Natural infection of Atlantic salmon (*Salmo salar* L.) with salmonid alphavirus 3 generates numerous viral deletion mutants

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Salmon pancreas disease virus (SPDV) also referred to as salmonid alphavirus (SAV) is a virus causing pancreas disease in Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*). Although the virus causes an economically important disease, relatively few full-length genome sequences of SAV strains are currently available. Here, we report full-length genome sequences of nine SAV3 strains from sites farming Atlantic salmon geographically spread along the Norwegian coastline. The virus genomes were sequenced directly from infected heart tissue, to avoid culture selection bias. Sequence analysis confirmed a high level of sequence identity within SAV3 strains, with a mean nucleotide diversity of 0.11%. Sequence divergence was highest in 6K and E2, while lowest in the capsid protein and the non-structural proteins (nsP4 and nsP2). This study reports for the first time that numerous defective viruses containing genome deletions are generated during natural infection with SAV. Deletions occurred in all virus strains and were not distributed randomly throughout the genome but instead tended to aggregate in certain areas. We suggest imprecise homologous recombination as an explanation for generation of defective viruses with genome deletions. The presence of such viruses, provides a possible explanation for the difficulties in isolating SAV in cell culture. Primary virus isolation was successfully achieved for only two of eight strains, despite extensive attempts using three different cell lines. Both SAV isolates were easily propagated further and concomitant viral deletion mutants present in clinically infected heart tissue were maintained following serial passage in CHH-1 cells.

INTRODUCTION

Pancreas disease (PD) is an economically important disease in European salmonid aquaculture affecting both Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*) (McLoughlin & Graham, 2007). The disease is characterized by degeneration and necrosis of cardiomyocytes with subsequent inflammation, pancreatic acinar cell loss and subsequent skeletal muscle degeneration (McLoughlin *et al.*, 2006; McVicar, 1987). PD is caused by salmon pancreas disease virus (SPDV) also referred to as salmonid alphavirus (SAV), and belongs to the genus *Alphavirus* within the family *Togaviridae*. The alphaviruses are small, spherical, enveloped viruses that are important pathogens of animals and humans worldwide. Their genomes consist of a monopartite, single stranded, positive-sense RNA genome of 11–12 kb.

Most alphaviruses are transmitted by highly specific arthropod vectors, of which mosquitoes are the most common, but others exist such as lice and mites (Durden *et al.*, 1992). This together with specific environmental conditions and reservoir hosts might lead to restricted geographical distribution (Griffin, 2007). Although SAV3 has been detected by real-time PCR from the salmon louse *Lepeophtheirus salmonis* collected from diseased fish (Petterson *et al.*, 2009), the role of lice or other invertebrate vectors is yet to be determined as SAV infections can be transmitted without the aid of an arthropod (McLoughlin *et al.*, 1996; Xu *et al.*, 2012).

Genetic analysis of salmonid alphaviruses has shown at least six genetically distinct subtypes, SAV1–6, which are also separated geographically (Fringuelli *et al.*, 2008; Graham *et al.*, 2012; Weston *et al.*, 2005). Recently, a comparative experimental study in Atlantic salmon, conducted as a freshwater cohabitation trial, showed that...
all subtypes caused pathological changes typical of PD, although the relative virulence of the strains varied (Graham et al., 2011).

SAV3 represents a subtype that so far has been detected only in Norway where it causes PD in Atlantic salmon and rainbow trout (Hodneland et al., 2005). However, following the first detection of a SAV2-related virus in 2011, this subtype is now frequently found in PD outbreaks in mid-Norway (Hjortaas et al., 2013). The common understanding has been that there is little genetic variation within subtypes (Fringuelli et al., 2008; Karlsen et al., 2006; Weston et al., 2005), and previously published sequence data from the Norwegian strains show only minor amino acid differences between the strains analysed (Jansen et al., 2010; Karlsen et al., 2006). However, very few full-length SAV sequences have been published, and the SAV3 full-length sequences previously reported come from the same geographical area, and might therefore not be representative of the entire Norwegian coast. Furthermore, most available full-genome SAV sequences are based on isolates cultured in cells prior to sequencing. As primary virus isolation from field outbreaks has been difficult and yielded relatively few numbers of isolates (McLoughlin & Graham, 2007), the understanding has been that there is need for some degree of selection or adaptation before virus can be isolated in cell culture (Castric et al., 1997; Nelson et al., 1995). A previous study has shown that mutations occur following serial passage in CHSE-214 cells, and four amino acid substitutions were identified in the genome between passage 3 and 20 of one defined isolate of SAV3 (Karlsen et al., 2006).

