Annual Report 2015

Annual report on health monitoring of wild anadromous salmonids in Norway

Abdullah S. Madhun, Åse Helen Garseth**,*
Ann Cathrine B. Einen, Ingrid U. Fiksdal*, Hilde Sindre**, Sten Karlsson***,*
*Eirik Biering**, Bjørn Barlaup****, Egil Karlsbakk**

** Institute of Marine Research, ** Norwegian Veterinary Institute,*
**** Norwegian Institute for Nature Research, ****Uni Research*

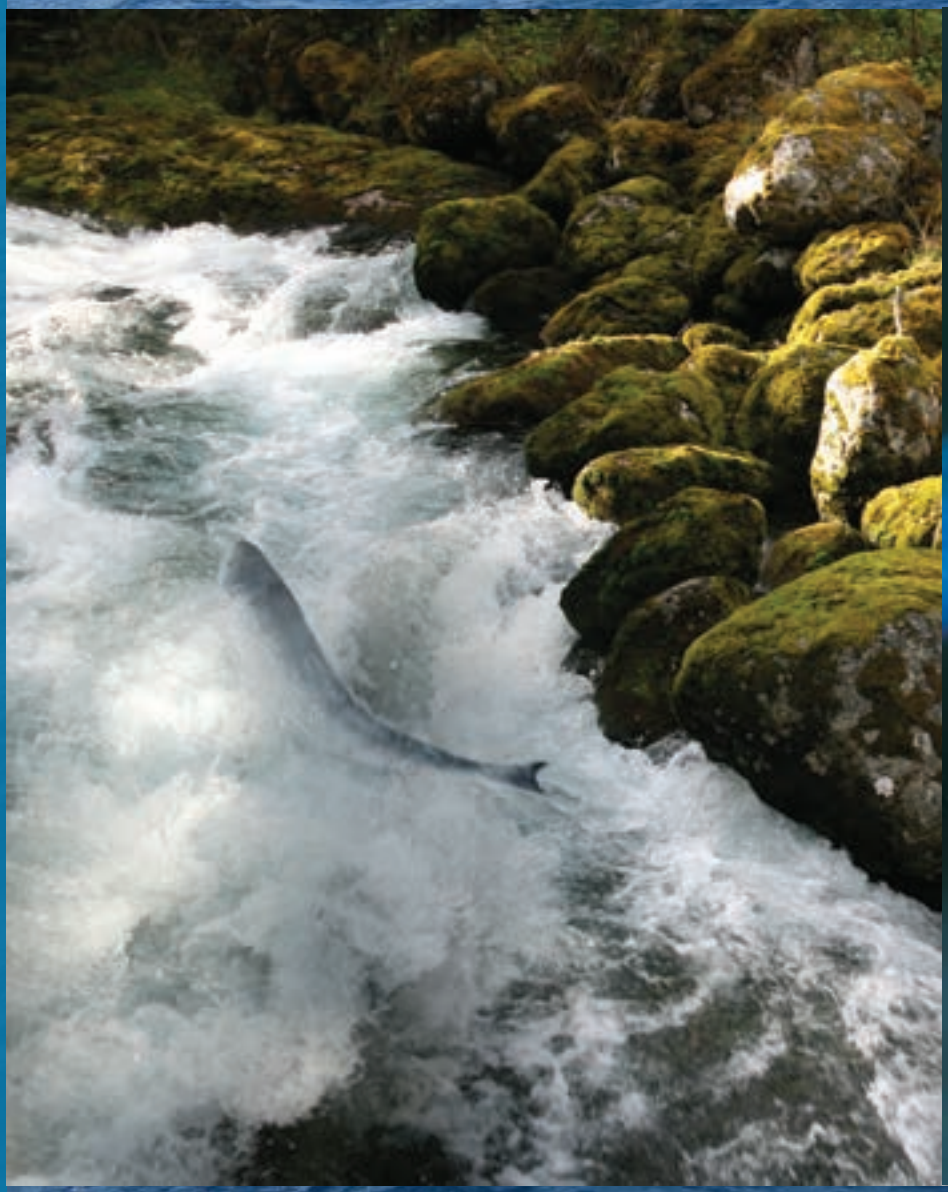

Foto og illustrasjon: John Ringstad

Institute of Marine Research No. 22-2016
Norwegian Veterinary Institute No. 7-2016

Date: 02.05.2016

www.imr.no
www.vetinst.no

HAVFORSKNINGSINSTITUTTET
INSTITUTE OF MARINE RESEARCH

Veterinærinstituttet
Norwegian Veterinary Institute

The Norwegian Veterinary Institute (NVI) and the Institute of Marine Research (IMR) were in 2015 commissioned by the Norwegian Food Safety Authority to carry out a health monitoring of salmonids in Norway.

Table of Contents

Health monitoring of salmon from western Norway

Abdullah S. Madhun*, Ann Cathrine Einen*, Ingrid Fiksdal*, Bjørn Barlaup** and Egil Karlsbakk*

**Institute of Marine Research*
***Uni Research Environment*

1. Introduction

Viral diseases represent a serious problem in fish farming in Norway and lead to huge economical losses. Disease outbreaks in fish farms may lead to a substantial increased infection pressure on neighbouring farms and on wild fish. This may cause increased infection levels (prevalence) and potentially also disease in susceptible wild stocks.

It is difficult to quantify disease incidence in wild fish because sick individuals in nature may be less catchable or may disappear unnoticed (e.g. due to predation). Therefore, we may only be able to collect infected but non-diseased fish such as individuals that has recently acquired or has survived an infection (carriers). Today, there is limited data on the prevalence of viral pathogens in wild salmonid populations in Norway. There is evidence for pathogen transmission from farmed to wild fish [1–4]. However, the frequency and the consequence of infections by many viral agents in wild fish are largely unknown.

Pathogens that cause disease in farmed salmon can also infect wild salmon. The effect of fish farming on the infection status of wild salmon stocks may be evaluated by comparing pathogen prevalence in wild fish populations captured from coastal areas that have different fish farming intensities and disease outbreak profiles.

ISA was a major problem in Norwegian aquaculture in the late 1980s. In the last 5 years, the annual number of cases has varied between 1 and 15. ISA virus may be classified based in the sequence of HE gene as HPR0 (avirulent) and HPRΔ (virulent) variants. HPR0 is highly prevalent in farmed Atlantic salmon [5, 6]. However, the virus causes non-clinical transient infections (2–4 months). There are increasing evidence that virulent HPRΔ variants originate from different HPR0 types. There is very limited data about the prevalence of ISAV HPR0 in wild salmon in Norway.

2. Aim

The aim of the current study was to investigate the prevalence of ISAV infections in returning Atlantic salmon originating from the Vosso and Dale rivers located in Hordaland, western Norway.

3. Materials and methods

Institute of Marine Research and Uni Research Environment have, for many years, smolt release projects in the rivers Dale and Vosso where thousands of cultivated smolts are released every year (for further information see [7, 8]). The smolts were tagged and towed in small pens to release sites at different locations between the rivers and the coast (Figure 1). These smolt release experiments have been used evaluate the effect of sea lice infection from fish farming on the wild salmon populations based on recapture rates [7, 8]. The tagging of the fish provides accurate information on the origin, year (i.e. age of returning salmon) and the site of release.

