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New subtype of salmonid alphavirus (SAV), *Togaviridae*, from Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* in Norway

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ABSTRACT: In Europe, 2 closely related alphaviruses (*Togaviridae*) are regarded as the causative agents of sleeping disease (SD) and salmon pancreas disease (SPD): SD virus (SDV) has been isolated from rainbow trout *Oncorhynchus mykiss* in France and the UK, while SPD virus (SPDV) has been isolated from salmon *Salmo salar* in Ireland and the UK. Farmed salmonids in western Norway also suffer from a disease called pancreas disease (PD), and this disease is also believed to be caused by an alphavirus. However, this virus has not yet been characterised at the molecular level. We have cultured a Norwegian salmonid alphavirus from moribund fishes diagnosed with cardiac myopathy syndrome (CMS) and fishes diagnosed with PD. The virus has also been found in salmon suffering from haemorrhagic smolt syndrome in the fresh water phase. The genomic organisation of the Norwegian salmonid alphavirus is identical to that in SPDV and SDV, and the nucleotide sequence similarity to the other 2 alphaviruses is 91.6 and 92.9%, respectively. Based on the pathological changes, host species and the nucleotide sequence, we suggest naming this virus Norwegian salmonid alphavirus (NSAV). Together with SPDV and SDV it constitutes a third subtype of salmonid alphavirus (SAV) species within the genus Alphavirus, family *Togaviridae*.

KEY WORDS: Norwegian salmonid alphavirus · Virus characterisation · Pancreas disease

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INTRODUCTION

In Europe, 2 closely related alphaviruses (*Togaviridae*) have been described from farmed salmonids. The salmon pancreas disease virus is the causative agent of pancreas disease in farmed Atlantic salmon *Salmo salar* in Ireland and Scotland (Nelson et al. 1995, McLoughlin et al. 1996, Rowley et al. 1998, Welsh et al. 2000, Weston et al. 1999) and sleeping disease virus the aetiological agent of sleeping disease in France and the UK (Boucher et al. 1995, Castric et al. 1997, Branson 2002, Villoing et al. 2000, Graham et al. 2003). In North America, pancreas disease was first described in 1987 (Kent & Elston 1987). In Canada in 1996, a togavirus-like agent was observed together with the infectious salmon anemia virus in association with a disease called haemorrhagic kidney syndrome (HKS) (Kibenge et al. 2000). However, this togavirus-like agent has not been identified and characterised.

Farmed salmonids (*Salmo salar* and *Oncorhynchus mykiss*) in Norway also suffer from a disease called pancreas disease, that seems to be caused by an alphavirus (Christie et al. 1998 and unpubl. data, Weston et al. 2002, 2003), but this virus has not yet been properly characterised at the molecular level. Affected fish often show an impaired swimming performance and loss of appetite. They tend to congregate at the surface in the cage corners, unable to maintain a normal position. Disease outbreaks have only been observed in sea water and usually 5 to 7 mo after the smolts have been transferred to the sea. Histopathological findings may include total or severe degeneration of exocrine pancreas and always various degrees of myopathy of skeletal and heart muscles (A. Nylund

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pers. obs., Poppe & Rimstad 1989). *Alphavirus* spp. have also been found, using RT-PCR and sequencing, in moribund farmed salmon in Norway diagnosed with cardiac myopathy syndrome, CMS (A. Nylund pers. obs.) and in salmon suffering from haemorrhagic smolt syndrome (HSS) in the fresh water phase (Nylund et al. 2003).

The majority of cases in which *Alphavirus* spp. have been detected associated with disease in salmon and rainbow trout have been in Hordaland and Sogn og fjordane (western Norway), but recently a few cases have been seen in northern Norway (Nordland, Troms and Finnmark) (A. Nylund pers. obs.). The genomes of the viruses from northern Norway have been partially sequenced and are identical to those from western Norway. This means that *Alphavirus* spp. have been found in the majority of Norwegian counties with salmon farming.

