Infectious salmon anaemia virus (ISAV): Evolution, genotyping, reservoirs and transmission.

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LIST OF PAPERS

Paper I

Plarre H, Devold M, Snow M, Nylund A (2005). Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in western Norway. Dis Aquat Org 66: 71 – 79.

Paper II

Nylund A, Plarre H, Karlsen M, Fridell F, Ottem KF, Bratland A, Sæther PA (2007). Transmission of infectious salmon anaemia virus (ISAV) in farmed populations of Atlantic salmon (*Salmo salar*). Arch Virol 152: 151 – 179 (E.pub. 2006).

Paper III

Plarre H, Nylund A, Karlsen M, Brevik Ø, Sæther PA, Vike S (submitted). Evolution of ISA virus (ISAV). Arch. Virol.

CONTENTS

ACKNOWLEDGEMENTS 3

LIST OF PAPERS 4

INTRODUCTION 6

History of infectious salmon anaemia (ISA) 6

Wild salmonids 14

ISA virus 16

Genotyping and molecular epizootiology 27

Virulence 36

Risk factors and transmission of ISA virus 38

AIMS OF STUDY 47

SUMMARY OF PAPERS 48

GENERAL DISCUSSION 53

Host species for ISA viruses 53

ISA virus evolution 59

Virulence 66

Molecular epizootiology 72

Reservoirs and transmission of ISA virus 76

FUTURE WORK 81

LITERATURE 82

APPENDIX A 109

INTRODUCTION

History of infectious salmon anaemia (ISA)

The first officially registered outbreak of infectious salmon anaemia (ISA) started in Norway in November 1984 (Thorud and Djupvik, 1988). It was a chronically proceeding disease among Atlantic salmon (*Salmo salar*) parr in a hatchery on the West Coast of Norway (Bremnes). Affected salmon were lethargic with pale gills, fin rot, exophthalmia, and haemorrhages in the anterior eye chamber and abdominal skin. Other clinical signs were a dark, pale or yellowish liver, a dark and swollen spleen, congested intestinal walls, petechia in periviceral fat, ascitic fluid, oedemas, haemorrhages in the swim bladder wall, and muscular haemorrhages. About 80% of the parr in the hatchery died during that winter and spring. Judging from the pathological findings, "Hitra disease" was suspected, however, no bacteria were isolated from diseased fish and oxytetracycline did not reduce mortality. In the following years a disease with similar clinical and pathological signs occurred in smolt and adult salmon in marine farms that had received smolt form this hatchery (Thorud 1991).

The first official registration of ISA came at a very bad time for Norwegian salmon production. One problem was an ongoing discussion of the causes of "Hitra disease". Some insisted it was caused by malnutrition (Poppe et al 1986; Salte et al 1987), while others believed it was a bacterial disease (Holm et al 1985; Egidius et al 1986; Totland et al. 1988) and even managed to culture the bacterium that was later named *Vibrio*

salmonicida (disease: cold water vibriosis). The dispute about the causes of "Hitra disease" goes back to the end of the seventies, and later studies have indicated that the reason for the disagreement was probably that "Hitra disease" encompassed at least three different diseases: cold water vibriosis, cardiomyopathy and ISA. There are several indications of this in articles published in Norwegian (cf. F*risk Fisk* brochure from this period, i.e. published reports from the Norwegian Research council). In some electron microscopic studies of tissues from salmon with "Hitra disease", the authors were not able to detect any bacteria and described pathological changes that later studies have shown to be associated with ISA. Even as late as in 1987 the three diseases were confused. One farm in Northern Norway was given the diagnosis Bremnes syndrome (i.e. ISA), but this was later changed to cardiomyopathy. The reason for the confusion was of course the lack of a safe diagnosis.

The first indication of ISA can probably be found as early as in 1977 (cf: Poppe *et al.* 1983). The authors give a review of haemorrhagic syndrome of Atlantic salmon in Norwegian aquaculture. The first case of haemorrhagic syndrome was registered in September/October 1977 in a fish farm close to Hammerfest, Northern Norway. They were not able to isolate any infective agent and therapy with oxytetracycline did not influence the mortality. The same disease was also registered in 1978 and in 1979 it was found in other areas in Northern Norway. Several explanations were given for this syndrome: stress, environmental stress, genetics, water temperature, malnutrition, virus (VHS, but it was not possible to isolate this virus), bacteria (it was not always possible to isolate bacteria, but in some farms *Vibrio* spp. were found), and physical factors.

Studies of tissues from fish with haemorrhagic syndrome have later revealed the presence of ISA virus particles in some of these tissues (A. Nylund, pers. com.).

ISA was first named "Bremnes syndrome" after the locality of the first official appearance. This was later changed to Salmon anaemia syndrome (SAS). However, protest from an airline company made another change of name necessary and in 1990 ISA (Infectious Salmon Anaemia) was recognised by the Office International des Epizooties.

In the period from the first official outbreak in 1984 until 1991 there was a steady increase in the number of outbreaks of the disease with around 80 in 1990 and 1991 (Figure 1). This increase lead to changes in the structure of salmon farming in Norway. The farmers introduced a system with separate generations at each site and the Norwegian Food Authorities introduced a number of regulatory actions like introduction of zones to combat outbreaks. This lead to a new situation in 1994 with only one official outbreak of ISA (cf. Håstein et al 1999). The number of outbreaks since 1994 up to the present has increased again to around 10 - 15 outbreaks every year. The number of outbreaks does not reflect the increase in the amount of salmon produced in the same period (1994 - 2010), indicating a certain degree of stabilisation.

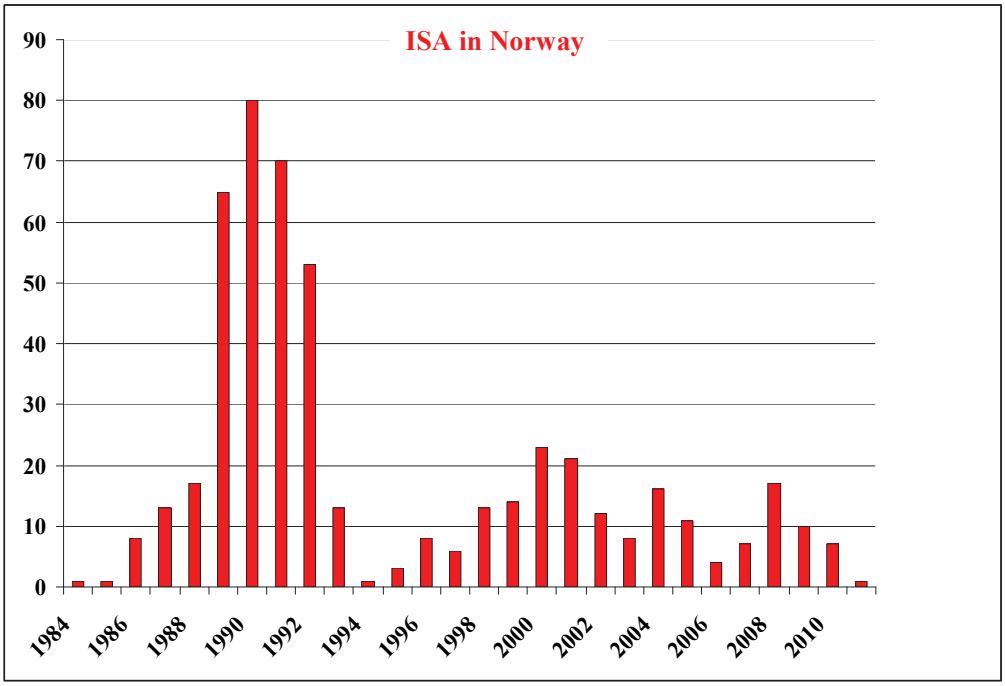


Figure 1. Number of ISA outbreaks each year in the period from 1984 to 2011 (Based on the official record).

For many years Norway was the only country that had Atlantic salmon suffering from ISA, but in 1997 the first official cases of ISA were registered in Canada and a year later in Scotland (Mullins et al 1998; Lovely et al 1999; Rodger et al 1998; Rowley et al 1999; Ritchie et al 2001a). Canada suffered repeated outbreaks of ISA in the years that followed, while in Scotland there was an apparent eradication of ISA in 1999. However, ISA virus was detected in both wild and farmed salmon in Scotland in the following years and in 2008 there were several outbreaks of ISA in the Shetland Islands (Raynard et al 2001; McBeath et al 2009; Murray et al 2010). ISA virus was detected in Chile in 1999 and in 2000 there were outbreaks of ISA in the Faeroe

Islands (Kibenge et al 2001a; Lyngøy 2003). In 2001 the first official outbreaks were registered on the East Coast of the USA and in 2002 ISA virus was detected in Ireland (Bouchard et al 2001). The first large scale outbreaks of ISA in Chile were recorded in 2007, with the majority of outbreaks occurring in region X (Godoy et al 2008; Vike et al 2008; Kibenge et al 2009). The ISA epidemics in Chile continued in 2008 and 2009, leading to a near collapse in the Chilean salmon industry. By 2010 the Chilean authorities seemed to have gotten the ISA epidemic under control, but outbreaks of ISA have also been registered in Chile in 2011.

The first transmission trial with material from salmon suffering from ISA was initiated on Friday 13th, November 1987, and indicated an infectious nature of the disease (Thorud & Djupvik 1988). In the following years several transmission trials were carried out and a pattern has emerged (Thorud 1991; Christie et al 1991, 1993; Dannevig et al 1993, 1994; Nylund et al 1994b). The transmission trials usually resulted in high mortalities where the start and duration of mortality seem to be dependent on genetics (differences in susceptibility between salmon stocks, Nylund et al 1995b), temperature (Thorud & Djupvik 1988), dose (number of virus/gram body weight) (Dannevig et al 1994; Nylund et al 1994b; Nylund et al. 1995c), time of year (yearly variation in hormone levels and immunological status, A. Nylund pers. com.), age (Glover et al 2006, A. Nylund pers. com.), and variation in ISA virus virulence (Kibenge et al 2007b, Mjaaland et al 2005).

The first transmission trials to verify a viral nature of the aetiological agent were carried out by Thorud (1991). In addition to challenge with infective material (liver from a natural outbreak of ISA) the same material was passed through several different pore-size cellulose ester membrane filters (450 nm, 220 nm and 100 nm) and injected into salmon. This study showed that the homogenate was still infective even after filtration through the 100 nm pore-size filter, which excludes any of the bacteria, isolated from farmed Atlantic salmon in Norway, from being the causative agent of ISA. Filtration through the 100 nm pore-size filter indicates an agent of viral nature. In addition, loss of infectivity of the homogenate after ether treatment indicated that the agent was an enveloped virus (Thorud 1991; Christie et al 1993). The study by Thorud also excluded IPN virus and EIBS virus as possible causative agents.

Transmission trials showed that the agent occurred in most tissues (Christie et al. 1991; Thorud 1991; Dannevig et al 1994; Nylund et al 1994b; Totland et al 1996), though some seemed to be more infective than others. In a comparison of tissues from liver, spleen, kidney, plasma, erythrocytes and head kidney leukocytes Dannevig et al. (1994) found the kidney to be most infective. Other studies have shown that mucus from the surface of the salmon and ascitic fluid are nearly as infective as blood (Rolland et al. 1998b; Nylund et al 1994b) and that freeze dried mucus is still infective when dissolved in cell culture media and injected into salmon (A. Nylund pers.com.). In a study of muscle tissue from salmon that were frozen at -20°C and thawed three times the infectivity increased (Thorud & Torgersen 1994). In 1992 it was shown that the

salmon louse *Lepeophtheirus salmonis* could act as a mechanical vector for the ISA virus (Nylund et al 1993a) and in 1993 it was shown that the virus was able to replicate in trout, *Salmo trutta* (Nylund et al 1993a, 1994a, 1995a, Nylund & Jakobsen 1995, Rolland & Nylund 1998a). However, the virus did not kill the trout. The ISA virus was also able to propagate in rainbow trout, *Oncorhynchus mykiss*, without causing any significant mortality (Nylund et al 1997). Later studies have confirmed that rainbow trout and a few other *Oncorhynchus* species are susceptible to ISA virus (Rolland & Winton 2003, MacWilliams et al 2007, Snow et al 2001b).

The only species that develops disease followed by mortalities when infected with the ISA virus is Atlantic salmon, *Salmo salar*. However, several other salmonid species are susceptible to ISA virus and some of these may end up as asymptomatic carriers of the virus (Nylund et al 1994a, b, 1995a, 1997, Nylund and Jacobsen 1995, Devold et al 2000, Snow et al 2001b, Rolland & Winton 2003, MacWilliams et al 2007), eg. *Salmo trutta*, *Salvelinus alpinus* and *Onchorynchus* spp. Mortality among ISA virus challenged rainbow trout has been observed (MacWilliams et al 2007). Among marine species, experimental challenge of herring, *Clupea harengus*, and Atlantic cod, *Gardus morhua*, has shown that these species may support replication of the ISA virus, but they are not able to transmit the virus to salmonid species in challenge trials (Nylund et al 2002, Grove et al 2007). Other marine fish species from the North Atlantic are not able to support replication of the ISA virus (Snow et al 2002, MacLean et al 2003, McClure et al 2004, A. Nylund pers.com). Several wild fish species from the coast of

Chile and Scotland, in areas with outbreaks of ISA, have also been tested and found to be negative for presence of ISA virus (Raynard et al 2001, Gonzales et al 2011). The only fish found to be positive in the study from Chile was escaped Atlantic salmon. In addition, three invertebrate species have also been tested for susceptibility for ISA virus: blue mussel, *Pecten maximus, Lepeophtheirus salmonis*, but none of these were able to sustain replication of the virus (Skår & Mortensen 2007; Nylund et al 1993b). Based on these data, it has been hypothesised that the natural host species for the ISA virus are salmonids in the north Atlantic.

The first detection of ISA virus in wild salmonids from several locations was registered in Scotland (Raynard et al 2001; Cunningham et al 2002). They found presence of ISA viruses in both Atlantic salmon (parr and adult salmon) and trout (brown trout and sea trout) at different locations in Scotland including a fresh water site distant to areas with marine salmon farms. This is the first evidence that ISA virus can be present in wild juvenile Atlantic salmon and brown trout in fresh water, i.e. before any exposure of these ISA virus hosts to sea water. A study of ISA virus in wild Atlantic salmon and trout, collected in 2001 – 2003, from rivers and a fjord in Sogn og Fjordane county was performed in Norway (Paper I). This paper shows a high prevalence of ISA virus in wild trout and Atlantic salmon in these rivers in 2001 and 2002. We also found three out of 45 sea trout, collected in Nordfjord, positive for ISA virus. These studies from Norway and Scotland show that ISA viruses are relatively

common in wild salmonids and suggest, together with challenge experiments, that these two species are the most likely natural hosts for ISA viruses.

Wild salmonids

One characteristic trait of Atlantic salmon and trout (*Salmo trutta*) is that they live in genetically distinct, small fragmented populations belonging to different river systems along the west coast of Europe and the East coast of North America. Both species spawn and have an early development in fresh water and when they reach the smolt stage, they migrate to sea. However, while the trout stays in the coastal waters, the Atlantic salmon migrates to the high seas, Barents and Norwegian Sea and some are also found in the North Sea. Trout also differ from salmon in that they may return to the rivers during the autumn every year, while salmon usually stay out at sea until they are ready to spawn, which may be 3 - 4 years. Common for both species is that the population density at sea is low compared to the high density found in the river systems during spawning. These two species are probably the main hosts for the ISA virus and it is expected that the ISA virus must have co-evolved with these and adapted to transmission in the fresh water phase, since the population density is highest in the rivers and interactions between individuals are highest during spawning. It has been documented (Paper I) that salmon and trout eat eggs on the spawning grounds during breeding (Figure 2). Hence, if the gonadal products, eggs and sperms, are infected with the virus, it can easily be transmitted to other life stages like fry, parr and smolt when these are feeding on the infected gonadal products.

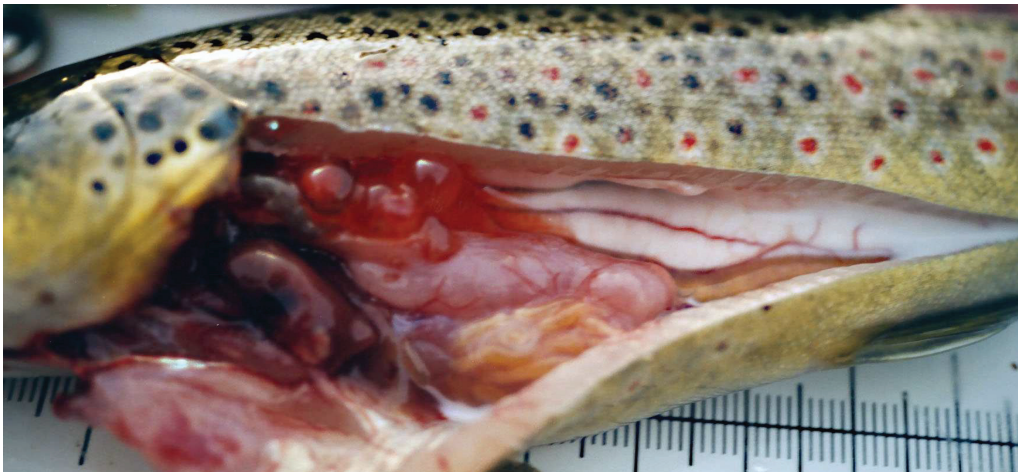


Figure 2. Mature male (*Salmo trutta*) that has been eating eggs during spawning in Bortne River.

Since the populations in fresh water are too small to sustain highly virulent viruses, it is expected that the ISA viruses must consist of low virulent strains in natural populations, or that the fresh water stages of these species are less susceptible or less likely to develop disease compared to the marine stages. It may also be expected that the viruses should be more adapted to the trout, which spends more time in the river systems and coastal areas compared to the Atlantic salmon. It has been shown that even though the ISA virus may replicate in the trout and cause slight disease, it does not cause any mortality (Nylund et al 1993a, 1994a, 1995a, Nylund & Jakobsen 1995). It has also been documented that the production of ISA virus in carrier trout increases during stress and sexual maturation (Nylund et al 1994a, 1995a; Rolland & Nylund 1998a; Devold et al 2000). Both species, salmon and trout, may be infected by ISA

virus in both fresh and sea water and throughout the year. However, it seems that the Atlantic salmon are less likely to develop disease during the period from September to December (A. Nylund pers.com.). This could be an adaptation for protection against the development of ISA after exposure to the virus during spawning. The existence of low virulent or avirulent ISA virus could be another explanation for how the ISA virus is able to survive in wild salmonid populations (Nylund et al 2003). When pathogenic strains develop from the avirulent HPR0 type in the river systems, they will probably soon be lost from the population, if the host dies before the virus is transmitted to a new host.

ISA virus

Several different virus-like particles have been found in salmon suffering from ISA (Thorud et al 1990; Christie et al 1991; Sommer & Mennen 1992), but none of these were identified as the causal agent of the disease. The first pictures of the ISA virus were presented in 1993 (Watanabe et al. 1993) and at the EAFP conference in Brest (September 1993, A Nylund, pers. com.). The virus was seen as early as 7 days after challenge. The morphology of the virion and the target cells have since been dealt with in several studies (Hovland et al 1994; Koren & Nylund 1997; Dannevig et al 1995; Nylund et al. 1995a, 1996; Sommer & Mennen 1997).

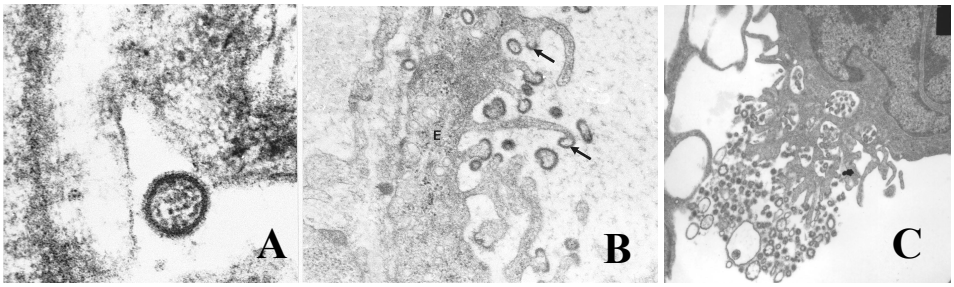


Figure 3. A) ISA virus virion, B) Budding of ISA virions from blood vessel endothelial cells, C) Budding of ISA virions from a polymorphonuclear leukocyte (Nylund et al 1995b, 1996).

The free ISA virus virions are spherical and about 100 nm in diameter, but some variation in size and morphology does occur (Watanabe et al 1993, Hovland et al. 1994; Koren & Nylund 1997, Nylund et al 1995a, 1996). The envelope of the virus is a unit membrane with an electron dense layer, 10 nm thick, on the outer surface (Figure 3). The virions may also be surrounded by an additional electron dense cloud about 50 nm thick. The virions contain electron dense granules, about 10 to 12 nm in diameter, believed to be transverse sections of the helical nucleocapsids. The diameter of negatively stained ISA virus particles ranges from 45 - 140 nm (majority range from 130 – 140 nm) and they are covered with surface projections about 10 nm in length (Dannevig et al 1995). The projections seem to have a knob-like thickening at the distal end. A few, large (up to 700 nm), highly pleomorphic particles could also be found in infected cell culture medium (Dannevig et al 1995).

Budding of the ISA virus has been observed from the surface of endothelial cells lining bloodvessels, endocardial cells and polymorphonuclear leukocytes (Watanabe et al 1993; Hovland et al 1994; Koren & Nylund 1997; Nylund et al. 1995a, 1996) (Figure 3). A few cases of what seems to be budding into intracellular vesicles in the endothelial cells have also been observed. The budding process seemed to start with increasing electron density and thickening of the cell membrane. Areas where this occurred usually protrude from the cell. These protrusions can extend more than 2 µm from the cell surface. Protrusions were not observed when the budding occurred on the abluminal side of the endothelial cells (Nylund et al 1995b).

The host cells seem to be morphological intact despite high budding activity. No viral components can be detected within the host cell cytoplasm until the characteristic electron-dense, crescent-shaped, thickening of the plasma membrane appears. However, destroyed endothelial cells are frequently found in moribund fish, but cells surrounding the blood vessels seem to disintegrate before the destruction of the endothelial cells. Aggregations of what seem to be virus envelopes (i.e. virions not yet matured or completely assembled) are frequently found on the abluminal side of the endothelial cells (Nylund et al 1995b). Large aggregations of viruses surrounded by amorphous substance and cell debris, which seem to be remnants of lysed polymorphonuclear leukocytes, have also been observed (Nylund et al 1995b). Protrusions from the endothelial cells are frequently seen surrounding erythrocytes or protruding into erythrocytes and macrophages can be seen adhering to the endothelial

cells in the salmon suffering from ISA (Nylund et al 1995b, 1996; Koren & Nylund 1997). Erythrocytes, which seemed to be adhered to infected endothelial cells, are frequently found (Figure 4). Erythrophagocytosis can be observed in most tissues of the infected salmon (Figure 4).

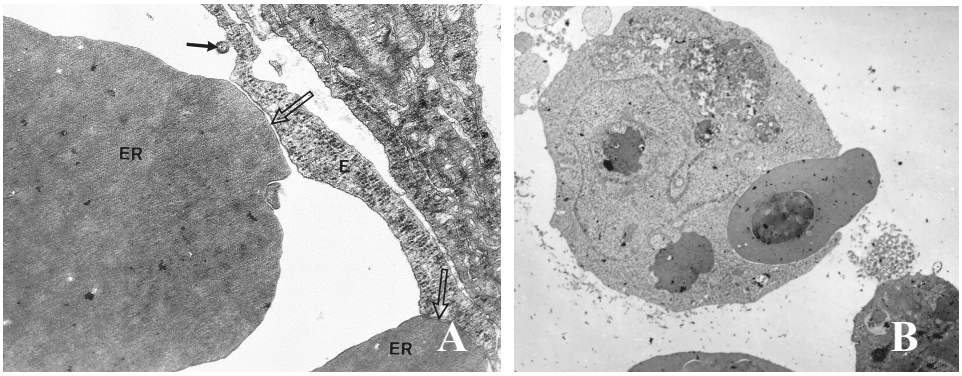


Figure 4. **A**) Erythrocyte attached to a blood vessel endothelial cell expressing ISA virus HE protein on its surface (Nylund et al 1995b). ISA virion budding from the cell surface (solid arrow), attachment area between the erythrocyte (ER) and the blood vessel endothelial cell (open arrow). **B**) Erythrophagocytosis. Macrophage eating erythrocytes with ISA virions attached to the surface.

The ISA virus was suggested as a possible member of the family *Orthomyxoviridae* based on size, morphology, biochemical and physiochemical characteristics, and presence of a segmented RNA genome (Sommer & Mennen 1996; Falk et al 1997; Koren & Nylund 1997; Mjaaland et al 1997). However, it was not until 1999, after analysis of segment 2 (the RNA dependent RNA polymerase, PB1), that the ISA virus was shown to represent a new genus in the family *Orthomyxoviridae* (Krossøy et al

1999). The slow development in the characterization and identification of the causative agent for ISA was partly due to the lack of susceptible cell cultures. The first isolation of the ISA virus in cell culture was obtained in a newly developed culture from salmon head kidney, SHK cells (Dannevig et al 1995), and a year later a similar approach, using head kidney leukocytes, also resulted in isolation of ISA virus (Sommer & Mennen 1996). In 1997 it was shown that the ISA virus could also be cultured in a commercially available established fish cell line, Atlantic salmon cells (AS cells) (Sommer & Mennen 1997). A culture of Atlantic salmon kidney cells (ASK cells) was also established at the University of Bergen and used for isolation of ISA viruses for studies of the virus genome (Devold et al 2000).

The buoyant density of the ISA virus was found to be 1.184 to 1.262 g/cm^3 according to Christie et al (1993), while Falk et al (1997) found the density to be 1.18g/ml in sucrose and CsCL gradients. Several studies have shown that the ISA virus is sensitive to chloroform, freon, heat and low pH (Thorud 1991; Christie et al 1993; Falk et al 1997). The optimal temperature for replication of the ISA virus is according to Falk et al (1997) 15 ºC. The tolerance and survival of the ISA virus, which is of importance both for the epizootic of the disease and for disinfection of infected farms and slaughterhouses, have been dealt with in several studies (Thorud 1991; Christie et al 1993; Nylund et al. 1993a; Torgersen 1993; Nylund et al 1994b; Thorud & Torgersen 1994). The ISA virus may survive for several hours at 20°C (Nylund et al 1993a, 1994b) but the infectivity is lost after exposure to 55°C for one minute (Torgersen

1993). According to the study by Torgersen (1993) the ISA virus is also inactivated by exposure to: a) formic acid at pH < 4.0 for 24 hours, b) sodium hydroxide (NaOH) at pH = 12 for 7 hours or more, c) 0.185% HCHO for 16 hours, d) 8 mg/min/l ozone for 4 - 6 minutes, and e) 20 mg/l sodium hypochlorite for 1 hour. The ISA virus is also inactivated by exposure to organic solvents, ether and chloroform (Christie et al 1993; Thorud 1991). Survival of the ISA virus in active sea water will depend on temperature, biological activty and UV-exposure (A. Nylund pers.com).

The ISA virus is an enveloped, single stranded, negative strand RNA virus where the genome consists of 8 segments with a total molecular size of approximately 14.5 kb (Mjaaland et al 1997; Clouthier et al 2002). The ISA virus is the only species in the genus *Isavirus*, family *Orthomyxoviridae* (Kawaoka et al 2005). The first sequence, segment eight, was published in 1997 (Mjaaland et al 1997) and in 1999 the sequence of the putative RNA-dependent RNA polymerase, PB1, was published (Krossøy et al 1999). Several studies on the putative haemagglutinin, segment six, of the ISA virus were published in 2001 (Krossøy et al 2001a,b; Devold et al 2001; Rimstad et al 2001; Griffiths et al 2001), and in 2002 the first study presenting the complete genome of ISA virus was available (Clouthier et al 2002). The first six segments contain one open reading frame (ORF) each (Krossøy et al 1999, 2001a,b; Devold et al 2001; Rimstad et al 2001; Ritchie et al 2001b; Griffiths et al 2001; Clouthier et al 2002; Snow et al 2003; Aspehaug et al 2004) while segment seven and eight contain at least two ORFs each (Biering et al 2002; Clouthier et al 2002; Ritchie et al 2002; McBeath et al 2006;

Kibenge et al 2007a; Garcia-Rosado et al 2008). Identification of the ISA virus proteins is still ongoing, but several of the proteins have been identified (Table 1).

The protein coded by segment one in the ISA virus genome contains a nuclear localization signal (and expression of the protein in a cell line exhibited a nuclear localization) and the protein size (ORF of 2169 nt) and amino acid composition is similar to the PB2 protein from othromyxoviruses (Snow et al 2003). The sequence of segment two contains an ORF of 2245 nucleotides coding for a putative protein showing the core polymerase motifs characteristic of all viral RNA-dependent RNA polymerases consistent with the properties of PB1 from members of the *Orthomyxoviridae* (Krossøy et al 1999). Segment three contains an ORF consisting of 1851 nt encoding a predicted protein of 616 amino acids (Ritchie et al 2001b; Snow & Cunningham 2001; Clouthier et al 2002). The amino acid sequence shows no or little homology with nucleoproteins (NP) from members of the *Orthomyxoviridae*, but functional analyses of the protein support the assumption that segment three is coding for a NP (Aspehaug et al 2004; Falk et al 2004; Goic et al 2008). Segment four contains an ORF consisting of 1737 nt. The putative protein (578 amino acids) contains no conserved motifs, but based on the predicted cytoplasmic location and the presence of putative sites of phosphorylation it was suggested to be the acid polymerase, PA, by Ritchie et al (2001b).

Two surface proteins are present on the ISA virions (Falk et al 2004), i.e. the fusion protein, F, and the heamagglutinin-esterase protein, (HE). Segment five contains an ORF consisting of 1332 nt coding for a protein consisting of 444 amino acids (Clouthier et al 2002; Aspehaug et al 2005). Analysis of the function of the protein coded by segment five shows that it is a pH-dependent F protein produced as a precursor protein, F0, that is proteolytically cleaved into F1 and F2 (Aspehaug et al 2005). It has been shown that inserts may occur in the vicinity of a putative cutting site for trypsin extending the ORF and resulting in ISA virus F proteins ranging from 444 to 455 amino acids (Devold et al 2006). The other surface protein is a heamagglutinin-esterase protein (HE) coded by the only ORF in segment six (Devold et al 2001; Griffiths et al 2001; Krossøy et al 2001b; Rimstad et al 2001; Kristiansen et al 2002; Falk et al 2004; Hellebø et al 2004; Müller et al 2010). The length of the ORF may range from 1164 to 1236 nt resulting in HE proteins ranging from 387 to 411 amino acids (**Paper II**). The variation in length is due to variation in a highly polymorphic region (stretching from nucleotide 1006 to 1117 in the ORF) next to the transmembrane region (Devold et al 2001, **Paper II** and **III**). Table 2 gives an overview of HPR observed in ISA viruses.

Table 1. Characterization /identification of the eight RNA segments in the ISA virus genome and the putative proteins coded by the ORFs.

Seg	ORF	Length	Protein		Literature
1	1	2169 nt	PB2	Polymerase	Clouthier et al 2002, Snow et al 2003
2	1	2245 nt	PB1	Polymerase	Krossøy et al 1999, Clouthier et al 2002
3	1	1851 nt	N	Nucleoprotein	Ritchie et al 2001, Falk et al 2004, Aspehaug et al 2004, Clouthier et al 2002, Goic et al 2008
4	1	1737 nt	PA	Polymerase	Ritchie et al 2001, Clouthier et al 2002
5	1	1332 - 1365 nt	F	Fusion	Aspehaug et al 2005, Clouthier et al 2002, Falk et al 2004, Devold et al 2006
6	1	1164 – 1236 nt	HE	Haemagglutinin-esterase	Krossøy et al 2001, Rimstad et al 2001, Devold et al 2001, Falk et al 2004, Kristiansen et al 2002, Clouthier et al 2002, Mikalsen et al 2005 Muller et al 2008, 2010
7	2 (3)	903 nt 526 nt	NS1 NEP (NS3)	Inf-sig-ant. Nuc exp prot ?	Biering et al 2002, Clouthier et al 2002, Ritchie et al 2002, McBeath et al 2006, Kibenge et al 2007, Garcia-Rosado et al 2008
8	2		M S8ORFs	Matrix Inf-sig-ant	Biering et al 2002, Clouthier et al 2002, Falk et al 2004, Garcia-Rosado et al 2008

Table 2. Overview of the different highly polymorphic region (HPR) groups observed in the stalk region of the HE from ISA viruses. St = surface tail, TMR transmembrane region.

HPR	St	HPR								TMR	strain
		1-2	3 - 7	8 - 12	13 - 17	18 - 22	23 - 27	28 - 32	33 - 37		
Europe											
HPR0a	NIT	DV	KIRVD	AIPPQ	LNQTF	NTNQV	EQPAT	SVLSN	IFISM	GVA	**SF83/04**
HPR0b	NIT	DV	KIRVD	AIPPQ	LNQTF	NTNQV	EQPA**N**	SVLSN	IFISM	GVA	**ISA440971**
HPR0a	NIT	DV	KIRVD	AIPPQ	LNQTF	NTNQV	EQPAT	SVLSN	IFISM	GVA	**CH28/08**
Chile	NIT	DV	KIRVD	AIPPQ	LNQTF	NT			ISM	GVA	**FJ594284**
HPR9	NIT	DV	KIRVD	AIPPQ	LNQTF	NT			M	GVA	**SF47/99**
Chile	NIT	DV	KIRVD	AIPPQ	LNQTF				ISM	GVA	**FJ594294**
HPR16	NIT	DV	KIRVD	AIPPQ	LNQTF				M	GVA	**T90/04**
HPR19a	NIT	DV	KIRVD	AIPPQ	LNQT**L**					GVA	**SF18/96**
HPR19b	NIT	DV	KIRVD	AIPPQ	LNQTF					GVA	**MR139/08**
HPR3	NIT	DV	KIRVD	AIPPQ	LNQT				FISM	GVA	**N9/93**
HPR2	NIT	DV	KIRVD	AIPPQ	LNQT				M	GVA	**H2/89**
HPR30	NIT	DV	KIRVD	AIPPQ	LNQ				ISM	GVA	**MR118/06**
HPR31	NIT	DV	KIRVD	AIPPQ	LN				IFISM	GVA	**T121/07**
HPR4a	NIT	DV	KIRVD	AIPPQ	L			SN	IFISM	GVA	**T10/93**
HPR4b	NIT	DV	KIRVD	AIPPQ	L			SN	**T**FISM	GVA	**H101/04**
HPR12a	NIT	DV	**GlG**VD	AIPPQ	L			N	IFISM	GVA	**N5/98**
HPR12b	NIT	DV	KIRVD	AIPPQ	L			N	IFISM	GVA	**EU625666**
HPR5	NIT	DV	KIRVD	AIPPQ	L				ISM	GVA	**SF14/95**
HPR8	NIT	DV	KIRVD	AIPPQ	L					GVA	**MR46/99**
HPR11a	NIT	DV	KIRVD	AIPP				RN	IFISM	GVA	**SF54/00**
HPR11b	NIT	DV	KIRVD	AIPP				RN	IF**V**SM	GVA	**SF63/01**
HPR6	NIT	DV	KIRVD	AI		QV	EQPAT	SVLSN	IFISM	GVA	**ST25/97**
HPR32	NIT	DV	KIRVD	A		TNQV	EQPAT	SVLSN	IFISM	GVA	**N128/07**
HPR14a	NIT	DV	KIRVD	A		NQV	EQPAT	SVLSN	IFISM	GVA	**ST21/96**
HPR14b	NIT	DV	KIRVD	A		NQV	EQPAT	SVLSN	**T**FISM	GVA	**FM168/10**
HPR14c	NIT	DV	K**T**RVD	A		NQV	EQPAT	SVLSN	**T**FISM	GVA	**FM174/11**
HPR14d	NIT	D**L**	KIRVD	A		NQV	EQPAT	SVLSN	**T**FISM	GVA	**FM174/11a**
HPR33	NIT	DV	KIRVD			DNV	GQPAT	SVLSN	IFISM	GVA	**N127/07**
HPR18	NIT	DV	KIRV				PAT	SVLSN	IFISM	GVA	**R111/05**
HPR17	NIT	DV	KIR			LEV	EQPAT	SVLSN	IFISM	GVA	**MR103/05**
HPR10	NIT	DV	KIK				QPAT	SVLSN	IFISM	GVA	**MR52/00**

Table 2. Continued....

