University of South Bohemia in České Budějovice

Faculty of Science

BIOLOGY, LIFE CYCLE AND PHYLOGENY OF MALACOSPOREANS IN FISH AND BRYOZOANS

Master thesis

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ANNOTATION

Malacosporeans (Myxozoa) comprising only three nominal species, cycle between vertebrate (fish) and invertebrate (bryozoans) host in freshwater aquatic ecosystems. This thesis is focused on *in vitro* cultivation of bryozoans, using algal cultures in order to investigate malacosporean life cycles via transmission experiments. Moreover, the biodiversity, prevalence, distribution, habitat/host preference and phylogenetic trends of malacosporeans in freshwater fish hosts are scrutinized using light microscopy and molecular methods. The potential existence of malacosporeans in marine bryozoans is also investigated.

DECLARATION [in Czech]

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

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Martina Hrabcová

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1. INTRODUCTION

1.1. Myxozoa

The Myxozoa Grassé, 1970 are a group of microscopic metazoan parasites (Canning and Okamura 2004), belonging to the phylum Cnidaria Hatschek, 1888. The Myxozoa consist of more than 2300 nominal species (Morris 2010). Most of them alternate between fish and invertebrate hosts, mostly annelids (oligochaetes and polychaetes) and bryozoans (summarized in Lom and Dyková 2006). Myxozoans have also been rarely detected in flatworms (Freeman and Shinn 2011), reptiles (Eiras 2005), amphibians (e.g. Hartigan *et al.* 2012), birds (Bartholomew *et al.* 2008) and mammals (Friedrich *et al.* 2000, Prunescu *et al.* 2007). Understanding of the biology of myxozoans is of big economic importance since infections caused by several representatives result in significant diseases and mortality of farmed fish.

Myxozoans are characterized by multicellular spores, which typically contain highly complex organelles called polar capsules. Their function is the attachment of the infective spore to the host (Canning and Okamura 2004). Life cycles are resolved only for some 50 species (summarized in Székely *et al.* 2014) and include two phases, *i.e.* myxospore and actinospore phase. They involve two types of spores, myxospores and actinospores, developing in the vertebrate and invertebrate host. Vertebrate hosts are intermediate hosts of myxozoans whereas invertebrates are final hosts, as sexual reproduction occurs. The myxospore phase always takes place in a fish (or another vertebrate host) while the actinospore phase takes place in an annelid or a bryozoan (Grabner and El-Matbouli 2010).

The Myxozoa were grouped with protistan taxa until the early 1990s. Nevertheless, more than a century ago, it was suggested that myxozoans were metazoans (Štolc 1899). This hypothesis was later confirmed by Weill (1938), who claimed, that myxozoans are close relatives of cnidarians. Due to the remarkable similarities of myxozoans to some parasitic cnidarians he proposed an affinity to narcomedusans. Some other authors also concluded that the Myxozoa are very similar to Cnidaria, based on their ultrastructure, particularly on the similarity of polar capsules and nematocysts (Lom and Dyková 1997). This relationship was later confirmed by using a combination of morphological and molecular data – the small subunit ribosomal DNA (SSU rDNA). It has been shown that Myxozoa is not a sister clade of Cnidaria but it is rather nested within the Cnidaria (Siddall and Whiting 1999). However, some molecular studies proposed myxozoan affinities with bilaterians (Smothers *et al.* 1994,

Hanelt *et al.* 1996). The phylogenetic position of the Myxozoa within the Cnidaria has been confirmed by phylogenomic analyses based on protein coding genes of *Myxobolus cerebralis* Hofer, 1903 (Nesnidal *et al.* 2013) and *Buddenbrockia plumatellae* Schröder, 1910 (Jiménez-Guri *et al.* 2007b) as well as by the presence of nematogalactin genes, which are exclusive to cnidarians (Evans *et al.* 2010). However, the exact origin of myxozoans within the Cnidaria remains unresolved.

The phylum Myxozoa is divided into two classes, the Myxosporea Bütschli, 1881 including most of the described genera alternating between vertebrates and annelids, and the Malacosporea Canning, Curry, Feist, Longshaw et Okamura, 2000 containing the genera *Buddenbrockia* and *Tetracapsuloides*, parasitizing fish and bryozoans (Canning and Okamura 2004).

1.2. Malacosporea

In contrast to the large class Myxosporea, little information exists about the early development, life cycles and species diversity of the Malacosporea. Only 3 nominal species have been described so far but the existence of further species is expected, considering new SSU rDNA sequence data and new spore morphologies (Morris *et al*. 2002, Tops *et al.* 2005, McGurk *et al.* 2006a, Jiménez-Guri *et al.* 2007b, Hartikainen *et al.* 2014). In contrast to the Myxosporea, which use annelids as definitive hosts, Malacosporea parasitize freshwater bryozoans (Phylactolaemata) in which they form worm-shaped or sac-like parasites containing infectious malacospores (Canning *et al.* 2002). These infect fish, where fish malacospores are produced. In the Myxosporea, a two-host life cycle seems to be obligatory with very few exceptions (Diamant *et al.* 1994, Redondo *et al.* 2004). In the case of the Malacosporea, different life-cycle strategies probably exist. They may not always include both, fish and invertebrate hosts as some studies suggested horizontal transfer of the parasites between zooids of bryozoan colonies and vertical transfer via statoblasts, dormant stages of bryozoans (Tops *et al.* 2004, Hill and Okamura 2007, Abd-Elfattah *et al.* 2014a). The vertebrate host is known only for *T. bryosalmonae* (Feist *et al.* 2001, Morris and Adams 2006), *B. plumatellae* and *Buddenbrockia* sp. (Grabner and El-Matbouli 2009). *Buddenbrockia plumatellae* was the first described malacosporean species (Schröder 1910) and parasitizes different freshwater bryozoan species, e.g. *Hyallinella punctata*, *Lophopodella carterii*, *Plumatella fungosa*, *Plumatella repens*, *Stollela evelinae*, *Cristatella mucedo*. The economically most important malacosporean is *T. bryosalmonae* (Canning *et al.*

2002), the causative agent of the proliferative kidney disease (PKD) in salmonid fish (Anderson *et al.* 1999a,b; Canning *et al.* 1999, Feist *et al.* 2001). The most recently described, third nominal species is *Buddenbrockia allmani* (Canning *et al.* 2007).

Buddenbrockia plumatellae and *T. bryosalmonae* differ morphologically (shape and size of the sacs) and also on the basis of their DNA sequence (about 20% sequence difference in the SSU rDNA) (Canning *et al.* 2007). Additional to sac-like stages, which have been detected only in *Cristatella mucedo* (Okamura 1996, Canning *et al.* 2002), *B. plumatellae* can develop a vermiform stage in the bryozoan hosts. In contrast to *B. plumatellae*, *T. bryosalmonae* has no vermiform stages. Despite there is a report of *T. bryosalmonae* myxoworm (malacosporean vermiform stage sensu Canning *et al.* 2008) in the bryozoan host (Taticchi *et al.* 2004), convincing data are lacking. Considering potential cryptic speciation, typical for many endoparasites, worm-like stage may have been lost or gained repeatedly during the evolution, as the parasites were forced to evolve new life strategies (Hartikainen *et al.* 2014).

1.2.1. Malacosporean history: The discovery of *Buddenbrockia*

Buddenbrockia is a malacosporean "worm" which was firstly observed in 1850 by Dumortier and van Beneden who found the intensively moving parasitic "worms" inside the body cavity of freshwater bryozoan colonies of *Plumatella fungosa*. Later, this animal was described and named as *B. plumatellae* Schröder, 1910 (Figure 1).

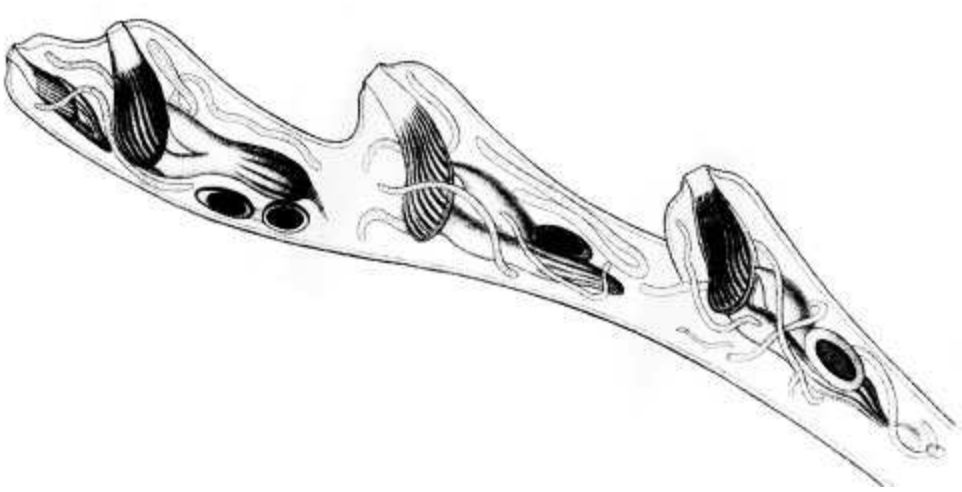

Fig. 1. *Buddenbrockia plumatellae* myxoworms in a colony of *Plumatella* sp. Drawning from the original species description (Schröder 1910).

Buddenbrockia was considered an enigmatic organism for a long time due to its questionable taxonomic affinities to other metazoan phyla. Schröder (1910) suggested that *Buddenbrockia* was a mesozoan. Later it was supposed to be a nematode or a trematode sporocyst (Braem 1911, Schröder 1912). Finally, 13 years ago, according to the ultrastructural studies (Okamura *et al.* 2002) and molecular analysis based on SSU rDNA it was finally concluded that *Buddenbrockia* is a myxozoan (Monteiro *et al.* 2002). The surprising aspect of this discovery was the complex morphology of this myxoworm with its differentiation into tissue layers, in contrast to the strongly reduced and simplified myxosporean plasmodia which are simple spore sacs that lack motility and tissue differentiation (Canning *et al.* 2002). Later, phylogenetic analysis of *Buddenbrockia* has shown that this myxozoan clusters within cnidarians as a sister branch to the Medusozoa (Jiménez-Guri *et al.* 2007a). The relationship to cnidarians was also confirmed by morphology as *Buddenbrockia* has a radial symmetry (Monteiro *et al.* 2002, Okamura and Canning 2003).

1.2.2. Malacosporean development and structure

First ultrastructural analysis discovered that the *Buddenbrockia* worm-like stage consists of an outer and inner epithelial tissue layer. Between them, four longitudinal muscle blocks composed of muscle cells are positioned (Okamura *et al.* 2002). Sac-like stages, e.g. those of *T. bryosalmonae,* are composed of an outer and inner epithelial tissue layer only. It has been proven molecularly that *B. plumatellae* forms both, worm- and sac-like stages in the bryozoans (Tops *et al.* 2005). These sac-like stages were previously named *Tetracapsula bryozoides* (Canning *et al.* 1999). However, based on later ultrastructural and molecular studies some authors have suggested that *B. plumatellae* and *T. bryozoides* are stages in the life cycle of the same organism (Monteiro *et al.* 2002, Canning *et al.* 2002). *Tetracapsula bryozoides* was therefore synonymized with the firstly described *B. plumatellae*. Other species, such as *B. allmani* (Figure 2) or *T. bryosalmonae* form only sac-like stages and were never reported to develop a motile, highly-differentiated myxoworm.

Fig. 2 Infected colony of *Lophopus crystallinus* with spherical sacs of *Buddenbrockia allmani* (Hill and Okamura 2007, Canning *et al.* 2007).

The development of *Buddenbrockia* can be divided into pre-sac stages, sac formation, myxoworm formation, followed by muscle and sporogonic cell differentiation. Noticeable features in development of malacosporeans are sporoplasmosomes present in the primary cells of the cell-in-cell stages and in the sporoplasms of malacospores (Schröder 1912, Morris and Adams 2007, Canning *et al.* 2008). Sac formation is accompanied by the permanent association of the cells with an external layer of mural cells. The coherence of the wall is provided by true cell junctions further developed into junctional complexes (Canning *et al.* 2002, Canning *et al.* 2008). The cellular wall surrounding the inner cells, is produced by the increasing number of mural cells. The elongation process during worm formation is dependent on the presence of longitudinal muscles, so the differentiation of muscle cell precursors controls the process of elongation. In the youngest worm stages, initial elongation is observed and the mural cells surround a core of undifferentiated cells. Finally, enlargement of the sacs and increased number of mural and inner cells result in differentiation of the inner cells into muscle primordia and sporogonic cells (Canning *et al.* 2008).

When mature, the myxoworm is filled with typical multicellular malacospores, which are composed of 8 valve cells, 4 polar capsules and 2 infectious sporoplasms, each consisting of a primary cell enveloping a secondary cell (McGurk *et al.* 2005a, Morris and Adams 2007, Morris and Adams 2008; Figure 3). In *Buddenbrockia* worms, polar capsules are found not only in infective spores but also in the epidermis of the worm. Malacospores seem to provide

some diagnostic features as they differ in size and may be ornamental (Canning *et al.* 2002, Gruhl and Okamura 2012, Morris *et al.* 2007), however, differentiation of sac- or worm-like stages for taxonomic reasons is difficult as their size differs according to stage of maturity. This may be a reason why only 3 nominal species exist to date.

In malacospores produced in the fish host, species identification is even more difficult as spores are extremely cryptic. Often, only polar capsules are detected and the shape of the soft-walled spores is difficult to estimate, especially in kidney squashes of infected hosts. Transmission electron microscopy was able to show that fish malacospores are composed of 4 valve cells, 2 polar capsules and 1 sporoplasm without a secondary cell (Morris and Adams 2008).

