'Who's who' in renal sphaerosporids (Bivalvulida: Myxozoa) from common carp, Prussian carp and goldfish – molecular identification of cryptic species, blood stages and new members of *Sphaerospora sensu stricto*

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SUMMARY

Myxozoans are a group of diverse, spore-forming metazoan microparasites bound to aquatic environments. *Sphaerospora dykovae* (previously *S. renicola*) causes renal sphaerosporosis and acute swim bladder inflammation (SBI) in juvenile *Cyprinus carpio carpio*, in central Europe. A morphologically similar species with comparably low pathogenicity, *S. angulata* has been described from *C. c. carpio, Carassius auratus auratus* and *Carassius gibelio*. To clarify uncertainties and ambiguities in taxon identification in these hosts we decided to re-investigate differences in spore morphology using a statistical approach, in combination with SSU and LSU rDNA sequence analyses. We found that developing spores of *S. angulata* and *S. dykovae* cannot be distinguished morphologically and designed a duplex PCR assay for the cryptic species that demonstrated *S. dykovae* is specific to *C. c. carpio*, whereas *S. angulata* infects *C. a. auratus* and *C. gibelio*. The molecular identification of myxozoan blood stages in common carp and goldfish, which had previously been ascribed to *Sphaerospora* spp. showed that approximately 75% of blood stages were from non-sphaerosporid coelozoic species infecting these cyprinids and more than 10% were from an alien species, *Myxobilatus gasterostei*, developing in sticklebacks. We hereby report non-selective myxozoan host invasion and multi-species infections, whose role in SBI still requires clarification.

Key words: *Sphaerospora*, Myxozoa, cyprinid, morphometry, cryptic speciation, ribosomal DNA, molecular identification, blood stages, multi-species infection.

INTRODUCTION

Myxozoans are diverse and widely distributed metazoan microparasites that alternate between vertebrate (predominantly fish) and invertebrate hosts (oligochaetes, polychaetes and bryozoans), in a variety of aquatic environments. Traditionally, myxozoan taxonomy has relied on spore morphology to define genera; however, molecular phylogenetics proved that most traditional myxozoan genera are polyphyletic (Holzer *et al*. 2004; Fiala, 2006). Thereby, the genus or morphotype *Sphaerospora* Thélohan, 1892 is probably the most polyphyletic of all, with representatives in 6 different phylogenetic clades of the Myxozoa (recently re-analysed by Bartošová *et al*. 2011*a*). Most *Sphaerospora* spp., including the type species *Sphaerospora elegans* Thélohan, 1892 infect the urinary tract and cluster together in a basal phylogenetic clade, termed *Sphaerospora* sensu stricto (Jirků *et al*. 2007). The members of *Sphaerospora* s. str. show as a common feature extremely long expansion segments in the shape of linear helices in 2 variable regions of the SSU rRNA (Holzer *et al*. 2007).

Renal sphaerosporosis in carp, *Cyprinus carpio carpio* L. in Europe was first described in Hungary by Molnár (1980*a*), who related the observed pathological changes in the kidney to *Sphaerospora angulata* Fujita, 1912, a myxozoan originally described from the renal tubules of carp *C. c. carpio* and goldfish *Carassius auratus auratus* (L.) in Sapporo, Japan (Fujita, 1912). Shortly afterwards Lom and Dyková (1982) described this intratubular myxozoan from

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carp kidney as a new species, *Sphaerospora renicola*, on the basis of morphological differences to *S. angulata*. Due to the demise of the genus *Leptotheca* Thélohan, 1895 (Gunter and Adlard, 2010) *S. renicola* became a senior secondary homonym of a morphologically distinct species from Atlantic mackerel *Scomber scombrus* L. and the species described from carp was re-named *Sphaerospora dykovae* (Lom and Dyková, 1982) in honor of Professor Dyková who initially described it (Gunter and Adlard, 2010). In morphological descriptions of spores from Hungarian carp, Molnár (1980*a*,*b*) stated that the spores were clearly angulate and similar to *S. angulata*, in contrast to *S. dykovae*, which was described as 'globular' (Lom and Dyková, 1982). Species differentiation in this case is of particular importance as *S. angulata* infections in goldfish have not been related to serious pathological changes, whereas *S. dykovae* causes renal sphaerosporosis in common carp and its proliferative, presporogonic stages are the agents of acute and serious swim-bladder inflammation (SBI) (Körting, 1982; Kovács-Gayer *et al.* 1982; Kovács-Gayer, 1983; Körting and Hermanns, 1984; Molnár and Kovács-Gayer, 1986). Another cycle of *S. dykovae* proliferation is supposed to occur in the blood (Csaba, 1976; Molnár, 1980*a*,*b*; Kovács-Gayer *et al.* 1982; Lom *et al.* 1983; Grupcheva *et al.* 1985) although the transmission of these blood stages to SPF receptor fish did not always result in *S. dykovae* infection in the kidney (Molnár, 1980*a*; Körting *et al.* 1989).

Sphaerospora spp. myxospores have few distinguishing morphological characteristics, and this has led to uncertainties and ambiguities in taxon identification. Molecular methods have considerably aided species differentiation in the myxozoans and have furthermore uncovered wrongly ascribed life-cycle stages (e.g. Holzer *et al.* 2004; Bartošová *et al.* 2011*b*). Only recently, the SSU rDNA sequence of *S. dykovae* was corrected in GenBank™ (named *S. renicola*, GenBank Accession number JF758875, Eszterbauer, 2011) resulting in *S. dykovae* becoming a member of the *Sphaerospora* s. str. group. To clarify the identities of the renal sphaerosporids in common carp, Prussian carp and goldfish we decided to re-investigate differences in spore morphology using a statistical approach for morphometrics, in combination with molecular data from the same samples used for measurements. Herein, we provide full SSU and LSU rDNA sequences from the renal sphaerosporids in common carp, Prussian carp and goldfish from different geographic locations, thus resolving the 'who's who' and contributing new data on intraspecific sequence variability in the *Sphaerospora* s. str. clade. Furthermore, we provide molecular evidence for the identity of diverse, cryptic myxozoan blood stages in goldfish and carp.

MATERIALS AND METHODS

Fish species and sampling localities (detailed in Table 1)

Common carp, *C. c. carpio* ($n=142$) were collected during September–November 2011, from 7 earthen ponds, most of which are used for commercial production of carp, in and around the South Bohemian region of the Czech Republic. Previously, 21 carp had been collected in a carp farm near Hortobágy (Hungary), in August 2009. Goldfish, *C. a. auratus* ($n=91$) were sampled from 6 localities: Czech Republic (earthen ponds), North America (Oregon pet shop, fish reared in California) and from Australia (report and photographs from Professor Jiří Lom, number of fish unknown). Prussian carp, *Carassius gibelio* (Bloch) ($n=25$) were sampled from 2 localities, one in the Czech Republic and another one in Hungary. All fish were transported to the laboratory whilst alive, euthanized on arrival by a Schedule 1 method and dissected.

