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Molecular features associated with the adaptive evolution of Infectious Salmon Anemia Virus (ISAV) in Chile

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ABSTRACT

Infectious salmon anemia virus (ISAV) is an Orthomyxovirus challenging salmon production, with a particular impact in Chile. During 2007–2010 a devastating and of unexpected consequences epizootic event almost destroyed a blooming industry in the country. The event was caused by an aggressive variant with a distinctive deletion in Segment 6, one of the eight genomic segments of the virus. After the outburst, although the infective viral variant seemed to have disappeared, a non-infective variant, not previously reported, was discovered and is characterized by a complete, non-deleted coding segment 6, which has prevailed in the fish population until now. This variant, known as HPR0, appears to be the ancestor strain of ISAV from which novel infective variants are generated. Additional variations in segment 5 have also been associated with the virulence observed in the field, an analysis of the differences in these two protein coding segments has been performed. It appears to us that a combinatorial effect exists between the features displayed by segments 5 and 6 which modulate the intensity of viral outbursts. As a result, a theoretical integrative model is presented which explains the different degree of virulence observed in the field based only on molecular data, this could help estimating the intensity of damage a given variant might exert over a productive farm.

1. Introduction

Most pathogenic RNA viruses consist of dynamic populations of ever-changing mutants resulting from rounds of replication with limited fidelity, a reflection of a robust and stable strategy for persistence, variability and adaptation (Más et al., 2010; Randall and Griffin, 2017). This evolutionary modality comprises synergistic clusters of cooperative individuals, each one displaying a distinctive genotypic/phenotypic condition, modulated by dynamic mutations constituting heterogeneous populations of viruses that confuse the host immune system to allow the selection of better fitted variants able to persist and propagate (Domingo et al., 1998; Domingo et al., 2012; Lauring and Andino, 2010; Ojosnegros et al., 2011). Salmon culture under confined conditions in Chile represents an ideal environment for a RNA virus to display its full adaptive potential becoming a highly aggressive pathogen. There are two main reasons to support this hypothesis: the first one is that salmon is an introduced species and when grown in confinement are subjected to a high level of stress in a completely novel and different environment; the second one is that, along with the introduced salmon species, came a novel Orthomyxovirus known as *infectious salmon anemia virus* (ISAV), causative of epizootic outbursts in salmon farms in other latitudes (Bouchard et al., 2001; Lovely et al., 1999; Thorud and Djupvik, 1988). Therefore, the Chilean environment is prone for ISAV to develop and adapt becoming a latent threat for the industry. In fact, a hard proof that this was the case came in 2007–2010 when a devastating ISAV epizootic event occurred for the first time in the country which almost destroyed a 30 year old blooming industry, generating significant losses both from a social as well as from an economics perspective (Mardones et al., 2009).

Thus, the purpose of this work is the evaluation of the present status of the virus in Chile from a molecular point of view in order to foresee if the evolution of distinctive molecular markers associated with pathogenicity and virulence allows estimating the pathogenic potential of the virus in Chilean waters.

From the eight sub genomic segments of the virus, segments 5 and 6 are the most variable ones and have been associated with the virulence displayed by different variants of ISAV (Devold et al., 2001; Kibenge et al., 2001a, 2001b; Markussen et al., 2008; Rimstad and Mjaaland,

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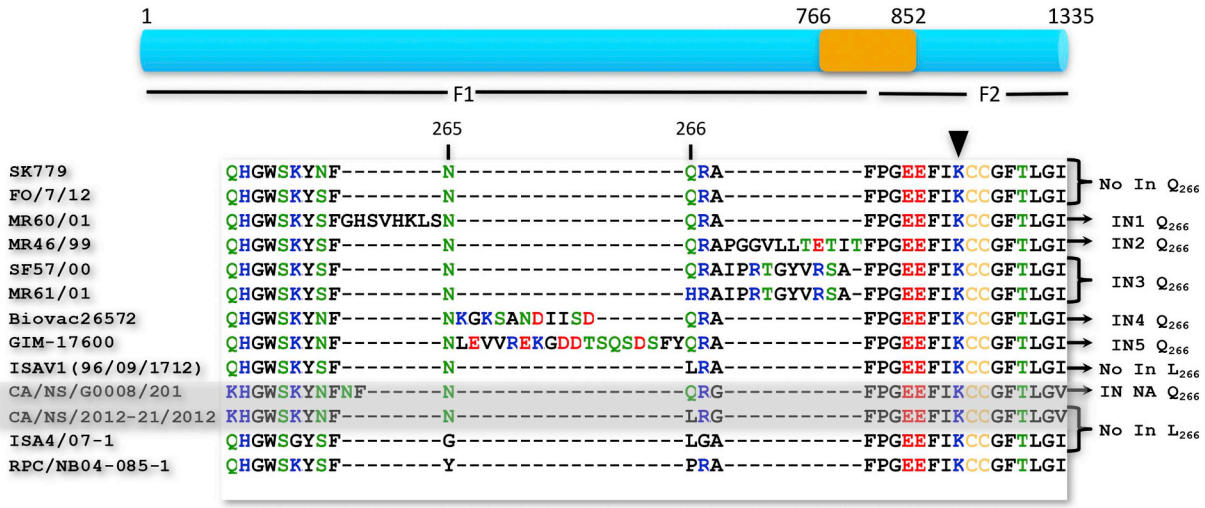

