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Nucleotide sequence variation in salmonid alphaviruses from outbreaks of salmon pancreas disease and sleeping disease

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ABSTRACT: We compared 18 salmonid alphaviruses (SAV) including the reference F93-125 salmon pancreas disease virus (SPDV) and S49p sleeping disease virus (SDV) isolates by nucleotide sequence analyses of regions within the E1, nsP4 and nsP3 genes, and found these to comprise 3 distinct groups, which we have designated Subtypes 1, 2 and 3: Subtype 1, which comprised SAVs with sequences closely similar to the reference SPDV isolate, included SAVs from pancreas disease (PD) outbreaks in farmed salmon in Ireland and Scotland over a 10 yr period; viruses from recent outbreaks of sleeping disease (SD) in freshwater-reared trout farmed in England, Scotland and France were closely similar to and were grouped with the reference SDV isolate in Subtype 2; 3 viruses isolated from PD-affected salmon in Norway were genetically different from viruses belonging to Subtypes 1 and 2 and have been assigned to Subtype 3; 1 virus isolated from PD-affected salmon in the Western Isles, Scotland, in 2003 showed consistent nucleotide sequence differences from SAV Subtypes 1, 2 and 3, but was more closely related to the Subtype 1 SAVs. The occurrence of the different subtype SAVs appeared to have a geographical basis, which may prove useful in future molecular epidemiology studies of SAV-induced disease outbreaks.

KEY WORDS: Salmon pancreas disease · Sleeping disease · Salmonid alphavirus · SAV · Subtypes 1, 2 and 3

INTRODUCTION

Outbreaks of sleeping disease (SD) in freshwater-reared rainbow trout have been described in Northern France, England and Scotland, whilst outbreaks of pancreas disease (PD) in farmed Atlantic salmon have been described throughout Western Europe and North America (Kent & Elston 1987, Poppe et al. 1989, Boucher et al. 1994, Castric et al. 1997, Christie et al. 1998, Rowley et al. 1998, Graham et al. 2003b). Both diseases are associated with similar histopathological lesions of the pancreas, heart and muscle of their respective species (Boucher & Baudin-Laurencin 1996, McLoughlin et al. 1996, Weston et al. 2002). The causal agents of PD, salmon pancreas disease virus (SPDV) and sleeping disease virus (SDV), have been identified as atypical alphaviruses, the first alphaviruses to be reported in fishes (Weston et al. 1999, Villoing et al. 2000).

Alphaviruses have positive sense RNA genomes of 10 to 12 kb. The 4 nonstructural proteins (nsP1 to nsP4) necessary for virus replication are encoded by the 5′ two-thirds of the genome with the virus structural proteins being encoded from the 3′-terminal one-third of the genome (Strauss & Strauss 1994). SPDV and SDV resemble mammalian alphaviruses in regard to genome organisation and the possession of nucleotide sequence elements and amino acid motifs. However, the levels of nucleotide and amino acid sequence identities shared with previously described alphaviruses

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are relatively low (Weston et al. 2002). In contrast, the reference SPDV isolate F93-125 (Nelson et al. 1995) and reference SDV isolate S49p (Castric et al. 1997) showed 91.1% nucleotide sequence identity along their 11919 nucleotide (nt) and 11900 nt genomes respectively (Weston et al. 2002). In a comparative study of alphavirus sequences, Powers et al. (2001) suggested that, on the basis of this limited nucleotide sequence divergence, the 2 reference SPDV and SDV isolates constitute 2 subtypes of a new alphavirus species, for which the name *Salmonid alphavirus* (SAV) was proposed (Weston et al. 2002).

To date, sequence data have been reported for the 2 reference isolates only. The objective of this study was to gain further insight into the molecular diversity exhibited by SAVs with the view to applying our findings epidemiologically. In this paper we compare partial nucleotide sequences of 16 additional SAVs originating in France, Ireland, Norway and the United Kingdom. Our results support the occurrence of 3 genetically different SAV groups, which we have called Subtypes 1, 2 and 3.