Morphometric analyses of spores

Fresh kidney smears were examined by light microscopy and digital images of sphaerosporid spores in the tubular lumen were taken at ×1000 magnification. The same tissue smears from which digital images of spores were taken were used for DNA extraction. Spores were measured on digital images using ImageJ v.1·44p (Wayne Rasband, http://imagej.nih.gov/ij) calibrated against a digital image of a graticule. Measurements ($n=10$–20 per fish locality) of spore length (SL), spore width (SW), spore thickness (ST), polar capsule length (PCL) and polar capsule width (PCW) were obtained following the guidelines of Arthur and Lom (1989) and Sitja-Bobadilla and Alvarez-Pellitero (1994). Statistical analyses were conducted to determine the significance of morphological differences between sphaerosporid spores from common carp, goldfish and Prussian carp from different hosts and sites (Table 1). Absolute measurements were ln-transformed, and the ratios SL:ST and PCL:SL were arcsine square-root transformed and subjected to a Principal Component Analysis (PCA). The Kruskal-Wallis one-way analysis of variance was used to conduct multiple comparisons and to determine significant differences between the morphometric data from different parasite species and localities. All statistical analyses were carried out using Statistica (StatSoft Inc.).

rDNA sequencing

DNA was extracted from tissue smears of all fish that had small, round to oval plasmodia in the tubular lumen, whether mature spores were present or not. DNA was also extracted from tissue smears of 39

Table 1. *Sphaerospora angulata* and *S. dykovae* sample data: origin of fish, parasite prevalence and sequence data

	Sampling locality	Coordinates	Host	Prevalence (microscopy)	Morphometry (spores/fish)	SSU rDNA GenBank Accession numbers	LSU rDNA GenBank Accession numbers
Sphaerospora angulata	Sasov, Jihlava/CZ	N49°22′48·5″ E15°36′07·5″	*C. a. auratus*	58·3% (7/12)	20/3	JQ801528	JQ801535 – 37
	Pet shop/CZ	Bred in CZ	*C. a. auratus*	33% (1/3)	15/1	JQ801525	JQ801539
	Tourov/CZ	N49°07′22″ E14°02′04″	*C. a. auratus*	10·7% (3/28)	–	JQ801527	JQ801534
	Chřešťovice/CZ	N49°19′24·8″ E14°17′11·7″	*C. a. auratus*	0% (0/36)	–		
	Australia	Bred in Australia	*C. a. auratus*	n/a	10/?		
	Pet shop/ USA	Bred in California/USA	*C. a. auratus*	58·3% (7/12)	12/1	JQ801529	
	Hluboká nad ./CZ	N49°05′03·0″ E14°44′24·5″	*C. gibelio*	12·5% (1/8)	20/1	JQ801526	JQ801538
			C. a. auratus	0% (0/3)	–		
	Százhalombatta/HU	N47°20′25·5″ E18°53′46·7″	*C. gibelio*	5·9% (1/17)	no	JQ801530	
Sphaerospora dykovae	Šnejdlík pond/CZ	N49°00′17″ E14°25′04″	*C. c. carpio*	9·5% (2/21)	–		
	Motovidlo pond/CZ	N48°59′57″ E14°22′45″	*C. c. carpio*	8·3% (2/24)	–	JQ801532	JQ801542 – 44
	Vodňany/CZ	N49°09′35″ E14°10′07″	*C. c. carpio*	17·4% (8/46)	20/2	JQ801533	JQ801540 – 41, JQ801545
	Tourov/CZ	N49°07′22″ E14°02′04″	*C. c. carpio*	25% (4/16)	20/2		JQ801546
	Chřešťovice/CZ	N49°19′23·8″ E14°17′11·8″	*C. c. carpio*	0% (0/20)	–		
	Velký Hvězdář pond/CZ	N49°03′40″ E14°25′48″	*C. c. carpio*	0% (0/6)	–		
	Jindřichův Hradec/CZ	N49°08′37″ E15°00′45″	*C. c. carpio*	0% (0/17)	–		
	Hortobágy/HU	N47°35′11·1″ E21°02′58·4″	*C. c. carpio*	42·8% (9/21)	no	JQ801531	

common carp and 66 goldfish that had no visible signs of infection. After microscopic examination, the cover slips were removed and tissues were collected in TNES urea buffer (Asahida *et al*. 1996) where they were stored for 1–60 days. DNA was extracted using a simplified phenol-chloroform extraction protocol (Holzer *et al*. 2004) and, after drying, DNA was re-dissolved in RNAse/DNAse-free water. rDNA sequences were amplified in nested PCRs using a series of primer combinations (Table 2). For complete SSU rDNA, 3 overlapping fragments were produced, whereas almost complete LSU rDNA was sequenced from 2 overlapping fragments. We used full SSU and partial LSU rDNA sequences for analysis of intraspecific sequence variability. PCRs were conducted in 10 μl reactions with 0·025 Uμl^{-1} Titanium Taq DNA polymerase and 10× buffer which contained 1·5 mM $MgCl_2$ (BD Biosciences Clontech), with 0·2 mM of each dNTP, 0·5 mM of each primer, and 10–150 ng of template DNA. Denaturation of DNA (95 °C for 2 min) was followed by 30 cycles of amplification (95 °C for 40 s, annealing temperature (Table 2) for 40 s, and 68 °C for 1 min 50 s) and ended by a 4 min extension (68 °C). Direct sequencing of PCR products was attempted preferentially; if this failed, PCR amplicons were cloned into the pDrive Cloning vector (Qiagen PCR Cloning Kit, Germany) and transformed into the competent *E. coli* strain XL-1. Plasmids from 6 clones were sequenced per DNA sample.

Design of a specific duplex PCR and diagnosis of infections

Based on the SSU rDNA sequences obtained from the sphaerosporids in common carp, Prussian carp and goldfish specific primer pairs (Table 2) were designed amplifying products of 1058 bp (*S. angulata*) and of 552 bp (*S. dykovae*). The primers were tested for their complementarity and specificity, before use in the final duplex PCR assay. This assay used cycle conditions as above, except had an annealing temperature of 67 °C for 40 s and an elongation time of 50 s. We tested specificity of the duplex PCR assay against 11 other members of the *Sphaerospora* s. str. clade belonging to the genera *Sphaerospora* and *Polysporoplasma* Sitjà-Bobadilla and Alvarez-Pellitero, 1995 (*unpublished data*).

The specific primers SangR and SdykR were used also to confirm the identity of parasite stages in histological sections by *in situ* hybridization according to the protocol of Holzer *et al*. (2003).

