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RELATIONSHIPS AMONG MEMBERS OF THE GENUS *MYXOBOLUS* (MYXOZOA: BILVALVIDAE) BASED ON SMALL SUBUNIT RIBOSOMAL DNA SEQUENCES

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ABSTRACT: Sequences representing ~1,700 base pairs of the 18S rRNA gene from 10 different species in the genus *Myxobolus* were found to group them into 3 clusters that showed little correlation with spore morphology and size or host specificity, criteria currently used for both higher and lower taxonomic placements in the Myxozoa. Of the phenotypic criteria examined, tissue tropism was most correlated with the rRNA groupings observed. Spores of similar size and shape (*Myxobolus cerebralis* vs. *Myxobolus squamalis*) were distantly related in some instances, whereas spores with divergent morphology and size were sometimes found to be closely related (*M. cerebralis* and *Myxobolus insidiosus*). These initial investigations into the phylogenetic relationships of putative members of the genus *Myxobolus* clearly indicate the potential limitations of groupings based on size and morphological properties of the spores and host species infected. We propose that 18S rRNA gene sequences, combined with information on tissue tropism, host species infected, and developmental cycles in the fish and alternate host (when and if known) be given greater consideration in taxonomic placements of myxosporeans.

The phylum Myxozoa represents an assemblage of more than 1,300 species known principally as parasites of fish and more rarely amphibians and reptiles (Grassé, 1970; Lom and Dyková, 1992; Moser and Kent, 1994). Myxozoans are identified by the final developmental stage found in the fish host, the spore. The spore is derived from the terminal differentiation of 3 cell lineages that form the polar capsules containing coiled extrusive filaments, 1 or more sporoplasm cells, and the surrounding protective shells or valves. The arrangement, number, size, and shape of these 3 elements are used as the principal criteria for higher taxonomy of the Myxozoa (Lom and Arthur, 1989; Lom et al., 1997). Host species infected and characteristics of the developmental cycles have also been used to assist at differentiation at the species level (Lom, 1987; Lom and Arthur, 1989; Lom and Dyková, 1992). These criteria, however, are subject to limitations as recently illustrated by Bahri and Marques (1996), who showed that species classified as *Myxobolus exiguus* based on spore morphological properties may represent up to 5 different species when ultrastructural and tissue tropism characteristics are included.

Over 450 species have been described in the genus *Myxobolus* (Lom and Dyková, 1992). Species have been assigned principally on the size and shape of the valves and polar capsules and filaments (Lom, 1987; Lom and Dyková, 1992; Moser and Kent, 1994). Additional criteria for species assignments include differences in tissue tropism, developmental cycles, and host species infected. Certain myxosporeans, however, are known to infect more than 1 host species (Mitchell, 1977; O'Grodnick, 1979). Furthermore, differences in size between spores even from the same cyst have been observed (Mitchell, 1989) and effects of environmental factors and host species on development and final spore morphology are unknown. The initial discovery of the stages of myxosporeans occurring outside of the fish host in aquatic oligochaetes (Wolf and Markiw, 1984), and now in polychaetes (Bartholomew et al., 1997), provides additional criteria of potential use in taxonomic assignments. Unfortunately, few myxosporeans have been coupled to their corresponding actinosporean stages (Kent et al., 1996), and if all *Myxobolus* spp. possess these stages, many are yet undiscovered. A useful method to match corresponding actinosporean and myxosporean stages for myxozoans is the polymerase chain reaction (PCR) amplification and sequencing of rDNA, as we have demonstrated with *Myxobolus cerebralis* (Andree et al., 1997).

Myxozoans currently pose an enigma to taxonomists, once being classified as parasitic protozoa (Grassé, 1970) but now placed with the metazoa either with the bilateria or the cnidarians (Smothers et al., 1994; Siddall et al., 1995; Schlegel et al., 1996). Functional relationships between the polar capsule/filaments and the nematocyst argue more for an association with cnidarians (Siddall et al., 1995). The subject of the current study was to examine the intrageneric relationships of 10 members of the genus *Myxobolus*, the largest grouping of species found in the phylum Myxozoa. We chose 10 species representing origins in 2 continents, from 8 host species, with various spore morphologies and tissue tropisms for a comparison of their relatedness using 18S rRNA gene sequences. Five species from 5 different genera within the myxosporea were used as outgroups for this study.

