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The Parasite that Causes Whirling Disease, *Myxobolus cerebralis*, is Genetically Variable Within and Across Spatial Scales

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Abstract:

Understanding the genetic structure of parasite populations on the natural landscape can reveal important aspects of disease ecology and epidemiology and can indicate parasite dispersal across the landscape. *Myxobolus cerebralis* (Myxozoa: Myxosporea), the causative agent of whirling disease in the definitive host *Tubifex tubifex,* is native to Eurasia and has spread to more than 25 states in the USA. The small amounts of data

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available to date suggest that *M. cerebralis* has little genetic variability. We examined the genetic variability of parasites infecting the definitive host *T. tubifex* in the Madison River, MT, and also from other parts of North America and Europe. We cloned and sequenced 18S ribosomal DNA and the internal transcribed spacer-1 (ITS-1) gene. Five oligochaetes were examined for 18S and five for ITS-1, only one individual was examined for both genes. We found two different 18S rRNA haplotypes of *M. cerebralis* from five worms and both intra- and interworm genetic variation for ITS-1, which showed 16 different haplotypes from among 20 clones. Comparison of our sequences with those from other studies revealed *M. cerebralis* from MT was similar to the parasite collected from Alaska, Oregon, California, and Virginia in the USA and from Munich, Germany, based on 18S, whereas parasite sequences from West Virginia were very different. Combined with the high haplotype diversity of ITS-1 and uniqueness of ITS-1 haplotypes, our results show that *M. cerebralis* is more variable than previously thought and raises the possibility of multiple introductions of the parasite into North America.

Keywords: Haplotype diversity, ITS-1, Myxozoa, variability across space, 18S.

MYXOZOANS are an economically important group of microscopic metazoan parasites (Kent et al. 2001), and most have a two-host life cycle. Alternating between annelid and bryozoan determinate hosts, the majority of myxozoans infect fish as their alternate host (Jimenez-Guri et al. 2007). Myxozoa represent an assemblage of over 2,000 described species. Emerging discoveries of myxosporean pathogens, combined with the approximately 800 currently described species in the genus *Myxobolus*, are suggesting that these parasites represent a serious threat to the development of pisciculture (Lom and Dykova 2006). The most studied myxo-zoan, *Myxobolus cerebralis*, is in the forefront of research because of its effect on endemic cutthroat trout, *Oncorhynchus clarkii,* and the dramatic declines of wild rainbow trout, *Oncorhynchus mykiss,* in the intermountain west of the USA, perhaps related to the impact of parasitism by *M. cerebralis* (Nehring, Thomson, and Hebein 1998; Vincent 1996).

Myxobolus cerebralis is responsible for whirling disease (Gilbert and Granath 2001). The Eurasian native parasite was introduced into North America through movement of infected brown trout, *Salmo trutta*, from Europe in 1958 (Hoffman 1962), and since has been reported in more than 25 states, from New York to Alaska (Arsan et al.

2007; Bartholomew and Reno 2002). The parasite has two distinct spore stages: (1) myxospores that develop in salmonids and are known to be infective to only one species of aquatic annelid *Tubifex tubifex* (Clitellata: Oligochaeta) and (2) triactinomyxons (TAMs) that develop in *T. tubifex* and are infective to numerous salmonid taxa (Wolf and Markiw 1984). Because some myxozoans are sympatric, accurate identification of the para- site is critical to understanding host-parasite dynamics in natural populations. The considerable morphological similarity in spores of *Myxobolus* spp. can lead to misidentification based on visual assessment (Lom and Hoffman 1971); however, molecular genetic tests based on nuclear DNA coding for the ribosomal 18S gene can verify if infections are due to *M. cerebralis* (Andree, MacConnell, and Hedrick 1998) or some other species.

Although detected in river drainages throughout the USA, disease severity among wild fish populations can be highly variable. This spatial variability results, in part, from variation in the distribution and abundance of the worm host (Krueger et al. 2006). Variation in susceptibility among fish taxa probably contributes little to spatial variation in fish disease (Vincent 2002). Previous studies concluded that there was little genetic variability in *M. cerebralis* (Andree et al. 1999; Whipps et al. 2004); however, these conclusions are based on small sample sizes.