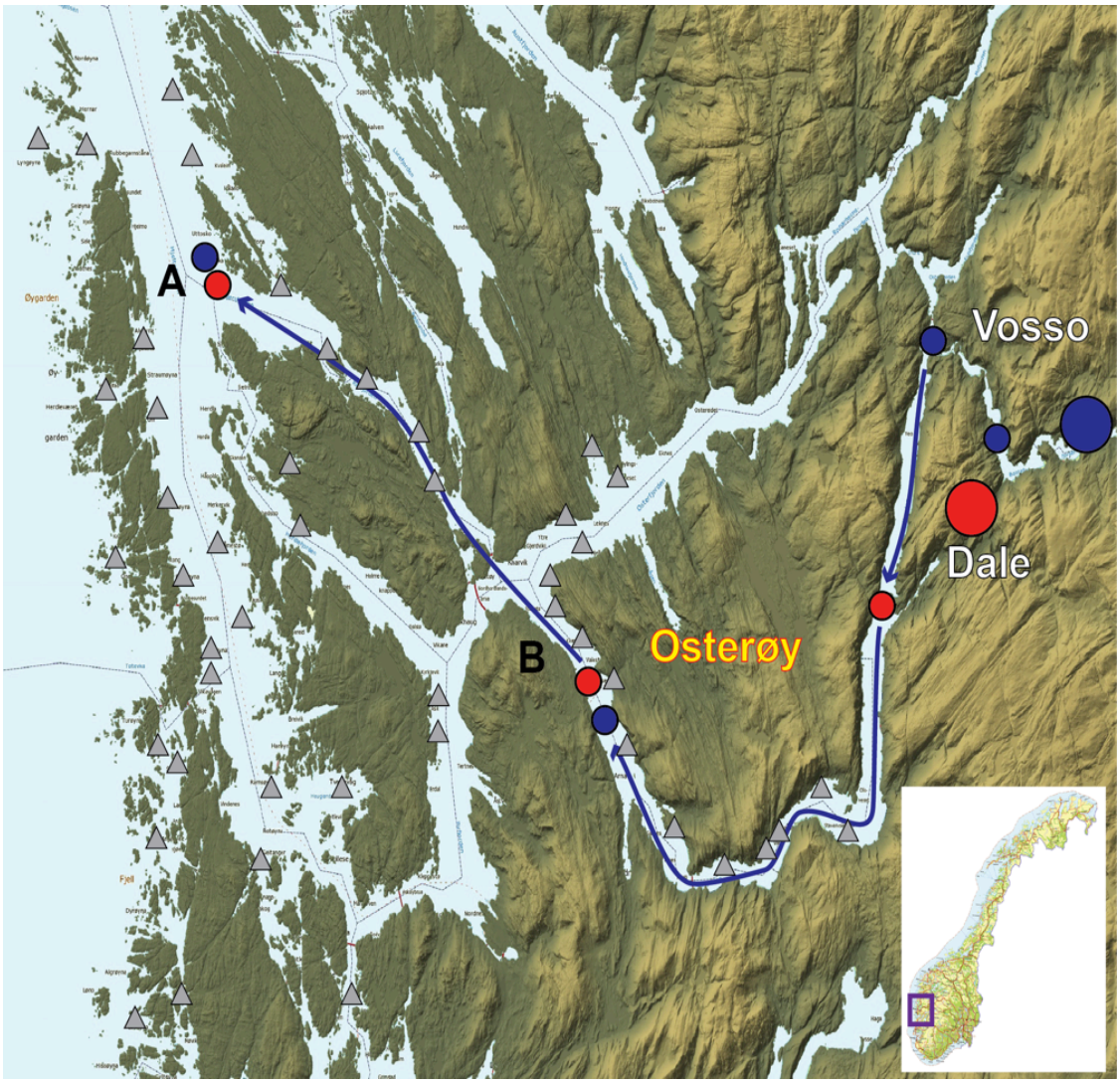

Figure 1: A map showing the Osterfjord system. Circles are showing the release sites for smolt from Vosso (blue) and Dale (red). Grey triangles are showing approved fish farming sites. The returning salmon were captured in inner part of the fjord (sea), the river mouth (estuary) or in the rivers (fresh water).

A total of 573 returning salmon were captured by angling in the rivers or by bag net in the fjord. The salmon were caught at three areas (sea, estuary and rivers) in the Osterfjord system in 2012–2014 (Table 1). The fish weight, length and sex were determined. Tags were read to determine release site, release date and the river of origin. The head of captured fish was cut off behind the pectoral fin and deep frozen (-20 ^{o}C) as soon as possible after capture.

At autopsy, gill tissue samples were taken from the fish while still frozen and transferred to tubes on dry ice. The gill samples were sent on dry ice to an accredited commercial laboratory for RNA extraction and virus testing (PatoGen Analyse AS). Analysis for ISA virus was performed by PatoGen using their in-house real-time PCR assays (for detection viral RNA). The ISAV assay used detects both HPR0 and HPRΔ. Samples with C_t (cycle-threshold) value below 37.0 were considered positive.

4. Results

ISAV was not detected in any of the gills from the tested salmon (N=573) originating from the rivers Vosso (N=526) and Dale (N=47).

Table 1. The numbers, the origin and the catch year of the tested salmon.

	Catch Year			
River	**2012**	**2013**	**2014**	**Total**
Dale	0	17	30	47
Vosso	149	186	191	526
Total	149	203	221	573

5. Discussion and Conclusion

The current study is part of a larger project including the aim of establishing baseline data on the prevalence of ISAV (both HPR0 and HPRΔ) in wild salmon in western Norway. We could not detect ISAV in any of the tested salmon. We have previously found that 7% of returning wild salmon and 5% of escaped salmon from northern Norway were infected with HPR0 ISAV (unpublished data). However, less than 1% of escaped salmon caught in western Norway was positive for HPR0-ISAV (manuscript in preparation). Plarre et al. (2005) have shown that high HPR0-ISAV is highly prevalent (6–100%) in salmonids from rivers in western Norway [8]. However, previous screening of limited numbers of brood salmon from Vosso failed to detect the virus [10]. ISA is seldom observed in farmed salmon in western Norway in the recent years [11]. Therefore, the probability that wild salmon have been exposed to HPRΔ-ISA virus from ISA outbreaks in salmon farms has been very low, both when migrating as smolt (2009–2013) and when returning as adult (2012, 2013 or 2014). On the other hand, HPR0-ISAV is highly prevalent in farmed Atlantic salmon [6]. However, the virus causes a non-clinical transient infection, rendering it unlikely that infections acquired as smolt could be detected in returning adult salmon. On the other hand, fish could be infected in the feeding areas from contact with other wild salmon or from escaped farmed salmon. When returning to the coast, salmon could also be infected by HPR0-ISAV released from fish farms undergoing infection.

The current results have shown that ISA virus infections were absent in samples of returning salmon originated from the rivers Vosso and Dale during the period 2012–2014, and therefore may suggest that the risk of acquiring HPR0-ISAV infection from fish farming is low. However, time series of samples from different rivers are necessary to better understand ISAV transmission and virus exchange between wild and farmed salmon.

6. References

1. Johnsen, B.O., Møkkelgjerd, P.I. & Jensen, A.J (1993). Furunkulose i norske vassdrag. Statusrapport, in NINA Forskningsrapport. NINA. p. 73.
2. Garseth, Å.H., Ekrem, T. & Biering, E. (2013) Phylogenetic Evidence of Long Distance Dispersal and Transmission of Piscine Reovirus (PRV) between Farmed and Wild Atlantic Salmon. *PLoS One,* **8.**
3. Raynard, R.S., Murray, A.G. & Gregory, A. (2001) Infectious salmon anaemia virus in wild fish from Scotland. *Dis Aquat Organ,* **46:** 93-100.
4. Raynard, R., et al. (2007) DIPNET - Review of disease interactions and pathogen exchange between farmed and wild finfish and shellfish in Europe. Oslo, Norway. p. 459.
5. Christiansen, D.H., et al. (2011) A low-pathogenic variant of infectious salmon anemia virus (ISAV-HPR0) is highly prevalent and causes a non-clinical transient infection in farmed Atlantic salmon (Salmo salar L.) in the Faroe Islands. *J Gen Virol*, **92:** 909-918.
6. Lyngstad, T.M., et al. (2012) Low virulent infectious salmon anaemia virus (ISAV-HPR0) is prevalent and geographically structured in Norwegian salmon farming. *Dis Aquat Organ*, **101:** 197-206.
7. Skilbrei, O.T., et al. (2013) Impact of early salmon louse, Lepeophtheirus salmonis, infestation and differences in survival and marine growth of sea-ranched Atlantic salmon, Salmo salar L., smolts 1997-2009. J Fish Dis,. **36**: 249-60.
8. Vollset, K.W., et al. (2014) Salmon lice increase the age of returning Atlantic salmon. Biol Lett, 2014. **10**: e20130896.
9. Plarre, H., et al. (2005) Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in western Norway. *Dis Aquat Organ,* **66:** 71-79.
10. Barlaup, B. (2013) Redningsaksjonen for Vossolaksen. Direktoratet for naturforvaltning. Trondheim. p. 224.
11. Moldal, T. & Bornø, G. (2016) Infectious salmon anemia (ISA). In: Hjeltnes H, Walde C, Bang Jensen B, Haukaas A (eds) (2016) The health situation in Norwegian aquaculture 2015. Veterinærinstituttet Rapportserie 2016(3):28-29.
    http://www.vetinst.no/Publikasjoner/Rapportserie/Rapportserie-2016/Fiskehelserapporten-2015
    (In Norwegian).

Health monitoring of wild anadromous salmonids in freshwater in Norway

Åse Helen Garseth*, Hilde Sindre*, Sten Karlsson** and Eirik Biering*

**Norwegian Veterinary Institute*
***Norwegian Institute for Nature Research (NINA)*

1. Introduction

The Norwegian Veterinary Institute has organized the Health service for stock enhancement hatcheries for several years and also has a substantial activity in the gene bank program for wild Atlantic salmon and sea-trout. These activities have generated data and samples that can be used in retrospective studies of potential disease interaction between wild and farmed salmonids. It has previously been shown that piscine orthoreovirus (PRV) is widely distributed among wild and farmed salmon in the marine environment, while the information concerning potential freshwater reservoirs is scarce. These topics are addressed in this report.

