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Faculty of Science

HIDDEN DIVERSITY AND EVOLITONARY TRENDS IN MALACOSPOREAN PARASITES (CNIDARIA: MYXOZOA) IDENTIFIED USING MOLECULAR PHYLOGENETICS

RNDr. Thesis

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ANNOTATION

Malacosporeans represent only a minor part of the huge myxozoan diversity, accounting for more than 2000 species. In contrast to the remainder of the Myxozoa, malacosporeans cycle between vertebrate (fish) and invertebrate (bryozoans) hosts and are restricted to freshwater habitats. The present study applies light microscopy and molecular methods to scrutinize the morphology, prevalence, diversity, distribution as well as host and habitat preference of malacosporeans in fish hosts. Comprehensive phylogenetic analyses based on newly obtained malacosporean SSU rDNA sequences and those available in GenBank reveal important evolutionary trends in this group. The significant increase in malacosporean species richness unveiled in the present study points to a hidden biodiversity. Reasons for this cryptic species diversity, the potential existence of malacosporean life cycles in marine environment as well as the evolution of worm- and sac-like morphology in bryozoan host are discussed.

DECLARATION [in Czech]

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České Budějovice, 22.4.2015 _____

Martina Hrabcová

AUTHOR´S CONTRIBUTION

The co-authors listed below fully acknowledge that Martina Hrabcová contributed substantially to above publication. She participated in the field sampling, preparation of samples for morphological and molecular analyses; carried out molecular characterisation (DNA isolation, amplification, sequencing, sequence assembling) for a significant part of the samples and participated in data interpretation.

Agreement of the co-authors

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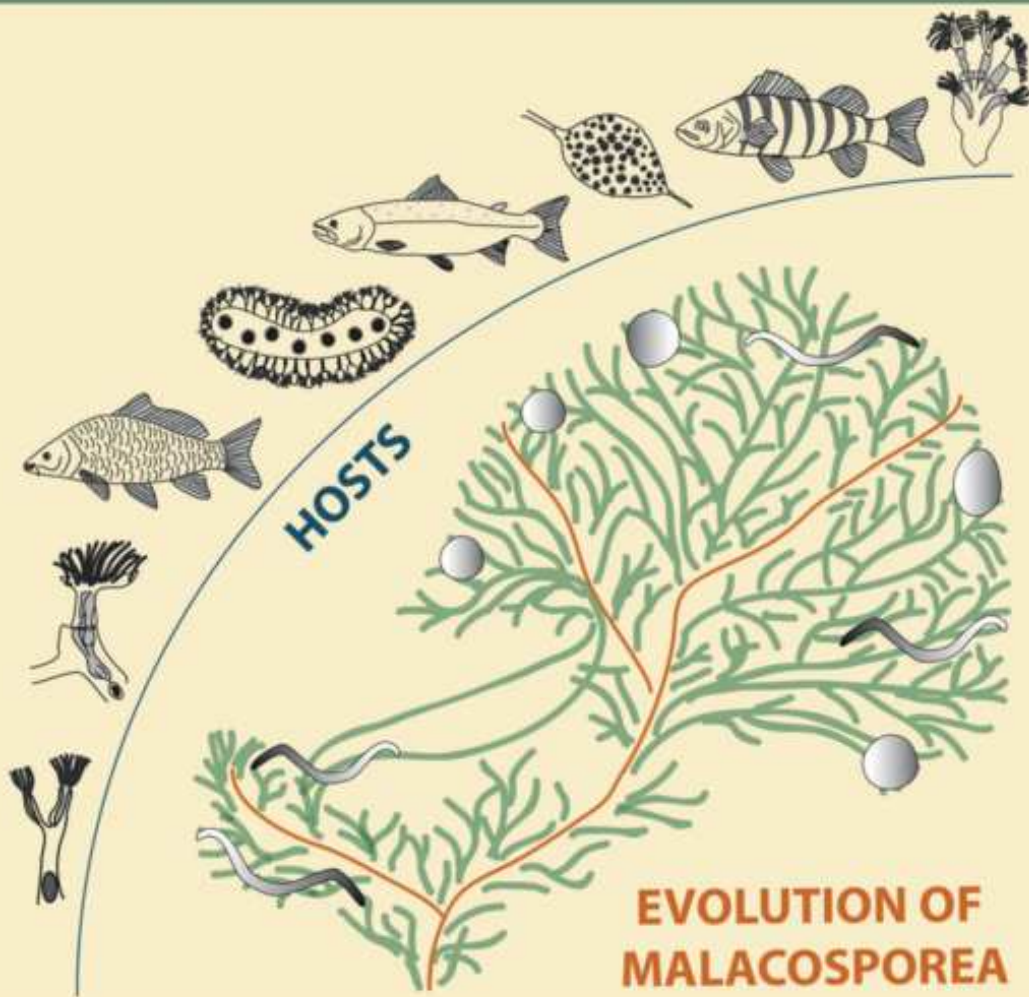RNDr. Hana Pecková   Sneha Patra, MSc.   RNDr. Tomáš Tyml

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

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A R T I C L E   I N F O

A B S T R A C T

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

1. Introduction

Myxozoans are an important parasite group of aquatic organisms, highly derived and morphologically simplified cnidarians (Jiménez-Guri et al., 2007b; Collins, 2009; Holland et al., 2011). The phylum Myxozoa covers some 2400 species of approximately 65 genera and thus significantly contributes to cnidarian diversity with over 10,000 species described (Zhang, 2011). These microscopic endoparasites have complex life cycles with developmental phases alternating between a vertebrate (intermediate) host and an invertebrate (final) host (Wolf and Markiw, 1984). The vast majority of myxozoan species infects fish and annelids and belongs to the class Myxosporea. Malacosporeans, the second class of the Myxozoa, represent a small fraction of myxozoan biodiversity with only three nominal species described which belong to two genera, Tetracapsuloides and Buddenbrockia. They parasitise body cavities of freshwater bryozoans (Bryozoa, Phylactolaemata) where sac- or worm-like stages containing malacospores are produced and infective stages are released. They alternately infect fish hosts where fish malacospores develop in the host's kidney tubules (Hedrick et al., 2004; Grabner and El-Matbouli, 2010).

To date, only one malacosporean life cycle has been both experimentally demonstrated and molecularly verified: Tetracapsuloides bryosalmonae infecting the bryozoan Fredericella sultana was able

to be transmitted to salmonid fishes (Feist et al., 2001) and vice versa (Morris and Adams, 2006). Two other life cycles, where *Buddenbrockia plumatellae* and an unknown malacosporean species both infecting the bryozoan *Plumatella repens* were able to be transmitted to minnow (*Phoxinus phoxinus*) and common carp (*Cyprinus carpio*), respectively, have only been partially resolved (Grabner and El-Matbouli, 2010). Their full elucidation would require successful transmission from fish to bryozoan.

Freshwater bryozoans are substrate-associated suspension-feeder organisms with 94 nominal species (Massard and Geimer, 2008) of which approximately 15 have been found to host malacosporeans (Okamura et al., 2001; Okamura and Wood, 2002; Canning and Okamura, 2004; Tops and Okamura, 2005).

Fish, being the most frequent vertebrate hosts of the Myxosporea, have been poorly studied as potential hosts for malacosporeans. The only exception is represented by economically important salmonid species *T. bryosalmonae* syn. PKX-organism (Seagrave et al., 1980) and syn. *Tetracapsula bryosalmonae* (Canning et al., 1999, 2002) which can under certain conditions cause serious damage to the fish kidney, a pathological condition called proliferative kidney disease (PKD; Canning et al., 1999). *Buddenbrockia plumatellae*, recently found in the kidney of minnow, was first described about a century ago as a motile worm-like organism (Schröder, 1910) also producing sac-like stages, previously named *Tetracapsula bryozoides* (Canning et al., 2002). The *Buddenbrockia* "worm" played a key role in revealing myxozoan affinities to the cnidarians (Jiménez-Guri et al., 2007b). The malacosporean, recently detected in common carp kidney and phylogenetically related to *Buddenbrockia* spp., has not yet been described (Grabner and El-Matbouli, 2010). Fish hosts of the remaining malacosporeans, *Buddenbrockia allmani* from the bryozoan *Lophopus crystallinus* and an undescribed malacosporean species from the bryozoan *F. sultana*, are unknown (Tops et al., 2005; Canning et al., 2007).

From a phylogenetic point of view, Myxozoa split into two sister lineages, the malacosporean and the myxosporean clade (Evans et al., 2010). The evolutionary history of the Myxozoa has never been studied in depth as myxozoan fossils are not available. Malacosporeans branch early in the myxozoan evolution, perhaps reflecting their development in bryozoans opposed to annelids (Kent et al., 2001) which commonly host myxosporeans. The malacosporean lineage would have diverged from the main evolutionary line of the Myxozoa before the radiation that gave rise to the many myxosporean species (Anderson et al., 1999). Ancestral cnidarian characters can still be found in certain malacosporean representatives with vermiform body organisation and radial symmetry (Jiménez-Guri et al., 2007b) in comparison to the considerable reduction of body complexity seen in the myxosporeans (Canning and Okamura, 2004).

The phylogeny of malacosporeans known to date is affected by poor taxon sampling and includes two groups, *Tetracapsuloides* and *Buddenbrockia*, which split into a few lineages with only partially resolved inter-relationships (Tops et al., 2005; Grabner and El-Matbouli, 2010).

It is likely that a more intense research of malacosporean hosts may show a much higher diversity of the Malacosporea than expected (Grabner and El-Matbouli, 2010) due to previous poor sampling in their fish hosts, worldwide distribution of bryozoans (Kipp et al., 2010) and ability of a wide variety of bryozoan species to host different malacosporeans (Canning and Okamura, 2004). Moreover, host-parasite associations of malacosporeans with bryozoans are presumably more ancient than those of myxosporeans with annelids, thus providing more evolutionary time for their radiation.

