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# Molecular characterization of *Sphaerospora molnari* (Myxozoa), the agent of gill sphaerosporosis in common carp *Cyprinus carpio carpio*

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ABSTRACT: *Sphaerospora molnari* Lom, Dyková, Pavlásková and Grupcheva, 1983 often causes severe infections in the gills and skin of common carp fingerlings *Cyprinus carpio carpio* in Central Europe. Although most *Sphaerospora* spp. are coelozoic and affect the excretory system of fish, *S. molnari* develops mature spores in the epithelia of gill filaments, making it a rare representative of histozoic freshwater species within the genus. On the basis of a partial 18S rDNA sequence assigned as belonging to *S. molnari*, previous phylogenetic studies located the species within the *Myxobolus* clade. In the present study, *S. molnari* isolates from Hungary and the Czech Republic were characterized based on morphology, DNA sequence analysis and phylogenetic comparison. The obtained 3714 bp final consensus 18S rDNA sequence of the parasite showed several, sometimes extremely long inserts in the variable regions of the gene and differed considerably from the one published in GenBank in 2002. *In situ* hybridization confirmed the validity of the obtained DNA sequence and detected pre-sporogonic blood stages in the interstitium and blood vessels of the kidney. Phylogenetic analysis showed that *S. molnari* clusters within the *Sphaerospora* sensu stricto clade with a high support, revealing it as the first known histozoic member of the *Sphaerospora* subclade comprising parasites of freshwater fish.

KEY WORDS: Sphaerosporosis · Histozoic myxozoans · Blood stages · Cyprinids · 18S rDNA · Phylogeny · *In situ* hybridization

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## INTRODUCTION

*Sphaerospora* spp. (Myxozoa) are common endoparasites of fish, with the highest diversity described in cyprinids (Baska & Molnár 1988, Sitjà-Bobadilla & Alvarez-Pellitero 1994). Most members of the genus are coelozoic (i.e. plasmodial stages of parasite locate in cavities of body organs) and infect the excretory system of fish. Because of their simple spherical or sub-spherical myxospore shape involving 2 valves, 2 polar capsules and usually 2 uninucleate sporoplasms, species identification based solely on morphological features is rather difficult. Thus, it is not surprising that particular emphasis is put on using molecular tools for the taxonomy of *Sphaerospora* spp. Following the description of the genus by Thélohan in 1892 (Thélohan 1895), it took more than a century until the first rDNA sequences of *Sphaerospora* spp. were published (Kent et al. 1998, 2001). Phylogenetic studies in combination with the examination of phenotypic and biological features (i.e. spore morphology, tissue tropism, host range, etc.) have become 'mainstream' in myxozoan research (e.g. Whipps et al. 2003, Eszterbauer 2004, Holzer et al. 2004, Fiala 2006, Molnár et al. 2010, Fiala & Bartošová 2010). Due to the unknown host specificity

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and geographical distribution of many myxozoan taxa, it is important to provide a combination of morphological and molecular features for a given myxozoan species. An upswing in *Sphaerospora* taxonomy was experienced when the number of known *Sphaerospora* 18S rDNA sequences increased significantly, and larger DNA fragments or complete sequences were used for phylogeny. Having studied the phylogeny of the genus, which is known to be highly polyphyletic, Jirků et al. (2007) introduced the term *Sphaerospora* sensu stricto for those species that are coelozoic and cluster together in a basal phylogenetic myxosporean clade. Recently, the clade expanded with 2 new members, *S. angulata* from Prussian carp and goldfish and *S. dykovae* from common carp, both of which develop spores in the kidney tubules (Holzer et al. 2013). However, a few 18S rDNA sequences of *Sphaerospora* spp. still cluster outside the *Sphaerospora* sensu stricto clade, i.e. *S. testicularis* Sitjà-Bobadilla and Alvarez-Pellitero, 1990, *S. dicentrarchi* Sitjà-Bobadilla and Alvarez-Pellitero, 1992, *S. elwhaiensis* Jones, Fiala, Prosperi-Porta, House and Mumford, 2011, and *S. molnari* Lom, Dyková, Pavlásková and Grupcheva, 1983; these species are mostly histozoic parasites, the plasmodia of which develop intra- or intercelullarly in tissues of fish.

