the subsequent publishing of the phylogenomic study which revealed Myxozoa as members of the Cnidaria (Jimenéz-Guri et al. 2007). Further research on this topic is maintained by my supervisor, Dr. Ivan Fiala, by his attempts to sequence the whole mitochondrial genome of a myxosporean representative to shed more light on the position of the Myxozoa within the Metazoa.

The major part of my Ph.D. thesis was focused on the investigation into the myxosporean phylogenetic relationships using the ribosomal data (paper I). This study is the first to analyze the comprehensive LSU rDNA dataset of the myxosporean species that represent a broad taxonomic sample across the main phylogenetic clades. The reconstruction of the trees is preceded by the elimination of ambiguous regions not only with the frequently used “by eye

exclusion" but also with the help of the specialized program (GBlocks, Castresana 2000). The latter approach creates the final dataset by applying various parameters to eliminate poorly aligned and divergent regions. Moreover, GBlocks´s selection facilitates the reproduction of the alignment preparation and subsequent phylogenetic analyses by other researchers. The analyses of the alignments prepared under the various methods and parameters were almost identical and the differences were only encountered within the few nodes with low supports. The comparison of the results based on our LSU rDNA data with the results of the SSU-based study of Fiala (2006) has revealed similar evolutionary patterns of both genes. This has been demonstrated not only by the identical lengths differences between the sequences of the same myxosporeans and the long-branch nature of sequences of identical species but mainly by the congruent topologies of the SSU- and LSU-based myxosporean trees. Higher informative content within the LSU rRNA gene than within the SSU rRNA one have been shown by the increased resolution, nodal supports and tree indexes in the single LSU-based trees and in the trees inferred from the combined ribosomal data. The comparison of the tree statitistics and tree topologies reconstructed from different parts of the LSU rRNA gene revealed second half (D5-3´end) of this LSU rDNA as the most informative region. Moreover, we recommend the combination of this LSU region with the complete SSU rDNA data as the most effective strategy to obtain a reliable and robust myxosporean phylogeny based on rDNA data. We also develop specific myxosporean LSU primers that facilitate the amplification of the D5-3´end LSU rDNA region. The results of this study support that SSU rDNA is a reliable phylogenetic marker for reconstructing myxosporean relationships and the polyphyletic and paraphyletic relationships revealed by ribosomal data most probably correspond to the true species evolution.

Further aim to support rDNA-based phylogenies by the protein-coding data with an independent evolution of the ribosomal genes has been reached up by the amplification of the HSP70 and EF2 data. Despite significant difficulties with obtaining of the sequences of both genes, we amplified seven HSP70 and twelve EF2 sequences. The phylogenetic analyses have revealed that different paralogs of HSP70 gene have been amplified (endoplasmic reticulum type in four myxosporean species and cytosolic type in three species). The MP analysis of available endoplasmic reticulum HSP70 paralogs have shown that the relationships among the myxosporeans correspond to the results of the rDNA-based phylogenies (appendix III). This suggests that HSP70 gene may be a useful molecular marker for the reconstruction of myxosporean relationships. However, before this can be fully assessed more HSP70 sequences of other myxosporean species should be obtained (appendix III).

The phylogenetic analyses of the significant number of myxosporean sequences of the second protein-coding gene (EF2) confirmed evolutionary trends within myxosporeans revealed by the ribosomal data. These findings definitely supported the statement that SSU-based phylogeny corresponds to the organismal phylogeny (paper II).

These results provided a stable ground for the subsequent tracing of evolution of the myxozoan morphological and bionomical characters on the SSU-based tree. The aim was to search for any synapomorphies of these characters following the myxozoan phylogenetic relationships. The finding of only one such morphological feature reflects the existence of a huge disagreement between the myxozoan taxonomy and phylogeny. This discrepancy is explained by an extreme myxospore plasticity occurring during the evolution of the Myxozoa. Certain bionomical characters congruent with the phylogenetic relationships of myxosporeans were found, providing additional source of data for future taxonomic revisions within the phylum Myxozoa that are desirable to resolve existing discrepancies. This may also be facilitated by the knowledge into the evolution of myxozoan morphotypes from their ancestral morphologies that has also been revealed in scope of paper II.

