Vol. 56: 11–24, 2003 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published August 15

Emergence and maintenance of infectious salmon anaemia virus (ISAV) in Europe: a new hypothesis

A. Nylund[1,*], M. Devold[1], H. Plarre[1], E. Isdal[2], M. Aarseth[1]

[1]Department of Fisheries and Department of Marine Biology, University of Bergen, 5020 Bergen, Norway
[2]Intervet Norbio, 5020 Bergen, Norway

ABSTRACT: The present study describes the use of molecular methods in studying infectious salmon anaemia virus (ISAV), an important pathogen of farmed salmon in Norway, Scotland, the Faeroe Islands, Canada, USA and Chile. The nucleotide sequences of the haemagglutinin gene (HA) from 70 ISAV isolates have been analysed for phylogenetic relationship and the average mutation rate of nucleotide substitutions calculated. The isolates constitute 2 major groups, 1 European and 1 North American group. The isolate from Chile is closely related to the North American isolates. The European isolates can be further divided into 3 separate groups reflecting geographical distribution, time of collection, and transmission connected with farming activity. Based on existing information about infectious salmon anaemia (ISA) and new information emerging from the present study, it is hypothesised that: (1) ISAV is maintained in wild populations of trout and salmon in Europe; (2) it is transmitted between wild hosts mainly during their freshwater spawning phase in rivers; (3) wild salmonids, mainly trout, possibly carry benign wild-type ISAV isolates; (4) a change (mutation) in virulence probably results from deletions of amino acid segments from the highly polymorphic region (HPR) of benign wild-type isolates; (5) ISA emerges in farmed Atlantic salmon when mutated isolates are transmitted from wild salmonids or, following mutation of benign isolates, in farmed salmon after transmission from wild salmonids; (6) farming activity is an important factor in transmission of ISAV between farming sites in addition to transmission of ISAV from wild salmonids to farmed salmon; (7) transmission of ISAV from farmed to wild salmonids probably occurs less frequently than transmission from wild to farmed fish due to lower frequency of susceptible wild individuals; (8) the frequency of new outbreaks of ISA in farmed salmon probably reflects natural variation in the prevalence of ISAV in wild populations of salmonids.

KEY WORDS: Infectious salmon anaemia virus · ISAV · Maintenance · Reservoir · Epizootics

INTRODUCTION

Molecular methods have become important tools in the study of the diversity, maintenance and dissemination of viral diseases (Hungnes et al. 2000). In order to understand the spreading of fish diseases, comparisons of gene sequences from different isolates of fish viruses have been presented in several publications (Aspehaug et al. 1999, Snow 1999, Dalla Valle et al. 2001, Krossøy et al. 2001b, Weston et al. 2002). In these studies, gene sequences were used to deduce phylogenetic relationships between the different isolates and thus trace viral evolution and routes of dissemination.

The infectious salmon anaemia virus (ISAV) has a worldwide distribution, occurring in most salmon-producing countries (Thorud & Djupvik 1988, Mullins et al. 1998, Rodger et al. 1998, Rowley et al. 1999, Bouchard et al. 2001, Kibenge et al. 2001a, Raynard et al. 2001a, Ritchie et al. 2001). It has a segmented genome (8 segments) and all segments have been sequenced, but the functional products of all genes have not yet been identified (Clouthier et al. 2002).

*Email: are.nylund@ifm.uib.no

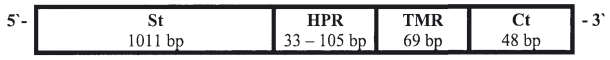

Fig. 1. Infectious salmon anaemia virus (ISAV) haemagglutinin gene. St: surface tail (5′-end flanking region or N-terminal region of the HPR); HPR: highly polymorphic region; TMR: transmembrane region; Ct: cytoplasmic tail (3′-end flanking region or carboxyl flanking region of TMR). Size of the different regions (except HPR) refers to the Norwegian ISAV Isolate H36/98 (Bømlo/Bremnes); HPR of European isolates may vary from 33 to 105 nucleotides (bp) or 11 to 35 amino acids

However, the ISAV haemagglutinin gene has been identified (Griffiths et al. 2001, Kibenge et al. 2001b, Krossøy et al. 2001a, Rimstad et al. 2001) and, based on other studies of orthomyxoviruses, this gene should be well suited for isolate comparisons (cf. Webster et al. 1992). The first studies using this gene from the ISAV have already been published (Devold et al. 2001, Kibenge et al. 2001b). The open reading frame (ORF) of the haemagglutinin gene of the ISAV ranges from about 1161 base pairs (bp) to about 1233 bp, depending on the length of a highly polymorphic region (HPR) (cf. Devold et al. 2001, Kibenge et al. 2001b, Krossøy et al. 2001a). This gene was used by Devold et al. to separate a selection of 36 European ISAV isolates. However, what seemed to be possible recombination events within the HA gene led Devold et al. (2001) to suggest that only about 900 bp at the 5′-end region of the gene, excluding the HPR and the 3′-end region, could be used in construction of the ISAV phylogeny.

The ISAV is considered to be a marine virus, causing disease in marine salmon farms and at smolt production sites that use seawater. It is also believed that transmission of the virus occurs in the marine phase of the salmon and, in most cases, is connected with farming activity. The present study presents a hypothesis suggesting that the maintenance of the ISAV is connected with wild salmonids in the freshwater phase and that the emergence of new isolates in salmon farms is a result of transmission from wild salmonids.

MATERIALS AND METHODS

The present study is based on sequences of the haemagglutinin gene (Fig. 1) from different ISAV isolates published by Devold et al. (2001), Kibenge et al.

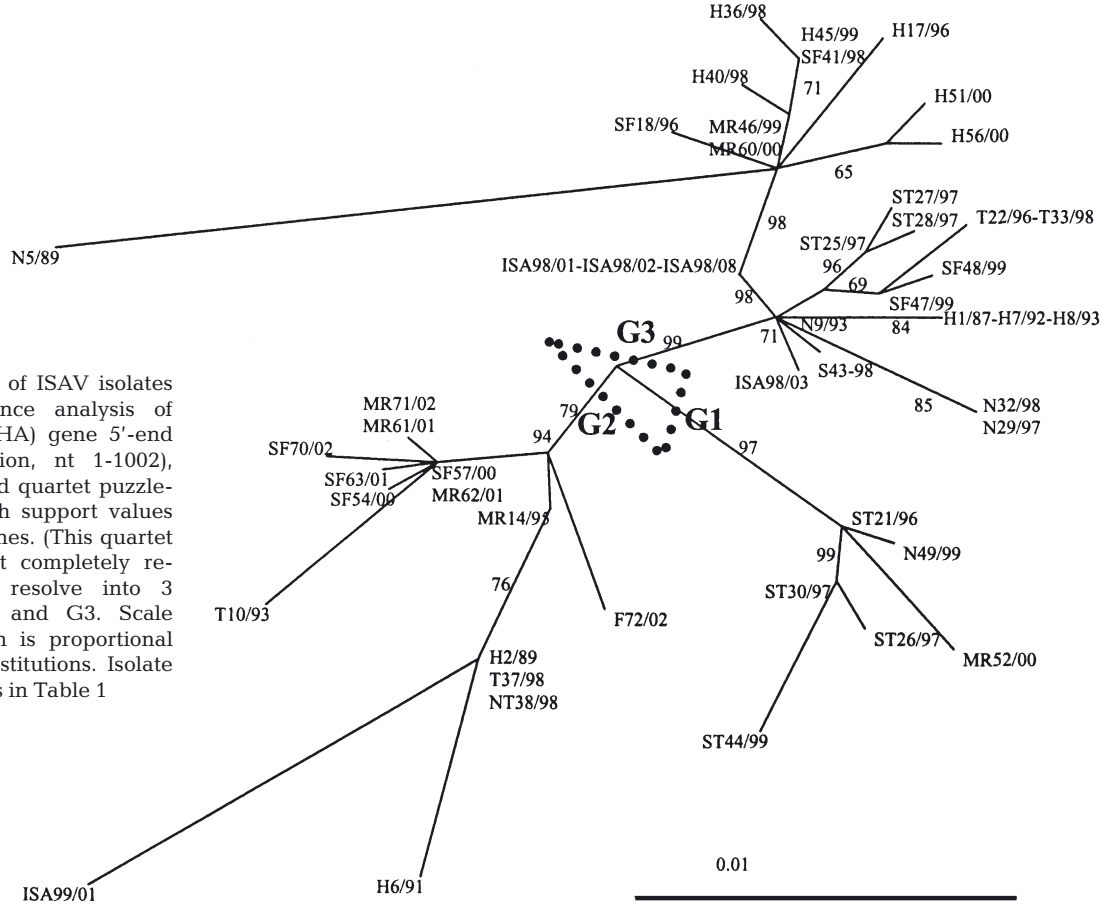

Fig. 2. Clustering of ISAV isolates based on sequence analysis of haemagglutinin (HA) gene 5′-end (surface tail region, nt 1-1002), showing unrooted quartet puzzle-tree topology with support values for internal branches. (This quartet puzzle-tree is not completely resolved.) Isolates resolve into 3 groups: G1, G2 and G3. Scale bar/branch length is proportional to number of substitutions. Isolate code nos. as in Table 1

