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Nilanjan Lodh

Marquette University, [nilanjan.lodh@marquette.edu](mailto:nilanjan.lodh@marquette.edu)

Lori Stevens

University of Vermont

Billie Kerans

Montana State University - Bozeman

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# Prevalence of *Myxobolus cerebralis* Infections Among Genetic Lineages of *Tubifex tubifex* at Three Locations in the Madison River, Montana

N. Lodh

*Department of Biology, The University of Vermont,  
Burlington, VT*

L. Stevens

*Department of Biology, The University of Vermont,  
Burlington, VT*

B. Kerans

*Department of Ecology, Montana State University,  
Bozeman, MT*

**Abstract:** Host biodiversity can impact disease risk and influence the transmission of parasitic disease. Stream sediment-dwelling worms, *Tubifex tubifex* (Clitellata: Oligochaeta), are the definitive host of the parasite *Myxobolus cerebralis* (Myxozoa: Myxosporidia), which causes whirling disease in salmonid fishes. Genetic diversity of *T. tubifex* is correlated with host susceptibility to *M. cerebralis*, and mitochondrial Lineage III is generally

shown to be more likely to be infected and produce the triactinomyxon (TAM) spores than other lineages. We determined the mitochondrial lineage, relative abundance, and prevalence of infection of *T. tubifex* collected at 3 sites in the Madison River, Montana, where previous study had shown variation in whirling disease prevalence and severity in caged trout fry. We also compared visual identification of TAMs released from cultured worms with a molecular genetic assay (diagnostic polymerase chain reaction [PCR]) for parasite detection of both infected and uninfected worms. We estimated that mitochondrial Lineage III was most abundant at the site previously shown to have high fish disease and was also most likely to be infected. The 2 techniques for detecting parasite infection did not always agree, and the likelihood of PCR (+) and spore (-) was not significantly different from PCR (-) and spore (+). Differences in the relative infection prevalence for these 2 lineages may explain the wide range of infection in natural streams.

Whirling disease was first described among European populations of farmed rainbow trout (*Oncorhynchus mykiss*), which is a native North American salmonid species that had been widely introduced into Europe and elsewhere as a food fish (Hofer, 1908). Whirling disease is caused by the Eurasian native myxozoan *Myxobolus cerebralis* (Mc), which first appeared in North America in the 1950s (Hoffman, 1970) and since has been reported in more than 25 states, from New York to Alaska (Bartholomew and Reno, 2002; Arsan et al., 2007). The disease has recently been recognized as the principal cause of major declines of wild rainbow trout populations in the intermountain region of the United States (Vincent, 1996).

The distribution of *M. cerebralis* and the severity of whirling disease among wild rainbow trout populations are locally and regionally variable. It is unlikely that differences in the parasite or rainbow trout host can account for all of the variation in disease severity seen in wild rainbow trout populations. Genetic variability of *M. cerebralis* among populations is very low (Andree et al., 1999; Whipps et al., 2004), and most rainbow trout strains differ little in their susceptibility to the triactinomyxon (TAM) spores that infect fish (Vincent, 2002). However, the distribution and abundance of *T. tubifex*, the aquatic oligochaete that serves as the definitive host of *M. cerebralis*, is a major factor in the spread, severity, and potential control of whirling disease among wild fish populations (Hedrick et al., 1998; Kerans and Stevens, 1998; Rasmussen et al., 1998, 1999; Beauchamp et al., 2005; Krueger et al., 2006).

*Tubifex tubifex* exhibits considerable variability in susceptibility to the parasite. There are multiple genetic lineages (I-VI) of *T. tubifex* (Sturmbauer et al., 1999; Beauchamp et al., 2001; Crottini et al., 2008) that can be distinguished with the use of mitochondrial (16S) and genomic (18S and ITS) DNA sequences. Susceptibility of *T. tubifex* lineages to infection by the myxospores of *M. cerebralis* ranges from highly susceptible (Lineage III) and moderately susceptible (Lineage I), to completely resistant (Lineages V and VI; Stevens et al., 2001; Beauchamp et al., 2002; Steinbach et al., 2006; Arsan et al., 2007; Rasmussen et al., 2008). Although much of the work identifying genetic lineages of *T. tubifex* is based on the mitochondrial 16S rDNA gene, it is unlikely that this gene directly confers resistance to the parasite (Arsan et al., 2007).