The complete genomes of SPDV from Ireland (Isolate F93-125) and SDV from France (Isolate S49P) have been sequenced and consist of about 12 kb (Weston et al. 2002). No other isolates have been completely sequenced, but partial sequencing of selected nonstructural and structural protein genes indicate that SPDV and SDV from the UK are identical to those sequenced from Ireland and France, respectively (Graham et al. 2003, Weston et al. 2003). Included in the studies by Weston et al. (2002, 2003) are also 2 Norwegian alphavirus isolates, PD97-N2 and PD97-N3 (supplied by K. E. Christie, Intervet, Bergen), originating from diseased salmon and rainbow trout, respectively. Both isolates were partially sequenced by Weston et al. (2003) from a virus harvested from Chinook salmon embryo CHSE-214 cell cultures, and the sequences were found to belong to the SPDV Alphavirus type (Subtype 1). It will, however, be shown in this study that the PD97-N2 and PD97-N3 isolates are in fact a new, different and distinct Norwegian subtype of salmonid alphavirus.

The first complete genome of the Norwegian salmonid alphavirus subtype (NSAV) from 4 separate outbreaks of disease, 2 diagnosed with cardiac myopathy syndrome and 2 with pancreas disease, are presented herein. The sequences are compared with those of the SDV and the SPDV from France and Ireland. The phylogenetic relationship between Norwegian salmonid alphavirus, SPDV and SDV is discussed. It is suggested that the Norwegian isolates be given status as a separate subtype, Norwegian salmonid alphavirus (NSAV), within the salmonid species alphavirus (Weston et al. 2002).

MATERIALS AND METHODS

The Norwegian salmonid alphavirus (NSAV) was collected from salmon and rainbow trout at 4 different farms in western Norway (Table 1). The first isolate was collected in 1997 from rainbow trout with pancreas disease (PD) in Hordaland (Isolate PD97-N3, Weston et al. 2002, 2003, K. E. Christie unpubl. data) and cultured in CHSE-214 cells. We collected 2 isolates (SavH10/02 and SavH20/03) from Atlantic salmon *Salmo salar* diagnosed with cardiac myopathy syndrome (CMS) in 2002 and 2003 in Hordaland. The 4th isolate (SavSF21/03) was collected from salmon diagnosed with PD in 2003 in Sogn og Fjordane. Selected tissues (gills, heart, pancreas, kidney and somatic muscle) were frozen in liquid nitrogen for storage at –80°C.

Tissue homogenates of the original material from which the 2 isolates PD97-N2 and PD97-N3 were isolated, and later cell culture passages of the same 2 isolates (supplied by K. E. Christie), have also been included in the present study. The PD97-N2 isolate was collected from Atlantic salmon in Hordaland in 1997 (Christie et al. 1998). The tissue homogenates and the cell culture passages had been stored in liquid nitrogen. To establish if the Irish/UK SPDV subgroup was present in Norway or if there could have been laboratory contamination of the cell cultures with an isolate (F93-125) from Ireland, we screened Passages 5, 6 and 12 of Isolate PD97-N2 and Passages 4, 6 and 12 of Isolate PD97-N3 for the presence of salmonid alphavirus (SAV), and sequenced a 899 bp segment from the structural genes from all passages and the tissue homogenates.

Cell culture. The Norwegian salmonid alphavirus was first isolated, using CHSE-214 cells, from tissue homogenates of heart and kidney from salmon suffering from CMS (SavH10/02). The CHSE-214 cells were cultured in 15 cm^2 tissue culture flasks (Nunc) at 20°C in Eagles minimum essential medium (EMEM) (Sigma) supplemented with 10% foetal bovine serum (FBS) (10% v/v), L-glutamine (4 mM) and gentamicin (50 µg ml^{-1}). The cells were then subcultured for 7 to 10 d

Table 1. Salmonid alphaviruses (NSAV) included in this study. PD97-N3 and the 3 Sav isolates were collected from 4 different farms in western Norway. *S*: *Salmo salar*; *O*: *Oncorhynchus mykiss*

Virus	Year	Country	Locality	Host	Accession No.
PD97-N2	1997	Norway	Osterøy	*S*	
PD97-N3	1997	Norway	Osterøy	*O*	AY604237
SavH10/02	2002	Norway	Øygarden	*S*	AY604236
SavH20/03	2003	Norway	Sotra	*S*	AY604235
SavSF21/03	2003	Norway	Gulen	*S*	AY604238
F93-125	<1995	Ireland	West Ireland	*S*	AJ316244
S49P	?	France	Atlantic coast	*O*	AJ316246

until the tissue flasks were covered with a 60 to 80% confluent monolayer. Homogenates from SMAV-infected tissues were diluted 1:100 in phosphate buffered saline and incubated for 1 h at 15°C in cell culture flasks with the monolayer of CHSE-214 cells. The inoculum was then removed and replaced by supplemented EMEM as described above, but with 2% FBS. The cells were incubated for 4 to 8 wk or until a cytopathic effect (CPE) could be observed. The cultures were supplied with fresh media at intervals of 1 to 2 wk.