(2001b), Krossøy et al. (2001a), Rimstad et al. (2001) and Clouthier et al. (2002). (For isolates and their respective accession numbers see Table 1.) In addition, the sequence of the HPR from an ISAV (accession no. AJ440971) present in wild salmon from Scotland (Scot-w), and the HA gene of 1 isolate from the Faeroe Islands and 8 new Norwegian isolates, are included. Sequences of the haemagglutinin gene from a total of 70 isolates were included in this study (see Table 1). The majority (79%) of these isolates were collected between 1996 and 2002, which means that the first 10 yr (1984 to 1993) of ISA infection in Norwegian aquaculture are poorly represented.

The new isolates included in this study were cultured in ASKV-cells (cf. Devold et al. 2000). The extraction of RNA, RT-PCR and sequencing were performed as described by Devold et al. (2001). It was, however, not possible to sequence the PCR products from Isolate N5/89: this product was cloned and 10 different clones from this isolate were sequenced.

The Vector NTI Suite software package (InforMax) was used for multiple alignment of partial nucleotide and protein sequences. To perform pairwise comparisons between the different sequences from the ISAV isolates, the multiple-sequence alignment editor GeneDoc was used.

In addition to software analysis of the sequences, the highly polymorphic region (HPR) of the haemagglutinin gene was manually aligned and compared for all isolates.

Phylogenetic analyses of the data sets were performed using PAUP* version 4.0 (Swofford 1998, Devold et al. 2001) and Puzzle 5.0 (cf. Aspehaug et al. 1999). All surface tail St regions (5′-end) of the HA sequences from the isolates (see Table 1) were included in the data set in the first phylogenetic analysis. This data set is not shown, but the 2 phylogenetic trees presented herein (see Figs. 2 & 3) are based on a selection from this data set. (Fig. 2 includes all isolates from Norway, Scotland and the Faeroe Islands presented in Table 1, while Fig. 3 is based on an analysis of only those isolates that are included in that figure.) Exclusion of the Canadian isolates did not change the internal relationship between the European isolates (see Fig. 2), nor did exclusion of isolates change the major branches of the tree presented (see Fig. 3). Phylogenetic analyses including both the St region and the 2 short regions transmembrane (TMR) and cytoplasmic tail (Ct) did not change the phylogenies (phylogenetic tree not presented). To secure homology of the sequences used in the phylogenetic analyses, only the St region was used. Inclusion of the HPR in the analysis would change the phylogeny and result in a tree that, to a large extent, would be dominated by the different HPR groups (Devold et al. 2001). Phylogenetic trees were drawn using TreeView (Page 1996).

A recommended method of estimating the rate of nucleotide substitutions is to examine the phylogenetic relationship of genes from different isolates and then use only isolates that are closely related by descent (Saitou & Nei 1986). The rate of nucleotide substitu-

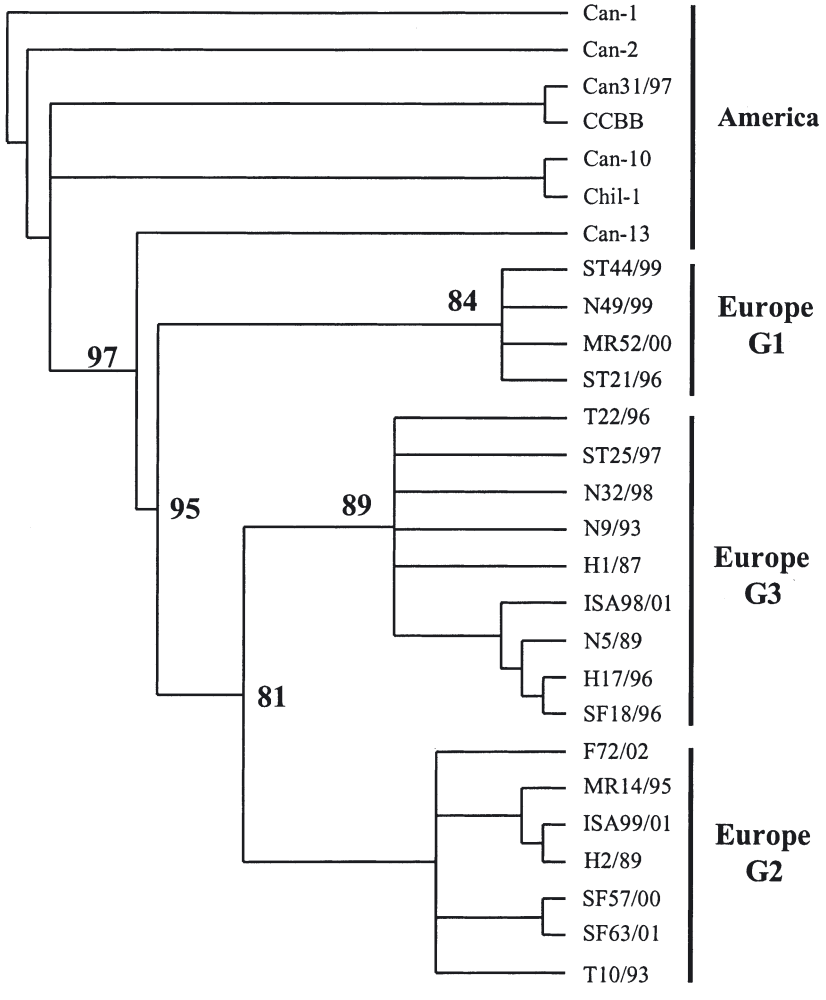

Fig. 3. Clustering of ISAV isolates based on sequence analysis of HA gene 5′-end (surface tail region, nt 1-1002). The unrooted quartet puzzle-tree topology with support values for major internal branches. Isolates resolve into 1 American group and 3 European groups (G1, G2 and G3). Isolate code nos. as in Table 1

Table 1. Overview of infectious salmon anaemia virus (ISAV) isolates, accession numbers of haemagglutinin gene sequences and number of highly polymorphic regions (HPR). Code number given to isolates and year of collection

