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Using real-time PCR and Bayesian analysis to distinguish susceptible tubificid taxa important in the transmission of *Myxobolus cerebralis*, the cause of salmonid whirling disease

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ABSTRACT

Aquatic oligochaetes have long been appreciated for their value in assessing habitat quality because they are ubiquitous sediment-dwelling filter feeders. Many oligochaete taxa are also important in the transmission of fish diseases. Distinguishing resistant and susceptible taxa is important for managing fish disease, yet challenging in practice. *Tubifex tubifex* (Oligochaeta: Tubificidae) is the definitive host for the complex life-cycle parasite, *Myxobolus cerebralis,* the causative agent of salmonid whirling disease. We developed two hydrolysis probe-based qualitative real-time PCR (qPCR) multiplex assays that distinguish among tubificid taxa collected from the Madison River, Montana, USA. The first assay distinguishes *T. tubifex* from *Rhyacodrilus* spp.; while the second classifies *T. tubifex* identified by the first assay into two genetic lineages (I and III). Specificity and sensitivity were optimized for each assay; the two assays showed specificity of 94.3% and 98.6% for the target oligochaetes, respectively. DNA sequencing verified the results. The development of these assays allowed us to more fully describe tubificid community composition (the taxa and their abundance at a site) and estimate the relative abundances of host taxa. To relate tubificid relative abundance to fish disease risk, we determined *M. cerebralis* infection prevalence in samples identified as *T. tubifex* using similar molecular techniques. Given prior information (i.e., morphological identification of sexually mature worms), Bayesian analysis inferred that the first qPCR assay improved taxonomic identification. Bayesian inference of the relative abundance of *T. tubifex*, combined with infection assay results, identified sites with a high prevalence of infected *T. tubifex*. To our knowledge, this study represents both the first assessment of oligochaete community composition using a qPCR assay based on fluorescent probes and the first use of Bayesian analysis to fully characterize the dominant infected taxa in streams where whirling disease is observed.

1. Introduction

Benthic oligochaetes, such as tubificid worms, are ubiquitous sediment filter feeders in streams, reflect important aspects of stream biology (Kerans and Karr, 1994) and can be important in the transmission of fish parasites (Brinkhurst, 1996). *Tubifex tubifex* (Oligochaeta: Tubificidae) is the definitive host for the parasite *Myxobolus cerebralis* (Myxozoa: Myxobolidae), the causative agent of salmonid whirling disease (Wolf et al., 1986; Vincent, 1996). The complex, two-host life-cycle of *M. cerebralis* (*T. tubifex* produces a spore infective to salmonids and salmonids produce a spore infective to *T. tubifex*) presents challenges to scientists and managers seeking to reduce disease in wild salmonid populations (Kerans and Zale, 2002; Gilbert and Granath, 2003). The distribution of *M. cerebralis* and the severity of whirling disease among wild fish populations are locally and regionally variable (Nickum, 1999; Kaeser et al., 2006; Kaeser and Sharpe, 2006; Krueger et al., 2006). For example, rainbow trout (*Oncorhynchus mykiss*) populations suffered severe declines in some drainage systems of the United States (USA), while other populations show little or no effect after establishment of the parasite (Vincent, 1996; Modin, 1998; Sandell et al., 2001). Susceptibility or resistance of the fish host is an important factor influencing the severity of *M. cerebralis* infections (Wagner et al., 2002a,b; Fetherman et al., 2011). However, much of the spatial variability is likely to be due to variation in oligochaete communities (Lodh et al., 2011), genetic variation in the parasite (Andree et al., 1999; Whipps et al., 2004a,b; Lodh et al., 2012), salmonid population dynamics (Downing et al., 2002) and the environment (Krueger et al., 2006; McGinnis and Kerans, 2012).

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Interactions among these factors (Kerans and Zale, 2002) also contribute to variation in disease risk.

Unfortunately, taxonomic identification of oligochaetes depends largely on the morphological characteristics of sexually mature worms. This life stage constitutes a relatively short period in the life cycle (Kathman et al., 1998) and may represent a small proportion of a sampled population depending on the season of collection. Molecular genetic assays based on DNA sequence provide a method to identify morphologically similar worms. Previously, species-specific primers were developed that identified immature *T. tubifex* (based on a single amplicon of 192 bp) from other taxa (indicated by multiple bands, lack of amplification or different sized PCR products) by amplifying the Internal Transcribed Spacer 1 region (ITS-1) of nuclear ribosomal DNA (Hallett et al., 2005). However, the non-coding ITS-1 region is highly variable and for distinguishing species, the ITS-1 region was found to be appropriate. Regions of DNA differ in their genetic variability and generally mitochondrial DNA is more variable than nuclear DNA. Assays, based on mitochondrial 16S ribosomal DNA (Beauchamp et al., 2001), are available to distinguish among genetic lineages of *T. tubifex* (Sturmbauer et al., 1999; Beauchamp et al., 2002) that vary in parasite susceptibility (Rasmussen et al., 2008; Lodh et al., 2011). The nuclear 18S rDNA gene is approximately 1,800 bp in length including regions that could be the basis of assays for identifying *Tubifex* spp. and other genera; less conserved regions can distinguish lineages within *T. tubifex*. Both previous assays were based on visualizing PCR products in agarose gels. Real-time PCR is generally more reliable than regular PCR and because it does not require visualizing PCR products in a stained agarose gel, it is less time consuming (Espy et al., 2006; Mackay, 2007). Finally, it has been well documented that in parasitological studies using this method, the minimum error is ~10% (Souaze et al., 1996) and the level of specificity increases (Bell and Ranford-Cartwright, 2002).

Using two regions of the 18S gene, one that varied among genera and one that varied among *T. tubifex* lineages, we developed two multiplex molecular genetic hydrolysis probe-based qPCR assays to qualitatively distinguish between tubificid taxa and facilitate the description of the tubificid community composition in the Madison River, Montana, USA. Whirling disease caused a 90% reduction in rainbow trout populations in the 40 km section where rainbow trout reside (Vincent, 1996) and the loss of millions of dollars in revenue from recreational fishing (Nickum, 1999). Tubificid communities are of major importance in the transmission of whirling disease to salmonid fish (Krueger et al., 2006; Elwell et al., 2009; Lodh et al., 2011).

The tubificid taxa present in our study reach of the Madison River are similar to other watersheds in North America (Brinkhurst and Jamieson, 1971; Stimpson, 1982; Brinkhurst, 1986). The majority of worms belong to five taxa: lineages I and III of *T. tubifex* (T_I and T_{III}, respectively), *Rhyacodrilus* spp., *Ilyodrilus* sp. and *Limnodrilus hoffmeisteri* (Lodh et al., 2011; McGinnis and Kerans, 2012). Only *T. tubifex* is a competent host for *M. cerebralis* (Kerans et al., 2004; Elwell et al., 2009) and there is significant variation in the susceptibility within and among *T. tubifex* lineages (Rasmussen et al., 2008). Most T_{III} worms are highly susceptible to *M. cerebralis* (Baxa et al., 2008) compared with T_I worms and other lineages that range from low susceptibility to complete resistance (Elwell et al., 2006; Arsan et al., 2007; Rasmussen et al., 2008; Lodh et al., 2012).

In our study reach, the tubificid community was almost exclusively comprised of T_I, T_{III}, *Rhyacodrilus* spp., *L. hoffmeisteri* and rarely *Ilyodrilus* sp. (Kerans et al., 2005; Lodh et al., 2011). *Ilyodrilus* sp. specimens comprise less than 0.1% of the oligochaete community and because *L. hoffmeisteri* specimens are easily distinguished from the other taxa morphologically, these two taxa were not included in these initial efforts to develop molecular genetic probes. Immature T_I, T_{III}, and *Rhyacodrilus* spp. specimens cannot be distinguished morphologically and even sexually mature T_I and T_{III} worms can only be differentiated by DNA sequences. Consequently, we developed two qPCR hydrolysis probe-based multiplex assays to help fully characterize the tubificid community: one distinguishes *T. tubifex* from the non-host *Rhyacodrilus* spp.; and a second distinguishes between T_I and T_{III} lineages. Using laboratory-reared worms of known taxonomic identity, assay amplification efficiency and reproducibility were assessed and the specificity of each probe was tested. The probe data were analyzed using simple Bayesian statistics to estimate the relative abundance of T_I, T_{III}, and *Rhyacodrilus* spp. and reveal relationships between host/non-host taxa and host-parasite dynamics. To our knowledge, this is the first study combining molecular data of host community composition and the associated parasite to address fish disease risk using qPCR assays and Bayesian statistics.

