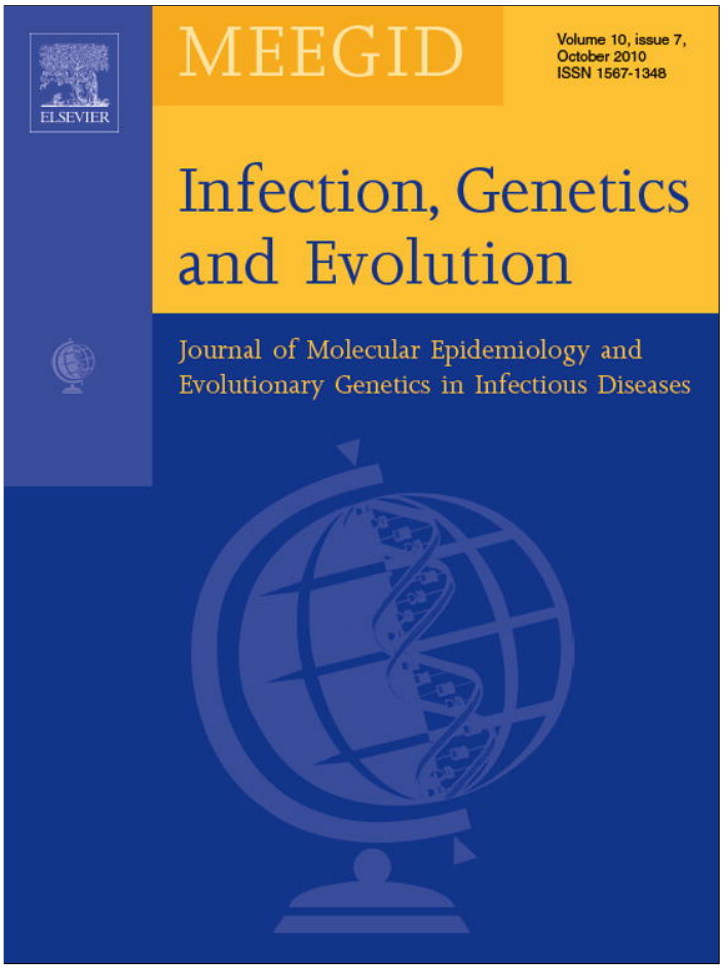

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Spatial, temporal and host factors structure the *Ceratomyxa shasta* (Myxozoa) population in the Klamath River basin

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ABSTRACT

The myxozoan parasite *Ceratomyxa shasta* is a virulent pathogen of salmonid fish in the Klamath River, Oregon/California, USA. We previously defined four principal genotypes of the parasite (O, I, II, III) based on a trinucleotide repeat $(ATC)_{0-3}$ in Internal Transcribed Spacer region 1 sequences. Genotypes occur in sympatry and show marked host preference: I in Chinook salmon (*Oncorhynchus tschawytscha*) and II in non-native rainbow trout (*O. mykiss*). In the present study, we sequenced the parasite from river water samples collected in May, June and September at three localities below, above and between the Klamath's five dams. We also sampled adult and juvenile coho salmon (*O. kisutch*), steelhead trout (*O. mykiss*, anadromous form) and native redband rainbow trout (*O. mykiss*, freshwater form) and additional Chinook salmon and non-native rainbow trout. We found that the *C. shasta* population was highly structured spatially, temporally and with respect to fish host species. Genotype O was present in water throughout the basin but detected almost exclusively in steelhead and native rainbow trout. Genotype I was in water only below the dams and detected only in Chinook salmon. Genotype II was detected in coho salmon below the dams, and in non-native rainbow trout exposed both above and below the dams. The same genotypes were detected in adult and juvenile fish of the same species. These findings have major implications for the design of effective surveillance and control programs for this economically and ecologically important fish parasite.

1. Introduction

The myxozoan *Ceratomyxa shasta* (Noble, 1950) is a common intestinal parasite of salmonid fishes in the Pacific Northwest of North America (Hoffmaster et al., 1988; Bartholomew et al., 1997). It can infect at least 10 species of salmon and trout (Bartholomew et al., 1989), however the severity of disease caused by *C. shasta* varies between species and strains, and can be affected by environmental conditions, especially high water temperature (Udey et al., 1975; Zinn et al., 1977). The parasite has an indirect life cycle with two obligate hosts: salmonid fish and the polychaete worm *Manayunkia speciosa* (Bartholomew et al., 1997). In each host, parasite development gives rise to a distinct waterborne spore stage: myxospores from the fish, actinospores from the polychaete.

The parasite has been regarded as a single taxon throughout both its geographic and host ranges based on site of infection in fish and morphology of the resultant myxospores. We recently identified multiple genetic types – genotypes – within the parasite population in the Klamath River, Oregon/California (Atkinson and Bartholomew, 2010). We observed differences in *C. shasta* infection prevalence and mortality in populations of Chinook salmon (*O. tshawytscha*, Iron Gate Hatchery strain) and non-native rainbow trout (*O. mykiss*, Roaring River hatchery strain) in the upper and lower Klamath River basin. We targeted the Internal Transcribed Spacer region 1 (ITS-1), which has been used to probe intra-specific population structure in many parasite taxa, for example Apicomplexa (Hnida and Duszynski, 1999), Platyhelminthes (Nolan and Cribb, 2005) but only a few Myxozoa (Whipps et al., 2004; Henderson and Okamura, 2004; Whipps and Kent, 2006). We sequenced *C. shasta* from fish and water, and resolved 4 ITS-1 genotypes (O, I, II, III) based on a trinucleotide repeat $(ATC)_{0-3}$.

Genotype I was present only in the lower basin and only in Chinook salmon. Genotype II was present in both the upper and lower basin, and infected the non-native rainbow trout. Genotype III was also found in the upper and lower basin, and in low prevalence in both species. Genotype 0 was found only in upper river water samples and its fish host was not determined. We hypothesised that host–parasite genotype affinities have arisen from barriers to gene flow imposed by the divergent host life histories, principally different temporal and spatial components of inter- and intra-basin migration. Host migration patterns and

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hence parasite distribution have also been profoundly affected by damming of the Klamath River.

