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Parallel phylogenetic analyses using the N, G or Nv gene from a fixed group of VHSV isolates reveal the same overall genetic typing

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ABSTRACT: Different genetic regions representing the viral phospho- (P), nucleocapsid- (N) or glyco-protein (G) gene have been used for phylogenetic studies of viral haemorrhagic septicaemia virus (VHSV). Since these analyses were performed on different virus isolates using various genomic regions, it has been difficult to evaluate how the choice of target region affects the output of the analyses. To address this, we sequenced and performed parallel phylogenetic analysis of an N gene fragment, the entire Nv (non-structural protein) and G genes, and 4 different fragments of the G gene from a fixed virus panel. The overall genotyping of the selected isolates was identical for the 7 target regions, but separation of Genotype I sub-lineages was best when the analysis was performed on the full length G gene (1524 nucleotides, nt). Good resolution was furthermore obtained using smaller sequencing windows represented by a G gene fragment (nt 360 to 720) or the Nv gene (366 nt), although these regions had different characteristics with respect to resolution of Genotype I sub-lineages and resolution within Sub-lineage Ia. Phylogenetic analysis based on the deduced amino acid sequences was also performed. The phylogenetic relationship between the nucleotide and amino acid sequences of the isolates corresponded best in the case of the N gene/protein. For the 6 other genomic regions, genetically distant isolates occasionally grouped together when compared at protein levels. No clear relationship between the G gene genotyping and serotyping with neutralising (G protein specific) antibodies was observed, stressing that epidemiological analysis based on phenotypic characteristics such as serotype could be misleading.

KEY WORDS: Genotyping · Epidemiology · Rhabdovirus · Functional protein · Amino acid

INTRODUCTION

The fish rhabdovirus viral haemorrhagic septicaemia virus (VHSV) is a significant viral pathogen in the European aquaculture industry, as disease outbreaks may result in losses that approach 100% in farmed rainbow trout (Jørgensen 1992). VHSV has also been isolated from many free-living anadromous and marine fish species from European, North American and Japanese waters, indicating a widespread occurrence of the virus in the marine environment of the northern hemisphere (reviewed by Skall et al. 2004).

VHSV is an enveloped negative strand RNA virus belonging to the *Novirhabdovirus* genus of the *Rhabdoviridae*. The viral genome consists of approximately 11200 nucleotides and contains 6 genes in the order 3′-N-P-M-G-Nv-L-5′, encoding 5 structural proteins—nucleocapsid- (N), phospho- (P), matrix- (M), glyco- (G) and RNA polymerase- (L) proteins—and a non-structural (Nv) protein with unknown function (Schutze et al. 1999).

Initial sequence studies and phylogenetic analyses based on 2 short regions within the G gene of European and North American VHSV isolates identified the existence of 3 major genotypes of the virus, and confirmed that significant differences existed between strains from North America and Europe (Benmansour et al. 1997, Stone et al. 1997). Further analyses based on the most variable region of the VHSV N gene identified 4 genotypes with isolates from European fresh-

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water and the Baltic Sea in Genotype I, isolates from a narrow region in the Baltic Sea in Genotype II, isolates from the North Sea/coastal waters of the United Kingdom in Genotype III, and isolates from the Eastern Pacific Ocean in Genotype IV (Snow et al. 1999). Recent phylogenetic analysis performed by Nishizawa et al. (2002) and Kim et al. (2003) based on parts of the G and P genes indicated that Japanese and Korean isolates group with the North American isolates. A recently developed genotyping assay based on restriction fragment length polymorphism (RFLP) analysis of the VHSV G gene has confirmed this observation (Einer-Jensen et al. 2005). This RFLP assay furthermore allowed typing of an isolate originating from the waters east of North America (Flemish Cap) as being Genotype III, indicating an overlapping migration pattern with European fish.

