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WIDESPREAD DISTRIBUTION OF *CERATONOVA SHASTA* (CNIDARIA: MYXOSPOREA) GENOTYPES INDICATES EVOLUTIONARY ADAPTATION TO ITS SALMONID FISH HOSTS

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ABSTRACT: The distribution of the freshwater myxozoan parasite *Ceratonova shasta* in the Pacific Northwest of North America is limited to overlap in the ranges of its 2 hosts: the polychaete *Manyunkia* sp., and Pacific salmonids. Studies in the Klamath River (Oregon/California) and Deschutes River (Oregon), showed that the parasite population is comprised of multiple sympatric genotypes, some of which correlate with particular salmonid host species and with differences in clinical disease in those hosts. The 3 primary genotypes O, I, and II are defined by the number of a specific tri-nucleotide repeat in the internal transcribed spacer-1 region. To understand the spatial extent of host–parasite genotype patterns, we sequenced the parasite from 448 salmonid fishes from river basins in California, Oregon, Washington, Idaho, and British Columbia, Canada. We sampled intestinal tissues from 6 species of salmon and trout, both those that exist naturally with the parasite (sympatric) and those that do not naturally co-occur with the parasite and were exposed artificially in cages (allopatric). In most river basins we detected the same primary *C. shasta* genotypes that were described from the Klamath and Deschutes rivers, and we did not detect any novel primary genotypes. Host–parasite genotype patterns were consistent with previous data: genotype O was found in sympatric trout only; genotype I predominantly in Chinook salmon, and genotype II in all 6 fish species but dominant in coho salmon. Our findings of widespread, consistent host–parasite genotype patterns support the hypothesis that *C. shasta* has a long evolutionary history with salmonid fishes in the Pacific Northwest, and impels additional studies to determine if these parasite genotypes should be considered different species.

The myxozoan parasite *Ceratonova shasta* (Noble, 1950; syn. *Ceratomyxa shasta*) is a significant myxozoan parasite of wild and cultured salmon and trout populations in the Pacific Northwest (PNW) of North America. It affects multiple fish species, including *Oncorhynchus tshawytscha* (Chinook), *Oncorhynchus kisutch* (coho), *Oncorhynchus gorbuscha* (pink), *Oncorhynchus keta* (chum), *Oncorhynchus nerka* (sockeye), and trout *Oncorhynchus clarkii* (cutthroat) and *Oncorhynchus mykiss* (rainbow/steelhead) (Hendrickson et al., 1989; Bartholomew, 1998). These fishes have different life histories and genetically diverse subpopulations (Waples et al., 2001), and vary widely in their susceptibility to the parasite (Zinn et al., 1977; Bartholomew, 1998). Generally, salmonids that evolved in sympatry with *C. shasta* have low susceptibility to disease, whereas allopatric, naïve fish strains tend to exhibit acute disease after exposure to the parasite even at very low doses, and parasite myxospores can develop in both cases (Bartholomew, 1998; Bartholomew et al., 2001; Bjork and Bartholomew, 2009).

For sympatric salmonids, observations of differences in disease severity led to the discovery of genetic variations in the parasite. For example, in the Klamath River, California/Oregon, differences in mortality in Chinook and coho salmon and rainbow trout held in different sections of the river correspond with different *C. shasta* genetic types ("genotypes") that are defined by variations in the parasite's internal transcribed spacer region 1 (ITS-1) DNA. These variants have been confirmed by phylogenetic analysis to represent a robust genetic structure within *C. shasta* (Atkinson and Bartholomew, 2010a, 2010b). Comparison of parasite sequences from different salmonid species revealed consistent associations: genotype O with rainbow/steelhead trout; type I with Chinook salmon; type II with coho salmon. Type III was detected less commonly and was recently determined to be indistinguishable from genotype II (Atkinson et al., 2018).

