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Occurrence and genetic typing of infectious hematopoietic necrosis virus in Kamchatka, Russia

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ABSTRACT: Infectious hematopoietic necrosis virus (IHNV) is a well known rhabdoviral pathogen of salmonid fish in North America that has become established in Asia and Europe. On the Pacific coast of Russia, IHNV was first detected in hatchery sockeye from the Kamchatka Peninsula in 2001. Results of virological examinations of over 10 000 wild and cultured salmonid fish from Kamchatka during 1996 to 2005 revealed IHNV in several sockeye salmon *Oncorhynchus nerka* populations. The virus was isolated from spawning adults and from juveniles undergoing epidemics in both hatchery and wild sockeye populations from the Bolshaya watershed. No virus was detected in 2 other watersheds, or in species other than sockeye salmon. Genetic typing of 8 virus isolates by sequence analysis of partial glycoprotein and nucleocapsid genes revealed that they were genetically homogeneous and fell within the U genogroup of IHNV. In phylogenetic analyses, the Russian IHNV sequences were indistinguishable from the sequences of North American U genogroup isolates that occur throughout Alaska, British Columbia, Washington, and Oregon. The high similarity, and in some cases identity, between Russian and North American IHNV isolates suggests virus transmission or exposure to a common viral reservoir in the North Pacific Ocean.

KEY WORDS: Fish virus · IHNV · Sockeye salmon · Russia · Virus typing · Rhabdovirus · Infectious hematopoietic necrosis virus

INTRODUCTION

Infectious hematopoietic necrosis (IHN; Amend et al. 1969) is an economically significant disease caused by a rhabdovirus (IHNV) in Pacific salmon *Oncorhynchus* spp., Atlantic salmon *Salmo salar*, and rainbow/steelhead trout *O. mykiss* (Wolf 1988, Bootland & Leong 1999). The disease was first observed in cultured sockeye salmon *O. nerka* in western North America (Rucker et al. 1953, Watson et al. 1954), and the first report of IHNV isolation in cell culture was by Wingfield et al. (1969). Economic losses from IHNV result directly from fish mortality, and indirectly from regulations restricting the movement of IHNV-infected fish or the destruction of infected fish stocks to control the spread of the virus.

In North America, IHNV is currently endemic throughout the Pacific Northwest, where it occurs in multiple Pacific salmon and trout species. An IHNV genetic typing system has been developed based on phylogenetic analysis of the 303 nucleotide (nt) 'midG' region within the viral glycoprotein (G) gene (Emmenegger et al. 2000, Troyer et al. 2000, Emmenegger & Kurath 2002, Garver et al. 2003, Kurath et al. 2003, Troyer & Kurath 2003). This system has revealed 3 major IHNV genogroups in North America, designated U, M, and L to correlate with their occurrence in the upper, middle, and lower portions of the virus geographic range (Kurath et al. 2003). The resolution of IHNV into these genogroups has recently been confirmed by analyses of a second genetic region, the '5'N', which is a 412 nt sequence

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near the 5′ end of the viral nucleocapsid (N) gene (Garver et al. 2003, Kurath et al. 2005). A set of 37 North American IHNV isolates has been selected to represent all genogroups and sub-genogroups currently known, and their midG and 5′N sequences comprise the GB37 data set for IHNV phylogenetic analyses (Kurath et al. 2005).

Although originally endemic to North America, IHN also occurs in Asia and Europe, where it is thought to have been spread during the 1960s and 1980s, respectively, via transport of infected fish eggs or fry from North America (Sano et al. 1977, Bovo et al. 1987). In Russia, IHN was confirmed from rainbow trout in an experimental hatchery near Moscow in 2000 (I. Shchelkunov pers. comm.). This virus had never been detected on the Pacific coast of Russia until it was isolated among adult sockeye salmon on the Kamchatka Peninsula in 2001 (Rudakova & Bochkova 2005). In Kamchatka, 5 fish hatcheries rear several species of Pacific salmon for release into rivers and streams. This report describes virological testing, IHNV isolations, and the first genetic characterizations of IHNV from cultured and wild Pacific salmon in Eastern Russia.

MATERIALS AND METHODS

Virological sampling and diagnosis of IHNV. A total of 7697 cultured and 2933 wild salmonid fish from Kamchatka were examined during 1996 to 2005 (Tables 1 & 2). Fish species sampled were *Salvelinus malma* and Pacific salmon *Oncorhynchus* spp.: sockeye salmon *O. nerka*, chum salmon *O. keta*, pink salmon *O. gorbuscha,* chinook salmon *O. tschawytscha,* and coho salmon *O. kisutch*. The fish were either caught on their natural spawning grounds by netting or collected from holding ponds used to maintain spawning adults or fry at the salmon hatcheries.