HPR36	NIT	D**L**	K		TF	NTNQV	EQPAT	SVLSN	IFISM	GVA	**CH09/08**
HPR13	NIT	DV	K				EQPA**N**	SVLSN	IFISM	GVA	**ISA440970**
HPR1	NIT	DV	K				PAT	SVLSN	IFISM	GVA	**H1/87**
HPR7a	NIT	DV	K				T	SVLSN	**T**FISM	GVA	**H17/96**
HPR7b	NIT	DV	K				T	SVLSN	IFISM	GVA	**N29/97**
HPR7c	NIT	DV	K				T	SVLSN	I**S**ISM	GVA	**R171/07**
HPR7d	NIT	DV	**R**				T	SVLSN	IFISM	GVA	**Chile***
HPR7e	NIT	DV	K				T	S**AP**SN	IFISM	GVA	**Chile***
HPR7f	NIT	DV	K				T	SV**S**SN	I**S**ISM	GVA	**CH03/08***
HPR7g	NIT	DV	K				T	SVLSN	IFI**Y**M	GVA	**FJ594307***
HPR7h	NIT	DV	K				T	SV**S**SN	IFISM	GVA	**CH03/08***
HPR7i	NIT	DV	K				T	SVlSN	I**S**ISM	GVA	**R171/07**
HPR15b	NIT	DV					A T	SVLSN	IFISM	GVA	**FJ594282**
HPR15a	NIT	DV					ET	SVLSN	IFISM	GVA	**H36/98**
HPR34	NIT						EQPAT	SVLSN	IFISM	GVA	**NT134/08**
America											
HPR00	NIT	DV	KIRVD	AIPPQ	LNQTF	NTNQV	EQPST	TVLSN	IFISM	GVA	**USA2004**
HPR00	NIT	DV	KIRVD	AIPPQ	LNQTF	NTNQV	EQPAT	SVLSN	IFISM	GVA	**AY646059**
HPR20	NIT	DV	NNRVD	AI	L	GVNQV	EQPST	SVPSN	IFISM	GVA	**CCBB**
HPR21	NIT	DV	NNRVD	AIPPQ	L			SN	IFISM	GVA	**Can31/97**

* = ISA viruses from Chile, but of European origin.

Segments seven and eight of the ISA virus both contain two ORFs (Biering et al 2002; Ritchie et al 2002; McBeath et al 2006; Garcia-Rosado et al 2008), and it has been suggested that there could be a third ORF in segment 7 (Kibenge et al 2007a). The first two ORFs of segment seven consist of 903 and 522 nt coding for proteins consisting of 300 and 159 amino acids, respectively, where the latter is a result of a splicing event

that removes a 526 nt intron during the formation of mRNA (Biering et al 2002). Both proteins share the first 22 amino acids. The largest ORF seems to be coding for a non-structural (NS1) protein, probably an interferon-signalling antagonist (Biering et al 2002; McBeath et al 2006; Garcia-Rosado et al 2008), while the second ORF could be coding for a nuclear export protein (NEP) (Kibenge et al 2007a). The first of the two ORFs in segment eight is coding for a major structural protein, probably the matrix (M) protein (Biering et al 2002), while the latter is suggested to be coding for a type I IFN antagonist (Garcia-Rosado et al 2008).

Genotyping and molecular epizootiology

All organisms, including viruses, are related by common ancestry and display a genetic diversity that reflects their evolutionary history. Changes in the virus genome occur when mutations are incorporated into the genome and passed to later generations. By comparing homological characters, homological nucleotide and amino acid sequences and changes in these, it is possible to reconstruct the evolutionary history of existing viruses. All viruses in farmed populations of fish originate from viruses present in wild populations and these viruses have coevolved with their hosts for millions of years and will, in most cases, not cause large mortalities among their natural hosts. This "equilibrium" will change when there are changes in the host, virus populations, or changes in the environment. An increased number of hosts and reduced distance between the hosts could increase the virulence of a well adapted host specific virus. The idea behind fish farming is to be able to produce a high number of individuals in a

smallest possible area (farm with nets or cages) and, in addition, the farmed fish populations will in most cases have a lower genetic variation compared to wild populations. Introduction of viruses into such farmed populations of fish could speed up the evolution (substitution rate) of viruses and virus genotypes with new or different characteristics may emerge.

Tracing the relationships between members of the same virus species will in practice involve the creation of alignments of homological sequences that can be used for reconstruction of the evolutionary history based on a model of evolution. Choice of sequences is important since each gene in a virus genome may evolve at a different speed, i.e. the substitution rate is in most cases expected to be higher for virus surface proteins exposed to the host immune system compared to genes coding for internal virus proteins like nucleoprotein and polymerases that are part of the ribonucleoprotein complex (Webster et al 1992, Wong et al 2011). The ISA virus genome consists of eight segments coding for a minimum of 10 proteins. Two of the major structural proteins are surface proteins, F and HE, coded in segments five and six.

The ISA virus differs from influenza A virus in the coding and function of the surface proteins where the HA, responsible for binding of virions to host cell receptors and for fusion between the virion envelope and the host cell of the influenza A, is coded by segment four, while segment six of the ISA virus is coding for a protein that functions

both as attachment to host cells and freeing the virions from host cell receptors (Krossøy et al 2001b; Hellebø et al 2004). The fusion activity of ISA viruses is provided by a separate surface protein coded by segment five (Aspehaug et al 2005). The segment six of the influenza A virus codes for a protein with neuraminidase activity (cleaves terminal sialic acid from glycoproteins or glycolipids). The HA0 of the influenza A is posttranslationally cleaved into two subunits (HA1 and HA2) and this cleavage is required for infectivity (virus-cell fusion) and is carried out by host-produced trypsin-like proteases. Inserts in front of the cutting site are known to change the virulence of the influenza A virus (Suarez et al 2004). Similar changes can also be seen in the ISA virus where the fusion protein is produced as a precursor protein, F0, which is posttranslationally, cleaved into F1 and F2 and inserts may occur in front of the cutting sites (Devold et al 2006; Markussen et al 2008; Vike et al 2008). However, the effect of an insert in front of the cutting site of the ISA virus F protein remains to be shown. Both the NA protein of influenza A viruses and the HE protein of the ISA virus may have deletions in the stalk region, and it has been shown that the deletion may affect the influenza NA protein's ability to release the virus from the cell (Matrosovich et al 1999). The region in the ISA virus segment six, where the deletions occur, is named the highly polymorphic region (HPR) and all pathogenic ISA viruses have a shorter HPR compared to what is believed to be the avirulent wild-type HPR0 ISA virus. Hence, in both influenza A and ISA viruses, the neuraminidase or esterase activity of the surface proteins seems to be affected by deletions in the stalk region, while inserts in front of the cutting site of the precursor proteins (HA0 and F0) may have an effect on attachment and fusion activity of the respective surface proteins.

The segment six of the ISA virus, the haemagglutinin (HA) gene, was first identified and characterized by Krossøy et al (2001b). They showed that the protein had haemagglutination activity, but unlike influenza virus HA they did not find posttranslational cleaving of the ISA virus HA. This study also revealed a highly polymorphic region (HPR) in the ectodomain close to the transmembrane region. Later the same year several other studies were published supporting the characterization given by Krossøy et al (2001b) (Devold et al 2001; Griffiths et al 2001; Kibenge et al 2001; Rimstad et al 2001). Receptor-destroying enzyme activity of the ISA virus was detected (possibly an acetylesterase) in 1997 (Falk et al 1997), and in 2004 it was shown that the protein coded by segment six carried receptor-destroying activity identified as an acetylesterase (Hellebø et al 2004), i.e. the protein was identified as a hemagglutinin-esterase (HE). The HE protein has been further characterized by Mikalsen et al (2005), and Müller et al (2010). The first phylogenetic analysis using the HE gene from the ISA virus was published by Devold et al (2001). The study included ISA viruses from 37 different salmon farms in Norway, Scotland and Canada and revealed several different variants of the HPR. The same year Kibenge et al (2001b) said that the shorter HPRs seen in European ISA viruses compared to isolates of Canadian origin suggested that the archetypal ISA virus was probably of Canadian origin, inferring a direction of evolution towards shorter HPRs. However, as shown by Devold et al (2001) there is a large variation in the length of HPRs from European ISA virus isolates and no indication of evolution towards shorter HPRs. Devold et al (2001)

suggested that the variation could be a result of recombination, with the recombination site within the HPR, and it was recommended that only the 5`-end flanking region to the HPR should be used in phylogenetic studies (Devold et al 2001).

The longest HPR observed was first obtained from ISA virus in a wild Atlantic salmon (Cunningham et al 2002), and it became obvious that all other HPRs could be derived from this HPR by recombination as suggested by Devold et al (2001). The long HPR was termed HPR0 by Nylund et al (2003) and they suggested that ISA viruses with HPR0 were low virulent wild-type viruses that could mutate into the virulent isolates with shorter HPRs found in farmed populations of Atlantic salmon. Hence, reservoirs of low virulent ISA viruses could be a constant source of new virulent ISA viruses. It was also suggested that the long HPR found by Cunningham et al (2002) could be an ancestral form of the ISA virus HE gene, and all the shorter European HPRs were a result of parallel evolution due to strong functional selection pressure leading to deletions in the HPR (Mjaaland et al 2002). Mjaaland et al (2002) refuted the suggestion by Devold et al (2001) that the short HPRs could be a result of recombination. HPR0 ISA viruses have been found in all areas with farming of Atlantic salmon, Norway (**Paper II** and **III**), Scotland (Cunningham et al 2002; McBeath et al 2009), USA (**Paper II**), Canada (Cook-Versloot et al 2004), Faroe Islands (Christiansen et al 2011), and Chile (Kibenge et al 2009, **Paper III**).

Nylund et al (2003) showed that the substitution rate of the HE gene was relatively low compared to the substitution rate for genes of surface proteins from other members of the *Orthomyxoviridae*, and they suggested that a possible explanation for this could be

that changes in the HPR could result in changes in virulence of ISA viruses. Hence, all HPR shorter than the HPR0 could be "offspring" of wild type, low virulent, HPR0 ISA viruses, i.e. shorter HPRs would represent a shift towards higher virulence (Nylund et al 2003). This would mean that the shorter HPRs are not a result of an evolutionary trend towards shorter HPRs, but a process where the low virulent, wild type, HPR0 ISA viruses may shift to high virulence. A similar mechanism has been described for changes in virulence of influenza A viruses (Zhou et al 2009; Sorrell et al 2010; Wu et al 2010; Jinling et al 2011). Deletions in segment six of ISA viruses or inserts in the HA gene of influenza A viruses do not change the phylogenetic position of the isolates but lead to changes in virulence. This means that the HPR will not contain any phylogenetic information, distantly related ISA viruses may have the same HPR, but that would be a result of similar recombination events changing wild type HPR0 ISA viruses into virulent ISA viruses with a shorter HPR. The shortening of the HPR seems to follow a fixed pattern.

Nylund et al (2003) launched a hypothesis to explain the maintenance of ISA viruses in Norway and the emergence of ISA in Norwegian salmon farms. The hypothesis was based on the assumption that ISA viruses must have existed for a long time in natural populations of salmonids in Norway. The salmonids (*S. salar* and *S. trutta*) live in small fragmented populations and a specific salmonid virus must have co-evolved with the host resulting in a low or avirulent wild type virus (HPR0 ISA viruses). The emergence of ISA in farmed salmon was suggested to result from transmission of wild

type ISA viruses from wild salmonids to populations of farmed salmon. Change in the HPR (from HPR0 to shorter HPRs) would make the ISA viruses visible in large farmed populations of salmon, and at the same time the virus was given the opportunity to spread within these populations. Transport of infected farmed salmon would also help spreading the virus along the Norwegian coast.

In 2005 it was shown that the ISA virus (probably an avirulent HPR0) could be vertically (or transgenerationally) transmitted (A. Nylund pers.com., Multiple authors 2005), and in a large study of transmission of ISA viruses in farmed populations of Atlantic salmon published in 2006 the importance of vertical transmission was given further support by results from molecular epizootics and detection of a high number of positive smolt populations in fresh water (**Paper II**). This study showed the presence of avirulent HPR0 ISA viruses in smolt populations in Norway and in Atlantic salmon brood fish, and in addition, it was shown that virulent ISA viruses from different areas along the Norwegian coast were closely related. The relationship seemed to reflect the origin of the eggs from which the salmon were hatched, i.e. salmon suffering from ISA caused by closely related ISA viruses came from the same brood fish populations. **Paper II** concluded that a limited number of ISA viruses seem to be circulated in the production cycle of farmed Atlantic salmon in Norway, and suggested that there is little or no transmission of ISA virus from wild salmonids to farmed salmon. The low substitution rate observed for the HE gene could be a result of vertical transmission since this type of transmission will put different constraints on the evolution of the ISA

virus genes compared to viruses that are only transmitted horizontally (**Paper II** and **III**).

The fusion (F) protein was first characterized by Aspehaug et al (2005) who showed that the protein coded for by segment five was a pH-dependent F protein produced as a precursor protein, F0, which was proteolytically cleaved into F1 and F2. Comparison of several F protein genes from several different ISA viruses has shown that inserts may occur in the vicinity of the putative cutting site for trypsin, thus extending the ORF and resulting in ISA virus F proteins ranging from 444 to 455 amino acids (Devold et al 2006). The authors suggested that the insert, which showed 100% identity with other parts of the ISA virus genome, must have been inserted as a result of recombination and could possibly influence the virulence of ISA viruses. Inserts in segment five of ISA viruses has later been demonstrated by several authors (Godoy et al 2008; Markussen et al 2008; Vike et al 2008; Kibenge et al 2009). Devold et al (2006) showed that the substitution rate of segment five, as that of segment 6, is low compared to that of influenza virus surface proteins. It was suggested that the low substitution rate of segments five and six could be understood if the variation in the ISA virus genome generated in marine salmon farms were lost at the time of slaughtering. This would mean that variation in the ISA virus genome generated during the sea water phase of farmed salmon is lost when commercial size is reached and that little variation is transmitted horizontally to other farms or wild reservoirs. This was interpreted as support for the hypothesis of vertical (transgenerational) transmission of

ISA virus in farmed brood fish populations (Devold et al 2006, **Paper II**). Phylogenetic analyses of segments five and six have been used by several authors in studies of ISA virus transmission (Devold et al 2001, 2006; **Paper II** & **III;** Markussen et al 2008; Kibenge et al 2009; Lyngstad et al 2008, 2011).

Phylogenetic analysis using the complete genome or sequences of all eight segments of the ISA virus have been performed, but only a few isolates were included, 12 and 17 respectively (Markussen et al 2008, Cottet et al 2010). These are the only studies that have included segments three (N protein gene) and four (putative PA gene) in a phylogenetic analysis of ISA viruses. The N and PA proteins are both internal proteins where the former coats the viral RNA and is involved in the nuclear export of the nucleocapsids during ISA virus replication. The putative PA protein is believed to be the acid polymerase and involved in the replication of the ISA virus genome. They are both important for ISA virus replication and their evolution should reflect this.

The combination of transmission of viruses with isolation of different host populations will contribute to the evolutionary divergence generating different virus gene pools. The barriers may be due to infrequent likelihood for transmission connected to partial geographical isolation, behaviour of the host, or that the virus may use several host species. The population structure and the behaviour of the natural hosts (*S. salar* and *S. trutta*) for the ISA virus suggest that a large variation in the number of virus genotypes

could have been maintained along the Norwegian coast reflecting the genetic distinctness of the host species populations. It is less likely that interspecies transmission of ISA virus between *S. salar* and *S. trutta* should have resulted in any differences in the surface protein genes (HE and F) within river systems, but the internal protein genes (PA and NP) may have adapted to the two respective hosts. Hence, the surface protein genes should, to a larger extent, reflect the variation in the different wild populations, while changes in the internal protein genes may, to a larger extent, be a result of adaptation to host species and show less variation. However, farming of Atlantic salmon during the last 30 years must have had a strong influence on the evolution of the ISA virus and the maintenance of virus gene pools. It is well documented that movement of embryos and fish have resulted in movement of ISA viruses along the Norwegian coast, but also as far as from Norway to Chile (Devold et al 2006, **Paper II** & **III**, Vike et al 2008, Kibenge et al 2009). This has resulted in a situation where the same ISA virus may be found as far apart as in contemporary ISA outbreaks in Northern Norway and Chile (**Paper III**). Chile has now imposed a ban on import of embryos from other salmon producing countries which should isolate the Chilean ISA viruses and should lead to the evolution of a separate gene pool.

Virulence

The virulence and host range of influenza A (family *Orthomyxoviridae*) are believed to be controlled by all genes in the genome (Webster et al 1992; Brown 2000). However, the surface proteins are believed to play a key role (Webster et al 1992). ISA virus HE proteins have a stalk region of variable length (Devold et al 2001; Krossøy et al 2001b;

Rimstad et al 2001; Griffiths et al 2001; **Paper II; Paper III**) and a variable stalk region is also present in the neuraminidase protein of influenza A viruses (Zhou et al 2009; Sorrell et al 2010; Wu et al 2010; Jinling et al 2011). The stalk region of the influenza viruses is known to play a role in determination of virulence. The longest stalk observed in ISA virus HE is called HPR0 and viruses of this type can not be cultured in any of the fish cell cultures tested and they do not cause any pathology or mortality in infected Atlantic salmon. Hence, it has been suggested that the HPR of the HE protein could be an important virulence factor for the ISA viruses (Nylund et al 2003; Kibenge et al 2007b; Markussen et al 2008; Mjaaland et al 2002; Ritchie et al 2008; **Paper II; Paper III**).

Inserts in front of the cutting site of the precursor protein F0 could also influence the virulence of ISA viruses (Devold et al 2006; Kibenge et al 2007b; Markussen et al 2008). As a comparison it has been shown that inserts in the HA protein of influenza A, in front of the cutting site for HA0, could increase the virulence (Hirst et al 2004; Suarez et al 2004). Hence, the HPR of segment six (HE gene) and inserts in segment five (F gene) may both be important virulence factors for ISA viruses.

Little is known about the possible importance of variation in the other six segments of ISA viruses, and one reason for this is the lack of information about the genetic variation in these segments. However, based on knowledge from other

Orthomyxoviruses there is every reason to believe that they play a part in the determination of virulence (Webster et al 1992; Brown 2000).

Virulence of viruses is not only dependent on the virus genome, but also, to a large extent, on the host and host factors. ISA viruses are able to multiply in both salmon and trout in the North Atlantic, but all isolates tested have only been able to cause mortality in salmon (Nylund et al 1994a,b, 1995a,b,c, 1997; Nylund and Jacobsen 1995; Devold et al 2000). The host factors responsible for this difference have not yet been identified.

Risk factors and transmission of ISA virus

The first official outbreak of ISA was registered at a smolt production site in 1984 in western Norway (Thorud & Djupvik 1988). The next year the disease was observed at marine sites that had received smolt from this hatchery (Thorud 1991), and in 1990 ISA (about 80 cases) was present in all counties with production of Atlantic salmon with the highest number of cases in western Norway (Jarp & Karlsen 1997). Most cases of ISA occur in sea water production of Atlantic salmon, during spring and late autumn, i.e. in periods with temperatures between 10 and 15 ºC.

It has been thoroughly documented that the ISA virus can be transmitted horizontally between individuals of Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*) and trout (*Salmo trutta*) kept in tanks or sea cages (Thorud & Djupvik 1988; Thorud 1991; Nylund et al 1993b, 1994a, 1995a, 1995c, 1997; Nylund & Jakobsen 1995; Dannevig et al 1994; Thorud & Torgersen 1994; Rolland & Nylund 1998a; Devold et al 2000; Griffiths & Melville 2000; Jones & Groman 2001; Snow et al 2001b,c; MacWilliams et al 2007), and in 1992 it was shown that the salmon louse (*Lepeophtheirus salmonis*) could play a role as a mechanical vector for transmission of ISA virus (Nylund et al 1992, 1993b; Rolland & Nylund 1998b). No other organisms have been shown to play a role in ISA virus transmission. The ISA virus may replicate in herring (*Clupea harengus*) and cod (*Gadus morhua*), but these species are not able to transmit the virus to Atlantic salmon during cohabitation (Nylund et al 2002; Grove et al 2007).

The ISA virus is present in mucus, blood and urine from positive Atlantic salmon (Thorud 1991; Nylund et al 1994b; Totland et al 1996; Rolland & Nylund 1998b) and these "fluids" may play an important role in the horizontal transmission between individuals. The distance the ISA virus can be transmitted passively through sea water will depend on the survival time of the virus and the dilution effect during dispersion. The virus will survive for a longer time at low temperatures (like 5 ºC) compared to high temperatures (15 ºC), but the survival time is even more dependent on biological activity in the sea water and exposure to UV light (Henrik Duesund pers. com.). In a

pilot study of the survival of ISA virus in active sea water the survival time was less than 48 hours (A. Nylund pers. com.).

Most cases of ISA occur about one year after sea transfer of Atlantic salmon, but the disease may occur in hatcheries and as early as one month after transfer to sea (Jarp & Karlsen 1997; Nylund et al 1999). Several studies have looked at possible ISA risk factors in marine production of Atlantic salmon, and the overall conclusion has been that the ISA virus is mainly transmitted from infected salmon in farms and slaughterhouses to clean sites through sea water (Vågsholm et al 1994; Jarp & Karlsen 1997; Hammell & Dohoo 2005; McClure et al 2005; Gustafson et al 2007a; Mardones et al 2009; Murray et al 2010). Scheel et al (2007) developed a stochastic space-time model to quantify risk factors for the transmission of ISA virus in Norway (period 2002 to 2005) and they found that seaway distance and local contact networks were factors that affected the transmission between sites. However, there was a large unidentified component of risk other than transmission of virus from infected sites (Scheel et al 2007). A similar result was obtained by Aldrin et al (2010) who found that seaway distance between farms was important for transmission of ISA (period 2003 to 2007), but with a dominance (70 to 80 %) of a non-defined transmission pathway for this disease.

Another important risk factor for introduction of ISA into a sea site is suggested to be purchase of smolts from several fresh water hatcheries (Vågsholm et al 1994; Jarp & Karlsen 1997). It was suggested that the risk was not due to transport of smolt with undiagnosed ISA, but that the smolt was infected during transportation (Jarp & Karlsen 1997). According to Vågsholm et al (1994) the risk factors are not consistent with a hypothesis that ISA has a ubiquitous marine reservoir, and they suggested that rapid sanitary slaughter of primary cases of ISA outbreaks could limit the spread of ISA in a district. In a study of ISA outbreaks in Scotland in 1998 to 1999 it was suggested that the spread of ISA virus was linked to well boat traffic through shipment of live fish (Murray et al 2002). The pattern of spread did not support a natural diffusive expansion of the ISA virus epizootic. The sources for the ISA viruses carried by well boats were believed to be processing plants and adjacent harvesting stations (Murray et al 2002). In a study of spatial and non-spatial risk factors connected to transmission of ISA virus between cages at salmon production sites a diffuse distribution of virus exposure throughout infected sites was observed i.e. limited relationship between cage adjacency and timing of disease (Gustafson et al 2007b). The authors suggested that disease manifestation could be related with host-susceptibility factors.

A method for detection of ISA virus in sea water (able to detect 5.5 viruses/ml) was developed and tested by Løvdal & Enger (2002). Using this method they were able to detect the virus inside cages with outbreaks of ISA, but not in samples collected downstream from the ISA cages (Løvdal & Enger 2002). McClure et al (2004b) tested

the prevalence of ISA virus in farms with and without outbreaks of ISA in the New Brunswick area in the period 2000 to 2002 using RT PCR. Samples were taken from moribund salmon and healthy salmon from cages with outbreaks of ISA, from healthy salmon in a non-outbreak cage on an outbreak farm, from healthy salmon in the non-outbreak neighbour farm, and from healthy salmon from non-outbreak farms distant from outbreak farms. The prevalence of ISA virus was highest in the salmon from cages in the outbreak farms, but ISA virus positive salmon were also found in the two latter groups.

These data are slightly contradictory where some studies indicate horizontal transmission over relatively long distances, while other studies suggest limited transmission between neighbouring cages. One explanation for this could be the fact that ISA virus can be present in salmon populations without causing outbreak of ISA and, hence, spreading of the virus to new sites or cages is not detected, but it also means that the source for the virus causing an ISA outbreak may not be detected. An important fact is that in none of the above mentioned studies were the ISA viruses identified, genotyped, and most of these studies seem to rest on the assumption that salmon infected with ISA virus will eventually develop ISA. Nor have any of these studies discussed the possible importance of avirulent ISA viruses in the spread of the virus and eventual development of ISA (cf: Nylund et al 2003, **Paper II** & **III**).

In a recent modelling study of transmission of ISA the phylogenetic relationships between virus isolates was combined with space–time data on disease occurrences (Aldrin et al 2011). The study period was 2003 – 2009 with the major emphasis on outbreaks of ISA in a small area in Troms County in 2007 – 2009. The authors found that the risk of infection decreases with increasing distance between sites and that the source of infection in nearly 50 % of the cases is most likely to be ISA viruses closely related to viruses from outbreaks in neighbouring farms (Aldrin et al 2011). The remaining half of the farms included in the study was infected with ISA virus from an unknown source.

The high number of ISA cases in the period 1987 – 1990 made it necessary for Norwegian salmon farmers to introduce measures to reduce the impact of ISA on Norwegian salmon culture. The salmon farmers changed their production from two or more generations at each site to only one generation of Atlantic salmon per site starting in 1990 – 1991. In addition, the authorities introduced a ban on use of sea water in hatcheries and a ban on moving fish already stocked into sea water in 1989, regulations on transport of salmonids in 1990, regulations on disinfection of waste water/offal from fish slaughterhouses, and regulations on disinfection of intake water to hatcheries in 1991 (Håstein et al 1999). After this the official number of ISA cases dropped to only one in 1993, and has since stabilised with about 10 to 20 cases every year. Use of oil vaccines (against bacterial diseases in the production of Atlantic salmon) was also introduced in the early 90s and it has been observed that the oil

adjuvant may give a certain protection against ISA virus. It is difficult to know which of these measures implemented were most important for the reduction in the number of ISA outbreaks from close to 100 every year to as few cases as 10 to 20 every year, however, separation of generations must have played a major role preventing horizontal transmission and a continuous production of virulent ISA viruses at infected sites (cf Gustafson et al 2007a).

The first study of vertical transmission of ISA virus was published by Melville & Griffiths (1999). They collected eggs from Atlantic salmon with ISA virus positive ovarian fluids, and the eggs and resulting offspring (alevins and parr) were tested for virus by cell culture and RT PCR. Healthy parr were also injected with homogenates from eyed eggs. All samples were negative for ISA virus and the homogenates did not induce any mortality in the parr. However, as pointed out by the authors, the detection limit for the RT PCR is not known and the amount of virus could be below the detection limit for the methods used (Melville & Griffiths 1999). The most sensitive method they employed was a PCR using FA-3/RA-3 primers (Devold et al 2000), but this assay will not detect ISA virus in carriers (**Paper I**). In addition they decreased the sensitivity of the RT PCR assays by pooling samples from individual fish. The only method that is sensitive enough to detect carriers of ISA virus is real time RT PCR used on individual samples (pooling of samples will also destroy the sensitivity of real time assays). It should also be added that this study was performed before the scientific

community was aware of the existence of avirulent ISA viruses which can not be cultured in cell cultures.

The first indication of possible vertical or transgenerational transmission of ISA virus came in 2004 (A. Nylund pers. com). Later that year a Norwegian Broodfish company, AS Bolaks (SalmoBreed AS), experienced an outbreak of ISA on brood fish of Atlantic salmon kept in Hordaland County (Multiple authors 2005). Offspring (eggs, embryos and fry) from the ISA virus positive fish were screened, with the help of real time RT PCR (cf. **Paper I**), for presence of ISA virus and the results showed that virus genome could be detected in all stages of development suggesting vertical transmission of the ISA virus, but the fry were killed and it was not verified if the infected fish would develop ISA at a later stage (Multiple authors 2005). Embryos of Atlantic salmon, produced in Norway, have for many years been transported to Chile and in 2007 a large epizootic of ISA started in the Chilean salmon industry. The virus and the disease had previously only been detected in countries around the North Atlantic. The causative ISA virus was identified as a Norwegian genotype and the presence of a Norwegian ISA virus in Chile, a country without natural populations of salmonids, was interpreted as evidence of vertical transmission (Vike et al 2008).

Genotyping of ISA viruses from outbreaks of ISA in Norway has also given support to a hypothesis suggesting that vertical/transgenerational transmission of ISA virus could

be of importance for the dissemination of the virus in Norwegian salmon production (**Paper II**). However, two other studies using genotyping as a tool for understanding the transmission of ISA virus (Lyngstad et al 2008, 2011) did not find any evidence of vertical transmission. But these studies did not include any information about the origin of the salmon (brood fish) at the different production sites suffering from ISA and they included material from a limited time period only. In the latter study it is stated that shared broodfish company was excluded from the analysis, because there are so few broodfish companies in Norway (Lyngstad et al 2011). Lyngstad et al (2008, 2011) arrived at the conclusion that seaway distance between salmon farms is the only trustworthy explanation for the genetic similarity between ISA viruses isolates collected during outbreaks of ISA in Norway.

AIMS OF STUDY

The goal of this project was to characterize the partial genome from a large selection of ISA viruses to gain knowledge about the evolution of this virus and a better understanding of the maintenance and dissemination of ISA viruses in farmed populations of Atlantic salmon.

Specific aims for this study were:

- Mapping of the variation in the nucleotide sequences of the genome segments coding for the two surface proteins, F and HE protein, and the two internal proteins, N and PA protein, constituting a part of the ribonucleoprotein complex of ISA viruses.
- Mapping of important mechanisms (mutations, recombination, and reassortment) for the evolution of ISA viruses in farmed populations of Atlantic salmon.
- Mapping of virulence indicators in the ISA virus genome.
- Establishing a genotyping system for ISA viruses.
- Using the genotyping system to gain understanding of the maintenance and dissemination of ISA viruses.

SUMMARY OF PAPERS

Paper I

Plarre H, Devold M, Snow M, Nylund A (2005). Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in western Norway. Dis Aquat Org 66: 71 – 79.

Studies of infectious salmon anaemia virus (ISA virus), an important pathogen of farmed salmon in Norway, Scotland, the Faeroe Islands, Ireland, Canada, USA and Chile, suggest that natural reservoirs for this virus can be found on both sides of the north Atlantic. Based on existing information about ISA virus it is believed that it is maintained in wild populations of trout and salmon in Europe. It has further been suggested that the ISA virus is transmitted between wild hosts mainly during their freshwater spawning phase in rivers, and that wild salmonids, mainly trout, are possible carriers of benign wild-type variants of ISA virus. Change in virulence is probably a result of deletions of amino acid segments from the highly polymorphic region (HPR) of benign wild-type isolates after transmission to farmed salmon. Hence, it has been suggested that the frequency of new outbreaks of ISA in farmed salmon could partly reflect natural variation in the prevalence of ISA virus in wild populations of salmonids.

The aims of the present study were to screen for ISA virus in wild salmonids during spawning in rivers and to determine the pathogenicity of resultant isolates from wild fish. Tissues from wild salmonids were screened by RT-PCR and Real Time PCR. The prevalence of ISA virus in wild trout (*Salmo trutta*) varied from 62 to 100% between tested rivers in 2001. The prevalence dropped in 2002 ranging from 13 to 36 % in the same rivers and only 6% in 2003. All ISA virus were non-pathogenic when injected into disease-free Atlantic salmon, but were capable of propagation as indicated by subsequent viral recovery. However, non-pathogenic ISA virus has also been found in farmed salmon where prevalences as high as 60% have been registered but with no mortalities occurring. Based on the results of the present and other studies it must be concluded that vital information about the importance of wild and man-made reservoirs for the emergence of ISA in salmon farming is still lacking. This information can only be gained by further screening of possible reservoirs combined with the development of a molecular tool for typing virulence and geographical origin of the virus isolates.

Paper II

Nylund A, Plarre H, Karlsen M, Fridell F, Ottem KF, Bratland A, Sæther PA (2007). Transmission of infectious salmon anaemia virus (ISAV) in farmed populations of Atlantic salmon (*Salmo salar*). Arch Virol 152: 151 – 179 (E.pub. 2006).

In the present study 24 smolt production sites were screened for the presence of infectious salmon anaemia virus (ISAV) with the help of a specific real time RT PCR assay, and 22 of these sites had smolts that were positive. If these smolt production sites are representative for the prevalence of ISAV in Norwegian smolts then most marine production sites must be considered to be positive for ISAV. In addition, 92 European ISAV isolates have been genotyped based on the hemagglutinin-esterase gene (HE), and the distribution pattern of these analysed. This pattern has been coupled to information about the origin of smolt, eggs and broodfish in those cases where it has been possible to obtain such information, and with information about ISAV in neighbouring farms. The pattern suggests that an important transmission route for the ISAV could be that the salmon farming industry in Norway is circulating some of the isolates in the production cycle, i.e. some sort of vertical or transgenerational transmission may occur. It has also been shown that avirluent ISAV isolates are fairly common in Norwegian farmed salmon. Based on this it is hypothesized that the change from avirulent to virulent ISAV isolates is a stochastic event dependent on the replication frequency of the virus and the time available for changes in a highly polymorphic region (HPR) of the HE gene to occur. This, and the possibility that only avirluent ISAV isolates are vertical transmitted, may explain why ISA most often occurs at marine sites and why no more than about 15 farms get ISA every year in Norway.

Paper III

Plarre H, Nylund A, Karlsen M, Brevik Ø, Sæther PA, Vike S (submitted). Evolution of Infectious Salmon Anaemia virus (ISA virus). Arch. Virol.

Infectious salmon anaemia virus, ISA virus (*Isavirus, Orthomyxoviridae*), emerged in Norwegian salmon culture in the mid-80s. The genome consists of eight segments coding for at least 10 proteins. ISA viruses show a lot of similarities with influenza A viruses, but differ in many important aspects like number of hosts, host population structure, and transmission. The only known hosts and reservoirs for ISA viruses are salmonids found in countries surrounding the North Atlantic. In this study four different segments in the genome of about 100 ISA viruses have been sequenced in an attempt to understand the evolution of ISA viruses and how these viruses are maintained in and transmitted between populations of farmed Atlantic salmon. The four gene segments are coding for the nucleoprotein (NP), the putative acid polymerase (PA), the fusion protein (F) and the haemagglutinin-esterase (HE).

Analyses of these four genes show that the substitution rates of the internal proteins (NP and PA) are lower compared to the two surface proteins (F and HE). All four segments are evolving at a lower rate compared to similar genes in influenza A viruses. The ISA virus populations consist of avirulent viruses and pathogenic strains with variable virulence in Atlantic salmon. Recombination resulting in inserts close to the proteolytic-cleavage site of the precursor F0 protein and deletions in the stalk region of the HE protein seem to be responsible for the transition from avirulent ISA viruses into

pathogenic strains. It is also shown that reassortment is a frequent event among the dominating ISA viruses in farmed Atlantic salmon.

The pattern that is obtained after phylogenetic analyses of the four gene segments from ISA viruses, suggests that the variation is limited to a few distinct clades and that no major changes have occurred in the ISA virus population in Norway since the first viruses were isolated. Calculation of the time of most recent common ancestor (TMRCA) suggests that the Norwegian ISA viruses separated from the European subtype found in North America between 1932 and 1959. The TMRCA data also suggest that the ISA viruses in Chile were transmitted from Norway in the period from 1995 to 2007, depending on which of the four genes are used in the analysis.

GENERAL DISCUSSION

Host species for ISA viruses

Two major approaches, experimental challenges and screening of wild species, have been used in the search for possible host species for ISA viruses in the North Atlantic. The challenge experiments have shown that naturally occurring salmonids, Atlantic salmon (*Salmo salar*), trout (*S. trutta*) and Arctic Char (*Salvelinus alpinus*), in countries around the north Atlantic can be infected with the virus and may sustain virus replication (Nylund et al 1994a,b, 1995a,b,c; Nylund and Jacobsen 1995; Devold et al 2000; Snow et al 2001a; Rolland & Nylund 1998a). However, while Atlantic salmon and trout can become carriers of ISA viruses the char seem to be able of viral clearance (Snow et al 2001a). Several species of pacific salmon, steelhead trout/rainbow trout (*Oncorhychus mykiss*), coho salmon (*O. kisutch*), chum (*O. keta*) and Chinook (*O. tschawytscha*), have also been tested in challenge experiments as possible hosts for ISA viruses (Nylund et al 1997; Snow et al 2001a; Rolland & Winton 2003; MacWilliams et al 2007). None of these species are naturally occurring in the North Atlantic, but rainbow trout have been introduced several times to countries around the North Atlantic. Some of these *Oncorhynchus* species can sustain replication of the ISA virus and the rainbow tout may even show signs of disease and some of these will end up as carriers (Nylund et al 1997; Snow et al 2001a; Rolland & Winton 2003; MacWilliams et al 2007). During screening of wild salmonids (Atlantic salmon and trout) ISA viruses have been detected in both species in Scotland and Norway in both fresh and sea water (Raynard et al 2001, **Paper I**). Hence, there is both experimental

and empirical support for the hypothesis that wild salmonids in the North Atlantic are the natural hosts for the ISA viruses (cf Nylund et al 2003).

Several other fish species in the marine environment and most often in connection with areas with farming of Atlantic salmon or areas experiencing outbreaks of ISA have been tested for presence of ISA viruses (table 3). All marine fish tested have been negative for presence of ISA virus with the exception of two Pollock (*Pollachius virens*) collected from inside a cage with ISA-diseased Atlantic salmon. It was not possible to culture the virus from these two specimens and the PCR product could not be sequenced (MacLean et al 2003). MacLean et al (2003) also found a pool of tissues (including gill tissues) from five Atlantic cod (*Gadus morhua*), taken from a well boat containing salmon exhibiting clinical ISA, positive for ISA virus using a cell culture. However, all five specimens were negative for ISA virus when they were tested by RT PCR. Both the ISA virus RT PCR positive Pollock and the isolation of ISA virus from Atlantic cod were obtained from populations kept together with Atlantic salmon suffering from outbreaks of ISA. The chances for contamination from the ISA virus positive Atlantic salmon must be extremely high under such conditions and the positive results obtained by MacLean et al (2003) are, in my opinion, best explained as detection of ISA virus contamination of the samples. The contamination hypothesis is also supported by a study performed by Snow et al (2002) who found, after experimental challenges, that *P. virens* was resistant to ISA virus infections and unable to support ISA virus replication. The same result, *P. virens* being resistant to ISA virus

infection, was obtained by McClure et al (2004a) who looked at 93 specimen cohabitating with Atlantic salmon suffering from ISA. All 93 were negative for ISA virus RNA. In a challenge experiment on Atlantic cod (weight = 6 gram) Grove et al (2007) found indication of ISA virus replication and detected virus RNA in the brain of 6 out of 8 Atlantic cod as late as 45 days post challenge (after intraperitoneal injection of ISA virus). The cod showed no signs of clinical disease and no mortality was observed. Atlantic cod cohabitating with Atlantic salmon suffering from ISA (cohabitation challenge) remained negative for presence of ISA virus RNA throughout the experimental period of 74 days (Grove et al 2007). No attempt was made to isolate the possible ISA virus from the brain tissues or inject it into susceptible salmon, which makes it impossible to decide if the virus was replicating in the brain or if the RNA in the brain was a result of selective retention. Based on the result from this study it can not be concluded that the ISA virus is able to replicate in Atlantic cod. The fact that cod cohabitating with salmon suffering from ISA, remained negative with respect to presence of ISA virus strongly suggests that cod is not a potential reservoir species for this virus.