Other papers (III, IV) were focused on the phylogenetic and morphological studies of several myxosporeans. Phylogenetic positions of *Sphaerospora testicularis* and *Latyspora scomberomori* are assessed in paper III. The phylogenetic placement of *L. scomberomori* along with its unique spore morphology impelled us to establish a new myxosporean genus *Latyspora*. The comparison of *L. scomberomori* with its morphologically similar species has shown that *Sphaerospora lobosa* corresponds to the definition of the newly described genus. Therefore, this species was transferred to the genus *Latyspora* as *L. lobosa* n. comb. Polyphyletic nature of the genus *Sphaerospora* was strengthened by the clustering of *S. testicularis*, a serious parasite of sea bass testes, out of the basal sphaerosporid clade that encompasses the type species *S. elegans*. Taxonomic remarks on this genus are provided within this paper along with critical comments on the correctness of certain sphaerosporid sequences available in the GenBank. We also highlighted that *S. testicularis* and *Parvicapsula minibicornis* are not typical species of their genera due to their unusual morphological and sequence characteristics.

The assessment of the phylogenetic position of the frog-infecting myxosporean, *Chloromyxum careni*, revealed that it neither clusters with other myxosporeans parasitizing amphibians nor with the type species of the genus *Chloromyxum* (*C. leydigi*) and other chloromyxids. It represents a distinct basal lineage of the group consisting of the *Myxobolus* and *Myxidium lieberkuehni* clades and contains extremely long inserts within the variable

regions of its SSU and LSU rDNA sequences. Besides its sequence characterization, we present detail morphological and biological data on *C. careni* gathered with the use of light microscopy, histology, SEM and TEM. Moreover, we provide some taxonomic comments on the genus *Chloromyxum* (paper IV).

We also used the SSU and LSU rRNA genes to study the different populations of three gall bladder parasites *Chloromyxum cristatum*, *C. fluviatile* and *Zschokkella nova* in order to investigate their presumed cryptic nature. Employing the comparison of genetic distances among the myxosporean rDNA sequences and performing phylogenetic analyses we demonstrate that cryptic species assemblages exist in *C. fluviatile* and *Z. nova*. On the other side, the application of the same methods on *C. cristatum* showed that this myxosporean is not an assemblage of more species and supported the synonymy of *Chloromyxum cyprini* with *C. cristatum*. We have developed a multiplex PCR to discriminate *Z. nova*, *C. cristatum* and *C. fluviatile*. It is especially advantageous for the distinction of the non-mature plasmodia of both *Chloromyxum* species. This method also helped to assess the exact prevalence of these parasites in examined samples and enabled to select single infected host samples for the intended population studies (paper V).

ZHRNUTIE (In Slovak)

V predkladanej Ph.D. práci je študovaná fylogenéza kmeňa Myxozoa na troch evolučných úrovniach prevedením fylogenetických analýz ribozomálnych a proteín-kódujúch sekvencií. Sú preskúmané fylogenetické vzťahy na vysokej evolučnej úrovni medzi jednotlivými metazoárnymi skupinami za účelom odhaliť pozíciu kmeňa Myxozoa v rámci Metazoa, ktorá je v súčasnosti široko diskutovaná. Cieľom štúdia fylogenézy na nižšej úrovni (v rámci triedy Myxosporea) bolo potvrdiť evolučné trendy založené na jednom géne pomocou iných molekulárnych markerov. Tieto výsledky pomohli odhaliť skutočnosť či zrekonštruované fylogenetické vzťahy zodpovedajú organizmálnej fylogenéze. Uvedený krok bol kľúčový pre budúce pokusy o vyriešenie nesúladu medzi fylogenézou a taxonómiou myxosporeí. Nakoniec boli preskúmané vzťahy myxosporeí na populačnej úrovni, ktoré smerovali k odhaleniu predpokladanej existencie kryptických druhov u vybraných druhov myxosporeí.