Ovarian fluid and pools of kidney and spleen from adult fish (5 fish per pool), or whole fry or viscera of juvenile fish (5 fish per pool), were collected and transported to the laboratory on ice as described by LaPatra (1994). In 2005, ovarian fluid and kidney/spleen specimens from adult sockeye were collected from individual fish. Ovarian fluid was centrifuged without dilution. Tissue samples were homogenized in a mortar with quartz sand, and minimal essential medium (MEM-0) was added to equal a 1:10 dilution (w/v). Dilutions were centrifuged at 6000 × *g* for

Table 1. Occurrence of IHNV in Kamchatka hatchery salmon. **Bold italics** indicate IHNV positive populations, with viral prevalences as described in text. nt: not tested for virus, fnc: fish not cultured

Hatchery site	Fish stock	Number of fish examined each year									
		1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Bolshaya River watershed											
Malkinskiy	Sockeye fry	60	60	60	120	120	120	***170***	230	111	175
	Sockeye adult	nt	nt	nt	nt	30	***30***	***185***	***65***	***60***	***60***
	Chinook fry	60	15	60	120	120	120	60	60	30	30
	Chinook adult	nt	nt	nt	nt	27	20	30	30	30	30
Ozerki	Sockeye fry	60	60	60	60	120	nt	135	120	***235***	100
	Sockeye adult	nt	nt	nt	nt	15	***30***	30	***30***	***90***	90
	Chum fry	60	60	60	60	60	nt	60	90	90	60
	Chum adult	nt	nt	nt	nt	20	30	30	30	30	30
Paratunka River watershed											
Paratunskiy	Chum fry	60	60	60	120	120	120	60	60	90	30
	Chum adult	nt	nt	nt	nt	15	20	20	30	30	30
	Coho fry	fnc	fnc	fnc	fnc	fnc	120	60	fnc	fnc	fnc
Viljuskiy	Chum fry	60	nt	nt	60	60	90	60	90	90	30
	Chum adult	nt	nt	nt	nt	nt	nt	30	30	30	30
	Coho fry	fnc	fnc	fnc	fnc	fnc	30	60	90	90	nt
	Coho adult	nt	nt	nt	nt	nt	nt	30	30	19	nt
Avacha River watershed											
Ketkino	Chum fry	60	60	120	120	120	90	90	90	90	30
	Chum adult	nt	nt	nt	nt	nt	30	30	30	30	30
	Sockeye fry	fnc	fnc	fnc	60	fnc	fnc	fnc	fnc	fnc	fnc

Table 2. Occurrence of IHNV in Kamchatka wild salmon

Site	Host and life stage	Years tested (no. fish tested each year)	Years IHNV positive (% prevalence)
Bolshaya River watershed			
Mouth of Bolshaya River	Chum fry	2003 (60), 2004 (22), 2005 (30)	None
	Pink fry	2003 (60), 2005 (30)	None
	Pink adult	2005 (30)	None
	Coho fry	2003 (42), 2005 (30)	None
	Coho adult	2005 (30)	None
Kluchovka River	Sockeye fry	2001 (30), 2002 (30), 2003 (135)	None
	Sockeye adult	2005 (6)	None
	Salvelinus malma fry	1997 (30), 2001 (30), 2002 (30), 2003 (5), 2005 (30)	None
	Coho fry	1998 (30), 2001 (18), 2002 (30), 2003 (50), 2005 (30)	None
Ganalskiy Vahtang River	Sockeye adult	2000 (20), 2002 (19), 2005 (21)	2002 (75)
Domashniy stream	Sockeye adult	2002 (13), 2003 (30)	2002 (67), 2003 (100)
	Sockeye fry	2003 (30), 2004 (30), 2005 (20)	None
Lake Nachikinskoe	Sockeye adult	2003 (40), 2004 (30), 2005 (60)	2003 (100), 2004 (67), 2005 (100)
	Sockeye fry	2003 (30), 2004 (80), 2005 (60)	2003 (13)
Plotnikova River	Sockeye adult	1998 (15), 2001 (7), 2002 (10)	
	Sockeye fry	2003 (60)	None
	Coho fry	1998 (20)	None
	Chum fry	1998 (20)	None
Paratunka River watershed			
Paratunka River	Sockeye fry	2002 (60), 2003 (30), 2004 (30)	None
	Sockeye adult	2004 (7)	None
	Chum fry	2001 (80), 2003 (60), 2004 (30)	None
	Chum adult	1999 (20), 2004 (30)	None
	Coho fry	1997 (30), 2001 (19), 2003 (90), 2004 (30)	None
	Coho adult	1999 (20), 2000 (20), 2003 (30), 2004 (30)	None
Lake Bolshoy Viljuy	Coho adult	1999 (20), 2001 (15), 2002 (7), 2003 (8), 2004 (23)	None
Avacha River watershed			
River Avacha	Chum fry	2001 (30), 2002 (60), 2003 (60), 2004 (30)	None
	Chum adult	1998 (20), 1999 (30), 2000 (15), 2001 (15), 2002 (30), 2004 (30)	None
	Coho fry	2000 (12), 2001 (59), 2002 (30), 2004 (30)	
	Coho adult	1998 (20), 1999 (20), 2000 (15), 2002 (30), 2003 (8), 2004 (30)	None
	Sockeye fry	2002 (60)	None
	Sockeye adult	2000 (10), 2002 (17), 2004 (30)	None
	Pink adult	2001 (25), 2004 (30)	None
	S. malma fry	1997 (30)	None
	S. malma adult	1997 (10), 1999 (10), 2000 (5), 2004 (10)	None

20 min. Serial $\log_{10}$ dilutions of supernatant for each pool were prepared with minimal essential medium supplemented with 10% fetal calf serum (MEM-10) and used to inoculate chinook salmon embryo (CHSE-214) and epithelioma papulosum cyprini (EPC) cell lines (Fijan et al. 1983) as described by Fried (1984). For each sample, 3 serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) were each added to 4 wells of a 96-well plate containing confluent cell monolayers. Cell cultures were incubated at 15°C, and tissue culture infective doses ($TCID_{50}$) were determined using the method of Reed & Muench as described by Musselius (1983).

