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Identification of a wild reservoir of salmonid alphavirus in common dab *Limanda limanda*, with emphasis on virus culture and sequencing

D. W. Bruno[1,*], P. A. Noguera[1], J. Black[1], W. Murray[1], D. J. Macqueen[2], I. Matejusova[1]

[1]Marine Scotland Science, Marine Laboratory, 375 Victoria Road, PO Box 101, Aberdeen AB11 9DB, UK
[2]Institute of Biological and Environmental Sciences, University of Aberdeen, Tillydrone Avenue, Aberdeen AB24 2TZ, UK

ABSTRACT: Common dab *Limanda limanda* from Scottish and international waters were examined by quantitative real-time RT-qPCR for evidence of viral RNA consistent with salmonid alphaviruses (SAV). SAV prevalence in heart tissue varied between sampling sites and reached up to 17% in fish collected near the Shetland Islands, Scotland. Raw Ct values ranging from 22.31 to 39.45 were obtained from the SAV-positive tissue material using the nsP1 RT-qPCR assay. Bayesian-, likelihood- and distance-based phylogenetic analyses performed with the amplified partial E2 gene sequence dataset suggest that the dab-derived virus belongs to SAV Subtypes I, II and V. A single SAV subtype was identified from the majority of sampling sites, apart from Shetland, where Subtypes II and V were also identified. The presence of SAV RNA from common dab in regions detached from salmon aquaculture lends support to the hypothesis that common dab are bone fide wild reservoirs of SAV, independent of fish farming activity. There was no link between the occurrence of viral RNA, length and sex of the dab, water depth, or health status as recorded using the International Council for the Exploration of the Sea (ICES) guidelines. In addition, the histological changes recorded in dab could not, with certainty, be attributed to infection with SAV. Finally, and for the first time, this study demonstrated that the dab-derived SAV Subtype V virus could be successfully cultured in a salmonid cell line.

KEY WORDS: Common dab · Salmonid alphavirus · SAV · Wild fish · Virus culture

INTRODUCTION

Pancreas disease (PD) was first recorded in Scotland from farmed Atlantic salmon *Salmo salar* in 1976 (Munro et al. 1984). A similar condition termed 'sleeping disease' (SD) was subsequently described from freshwater-reared rainbow trout *Oncorhynchus mykiss* (Boucher & Baudin-Laurencin 1996). The 6 salmonid alphavirus (SAV) subtypes described (including SAV2/SDV) belong to 1 viral species. The name of the virus is salmon pancreas disease virus (SPDV) (Togaviridae), commonly called SAV. The 6 SAV subtypes have been distinguished using phylogenetic analysis with partial E2 and nsP3-gene sequence data (Fringuelli et al. 2008), providing evidence that some subtypes are dominant in certain geographical regions. For example, SAV II is the dominant subtype from Atlantic salmon reared in the Shetland Isles, whereas salmon from the northeast of Scotland and the Western Isles show Subtype V to be dominant (Graham et al. 2012). However, SAV II strains can now be found in saltwater (Graham et al. 2012), and, similarly, SAV I strains occur in freshwater salmonids (Lester et al. 2011). Salmonid alphaviruses do not require an arthropod vector to complete their life cycle, which is unusual compared to mammalian

*Corresponding author: david.bruno@scotland.gsi.gov.uk

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alphaviruses. Considerable existing data suggest that horizontal transmission represents an important route of spread of PD and that infection pressure has a strong effect on the probability of recording an outbreak in a single cohort (McLoughlin et al. 1996, Kristoffersen et al. 2009, Viljugrein et al. 2009). Jansen et al. (2010) reported that if infection occurs in a population, disease is highly likely to develop, assuming fish spend an adequate time in that environment. Through experimental studies, SAV has been shown to survive for 5.7 d at 10°C in sterile saltwater with organic loading (Graham et al. 2007). Moreover, viral RNA has been shown to persist in tissues for extended periods (Andersen et al. 2007, Christie et al. 2007, Graham et al. 2010, Jansen et al. 2010), potentially posing an infection risk to healthy fish.

Despite fallowing, re-stocking of salmonids at farms has resulted in re-infection (McLoughlin et al. 2003), suggesting that natural reservoirs of this virus may exist. This is supported by Karlsen et al. (2014) who suggested all subtypes diverged prior to the introduction of rainbow trout to Europe and, therefore, existed in a wild reservoir. A previous study using RT-qPCR detected a potential wild reservoir of SAV in several flatfish species, but failed to provide follow-up evidence in support of this result, e.g. successful isolation of the virus (Snow et al. 2010). The objectives of the current study were (1) to determine the prevalence of SAV subtypes in common dab from Scottish and international waters, (2) to culture any identified SAVs *in vitro* and (3) to examine common dab tissues from selected sampling areas for the presence of histopathological changes consistent with pancreas disease.

