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Detection and phylogenetic assessment of PRV-1 via sampling of biological materials released from salmon farms in British Columbia

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Abstract

The growth of the net pen salmon farming industry and the concurrent decline of many wild salmon populations have generated an ongoing call for research into the potential role of infectious agents associated with salmon farming. Here, we describe a method to screen for the presence of viral nucleic acids by sampling biological waste in the vicinity of farms. We collected biological samples adjacent to 56 marine net pens from five different companies, and two farm salmon processing plants (n = 230), 70% of samples were positive for *Piscine orthoreovirus*-1 (PRV-1). Viral genome segments S1 (n = 68) and M2 (n = 39) were sequenced and subjected to phylogenetic analysis. Sequences were all monophyletic to the clade of PRV-1 routinely found in the region. Consistent with previous studies, samples collected near Atlantic salmon (*Salmo salar*) farms were related to a lineage of PRV-1a commonly detected in both farmed and wild salmon in British Columbia, while a related lineage of PRV-1a was detected near Chinook salmon (*Oncorhynchus tshawytscha*) farms. Similarity in sequence within companies suggests that Atlantic salmon freshwater hatcheries are a probable source of this virus to the marine environment, concordant with recent detections of PRV-1 within those hatcheries.

Key words: *Piscine orthoreovirus*, PRV-1, aquaculture, salmon farm, phylogenetics

Introduction

Open-net salmon farming creates opportunities for pathogens to be exchanged between farmed and wild fish. Open-net salmon farming facilities in British Columbia (BC) are situated in the nearshore marine habitat frequented by wild fishes, and rely on ocean currents to flush biological waste out of their net pens into the surrounding ocean. Thus, pathogens amplified in these dense farmed populations are continually released into the marine environment (Taranger et al. 2014; Shea et al. 2020). The free movement of seawater directly links the farm environment with wild salmon habitat, exerting anthropogenic influence on the local virome.

Introductions of non-native viruses have potential negative implications to both wild and farmed populations. Farm salmon populations differ from wild populations in epidemiologically salient ways, as farmed salmon are high-density, free of predation pressure, stationary and do not need to seek food. Further, in the eastern Pacific, the Atlantic salmon (*Salmo salar*) raised in farms are members of an exotic species. Thus, their introduction exposed native Pacific species to exotic infectious disease (Kibenge et al. 2019; Siah et al. 2020; Mordecai et al. 2021). There have been multiple introductions of Atlantic salmon to the Pacific to enable Atlantic salmon aquaculture. Bakke and Harris (1998) recognised that disease spill-over from farms could be detrimental to the conservation of wild salmon. Peterman and Dorner (2012) reported that although declines in wild salmon pre-date farming in the region, declines have intensified since 1990, and offered marine mortality from pathogens as a leading hypothesis.

Piscine orthoreovirus (PRV) is omnipresent in farm salmon in BC and present in wild Pacific salmon at varying levels (Morton et al. 2017; Polinski et al. 2020; Mordecai et al. 2021). PRV has been linked to disease in Chinook salmon (*Oncorhynchus tshawytscha*) (Di Cicco et al. 2018), Therefore, the virus presents an important case study to identify the temporal and spatial relationship between salmon farms and presence of the virus in the region. PRV, a member of the virus family, *Reoviridae*, has a genome of 10 segments of double-stranded RNA enclosed in a non-enveloped double-layered protein capsid. Among closely related viruses, these are thought to be highly stable (King et al. 2012). Thus,

PRV-1 is thought to be capable of spreading over long distances between infected salmon farms (Kristoffersen et al. 2013).

Analysis of whole-genome and individual-segment sequences has resolved the PRV species into three distinct strains (also known as genotypes), named PRV-1, PRV-2 and PRV-3 (Vendramin et al. 2019*a*). The S1 segment can be used to differentiate strains of PRV, as well as substrains (also known as subgenotypes or subtypes) of PRV-1 (Kibenge et al. 2013). Efforts to fully characterize variation within the PRV-2 and PRV-3 strains are ongoing (Sørensen et al. 2020).

To date, only the PRV-1a substrain has been detected in the Northeast Pacific Ocean, where it infects both farmed Atlantic salmon and wild Pacific salmon (*Oncorhynchus* spp.; Kibenge et al. 2013; Siah et al. 2015, 2020; Morton et al. 2017; Di Cicco et al. 2018; Purcell et al. 2018; Mordecai et al. 2021). Recent and comprehensive analyses of PRV-1 diversity concluded that all the circulating sequence isolates in the Eastern Pacific appear to descend from a single introduction event approximately 30 years ago (Mordecai et al. 2021). However, there is also evidence of at least one, more recent, introduction of a different lineage of PRV-1a into the region (Kibenge et al. 2019; Siah et al. 2020; Mordecai et al. 2021). A putative detection from an archived sample from 1977 suggests earlier introductions are also possible (Marty et al. 2015; Kibenge et al. 2017; Siah et al. 2020). Regardless of the inferred date of introduction, all studies agree that the origin of PRV-1 is the North Atlantic (Kibenge et al. 2013; Siah et al. 2020). Now that it is in the Northeast Pacific, there is concern that salmon farms, which have high rates of infection, pose a transmission risk to wild Pacific salmon (Bateman et al. 2021; Mordecai et al. 2021).

Via laboratory challenge with purified virus, all three strains of PRV are known to cause disease (Takano et al. 2016; Wessel et al. 2017; Vendramin et al. 2019*a*), and all primarily target the red blood cells of salmonid fish (Finstad et al. 2014; Wessel et al. 2015, 2017; Takano et al. 2016; Vendramin et al. 2019*b*). Infections are associated with distinctly different disease manifestations across different salmonid host species, which is thought to be a result of how the red blood cells of each species respond to the virus (Olsen et al. 2015; Wessel et al. 2015, 2017; Godoy et al. 2016; Takano et al. 2016; Hauge et al. 2017; Cartagena et al. 2018; Di Cicco et al. 2018; Vendramin et al. 2019*a*). Further, in a specific Icelandic strain of Atlantic salmon, there is variation in virulence across substrains of PRV-1 (Wessel et al. 2020). Importantly, however, the potential virulence (or pathogenicity) of a viral lineage in one host species does not predict the virulence of the same lineage in other species (Longdon et al. 2015). Therefore, the risk posed by PRV-1 to Pacific salmon cannot be determined by experimental evidence from Atlantic salmon.

