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USE OF MOLECULAR EPIDEMIOLOGY TO TRACE TRANSMISSION PATHWAYS FOR INFECTIOUS SALMON ANAEMIA VIRUS (ISAV) IN NORWEGIAN SALMON FARMING

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ABSTRACT

Infectious Salmon Anaemia (ISA) may be a disease affecting farmed Atlantic salmon, and most salmon-producing countries have experienced ISA outbreaks. The current study aimed to use epidemiological and viral sequence information to trace transmission pathways for ISA virus (ISAV) in Norwegian salmon farming.

Methods

The study covers a period from January 2007 to July 2009 with a comparatively high rate of ISA outbreaks, including an outsized cluster of outbreaks that emerged in Northern Norway (the North-cluster). Farms with ISA outbreaks and neighboring salmon farms (At-risk-sites) were tested for the presence of ISAV, and epidemiological information was collected. ISAV hemagglutinin-esterase (HE) and fusion (F) protein genes were sequenced and phylogenetic analyses were performed. Associations between sequence similarities and salmon population data were analyzed to substantiate possible transmission pathways.

Results

There was a high degree of genetic similarity between ISAV isolates within the North-cluster. ISAV was detected in 12 of 28 At-risk-sites, and a high proportion of the viruses were identified as putative low virulent genotypes harboring the complete length highly polymorphic region (HPR); HPR0 of the HE protein and therefore the organic compound glutamine (Q) within the F protein at position 266. The sequences from HPR0/F (Q266) genotypes revealed larger genetic variation, lower viral loads, and lower prevalence of infection than HPR-deleted genotypes. Seaway distance between salmon farms was the sole robust explanatory variable to clarify the genetic similarity between ISAV isolates.

Discussion

We suggest that one HPR-deleted genotype of ISAV has spread between salmon farms within the North-cluster. Furthermore, we discover that HPR0/F (Q266) genotypes are frequently present in farmed populations of Atlantic salmon. From this, we anticipate a population dynamics of ISAV portrayed by low virulent genotypes occasionally transitioning into virulent genotypes, causing solitary outbreaks or local epidemics through local transmission.

KEYWORDS: Hemagglutin in-esterase; Fusion protein; HPR0 ;Virulence.

INTRODUCTION

Infectious salmon anemia (ISA) was first described in Norway in 1984 (Thorud and Djupvik, 1988). Around 1990, a large-scale epidemic of ISA emerged in Norwegian salmon farming (Thorud and Håstein, 2003). Disease control measures and regulations were implemented within the early 1990s, followed by a discount in disease incidence. Since 1994, annual numbers of ISA outbreaks in salmon farms have varied between 1 and 20 (Thorud and Håstein, 2003, Lyngstad et al., 2008). ISA has imposed large economic in addition as welfare-related challenges in most salmon-producing countries. A large-scale epidemic of ISA is currently unfolding in Chilean salmon farming (Godoy et al., 2008, Mardones et al., 2009).

In Norway, salmon farms contracting ISA have attended cluster in space and time, although most disease outbreaks have appeared isolated from other outbreaks (Scheel et al., 2007, Aldrin et al., 2010). because of the occurrence of isolated outbreaks of ISA in Norwegian salmon farming, the transmission pathways for the ISA virus (ISAV) is a problem under debate. this can be illustrated by recent literature advocating either horizontal transmission of ISAV between Atlantic salmon farms (Scheel et al., 2007, Lyngstad et al., 2008, Aldrin et al., 2010) or vertical transmission, i.e. from parent fish to offspring (Nylund et al., 2003, Nylund et al., 2007, Vike et al., 2009).

ISAV is assigned to the genus Isavirus within the family Orthomyxoviridae and shares several similarities with influenza viruses (Kawaoka et al., 2005). Like influenza A and B viruses, it's a segmented genome consisting of eight single-stranded RNA segments (Lamb and Krug, 2001, Mjaaland et al., 1997). Gene segments 5 and 6 encode the surface glycoproteins and are believed to be important for the pathogenicity of ISAV (Rimstad et al., 2001, Aspehaug et al., 2005, Markussen et al., 2008). The hemagglutinin-esterase (HE), encoded by segment 6, displays both receptor-binding and receptor-destroying enzyme activities (Rimstad et al., 2001, Krossøy et al., 2001a, Falk et al., 2004). The HE contains a highly polymorphic region (HPR) of a maximum of 35 amino acids near the transmembrane domain of the encoded protein, while the sequence within the remainder of the gene is comparatively conserved (Krossøy et al., 2001a, Mjaaland et al., 2002, Cunningham et al., 2002). Mjaaland et al. (2002) suggested that the sequence variability within the HPR of the HE gene arose from differential deletions of a full-length low virulent precursor gene (HPR0), leading to more or less pathogenic viruses. Since then, the HPR0 genotype has been confirmed in both wild- and farmed Atlantic salmon (Cunningham et al., 2002, Cook-Versloot et al., 2004, Nylund et al., 2007, Markussen et al., 2008, McBeath et al., 2009). Gene segment 5 encodes the fusion (F) protein which is liable for the fusion of viral and cellular membranes (Aspehaug et al., 2005). Markussen et al. (2008) suggested a virulence marker immediately upstream of the arginine at the putative

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cleavage site within the F gene (R267), where either glutamine to leucine substitution at position 266 (Q266 → L266) or a brief sequence insertion near this site, was necessary for acquiring virulence. it's likely that these alterations determine the kinds of host proteases capable of cleaving this protein, possibly altering host range and/or tissue tropism, analog thereto seen for highly pathogenic influenza an epidemic H5 and H7 when these circulate in poultry populations (Steinhauer, 1999, Markussen et al., 2008). The HE- and F genes are thus important in phylogenetic and epidemiological studies of the spread of ISAV (Krossøy et al., 2001b, Nylund et al., 2007, Markussen et al., 2008, Lyngstad et al., 2008, Vike et al., 2009). The forces driving the deletions within the HPR is also analog to the varying stalk lengths described for the influenza virus A and B receptor-destroying enzyme, the neuraminidase (NA), a property that has been related to host range (Air et al., 1990, Castrucci and Kawaoka, 1993).

