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MOLECULAR PHYLOGENY OF THE KIDNEY-PARASITIC *SPHAEROSPORA RENICOLA* FROM COMMON CARP (*CYPRINUS CARPIO*) AND *SPHAEROSPORA* SP. FROM GOLDFISH (*CARASSIUS AURATUS AURATUS*)

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The genetic relatedness of two kidney-parasitic *Sphaerospora* species was studied. Although *S. renicola*, the causative agent of swimbladder inflammation of common carp fingerlings *(Cyprinus carpio)*, and *Sphaerospora* sp. originating from goldfish *(Carassius auratus auratus)* were indistinguishable on the basis of spore morphology, they were found to be genetically different as their 18S rDNA sequences shared only 71.9% identical nucleotides. In the phylogenetic trees, *Sphaerospora* sp. from goldfish grouped with *Myxidium truttae* (AJ582061) within the clade of the coelozoic freshwater species. *Sphaerospora renicola* clustered with *S. molnari* (AF378345) within the group of myxosporeans histozoic in gills. The topology of the six *Sphaerospora* species on the phylogenetic trees implied that myxospore morphology does not correlate with the genetic relationships, and the genus seems to be polyphyletic.

Key words: Myxosporeans, *Sphaerospora* spp., carp, goldfish, kidney tubules, swimbladder inflammation, 18S rDNA, molecular phylogeny

About 50 species of myxosporeans in the genus *Sphaerospora* have been described from several freshwater and marine fish (Lom and Dyková, 1992). The general morphological features in this genus are spherical or subspherical spore shape, prominent sutural ridge and subspherical, sometimes pyriform polar capsules. Most of the species are coelozoic, developing mature myxospores in the renal tubules.

The best known freshwater species is *Sphaerospora renicola* Dyková and Lom, 1982, which is widely distributed in cultures of common carp *(Cyprinus carpio)*. The extrasporogonic developmental stages emerging seasonally in blood cause swimbladder inflammation (SBI) in carp fingerlings. A number of reports have been published about the complex developmental cycle of this parasite since the 1980s. The aetiology of parasitic SBI was first suggested by Molnár (1980*b*), who hypothesised that the developmental stages found in the blood by

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Csaba (1976) and the proliferating stages detected in the swimbladder by Kovács-Gayer et al. (1982) and Körting (1982) might be the early stages of the kidney-parasitic *S. renicola*. Several authors have also studied the pathogenicity in detail (Molnár, 1980*a*; Odening, 1987; Dyková and Lom, 1988). Al-Samman et al. (2003) reported that swimbladder changes were accompanied by reno-, hepato- and splenomegaly in carp fry suffering from SBI.

Until recently, five *Sphaerospora* species have been studied genetically on DNA level. The sequence of the 18S ribosomal RNA gene (18S rDNA) of kidney-parasitic *S. oncorhynchi* (AF201373, Kent et al., 2001) and *S. truttae* (AJ581915, Holzer et al., 2003*a*) have been studied from salmonids, while *S. dicentrarchi* (AY278564) originating from sea bass *(Dicentrarchus labrax)* has been described genetically by Caffara et al. (unpublished). The partial 18S rDNA sequence of *S. elegans* (AJ609590) from stickleback *(Gasterosteus aculeatus)* has also been submitted to GenBank by Holzer et al. (2003*b*). The only species parasitising cyprinids that has been examined to date is *S. molnari* (AF378345) collected from the gills of goldfish *(Carassius auratus)* in Japan (Kent et al., 2001).

In the present paper, the partial 18S rDNA sequence of two kidney-parasitic *Sphaerospora* species, *S. renicola* from common carp and *Sphaerospora* sp. from goldfish *(Carassius auratus auratus)* was determined in order to estimate their phylogenetic positions among other myxosporeans.

Materials and methods

Myxospores of two *Sphaerospora* species were collected from cyprinids in Hungary (Table 1). After collection and transportation to the laboratory, several dozens of common carp fingerlings and seven goldfish were sacrificed by an overdose of the anaesthetic MS 222, followed by severance of the spinal cord. Squash preparations of gills, kidney, swimbladder and the rete mirabile in the eye were examined for the presence of mature spores and blood stages of *Sphaerospora*. Mature spores were measured and morphologically identified. Digital pictures of the myxospores were also taken with an Olympus DP 10 camera. Kidney samples collected were preserved deep frozen in 1.5-ml Eppendorf tubes until further use.