In Atlantic salmon challenges carried out in Norway, all tested isolates of PRV-1 (including both PRV-1a and PRV-1b) caused inflammatory heart lesions (Wessel et al. 2020), with the most severe lesions manifested after infection by some, but not all, isolates of PRV-1b (Wessel et al. 2017, 2020). These results from Norway support experimental studies in Canada which did observe lesions but did not confirm the cause. For instance, in a challenge study in BC, PRV-1a was associated with mild to moderate lesions in Atlantic salmon (Polinski et al. 2019), and in a separate epidemiological study, heart and skeletal muscle inflammation (HSMI) was reported on Atlantic salmon farms in BC (Di Cicco et al. 2017). Important to the potential risk posed to wild Pacific salmon, PRV-1a has been associated with mild heart lesions in sockeye salmon (*Oncorhynchus nerka*) and Chinook salmon (Garver et al. 2016), jaundice/anemia in farmed Chinook (Di Cicco et al. 2018), and lesions consistent with jaundice/anemia in wild Chinook salmon (Wang 2018) (although we note that due to the nature of these studies the etiological role of PRV in these lesions has not been determined). Other challenge studies of PRV-1 in Chinook salmon resulted in mild disease lesions and changes to the hematocrit of infected individuals (Purcell et al. 2020). The absence of overt disease in challenge studies does not rule out the possible existence of a cause-and-effect relationship between the virus and disease under different conditions. A recent study found that increasing rates of PRV positivity in a population are associated with decreasing survival for Chinook salmon, and body condition (in terms of weight, a key health metric for wild fish) decreases as PRV infections become more intense in individuals (Bass et al. 2022). There is increasing evidence of PRV-1a being associated with, or being the cause of, disease (Di Cicco et al. 2017, 2018; Wessel et al. 2020). In contrast, the Canadian governmental organization that is responsible for regulating PRV on salmon farms does not currently classify PRV as a "disease agent" (BC Aquaculture Regulatory Program 2019).

PRV-1 prevalence in wild Atlantic salmon is relatively higher in areas with Atlantic salmon farming activity (Vendramin et al. 2019*a*); and similarly, in BC, Canada, PRV-1a infection is common in farmed Atlantic salmon, with infection becoming almost ubiquitous over the production cycle (Bateman et al. 2021; Mordecai et al. 2021). Genomic sequencing of PRV-1 in farmed and wild salmon in BC has found evidence of transmission of PRV-1 between farmed and wild salmon, and the probability of PRV-1 infection is elevated in wild Pacific salmon in proximity to salmon farms (Morton et al. 2017; Mordecai et al. 2021).

Recent studies with access to farm salmon found that certain infectious agents (including PRV-1) are detected in freshwater hatcheries, and these are likely translocated to the marine environment via transfer of fish from infected hatcheries into marine farms (Bateman et al. 2021). There is minimal sequence data available of PRV-1 from hatcheries, but genomic surveillance of PRV in BC salmon farms could help to determine the source of infection, as similar lineages of PRV would be expected in farms stocked from the same hatcheries. The alternative hypothesis is that PRV is transmitted from the marine environment (either via wild fish, or other nearby farms) to net pens, where the infection then spreads within the farm. This issue remains uncertain because there is little publicly available data on the infection rate in hatcheries.

To address the question of whether farms play a role in disease transmission, it is necessary for researchers to have access to the farms, as well as to freshwater hatcheries.

Sequence-based molecular epidemiology relies on identifying samples to specific farm sites. To date, only limited access has been provided to a few selected research teams. A handful of previous studies relied on samples obtained from markets, and for these samples the company and farm site where the fish was grown were unknown (Kibenge et al. 2013; Morton et al. 2017). Studies which did have a degree of access were limited to only certain farms at certain times (Di Cicco et al. 2018; Mordecai et al. 2021).

In the absence of access to samples from within farms, environmental detection is a useful method for identifying the local presence of a pathogen. In this study, we demonstrate a method to access biological materials for virus surveillance and sequencing without requiring direct access to fish in the farms. The study was designed to probe the potential for using samples of detritus drifting out of the net pens (*i*) to monitor the relative prevalence of PRV within the net pens, (*ii*) to obtain sequences for genetic epidemiological analysis, and (*iii*) to gain preliminary insight on the potential role of such detritus in transmitting the virus from the farms to the external environment. This allowed us to link samples to individual farm companies, and describe viral genomic variation between aquaculture companies in terms of farm company, geographical location and species.

Materials and methods

Sampling

The study area spanned 650 km from Puget Sound, Washington State, USA, through to the central coast of BC, Canada. Sixty-nine salmon farms were operating in this area at some point during the sampling period from 2018 to 2020. Samples were collected at 56 salmon farms that were sited in clusters within seven geographic areas labeled: (*i*) Salish Sea, (*ii*) Discovery Islands/Johnstone Strait, (*iii*) Broughton Archipelago, (*iv*) Port Hardy, (*v*) Nootka Sound/Esperanza, (*vi*) Clayoquot Sound, and (*vii*) Central Coast (Fig. 1).

While the majority of sampling took place in 2018, 2019, and 2020, additional samples were included from 2016 to 2017. The 2017 sampling event occurred as Atlantic salmon smolts were introduced to the net pens via large diameter hoses from a smolt transport vessel. The transfer process caused a large number of scales to drift out from the farm. For all sampling events, a small vessel was used to circle each farm at slow speed with observers looking into the water to identify and collect biological material. In most cases, tidal flushing action was observed along one side of the farm perimeter causing the farm containment nets to billow outward with biological material visibly drifting from the farm in this tidal current.

Additionally, we collected "Effluent" from the farm salmon processing plants: Browns Bay Packing in Campbell River, and Lions Gate Fisheries in Tofino. To the best of our knowledge, Brown's Bay exclusively processes farmed salmon. At the time of sampling, we believe the Lions Gate Fisheries plant solely processed farmed Chinook, and a farm salmon delivery vessel was present at the plant at the time of sampling. Since the time of sampling, this plant has also been processing wild salmon. The effluent samples were collected via a 150 μm mesh plankton net, held by a diver over the submerged pipe draining from the plants. These samples exhibited the color and texture of kidney or semicoagulated blood and a putative host was designated based on the species of salmon known to be processed in each plant at the time of sampling.

There are no permits required in BC to sample marine water or free-floating detritus.

A small number of farm salmon were collected whole, including 5 Atlantic salmon morts provided from a Mowi farm and 6 Chinook salmon purchased from Creative Salmon. Additionally, 2 olive rockfish (*Sebastes serranoides*) were collected while feeding on the effluent during the sampling event from the processing plant in Tofino and, therefore, were categorized as "Creative" samples. For all these fish, organs (gills, heart, head kidney, and liver) were dissected, pooled, and processed for RNA extraction.