Anadromous fish are prevented from migrating into the upper basin by a series of dams that have partitioned the river basin for more than 90 years. These species, which include multiple strains of Chinook salmon, coho salmon (*Oncorhynchus kisutch*) and steelhead trout (*Oncorhynchus mykiss*, anadromous form), hatch in fresh water then migrate to the ocean to complete their development, before they return to specific parts of the river at specific times of the year to spawn and die – though steelhead trout can spawn several times (NRC, 2004). Above the dams, principally freshwater salmonid species are found, which include rainbow trout – both wild, native redband and stocked non-natives – and introduced brown (*Salmo trutta*) and brook (*Salvelinus fontinalus*) trout. These species live their entire lives within the river basin and can spawn multiple times. Non-native rainbow trout are stocked into an upper basin tributary every week from late May until the end of August, but have only a short life in the system due to angling, predation and their susceptibility to *Ceratomyxa shasta* once they enter the Williamson River (W. Tinniswood, ODFW, pers. comm.). The dams have likely been a strong driver of genetic drift between upper and lower river basin parasite assemblages by partitioning the host populations.

To expand our understanding of *C. shasta* genotype relationships with Klamath River salmonids, especially species considered most 'at risk' in the basin, we analysed samples from coho salmon, steelhead trout and native redband rainbow trout. We compared results from different sub-populations of fish: upper versus lower basin, adult versus juvenile, Iron Gate versus Trinity River hatchery strains. Parasite genotypes in river water were also assessed at the same localities where sentinel fish were held in May, June and September, to examine temporal patterns of parasite genotype. Overall, a better understanding of the genetic structure of the *C. shasta* population and how it relates to its fish hosts should permit development of improved parasite monitoring and management strategies in the Klamath River basin, which include what strains of fish are most informative in sentinel studies and whether specific parasite control measures could be developed, such as carcass removal.

2. Materials and methods

2.1. Klamath basin overview and field localities

The Klamath River spans the Pacific Northwest states of Oregon and California, U.S.A., is about 425 km long and is partitioned into upper and lower sections by five dams (Fig. 1). Only the "lower basin" below the dams is accessible to anadromous salmonids, which include Chinook salmon, coho salmon, steelhead trout and coastal cutthroat (*O. clarkii*) trout (NRC, 2004). The upper basin comprises reaches and reservoirs between each of the dams, and Klamath Lake and tributaries above the uppermost dam. Resident upper basin salmonids include kokanee salmon (resident *O. nerka*) and trout species: redband rainbow, bull (*Salvelinus confluentus*), brown, brook and non-native rainbow trout.

In the present study, we genotyped additional parasite samples from water and fish samples from upper and lower basin localities previously studied (Atkinson and Bartholomew, 2010), and added a locality between the dams, with a lower diversity of salmonid hosts and presumable correspondingly lower parasite diversity. Upper Klamath River basin localities included: Williamson River (WR, 42°30′48″N, 121°55′0″W) close to its entry into Klamath Lake (KL); other tributaries of KL (Spring Creek, Sprague and Wood Rivers); Keno Eddy (KED, 42°8′58″N, 122°0′55″W) on the Klamath River mainstem between two dams and nearby Spencer Creek, a *C. shasta*-negative tributary, and source of naïve native redband rainbow trout.

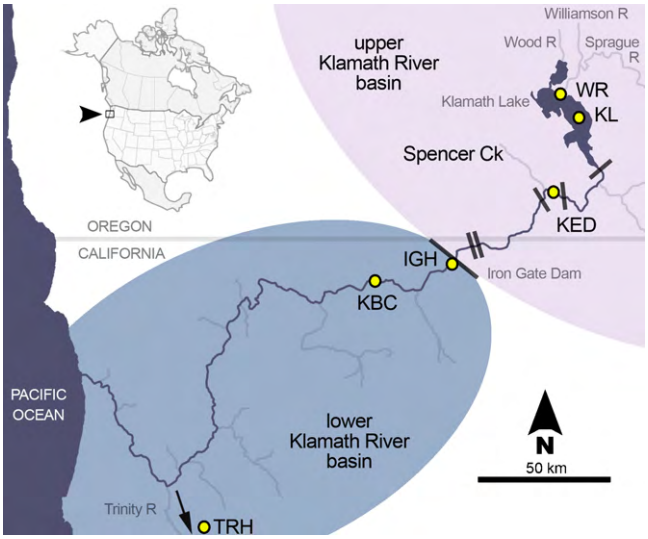

Fig. 1. Map of Klamath River basin showing sample localities (dots with locality codes), tributaries and the dams (black bars) that divide the basin into upper and lower regions (shading). Locality codes: WR Williamson River, KL Upper Klamath Lake, KED Keno eddy, IGH Iron Gate Hatchery, KBC Klamath mainstem near Beaver Creek, TRH Trinity River Hatchery (which is approximately 70 km south of the junction of the Trinity and Klamath rivers).

Lower Klamath River basin localities: Klamath River mainstem near Beaver Creek (KBC, 41°52′1″N, 122°48′33″W); Iron Gate State Fish Hatchery (IGH, 41.929892N, 122.442112W) on Bogus Creek, immediately below Iron Gate Dam, at the upstream limit of salmon migration and Trinity River State Fish Hatchery (TRH, 40.725681N, 122.795198W) on the Trinity River mainstem, a major tributary of the lower Klamath River.