DNA extraction from the samples was performed by different methods. Samples containing mature myxospores (samples no. 1 and 2) were homogenised in distilled water and then spores were separated from the host cells using a two-phase system of 4.8% dextran and 3.76% polyethylene glycol (PEG, MW 6000) as described by Holzer et al. (2003*a*). As this separation method is based on the surface differences between mature myxospore and host cell, it is not efficient for the separation of blood stages and immature myxospores. The PEG phase containing the spores was washed twice with distilled water and centrifuged at

2000 *g* for 10 min. The spore pellet was suspended in 500 µl lysis buffer (100 mM NaCl, 10 mM Tris, 10 mM EDTA, 0.2% SDS, and 0.4 mg/ml proteinase K) and incubated at 55 °C for 3–4 h. DNA was extracted using the Miniprep Express Matrix (BIO 101) as described previously by Eszterbauer (2004). Samples containing immature myxospores were homogenised in distilled water and digested in lysis buffer containing proteinase K (as mentioned above) overnight. DNA extraction was carried out using phenol and chloroform followed by precipitation with sodium acetate in ethanol (Molnár et al., 2002). DNA was resuspended in MilliQ-purified water. DNA content was estimated by electrophoresis of known amount of λ phage DNA cut with *Pst*I.

Table 1

Sphaerospora species examined. Samples were collected from different fish farms in Hungary

Species	Fish host	Locality	Sampling date	Host organ	Developmental stage	Sample no.
S. renicola Dyková and Lom, 1982	Common carp (*Cyprinus carpio*)	Százhalombatta	29/08/2002	kidney tubules	mature spores	1
		Százhalombatta	15/08/2003	kidney tubules	mostly mature spores	2
		Hortobágy	19/07/2002	kidney tubules	immature spores	3
S. sp.	Goldfish (*Carassius auratus auratus*)	Százhalombatta	24/02/2004	kidney tubules	mature and immature spores	4
		Százhalombatta	25/02/2004	kidney tubules	mature and immature spores	5

For PCR amplification of the 18S rDNA of *Sphaerospora* species examined, a primer pair named SphF–SphR was designed on the basis of the alignment of *Sphaerospora* sequences submitted to GenBank by February 2003. Primer sequences and their references are summarised in Table 2. The total volume of PCR was 50 µl that contained 10–50 ng extracted DNA, 1 × Taq PCR reaction buffer (MBI Fermentas), 1.25 mM $MgCl_2$, 0.2 mM dNTP mix (Sigma), 50 pmol of each primer and 2 units of Taq DNA Polymerase (MBI Fermentas). MJ Research PTC-200 and a Biometra T1 thermocycles were used for amplifi-

cation. Amplification conditions were 95 °C for 50 sec, 56 °C for 50 sec and 72 °C for 80 sec for 35 cycles, with a terminal extension at 72 °C for 7 min. PCR products were electrophoresed in 1.0% agarose gel and purified with Geneclean III Kit (Bio 101).

Except *S. renicola* sample no. 1, PCR fragments purified were cloned into pGEM-T vector with pGEM-T Vector System I (Promega) following the manufacturer's manual. Positive clones were selected using the blue-white colour screening method. Positive clones were sequenced with universal forward primer.

The PCR product of sample no. 1 and positive clones were then sequenced in both directions with six primers listed in Table 2 using the Applied Biosystems (ABI) BigDye Terminator v3.1 Cycle Sequencing Kit with an ABI 3100 Genetic Analyzer automated DNA sequencer (Applied Biosystems). Only sample no. 1 was sequenced directly. For sequence assembling, the STADEN Sequence Analysis Package version 2001.0 (Staden, 1996) was used.

Table 2

Primers used for PCR and/or sequencing

Name	Sequence	Reference
SphF	5'-ACT CGT TGG TAA GGT AGT GGC T-3'	Present study
SphR	5'-GTT ACC ATT GTA GCG CGC GT-3'	Present study
MB5r	5'-ACC GCT CCT GTT AAT CAT CAC C-3'	Eszterbauer, 2004
MB3f	5'-GAT GAT TAA CAG GAG CGG TTG G-3'	Eszterbauer, 2004
MC5	5'-CCT GAG AAA CGG CTA CCA CAT CCA-3'	Molnár et al., 2002
MC3	5'-GAT TAG CCT GAC AGA TCA CTC CAC GA-3'	Molnár et al., 2002