To further investigate spatial variation in fish disease, we assessed the genetic variability among samples of *M. cerebralis* using 18S rDNA and internal transcribed spacer-1 (ITS-1) DNA sequences and within samples of *M. cerebralis* using ITS-1, isolated from *T. tubifex* collected from a small stretch of the Madison River, MT. We also assess the genetic related- ness among *M. cerebralis* from MT, West Virginia (WV), Virginia (VA), California (CA), Oregon (OR), and Alaska (AK) in the USA; and from Munich, Germany (DEU) and Russia (RUS) using available GenBank sequences. The study questions were (1) Is *M. cerebralis* within a small stretch, within a single sampling location of the Madison River, or within a single worm, genetically variable? (2) How is genetic variability partitioned on a geographic scale? (3) How does *M. cerebralis* genetic variability in MT compared with other regions? These results provide information on the introduction of the parasite into North America and form the basis for

examining the contribution of genetic variation in the parasite to spatial variability in fish disease.

MATERIALS AND METHODS

Worm collection

We examined myxozoans infecting tubificid worms collected from two side channels (North Slide, MT: lat. 44.8263 and long. —111.45103; Lyons Bridge, lat. 44.90426 and long. —111.59843) of the Madison River, MT, in 2005. Oligochaetes were collected with a kick net and stored on ice while transported to the laboratory (Krueger et al. 2006). Individuals were put in 4-ml well plates with dechlorinated tap water at 15 °C on 12:12 L:D light regime. Infection was determined by scanning each well for the presence of TAMs using

Table 1. Primers used for sequencing and cloning of *Myxobolus cerebralis.*

Primer name	Primer sequence	Target	PCR product size
PM9[a]	GCA TTG GTT TAC GCT GAT GTA GC	Myxozoan 18S	500 bp
PM4	GGC ACA CTA CTC CAA CAC TGA ATT TG		
Mc18S1F[b]	AAT ACG CTG GGA TCG ATG	*Myxobolus cerebralis* ITS-1	600 bp[c]
Mc5S1R	ATG ACT CAC TAG GCT TGC		

[a]Andree et al. (1998).
[b]Whipps et al. (2004).
[c]The ITS-1 region was ~ 420 bp of the 600 bp PCR amplicon.

a dissecting microscope (40X) 2 times over a 2-wk period. After the 2-wk period, the anterior section of the oligochaete was slide-mounted for morphological identification, and the posterior section was preserved in lysis buffer for later DNA extraction and sequence analysis.

DNA extraction, amplification, cloning, and sequencing

DNA was extracted using Nucleospin® extraction kits (BD Biosciences Clontech, Foster City, CA). Oligochaetes were confirmed as *T. tubifex* lineage III by sequencing of 16S rDNA (Beauchamp et al. 2002). Parasite DNA was PCR-amplified for cloning in 25-µl reactions with 10 µM of each primer (Table 1) using EconoTaq® Plus Green 2X Master Mix (VWR, Radnor, PA). The PCR cycling parameters for both 18S rDNA and ITS-1 were 95°C for 5 min, followed by 35 cycles of 94°C for 30s, 53°C for 45s, 72°C for 90s, and 72°C for 7 min.

Amplified products were visualized in 2% agarose gels stained with ethidium bromide. Because worms in the natural stream environment may be infected multiple times, by genetically identical or genetically different *M. cerebralis,* PCR fragments were cloned using the pGEM®-T Easy Vector Systems (Promega, Madison, WI). We examined a single clone of the relatively invariant 18S rRNA gene from each of five individual worms. For one of these individuals, and for another four individuals, we sequenced 1–6 clones of the more variable ITS-1 region (Table 2). Sequencing was performed in both directions for each clone by Agencourt (Beckman Coulter Genomics, Danvers, MA).

Sequence alignment, overview of genetic diversity, and haplotype network analysis

The 18S rRNA sequences were trimmed to the ~460 nucleotides between the primers and the ITS-1 sequences to the ~420 nucleotide region between 18S rRNA and 5.8S rRNA (Whipps et al. 2004). The sequences were edited and aligned using Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, MI), confirmed as *M. cerebralis* using the BLAST search algorithm and have been deposited in GenBank (Table 2). Additional *M. cerebralis* sequences from GenBank (Table 2) were included in our analysis. For 18S rRNA, these included ten sequences from a wide geographic range (i.e. WV, VA, CA, OR, AK, and DEU); however, for ITS-1, only three additional sequences were available, all from CA. The CA haplotypes for ITS-1 were also reported to be found in *M. cerebralis* from WV, DEU, and RUS (Whipps et al. 2004). *The haplotypes are identified based on acronyms, XX_YY_ZZ, where XX = country (USA = United States, DE = Deutschland [Germany]); YY = state (or Munich for the sample from Eurasia); ZZ = collection location (LB = Lyons Bridge; NS = North Slide; LH = Leetown Hatchery; MWH = Mt. Whitney Hatchery; LRH = Lostine River; EH = Elmendorf Hatchery). Tt = sample isolated from the definitive host, *T. tubifex*, Fish = sample from a fish; actinospore = a single actinospore.

