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Disparate infection patterns of *Ceratomyxa shasta* (Myxozoa) in rainbow trout (*Oncorhynchus mykiss*) and Chinook salmon (*Oncorhynchus tshawytscha*) correlate with internal transcribed spacer-1 sequence variation in the parasite ☆

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ABSTRACT

Ceratomyxa shasta is a virulent myxosporean parasite of salmon and trout in the Pacific Northwest of North America. The parasite is endemic in the Klamath River, Oregon/California, where a series of dams prevent movement of fish hosts between the upper and lower parts of the basin. *Ceratomyxa shasta* exhibits a range of infection patterns in different fish species above and below the dams. We hypothesised that the variations in infection and disease are indicators that different strains of the parasite exist, each with distinct host associations. Accordingly, we sought to identify strain-specific genetic markers in the ssrRNA and internal transcribed spacer region 1 (ITS-1). We examined 46 *C. shasta* isolates from water samples and two fish hosts, from June 2007 field exposures at upper and lower Klamath River sites with similarly high parasite densities. We found 100% of non-native rainbow trout became infected and died at both locations. In contrast, mortality in native Chinook salmon was <10% in the upper basin, compared with up to 40% in the lower basin. Parasite ssrRNA sequences were identical from all fish. However, ITS-1 sequences contained multiple polymorphic loci and a trinucleotide repeat $(ATC)_{0-3}$ from which we defined four genotypes: 0, I, II and III. Non-native rainbow trout at both sites were infected with genotype II and with a low level of genotype III. Chinook salmon in the upper basin had genotypes II and III, whereas in the lower basin genotype I predominated. Genotype I was not detected in water from the upper basin, a finding consistent with the lack of anadromous Chinook salmon there. Genotype O was only detected in water from the upper basin. Resolution of *C. shasta* into sympatric, host-specific genotypes has implications for taxonomy, monitoring and management of this significant parasite.

Nucleotide sequence data reported in this paper are available in the GenBank™ database under the Accession No. GQ358729.

1. Introduction

Ceratomyxa shasta (Noble, 1950) is a common myxosporean parasite of salmonid fishes in the Pacific Northwest of North America (Hoffmaster et al., 1988; Bartholomew et al., 1997). It is regarded as a single taxon across multiple species of salmon and trout, based on myxospore morphology and the site of infection in fish. The parasite develops in the intestine and is highly virulent in susceptible fish. *Ceratomyxa shasta* is responsible for severe mortalities in juvenile salmon in the Klamath River, Oregon-California, USA (Foott et al., 2004) and researchers are exploring ways to mitigate its effects in this system.

The Klamath River has been divided for more than 90 years by a series of dams that partition the ecosystem and isolate host and parasite populations. Anadromous Chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*Oncorhynchus kisutch*) and steelhead trout (*Oncorhynchus mykiss*) are prevented from migrating into the upper basin. Above the dams, salmonid populations are dominated by naturally reproducing redband rainbow trout and stocked non-native rainbow trout (which have a limited life in the system due to angling and their susceptibility to *C. shasta*). The dams also interrupt parasite spore movement downstream (Stocking et al., 2006; Bartholomew et al., 2007) and may drive genetic drift between upper and lower river parasite populations. Removal of the dams has been proposed and multidisciplinary assessments are being made of potential consequences. Research in our laboratory has established baseline data on the spatial and temporal

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distribution of *C. shasta* in the river, before dam removal allows reintroduction of anadromous fish hosts and their parasites into the upper basin (Hallett and Bartholomew, 2006; Stocking et al., 2006).

Water sample analyses since 2005 have shown two sites with consistently high parasite levels, the Williamson River in the upper basin (WR) and the Klamath River mainstem near Beaver Creek (KBC) some 100 river kilometres downstream from WR and below the dams (see detailed map in Bartholomew et al., 2007). Since 2004, 'sentinel' fish have been exposed in cages at both sites and these have shown different responses to the parasite. Nearly 100% of non-native rainbow trout become infected and die at both sites. In contrast, few native Chinook salmon become infected at WR, but a higher prevalence of infection and mortality occurred in groups exposed at KBC (Stocking et al., 2006; Maule et al., 2009). This disparate infection pattern suggested a factor other than spore dose was affecting disease.

