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ORIGINAL ARTICLE

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

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## Abstract

The environmental DNA (eDNA) metabarcoding approach has become a useful tool for detecting the species diversity of different animal groups, including parasites. Myxozoa (Malacosporea and Myxosporea) represent a unique group of morphologically simplified endoparasites that mainly infest fish and whose diversity remains largely unexplored. Metabarcoding of DNA from the aquatic environment is a promising non-invasive method that allows us to assess myxozoan biodiversity at a given site. This is essential not only for describing myxozoan communities, but also for the development of disease control methods. Using an alignment of 330 sequences, we employed *in silico* PCR to score primer pairs, designed to amplify the V4 region of the SSU rDNA of different myxosporean sublineages comprising the entire diversity of oligochaete-infecting (freshwater) myxosporeans. We selected eight clade-specific primer sets for metabarcoding, avoiding amplification of DNA from other organisms present in eutrophic freshwaters. The metabarcoding approach used in the analysis of eDNA sediment samples detected a high myxosporean diversity even in small sample volumes (in total 44 OTUs). Furthermore, metabarcoding analysis of myxosporeans in fish tissue samples selected for primer testing revealed 91 different myxosporean OTUs, more than double the number obtained by classical PCR screening and Sanger sequencing with general myxozoan primers and almost seven times higher detection than by microscopic examination. Our results further suggest quantitative sampling requirements for realistic future diversity estimates by comparing OTUs from fish tissue metabarcoding and eDNA samples. The use of specific primer sets enabled the detection of a high proportion of myxosporean reads (63–100%) in all datasets, even in highly eutrophic habitats. This shows our metabarcoding approach as an excellent tool for non-invasive and sensitive detection of myxosporean biodiversity in aquatic sediments, potentially useful for monitoring myxozoan disease agents that threaten economically important fish in aquaculture.

**KEYWORDS**
comparative diversity, environmental DNA, eutrophic, fish parasites, phylogeny, sediment

## 1 | INTRODUCTION

Assessing the biodiversity of life on Earth in terms of species and genetic diversity is one of the most difficult topics in ecological studies (Myers et al., 2000). Despite constant research, we still know very little about organismal biodiversity. In the last decade, amplicon sequencing and metabarcoding of various environmental samples have been performed to determine the diversity of microbes, protists, fungi, parasites, or fishes and to monitor endangered species or infections by parasites and other pathogens (Bass et al., 2015; Català et al., 2015; Pawlowski et al., 2014; Rees et al., 2014). In addition, these next-generation methods have also been used to detect rare, low-density animal populations (Furlan et al., 2019; Jerde et al., 2011).

The biodiversity of parasitic organisms is thought to be unexplored. Myxozoa is one such parasitic group with a potentially highly underestimated diversity (Holland et al., 2011; Okamura et al., 2018). These endoparasitic cnidarians infect annelids and bryozoans as definitive hosts and fish, amphibians, rarely reptiles, and even birds and mammals as intermediate hosts (Aguiar et al., 2017; Bartholomew et al., 2008; Dyková et al., 2007; Fiala et al., 2015; Lisnerová, Fiala, et al., 2020). Myxozoan infections are often inconspicuous, but some cause severe damage to their hosts, including economically important fishes (Eszterbauer et al., 2015; Hartikainen & Okamura, 2015). Research focuses on these organisms not only because of their economic and veterinary importance but also because of their ultimate uniqueness among animals in terms of development (e.g., endogenous budding and spore formation; Lom & Dyková, 2006), evolutionary origin (Holzer et al., 2018), nuclear and mitochondrial genome characteristics (smallest metazoan nuclear genomes with important gene reductions and loss of basic biological functions and highly divergent mitochondrial genomes with the fastest rate of evolution; Alama-Bermejo & Holzer, 2021) and by unique functional molecular adaptations to the parasitic lifestyle, such as the loss of mitochondrial respiration in one species (Yahalomi et al., 2020).

The known diversity of myxozoans reaches about 2600 described species classified in 65 genera (Okamura et al., 2018). However, it is believed that the diversity of myxozoans is greatly underestimated (Bartošová-Sojková et al., 2014; Hartikainen et al., 2016; Okamura et al., 2018). Some authors even estimate that there are up to 16,000 myxozoans parasitizing fishes in the Neotropical part of the world alone. This estimate is based on the high host specificity of most myxozoans and the great diversity of freshwater fishes in the Amazon (Eiras et al., 2011). Traditionally, research on myxozoan diversity has been based mainly on the dissection and examination of their typical intermediate hosts—fishes, for which myxozoan infections have been reported worldwide (e.g., Bartošová-Sojková et al., 2018; Kaur et al., 2017; Kodádková et al., 2014). Myxozoan life cycles which are characterized by transmission stages in the form of spores, released by both definitive and intermediate hosts into the environment (water, aquatic sediments), where they can be detected by eDNA metabarcoding methods.

The introduction of the eDNA metabarcoding technique for assessing the biodiversity of a given group of organisms may present many methodological difficulties that affect the detection of diversity in all steps of sample processing: study design, sample collection, laboratory methods and bioinformatic analyses (summarized in Alama-Bermejo & Holzer, 2021). First, an appropriate technique for eDNA capture of the group of organisms in question from the environment is essential for the successful assessment of eDNA diversity, as is the capture of developmental stages in organism-rich waters (without the possibility of filtration) with potential dilution effect (Alama-Bermejo & Holzer, 2021), and optimized DNA isolation with an inhibitor removal step (Eichmiller et al., 2016). The selection of an appropriate marker is another important criterion for metabarcoding studies. Different genes and gene regions are used, such as ITS mainly for fungi (Riit et al., 2016; Schoch et al., 2012) or rbcl for plants (Fahner et al., 2016). COI (e.g., Lacoursière-Roussel et al., 2018) and SSU rDNA (e.g., Rojas-Velázquez et al., 2019) are commonly used markers for a variety of organisms, from protists to metazoans. SSU rDNA is a multi-copy gene and consists of variable and conserved regions of varying lengths, with region V4 being the largest variable part of this gene in eukaryotes, including myxozoans (Holzer et al., 2007; Nickrent & Sargent, 1991), suggesting it as a good candidate marker for biodiversity assessment using eDNA and metabarcoding.

Selection and comprehensive evaluation of PCR primers that amplify the target gene marker of the desired group of organisms is a fundamental step in eDNA metabarcoding assays (Alberdi et al., 2018; Collins et al., 2019; Zhang et al., 2020). Efficient primer binding is a prerequisite for the correct assessment of the taxon group of interest. Less efficient primer binding may result in no amplification or amplification of DNA fragments from different taxon groups that co-occur in the environment with the target group. The ideal primer pair should be designed to bind a conserved (group-specific) sequence region and amplify the target sequence with variable sites that allow taxonomic identification of very closely related species.

The assessment of myxozoan diversity using the eDNA metabarcoding approach is still in its early stages. Hartikainen et al. (2016) published a pioneering study showing that eDNA analysis is a very useful tool for myxozoan detection. They introduced myxozoan-lineage-specific primers that were able to amplify myxozoan DNA from water filtrates and, interestingly, from cormorant and otter feces, in which myxozoan detection was unexpectedly high. A species-specific eDNA approach has also been used to detect and monitor the density of *Ceratonova shasta*, a pathogen of salmonids (Richey et al., 2020) and *Kudoa thyrsites*, a pathogen of marine fish (Marshall et al., 2022).

Our preliminary unpublished investigation of myxozoan diversity in freshwater eutrophic waters using the eDNA metabarcoding approach of Hartikainen et al. (2016) revealed very low detection of myxozoans at sites where fishes are known to have multi-species infections. This lack of success could be related to the eutrophic characteristics of the sampled aquatic habitats, characterized by dense

algal and protist communities typical of warmer sites in Central Europe and elsewhere. Only small amounts of water samples could be filtered before the filter became clogged due to high numbers of planktonic organisms. PCR using the published primers (Hartikainen et al., 2016) non-specifically amplified a large variability of the very abundant organisms and only a small fragment of the myxozoan diversity.