The present study aimed to use epidemiological and viral sequence information to trace transmission pathways for ISAV in Norwegian salmon farming. The study covers a period with a comparatively high rate of ISA outbreaks in salmon farms, and most significantly, an oversized cluster of outbreaks that emerged in Northern Norway. In parallel with this emergence of ISA outbreaks, neighboring salmon farms to farms with ISA were tested for the presence of ISAV and epidemiological information was collected. Positive findings of ISAV were characterized through sequencing within the HE- and F genes. Associations between sequence similarities and epidemiological data from infected salmon populations were analyzed to substantiate possible transmission pathways. Data are presented to suggest that an HPR-deleted/F (Q → L266) genotype of ISAV has spread between salmon farms located in proximity. Our findings strengthen the hypothesis that ISAV of HPR0 genotypes are of low virulence, and that we find that HPR0/F (Q266) genotypes are frequently present in farmed populations of Atlantic salmon. From this, we anticipate a population dynamics of ISAV portrayed by low virulent genotypes occasionally transitioning into virulent genotypes, causing solitary outbreaks or local epidemics through local transmission.

METHODS

Fish population data and sampling

The study population consisted of farmed Atlantic salmon from sites with confirmed ISA (ISA-sites) and neighboring sites to ISA-sites equipped Atlantic salmon (At-risk-sites). At-risk sites included both smolt producers and marine grow-out sites. The study period was from January 2007 to July 2009. Site IDs and geographical coordinates were obtained from the aquaculture register of the Directorate of Fisheries (DF) (http://www.fiskeridir.no/fiskeridir/akvakultur/registre). Collection of epidemiological data from ISA- and At-risk-sites was performed utilizing the same questionnaire. the info covered fish origin (smolt producing site and broodfish company) and management operations (identity of visiting well boats/service boats, diving companies, companies delivering feed and corporations collecting animal waste, identity, and site of land base/feed barge, and site for storage of animal waste). Samplings of fish from ISA-sites and At-risk-sites were performed by the Norwegian Food Safety Authority (NFSA). From ISA-sites, head kidney tissue was sampled in RNA later (Ambion) and transport medium, from 10 fish. Gill tissue was sampled additionally in 2009. From At-risk-sites, head kidney-, heart- and gill tissues were sampled as above, from 30 fish. The samples were sent express overnight to the National Veterinary Institute (NVI) and stored at − 70 °C. Altogether 302 fish from 30 ISA-sites and 832 fish from 28 At-risk-sites were analyzed for ISAV(Appendix A, Appendix B). ISA-sites were confirmed in and of itself by the NFSA per the contingency plan for control of ISA in Norway (see Lyngstad et al., 2008).

Virus detection and sequencing

All samples were homogenized in lysis buffer and total RNA was extracted on automated NucleotideliSens®easyMAGTM (BioMérieux, Norge AS, Oslo, Norway) following the manufacturer's protocol for

off-board lysis. The concentration of RNA was measured using Nano Drop ND-1000 (Saveen). Isolated RNA was stored at − 80 °C. Amplification and detection of ISAV from samples collected in 2008–2009 was performed on a Strata gene Mx3005P (LaJolla, CA, USA) by one-step real-time RT-PCR using primers and probes targeting ISAV gene segment 7 (Plarre et al., 2005). Reactions were conducted in an exceedingly final volume of 20 μl with a One Step RT-PCR kit (Qiagen), ROX passive reference RT-PARE_03 (Eurogentec), and R Nase Out (Invitrogen). 300–600 ng total RNA was mixed with 1 × RT-PCR buffer, 30 nM ROX passive reference, 400 μM dNTPs, 0.5 μM of every primer, 0.3 μM MGB probe (Applied Biosystems), 1.25 mM MgCl2, 4 U RNase Out and 0,8 μl One Step RT-PCR Enzyme mix. The RT step conditions were 50 °C/30 min followed by 95 °C/15 min. A three-step PCR cycling protocol was used as follows: 45 cycles of 94 °C/30 s, 55 °C/30 s, and 72 °C/45 s. The initial screening results of samples from At-risk-sites showed that the cycle threshold values (Ct-values) obtained from gill samples were lower compared to kidney samples, or the combination of the organs. Analyses were therefore mainly performed on gill samples. Samples from At-risk-sites having Ct-values below 34 were considered positive, while Ct-values within the region 34–39 were retested through new RNA isolation and real-time RT-PCR. Here, samples were considered ISAV positive if a Ct-value below 39 was obtained within the duplicate assay.

Amplification of ISAV from ISA-sites collected in 2007 was performed by one-step RT-PCR using primers targeting ISAV gene segment 8 (Mjaaland et al., 1997). Reactions were conducted in a very final volume of fifty μl with a One Step RT-PCR kit (Qiagen), and RNase Out (Invitrogen). 500 ng total RNA was mixed with 1 x RT-PCR buffer, 1 × Q − solution, 400 μM dNTPs, 0.6 μM of every primer, 4 U RNase Out and a pair of μl One Step RT-PCR Enzyme mix. The RT step conditions were 50 °C/30 min followed by 95 °C/15 min and PCR cycling: 40 cycles of 94 °C/1 min, 55 °C/1 min and 72 °C/1 min and a final extension at 72 °C/7 min. the ultimate PCR products, 155 bp, were visualized by gel electrophoresis and ethidium bromide staining.

Sequencing of ISAV HE- and also the F genes was performed on samples from kidney from two individual fishes from each ISA-site and gill samples from one fish from each At-risk-site. Besides, sequencing of the kidney samples from At-risk-sites was attempted. The HE- and F gene segments were amplified either by a One-step RT-PCR kit (Qiagen) where cDNA synthesis was dole out using gene segment-specific primers (see Supplement) or by a two-step RT-PCR kit with qScript cDNA super mix and AccuStart PCR Supermix (Qantas) where reverse transcription was performed employing a mixture of random hexamers and oligo (dT). The conditions for the One Step method were as follows: cDNA synthesis 50 °C/30 min followed by 95 °C/15 min and PCR cycling: 45 cycles of 94 °C/1 min, 55 °C/1 min and 72 °C/90 s. within the two-step method, the RT conditions were 25 °C/5 min, 42 °C/30 min and 85 °C/5 min followed by PCR cycling: 94 °C/1 min, 40 cycles of 94 °C/30 s, 53 °C/30 s, 72 °C/40–60 s. because of the commonly low amounts of viral RNA in samples from At-risk-sites, sequences were obtained by producing small overlapping fragments, starting from 340 to 591 nucleotides in size (see Supplement). In samples where PCR alone failed to produce enough templates for sequencing, nested PCR was performed on a 2 μl purified PCR product (Montage DNA gel extraction kit, Millipore) from the primary round. PCR was conducted using Accustart PCR Super mix with cycling conditions as described previously. The PCR products from ISA-sites were sent to Macro gen Inc. in Korea for sequencing, while products from At-risk-sites were sequenced using an ABI Prism Big Dye Terminator Cycle sequencing kit on ABI 3130XL Genetic Analyzer at the NVI. Sequences were assembled using the Sequencher 4.5 software from Gene Codes. Multiple sequence alignments of HE- and F genes were performed in MEGA 4 software (Tamura et al., 2007). The HPR of the HE gene was aligned manually. some nucleotides can be placed equally on either side of the gap (Mjaaland et al., 2002) and every one possible positions were included within the alignment.