2.2. River water samples

At each of the three sentinel fish exposure localities (WR, KED, KBC), triplicate 1 L river water samples were collected at the beginning and end of caged fish exposures in 2007: 15 and 18 May, 19 and 22 June, 11 and 14 September; only 2 samples were taken at WR on 11 Sept. Water was vacuum-filtered through a 5 μm membrane per the protocol of Hallett and Bartholomew (2006). Total DNA was extracted from material retained on the membrane, then analysed with a *C. shasta*-specific qPCR assay (Hallett and Bartholomew, 2006). The qPCR results were expressed as cycle quantification values (Cq), which are inversely proportional to DNA amount and a proxy for spore density in the water. qPCR-positive samples ($Cq < 40$) were then genotyped. As both waterborne spore stages of the parasite are smaller than 20 μm and are indistinguishable by current molecular assays, this water sampling protocol gave a measure of total parasite density, not just that fraction (actinospores) infective to fish. Statistical analyses were performed using S-plus 8.0 (TIBCO Software Inc., Palo Alto, CA).

2.3. Fish

Parasite samples were genotyped from juvenile fish that we exposed in the river (sentinel fish), and from frozen, archived juvenile and adult fish from previous studies and collections. Juvenile fish were held in the river at three sentinel localities to assess actual infectivity of water, i.e. presence of actinospores (Stocking et al., 2006; Atkinson and Bartholomew, 2010). Briefly, fingerlings, $n = 40–80$, were held in cages anchored in the river for 72 h, then transferred to the laboratory where they were monitored at 18 °C for 90 days post-exposure (d.p.e.). The protocol for the use of the animals in this study (Animal Care and Use

Protocol 3850) was reviewed and approved by the Institutional Animal Care and Use Committee of the Office of Research Integrity at Oregon State University, Corvallis, OR, USA. Fish were checked daily and any that were clinically diseased were euthanised with an overdose (500 ppm) of buffered MS222 anaesthetic, necropsied and an intestinal swab examined microscopically for distinctive *C. shasta* myxospores or developmental stages. If no parasite spores were visible, a small section of intestine was removed and DNA extracted (Stocking et al., 2006), diluted 1:100, then assayed by a standard PCR (Palenzuela et al., 1999; USFWS and AFS-FHS, 2007). Fish that died after 5 d.p.e. were included in the analysis; earlier mortalities were ascribed to non-*Ceratomyxa shasta* causes. All fish that survived to 90 d.p.e. were killed by overdose with MS222, then processed as for the non-survivors. For each group, up to 25 visually negative fish were assayed by PCR. Prevalence data included all examined fish that were visually and/or PCR-positive for the parasite. For some groups, the prevalence at 90 d.p.e. was likely less than the prevalence within the first month post-exposure, as resistant strains can clear the parasite infection in 30 days (S. Bjork., OSU, pers. comm.). Mortality data was regarded as the more important data set and included only those fish that died prior to 90 d.p.e. and were positive for the parasite. The following are details of fish species and strains we sampled.

Chinook salmon (*O. tshawytscha*, IGH and TRH strains): $n = 40$–45 IGH strain juveniles exposed at KWR and KBC in May, June and September 2007 as previously reported (Atkinson and Bartholomew, 2010), unexposed fish held in parallel in the laboratory as a control; *C. shasta*-positive adult fish 10 IGH strain and 10 TRH strain that had returned to spawn in November–December 2005.

Steelhead trout (*O. mykiss*, anadromous form, IGH and TRH strains): 40 TRH strain juveniles exposed at KBC June 2007, unexposed fish held in parallel in the laboratory as a control; archived *C. shasta*-positive adult fish 10 IGH strain and 10 TRH strain that had returned to spawn in February–March 2006.

Rainbow trout (*O. mykiss*, freshwater form, non-native Roaring River hatchery strain and native redband strains): 40 non-native juveniles exposed at WR, KED and KBC in May, June and September 2007; unexposed fish held in parallel in the laboratory as a control. Archived juveniles, 14 native strain wild fish caught in Spencer Ck and then exposed at KBC in May 2005, unexposed fish were analysed for controls; 36 adult native strain wild fish captured in KL by Oregon Department of Fish and Wildlife, October 2007.

Coho salmon (*O. kisutch*, IGH and TRH strains): juveniles, 40–45 IGH strain fish exposed at KBC in May, June and September 2007, unexposed fish held in parallel in the laboratory as a control; 10 archived *C. shasta*-positive adult IGH strain and 10 TRH strain fish that had returned to spawn in October–December at IGH (2006) and TRH (2005).

Brook trout (*S. fontinalis*): adults, 2 fish netted by ODFW 2008 in Wood R. Brown trout (*S. trutta*): adults, 4 fish netted by ODFW 2008 in Wood R.

2.4. DNA extraction and C. shasta genotyping PCR assay

In the present study we modified the previously reported 2-round genotyping PCR by use of a novel *C. shasta*-specific ITS-1 reverse primer, CsGenR1 (AGGGATCCACCGTTAAC). PCR chemistry and instrumentation were unchanged, but cycling conditions were modified: initial denaturation at 95 °C for 3 min, then 35 cycles of 94 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s, and terminal extension at 72 °C for 10 min.

DNA sequencing was undertaken as described previously, with substitution of primer CsGenR1 in the sequencing reaction. Forward sequencing reads were made with primer Cs1482F (Atkinson and Bartholomew, 2010) to verify unique genotypes. Sequence chromatograms were interpreted by eye, and primary genotypes O, I, II, III, were ascribed on the basis of the number of trinucleotide repeats $(ATC)_{0-3}$ at approximate position 450 (1968 in reference *C. shasta* SSU-ITS1 sequence GQ358729). Subgenotypes were resolved by incorporation of additional single nucleotide polymorphisms (SNPs) and insertions/deletions (INDELS) into the analysis. All water samples and many fish samples contained multiple parasite genotypes that, where possible, were resolved by visual inspection of sequence chromatograms and relative proportions quantified by measurement of peak heights as described previously (Atkinson and Bartholomew, 2010).

2.4.1. Phylogenetic analyses

DNA sequences were aligned by eye and pairwise comparisons made with BioEdit (Hall, 1999). Sequences that occurred only once were excluded from the analysis. Gaps were reduced to binary characters, concatenated to the start of the alignment. The optimum evolutionary model for the dataset was selected by the Akaike Information Criterion using MrModelTest v2.3 (Nylander, 2004). Bayesian analysis was conducted using MrBayes (v3.1.2, Huelsenbeck and Ronquist, 2001). Phylograms were visualised in MEGA (v4.4028; Tamura et al., 2007) then annotated in Adobe Photoshop (Version CS3, Adobe Systems, Palo Alto, California).

