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Evolution of the fish rhabdovirus viral haemorrhagic septicaemia virus

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Viral haemorrhagic septicaemia (VHS) caused by the rhabdovirus VHSV is economically the most important viral disease in European rainbow trout farming. Until 1989, this virus was mainly isolated from freshwater salmonids but in the last decade, it has also been isolated from an increasing number of free-living marine fish species. To study the genetic evolution of VHSV, the entire G gene from 74 isolates was analysed. VHSV from wild marine species caught in the Baltic Sea, Skagerrak, Kattegat, North Sea, and English Channel and European freshwater isolates, appeared to share a recent common ancestor. Based on the estimated nucleotide substitution rate, the ancestor of the European fresh water isolates was dated some 50 years ago. This finding fits with the initial reports in the 1950s on clinical observations of VHS in Danish freshwater rainbow trout farms. The study also indicates that European marine VHSV and the North American marine line separated approx. 500 years ago. The codon substitution rate among the freshwater VHSV isolates was found to be 2·5 times faster than among marine isolates. The data support the hypothesis of the marine environment being the original reservoir of VHSV and that the change in host range (to include rainbow trout) may have occurred several times. Virus from the marine environment will therefore continue to represent a threat to the trout aquaculture industry.

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INTRODUCTION

Viral haemorrhagic septicaemia (VHS) is best known as an infectious disease of rainbow trout (*Oncorhynchus mykiss*) in Western Europe, where it causes extensive losses among commercial farms (Wolf, 1988; Smail, 1999). The causative virus (VHSV) is an enveloped negative-strand RNA virus belonging to the *Novirhabdovirus* genus of the *Rhabdoviridae*. The VHSV genome consists of approximately 11 200 nucleotides and contains six genes in the order 3′-N-P-M-G-Nv-L-5′, encoding a non-structural protein (Nv) with unknown function and five structural proteins: nucleocapsid- (N-), phospho- (P-), matrix- (M-), glyco- (G) and RNA polymerase (L) protein, respectively (Schutze *et al.*, 1999). The G protein has been shown to be the target molecule for neutralizing and protective antibodies (Bearzotti *et al.*, 1995; Lorenzen *et al.*, 1990, 1999), which has also been reported for other rhabdoviruses (Huang *et al.*, 1994; Kelley *et al.*, 1972; Wiktor *et al.*, 1973). The first isolation of VHSV and identification of this virus as the causal agent of severe disease outbreaks among cultured rainbow trout was carried out in Denmark in 1962 and, subsequently, a voluntary disease eradication campaign was started in Danish trout farms in 1965 (Jensen, 1965; Jørgensen, 1974). From 1980 to 1997, 20–40 farms carried out a controlled stamping out procedure each year and, as a consequence, the total numbers of infected farms in Denmark have decreased from approximately 400 to 40 over that period (Olesen, 1998). After the initial Danish isolation and identification, the virus was also reported in other European countries (e.g. Austria, Belgium, France, Germany, Italy and The Netherlands) (Olesen, 1998).

In the last decade, VHSV has been isolated from an increasing number of free-living marine fish species in the Northern hemisphere waters near Japan, Europe and North America (Hedrick *et al.*, 2003; Mortensen *et al.*, 1999; Takano *et al.*, 2000), indicating a widespread occurrence of the virus in the marine environment. The isolation of VHSV at new geographical locations and in new hosts might reflect either the spread of the virus into new ecological niches or the existence of populations of virus that have not previously been discovered.

The virus isolates from wild marine fish are serologically indistinguishable from the continental isolates (Benmansour *et al.*, 1997; Einer-Jensen *et al.*, 1995; Winton *et al.*, 1989), but all marine isolates tested so far typically produce little

or no mortality in rainbow trout fry following waterborne challenge (Dixon *et al.*, 1997; Skall *et al.*, 2004). However, some of the marine isolates have been shown to be highly pathogenic to turbot (*Scophthalmus maximus*) and Pacific herring (*Clupea pallasii*) (Kocan *et al.*, 1997; King *et al.*, 2001a). Genetic similarity between marine isolates from the North Sea and those responsible for outbreaks of disease in cultured turbot farms in Scotland has also been reported (Ross *et al.*, 1995; Snow *et al.*, 1999). Previous phylogenetic studies of VHSV isolates have identified the existence of discrete genetic groupings, related to geographical areas (Benmansour *et al.*, 1997; Thiéry *et al.*, 2002; Stone *et al.*, 1997; Nishizawa *et al.*, 2002). Although speculated (reviewed by Dixon, 1999), a close link between marine isolates and freshwater isolates has so far not been demonstrated. The present study focuses on a detailed phylogenetic characterization of the four known genotypes (Snow *et al.*, 1999), with emphasis on determination of the origin of rainbow trout pathogenic isolates.

METHODS

Virus isolates and RNA extraction. A panel of 74 virus isolates was selected in order to represent variations in geographical area, host species and year of isolation (Table 1 and Fig. 1). The analysed panel comprises 38 years of VHSV isolation and includes 40 freshwater isolates from rainbow trout, three isolates from sea-reared rainbow trout that had been infected before transfer to the sea, four rainbow trout isolates from sea-reared fish that were uninfected before transfer to the sea and 26 isolates recovered from a wide range of pelagic and bottom-dwelling wild marine fish species. The 74 isolates in this analysis represented 65 different collection sites covering wide geographic areas, although 34/43 rainbow trout isolates and 7/26 marine isolates came from Denmark or surrounding waters.

Propogated virus isolates with a low (usually 3–7) passage number were inoculated at low multiplicity on monolayers of BF-2 cells (Wolf *et al.*, 1966; Lorenzen *et al.*, 1988). When a total cytopathic effect was evident, the cell culture was centrifuged at 4000 ***g*** for 15 min. A 5 ml sample of each supernatant was ultracentrifuged (86 000 ***g***, 1 h) and the pelleted virus particles were suspended in 95 µl RNase-free water. Viral RNA was extracted from the pellet by the use of RNA affinity spin columns (RNeasy Total RNA kit; Qiagen), according to the manufacturer's instructions. Purified RNA was eluted in 30 µl RNase-free water, aliquoted into six tubes and stored until used at −80 °C.