Identification of blood stages

Blood smears of 122 carp and 91 goldfish were Giemsa stained and screened for the presence of myxozoan blood stages. Blood from all these fish was

Table 2. Primers and PCR conditions

Primer name	Sequence	Relative position	rDNA region	Amplicon size (PCR primer combination)	PCR type	Annealing temp.	Reference
ERIB 1	5′-ACCTGGTTGATCCTGCCAG-3′	1 (JQ801532)	SSU	3163 (ERIB1&ERIB10)	1st round	60 °C	Barta *et al*. (1997)
ERIB 10	5′-CTTCCGCAGGTTCACCTACGG-3′	3163 (JQ801532)	SSU				Barta *et al*. (1997)
SphSSUR680	5′-AGCACTGTTTTAGGTTGTAGTCTATC-3′	633 (JQ801532)	SSU	633 (ERIB1&SphSSUR80)	nested	62 °C	Present study
MyxGP2F	5′-TGGATAACCGTGGGAAA-3′	162 (JQ801532)	SSU	1740 (MyxGP2F&Act1R)	nested	58 °C	Kent and Lom, (1998)
Act1R	5′-AATTTCACCTCTCGCTGCCA-3′	1902 (JQ801532)	SSU				Hallett *et al*. (2002)
Myxgen4F	5′-GTGCCTTGAATAAATCAGAG-3′	1091 (JQ801532)	SSU	2072 (Myxgen4F&ERIB10)	nested	60 °C	Diamant *et al*. (2004)
NLF164	5′-ACCTCCACTCAGGCAAGATTA-3′	n/a	LSU	n/a (NLF164&NLR1694)	1st round	45 °C	Bartošová *et al*. (2009)
NLR1694	5′-TCTYAGGAYCGACTNAC-3′	3313 (JQ801540)	LSU				Van der Auwera *et al*. 1994
NLF184	5′-ACCCGCTGAAYTTAAGCATAT-3′	1 (JQ801540)	LSU	2721 (NLF184&NLR1270)	nested	57 °C	Van der Auwera *et al*. 1994
NLR1270	5′-TTCATCCCGCATCGCCAGTTC-3′	2721 (JQ801540)	LSU				Bartošová *et al*. (2009)
NLF1050	5′-AATCGAACCATCTAGTAGCTGG-3′	1916 (JQ801540)	LSU	approx. 3600 (NLF1050&NLR3284)	1st round	50 °C	Bartošová *et al*. (2009)
NLR3284	5′-TTCTGACTTAGAGGCGTTCAG-3′	n/a	LSU				Van der Auwera *et al*. 1994
NLF1126PB	5′- CGAAATATCCCTCAGGAT-3′	1945 (JQ801540)	LSU	2866 (NLF1126PB&NLR2571)	nested	52 °C	Present study
NLR2571	5′- CTCAACAGGGTCTTCTTTCC-3′	4811 (JQ801540)	LSU				Van der Auwera *et al*. 1994
SangF	5′-GTGGTGGTGCGTGATGTGTG-3′	894 (JQ801528)	SSU	1091 (SangF&SangR)	duplex, single round	67 °C	Present study
SangR	5′-CACGTGCACACATGAACCGAG-3′	1985 (JQ801528)	SSU				Present study
SdykF	5′-TGATGTGCGTGTCAGTTGTGC-3′	897 (JQ801532)	SSU	588 (SdykF&SdykR)			Present study
SdykR	5′-ACGCAAAGATGCACACACACTGGAC-3′	1485 (JQ801532)	SSU				Present study

also used for DNA extraction. We used a nested PCR assay which non-specifically amplifies partial myxozoan SSU rDNA to identify these stages and to compare the thresholds of PCR versus microscopical detection. First-round primers were ERIB1 and ERIB10, followed by a nested second round that used primers MyxGP2F and Act1R (Table 1). The same PCR assay was used to amplify SSU rDNA for sequencing of the other myxozoan taxa that were detected in the fish.

Sequence alignments and phylogenetic analyses

For analysis of intraspecific variability, full SSU and partial LSU rDNA sequences of different isolates of each sphaerosporid species were self-assembled and aligned in SeqMan II (DNASTAR, Inc.). Alignments were checked and manually corrected in BioEdit 7.0.5.2 (Hall, 1999). For phylogenetic analyses, the newly obtained SSU rDNA sequences of *Sphaerospora* spp. and of the other myxozoan taxa were aligned with published sequences of representatives of the four principle myxozoan phylogenetic clades (Fiala, 2006; Holzer *et al*. 2007; Fiala and Bartošová, 2010; taxa and GenBank Accession numbers given in Fig. 3), using Clustal X ver. 1·83 (Thompson *et al*. 1997) with default parameters. The alignment was edited and ambiguous regions of the final alignment were identified by eye and excluded. The malacosporeans *Tetracapsuloides bryosalmonae* Canning *et al*. 1999 and *Buddenbrockia plumatellae* Schröder, 1910 were used as outgroup. Maximum parsimony (MP) analysis was performed in PAUP* 4.b10 (Swofford, 2001) using a heuristic search with random addition of taxa, the ACCTRAN option and the TBR swapping algorithm. All characters were treated as unordered, Ts/Tv ratio was set to 1:2 and gaps were treated as missing data. Maximum likelihood (ML) analysis was performed in RA×ML (Stamatakis, 2006) using the GTRGAMMA model (log likelihood = 14761·041543, gamma shape = 0·483849). Bootstrap support values were based on 1000 (MP) and 500 (ML) replicates, with random sequence additions. Bayesian inference (BI) was conducted in MrBayes v. 3.0 (Ronquist and Huelsenbeck 2003) employing the GTR + Γ + I evolutionary model. Posterior probabilities were estimated from 1 000 000 generations via 2 independent runs of 4 simultaneous Markov chain Monte Carlo simulations with every 100th tree saved. Tracer v.1.4.1 (Drummond and Rambaut, 2007) was used to ascertain the length of the burn-in period, which was set to 12 000 generations.

RESULTS

Infection prevalence (Table 1)

Pseudoplasmodia containing refractile granules and/or 2 sphaerosporid spores were observed in the renal tubules of 16 common carp from 4 localities, in 15 goldfish (+ unknown number of goldfish from Australia, images of Professor Lom) from 4 localities and in 2 Prussian carp from 2 localities. Infection prevalences at the different localities was 8·3% – 42·8% in common carp, 10·7% – 58·3% in goldfish and 5·9% – 12·5% in Prussian carp.

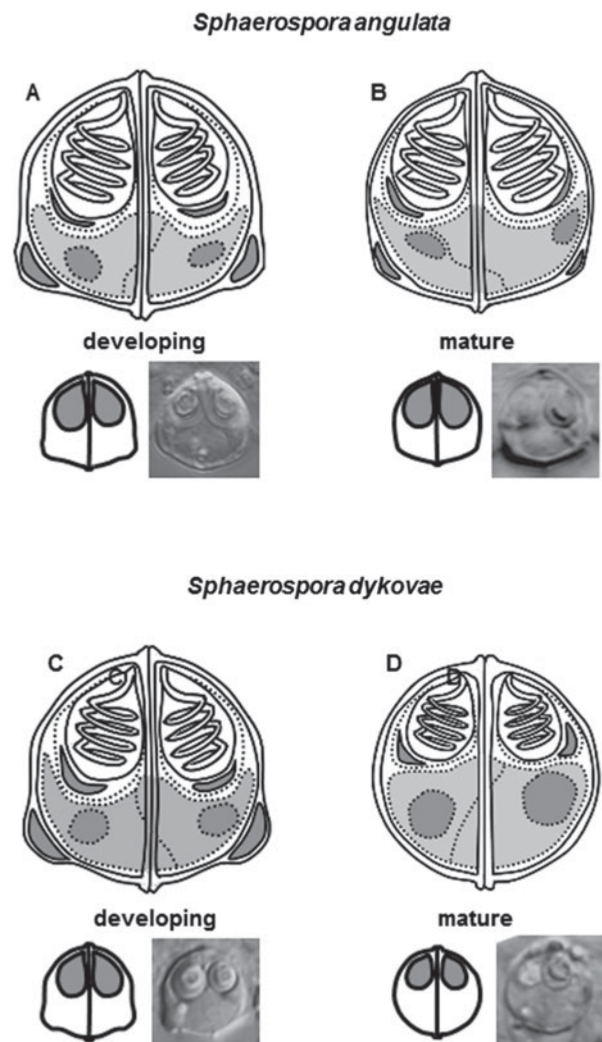

Fig. 1. Spore morphology of *S. angulata* (A and B) and *S. dykovae* (C and D). (A and C) Maturing spores with bulges on posterior end of spore due to the location of the large valvular nuclei. (B and D) Fully mature spores with no visible (*S. dykovae*) or only small (*S. angulata*) valvular nuclei remnants. Small schematic drawings showing overall shape and relationship of size of polar capsules to size of spore for both spore developmental stages of both species; notice similarity of maturing spore stages.