2. Aim

In 2015, The Norwegian Veterinary Institute organized the health monitoring program with a threefold objective. The two first objectives were to perform a closer follow-up of previously virus positive samples by performing phylogenetic analysis on virus sequences (Study 1) and by investigating the genetic integrity of virus positive hosts (study 2). The third objective was to investigate the existence of salmonid fresh-water reservoirs of piscine orthoreovirus (PRV) (Study 3):

Study 1. Phylogenetic analyses of virus sequences from wild caught and farmed salmonids
Study 2. Evaluating the genetic integrity of virus positive wild salmon
Study 3. Salmonid freshwater reservoirs of Piscine orthoreovirus (PRV)

3. Conclusions

Study 1. Phylogenetic analyses of virus sequences from wild caught and farmed salmonids

The phylogenetic analyses show that virus sequences derived from wild salmon and sea trout group together with virus sequences from farmed salmon, indicating transmission between farmed and wild salmon. For SAV and IPNV, the detected subtype is endemic in farmed salmon in the area where the fish were captured. However, IPNV from sea trout (Fusta, Nordland) differ slightly from IPNV seen in salmon. All PMCV detected in farmed and wild salmon have so far belonged to the same genogroup. The PMCV detected in salmon captured in Årøy (2012) and Numedalslågen (2012) also belong to this genogroup, but it is somewhat surprising that the two fish, sampled in completely different places in Norway, contain PMCV which are closely related.

Study 2. Evaluating the genetic integrity of virus positive wild salmon.

The study was not able to detect a statistically significant association between virus carrier state and the farmed origin index P(wild). However, there is a trend toward more virus carriers among salmon classified as non-wild, encouraging further studies with a larger dataset.

Study 3. Salmonid freshwater reservoirs of Piscine orthoreovirus (PRV)

None of the tested wild relict salmon were PRV-positive, while 4 of 272 brown trout were PRV positive, albeit with low viral levels. It seems likely that a fresh water reservoir of PRV is of minor importance compared to the marine reservoirs.

4. Study 1. Phylogenetic analyses of virus sequences from wild caught and farmed salmonids

Introduction

It is suspected that disease outbreaks in fish farms lead to increased infection pressure on wild salmon stocks. The investigation of how infectious diseases in farmed fish can have an impact on wild populations consists of several steps. However, the most critical step in this investigation is to confirm that pathogens are transmitted between farmed and wild populations. Molecular epidemiology is an important tool in this context and has been used in epidemiological research within the aquaculture industry [1] and in the investigate of wild-farmed disease interaction [2-5]. The objective of Study 1 was thus to trace pathogen transmission between farmed and wild salmonids by the use of molecular epidemiology.

Materials and methods

The Norwegian veterinary institute (NVI) organizes health control of wild Atlantic salmon brood fish used for stock enhancement and for the gene bank of wild Atlantic salmon. Disease surveillance and research projects have provided additional data and samples. A selection of virus positive salmonids from previous activities was included in the study as displayed in Table 1. The samples represent wild sea-trout and salmon of wild, hatchery-reared and escaped farmed origin, all captured in Norwegian rivers. The virus positive organ material from was shipped to the NVI from Patogen Analyse in three separate packages. Two PMCV sequences and one ISAV sequence from previous project were included in the study. Sequences from farmed salmon were either from the NVI biobank or from GenBank.

Table 1. Overview of selected virus positive samples.

Sample no	Virus	Year	County	River	Species	Sample	Ct-value	NVI Ref.
1	SAV	2012	Hordaland	Vosso	Salmon (Hatchery reared?)	RNA	28.7	Vir3257
2	IPNV	2011	Nordland	Fusta	Sea trout	RNA	31.6	Vir3258
3	IPNV	2011	Hordaland	Vosso	Salmon (Wild)	Kidney	31.6	Vir3259
4	IPNV	2011	Møre og Romsdal	Surna	Salmon (Wild)	Kidney	30.4	Vir3260
5	PMCV	2012	Vestfold	Numedalslågen	Salmon (Escaped)	RNA	15	Vir805
6	PMCV	2012	Sogn og Fjordane	Årøy	Salmon (Wild)	RNA	28.7	Vir806
7	PMCV	2008	Sogn og Fjordane	Nausta	Salmon (Wild)	-	29	
8	PMCV	2007	Sogn og Fjordane	Årøy	Salmon (Wild)	-	29	
9	ISAV	2012	Møre og Romsdal	Måna	Salmon (Wild)	RNA	34.6	Vir804
10	ISAV	2008	Nord-Trøndelag	Moelva	Salmon (Wild)	-	-	

Infectious salmon anaemia virus (ISAV)

Attempts made to amplify and sequence the HPR –region of the HE-gene with primers published in [6] yielded no results. The sample was therefore analyzed by common diagnostic real time RT-PCR targeting segment 8. ISAV RNA could not be detected in the sample. A possible reason for this is the degradation of RNA during transport and thawing of the material. Based on the negative real time RT-PCR, no further attempts were made to sequence the content of this sample. One ISAV sequence from a previous project was included in the study. The Phylogenetic tree including sample no 1 from a wild salmon captured in Moelv, Nord-Trøndelag 2008 is shown in Figure 1.

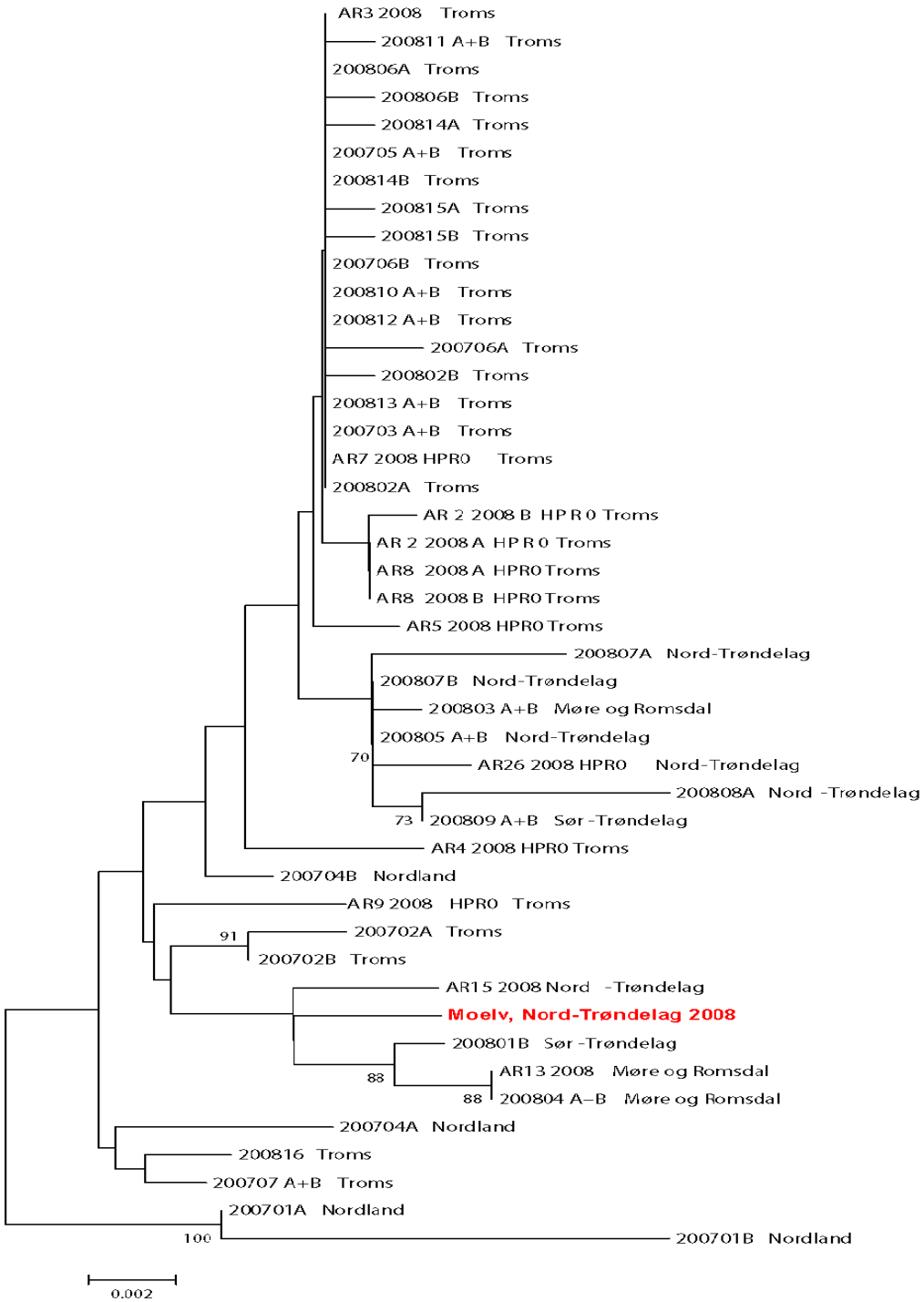

Figure 1. Displays a phylogenetic tree including ISAV segment 6 sequences from farmed salmon and one sequence from a wild salmon captured in Moelv, Nord-Trøndelag in 2008.