MATERIALS AND METHODS

Collection of wild common dab

Common dab *Limanda limanda* were collected from Cragmylie, Garron and Back o Garron (Stonehaven Bay, Kincardineshire, Scotland) (Fig. 1) using the MRV 'Temora' (10 m Blyth catamaran) between January 2009 and June 2010. Fish were collected using a demersal trawl, towed for short periods in 12 to 36 m of water across the 3 areas. The sampling plan, with respect to number of fish, was designed to achieve a 95 to 99% chance of detecting 1 or more infected dab per site. The fish were kept alive in a covered tank supplied with ambient temperature seawater for a maximum of 4 h before transportation from Stonehaven Bay to the laboratory (a distance of ~24 km) in bins supplied with pumped air or oxygen. The fish were transferred to tanks that received natural seawater, which was mechanically filtered and ultraviolet irradiated, they were held in a bio-secure area where no infection studies were being carried out, and were examined within 4 d of arrival as detailed in the following subsection.

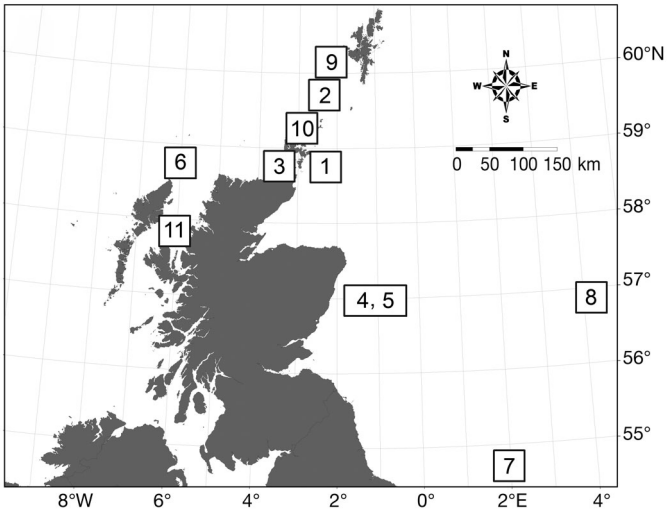

Fig. 1. Location of sampling sites (1–11) around Scotland

Common dab were also fished with a bottom trawl net for 30 min using the RV 'Scotia'. The same statistical approach was implemented for designing the sampling plan. Fishing was conducted in 7 areas around the Scottish coastline and in 2 areas in the northern North Sea, including international waters. The nominal centre of each trawl area is presented in Fig. 1. Common dab were laid flat in a blast freezer on board the vessel, and each fish was then individually bagged and labelled. The fish were returned to the laboratory and stored at −80°C until processed.

Tissue sampling

For molecular investigations, tissues from all fish (heart, kidney, brain and skeletal muscle) were placed into individually labelled cryotubes containing RNA*later* (VWR International) and stored at −80°C. The samples of skeletal muscle did not include the scale layer. For virus isolation, heart and kidney tissues from individual dab were taken, stored in cryotubes and snap frozen at −80°C. For histopathological investigation, fish from Stonehaven Bay were examined following terminal anaesthetization, using tricaine methane sulphonate (MSS, Sigma) at 0.2 g l^{-1}, whereas fish frozen at other loca-

tions were allowed to thaw in the laboratory but were dissected while partially frozen. Signs attributed to epidermal hyperplasia/papilloma, lymphocystis, and acute and healing skin ulcers were recorded from all dab and prepared according to ICES standard quality assurance procedures (Bucke et al. 1996, Lang 2002). Hyperpigmentation on the ocular side was recorded according to the categories of the Biological Effects Quality Assurance Monitoring Programme (BEQALM): 1 being least severe, 2 being moderately severe and 3 being most severe. Total length (measured to the nearest centimetre below) was recorded for each dab (both frozen and freshly killed), and sex was noted externally from the shape of the gonad.

Tissues (skin including musculature, gills, heart, kidney, spleen, liver, pancreas, gut) were removed from anaesthetised fish (Stonehaven area only), fixed in 10% buffered neutral formalin solution for a minimum of 24 h and embedded in paraffin, and sections were stained with haematoxylin and eosin. A minimum of 30 fish from each of the 2 locations at Stonehaven Bay, including fish shown to be SAV positive by PCR, were examined histologically. Sections were reviewed without knowledge of the PCR results, and all tissues were assessed with a particular focus on pancreas, heart and red and white skeletal muscle, as these are the tissues most severely affected during SAV infection in salmonids (Christie et al. 2007, McLoughlin & Graham 2007). Histological changes attributed to PD include a severe loss of exocrine pancreatic tissue combined with chronic myositis, with red and white skeletal myocytic degeneration and hypercellularity within the heart (McVicar 1987, McLoughlin et al. 1996, Murphy et al. 1992). A SAV infection in dab, however, may not necessarily cause histological changes in the same tissues as those noted for salmon.