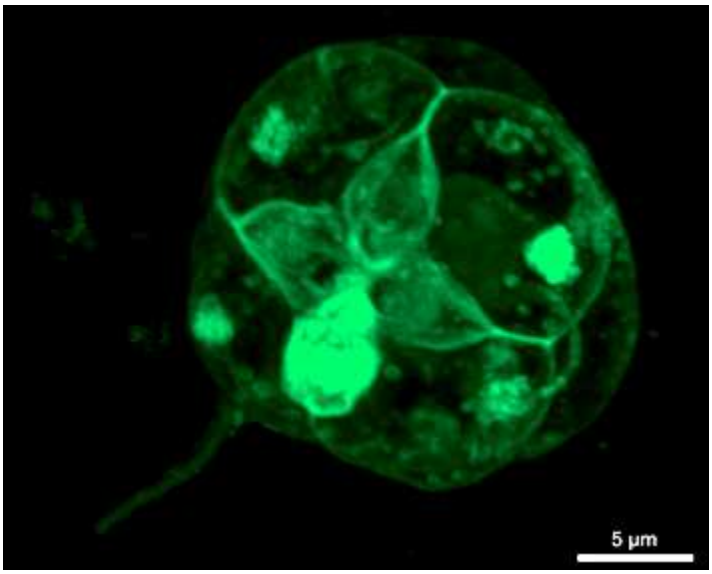

Fig. 3. Confocal laser scanning microscopy 3D reconstruction of a malacospore of *Tetracapsuloides bryosalmonae* (McGurk *et al.* 2005b).

1.2.3. Species diagnosis and malacosporean diversity

Due to the abovestated difficulties in differentiating between malacosporean species, SSU rDNA sequences have been used to aid species diagnosis in this group. Using molecular methods, sequences probably representing several new species have been discovered. Two new sequences were obtained during the systematic study of the Malacosporea (Tops *et al.* 2005), *i.e.* one from a sac-like stage infecting the rare bryozoan *Lophopus crystallinus* and the second one from a vermiform stage infecting *Fredericella sultana*. Homology between them and the SSU rDNA sequence of *B. plumatellae* was approximately 94% (Tops *et al.* 2005). The *Buddenbrockia* isolate from *L. crystallinus* was thereafter established as a new

species *B. allmani* (Canning *et al.* 2007) and a parasite from *F. sultana*, recently repeatedly found in *F. sultana* (Hartikainen *et al.* 2014) remains so far undescribed.

Furthermore, during laboratory experiments focused on malacosporean transmission from *Plumatella repens* to different fish host species, new sequences of *Buddenbrockia* spp. from cyprinid fish, *Cyprinus carpio* and *Phoxinus phoxinus* were obtained. A sequence amplified from the kidney of Eurasian minnow *P. phoxinus* was identified as *B. plumatellae*. The second malacosporean parasite transmitted from *P. repens* to common carp is likely a further undescribed species of *Buddenbrockia* (Grabner and El-Matbouli 2010).

Moreover, recent molecular studies on malacosporean isolates from bryozoans showed that malacosporean diversity is much higher than expected as they unveiled 4 additional malacosporean lineages (Hartikainen *et al.* 2014), *i.e. Buddenbrockia* sp. 1, Malacosporea sp. 1, Malacosporea sp. 2 and Malacosporea sp. 3. Novel lineage Malacosporea sp. 1 is represented by a parasite infecting *F. indica* and *F. sultana* found at two sites in Germany (the Rivers Lohr and Lohrbach) and also in North America (Lake Aberdeen, Washington). This new malacosporean exhibits an intermediate morphology between the sacs and vermiform stages, *i.e.* a lobey structure. The sacs are elongated, non-motile, irregularly shaped with lack of musculature and fine structure. Unfortunately, no ultrastructural studies are available for this malacosporean, which most probably represents a new malacosporean genus (Hartikainen *et al.* 2014). The novel lineage Malacosporea sp. 2 includes the sequence of a motile, vermiform parasite that was detected in colonies of *F. sultana*. The third novel lineage Malacosporea sp. 3 forms a sister clade to genus *Tetracapsuloides* and was found in the colonies of *P. repens*, in Borneo as a motile worm (Hartikainen *et al.* 2014).

Although high species diversity in the Malacosporea was recently revealed in bryozoans (Hartikainen *et al.* 2014), little information is available about malacosporean diversity in fish hosts. It is likely that more intense research in fish will show a much higher diversity as well. Most importantly, the marine environment still remains unexplored for malacosporeans, but since bryozoans are predominantly marine, the existence of marine malacosporeans can be expected.

1.2.4. Malacosporean life cycles

The first malacosporean life-cycle was proven via experimental studies focused on transmission of *T. bryosalmonae* from the bryozoan *F. sultana* to brown trout (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*) and its transmission from these infected fish back to *F. sultana* (Canning *et al.* 1999, Morris and Adams 2006, Grabner and El-Matbouli 2008).

Another experiment confirmed that fish can get infected after very short exposure (even less than 10 minutes) to *T. bryosalmonae* spores released from disrupted bryozoans (Longshaw *et al.* 2002). *Tetracapsuloides bryosalmonae* infects most salmonid fish species and a number of freshwater bryozoans are also susceptible, *i.e. Pectinatella magnifica, Plumatella rugosa, Plumatella emarginata, Cristatella mucedo* and *F. sultana*. The last one is probably utilized by *T. bryosamonae* as its main bryozoan host (Tops and Okamura 2005, Hartikainen *et al.* 2014). Spores released from bryozoans into the water attach to the fish by eversion of their polar filaments from polar capsules. It has been proven that a single spore is sufficient to develop infection and launch a host response (McGurk *et al.* 2006b). The attachment of spore to the skin or gills with polar filaments enables invasion of the parasites via the epidermal and mucus cells (Morris *et al.* 2000, Longshaw *et al.* 2002). Thereafter, the infectious stages proliferate in the bloodstream and reach the kidney where they replicate again, producing cell doublets in the interstitial tissue. These penetrate the renal tubules and further cell multiplication and differentiation results in the production of the malacospores in the kidney tubules (Morris and Adams 2008). Interstitial stages can probably transform back into blood stages (<25 µm in diameter) under unknown conditions and remain in the host for a long time (Abd-Elfattah *et al.* 2014b). This implies that the endurance of the blood stages in the fish host is closely linked with parasite persistence and possible relapse of *T. bryosalmonae* infection (Dash and Vasemägi 2014, Abd-Elfattah *et al.* 2014b). Circulation of *T. bryosalmonae* blood stages explains the presence of the parasite in other organs (liver, spleen, heart, gills, brain, intestine) long after exposure (Holzer *et al.* 2006, Abd-Elfattah *et al.* 2014b). The spores from the kidney tubules are subsequently released in the urine and into the water, later infecting bryozoans (Hedrick *et al.* 2004, Morris and Adams 2006, Grabner and El-Matbouli 2008) (Figure 4).

Fig. 4. Sac-like stages of *T. bryosalmonae* inside the colony of *Fredericella sultana* (Silvie Tops).

While the fish-bryozoan life cycle is expected to be the general rule, with regard to bryozoans, the infection can be spread to the new bryozoan colonies by colony fragmentation (Morris and Adams 2006) and to new sites by vertical tranfer via bryozoan dormant stages, statoblasts (Hill and Okamura 2007, Abd-Elfattah *et al.* 2014a).

1.2.5. Malacosporean pathogens of fish and bryozoans

Tetracapsuloides bryosalmonae can cause PKD, an important pathological condition. It is the only malacosporean pathogen known to date, though other species may participate in pathology (e.g. Holzer *et al.* 2014). PKD affects wild and farmed salmonid fish (Feist *et al.* 2001). It is also one of the most economically important fish diseases (Hedrick *et al.* 1993). Since the first record of PKD in Germany (Plehn *et al.* 1924), PKD has been detected in most European countries, in Canada and several western states of the USA (Hedrick *et al.* 1993). The etiological agent of PKD in salmonid fish was identified as a myxozoan on the basis of spores present in the kidney tubules (Kent and Hedrick 1985). Previously identified as the enigmatic PKX organism (Seagrave *et al.* 1980) it was later named as *Tetracapsula bryosalmonae* (Anderson *et al.* 1999a,b, Canning *et al.* 1999) and thereafter renamed to *Tetracapsuloides bryosalmonae* (Canning *et al.* 2002). The invasion involves cycles of cell divisions and multiplications in the blood, kidney interstitium and other organs. As a response to infection, the fish develops a massive immune reaction – the actual disease (Okamura and Canning 2003).

The course of the disease depends on the season (temperature). Usually, first infections appear when the water temperature rises above 15 °C. Thus, the infection typically peaks during the summer and fall (Hedrick *et al.* 1993). It has been reported that even lower temperatures around 12 °C may induce clinical PKD (Morris *et al.* 2005, Schmidt-Posthaus *et al.* 2012). Clinical signs of PKD include swollen kidney and spleen, bulging eyes, blackened fins and tail and subsequent accumulation of abdominal fluid (ascites) (Okamura and Canning 2003). The mortality caused by PKD approximately reaches up to 20% but with secondary pathogens or unfavourable conditions in fish farms and hatcheries can even reach up to 95–100% (Hedrick *et al.* 1993). The higher percentage of mortality and organ damage can also be attributed to co-infections caused by other myxozoans, for example *Chloromyxum schurovi* (Feist *et al.* 2002). Epizootiological studies imply that once the host fish is exposed to *T. bryosalmonae* and survives, it develops resistance for following years (Ferguson and Ball 1979, Foott *et al.* 1987). It has been claimed that PKD mainly affects

young salmonids, especially yearlings (des Clers 1993) but new research implies that higher prevalence in 1+ fish compared to 0+ fish might be caused by the re-infection before immunity is acquired (Schmidt-Posthaus *et al.* 2013, Dash and Vasemägi 2014). It is still not clear whether *T. bryosalmonae* persists in host kidneys in some salmonids when they return to the rivers to spawn and thus enable relapse of infection (Mo *et al.* 2011, Dash and Vasemägi 2014). All salmonid fish seem to be susceptible. This includes farmed fish and hatcheries but PKD may influence also population dynamics of wild fish populations, as it has been reported in brown trout *Salmo trutta fario* populations in Switzerland (Wahli *et al.* 2002) and Atlantic salmon *Salmo salar* population in the Central Norway (Sterud *et al.* 2007).

The spread of *T. bryosalmonae* is closely linked to the presence and distribution of bryozoans (Okamura *et al.* 2001). *Tetracapsuloides bryosalmonae* produces large amounts of spores with each parasite sac containing between 2800 to 4000 infectious spores (malacospores) (Okamura *et al.* 2011) and it is also relatively host-unspecific: *T. bryosalmonae* has been identified in several species of the genera *Plumatella*, *Hyalinella*, *Lophopodella*, *Fredericella* and *Stolela* from Brazil, Bulgaria, Japan, and Austria (Marcus 1941, Grancarova 1968, Oda 1978). A recent study showed that high dispersion of PKD might be caused by vertical transmission of *T. bryosalmonae* by the dormant stages of bryozoans, statoblasts, inasmuch as the buoyant statoblasts are likely to be dispersed over great distances (Abd-Elfattah *et al.* 2014a). Apart from that, due to climate change and global warming, a higher frequency of the occurrence of PKD is expected. Not only does the higher water temperature affect the onset of infection in the fish but also the earlier development and greater bryozoan biomass production can also contribute to this process (Okamura and Canning 2003, Tops *et al.* 2006).

While this parasite causes significant economic losses in aquaculture and wild fish populations (Anderson *et al.* 1999a, Feist *et al.* 2001), the presence of *T. bryosalmonae* in freshwater bryozoans has a relatively small effect on their fitness (Tops *et al.* 2009). Anyway, under specific conditions parasitized bryozoans can develop a slowdown in growth, statoblasts reduction, decline in hatching of colonies or, on the contrary, flourishing and gigantism at the zooid level in a way that should enable increased transmission of the parasite (Hartikainen *et al.* 2013). These effects are also expected to worsen with temperatures on the rise.

1.3. Bryozoans – known and other potential malacosporean hosts

Taking into account that bryozoans are definitive host of malacosporeans and that some malacosporean parasites can be spread through vertical transmission from one bryozoan colony to another (Hill and Okamura 2007, Abd-Elfattah *et al.* 2014a), it may be assumed that some of malacosporeans utilize bryozoans as their only host and that this only host is responsible for transmission and dispersion of the parasite into the environment. That makes the bryozoans an essential prerequisite for studying malacosporean life cycle strategies.

Bryozoans (or Ectoprocta) are small marine and freshwater invertebrate animals that live on submerged surfaces, such as plants, wood, rocks and a wide range of synthetic materials. They significantly participate in species diversity in aquatic ecosystem and play a role as bioconstructors, providing habitat for numerous invertebrate taxa (Cocito 2004) including micropredators (Lidgard 2008). Moreover, marine species, due to their ability of forming mineralized skeletons consising of calcium carbonate are considered significant contributors of carbonate sediments in many marine areas (Bone and James 1993). The mainly marine phylum Ectoprocta includes almost 4000 described species and only about 100 of them live in freshwater (Wood 2005, Wood *et al.* 2006). It is furthermore estimated that there are more than 5700 (d'Hondt 2005) or even 8000 extant and 15 000 extinct bryozoan species known only from fossils (Ryland 2005). They are divided into the three classes Stenolaemata, Gymnolaemata, and Phylactolaemata (McKinney and Jackson 1989). Stenolaemata include marine bryozoans with tubular zooids with strongly calcified walls (Barns 1982). The class Gymnolaemata is mainly composed of fossil species with cylindrical and flattened chitinous or calcified zooids (Ryland 2005). Phylactolaemata represents the smallest group of Ectoprocta. In January 2006, there were 88 valid freshwater bryozoan species spread worldwide (Massard and Geimer 2008) but after the discovery of new species in Thailand the number has risen up to 94 species living exclusively in freshwater (Wood *et al.* 2006). Bryozoans are often called "moss animals". This name refers to the appearance of certain species. Colonies are composed of many genetically identical zooids that are connected to each other. The individual zooids of bryozoan colonies are associated to the extent that it is impossible to distinguish where one zooid finishes and the new one begins (Wood 1989). They are suspension feeders capturing organic particles by using a special device, called lophophore placed on each zooid. This apparatus works on the principle of the filtration feeding (Massard and Geimer 2008). Other organs such as mouth, gut, muscles, nervous and reproductive system are also present in the zooid (Figure 5).