We have hypothesized that *C. shasta* evolved into genetic lineages/genotypes that have particular fish host specificities from pressures to adapt to hosts' different life histories, including anadromous vs. freshwater resident forms, and different seasonal spawning migration (i.e., spring/fall; Atkinson and Bartholomew, 2010b; Stinson and Bartholomew, 2012). For anadromous fish, we assume that juvenile salmon and steelhead trout become infected as they migrate downriver to the ocean, and parasite-induced mortality during migration results in deposition of myxospores in lower river reaches. As fish re-enter freshwater to spawn, they become infected as they migrate through the lower reaches, then transport the parasite back upriver, and infect the polychaete host in areas where the next generation of fish will be exposed. We hypothesize that this finely tuned relationship with the strong life-history timing in different salmonids has shaped the evolution of different *C. shasta* genotypes, and indicates a long history of host–parasite co-evolution in the endemic regions of the PNW. We predict therefore that we should find the same *C. shasta* genotypes in the same hosts across the parasite's geographic range. Alternatively, if we discover additional host–parasite relationships within the parasite's range, this would suggest that parasite diversification into different host species has a relatively recent origin. Regardless of the wider geographic findings, we predict that parasite spatial diversity within individual river basins will reflect more recent anthropogenic effects: for example, in both the Klamath and Deschutes rivers, barrier dams prevent anadromous Chinook salmon passage; hence *C. shasta* genotype I is absent from upper portions of the basins (Atkinson and Bartholomew, 2010a; Hurst and Bartholomew, 2012; Stinson and Bartholomew, 2012).

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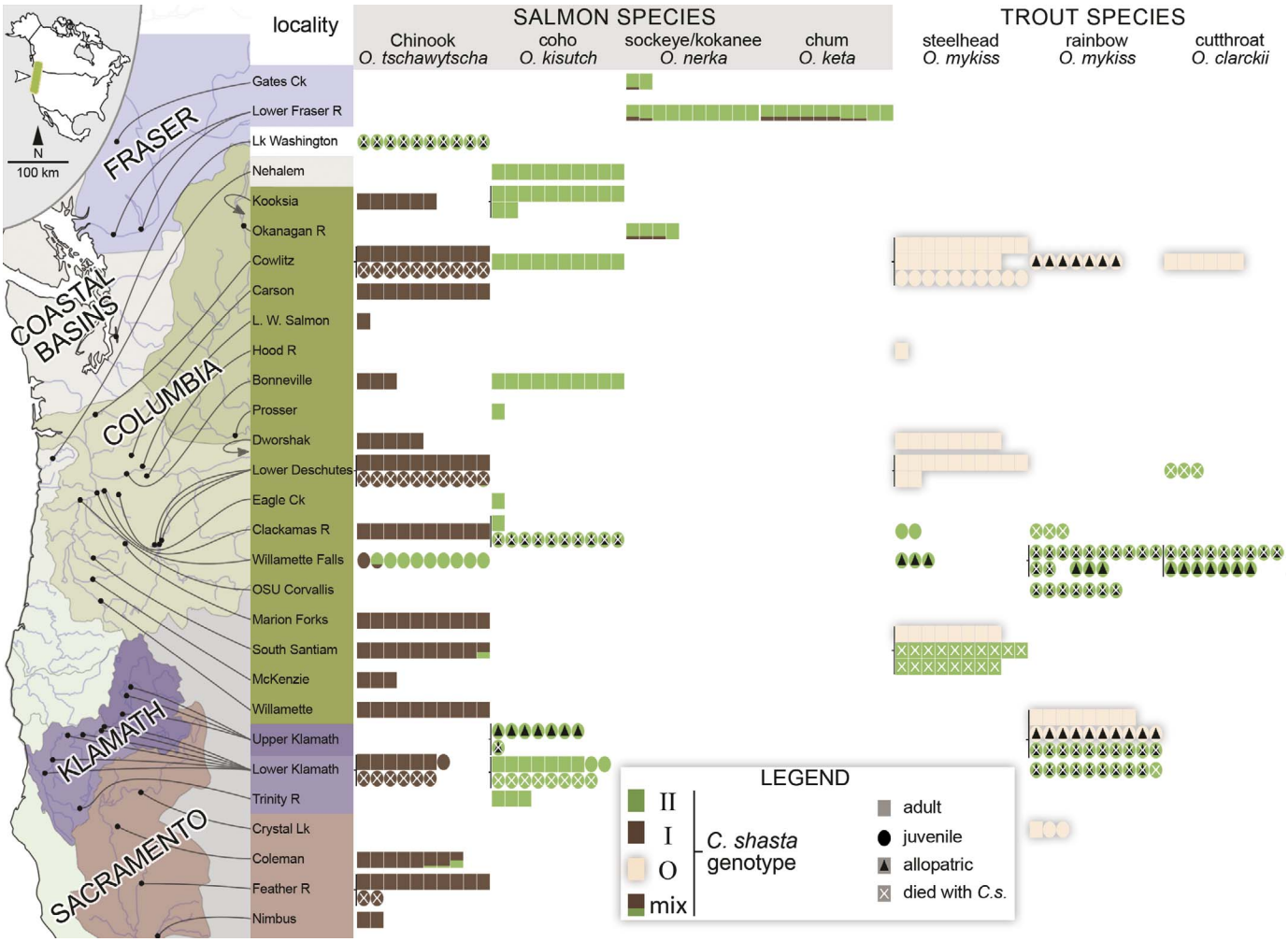