MATERIALS AND METHODS

Viruses. The SAVs compared by nucleotide sequencing in this study mainly comprised viruses present in viraemic serum samples that were submitted to the Veterinary Sciences Division for serological diagnosis, and cell culture isolates (Table 1). In the case of 2 SAVs (F02-67, F02-85), viruses were present in tissue homogenates obtained from clinical outbreaks of SD. The reference F93-125 SPDV isolate, which was isolated in our laboratory from an outbreak of PD in Ireland (Nelson et al. 1995), was used for control purposes in RT-PCR reactions. This isolate and additional cell culture isolates produced from clinical material from PD-affected salmon or SD-affected trout by the Veterinary Sciences Division, Belfast (Virus F02-64), AFSSA, Brest, France (Viruses S49p and VF03; kindly supplied by Dr. J. Castric, AFSSA), and Intervet Norbio, Bergen, Norway (Virus N98-05 and N03-08; kindly supplied by K. Christie, Intervet, Bergen, Norway), were grown in Chinook salmon embryo (CHSE-214) cells as described by Nelson et al. (1995). Viraemic sera were identified using the method of Jewhurst et al. (2004). Tissue homogenates of heart, kidney and spleen were prepared as described by Nelson et al. (1995) from SD-affected trout in Scotland (Virus F02-85) and England (Virus F02-67) following outbreaks during 2002. Information relating to host species, source and country of origin is summarised in Table 1.

RNA extraction. RNA was extracted from SAV-infected CHSE-214 cells, from viraemic serum samples and from homogenised pools of kidney, spleen and heart using the Qiagen RNeasy Kit (Qiagen) according to the manufacturer's instructions.

Reverse-transcription polymerase chain reaction. In the case of 3 SAVs, F02-64, F02-67 and F02-85, amplicons of 426, 484 and 515 bp were produced for the nsP3, nsP4 and E1 gene regions respectively. The nsP3 primer pair 5′-AGACCGCCTTCTAGCCTAC-3′ (forward), 5′-AACGGATCCCGCTGATGA-3′ (reverse)

Table 1. Salmonid alphaviruses investigated and sequence accession numbers. Values in parentheses are sample numbers of serum samples received and are not referred to hereafter. CHSE: Chinook salmon embryo. S: Atlantic salmon; T: rainbow trout

Virus	Year and country of origin	Source	Fish type	Accession No.		
				nsP4	E1	nsP3
F02- 143 (6)	2002 Ireland	Serum	S	AJ811575	AJ811947	AJ812286
F02-73 (8)	2002 Ireland	Serum	S	AJ811576	AJ811948	AJ812287
F02-148 (7)	2002 Ireland	Serum	S	AJ811577	AJ811949	AJ812288
F02-153 (107)	2002 Ireland	Serum	S	AJ811578	AJ811950	AJ812289
F02-194 (11)	2002 Ireland	Serum	S	AJ811579	AJ811951	AJ812290
F03-136 (20)	2003 Ireland	Serum	S	AJ811580	AJ811952	AJ812291
F02-64	2002 Scotland	CHSE-214 cells	S	AJ583019	AJ583020	AJ583019
F93-125	1993 Ireland	CHSE-214 cells	S	AJ316244	AJ316244	AJ316244
N98-05	1999 Norway	CHSE-214 cells	S	AJ811582	AJ811954	AJ812293
N03-08	2003 Norway	CHSE-214 cells	S	AJ811583	AJ811955	AJ812294
F03-195	2003 Norway	Serum	S	AJ811584	AJ811956	AJ812295
F03-91 (2)	2003 England	Serum	T	AJ811585	AJ811957	AJ812296
F04-19 (2)	2004 England	Serum	T	AJ811586	AJ811958	AJ812297
VF03	2002 France	CHSE-214 cells	T	AJ811587	AJ811959	AJ812298
F02-85	2002 Scotland	Tissue homogenate	T	AJ577781	AJ583022	AJ583021
F02-67	2002 England	Tissue homogenate	T	AJ582969	AJ582971	AJ582970
S49p	1995 France	CHSE-214 cells	T	AJ316246	AJ316246	AJ316246
F03-209 (3)	2003 Scotland	Serum	S	AJ811581	AJ811953	AJ812292