Piscine myocarditis virus (PMCV)

The PMCV genome contains three open reading frames (ORFs). ORF2 encodes the polymerase, whereas ORF1 encodes the coat protein. ORF3 is also a putative structural protein, but the function of this is not clear. Previous analyzes of various PMCV variants shows that genetic variation within Norwegian aquaculture is limited and all findings are within the same genogroup. A variant of PMCV has been found in the marine fish species Greater argentine, but this variant differs from PMCV in salmon.

Sequencing

The gene of the coat protein ORF1, and the gene of another presumed structural protein, ORF3 were selected for sequencing, as they are better suited to display differences between isolates than ORF2, encoding the polymerase. ORF 1 and ORF 3 were sequenced for both positive samples with a total of seven primer sets as described in [7]. Primer set 2 for ORF 1 did not work for any of the samples, but the sequence was obtained with primer 1 and 3-5 (ORF1). Sequencing of ORF3 was obtained with both primer sets. ORF 1 and ORF 3 sequences from two wild salmon were recruited from a previous PMCV project [8].

Results

For ORF 1, 1619 base pairs from the 5' end of the reading frame were first selected for the phylogenetic analyses. PMCV ORF1 sequences from farmed salmon were obtained from GenBank by a Blast search. The two positive samples from Nausta 2008 and Årøy 2007 were not includes as they were too short (798 bp). Then 789 bp from ORF 1 was used to estimate a phylogenetic tree including the Nausta 2008 and Årøy 2007 samples. The estimated phylogenetic tree was concordant with the 1619 bp tree and is presented in Figure 2. The analyzes show that in the investigated area of ORF1, the two positive samples from the monitoring program; PMCV detected in farmed salmon from Numedalslågen and from wild salmon from Årøy, Sogn og Fjordane group together. The two positive samples detected in wild salmon from Nausta 2008 and Årøy 2007 in a previous research project [9] do not stand out from sequences derived from farmed salmon.

The full length of ORF3 (909 bases) was analyzed in a matrix containing ORF3 sequences reported to GenBank. The estimated tree show that both the sample from escaped farmed salmon from Numedalslågen and from the wild salmon from Årøy, Sogn og Fjordane clustered together. This is in accordance with analysis of ORF1. (See Figure 2).

PMCV detected in Norway have previously been shown to have little genetic variation and all belong to the same genogroup. The variation within ORF3 is somewhat higher than in ORF1 [7]. Variation in gene sequence can be detected between different samples from the same outbreak and hampers molecular epidemiology. However, two of the "Wild" samples grouped together with "farmed" samples indicating pathogen transmission. Nevertheless it is somewhat surprising that samples originating from very different places in the country show nearly identical sequence. The significance of this finding should be further investigated.

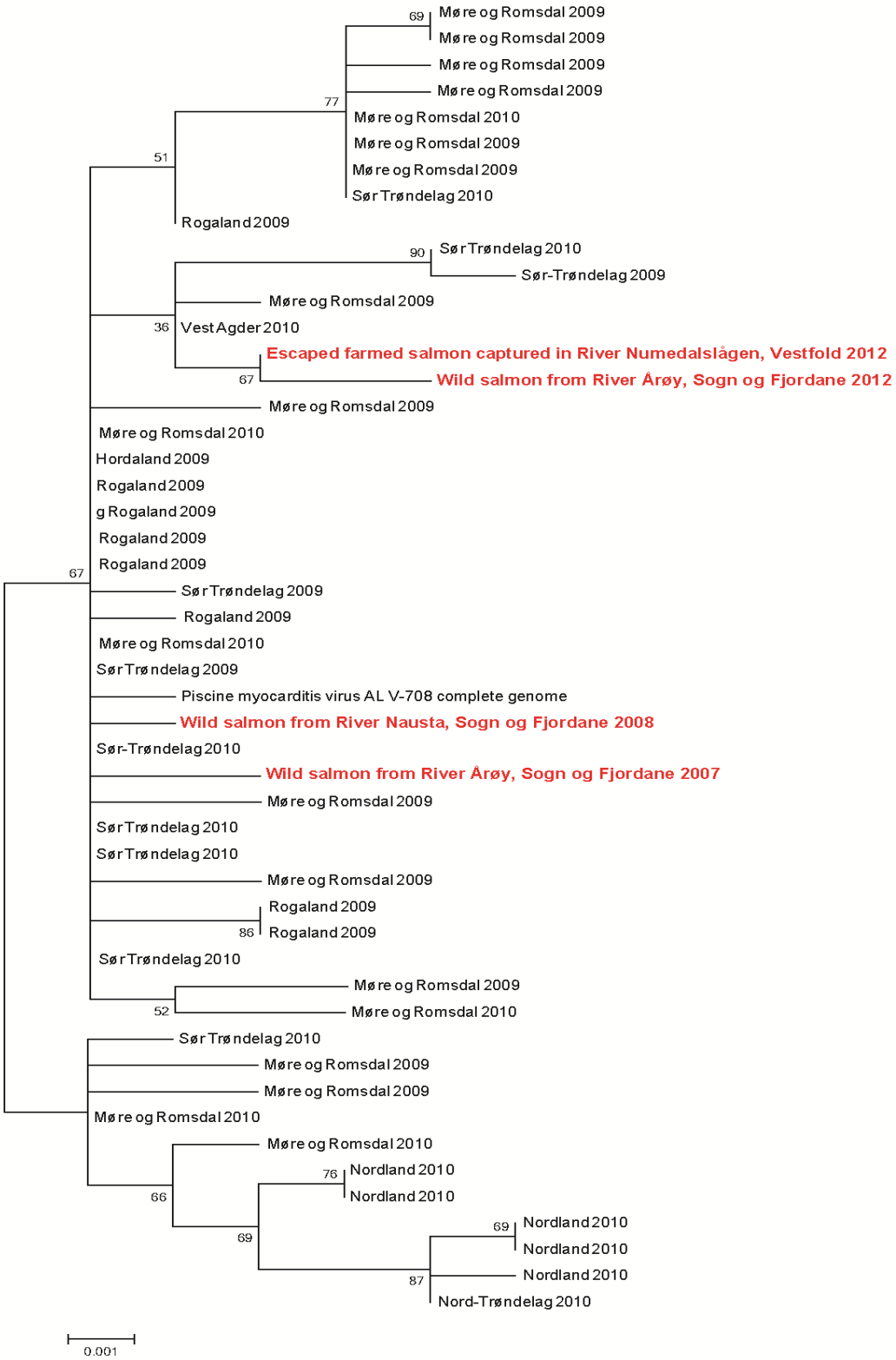

Figure 2. Displays a phylogenetic tree of PMCV ORF1 (798 bp) including PMCV sequences from farmed salmon in Norway, three sequences from wild Atlantic salmon and one sequence from an escaped farmed salmon.

Salmonid alphavirus (SAV)

The SAV genome codes for a total of 8 proteins, four structural and four non-structural proteins. The two genes with most variation and thus most suitable for phylogenetic analyses are E2 and nsP3. A total of six different SAV genotypes are defined by differences in these genes (SAV1 to SAV6). Within SAV2 we find two subgroups, marine SAV2 and freshwater SAV2.

Genotype SAV3 is endemic in counties Rogaland, Hordaland and Sogn o Fjordane in the south-western part of Norway and has so far not been detected in other countries. Moreover, marine SAV2 has been detected in mid-Norway since 2010, with Hustadvika (Romsdal) as the southernmost border.

Sequencing

An area of the SAV E2 gene is routinely used for sequencing and genotyping of SAV at the Norwegian Veterinary Institute, as there are significant differences in gene sequence between the different subtypes in this area. The SAV-positive sample was sequenced according to the method and primers described in [10].

Results

357 bases from SAV E2 gene were used in the phylogenetic analyses / genotyping. The results are presented in Figure 3. The SAV-positive fish is typed as SAV3 and is genetically closely related to the other SAV3 isolates, whereof many are from the same geographic area. This is as expected, based on the fact that marine SAV2 has never been detected in Hordaland, whereas SAV3 is endemic in this county.