This paper describes sequence analysis of full-length viral genomes of SAV3 from nine sites geographically spread along the Norwegian coastline. To avoid culture selection bias we performed whole genome sequencing directly from infected fish tissues. The sequence analysis confirmed little divergence between the Norwegian strains, but revealed relatively few numbers of isolates (McLoughlin & Graham, 2007), the understanding has been that there is need for some degree of selection or adaptation before virus can be isolated in cell culture (Castric et al., 1997; Nelson et al., 1995). A previous study has shown that mutations occur following serial passage in CHSE-214 cells, and four amino acid substitutions were identified in the genome between passage 3 and 20 of one defined isolate of SAV3 (Karlsen et al., 2006).

RESULTS

PD confirmed in Atlantic salmon study samples

Nine Atlantic salmon farms with a previous diagnosis of PD were available and included in the study (Fig. 1). The PD diagnosis was confirmed by histopathological evaluation of haematoxylin and eosin (H/E) stained sections, showing changes typical for PD in heart, pancreas and muscle tissue of the individuals sampled from all locations. The degree of pathological changes ranged from mild to severe and chronic. Presence of SAV in parallel heart tissue was shown by real-time reverse-transcription PCR (RT-PCR) and ranged from 30% SAV positive individuals in SAV3-2-MR/10 to 80% in SAV3-1-T/10 (supplementary details are available from the authors on request), and supported the PD diagnosis. Based on Cp values from real-time RT-PCR one fish from each site was selected for genome sequencing and virus isolation in cell culture. The same field samples were used for generating the genome sequences and to attempt virus isolation in cell culture. As sequenced viral strains were generated from individual fish, variability observed represent intra-host variation.

Natural infection of SAV generates numerous virus variants with internally deleted genomes

At present, few SAV full-length genome sequences are available and most of these virus strains have been cultured in cells prior to sequencing. To avoid genetic changes due to cell-culture adaptation, virus genome sequencing was performed directly from clinical samples of heart tissue preserved in RNAlater. The strategy for sequencing was amplification of the entire viral genome in six overlapping PCR products. To bypass difficulties with direct sequencing of the PCR products, all PCR products were cloned and at least three clones from each PCR product were sequenced. Analysis of sequence data revealed the presence of genome deletions in many subsets of clones sequenced from each PCR product. The deletions varied in length from 1 to almost 400 nt. A closer study of the original gel electrophoresis photos of the initial PCR products revealed that deletion variants were sometimes visible as multiple bands prior to cloning. As many deletions were relatively short, some bands on gel also appeared thick, and probably consisted of two or several products of about the same size. A complete graphical presentation showing the distribution of deletions is shown in Fig. 2. The number of deletions varied between strains, ranging from only three in SAV3-9-R/10 up to 35 deletions in SAV3-8-R/10. As the majority of the deletions lead to frameshift and premature stop codons, many were assuming encoding non-viable virus variants. The deletions were observed in all strains and in all parts of the genome, but were not distributed randomly throughout the genome but tended to be aggregated. Deletions found in nsP2 (position 2307–2366) and E1 (position 858–1006) were identical in virus strains from five and four different locations, respectively. The sequences in close proximity to the deletions were identical for the strains, and an inverted motif TGGCCCTC was found upstream and downstream of the deletion in E1. This suggests these deletions were due to sequence-dependent mechanisms, although evolution from an ancestor containing the deletion cannot be ruled out.

Genome deletions were much more common than insertions, which were found in virus strains from six
different locations. Insertions were typically of one nucleotide, except for an insertion of 4 nt found in the 3’ end of the E1 gene in one of the strains (SAV3-9-R/10).

The same insertion was found in three out of four clones sequenced. The insertion interfered with the standard stop codon of E1 and resulted in an extension of 21 amino acids.

Fig. 1. Map showing the location of farms included for sequencing of SAV strains in this study. The strains are numbered from north to south along the Norwegian coastline (1–9). Capital letters denotes county of origin: T, Troms; MR, Møre og Romsdal; SF, Sogn og Fjordane; H, Hordaland; R, Rogaland. Finally, the time of sampling is given (2009/2010).
compared to the standard size of E1. Although the insertion was found in the majority of clones sequenced from this virus strain, it is not known whether this version of E1 has the ability to assemble into viral glycoprotein spikes.