Primer design. Primers for full-length RT-PCR amplification and sequencing of the G gene (Table 2) were designed on the basis of the published genomic sequences (GenBank accession nos: AF143863, AF143862, Z93414, Z93412, Y18263 and AJ233396), but the high level of genetic variation up- and downstream of the target gene necessitated the design of additional primer sets. Primers for sequencing were designed on the basis of the G gene sequence of the DK-3592B isolate (GenBank X66134) as well as on basis of the generated sequence data.

RT-PCR amplification and DNA sequencing. RT-PCR-amplified products were sequenced directly without cloning in order to minimize the bias associated with minor heterogeneities in the virus population. The products were synthesized by single tube RT-PCR (Titan One Tube RT-PCR System; Roche) according to the manufacturer's instructions. Briefly, 5 µl extracted viral RNA (approx. 2 µg), 50 pmol of each of the desired primers (Table 2), 1 µl 10 mM dNTP, 10 µl 5 × RT-PCR reaction buffer (enclosed buffer with 7·5 mM $MgCl_2$ and DMSO), 2·5 µl 100 mM DTT, 1 µl enzyme mix and MilliQ water was used per 50 µl one-tube RT-PCR. The RT-PCRs were performed using a NMJ Research PCT-200 Peltier thermal cycler.

Quantity and quality of the RT-PCRs were evaluated by 1 % agarose gel electrophoresis of 5 µl PCR product (data not shown). In a few samples with multiple bands, the remaining 45 µl PCR product was run on a new gel. The band of expected size was then excised from the gel and the DNA was purified by affinity spin columns (QIAquick Gel Extraction; Qiagen) and eluted in 10 mM Tris/HCl pH 8·5. DNA in samples showing a single band of the expected size was purified immediately on affinity spin columns. The amplicons were additionally purified using QIAqiuck spin columns (Qiagen) and sequenced on ABI 373A or ABI 377 automatic sequencers using the PRISM BigDye Terminator cycle sequencing kit (Applied Biosystems).

Computer analysis. Multiple sequence alignments of nucleotide sequences were performed with the CLUSTAL W program (Higgins & Sharp, 1989). Phylogenetic analysis was performed using the PAUP* software package, version 4.10 (Swofford, 2000). A starting tree was produced by the neighbour-joining algorithm with PAUP* default parameters. Using this tree, parameters of a general time reversible model of substitution (GTR) with invariable sites and a gamma distribution of rate heterogeneity were estimated from the data by maximum-likelihood. These parameters were: three base frequency parameters, six parameters of the substitution matrix, the proportion of invariable sites (PInvar), the transition/transversion ratio (Tratio) and one shape parameter from the gamma distribution describing rate heterogeneity among sites (Yang, 1993). This optimized substitution model was then used to re-estimate the tree and to assess the robustness of each node on the tree by the bootstrap resampling method with 1000 replicates (both by neighbour-joining). Bootstrap values exceeding 70 % were considered to indicate significant relatedness.

The US-Makah VHSV isolate was used as an outgroup root, as it has previously been established that the North American VHSV isolates are phylogenetically distinct from the European isolates (Benmansour *et al.*, 1997; Nishizawa *et al.*, 2002; Thiéry *et al.*, 2002). Phylogenetic trees were displayed using TreeView (Page, 1996) and nucleotide identity within the VHSV population was determined using GeneDoc (Nicholas *et al.*, 1997).

Nucleotide substitution rates and likelihood estimates for molecular clock tests were estimated using the TipDate model for evolution of a molecular clock (Rambaut, 2000) as implemented in the CODEML program (Yang, 1997; Goldman & Yang, 1994). This model incorporates the time-structure of serial samples, which means that the rate of substitution can be estimated directly from the data without the use of external calibration. The analyses were performed on fixed phylogenetic trees obtained by the neighbour-joining method (see above).

RESULTS

RT-PCR amplification and sequence analyses

Nucleotide sequencing was performed on both strands for 66 G genes (1524 nt). Subsequent alignments, including available sequence from GenBank, revealed that 62/74 of the G gene sequences were unique (Table 1) and identical G gene sequences were only found in isolates originating

Table 1. Data related to the 74 viral haemorrhagic septicaemia virus isolates used in this study

GenBank accession numbers representing available full-length G genes have been included. Only unique sequences (shown in bold) were included in the phylogenetic analysis. Isolates with identical sequences: **DK-1p52** = DK-1p55; **DK-1p8** = DK-1e62 = DK-1p12; **DK-5727** = DK-5740 = DK-5741; **DK-9995361** = DK-2000511 = DK-2000791; **DK-2000201** = DK-2000273 = DK-2000291 = DK-2000704; **SE-SVA29** = SE-SVA31; **SE-SVA30** = SE-SVA32.

