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Genotyping and Pathogenicity of Viral Hemorrhagic Septicemia Virus from Free-Living Turbot (*Psetta maxima*) in a Turkish Coastal Area of the Black Sea

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Viral hemorrhagic septicemia (VHS) is one of the most serious fish viral diseases for cultured rainbow trout (*Oncorhynchus mykiss*), although VHS virus (VHSV) seems to be ubiquitous among marine fishes. In the present study, VHSV isolation was performed with free-living and cultured turbot (*Psetta maxima*) in the Trabzon coastal area of the Black Sea to evaluate participation of VHSV in mass mortalities of seed-produced turbot larvae. VHSV was detected in 14 of 66 free-living spawners (positive ratio, 21.2%), 1 of 65 free-living immature fish (1.5%) and 7 of 40 cultured brood stock (17.5%), respectively. Based on a partial glycoprotein gene nucleotide sequence, Turkish VHSV isolates were classified into the class I-e of genotype I and were the most closely related to the GE-1.2 isolate (>98% identity), which was found >20 years ago in Georgia. Thus, it was revealed that Turkish VHSV isolates were not introduced from European countries, it could be an indigenous type of VHSV distributing in the Black Sea environment. In pathogenicity tests, the Turkish isolates did not induce mortality in turbot larvae and rainbow trout fingerlings. Mass mortalities at a rate of approximately 90% occurred in turbot larvae produced by experimental seeding, although VHSV was not detected in any dead fish. Thus, it was concluded that mass mortality in the seed-produced turbot larvae was not caused by VHSV infection.

Viral hemorrhagic septicemia (VHS) is one of the most serious viral diseases of farmed rainbow trout (*Oncorhynchus mykiss*) in continental European countries (22, 36), and it is listed as a notifiable disease in the World Organisation for Animal Health's Office International des Epizooties (http://www.oie.int/eng/maladies/en_classification.htm). Near the end of the 1980s, VHS virus (VHSV) was first reported in various marine fishes in Europe (16, 20) and then in anadromous salmon (34) and marine fish in North America (12, 13). Moreover, it was also found in wild and cultured Japanese flounder (*Paralichthys olivaceus*) in far eastern Asia (27, 28, 33). The virus seems to be ubiquitous among marine fishes (3, 22, 35). VHSV is a member of the genus *Novirhabdovirus* of the family *Rhabdoviridae*, and it has a single molecule of linear, negative-sense, single-stranded RNA (approximately 11.1 kb) with six genes in the order 3′-N-P-M-G-NV-L-5′ (32). Previous studies of the molecular epidemiology of VHSV isolates based on the nucleoprotein (N) and glycoprotein (G) gene sequences have revealed the existence of four genotypes among freshwater and marine isolates. Genotype I includes a wide range of viruses originating from freshwater rainbow trout in continental Europe and marine species within the Baltic Sea, Skagerrak, Kattegat, and the English Channel. Genotype II is composed of marine isolates recovered from the Baltic Sea. Genotype III includes isolates originating in cultured turbot (*Scophthalmus maximus*) from the British Isles, from a variety of marine species captured in Scottish waters. Genotype IV comprises isolates from marine fish species found in the Pacific Northwest and Japan (1, 4, 5, 17, 23, 24, 26, 29).

Turbot *Psetta maxima* is one of the most valuable fish marketed in seaside countries of the Black Sea and Europe. Since 1997, a research and development project for seed production techniques of turbot in the Black Sea was started at the Central Fisheries Research Institute (CFRI) in Trabzon, Turkey, as a collaboration between the Turkish Ministry of Agriculture and Rural Affairs and the Japan International Cooperation Agency. In 2002, seed production of 139,000 larvae was carried out at the CFRI hatchery. However, difficulties resulted in a mortality rate of >90% (daily, approximately 7%) in the larvae at 7 to 21 days posthatching in 2003 and 2004. VHSV was also isolated from some of fish in the hatchery in 2004 (H. Savaş et al., unpublished data). This was the first time that VHSV had been detected in Turkey. Thus, in the present study, we performed a survey of VHSV in free-living turbot in the Black Sea and cultured brood stock in the CFRI hatchery to detect VHSV. Partial nucleotide sequences of the viral G gene were analyzed to elucidate the genetic relatedness of the Turkish isolates to other known VHSV isolates.