Full-genome sequences showed homogeneity and were of the SAV3 subtype

The consensus sequences for full-length genomes of nine SAV strains, sampled from sites spread along the Norwegian coastline, were generated based on sequence information from a minimum of three clones per cloned fragment and did not contain deletions. Phylogenetic analysis revealed that all included virus strains were of the SAV3 subtype (results not shown), and assembled full-length sequences have been submitted to GenBank (accession numbers KC122918–KC122926). The mean nucleotide diversity among currently available full-length SAV3 sequences (nine reported in this study and four previously reported [AY604235–AY604238]) was 0.11 %, and ranged from 0.04 % to 0.21 % between the viral proteins. The corresponding numbers for each of the non-structural and structural proteins are shown in Table 1. The highest nucleotide divergence was found within the 6K gene followed by the structural protein E2, while lowest divergence was found for nsP4, nsP2 and the capsid protein.

Amino acid substitutions within available full-length consensus virus sequences, are summarized in Table 2, together with the four previously published full-length SAV3 sequences (AY604235–AY604238) which have all been collected from the same geographical region; some were based on isolates passaged on cell culture. A total of 29 amino acid positions (0.74 % of the total positions) were found to differ in at least one of the virus strains, with 22 substitutions not previously reported. However, 15 of these substitutions were infrequent and only found in one virus strain. In contrast, variability in three positions showed substitutions present in more than two strains. These include position 88 in nsP1 with an almost equal distribution of aspartic acid and valine being observed. Similarly, two positions in nsP3 showed either glutamic acid or valine in position 171, and methionine or threonine in position 517. Substitutions were also observed in 6K (residue 7), where two virus strains showed substitutions of isoleucine with threonine. This substitution has been described previously (Jansen et al., 2010). Interestingly, none of the four amino acid substitutions reported to...
occur in vitro during 20 passages of a SAV3 isolate in CHSE-214 cells were present in any of the virus strains included in this study (Karlsen et al., 2006).

Intra-host genome diversity was observed as differences between sequenced clones from the same fish, and was present in all but one strain. The extent of within-fish variability varied considerably between strains, and the number of nucleotide positions showing variation ranged from none in SAV3-9-R/10 to 96 in SAV3-6-H/10. Non-synonymous substitutions within individual fish and present in more than one virus strain were found in nsP3 position 517, nsP4 position 454, E2 position 136, E2 position 220 and E1 position 454.

Phylogenetic analysis of Norwegian full-length SAV3-strains

Due to the high degree of similarity among the studied SAV3 sequences, analysis was based on full-length nucleotide sequence data for optimal resolution. The phylogenetic tree displays two clusters with acceptable bootstrap values (Fig. 3). The four northernmost virus strains formed a separate cluster with a bootstrap value of 92 %, while two previously deposited sequences (AY604237 and AY604238) formed a second cluster with a bootstrap value of 97 %. However, a geographical pattern was not present when limiting the analyses to a 451nt sequence in the E2 and including all available sequence information in GenBank (results not shown).

Primary virus isolation was achieved for only two out of eight SAV3 strains

Even though SAV can be grown in cell culture, primary virus isolation from clinically infected material can be difficult (Rowley et al., 1998). Virus isolation was attempted for each of the eight strains, as organ material was conserved in RNAlater only from one site and could thus not be examined by cultivation in cell culture. Heart tissue homogenate from the one or two individuals used for generating the full-genome sequences. Tissue homogenate was inoculated on three different cell lines (CHSE-214, CHH-1 and AGK (Munang’andu et al., 2012). Primary virus isolation in CHSE-214 cells was successfully achieved from one of the eight tested strains (SAV3-9-R/10). The cytopathic effect (CPE) appeared in the third passage, with cells exhibiting CPE characteristics such as shrinkage and vacuole formation. With subsequent passages, CPE occurred earlier. The presence of SAV was confirmed by real-time RT-PCR while tests for infectious pancreatic necrosis virus (IPNV) infection were negative.