Fig. 1. Summary of the main sequence variations in the fusion protein coded by segment 5 of ISAV. North American isolates are shaded in grey. Right column indicates the feature as presented in Table 1. Amino acids color code: blue: basic; red: acid; green: polar; yellow: cysteine; black: hydrophobic. Numbers are based on isolate SK779 sequences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2002; Snow et al., 2006). Initially, viral strains were classified exclusively according to the integrity of a highly polymorphic region (HPR) in the coding sequence of Segment 6 (Devold et al., 2001; Kibenge et al., 2007b; Nylund et al., 2003; Nylund et al., 2007). The HPR region codes for an in-frame epitope of 35 amino acids towards the C terminal end of the viral, transmembrane glycoprotein, hemagglutinin-esterase (HE) glycoprotein. Surprisingly, the HPR region is found to be complete in nonpathogenic strains of the virus (named HPR0), while differential deletions in this epitope, which do not alter the coding frame of the HE protein, defines the pathogenic variants (known as HPR- followed by a number) (Devold et al., 2001; Nylund et al., 2003). Thus, HPR0 is universally considered to be the ancestor of pathogenic strains as recently suggested (Christiansen et al., 2017) supported by the demonstration that HPR0 co-exists with HPR-deleted variants in diseased fish (Cárdenas et al., 2014). Segment 5 codes for a fusion polypeptide (F protein) and represents the second transmembrane glycoprotein found in the surface of the virus. There are two distinctive molecular features associated with this protein: either an insertion derived from other segments of the virus, or transpositions of sequences among the same segment, and/or a change between two specific amino acids in position 266 (Q/L) in the primary sequence of the F protein (Markussen et al., 2008). A correlation between the variability observed in these two segments seems to define the virulence potential of a given viral HPR-deleted variant while the HPR0 variant conserves the full HPR epitope, lacks any insertion in segment 5 and consistently displays Q in position 266.

The relationship between the features of these two segments has been addressed before (Plarre et al., 2012). However, it has not been robustly demonstrated experimentally, and the level of virulence of the variants observed in the field suggests that such a cooperative effect exists. This correlation can be used as a way of monitoring the coexistence of variants or possible transitions from a non-pathogenic strain to a pathogenic one, constituting a useful diagnostic tool for defining the management strategy to apply in an affected farm.

Based upon the distinctive sequence variations observed in time between these two coding segments of ISAV, we have established a hypothetical, although sustainable, potential pattern of virulence in the field after a thorough review of the available entries in the GenBank database for both viral segments, complemented with information from Chilean isolates derived from our reference laboratory in Chile. Our proposal indicates that a combination of synergistic features involving the primary sequence of either coding segment and their functional protein constitute the key elements defining the potential aggressiveness a given ISAV might express in the field.

Although it is clear that the development and severity of the disease depends not only on the molecular markers, there is also an interrelation of genetic susceptibility (Biacchesi et al., 2007; Kjøglum et al., 2008; Li et al., 2011; Mjaaland et al., 2005), environmental factors and management (Gustafson et al., 2016; Mardones et al., 2013), hence this work seeks a pattern of behavior in the two main markers of virulence described for the virus so far. It should be noted that there is a lack of metadata on many of the samples used in this work, due to the period of time in which they were collected associated with the changes in regulations and management of the ISAV infections in the field. It is also important to note that variable HPR0 was considered only after the outbreak of 2007, when it was discovered and detected, its presence before this date cannot be established since its difficult detection means that no data were available about its existence.

2. Materials and methods

2.1. Analysis of Samples

Data for variability in coding segments 5 and 6 of ISAV isolates were obtained from three different sources as indicated below:

2.1.1. Field Isolates

Field samples are routinely received in the Reference Laboratory as part of the Specific Health Surveillance and Control of the Infectious Salmon Anemia Virus (PSEVC-ISA) program of Sernapesca. Those determined positive for ISAV by the official procedure (Snow et al., 2006) were further analyzed to specifically amplify the corresponding ORFs, amplifying the full ORF when possible or the variable region in other cases, of segments 5 and 6. Full ORFs were cloned using the Thermo Scientific CloneJET PCR Cloning Kit and all samples were validated by DNA sequencing (Macrogen, Seoul, Korea). The origin of the samples is indicated in the maps presented in Supplementary Fig. S1.

