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NOTE

First confirmation of salmonid alphavirus infection in Arctic char *Salvelinus alpinus* and in Austria

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ABSTRACT: To date, sleeping disease (SD) caused by salmonid alphavirus 2 (SAV 2) has been reported in freshwater rainbow trout *Oncorhynchus mykiss* and Atlantic salmon *Salmo salar*. This study describes for the first time the occurrence of SD in farm-reared Arctic char *Salvelinus alpinus* and the occurrence of SAV in Austria. Clinical symptoms were indicative of the disease, and the diagnosis was confirmed by histopathology, infectivity in first passages of CHSE-214 cells and PCR. The phylogenetic analysis of the amplified SAV-nonstructural protein-3 (nsP3) fragment revealed the affiliation to the SAV 2 genotype.

KEY WORDS: Salmonid alphavirus · Arctic char · *Salvelinus alpinus* · Sleeping disease · Pancreas disease

INTRODUCTION

The salmonid alphavirus (SAV) species comprises at least 6 virus genotypes, based on sequencing data of the genes encoding the glycoprotein E2 and the nonstructural protein-3 (nsP3). The subtypes correspond with their geographic distribution (Fringuelli et al. 2008). Among them, SAV 1 is the causative agent of pancreas disease (PD) of farmed Atlantic salmon *Salmo salar* (Nelson et al. 1995), whereas SAV 2 causes sleeping disease (SD) in freshwater-reared rainbow trout *Oncorhynchus mykiss* in continental Europe and Atlantic salmon in the UK and Norway (Castric et al. 1997, Villoing et al. 2000, Hjortaas et al. 2013). Another genotype (SAV 3) causing PD has been characterized from Atlantic salmon and sea-reared rainbow trout in Norway (Christie et al. 1998). The genotypes SAV 4, 5 and 6 have been isolated from seawater Atlantic salmon in Ireland, Northern Ireland and Scotland and also cause PD (Fringuelli et al. 2008). Brown trout *Salmo trutta* has been infected experimentally with SAV 1 but showed minor alterations in the pancreatic tissue compared to changes seen in Atlantic salmon and rainbow trout (Boucher et al. 1995). Information on susceptibility of other salmonid species has not been available until now. Disease patterns of PD and SD are similar, with reduced growth rates, lethargy, fecal casts and swimming disturbances, which resemble 'sleepy behavior' in the case of SD, thus leading to the name. Histological lesions include necrosis of the pancreatic tissue as well as alterations in the heart and the skeletal muscle (Munro et al. 1984, Ferguson et al. 1986). Horizontal transmission of the virus has been demonstrated in cohabitation experiments (Graham et al. 2011), while vertical transmission is still a matter of discussion (Bratland & Nylund 2009). Mortality rates for PD and SD vary considerably, ranging from 1–48% and from negligible to >22%, respectively (McLoughlin & Graham 2007).

Lake char *Salvelinus umbla* is a species native to Alpine and subalpine lakes in Italy, France, Switzerland, Germany and Austria, where different local

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strains have developed. Since the 1980s, the Nordic strain of the species, *S. alpinus* has been imported as fertilized eggs from Northern Europe for niche marketing at suitable locations. Total production in Austria for 2016 was 192.6 t (https://www.statistik.at/web_de/statistiken/wirtschaft/land_und_forstwirtschaft/viehbestand_tierische_erzeugung/aquakultur/index.html). Live fish of various age classes are translocated between the farms.

MATERIALS AND METHODS

During early spring 2018, we tested 12 arctic char (in their second year, total length 24–33 cm, body weight 112.3–286.9 g) for SAV. The fish originated from a farm which had introduced 5 t of the fish 2 mo prior to the onset of elevated mortalities. At the time of submission, cumulative mortality over the last 3 mo had reached 18%. Water temperature was constant at 7.0–7.5°C. Two of the 12 fish were submitted alive and were subsequently euthanized, while the others were already dead. Upon arrival, total length and weight of each fish was determined and Fulton's condition factor (CF = 100 $W L^{-3}$, where W = weight and L = length) was calculated. Necropsy followed a standard protocol.

For bacteriological examination, a kidney swab was taken from the 2 euthanized fish (MS 222, Sigma Aldrich, 1 g l^{-1}), spread on Columbia blood agar plates (Thermo Fisher Scientific, Oxoid) and inoculated at 15°C.