RNA extraction. RNA was extracted from infected cell cultures and tissues (heart and kidney) and reverse transcribed into cDNA as described by Devold et al. (2000).

RT-PCR. PCR products of the 4 Norwegian alphavirus isolates were obtained using primers directed against conserved areas of the SPDV (F93-125, Accession No. AJ316244) and SDV (S49P, Accession No. AJ316246) genome. The same primers were also used for sequencing.

The primers F4 (5′-AGC GAC TCC CAG ACG TTT ACG-3′) and R1 (CGG TTT ATC ACT GCT TCG TAC GA-3′) amplify an 899 bp fragment in the E2-6K-E1 junction. This primer set was used to obtain a fragment from tissue homogenates and from different cell culture passages of the 2 SAV isolates PD97-N2 and PD97-N3. The PCR amplifications were performed in a total volume of 50 µl using 2 µl of template cDNA, and the reaction mixture consisted of: 1× PCR buffer, 0.8 mM dNTP, 0.2 µM of the reverse and forward primer, 2 U of thermostable DNA polymerase (Qiagen), and dH_2O. The PCR conditions were as follows: after an initial 5 min denaturation step at 95°C, samples were taken through 35 amplification cycles, each consisting of a 30 s denaturation step at 94°C, a 30 s primer annealing step at 55°C, and a 1 min and 30 s extension step at 72°C. A prolonged extension step of 10 min at 72°C completed each reaction.

Real-time RT-PCR. Real-time RT-PCR assays were designed to separate the Norwegian salmonid alphaviruses and the SPDV isolate (F93-125). These assays determined whether the SPDV isolate was present in addition to the NSAV. They have also been used to screen for the presence of NSAV in fish diagnosed with CMS. The design of the primers and TaqMan TM probes was carried out using Primer Express Software (PE Applied Biosystems). The primer pair and probes specific for SPDV Subtype 1 were 5′-ACAGTGAAATTCGACAAGAAATGC-3′ (forward), 5′-TGGGAGTCGCTGGTGAAAGT-3′ (reverse) and FAM-5′-AGAGCGCTGACTCGGCAACCGT-3′ –MGB (probe), whereas the primer pair and probe specific for NSAV were 5′-CAGTGAAATTCGATAAGAAG-TGCAA-3′ (forward), 5′-TGGGAGTCGCTGGTAAA-GGT-3′ (reverse) and FAM-5′-AGCGCTGCCCAA-GCGACCG-3′ –MGB (probe). These 2 assays amplify a region in the E2 gene. All primers and probes were obtained from PE Applied Biosystems. The TaqMan assays were performed with 2 µl of cDNA, 900 µM of each primer, and 200 µM of probe in a total volume of 50 µl using the TaqMan Universal PCR Master Mix w/AmpErase® UNG (PE Applied Biosystems). Amplification and fluorescence detection were performed with the ABI Prism 7000 sequence detection system instrument as recommended by the manufacturer (PE Applied Biosystems).

Sequencing. The PCR products were purified using Qia-quick PCR purification columns (Qiagen) and then sequenced using the Big Dye terminator sequencing kit (Applied Biosystems). The complete genomes from all 4 salmonid alphaviruses were sequenced in both directions (SavH20/03–AY604235, SavH10/02–AY604236, PD97-N3–AY604237, SavSF21/03–AY604-238). Sequence data were assembled with the help of Vector NTI software (InforMax) and GenBank searches were performed using BLAST (2.0).

The sequences of the 4 NSAV isolates were aligned in Clustal X, in Vector NTI software, with full length sequences from SPDV (F93-125) and SDV (S49P) already available in the EMBL nucleotide database. To perform pairwise comparisons between the different SAV isolates, the multiple sequence alignment editor GeneDoc (available at www.psc.edu/biomed/genedoc) was used. In addition to software analysis of the sequences, polymorphic regions were manually aligned and compared.