FIGURE 1. Graphical summary of collecting sites, *Ceratonova shasta* genotypes, and fish samples (species, age, sympatric/allopatric relative to the parasite, and if the fish died with *C. shasta*).

To examine host associations of the *C. shasta* primary genotypes (on the basis of the tri-nucleotide repeat) across the parasite's geographic range, we collected and genotyped 448 samples from 6 species of salmonids across the PNW. Our data confirmed the widespread presence of the same *C. shasta* genotypes and were consistent with previously observed specific host–parasite associations (i.e., O, steelhead trout; I, Chinook salmon). We showed that genotype II infected a wide range of species, typically coho salmon and allopatric rainbow trout. We did not detect any additional primary genotypes. The widespread, consistent nature of both parasite presence and relationship with fish hosts provides insights into host–parasite evolution and the role of humans in shaping host and parasite distribution.

MATERIALS AND METHODS

Fish samples

Salmonids were either trapped at hatcheries, held in cages, or collected post-spawning. We classified fish by origin relative to the parasite: fish "sympatric" with the parasite came from water bodies endemic for *C. shasta*, and included spawning adult fish and hatchery-reared juvenile fish. "Allopatric" fish derived from non-enzootic watersheds and were mostly juvenile fish exposed as sentinels in cages at localities endemic for *C. shasta*. Fish were examined for clinical signs of disease (hemorrhagic intestine, swollen vent, and ascites) and intestines were scraped for *C. shasta* myxospores (Bartholomew, 2012). Fish with either disease or spores were PCR assayed using *C. shasta*–specific primers and sequenced for genotype (Atkinson et al., 2018). Intestinal tissue (~5-mm section of the posterior gut) was either frozen after collection, processed fresh, or preserved in ethanol. Sample localities are shown in Figure 1, with a comprehensive list of sites, species, and numbers of *C. shasta*–positive fish given in Suppl. Data, Tables S1 and S2.

Sample contributors

Multiple agencies contributed samples: Department of Fisheries and Oceans, Canada (DFO); Washington Department of Fish and Wildlife (WDFW); Oregon Department of Fish and Wildlife (ODFW); California Department of Fish and Game (CDFG); Salmon River Council; U.S. Fish and Wildlife Service (USFWS); U.S. Geological Survey; Oregon State University (OSU); and Yakama Nation.