In this study, we focus on screening fish as potential hosts of malacosporeans to improve our understanding of the biodiversity,

distribution, prevalence, habitat and host preference, as well as unveiling evolutionary trends in the malacosporeans by (i) microscopical examination and screening of kidney and urinary bladders of different freshwater/marine fish species from various geographic locations and habitats for the presence of malacosporeans using PCR; (ii) describing the morphology of the malacosporean stages observed; (iii) performing phylogenetic analyses of the newly gathered ribosomal and protein-coding data together with all available malacosporean sequence data in GenBank; (iv) investigation of the distribution, habitat preference and prevalence of malacosporeans in fish; and (v) by studying the evolutionary trends of malacosporeans by mapping the morphology of bryozoan-related stages, vertebrate/invertebrate host species, habitat preference and geographic data from the small subunit (SSU) rDNA-based phylogenetic tree.

2. Materials and methods

2.1. Sample collection, study area and time schedule

In total, 534 fish were screened for the presence of malacosporean DNA including 32 freshwater fish species (469 samples) and 29 marine fish species (105 samples) together belonging to 19 fish families (Osteichthyes). Most of the fish examined belonged to the families Cypriniformes and Perciformes. In addition to a few urinary bladders (17 samples), the majority of samples screened were kidneys (557) (Supplementary Table S1).

The fish sampling was conducted at 34 localities situated primarily in central Europe (Czech Republic (CR), Hungary, Slovak Republic) but also in other parts of Europe and around the world (Italy, Croatia, the United Kingdom (UK), South Africa and the United States of America (USA)) between 2011 and 2013. Additional DNA samples from the collection of the Laboratory of Fish Protistology, Biology Centre (BC) of the Academy of Sciences of the Czech Republic (ASCR) were archived from previous sampling conducted during trips to the North Sea in 2004 (Fiala, 2006), the Norwegian Sea, Vevang in 2007 and Italy in 2009 (Bartošová et al., 2013). The freshwater localities included semi-intensively farmed ponds, fish farms with outdoor ponds, decorative ponds, lakes, dams and rivers. All "static" sites had a small amount of inflowing water; often through water pipes covered with bryozoans inside. Sampling of fish at the marine localities was performed in oyster beds (South Carolina, USA) and the shallows near the coast (Florida, USA; South Africa; Croatia), at the open sea (North Sea, Norwegian Sea) and at extensive fish farms (Italy). Besides single or irregular samplings at most of the localities, sampling at some freshwater sites (Chřešťovice, CR; Jindřiš, CR; Strmilov, CR) was performed at regular intervals throughout the year (Supplementary Table S1).

2.2. Host species identification

Most of the fish species were determined by morphology (Froese and Pauly, 2013). Species of similar morphology were identified by sequencing of the taxonomically informative gene cytochrome b (*cytb*) using previously described primers (Boore and Brown, 2000; Supplementary Table S1).

2.3. Processing of samples

A sterile scalpel blade was used for the removal of the kidney from each fish. Forceps and scissors for dissection were cleaned with 10% hydrogen peroxide in order to prevent cross-contamination. Fish kidneys were the target organ of examination with some urinary bladders also screened. The squashed samples were examined by light microscopy on an Olympus BX51 microscope.

Plasmodia and spore morphology were documented with an Olympus DP70 digital camera. All samples, including microscopically negative samples, were stored in 400 µl of TNES urea buffer (10 mM Tris–HCl with pH 8, 125 mM NaCl, 10 mM EDTA, 0.5% SDS, 4 M urea) for subsequent DNA extraction.

2.4. DNA extraction, PCR amplification, cloning and sequencing

Total DNA was extracted using a standard phenol–chloroform protocol, after an overnight digestion with proteinase K (50 µg/ml; Serva, Germany) at 55 °C. DNA was resuspended in 50–100 µl of DNAse-free water and left to dissolve overnight at 4 °C.

Malacosporean-specific mala-f and mala-r primers (Grabner and El-Matbouli, 2010) were used in PCRs for all samples, amplifying approximately 680 bp of the SSU rDNA. Malacosporean-specific budd-f and budd-r primers (Grabner and El-Matbouli, 2010) were used to amplify almost complete length SSU rDNA. Composition of the PCR mixture was used as previously described by Bartošová et al. (2009) while PCR cycling conditions for both primer pairs were as described by Grabner and El-Matbouli (2010) but with different annealing temperatures (64 °C for mala-f/mala-r; 61 °C for budd-f/budd-r), and optimised to ensure higher specificity of the primers. The partial large subunit (LSU) rDNA sequence of *Buddenbrockia* sp. 2 was obtained by applying NLF-Tetr1 (Bartošová et al., 2009) and NLR1126 primers (Van der Auwera et al., 1994) with identical PCR mixture and cycling conditions as previously described by Bartošová et al. (2009). Elongation factor-2 (EF-2) sequences were amplified by nested PCR using identical primer combinations, PCR mixture and cycling conditions as in the Taq-Purple DNA polymerase protocol described by Bartošová et al. (2013).

PCR products were purified using a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., USA) and preferentially sequenced. Problematic amplicons with low DNA concentration were re-amplified using identical primer pairs and conditions as before. When mixed sequence composition of the PCR products was detected in chromatograms, amplicons were cloned into the pDrive vector with a PCR Cloning Kit (Qiagen, Germany) and transformed into TOP10 chemically competent *Escherichia coli* cells (Life Technologies, Czech Republic). Plasmid DNA was isolated using a High Pure Plasmid Isolation Kit (Roche Applied Science, Germany) and three colonies of each PCR product were sequenced on an ABI PRISM 3130x1 automatic sequencer (Applied Biosystems, Czech Republic) in the sequencing facility of the Faculty of Science and BC ASCR.

2.5. Alignments, phylogenetic analyses and p distances

The SSU and LSU rDNA and EF-2 (both nucleotide "EF-2nt" and amino acid "EF-2aa") sequences were aligned in MAFFT v6.864b (Katoh et al., 2002) using the E-INS-i method, with a gap opening penalty (−op) 5.0 and gap extension penalty (−ep) 0.0. The alignment was manually edited in BioEdit v7.0.5.2 (Hall, 1999) and in the LSU rDNA and EF-2 alignments the ambiguous sections and introns were removed manually.

The SSU rDNA-based alignment included 104 ingroup sequences comprising newly obtained malacosporean sequences and all malacosporean sequences of sufficient length for the analysis available in GenBank to date (Supplementary Table S4). Outgroups were either cnidarians or myxosporeans (Supplementary Table S2).

When cnidarians were set as the outgroup, the LSU rDNA-based analysis included eight ingroup taxa. Three myxosporean species, *Sphaeromyxa hellandi*, *Henneguya salminicola* and *Chloromyxum leydigi*, were used as outgroups in the next analysis with five ingroup malacosporean LSU rDNA sequences (Supplementary Table S2).

The EF-2 dataset used to assess the position of malacosporeans within metazoans included all five novel malacosporean sequences and then members of the marine and freshwater myxosporean lineage, cnidarians, vertebrates, nematodes, hemichordates, echinoderms, arthropods, poriferans and angiosperms (Supplementary Table S2). The EF-2 dataset used to infer the phylogenetic position of malacosporeans within the Myxozoa included 27 taxa represented by cnidarians as the outgroup and myxozoans as the ingroup with identical sequences as for the previous metazoan dataset (Supplementary Table S2). Another myxozoan EF-2 dataset (28 taxa) had the same taxon representation as the aforementioned dataset but additionally included *C. leydigi*, a myxosporean with an unstable phylogenetic position in the myxozoans (Supplementary Table S2). For the assessment of the length and position of introns in the EF-2 gene, comparison among novel malacosporean EF-2s with several taxa, for which their EF-2 sequences have been obtained from genomic libraries, were used (Supplementary Table S2). Additionally, all 23 EF-2 sequences of the Myxosporea, for which EF-2 is available in GenBank to date, were used for this comparison.

The SSU and LSU rDNA and EF-2 alignments were analysed as single datasets. EF-2nt data were analysed with all codon positions included (EF-2ntALL) and with the exclusion of the third codon position (EF-2ntEX3rd) in all types of analyses and additionally as codons (EF-2ntCOD) in Bayesian Inference (BI) analysis. Maximum Parsimony (MP) analyses were performed in PAUP* v4.b10 ((Swofford, D.L., 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, MA)), 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. We performed Maximum Likelihood (ML) analyses in RAxML v7.0.3 (Stamatakis, 2006) with the GTR + Γ model for rDNA /EF-2nt data and the WAG model for EF-2aa data. Bootstraps were based on 1,000 replicates for both MP and ML analyses. BI analyses were performed in MrBayes v3.0 (Ronquist and Huelsenbeck, 2003), using the GTR + Γ + I model of evolution for rDNA /EF2nt data and the WAG model EF-2aa data. Posterior probabilities were estimated from 1,000,000 generations via two independent runs of four simultaneous Markov Chain Monte Carlo algorithms with every 100th tree saved with burn-in set to 10% (100,000 generations). To ensure convergence and an effective sample size, results were verified with Tracer v1.4.1 (Rambaut, A., Drummond, A.J., 2007. Tracer v1.4, Available from http://beast.bio.ed.ac.uk/Tracer).

The *p* distances were calculated from 1713 bp alignment containing mostly complete SSU rDNA sequences of malacosporeans in PAUP* v4.b10. The exceptions were *Buddenbrockia* sp. 1 and *Tetracapsuloides* sp. 2 for which only single partial sequences were available.