We hypothesised that the range of infection patterns of *C. shasta* reflected the presence of multiple strains with different host associations. To resolve the genetic structure in the parasite populations, we sought strain-specific polymorphic loci in the ssrRNA and internal transcribed spacer-1 (ITS-1) regions of *C. shasta* isolates from river water, 'sentinel' Chinook salmon and rainbow trout from upper and lower river sites. We found consistent markers that defined both sympatric and allopatric parasite strains and that correlated with observed infection patterns in the different fish species and locations.

2. Materials and methods

2.1. Field locations

The Klamath River is ~425 river kilometres (Rkm) long and spans southern Oregon and northern California, USA. In this study we refer to two geographic divisions: the upper Klamath River basin which encompasses the five dams and river above them, and the lower Klamath River basin which extends from the lowest dam, Iron Gate, to the estuary, and is accessible to anadromous fish. The study sites were Williamson River (WR) in the upper basin (Rkm 441.0; 42°30′48″N, 121°55′0″W) and Beaver Creek (KBC) in the lower basin (Rkm 257.8, 41°52′1″N, 122°48′33″W). A detailed map of the river and collection sites has been published previously (Bartholomew et al., 2007).

2.2. River water samples

Based on the protocol of Hallett and Bartholomew (2006), three or four 1 L river water samples were collected coincident with the placement and removal of the sentinel fish cages (15 and 18 May, 19 and 22 June, 11 and 14 September, 2007; 17 samples from WR, 19 from KBC). Water was vacuum filtered through a 5 μm membrane which was then frozen. Total DNA was extracted from the filter membranes and subjected to a *C. shasta*-specific quantitative PCR (qPCR) assay (Hallett and Bartholomew, 2006). For the genotyping PCR (see Section 2.5) samples were tested undiluted, then if no amplification occurred, were diluted 1:5 and 1:10, to reduce the concentration of PCR inhibitors which had copurified with the parasite DNA.

2.3. Fish stocks and field exposures

River water contains both actinospore and myxospore stages of the parasite but only actinospores are infective to fish. As PCR analysis does not distinguish between the spore types, we also exposed naïve sentinel fish in the river. Different salmonid species and strains have different susceptibilities to *C. shasta* (Zinn et al., 1977); in this study we used susceptible "non-native" rainbow trout (*O. mykiss*; Roaring River stock) to confirm the presence of the parasite in the water, and resistant "native" Chinook salmon (*O. tshawytscha*; Iron Gate stock) to monitor disease effects in a species of interest.

Fish were used as fingerlings (weight ~1–20 g) and exposed according to the protocol of Stocking et al. (2006): cages of each species of fish were placed at the two sites; each cage contained 40–80 fish. Separate cohorts of fish were exposed in the river for ~72 h on each of 15–18 May, 19–22 June and 11–14 September 2007. Fish were then transported to Oregon State University's (OSU) J.L. Fryer Salmon Disease Laboratory, in Corvallis, Oregon, where they were maintained in flowing well-water and monitored for 3 months. Any moribund fish ("mortalities") were euthanised with an overdose of MS222 anaesthetic and an intestinal swab examined microscopically for distinctive *C. shasta* myxospores or developmental stages. From visually negative fish, total DNA was extracted from a sample of intestine (Stocking et al., 2006) and assayed by PCR (Palenzuela et al., 1999; USFWS and AFS-FHS 2007). At about 90 days post-exposure, all remaining fish were killed ("terminations") and up to 25 (depending on survival) examined. Mortalities ⩽5 days post-exposure were regarded as due to handling stress and were not included in final sample numbers and mortality data. Unexposed control groups of at least 25 fish of both species were assayed using PCR to assess background *C. shasta* infection in the hatchery stocks.