MATERIALS AND METHODS

Sources of spores

Spores of *M. cerebralis* (Höfer, 1903) from rainbow trout (*Oncorhynchus mykiss*) were obtained from Maria Markiw from experimental infections generated in the laboratory at the Fish Health Research Center, National Biological Service, Leetown, West Virginia.

The *Myxobolus squamalis* (Iversen, 1954) spores were collected from the scale pockets of adult chinook salmon (*Oncorhynchus tshawytscha*) returning to the Nimbus Fish Hatchery, Rancho Cordova, California (California Department of Fish and Game, Rancho Cordova, California). Cysts were ruptured and the contents collected in a 1.5-ml microfuge tube. The spores (3.0×10^7) were then washed by multiple rinses in tap water and collected using low-speed centrifugation (500 *g*).

Myxobolus arcticus (Pugachev and Khokhlov, 1979) spores were recovered from the hind-brain tissue of sockeye salmon (*Oncorhynchus nerka*) that were heavily infected. This tissue was dissected from infected fish and homogenized, then placed on a stepped Percoll gradient (100%, 75%, 50%, and 25% Percoll) and centrifuged at 6,000 *g* for 10 min. The purified spores that formed a band were removed, rinsed once in tap water, and collected by low-speed centrifugation (500 *g*).

Myxobolus insidiosus (Wyatt and Pratt, 1963) spores from juvenile chinook salmon (*O. tshawytscha*) were acquired from Aumsville Ponds, near Salem, Oregon. These spores (2.0×10^7) were dissected from

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infected skeletal muscle and cleaned by the same procedure described for *M. arcticus* but using sucrose rather than Percoll gradients.

Myxobolus neurobius (Schuberg and Schroeder, 1905) spores were purified from neural tissue of infected rainbow trout (*O. mykiss*) from the upper Owens River near Crestview, California. The infected tissue was processed and the spores purified as described for *M. arcticus*.

Several *Myxobolus* spp. were collected from infected fish in the waters of Lake Balaton, Hungary. The spores of each *Myxobolus* sp. were collected from cysts dissected from the tissues of various fish species as follows. *Myxobolus djragini* (Akhmerov, 1954) spores were collected from the head of silver carp (*Hypophthalmicthys molitrix*); *Myxobolus portucalensis*, from the fins of the European eel (*Anguilla anguilla*); *Myxobolus sandrae* (Molnár and Székely, 1995), from the skeletal muscle of pike perch (*Stizostedion lucioperca*); *Myxobolus ellipsoides*, from the gills of roach (*Rutilus rutilus*); *Myxobolus bramae* (Molnár and Székely, 1995), from the gills of bream (*Abramis brama*). These spores were purified, washed, and collected as described above for *M. arcticus*.

DNA extractions

The DNA was extracted from myxosporean spores by suspending them in 500 µl of lysis buffer (100 mM NaCl, 10 mM Tris, pH 7.6, 10 mM EDTA, 0.2% sodium dodecyl sulfate, 0.2 mg/ml proteinase K) and incubating at 55 C for 4 hr. Phenol and chloroform were then added to the digested sample and mixed on a rocker platform for 10 min. The upper phase was removed following centrifugation for 10 min at 5,220 *g* in a microcentrifuge. This extraction was repeated a second time followed by a single treatment with isoamyl alcohol/chloroform (1:24). Sodium acetate (3 M, pH 6.9) was added with 2 volumes of 100% cold ethanol to precipitate the DNA. The DNA was collected by centrifugation for 10 min at 16,000 *g* in a microcentrifuge. The pellet was washed once in 70% ethanol and air-dried for 15 min prior to resuspension in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). The DNA content was determined by spectrophotometry. The DNA for sequencing was obtained from individual bacterial clones.