2.1.2. Recovered from in vitro infected susceptible tissue culture cells

Four stable virulent strains (HPR-3; HPR-7a; HPR-7b and HPR-14) were originally recovered from the field and passaged *in vitro* using the macrophage-derived SHK-1 cell line by protocols standardized in our laboratory to validate their pathogenic potential as well as to confirm their different sequences in coding segments 5 and 6 (Sepúlveda et al., 2012).

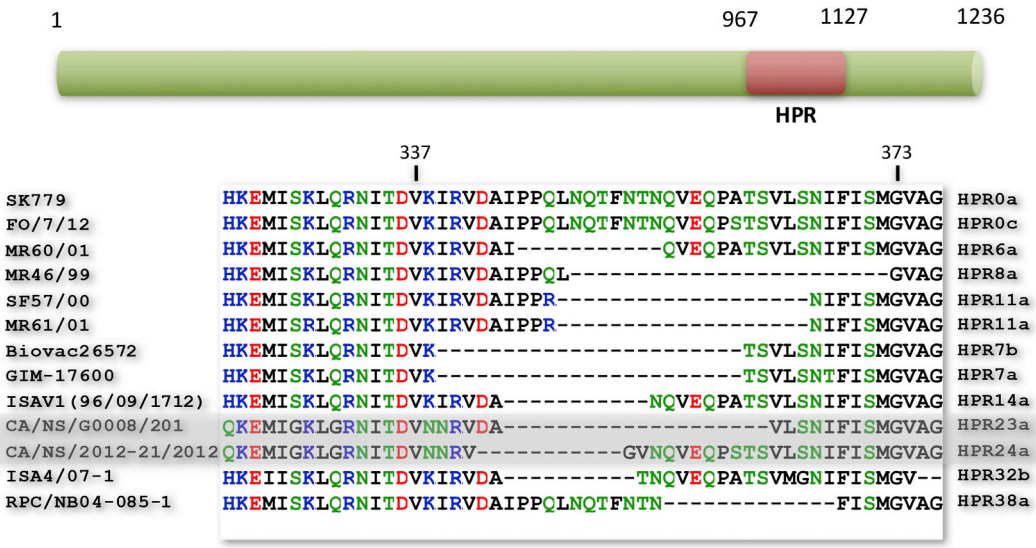

Fig. 2. Example of HPR sequences of glycoprotein HE coded by segment 6 of ISAV for the same isolates presented in Fig. 1. North American isolates are shaded in grey. Right column indicates segment 6 strain label as presented in Table 1. Amino acids color code: blue: basic; red: acid; green: polar; yellow: cysteine; black: hydrophobic. Numbers are based on isolate SK779 sequences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.1.3. Entries available at GenBank database

The GenBank review was centered on reliable entry sequences for ISAV segments 5 and 6. Additionally, target sequences reported on ISI papers were also reviewed as a complementary procedure to strengthen the specific sequence pattern associated with either one of the two target coding viral segments.

2.2. Distinctive features of coding segments 5 and 6

Additionally, each segment was analyzed according to the specific behavior described until now; these features are summarized below:

2.2.1. Segment 5

For the fusion glycoprotein (F) encoded in segment 5, several sites have been associated with its processing and virulence. F protein is cleaved to form the F1 and F2 sub-units at K_{276} to form the functional dimer of the protein (Fourrier et al., 2015). The amino acid in position 266, has been postulated as a virulence marker (numbers are in reference to the isolate SK779, EU118819 accession number for segment 5), with the presence of the amino acids Glutamine (Q) and Leucine (L) mainly, but with other amino acid changes as well (Fig. 1 summarize the main features of segment 5). In this site, the presence of inserted sequences, all coming from some segment of the same virus, has also been reported (Table S1 and Fig. S2).

Table 1
Summarized correlations between distinctive features of segment 5 and segment 6 of reported GenBank sequences.

Segment 6	Segment 5	AA_{266}	Reported sequences	Associatted ISAV strain[a]
S_6	S_5	S_5		
HPR Non-deleted	No insert	**Q**	35	HPR0a, HPR0c
	No insert	**L**	0	–
	Any insert	**Q/L**	0	–
HPR Deleted	No insert	**Q**	6	HPR14b, HPR3a, HPR7b[b]
	No insert	**L**	206	Not HPR0
	No insert	**P**	1	HPR38a
	Any insert	**Q**	52	HPR6a, HPR8a, HPR7a, HPR7b, HPR12b, HPR11a, HPR23a
	Any insert	**L**	0	–
	Any insert	**H**	2	HPR11a

[a] HPR (Cárdenas et al., 2017).
[b] coexistence of HPR0 and HPR7b. (Cárdenas et al., 2014).