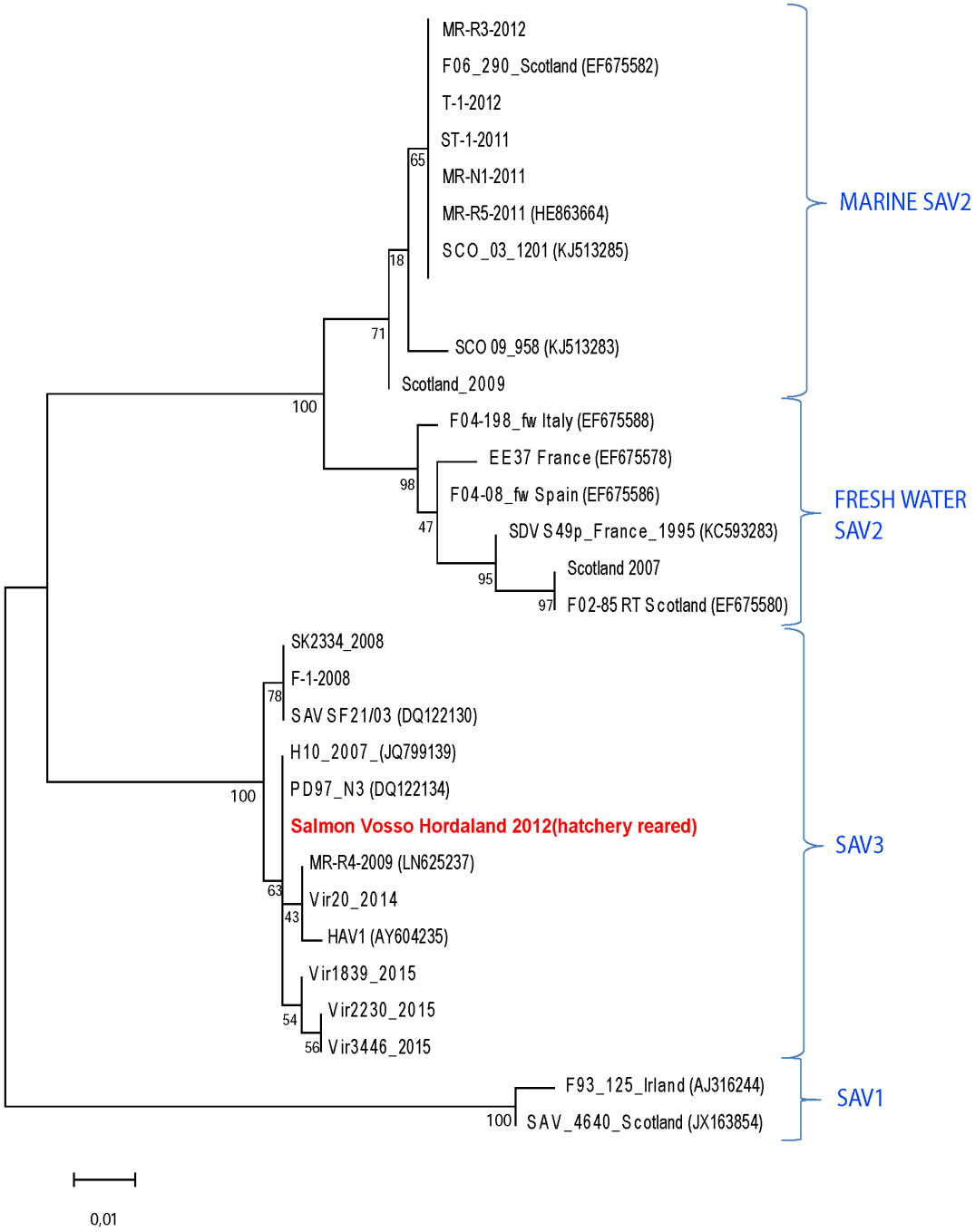

Figure 3. Displays a phylogenetic tree of SAV E2 gene (356 bp) including sequences from farmed salmon in Norway and one sequences from a hatchery reared wild salmon from River Vosso.

Infectious pancreatic necrosis virus (IPNV)

IPNV is an aquatic birnavirus belonging to serogroup A. Within serogroup A, there are 9 different serotypes, where the Sp type dominates in farmed Atlantic salmon in Norway. This serotype has also been demonstrated in other species, including halibut. The IPNV genome consists of two segments, segment A and segment B. Segment B encodes the polymerase (VP1) and is not suitable for phylogenetic analyses.

Sequencing

An area of Segment A was selected for sequencing with 4 in house primers (unpublished). Only two of the samples, IPNV from sea-trout captured in Fusta, Nordland and wild salmon captured in Vosso, Hordaland were sequenced, as IPNV was not detected by real time RT-PCR on isolated RNA from the sample from Surna, Møre og Romsdal. We were able to sequence a total of 1551 bp from the sea trout sample, only 387 pb were sequenced from the wild Vosso salmon.

Results

A total of 387 bp of segment A was used in the phylogenetic analyses and the estimated tree is presented in Figure 4. The analyses show that both samples grouped with the Sp serotype. This is as expected since this serotype is very widespread in Norwegian salmon farming. IPNV from a wild salmon captured in Vosso, Hordaland, is identical or very closely related to IPNV isolates from outbreaks of IPN in farmed salmon. IPNV from the sea-trout captured in river Fusta, Nordland, is somewhat different from salmon isolates in the NVI biobank, although even this belongs to the Sp serotype. While this may be a random variation, it is also possible that the differences in the sequence are linked to shift to a different species than salmon.

To confirm the results of the sea-trout IPNV, a phylogenetic analysis was also carried out based on the entire sequence (1551bp). The tree from this analysis was in accordance with the tree in Figure 4, and is therefore not presented in this report.

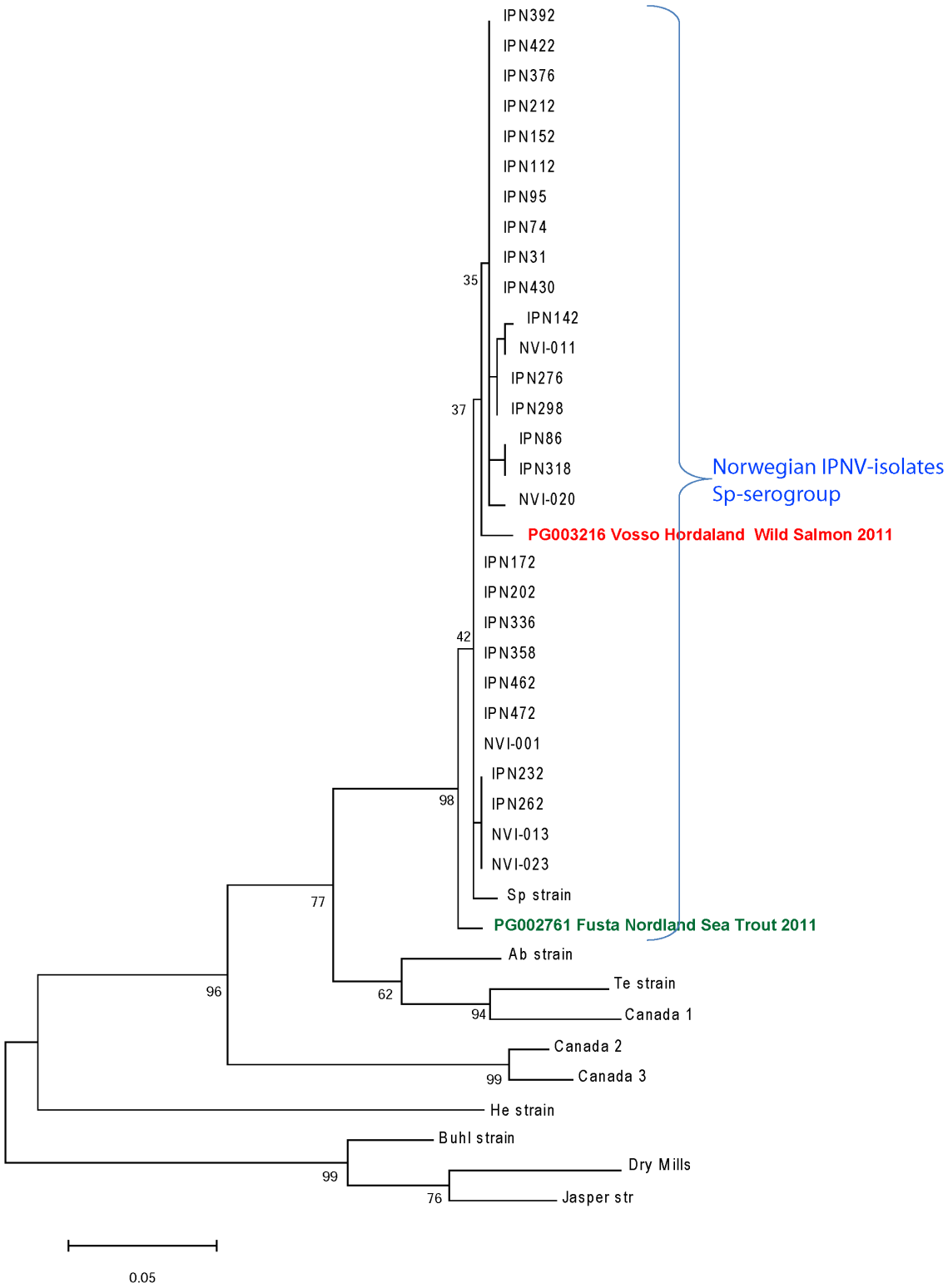

Figure 3. Displays a phylogenetic tree of IPNV segment A (387 bp) including sequences from farmed salmon, one sequences from a wild salmon from River Vosso, Hordaland and one sequence from a sea trout captured in River Fusta, Nordland**.**

Summary and conclusion

The ISAV-positive sample from Måna; Møre og Romsdal and one of the IPNV-positive samples were negative for the specified agent when tested at the Norwegian Veterinary Institute; hence it was not possible to obtain sequences from these samples. For the remaining samples, sequences were obtained and subtyping / phylogenetic analyses were performed. For both SAV and IPNV, the detected subtype is endemic in farmed salmon in the area where the fish were captured. However, IPNV from sea trout (Fusta, Nordland) differ slightly from IPNV seen in salmon. PMCV is widespread in salmon farming, and all virus detected during outbreaks have so far belonged to the same genogroup. The PMCV detected in salmon captured in Årøy (2012) and Numedalslågen (2012) also belong to this genogroup, but it is somewhat surprising that the two fish, sampled in completely different places in Norway, contain PMCV which are closely related.