For virological examination in cell culture and PCR, heart and trunk kidney samples were aseptically removed individually and immediately stored in MEM-Glutamax (Gibco Life Technologies) containing 2% fetal bovine serum (FBS), 0.1% gentamycin and RNA stabilization reagent (RNAlater®, Qiagen).

For cell culture infection trials, the established adherent fish cell lines epithelioma papillosum cyprini (EPC, Fijan et al. 1983), bluegill fry (BF-2, Wolf et al. 1966) and chinook salmon embryo (CHSE-214, Fryer et al. 1965) cells grown in MEM-Glutamax containing 2% FBS were used. Four pools of heart and kidney from individual fish were suspended in MEM-Glutamax using a glass homogenizer and filtrated through a 0.4 µm syringe filter. Dilutions (100- and 1000-fold) of the 4 suspensions were inoculated into confluent monolayers of the cell lines about 16 h after seeding, incubated at 15°C and inspected on 5 days each week. After 1–3 wk, the supernatants of these first passages were subcultivated twice up to passage number 3.

For genetic analysis, total RNA was extracted from 25 mg fish tissues (kidney, heart) using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Purified RNA was quantified using a NanoDrop-ND-1000 (Thermo Scientific) and then stored in aliquots at –80°C until required. The real-time RT-PCR for detection of SAV was performed according to Hodneland & Endresen (2006) with some modifications. Briefly, the reaction was carried out using the QuantiTect Probe RT-PCR Kit (Qiagen), 900 nM from each primer, 200 nM probe and 5 µl RNA. Cycling conditions were as follows: reverse transcription at 50°C for 30 min, enzyme activation at 95°C for 15 min, followed by 45 cycles at 94°C for 15 s and 55°C for 60 s. Fluorescence data for the real-time RT-PCR assay were acquired during the annealing and extension phase in the reaction using the FAM detection channel. A positive control, as well as negative extraction and no-template controls were included in each test run.

To generate material for sequencing and phylogenetic analysis, conventional RT-PCR using a Qiagen One Step RT-PCR Kit was performed to amplify the SAV-*nsP3* 490 bp fragment according to Fringuelli et al. (2008). The obtained sequences were aligned with SAV-*nsP3* gene sequences available in GenBank using the multiple sequence alignment tool Clustal-X (Thompson et al. 1997). An unrooted phylogenetic tree was constructed using the neighbor-joining method according to Saitou & Nei (1987).

To rule out infection with infectious pancreatic necrosis virus (IPNV), the extracted RNA of each individual fish sample was tested by PCR using a protocol according to Wang et al. (1997).

For histology, samples from the gills, heart, spleen, liver, pyloric caeca with pancreas, trunk kidney, head kidney and red and white muscle with the lateral line organ were stored in 10% neutral buffered formalin, submitted to routine histology procedures and HE stained.

RESULTS AND DISCUSSION

Fulton's condition factor of the investigated fish was 0.68–0.96. Johnston (2007) reported a condition factor of 1.2–1.4 for well-fed market-ready cultured Arctic char. Necropsy did not reveal any alterations except for reduced fat deposits in the diseased fish (Fig. 1). A low condition factor and the loss of body fat are important economic features of SAV-infected fish (Christie et al. 1998, Taksdal et al. 2015). Other signs indicative of SAV infection were not observable, with