Štúdiom vzťahov myxozoí a metazoí bola odhalená existencia konfliktných fylogenetických signálov medzi ribozomálnymi a proteín-kódujúcimi génmi. SSU rDNA dataset pozostávajúci zo sekvencií myxozoí dostupných v Génovej Banke bol rozšírený o kompletné LSU rDNA data. Myxozoa sa v konkatenovaných analýzach rDNA dat umiestnili ako sesterská skupina bilatérií (príloha I), čo podporilo jednu z dvoch najviac prevažujúcich hypotéz týkajúcich sa pozície Myxozoa v rámci Metazoa (Siddall a Whiting 1999, Evans a kol. 2010). Kvôli podozreniu z možného vplyvu LBA na spomínané analýzy boli získané sekvencie proteín-kódujúcich génov (EF1, EF2, α-tubulin), ktoré boli zanalyzované v kombinovanej matici. Fylogenetické analýzy prekvapivo ukázali, že Myxozoa tvoria včasne sa vetviacu bazálnu skupinu metazoí (príloha II), čo je v protiklade s výsledkami fylogenéz založených na rDNA sekvenciach. Hľadanie iných proteín-kódujúcich génov bolo ukončené po následnom publikovaní fylogenomickej štúdie, pri ktorej bolo odhalené, že Myxozoa sú členmi pŕhlivcov (Jimenéz-Guri a kol. 2007). Môj školiteľ, Dr. Ivan Fiala, pokračuje vo výskume zameranom na odhalenie pozície myxozoí v rámci metazoí, pri ktorom sa pokúša získať kompletný mitochondriálny genóm zástupcu myxosporeí.

Väčšina mojej Ph.D. práce bola zameraná na výskum fylogenetichých vzťahov myxosporeí pomocou ribozomálnych dat (článok I). Ide o prvú štúdiu analyzujúcu komplexný LSU rDNA dataset zahrňujúci myxosporeové druhy reprezentujúce širokú taxonomickú vzorku naprieč hlavnými fylogenetickými skupinami myxosporeí. Rekonštrukcii fylogenetických stromov predchádzala eliminácia problematických úsekov z alignmentu

nielen pomocou často používaného „vylúčenia od oka“ ale aj s využitím špeciálneho programu (Gblocks, Castresana 2000). Neskoršie zmienená metóda aplikuje pri vytváraní konečného alignmentu rôzne parametre za účelom vylúčenia ťažko alignovateľných a variabilných pozícií. Okrem toho uľahčuje reprodukovateľnosť tvorby alignmentov a nasledných fylogenetických analýz ostatnými výskumníkmi. Výsledky analýz alignmentov pripravených pomocou rôznych metód a parametrov boli veľmi podobné a rozdiely v topológii stromov sa prejavili iba v niektorých, slabo podporených uzloch. Porovnania výsledkov analýz založených na našich LSU rDNA datach s výsledkami štúdie Fialy (2006) vychádzajúcej z analýz SSU rDNA dat odhalili podobný trend v evolúcii oboch génov.

Toto tvrdenie je postavené nielen na rozdieloch v dĺžkach SSU a LSU sekvencií u rovnakých druhoch myxosporeí a long-branch povahe pozorovanej u sekvencií identických druhov, ale hlavne na zhode v topológiach fylogenetických stromov založených na SSU a LSU datach. Na základe vyššieho rozlíšenia, vyššiej podpory uzlov a lepších indexov fylogenetických stromov vychádzajúcich zo samostatných rDNA aj kombinovaných ribozomálnych datach bolo preukázané, že obsah informácie v LSU rRNA géne je vyšší než v SSU rRNA géne. Porovnania štatistík a topológií stromov zrekonštruovaných na základe rôznych úsekov LSU rRNA génu odhalili, že najviac informatívna je jeho druhá polovica (D5-3´koniec). Okrem toho kombinácia tohto LSU úseku s kompletnými SSU rDNA datami bola odporučená ako najefektívnejšia stratégia, pri ktorej je možné získať spoľahlivú a stabilnú fylogenézu myxosporeí. Vyvinuli sme tiež špecifické myxosporeové LSU primery, ktoré uľahčujú amplifikáciu „D5-3´koniec“ LSU rDNA úseku. Výsledky tejto štúdie potvrdili, že SSU rDNA je vhodný fylogenetický marker pre rekonštrukciu vzťahov myxosporeí, a že polyfyletické a parafyletické vzťahy odhalené pomocou ribozomálnych dat veľmi pravdepodobne zodpovedajú evolúcii skutočných myxosporeových druhov.