Results are summarized in Table 2.

Table 2. Summary of results

Sample no	Virus	Year	River	Species	Typing of sequence	NVI Ref.
1	SAV	2012	Vosso	Salmon (Hatchery reared?)	SAV 3	Vir3257
2	IPNV	2011	Fusta	Sea trout	Sp	Vir3258
3	IPNV	2011	Vosso	Salmon (Wild)	Sp	Vir3259
4	IPNV	2011	Surna	Salmon (Wild)	Not detected	Vir3260
5	PMCV	2012	Numedalslågen	Salmon (Escaped)	PMCV*	Vir805
6	PMCV	2012	Årøy	Salmon (Wild)	PMCV*	Vir806
7	PMCV	2008	Nausta	Salmon (Wild)	PMCV*	
8	PMCV	2007	Årøy	Salmon (Wild)	PMCV*	
9	ISAV	2012	Måna	Salmon (Wild)	Not detected	Vir804
10	ISAV	2008	Moelva	Salmon (Wild)	-	

* All PMCV-isolates belong to the same genogroup.

5. Study 2. Evaluating the genetic integrity of virus-positive wild salmon.

Introduction

Since the 1970's, selective breeding in farmed salmon has focused on commercially important traits such as growth rate and early sexual maturation in addition to resistance against important infectious diseases. Selective breeding and the domestication process has thus lead to significant genetic differences between farmed salmon and their wild conspecifics [11, 12]. Every year, escaped farmed salmon enter rivers [13] and breed with wild salmon . This flow of genes from farmed to wild salmon is rated as one of the most serious negative impact of salmon farming [14]. It has been shown that farmed salmon have lower genetic variation compared to wild salmon populations [15-18]that offspring of farmed salmon have lower fitness in the natural environment than their wild counterpart [19-21]. A panel of single nucleotide polymorphisms (SNPs) has been identified and shown to collectively be diagnostic in identifying individual salmon as having farmed salmon in their pedigree, regardless of their populations of origin [22, 23]. The method generates a P(wild) value that reflects the "probability of being wild".

It has previously been shown that returning of escaped farmed origin are more prone to be virus carriers than salmon of wild origin [24]. This may reflect the ease of pathogen transmission within the farmed environment. However, the aim of this study was to investigate if offspring of escaped farmed salmon, born in nature, also are more prone to be virus carriers than salmon of pure wild origin

Materials and methods

The Norwegian veterinary institute conducts scale reading and organizes health control of wild Atlantic salmon *(Salmo salar)* brood fish used for stock enhancement and for the gene bank of wild Atlantic salmon. Disease surveillance and research projects have provided additional data. A collection of 49 virus positive spawners of Atlantic salmon were included in the study. All were classified as wild by scale reading [25, 26]. The distribution of infections in a population typically varies across factors such as sex, age and breed. An uneven distribution of these *confounding factors* between the test and control group will thus accidentally introduce or mask associations in the study. To control confounding, each virus positive wild salmon was matched with a virus negative wild salmon from the same river with regards to potential confounders, in order of priority sex, catch-year, winter sea-age and smolt age [24, 27], although it should be noted that the match was not perfect for all pairs. Scales were sent to Norwegian institute of nature research (NINA) for genetic analyses to estimate level of wild (P(wild)) versus farmed (1 – P(wild)) origin for each fish. A high value reflects a high probability of being wild. Salmon with P(wild) values < 0.71 are unlikely of pure wild origin . Researchers at NINA were informed about the purpose of the testing, the river of origin and catch year, but not about the virus carrier status of the individual salmon, the test was thus blinded. Test result were returned as Excel sheets and imported to STATA 12 for data analysis. In stock enhancement hatcheries and the gene bank for wild Atlantic salmon, salmon with P(wild) values < 0,71 are disqualified as brood fish [28]. For the purpose of this study this value is used as to generate a genetic integrity classification. Salmon with P(wild) values > 0,71 are thus classified as wild (wild=1) and salmon with P(wild) values < 0.71 are classified as non-wild (wild=0). The Pearson chi square test and the non-parametric methods sign test and Wilcoxon matched-pairs signed-rank test were used to test the existence of a statistical association between virus carrier state and the two variables P(wild) value and genetic integrity classification. The null hypothesis is that the P(wild) and classification distribution is the same in virus positive and virus negative salmon. The study was restrained by the limited number of samples and by including several salmon stocks (rivers) and virus with very different characteristics. The analyses were performed without attempts to adjust for these factors, and should be interpreted carefully.

Results

Table 3 displays information about the matched pairs including P(wild) values, genetic integrity classification for all 98 tested individuals and value for each matched pair in the sign test. In one of the matched pairs, No 5 Fusta 2009, both fish were diagnosed as brown trout (*Salmo trutta)* and omitted from further analyses. Altogether 9 of 48 virus positive salmon were classified as non-wild, while 4 of 48 virus negative salmon were classified as non-wild (χ^2 (1df): 2.22, p= 0.136). The non-significant chi-square statistics imply that the null hypothesis should be kept. It is however noteworthy that the p-value is quite low.

Results from the non-parametric test also implied that the null hypothesis should be kept. Based on the p(wild) values, 24 pairs were positive and 23 pairs were negative and the rank sums were not significantly different: z=1.11, p=0.27. The sign test was used to analyse the association between virus carrier state and the genetic integrity classification. As displayed in Table 3, 11 matched pairs had non-zero values, 8 pairs are "negative" and 3 are "positive". Under the one-sided alternative hypothesis that more virus positive salmon are classified as non-wild (wild=0), the probability of detecting 8 or more "negative" pairs by chance is 0.11. Under the two-sided hypothesis, that the probability of detecting 8 or more "positive" or "negative" pairs by chance is 0.22.

Discussion and conclusion

The study was restrained by the limited number of samples and by including several salmon stock and virus with very different characteristics. Furthermore, the estimated level of farmed introgression is uncertain at the individual level and identification of specific admixed proportions, or hybrid groups cannot be done. A larger dataset is therefore likely needed to detect a possible association between carrying viruses and farmed origin. A larger dataset would also enable more sophisticated statistical testing. By the use of non-parametric tests, this study was not able to detect a statistically significant association between virus carrier state and farmed origin index P(wild). However, there is a trend toward more virus carriers among salmon classified as non-wild, encouraging further studies with a larger dataset.