In the present study, we propose a methodological approach to assess the diversity of myxosporean parasites in freshwater, tailored to mesotrophic and eutrophic waters with abundant phytoplankton and other eukaryotic microorganisms. We developed and validated a set of barcode primer pairs for the detection of myxosporean organisms with high taxonomic specificity and broad coverage. First, we analyzed a wide range of SSU rDNA myxosporean sequences to select candidate primer pairs. Then, we performed *in silico* PCR analysis to select the best-fitting primers. We then tested PCR sensitivity and primer specificity on myxosporean-infected fish tissue samples and finally performed a pilot eDNA metabarcoding analysis.

## 2 | MATERIALS AND METHODS

### 2.1 | Primer development and *in silico* PCR testing

We downloaded SSU rDNA sequences of all phylogenetic groups of the oligochaete-infecting (freshwater) lineage of myxosporeans (Holzer et al., 2018) available in GenBank. Full-length SSU rDNA sequences including all variable regions were aligned using MAFFT v7.450 (Katoh et al., 2005) with the E-INS-i algorithm in Geneious Prime 2019.0.4 (Biomatters Ltd., New Zealand). The final alignment consisted of 330 sequences that were ordered by phylogenetic clades of freshwater myxosporeans. Using an alignment view in Geneious Prime, we manually selected the best sites for primer pairing and designed primers specific to oligochaete-infecting (freshwater) myxosporean clades in the V4 region. Primer properties were checked using Primer3 v.2.3.7, which is implemented in Geneious Prime.

Based on the final alignment, we created a sequence database for *in silico* PCR evaluation of selected SSU rDNA sequences covering representatives of all freshwater myxosporean clades (including all eight subclades of the *Myxobolus* clade defined by Liu et al. (2019)). We evaluated the taxonomic resolution of the primer candidate pairs for each myxosporean clade by *in silico* testing of the primers on each sequence with three to five allowed mismatches in the binding region using Geneious Prime. We further tested the primer pairs published by Hartikainen et al., 2016.

### 2.2 | Fish sample collection

To test the candidate primers selected by *in silico* PCR and to differentiate between the detection of myxosporean diversity from fish vs eDNA samples, using metabarcoding, we dissected fish to obtain tissue samples infected with myxosporeans. A total of 166 fish from 17 species were collected: 100 fish (12 species) in Švihov reservoir (area 1602.6 ha; 49°40′27.480″N; 15°9′48.600″E), 33 fish (six species) in Rájský pond (area 0.2 ha; 49°49′45.840″N; 15°28′5.880″E), and 33 fish (four species) in Hostačovka brook (length 23.7 km; 49°48′56.707″N; 15°31′48.786″E) during the period from 2017 to 2019. The details of fish dissection are summarized in Table S1. All fish were euthanized with an overdose of buffered MS-222. The fish were dissected and obtained tissues examined for myxosporean infection by screening for spores, using an Olympus BX51 light microscope (Tokyo, Japan). Myxospore morphology was documented using an Olympus DP70 digital camera (Tokyo, Japan). All tissue samples were stored in 400 µl TNES-urea buffer (10 mM Tris–HCl with pH 8; 125 mM NaCl; 10 mM EDTA; 0.5% SDS and 4 M urea). Genomic DNA was extracted using the standard protocol for phenol–chloroform extraction with overnight digestion with proteinase K (50 µg ml-1; Serva, Heidelberg, Germany) at 55°C, and dissolved in 100 µl DNAse-free water (Holzer et al., 2004). DNA samples were combined to obtain one DNA sample for all tissues of all individuals of a single host species per site (=22 pooled DNA samples). These DNA pooled samples were prepared from both microscopically positive and negative tissues.

### 2.3 | eDNA preparation

To evaluate the capacity of our primers to amplify myxosporean DNA from environmental samples (aquatic sediments) and compare diversity estimates from fish and environments, we conducted a pilot eDNA metabarcoding analysis of test samples from aquatic sediments collected from the same sites from which fish had been sampled for parasite screening, in April 2020. To extend the diversity of aquatic habitats, we included a single aquatic sediment sample collected in September 2019 in the Douro River estuary, Porto, Portugal (GPS: 41°8′51.000″N, 8°39′11.000″W). Aquatic sediment samples of 300 ml (Hostačovka brook, Švihov reservoir), 150 ml (Rájský pond) and 50 ml (Douro River estuary) were used. The samples were taken according to the type of ecosystem: (i) from the bank of the Rájský pond; (ii) from the main stream as well as from the bed of the main stream in the case of the Hostačovka brook; (iii) and iv from the bank of the Švihov reservoir and Douro River. Each sample contained a certain volume of sediment (muddy sediment without pebbles) and was taken from the first 4 cm of top sediment layer. The sediment was processed immediately after collection. Tap water, free of myxozoan DNA (600 ml) was added to the sediment. The mixture was thoroughly agitated and filtered through 200 µm and 100 µm sieves to avoid large particles. The sample was mixed again, and the supernatant (around 600 ml) with floating particles (including myxospores and actinospores) was collected (after a settling period of the large and heavy particles, approximately 4 min) and centrifuged in a large volume centrifuge, at 4000×*g* for 20 min. The remaining water was aspirated off, and the pellet samples were stored in a freezer

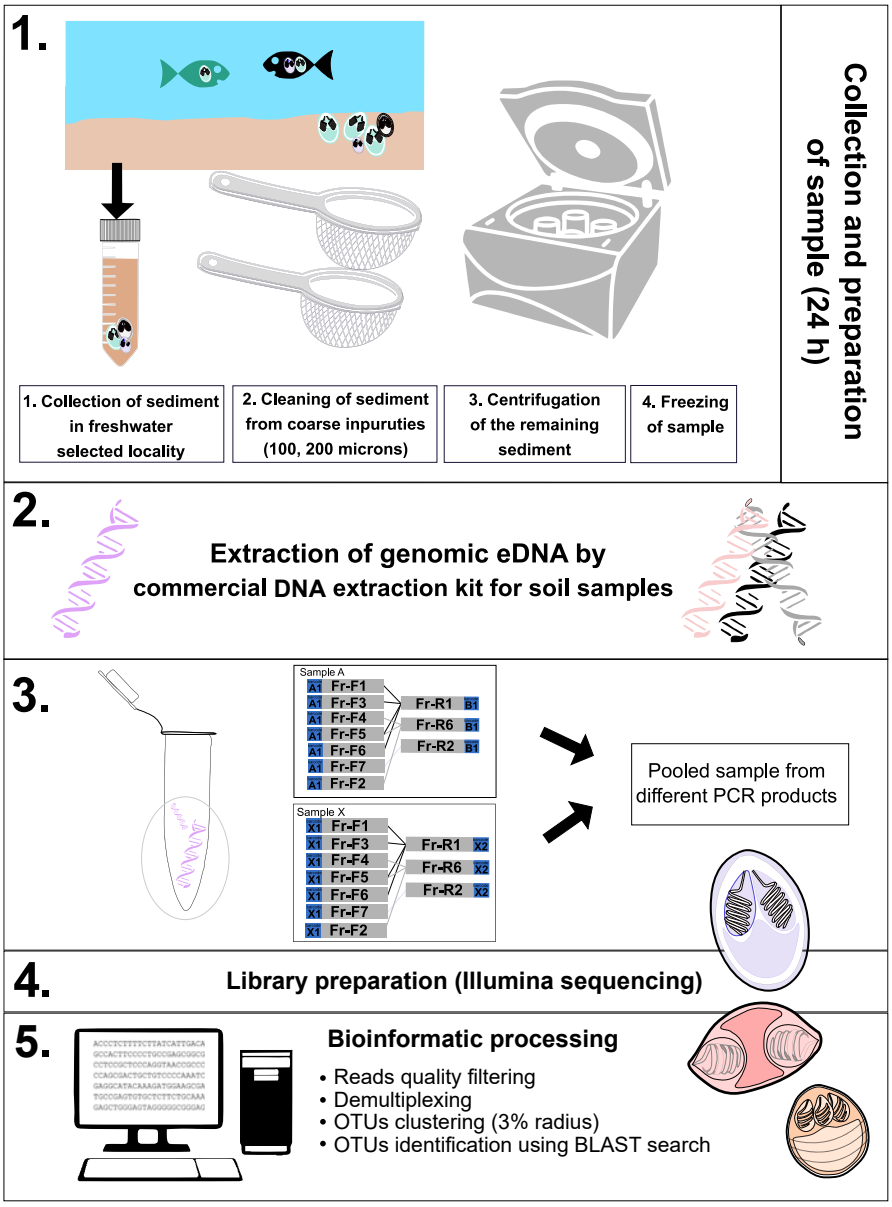



**FIGURE 1** Illustration of eDNA sample processing. Step 1: Collection of sediment sample; step 2: Isolation of genomic DNA; step 3: PCR scheme using myxosporean group-specific barcoded primer pairs; step 4: Library preparation for Illumina mi-Seq; step 5: Final bioinformatic processing resulting in determining myxosporean OTUs in the sample

(−20°C). The procedure is shown in Figure 1, step 1. Each pellet of the sediment sample was divided into Eppendorf tubes, each containing up to 500 mg of sediment from the soil sample. The FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, Ohio) was used for the extraction of genomic eDNA from sediment samples according to the manufacturer's instructions. The purified nucleic acids were eluted from the silica in 100 µl DES (DNase/pyrogen-free water) (Figure 1, step 2).