MATERIALS AND METHODS

Fish specimens. A total of 171 turbot, including 131 free-living fish captured between April and June 2005 by trawls on the Trabzon coast of the Black Sea in Turkey and 40 brood stock fish (each, 3 to 5 years old) in the CFRI hatchery were examined for virus isolation. The captured free-living fish were immediately transferred to the CFRI hatchery for determination of sexual maturity to select spawners mature enough for seed production; immature fish were subjected to virus isolation targeting four different tissues (brain, kidney, heart, and gonads).

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The mature fish were also subjected to virus isolation targeting those tissues after the fish had been stripped of sexual products. The 40 brood stock fish in the CFRI hatchery were randomly sacrificed for virus isolation by the same procedure as that used for the free-living fish.

Virus isolation. Two established fish cell lines, bluegill fry (BF-2) and rainbow trout gonad (RTG-2) cells, were used for virus isolation. BF-2 and RTG-2 cells were maintained at 18°C with Eagle's minimum essential medium (MEM; Gibco); supplemented with 10% (vol/vol) fetal bovine serum, 100 IU/ml penicillin G, and 100 μg/ml streptomycin; and subcultured every 10 to 14 days. Four tissues of adult fish (brain, heart, kidney, and gonad) and an entire larval body were subjected to virus isolation tests. Briefly, tissue homogenate with 9 volumes of Hanks' balanced salt solution was filtered with HA membrane (0.45 μm; Millipore) and was inoculated onto BF-2 and RTG-2 cells seeded in 24-well tissue culture plates (2 wells per sample). Inoculated cells were incubated at 18°C for 10 days, and the supernatant of the cells showing cytopathic effect was subjected to reverse transcription-PCR (RT-PCR) and neutralization tests for virus identification as described below. Titration of virus infectivity was performed with BF-2 cells seeded in 96-well tissue culture plates, and infectivity titers were read after 10 days of incubation at 18°C. The representative Turkish VHSV isolates, TR-Bs13/15H and TR-WS13G, with low passage numbers (maximum, three passages) were used for sequence analysis and pathogenicity tests.

Virus neutralization test. Representative virus isolates were subjected to quantitative neutralization tests with antisera against VHSV (Obama25) and aquabirnavirus (Obama10) (27). Briefly, 10-fold serial dilutions of isolated viruses were prepared, and each dilution was mixed with an equal volume of diluted antisera at 1:50 with Hanks' balanced salt solution. After incubation at 18°C for 1 h, an aliquot of each mixture (100 μl/well) was transferred to 2 wells of 96-well plates seeded with BF-2 cells and incubated at 18°C for 7 to 10 days for observation of viral neutralization.

PCR amplification. Viral RNA was extracted using an RNA extraction kit (Trizol; Invitrogen) according to the manufacturer's instructions for RT-PCR amplification with four different PCR primer sets. The first primer set, VM1sense (5′-CAC ATG RCT GAT ATT GAG ATG AG-3′) and VM1anti (5′-CTT GTC CAM STC CGC CTT G-3′), is for amplification of a 663-base region of the VHSV M1 gene (28), while the second primer set consists of VGsense (5′-CCA GCT CAA CTC AGG TGT CC-3′) and VGanti (5′-GTC ACY GTG CAT GCC ATT GT-3′), targeting a 587-base region of the VHSV G gene (17). The third and fourth primer sets consisted of IG1-ID3, targeting the G gene of infectious hematopoietic necrosis virus (IHNV, a fish novirhabdovirus) (14) and ABV-P1 and -P2 for the aquabirnavirus VP2/NS junction region (7, 18), respectively. For reverse transcription, extracted RNAs were heat denatured at 95°C for 5 min and then incubated at 42°C for 30 min in 10 μl of PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl) containing 50 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen), 2.5 μM reverse primer, 1 mM deoxynucleoside triphosphates, and 5 mM $MgCl_2$. After incubation at 99°C for 10 min, a targeted DNA was amplified in 50 μl of PCR buffer containing 0.5 μM each primer, 1.25 U of *Taq* DNA polymerase (Invitrogen), 0.2 mM deoxynucleoside triphosphates, and 2 mM $MgCl_2$ with a thermal cycler programmed for 1 cycle at 72°C for 10 min; 95°C for 2 min; 30 cycles, each consisting of 95°C for 40 s, 52°C (64°C in the case of VGsense-VGanti) for 40 s, and 72°C for 40 s; and a final hold step at 72°C for 5 min. The amplified products were analyzed by 2.0% agarose–40 mM Tris-acetate (pH 8.0)–1 mM EDTA gel electrophoresis and visualized under UV irradiation after being stained with ethidium bromide.