Experimental challenge of herring, *Clupea harengus*, has shown that this species may support replication of the ISA virus after bath challenge, but they were not able to transmit the virus to Atlantic salmon (Nylund et al 2002). However, when tissue samples, taken from herring 26 days post challenge (bath challenge), were homogenized, sterile filtered, and injected into salmon all the challenged individuals

ended up as RT PCR positive for ISA virus and one salmon died showing clinical signs of ISA (Nylund et al 2002). This study shows that the ISA virus is able to infect herring and this species is also able to sustain replication of the virus, but the ISA virus was not transmitted from infected herring to Atlantic salmon. It was also shown that the herring is able of viral clearance 42 day after a bath challenge (Nylund et al 2002). Based on this study it can not be excluded that the wild herring could play a role as a vector for ISA viruses in the marine environment. This could be of importance during outbreaks of ISA in salmon farms in areas with dense populations of herring. However, screening of wild herring for presence of ISA virus, including spawning individuals, has not resulted in any positive samples (table 3). Hence, there are no studies suggesting that herring may constitute a reservoir host for ISA viruses in the marine environment.

Other marine fish species (*Scophthalmus maximus, Anguilla anguilla, Ctenolabris rupestris, Labrus berggylta, Pollachius virens*) have also been challenged with ISA virus, but it has not been possible to show replication of the ISA virus in these species (Kvenseth 1997; Snow et al 2002; MacLean et al 2002; McClure et al 2004a; A. Nylund pers.com). Several wild fish species from the coast of Chile, in areas with outbreaks of ISA, have also been tested and found to be negative for presence of ISA virus (Gonzales et al 2011). The only fish found to be positive in the study from Chile was escaped Atlantic salmon. In addition, three invertebrate species have also been

Table 3. Wild marine fish species tested for presence of ISA virus.

Species	N	Pos	Authors
Scyliorhinus canicula	4	0	Raynard et al 2001
Raja clavata	35	0	Raynard et al 2001
Clupea harengus	1037	0	Raynard et al 2001 MacLean et al 2003, Pers. obs.
Sprattus sprattus	192	0	Raynard et al 2001, Pers. obs.
Ciliata mustela	21	0	Raynard et al 2001
Melanogrammus aeglefinus	321	0	Raynard et al 2001 MacLean et al 2003
Merlangius merlangus	152	0	Raynard et al 2001
Trisopterus esmarki Norway	229	0	Raynard et al 2001
Trisopterus minutus	79	0	Raynard et al 2001
Merluccius merluccius	86	0	Raynard et al 2001
Lophius piscatorius	1	0	Raynard et al 2001
Pleuronectes platessa	48	0	Raynard et al 2001
Solea solea	30	0	Raynard et al 2001
Zeus faber	1	0	Raynard et al 2001
Eutrigla gurnardus	18	0	Raynard et al 2001
Aspitrigula cuculus	30	0	Raynard et al 2001
Myoxocephalus scorpius	2	0	Raynard et al 2001
Agonus cataphractus	1	0	Raynard et al 2001
Callionymus lyra	6	0	Raynard et al 2001
Pomatoschistus microps	2	0	Raynard et al 2001
Lepidorhombus whiffiagonis	25	0	Raynard et al 2001
Zeugopterus punctatus	1	0	Raynard et al 2001
Hippoglossoides	21	0	Raynard et al 2001
Limanda limanda Common	20	0	Raynard et al 2001
Micromesistius poutassou	40	0	Raynard et al 2001
Pollachius virens	232	2[a]	McClure et al 2004a, MacLean et al 2003
*Alosa pseudoharengus**	1059	0	MacLean et al 2003
*Anguilla rostrata**	297	0	MacLean et al 2003
Gadus morhua	235	0[b]	MacLean et al 2003
Pseudupleurinectes	259	0	MacLean et al 2003
Scomber scombrus	211	0	MacLean et al 2003
Cyclopterus lumpus	26	0	MacLean et al 2003
Alosa sapidissima	3	0	MacLean et al 2003
Hippoglossus hippoglossus	2	0	MacLean et al 2003

* = occur in both fresh and sea water.

a = Two positives (by RT PCR) Pollock collected inside a cage with ISA diseased salmon, but it was not possible to culture or sequence the ISA virus (MacLean et al 2003).

b = ISA virus was cultured from a pool of tissues (including gill tissues) from five Atlantic cod taken from a well boat containing salmon exhibiting clinical ISA. However, all five specimens were negative when tested by RT PCR.

Table 4. ISA virus in wild salmonids from selected rivers and fjords in Norway (unpublished). This study is based on testing of gill tissues from Atlantic salmon and trout using real time RT PCR (**Paper I**).

River / fjord	*Salmo salar*	*Salmo trutta*	Year
Møre og Romsdal county			
Eira	0/6 (0.0 %)	0/4 (0.0 %)	2008
Eira	0/31 – (0.0 %)	-	2009
Sogn og Fjordane county			
Nordgulen	-	**1/33 (3.0 %)**	2005
Nordgulen	-	0/30 (0.0 %)	2006
Nordgulen	-	**1/30 - (3.3 %)**	2009
Brekke	-	**6/63 (10.3 %)**	2004
Brekke	-	0/35 (0.0 %)	2005
Brekke	-	0/30 (0.0 %)	2006
Brekke	-	0/30 - (0.0 %)	2009
Hordaland county			
Vosso	0/29 – (0.0 %)	-	2009
Vosso	0/30 – (0.0 %)	-	2010
Øystese (fjord)	**1/4**	0/11 – (0.0 %)	2009
Eidfjord	**1/15 – (6.7 %)**	-	2006
Etnefjord	-	0/54 – (0.0)	2003/2005
Etne-elva	0/21 – (0.0 %)	-	2009
Dale-elva	0/30 – (0.0 %)	-	2009
Rogaland county			
Lyse	0/19 – (0.0 %)	0/11 – (0.0 %)	2007
Lyse	**1/19 – (5.3 %)**	0/10 – (0.0 %)	2008
Lyse	0/13 – (0.0 %)	**1/17 - (5.9 %)**	2009
Lyse	**1/21 - (4.8 %)**	0/18 – (0.0 %)	2010
Lyse	0/13 – (0.0 %)	0/15 – (0.0 %)	2011
Vestbøelva	-	**2/10 – (20.0 %)***	2009
Denmark			
Skjern	0/18 – (0.0 %)	-	2010

* Segment six of the ISA virus has been sequenced from one individual. This is the first ISA virus from wild salmonids that does not have an HPR0 stalk region in the HE protein.

tested for susceptibility to ISA virus: blue mussel (*Mytilus edulis*), *Pecten maximus, Lepeophtheirus salmonis*, but none of these were able to sustain replication of the virus (Skår & Mortensen 2007; Nylund et al 1993a). Based on the existing data about

susceptibility to and presence of ISA virus in wild fish it seems as if the only natural hosts for this virus in the North Atlantic are Atlantic salmon (*S. salar*) and trout (*S. trutta*) (cf. **Paper I**; Raynard et al 2001; table 4).

ISA virus evolution

To fully understand the mechanisms that cause variation and evolution of ISA viruses it is necessary with basic knowledge of the structure and function of the ISA virus genome. The genome consists of eight segments of single stranded RNA which encode 10 or 11 polypeptides (Mjaaland et al 1997; Clouthier et al 2002; Kibenge et al 2007a). All proteins of ISA viruses have not yet been identified, but existing knowledge is presented in Table 1. In addition to the two surface proteins (HE and F) the encoded polypeptides include the same three polymerase (PB1, PB2 and possibly PA) proteins as seen in influenza A viruses, a nucleoprotein (NP), a matrix protein (M), two interferon antagonists (NS1 & NS2) and nuclear export protein (NEP). Segment seven encodes two proteins (NS1 & NEP), but the presence of a third protein (NS3) encoded by this segment has been suggested by Kibenge et al (2007a). However, this has yet to be confirmed by other studies.

The surface protein, HE, is responsible for binding the virions to residues (sialic acid) on the surface of the host cell and releasing the virions from infected cells through cleavage of the host cell residues, while the other surface protein, F, provides fusion of

the virion envelope and the host cell membrane. The three polymerase proteins and the NP constitute the ISA virus replication machinery, the ribonucleoprotein complex (RNP). The major focus of the present study is on segments three, four, five and six, encoding two surface proteins (HE & F) and two internal proteins (NP & PA). The evolution of these four proteins is compared with proteins with a similar function in influenza A viruses.

The ISA viruses, like the other members of the family *Orthomyxoviridae*, lack "proofreading" mechanisms and are therefore unable to repair errors occurring during the replication process, which may lead to substantial genetic diversity and result in a high substitution rate. The segmented genome of ISA viruses also allows for exchange of RNA segments between genotypically different ISA viruses resulting in generation of new genotypes/strains, a process termed "shift" in the evolution of influenza viruses. In addition to mutations and reassortment causing evolutionary change in ISA virus populations, the evolution of the ISA virus genome is also influenced by recombination events (cf. Devold et al 2001, 2006; **Paper II** and **III**). Recombination is considered as a very rare event in the evolution of influenza A viruses, while it seems to occur more frequently in the evolution of ISA viruses (Forrest & Webster 2010; Devold et al 2001, 2006; **Paper II** and **III**). Another important difference between influenza A viruses and ISA viruses is the number and variation of host species. Influenza A viruses have been found in a large number of aquatic bird species and domestic poultry, and in humans, pigs, horses, dogs, cats, mink, seals and whales, while only two closely related

fish species (*Salmo salar* and *S. trutta*) are recognized as natural hosts for the ISA viruses (Raynard et al 2001; Nylund et al 2003; MacLean et al 2003; **Paper I;** see discussion above). The difference in number of hosts and the sizes of the host populations should have a large influence on the evolution of these two viruses where the genetic variation maintained in ISA virus populations is expected to be much smaller compared to that of influenza A viruses.

Antigenic drift, caused by point mutations selected for because of increased genetic fitness, is considered as a continuous process in influenza A viruses (Forrest & Webster 2010; Nelson & Holmes 2007). Accumulation of such changes is expected to be most pronounced in the surface proteins (HA and NA) of influenza A viruses at the antibody binding sites, while the changes in internal proteins should occur at a lower rate (Webster et al 1992). However, the substitution rate of influenza A viruses is also influenced by reassortment (shift), which may speed up the substitution rate of internal proteins like NP and PA (Wong et al 2011). Reassortment occurs when genotypically different viruses infect the same host cell and an exchange of RNA segments occurs, resulting in a new combination of gene segments. For influenza A viruses this may occur in avian reservoir species or any of the other hosts resulting in a virus to which the host population is immunologically naive (Nelson & Holmes 2007). For the ISA virus the situation is different with only two known host species, *S. salar* and *S. trutta*, in the north Atlantic area, and with very small fragmented wild populations of these two species. The highest number of hosts for the ISA viruses is found as farmed

Atlantic salmon, which means that the host populations' structure for ISA viruses are very different from that of influenza A viruses. Farmed Atlantic salmon are slaughtered if experiencing an outbreak of ISA or when the commercial size is reached, meaning that all variation accumulated in ISA virus populations during the production of the Atlantic salmon is lost unless the virus is transmitted to neighbouring salmon populations (horizontal transmission) or to the offspring of Atlantic salmon brood fish (transgenerational/vertical transmission). The difference in population size and life expectancy of the hosts for ISA viruses and influenza A viruses should influence the substitution rates and the survival of new genotypes generated as a result of reassortment and mutations.

The substitution rates will also reflect differences in methodology and number of virus sequences used for analysis (Table 5). The substitution rate for the ISA virus HE gene ranges from 1.13×10^{-3} per site per year, N = 13 sequences (Kibenge et al 2007b) to 6.0×10^{-6}, N = 54 sequences (Devold et al 2006). The substitution rates for this gene obtained in **Paper II** and by Devold et al (2006) are nearly identical while the substitution rate for the HE gene obtained in **Paper III** is significantly higher, but lower than the rate given by Kibenge et al (2007b). The number of sequences used in **Paper II** and **III** in the calculation of the substitution rate for the HE gene is nearly the same, but the time span for the collection of the viruses differs with six years and two different methodologies have been used, which may have influenced the results. The relatively large differences in substitution rates between influenza A viruses and ISA

viruses could be explained by many factors (population size, transmission routes, number of host species, etc) where one explanation could be that the ISA viruses are "old" viruses in salmonid hosts which evolve more slowly compared to influenza A viruses that may change host from birds to humans and evolve more rapidly to evade host immunity and achieve efficient transmission in new host species (cf. Nelson & Holmes 2007).

Table 5. Substitution rates per site per year of four ISA virus genome segments. N = number of sequences included in the analysis.

N	Year span	Seg. 3	Seg. 4	Seg. 5	Seg. 6	Publication
54	1987-2004	-	-	8.60×10^{-5}	6.10×10^{-6}	Devold et al 2006
93	1987-2005	-	-	-	0.70×10^{-5}	**Paper II**
13	1997-2004			0.67×10^{-3}	1.13×10^{-3}	Kibenge et al 2007b
96	1987-2011	1.9×10^{-4}	2.2×10^{-4}	3.9×10^{-4}	3.5×10^{-4}	**Paper III**

Recombination can occur as non-homologous recombination between two different RNA segments or as homologous recombination involving template switching while the polymerase is copying the RNA. Both types of recombination may occur in influenza A and ISA viruses, but the latter type of recombination will be more difficult to document. Non-homologous recombination does occur between the HA and NP genes of influenza A viruses and is thought to be responsible for outbreaks of some types of avian influenza (Suarez et al 2004; Perdue & Suarez 2000; Hirst et al 2004). This type of recombination has also been observed in the F protein gene of ISA viruses where inserts are introduced ahead of the trypsin cutting site of the precursor F protein, F0 (Devold et al 2006). The inserts have so far been obtained from the N, F and PB1

proteins of the ISA virus genome. It is believed that the inserts in the F protein influence the virulence of ISA viruses (Devold et al 2006; Godoy et al 2008; Markussen et al 2008; Vike et al 2008; **Paper III**). Evidence of homologous recombination in influenza A viruses has not yet been definitely shown according to Nelson & Holmes (2007), but it has recently been demonstrated that intragenic, homologous, recombination plays a role in driving the evolution of influenza A viruses (He et al 2008, 2009). In studies of ISA viruses it has also been suggested that the variation seen in the stalk region of the HE protein is a result of homologous recombination (Devold et al 2001; **Paper II**). A major argument for the hypothesis of recombination in the stalk region (HPR) of the ISA virus HE proteins is the deletion pattern observed in this region of the protein (Devold et al 2001; **Paper II & III;** Table 2).

Studies of influenza A have shown that the N proteins evolve into host-specific lineages and that the evolutionary rates may vary among the lineages (Gorman et al 1990a, 1991; Xu et al 2011). The substitution rate of ISA virus N proteins is relatively low compared to that of influenza A viruses, and the number of variable positions in the nucleotide and putative amino acid sequences is significantly lower compared to that of the surface proteins from the same viruses (Table 6, **Paper III**). The PA nucleotide and amino acid sequences have an even lower number of variable positions, but a slightly higher substitution rate compared to the N protein genes from these ISA viruses. All ISA virus sequences of the NP and PA have been obtained from farmed

Atlantic salmon (**Paper III**), which means that we are probably only looking at two proteins already adapted to this host. The phylogenetic analysis of the N proteins shows poor resolution with only a few well supported clades and no indications of more than one distinct lineage (cf. Xu et al 2011; **Paper III**). The pattern obtained after analysis of the PA protein gene is different from that of the N protein gene where the former has evolved into two separate lineages in European farmed Atlantic salmon (**Paper III**). It could be that this is a result of reassortment between Atlantic salmon ISA viruses and trout ISA viruses where a trout ISA virus segment 4 (PA) has been introduced into the former. Another possibility is, of course, that one of the PA lineages has been introduced from an Atlantic salmon ISA virus from a different geographical area in Europe. It can not be excluded, but I find it less likely that one of the lineages comes from an unidentified marine reservoir or from herring.

Table 6. Percentage of variable positions in the nucleotide and amino acid sequence alignments of the nucleoprotein (N), acid polymerase (PA), fusion protein (F) and the hemagglutinin-esterase (HE) protein from 96 ISA viruses (based on **Paper III**).

	NP	**PA**	**F**	**HE**
Nucleotides	12.7	11.7	15.1	16.9
Amino acids	11.8	9.3	15.1	16.2
	Internal proteins		**Surface proteins**	

Virulence

In their natural reservoir, influenza A viruses are largely benign and cause asymptomatic infections (Forrest & Webster 2010), and the same observation is typical for a large number of ISA viruses from wild and farmed Atlantic salmon in fresh and sea water (Cunningham et al 2002, **Paper II, Paper III**, A. Nylund pers.com). In avian species lethal influenza A causes systemic infections, and the major change in these viruses is the acquisition of basic amino acids in the cleavage site of the HA (cf. Forrest & Webster 2010). High pathogenicity of influenza A in mammals is associated with polygenic traits and specific mutations have been identified in the two polymerases, PB1 and PB2, and in the NS, HA (basic amino acid inserted) and NA (deletions in the stalk region). Hence, the virulence of influenza A viruses is polygenetic. Even though it has yet to be documented there is every reason to believe that the virulence of ISA viruses is also polygenetic.

Like the influenza A viruses the ISA viruses show some of the same characteristics in the surface proteins: a) inserts of amino acids ahead of the cutting site of the HA0 (influenza A) and F0 (ISA viruses), and b) a variable length of the stalk region of the other surface protein, neuraminidase, NA (influenza A) and haemagglutinin-esterase, HE (ISA viruses). It is well documented in the literature of influenza A viruses that these characteristics play a role in the determination of virulence (Matrosovich et al 1999; Hirst et al 2004; Cheung & Poon 2007; Zhou et al 2009; Wu et al 2010; Sorrell et al 2010; Spackman et al 2010; Li et al 2011). It is also acknowledged that ISA

viruses with a full length stalk region, HPR0, in the HE protein are of low (avirulent) virulence, while all ISA viruses, detected so far, with a shorter HPR have the ability to cause disease in Atlantic salmon (Nylund et al 2003; Kibenge et al 2007b; Markussen et al 2008; Mjaaland et al 2002; Ritchie et al 2008; Christiansen et al 2011; **Paper II** & **III**). The ISA viruses with shorter HPRs can be cultured in available cell cultures, while the HPR0 ISA viruses can only be cultured in salmonid hosts (McBeath et al 2011; A. Nylund, pers. com.). However, it has been shown that the HPR0 HE protein is fully functional in terms of both receptor-binding and receptor-destroying activity (McBeath et al 2011), which raises a question about the role of the HPR in determination of virulence of ISA viruses. In a study of cells transfected with F protein Aspehaug et al (2005) found that the fusion activity was much more efficient in cells co-expressing the HE and F proteins. This led them to suggest that the ISA virus HE protein could be required in the fusion process (Aspehaug et al 2005). A comparison of F proteins from avirulent (low virulent) HPR0 ISA viruses shows that they all share the same residue at position 266 (Q^{266}) while all pathogenic ISA viruses have L^{266} at this position or an insert close to this residue (**Paper III**). The ISA viruses with an insert close to the putative trypsin cleavage sites all have a Q or H at position 266 (**Paper III**). Only one low virulent ISA virus deviating from this pattern, with proline (P^{266}) at this position, has been found (Kibenge et al 2007b). The fact that all avirulent HPR0 ISA viruses, detected so far, have a unique amino acid (Q) at position 266, only shared by virulent ISA viruses with an insert close to this residue, makes it impossible to decide, at the present, if the HPR0 is an important factor for virulence or if the virulence is determined by the residue at position 266 in the F protein, or if a

combination of these two markers determines if ISA viruses are of low or high virulence.

The study published by McBeath et al (2011) clearly shows that the HPR0 stalk region of ISA viruses does not affect the functional activity of the HE protein. Hence, if the fusion activity of the ISA virus F protein is dependent on the HE protein stalk length then this may explain what seems to be a coupling between HPR0 in the HE protein and the residue Q^{266} in the F protein. This hypothesis requires that a change in one of these characters requires a complementary change in the other. Another explanation for what seems to be a coupling between the two characters could be that the sampling size is small and not a real representation of naturally occurring combinations of these characters. Low virulent (or avirulent) ISA viruses could exist that have a short HPR and Q^{266} in the F protein or *vice versa*, however, the latter combination is probably less likely to occur compared to the former. It should be added that while Kibenge et al (2007b), Johnsen et al (2008), and Ritchie et al (2008) consider the HPR as an important virulence factor based on field data and challenge experiments, Cottet et al (2010) hypothesized that the HPR is not by itself a virulence factor, but that virulence is the consequence of multiple factors rather than a specific change in only one segment. They suggest that the HPR should be discarded as a virulence marker (Cottet et al 2010). It is not difficult to agree with the assumption that virulence is a consequence of multiple factors including the host and all eight gene segments of the ISA virus, however, it is still a fact that all HPR0 ISA viruses with a Q^{266} residue in the

F protein are of low (avirulent) virulence. Whether it is the residue in the F protein (Q^{266}) or the HPR in the HE protein, or a combination of both, that is responsible for the virulence of these ISA viruses remains to be shown.

It is also known that glycosylation sites in the surface proteins may have an influence on the virulence of influenza viruses by affecting the level of binding to the host cells receptor, i.e. influencing structure and function of the surface proteins (Lebarbenchon & Stallknecht 2011; Matrosovich et al 1999; Zaraket et al 2009). The NetNGlyc 1.0 and the NetOGlyc 3.1 servers were used to identify the N-Glycosylation sites and for predictions of type GalNAc O-glycosylation sites in the F and HE proteins from ISA viruses (http://www.cbs.dtu.dk/services/NetOGlyc/ and http://www.cbs.dtu.dk/services/NetNGlyc/) (Julenius et al 2005). The predictions of N-glycosylation sites are done only on the Asn-Xaa-Ser/Thr sequons, including the Asn-Pro-Ser/Thr. This software identified one O-glycosylation and two possible N-Glycosylation sites in the HE protein from European ISA viruses (Table 7). The position of the O-glycosylation site was at residue T^{125} and this site was present in all European ISA viruses. Two putative N-Glycosylation sites were identified in the surface part of the HE protein of the European ISA viruses, but only one ($^{333}NIT^{335}$) of these was present in all viruses with two exceptions: one virus from Norway, Vir28 (Ac. no. DQ785257), and one from Chile, VT11282007-042, (Ac.no. EU625679), which both lack this glycosylation site. The putative amino acid sequence of the HE from Vir28 lacks N-Glycosylation sites completely, while the Chilean ISA virus has

one in position ^{154}NSS^{156}. The ^{154}NSS^{156} residue is shared with the majority of the other Chilean ISA viruses, and ISA viruses belonging to the North American genotype have a putative glycosylation site in nearly the same position, ^{155}NPT^{157} (Krossøy et al 2001; Kibenge et al 2001b). Both Krossøy et al and Kibenge et al suggest that this N-Glycosylation site may not be used. The other N-Glycosylation site (^{349}NQT^{351}) of the European ISA viruses was located in the stalk region of the HE protein and, hence, not present in all viruses. However, this is considered as a weak glycosylation sequon and probably not in use.

Table 7. O-glycosylation and N-Glycosylation sites in the F and HE protein from European genotype of ISA viruses (cf. Julenius et al 2005).

Protein	**Protein**	**N-Glycosylation, aa no**			**O-glycosylation, aa no**	
F	Position	24 - 26	110 - 112	359 - 361	48	50
	Sequence	NPT++	NLT+++	NIS+	T	T
HE	Position	154 - 156	333 - 335	349 - 351	125	
	Sequence	NSS+	NIT ++	NQT-	T	

Two and three O-glycosylation and N-glycosylation sites were identified in the F proteins from European ISA viruses, respectively (Table 7). The NetOGlyc 3.1 server identified two O-glycosylation sites (T^{48} and T^{50}) in the F proteins of ISA viruses belonging to the European genotype, while the two sites given in an earlier publication are T^{52} and T^{64} (Aspehaug et al 2005). All four of these putative O-glycosylation sites are highly conserved with three exceptions, H93/04, N29/97, and N32/98. The first lacks a glycosylation site in position 48, while the latter two (N29/97, N32/98) lack a

glycosylation site in position 52. It remains to be shown which of these four putative O-glycosylation sites are functional. All three putative N-glycosylation sites detected are present in both the European and in the North American ISA virus genotypes. The conservation of ^{110}NLT^{112} and ^{359}NIS^{361} suggests, according to Cottet el at (2010), that these two sites are indispensable for the function of the fusion protein. The first putative N-glycosylation site (with a proline, P^{25}), ^{24}NPT^{26}, is also shared by all members of the European genotype, but was not considered as a functional site by Aspehaug et al (2005) and Cottet et al (2010). The glycosylation sites in the F and HE proteins from ISA viruses belonging to the European genotype are conserved among both avirulent (HPR0 ISA viruses) and virulent ISA viruses with only a few possible exceptions (see above).

The three polymerase proteins (PB1, PB2, PA) and the NP constitute the ISA virus replication machinery, the ribonucleoprotein complex (RNP), and single amino acid substitutions in these proteins may lead to an increased virulence in influenza A viruses (Forrest & Webster 2010; Cheung & Poon 2007). This may also be the case for ISA viruses, but the PA and NP from avirulent ISA viruses do not differ from any of the virulent isolates (**Paper III**), and the information about PB1 and PB2 is limited to a few isolates only, giving no solid bases for any conclusions. However, it has been suggested that mutations in the RNP of ISA viruses from Chile could be an adaptation to a new environment allowing the wide distribution observed (Cottet et al 2010). One such substitution is the I^{67} – T^{67} in the NP of the Chilean ISA viruses. However, the

change of residue 67 in the NP is also found in avirulent ISA viruses from Chile and in a virulent ISA virus (isolate FM168/10) from Norway (**Paper III**), hence, this is not a unique adaptation for spreading of virulent ISA virus along the Chilean coast as suggested by Cottet et al (2010). The two residues S^{105} and S^{558} in the NP of ISAV901 from Chile are unique substitutions found in no other ISA viruses including other viruses from Chile. The only unique amino acid substitutions (T^{204}, A^{328}) in the PA protein from Chile, mentioned by Cottet et al (2010), are found in the isolate ISAV901, but are not present in any of the other ISA viruses from Chile (cf. **Paper III**). Two of the substitutions in the PA protein (L^{292}, E^{471}) mentioned by Cottet et al are commonly occurring in European ISA viruses and the residue R^{531} is also present in avirulent ISA viruses from Chile and a virulent ISA virus isolate (FM168/10) from Norway (cf. **Paper III**). Hence, segment three (NP gene) and segment four (PA gene) of ISA viruses from Chile provide no evidence for unique substitutions in these segments adapting the virus to new environments explaining the wide distribution of these along the Chilean coast.

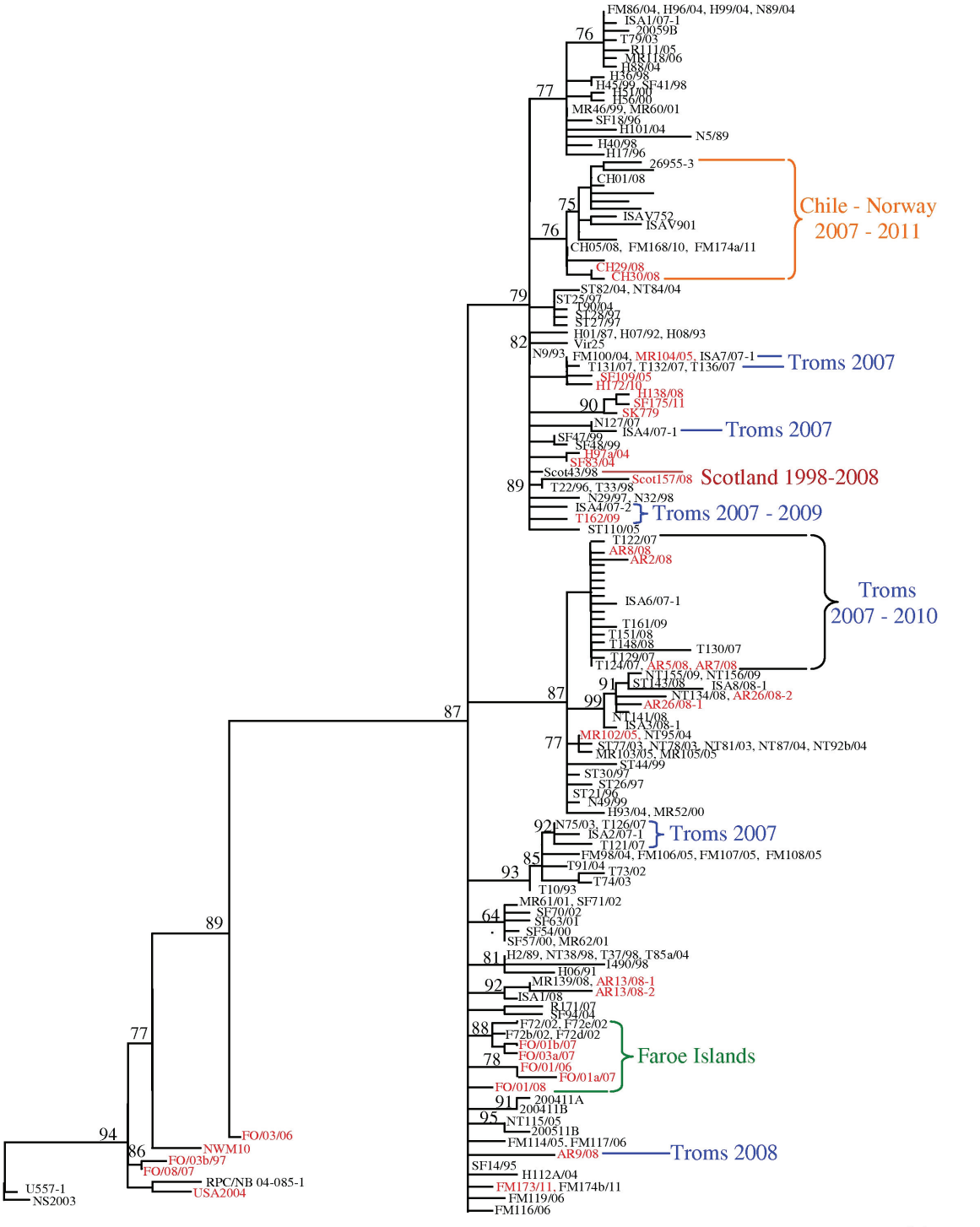
Molecular epizootiology

Viruses are best characterized and identified by sequencing of their respective genomes. Single stranded RNA viruses have a relatively high mutation rate, resulting in high substitution rates, due to lack of enzymes for proofreading, and hence, information from such genomes can be used to identify and follow the spreading of specific virus isolates (Gorman et al 1990a, 1990b, 1991; Subbarao & Shaw 2000;

Ikonen et al 2005; Bahl et al 2009; McHardy & Adams 2009; Zaraket et al 2009; Fourment et al 2010; Bataille et al 2011; Lebarbenchon & Stallknecht 2011; Xu et al 2011). For most RNA viruses the largest variation is found in genes coding for virus surface proteins, and such genes have been used in several studies of phylogeny and molecular epizootiology of fish viruses (Einer-Jensen et al 2004, 2005a,b; Kurath et al 2003; Snow et al 2004; Thiery et al 2004; Nylund et al 2008; Karlsen et al 2006; Duesund et al 2010). In phylogenetic studies of ISA viruses the main targets have been the two surface protein genes, F and HE (segments five and six) and have included ISA viruses from most salmon producing countries around the North Atlantic and from Chile (**Paper II** & **III;** Devold et al 2001, 2006; Krossøy et al 2001a; Nylund et al 2003; Vike et al 2008; Kibenge et al 2009; Cottet et al 2010; Christiansen et al 2011; Lyngstad et al 2011). These studies show that the best resolution between ISA virus isolates is obtained by using the surface protein genes (F and HE) in the phylogenetic analysis, but they also show that reassortment of the gene segments of ISA viruses are frequent events among the dominating isolates in farmed Atlantic salmon (**Paper III**). Hence, a safe identification of the isolates depends on sequencing of a large part of the genome. Another requirement is that as many ISA virus isolates as possible should be included in the phylogenetic analysis to make sure that the correct affinities of the isolates in question are identified. Figure 5 gives an example of a phylogeny based on the majority of variable HE gene sequences available in the GenBank. This figure also shows that the outbreaks of ISA in Troms in the period 2007 – 2010 were caused by several different ISA viruses, i.e. ISA viruses belonging to six different strains, which

show that the epizootic was not caused by the introduction of a single virus spreading horizontally between farms (cf. **Paper III**).

Figure 5. Phylogenetic tree showing the relationship between ISA viruses from Europe and North America based on 942 nucleotides that code for the HE protein excluding the highly polymorphic region (HPR), the transmembrane region and the cytoplasmic tail region. The best-fitting nucleotide substitution model was used during maximum likelihood analysis and the tree was bootstrapped (50 000 quartet puzzling steps) in TREE_PUZZLE. The scale bar shows the number of nucleotide substitutions as a proportion of branch lengths. Information about the sequences can be found in Appendix A.


FM86/04, H96/04, H99/04, N89/04
ISA1/07-1
20059B
T79/03
R111/05
MR118/06
H88/04
H36/98
H45/99, SF41/98
H57/00
H56/00
MR46/99, MR60/01
SF18/96
H101/04
N5/89
H40/98
H17/96
26955-3
CH01/08
ISAV752
ISAV901
CH05/08, FM168/10, FM174a/11
CH29/08
CH30/08
Chile - Norway
2007 - 2011
ST82/04, NT84/04
ST25/97
T90/04
ST28/97
ST27/97
H01/87, H07/92, H08/93
Vir25
N9/93
FM100/04, MR104/05, ISA7/07-1
T131/07, T132/07, T136/07
Troms 2007
SE109/05
HI72/10
HI138/08
SF175/11
SK779
N127/07
ISA4/07-1
Troms 2007
SF47/99
SF48/99
H97a/04
SF83/04
Scot43/98
Scot157/08
Scotland 1998-2008
T22/96, T33/98
N29/97, N32/98
ISA4/07-2
T162/09
Troms 2007 - 2009
ST110/05
T122/07
AR8/08
AR2/08
ISA6/07-1
T161/09
T151/08
T148/08
T130/07
T129/07
T124/07, AR5/08, AR7/08
Troms
2007 - 2010
NT155/09, NT156/09
ST143/08
ISA8/08-1
NT134/08, AR26/08-2
AR26/08-1
NT141/08
ISA3/08-1
MR102/05, NT95/04
ST77/03, NT78/03, NT81/03, NT87/04, NT92b/04
MR103/05, MR105/05
ST44/99
ST30/97
ST26/97
ST21/96
N49/99
H93/04, MR52/00
N75/03, T126/07
ISA2/07-1
T121/07
Troms 2007
FM98/04, FM106/05, FM107/05, FM108/05
T91/04
T73/02
T74/03
T10/93
MR61/01, SF71/02
SF70/02
SF63/01
SF54/00
SF57/00, MR62/01
H2/89, NT38/98, T37/98, T85a/04
I490/98
H06/91
MR139/08, AR13/08-1
AR13/08-2
ISA1/08
R171/07
SF94/04
F72/02, F72e/02
F72b/02, F72d/02
FO/01b/07
FO/03a/07
FO/01/06
FO/01a/07
Faroe Islands
FO/01/08
200411A
200411B
NT115/05
200511B
FM114/05, FM117/06
AR9/08
Troms 2008
SF14/95
H112A/04
FM173/11, FM174b/11
FM119/06
FM116/06
FO/03/06
NWM10
FO/03b/97
FO/08/07
RPC/NB 04-085-1
USA2004
U557-1
NS2003
0.01

Reservoirs and transmission of ISA virus

The only wild species that seem to be able to sustain replication of ISA viruses in Norway are the salmonids, *Salmo salar* (Atlantic salmon) and *Salmo trutta* (Trout). The virus has been found in these species in Scotland and Norway and positives have also been found in both fresh and sea water (Raynard et al 2001, **Paper I**). The prevalence of ISA virus in trout in a few selected rivers in western Norway (Sogn og Fjordane) was relatively high in 2001 – 2002 (**Paper I**). In the years since 2003, however, it has only been possible to find a few ISA virus positive salmonids in rivers and fjords on the west coast of Norway (Table 4). Only a few outbreaks of ISA in farmed Atlantic salmon have occurred in this area in the same period. This raises the question about which reservoir for ISA viruses is most important in the present situation in Norway. Is it wild salmonids or farmed Atlantic salmon? Based on the population size of wild salmonids compared to the number of farmed Atlantic salmon it is obvious that farmed salmon has the largest potential as reservoir for this virus. The relatively low prevalence of ISA virus in wild salmonids, the low population sizes, the behaviour of these species in sea water, and the fact that all those found positive so far are carrying avirulent ISA viruses, suggest that wild salmonids are not important as reservoir for ISA virus and for transmitting this virus to farmed Atlantic salmon.