As mixed myxozoan infections are common, especially in cyprinid fish, with >20 species reported in common carp (Lom & Dyková 2006), we recently started to confirm the identity of published 18S rDNA sequences of sphaerosporids clustering outside the *Sphaerospora* sensu stricto clade. The need to reanalyze these *Sphaerospora* spp. has already been highlighted in studies dealing with myxozoan phylogeny and the relations between their development and phylogenetic data (Fiala 2006, Morris & Adams 2008, Bartošová et al. 2011). By hybridization of specific oligonucleotide probes to target DNA in histological sections (*in situ* hybridization [ISH]) we were able to identify erroneously ascribed *Sphaerospora* sp. entries in GenBank (e.g. Eszterbauer 2011, Holzer et al. 2013). Contrary to the majority of *Sphaerospora* spp. and most of the sequenced members of *Sphaerospora* sensu stricto, *Sphaerospora molnari* is a histozoic species that develops myxospores in the epithelia of gills and skin, causing gill sphaerosporosis in common carp *Cyprinus carpio carpio* fingerlings in Europe (Iskov 1969, Hámory & Molnár 1972, Molnár 1979, 1980, Waluga 1983). Transelectromicroscopic studies by Kaup et al. (1995) confirmed the histological observations that the monosporic sporoblasts of *S. molnari* surrounded by an envelope cell lay isolated in the gill epithelium. Before 1983, *S. molnari* was identified as *S. carassii*; thereafter, it was distinguished from *S. carassii* Kudo, 1919 and *S. chinensis* Lom, Dyková, Pavlásková and Grupcheva, 1983 based on spore morphology, host species and geographic locality (Lom et al. 1983). *S. molnari* invades the epithelia of gill filaments and lamellae and secondarily also those of the skin surrounding the gill cavity and the nasal pits. Affected epithelia show marked dystrophic changes and necrosis, causing secondary bacterial infections and leading to discharge of spores into the environment Molnár (1979). This is why *S. molnari* is regarded as a serious pathogen of common carp fingerlings in Central Europe.

In the present study, we provide 18S rDNA sequences of *Sphaerospora molnari* isolates collected from Hungary and the Czech Republic. Using *in situ* hybridization, we confirm the validity of the obtained sequences and provide insights into the location of pre-sporogonic stages of *S. molnari*. Furthermore, we clarify the phylogenetic position of the first histozoic member of the freshwater subclade of *Sphaerospora* sensu stricto sequenced to date.

## MATERIALS AND METHODS

### Source of samples

Common carp *Cyprinus carpio carpio* fingerlings were captured by hand with a net in an earthen pond of a carp hatchery in Hortobágy, Hungary (47° 35′ 11.1″ N, 21° 02′ 58.4″ E). Fifty individuals were subjected to parasitological examination. As a second sampling source, 0+ carp fingerlings were collected between March and October 2011 and 2012 in Chřešťovice (n = 23) and Jindřichův Hradec (n = 46), Czech Republic (49° 19′ 25″ N, 14° 17′ 12″ E and 49° 09′ 35″ N, 14° 10′ 07″ E). In the Hungarian samples, gill arches, gill scrapings and kidney smears were examined by light microscopy. Photomicrographs were taken of the observed myxospores using a Moticam 2000 (Motic, VWR) digital microscope camera mounted on a Zeiss Axiostar Plus phase contrast microscope (Carl Zeiss). *Sphaerospora molnari* myxospores (n = 50) from Hungarian carp were measured on digital images using the software Motic Images Plus 2.0 calibrated against a digital image of a graticule. Measurements were obtained following the guidelines of Arthur & Lom (1989) and Sitjà-Bobadilla & Alvarez-Pellitero (1994). The tissue samples observed by microscopy on slides were collected

for subsequent molecular analysis and stored at −20°C. Gill arches affected by myxosporean infection were fixed in 10% neutral buffered formalin for ISH. Kidney and blood samples from carp fingerlings originating from the Czech Republic were fixed in TNES-urea (Asahida et al. 1996) for DNA analysis, and several kidneys were also fixed for ISH as described above.