## 2.4 | Control PCR amplification and sanger sequencing

Before PCR amplification for metabarcoding analysis, we screened individual DNA fish tissue samples (Table S1) for the presence of myxozoan infections using nested PCR with the universal eukaryotic primer combination set ERIB1 (5′-ACCTGGTTGAT CCTGCCAG-3′)+ERIB10 (5′-CTTCCGCAGGTTCACCTACGG-3) (Barta et al., 1997) in the first run and the general myxozoan primer set MyxGP2F (5′-TGG ATA ACC GTG GGA AA-3′) (Kent et al., 1998)+ACT1r (5′-AAT TTC ACC TCT CGC TGC CA-3′) (Hallett & Diamant, 2001) in the second run. PCRs and cycling parameters were set up as in Lisnerová, Blabolil, et al. (2020). Obtained PCR products were cleaned by Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., New Taipei, Taiwan) and sequenced directly by Sanger sequencing (SEQme, Dobříš, Czech Republic). Cloning was performed in order to obtain single species sequences in cases when mixed infections were suspected, or direct sequencing resulted in chromatograms with overlapping peaks. We used the PCR Cloning Kit (Qiagen, Hilden, Germany) with the pDrive vector to clone PCR fragments and competent *Escherichia coli* cells (Life Technologies, Prague, Czech Republic) for vector transformation. We extracted and purified cloned DNA plasmids using the High Pure Plasmid Isolation Kit (Roche Applied Science, Penzberg, Germany), and we

sequenced three plasmid colonies from each cloned product by Sanger sequencing (SEQme, Dobříš, Czech Republic).

## 2.5 | Metabarcoding

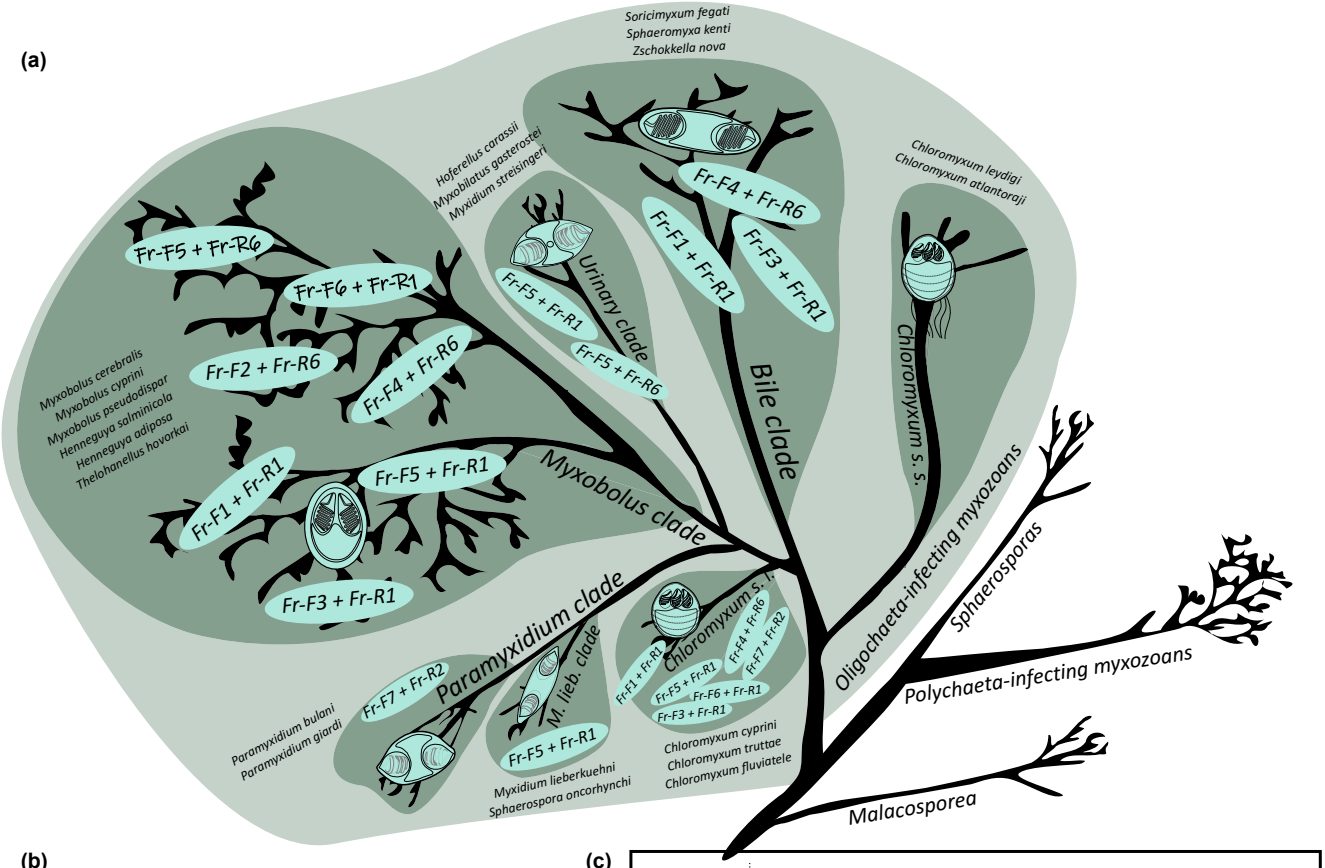
We determined the specific part of the V4 region of the SSU rDNA as the best for metabarcoding analysis of myxozoans, and we identified the position for the forward and reverse primer annealing sites (22 selected primer pairs, Table S2) based on a manual evaluation of the SSU rDNA of all available myxosporean sequences clustered in the freshwater/oligochaete-infecting lineage. The specific primers delineate the part of V4 SSU rDNA sequence that meets the criteria of expected amplified product size of approximately 350 bp and is suitable for metabarcoding as it covers a variable informative region (Figure 2b). More importantly, primer binding sites in the variable region were selected for highly specific primer annealing, allowing selective amplification of DNA from specific phylogenetic sublineages (clades) of freshwater myxosporeans. Based on the alignment and known phylogenetic relationships of freshwater myxosporean species (Fiala et al., 2015; Holzer et al., 2018), we designed seven forward and six reverse primers suggested to be specific to the defined myxosporean phylogenetic groups (Table S2). From these primers, we selected 22 paired combinations for *in silico* PCR analysis. Based on the results of *in silico* PCR, we were able to select a final set of eight PCR primer pairs that *in silico* amplified DNA from all 93 freshwater myxosporean sequences tested, covering the diversity of freshwater myxosporeans (Table S2). We also tested the *in silico* amplification of DNA feom several representatives of groups commonly found in freshwater environments, such as algae, Excavata, Amoebozoa and Alveolata. None of these organisms showed positive *in silico* amplification by our original 22 candidate primer pairs.