SAV RT-qPCR and sequencing

Several studies have shown heart tissue to be one of the most appropriate tissues for detecting SAV during all stages of infection (also persistent infections) (Graham et al. 2006, Christie et al. 2007, Andersen et al. 2007); for this reason, only heart tissue was processed for molecular testing. Approximately 5 mg of homogenised tissue was used for extraction of total RNA (MagAttract M48 RNA Tissue Kit, Qiagen) using the M48 BioRobot (Qiagen). Total RNA was eluted in a final volume of 100 µl. cDNA synthesis was performed using the Taqman Reverse Transcription Reagent Kit and random hexamers (Applied Biosystems) according to Snow et al. (2010). For detection of the SAV target in heart tissue an nsP1 RT-qPCR assay was used, with a cut off Ct value of 39.3 (see Hodneland & Endresen 2006 for details on assay validation). RT-qPCR reactions were run on an ABI Prism 7000 detection system, using cycling conditions and the reaction set up according to Snow et al. (2010). Negative controls were routinely included at the stages of extraction, reverse transcription and RT-qPCR. RT-qPCR was performed in triplicate from each sample, with positives only being recorded where a raw Ct value below the assay cut off point was generated from all 3 independent reactions.

Partial E2 gene amplification was conducted in a final volume of 50 µl with 1 µl of cDNA tested SAV positive by RT-qPCR (as described above) according to Fringuelli et al. (2008). For cDNA exhibiting nsP1 RT-qPCR Ct values >33, a nested PCR was performed using the primers E2N Forward (5′ AGG CCA CTG GCC ACT ACA 3′) and E2N Reverse (5′ GGA AAC CAA GGT TCC GTG 3′) and 35 cycles of 94°C for 20 s, 50°C for 20 s and 72°C for 50 s, followed by second-round amplification according to Fringuelli et al. (2008). PCR product was either purified directly using ExoSAP IT (GE Healthcare) or excised from agarose gel and purified using MinElute (Qiagen). Approximately 10 ng of purified product was sequenced (GenomeLab DTCS Quick Start Kit, Beckman Coulter) in both directions on an automated CEQ8800 DNA sequencer (Beckman Coulter) using primers according to Fringuelli et al. (2008). Sequences were analysed using Sequencher software (Gene Codes).

Sequence and phylogenetic analyses

A single representative of each redundant dab E2 sequence type was retained for sequence analysis, which initially involved *blastn* searches (Altschul et al. 1990) against the non-redundant NCBI nucleotide database. The final dataset covered 55 sequences spanning 350 bp (Table 1), which was aligned using Clustal W (Thompson et al. 1994) within BioEdit Version 7.0.5.3 (Hall 1999). An established statistical test (Xia et al. 2003) performed in DAMBE V. 5.3 (Xia 2013) demonstrated an absence of mutational saturation for all codon positions. Prior to tree-building, the alignment was loaded into Mega 5.0 (Tamura et al. 2011) to identify the best-fitting model of nucleotide substitution by maximum likelihood (ML), which was K80+G (i.e. Kimura 1980, modelled assuming a gamma distribution of among-site rate variation).

Table 1. Details of salmonid alphavirus isolates from which a partial E2 gene sequence was used in the present study. AS: Atlantic salmon; RT: rainbow trout