### DNA extraction, PCR and DNA sequencing

Two samples from each sampling site were thawed and then homogenized in 1.5 ml microtubes with a sterile plastic pestle (Eppendorf). Then, tubes containing the homogenates were filled with $dH_2O$, mixed by vortexing and centrifuged at 7000 × *g* for 5 min on a tabletop centrifuge. Pellets were dissolved in 500 µl lysis buffer (100 mM NaCl, 10 mM Tris, 10 mM EDTA, 0.2% sodium dodecyl sulphate, and 0.4 mg $ml^{-1}$ Proteinase K) and incubated at 55°C for 5 h. DNA was then purified using the MiniPrep Express Matrix (BIO101, Qbiogene) as per Eszterbauer (2004). TNES-urea fixed samples were lysed with 0.1 mg $ml^{-1}$ Proteinase K (Sigma-Aldrich) overnight and extracted using a general phenol-chloroform extraction protocol. Genomic DNA was amplified for the first round with the primer pair ERIB1-ERIB10 (Table 1). The total volume of the PCR reactions was 50 µl, which contained ~50 ng DNA, 1× *Taq* PCR reaction buffer (Fermentas, Thermo Fisher Scientific), 1.5 mM $MgCl_2$, 0.2 mM dNTP mix (Sigma-Aldrich), 25 µM of each primer and 2 units of Taq DNA Polymerase (Fermentas, Thermo Fisher Scientific). Amplification conditions were 95°C for 50 s, 59°C for 50 s and 72°C for 180 s for 35 cycles, with an initial denaturation at 95°C for 5 min and terminal extension at 72°C for 7 min. As a second round, PCR assays with the same cycling conditions but with a shorter (80 s) elongation step and, in some cases, at a different annealing temperature (given in parenthesis) were performed with the following primer combinations: MB5-ERIB10, ERIB1-Act1R (52°C), ERIB1-Smol800R, Smol3130F-ERIB10, Act1F-ERIB10 (52°C) and Act1F-Myx4R (Table 1). PCR products were purified with a MEGAquick-Spin PCR and Agarose Gel DNA Extraction System (INtRON Biotechnology).

The purified PCR products were used for direct sequencing with an ABI BigDye Terminator v. 3.1 Cycle Sequencing kit with an ABI 3100 Genetic Analyzer automated DNA sequencer (Applied Biosystems, Life Technologies and Macrogen Europe). Besides the amplification primers, the primers listed in Table 1 were used. For 2 samples, PCR products amplified with ERIB1-ERIB10 were cloned using CloneJet PCR Cloning Kit as per manual (Fermentas, Thermo Fisher Scientific). Alkaline miniprep was used for plasmid purification. Five clones of each sample were subjected to partial DNA sequencing using the universal plasmid primers supplied with the cloning kit. One clone was sequenced entirely with overlaps in both directions using the primers listed in Table 1. A specific PCR assay for *S. molnari*

Table 1. Oligonucleotides used for PCR (P), DNA sequencing (S) and *in situ* hybridisation (I)

| Name | Sequence (5′→ 3′) | Use | Reference |
|---|---|---|---|
| ERIB1 | ACC TGG TTG ATC CTG CCA G | P, S | Barta et al. (1997) |
| ACT1F | GGC AGC AGG CGC GCA AAT TAC CCA A | P, S | Hallett & Diamant (2001) |
| ACT1R | AAT TTC ACC TCT CGC TGC CA | P, S | Hallett & Diamant (2001) |
| MYX4R | CTG ACA GAT CAC TCC ACG AAC | P, S | Hallett & Diamant (2001) |
| SPHR | GTT ACC ATT GTA GCG CGC GT | S | Eszterbauer & Székely (2004) |
| ERIB10 | CTT CCG CAG GTT CAC CTA CGG | P, S | Barta et al. (1997) |
| MB5 | GGT GAT GAT TAA CAG GAG CGG T | P, S | Eszterbauer (2004) |
| Smol700F | GGA GAT GGT GAC GAG ACA TA | S | Present study |
| Smol800R | TGG TAT TAC CGC GGC TGC TG | P, S | Present study |
| Smol1200F | GCC AAG CGA GCG TCC AAT CA | S | Present study |
| Smol1200R | TTG GAC GCT CGC TTG GCT GT | S | Present study |
| Smol1400F | CTC CTT CAC TGT GAG CAA CG | S | Present study |
| Smol1900R | GTA CCG AAG AAC GCA CAC GA | S | Present study |
| Smol1800F | GTC CAA TTG CTT GAA CCA CC | S | Present study |
| Smol2500R | GCA TGT GTG AGC GTG ATT GT | S | Present study |
| Smol3130F | TGT ACT GTT GCC GGT GGA TT | P, S | Present study |
| 115FOA | CGT GAA CGA GCG CAA CCA CA | S | Present study |
| SmSSU487F | GCC TCT CCA CCT GTG TAT G | P, I | Present study |
| SmSSU1307R | ACC GTG AGC CAC GCG TAA TG | P, I | Present study |