Spore morphology and morphometrics (Table 3; Figure 1)

In goldfish and Prussian carp sphaerosporid spores (Fig. 1A and B) were triangular, had convex sides and were slightly pointed at the mid-posterior margin of the spore. This triangular shape is caused by

valvulogenic cell nuclei, located laterally on posterior part of the spore, and is distinct in both semi-mature and mature spores, in which the nuclei were considerably smaller but were still visible and shape-determinant. The sporoplasm consisted of 2 separate cells, each with 1 nucleus. Polar capsules were pyriform, of slightly different sizes, and each contained a filament that formed 4–5 coils. After comparison with published reports (Table 3) we ascribed this species to *S. angulata* with which our data were identical, also regarding size and shape. The present study and previous reports show that the polar capsule length constitutes 46–51% of the full length of the spore. Molecular data provided proof that the sphaerosporids from goldfish and Prussian carp are conspecific (see below).

In common carp, the shape of the sphaerosporid spores varied greatly (Fig. 1C and D, Table 3): Spores in early stages of maturation were angulate and had relatively large valve cell nuclei at the posterior part of the spore, as observed in *S. angulata* (Fig. 1C). The pyriform polar capsules of these spores were of equal size and made up 44–45% of the spore length. Fully mature spores were completely spherical (Fig. 1D) and occasionally demonstrated 2 tiny 'tubercules' laterally on the posterior part of the spores. Polar capsules in the spherical spore type were considerably smaller in relation to the spore size, measuring only 36% of the spore length (Fig. 1D, Table 3). This morphology matched with *S. dykovae* (Lom and Dyková, 1982). Our molecular analyses showed that all spore morphologies observed in the renal tubules of *C. c. carpio* represent the same species, *S. dykovae*.

Mature spores of both species were more compact and thus slightly smaller than immature spores. The schemata provided in Fig. 1 show that developing spores of *S. dykovae* and *S. angulata* are virtually indistinguishable, which was confirmed in the statistical analysis of the morphometrical data (see below). In both species, *S. angulata* and *S. dykovae*, an ephemeral vacuole was frequently observed at the posterior end of the developing spore, which disappeared on full maturation. This feature was previously mentioned in the original description of *S. dykovae* only (Lom and Dyková, 1982).

Statistical analysis of morphometric data

Analyses of variance of 5 metrical features and 2 relative proportions/ratios showed that the sphaerosporids from common carp, Prussian carp and goldfish are morphometrically indistinguishable with respect to spore length, spore width, spore thickness and the ratio between spore length and thickness. However, significant differences were observed in both polar capsule length ($P = 0{\cdot}0004$) and width ($P = 0{\cdot}0003$), and the ratio between polar capsule length and spore length ($P = 0{\cdot}0001$), between mature (spherical) spores of *S. dykovae* and all other spores: immature *S. dykovae* and immature and mature *S. angulata*. The PCA included all absolute measurements and ratios and showed that the first 2 principal components explained 89·51% of the variation in the dataset (component I: 58·16%, component II: 31·35%). A plot of the data in the first plane of the PCA (Fig. 2) confirmed that *S. angulata* and *S. dykovae* are morphologically inseparable. Only the group that corresponds to *S. dykovae* mature spores is clearly separated from the remainder of *S. dykovae* and *S. angulata* (Fig. 2, white triangles). The PCA shows that the measurements were widely dispersed, especially along the first principal component, which is mainly related to size. Polar capsule length and width had the highest coefficient on the first component, while the ratio between spore length and polar capsule length was most influential for the distribution of the data along the second principal component.

rDNA sequence data, sequence variability and duplex PCR assay results

Complete SSU rDNA sequences were obtained from 6 isolates of *S. angulata* and 3 isolates of *S. dykovae*. Almost complete LSU rDNA sequences were produced for 1 isolate of each species, and partial LSU rDNA sequences including variable domains D1–D6 were produced from 6 *S. angulata* isolates and from 7 *S. renicola* isolates (Table 1). The SSU rDNA sequences demonstrated extremely long expansion segments in variable regions V4 and V7, resulting in total lengths of 3469 bp for *S. angulata* and 3163 bp for *S. dykovae*. These sequences represent the longest myxozoan SSU rDNA sequences published to date. The partial SSU rDNA sequence provided for *S. dykovae* (Eszterbauer, 2011) was found to be almost identical with the ones provided in the present study.

In the SSU rDNA of *S. angulata*, within species sequence variability was found to be lower between the European isolates (0·1–0·4% sequence divergence; 3–10/3383 bp) than between European and Californian isolates with (0·5–0·7% divergence; 17–24/3383 bp), independent from the host species (*C. a. auratus* and *C. gibelio*). In *S. dykovae,* SSU rDNA sequences differed by 0·1–0·4% (3–15/3092 bp) between European isolates, which was in the same range as for *S. angulata*. LSU rDNA data were obtained only for European isolates. LSU rDNA sequences differed by 0–0·7% (0–16/2725 bp) for *S. angulata* and 0·2–0·8% (3–22/2721 bp) for *S. dykovae*. Hence the LSU rDNA gene region had twice the variation as the SSU. LSU rDNA sequence divergence was the same within hosts from the same pond as between localities.

Table 3. Description and morphometrical data of *Sphaerospora angulata* and *S. dykovae* myxospores

(Comparison of measurements obtained in the present study with published records (SL, spore length; SP, spore width; ST, spore thickness; PC, polar capsule; PCL, polar capsule length; PCW, polar capsule width; PF, polar filament.)