The major reservoirs for ISA viruses seem to be farmed Atlantic salmon in fresh and sea water (**Paper II** & **III**). The majority of smolt production sites tested contain smolt positive for ISA viruses (**Paper II**) and the virus is also very often present in Atlantic

salmon in the marine production phase without causing any outbreaks of ISA (**Paper II** and **III,** Nylund et al 2011, A. Nylund pers. com). Sequencing of ISA virus from Atlantic salmon kept in farms with no outbreaks of ISA will in most cases result in identification of HPR0 ISA viruses. These avirulent ISA viruses seem to be dominating in farmed Atlantic salmon and can be found in farms in all parts of Norway. Based on the population size and distribution of farmed Atlantic salmon along the Norwegian coast it is relatively safe to assume that they constitute the major reservoir for this virus in Norway. Even if herring may sustain replication of ISA virus after experimental challenge it has not been possible to detect the virus in wild herring (see Table 3), and no evidence exists to suggest that this species may play a role as reservoir for ISA viruses.

It is obvious from challenge experiments and empirical data from the field that ISA viruses can be horizontally transmitted between individuals within a farm site during an outbreak of ISA. However, the distance of transmission is more difficult to document, and in several farms it has been observed that if salmon in a cage suffering outbreak of ISA are slaughtered, transmission to neighbouring cages can be prevented. Still, when several outbreaks of ISA occur in the same area it is not uncommon to find nearly identical ISA viruses in Atlantic salmon in neighbouring farms (Lyngstad et al 2011, **Paper III**). The hypothesis promoted by the Norwegian Food Authorities, to explain several local outbreaks of ISA, is that all spreading of ISA virus is due to horizontal transmission in the marine production phase of Atlantic salmon (cf.

Lyngstad et al 2011), and that the origin of "new" ISA viruses comes from unidentified marine reservoirs. That ISA viruses can be transmitted horizontally between farms in a small area when escaped infected salmon are present is not very controversial (cf. Lyngstad et al 2011, **Paper III**). However, the studies of horizontal transmission based on genotyping of the ISA virus are few and,so far, it has not been possible to detect any natural marine reservoirs for ISA viruses in such areas. One area where possible horizontal transmission has been studied is in northern Norway, southern Troms, in the time period from 2007 - 2010 (Lyngstad et al 2011, **Paper III**). It is well documented that in the majority of salmon farms suffering outbreaks of ISA the salmon were infected with nearly identical ISA viruses. However, all these farms had also received salmon that originated from the same brood fish company (**Paper III**). In addition, to get the complete picture of the situation in this area, one will also have to include all ISA virus isolates detected in all farms during the mentioned period. A complete overview of the ISA outbreaks in this area shows that a minimum of six very different ISA viruses were the causes for the outbreaks, and hence, horizontal transmission of ISA virus from a primary outbreak can not explain the observations (Figure 5, **Paper III**). Admittedly, based on the fact that the majority of the ISA viruses identified had nearly identical genomes (four segments sequenced), the same HPR, and the presence of escaped salmon infected with ISA virus, the hypothesis of horizontal transmission can be supported (**Paper III**). However, it should be added that the dominating ISA virus in southern Troms (2007 – 2010) was closely related to ISA viruses from earlier outbreaks in western and mid Norway, and not a new emerging ISA virus with no previous history in Norway (cf. figure 4, **Paper III**). Phylogenetic analysis of segment

6 (the HE protein gene) from ISA viruses shows that the clade including the dominating ISA viruses from Southern Troms (2007 – 2010) also contains several HPR0 ISA viruses, including one from a fresh water site (western Norway) belonging to the brood fish company from where salmon in the infected farms originated (**Paper III**).

A large amount of evidence, supporting the hypothesis of vertical/transgenerational transmission of ISA viruses, has accumulated during the last six years (Multiple authors 2005, Vike et al 2008, **Paper II** & **III,** A. Nylund pers. com.). Brood fish companies in Norway have experienced several outbreaks of ISA in their brood fish stock during the last decades, and screening of brood fish in normal production has on several occasions shown that close to 100 % of the fish were infected with HPR0 ISA viruses (A. Nylund pers.com., Multiple authors 2005, **Paper II**). HPR0 ISA viruses belonging to the same clades as the brood fish ISA viruses can also be found in salmon smolt in fresh water and in salmon in marine farms (**Paper II** & **III**), and the evidence from existing literature clearly suggests that the pathogenic ISA viruses originate from these HPR0 ISA viruses (**Paper II** & **III**, Christiansen et al 2011). The emergence of ISA in Chile, caused by a Norwegian ISA virus, i.e. ISA virus in a country on the southern hemisphere with no natural populations of salmonids importing large amounts of salmon embryos every year for industrial production, strongly supports the hypothesis of vertical/transgenerational transmission of ISA viruses (Vike et al 2008).

Based on the existing evidence of vertical/transgenerational transmission of ISA viruses, presence of ISA viruses in smolt in fresh water, and outbreaks of ISA in geographically very distant areas caused by nearly identical ISA viruses, strongly suggests that vertical transmission plays an important role in the maintenance and spreading of ISA virus in Norwegian salmon production (**Paper II** & **III**). This does not, of course, exclude the possibility that horizontal transmission may play a role during outbreaks of ISA in local areas, but based on the wide distribution of the few ISA virus clades present in farming of Atlantic salmon in Norway, the most important transmission route seems to be movement of HPR0 ISA viruses in connection with smolt transport (cf. **Paper II**). Since HPR0 ISA viruses are present in both brood fish and smolt of Atlantic salmon in pure fresh water, and since offspring (embryos and fry) from ISA virus positive brood fish stay positive throughout production in fresh water, it seems highly likely that the salmon industry is circulating a small number of ISA viruses in the production system (**Paper II** & **III**).

The existing knowledge about hosts susceptible to ISA viruses in the North Atlantic, results from studies of genotyping of ISA viruses from healthy and ISA suffering salmon, and presence of vertical transmission of ISA viruses strongly suggests that the main reservoir for ISA viruses in Norway is farmed Atlantic salmon. In this system the brood fish seem to play a central role in the dissemination of these viruses.

FUTURE WORK

A future aim of ISA virus research is to fully understand the molecular basis for interactions of the virus and the host. The sequencing of the complete genome of Atlantic salmon will soon be finished and the sequences of some ISA viruses have already been established. Hence it should be possible in the coming years to resolve the genetic control of the interactions of ISA viruses and the host (Atlantic salmon), which should give a new and better understanding of pathogenesis and possibly new insight into how to control the spreading of ISA viruses and the mechanisms behind outbreaks of ISA. It is difficult to imagine that the ISA virus can be eradicated from the natural populations of salmonids in the North Atlantic, but strategies to eradicate the virus from farmed populations can already be envisioned. The remarkable advances in molecular biology, including reverse genetics, during the last decade also point towards new strategies for vaccine development as another option in the control of ISA viruses and development of ISA in farmed populations of Atlantic salmon.

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APPENDIX A.

Overview of ISA virus sequences used in figure 5.

ISA virus	Year	HPR	Segment 6
Rogaland			DQ108609
R111/05	2005	18	-
R171/07	2007	7i	-
R170/09	2009	32	-
Hordaland			
H1/87	1987	1	AF364893
H2/89	1989	2	AF220607
H6/91	1991	2	AF364894
H7/92	1992	1	AF364898
H8/93	1993	1	AF309075
H17/96	1996	7a	AF364891
H36/98	1998	15	AF302799
H40/98	1998	7b	AF364877
H45/99	1998	15	AF364870
H51/00	2000	7b	AF364882
H56/00	2000	15	AF364880
H88/04	2004	1	AY973178
H93/04	2004	10	AY97379
H96/04	2004	7	AY973180
H97/04	**2004**	**0-7b**	**DQ108604**
H99/04	2004	7b	AY973181
H101/04	2004	4b	DQ108602
H112A/04	2004	8	DQ108603
H138/08	**2008**	**0**	**JN711063**
H172/10	**2010**	**0**	**JN711064**
Sogn og Fjordane			
SF14/95	1995	5	AF364873
SF18/96	1996	2	AF364869
SF41/98	1998	15	AF364871
SF48/99	1999	8	AF364878
SF47/99	1999	9	AF364888
SF54/00	2000	11a	AF364884
SF57/00	2000	11a	AF364890
SF63/01	2001	11b	AY127879
SF70/02	2002	11a	AY127880
SF71/02	2002	11a	AY127881
SF83/04	**2004**	**0**	**AY973190**
SF94/04	2004	14	DQ108611
SF109/05	**2005**	**0**	**DQ108610**
SF175/11	**2011**	**0**	**-**

Møre og Romsdal			
MR46/99	1999	8	AF364896
MR52/00	2000	10	AF364892
MR60/01	2001	6	AY127876
MR61/01	2001	11a	AY127877
MR62/01	2001	11a	AY127878
MR102/05	**2005**	**0**	**DQ108605**
MR103/05	2005	17	DQ108606
MR104/05	**2005**	**0**	**DQ108607**
MR105/05	2005	17	DQ108608
MR118/06	2006	30	-
MR139/08	2008	35	JN711065
AR13/08-1	**2008**	**0**	**FN687354**
AR13/08-2	**2008**	**0**	**FN687355**
ISA3/08-1	2008		FN687308
Sør Trøndelag			
ST21/96	1996	14	AF364886
ST26/97	1997	14	AF364879
ST25/97	1997	6	AF364885
ST27/97	1997	6	AF364897
ST28/97	1997	6	AF364875
ST30/97	1997	14	AY127875
ST44/99	1999	14	AF302803
ST77/03	2003	7b	AY973191
ST82/04	2004	8	AY973192
ST110/05	2005	3	DQ108598
ST143/08	2008	35	-
ISA1/08	2008		FN687305
Nord Trøndelag			
NT38/99	1998	2	AF364874
NT78/03	2003	7b	AY973183
NT8103	2003	7b	AY973184
NT84/04	2004	8	AY973185
NT87/04	2004	7b	AY973186
NT92A/04	2004	7b	AY973187
NT92B/04	2004	7b	AY973188
NT95/04	2004	5	AY973189
NT115/05	2005	3	-
NT134/08	2008	34	JN711069
NT141/08	2008	2	JN711070
AR26/08-1	**2008**	**0**	**FN687356**
AR26/08-2	**2008**	**0**	**FN687357**
ISA8/08-1	2008		FN687318
NT155/09	2009	35	JN711071
NT156/09	2009	35	JN711072

Nordland			
N5/89	1989	12	AY127882
N9/93	1993	3	AF364895
N29/97	1997	7b	AF364872
Vir25	1997	35	DQ785256
N32/98	1998	7b	AF364883
N49/99	1999	7b	AF364876
N75/03	2003	4	AY971661
N89/04	2004	14	AY971662
N127/07	2007	33	JN711066/67
ISA1/07-1	2007		FN687284
Troms			
T10/93	1993	4a	AF302801
T22/96	1996	2	AF364889
T33/98	1998	2	AF364887
T37/98	1998	2	AF364881
T73/02	2002	14	AY971663
T74/03	2003	14	AY971664
T79/03	2003	14	AY971665
T85/04	2004	?	AY971693
T90/04	2004	16	AY971666
T91/04	2004	3	AY971667
T121/07	2007	31	JN711074
T122/07	2007	5	JN711075
T124/07	2007	5	-
T126/07	2007	31	JN711076
T129/07	2007	5	JN711077
T130/07	2007	5	-
T131/07	2007	2	JN711078
T132/07	2007	2	-
ISA4/07-1	2007		FN687290
ISA4/07-2	2007		FN687291
ISA2/07-1	2007		FN687286
ISA6/07-1	2007		FN687294
ISA7/07-1	2007		FN687303
T136/07	2007	2	-
T140/08	2008	5	JN711079
T144/08	2008	5	JN711080
T145/08	2008	5	JN711081
T147/08	2008	5	JN711082
T148/08	2008	5	JN711083
T149/08	2008	5	JN711084
T151/08	2008	5	JN711085
AR2/08	**2008**	**0**	**FN687348**
AR5/08	**2008**	**0**	**FN687350**
AR7/08	**2008**	**0**	**FN687351**
AR8/08	**2008**	**0**	**FN687352**
AR9/08	**2008**	**0**	**FN687353**

T152/09	2009	5	JN711086
T154/09	2009	5	JN711087
T158/09	2009	5	JN711088
T160/09	2009	5	JN711089
T161/09	2009	5	JN711090
T162/09	**2009**	**0**	**JN711091**
T167/10	2010	5	JN711092
Finnmark			
FM86/04	2004	1	AY971659
FM98/04	2004	14	AY971660
FM100/04	2004	7b	AY971658
FM106/05	2005	14	DQ108599
FM107/05	20055	14	DQ108600
FM108/05	2005	14	DQ108601
FM114/05	2005	3	JN711057
FM116/06	2006	9	JN711058
FM117/06	2006	3	-
FM119/06	2006	3	-
FM168/10	2010	14	JN711059
FM173/11	**2011**	**0**	**JN711060**
FM174a/11	2011	14b	JN711061
FM174b/11	2011	14b	JN711062
Unknown			
SK779	**2006**	**0**	**EU118820**
20059B	2005		FM203273
200411A	2004		FM203262
200411B	2004		FM203263
200511B	2005		FM203297
Chile			
CH01/08	2008	7b	EU851043
CH05/08	2008	35	JN711093
CH29/08	**2008**	**0**	**JN711094**
CH30/08	**2008**	**0**	**JN711095**
ISAV752	2009	7b	GU830900
ISAV901	2009	15b	GU830908
26955-3	2007		EU625679
Faeroe Islands			
F72/02	2002	3	AF526263
F72b/02	2002		AY971656
F72d/02	2002		AY971657
F72e/02	2002		DQ108612
FO/01/06	**2006**	**0**	**HQ664992**
FO/03/06	**2006**	**0**	**HQ664993**
FO/01b/07	**2007**	**0**	**HQ664995**
FO/03b/07	**2007**	**0**	**HQ664997**

FO/03a/07	**2007**	**0**	**HQ664996**
FO/01a/07	**2007**	**0**	**HQ664994**
FO/08/07	**2007**	**0**	**HQ664998**
FO/01/08	**2008**	**0**	**HQ664999**
Scotland			
Scot43/98	1998	7b	AF302802
1490/98	1998		AF391126
NWM10	**2007**	**0**	**FJ178189**
Scot157/08	**2008**	**0**	**JN711096**
Canada			
NS2003	2003	3	AY601904
RPC/NB 04-085-1	2004		AY963263
U5571-1			AF294881
USA			
USA2004	**2004**	**0**	**AY973194**

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NOTE

Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in western Norway

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ABSTRACT: Studies of infectious salmon anaemia virus (ISAV), an important pathogen of farmed salmon in Norway, Scotland, the Faeroe Islands, Ireland, Canada, the USA and Chile, suggest that natural reservoirs for this virus can be found on both sides of the North Atlantic. Based on existing information about ISAV it is believed to be maintained in wild populations of trout and salmon in Europe. It has further been suggested that ISAV is transmitted between wild hosts, mainly during their freshwater spawning phase in rivers, and that wild salmonids, mainly trout, are possible carriers of benign wild-type variants of ISAV. Change in virulence is probably a result of deletions of amino acid segments from the highly polymorphic region (HPR) of benign wild-type isolates after transmission to farmed salmon. Hence, it has been suggested that the frequency of new outbreaks of ISA in farmed salmon could partly reflect natural variation in the prevalence of ISAV in wild populations of salmonids. The aims of the present study were to screen for ISAV in wild salmonids during spawning in rivers and to determine the pathogenicity of resultant isolates from wild fish. Tissues from wild salmonids were screened by RT-PCR and real-time PCR. The prevalence of ISAV in wild trout *Salmo trutta* varied from 62 to 100% between tested rivers in 2001. The prevalence dropped in 2002, ranging from 13 to 36% in the same rivers and to only 6% in 2003. All ISAV were non-pathogenic when injected into disease-free Atlantic salmon, but were capable of propagation, as indicated by subsequent viral recovery. However, non-pathogenic ISAV has also been found in farmed salmon, where a prevalence as high as 60% has been registered, but with no mortalities occurring. Based on the results of the present and other studies, it must be concluded that vital information about the importance of wild and man-made reservoirs for the emergence of ISA in salmon farming is still lacking. This information can only be gained by further screening of possible reservoirs, combined with the development of a molecular tool for typing virulence and the geographical origin of the virus isolates.

KEY WORDS: ISA virus · Wild salmonids · Natural reservoir

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INTRODUCTION

Infectious salmon anaemia virus (ISAV) is the etiological agent of a disease that has occurred in most salmon *Salmo salar*-producing countries worldwide (Thorud & Djupvik 1988, Mullins et al. 1998, Rodger et al. 1998, Rowley et al. 1999, Bouchard et al. 2001, Kibenge et al. 2001, Ritchie et al. 2001). The official history of ISA dates back to 1984 in Norway, but the 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) and hemagglutinin genes (Segment 6), North American and European isolates are thought to have separated >100 yr ago (Krossøy et al. 2001, Krossøy 2002, Nylund et al. 2003). The demonstrated existence of ISAV on both the European and North American continents

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prior to the advent of intensive salmon aquaculture, suggests the long-term maintenance of ISAV in a wild reservoir. ISAV and raised levels of ISAV-specific antibodies have been found in wild salmonids with no clear relationship to aquaculture activity (Raynard et al. 2001a, K. Falk pers. comm.). Furthermore, studies based on sequencing of a so-called highly polymorphic region (HPR) of the haemagglutinin gene have suggested that all pathogenic ISAV isolates may be derived by differential deletions or by recombination of a potential ancestral sequence obtained from wild fish (Devold et al. 2001, Cunningham et al. 2002, Mjaaland et al. 2002, Nylund et al. 2003). Coupled to the fact that a range of salmonids have been experimentally shown to propagate ISA (Nylund et al. 1994a, 1995a,c, 1997, Nylund & Jakobsen 1995, Rolland & Nylund 1998a, Devold et al. 2000, Snow et al. 2001a,b, Rolland & Winton 2003), this evidence suggests that wild salmonids may constitute natural host species within which an avirulent ISAV subtype may have co-evolved.

The most frequent interactions between individual wild salmonids and, hence, opportunities for ISAV transmission between hosts 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 the acquired immunity towards viral diseases. 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. 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). It has also 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 the mucus, urine, faeces and ovarian fluids of infected individuals (Thorud & Djupvik 1988, Thorud 1991, Nylund et al. 1993, 1994a,b, 1995a,b, 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. 2001a,b).

Wild trout and salmon from 5 different rivers were collected and screened for the presence of ISAV. Two different areas in western Norway with frequent outbreaks of ISA in farmed salmon were selected, and fish were collected during spawning in the rivers in late October of 2001, 2002 and 2003. Pathogenicity characteristics of resultant ISAV were tested by intraperitoneal injection into disease-free salmon.

MATERIALS AND METHODS

Sample collection. Wild salmonids, trout *Salmo trutta* and salmon *Salmo salar*, from 5 selected rivers (Austgulen, Nordgulen, Takle, Brekke and Bortne) in western Norway were collected on 23 October 2001, on 29/30 October 2002 and on 28/29 October 2003 (Fig. 1). All 5 rivers are located in Sogn- og Fjordane county, in areas that traditionally have had a high density of salmon farms. Bortne River is located in the outer part of Nordfjord, while the other 4 rivers are located in the outer part of Sognefjord. Both areas have experienced frequent outbreaks of ISA since the early 1990s. Fish from these rivers were obtained by electrofishing from the river mouth and a maximum of 1 km up the rivers (Table 1). In the spring of 1998, 45 sea trout *S. trutta* were collected by trawl from the outer part of Nordfjord, i.e. the same fjord where the Bortne River is located.

Tissue samples from gills, heart, liver, kidney, pseudobranch, gut, spleen and gonads were taken from the newly killed fish and immediately frozen in

Fig. 1. Location of the rivers in western Norway where the trout and salmon were collected in 2001, 2002 and 2003

Table 1. *Salmo trutta, S. salar, Oncorhynchus mykiss*. Material collected from 5 rivers in 2001, 2002 and 2003 and 1 fjord in 1998 (L-range: size range [cm] of the collected fish)

	Species	N	Male	Female	Not mature	L-range	Year
Rivers							
Nordgulen	*S. trutta*	21	10	10	1	18.5–47.0	2001
	S. trutta	24	14	6	4	18.5–50.0	2002
	S. trutta	25	15	10	11	10.0–41.5	2003
Brekke	*S. trutta*	12	5	5	2	17.5–47.5	2001
	S. salar	4	–	4	–	50.0–60.0	2002
	S. trutta	26	8	8	10	19.0–50.0	2002
	S. trutta	27	20	7	7	13.5–65.0	2003
Austgulen	*S. trutta*	13	3	4	6	17.5–35.0	2001
	O. mykiss	1	1			22.5	2001
Takle	*S. trutta*	4	2	2	–	16.5–21.0	2001
Bortne	*S. salar*	3	3	–	–	60.0–80.0	2002
	S. trutta	24	10	4	10	20.0–60.0	2002
	S. salar	1	–	1	1	10.5	2003
	S. trutta	16	10	6	7	10.5–31.0	2003
Fjord							
Nordfjord	*S. trutta*	45	–	–	45	–	1998

liquid nitrogen. On arrival at the laboratory, the tissues were transferred to –80°C.

Virus isolation and detection. Culture of ISAV was attempted by inoculation of positive tissues on Atlantic salmon kidney (ASK) cells (Devold et al. 2000) using 3 passages. Positive cell cultures, i.e. cell cultures showing a cytopathic effect (CPE), and negative cell cultures were all tested for possible presence of ISAV by RT-PCR (primers: FA-3/RA-3) (Devold et al. 2000).

ISAV-positive homogenates of tissues from salmon in each river (with the exception of the Takle River) were injected intraperitoneally into 30 disease-free salmon, to see if any mortality would occur and if the virus could be cultured in salmon. The tissue samples from wild and experimentally challenged fish were screened by RT-PCR using the diagnostic primers FA-3/RA-3 (Devold et al. 2000) and by real-time PCR. Extraction of RNA from the tissues, reverse transcription polymerase chain reaction (RT-PCR) and sequencing of PCR products were performed as described by Devold et al. (2000). The results of the RT-PCR and real-time PCR assays on the wild fish were tested by contingency table analysis to see if prevalence was significantly different in the 3 following years.

The reaction mix for the real-time PCR was as follows: 1 µl of single-stranded cDNA from the reverse transcription, 12.5 µl TaqMan Universal Master Mix (2×), 900 nM of each primer and 200 nM of the MGB probe. The total volume was adjusted to 25 µl using RNAse-free water. The reaction cycle was 2 min at 50°C (UNG activation), 10 min at 95°C and 45 cycles of 95°C for 15 s, and 1 min at 60°C. Two different real-time assays were designed for detecting the ISAV genome: 1 assay for Segment 7 (the tentative nonstructural protein genes) and 1 assay for Segment 8 (the tentative matrix protein genes) (Table 2). An 84 bp and a 63 bp region were amplified from Segments 7 and 8, respectively. The labelled fluorogenic probes are specific for the cDNA region between the forward and reverse primer sets. The probes contain a fluorescent reporter dye and a quencher dye; their physical proximity to each other suppresses any light emitted by the reporter. During PCR, the 5' → 3' nuclease activity of the DNA polymerase releases the reporter dye from the probe, which results in an increase in fluorescence. The reporter dye, 6-FAM, has an emission wavelength maximum of λ_{max} = 518 nm and is measured by the ABI 7700 sequence detection system (Applied Biosystems).

The sensitivity of the real-time PCR assays was compared with the sensitivity of the diagnostic PCR using the primer set FA-3/RA-3 (Devold et al. 2000). A dilution series of cDNA from ISAV was used as templates in the comparison of the different assays.

In 2003 the tissue distribution of virus in ISAV-positive fish was tested by real-time PCR. Originally, only kidney tissue was tested for the presence of virus, as was done during 2001 and 2002 as well.

Table 2. Primer and probes in the assay mix were produced by Applied Biosystems (ABI). The probes are specific for the region between the primer sets and are targeted against Segments 7 and 8 from the ISAV (infectious salmon anaemia virus) genome

Primers/probes	Name	Sequence
Segment 7		
Probe	S7-P1	6FAM-cac atg acc cct cgt c-MGBNFQ
Forward primer	S7-F1	5'-tgg gat cat gtg ttt cct gct a
Reverse primer	S7-R1	5'-gaa aat cca tgt tct cag atg caa
Segment 8		
Probe	S8-P1	6FAM-acg gtg gat ctt tc-MGBNFQ
Forward primer	S8-F1	5'-cga cga tga ctc tct act gtg tga t
Reverse primer	S8-R1	5'-tca tca gtg tcg cca tgc tt

Sequencing and sequence analysis. PCR products were purified using Qia-quick PCR purification columns (Qiagen) and then sequenced using the Big Dye terminator Sequencing kit (Applied Biosystems). The templates were sequenced in both directions. Sequence data were assembled with the help of Vector NTI software (InforMax), and GenBank searches were performed using BLAST (2.0).

The Vector NTI Suite software package (InforMax) was used for the multiple alignments of partial nucleotides. To perform pairwise comparisons between the different sequences from the ISAV isolates, the multiple sequence alignment editor GeneDoc was used. Phylogenetic analyses of the data sets were performed using PAUP* Version 4.0 (Swofford 1998) and TREE-PUZZLE 5.0 (available at: www.tree-puzzle.de). TREE-PUZZLE reconstructs phylogenetic trees from molecular data by maximum likelihood, and computes maximum-likelihood distances and branch lengths. Phylogenetic trees were drawn using TreeView (Page 1996).

RESULTS

Trout *Salmo trutta* were the dominating salmonid species in the rivers and fjords where the fish were collected and screened for the presence of ISAV. The sample material also included a few Atlantic salmon *Salmo salar* from 2 rivers (Bortne & Brekke) and 1 individual of rainbow trout *Oncorhynchus mykiss* collected in the Austgulen River. The majority of fish from the rivers were spawning or had just spawned, but a few parr and smolt were also included in the collected material. Except for the rainbow trout, none of the fish seemed to be escapees from fish farms. The weight of the fish was not measured, but their length, the number of fish caught, their sex and the number of immature fish are given in Table 1. The mean length of the fish caught in 2003 was much smaller and more fish were immature compared to those caught during the previous years.

None of the fish showed any clinical signs of ISA. However, 1 spawning salmon from the Bortne River (2002) had skin ulcers, and 1 immature trout from the Brekke River (2003) had white granulomas and an enlarged kidney. In 2003 5 trout (4 immature, 1 spawning) had nodules/vesicles on their spleens, while 1 spawning trout from the Brekke River was infected with *Anisakis simplex*, and 1 spawning trout from the Bortne River was heavily infected with *Eubothrium* sp. All the fish were screened for the presence of ectoparasites. A few of the fish had salmon lice, *Lepeophtheirus salmonis*, *Trichodina* spp. and *Riboscyphidia* sp., but the intensity was low, and no lesions due to parasites were observed.

Virus detection

It was not possible to culture any ISAV in ASK cells from any of the fish included in this study. However, it was possible to detect ISAV in salmon, challenged with tissue homogenates from ISAV-positive fish, 4 wk after challenge, but no mortality was observed. Sequencing of the PCR products and phylogenetic analysis of the sequences, confirmed them to be ISAV genomes of European origin.

The kidneys from all the collected fish were screened for the presence of ISAV with the help of RT-PCR (primers: FA-3/RA-3), and fish collected in 2002 and 2003 were also screened by real-time PCR using primers and probes targeting Segment 8. Both the Segment-7 and -8 real-time PCR assays were used in the screening of the material from 2003. The real-time PCR assays were more analytically sensitive than the RT-PCR (FA3/RA3), and gave positive reactions with 1000 times less template than the RT-PCR (Fig. 2). The results of the RT-PCR and real-time tests are presented in Table 3, and this table shows a clear trend: the highest prevalence of ISAV was detected in 2001 (up to 100%), it decreased during 2002 to a maximum of 81% and reached a low point in 2003, with no more than 1 fish testing positive for ISAV in each river. This trend can be observed in all rivers and is confirmed by both RT-PCR and real-time PCR. The results of the RT-PCR and real-time PCR assays on the wild fish were tested by contingency table analysis (Table 3). Only 3 of the 45 sea trout collected in 1998 were positive for ISAV.

Unfortunately, only 3 fish were available for testing the tissue distribution of virus in ISAV-positive fish in 2003, 2 that were immature and 1 that was spawning. The results are presented in Table 4 and show that ISAV was only detected in 4 of the 8 tissues tested.

DISCUSSION

Molecular methods have become important tools in viral diagnostics and in the study of the diversity, maintenance and dissemination of viral diseases (Hungnes et al. 2000). The latest revolution in viral diagnostics came with the development of real-time PCR due to its rapidity, sensitivity (i.e. low detection limit), reproducibility and the reduced risk of carry-over contamination (Mackay et al. 2002). In addition, it provides the possibility for relative and absolute quantification. The high sensitivity of real-time PCR makes it a good method for detection of carriers of viral agents like the ISAV, since it must be expected that orthomyxoviruses have to produce a low number of infective particles in order to persist in the host (Knipe & Howley 2001). PCR has established itself during recent years as the gold

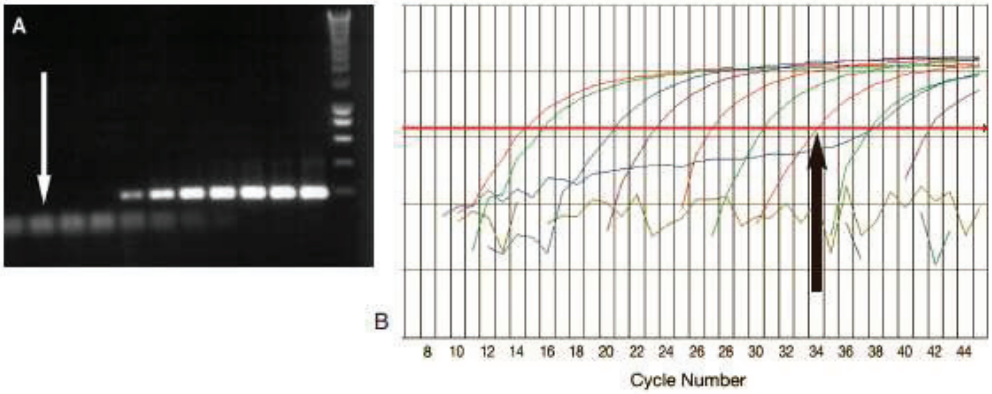

Fig. 2. (A) Demonstrating the difference in threshold of detection of infectious salmon anaemia virus by RT-PCR (FA3/RA3) and the 2 real-time PCR assays targeting Segments 7 and 8. Agarose gel showing RT-PCR amplification products (10 µl) stained by ethidium bromide. The lanes represent 10-fold dilutions. The white arrow marks the dilution that could be detected by the real-time PCR assays. (B) Results of the real-time PCR assay targeting Segment 7 using the same dilution series as in the RT-PCR. The black arrow shows the detection limit for the RT-PCR

standard for detecting nucleic acids from a number of origins, and RT-PCR has become an important tool in the diagnostics of ISA in both Europe and North America (Mjaaland et al. 1997, Devold et al. 2000, Griffiths & Melville 2000, Mikalsen et al. 2001, Raynard et al. 2001a,b, Snow et al. 2001a, 2003, Løvdal & Enger 2002, McClure et al. 2004). The dominating assays for RT-PCR detection of ISAV have been an assay published by Mjaaland et al. (1997) and another assay developed by Devold et al. (2000). Both assays target Segment 8, presumably the matrix protein genes from the ISAV (Biering et al. 2002). The real-time PCR used in the present paper is able to detect template 1000 times more dilute than the RT-PCR assay (FA3/RA3) developed by Devold et al. (2000). This difference in sensitivity is also reflected by the results presented in this study, in which the real-time PCR in each case gives a higher number of ISAV-positive individuals.

Table 3. *Salmo trutta, S. salar, Oncorhynchus mykiss*. Results of the RT-PCR and real-time PCR screening of kidney tissues from wild trout *S. trutta* and salmon *S. salar* caught in rivers located in western Norway (nt: not tested; n: number of positive fish; %: percentage of positive fish). Results of the RT-PCR and real-time PCR assays on the samples of *S. trutta* collected in 2001, 2002 and 2003 from the Nordgulen, Brekke and Bortne Rivers were tested by contingency table analysis (χ^2/p<). There was a significant decline of ISAV-positive trout in all 3 rivers using both assays for detection

	Species	N	RT-PCR n/%	χ^2/p<	Real-time PCR n/%	χ^2/p<	Year
Rivers							
Nordgulen	*S. trutta*	21	14/67		nt		2001
	S. trutta	24	3/13	30.3/0.001	9/38		2002
	S. trutta	25	0/0		1/(4)	8.5/0.01	2003
Brekke	*S. trutta*	12	8/67		nt		2001
	S. salar	4	3/75		4/(100)		2002
	S. trutta	26	7/27	21.1/0.001	21/81	32.4/0.001	2002
	S. trutta	27	0/0		1/(4)		2003
Austgulen	*S. trutta*	13	13/100		nt		2001
	O. mykiss	1	1/100		nt		2001
Takle	*S. trutta*	4	3/75		nt		2001
Bortne	*S. salar*	3	0/0		2/(67)		2002
	S. trutta	24	8/34		15/63		2002
	S. trutta	16	0/0	6.6/0.01	1/(6)	12.7/0.001	2003
	S. salar	1	0/0		0/(0)		2003
Fjord							
Nordfjord	*S. trutta*	45	3/6.7		nt		1998

The official history of the ISAV in salmon farming goes back to 1984 in western Norway (Thorud & Djupvik 1988). However, based on the mutation rate of the RNA-dependent RNA polymerase gene, Krossøy et al. (2001) estimated that the European and North American isolates of ISAV must have separated at least 100 yr ago. More than 100 yr of separation between European and North American ISAV isolates was also supported by a study of the mutation rate of the haemagglutinin gene (Segment 6) of the ISAV (Nylund et al. 2003). A dis-

Table 4. *Salmo trutta, S. salar, Oncorhynchus mykiss*. Tissue distribution of ISAV in positive carrier fish from 3 rivers (Bortne, Brekke, Nordgulen) using real-time PCR. The first PCR was done on kidney tissue only to check for absence/presence of ISAV; the second PCR was done on various tissues from ISAV-positive fish in order to determine tissue distribution of virus. Superscript 7 and 8; PCR for Segment 7 and 8, respectively. nt: not tested; Ct: cycle thresholds of the growth curves

	Tissue	Ct		
		Bortne	Brekke	Nordgulen
1st PCR	Kidney	36.96[7]	38.12[7]	39.29[8]
2nd PCR	Kidney	30.88[7]/29.16[8]	–	–
	Pseudobranch	–	–	–
	Gill	–	–	–
	Heart	–	36.95[8]	38.08[8]
	Liver	–	–	–
	Spleen	32.51[7]	–	–
	Gonads	nt	nt	–
	Gut	40.22[8]	–	–

tant relationship to the other members in the family Orthomyxoviridae, the influenza viruses (Krossøy et al. 1999), suggests a very old and probably a marine origin for the ISAV. This has led to a hunt for the marine reservoirs of ISAV, and many marine fish species have been challenged to see if they could be hosts for the ISAV (Thorud & Torgersen 1994, Kvenseth 1998, Nylund et al. 2002, Snow et al. 2002). So far, only limited replication has been found in herring *Clupea harengus* (Nylund et al. 2002). On the other hand, the ISAV is able to replicate in most salmonid species, *Salmo* spp., *Oncorhynchus* spp. and *Salvelinus alpinus* (Nylund et al. 1994a, 1995a,b, 1997, Nylund & Jakobsen 1995, Rolland & Nylund 1998a, Devold et al. 2000, Snow et al. 2001a,b, Rolland & Winton 2003), causing mortality in Atlantic salmon only (Thorud & Djupvik 1988, Mullins et al. 1998, Rodger et al. 1998, Bouchard et al. 2001). Based on empirical and experimental evidence, everything points towards salmonids as the natural hosts of ISAV, and, in Norway, this means in most cases Atlantic salmon *S. salar* and trout *S. trutta*.

All salmonids have a freshwater phase when they are young, and this early phase is, for most salmonid species, followed by a marine phase (growth phase). At the start of sexual maturation the salmonids at sea return to freshwater, usually rivers, to spawn. Hence, the highest population densities of hosts will be found in rivers during spawning, where, in addition to mature salmonids, there will be an abundance of younger, susceptible stages of the same species (cf. Nylund et al. 2003). In a natural system, the most obvious place to look for the ISAV would, therefore, be in salmonid hosts during spawning. The present study, based on the material collected in 2001 and 2002, strongly supports such a view. Both RT-PCR and real-time PCR resulted in a high number of positive individuals of trout in both years, but higher in 2001 compared to 2002. However, the number of positive individuals dropped significantly in 2003 when only 1 individual fish was found positive in each of the 3 rivers that were screened. In 1998 a similar screening for ISAV was performed (RT-PCR using FA3/RA3) on sea trout (N = 45) collected in Nordfjord (the fjord where the Bortne River empties) and 3 individuals, 6.7%, were found to be positive. Salmonids in freshwater in the UK have also been found positive for ISAV, and it was suggested that they were infected by transmission of virus from ISAV-positive marine farming sites (Raynard et al. 2001a). Hence, important questions are: What connections exist between wild and farmed salmonids? Can changes in infection pressure from ISAV-positive salmon farms explain the large drop in number of ISAV-positive wild fish from 2001 to 2003? Does the pattern of ISAV prevalence in wild fish reflect the infection pressure from marine salmon farms or vice versa, or is the observed pattern just reflecting natural variation within these populations?