was designed to amplify a 426 bp cDNA product corresponding to the 5280 to 5706 nt region of the genome, the nsP4 primer pair, 5′-TGTAAGGACACCGACCTGTGGA-3′ (forward); 5′-TCAGCAGCATCAGCGCTGTAA-3′ (reverse), was designed to amplify a 484 bp cDNA product corresponding to the 6697 to 7180 nt region of the genome; the E1 primer pair, 5′-GAAGTGGTGACGGCAGTCCAC-3′ (forward), 5′-TCGCAGCTGTCCACCACGCAT-3′ (reverse), was designed to amplify a 515 bp cDNA product corresponding to the 10898 to 11413 nt region of the SAV genome. On the basis of the sequences of these 3 SAVs and those of the 2 reference SAVs (F93-125 and S49p), new primer pairs to conserved sequences were selected to amplify smaller amplicons that were encompassed by each of these 3 amplicons. Thus, the nsP3 primer pair, 5′-GCCATGACCCCGGAACGGGTC-3′ (forward), 5′-CGGAACGGATCCCGCTGATGA-3′ (reverse), was designed to amplify a 393 bp cDNA product corresponding to the 5146 to 5538 nt region of the SAV genome; the nsP4 primer pair, 5′-GTGATGCACAGCAGAGTCGAA-3′ (forward), 5′-TCGAATGAGGAGATGTCCGTT-3′ (reverse), was designed to amplify a 332 bp cDNA product corresponding to the 6802 to 7133 nt region of the SAV genome; the E1 primer pair, 5′-ATCGGCGAAGAGGTCTATAA-3′ (forward), 5′-CACAATCGAGGGCCAGGAGCG-3′ (reverse), was designed to amplify a 286 bp cDNA product corresponding to the 11020 to 11307 nt region of the SAV genome. The 393 bp (nsP3), 332 bp (nsP4) and 286 bp (E1) RT-PCR amplicons were produced for all SAVs except F02-64, F02-67, F02-85 and the 2 reference isolates.

First strand cDNA synthesis was performed on 2 µg of RNA using Superscript II RNase H^- reverse transcriptase (Invitrogen) using the reverse primers. Second strand synthesis and PCR was carried out using both forward and reverse primers using the 'Expand long template' PCR system (Roche) with cycle conditions of 94°C for 30 s, 60°C for 30 s and 68°C for 1 min for 35 cycles. The same cycle conditions were used for all 6 sets of primer pairs.

Cloning, sequencing and sequence analysis. cDNA fragments of 426 (nsP3), 484 (nsP4) and 515 bp (E1), which were produced with RNA extracted from the F02-64, F02-67 and F02-85 SAVs, were cloned into pCR2.1® TOPO (Invitrogen). cDNA fragments of 393 (nsP3), 332 (nsP4) and 286 bp (E1) were sequenced directly from RT-PCR products. Cycle sequencing was performed with the ABI BigDye™ terminator ready reaction kit on purified plasmid DNA following the manufacturer's protocol (Applied Biosystems). Electrophoresis was carried out using an ABI310 analyser, and electropherograms were interpreted using Vector NTI software (Informax, Invitrogen). With cloned cDNA fragments, sequencing reactions were performed using primers specific to the M13 forward and reverse sequences within the pCR2.1®TOPO plasmid vector. Sequencing reactions were also performed directly on RT-PCR products using the amplification primers specified above. Sequencing was carried out on 2 or more clones or RT-PCR products for each virus, with both strands being sequenced at least twice. Pairwise local alignments of derived sequences, which excluded the primer sequences, were performed using the Lalign programme (Myers & Miller 1988). Phylogenetic analysis was carried out using the Phylip package (Department of Genetics, University of Washington). Sequences were aligned using CLUSTAL and data sets were statistically analysed using the SEQBOOT algorithim, DNADIST, and NEIGHBOR. Phylogenetic trees were calculated using the CONSENSE algorithim and drawn using the NJ plot software (Thompson et al. 1997). The bootstrap probabilities of each node were calculated using 100 replicates. GenBank accession numbers for the sequences are in Table 1. The nucleotide sequences of the reference SDV S49p isolate, which was isolated from SD-affected trout in France in 1997 (Castric et al. 1997), and the reference SPDV F93-125 isolate were accessed from the GenBank database (S49p, AJ316246; F93-125, AJ316244).