The PCR scheme using barcoded primers is described in Figure 1 (step 3). Each sample (eDNA or fish tissue) was amplified with the set of eight primer pairs tagged with a forward and reverse barcode unique for each eDNA locality or fish tissue. The PCR reactions were performed using the AccuPower PCR PreMix premix (Bioneer, Daejeon, South Korea) containing a lyophilized PCR master mix, 0.5 µl of each primer (25 pmol), 18 µl of DNAse-free water, and 1 µl of extracted DNA in a concentration around 50 ng/µl. We amplified PCR products using the nested PCR approach. Universal eukaryotic primers ERIB1 and ERIB10 were used in the first run (cycling parameters set up as in Lisnerová, Blabolil, et al. (2020)). Barcoded myxosporean-group-specific sets of primers were used in the second run (Table S2, Figure 2a). The cycling parameters were set up as follows: initiation 95°C for 3 min, followed by 40 cycles of 94°C for 1 min, 58°C for 30 s, 72°C for 30 s, with a terminal extension at 72°C for 10 min. In addition, Hartikainen et al. (2016) approach was evaluated using the sample from the estuary of the Douro River. PCR products were extracted by a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan). PCR product libraries were prepared for the Czech eDNA, fish host samples and the eDNA sample from the Douro River estuary. Amplicons were paired-end sequenced on an Illumina Mi-Seq (250 bp) (SEQme, Dobříš, Czech Republic).

## 2.6 | Bioinformatic processing and phylogenetic analyses

Forward and reverse reads were merged using FastqJoin (Aronesty, 2011) with a minimum of 20 bp and maximum of 40 bp overlap. Reads were quality-controlled and low-quality sequences were filtered with a minimum quality value threshold set to 33 with a minimum of 50% of bases required to have a given quality using the FastX Toolkit (Gordon & Hannon, 2010). We demultiplexed the data based on the barcode sequences using a Python script (File S1) with subsequent barcode trimming. Chimaera removal was done by UCHIME (Edgar et al., 2011). Operational taxonomic units (OTUs) were clustered to generate sets of unique sequences at an OTU radius of 3% and the OTU table was constructed using USEARCH (Edgar, 2010). Only OTUs containing more than 50 reads were included in the analysis to avoid false-positive detections, with the exception of OTU62 (22 reads), which we considered to be a valid OTU based on phylogenetic analysis and ecological context. Finally, a BLAST search was performed using the NCBI nucleotide database to identify each OTU in relation to the group or species, if known. We compared exclusive and shared OTUs by sample type (eDNA localities and fish species) using Venn diagrams (Oliveros, 2007).

To reveal the taxonomic position of the new myxosporean OTUs, we used a large SSU rDNA dataset representing all clades of the myxosporean freshwater lineage including eDNA OTU sequences from the study of Hartikainen et al. (2016). We used *Chloromyxum* spp. sequences from sharks and rays as close outgroups. For GenBank accession numbers concerning all taxa see Figure S1. Sequences were aligned using the E-INS-i multiple alignment method in MAFFT v7.017 (Katoh et al., 2005). Ambiguously aligned positions were removed. Phylogenetic trees were generated by two phylogenetic methods: Bayesian inference (BI) and maximum likelihood (ML) implemented in Geneious prime 2019.0.4 (Biomatters Ltd., New Zealand). ML analysis was performed using RAxML v7.2.8 (Stamatakis, 2006) with a GTR+Γ model. Bootstrap supports were calculated from 1000 replications. BI was done using MrBayes v3.0 (Ronquist & Huelsenbeck, 2003) with the GTR+Γ model of evolution. MrBayes was run to estimate posterior probabilities over 5 million generations via two independent runs of four simultaneous Markov Chain Monte Carlo (MCMC) algorithms with every 100th tree saved. Species-specific divergences were identified from proportional distances (in %) calculated in Geneious prime based on the dataset used for the ML analysis.




(c)

| Primer combinations | *Myxobolus* clade iv | vii | viii | *Chloromyxum s.l.* | Bile clade | Urinary clade | *M. lieber.* clade | Actinosporean clade |
|---|---|---|---|---|---|---|---|---|
| FR-F1 + FR-R1 | 3 | 9 | 21* | 1 | - | - | - | - |
| FR-F3 + FR-R1 | - | 2 | 5* | 1 | 3 | | 2 | 1* |
| FR-F4 + FR-R6 | - | - | 12 | - | - | - | - | - |
| FR-F5 + FR-R1 | - | 1 | 5* | 1 | 3 | 4 | 6 | 6 |
| FR-F5 + FR-R6 | 1* | 2* | 4 | 6* | 1* | - | - | - |
| FR-F6 + FR-R1 | - | 7 | 4* | 3 | 1 | - | - | 1* |

FIGURE 2 Primer design strategy. (a) Illustration of oligochaete-infecting (freshwater) myxosporean phylogenetic tree with selected specific primer sets designed for individual phylogenetic clades. (b) Illustration of SSU rDNA gene with variable regions V1-V9. Selected part of V4 region is highlighted. (c) Number of OTUs amplified in eDNA and fish tissue samples, using defined primer combinations, excluding those that did not amplify any target sequences

## 3 | RESULTS

### 3.1 | Metabarcoding of fish host tissue infected by myxosporeans proved efficiency of group-specific primers

Microscopically, the fish were infected with 13 myxosporean species assigned to four genera (Figure 3; Table S1). PCR of pooled fish tissue DNA samples revealed positive products of the expected weight size for six of eight primer combinations selected by *in silico* PCR analysis. The primer combinations FR-F2+FR-R6 and FR-F7+R-R2 gave negative results in all fish PCR samples examined. Comparative Sanger sequencing analysis of the cloned PCR products (three clones per PCR product) amplified with myxozoan-specific primers (MyxGP2F and ACT1r) and the same set of fish tissue samples (non-pooled) resulted in the identification of 36 different SSU rDNA myxosporean sequences. Metabarcoding analysis revealed 84 myxosporean OTUs from all defined clades of the freshwater lineage, with the highest number of OTUs found in the *Myxobolus* clade (Figures 4 and 5).

### 3.2 | Pilot eDNA metabarcoding approach specifically detected myxosporean OTUs in sediment samples

The eight barcoded primer pairs selected by *in silico* PCR and tested on fish DNA samples containing myxosporean DNA were used in a preliminary eDNA metabarcoding study to demonstrate the