Oligonucleotide synthesis

Primers for the specific amplification of myxozoan sequences were selected and tested for possible secondary structure, self-complementarity, and absence of cross reactions to host sequences using "Amplify" software (University of Wisconsin Genetics, Madison, Wisconsin).

Primers for DNA sequencing and PCR were prepared using an ABI (Applied Biosystems Inc., Branchburg, New Jersey) DNA synthesizer, model 394. Oligonucleotides were then desalted on a NAP 5 desalting column (Pharmacia Biotech, Inc., Alameda, California) and diluted in water to working concentrations for PCR and DNA sequencing, 20 pmol/µl and 10 ng/µl, respectively.

Primer sequences: 18e, 5′-CTGGTTGATTCTGCCAGT-3′; 18g′, 5′-CGGTACTAGCGACGGGCGGTGTG-3′; MX5, 5′-CTGCGGACGGC TCAGTAAATCAGT-3′; and MX3, 5′-CCAGGACATCTTAGGGCAT-CACAGA-3′.

PCR amplification of rDNA

The myxosporean 18S rDNA of *M. cerebralis*, *M. insidiosus*, and *M. squamalis* were amplified using universal primers 18g′ and 18e as described by Hillis and Dixon (1991). These yielded a PCR product of ~1,940 base pairs (bp). Consensus primers were chosen from the alignment of these 3 sequences. The remaining myxosporean 18S rDNA sequences were amplified using these primers (designated as MX5 and MX3) resulting in a PCR product of ~1,715 bp.

The 18S rDNA fragments were amplified in standard 50-µl reactions containing 10 mM Tris-HCl, pH 8.3 (at 25 C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% w/v gelatin, 400 µM dNTPs, 5 µM tetramethyl ammonium chloride, 40 pmol of each primer, and 2 U Taq polymerase. The thermal cycler used was a model PTC-100 (manufactured by MJ Research, Watertown, Massachusetts). Forty cycles of 1 min at 95 C, followed by 2 min at 45 C, followed by 4.5 min at 72 C were used in the amplification. The amplification cycles were preceded by a denaturation step where samples were held at 95 C for 5 min. The thermal cycler program finished with an extended elongation step where samples were held at 72 C for 10 min.

Cloning of PCR products

The myxosporean 18S rDNA genes were cloned into pNoTA using the TA Cloning Kit (Invitrogen, Carlsbad, California) or into pCRII using the Prime PCR Cloner Kit (5 Prime > 3 Prime, Boulder, Colorado). Inserts were confirmed by screening transformant colonies by PCR using the same primers (MX5 and MX3) as those that were used in the original amplification. Transformant colonies were picked using sterile toothpicks and inoculated into 10 µl of sterile molecular biology grade water (Sigma Chemical Co., St. Louis, Missouri). Three microliters of the inoculum was used in a PCR assay as described above. The individual positive clones were restreaked to purify them from any co-transformants. Cultures of these clones were grown in LB broth containing 300 µg/ml of ampicillin and plasmid DNA obtained as described by Sambrook et al. (1989).

Sequencing of rDNA

Plasmid DNA from transformant colonies was prepared according to Sambrook et al. (1989). Sequencing primers were made to hybridize to the more conserved regions of the 18S rRNA gene and sequence was obtained by gene walking. Sequencing proceeded in both directions from either end of the genes. The 18S rDNA sequence was derived by oligonucleotide-directed dideoxynucleotide chain-termination sequencing using the TAQuence sequencing kit (United States Biochemical Corporation, Cleveland, Ohio). Sequencing reactions were electrophoresed on 0.4-mm-thick 6% polyacrylimide gels in a Sequi-Gen II Sequencing System (Bio-Rad Inc., Hercules, California) or determined on an ABI 377 (Applied Biosystems Inc., Branchburg, New Jersey) automated sequencer using fluorescently labeled dye terminators. The determined sequences of *M. arcticus*, *M. bramae*, *M. djragini*, *M. ellipsoides*, *M. neurobius*, *M. portucalensis* and *M. sandrae* have been submitted to GenBank under the following respective accession numbers: AF085176, AF085177, AF085179, AF085178, AF085180, AF085182, and AF085181. The 18S rRNA sequences for the outgroups *Henneguya salminicola* (Ward, 1919), *Myxidium* sp., *Kudoa thyrsites*, *Ceratomyxa shasta*, and PKX were retrieved from GenBank using the following respective accession numbers: AF031411, MSU13829, AF031413, AF001579, and U70623.