was designed, using primers SmSSU487F and SmSSU1307R (Table 1) with all PCR parameters as described above apart from the annealing temperature, which was set as 66°C. For sequence assembly, the STADEN Sequence Analysis Package v. 2001.0 (Staden 1996) was used.

### *In situ* hybridization

Samples for ISH were fixed in 10% neutral-buffered formalin for 24 to 48 h, dehydrated in an ethanol series and embedded in paraffin. Six µm sections were cut and adhered to salinized slides. The ISH method followed established protocols (Holzer et al. 2003, 2010) applying a DIG-alkaline phosphatase detection system. Briefly, sections were deparaffinized and rehydrated in a graded ethanol series and then equilibrated in Tris-buffered saline (TBS) (pH 8.0). Thereafter, sections were permeabilized with 100 µg $ml^{-1}$ of Proteinase K in TBS for 25 min at 37°C. Sections were then washed in phosphate-buffered saline (pH 7.4) and post-fixed for 15 min with 0.4% paraformaldehyde in phosphate-buffered saline. Fixative was rinsed off with distilled water, and the sections were dried at 45°C. The primers SmSSU487F and SmSSU1307R (Table 1) were commercially DIG-labeled at the 3′-end (Sigma-Aldrich) and used as specific probes. The specificity of the probes was confirmed by DNA sequence comparison via BLAST search because no match was found with any other DNA sequences in GenBank. Gene Frames (Thermo Fisher Scientific) were adhered to the slides, and hybridization buffer (4× SSC in TBS containing 0.5% Ficoll, 0.5% polyvinylopyrrolidone, 0.5% bovine serum albumin and 100 µg $ml^{-1}$ calf thymus DNA) containing 1.5 ng $µl^{-1}$ of each oligonucleotide probe was added. After covering the Gene Frames, the sections were denatured for 4 min at 90°C and hybridized with the labeled probes for 2 h at 45°C. The incubation was followed by a non-stringent washing in 2× SSC and highly stringent washing in 0.1× SSC containing 0.1% Tween 20 at 45°C. Sections were then transferred into TBS (pH 7.5) and incubated with 1:5000 anti-DIG-alkaline phosphatase Fab fragments (Roche Diagnostics) in TBS for 2 h at room temperature. Following a wash in TBS (pH 8.0), Vector Blue substrate (Vectorlabs) was used to visualize the alkaline phosphatase linked DIG-labeled probe according to the manufacturer's recommendations. Counterstaining was conducted using 0.1% nuclear fast red. Finally, sections were dehydrated through an alcohol series and transferred into Xylene Substitute (Sigma-Aldrich) and permanently mounted with VectaMount (Vectorlabs).