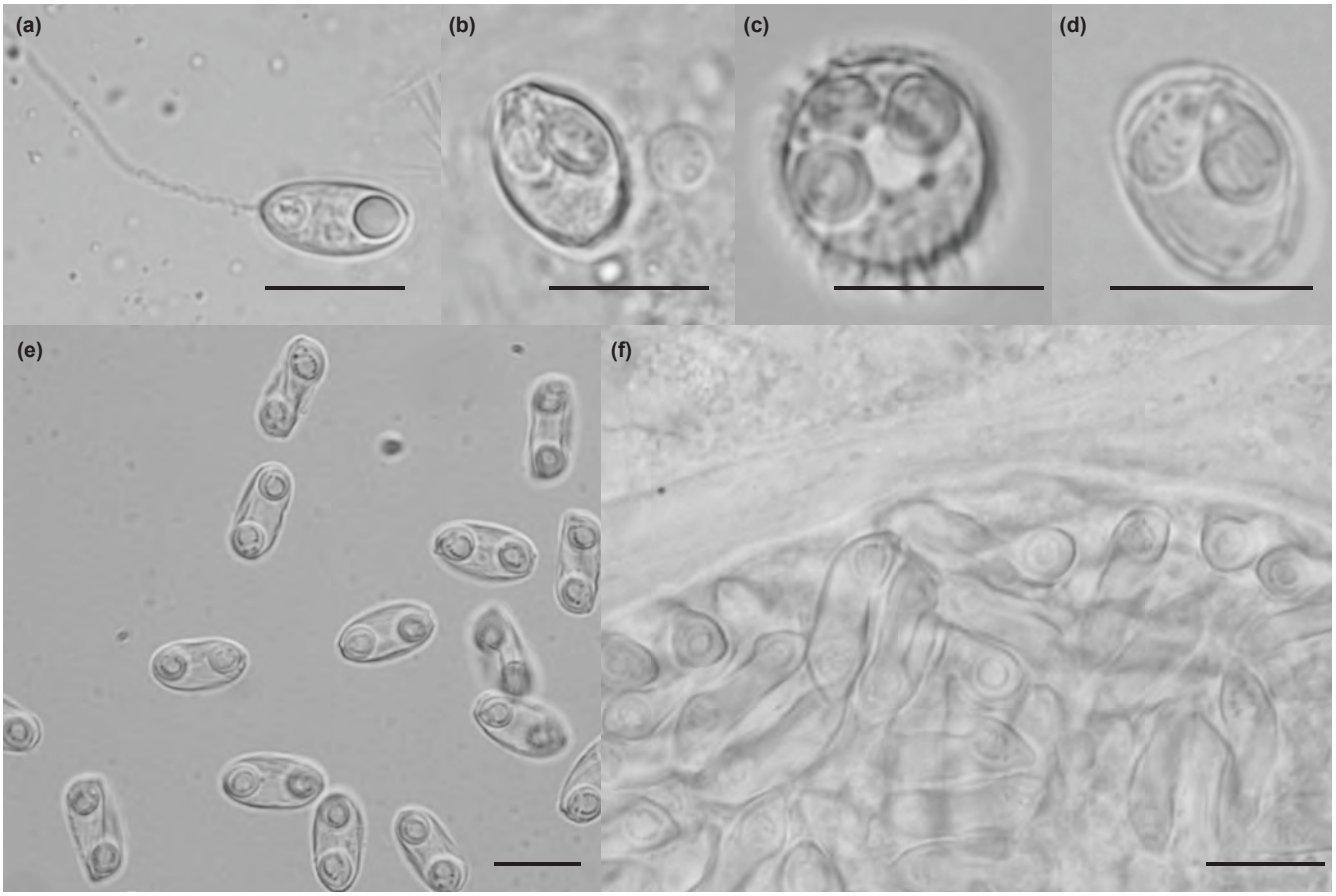



**FIGURE 3** Examples of mature myxosporean spores obtained from the different tissues/organs of different fish hosts from samplings sites in the Czech Republic. (a) Spore of *Zschokkella* sp. from gall bladder of *Abramis brama* from Švihov reservoir; (b) Spore of *Myxobolus* sp. from kidney of *Phoxinus phoxinus* from Hostačovka brook; (c) Spore of *Chloromyxum* sp. from gall bladder of *Ctenopharyngodon idella* from Rájský pond; (d) Spore of *Myxobolus* sp. from kidney of *Alburnus alburnus* from Švihov reservoir; (e) Spores of *Zschokkella* sp. from gall bladder of *Ctenopharyngodon idella* from Rájský pond; F spores of *Myxidium* sp. from kidney of *Scardinius erythrophthalmus* from Švihov reservoir. Scale 10 μm

specificity and functionality of our newly developed assay for assessing myxosporean diversity in eDNA samples. Metabarcoding analysis of four sets of eDNA samples revealed 27 OTUs in the three Czech localities (Figures 4 and 5) and 17 OTUs in the single sample from the Douro River estuary (Portugal) (Figure 6). The samples from the Švihov reservoir and the Rájský pond contained both 14 OTUs and seven OTUs were detected in the sample from the Hostačovka brook. The comparative metabarcoding of the sediment sample from the Douro River estuary using primers published in Hartikainen et al. (2016) did not detect any myxozoan OTU.

The specificity of amplification of myxosporean sequences was high in the environmental samples. We detected 93.8% (1,030,330 of 1,098,531) of OTUs from eDNA that were assigned to myxosporeans. Interestingly, Rájský pond showed 100% of myxosporean reads (355,219 out of 355,219) and Švihov reservoir 99.5% of myxosporean reads (550,599 out of 553,391), while Hostačovka brook had a much lower percentage of myxosporean reads (65.6%; 124,512 out of 189,921). The smaller percentage of myxosporean reads belongs to the sample from the Douro River estuary (63.1%; 260,720 out of 413,486). The *Myxobolus* clade was most abundant in eDNA samples from the Czech localities. Eighteen different OTUs belonged to the *Myxobolus* subclade VIII, which contains almost exclusively myxobolids infecting cyprinids (Figures 4 and 5). Six OTUs belonged to the *Chloromyxum s. l.* clade and one OTU belonged to both the gall and urine clades. Fifteen OTUs had a sequence distance of more than 2% from the most closely related sequences from GenBank.

Figures 4 and 5 show the Venn diagrams with the OTU detections by three different approaches (metabarcoding of infected fish tissue/Sanger sequencing of fish tissue/metabarcoding of the environment). The highest number, 70 OTUs, was detected in the water reservoir, with 80% (56/70) of OTUs detected by metabarcoding of fish tissues and 20% (14/70) of OTUs detected by eDNA metabarcoding. Only 11.4% (8/70) of OTUs were detected by both methods. In the much smaller pond system, the ratio of OTUs detected in the environment and fish was higher: 48.3% (14/29) of OTUs in the environment and 69% (20/29) of OTUs in fish tissues; 37.9% (11/29) of OTUs were found by both methods. The analysis of the Hostačovka brook revealed 19 myxosporean OTUs, of which 89.5% (17/19) were detected in the fish tissues and 36.8% (7/19) in the eDNA samples, while 26.3% (5/19) were found by both methods. Sanger control

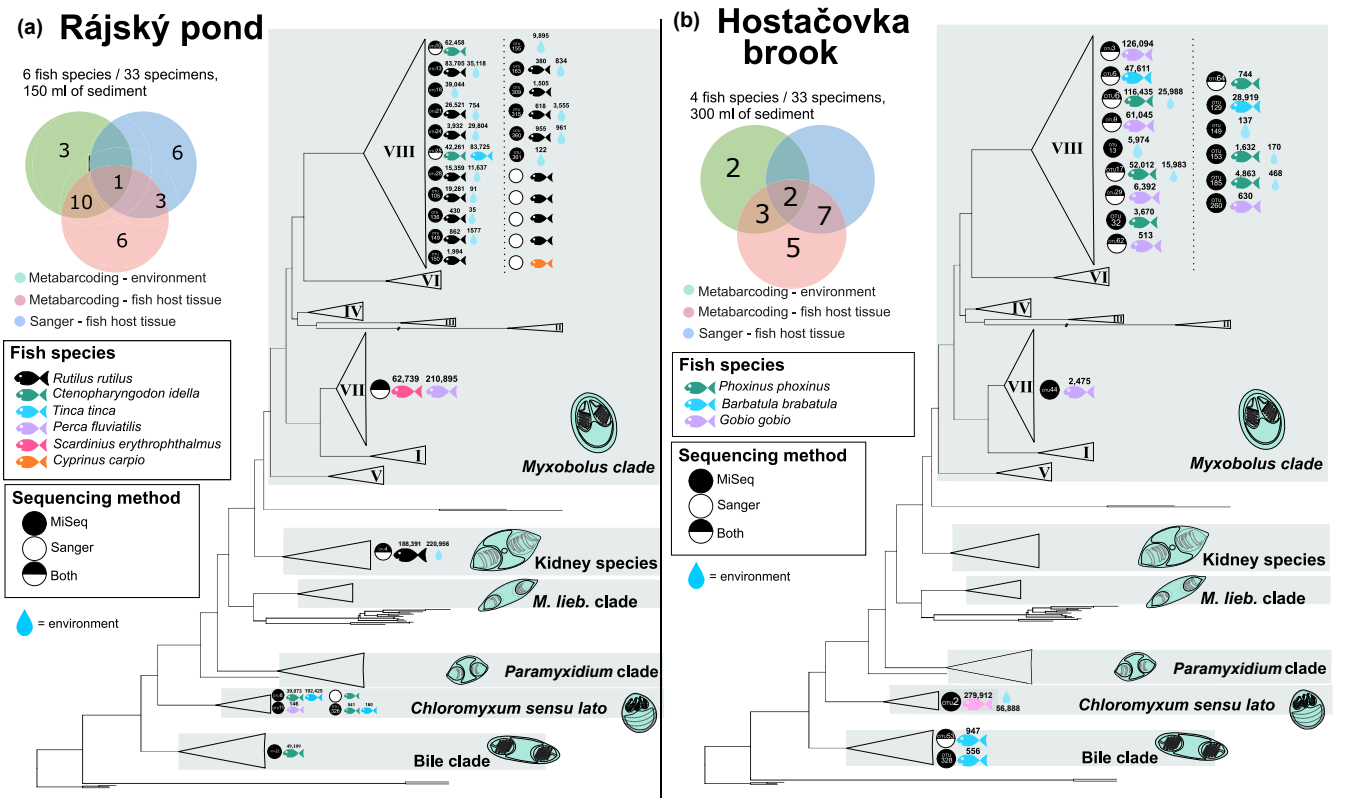



FIGURE 4 SSU rDNA-based phylogenetic tree of oligochaete-infecting (freshwater) myxosporean lineage with individual taxon branches collapsed in the main phylogenetic clades. Individual OTUs obtained from environmental and fish tissue metabarcoding and Sanger sequencing of fish tissues are shown in circles in the corresponding clades. Fish host species are represented by colored fish pictograms with the number of reads found for each OTU specified. The Venn diagrams show the exclusive and shared OTUs obtained from the three different processing types. (a) Rájský pond; (b) Hostačovka brook