We calculated both qualitative measures of genetic diversity (i.e. those that consider haplotypes as the same or different)

Table 2. List of Myxobolus cerebralis DNA sequences including information on location of collection, clone identification, GenBank accession number, and reference.

Mc source Id[f]	Clone Id	18S	ITS-1
USA_MT_LB6 + 3	W54	JN134160	
USA_MT_LB6 - 3	W56	JN134161	
USA_MT_LB6 + 4	W65	JN134162	
USA_MT_LB6 - 4	W70	JN134163	
USA_MT_LB7 + 11	W53	JN134164	
	A23		JN134165
	A24		JN134166
	A29		JN134167
	W16		JN134168
	W18		JN134169
	W19		JN134170
USA_MT_LB6 + 10	A56		JN134171
	A57		JN134172
	A58		JN134173
	A59		JN134174
USA_MT_LB6 + 11	A117		JN134175
USA_MT_NS5 + 147	A41		JN134176
	A42		JN134177
	A44		JN134178
	A45		JN134179
USA_MT_NS6 + 6	W34		JN134180
	W36		JN134181
	W37		JN134182
	W38		JN134183
	W40		JN134184
USA_WV_LH_Fish1		U96492[a]	
USA_WV_LH_Tt		U96493[a]	
USA_WV_ LH_Fish1		AF115254[b]	
DEU_Munich_Tt		AF115255[b]	
USA_CA_MWH_Fish		AF115253[b]	
USA_OR_ OSU_Fish		EF370481[c]	
USA_OR_LR_Fish		EF370480[c]	
USA_AK_EH_Fish1		EF370478[c]	
USA_AK_EH_Fish2		EF370479[c]	
USA_VA		AY040634[d]	
USA_CA_actinospore1			AY479922[e]
USA_CA_actinospore2			AY479923[e]
USA_CA_actinospore3			AY479924[e]
Mixture from CA and WV, USA; DEU, Russia			AY479925[e]

[a]Andree et al. (1997).
[b]Andree et al. (1999).
[c]Arsan et al. (2007).
[d]Schill, unpubl. observ.
[e]Whipps et al. (2004).
[f]XX_YY_ZZ: XX = country (USA = United States, DE = Deutschland [Germany]); YY = state (or Munich for the sample from Eurasia); ZZ = collection location (LB = Lyons

Bridge; NS = North Slide; LH = Leetown Hatchery; MWH = Mt. Whitney Hatchery; LRH = Lostine River; EH = Elmendorf Hatchery). Tt = sample isolated from the definitive host, *Tubifex tubifex*, Fish = sample from a fish; actinospore = a single actinospore.

and quantitative, or distance, measures that quantify the dif- ferences between haplotypes (i.e. sequences that differ by one nucleotide are more similar than sequences that differ by five nucleotides). Qualitative measures include haplotype diversity, *h* (Nei and Tajima 1981), and the number of effective alleles, *Ne* (Johnson 1974), estimated using GenAlEx 6.1 (Peakall and Smouse 2006). Allelic richness was calculated using HP-RARE 1.0 (Kalinowski 2005). The two quantitative measures of genetic diversity were the percent polymorphic sites, estimated using GenAlEx 6.1 (Peakall and Smouse 2006) and nucleotide diversity, p (Nei 1987), calculated using DnaSP v5 (Librado and Rozas 2009). Haplotype networks were created using Net- work 4.516 (fluxus-engineering.com).

Genetic distance and AMOVA

Pairwise genetic distances were calculated using the Jukes–Cantor method (Jukes and Cantor 1969) for 18S rRNA sequences using MEGA 5.04 (Tamura et al. 2011). The hierarchical genetic variation for ITS-1 was analyzed with AMOVA by partitioning the variation among three geographic groups (i.e. the pooled CA sequences, and LB and NS in MT), among worms, and within worms based on Φ_{ST} values in GenAlEx (Peakall and Smouse 2006).