Sphaerospora angulata	Authors	Host	Locality	Spore description	SL	SW	ST	PCL	PCW	PF turns	Average PCL/SL
	[A]Fujita (1912)	*C. carpio?* *C. a. auratus?*	Japan	Spores triangular with swollen sides, PCs of unequal size	7–8	6–7	5	max. 3·8	–		51%
	[B,C]Shulman (1966)	*C. carpio haematopterus* *C. a. auratus* *C. gibelio*	Basin of Amur River (Russia), Basin of Liaohe River (Korea)	Spores noticeably differ from spherical shape, shell valves proturberant. PCs unequal in size	7–12	6–9	5	4–5·5/3–4·5	3·5–5		53%
	Present study	*C. a. auratus*	Sasov, Jihlava and Tourov (Czech Republic)	Spores angular, PCs differ slightly in size	6·53 (5·85–7·5)	5·78 (5·46–5·85)	6·39 (5·54–7·12	3·14 (2·85–3·48)/ 2·76 (2·36–3·22)	2·41 (2·37–2·71)/ 2·18 (1·79–2·67)	4–5	49%
	Present study	*C. gibelio*	Hluboká nad V. (Czech Republic)	Spores angular, PCs pyriform, differ slightly in size	6·94 (6·33–8·02)	6·0 (5·69–6·22)	6·78 (6·51–8·11)	3·32 (2·98–3·69)/ 3·11 (2·51–3·29)	2·72 (2·6–2·84)/ 2·28 (2·00–2·7)	4–5	46%
	Present study	*C. a. auratus*	California, USA	Spores angular, PCs differ slightly in size	7·1 (6·8–7·5)	6·2 (5·7–6·7)	7·0 (6·1–7·6)	3·2 (2·7–3·7)/ 2·9 (2·6–3·3)	2·5 (2·3–2·8)/ 2·2 (1·9–2·5)	4	44%

Sphaerospora dykovae	Authors	Host	Locality	Spore description	SL	SW	ST	PCL	PCW	PF turns	Average PCL/SL
	[D]Molnar (1980*a*)	*C. carpio*	Ponds in Hungary	Characteristic angular shape, PCs nearly equal in size	6–7·5	6–6·5	–	3·5–4	2–2·5		56%
	[A]Lom and Dyková (1982)	*C. carpio*	Ponds in Czech Republic	Spores spherical. Tubercules on posterior part of spore diminish during development. PCs of identical size	7·3 (6·0–8·0)	7·2 (6·4–8·3)	–	1·7–2·3	1·3–1·6	4–5	27%
	Odening 1987	*C. carpio*	Ponds in Germany		6·5 (5·8–8)			1·7–2·3	1·3–1·6		31%
	Present study	*C. c. carpio*	Ponds in Tourov, Motovidlo and Vodnany (Czech Republic)	Spores angular to spherical, PCs of identical size	7·0 (6·33–7·65)	7·18 (6·23–7·50)	7·02 (5·88–7·90)	3·11 (2·13–3·67)	2·42 (1·46–2·88)	4–5	30–45%

Notes: (A) type record, (B) includes the data of Fujita (1912) and Molnár (1980*a*), (C) probably *S. angulata* in *C. a. auratus* and *C. gibelio* and *S. dykovae* in *C. carpio haematopterus,* (D) originally called *S. angulata*.

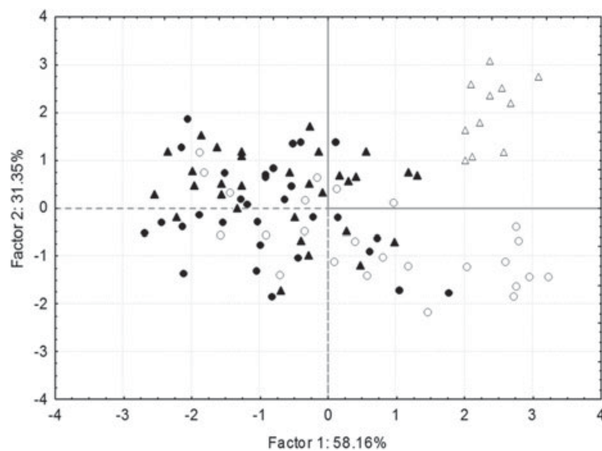

Fig. 2. Plot of the first two principal components of a Principal Component Analysis (PCA) of 5 measurements (spore length, spore width, spore thickness, polar capsule length, polar capsule width; ln-transformed) and 2 ratios (spore length:spore thickness, polar capsule length:spore length; arcsine square-root transformed) obtained from *S. angulata* (immature spores, black dots; mature spores, white dots) and *S. dykovae* (immature spores, black triangles; mature spores, white triangles).

The duplex PCR assay designed for the 2 sphaerosporids did not cross-react with any of the 11 members of *Sphaerospora* s. str. we tested, and it specifically amplified only *S. dykovae* and *S. angulata*. The assay demonstrated that goldfish and Prussian carp were infected exclusively with *S. angulata* and common carp exclusively with *S. dykovae*. *In situ* hybridization showed that the specific primers only bound to intratubular stages of the parasite in the respective hosts (data not shown).

Molecular identification of myxozoan blood stages and other myxozoan taxa

Myxozoan blood stages also called C-cells or unidentified blood objects (UBOs) were detected in Giemsa-stained blood smears of 20% (28/142) of common carp and in 35% (15/43) of goldfish. PCR proved to be more sensitive and detected myxozoan DNA in the blood of 43% (61/142) of common carp and 58% (25/43) of goldfish. However, the non-specific PCR assay did amplify myxozoan DNA in all microscopically positive samples. We matched all but 1 of the sequences obtained from the blood with myxozoan sequences in GenBank or from the present study (Table 4). Thereby, the SSU rDNA sequences obtained from blood isolates were found to be identical with a maximum of 3 bp difference from published sequences. *Sphaerospora* s. str. blood stages were usually detected together with other myxozoans in the same sample as evidenced by different amplicon sizes on the gel (~ 1500 bp versus ~ 900 bp). Sequence chromatograms of the 900 bp sequences had mixed peaks (i.e. multiple amplicons) in 9 carp and 11 goldfish, which were then excluded from the analyses.

S. dykovae and *S. angulata* made up no more than 24·6% and 19·3%, respectively, of the blood stages in their respective hosts. The only evidence of cross-infection between the two species was the detection of *S. angulata* DNA in the blood of a single carp. The majority of myxozoan blood stages were identified as coelozoic species inhabiting the urinary and biliary systems of carp and goldfish. *Buddenbrockia* sp. Schröder, 1910 represented the most common blood-stage prevalences of 26·5% (carp) and 27·7% (goldfish). Bile-inhabiting myxozoans accounted for 4·3–8·8% of myxozoan blood stages and comprised the species *Zschokkella nova* Klokacewa, 1914, *Zschokkella* sp., *Chloromyxum cristatum* Léger, 1906 and *Chloromyxum auratum* Hallett *et al*. 2006. Surprisingly, *Myxobilatus gasterostei* (Parisi, 1912), a myxozoan from the urinary system of the three-spined stickleback, represented more than 10% of blood stages in both hosts. Only a single histozoic species, i.e. *Myxobolus encephalicus* (Mulsow, 1911) was detected in the blood, a myxozoan which was present exclusively in carp. In carp, blood stages of an unidentified myxozoan with phylogenetic affinities to *S. dykovae* and *S. angulata* were relatively common (14·7%).

We obtained partial SSU rDNA sequences of *Hoferellus carassii* Akhmerov, 1960 from large spore-forming plasmodia detected in the urinary bladder of carp and goldfish, and from *M. encephalicus* spores that occurred in large masses in the brain of 1 carp. The sequence of *M. encephalicus* was found to be nearly identical (3/820 bp divergence) with that of the 'old' *S. dykovae* isolate in GenBank (Myxosporea sp. EE-2004 AY735410, Eszterbauer and Székely, 2004).