The appearance of rounded and granular cells in grape-like clusters within the inoculated cell lines was indicative of cytopathic effect (CPE) induced by IHNV (Meyers 2000). Virus was identified as IHNV by serum neutralization assays as described by LaPatra (1994), using a rabbit antiserum to IHNV provided by J. Kaufman (Oregon Department of Fish and Wildlife, Corvallis, OR, USA). When IHNV-infected cell mono-

layers were almost destroyed, supernatant containing the virus was harvested and frozen at –70°C or lyophilized for long-term storage. This was done after the second or third passage of virus on EPC or CHSE-214 cell lines.

Nomenclature for individual virus isolates was designed to indicate the site of isolation (Ryb, Rybnoe; MH, Malkinskiy Hatchery; OH, Ozerki Hatchery; LN, Lake Nachikinskoe), the last 2 digits of the year of isolation (20XX), host species (T, rainbow trout; S, sockeye), and host life stage (A, adult; F, fry). Thus, for example, the virus isolated from Malkinskiy Hatchery sockeye adults in 2001 was designated MH01SA. When more than one isolate was generated from sampling the same host population on the same date, individual isolate names included a numerical suffix (e.g. LN03SA-1, LN03SA-2). The isolate from rainbow trout in an experimental hatchery near Moscow (Ryb00TF) was kindly provided by Dr. I. Shchelkunov (All-Russian Research Institute of Freshwater Fishes).

IHNV genetic typing. Genetic typing was conducted on selected Russian IHNV isolates from cultured or wild adult and young sockeye salmon from the watershed of the Bolshaya River (Table 3). Viral supernatant prepared as described above was used as a template for reverse-transcriptase polymerase chain amplification (RT-PCR) and nucleotide sequencing of the midG and 5′N regions of the viral genome (Garver et al. 2003, Troyer & Kurath 2003). Raw sequence data were edited using Sequencher 4.1 software (Gene Codes), and sequence files were aligned and analyzed with MacVector 6.5.3 and AssemblyLIGN 1.0/9 software (International Biotechnologies). The 303 nt midG sequences are homologous to nt 686–988 of the IHNV G gene sequence in GenBank accession no. U50401, and the 412 nt 5′N sequences are homologous to nt 1–412 of the IHNV N gene sequence in GenBank accession no. U50402.

Phylogenetic analyses were done with PAUP* version 4.0b (Swofford 1998) using 1000 bootstrapped replicates of the infile data. Russian IHNV midG and 5′N sequences were analyzed in the context of North American IHNV sequences in the GB37 data set (Kurath et al. 2005). Phylogenetic trees (see Fig. 2) are neighbor-joining phylograms with branch lengths accurately indicating genetic distance, and all nodes with bootstrap values less than 70 have been collapsed to polytomies. Trees were drawn using the SRCV isolate of IHNV as the outgroup.

RESULTS

Isolation of IHNV in hatchery salmonids from Kamchatka

Virological examination of fry and fingerlings cultured at the 5 hatcheries on Kamchatka Peninsula (Fig. 1) has been done annually since 1996, and the spawning adult fish used for reproduction at the hatch-

Table 3. Russian IHNV isolates characterized by genetic typing. Isolate names are as described in the methods. MidG and 5′N sequence types are shown as universal sequence designations from the IHNV database of the Western Fisheries Research Center (E. J. Emmenegger & G. Kurath unpubl.)

Isolate name	Site of origin	Isolation month/year	Host, life stage	Notes	MidG type	5′N type
Ryb00TF	Rybnoe Hatchery	4/2000	Trout fry	Epidemic in experimental trout hatchery	mG142U	5N048U
MH01SA	Malkinskiy Hatchery	9/2001	Sockeye adult	First IHNV isolation in Kamchatka	mG143U	5N001U[a]
MH02SF	Malkinskiy Hatchery	2/2002	Sockeye fry	Epidemic in progeny of 9/2001 adults	mG002U[a]	5N049U
MH02SA	Malkinskiy Hatchery	9/2002	Sockeye adult	Same host stock as MH01SA	mG143U	5N001U[a]
OH01SA	Ozerki Hatchery	9/2001	Sockeye adult	Second IHNV isolation in Kamchatka	mG003U[a]	5N001U[a]
LN03SA-1	Lake Nachikinskoe	7/2003	Sockeye adult	3 different 5-fish pooled samples collected on the same date from wild fish at their natural spawning ground	mG144U	5N050U
LN03SA-2	Lake Nachikinskoe	7/2003	Sockeye adult		mG003U[a]	5N001U[a]
LN03SA-3	Lake Nachikinskoe	7/2003	Sockeye adult		mG144U	5N050U

[a]Sequences from Russian IHNV identical to sequences previously observed in the database (see 'Results: Genetic typing…')