RESULTS

Pairwise comparison of the 18 SAVs using percentage nucleotide identities determined following alignments for each of the 3 regions, showed that the greatest variation was in the nsP3 region (range 0 to 18.5%), followed by the nsP4 (range 0 to 10.0%) and E1 (range 0 to 7.3%) regions (Table 2). Based on the nucleotide identity differences observed in each of the 3 regions, our analysis indicated that 17 of the 18 SAVs could be divided into 3 groups, which we have called Subtypes 1, 2 and 3. Subtype 1 comprised 7 SAVs from PD-affected salmon farmed in Ireland (F02-73, F02-143, F02-148, F02-153, F02-194, F03-136) or Scotland (F02-64) in 2002 or 2003 in addition to the reference SPDV isolate, F93-125, which was obtained from diseased salmon farmed in Ireland in 1993. Subtype 2 comprised 6 SAVs from SD-affected freshwater trout farmed in Scotland (F02-67), England (F02-85, F03-91, F04-19) and France (VF03) in 2002 to 2004 and the reference SDV isolate, which was obtained in France in 1995. Subtype 3 comprised 3 cell culture isolates (N98-5, N03-8, F03-195) obtained from PD-affected salmon farmed in Norway in 1998 to 2003. The remaining virus, F03-209, which was obtained from PD-affected salmon reared in the Western Isles, Scotland, showed

Table 2. Summary of nucleotide sequence identity differences between subtypes of salmonid alphaviruses (SAVs) showing ranges of % nucleotide identity observed by comparing all SAVs within subtypes

Subtype compared	Gene fragment region	Subtype 1	Subtype 2	Subtype 3	F03-209
Subtype 1 (8 SAVs)	nsP3	99.4–100	81.5–81.8	85.8–86.3	92.6–92.9
	nsP4	99.0–100	89.7–91.4	90.0–91.4	94.5–95.5
	E1	98.8–100	94.7–95.9	92.7–93.5	96.7–97.6
Subtype 2 (6 SAVs)	nsP3	81.5–81.8	100	86.6	82.3
	nsP4	89.7–91.4	99.0–100	92.8–93.4	91.0–91.7
	E1	94.7–95.9	99.6–100	93.9–94.7	92.7–93.1
Subtype 3 (3 SAVs)	nsP3	85.8–86.3	86.6	99.7–100	87.5–87.7
	nsP4	90.0–91.4	92.8–93.4	100	93.4
	E1	92.7–93.5	93.9–94.7	99.6–100	91.4–91.8
F03–209 (1 SAV)	nsP3	92.6–92.9	82.3	87.5–87.7	100
	nsP4	94.5–95.5	91.0–91.7	93.4	100
	E1	96.7–97.6	92.7–93.1	91.4–91.8	100

consistent differences from SAVs of the other 3 subtypes, but was more closely related to Subtype 1 SAVs.

For each of the 3 regions investigated, the intra-subtype nucleotide identity variation was relatively small. For example, the 8 Subtype 1 SAVs displayed nucleotide identities of 99.4 to 100%, 99.0 to 100% and 98.8 to 100% for the nsP3, nsP4 and E1 regions respectively, while the 6 Subtype 2 SAVs displayed identities of 100% (nsP3), 99.0 to 100% (nsP4) and 99.6 to 100% (E1) (Table 2). In contrast the nucleotide identity differences between viruses belonging to different subtypes were relatively large (Tables 2 & 3). For example, sequence data obtained with the nsP3 region indicated that Subtype 1 SAVs displayed identities of between 81.5 and 81.8% with Subtype 2 SAVs, of between 85.8 and 86.3% with Subtype 3 SAVs and of between 92.6 and 92.9% with the Subtype 1 variant, F03-209 (Table 2). The most notable distinguishing feature between subtypes was found in the 3′-terminus of the nsP3 gene, where the 6 Subtype 2 SAVs were shown to contain a 24 nt deletion, which was not possessed by the other 12 SAVs investigated.

Smaller differences were observed when sequence data obtained with the nsP4 and E1 regions were compared (Tables 2 & 3). With minor exceptions, the variations among subtypes were usually consistent irrespective of the genome region used for comparison. One exception concerned the Subtype 1 SAVs, which differed most from the Subtype 2 SAVs using sequence data obtained with the nsP3 and nsP4 regions but, when sequence data obtained using the E1 region was considered, the Subtype 1 SAVs differed most from the Subtype 3 SAVs (Table 2). The SAVs belonging to Subtypes 2 and 3 displayed similar levels of variation compared to any of the other subtypes, irrespective of the genome region used for sequence comparison. The F03-209 SAV showed least variation compared to the Subtype 1 SAVs.