RESULTS

We described in detail below our findings that 18S rRNA sequences showed little variability and that the most common haplotype was found over a broad geographic area; however, 18S rRNA sequences from WV were distinct from other North American sequences, as well as the sequence from Eurasia. The ITS-1 sequences were highly variable, but genetic differentiation was not correlated with geographic location (data not shown). All of the sequences were identified as *M. cerebralis* based on similarity (i.e. 99–100% for 18S rRNA and 98–99% for ITS-1) to sequences in GenBank.

Genetic variation in the 18S rRNA gene

The combined 15 sequences of the 18S rRNA gene showed four haplotypes and three of the four differed by 1–2 nucleotides and a single deletion (Table 3). The common haplotype, represented by 4 of the 5 sequences from MT and 10 of 15 sequences overall, was found over a wide geographic range, including MT, AK, OR, VA, and DEU; the other haplotype from MT differed by a single nucleotide from the common MT haplotype and by a nucleotide substitution and a single insertion/deletion from a haplotype from CA. However, sequences from WV differed at five of the seven variable sites, yet were identical to each other (Table 3). The haplotype network based on all 15 of the 18S rRNA sequences highlights the differences between the WV haplotype and the other haplotypes (Fig. 1).

Qualitative measures of genetic diversity show a little less variation among the MT sequences relative to the broader geographic area (Table 4). Haplotype diversity was 0.54 over- all and 0.40 for the MT sequences, a similar pattern was seen for the effective number of alleles (2.03 vs. 1.47) and allelic richness (2.40 vs. 2.00).

Quantitative measures that consider the number of nucleotide differences between sequences highlight the divergence of the WV sequences. For example, comparison of the percent polymorphic sites for the entire group of 15 sequences was about 7 times that of the five sequences from the Madison River (i.e. 1.5% vs. 0.2%) and the nucleotide diversity was almost 5 times higher over the broad geographic range (i.e. 0.0043 vs. 0.0009). The genetic distances between Haplotype 1 of 18S rRNA (18S_H1) from WV and the haplotypes from other geographic locations (i.e. 18S_H2, 18S_H3, and 18S_H4) were significantly greater than 0 (i.e. 0.011, 0.011, and 0.015, respectively), emphasizing the genetic distinctness of 18S_H1 (Table 5).

ITS-1 variability

As expected, the ITS-1 sequences were more variable than the 18S rRNA sequences; however, there was not much divergence between MT and CA. The three sequences from GenBank, all from CA, but also reported from WV and RUS (see Whipps et al. 2004) are different from the sequences from MT (Table 6). Almost all the ITS-1

sequences were unique and usually multiple clones from the same worm were different; for example, five clones from MT_NS2 were all unique (Table 7); however, in three cases more than one clone from the same worm (MT_LB1, MT_LB2, and MT_NS1) had identical ITS-1 sequences (Table 7). One ITS-1 sequence was found in worms (MT_LB1 and MT_NS1) from the two different MT locations 15 km apart.

Table 3. Variance table for ~460 bp of 18S rDNA sequence for *Myxobolus cerebralis* infecting worms or fish from six states in the USA and one sequence from Europe. Included are 18S sequences for *M. cerebralis* from GenBank and from this study. Nucleotide positions are measured relative to the 5′ position of primer PM9.

			Variable sites						
Mc source Id[a]	Haplotype	GenBank accession	26	43	51	97	98	122	269
USA_WV_LH_Fish1	18S_H1	U96492	T	T	C	A	A	A	T
USA_WV_LH_Tt	18S_H1	U96493	T	T	C	A	A	A	T
USA_WV_LH_Fish2	18S_H1	AF115254	T	T	C	A	A	A	T
USA_CA_MWH Fish	18S_H2	AF115253	G	A	G	T	T	:	T
USA_VA	18S_H3	AY040634	G	G	G	T	T	A	T
DEU_Munich_Tt	18S_H3	AF115255	G	G	G	T	T	A	T
USA_AK_EH_Fish1	18S_H3	EF370478	G	G	G	T	T	A	T
USA_AK_EH_Fish2	18S_H3	EF370479	G	G	G	T	T	A	T
USA_OR_LR_Fish	18S_H3	EF370480	G	G	G	T	T	A	T
USA_OR_ OSU_Fish	18S_H3	EF370481	G	G	G	T	T	A	T
USA_MT_LB_Tt W53	18S_H3	JN134160	G	G	G	T	T	A	T
USA_MT_LB_Tt W54	18S_H3	JN134161	G	G	G	T	T	A	T
USA_MT_LB_Tt W56	18S_H3	JN134162	G	G	G	T	T	A	T
USA_MT_LB_Tt W65	18S_H3	JN134163	G	G	G	T	T	A	T
USA_MT_LB_Tt W70	18S_H4	JN134164	G	G	G	T	T	A	C

[a]See Table 2 for information on the source of the M. cerebralis sequence.