3. Results

3.1. River water analyses

Waterborne *Ceratomyxa shasta* was detected at all time points at the three field localities (Fig. 2). June parasite density data for WR and KBC were reported previously (Atkinson and Bartholomew, 2010) and are included here for comparison. There were significant changes in parasite density between 15 and 18 May at both KED (mean Cq 38.0 ± 0.87 versus 35.2 ± 0.17, p-value < 0.01) and KBC (36.1 ± 0.65 versus 31.5 ± 0.46, p-value $< .001$). No other intra-month means were significantly different from each other. The highest parasite densities were seen at WR in May (mean Cq 31.4 ± 1.14) and June (29.7 ± 0.42). At the same time points, KBC mean densities were 10 times lower (about 3 Cq) than WR, and KED were about 50 times lower (5 Cq) than WR. September parasite densities were significantly lower than June at both WR (32.6 ± 0.70 versus 29.7 ± 0.42, p-value < 0.001) and KED (38.9 ± 1.8 versus 35.6 ± 1.7, p-value < 0.02) but not KBC (35.1 ± 1.54 versus 33.1 ± 2.79, p-value > 0.3).

Genotype profiles were obtained for 48 water samples (Fig. 2), all of which had a Cq < 38; 4 samples at KED did not contain sufficient DNA for genotyping and only two samples were obtained on 11 September at WR and KED. Novel *C. shasta*-specific ITS-1 reverse primer CsGenR1 was less sensitive to the inhibition present in many samples but it did not alleviate the need for 2-rounds of PCR. At the furthest up-river locality, KWR, only genotypes O and II were detected, with type II always dominant. The proportion of type O was significantly higher on 14 September (~35% O 45% II, p-value < 0.05) than any other time point (~10% O, 90% II). Types I and III were not detected. At KED, between the dams, type O dominated at all time points; in one May sample and in four June samples we detected a '*C. shasta*-like' amplicon ("genotype X") which pairwise comparison showed was only 87% similar to genotypes O, I, II and III (97–99% similar with each other over ~550 nucleotides; refer also to the results of the phylogenetic analysis). At KBC, below the lowermost dam, only genotypes I and II were detected in May and June and proportions were fairly constant: ~55% I 45% II. In September, type O was detected at up to ~35%, at the expense of type I. Genotype III was not detected in water at any locality, which suggests it was never present at a proportion of >5%.

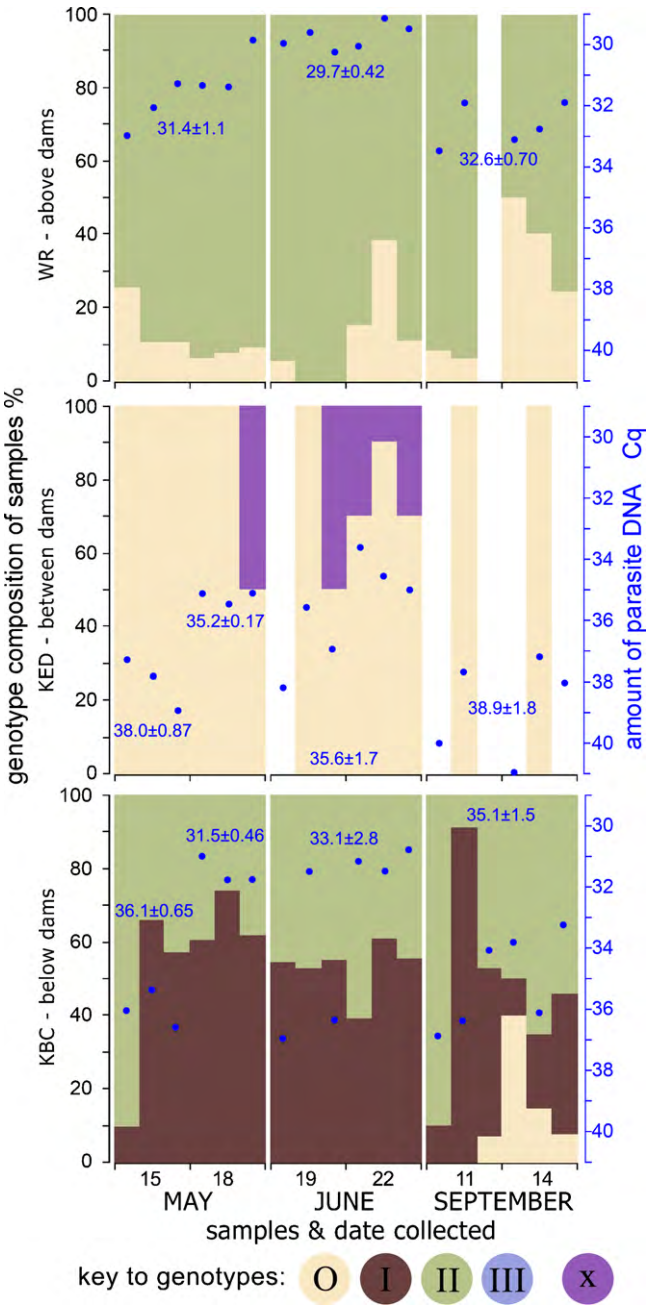

Fig. 2. *Ceratomyxa shasta* in Klamath River water. Triplicate 1 L samples were taken on two dates in each of May, June and September, 2007, at upper river basin localities WR and KED and lower river locality KBC. Parasite DNA amount (a proxy for spore density; dots) is shown as qPCR assay quantification cycle (Cq), which is inversely proportional to DNA amount. Mean and standard deviation are shown for the entire month unless there was a significant difference (p-value < 0.05) between sample days, in which case mean and standard deviation are given for each date. Genotypes (stacked bars) are shown as percentage composition of each sample. Blank bars indicate no sample recovered or DNA amount was too low to genotype. Genotype III was not detected in any water sample. Genotype X was detected at KED only and was not seen in any fish.