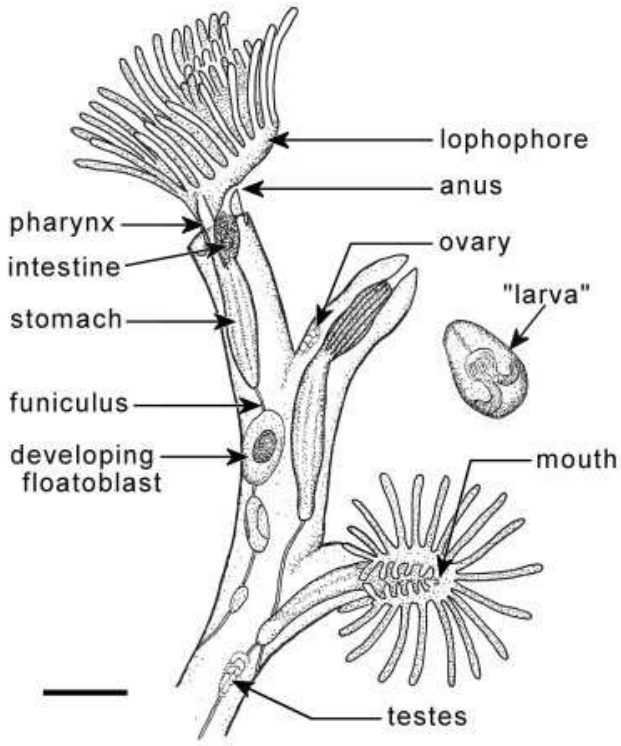

Fig. 5. Basic anatomy of a bryozoan (Wood 2001)

Although marine and freshwater bryozoans are similar in structure, freshwater bryozoans are larger and easier to study. In general, warm waters support greater and faster growth of colonies than cold and clear waters (Wood 2005). Even though they are ubiquitous, bryozoans have often been overlooked due to their colonial growth in cryptic, protected places such as undersides of submerged branches, macrophytes and stones. The life cycle includes hatching of small colonies from statoblasts during late spring or early summer when the temperature increases. The statoblasts are asexually produced, small, seed-like structures, which are composed of two chitinized valves that enclose dormant germinal tissues (Reynolds 2000, Okamura and Wood 2002). Morphologically, statoblasts are divided into three categories: Floatoblasts, sessoblasts, and piptoblasts (Wood 1979, Mukai 1982). Floatoblasts have their chambers filled with gas; sessoblasts are larger than the previous ones with empty chambers but being firmly cemented to the base. The last type of statoblasts are piptoblasts which have chambers without gas so they neither float nor adhere to the substrate because they have no annular float and adhesive apparatus (Reynolds 2000). Statoblasts can be released into the environment to start a new colony anywhere (Okamura and Wood 2002). The reproduction includes asexual and sexual part. Asexual reproduction in freshwater bryozoans includes simple fragmentation, fission, and several types of budding. When the

new colony is established, statoblasts are also formed. All freshwater and most marine bryozoans are hermaphrodites (Barnes 1982). Some species produce both sperms and eggs at the same time, others are protandric hermaphrodites (Zrzavý 2006). The sperm develops in special clusters in the funiculi, and after releasing into the coelom, the sperm moves passively. Egg clusters consisting of 20–40 cells are hatched at the inner colony wall (Wood 2005). After fertilization a trochophore larva develops and transformes into a primary zooid by methamorphosis (Zrzavý 2006).

As the majority of bryozoans live in the marine environment but malacosporeans have so far only been described in freshwater bryozoan species belonging to the Phylactolaemata. Research on other groups, especially the highly diverse marine bryozoans could unveil a higher diversity of malacosporeans than previously expected.

1.3.1. Bryozoans in the Czech Republic

In the Czech Republic, 10 species of bryozoans belonging to two classes (Gymnolaemata and Phylactolaemata) are present (Korábek 2009). In the Gymnolaemata, polymorphism of individual zooids within one colony can be observed and their lophophore is circle-shaped. In contrast, Phylactolaemata have no distinguished zooids. They are tightly connected and the lophophore is U-shaped. The most common species are represented by *P. emarginata, P. fruticosa, P. fungosa, P. repens* and *P. punctata*. All of the abovementioned *Plumatella* species can be distinguished from each other on the basis of different size of colonies and zooids, different shape of lophopore, different number of tentacles on the lophopore, and by different morphology of statoblasts. Other species that are included in the class Phylactolaemata are *F. sultana, L. crystallinus, C. mucedo* and *P. magnifica*. The latter species has its origin in America and it was artificially introduced into Czech basins during the 20th century (Balounová *et al.* 2011). *Pectinatella magnifica* is distributed widely in South Bohemia (Šetlíková *et al.* 2005) and along with *F. sultana* they are the only two species that can survive the winter without production of statoblasts. *Pectinatella magnifica* maintains colonies during the whole year. Gymnolaemata are represented by only one species in the Czech Republic, which is *Paludicella articulata* (Korábek 2009).

2. OBJECTIVES

- To establish a method for *in vitro* culturing of specific pathogen-free (SPF) colonies of *Plumatella repens* and *Fredericella sultana* using laboratory-grown algae cultures.

- To study malacosporean life cycles via cohabitation transmission experiments.

- To examine fish from different freshwater localities, predominantly in the Czech Republic and Central Europe for malacosporean infections using light microscopy and molecular methods in order to investigate the prevalence, diversity, distribution and habitat and host preference of malacosporeans in fish hosts.

- To perform the phylogenetic analyses of newly obtained sequences together with all malacosporean sequences available on GenBank to study the evolutionary trends within the Malacosporea.

- To investigate malacosporean diversity in marine bryozoans.

3. MATERIALS AND METHODS

3.1. Sampling

3.1.1. Bryozoan sampling

The bryozoan species *Fredericella sultana* and *Plumatella repens* were sampled at a commercial carp production pond (Motovidlo; Figure 6) and at small ponds in Chřešťovice, which are used for ornamental fish culture (Table I). These bryozoans, the most common representatives of the class Phylactolaemata in the South Bohemian water bodies (Kafka 1886), were found attached to the submerged stones and branches on the bottom of the ponds. The bryozoan colonies (8–10 zooids per one sample) were investigated under an Olympus SZX7 stereomicroscope and screened for the presence of malacosporean infections by molecular methods (see section 3.6.).

Fig. 6. Sampling of bryozoans at Motovidlo pond.

In order to gain SPF bryozoan colonies, necessary for following infection/cohabitation experiments, some colonies were split into groups of three zooids, using a scalpel and cleaned thoroughly from any attached epibionts, by water current created with a pipette, under the abovementioned stereomicroscope. Afterwards, the zooids were superglued to plastic Petri dishes and attached to a plastic grid that was placed into the tank so that the Petri dishes were facing upside down. The tank was filled with declorinated tap water,

aerated and the bryozoans were fed weekly with 500 ml of laboratory-cultured algae (see section 3.3.).

Four species of marine bryozoans were sampled, *i.e. Bugula neritina*, *Zoobotryon verticillatum*, and two undetermined bryozoan species (Bryozoa sp. 1 and Bryozoa sp. 2). A total of 97 colonies were sampled in two localities at the Gulf of Mexico, Florida (Table I).

3.1.2. Fish sampling

Fish were collected at 16 freshwater localities during the years 2011–2013. Two localities were in the Slovak Republic, one in Hungary and all others in the Czech Republic (Table I). Localities included semi-intensively farmed ponds, fish farms with outdoor ponds, decorative ponds, lakes and rivers.

Table I: List of the localities where bryozoan and fish samples were collected.

Bryozoan sampling localities	Fish sampling localities
Chřešťovice fish farm, CR	*Bavorov, CR*
Motovidlo Pond, Čejkovice, CR	*Dyje River, South of Břeclav, CR*
Lido Key, Sarasota, Florida	*Horní Hluboký Pond, Strmilov, CR*
City Island, Sarasota, Florida	*Hluboká nad Vltavou, CR*
	Chřešťovice fish farm, CR
	Jihlava, CR
	Jindřiš fish farm, CR
	Malá Outrata Pond, Vodňany, CR
	Motovidlo Pond, Čejkovice, CR
	Rožmberk Pond, CR
	Tourov, CR
	Šnejdlík, České Budějovice, CR
	Vodňany, CR
	Danube River at Štúrovo, SR
	Hron River at Štúrovo, SR
	Hortobágy, HU

Note: (CR=Czech Republic, SR=Slovak Republic, HU=Hungary).

In total, 278 fish individuals belonging to 4 orders and 25 species (Table II) were sampled. As a result, 278 kidneys, 47 blood samples, 10 swim bladders, 6 urinary bladders, 4 eyes, 3 bile samples, 2 brains and 1 heart were screened for the presence of malacosporeans.

Table II: List of the sampled fish species divided according to fish orders.

Cypriniformes	Perciformes	Salmoniformes	Gasterosteiformes
Abramis brama	*Lepomis gibbosus*	*Oncorhynchus mykiss*	*Gasterosteus aculeatus*
Alburnoides bipunctatus	*Perca fluviatilis*	*Salvelinus fontinalis*	
Alburnus alburnus	*Sander lucioperca*		
Aspius aspius			
Ballerus sapa			
Barbus barbus			
Blicca bjoerkna			
Carassius auratus auratus			
Chondrostoma nasus			
Cyprinus carpio			
Gobio gobio			
Leucaspius delineatus			
Leuciscus idus			
Leuciscus leuciscus			
Rhodeus sericeus amarus			
Rutilus rutilus			
Scardinius erythrophthalmus			
Squalius cephalus			
Tinca tinca			

3.2. Dissection

Before dissection, each fish was weighted and measured and blood sample was taken with a BD Ultra Fine Insulin syringe. This syringe was rinsed with the heparin before use, in order to prevent blood coagulation. Blood was taken from the caudal vein in the area of the rear lateral line. To prevent contamination, dissection equipment was cleaned with 10% hydrogen peroxide, after each dissected fish. Sterile scalpel blades were used to remove the kidney. The drop of the blood and the kidney sample were examined under the Olympus BX51 light microscope. Four microliters of blood and small kidney samples were mixed with 400 µl TNES urea buffer (10 mM Tris-HCl with pH 8, 125 mM NaCl, 10 mM EDTA, 0.5% SDS, 4 M urea) (Asahida *et al.* 1996), for the molecular use. Plasmodia and spore morphologies were documented with an Olympus DP70 digital camera.

All animal procedures were performed in accordance with Czech legislation (section 29 of Act No. 246/1992 Coll., on Protection of animals against cruelty, as amended by Act No. 77/2004 Coll.)

3.3. Algal cultivation

Algal cultivation had to be established in order to provide food for SPF bryozoans which were used in cohabitation experiments planned in this thesis. Three algal cultures consisting of the genera *Chlamydomonas*, *Cryptomonas* and *Fragilaria* were obtained from the Institute of Hydrobiology in České Budějovice, BC CAS. All cultures were handled in a sterile environment to avoid bacterial or other contaminations so that all manipulations were done in the flow chamber with sterile equipment. For culturing algae, Wright's cryptophyte medium (WC medium; Guillard and Lorenzen 1972; Table III, IV, V), which had been recommended by the staff of Institute of Hydrobiology, was used. The algal cultures were grown in 500 ml Erlenmeyer flasks that were plugged with an autoclaved cellulose plug, surrounding a glass pipette. Aeration of the cultures in the flask was achieved by connecting an electric aerator to the glass pipettes. The algal cultures were maintained under medium light intensity at 20 °C. After 10 days, the cultures were fully grown and subcultured. For subculturing, 5 ml of the fully-grown culture was transferred in to 500 ml of fresh medium.

Table III: Composition and quantity of the stock solutions for the WC medium preparation.

Component	Stock solution $g \cdot l^{-1}$ dH_2O	Quantity	Final Medium conc.
Tris Buffer	----------	500 mg	4.13×10^{-3}
$NaNO_3$	85.01	500 mg	1.00×10^{-3}
$CaCl_2 \cdot 2H_2O$	36.76	1 ml	2.50×10^{-4}
$MgSO_4 \cdot 7H_2O$	36.97	1 ml	1.50×10^{-4}
$NaHCO_3$	12.60	1 ml	1.50×10^{-4}
$Na_2SiO_3 \cdot 9H_2O$	28.42	1 ml	1.00×10^{-4}
K_2HPO_4	8.71	1 ml	5.00×10^{-4}
Trace metal solution	Following Table II	1 ml	----------
Vitamins solution	Following Table III	1 ml	----------

For culture medium preparation, Tris buffer was dissolved in 900 ml of dH_2O, then the other solutions (Table III) were added and the final volume was brought to 1000 ml with dH_2O. The required pH of 7.6–8.0 was checked with Litmus paper and the whole solution was autoclaved.

Table IV: Composition and quantity of the stock solution for Trace metal solution.

Component	Stock solution g·l^{-1} dH_2O	Quantity	Final Medium conc.
$Na_2EDTA \cdot 2H_2O$	----------	4.36 g	1.17×10^{-5}
$FeCl_3 \cdot 6H_2O$	----------	3.15 g	1.17×10^{-5}
$CuSO_4 \cdot 5H_2O$	10.00	1 ml	4.01×10^{-8}
$ZnSO_4 \cdot 7H_2O$	22.00	1 ml	7.65×10^{-8}
$CoCl2 \cdot 6H_2O$	10.00	1 ml	4.20×10^{-8}
$MnCl_2 \cdot 4H_2O$	180.00	1 ml	9.10×10^{-7}
$Na_2MoO_4 \cdot 2H_2O$	6.00	1 ml	2.48×10^{-8}
H_3BO_3	----------	1.00 g	1.62×10^{-5}

All solutions necessary to prepare the Trace metal solution were added into 950 ml of dH_2O and afterwards the final volume was transferred into 1000 ml with dH_2O and autoclaved.

Table V: Composition and quantity of the stock solution for Vitamin solution.

Component	Stock solution g·l^{-1} dH_2O	Quantity	Final Medium conc.
Thiamine·HCl	----------	100 mg	2.96×10^{-7}
Biotin	0.50	1 ml	2.05×10^{-9}
Cyanocobalamin	0.50	1 ml	3.69×10^{-10}

For preparation of the Vitamin solution, Thiamine·HCl was dissolved in 950 ml of dH_2O and 1 ml of the stock solutions were added and afterwards the final volume was transferred into 1000 ml with dH_2O and filter-sterilized and stored in the -20 °C freezer. Bryozoans were fed, weekly, with 500 ml of cultured, fully-grown algae.

3.4. Cohabitation experiments

Two cohabitation experiments were set up for the study of malacosporean life cycles (malacosporean transmission from fish to bryozoan colonies and *vice versa*).