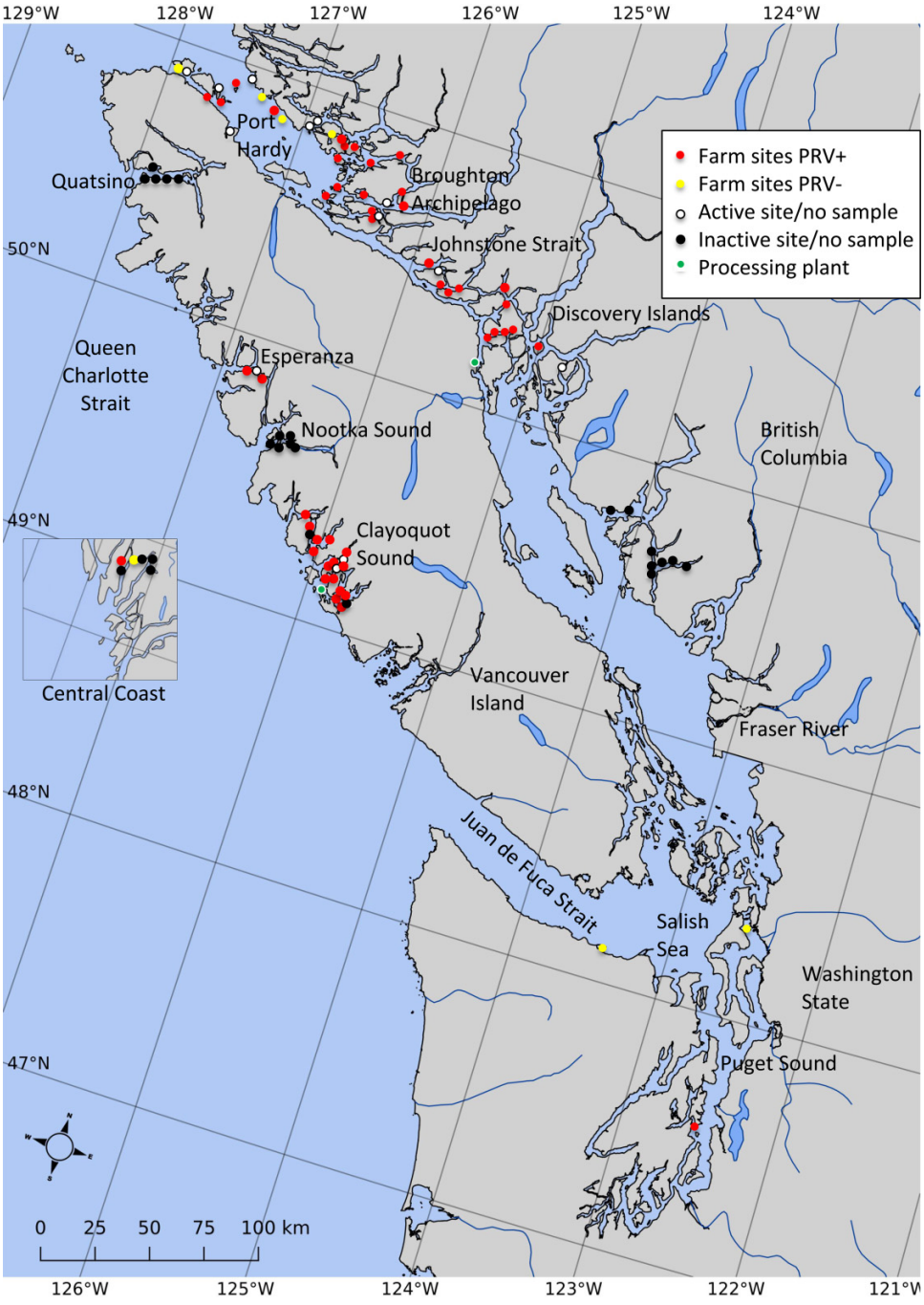
Environmental samples of biological material found adjacent to the farms were collected with a 2 mm mesh "aquarium" net attached to a telescoping 4 m pole. A fresh pair of sterile tweezers was used per sample to transfer approximately 0.1 g material from the net into 1.5 mL vials filled with RNALater (TM). The vials were stored at 4 °C in a refrigerator for 1–3 weeks, the duration the crew were at sea, until they could be shipped on ice to the testing laboratory.

Several types of biological material were collected adjacent to the farms. We categorized these as "lipid-like"—which consisted of globular fragments of white or yellowish fatty matter floating on the surface, "unidentified biological material"—frayed fragments of fish tissues, "feces-like"—orange or brown fish feces, fish "scales" and "sea lice" (or combinations thereof) (Fig. S1). We differentiated the sample types by buoyancy; "unidentified biological material", "feces-like", and "scales" were suspended below the surface, while biological material floating on the surface was categorized as "lipid-like". Samples were typically <5 mm in any dimension, and hence material from each site was pooled within vials to provide a sufficient amount (approximately 0.1 g) of material for testing. Collections were opportunistic. Most sites were sampled more than once. The number of samples was limited by sample availability. Samples could not be found at every active salmon farm.

RNA extraction and RT-qPCR

Molecular analysis (RT-PCR, amplicon sequencing) was carried out by Frederick and Molly Kibenge (Department of Pathology and Microbiology, University of Prince Edward Island). Total RNA was isolated using a modified total RNA extraction protocol with Trizol Reagent (Life Technologies Inc.) and RNeasy mini Kit (QIAGEN) as previously described (Kibenge et al. 2013). Total RNA from fat samples was extracted using the RNeasy Lipid Tissue mini kit (QIAGEN) which includes Qiazol. RT-qPCR was run on the LightCycler 480 (Roche Applied Science), version 4.0. The crossing point (Cp) or threshold cycle (Ct) was determined by use of the maximum-second-derivative function on the LightCycler

Fig. 1. The study area spanned 650 km of coastline from Washington State, USA, through central BC, Canada. Some farms were inactive during sampling, and at some active sites floating detritus was not observed. Samples were also collected from submerged marine effluent pipes from two farm salmon processing plants. (The map projection is "NAD83 North America Albers Equal Area Conic". The hydrography data are from the USGS North American Atlas data set and the coastline data are a combination of Canadian Administrative boundaries and the Washington State Department of Ecology layer.)



software release 1.5.0. The OneStep RT-PCR kit (QIAGEN) was employed for all RT-qPCR reactions according to the manufacturer's specifications.

The RT-qPCR assay for PRV used the primer-probe set sequences and reaction conditions as previously described (Kibenge et al. 2013) with minor modifications. Total RNA was denatured at 95 °C for 5 min and immediately placed on ice before use in the RT-qPCR. The PRV assay was performed in 1 well per sample, and samples considered to be PRV-positive had Ct values $\leq$ 40 and with an exponential curve; Ct values $>$ 40 were considered negative.

Up to 8 vials of material (and thus 8 individual Ct results) were obtained per farm, including multiple samples collected on the same date and multiple visits to the site (Table S1). A farm site was designated as PRV-positive if any of the samples from that site produced a Ct value of $\leq$40.

Conventional RT-PCR and sequence analysis of PRV segments S1 and M2

Selected samples with Ct values $\leq$ 40 in the PRV RT-qPCR assay were tested in conventional RT-PCR targeting the full-length genome segments S1 and M2 with the PCR primer pairs and reaction conditions; and the PCR products were cloned and sequenced as previously described (Kibenge et al. 2013).

Sample quality and confirmation of salmon origin

Identification of the material as salmon and assessment of RNA quality were based on RT-qPCR for elongation factor 1 alpha (ELF-1α) carried out using Roche LightCycler 480 RNA master Hydrolysis Probe kit (Roche Diagnostics). The primers, probes, and reaction conditions used were as previously described (Sepúlveda et al. 2013), and were designed to specifically amplify and detect selected ELF-1α coding sequences in Atlantic salmon, coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*). The ELF-1α assay was performed in duplicate to determine the quality of the RNA (Madhun et al. 2018).

Phylogenetic analysis

Full genome analysis of PRV-1 has shown that, due to reassortment of viral genome segments, the perceived evolutionary history of a viral lineage can differ between segments, and a comprehensive analysis requires construction of individual phylogenies for each segment. These are typically accompanied by an analysis with a single tree representative of all segments (Dhamotharan et al. 2019; Mordecai et al. 2021). However, multiple studies have used solely one or two segments to characterize the virus, and it has been demonstrated that this is adequate to differentiate between substrains of PRV-1 (Dhamotharan et al. 2019). In this study, solely the S1 (and in some cases, the M2) segments were sequenced. Additionally, since sequences were amplified from environmental samples, separate PCR amplicons of different segments cannot be attributed to the same individual host, and therefore sequences from the same sample were not concatenated and not analyzed together.