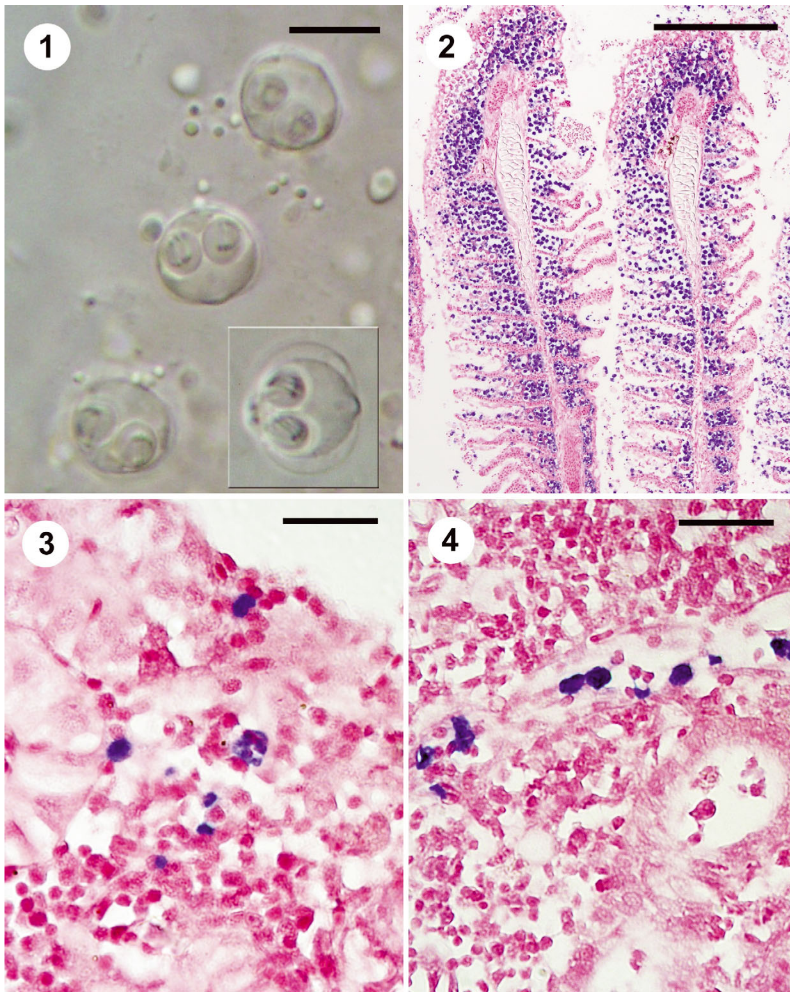
### Phylogenetic analysis

The SSU rDNA sequences of all available *Sphaerospora* s. str. spp. and selected members of freshwater and marine myxosporean lineages were aligned in Clustal X v. 1.83 (Thompson et al. 1997) using default parameters. The alignment was manually edited, and the ambiguous regions were excluded in BioEdit (v. 7.0.5.2; Hall 1999). *Chloromyxum leydigi* was used as outgroup in all analyses. We performed maximum parsimony analysis in PAUP* (v4.b10; Swofford 2001), using a heuristic search with random taxa addition, the ACCTRAN option, TBR swapping algorithm, all characters treated as unordered and gaps treated as missing data. Clade support values were calculated from 1000 bootstrap replicates with random sequence additions. Maximum likelihood analysis was calculated in RAxML (Stamatakis 2006) using the GTR + $\Gamma$ model. Bootstraps were based on 1000 replicates. The Bayesian inference (BI) analysis was performed in MrBayes v. 3.0 (Ronquist & Huelsenbeck 2003) using the GTR + $\Gamma$ + I model of evolution. Posterior probabilities were estimated from 1 000 000 via 2 independent runs of 4 simultaneous Markov chain-Monte Carlo chains with every 100th tree saved. The burn-in period (100 000 generations) was determined in Tracer v. 1.4.1 (Rambaut & Drummond 2007).

## RESULTS

### Characteristics of parasite infection

Of the 50 examined fish originating from Hungary, gills of 6 specimens (12% prevalence) were infected with subspherical myxospores developing in monosporous pseudoplasmodia in the epithelium of gill filaments. The morphological examination of myxospores in gill scrapings confirmed that the observed spores belonged to *Sphaerospora molnari* (Fig. 1), and their measurements resembled those described in the original description by Shulman (1966) (Table 2). In 2 fish specimens, a massive infection was observed in most gill filaments, where the entire filament was affected by the parasite. In the 2 fish whose gills were used for ISH and DNA sequence analysis, no other myxozoans were detected in the course of dissection.




The gill smears of carp from the Czech Republic were not studied microscopically; however, the 18S rDNA of *Sphaerospora molnari* was sequenced from 2 blood samples of carp. The specific *S. molnari* PCR assay showed that 34.8% (8 of 23) of common carp in Chřešťovice and 85% (39 of 46) of common carp in Jindřichův Hradec were positive for *S. molnari* DNA in the blood. Positive samples were found all through the sampling period.