Locality	Year	Code	HPR	Accession no.	Source
NORWAY					
Hordaland					
Eikelandsosen	1987	H1/87	1	AF364893	Devold et al. (2001)
Golten	1989	H2/89	2	AF220607	Rimstad et al. (2001)
Sotra	1991	H6/91	2	AF364894	Devold et al. (2001)
Sotra	1992	H7/92	1	AF364898	"
Sotra	1993	H8/93	1	AF309075	Krossøy et al. (2001a)
Varaldsøy	1996	H17/96	7	AF364891	Devold et al. (2001)
Bømlo (Bremnes)	1998	H36/98	7	AF302799	Krossøy et al. (2001a)
Strandebarm	1998	H40/98	7	AF364877	Devold et al. (2001)
Mundheim	1998	H45/99	7	AF364870	"
Øygarden	2000	H51/00	7	AF364882	"
Sørnes	2000	H56/00	7	AF364880	"
Sogn og Fjordane					
Landøy	1996	SF18/96	2	AF364869	"
Gulen	1998	SF41/98	7	AF364871	"
Skatestraumen	1999	SF48/99	8	AF364878	"
Nordfjord	1999	SF47/99	9	AF364888	"
Solund	2000	SF54/00	11	AF364884	"
Fjaler	2000	SF57/00	11	AF364890	"
Segløy (Gulen)	2001	SF63/01	11	AY127879	Present study
Leiholmane (Gulen)	2002	SF70/02	11	AY127880	"
Møre og Romsdal					
Selje	1995	MR14/95	5	AF364873	Devold et al. (2001)
Misund	1999	MR46/99	8	AF364896	"
Lepsøy	2000	MR52/00	10	AF364892	"
Ørskog	2001	MR60/01	6	AY127876	Present study
Smøla	2001	MR61/01	11	AY127877	"
Herøy	2001	MR62/01	11	AY127878	"
Brunsvik	2002	MR71/02	11	AY127881	"
Sør Trønderlag					
Hitra	1996	ST21/96	6	AF364886	Devold et al. (2001)
Hitra	1997	ST26/97	6	AF364879	"
Frøya	1997	ST25/97	6	AF364885	"
Frøya	1997	ST27/97	6	AF364897	"
Åfjord	1997	ST28/97	6	AF364875	"
Hestvika	1997	ST30/97	6	AY127875	Present study
Hitra	1999	ST44/99	6	AF302803	Krossøy et al. (2001a)
Nord Trønderlag					
Nærøy	1998	NT38/99	2	AF364874	Devold et al. (2001)
Nordland					
Meløy	1989	N5/89	12	AY127882	Present study
Vestvågøy	1993	N9/93	3	AF364895	Devold et al. (2001)
Torgnes	1997	N29/97	7	AF364872	"
Dønna	1998	N32/98	7	AF364883	"
Henningsvær	1999	N49/99	7	AF364876	"
Troms					
Gullesfjord	1993	T10/93	4	AF302801	Krossøy et al. (2001a)
Senja	1996	T22/96	2	AF364889	Devold et al. (2001)
Senja	1998	T33/98	2	AF364887	"
Blåmannsvik	1998	T37/98	2	AF364881	"
Faeroe Islands					
Færøyene	2002	F72/02	3	AF536263	Present study
SCOTLAND					
Loch Nevis	1998	Scot43/98	7	AF302803	Krossøy et al. (2001a)
390/98	1998	ISA98/01	7	AF283997	Kibenge et al. (2001b)
390/98	1998	ISA98/01	7	AJ276859	Rimstad et al. (2001)
832/98	1998	ISA98/02	7	AF388582	Kibenge et al. (2001b)
912/99	1999	ISA98/08	7	AF395337	"
301/98	1998	ISA98/03	7	AF388581	"
1490/98	1998	ISA99/01	2	AF391126	"
Wild salmon	1999	Scot-w	0	AJ440971	Cunningham et al. (2002)

Table 1 (continued)

Locality	Year	Code	HPR	Accession no.	Source
CANADA					
Bay of Fundy	1997	Can31/97	21	AF302800	Krossøy et al. (2001a)
CCBB		CCBB	21	AF404342	Clouthier et al. (2002)
RPC/NB-980 280-2		Can-1	20	AF294870	Kibenge et al. (2001b)
RPC/NB-980 028-10		Can-2	21	AF294871	"
DFO-1		Can-3	21	AF294872	"
RPC/NB-980 458-1		Can-4	21	AF294873	"
RPC/NB-990 508-3		Can-5	21	AF294874	"
RPC/NB-990 002-1		Can-6	21	AF294875	"
RPC/NB-980 049-1		Can-7	21	AF284876	"
RPC/NB-970 877-2		Can-8	21	AF294877	"
Back Bay 98	1998	Can-9	21	AF283995	"
HKS-36		Can-10	21	AF294878	"
NBISA01		Can-11	21	AF283996	"
RPC/NB-990 681-3		Can-12	21	AF294880	"
U5575-1		Can-13	3[a]	AF294881	"
GA/TO Fish03		Can-16	21	AF297551	"
Canada		Can-15	21	AX083268	Griffiths & Ritchie (GenBank patent)
CHILE					
7833-1		Chil-1	21	AF294879	Kibenge et al. (2001b)

[a]Similar to European isolate HPR3

tions, based on all nucleotide mutations in the flanking regions (St, TMR and Ct) of the HPR of the haemagglutinin gene, for isolates that clustered together in the phylogenetic tree was calculated using the Kimura 2- and 6-parameter test (Gojobori et al. 1990). The estimated average mutation rate (assumed to represent a constant rate of nucleotide substitutions) was then used to calculate a divergence date between the Norwegian and Canadian isolates based on this gene.

The nomenclature for classification of the different ISAV isolates based on the HPR is as described by Devold et al. (2001).

RESULTS

The accession numbers of the new ISAV isolates from Norway and the Faeroe Islands are given in Table 1. The highly polymorphic region (HPR) of the European isolates varies in length between 11 and 35 amino acids, where the maximum number of amino acids is found in HPR0 (ISAV from wild salmon in Scotland, Scot-w) and the lowest number in HPR8 (Isolates SF48/99 and MR46/99 from Norway). In a study of Canadian ISAV isolates, 2 HPR groups (HPR20 and HPR21) consisting of 28 and 18 amino acids, respectively, were found (Griffiths et al. 2001, Kibenge et al. 2001b). The amino acid sequences of the HPR groups from Canadian isolates are slightly different from those of the European isolates. KIRVDAI is replaced by NNRVDAI, FNTN by LGVN, PA by PS, and TVSL by TVSP (Table 2).

The amino acid sequence of the 5′-end flanking region (St) to the HPR from European isolates (cf. Table 1) contains 31 variable sites (9.3%) of a total of 334 amino acids. The first 3 amino acids of the protein are not included in the alignment. Most of the amino acid variation is accounted for by 8 variable sites located between Sites 9 and 49 and at 10 variable sites between 313 and 338 relating to the haemagglutinin protein of Isolate H36/98. The number of variable amino acid sites between isolates within the different HPR groups are given in Table 3.

The number of variable nucleotide sites within the 5′-end flanking region (St) was 83 out of 1002 (8.3%). The first 9 nucleotides of the ORF are not included in the alignment. Much of the nucleotide variation in the 5′-end flanking region is concentrated between Positions 82 and 141 (12 variable sites) and between 936 and 1011 (19 variable sites) relating to the ORF of Isolate H36/98.

Calculations of the mutation rate for the isolates that clustered together in the phylogenetic analysis (Fig. 2) indicate an average substitution rate of 5.0×10^{-4} nucleotides site^{-1} yr^{-1} for both the 2- and 6-parameter test, which is to be expected when the data set contains a small number of substitutions (Gojobori et al. 1990). When this mutation rate is used to calculate the divergence date between the Norwegian and Canadian isolates, indications are that this divergence might have happened around 200 yr ago.

The ISAV isolates can be classified into 2 major groups, an American group and a European group

Table 2. Overview of different highly polymorphic region (HPR) groups of infectious salmon anaemia virus (ISAV) and HPR lengths. St: surface tail; TMR: transmembrane region. HPR0 is from wild salmon in Scotland. aa: amino acid; aa no.: amino acid number. Bold-face indicates amino acid substitutions

	St	Highly polymorphic region (HPR)																													TMR		
Europe	T	D	V	K	I	R	V	D	A	I	P	P	Q	L	N	Q	T	F	N	T	N	Q	V	E	Q	P	A	T	S	V	L	S	N	I	F	I	S	M	G	V	A
aa no.:				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35			
HPR0	T	D	V	K	I	R	V	D	A	I	P	P	Q	L	N	Q	T	F	N	T	N	Q	V	E	Q	P	A	**N**	S	V	L	S	N	I	F	I	S	M	G	V	A
HPR1	T	D	V	K																						P	A	T	S	V	L	S	N	I	F	I	S	M	G	V	A
HPR2	T	D	V	K	I	R	V	D	A	I	P	P	Q	L	N	Q	T																					M	G	V	A
HPR3	T	D	V	K	I	R	V	D	A	I	P	P	Q	L	N	Q	T																		F	I	S	M	G	V	A
HPR4	T	D	V	K	I	R	V	D	A	I	P	P	Q	L	S	N	I																		F	I	S	M	G	V	A
HPR5	T	D	V	K	I	R	V	D	A	I	P	P	Q	L																						I	S	M	G	V	A
HPR6	T	D	V	K	I	R	V	D	A	I												Q	V	E	Q	P	A	T	S	V	L	S	N	I	F	I	S	M	G	V	A
HPR7	T	D	V	K																								T	S	V	L	S	N	I	F	I	S	M	G	V	A
HPR8	T	D	V	K	I	R	V	D	A	I	P	P	Q	L																										G	V	A
HPR9	T	D	V	K	I	R	V	D	A	I	P	P	Q	L	N	Q	T	F	N	T																		M	G	V	A
HPR10	T	D	V	K	I	**K**																Q				P	A	T	S	V	L	S	N	I	F	I	S	M	G	V	A
HPR11	T	D	V	K	I	R	V	D	A	I	P	P																				**R**	N	I	F	I	S	M	G	V	A
HPR12	T	D	V	G	I	R	V	D	A	I	P	P	Q	L	N	Q	T																N	I	F	I	S	M	G	V	A
HPR13	T	D	V	K																				E	Q	P	A	N	S	V	L	S	N	I	F	I	S	M	G	V	A
America																																										
HPR20	T	D	V	**N**	**N**	R	V	D	A	I								**L**	**G**	**V**	N	Q	V	E	Q	P	**S**	T	S	V	**P**	S	N	I	F	I	S	M	G	V	A
HPR21	T	D	V	**N**	**N**	R	V	D	A	I	P	P	Q	L																		S	N	I	F	I	S	M	G	V	A

(Fig. 3). The similarity between the European isolate N49/99 and the Canadian isolate CCBB, at the protein and nucleotide level, is less than 90% (Table 3). One Canadian isolate, Can-13 (Nova Scotia HPR3), is however similar to the European isolates. This Canadian isolate is not included in the phylogeny of the European isolates since the sequence deposited in the GeneBank (Accession No: AF294881) is shorter that those used in the phylogenetic analysis of the European isolates. The numbers of amino acid and nucleotide differences between Can-13 and F72/02 are given in Table 3.

