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Myxobolus cerebralis internal transcribed spacer 1 (ITS-1) sequences support recent spread of the parasite to North America and within Europe

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ABSTRACT: Molecular approaches for resolving relationships among the Myxozoa have relied mainly on small subunit (SSU) ribosomal DNA (rDNA) sequence analysis. This region of the gene is generally used for higher phylogenetic studies, and the conservative nature of this gene may make it inadequate for intraspecific comparisons. Previous intraspecific studies of *Myxobolus cerebralis* based on molecular analyses reported that the sequence of SSU rDNA and the internal transcribed spacer (ITS) were highly conserved in representatives of the parasite from North America and Europe. Considering that the ITS is usually a more variable region than the SSU, we reanalyzed available sequences on GenBank and obtained sequences from other *M. cerebralis* representatives from the states of California and West Virginia in the USA and from Germany and Russia. With the exception of 7 base pairs, most of the sequence designated as ITS-1 in GenBank was a highly conserved portion of the rDNA near the 3-prime end of the SSU region. Nonetheless, the additional ITS-1 sequences obtained from the available geographic representatives were well conserved. It is unlikely that we would have observed virtually identical ITS-1 sequences between European and American *M. cerebralis* samples had it spread naturally over time, particularly when compared to the variation seen between isolates of another myxozoan (*Kudoa thyrsites*) that has most likely spread naturally. These data further support the hypothesis that the current distribution of *M. cerebralis* in North America is a result of recent introductions followed by dispersal via anthropogenic means, largely through the stocking of infected trout for sport fishing.

KEY WORDS: Myxozoa · Ribosomal DNA · *Myxobolus cerebralis* · Whirling disease · Internal transcribed spacer

INTRODUCTION

One of the most frequently cited examples of myxozoan parasitism is whirling disease, caused by *Myxobolus cerebralis*. Symptoms of the disease include skeletal deformities, blackened tails and characteristic whirling behavior (Gilbert & Granath 2003). The parasite has been found in 26 countries around the world (Bartholomew & Reno 2002), and it is widely accepted that this distribution is a result of anthropogenic movement of fish (Wolf 1986, Hedrick et al. 1998). Hoffman (1970) proposed that brown trout in Germany were the natural host for the parasite and that it was through movement of infected rainbow trout from this area that the spread to North America resulted. Presumably, this same mechanism was involved in the spread to other

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countries such as New Zealand and South Africa. Determining whether population structure exists for this parasite, and therefore genetically distinct populations, may be important in the identification of potential hot spots as reservoirs of infection, especially within the USA. *M. cerebralis* has been observed in 22 states in the USA (Bartholomew & Reno 2002) and there is great concern for wild trout populations as this parasite becomes enzootic in streams where fish were previously uninfected.

MATERIALS AND METHODS

Molecular approaches for resolving relationships among the Myxozoa have relied mainly on small subunit (SSU) ribosomal DNA (rDNA) sequence analysis (Kent et al. 2001). The conservative nature of this gene, however, may make it inadequate for intraspecific comparison. Generally, the internal transcribed spacer (ITS) regions of the rDNA are usually much more variable than the SSU, and are therefore useful for intraspecific comparisons (van Herwerden et al. 2000). Andree et al. (1999) compared the SSU and ITS sequences from representatives of *Myxobolus cerebralis* from Germany, West Virginia, and California and reported that there were more sequence differences within the SSU than in the ITS.

To verify this result, we reexamined the ITS-1 sequences of Andree et al. (1999) (GenBank accession numbers AF115256–AF115260) using the basic local alignment search tool (BLAST) (Altschul et al. 1990) and sequence alignments. Searches were conducted in GenBank using the nucleotide-nucleotide BLAST. Sequence alignments of the above *Myxobolus cerebralis* sequences to metazoan rDNA were performed in Clustal X (Thompson et al. 1997) using the following sequences: *Kudoa thyrsites* (AY078430), *Homo sapiens* (HSU13369), *Drosophila melanogaster* (M21017), *Aurelia aurita* (AY039208), *Oncorhynchus mykiss* (AF308735).

The best matches with the BLAST were the SSU sequence of several myxozoans and other invertebrate taxa. We verified these results with our SSU rDNA alignments (Fig. 1). Clearly, a majority of the sequence identified as ITS-1 by Andree et al. (1999) (all but 7 base pairs, bp) corresponds to the highly conserved 3-prime region of the SSU rDNA. These results prompted us to reexamine the ITS region of *Myxobolus cerebralis*.