CHH-1 and AGK cells were used in addition to CHSE cells, testing different variables such as volume of inoculum and culture plate formats (results not shown). Despite repeated attempts, no CPE was observed in AGK cells, while primary virus isolation was successfully managed in one more isolate (SAV3-2-MR/10) using CHH-1 cells. Here CPE was present from passage 2, and resulted in complete lysis of the cell population. This isolate was passaged four times, resulting in total cell lysis in every passage. Presence of SAV in the cell-culture medium was confirmed by real-time RT-PCR from supernatant after the second passage, while tests for IPNV infection were negative. Real-time PCR indicated no virus replication in cultures without CPE.

Defective viruses present in clinically infected heart tissue were maintained following serial passage in CHH-1 cells

Non-viable virus variants present in infected fish tissues will likely interfere with successful primary virus isolation of SAV (Roux et al., 1991). As we were able to recover two of the virus strains in cell culture, we went on to study whether defective viruses containing internal genome deletions were maintained following serial passage in cells. Tissue homogenate supernatant from one of the culturable virus strains (SAV3-2-MR/10) were passaged six times in CHH-1 cells, and partially sequenced after each passage (from passage 2). The experiment was performed twice using slightly different protocols for the initial inoculum (Table 3), giving similar results. For practical reasons the analysis was limited to 2.3 kb at the 3’ end of the SAV3 genome (including 650 nt of E2, 6K and E1 gene), and CPE was present in both experiments from passage 2, resulting in complete cell lysis. Deletions were found in all (five out of five) clones amplified directly from infected fish (Table 3 and Fig. 2). After passage in cell culture, less than 20 % of the sequenced clones contained deletions. Even though virus variants with internally deleted genomes were reduced after serial passage in CHH-1 cells, there was no clear trend with regard to passage number and the frequency of sequences carrying deletions. However, it

| Table 1. Comparison of mean- and maximum nucleotide diversity (%) among viral proteins of SAV3. The analyses were done using MEGA5 software, and were based on available full-length sequences: nine reported in this study and four previously reported sequences (AY604235–AY60438) |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                | nsP1            | nsP2            | nsP3            | nsP4            | Cp              | E3              | E2              | 6K              | E1              |
| Length, nucleotides            | 1683            | 2577            | 1674            | 1827            | 843             | 213             | 1314            | 204             | 1383            | 11756           |
| Maximum difference             | 0.430           | 0.155           | 0.358           | 0.219           | 0.237           | 0.469           | 0.609           | 0.980           | 0.289           | 0.171           |
| Mean difference                | 0.131           | 0.055           | 0.168           | 0.042           | 0.052           | 0.132           | 0.180           | 0.214           | 0.111           | 0.108           |
remained evident that defective viruses carrying deletions over the first six passages. Defective viruses with the characteristic 149 nt deletion in E1 as described above were present in two clones sequenced directly from infected tissue homogenate from which strain SAV3-2-MR/10 originated. This viral deletion variant was maintained during cell passage in CHH-1 cells and found after both parallel virus passages (present after both passages 3 and 4).

### DISCUSSION

This study provides the first comprehensive full-length genome comparison of nine salmonid alphavirus (SAV) strains geographically spread along the Norwegian coastline. All sequenced virus strains in this study were SAV3, the predominant subtype found in Norway at the time of sampling (Farmed fish health report 2012; www.vetinst.no). The virus genomes were sequenced directly from heart tissues, as it is well known that during passage in cell culture, adaptation can occur and genetic changes can be introduced into the virus genome (Karlsen et al., 2006). Due to technical difficulties with direct sequencing, all PCR products were cloned prior to sequencing. This approach allowed us to generate legible sequences from the population of sequences carrying multiple deletions.

An important finding of our study was the presence of defective viruses carrying numerous indel mutations throughout the genome of SAV. With a total of 3–35 deletions within individual clones from the same virus strain, ranging in size from 1 to 400 nt, deletions were a predominant finding over genome insertions. The presence of larger genome deletions cannot be excluded, as sequence data were based on genome fragments of approximately 2300 nt. Deletion mutants have previously been identified in many RNA virus families both from field samples and in vitro (Kumaria et al., 2011; Pesko et al., 2012; Tsai et al., 2007), and have also been described in alphaviruses (Forrester et al., 2011). Virus variants containing deletions in the 6K region have previously been reported in salmonid alphavirus (SAV-1/SPDV) but were believed to be cell-culture artefacts (Weston et al., 2002). The first SAV-1 sequence published represented a deletion mutant lacking 108 nt in the 6K region (Weston et al., 1999), and was later corrected (Weston et al., 2002). In contrast, our data provide evidence that deletions are present throughout the genome of SAV3 and that they are not caused by cell-culture artefacts. We also observed deletions in the 6K region, but these were not more prevalent than in other parts of the genome.