PRV-1 sequences were aligned with publicly available PRV sequences using MAFFT (Katoh and Standley 2013). Model selection was carried out using MODELTEST (Posada and Crandall 1998), and the GTRCATI model was used to construct ML trees within RaxML using 100 distinct starting trees and 1000 bootstraps (Stamatakis 2014). Trees were midpoint rooted, which placed the root between PRV-1a and PRV-1b. Trees were displayed and annotated using ggtree (Yu et al. 2017). Sequences belonging to the PRV-1b substrain were collapsed (black triangle at bottom of Fig. 3), and the sublineages (clades 1 and 2) within were attributed as previously described (Mordecai et al. 2021). Clade 2 was further subdivided into clades 2.1 and 2.2. These clades were used to describe the various lineages of PRV-1 geographically and by company.

Statistical analysis

We analyzed the relationship between detections of the host ELF-1 gene and PRV-1 by comparing positive detections (Ct values $\leq$ 40; Fig. 2A). Because multiple samples were frequently obtained on single sampling trips to individual farms, samples obtained from the same sampling trip to the same farm could not be viewed as independent. To account for this potential dependence when assessing the significance of the relationship, we used mixed effects, linear modeling using the function, "glmer", in package, "lme4" (Bates et al. 2015) within R (R Core Team 2021). As in Fig. 2A, the dependent variable was "PRV Ct", and the independent variable was "ELF-1α Ct." To account for the dependence, we introduced a random effects term (which we refer to as the "sampling event" term) corresponding to each set of multiple samples. These random effects were assumed to be independent, identically distributed random variables with constant variance. There was one term for each sampling event. By specifying the "family" in the function, "glmer", to be "Gaussian", we ensured that the model was otherwise identical to a simple linear regression model.

We calculated confidence intervals for the prevalence at the various Ct thresholds applied (Table S2). We calculated these using a 1-sample proportion test, and we also calculated the intervals to account for potential variation in prevalences between sampling events by the standard profile likelihood methodology (Johnson et al. 1995). Within-sample-event prevalences were assumed to have a beta-distribution (Bolker 2008).

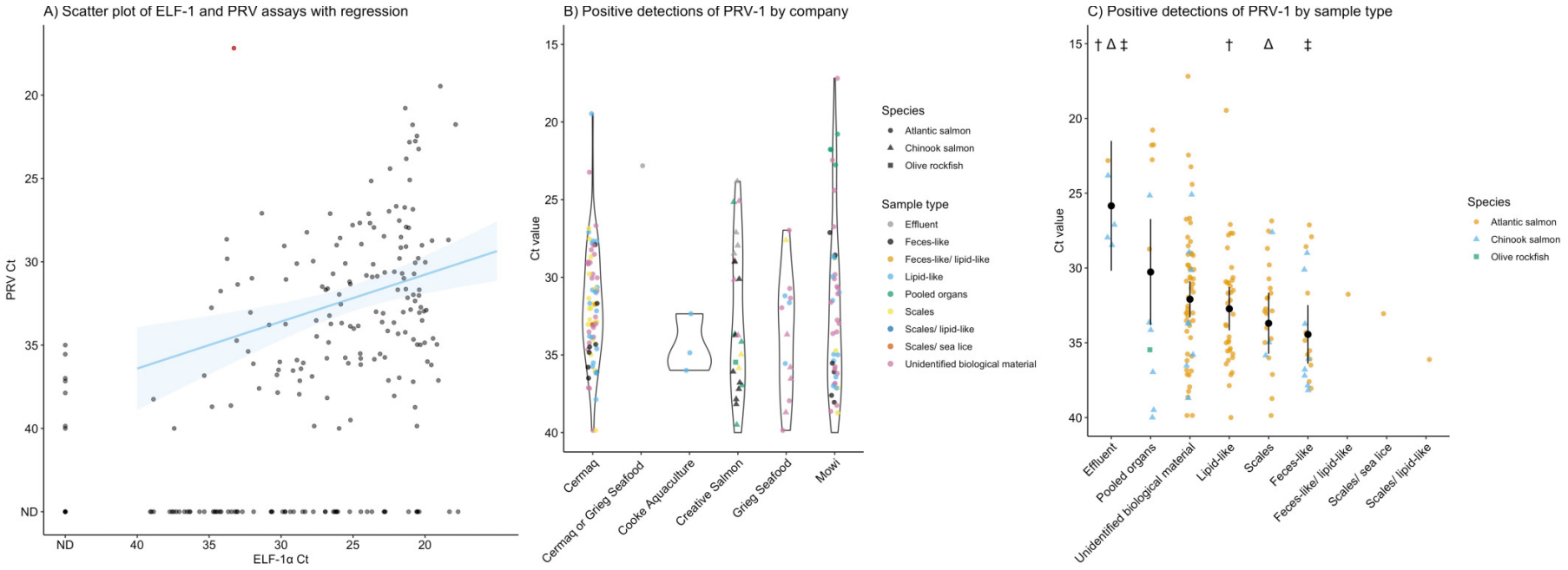
We also analyzed both the prevalence (proportion of positive detections) and the intensity (as gauged by the Ct value of the positive detections) for potential differences between companies and sample types, again using mixed-effects modeling (Figs. 2B-C). To draw inferences on proportions of positive test results, we used mixed-effects logistic regression (specifying the family as "binomial"). To draw inferences on Ct-values (conditional upon these being positive), we used mixed-effects linear regression (specifying the family as "Gaussian"). Because we detected no evidence of severe skewness in the data, we are confident that the implicit assumption of normality was warranted.

Our primary inferences were on potential differences in prevalence and intensity between tissue types and aquaculture companies. To this end, we fit models containing both factors together, others containing each factor separately, and null models containing no factors. All tests of significance were based on likelihood ratios for restricted vs. unrestricted models using the R "anova" function on fits generated by "glmer".

Where feasible, we followed these up with multiple comparisons and confidence bounds on individual estimated means. Bonferroni adjustments were applied to the multiple comparisons. To produce the confidence bounds, we used the Wald method for constructing confidence intervals for the relevant parameter estimates. To assess the reliability of this approach, we used zeta plots, available in "lme4" (Fig. S2).