Most marine salmon farms close to these rivers were empty in the period summer 2002 to summer 2003 due to earlier problems with ISA. They have now been restocked. On the other hand, it is also known that the prevalence of virus in natural populations may vary from year to year depending on the status of acquired immunity within the respective populations (Knipe & Howley 2001). Testing of the ISAV from the wild fish showed that none of these gave any pathology or mortality when injected into disease-free salmon, but they did replicate, i.e. the ISAV were still detected 4 wk after the challenge. Avirulent ISAV have also been detected in farmed salmon in western Norway (A. Nylund unpubl. data), and, hence, farmed salmon could have been responsible for the high prevalence of ISAV in the rivers in 2001 and 2002. However, this would not be the pathogenic ISAV isolates collected from farms in these 2 areas in the period from 2000 to 2003. To better understand the relationship between the prevalence of ISAV in wild salmonids and the occurrence of ISA in farmed salmon, it will be necessary to screen wild populations of salmonids over a longer period (maybe 10 yr). The results from such a study should be correlated with data on ISA from salmon farms in the same areas. In addition, if possible, a molecular typing system for geographical origin and virulence should be developed to aid the interpretation of the results from such a study.

It is obviously too early to have any firm opinions about what is happening in the western Norwegian river systems included in this study. However, the

occurrence of ISAV in Norway, Canada, the UK, the Faeroe Islands, the USA and Ireland represent separate events, including different ISAV isolates (Nylund et al. 2003). In all these cases, the emergence of ISAV must have local natural reservoirs (nothing is known about the Irish ISAV). This assumption is further supported by the discovery of ISAV, in Scotland and Canada, with full-length HPRs in the HA gene (Segment 6), i.e. sequences not previously observed in any other countries (Cunningham et al. 2002, Cook-Versloot et al. 2004). Full-length HPRs were later also found in ISAV from farmed salmonids in the USA and Norway (A. Nylund unpubl. data), and these viruses are different from each other and from those described in the UK and Canada. A study of ISAV isolates from Norway, based on sequences of the HA gene, shows that there are several distinct groups of ISAV in Norway (Devold et al. 2001, Nylund et al. 2003). These isolates reflect geographical origin and time of collection, and, based on this information, Nylund et al. (2003) suggest that at least 21 transmissions from wild to farmed salmonids are represented in the material presented. Still, it is a long way from the estimated number of transitions from wild to farmed fish that can be documented to the hundreds of outbreaks of ISA in salmon farming through the years. A number of these outbreaks can be explained as horizontal transmissions between salmon farms and spreading of ISAV via transportation of infected smolts, but the tools needed to quantify this type of transmission are not available. Hence, there are large numbers of ISA cases in salmon farming for which transmission from wild fish or between salmon farms cannot be determined. Future management of ISA does, however, require knowledge about the source of the ISAV responsible for new outbreaks. We, therefore, recommend that more resources should be put into the study of natural reservoirs, man-made reservoirs (farmed stocks of salmonids) and the development of better molecular tools for typing of ISAV isolates with respect to geographical origin and virulence.

The work on tissue distribution, presented in this study, is limited to 3 individuals only (*Salmo trutta*). Two of these specimens were young fish (parr), and the gonads were not collected from these. The results from the tissues screened provides no clear pattern that can be used to support or reject any hypothesis about tissue/cell tropism in carriers, nor can the results be used to test any hypothesis about interspecies transmission of the ISAV. Cycle threshold (Ct) values from 38 and up are close to the detection limit for this real-time PCR and are not always reproducible. This explains why the second PCR on the kidney samples from Brekke and Nordgulen was negative. Further studies on tissue distribution of ISAV in carriers are needed.

The present study has shown that trout and salmon in Norwegian rivers are carrying ISAV and that these species could be the natural reservoirs for this virus. The importance of this natural reservoir, with respect to ISA in farmed salmon, has not been determined, nor can any conclusions be made about the influence of ISAV-infected farmed salmon on wild populations and their carrier status. These important questions for the management of ISA will require comparative studies of natural and man-made reservoirs over a longer timescale. Such studies will also rely upon the development of a typing system for the ISAV with respect to geographical origin and virulence.

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Transmission of infectious salmon anaemia virus (ISAV) in farmed populations of Atlantic salmon (*Salmo salar*)

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Summary. In the present study, 24 smolt production sites were screened for the presence of infectious salmon anaemia virus (ISAV) with the help of a specific real-time RT PCR assay, and 22 of these sites had smolts that were positive. If these smolt production sites are representative for the prevalence of ISAV in Norwegian smolts, then most marine production sites must be considered to be positive for ISAV. In addition, 92 European ISAV isolates have been genotyped based on the hemagglutinin-esterase gene (HE), and their distribution pattern was analysed. This pattern has been coupled to information about the origin of smolt, eggs, and broodfish in those cases where it has been possible to obtain such information, and with information about ISAV in neighbouring farms. The pattern suggests that an important transmission route for the ISAV could be that the salmon farming industry in Norway is circulating some of the isolates in the production cycle, i.e. some sort of vertical or transgenerational transmission may occur. It has also been shown that avirluent ISAV isolates are fairly common in Norwegian farmed salmon. Based on this, it is hypothesized that the change from avirulent to virulent ISAV isolates is a stochastic event that is dependent on the replication frequency of the virus and the time available for changes in a highly polymorphic region (HPR) of the HE gene to occur. This, and the possibility that only avirluent ISAV isolates are vertically transmitted, may explain why ISA most often occurs at marine sites and why no more than about 15 farms get ISA every year in Norway.

Introduction

A central dogma, which is the guideline for the official management of ISA in Europe and Norway, is that the main reservoir for ISAV is in the marine environment (most likely farmed salmonids) and that the dominant transmission route is horizontal transmission in sea water. The transmission is believed to

occur by passive transmission via sea water, transmission via escaped infected salmonids or wild salmonids, and transmission via boat transport between marine sites (well boats moving fish or used for delousing, or ballast water from supply boats). The strong belief in this dogma and suggested transmission routes, has led the bureaucracy in the EU and Norway to decide to eradicate ISA from salmon farming in Europe by killing all fish in farms experiencing outbreaks of ISA. This has been a very expensive and often fatal strategy for small farm companies and, unfortunately, the strategy has not succeeded in Norway, where the salmon farming industry is still suffering from about 15 outbreaks of ISA every year.

Knowledge about infectious salmon anaemia virus (ISAV) and the disease ISA has accumulated with increasing speed during the last 10 years. Emergence of the virus in Norway, Canada, Chile, Faeroe Islands, Ireland, Scotland, and USA has given this eight-segmented orthomyxovirus the status of one of the major viral diseases in the production of farmed Atlantic salmon (*Salmo salar*) [1–3, 15, 20, 21, 29, 31, 34, 35]. Studies so far have indicated that there are at least two major geographical reservoirs for ISAV, represented by a European subtype (EU-ISAV) of ISAV including isolates from Norway, Scotland, Faeroe Islands, and North America, and a North American subtype (NA-ISAV) found in Canada, USA, and Chile [15, 24]. Most information about the evolution of ISAV isolates comes from studies of the EU-ISAV subtype, and mainly from outbreaks of ISA in Norway [5, 7, 13, 16, 24]. This is not surprising, taking into consideration that Norway is the major salmon-producing country and that ISAV was discovered more than 10 years earlier in Norway than in other countries where the virus has been found.

The observation of several distinct ISAV isolates in Norway requires, according to Nylund et al. [24], that most of the transmission of viruses occurs in the freshwater phase in natural populations of salmonids. It has been suggested that ISAV is mainly transmitted during spawning in rivers, where the local population density is high and the interactions between infected and non-infected individuals are frequent [24, 29]. This implies horizontal transmission between individuals at the spawning ground, but it also opens the possibility of vertical or transgenerational transmission, i.e. transmission via gonadal products. It has been shown that ISAV is present in gonadal fluids of infected salmon [25] and that the virus can be transmitted from Atlantic salmon broodfish to offspring [22]. Vertical transmission of ISAV represents a new route of transmission within farmed populations of salmonids.

Could the salmon farming industry have been circulating isolates of ISAV since the start of the industry? If vertical transmission of ISAV occurs within the salmon farming industry, this should be reflected in the relationship between isolates from ISA outbreaks in the sea phase of the salmon, i.e. the relationship of ISAV should reflect the origin of the broodfish, eggs, and smolt. On the other hand, horizontal transmission in the sea phase should result in a different pattern where distinct geographical ISAV isolates should be dominating, i.e. spreading of ISAV between farms in the seawater phase. The aim of the present study is to look at the possible importance of transmission of ISAV via smolt from the freshwater phase in addition to possible horizontal transmission in the sea phase.

The study focuses on ISA outbreaks in Norway during the last five years. The open reading frame (ORF) of the heamagglutinin-esterase (HE) gene (segment 6) of ISAV isolated from these outbreaks has been sequenced and compared. The result of the genotyping is discussed with respect to possible transmission of ISAV from fresh water to sea as opposed to only horizontal transmission in the seawater phase of the salmon.

Materials and methods

Host and ISAV

This study is based on published sequences of segment 6, the haemagglutinin-esterase (HE) gene, of the infectious salmon anaemia virus (ISAV) [7, 16, 18, 24], in addition to new sequences from ISAV HE genes collected from Atlantic salmon (*Salmo salar*) during the last four years (Table 1). The new sequences were obtained directly from tissues (gills, heart and kidney) of salmon suffering from ISA in farms with official diagnostics (published by the The Norwegian Food Safety Authority, http://www.mattilsynet.no) and from salmon in farms with no signs of ISA [8]. The latter were collected from fish in both fresh and sea water.

Table 1. An overview of ISAV, the accession numbers of the HE gene sequences, and the HPR number. The names of the different localities and the county they are located in are given in the first column. Eggs refers to brood fish companies, i.e. the origin of the egg used for smolt production. All ISAV isolates are from Norway unless otherwise stated. The code includes the number given to the virus and the year of collection. The ISAV, from farmed salmon in countries around the north Atlantic, are grouped into phylogenetic groups

Group	Year	Code	HPR	ISA	Eggs	Accession no.	Author
NA-ISAV							
Bay of Fundy, Canada	1997	Can31/97	21	Y	–	AF302800	Krossøy et al. [17, 18]
CCBB, Canada		CCBB	21	Y	–	AF404342	Clouthier et al. [3]
Maine, USA	2001	ME/01	21	Y	–	AY059402	Clouthier et al. [3]
EU-ISAV							
EU-G1							
Hitra	1996	ST21/96	14	Y	–	AF364886	Devold et al. [7]
Hitra	1997	ST26/97	14	Y	–	AF364879	Devold et al. [7]
Hestvika	1997	ST30/97	14	Y	A	AY127875	Nylund et al. [24]
Hitra, Vedøy	1999	ST44/99	14	Y	A	AF302803	Krossøy et al. [17, 18]
Henningsvær	1999	N49/99	7b	Y	A, D	AF364876	Devold et al. [7]
Lepsøy	2000	MR52/00	10	Y	A	AF364892	Devold et al. [7]
Åfjord	2003	ST77/03	7b	Y	–	AY973191	Present study
Astridholmen, Nærøy	2003	NT78/03	7b	Y	A, B	AY973183	Present study
Storbrannholmen	2003	NT81/03	7b	Y	A, B	AY973184	Present study
Kvernøyvika, Flatanger	2004	NT87/04	7b	Y	–	AY973186	Present study
Bjørøya, Flatanger	2004	NT92B/04	7b	Y	A, B	AY973188	Present study
Eidsvikøy	2004	H93/04	10	Y	A, B	AY973179	Present study
Steinsøya, Vikna	2004	NT95/04	5	Y	A, B	AY973189	Present study
Smolt production site I	2005	MR102/05	0	N	A	DQ108605	Present study
Lid, Tingvoll	2005	MR103/05	17	Y	A, B	DQ108606	Present study
Lid, Tingvoll	2005	MR105/05	17	Y	A, B	DQ108608	Present study

(*continued*)

Table 1 (*continued*)

Group	Year	Code	HPR	ISA	Eggs	Accession no.	Author
EU-ISAV							
EU-G2							
EU-NS							
Nova Scotia, U5575-1	2001	Can-13	3	Y	–	AF294881	Kibenge et al. [15, 16]
Nova Scotia, Canada	2003	NS2003	3	Y	–	AY601904	Present study
Maine, USA	2004	USA2004	0	N	–	AY973194	Present study
EU-F							
Faeroe Islands	2002	F72/02	3	Y	–	AF536263	Nylund et al. [24]
Faeroe Islands	2002	F72b/02	2	Y	–	AY971656	Present study
Faeroe Islands	2002	F72e/02	3	Y	–	AF536263	Present study
Faeroe Islands	2002	F72d/02	2	Y	–	AY971657	Present study
EU-NN							
Gullesfjord	1993	T10/93	4a	Y	–	AF302801	Krossøy et al. [17, 18]
Eidebukt, Rotsund	2002	T73/02	14	Y	A, B, D	AY971663	Present study
Uløy, Rotsund	2003	T74/03	14	Y	C, D	AY971664	Present study
Storurdvika	2003	N75/03	4a	Y	C, D	AY971661	Present study
Strandmo	2004	T91/04	3	Y	A, B	AY971667	Present study
Tindlandet, Hammerfest	2004	FM98/04	14	Y	A, E	AY971660	Present study
Vegglandet	2005	FM106/05	14	Y	A, E	DQ108599	Present study
Vinnaeidet	2005	FM107/05	14	Y	E	DQ108600	Present study
Vinnalandet	2005	FM108/05	14	Y	A	DQ108601	Present study
EU-NW							
Solund	2000	SF54/00	11a	Y	–	AF364884	Devold et al. [7]
Fjaler	2000	SF57/00	11a	Y	–	AF364890	Devold et al. [7]
Smøla	2001	MR61/01	11a	Y	–	AY127877	Nylund et al. [24]
Herøy	2001	MR62/01	11a	Y	–	AY127878	Nylund et al. [24]
Segløy (Gulen)	2001	SF63/01	11b	Y	–	AY127879	Nylund et al. [24]
Leiholmane (Gulen)	2002	SF70/02	11a	Y	–	AY127880	Nylund et al. [24]
Brunsvik	2002	SF71/02	11a	Y	A	AY127881	Nylund et al. [24]
Smolt production site II	2004	H112A/04	8	N	B	DQ108603	Present study
EU-H1							
Golten, Sotra	1989	H02/89	2	Y	–	AF220607	Rimstad et al. [32]
Sotra	1991	H06/91	2	Y	–	AF364894	Devold et al. [7]
Selje	1995	SF14/95	5	Y	–	AF364873	Devold et al. [7]
Blåmannsvik	1998	T37/98	2	Y	–	AF364881	Devold et al. [7]
Nærøy	1998	NT38/98	2	Y	–	AF364874	Devold et al. [7]
Smolt production site III	2004	T85A/04	–	N	B	AY971693	Present study
EU-G3							
EU-H2							
Meløy	1989	N05/89	12	Y	–	AY127882	Nylund et al. [24]
Varaldsøy	1996	H17/96	7a	Y	B	AF364891	Devold et al. [7]
Landøy	1996	SF18/96	19	Y	–	AF364869	Devold et al. [7]
Bømlo (Bremnes)	1998	H36/98	15	Y	–	AF302799	Krossøy et al. [17, 18]
Strandebarm	1998	H40/98	7b	Y	B	AF364877	Devold et al. [7]
Gulen	1998	SF41/98	15	Y	B	AF364871	Devold et al. [7]
Mundheim	1999	H45/99	15	Y	B	AF364870	Devold et al. [7]
Midsund	1999	MR46/99	8	Y	–	AF364896	Devold et al. [7]
Øygarden	2000	H51/00	7b	Y	–	AF364882	Devold et al. [7]

(*continued*)

Table 1 (*continued*)

Group	Year	Code	HPR	ISA	Eggs	Accession no.	Author
Sørnes	2000	H56/00	15	Y	–	AF364880	Devold et al. [7]
Ørskog	2001	MR60/01	6	Y	–	AY127876	Nylund et al. [24]
Kattfjord	2003	T79/03	14	Y	B	AY971665	Present study
Smolt production site III	2004	T85B/04	15	N	B	DQ118254	Present study
Lille Torskefjord	2004	FM86/04	1	Y	B	AY971659	Present study
Ure, Lofoten	2004	N89/04	14	Y	B	AY971662	Present study
Hordaland-I	2004	H88/04	1	N	B	AY973178	Present study
Bjørøya, Flatanger	2004	NT92A/04	7b		A, B	AY973187	Present study
Broodsfih company B	2004	H96/04	7b	Y	B	AY973180	Present study
Bømlo	2004	H99/04	7b	Y	B	AY973181	Present study
Dysvik	2004	H101/04	4b	Y	B	DQ108602	Present study
Smolt production site IV	2004	SF113/04	15	N	B	DQ118255	Present study
Finnøy	2005	R111/05	18	Y	B	DQ108609	Present study
EU-S1							
390/98	1998	ISA98/01	7b	Y	–	AJ276859	Rimstad et al. [32]
832/98	1998	ISA98/02	7b	Y	–	AF388582	Kibenge et al. [15, 16]
912/99	1998	ISA98/08	7b	Y	–	AF395337	Kibenge et al. [15, 16]
EU-MN							
Senja	1996	T22/96	2	Y	–	AF364889	Devold et al. [7]
Frøya	1997	ST25/97	6	Y	–	AF364885	Devold et al. [7]
Frøya	1997	ST27/97	6	Y	–	AF364897	Devold et al. [7]
Åfjord	1997	ST28/97	6	Y	–	AF364875	Devold et al. [7]
Senja	1998	T33/98	2	Y	–	AF364887	Devold et al. [7]
Nordfjord	1999	SF47/99	9	Y	A	AF364888	Devold et al. [7]
Skatestraumen, Nordfjord	1999	SF48/99	8	Y	–	AF364878	Devold et al. [7]
Bjugn	2004	ST82/04	8	Y	A	AY973192	Present study
Marine site 1	2004	NT84/04	8	N	A	AY973185	Present study
Kråksølvnes, Senja	2004	T90/04	16	Y	A, B, D	AY971666	Present study
Olderfjorden	2005	FM100/04	7b	Y	A	AY971658	Present study
Smolt production site I	2005	MR104/05	0	N	A	DQ108607	Present study
Marine site 2	2005	SF109/05	0	N	A	DQ108610	Present study
Flesa, Hitra	2005	ST110/05	3	Y	A	DQ108598	Present study
EU-H3							
Eikelandsosen	1987	H01/87	1	Y	–	AF364893	Devold et al. [7]
Sotra	1992	H07/92	1	Y	C	AF364898	Devold et al. [7]
Sotra	1993	H08/93	1	Y	C	AF309075	Krossøy et al. [17, 18]
EU-H4							
Torgnes	1997	N29/97	7b	Y	–	AF364872	Devold et al. [7]
Dønna	1998	N32/98	7b	Y	–	AF364883	Devold et al. [7]
Marine site 3	2004	SF83/04	0	N	B	AY973190	Present study
Broodfish company C	2004	H97/04	0	N	C	DQ108604	Present study
EU-S2							
Loch Nevis	1998	Scot43/98	7	Y	–	AF302803	Krossøy et al. [17, 18]
301/98	1998	ISA98/03	7	Y	–	AF388581	Kibenge et al. [15, 16]
EU-uncertain							
Vestvågøy	1993	N09/93	3	Y	A, D	AF364895	Devold et al. [7]

The production of Atlantic salmon (*Salmo salar*) in Norway is structured as follows: A) Brood fish are kept at separate marine sites (2.5 years), but brood fish may also be collected from marine productions sites. After the marine period, the brood fish are taken into freshwater tanks, on land, in August and are kept in fresh water until stripping of eggs and milt in November–December. Each brood fish company keeps four separate year classes (generations) of salmon. B) Fertilized eggs and embryos, are kept at other freshwater localities (usually not the same localities as the brood fish) until hatching and start-feeding. C) Embryos or salmon fry/juveniles are usually moved, before hatching or after start-feeding, to new freshwater localities for production of smolts. D) Smolts are moved in May/June, 18 months after stripping of the brood fish, to marine production sites and marine brood fish sites. The fish in the marine sites usually reach commercial size after 1.5–2 years in sea cages, while the brood fish may be kept for 2.5 years in the sea. Brood fish companies may sell eggs and milt, embryos, fry, or smolt to other companies, which means that smolt from different smolt producers, may have the same origin, i.e. come from the same brood fish.

The majority of marine production sites for salmon in Norway receive smolt from more than one smolt producer. Deliveries from as many as four different smolt production sites are common. From all farms with ISAV-positive salmon (in 2000–2005) we tried to trace the origin of smolts and eggs, and the broodfish from which they came. It has been difficult to obtain such information, but in many cases (51 farms, i.e. 61.4% of the Norwegian isolates included in this study) we were able to trace the fish back to fresh water (smolt production site) and to the broodfish.

All broodfish production sites have been coded so that they can only be identified according to the county in which they are located. Eggs included in this study were produced by five different broodfish companies (BF-A, BF-B, BF-C, BF-D and BF-E). Each broodfish company has their broodfish at several different locations, and to protect the companies, these locations are not given. Salmon farms in Norway with clinical outbreaks of ISA can be identified from the official record on the homepage of The Norwegian Food Safety Authority (http://www.mattilsynet.no). Hence, these ISA-positive sites are identified according to geographical location (Table 1).

In addition to collection of material from ISAV-infected marine farms, several smolt populations from counties in Norway were screened for the presence of ISAV using a real-time RT PCR assay (29) (Table 2). Negative tissues, RT, and PCR controls were included in all real-time RT PCR runs. The presence of ISAV in smolt was not suspected before screening, but some of the smolts were suffering from haemorrhagic smolt syndrome (HSS) or proliferative gill disease (PGD). However, the majority of the smolts were screened as part of a project with the aim of detecting ISAV in Norwegian smolt populations. Each sample consisted of 30–60 specimens, but only a few individuals (cf. Table 2) from these populations were tested in the first screening. Further testing of the populations was stopped if ISAV-positive individuals were found among these first individuals. All smolt locations have been coded and can only be identified by county.

Sequencing

The extraction of RNA, RT-PCR, real-time RT-PCR, and sequencing were performed as described by Devold et al. [7] and Plarre et al. [29]. All PCR products were sequenced in both directions. The Vector NTI Suite software package (InforMax) was used to assemble sequences and for construction of sequence alignments.

Phylogeny

In the present study, phylogenetic analysis of selections of ISAV isolates were performed using 1002 nt of the 5′-end flanking region or N-terminal region (N-term) of the HPR of

Table 2. Real-time RT PCR screening of Atlantic salmon par and smolt populations in fresh water for presence of ISAV. None of these farms had any outbreaks of ISA

County	Year	N	Positive	HPR	Disease
Hordaland					
H03a	2003	9	1	–	
H04a	2004	6	5	–	HSS
H04b	2004	9	9	–	
H04c	2004	12	3	7b	
H05a	2005	30	6	–	
Sogn og fjordane					
SF04a	2004	6	6	0	PGD
SF04b	2004	15	6	–	PGD
SF04c	2004	4	2	–	
Møre og Romsdal					
MR04a	2004	12	2	–	
Sør-Trønderlag					
ST04a	2004	10	4	–	
Nord-Trønderlag					
NT04a	2004	30	0	–	
Nordland					
N04a	2004	8	3	–	
N04b	2004	10	1	–	
N04c	2004	30	0	–	
N04d	2004	31	4	–	
N04e	2004	8	1	–	
N05a	2005	20	1	–	
N05b	2005	19	1	–	
N05c	2005	60	5	–	
Troms					
T04a	2004	15	4	–	
Finnmark					
FM04a	2004	3	1	–	
FM04b	2004	15	2	–	
FM05a	2005	10	5	–	
FM05b	2005	61	18	–	

N The number of fish screened for the presence of ISAV; *positive* number of ISAV positive salmon; *disease* disease suspected in the farm; *HSS* haemorrhagic smolt syndrome; *PGD* proliferative gill disease

the HE gene sequences (Fig. 1). In addition to the new HE gene sequences presented in this study, a large number of sequences were obtained from the GenBank (Table 1). The alignment was manually adjusted using the sequence alignment editor GeneDoc (Available at: www.psc.edu/biomed/genedoc). The highly polymorphic region (HPR) of the HE gene was manually aligned and compared for all isolates.

	N-term	HPR	TMR	Ct	
5′-	1011 nt 337 aa	33–105 nt 11–35 aa	69 nt 23 aa	48 nt 16 aa	-3′

Fig. 1. Schematic drawing of the heamagglutinin-esterase gene (open reading frame, ORF) and the putative protein. N-term (5′-end flanking region or N-terminal region of the HPR), HPR (highly polymorphic region with variable length in different ISAVs), TMR (transmembrane region), Ct (cytoplasmic tail or 3′-end flanking region or carboxyl flanking region of the TMR). The sizes of the different regions are constant with the exception of the HPR. The HPR from European ISAV isolates may vary from 33 to 105 nucleotides or 11 to 35 amino acids

Analyses were performed using TREE-PUZZLE 5.2 (Available at: http://www.tree-puzzle.de) and PAUP v4.0 [37] and were based on the N-term region to secure homology of the sequences used [cf. 24].

Phylogenetic analysis in PAUP was performed using likelihood as follows: 1) Modeltest v. 3.6 [28] was used to identify one of 56 models best fitting the dataset based in the Akaike Information Criterion (AIC). 2) A model with base frequencies (A = 0.2877, C = 0.2035, G = 0.2600, T = 0.2488), six substitution types with six-parameter instantaneous rate (A–C = 2.1994, A–G = 4.8895, A–T = 0.7423, C–G = 4.8895, G–T = 1.0000), and among-site rate variation with gamma shape value 0.5206 was employed. 3) A maximum-likelihood tree was obtained, but it was not bootstrapped due to lack of computer capacity.

The trees presented were obtained using TREE-PUZZLE 5.0, and 50 000 quartet puzzling (QP) steps were carried out using the HKY model of substitution and gamma distribution rates [11]. Phylogenetic trees were drawn using TreeView [26].

Nucleotide substitution rates

The rate of nucleotide substitutions was calculated based on all nucleotide mutations in the N-term region (1002 nucleotides) for isolates that clustered together in the phylogenetic tree [cf. 24]. A substitution rate with 0.95 confidence intervals was calculated by BASEML in the PAML v 3.14 package [38], using the single rate dated tips (SRDT) model [30]. This model assumes that a single rate of substitution applies for every branch in a rooted phylogenetic tree (molecular clock) and optimizes length of the branches so that relative tip-positions correlate with sampling dates. The molecular clock was tested by a likelihood ratio test [cf. 27], comparing the likelihood of the SRDT model with the likelihood of a different rate (DR) model (also calculated by BASEML, PAML), in which the branches are allowed to evolve with independent rates.

Results

Subtypes and groups

Phylogenetic analysis of all sequences included in this study, using three North American isolates (NA-ISAV) as outgroup (Accession nos: AF404342, AF302800, AY059402), resulted in several phylogenetic groups with high support values (Fig. 2). The two ISAV isolates from Nova Scotia, Canada (Accession nos: AF294881, AY601904), and the ISAV from Maine, USA (Accession no: AY973194), all group within the European branch, EU-ISAV. The European ISAV (EU-ISAV) has been divided into three major groups (Fig. 2) [cf. 24]. EU group 1

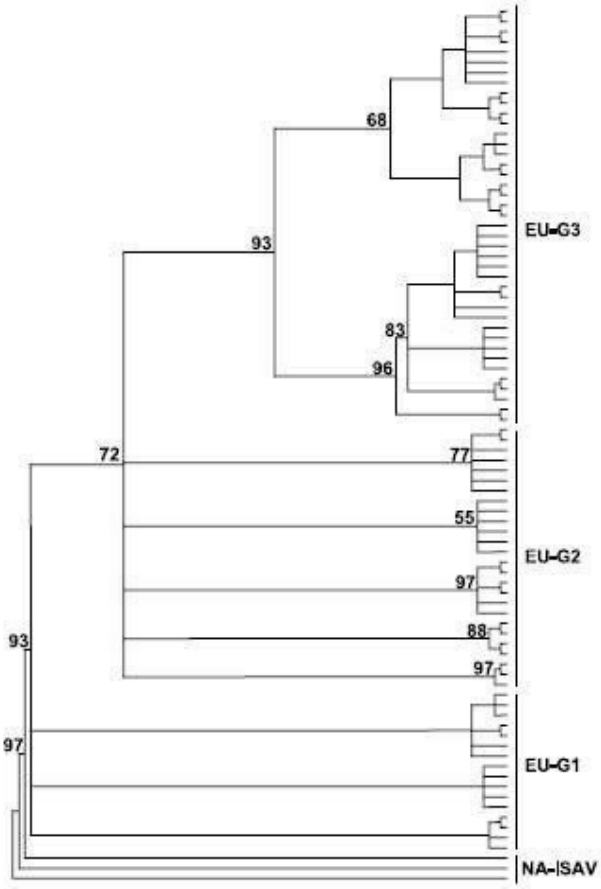

Fig. 2. Cladogram showing the major ISAV groups included in this study. One group from North America (NA-ISAV) and one European group (EU-ISAV) divided into three subgroups (EU-G1, EU-G2 and EU-G3)

(EU-G1), the most basic branch in the phylogeny, consists mainly of isolates from counties in central Norway (Trøndelag). The relationships between the isolates in EU-G1 are not fully resolved (Fig. 3). The second group, EU-G2, consists of several subgroups (EU-NS, EU-F, EU-NN, EU-NW, and EU-H1, Table 1 and Fig. 4). The third group, EU-G3 (support value = 93), consists of ISAV mainly from western Norway and a few isolates from Scotland. This group can also be divided into subgroups (EU-H2, EU-S1, EU-MN, EU-H3, EU-H4, EU-S2) (Fig. 5). Several of the subgroups reflect, to some extent, geographical origin. However, most of these subgroups include ISAV collected from different counties in Norway. All phylogenies were analyzed using PAUP and TREE-PUZZLE and

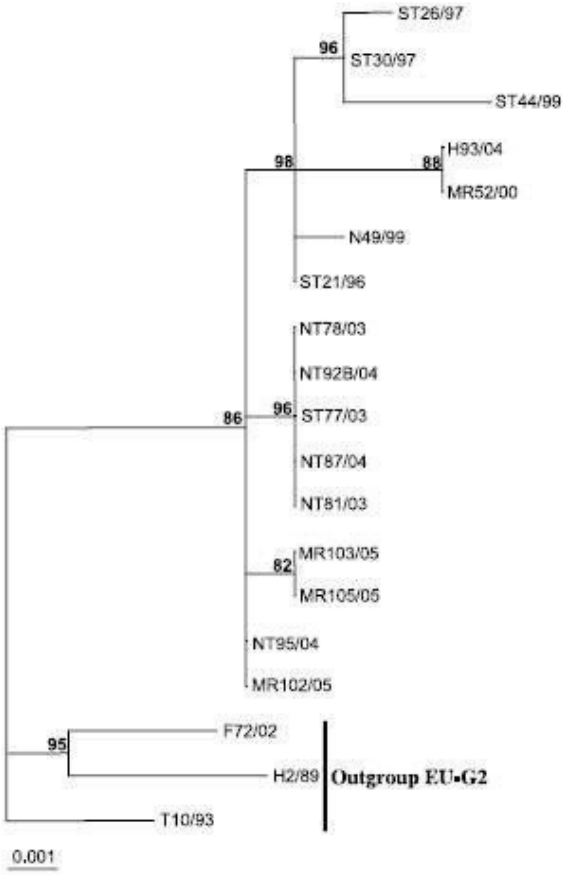

Fig. 3. The phylogentic relationship between ISAV isolates in the EU-G1 group. Isolates (F72/02, H2/89 and T10/93) from EU-G2 have been used as outgroup

both methods resulted in the same trees. However, the trees were bootstrapped in TREE-PUZZLE only.

Group EU-G1

The earliest EU-ISAV branch on the phylogenetic tree is defined as EU-G1 and consists mainly of ISAV isolates from the Trøndelag counties (central Norway). This group consists of 16 ISAV isolates that were collected during a ten-year period from 1996 to 2005 (Table 1, Figs. 3 and 6). In EU-G1 there is variation in 12 nt positions of the 1002 nt (overall similarity = 98.8%) included in the phylogenetic analysis (Fig. 3). In the same part of the HE there is variation in 6 amino acid positions.

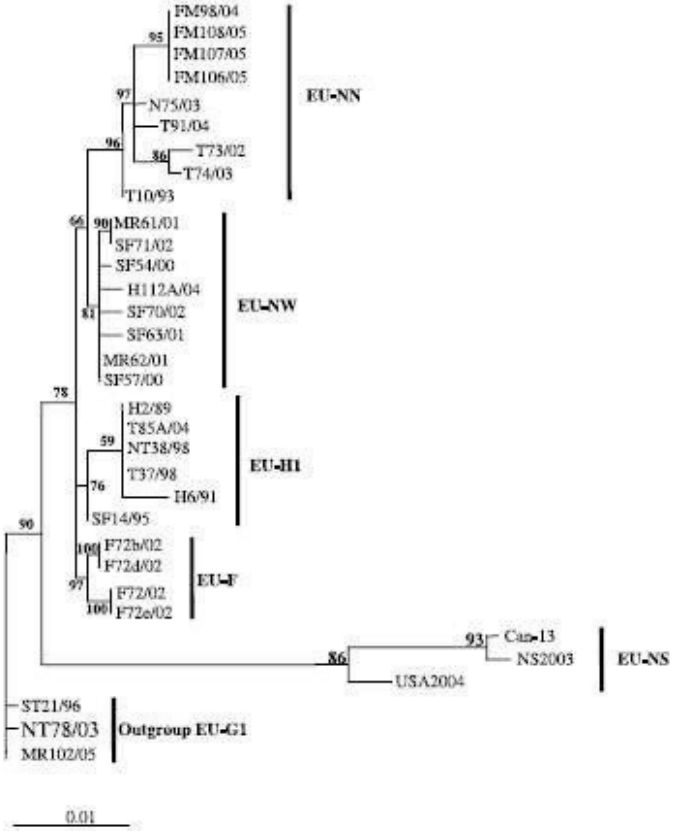

Fig. 4. The phylogentic relationship between ISAV isolates in the EU-G2. Isolates (ST21/96, NT78/03, MR102/05) from EU-G1 have been used as outgroup

Most of the isolates in EU-G1 were collected from marine farms owned by different companies. However, the isolates MR102/05, MR103/05, and MR105/05 were from two different farms owned by the same company. Isolate MR102/05 came from a smolt production site (I) without clinical ISA, while the other two came from a marine site, with clinical ISA, in the same fjord. Some of the farms in EU-G1 have received smolt from the same smolt production sites, and according to the Norwegian Food Safety Authority (NFSA), ISAV could have been transported by well boat from site ST77/03 to site NT81/03 during smolt transport (Figs. 3 and 6).

Broodfish company A (BF-A) has produced eggs for the fish in all the 12 farms in EU-G1 that we have been able to get data from (Table 1). Among these 12 farms, seven have also received fish from eggs produced by BF-B, and one farm has received fish hatched from eggs from BF-A and BF-D. A total of 20 different

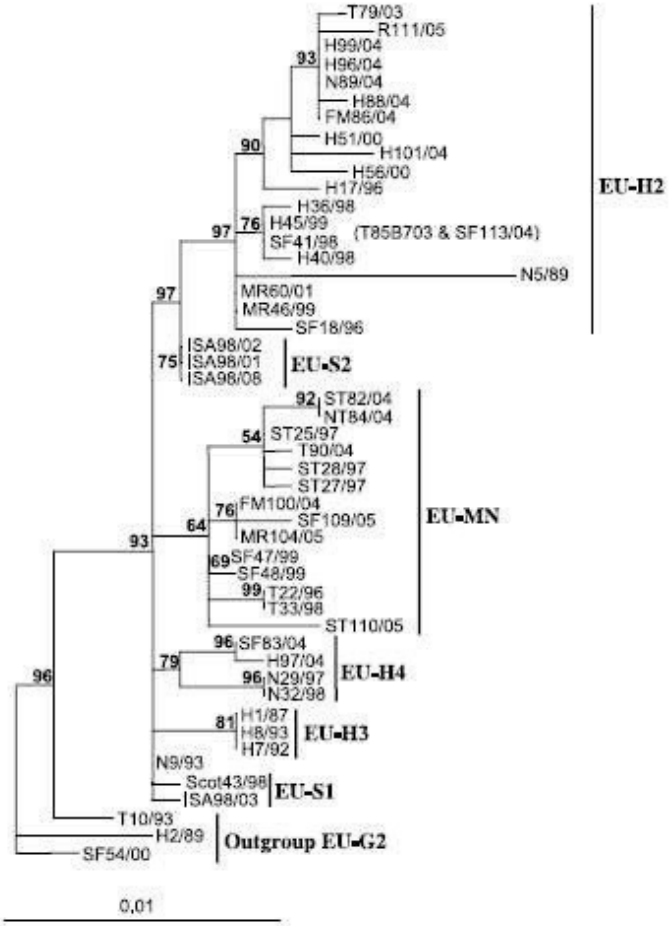

Fig. 5. The phylogentic relationship between ISAV isolates in the EU-G3. Isolates (T10/93, H2/89, SF54/99) from EU-G2 have been used as outgroup. The isolates T85B/04 and SF113/04 have not been included in the phylogenetic analysis, but their 600 nt, including the HPR, are identical to that of the isolates H36/98, SF41/98 and H45/99

smolt production sites have delivered smolt hatched from eggs produced by the three broodfish companies identified in EU-G1. There are two different ISAV isolates present at locations NT92/04 and smolt production site I. The isolates NT92B/04 and MR102/05 belong to EU-G1, while the isolates NT92A/04 and MR104/05 belong to EU-G3, subgroups EU-H2 and EU-MN, respectively. Six different HPR groups are present within EU-G1 (Table 1).