Viral mutants containing deletions or insertions emerge from aberrant or imprecise homologous recombination (Lai, 1992), likely produced through a copy choice mechanism (Lazzarini et al., 1981). Copy choice recombination occurs when two or more viruses infect the same host cell, and the RNA polymerase dissociates from the
template strand and switches to a homologous template at a different genomic position during synthesis, while the nascent string remains (Lai, 1992). Distribution of the observed deletions presented graphically in Fig. 2 clearly shows that the deletion variants were not randomly distributed throughout the genome, but typically clustered in certain areas. This is in conformity with publications reporting that factors such as secondary RNA structure, sequence identity and the kinetics of transcription influence template switching (Baird et al., 2006; Simon-Loriere & Holmes, 2011). The presence of an inverted sequence motif immediately upstream and downstream of a deletion variant frequently found in E1 further strengthens the importance of sequence-dependent mechanisms for generation of these deletion mutants. It is not, however, possible to ensure that identical deletion variants found in several strains and collected from different locations have arisen from independent deletion events, as it is possible that these deletion variants occurred through a single ancestral deletion with subsequent spread in the viral population.

Although nucleotide insertion and deletion (indel) events, together with substitutions, represent the major mutational processes of gene evolution (Söding & Lupas, 2003), it is theorized that deletions are generated as a by-product of replication and are of no advantage to the viral population (Forrester et al., 2011). Defective viruses containing only some portion of the infectious virus genome might, however, also be defined as defective interfering particles (DIPs) because they can interfere with replication of non-defective genomes through competition for cellular resources (Roux et al., 1991; Stauffer Thompson et al., 2009). DIPs have also been related to persistent infections in both flavivirus and alphavirus (La et al., 1996; Poidinger et al., 1991; Tsai et al., 2007; Weiss et al., 1983), and a role in maintaining persistent infections in vivo has been proposed (Noppornpanth et al., 2007). As the SAV genome seems highly prone to internal deletions giving rise to defective viruses, the role of DIPs for persistence of PD should be further investigated.

Primary isolation of SAV poses a significant challenge (Graham et al., 2008; López-Doriga et al., 2001; Rowley et al., 1998), likely due to several factors including both low viral load and presence of virus neutralizing antibodies in tissues (Graham et al., 2010). Even though viral RNA is present in tissues over a 9 month period indicating a carrier state, growth of SAV in cells detected by immunostaining was only successful in the acute phase (Graham et al., 2010). The present work is in conformity with these observations as we were only able to recover two out of eight field strains in any of the three cell lines tested, and it is tempting to speculate that defective viral genomes being abundantly present in tissue homogenates might explain

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difficulties related to primary virus isolation. Despite the confirmed presence of viral RNA by PCR in infected tissues prior to isolation in cell culture, we observed that a large number of these viral genomes were incomplete, being unable to sustain an infection by themselves. These defective viral variants will also be potent activators of the interferon-induced responses, and it is well known that alpha interferon as part of the immune response is a strong inhibitor of SAV3 replication in vitro (Xu et al., 2010). DIPs cannot sustain infection themselves but are efficiently replicated and encapsidated using proteins produced by wild-type viral genomes (García-Arizaga et al., 2004), which was also the case for SAV. When SAV was successfully recovered in cell culture, defective viral genomes were maintained after six passages in CHH-1 cells.

It has been speculated that only minor populations of viral quasispecies present in clinical SAV samples have possibilities of growth in cell cultures (Weston et al., 2002), and a serine to proline substitution at position 206 in the E2 protein has been associated with the appearance of CPE in CHSE-214 cells (Karlsen et al., 2006). It was also suggested to affect in vivo virulence although this has not been documented experimentally. In contrast, Fringuelli et al. (2008) suggested that other factors were responsible for in vivo virulence after finding proline in all E2 sequences generated. Our study confirmed that proline at position 206 in the E2 protein is not a prerequisite for viral propagation in cell culture as the SAV3-2-MR/10 isolate successfully recovered in cells revealed serine at position 206 both prior to and after serial passage in cells.