Phylogeny. The evolutionary relationships between alphaviruses have been presented by Powers et al. (2001) using partial E1 envelope glycoprotein gene sequences and complete structural polyprotein sequences. We performed a study of the nonstructural polyprotein amino acid sequences of a selection of alphaviruses including the NSAV isolates, and obtained a similar phylogeny (data not presented). In the present study, a phylogenetic analysis of a selection of SAV isolates using the complete nucleotide genome sequence was performed: Accession Nos. AJ316244 (F93-125), AJ316246 (S49P), AY604235 (SavH20/03), AY604236 (SavH10/02), AY604237 (PD97-N3), AY604-238 (SavSF21/03). The alignment was manually adjusted using the sequence alignment editor GeneDoc (available at www.psc.edu/biomed/genedoc) and gaps were deleted before analysing. The analyses were performed using TREE-PUZZLE 5.0 (available at www.tree-puzzle.de). TREE-PUZZLE reconstructs phylogenetic trees from molecular data by maximum likelihood, and computes maximum likelihood distances and branch lengths. In this study, 1000 quartet puzzling (QP) steps were carried out. The QP tree search

estimates support values for each internal branch. Branches showing QP reliability from 90 to 100% can be considered very strongly supported. Branches with lower reliability (>70%) can, in principle, be trusted. Phylogenetic trees were drawn using TreeView (Page 1996).

RESULTS

The almost complete genome of 4 alphaviruses (Norwegian salmonid alphavirus) associated with disease in Norwegian farmed Atlantic salmon *Salmo salar* and Rainbow trout *Oncorhynchus mykiss* in sea water have been sequenced. The 4 NSAV sequences deposited in the GenBank lack 8 to 53 nucleotides (nt) at the 5′-end of the first open reading frame (ORF). Two isolates, SavH10/02 (Accession No. AY604236) and SavH20/03 (Accession No. AY604235), were obtained from salmon diagnosed with cardiac myopathy syndrome (CMS) by the Norwegian Veterinary Service; 1 (SavSF21/03, Accession No. AY604238) was associated with 'classical' pancreas disease (PD), and 1 was isolated from rainbow trout (PD97-N3, Accession No. AY604237). Of these isolates, 2 (PD97-N3 and SavH10/02) were sequenced after growing in cell culture, while the latter 2 (SavH20/03 and SavSF21/03) were sequenced using RNA extracted from host tissues (mainly heart). SavH10/02 and N3-1997 grew well in cell cultures (CHSE-214), but produced no prominent cytopathic effect (CPE).

The genome organisation of the 4 NSAV isolates is similar to that of the Irish salmon pancreas disease virus, SPDV (Isolate F93-125), and the French sleeping disease virus, SDV (Isolate S49P). However, comparisons over the complete genome between the Norwegian isolates (NSAV), SPDV (F93-125) and SDV (S49P) revealed large sequence differences (Tables 2 & 3). Comparing 11 742 nucleotides from NSAV with SPDV and SDV revealed 91.6 and 92.9% similarity, respectively.

The first ORF encoding the nonstructural proteins (nsP1, nsP2, nsP3 and nsP4), in the NSAV isolates is shorter than that of SPDV and SDV. The ORF of the 4 NSAV isolates has the same length (7761 nt). The major reason for the size difference is insert/deletions in nsP3 (Table 4). The sequence similarity over the nonstructural subgenomic part is higher than 99% between the NSAV isolates, while their similarity to SPDV and SDV is 90 and 92%, respectively. The nonstructural polyproteins of the 4 NSAV isolates are predicted to be 2587 amino acids. The amino acid (AA)

Table 2. Salmonid alphavirus (SAV). Percent nucleotide (nt) sequence similarities between the 3 subtypes in Europe, comparing the different ORFs (open reading frame of SavH10/02 isolate) on the genomic strand. SPDV: salmon pancreas disease virus; SDV: sleeping disease virus

Sub-type	Isolate	NSAV SavH10/02								
		nsP1[a]	nsP2	nsP3	nsP4	C	E3	E2	6K	E1
NSAV	N3-1997	99	99	99	100	99	99	99	99	100
NSAV	SavH20/03	100	99	99	99	100	100	99	100	100
NSAV	SavSF21/03	99	100	99	100	99	99	99	100	100
SPDV	F93-125	94	91	85	92	91	89	89	94	93
SDV	S49P	95	93	88	94	88	92	92	94	93
ORF/nt		1631[a]	2577	1674	1829	845	210	1316	206	1385

[a]A few nucleotides are missing at the beginning of the ORF

Table 3. Salmonid alphavirus (SAV). Percent amino acid (aa) sequence similarities between the 3 subtypes in Europe, comparing the different proteins