Phylogenetic relationships

Our final alignment consisted of 1466 characters (33·8% of the original alignment), from which 817 were variable and 688 were parsimony-informative. The MP analysis resulted in a single most parsimonious tree with a length of 3801 steps. Tree topology (Fig. 3) comprised 4 well-defined clades, previously defined as malacosporean, *Sphaerospora* s. str., marine myxosporean and freshwater myxosporean. In the present analysis, *Sphaerospora* s. str. appears as a sister clade to the marine myxosporeans; however, this position receives low support. The analyses clearly demonstrate that *S. dykovae* and *S. angulata* as well as the unidentified myxozoan SSU rDNA sequence detected in the blood are well-supported members of the *Sphaerospora* s. str. clade. They form a group of closely related cyprinid-infecting sphaerosporids in a sister relationship with *Sphaerospora truttae* Fischer, Scherl, El-Matbouli & Hoffmann, 1986 and *Sphaerospora elegans* (Parisi, 1912) from the

Table 4. Molecular analysis of blood stages detected in *C. c. carpio* and *C. a. auratus* – species identification and prevalence

Myxozoan species	Match with GenBank Acc. no.	Reference	Percentage of total blood stages (host)
Sphaerospora angulata	JQ801525-30	Present study	24·6% (*C. a. auratus*), 2·1% (*C. c. carpio*)
Sphaerospora dykovae	JQ801531-33	Present study	19·3% (*C. c. carpio*)
Unknown myxozoan isolate	JQ801548	Present study	14·7% (*C. c. carpio*)
Buddenbrockia sp.	FJ939290	Grabner and El Matbouli (2010)	26·5% (*C. c. carpio*), 27·7% (*C. a. auratus*)
Hoferellus carassi	JQ801547	Present study	4·3% (*C. a. auratus*)
Zschokkella nova	GU471279	Bartošová and Fiala (2010)	6·4% (*C. a. auratus*)
Zschokkella sp.	DQ118776	Zielinsky *et al*. (*unpublished*)	8·5% (*C. a. auratus*)
Chloromyxum cristatum	AY604198	Fiala and Dyková (2004)	8·8% (*C. c. carpio*)
Chloromyxum auratum	AY971521	Hallett *et al*. (2006)	4·3% (*C. a. auratus*)
Myxobolus encephalicus	JQ801549	Present study	17·6% (*C. c. carpio*)
Myxobilatus gasterostei	AJ582063	Holzer *et al*. (2004)	11·8% (*C. c. carpio*), 10·6% (*C. a. auratus*)

kidney tubules of trout and stickleback. The other myxozoans identified from the blood of carp and goldfish are of diverse phylogenetic origins and cluster amongst the members of the malacosporean, the *Sphaerospora* s. str. and the freshwater myxosporean clade (Fig. 3, arrows). Our partial *H. carassii* SSU rDNA sequence was excluded from the analysis as it was found to be unstable with regard to its exact position in the freshwater clade.

DISCUSSION

Molecular versus morphological data indicate the existence of cryptic sphaerosporid species

Myxozoan taxonomy has typically followed a morphological species concept, based on characteristics of the myxosporean spore, formed in the vertebrate host. As extremely reduced parasitic organisms, myxozoans demonstrate a simple spore morphology with an average of 6 cells that limit the number of physical characteristics and measurements that can be taken. Most characters are continuous (e.g. length measurements or ratios), and overlapping character variation can encumber species delimitation. As distinguishing traits are rare, re-identification of previously described taxa is sometimes problematic. Our results show that it is not possible to distinguish species of maturing, angular, sphaerosporid spores in the renal tubules of goldfish, Prussian carp and carp. Hence, we incorporated rDNA sequences as an additional data set, which clearly resolved 2 distinct *Sphaerospora* spp. in these hosts. Our study is a further example of the usefulness of molecular data for determining species limits in taxa which are poor in reliable morphological characters as, for example, malaria parasites (Martinsen *et al*. 2007; Singh and Simon, 2009; Valkiūnas *et al*. 2010).

Within the myxozoans, a similar case of cryptic speciation as in the present study, with extremely similar spore morphotypes representing different genotypes has been reported for gill-infecting *Myxobolus* spp. (Molnár *et al*. 2010) and bile-inhabiting *Chloromyxum* and *Zschokkella* spp. (Bartošová and Fiala, 2011). On the other hand, different spore morphotypes have been related to the same genotype (Hallett *et al*. 2002; George-Nascimento *et al*. 2004; Eszterbauer *et al*. 2006). This stresses the importance of providing a characterization for myxozoan taxa which is based on the synopsis of morphology and molecular data.

Morphological convergence among taxa can be expected as a response to similar selection pressures (Price *et al*. 2000; Van Buskirk, 2002). As most *Sphaerospora* spp. infect the urinary system, predominantly the tubular lumen, it can be expected that adaptation to this environment favours the spherical shape and thus morphological convergence, as in the present case. The supposed ancient spore shape and site of infection of sphaerosporids (Fiala and Bartošová, 2010) have received little modification during the evolution of myxozoan taxa, possibly due to continuing success. Thereby, the success of sphaerosporids may be based on several factors such as, for example, rapid initial proliferation in the blood, which provides a rich source of amino acids without penetration of host cells, or spore formation in an immunoprivileged coelozoic site (Sitja-Bobadilla, 2008) with easy release into the environment via the urine of the host.

The present study provides the first evidence for speciation of *Sphaerospora* s. str. within cyprinids. High species diversity seems to occur in this host family (e.g. Lom and Dyková, 1982; Baska and Molnár, 1988) and recently obtained molecular data confirm this (*unpublished observations*).