Table 3. Pairwise comparison of nucleotide identities exhibited by SAVs in amplified E1 and nsP4 gene fragments. Primer sequences were not included in comparisons. Above diagonal: % nucleotide identity over E1 region; below diagonal: % nucleotide identity over nsP4 region

Virus	F93-125	F02-64	F02-73	F02-143	F02-148	F02-153	F02-194	F03-136	S49p	F02-67	F02-85	F03-91	VF03	F04-19	N98-05	N03-08	F03-195	F03-209	
F93-125	–	99.0	99.0	99.0	99.0	99.0	99.0	99.0	95.1	94.7	94.7	94.7	95.1	94.7	93.1	92.7	93.1	97.1	
F02-64	100	–	100	100	100	100	100	100	95.1	94.7	94.7	94.7	95.1	94.7	93.1	92.7	93.1	97.1	
F02-73	99.6	99.6	–	100	99.6	99.2	99.6	99.6	95.5	95.1	95.1	95.1	95.5	95.1	93.5	93.1	93.5	97.6	
F02-143	99.6	99.6	100	–	99.6	99.2	99.6	99.6	95.5	95.1	95.1	95.1	95.5	95.1	93.5	93.1	93.5	97.6	**Subtype 1**
F02-148	99.2	99.2	100	100	–	99.6	99.2	100	95.9	95.5	95.5	95.5	95.9	95.5	93.1	92.7	93.1	97.1	
F02-153	98.8	98.8	100	100	100	–	98.8	99.6	95.5	95.1	95.1	95.1	95.5	95.1	92.7	93.1	92.7	96.7	
F02-194	99.2	99.2	100	100	100	100	–	99.2	95.1	94.7	94.7	94.7	95.1	94.7	93.1	92.7	93.1	97.1	
F03-136	99.2	99.2	100	100	100	100	100	–	95.9	95.5	95.5	95.5	95.9	95.5	93.1	92.7	93.1	97.1	
S49p	89.7	90.7	90.7	90.7	90.7	90.7	90.7	90.7	–	99.3	98.6	99.3	99.7	99.3	92.8	92.8	92.8	93.1	
F02-67	90.3	91.4	91.4	91.4	91.4	91.4	91.4	91.4	99.6	–	99.3	100	99.7	100	93.4	93.4	93.4	92.7	
F02-85	89.7	90.7	90.7	90.7	90.7	90.7	90.7	90.7	99.6	100	–	99.3	99.0	99.3	92.8	92.8	92.8	92.7	**Subtype 2**
F03-91	90.3	91.4	91.4	91.4	91.4	91.4	91.4	91.4	99.6	99.6	99.6	–	99.6	100	93.4	93.4	93.4	92.7	
VF03	90.0	91.0	91.0	91.0	91.0	91.0	91.0	91.0	100	99.6	99.6	99.7	–	99.7	93.1	93.1	93.1	93.1	
F04-19	90.3	91.4	91.4	91.4	91.4	91.4	91.4	91.4	99.6	99.6	99.6	100	99.6	–	93.4	93.4	93.4	92.7	
N98-5	91.4	92.4	92.4	92.4	92.4	92.4	92.4	92.4	94.7	94.3	94.3	94.3	94.7	94.3	–	99.6	100	91.8	
N03-08	91.4	92.4	92.4	92.4	92.4	92.4	92.4	92.4	94.3	94.3	94.3	93.9	94.3	93.9	100	–	99.6	91.4	**Subtype 3**
F03-195	91.4	92.4	92.4	92.4	92.4	92.4	92.4	92.4	94.7	94.3	94.3	94.3	94.7	94.3	100	100	–	91.8	
F03-209	94.5	95.5	95.5	95.5	95.5	95.5	95.5	95.5	91.0	91.7	91.0	91.7	91.4	91.7	93.4	93.4	93.4	–	

Phylogenetic analysis, based on sequence comparisons performed with the E1 region (Fig. 1a) placed the 18 SAVs in 3 groups that corresponded to Subtypes 1, 2 and 3 that had been identified using pairwise nucleotide identity comparisons (Table 2). Similar results were achieved with analyses based on nsP3 (not shown) and nsP4 (Fig. 1b) genome regions, although in the nsP4-based analysis, the Subtype 1 SAVs were not monophyletic. In the analyses based on E1 and nsP4 regions, the F03-209 SAV was positioned within or close to the SAVs belonging to Subtype 1. In the phylogenetic analyses, the Subtype 2 SAVs formed clusters (Fig. 1a,b), while the 3 Norwegian SAVs comprising Subtype 3 were closely related relative to the other SAVs.