Nucleotide sequence analysis. PCR products from representative isolates TR-WS13G and TR-Bs13/15H were purified with a PCR purification kit (Stratagene) and subjected to nucleotide sequence analysis. Triplicate PCR products originating from independent RT reactions were sequenced for each isolate. Sequencing reactions were performed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) with ABI PRISM dye terminator sequencing chemistry, according to the manufacturer's instructions. The resulting sequences were assembled with DNASIS (Hitachi) to identify and exclude duplicate sequences from the data set. Based on single representatives of each sequence, a multiple alignment of the sequences was constructed using Clustal X (30, 31) to infer genetic relationships among each sequence with neighbor-joining criteria, and a final radial tree was drawn with the programs NJplot and Unrooted (19). The deposited nucleotide sequences of 91 isolates of VHSV in DDBJ were used for comparative purposes: AB060725, AB060727, AF143862 to -863, AF345857 to -859, AY546575 to -593, AY546596 to -605, AY546614 to -625, AY546627 to -632, U28747, U28800, U88050 to -056, X66134, Z93405 to -407, Z93409, Z93411 to -414, and Z93417 to -431 (4, 17).

Viral pathogenicity. The isolate TR-WS13G was used for experimental infections with turbot larvae (15 days old, approximately 5 mm of total body length) and rainbow trout fingerlings (146 days old with approximately 40 mm of total body length). A total of 400 turbot larvae were reared in four 200-liter aquaria at a density of 100 fish per tank and allowed to acclimate for 3 days prior to infection. Turbot larvae were challenged via an immersion root. Briefly, water flow was stopped, and the tanks were drained to a volume of 50 liters for the addition of TR-WS13G isolate to two tanks to achieve a dose of 3.0 log 50% tissue culture infective doses ($TCID_{50}$)/ml (groups V-a and V-b). After 1 h, the tank volumes were allowed to return to 200 liters each and maintained at approximately 20°C with a daily flow ratio of 600% for the duration of the experiment. Turbot larvae in the remaining two tanks received a control immersion infection carried out in a similar manner with an equivalent volume of MEM containing no virus (groups C-a and C-b). For the experimental challenge of rainbow trout fingerlings, a total of 20 fish were stocked in 4 liters in two aquaria at 10 fish per tank. Rearing water was kept at 13°C and maintained with a daily flow ratio of 80%. The TR-WS13G isolate at 6.1 log $TCID_{50}$/100 μl/fish or MEM (control) was injected into each fish via the intraperitoneal root. During experimental infections, dead fishes were removed daily and kept at −80°C; surviving fish were collected at 14 days after challenge. The dead turbot larvae (4 to 6 fish) or 10 surviving fish were pooled per each sample and subjected to virus isolation for VHSV recovery. All of the rainbow trout in the challenge and control groups were individually subjected to VHSV recovery.

Seed production trial. Eleven males and five females were selected from free-living spawner candidates captured in the Trabzon coastal area of the Black Sea, and a tag was driven into a pectoral fin for the identification of each fish. After stripping and artificial insemination, fertilized eggs were disinfected at a rate of 100 ppm of iodophor for 10 min by following data by Kurita et al. (10) and Hatori et al. (6). Hatched larvae were reared for 25 days in different aquaria, with each batch at a density of approximately 35,000 larvae per 2,000-liter tank, and larval mortality rates were monitored daily. Dead fish were collected every day and kept at −80°C until used for virus isolation and RT-PCR tests. After being stripped, all spawners were dissected to remove brain, heart, kidney, and gonad tissues for virus isolation as described above.

