(Short paper)
Genetic Diversity of Infectious Hematopoietic Necrosis Virus Isolated in Hokkaido, Japan

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Abstract　The NV gene sequence of the IHN virus isolated in Hokkaido, Japan, was investigated in order to obtain knowledge about the genetic evolution of the virus. Four strains isolated in the 1980s (HLR-1, HSR-3, HLM-3 and HSM-1) and four strains isolated in the 2000s (Hen 00, Hhi 01, Hni 00 and Hch 01) were used in this study. All the NV genes were found to be 336 in nucleotide length. The genetic distances (substitutes / positions) of the combinations of the isolates in the 1980s were 0.015 to 0.033. The genetic distances of the isolates in the 2000s were 0.039 to 0.086. The phylogenic tree constructed from the sequence data indicated that the diversity of NV genes of the isolates in the 2000s was higher than that of the isolates in the 1980s. Through an analysis including of other NV gene sequences deposited in GenBank, strain RB-76 isolated in the United States was found to be similar to strain HSR-3. The substitutes between strain RB-76 and strain HSR-3 were 2 nucleotides per 336 positions (0.006 in genetic distance). Each sequence data of eight strains isolated in Hokkaido showed highest similarity with the data of strain RB-76. The results suggest that the IHN virus in Hokkaido evolved from an origin similar to that of strain RB-76.

Key words: IHN, genetic diversity, NV gene

Infectious hematopoietic necrosis (IHN) is still a serious problem in salmonid culture. The IHN virus may have high variation because the distribution of the virus has spread in the world since the 1950s. Techniques for researching the polymorphisms of the IHN viral gene have been reported. Oshima *et al*. (1995) researched the variety by T1 ribonuclease fingerprinting. Kurath *et al*. (1995) reported the utility of RNase protection assay. In the techniques for researching genetic variations, nucleotide sequencing may bring the most detailed data. The IHN virus genome is a single-stranded antisense RNA encoding the N, P, M, G, NV and L gene (Kurath *et al*., 1985). Nichol *et al*. (1995) researched the nucleotide divergence of the G and NV gene of the virus isolated in the United States. They concluded that the phylogenic relationships of the virus correlated with the localities of the isolate origins. In this report, the NV gene sequences of four strains isolated in the 1980s (1986-1989) and the four isolated in the 2000s (2000- 2001) were investigated in order to obtain knowledge about the genetic evolution of the IHN virus in Hokkaido, Japan.

The IHN virus strains used for this study were isolated from salmonid reared at private farms in Hokkaido, Japan (Table 1). The details of the origins including location or farm name have been eliminated from Table 1 because of commercial privacy. For virus multiplication, RTG-2 cells (Wolf and Quimby, 1962) were cultured at 20℃ with Eagle's minimum essential medium (MEM) supplemented with fetal bovine serum

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at 10 %, penicillin at 100 I.U. per ml and streptomycin at 100 μg per ml. RTG-2 cells cultured in 25 cm^2 flask were infected with IHN virus at MOI 1, and incubated at 15℃ for 72 h. Infected cells were harvested by mechanically detaching from the flask bottoms, and centrifugation at 1000 X *g* for 10 min. Nucleic acid was purified from cell pellets by SepaGene (Sankyo Junyaku Co., Ltd., Tokyo, Japan) according to the manual. The obtained pellets containing the IHN virus genome were dissolved in distilled water, and used for RT-PCR. RT-PCR was conducted by Titan One Tube RT-PCR (Roche Diagnostic Inc., Indianapolis, Indiana, U. S.). The primers for NV gene amplification were designed from the published sequence of strain WRAC (Morzunov *et al*., 1995; Gene Bank accession number L40883). The nucleotide sequences of the primers were
Forward (5'-CAAAAAGAGACAATGGACCAC-3')
and Reverse (5'- CTTTCTGTGATGGGGTGCTGT-3').
RT-PCR was performed in a mixture (50 μl in the total volume) containing 1 μl RNA sample, 1 μl primers (20 μM), 10 μl 5 X buffer (of the kit), 5 μl dNTPs (2 mM each, Perkin-Elmer Inc., Boston, Massachusetts, U. S.), 2.5 μl dithiothreitol (100 mM, of the kit), 1 μl of the enzyme containing reverse transcriptase and DNA polymerase (of the kit), and 1.5 μl RNasin (RNase inhibitor, 40 units per μl, Promega Inc., Madison, Wisconsin, U.S.). Temperature cycling was performed by Gene Amp 2400 (Perkin-Elmer). The reaction mixtures were incubated for reverse transcription (50 ℃ for 30 min), and were then incubated for 35 cycles composed of denaturation (94 ℃ for 30 sec), annealing (55 ℃ for 30 sec) and extension (68℃ for 45 sec). Finally, the reaction tubes were incubated for additional extension (68℃ for 7 min). The PCR products obtained were ligated into plasmid vector pCR 2.1 (Invitrogen Inc., Carlsbad, California, U. S.). The plasmid vector was transfected into competent *E.coli* cells (strain Top 10 F'). In order to determine the sequences, the plasmid was purified from the transformed *E.coli* clone using FlexiPrep kit (Amersham Pharmacia Biotech AB., Uppsala, Sweden). The sequences of the NV genes were determined by an automatic sequencer, ALF-2 (Amersham Pharmacia Biotech), using Autocycle sequencing kit (Amersham Pharmacia Biotech). The sequence edits were performed by a software package, GeneWorks 2.45 (Teijin Science Technology Co., Ltd., Yokohama, Japan). A phylogenetic tree was constructed by Clustal W 1.7 (Thompson *et al*., 1994), and drawn by TreeView (Page, 1996).