Phylogenetic analysis

Phylogenetic analyses were performed on 881 nucleotides of the 5′-part of the HE gene (excluding the HPR), and on 561 nucleotides of the F gene (excluding the nucleotide's liable for the Q266 → L266 substitution) by maximum likelihood (ML) using the program PHYML (Guindon and Gascuel, 2003) (http://www.phylemon.bioinfo.cipf.es/cgi-bin/phyml.cgi), and using the Kimura 2 parameter model for nucleotide substitution (Kimura, 1980). Support was assessed through 1000 bootstrap replicates. The constructed trees were visualized by MEGA4 software (Tamura et al., 2007)

The nucleotide sequences from this study have the subsequent accession numbers in Genbank: FN687284–FN687425FN687284FN687285FN687286FN687287FN687288FN687289FN687290FN687291FN687292FN687293FN687294FN687295FN687296FN687297FN687298FN687299FN687300FN687301FN687302FN687303FN687304FN687305FN687306FN687307FN687308FN687309FN687310FN687311FN687312FN687313FN687314FN687315FN687316FN687317FN687318FN687319FN687320FN687321FN687322FN687323FN687324FN687325FN687326FN687327FN687328FN687329FN687330FN687331FN687332FN687333FN687334FN687335FN687336FN687337FN687338FN687339FN687340FN687341FN687342FN687343FN687344FN687345FN687346FN687347FN687348FN687349FN687350FN687351FN687352FN687353FN687354FN687355FN687356FN687357FN687358FN687359FN687360FN687361FN687362FN687363FN687364FN687365FN687366FN687367FN687368FN687369FN687370FN687371FN687372FN687373FN687374FN687375FN687376FN687377FN687378FN687379FN687380FN687381FN687382FN687383FN687384FN687385FN687386FN687387FN687388FN687389FN687390FN687391FN687392FN687393FN687394FN687395FN687396FN687397FN687398FN687399FN687400FN687401FN687402FN687403FN687404FN687405FN687406FN687407FN687408FN687409FN687410FN687411FN687412FN687413FN687414FN687415FN687416FN687417FN687418FN687419FN687420FN687421FN687422FN687423FN687424FN687425.

Analyses of transmission pathways

To use genetic information to trace probable transmission pathways for ISAV, it had been assumed that any pair of virus isolates from different sites may or might not share a standard source of infection. within the present analyses, we depict the three alternative transmission pathways: 1. transmission mechanism between sites over short seaway distances; 2. transmission mechanism between sites through shared management; or 3. sites being directly infected by having shared one or more smolt producing sites presumed to deliver infected fish. for every of the depicted transmission pathways, it had been assumed that directly sharing an infected source would imply that ISAV isolates would be genetically similar, whereas not sharing an infected source wouldn't imply any expectations during this regard. Hence, a cut-off between genetic similarity vs. non-similarity between ISAV isolates was constructed and also the binary dependent genetic similarity variable was accustomed analyze the results of the three transmission pathway variables. Also, a fourth explanatory variable: the quantity of days between positive ISAV samples was analyzed to regulate for a possible effect of genetic drift in ISAV isolates. The explanatory variable: shared Broodfish Company, was excluded because there are only two major and some minor brood fish companies in Norway (Paisley et al., 2010). A majority of the sites within the present study received smolts originating from both of the main brood fish companies.

As a basis for constructing the dependent genetic similarity variable, time directed matrices of the genetic distances between ISAV isolates were calculated for all pairs of websites for the 5′-part of the HE gene and also the F gene, respectively, using the Kimura 2 parameter model for nucleotide substitution (Kimura, 1980). The minimum genetic distance for a given pair of web sites was used if sequences from quite one ISAV isolate from the identical site were available. This yielded matrices for the HE- and F genes of all possible combinations of pairwise

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minimum genetic distances between ISAV isolates, where both ISA-sites and At-risk-sites were included. For HPR sequences, a binary variable was calculated within which the association between any pair of ISAV isolates was denoted as either compatible or non-compatible with transmission mechanism. This categorization relies on the idea that deletions may occur within the HPR, but insertions don't occur during this region (Mjaaland et al., 2002). Hence, a compatible association is defined as either a similar deletion pattern within the HPR or when the latter isolate in a very given pair had a deletion pattern that spanned the overall deleted nucleotide positions within the first isolate.

Similar time-directed matrices between all pairs of web sites with positive ISAV findings were calculated for the explanatory variables. Seaway distances between pairs of web sites with a maximum distance of 100 km were obtained from Krist off ersen et al. (2009). For sites located over 100 km apart, we used Euclidean distances as an approximation to seaway distance. Shared management and shared smolt producing site were calculated as binary variables, indicating whether pairs of web sites shared any type of management operations (see the previous discussion) or shared smolt producing sites. The period of time (days) between the dates when samples were received at the NVI was also tabulated. the ultimate dataset contained information on 30 ISA-sites and 10 At-risk-sites from which the HE gene was sequenced, leading to a complete of 780 pairs of websites directed in time from the start to the tip of the study period. Since two of the At-risk-sites were smolt producers these had by definition no smolt suppliers.

Due to missing F gene sequences from 1 ISA site and a couple of At-risk-sites (Appendix A, Appendix B) and therefore the more limited genetic variation within the F gene (see Figs. 3a and b),

These sequences weren't included within the analysis of transmission pathways. The inclusion of F-gene sequences, however, didn't qualitatively affect the results (analysis not shown).