2.4. Sequencing primers

An initial assessment was made of the entire *C. shasta* ribosomal gene array (ssrRNA, ITS-1, 5.8S, ITS-2, lsrRNA), using the *C. shasta*-specific primer Cs2 (ATTACAAGGGTCAATACTTTGC) (Palenzuela et al., 1999) with either NC13R (GCTGCGTTCTTCATCGAT) (Gasser et al., 1993) to encompass ssrRNA, ITS-1 and part of 5.8S (Fig. 1), or NC2 (TTAGTTTCTTTTCCTCCGCT) (Gasser et al., 1993) to amplify ITS-2 and the 5′ part of lsrRNA. The starting locus of the *C. shasta* ITS-1 region was determined by comparison with the alignment in Whipps et al. (2004) of the closely related myxosporean *Kudoa thyrsites*. All loci were numbered from the 5′ end of primer ERIB1, near the start of the ssrRNA gene.

The ssrRNA and ITS-1 had contrasting amounts of variation and thus became our principle targets. Accordingly, universal ssrRNA 5′ primer ERIB1 (ACCTGGTTGATCCTGCCAG) (Barta et al., 1997) was paired with a novel primer Cs1591R (CTCATGTTGAATACGTCTCTG) to amplify ~1,610 nucleotides (nt) of ssrRNA. An overlapping ~600 nt fragment of ssrRNA-ITS-1-5.8S was generated with the novel *C. shasta*-specific primer Cs1482F (CCTGCTCTGAGAAGAGTGG) and NC13R. This Cs1482F–NC13R fragment was used for all ITS-1 genotyping. Both Cs1591R and Cs1482F were designed in a relatively conserved region of the ssrRNA to encompass intraspecific genotypes and closely related species.

2.5. Genotyping PCR and sequencing

Two-round PCRs were necessary to amplify ITS-1 DNA from the majority of samples, given residual inhibition present in fish samples and loss of DNA during processing of water samples. Reactions were performed in 20 μL vols.: 1 μL template DNA; 0.25 μM each primer; 1.25 U Go Taq Flexi polymerase (Promega, San Luis Obispo, California, USA); 4 μL 5× Go Taq Flexi clear buffer; 1.5 mM $MgCl_2$; 1 μL Rediload dye (Invitrogen, Carlsbad, CA); 25 ng/μL BSA; 0.2 mM each of dNTP; 10.55 μL of water. Both PCR rounds used the same primers.

PCR was performed on a PTC-200 thermocycler (MJ Research Inc., Watertown, Massachusetts, USA) with initial denaturation at 95 °C for 2 min, then 35 cycles of 94 °C for 20 s, 53 °C for 30 s,

72 °C for 120 s, and terminal extension at 72 °C for 10 min. PCR products were electrophoresed through 1% agarose gels stained with 1% SYBRsafe (Invitrogen) alongside a 1 kb + DNA ladder (Invitrogen) to verify production of correctly sized amplicons. PCR products were purified using a Qiagen PCR Purification kit as per the manufacturer's instructions, then quantified using a DNA spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

ssrRNA gene amplicons were sequenced with both ERIB1 and Cs1591R. After initial validation of the assay, ITS-1 amplicons were sequenced in one direction only, with Cs1482F. Reactions were carried out with an ABI BigDye Terminator Cycle Sequencing Kit v3.1 and ABI3730 Genetic Analyzer (Applied Biosystems, Foster City, CA USA) at the OSU Center for Genome Research and Biocomputing. Sequence fragments were aligned by hand in BioEdit (Hall, 1999).

2.6. Calibration and interpretation of chromatograms from mixed genotype samples

Chromatograms were examined to identify polymorphic loci, indicated by two or more coincident peaks, which signalled the presence of mixed alleles/genotypes. All water samples and many fish contained multiple genotypes. To verify that a linear relationship existed between chromatogram peak height and genotype proportion, we sequenced known mixtures of pure genotype I and II ITS-1 amplicons from fish using a methodology analogous to Hall and Little (2007). DNA was quantified using a spectrophotometer, then mixed in 20 µL vols. at I:II molar ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 7:3 and 9:1, respectively, and sequenced with primer Cs1482F. Chromatograms were examined in BioEdit and relative peak heights at five loci were measured. Data were analysed with a paired *t*-test and the coefficient of determination calculated. The locus that gave the best fit to the expected curve was then used to estimate genotype ratios in mixed genotype field samples (Fig. 4). Compositions of mixed genotype samples were determined visually by comparison with unmixed reference samples.