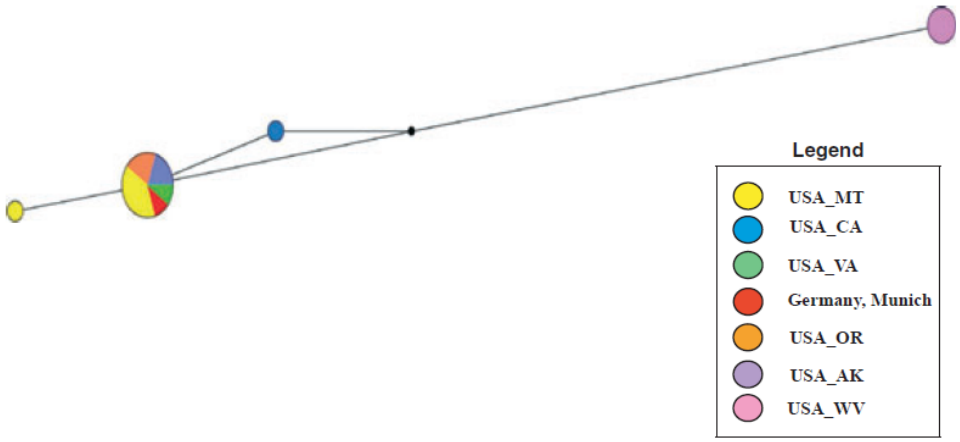

Fig. 1. Haplotype network for *Myxobolus cerebralis* based on the analysis of ~ 460 bp of the18S rDNA sequence. Parasite DNA was examined from worms from six states in the USA and one sequence from Europe. Size of circles is proportional to the

number of individuals sharing a given haplotype. Colors in each haplotype pie indicate the location of collection. Branch lengths are proportional to the number of mutations.

Table 4. Genetic variability for both ~ 460 bp of the 18S rDNA and the ~ 420 bp ITS-1 region.

Gene	No. of sequences	No. of variable sites	No. of haplotypes	% Polymorphic sites	*h*	π	*Ne*	Allelic richness
18S rRNA	5[a]	1	2	0.002	0.400	0.00087	1.471	2.00
18S rRNA	15[b]	7	4	0.015	0.543	0.00425	2.027	2.40
ITS-1	20[a]	22	16	0.053	0.963	0.01157	11.765	8.94
ITS-1	23[b]	23	19	0.055	0.971	0.01168	14.400	9.00

[a]Madison River, MT sequences.
[b]All sequences.
h = haplotype diversity; $h = (n/(n-1))^* \sum p_i^2$, where *n* is the number of sequences examined and p_i is the frequency of the *i*th haplotype.
π = nucleotide diversity, average proportion of nucleotides that differ between two sequences; $\pi = \frac{n}{n-1}\sum_{i=1}^{k}\sum_{j=1}^{k} p_i p_j \pi_{ij}$, where p is the proportion of nucleotides that differ between the ith and jth sequences.
Ne = Number of effective alleles, corrects for differences in the number and distributions of alleles (e.g., two equally frequent alleles vs. 90% of one allele and 10% of the other); $Ne = 1/\sum p_i^2$.

Table 5. The haplotype from WV (18S_H1) is very different from the haplotypes from the other geographic locations. Values are the pair-wise genetic distances (Jukes–Cantor distance—lower triangle) and standard error (upper triangle) for *Myxobolus cerebralis* 18S rRNA.

	18S_H1	18S_H2	18S_H3	18S_H4
18S_H1		0.005	0.005	0.006
18S_H2	**0.011**		0.002	0.003
18S_H3	**0.011**	0.002		0.002
18S_H4	**0.015**	0.005	0.002	

Significant genetic distances are in bold case.

In contrast to the results for 18S rRNA, both the qualitative and quantitative measures of genetic diversity of the 20 sequences from MT are only slightly different from the combined 23 sequences from CA and MT (e.g. 22 vs. 23 of the ~ 420 nucleotide sites were variable, 16 vs. 19 haplotypes, 9.00 vs. 8.94 allelic richness, Table 4). The number of mutations among haplotypes varies from one to four. There was no statistically significant differentiation among CA, MT_LB, and MT_NS based on AMOVA ($P > 0.05$); however, there was significant variation

among the *M. cerebralis* sequences from different worms ($P < 0.001$, Table 8).