Isolate code	Synonym	Year of isolation	Origin	Genotype*	Host species	Primer set	References	Accession no.
AU-8/95	DK-8076	1995	Austria	I-a	Rainbow trout	GA	–	AY546570
CH-FI 262 BFH	DK-9995345	1999	Switzerland	I-a	Rainbow trout	GA	–	AY546571
DE-Fil3	Fil3	1983	Germany	I-a	Rainbow trout	–	Schutze *et al.* (1999)	Y18263
DK-F1	F1	1962	Denmark	I	Rainbow trout	GB	Batts *et al.* (1993); Benmansour *et al.* (1997); Jørgensen *et al.* (1995); Nishizawa *et al.* (2002); Snow *et al.* (1999); Stone *et al.* (1997)	AF345857
DK-Hededam	Hededam	1972	Denmark	I	Rainbow trout	–	Batts *et al.* (1993); Benmansour *et al.* (1997); Betts & Stone (2000); Dixon *et al.* (1997); Nishizawa *et al.* (2002); Stone *et al.* (1997); Thiéry *et al.* (2002)	Z93412 U28798‡
DK-M.rhabdo	Cod Ulcus 79	1979	Baltic Sea, Little Belt 39F9†	I-b	Cod	–	Batts *et al.* (1993); Betts & Stone (2000); Dixon *et al.* (1997); Nishizawa *et al.* (2002); Snow *et al.* (1999); Stone *et al.* (1997)	Z93414
DK-1e62	DK-9695333	1996	Baltic Sea 39G5†	I-b	Cod	GB	King *et al.* (2001a); Skall *et al.* (2004)	AY546572
DK-1p8	DK-9695287	1996	Baltic Sea 40G7†	I-b	Herring	GA	King *et al.* (2001a); Skall *et al.* (2004); Snow *et al.* (1999)	AY546573
DK-1p12	DK-9695287	1996	Baltic Sea 40G8†	I-b	Herring	GA	King *et al.* (2001a); Skall *et al.* (2004); Snow *et al.* (1999)	AY546574
DK-1p40	DK-9695303	1996	Baltic Sea 43G9†	I-b	Rockling	GA	King *et al.* (2001a); Skall *et al.* (2004); Snow *et al.* (1999)	AY546575
DK-1p52	DK-9695305	1996	Baltic Sea 41G9†	II	Sprat	GC	Skall *et al.* (2004); Snow *et al.* (1999)	AY546576
DK-1p53	DK-9695305	1996	Baltic Sea 41G9†	II	Herring	GC	Skall *et al.* (2004); Snow *et al.* (1999)	AY546577
DK-1p55	DK-9695305	1996	Baltic Sea 41G9†	II	Sprat	GC	Skall *et al.* (2004); Snow *et al.* (1999)	AY546578
DK-1p86	DK-9695324	1996	Baltic Sea 38G9†	I-b	Sprat	GB	King *et al.* (2001a); Skall *et al.* (2004)	AY546579
DK-4p37	DK-9795405	1997	North Sea 45F5†	I-b	Blue whiting	GB	King *et al.* (2001a); Skall *et al.* (2004); Snow *et al.* (1999)	AY546580
DK-4p101	DK-9795427	1997	North Sea 43F7†	III	Whiting	GD	Skall *et al.* (2004); Snow *et al.* (1999)	AY546581
DK-4p168	DK-9795431	1997	Skagerrak 44G0†	III	Herring	GD	King *et al.* (2001a)	AY546582

Table 1. cont.

Isolate code	Synonym	Year of isolation	Origin	Genotype*	Host species	Primer set	References	Accession no.
DK-5e59	DK-9895255	1998	Kattegat 43G1†	I-b	Dab	GB	–	AY546583
DK-6p403	DK-9995324	1999	Skagerrak 44F9†	I-b	Herring	GA	–	AY546584
DK-2835	Vejen Lille V.	1982	Denmark	I-c	Rainbow trout	GA	–	AY546585
DK-3592B	Voldbjerg	1986	Denmark	I-a	Rainbow trout	–	Einer-Jensen *et al.* (1995); Jørgensen *et al.* (1995); Snow *et al.* (1999)	X66134
DK-3946	Kideris	1987	Denmark	I-a	Rainbow trout	GB	–	AY546586
DK-3971	Musholm	1987	Denmark	I-a	Rainbow trout¶	GB	–	AY546587
DK-5123	Vejen Lille V.	1988	Denmark	I-c	Rainbow trout	GB	–	AY546588
DK-5131	Klapmølle	1988	Denmark	I-c	Rainbow trout	GA	Batts *et al.* (1993); Einer-Jensen *et al.* (1995); Lorenzen *et al.* (1988); Nishizawa *et al.* (2002); Stone *et al.* (1997)	AF345858
DK-5151	Rindsholm	1988	Denmark	I-a	Rainbow trout	GA	Batts *et al.* (1993); Einer-Jensen *et al.* (1995); Nishizawa *et al.* (2002); Stone *et al.* (1997)	AF345859
DK-5727	Nielsby	1989	Denmark	I-a	Rainbow trout	GA	Jørgensen *et al.* (1995)	AY546589
DK-5740	Vejen Lille V.	1990	Denmark	I-a	Rainbow trout	GA	–	AY546590
DK-5741	Vejen Store V.	1990	Denmark	I-a	Rainbow trout	GB	–	AY546591
DK-6045	Nørre Å	1991	Denmark	I-a	Rainbow trout	GB	Jørgensen *et al.* (1995)	AY546592
DK-6137	Hjarnø	1991	Denmark	I-a	Rainbow trout¶	GA	Jørgensen *et al.* (1995)	AY546593
DK-7380	Agersø	1994	Denmark	I-a	Rainbow trout¶	GA	–	AY546594
DK-7974	Fole	1995	Denmark	I-a	Rainbow trout	GA	–	AY546595
DK-9595168	Agersbæk	1995	Denmark	I-a	Rainbow trout	GA	–	AY546596
DK-9695377	Sdr. Lyngvig	1996	Denmark	I-a	Rainbow trout	GA	–	AY546597
DK-9795568	St. Karlskov	1997	Denmark	I-a	Rainbow trout	GA	–	AY546598
DK-9895024	Kodbølgård	1998	Denmark	I-a	Rainbow trout	GA	–	AY546599
DK-9895093	Åbro	1998	Denmark	I-a	Rainbow trout	GB	–	AY546600
DK-9995007	Kongeåen	1999	Denmark	I-a	Rainbow trout	GA	–	AY546601
DK-9995144	Voldbjerg	1999	Denmark	I-a	Rainbow trout	GA	–	AY546602
DK-9995174	Tim Mølle	1999	Denmark	I-a	Rainbow trout	GA	–	AY546603
DK-9995361	Kodbølgård	1999	Denmark	I-a	Rainbow trout	GA	–	AY546604
DK-200098	Bratbjerg	2000	Denmark	I-a	Rainbow trout	GA	–	AY546605
DK-200148	Gårdsdal	2000	Denmark	I-a	Rainbow trout	GA	–	AY546606
DK-200149	Ravnstrup	2000	Denmark	I-a	Rainbow trout	GA	–	AY546607
DK-200020-1	Sdr. Omme	2000	Denmark	I-a	Rainbow trout	GB	–	AY546608
DK-200027-3	Grønbjerg	2000	Denmark	I-a	Rainbow trout	GB	–	AY546609
DK-200029-1	Langelund	2000	Denmark	I-a	Rainbow trout	GB	–	AY546610
DK-200051-1	Nærild	2000	Denmark	I-a	Rainbow trout	GB	–	AY546611

Table 1. cont.