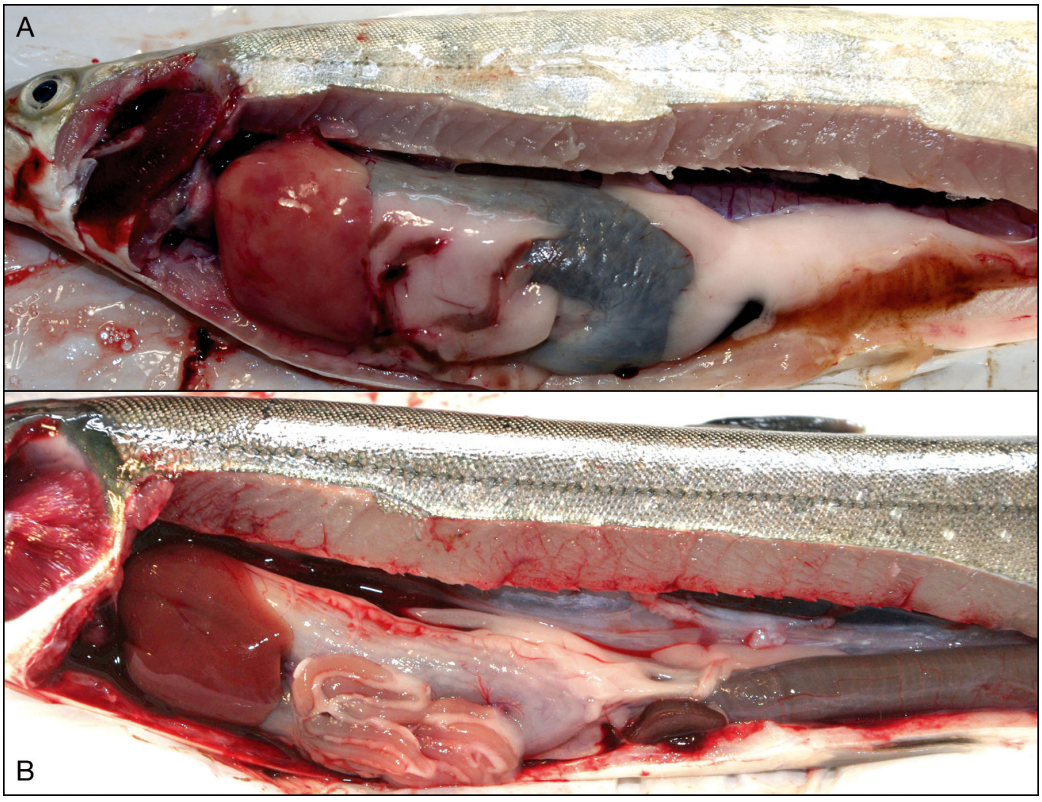

Fig. 1. Two Arctic char from the same tank, (A) negative and (B) positive for salmonid alphavirus. The only obvious indication of the disease is the loss of body fat in the region of the pyloric caeca. In both fish the gut is filled with food. Note the different development of the gonads. Yellow fluid contents of the hindgut in (A) are a result of early decay

all fish having the gut filled with food. Given the severe histological findings (see below), it is surprising that the fish had been feeding. This observation is also in contrast to Munro et al. (1984), who found that, in surviving fish which had returned to normal feeding, the pancreatic tissue had recovered.

Examination of the histological slides revealed necrosis of myocytes in skeletal and heart muscle and of acinar cells in the pancreas, as described by Boucher & Baudin Laurencin (1996) and Graham et al. (2007) with only mild inflammatory reactions (Fig. 2A). Alterations of the red muscle were far more severe than in the heart (Fig. 2B,D). In the white muscle, we observed focal necrosis with mild inflammation and evidence of large cells containing eosinophilic material (Fig. 2C). In the trunk kidney, massive focal hyperplasia of the hematopoietic interstitial cells, compressing tubuli and glomeruli, was evident. Cells with eosinophilic contents, as described by McLoughlin & Graham (2007), could not be observed (Fig. 3A). Occasional edema of the Bowman space and total degeneration of glomeruli as well as disseminated basophilic tubuli indicating regeneration processes were evident (Fig. 3B,C). Eosinophilic and basophilic casts filled up the lumen of numerous tubuli. In the interstitium, homogenous heterophilic masses and rounded cells were observed (Fig. 3D). Throughout the section, melanomacrophages were scattered. Some of the alterations of the kidney suggest chronic damage, but extravascular fluid more likely was due to cardiac failure. An observation of edema ambient to larger vessels was also observed in the liver. The other studied organs did not show any histological alterations.

Cultures of bacterial swabs did not result in any growth.

Results of cell culture varied for the different cell lines. During the first incubation period in EPC cells, only a toxic effect of the 4 samples could be observed. In BF-2 and CHSE-214 cells, 1 sample induced CPE at 5 and 14 d post infection, respectively. The lower dilution of the sample caused increasing vacuolization in BF-2 cells leading to complete destruction of the monolayer 2 d later. However, this effect was not reproducible in further BF-2 passages. CHSE-214 cells incubated with the same sample