Sample genotyping

Our aim was to genotype *C. shasta* from at least 10 fish from each locality and species. This was not always possible if fewer fish were provided by collaborating agencies or if a smaller number of fish in a group was infected. Total DNA was extracted from intestinal samples using a modified "boiled-crude" method of Palenzuela et al. (1999): incubation at 56 C for 1–2 hr with 180 µl of Qiagen buffer ATL (Qiagen, Germantown, Maryland) and 20 µl of proteinase K to digest tissue, followed by heat denaturation at 95 C for 15 min. These crude extracts were diluted 1:100 with water or Qiagen buffer AE before amplification in a PCR using Promega GoTaq Flexi polymerase (Promega, Madison, Wisconsin), with *C. shasta*–specific primers: either Cs1482F and CsGenR1 (Atkinson and Bartholomew, 2010b) or the slightly modified genotyping primers Cs1479F and Cs2067R (Atkinson et al., 2018), using chemistry and cycling conditions given in that paper. Amplified DNA was purified using either a Qiagen PCR purification kit or ExoSAP-IT (USB, Cleveland, Ohio). All samples were sequenced in 1 direction with the appropriate forward primer (Cs1482F or Cs1479F) at OSU's Center for Genome Research and Biocomputing, using an ABI Prism® 3730 genetic analyzer (Applied Biosystems, Foster City, California). We assigned genotypes by counting tri-nucleotide (ATC) repeats at position ~460 in sequence chromatograms (Atkinson and Bartholomew, 2010a) using 4Peaks (v. 1.7.2) or BioEdit (Hall, 1999). We confirmed that the different primer pairs resulted in the same genotype assignment by sequencing several samples with both primer pairs. For samples with mixed *C. shasta* genotypes (visible as multiple, stacked peaks in typically the downstream part of the sequence), the percentage of each genotype was estimated from the average height ratios of coincident peaks (Atkinson and Bartholomew, 2010a). The lower limit for detecting a genotype in a mixed sample was regarded as ~5% of the signal.

RESULTS

We genotyped *C. shasta* from 448 fish samples, from 6 species of salmon and trout: 161 Chinook salmon, 153 rainbow/steelhead trout, 83 coho salmon, 10 chum salmon, 25 coastal and west slope cutthroat trout, and 16 sockeye salmon. Sampling intensity varied between species and locality (Fig. 1; Tables SI, S2), with samples from Chinook and coho salmon accounting for 55% of the data set. We found that the 3 genotypes identified originally from the Klamath River basin (O, I, II) were widely distributed throughout the PNW. Genotypes I and II were found in fish sampled from all river basins. Genotype O was detected in fish sampled from almost all locations except the Fraser River (where we were sampled only sockeye and chum salmon) and the Sacramento River (where we sampled only Chinook salmon). Mixed infections with genotype II (i.e., O and II, I and II) were detected in ~5% of fish samples (22/448); mixtures of genotypes O and I were not detected. We did not encounter any novel genotypes based on the number of repeats of the trinucleotide sequence ATC in the ITS-1 region; however, SNPs were observed in chromatograms of many sequences, as has been noted previously (Atkinson and Bartholomew, 2010b).

Genotype O was detected in trout only, from all river basins where trout were sampled (Fig. 1). We detected this genotype in the Klamath River, and throughout the Columbia River basin in the Cowlitz River (Washington), the Clearwater River (Idaho), and the Willamette and Deschutes rivers (Oregon). Although rainbow/steelhead trout are native to both the Fraser and Sacramento rivers, we were unable to obtain fish from these systems. Genotype O was dominant in samples of sympatric rainbow/steelhead and coastal cutthroat trout. These fish did not usually have clinical signs of disease, and parasite myxospores were present at low to moderate intensity. Sympatric, adult steelhead trout from the South Santiam Hatchery (Willamette River, Oregon) that successfully spawned were infected with genotype O, but steelhead trout that died before spawning displayed clinical disease and were infected with genotype II.