Ďalší cieľ, ktorým bolo podporiť fylogenézu myxosporeí založenú na rDNA sekvenciach pomocou proteín-kódujúcich dat s nezávislou evolúciou od ribozomálnych génov, bol dosiahnutý úspešnou amplifikáciou HSP70 a EF2 dat. Napriek značným problémom so získavaním sekvencií oboch génov sa nám podarilo naamplifikovať sedem HSP70 a dvanásť EF2 sekvencií. Fylogenetické analýzy odhalili, že došlo k amplifikácii rozličných paralógov HSP70 génu (paralóg z endoplazmatického retikula u štyroch druhov myxosporeí a cytosolický paralóg u troch druhov). MP analýza prevedená na základe novo získaných HSP70 paralógov pochádzajúcich z endoplazmatického retikula a sekvencií dostupných v Génovej Banke ukázala, že zrekonštruované vzťahy medzi druhmi myxosporeí zodpovedajú topológii stromov odvodených z rDNA dat (príloha III). To naznačuje, že HSP70 gén by

mohol byť vhodný molekulárny marker použiteľný pre rekonštrukciu fylogenetických vzťahov myxosporeí. Pre potvrdenie tejto hypotézy je však potrebné získať oveľa viac HSP70 sekvencií od iných druhov myxosporeí.

Fylogenetické analýzy myxosporeových sekvencií druhého proteín-kódujúceho génu, (EF2) potvrdili evolučné trendy v rámci myxosporeí odhalené pomocou ribozomálnych dat. Tieto výsledky definitívne podporili tvrdenie, že fylogenéza založená na SSU rDNA datach zodpovedá organizmálnej evolúcii (článok II).

Získané data poskytli stabilný základ pre nasledovné mapovanie evolúcie morfologických a bionomomických znakov myxozoí na strom založený na SSU datach. Cieľom bolo nájsť také synapomorfie znakov, ktoré by zodpovedali fylogenetickým vzťahom myxosporeí. Iba jeden nález takéhoto morfologického znaku odráža, že medzi taxónomiou a fylogenézou myxozoí je veľký nesúlad. Ten vysvetľujeme extrémnou plasticitou objavujúcou sa počas evolúcie myxozoí. Okrem toho nález niektorých bionomických znakov zodpovedajúcich fylogenetickým vzťahom myxosporeí poskytuje ďalší zdroj dat pre budúce taxonomické revízie kmeňa Myxozoa, ktoré sú nevyhnutné pre vyriešenie existujúcich rozporov. Tento cieľ môžu uľahčiť poznatky o evolúcii morfotypov myxozoí z morfológií ich predkov, čo bolo tiež predmetom výskumu v článku II.

Ďalšie články (III, IV) boli zamerané na fylogenetické a morfologické štúdium určitých druhov myxosporeí. V článku III je ozrejmená fylogenetická pozícia myxosporeí *Sphaerospora testicularis* a *Latyspora scomberomori*. Poznatky o fylogenetickej pozícii *L. scomberomori* spolu s unikátnou morfológiou jej spór nás priviedli k založeniu nového myxosporeového rodu *Latyspora*. Porovnaním morfologických charakteristík *L. scomberomori* s morfologicky príbuznými druhmi sme zistili, že *Sphaerospora lobosa* zodpovedá definícii novo opísaného rodu. Z tohto dôvodu bola *S. lobosa* prevedená do rodu *Latyspora* ako *L. lobosa* n. comb. Polyfyletická povaha rodu *Sphaerospora* bola dokonca posilnená odhalením pozície *S. testicularis* (závažného parazita semenníkov ryby *Dicentrachus labrax*) mimo skupinu bazálnych sphaerosporidov zahŕňajúcu typový druh, *S. elegans*. V článku III naviac upozorňujeme na nesprávnosť niektorých sekvencií sphaerospor uvedených v Génovej Banke. Okrem toho poukazujeme na to, že *S. testicularis* a *Parvicapsula minibicornis* nie sú typickými zástupcami svojich rodov kvôli ich nezvyčajným morfologickým a molekulárnych charakteristikám.