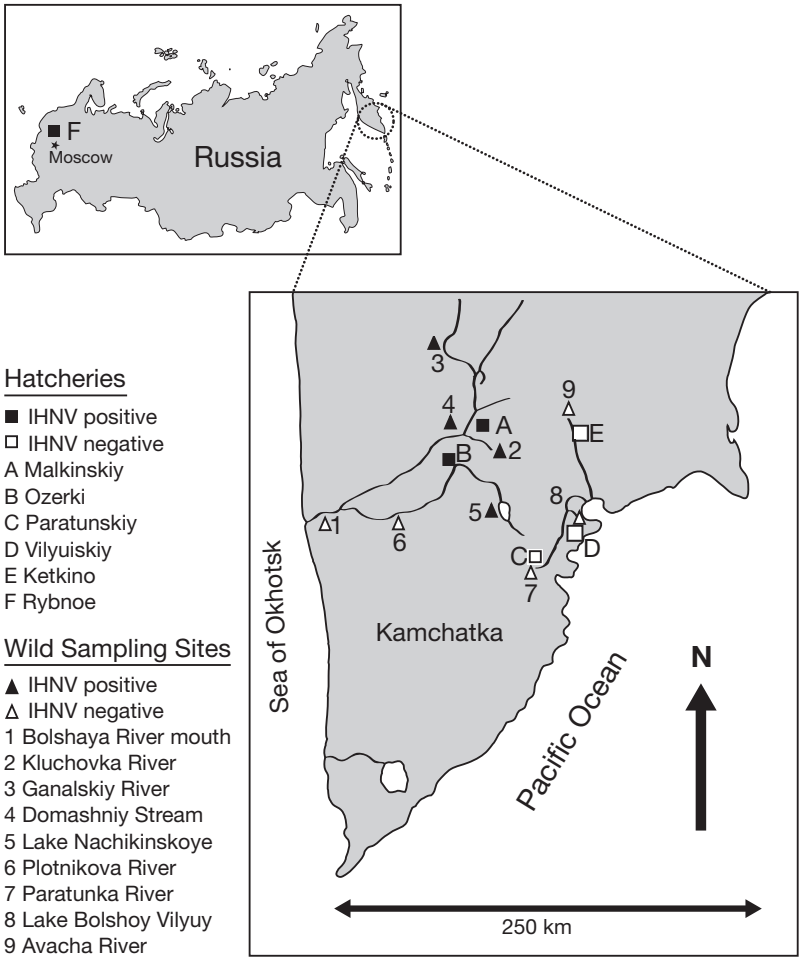

Fig. 1. Southern end of the Kamchatka Peninsula indicating the collection sites for Russian salmon examined for presence of IHNV

eries have been examined annually since 2000 (Table 1). In cultured populations of young or adult chum, coho, and chinook salmon, no IHN virus was detected during 1996 to 2005, although cytoplasmic inclusions tentatively indicating viral erythrocytic necrosis (VEN) or erythrocytic inclusion body syndrome (EIBS) were occasionally observed (S. Rudakova unpubl. data). The first confirmed IHNV detection in Kamchatka occurred in sockeye salmon hatchery adults in 2001 (Rudakova 2004, Rudakova & Bochkova 2005).

In September 2001, 30 adult sockeye salmon returning to each of the 2 streams near Malkinskiy and Ozerki hatcheries were examined, and IHNV was detected in 50 and 75% of the fish, respectively. These fish were used as brood stock at the Malkinskiy and Ozerki salmon hatcheries, and their eggs were disinfected after fertilization in 0.1% active iodine for 15 min. Upon positive identification of IHNV in the adult broodstock, the disinfection was repeated at the eyed-egg stage. An epidemic of IHN occurred from February through April 2002, only at the Malkinskiy hatchery, among 5 to 6 cm progeny (age 60 to 90 d) (Rudakova 2004). Cumulative percent mortality was 79% at the point when the remaining fish were destroyed in order to avoid further contagion. External signs were typical for this disease (Wolf 1988).

Due to this first detection of IHNV, more extensive testing of the sockeye broodstock at the Malkinskiy and Ozerki salmon hatcheries was done in 2002 to 2005, and virus was detected at both facilities. At the Malkinskiy Hatchery, prevalence and individual fish virus titers increased with time during the spawning season from 16.6% to 66.7% in 2002 and from 66.7% to 83.3% in 2003 (Rudakova & Bochkova 2005). At the Ozerki Hatchery, IHNV was not detected among adult sockeye in 2002 but prevalence of IHNV-positive fish was 50% in 2003. A second epidemic of IHNV occurred from March to June 2004 among 3 to 5 cm (age 20 to 90 d) sockeye progeny reared at the Ozerki Hatchery. Mortality was 48%, and all fish from infected lots were destroyed to avoid further contagion. In 2004, sockeye used for reproduction at the 2 hatcheries were examined during the spawning season, and the prevalence of IHNV was 16.6% at the Ozerki Hatchery and 16.6% at the Malkinskiy Hatchery. In 2005, sockeye broodstocks were IHNV positive only at the Malkinskiy Hatchery, with a prevalence of 6.9% by testing of individual fish.

In all cases of positive virus diagnosis, typical IHNV-induced CPE (Meyers 2000) was observed in cell lines between 3 and 6 d post-inoculation (PI), and destruction of the cell monolayer was nearly complete at all dilutions by 10 d PI. The presence of IHNV was confirmed from each of these cell cultures by using the serum neutralization assay (LaPatra 1994). The titer of IHNV varied from 10^6 to 10^9 $TCID_{50}$ ml^{-1}.