Examination of the pairwise Φ_{ST} values for ITS-1 emphasizes the lack of geographical population structure for this locus (Table 9). For example, MT_NS2 is more different from MT_NS1 (0.345, $P < 0.001$) than from CA (0.075). The haplotype network based on the 23 ITS-1 sequences shows the CA sequences mixed in among the MT sequences, highlighting the lack of geographic differentiation (Fig. 2).

DISCUSSION

The genetic structure of parasites in the natural landscape can reveal information critical to understand host-parasite interactions (Real and Biek 2007). In particular, one can learn about parasite invasion strategies for a particular geographical area by looking at the genetic structure at the population level. Although previous studies did not detect genetic population structure for *M. cerebralis*, our addition of data from MT revealed genetic population structure. For 18S rDNA, there was genetic structure among geographic regions; however, the genetic structure did not suggest a single recent invasion by a genetically homogeneous inoculum as previously hypothesized (Whipps et al. 2004). There is also the possibility of the presence of cryptic species (Bartošová and Fiala 2011). However, 18S rRNA sequences for both myxosporean and TAM actinosporean stages of *M. cerebralis* from WV were 99.8% identical and substantially different (89.9% and 77.8%) from two other *Myxobolus* spp. found in salmonid fish (Andree, Gresoviac, and Hedrick 1997). Arsan et al. (2007) found 20 variable nucleotides out of 2,120 in 18S rRNA of *M. cerebral- is* from two fish from Alaska, one fish from the Lostine River, OR, and one fish from the Oregon State University Salmon Disease Lab. Moreover, there were 6 bp differences between the Alaska sequences,

Table 6. Variance table for ~ 420 bp of ITS-1 DNA sequence of *Myxobolus cerebralis* infecting worms (MT) or collected as actinospores (CA), along with DNA sequence from a mix of spores that includes sequences from Eurasia (Germany, DEU and Russia, RUS). Nucleotide positions are measured relative to the 5′ position of primer PM9.

Source of Mc DNA (number of haplotypes)	N	Nucleotide position																											
		42	55	66	79	85	126	127	138	210	212	226	257	259	321	333	335	338	340	346	361	367	412	413	414	415	416	417	418	419
MT_LB1 (2), MT_NS1 (2)[a]	4	G	A	G	C	T	T	T	G	C	A	A	T	T	T	C	T	A	A	A	A	G	A	T	A	T	T	C	A	T
MT_LB2	2	G	A	G	C	T	T	T	G	T	A	A	T	T	T	C	A	A	A	A	A	G	A	T	A	T	T	C	A	T
MT_LB1	1	G	A	G	C	T	T	T	T	C	A	A	T	T	T	C	T	A	A	A	A	G	A	T	A	T	T	C	A	T
MT_LB1	1	T	A	G	G	C	T	T	G	C	A	A	T	T	T	T	T	A	A	A	A	G	A	T	A	T	T	C	A	T
MT_LB1	1	T	A	G	C	T	T	T	G	C	A	A	T	T	T	C	T	A	A	A	A	G	A	T	A	T	T	C	A	T
MT_LB1	1	T	A	G	G	T	A	C	G	T	A	A	T	T	T	T	A	A	A	A	A	G	A	T	A	T	T	C	A	T
MT_LB2	1	G	A	A	C	T	T	T	G	C	A	G	A	T	T	C	A	A	A	A	A	G	A	T	A	T	T	C	A	T
MT_LB2	1	G	A	G	C	T	T	T	G	T	G	A	T	T	T	C	A	A	A	A	A	G	A	T	A	T	T	C	A	T
MT_LB3	1	G	A	G	C	T	A	C	G	T	A	A	T	T	C	C	A	A	A	A	A	G	A	T	A	T	T	C	A	T
MT_NS1	1	G	A	A	C	T	T	T	G	C	A	A	A	T	T	C	A	A	A	A	A	G	A	T	A	T	T	C	A	T
MT_NS1	1	G	A	G	C	T	T	T	G	T	A	A	T	C	T	C	A	A	A	A	A	G	A	T	A	T	T	C	A	T
MT_NS2	1	T	A	A	G	T	T	T	G	C	A	A	A	T	T	T	A	A	A	A	A	G	A	T	T	C	A	T	:	:
MT_NS2	1	T	T	A	G	T	T	T	G	C	A	A	A	T	T	T	A	T	A	A	A	G	A	T	T	C	A	T	:	:
MT_NS2	1	T	A	G	G	T	T	T	G	T	A	A	T	T	T	T	A	A	G	A	A	G	A	T	A	T	T	C	A	T
MT_NS2	1	T	A	G	G	T	A	C	G	T	A	A	A	T	T	T	A	A	A	A	A	G	A	T	A	T	T	C	A	T
MT_NS2	1	T	A	G	G	T	T	T	G	C	A	A	T	T	T	T	T	A	A	G	A	A	A	T	A	T	T	C	A	T
CA_A1	1	T	A	A	G	T	T	T	G	C	A	A	T	T	T	T	A	A	A	A	C	G	T	C	A	T	T	C	A	T
CA_A2	1	T	A	A	G	T	T	T	G	T	A	A	T	T	T	T	A	A	A	A	A	G	A	T	A	T	T	C	A	T
CA_A3	1	T	A	G	G	T	T	T	G	C	A	A	T	T	T	T	A	A	A	A	C	G	A	T	A	T	T	C	A	T
Mix: CA, WV, USA; DEU; RUS[b]	1	T	A	R	G	T	T	T	G	Y	A	A	T	T	T	T	A	A	A	A	M	G	A	T	A	T	T	C	A	T