3.2. Fish samples – infection prevalence, mortality and parasite genotypes

Caged juvenile fish exposed at WR, KED and KBC, and wild-caught adults in KL (adjacent to WR) provided infection prevalence and mortality data (Fig. 3). A subset of prevalence, mortality and genotype data for Chinook salmon and non-native rainbow trout was published in Atkinson and Bartholomew (2010) and is included here with addition of new data: some prevalence data are now higher due to inclusion of additional PCR results and some mortality data are lower as "mortality" now comprises only those fish which died and tested positive for the parasite, not total mortality. Infected Chinook salmon ($n = 12$) that survived to 90 d.p.e. were genotyped for the first time (no non-native rainbow trout survived to 90 d.p.e.).

Ceratomyxa shasta samples were genotyped from 178 fish of 3 species and 8 strains (Fig. 4). Genotypes O, I, II, III were detected; genotype X was not. Types O, I, and II had distinct host affinities (see subsequent paragraphs). Type III was detected in multiple fish species at multiple localities, but only at low prevalence and as a minor proportion of total parasite DNA present: a mixed infection in 5/48 Chinook salmon, and 3/40 coho salmon and 2/32 non-native rainbow trout, but was not detected in any of 55 native *O. mykiss* (redband rainbow trout or steelhead).

Chinook salmon IGH strain juveniles at WR had very low infection prevalence (0–24%) and mortality (0–3%) compared with KBC (40–77% and 3–40%, respectively; Fig. 3). The 6 WR fish we genotyped had all survived to 90 d.p.e. and were type II or III (Fig. 4). Genotype I was only detected in Chinook held at KBC and was the only genotype present in the 9 fish that died <90 d.p.e. Of the 11 genotyped fish that survived to 90 d.p.e., 7 had mixed infections; type I was dominant in 4/7 with types II and III also detected. Adult Chinook salmon that returned to IGH and TRH were all infected with type I; the only mixed infections (<10% type II) were detected in 10/12 TRH strain fish. Control IGH strain Chinook salmon were PCR-negative for *C. shasta* in May (0/25) and June (0/26), but in September 1/18 was PCR-positive.

Non-native rainbow trout had >97% prevalence of infection and mortality at WR and KBC at all time points (Fig. 3), with genotype II in all fish (Fig. 4) (Atkinson and Bartholomew, 2010). At KED, infection prevalence was high (>92%) at all time points but mortality was never >36%. Type O was the only parasite genotype detected in the non-native rainbow trout at KED, in stark contrast to the genotypes detected at WR and KBC. Mortality in the KED fish appeared to be dose-dependent: it dropped in September with decreased waterborne parasite density. Non-native rainbow trout controls were PCR-negative in May (0/25) and June (0/25), but in September 1/25 was positive.

Native redband rainbow trout juveniles were only held at KBC and had 23% prevalence of infection (14/62) and 9% (2/23) mortality. Of wild adult native rainbow trout captured in KL, 31% (11/36 fish) were infected but mortality was unknown. Genotype O dominated both in juveniles (100% in 13/14), and adults (100% in 9/11); the few other fish had 50-100% genotype II (Fig. 4). All 21 wild-caught juvenile native control fish were negative.

Steelhead trout TRH strain juveniles were only held at KBC and had 96% prevalence of infection (24/25 fish) but no mortality, and all infections were type O (Figs. 3 and 4). All IGH and TRH adult fish were infected with type O. An A/G polymorphism at position 1916 (subgenotype Om in Fig. 5) was observed in 9/10 TRH strain adults but only 1/10 IGH strain. In the control juvenile TRH strain steelhead, 1/26 tested positive.

Coho salmon IGH strain juveniles were only exposed at KBC and had >80% prevalence of infection and mortality in May and June, but <45% in September (Fig. 3). Of the 11 juvenile fish that died <90 d.p.e., all had pure infections of type II whereas 4/9 fish that survived to 90 d.p.e. had mixed infections of 44–81% II with the remainder III or I (Fig. 4). Control fish in May and June were negative (0/25, 0/25); >20% (6/25) September controls tested positive; the source of infection in the control positives was not identified.

None of the 6 wild-caught brook and brown trout from upper basin tributaries were infected with *C. shasta*. No control fish of any strain died from *C. shasta*.

