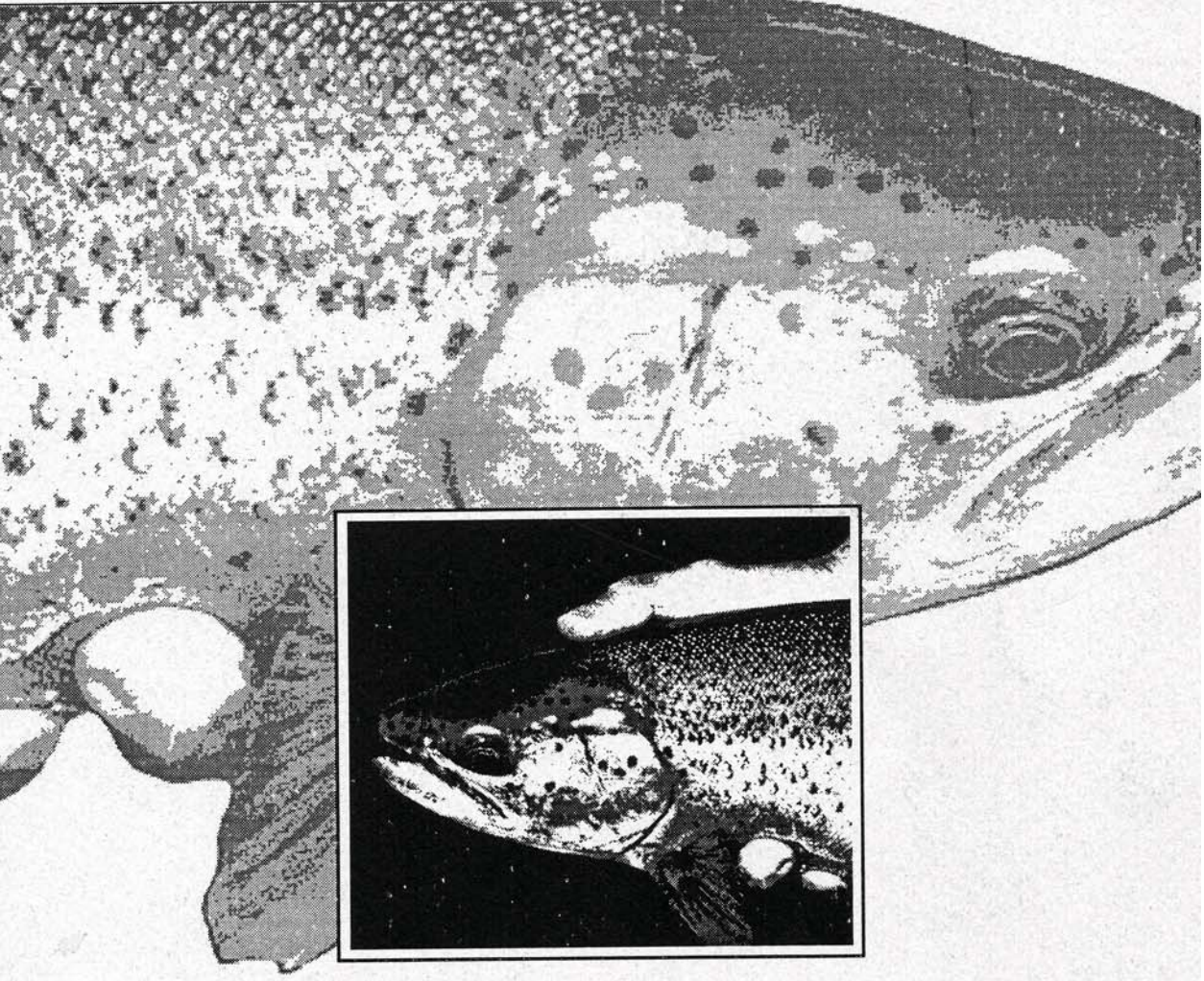

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INVESTIGATIONS OF INTRASPECIFIC GENETIC VARIATION OF *MYXOBOLUS CEREBRALIS*