3. Results

3.1. Light microscopy, prevalence, co-infections and distribution

The microscopic detection of the malacosporean parasites was very difficult. More than half of all samples (64%) examined under the light microscope did not contain recognisable myxozoan stages. Subsequent malacosporean-specific PCR screening revealed that 29% of microscopically negative samples were PCR-positive for malacosporean DNA. Under light microscopy, developmental stages and/or spores of myxozoans (samples labelled as microscopically positive) were recognised in 36% of all samples. Half of the microscopically positive samples were PCR-positive for malacosporean DNA (Supplementary Table S3). Malacosporean spores were found only in *Buddenbrockia* sp. 2 in common carp and goldfish,

where they were scarce (Fig. 1). Images in Fig. 1 were taken exclusively from fish without other myxozoan infections as determined by PCRs using general myxozoan primers. Interstitial PKX organisms were accompanied by pathological PKD and intratubular stages, which were subsequently identified by PCR as *T. bryosalmonae*, in salmonids *Oncorhynchus mykiss* and *Salmo trutta* from Jindřiš, CR and from Howietoun, UK. No signs of PKD were detected in the kidney of non-salmonid fishes, neither at PKD-positive localities nor at other sites (Supplementary Table S1).

Malacosporean pseudoplasmodia in the kidney tubules were usually immature; spores were detected only occasionally inside these monosporic pseudoplasmodia. Intratubular pseudoplasmodia possessed thin walls and greatly resembled early sporogonic stages of myxosporeans e.g. *Sphaerospora* spp. The early plasmodial stages of malacosporeans were small in size, globular in shape and rich in refractile granules of unequal size. In more developed stages, that looked elongate in squash preparations, formation of two spherical polar capsules was visible. The spores of *Buddenbrockia* sp. 2 observed in the kidney tubules were globular, elongate to ovoid shape and possessed thin walls (fragile appearance) which are typical of malacosporeans. In most cases, the shell valves were hardly recognisable by light microscopy and their typical features were two small conspicuous spherical polar capsules of equal size.

The most common malacosporean parasites were *B. plumatellae*, *Buddenbrockia* sp. 2 and *Tetracapsuloides* sp. 3. Malacosporeans *Buddenbrockia* sp. 3, *T. bryosalmonae*, *Tetracapsuloides* sp. 2, and *Tetracapsuloides* sp. 4 through sp. 5 were found only in one to two fish species. The prevalence of malacosporeans reached 100% in some fish species and localities (Table 1). No seasonal effect on the prevalence of malacosporeans in their fish hosts was observed (Supplementary Table S1).

Regarding the localities from the freshwater environment, most of them (19/26) were positive for malacosporeans in a comparison to the marine localities ($n = 7$) which were all negative (Supplementary Table S1). The highest number of malacosporean species ($n = 5$) was found at two Czech localities i.e. a fish farm in Jindřiš and in the Dyje River south from the town Břeclav (Fig. 2).

Most of the positive samples contained single malacosporean infections. Cloning of the PCR products with double signals in the chromatograms revealed co-infections of up to three malacosporean species in the same individual fish (*Alburnus alburnus* and *Rutilus rutilus* from Dyje River, CR; *Gobio gobio* from Jindřiš, CR; Fig. 2).

3.2. Molecular identification of fish hosts and malacosporeans

The obtained fish host *cytb* sequences (NCBI accession numbers: KF731736 – KF731756) showed 100% base similarity with GenBank reference data i.e. *A. alburnus*, *Ballerus sapa*, *Barbus barbus*, *Blicca bjoerkna*, *Carassius auratus auratus*, *Cyprinus carpio*, *G. gobio*, *Chelon labrosus*, *Leucaspius delineatus*, *Leuciscus leuciscus*, *Liza ramada*, *Lutjanus griseus*, *R. rutilus*, *Squalius cephalus*, and *Strongylura notata* (for GenBank accession numbers see Supplementary Table S1).

We obtained 50 malacosporean SSU rDNA (NCBI: KF731680–KF731729), one partial LSU rDNA (NCBI: KF731730) and five EF-2 sequences (NCBI: KF731731–KF731735) belonging to *B. plumatellae*, *Buddenbrockia* sp. 2, *T. bryosalmonae* and five new, yet undescribed *Buddenbrockia* and *Tetracapsuloides* spp. detected exclusively in freshwater fish (Table 1; Supplementary Table S1).

3.3. Species boundaries and molecular distances

Due to the problematic morphological discrimination of malacosporean species, parasites were determined as different species using a combination of molecular data and biological traits (host species, host habitat and geographic locality). Based on the known inter-species variation in myxosporeans, we arbitrarily applied 1% nt sequence difference in the SSU rDNA as a genetic yardstick for species discrimination (see the Discussion regarding species boundaries). Intraspecific variability in the SSU rDNA of malacosporean species ranged from 0.00% to 0.85%. Generally, *Tetracapsuloides* spp. had lower intraspecific divergences (up to 0.69% in *Tetracapsuloides* sp. 3) than *Buddenbrockia* spp. (up to 0.85% in *B. allmani*). The lowest interspecific divergence in malacosporeans (1.67%) was calculated between *T. bryosalmonae* and *Tetracapsuloides*
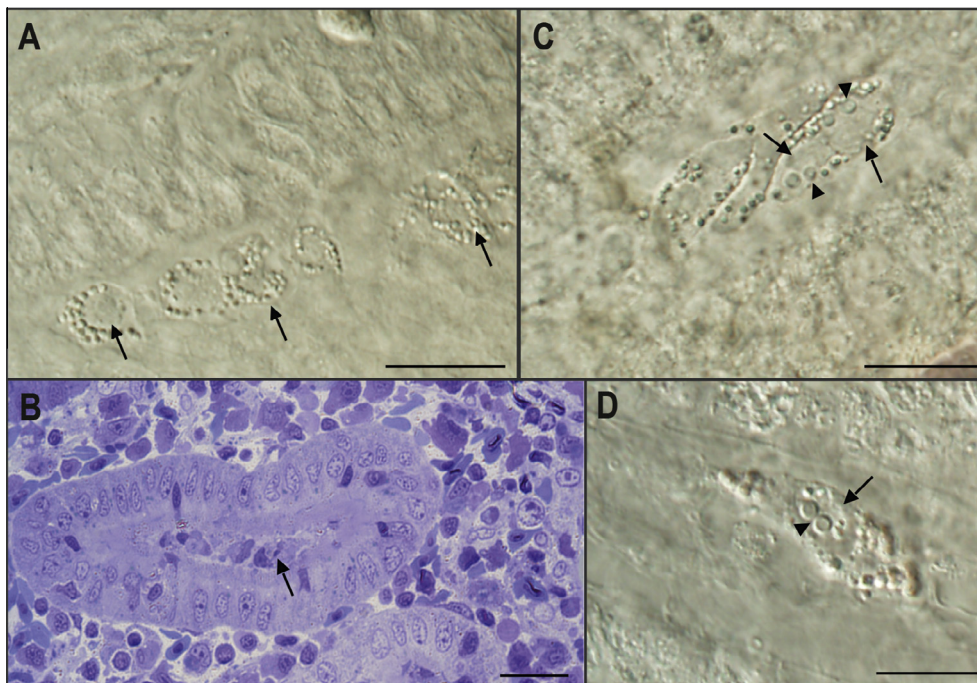
Fig. 1. Morphology of the *Buddenbrockia* sp. 2 stages in the kidney tubules of goldfish *Carassius auratus auratus* from Chřešťovice, Czech Republic. Young pseudoplasmodia (arrows) observed by (A) light microscopy and (B) in the Toluidine blue stained semi-thin section. (C, D) Mature fish malacospores (arrows) with spherical polar capsules (arrowheads) and soft shell valves. Scale bars = 20 μm.

Table 1
List of malacosporeans found in this study with data on their hosts, localities, sequences and prevalence.