The European isolates can be further divided into distinct groups with high support values. The phylogeny resulting from comparison of the St sequences (5′-end) shows that European isolates resolve into 3 groups, G1 to G3 (Fig. 2); 2 of these groups can be further divided into 2 subgroups each.

The smallest group (G1) consists of 6 Norwegian isolates collected between 1996 and 2000 (support value SV = 97). The majority of these isolates (N = 4) come from Sør-Trønderlag (ST) and belong to HPR6; one of the other isolates comes from a neighbouring area (Møre og Romsdal, MR52/00: HPR10), while the last isolate (N49/99: HPR7) comes from Nordland (N), northern Norway.

Group G2 (SV = 79) consists of 15 isolates including 1 from Scotland (ISA99/01). This group can be subdivided into 1 group consisting of 7 isolates and another consisting of 8 isolates. The latter group contains 7 isolates (all HPR11), mostly from western Norway, collected between 2000 and 2002, and 1 isolate from northern Norway collected in 1993 (T10/93: HPR4). The support value for this subgroup is only 54. The other subgroup (SV = 94) contains isolates collected between 1989 and 2002 from Scotland, the Faeroe Islands and several different areas in Norway (N, NT, MR, H). This group includes 3 different HPR groups (HPR2, 3, 5). The isolates from the Faeroe Islands (F72/02: HPR3) and Møre og Romsdal (MR14/95: HPR5) emerge as a sister group to the remaining isolates in this clade (all HPR2). Within the latter group the Scottish isolate (ISA99/01) contains most nucleotide substitutions (Table 3).

The largest of the 3 groups (G3, SV = 99) consists of 29 isolates with 15 in one subgroup and 14 in the other subgroup. The isolates were collected between 1987 and 2000. The larger of these 2 subgroups contains the oldest isolate available (H1/87). This isolate can be connected to the first official outbreak of ISA in Norway and groups closely together with 2 other isolates from the same county (H7/92 and H8/93), all 3 belonging to HPR1. The other isolates within this subgroup come from different parts of Norway (T, N, ST and SF) and Scotland, and belong to 6 different HPR groups (2, 3, 6, 7, 8, and 9). The relationship between these isolates is not fully resolved, but some of them group together with high support values. The smaller of the 2 subgroups contains isolates from both Norway and Scotland. Most of the isolates within this subgroup belong to HPR7 (Table 1, Fig. 2). The Scottish isolates, which all belong to HPR7, stand apart

from the Norwegian isolates within the same HPR group. Isolates ISA98/01, ISA98/02 and ISA98/08 are nearly identical and slightly different from 2 other Scottish isolates (Scot-43/98 and ISA98/03) that group together. The Norwegian isolates that do not belong to HPR7 belong to 4 different HPR groups (HPR 2, 6, 8 and 12) and were collected from 3 different counties in Norway (SF, MR, N).

The HPR7 contains 15 different isolates collected in Norway and Scotland. The relationships among these isolates reflect their geographical origin. The isolates from western Norway (H17/96, H36/98, H40/98, SF41/98, H45/99, H51/00 and H56/00) group together and are more closely related to the Scottish isolates within the same HPR group than to the isolates from northern Norway (N29/97, N32/98 and N49/99).

Table 3. Overview of amino acid and nucleotide variation in surface tail region of HA within different HPR groups containing several isolates. Support values (SV) are from phylogenetic analysis of all European ISAV isolates using Puzzle (ver 5.0). No.: number of nucleotides and amino acids compared; %: percent similarity among isolates. Isolate code nos. as in Table 1. SV < 70 are not given(marked by dashes) as only SV > 70 should be trusted

HPR group	No. of isolates	SV	Amino acids No.	Amino acids %	Nucleotides No.	Nucleotides %	Years of isolation
HPR1							
H1/87, H7/92, H8/93	3	84	0–334	100	0–1002	100	1987–1993
HPR2							
H2/89, H6/91, SF18/96, T22/96, T33/96, T37/98, NT38/98, ISA99/01	8	–	13–334	96.10	28–1002	97.02	1989–2001
H2/89, H6/91, SF18/96, T22/96, T33/96, T37/98, NT38/98	7	–	8–334	97.60	19–1002	98.10	1989–1998
H2/89, H6/91, T37/98, NT38/98, ISA99/01	5	76	7–334	97.90	12–1002	98.80	1989–1999
H2/89, H6/91, T37/98, NT38/98	4	–	2–334	99.40	4–1002	99.60	1989–1998
HPR6							
ST21/96, ST25/97, ST26/97, ST27/97, ST28/97, ST30/97, ST44/99, MR60/01	8	–	9–334	97.30	20–1002	98.00	1996–2001
ST21/96, ST26/97, ST30/97, ST44/99,	4	91	3–334	99.10	5–1002	99.50	1996–1999
ST25/97, ST27/97, ST28/97	3	96	2–334	99.40	2–1002	99.80	1997
HPR7							
H17/96, H36/98, H40/98, H45/99, H51/00, H56/00, SF41/98, N29/97, N32/98, N49/99, S43/98, ISA98/01, ISA98/02, ISA98/03, ISA98/08	15	–	9–334	97.30	27–1002	97.30	1996–2000
H17/96, H36/98, H40/98, H45/99, H51/00, H56/00, SF41/98	7	–	2–334	99.40	8–1002	99.20	1996–2000
N29/97, N32/98	2	85	0–334	100.00	0–1002	100	1997–1998
S43/98, ISA98/01, ISA98/02, ISA98/03, ISA98/08	5	–	2–334	99.40	3–1002	99.70	1998
HPR11							
SF54/00, SF57/00, SF63/01, SF70/02, MR61/01, MR62/01, MR71/02	7	–	1–334	99.70	5–1002	99.50	2000–2002
Mixed HPR							
ST21/96, ST26/97, ST30/97, ST44//99, N49/99, MR52/00	6	97	5–334	98.50	9–1002	99.10	1996–2000
H2/89, H6/91, MR14/95, T37/98, NT38/98, ISA99/01, F72/02	7	94	10–334	97.00	19–1002	98.10	1989–2002
H17/96, H36/98, H40/98, H45/99, H51/00, H56/00, SF41/98, SF18/96, MR46/99, MR60/00, N5/89	11	98	9–334	97.30	23–1002	97.70	1989–2000
N49/99, CCBB	2	–	45–334	86.52	112–1002	88.82	1998–1999
F72/02, Can-13	2	–	11–321	96.57	20–966	97.92	1998–2002

Isolates within HPR11 (N = 7) have a limited geographical distribution, being present in 2 neighbouring counties (SF and MR) only. They have been collected between 2000 and 2002. Other HPR groups (HPR2 and HPR6) that contain several isolates show a slightly different pattern. HPR6 contains 8 isolates collected from a very small area in Sør-Trønderlag (ST) and Møre og Romsdal (MR). These isolates belong to 3 different distinct phylogenetic groups; ST25/97, ST27/97, ST 28/97 (larger subgroup within G3), MR60/01 (smaller subgroup within G3), and ST21/96, ST26/97, ST30/97, ST44/99 (within G1). The 8 isolates that belong to HPR2, with the exception of SF18/96, T22/96 and T33/98, group together (SV = 76), but come from different geographical areas (H, NT, T and Scotland) (Fig. 2). The Scottish isolate (ISA99/01) stands somewhat apart from the other 4 isolates.