Our data suggest that recombination events occur frequently during replication of salmonid alphavirus, leading to viral mutants containing deletions and insertions. Recombination appears more frequently in positive-sense ssRNA viruses, than in negative sense ssRNA viruses (Simon-Loriere & Holmes, 2011), and is well known to occur in alphaviruses (Schlesinger & Weiss, 1994). Evidence for homologous recombination in alphaviruses was confirmed in sequence analysis of Western equine encephalitis virus (WEEV) that appears to have arisen by homologous double crossovers during natural infection (Simon-Loriere & Holmes, 2011), and is well known to occur in positive ssRNA viruses (Schlesinger & Weiss, 1994). The geographical distribution of the sites included in this study is shown in Fig. 1. The farms were chosen based on prior PD diagnosis. From each site, 10–20 fish were killed by a sharp blow to the head prior to necropsy. Heart specimens were collected and parallel samples preserved on RNAAlater (Invitrogen) and transport medium containing Leibovitz’s L-15 (Invitrogen) supplemented with 100 μg gentamicin ml⁻¹ (Sigma Aldrich). Parallel samples from heart, pancreas and skeletal muscle were submerged in 10% phosphate-buffered formalin and processed for paraffin-embedding and sectioning according to standard methods. Tissue specimens were stained with haematoxylin and eosin using standard methods. From one of the sites however, only samples preserved in RNAAlater were available.

**METHODS**

**Sampling and background information.** Farmed Atlantic salmon (Salmo salar L.) were sampled during the 2009–2010 grow-out season from nine different seawater sites spread along the Norwegian coastline. The geographical distribution of the sites included in this study is shown in Fig. 1. The farms were chosen based on prior PD diagnosis. From each site, 10–20 fish were killed by a sharp blow to the head prior to necropsy. Heart specimens were collected and parallel samples preserved on RNAAlater (Invitrogen) and transport medium containing Leibovitz’s L-15 (Invitrogen) supplemented with 100 μg gentamicin ml⁻¹ (Sigma Aldrich). Parallel samples from heart, pancreas and skeletal muscle were submerged in 10% phosphate-buffered formalin and processed for paraffin-embedding and sectioning according to standard methods. Tissue specimens were stained with haematoxylin and eosin using standard methods. From one of the sites however, only samples preserved in RNAAlater were available.

**RNA isolation.** Total RNA was extracted from heart tissue of individual fish using the RNeasy Fibrous tissue mini kit (Qiagen), according to the kit protocol. The tissue was homogenized in RLT buffer with β-mercaptoethanol using steel beads in a mixer mill MM301 (Retsch) for 2 min at 20 Hz. RNA was quantified using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). From cell-culture supernatants RNA was purified using QIAamp Viral RNA Mini kit (Qiagen) including optional on-column DNase digestion according to the manufacturer’s specifications.

**cDNA synthesis and real-time PCR.** cDNA synthesis was performed using the Transcriptor High Fidelity cDNA Synthesis kit (Roche Applied Science), which includes proofreading activity. In general, 1–4 μg RNA was denatured at 65 °C for 10 min, and cDNA synthesis was carried out at 50 °C for 30 min using a combination of random hexamers and oligo (dT) primers. Initially, all fish were screened for presence of SAV by real-time PCR analysis using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) in a LightCycler 480 Real-time PCR system (Roche Applied Science). In short, 2 μl undiluted cDNA was used as template in a 20 μl reaction with primers SAV forward (5’-CAGTGGAAATTCGATAAGAAGTGCAA) and SAV reverse (5’-TGGAGGATCCTGCGTTAAGG). The primer annealing temperature was 60 °C and the real-time PCR was run for 40 cycles according to the manufacturer’s recommendations.