Isolate code	Synonym	Year of isolation	Origin	Genotype*	Host species	Primer set	References	Accession no.
DK-200070-4	Dyrvig	2000	Denmark	I-a	Rainbow trout	GB	–	AY546612
DK-200079-1	Nærild	2000	Denmark	I-a	Rainbow trout	GA	–	AY546613
FI-ka-66	DK-200198-2	2000	Gulf of Bothnia 49H1†	I-d	Rainbow trout§	GA	–	AY546614
FI-ka-422	DK-200240	2000	Gulf of Bothnia 49H6†	I-d	Rainbow trout§	GA	–	AY546615
FR-02-84	02-84	1984	France	I-a	Rainbow trout	–	Benmansour *et al.* (1997); Nishizawa *et al.* (2002); Thiéry *et al.* (2002)	U28800
FR-07-71	07-71	1971	France	I-a	Rainbow trout	–	Batts *et al.* (1993); Benmansour *et al.* (1997); Nishizawa *et al.* (2002); Snow *et al.* (1999); Thiéry *et al.* (2002)	AY546616 X59148‡
FR-14-58	14-58	1990	France	I-a	Rainbow trout	–	Betts & Stone (2000); Nishizawa *et al.* (2002); Thiéry *et al.* (2002)	AF143863
FR-23-75	23-75	1975	France	I-a	Brown trout	GA	Batts *et al.* (1993); Benmansour *et al.* (1997); Einer-Jensen *et al.* (1995); Nishizawa *et al.* (2002); Stone *et al.* (1997); Thiéry *et al.* (2002)	AY546617 U28799‡
FR-L59X	$L59_X$	1987	France	III	Eel	GD	Castric *et al.* (1992); Jørgensen *et al.* (1994); Thiéry *et al.* (2002)	AY546618
GE-1.2	VHS-1.2	1981	Georgia	I	Rainbow trout	GA	–	AY546619
IR-F13.02.97	DK-9795386	1997	Ireland 31E0†	III	Turbot	GD	Snow *et al.* (1999)	AY546620
NO-A163-68 EG46	DK-201311	1968	Vestrefjord, Norway	I-d	Rainbow trout#	GB	–	AY546621
SE-SVA-14	DK-9895195	1998	Kattegat 45G1†	I-b	Rainbow trout§	GB	Nordblom (1998)	AY546622
SE-SVA-1033	DK-200141	2000	Kattegat 45G1†	I-b	Rainbow trout§	GA	Nordblom & Norell (2000)	AY546623
SE-SVA-29	DK-201226-29	2000	Kattegat 45G1†	I-b	Herring	GB	–	AY546624
SE-SVA-30	DK-201226-30	2000	Kattegat 45G1†	I-b	Sprat	GB	–	AY546625
SE-SVA-31	DK-201226-31	2000	Kattegat 45G1†	I-b	Herring	GB	–	AY546626
SE-SVA-32	DK-201226-32	2000	Kattegat 45G1†	I-b	Bottom-living species‖	GB	–	AY546627

Table 1. cont.

Isolate code	Synonym	Year of isolation	Origin	Genotype*	Host species	Primer set	References	Accession no.
UK-860/94	TurbGigha94	1994	Gigha, W Scotland 30F0†	III	Turbot	GD	Ross *et al.* (1995); Snow *et al.* (1999)	AY546628
UK-96-43	Phrg Eng.Chan.	1996	English Channel	I-b	Herring	–	Betts & Stone (2000); Dixon *et al.* (1997); Nishizawa *et al.* (2002)	AF143862
UK-H17/2/95	HaddShetl95	1995	North Sea, E. Shetland 50F1†	III	Haddock	GC	King *et al.* (2001a); Smail (1995); Snow *et al.* (1999)	AY546629
UK-H17/5/93	CodShetl93	1993	North Sea, E. Shetland 49F0†	III	Cod	GC	Einer-Jensen *et al.* (1995); King *et al.* (2001a); Nishizawa *et al.* (2002); Smail (1995); Snow *et al.* (1999); Stone *et al.* (1997)	AY546630
UK-MLA98/6 HE1	HE503	1998	North Sea 50E9†	I-b	Herring	GB	King *et al.* (2001b)	AY546631
UK-MLA98/6 PT11	PT431	1998	North Sea 48F1†	III	Norway pout	GD	King *et al.* (2001b)	AY546632
US-Makah	RBV	1988	Washington, USA	IV	Coho salmon	–	Batts *et al.* (1993); Benmansour *et al.* (1997); Dixon *et al.* (1997); Einer-Jensen *et al.* (1995; 1993); Stone *et al.* (1997); Thiéry *et al.* (2002); Nishizawa *et al.* (2002)	U28747

*Genotype based on phylogenetic tree (Fig. 2).

†Coordinates of ICES statistical squares from which the infected European marine fish was caught (see also map, Fig. 1).

‡Available sequence information not included in phylogenetic analysis.

§Rainbow trout were certified VHS free before transfer from freshwater.

‖Virus was isolated from pool of *Pholis gunellus, Gobiidae* species, *Zoarces viviparous* and *Acanthocottus scorpius.*

¶Virus isolated from sea-reared rainbow trout, infected before transfer to the sea.

#Virus was isolated from rainbow trout imported from Denmark (T. Håstein, personal communication).