Sub-type	Isolate	NSAV SavH10/02								
		nsP1[a]	nsP2	nsP3	nsP4	C	E3	E2	6K	E1
NSAV	N3-1997	100	99	99	100	100	98	99	98	100
NSAV	SavH20/03	100	100	100	100	100	100	100	100	100
NSAV	SavSF21/03	100	100	99	100	100	98	99	100	100
SPDV	F93-125	95	96	88	97	95	94	95	97	98
SDV	S49P	97	97	90	98	88	95	94	95	96
No. of aa		543	859	558	609	281	71	438	68	461

[a]A few amino acids are missing at the beginning of the protein

Table 4. Salmonid alphaviruses (SAV). Location of gaps/insertions in complete genome in relation to complete genome of S49P (SDV), Accession No. AJ316246, which is used as reference

Subtype	Isolate	Insert	Insert	Gap	Gap	Gap	Gap	Gap	Gap
NSAV	SavH10/02	–	24 nt/nsP3	3 nt/nsP3	12 nt/nsP3	27 nt/nsP3	3 nt/C	3 nt/C	3 nt/E1
SPDV	F93-125	3 nt/nsP1	24 nt/nsP3	3 nt/nsP3	–	–	–	3 nt/C	3 nt/E1
SDV	Position	1662-1663	5373-5374	5595-5599	5695-5707	5715-5743	8177-8181	8486-8490	11723-11727

sequence similarity is higher than 99% between the NSAV isolates, while their similarity to SPDV and SDV is 95% and 96%, respectively.

The cleavage sites in the NSAV nonstructural polyproteins were deduced from amino acid sequence homology with the SPDV and the SDV. The size of nsP1, nsP2, nsP3 and nsP4 are 561, 859, 558 and 609 AA, respectively. The sequence similarities between the nonstructural proteins from the different salmonid alphaviruses are given in Tables 2 and 3. The nucleotide sequence similarity between the different nonstructural proteins of the 3 virus groups varies from 85 to 95%, with the largest difference between NSAV and SPDV in nsP3 and the highest similarity between NSAV and SDV in nsP1 (Table 2). The ORF of Proteins nsP2 and nsP4 has the same length in all 3 (NSAV, SPDV and SDV) of the salmonid alphaviruses. As in other alphaviruses the nsP4 protein contains the conserved motif GDD at Residues 466 to 468 present in all 3 salmonid alphavirus subtypes (SDV, SPDV and NSAV). The SPDV has longer ORFs than the other 2 SAV subtypes when comparing nsP1 and nsP3 (Table 4). The opal termination codon found in some alphaviruses between the nsP3 and nsP4 proteins (Strauss & Strauss 1994) is not found in the NSAV isolates, where the nsP3 ends with the codon GGG (gly). The TGA codon is replaced by a sense codon CAA (glutamine) followed by CGA (arginine), and hence 2 nucleotides are changed from the tetranucleotide TGAC.

The second ORF of the 4 NSAV isolates, encoding the structural proteins, is 3960 nt long including the stop codon (TAA). Hence, it is slightly shorter than the ORF of both the SPDV and SDV. The NSAV isolates show more than 99% nucleotide identity over their structural polyprotein region, while their similarity to SPDV and SDV is 91 and 92%, respectively. When the individual structural protein genes of the NSAV isolates are compared with SPDV and SDV, the nucleotide similarities vary from 88% for the capsid protein gene (SDV) to 94% for the 6K gene (SPDV and SDV) (Table 2). The nucleotide variation is fairly evenly distributed. However, 2 gene regions with relatively high nucleotide difference occur in the capsid gene and 1 region at the C terminus of the E1 protein. The similarity at the amino acid level between the NSAV isolates and SDV and SPDV vary from 88% for the capsid protein (SDV) to 98% for the E1 protein (SPDV) (Table 3). The structural polyproteins of the NSAV isolates are comprised of 1320 amino acids and show highest similarity to SPDV, with the exception of the E3 gene, which is most similar to E3 from the SDV isolate (Table 3). The ORF of Proteins E3, E2, and 6K is the same length in all 3 (SMAV, SPDV and SDV) of the salmonid alphaviruses. The ORFs of C and E1 from SDV are longer than that of NSAV and SPDV.