2. Materials and methods

2.1. Study design

Our overall goal was to estimate site-specific prevalence of infected worm hosts (our measure of whirling disease risk) using the relative abundance of T_I, T_{III} and *Rhyacodrilus* spp. (proportion of each taxon of the total numbers of the three taxa) at six sites along the Madison River. To accurately identify the tubificid taxa, we developed two hydrolysis (i.e. dual-labeled) probe qPCR assays. Assays were verified by morphological identification and DNA sequence analysis. The first assay (hereafter referred to as the "Tt or Rhy" assay) distinguished between *T. tubifex* and *Rhyacodrilus* spp.; the second (hereafter referred to as the "T_I or T_{III}" assay) further examined the *T. tubifex* samples identified by the first assay to distinguish between T_I and T_{III}. In the samples identified as *T. tubifex*, we compared two approaches to assay infection with *M. cerebralis*, a melting curve analysis and a hydrolysis probe PCR.

To verify assay results, we selected ~20% of samples tested in each assay for DNA sequencing. More specifically, we sequenced all samples that were ambiguous in each assay or differed from morphological identifications and then randomly selected samples to reach the goal of 20%. Simple Bayesian statistics were used to predict: (i) the relative abundance of *T. tubifex* and *Rhyacodrilus* spp. at six sites on the Madison River, and (ii) site-specific prevalence of infected hosts at these sites by combining the results from the two taxa assays with the *M. cerebralis* infection data.

2.2. Sample acquisition, handling and preparation

Tubificid communities were sampled in the upper Madison River watershed between Earthquake Lake and Ennis Lake in Madison County, Montana, USA in June and August of 2009. Six sites (side channels or tributary reaches, Fig. 1) previously showed variation in fish disease risk (Krueger et al., 2006) and tubificid communities. Oligochaetes were collected with at least three timed (2 min) kick-net (120 μm mesh) samples along the stream banks. At each site, sampling was repeated until 250 worms had been collected or 2.5 h of sorting time was reached. Live worms were separated from sediment, placed in jars with stream water and stored on ice for transportation to the laboratory for examination under a dissecting microscope. At least 88 worms with hair and pectinate chaetae (i.e. *T. tubifex*, *Rhyacodrilus* spp. and *Ilyodrilus* sp.) from each site were randomly selected for the genetic assays, which were run in 96-well plates (88 samples and eight controls). The anterior half of each worm was slide mounted and morphologically identified using taxonomic keys (Kathman et al., 1998). Genomic DNA was extracted from the posterior half using the E.Z.N.A 96 Tissue DNA

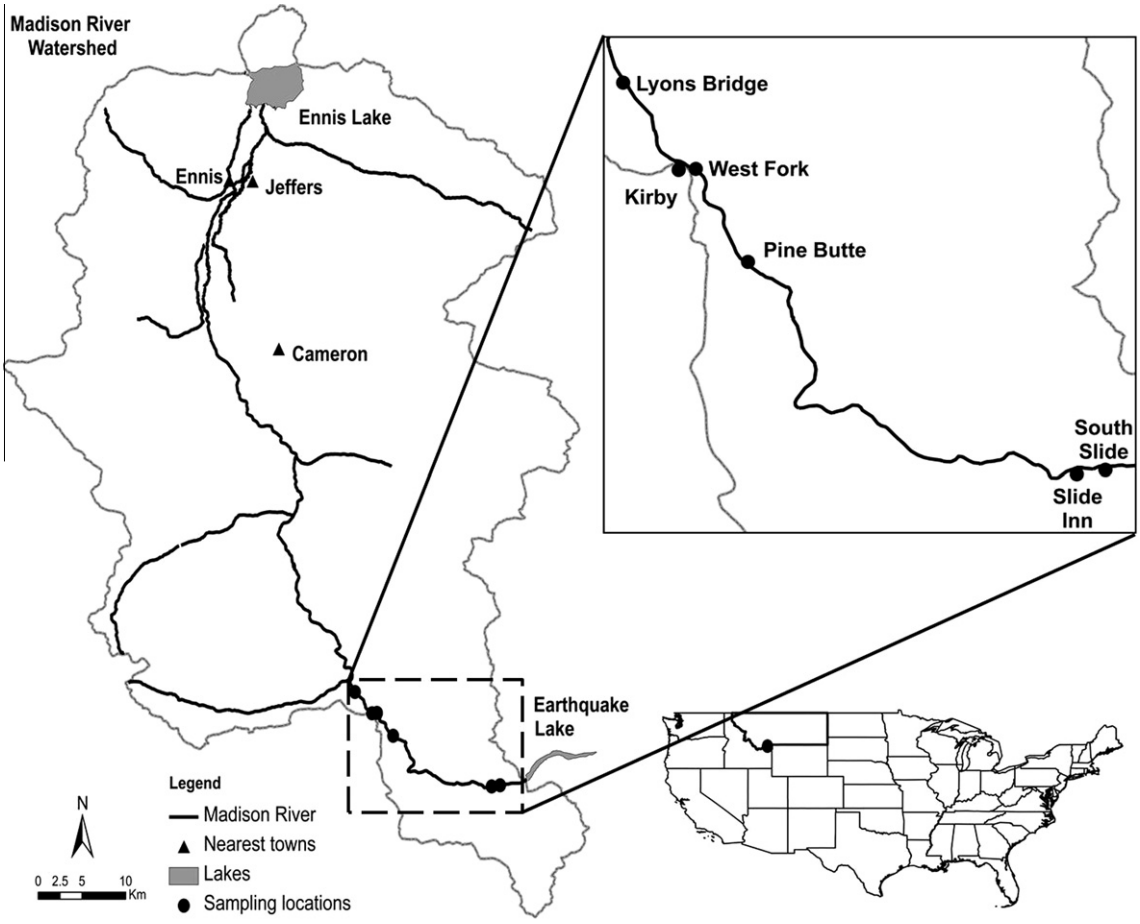


Fig. 1. Study area map of the Madison River watershed in southwestern Montana, USA. Black circles indicate the six 2009 collection sites of tubificid taxa (lineages I and III of *Tubifex tubifex*, *Rhyacodrilus* spp., *Ilyodrilus* sp. and *Limnodrilus hoffmeisteri*). The black dot within the United States map (bottom right corner) shows the watershed location within the United States and the state of Montana. Nearest towns (black triangles) and lakes are presented.

Kit (Omega Bio-tek, Inc., USA), quantified using spectrophotometry (Nanodrop; Thermo Scientific, USA) and stored at −20 °C.

2.3. PCR primers and probes design

2.3.1. Taxa assays

Previous studies (Lodh et al., 2011) and morphological data (this study) indicated that >95% of the worms were T_I, T_{III} or *Rhyacodrilus* spp.; therefore, RealTimeDesign™ software (Biosearch Technologies, Inc., USA) was used to design the two assays based on ~100 bp regions of the 18S rRNA gene that varies among, but not within, these taxa. Multiple DNA sequences were used to design the probes including: (i) 20 worms collected in 2009, (ii) worms cultured in the laboratory from individuals previously collected at these sites, and (iii) GenBank sequences. The 18S gene was sequenced following previously described protocols (Erseus et al., 2002).