Figs. 1 to 4. *Sphaerospora molnari* and *Cyprinus carpio carpio*. Fig. 1. *S. molnari* myxospores from gill of common carp fingerling C. *carpio carpio*. Fresh preparation. Bar = 10 µm. Inset: *S. molnari* myxospore with mucous envelope. Fig. 2. Gill of common carp fingerlings heavily infected with *S. molnari*. The monosporous pseudoplasmodia develop in high numbers in the epithelium of the gill filaments. Parasites show dark blue coloration. *In situ* hybridization. Bar = 200 µm. Fig. 3. Kidney parenchyma of common carp fingerling with *S. molnari* developmental stages. Parasite stages are dark blue. *In situ* hybridization. Bar = 10 µm. Fig. 4. *S. molnari* blood stages (stained dark blue) detected in a kidney capillary of common carp fingerling. *In situ* hybridization. Bar = 10 µm

Table 2. Measurements of the myxospores of *Sphaerospora molnari* and other relevant, closely related *Sphaerospora* spp. developing in cyprinids. Mean values ± standard deviations are given in µm. Minimum and maximum values are in parentheses. SL: spore length; SW: spore width, PCL: length of polar capsule; PCW: width of polar capsule; PF: polar filament; nd: no data

| Species | Authors | Locality | Host | Tissue | SL | SW | PCL | PCW | PF turns |
|---|---|---|---|---|---|---|---|---|---|
| *Sphaerospora molnari* (n = 50) | Present study | Hungary | *Cyprinus carpio carpio* | Gill epithelium | 9.9 ± 0.59 (8.7–11.1) | 10.0 ± 0.51 (9.0–10.9) | 4.68 ± 0.39 (3.9–5.8) | 3.9 ± 0.28 (3.3–5.0) | 4–5 |
| *S. molnari* | Lom et al. (1983) | Czech Republic | *C. carpio carpio* | Gill and skin epithelium | 10.3 (9.5–11) | 10.5 (10–11) | 4–5 | 4–4.5 | 4 (3–5) |
| *S. chinensis* | Lom et al. (1983) | Czech Republic | *C. carpio carpio* | Gill epithelium | 7.4 | 7 | nd | nd | 5 |
| *S. carassii*[a] | Shulman (1966) | Former USSR & Japan | *Carassius carassius, Carassius gibelio, C. carpio carpio, Rutilus rutilus* | Gill epithelium | 8–13 | 8–13 | 4–5 | nd | nd |
| *S. dykovae*[b] | Dyková & Lom (1982) | Czech Republic | *C. carpio carpio* | Kidney tubules | 7.3 (6.0–8.0) | 7.2 (6.4–8.3) | 1.7–2.3 | 1.3–1.6 | 4–5 |

[a]Originally described by Kudo (1919)
[b]Originally described as *S. renicola*

### DNA sequence analysis

Almost complete 18S rDNA sequences were obtained from 2 Hungarian and 2 Czech isolates of *Sphaerospora molnari*. The 3693 and 3714 bp final consensus DNA sequences both possessed several, sometimes extremely long expansions in the variable regions V2, V4, V5 and V7 of the 18S rDNA gene region. The 18S rDNA of the 2 Hungarian isolates (GenBank accession number JX431510) were identical, while the Czech isolates (GenBank accession no. JX431511), which were also identical with each other, differed from the Hungarian isolates at 7 nucleotide positions (99.7% similarity). The 1794 bp partial 18S rDNA sequence of Myxozoa sp. ASH-2012 isolate bloodcarpCZ that has recently become available in GenBank (JQ801548) was identical to the Czech isolate of *S. molnari*. The presently examined *S. molnari* isolates and the one (AF378345) previously submitted to GenBank by Kent et al. (2001) differed considerably in primary sequence characteristics and in the absence of relevant expansion segments, except in region V7. The two 18S rDNA sequences possess only 48.5 to 48.6% similarity in a much shorter, 2080 bp long alignment of the gene.

### ISH

ISH using a *Sphaerospora molnari*-specific DNA probe confirmed the great parasite load in the epithelium of gill filaments and clearly linked the *S. molnari* spore morphotype to the amplified 18S rDNA sequence. Especially in the apical part of the gill filaments, epithelial hyperplasia and necrosis of gill tissue was also observable (Fig. 2). Interestingly, large numbers of pre-sporogonic stages of the parasite, measuring between 3.5 and 5 µm were detected in the blood vessels (Fig. 4). Pre-sporogonic stages were also commonly found in the interstitial tissue of the kidney (Figs. 3 & 4).