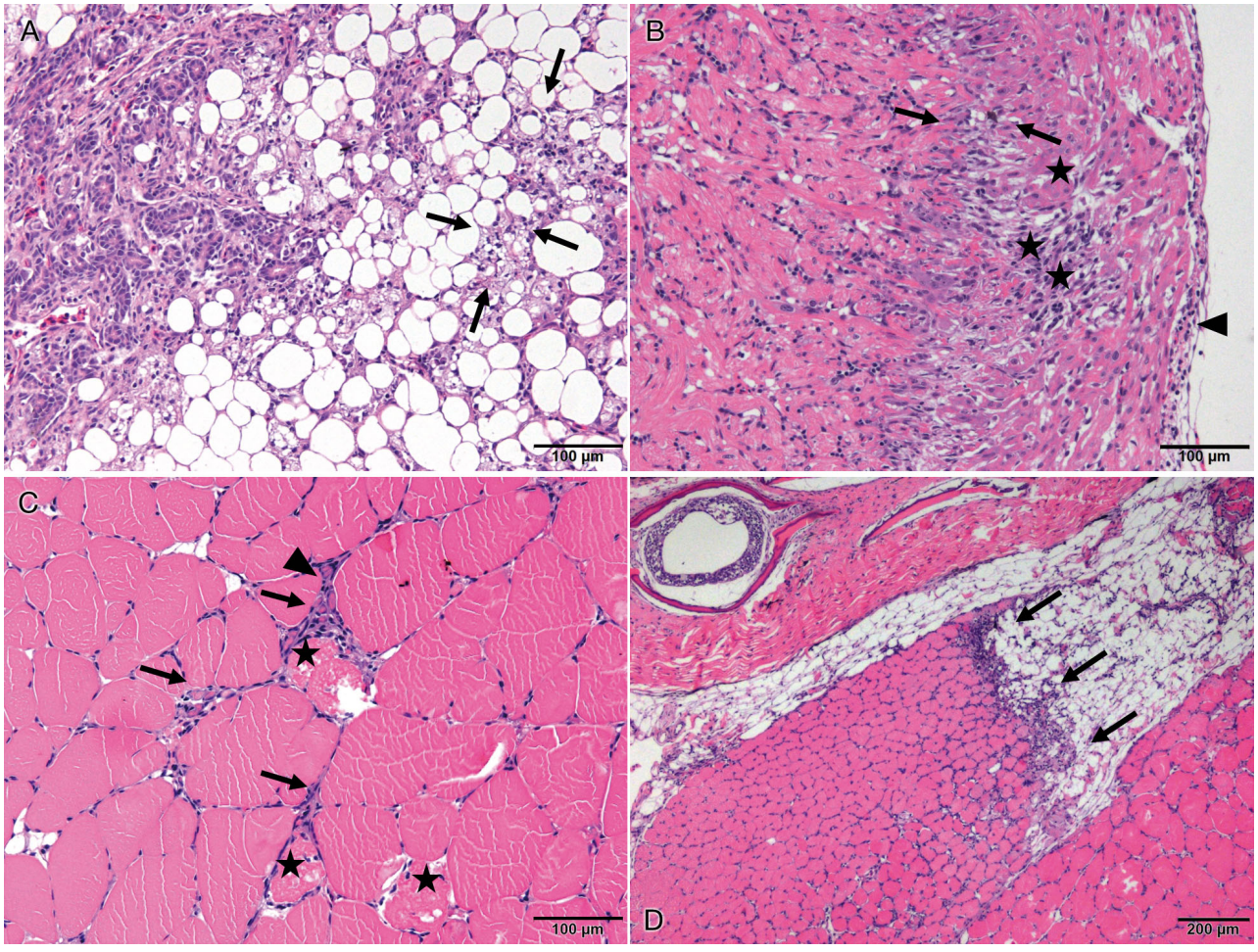

Fig. 2. Histology (HE stain) of Artic char infected with salmonid alphavirus. (A) Pancreas: on the left side, islets of intact pancreatic tissue and fibrosis are evident; on the right side, note the remains of necrotic acinar cells and moderate mononuclear cell infiltration (arrows). (B) Heart: generalized infiltration of all muscle layers with mononuclear cells. Aggregations of inflammatory cells can be seen between the compact and the spongy layers (arrows) and in an area of acute necrosis (asterisks). Mild inflammation in the pericardium is evident (arrowhead). (C) White muscle: focal necrosis of muscle fibers (asterisks), mononuclear cell infiltration (arrows), large cells containing eosinophilic material (arrowhead). (D) Red muscle: massive necrosis of red muscle accompanied by mononuclear cell infiltration (arrows); endomysium sheets are left empty

slowly became pyknotic during the first 3 wk of incubation. In the second passage, groups of cells became temporarily vacuolated up to Day 4. A CPE in SD virus-infected CHSE-214 cells, i.e. pyknosis and vacuolization, was described by Nelson et al. (1995). However, in our case, vacuolization disappeared during the following days of the second passage. In further subcultures, no infectivity was detectable.