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Introduction

Myxobolus cerebralis (Hofer 1903), the causative agent of whirling disease has been proposed to be of European origin. It appears to have arrived in the United States in the 1950's via shipments of frozen "table trout" (Hoffman, 1970; Wolf, 1985). The parasite has since spread and is currently found in 22 states. For many years fish hatcheries have managed this disease by keeping their juvenile fish in concrete raceways instead of earthen ponds which may provide habitat for the parasites alternate host, *Tubifex tubifex* (Wolf et al, 1986). Recently, fishery biologists from Montana and Colorado have experienced dramatic losses of wild rainbow trout that were attributed to *M. cerebralis*. This was not the case on the east coast where the parasite was first documented in this country, or on the west coast. This has raised questions about the possibility that these new epizootics in the intermountain western states might represent a new more virulent strain of *Myxobolus cerebralis.* The taxonomy of the entire phylum Myxozoa is currently under review. Morphometry, tissue tropism, and host species are the primary criteria for assigning species. A myxospore identified in a previously unreported host species may be considered a new species. Yet it is well known that *M. cerebralis* can infect more than one species (O'Grodnick, 1989). The criteria presently used to classify the myxozoa may mask the underlying genetic diversity of myxozoans.

For these reasons we decided to investigate the molecular taxonomy of *M. cerebralis* and related species in the genus *Myxobolus*. Initial work investigating genetic sequences of *M. cerebralis* have focused on the 18S ribosomal RNA. This was part of a project comparing the 18S rRNA sequences of the actinosporean stage and the myxosporean stage to confirm the proposed life cycle of this parasite (Wolf and Markiw, 1984; Wolf and Markiw, 1986). Using archived collections of spores to purify DNA we PCR amplified, cloned, and sequenced the 18S rRNA gene. Specific PCR primers were developed to more easily amplify this gene from other sources of *M. cerebralis*. We have used these primers to amplify, clone, and sequence the 18S rRNA gene from isolates of *M. cerebralis* which originated in California, West Virginia, and Germany. We have since included the ITS-1 region of the ribosomal RNA in our analyses. If the theory of how *M. cerebralis* was introduced to this country is correct then sequences from each side of the Atlantic Ocean should be nearly identical. On the other hand, if a similar species already existed on this continent that arose from a common ancestor we should see a reasonable amount of genetic variation in the 18S sequences. A third possibility, is that enough genetic diversity is inherent in this genus to provide the raw material for strain types to develop. To investigate this last possibility further we compared 18S rRNA sequences from 10 different species of the genus myxobolus to see how closely related these species are. Our findings suggest at the very least that the current morphometric methods used to classify this phylum may be inappropriate given the amount of genetic diversity between species within the same genus.

Materials and Methods

Source of spores

M. cerebralis spores from Virginia were provided by Dr. Maria Markiw at the Fish Health Research Center, National Biological Survey, Leetown, West Virginia. Spores from Germany were provided by Dr. Mansour El

Matbouli, University of Munich. Spores from California were purified from infected rainbow trout tissues using the pepsin trypsin digest described by Markiw and Wolf (1974). The *M. squamalis* (Iversen, 1954) spores were collected from the scale pockets of adult chinook salmon (*O. tshawytscha*) returning to the Nimbus Fish Hatchery, Rancho Cordova, California by Dr. W. Cox. Cysts were ruptured and the contents collected in a 1.5ml microfuge tube. The spores (3.0×10^7) were then washed in tap water by rinses and low speed centrifugations (500 x g). *Myxobolus insidiosus* (Wyatt and Pratt, 1963) spores from juvenile chinook salmon were a kind gift from Dr. J. Bartholomew and Dr. A. Amandi (Oregon State University, Corvallis, Oregon). These spores (2.0×10^7) were dissected from infected skeletal muscle and cleaned by the same procedure described for *M. squamalis*. The *M. arcticus* spores were donated by Dr. Mike Kent. *M. neurobius* spores were collected and donated by Dr. J. Bartholomew (Oregon State University, Corvallis, Oregon). Spores from *M. djragini* (from the head of silver carp), *M. elipsoides* (from gill cysts of bream), *M. bramae* (from gill cysts of bream), *M. sandrae* (from muscle cysts of pike perch), and *M. portucalensis* (from cysts in the fins of European eel) were all provided by Dr. C. Szekely and Dr. K. Molnar (Hungarian Academy of Sciences, Budapest, Hungary)