The fact that various genes and fragments have been used during the described studies has led to some confusion. When using the G or P gene fragments, 3 genotypes were identified (Benmansour et al. 1997, Stone et al. 1997, Nishizawa et al. 2002, Kim et al. 2003), whereas 4 types were found when using the N gene fragment (Snow et al. 1999). A recent comprehensive evolutionary study based on the entire G gene of VHSV did, however, confirm the presence of 4 major genotypes (Einer-Jensen et al. 2004). This finding brought attention to the basic problem that the above-mentioned phylogenetic analyses had been performed on different groups of VHSV isolates using different regions of the genome. The present study was initiated in order to clarify how the choice of target region and fragment length affects the phylogenetic analyses when performed on a representative panel of VHSV isolates. The selected regions herein correspond to those previously reported by other authors (G gene, nt 220 to 1293 [Benmansour et al. 1997]; G gene, nt 360 to 720 [Stone et al. 1997]; G gene, nt 710 to 995 [Thiéry et al. 2002]; G gene, nt 175 to 761 [Nishizawa et al. 2002]; full length G gene, nt 1 to 1524 [Einer-Jensen et al. 2004]; N gene, nt 23 to 445 [Snow et al. 1999]. This is the first study to include phylogenetic analyses of the Nv gene.

MATERIALS AND METHODS

The virus panel consisted of 16 isolates representing the 4 known genotypes (Table 1). Virus isolates were propagated on monolayers of BF-2 cells in low (usually 3 to 7) passage numbers and viral RNA was extracted by the use of affinity spin columns (as described by Einer-Jensen et al. 2004). Purified RNA was eluted in 30 µl RNase free water, aliquoted into 6 tubes and stored at –80°C until used.

Table 1. Genotype and serotype data for the 16 analysed VHSV isolates. Additional information on year of isolation, geographic origin and host species has been reported elsewhere (Einer-Jensen et al. 2004) and the genotype data listed here were acquired in the same study by phylogenetic analysis of the entire G gene. Serotype data generated according to Olesen et al. (1993) and kindly provided by Dr. N. J. Olesen, Danish Institute for Food and Veterinary Research). nd: no data

Isolate code	Geno type	Sero-group	GenBank Accession No.
AU-8/95	Ia	III	DQ159192 (N fragment) AY546570 (G) DQ159197 (Nv)
DE-Fil3	Ia	I	Y18263 (N fragment, G, Nv)
DK-Hededam	I	I	Z93412 (N fragment, G, Nv)
DK-M.rhabdo	Ib	I	Z93414 (N fragment, G, Nv)
DK-1p40	Ib	nd	AJ130919 (N fragment) AY546575 (G) DQ159200 (Nv)
DK-1p53	II	nd	AJ130921 (N fragment) AY546577 (G) DQ159195 (Nv)
DK-3592B	Ia	I	AF012093 (N fragment) X66134 (G) DQ159198 (Nv)
DK-6137	Ia	III	DQ159190 (N fragment) AY546593 (G) DQ159199 (Nv)
FR-07-71	Ia	I	D00687 (N fragment) AY546616 (G) AJ233396 (Nv)
FR-14-58	Ia	nd	AF143863 (N fragment, G, Nv)
FR-23-75	Ia	II	DQ159191 (N fragment) AY546617 (G) DQ159196 (Nv)
GE-1.2	Ie	I	DQ159189 (N fragment) AY546617 (G) DQ159201 (Nv)
UK-96-43	Ib	nd	AF143862 (N fragment, G, Nv)
UK-860/94	III	I	AJ130915 (N fragment) AY546628 (G) DQ159203 (Nv)
UK-H17/2/95	III	II	AJ130924 (N) AY546629 (G) DQ159202 (Nv)
US-Makah	IV	I	X59241 (N fragment) U28747 (G) DQ159204 (Nv)

Primers for full length RT-PCR amplification of the N or Nv genes were designed on the basis of available genomic sequences of VHSV. The 635 bp RT-PCR amplicons encoding the Nv gene were generated using the Nv sense (5′ ATGCAGCAGTTCTCCAGAA 3′) and Nv anti-sense (5′ TGTACTTCCCGGTC-TAGTTC 3′) primers, whereas the N1 sense (5′ TGGAAAGTGAAAGTTGAAC 3′) and N1 anti-sense (5′ AACGGCACGATTATAAGAAT 3′) primers were used for amplification of a 1340 bp fragment encoding the N gene.