Subtype	Virus	Isolation year	Country of origin	Host species	Accession number
I	IRE F93125	1993	Ireland	AS	AJ316244
	IRE F03123	2003	Ireland	AS	EF675548
	SCO 08494	2008	Scotland	AS	KJ513287[a]
	IRE F02194	2002	Ireland	AS	EF675552
	SCO 07887	2007	Scotland	AS	KJ513284[a]
	IRE F06182	2006	Ireland	AS	EF675549
	SCO 10682	2010	Scotland	Dab	KJ513276[a]
II	FR S49P	1995	France	RT	AJ316246
	FR EE37		France	RT	EF675578
	NO SAV2011A	2011	Norway	AS	HE863662
	FR	1999	France	RT	AJ238578
	IT F04198	2004	Italy	RT	EF675590
	SPA F0408	2004	Spain	RT	EF675587
	SCO 02798	2002	Scotland	RT	KJ513286[a]
	ENG F04212	2004	England	RT	EF675585
	SCO 031201	2003	Scotland	AS	KJ513285[a]
	SCO 09958	2009	Scotland	AS	KJ513283[a]
	SCO 10684/51	2010	Scotland	Dab	KJ513277[a]
III	NO SAVR31/04	2004	Norway	AS	DQ122146
	NO SAVH05/01	2001	Norway	AS	DQ122141
	NO SAVH21/03	2003	Norway	AS	AY604238
	NO SAVF29/03	2003	Norway	AS	DQ122127
	NO SAVT09/10/2	2009	Norway	AS	HM208123
	NO SAVH06/1/1	2006	Norway	AS	HM208094
	NO SAVH20/03	2003	Norway	AS	AY604235
	NO SAVT09/10/1	2009	Norway	AS	HM208122
	NO SAVF0711/2	2007	Norway	AS	HM208092
	NO SAVF07/11/1	2007	Norway	AS	HM208091
	NO SAVH07/2/2	2007	Norway	AS	HM208098
IV	SCO F06139	2006	Scotland	AS	EF675569
	SCO F03209	2003	Scotland	AS	EF675565
	IRE F91116	1991	Ireland	AS	EF675564
	SCO F06243	2006	Scotland	AS	EF675566
	IRE F0444	2004	Ireland	AS	EF675560
V	SCO F0702	2007	Scotland	AS	EF675571
	SCO F0693	2006	Scotland	AS	EF675567
	SCO F05310	2005	Scotland	AS	EF675575
	SCO F06267	2006	Scotland	AS	EF675574
	SCO F0617	2006	Scotland	AS	EF675568
	SCO 09913	2009	Scotland	Dab	KJ513288
	SCO 10406	2010	Scotland	Dab	KJ513278[a]
	SCO 10407	2010	Scotland	Dab	KJ513279[a]
	SCO 10408	2010	Scotland	Dab	KJ513280[a]
	SCO 10525	2010	Scotland	Dab	KJ513281[a]
	SCO 10411	2010	Scotland	Dab	KJ513282[a]
	SCO 10684/17	2010	Scotland	Dab	KJ513267[a]
	SCO 10684/20	2010	Scotland	Dab	KJ513268[a]
	SCO 10648/27	2010	Scotland	Dab	KJ513269[a]
	SCO 10684/60	2010	Scotland	Dab	KJ513270[a]
	SCO 10684/70	2010	Scotland	Dab	KJ513271[a]
	SCO 10684/49	2010	Scotland	Dab	KJ513273[a]
	SCO 10684/71	2010	Scotland	Dab	KJ513274[a]
	SCO 10684/73	2010	Scotland	Dab	KJ513275[a]
	SCO 10685	2010	Scotland	Dab	KJ513272[a]
VI	IRE F104596	1996	Ireland	AS	EF675547

[a]Sequences generated in the present study

A Bayesian phylogenetic analysis was performed in BEAST V. 1.7 (Drummond et al. 2012), incorporating the best-fit substitution model, a relaxed molecular clock model (Drummond et al. 2006), a Yule Speciation prior (Gernhard 2008) and a UPGMA starting tree. The sequence data were split into 2 partitions (Codon Positions 1 & 2, separate from Position 3). The Markov chain Monte Carlo (MCMC) method was run for 100 000 000 generations, logging parameters every 1000 generations. Tracer (tree.bio.ed.ac.uk/software/tracer/) was used to confirm the convergence of the MCMC, evidenced by effective sample sizes >4000 for all parameters. A maximum clade credibility tree was generated using TreeAnnotator (Drummond et al. 2012), discarding the first 10% of trees sampled in MCMC. ML phylogenetic analysis was performed in Mega 5.0, using K80+G along with non-parametric bootstrapping (1000 replicates) to gain branch support values. A neighbour joining (NJ) analysis was also performed in Mega 5.0 using the composite ML+G model (Tamura et al. 2004), again with 1000 bootstrap replicates. Supporting neighbour joining and maximum likelihood phylogenetic analyses was performed with the 350 bp partial E2 gene sequence and are available as Fig. S1 in the Supplement at www.int-res.com/articles/suppl/q005p089_supp.pdf.

Virus culture

Heart and kidney tissue from individual dab were taken and stored in cryotubes and snap frozen at –80°C. Samples were thawed, diluted 1:5 in transport media (Liebovitz L-15, Lonza; 10% new born calf serum, Invitrogen; gentamicin at 50 mg l^{-1}; polymyxin B at 10 000 U ml^{-1}, Sigma) at a final pH of 7.4 to 7.8, homogenised and clarified by centrifugation at 2000 *g* for 15 min. Samples were inoculated at 1:10 and 1:20 dilutions with a 3 h absorption

step onto 6-well plates containing monolayers of CHSE-214 cells (Fryer et al. 1965). The cell monolayers were at a 60 to 80% confluence, were 24 to 48 h old and were incubated at 15°C following inoculation.

Cultures were read every 7 d for appearance of cytopathic effect (CPE) and were sub-cultivated on Days 14 and 28. At Day 42, cell monolayers were scraped into RLT buffer (Qiagen) containing 0.1% β-mercaptoethanol (Sigma). The partial SAV E2 gene sequence was generated from the cell culture positive material as described above. Virus cultivation was not attempted if raw Ct values of less than approximately 36 were noted. Hence, tissue with low Ct values from SAV Subtype V positive fish were inoculated onto monolayers of CHSE-214 cells. Cultures were passed, and no CPE signs were observed.