3. Results

3.1. Water samples

In May, June and September, 2007, WR water samples had very high levels of *C. shasta* with average quantification cycle (Cq) values of 33.8, 29.7 and 32.6, respectively, which corresponded to 10–100 spores L^{-1}, based on comparison with reference samples of known numbers of spores (Hallett and Bartholomew, 2006). KBC water samples had average Cq values of 33.9, 33.0 and 35.1 which corresponded to about 10 spores L^{-1}. Parasite DNA was successfully sequenced from all water samples.

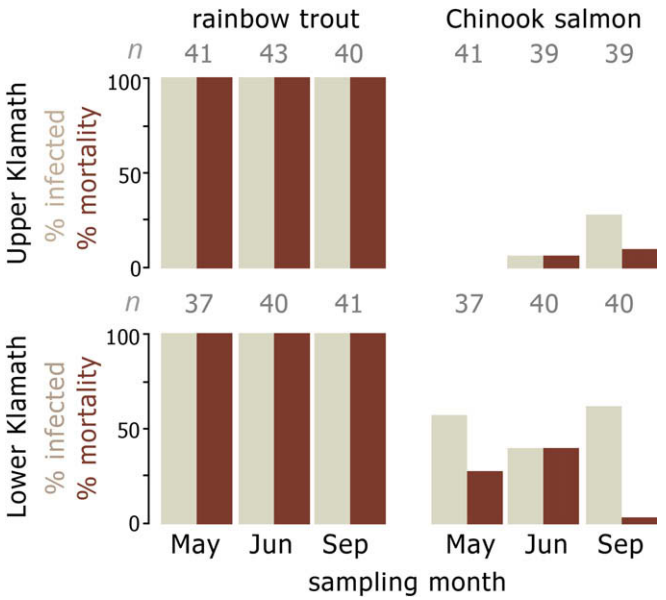

Fig. 2. Infection prevalence of *Ceratomyxa shasta* (light bars), and mortality (dark bars) in non-native rainbow trout and native Chinook salmon exposed in May, June and September 2007 at two sites in the upper and lower Klamath River basin. Only fish that survived ⩾5 days post-exposure are included (*n*). In September only, infections were detected in 3% of Chinook salmon controls which has potentially inflated the infection data for that time point.

3.2. Sentinel fish

Ceratomyxa shasta infection prevalence and mortality in non-native rainbow trout were 100% at all time points and locations (Fig. 2). Native Chinook salmon had lower infection prevalences (WR: May 0/25, June 1/25 (4%), September 6/25 (24%); KBC: May 20/35 (57%), June 16/40 (40%), September 16/26 (62%)) and mortality (WR: May 0/41, June 2/39 (5%), September 3/39 (8%); KBC: May 10/37 (27%), June 16/40 (40%), September 1/40 (3%)). Control fish of both species examined in May and June were negative; in September, 3/23 (13%) of rainbow trout and 1/15 (7%) of Chinook salmon assayed were positive.

3.3. DNA sequencing and genotypes

A reference sequence that included ssrRNA, ITS-1, 5.8S, ITS-2 and part of the lsrRNA was produced (GenBank Accession Number GQ358729). Four single nucleotide polymorphisms (SNPs) were apparent between all *C. shasta* isolates from Chinook salmon and rainbow trout and the original *C. shasta* ssrRNA sequence in GenBank (AF001579). The ssrRNA sequences from 14 *C. shasta* isolates from Chinook salmon and non-native rainbow trout were identical