sequencing of PCR products (directly sequenced or after cloning) revealed about half (47.4%, 9/19), of the diversity of myxosporean OTUs in the fish tissue samples from the Hostačovka brook, determined by metabarcoding, and only one third of myxosporean OTUs diversity in the fish tissue samples from the Rájský pond (34.5%, 10/29) and the Švihov reservoir (28.6%, 20/70).

## 3.3 | Phylogenetic relationships of detected myxosporean OTUs

Phylogenetic analysis of all OTUs recovered from the three PCR libraries revealed the relationship of 108 unique OTUs identified by metabarcoding: 91 OTUs from metabarcoding of infected fish tissue samples and eDNA from three Czech localities and 17 OTUs from eDNA from the Douro River estuary (Figure S1). Based on the results of metabarcoding of infected fish tissue samples and eDNA from three Czech localities, several identical OTUs were detected in tissues from different fish species, and identical OTUs were also found in both fish and aquatic sediment samples from the same study sites. (Figures 4 and 5). Sixteen OTUs were identical to sequences from GenBank, and seven OTUs were identical to OTUs from the eDNA study by Hartikainen et al. (2016). Moreover, we detected three OTUs that have identical sequences in both GenBank and OTUs dataset from the Hartikainen et al. study. Eighty-two OTUs were not identical to any known myxozoan sequence and represent unexplored myxozoan diversity.

The phylogenetic relationships of the OTUs from the Czech localities and the Douro River estuary with the available myxosporean sequences from the freshwater lineage are shown in the large phylogenetic tree (Figure S1). Newly generated OTUs are present in all phylogenetic groups of the myxosporean freshwater lineage, with the highest representation in the *Myxobolus* clade. The largest number of OTUs that are placed within this clade belong to the *Myxobolus pseudodispar* species complex as well as to a clade containing *Myxobolus parviformis*, *M. muellericus* and *M. diversicapsularis*. We discovered a relatively high number of new OTUs also in non-myxobolid clades such as the *Chloromyxum* clade and the *Myxidium lieberkuehni* clade. Eight OTUs are present in the *Paramyxidium* clade (Environmental clade as defined by Hartikainen et al. (2016)), although the primer pair specific for this group did not amplify any PCR product. Almost all newly obtained sequences from the amplicon data of the Douro River sample were phylogenetically close to the sequences of *Myxobolus* spp. from mullets and formed a well-supported clade (Figure 6a). A single OTU with low abundance was found outside this cluster near *Myxobolus portucalensis* (Figure 6b). None of the 17 newly obtained sequences is completely identical to those previously published.

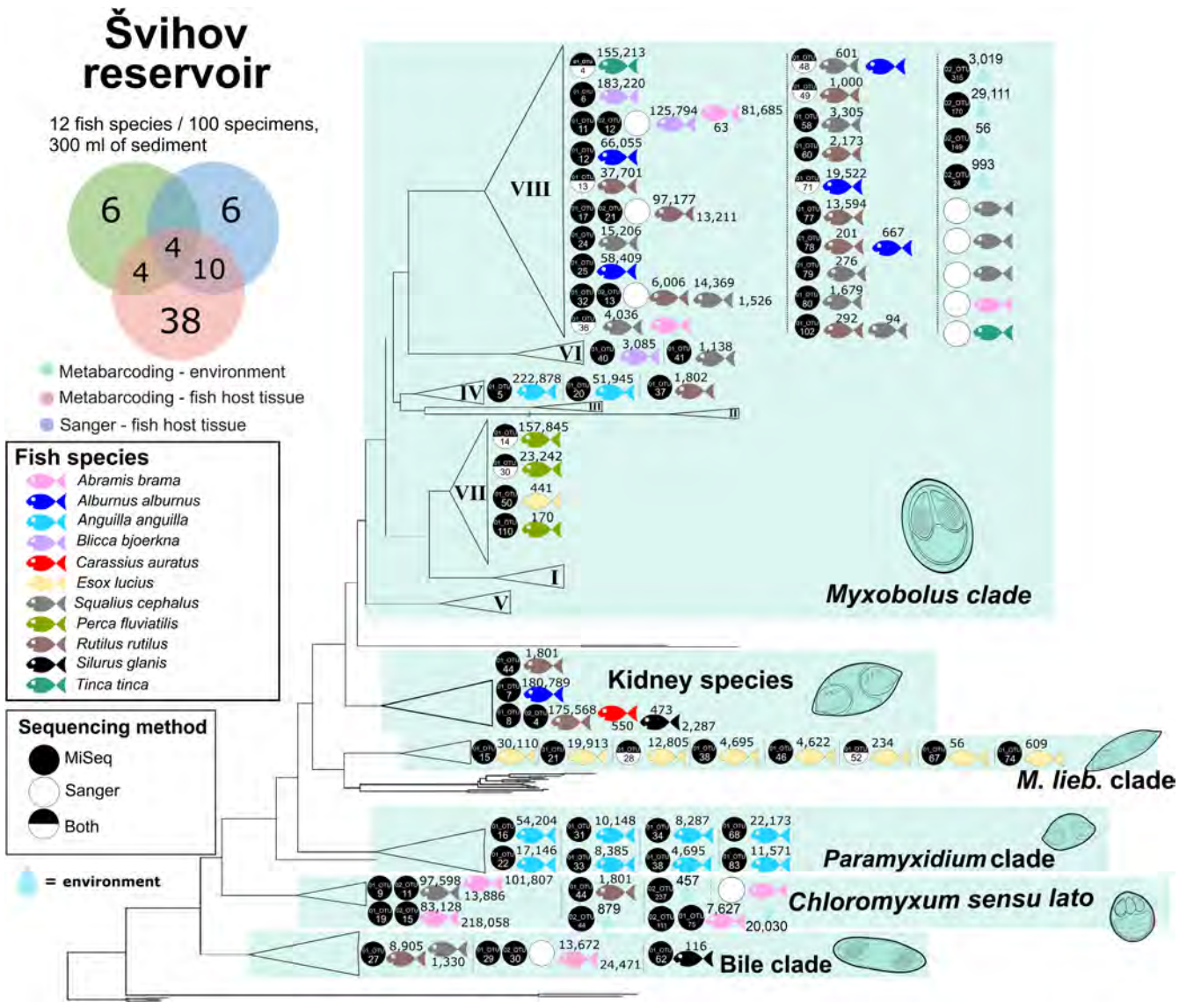



FIGURE 5 SSU rDNA-based phylogenetic tree of oligochaete-infecting (freshwater) myxosporean lineage with individual taxon branches collapsed in the main phylogenetic clades. Individual OTUs obtained from environmental and fish tissue metabarcoding and sanger sequencing of fish tissues from Švihov reservoir are shown in circles in the corresponding clades. Fish host species are represented by colored fish pictograms with the number of reads found for each OUT. The Venn diagram shows the exclusive and shared OTUs obtained from the three different sample types

Figure 2c summarizes the ability of specific primer pairs to amplify products in all metabarcoding assays performed. The highest number of 34 OTUs was amplified by FR-F1 + FR-R1, with almost all OTUs belonging to the *Myxobolus* clade. The second highest score had FR-F5 + FR-R1 with 26 OTUs amplifying myxosporeans from all six defined freshwater myxosporean clades. The other four primer combinations yielded between 12 and 16 OTUs.