2.2.2. Segment 6

Hemaglutinin esterase (HE), the second viral surface glycoprotein and encoded by segment 6, has been proposed to be one of the main virulence factors. Until now the classification of ISAV strains has been made based on the high polymorphic region of genomic segment 6 (HPR), and specifically in the quality of the deletion (extension and location) of the resulting HE (Fig. 2). The strain HPR0, considered as the ancestor (Christiansen et al., 2011; Lyngstad et al., 2012; Mjaaland et al., 2002), does not present deletions, and does not lead to the development of the disease in the field. All pathogenic strains have deletions of different magnitude from 7 to 24 amino acids (21 to 72 base pairs). Most of the deletions are located within HPR region; however, there are some reported sequences not following this pattern. It should be noted that the deletions comprise complete codons so that the open reading frame is conserved. ISAV variants have been classified according to the HPR region, but due to the rapid spread of the information a high degree of disorder was generated in the denomination of new variants. A reorganization of the label of the strains was proposed by our group (Cárdenas et al., 2017) based in GenBank sequences, and related publications.

2.3. RNA extraction, cDNA synthesis and specific amplification

Total RNA, either from infected tissues and/or from tissue culture infected cells, was obtained as previously described (Sepúlveda et al., 2012). Initial cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) with random hexamer primers, followed the protocol recommended by the supplier. Polymerase Chain Reaction (PCR) was performed using One *Taq* DNA Polymerase (New England Biolabs) according to the manufacturer's instructions, with 200 nM of each specific primer, and 1 µL of cDNA as template. Thermal conditions used were: initial denaturation: 3 min at 94 °C, followed by 40 cycles of 94 °C for 30 min, 55 °C for 30 min and 68 °C for 2 min, with a final extension of 4 min at 72 °C.

Table 2
Chi-Square test results for segment 5 and segment 6 main features. Observed values are indicated in bold letters, expected values in italics, individual chi-squared values are in parentheses. Table on the bottom right has the result of the test.

S6	HPR Non-deleted	HPR Deleted	
S5			
No insert Q_{266}	**35** *4.80* (190.04)	**6** *36.20* (25.20)	***41***
Insert Q_{266}	**0** *6.09* (6.09)	**52** *45.91* (0.81)	***52***
No Insert L_{266}	**0** *24.11* (24.11)	**206** *181.89* (3.20)	***206***
	35	***264***	***299***

χ^2	249.443
df	2
p-value	0.0000
Yates´ χ^2	240.168
Yates p-value	0.0000

Table 3
Summarized correlation between distinctive features of segment 5 and segment 6 of Chilean isolates.

Segment 6	Segment 5	AA_{266}	Associatted ISAV strain
S_6	S_5	S_5	
HPR Non-deleted	No Insert	Q	HPR0a
HPR Deleted	No insert	L	HPR2a, HPR3a, HPR7a, HPR7b, HPR8a, HPR9a, HPR14a, HPR35a
	IN4	Q	HPR7b
	IN5	Q	HPR7a

2.4. Design of specific primers

Oligonucleotides were designed based on multiple sequences alignments to selectively amplify the full ORFs as well as the variable regions of segment 5 and 6; CLC Main Workbench 6.9.1 (https://www.qiagenbioinformatics.com/) was used for the design and OLIGO 4.1 software (National Biosciences Inc.) was used to optimize thermodynamic properties of the primers. The primers designed and used are shown in Table S2.

2.5. Sequencing

All resulting amplicons were resolved by 1% agarose gel electrophoresis in Tris-Borate-EDTA (TBE) Buffer (Winkler), purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific) and subsequently cloned in the pJET 1.2 vector using the CloneJET PCR Cloning Kit. Chemically competent TOP10 cells (Thermo Fisher Scientific) were transformed using the heath-shock method. Cells were plated in LB plates containing 50 µg/mL of ampicillin. After overnight culture at 37 °C, selected colonies were grown in liquid LB media containing the antibiotic. After 16 h, cells were pelleted at 5000 x *g* and pDNA was extracted using GeneJET Plasmid Miniprep Kit according to the manufacturer's instructions. Plasmids were sequenced using the pJET1.2F and pJET1.2R primers using Sanger Sequencing (Macrogen, Seoul, Korea).

2.6. Bioinformatics analysis

All DNA sequences for segments 5 and 6 from the same isolate were analyzed using the CLC MainWorkbench 6.9.1 tool (https://www.qiagenbioinformatics.com/). GenBank entries were uploaded into the software and compared with the sample sequences obtained either from the field, from ISI journal reports and/or from the *in vitro* passages. Alignments of the variable region for the reported sequences, along with a sample of the sequences found in GenBank, for each segment are shown in Supplementary Figs. S3 and S4.