Nucleotide sequences were aligned with the MultAlin program (Corpet, 1988). The alignment was corrected manually using the GeneDoc sequence alignment editor program. Ambiguously aligned regions and gaps longer than 5 nucleotides were excluded. Phylogenetic calculations were performed with PHYLIP v3.6a (Felsenstein, 1997). The data were analysed with maximum likelihood (DNAML with transversion/transition ratio 1:1, 1:2 or 1:3, empirical base frequencies, one rate class for nucleotide substitution and global re-arrangements) and distance matrix analysis (DNADIST using Kimura-2 parameter followed by FITCH with global re-arrangements). Clade support was assessed with bootstrapping (100 replicates for maximum likelihood and 1000 replicates for distance matrix method). *Polypodium hydriforme* was chosen as outgroup.

Results

During the morphological examination of fish kidneys collected for molecular study, only *Sphaerospora* species were identified and no other myxosporeans were observed in squash preparations. In carp kidney samples, the shape and size of mature myxospores found in renal tubules were identical with those of *S. renicola* described by Dyková and Lom (1982). The myxospores found in the kidney tubules of goldfish also showed very similar body shape and size, and were morphologically indistinguishable from *S. renicola* (Fig. 1).

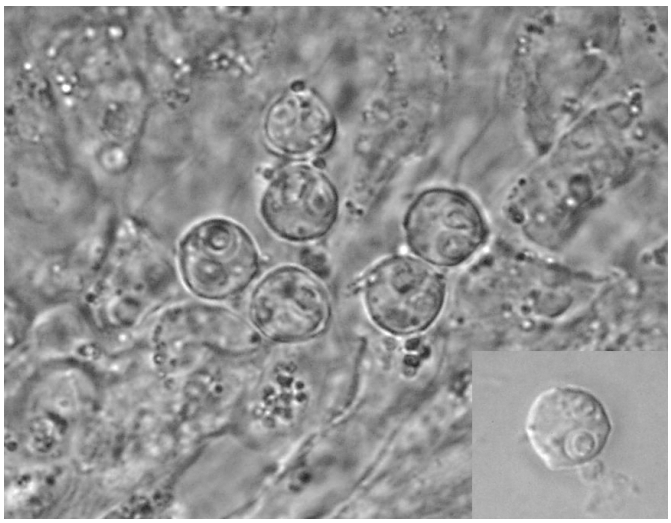

Fig. 1. Myxospores of *Sphaerospora* sp. from renal tubules of goldfish (*Carassius auratus auratus*). Squash preparation. The scale bar is 10 μm

The SphF-SphR primers successfully amplified approx. 1400 bp fragments of 18S rDNA from every sample. The 1413 bp sequence of *S. renicola* (AY735410) and the 1349 bp long sequence of *Sphaerospora* sp. (AY735411) from goldfish were deposited to GenBank. In the case of sample no. 3, one of the clones gave different sequence. With BLAST search, this clone was determined as an 18S rDNA sequence belonging to myxosporeans, but it was not identical with any species available in GenBank. The most similar species was *Myxobolus pavlovskii* (AF507973), although their genetic similarity was only 86.5%. The sequences of cloned fragments of *S. renicola* were generally 100% identical, except for three out of the eleven clones, where 0.07–0.21% differences at different positions were observed among sequences. In the case of *Sphaerospora* sp., only six clones were sequenced, and 0.07–0.14% nucleotide differences also occurred in two clones.