Nucleotide sequence accession numbers. The determined nucleotide sequences were registered with the DNA Data Bank of Japan (DDBJ) as accession numbers AB231160 and AB231161

RESULTS

VHSV isolation and detection. As a result of virus isolation testing for a total of 66 free-living spawners captured in the Trabzon coastal area, VHSV was isolated from 14 fish (positive ratio, 21.2%), and unknown virus isolates were isolated from two fish kidneys (Table 1). All of the VHSV isolates exhibited a cytopathic effect typical of fish rhabdoviruses on BF-2 cells and were well neutralized by antiserum against VHSV (data not shown). VHSV isolation ratios from heart and gonad tissues were, respectively, 21.5% and 21.2%, which were higher than those from brain (7.1%) and kidney (11.8%) tissues. In comparing the VHSV detection ratio, BF-2 cells were 7 to 14 times more sensitive than RTG-2 cells in each tissue (Table 1). All of the VHSV isolates exhibited positive reactions by PCR with primers VM1sense-VM1anti and VGsense-VGanti but not with primers IG1-ID3 (for IHNV) or ABV-P1 and ABV-P2 (for aquabirnaviruses) (data not shown). Sizes of the obtained PCR products from each VHSV isolate were 660 bp by VM1sense-VM1anti and 590 bp by VGsense-VGanti; those sizes corresponded to the target region of each primer set. No PCR product was amplified from the unknown virus isolates by any primer sets used in the present study.

Results of virus isolation from free-living immature turbot in the Trabzon coastal area and cultured brood stock fish in the CFRI hatchery are shown in Table 2. VHSV was isolated from only 1 fish of 65 free-living immature fish (positive ratio, 1.5%), while 7 of 40 brood stock fish were VHSV positive (17.5%) by BF-2 cell culture. One unknown virus was isolated from free-living fish by RTG-2 cell culture. The VHSV isolation ratio of

TABLE 1. VHSV isolation with RTG-2 and BF-2 cells from four tissues of free-living turbot spawners caught in the Trabzon coastal area

Fish	*n*	Mean body wt (g)	Mean body length (cm)	% of virus isolated (no. of samples/total) from:							
				Brain		Heart		Kidney		Gonad	
				RTG-2	BF-2	RTG-2	BF-2	RTG-2	BF-2	RTG-2	BF-2
Male	37	1,764	44.9	0 (0/24)	12.5 (3/24)	2.7 (1/37)	29.7 (11/37)	3.4[a] (1/29)	20.7 (6/29)	2.7 (1/37)	24.3 (9/37)
Female	27	2,156	46.5	0 (0/17)	0 (0/16)	0 (0/27)	11.5 (3/26)	0[a] (0/19)	0 (0/20)	3.7 (1/27)	18.5 (5/27)
Unknown	2			0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)
Total	66	2,767	51.4	0 (0/43)	7.1 (3/42)	1.5 (1/66)	21.5 (14/65)	2.0 (1/50)	11.8 (6/51)	3.0 (2/66)	21.2 (14/66)

[a] Additional unknown virus isolates were detected from kidney of two fish by RTG-2 cells but not counted here.

culture brood stock fish was slightly lower than that for free-living spawners but obviously higher than that of free-living immature fish (Table 2). Also, a great discrepancy was observed in VHSV isolation ratios between RTG-2 and BF-2 cells, as well as that of free-living spawners.

Nucleotide sequence analysis. A partial G gene of the representative two VHSV isolates, TR-Bs13/15H and TR-WS13G, was amplified with VGsense-VGanti for nucleotide sequence analysis, and both of the PCR products were 587 bp, corresponding to nucleotides (nt) 175 to 761 of the VHSV G gene open reading frame. In a comparison of sequences between TR-Bs13/15H and TR-WS13G isolates, four bases of nucleotide substitution and one residue of amino acid substitution were observed, and sequence identities of >99% at nucleotide and amino acid levels were observed. Against known VHSV isolates, both new isolates exhibited a >83% sequence identity at the nucleotide level and >94% identity at the amino acid level. The present radial tree based on nt 361 to 720 of the G gene open reading frame revealed the existence of four major clusters for genotypes I to IV; moreover, five minor clusters for classes I-a to I-e were observed for genotype I, as described in previous studies (4, 5). I-a represented European freshwater isolates from Austria, Denmark, France, and Switzerland; I-b included isolates originating from the Baltic Sea, Skagerrak, Kattegat, the North Sea, and the English Channel; I-c included Danish freshwater isolates; I-d was for Norwegian and Finnish isolates; and I-e included an isolate from Georgia. The Turkish isolates, TR-Bs13/15H and TR-WS13G, appeared in class I-e of genotype I in the present tree (Fig. 1) and were the most closely related to the GE-1.2 isolate (AY546619), which was isolated from rainbow trout in Georgia in 1982 (4). Sequence identities between GR-1.2 and TR-Bs13/15H or TR-WS13G were >98% at the nucleotide level and >99% at the amino acid level.