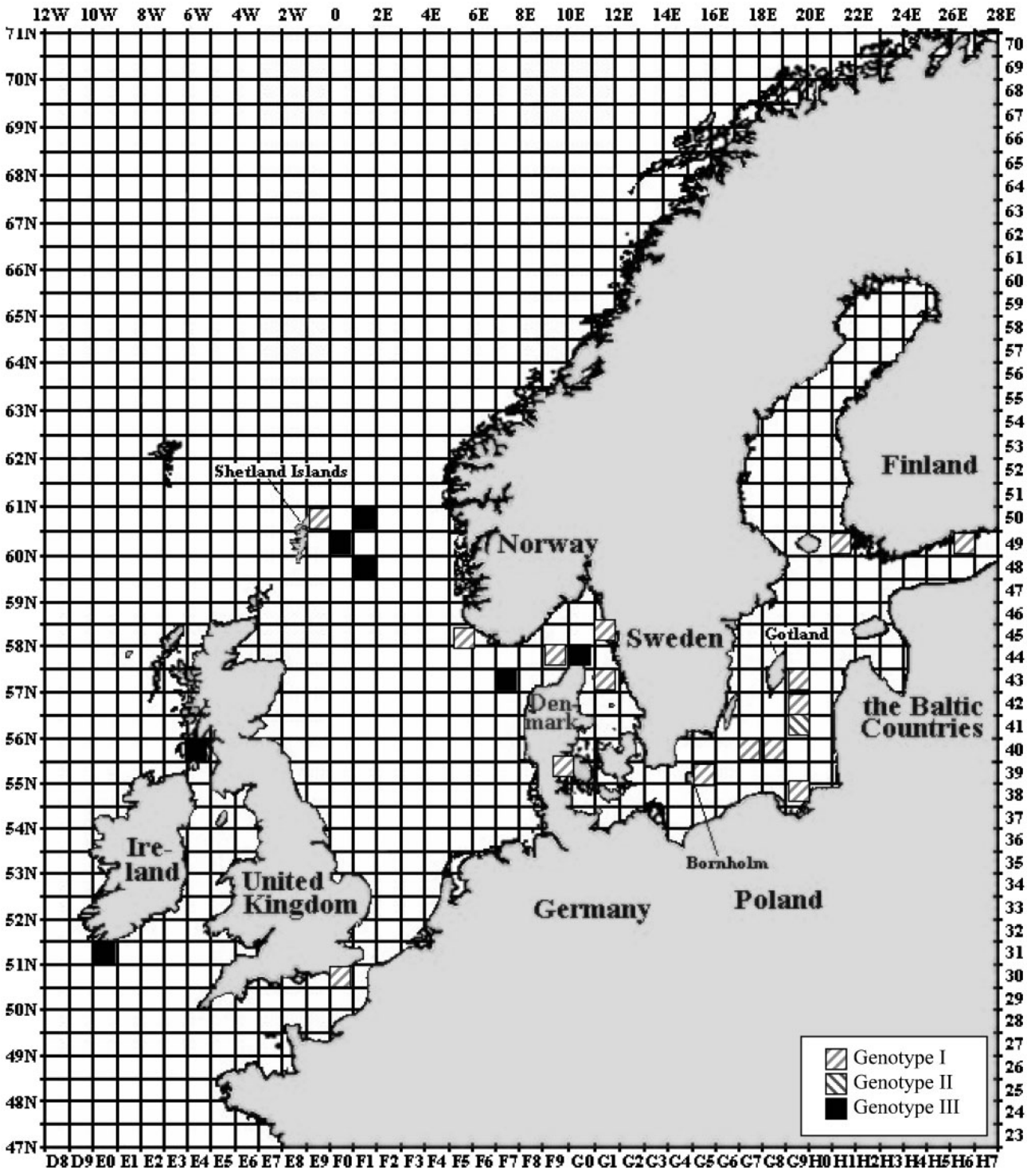

Fig. 1. Map of the ICES statistical squares charted, from which the included European marine VHSV have been isolated. Relations between isolate code and ICES square coordinates are given in Table 1. Genotype distributions of the isolates are indicated by shaded or striped squares.

from the same geographical region or from farms infected in the same epidemic. The identity level within the four observed genotypes varied from 94 to 99 % whereas it was 84 to 91 % between genotypes (Table 3). The North American isolate (genotype IV) had the lowest degree of similarity to the other isolates (Table 3).

Table 2. Primers used for RT-PCR amplification and sequence analysis

Primers for sequence analysis were designated GA–GD and GSeq. The + or − refer to orientation (sense or antisense, respectively).

Primer	Position (nt)*	Sequence (5′→3′)	PCR product (bp)
GA+	585 in M gene	CTCCTCTGTCCGACCTT	–
GA−	9 in Nv gene	GGTCGCCATGTTTCTTTATC	1717
GB+	532 in M gene	GTCGAAGAAGAGATAGGC	–
GB−	8 in Nv gene	GTTGGGTCGCCATGTTTCT	1757
GC+	381 in M gene	CATTAGACATGGGAGTGTG	–
GC−	216 in Nv gene	CTAGGAGACTTATCCTCATG	2121
GD+	511 in M gene	GAAGGACTACTACAATCGTG	–
GD−	48 in Nv gene	GGAGGACGAGTGGAGAAA	1827
GSeq1+	235	CCAACCAAGATCATCCAT	–
GSeq2+	637	GCCCATTGCCCCACG	–
GSeq3+	954	CCATAGTGATATCACCGC	–
GSeq4+	1272	CCTTGTGGAAGTCCCTC	–
GSeq5+	1300	GTGTTTGTCTCCAACACATC	–
GSeq6−	303	GCACAGAGTGACTTATCG	–
GSeq7−	424	CACGAGTACCCGTTCTT	–
GSeq8−	441	CCCTGAACCCTCCTGC	–
GSeq9−	954	CCATAGTGACATCACTGC	–
GSeq10−	1020	CCCTGGACCCGGCAA	–

*The target gene and positions of the first nucleotide in the 5′ end of the primer are given.