[a]This haplotype was found in worms from both MT locations. LB = Lyons Bridge and NS = North Slide.
[b]DNA sequence was from a mix of actinospores, myxospores, and M. cerebralis isolated from rainbow trout heads.

Table 7. Worm identification, number of ITS-1 clones sequenced per worm, and number of unique haplotypes found in each worm.

Mc source Id[a]	No. of clones/worm	Accession #	Haplotypes
MT_LB1	6	JN134165-JN134170	ITS_H1 (2)[b], ITS_H13, ITS_H14, ITS_H15, ITS_H16
MT_LB2	4	JN134171-JN134174	ITS_H2 (2), ITS_H10, ITS_H11
MT_LB3	1	JN134175	ITS_H12
MT_NS1	4	JN134176-JN134179	ITS_H1 (2)[b], ITS_H8, ITS_H9
MT_NS2	5	JN134180-JN134184	ITS_H3, ITS_H4, ITS_H5, ITS_H6, ITS_H7

[a]See Table 2 for information on the source of the M. cerebralis sequence.
[b]Haplotype ITS_H1 was found in worms from both LB and NS locations.

Table 8. AMOVA indicates that genetic variation among ITS-1 sequences is highest within worms, followed by between worms at the same location. There is no significant genetic variation among the two MT locations (LB and NS) and the CA sequences.

Source	df	SS	MS	%	*P*
Among CA, MT_LB, MT_NS	2	10.570	5.285	0	>0.05
Among worm	2	14.617	7.308	33	<0.001
Within worms	17	37.450	2.203	67	<0.001
Total	21	62.636		100	

Table 9. Pairwise Φ_{ST} values (below diagonal) of *Myxobolus cerebralis* ITS-1 DNA isolated from four worms (4–6 cloned sequences per worm) from MT and three sequences from CA. Probability values are shown above the diagonal.

	CA	MT_LB1	MT_LB2	MT_NS1	MT_NS2
CA		0.049	0.035	0.030	0.270
MT_LB1	0.380*		0.008	0.272	0.007
MT_LB2	0.544*	0.270**		0.643	0.018
MT_NS1	0.534*	0.058	0.016		0.001
MT_NS2	0.075	0.278**	0.357*	0.345***	

*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

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and they differed from the Oregon lab sequence by 6–8 bp, and the Oregon Lostine River sequence by 3–5 bp. In summary, our findings based on the 18S rRNA gene suggest that *M. cerebralis* may have been introduced to North America more than once, and this conflicts with previous reports based on ITS-1 sequences (Whipps et al. 2004).

Surprisingly, the comparison of the ITS-1 gene sequences between MT and other localities (i.e. CA, WV, DEU, and RUS) did not support the idea of distinct lineages. We found that ITS-1 is highly variable both between and within individuals from two sites of the Madison River, MT. This statistically significant inter- and intraindividual variation resulted from 16 different haplotypes from 20 sequences. Most haplotypes in the Madison River, MT, were unique, and comparison with the ITS-1 sequences from CA, WV, DEU, and RUS (Whipps et al. 2004) showed that there was no geographic pattern in the variation. It is curious the 18S rRNA sequences from WV showed geographic differentiation, while the single ITS-1 sequence from the same geographic area did not. The lack of geographic differentiation of ITS-1 may be due to our small sample size.