TABLE 2. VHSV isolation from heart of cultured brood stock in the CFRI hatchery and free-living turbot in the Trabzon coastal area

Fish	*n*	Mean body wt (g)	Mean body length (cm)	% Isolation (no. of samples/total)	
				RTG-2	BF-2
Free-living spawners	66	2,767	51.4	1.5 (1/66)	21.5 (14/65)
Free-living turbot	65	714	35.1	0[a] (0/65)	1.5 (1/65)
Cultured brood stocks	40	1,189	39.3	2.5 (1/40)	17.5 (7/40)

[a] An additional unknown virus isolate was detected from the free-living fish but not counted.

Virus pathogenicity tests. Cumulative mortality curves of turbot larvae and rainbow trout fingerlings in the VHSV infection experiments with the TR-WS13G isolate are shown below (see Fig. 2). Although no typical symptom for VHS disease was externally observed in any fish in the four groups, cumulative mortalities of turbot larvae in the two VHSV-challenged groups, V-a and V-b, were 7% and 23% (average, 15%) at 14 days postchallenge, levels similar to as those of the control groups (C-a, 8%; C-b, 14%; average, 11%). VHSV was recovered from only two samples of dead fish at 7 to 12 days postchallenge from the challenged group V-a, while the virus was not recovered from any fish in another challenged group (V-b) or two control groups (C-a and C-b). In the experimental infection with rainbow trout fingerlings, no mortality was observed in either the challenge or the control group within 14 days postchallenge; moreover, no VHSV was isolated from any survival fish in the challenged and control groups.

Seed production of larvae in the CFRI hatchery. The six batches of offspring from 15 free-living spawners were reared in 12 separate tanks in the CFRI hatchery to monitor mortalities and VHSV existence. Although VHSV was detected in 3 of 11 males and one of four females, all spawners seemed apparently healthy. After 7 days of hatching, daily moralities of 6 to 7% occurred in all batches and continued until the 21st day after hatching. Cumulative mortalities in tanks V-1 to V-6 for larvae from VHSV-positive spawners ranged from 78.0% to 95.3% (average, 87.5%), while those in tanks C-1 to C-6 for larvae from VHSV-negative spawners were from 81.3% to 96.7% (average, 91.0%). There was no significant difference in cumulative mortalities between larvae from VHSV-positive and -negative spawners; moreover, no VHSV was detected from any dead larvae by virus isolation or RT-PCR tests (Table 3).

DISCUSSION

A total of 171 turbot, including 66 free-living spawners, 65 free-living immature fish, and 40 cultured brood stock fish, were tested for virus isolation to evaluate a participation of VHSV in mortality of turbot larvae in the CFRI hatchery. The VHSV isolation ratios from free-living spawners and immature turbot were 21.5% and 1.5%, respectively (Tables 1 and 2), although all of the VHSV-positive fish exhibited no clinical

TABLE 3. Results of VHSV detection and cumulative mortalities of turbot larvae of seed production in the CFRI in 2005

Spawner VHSV status	Tank no.	Date of hatching	Spawner[a]		Virus isolation[b]		RT-PCR[b]		Cumulative mortality (%)	
			Male	Female	RTG-2	BF-2	Test 1	Test 2	At 25 days old	Avg
Positive	V-1	13 May	**60G, 62G**	35G	—	—	—	—	84.6	87.5
	V-2	13 May	**60G, 62G**	35G	—	—	—	—	89.6	
	V-3	15 May	57G, **61G**	35G	—	—	—	—	78.0	
	V-4	15 May	57G, **61G**	35G	—	—	—	—	82.8	
	V-5	9 May	8G, 9G	**27G**	—	—	—	—	95.3	
	V-6	9 May	8G, 9G	**27G**	—	—	—	—	94.4	
Negative	C-1	10 May	5G, 7G, 8G, 9G	35G	—	—	—	—	81.3	91.0
	C-2	10 May	5G, 7G, 8G, 9G	35G	—	—	—	—	88.0	
	C-3	10 May	5G, 7G, 8G, 9G	35G	—	—	—	—	95.6	
	C-4	5 May	6G, 10G	2G	—	—	—	—	96.7	
	C-5	8 May	5G, 7G, 22G	24G	—	—	—	—	92.6	
	C-6	8 May	5G, 7G, 22G	24G	—	—	—	—	91.5	