The ISAV isolates collected between 1996 and 2000, plus one isolate collected in 2004 (H94/04), group together with high support (support value = 98), and among these the nucleotide sequences of MR52/00 and H93/04, separated in time

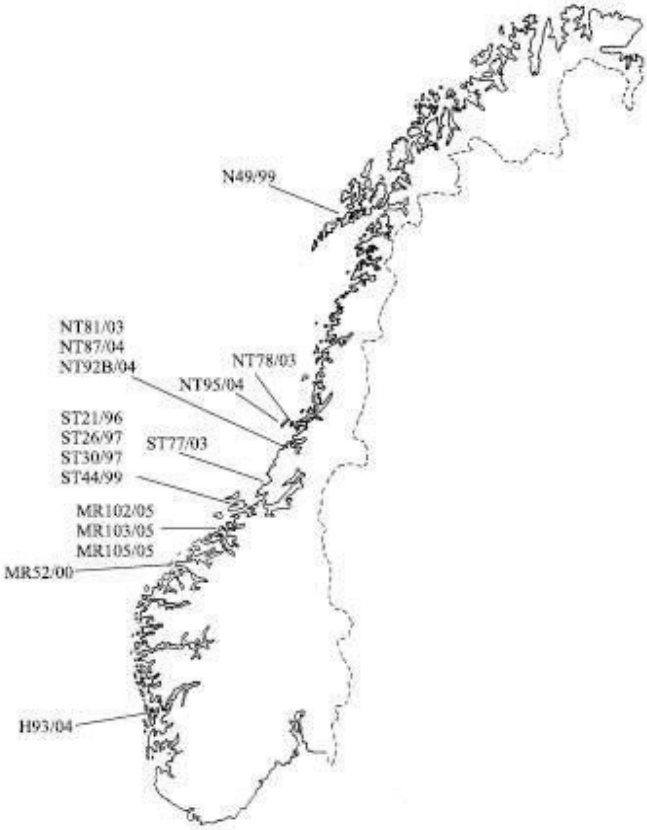

Fig. 6. The geographical distribution of Norwegian farm locations where 16 ISAV isolates in EU-G1 were collected in the period from 1996 to 2005

of isolation by four years, are identical. The isolate N49/99, from northern Norway, has a different HPR compared to ST21/96, but differs by only one nucleotide (nt) when comparing the first 1002 nt of the HE gene. The remaining nine isolates in EU-G1 were collected between 2003 and 2005 and show variation at only two nucleotide positions. The following isolates have an identical nucleotide sequence in the analysed segment; a) MR103/05 and MR105/05, b) NT95/04 and MR102/05, and c) ST77/03, NT78/03, NT81/03, NT87/04, and NT92B/04. However, the isolates NT95/04 and MR102/05 have different HPRs.

Group EU-G2

This European group of ISAV contains 30 isolates collected from salmon farms in North America (EU-NS), Faeroe Islands (EU-F), and Norway (EU-NN, EU-NW and EU-H1) (Table 1, Figs. 4 and 7). These isolates were collected during a period

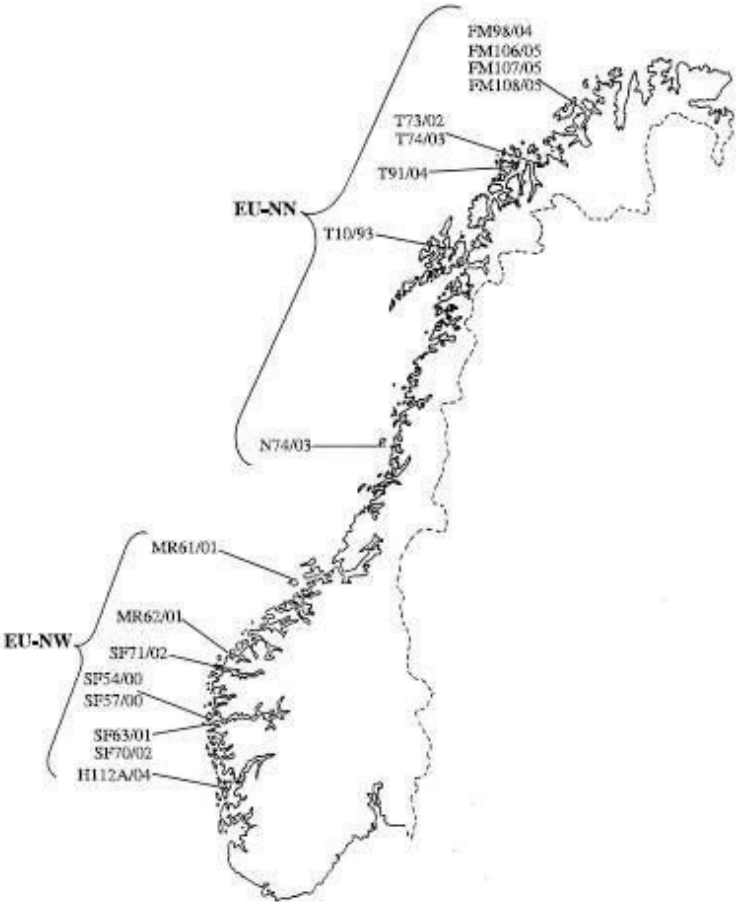

Fig. 7. Geographical distribution of Norwegian farm locations where a selection of ISAVs in EU-G2 were collected in the period from 1993 to 2005

of 17 years from 1989 to 2005. Two of the isolates in the EU-NS subgroup are from Nova Scotia (Canada), while the third is from Maine, USA. The latter was from a farm without clinical outbreak of ISA and the virus had an HPR0 (Tables 1, 4). Within EU-G2, there is variation at 75 nt positions of the 1017 nt (overall similarity = 92.4%) included in the phylogenetic analysis. In the same part of the HE, there is variation at 27 amino acid positions. Isolate NS2003 differs from the oldest European isolate (H2/89) in EU-G2 at 39 positions (similarity = 96.2%). Between the Norwegian isolates in EU-G2 there is variation in 37 nt (overall similarity = 96.5%), resulting in 16 amino acid substitutions. Little information is available about 12 of the 23 Norwegian ISAV isolates in EU-G2.

EU-NN

A subgroup of 9 isolates from marine salmon farms in northern Norway constitutes a well-defined phylogenetic assembly of ISAV (EU-NN) found only in this part of the country (Table 1, Figs. 4 and 7). These isolates were collected during a 13-year period from 1993 to 2005, from all three counties in northern Norway (Nordland, Troms, and Finnmark). The first 1017 nt of the HE gene from these ISAV isolates show variation in 13 nucleotide sites (similarity = 98.7%), resulting in 7 amino acid substitutions. Three different HPR groups (HPR3, 4a and 14) are present in EU-NN.

Four of the ISAV isolates in EU-NN (FM 98/04, FM106/05, FM107/05, FM108/05), which come from four different farms owned by the same company, have identical N-term sequences and HPR groups. These four farms are located very close to each other, and two of them are connected by a walking bridge. The eggs were delivered by two different broodfish companies (BF-A and BF-E) and the smolt from four different smolt producers.

The other ISAV isolates in EU-NN come from marine salmon farms where the smolt were produced from eggs delivered by four different broodfish companies (Table 1). The smolts were produced by 16 different companies at 16 different locations. It has not been possible to find any single factor that connects these isolates together except that they all are from northern Norway.

EU-NW

The majority of the 8 isolates in EU-NW come from the northwestern part of Norway and represent three different HPR groups (Table 1 and Fig. 7). The isolates, collected in the period between 2000 and 2002, were all from clinical outbreaks of ISA, while the isolate from 2004 (H112A/04) was collected at a smolt production site (II) with no outbreak of ISA. The smolt production site (H112A/04) had received eggs from a broodfish company (BF-B) in Hordaland county. The first 1018 nt of the HE gene (N-term region) from these ISAV isolates show variation in 8 nucleotide sites (similarity = 99.2%), resulting in 3 amino acid substitutions. Two of the amino acid substitutions are found in one isolate (SF63/01) only, while the third is shared by MR61/01 and SF71/02. The isolates MR61/01 and SF71/02 are identical to each other despite the large distance between the farms from which they were collected. A large distance also separates the other two identical isolates, SF57/00 and MR62/01 in this subgroup (Fig. 7).

EU-H1

The third Norwegian subgroup, EU-H1, contains 6 isolates collected from farms in northern, central, and western Norway during a period of 16 years, 1989–2004 (Table 1, Fig. 4). The first ISAV (H2/89) in this subgroup was isolated as early as 1989 from farms in Hordaland, western Norway, and two years later a second isolate (H6/91) in this group was found in the same county. In 1995, a related ISAV (SF14/95) was found in the neighbouring county, Sogn og Fjordane. Later,

ISAV isolates identical to H2/89 were found) in 1998 in two other counties in Norway that are farther north (T37/98 and NT38/98. The last isolate (T85A/04) in this group was found in 2004 at a smolt production site (III), with no outbreak of ISA, in northern Norway. This farm had received eggs from a broodfish company (BF-B) located in Hordaland. The sequences of the N-term region (1031 nt) of the isolates H02/89, T37/98, NT38/98, and T85A/04 are identical, while H06/91 and SF14/95 differ in 4 and 3 nucleotides, respectively. Three different HPR groups are present within subgroup EU-H1 (Table 1). SF14/95 differs from the closest Faeroe isolate at 3 nucleotide positions only.

Group EU-G3

This group contains 49 ISAV isolates from Norway (subgroups EU-H2, EU-MN, EU-H3, EU-H4) and Scotland (subgroups EU-S1, EU-S2) (Table 1, Figs. 5, 8, 9). The isolates were collected during a 19-year period from 1987 to 2005. The subgroup EU-S2 is sister group to a large collection of isolates mainly from western Norway, EU-H2 (SV = 97). The relationships between the other subgroups (EU-MN, EU-H3, EU-H4, EU-S1) are unresolved. Within EU-G3 there is variation in 60 nt positions of the 1003 nt used from the N-term region (overall similarity = 94.0%) in the phylogenetic analysis. In the same part of the HE gene there is variation at 25 amino acid positions. All outbreaks of ISA since 1999, involving ISAV in EU-G3, have been isolates belonging to only two subgroups (EU-H2 and EU-MN). The brood stock that have been the source of salmon in these farms come mainly from two different broodfish companies, BF-A and BF-B which were the stocks for the salmon carrying ISAV in subgroups EU-MN and EU-H2, respectively.

Information about the two subgroups from Scotland, EU-S1 and EU-S2, is limited and so is the information about the two Norwegian subgroups EU-H3 and EU-H4. However, subgroup EU-H4 is interesting since it contains an ISAV isolate from a small broodfish company in western Norway.

EU-H2

The majority of the 22 ISAV isolates in this subgroup were collected from farms in western Norway, mainly Hordaland county. However, EU-H2 also contains isolates (T79/03, T85B/04, FM86/04, N05/89, and N89/04) collected from the three northernmost counties in Norway (Table 1, Figs. 5 and 8). Three of the isolates, T85B/04, H88/04, and SF113/04, in this subgroup are from a marine farm and two smolt production sites (III and IV) that have not been diagnosed with ISA. It has only been possible to obtain about 600 nt, including the HPR, from isolates T85B/04 and SF113/04, and hence they were not included in the phylogenetic analysis. However, their sequences (about 600 nt) are identical to each other and to the isolates H36/98, H45/99, and SF41/98. This subgroup also includes an isolate from one of the localities belonging to brood fish company B (isolate H96/04) that was diagnosed with ISA in 2004.

Fig. 8. Geographical distribution of Norwegian farm locations where ISAVs in EU-G3 (subgroup EU-H2) were collected in the period from 1989 to 2005

The EU-H2 isolates were collected during a 17-year period (1989–2005), but most of the isolates were collected from 1998 to 2005. The sequence variation (1002 nt of the N-term region) within the EU-H2 subgroup includes 27 nucleotide substitutions (overall similarity = 97.3%), resulting in 11 amino acid substitutions. If the isolate N5/89 is excluded from the alignment, the sequence variation is reduced to 16 nt (overall similarity = 98.4%). The isolate SF18/96 differs from the Scottish isolates (ISA98/01, ISA98/02, ISA98/08) at only 4 nt positions (similarity = 99.6%). The subgroup EU-H2 contains 11 different HPR groups (Tables 1 and 4).

It has not been possible to get information about all broodfish and smolt companies that have delivered salmon to these farms, but we have been able to trace the fish from 15 of these locations. These farms are widely dispersed in

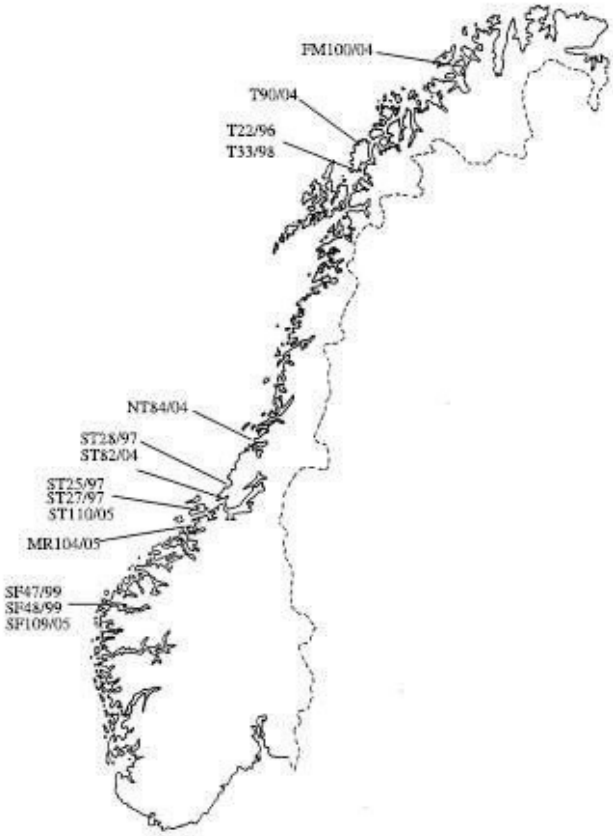

Fig. 9. Geographical distribution of Norwegian farm locations where ISAVs in EU-G3 (subgroup EU-MN) were collected in the period from 1997 to 2005

Norway, from southwestern Norway (Rogaland, R111/05) to northeastern Norway (Finnmark, FM86/04) (Table 1, Fig. 8). All of these 15 locations have received eggs or salmon hatched from eggs from broodfish company BF-B (three different localities) in western Norway. One farm (isolate NT92A/04) had received fish hatched from eggs from both BF-A and BF-B. The salmon at this location were infected with two different ISAV isolates, one belonging to EU-G1 and the other to EU-H2. The salmon at smolt production site III, located in northern Norway, were also infected by two different isolates, T85A/04 (subgroup EU-H1) and T85B/04 (subgroup EU-H2). These two subgroups contain the majority of isolates from western Norway, and the eggs were delivered to smolt production site III from BF-B in western Norway.

The smolts of the marine sites in EU-H2 were delivered from 17 different smolt production sites. The isolates N89/04, FM86/04, H96/04, and H99/04, collected

in three different counties, have an identical N-term sequence, while the isolates T79/03, H88/04, and R111/05 differ from these four isolates at 1, 1, and 2 nucleotide positions, respectively. The isolates SF41/98 and H45/99 have an identical N-term sequence, while H40/98 differs from these two isolates at 1 nucleotide position.

EU-MN

The majority of the 14 isolates in this subgroup come from the middle part of Norway (the Trøndelag counties), but ISAV from farms in northern and western Norway are also represented (Table 1, Fig. 9). They were collected during a 10-year period from 1996 to 2005. EU-MN also includes two isolates from two different marine sites without ISA and one isolate from a smolt production site (I) without ISA. The salmon at marine site 1 were suffering from heart and skeletal muscle inflammation (HSMI), while the salmon at marine site 2 were dying from pancreas disease. The smolt at smolt production site I did not experience any increased mortalities. The sequence variation (1002 nt of the N-term region) between the ISAV isolates from farms in subgroup EU-MN includes 15 nucleotide substitutions (overall similarity = 98.5%) resulting in 6 amino acid substitutions. The following isolates have an identical sequence; T22/96 and T33/98, ST82/04 and NT84/04, and FM100/04 and MR104/05. The latter of these three pairs received smolt from different smolt production sites, but the smolt were hatched from eggs delivered by BF-A. There are eight different HPR groups represented in EU-MN.

It has been possible to obtain information about the origin of eggs and smolt, which made up the salmon populations, in eight of the twelve farms that had ISAV isolates of subgroup EU-MN. All of these eight farms had received salmon hatched from eggs delivered from broodfish company BF-A. One farm (T90/04) also had received salmon cultured from eggs delivered by BF-B and BF-D. The smolts in these eight farms were delivered by 13 different smolt production sites.

EU-H4

This is a small subgroup of ISAV with no recent outbreaks of ISA. ISAV H97/04 was collected from broodfish in western Norway (BF-C) with no clinical signs of ISA and sequenced. This isolate and SF83/04 have an HPR0 (Tables 1 and 4). The latter was detected in smolt in Sogn- og Fjordane county, and the smolt had been delivered from a smolt production site located in the same fjord as BF-C. The eggs were delivered by broodfish company BF-B from a locality that was also close to BF-C. The other two ISAV in this subgroup are from two marine farms in northern Norway (Nordland). It has not been possible to obtain information about smolt deliveries to these farms. The sequence variation (1002 nt of N-term region) between the ISAV isolates from these four farms (subgroup EU-H4), collected in the period from 1997 to 2004, includes 6 nucleotide substitutions (overall similarity = 99.4%), resulting in 2 amino acid substitutions. EU-H4 contains 2 different HPR groups (Tables 1 and 4).

Table 3. Overall substitution rates for the EU-ISAV subtype and the three ISAV groups, EU-G1, EU-G2, and EU-G3, based on the N-term sequence (1002 nt) of the HE gene

Group/subgroup	N	Years	Rate	Clock
EU-ISAV (G1+G2+G3)	93	1987–2005	$0.7 \pm 0.1 \times 10^{-5}$	nc
EU-G1	16	1996–2005	NA	–
EU-G2*	27	1989–2005	$1.44 \pm 0.35 \times 10^{-4}$	nc
EU-G3	47	1987–2005	$2.15 \pm 0.68 \times 10^{-4}$	nc

*The calculation of the substitution rate in subgroup EU-G2 does not include the three isolates in subgroup EU-NS

nc No molecular clock

Substitution rate of the HE gene

Overall substitution rates for the EU-ISAV subtype and the EU-ISAV groups (EU-G1, EU-G2 and EU-G3) were calculated based on the N-term region (1002 nt) of the hemagglutinin-esterase gene (HE) (Table 3). The resulting rates varied between $0.7 \pm (0.1) \times 10^{-5}$ nucleotides site^{-1} yr^{-1} (EU-ISAV) and 2.15 ($\pm$0.68) $\times 10^{-4}$ nucleotides site^{-1} yr^{-1} (EU-G3). A molecular clock was rejected in all cases.

Highly polymorphic region (HPR)

The number of different HPR groups detected so far has increased to 24 in Europe and four in North America (Table 4). The pattern of variation is constrained to the 35 amino acids defined as the HPR [cf. 24]. With the exception of HPR12 (isolate N05/89), there are few amino acid substitutions in the different positions of the HPR. In HPR11 the amino acid in position 29 (serine, S) has been substituted by arginine (R). However, this change does not require any nucleotide substitution but could be a result of a recombination/deletion event that splits two codons, one coding for glutamine (Q–C/AG) and the latter for serine (S–A/GC), resulting in an arginine (R–CGC). The following HPR groups are separated by substitutions that are not a result of recombination/deletion; HPR4a and HPR4b and HPR7a and HPR7b are separated by a substitution at position 31 (isoleucine I–threonine T), and HPR11a and HPR11b are separated by a substitution at position 33 (isoleucine I–serine S). In these cases a non-polar amino acid is substituted by another non-polar one.

Two different HPR0s have been found in Europe – one in Scotland and one in Norway. They differ at amino acid number 25, where the aspargine (N) in the Scottish ISAV is replaced by threonine (T) in the Norwegian strain. Two different HPR0s have also been detected in ISAV from North America. The North American HPR0 (isolate USA 2004) presented in this study, comes from Maine, USA, and this virus was not associated with ISA mortality. It differs from the Norwegian HPR0 by having a serine (S) in position 24 instead of the alanine (A) found in Norwegian ISAV. The other HPR0 is from Nova Scotia, Canada, and has been sequenced from cell cultures in which no cytopathic effect was observed

Table 4. Overview of the different highly polymorphic region (HPR) groups of infectious salmon anaemia virus (ISAV)

HPR aa no	N-term	HPR 1–5	6–10	11–15	16–20	21–25	26–30	31–35	TMR	ISAV
Europe										
HPR0a	TDV	KIRVD	AIPPQ	LNQTF	NTNQV	EQPAT	SVLSN	IFISM	GVA	SF83/04
[1]HPR0b	TDV	KIRVD	AIPPQ	LNQTF	NTNQV	EQPAN	SVLSN	IFISM	GVA	ISA440971
HPR1	TDV	K				PAT	SVLSN	IFISM	GVA	H1/87
HPR2	TDV	KIRVD	AIPPQ	LNQT				M	GVA	H2/89
HPR3	TDV	KIRVD	AIPPQ	LNQT				FISM	GVA	N9/93
HPR4a	TDV	KIRVD	AIPPQ	L			SN	IFISM	GVA	T10/93
HPR4b	TDV	KIRVD	AIPPQ	L			SN	TFISM	GVA	H101/04
HPR5	TDV	KIRVD	AIPPQ	L				ISM	GVA	SF14/95
HPR6	TDV	KIRVD	AI		QV	EQPAT	SVLSN	IFISM	GVA	ST25/97
HPR7a	TDV	K				T	SVLSN	TFISM	GVA	H17/96
HPR7b	TDV	K				T	SVLSN	IFISM	GVA	N29/97
HPR8	TDV	KIRVD	AIPPQ	L					GVA	MR46/99
HPR9	TDV	KIRVD	AIPPQ	LNQTF	NT			M	GVA	SF47/99
HPR10	TDV	KIK				QPAT	SVLSN	IFISM	GVA	MR52/00
HPR11a	TDV	KIRVD	AIPP				RN	IFISM	GVA	SF54/00
HPR11b	TDV	KIRVD	AIPP				RN	IFVSM	GVA	SF63/01
HPR12	TDV	GIGVD	AIPPQ	L			N	IFISM	GVA	N5/98
[1]HPR13	TDV	K				EQPAN	SVLSN	IFISM	GVA	ISA440970
HPR14	TDV	KIRVD	A		NQV	EQPAT	SVLSN	IFISM	GVA	ST21/96
HPR15	TDV					ET	SVLSN	IFISM	GVA	H36/98
HPR16	TDV	KIRVD	AIPPQ	LNQTF				M	GVA	T90/04
HPR17	TDV	KIR			LEV	EQPAT	SVLSN	IFISM	GVA	MR103/05
HPR18	TDV	KIRV				PAT	SVLSN	IFISM	GVA	R111/05
HPR19	TDV	KIRVD	AIPPQ	LNQTL					GVA	SF18/96
America										
HPR00	TDV	KIRVD	AIPPQ	LNQTF	NTNQV	EQPST	TVLSN	IFISM	GVA	USA2004
[2]HPR00	TDV	KIRVD	AIPPQ	LNQTF	NTNQV	EQPAT	SVLSN	IFISM	GVA	AY646059
HPR20	TDV	NNRVD	AI	L	GVNQV	EQPST	SVPSN	IFISM	GVA	CCBB
HPR21	TDV	NNRVD	AIPPQ	L			SN	IFISM	GVA	Can31/97

[1]These two HPR groups have only been found in Scotland [6]
[2]This HPR0 has been found in Nova Scotia, Canada [4]
N-term N terminal region with respect to the HPR; *TMR* transmembrane region

(cf. Versloot et al., 2004). This HPR0 (accession nos: AY646058, AY646059) is identical to that found in Norwegian ISAV (SF83/04, H97/04, MR102/05, MR104/05, SF109/05).

The shortest HPR consists of 11 amino acids (HPR8), while the longest HPR associated with pathology and disease in farmed salmon in Europe consists of

24 amino acids (HPR6 and HPR14). The largest HPR from a virulent strain in North America consists of 28 amino acids (HPR20).

Prevalence of ISAV in smolt populations

From 24 smolt production sites, about 30 to 60 individuals were collected, but only about 10 (in a few cases fewer) from each site were included in the first screening for ISAV. The majority of the fish were collected in 2004, and none of them showed any signs of ISAV (Table 2). Smolt from all 24 farms, with the exception of two, contained ISAV-positive individuals (real-time RT PCR). The negative smolt farms were located in the counties Nord Trøndelag and Nordland, while all farms in western Norway were positive for ISAV. The HE genes of ISAV from two of the positive farms in western Norway were sequenced and one was an ISAV with HPR0, i.e. an avirulent virus [cf. 29].

Discussion

The phylogenetic analysis of ISAV from the north Atlantic countries confirms the division of ISAV into two subtypes, a North American subtype (NA-ISAV) and a European subtype (EU-ISAV) [1, 7, 17, 18, 24]. However, unlike previous studies in which isolates from Nova Scotia were presented as close relatives of the EU-ISAV subtype, the present study gives support for placing these isolates within EU-ISAV. The close relationship between EU-NS and other EU isolates can be interpreted as a result of introduction of ISAV viruses from Europe to North America. This transmission could have occurred as a result of transport of ISAV-infected fertilized eggs from Europe to North America [cf. 22]. The host in North America is the same as in Europe, i.e. Atlantic salmon (*Salmo salar*), but external factors may have introduced a different selection pressure on these isolates in North America than in Europe. In addition, genetic drift may have played a role in the substitution rate of the isolates in the EU-NS subgroup, which means that the substitution rate observed within EU-ISAV may not be correct for the rate of change in isolates introduced to North America.

Transmission of ISAV in farmed populations

It has been suggested that transportation of infected salmon in well boats may play an important role in the spread of ISAV [24]. Well boat transports include transportation of smolt to marine farms and salmon from marine farms to slaughterhouses. The latter is mainly short-distance transport to the nearest slaughterhouse, especially if the salmon is suspected to be infected with ISAV, i.e. salmon suffering from ISA have to be slaughtered, at a slaughterhouse close to the infected farm, as soon as possible after the disease has been diagnosed (to prevent possible spread of the ISAV). Smolt transport will also, in most cases, be short-distance transport. However, lack of smolt in the three northernmost counties in Norway means that large numbers of smolts are transported from western and central Norway to

the north. If a large number of Norwegian smolt populations in the freshwater phase are infected with ISAV, then this should be reflected in the spreading pattern of ISAV isolates, i.e. isolates from western and central Norway should be present in northern Norway but not *vice versa* since smolt are not transported from northern Norway to the south. The present study shows that there is indeed a high prevalence of ISAV-positive smolt production sites in Norway. Only 2 of the 24 smolt populations that were screened for the presence of ISAV were negative. However, in each of these two negative populations, only 30 salmon smolts were checked for the presence of ISAV, which means that a prevalence of ISAV lower than 10% within these populations may not have been detected. It is also common that marine production sites for Atlantic salmon contain smolt from as many as four different smolt production sites. Hence, if the smolt production sites discussed in this paper are representative for the situation in Norway, then one can assume that nearly all marine production sites contain salmon that are positive for ISAV. In some marine sites one should also expect to find more than one type of ISAV isolate, depending on the origin of the salmon. In the present study, two different ISAV isolates were found within three smolt populations (smolt production site I, II, and III) and at one marine site (NT92/04). The ISAV in the smolt populations were not associated with outbreaks of ISA. This means that studies on spreading of ISAV require detailed information about the origin of all salmon at the marine sites, and the fact that more than one ISAV isolate can be present in one smolt population has to be taken into consideration, in addition to information about ISAV in neighbouring farms.

Little information is available for 32 of the 83 ISAV isolates of the Norwegian groups and subgroups in EU-ISAV. However, all of the information about ISA in neighbouring farms and the origin of broodfish, eggs, and smolt is available from the 51 (61.4%) Norwegian farms included in this study (Table 1). The information obtained for isolates in EU-G1, EU-H2 and EU-MN points towards a common pattern for the spread of these ISAV isolates. The factor that best explains the origin of these isolates is the source of the eggs, i.e. the broodfish companies. Within EU-G1, EU-H2, and EU-MN, it is possible to find closely related ISAV at geographically distant marine sites (Figs. 5–8). The distance, in itself, excludes the possibility of horizontal transmission in the sea phase caused by passive transmission in sea water or through infected wild or escaped salmonids. Some of these sites (e.g. T79/03, FM86/04, and N89/04) had received smolt from the same smolt production site, while other similar ISAV isolates were found in farms that had received smolt from different producers, cf. EU-G1, EU-H2, and EU-MN (Table 1, Figs. 4, 7 and 8). The smolt can be a possible primary source of some of the ISAV isolates, but if all isolates within the respective groups/subgroups are considered, another source, the broodfish companies, emerges as a more likely primary source of the different isolates. Vertical transmission of ISAV, as an explanation for the observed pattern of distribution of ISAV isolates, is also supported by a recently published Norwegian study that documents transmission of ISAV from broodfish via embryos to newly hatched fry [22]. In EU-G1, all ISAV isolates have a possible connection to broodfish company BF-A. In five of

the farms in this group there are also salmon grown from eggs produced by BF-B and in one of these there are two ISAV isolates (NT92/04) present. The isolate NT92B/04 belongs to group EU-G1, which is dominated by salmon grown from eggs produced by BF-A, while the other isolate from the same farm, NT92A/04, belongs to subgroup EU-H2, which is dominated by salmon grown from eggs produced by BF-B.

In subgroup EU-H2 we have been able to obtain information from 15 different farms, which include 12 marine sites, two smolt production sites (III and IV), and one location belonging to broodfish company BF-B. This broodfish company keeps the fish at four different localities, and three of these delivered the eggs from which the salmon in EU-H2 were hatched. Broodfish company BF-A also keeps the broodfish at different localities. This company is associated with two very different ISAV isolates found in EU-G1 and EU-MN. Isolates in these two groups are mainly responsible for outbreaks of ISA in central Norway, which is an area dominated by salmon production based on eggs from BF-A. However, these isolates can be found as far north as Finnmark and as far south as Hordaland, but always associated with eggs delivered from BF-A when such information has been made available. At a smolt production site (I) used by BF-A, two different ISAVs, MR102/05 and MR104/05, belonging to EU-G1 and EU-MN, were present. Both isolates had an HPR0 and were not associated with ISA. All smolts at this smolt production site were hatched from eggs from BF-A, and there was no addition of sea water before the samples were taken.

The correlation between the phylogenetic position of the ISAV isolates within the group/subgroups EU-G1, EU-H2, and EU-MN and the broodfish companies delivering the eggs from which the salmon were hatched, indicates possible "vertical" or transgenerational transmission of ISAV. Another strong support for the hypothesis of vertical transmission of ISAV or spread of ISAV via eggs is found at two different smolt production sites, III and IV. The sites had received eggs from the same broodfish company, BF-B, located in Hordaland. The smolt sites are located about 1000 km apart in Troms, northern Norway (isolate T85B/04) and Sogn og Fjordane, western Norway (isolate SF113/04). The smolt/parr, still kept in fresh water, were harbouring the same ISAV isolate. The smolt at site III (Troms) contained an additional ISAV, T85A/04, which belongs to a subgroup of ISAV containing isolates mainly from Hordaland, EU-H1. These data support a hypothesis of circulation of ISAV in the production cycle of salmon in Norway. In such a hypothesis, ISAV-infected broodfish, carriers of ISAV, seem to play a key role as a source of infection in the next generation.

Rate of evolution

Information about the rate of nucleotide substitutions in the genome of the ISAV is central for the understanding of the evolution of the ISAV in farmed populations of Atlantic salmon (*Salmo salar*). Such information will be vital for predicting the response to treatment with vaccines, but will also be important for the management of the disease, i.e. at what rate can one expect changes in the

different genotypes? Genotyping can be a powerful tool in studies of epizootics of ISAV isolates and can give valuable information about the source/origin of ISAV that causes disease in farmed populations of salmon [cf. 9, 12, 19, 36]. However, this is dependent on detailed knowledge about nucleotide substitution rates. It is assumed that because of their error-prone replication (due to the RNA-dependent RNA polymerase), RNA viruses will mutate quickly and hence evolve quickly, which may result in a molecular clock. However, the rate of nucleotide substitution is a function of both the rate of mutation and the rate of replication, and if viral RNA is not evolving neutrally, due to undetermined effects of codon usage and RNA secondary structures, fluctuations in adaptive environments and population size will also affect substitution rates and their constancy over time [cf. 14]. Molecular evolution will only follow a molecular clock if mutation and replication rates are constant and most substitutions are neutral. Hence, it should not be expected that the molecular evolution of the HE gene follows a molecular clock.

It was not possible to calculate the rate of molecular evolution of the ISAV in all European groups based on the N-term region (about 1000 nt) of the hemagglutinin esterase gene (HE) (cf. Table 3), but we were able to obtain substitution rates for the whole EU-ISAV subtype and the groups EU-G2 and EU-G3. However, the requirement for a molecular clock was not satisfied, hence it is not possible to use the substitution rate to calculate time of divergence for the different groups of ISAV. The resulting rates varied from 0.7×10^{-5} ($\pm 0.1 \times 10^{-5}$) to 2.15×10^{-4} ($\pm 0.68 \times 10^{-4}$) nucleotides site^{-1} yr^{-1}, which is significantly lower than that of the HA from influenza B (16.0×10^{-4}), but the rate for group EU-G3 is close to that observed for the HE from human influenza C (2.4×10^{-4}) [14]. The relatively low rate of nucleotide substitutions found is also supported by the presence of identical HE sequences from isolates separated in time by many years. The isolates H2/89, T37/98, and NT38/98 have identical HE gene sequences but are separated in time by 9 years and were collected in very different geographical areas.

Despite of the lack of a molecular clock, there is a relationship between genetic divergence and time, and it has been shown that even if the molecular clock is rejected, the maximum likelihood estimates of substitution rates can be valid when sufficiently large data sets are analyzed [14]. However, the presence of identical ISAV isolates, separated in time by 9 years (H2/89 and T37/98), clearly shows that the substitution rate calculated, based on isolates collected during the last 19 years, 1987–2005, must be used with great care. Hence, it can be concluded that either mutation rates, replication rates, or undefined selective constraints vary to some extent, which might be expected since ISAV infects more than one host species. An important constraint on the evolution of ISAV must be the loss of variation in ISAV populations due to slaughtering of infected fish. Only a few of the total of all salmon produced in Norway are used as broodfish. The rest are slaughtered, and the ISAV infecting these are lost unless there is a significant horizontal transmission of the ISAV in the sea phase, between farms, and from farms to wild salmonids. However, as the present study shows, most of the spreading of ISAV isolates is

best interpreted as a result of transmission from freshwater, via smolt transport, to the marine farms, i.e. transmission through broodfish, eggs, fry, and smolt. The possible vertical or transgenerational transmission of ISAV isolates constitutes a narrow bottleneck for transmission of genetic variation accumulated in ISAV populations in the marine environment.

The relatively low substitution rate of the ISAV HE gene should also be viewed with respect to general information about the substitution rates of RNA viruses. Comparing the synonymous substitution rates of RNA viruses with replication frequencies and replication error rates, Hanada et al. [10] found that the main source of the rate variation was differences in the replication frequency, because the rates of replication error were roughly constant for different RNA viruses. In a farm situation, ISAV is given the opportunity for a high replication frequency, hence one should expect a high substitution rate. Since a high substitution rate was not observed, this could again be explained as a loss of genetic variation due to stamping out of infected populations of salmon and removal of all salmon when a commercial size is reached. Such a scenario suggests that horizontal transmission of ISAV between marine production sites, at least under Norwegian farming conditions, must be very limited.

According to Hanada et al. [10], viral life strategies will also affect substitution rates because infection and transmission modes will strongly affect the replication frequency. A virus that is vertically or sexually transmitted will have a lower replication rate, and hence, a lower substitution rate, compared to viruses that are solely dependent on horizontal transmission in large populations. This leads to an important constraint on the evolution of ISAV in natural populations of salmonids, i.e. the biology of the hosts. The salmonid hosts live in small, dense, and fragmented populations in freshwater and at very low densities in the marine environment (cf. Nylund et al., [24]). Since the ISAV is only known to replicate in salmonids, this must have limited the rate of replication (virulence) of ISAV within the individual hosts, i.e. the ISAV must be adapted to co-exist with the host in natural populations without reducing the survival of the host. This may explain the presence of the avirluent ISAV found in farmed populations of salmonids in both fresh and salt water [24, 29]. These viruses probably represent the "wild-type" ISAV, which recombines, resulting in the known virulent isolates connected to clinical outbreaks of ISA in farmed populations of salmon. This hypothesis is strongly supported by the presence of several different HPR groups within each EU group and subgroup (Tables 1 and 4). In the subgroups EU-H2 (22 isolates) and EU-MN (14 isolates), 11 and 8 different HPR groups are present, respectively. Some of the closely related isolates may have an identical HE sequence, differing only in the HPR.