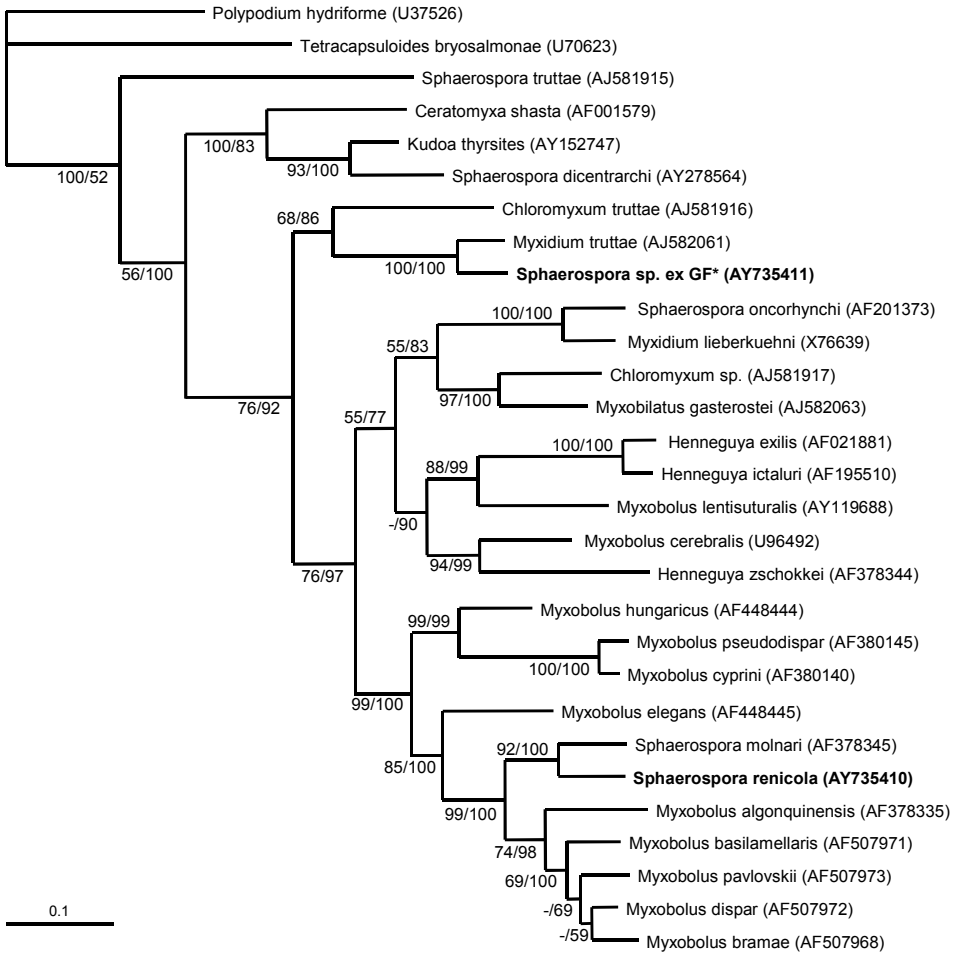

Fig. 2. Maximum likelihood tree of the 18S rDNA sequences of myxosporeans, rooted at *Polypodium hydriforme*. Numbers at nodes indicate bootstrap confidence levels in percent (maximum likelihood: 100 repetitions, Tv/Ts 1:2; distance matrix method: 1000 repetitions). GenBank accession numbers are in parentheses. The scale bar indicates a branch length of 0.1 inferred substitutions per site. *GF: goldfish

Phylogenetic analyses were based on the final, edited alignment that was 1361 bp in length and contained 29 myxosporean species. Interestingly, the two *Sphaerospora* species sequenced newly occupied distant places on phylogenetic trees (Fig. 2). The species most closely related to *S. renicola* was *S. molnari* (AF378345) with 81.8% identity in the 1413 bp sequences. They clustered with high bootstrap values to the group containing gill-infecting *Myxobolus* species. *Sphaerospora* sp. from goldfish was the most similar to *Myxidium truttae* (AJ582061) and 88.4% genetic identity was obtained on 1349 bp. This sub-clade,

supported by a high bootstrap value, was found close to other coelozoic freshwater parasites like *Chloromyxum truttae* (AJ581916). Distance matrix analysis confirmed the clustering pattern of maximum likelihood analysis, in several cases with different bootstrap values (Fig. 2).

In spite of the morphological similarity, *Sphaerospora* species occupied very different positions on the phylogenetic tree. Within the genus *Sphaerospora*, *S. truttae* was the closest to the outgroup, and its similarity to other *Sphaerospora* species was less than 40%. The differences in 18S rDNA sequences are summarised in Table 3.

Table 3

Percentage of base pair identities of 18S rDNA sequences obtained from *Sphaerospora* species used for phylogenetic analysis (on the basis of a 960 bp long, unedited alignment). S trut: *Sphaerospora truttae*, S dicent: *S. dicentrarchi*, S sp-GF: *Sphaerospora* sp. from goldfish, S onco: *S. oncorhynchi*, S ren: *S. renicola*, S mol: *S. molnari*

	S trut	S dicent	S sp-GF	S onco	S ren	S mol
S trut	100.0	31.3	36.7	39.0	35.5	36.4
S dicent		100.0	52.1	51.6	53.0	50.4
S sp-GF			100.0	61.8	58.1	57.4
S onco				100.0	66.6	64.2
S ren					100.0	79.6
S mol						100.0