[a] Spawner numbers in boldface type were VHSV positive.
[b] —, negative results.

signs of disease. The present results revealed that VHSV was widely distributed among free-living turbot in the Trabzon coastal area of the Black Sea. This is the first detection of VHSV in free-living fish in Turkey, although IHNV and infectious pancreatic necrosis virus have been isolated from cultured rainbow trout and Black Sea trout (*Salmo trutta labrax*) at sea cages in the Black Sea (Savaş et al., unpublished). An obvious difference in VHSV isolation ratio was observed between free-living immature fish and spawners, which were captured at the same time and in the same area. Thus, it is suggested that the infection level of VHSV in turbot could drastically increase by spawning maturation, even if it was at an undetectable level when fish were immature.

In a comparison of the detection ratios among four different tissues, VHSV was detected at a higher level in heart and gonad tissues than in kidney and brain tissues (Table 1). Although heart tissue has generally not been employed as a target of VHSV isolation in previous studies, Iida et al. (8) suggested that heart tissue was the most probable hiding site of VHSV, based on the data for the fate of VHSV in Japanese flounder experimentally infected via an immersion root. Moreover, necrotizing myocarditis could be considered a pathognomonic sign of VHSV infection in Japanese flounder (9). Thus, heart tissue is also considered to be an important target for VHSV detection in potential carrier fishes.

Though RTG-2 cells are generally used for the diagnosis VHS (22), the VHSV detection ratio by RTG-2 cells was much lower than the ratio for BF-2 cells (Table 1). Mori et al. (15) reported that it was difficult to multiply a Japanese isolate of VHSV in RTG-2 cells. It is confirmed that susceptibility of RTG-2 cells to VHSV was $>10^2$ times lower than that of BF-2 cells in in vitro experiments (data not shown). We believe that the low level of susceptibility of RTG-2 cells to VHSV could be a result of the conditions of our RTG-2 cells, not caused by the virus isolates, because susceptibilities of cells against viruses are liable to change by passage numbers and/or maintenance conditions (11).

Partial G gene nucleotide sequences of the representative two isolates, TR-WS13G and TR-Bs13/15H, were analyzed to evaluate the genetic relatedness among known VHSV isolates. As shown in Fig. 1, the present radial tree of 99 VHSV isolates revealed four separate clades for genotypes I to IV; moreover, five minor clades for classes I-a to I-e were observed in genotype I, as previously identified by Einer-Jensen et al. (4, 5). Both TR-WS13G and TR-Bs13/15H isolates appeared in class I-e of genotype I and exhibited >98% sequence identity at both the nucleotide and amino acid levels with the GE-1.2 isolate, which was first isolated in 1981 from rainbow trout in Georgia, a country neighboring Turkey (4). In the present analysis, five VHSV isolates from turbot (*S. maximus*) (814, UK-860/94, Sco95, IR-F13.02.97, and Ger7321) were included; the first four isolates appeared in a clade for genotype III, while the remaining isolate, Ger7321, appeared in class I-a of genotype I (Fig. 1). Thus, it was confirmed that Turkish isolates of VHSV were genetically distinguishable from known turbot (*S. maximus*) isolates. Furthermore, we considered that the Turkish isolates could be an indigenous type of VHSV distributed in the eastern Black Sea area and not introduced from European countries. This assumption was strongly supported by the fact that a GE-1.2 isolate, genetically quite similar to TR-WS13G, existed >20 years ago in Georgia.