## 4 | DISCUSSION

We successfully developed a metabarcoding assay for the detection of myxosporeans that effectively revealed parasite OTUs even in eutrophic freshwater ecosystems with high detection and specificity of myxosporean OTUs. We have shown that our strategy based on specific PCR primer-pairs for different subclades is able to specifically detect myxosporean parasites in habitats shared by many different eukaryotic organisms. Direct comparison of our specific primer-pair approach to another approach based on general myxozoan primers (Hartikainen et al., 2016) proved that the usage of specific myxosporean primer is more effective strategy for metabarcoding, which in this study allowed detection of 17 different myxosporean OTUs from an eDNA sample (Douro River) that yielded no results when using general myxozoan primers (only a variety of other eukaryotic OTUs were detected). Our metabarcoding assay, used in the test phase to screen fish tissue, revealed a 2.5-fold higher number of myxosporean-containing OTUs in infected fish tissue samples than screening with regular Sanger sequencing, and a 3.1-fold higher

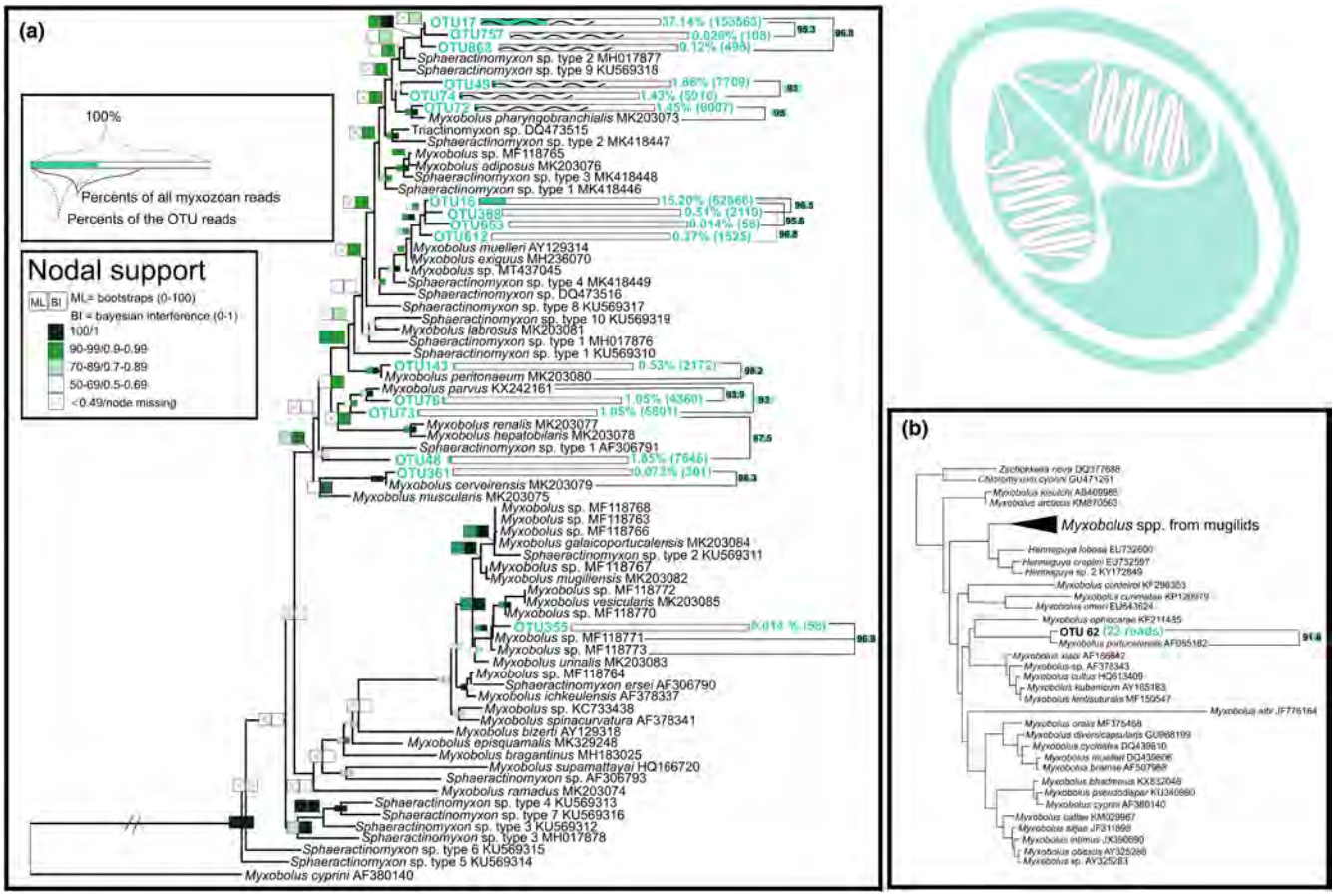



**FIGURE 6** Phylogenetic relationships of OTUs obtained from an environmental sample from the Douro River sediment. (a) The SSU rDNA-based myxosporean phylogenetic tree containing all sequences of *Myxobolus* spp. from the mugilid group, with highlighted newly obtained OTUs (in green and bold) with details of each OTU (number and percentage of reads and similarity to the most related sequence). Maximum likelihood/Bayesian inference nodal supports are shown at each node by colored squares according to the scale given in the legend. (b) The SSU rDNA-based myxosporean phylogenetic tree, including the newly found OTU (green and bold) and the closely related *Myxobolus* spp.

number of myxosporean-containing OTUs in infected fish tissue samples than in environmental samples. Analysis of our assay using eDNA samples isolated from freshwater sediments showed a high abundance of myxosporeans in samples of small volumes, suggesting that sediment samples are suitable for assessing myxozoan biodiversity in freshwater ecosystems.

To avoid problems with clogging of filters by eutrophic water samples rich in algae, we used a new method for obtaining eDNA material from freshwater sediments based on rinsing the sediments, centrifuging the washed material, and isolating DNA from the pellet with myxospores and other developmental stages. Our method is based on knowledge of the myxosporean life cycle when myxospores released from fish sink to the bottom to potentially infect benthic annelid hosts (Lom & Dyková, 2006). Therefore, freshwater sediments contain myxospores, which can be detected thanks to their longevity (up to 30 years; Halliday, 1976; Okamura et al., 2015) when compared with short-lived actinospores released from oligochaete hosts (maximum 14 days; Xiao & Desser, 2000). The longevity of myxospores allows us to detect myxosporean biodiversity at the studied site independent of seasonal occurrence of spores inside fish hosts, as documented in Hartikainen et al. (2016). Therefore, our method can be useful for myxosporean pathogen monitoring, as it can detect the potential pathogen at the myxospore stage at a given site or warn against the use of susceptible fish at sites where a certain pathogen is present.

Freshwater ecosystems contain approximately 200,000–250,000 species of eukaryotic microorganisms (Debroas et al., 2017), thus the selection of appropriate molecular markers and primers is crucial for the successful detection of target organisms. Myxozoans are cnidarians with specific, taxonomically restricted genes (minicollagenes) that were discovered by Holland et al. (2011) and would make an ideal DNA target. However, myxozoan spores consist of only a few cells and minicollagens are single copy genes, hence their use for the detection of myxozoans is questionable. Therefore, multi-copy SSU rDNA is the target of choice and its hypervariable V4 region has been shown to be the best region for myxozoan metabarcoding sequencing (Hartikainen et al., 2016; this study). The pairwise distances of the V4 region closely match the pairwise distances of almost complete length of the SSU rDNA (Dunthorn et al., 2012), suggesting that this relatively short sequence is suitable for species

diversity assessment. Moreover, V4 is present in almost all myxozoan sequences available in GenBank, allowing direct comparison of the obtained OTUs with known species. Amplification of PCR products with universal primers is often the first choice for eDNA studies (e.g., Lim et al., 2016). However, detection of rare species with universal primers can be problematic, as documented by Hartikainen et al. (2016) in the case of myxozoan species.