DISCUSSION

Prior to this study, published comparative sequence data was only available for the reference SPDV and SDV isolates (Weston et al. 2002). The present study has extended this data to include 16 additional SAVs that differ with regard to salmonid species infected, country of origin and year of isolation. Our analyses, involving 3 different genomic regions, have shown that the 18 SAVs do not exhibit a spectrum of sequence diversity but, instead, can be assigned to 3 genetically different groups which, adopting the nomenclature suggested by Powers et al. (2001), we have called subtypes. As a consequence, we propose that the SAVs should not be labelled as 'SPDV' or 'SDV', which largely reflects the salmonid species affected, but rather that they should be grouped according to nucleotide sequence criteria, such that viruses with sequences closely related to the reference SPDV F93-125 isolate are grouped in Subtype 1 and viruses with sequences closely related to the reference SDV S49p isolate are grouped in Subtype 2. Nucleotide sequence analysis performed in this study and that performed in University of Bergen, Norway (A. Nylund, University of Bergen, Norway, pers. comm.) have indicated that SAVs from infected salmon in Norway are genetically distinct and should be assigned to Subtype 3. The remaining SAV (F03-209) investigated, which was detected in the Western Isles, Scotland, in 2003, differs at the nucleotide sequence level from viruses belonging to the other 3 SAV subtypes, but is more closely related to the Subtype 1 SAVs (Table 2). The future identification of additional SAVs with sequences similar to F03-209 may lead to such viruses being designated Subtype 1a.