				Virus positive			Virus negative			
Match no	Virus	County	River	Year	P(wild)	Wild	Year	P(wild)	Wild	Sign test
1	PMCV	Sogn og Fjordane	Nausta	2008	.731	1	2008	.979	1	0
2	PMCV	Sogn og Fjordane	Årøy	2007	.95	1	2007	.949	1	0
3	PMCV	Sogn og Fjordane	Årøy	2012	.868	1	2012	.952	1	0
4	IPN	Møre og Romsdal	Bævra	2010	.038*	0	2010	.903	1	-1
5	IPN	Nordland	Fusta	2009	.99	1	2009	.988	1	0
6	IPN	Vestfold	Numedalslågen	2010	.969	1	2010	.953	1	0
7	IPN	Nord-Trøndelag	Stjørdal	2005	.268*	0	2005	.881	1	-1
8	IPN	Nord-Trøndelag	Stjørdal	2005	.855	1	2005	.972	1	0
9	IPN	Nord-Trøndelag	Stjørdal	2008	.962	1	2008	.962	1	0
10	IPN	Aust Agder	Storelva	2008	.975	1	2008	.953	1	0
11	IPN	Sogn og Fjordane	Vikja	2005	.958	1	2005	.954	1	0
12	ISA	Nord-Trøndelag	Moelva	2008	.973	1	2008	.956	1	0
13	ISA	Nord-Trøndelag	Moelva	2008	.984	1	2008	.983	1	0
14	ISA	møre og romsdal	Måna	2012	.948	1	2012	.99	1	0
15	ISA	Nord-Trøndelag	Øyensåa	2008	.986	1	2008	.964	1	0
16	ISA	Nord-Trøndelag	Øyensåa	2008	.989	1	2008	.962	1	0
17	PRV	Møre og Romsdal	Eidsdals	2008	.968	1	2008	.865	1	0
18	PRV	Nord -Trøndelag	Moelva	2008	.343*	0	2008	.966	1	-1
19	PRV	Nord -Trøndelag	Namsen	2008	.984	1	2008	.807	1	0
20	PRV	Nord -Trøndelag	Øyensåa	2008	.939	1	2008	.954	1	0
21	PRV	Hordalan	Ekso	2007	.121*	0	2007	.296*	0	0
22	PRV	Hordalan	Ekso	2007	.501*	0	2007	.937	1	-1
23	PRV	Hordalan	Ekso	2007	.971	1	2007	.94	1	0
24	PRV	Hordalan	Ekso	2008	.836	1	2008	.961	1	0
25	PRV	Aust-Agd	Storelva	2009	.938	1	2009	.977	1	0
26	PRV	Møre og Romsdal	Eira	2007	.982	1	2007	.611*	0	1
27	PRV	Møre og Romsdal	Eira	2008	.85	1	2008	.894	1	0

				Virus positive				Virus negative			
Match no	Virus	County	River	Year	P(wild)	Wild		Year	P(wild)	Wild	Sign test
28	PRV	Møre og Romsdal	Eira	2008	.987	1		2009	.917	1	0
29	PRV	Møre og Romsdal	Eira	2008	.435*	0		2008	.957	1	-1
30	PRV	Møre og Romsdal	Eira	2009	.986	1		2009	.961	1	0
31	PRV	Møre og Romsdal	Eira	2008	.979	1		2009	.911	1	0
32	PRV	Møre og Romsdal	Surna	2008	.907	1		2008	.974	1	0
33	PRV	Møre og Romsdal	Surna	2007	.9	1		2007	.322*	0	1
34	PRV	Møre og Romsdal	Surna	2007	.974	1		2007	.981	1	0
35	PRV	Møre og Romsdal	Surna	2009	.487*	0		2009	.957	1	-1
36	PRV	Møre og Romsdal	Surna	2007	.652*	0		2008	.785	1	-1
37	PRV	Møre og Romsdal	Surna	2009	.962	1		2009	.839	1	0
38	PRV	Møre og Romsdal	Surna	2009	.98	1		2009	.055*	0	1
39	PRV	Møre og Romsdal	Surna	2009	.91	1		2009	.959	1	0
40	PRV	Møre og Romsdal	Surna	2007	.927	1		2007	.985	1	0
41	PRV	Møre og Romsdal	Surna	2008	.959	1		2008	.897	1	0
42	PRV	Nord -Trøndelag	Stjørdal	2009	.971	1		2009	.907	1	0
43	PRV	Nord -Trøndelag	Stjørdal	2009	.976	1		2009	.773	1	0
44	PRV	Nord -Trøndelag	Stjørdal	2007	.948	1		2007	.97	1	0
45	PRV	Nord -Trøndelag	Stjørdal	2008	.862	1		2008	.963	1	0
46	PRV	Nord -Trøndelag	Stjørdal	2008	.959	1		2008	.82	1	0
47	PRV	Sogn og fjordane	Vikja	2008	.036*	0		2009	.774	1	-1
48	PRV	Sogn og fjordane	Vikja	2007	.974	1		2007	.971	1	0
49	PRV	Sogn og fjordane	Vikja	2008	.751	1		2008	.969	1	0

* P(wild) < 0.71, meaning that they are classifies as non-wild.

6. Study 3. Salmonid freshwater reservoirs of Piscine orthoreovirus (PRV)

Introduction

Since the detection of piscine orthoreovirus (PRV) in 2010, a number of studies have been conducted to describe the occurrence and distribution of the virus. These studies have focused on marine reservoirs and have shown that PRV is a common and widely distributed virus among wild and farmed salmon in this environment. There is however limited knowledge with regards to the occurrence of PRV in salmonid freshwater habitats. The aim of this study was therefor to investigate the existence of salmonid freshwater reservoirs of PRV.

Materials and methods

Nine stock enhancement hatcheries producing non-anadromous brown trout (*Salmo trutta*) for restocking purposes were recruited to the study. In addition, samples were collected from wild-caught brood fish of the two relict salmon (*Salmo salar*) stocks, Småblanken in Namsen, Nord Trøndelag County and Byglandsblega in Byglandsfjorden, Aust Agder County.
Autopsies and sampling of kidney were performed by authorized fish health personnel contracted to the individual hatchery or from NVI. In three hatcheries kidney samples were collected by skilled personnel from the hatcheries after careful instructions from NVI. Kidney samples were fixed in RNAlater™ and shipped chilled to analysis immediately after autopsy, or alternatively stored in the refrigerator for at least 24 hours for fixation before freezing and shipping. All PCR assays were performed by PatoGen Analyse AS (http://www.patogen.no). PatoGen Analyse is an ISO 17025 accredited laboratory. PRV-positive tissue samples or RNA extracted from the samples were sent to NVI for sequencing.

Results

None of the 52 relict salmon were PRV positive. However, a small amount of viral RNA from PRV was detected in altogether 4 of 271 tested brown trout (Table 4). This constitutes 1.5 % (0.6 to 3.7%) of the brown trout. Two PRV positive individuals were detected in a hatchery from the northern part of Norway, and the remaining two were detected in a hatchery situated in central parts of Norway. In each facility this represents a prevalence of 7% (95% CI 1.9 to 21.3%). The Ct-values ranged from 34.4 to 36.5. Only one of the four PRV-positive samples had tissue left after completion of the PCR assay. This tissue sample and extracted RNA from the remaining three positive samples were sent to NVI for sequencing. The NVI was not able to reproduce the detection of PRV, with the exception of one sample. This sample had a Ct value of 40.5 in the NVI PCR, above the cut-off value, and was therefore considered unsuitable for sequencing.

Table 4. Results from PCR analyses of non-anadromous *Salmo trutta* and *Salmo salar* are presented. All 52 non-anadromous S*almo salar* were negative, while four of 272 tested *Salmo trutta* were PRV positive, albeit with low viral loads.

PCR analysis	Number tested	Virus positives	Comments
***Salmo trutta*, (non-anadromous)**	272	4	From two hatcheries. Ct-value range 34.4 - 36.5 (40.5 at NVI)
***Salmo salar*, (non-anadromous)**	52	0	
Total no. analyses	324	**4**	

Discussion and conclusion

The probability of detecting a pathogen present in a population depends on the population size, the number of infected individuals and the sample size. None of the tested wild relict salmon were PRV-positive, while 4 of 272 brown trout were PRV positive, albeit with low viral levels.

Sampling of brown trout took place in hatcheries. The rationale behind this study design was that given that the virus is present in a non-anadromous brown trout reservoir, it is more likely to be detected in a hatchery where fish are kept together in tanks for a prolonged period, hence facilitating transmission of pathogens between individuals. The overall prevalence in non-anadromous brown trout in this study was at par with previous studies in sea-trout [29, 30]. The question is whether few positives is due to a low susceptibility of brown trout, low infection pressure or if infected fish die and thus avoid being sampled [31]. Mortalities directly attributable to PRV-infection were not reported by the hatcheries, although targeted investigations to uncover this have not been conducted. With one exception, the viral load have been low (high ct-values) in all PRV-positive *Salmo trutta*. This may reflect that this species is a less suitable host for PRV than Atlantic salmon. The aforementioned exception, a sea trout captured in Moelva, Nord-Trøndelag in 2008, had a Ct value of 25.9 and the sequenced virus grouped together with PRV from Atlantic salmon [5]. The susceptibility of *Salmo trutta* to PRV infection is not known and should be further studied.

Given that PRV is a virus with low virulence in both brown trout and salmon, and that the measured prevalence thus is a reflection of the actual prevalence, it seems likely that a fresh water reservoir of PRV is of minor importance compared to the marine reservoirs. This supports previous conclusions that the main reservoir of PRV is in farmed salmon in the marine environment.

7. Acknowledgements

Thanks to the stock enhancement hatcheries participating in the study and to the gene bank program for salmon for providing the samples. Also thanks to the fish health personnel that autopsied the fish and secured tissue samples. Thanks to Monika J. Hjortaas at NVI for providing the phylogenetic analyses and tree of ISAV segment 6 in Study 1.