The first ORF of the NSAV isolates is followed by a non-translated region of 38 nt before the start of the second ORF encoding the structural proteins. This non-translated region has a nucleotide sequence identical to that of the SDV (S49P). One of the conserved nucleotide sequence elements (CSE) of alphaviruses, CSE3 (24 nt), is also present in this region of the NSAV isolates, and includes the last 12 nt from the end of the nsP4 sequence and the first 12 nt of the non-translated region. The CSE3 sequence of the NSAV isolates is identical to that found in S49P (Villoing et al. 2000) and differs by 1 nt from that of the SPDV isolate F93-125 (Weston et al. 2002).

The CSE2 of the NSAV isolates were identified by aligning nsP1 with the complete sequence of Sindbis virus (Accession No. NC_001547). The nucleotide and amino acid sequence similarities of nsP1 from the NSAV isolates compared to the SIN nsP1 are 49 and 41%, respectively. However, the nucleotide and amino acid sequence similarities of the CSE2 motif of the NSAV and SIN are 74 and 71%, respectively. CSE2 is a 48 nt sequence stretching from Positions 153 to 200 on the complete genome of SDV (Accession No. NC_003433) (Table 5). In the NSAV isolates, 2 hypothetical stem-loop structures are suggested for the CSE2 (Fig. 1), and were constructed using Mfold 3.1 (Zuker 2003).

The F4-R1 amplicons, from the tissue homogenate of Atlantic salmon with NSAV PD97-N2, were almost identical to the same gene from the other 4 NSAV isolates. However, in Cell Culture Passage 5, the Norwegian SAV, PD97-N2, was found to have been substituted by the Irish SPDV (F93-125). Use of the specific real time RT-PCR assay for Isolate F93-125 could not detect this isolate before Passage 5.

Table 5. Alphavirus. The '51-nucleotide' conserved sequence element (CSE2), showing linear nucleotide sequences of 4 isolates sequenced through this region. Upper row is CSE2 sequence from Sindbis virus, SIN (Niesters & Strauss 1990), the other 3 viruses are salmonid alphaviruses (NSAV, SDV and SPDV). Nucleotides that are similar to Sindbis virus CSE2 sequence are represented by dots. aa = amino acids. There are 2 deletions (–) in the salmonid alphaviruses compared to SIN. Nucleotide numbers refer to full length sequences of Sindbis virus (NC_001547) and SDV (AJ316246)

aa		Q	V	T	P	N	D	H	A	N	A	R	A	F	S	H	L	A	
nt (SIN)	155																	205	
SIN	CAG	CAG	GUC	ACU	CCA	AAU	GAC	CAU	GCU	AAU	GCC	AGA	GCA	UUU	UCG	CAU	CUG	GC	CAG
NSAV	..A	...	...	G–.	...	.C–	...	...	..C	GCC	...	...	..U	..C	..C	..C	U..	..	...
SDV	..A	.C.	...	G–.	...	.C–	...	...	..C	GCC	...	...	..U	..C	..C	..C	U..	..	...
SPDV	..A	U..	...	G–.	.U.	.C–	...	...	..C	GCC	...	...	..U	..C	..C	..C	U..	..	...
nt(SDV)		153																200	
aa	N	R	S		S	N	D	H	A	A	A	R	A	F	S	H	L	A	

Complete genome sequences of the 4 NSAV, the SPDV and SDV were used to compare the genetic relationships between these viruses (Fig. 2). The genetic distance tree shows that the salmonid alphaviruses constitute 3 different subtypes within this species.

DISCUSSION

This paper comprises the first presentation of the complete nucleotide sequence from a new subtype of salmonid alphavirus. This new subtype has, so far, only been found in Norwegian aquaculture of salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss*. Including this Norwegian salmonid alphavirus, the SAV group now consists of 3 distinctly different but related viruses: (1) the sleeping disease virus (SDV) isolated from rainbow trout in France and UK (Villoing et al. 2000, Graham et al. 2003); (2) the salmon pancreas disease virus (SPDV) isolated from salmon in Ireland and the UK (Weston et al. 1999, Welsh et al. 2000); (3) the Norwegian salmonid alphavirus (NSAV) isolated from salmon and rainbow trout in Norway (Christie et al. 1998, and present study). NSAV isolates cause heart and skeletal muscle myopathy and have been associated with diseases like pancreas disease (Christie et al. 1998), haemorrhagic smolt syndrome (Nylund et al. 2003), and cardiac myopathy syndrome (present study). The detection of NSAV from smolt with HSS was probably an accidental finding and not the causative agent of this disease (cf. Nylund et al. 2003).