Malacosporean species	Fish species	Locality	GenBank acc. No.	Parasite prevalence
Buddenbrockia plumatellae	*Abramis brama*	Danube River, at Štúrovo, SR	KF731680[a]	25% (1/4)
	Alburnus alburnus	Jindřiš Farm, CR	KF731681[a]	46% (5/11)
		Dyje River, south of Břeclav, CR	KF731682[a]	33% (1/3)
		Hron River, at Štúrovo, SR	KF731683[a]	20% (1/5)
	Aspius aspius	Dyje River, south of Břeclav, CR	KF731684[a]	50% (4/8)
	Blicca bjoerkna	Dyje River, south of Břeclav, CR	KF731685[a]	65% (13/20)
		Oxbows of Dyje River, between Břeclav and confluence of Dyje and Morava r., CR	KF731686[a]	100% (3/3)
		Rožmberk Pond, CR	KF731687[a]	40% (2/5)
		Danube River, at Štúrovo, SR	KF731688[a]	14% (1/7)
	Chondrostoma nasus	Hron River, at Štúrovo, SR	KF731689[a]	100% (2/2)
	Leuciscus idus	Dyje River, south of Břeclav, CR	KF731690[a]	83% (5/6)
	Leuciscus leuciscus	Oslava River, south of Oslavany, CR	KF731691[a] KF731735[c]	100% (1/1)
		Dyje River, south of Břeclav, CR	KF731692[a]	100% (1/1)
	Perca fluviatilis	Rožmberk Pond, CR	KF731693[a]	50% (1/2)
	Rutilus rutilus	Dyje River, south of Břeclav, CR	KF731694[a]	60% (6/10)
		Rožmberk Pond, CR	KF731695[a]	50% (1/2)
	Scardinius erythrophthalmus	Jihlava, CR	KF731696[a], KF731734[c]	50% (1/2)
	Squalius cephalus	Oslava River, south of Oslavany, CR	KF731697[a], KF731733[c]	100% (1/1)
		Dyje River, south of Břeclav, CR	KF731698[a]	20% (1/5)
Buddenbrockia sp. 2	*Carassius auratus auratus*	Jihlava, CR	KF731699[a]	50% (4/8)
		Chřešťovice Farm, CR	KF731700[a], KF731730[b], KF731731[c]	73% (27/37)
	Carassius gibelio	Jihlava, CR	KF731701[a]	56% (5/9)
	Cyprinus carpio	Hortobágy, Hungary	KF731702[a]	27% (3/11)
		Horní Hluboký Pond, Strmilov, CR	KF731703[a]	60% (12/20)
		Malá Outrata Pond, CR	KF731704[a]	50% (1/2)
		Motovidlo Pond, CR	KF731705[a]	100% (2/2)
		Chřešťovice Farm, CR	KF731706[a], KF731732[c]	77% (13/17)
		Vodňany, CR	KF731707[a]	100% (3/3)
Buddenbrockia sp. 3	*Barbus barbus*	Dyje River, south of Břeclav, CR	KF731708[a]	67% (2/3)
		Oxbows of Dyje River, between Břeclav and confluence of Dyje and Morava r., CR	KF731709[a]	100% (1/1)
	Rutilus rutilus	Dyje River, south of Břeclav, CR	KF731710[a]	10% (1/10)
Tetracapsuloides bryosalmonae	*Oncorhynchus mykiss*	Jindřiš Farm, CR	KF731711[a]	93% (67/72)
	Salmo trutta	Howietoun fishery, UK	KF731712[a]	92% (11/12)
Tetracapsuloides sp. 2	*Gobio gobio*	Jindřiš Farm, CR	KF731713[a]	25% (1/4)
Tetracapsuloides sp. 3	*Ballerus sapa*	Danube River, at Štúrovo, SR	KF731714[a]	11% (1/9)
	Barbus barbus	Dyje River, south of Břeclav, CR	KF731715[a]	33% (1/3)
	Cyprinus carpio	Hortobágy, Hungary	KF731716[a]	9% (1/11)
	Gobio gobio	Jindřiš Farm, CR	KF731717[a]	67% (4/6)
		Morava River, under Lanžhot, CR	KF731718[a]	25% (1/4)
		Dyje River, south of Břeclav, CR	KF731719[a]	9% (1/11)
		České Budějovice, CR	KF731720[a]	50% (1/2)
	Leucaspius delineatus	Jindřiš Farm, CR	KF731721[a]	100% (2/2)
	Leuciscus idus	Oxbows of Dyje River, between Břeclav and confluence of Dyje and Morava r., CR	KF731722[a]	75% (3/4)
	Perca fluviatilis	Iseo Lake, Italy	KF731723[a]	25% (2/8)
	Rutilus rutilus	Dyje River, south of Břeclav, CR	KF731724[a]	10% (1/10)
Tetracapsuloides sp. 4	*Alburnus alburnus*	Dyje River, south of Břeclav, CR	KF731725[a]	67% (2/3)
		Hron River, at Štúrovo, SR	KF731726[a]	20% (1/5)
	Rutilus rutilus	Svratka River, Rajhradice, CR	KF731727[a]	100% (2/2)
Tetracapsuloides sp. 5	*Gobio gobio*	Jindřiš Farm, CR	KF731728[a]	33% (2/6)
		Dyje River, south of Břeclav, CR	KF731729[a]	91% (10/11)

Note: Buddenbrockia sp. 1, *Buddenbrockia allmani*, *Tetracapsuloides* sp. 1 and a member of a novel malacosporean lineage are not included in the table as no new data for these species were obtained in the present study. Their phylogenetic position is shown in Fig. 3. CR, Czech Republic; SR, Slovak Republic; UK, United Kingdom.
GenBank accession numbers:
[a] small subunit rDNA sequence.
[b] large subunit rDNA sequence.
[c] elongation factor 2 nucleotide sequence.

sp. 5. The sequence difference between *T. bryosalmonae* and its closely related species *Tetracapsuloides* sp. 1 was 2.16%. The largest interspecific divergence in the genus *Tetracapsuloides* (6.22%) was calculated for *Tetracapsuloides* sp. 3 and *Tetracapsuloides* sp. 4. The worm-like stages of *B. plumatellae* had similar intraspecific variability (0.43%) as the sac-like stages (0.55%) with a 1.29% sequence difference between the two forms. The largest interspecific divergence in the genus *Buddenbrockia* (5.54%) was calculated for
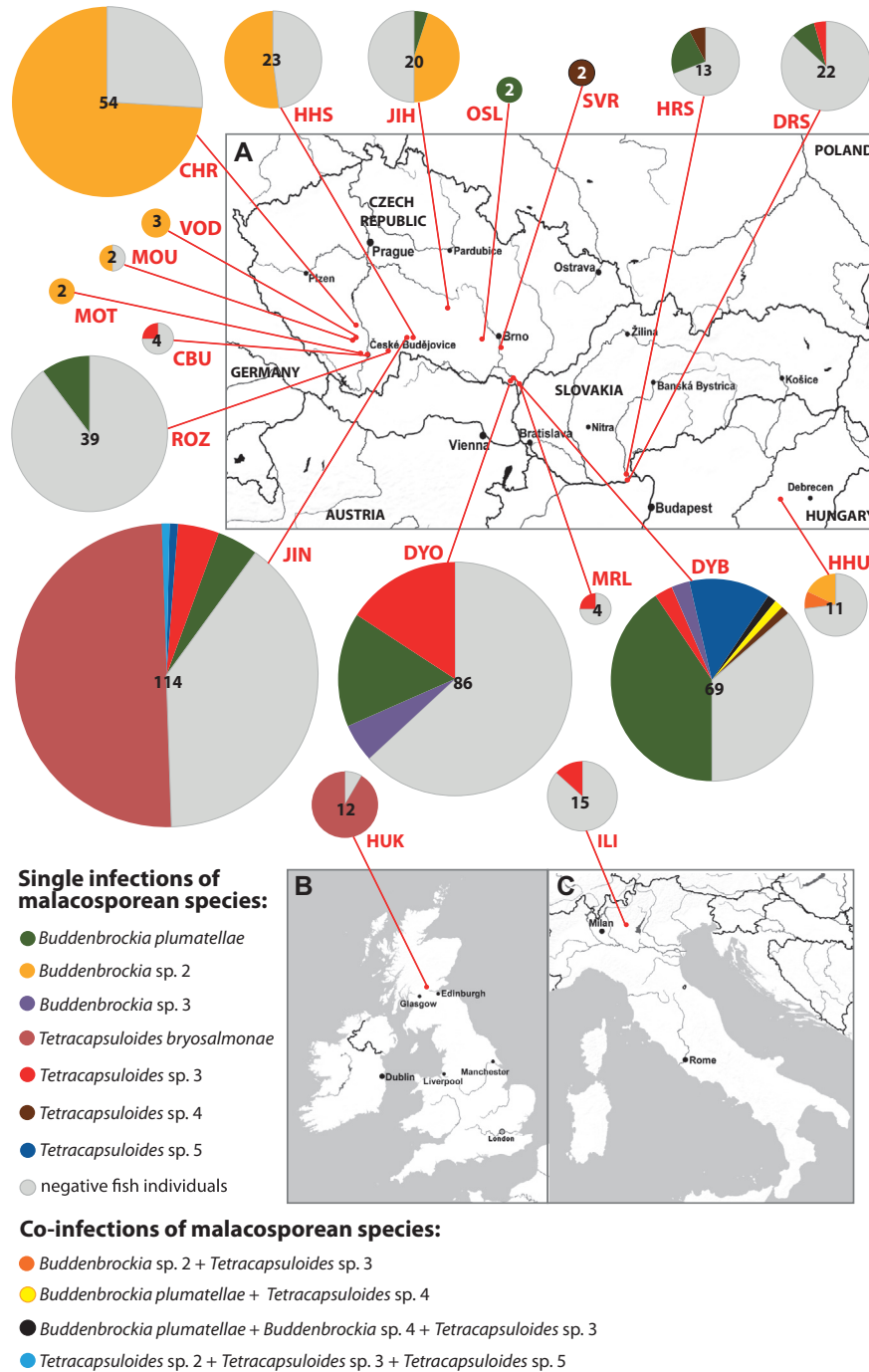
Fig. 2. Malacosporean prevalence and distribution in PCR-positive fish hosts and localities in (A) the Czech Republic (CR), Hungary and Slovak Republic (SR), (B) the UK and (C) Italy. The maps were constructed in MapCreator. The number inside each pie chart indicates the total number of fish examined at the locality. CBU, České Budějovice, CR; CHR, Chřešťovice, CR; DRS, Danube River, at Štúrovo, SR; DYB, Dyje River, south of Břeclav, CR; DYO, Oxbows of Dyje River, between Břeclav and confluence of Dyje and Morava rivers, CR; HHS, Horní Hluboký Pond, Strmilov, CR; HHU, Hortobágy, Hungary; HRS, Hron River, at Štúrovo, SR; HUK, Howietoun, UK; ILI, Iseo Lake, Italy; JIH, Jihlava, CR; JIN, Jindřiš, CR; MOT, Motovidlo Pond, CR; MOU, Malá Outrata Pond, CR; MRL, Morava River, Lanžhot, CR; OSL, Oslava River, south of Oslavany, CR; ROZ, Rožmberk Pond, CR; SVR, Svratka River, Rajhradice, CR; VOD, Vodňany, CR.

Buddenbrockia sp. 1 and *B. plumatellae* sacs. The maximum interspecific divergence in malacosporeans (19.14%) was found between *Buddenbrockia* sp. 1 and a malacosporean sequence (NCBI: FJ981824) previously identified as *B. plumatellae*. The percentage difference of the mentioned malacosporean sequence was very similar when compared either with *Buddenbrockia* spp. or with *Tetracapsuloides* spp. (Fig. 3).