3.4.1. Cohabitation experiment 1

In Cohabitation experiment 1, the potential transmission of malacosporean spores from bryozoans to fish hosts was sought. Stones and sticks with bryozoan colonies of *P. repens*, collected in the wild were submerged in the water of the aquaria. Then, a plastic basket was placed into the aquarium, which was held in place by wires so that it would be partly under and partly above the water level. Aeration was added, too. Fifteen one-year-old SPF

common carp *Cyprinus carpio* individuals were placed into the basket to prevent them from feeding on the bryozoan colonies (Figure 7). The fish were hatched from the eggs in the aquaculture system at the animal facility of the Institute of Parasitology. Each aquarium was covered with black foil due to the light sensitivity of the bryozoans. Every third day, 250 ml of fully-grown algal cultures representing a mix of the three abovementioned algal species was added into the tank to feed the bryozoans. Cohabitation experiment 1 was performed for one month with three fish being dissected every week. Blood and kidney smears were investigated under the Olympus BX51 light microscope and then taken into TNES buffer for further molecular screening. After the cohabitation experiment was terminated all bryozoan colonies used in the experiment were investigated under the abovementioned microscope and subsamples of 20 bryozoan colonies were screened for malacosporean infection.

Fig. 7. Arrangement of Cohabitation experiment 1.

3.4.2. Cohabitation experiment 2

In Cohabitation experiment 2, the potential transmission from fish suspected to harbour malacosporean infections to SPF bryozoan colonies of *P. repens* and *F. sultana* was investigated. For this experiment, fish collected in Chřešťovice were used. Previous screening performed by the members of our laboratory had shown that the prevalence of malacosporean infections in fish at this locality was more than 80%. Six one-year-old common carp individuals were used for the experiment. The arrangement of the cohabitation tank was the same as in Cohabitation experiment 1. SPF bryozoan colonies placed on plastic Petri dishes prepared as described in 3.1.1. were used. These Petri dishes were attached to the sides of aquarium with velcro. The whole aquarium was covered with black foil. The bryozoans were investigated under the Olympus SZX7 stereomicroscope for the presence of malacosporean spore sacs or worms, every third day. The feeding regime of the bryozoans

with the algal cultures was the same as in Cohabitation experiment 1. The Cohabitation experiment 2 was performed for three months after which all fish used for the experiment were dissected. Blood, kidney and bryozoan colonies samples were checked under the light microscope and taken into TNES buffer for molecular screening.

3.5. DNA extraction

For extraction of the DNA two methods were used. Phenol-chloroform extraction was used for fish tissue samples and the QIAmp DNA Stool Mini Kit (QIAGEN) was used for bryozoan samples, as we expected a large amount of PCR inhibitors in the latter samples.

3.5.1. QIAmp DNA Stool Mini Kit (QIAGEN) extraction

During extraction of the bryozoan samples 200 mg of bryozoans were put into a microtube filled with glass beads of 0.5 mm in diameter (BioSpec Products, Inc.) and with 1 ml of ASL Buffer (QIAmp DNA Stool Mini Kit, QIAGEN). The sample was homogenized with beadbeater (FastPrep – 24, M.P. Biomedicals) for 1 minute at 5.5 m/s. All other steps of the extraction were carried out according to the manufacturer's protocol. Extracted DNA was stored in nanopure water at -20 °C.

3.5.2. Phenol-chloroform extraction

Samples of kidneys, blood, brains, eyes, swimm bladders, urinary bladders, heart and bile were extracted with a simple phenol-chloroform extraction. Samples stored in 96% ethanol were processed by removing the alcohol by decanting and evaporating the remainder of the liquid on thermoblock set at 37 °C. Fresh (unfixed) as well as fixed, ethanol-free samples were dissolved in 400 µl of TNES urea buffer. DNA was digested with 100 µg/ml of Proteinase K (Serva, Germany). The samples were incubated with Proteinase K at least for 16 hours or overnight at 55 °C. After incubation, 400 µl of phenol was added in the laboratory fume hood. The tubes were inverted repeatedly for 5 minutes and mixed properly with 400 µl of chloroform by overend turning of the tubes. Thereafter, the samples were centrifuged at 15 000 g for 5 minutes at room temperature. After centrifugation, two layers had separated in the tubes. The top aqueous layer containing DNA was removed to a new tube. DNA was precipitated by mixing the aqueous layer with a triple amount of ice-cold 92% ethanol. Then, the tubes were centrifuged again at 15 000 g for 20 minutes at 4 °C to pellet the DNA. Ethanol was decanted after centrifugation and the DNA pellet was washed

with 1000 µl of 70% ethanol. The tubes were centrifuged for the last time at 15 000 g for 4 minutes at 4 °C, alcohol was decanted and the remainder of the ethanol was evaporated on the thermoblock at 50 °C, for 10 minutes. After the final drying of pellets they were re-suspended in nanopure water (50–500 µl depending on DNA quantity). Samples were left to dissolve overnight at 4 °C and then directly used as a template for PCR.

3.6. Polymerase chain reaction

The polymerase chain reaction (PCR) was used for detection of malacosporean DNA in kidneys, blood, brains, urinary bladders, swim bladders, bile, eyes and heart, from freshwater fish and in parts of colonies of freshwater and marine bryozoans. For detection of malacosporean DNA, specific primers amplifying a partial (mala-f, mala-r) or the almost complete sequence of malacosporean SSU rDNA (budd-f, budd-r) were used. Additionally, less specific primers (Erib1, Erib10), that amplify eukaryotic SSU rDNA, were used. More specific primers (Myxgp2f, ACT1r), that amplify myxozoan SSU rDNA, were applied in a second nested step. The latter approach was used for the marine bryozoans, as it was expected that marine malacosporeans (if existing) may have somewhat divergent sequences from their freshwater counterparts. All used primers with their corresponding data and annealing temperatures are listed in Table VI.

Table VI: List of primers used for PCR with their annealing temeratures and corresponding information.

Name of primer	Annealing temperatures	Sequence (5´→ 3´)	Length of fragment (bp)	References
mala-f	64 °C	AAACGARTAAGGTCCAGGTC	640	Grabner and El-Matbouli 2010
mala-r	64 °C	CACCAGTGTAKCCCGCGT		Grabner and El-Matbouli 2010
budd-f	61 °C	CTGCGATGTACTCGTCTTAAAG	1780	Grabner and El-Matbouli 2010
budd-r	61 °C	CGACCAAGCTCAAACAAGTTT		Grabner and El-Matbouli 2010
Erib1	60 °C	ACCTGGTTGATCCTGCCAG	2000	Barta *et al.* 1997
Erib10	60 °C	CTTCCGCAGGGTTCACCTACGG		Barta *et al.* 1997
Myxgp2f	58 °C	WTGGATAACCGTGGGAAA	1600	Kent *et al.* 1998
ACT1r	58 °C	AATTTCACCTCTCGCTGCCA		Hallet and Diamant 2001

Note: All primers amplify partial to complete SSU rDNA. The PCR product lengths using Erib1-Erib10, ACT1r – Myxgp2f are stated for Myxozoa in general as the length of the expected product in Malacosporea is unknown.

PCR stock solutions:

- dd H_2O
- 10x Taq purple Buffer complete (Top-Bio, CR)/ 10x Titanium Taq Buffer complete (Clontech Laboratories, USA)
- dNTP mix
- Forward primer
- Reverse primer
- Taq Purple polymerase (Top-Bio, CR)/Titanium Taq polymerase (Clontech Laboratories, USA)

The PCR reaction protocol with mala-f/r and budd-f/r primers consisted of primary denaturation at 95 °C for 5 minutes, amplification of 40 cycles at 95 °C for 45 seconds, annealing temperature for primers in the above mentioned Table VI for 45 seconds, 72 °C for 45 seconds or 140 seconds for elongation, respectively. Final extension was performed at 72 °C for 5 minutes. Annealing temperatures for mala-f/r and budd-f/r primers were adjusted beside from recommended annealing temperatures (Grabner and El-Matbouli 2010) and optimized using gradient PCR to avoid nonspecific PCR products.

The PCR reaction protocol with Erib1/Erib10 and Myxgp2f/ACT1r consisted of primary denaturation at 95 °C for 3 minutes, amplification of 30 cycles composed of 94 °C for 50 seconds, recommended annealing temperatures for primers in the abovementioned Table VI for 50 seconds, 68 °C for 150 seconds or 90 seconds for elongation, respectively. Final extension was performed at 68 °C for 8 minutes. The exact compositions of individual PCR reactions are listed in Table VII.

Table VII: Composition of PCR reaction mixtures.

Individual PCR components	**PCR composition with mala-f/r**		**PCR composition with budd-f/r**		**Primary PCR composition with Erib 1/Erib10**		**Secondary PCR composition with Myxgp2f/ACT1r**	
10x buffer	--------	1.00 µl	--------	1.00 µl	--------	1.00 µl	--------	1.00 µl
dNTP	10 mM	0.20 µl	10 mM	0.20 µl	10 mM	0.20 µl	10 mM	0.20 µl
Forward primer	10 µM	0.20 µl	10 µM	0.20 µl	10 µM	0.20 µl	10 µM	0.20 µl
Reverse primer	10 µM	0.20 µl	10 µM	0.20 µl	10 µM	0.20 µl	10 µM	0.20 µl
Taq Purple polymerase	1U/1µl	0.40 µl	1U/1µl	0.40 µl	--------	--------	-------	--------
Taq Titanium polymerase	--------	--------	--------	--------	1U/1µl	0.10 µl	1U/1µl	0.10 µl
dd H_20	--------	7.50 µl	--------	7.50 µl	--------	7.30 µl	--------	7.30 µl
DNA	--------	0.50 µl	--------	0.50 µl	--------	1.00 µl	--------	1.00 µl
Final volume	**10.00 µl**		**10.00 µl**		**10.00 µl**		**10.00 µl**	

PCR products prepared with Purple Taq Polymerase were directly loaded onto the gel. PCR products prepared with Titanium Taq Polymerase were mixed with 6x Gel Loading Dye blue to a final concentration 1.6 µl/10 µl of PCR product, before loading.

3.7. Gel electrophoresis

Gel electrophoresis was used to visualize the PCR products. 1% agarose gels were prepared by mixing agarose and TAE buffer, then heated in the microwave for 2 minutes and cooled down to approximately 45 °C. Then, ethidium bromide in final concentration 0.5 µg/ml was mixed with the gel. A gel tray with a comb was filled with gel solution and was left to solidify for 30 minutes. Thereafter, the comb was taken out and the gel tray was placed into the electrophoresis tank containing TAE buffer. Each gel well was filled with 10 µl of PCR product. The first well was equipped with a 1kb ladder marker or a 100 bp ladder marker, depending on the estimated size of the PCR product. The gels were run at 80 V for one hour. The DNA fragments were finally visualized under ultraviolet light and PCR amplicon sizes were compared with the ladders. Desired fragments were cut off the gel and used for PCR product gel extraction.

3.8. PCR product purification

The PCR product extraction from the gels was done with the commercial kit Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan), according to manufacturer's protocol. The amplicons were eluted from the spin columns in 50 µl of nanopure water and stored until used for DNA sequencing.

3.9. Cloning

Cloning was used in order to gain better quality sequences and single species sequences. The PCR Cloning Kit (QIAGEN, Germany) was used for cloning. At first, ligation-reaction master mix was prepared, composed of 0.5 µl Cloning vector, 2 µl PCR product (isolated from gel), and 2.5 µl Ligation Master Mix. This ligation reaction was incubated in the thermocycler (BIOERXpCycler) at 14 °C for 2 hours. After the incubation, the vector was transformed into the DH5α competent cells. 50 µl of freshly thawed competent cells were gently mixed with the ligation reaction and then incubated for 8 minutes on ice. Thereafter, they were exposed to heat shock in the water bath preheated at 42 °C for 40 seconds. Afterwards the tubes were incubated for 2 minutes on the ice. Then 200 µl of SOC medium

was added to the mixture and the tubes were shaken at 37 °C for 1 hour. Meanwhile LB agar plates were preheated on 37 °C and 40 µl of X-Gal was spread all over the agar plate. After 1 hour the mixture was spread on the agar plate with a glass hockey stick spreader. The agar plates were incubated at 37 °C overnight. The following day white colonies were tested for the presence of desired template using PCR screening. Four colonies were chosen, scrubbed with a micropipette tip and dissolved in 30 µl of nanopure water, then shaken for 10 minutes at 37 °C. These samples were used as a template for subsequent PCR screening. Master mix was prepared as described in Table VIII. Universal M13f (5´GTTTTCCCAGTCACGAC3´) and M13r (5´AACAGCTATGACCATG3´) primers were used for amplification of the PCR product.

Table VIII: Composition of PCR reaction mixture and annealing temperature for M13f/r primers.

PCR reaction		**54 °C**
10×buffer	---------	1.30 µl
dNTP	250 µM	1.00 µl
M13-f	10 µM	0.50 µl
M13-r	10 µM	0.50 µl
Taq purple polymerase	1U/1µl	0.50 µl
dd H_20	---------	7.20 µl
Bacteria cell suspension	---------	2.00 µl
Final volume	---------	**13.00** µl

The PCR reaction protocol with M13f/r included primary denaturation at 95 °C for 10 minutes, amplification of 20 cycles composed of 95 °C for 30 seconds, annealing at 54 °C for 1 minute and 72 °C for 1 minute for elongation. Final extension was performed at 72 °C, for 5 minutes. Afterwards amplicons were visualized on the gel. Colonies that contained vectors with inserts of the expected size were mixed with 3 ml of LB medium and ampicillin with final concentration 75 µg/ml. This solution was shaken at 37 °C overnight. The plasmids were subsequently isolated with High Pure Plasmid Isolation Kit (Roche, Switzerland) according to manufacturer's protocol.

3.10. Sequencing

The amplicons obtained by PCR were sequenced commercially, using the Sanger sequencing method (SEQme s.r.o., Czech Republic). Sequencing reactions consisted of 1 µl of a single primer used for PCR (forward or reverse) and 9 µl of the isolated PCR amplicon

with the concetration required 10 ng/1 µl. For plasmids, the required concentration for sequencing was 50 ng/1 µl. PCR product and plasmid concentrations were measured on the Biochrom Libra S12 spectrophotometr.