The probes and primers for each assay (Table 1) were purchased from Biosearch Technologies, Inc., USA. One advantage of the two-step assay design is that the "T_I or T_{III}" assay needs only to be performed on those worms identified as *T. tubifex* in the "Tt or Rhy" assay. Also, identification of the three taxa using two multiplex qPCR assays in tandem minimizes crosstalk (i.e., fluorescent signal overlap) between dyes in the reaction mix (Supplementary Table S1); and each assay could be optimized for the particular set of primers and probes.

Table 1
PCR primer and probe sequences used in the two taxa assays (identifying *Tubifex tubifex*, (Tt with lineages T_I and T_{III}) and *Rhyacodrilus* spp. (Rhy)), and designed using RealTimeDesign™ software and worm sequences from worms cultured in the laboratory, collected in 2009, and available GenBank sequences (AY334473, AF362440, DQ284761 and GQ355437).

Assays	Primer	Primer sequence 5′–3′
"Tt or Rhy"	Primer 1&3A	AGC TCG TAG TTG GAT CTC
	Primer 1&3B	CTG CTT TGA GCA CTC TAA
	Probe P1 (Rhy)	AAA GCA CTC AAC GAA GAG CAC
	Probe P3 (Tt)	AAA GCA CTC AGC GAA GAG CAC
"T_I or T_{III}"	PrimerF	AAA CGC CAC TTG TCC CTC TA
	PrimerR	GGT GCA TGG CCG TTC TTA G
	Probe T_I–T	TAA CAC CGA CAA AGG C
	Probe T_{III}–C	ACA CCG ACA GAG GCA

2.3.2. Parasite assays

To detect the presence of *M. cerebralis* (hereafter referred to as Mc infection assays), we compared a melting curve assay and a dual-labeled probe qPCR assay on a segment of the parasite's 18S gene (Kelley et al., 2006). Primer and probe sequences are listed in Table 2.

2.4. Assay optimization and validation

The qPCR experiments were performed and interpreted using the Minimum Information for Publication of Quantitative Real-

Table 2
Sequences of the primers and probes used in the real-time TaqMan and conventional PCR *Myxobolus cerebralis* infection assays (from Kelley et al., 2006).

	Primer/probe	Primer/probe sequence 5′–3′
Primers	Myx18-909f	CTTTGACTGAATGTTATTCAGTTACAGCA
	Myx18-996r	GCGGTCTGGGCAAATGC
Probe	Myx18-953p	ACCGGCCAAGGACTAACGAATGCG

Time PCR Experiments (MIQE) guidelines for the case of the qualitative analysis of qPCR (Bustin et al., 2009). Thus, we present information on how we optimized our assays regarding the low-end sensitivity. Assays were run in 96-well plates using 88 wells for the collected samples and the remaining eight wells for positive controls consisting of laboratory cultured or field collected samples verified by DNA sequencing and negative (H_2O instead of DNA template) controls. Fluorescence data were collected with a LightCycler® 480 (Roche Applied Science, USA) thermocycler.

2.4.1. Taxon assay

Uniplex and multiplex assays, each with target DNA and/or a mixture of the remaining target DNAs, confirmed specificity. Optimization included varying the annealing temperatures for both taxa, testing the primer concentrations at 0.1, 0.5 and 1.0 μl of 20 μM for the "Tt or Rhy" assay and 0.5, 1.0 and 1.5 μl of 10 μM for the "T_I or T_{III}" assay, and varying the probe concentration.

The "Tt or Rhy" assay included 10 μl reactions with 5 μl of PerfeCTa™ multiplex PCR superMix (2X) (Quanta BioSciences, Inc., USA), 0.1 μM of each primer, 0.5 μM of each probe, ~30 ng of DNA and PCR grade water. The cycling profile consisted of a 10 min denaturation at 95 °C, followed by 45 cycles of denaturation for 10 s at 95 °C, primer and probe annealing for 30 s initially at 66 °C and for the first 20 cycles decreased by 0.2 °C per cycle until 62 °C and finally followed by extension for 10 s at 72 °C. The "T_I or T_{III}" assay reactions used 5 μl of PerfeCTa™ multiplex PCR superMix (2×), 1 μM of each primer, 2.5 μM of each probe, ~30 ng of DNA and PCR grade water. The temperature–time profile for the "T_I or T_{III}" assay included 5 min initial denaturation at 95 °C followed by 40 cycles of 20 s at 95 °C, 1 min at 60 °C and 10 s at 72 °C.

2.4.2. Mc infection assay

For the Mc infection assays based on melting curve analysis, each qPCR contained 5 μl of 2× High Resolution Master Mix (Roche Applied Science, USA), 0.1 μM of each primer, 3.5 mM $MgCl_2$, PCR grade water and ~60 ng of DNA. The thermo cycling conditions were 10 min at 95 °C, followed by 60 cycles of 10 s at 95 °C, 15 s at 60 °C and 10 s at 72 °C. The dual-labeled probe PCR assay was modified from Kelley et al. (2006) and contained 5 μl of PerfeCTa™ multiplex PCR superMix (2×), 0.9 μM of each primer, 1 μM of the probe, PCR grade water and ~60 ng of DNA. The temperature–time profile was 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 5 s at 64 °C decreased to 62 °C during the first 20 cycles, followed by the final extension at 60 °C for 10 s.

2.5. Data and statistical analysis

For the "Tt or Rhy" and "T_I or T_{III}" assays, individual samples were categorized using scatter plots of fluorescence signals from two probes (Supplementary Fig. S1) using Endpoint Genotyping Analysis (Roche Applied Science LightCycler® 480 Real-Time PCR System software, version 1.5). Samples with strong fluorescence bias from either probe were classified as that probe's taxon. Samples with low values for both probes were classified as negative. A few samples showed relatively strong fluorescence from both probes and were classified as unknown.

Mc infection assay data were analyzed using either the melting curve analysis or absolute quantification algorithms of the LightCycler® software. Samples were classified as infected (positive for carrying the parasite and potentially capable of releasing fish-infective spores) or uninfected (negative for infection) with melting curve analysis by comparison with known infected and uninfected samples. The dual-labeled probe qPCR data were categorized using the Absolute Quantification analysis. Approximately 20% of the samples from each assay were validated by DNA sequencing.

2.5.1. Bayesian statistical inference

We examined the data from each site separately and used simple Bayesian statistical inference to assess the likelihood of the following hypotheses:

Hypothesis 1. Site-specific estimates of the relative abundance (i.e., proportion of *T tubifex or Rhyacodrilus* spp. of the entire number of *T. tubifex* and *Rhyacodrilus* spp.) of the two taxa (i.e., prior data based on the number of sexually mature worms identified morphologically as *T. tubifex* or *Rhyacodrilus* spp. using the subset of the 88 worms randomly selected for the genetic assays) can be improved using the "Tt or Rhy" assay information.

Hypothesis 2. Site-specific prevalence of infected *T. tubifex* can be estimated based on the Mc infection assays results (percent of infected *T. tubifex*) and the relative abundance of *T. tubifex* (i.e., proportion of *T. tubifex* of the entire number of *T. tubifex* and *Rhyacodrilus* spp.).

Using Bayes' Theorem, we predict the posterior distribution function, $P(A|B)$, as:

$$P(A|B) = P(A) \times P(B|A), \quad (1)$$

where the prior probability distribution, $P(A)$, represents additional information (external prior belief) available to the investigator about some parameter (A) of interest. The likelihood function, $P(B|A)$, is a mathematical representation of the sample data and represents the probability of observing the data, B, conditioned on the parameter of interest. The objective is to obtain the posterior probability distribution, $P(A|B)$, (i.e., scaled product of the prior distribution and likelihood function) to better express what is known about A based on both the sample data and the prior information. Generally, the more high-quality information accrued, the less uncertainty associated with the posterior probability distribution.