3.4. Phylogenetic analyses

3.4.1. SSU rDNA data phylogeny

All sequences obtained by screening of fish samples clustered together within a single, strongly supported malacosporean lineage, forming a sister clade to the myxosporeans. The malacosporean lineage was found to split into three strongly supported clades:

% of dissimilarity	B. plumatellae "worm"	B. plumatellae sac	B. allmani	Buddenbrockia sp. 1 *	Buddenbrockia sp. 2	Buddenbrockia sp. 3	Tetracapsuloides bryosalmonae	Tetracapsuloides sp. 1	Tetracapsuloides sp. 2 *	Tetracapsuloides sp. 3	Tetracapsuloides sp. 4	Tetracapsuloides sp. 5	Nov. lineage
Buddenbrockia plumatellae "worm"	0.428												
Buddenbrockia plumatellae sac	1.285	0.551											
Buddenbrockia allmani	5.235	5.348	0.846										
Buddenbrockia sp. 1 *	5.130	5.539	3.594	-									
Buddenbrockia sp. 2	3.652	3.962	2.235	3.580	0.061								
Buddenbrockia sp. 3	4.767	4.698	2.436	3.981	2.436	0.000							
Tetracapsuloides bryosalmonae	15.524	15.253	16.239	16.263	14.044	13.969	0.617						
Tetracapsuloides sp. 1	15.953	15.683	17.693	17.454	14.667	14.593	2.158	0.349					
Tetracapsuloides sp. 2 *	11.983	11.611	5.997	6.548	11.116	11.247	4.085	4.566	-				
Tetracapsuloides sp. 3	16.270	16.128	17.418	17.446	15.110	14.846	5.870	5.784	4.048	0.688			
Tetracapsuloides sp. 4	15.712	15.444	17.252	16.688	14.427	14.162	2.846	3.139	5.119	6.219	0.000		
Tetracapsuloides sp. 5	15.595	15.198	16.669	16.204	14.057	13.980	1.672	2.341	3.396	5.417	2.524	-	
Novel malacosporean lineage	18.765	18.328	18.649	19.137	17.302	17.219	16.447	16.590	12.392	16.617	16.218	15.848	-

Fig. 3. Distance matrix showing the percentage of maximum small subunit rDNA sequence divergence among the malacosporean taxa. Dash indicates that interspecific variability was not possible to calculate as only one sequence was available; * partial small subunit rDNA sequence compared; a novel malacosporean lineage corresponds to a sequence NCBI: FJ981824.

the *Buddenbrockia* clade, the *Tetracapsuloides* clade and a new malacosporean lineage (Fig. 4).

The *Buddenbrockia* clade included *B. plumatellae*, *B. allmani* and *Buddenbrockia* sp. 1 through 3. The position of each species in the clade was very unstable in all performed analyses which was reflected by weak nodal supports corresponding to the mutual relationships of *Buddenbrockia* spp. (see Section 3.4.2.; Fig. 4).

The *Tetracapsuloides* clade comprised *T. bryosalmonae* and *Tetracapsuloides* sp. 1 – sp. 5. The malacosporean *Tetracapsuloides* sp. 2 clustered with *Tetracapsuloides* sp. 3 in a well-supported group, other species had an unstable phylogenetic position in the *Tetracapsuloides* clade (Fig. 4). *Tetracapsuloides* sp. 1 clustered either with *T. bryosalmonae* in one group splitting into two separate, closely-related clades (using ML, BI) or within the *Tetracapsuloides* clade but not sister to *T. bryosalmonae* (using MP). The novel malacosporean lineage represented by a single sequence of malacosporean from the bryozoan *Plumatella fungosa* from Ohio, USA (NCBI: FJ981824), clustered either sister to the *Tetracapsuloides* clade (all types of analyses with Cnidaria as outgroup; Fig. 4) or basal to the *Buddenbrockia + Tetracapsuloides* clade (all types of analyses with Myxosporea as outgroup, trees not shown).

3.4.2. Evolutionary trends in malacosporeans

The *Buddenbrockia* clade included both sacs and worm-like morphotypes from the bryozoan hosts. The *B. plumatellae* group split into the "worm" and sac subclades which were well-resolved and highly supported in all analyses performed. The *Tetracapsuloides* clade contained only sacs and the new malacosporean lineage was represented by a "worm" (Fig. 4).

As for fish hosts, *B. plumatellae* "worm-like" clade and *Tetracapsuloides* sp. 3 clade included members identified in various fish species from the families Cypriniformes and Perciformes. *Tetracapsuloides bryosalmonae* was restricted to salmonids. *Buddenbrockia* sp. 3 and *Tetracapsuloides* sp. 4 were found in two cyprinid species as well as *Buddenbrockia* sp. 2 which exclusively infected the cyprinid genera *Cyprinus* and *Carassius*. Rather strict host specificity was revealed for *Tetracapsuloides* sp. 2 and 5 infecting only one cyprinid, *G. gobio*. Fish hosts for other malacosporean species in the tree are unknown (Fig. 4, Supplementary Tables S1, S3).

Inclusion of the GenBank malacosporean data from bryozoan as well as fish hosts together with newly obtained data in the SSU rDNA-based analyses revealed positioning of several fish-related sequences in the *B. plumatellae* "worm-like" clade, which encompassed isolates from bryozoans of the family Plumatellidae. Moreover, fish isolates clustered within the *T. bryosalmonae* clade comprising isolates from a broad spectrum of bryozoan hosts (crist-

atellids, fredericellids, plumatellids and pectinatellids). No matches of novel *Buddenbrockia* and *Tetracapsuloides* spp. recognised in this study with the malacosporean sequences known explicitly from bryozoans were found. Regarding the remaining malacosporeans with GenBank data and which have unknown fish hosts, they infected either one bryozoan host species i.e. *B. plumatellae* sac-like isolates in *Cristatella mucedo*, *B. allmani* in *L. crystallinus*, *Buddenbrockia* sp. 1 in *F. sultana* and a member of the novel malacosporean lineage in *P. fungosa* or had a wide host range i.e. *Tetracapsuloides* sp. 1 infecting cristatellids, plumatellids and pectinatellids (Fig. 4, Supplementary Table S4).

No general pattern was observed in regard to malacosporean clustering according to host habitat as both *Buddenbrockia* and *Tetracapsuloides* spp. were present in different lentic (static water) and in lotic (flowing water) ecosystems. *Buddenbrockia plumatellae*, *B. allmani*, *T. bryosalmonae*, *Tetracapsuloides* sp. 3 and sp. 5 were present at both static and riverine habitats whereas *Buddenbrockia* sp. 2, *Tetracapsuloides* sp. 1 and sp. 2 and the member of a new malacosporean lineage originated from static water sites. The flowing waters were exclusively inhabited by *Buddenbrockia* sp. 1 and sp. 3 and *Tetracapsuloides* sp. 4 (Fig. 4, Supplementary Table S4). If we additionally evaluated the appearance of bryozoan colonies, malacosporeans inhabiting bryozoan species producing gelatinous colonies were reported only from static habitats (lakes and ponds) in Europe (*B. plumatellae* sacs in *C. mucedo*) and North America (*Tetracapsuloides* sp. 1 and *T. bryosalmonae* in *C. mucedo* and *Pectinatella maginifica*) (Supplementary Table S4).

A general conclusion of malacosporean clustering according to the morphology of their bryozoan-related stages and host habitat could not be drawn as both sacs and worm-like morphotypes occurred in lentic and lotic ecosystems. These characteristics were rather important for discrimination of closely related malacosporeans i.e. *B. plumatellae* "worms" had a broader habitat preference (static and flowing waters) whereas *B. plumatellae* sacs were present only at static water habitats (Fig. 4, Supplementary Table S4).