The other HPR groups (HPR 4, 5, 9, 10 and 12) contain only 1 isolate each, while HPR1, HPR3 and HPR8 contain 3, 2 and 2 isolates, respectively. The isolates in HPR1 group closely together, while the 2 geographically closely related isolates in HPR8 belong to 2 different subgroups within the G3 group. The 2 isolates within HPR3 come from the Faeroe Islands (F72/02) and Nordland (N9/93) and show no close phylogenetic relationship (Fig. 2).

DISCUSSION

The phylogenetic analysis based on the 5′-end flanking (St) region to the highly polymorphic region (HPR) of the haemagglutinin gene shows that the ISAV isolates cluster in 2 major groups. One group contains all the North American isolates with the exception of 1 isolate from Nova Scotia (Kibenge et al. 2001b, Ritchie et al. 2001), while the other contains European isolates collected from Norway, Scotland, the Faeroe Islands and Nova Scotia, Canada. This is consistent with earlier studies of ISAV isolates (Blake et al. 1999, Cunningham & Snow 2000, Inglis et al. 2000, Devold et al. 2001, Kibenge et al. 2001b, Krossøy et al. 2001a,b). The European isolates can be further divided into 3 groups with relatively high support values (Fig. 2). The first group (G1) contains a few Norwegian isolates only, while the second group (G2) contains, in addition to Norwegian isolates, 1 isolate from the Faeroe Islands and 1 from Scotland (ISA99/01). The third group contains Scottish and Norwegian isolates, but the relationships among these isolates are partly unresolved. The isolate from Nova Scotia (Can-13) has not been included in the phylogeny of European isolates, but is a sister-group to the European isolates (Fig. 3).

Possible mechanism for change in virulence of ISAV

The mutation rate for the HA gene (based on the flanking regions to the HPR) was calculated to be $5.0 ¥ 10^{-4}$ nucleotides site^{-1} yr^{-1}, which is slightly lower compared to the mutation rate reported for the polymerase gene PB1 ($0.96 ¥ 10^{-3}$ nucleotide yr^{-1}) from the ISAV (Krossøy et al. 2001b). Differences in mutation rate are not surprising since each virus gene may evolve differently because of different selective pressures and evolutionary constraints (Webster et al. 1992). The HA may be subjected to strong selection pressure from neutralising antibodies of the host immune system and may evolve more rapidly compared to the polymerases. Hence, it is surprising that the HA gene from ISAV evolved more slowly than PB1. This low mutation rate of the St region (part of the HA, protruding from the surface of infective virus particles, exposed to antibodies, possibly neutralising, produced by the host) of the HA gene could indicate that the ISAV has a different mechanism of counterselection by the host immune system. One possible explanation is that changes in the HPR result in changes in the virulence of the ISAV. Specifically, that deletion of amino acid segments from what may be a non-pathogenic wild-type HPR0 may change the virulence. This wild-type virus may have 'molecular mimicry', or only be able to propagate in a limited number of target cells (partly hidden from the host immune system), or simply produce a very low number of infective particles. Deletion, or a series of deletions, of amino acids from the HA may change the charge of the protein or the tertiary structure resulting in a change in the exposure of epitopes and binding to target cells.

HPR0 has been sequenced from ISAV in wild Atlantic salmon from Scotland and consists of 35 amino acids, representing the longest stretch of amino acids among the HPR groups (Cunningham et al. 2002) (Table 2). Comparison of the other HPR groups with HPR0 reveals that all HPR groups can be understood as a result of deletions of amino acid segments from the full-length HPR0 sequence. There is no longer a need for possible recombination events to explain the variability of HPR found in European isolates (cf. Devold et al. 2001). Each HPR group could represent separate mutation events, i.e. deletions of nucleotide segments from a wild-type HPR0 isolate. If the same sequences are deleted in distantly related isolates, the result would be isolates with similar HPR group, but not closely related when comparing the 5′-end flanking regions. This would explain why isolates within HPR7 from different regions in Norway do not group closely together. These isolates could represent more than 1 mutation event in different geographical areas with different HPR0 isolates as a starting point. The result-

ing pattern would be slightly confused by transportation of ISAV isolates due to farming activity. Transportation by human activity may explain why isolates with the same HPR in the same area may be distantly related (e.g. HPR6), while isolates from different geographical areas but with the same HPR may be closely related.

So far, 13 different HPR groups (HPR0 to HPR12) have been found in Europe. The HPR0 was obtained from a wild salmon with no clinical signs of ISA (Cunningham et al. 2002). Benign ISAV isolates (non-pathogenic when injected into ISAV-free salmon) have been found in salmon farms and rivers on the west coast of Norway, but these isolates have so far not been characterised (A. Nylund pers. obs.). If the HPR0 represents a non-pathogenic wild type, then this will have important implications for the understanding and interpretation of the pathogenic ISAV isolates from commercial salmon farms. The European HPR groups (the pathogenic isolates from salmon farms) can be interpreted as 'offspring' from such a wild-type and may represent a shift towards more virulent types of isolates. This would mean that each new HPR group could represent a new transmission from wild fish to farmed fish. If this hypothesis is correct, then the European ISAV isolates from farmed salmon studied so far would represent a minimum of 21 transitions from wild salmonids to farmed salmon according to the recorded HRP groups and the phylogeny (Fig. 2). Those cases where isolates with similar HPR show close phylogenetic relationships could represent a single transmission from wild to commercial fish, and later transmission of the isolate between commercial sites or several separate transmission from the same wild-type stock. If this hypothesis is correct, then one would also expect to find isolates that are distantly related within the same HPR group, i.e. several distinct wild-type isolates/strains occurring in the wild populations of salmonids in Europe. A support for this hypothesis can be found in the pattern (time and space) of HPR groups and the relationship within the HPR groups.

Based on the calculated substitution rate (HA gene), the divergence date between the European and American isolates was calculated to be around 1800, which is 100 yr earlier than the date suggested by Krossøy et al. (2001b). However, one should be cautious about this dating for several reasons: evolutionary rates are not always linear with time (Scholtissek et al. 1993); the timescale of virus isolation and the number of isolates included will probably affect estimated evolutionary rates; different gene segments may also have different evolutionary rates, and genetic shift may occur in viruses with segmented genomes (Webster et al. 1992). As suggested above, the HA gene of the ISAV may have a mechanism for overcoming the host immune system that differs from that of other orthomyxoviruses. Changes in the HPR may buffer the selections pressure on the HA gene and result in a lower mutation rate of the St region of this gene. If, on the other hand, the mutation rate calculated for the HA gene is valid, then the ISAV in America and Europe separated before transportation (1883 to 1884) of salmonid fishes between these 2 regions took place (Rasmussen 1967, Courtney et al. 1984, Welcomme 1988, Krossøy et al. 2001b). Future studies on other gene segments from ISAV isolates may give additional information about the separation of American and European isolates.

New hypothesis for maintenance and emergence of ISAV in Europe

A good understanding of how new isolates of ISAV emerge in salmon culture systems and how ISAV isolates are maintained in salmonid populations (wild and cultured) is a prerequisite for a proper management of this disease. Since ISA first emerged in Norwegian salmon culture in 1984 (Thorud & Djupvik 1988), much knowledge has accumulated, and studies of the haemagglutinin gene have demonstrated a pattern of relationship among isolates from different outbreaks. Based on this information it is now possible to build a hypothesis that may explain the maintenance of the ISAV in wild and cultured salmonid populations and the emergence of ISA in the different affected salmon-farming areas.

Possible wild hosts and maintenance of the ISAV

The official history of the ISAV goes back to 1984, but this virus must be much older, being a distant relative of other viruses in the family *Orthomyxoviridae* (Krossøy et al. 1999). Based on the mutation rate of the RNA-dependent RNA polymerase (ISAV Segment 2) the separation between the North American and European isolates was suggested to have occurred between 1840 and 1920 (Krossøy et al. 2001b, Krossøy 2002). The present study indicates an even earlier separation. It is also well documented that most salmonids (*Salmo* spp., *Oncorhynchus* spp. and *Salvelinus alpinus*) may serve as hosts for the ISAV (Nylund et al. 1995a,b,c, 1997, Rolland & Nylund 1998a, Devold et al. 2000, Kibenge et al. 2001a, Snow et al. 2001). Based on this knowledge, it should be fairly safe to assume that the ISAV has a long history connected to 1 or several of these salmonids.