Low cytopathogenicity of SAV from Arctic char *in vitro* is in accordance with previous reports on samples from other salmonid species (Nelson et al. 1995, Graham et al. 2003). Infection with IPNV was ruled out by PCR as described above.

Real-time RT-PCR was positive for the SAV in 11 out of 12 fish samples, with quantitation cycle (Cq) values ranging from 26.36 to 35.34. This result was confirmed by conventional RT-PCR and sequencing. The sequence obtained from the SAV-PCR product was deposited in GenBank under accession number MH507515. The BLASTn search revealed a 98–99% similarity among the obtained PCR products and 98% identity with the SAV (GenBank accession number EF675532). The phylogenetic analysis revealed that the amplified sequences were affiliated with the SAV 2 genotype (Fig. 4). In conclusion, SAV RNA was detected for the first time in Arctic char, associated with clinical signs, mortalities and histopathological changes indicative for SD. Moreover, this is also the first report of SD and evidence of SAV infection in Austria.

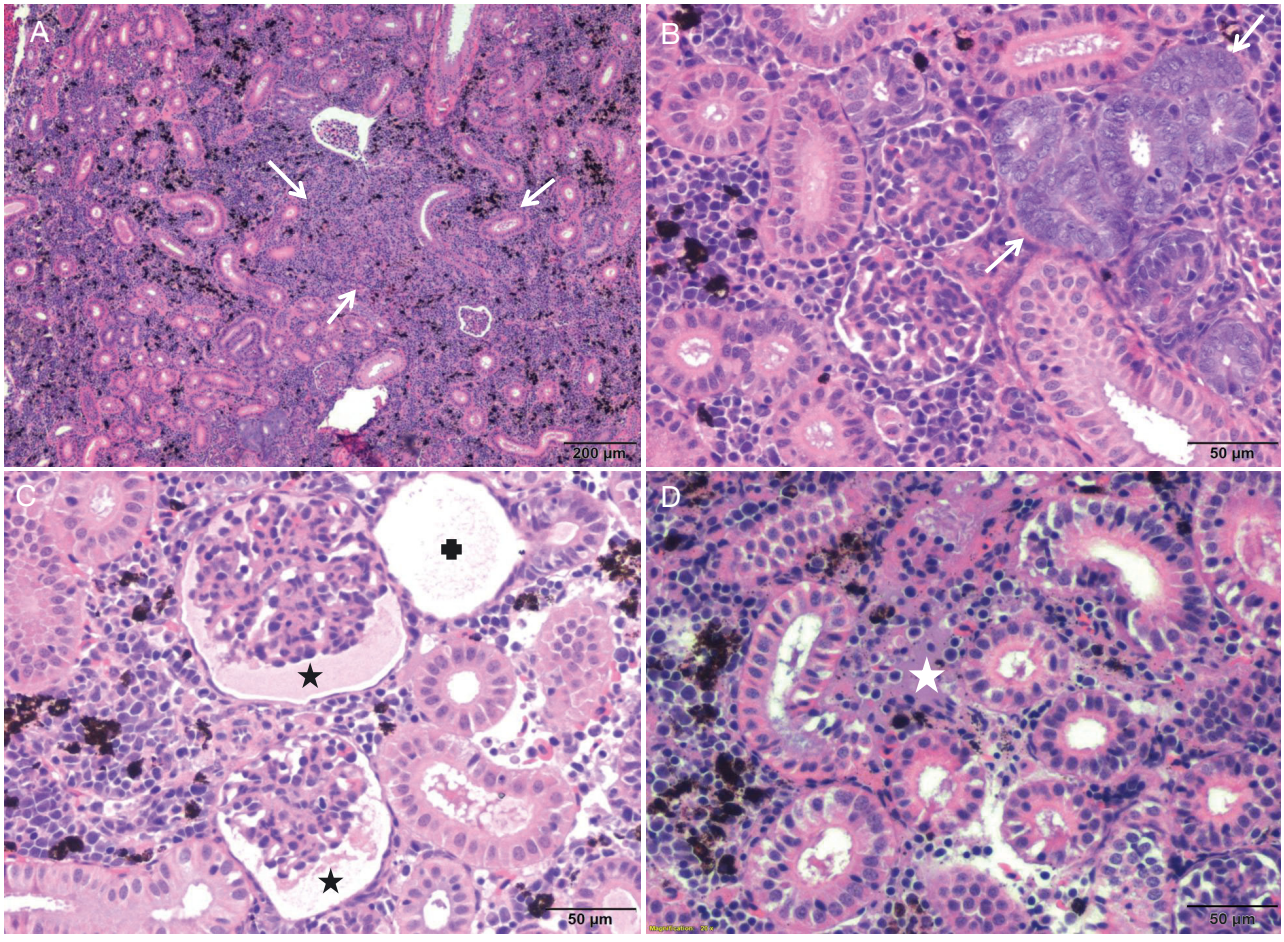