3.11. Phylogenetic analyses

The preliminary analyses (results not shown) were based on the SSU rDNA alignment, which included newly obtained sequences and all malacosporean sequences available on GenBank. Further analyses (Figures 11–13 in 4.4.) were based on the taxa-reduced alignment composed of 17 selected sequences of maximum length and of sufficient quality. Each single sequence was a representative of the particular clade/lineage as found out by preliminary analyses. All individual steps of phylogenetic analyses were performed in Geneious v8.1.2 (Biomatters Limited, Auckland, New Zealand) including all programmes required. The SSU rDNA sequences of malacosporeans were aligned in MAFFT v6.864 b (Katoh *et al.* 2002) using the E-INS-i, with gap opening penalty (-op) 1.53 and gap extension penalty (-ep) 0.0 and also using the L-INS-i method with gap opening penalty (-op) 1.53 and gap extension penalty (-ep) 0.123. Third types of alignments were created in Geneious using Geneious alignment with 65% cost matrix similarity. The alignments were manually edited in BioEdit v7.0.5.2 (Hall 1999). Maximum Parsimony (MP) analyses were performed in PAUP* v4.b10 (Swofford 2003) using a heuristic search with random taxa addition, the ACCTRAN option, TBR swapping algorithm, all characters treated as unordered, a Ts/Tv ratio of 1:2, and gaps treated as missing data. Maximum Likelihood (ML) analyses were performed in RAxML v7.0.3 (Stamatakis 2006) with the GTR+G model. Bootstraps were based on 1,000 replicates for both MP and ML analyses. Bayesian inference (BI) analyses were performed in MrBayes v3.0 (Ronquist and Huelsenbeck 2003), using the GTR+G+I model of evolution. Posterior clade probabilities were estimated from 1,000,000 generations via two independent runs of four simultaneous Markov Chain Monte Carlo simulations with every 100th tree saved and burn-in set to 10% (100,000 generations). P-distances were calculated in PAUP* v4.b.10 from a 1713 bp alignment containing almost complete SSU rDNA sequences of malacosporeans.

4. RESULTS

4.1. Bryozoan cultivation

Bryozoan cultivation using the abovedescribed algae cultures and method (3.3.) was successful for sustaining the bryozoan colonies of *Plumatella repens* for the restricted time period (from March to August) required for cohabitation experiments. The colonies of *P. repens* were growing and increasing the number of zooids. After half a year they started to be of whiter color and were losing their fitness with no apparent cause. Finally the colonies died without producting statoblasts.

4.2. Cohabitation and transmission experiments

The cohabitation experiments established to investigate the transmission of malacosporean infections from bryozoans to fish and *vice versa* were unsuccessful.

In Cohabitation experiment 1 (3.4.1.) malacosporean infection was not detected by PCR screening of blood and kidney samples of 15 fish as well as by screening of 20 bryozoan colonies that were collected from the experimental tank after the termination of cohabitation experiment.

In Cohabitation experiment 2 (3.4.2.) two kidney samples and one blood sample of two out of 6 cohabited carps were PCR positive for malacosporean DNA, but negative for infection by light microscopy. Consecutive sequencing of the obtained amplicons revealed the presence of a new malacosporean species (in this thesis named as *Buddenbrockia* sp. 2) in the infected fish used to infect bryozoans. The rest of the carp (four) from the experimental tank as well as 20 samples of bryozoans were microscopically and PCR negative.

4.3. Field samples

4.3.1. Light microscopy

The microscopic detection of malacosporean parasites was very difficult. More than three quarters of kidney samples (79%) did not contain myxozoan stages recognizable by light microscopy. However, subsequent Malacosporea-specific PCR screening of these microscopically negative samples revealed that 32% were positive for malacosporean DNA. From the total of 21% microscopically positive samples (containing various stages of parasite development), 8% were PCR negative (Table IX). Microscopically positive samples

included young *Sphaerospora*-like or malacosporean-like plasmodia in kidney tubules as well as spores of various myxozoan species, *i.e. Myxobolus* sp., *Sphaerospora* sp., *Buddenbrockia* sp. and *Myxidium* sp.

Table IX: Results of light microscopic (LM) observation and PCR screening of fish kidney samples.

Fish species	Nr. of all samples	LM-/ Mala PCR-	LM-/ Mala PCR+	LM+/ Mala PCR-	LM+/ Mala PCR+
Abramis brama	4	3	1	0	0
Alburnoides bipunctatus	1	1	0	0	0
Alburnus alburnus	18	9	2	1	6
Aspius aspius	8	7	0	1	0
Ballerus sapa	9	8	1	0	0
Barbus barbus	4	3	0	0	1
Blicca bjoerkna	30	16	14	0	0
Carassius auratus auratus	53	33	15	4	1
Chondrostoma nasus	2	0	2	0	0
Cyprinus carpio	61	22	21	10	9
Gasterosteus aculeatus	1	0	0	1	0
Gobio gobio	9	3	4	1	1
Lepomis gibbosus	3	3	0	0	0
Leucaspius delineatus	2	0	0	0	2
Leuciscus idus	3	0	2	0	1
Leuciscus leuciscus	3	2	1	0	0
Oncorhynchus mykiss	15	0	2	0	13
Perca fluviatilis	6	5	1	0	0
Rhodeus sericeus amarus	2	2	0	0	0
Rutilus rutilus	6	2	3	1	0
Salvelinus fontinalis	2	2	0	0	0
Sander lucioperca	28	27	0	1	0
Scardinius erythrophthalmus	2	1	0	0	1
Squalius cephalus	3	0	0	2	1
Tinca tinca	3	3	0	0	0

Malacosporean spores were found only sporadically and only in common carp and goldfish (Figure 8). Subsequent PCR screening of the malacosporean light microscope-positive samples from common carp and goldfish revealed that malacosporean spores belonged to *Buddenbrockia* sp. 2 and no other myxozoan infection was confirmed using general myxozoan primers thereafter. Nevertheless, the plasmodia in these samples were not numerous. Malacosporean plasmodia in the kidney tubules were usually immature; spores were detected only occasionally inside monosporic pseudoplasmodia. Intratubular pseudoplasmodia possessed thin walls. The early plasmodial stages of malacosporeans were small in size, globular in shape and rich in refractile granules of unequal size. Formation of two spherical polar capsules was visible within more developed elongate stages. The spores of *Buddenbrockia* sp. 2 observed in kidney tubules were globular, elongate to ovoid in shape and possessed thin walls, typical for malacosporeans. The shell valves were generally difficult to recognize.

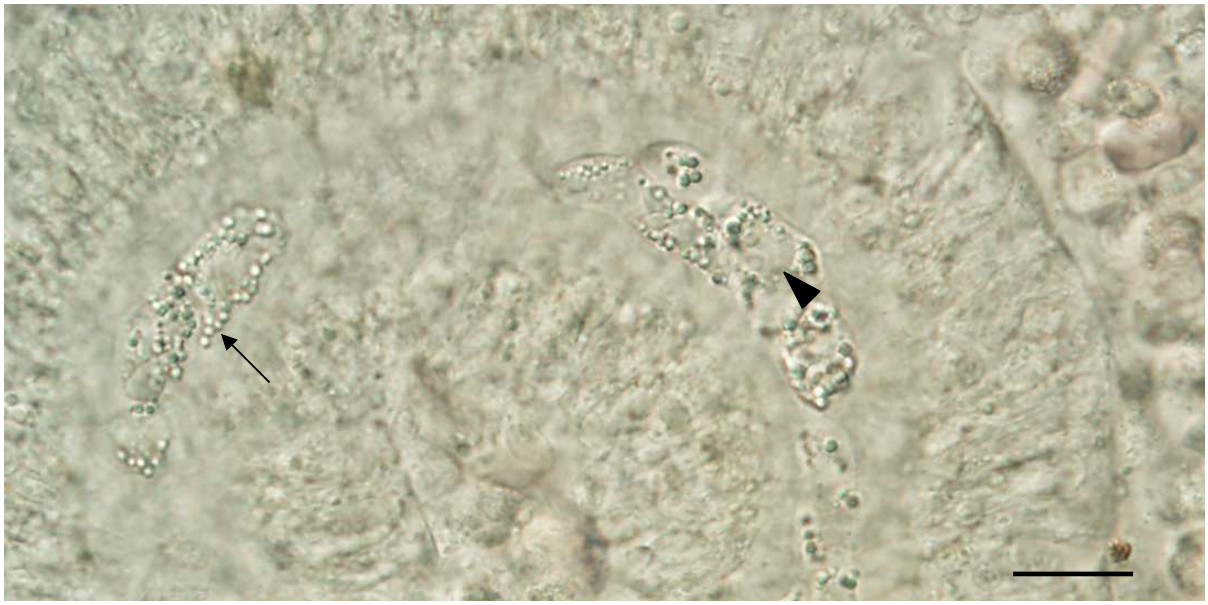

Fig. 8. Morphology of *Buddenbrockia* sp. 2 in the kidney tubules of goldfish *Carassius auratus auratus* from Chřešťovice fish farm, CR as observed by light mictroscopy. Intratubular sporogonic plasmodia containing numerous refractile granules (arrow) observed by light microscopy, mature fish malacospore (arrowhead) with spherical polar capsules and soft shell valves. Scale bar 20 µm.

4.3.2. Molecular identification and distribution of malacosporeans in fish

In total, 351 fish samples were screened using Malacosporea-specific primers. In total, 123 samples, consisting of 108 kidneys, 7 blood samples, 5 urinary bladders, 2 brains and 1 heart were positive for malacosporean DNA. In addition to two previously described nominal species, *i.e. Buddenbrockia plumatellae* and *Tetracapsuloides bryosalmonae*, one so far undescribed but previously reported malacosporean species (Grabner and El-Matbouli 2010), *i.e. Buddenbrockia* sp. 2, was detected in the screened fish. Due to the DNA

sequences obtained, five new species of Malacosporea were detected in our samples, *i.e.* *Buddenbrockia* sp. 3, *Tetracapsuloides* sp. 2, *Tetracapsuloides* sp. 3, *Tetracapsuloides* sp. 4 and *Tetracapsuloides* sp. 5. The prevalence of certain species was relatively high in the screened samples and even reached 100% in some fish and localities (Table X).

Table X: List of malacosporean species found in fish by PCR screening of field samples with data on their localities, sequences and prevalence.

Malacosporean species	Fish species	Locality	GenBank acc. No.	Parasite prevalence
Buddenbrockia plumatellae	*Abramis brama*	Danube River, at Štúrovo, SR	KF731680	25% (1/4)
	Alburnus alburnus	Jindřiš fish farm, CR	KF731681	44% (5/11)
		Hron River, at Štúrovo, SR	KF731683	20% (1/5)
	Blicca bjoerkna	Dyje River, south of Břeclav, CR	KF731685	61% (11/18)
		Rožmběrk Pond, CR	KF731687	40% (2/5)
		Danube River, at Štúrovo, SR	KF731688	14% (1/7)
	Chondrostoma nasus	Hron River, at Štúrovo, SR	KF731689	100% (2/2)
	Leuciscus idus	Dyje River, south of Břeclav, CR	KF731690	100% (3/3)
	Leuciscus leuciscus	Dyje River, south of Břeclav, CR	KF731692	100% (1/1)
	Perca fluviatilis	Rožmberk Pond, CR	KF731693	50% (1/2)
	Rutilus rutilus	Dyje River, south of Břeclav, CR	KF731694	50% (2/4)
		Rožmberk Pond, CR	KF731695	50% (1/2)
	Scardinius erythrophthalmus	Jihlava, CR	KF731696	50% (1/2)
	Squalius cephalus	Dyje River, south of Břeclav, CR	KF731698	33% (1/3)
***Buddenbrockia* sp. 2**	*Carassius auratus auratus*	Jihlava, CR	KF731699	14% (1/7)
		Chřešťovice fish farm, CR	KF731700	65% (15/23)
	Cyprinus carpio	Hortobágy, Hungary	KF731702	27% (3/11)
		Horní Hluboký Pond, Strmilov, CR	KF731703	60% (9/15)
		Malá Outrata Pond, CR	KF731704	50% (1/2)
		Motovidlo Pond, CR	KF731705	100% (2/2)
		Chřešťovice fish farm, CR	KF731706	76% (13/17)
		Vodňany, CR	KF731707	100% (2/2)
***Buddenbrockia* sp. 3**	*Barbus barbus*	Dyje River, south of Břeclav, CR	KF731708	100% (1/1)
Tetracapsuloides bryosalmonae	*Oncorhynchus mykiss*	Jindřiš fish farm, CR	KF731711	100% (15/15)
***Tetracapsuloides* sp. 2**	*Gobio gobio*	Jindřiš fish farm, CR	KF731713	25% (1/4)
***Tetracapsuloides* sp. 3**	*Ballerus sapa*	Danube River, at Štúrovo, SR	KF731714	11% (1/9)
	Cyprinus carpio	Hortobágy, Hungary	KF731716	9% (1/11)
	Gobio gobio	Jindřiš fish farm, CR	KF731717	75% (3/4)
		České Budějovice, CR	KF731720	100% (1/1)
	Leucaspius delineatus	Jindřiš fish farm, CR	KF731721	100% (2/2)
***Tetracapsuloides* sp. 4**	*Alburnus alburnus*	Dyje River, south of Břeclav, CR	KF731725	100% (1/1)
		Hron River, at Štúrovo, SR	KF731726	20% (1/5)
***Tetracapsuloides* sp. 5**	*Gobio gobio*	Jindřiš fish farm, CR	KF731728	20% (1/5)
		Dyje River, south of Břeclav, CR	KF731729	100% (2/2)

Although most of the PCR positive samples contained single malacosporean infection, coinfections were also detected in two kidney samples, *i.e. Buddenbrockia* sp. 2 + *Tetracapsuloides* sp. 3 in *Cyprinus carpio* from Hungary and *Tetracapsuloides* sp. 2 + *Tetracapsuloides* sp. 3 + *Tetracapsuloides* sp. 5 in *Gobio gobio* from Jindřiš fish farm (Figure 9).