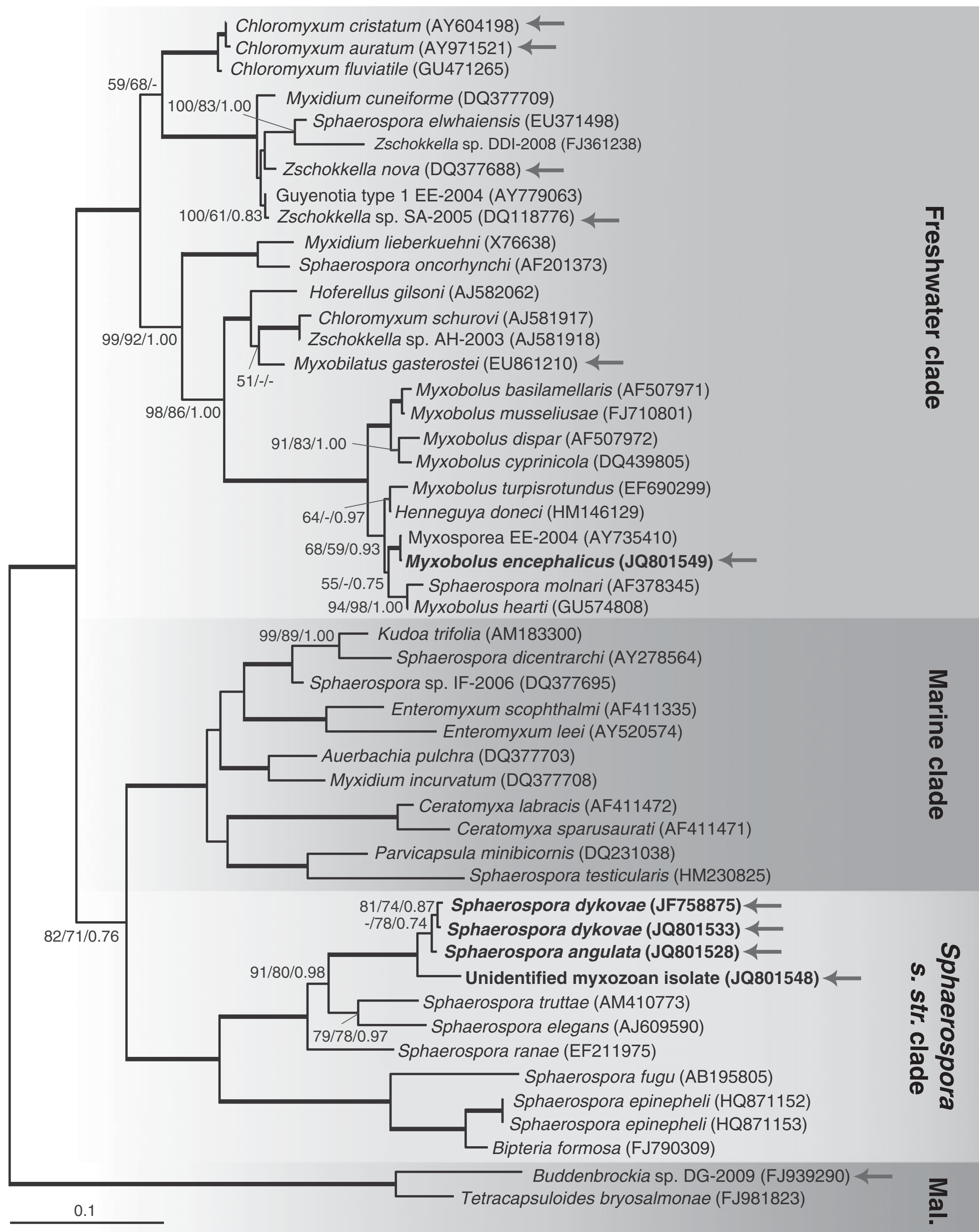

Fig. 3. Maximum likelihood phylogenetic tree of 49 myxozoan SSU rDNA sequences; GenBank Accession numbers in parenthesis; taxa with new sequences are in bold and myxozoans identified in the blood are marked with an arrow. Note that *S. dykovae* and *S. angulata* as well as the unidentified myxozoan detected in the blood cluster within the *Sphaerospora* s. str. clade. Maximum parsimony and maximum likelihood bootstrap values (BS) and Bayesian inference posterior probabilities (BPP) are shown at nodes. Nodes with BS ⩾ 95 and BPP ⩾ 0·98 have bold branches. Weakly supported nodes (BS < 50, BPP < 0·70) are represented with dashes or blanks. Malacosporeans (Mal.) were used as outgroup. Scale bar = 0·1 substitution per site.

'Who's who' in past and present reports?

Our current investigations into *S. angulata* and *S. dykovae* allow us to comment on previous studies of sphaerosporid parasites in goldfish and carp. In the original description of *S. angulata,* Fujita (1912) stated, in the first section, related to *Hoferellus carassii* Akhmerov, 1960 (synonym *Mitraspora cyprini* Fujita, 1912) that he studied common carp and goldfish from small ponds in Japan. However, he gave no further host reference when providing the descriptive details of *S. angulata*. Fujita (1912) described mature spores as angulate with polar capsules that differ in size. We thus consider the kidney sphaerosporid that we encountered in goldfish and Prussian carp to be *S. angulata* based on similar features. We considered the parasite from common carp to be *S. dykovae* which was originally described from this host (Lom and Dyková, 1982), even though we observed some differences in immature spores compared with the original description. We found that immature *S. dykovae* spores had an angular shape and larger polar capsules than mature spores; we used SSU and LSU rDNA sequencing to show both, immature and mature forms represent the same taxon. It remains unclear why the polar capsules in *S. dykovae* exhibit large size variation during spore development while those of *S. angulata* does not.

Previously, another SSU rDNA sequence was provided from the kidney of goldfish (initially named *Sphaerospora* sp., now re-named as Myxosporea sp. 2 EE-2004, AY735411; Eszterbauer and Székely, 2004), which is identical with that of a *Zschokkella* sp. from the bile ducts of goldfish (DQ118776) and that of its alternate life-cycle stage, a guyenotia type actinosporean spore (Eszterbauer *et al*. 2006; AY779063). The same species was identified as a blood stage in the present study. It is likely that, in the original study, *Zschokkella* sp. was preferentially amplified from the blood of goldfish kidney and mistaken for the sequence of *S. angulata*, which had visible myxospores in the kidney (Eszterbauer and Székely, 2004). To avoid similar problems, considering the large number and diversity of myxozoan blood stages in carp and goldfish, we employed *in situ* hybridization with specific probes to confirm the identity of the sequences of *S. dykovae* and *S. angulata*. This method has also been applied by Eszterbauer (2011) to clarify the ambiguities with the SSU rDNA of *S. dykovae* (referred to as *S. renicola*), and it seems to be a reliable tool for confirming the localization and identity of myxozoan species.

Sphaerospora *s. str. host specificity*

Limited data are available on vertebrate host specificity of *Sphaerospora* spp. (Molnár, 1994). However, previous studies on *Sphaerospora truttae* Fischer-Scherl *et al*. 1986 showed that this parasite can develop in brown trout but not in rainbow trout (McGeorge *et al*. 1996). We suggest that most renal *Sphaerospora* spp. have a rather strict host specificity (*unpublished data*), similar to that in several other myxozoan genera, e.g. *Ceratomyxa* (Gunter *et al*. 2009; Alama-Bermejo *et al*. 2011). We demonstrated, by species-specific duplex PCR, that *S. angulata* parasitizes goldfish and Prussian carp, two very closely related host species (Papoušek, 2008) which were long believed to be subspecies (*C. auratus auratus* and *C. auratus gibelio*), whereas *S. dykovae* infects exclusively the common carp. While we only studied a single site where goldfish and carp are cultured together, it is likely that Prussian carp is present also in most of the carp ponds studied (Lusková *et al*. 2010) so that cross-infection could be possible. We demonstrated the presence of *S. angulata* blood stages in 1 carp specimen but no kidney infection was detected in this fish, which suggests that this parasite can enter common carp but is not able to complete its development in the atypical host (see section on non-selective host invasion). Thus, in the case of *S. dykovae* and *S. angulata*, species distinction can probably be based on host record. We suggest that this host specificity is kept in mind when assessing previous reports (Table 3) of kidney sphaerosporids from goldfish, Prussian carp and common carp were addressed under the same name, *S. angulata*, prior to the description of *S. dykovae* (Lom and Dyková, 1982), while from 1982 onwards all reports referred to *S. dykovae*.

S. angulata appears to be a pan-global species, described from Asia in 1912 (Fujita, 1912) and from 3 other continents in the present study. This implies that the same invertebrate host species should be present all over the world as, at least for myxozoans developing in oligochaetes, host specificity is believed to be rather high (Özer *et al*. 2002; Xiao *et al*. 1998; Yokoyama, 1997). However, the alternate hosts of *Sphaerospora* s. str. are yet to be discovered as experimentally determined life-cycle stages (Molnár *et al*. 1999; Özer and Wootten, 2000) were later proven wrong by DNA sequencing of the supposed alternating life-cycle stages (Holzer *et al*. 2004; Eszterbauer *et al*. 2006).