Table 1 Strains of IHN virus isolated in Hokkaido, Japan

Strain	Year	Host
HLR-1*	1989	Rainbow trout
HSR-3*	1986	Rainbow trout
HLM-3**	1988	Masu salmon
HSM-1**	1988	Masu salmon
Hen 00	2000	Rainbow trout
Hhi 01	2001	Rainbow trout
Hni 00	2000	Rainbow trout
Hch 01	2001	Sockeye salmon

*: The strain was reported by Suzuki and Sakai (1989).
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The nucleotide sequences of the NV gene were shown in Table 2. All the NV genes were found to be 336 in nucleotide length including the start and stop signal. The genetic distances (substitutes / positions) of the strains are shown in Table 3. The genetic distances of all combinations of the isolates in the 1980s (HLR-1, HSR-3, HLM-3 and HSM-1) were 0.015 to 0.033. The genetic distances of the isolates in the 2000s (Hen 00, Hhi 01, Hni 00 and Hch 01) were 0.039 to 0.086. The phylogenic tree constructed from the sequence data is shown in Fig. 1. The tree clearly indicates that the diversity of the NV genes of the isolates in the 2000s was higher than that of the isolates in the 1980s. These findings indicate that the diversity of the virus may have increased in Hokkaido, Japan.

Nicol *et al*. (1995) have researched the divergence of the IHN virus isolated in the United States by an analysis of sequence data including the NV gene (GenBank accession number L40871- L40882). The genetic distances of the NV gene between the isolates used in their study and the isolates in this report were calculated (Table 4). Out of 12 strains isolated in the United States, strain RB-76 was found to be similar to strain HSR-3. The substitutes of the NV gene between strain RB-76 and strain HSR-3 were only 2 nucleotides

Table 2 Nucleotide sequences of NV gene encoded in the IHN virus strains isolated in Hokkaido, Japan

	1 10	20	30	40	50	60	70	80	90
	atggaccacc	gygamayaaa	cacgmwcatg	gargcactca	gagamgytct	gcgrtacaag	aacraggtgg	ccggacacgs	yttcctcttt
HLR-1	**********	*t**c*c***	****aa****	**g*******	****a*t***	***a******	***g******	*********g	c*********
HSR-3	**********	*t**c*c***	****aa****	**g*******	****a*t***	***a******	***g******	*********g	c*********
HLM-2	**********	*t**c*c***	****at****	**g*******	****a*t***	***a******	***g******	*********c	t*********
HSM-1	**********	*t**c*c***	****aa****	**g*******	****a*t***	***g******	***g******	*********g	c*********
Hen00	**********	*t**a*t***	****ca****	**g*******	****c*c***	***a******	***g******	*********g	c*********
Hhi01	**********	*c**a*c***	****aa****	**a*******	****a*c***	***a******	***g******	*********g	c*********
Hni00	**********	*c**a*c***	****aa****	**g*******	****a*c***	***a******	***g******	*********g	c*********
Hch01	**********	*t**a*t***	****aa****	**g*******	****a*t***	***a******	***a******	*********g	c*********