Fig. 3. Histology (HE stain) of Artic char kidney infected with salmonid alphavirus. (A) Massive hyperplasia of the interstitial cells (arrows). (B) Regeneration of nephrons (arrows). (C) Extended Bowman space, filled with eosinophilic fluid (asterisk) and glomerular degeneration (cross). (D) Heterophilic fluid and rounded cells (asterisk)

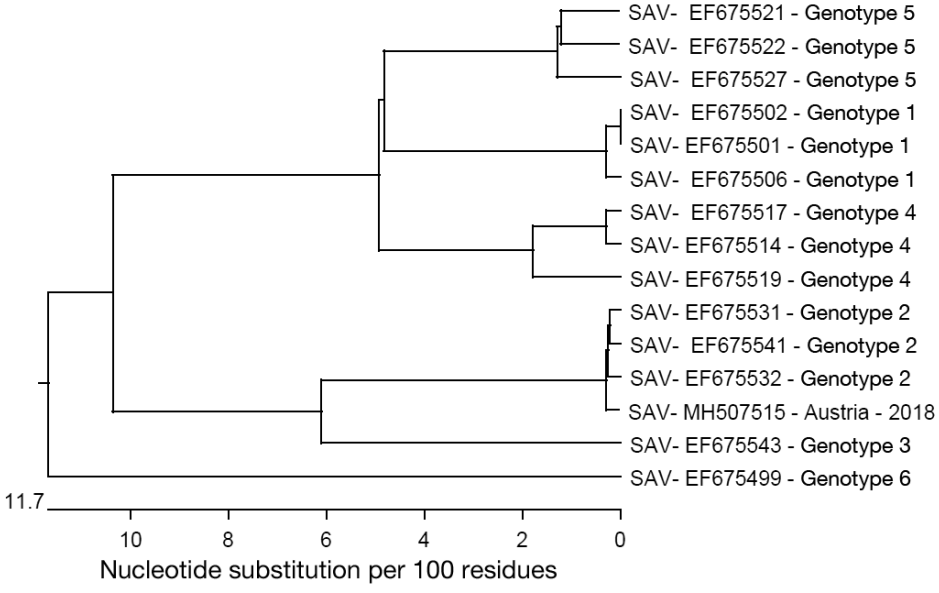

Fig. 4. Unrooted phylogenetic tree showing the relative position of Austrian salmonid alphavirus (SAV) isolate to other SAV genotypes. The tree is based on nucleotide sequence comparisons of the *nsP3* gene fragment and was generated using the neighbor-joining method

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LITERATURE CITED

Boucher P, Baudin Laurencin F (1996) Sleeping disease and pancreas disease: comparative histopathology and acquired cross-protection. J Fish Dis 19:303–310

Boucher P, Raynard RS, Houghton G, Baudin Laurencin F (1995) Comparative experimental transmission of pancreas disease in Atlantic salmon, rainbow trout and brown trout. Dis Aquat Org 22:19–24

Bratland A, Nylund A (2009) Studies on the possibility of vertical transmission of Norwegian Salmonid Alphavirus in production of Atlantic salmon in Norway. J Aquat Anim Health 21:173–178