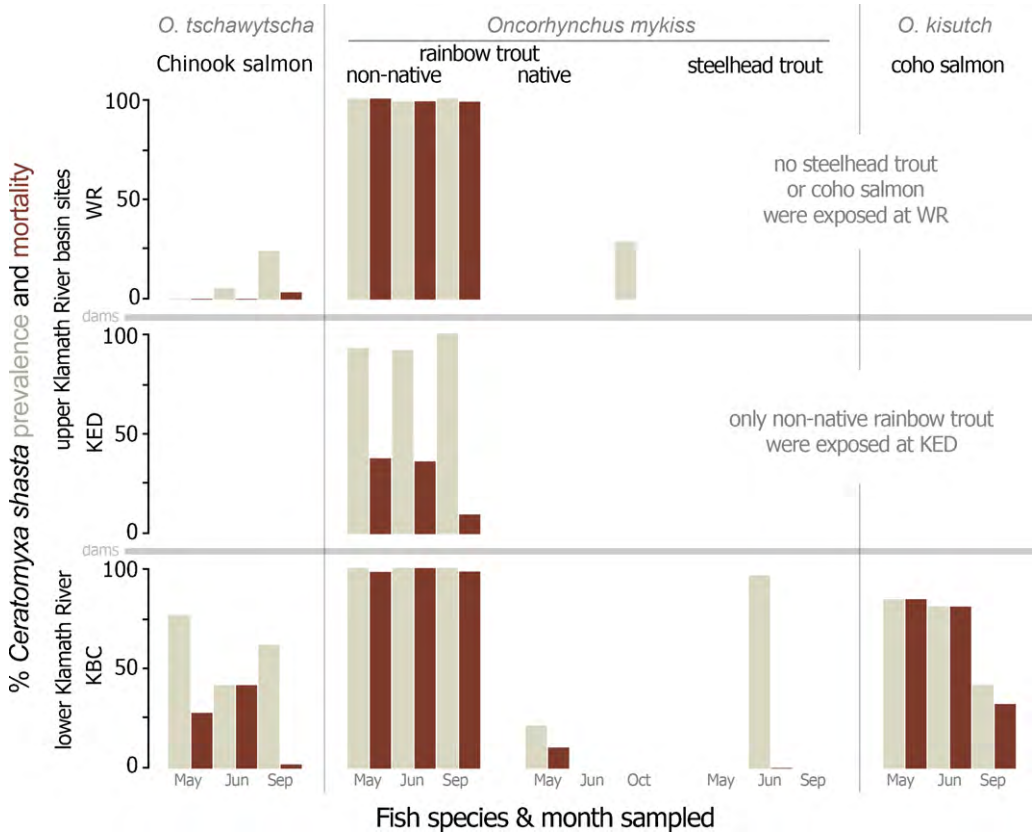

Fig. 3. Prevalence of and mortality due to *Ceratomyxa shasta* in five strains of juvenile salmonids exposed in cages in the Klamath River. The greatest range of fish was exposed in the lower river at KBC where natural populations of these species exist. Infection prevalence = light bars. Mortality due to *C. shasta* = dark bars.

3.3. Phylogenetic analysis of parasite samples

Sequence reads of genotypes O and I usually contained INDELS and SNPs in addition to the primary ATC repeats, and several subgenotypes could be distinguished that occurred in multiple samples. It was often difficult to determine the exact sequences of subgenotypes using the current methodology and this probably led to underestimation of alleles in the parasite population. The best-fit substitution model was determined to be F81+I, with base frequencies A 0.3193, C 0.1724, G 0.1816, T 0.3267 and the proportion of invariable sites = 0. Unrooted phylograms are shown in Fig. 5.

4. Discussion

We have shown the Klamath River *Ceratomyxa shasta* population was highly structured spatially and with respect to fish host species. Parasite genotypes occurred in sympatric mixes in water samples and their proportions varied over time. When exposed to mixed genotypes, host fish species became infected only with particular types. We found consistent patterns of specific *C. shasta* genotypes that infected a particular host species/strain, and in each strain a single primary genotype was dominant. Further, the presence or absence of genotypes in each of the three localities we studied was influenced by the species of salmonid naturally present at each locality, at each time point.

For more than 80 years, dams have made the upper basin localities KED, KL and WR inaccessible to anadromous Chinook and coho salmon (Fig. 1). This has had a direct effect on the distribution of *C. shasta* genotypes in the river. For example genotype I, which we have shown to infect Chinook salmon almost exclusively, was only detected in the lower. We did not detect type I in water at either upper basin locality, nor did naïve caged Chinook become infected with it there. Genotype II has now been shown to be associated with both adult and juvenile anadromous coho salmon in the lower basin, where it was detected in the water. Type II was also detected in water from the uppermost locality WR, where no coho salmon are present. We showed genotype II can also infect susceptible non-native rainbow trout, and herein is the reason for its persistence in the upper basin: susceptible rainbow trout are stocked there between May and August to provide fish for anglers. As these fish die, they release type II myxospores (C. Hurst, OSU, pers. comm.) which maintain infections in the polychaete host population and create an artificial 'hot spot' of infectivity. This unnaturally high parasite level in the upper basin could lead to disease in reintroduced native coho which would otherwise have had a manageable infection. A cessation to stocking non-native fish would probably decrease levels of genotype II in the upper basin and may be a necessary management action if native coho salmon are reintroduced, following removal of the dams (BOR, 2010).

Genotype O had previously been detected only in water from the upper basin – its fish host was unknown (Atkinson and Bartholomew, 2010). We have now shown that genotype O is dominant in native rainbow and steelhead trout (both *O. mykiss*). While anadromous steelhead are only present in the lower river (i.e. KBC), native rainbow trout are present throughout the Klamath river basin, which explains the presence of type O in all water samples. The KED locality between dams was the only location where we detected type O in the absence of type II, most likely due the lack of any suitable host for type II (coho salmon or non-native rainbow trout). KED was therefore the only location where we could expose non-native rainbow trout to type O without them dying as a result of type II infection. And indeed, the non-native rainbow trout became infected with type O at KED,