The prior probability distribution, $P(A)$, of Hypothesis 1 is the probability of observing *T. tubifex* or *Rhyacodrilus* spp. using morphological identifications of the sexually mature worms at a particular site described assuming a Beta(α, β) distribution. The site-specific likelihood function, $P(B|A)$, is the probability (generated using a binomial distribution) of classifying an immature worm as *T. tubifex* or *Rhyacodrilus* spp. using the probe analysis (qPCR data) at that same site. Thus, the posterior predictive probability distribution ($P(A|B)$ expressed as a Beta-binomial distribution) is our updated belief of the expected frequency of either taxon (*T. tubifex* or *Rhyacodrilus* spp.), given the observed data (probabilities of *T. tubifex* and *Rhyacodrilus* spp. using qPCR data) and the prior information (probabilities of *T. tubifex* or *Rhyacodrilus* spp. using morphological identifications) at given sites and for a worm community solely comprised of *Rhyacodrilus* spp. and *T. tubifex* worms.

For Hypothesis 2, the prior probability distribution, $P(A)$, is the estimated proportion of infected (or uninfected) *T. tubifex* worms at a specific site based on the Mc infection assays. Here, we have one likelihood function, $P(B|A)$, which represents the estimated relative abundance of *T. tubifex* at a particular site calculated after the presence of all other taxa were accounted for (unpublished data,

hereafter referred to as best site-specific estimates of the entire community composition). Thus, the posterior probability distribution expresses our belief that a sampling site has a certain prevalence of infected *T. tubifex* (derived as the proportion of infected *T. tubifex* over the total population of *T. tubifex*). For this second hypothesis, we placed a prior distribution of Beta(α, β) on the infection probability (binomial distribution) and the estimated posterior probability is expressed as Beta-binomial.

3. Results

3.1. Taxon assay performance

The two assays were located in different regions of the 18S ribosomal DNA, each region having conserved primer annealing regions and a single nucleotide polymorphism (Fig. 2). Both assays work over a range of 0.003–30 ng of DNA (Supplementary Fig. S2). No abnormal amplifications (i.e., sigmoidal shape or straight line) were observed. Based on the highly reproducible amplification, ~30 ng/ul of DNA was selected for further assays. Varying the primer and probe concentrations showed efficient results with high amplification peaks at the selected primer and probe concentration for each assay. Lowering the annealing temperature by 2 °C after 20 cycles increased end point fluorescence for the "Tt or Rhy" assay. Uniplex and multiplex reactions using laboratory-reared worms confirmed the primer and probe specificities and showed clear discrimination (Supplementary Fig. S3). Neither probe detected template of the other taxa, nor did the probes impair the target sequence amplification or show co-amplification.

3.2. Mc infection assay performance

The melting curve analysis assay (Supplementary Fig. S4A), identified infected samples as having a peak at ~84 °C, based on comparison with known infected and uninfected worms. The dual labeled probe assay, previously confirmed in laboratory trials using infected fish (Andree et al., 1999), showed similar sensitivity for field collected worms (Supplementary Fig. S4B). For both assays, the DNA sequence for 44 of the positive samples confirmed *M. cerebralis* infection, while attempts to sequence negative samples were unsuccessful. A positive test could result from an infected worm that produces actinospores, one that does not, or a worm with actinospores in the digestive tract. A previous study indicated ~80% of the worms that test positive for PCR produce actinospores (Lodh et al., 2011).

3.3. "Tt or Rhy" assay

Originally the 925 worms were separated into two categories: immature (IMHP) samples (598 samples or 64.6%) and mature *Rhy-*

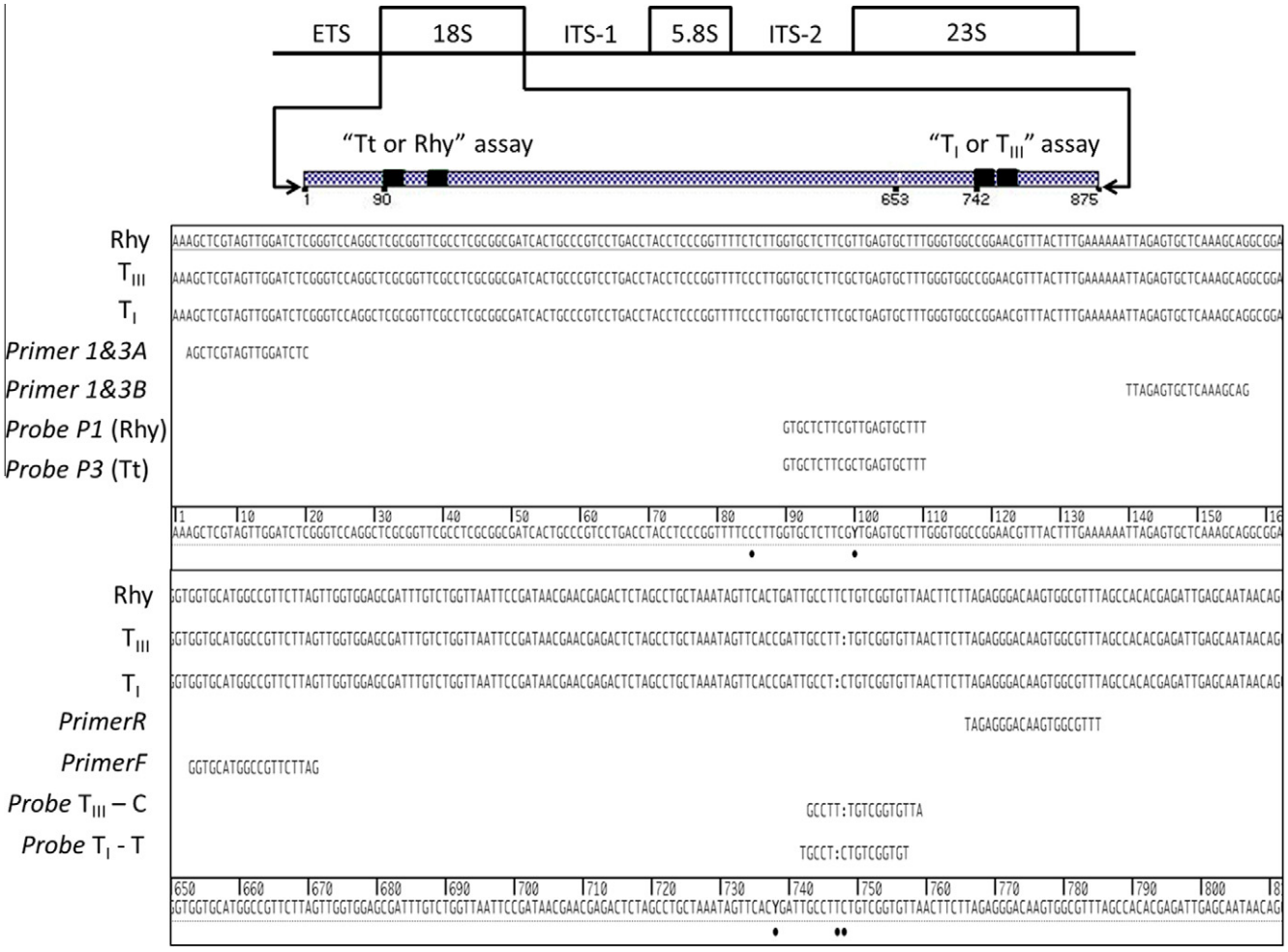


Fig. 2. DNA sequences from laboratory-reared worms of known taxa used to design primers and probes for the "*Tubifex tubifex*, (Tt) or *Rhyacodrilus* spp. (Rhy)" and *T. tubifex* lineage "T_I or T_{III}" assays. The position of the nuclear 18S gene relative to the other genes (e.g. external transcribed sequence (ETS) and internal transcribed sequence-1 (ITS-1) is shown at the top of the figure.

Table 3
Comparison of morphological and inferred identifications for worms collected in 2009 for each of the six sites (listed upstream to downstream) from the "*Tubifex tubifex* (Tt) or *Rhyacodrilus* spp. (Rhy)" assay. The shaded boxes show agreement between the two methods. Visual identification is based on morphology and can only be determined for sexually mature adults; immature worms (IMHP) could only be identified by DNA. The inferred identification is based on real-time PCR data and DNA sequencing.