Experimental challenges of salmon using NSAV isolates seem to result in pathological changes associated with PD (Poppe & Rimstad 1989, Christie et al. 1998, authors' pers. obs.) and CMS in western Norway (Ferguson et al. 1990, Nylund 2001). Because of a lack of sensitive and specific diagnostic tools, these 2 diseases have been confused in salmon aquaculture in western Norway. However, NSAV isolates are not commonly found in salmon aquaculture north of western Norway, except for a few documented cases in which the virus

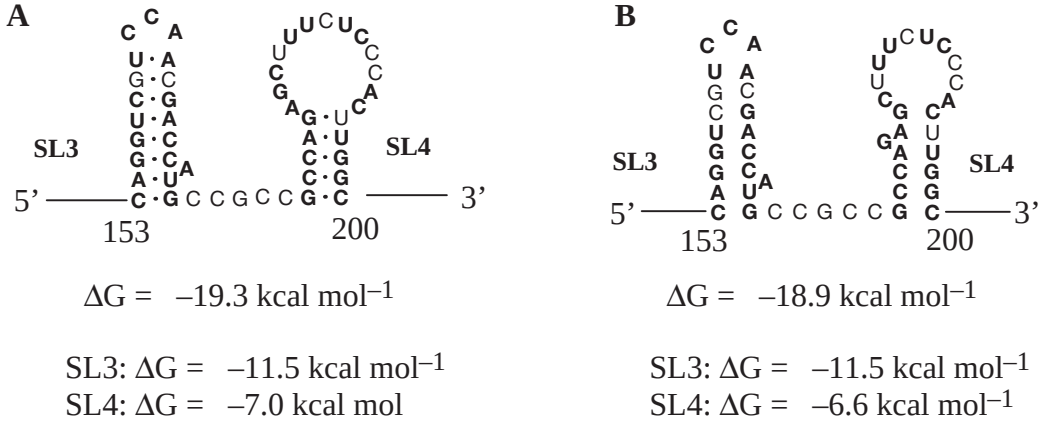

Fig. 1. Norwegian salmonid alphavirus (NSAV), showing 2 possible hypothetical stem-loop structures of conserved sequence element CSE2. ΔG values calculated at 15°C. Nucleotides that seem to be conserved between NSAV isolates and Sindbis virus are in boldface; numbers refer to complete genome of sleeping disease virus (Accession No. AJ316246). Stem-loop structures (SL3 and SL4) after Frolov et al. (2001)

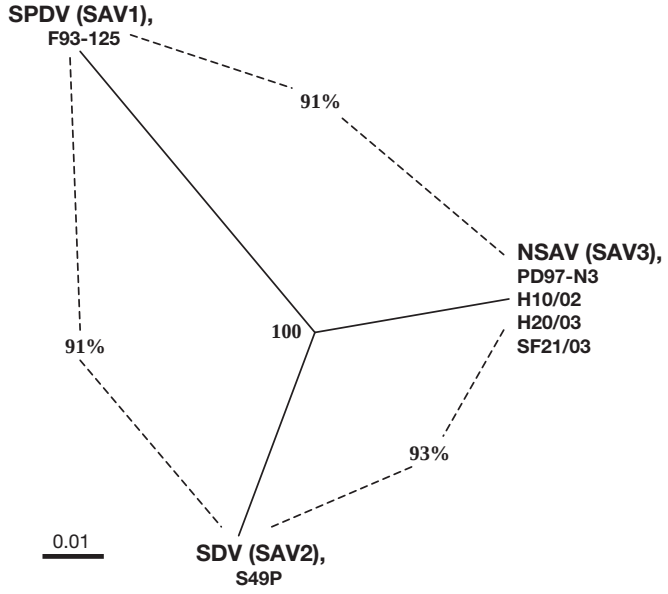

Fig. 2. Salmonid alphaviruses (SAV). Genetic distance of the SAV subtypes in relation to each other. Evolutionary relationship based on alignment of complete genome (11720 nucleotides) of 6 SAV isolates including all 3 subtypes (SAV1, SAV2 and SAV3). Scale bar: number of nucleotide substitutions as a proportion of branch length. Percent nucleotide similarity between the subtypes is shown. SPDV: salmon pancreas disease virus; SDV: sleeping disease virus

was most likely introduced to northern Norway via transport of infected smolts (A. Nylund pers. obs.). There have also been cases of CMS in western Norway, where we have been unable to detect NSAV using the 2 real-time PCR assays. These assays are very sensitive and can detect a carrier status in infected salmon. Hence, the reports of NSAV in salmon suffering from CMS in western Norway could be accidental findings, or merely reflect the lack of satisfactory official diagnostic methods for separating pancreas disease from CMS in Norway. Except for a few cases in which NSAV was present in fishes diagnosed with CMS, CMS may be considered as a separate disease not caused by the NSAV.