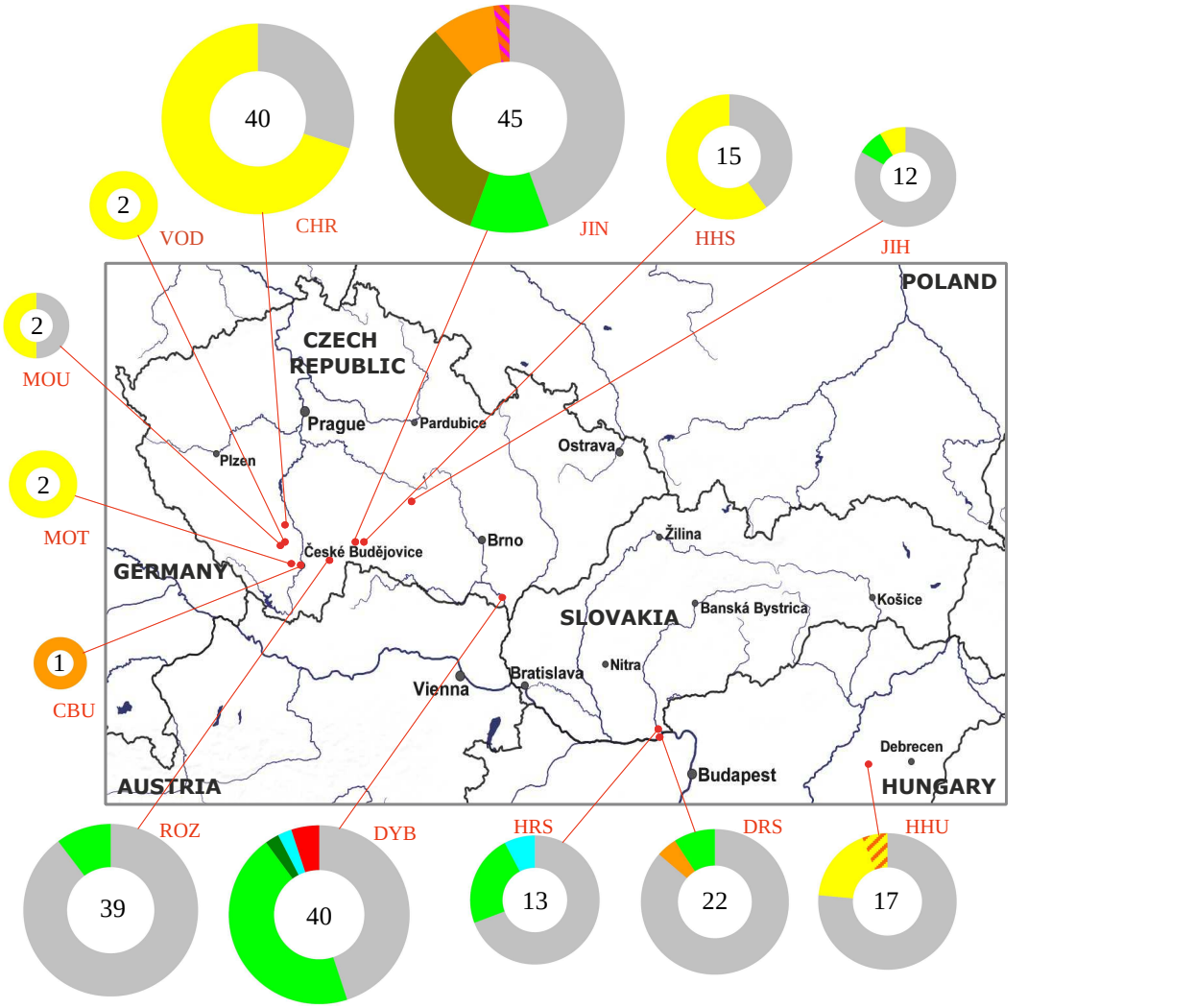

Fig. 9. Malacosporean prevalence and distribution as determined by PCR of fish hosts and localities in the Czech Republic, Hungary and Slovak Republic. The number inside each pie chart indicates the total number of fish examined at each locality. CBU, České Budějovice, CR; CHR, Chřešťovice, CR; DRS, Danube River, at Štúrovo, SR; DYB, Dyje River, South of Břeclav, CR; HHS, Horní Hluboký Pond, Strmilov, CR; HHU, Hortobágy, HU; HRS, Hron River, at Štúrovo, SR; JIH, Jihlava, CR; JIN, Jindřiš, CR; MOT, Motovidlo Pond, CR; MOU, Malá Outrata Pond, CR; ROZ, Rožmberk Pond, CR; VOD, Vodňany, CR.

Malacosporean parasites were abundant in the examined fish from Central European localities. In total, fish from 13 out of 16 localities were PCR positive for malacosporean DNA. The highest number of malacosporean species was observed at Jindřiš (*n*=5) and at Štúrovo in the Danube River (*n*=4).

No preference of malacosporeans for certain aquatic ecosystem was observed. For example, *B. plumatellae*, *Tetracapsuloides* sp. 3 and *Tetracapsuloides* sp. 5 were found not only in lentic (static) water but also in lotic (flowing) ecosystems. *Tetracapsuloides bryosalmonae* was detected only in static water in this study (Table XI). Even though *Buddenbrockia* sp. 2 and *Tetracapsuloides* sp. 2 were found only in ponds, malacosporeans *Buddenbrockia* sp. 3 and *Tetracapsuloides* sp. 4 were detected in fish collected from flow habitats.

Table XI: Malacosporean occurence according to host habitat.

Aquatic system	Exclusively lotic	Exclusively lentic	Both lotic and lentic
Malacosporean species	*Buddenbrockia* sp. 3 *Tetracapsuloides* sp. 2 *Tetracapsuloides* sp. 4 *Tetracapsuloides* sp. 5	*Buddenbrockia* sp. 2 *T. bryosalmonae*	*Tetracapsuloides* sp. 3

4.3.3. Increase of the diversity and host species spectrum of the Malacosporea

This study revealed five new malacosporean species of the Malacosporea, *i.e. Buddenbrockia* sp. 3 and *Tetracapsuloides* sp. 2–5. PCR screening of our fish samples from different fish host species revealed that malacosporeans detected in this study have a wide fish host species spectrum. For example, 11 fish species from the families Cypriniformes and Perciformes are new host records for *Buddenbrockia plumatellae* (the only previously known fish host was *Phoxinus phoxinus*, Grabner and El-Matbouli 2010). Another example is *Tetracapsuloides* sp. 3, which was found in 8 fish host species from the families Cypriniformes and Perciformes. *T. bryosalmonae* was restricted to salmonid fish only. *Buddenbrockia* sp. 3 and *Tetracapsuloides* sp. 4 were found in two cyprinid species as well as *Buddenbrockia* sp. 2 which exclusively infected the genera *Cyprinus* and *Carassius.* Rather strict host specificity was revealed for *Tetracapsuloides* sp. 2 and *Tetracapsuloides* sp. 5 infecting only one cyprinid species, *Gobio gobio* (Table XII).

Table XII: Vertebrate and invertebrate host spectra for malacosporeans detected in this study and previously (Anderson *et al.* 1999, Tops *et al.* 2005, Canning *et al.* 2007, Grabner and El-Matbouli 2010, Evans *et al*. 2010, Bartošová-Sojková *et al.* 2014, Hartikainen *et al.* 2014).

Malacosporean species	Fish host	Bryozoan host	Shape of related stage in bryozoa
Buddenbrockia plumatellae worm-like	***Abramis brama*** ***Alburnus alburnus*** ***Aspius aspius*** ***Blicca bjoerkna*** ***Chondrostoma nasus*** ***Leuciscus idus*** ***Leuciscus leuciscus*** ***Perca fluviatilis*** *Phoxinus phoxinus* ***Rutilus rutilus*** ***Scardinius erythrophthalmus*** ***Squalius cephalus***	*Hyallinella punctata* *Lophopodella carterii* *Plumatella fungosa* *Plumatella repens* *Stolella evelinae*	worm
Buddenbrockia plumatellae sac-like	unknown	*Cristatella mucedo*	sac
Buddenbrockia sp. 1	unknown	*Cristatella mucedo*	sac
Buddenbrockia sp. 2	***Carassius auratus auratus*** ***Carassius gibelio*** *Cyprinus carpio*	unknown	unknown
***Buddenbrockia* sp. 3**	***Barbus barbus*** ***Rutilus rutilus***	unknown	unknown
Buddenbrockia sp.	unknown	*Fredericella sultana*	worm
Novel lineage	unknown	*Plumatella fungosa*	worm
Buddenbrockia allmani	unknown	*Lophopus crystallinus*	sac
Tetracapsuloides bryosalmonae	*Oncorhynchus mykiss* *Salmo salar* *Salmo trutta*	*Cristatella mucedo* *Fredericella sultana* *Pectinatella magnifica* *Plumatella emarginata* *Plumatella rugosa*	sac
Tetracapsuloides sp. 1	unknown	*Cristatella mucedo* *Pectinatella magnifica* *Plumatella rugosa*	sac
***Tetracapsuloides* sp. 2**	***Gobio gobio***	unknown	unknown
***Tetracapsuloides* sp. 3**	***Ballerus sapa*** ***Barbus barbus*** ***Cyprinus carpio*** ***Gobio gobio*** ***Leucaspius delineatus*** ***Leuciscus idus*** ***Perca fluviatilis*** ***Rutilus rutilus***	unknown	unknown
***Tetracapsuloides* sp. 4**	***Alburnus alburnus*** ***Rutilus rutilus***	unknown	unknown
***Tetracapsuloides* sp. 5**	***Gobio gobio***	unknown	unknown
Malacosporea sp. 1	unknown	*Fredericella indica* *Fredericella sultana*	lobey
Malacosporea sp. 2	unknown	*Fredericella sultana*	worm
Malacosporea sp. 3	unknown	*Plumatella sp.*	worm

Note$_1$: Species highlighted in dark blue show new malacosporean species obtained in this study. The light blue highlighted hosts represent new hosts confirmed by members of the Laboratory of Fish Protistology, PAU, BC CAS without contribution of the author. Hosts highlighted in green represent newly confirmed hosts by members of the Laboratory of Fish Protistology. PAU, BC CAS with contribution of the author.

Note$_2$: References – *Buddenbrockia* sp. 1 (Hartikainen *et al.* 2014); *Buddenbrockia* sp. (Tops *et al.* 2005) corresponds to *Buddenbrockia* sp. 2 in Hartikainen *et al.* (2014) and to *Buddenbrockia* sp. 1 in Bartošová-Sojková *et al.* (2014); *Buddenbrockia* sp. 2 (Bartošová-Sojková *et al.* 2014) corresponds to *Buddenbrockia* sp. in Grabner and El-Matbouli (2010); *Buddenbrockia* sp. 3 (Bartošová-Sojková *et al.* 2014); *Tetracapsuloides* sp. 1 (Hartikainen *et al.* 2014) corresponds to an unidentified myxozoan parasite of Anderson *et al.* (1999); *Tetracapsuloides* sp. 2 through *Tetracapsuloides* sp. 5 (Bartošová-Sojková *et al.* 2014); Malacosporea sp. 1 through Malacosporea sp. 3 (Hartikainen *et al.* 2014); Novel lineage (Bartošová-Sojková *et al.* 2014) correspons to *B. plumatellae* in Evans *et al.* (2010) and to Buddenbrockia sp. 4 in Hartikainen *et al.* 2014).

4.4. Genetic distances and phylogenetic analyses

Taking into account the difficulty of morphology-based species determination of the Malacosporea by light microscopy, the identification of species in this study was principally carried out on the basis of molecular analyses.

Based on known inter-species variations in myxosporeans (Bartošová and Fiala 2011), the 1% nt sequence divergence in the SSU rDNA was established as a genetic yardstick to discriminate individual species. Intraspecific variability in the SSU rDNA of malacosporean species ranged from 0.00% to 0.76%. The exception was represented by *Buddenbrockia plumatellae* as the variability between the worm- and sac-like stages was 1.22%, and they have been considered different species in the past (see Section 5.3.). The lowest interspecific variability within the genus *Tetracapsuloides* (1.67%) was found between *T. bryosalmonae* and *Tetracapsuloides* sp. 5. This percentage represented also the lowest interspecific variability within the whole class Malacosporea. The largest interspecific divergence in the genus *Tetracapsuloides* (6.22%) was found between *Tetracapsuloides* sp. 3 and *Tetracapsuloide*s sp. 4. The lowest interspecific variability in genus *Buddenbrockia* was represented by 1.22% between *B. plumatellae* worm- and sac-like stages. The highest dissimilarity within the *Buddenbrockia* (6.47%) was found between *Buddenbrockia* sp. and *B. plumatellae* sac form. The maximum interspecific variability in malacosporeans (24.06%) was calculated between *B. plumatellae* sac form and Malacosporea sp. 1 (Figure 10).

% of dissimilarity	*Buddenbrockia plumatellae* worm																
Buddenbrockia plumatellae worm	0.637	*Buddenbrockia plumatellae* sac															
Buddenbrockia plumatellae sac	1.221	0.538	*Buddenbrockia allmani*														
Buddenbrockia allmani	5.989	5.545	0.727	*Buddenbrockia* sp.													
Buddenbrockia sp.	5.107	6.471	4.883	0.764	*Buddenbrockia* sp. 1												
Buddenbrockia sp. 1	3.896	4.203	3.242	4.837	-	*Buddenbrockia* sp. 2											
Buddenbrockia sp. 2	3.829	4.162	2.594	4.336	3.252	0.061	*Buddenbrockia* sp. 3										
Buddenbrockia sp. 3	4.767	5.382	3.172	5.214	3.680	2.436	0.000	*Tetracapsuloides bryosalmonae*									
Tetracapsuloides bryosalmonae	19.581	18.071	20.057	20.642	18.279	14.044	13.969	0.617	*Tetracapsuloides* sp. 1								
Tetracapsuloides sp. 1	19.813	19.106	21.846	23.632	20.592	14.667	14.593	2.158	0.339	*Tetracapsuloides* sp. 2							
Tetracapsuloides sp. 2	14.716	6.723	6.372	15.107	15.935	11.116	11.247	4.085	4.566	-	*Tetracapsuloides* sp. 3						
Tetracapsuloides sp. 3	19.789	16.128	17.272	19.298	15.285	15.110	14.846	5.870	5.784	4.048	0.688	*Tetracapsuloides* sp. 4					
Tetracapsuloides sp. 4	18.247	15.444	16.523	20.773	20.651	14.427	14.162	2.846	3.139	5.119	6.219	0.000	*Tetracapsuloides* sp. 5				
Tetracapsuloides sp. 5	17.862	15.198	17.733	20.384	20.477	14.057	13.980	1.672	2.341	3.396	5.417	2.524	-	Novel lineage			
Novel lineage	21.871	18.765	23.721	23.431	22.518	17.302	17.219	16.447	16.590	12.392	16.617	16.218	15.848	-	Malacosporea sp. 1		
Malacosporea sp. 1	21.372	24.056	22.831	22.175	22.041	17.838	18.241	20.526	21.083	13.894	22.482	21.225	21.168	23.116	0.076	Malacosporea sp. 2	
Malacosporea sp. 2	19.046	21.329	20.504	22.674	22.688	16.450	16.066	17.263	18.016	11.149	18.992	17.847	17.969	20.893	9.807	0.155	Malacosporea sp. 3
Malacosporea sp. 3	19.885	19.913	20.260	20.615	22.936	15.116	15.282	9.594	9.683	5.604	7.238	10.295	9.380	20.437	20.410	19.490	-

Fig. 10. Distance matrix showing the maximum percentage of SSU rDNA sequence dissimilarity among the malacosporean taxa. Dash indicates that interspecific variability was not possible to calculate as only one sequence was available.