Identity of myxozoan blood stages and common pathways in the fish host

The proliferative stages of myxozoans in the blood (short 'blood stages') were originally described by Csaba (1976) and called C blood protozoans by Molnár (1980*a*) and 'unidentified blood objects' (UBOs) by Lom *et al*. (1983). These stages have been observed consistently in the blood of common carp before renal sphaerosporosis. Proliferative blood stages have been reported from other

Sphaerospora-infected cyprinids (Lom *et al*. 1985; Baska and Molnár, 1988) and were long thought to be characteristic only for the genus *Sphaerospora*. However, Csaba *et al*. (1984) showed that 15–28% of common carp with myxozoan blood stages showed neither signs of SBI nor *S. dykovae* infection in the kidney. Furthermore, inoculation of SPF carp with blood stages did not always lead to *S. dykovae* development in the renal tubules (Molnár 1980*a*; Molnár and Kovács-Gayer, 1986). These data already indicated that not all blood stages were *Sphaerospora* spp. More recently, it has been demonstrated that species from all other major myxozoan phylogenetic lineages use the blood for proliferation or transport, e.g. Kent and Lom (1986), Holzer *et al*. (2006) and Björk and Bartholomew (2010).

In a study of 3100 common carp from Czechoslovakia, 13 myxozoan species were detected in different organs and tissues (Dyková and Lom, 1988). In the light of these findings, blood stages from a variety of species could be expected to occur. We demonstrated by SSU rDNA sequencing that only 19·3% and 24·6% of the blood stages present in goldfish and carp belong to *S. angulata* and *S. dykovae*, respectively, and that the remaining 75·4–80·7% of blood stages were from 9 other myxozoan species.

In some cases, the general myxozoan primers that we used amplified multiple species simultaneously. *Sphaerospora* s. str. species produced distinct high molecular bands relative to other myxozoans (~1500 bp versus ~900 bp) and it is likely that the ~900 bp band may be comprised of more than one myxozoan species. Our prevalence data may have been skewed by preferential PCR amplification of one species over another, as has been shown in studies of mixed infections of malaria parasites in birds (Valkiūnas *et al*. 2006). However, our intention was not to provide absolute prevalences for each species, rather to demonstrate that the blood stages previously ascribed to *S. dykovae* in common carp (Csaba *et al*. 1984; Ter Hoefte *et al*. 1984, Grupcheva *et al*. 1985) may be proliferative stages of other myxozoan species. In 2 carp the number of blood stages was very high on Giemsa-stained smears, and both samples represented stages of *M. encephalicus*. *Myxobolus* spp. are histozoic and despite being very diverse in carp we only detected 1 species in the blood, which suggests that they do not generally use the circulatory system as a proliferation site or as a medium of transport to their target organ. A non-blood route for myxobolids is supported by the study of El-Matbouli *et al*. (1995) on the intrapiscine pathway of *Myxobolus cerebralis* Hofer 1903, which proliferates in the subcutis and migrates along nerve fibres to the brain and the cartilage of the head. Our results indicate that the blood stream is predominantly a pathway for coelozoic species, as previously demonstrated in other hosts (Holzer *et al*. 2003, 2006). The presence of blood stages of histozoic *M. encephalicus* was an exception to this rule. This parasite forms plasmodia in the blood vessels of the brain (Dyková *et al*. 1986; Lom and Dyková, 2006) and we suspect that *M. encephalicus* plasmodia may become dislodged and are transported through the whole body via the blood. The presence of large numbers of *M. encephalicus* blood stages may explain why this species was preferentially amplified from kidney samples of common carp by Eszterbauer and Székely (2004).

It has to be pointed out that most of the fish analysed in the present study were obtained in October/November 2011, and we suspect that the presence and composition of blood stages is likely to vary both throughout the year (e.g. Grupcheva *et al*. 1985; Sedlaczek *et al*. 1990; Holzer *et al*. 2006) and according to cycles of vegetative reproduction of the parasites (Björk and Bartholomew, 2010). The latter was demonstrated also by Molnár and Kovács-Gayer (1986) who observed large numbers of *S. dykovae* blood stages ⩾25 days after infection of receptor fish with swim-bladder isolates from SBI-affected fish, i.e. after spore formation (12 days p.i.) and after clearance of the infection from the kidney (32 days p.i.). This indicates that *S. dykovae* spore production in the kidney is followed by a new cycle of proliferation in the blood.

Proof for non-selective host invasion of myxozoans and multi-species infections

We identified ~11% of myxozoan blood stages in both common carp and goldfish as *M. gasterostei*, a renal, intratubular parasite of the three-spined stickleback *Gasterosteus aculeatus*. To the best of our knowledge, this is the first time that developmental myxozoan stages of a myxozoan infecting a distantly related host, were detected in an atypical host fish. This is a rather surprising result but may be explained by the recent findings of Kallert *et al*. (2011) who identified common fish mucus nucleosides as the main chemical stimulants for actinospore polar filament discharge, required for the initial step of the infection process. Kallert *et al*. (2009 and 2011) and Yokoyama *et al*. (1995) furthermore demonstrated that the subsequent entry of the fish-infective sporoplasms is non-host specific. Körting *et al*. (1989) injected myxozoan blood stages from goldfish into common carp, and found that these stages proliferated in the blood of the new host. While this data prompted Körting *et al*. (1989) to state that the renal sphaerosporids from carp and goldfish were conspecific, he never observed spore formation in the renal tubules of the receptor fish. In the present study, we did not observe *Myxobilatus*-type spores in any of the cyprinids studied, which strongly suggests that the development of this stickleback-specific

myxozoan in the unsuitable hosts (carp and goldfish) is arrested before spore formation can take place. Körting's results (Körting *et al*. 1989) suggest that some physiological triggers and nutritional adequacies are given and that these nutrients can be acquired by the parasites, thus allowing initial proliferation. Further studies are required to understand where and how the development of these incidental parasites is arrested in their unsuitable hosts and whether the alien parasite stages can cause pathological effects as known from other aquatic parasites, e.g. schistosomes causing cercarial dermatitis in humans (see review by Kolářová, 2007).

Most studies of myxozoan host-parasite interactions focus on the effects of a single parasite on a single host, neglecting the host's interactions with other species. Multi-species infections may lead to either competition within the host and thus an increasing virulence over time (Read and Taylor, 2001) or to co-existence with some intermediate virulence levels being established (May and Nowak, 1955). Considering our evidence of many different species in the blood of carp, it would be interesting to re-investigate the myxozoan agent(s) involved in SBI in common carp using molecular methods. SBI is still a problem in central Europe (Eszterbauer and Steinhagen, *personal communication*) although most reports date back to the 1980/90s when the aetiological agents of the disease were first investigated. The superior sensitivity and specificity associated with the use of molecular assays has greatly improved the field of infectious disease diagnostics by providing rapid, accurate results that permit identification of myxozoan species, independent from the stage of development. Using such tools, in the present study, we have characterized new members of *Sphaerospora* s. str. in carp, Prussian carp and goldfish, we shed light on the identity of myxozoan blood stages in these hosts and we provide the diagnostic tools to further analyse the relationship between these species and SBI in common carp.

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