Norwegian lakes and river systems constitute a natural environment for 3 of these salmonids (*Salmo salar*, *S. trutta* and *Salvelinus alpinus*), and the Norwegian

natural populations of salmon and trout are probably the largest in Europe. It should be fairly safe to assume that the ISAV has been present in 1 or several of the local populations of salmon and trout from different Norwegian river systems and that there has been a co-evolution between the virus and its hosts. This must have been the situation before salmon farming became an important industry in Norway.

Enveloped viruses are relatively unstable outside a competent host, and for a virus to remain in circulation it must be able to move efficiently among different hosts and/or to persist in an individual host (Knipe & Howley 2001). Requirements for the maintenance of a virus in an area include a steady supply of susceptible hosts or mechanisms that can secure persistence of the virus within infected individuals. In the individual host, evasion of immunity is necessary to permit virus persistence. Hence, the number of host species and the density, size and distribution pattern of host populations are important factors for the maintenance and spread of viruses. The population structure of salmonids is strongly influenced by anadromous behaviour. The highest population densities are found in rivers and lakes, which means that salmon and trout in Norway (and other Europeans countries) occur in a multitude of relatively small and partly isolated populations. The most frequent interactions between salmonids occur during spawning in rivers. The rivers are also the rearing areas for susceptible fry, parr and smolts, i.e. individuals that can be expected to lack acquired immunity towards viral diseases. The population densities in the open ocean (salmon) and in fjords (trout and salmon) will in most cases be significantly lower than in rivers. Based on these facts it is expected that a virus which has co-evolved with salmon/trout should be adapted to transmission in the freshwater phase, i.e. in the rivers and preferably during spawning when the chances of interactions between individuals are highest. In addition to breeding interactions, it is also well documented that eggs are eaten by other fishes present in the breeding grounds. This includes all individuals from breeding fish to fry, parr and smolts.

It has been thoroughly documented that the ISAV can be transmitted in both fresh- and seawater and it has been shown that the virus can be present in mucus, urine, faeces and ovarian fluids of infected individuals (Thorud & Djupvik 1988, Thorud 1991, Nylund et al. 1993, 1994a,b, 1995a,b,c, 1997, 1999, Hovland et al. 1994, Nylund & Jakobsen 1995, Totland et al. 1996, Rolland & Nylund 1998a,b, Jones et al. 1999, Melville & Griffiths 1999, Devold et al. 2000, Jones & Groman 2001, Raynard et al. 2001b, Snow et al. 2001). These studies have also shown that the ISAV may enter via the gills and probably also through wounds involving salmon lice. It should therefore be expected that before the start of salmon culture, transmission of the ISAV most probably occurred mainly in rivers. The virus may also have been transmitted via pre-adult and adult salmon lice in fjords with high densities of trout. The latter could have been an important mechanism for transmission of ISAV between different river systems or river populations of salmonids. Another mechanism for transmission between river systems would be the return of ISAV-carrying salmonids to rivers other than their home river.

Growth and onset of maturation in the open ocean is an important character separating salmon from trout, and means that exposure of the salmon to fresh infection of ISAV will occur at a much lower frequency than for trout. Hence, the ISAV could be closer adapted to trout as a host species than to salmon (Nylund et al. 1994a, 1995a, Nylund & Jakobsen 1995, Rolland & Nylund 1998a, Devold et al. 2000). It has been documented that the production of ISAV in trout increases during stress and sexual maturation (Nylund et al. 1994a, 1995a, Rolland & Nylund 1998a, Devold et al. 2000). However, if adult salmon in some rivers become infected during spawning, then subsequent mortality will be of little consequence, since most salmon spawn only once. In addition, the salmon in most Norwegian rivers spawn approximately 1 mo later than trout. However, salmon fry, parr and smolt should have evolved some mechanism to increase the possibility of survival during ISAV exposure in rivers. These mechanisms could be behavioural (avoiding the breeding grounds) or innate, i.e. reduced susceptibility to ISAV infection in the river phase. At present there is little information available about the susceptibility of fry in freshwater, but in one experiment at our laboratory, first-feeding fry seemed to be less susceptible to ISAV than smolt and adult salmon (A. Nylund unpubl. data). Several researchers have also observed that salmon seem to be less susceptible to ISAV (or less likely to develop ISA) during the period September to December which covers the period when spawning occurs in the autumn (K. Falk pers. comm., A. Nylund pers. obs.). This could be an adaptation for protection against ISAV exposure during spawning. Another possible explanation is that all dominating ISAV isolates in wild fishes are benign to all salmonids (see above). When a pathogenic strain emerges in the river system, after mortality of the infected salmon it will soon be lost due to lack of a susceptible host because of acquired immunity of trout and surviving salmon. The pathogenic ISAV isolate will thus die out before it can be transmitted to new river systems.

The population structure of wild trout (*Salmo trutta*), small and partly isolated populations with highest density and interactions in rivers) should have had an important influence on the distribution pattern of ISAV

isolates. If transmission of ISAV has mainly occurred during spawning in rivers, then distinct river or river-system isolates may have developed. It is also well established that a virus that uses several hosts may develop distinct isolates/strains that are specific for each host as a result of co-evolution with the host (Knipe & Howley 2001). Since salmon and trout co-exist closely in the river systems, the evolution of host-specific strains are less likely in Europe. It is more likely that host-specific strains may have developed separately in North America and Europe, since the former is dominated by introduced *Oncorhynchus mykiss* and resident *S. salar*, while in the latter, rivers are dominated by both *S. trutta* and *S. salar*. This is partly supported by the large difference between American and European isolates (Blake et al. 1999, Cunningham & Snow 2000, Inglis et al. 2000, Devold et al. 2001, Kibenge et al. 2001b, Krossøy et al. 2001a,b, present study). However, this difference may simply be a result of very little gene flow between European and North American ISAV isolates. A hypothesis that places a major emphasis on river transmission of ISAV would result in a distribution pattern of ISAV isolates in Europe comprising mainly river-system-specific isolates. At the very least, the hypothesis would be supported by a cline of increasing difference between isolates from the south to the north of the distribution area of trout due to opposing evolutionary mechanisms such as mutations/drift/selection on the one hand and gene flow on the other. Support for this can be found in the presence of area-specific ISAV isolates in Europe (present study). The basic distribution pattern of ISAV isolates that emerges reflects both geographic area and time of isolation. In addition, claims of transmission of ISAV isolates between farms are supported (cf. Devold et al. 2001).

In our phylogenetic studies, some of the isolates within HPR groups are very similar, and cluster together with high support values, while other isolates within the same HPR show no close phylogenetic affinities (Table 1, Fig. 2). The pattern (distribution in time and space) of the HPR groups indicate, that the hypothesis of river-system-specific isolates could be correct. When isolates with a new HPR group are found within an area, later isolates of the same HPR-type within the same area are always closely related. A recent example is HPR11, which was first discovered in 2000 in Sogn og Fjordane. All later isolates with the same HPR in this county and in the neighbouring county (Møre og Romsdal) have been closely related. HPR7 is an example in which the isolates fall into several distinct phylogenetic groups. This HPR has been found in 16 isolates collected between 1996 and 2000 in 3 Norwegian counties and in Scotland. Seven of these isolates (H17/96, H36/98, H40/98, SF41/98, H45/98, H51/00 and H56/00) group together with a high support value; the Scottish isolate Scot43/98 shows no clear affinity with any of the Norwegian isolates, and the 3 isolates from Nordland fall into 2 separate groups, with 2 isolates (N29/97 and N32/98) from the southern/middle part of Nordland comprising 1 group and 1 isolate (N49/99), from the northern part of Nordland grouping with isolates from Hitra (HPR6). HPR2 constitutes a similar example.

Emergence of ISAV in salmon farms

Salmon farming within the living/growing areas of trout may have changed the 'balance' between the ISAV and wild salmonids. The increasing numbers of susceptible hosts (salmon) within the migration areas of wild salmon and trout must have increased the chances of transmission of pathogens from wild to farm fish, which resulted in the emergence of ISA.

Another influence from salmon farming is the increased production of salmon lice (*Lepeophteirus salmonis*) in the feeding areas of the sea trout. Several studies have documented the early return of sea trout with heavy lice infections in areas of salmon farming (Tully et al. 1993a,b, Birkeland & Jakobsen 1997), while lice pressure seems to be lower in areas without salmon farming. Other studies have shown that a high number of salmon lice constitute an important stress for trout (Birkeland 1996, Grimnes & Jakobsen 1996, Dawson et al. 1998, Bowers et al. 2000). It has also been documented that stress increases the production of ISAV in infected trout (carrier trout) and that salmon lice may be effective vectors transmitting the ISAV from infected fish to susceptible individuals (Nylund et al. 1993, 1994a, 1995a, Rolland & Nylund 1998b, Devold et al. 2000). Hence, salmon farming through the introduction of a high number of susceptible individuals and increased production of salmon lice may have opened a new transmission route for the ISAV, from wild to farmed fish and between farms. Transmission of ISAV between farms may result from farming activity.