In the pathogenicity tests, TR-WS13G induced 7% and 23% of cumulative mortalities in challenged groups, and VHSV was recovered from some of the dead fish in group V-a but not from any dead fish in another challenged group, V-b (Fig. 2). Thus, turbot larvae were confirmed to have low susceptibility to Turkish VHSV, and susceptibility of turbot larvae seemed to be dependent on the condition of individual fish. Cumulative mortalities in challenged groups were similar to those of control groups (Fig. 2). Moreover, TR-WS13G induced no mortality in rainbow trout fingerlings infected by the intraperitoneal root, and no VHSV was recovered from challenged fish. It is possible to conclude that Turkish VHSV has no or little pathogenicity against at least turbot larvae and rainbow trout fingerlings. Previous studies (2, 25) reported that VHSV isolates from farmed turbot (*S. maximus*) exhibited high levels of pathogenicity to *S. maximus* by a challenge via the intraperitoneal root but relatively low pathogenicity after challenge via an immersion root. Skall et al. (21) concluded that VHSV isolates from wild marine fish were generally nonpathogenic or had very low levels of pathogenicity by immersion challenge for rainbow trout; they also reported that two isolates from farmed

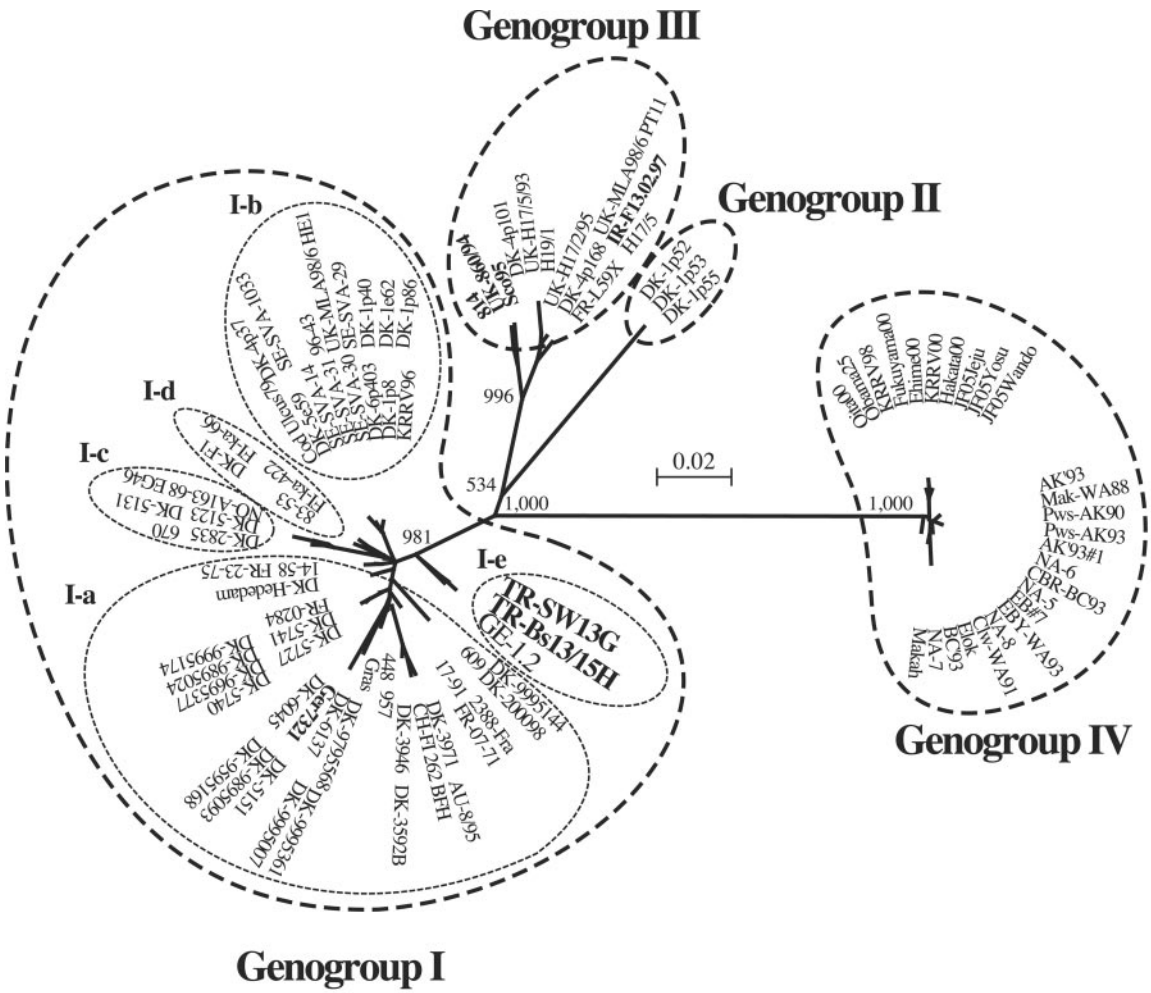

FIG. 1. Radial tree based on nucleotide sequences of a partial G gene nucleotide sequence (nt 361 to 720) among 99 worldwide isolates of VHSV. Isolates shown in boldface type originated from turbot (*Scophthalmus maximus* and *Psetta maxima*). Bootstrap values at 1,000 times construction are shown at major nodes. The scale bar is for a genetic distance marker (number of replacement nucleotides per site).

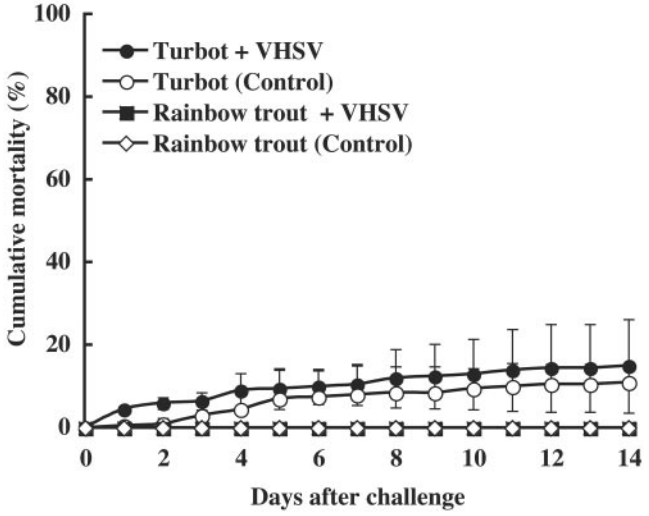

FIG. 2. Cumulative mortality curves of turbot larvae and rainbow trout fingerlings in the experimental infection with Turkish VHSV isolate TR-WS13G. The turbot and rainbow trout were challenged via an immersion root and an intraperitoneal root, respectively. Cumulative mortalities of turbot larvae are shown as averages of data from each of two experimental groups.