The RT-PCR products were synthesised by single tube RT-PCR and sequenced as described previously (Einer-Jensen et al. 2004). The Nv amplification primers were also used for sequencing the Nv gene, whereas the N1 sense primer and N2 anti-sense primer (5′ TGGGGAACTGCAGCACT 3′) were used for sequencing an N gene fragment (nt 23 to 445 of 1209) similar to that described by Snow et al. (1999). All data related to RT-PCR amplification and sequencing of the entire G gene (nt 1 to 1524) have recently been published elsewhere (Einer-Jensen et al. 2004).

Sequence alignments were performed using CLUSTAL W (Higgins et al. 1994) from the Genetics Computer Group (GCG) package (Wisconsin Package Version 10.3, Accelrys). The MODELTEST (Version 2.0) program (Posada & Crandall 1998) was then used to identify the model that best fits the nucleotide sequence data, from 24 different models, using the Akaike information criterion (AIC) (Akaike 1974). The estimated parameters were subsequently used during the phylogenetic analyses of nucleotide sequences using the neighbour-joining distance method in the PAUP* (Version 4.0) program (Swofford 2000). The predicted amino acid sequences were analysed using the seqboot, protdist and neighbor commands (default parameters) within the Phylip (Version 3.5c) program (Felsenstein 1989) also from the GCG package. All analyses were based on 1000 replicates to assess the robustness of the branch order, and nodes with bootstrap values less than 70% were collapsed. The phylogenetic trees were displayed using the drawing software TreeView (Page 1996) and nucleotide identity within the VHSV population was determined using GeneDoc (Nicholas et al. 1997).

RESULTS

Complete G gene sequences for the selected set of 16 VHSV isolates have been previously described (Einer-Jensen et al. 2004). The N and Nv primer sets were used for RT-PCR amplification of the desired target genes, and DNA fragments showing the expected sizes (1340 and 635 bp, respectively) were successfully obtained (data not shown). Nucleotide sequencing was performed on both strands, and subsequent alignments revealed that the obtained sequences of the respective partial N and complete Nv genes were unique for the 16 virus isolates. The level of nucleotide divergence appeared to be dependent on the target region, as 85 to 100, 73 to 99 and 84 to 98% identities were observed within the full length G, Nv genes and the partial N gene fragment, respectively. The North American isolate (US-Makah) had the lowest degree of similarity with the other isolates and was therefore used as an outgroup root in the trees.

Sequence alignments of the Nv genes revealed that 2 different lengths of the open reading frame (ORF) existed among the analysed isolates: the Genotype II isolate had a 390 nucleotide (nt) ORF, whereas all other isolates had a 366 nt ORF. Both stop codons were present within Genotype I and IV isolates, whereas only the first or the second were present within Genotype III or II isolates, respectively. Additional sequencing of 2 more Genotype II isolates (DK-1p49 and DK-1p55, GenBank Accession No. DQ159193 and DQ162801, respectively) confirmed the longer ORF. The importance of this observation is presently unknown, as Genotype II isolates have not been characterised by phenotypic properties (e.g. pathogenicity) that differ

Table 2. Identification of model. MODELTEST analysis by maximum-likelihood allowed identification of models that best fit the individual nucleotide data set. Estimated parameters were base frequency, rates of substitution matrix, proportion of invariable sites (PInvar) and gamma distribution of rates variation (gamma shape) among sites. Model selected: general-time-reversible (GTR+G) or symmetrical model (SYM+G). Eq. all: equal rates for all sites