We also eliminated the following sets of observations from our formal statistical tests associated with these primary

Fig. 2. (A) Scatter plot of *Piscine orthoreovirus*-1 (PRV-1) and elongation factor 1 alpha (ELF-1α) Ct values. A single, notable outlier is shown in red. This outlier had no influence on the significance of the correlation between these 2 variables. Negative detections (threshold cycle, Ct $>$ 40) are plotted as "ND", but these were not included in the regression analysis. (B) Violin plots of Ct value of positive samples from each company. Points are colored by sample type and shaped by species. There were no significant differences between these groups. (C) Plots of Ct value of positive samples from each sample type. Points are colored and shaped by species. Environmental samples are categorised as effluent ($n = 5$) (i.e., processing plant effluent samples, collected via a 150 μ mesh plankton net, which had the color and texture of kidney or semicoagulated blood), lipid-like ($n = 54$), feces-like ($n = 49$), unidentified biological material ($n = 75$), pooled organs ($n = 11$), scales ($n = 32$), feces-like/lipid-like ($n = 1$) (feces and lipid mixed together), scales/sea lice ($n = 1$) (scales and sea lice mixed together), and scales/lipid-like ($n = 1$) (scales and lipid mixed together). There were significant differences among the means of the 6 primary samples for which n was greater than 1. The symbols indicate significant differences between pairs of samples; i.e., any 2 sample types sharing the same symbol are significantly different.



inferences (but included them in Figs. 2 and S3): (*i*) all Cooke Aquaculture samples because there were only three samples producing positive Ct values from a total of 8 samples, all from a single sampling event, and (*ii*) the 3 combined tissue-type categories containing only 1 or 2 samples in each category. Furthermore, for all analyses that included the factor "Company", the Brown's Bay effluent sample was excluded because the facility processed fish from more than 1 company and we could not assign a company to the effluent.

Results

Detection of PRV-1 at farm sites

Biological materials were collected in 2016–2020 from 56 of the approximately 85 salmon farms that were active during this time period. These sites were distributed along the length of the study area (Fig. 1). The farm sites belonged to five different aquaculture companies Mowi, Cermaq, Grieg Seafoods, Creative Salmon and Cooke Aquaculture. The biological detritus samples were identified as lipid-like, feces-like, scales, sea lice or unidentified biological material (Fig. S1). We also included a small number of mortalities provided from Mowi and Creative Salmon. Samples were also collected from the submerged marine effluent pipes from two farm salmon processing plants and also from olive rockfish visibly feeding on this effluent.

In total, 230 samples were initially screened for PRV-1 and positive samples were selected for full length amplification of the S1 and M2 segments for sequencing. In an effort to ensure that the materials detected were indeed from a salmonid host, and to evaluate the quality of the nucleic acids and to detect possible sample degradation, we deployed an RT-qPCR assay for the ELF-1α (Sepúlveda et al. 2013) (Table S1). Of the 17 samples where there was no positive detection for the ELF-1α control, 6 of these were positive for PRV. We assumed that these cases were a result of host RNA degrading faster than the encapsulated and therefore more stable viral RNA. On the other hand, there were numerous cases where the ELF-1α control was positive, but PRV was not detected (Table S1 and Fig. 2A). This assay detects the ELF-1α gene of salmonid species including that of Chinook and Atlantic salmon, the two salmonid farmed host species in our study. We analyzed the relationship between detections of the host ELF-1 gene and PRV-1 by comparing positive detections (Ct-values $\leq$ 40; Fig. 2A) with a mixed-effects model that accounted for potential dependence among samples collected on the same day from the same farm site. The relationship between positive detections was strongly significant ($p < 0.001$). We detected a single outlier in the relationship depicted in Fig. 2A. Although the outlier is far from the bulk of the data in the vertical direction, but not in the horizontal direction, we did not anticipate that it would have substantial influence. By re-analyzing the data with this outlier removed, we confirmed that this was indeed the case (p-value reduced from 0.00063 to 0.000053). Negative detections were plotted, but not included in the regression.

Overall prevalence of PRV-1, as estimated by the proportion of samples with a positive PCR result, was 70% (Fig. S3), overall confidence intervals and Ct thresholds are shown in Table S2. Of the positive detections 49 had a Ct value of >35. Therefore, a more conservative estimate of prevalence discounting the lower load detections would be closer to 50%. However, 9 of these lower load positive detections (with a Ct > 35) had at least one segment sequenced, suggesting that discounting samples with a high Ct is not representative of the true prevalence. We recognise that samples with a high Ct value are more likely to be false positives, and this becomes more likely at higher Ct values. However, by including the higher Ct value positives in our comparisons we increase the sample size and thereby the power of the associated tests of significance. Since any overestimates of prevalence would be comparable between sites, we have applied a cutoff Ct threshold of 40, while recognising the possibility that a small number of these could potentially be false positives.

We analyzed both the prevalence (proportion of positive detections) and the intensity (as gauged by the Ct value of the positive detections) for potential differences between companies and tissue types (also using mixed-effects modeling; Figs. 2B and 2C). For both prevalence and intensity, we found no significant evidence of differences between aquaculture companies ($p = 0.64$ for intensity and $p = 0.08$ for prevalence). Moreover, this lack of significance persisted for the intensity component even after accounting for potential differences between sample types ($p = 0.16$). (For prevalence, the program failed to converge on a reliable solution with sample type in the model.)

By contrast, for differences between sample types, we found significant evidence of intensity differences ($p = 0.00006$), with this significance persisting after accounting for potential differences between aquaculture companies ($p = 0.01$). (Convergence problems again blocked the corresponding assessments for the prevalence.)

In addition, we tested each pair of primary types for significant differences in intensity. The only significant comparisons ($p < 0.05$ after Bonferroni adjustment for the multiple comparisons) were between effluent and each of lipid-like, feces-like, and scales (Fig. 2 and Table S3).

Thus, any perceived differences in prevalence between companies (Fig. S3) are attributable to a combination of (*i*) chance sampling fluctuations and potentially and (*ii*) confounding with differences between tissue types. The same applies to the relatively minor differences in intensity between companies (Fig. 2B). As is apparent in this figure, they are not statistically significant. Yet by contrast, there is evidence of substantive differences in intensity between sample types. The pairwise comparisons of intensity levels between all pairs of the six primary sample types are summarized in Fig. 2C. These show that the elevated mean intensity in the effluent samples was significantly higher than the mean for many of the other tissue types.

Phylogenetic analysis of PRV-1 S1 and M2 sequences

PRV S1 segment sequences ($n = 68$) were obtained from 4 of the 5 companies in this study. No sequence was obtained

from Cooke Aquaculture, where only 3 (lower load) positive samples were collected (Fig. 2B).

A maximum likelihood phylogenetic approach was used to compare S1 sequences from this study with those that are publicly available from Genbank. Some of these originate from aquaculture companies not sampled in this study, labeled companies X, Y, and Z. To increase resolution of the overall phylogeny, and to be able to differentiate PRV-1a lineages in the Northeast Pacific as well as possible, we aligned all the S1 sequences from the current study with all other available S1 sequences and full concatenated genome sequences (Figs. 3 and S4). Sequences collected from wild Pacific salmon in BC are known to be interspersed on the phylogeny with sequences from farms in BC (Mordecai et al. 2021), and this observation is maintained with the environmental sequences from this study.

As expected, all of the PRV-1 S1 segment sequences from this study fall within a monophyletic clade which contains the majority of PRV-1 sequences collected in the Northeast Pacific. Sequences from the Northeast Pacific that fall outside this monophyletic clade are from a previous study and were collected from escaped farmed Atlantic salmon after a Cooke Aquaculture net pen farm collapsed in Washington State (Kibenge et al. 2019). These sequences represent a secondary, more recent introduction linked to Iceland (Fig. 3) (Kibenge et al. 2019; Mordecai et al. 2021). This distinct lineage of PRV-1a has not since been detected in the study region, suggesting there was limited or no onward transmission to farms and wild fish in BC.

