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Sphaerospora sensu stricto: Taxonomy, diversity and evolution of a unique lineage of myxosporeans (Myxozoa)

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ABSTRACT

Myxosporeans (Myxozoa) are eukaryotic parasites, primarily of fish, whose classification is in a state of flux as taxonomists attempt to synthesize the traditional morphology-based system with emerging DNA sequence-based phylogenies. The genus *Sphaerospora* Thélohan, 1892, which includes pathogenic species that cause significant impacts on fisheries and aquaculture, is one of the most polyphyletic taxa and exemplifies the current challenges facing myxozoan taxonomists. The type species, *S. elegans*, clusters within the *Sphaerospora sensu stricto* clade, members of which share similar tissue tropism and long insertions in their variable rRNA gene regions. However, other morphologically similar sphaerosporids lie in different branches of myxozoan phylogenetic trees. Herein, we significantly extend taxonomic sampling of sphaerosporids with SSU + LSU rDNA and EF-2 sequence data for 12 taxa including three representatives of the morphologically similar genus *Polysporoplasma* Sitjà-Bobadilla et Álvarez-Pellitero, 1995. These taxa were sampled from different vertebrate host groups, biogeographic realms and environments. Our phylogenetic analyses and statistical tests of single and concatenated datasets revealed *Sphaerospora s. s.* as a strongly supported monophyletic lineage, that clustered sister to the whole myxosporean clade (freshwater + marine lineages). Generally, *Sphaerospora s. s.* rDNA sequences (up to 3.7 kb) are the longest of all myxozoans and indeed metazoans. The sphaerosporid clade has two lineages, which have specific morphological, biological and sequence traits. Lineage A taxa (marine *Sphaerospora* spp.) have a single binucleate sporoplasm and shorter AT-rich rDNA inserts. Lineage B taxa (freshwater/brackish *Sphaerospora* spp. + marine/brackish *Polysporoplasma* spp.) have 2–12 uninucleate sporoplasms and longer GC-rich rDNA inserts. Lineage B has four subclades that correlate with host group and habitat; all *Polysporoplasma* species, including the type species, cluster together in one of these subclades. We thus suppress the genus *Polysporoplasma* and the family Polysporoplasmidae and emend the generic diagnosis of the genus *Sphaerospora*. The combination of morphological, biological and DNA sequence data applied in this study helped to elucidate an important part of the taxonomic puzzle within the phylum Myxozoa.

1. Introduction

Myxosporeans (Myxozoa) are eukaryotic, obligate endoparasites, closely related to medusozoans (Cnidaria; Jiménez-Guri et al., 2007; Reft and Daly, 2012). They have radiated extensively in both freshwater and marine habitats, and have life cycles that alternate between intermediate vertebrate (mainly fish) and definitive invertebrate (mainly annelid) hosts. Two morphologically distinct waterborne spore types are involved in the life cycle: myxospores released from the vertebrate host then infect the invertebrate host, from which actinospores are released to infect the vertebrate host. Spores develop typically in cavities of host organs (coelozoic species) or within tissues (histozoic species). Myxosporeans are a morphologically and biologically diverse group, and there is persistent taxonomic controversy between the classical spore-morphology-based system and emerging DNA sequence-based phylogenies. The present lack of a single, consistent taxonomic approach has led to numerous paraphyletic and polyphyletic nominal taxa being distributed throughout the myxosporean phylogenetic tree (Fiala, 2006; Bartošová et al., 2009; Fiala and Bartošová, 2010).

The genus *Sphaerospora* Thélohan, 1892 (Variisporina, Sphaerosporidae) is polyphyletic, but clustering of its nominal species

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resembles some general patterns documented for other myxosporeans, especially the grouping of taxa by site of infection (Holzer et al., 2004; Fiala, 2006; Jirků et al., 2007). The genus comprises ~80 nominal species, most of which are coelozoic parasites of the urinary system of fish and amphibians, and some of which cause severe diseases (Lom and Dyková, 2006). In addition, the genus includes eighteen species formerly regarded as *Leptotheca* spp. (Gunter and Adlard, 2010). Phylogenetically, most sphaerosporids group into the *Sphaerospora sensu stricto* clade sensu Jirků et al. (2007; *Sphaerospora s. s.*, alternately, the "sphaerosporid clade", or "true sphaerosporids"), which includes the type species *Sphaerospora elegans*. Several other nominal *Sphaerospora* spp., "*Sphaerospora sensu lato*" sensu Jirků et al. (2007), cluster in different clades (Bartošová et al., 2011). *Sphaerospora s. s.* is the first case in the phylum Myxozoa, in which a molecular trait, the presence of extensive nucleotide insertions in the V4 region of the SSU rRNA gene, complements morphological taxonomic diagnosis (Jirků et al., 2007).

Presently, the genus *Polysporoplasma* Sitjà-Bobadilla et Álvarez-Pellitero, 1995 (Variisporina, Sphaerosporidae) comprises two nominal species, both of which infect the urinary system of their fish hosts. The type species, *Polysporoplasma sparis*, causes glomerular disease of gilthead sea bream, *Sparus aurata* (Palenzuela et al., 1999). *Polysporoplasma* differs from *Sphaerospora* only in the number of uninucleate sporoplasms per myxospore: two in *Sphaerospora* spp. vs. four to twelve in *Polysporoplasma* spp. (generic diagnoses sensu Sitjà-Bobadilla and Álvarez-Pellitero, 1995 and Lom and Dyková, 2006, respectively). Several nominal *Sphaerospora* species also possess one binucleate sporoplasm (Sitjà-Bobadilla and Álvarez-Pellitero, 1994). The two genera may be closely related, possibly synonymous, but no sequence data are available for either *Polysporoplasma* species, which prevents genetic and phylogenetic comparison.