Genotype I was detected almost exclusively in sympatric strains of Chinook salmon, where it was dominant in both adult and juvenile fish, and in spring and fall run stocks. Clinical signs of disease and mature genotype I myxospores were observed in both adult and juvenile Chinook salmon, with only 17% of cases where fish died of enteronecrosis. We detected genotype I as the lowest fraction of mixed genotype infections in sockeye (5/16) and chum salmon (10/10) from the Fraser and Okanagan river sites; Chinook salmon samples were not obtained from these localities.

Genotype II was detected in 6 species and across all river basins. For coho salmon, 100% genotype II was detected in 20/20 sympatric juveniles and 55/55 adults. In other sympatric fishes, genotype II was often detected in mortality events (e.g., 10/10 Chinook salmon from a facility fed by Lake Washington, 17/17 adult steelhead trout at South Santiam Fish Hatchery. Sockeye and chum salmon had genotype II as the dominant genotype in all 26 samples but mixed with genotype I in 5/16 sockeye and 10/10 chum. Only chum salmon had clinical signs of disease and visible myxospores. Genotype II caused mortality in allopatric rainbow trout held in the Willamette and Klamath rivers, and allopatric cutthroat trout in the Willamette and Deschutes rivers.

DISCUSSION

Our survey of *C. shasta* genotypes from 6 Pacific salmonid species in rivers from California to British Columbia, Canada demonstrated that the parasite population had a similar genetic composition and host associations across a large spatial scale. Specifically, we identified the same primary ITS-1 parasite genotypes across the PNW and no novel primary genotypes in the ATC-repeat genotyping region of the ITS-1. Overall, the population structure of *C. shasta* primary genotypes correlated consistently to fish host species for sympatric samples, rather than to geographic locality. The fish host character of sympatric/allopatric overrode any life-history trait (adult/juvenile, spring/fall run, anadromous/freshwater forms). With few exceptions, we found that the previously observed associations between parasite genotype and host salmonid species were consistent at this large spatial scale: type O in rainbow/steelhead trout, type I in Chinook salmon, type II in coho salmon and other species (Atkinson and Bartholomew, 2010a, 2010b; Stinson and Bartholomew, 2012). These observations supported the hypothesis that host–parasite relationships of *C. shasta* are well established. More in-depth phylogeographic analyses, incorporating SNP and INDEL variations within the ITS-1 region, or preferably a nuclear marker gene, may reveal higher spatial resolution of parasite populations and provide greater insight to the timing of the parasite

genotypes' most recent common ancestor and subsequent evolution events.

We confirmed that genotype O was observed in trout specifically, and never detected in salmon, even when present in the water during exposure. We take this as further evidence that genotype O has evolved solely as a parasite of trout, i.e., evolutionarily distant enough to be unable to infect salmon. Genotype O was present throughout the parasite range, which likely parallels the wide distribution of sympatric Pacific trout in river systems across the region (Waples et al., 2001). We observed that sympatric rainbow/steelhead and coastal cutthroat trout infected with genotype O showed few clinical signs of disease, and saw myxospores infrequently. Allopatric rainbow trout exposed simultaneously to genotypes O and II died rapidly from the proliferation of genotype II, overshadowing any effects of genotype O. However, as with the original Klamath River observations of Atkinson and Bartholomew (2010a, 2010b), when genotype II was absent, these fish survived with genotype O infections. We observed that 1 group of sentinel-exposed allopatric rainbow trout exposed in the mid-Klamath River basin in June 2016 survived for almost 2 yr (until euthanized), with mature myxospores seen in feces throughout this period. The lack of mortality associated with genotype O suggests that selection for virulence is not strong when the parasite utilizes a solely freshwater host. Thus, the iteroparous nature of Pacific trout provides an advantage to the parasite by the alternate host being physically present in sympatry longer than semelparous salmon.