Phylogenetic analysis

Mac DNAsis version 3.5 (Hitachi Software Engineering America, Ltd., San Bruno, California) was used to align sequences manually. The *M. cerebralis*, *M. squamalis*, and *M. insidiosus* sequences were truncated to compare only homologous sequences, as the other myxosporean sequences were obtained using a more myxosporean-specific set of internal 18S primers (MX5 and MX3). The aligned sequences were subjected to distance analysis as well as maximum parsimony analysis using Molecular Evolutionary Genetic Analysis version 1.01 (MEGA, Pennsylvania State University, University Park, Pennsylvania) developed by Kumar et al. (1993). The Jukes–Cantor methodology (1969) was performed with pairwise elimination of deletion/insertion sites. Phylogenetic relationships were inferred from this distance data using the neighbor-joining method (Saitou and Nei, 1987), with estimation of statistical confidence in the branch points of the tree being obtained using bootstrap resampling (500 replications). Using the maximum parsimony analysis we obtained a consensus tree from 15 possible trees with a length of 1,987, which strongly resembled the distance tree in its topology (data not shown). In addition, a pairwise analysis of the 1,700 bp of sequence from each clone was done using PAUP, version 1.0 (Phylogenetic Analysis Using Parsimony) to obtain values for percent sequence similarity.

RESULTS

The original primers 18e and 18g′ amplified the 18S rDNA of *M. cerebralis* (Höfer, 1903), *M. squamalis* (Iversen, 1954), and *M. insidiosus* (Wyatt and Pratt, 1963) but not that of several *Myxobolus* spp. originating from fish from Hungary. However, the consensus primers MX5 and MX3 successfully amplified the 18S rRNA gene from most of the remaining *Myxobolus* spp.