Because laboratory infection experiments show considerable variability in susceptibility of *T. tubifex* strains (e.g., Kerans et al., 2004), local and regional variability in whirling disease severity in rainbow trout is likely related to the particular lineage of *T. tubifex* and its density at a location (Beauchamp et al., 2005). In the Madison River, Montana, where *M. cerebralis* is epizootic (Vincent, 1996), whirling disease risk is positively correlated with density of infected *T. tubifex* (Krueger et al., 2006); however, studies of the mitochondrial lineages present in various habitats and their relative infection prevalence are lacking. Determining the relative abundance of genetic lineages of *T. tubifex* in the Madison River and their associated parasite prevalence can pinpoint areas to target disease control efforts. In the present study, we determined the lineages of *T. tubifex* present at 3 sites in the Madison River that had previously shown significant differences in caged trout fry infection prevalence and severity (Krueger et al., 2006) and measured the prevalence of infection with *M. cerebralis* in these worms.

Lineages of *T. tubifex* can only be distinguished based on DNA sequence (Sturmbauer et al., 1999; Beauchamp et al., 2001); however, infection with *M. cerebralis* can be detected by both visual observation of TAMs and by using a polymerase chain reaction (PCR) assay specific for *M. cerebralis* (Andree et al., 1998; Granath et al., 2007; Gilbert and Granath, 2008). In fish, the PCR test improved the detection of *M. cerebralis* compared to visual observation because it can detect the presence of the parasite at lower thresholds (Andree et

al., 1998). In addition, the PCR test can confirm infected worms are producing TAMs of *M. cerebralis* and also identify worms that are infected and not yet producing TAMs (Granath and Vincent, 2010), as well as worms that may have stopped producing TAMs, but remain infected (Gilbert and Granath, 2001). For the best understanding, both methods should be used.

The present study directly compared the lineages of both infected and uninfected worms and examined infection prevalence with the use of 2 methods, i.e., visual identification of TAM production and PCR detection. To examine the role of spatial variation in the abundance of the different lineages, the following questions were addressed: (1) What is the relative abundance of different lineages of *T. tubifex* at various locations in the Madison River? (2) What is the parasite prevalence among different lineages? (3) Is the diagnostic PCR test equivalent to visual observation of TAMs for assessing infection?

The study locations were 3 side channels, North Slide, Pine Butte, and Lyons Bridge in the upper 39 km of the Madison River between Quake Lake and Ennis Lake in Madison County, Montana, where whirling disease risk had been measured in 1999 and 2000 (Krueger et al., 2006). In 2005, live oligochaetes were collected in May, June, and July with the use of kick nets, and were stored on ice for transportation to the laboratory. Specimens with hair chaetae were likely to be *T. tubifex* and were placed in 4-ml well plates with dechlorinated tap water at 15 C on 12:12 light:dark light regime. For 1,253 worms, infection was determined by scanning each well for TAMs twice over a 2-wk period with the use of a dissecting microscope (40×). We found 46 worms producing TAMs and 1,207 that were not.

After the 2-wk period, we used molecular genetic assays to test 102 worms, which included 46 worms that produced TAMs and 56 randomly selected worms that did not produce TAMs. The remaining 1,151 non-TAM producing worms were not genetically tested. DNA was extracted from these 102 worms with the use of Nucleospin® kits (BD Biosciences Clontech, Foster City, California). The DNA was used to amplify and sequence host DNA to determine the genetic lineage of *T. tubifex* (Sturmbauer et al., 1999) and parasite DNA was also amplified to assess the prevalence of infection for each lineage and to

examine the agreement between the PCR test for *M. cerebralis* infection and visual observation of TAMs produced by infected worms. The PCR assay also served to confirm that worms producing TAMs were infected with *M. cerebralis* and not another myxozoan. Approximately 350 base pairs (bp) of the 16S rRNA gene were sequenced to determine the mitochondrial lineage of *T. tubifex* with the use of previously described procedures (Beauchamp et al., 2001). The 16S rDNA sequences were then compared to sequences previously published in GenBank with the use of the NCBI BLAST algorithm, which identified the samples from this study as Lineage III and Lineage I of *T. tubifex* and *Rhyacodrilus* sp. Of the 102 worms that were tested, 61 were Lineage III, 29 were Lineage I, and 12 were *Rhyacodrilus* sp.