The DNA from actinospore (triactinomyxon) and myxospore stages of *Myxobolus cerebralis* or from tissues of infected rainbow trout were obtained from the following locations: actinospores in 95% ethanol from California and Russia, rainbow trout heads from West Virginia, and myxospore DNA from Germany. For polymerase chain reactions (PCR), DNA was extracted via the DNeasy Tissue kit (QIAGEN), or individual actinospores were collected for direct use in PCR reactions. The ITS-1, 5.8S, and ITS-2 region from the California *M. cerebralis* was amplified in a PCR reaction using standard conditions (Whipps et al. 2003) with the following primers. Primer Mc18S1F (5′ AAT ACG CTG GGA TCG ATG) was complementary to the 3-prime region of the SSU and used with the reverse primer 28S1R (5′ GTG TTT CAA GAC GGG TCG), a general large subunit (LSU) primer of Whipps et al. (2004). The resulting 1670 bp fragment was cloned into plasmids (QIAGEN) and 3 clones were sequenced in both directions.

RESULTS AND DISCUSSION

Clone sequences were almost identical and have been deposited in GenBank (AY479922–AY479924). Start and stop positions (Fig. 2) of ITS-1, 5.8S and ITS-2 regions were approximated from alignments of SSU, 5.8S and LSU sequences of a broad range of metazoan taxa. The ITS-1 sequence was approximately 410 bp long with an AT content of 65% (as opposed to a 50% AT content in the SSU). From *Myxobolus cerebralis* ITS-1 clone sequences, we observed 3 variable nucleotide positions and a single insertion-deletion (indel), representing a sequence variability of 1.7% between clones from a single sample. The 5.8S rDNA was 176