2.7. Phylogenetic analysis

For the construction of phylogenetic trees, isolates with 800 base pairs or more in each segment were chosen. The sequences were trimmed to have the same number of base pairs in all the isolates, and the variable regions were eliminated, conserving the ORF. For the construction of the trees this two sets of data were used, with 662 base pairs for segment 5 and 858 for segment 6, and a total of 256 isolates. MEGA 6 software (Tamura et al., 2013) was used for determining the best model to construct phylogenetic trees with the maximum likelihood method. Segment 5 sequences were analyzed with the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985) with gamma distribution and with 1000 bootstrap iterations, while segment 6 sequences were analyzed with the Kimura 2-parameter model (Kimura,

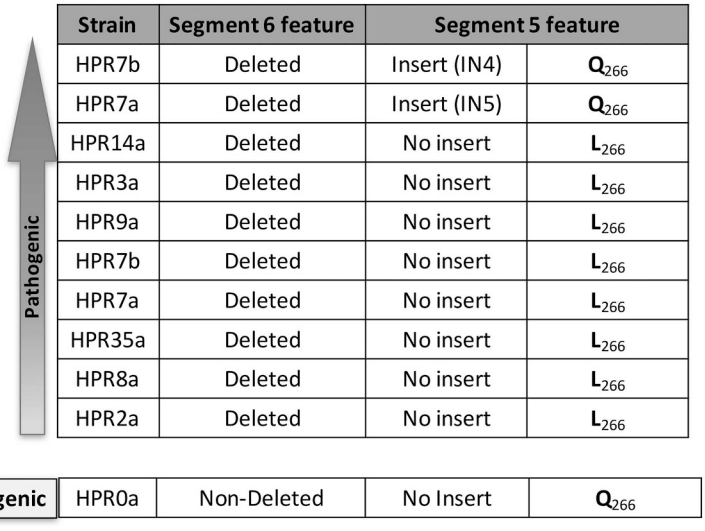

Fig. 3. Proposed scheme of the pathogenicity for Chilean strains behavior in the field, and their correlation with segments 5 and 6 features.

1980) with gamma distribution and with 1000 bootstrap iterations.

2.8. Ethics statement

Field samples used in this study were provided by Sernapesca, the official national Chilean entity responsible for aquatic management control as part of the Specific Health Surveillance and Control of the Infectious Salmon Anemia Virus (PSEVC-ISA) and processed by standardized procedures validated in the Reference Laboratory for ISAV Diagnosis and in compliance with the procedures and standards established by Sernapesca as well as by Chapter 7.4 of "The Aquatic Animal Health Code" of the World Organization for Animal Health (OIE).

3. Results

3.1. Sequence validation of relevant regions in segments 5 and 6.

To perform our comparative analysis, 50 new different isolates were sequenced for segments 5 and 6 (Table S3), plus the sequences of 248 isolates already available in GenBank (Supplementary Table S4, Figs. S3 and S4). The subject of analysis consisted in the comparative sequence profiles of the variable regions of segment 5 as well as in the integrity of the HPR region of segment 6. Two target features were selected for segment 5: the variable region where insertions tend to occur, and the amino acid nature in position 266 (Fig. 1). On the other hand, one single target was selected for segment 6: the intact or deleted nature of the HPR region (Fig. 2).

Since a specific correlation regarding the virulence potential of a given variant between these two segments has not been established yet, a pattern correlating the integrity of the HPR region in segment 6 with the two main features described for segment 5 was proposed. The proposed pattern was firstly supported by the fact that, without exception, all pathogenic variants harbor deletions to different extent in the HPR region (HPR-deleted), lack insertions in segment 5 and contain a Leucine residue in position 266. Secondly, all variants lacking deletions in segment 6 HPR region (HPR0) harbor a Glutamine residue in position 266. Thirdly, all variants harboring an insertion in segment 5, normally contain a Glutamine residue in position 266 and in all cases matching with a deletion in the HPR region. Notwithstanding, the available data in GenBank indicate that some exceptions occur: (a) two isolates have been reported bearing Histidine in position 266, an insertion in segment 5 and a deletion in the HPR region of segment 6; (b) one isolate with Proline in position 266, and a change of Tyrosine by Asparagine in position 265 (Fig. 1), (c) six isolates bearing Glutamine in position 266 and no insertion in segment 5 although bearing a deletion in the HPR region of segment 6. Interestingly enough, three of these sequences were reported by our laboratory corresponding to field samples recovered from the same farm where co-existence of HPR0 and HPR7b variants was detected in the same fish. These combinations are summarized in Table 3.

Additionally, a Chi-square (X^2) analysis was made to establish the dependence or not of the segment 5 and segment 6 distinctive features indicated in Table 1, and the result showed an interdependence of the two segments as summarized in Table 2.