All of the PRV-1 S1 sequences from the Northeast Pacific (i.e., excluding the Washington escapees) can be separated into two distinct clades (Mordecai et al. 2021). Clade 1 is primarily made up of Chinook salmon from the Columbia River and farmed Chinook salmon (Fig. 3). Clade 2 contains the majority of farmed Atlantic salmon, as well as wild Pacific salmon originating from BC rivers that share marine habitat with farmed Atlantic salmon (Fig. 3).

We also constructed a tree solely based on the S1 segment (Figs. S5 and S8). This tree contains multiple polytomies (where more than one branch descends from a single node) and many identical sequences—some of which were collected over several years (Fig. S6). A striking example of this are samples KR478643 (Siah et al. 2015) and MH581202 (Browns Bay processing plant), collected 17 years apart but with 100% nucleotide identity.

Additionally, for a selection of samples, the M2 segment was also sequenced ($n = 39$), although a subset of these were only partial sequences ($n = 21$), and a similar phylogenetic analysis was performed (Figs. S7 and S8). Many of the partial sequences represented a clade distinct from any previously detected in BC (branch support value: 87). This is perhaps not surprising, as the number of M2 sequences available from the region is relatively low. We expect this clade to become more populated as more M2 sequences become available. Interestingly, this clade falls outside of a Norwegian sample from 1988, which groups with the rest of the BC sequences.

Based on the clades shown in Fig. 3, each sample originating from a farm was plotted on a map according to sampling location (Fig. 4). Detections of viruses within clade 1 samples (i.e., the same lineage of PRV hypothesized to originate from Chinook salmon broodstock in hatcheries within the Columbia River) were largely detected in farmed Chinook on the west coast and Sechelt inlet (Mordecai et al. 2021). The same lineage of PRV was often shared between sites from the same company, while some companies shared a mixture of lineages, with clade 2.1 being the most common, making up almost half of all aquaculture samples.

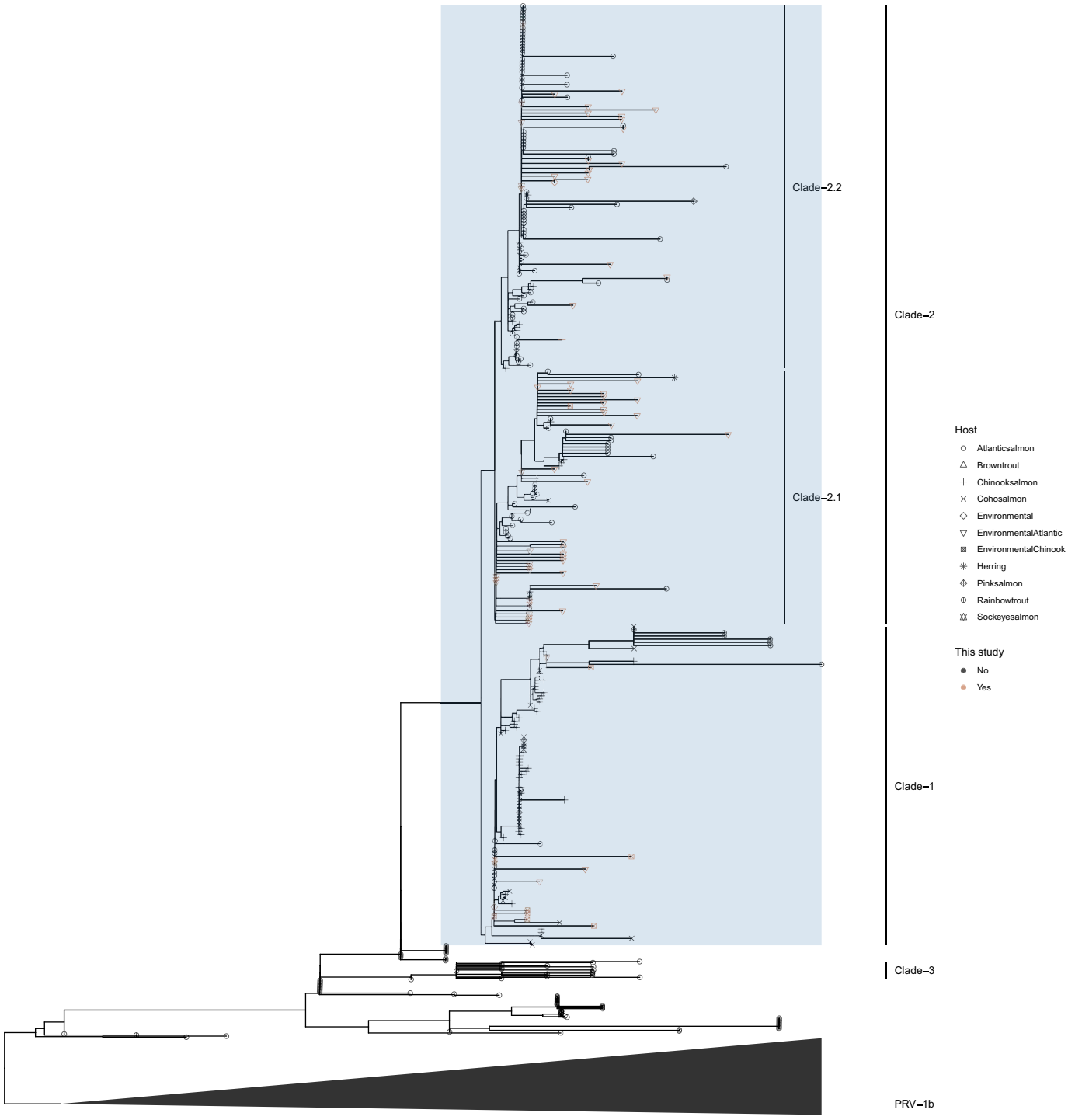
Discussion

Not unexpectedly, in this study, PRV-1 was detected in all types of biological samples collected adjacent to the net pens of all the various companies we tested, as well as in the effluent from salmon processing plants.

The source of PRV in marine farmed fish remains debated, but detections of PRV-1 (as well as other infectious agents) in Atlantic salmon in freshwater hatcheries suggest that as these infected fish are transferred into marine farms, they can become a source of the virus to the marine environment (Bateman et al. 2021). Similarity and grouping of sequences within individual companies suggests hatcheries may be the source of infection. Whether sterilization of eggs by the industry has addressed issues of PRV infection in freshwater hatcheries is an open research question which has not been addressed in the peer reviewed literature. Of note, the 2017 positive result was from a scale that was collected during the transfer of young Atlantic salmon directly from a hatchery into a farm via a wellboat. The most recent sampling suggests that infection remains high in marine farms despite lower rates of infection reported in hatcheries. This suggests infection in net pens may be maintained by nearby infected pens, or by wild reservoirs. Differences between farms may be attributed to adaptation to a specific host. For example, all current sequences suggest that the PRV lineage in farmed Chinook salmon is distinct from the lineage detected in farmed Atlantic salmon. Further research is needed to determine whether different lineages of PRV-1 are either better adapted to a specific host, or, alternatively, are more generalist and able to move freely between different species.