Isolation of IHNV in wild salmonids from Kamchatka

The examination of wild populations of salmon in watersheds of Kamchatka began in 1997, but inspections were not systematic until after 2000, when sampling became more regular (Table 2). IHNV can be isolated most easily during epidemics among young

fish or at the end of the fish life cycle during spawning (Mulcahy et al. 1982). For this reason, adult fish were caught on their natural spawning grounds for sampling. Due to detection of IHNV in adult hatchery sockeye in 2001, a more extensive survey of wild fish, including spawning sockeye, was conducted in 2002 to 2005 at 6 sites in the Bolshaya River watershed: mouth of the Bolshaya River, Kluchovka River, Ganalskiy Vahtang River, Domashniy stream, Lake Nachikinskoe, and Plotnikova River. Wild fish were also sampled from 2 other independent watersheds: Avacha River and Paratunka River (including Bolshoy Viljuy Lake). These watersheds contain the Ketkino, Paratunskiy, and Viljuskiy hatcheries (Fig. 1; Table 3). In wild populations of adult and juvenile chum, coho, pink salmon, and *Salvelinus malma*, no IHNV was detected during this period of observation (Table 2). IHNV was found in adult sockeye from 3 of the 6 sampling sites within the Bolshaya River watershed, including the Ganalskiy Vahtang River, Domashniy stream, and Lake Nachikinskoe. Sockeye fry were also IHNV positive in Lake Nachikinskoe. In this lake, a natural epidemic was ongoing among sockeye fry, and the histopathology of collected fish was consistent with IHNV infection. This is the first confirmation of an epidemic of IHN in wild salmon in Russia, as this sockeye population has never been supplemented with hatchery fish.

As above, in all cases, typical IHN-induced CPE (Meyers 2000) was observed in cell lines between 3 and 6 d PI, destruction of the cell monolayer was nearly complete at all dilutions by 10 d PI, and IHNV was confirmed by serum neutralization assay (LaPatra 1994). The titer of the virus was quite high (10^6 to 10^9 TCD_{50} ml^{-1}).

Genetic typing of IHNV from Kamchatka

Genetic typing of 8 Russian IHNV isolates (Table 3) was done by determining their midG and 5′N nucleotide sequences. Among these isolates were 5 from hatchery fish, including the first confirmed Russian IHNV from Rybnoe in eastern Russia in 2000, and isolates from the Malkinskiy and Ozerki hatcheries in 2001 to 2002. The other 3 isolates were from 3 different 5-fish pools sampled on the same date in 2003 from a wild spawning sockeye salmon population in Lake Nachikinskoe. With the exception of the Rybnoe isolate, all hatchery and wild IHNV isolates that were typed originated in the Bolshaya River watershed in Kamchatka (Fig. 1).

Analyses of the midG sequences from these 8 IHNV isolates revealed 3 pairs of isolates with identical sequences; thus there were 5 unique midG sequences from the Russian isolates (Table 3). Alignment of the 5 unique midG sequence types from Russian IHNV showed 5 sites of sequence variability distributed apparently randomly along the sequence. The sequences differed from each other by 1 to 3 nt out of 303 nt, so the maximum pair-wise divergence was 1.0% (Fig. 2). Among the Russian IHNV, the Ryb00TF isolate from eastern Russia had the most divergence, with 3 nt difference from most other sequences. Excluding this isolate, the Kamchatka IHNV midGs differed by only 0 to 2 nt (0.7% maximum divergence). The 3 isolates representing different 5-fish pooled samples from the wild sockeye in Lake Nachikinskoe had 1 of 2 different consensus sequences, and within 2 of these isolates there was clear sequence heterogeneity in which nucleotide position 21 had 2 peaks indicating both C and A. Two of the Russian IHNV midG sequence types were identical to common midG sequence types described previously from North American IHNV. The identical midG sequence from Russian isolates OH01SA and LN03SA-2 matched the midG sequence type designated mG003U, previously found in 33 IHNV isolates from Alaska, British Columbia, and Washington (Emmenegger & Kurath 2002, E. J. Emmenegger & G. Kurath unpubl. data). Similarly, the midG of the Russian MH02SF was identical to sequence type mG002U, which has been found in 57 IHNV isolates, again from Alaska, British Columbia, and Washington (Emmenegger et al. 2000, E. J. Emmenegger & G. Kurath unpubl. data).

Phylogenetic analyses of the Russian midG sequences combined with midG sequences from 37 IHNV isolates selected to represent all currently known IHNV genetic types (the GB37 database, Kurath et al. 2005) showed all Russian IHNV to be within the major U

	Ryb00TF	MH01SA	MH02SF	MH02SA	OH01SA	LN03SA-1	LN03SA-2	LN03SA-3
Ryb00TF	X	3, 8	3, 10	3, 8	2, 8	3, 10	2, 8	3, 10
MH01SA		X	2 ,2	0, 0	1, 0	2, 2	1, 0	2, 2
MH02SF			X	2, 2	1, 2	2, 4	1, 2	2, 4
MH02SA				X	1, 0	2, 2	1, 0	2, 2
OH01SA					X	1, 0	0, 0	1, 2
LN03SA-1						X	1, 2	0, 0
LN03SA-2							X	1, 2
LN03SA-3								X

Fig. 2. Number of nucleotide (nt) differences identified among sequences from 8 IHNV isolates obtained from salmonid fish in Russia. Isolate names are as in Table 3, and entries indicate the pair-wise number of nt differences in the midG sequences, followed by the number of nt differences in the 5′N sequences