The faster rate of evolution (Holzer et al., 2018) complicates the design of primers universal to all myxozoan phylogenetic lineages—the variable regions are too variable in myxozoans, and the more conservative parts are homologous with other eukaryotes. We, therefore, designed primers specific to each myxosporean phylogenetic group and we tested the resulting primer combinations by *in silico* PCR. The percentage of myxosporean reads in the data was very high, demonstrating the ability of our assay for detecting myxosporean diversity. The amplicons of the eDNA samples from two habitats, including a heavily eutrophic small pond, consisted of almost 100% myxosporean reads. In addition, 17 different myxosporean OTUs were detected in a single sample from the Douro River, which had the lowest percentage of myxosporean reads (63.1%). Our pilot experiments demonstrated that the designed primers and their combinations are suitable to detect the less abundant myxosporean DNA sequences in DNA-rich eDNA samples.

The high specificity of the chosen primers for known myxosporean subgroups may yet overlook unknown lineages of freshwater myxosporeans. Although our primer design is based on almost 1000 SSU rDNA sequences available in GenBank, there might be further phylogenetic lineages that could differ in primer regions and therefore might not be recognized. However, this can be compensated by the fact that our primers designed for specific groups often also amplify other myxosporean groups (Figure 2c). For example, the primer combination FR-F1 + FR-R1 amplified 21 OTUs from *Myxobolus* subclade viii, although it was specifically designed for *Myxobolus* subclades iii - vi, as shown by *in silico* PCR analysis (Table S3). Another example is the successful recognition of eight OTUs from the *Paramyxidium* clade (environmental clade as defined by Hartikainen et al. (2016)), although we could not amplify a PCR product with the specific primer combination (FR-F7 + FR-R2) designed for this phylogenetic group. This primer pairing plasticity shows the suitability of these primers to reveal the entire species diversity of freshwater myxosporeans at selected locality and, on the other hand, could also explain the relatively high percentage of non-myxosporean reads in the eDNA samples from the Hostačovka brook and the Douro River. Our results are influenced by the specific composition of Myxosporea at the studied sites. Our primer pair combinations did not detect Myxosporea species from five *Myxobolus* subgroups (i-iii, v, and vi). The number of myxosporean species in these groups is comparatively low suggesting that these species very likely do not occur at the sites studied. We, therefore, recommend the use of a full set of eight primer pairs selected by *in silico* PCR for the assessment of myxosporean species diversity, including the primer pair Fr-F2 + FR-R6, which, however, did not yield a PCR product, although *in silico* PCR suggested its specificity for *Myxobolus* subclades i and viii.

In environmental samples from four different habitats selected to test our new methodological approach to eDNA metabarcoding, over 50 different OTUs were found. This indicates a very high sensitivity of our metabarcoding approach as well as a high diversity of myxosporeans at each site, considering that eDNA analysis was only performed on a small number of pilot samples composed of small volumes of sediment. Our bioinformatic pipeline generated OTUs based on a threshold of 97% sequence similarity, which gives us strong confidence that the OTUs represent species, as the V4 sequence variability is comparable to the full SSU rDNA gene (Dunthorn et al., 2012). However, there are many examples of myxosporean species with sequence identity greater than 97% (Lisnerová, Blabolil, et al., 2020). Comparing the sequence similarities between the described (morphologically defined) species and the similarities between the OTU sequences can help answer the question of whether the OTUs represent individual species or reflect intragenomic variance. For example, the two formally described species *Myxobolus galaicoportucalensis* (MK203084) from the gut of *Mugil cephalus* and *Myxobolus mugiliensis* (MK203082) from the gills of the same host from Portugal are 97.95% similar in the V4 region examined (341 bp in length). These two species were described based on differences in spore morphometry, different tissue specificity and dissimilarity of SSU rDNA (Rocha et al., 2019). Therefore, we hypothesize that sequence dissimilarity of more than 2% in the V4 region studied could be the limit for delimiting species in the sublineages of the phylogenetic tree studied. The sequence similarity of full-length SSU rDNA of these two species (comparison of 2038 bp) was 97.20%, suggesting that the genetic distances revealed by the rDNA region selected for our metabarcoding assay reflect the genetic distances of the full-length SSU rDNA gene.

In Portugal, the diversity of myxozoans has long been studied (Cruz et al., 2003; Rocha et al., 2019; Saraiva et al., 2000), including the investigation of mugilid fishes for myxozoan infections through morphological (Eiras et al., 2007) and molecular approaches (Rocha et al., 2019). All obtained OTUs from the Douro River differ from the most closely related sequence by more than 1.7% sequence dissimilarity (87.5%–98.3% sequence similarity). OTU361 has the highest similarity to formally described species *Myxobolus cerveirensis* MK203079 (98.3% similarity) and OTU143 with *Myxobolus peritonaeum* MK203080 (98.20% similarity). These two OTUs were hence associated with existing species. The other 14 identified OTUs displayed genetic differences higher than 2% to the sequences of the described species. These may be assumed to represent a considerably high diversity of undescribed *Myxobolus* species, mostly likely infecting mugilids (Figure 6a). One OTU (OTU62) cluster within *Myxobolus* subclade IV near to *Myxobolus portucalensis* AF085182 (similarity 91.8%). Our results further showcase that myxosporean diversity, namely of *Myxobolus*, remains to be described even in geographical locations where myxozoan research has been ongoing for a long time.

Although our pilot metabarcoding analysis of selected eDNA samples revealed that the amount of analyzed material contained a relatively high myxosporean diversity, the number of eDNA samples (sediment volume) was very low for a thorough screening of selected

localities. To map the actual diversity at a given site, effective eDNA sampling needs to be evaluated (Sakata et al., 2020) and an adequate number of replicate sediment samples should be processed to assess the true myxosporean diversity. From the Venn diagrams of our study, we can conclude that undersampling is evident at the large water reservoir (Švihov), as the number of OTUs detected in fish and eDNA samples from this locality is significantly disproportional (53/14 OTUs). When analyzing a much smaller habitat (Rájský pond), the ratio of OTUs in fish and eDNA samples was much lower (20/14 OTUs). Several sites need to be sampled to get the best estimate of myxosporean diversity and the amount of sediment (number of samples) that needs to be collected for an accurate determination of diversity should be adapted to the size of the habitat. Processing the samples and PCR with the recommended eight primer pairs could be labor intensive and time consuming for large water bodies. Therefore, we recommend pooling DNA samples from several sites at the same location before PCR processing. The sampling strategy should also be optimized for the type of ecosystem, for example, lake, pond, brook, or river, or other environmental conditions such as the type of sediment or the flow of water.

The high number of OTUs detected by metabarcoding in fish samples probably reflects the sensitive metabarcoding approach, which can detect infections with a very low intensity that are often not detected by Sanger sequencing, which only recovers the dominant amplicon sequence in the PCR sample. The overestimation is minimized by our methodological approach, which uses a bioinformatics pipeline that includes read sequence quality filtering, chimaera removal and the criterion of >97% similarity when clustering reads.

Here, we have demonstrated that eDNA metabarcoding of sediment samples is an effective tool for exploring myxosporean diversity without the need for host examination, which is an invasive technique requiring access to often rare, threatened, or endangered fish species. Seasonal variation of infections in the fish host is another problem in biodiversity assessment that is overcome by eDNA analysis of samples containing resistant myxospores spores that survive for long periods in sediments. Our new metabarcoding approach, using eDNA from sediments and clade-specific myxosporean primers, opens a new perspective for a more realistic estimate of the diversity of myxosporeans. Future research aimed at collecting eDNA samples for myxosporeans from previously unexplored geographical areas can elucidate the largely unexplored species diversity and contribute to the understanding of myxozoan phylogeny and evolutionary history.

## AUTHOR CONTRIBUTIONS

IF conceived and designed the present study. PB was responsible for the fish sampling. ML and IF prepared dissection, microscopic examination, and environmental sampling, IF performed primer design, ML performed DNA extraction, PCR screening and sequencing. IF and ML conducted phylogenetic analyses, ML prepared Figs, funding acquisition was prepared by ML, IF, and ASH, ML, and IF drafted the manuscript. All authors have read and agreed to the published version of the manuscript.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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