Considering that ISAV was detected for the first time in Chile only in the early 2000′, it was logical to think that the pathogenic variants were in agreement with those reported in Norway, country from where the salmon species were introduced. Notwithstanding, significant changes were detected. The highly pathogenic variant HPR7b, although sharing the same deletion in segment 6 as the Norwegian strain, has an insertion in segment 5, and a glutamine as amino acid 266, while in the Norwegian strain is leucine. Eventually, new outbursts occurring in Chilean productive farms displaying different degree of aggressiveness have yielded novel isolates sharing alternative and/or novel combinations in the key sites of segments 5 and 6, including new insertions in

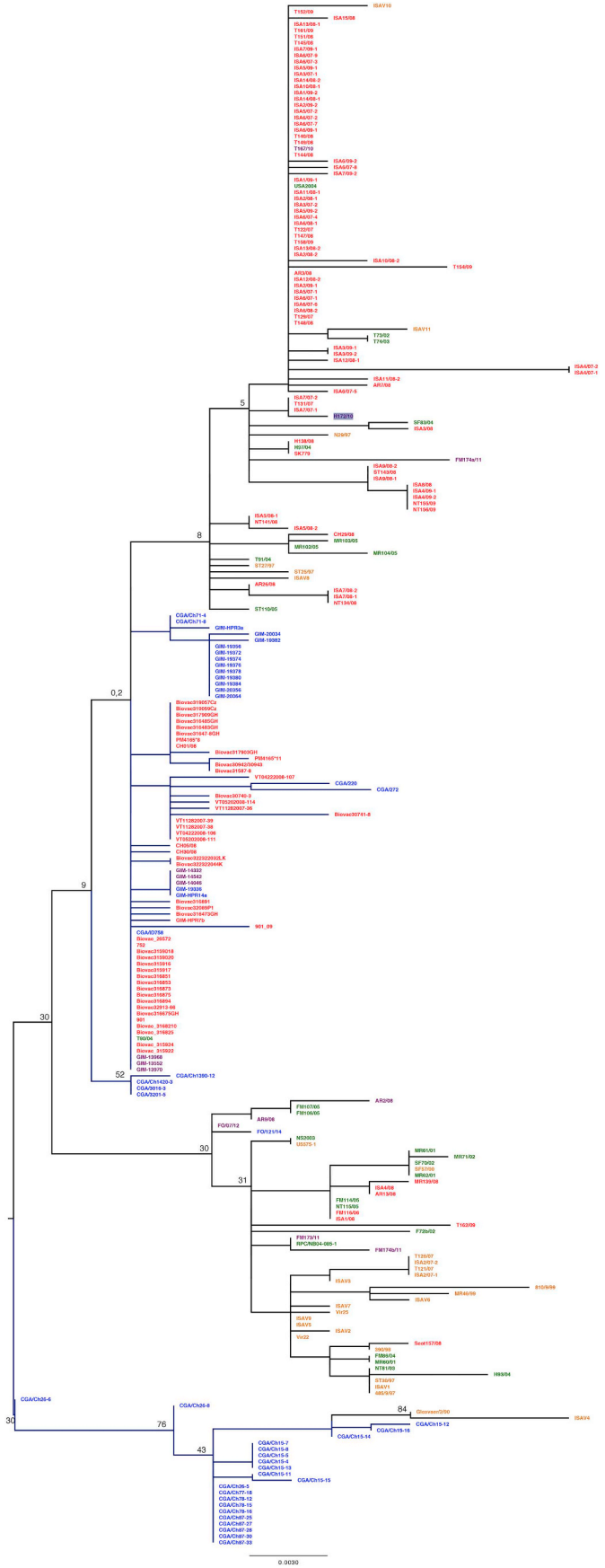

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Fig. 4. Phylogenetic tree for segment 5. 248 sequences of European genogroup were used. The tree was constructed by using the Maximum Likelihood method. The tree with the highest log likelihood (−2233.0578) is shown. The Boostrap value for the main branches is shown. The analysis involved 248 nucleotide sequences and a total of 662 positions in the final dataset. Branches in dark blue correspond to Chilean isolates. Code color indicate isolation years: Orange 1980–2000, green 2001–2005, red: 2006–2009, purple: 2010–2012, and blue: 2013-onwards. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013) and the trees were edited with FigTree v.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the former and different extent deletions in the latter. Thus, the plasticity observed in the analyzed sequences and the correlation of features existing among these two segments represents a clear indication that the virus is evolving in the new environment.

With the accumulated molecular data, we have generated both; a table that summarizes the specific features in coding segments 5 and 6, that differentiates virulent from nonvirulent isolates; as well as a proposal that reflects the differential aggressiveness observed subjectively in the field by the viral variants officially reported until our days (Table 3 and Fig. 3[1] respectively).

3.2. Phylogenetic analysis

Phylogenetic trees for both segment 5 and segment 6 showed a clear separation of North American and European genogroup, with a bootstrap value of 100 (data not shown), so the analysis was done only with European genogroup sequences, with 228 isolates in total. For both segments, the Chilean isolates were clustered together (Figs. 4 and 5). The isolates are indicated in color according to the isolation years; with orange for isolates of year 2000 and before, green for 2001–2005, red for 2006–2009, purple for 2010–2012 and blue for 2013 onwards.