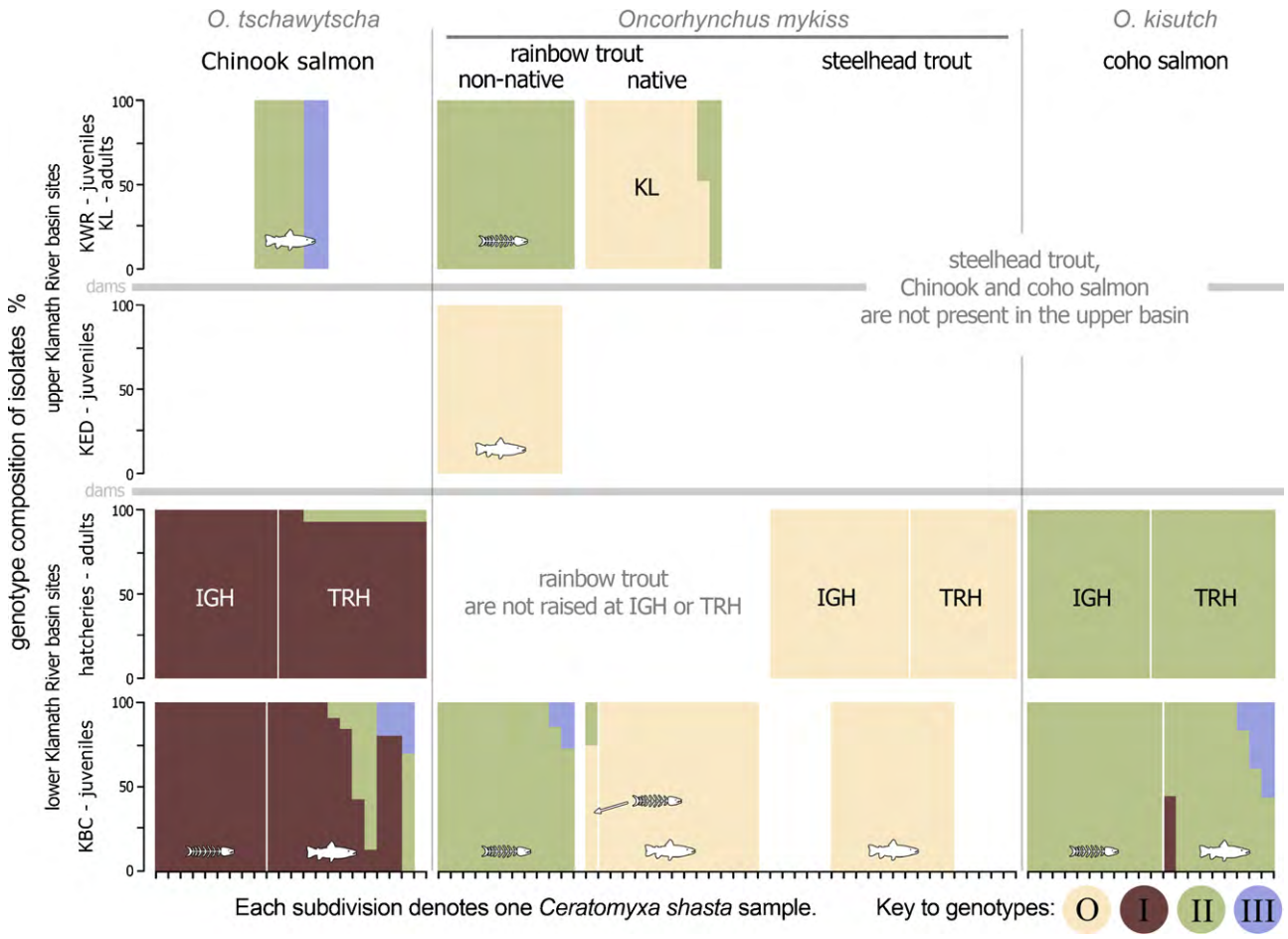

Fig. 4. Genotype profiles of *Ceratomyxa shasta* in 8 salmonid strains from the Klamath River. Juvenile fish were held in cages at three localities in the upper (WR, KED) and lower (KBC) river basin. Parasite samples were genotyped both from juvenile fish that died from the parasite (skeleton icon) and those that became infected but survived to 90 days post-exposure (solid icon). Infected adult fish were genotyped from Iron Gate (IGH) and Trinity River (TRH) hatcheries, and from Klamath Lake (KL).

which showed this genotype can infect the three strains of *O. mykiss* that we tested. Type O was detected at high prevalence but infection resulted in low or no mortality in *O. mykiss*, even in the non-native strain, which suggests adaptations have occurred between *O. mykiss* and type O to reduce the incidence of fatal disease while maintaining the parasite.

Anadromous *O. mykiss* (steelhead trout) held at KBC had a 96% prevalence of infection with type O but did not die from the parasite. The river water during the fish exposure (19–22 June 2007, Fig. 2) did not have detectable levels of genotype O, instead it contained high levels of types I and II. These data show both the extraordinary sensitivity of steelhead trout to genotype O despite it being present in the water at levels below our detection limit, and simultaneously, the apparent inability of genotypes I or II to infect steelhead. The infections with type O that did develop did not cause mortality in these juvenile steelhead, in stark contrast to almost total mortality (due to type II) in non-native rainbow trout exposed contemporaneously. Native steelhead may be demonstrating tolerance for both type O and the other genotypes of the parasite.

Within different steelhead trout sample groups, we observed a unique SNP that varied between *C. shasta* sequences from IGH strain and TRH strain adults (that returned to Iron Gate and Trinity River hatcheries, respectively). This could be used as a parasite-derived biological tag to distinguish IGH and TRH strains. It also suggests that not all of the parasite burden of the TRH fish is derived from a source in the lower reaches of the Klamath River (i.e. in common with IGH fish), instead TRH fish become infected with a unique parasite population after they have entered the Trinity River.

While temporal analysis of the water sample data was limited by small sample sizes and an inability to discriminate between contributions of the two parasite spore stages, actinospores (from polychaetes) and myxospores (from fish), several patterns were evident. For example, parasite densities were high in May and June at all localities, coincident with warming water temperatures favourable to development and release of actinospores from the polychaete host. This is probably an adaptive response of the parasite, timed to coincide with out-migrating juvenile Chinook salmon in the lower basin and with stocking of non-native rainbow trout in the upper basin. Myxospores probably also contributed to the high upper basin densities we observed, both from spawning native rainbow trout (genotype O) and from diseased non-native rainbow trout which had been stocked earlier in the year (genotype II). In September, parasite densities were generally lower, and the genotypic compositions had changed: at WR, the final stocking of non-native rainbow trout was in August and few would have survived through September, which we suspect lowered the type II myxospore contribution; at KBC, higher type O in September could have reflected myxospore contributions from steelhead returning to spawn. We surmised that the complex month-to-month changes in parasite density and genotype composition in the river likely reflected contributions from multiple hosts, each with different life histories to which the parasite has adapted. Resolution of more precise causal relationships between parasite genotype occurrence and fish migrations