"Tt or Rhy" assay		Morphological identification				Sites
		Rhy	Tt	IMHP	Total	
Inferred taxa	Rhy	38	1	28	67	South Slide
	Tt	10	28	62	100	
	Total	48	29	90	167	
	Rhy	32	0	31	63	Slide Inn
	Tt	0	0	0	0	
	Total	32	0	31	63	
	Rhy	16	0	11	27	Pine Butte
	Tt	2	15	124	141	
	Total	18	15	135	168	
	Rhy	0	0	0	0	West Fork
	Tt	0	57	112	169	
	Total	0	57	112	169	
	Rhy	67	0	58	125	Kirby
	Tt	5	11	20	36	
	Total	72	11	78	161	
	Rhy	2	0	3	5	Lyons Bridge
	Tt	0	23	105	128	
	Total	2	23	108	133	

Table 4
Infection of *Tubifex tubifex* T_I and T_{III} worms based on the *Myxobolus cerebralis* (Mc) infection assay for each of the six sites.

	Taxon inferred from "TI or TIII" assay	Mc assay			Sites
		Positive	Negative	Total	
assay	T_I	7	25	32	South Slide
	T_{III}	14	45	59	
	Total	21	70	91	
	T_I	0	0	0	Slide Inn
	T_{III}	0	0	0	
	Total	0	0	0	
	T_I	3	42	45	Pine Butte
	T_{III}	12	82	94	
	Total	15	124	139	
	T_I	22	87	109	West Fork
	T_{III}	19	40	59	
	Total	41	127	168	
	T_I	1	24	25	Kirby
	T_{III}	5	6	11	
	Total	6	30	36	
	T_I	27	21	48	Lyons Bridge
	T_{III}	39	41	80	
	Total	66	62	128	

acodrilus spp. (Rhy) (175 samples or 19%) and *T. tubifex* (Tt) (152 samples or 16.4%) worms (Supplementary Table S2). The software classified each of these 925 worms into four categories (Supplementary Fig. S1A): *Rhyacodrilus* spp. (270 samples or 29.2%), *T. tubifex* (538 samples or 58.16%), unknown (53 samples or 5.72%) and negative (64 samples or 6.9%). To identify samples categorized as "unknown" or "negative" and verify samples categorized as *Rhyacodrilus* spp. or *T. tubifex*, the qPCR products were sequenced for 123 of the 598 immature worms and 65 of the 327 sexually mature worms (~20% of the 925 worms). The sequencing data revealed that all samples categorized as negative were taxa not previously found in the area (*T. tubifex* lineage VI and *Tubifex ignotus*). Also, the samples categorized as unknown were either *Rhyacodrilus* spp. or *T. tubifex*. Finally, sequencing also confirmed that the samples categorized as *Rhyacodrilus* spp. or *T. tubifex* (87.35% or 808 worms) by the qPCR assay were identified correctly as *Rhyacodrilus* spp. or *T. tubifex*, even for the immature worms not visually identified as *Rhyacodrilus* spp. or *T. tubifex*.

Table 5
Bayesian Inference: Hypothesis 1. The data from Table 3 are used to calculate relative abundances of *Tubifex tubifex* (Tt) and *Rhyacodrilus* spp. (Rhy) (Posterior) at each site; sample *n* values are shown in parenthesis.

Taxon	Prior (*n*) *P*(*A*)		Likelihood (*n*) *P*(*B*|*A*)		Posterior *P*(*A*|*B*)	Inferred	Sites
Rhy	0.630	(77)	0.311	(90)	0.434	0.401	South
Tt	0.370		0.689		0.566	0.599	Slide
Rhy	1.000	(32)	1.000	(31)	1.000	1.000	Slide Inn
Tt	0		0		0	0	
Rhy	0.545	(33)	0.081	(135)	0.096	0.161	Pine Butte
Tt	0.455		0.919		0.904	0.839	
Rhy	0	(57)	0	(112)	0	0	West Fork
Tt	1.000		1.000		1.000	1.000	
Rhy	0.847	(83)	0.744	(78)	0.941	0.776	Kirby
Tt	0.153		0.256		0.059	0.224	
Rhy	0.059	(25)	0.028	(108)	0.002	0.038	Lyons
Tt	0.941		0.972		0.998	0.962	Bridge

Both *Rhyacodrilus* spp. and *T. tubifex* were found at four sites (South Slide, Pine Butte, Kirby and Lyons Bridge), while Slide Inn had only *Rhyacodrilus* spp. and West Fork had only *T. tubifex* (Table 3). The columns of Table 3 present our morphological identifications based on morphological characteristics present only in sexually mature worms. Table 3 also includes the final taxonomic identifications (inferred taxa) which are based on combining the sequence and amplification data for all of the samples except those categorized as negative (showing 861 out of 925 worms). The diagonals of Table 3 (shaded grey boxes) show the number of worms that were identified as the same taxon by both identification methods (morphological and qPCR). Comparison of these two methods shows good agreement (18 mismatches at South Slide, Pine Butte and Kirby). The "Tt or Rhy" assays showed high specificity (94.27%) with only ~5% of the 925 samples categorized as unknown. The first qPCR assay allowed us to identify 33.3% of the 861 field sampled worms (total number of worms minus the negative samples) as *Rhyacodrilus* spp. and 66.6% as *T. tubifex*.

3.4. "T_I or T_{III}" assay

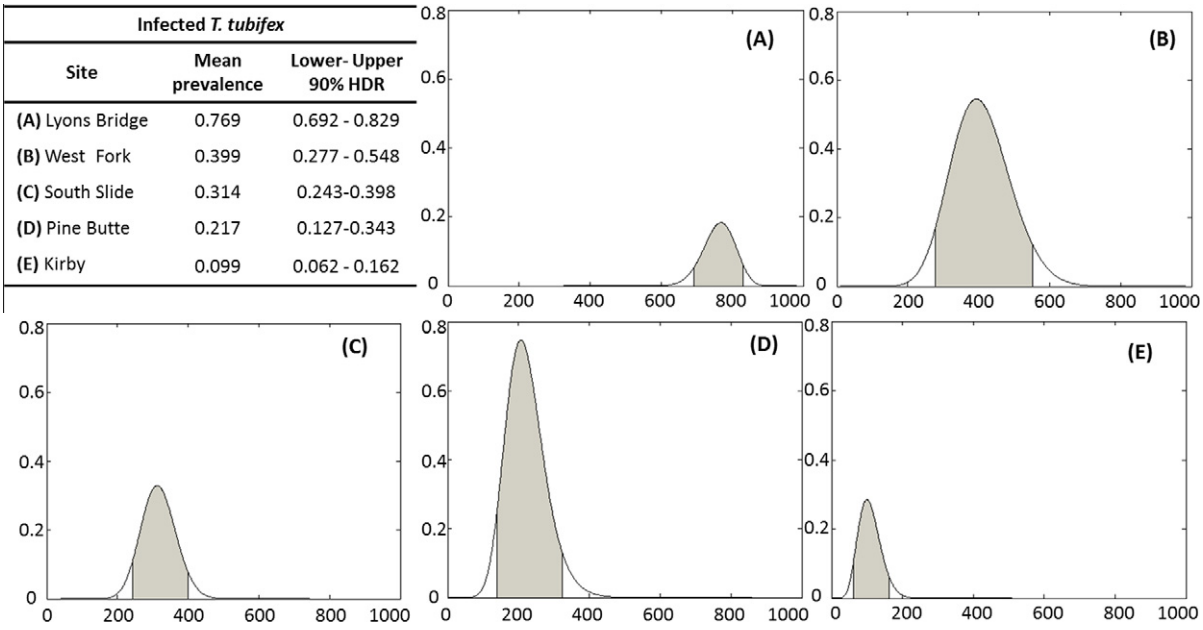
Assays of 574 *T. tubifex* from the first qPCR assay identified both T_I (256 samples or 44.6%) and T_{III} (298 samples or 51.9%) lineages at five of the six sites, the exception being Slide Inn (Table 4). T_I was more prevalent in West Fork and Kirby, while T_{III} was more prevalent in South Slide, Pine Butte and Lyons Bridge. DNA sequencing of 110 of the 554 T_I and T_{III} worms showed 100% agreement with the probe data. Twenty samples were classified as "unknown" or "negative". After sequencing, the eight "unknown" included three T_I and five T_{III} lineages. Similarly, the 12 "negative" samples included other lineages of *T. tubifex* (T_{VI}) and *Tubifex ignotus*. The "T_I or T_{III}" assays showed high specificity (98.6%) with ~2% of the 574 samples categorized as unknown. *Supplementary* Table S3 summarizes both the Mc and the raw "T_I or T_{III}" infection assay data. We should note that some *Rhyacodrilus* spp. samples were included in both assays as negative controls to confirm the inability of the second assay to detect this taxon.