DNA Extractions

Myxosporean stages of *M. cerebralis* as collected from fish cartilage by the pepsin/trypsin digestion method proved impervious to the lysis buffer containing proteinase K as judged by the inability to open the two valves that protect the sporoplasm and the failure to recover DNA after such treatments. Improved recovery of genomic DNA was obtained from these spores (2×10^6) by heating in a microwave oven. The spores were pelleted briefly in a microfuge tube and air dried for 15 min. The dried pellet was placed in an 800 watt microwave oven and heated for 1 min. then were resuspended in lysis buffer (100 mM NaCl, 10 mM Tris pH 7.6, 10 mM EDTA, 0.2% SDS, 0.2 mg/ml proteinase K) and incubated at 55 ^{0}C for 16 h. Phenol/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 8,000 rpm in a microcentrifuge. This extraction was repeated a second time followed by a single treatment with isoamyl alcohol/chloroform. Sodium acetate (3 M pH 6.9) was added with 2 volumes of 100% cold ethanol to precipitate the DNA. DNA was collected by centrifugation for 10 min at 14,000 rpm 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). DNA content was determined by spectrophotometry.

Oligonucleotide synthesis

Universal primers (18e and 18g') (Hillis and Dixon,1991) and specific primers for amplification of myxozoan sequences (Tr) were selected and tested for possible secondary structure, self-complementarity, and absence of cross reactions to host sequences using Amplify from University of Wisconsin Genetics (Madison, WI).
Primers for DNA sequencing and PCR were prepared using an ABI DNA synthesizer, model #394 .
Oligonucleotides were then desalted on a NAP 5 desalting column from Pharmacia Biotech, Inc. (Alameda, CA) and diluted in water to working concentrations for PCR and DNA sequencing, of 20 pmoles/µl and 10 ng/µl , respectively.

Primer Sequences:

18e	5' - CTGGTTGATTCTGCCAGT - 3'
18g'	5' - CGGTACTAGCGACGGGCGGTGTG - 3'
Tr5-3	5' - CGTGAGACTGCGGACGGCTCAG - 3'
Tr3-1	5' - CGGTGTGTACAAAGGGCAGGGAC - 3'
Tr5-6	5' - GGCAGCGTTAAAACTGTCTCACG - 3'
Tr3-6	5' - CCTCACAGTCTCTCCATGACAC - 3'
5.8c	5' - GTGCGTTCGAAATGTCGATGATC - 3'
18-3	5' - GTACACACCGCCCGTCGCTAC - 3'
MX5	5' - CTGCGGACGGCTCAGTAAATCAGT - 3'
MX3	5' - CCAGGACATCTTAGGGCATCACAG - 3'

PCR Amplification of rDNA

The actinosporean 18S rDNA was amplified using universal primers 18e and 18g' (Hillis and Dixon,1991). These primers gave rise to a 1934 bp product. The myxosporean 18S rDNA was amplified using a nested PCR and species- specific primers (Tr) developed using the sequences obtained from the actinosporean. The actinospore stag was used as a source of DNA initially because this stage could be purified from host tissue with more certainty that it was free of host DNA contamination. This also allowed comparison of homologous sequences from both stages. In the first round, primer Tr 5-3 and primer Tr 3-1 were used. In the second round of the nested PCR, primer Tr 5-6 and primer Tr 3-6 were used. These primers gave rise to a 1544 bp product. After the sequences from two different species had been obtained using primers 18e and 18g' we aligned all the homologous regions and found a region that they all appeared to share. Primers MX5 and MX3 were made to specifically hybridize to these regions. These primers allowed the amplification of a 1700 bp portion of the 18S rRNA gene from seven more species. The ITS-1 region was amplified using one universal primer (5.8c) (Hillis and Dixon, 1991) and one primer specific to the 3' end of the 18S rRNA gene of *M. cerebralis* as determined by sequencing (18-3). These primers give rise to a 180 bp fragment from *M. cerebralis*.

The rDNA fragments were amplified in standard 50 :l reactions containing 10 mM Tris-HCl pH 8.3 (at 25 ^{0}C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % w/v gelatin, 400 µM dNTP's, 5 µM tetramethyl ammonium chloride, 40 pmoles of each primer, and 2 U Taq polymerase. The PCR thermal cycler used was a model PTC-100 manufactured by MJ Research (Watertown, MA). Forty cycles of 1 min at 95 ^{0}C, followed by 2 min at 45 ^{0}C, followed by 4.5 min at 72 ^{0}C were used in the amplification. The amplification cycles were preceded by a denaturation step where samples were held at 95 ^{0}C for 5 min. The thermal cycler program finished with an extended elongation step where samples were held at 72 ^{0}C for 10 min.