	G gene					Nv gene	N gene
	nt 1–1524	nt 220–1293	nt 360–720	nt 175–761	nt 710–995	nt 1–366	nt 23–445
Model selected	GTR+G	GTR+G	GTR+G	GTR+G	SYM+G	GTR+I	GTR+G
Base frequencies							
A	0.2659	0.2738	0.2841	0.2680	0.25	0.2628	0.2347
C	0.2782	0.2737	0.2858	0.2987	0.25	0.3201	0.2462
G	0.2301	0.2436	0.2265	0.2272	0.25	0.1994	0.3015
T	0.2259	0.2089	0.2036	0.2061	0.25	0.2177	0.2176
Rate matrix							
(a) [A-C]	1.9185	2.4466	2.1339	3.3137	2.4408	3.4971	1.4935
(b) [A-G]	8.0752	9.2788	7.9580	12.5618	7.9065	20.0658	3.7799
(c) [A-T]	0.3518	0.6410	0.0000	0.0000	0.9892	1.7568	0.4885
(d) [C-G]	0.1346	0.1023	0.0000	0.0000	0.0000	0.3033	0.1254
(e) [C-T]	12.1500	15.8323	12.6240	17.7114	11.4614	21.5787	5.2727
(f) [G-T]	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
PInvar	0	0	0	0	0	0.4684	0
Gamma shape	0.3410	0.3336	0.3484	0.3294	0.3630	Eq. all	0.3005

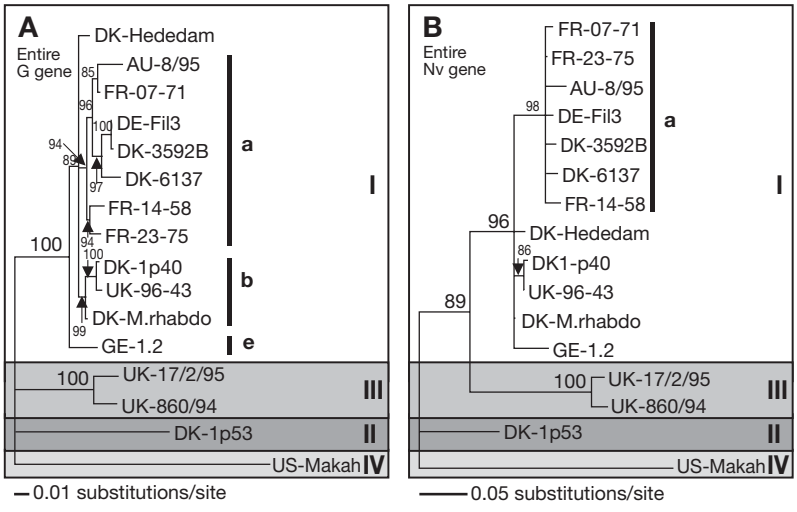

Fig. 1. Neighbour-joining distance trees of full length genes with bootstrap values >70% (from 1000 data set). Sub-lineages Ia,b,e corresponding to those described by Einer-Jensen et al. (2004) (see 'Genotype' column, Table 1) are indicated. Full length (A) G gene (1521 nt) and (B) Nv gene (366 nt). Isolate codes as in Table 1

from other marine isolates (Skall et al. 2004). The lengths of the Nv gene sequences used for the following phylogenetic analysis corresponded to the protein encoded by the short ORF.

The best-fit model and parameters for phylogenetic analysis of the individual nucleotide sequence data were estimated using the MODELTEST program (Table 2). In the trees generated, the number of bootstrap values above 70% from the 2 full length genes were 11 for the G gene (1521 nt), and 4 for the Nv gene (366 nt) (Fig. 1). The 4 genetically distinct groups had significant branches and matched the genotyping previously identified through the full length G gene based study by Einer-Jensen et al. (2004) (summarised in Table 1, Fig. 1). Similarly the sub-lineage pattern (Ia, b, e) within the full