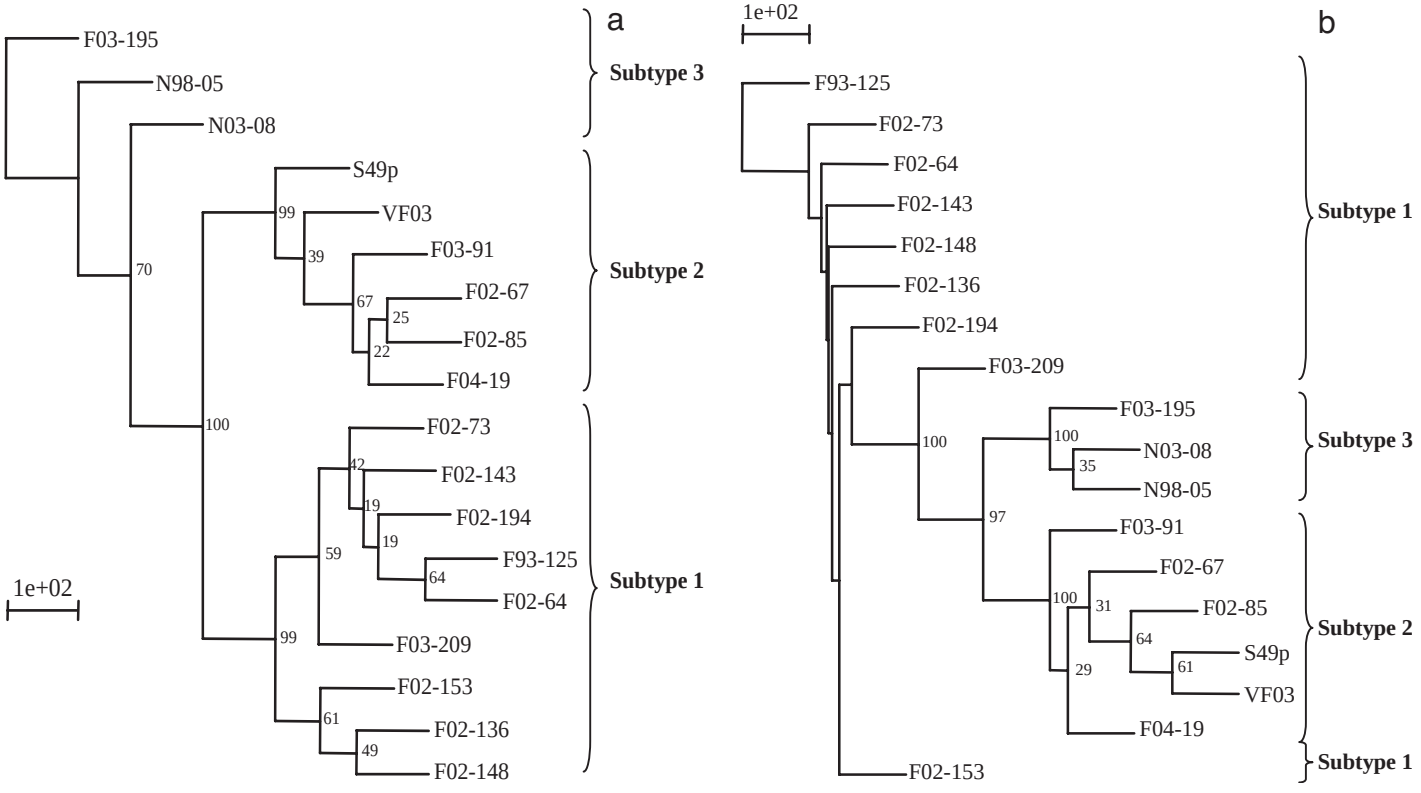

Fig. 1. Phylogenetic analyses of nucleotide sequences of salmonid alphaviruses based on sequence comparisons with (a) E1 245 bp and (b) nsP4 290 bp gene fragment regions. Percentage bootstrap support values for neighbour joining are given at each fork; branch lengths are informative and drawn to scale

The ability to differentiate subtypes using any of the 3 genomic regions suggested that the genetic differences between subtypes occur throughout the SAV genome. In this investigation the greatest sequence variation between subtypes was obtained using the 393 bp nsP3 region, which was selected for comparison purposes because it encompassed the 24 nucleotide deletion identified in the reference SDV isolate compared to the reference SPDV isolate. Our study showed that all 6 Subtype 2 SAVs possessed the same deletion. The 332 bp region within the nsP4 gene, which is the most conserved alphavirus gene, and the 286 bp region within the E1 glycoprotein gene, which encompasses the hydrophobic fusion domain that is conserved in alphaviruses, were selected on the basis of preliminary work that showed that these primer sequences were conserved when other SAV isolates were compared (authors' unpubl. results). Our results showed that the intra-subtype differences were very small in comparison to the differences between subtypes, and more extensive sequencing of additional SAVs will be required to determine if other genomic regions are better for differentiating individual SAVs from within the same subtype.

Of the 18 SAVs investigated in this study, 12 were obtained from outbreaks of PD in salmon and these included SAVs from Subtypes 1 and 3 as well as F03-209. Our results indicate that there is likely to be a geographical basis for the occurrence of these groupings. Thus, Subtype 1 SAVs occurred in Ireland and Scotland, Subtype 3 SAVs occurred in Norway, while the F03-209 (which showed consistent differences with SAVs belonging to Subtypes 1, 2 and 3) had a different geographic origin, namely the Western Isles, off the west coast of Scotland. Earlier work from this laboratory had shown that 2 Norwegian isolates, known as N2 and N3, belonged to Subtype 1 (Weston et al. 2003). However, the provenances of these 2 Norwegian isolates are now considered questionable and they have not been included in the present study. The 3 Norwegian SAVs (N98-05, N03-08, F03-193) investigated in this study are considered to be representative of Norwegian SAVs in general (S. Villoing, Intervet, Bergen, Norway, pers. comm.), and our results with these are consistent with unpublished results obtained by researchers in Norway who have shown that all Norwegian SAVs investigated to date are closely related at the nucleotide sequence level and differ from the reference SPDV and SDV isolates (A. Nylund, University of Bergen, Norway, pers. comm.). The occurrence of the Subtype 2 SAVs may also have a geographical basis, but, since these SAVs were all detected in SD-affected freshwater trout, a host–species influence cannot be ruled out. Of the 6 Subtype 2 SAVs, 2 (S49p and VF03) were obtained from Brittany, France, while the remaining 4 included viruses detected in the first and subsequent outbreaks of SD in England and Scotland (Graham et al. 2003b). Since it is considered possible that the SAVs responsible for first UK SD outbreaks may have been introduced with trout imported from France, the 6 SAVs investigated in our study may have closely related geographic origins. Analysis of additional viruses with different geographic origins will be required to determine the molecular diversity of SAVs that infect freshwater-reared rainbow trout. Recent unpublished work has shown that an SAV isolated from an outbreak of PD in seawater rainbow trout, which were farmed in Norway in proximity to PD-affected salmon, belonged to Subtype 3 (K. Christie, Intervet, Bergen, Norway, pers. comm.). Since Norwegian salmon are infected by Subtype 3 SAVs, this finding suggests that transfer of infection between salmonid species can occur naturally as well as experimentally, as described by Weston et al. (2002). Although most of the viruses compared in this study were detected in recent years (2002 to 2003), results obtained with the Subtype 1 SAVs indicate that viruses infecting salmon farmed on the west coast of Ireland over a 10 yr period were very closely related and thus suggest that there has been little genetic change over a 10 yr period. Similarly, Subtype 2 viruses (S49p and VF03), detected 8 yr apart, were also found to be very closely related.