Discussion

Sphaerospora renicola previously identified by Molnár (1980*b*) as *S. angulata* Fujita, 1912 is a common parasite in intensive carp cultures in Europe. Although this species has been described from common carp, it was indistinguishable morphologically from *Sphaerospora* sp. developing in the renal tubules of goldfish. Therefore, the genetic identity of the two myxospores infecting different hosts has also been expected. This theory seemed to be confirmed by Lom and Dyková (1992) who have reported that goldfish also could become infected by *S. renicola*. The present study on 18S rDNA sequences proved that in spite of the morphological similarity, these two species are genetically distinct, and they share only 71.9% identical nucleotides. Morphologically very similar species of the genus *Myxobolus* have already been differentiated by molecular biological methods (Andree et al., 1999; Eszterbauer, 2002). However, members of the genus *Sphaerospora* have not been studied genetically as intensively as other myxosporean genera, and the five *Sphaerospora* species, the 18S rDNA sequences of which have previously been submitted to GenBank, differed on the basis of myxospore morphology.

Interestingly, among the clones of *S. renicola* that were sequenced in this study, one clone of an unidentified myxosporean species was determined. Since no other myxospores could be seen in squash preparations, the occurrence of this unknown sequence cannot be readily explained. The kidney of cyprinids and other advanced bony fish contains melano-macrophage centres. The general functions of these centres are detoxification and relocation of debris of damaged cells, and they also play an important role in the response of fish to infectious agents (Agius and Roberts, 2003). Thus, the kidney of cyprinids may be an 'accumulation centre' of myxosporeans, which develop in different organs of fish and may reach the kidney via the blood system [e.g. *Myxobolus pseudodispar* originating from muscle (Baska, 1987)]. To avoid contamination by other myxosporeans, it is important to collect samples very carefully, and to use only separated cysts for molecular biological examinations (Eszterbauer, 2004). Unfortunately, coelozoic parasites such as *Sphaerospora* species examined in the present study do not form cysts, and thus this collection procedure cannot be followed. Therefore, molecular cloning of amplified PCR products is necessary, especially if primers used for PCR are not species specific. Molecular cloning of samples also gives an opportunity for a more detailed examination of intraspecific variability. The majority of the clones of *S. renicola* were genetically identical, and only in three clones was a 0.07–0.21% DNA sequence difference determined. In the case of *Sphaerospora* sp. the difference in the nucleotide sequence was 0.07–0.14%. This diversity was related to different nucleotide positions at highly conserved regions of the 18S rRNA gene, therefore nucleotide differences might be caused by the infidelity of *Taq* DNA polymerase.

The present phylogenetic analyses have shown that the *Sphaerospora* species examined to date are not closely related genetically. *Sphaerospora* sp. from goldfish grouped with *Myxidium truttae* within the clade of ancient freshwater species. Their development is coelozoic in the renal tubules as in the case of most other *Sphaerospora* species (e.g. *S. oncorhynchi*, *S. dicentrarchi*, *S. truttae*), thus we interpret that the phylogenetic position of *Sphaerospora* sp. from goldfish reflected its site preference. *S. renicola* clustered with *S. molnari* within the clade of myxosporeans histozoic in gills. This clade contained mostly *Myxobolus* species, which grouped by their tissue tropism as previously reported by Eszterbauer (2004). The phylogenetic position of *S. renicola*, which is a typical coelozoic parasite, might suggest that coelozoic development as an ancestral feature first disappeared and/or was replaced by histozoic development and at a later point of evolution this feature re-appeared within the clade of myxosporeans histozoic in gills.

The positions of the six *Sphaerospora* species involved in phylogenetic analyses showed that the morphology of myxospores does not correlate with their genetic relationship. Kent et al. (2001) suggested that most of the myxosporean genera studied up to that time (e.g. the genera *Myxobolus, Henneguya,*

Myxidium) were para- or polyphyletic, and the taxa cluster by development and tissue tropism rather than by spore morphology. Although in Kent's phylogenetic analyses, only two *Sphaerospora* species, *S. oncorhynchi* and *S. molnari* have been used, their clustering was similar to that found in the present study. We also studied the phylogeny of *Sphaerospora* species in relation with their geographical locations and fish hosts. These six species originated from different locations (Canada, Hungary, Japan, UK, USA) and different hosts (mostly salmonids and cyprinids), but no correlations were found to their phylogenetic positions.

The phylogenetic relationships observed among *Sphaerospora* species seem to clearly confirm that this genus is polyphyletic. Genetic study of additional *Sphaerospora* species is needed to define the different factors influencing the evolution of this genus.

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