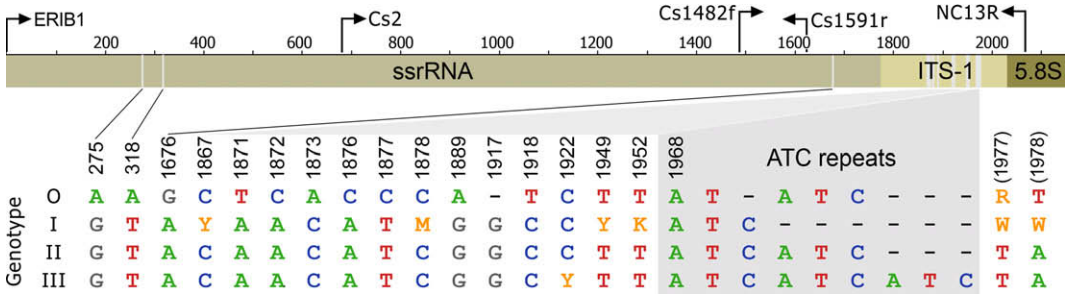

Fig. 1. Schematic representation of part of the *Ceratomyxa shasta* ribosomal gene array which shows inferred gene boundaries, primer binding locations and polymorphic loci, with the trinucleotide repeat highlighted.

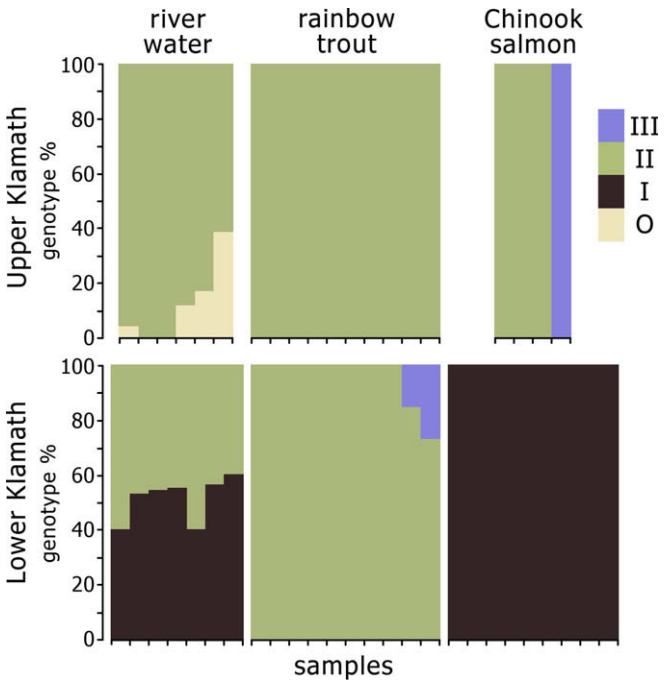

Fig. 3. Proportions of *Ceratomyxa shasta* genotypes in June 2007 isolates at two sites in the upper and lower Klamath River basin. Water data are for replicate 1 L samples taken when fish were placed in the river (19 June) and when they were retrieved (22 June). Fish data are shown for individual non-native rainbow trout and native Chinook salmon. Only four Chinook salmon were genotyped from the upper river site, where infection levels were low in this species.

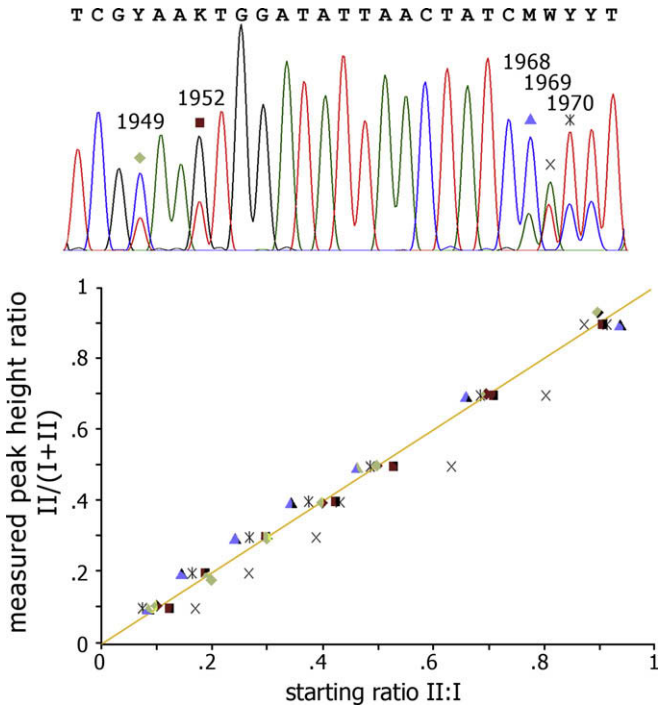

Fig. 4. Correlation of genotype proportion with relative peak height in a mixed sample. The chromatogram shows the five loci where peak heights were measured. The chart shows correlation of measured peak height ratios against starting molar ratio. A linear relationship was confirmed, and loci 1970 and 1949 correlated best with expected values (line).