Genetic similarity between pairs of isolates was defined by

1. compatible deletion patterns within the HPR, and 2. a genetic distance of ≤ 1 within the 5′-part of the HE gene. The sensitivity of the cut-off within the genetic distance was tested by varying this within the analyses. Pairs of isolates with non-compatible deletion patterns within the HPR or larger genetic distance within the 5′-part of the HE gene were categorized as non-similar.
2. The association between the dependent genetic similarity variable and therefore the explanatory variables was analyzed employing a logistic regression model:

$$\text{logit}\,(\mathrm{p_i}) = \beta_0 + \sum \beta_i \mathrm{X_i}$$

Where pi is that the probability of similarity between ISAV isolates, β0 is that the intercept, βi is that the vector of regression parameters, and Xi is that the vector of covariates for the ith pair of ISAV isolates.

Our variables weren't independent observations since a given site was paired with all other sites. Accordingly, the importance of the regression coefficients was assessed with randomized distributions (Legendre, 2000). We used a Mantel test where we permuted data (2000 permutations), and therefore the p-values were calculated from the z-values obtained when analyzing the permuted data. an analogous approach was accustomed test the difference in sequence variation between HPR0 and HPR-deleted genotypes, where the Wilcoxon test was applied.

The descriptive and statistical analyses were conducted in R version 2.10.0 (The R Foundation for Statistical Computing 2009).

RESULTS

ISA-sites

ISA was confirmed on 30 sites during the study period. All ISA-sites were marine fish farms holding Atlantic salmon. The genetic distances between ISAV sequences within the

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ISA-sites were generally low, but larger within the 5′-part of the HE gene than within the HPR or the F gene (Appendix A). All ISAV isolates from ISA-sites displayed deletions within the HPR and a leucine at position 266 (L266) within the encoded F protein.

Except for site ISA4/07, all within site isolates of ISAV shared identical deletion patterns within the HPR. On ISA4/07, isolates were found containing two different HPR deletion patterns, none of which could are produced through an additional deletion of the opposite. The F genes sequenced from ISA4/07 were identical, but in contrast to the opposite F genes during this study and available F gene sequences from Genbank the encoded aminoalkanoic acid in position 267 was glycine (G) instead of arginine (R) which constitutes the putative cleavage site all told other known ISAV.

Most of the ISA-sites (17) were located within the North-cluster (Fig. 1). the primary ISA site during this area was sampled for ISAV in June 2007 (ISA3/07). This site was stocked Atlantic salmon in 2006. Throughout the study period 0/6 sites with Atlantic salmon stocked to sea in 2005 (2005 G), 4/8 of the 2006 G, 7/9 of the 2007 G, and 6/6 of the 2008 G contracted ISA within the North-cluster. Among these ISA sites, a maximum of 9 sites shared some type of management operations and a maximum of seven sites shared a selected smolt producing site.

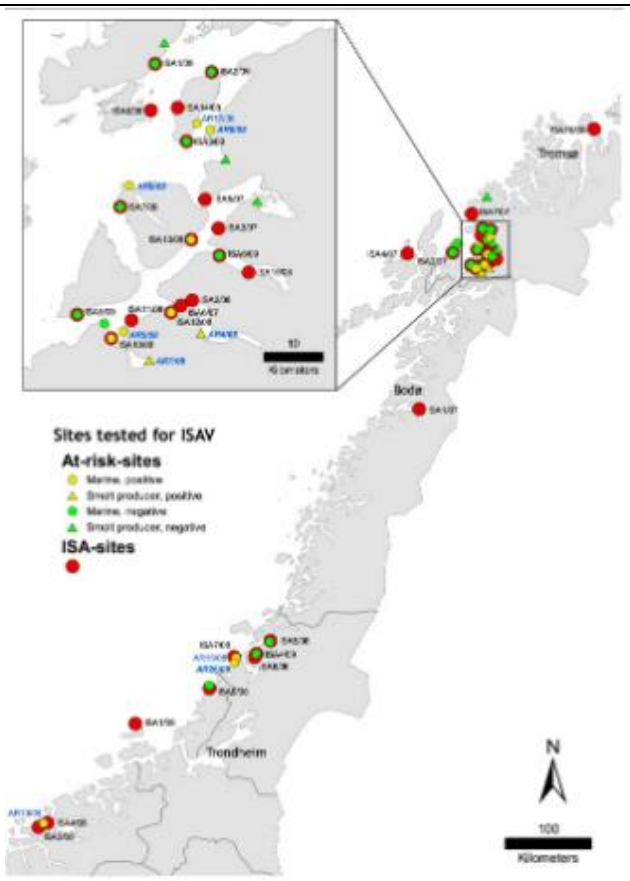

Fig. 1. Map of ISA- and At-risk-sites sampled within the period January 2007 to July 2009. The enlarged area shows the 17 ISA-sites clusterings in an exceedingly relatively limited area in Northern Norway (The North-cluster). Site identities are labeled in keeping with Appendix A, Appendix B (ISA-sites are in black; At-risk-sites in blue; and HPR0 genotypes in italic bold).

At-risk-sites

In total, 28 At-risk-sites (22 marine and 6 smolt producing sites) were sampled for ISAV. ISAV was detected in 12 At-risk-sites, two of which were smolt producers (Fig. 1, Appendix B). The HPR0 genotype was found in 8 At-risk-sites, an HPR-deleted genotype in 3, and only partial sequence information was obtained from one At-risk-site. F gene sequences related to the HPR0 genotypes all encoded glutamine in position 266 (Q266), whereas F gene sequences related to HPR-deleted genotypes all encoded leucine during this position (L266, Appendix B). A high proportion of the marine At-risk-sites (11/22) developed

clinical ISA subsequently to the primary sampling. These sites were sampled a second time as ISA sites (Fig. 1). In 7 of those sites, the primary samples of 30 fish were negative (Fig. 1, Appendix B), including all sites farming the 2008 G Atlantic salmon within the North-cluster. Three At-risk-sites where the HPR0 genotype was found failed to report ISA, while two of the At-risk-sites with HPR-deletions failed to report ISA.