The geographic distribution pattern of malacosporeans was evaluated only in the Northern hemisphere as all malacosporean sequences analysed during this comprehensive study originated from this part of the world. *Buddenbrockia plumatellae* "worm" clade included both isolates from Europe (*Plumatella* spp. and fish hosts) and USA (*Hyalinella punctata*) whereas *B. plumatellae* sac, *B. allmani* and *Buddenbrockia* sp. 1 sequences originated from Europe. Sequences of *T. bryosalmonae* from North America (*C. mucedo*, *Pectinatella magnifica*, *O. mykiss*) and Europe (*F. sultana*, *Plumatella emarginata*, *O. mykiss*, *Salmo* spp.) grouped in a single
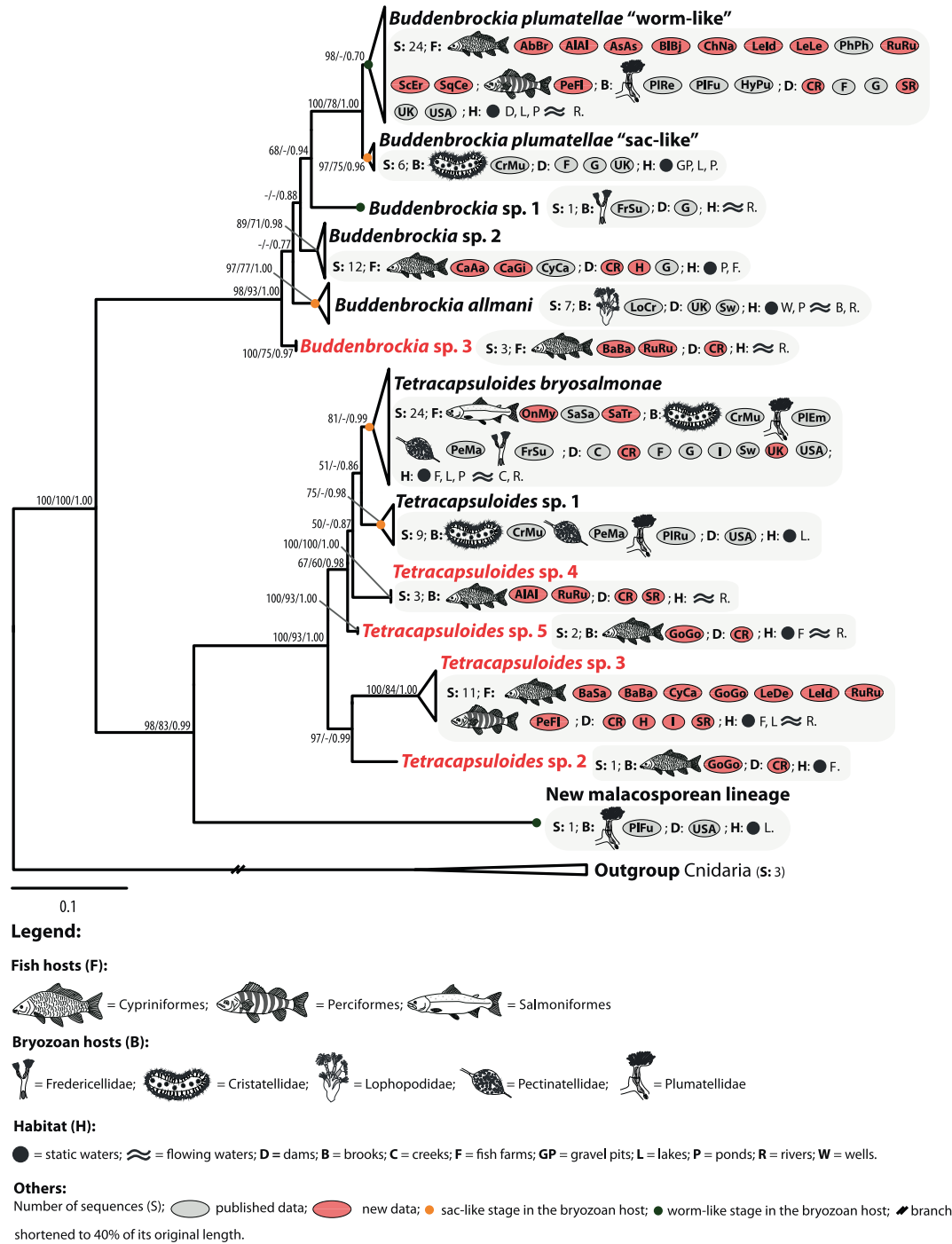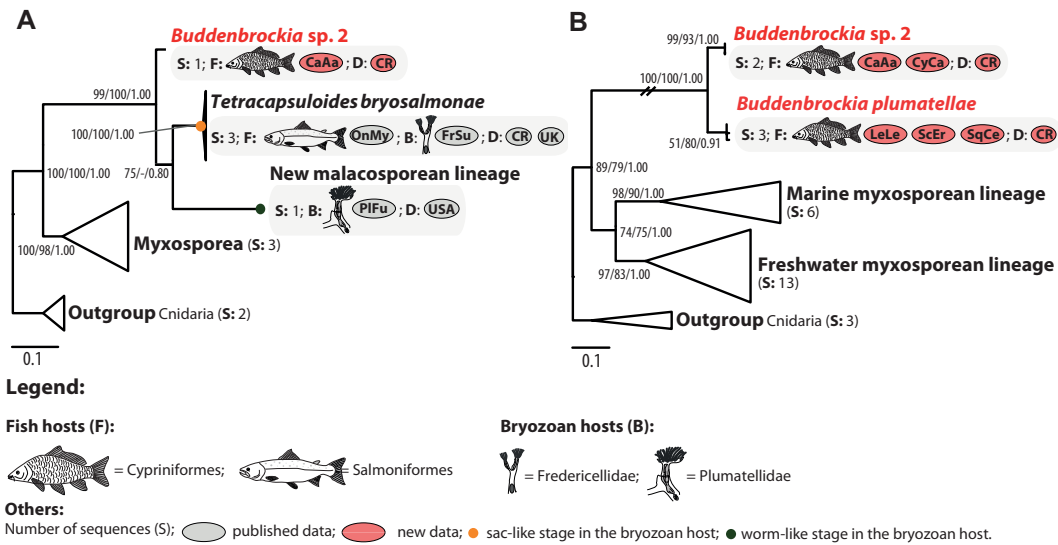
Fig. 4. Maximum likelihood phylogenetic trees based on small subunit rDNA data showing the phylogenetic trends in clustering of malacosporeans. Numbers at nodes indicate Maximum Likelihood/Maximum Parsimony/Bayesian Inference nodal supports. Bootstraps calculated from 1,000 replicates; nodal supports <50% not shown. Fish hosts: AbBr, *Abramis brama*; AlAl, *Alburnus alburnus*; AsAs, *Aspius aspius*; BaBa, *Barbus barbus*; BaSa, *Ballerus sapa*; BlBj, *Blicca bjoerkna*; CaAa, *Carassius auratus auratus*; CaGi, *Carassius gibelio*; ChNa, *Chondrostoma nasus*; CyCa, *Cyprinus carpio*; GoGo, *Gobio gobio*; LeDe, *Leucaspius delineatus*; LeId, *Leuciscus idus*; LeLe, *Leuciscus leuciscus*; OnMy, *Oncorhynchus mykiss*; PhPh, *Phoxinus phoxinus*; PeFl, *Perca fluviatilis*; RuRu, *Rutilus rutilus*; SaSa, *Salmo salar*; SaTr, *Salmo trutta*; ScEr, *Scardinius erythrophthalmus*; SqCe, *Squalius cephalus*. Bryozoan hosts: CrMu, *Cristatella mucedo*; FrSu, *Fredericella sultana*; HyPu, *Hyalinella punctata*; LoCr, *Lophopus crystallinus*; PeMa, *Pectinatella magnifica*; PlEm, *Plumatella emarginata*; PlFu, *Plumatella fungosa*; PlRe, *Plumatella repens*; PlRu, *Plumatella rugosa*. Distribution (D): C, Canada; CR, Czech Republic; F, France; G, Germany; H, Hungary; I, Italy; SR, Slovak Republic; Sw, Switzerland; UK, United Kingdom; USA, United States of America.

clade irrespective of geographic distribution. *Tetracapsuloides* sp. 1 and a member of the novel malacosporean lineage originated from USA. As our screening of freshwater localities was performed only in Europe we could not evaluate the general distribution of novel *Buddenbrockia* and *Tetracapsuloides* spp. (Fig. 4, Supplementary Table S4).

3.4.3. LSU rDNA data confirm the SSU rDNA phylogeny

The same clades as in the SSU rDNA-based phylogeny, identifying the *Buddenbrockia*, *Tetracapsuloides* and the novel malacosporean lineage (NCBI: FJ981817), but with a lower taxon sampling, were present in the LSU rDNA tree. Similarly to the SSU rDNA-based tree, the new malacosporean lineage clustered either

Fig. 5. Maximum likelihood phylogenetic trees based on (A) large subunit rDNA and (B) EF-2 data showing the phylogenetic trends in clustering of malacosporeans. Numbers at nodes indicate Maximum Likelihood/Maximum Parsimony /Bayesian Inference nodal supports. Bootstraps calculated from 1,000 replicates; nodal supports <50% not shown. Fish hosts: CaAa, *Carassius auratus auratus*; CyCa, *Cyprinus carpio*; LeLe, *Leuciscus leuciscus*; OnMy, *Oncorhynchus mykiss*; ScEr, *Scardinius erythrophthalmus*; SqCe, *Squalius cephalus*. Bryozoan hosts: FrSu, *Fredericella sultana*; PlFu, *Plumatella fungosa*. Distribution (D): CR, Czech Republic; UK, United Kingdom; USA, Unites States of America.

sister to the *Tetracapsuloides* clade (ML and BI analyses with Myxosporea or Cnidaria as outgroups; Fig. 5A) or basal to the *Buddenbrockia + Tetracapsuloides* clade (MP analyses with Cnidaria or Myxosporea as outgroups; trees not shown).

3.4.4. EF-2 data of malacosporeans exhibit unique sequence patterns

We produced five new partial EF-2 gene sequences of *Buddenbrockia* sp. 2 and *B. plumatellae*. These differed substantially from all myxosporean EF-2 sequences. The BLAST search showed 66 – 76% identity to myxosporeans (*S. hellandi, Kudoa crumena, C. leydigi*) and cnidarians (*Hydractinia, Hydra, Aurelia*) but similar identity was also recorded with different vertebrate, arthropod, plant taxa etc.

All malacosporean EF-2 sequences possessed two introns located at positions 196 and 606/614 of their partial EF-2. Intragenomic variability in the EF-2 of *Buddenbrockia* sp. 2 isolates from common carp and goldfish was 9 nt located in the second intron region. Similarly, the EF-2 sequence of *B. plumatellae* isolates differed by 1 nt in the second intron (Table 2). In-depth study of the alignment of different metazoan EF-2 sequences obtained from genomic data (introns included) showed that introns in the EF-2 gene are a common feature and the location of the introns is variable in metazoans including free-living cnidarians and myxozoans. The location of the first intron located at position 196 seems to be unique for malacosporeans as no inserts were found in this location for other metazoan EF-2 sequences. The second intron location of malacosporeans occurs in the same location as in *Nematostella vectensis*, the only cnidarian for which genomic data is available (Putnam et al., 2007), and in the European hamster *Cricetus cricetus*. Their inserts were highly divergent in nt composition and much longer in the cnidarian (285 nt) and the mammal (88 nt)

than in the malacosporeans (35–44 nt). Only two out of 23 myxosporeans species contained inserts in their EF-2 sequences. *Myxidium incurvatum* contains a single insert with a length of 115 nt located in a different region than malacosporeans but with the same location as in the nematode *Wuchereria bancrofti* (140 nt). The myxosporean *Zschokkella nova* has a single 35 nt insert located in the same region as *C. cricetus* (210 nt) and *W. bancrofti* (208 nt).

The EF-2ntALL, EF-2ntEX3rd and EF-2aa-based phylogenetic analyses with different metazoan taxa confirmed that the novel EF-2 sequences belong to malacosporeans which clustered sister to myxosporeans in a well-supported myxozoan lineage (trees not shown). In some analyses, malacosporeans clustered not basally but inside the myxosporean lineage sister to either freshwater or marine lineages. However, these relationships were always weakly supported (trees not shown). In the EF-2 analysis with cnidarians as outgroup and myxozoans as ingroup taxa, the malacosporean clade always clustered sister to myxosporeans with high support. The malacosporean clade that includes all novel *Buddenbrockia* sp. 2 and *B. plumatellae* EF-2 sequences created a long branch in the tree (Fig. 5B). When *C. leydigi* was included in the analyses, malacosporeans surprisingly clustered sister to this taxon in a single branch further grouping with other myxosporeans, however bootstrap support for this clustering remained low in all analyses (trees not shown).