The distance analysis showed the *Myxobolus* spp. represented

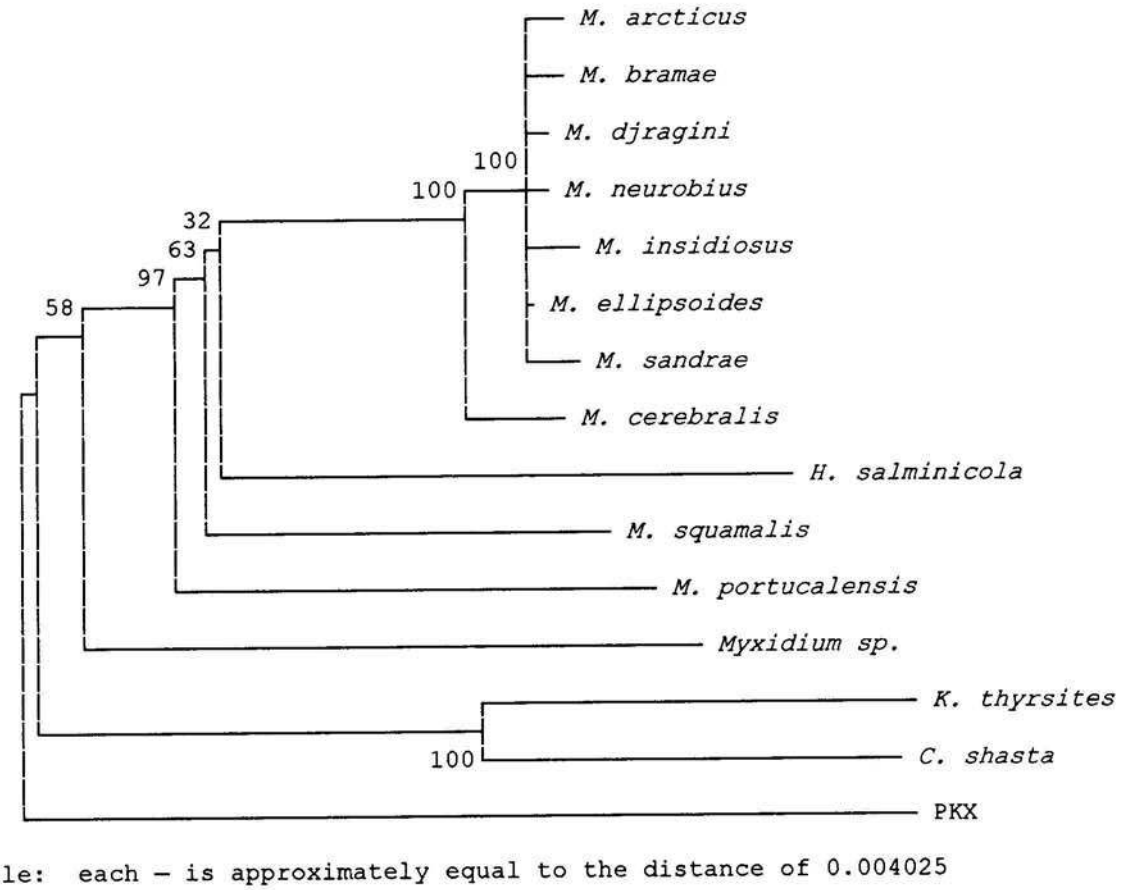

FIGURE 1. Phylogram generated using neighbor-joining analysis showing relationships among *Myxobolus* spp. based on 18S rDNA. Comparison was done using approximately 1,760 bp of sequence amplified from the 18S rDNA of 1 marine and 14 freshwater myxosporean species. Outgroups include *Henneguya salminicola* (GenBank accession no. AF031411), *Kudoa thyrsites* (GenBank accession no. AF031412), *Ceratomyxa shasta* (Genbank accession no. AF001579), *Myxidium* sp. (Genbank accession no. U13829), and PKX (GenBank accession no. U70623). Numbers at nodes indicate bootstrap confidence levels.

3 overlapping clades (Fig. 1). The first clade is composed of *M. arcticus, M. bramae, M. djragini, M. neurobius, M. insidiosus, M. ellipsoides,* and *M. sandrae.* The second clade is composed of *M. cerebralis* and the above-mentioned species. The third clade is composed of all of the above-mentioned species with *M. portucalensis, M. squamalis,* and *H. salminicola* as deeply rooted outliers. Bootstrap confidence levels for the branch nodes of *M. squamalis* and *H. salminicola* are weak, but they branch close together as part of a clade. Similar tree topology was found when the data were subjected to maximum parsimony analysis giving further support to these clades. Interestingly, the outgroups *C. shasta* and *K. thyrsites* branched together.

A phenetic comparison of sequence similarity also shows a similar grouping into 3 clusters (Table I). Using *M. cerebralis* for comparison, the 2 other groups have sequence similarities averaging 91.6% and 75.4%, respectively. The outgroup *H. salminicola* used in this study, unlike all the *Myxobolus* spp., has caudal appendages typical of this genus and had a slightly higher degree of sequence similarity to *M. cerebralis* (74.7%) than did *M. portucalensis* (72.8%). We found a marginal correlation with tissue tropism when standard taxonomic criteria employed in classification of this phylum was compared to the clades of the phylogram we obtained from the sequence data (Table II).

DISCUSSION

The alpha taxonomy of the myxosporeans has been enigmatic from the earliest assignments to the Kingdom Protista (Müller, 1838). Criteria currently used for classification among myxosporeans include spore morphology, characteristics of developmental stages, and host and tissue specificity. In the past, spore morphology was often used as a sole criterion for higher taxonomy (Lom and Arthur, 1989; Lom and Dyková, 1992) for separation of the phylum Myxozoa into classes, orders, suborders, and genera (Grassé, 1970). Using the older taxonomic scheme, *Myxobolus* spp. represent the largest of the 3 genera (*Henneguya* and *Thelohanellus* being the other 2) within the class Myxosporea, order Bivalvulida, suborder Platysporina (Grassé, 1970). Despite improvements in new species descriptions, a more precise accounting of previously described species is needed.