3.5. Mc infection assay

Assays of 574 worms found 26.3% (151 samples) to be infected and indicated presence of the parasite is present at all sites except Slide Inn (Table 4). At most of the sites, the proportion of negative

Table 6
Bayesian inference: Hypothesis 2. Relative abundance of *Tubifex tubifex* and availability of infectious myxospores (expressed as proportion of infected *T. tubifex*) are used to estimate the mean prevalence of infected *T. tubifex* at each site (*x* axis). The shaded High Density Regions (HDR) indicate the 90% upper and lower limits of the most propable prevalence of infected *T. tubifex* at each site while the associated uncertainty is captured by the skewness of the posterior distribution.

Infected *T. tubifex*		
Site	Mean prevalence	Lower- Upper 90% HDR
(A) Lyons Bridge	0.769	0.692 - 0.829
(B) West Fork	0.399	0.277 - 0.548
(C) South Slide	0.314	0.243-0.398
(D) Pine Butte	0.217	0.127-0.343
(E) Kirby	0.099	0.062 - 0.162



samples (423 samples or 73.7%) was higher than the proportion of infected samples with the exception of Lyons Bridge. Samples sent for sequencing (30 positive and 70 negative) verified the qPCR data.

3.6. Bayesian statistical inference

The proportion of *Rhyacodrilus* spp. and *T. tubifex* varied between sexually mature (Table 5, column labeled "Prior") and immature (Table 5, column labeled "Likelihood") worms. This observation led us to hypothesize that Bayesian analysis of these datasets might improve the site-specific "prior" relative abundance estimates of *Rhyacodrilus* spp. and *T. tubifex* based only on morphology (Hypothesis 1). The column "Posterior" presents the updated estimate of relative abundance of either taxon generated using Bayesian analysis at each site. The "Inferred taxa" (Table 5) reflects our final estimate of the worm community composition based on DNA sequencing. Estimates show Slide Inn and Kirby have high relative abundance of *Rhyacodrilus* spp.; South Slide has a slight bias of *Rhyacodrilus* spp. over *T. tubifex*; whereas West Fork, Pine Butte and Lyons Bridge have high relative abundance of *T. tubifex*.

The Bayesian estimates of the prevalence of infected *T. tubifex* (Table 6, "Mean prevalence") are based on the Mc infection assay results (Table 4). To test Hypothesis 2, we used the best site-specific *T. tubifex* relative abundance estimates of the entire community composition. These estimates were calculated using the *Rhyacodrilus* spp. and *T. tubifex* information of Table 3 and interpolation methods described in Zendt and Bergersen (2000) given the entire sampled worm community. The mean prevalence of infected *T. tubifex*, together with the relative abundances of T_I and T_{III}, are the best indicators for the existence of fish infecting spores. Previous studies (Kerans et al., 2004; Rasmussen et al., 2008) have shown that T_{III} worms can produce 100–1,000 times as many fish infecting spores as T_I. When rank ordering sites from higher to lower probability of infected *T. tubifex* estimates (Table 6), we observed that the order does not match well with the order of mean prevalence of *T. tubifex* releasing fish-infective spores presented in Fig. 2B in Krueger et al. (2006). Note that West Fork was not included in Krueger et al. (2006). On the other hand, our ranking better matches the density of *T. tubifex* releasing fish-infective spores as well as the site-specific contribution estimates of whirling disease risk (see Figs. 2C and 3, respectively, in Krueger et al. (2006)).

4. Discussion

We developed two highly specific and sensitive multiplex qPCR assays to discriminate among three taxa (*Rhyacodrilus* spp., T_I and T_{III}) that vary in their ability to transmit whirling disease and account for >95% of such tubificids along a reach of the Madison River. Determining tubificid community composition is important because these worms range from non-hosts to hosts that vary 100-fold in their ability to produce fish-infective spores. The assays can determine the taxon of immature tubificids and verify the morphological identifications of mature tubificids. Furthermore, they provide a significant tool for assessing stream biodiversity because sexually mature tubificids, which are the only ones that can be identified morphologically, are often a small percentage of the community (Krueger et al., 2006). Thus, this study represents both the first assessment of tubificid community composition using a qPCR assay based on fluorescent probes and the first use of Bayesian analysis to fully characterize the dominant infected taxa in streams where whirling disease has been observed.

Developing such assays depends on prior knowledge of the tubificid community and their DNA sequences. This information is often available in public databases (i.e., GenBank) or can be obtained by sequencing DNA from morphologically identified, sexually mature worms. Assays can be developed to target taxa of ecological interest. The first assay developed in this study has a dual purpose. First, by identifying only the *T. tubifex* samples needed in the second assay, we minimized the cost (fewer samples) and the risk of misidentification (fewer negative samples from other taxa) associated with the second assay. Second, even though *Rhyacodrilus* spp. samples do not directly affect the transmission of whirling disease, they are extremely abundant in our

study area (across the locations, the second most abundant taxon that was investigated). Ecological interactions among these taxa likely influence fish disease risk.

Development of these qPCR assays resolves a problem common to species identification. Visual distinction between *Rhyacodrilus* spp. and *T. tubifex* is dependent on the abilities of individual researchers and the proportion of sexually mature worms. In addition, because there are no known external morphological differences between T_I and T_{III}, the multiplex qPCR assays allow more accurate and precise identification. For example, the "T_I or T_{III}" assay examines variation among lineages in their ability to produce fish-infective spores. T_{III} strains can produce 100–1,000 times more fish-infective spores than T_I (Rasmussen et al., 2008) and T_{III} are also somewhat variable in spore production (Baxa et al., 2008). An assay could be developed to distinguish among such T_{III} strains, if a DNA polymorphism associated with the spore productivity difference was known.

Numerous variables and experimental factors influence the efficiency and accuracy of multiplex qPCR assays. The concentrations of the primers and probes were optimized so that the appropriate PCR products were clearly detected and distinguished. The assay worked well over a wide range of DNA concentrations (0.003–30 ng/μl). A technique commonly used to improve PCR efficiency, a step-size decrease of the annealing temperature (touchdown PCR), improved results. Uniplex and multiplex assays produced comparable results. Amplification curves were contrasted with curves from known positive and negative samples for each assay to support our identifications.

The ability to assay large numbers of both sexually mature and immature worms makes it feasible to determine the relative abundance of these taxa at multiple sites. The "Tt or Rhy" assay classified ~1,000 worms (Table 3) showing that almost 30% were *Rhyacodrilus* spp. and ~60% were *T. tubifex.* After DNA sequencing of the 53 unknowns, the final composition of the collected worm community was 31% *Rhyacodrilus* spp., 62% *T. tubifex* and the remaining were other taxa (*T. tubifex* lineage VI and *T. ignotus*) not used in our estimation of infection prevalence. All of the misclassifications between morphological and qPCR identifications were a result of the sampled worm not being *Rhyacodrilus* spp. or *T. tubifex* (10 samples), or due to human error in morphological identification or labeling (eight samples). Reanalysis of the eight samples confirmed the morphological identifications, suggesting a problem with labeling. The fluorescence data produced 64 (6.9%) negative samples that were not *Rhyacodrilus* spp. or *T. tubifex* and their DNA sequence showed matches in GenBank identifying other taxa. Developing probes for additional taxa for this type of study is a progressive process and in our case, would depend on their importance in the transmission of the parasite.