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Mc 122  AAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATC gacattt 175
Kt 1673 ----------------------------------------ATC attgaaa 1726
Dm 1948 ----------------------------------------ATTA ttgtat 2001
Hs 1824 ----------------------------------------ATTA acggag 1877
Om 1786 ----------------------------------------ATTAAC gggt 1839
Aa 1764 --------------------------------A--------attaccggaa 1817
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Fig. 1. Alignment of 3-prime end of small subunit (SSU) ribosomal DNA (rDNA) to *Myxobolus cerebralis* (Mc) sequence of Andree et al. (1999) (AF115256), illustrating its identity to this highly conserved region. Other taxa in alignment were: Kt, *Kudoa thyrsites* (AY078430); Dm, *Drosophila melanogaster* (M21017); Hs, *Homo sapiens* (HSU13369); Om, *Oncorhynchus mykiss* (AF308735); and Aa, *Aurelia aurita* (AY039208). ITS-1 start indicated by lowercase letters in **bold**

```
     SSU-->
     Mc18S1F---------->
1    AATACGCTGG GATCGATGTA AAATGGTGCA ATTTCGAGGR AGTAAAAGTC GTAACAAGGT  60
                                  ITS-1-->
61   TTCCGTAGGT GAACCTGCGG AAGGATCatt gacgttttac acaaacattt tgtacgttgt  120

121  acgtattatt cgagaaatta catctgttgc tgragctgtg tctcagacta gttgaggaga  180
181  gtcgcattac gagaaagcat tcttctagtc gcttcagcaa cacagagaaa tcaagacgac  240

241  ctccactttt aatataaatc actgagagta gactacttat ttcgtctatt tctgttygac  300
301  attgatgaga caatagtgaa ctttagagta atattagtct ttgtttttgt gaatttttaa  360
361  tcgtgttgta taaagctgtt gtgtttataa aatgtgaatg ataagtattt cctgttcgtt  420

421  gagtatatgt gtaatgcgtg tattcaamgt ttaggggtgc tctattttca cagagtgaga  480
                          5.8S-->
481  ttttacgagt tgatatatat attCATAACC ATTAACGGTG GATCACTTGG CTCGAGGGAC  540
                                              <-------------Mc5S1R
541  GATGAAGAAC GTGGCAAAAT GCGATAAGTG ATGCGATTCG CAAGCCTAGT GAGTCATCAA  600
601  GTTTTTGAAC GCAAATGGCA CTCTTGATTG CATCAGGAGT ATGTCTGGTT GAGGGTTGTT  660
                       ITS-2-->
661  TTTTGAGATA ACGTCACACg gttggctttg caattgatgt gcgaatatac actagttgta  720
721  tattagagtt ggatgtgaac caagcgcggg ttctcatctc aaatgattgg acaacacctg  780
781  taacactttg gtagatgtat taattgttga atttgttggt gaaaacttta gagtacaaca  840
                                                          LSU-->
841  atgtgtgcac ctattaaaaa gatacagtag ttataattat aaatttacgg caACCTCAAC  900

901  TCAGGCAAGA TTACGCCGTG AACTAAAGCA TTTCAGTAAC GGCAGGAAAA GAAAATAACT  960
961  ATGATTCCCT CAGTAACTGC GAGTGAAGTG GGAAGAGCCC AACGTTGAAA GCTACATCTT 1020
1021 TAACCGGGTG TCGCGTTGTA ACGTATAGAT GCAACATCGA GACGTGAACC AGATTTGAAG 1080
1081 TCGCTTAGAA TAGCGCACCA TAGAGGGTTT TAGTCCCGTA CATGAATTTG TGTGTCACAT 1140
1141 CAAGTATGTT GTCTTCTAAG AGTCGGATTG TTTGGGAATA CAGTCTGAAG TGGGTGGTAA 1200
1201 ACTCCATCTA AGGCTAAATA TAACCTCGAG ACCGATAGCG AACAAGTACC GTGAGGGAAA 1260
1261 GTTGAAAAGC ACTCTGAAAA GAACGTGAAA AGGGCGTGAA ACCGTTAATG GGGAAGCGTA 1320

1321 CGGTAGAGTC GAAGTAGAGC AGCGTGGGTG CGCGTATTTT GTGGCAACAC AGRATGCGTG 1380
1381 GCAGGTAGCG TTACTCTACG CGCCGGACTC ATTGTTAAAT GACTGGCGTA GTAAGTGGTT 1440
1441 GGTAGTAACA GTGCATCCCT GAGGATGTTC TGTGCTATTA GCATTTACAA TATGTTTTAG 1500
1501 AGTTGTTCGA CAATGATGTG TAGAGCGTAA TATCAATGTG TAGTGGCTAT CGGTAGAGTG 1560
1561 CAAGTTTAGG CAAACACTCT ATTGACAACC ATTTCGTATT GGTGTTACAA GACAAGTCGC 1620
                                          <-------------28S1R
1621 ATACGATTTT CCGGCGAGAC ATGCTACTGT TCGACCCGTC TTGAAACAC 1669
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Fig. 2. *Myxobolus cerebralis* small subunit (SSU), internal transcribed spacer (ITS) regions (lower case letters), and large subunit (LSU) ribosomal DNA sequence. Primer sites are shown in **bold**, downward arrows mark regions of variability within the ITS-1, insertion/deletion is shaded

bp long and ITS-2 was 213 bp long. No sequence variability was observed within the ITS-2 or 5.8S, and only a single ambiguous base was found in the LSU. Given that some variation was detected in the ITS-1, we decided to continue using this region for comparisons of geographically distinct representatives of *M. cerebralis*.

Another primer, Mc5S1R (5′ ATG ACT CAC TAG GCT TGC), complementary to the 5.8S rDNA of *Myxobolus cerebralis* was used with Mc18S1F to amplify a 600 bp fragment containing the ITS-1. We used these PCR primers to amplify and sequence the ITS-1 from a pool of actinospores as well as from 5 individual spores. Sequences were similar to those obtained by cloning as described above. The ambiguities were clearly visible on the sequence reads, and the sequences became unreadable at the point of the indel near the end of the ITS-1 in both the pooled actinospore sequence and in the sequence from individual actinospores (GenBank accession number AY479925). These data suggest that there is some ITS-1 sequence variation within a single actinospore. As an actinospore contains multiple cells, we cannot say whether this observed variation occurs within individual cells or between cells of the actinospore. This within-isolate variation has been observed in other invertebrates such as *Echinococcus* sp. (Bowles & McManus 1993).

Using the Mc18S1F/Mc5S1R primers, we amplified and sequenced the ITS-1 from *Myxobolus cerebralis* representatives from West Virginia, Germany and Russia. These sequences were identical to those obtained from the California *M. cerebralis*, including the same ambiguous bases and 3-prime indel. Although this lack of intraspecific variation within the ITS is not uncommon (Hoste et al. 1993, van Herwereden et al. 2000), it seems unlikely that these sequences would be identical for such geographically distinct representatives had the parasite spread naturally over time. For example, it is most likely that *Kudoa thyrsites* has dispersed naturally over time, and there is as much as

43.5% sequence difference in the ITS-1 region between different geographic representatives of this cosmopolitan marine myxozoan around the world (Whipps & Kent 2003).

Our reevaluation of the ITS rDNA of *Myxobolus cerebralis* provides strong support for the already widely accepted hypothesis that the parasite has spread from a single source in recent times. We also observed identical *M. cerebralis* ITS-1 sequences from Germany and Russia, the area that Hoffman (1970) considered to be the parasite's endemic range. This suggests there had been recent introductions and/or mixing of *M. cerebralis* populations throughout Eurasia because we assume that populations with a long history of reproductive isolation would have very different ITS-1 sequences (as seen for *Kudoa thyrsites*).

We have determined through reevaluation of the existing ITS sequences on GenBank that nearly all of reported sequence by Andree et al. (1999) is a conserved region near the 3-prime end of the SSU rDNA. Despite this initial misidentification, comparisons of the true ITS-1 sequences we generated provide a similar conclusion, and only minor variations occurred in all of the geographic representatives we examined. These results provide further evidence to support the hypothesis for a recent introduction of *Myxobolus cerebralis* from Europe to the USA

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