ISAV prevalence and Ct-value comparisons

The prevalence of ISAV positive fish was 100% in most samples from ISA-sites and usually much above in samples from At-risk-sites (Appendix A, Appendix B). all-time low Ct-value obtained from HPR0 genotypes was 24, which was markedly above the bottom values obtained for HPR-deleted genotypes found in ISA-sites additionally as in At-risk-sites (Fig. 2).

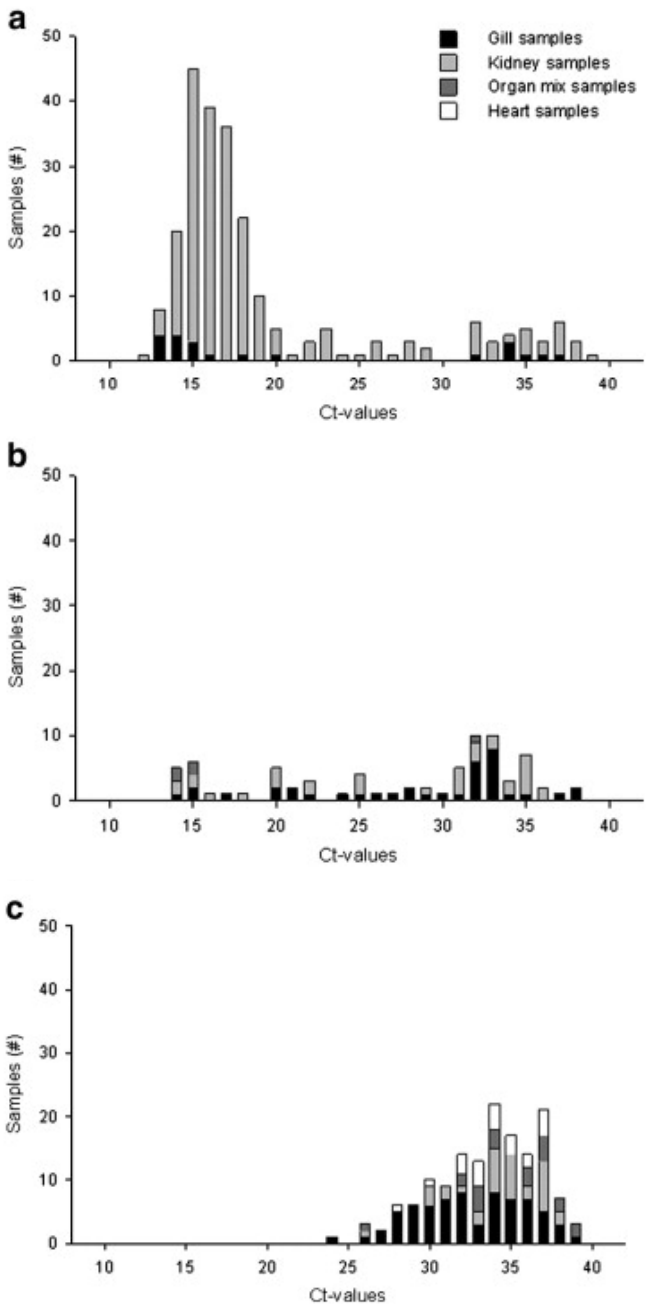

Fig. 2. Frequency distributions of the cycle threshold values (Ct-values) from all positive samples and every one different tissue combinations tested with real-time RT-PCR from ISA- and At-risk-sites sampled within the period January 2008 to July 2009 (ISA-sites (a); At-risk-sites with HPR-deleted genotypes (b); and At-risk-sites with HPR0 genotypes (c)).

ISAV phylogeny

The variation in genetic distance between ISAV isolates was slightly larger within the 5′-part of the HE gene than within the F gene (Figs. 3a and b). The genetic distance between

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isolates was highly correlated between the HE- and F genes (Spearman r = 0.7).

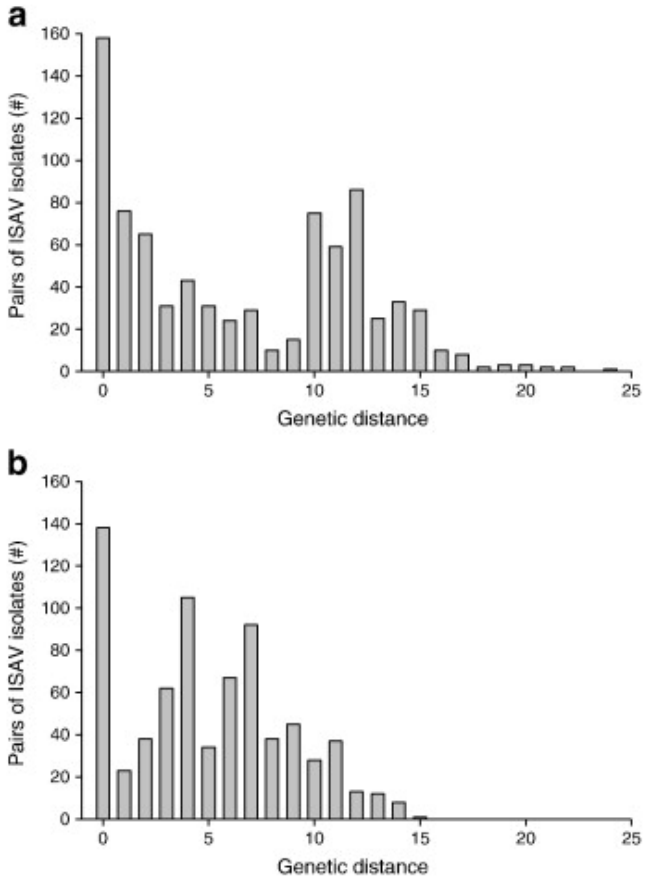


Fig. 3. Frequency distributions of genetic distances between ISAV isolate within the matrix of all pairwise combinations of websites (both ISA-sites and At-risk-sites). The genetic distances represent the minimum distance for a given pair of web sites if sequences from over one ISAV isolate were available. The genetic distances were calculated using the Kimura 2 parameter model for nucleotide substitution (5′-part of the HE gene (a); and F gene(b)).