The relative abundance of the 2 *T. tubifex* lineages and *Rhyacodrilus* sp. was variable among the 3 study locations and Lineage III was most abundant at the site that previously had highest fish disease, Lyon Bridge (likelihood ratio:  $\chi^2 = 47.11$ ,  $df = 4$ ,  $P < 0.0001$ ; JMP® Version 8, SAS Institute, Inc., Cary, North Carolina). The relative abundance did not vary among the sampling dates (likelihood ratio:  $\chi^2 = 7.01$ ,  $df = 4$ ,  $P > 0.05$ ; JMP® Version 8, SAS Institute). Most Lineage III (40/61) worms were collected from Lyon Bridge, whereas most Lineage I (14/29) worms were from North Slide and all 12 *Rhyacodrilus* sp. were collected from Pine Butte (Table I). *Rhyacodrilus* sp. was not included in the analysis hereafter.

Of the 46 worms that produced TAMs, 38 (83%) were Lineage III and 8 (17%) were Lineage I (Table II). In contrast, 23 (52%) of the 44 non-TAM producing worms were Lineage III; 21 (48%) were Lineage I. Thus, based on TAM production of field-collected worms, more Lineage III worms were producing TAMs than Lineage I worms (likelihood ratio:  $\chi^2 = 9.723$ ,  $P < 0.002$ ; JMP® Version 8, SAS Institute).

Forty-eight of the 90 individuals examined were positive for parasite infection by PCR (Table II). Of these, only 7 (15%) were Lineage I, whereas 41 (85%) were Lineage III. For the PCR negative worms, 22 (52%) were Lineage I, and 20 (48%) were Lineage III. Thus, by the PCR test, more Lineage III worms were infected than Lineage I worms (likelihood ratio:  $\chi^2 = 15.127$ ,  $P < 0.0001$ ; JMP® Version 8, SAS Institute).

Both ways of assessing infection showed that Lineage I worms had a lower prevalence of infection than worms of Lineage III; however, the 2 methods did not always agree. Nine individuals were scored positive by Mc diagnostic PCR and did not produce TAMs (Table III), presumably because either PCR can detect low levels of infection that cannot be detected visually, PCR can detect infection before worms start producing TAMs, or some infected worms do not produce TAMs. Eight worms produced TAMs and were not positive by Mc diagnostic PCR. Attempts to amplify and sequence the 18S rDNA (Andree et al., 1998, 1999) gene were successful for 4 of these worms, which were identified as *M. cerebralis* by BLAST search algorithm (NCBI BLAST). Because all samples were PCR amplified and sequenced for *T. tubifex* lineage identification as described above, we rule out PCR inhibition. We speculate that perhaps there is another myxozoan present that fails to amplify with the genera specific primers or that the worms shed almost all of their TAMs and the infection was below the limits of PCR detection.

Lineage I showed a relatively high degree of agreement ( $\kappa = 0.73$ ;  $SE = 0.15$ ) between the PCR screen and TAM presence, whereas Lineage III had a lower degree of agreement ( $\kappa = 0.53$ ,  $SE = 0.11$ ; JMP® Version 8, SAS Institute). Kappa values show the agreement of the PCR and visual observation tests (-1 = negative association, 0 = random, 1 = complete agreement). The symmetry for lack of agreement (Bowker Symmetry; JMP® Version 8, SAS Institute) for Lineage I was lower (0.33) than Lineage III (0.69). This test checks for symmetry in 2-way tables and the test decision is based on a  $\chi^2$  approximation of the distribution of the test statistic (Krampe and Kuhnt, 2007). The Bowker Symmetry is not significantly different from random ( $P > 0.05$ ) for either lineage, indicating that PCR (+) and TAM (-) is as likely as PCR (-) and TAM (+).