DNA Electrophoresis

PCR products were loaded into 1.5% agarose submarine gels and run for 50 minutes at 35 volts/cm . The DNA bands were visualized by staining with ethidium bromide for 25 minutes and transillumination with short wavelength (254 nm) ultraviolet light.

Cloning of PCR Products

The actinosporean 18S rDNA was cloned into pCR II using the TA cloning kit from Invitrogen (San Diego, CA). All the myxosporean 18S rDNA were cloned into pNoTA using the Prime PCR Cloner Kit from 5 Prime > 3 Prime (Boulder, CO). Inserts were confirmed by screening transformant colonies by PCR using the same primers 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 from Sigma Chemical Co.(St. Louis, MO). Three microliters of the inoculum was used in a PCR assay as described above.

Sequencing of rDNA

Plasmid DNA from transformant colonies was prepared according to Sambrook et al. (1989). The 18S rDNA sequence was derived by oligonucleotide directed dideoxynucleotide chain termination sequencing using the TAQuence sequencing kit from United States Biochemical Corporation (Cleveland, OH). Sequencing reactions were run on 0.4 mm thick 6 % polyacrylimide gels in a Sequi-Gen II Sequencing System from BioRad (Hercules, CA) or on an ABI 377 (Branchburg, New Jersey) automated sequencer using fluorescently labeled dye terminators. The sequence of Henneguya salminicola was provided by Dr. Bob Devlin.

Sequencing Alignments and Analysis

Mac DNAsis v 3.5 from Hitachi Software Engineering America, Ltd. (San Bruno, CA) and GeneWorks v 2.4 from Intelligenetics Inc. (Mountain View, CA) were used to align sequences. Phylogenetic analysis was done using PAUP ver.3.0 (Illinois Natural History Survey, 1991). For phylogenetic analysis all sequences were truncated to include only the 1700 bp encompassed by sequences MX5 and MX3.

Results

The complete sequence of the 18S rRNA gene was determined for clones from Germany, California, and Virginia. The sequence differences observed among these three clones were small and could be accounted for when considering microheterogeneity and the mechanisms that give rise to the multiple copies of this gene that typically

exist in a eukaryotes genome (East et al,1992) (Gunderson et al, 1987). We considered the ITS-1 region the next logical extension to continue our search. Since this sequence is found abutting the 18S rDNA we already had one primer sequence from the 18S rRNA gene to amplify this region. In addition this region is noted for being under less stringent natural selection since it does not code for a functional component of the ribosome therefore it can provide useful genetic markers for classification at both lower and higher taxonomic levels. (Hillis and Dixon, 1991) (Gutell et al,1985). At present we have cloned and sequenced the ITS-1 region from California and Virginia isolates. We have found the 180 bases of sequence of the ITS-1 region differs between these two at only four positions. We are continuing to look at this region of sequence from populations from Germany and the Rocky Mountain region of the United States.

The phylogenetic analysis showed *M. arcticus*, *M. bramae*, *M. djragini*, *M. elipsoides*, *M. neurobius*, and *M. sandrae* compose a single clade. *M. cerebralis* though separate is probably in the same genus as the above species while *M. squamalis*, and *M. portucalensis* were as distant as *Henneguya salminicola* (Fig.1*)*. These last two should most likely be assigned a separate genus.

Discussion

Disease caused by any parasite is due to a complex interaction of factors from the host and from the parasite under specific environmental influences. The disease state is brought on in successive stages. First there is attachment of the parasite to the host. Then the parasite must penetrate the host. And to be successful it must be able to replicate in the host. The specific factors that are key to each of these stages are referred to as virulence factors. In the case of *Myxobolus cerebralis* these virulence factors are currently unknown. Possibilities include proteases used to penetrate host tissues; cartilaginase- type enzymes used to digest the host skeletal elements; and receptor proteins used in attaching to the host epidermis or components involved in the mechanochemical stimulus that leads to attachment. Until the identity of the virulence factors are known we can look for different strains of *M. cerebralis* by utilizing easily accessible genetic markers in the ribosomal RNA motifs and confirming if these genetic markers are associated with observed differences in virulence of geographic isolates of the parasite.