RESULTS

Detection of SAV RNA in heart of common dab

All sampling sites, with the exception of Study Site 8 on the east coast of Scotland (Table 2, Fig. 1), revealed a positive detection of SAV in common dab *Limanda limanda*-derived RNA using the nsP1 RT-qPCR assay. The prevalence of SAV at the positive sampling sites varied between 0.8 and 16.8% (Table 2). Generally, lower prevalence of SAV in common dab was detected on the east and southeast coasts of Scotland (≤4%) compared to the west coast of Scotland, including the Shetland and Orkney Islands (Table 2). Negative controls applied during RNA extraction, cDNA synthesis and RT-qPCR did show detection of the pathogen target in any case.

Sequencing and phylogenetic analysis

A partial E2 gene was amplified from the SAV-positive dab from each sampled locality. Material from a minimum of 1 SAV-positive dab heart was sequenced for the partial E2 gene region, except for Sites 4 and 11 (Table 2). Three different subtypes of dab SAV were tentatively identified based on amino acid sequences: I (1 fish), II (1 fish) and V (19 fish) (Table 2). SAV nucleic acid variability among the individual study sites and within Site 9 was investigated, showing a 0.27 to 1.89% divergence in Subtype V originating from dab (Fig. 2). No amino acid substitutions resulted from the observed variation in the analysed partial E2 region.

Phylogenetic analyses using Bayesian, ML and NJ methods (Fig. 3) provided maximum statistical support for monophyletic SAV Subtypes III and II, using nomenclature introduced by Fringuelli et al. (2008).

Table 2. Prevalence of salmonid alphavirus (SAV), and summary information for common dab *Limanda limanda*. qPCR was conducted in triplicate, with positives only being considered if all 3 independent reactions showed a SAV target. Samples sequenced for the partial E2 gene are shown in **bold**. See Fig. 1 for the location of sampling sites. F: female; M: male (sex of SAV-positive fish is given in parentheses; Ct values for these individuals are given in the same order)

Sampling site	Date of collection	No. fish sampled	Male/ female fish	No. of SAV-positive fish	Prevalence of SAV (%)	Ct values (average)	SAV subtype
1	Feb 10	49	18/31	3 (F/M/F)	6.1	37.82/37.77/**34.41**	V
2	Feb 10	45	12/33	3 (F/F/F)	6.7	35.52/37.43/**36.43**	V
3	Feb 10	96	67/29	5 (M/F/F/F/M)	5.2	34.3/**27.1**/36.5/35.47/37.71	V
4	Mar 10	120	60/60	1 (F)	0.8	34.89	No sequence
5	May 10	118	11/107	1 (F)	0.8	**34.74**	V
6	Jul 10	77	44/33	1 (F)	1.3	**36.64**	V
7	Aug 10	119	60/59	1 (F)	0.8	**36.43**	I
8	Sep 10	120	72/48	0	0		
9	Oct 10	119	87/32	20 (F/F/M/M/F/F/F/M/F/ M/M/M/F/M/F/F/F/M/M/M)	16.8	36.03/**32.54/35.78/28.05/36.46/** 35.74/**30.13**/32.96/33.75/**37.51/ 36.24**[a]**/32.51/22.31**/39.45/**26.46**[b]/ **36.23/ 38.33/34**.7/**32.5**/35.6	II[a]V
10	Nov 10	120	44/76	3 (M/F/F)	2.5	37.09/37.52/**36.07**	V
11	Feb 12	40	23/17	2 (F/F)	5	32.5/31.12	No sequence

[a]SAV Subtype II as identified based on partial E2 gene sequencing
[b]Ct value of the cultured samples