Štúdia zameraná na určenie fylogenetickej pozície myxosporeového druhu *Chloromyxum careni* infikujúceho obojživelníky odhalila, že tento druh sa v prevedených analýzach nezoskupuje ani s ďalšími parazitmi obojživelníkov ani s typovým druhom rodu

Chloromyxum (*C. leydigi*) ani inými druhmi tohto rodu. *Chloromyxum careni* reprezentuje samostatnú líniu s bazálnou pozíciou k skupine tvorenej *Myxobolus* a *Myxidium lieberkuehni* kladmi a obsahuje extrémne dlhé inzerty vo variabilných úsekoch svojich SSU a LSU rDNA sekvencií. Spolu so sekvenčnou charackteristikou v článku poskytujeme aj morfologické a biologické data o *C. careni* získané pomocou svetelnej mikroskopie, histologie, SEM a TEM. Okrem toho uvádzame taxonomické poznámky týkajúce sa rodu *Chloromyxum* (článok IV).

SSU a LSU rRNA gény boli naviac použité k štúdiu rozličných populácií troch parazitov žlčového mechúra rýb, *Chloromyxum cristatum*, *C. fluviatile* a *Zschokkella nova* s cieľom preskúmať ich predpokladanú kryptickú povahu. Pomocou porovnania genetických vzdialeností medzi rDNA sekvenciami myxosporeí a prevedením fylogenetických analýz sme odhalili existenciu kryptických druhov u *C. fluviatile* a *Z. nova*. S využitím rovnakých metód naopak ukazujeme, že *C. cristatum* nie je zmesou viacerých druhov a podporujeme synonýmiu *Chloromyxum cyprini* s *C. cristatum*. Pre rozlíšenie *Z. nova*, *C. cristatum* a *C. fluviatile* je vyvinutá multiplex PCR, ktorej hlavná výhoda spočíva v rozlíšení nezrelých plazmódií oboch druhov rodu *Chloromyxum*, nerozlíšiteľných pomocou svetelnej mikroskopie. Táto metóda okrem toho pomohla určiť presnú prevalenciu *Z. nova*, *C. cristatum* a *C. fluviatile* vo vyšetrených vzorkách a umožnila vytriedenie hostiteľských vzoriek infikovaných iba jedným druhom pre zamýšľané populačné štúdie (článok V).