Across the PNW, genotype I had an almost exclusive association with Chinook salmon, with the capability of causing mortality (Hallett et al., 2012). We detected genotype I as a minor component of mixed infections in sockeye and chum salmon (current study), and previously in non-native brook trout (*Salvelinus fontinalis*; Stinson and Bartholomew, 2012), but never in coho salmon or any Pacific trout species, which is further evidence of its host specificity. Under atypical circumstances, where Chinook salmon were exposed to *C. shasta* genotype II in the absence of genotype I, Chinook salmon could become lethally infected with genotype II (e.g., Upper Klamath Lake; Atkinson and Bartholomew, 2010a). However, as with genotypes O and II in allopatric rainbow trout, if Chinook salmon were exposed where there was any genotype I detectable in water, genotype I would predominate in the subsequent fish samples, e.g., Klamath and Deschutes rivers where data show multiple sympatric genotypes (Atkinson and Bartholomew, 2010a, 2010b; Stinson and Bartholomew, 2012; Bartholomew et al., 2016). We detected mixed genotypes in a low number of adult Chinook salmon samples from the South Santiam Fish Hatchery and Coleman National Fish Hatchery, although we could not determine if both genotypes produced mature parasite spores. This concurs with previous studies that show that Chinook salmon can be simultaneously infected by multiple genotypes (Stinson and Bartholomew, 2012; Hurst et al., 2014), but that typically only the host-specific genotype I is able to persist and sporulate. The effects of mixed infections on disease development and parasite success are not well understood, and work is needed to better understand in-fish interactions between parasite genotypes. The fact that migratory steelhead trout are present in many rivers at similar times as Chinook salmon, but were not infected with genotype I, indicates that there is high affinity of each parasite type for either trout or salmon hosts, and suggests that these could even be regarded as different species.

In contrast to the host specificities of genotypes O and I, genotype II was detected in 6 salmonid species, and in all river basins. It was the only genotype detected in coho salmon and the dominant genotype in sockeye and chum salmon. We observed mortality in sympatric juvenile coho salmon only in the lower Klamath River, where it has been shown previously to be associated with exposure to high densities of genotype II (Hallett and Bartholomew, 2006; Hallett et al., 2012). Rainbow trout have been widely introduced for fishing without regard for their resistance to *C. shasta*, and in some cases have been stocked precisely because they are susceptible and do not survive to compete with native stocks (Hurst et al., 2012). As has been observed previously in allopatric rainbow/steelhead trout, we determined that diseased allopatric Chinook salmon and cutthroat trout were most often infected with genotype II (Bjork and Bartholomew, 2010; Stinson and Bartholomew, 2012). Allopatric trout were more susceptible to genotype II than genotype O even when they co-occurred, and the high mortality in these exposures demonstrates the selective pressure that the parasite exerts on its host. We observed sporulation of genotype II in 6 salmonid species, indicating completion of parasite development in the fish host (Shul'man, 1966). Together with previous observations of genotype II sporulation in brown (*Salmon trutta*) and brook trout (Stinson and Bartholomew, 2012), these data demonstrate that this genotype is a true generalist, able to complete its life cycle in multiple fish host species. We detected genotype II in DNA extracted from pink salmon (*O. gorbuscha*) kidney samples, though without intestinal samples from these fish we do not know whether this represented an infection capable of producing spores, or was DNA from blood-borne intermediate stages (data not shown). The ability of genotype II to infect and cause disease across multiple species suggests that it has either evolved more recently or succeeds via a different host strategy. Significantly, the practice of stocking allopatric salmonids provides a mechanism for sustaining this generalist genotype in areas presently inaccessible to coho salmon and other sympatric host species.