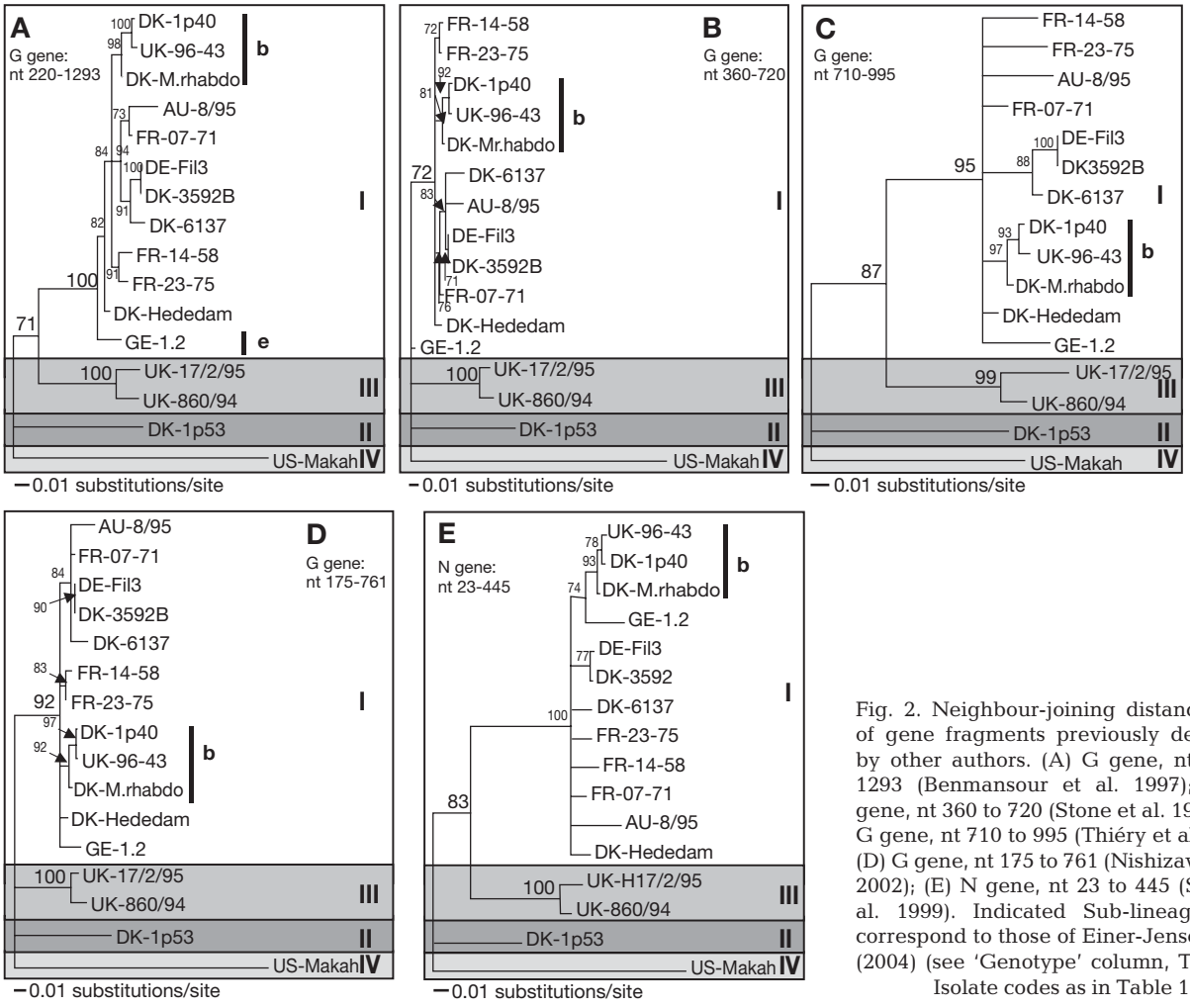

Fig. 2. Neighbour-joining distance trees of gene fragments previously described by other authors. (A) G gene, nt 220 to 1293 (Benmansour et al. 1997); (B) G gene, nt 360 to 720 (Stone et al. 1997); (C) G gene, nt 710 to 995 (Thiéry et al. 2002); (D) G gene, nt 175 to 761 (Nishizawa et al. 2002); (E) N gene, nt 23 to 445 (Snow et al. 1999). Indicated Sub-lineages Ib,e correspond to those of Einer-Jensen et al. (2004) (see 'Genotype' column, Table 1). Isolate codes as in Table 1