Because T_{III} is more susceptible to *M. cerebralis* than T_I, we developed the "T_I or T_{III}" assay. We were able to classify 554 of the 574 samples identified as *T. tubifex* in the "T_I or T_{III}" assay (Table 4) and after sequencing 110 of the 574 samples, we concluded that 259 were T_I and 303 were T_{III} of the total 562 *T. tubifex* samples. The DNA sequences of the eight samples initially categorized as unknown as well as the 12 samples categorized as negative were examined. The eight unknown samples were identified as either T_I or T_{III}. On the other hand, the 12 samples initially categorized as negative that were identified as other *T. tubifex* lineages, were not included in Table 4 and excluded from the Bayesian analysis. The Mc infection assay detected the parasite in worms collected at five of the six sites (Table 4); the one site without parasite detection, Slide Inn, previously had a low number of *T. tubifex* (and for this study there were none) and negative site-specific contribution to whirling disease risk (Krueger et al., 2006).

The comparison of the prior distribution (morphological identification of sexually mature tubificids) and the likelihood (qPCR identification of both sexually mature and immature tubificids) confirms that the qPCR data better match the inferred data at sites with a large proportion of immature tubificids (Pine Butte, South Slide, West Fork and Lyons Bridge; see *n* values of column labeled "Likelihood" in Table 5). That was expected since Bayesian inference uses complementary data to improve predictions (e.g., the relative abundance of *Rhyacodrilus* spp. in Pine Butte would be calculated erroneously since >50% of the samples are based on morphological identification). At sites where the ratio of sexually mature to immature worms is approximately equal, the qPCR data are equally informative to the morphological classification (Slide Inn and Kirby, Table 5). Although not shown in this work, ecologists have the ability to use the relative abundance of either taxon (*Rhyacodrilus* spp. or *T. tubifex*) with the 90% High Density Region (HDR) to generate a better representation of the taxa relative abundance associated with the real tubificid community composition.

Comparison of the prevalence of infected *T. tubifex* Bayesian estimates (Table 6) with the 1999 data of Krueger et al. (2006) reveals an interesting point. Even though our prevalence of infected *T. tubifex* estimates do not exactly match those reported in Krueger et al. (2006), our site-specific estimates follow the same rank (from high to low) as the density of infected *T. tubifex* estimates (see the Fig. 2C in Krueger et al. (2006)). In the study of Krueger et al. (2006), the fish infection prevalence was better correlated with the density of infected *T. tubifex* than the prevalence of infected *T. tubifex*. Our genetic estimates of *T. tubifex* relative abundance are not directly comparable with the estimates of Krueger et al. (2006). First, their estimate is based on the entire tubificid community and includes species that are not morphologically confused with *T. tubifex* (e.g., *Limnodrilus* sp.). Second, our estimate is more precise than that of Krueger et al. (2006) because the probes allowed us to identify immature tubificids, whereas Krueger et al. (2006) used the relative abundances of the tubificid adults to assign immature tubificids to species. Thus, our results suggest that combining our genetic analysis with a Bayesian analysis captures the dynamics of infection in the Madison River.

The posterior estimates of prevalence of infected *T. tubifex* worms could be used to analyze data collected in the future. We could enhance our analysis by combining the relative abundance of T_I and T_{III} with the infected *T. tubifex* from prior data collections. For field ecologists, determining the prevalence of infected *T. tubifex* is a time consuming process, requiring microscopic identification of the spores. The 90% HDR (Table 6) estimated the uncertainty associated with each site-specific prediction and the skewness of the associated curves provides an indication/tendency of the number of infected *T. tubifex*. Managers should be cautious in cases where the prevalence of infected *T. tubifex* lies in the lower or upper boundary of the estimated probability distribution since this fact may lead to changes in the implementation of management strategies.

To summarize, whirling disease dynamics are probably influenced by the absolute and relative abundance of the worm host (*T.tubifex*), the biological community (*T.tubifex* + *Rhyacodrilus* spp. + other taxa), the density of infectious spores and abiotic environment. To more fully describe the tubificid community, we developed qPCR assays to distinguish between hosts and non-hosts that vary 100-fold in their ability to produce fish-infective spores. These assays are original and were verified by DNA sequence data after fine-tuning the key parameters in the qPCR protocols. The ability to classify immature worms is exciting as these are usually more abundant than sexually mature worms in our study area, and the ratio of *Rhyacodrilus* spp. and *T. tubifex* in the sexually mature and immature worms varies among sites at fixed sampling times.

Our assay development used a more conserved gene than previous studies and resulted in molecular genetic data that were used to estimate worm community composition and prevalence of in-

fected worms for the first time using Bayesian analysis. Proof-of-concept is provided by comparing worm host community composition and infected *T. tubifex* prevalence at six geographic sites, identifying areas more likely to have released spores. Bayesian analysis provided a rich method for including multiple types of information in a straightforward manner to better identify potential areas of disease risk.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2013.01.006.

References

Andree, K.B., El-Matbouli, M., Hoffman, R.W., Hedrick, R.P., 1999. Comparison of 18S and ITS-1 rDNA sequences of selected geographic isolates of *Myxobolus cerebralis*. Int. J. Parasitol. 29, 771–775.

Arsan, E.L., Hallett, S.L., Bartholomew, J.L., 2007. *Tubifex tubifex* from Alaska and their susceptibility to *Myxobolus cerebralis*. J. Parasitol. 93, 1332–1342.

Baxa, D.V., Kelley, G.O., Mukkatira, K.S., Beauchamp, K.A., Rasmussen, C., Hedrick, R.P., 2008. Arrested development of the myxozoan parasite, *Myxobolus cerebralis*, in certain populations of mitochondrial 16S lineage III *Tubifex tubifex*. Parasitol. Res. 102, 219–228.

Beauchamp, K.A., Gay, M., Kelley, G.O., El-Matbouli, M., Kathman, R.D., Nehring, R.B., Hedrick, R.P., 2002. Prevalence and susceptibility of infection to *Myxobolus cerebralis*, and genetic differences among populations of *Tubifex tubifex*. Dis. Aquat. Organ. 51, 113–121.

Beauchamp, K.A., Kathman, R.D., McDowell, T.S., Hedrick, R.P., 2001. Molecular phylogeny of tubificid oligochaetes with special emphasis on *Tubifex tubifex* (Tubificidae). Mol. Phylogenet. Evol. 19, 216–224.

Bell, A.S., Ranford-Cartwright, L.C., 2002. Real-time quantitative PCR in parasitology. Trends Parasitol. 18, 337–342.

Brinkhurst, R., 1996. On the role of tubificid oligochaetes in relation to fish disease with special reference to the Myxozoa. Annu. Rev. Fish Dis. 6, 29–40.

Brinkhurst, R.O., 1986. Guide to the Freshwater Aquatic Microdrile Oligochaetes of North America. Fisheries and Oceans Canada, Ottawa.

Brinkhurst, R.O., Jamieson, B.G.M., 1971. Aquatic Oligochaeta of the World. University of Toronto Press, Toronto, Buffalo.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622.

Downing, D.C., McMahon, T.E., Kerans, B.L., Vincent, E.R., 2002. Relation of spawning and rearing life history of rainbow trout and susceptibility to *Myxobolus cerebralis* infection in the Madison River, Montana. J. Aquat. Anim. Health 14, 191–203.

Elwell, L.C.S., Kerans, B.L., Rasmussen, C., Winton, J.R., 2006. Interactions among two strains of *Tubifex tubifex* (Oligochaeta: Tubificidae) and *Myxobolus cerebralis* (Myxozoa). Dis. Aquat. Organ. 68, 131–139.

Elwell, L.C.S., Kerans, B.L., Zickovich, J., 2009. Host–parasite interactions and competition between tubificid species in a benthic community. Freshwater Biol. 54, 1616–1628.