Although surveillance of market farm fish yielded important insights into the emergence and origin of PRV in BC (Kibenge et al. 2013), it cannot link a virus to a specific site or hatchery, especially since aquaculture companies do occasionally stock their pens with smolts purchased from different companies. For instance, in 2019 Atlantic salmon smolts were transferred from two Cermaq hatcheries into Grieg's marine farms (transfer licences #120338 and #120231; Access to Information Requests 2020). Therefore the full transmission history of pathogens cannot be monitored using market fish. In the absence of access to salmon in the farms, collecting the biological waste flushed out of the farms by marine currents can provide information on the point-source release of pathogens. Despite the fact that the relatively small sample size of our study was not statistically robust, we note that, while a negative result is not sufficient evidence to conclude that an entire farm population is totally free of an agent, a positive test result is evidence that the agent is present.

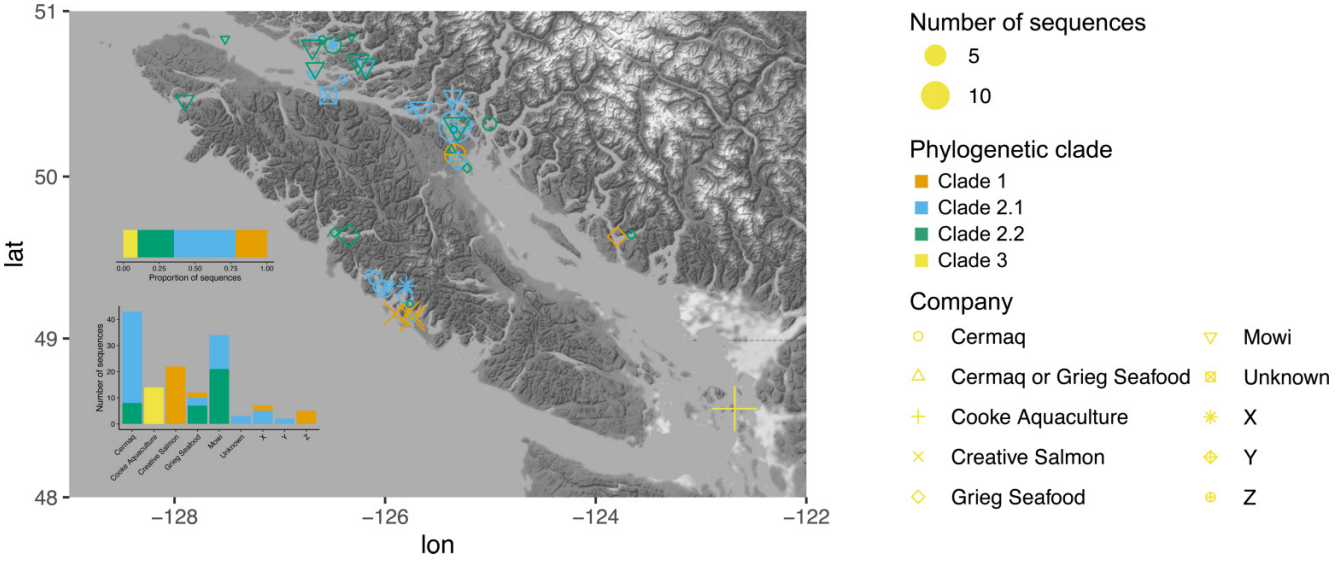
Fig. 3. Maximum likelihood phylogeny based on an alignment of the S1 genome segment ($n = 339$) and concatenated full genome sequences ($n = 122$). Branch tip points are shaped by the species of the host from which they were isolated, and samples originating from this study are colored orange. PRV-1b sequences are collapsed (black triangle). The monophyletic lineage which is typically seen in North America is shaded in blue. A tree with branch labels for all sequences is included in the supplementary figures (Fig. S4).



Moreover, repeated, intensive sampling at the same farm can provide evidence on the prevalence of PRV-1 over time. More focused sampling designs may go some way to address potential confounding effects on the prevalence of PRV, such as the length of time the pens have been stocked.

Our study highlights some of the limitations of collecting biological materials adjacent to farms. As we used a nucleic acid based detection method, we cannot conclude that we are detecting infectious viruses. However, we suggest that despite the limitation, detection of viral RNA is a valid indicator

Fig. 4. Map of *Piscine orthoreovirus*-1a (PRV-1a) clades (described in Fig. 3) detected from farms. Points are sized according to the number of samples at each unique site, and colored according to the lineage of PRV, that is, clades 1 (orange), 2.1 (blue), 2.2 (green), and 3 (yellow). Included are sequences from a previous study in which 3 other farm companies were sampled; these are labeled companies X, Y, and Z). Inset plots shows the proportion of sequences attributed to each clade (top) and the number of sequences attributed to each clade by company (bottom). The basemap is from Stamen Maps (Stamen Maps n.d.), and the map was made using the ggmap package within R (Kahle and Wickham 2013).



of infection on the farm itself. In an ideal study, we would have been able to validate our detections by conducting the same sampling methods within the farm, and combine these with monitoring of the disease status of the salmon being raised in the farms via histopathological investigation (Di Cicco et al. 2017). There is uncertainty over whether the materials pooled per vial originated from one or more hosts. We cannot be certain if the samples that had low-level detections (Ct > 35) and were not successfully sequenced were the result of nonspecific amplification and are false positives.

We caution that any inferences on intensity differences among types of biological material must be regarded as tentative. Formal inferences can be performed through mixed-effects modeling to account for the potential for dependence among samples caught on the same sampling trip to an individual farm. Nevertheless, the study was not designed to provide confirmatory evidence of, e.g., potential differences in infection levels between sample types. In particular, given the limited sample sizes and opportunistic timing and location of the sampling events, we were unable to deal effectively with potential confounding factors. For example, a higher viral prevalence in lipid-like samples might be expected, as this sample type was found only outside farms stocked with older fish. Because it was sampled later in the farm production cycle, the PRV prevalence in these farms would likely have been considerably higher (Mordecai et al. 2021). Our tests address solely the potential for chance sampling fluctuations to account for any such differences.

We found that PRV intensity was significantly higher in the samples of the effluent from salmon processing plants. While these were the oldest fish sampled, the lower Cts in these samples was likely because coagulated blood was collected and erythrocytes are the main target of PRV infection (Finstad et al. 2014).

As PRV-1 is known to infect the intestine and be shed via the feces (Hauge et al. 2016), positive detections in feces support the hypothesis that feces may serve as a route of transmission between salmon farms and the surrounding environment. Similarly, the detection of PRV-1 in a sea-lice/scale sample raises the question of whether sea-lice play a role in the transmission of PRV-1 between farmed and wild fish. Alternatively, this detection could be the result of "environmental contamination" of PRV-1 in the water column which could be responsible for some of the low level positive detections from this study. As previous research has shown that PRV-1 can be detected in water collected adjacent to farms (Shea et al. 2020), contamination from "free-floating" PRV remains a possibility in any of the materials we collected.

We propose that our method of sampling for biological materials in the marine environment adjacent to salmon farms provides an additional tool to the environmental-DNA (eDNA) studies which rely on sampling of the water itself (Shea et al. 2020). While eDNA sampling detects pathogens suspended in the water, biological waste sampling detects pathogens that may adhere to, or are within the cells of, biological detritus. For example, Shea et al. (2020) detected PRV-1 in waters around salmon farms, but detections were surprisingly infrequent considering how common PRV-1 is on farms (Mordecai et al. 2021); this low detection level was attributed to either biological or technical artifacts.