One approach is the use of 18S rRNA gene sequences. When beginning such a study, only the best-characterized *Myxobolus* species and spores collected only from the type hosts and type

TABLE I. A pairwise comparison of the percent sequence similarity for 1,700 base pairs of 18S rDNA sequences from 10 myxosporeans isolated from the genus *Myxobolus* and the outgroup, *Henneguya salmonicola.*

	M. cereb	*M. neur*	*M. ellips*	*M. djrag*	*M. arct*	*M. insid*	*M. bram*	*M. sand*	*M. squam*	*M. port*	*H. salm*
M. cerebralis	100	92.1	92	91.7	91.6	91.5	91.3	90.7	78	72.8	74.7
M. neurobius		100	97.7	97.2	97.1	96.4	96.9	95.8	77.6	72.9	74.9
M. ellipsoides			100	97.4	97.2	96.4	96.8	95.8	77.7	73	74.6
M. djragini				100	96.7	96.1	96.7	95.5	77.2	72.9	74.4
M. arcticus					100	95.9	96.5	95.2	77.9	72.8	73.9
M. insidiosus						100	95.7	95	76.8	72.4	74.6
M. bramae							100	95.6	76.8	72.4	74.1
M. sandrae								100	77	72.2	73.8
M. squamalis									100	71.9	72.6
M. portucalensis										100	72.9
H. salminicola											100

TABLE II. A comparison of morphology, host species, and tissue tropism of the 10 species in the genus *Myxobolus* used in the 18S rDNA phylogenetic study.

Myxosporean species	Actinospore type	Host specificity	Host species	Organ specificity	Tissue specificity	Spore morphology*
M. cerebralis	Triactinomyxon (Markiw and Wolf, 1984)	Salmonids†	*Salmo* spp., *Oncorhynchus* spp., *Salvelinus* spp.	Head region	Cartilaginous and nervous tissue	●
M. squamalis	Not known	Salmonids†	*Salmo* spp., *Oncorhynchus* spp., *Salvelinus* spp.	Epidermis	Connective tissue	●
M. arcticus	Triactinomyxon (Kent et al., 1993)	Salmonids†	*Oncorhynchus* spp., *Salvelinus* spp.	Brain	Nervous tissue	●
M. insidiosus	Not known	Salmonids†	*Salmo* spp., *Oncorhynchus* spp., *Salvelinus* spp.	Muscle	Connective tissue	●
M. neurobius	Not known	Salmonids†	*Oncorhynchus* spp., *Salvelinus* spp., *Thymallus* spp.	Brain	Nervous tissue	●
M. drjagini	Triactinomyxon (El-Mansy and Molnár, 1997)	*Hypophthalmichthys molitrix*‡	*Hypophthalmichthys molitrix*	Head	Nervous tissue	■
M. portucalensis	Triactinomyxon (El-Mansy et al., 1998)	Anguillids†	*Anguilla anguilla*, possibly other *Anguilla* spp.	Epidermis of fins	Connective tissue	●
M. sandrae	Not known	*Stizostedion* genus§	*Stizostedion lucioper-cae, S. volgense*	Muscle	Connective tissue	●
M. ellipsoides	Not known	Cyprinids‖¶	*Tinca tinca*¶ and 56 other cyprinid spp.	Gills	Not known	◆
M. bramae	Not known	Cyprinids‖¶	*Abramis brama*¶ and 58 other cyprinid spp.	Gills	Endothelium	●
Henneguya salminicola	Not known	Salmonids†	Salmonidae and some coregonids	Muscle	Connective tissue	▲

* Key to morphology: ●, round to ovoid with 2 valves and 2 polar capsules; ■, round to ovoid with 2 valves and 2 polar capsules of unequal size; ◆, ovoid with 2 valves and 2 polar capsules; ▲, ovoid having 2 long caudal appendages with 2 valves and 2 polar capsules.
† Loose host specificity.
‡ Very strong host specificity.
§ Strong host specificity.
‖ Weak host specificity.
¶ Described as weak host specificity but possibly due to wrong species identification.

locations should be used. Realizing these requirements, we selected for our study several well known species, including *M. cerebralis, M. squamalis, M. drjagini, M. portucalensis, M. sandrae,* and *M. bramae* collected from the type hosts. Also, *M. arcticus, M. insidiosus,* and *M. neurobius* were obtained from closely related fish species from the type locations. Only *M. ellipsoides,* a parasite of tench (*Tinca tinca*), was derived from another host, the roach (*R. rutilus*), that belongs to a second genus of cyprinid fishes.