4. Discussion

Acute diseases driven by RNA viruses are normally expected to be controlled by the innate and adaptive immune responses of the host, ideally resulting in a full clearance of them. Notwithstanding, viruses take advantage of this situation designing strategies that mislead the host, persisting and allowing them to express their full pathogenic potential in time. RNA viruses, other that the Retroviridae family, establish what it is known as 'within host' persistent infections, a chronic condition in an apparently virus-free host, silently replicating to a low threshold level and being able to reactivate the disease at later times.

ISAV is a novel Orthomyxovirus that clinically affects *Salmo salar* species kept under confined rearing conditions. This virus was introduced in Chile altogether with salmon species for commercial purposes, resulting in an unbalance of the new environment and clearly creating an ongoing evolutionary opportunity for both host and pathogen, to trigger and develop molecular strategies to adapt, mutate and persist. ISAV virus is known to display different degree of virulence when harboring a defined deletion in a highly polymorphic region (HPR) in coding segment 6 (Devold et al., 2001; Kibenge et al., 2001a, 2001b; Mjaaland et al., 2002), one of the eight segments comprising the viral genome. Nevertheless, other coding sequences have also demonstrated a dynamic fluidity associated with the pathogenicity of a given viral variant. Indeed, coding segment 5 seems to be the target for internal rearrangements as well as for hosting insertion sequences coming from other viral coding segments (Aspehaug et al., 2005; Devold et al., 2006; Kibenge et al., 2007a). Surprisingly, none of the alterations, *i.e.* deletions in segment 6 and or insertions in coding segment 5, affected the original open reading frame (ORF) of the corresponding resulting proteins. Therefore, these are not random events; instead they seem to be components of a well-defined, concerted and highly regulated ongoing survival strategy. We concluded that the variability in coding segments, 5 and 6 represents to a large extent the plasticity of the virus resulting in an ever-changing dynamic adaptation in full agreement with the quasispecies theory known to rule variability and persistence in RNA viruses. Here it is proposed that the molecular changes affecting these two coding segments are not random or independent events. On the contrary, there seems to be an additive association between the independent changes, resulting in attenuated or exacerbated expression of target viral variants, although always controlled by a strain, devoid of a deletion in segment 6, with no insertion in segment 5 and with a conserved amino acid residue in position 266 in the boundary region in segment 5 where insertions tend to occur. Thus, *in vivo* infection in the field is a combination of an unstable viral population where one particular variant seems to outstand defining the detectable clinical features.

The phylogenetic analysis of the data set showed the association of the Chilean isolates, and, although the bootstrap values were low, there seems to be a temporary association according to the year of isolation. Chilean isolates from 2006 to 2009 (in red in Figs. 4 and 5), are clustered together and most of them correspond to the highly virulent variant HPR7b. More subtle relationships were not established taking into account that the data set was limited.

The ISAV study has evolved, as the pathogen, together with the surveillance programs and the management of the disease in the field; as described by Gustafson (Gustafson et al., 2016). The presence of pathogenic strains of the virus have been decreasing over time, but the HPR0 variant is still present and since 2012, when it began to be included in the official reports (SERNAPESCA, 2012, 2013, 2014, 2015, 2016, 2017); 65, 73, 33, 69, 41 and 49 cases have been reported annually in the country. However, it is not possible to establish a correlation of the pathogenic behavior with the location and date of collection of the samples analyzed, maybe due to the low number of samples with sequence information for both segments.

5. Conclusions

In this scenario, ISAV infections involves the HPR0 variant as the ancestor virus; responding to either external or internal signals, infective variants are generated by first inducing deletions in the HPR region in coding segment 6 and jointly or in time, specific modifications in coding segment 5, resulting in pathogenic variants of different virulence.

Therefore, HPR0 represents a transient form of ISAV which produces an intermediate infection without clinical signs and with a ubiquitous presence in almost all productive farms in Chile (SERNAPESCA, 2016) as well as in other salmon producing countries (Christiansen et al., 2011; Lyngstad et al., 2012).

Currently, although it is not considered in the norm, most of the detected variants are sequenced for both segment 5 and segment 6, which can be an advantage in the case of finding coexistence, or detecting a case of a variant in "transition", which it can have a complete segment 6 and be considered HPR0, but a segment 5 with some modification associated with a pathogenic variant.

Nonetheless, it has to be kept in mind that ISAV is an 8 sub-genomic segments containing pathogen, and that the dynamic variability observed in natural populations might not be circumscribed only to the changes observed in coding segments 5 and 6. We have already described that insertions, mimicking transposition-like events, occur over segment 5, derived from other viral coding segments, altogether with deletions occurring in the HPR region in segment 6. Additionally,

[1] It should be noted that: 1.- The periods of time in which the sick fish were taken out of the water are different due to the updating of the regulations from 2007 to 2017. This implies that, the evolution time of the disease was different and in some cases, it may have some relation to the clinical presentation.