Conclusions

The geographical pattern of related ISAV isolates presented in this paper shows that the number of isolates in circulation during the last five years is limited, and that these are transported over longs distances in connection with egg and

smolt transports. The substitution rate of the ISAV HE gene is low compared to most other RNA viruses [cf. 14]. Despite the large potential for replication and evolution, represented by the large number of hosts available in salmon farms, strong constraints exist on the nucleotide substitution rate and the resulting rate of evolution of ISAV. This means that the genotypes of ISAV present in farmed salmonids are slow-evolving and well suited for studies on epidemics. The nucleotide variation observed between different subgroups of ISAV is changing very slowly, and the presence of many European subgroups is best interpreted as a result of many years of evolution of ISAV connected to fragmented natural populations of salmonid hosts. ISAV has probably gone through little evolutionary change since the introduction in farmed populations of salmonids. Since only a few of the ISAV subgroups are connected to outbreaks of ISA in Norway during the last five years, it is tempting to speculate that the reduction in the number of broodstock locations/broodfish companies has resulted in a loss of some of the subgroups from farmed salmonids in Norway. Future studies of ISAV epidemics should include studies of possible vertical transmission and spreading of isolates via the production cycle i.e. via broodfish, eggs/embryos, and smolt transportation.

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III

Evolution of Infectious Salmon Anaemia virus (ISA virus).

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Abstract

Infectious salmon anaemia virus, ISA virus (*Isavirus, Orthomyxoviridae*), emerged in Norwegian salmon culture in the mid-80s. The genome consists of eight segments coding for at least 10 proteins. ISA viruses show a lot of similarities with influenza A viruses, but differ in many important aspects like number of hosts, host population structure, and transmission. The only known hosts and reservoirs for ISA viruses are salmonids found in countries surrounding the North Atlantic. In this study four different segments in the genome of about 100 ISA viruses have been sequenced in an attempt to understand the evolution of ISA viruses and how these viruses are maintained in and transmitted between populations of farmed Atlantic salmon. The four gene segments are coding for the nucleoprotein (NP), the putative acid polymerase (PA), the fusion protein (F) and the haemagglutinin-esterase (HE).

Analyses of these four genes show that the substitution rates of the internal proteins (NP and PA) are lower compared to the two surface proteins (F and HE). All four segments are evolving at a lower rate compared to similar genes in influenza A viruses. The ISA virus populations consist of avirulent viruses and pathogenic strains with variable virulence in Atlantic salmon. Recombination resulting in inserts close to the proteolytic-cleavage site of the precursor F0 protein and deletions in the stalk region of the HE protein seem to be responsible for the transition from avirulent ISA viruses into pathogenic strains. It is also shown that reassortment is a frequent event among the dominating ISA viruses in farmed Atlantic salmon.

The pattern that is obtained after phylogenetic analyses of the four gene segments from ISA viruses, suggests that the variation is limited to a few distinct clades and that no major changes have occurred in the ISA virus population in Norway since the first viruses were isolated. Calculation of the time of most recent common ancestor (TMRCA) suggests that the Norwegian ISA viruses separated from the European subtype found in North America between 1932 and 1959. The TMRCA data also suggest that the ISA viruses in Chile were transmitted from Norway in the period from 1995 to 2007, depending on which of the four genes are used in the analysis.

Key words: ISA virus, Salmo salar, evolution, phylogeny

Introduction

The rapid evolution of viruses together with recent advances in nucleic acid sequencing and bio-computing, makes it possible to distinguish between related strains, study the diversity, and deduce the relationships between viruses from different regions and different outbreaks (Devold et al 2006; Nylund et al 2007, 2008; Kibenge et al 2008). Molecular studies are particularly valuable for understanding the dissemination and evolution of viruses. The knowledge obtained can be used in epizootical reconstructions, in real-time surveillance, and may even enable possible future predictions about development of viral diseases (cf. Hungnes et al 2000). Variation between isolates results from accumulation of molecular changes that can occur by a number of different mechanisms including point mutations, gene reassortment, deletions, and recombination (Webster et al 1992). Hence, it is of vital importance to understand how the different segments of the Infectious salmon anaemia virus (ISAV), used in epizootic reconstructions and surveillance, evolve, i.e. the substitution rates for the segments and importance of reassortments and recombination events.

Use of molecular tools is now becoming more important in the management of viral diseases, and the first studies of epizootics of viruses connected to fish farming have been published (Kurath et al 2003; Einer-Jensen et al 2004, 2005a,b; Snow et al 2004; Thiery et al 2004; Hodneland et al 2005; Karlsen et al 2006; Devold et al 2006; Nylund et al 2003, 2007, 2008; Godoy et al 2008; Markussen et al 2008; Vike et al 2009; Kibenge et al 2009). ISA virus is one of the fish viruses that have been studied by a molecular approach to elucidate transmission routes and diversity (cf. Nylund et al 2007). The ISAV has a wide distribution - Norway, Canada, Scotland, Ireland, Faroe Islands, Chile, USA (Mullins et al 1998; Rodger et al 1998; Kibenge et al 2001a, 2009; Bouchard et al 2001; Godoy et al 2008; Vike et al 2009), and is able to propagate in several different host species, *Salmo salar, S. trutta, Salvelinus alpinus, Oncorhynchus mykiss, Oncorhynchus* spp, *Clupea harengus* (Nylund et al 1995a,b,c, 1997, 2002, pers. obs.; Devold et al 2000; Kibenge et al 2001a; Snow et al 2001a,b; Rolland & Winton 2003), where *S. salar* and *S. trutta* seem to be the major hosts in the North Atlantic. However, little is known about the host specificity of different isolates.

The genome of ISA viruses contains eight segments of negative stranded RNA. In the present study four segments, the nucleoprotein (NP) gene - segment 3, the tentative acidic polymerase (PA) gene – segment 4, the fusion protein gene (F) – segment 5, and the

haemagglutinin-esterase gene (HE) – segment 6) from a selection of ISA viruses have been sequenced and compared. Studies of virus genes from influenza viruses have shown that each gene may evolve differently because of different selective pressures and evolutionary constraints (Gorman et al 1992; Webster et al 1992). Genes that code for surface proteins, like the HE and F protein genes, may be subjected to strong selection pressures by neutralising antibodies of the host immune systems, while genes coding for internal proteins, like the NP and PA, are not expected to be subjected to strong host immune selection pressures but are thought to undergo significant host-specific adaptive evolution (Gammelin et al 1990; Gorman et al 1992; Xu et al 2011). Because of the selection pressure from the host immune system, surface proteins are expected to evolve more rapidly and to be replaced by reassortment more frequently. Internal protein genes that show a high degree of host-specific evolution are not expected to reassort frequently (Webster et al 1992). The reason for this being that loss of host-adapted genes through reassortment may decrease the fitness of progeny virions in the respective hosts. It has been shown that NP genes of influenza viruses fall into host-specific groups (Bean 1984; Gorman et al 1990, 1991; Xu et al 2011).

The natural hosts for ISA viruses seem to be Atlantic salmon (*S. salar*) and trout (*S. trutta*) in the North Atlantic (Raynard et al 2001; Plarre et al 2005). These two species live in small fragmented, genetically distinct, populations where spawning only occurs in their home river system. If these are the only host species one should expect to find variation in the two surface proteins reflecting the river of origin, while the variation in the internal proteins (NP and PA) should, to a larger extent reflect the host species, i.e. salmon or trout. All sequences from ISA viruses are unfortunately from farmed Atlantic salmon and, hence, one should expect to find little variation in the internal proteins unless there has been transmission from marine sources (not Atlantic salmon) to farmed salmon. The present study, focusing on two internal and two surface proteins for ISA viruses from farmed Atlantic salmon, that give new knowledge about the evolution of ISA viruses, the reservoirs and transmission routes.

Materials and methods

This study includes ISA viruses collected in the period from 1987 until 2011, and consists of ISA viruses from Norway (N = 86), Scotland (N = 2), Faroe Islands (N = 1), North America (N = 2) and Chile (N = 5) (Table 1). The viruses have been obtained from salmon in both fresh and sea water. Atlantic salmon does not occur naturally in Chile and the ISA viruses from this country were all obtained from individuals that originally came as embryos from Europe or North America (east coast). ISA viruses can be separated into two major genotypes, a North American and a European genotype, where a subtype of the European genotype is also present in Eastern North America (Devold et al 2006; Nylund et al 2007). This study includes only ISA viruses belonging to the European genotype.

Sequences of four of the eight ISA virus genome segments (three, four, five and six) from these viruses have been obtained both from cultures and directly from tissues of infected salmonids. Information about and sequences from some of these ISA viruses have already been presented in other publications (Devold et al 2006;Nylund et al 2007; Markussen et al 2008; Cottet et al 2010; Christiansen et al 2011; Lyngstad et al 2011). In addition, some sequences of segment six from avirulent ISA viruses have been collected from the GenBank and are included in the phylogenetic analysis of this segment (Table 2).

RNA extraction and sequencing

The extraction of RNA, RT PCR and sequencing were performed as described by Devold et al (2000). All PCR products of ISA viruses obtained from heart or gill tissues or from cell cultured isolates, were sequenced in both directions. The primers used for PCR and sequencing of segments three and four are presented in table 3. Primers for sequencing of segments five and six have been published earlier (Devold et al 2001, 2006).

Phylogeny

The sequence data were assembled with the help of Vector NTI software (InforMax, Inc.) and the GenBank searches were done with BLAST (2.0). The Vector NTI Suite software package (InforMax, Inc.) was used for the multiple alignments of nucleotide and deduced amino acid sequences. To perform pairwise comparisons of the different sequences from the four ISA virus segments, the multiple sequence alignment editor GeneDoc (Available at: www.psc.edu/biomed/genedoc) was used. Sequences already available on the EMBL nucleotide database were also included in the comparisons (cf. Tables 1 & 2).

The phylogenetic trees obtained by analyses of segment three, NP, (positions 12 - 1851 in the ORF) were compared to phylogenetic trees obtained by analyses of segment four, PA, (positions 65 - 1722 in the ORF), segment five, F (positions 37 - 1335 in the ORF, Accession no: AY853958), and segment six, HE, (positions 64 – 1005 in the ORF, Accession no: AF302799) from the same viruses. These trees were constructed using TREE-PUZZLE 5.2 (Available at: http://www.tree-puzzle.de), maximum likelihood (ML). The evolutionary model and substitution rates for the ML analyses were calculated for all datasets using jModelTest (Posada 2008) with the Akaike Information Criterion option. All four segments were analysed using a GTR nucleotide evolution model with four category gamma rate. The maximum likelihood trees were bootstrapped (50 000 puzzling steps) in TREE_PUZZLE. The ISA virus isolate NS2003, from Nova Scotia, Canada, has been used as an out-group in the phylogenetic analysis of all four segments. Phylogenetic trees were drawn using TreeView (Page, 1996).

Substitution rates and time of most recent common ancestor (TMRCA)

The substitution rates and the time of most recent common ancestor (TMRCA) of each segment were estimated using a Bayesian Markov Chain Monte Carlo method as implemented in BEAST version 1.6.2 package (Drummond & Rambaut 2007). In all cases the HKY+G+I nucleotide substitution model was used with the relaxed clock assumption (Posada & Crandal 1998). Each run was analysed by Tracer to ensure that sufficient ESS values (>200) and

convergence was reached. The maximum clade credibility tree was computed using the TreeAnnotator program, with the first 10% of trees removed as burn-in.

Results

The ISA virus isolate, NS2003, from Nova Scotia, Canada, has been used as an out-group in the phylogenetic analysis of all four segments.

Segment 3, nucleoprotein (NP) gene

The phylogenetic analysis of the NP gene included 1840 nt in the ORF showing variation in 234 nt positions (12.7 %) resulting in 72 amino acid substitutions (11.8 %). The resolution between the sequences was relatively poor with only five clades (3A, 3B, 3D, 3G & 3H) showing strong support values (>85). Three clades (3C, 3E & 3F) had support values above 69 (Figure 1). Clade 3A contains two HPR0 ISA viruses (H172/10, SK779) and 10 pathogenic isolates collected in the period from 1993 – 2010 at different salmon farming sites in four different counties in Norway. H172/10 was collected from smolt at a fresh water site in Hordaland county, western Norway, while the other HPR0, SK779, is from salmon at a marine site. Members of this clade have identical nucleotides at 98 % of the positions in the sequence and share 98.9 % of the amino acids. Pathogenic and HPR0 ISA viruses from Chile and Norway group together in clade 3B. This clade consists of 16 sequences (collected in 1997 – 2011) where four (CH29/08, CH30/08, MR102/05, MR104/05) are from HPR0 ISA viruses. These four viruses are from salmon smolt at two fresh water sites in Chile and Norway. The fresh water site in Norway belongs to a Norwegian brood fish company in western Norway. In these viruses 99 % of the nucleotides and amino acids are identical. The sequence of the NP from the HPR0 CH29/08 is identical to that from the pathogenic isolate ISAV752 also from Chile. Clade 3C, containing 16 ISA viruses, is poorly supported (SV = 69) and consists of viruses from the two northernmost counties in Norway only. All these isolates were collected in the period from 2005 – 2010 and the majority were obtained in a short period with several outbreaks of ISA in a small area in Troms county. Members of this clade differ in 3.5 % and 5.1 % of the nucleotide and amino acid positions, respectively. However, if the isolate T154/09 is excluded from the group the differences drop to 0.8 % for both the nucleotide sequences and the putative amino acid sequences. Three HPR0 ISA viruses collected from salmon in sea water are present in clade 3D where one is from the east coast of USA while the other two are from different geographical areas in Norway. This clade also includes 12 pathogenic ISA viruses, collected in the period 1989 – 2011, from different

sites along the Norwegian coast. Members of this clade have identical nucleotides at 98.7 % of the positions in the sequence and share 99.2 % of the amino acids. The NP sequence from the isolates, H02/89, NT38/99, T37/98 and ISAV4, collected in the period from 1989 – 1999, are identical. Two of the 10 ISA viruses in clade 3E are HPR0 viruses, one from smolt in fresh water (H138/08) and one from mature brood fish (H97/04) kept in fresh water. The viruses in this clade have been collected in the period from 2000 – 2008. Members of this clade differ in 1.8 % and 2.1 % of the nucleotide and amino acid positions, respectively. Clades 3F, 3G and 3H consist each of a few ISA viruses only. The three viruses in clade 3F are all from western Norway and were collected in the period 1996 – 2001, while clade 3G consists of two viruses from Scotland (Scot43/98, Scot157/08) and clade 3H of one virus from Faroe Islands (F72/02) and one from Northern Norway (T162/09). Scot157/08 is an HPR0 ISA virus from mature broodfish kept in fresh water, while the other HPR0 virus, T162/09, was obtained from smolt in fresh water in Northern Norway. The two isolates SF18/96 and MR46/99, in clade 3F, are identical while MR60/01 differs at three nucleotide positions where one substitution results in an amino acid substitution. The two Scottish ISA viruses in clade 3G differ in 8 nt positions (0.4 %) resulting in one amino acid substitution. The NP gene sequence of the isolate from Faroe Islands (F72/02) differs from the other member in clade 3H, a virus (T162/09) from fresh water in northern Norway, in 7 (0.4 %) positions resulting in two amino acid substitutions.

Segment 4, putative acid polymerase (PA) gene

The phylogenetic analysis of the putative PA gene included 1660 nucleotides in the ORF showing variation in 195 nucleotide positions (11.7 %) resulting in 51 amino acid substitutions (9.3 %). The PA gene sequences constitute two well supported major groups (SV = 98 for both groups) with a few supported clades within each group (Figure 2). The well supported (SV > 84) clades are 4A, 4B, 4F, 4G, 4H and 4I, while the support for the clades 4C, 4E, and 4D, are between 72 and 81. ISA viruses from both Norway and Chile are present in clade 4B, and, in addition to five pathogenic isolates, the clade also contains two HPR0 ISA viruses (CH29/08, CH30/08) from smolt in freshwater in Chile. Members of this clade differ in 0.2 % and 0.4 % of the nucleotide and amino acid positions, respectively. Only two nucleotide substitutions separate the two pathogenic ISA virus isolates (FM168/10,

FM174a/11) collected in northern Norway from four identical sequences (CH05/08, CH29/08, CH30/08, ISAV752) collected from salmon in Chile. Clade 4C, containing 15 ISA viruses, consists of viruses from Troms county in northern Norway only. All these isolates were collected in the period from 2007 – 2010 and the majority were obtained from farmed salmon in a small area in southern Troms. Members of this clade differ in 0.7 % and 1.3 % of the nucleotide and amino acid positions, respectively. Six of the PA gene sequences are identical in this clade. Clade 4E consists of sequences from 11 ISA viruses collected at farming sites for Atlantic salmon along the Norwegian coast. Both of the HPR0 ISA viruses in this clade (H138/08, H172/10) were collected from smolt in fresh water in western Norway at two different smolt production sites. Members of this clade differ in 1.3 % (22 nucleotide positions) and 1.4 % of the amino acid positions, respectively. Clade 4H contains five sequences from ISA viruses, collected in Norway in 1996 - 1999, showing variation in 5 nt positions. These substitutions do not result in any amino acid changes in the putative protein. The other clades (4A, 4D, 4F, 4G, 4I) consist of a few sequences only. Clade 4D consist of five sequences from Norwegian ISA viruses collected in the period 2005 - 2009, showing variation at five nucleotide sites resulting in three amino acid substitutions. The four ISA viruses in clade 4F were collected in 1996 – 2004 from different regions along the Norwegian coast. These viruses show variation in six nucleotide positions, but the putative amino acid sequences are identical for all four isolates. The three sequences in clade 4A come from ISA viruses collected in the southern part of Troms County, and the two isolates T121a/07 and T121b/07 were collected in the same farm. These two sequences differ in three nucleotide positions, while T121b/07 differs from T126/07 in one nucleotide position only. All four substitutions result in amino acid changes. The two ISA viruses in clade 4G were both collected in Scotland. Scot43/98 was collected from salmon during an outbreak of ISA in sea water in 1998, while the HPR0 ISA virus, Scot157/08, was collected 10 years later from mature broodfish in fresh water. These two viruses differ in five nucleotide positions resulting in one amino acid substitution. Clade 4I consists of two ISA virus isolates from Troms county (2002 – 2003) that differ in three nucleotide positions resulting in one amino acid substitution.

Segment 5, fusion protein (F) gene

The phylogenetic analysis of the F protein gene included 1299 nt in the ORF showing variation in 199 nt positions (15.1 %) resulting in 66 amino acid substitutions (15.1 %). The F protein gene sequences group into two major groups with several clades in each group (Figure 3). Clade 5B consists of 18 sequences, collected in the period from 1997 – 2011, including four (MR102/05, MR104/05, CH29/08, CH30/08) HPR0 ISA viruses sampled in fresh water in Norway and Chile. There is variation in 40 nucleotide positions (3.1 %) resulting in 11 amino acid substitutions (2.5 %) within the clade. Only ISA viruses from Troms county, collected in the period 2007 – 2010, are present in clade 5C. These 14 virus sequences show variation in 23 nucleotide positions (1.8 %) resulting in 13 amino acid substitutions (3.0 %). However, if the isolate T154/09 is removed from the alignment the number of variable nucleotide and amino acid positions drops to three and two, respectively. The seven ISA viruses in clade 5H were collected in the period 1996 – 2004 from salmon in four different counties. Among the members of this clade there is variation in 13 (1.0 %) nucleotide positions resulting in changes in four amino acid residues (0.9 %). All amino acid substitutions are found in the H93/04 ISA virus. The remaining supported clades contain only a few ISA virus sequences each and the support values range from 70 – 98. Clade 5M consists of three sequences from ISA viruses collected in Northern Norway and two of these are HPR0 viruses. One is from smolt in fresh water in Troms county while the other (FM173/11) is from salmon in sea water in Finnmark county. There are no known connections between these two populations.

The sequences of segment five from 14 different HPR0 ISA viruses (avirulent viruses) are included in this study and they all share the same amino acid in position 266 (glutamine, Q_{266}) in the F protein. There is no insert in segment five of any of these viruses. A total of nine virulent ISA viruses with an insert in segment five are included in this study and all, but two (MR61/01, SF71/02) share a glutamine in position 266. The other two have a histidine (H) in this position. All other virulent ISA viruses from Europe and most ISA viruses from North America share a leucine (L_{266}) at position 266 of the fusion protein. The only know exception is an ISA virus (RPC/NB 04-085-1) from Canada, considered to be of low virulence (Kibenge

et al 2007). This isolate differs from all other known ISA viruses in having a proline (P) in position 266 in segment five.

Segment 6, haemagglutinin-esterase (HE) gene

The phylogenetic analysis of segment six from ISA viruses, including extra sequences from avirulent (HPR0) viruses, is presented in figure 4. This shows that HPR0 ISA viruses from healthy salmon are present in the same clades as the virulent ISA viruses from Atlantic salmon suffering from ISA. HPR0 ISA viruses are present in both fresh and sea water, and they have also been obtained from salmon kept at brood fish sites in fresh water (MR102/05, MR104/05, H97/04, Scot157/08). The two fresh water HPR0 ISA viruses from Chile (CH29/08, CH30/08) were obtained from salmon smolt kept in fresh water. A large number of HPR0 ISA viruses are from salmon in sea water (SF83/04, AR2/08, AR8/07, AR26/08-1, FO/01/06, FO/01b/07, FO/03/07, FO/03b/07, NWM10, USA2004), while the Norwegian HPR0 viruses, H138/08, SK779, H172/10, and T162/09, are all from salmon at smolt production sites in fresh water. The HPR0 ISA virus, AR7/08, is from a smolt production site in Troms county using addition of sea water.

The phylogenetic analysis of the HE protein gene included 942 nucleotides in the ORF showing variation in 159 nucleotide positions (16.9 %) resulting in 51 amino acid substitutions (16.2 %). Several supported (SV > 70) clades were obtained after the analysis of this gene, but the position of some of the sequences was unresolved (Figure 4). Clade 6A (SV = 94) consists of eight ISA viruses collected in northern Norway in the period from 1993 – 2007. There is variation in 16 nucleotide positions (1.7 %) resulting in 8 amino acid substitutions (3.1 %) within the clade. Five different HPRs are present within this clade where the isolates closest in time share HPR (Table 1). The seven ISA viruses in clade 6B have been collected in Norway and Chile in the period from 2008 – 2011. The HE gene sequences from two isolates, one from Norway (FM168/10) and one from Chile (CH05/08), are identical. The number of variable nucleotide positions within the clade was 10 (1.1 %) resulting in seven amino acid substitutions (2.2 %). The HPR0 ISA viruses (CH29/08, CH30/08) were collected from smolt in fresh water in Chile, while the other ISA viruses in this clade are from Atlantic

salmon in sea water and all with different HPRs. Both HPR0 and pathogenic ISA viruses are found in clade 6C which contains sequences from 33 viruses collected in Norway in the period from 1996 – 2010. The number of variable nucleotide positions within the clade is 26 (2.8 %) resulting in 12 amino acid substitutions (3.8 %). Several of these sequences (T140/08, T144/08, T145/08, T147/08, T149/08, T152/09, T154/09, T158/09, T160/09, T167/10) are identical including one HPR0 virus (AR7/08) from salmon in a marine farm. They have all been collected in a small area in Troms county. In addition, clade 6C also contains three more HPR0 ISA viruses where one (MR102/05) is from smolt kept in fresh water at a brood fish location. The HPRs reflect to a large extent the time of collection (table 1). Clade 6J, which contains sequences from six ISA viruses collected in middle Norway in the period 2008 – 2009, constitutes a well supported clade within 6C. This clade shows variation in four (0.4 %) nucleotide positions leading to three (1.0 %) amino acid substitutions. One of the viruses in this clade is an HPR0 virus (AR26/08-1). The members of clade 6F were collected from different sites along the Norwegian coast in the period 1989 – 2004. Within the clade there is variation in 18 (1.9 %) nucleotide positions resulting in 6 (1.9 %) amino acid substitutions. The segment six sequences used in the phylogenetic analysis of the ISA viruses, FM86/04 - N89/04 and MR46/99 – MR60/01, are identical, but the HPR of the two pairs are different. The remaining supported clades contain a few isolates only. The clade 6G contains one HPR0 ISA virus from a brood fish site in Scotland and two pathogenic isolates from northern Norway, while the two viruses in clade 6H, one avirulent and one pathogenic, both come from Faroe Islands. The latter two were collected five years apart and differ in four nucleotide positions, while two (H02/89, NT38/99) of the four identical members in clade 6D were collected 10 years apart and share the same HPR.

Reassortment in the ISA virus

This study includes four of the eight segments that constitute the ISA virus genome, i.e. genes for two surface proteins (F and HE) and two internal proteins (NP and PA) that constitute a part of the ribonucleoprotein (RPN) complex. Comparing the four phylogenies based on the four segments shows that reassortment is a frequent event in the evolution of the ISA virus genome from farmed Atlantic salmon. However, clade C, as defined by 3C, consists of ISA viruses collected during a short period (2005 – 2010) in a small geographical area and in this

clade only two viruses (FM106/05, FM107/05) show reassortment in segments five and six. There is considerable reassortment occurring among the viruses that are defined by clade 5B, but a core of viruses, consisting of a mixture of Chilean and Norwegian viruses (CH05/08, CH29/08, CH30/08, ISAV752, ISAV901, FM168/10, FM174a/11) collected in the period 2008 – 2011, show the same affinities in all four phylogenies. Another group of ISA viruses (H02/89, H06/91, ISAV4, T37/98, NT38/99) collected during a 10 year period (1989 – 1999) constitutes also a core of viruses with no reassortment being detected in the analyses of the four segments.

Time of most recent common ancestor (TMRCA)

The estimated time of most recent common ancestor (TMRCA) for each of the four lineages obtained from analysis of the four ISA virus segments are presented in Table 4. The consensus lower and upper 95% highest probability density (HPD) is 1959 and 1932, respectively. Analysis of the genes for the two internal proteins, NP and PA, demonstrates an earlier origin with a mean TMRCA estimated to be 1918 and 1898, respectively, compared to the mean TMRCA estimated for the F (1960) and HE (1934) genes.

The estimated time of separation between the Chilean and Norwegian ISA viruses depends on which segment is used in the analysis (Table 4b). Based on the NP and the PA genes the consensus lower and upper 95% highest probability density (HPD) is 2007 and 2001, respectively. The upper and lower 95 % HPD for the F protein gene is 1996 and 2002, respectively, while it is 1995 and 1999 for the HE gene (figure 5).

Several ISA viruses collected from Atlantic salmon during outbreaks in southern Troms in the period 2007 – 2010 group together in clade C in the analyses of all four segments (figures 1-4). The consensus lower and upper 95% HPD for this group of viruses is 2005 and 2001, respectively (Table 4b). Other ISA viruses collected in the same areas in the same time period separated from this group much earlier and are members of different clades.

The TMRCA for clade 3A and the nearest relatives was estimated to be found in 1964, i.e. about 20 years before the first official outbreak of ISA in Norway. Due to reassortment it was not possible to recognize the same clade in the analysis of the other three segments.

Two North American ISA viruses have been included in this study: one from USA collected in 2004 and another from Nova Scotia collected in 2003. Based on analysis of the NP gene these separated from the closest European relatives in about 1973 and 1997, respectively. The PA gene suggests the separation between the two North American ISA viruses and their closest relative, a virus from Faroe Islands (F72/02), to have occurred in 1992, while analyses of the HE gene suggest a separation between these two North American ISA viruses and the closest Norwegian ISA viruses to have occurred in 1943.

Substitution rates

The average substitution rates of the four phylogenetic lineages obtained by analysis of the four ISA virus protein genes were estimated as substitutions per site per year (Table 5). The two genes coding for proteins (NP and PA) playing a role in virus replication and host adaptation had a lower mean rate compared to the two surface proteins (F and HE).

Discussion

Phylogenetic analyses of the four segments from the about 100 ISA viruses included in this study show that the affinities between the different viruses is to a large extent dependent on the segment used in the analyses. Reassortment seems to be a very commonly occurring event in ISA viruses from farmed Atlantic salmon (*Salmo salar*). These results confirm the findings by Devold et al (2006) who looked at two segments from 54 ISA viruses. This means that the dominating ISA viruses in farmed salmon must occur relatively frequently in the same individuals. The mixing of the ISA viruses could occur in salmon of different origin, carrying different ISA viruses, at smolt and marine production sites or in broodfish populations. Alternatively, ISA viruses could be present in wild marine fish, however, at present there are no results supporting such an assumption and local marine reservoir can not explain the distribution patterns of ISA viruses in Norway or in farmed salmon in Europe and in Chile (Nylund et al 2007, Vike et al 2009).

Even if reassorting is a common event in the evolution of ISA viruses in farmed populations of salmon some viruses group together in well supported clades independent of which of the four segments that are used in the analyses. One example is a group of ISA viruses (clade C) from outbreaks of ISA in a small area in Troms County, Northern Norway. These viruses were collected in the period from 2007 – 2010. They all have the same HPR in the HE protein and, with the exception of T154/09, they all share nearly identical sequences of the four protein genes included in this study (see clade C). All salmon carrying these viruses in farms in Troms have received some salmon hatched from eggs delivered by one specific broodfish company. The virus could therefore have arrived in the populations as a result of transgenerational transmission of HPR0 ISA viruses (cf Nylund et al 2007; Vike et al 2009). The presence of avirulent HPR0 ISA viruses grouping together with the pathogenic strains (see figure 4) shows that the potential for a shift from avirulent to virulent ISA viruses was present in these populations. However, the most favoured explanation, by the Norwegian Food Safety Authority and the National Veterinary Institute is that the viruses have been spread in this small area as a result of horizontal transmission connected to farming activities in the areas and a high number of escaped ISA virus infected salmon (Lyngstad et al 2011). This explanation is supported by the fact that they all share the same HPR. However, the viruses in

clade C do not give the complete story of the outbreaks of ISA in Troms County in the mentioned time period. Other ISA viruses (N127/07, T121/07, T126/07, T131/07, T162/09), distinct from the viruses in clade C were present in other farms in the same area at the same time. These ISA viruses group in two different clades distinct from other viruses in this area. The outbreaks of ISA in this area therefore seem to be a result of at least three different introductions of ISA viruses. The phylogeny based on the HE gene also shows that the viruses (clade 6C) from the 2007 – 2010 ISA outbreaks in this region are closely related to ISA viruses from earlier outbreaks in other geographical regions in Norway where the oldest virus in the clade was collected in 1996. This clade also contains an HPR0 ISA virus (MR102/05) collected from a fresh water site belonging to the broodfish company from where salmon in the farms suffering ISA outbreaks in 2007 – 2010 originated. The emergence of the closely related ISA viruses (clade C) in different farms in Troms county (2007 – 2010) could have been a result of horizontal transmission between the farms playing an important role, but the origin of the viruses in clade C seems to be in western and middle Norway and very distant from the Troms region.

Another group of ISA viruses that deserves some attention can be found in clade B and consists of viruses from both Chile and Norway. These viruses (CH05/08, CH29/08, CH30/08, ISAV901, ISAV752, FM168710, FM174a/11) group together in the same clade in the phylogenies resulting from analyses of all four segments. In clade 3B and 5B a number of additional Norwegians ISA viruses group together with the Chilean viruses and the oldest of these were collected in 1997 in middle Norway. Clades 3B and 5B contain HPR0 ISA viruses from both Norway and Chile where the Norwegian viruses, MR102/05 and MR104/05, were collected at a fresh water site belonging to a Norwegian Broodfish company. This company has for many years been supplying salmon embryos to the Chilean salmon industry. It has been suggested that ISA viruses were introduced to Chile as a result of salmon embryo transport (Vike et al 2009). Our data suggest that ISA viruses have been introduced more than once from Norway to Chile (figure 4, table 4b). The introductions seem to have occurred in the period from 1995 – 2007 where the lower and upper 95 % HPD is dependent on which segment is used for calculation of TMRCA. The fact that a North American ISA virus was detected in Chile in 1999 (Kibenge et al 2001a), in addition to the more recent introductions of ISA viruses from Norway to Chile (Vike et al 2009, present study), show that movement of

ISA viruses through movement of salmon embryos is a relatively large risk factor for the industry. Recent literature also suggests that other viruses like the infectious pancreas necrotic virus (IPNV) and the piscine reovirus (PRV) could have been introduced to Chile via movement of salmon embryos from producers in the North Atlantic region (Mutoloki & Evensen 2011, Bustos et al 2011).

In addition to reassortment the evolution of ISA viruses is also influenced by mutations, and reassortment may also influence the substitution rates resulting from mutations (Wong et al 2011). As a null hypothesis each of the eight genome segments of ISA viruses may be considered as evolving independently, accumulating substitutions at their own rate which may be assumed to be roughly equal across all segments. However, looking at the substitution rates of the eight segments of influenza A viruses it is clear that differences in substitution rates for the different segments is to be expected (Chen & Homes 2006, Bahl et al 2009, Qu et al 2011, Wong et al 2011). The substitution rates for the genes of the two proteins that are part of the ISA virus replication machinery (NP and PA) are slightly lower compared to the substitution rates for the two surface proteins (F and HE). This difference in substitution rate, with the PA and NP as the most constrained proteins, is to be expected if one compares with similar studies of influenza A viruses (Bahl et al 2009). However, the substitution rates for the four segments from ISA viruses are lower compared to similar rates from influenza A viruses. A discussion of possible explanations for the relatively low substitution rates seen in ISA virus genes has been presented by Nylund et al (2007), who suggest that variation accumulating in ISA virus populations could be lost when the host is slaughtered, suggesting limited importance of horizontal transmission between salmon farms. It was also discussed that infection and transmission modes (viral life strategies) could strongly affect the replication frequency and hence, the substitution rates. If the dominating transmission route for ISA viruses is vertical or transgenerational transmission of avirulent HPR0 ISA viruses, that later mutate into pathogenic viruses, then this should result in an important constraint on the evolution of ISA viruses (Nylund et al 2007). The difference in substitution rates between influenza A viruses and ISA viruses could also be due to differences in mutation rates at the molecular level (cf. Nobusawa & Sato 2006). Hence, more knowledge about the factors that influence the substitution rate of ISA virus genomes is necessary before any conclusion can be drawn.

In 2005 it was shown that the fusion activity of the F protein from ISA viruses could be initiated by treatment with trypsin and low pH (Aspehaug et al 2005), and the two most likely trypsin cleavage sites were considered to occur after residue R^{267} and K^{276}. This view was also supported by Devold et al (2006) who looked at the fusion protein from 54 different ISA viruses. They also discovered that inserts, of varying length, occurred in front of and after the residue R^{267} in the fusion protein gene of some ISA viruses and that in all viruses with inserts the residue L^{266} was substituted by Q^{266} or H^{266}. Based on similar changes in influenza A viruses it was suggested that the insert could be important for the accessibility of these sites to trypsin and, hence, influence the virulence of the ISA virus (Devold et al 2006). This view was later supported by another study of the complete genome from 11 ISA viruses (Markussen et al 2008). The most favoured cleavage site was considered to be located after residue K^{276} (Aspehaug et al 2005) and one ISA virus used in this study lacks a possible cleavage site after position 267 supporting the view held by Aspehaug et al (2005). All virulent ISA viruses included in this study have the residue L^{266} with the exception of viruses with an insert in this region of the fusion protein which have a Q or H in this position. Only one ISA virus (RPC/NB04-085-1, Accession no: EF432567), suggested to be of low virulence and with the residue P^{266}, is known to deviate from this pattern (Kibenge et al 2007). All the avirulent or HPR0 ISA viruses included in this study have Q at position 266. The present data strengthen the hypothesis that the amino acid at position 266 in the F protein is an important virulence indicator for ISA viruses. A high number of sequences are now available from both segments five and six from HPR0 ISA viruses and without any exception they all have the residue Q^{266} in the F protein and a full length HPR (HPR0) in the HE protein. This suggests that it is not enough with deletions of a part of the HPR for the virus to become virulent, but that changes in the F protein in front of the cutting site (K276) are also necessary for a transition to a virulent type. This change could result from recombination in both segments leading to a shorter HPR in the HE protein and an insert in the F protein (Devold et al 2006). Another possibility is reassortment where one or both segments are replaced with homologous segments from virulent ISA viruses. A simple mutation could also change the residue at position 266 in the F protein. In the present study we did not find amino acid residues in the NP and PA proteins that separated the virulent ISA viruses from the HPR0 ISA viruses.