S. maximus fish did not induce mortality in rainbow trout by immersion, supporting the view that they originated from the marine environment. Interestingly, TR-WS13G did not induce mortality in brood stock turbot (*P. maxima*) by intramuscular injection at 5.0 log $TCID_{50}$/fish (Işıdan et al., unpublished data). The present findings also support the assumption, described above, that the Turkish VHSV isolates originated from the Black Sea environment as an indigenous type.

As the results of virus isolation from cultured brood stock fish in the CFRI hatchery show, VHSV was detected in 7 of 40 fish (17.5%); the positive ratio was slightly lower than that of free-living spawners (21.5%) but much higher than that of free-living immature fish (1.5%) (Table 2). Although none of the CFRI brood stock fish exhibited clinical symptoms of VHS disease, there was concern that the VHSV-positive ratio of the brood stock fish might increase with spawner maturation as described above; therefore, the CFRI brood stock fish were not employed in 2005 for seed production. The seed production and rearing of turbot larvae were performed by taking care to avoid VHSV contamination, following a control strategy for fish viral diseases by Yoshimizu (37). Unfortunately, VHSV was detected

in 4 of 11 spawners for the seed production; approximately 7% of daily mortalities occurred in all of the tanks from the 7th to the 21st day after hatching. However, there was no significant difference in cumulative mortalities of larvae between from VHSV-positive spawners (tanks V-1 to V-6) and VHSV-negative spawners (tanks C-1 to C-6); no VHSV was detected in any dead larvae (Table 3). Therefore, it could be concluded that the mortalities in the seed production of turbot larvae were not caused by VHSV infection, although a main factor(s) has not yet been determined. Furthermore, the present results suggest that VHSV-free turbot larvae could be produced by disinfection of fertilized eggs with iodophor and taking enough care to avoid horizontal infection, even though VHSV-positive fish crept into spawner candidates.

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