There was a high degree of genetic similarity between ISAV isolates from ISA-sites within the North-cluster in both the HE- and therefore the F genes (Figs. 4a and b). All ISAV isolates from ISA-sites within the North-cluster shared the same deletion pattern within the HPR. The deletion within the nucleotide sequence of the HPR stretched from nucleotide position number 1045 to 1107 relative to the HPR0 genotype sequence EU118820 (Genbank). No sequences with an analogous deletion pattern were identified after the alignment of the nucleotide sequences from this study with 331 ISAV HE nucleotide sequences presently available from Genbank.

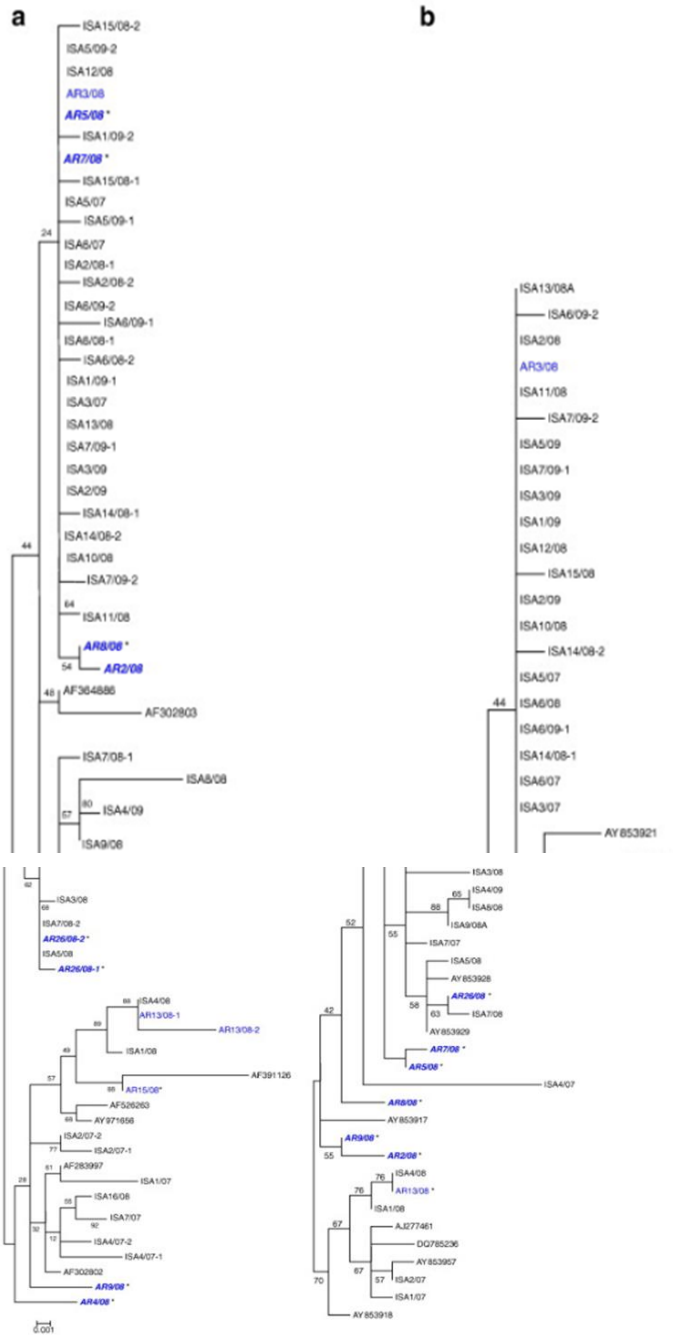


Fig. 4. Phylogenetic tree showing the 5′-part of the ISAV HE gene (a) and therefore the ISAV F gene (b) from ISA- and At-risk-sites sampled within the period January 2007 to July 2009. The phylogenetic tree was constructed using maximum likelihood (ML) and also the Kimura 2 parameter model for nucleotide substitution. The analyses were

performed using 881 nucleotides of the 5′-part of the HE gene (excluding the HPR) and on 561 nucleotides of the F gene (excluding the nucleotides chargeable for the Q266 → L266 substitution). Support was assessed through 1000 bootstrap replicates. Site identities are labeled in step with Appendix A, Appendix B (ISA-sites are in black; At-risk-sites in blue; and HPR0 genotypes in italic bold). All sequences originate from kidney samples, apart from those marked with an asterisk that originates from gill samples.

The sequences from HPR0 genotypes vs. HPR-deleted genotypes from the North-cluster area revealed larger overall genetic variation for the HPR0 genotypes (Figs. 4a and b). The genetic distances within the 5′-part of the HE gene were significantly larger between HPR0 genotypes compared to HPR-deleted genotypes from this area (Wilcoxon statistic, 2000 permutations; $p \leq 0.001$). The genetic distances within the F gene sequences between HPR0 genotypes and HPR-deleted genotypes isolated subsequently from the identical stocks of salmon (i.e. AR2/08-ISA13/08; AR5/08-ISA11/08) were relatively large (8 and three respectively; Fig. 4b).

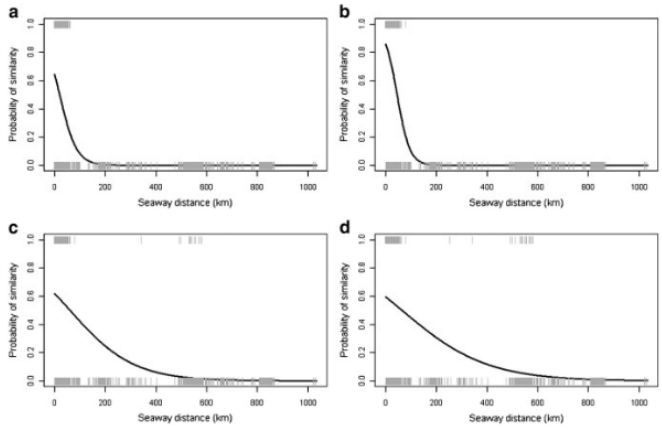
Analyses of transmission pathways

Univariate logistic regression analyses gave significant effects for all the tested variables. The AIC value for seaway distance was markedly less than for the opposite variables, indicating that this variable was a significantly better predictor of genetic similarity than the opposite variables (Table 1). Seaway distance was negatively correlated to shared management (spearman r = − 0.19) and shared smolt producing site (spearman r = − 0.27). Models of genetic similarity entering seaway distance along with shared management operations or shared smolt producing site resulted in non-sensible changes in signs (+/−) of parameter estimates, for the latter two variables. Hence, these variables weren't further analyzed. A model of genetic similarity entering seaway distance and days between diagnoses canceled out the effect of days. It follows that the sole model yielding a strong explanatory effect entered only genetic similarity as a function of seaway distance. Changing the cut-off within the genetic distance within the 5′-part of the HE gene changed the form of the logistic functions (Figs. 5a–d). The sharpest decrease within the probability of genetic similarity was attained when the cut-off allowed for a genetic distance of max 1 for the similar category. These figures reflect the strong tendency for genetically similar ISAV isolates to cluster close.