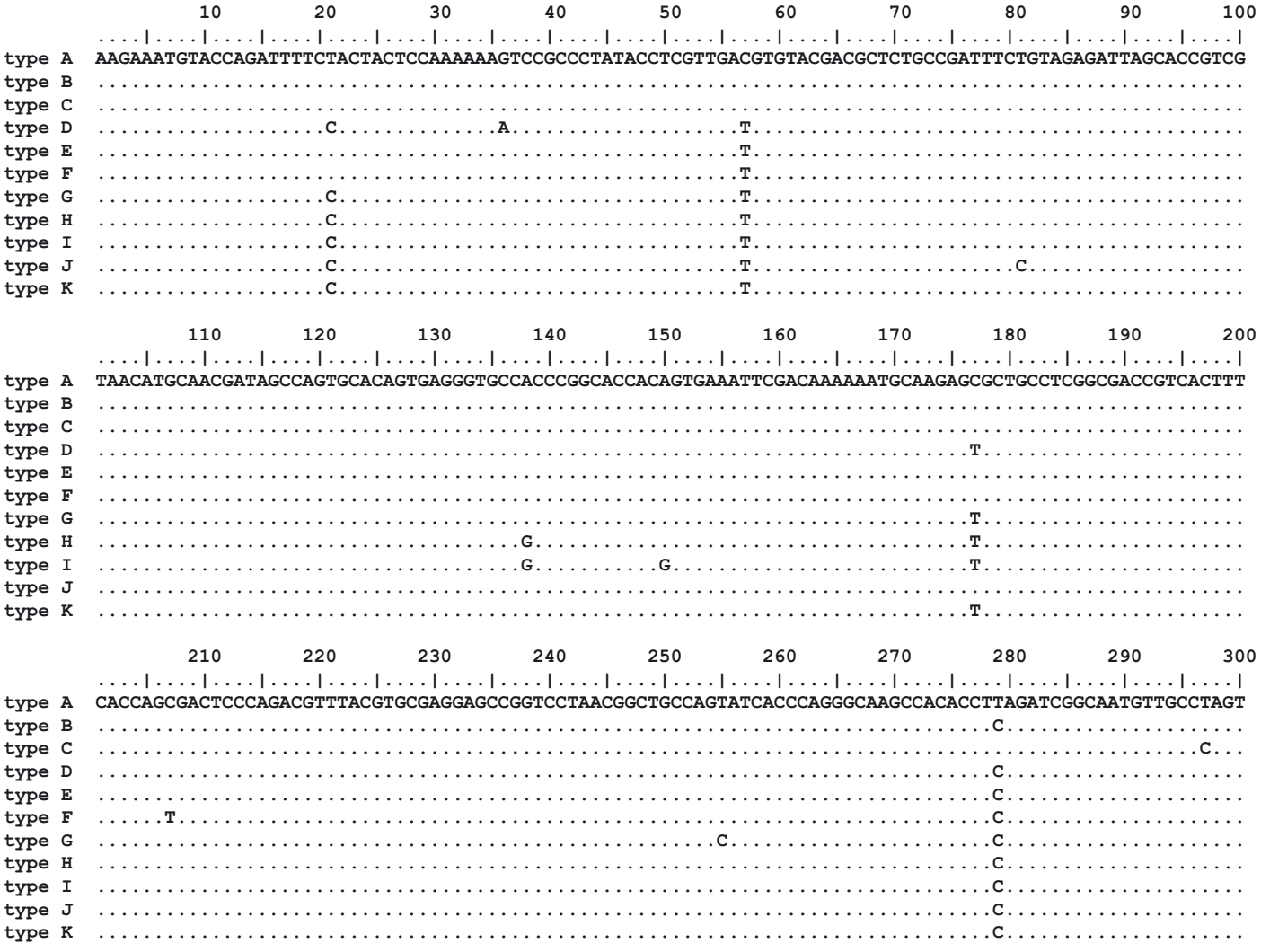

Fig. 2. Nucleic acid variability (type A to K) observed in the partial E2 gene of salmonid alphavirus Subtype V originating from common dab *Limanda limanda* observed in the present study. Numbers are base pairs

The split between SAV Subtypes III and II and all other SAV sequences received maximal statistical support as the true root of the tree under the Bayesian analysis (Fig. 3). While there is also strong support for monophyletic clades of characterised SAV sequence Subtypes I, IV and V, there is large statistical uncertainty surrounding their relationships (Fig. 3).

These analyses also confirmed that the dab 2010 684 51 isolate belongs to SAV Subtype II; this sequence forms the basal branch in a monophyletic cluster of sequences including marine isolates from Scotland and Norway (Fig. 3). Similarly, the dab 2010 682 isolate formed the most basal branch in the SAV Subtype I cluster (Fig. 3). In contrast, multiple dab SAV Subtype V sequences were clustered together with salmon Subtype V sequences (Fig. 3), a pattern consistent with relatively recent horizontal transmission of SAV.

Dab SAV isolation on CHSE-214 cell line

Total RNA was extracted from infected cells at Day 42, and the nsP1 RT-qPCR assay confirmed the presence of the SAV target, with an average Ct value of 21.40 from triplicate samples of individual fish.

Histological examination of wild caught common dab

There was no evidence of lesions that could be attributed specifically to that described for PD in the common dab examined from Stonehaven Bay, including those fish identified as positive for SAV RNA by RT-qPCR. Lesions associated with the heart occurred in 43% of the dab examined and included mild epicarditis involving the bulbous and a mild focal