Observations of mortalities resulting from *M. cerebralis* in the field has lead to speculation that various strains of *M. cerebralis* may be more lethal to trout than others. Additionally, evidence that is mostly anecdotal suggests that strains of trout from different regions may have natural immunity to the agent of whirling disease, *M. cerebralis*. Trout stocks of the Pacific coast while testing positive for *M. cerebralis* don't show the high mortalities of the infected feral stocks of the inter mountain western states. If less virulent strains do exist this may provide a means of stemming the decline of trout populations by displacing the more virulent strain with a less virulent form of the parasite or conversely replacing the strain of trout stocked by the hatcheries. Finding sufficient genetic variation within the gene pool may indicate the strain variation we are looking for actually exists. This would have to be confirmed at some point by collecting data from transmission studies that correlated with the genetic evidence. It is for this reason that we are looking for genetic diversity within the species *M. cerebralis* and attempting to identify readily recognizable genetic markers. Direct comparisons of rRNA genes have given us a means of investigating this possibility until such time as the actual virulence genes are identified. The differences thus far observed can only be determined to be meaningful after the proper transmission studies have been done to see if the observed sequence differences correlate with differences in virulence. From the 18S rRNA comparisons of other species in the genus we can see that considerably more genetic diversity exists than one would expect from a single genus. Additionally, little is known about sexual modes of reproduction among the myxosporeans. Since documentation of multiple myxosporean species infecting a single host exists (Hedrick et al, 1991), horizontal transfer of genes remains a possibility as well.

Acknowledgments

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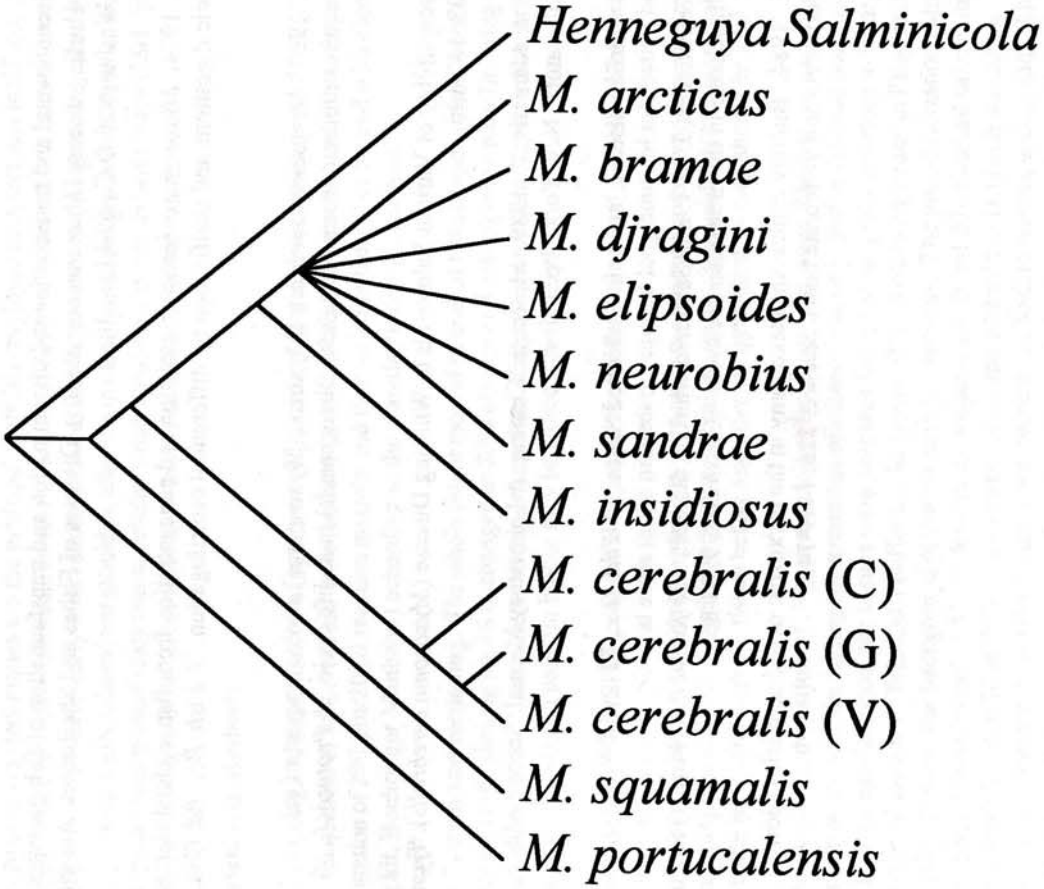
Figure 1. *Myxobolus* spp. 18S rRNA Phylogram



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