Differences between sequences published by different laboratories for the same virus isolate were observed in a number of cases, e.g. two GenBank sequences of DK-Hededam (U28798 and Z93412) differed in five positions, which resulted in four amino acid substitutions. Propagation on different cell lines, polymerase with or without proofreading and/or sequencing of cloned gene products rather than PCR amplicons might explain these differences. When available, sequence data from the present study were used for the phylogenetic analysis in order to minimize this bias (Table 1). The gene sequences obtained in this study were distinct, but mixed nucleotide positions occurred in nine isolates despite repeated amplification and subsequent sequence analysis (two or three times). This finding may indicate the presence of heterogeneous genetic populations within some of the virus samples. Sequencing of plaque-cloned virus populations remains to be performed to clarify such sequence differences.

Table 3. Sequence identity between the VHSV isolates of the four genotypes as defined by Snow *et al.* (1999)

Genotype	I	II	III	IV
I	94–99	–	–	–
II	87–89	99	–	–
III	89–91	88–89	96–99	–
IV	85–87	84–85	84–85	–*

*Only one isolate included.

Phylogenetic analysis

The genetic relationships among the 62 unique VHSV sequences are illustrated in the tree (Fig. 2), in which the four genotypes (I–IV; previously identified by Snow *et al.*, 1999) were supported by significant bootstrap values. Trees with similar topology were consistently produced by both distance and parsimony methods (data not shown). The freshwater isolates all belonged to genotype I, whereas sea water isolates were represented in all of the four genotypes. Five sublineages were identified within the genotype I isolates (Fig. 2): sublineage I-a represented a continuous evolution of European freshwater VHSV isolates from Austria, Denmark, France and Switzerland, with the most recent isolates situated at the branch tips; I-b included isolates originating from the Baltic Sea, Skagerrak, Kattegat, North Sea and English Channel; I-c included a small group of older Danish freshwater isolates; I-d was represented by an old Norwegian isolate [NO-A163-68 EG46, supposedly imported with rainbow trout from Denmark; T. Håstein (personal communication)] and two recent isolates from rainbow trout farmed in brackish water in Finland; I-e included a Georgian isolate as the only representative. Among the rainbow trout isolates, the oldest isolates as well as the two recent isolates from brackish water (Finland) were positioned close to the common ancestor of marine (sublineage I-b) and freshwater isolates (indicated by asterisk in Fig. 2).

Genotype II included only isolates from fish caught in a narrow region called the Eastern Gotland Basin (Baltic Sea)