Note: Coloured species with new data obtained in this study corresponds to colouring in legend in Figure 9, data for grey coloured species were obtained from Genbank.

All sequences obtained by screening of fish samples clustered together within a single, strongly supported malacosporean lineage. In all three phylogenetic analyses performed using different multiple alignments (E-INS-i, L-INS-i and Geneious alignment) the malacosporean lineage was found to split into two strongly supported clades, *i.e.* the *Buddenbrockia* clade and the *Tetracapsuloides* clade, and three weaker supported lineages, *i.e.* The novel malacosporean lineage, Malacosporea sp. 1 and Malacosporea sp. 2 (Figure 11, 12, 13).

The *Buddenbrockia* clade included *Buddenbrockia plumatellae* sac and worm stage, *Buddenbrockia allmani*, *Buddenbrockia* sp., *Buddenbrockia* sp. 1–3. On one hand, some phylogenetic analyses (E-INS-i, L-INS-i alignment) using ML showed sister clustering of *Buddenbrockia* sp. 3 and *B. allmani*, nevertheless the support of this formation was very low. On the other hand, one analysis (Geneious alignment) using ML placed *Buddenbrockia* sp. 3 within the *Buddenbrockia* clade but not sister to *B. allmani*. Considering the weak nodal support values, the position of the species in the *Buddenbrockia* clade was very unstable except for *B. plumatellae* sac and worm stage that clustered together in a well supported group (Figure 11, 12, 13).

The *Tetracapsuloides* clade contained *T. bryosalmonae*, *Tetracapsuloides* sp. 1–5 and Malacosporea sp. 3. The SSU-based phylogenies robustly placed Malacosporea sp. 3 represented by the only sequence obtained from a motile worm from *Plumatella repens* in Borneo, MYS (NCBI: KJ150277) and *Tetracapsuloides* sp. 3 as one sister group splitting into two separate, closely related clades. In all analyses performed, the clustering of *Tetracapsuloides* sp. 2 with sister group of *Tetracapsuloides* sp. 3 and Malacosporea sp. 3 was also well supported using ML. Considering bootstrap values, the position of other species in the *Tetracapsuloides* clade was unstable in all nine performed analyses (Figure 11, 12, 13).

The novel lineage represented by a single sequence of malacosporean from the bryozoan *Plumatella fungosa* from Ohio, USA (NCBI: FJ981824). This lineage clustered with low support either as a sister group to the *Tetracapsuloides* clade (all three aligments performed using ML and using MP within the E-INS-i alignment) or as a sister group to the *Buddenbrockia* clade (Geneious and L-INS-i aligments using MP method) (trees not shown). All sequences used in phylogenetic analyses are shown in Table XIII.

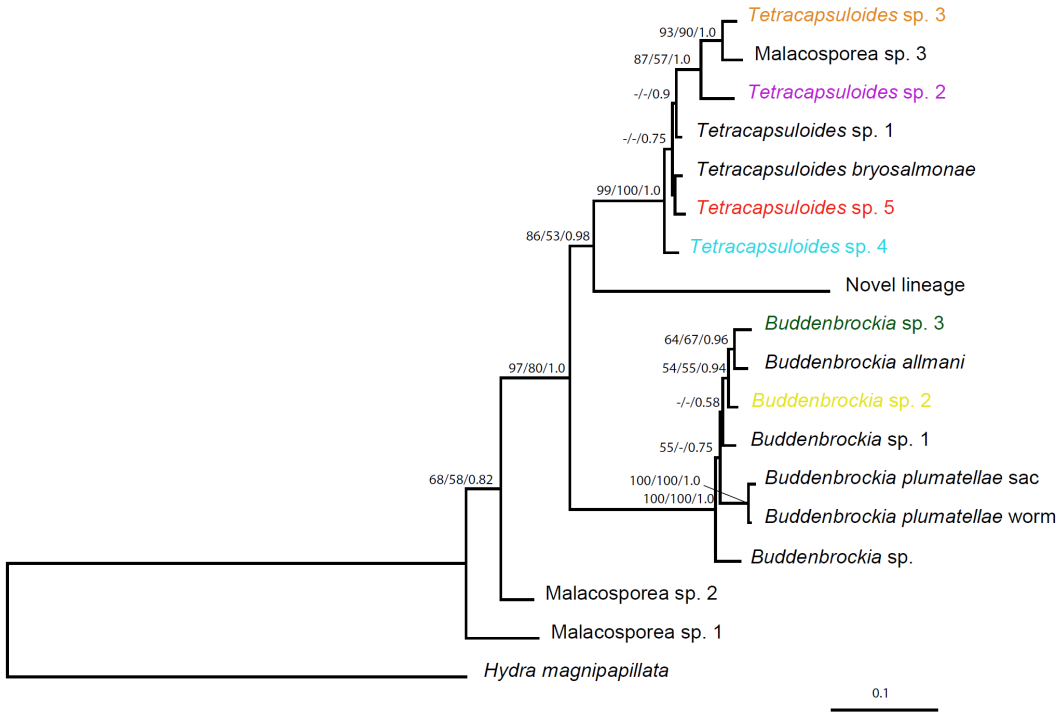

Fig. 11. Maximum Likelihood (using E-INS-i alignment) phylogenetic tree based on SSU rDNA data showing the phylogenetic trends in clustering of malacosporeans. Numbers at nodes indicate nodal supports for Maximum Likelihood/Maximum Parsimony/Bayesian Inference. Bootstraps calculated from 1,000 replicates; nodal supports < 50% not shown. *Note:* Colouring corresponds to legend in Figure 9.

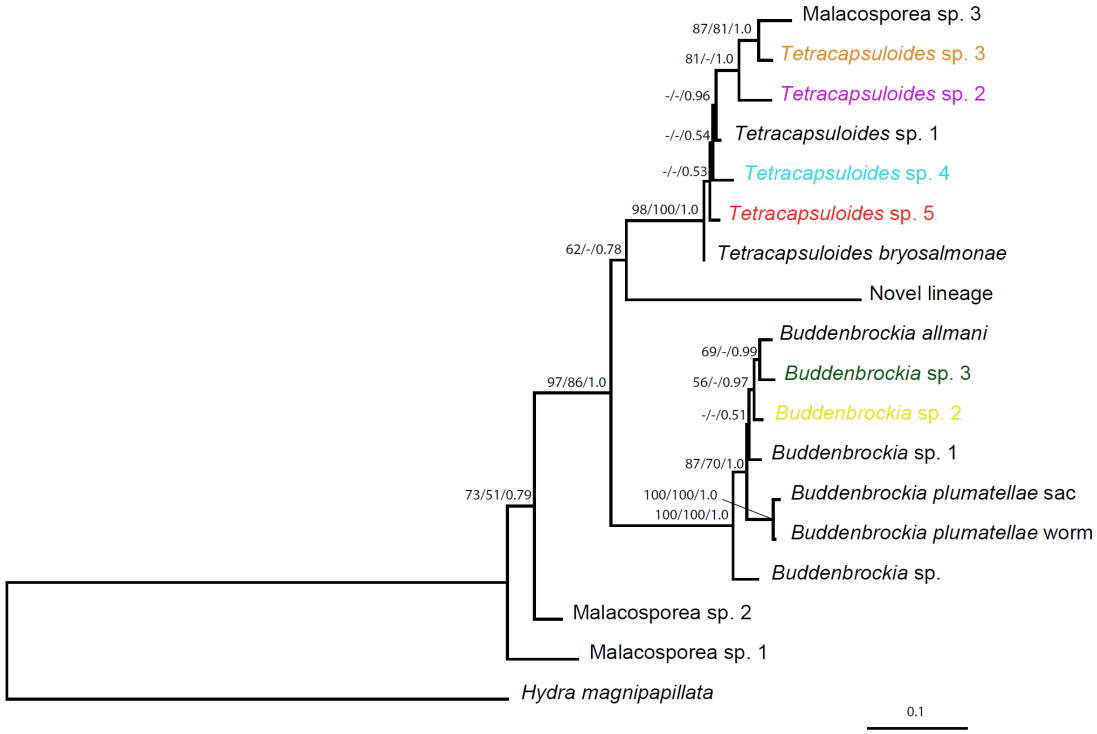

Fig. 12. Maximum Likelihood (using L-INS-i alignment) phylogenetic tree based on SSU rDNA data showing the phylogenetic trends in clustering of malacosporeans. Numbers at nodes indicate nodal support for Maximum Likelihood/Maximum Parsimony/Bayesian Inference. Bootstraps calculated from 1,000 replicates; nodal supports < 50% not shown. *Note:* Colouring corresponds to legend in Figure 9.

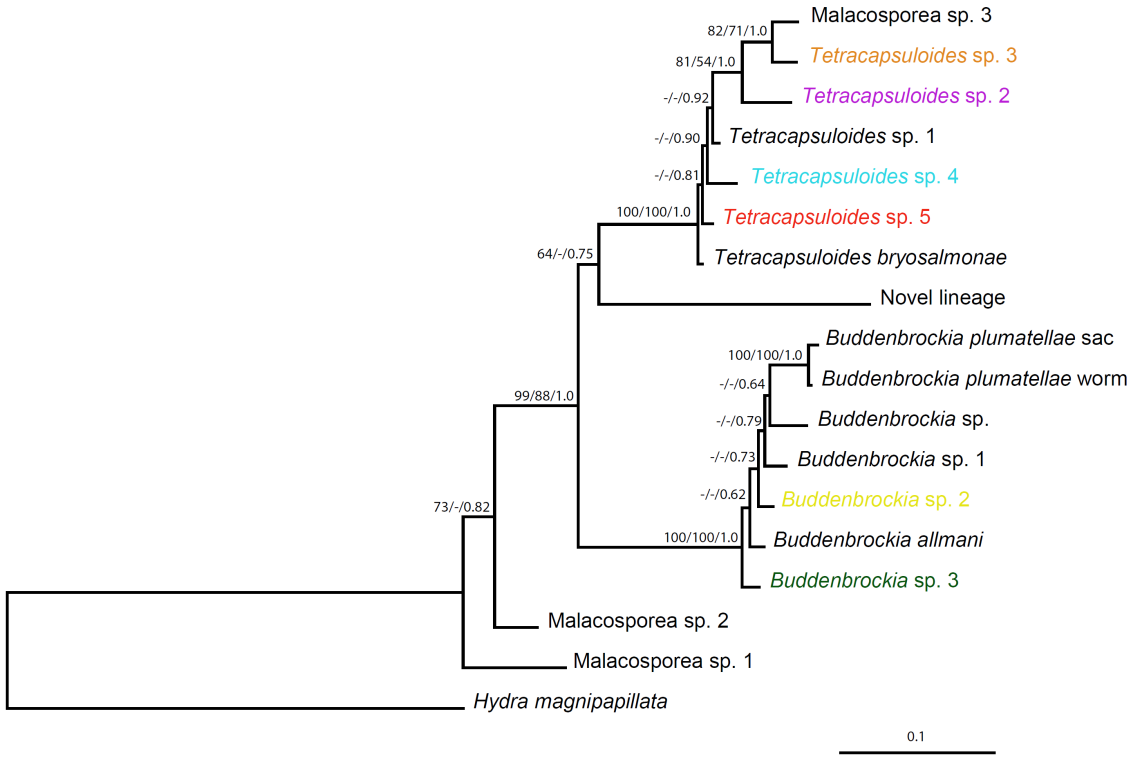

Fig. 13. Maximum Likelihood (using Geneious alignment) phylogenetic tree based on SSU rDNA data showing the phylogenetic trends in clustering of malacosporeans. Numbers at node indicates nodal support for Maximum Likelihood/Maximum Parsimony/Bayesian Inference. Bootstraps calculated from 1,000 replicates; nodal supports < 50% not shown. *Note:* Colouring corresponds to legend in Figure 9.

Table XIII: List of sequences with their supporting data used for phylogenetic analyses.

Malacosporean species	**Code**	**Length (bp)**	**Author**
Buddenbrockia plumatellae-sac	AJ937882	1042	Tops *et al.* 2005
Buddenbrockia plumatellae-worm	KF731698	1745	Bartošová-Sojková *et al.* 2014
Buddenbrockia allmani	AJ937880	1043	Tops *et al.* 2005
Buddenbrockia sp.	AJ937879	1048	Tops *et al.* 2005
Buddenbrockia sp. 1	KJ150261	1603	Hartikainen *et al.* 2014
Buddenbrockia sp. 2	KF731700	1742	Bartošová-Sojková *et al.* 2014
Buddenbrockia sp. 3	KF731708	1741	Bartošová-Sojková *et al.* 2014
Malacosporea sp. 1	KJ150272	1504	Hartikainen *et al.* 2014
Malacosporea sp. 2	KJ150275	1454	Hartikainen *et al.* 2014
Malacosporea sp. 3	KJ150277	1296	Hartikainen *et al.* 2014
Novel lineage	FJ981824	1734	Evans *et al.* 2010
Tetracapsuloides bryosalmonae	FJ981823	1801	Evans *et al.* 2010
Tetracapsuloides sp. 1	KJ150278	1438	Hartikainen *et al.* 2014
Tetracapsuloides sp. 2	KF731713	590	Bartošová-Sojková *et al.* 2014
Tetracapsuloides sp. 3	KF731715	1725	Bartošová-Sojková *et al.* 2014
Tetracapsuloides sp. 4	KF731725	1725	Bartošová-Sojková *et al.* 2014
Tetracapsuloides sp. 5	KF731729	1724	Bartošová-Sojková *et al.* 2014

Note: Hydra magnipapillata sequence HQ392522 was used as an outgroup.

4.5. Molecular identification of malacosporeans in marine bryozoans

In total, 97 samples of 4 marine bryozoan species were screened to detect potential malacosporean infection. Bands obtained in 28 samples amplified with general eukaryotic (Erib1/Erib10) and Myxozoa-specific primers (Myxgp2f/ACT1r) were sequenced. From this number, 7 amplicons belonged to myxosporean species (Table XIV). Malacosporean infection was neither found using mala-f/r primers nor with abovementioned general eukaryotic and Myxozoa-specific primers.

Table XIV: Myxosporeans detected with PCR in marine bryozoans samples.