### Phylogeny

Phylogenetic analysis showed that the histozoic, gill-dwelling *Sphaerospora molnari* clusters within the *Sphaerospora* sensu stricto clade and groups with a high support with the coelozoic kidney parasites *S. hankai* Lom, Desser and Dykova, 1989, *S. dykovae* (Dyková & Lom 1982) and *S. angulata* Fujita, 1912, from freshwater fish. The exact relationships among these 4 species could not be clearly resolved. Confirming the findings of prior DNA sequence analysis, the 18S rDNA of the examined *S. molnari* isolates and the one submitted by Kent et al. (2001) were located distantly on the phylogenetic tree, as the latter one grouped outside the *Sphaerospora* sensu stricto clade with the histozoic *Myxobolus* clade (Fig. 5).

## DISCUSSION

The molecular study of *Sphaerospora molnari*, the agent of gill and skin sphaerosporosis in common carp in Central Europe, provided several novel findings. We proved that *S. molnari* is in fact a member of the *Sphaerospora* sensu stricto clade and most likely has evolved from coelozoic kidney parasites of freshwater fishes, with a close relationship to all species hitherto sequenced from cyprinids. For a long time, the phylogenetic position of *S. molnari* was thought to be different: a partial 18S rDNA assigned as *S. molnari* (AF378345) but sequenced from goldfish *Carassius auratus auratus* by Kent et al. (2001) was one of the sequences that rendered the genus *Sphaerospora* as polyphyletic, as it clusters with histozoic *Myxobolus* species from freshwater environments (e.g. Kent et al. 2001, Eszterbauer & Székely 2004, Holzer et al. 2004, Fiala 2006, Bartošová et al. 2011). Lom et al. (1983) stated that *S. molnari* is a gill parasite exclusively occurring in common carp *Cyprinus carpio carpio* and differs morphologically from *S. chinensis* from the gills of *C. carpio carpio* in the Far East. Furthermore, they distinguished *S. molnari* from *S. carassii*, which was originally described from the gills of *Carassius carassius* but also reported from *C. gibelio* and *Rutilus rutilus* and which, thereby, may represent a species complex. While the relation of *S. molnari* to these species is still unclear as no DNA sequences are available, we suggest that they may possess a similar evolutionary history, originating from sphaerosporids in the excretory system of cyprinids. The isolate of Kent et al. (2001) was collected from the gills of goldfish. Usually, shorter 18S rDNA sequences, like the ones belonging to the freshwater clade as defined by Fiala (2006), amplify preferentially over the extremely long 18S rDNA sequences of *Sphaerospora* sensu stricto members. Due to commonly occurring mixed infections, we suggest that the sequence of Kent et al. (2001) requires further validation before its potential adscription to *S. carassii*.

Holzer et al. (2013) recently reported that *Sphaerospora angulata*, which forms spores in the kidney of goldfish and Prussian carp, possesses the longest 18S rDNA (3469 bp) known so far. Our findings con-