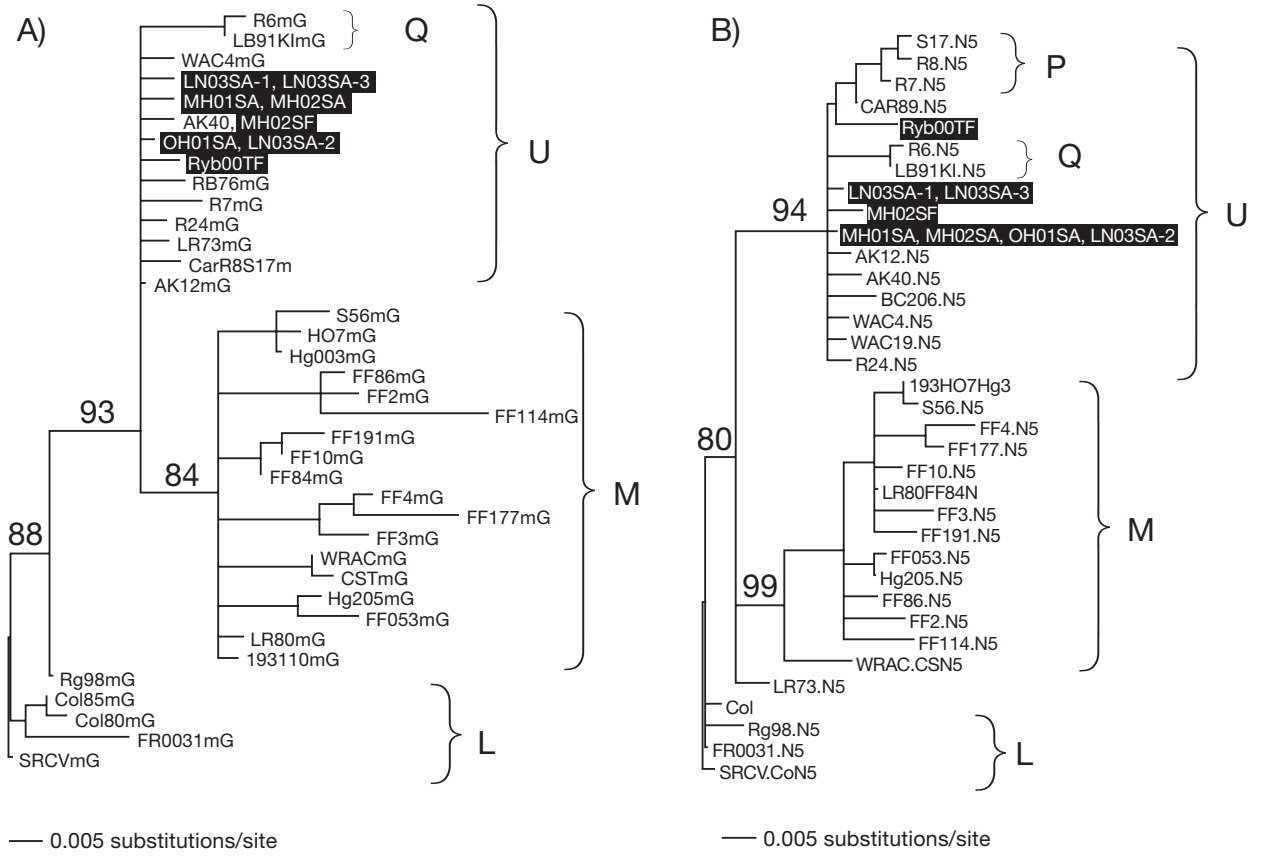

Fig. 3. Phylogenetic analysis of (A) midG and (B) 5′N sequences from 8 IHNV isolates obtained from salmonid fish in Russia. Trees shown are neighbor-joining distance phylograms with horizontal branch lengths accurately corresponding to genetic distance. Bootstrap values from 1000 bootstrap data sets are shown at major nodes, and all branches with values less than 70 have been collapsed to polytomies. Nomenclature for North American sequence types from the GB37 data set are as in Kurath et al. (2005), and Russian isolate names, as in Table 3, are highlighted in black boxes. The known major genogroups (U, M, L) and sub-groups (P, Q) of IHNV are indicated by brackets

genogroup of IHNV (Fig. 3A). The Russian sequences were indistinguishable from North American U genogroup sequences in that they fell on short individual branches from a common ancestral node, with no branching sub-structure. This placement was consistent in phylogenetic trees made with either neighbor-joining distance or parsimony algorithms, and in trees with larger databases including all currently known IHNV midG sequences (data not shown).

In phylogenetic analyses of a different gene region, 5′N sequences of IHNV have previously shown more structure within the U genogroup (Garver et al. 2003, Kurath et al. 2005). The 8 Russian IHNV isolates grouped into a total of 4 different 5′N sequences that correlated well with the groupings by midG identities (Table 3). The only difference was that 4 Russian isolates had identical 5′N sequences, and these isolates were resolved into 2 pairs of identical isolates by midG analyses. Alignment of the Russian 5′N sequences revealed 13 sites of variability, again distributed along the length of the sequence. The pair-wise differences between 5′N sequences ranged from 0 to 10 nt out of 412 nt, for a maximum divergence of 2.4% (Fig. 2). Again the Ryb00TF isolate was most divergent, and when it was excluded, the Kamchatka IHNV 5′N sequences differed by only 0 to 4 nt (maximum 1.0% divergence). The 5′N sequence that was identical in 4 of the Russian IHNV isolates matched the 5′N sequence type designated 5N001U, which was found in the North American isolate RB76 (Nichol et al. 1995). The other 3 Russian 5′N sequence types did not match previously identified sequence types. Sequence heterogeneity among and within the 3 isolates from Lake Nachikinskoe wild spawning sockeye was again evident, with 1 of 2 nucleotides at each of 3 sites: nt 222, 319, and 378.