Bryozoan sp.	**Locality**	**Date of collection**	**Myxosporea sp.**
Zoobotryon verticillatum	*City Island Sarasota, Florida*	17.09.2014	*Kudoa* sp. 1
Zoobotryon verticillatum	*City Island Sarasota, Florida*	17.09.2014	*Kudoa* sp. 1
Zoobotryon verticillatum	*City Island Sarasota, Florida*	17.09.2014	*Kudoa* sp. 2
Bugula neritina	*City Island Sarasota, Florida*	17.09.2014	*Myxobolus* sp. 1
Zoobotryon verticillatum	*Lido Key Sarasota, Florida*	27.10.2014	*Myxobolus* sp. 2
Bryozoa sp. 2	*Lido Key Sarasota, Florida*	27.10.2014	*Myxobolus* sp. 3
Bryozoa sp. 2	*Lido Key Sarasota, Florida*	27.10.2014	*Kudoa* sp. 3

5. DISCUSSION

5.1. Bryozoan cultivation and cohabitation experiments

An important aim of this study was to hatch and culture bryozoans *in vitro* by establishing suitable algae cultures as a source of food for freshwater bryozoans. Live, SPF bryozoan colonies raised under laboratory conditions are an essential prerequisite for the carrying out of transmission experiments. Culturing of bryozoans under laboratory conditions has been problematic in the past and in the case of certain species (e.g. *Cristatella* sp. and *Pectinatella* sp.) it has never been achieved for more than a few days (Wood 2005). One of the most efficient, simple and minimum time-consuming culture systems is the maintenance of bryozoan colonies in aged pond water with fish present in the same tank (Mukai 1980). Even though such water contains enough nutrients for bryozoans, the main problem for the study of infectious agents is that one cannot be sure that fish as well as bryozoan colonies, which germinate from statoblasts, are pathogen-free (SPF).

Thus, autoclaved WC medium containing only salts, metals and vitamins commonly used to culture algae (Guillard and Lorenzen 1972) was used for the cultivation of algae, which were later added into the bryozoan tank as a source of food. In our study, the medium has been found to be appropriate for culturing of *Chlamydomonas*, *Cryptomonas* and *Fragilaria.* Nevertheless, utilization of algae alone as a food source for bryozoans was not efficient for long-term maintenance. Bryozoan colonies flourished only for a few months and started to die after half a year. However, cultured algae as a food source for bryozoans are suitable for short-term maintenance and experiments, such as the ones performed in this study. Another option of bryozoan culturing in the future may be a recently published BMC medium that enabled the maintenance of *Fredericella sultana* colonies under laboratory conditions for more than 12 month (Kumar *et al.* 2013). The BMC medium is likely also suitable for culturing other bryozoan species such as *Plumatella repens*. Another method for long-term maintenance of bryozoans can possibly include zooplankton species such as e.g. rotifers.

Unfortunately, none of the aquaria-based transmission experiments was successful. The reason why Cohabitation experiment 1 failed was probably due to missing malacosporean infection in the bryozoans, despite their collection at a malacosporean-positive locality in Chřešťovice (data from preliminary screening of fish). In Cohabitation experiment 2, infections from *Buddenbrockia* sp. 2-positive fish were not transmitted to the bryozoans situated in the same experimental tank. All bryozoan colonies checked microscopically (stereomicroscope) were negative, however, not all colonies were screened and early

infectious stages may well remain uncovered. The conditions under which malacosporean stages mature and are best transmitted are unknown and laboratory infections have been notoriously difficult. Only after years of experimental trials by different research groups (Feist *et al.* 2001, Tops *et al.* 2004) the study of Morris and Adams (2006) provided evidence of transmission of mature fish malacospores of *Tetracapsuloides bryosalmonae* to the bryozoan *F. sultana*. The transmission of malacospores from bryozoan sac-like stages is influenced by temperature and water flow (Hedrick *et al.* 1993, Morris *et al.* 2005, Schmidt-Posthaus *et al.* 2012) but it is not yet clear which conditions are required for the *vice versa* transmission. Moreover, it is difficult to estimate if the fish used for experimental infection in the present study were still infectious, *i.e.* if the plasmodia were producing spores later released into the aquatic environment (experimental tank) via the urine of the host. It is also unclear whether the timing of the experimental exposure and the screening for infections was correct. In some Myxosporea (e.g. *Myxobolus* sp.) the maturation of spores take up to 3 months after infection of fish via infective triactionomyxon spore stages (Lom and Dyková 2006). Our effort to elucidate the bryozoan host of *Buddenbrockia* sp. 2 was not successful. We used the same fish (*Cyprinus carpio*) and bryozoan host (*P. repens*) as Grabner and El-Matbouli (2010). These authors demonstrated that *P. repens* colonies collected from a malacosporean-positive locality were PCR positive for *Buddenbrockia* sp. 2. Even though, in their following transmission experiment, 4–5 zooids of statoblast-raised bryozoans of *P. repens* cohabitated with *Buddenbrockia* sp. 2-infected carp were PCR-positive, no overt (visible) infection was observed in the zooids of the remaining colony. They thus concluded that a cryptic infection of *Buddenbrockia* sp. 2 might have already been present in the statoblasts of *P. repens* used for germination and this parasite infectious for carp may be specific to other species of bryozoans. They note that other bryozoan species (*P. fruticosa* and *C. mucedo*), which could serve as potential hosts of *Buddenbrockia* sp. 2, were also found in the pond from which the infected colony of *P. repens* was collected. However, *P. repens* may well be the definitive host of *Buddenbrockia* sp. 2 as it is likely that the conditions in the transmission experiments (ours and of Grabner and El-Matbouli 2010) did not correspond to the natural conditions in the life cycle of this parasite. Moreover, to the best of our knowledge, only *P. repens* was present in our *Buddenbrockia* sp. 2-positive locality.

5.2. Hidden diversity and host spectrum expansion

The PCR screening of fish kidneys and DNA sequencing of amplicons revealed the existence of five new malacosporean species of *Buddenbrockia* and *Tetracapsuloides*. These results provide strong evidence of significantly higher malacosporean diversity in Central European freshwater habitats than previously expected. The fish host spectrum was considerably extended for *B. plumatellae*, which had been reported so far only from *Phoxinus phoxinus* (Grabner and El-Matbouli 2010) as well as for *Buddenbrockia* sp. 2 that was previously known only from *Cyprinus carpio* (Grabner and El-Matbouli 2010). In total, the vertebrate host spectrum of malacosporeans was enriched to 18 additional fish species. It is surprising that despite the intensive research on fish parasites only a low number of malacosporean species had been described. The reasons are probably the difficulty of microscopical detection and identification, resulting from i) the exceptional occurrence of mature malacospores, which ii) additionally lack taxonomically informative spore characters and iii) the relatively small size of plasmodial stages which are easy to overlook, iv) the resulting difficulties in differentiating them from infections caused by other renal tubule-inhabiting myxozoans (e.g. *Hoferellus* spp., *Sphaerospora* spp.), especially if immature, as well as v) mostly asymptomatic infections, with infected fish exhibiting no external signs of disease. In this study, we found a disagreement between the number of malacosporean-positive samples identified by light microscopy and by PCR screening.

Determination of malacosporean stages was difficult due to the abovementioned reasons. The lack of intratubular plasmodia and malacospores in the vast majority of fish kidneys examined in this study may have been caused by parasites entering accidental fish hosts, which represent a dead end. These species may be able to enter the circulatory system (Kallert *et al.* 2011, Holzer *et al.* 2013) but are unable to migrate to the target site and form spores. This has to be taken into the account when considering the numerous new host records found in this study, especially regarding the co-infections detected in several fish. Due to the observation of spores of *Buddenbrockia* sp. 2 in kidney tubules of *Cyprinus carpio* and *Carassius auratus auratus* it can be assumed that both fish species represent true hosts. On the contrary, it is not clear whether *Buddenbrockia* sp. 3 and *Tetracapsuloides* spp. identified in this study are able to form mature fish malacospores in the fish where they were observed since no spores of these malacosporean species were microscopically detected in kidney tubules.

In the present study, PKD was observed only in salmonids and no pathology was found in non-salmonid fish infected with *T. bryosalmonae*. Furthermore, fish kidneys infected with malacosporean species other than *T. bryosalmonae* did not exhibit signs of pathology.

No lotic or lentic environment preference has been observed for individual malacosporean species as well as for their sac- or worm-like bryozoan-related stages. Our expectation, that the sac formations would prefer the riverine habitats (advantage of water flow for dispersal of spores) and that motile worm stages would inhabit static waters, was not supported. Two different bryozoan-related morphotypes in *B. plumatellae* are thus rather the result of evolutionary loss/gain of the vermiform shape that evolved as an adaptation to endoparasitism (Hartikainen *et al.* 2014).

5.3. Phylogenetic analyses and distance matrix

A universal rule for defining species boundaries based on molecular data is problematic as diverse organismal groups differ in the speed of evolution of their genes. As myxozoan SSU rDNA is fast evolving (Evans *et al.* 2010), a general level of SSU rDNA sequence variation has not been established to define the species concept in the Myxozoa. The 1% SSU rDNA sequence divergence used as a genetic yardstick to define malacosporean species in this study was based on the known genetic differences in myxosporeans for which interspecific variation is typically >1% (Bartošová and Fiala 2001, Whipps and Kent 2006).

The distance matrix revealed a sequence divergence of 1.22% between the *B. plumatellae* worm- and sac-like stages, which thus suggests that these two stages represent two species rather then one species with two different shapes. This percentage was significantly higher than in previous analyses using a more limited dataset (0.7%, Tops *et al.* 2005). Some previous studies suggested that the worm and sac stage are conspecific, being expressed as facultative polymorphisms in different bryozoan host (Monteiro *et al.* 2002, Tops *et al.* 2005). On the other hand, SSU rDNA phylogenies provided strong support for separation of the two forms as they created two well-supported separate clades (Monteiro *et al.* 2002, Tops *et al.* 2005, Hartikainen *et al.* 2014). Another fact supporting the "two species interpretation" is the strict occurence of these stages in different bryozoan hosts. While sac-forming parasites occur exclusively in *C. mucedo*, the vermiform parasites occur in plumatellids (*Plumatella* spp. and *Hyalinella punctata*) (Hartikainen *et al.* 2014). Nevertheless, the relatively low sequence divergence between the two forms might be the result of a recent or ongoing speciation or a recent host switching (Hartikainen *et al.* 2014).

Our screening of a large number of marine bryozoans did not reveal any marine malacosporeans in the examined hosts. The individual screening was difficult due to lots of contaminating material (mainly algae and ciliates) in the samples. Moreover, the Malacosporea-specific primers may not have worked as they are primarily designed for freshwater malacosporeans. Nevertheless, the general myxozoan primers designed to target also conservative regions of myxozoan SSU rDNA would most probably amplify the parasite if present in the sample. However, we still believe in a marine origin of the most basal myxozoans, as they emerged from free-living cnidarians, which occure predominantly in marine habitats. These may be parasitizing bryozoans, supported by the fact that the vast majority of bryozoans are marine species (Gordon 1999). Reports of vermiform stages reminiscent of malacosporean parasites in marine bryozoans exist from the Falkland Islands and the Patagonia shelf *i.e. Beania magellanica*, *Camptoplites giganteus*, *Notoplites drygalskii*, *Notoplites vanhoffeni*, *Notoplites tenius* and *Menipea Flagellifera* (Hastings 1943). Considering the extremely low prevalence of infections of myxosporeans in their definitive hosts (annelids), screening of 97 samples might not have been sufficient to detect the parasites in marine bryozoans. Another hypothesis is that marine malacosporeans may utilize different invertebrate hosts, e.g. phoronids or brachiopods. The latter two groups are phylogenetically older than bryozoans and freshwater bryozoans, presently the only known hosts of malacosporeans, represent the earliest lineage of bryozoans (Fuchs *et al.* 2009, Waeschenbach *et al.* 2012). Thus, the existence of malacosporeans in the marine environment cannot be ruled out and further screening of more marine hosts (not only bryozoans) is required. Our findings of myxosporeans in marine bryozoans are explained by filtration of the myxospores by the zooids of the bryozoans along with food particles rather than the bryozoans being a part of their life cycles. This idea is supported by the finding of individual *Myxobolus* myxospores in some of the samples of marine bryozoans during their light microscopy examination.

In summary, this study suggests that the malacosporean biodiversity in freshwater is much wider than expected. Taking into account that more than 80% of all investigated localities contained at least one malacosporean species it can be assumed that the globally distributed bryozoans will be infected by an even wider diversity of malacosporeans. However, there is still a missing gap in the knowledge of the host species spectra and life cycles of individual malacosporean species. Moreover, malacosporeans in marine environments (if existing) are still hidden in the oceans, but future investigations into marine

bryozoans and other invertebrate groups could solve this puzzle and contribute important information on the origins of the myxozoans.

6. CONCLUSIONS

- Wright's cryptophyte medium proved to be easy to prepare and suitable for culturing different species of algae *i.e. Chlamydomonas*, *Cryptomonas* and *Fragilaria*.

- The use of a mixture of *Chlamydomonas*, *Cryptomonas* and *Fragilaria* for bryozoan feeding resulted in successful short-term maintenance of bryozoans under laboratory conditions but different methods on bryozoan diets for long-term maintenance are recommended for future experiments.

- None of experimental transmissions was successful most probably due to missing malacosporean infection in the bryozoans used to infect fish in Cohabitation experiment 1 and by the fact that infected fish probably did not release mature spores into the water to infect bryozoans in Cohabitation experiment 2.

- Molecular screening of samples revealed the high prevalence and hidden diversity of malacosporeans in cypriniform and perciform fish host species from Central European freshwater habitats by adding five new species of *Buddenbrockia* and *Tetracapsuloides*.

- Fish host species spectrum was extended for *B. plumatellae*, *i.e. Abramis brama*, *Alburnus alburnus*, *Aspius aspius*, *Blicca bjoerkna*, *Chondrostoma nasus*, *Leuciscus idus*, *Leuciscus leuciscus*, *Perca fluviatilis*, *Rutilus rutilus*, *Scardinius erythrophthalmus*, *Squalius cephalus*; and for *Buddenbrockia* sp. 2, *i.e. Carassius auratus auratus* and *Carassius gibelio*.

- Overall malacosporean phylogenetic analysis revealed a new lineage in the class Malacosporea and determined the position of newly identified species.

- Molecular screening did not reveal any malacosporean infection within the marine bryozoans.

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