The extent of the biological differences that may exist between SAVs belonging to the different subtypes remains unknown. On the basis that the 12 SAVs from salmon investigated in this study were obtained from salmon farms that were experiencing clinically and histologically-diagnosed PD, there are grounds for believing that SAVs belonging to Subtypes 1 and 3 and the F03-209 SAV are similar in terms of their disease-causing abilities. Preliminary experimental infections of Atlantic salmon have shown that SAVs belonging to Subtypes 1 and 3 produce very similar histopathological effects (K. Christie, Intervet, Bergen, Norway, pers. comm.), but more detailed pathogenesis studies are required to determine whether minor differences exist between representative viruses. It has been known for some time that the histopathologies associated with PD and SD in their respective species are very similar, and this was confirmed in cross-infection experiments in which salmon and trout were each separately infected with the reference F93-125 SPDV Subtype 1 isolate and the reference S49p SDV Subtype 2 isolate (Weston et al. 2002).

Earlier comparative work also showed that SAVs belonging to Subtypes 1 and 2 displayed variation in terms of their reactivity with monoclonal antibodies (MAb) (Weston et al. 2002, Jewhurst et al. 2004). Thus, although 7 MAbs that had been raised to the reference

F93-125 SPDV (Subtype 1) isolate were found to react with examples of Subtype 1 and 2 SAVs, 2 of 3 MAbs that had been raised to the reference S49p SDV isolate were found to be unreactive with the Subtype 1 SAVs investigated (Jewhurst et al. 2004). A larger panel of MAbs and a more diverse range of SAV isolates are currently being assembled to investigate the antigenic diversity of SAV. Monoclonal antibodies that react with all SAVs will be of particular use as diagnostic reagents. Although SAVs exhibit some antigenic diversity, detection using a modified virus neutralisation test of SAV-specific antibody in serum samples from salmon and trout farmed in Ireland, France, Norway and the UK indicates that the SAVs encountered to date, which include those of Subtypes 1, 2 and 3, belong to the same serotype (Graham et al. 2003a). Cross-neutralisation testing using SAVs representative of each subtype and homologous antisera, preferably from experimentally inoculated fishes, will be required to determine the extent of antigenic variation. Should it be shown that substantial differences exist between the efficiencies with which SAVs are neutralised by heterologous antisera, there would be implications for the development and use of SAV vaccines.

In conclusion, our analysis with 18 SAVs, based on 3 partial gene sequences, has revealed the existence of 3 genetically different subtypes that may have a geographical basis for their occurrence. Increased investigation of the molecular diversity displayed by these alphaviruses is likely to identify additional subtypes, and may identify genome regions better suited for differentiating SAVs from the same subtype. From an epidemiological point of view, this might prove useful for tracking virus spread between farms. The results of this and future molecular diversity studies with SAV should be taken into account when developing diagnostic RT-PCR tests such that the oligonucleotide primers selected for test development should be based on conserved genomic sequences.

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