4. Discussion

Using PCR of fish kidneys and DNA sequencing, we revealed high prevalence and diversity of malacosporeans in several fish host species from European freshwater habitats, adding five new

Table 2
Length and positions of introns in the partial elongation factor 2 gene sequences of malacosporeans obtained in this study.

GenBank acc. No.	Malacosporean species	Sequence length (bp)	Intron 1 length (position)	Intron 2 length (position)
KF731731	*Buddenbrockia* sp. 2 ex *Carassius auratus auratus*	790	32 (196–227)	44 (606–649)
KF731732	*Buddenbrockia* sp. 2 ex *Cyprinus carpio*	781	32 (196–227)	35 (606–640)
KF731733	*Buddenbrockia plumatellae* ex *Squalius cephalus*	788	40 (196–235)	37 (614–650)
KF731734	*Buddenbrockia plumatellae* ex *Scardinius erythrophthalmus*	789	40 (196–235)	38 (614–651)
KF731735	*Buddenbrockia plumatellae* ex *Leuciscus leuciscus*	789	40 (196–235)	38 (614–651)

species of *Buddenbrockia* and *Tetracapsuloides* to the existing three species described and an additional three species for which only molecular data are available. We extended the fish host species spectrum for *B. plumatellae* and *Buddenbrockia* sp. 2. We were able to detect malacosporeans in a large spectrum of fish host species (21 fish species) which corresponds to 45% of freshwater fish samples. Intensive phylogenetic analyses revealed that apart from *Buddenbrockia* and *Tetracapsuloides*, a novel malacosporean lineage (likely a new genus) exists.

The significant increase in malacosporean species richness revealed in the present study points to a hidden biodiversity within this parasite group. Why, despite intensive research performed on fish parasites, especially in cyprinids, are only a low number of malacosporean species described from their fish hosts? The main reason for this is likely the cryptic nature of malacosporean young developmental and sporogonic stages due to (i) the small size and hyaline appearance of pseudoplasmodia which are difficult to differentiate and easy to overlook in samples; (ii) the similarity, thus easy misidentification, of malacosporean pseudoplasmodia and vegetative stages of other myxozoans, especially with early pseudoplasmodia of sphaerosporids; (iii) the exceptional occurrence of mature fish malacospores with taxonomically informative characters (soft shell valves, small spherical polar capsules); and (iv) mostly asymptomatic infections, with infected fish exhibiting no external signs of disease or compromised health. These reasons most probably explain the significant differences in the percentage of malacosporean infections detected by PCR but not by light microscopy (22% of all samples) and vice versa (21% of all samples) (Supplementary Table S3).

The rare occurrence of spores in fish kidneys may possibly be explained if fish were a blind developmental lineage (accidental hosts) of malacosporeans which mostly cycle in bryozoans. This is supported by the occurrence of *T. bryosalmonae* in lakes lacking salmonids (Anderson et al., 1999; Okamura et al., 2001). Another more likely explanation is that malacosporeans are unable to complete their development in aberrant fish hosts. Arrest of spore development occurs when atypical hosts are invaded by myxozoan sporoplasms which are non-host specific (Kallert et al., 2011, 2012) but are able to enter the circulatory system (Holzer et al., 2013) and potentially the kidney. Previous studies have shown that *Buddenbrockia* sp. 2 represented the most common blood-stage in common carp (26.5%) and goldfish (27.7%; Holzer et al., 2013). We expect that common carp and goldfish are the natural fish hosts for *Buddenbrockia* sp. 2 as formation of mature spores has repeatedly been observed to occur in kidney tubules. Conversely, whether the other new *Tetracapsuloides* spp. and *Buddenbrockia* sp. 3 are capable of forming spores in their hosts is questionable. Moreover, findings of co-infections with multiple malacosporean species in a single fish in this study bring into question whether, in the case where a particular fish species is a natural host for all these malacosporeans, all of them are capable of forming sporogonic stages in one fish individual and expressing similar infection dynamics as other myxozoan species (Holzer et al., 2010).

Similarly, PKX-like stages have rarely been reported from nonsalmonid fishes (*G. gobio*, *L. leuciscus* and *R. rutilus*; Bucke et al., 1991) but usually macroscopic signs of kidney disease were not seen in these hosts (Bucke et al., 1991) or reports of PKD in nonsalmonid hosts were sporadic (*Esox lucius* and *R. rutilus*; Seagrave et al., 1981; Tops and Okamura, 2005). Immunohistochemical and PCR study on the wild fish population in PKD enzootic rivers confirmed previous findings of *T. bryosalmonae* in *E. lucius* as well as its known host range in salmonids in the UK (Morris et al., 2000). In the present study, PKD was observed only in salmonids and no pathology was found associated with any of our *T. bryosalmonae* PCR-positive non-salmonid fishes. Taking into account our findings, it is very likely that *T. bryosalmonae* is the agent of PKD

exclusively in salmonid fishes. It is also likely that the past reports from fish species harbouring PKX-like stages do not correspond to *T. bryosalmonae* but to other malacosporean species (potentially to *B. plumatellae*, *Buddenbrockia* sp. 3, *Tetracapsuloides* sp. 2 through 5 from this study). Moreover, our data suggest that PKX-like stages with sporoplasmosomes typical for Malacosporea reported from the gills, blood, brain and kidney of common carp (Voronin, 1993; Voronin and Chernysheva, 1993) possibly belong to *Buddenbrockia* sp. 2, the predominant species in common carp, or alternatively to *Tetracapsuloides* sp. 3.

In the present study, we did not find any malacosporeans in marine fish samples independently of fish species, habitat, latitude, depth (e.g. oyster beds and shallows near the sea coast, open sea), and water temperature (subtropics with higher temperatures i.e. Florida, USA, South Carolina, USA and with lower temperatures i.e. South Africa; temperate zones with higher temperatures i.e. Croatia, Italy and with lower temperatures i.e. North Sea and Norwegian Sea) (Supplementary Table S1). We either did not screen enough fish to detect malacosporeans or malacosporean life cycles may involve only marine bryozoans (Anderson et al., 1999; Canning and Okamura, 2004) or other invertebrate groups (phoronids or brachiopods) without a necessity to circulate in vertebrate hosts. The existence of malacosporeans in the marine environment is highly probable due to the fact that the vast majority of bryozoans are marine species (Gordon, 1999). Furthermore, findings of vermiform stages reminiscent of malacosporean parasites in marine bryozoans off the Falkland Islands and the Patagonian shelf i.e. *Beania magellanica*, *Camptoplites giganteus*, *Notoplites drygalskii*, *Notoplites vanhoffeni*, *Notoplites tenuis*, and *Menipea flagellifera* (Hastings, 1943) provide support for this hypothesis.

The present comprehensive phylogenetic analyses revealed the existence of several novel malacosporean species/lineages. No strict separation of malacosporean clades according to host species (both fish and bryozoan), host habitat, morphology of the bryozoan-related stages, and biogeography was found, most probably due to the frequent host-parasite switches during evolution (Jackson, 1999). Moreover, the real evolutionary trends in malacosporeans are difficult to assess as some deductions may be affected by (i) the lack of molecular data for malacosporeans in hosts in which they have been previously recognised e.g. *T. bryosalmonae* and *B. plumatellae* (Supplementary Table S4), (ii) poor taxon sampling e.g. findings of *B. allmani* in single bryozoan species collected at distant European localities suggest its strict host specificity versus insufficient sampling is rather indicated by findings of *Buddenbrockia* sp. 1 and a member of the novel malacosporean lineage in a single bryozoan species (Supplementary Table S4), (iii) missing information about the morphology of both fish- and bryozoan-related stages for some malacosporeans and by (iv) the constraint given by the natural occurrence of hosts in certain habitats e.g. the findings of sac-forming *B. plumatellae*, *Tetracapsuloides* sp. 1 and *T. bryosalmonae* in *C. mucedo* and *P. magnifica* at static sites reflects the general association of gelatinous bryozoans with ponds and lakes rather than with rivers and streams (Okamura and Wood, 2002). Our original expectations of the presence of sac-like malacosporean stages at the riverine habitats, in which water flow would facilitate the dispersal and spatial distribution of the parasites in the environment, and the presence of motile vermiform stages in the static water sites was not supported. Their dispersal is rather conveyed via their motile fish hosts, by fission of bryozoan colonies and via infected bryozoans' statoblasts (Henderson and Okamura, 2004; Hill and Okamura, 2007; Abd-Elfattah et al., 2014).

Particularly crucial for this study is the definition of a species which has always been a difficult task (Balakrishnan, 2005; Tibayrenc, 2006). Universal delimitation of species boundaries based on molecular data is problematic as diverse organismal

groups differ in the speed of evolution of their genes. Based on their SSU rDNA, myxozoans appear to be fast-evolving (Evans et al., 2010). There is no universal criterion regarding what constitutes a sufficient level of SSU rDNA sequence variation to represent distinct species in this parasite group. 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, 2011; Whipps and Kent, 2006). However, it can reach >2.6% in extreme cases (Schlegel et al., 1996). A 2–3% difference in SSU rDNA was set as a cut-off between the values for intra- and interspecies variation of myxosporeans in the genus *Cystodiscus* (Hartigan et al., 2011). Nevertheless, this threshold was based on intra-specific variations of the cytochrome c oxidase subunit 1 (*Cox*I) gene (Hebert et al., 2004; Waugh, 2007) and the mitochondrial (mt)DNA mutation rate is typically one order of magnitude higher than the nuclear one (Ballard and Whitlock, 2004). If a low genetic variation prevents reliable distinction between inter- and conspecific, e.g. *Myxobolus fryeri* and *Myxobolus insidiosus* differing by only 0.5% in their SSU rDNAs (Ferguson et al., 2008), and morphological characteristics as important aspects of species recognition are lacking, biological characters (host species, host habitat, geographic location) can significantly aid species distinction. As this was our case, molecular data were accompanied by aforementioned biological criteria to discriminate species in this study.