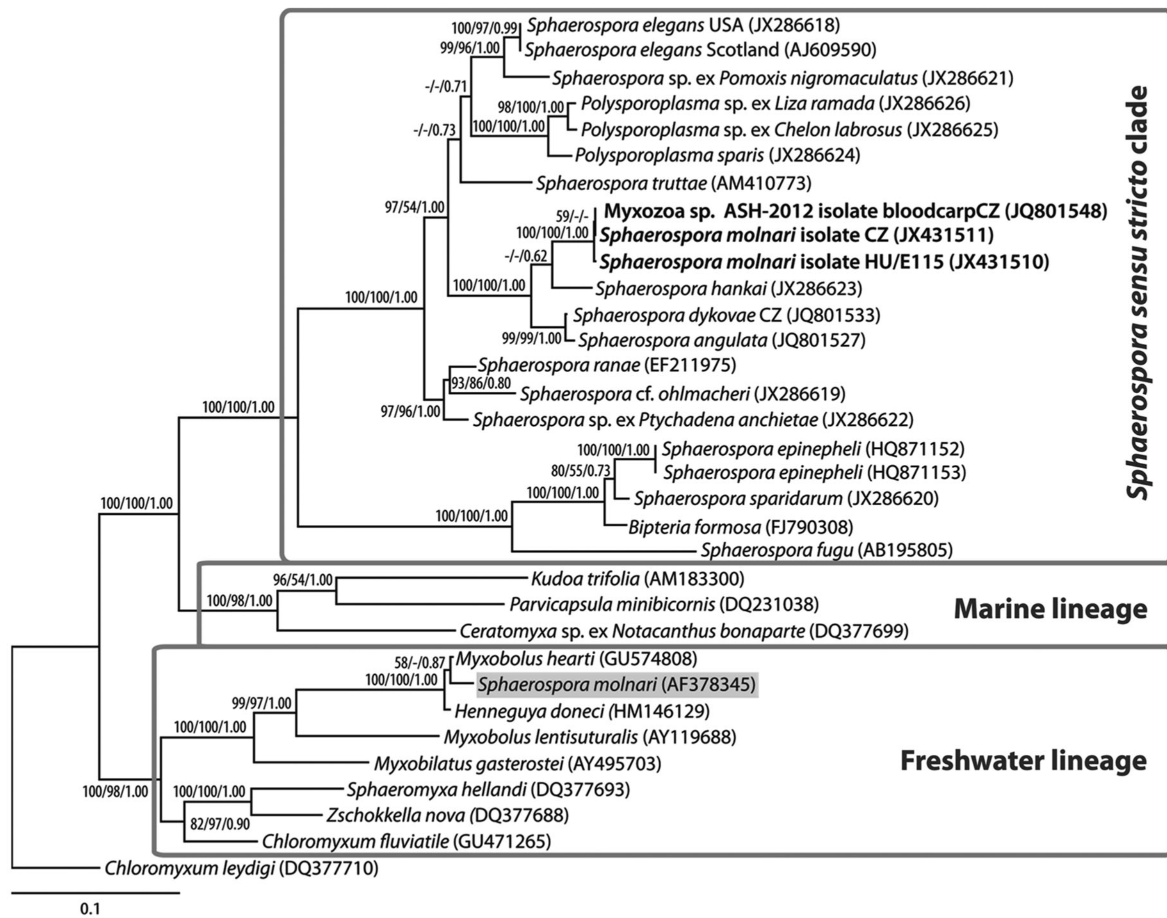



Fig. 5. 18S rDNA-based maximum likelihood (GTR + Γ model) tree of 33 myxosporean sequences showing *Sphaerospora molnari* isolates clustering within the *Sphaerospora* sensu stricto clade. Newly sequenced taxa in **bold**. *Chloromyxum leydigi* was used as outgroup. Maximum likelihood/maximum parsimony bootstraps/Bayesian posterior probabilities shown at nodes. Dashes indicate bootstrap values <50 or not present in the maximum parsimony or Bayesian tree. NCBI accession numbers shown in brackets. 18S rDNA sequence of *S. molnari* (AF378345) previously submitted by Kent el al. (2001) is shaded grey

firmed that the 18S rDNA of *S. molnari* is even longer (3714 bp were obtained from the Czech isolates), which is highly unusual, even among *Sphaerospora* spp., which are known to contain sometimes extremely long nucleotide inserts compared to other myxozoan genera (Fiala 2006, Holzer et al. 2007, 2013). In *S. molnari*, these inserts were 50 to 100 bp longer than in *S. angulata*, and some of them even affected unique regions where other sphaerosporids do not exhibit inserts.

Using ISH, we were able to specifically detect proliferative stages of *Sphaerospora molnari* in kidney capillaries and also in the parenchyma. The species-specific assay had a great advantage over histology as the misidentification of blood stages of e.g. *S. dykovae*, a frequently occurring kidney parasite of common carp, undoubtedly could be avoided. The surprisingly high number of blood stages of *S. molnari* and the intercellular, histozoic occurrence of *S. molnari* in the kidney interstitial tissue, detected by ISH, presents clear similarities with coelozoic sphaeroporids forming spores in the renal tubules of freshwater fish, as they are known to proliferate in the blood (Baska & Molnár 1988) and invade the interstitial tissue of the kidney in order to penetrate the tubular epithelia (Holzer et al. 2003). However, we never observed *S. molnari* in the intratubular space. *S. molnari* may have evolved from a coelozoic kidney parasite and may have become histozoic in the gills by parasite competition in the kidney tubules of carp (i.e. *S. dykovae*). It is likely that competitive exclusion and separation forced *S. molnari* to explore another niche in the host, and thereby the parasite found an easy option for spore release.

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