to each other over their entire length (1,776 nt; which is shorter than most myxozoans). *Ceratomyxa shasta* ssrRNA sequences from WR water samples revealed three SNPs not present in parasite isolates from the sentinel fish (Figs. 1 and 3).

The *C. shasta* ITS-1 region began at 1,776, 5 nt downstream from the end of the universal ssrRNA primer ERIB10 and was 240–246 nt long (Fig. 1), shorter than that of other myxozoans (393–493 nt in *K. thyrsites* (Whipps and Kent, 2006), 410 nt in *Myxobolus cerebralis* (Whipps et al., 2004), ~400 nt in *Tetracapsuloides bryosalmonae* (our estimation based on data of Henderson and Okamura, 2004)). The *C. shasta* ITS-1 had a nucleotide composition of 73.5% AT (ssrRNA was ~50% AT). Parasite ITS-1 was sequenced from the following sample groups collected in June: 13 river water samples (six WR; seven KBC); 20 rainbow trout (10 WR; 10 KBC); 13 Chinook salmon (the only four WR; nine KBC) (Fig. 3).

Multiple SNPs and several insertions/deletions (INDELs) were apparent, as well as a trinucleotide repeat $(ATC)_{0-3}$ at locus 1968 (Fig. 1). Total ITS-1 sequence variation was 0.4–7.1% between isolates. Genotypes were named after the number of consecutive ATC repeats, O, I, II, III. *Ceratomyxa shasta* from all water samples and from 21/23 KBC Chinook salmon comprised a mixture of genotypes. Some 10% of isolates had unique polymorphisms.

3.4. Calibration of chromatograms from mixed genotype samples

Sequence chromatograms of artificial mixtures of genotypes confirmed peak height was proportional to the amount of genotype (Fig. 4), although when compared with expected values, the linearity and fit of experimental data varied according to which SNP locus was used: 1,949 ($R^2 = 0.998$, $P = 0.482$), 1,952 ($R^2 = 0.997$, $P = 0.063$), 1,968 ($R^2 = 0.992$, $P = 0.070$), 1,969 ($R^2 = 0.967$, $P = 0.006$), 1,970 ($R^2 = 0.999$, $P = 0.534$). Hence loci 1,970 and 1,949 were used for estimation of genotype proportions in field samples. Distinct peaks were visible at all ratios tested, which indicated that we could resolve genotype components at least to 10% using direct sequencing. Noise was typically ~5% in the chromatograms.

3.5. Population genetic structure

Genotype I was only detected in lower river (KBC) water samples and was specific to Chinook salmon (Fig. 3). Chinook salmon also became infected with genotypes II and III at low levels. Genotype II was detected at both sites and showed a strong association with non-native rainbow trout, which were also infected with genotype III at a low level. Genotype III was not detected in water samples but occurred at low prevalence in both fish hosts. WR water contained on average 80% genotype II and 20% genotype O. KBC water averaged 50% genotype I and 50% genotype II.

4. Discussion

Genetic differences were found between *C. shasta* isolates from Chinook salmon and rainbow trout exposed sympatrically at sites in the upper and lower Klamath River basin. We resolved four primary parasite genotypes (O, I, II, III) based on a trinucleotide repeat $(ATC)_{0-3}$ in the ITS-1. These genotypes exhibited both host and geographic patterns of occurrence that correlated with observed patterns of infection and mortality in sentinel fish and with known distributions of wild fish hosts. Our findings also explain previously observed disparities between parasite density in river water and fish infections (Hallett and Bartholomew, 2006; Stocking et al., 2006; Maule et al., 2009).