2. The timing in terms of fish size and weight of the different cases may also be related to the severity of the clinical presentation.

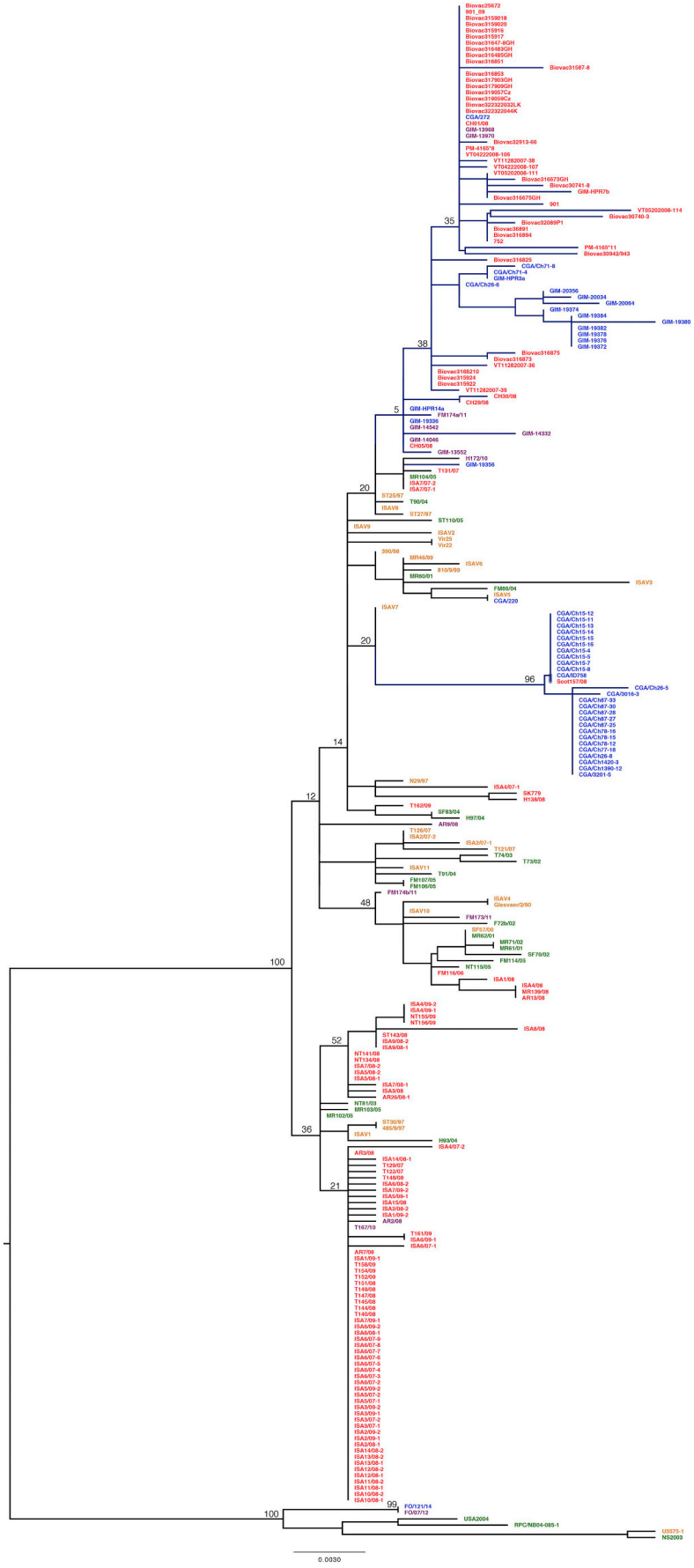

Fig. 5. Phylogenetic tree for segment 6. containing Chilean isolates. Isolates from this report are indicated by a red diamond, other Chilean isolates are indicated by a black triangle. The tree was constructed by using the Maximum Likelihood method. The tree with the highest log likelihood (−3146.2180) is shown The Boostrap value for the main branches is shown. The analysis involved 248 nucleotide sequences and a total of 857 positions in the final dataset. Branches in dark blue correspond to Chilean isolates. Code color indicate isolation years: Orange 1980–2000, green 2001–2005, red: 2006–2009, purple: 2010–2012, and blue: 2013-onwards. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013) and the trees were edited with FigTree v.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

different ISAV variants have been reported coexisting in infected individuals, especially under strong selective pressure, a clear demonstration that other rearrangements might also exist impacting the pathogenic potential of the virus population in a given epizootic outburst. In this framework, additional strategies might also be involved in defining the variability observed, such as genetic assortment and reciprocal and non-reciprocal recombination among the distinct variants in the quasispecies population, which are important mechanisms known to have pivotal roles in the evolution of other segmented RNA viruses.

Thus, based on the information available today, we have proposed a model that correlates the field pathogenicity of ISAV variants in Chile, as an operational tool for estimating the impact they may have on the suitability of national and foreign production centers.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.12.028.

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