Phylogenetic analyses of the Russian 5′N sequences combined with the 5′N sequences of the GB37 database again showed that all Russian IHNV fell clearly within the U genogroup (Fig. 3B). The Ryb00TF isolate from Eastern Russia appeared to be basal to the sub-

group U-P within the U genogroup (Kurath et al. 2005), and the Kamchatka isolate types formed short individual branches to the ancestral node, similar to all other North American sequence types. Again, this placement was consistent in trees generated with other algorithms or databases (data not shown).

DISCUSSION

Observations of fish in Russia for viral pathogens have not been extensive due to the comparatively low level of aquaculture development and long economic crisis in the country. We began to carry out purposeful investigation of both cultured and wild populations of salmon to define prevalence of IHNV in Kamchatka in 2000. Prior to that time, sampling of salmon hatchery fry since 1996 had not detected IHNV. Between 2000 and 2005, over half of the observed populations of sockeye salmon were IHNV positive (Tables 1 & 2). Although this observation suggests a possible recent increase in IHNV prevalence, we cannot conclude that IHNV was not present prior to 2001. A lack of information does not mean that fish in watersheds are free from viral pathogens. It is very important to know about the presence of virulent pathogens in order to prevent their spread via human activity.

Aquaculture in Kamchatka began in 1926, when the first Kamchatka hatchery opened on Lake Ushkovskoe in the watershed of the Kamchatka River. This hatchery used a spring water supply to rear fry of sockeye, coho, and chum. However, the facility was small and economically unsuccessful, and it was closed at the beginning of the 1980s. Examination of documents about the work of the Ushkovskiy Hatchery during the 1920s to 1980s revealed different mortality levels (3.2 to 92.1%) for fry of all salmon species during all periods of rearing. Managers and scientists have written only about poor fish culture technology or inadequate water supply, and nothing about pathological changes among fish. Hatcheries currently rearing salmon on Kamchatka peninsula are relatively new. The oldest is Malkinskiy Hatchery, which began work in 1982. The other 4 hatcheries were opened in the 1990s. These hatcheries did not use eggs of sockeye or other species of salmon from the USA or other countries.

Historical literature suggests that IHNV in North America was originally restricted to sockeye salmon hosts (Rucker et al. 1953, Guenther et al. 1959, Wingfield et al. 1970), and that the virus was endemic in Alaskan sockeye before it became widespread in more southern states of Washington, Oregon, and California (Amend & Wood 1972, Grischkowsky & Amend 1976, Mulcahy et al. 1980). In light of these observations, it is interesting that IHNV in Kamchatka was only found in sockeye salmon and that it is a U genogroup virus very similar to IHNV from northwestern North America. In North America, IHNV may have been inadvertently spread throughout the lower states by the historically common practices of salmon transplantations (Wolf 1988, Roppel 1982, Burgner 1991) and/or use of raw, unpasteurized salmon viscera in feed for salmon fry in hatcheries during the 1950s and 1960s (Watson et al. 1954, Guenther et al. 1959, Wolf 1988). The suggestion that IHNV was introduced to Japan via a shipment of contaminated sockeye salmon eggs from Alaska (Sano et al. 1977) is consistent with the typing of a Japanese IHNV isolate within the U genogroup (Kurath et al. 2003), since all IHNV analyzed to date from Alaska are in the U genogroup (Emmenegger et al. 2000). Similarly, the finding that western European IHNV isolates are all within the M genogroup (Enzmann et al. 2005) agrees with the suggestion that IHNV was introduced to Europe in infected trout eggs from the United States (Bovo et al. 1987, 1991, Laurencin 1987), since rainbow trout in North America have almost exclusively M genogroup IHNV (Troyer et al. 2000, Troyer & Kurath, 2003).

We do not know what role Kamchatka sockeye play in the global picture of IHNV. Is this virus common in Russian populations of sockeye? Has it been in those host populations for many years or was it recently introduced? It is interesting to consider the possible source of the virus detected since 2001 in Kamchatka sockeye. Russian hatcheries in Kamchatka have never imported eggs or fry from North America or other countries, so we assume the presence of IHNV is due to more natural causes. Many authors (Barnaby 1944, Hartman & Raleigh 1964, Burgner 1991, Quinn 1993) have described 'homing' for sockeye, and it is highly unlikely that infected American fish stray to Russian rivers to spawn. However, phylogenetic analysis of Russian IHNV isolates presented here showed that they clearly fall within the U genogroup and are very similar, or even identical by our typing methods, to common American isolates from the northern U genogroup range. A basic principle of evolutionary biology is that separation and isolation of distinct populations of a species leads over time to their divergence, either by genetic drift or differing selection in their respective environments. The lack of evolutionary divergence between IHNV in sockeye from Russia and North America suggests that the virus in these 2 host populations comes from a common source relatively recently and/or continuously. This in turn suggests that the source of IHNV is in the Pacific Ocean. The nature of a putative ocean source of IHNV is not known, but possible candidates include salmonid or non-salmonid fishes, parasites, or prey organisms. Groot & Margolis (1991) have shown that marine migration ranges of Kamchatka and American populations of sockeye