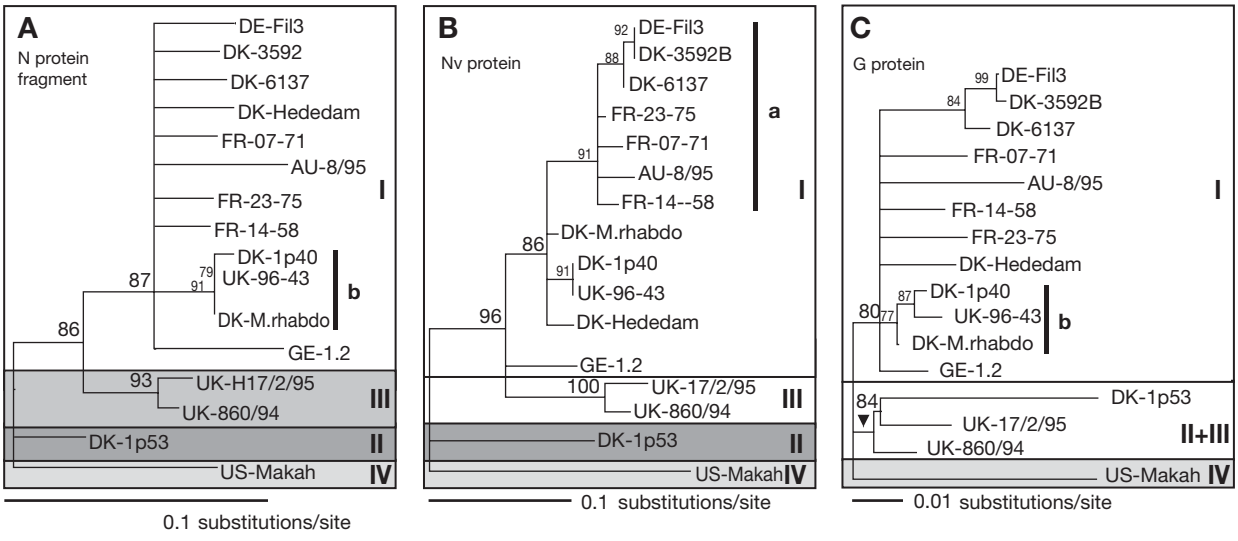

Fig. 3. Neighbour-joining distance trees based on amino acid sequences of (A) N protein fragment, (B) Nv protein, and (C) G protein, with bootstrap values >70%. Isolate codes as in Table 1

length G-based trees corresponded with those previously reported (Einer-Jensen et al. 2004). Only Sub-lineage Ia was found when using the Nv gene as target sequence, since the DK-M.rhabdo isolate was not grouped with the 2 other Sub-lineage Ib isolates, and a low bootstrap value (<70%) at the node of isolate GE-1.2 hindered identification of Sub-lineage Ie.

The overall genotyping, based on the 5 sub-genic regions (1 N and 4 G gene fragments, Fig. 2), was also in agreement with that in the study of Einer-Jensen et al. (2004) (Table 1). The number of nodes with bootstrap values above 70% was 12 (G gene, nt 220 to 1293), 8 (G gene, nt 360 to 720), 7 (G gene, nt 710 to 995), 7 (G gene, nt 175 to 761) and 7 (N gene, nt 23 to 445). The large G fragment, nt 220 to 1293 (Fig. 2A), therefore had comparable resolution to the full length based tree, whereas the nt 360 to 720 fragment (Fig. 2B) appeared to be the most informative of the smaller gene fragments with respect to number of significant branches. However, whether Sub-lineage Ia was grouped as a separate clade with a common ancestor distinct from the other Genotype I sub-lineages depended on the selected target region. Similarly, the resolution of sub-grouping within Genotype Ia isolates also depended on the selected region.

To analyse how genotypic variability was reflected by phenotypic variation and clustering, phylogenetic trees based on the deduced amino acid sequences were subsequently determined (Fig. 3). The grouping based on amino acid sequences had comparable resolution to the nucleotide sequence based grouping for the N gene/protein fragment. For the Nv gene, analysis at the amino acid level resulted in a low bootstrap value (<70%) for isolates GE-1.2 (Fig. 3B). The G protein tree also lost resolution, as only Sub-lineage Ib was observed within the group of Genotype I isolates. Furthermore Genotype II isolate (DK-1p53) was included within the group of Genotype III isolates. For the G gene fragments, phylogenetic analyses at amino acid level did not allow any significant grouping, as the bootstrap values in general were very low (<50%) (data not shown).