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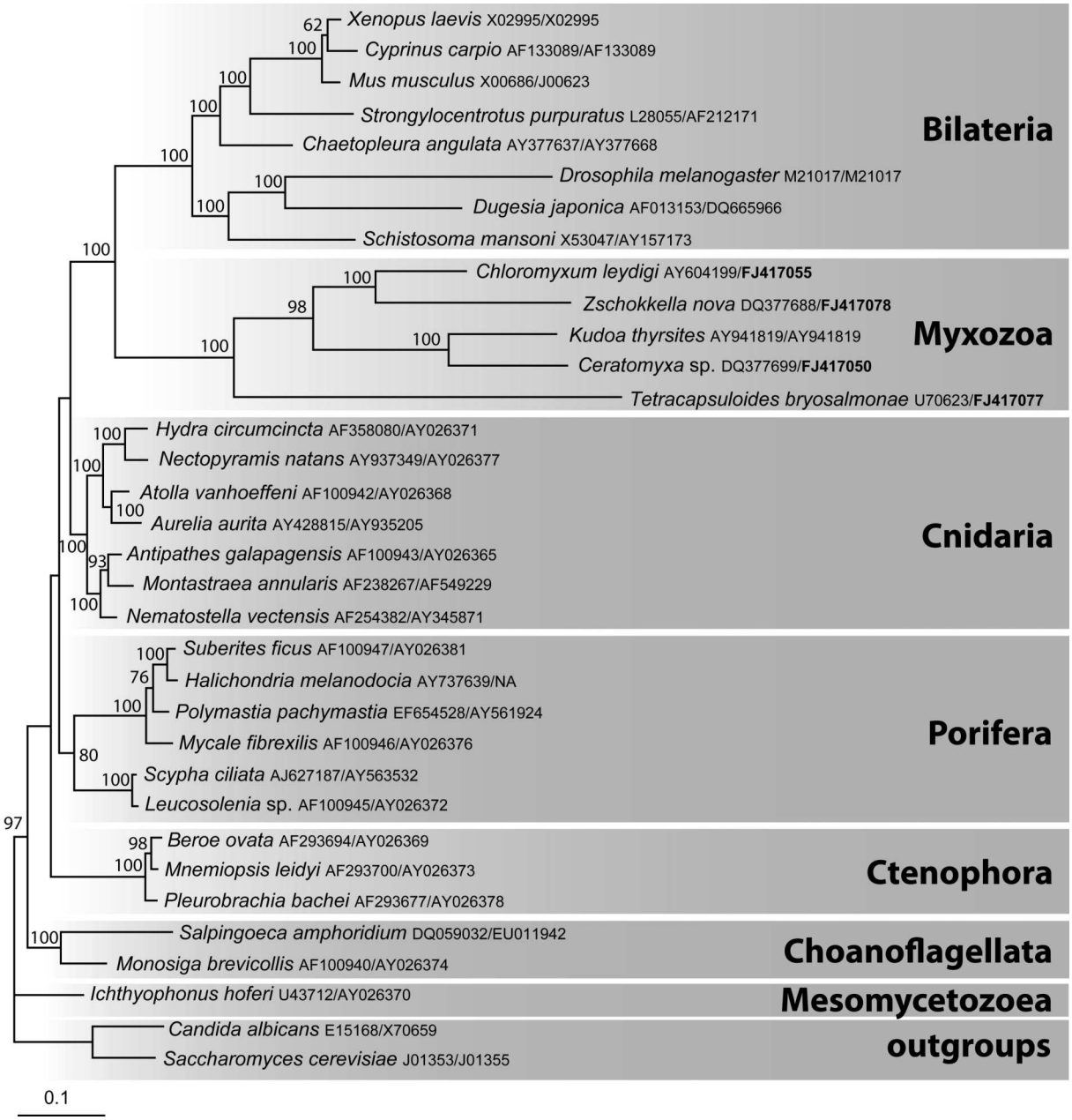
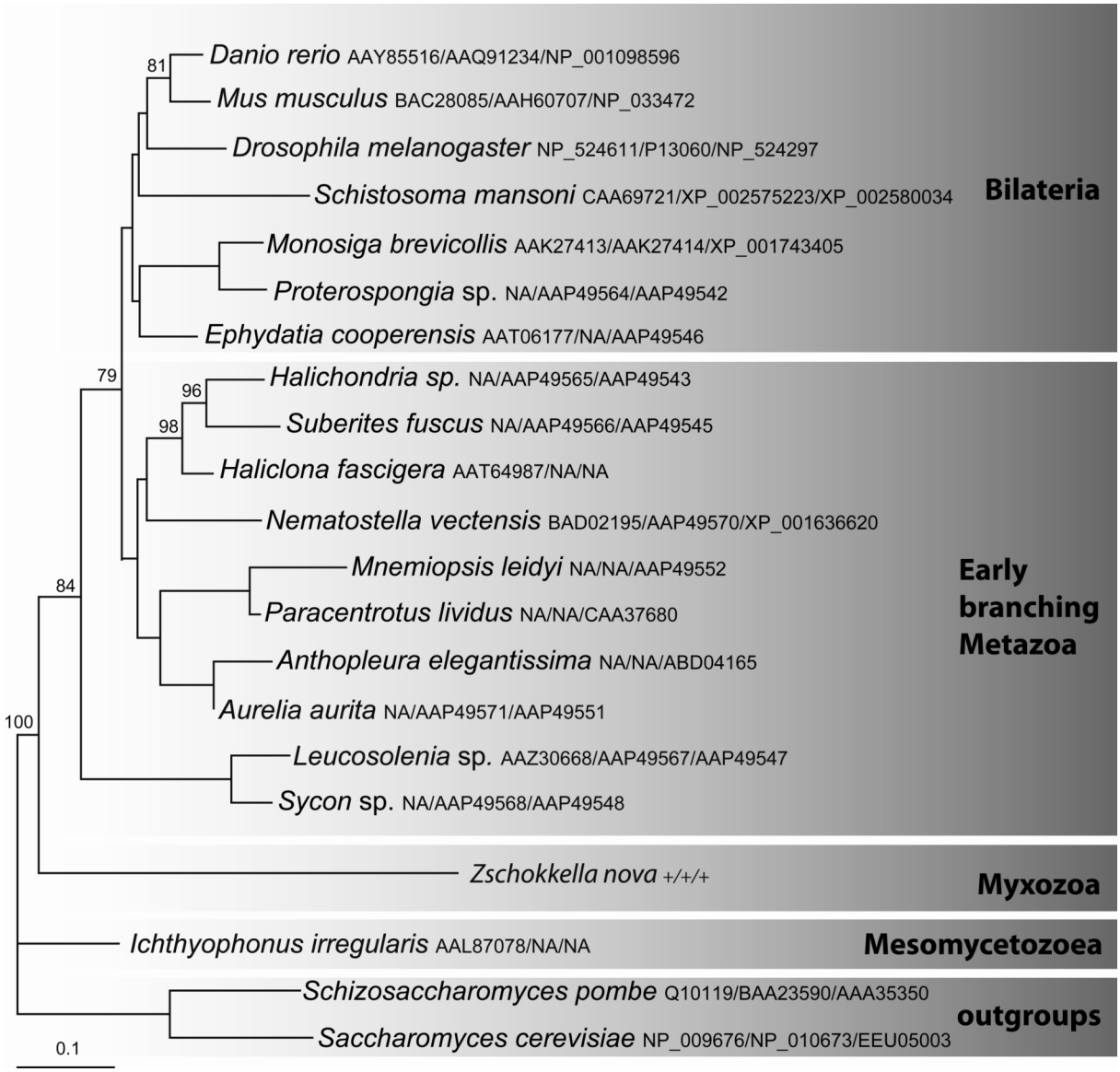
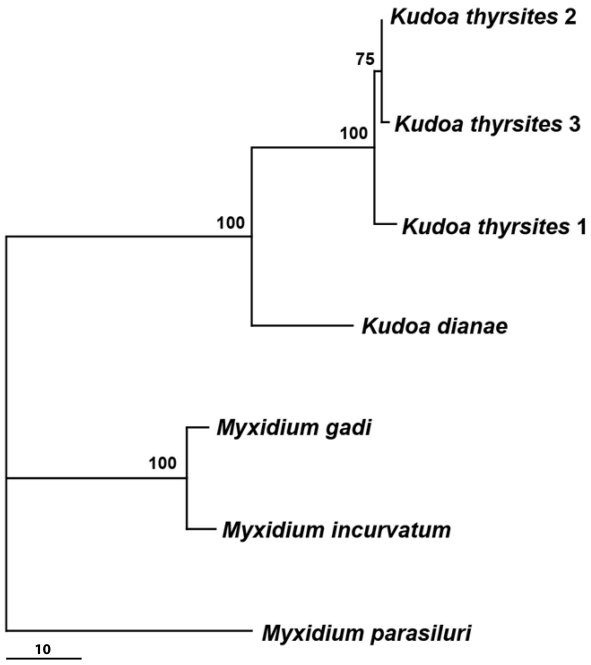
APPENDIX