The segment six of the ISA virus, the haemagglutinin (HA) gene, was first identified and characterized by Krossøy et al (2001). They showed that the protein had haemagglutination activity, but unlike influenza virus HA they did not find posttranslational cleaving of the ISA virus HA. This study also revealed a highly polymorphic region (HPR) in the ectodomain close to the transmembrane region. Later the same year several other studies were published supporting the characterization given by Krossøy et al (2001) (Devold et al 2001; Griffiths et al 2001; Kibenge et al 2001b; Rimstad et al 2001). In 2004 it was shown that the protein coded by segment six also carried receptor-destroying activity, which was identified as an acetylesterase (Hellebø et al 2004), i.e. the protein was identified as a hemagglutinin-esterase (HE). The HE protein has been further characterized by Kristiansen et al (2002), Mikalsen et al (2005), and Müller et al (2010). The first phylogenetic analysis using the HE gene included ISA viruses from 37 different salmon farms in Norway, Scotland and Canada and revealed several different variants of the HPR (Devold et al 2001). The same year Kibenge et al (2001b) said that the shorter HPRs seen in European ISA viruses compared to isolates of Canadian origin suggested that the archetypal ISA virus was probably of Canadian origin, inferring a direction of evolution towards shorter HPRs. However, as shown by Devold et al (2001) there is a large variation in the length of HPRs from European ISA virus isolates and no indication of evolution towards shorter HPRs. Devold et al (2001) suggested that the variation could be a result of recombination, with the recombination site within the HPR, and it was recommended that only the 5`-end flanking region to the HPR should be used in phylogenetic studies (Devold et al 2001). The longest HPR observed was first obtained from ISA virus in a wild Atlantic salmon (Cunningham et al 2002), and it became obvious that all other HPRs could be derived from this HPR by recombination as suggested by Devold et al (2001). The long HPR was termed HPR0 by Nylund et al (2003) and they suggested that ISA viruses with HPR0 were low virulent wild-type viruses that could mutate into the virulent isolates with shorter HPRs found in farmed populations of Atlantic salmon. Hence, natural reservoirs or reservoirs connected to farmed brood fish, of low virulent ISA viruses, could be a constant source of new virulent ISA viruses. HPR0 ISA viruses have been found in all areas with farming of Atlantic salmon, Norway (Nylund et al 2007), Scotland (Cunningham et al 2002; McBeath et al 2009), USA (Nylund et al 2007), Canada (Cook-Versloot et al 2004), Faroe Islands (Christiansen et al 2011), and Chile (Kibenge et al 2009), and in the present study HPR0 ISA viruses have been found in nearly all clades resulting from the analysis of segment six, HE gene, (Figure 4). This supports the suggestion that changes in the HPR could

result in changes in virulence of ISA viruses (Nylund et al 2003), and that all HPR shorter than the HPR0 could be "offspring" of wild type, low virulent, HPR0 ISA viruses, i.e. shorter HPRs would represent a shift towards higher virulence (Nylund et al 2003). This would mean that the shorter HPRs are not a result of an evolutionary trend towards shorter HPRs (cf. Kibenge et al 2001b), but a continuous stochastic process where the low virulent, wild type, HPR0 ISA viruses may shift to high virulence. A similar mechanism has been described for changes in virulence of influenza A viruses (Hirst et al 2004). Deletion in segment six (HE gene), inserts or mutations in segment five (F protein gene) of ISA viruses, or inserts in the HA gene or the stalk region of the neuraminidase (N) protein of influenza A viruses do not change the phylogenetic position of the isolates but lead to change in virulence.

Conclusion

This paper shows that the substitution rates calculated for the four genes coding for two surface proteins (F and HE) and two internal proteins (NP and PA) of ISA viruses, are lower compared to similar rates obtained from studies of comparative gene sequences from influenza A viruses. However, reassortment seems to be frequently occurring among the ISA viruses from Norway, and recombination of the F and HE protein genes seems to be the cause for transition from low virulent (avirulent) ISA viruses to pathogenic strains with different virulence. Recombination in ISA viruses affects the stalk region of the HE protein and gives an insert close to the proteolytic-cleavage site for the precursor protein F0. It is suggested that one of the causes for the difference between influenza A viruses and ISA viruses is due to large differences in host population structure and host numbers. The natural reservoirs for ISA viruses (wild Atlantic salmon and trout) seem to be of little or no importance for the spreading and emergence of ISA viruses in farmed Atlantic salmon. The reservoir for the ISA viruses, that are associated with outbreaks of ISA, can be found in the farmed populations of Atlantic salmon as low or avirulent strains (HPR0 ISA viruses). Transmission of the avirulent strains of ISA viruses seems to occur through vertical (transgenerational) transmission followed by short and long distance movement of embryos and infected smolt from fresh water sites. Virulent strains emerge in farmed Atlantic salmon after mutations (substitutions and recombination) in the F and HE protein genes. These strains can be transmitted over long distances in connection with movements of smolt, horizontally between salmon within farms,

and possibly also between farms in connection with human activity (farming operations involving several farms in an area) and escaping of ISA virus infected salmon moving between farming sites.

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Figure legends

Figure 1. Phylogenetic tree showing the relationship between ISA virus isolates from Europe and North America based on 1840 nucleotides that code for the N protein. The best-fitting nucleotide substitution model was used during maximum likelihood analysis and the tree was bootstrapped (50 000 quartet puzzling steps) in TREE_PUZZLE. The scale bar shows the number of nucleotide substitutions as a proportion of branch lengths.

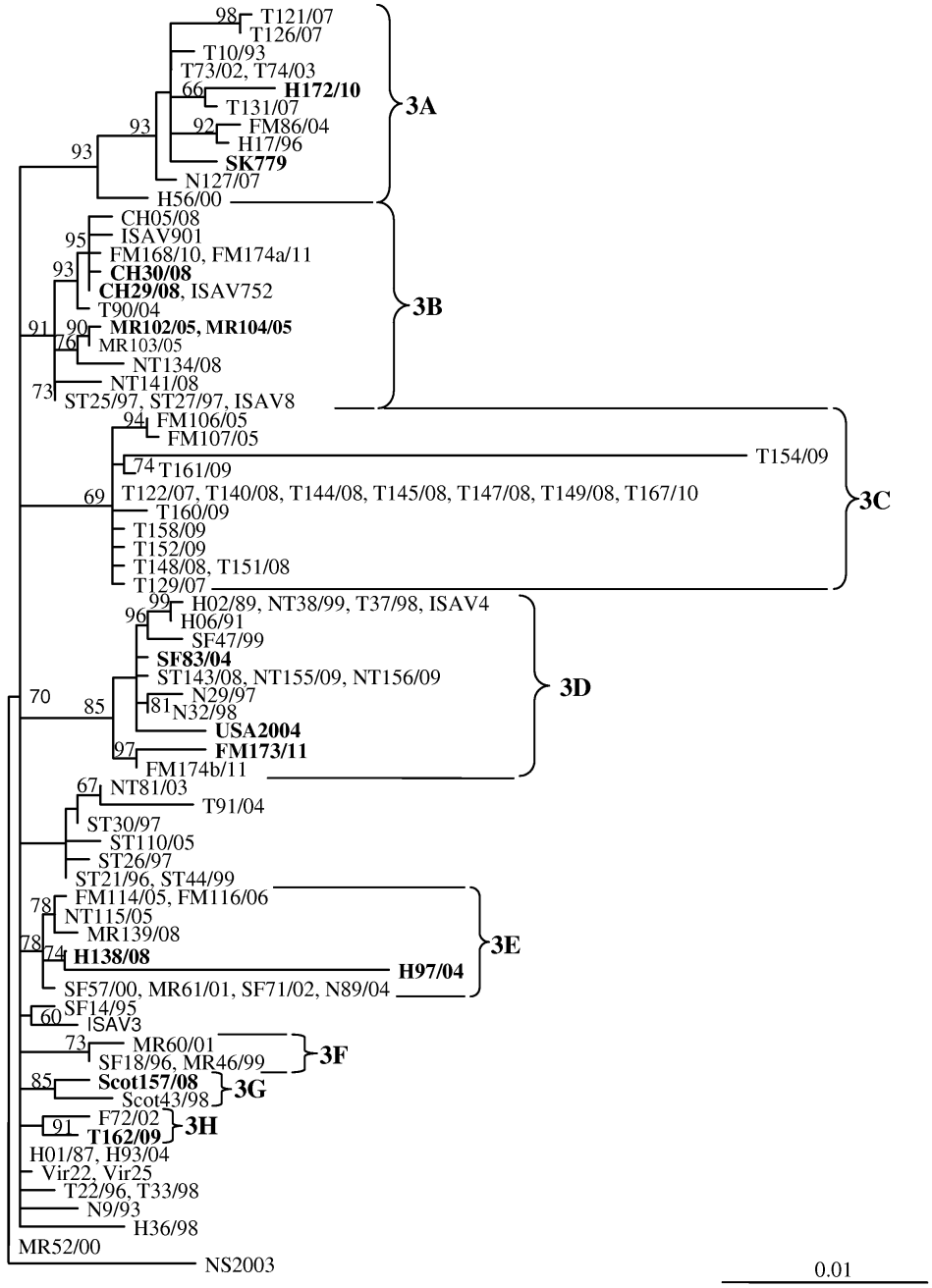
Figure 2: Phylogenetic tree showing the relationship between ISA viruses from Europe and North America based on 1660 nucleotides that code for the putative PA protein. The best-fitting nucleotide substitution model was used during maximum likelihood analysis and the tree was bootstrapped (50 000 quartet puzzling steps) in TREE_PUZZLE. The scale bar shows the number of nucleotide substitutions as a proportion of branch lengths.

Figure 3. Phylogenetic tree showing the relationship between ISA viruses from Europe and North America based on 1299 nucleotides that code for the F protein excluding the inserts that occur in some of the viruses. The best-fitting nucleotide substitution model was used during maximum likelihood analysis and the tree was bootstrapped (50 000 quartet puzzling steps) in TREE_PUZZLE. The scale bar shows the number of nucleotide substitutions as a proportion of branch lengths.

Figure 4. Phylogenetic tree showing the relationship between ISA viruses from Europe and North America based on 942 nucleotides that code for the HE protein excluding the highly polymorphic region (HPR), the transmembrane region and the cytoplasmic tail region. The best-fitting nucleotide substitution model was used during maximum likelihood analysis and the tree was bootstrapped (50 000 quartet puzzling steps) in TREE_PUZZLE. The scale bar shows the number of nucleotide substitutions as a proportion of branch lengths.

Figure 5. Maximum clade credibility (MCC) tree of the ISA virus HE gene lineage. The tree is scaled to time (horizontal axis), with horizontal bars at nodes representing the 95 % HPDs of TMRCA.

Figure 1.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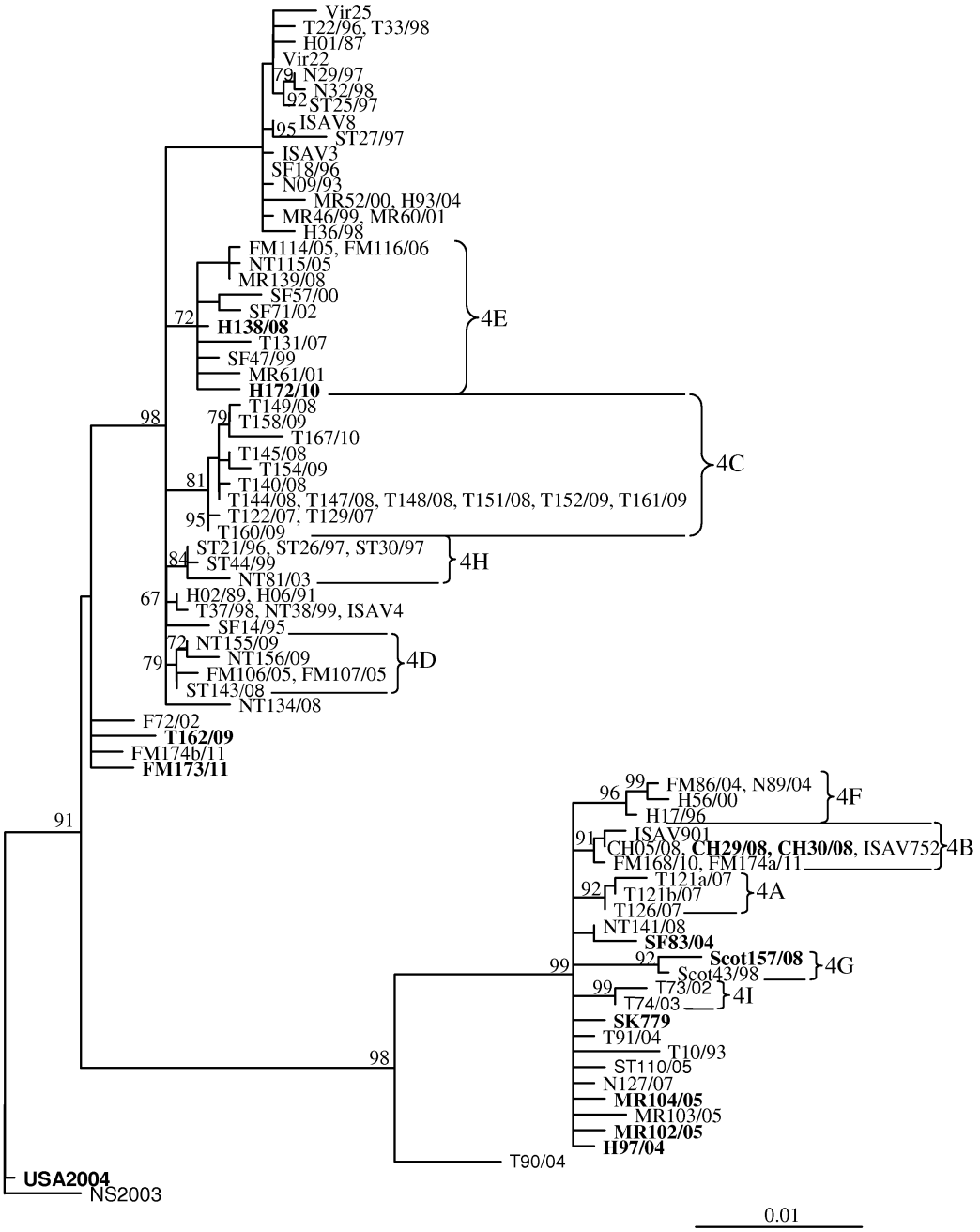
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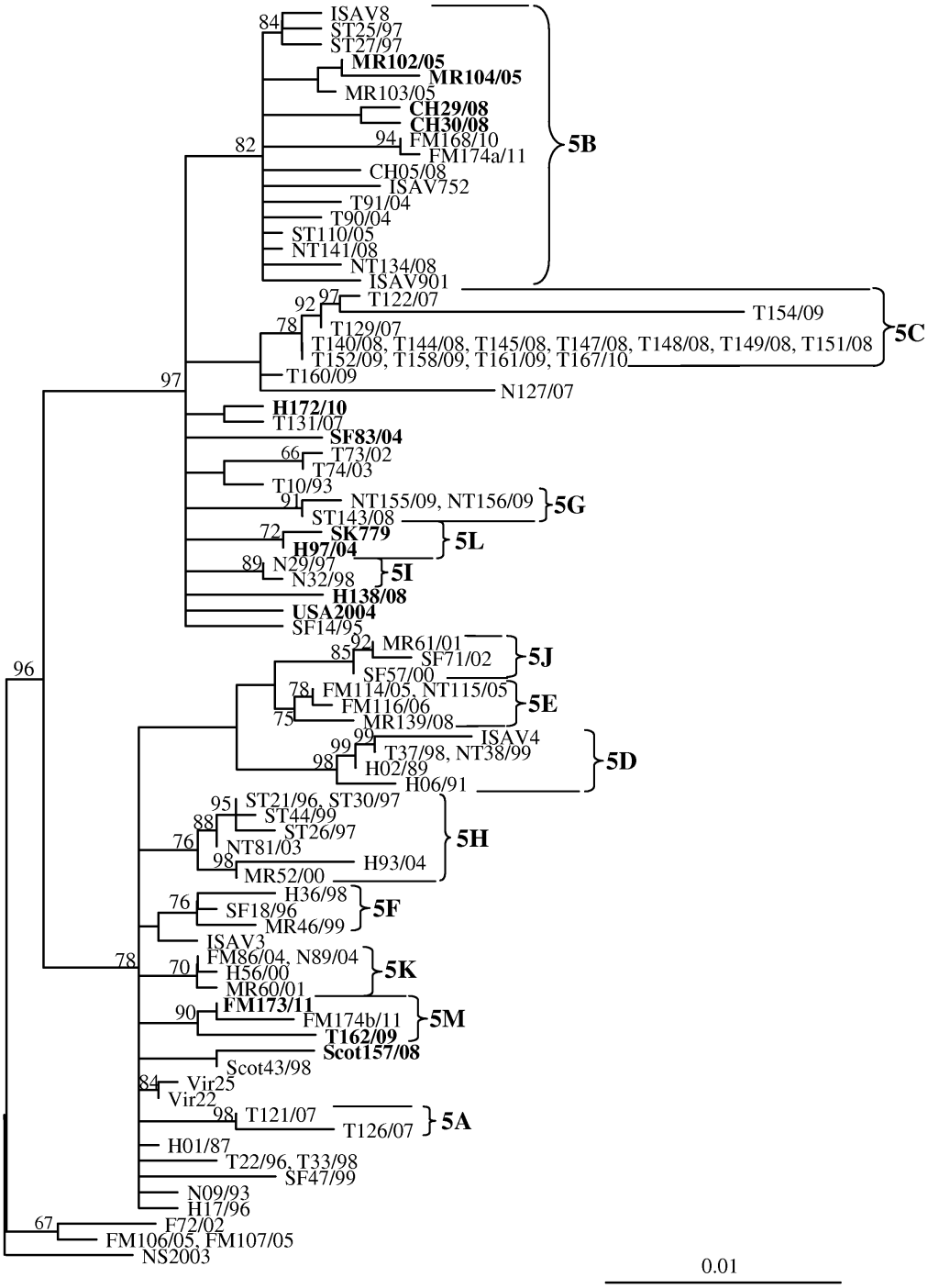
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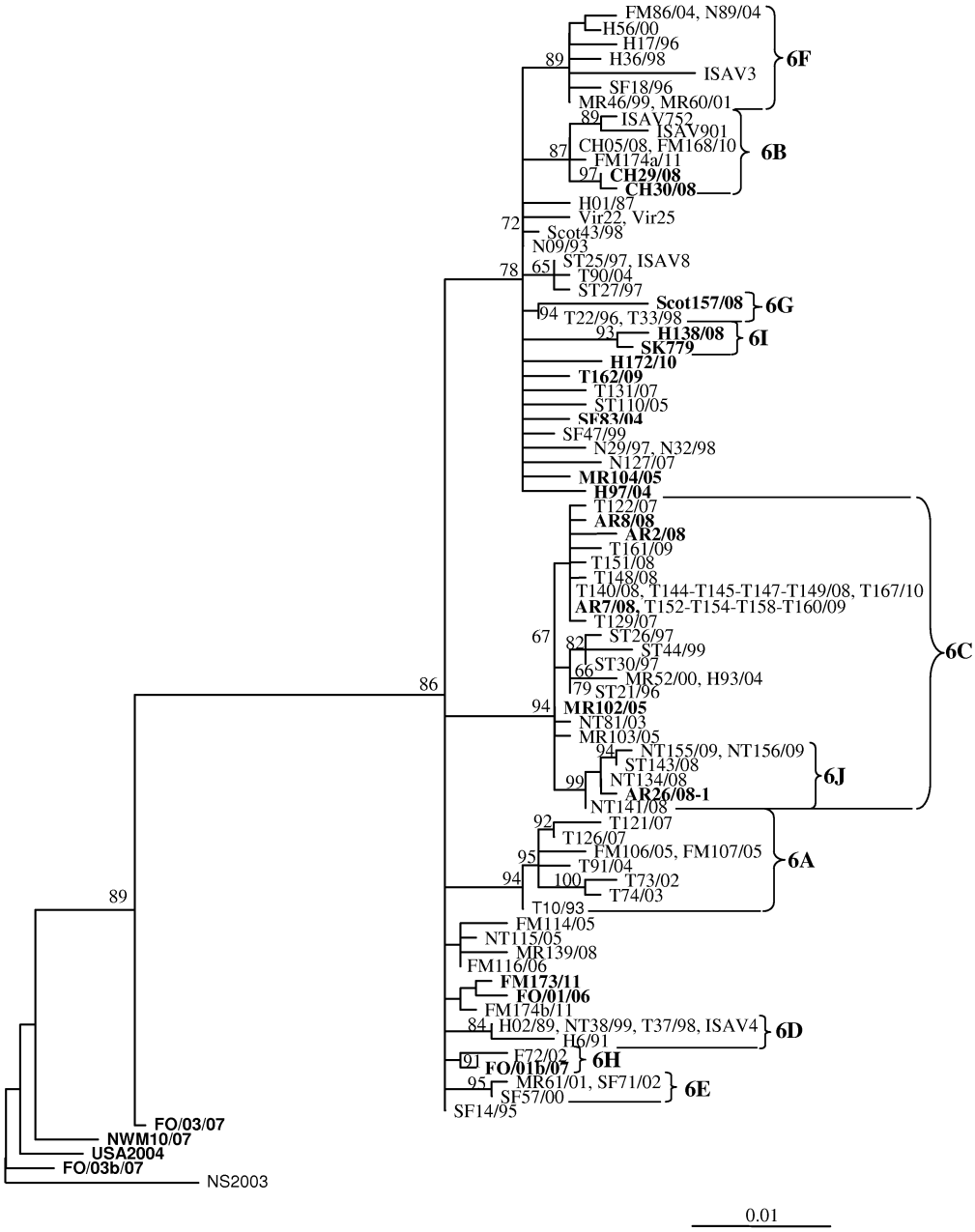
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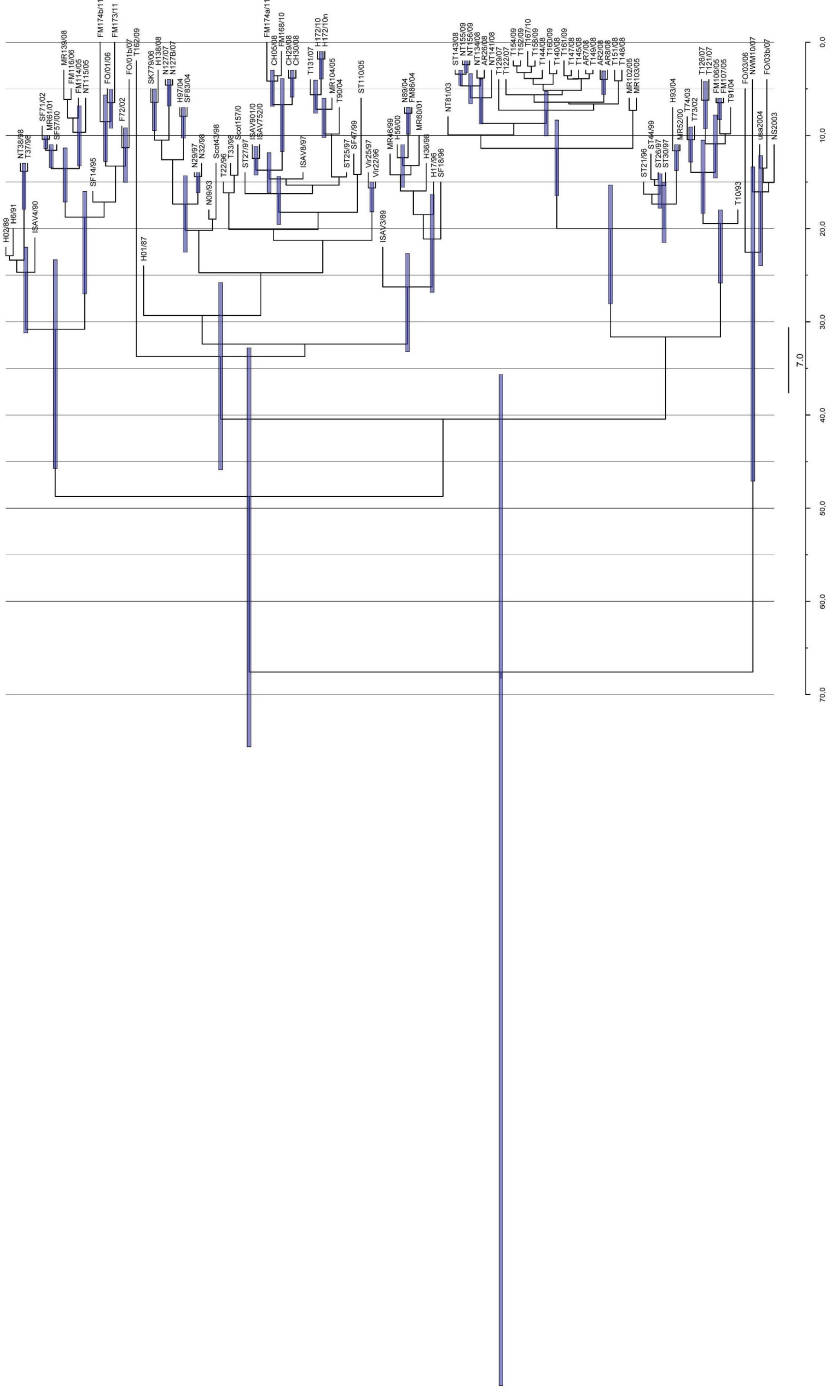
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MR13/98
SF5700
FM11506
H1405
NT11505
SF1455
FM174/11
FO01/06
FM173/11
F7202
FO01b07
T152/09
H01/87
SK7/00
H129/98
N27/97
N27/07
H0704
SF9304
N32/97
N32/08
N38/93
Scot4398
T22/98
T33/98
ST27/97
Scot15/70
ISAV9/10
ISAV7/05
FM174/11
CH0308
FM16/10
CH0308
CH3/08
T13/07
H72/10
H72/10n
MR104/05
T9/04
ISAV9/07
ST3/97
SF7/99
ST11/05
Vr2597
Vr2298
ISAV3/99
MR4/08
T9/00
N0/04
FM99/04
MR6/01
H3/98
ST7/98
SF1/98
NT91/03
ST143/08
NT15/03
NT15/06
NT134/06
AS2/08
NT141/08
T1/07
T122/07
T122/07
T129/09
T14/09
T128/09
T128/09
T160/09
T160/09
T161/09
T161/09
T145/08
T145/08
T140/08
T140/08
AS2/08
AS2/08
T13/08
T13/08
MR113/05
MR113/05
ST2/10/96
ST4/99
ST2/97
ST2/97
SF1/97
MR5/00
H3/04
T74/03
T7/05
T129/07
T10/07
FM109/05
NT107/05
T9/04
T1/03
FO03/06
NW4/07
ISA2/04
NS2/03
FO03b07
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
7.0

Table 1. An overview of ISA viruses and the accession numbers of segments three, four, five and six gene sequences. The names of the different localities (country and county) and virus codes are given in the first column. The second and third column give the year of collection and the HPR in segment six and presence of insert in segment five, respectively.

Country/virus-id	Year	HPR -IN	Ac. no Seg 3	Ac. no Seg 4	Ac. no Seg 5	Ac. No Seg 6
Faeroe Islands						
F72/02	2002	3	JN710838	JN710925	AY853917	AF526263
Scotland						
Scot43/98	1998	7b	JN710880	JN710967	AY853947	AF302802
Scot157/08	2008	0	JN710879	JN710966	JN711010	JN711096
Canada						
NS2003	2003	3	JN710871	JN710958	AY853919	AY601904
USA						
USA2004	2004	0	JN710921	JN711009	JN711056	AY973194
Chile						
CH05/08	2008	35-IN	JN710835	JN710922	JN711011	JN711093
CH29/08	2008	0	JN710836	JN710923	JN711012	JN711094
CH30/08	2008	0	JN710837	JN710924	JN711013	JN711095
752		7b-IN	GU830897	GU830898	GU830899	GU830900
901		15b	GU830905	GU830906	GU830907	GU830908
Norway						
Hordaland						
H01/87	1987	1	JN710848	JN710936	AY853942	AF364893
H02/89	1989	2	JN710852	JN710940	AY853930	AF220607
ISAV4	1990		DQ785206	DQ785220	DQ785234	DQ785248
H06/91	1991	2	JN710855	JN710935	AY853934	AF364894
H17/96	1996	7a	JN710850	JN710938	AY853956	AF364891
H36/98	1998	15	JN710853	JN710941	AY853958	AF302799
H56/00	2000	15	JN710854	JN710942	AY853946	AF364880
H93/04	2004	10	JN710856	JN710943	AY853948	AY973179
H97/04	2004	0	JN710857	JN710944	JN711024	DQ108604
H138/08	2008	0	JN710849	JN710937	JN711022	JN711063
H172/10	2010	0	JN710851	JN710939	JN711023	JN711064
Sogn og Fjordane						
SF14/95	1995	5	JN710881	JN710968	AY853925	AF364873
SF18/96	1996	19	JN710882	JN710969	AY853963	AF364869
SF47/99	1999	9	JN710883	JN710970	AY853965	AF364888
SF57/00	2000	11b-IN	JN710884	JN710971	AY853939	AF364890
SF71/02	2002	11b-IN	JN710885	JN710972	AY853936	AY127881
SF83/04	2004	0	AY744390	AY744391	AY744392	AY973190
Møre og Romsdal						
MR46/99	1999	8-IN	JN710862	JN710949	AY853962	AF364896
MR52/00	2000	10	JN710863	JN710950	AY853949	AF364892
MR60/01	2001	6-IN	JN710864	JN710951	AY853944	AY127876
MR61/01	2001	11a-IN	JN710865	JN710952	AY853935	AY127877
MR102/05	2005	0	JN710858	JN710945	EU851044	DQ108605
MR104/05	2005	0	JN710860	JN710947	JN711026	DQ108607
MR103/05	2005	17	JN710859	JN710946	JN711025	DQ108606
MR139/08	2008	35	JN710861	JN710948	JN711027	JN711065

Table 1 continued…

Country/virus-id	Year	HPR -IN	Ac. no Seg 3	Ac. no Seg 4	Ac. no Seg 5	Ac. No Seg 6
Sør Trønderlag						
ST21/96	1996	14	JN710888	JN710975	AY853952	AF364886
ST25/97	1997	6	JN710889	JN710976	AY853926	AF364885
ST26/97	1997	14	JN710890	JN710977	AY853951	AF364879
ST27/97	1997	6	JN710891	JN710978	AY853929	AF364897
ST30/97	1997	14	JN710892	JN710979	AY853953	AY127875
ISAV8	1997	14	DQ785210	DQ785224	DQ785238	DQ785252
ST44/99	1999	14	JN710893	JN710980	AY853954	AF302803
ST110/05	2005	3	JN710886	JN710973	JN711034	DQ108598
ST143/08	2008	35	JN710887	JN710974	JN711035	JN711073
Nord Trønderlag						
NT38/99	1998	2	JN710877	JN710964	AY853932	AF364874
NT81/03	2003	7b	JN710878	JN710965	AY853955	AY973184
NT115/05	2005	3	JN710872	JN710959	JN711029	JN711068
NT134/08	2008	34	JN710873	JN710960	JN711030	JN711069
NT141/08	2008	2	JN710874	JN710961	JN711031	JN711070
NT155/09	2009	35	JN710875	JN710962	JN711032	JN711071
NT156/09	2009	35	JN710876	JN710963	JN711033	JN711072
Nordland						
ISAV3	1989	12a	DQ785205	DQ785219	DQ785233	DQ785247
N9/93	1993	3	JN710870	JN710953	AY853967	AF364895
Vir22	1996	35	DQ785216	DQ785230	DQ785244	DQ785258
Vir25	1997	35	DQ785214	DQ785228	DQ785242	DQ785256
N29/97	1997	7b	JN710867	JN710955	AY853920	AF364872
N32/98	1998	7b	JN710868	JN710956	AY853921	AF364883
N89/04	2004	14	JN710869	JN710957	AY853945	AY971662
N127/07	2007	33	JN710866	JN710954	JN711028	JN711066/67
Troms						
T10/93	1993	4a	JN710894	JN710981	AY853922	AF302801
T22/96	1996	2	JN710914	JN711002	AY853968	AF364889
T33/98	1998	2	JN710915	JN711003	AY853969	AF364887
T37/98	1998	2	JN710916	JN711004	AY853933	AF364881
T73/02	2002	14	JN710917	JN711005	AY853923	AY971663
T74/03	2003	14	JN710918	JN711006	AY853924	AY971664
T90/04	2004	16	JN710919	JN711007	JN711055	AY971666
T91/04	2004	3	JN710920	JN711008	AY853928	AY971667
T121/07	2007	31	JN710895	JN710982/83	JN711036	JN711074
T122/07	2007	5	JN710896	JN710984	JN711037	JN711075
T126/07	2007	31	JN710897	JN710985	JN711038	JN711076
T129/07	2007	5	JN710898	JN710986	JN711039	JN711077
T131/07	2007	2	JN710899	JN710987	JN711040	JN711078
T140/08	2008	5	JN710900	JN710988	JN711041	JN711079
T144/08	2008	5b	JN710901	JN710989	JN711042	JN711080
T145/08	2008	5	JN710902	JN710990	JN711043	JN711081
T147/08	2008	5	JN710903	JN710991	JN711044	JN711082
T148/08	2008	5	JN710904	JN710992	JN711045	JN711083
T149/08	2008	5	JN710905	JN710993	JN711046	JN711084
T151/08	2008	5	JN710906	JN710994	JN711047	JN711085
T152/09	2009	5b	JN710907	JN710995	JN711048	JN711086
T154/09	2009	5	JN710908	JN710996	JN711049	JN711087
T158/09	2009	5	JN710909	JN710997	JN711050	JN711088
T160/09	2009	5	JN710910	JN710998	JN711051	JN711089
T161/09	2009	5	JN710911	JN710999	JN711052	JN711090
T162/09	2009	0	JN710912	JN711000	JN711053	JN711091
T167/10	2010	5	JN710913	JN711001	JN711054	JN711092

Table 1 continued...

Finnmark						
FM86/04	2004	1	JN710847	JN710934	AY853943	AY971659
FM106/05	2005	14	JN710839	JN710926	JN711014	DQ108599
FM107/05	2005	14	JN710840	JN710927	JN711015	DQ108600
FM114/05	2005	3	JN710841	JN710928	JN711016	JN711057
FM116/06	2006	9	JN710842	JN710929	JN711017	JN711058
FM168/10	2010	14c	JN710843	JN710930	JN711018	JN711059
FM173/11	2011	0	JN710844	JN710931	JN711019	JN711060
FM174a/11	2011	14b	JN710845	JN710932	JN711020	JN711061
FM174b/11	2011	14b	JN710846	JN710933	JN711021	JN711062
Unknown origin						
SK779	2006	0	EU118817	EU118818	EU118819	EU118820

Table 2. Additional sequences of HPR0 ISA virus used in the analysis of segment six. The name of the country of origin and the sequence codes are given in the first column followed by year of collection, location (fresh water or sea water) of the host (Atlantic salmon), and Accession number.

Country/virus -id	Year	Fw/Sw	Accession no
Norway			
AR2/08	2008	Sw	FN687348
AR7/08	2008	Fw*	FN687351
AR8/08	2008	Sw	FN687352
AR26/08-1	2008	Sw	FN687356
Faroe Islands			
FO/01/06	2006	Sw	HQ664992
FO/03/06	2006	Sw	HQ664993
FO/01b/07	2007	Sw	HQ664995
FO/03b/07	2007	Sw	HQ664997
Scotland			
NWM10	2007	Sw	FJ178189

Fw* = fresh water site using addition of sea water.

Table 3. Primers used for PCR and sequencing of segments three and four of ISA viruses. The position is given with respect to the ORF of the two segments.

Primer	Sequence	Position
Segment three		
S3F1	TCA AAT CCC AAA AAT AAT ACA GAA AAC G	upstream
S3F7	GGT TGT AGA AGA TCT ACC GC	54 - 74
S3F2	CAA TCA CCA CGT TCT TCA GCG	452 - 472
S3F3	TGC GGA GGA GTT GCT CTA GAT	913 - 933
S3F4	CAA CCA AGG CGC TAT CAG GGA	1566 - 1586
S3R7	TCC CAC CTG CTT CTT CCT CG	364 - 345
S3R2	TGC CTC TTT CCG TCT TCA TCA	563 - 543
S3R4	TCC TCA GTG CGA CAC CTT CC	1034 - 1015
S3R3	ACC AGA GCC CTG ACA TTC ACA C	1640 - 1619
S3R10	TGT AAA TAT CGT CAA TAC CG	downstream
Segment four		
S4F12	ATG GTG TGC TTG ACA TGG G	398 - 416
S4F16	CCT GCA AGA ATT GCT GCT CAC AAC C	750 - 774
S4F10	GAT GGA TAA CCT CCG TGA ATG C	(-)1 - 21
S4F11	GTT TTG GCG AAC AGG AGA AG	893 - 912
S4F13	CGG AAC TCG TAA CAG ATG GAG	1397 - 1417
S4R12	ATT CTT TGC AGG GTC TGC	661 - 644
S4R14	TTT CTT CTC CTG TTC GCC	915 - 898
S4R10	CCT CGT TGT TGT TGC CC	1143 - 1127
S4R13	GCT CTC CAT CTG CTA ATT GTA GC	1475 - 1453
S4R11	GGA AAA TAC ATA TCG TCA AAC ACA C	downstream

Table 4. The estimated time of most recent common ancestor (TMRCA) for each of the four ISA virus segments for the viruses included in the analysis.

Segment	Mean	95% HPD lower	95% HPD upper
3 (NP)	1918	1969	1840
4 (PA)	1898	1959	1808
5 (F)	1960	1980	1932
6 (HE)	1934	1976	1867
Consensus		**1959**	**1932**

Table 4b. The estimated time of most recent common ancestor (TMRCA) for the Chilean ISA viruses and for a group of closely related ISA viruses from southern Troms based on each of the four ISA virus segments.

TMRCA Norway-Chile

Segment	95% HPD lower	95% HPD upper
3 (NP)	2007	2001
4 (PA)	2007	2001
5 (F)	2002	1996
6 (HE)	1999	1995
Consensus	-	-

TMRCA Southern Troms clade C

Segment	95% HPD lower	95% HPD upper
3 (NP)	2005	1999
4 (PA)	2006	2000
5 (F)	2005	1998
6 (HE)	2006	2001
Consensus	**2005**	**2001**

Table 5. The mean substitution rates for ISA virus of four ISA virus segments.

Segment	Mean Substitution rate	95% lower	95% upper
3 (NP)	1.9×10^{-4}	1.2×10^{-4}	2.6×10^{-4}
4 (PA)	2.2×10^{-4}	1.4×10^{-4}	3.0×10^{-4}
5 (F)	3.9×10^{-4}	2.8×10^{-4}	5.1×10^{-4}
6 (HE)	3.5×10^{-4}	2.3×10^{-4}	4.8×10^{-4}