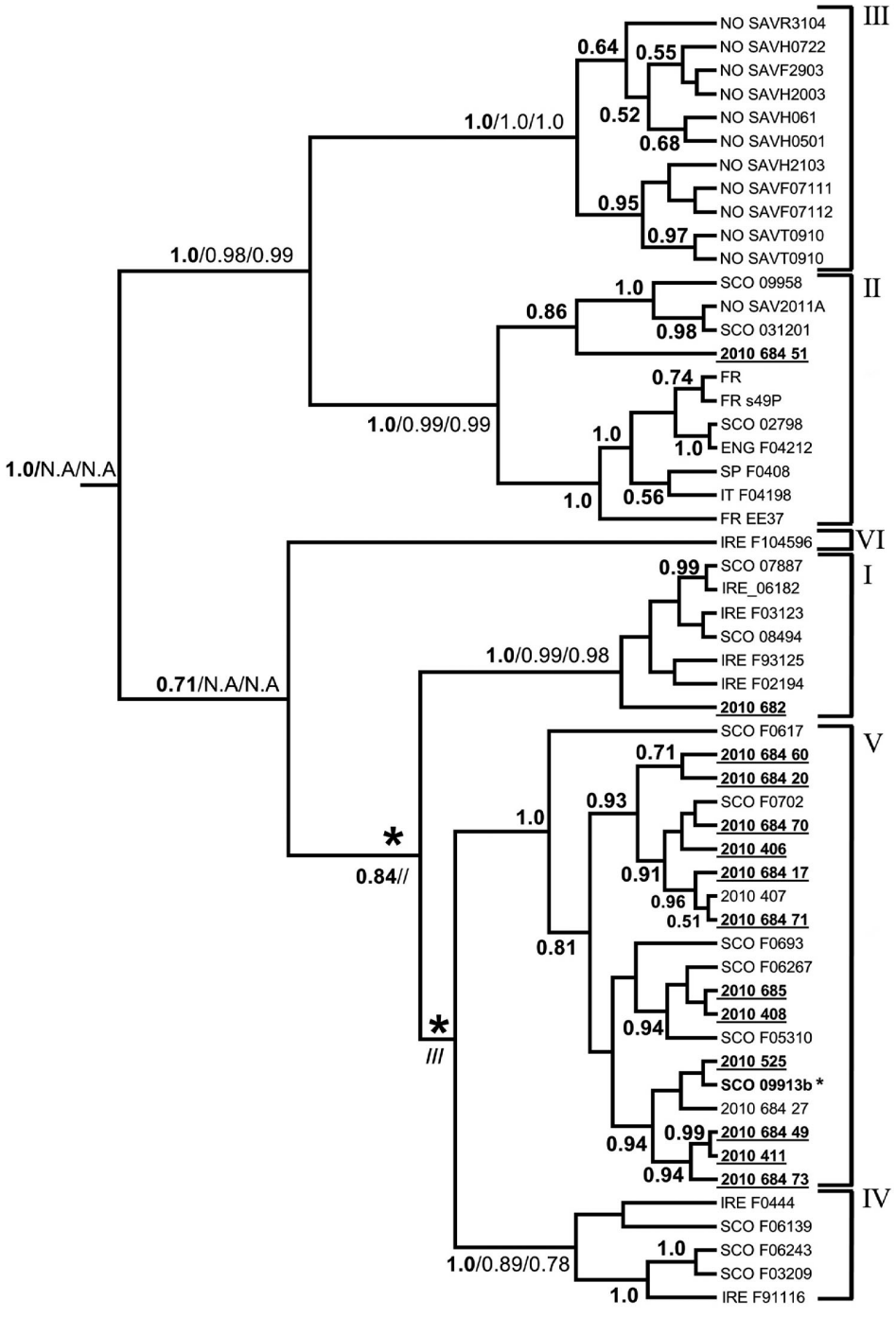

Fig. 3. Bayesian phylogenetic analyses performed with the 350 bp partial E2 gene sequence. The analysis was performed using the best-fitting model of nucleotide substitution (K80+G). The maximum clade credibility tree shown is based on 90 000 trees sampled from the MCMC chain after convergence was reached. Numbers at each node are support values from the different tree-building methods, ordered as: Bayesian posterior probability/ML bootstrap proportion/NJ bootstrap proportion (in all cases, only values >0.5 shown). Branch lengths are scaled to be relative in time according to an uncorrelated lognormal relaxed clock model. The asterisks on 2 internal nodes highlights the weak statistical support at branches separating defined subgroups I, IV and V. Bold underlined branch labels are sequences from dab isolates generated during this study

infiltration in the ventricle. Two dab showed mild infiltration into the red muscle tissue and extensive infiltration into the white muscle tissue, respectively. Nematodes were located in the pancreas and musculature in 26% of the fish examined.

DISCUSSION

This paper constitutes the first isolation of a salmonid alphavirus from a wild-caught non-salmonid marine fish species, namely the common dab *Limanda limanda.* A long time after the first isolation of SAV from farmed salmonids, the virus can now also be linked to subclinical infection in dab. The generally high Ct values reported in the current study are indicative of a low level of viral RNA in heart tissue. This confirms and extends previous work that identified the presence of SAV RNA in heart and kidney tissue collected from flatfish in Scottish coastal waters, but which failed to isolate the virus (Snow et al. 2010). Based on the presence of SAV in dab in a locality remote from salmonid aquaculture, we concluded that a reservoir of SAV in non-salmonid wild fish probably existed prior to the large-scale development of salmonid aquaculture in Scotland (Nash 2011). This is supported by a study on the evolutionary rate of SAV that suggested the location of a wild reservoir would be in or around the North Sea (Karlsen et al. 2014).