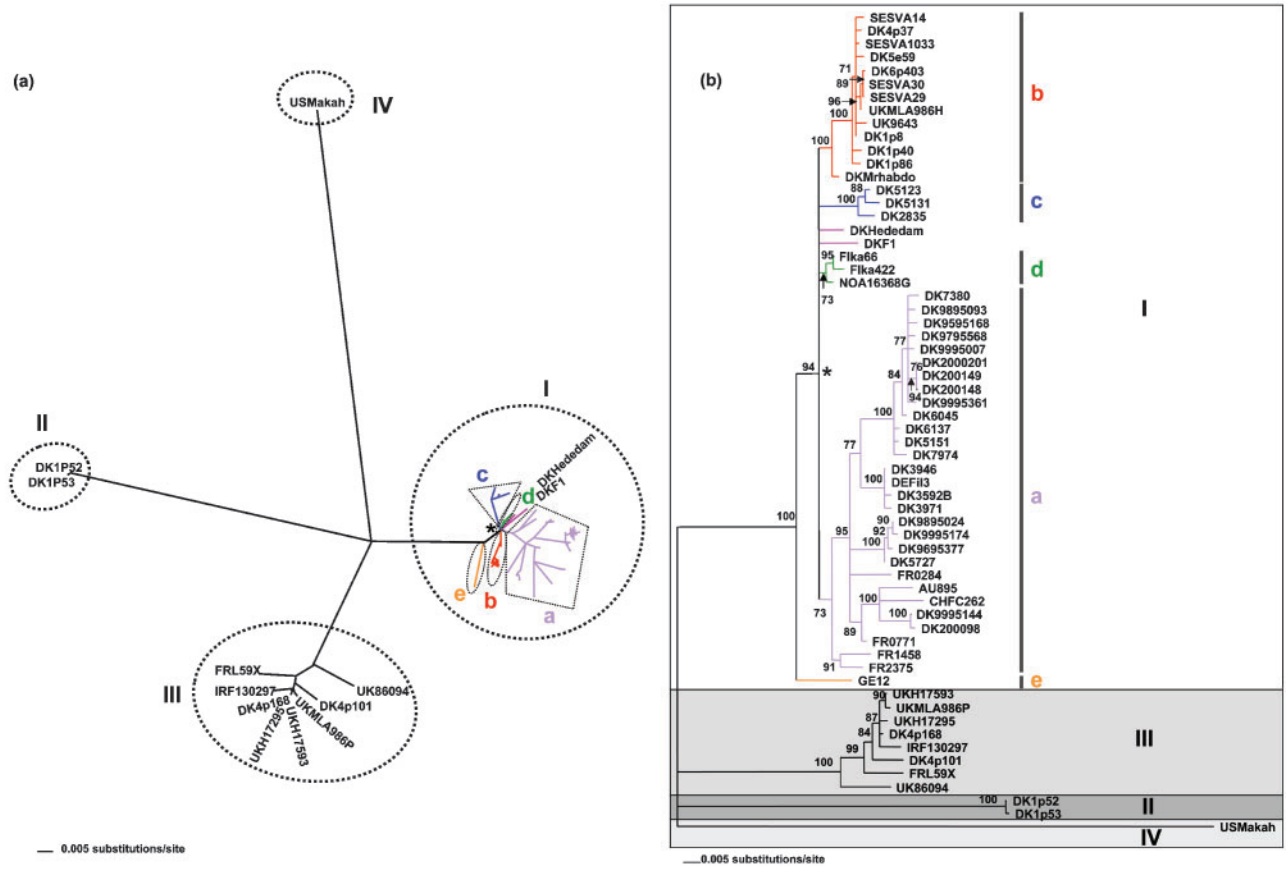

Fig. 2. Phylogenetic trees showing the relationship of the 62 unique full-length VHSV G gene sequences (Table 1) illustrated by a radial tree (a) and a phylogram using an American isolate as outgroup (b). The best-fitting nucleotide substitution model (parameters: distance = GTR, rates = gamma, class = (a, b, c, d and e), Tratio = 4·3, shape = 1·235069 and PInvar = 0·422062) was used during neighbour-joining distance analysis and the branch lengths in (a) and the horizontal branch lengths in (b) reflect genetic distances. Bootstrap values were obtained from 1000 resampled datasets and nodes with bootstrap values less than 70 % have been collapsed in (b). Genotype I sublineage identified by significant bootstrap values (> 70 %) have been designated I-a to I-e. The asterisk denotes the shared ancestral node of European freshwater isolates and marine isolates. Scale bars refer to the expected numbers of substitutions per site.

(Fig. 1). Due to very deep water in this area, the location holds a separate biotope with high salinity and is believed to host an endemic fish population. The presence of two relatively different genotypes (87 to 89 % identity, Table 3) within the Baltic Sea could therefore be a result of different genetic evolution within the biotopes or be a result of two separate introduction events in this area.

Genotype III included isolates from the North Sea/coastal waters of the UK and Ireland, as well as an isolate originating from an eel caught in northern France. As with genotype I, several distinct sublineages existed within genotype III. However, these lineages were not designated with letters, as only a few isolates covering a broad geographical zone were included. Virus genotypes I and III were both isolated from fish caught in the North Sea and Skagerrak, a finding that may reflect migration patterns of the host fish populations (Fig. 1).

Molecular clock analysis

A likelihood ratio test for the presence of a molecular clock, including all unique sequences, rejected the hypothesis, indicating that the rate of substitution is not constant throughout the tree (Table 4). Fig. 2 shows that a large group of freshwater isolates (I-a) protrudes further from the root than the marine isolates (I-b). This indicates that the rate of substitution varies as a function of the environment. On that assumption, the data were separated into two environmentally related phylogeny groups for further analysis: the large group of monophyletic freshwater isolates (denoted FW, corresponding to sublineage I-a) and all sea-water isolates (SW corresponding to I-b, II, III and IV). For these groups, the likelihood ratio test gave acceptance of the hypothesis of molecular clocks with substitution rates of $1{\cdot}74 \times 10^{-3}$ and $7{\cdot}06 \times 10^{-4}$ substitution per codon per year, respectively (Table 4). When the remaining genotype

Table 4. Molecular clock analysis

Group of virus isolates and model*	*n*†	Log-likelihood	p‡	χ^2	Estimated rate of substitution per codon per year
All_{clock}	62	−7093·42	68	$P \ll 0{\cdot}001$§	NA
$All_{no\text{-}clock}$		−6443·19	127		
FW_{clock}	29	−3483·34	35	$P = 0{\cdot}79$	$1{\cdot}74 \times 10^{-3}$ ($\pm 3{\cdot}17 \times 10^{-4}$)
$FW_{no\text{-}clock}$		−3473·32	61		
$FW + O_{clock}$	38	−4381·46	44	$P \ll 0{\cdot}001$§	NA
$FW + O_{no\text{-}clock}$		−4196·32	79		
SW_{clock}	24	−4196·92	30	$P = 0{\cdot}12$	$7{\cdot}06 \times 10^{-4}$ ($\pm 4{\cdot}51 \times 10^{-5}$)
$SW_{no\text{-}clock}$		−4936·43	51		
$SW + O_{clock}$	33	−4961·92	39	$P = 0{\cdot}01$§	NA
$SW + O_{no\text{-}clock}$		−4936·44	69		

*Defined groups of isolates: All, all isolates with unique sequences; FW, monophyletic freshwater isolates from genotype I-a and SW, sea-water isolates from genotypes I-b, II, III and IV. O, other genotype I isolates including sublineage I-c, I-d, I-e and single representatives GE-1.2, DK-F1 and DK-Hededam.

†Number of isolates in each group. The number of parameters in the substitution model (m) is six and includes one parameter for the ratio of the rate between transitions and transversions, three parameters of the ratio of the rate between non-synonymous and synonymous substitutions to allow for heterogeneous selection pressure and two accompanying rate-class probability parameters. For the clock models the number of tree parameters (T_{clock}) equals the number of node height parameters plus a substitution rate parameter [$T_{clock} = (n-1) + 1 = n$] and, for the no-clock models the number of tree parameters ($T_{no\text{-}clock}$) equals the number of branch lengths in an unconstrained tree ($T_{no\text{-}clock} = 2n-3$).

‡The molecular clock hypothesis was tested statistically by evaluating twice the difference in the log-likelihoods [$-2(\ln L_{clock} - \ln L_{no\text{-}clock})$] in a $\chi^2_{(d.f.)}$ distribution. Where the number of degrees of freedom (d.f.) is given by the difference in the total number of parameters (p) in the clock model ($p_{clock} = m + T_{clock}$) and the total number of parameters in the no-clock model ($p_{no\text{-}clock} = m + T_{no\text{-}clock}$).

§Molecular clock hypotheses rejected at the 5 % level.

NA, Not applicable.

I isolates were included in either the fresh- or sea-water group, the molecular clock hypothesis was again rejected (Table 4). The estimated substitution rates of the fresh- and sea-water isolates were subsequently extrapolated onto the phylogenetic trees in order to identify the time of the most recent common ancestors (MRCA). This gave an estimated MRCA of the large group of freshwater isolates (I-a) approximately 50 ($\pm 4{\cdot}8$) years ago and an estimated MRCA of the marine isolates approximately 500 ($\pm 25{\cdot}9$) years ago.