In most cases, transmission of ISAV to farmed salmon will be a dead-end for the virus, at least in areas where infected populations are culled and the farm sites fallowed before the virus is transmitted further. Transmission back to wild populations of salmon and trout is probably negligible, since the number of susceptible individuals will be low and some individuals will already have acquired some immunity against the ISAV. The situation will be somewhat different if the ISAV is transported via farm activity (transportation of smolt, equipment etc.) to areas where the wild populations are ISAV-free. In such areas there may be a possibility of transmission back to wild fish via escaped

farm fish, salmon lice etc., and new ISAV loci may be established. There exists little or no documentation of transmission of pathogenic ISAV isolates from farmed to wild populations of salmonids and there are no reports of mortality due to ISA in wild populations. Admittedly, the chances of finding moribund and dead fish in the wild is minute. Epizootiological studies of the ISAV in farmed and wild populations of salmonids may, however, give an insight into the importance of transmission via farm activity. The presence of 2 very different groups of ISAV isolates in Sør-Trønderlag (ST) may indicate that 1 of these isolate groups has been transported to the area by farming activity (Table 1, Fig. 2).

An observation supporting the hypothesis that ISAV in wild populations of salmonids may be the driving force behind emergence of ISA in farmed populations of salmon is the cycle of new ISA outbreaks (cf. Håstein et al. 1999, their Fig. 1, p. 222). The major peak of new ISA outbreaks in Norway occurred between 1988 and 1992 and fell to zero in 1994. A recommencement of ISA outbreaks in 1995 was followed by an increase in new outbreaks in the following years. At the end of the 1970s and beginning of the 1980s, 'haemorrhagic syndromes' (HS) were quite common observations in Norwegian salmon farming (Poppe et al. 1983). It has now been established that HS must have been caused by several pathogens, resulting in cold-water vibriosis, the cardiomyopathy syndrome, and the haemorrhagic smolt syndrome. In addition, ISAV-like particles have been found in tissues from salmon that died in 1977/1978 with haemorrhagic syndrome (A. Nylund pers. obs.). Hence, we can speak of 3 peaks of ISA in Norwegian salmon farming. An explanation for these cycles in ISA outbreaks could be that the prevalence of ISAV builds up in wild populations of salmonids, increasing the possibility of transmission to farmed populations. When the number of susceptible wild individuals drops, due to natural acquired immunity and natural mortality of infected individuals, the chances for transmission of ISAV to farmed populations also drop. The cycle will be repeated when new susceptible individuals increase in number in the wild populations. Admittedly, testing this hypothesis requires screening of wild populations over decades and correlating these results with outbreaks of ISA in farmed populations in the same areas. Still, if this hypothesis proves to be correct it will have important implications for future management of this disease and for choice of combat strategy, culling or vaccination. Future isolation of ISAV from wild salmonids and challenge experiments will show if isolates with an HPR0 haemagglutinin represent the typical benign wild type as opposed to the known pathogenic farmed isolates.

LITERATURE CITED

Aspehaug V, Devold M, Nylund A (1999). The phylogenetic relationship of nervous necrosis virus from halibut (*Hippoglossus hippoglossus*). Bull Eur Assoc Fish Pathol 19:196–202

Birkeland K (1996) Salmon lice, *Lepeophtheirus salmonis* Krøyer, infestations and implications for anadromous brown trout, *Salmo trutta* L. PhD thesis, University of Bergen

Birkeland K, Jakobsen PJ (1997) Salmon lice, *Lepeophtheirus salmonis*, infestation as a causal agent of premature return to rivers and estuaries by sea trout, *Salmo trutta*, juveniles. Environ Biol Fish 49:129–137

Blake S, Bouchard D, Keleher W, Opitz M, Nicholson BL (1999) Genomic relationships of the North American isolate of infectious salmon anemia virus (ISAV) to the Norwegian strain of ISAV. Dis Aquat Org 35:139–144

Bouchard DA, Brockway K, Giray C, Keleher W, Merrill PL (2001) First report of infectious salmon anemia (ISA) in the United States. Bull Eur Assoc Fish Pathol 21:86–88

Bowers JM, Mustafa A, Speare DJ, Conboy GA, Brimacombe M, Sims DE, Burka JF (2000) The physiological response of Atlantic salmon, *Salmo salar* L., to a single experimental challenge with sea lice, *Lepeophtheirus salmonis*. J Fish Dis 23:165–172

Clouthier SC, Rector T, Brown NEC, Anderson ED (2002) Genomic organization of infectious salmon anemia virus. J Gen Virol 83:421–428

Courtenay WR Jr, Hensley DA, Taylor JN, McCann JA (1984) Distribution of exotic fishes in the continental United States. In: Courtenay WR Jr, Stauffer JR Jr (eds) Distribution, biology and management of exotic fishes. John Hopkins University Press, Baltimore, p 41–77

Cunningham CO, Snow M (2000) Genetic analysis of infectious salmon anaemia virus (ISAV) from Scotland. Dis Aquat Org 41:1–8

Cunningham CO, Gregory A, Black J, Simpson I, Raynard, RS (2002) A novel variant of the infectious salmon anaemia virus (ISAV) haemagglutinin gene suggests mechanisms for virus diversity. Bull Eur Assoc Fish Pathol 22:366–374

Dalla Valle L, Negrisolo E, Patarnello P, Zanella L, Maltese C, Bovo G, Colombo L (2001) Sequence comparison and phylogenetic analysis of fish nodaviruses based on the coat protein gene. Arch Virol 146:1125–1137

Dawson LHJ, Pike AW, Houlihan DF, McVicar AH (1998) Effects of salmon lice *Lepeophtheirus salmonis* on sea trout *Salmo trutta* at different times after seawater transfer. Dis Aquat Org 33:179–186

Devold M, Krossøy B, Aspehaug V, Nylund A (2000) Use of RT–PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout *Salmo trutta* after experimental infection. Dis Aquat Org 40:9–18

Devold M, Falk K, Dale OB, Krossøy B, Biering E, Aspehaug V, Nilsen F, Nylund A (2001) Strain variation, based on the haemagglutinin gene, in Norwegian ISA virus isolates collected from 1987 to 2001: indications of recombination. Dis Aquat Org 47:119–128

Gojobori T, Moriyama EN, Kimura M (1990) Statistical methods for estimating sequence divergence. Methods Enzymol 183:532–550

Griffiths S, Cook M, Mallory B, Ritchie R (2001) Characterisation of ISAV proteins from cell culture. Dis Aquat Org 45: 19–24

Grimnes A, Jakobsen PJ (1996) The physiological effects of salmon lice infection on post-smolt of Atlantic salmon. J Fish Biol 48:1179–1194

Håstein T, Hill BJ, Winton JR (1999) Successful aquatic ani-

mal disease emergency programmes. Rev Sci Tech Off Int Epizoot 18:214–227

Hovland T, Nylund A, Watanabe K, Endresen C (1994) Observation of infectious salmon anaemia virus (ISAV) in Atlantic salmon (*Salmo salar* L.). J Fish Dis 17:291–296

Hungnes O, Jonassen TØ, Jonassen CM, Grinde B (2000) Molecular epidemiology of viral infections: how sequence information helps us understand the evolution and dissemination of viruses. APMIS 108:81–97

Inglis JA, Bruce J, Cunningham CO (2000) Nucleotide sequence variation in isolates of infectious salmon anaemia virus (ISAV) from Atlantic salmon *Salmo salar* in Scotland and Norway. Dis Aquat Org 43:71–76

Jones SRM, Groman DB (2001) Cohabitation transmission of infectious salmon anemia virus among freshwater-reared Atlantic salmon. J Aquat Anim Health 13:340–346

Jones SRM, MacKinnon AM, Salonius K (1999) Vaccination of freshwater-reared Atlantic salmon reduces mortality associated with infectious salmon anaemia virus. Bull Eur Assoc Fish Pathol 19:98–101

Kibenge FSB, Garate ON, Johnson G, Arriagada R, Kibenge MJT, Wadowska D (2001a) Isolation and identification of infectious salmon anaemia virus (ISAV) from Coho salmon in Chile. Dis Aquat Org 45:9–18