Sequencing ribosomal RNA genes has only recently been applied to the taxonomy of the myxosporeans (Kent et al., 1994; Smothers et al., 1994; Siddall et al., 1995; Schlegel et al., 1996; Andree et al., 1997; Hervio et al., 1997), including identification of different life stages as they occur alternately in fish and annelid hosts (Andree et al., 1997; Bartholomew et al., 1997). Comparisons of the 18S rRNA gene sequences have also been applied to the taxonomy of the relationship between myxosporeans and other multicellular organisms (Smothers et al., 1994; Siddall et al., 1995; Schlegel et al., 1996). Recent molecular phylogeny places them more closely with the metazoa (Schlegel et al., 1996) but with some disagreement (Cavalier-Smith, 1993).

The use of 18S rRNA gene sequences as a molecular "chronometer" is discussed elsewhere (Hillis and Dixon, 1991). Kent et al. (1996) mentions a "fast clock" mode of evolution in regard to the rDNA of myxosporeans. Whereas this may explain a high degree of variability among closely related species, it does not explain why, in this work, *M. squamalis* and *M. portucalensis* branch basal to *H. salminicola,* a member of a different genus with very different spore morphology (Fig. 1), unless they are all 3, in fact, closely related, and spore morphology is simply a poor indicator of relatedness. The *Myxobolus* spp. examined do form 3 overlapping clades that can be viewed as 1 single clade with a high degree of diversity within it. However, the inclusion within this single clade of an organism from another genus and the high degree of genetic diversity suggests that the genus *Myxobolus* is not monophyletic. Additionally, there is *K. thyrsites,* a marine member of the myxosporean order Multivalvulida branching together with *C. shasta,* a freshwater member of the myxosporean order Bivalvulida, which suggests that the higher order taxonomy of the myxosporea is in disarray as well.

In a study of more than 700 species from 30 myxosporean genera, Moser (1977) found spore morphology to be more constant among isolates of the same myxosporean species of disparate geographic origin, whereas species from different tissues and different hosts exhibited greater diversity of spore morphology. He concluded that spore size and shape is determined by selective forces imposed by host behavior and the particular environment within the respective host tissues. Hervio et al. (1997), in their analysis of 4 species of *Kudoa* using 18S rDNA, found organisms grouped according to geographic location rather than spore morphology.

In regard to tissue specificity of myxosporeans, relatively little is known. Molnár (1991) reports the degree of specificity of tissue tropism among myxosporeans can be variable and detailed studies of tissue tropism and host specificity have been neglected. For some *Myxobolus* spp., e.g., *Myxobolus muelleri, M. bramae,* and *Myxobolus musculi,* Donetz and Shulman (Donetz and Shulman, 1984) have listed more than 40 hosts and several locations in the fish body where the parasites are found. Many descriptive records for myxosporeans have been assessed by describing spores as resembling only morphologically those of the type specimens with little mention of tissue specificity. Unfortunately, this has led to species assignments that were inaccurate, confusing, or duplicative (Bahri and Marques, 1996).