Parasite susceptibility varies among *T. tubifex* lineages, but has not been directly linked to the 16S mitochondrial gene. Variation within lineages has also been documented (Beauchamp et al., 2002; Arsan et al., 2007; Rasmussen et al., 2008). Previous assessment of parasitic infection in *T. tubifex* has been done by observing TAM production (Beauchamp et al., 2002; Kerans et al., 2004, 2005; Rasmussen et al., 2008; Hallett et al., 2009) and by diagnostic PCR originally developed for fish and later used in *T. tubifex* (Gilbert and

Granath, 2001). More recently, qPCR was used to detect the presence of *M. cerebralis* in *T. tubifex* (Arsan et al., 2007). In addition, many studies (Gilbert and Granath, 2001; DuBey and Caldwell, 2004; Koel et al., 2006; Arsan et al., 2007; Baxa et al., 2008; Hallett et al., 2009; Granath and Vincent, 2010) used *M. cerebralis* diagnostic PCR to confirm infection in worms, which had been visually identified as TAM producers.

In laboratory experiments using worms collected from California, Montana, Utah, and Argentina, Rasmussen et al. (2008) observed higher Lineage III susceptibility in comparison to Lineage I. In comparison, Beauchamp et al. (2002) found variation between 2 locations of the Colorado River, Colorado. At 1 site, they found infection higher in Lineage I than Lineage III, whereas only Lineage III was infected at the other location. Other field observations (DuBey et al., 2004) and combined field and experimental studies (Arsan et al., 2007), show Lineage III had higher infection prevalence than Lineage I. Our investigation thus supports previous work in that it showed infection is more prevalent in Lineage III than in Lineage I, consistent with the reported higher susceptibility. Forty-one Lineage III worms (from an estimate of 614 Lineage III worms) were positive by PCR for parasitic infection, suggesting that the prevalence for Lineage III is ~7%, whereas only 7 Lineage I worms (from an estimated 580 Lineage I worms) were infected (~1%).

Higher prevalence for Lineage III over Lineage I in these 3 side channels of the Madison River suggest that, in a natural setting, local whirling disease prevalence is influenced by the specific lineage of *T. tubifex* present, along with other ecological and physiological parameters. These data from 2005, showing high abundance of Lineage III at Lyon Bridge, a preponderance of Lineage I at North Slide, and collection of *Rhyacodrilus* sp. only at Pine Butte, reflect the whirling disease risk measured with the use of sentinel cages in 1999-2000, where Lyon Bridge had the highest risk and Pine Butte the lowest (Krueger et al., 2006).

These results are important for management of wild fish populations because high Lineage III abundance provides more host habitat for *M. cerebralis*. Determination of site-specific contribution is important to implement small spatial scale management strategies,



which may have a large impact on reducing whirling disease throughout the river system (Krueger et al., 2006).

Worm habitat variability may also play a key role in disease dynamics if different environmental tolerances and physiological parameters are also correlated with genetically based differences in susceptibility (Kerans et al., 2005). Disease can be variable among side channels and the main channel (Krueger et al., 2006) and myxospore dormancy and *T. tubifex* life span are affected by ecological phenomena, such as changes in water temperature, sedimentation, and pH. In addition, *T. tubifex* can tolerate low oxygen levels, desiccation, and a wide range of temperatures (Reynoldson, 1987; Brinkhurst, 1996). However, it is still an open question with respect to the manner in which genetic differences among lineages contribute to difference in infection prevalence or determine the abundance of the lineages in different habitats.

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## Author Affiliation

N. Lodh, L. Stevens, and B. Kerans\*, Department of Biology, The University of Vermont, 109 Carrigan Drive, 120A MLS, Burlington, Vermont 05405;  
\*Department of Ecology, 310 Lewis Hall, Montana State University, Bozeman, Montana 59717. e-mail: nlodh@uvm.edu