The present study was designed to determine the presence and diversity of PRV-1 released from marine farmed fish in BC, Canada, and to investigate any relationship between different farm sites operated by aquaculture

companies in the area. Our findings add to the number of publicly available sequences, and the phylogenetic placement of the sequences from this study are concordant with previous studies (Di Cicco et al. 2018; Siah et al. 2020; Mordecai et al. 2021). Earlier studies based on limited sequences obtained from farmed salmon purchased from markets, suggested that the virus originates from Norway (Kibenge et al. 2013). The virus's European origin has since been confirmed with extensive sequencing and phylogeographic analysis by separate groups (Siah et al. 2020; Mordecai et al. 2021). The majority of farmed salmon samples acquired in Western Canada markets were PRV-positive (Kibenge et al. 2013) and this has also been corroborated by more recent studies (Bateman et al. 2021; Mordecai et al. 2021). Laurin et al. (Laurin et al. 2019), which received access to government audit samples of farmed salmon (identified by region, but not site) reported that ~70% of salmon in farms throughout BC were PRV-positive. More recently, a longitudinal study of 4 Atlantic salmon cohorts, as well as an analysis of aquaculture audit samples, found that PRV infection becomes almost ubiquitous by the end of the production cycle (Bateman et al. 2021; Mordecai et al. 2021).

It is possible that a substantial portion of the negative detections came from farms early in their production cycle, when prevalence of PRV is at its lowest (Bateman et al. 2021; Mordecai et al. 2021). Additionally, there is an observed seasonality to PRV infection on farms, with increased detections over the winter (Di Cicco et al. 2017). Detections of PRV in freshwater hatcheries suggest that the source hatchery may exert significant influence on the presence/absence of PRV in farmed salmon upon ocean entry, but it seems that eventually, almost all fish on farms become infected regardless (Bateman et al. 2021; Mordecai et al. 2021), likely via infection from neighbouring sites stocked with different cohorts, or alternatively, from wild fish migrating past multiple farms and thus acting as carriers between farms. In most cases, the aquaculture industry maintains geographic distance between companies, with the exception of one area in the Discovery Islands where Mowi, Cermaq, and Grieg Seafoods operated 4 farms within 7 km of the narrow Okisollo Channel, increasing the potential for transfer of pathogens between companies (Fig. 1).

Sequences collected from wild salmon in BC are phylogenetically interspersed with sequences from farms in BC (including those collected in this study) (Fig. 3) and this is indicative of transmission between these populations. We suggest that the direction of transmission is mainly from farmed fish to wild. This is supported by two independent studies, both of which reported that PRV-1 prevalence in wild salmon increases with exposure to salmon farms (Morton et al. 2017; Mordecai et al. 2021). Host density, biomass and infection rates are typically much greater inside net pens than outside the pens, therefore it is likely that net pens located on the migration routes of wild salmon act as powerful reservoirs of infection for in-migrating adult wild salmon and out-migrating juvenile wild salmon. Furthermore, as the biomass of infected fish is highest within net pens, compared to the relatively low infection rates in the more dispersed wild fish which are subject to predation pressure that would target weaker individuals (Furey et al. 2021), the direction of the majority of transmission between these populations is more likely to be from farmed to wild fish.

In our study, several PRV-1a sequence variants were detected with 100% sequence identity to isolates that pre-date this study by many years. An interesting observation regarding the PRV sequences in the Northeast Pacific is the relatively low amount of sequence diversity, especially when analysis is restricted to solely one segment (Figs. S5 and S7), with sequences often identical between multiple years. This apparent lack of diversity is consistent with a relatively recent introduction of the virus to the region, and thus a subsequently lower amount of time for diversity to arise. As expected, the diversity of PRV in the Northeast Pacific appears to be gradually increasing, with new variants accruing proportional to time (Siah et al. 2020; Mordecai et al. 2021). A similar phenomenon has been described for ebola virus: there was no temporal signal when only one gene was analyzed, whereas a clock-like accumulation of mutations was observed when full genomes were analyzed (Zhukova et al. 2020). Whole genome sequencing is clearly the most reliable way to assess the diversity and inferred transmission of viruses.

Recent evidence of transmission of infectious agents between farmed and wild salmon (Mordecai et al. 2021; Bateman et al. 2022), and the growing body of evidence linking individual agents to disease (Di Cicco et al. 2018; Santos et al. 2019; Valdes et al. 2021), confirm that farm-origin pathogens must be considered when assessing the cause of poor early marine survival of wild BC salmon and associated population declines (Noakes et al. 2000; Peterman and Dorner 2012; Riddell et al. 2013; Bendriem et al. 2019) that have failed to rebound despite significant reduction in fishing pressure (Walters et al. 2019). Passive release of infectious agents directly into wild salmon habitat is an important transmission pathway that can be measured through filtration of water samples. However, the release of infected organic material could be an alternative transmission pathway with a potential role in dispersal, since infected tissues adrift in marine currents attract motile scavengers to the farms. For example, Pacific herring (*Clupea pallasii*) aggregations in the order of thousands of fish were observed feeding along the outside of the nets at locations selected for sampling (Fig. S1). Wild salmon smolts (*Oncorhynchus* spp.) and gulls (*Larus* spp.) were observed targeting and consuming the same type of biological material as we collected for this study. Additionally, the caprellid amphipod *Caprella kennerlyi* heavily populated the nets, and were observed feeding on the free-floating biological matter (Fig. S1). While the impact of PRV on non-salmonid species is unknown, observations of native species consuming farmed Atlantic salmon detritus from farms where PRV has been detected in detritus provide evidence of a pathway that aquaculture-source pathogens are entering the marine food chain with undetermined consequences. Pacific herring are an important mid-trophic level prey species in the Northeast Pacific food web (Surma et al. 2018), and are regularly consumed by Chinook salmon (Duffy et al. 2010). The abundant caprellids observed on the farms are consumed by a variety of coastal marine fish (Woods 2009), including shiner perch, (*Cymatogaster aggregate*) (Caine 1991), which were

observed in large schools in and around the farms. At times these amphipods were observed drifting alive en masse away from the farms during sample collection.

The observations of gulls swimming among farm-source detritus and eating it indicates the potential for long-distance dispersal of aquaculture pathogens on their feathers and through their digestive tract, although we note that whether the virus remains infective via this potential route of transmission needs to be assessed. Nevertheless, the uptake of PRV-infected material into the Northeast Pacific food chain warrants serious consideration, particularly in light of evidence that this virus is exotic to this region (Kibenge et al. 2013; Siah et al. 2020; Mordecai et al. 2021).

We suggest that the influence of pathogens released from salmon farms on the health of wild salmon should be more fully examined as a matter of urgent Pacific salmon conservation concern. This work reaffirms that a precautionary approach to managing PRV in BC is highly warranted, especially in light of the evidence that PRV-1 is a disease agent (Di Cicco et al. 2017, 2018; Wessel et al. 2020) and is transmitted between farmed and wild salmon populations (Mordecai et al. 2021).

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Data availability

Data generated during this study are provided within the published article and its supplementary materials. Please note that sequence data are available at GenBank; accession numbers are available in Table S1.

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Competing interests

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Supplementary material

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