**Amplification of viral genome, cloning and sequencing.** PCR amplification of the full viral genome was performed using Advantage 2 Polymerase Mix (Clontech). For practical reasons amplification of the entire genome was performed in six fragments, each overlapping reports for the first time that numerous viral deletion mutants, in all parts of the genome, are generated during natural infections with SAV. We suggest recombination as an explanation for generation of these virus variants. Defective viruses, missing parts of their genome and present in the infected tissues, provide a possible explanation for difficulties regarding primary virus isolation. Novel DNA sequencing techniques providing in-depth viral genome sequence data should shed light on the variety of mutants present in SAV populations and the dynamics of these during infection processes. Future work should address whether the defective viral genomes behave as DIPs or are merely a by-product of replication, and explore their role in primary virus isolation and in sustaining persistent infections of SAV.
the next fragment by approximately 150 nt. The primer sets were designed using Vector NTI advance 11.0 software (Invitrogen) and defined products between 2000 and 2300 base pairs. Primer sequences are shown in Table S1 (available in JGV Online). PCR was performed using 2 μl undiluted cDNA as template at standard conditions: 95 °C for 1 min, 30 cycles at 95 °C for 10 s, 60–68 °C for 1 min and final extension at 68 °C for 3 min. The resulting products were visualized in a 1 % agarose gel with SYBR Safe staining (Invitrogen). The products were excised from the gel and purified with Qiagen gel extraction kit (Qiagen). PCR products were ligated into the pCR 2.1 vector using TOPO TA cloning kit (Invitrogen) before subsequently being transformed into competent OneShot TOP10 bacterial cells (Invitrogen). The insert of purified plasmids of a minimum of three clones from each fragment was sequenced using commercial services (Eurofins MWG operon or GATC) using both standard vector primers and internal forward and reverse primers designed for each fragment.

**Virus propagation in cell culture.** From each site heart tissue samples with the highest virus load shown by real-time PCR were selected for cell-culture propagation. Heart tissues were homogenized in Leibovitz’s L-15 medium (Invitrogen) supplemented with 50 μg gentamicin ml−1 (Sigma Aldrich), 1/10 (w/v). After centrifugation, supernatant was further diluted 1/10 in Leibovitz’s L-15 medium containing 2 % FBS (Sigma Aldrich) and 50 ng ml−1 gentamicin before being inoculated on Chinook salmon embryo cells (CHSE-214). Extensive optimizations with various combinations of amount inoculate and culture plate formats were performed. Two other cell lines were also inoculated in parallel: Asian Grouper Kidney (AGK) and Chum salmon heart (CHH-1) cells. All cells were grown at 15 °C up to 21 days post-inoculation, frozen and thawed, and cell-culture supernatant was harvested after centrifugation. Supernatants were passaged 2-6 times in all cell lines. Supernatant from non-CPE cultures was also analysed by real-time PCR for SAV infection after 10–14 days. Cultures showing CPE were tested for both IPNV and SAV. IPNV testing was performed using the same real-time PCR method as described earlier, with forward primer GACTGGAG-CTGCTGTGGT and reverse CCGAATCTCGGCACATGGTGT.

**Serial passage of isolate SAV3-2-MR/10.** Isolate SAV3-2-MR/10 was serial passed in CHH-1 cell line in two parallel experiments (Table 3). In experiment 1, cells in a 24-well plate were initially inoculated with 250 μl of an undiluted homogenate per well in the first passage, 250 μl supernatant transferred to new wells in the second and then 100 μl supernatant was used as inoculum from the third to the sixth passage. In experiment 2, 5 μl inoculum was used per well in all passages. Cell-culture supernatant was harvested after 7–9 days when total cell lysis had occurred. SAV3 genome sequence analysis was performed on the supernatants after each passage as described earlier. The analysis was limited to fragment 6 covering 2.3 kb at the 3′ end of the SAV3 genome.

**Bioinformatics.** Consensus full-length sequences for the nine virus strains, all from different sites, were aligned in Contig Express of Vector NTI advance 11.0 software. The consensus sequences were generated based on sequence information from a minimum of three clones per fragment and consensus was determined on the basis of simple majority. The full-length nucleotide sequences from the nine fish farms were imported into the MEGA5 software for further analysis. In addition four previously published full-length SAV3 sequences were downloaded from GenBank. The sequences were aligned using the MUSCLE (Edgar, 2004) algorithm within MEGA5. To find the optimal substitution model the statistical program R (R Development Core Team, 2010) was used together with the ‘ape’ library (Paradis et al., 2004). In addition, the PhyML program (Guindon et al., 2009) was run in R to carry out maximum-likelihood estimation. The model that obtained the lowest AIC (Akaike Information Criterion) was chosen. According to the AIC a maximum-likelihood phylogenetic tree with 1000 bootstrap replications was constructed using Hasegawa–Kishino–Yano model with invariant sites. Bootstrap values of 60 and above was included in the output. The tree was constructed from 11756 nt from nsP1 to E1.

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**REFERENCES**