We observed that host origin, whether sympatric or allopatric with the parasite, was the main factor that correlated with clinical disease. The majority of our samples were from fish populations sympatric with the parasite, and often the infected fish we sampled were not clinically diseased. Sympatric rainbow/steelhead and coastal cutthroat trout usually showed few disease signs, with relatively low numbers of genotype O myxospores detected in intestinal scrapes. Sockeye salmon are the only sympatric fish that showed little to no disease signs, and Stinson and Bartholomew (2012) demonstrated that kokanee (landlocked sockeye salmon) cleared infection 3 wk after being exposed to the parasite. Disease signs and mortality in sympatric fish were observed either when some other stressor was present, e.g., prespawn mortality in adult steelhead trout at South Santiam Fish Hatchery, or when parasite levels were high. In contrast, allopatric fishes exposed in the same localities (Atkinson and Bartholomew, 2010b; Hurst and Bartholomew, 2012; Stinson and Bartholomew, 2012) typically displayed gross signs of disease and produced large numbers of genotype II spores. The life stage of the host is also an important factor that correlates with severity of the disease. Sympatric adult Chinook and coho salmon generally have a higher prevalence *C. shasta* infection than their juvenile

counterparts (Bartholomew, 1998), which is presumably a result of the decrease in immune function at this life stage as the fish stop feeding and shift their energy into reproduction (Dolan et al., 2016). Infecting anadromous fish hosts at the end of their life cycles is a critical part of the parasite's life-cycle strategy, allowing it to be transported upriver to infect polychaete populations (Bartholomew, 1998; Kent et al., 2014).

Patterns of *C. shasta* genotypes in different fish species and life stages suggest that 2 broad parasite strategies have evolved—specialist and generalist. Genotypes O and I are specialists, having host-specific relationships with Pacific trout and Chinook salmon respectively, whereas genotype II is a generalist, opportunistically infecting a range of both trout and salmon species. These relationships may have been shaped by the life-history characteristics of the various fish species. For example, specialist genotypes I and O infect fish with highly plastic life histories (e.g., variable maturation ages and non-migrating freshwater resident forms (Waples et al., 2001)), which may provide the parasite with a more stable environment, i.e., longer periods when susceptible hosts are present in the river for the parasite to complete its life cycle. In contrast, coho, chum, and pink salmon have a nearly fixed life history (Groot and Margolis, 1991), which can leave periods when no hosts are present. Whereas coho salmon can be considered a stable host, as juveniles reside in freshwater for a year, chum and pink salmon are riskier hosts, as juveniles of these species spend less than half a year in freshwater. Thus, the different hosts' life-cycle patterns may select for a generalist parasite, which is buffered against the absence of 1 specific host. Further, a generalist parasite may fundamentally be able to infect new hosts opportunistically.

This study, taken together with our previous work, has demonstrated the existence of 3 primary *C. shasta* genotypes across a large spatial scale, and over at least 13 yr (2005–2018; Atkinson and Bartholomew, 2010b; Stinson and Bartholomew, 2012). These data support the hypothesis that long-term co-evolution of hosts and *C. shasta* have given rise to distinct genotypes with specificity for particular sympatric hosts. We further hypothesize that host specificity is the overriding driver of parasite diversity, but that the net, long-term effect of straying of infected fish hosts has resulted in parasite gene flow throughout the PNW. An additional study of the sub-genotype parasite variations is underway and should prove useful for both finer-scale mapping of parasite spatial variation and as a tag for better characterization of host populations, as has been demonstrated for other fish parasites (e.g., Mackenzie, 2002; Criscione et al., 2006). Finer patterns in the ITS sequence data, or development of nuclear gene markers, should better characterize the nature of the parasite population, i.e., best regarded as genotypes of *C. shasta*, or different species altogether. Additional genetic markers will help define the long-term timing of host–parasite evolutionary events, and to characterize presence and rates of gene flow between parasite sub-populations, particularly if combined with data from other fish pathogens (e.g., IHNV; Kurath et al., 2003) that share the host and geographic range of *C. shasta*. The parasite's distribution in the northern part of its current range and seasonal genotype distributions are of interest given changing climate patterns that will result in warming waters and changes in discharge (Ray et al., 2015). We predict that timing of parasite emergence will change, potentially affecting fish runs that previously had avoided parasite encounter (Margolis and Evenlyn, 1975). Hence there is a need to characterize present host/parasite distribution and timing in rivers where the parasite is currently endemic, to predict future disease risks.

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