We speculate that *C. shasta* genotypes have arisen from fragmentation of a once homogeneous population by physiological and physical barriers which have restricted gene flow. The original drivers of sympatric *C. shasta* diversification were physiological constraints imposed by different salmonid hosts with varying life

histories – anadromous versus freshwater and summer versus winter spawners. More recent population fragmentation has occurred through construction of five dams which imposed a physical barrier to upstream migration of Chinook salmon and which explains the lack of genotype I in the upper basin. The dams also directly prevent downstream passage of spores from the upper basin parasite population (Stocking et al., 2006). Removal of the dams would allow mixing of upper and lower basin parasite populations which makes it necessary to ascertain the relationships of *C. shasta* genotypes with other host species, which include coho salmon, steelhead and native rainbow trout.

Genotaxonomy of myxozoans relies almost exclusively on ssrRNA sequences, which show variation between morphospecies ranging from more than 40% to less than 1% (e.g. Fiala, 2006; Ferguson et al., 2008). Morphologically distinct marine *Ceratomyxa* spp. have 2–25% variation in their ssrRNA (Gunter et al., 2009). Low level ssrRNA variation by itself has not been considered sufficient grounds to divide taxa which infect the same fish host and tissues, for example in *M. cerebralis* (Whipps et al., 2004; Arsan et al., 2007). We found no difference in *C. shasta* ssrRNA sequences from Chinook salmon and non-native rainbow trout and would conclude that the isolates are conspecific based on this gene. However, persistent differences in infection prevalence and mortality of fish at the different sites impelled us to examine a more variable gene region, the ITS-1, on the assumption that ssrRNA alone cannot resolve putative closely related species/strains.

The ITS-1 has been used to examine inter- and intraspecific differences in other taxa, e.g. Arthropoda (Harris and Crandall, 2000), Platyhelminthes (Nolan and Cribb, 2005) and Apicomplexa (Hnida and Duszynski, 1999). In the Myxozoa, ITS-1 has been used to assess cryptic speciation and phylogeographic distribution in three widespread, problematic species: *K. thyrsites* (Whipps and Kent, 2006), *M. cerebralis* (Whipps et al., 2004) and *T. bryosalmonae* (Henderson and Okamura, 2004). ITS-1 variation in these taxa ranged from 4.8% (*T. bryosalmonae*) to 43.5% (*K. thyrsites*) between widely separated geographic isolates across thousands of kilometres, and resolved genotypes or clades with different host affinities or geographic distribution on a global scale. We found *C. shasta* had ITS-1 variation of 0.4–7.1% between isolates from different fish hosts at two sites in the Klamath River basin. This genetic variation is comparatively very high given the small geographic scale of the sampling. We consider that the level of sympatric variation, coupled with host specificity, is strong evidence to support a taxonomic review of *C. shasta*.

Regardless of the taxonomic implications, the discovery of discrete, host-specific *C. shasta* genotypes has immediate relevance for fisheries management in the Klamath River. In the upper basin, we detected high levels of genotype II, which we suspect are maintained by the practice of stocking susceptible, non-native rainbow trout. If the dams are removed, Chinook salmon and other anadromous fish will re-enter this region and both be exposed to existing high levels of genotype II and introduce genotype I (and possibly other genotypes). While the effects of genotype II on Chinook salmon appear to be minimal, its impact on sensitive populations of anadromous coho salmon and steelhead trout are unknown. Genotypes O and III were both detected in low abundance in the upper basin and their potential effects are unclear as the host was not identified for genotype O and genotype III did not appear to be host-specific or result in mortality.

The connection between *C. shasta* genotypes and host species calls into question the validity of *C. shasta* as a single taxon. As the accurate identification of parasites is fundamental to understanding their spatial, temporal and host distribution, we need to assess whether basic morphotaxonomic support exists for maintaining *C. shasta* as a single entity, i.e. do myxospores of different genotypes have distinct morphologies? To further understand the population structure of the parasite, we need to extend assessment of *C. shasta* genotypes to other host species in the Klamath River – native redband rainbow trout and steelhead and coho salmon – and to hosts from across the Pacific Northwest.

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