Table 1. Summary of descriptive statistics for the explanatory variables for pairs of ISA-sites and ISAV positive At-risk-sites from January 2007 to July 2009. Pairs of websites are categorized as similar for 1. genetic distances of ≤ 1 within the 5'-part of the HE gene, and 2. compatible deletion patterns within the HPR.

Risk variable	Level	Non-similar n = 571	Similar n = 209	*p*-value	AIC
Seaway distance (km)	Mean (5%, 95%)	403 (20, 844)	26 (5, 52)	< 0.001	448
	Sd	291	15		
Days between positive ISAV samples	Mean (5%, 95%)	229 (9, 542)	260 (7, 678)	0.05	907
	Sd	191	199		
Shared management operations	0	228	45	< 0.001	802*
	1	282	148		
Shared smolt producing site	0	463	146	< 0.001	805*
	1	47	47		

* Two smolt producing sites were not included in the univariate analysis for the explanatory variables shared management and shared smolt producer.



Two smolt producing sites weren't included within the un ivariate analysis for the explanatory variables shared management and shared smolt producer. Fig. 5. Estimated probability of genetic similarity between

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pairs of ISAV isolates as a function of seaway distance between infected salmon farms. Four different bring to an end values for genetic distance within the HE gene sequence are shown (genetic distance = 0 (a); ≤ 1 (b); ≤ 2 (c); and ≤ 3 (d)). Actual data are shown for the similar category at the highest x-axis, and therefore the non-similar category at the underside x-axis.

DISCUSSION

Virulent and low virulent ISAV

Altogether 30 ISA outbreaks were recorded during the current study, and 17 of those appeared clustered during a relatively limited area in Northern Norway. Following the primary outbreak of ISA within the North-cluster within the summer of 2007, an increasing proportion of salmon put to sea contracted the disease, including all 2008 G stocks of farmed salmon. Hence, the history of 17 consecutive ISA outbreaks within a limited area could also be described as a neighborhood epidemic of ISA.

The ISAV isolates from the 17 consecutive ISA outbreaks within the North-cluster showed a high degree of genetic similarity within the 5′-part of the HE gene and also the F gene. They also shared a similar deletion pattern within the HPR, suggesting a typical origin of the ISAV causing the North-cluster of outbreaks. The high degree of genetic similarity between ISAV isolated from aggregated ISA-sites accords with earlier findings (Lyngstad et al., 2008), albeit only smaller aggregations of ISA outbreaks was reported during this study.

Sequences of the HE and F genes, respectively, that were isolated within ISA-sites, generally displayed a high degree of similarity, indicating that ISA outbreaks were caused by single strains of ISAV. The HPR was identical within all sites apart from site ISA4/07. Here the 2 HPR-deleted genotypes were incompatible since none of them could are produced through an additional deletion of the opposite, while the 2 F gene sequences from this site were identical, sharing a special feature at the cleavage site of the protein (G267). the 2 HE sequences clustered most closely together, and also the data thus indicate a typical origin both for the F- and HE genes. the various HPR deletion patterns suggest, however, two separate deletion events indicating that the 2 closely related outbreak strains during this site originate from a typical ancestor.

A remarkable finding within the present study was the high proportion of At-risk-sites within which HPR0 genotypes were identified. These sites had no previous suspicion of ISA. The low viral loads related to HPR0 genotypes, as indicated by high Ct-values, together with the widely low prevalence of such infections in farmed salmon populations, support the hypothesis that HPR0 genotypes are of low virulence (Markussen et al., 2008, Kibenge et al., 2009, McBeath et al., 2009). Furthermore, the presence of Q266 within the F gene of all HPR0 genotypes within the present study, as hostile the L266 present altogether HPR-deleted genotypes, provides further evidence for the Q266 → L266 substitution being related to the acquisition of virulence in ISAV (Markussen et al., 2008).

The present results, together with the reports from the Faeroe Islands (Christiansen and Østergård, 2007), suggest that HPR0 genotypes are cosmopolitan. However, a blast search in Genbank revealed that only 13 of 354 sequences from the HE gene were of the HPR0 genotypes. This probably reflects that the sampling of ISAV mainly has been done on diseased fish which low viral loads related to HPR0 genotypes imply that sequencing of positive findings is challenging. The HPR0 genotypes within the present study were also genetically more heterogenic than HPR-deleted genotypes from local disease clusters, as was shown for HPR0 genotypes from the North-cluster area.

Transmission pathways.

The logistic regression model of associations between sequence similarities and epidemiological data singled out seaway distance because the only robust explanatory

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variable. When sequence similarity was constrained to a genetic distance of but two between pairs of isolates, the probability of similarity decreased sharply with increasing distance between sites. From this, we conclude that ISAV is spread directly between proximate salmon farms which transmission mechanism between sites implies that the virus isolated from them is genetically nearly identical. Transmission between proximate sites may be by passive drift within the water current, as has been substantiated both for ISAV (Gustafson et al., 2007) and salmonid alphavirus (Viljugrein et al., 2009). there have been also reports of escaping fish from two of the ISA-sites within the North-cluster which could have contributed to local transmission. However, when considering all ISA sites featured within the present study, transmission between proximate sites may only reasonably explain about half the outbreaks.