Castric J, Baudin Laurencin F, Brémont M, Jeffroy J, Le Ven A, Bearzotti M (1997) Isolation of the virus responsible for sleeping disease in experimentally infected rainbow trout (*Oncorhynchus mykiss*). Bull Eur Assoc Fish Pathol 17:27–30

Christie KE, Fyrand K, Holtet L, Rowley HM (1998) Isolation of pancreas disease virus from farmed Atlantic salmon, *Salmo salar* L., in Norway. J Fish Dis 21:391–394

Ferguson HW, Roberts RJ, Richards RH, Collins RO, Rice DA (1986) Severe degenerative cardiomyopathy associated with pancreas disease in Atlantic salmon, *Salmo sala*r L. J Fish Dis 9:95–98

Fijan N, Sulimanovi D, Bearzotti M, Muzini D and others (1983) Some properties of the *Epithelioma papulosum cyprini* (EPC) cell line from carp *Cyprinus carpio*. Ann Inst Pasteur Virol 134:207–220

Fringuelli E, Rowley HM, Wilson JC, Hunter R, Rodger H, Graham DA (2008) Phylogenetic analyses and molecular epidemiology of European salmonid alphaviruses (SAV) based on partial E2 and nsP3 gene nucleotide sequences. J Fish Dis 31:811–823

Fryer JL, Yusha A, Pilcher KS (1965) The *in vitro* cultivation of tissue and cells of Pacific salmon and steelhead trout. Ann N Y Acad Sci 126:566–586

Graham DA, Rowley HM, Walker IW, Weston JH, Branson EJ, Todd D (2003) First isolation of sleeping disease virus from rainbow trout, *Oncorhynchus mykiss* (Walbaum), in the United Kingdom. J Fish Dis 26:691–694

Graham DA, Jewhurst HL, McLoughlin MF, Branson EJ, McKenzie K, Rowley HM, Todd D (2007) Serological, virological and histopathological study of an outbreak of sleeping disease in farmed rainbow trout *Oncorhynchus mykiss*. Dis Aquat Org 74:191–197

Graham DA, Frost P, McLaughlin K, Rowley HM, Gabestad I, Gordon A, McLoughlin MF (2011) A comparative study of marine salmonid alphavirus subtypes 1–6 using an experimental cohabitation challenge model. J Fish Dis 34:273–286

Hjortaas MJ, Skjelstad HR, Taksdal T, Olsen AB and others (2013) The first detections of subtype 2-related salmonid alphavirus (SAV2) in Atlantic salmon, *Salmo salar* L., in Norway. J Fish Dis 36:71–74

Hodneland K, Endresen C (2006) Sensitive and specific detection of Salmonid alphavirus using real-time PCR (TaqMan®). J Virol Methods 131:184–192

Johnston G (2007) Growth nutrition and feeding: growth in Arctic charr. In: Johnston G (ed) Arctic charr aquaculture. Blackwell Scientific, Oxford

McLoughlin MF, Graham DA (2007) Alphavirus infections in salmonids — a review. J Fish Dis 30:511–531

Munro ALS, Ellis AE, McVicar AH, McLay HA, Needham EA (1984) An exocrine pancreas disease of farmed Atlantic salmon in Scotland. Helgol Meeresuntersuch 37:571–586

Nelson RT, McLoughlin MF, Rowley HM, Platten MA, McCormick JI (1995) Isolation of a toga-like virus from farmed Atlantic salmon *Salmo salar* with pancreas disease. Dis Aquat Org 22:25–32

Saitou N, Nei M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Taksdal T, Bang Jensen B, Böckerman I, McLoughlin MF, Hjortaas MJ, Ramstad A, Sindre H (2015) Mortality and weight loss of Atlantic salmon, *Salmon salar* L., experimentally infected with salmonid alphavirus subtype 2 and subtype 3 isolates from Norway. J Fish Dis 38: 1047–1061

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Villoing S, Bearzotti M, Chilmonczyk S, Castric J, Bremont M (2000) Rainbow trout sleeping disease virus is an atypical alphavirus. J Virol 74:173–183

Wang WS, Wi YL, Lee JS (1997) Single-tube, non-interrupted reverse transcription PCR for detection of infectious pancreatic necrosis virus. Dis Aquat Org 28:229–233

Wolf K, Gravell M, Malsberger RG (1966) Lymphocystis virus: isolation and propagation in centrarchid fish cell lines. Science 151:1004–1005

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