Buddenbrockia plumatellae split into two closely-related clades according to the bryozoan-related morphology, differences in habitat and host preference (both bryozoan and fish) and distribution. Worm-like stages seem to have a broader geographic distribution and host preference as they occurred in lentic and lotic ecosystems in Europe and the USA and infect plumatellids (Bryozoa). The worm-like clade also encompassed sequences of isolates originating from cyprinid and perciform fishes. Sac-like stages originated only from gelatinous cristatellids (Bryozoa) present at static water habitats in Europe. The worm-like stages have never been observed in *C. mucedo* (Canning et al., 2002). Increased taxon sampling in this study has revealed that the level of sequence divergence between the two stages (1.29%) is not as low as previously reported (0.7%; Tops et al., 2005). This difference is highly consistent and sufficient to produce two well-supported clades as recognised previously (Tops et al., 2005). "Worms" and sacs were proposed to be conspecific, being expressed as facultative polymorphisms in different bryozoan hosts (Monteiro et al., 2002; Tops et al., 2005). On the other hand, protein-coding data (rpl23a) did not support the conspecificity of the two stages (Jiménez-Guri et al., 2007a) which would indicate that these stages represent the same stage in the life cycle of two different malacosporean parasites (Tops et al., 2005). The relatively high SSU rDNA sequence divergence of the two stages accompanied by the evidence from other genes and differences in their distribution, habitat and host preference (Supplementary Table S4) indicate that the two stages probably belong to two different parasites that have undergone recent speciation. We conclude that the possible future resurrection of *T. bryozoides* for the sac-like stages would require further investigation and more evidence.

Past studies did not determine whether the sequence variation (1.9–2.4%) between *Tetracapsuloides* sp. 1 and *T. bryosalmonae* is due to the existence of two very closely-related species or to intra-specific variation in *T. bryosalmonae* SSU rDNA (Anderson et al., 1999). We suggest that sequence dissimilarity of the two taxa (2.16%) is significant enough to consider them as two different closely-related species which is further supported by their constant clustering in two separate, strongly-supported clades. It seems that *Tetracapsuloides* sp. 1 may be a recently evolved species that diverged from *T. bryosalmonae* in North America; this is shown by close phylogenetic relationships of the two species together with significant overlap in host species spectrum (gelatinous *C. mucedo*, *P. magnifica*), habitat preference (lakes) and distribution (USA) of *Tetracapsuloides* sp. 1 and North-American isolates of *T. bryosalmonae*.

Although phylogenetic analyses of the available malacosporean SSU rDNA sequences suggest that malacosporeans group according to the biogeographic distribution and their host species preference, this finding is affected by the lack of sequence data for particular malacosporean species in hosts from different geographic regions e.g. *T. bryosalmonae* was reported in fredericellids from both the USA (*Fredericella browni*, *Fredericella indica*) and Europe (*F. sultana*, *F. indica*) but sequence data are available only for the malacosporean from *F. sultana* (Okamura et al. 2001; Okamura and Wood, 2002; Supplementary Table S4). Even though malacosporeans have been reported from the Southern hemisphere i.e. Brazil (Marcus, 1941), the fact that all sequence data of malacosporeans originate from the Northern hemisphere prevents conclusions about the clustering of malacosporeans according to their biogeographic distribution. Although most malacosporeans were recorded in the Northern hemisphere, i.e. the USA, Europe, Japan, Turkestan (revised in Canning and Okamura, 2004) and PKD is absent in salmonid farms in the Southern hemisphere (Okamura and Wood, 2002), the real distribution of malacosporeans is hardly limited to the Northern hemisphere. Many bryozoan species, proven to be hosts for malacosporeans, are spread worldwide (Kipp et al., 2010). Therefore, we suppose the malacosporean distribution is rather influenced by poor taxon sampling of their hosts in the Southern hemisphere and more accurate conclusions would require further investigations in unstudied areas.

For the first time, the present study included the rDNA data of a worm-like malacosporean from *P. fungosa* collected in Cowan Lake, Ohio, USA in a comprehensive phylogenetic analysis with other malacosporeans. We reveal its surprising phylogenetic position as a separate malacosporean lineage, independent from the *Tetracapsuloides* and *Buddenbrockia* clades even though this malacosporean had originally been identified as *B. plumatellae* and the same name has been maintained in follow-on studies (Jiménez-Guri et al., 2007b; Evans et al., 2010). While the correct classification of a new malacosporean is irrelevant for its positioning in the metazoan tree, which was the aim of the two studies, it is noteworthy that the organism is definitely not conspecific with *B. plumatellae*. Moreover, the "real" *B. plumatellae* clade includes the SSU rDNA of the "worm" from *P. repens* collected in Germany (Supplementary Table S4), the host species and country from which *B. plumatellae* was described (Schröder, 1910). We assume that the new malacosporean is probably a member of a new genus. However, determining its definitive systematic position within the myxozoans would require a detailed morphological description of the original material supported by further ecological (e.g. fish host) and ultrastructural observations.

Based on the findings of this study, one may hypothesise that this novel malacosporean lineage is a member of the old evolutionary malacosporean line still retaining the features of the cnidarian ancestors (vermiform bodies with tetraradial symmetry and muscle blocks; Jiménez-Guri et al., 2007b). Members of the *Buddenbrockia* clade possess both ancestral features but also evolutionary younger features (sac-like stages). The *Tetracapsuloides* clade only contains parasites with sac-like stages. If *T. bryosalmonae* is an evolutionary young parasite, its high pathogenicity in salmonid fish (compared with no pathogenicity found for *Buddenbrockia* spp.; Grabner and El-Matbouli, 2010) may be explained by short co-evolution of host-parasite interactions.

As only one malacosporean life cycle has been fully resolved to date (Feist et al., 2001; Morris and Adams, 2006) it would be

of particular interest to discover which bryozoan hosts are included in the life cycle of novel fish-related malacosporeans and what kind of morphology (worm-, sac-like) their bryozoan-related stages express. We expect that the bryozoan *P. repens*, which is a cosmopolitan species (Kipp et al., 2010), may be the final host of *Buddenbrockia* sp. 2. *Plumatella repens* is highly abundant in the Czech Republic (Opravilová, 2006) and was the only bryozoan species present at the *Buddenbrockia* sp. 2-positive locality in the Chřešťovice, CR. Moreover, the successful co-habitation of *P. repens* from Germany overtly infected with worm-shaped malacosporean/s morphologically similar to the parasites described previously (Canning et al., 2002; Morris et al., 2002; McGurk et al., 2006) resulted in the infection of minnow by *B. plumatellae* and of common carp by *Buddenbrockia* sp. 2 (Grabner and El-Matbouli, 2010). Therefore, *P. repens* seems to host two different malacosporean species: *B. plumatellae* (McGurk et al., 2006) and a yet undescribed malacosporean species (Grabner and El-Matbouli, 2010), named *Buddenbrockia* sp. 2 in this study.

In this study, phylogenetic clustering of malacosporeans revealed by SSU rDNA data was confirmed by the LSU rDNA and EF-2 data as similarly shown previously for myxosporeans (Bartošová et al., 2009, 2013; Fiala and Bartošová, 2010). This study provides the first known EF-2 sequences of the Malacosporea as all 23 myxozoan EF-2 sequences available in GenBank to date belong to the myxosporean representatives. The unique nucleotide composition of malacosporean EF-2s is most probably the reason why they create a long branch in a sister relationship to the myxosporeans. The long branch attraction artifact is unlikely due to the use of different tree-building methods (MP, ML, BI) showing that the novel EF-2 sequences group within the Myxozoa, even in the taxon-rich metazoan tree.

Buddenbrockia spp. EF-2 gene sequences contain two introns of different nucleotide composition, length and location compared with other metazoans, even to cnidarians including other myxosporeans. Therefore, they cannot be considered taxonomically unique characters such as long inserts in the SSU rDNA typical for *Sphaerospora* spp. (Jirků et al., 2007; Bartošová et al., 2013). Interestingly, no intron was found in any of the *Sphaerospora* spp. EF-2s investigated. The different length and location of the EF-2 introns in metazoans, even in the same species (*Buddenbrockia* sp. 2 or *B. plumatellae*), indicate that these regions are under weak selection constraint. However, they still may play important roles in certain processes (Hesselberth, 2013). Malacosporeans, presumed to be the phylogenetically more ancient myxozoan group (Anderson et al., 1999; Kent et al., 2001), and only two myxosporean species possess inserts in their EF-2 sequences. These are of shorter length than those of their free-living cnidarian relatives which correlates with the hypothesis of genome reduction in parasitic organisms (Gil et al., 2003).

The present molecular study provides new insights into malacosporean diversity and evolutionary trends by identifying three main phylogenetic lineages (genera) with five new species of *Buddenbrockia* and *Tetracapsuloides* from cyprinid and perciform fishes. The significant increase in malacosporean species richness points to a hidden biodiversity within this parasite group. Addressing the biodiversity of parasites and understanding their role in the environment is crucial for conservation purposes. Moreover, the knowledge of host species spectra, distribution and evolutionary relatedness of novel malacosporean species, especially of *Tetracapsuloides* spp., potential fish pathogens such as *T. bryosalmonae*, are important for future diagnosis and control of relevant diseases in aquaculture. We expect that future research on fish as well as bryozoan hosts is likely to reveal an even higher diversity in the Malacosporea as well as to elucidate putative counterparts in the life cycle of novel malacosporean species.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2014.04.005.

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