Of the phenotypic properties evaluated for the *Myxobolus* spp. tissue tropism correlated best with groupings in the 3 overlapping clades based on rDNA sequences (Table II). *Myxobolus squamalis* and *M. portucalensis* are primarily parasites of the skin, whereas *M. ellipsoides, M. neurobius, M. arcticus, M. djragini, M. bramae, M. insidiosus,* and *M. sandrae* are parasites that seek deeper soft tissues in which to replicate and sporulate. *Myxobolus cerebralis* is a parasite of cartilage primarily, apparently using the central nervous tissue as a pathway to invade its particular niche (El-Matbouli et al., 1995, 1998). Among all the species examined, *M. cerebralis* is the only one with an inherent ability for cartilage necrosis (Lom and Dyková, 1992). In a pairwise comparison of simple sequence homology, the deeper soft tissue species, *M. ellipsoides, M. neurobius, M. arcticus, M. djragini, M. bramae, M. insidiosus,* and *M. sandrae* represented a homogeneous group with an average of 97.3% similarity in their 18S rDNA sequences, greater than that of the other myxobolids examined (Table I). In contrast, *M. squamalis* and *M. portucalensis* from the skin were approximately 78.0% and 73.0% similar to the other myxobolids.

Lom and Arthur (1989) and the Second International Symposium on Fish Parasitology stressed the need to characterize new species according to host species, morphology, and tissue tropism of the vegetative stages as well as spore morphology (Lom et al., 1997). With the same purpose, Landsberg and Lom (1991) listed the existing species according to type hosts. Additionally, the use of developmental stages, the actinosporean stage in particular, for species descriptions has been proposed (Yokoyama et al., 1995).

Prior to Wolf and Markiw (1984), demonstrating that actinosporeans were alternate developmental stages of myxosporeans, actinosporeans were believed to comprise a second class (Actinosporea) in the phylum Myxozoa that were parasitic for oligochaetes. The observations by Wolf and Markiw (1984) have led to taxonomic revisions of the phylum (Corliss, 1985; Kent et al., 1994). Clearly, more actinosporean stages will be associated with their myxosporean counterparts, which, in our view, will be aided by PCR amplification and sequencing of rDNA (Andree et al., 1997; Bartholomew et al., 1997). Although few myxosporean–actinosporean associations are now known, it is apparent that among those in the genus *Myxobolus,* there are quite different actinosporeans both in size and morphology. Using past taxonomic schemes, they would be placed in different genera in the class Actinosporea (Kent et al., 1993; Yokoyama et al., 1993, 1995; El-Mansy et al., 1998). As we have shown with the rRNA gene sequencing of the myxosporean stages, morphological properties may not be good indicators of genetic relationships, a feature that extends perhaps to the actinosporean stages as well. Therefore, the proposal that the proper identification of the actinosporean stage will clarify the relationship among members of this genus (Yokoyama et al., 1995) may not be of much assistance for taxonomic purposes.

Our examination of the relationships among 10 species in the

genus *Myxobolus* by rDNA comparisons grouped them into 3 clades. Of the criteria examined, membership in a clade did not correlate with the phenotypic property of spore morphology or host species, the criteria most often employed for assigning species (Lom, 1987; Moser and Kent, 1994; Lom et al., 1997). Though not a strict criterion, tissue tropism showed a more close association with the groupings based on sequence data. These initial investigations suggest that spore morphology is influenced by factors that render structural features of myxozoans poor indicators of phylogenetic relationships at least among the *Myxobolus* spp. we examined. Tree topology, genetic differentiation, and tissue tropism argue for a regrouping of these organisms into separate genera. Although additional members of the genus must be examined and then compared to many other outgroups from the phylum, initial evidence points toward the need to divide the genus.

The growing body of knowledge of myxosporean parasites surely indicates that major revisions in the taxonomy of this phylum are necessary. As new data emerge, the lack of a consensus regarding the taxonomy of this phylum seems clear and new questions emerge. Do myxosporeans have their origins in a freshwater environment or a marine habitat? Are freshwater and marine *Myxobolus* spp. truly members of the same genus? Do all myxosporeans have an actinosporean counterpart (Diamant, 1997)? Is more than 1 species of aquatic invertebrate host to a single myxosporean species? More DNA sequences (both nuclear and mitochondrial) and other data must be gathered to compare further with established morphometric criteria to help clarify the taxonomy of this phylum. When coupled with data such as geographic location, host species, tissue tropism, ultrastructure, life cycle, and alternate host, new patterns may begin to emerge providing insights into the evolution, life history, and dispersal of these important parasites of fish.

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