	100 110	120	130	140	150	160	170	180
	raygacggwg	acctggtmtg	gckkgargag	gacgacgmrm	crtggaggcg	gyyttacgat	gtcgtcammg	sactgatytb	ytccaagagg
HLR-1	g*c*****t*	*******a**	**gt**a***	*******cac	*a********	*ct*******	*******cc*	c******t*c	c*********
HSR-3	g*c*****t*	*******a**	**gt**a***	*******caa	*a********	*ct*******	*******ac*	c******c*c	c*********
HLM-2	g*c*****a*	*******a**	**gt**a***	*******caa	*a********	*ct*******	*******ac*	c******c*c	c*********
HSM-1	g*c*****a*	*******a**	**gt**a***	*******aaa	*a********	*ct*******	*******ac*	c******c*c	c*********
Hen00	g*c*****a*	*******a**	**gt**a***	*******caa	*g********	*ct*******	*******ac*	c******c*c	c*********
Hhi01	a*c*****a*	*******a**	**gt**a***	*******caa	*a********	*ct*******	*******ac*	c******c*g	c*********
Hni00	g*c*****a*	*******a**	**gt**g***	*******aaa	*a********	*tc*******	*******ac*	g******c*c	t*********
Hch01	g*t*****a*	*******c**	**tg**a***	*******cga	*a********	*ct*******	*******aa*	c******c*t	c*********

	190 200	210	220	230	240	250	260	270
	atgcagcsag	taytgtacat	ggacctcagy	atcaccaagg	gcgargggya	tytaytttk	gtggatctcc	arggrvmyaa	gaaccgcytg
HLR-1	*******g**	**t*******	*********c	**********	****g***c*	*c**c****t	**********	*a**gacc**	*******c**
HSR-3	*******g**	**t*******	*********c	**********	****g***c*	*c**c****t	**********	*g**gacc**	*******c**
HLM-2	*******g**	**t*******	*********c	**********	****g***c*	*c**c****t	**********	*g**gacc**	*******t**
HSM-1	*******g**	**t*******	*********c	**********	****g***c*	*c**c****t	**********	*g**gacc**	*******t**
Hen00	*******g**	**c*******	*********t	**********	****g***c*	*c**c****t	**********	*g**gacc**	*******c**
Hhi01	*******g**	**t*******	*********c	**********	****g***c*	*c**c****t	**********	*g**acac**	*******t**
Hni00	*******g**	**t*******	*********c	**********	****g***c*	*c**c****t	**********	*g**ggac**	*******c**
Hch01	*******c**	**t*******	*********c	**********	****a***t*	*t**t****g	**********	*g**gact**	*******c**

	280 290	300	310	320	330 336
	tacaaagarc	mccgrttcag	gagacatctg	atmctgattg	argamtttct	tgcttatcyc	agatag
HLR-1	********a*	c***a*****	**********	**c*******	*a**c*****	********c*	******
HSR-3	********a*	a***a*****	**********	**c*******	*a**c*****	********c*	******
HLM-2	********a*	c***g*****	**********	**c*******	*g**c*****	********c*	******
HSM-1	********a*	c***a*****	**********	**c*******	*a**c*****	********c*	******
Hen00	********a*	c***a*****	**********	**a*******	*a**c*****	********t*	******
Hhi01	********a*	c***a*****	**********	**c*******	*a**c*****	********c*	******
Hni00	********g*	c***a*****	**********	**c*******	*a**c*****	********c*	******
Hch01	********a*	c***g*****	**********	**c*******	*a**a*****	********c*	******

per 336 positions (0.006 in genetic distance). Furthermore, each sequence data of eight strains isolated in Hokkaido showed highest similarity with the data of strain RB-76. Through the analysis, it was suggested that strain HSR-3 and strain RB-76 evolved from the same origin. The results also suggest that the IHN virus in Hokkaido evolved from the origin similar to strain HSR-3. Winton (1991) stated that worldwide distribution of the IHN virus was due to the movement of infected fish and eggs. Yoshimizu (1996) also reported

Table 3 Genetic distance among the strains

	HLR-1	HSR-3	HLM-3	HSM-1	Hen 00	Hhi 01	Hni 00	Hch 01
HLR-1	—	0.015	0.033	0.024	0.045	0.036	0.051	0.071
HSR-3	0.015*	—	0.024	0.015	0.036	0.036	0.042	0.063
HLM-3	0.033	0.024	—	0.021	0.048	0.042	0.054	0.068
HSM-1	0.024	0.015	0.021	—	0.039	0.033	0.039	0.065
Hen 00	0.045	0.036	0.048	0.039	—	0.048	0.054	0.074
Hhi 01	0.036	0.036	0.042	0.033	0.048	—	0.039	0.077
Hni 00	0.051	0.042	0.054	0.039	0.054	0.039	—	0.086
Hch 01	0.071	0.063	0.068	0.065	0.074	0.077	0.086	—

*: Genetic distance is shown as value of substitutions per positions (336).