DISCUSSION

The present study focuses on the genetic evolution of the fish rhabdovirus VHSV in wild reservoir hosts and in cultured fish species. Previous findings of four main genotypes of VHSV (Snow *et al.*, 1999), which correlated with the geographical regions of isolation, were confirmed (Fig. 2). However, this study is the most detailed to date with respect to the number of isolates included and the diversity of their historical and geographical background. The selected target gene region was (in contrast to previous studies, which focussed on gene fragments) the entire G gene (1524 nt) including several hypervariable regions. The data allowed a detailed description of the evolution of VHSV and also a comparison of codon substitution rates among freshwater and marine VHSV isolates.

Marine VHSV isolates were represented in all four genotypes, whereas all freshwater isolates belonged to genotype I (Fig. 2). The marine virus group therefore possesses the majority of diversity and has the freshwater isolates included in their phylogeny. This finding supports the assumption that freshwater VHSV originates from the marine environment.

Phylogenetic analyses indicated that the European genotype I lineages had a common ancestral source. The isolates closest to the ancestral source were either old freshwater isolates from rainbow trout or isolates from the marine environment. Recently, outbreaks of VHSV without physical linkage to freshwater VHSV have occurred in Scandinavian marine-reared rainbow trout. These VHSV isolates were located close to the ancestral source in the phylogenetic diagram (Fig. 2), and therefore most likely represent new introductions/adaptations to rainbow trout. This theory was further supported by the finding that virus isolated from outbreaks of VHS in Swedish net pens in Kattegat in the early spring of 1998 and again in 2000 (SE-SVA-14 and SE-SVA-1033, sea coordinate 45G1 in Fig. 1) were almost identical to isolates found in the surrounding marine environment (99 % identity within the G genes from DK-5e59, DK-4p37, SE-29SVA, SE-30SVA, SE-31SVA and SE-32SVA). The source of infection is unknown, but it has been demonstrated that VHSV can multiply to high titres in herring and other pelagic species (Hedrick *et al.*, 2003; Hershberger *et al.*, 1999). It may thus be speculated

that virus excreted by shoals of migrating fish or the fish themselves have been the origin of the infection. Accordingly, raw marine fish was used for the feeding of rainbow trout in Finland, and could have been the source of the infection, as Dixon (1999) suggested for the first cases of VHS in Denmark. The isolation of a phylogenetically distinct VHSV isolate from a rainbow trout farm in Georgia, in the Caucasus, might represent an equivalent example of infection from wild fish, but it remains to be determined whether an uncharacterized marine reservoir of VHSV exists in the Black Sea.

Populations of RNA viruses are often complex mixtures of different, but closely related variants, and, as a consequence, they have a high potential for continuous adaptation (Quer *et al.*, 1996). In light of this, it is interesting that Danish freshwater VHSV isolates grouped discontinuously into two separate sublineages (I-a and I-c) or as single representatives branching out from the same ancestral point as the Baltic marine lineage (I-b) (Fig. 2a). Taken together, the above findings indicate that host adaptation from marine environment/species to rainbow trout has occurred three or four times in freshwater farms in Denmark and more recently in fish reared at the coast of Finland and Sweden. Genetic relatedness between a virulent sea-water isolate from farmed turbots and VHSV isolated from marine fish species near the Shetland Islands was also reported by Stone *et al.* (1997) and Snow *et al.* (1999). VHSV in wild marine fish therefore represents a continuous potential threat to farming of VHSV-susceptible species in the coastal zones.

Different nucleotide substitution rates were identified within the VHSV isolates from sea-water and the dominating freshwater sublineage I-a (Table 4). The rates of substitutions per codon per year were determined to be $7{\cdot}06 \times 10^{-4}$ and $1{\cdot}74 \times 10^{-3}$, respectively. This is within the range of evolutionary rates estimated for other RNA viruses (Jenkins *et al.*, 2002).

The hypothesis of divergent substitution rates would be strengthened by performing maximum-likelihood estimation with a model allowing for local clocks in the tree and then by comparing this to estimates obtained with a model of no molecular clock, in analogy with the model of Yoder & Yang (2000). However, there is currently no such model that allows for time-structured data and the study was therefore restricted to the described exploratory analyses. The rejection of the molecular clock hypothesis for the full dataset and its subsequent recovery when the isolates from different environments are separated does, however, along with the more than twofold difference in the estimates of the rate of substitution, give a strong indication that the rate of substitution differs within the two environments.

The observed nucleotide substitution rate in the G gene of VHSV from farmed freshwater fish was approximately 2·5 times faster than in the G gene of VHSV from free living fish in sea-water. It could be speculated that rainbow trout is an unnatural host for VHSV and that host species difference in immune response and/or physiology have influenced the evolutionary rate of the virus (Moya *et al.*, 2000). Alternatively, the higher substitution rate in freshwater may be related to the intense production procedures used in fish farms, where monocultures of rainbow trout are kept at high mean densities and stress levels, along with repeated introduction of naïve fish into the farms. Also, the generally higher temperatures within the freshwater farms than in the marine environment may have increased the speed of virus replication, as observed during *in vitro* replication. The elevated nucleotide substitution rates in freshwater isolates may therefore be a result of increased numbers of virus replications per year. A similar pattern has been observed for IHNV (Infectious Hematopoietic Necrosis Virus) in North America, where the evolution rate in a geographical zone with intense aquaculture was found to be three to four times higher compared with other regions (Troyer & Kurath, 2003).

The estimated mean substitution rates were related to the phylogenetic data and it was estimated that European freshwater VHSV had a common marine ancestor approximately 50 years ago, whereas European marine VHSV shared an ancestor with the North American type approximately 500 years ago. The first estimate coincides well with the initial reports of VHS-like symptoms in Denmark in the 1950s, followed by the first isolation of the causal virus in 1962 (Jensen, 1965). The second estimate is more difficult to confirm, but it could well be that the genotypes were separated a long time before fish farming was established on the two continents.

The present study demonstrates a close genetic linkage between VHSV in the marine environment and VHSV in farmed rainbow trout in Europe. Adaptation of the virus to rainbow trout is suggested to be a relatively recent event that has occurred more than once during the past 50 years in European rainbow trout farming.

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