Generally, myxozoans cluster into four main clades: the malacosporeans, the freshwater myxosporean lineage, the marine myxosporean lineage and *Sphaerospora s. s.* (Fiala, 2006). While the relationships of *Sphaerospora s. s.* with marine or freshwater myxosporean lineages were revealed in several analyses (Holzer et al., 2004, 2013; Bartošová et al., 2009; Karlsbakk and Køie, 2009), the basal position of *Sphaerospora s. s.* to all myxosporeans has been shown repeatedly in SSU rDNA and concatenated SSU + LSU rDNA-based analyses (Fiala, 2006; Jirků et al., 2007; Holzer et al., 2007, 2010; Bartošová et al., 2009, 2011; Evans et al., 2010; Fiala and Bartošová, 2010; U-taynapun et al., 2012). Little is known about evolutionary trends within the *Sphaerospora s. s.*, as few representatives have been sequenced (eight SSU rDNA + one partial LSU rDNA sequence are available in GenBank).

Our aims in this study were: (i) to sample additional *Sphaerospora s. s.* taxa; (ii) to determine the phylogenetic position of *Polysporoplasma* spp., including the type species *P. sparis*; (iii) to determine the phylogenetic position of the *Sphaerospora s. s.* within the Myxozoa using both rRNA and protein-coding genes; (iv) to determine within-clade relationships for *Sphaerospora s. s.* members; (v) to compare the location, lengths and base composition of expansion segments in the rRNA gene sequences of *Sphaerospora s. s.* and other myxozoan lineages; and (vi) to resolve the taxonomy using the evidence from aims i to v.

2. Materials and methods

2.1. Molecular markers and taxonomic sampling

Three different molecular markers were chosen: protein-coding gene EF-2, and non-protein-coding SSU rDNA (SSU) and LSU rDNA (LSU). These loci exhibit intra-specific variation and have orthologous sequences available in GenBank. Our goal was to sequence these loci from eight known *Sphaerospora* and *Polysporoplasma* species and four taxa that await formal description (Fig. 1 and Table 1). The samples originated from different vertebrate host groups (fish and amphibians), from various biogeographic regions (Afrotropical, Nearctic, Palearctic), and different host environments (freshwater/brackish/marine).

For comparative purposes, we sequenced the EF-2 gene from non-*Sphaerospora s. s.* myxosporeans *Chloromyxum cristatum*, *C. fluviatile*, *Myxidium lieberkuehni*, *Myxobolus longisporus*, *Sphaeromyxa balbianii*, and *Sphaerospora dicentrarchi*, from our archives (Laboratory of Fish Protistology, BC ASCR). SSU and LSU data from these species are available in GenBank (Fiala, 2006; Bartošová et al., 2009, 2011). We sequenced the EF-2 gene of *Kudoa trifolia* from *Liza ramada* (Risso, 1826) purchased from a supermarket in Castenaso (Bologna, Italy).

2.2. Host species determination

To identify the morphologically similar mugilid hosts of the two *Polysporoplasma* spp., we sequenced taxonomically informative genes cytochrome c oxidase I (COI) and cytochrome b (cytb), using existing primers (Folmer et al., 1994; Boore and Brown, 2000). Hosts of other myxosporean species were determined by morphology.

2.3. Material preservation, DNA extraction, amplification and sequencing

Host kidneys were preserved in 400 µl TNES urea buffer (10 mM Tris–HCl with pH = 8, 125 mM NaCl, 10 mM ethylenediamine tetraacetic acid, 0.5% sodium dodecyl sulfate, 4 M urea). Total DNA was extracted using a standard phenol–chloroform protocol, after digestion with proteinase K (50 µg/ml) overnight at 55 °C. DNA was re-suspended in 50–100 µl of RNAse/DNAse-free water and left to dissolve overnight at ~4 °C. We found that this extraction method gave a higher yield and quality of genomic DNA than commercial kits.

Cycling conditions and PCR primers are given in Supplementary Tables 1 and 2. Single round or nested PCRs were used as required. The SSU and LSU was amplified in two or three overlapping fragments, and then assembled in DNA Star SeqMan II v5.05 (DNASTAR Inc., Madison, Wisconsin, USA). When general myxosporean primers did not amplify the target genes, we designed species-specific primers. We used initially Taq-Purple DNA polymerase (Top-Bio, Czech Republic), then TITANIUM Taq DNA polymerase (Takara Bio Europe/Clontech, France) for problematic samples (26% of all PCR reactions). We also used LA DNA polymerase (Top-Bio, Czech Republic) to amplify long fragments of rDNA. EF-2 sequences were amplified in nested PCR with Taq-Purple or TITANIUM Taq DNA polymerase (Supplementary Tables 1 and 2). PCR products were purified using a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., USA) and preferentially sequenced directly.

Problematic amplicons with low DNA concentration or mixed composition were cloned into the pDrive Vector with a PCR Cloning Kit (Qiagen, Germany) and transformed into XL-1 *E. coli* strain competent cells. Positive colonies were PCR screened with M13 forward and reverse primers. Plasmid DNA was isolated using a High Pure Plasmid Isolation Kit (Roche Applied Science, Germany) and three colonies were sequenced in the sequencing facility of the Faculty of Science and BC ASCR.

The possibility of confusing host and parasite gene sequences was minimized by using myxozoan-specific primers and BLAST searching all sequences against GenBank.