Erseus, C., Kallersjo, M., Ekman, M., Hovmoller, R., 2002. 18S rDNA phylogeny of the Tubificidae (Clitellata) and its constituent taxa: dismissal of the Naididae. Mol. Phylogenet. Evol. 22, 414–422.

Espy, M.J., Uhl, J.R., Sloan, L.M., Buckwalter, S.P., Jones, M.F., Vetter, E.A., Yao, J.D.C., Wengenack, N.L., Rosenblatt, J.E., Cockerill, F.R., Smith, T.F., 2006. Real-time PCR in clinical microbiology: applications for a routine laboratory testing. Clin. Microbiol. Rev. 19, 165–256.

Fetherman, E.R., Winkelman, D.L., Schisler, G.J., Myrick, C.A., 2011. The Effects of *Myxobolus cerebralis* on the physiological performance of whirling disease resistant and susceptible strains of rainbow trout. J. Aquat. Anim. Health 23, 169–177.

Gilbert, M.A., Granath, W.O., 2003. Whirling disease of salmonid fish: Life cycle, biology, and disease. J. Parasitol. 89, 658–667.

Hallett, S.L., Atkinson, S.D., Bartholomew, J.L., 2005. Countering morphological ambiguities: development of a PCR assay to assist the identification of *Tubifex tubifex* oligochaetes. Hydrobiologia 543, 305–309.

Kaeser, A.J., Rasmussen, C., Sharpe, W.E., 2006. An examination of environmental factors associtaed with *Myxobolus cerebralis* infection of wild trout in Pennsylvania. J. Aquat. Anim. Health 18, 90–100.

Kaeser, A.J., Sharpe, W.E., 2006. Patterns of distribution and abundance of *Tubifex tubifex* and other aquatic oligochaetes in *Myxobolus cerebralis* enzootic areas in Pennsylvania. J. Aquat. Anim. Health 18, 64–78.

Kathman, R.D., Brinkhurst, R.O., Aquatic Resources Center (College Grove Tenn.), 1998. Guide to the freshwater oligochaetes of North America. Aquatic Resources Center, College Grove, Tennessee, USA.

Kelley, G.O., Adkison, M.A., Zagmuff-Vergara, F.J., Leutenegger, C.M., Bethel, J.W., Myklebust, K.A., McDowell, T.S., Hedrick, R.P., 2006. Evaluation of quantitative real-time PCR for rapid assessments of the exposure of sentinel fish to *Myxobolus cerebralis*. Parasitol. Res. 99, 328–335.

Kerans, B.L., Karr, J.R., 1994. A benthic index of biotic integrity (B-Ibi) for Rivers of the Tennessee Valley. Ecol. Appl. 4, 768–785.

Kerans, B.L., Rasmussen, C., Stevens, R., Colwell, A.E.L., Winton, J.R., 2004. Differential propagation of the metazoan parasite *Myxobolus cerebralis* by *Limnodrilus hoffmeisteri*, *Ilyodrilus templetoni*, and genetically distinct strains of *Tubifex tubifex*. J. Parasitol. 90, 1366–1373.

Kerans, B.L., Stevens, R.I., Lemmon, J.C., 2005. Water temperature affects a host–parasite interaction: *Tubifex tubifex* and *Myxobolus cerebralis*. J. Aquat. Anim. Health 17, 216–221.

Kerans, B.L., Zale, A.V., 2002. The ecology of *Myxobolus cerebralis*. In: Bartholomew, J.L., Wilson, J.C. (Eds.), Whirling Disease Reviews and Current Topics. American Fisheries Society Symposium, pp. 145–166.

Krueger, R.C., Kerans, B.L., Vincent, E.R., Rasmussen, C., 2006. Risk of *Myxobolus cerebralis* infection to rainbow trout in the Madison River, Montana, USA. Ecol. Appl. 16, 770–783.

Lodh, N., Stevens, L., Kerans, B., 2011. Prevalence of *Myxobolus cerebralis* infections among genetic lineages of *Tubifex tubifex* at three locations in the Madison River, Montana. J. Parasitol. 97, 531–534.

Lodh, N., Stevens, L., Kerans, B.L., 2012. The parasite that causes whirling disease, *Myxobolus cerebralis*, is genetically variable within and across spatial scales. J. Eukaryot. Microbiol. 59, 80–87.

Mackay, I.M., 2007. Real-Time PCR in Microbiology: From Diagnosis to Characterization. Caister Academic, Norfolk, UK.

McGinnis, S., Kerans, B., 2012. Land use and host community characteristics as predictors of disease risk. Landscape Ecol., 1–16.

Modin, J., 1998. Whirling disease in California: a review of its history, distribution, and impacts, 1965–1997. J. Aquat. Anim. Health 10, 132–142.

Nickum, D., 1999. Whirling disease in the United States: a summary of progress in research and management. Trout Unlimited, <http://whirlingdisease.montana.edu/pdfs/TU_report_99.pdf>.

Rasmussen, C., Zickovich, J., Winton, J.R., Kerans, B.L., 2008. Variability in triactinomyxon production from *Tubifex tubifex* populations from the same mitochondrial DNA lineage infected with *Myxobolus cerebralis*, the causative agent of whirling disease in salmonids. J. Parasitol. 94, 700–708.

Sandell, T.A., Lorz, H.V., Stevens, D.G., Bartholomew, J.L., 2001. Dynamics of *Myxobolus cerebralis* in the Lostine River, Oregon: implications for resident and anadromous salmonids. J. Aquat. Anim. Health 13, 142–150.

Souaze, F., NtodouThome, A., Tran, C.Y., Rostene, W., Forgez, P., 1996. Quantitative RT–PCR: limits and accuracy. Biotechniques 21, 280–285.

Stimpson, K.S., 1982. A guide to the freshwater *Tubificidae* (Annelida: Clitellata: Oligochaeta) of North America. U.S. Gov. Print. Off, Washington.

Sturmbauer, C., Opadiya, G.B., Niederstatter, H., Riedmann, A., Dallinger, R., 1999. Mitochondrial DNA reveals cryptic oligochaete species differing in cadmium resistance. Mol. Biol. Evol. 16, 967–974.

Vincent, E.R., 1996. Whirling disease and wild trout: the Montana experience. Fisheries 21, 32–33.

Wagner, E., Arndt, R., Brough, M., Roberts, D.W., 2002a. Comparison of susceptibility of five cutthroat trout strains to *Myxobolus cerebralis* infection. J. Aquat. Anim. Health 14, 84–91.

Wagner, E., Arndt, R., Brough, M., Wilson, C., Nelson, G., 2002b. Survival, performance, and resistance to *Myxobolus cerebralis* infection of lake trout x brook trout hybrids. N. Am. J. Fish. Manage. 22, 760–769.

Whipps, C.M., El-Matbouli, M., Hedrick, R.P., Blazer, V., Kent, M.L., 2004a. *Myxobolus cerebralis* internal transcribed spacer 1 (ITS-1) sequences support recent spread of the parasite to North America and within Europe. Dis. Aquat. Organ. 60, 105–108.

Whipps, C.M., Grossel, G., Adlard, R.D., Yokoyama, H., Bryant, M.S., Munday, B.L., Kent, M.L., 2004b. Phylogeny of the multivalvulidae (Myxoza: Myxosporea) based on comparative ribosomal DNA sequence analysis. J. Parasitol. 90, 618–622.

Wolf, K., Markiw, M.E., Hiltunen, J.K., 1986. Salmonid whirling disease - *Tubifex-Tubifex* (Muller) identified as the essential oligochete in the protozoan life-cycle. J. Fish Dis. 9, 83–85.

Zendt, J.S., Bergersen, E.P., 2000. Distribution and abundance of the aquatic oligochaete host *Tubifex tubifex* for the salmonid whirling disease parasite *Myxobolus cerebralis* in the upper Colorado River basin. N. Am. J. Fish. Manage. 20, 502–512.