Appendix I. The maximum likelihood tree based on the combined SSU + LSU rDNA data (5000 nt positions) showing the phylogenetic position of the Myxozoa within the Metazoa. The numbers at the nodes indicate the maximum likelihood bootstrap supports (500 replicates). Bootstrap percentages with less than 50% are not shown. GenBank SSU/LSU accession numbers are provided for each taxon. The new accession numbers are shown in bold. NA: sequence not available in the GenBank. Scale bar is given under the tree (substitution/site).



Appendix II. The maximum likelihood tree based on the combined EF1 + EF2 + α-tubulin data (955 AA positions) showing the phylogenetic position of the Myxozoa within the Metazoa. The numbers at the nodes indicate the maximum likelihood bootstrap supports with 500 replicates. Bootstrap percentages with less than 50% are not shown. Scale bar is given under the tree (substitution/site). GenBank EF1/EF2/α-tubulin accession numbers are provided for each taxon. +: the newly obtained sequences. NA: sequence not available in the GenBank. Scale bar is given under the tree (substitution/site).



Appendix III. The maximum parsimony tree based on the amino-acid sequences of the myxosporean HSP70 endoplasmic reticulum paralogs. The HSP70 sequences of *Kudoa thyrsites* were retrevied from the GenBank under the following accession numbers: AY924198 for *K*. *thyrsites* 1, AY92419 for *K*. *thyristes* 2 and AY924200 for *K*. *thyrsites* 3. Numbers at nodes represent bootstrap values for maximum parsimony (1000 replicates). Scale bar is given under the tree (substitution/site).