8. References

1. Lyngstad, T.M., et al., *Use of Molecular Epidemiology to Trace Transmission Pathways for Infectious Salmon Anaemia Virus (ISAV) in Norwegian Salmon Farming.* Epidemics, 2011. **3**(1): p. 1-11.
2. Mladineo, I., T. Segvic, and L. Grubisic, *Molecular evidence for the lack of transmission of the monogenean Sparicotyle chrysophrii (Monogenea, Polyopisthocotylea) and isopod Ceratothoa oestroides (Crustacea, Cymothoidae) between wild bogue (Boops boops) and cage-reared sea bream (Sparus aurata) and sea bass (Dicentrarchus labrax).* Aquaculture, 2009. **295**(3-4): p. 160-167.
3. Vendramin, N., et al., *Viral Encephalopathy and Retinopathy in groupers (Epinephelus spp.) in southern Italy: a threat for wild endangered species?* Bmc Veterinary Research, 2013. **9**.
4. Ruane, N.M., et al., *Molecular differentiation of infectious pancreatic necrosis virus isolates from farmed and wild salmonids in Ireland.* Journal of Fish Diseases, 2009. **32**(12): p. 979-987.
5. Garseth, A.H., T. Ekrem, and E. Biering, *Phylogenetic Evidence of Long Distance Dispersal and Transmission of Piscine Reovirus (PRV) between Farmed and Wild Atlantic Salmon.* Plos One, 2013. **8**(12).
6. Markussen, T., et al., *Ultra-deep pyrosequencing of partial surface protein genes from infectious Salmon Anaemia virus (ISAV) suggest novel mechanisms involved in transition to virulence.* PLoS One, 2013. **8**(11): p. e81571.
7. Wiik-Nielsen, J., et al., *Genetic variation in Norwegian piscine myocarditis virus in Atlantic salmon, Salmo salar L.* Journal of Fish Diseases, 2013. **36**(2): p. 129-139.
8. Garseth, A.H., E. Biering, and T. Tengs, *Corrigendum: Piscine myocarditis virus (PMCV) in wild Atlantic salmon Salmo salar.* Dis Aquat Organ, 2013. **104(3):**(3).
9. Garseth, Å.H., E. Biering, and T. Tengs, *Piscine myocarditis virus (PMCV) in wild Atlantic salmon Salmo salar.* Diseases of aquatic organisms, 2012. **102**(2): p. 157-161.

10. Hjortaas, M.J., et al., *The first detections of subtype 2-related salmonid alphavirus (SAV2) in Atlantic salmon, Salmo salar L., in Norway.* J Fish Dis, 2013. **36**(1): p. 71-4.
11. Anonymous, *Klassifisering av 104 laksebestander etter kvalitetsnorm for villaks* in *Temarapport nr 4*. 2016. p. 85 s. .
12. Glover, K.A., et al., *Atlantic salmon populations invaded by farmed escapees: quantifying genetic introgression with a Bayesian approach and SNPs.* Bmc Genetics, 2013. **14**.
13. Fiske, P., T. Aronsen, and K. Hindar, *Overvåkning av rømt oppdrettslaks i elver om høsten 2013*, in *NINA Rapport* 2014. p. 44 s.
14. Anonymous, *Status for norske laksebestander i 2010.* , in *Rapport fra Vitenskapelig råd for lakseforvaltning nr 2,*. 2010. p. 213 s.
15. Karlsson, S., T. Moen, and K. Hindar, *Contrasting patterns of gene diversity between microsatellites and mitochondrial SNPs in farm and wild Atlantic salmon.* Conservation Genetics, 2010. **11**(2): p. 571-582.
16. Skaala, O., et al., *Microsatellite analysis in domesticated and wild Atlantic salmon (Salmo salar L.): allelic diversity and identification of individuals.* Aquaculture, 2004. **240**(1-4): p. 131-143.
17. Skaala, O., J.B. Taggart, and K. Gunnes, *Genetic differences between five major domesticated strains of Atlantic salmon and wild salmon.* Journal of Fish Biology, 2005. **67**: p. 118-128.
18. Mjolnerod, I.B., et al., *Genetic differences between two wild and one farmed population of Atlantic salmon (Salmo salar) revealed by three classes of genetic markers.* Hereditas, 1997. **127**(3): p. 239-248.
19. Fleming, I.A., et al., *Lifetime success and interactions of farm salmon invading a native population.* Proceedings of the Royal Society of London Series B-Biological Sciences, 2000. **267**(1452): p. 1517-1523.
20. McGinnity, P., et al., *Fitness reduction and potential extinction of wild populations of Atlantic salmon, Salmo salar, as a result of interactions with escaped farm salmon.* Proceedings of the Royal Society B-Biological Sciences, 2003. **270**(1532): p. 2443-2450.
21. Skaala, O., et al., *Performance of farmed, hybrid, and wild Atlantic salmon (Salmo salar) families in a natural river environment.* Canadian Journal of Fisheries and Aquatic Sciences, 2012. **69**(12): p. 1994-2006.
22. Karlsson, S., et al., *Generic genetic differences between farmed and wild Atlantic salmon identified from a 7K SNP-chip.* Molecular Ecology Resources, 2011. **11**: p. 247-253.
23. Karlsson, S., et al., *A standardized method for quantifying unidirectional genetic introgression.* Ecology and Evolution, 2014. **4**(16): p. 3256-3263.
24. Garseth, A.H., E. Biering, and A. Aunsmo, *Associations between piscine reovirus infection and life history traits in wild-caught Atlantic salmon Salmo salar L. in Norway.* Preventive Veterinary Medicine, 2013. **112**(1-2): p. 138-146.
25. Fiske, P., R.A. Lund, and L.P. Hansen, *Identtifying fish farm escapees*, in *Stock identification methods*, S. Cadrin, K. Friedland, and J. Waldman, Editors. 2004, Elsevier. p. 659-680.
26. Lund, R.A. and L.P. Hansen, *Identification of wild and reared Atlantic salmon, Salmo salar L., using scale characters.* Aquaculture and Fisheries Management, 1991. **22**: p. 499-508.
27. Madhun, A.S., et al., *Potential disease interaction reinforced: double-virus-infected escaped farmed Atlantic salmon, Salmo salar L., recaptured in a nearby river.* Journal of Fish Diseases, 2014: p. n/a-n/a.
28. Karlsson, S., et al., *Stamlakskontroll 2014*. 2015: www.nina.no.
29. Garseth, A.H., et al., *Piscine reovirus (PRV) in wild Atlantic salmon, Salmo salar L., and sea-trout, Salmo trutta L., in Norway.* Journal of Fish Diseases, 2013. **36**(5): p. 483-493.
30. Biering, E., et al., *Annual report on health monitoring of wild anadromous salmonids in Norway*. 2013, Norwegian Veterinary Institute and Institute of Marine research: www.vetinst.no.
31. McVicar, A.H., *Disease and parasite implications of the coexistence of wild and cultured Atlantic salmon populations.* Ices Journal of Marine Science, 1997. **54**(6): p. 1093-1103.

The Norwegian Veterinary Institute (NVI) is a nationwide research institute in the fields of animal health, fish health, and food safety. The primary mission of the NVI is to give research-based independent advisory support to ministries and governing authorities. Preparedness, diagnostics, surveillance, reference functions, risk assessments, and advisory and educational functions are the most important areas of operation.

The Norwegian Veterinary Institute has its main laboratory in Oslo, with regional laboratories in Sandnes, Bergen, Trondheim, Harstad and Tromsø, with about 360 employees in total.

www.vetinst.no

Veterinærinstituttet
Norwegian Veterinary Institute

The Institute of Marine Research (IMR) is the largest marine science community in Norway with more than 700 employees. Our main task is providing advice to the Norwegian authorities on aquaculture and on the ecosystems of the Barents Sea, Norwegian Sea, North Sea and the Norwegian coastal zone. Around half of our activities are funded by the Ministry of Trade, Industry and Fisheries.

The Institute of Marine Research has headquarters located in Bergen, but important aspects of our work are done at our department in Tromsø, at our research stations in Matre, Austevoll and Flødevigen and on board our research vessels.

www.imr.no

HAVFORSKNINGSINSTITUTTET
INSTITUTE OF MARINE RESEARCH

The Norwegian Food Safety Authority (NFSA) is a governmental body whose aim is to ensure through regulations and controls that food and drinking water are as safe and healthy as possible for consumers and to promote plant, fish and animal health and ethical farming of fish and animals. We encourage environmentally friendly production and we also regulate and control cosmetics, veterinary medicines and animal health personnel. The NFSA drafts and provides information on legislation, performs risk-based inspections, monitors food safety, plant, fish and animal health, draws up contingency plans and provides updates on developments in our field of competence.

The NFSA comprises three administrative levels, and has some 1300 employees.
The NFSA advises and reports to the Ministry of Agriculture and Food, the Ministry of Trade, Industry and Fisheries, and the Ministry of Health and Care Services.

www.mattilsynet.no

Mattilsynet