A problem within the present analysis of transmission pathways was that neighboring salmon farms not only were located in proximity but also cared-for share management operations and native smolt producing sites. About the origin of the fish on a given salmon farm, most of the farms are stocked fish from several smolt-producing sites (Lyngstad et al., 2008). The smolt producers may besides produce fish that originates from different broodstock companies. Hence, sharing a smolt-producing site isn't a conclusive variable since infection could hypothetically be introduced from any infected smolt-producing site to a given marine farm. an analogous weakness applies for the current shared management variable since infection could hypothetically be spread by any of the management operations pooled during this variable. Hence, with this data, it's unfeasible to totally disentangle the possible transmission pathways of ISAV. However, among the ISA sites within the North-cluster, there have been no management operations or smolt-producing sites that were shared by over 9 sites. From this, we deduce that neither of the transmission pathways depicted by shared management operations nor shared smolt producing sites may explain the transmission of ISAV in its title. ISAV on a given ISA-site may basically originate from infectious sources hosting either virulent virus or low virulent virus that later evolves to a virulent virus. The latter pathway accords thereupon suggested for vertical transmission, with the extra requirement that transmission from parent to offspring would happen in freshwater (Nylund et al., 2003). Vertical transmission of the low virulent virus doesn't suits the genetics of ISAV isolated within the North-cluster of outbreaks. Here, all ISAV isolates shared a typical and unique deletion pattern within the HPR and it's not going that these were products of independent transitions from low virulent to virulent ISAV. Moreover, all 6 ISA-sites holding the 2008 G salmons within the North cluster were tested negative for ISAV shortly after the smolts had been transferred to seawater, suggesting that these fish contracted ISAV infection within the marine environment. Nevertheless, it's interesting to notice that HPR0 genotypes were identified from two local smolt-producing sites within the North-cluster area. These smolts had partly been exposed to seawater within the production, so infection through seawater exposure can not be ruled out. However, if ISAV can transmit for instance via salmon eggs, this might have serious consequences for long-distance transport of ISAV (Vike et al., 2009).

The role of virulent and low virulent genotypes within the spread of ISAV

Virulent and low virulent genotypes of ISAV evidently play different roles within the spread and population dynamics of ISAV. Virulent ISAV triggers disease outbreaks and tends to spread locally between neighboring salmon farms, giving rise to local ISA epidemics. The extent of such epidemics probably depends on the local density of farmed Atlantic salmon populations similarly as disease control measures. Results from Norwegian risk factor studies dispensed within the 1990s indicated that ISAV was mainly transmitted from local salmonid sources (Vågsholm et al., 1994, Jarp and Karlsen, 1997). it's also been shown that early removal of

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infected cages effectively limits the spread of ISAV (Gustafson et al., 2006), and delayed depopulation is believed to possess played a vital role within the emergence of the large ISA epidemic currently unfolding in Chile (Mardones et al., 2009).

Phylogeny and sequence analyses identified several cases within which the HE- and F genes of HPR0 genotypes were the same as that of local HPR-deleted genotypes, indicating that HPR0 and HPR-deleted genotypes were related within the identical area. However, there have been also samples of virulent ISAV isolates that probably didn't descend directly from HPR0 genotypes identified at the identical site before a disease outbreak. This was especially apparent within the F gene sequence AR2/08 with an HPR0 genotype. This isolate differed by a genetic distance of 8 from the subsequently isolated virulent genotype (ISA 13/08). the foremost likely explanation for this can be that the HPR0 and HPR-deleted genotypes represent independent infection events which the HPR-deleted genotype was acquired from the continued neighborhood epidemic of ISA. This accords with expectations, given the apparently high local transmissibility of virulent ISAV which transitions from low virulent to virulent strains are rare events. The latter is supported by the frequent observations of HPR0 genotypes reported from the Faeroe Island, although no ISA disease outbreaks are recorded since 2005 (Debes Christiansen personal communication).

At present, whether the putative natural host reservoirs for HPR0 genotypes are salmonids, as suggested by Nylund et al. (2003), or non-salmonids, has yet to be determined. Still, the apparent analogies with regards to mechanisms for virulence acquisition and population dynamics of ISA outbreaks in farmed salmon and influenza outbreaks in poultry caused by avian influenza viruses H5 or H7, that have wild waterfowls as natural host reservoirs, might point to a non-salmonid reservoir for ISAV. However, it's going to even be that salmon farm conditions with unnaturally large and dens host populations greatly enhance transmission, which can select for rapid replication and increased virulence in ISAV. The evolution of pathogen virulence in intensive fish farming has been substantiated for the case of Columnaris disease in Finland (Kunttu et al., 2009, Pulkkinen et al., 2010).

CONCLUSIONS

HPR0 genotypes were found in an exceedingly high proportion of salmon farms where the disease wasn't suspected. Low virus loads, moreover as low prevalence in HPR0, infected salmon populations, provide further support for HPR0 genotypes being of low virulence. Previously reported markers of low virulence were found consistently in HPR0 genotypes, and suggested mechanisms for transitions from low virulent to virulent strains show a striking analogy to avian influenza viruses H5 and H7. Among the heterogeneous HPR0 genotypes, some candidates could are directly ancestral to local virulent genotypes, given the genetic changes necessary for virulence acquisition (Q → L266 and HPR deletions). Although we don't present evidence for the transition from low virulent- to virulent genotypes of ISAV, all the evidence presented supports this path of virulence acquisition. Hence, we advise that the HPR0 genotypes play a very important role within the population dynamics and evolution of ISAV.

1. The population dynamics of ISAV as portrayed by low virulent genotypes occasionally transitioning into virulent genotypes, causing solitary outbreaks or local epidemics, fits the space-time occurrence of partly isolated and partly small clusters of outbreaks in Norway (Scheel et al., 2007, Lyngstad et al., 2008, Aldrin et al., 2010). We believe that the relatively low annual numbers of ISA outbreaks in Norwegian salmon farming in recent years are because of the regulations implemented to regulate this disease, demanding as an example depopulation of diseased fish. Relaxing

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such regulations may result in large-scale epidemics as has been experienced in most countries farming Atlantic salmon. Future research should target the source of low virulent HPR0 genotypes and therefore the risk of transition to virulent strains.

ISA-sites in Norway sampled from January 2007 to July 2009 with site identification (Site ID); dates when samples were received at the Norwegian Veterinary Institute (Date); several fish sampled from each site (n); the prevalence of positive samples (Prev); the amount of sequences (No seq) obtained from individual fish and ISAV isolates from the fusion protein gene (F gene) and also the hemagglutinin-esterase gene (HE gene); within site maximum genetic distance (Gen dist) between sequences were calculated using the Kimura 2 parameter model for nucleotide substitution.

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