Kibenge FSB, Kibenge MJT, McKenna PK, Stothard P, Marshall R, Cusack RR, McGeachy S (2001b) Antigenic variation among isolates of infectious salmon anaemia virus correlates with genetic variation of the viral haemagglutinin gene. J Gen Virol 82:2869–2879

Knipe DM, Howley PM (2001) Fields virology. Lippincott Williams & Wilkins, Philadelphia

Krossøy B (2002) A genetic characterisation of infectious salmon anaemia virus (ISAV). PhD thesis, University of Bergen, Norway

Krossøy B, Hordvik I, Nilsen F, Nylund A, Endresen C (1999) The putative polymerase sequence of infectious salmon anemia virus suggests a new genus within the *Orthomyxoviridae*. J Virol 73:2136–2142

Krossøy B, Devold M, Sanders L, Knappskog PM and 6 others (2001a) Cloning and identification of the infectious salmon anaemia virus haemagglutinin. J Gen Virol 82:1757–1765

Krossøy B, Nilsen F, Falk K, Endresen C, Nylund A (2001b) Phylogenetic analysis of infectious salmon anaemia virus isolates from Norway, Canada and Scotland. Dis Aquat Org 44:1–6

Melville KJ, Griffiths SG (1999) Absence of vertical transmission of infectious salmon anemia virus (ISAV) from individually infected Atlantic salmon *Salmo salar*. Dis Aquat Org 38:231–234

Mullins JE, Groman D, Wadowska D (1998) Infectious salmon anaemia in salt water atlantic salmon (*Salmo salar* L.) in New Brunswick, Canada. Bull Eur Assoc Fish Pathol 18: 110–114

Nylund A, Jakobsen P (1995) Sea trout as a carrier of infectious salmon anaemia virus. J Fish Biol 47:174–176

Nylund A, Wallace C, Hovland T (1993) The possible role of *Lepeophtheirus salmonis* in the transmission of infectious salmon anaemia. In: Boxshall G (ed) Pathogens of wild and farmed fish: sea lice. Ellis Horwood, Chichester, p 367–373

Nylund A, Alexandersen S, Løvik P, Jakobsen P (1994a) The response of brown trout (*Salmo trutta* L.) to repeated challenge with infectious salmon anaemia (ISA). Bull Eur Assoc Fish Pathol 14:167–170

Nylund A, Hovland T, Hodneland K, Nilsen F, Løvik P (1994b) Mechanisms for transmission of infectious salmon anaemia (ISA). Dis Aqat Org 19:95–100

Nylund A, Alexandersen S, Jakobsen P, Rolland JB (1995a) Infectious salmon anaemia (ISA) in brown trout. J Aquat Anim Health 7:236–240

Nylund A, Hovland T, Watanabe K, Endresen C (1995b) Presence of infectious salmon anaemia virus (ISAV) in different organs of *Salmo salar* L. collected from three fish farms. J Fish Dis 18:135–145

Nylund A, Kvenseth AM, Krossøy B (1995c) Suceptibility of wild salmon (*Salmo salar* L.) to infectious salmon anaemia (ISA). Bull Eur Assoc Fish Pathol 15 (5): 152–156

Nylund A, Kvenseth AM, Krossøy B, Hodneland, K (1997) Rainbow trout (*Onchorhynchus mykiss*, Walbaum, 1792): a carrier of infectious salmon anaemia (ISAV). J Fish Dis 20: 275–279

Nylund A, Krossøy B, Devold M, Aspehaug V, Steine NO, Hovland T (1999) Outbreak of ISA during first feeding of salmon fry (*Salmo salar*). Bull Eur Assoc Fish Pathol 19: 70–74

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 12:357–358

Poppe TT, Håstein T, Salte R (1983) Haemorrhagic syndrome in salmon: an overview (in Norwegian). Nor Veterinærtidsskr 95:317–323

Rasmussen CJ (1967) Håndbog i ørretopdræt. Rhodos International Science Publishers, Copenhagen (in Danish)

Raynard RS, Murray AG, Gregory A (2001a) Infectious salmon anaemia virus in wild fish from Scotland. Dis Aquat Org 46:93–100

Raynard RS, Snow M, Bruno DW (2001b) Experimental infection models and susceptibility of Atlantic salmon *Salmo salar* to a Scottish isolate of infectious salmon anaemia virus. Dis Aquat Org 47:169–174

Rimstad E, Mjaaland S, Snow M, Mikalsen AB, Cunningham CO (2001) Characterisation of the infectious salmon anemia virus genomic segment that encodes the putative haemagglutinin. J Virol 75:5352–5356

Ritchie RJ, Cook M, Melville K, Simard N, Cusack R, Griffiths S (2001) Identification of infectious salmon anaemia virus in Atlantic salmon from Nova Scotia (Canada): evidence for functional strain differences. Dis Aquat Org 44:171–178

Rodger HD, Turnbull T, Muir F, Millar S, Richards RH (1998) Infectious salmon anaemia (ISA) in the United Kingdom. Bull Eur Assoc Fish Pathol 18:115–116

Rolland JB, Nylund A (1998a) Sea running trout: carrier and transmitter of the infectious salmon anemia virus (ISAV). Bull Eur Assoc Fish Pathol 18:50–55

Rolland JB, Nylund A (1998b) Infectiousness of organic materials orienting in ISA-infected fish and transmission of the disease via salmon lice (*Lepeophtheirus salmonis*). Bull Eur Assoc Fish Pathol 18:173–180

Rowley HM, Campell SJ, Curran WL, Turnbull T, Bryson DG (1999) Isolation of infectious salmon anaemia virus (ISAV) from Scottish farmed Atlantic salmon, *Salmo salar* L. J Fish Dis 22:483–487

Saitou N, Nei M (1986) Polymorphism and evolution of influenza A virus genes. Mol Biol Evol 3:57–74

Scholtissek C, Ludwig S, Fitch WM (1993) Analysis of influenza A virus nucleoproteins for assessment of molecular genetic mechanisms leading to new phylogenetic virus lineages. Arch Virol 131:237–250

Snow M (1999) Molecular epidemiology and molecular biology of viral haemorhagic septicaemia virus (VHSV). PhD thesis, University of Aberdeen

Snow M, Raynard RS, Bruno DW (2001) Comparative susceptibility of Artic char (*Salvelinus alpinus*), rainbow trout

(*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) to the Scottish isolate of infectious salmon anaemia virus. Aquaculture 196:47–54

Swofford DL (1998) Phylogenetic analysis using parsimony and other methods, Version 4.0. Sinauer, Sunderland, MA

Thorud KE (1991) Infectious salmon anaemia: transmission trials — haematological, clinical chemical and morphological investigations. PhD thesis, National Veterinary Institute, Oslo

Thorud KE, Djupvik HO (1988) Infectious anaemia in Atlantic salmon (*Salmo salar* L.). Bull Eur Assoc Fish Pathol 8: 109–111

Totland GK, Hjeltnes BK, Flood PR (1996) transmission of infectious salmon anaemia (ISA) through natural secretions and excretions from infected smolts of Atlantic salmon *Salmo salar* during their presymptomatic phase. Dis Aquat Org 26:25–31

Tully O, Poole WR, Whelan KF (1993a) Infestation parameters for *Lepeophtheirus salmonis* (Krøyer) (Copepoda: Caligidae) parasitic on sea trout (*Salmo trutta* L.) off the west coast of Ireland during 1990 and 1991. Aquacult Fish Manag 24:545–555

Tully O, Poole WR, Whelan KF, Merigoux S (1993b) Parameters and possible causes of epizootics of *Lepeophtheirus salmonis* (Krøyer) parasitic on sea trout (*Salmo trutta* L.) off the west coast of Ireland. In: Boxshall GA, Defaye D (eds) Pathogens of wild and farmed fish: sea lice. Ellis Horwood, London, p 201–213

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992) Evolution and ecology of influenza A viruses. Microb Rev 56:152–179

Welcomme RL (1988) International introductions of inland aquatic species. FAO Fish Tech Pap 294

Weston J, Villoing S, Bremont M, Castric J and 7 others (2002) Comparison of two aquatic Alphaviruses, salmon pancreas disease virus and sleeping disease virus, by using genome sequence analysis, monoclonal reactivity, and cross-infection. J Virol 72:6155–6163

Editorial responsibility: Jo-Ann Leong, Kaneohe, Hawaii, USA

Submitted: July 5, 2002; Accepted: April 4, 2003
Proofs received from author(s): July 10, 2003