Analysis of the genomic sequences from the 4 NSAV isolates shows that the NSAV possesses a genome organisation identical to that observed for the other 2 SAV isolates, SPDV and SDV (Weston et al. 2002), and for mammalian alphaviruses (Strauss & Strauss 1994). NSAV, SPDV and SDV are also very similar at both the nucleotide level and the amino acid sequence level. The nucleotide sequence identity of the 3 viruses is above 90% over the complete genome and the amino acid identity about 95%. The NSAV isolates are more similar to the SDV (93%) than the SPDV (91%). The similarity to the mammalian alphaviruses is much lower, and a phylogenetic analysis based on the amino acid sequence of the structural polyproteins show that the SAV isolates constitute a distinct species (salmonid alphavirus, SAV) within the genus Alphavirus of the family *Togaviridae* (Powers et al. 2001, cf. Weston et al. 2002).

Most alphaviruses contain an opal codon (UGA) in the ORF between nsP3 and nsP4, and this is leaky when it is followed by a single C residue downstream, i.e. UGAC (Strauss & Strauss 1994). This opal codon is believed to regulate the production of nsP4 in infected cells, but it is not present in the SAV isolates. The conserved GDD motif characteristic of viral RNA polymerases (Strauss & Strauss 1994) is, on the other hand, present in the 3 subtypes of SAV.

Although the SAV isolates show no close relationship to any of the mammalian alphaviruses, they probably contain all the conserved sequence elements believed to play important roles in virus replication. Comparisons of Alphavirus genomes and mutagenesis of conserved RNA elements have identified a number of *cis*-acting sequences including (1) a 19 nt conserved sequence element (CSE4) which immediately precedes the 3′-terminal poly(A) tract, (2) a 24 nt sequence (CSE3) that spans the start site of the subgenomic RNA, (3) the 5′UTR with low overall homology between different alphaviruses but a conserved predicted secondary structure, and (4) the 51 nt (CSE2) believed to enhance RNA replication (Niesters & Strauss 1990, Strauss & Strauss 1994, Frolov et al. 2001). Since the sequences of the NSAV isolates are not complete at the 5′- and 3′-ends, we have only been able to locate what we believe are the CSE2 and CSE3. These CSE show a high sequence similarity to the mammalian alphaviruses.

It has been suggested, based on nucleotide sequences and reactivities of SPDV- and SDV-derived monoclonal antibodies with SPDV and SDV isolates, that NSAV, PD97-N2 and PD97-N3 are almost identical to the Irish SPDV isolate, F93-15 (Weston et al. 2002, 2003). As the present study shows, the tissue homogenates from which these 2 isolates (PD97-N2 and PD97-N3) were obtained contained only SAV isolates that are almost identical to the other Norwegian NSAV isolates. However, the PD97-N2 isolate is identical to SPDV (F93-125) in Passages 5 and 6 (material supplied by K. E. Christie). We believe this change could be a result of contamination by the Irish isolate (F93-125) that was in the laboratory at the time of isolation. Another possible explanation is that both isolates were present at the same time in the salmon and that 1 isolate became dominant after Passage 4. However, we have not been able to detect the F93-125 isolate in the tissue homogenates using a sensitive and specific real time RT-PCR assay. Nor has the F93-125 isolate or isolates similar to this been found in farmed salmonids from Norway. All cell culture passages (supplied by

K. E. Christie) of Isolate PD97-N3 were of the NSAV type. Since this isolate also emerged as nearly identical to the Irish SPDV (F93-125) in the studies of Weston et al. (2002, 2003), the change of isolate must have occurred at a later point.

The present study supports the classification of SPDV, SDV and NSAV as a new species, salmonid alphavirus (SAV), in the genus *Alphavirus* of the family *Togaviridae*, as suggested by Weston et al. (2002). This new species can further be divided into 3 distinct subtypes: (1) SPDV or SAV1 from salmon in Ireland and the UK (Weston et al. 2002); (2) the SDV or SAV2 from rainbow trout in France and the UK (Weston et al. 2002), and the Norwegian NSAV or SAV3 from salmon and rainbow trout in Norway (present study).

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