DISCUSSION

The present study is the first to perform parallel phylogenetic analysis of different genomic regions for a representative panel of VHSV isolates. The variability of 2 full length genes (G and Nv), 1 N gene fragment and 4 G gene fragments was phylogenetically analysed using optimised parameters. We identified 4 distinct clades corresponding to the major genotypes of VHSV from each of the 7 sets of nucleotide data (Figs. 1 & 2). This indicates that overall genotyping of VHSV can be performed with any of the tested genomic regions, and supports the general observation that recombination does not occur among rhabdoviruses (Badrane & Tordo 2001).

The resolution level with respect to identifying Genotype I sub-lineages was also evaluated: both trees based on full length genes (G and Nv) allowed genetic grouping of Sub-lineage Ia isolates (Fig. 1, Table 1), but further grouping was not observed when using the Nv gene. In contrast, Sub-lineage Ib was identified within the trees based on N or G gene fragments, whereas the Ia isolates, and in many cases also the Ie

isolate, were not grouped (Fig. 2). However, more epidemiological information, with respect to the number of significant nodes, was observed within Genotype I isolates when using the various gene fragments or full length G gene sequences. In summary, these findings suggest that detailed epidemiological studies for tracing the origins of individual isolates should preferably be based on the full length G gene (1524 nt). Since a smaller sequencing window is often preferable, this study also shows that the overall genotyping of the selected isolates was identical for all the target regions tested and that the potential information obtained is dependent on choice of target region. If the required capability of the analysis is to identify Sub-lineage Ia isolates, then use of the Nv gene is preferable, whereas higher resolution within Genotype I isolates may be obtained by using the G gene nt 360 to 720 fragment. However, these 2 analyses differ as to whether Sub-lineage Ia is grouped as a separate clade with a common ancestor distinct from the other Genotype I sub-lineages, as previously shown using full length G gene data (Fig. 1A, and Einer-Jensen et al. 2004).

Parallel phylogenetic analyses of the nucleotide and deduced amino acid sequences showed that the N fragment was the only target region that provided identical groupings. In contrast, bootstrap values lower than 70% were observed at 1 or more critical branch points within the full length G and Nv proteins and in all cases in the G fragment analyses, resulting in (e.g.) combining of Genotypes II and III when analysing the entire G protein. These findings could reflect the possibility that the G and Nv proteins ensure functionality as a result of positive selection/selective pressure beyond the level of random mutation on the RNA genome. The functionality of the non-structural Nv protein is still unknown, but the G protein is the only protein within the virus particle that is directly exposed to the host immune response and immunoselection on distinct regions is therefore likely. The tree representing the G gene region nt 710 to 995 (Fig. 2C) including important neutralising epitopes illustrates this: despite the highest number of substitutions per site, this region was not the best target region for detailed phylogenetic analysis. Accordingly, there was no clear relationship between the G gene genotyping and serotyping with neutralising (G protein specific) antibodies, as few nucleotide substitutions in the region of the major neutralising epitope changed the antibody recognition pattern. Epidemiological analyses based on phenotype characteristics such as serotyping would therefore be misleading.

Marine VHSV isolates typically produce little or no mortality in rainbow trout fry following waterborne challenge, although several isolates have been shown to have some pathogenicity for rainbow trout when delivered by intra-peritoneal injection (Skall et al. 2004). In the present analyses, differentiation of fresh and marine water Genotype I isolates correlated with Sub-lineages Ia and Ib, respectively. However, 1 Sub-lineage Ib isolate has previously been found to be pathogenic to rainbow trout (Einer-Jensen et al. 2004), and it is therefore important to stress that applied genetic analyses do not necessarily correlate with phenotypic properties such as pathogenicity to trout. Further efforts to identify a pathogenicity marker are therefore still required.

Finally, in order to avoid confusion, we would like to encourage future phylogenetic studies on VHSV to use the 4 genotypes initially defined by Snow et al. (1999). Additional genotypes can then be added when needed.

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