Table 4 Genetic distances of NV genes among the isolates in Hokkaido and the isolates in the United States

Isolates in U. S. *	Isolates in Hokkaido							
	HLR-1	HSR-3	HLM-3	HSM-1	Hen 00	Hhi 01	Hni 00	Hch 01
RB-76	0.015 **	0.006	0.018	0.009	0.030	0.030	0.036	0.057
LWS-87	0.027	0.018	0.030	0.021	0.042	0.042	0.048	0.068
LR-73	0.030	0.021	0.033	0.024	0.045	0.039	0.039	0.071
Carson-89	0.030	0.021	0.033	0.024	0.045	0.045	0.051	0.071
SRCV	0.036	0.027	0.039	0.030	0.039	0.045	0.051	0.068
Col-80	0.036	0.027	0.039	0.030	0.039	0.045	0.051	0.068
WRAC	0.033	0.030	0.042	0.033	0.036	0.042	0.048	0.068
CST-82	0.033	0.030	0.042	0.033	0.036	0.042	0.048	0.068
LR-80	0.042	0.033	0.045	0.036	0.045	0.051	0.057	0.077
Col-85	0.042	0.033	0.045	0.036	0.045	0.051	0.057	0.077
HO-7	0.048	0.039	0.051	0.042	0.051	0.057	0.063	0.083
193-11	0.045	0.042	0.054	0.045	0.054	0.051	0.065	0.086

*: The neuleotide sequences of NV gene of U. S. isolates were researched by Nichol et al. (1997) and deposited in GenBank (Number L40871-L40882).
**: Genetic distance is shown as value of substitutions per positions (336).

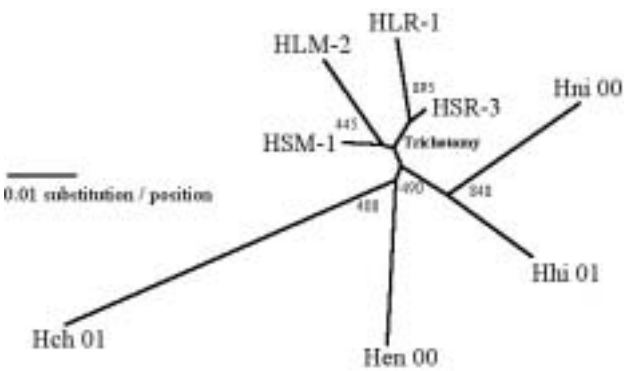

Fig. 1 The phylogenic tree (neighbor jointing method) constructed from sequence data of NV gene. Bootstrap values for 1,000 replicates are shown. Bar indicates genetic distance (0.01 substitutions per positions).

that IHN that took root in some salmonid fish in Japan might originate from infected eggs brought by international trade. The similarity found in strain HSR-3 isolated in Hokkaido and strain RB-76 isolated in the United States is strong evidence for their hypothesis.

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和文摘要

北海道で分離された伝染性造血器壊死症ウイルスの遺伝的多様性

畑山　誠

北海道におけるIHNウイルスの変異について知るため，ウイルス遺伝子の一つNV遺伝子の塩基配列を調査した．1980年代に分離されたIHNウイルス4株（HLR-1, HSR-3, HLM-3, HSM-1）ならびに2000年以降に分離された4株（Hen 00, Hhi 01, Hni 00, Hch 01）を解析の対象とした．1980年代に分離された4株の遺伝的距離（塩基置換率）は0.015から0.033であった．一方，2000年以降に分離された4株の遺伝的距離は0.039から0.086であり約十数年の間にもIHNウイルスの変異が進んでいることがうががえた．さらにGenBank に登録されているNV遺伝子の塩基配列を含め解析を行ったところ，米国で分離されたRB-76株が北海道のHSR-3株と非常に近似していることがわかった．この2株の塩基置換は336塩基中2塩基であった．そして北海道で分離された8株全てが，GenBankに登録されているNV遺伝子のなかRB-76株に近似していることがわかった．これらの結果から北海道のIHNウイルスはRB-76株に近似したものから派生したことが考えられた．