The high variability of ITS-1 for M. cerebralis from within a stretch of about 10 km of the Madison River, MT, was unexpected; however, even higher ITS-1 sequence difference (43.5%) was found for the cosmopolitan myxozoan species Kudoa thyrsites (Whipps et al. 2003). ITS-1 sequences are tandemly repeated in most genomes and have hundreds or thousands of copies (Hillis 1998). The variability in the M. cerebralis ITS-1 gene could be because concerted evolution has not yet homogenized the variable ITS-1 sequences. Concerted evolution homogenizes repetitive DNA sequences of a multigene family, so they share much higher sequence identity within a species than between two different species (Liao 2003). Another possibility is that the ITS-1 variability was already present in the population when the 18S rRNA sequences began to diverge, as is sometimes seen when comparing gene trees and species trees (Page and Charleston 1997). Because of the small genetic distances among ITS-1 sequences, the presence of pseudo-genes is unlikely.

The within-worm variation in ITS-1 may result from multiple infections with distinct genotypes of *M. cerebralis*. Nevertheless, the

possibility of polyploidy cannot be ruled out; however the only published study of myxozoan chromosomes did not find *Zschokkela nova* to be polyploid (2N = 6; Tiutiaev 2008). ITS-1 has been used to examine within- individual differences for other taxa, such as the fungus *Pythium* sp., where multiple ITS sequences were found in a single isolate (Belbahri et al. 2008) and the marine annelid *Platynereis dumerilli*, which was documented for polymorphic ITS sequence from a single individual (Hui et al. 2007). In addition, polyploidy has also been reported in fungi (Ko and Jung 2002).

Despite its importance, no studies have yet been performed to investigate the degree of genetic diversity and virulence of different lineages of myxozoans. Our data suggest at least two introductions of *M. cerebralis* to the USA. Additional data from Eurasia would strengthen this hypothesis. Our discovery of two divergent 18S groups in North America raises the possibility of variation in virulence of *M. cerebralis* within the USA and perhaps in Eurasia. Our study forms the basis for further study examining the contribution of genetic variation in the parasite to fish disease variability.

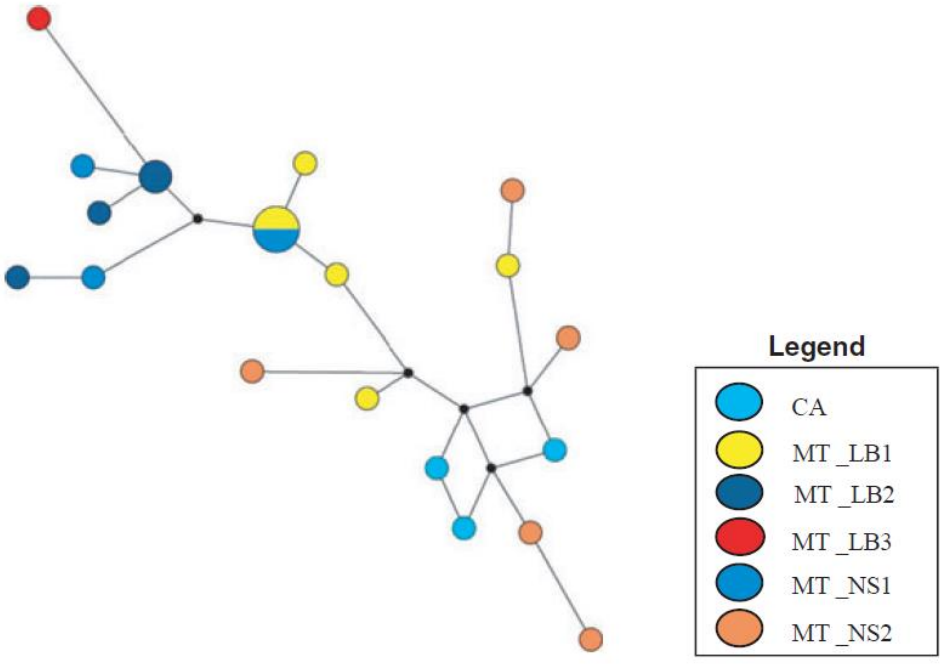

Fig. 2. Haplotype network of ITS-1 sequences (~ 420 bp) from the current study and from Whipps et al. (2004). Size of circles is proportional to the number of

sequences sharing a given haplotype. Clones from the same individual have the same color. Branch lengths are proportional to the number of mutations.

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