For SAV Subtype V, the present phylogenetic data are consistent with a history of repeated instances of horizontal transmission between dab and salmon. Wild reservoirs have

been well documented for a variety of viruses affecting marine fish farms, including viral haemorrhagic septicaemia virus (Snow et al. 2004, Gagné et al. 2007, Garver et al. 2013), piscine myocarditis virus (Poppe & Seierstad 2003, Böckerman et al. 2011, Tengs & Böckerman 2012) and piscine reovirus (Wiik-Nielsen et al. 2012). Nevertheless, the risk of transmission of viruses between wild and farmed fish is often difficult to ascertain (Kurath & Winton 2011). Fringuelli et al. (2008) reported identical sequences at individual farm sites over several years, implying that this is a slowly evolving virus. Nevertheless, this finding might also be explained by a consistent reservoir of infection associated with either environmental or anthropogenic processes. Common dab may play a role as a natural host for SAV, or, alternatively, the virus might be self-sustaining in aquaculture through farming practices; thus, areas of bio-security measures are required to control horizontal transmission (Kristoffersen et al. 2009). However, current practices of fallowing sea sites would not support this theory. Graham et al. (2012) suggested that bio-security to prevent spread between sites has not been sufficient in the past, as SAV Subtype II strains from farmed marine Atlantic salmon shared 100% identity with some freshwater strains. Similarly, Lester et al. (2011) reported the isolation of SAV Subtype I from rainbow trout reared in freshwater. It is also plausible that amplification and spread of SAV from infected fish farms could lead to the establishment of potential reservoirs of re-infection through the complexities of trade (McLoughlin et al. 2003). The saithe *Pollachius virens* occurs in the vicinity of sea cages (Bruno & Stone 1990), and later studies have shown virus-neutralising antibodies, but have failed to record disease, suggesting that inter-species transmission from wild to farmed fish, or vice versa, can take place (Graham et al. 2006). Finally, in terrestrial alphaviruses, transmission of infection is linked to an arthropod vector; however, in the fish to fish transmission of SAV, as demonstrated by Boucher et al. (1995), no vector is needed as a routine route of infection, and none has been identified to date. Salmon lice have been suggested as a potential vector for SAV. Although SAV may be detected in salmon lice by PCR (Karlsen et al. 2006, Petterson et al. 2009), viral replication in lice and the consequent transfer of the virus to a new host has not been proven.

The discovery of viral RNA in areas remote from fish farming would suggest that common dab are natural carriers of the virus and that the viral RNA is not the result of fish farming activity. However, explanations are required as to why the prevalence of SAV viral RNA was higher in some areas than in others, with a general trend of increasing prevalence towards the Shetland Isles. The distribution of SAV subtypes from farmed salmon by geographical region indicates that the northern Western Isles of Scotland have a higher number of Subtype V isolates than the Shetland Isles (Graham et al. 2012). However, these authors were aware of the fact that the number of strains available for analysis in each region would have varied.

These data might also be related to the kinetics of infection with SAV isolates of differing subtypes, as persistence of viral RNA in the tissues of farmed Atlantic salmon from which the virus was not isolated has been described (Christie et al. 2007, Graham et al. 2010, Jansen et al. 2010). It has also been noted that initial isolation of SAV can be complex, and a degree of cell-culture selection or adaptation may be necessary in some cases (McLoughlin & Graham 2007).

There was no histological evidence of PD in common dab from Stonehaven Bay. However, heart lesions were described in this species, which were independent of the presence of SAV RNA. The reason why lesions were located in this organ in what were believed to be healthy fish is unknown. We hypothesise that viruses other than SAV could be targeting this tissue and may have contributed to the lesions recorded, as no persistent subclinical SAV infection would be detectable by RT-qPCR. A comprehensive cohabitation challenge study by Graham et al. (2011) compared isolates from all 6 subtypes using parameters that included lesion score and virus load. In that study the authors demonstrated that there are differences between isolates with regards to replication and shedding dynamics; for example, Subtype V fish were found at a lower frequency than Subtype I and III fish, and virus load was initially lower than in the latter subtypes, but exceeded Subtype I at later times. From the dab examined in the current study only Subtype V was recovered from fish collected in Stonehaven Bay; hence, no comparable histology was available to examine the frozen dab that were PCR positive for Subtype I or II. As common dab are able to tolerate brackish waters and may enter freshwater river systems in coastal areas, they could encounter subtypes other than those frequently attributed to marine salmon. Snow et al. (2010) noted the occurrence of SAV RNA by RT-qPCR in pooled tissue from the long rough dab *Hippoglossoides platessoides*, common dab and the plaice *Pleuronectes platessa*; therefore, further research is required to understand the relevance of common dab and other flatfish as a wild reservoir of SAV and to assess the

potential of horizontal transmission with other wild or farmed fish. Such understanding would be advantageous in order to improve management practices on farms, as well as to interpret virus evolution and adaptation to new hosts.

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