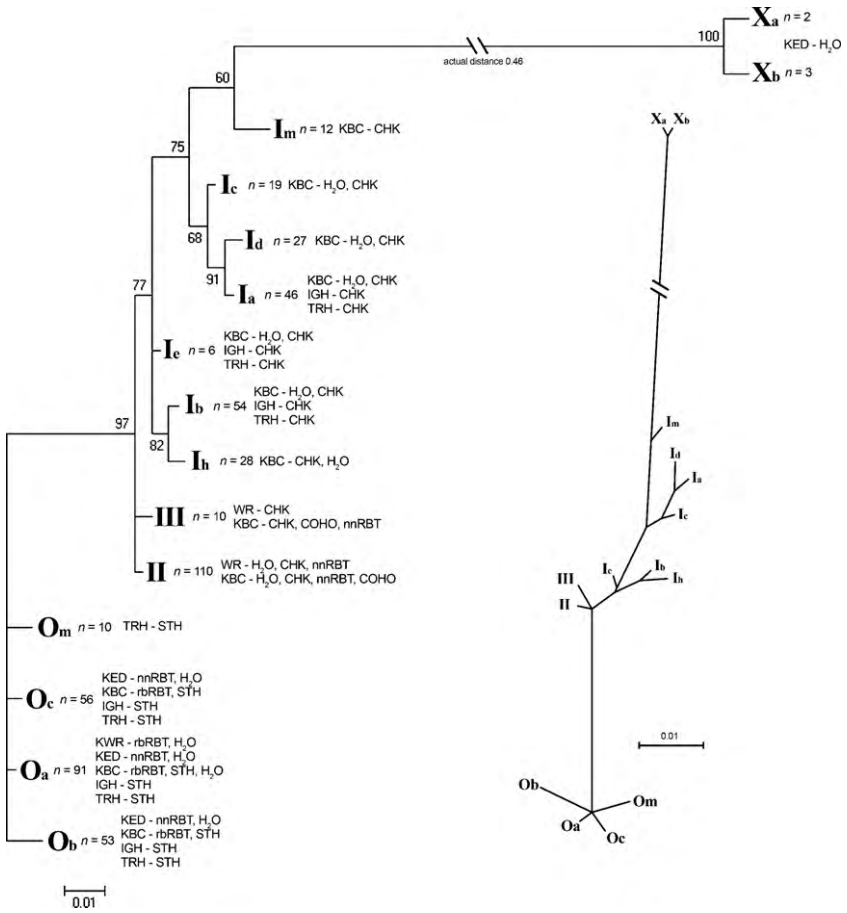

Fig. 5. Unrooted Bayesian inference phylograms of *Ceratomyxa shasta* samples: rectangular and radial plots. Rectangular plot has posterior probability values at nodes. Taxon labels comprise: genotype, number of occurrences, sample field location, sample type (H_2O river water, CHK Chinook salmon, nnRBT non-native rainbow trout, rbRBT native redband rainbow trout, COHO coho salmon, STH steelhead trout).

would require more species-specific data, especially in the lower basin where many host species coexist.

Phylogenetic analysis and pairwise sequence comparisons showed type X, which was detected only in water, was distinct from genotypes O, I, II and III found both in water and in salmonids. Although additional sampling is required to determine its fish host, genotype X has a relatively large genetic distance from the salmonid genotypes which may indicate a non-salmonid host, and its limited distribution to a locality between dams strongly suggests a resident species. While the four genotypes found in salmonids were 97–99% similar to each other, type O samples clustered apart from genotypes I–III. The genetic distance of genotype O, while not extreme, may imply it was the first to diverge from a common ancestor, perhaps a marine species that parasitised a salmonid ancestor and a marine polychaete. We suggest that subsequent salmonid differentiation into coho, Chinook *et cetera* and their invasion of freshwater habitats drove diversification of the parasite. Spatial and temporal barriers to gene flow were imposed by the different host life histories: strongly seasonal spawning, migration, and adoption of a solely freshwater life cycle by rainbow trout. Infected populations of rainbow trout have possibly been the most effective isolating mechanism and driver of genetic drift in the parasite, which is reflected in the genetic distance of the '*O. mykiss*' genotype O from the rest. Host-specificity of the genotypes now enables them to exist in sympatry.

Given their sympatric diversity, should the *C. shasta* genotypes be regarded as separate species? Several other myxozoans have been shown to encompass multiple genotypes with distinct geographic distributions and host preferences (Whipps et al., 2004; Whipps and Kent, 2006; Henderson and Okamura, 2004). Proposal of new species based on host factors alone has been discouraged in myxozoan taxonomy (Lom and Arthur, 1989) and delineation of myxozoan taxa solely on the basis of genetic characters does not yet have widespread support. Myxozoan taxonomy has traditionally relied on morphology (genus) and morphometry (species) of the myxospore stage (Lom and Arthur, 1989). For *C. shasta*, we have not undertaken morphometric analysis of myxospores of the different genotypes to determine if they can be distinguished in this manner. Given the similar tissue tropism and disease characteristics of genotypes O–III, we suggest they continue to be considered a single species, similar to the consideration of different bacterial or viral strains (e.g. infectious hematopoietic necrosis virus, Kurath et al., 2003) pending analysis of additional informative characters. We consider also that maintenance of a single taxon name facilitates communication among fisheries managers who are familiar with the parasite.

This study revealed genetic structure in the population of *C. shasta* in the Klamath River, the distribution pattern of its ITS-1 genotypes and their associations with different salmonid hosts. One of the primary implications of these findings is that it is now necessary to redesign the standard *C. shasta* PCR and qPCR screening assays, which cannot resolve the different parasite genotypes. Accurate parasite assays are an essential predictive tool for fisheries management to determine what hosts are likely to become infected where, and what level of mortality could be expected. Development of an additional method to better resolve subgenotypes may reveal additional markers for intra-basin

parasite populations, which could be used as a metric of parasite spread and a proxy indicator of host migration patterns. The discovery of multiple *C. shasta* genotypes with affinities to different fish hosts has spawned several questions: what are the relationships of parasite genotypes to their other obligate host, the benthic polychaete *Manayunkia speciosa*? Do additional genotypes exist elsewhere in its range across the Pacific Northwest? How are other salmonids, which include sockeye (*O. nerka*), pink (*O. gorbuscha*) and chum salmon (*O. keta*) affected by different genotypes?

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