overlap in the northern Pacific Ocean, so direct transmission of virus between Russian and American sockeye in the ocean could occur. Although this is contrary to the concept that IHNV transmission occurs mostly in fresh water (Bootland & Leong 1999), recent IHN epidemics among Atlantic salmon *Salmo salar* reared in marine net-pen farms (Armstrong et al. 1993) confirm that IHNV infections can be transmitted in sea water. In the marine environment, IHNV has been isolated from an adult sockeye salmon (Traxler et al. 1997) and from 3 non-salmonid finfish species (Kent et al. 1998). Additional non-salmonid fishes have been shown to be susceptible to experimental IHNV infection (Castric & Jeffroy 1991, LaPatra et al. 1995). With regard to possible non-fish sources, Foerster (1968) and French et al. (1976) summarized the stomach analysis data for sockeye reported by various investigators for many different areas of the ocean. The ability of IHNV to reproduce in these prey organisms is unknown. Ectoparasites of sockeye salmon have also been suggested as possible vectors for transfer of IHNV between fish (Mulcahy et al. 1990). At present it is impossible identify the source of the virus in the Pacific Ocean, but results of phylogenetic analysis of Russian and American IHNV isolates suggest either direct transmission between sockeye populations in the ocean, or existence of a non-sockeye seawater reservoir.

In addition to viral reservoirs, the data presented here suggest IHNV host specificity. Despite sampling many salmonid species of both wild and hatchery fish in Kamchatka, IHNV was only isolated from sockeye. In North America, the IHNV that caused the first explosive epidemics in hatchery sockeye during the 1950s did not cause disease in other fish species reared at the same facilities, and it did not cause disease in chinook or rainbow trout in severe experimental challenges (Rucker et al. 1953, Watson et al. 1954). Our current hypothesis is that in North America this first sockeye-specific virus was an ancestral U genogroup virus, and a host jump into rainbow trout may have resulted in divergence of the M genogroup (Kurath et al. 2003). In the Kamchatka hatcheries, when chum or chinook salmon, at either juvenile or adult life stages, were reared in the same facility as infected sockeye for multiple years they had no detectable virus (Table 1). The U genogroup IHNV in Russia may be sockeye-specific, although this hypothesis requires experimental testing. If true, it would be wise to conduct Russian aquaculture in such a manner as to prevent host jumps to other species. Among the wild salmonids sampled in Kamchatka, sockeye was the only species known to be a common host for IHNV. The other species (pink, chum, coho, and malma) are more refractory to IHNV (Wolf 1988, Bootland & Leong 1999), and our study confirmed this for Kamchatka.

In the genetic typing data presented here, the low level of divergence observed among the Kamchatka IHNV isolates (0 to 2 nt in the midG and 0 to 4 nt in the 5′N) was typical of the diversity seen in U genogroup isolates from North America (Kurath et al. 2003). The underlying concepts suggested previously for homogeneity of North American U genogroup IHNV isolates included high fitness of U virus in sockeye due to a long established host-pathogen association, and/or continuous direct or indirect exchange of virus among a geographically broad range of host stocks as discussed above. These concepts can now be extended to Russian IHNV. In practical terms, although the genetic homogeneity and the analysis of short genomic regions limits our ability to make conclusions regarding epidemiology within the U genogroup, several interesting observations can be made from the genetic typing data. Among the IHNV isolates characterized here, those with identical sequences came from the same sampling site or from sites within the same sub-basin (Table 3). At Malkinskiy Hatchery, IHNV from the adult sockeye in 2001 and 2002 were identical by our typing methods. However, the IHNV from the 2002 fry epidemic differed from the adult virus isolates by 2 nt in the midG and 2 nt in the 5′N. Although we must be conservative in our interpretations due to the small number of nucleotide differences involved, this observation suggests that the source of the IHNV that caused the fry epidemic was not the progenitor adults, so alternative sources should also be considered. Another interesting result from the typing data was the sequence difference found within and among virus isolates from wild sockeye in Lake Nachikinskoe. This difference demonstrates detectable genetic heterogeneity within the virus population circulating in one wild host population at the same time, but not necessarily within an individual fish, because the samples were 5-fish pools. As a third point, it is worth considering the possible origin of the Rybnoe isolate from a trout hatchery in eastern Russia. This isolate appeared uniquely divergent from the IHNV in Kamchatka, and it is not likely to be from western Europe because all European IHNV characterized to date are in the M genogroup (Enzmann et al. 2005). Although the original source is unknown, the Rybnoe isolate may represent IHNV endemic in a region of Russia other than Kamchatka. Finally, from a phylogenetic standpoint, the data presented here show no evidence of a monophyletic origin of IHNV in Kamchatka, or in Russia in general. This differs from IHNV from western Europe, which appeared to comprise a monophyletic group within the M genogroup (Enzmann et al. 2005). It may be that the phylogenetic analyses presented here cannot resolve a common ancestor for Russian IHNV due to the short genome regions analyzed and the low

genetic diversity in the U genogroup. Alternatively, the absence of a common ancestor is consistent with the hypothesis that the presence of IHNV in Kamchatka is not due to an introduction from a North American source, but rather that it represents an endemic virus and that Kamchatka is part of a larger contiguous geographic range of the U genogroup.

In the data presented here, IHNV was found in both hatchery and wild sockeye, but only in 1 of the 3 large watersheds where sampling was conducted. In the future, it will be important to survey IHNV from a wide range of Russian salmon populations to develop a more thorough understanding of the prevalence patterns, host specificity, and genetic types of the virus in Russia. This survey will determine whether the virus is limited to sockeye and to the Bolshaya watershed, and provide essential information for mitigating the disease impacts that have occurred in Kamchatka sockeye since 2001.

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