

Phylogeography of infectious haematopoietic necrosis virus in North America

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Infectious hematopoietic necrosis virus (IHNV) is a rhabdoviral pathogen that infects wild and cultured salmonid fish throughout the Pacific Northwest of North America. IHNV causes severe epidemics in young fish and can cause disease or occur asymptotically in adults. In a broad survey of 323 IHNV field isolates, sequence analysis of a 303 nucleotide variable region within the glycoprotein gene revealed a maximum nucleotide diversity of 8.6%, indicating low genetic diversity overall for this virus. Phylogenetic analysis revealed three major virus genogroups, designated U, M and L, which varied in topography and geographical range. Intragenogroup genetic diversity measures indicated that the M genogroup had three- to fourfold more diversity than the other genogroups and suggested relatively rapid evolution of the M genogroup and stasis within the U genogroup. We speculate that factors influencing IHNV evolution may have included ocean migration ranges of their salmonid host populations and anthropogenic effects associated with fish culture.

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INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus that causes acute, systemic disease in salmonid fish and also occurs in asymptomatic fish hosts. The virus is currently endemic throughout the Pacific Northwest of North America, with a contiguous range extending from Alaska to California and inland to Idaho. Within this geographical area the host range of IHNV includes five species of Pacific salmon, Atlantic salmon and several trout species (Wolf, 1988; Bootland & Leong, 1999). The first reported epidemics of IHNV occurred in sockeye salmon (*Oncorhynchus nerka*) fry at Washington and Oregon fish hatcheries during the 1950s (Rucker *et al.*, 1953; Guenther *et al.*, 1959; Wingfield *et al.*, 1969). Surveys indicated that IHNV was endemic in sockeye throughout Alaska by 1974 (Grischkowsky & Amend, 1976), but the virus was not widespread in Washington and Oregon through the 1970s (Amend & Wood, 1972; Mulcahy *et al.*, 1980; Pilcher & Fryer, 1980). Subsequently, two virus emergence events occurred in which IHNV became endemic in rainbow trout (*O. mykiss*) throughout the Hagerman Valley trout farming industry in southern Idaho between 1977 and 1980 (Busch, 1983) and in salmonids of the middle and lower Columbia River basin in the early 1980s (Groberg, 1983; Groberg & Fryer, 1983). In addition to cultured fish, IHNV is endemic in many wild salmonid stocks in the Pacific Northwest (Bootland & Leong, 1999).

Due to the extensive economic losses caused by IHNV in fish culture facilities, the virus has been well characterized in biological, immunological and molecular biological studies (for reviews, see Wolf, 1988; Bootland & Leong, 1999). IHNV is the type species of the genus *Novirhabdovirus*, within the family *Rhabdoviridae*. Similar to other rhabdoviruses, IHNV has a linear single-stranded, negative-sense RNA genome of approximately 11 000 nucleotides. The IHNV genome contains six genes in the order 3'-N-P-M-G-NV-L-5', representing the nucleocapsid, phosphoprotein, matrix protein, glycoprotein, non-virion protein and polymerase protein genes, respectively (Kurath *et al.*, 1985; Morzunov *et al.*, 1995). Phenotypic and genetic diversity among IHNV isolates from different geographical sources have been investigated since the 1980s using various methods, including protein electrophoretotyping (Hsu *et al.*, 1986), monoclonal antibody reactivity (Ristow & Arnzen, 1989; Ristow & Arnzen de Avila, 1991; Winton *et al.*, 1988; LaPatra *et al.*, 1994) and RNase T1 fingerprinting (Oshima *et al.*, 1995). Phylogenetic analyses of the complete glycoprotein and non-virion protein gene sequences of twelve IHNV isolates confirmed the earlier studies in concluding that IHNV genetic types correlate with geography (Nichol *et al.*, 1995).

In an effort to broaden and refine our understanding of the genetic diversity and phylogeny of IHNV throughout its entire geographical range, we have applied both RNase

protection and nucleotide sequence analysis methods in localized and regional studies of IHNV field isolates. Surprisingly low genetic diversity was found in large regional studies of IHNV throughout Alaska (Emmenegger *et al.*, 2000), British Columbia (E. D. Anderson, G. S. Traxler & G. Kurath, unpublished data) and coastal Washington (Emmenegger & Kurath, 2002). These three studies each involved characterization of over 40 IHNV isolates from various sites throughout large geographical areas and over approximately 20 years, and each found an intrapopulation nucleotide diversity (π , average number of nucleotide differences per nucleotide site between sequences) of less than 0.007 for the partial glycoprotein gene sequence analysed. In contrast, a localized study of IHNV isolates from four trout farms within 12 miles on the Snake River in Idaho revealed co-circulating IHNV lineages and unusually high IHNV genetic diversity in the trout aquaculture setting, with a π value of 0.036 (Troyer *et al.*, 2000). The goal of the current work is to present a complete, high-resolution picture of the IHNV genetic diversity and phylogeny throughout its geographical range. To do this, we have used sequence analysis of the 303 nucleotide mid-G gene region to characterize IHNV isolates from throughout the remaining regions of the range not previously examined. The 'mid-G' is a variable region within the IHNV G gene (from data in Nichol *et al.*, 1995) that contains putative antigenic determinants (Huang *et al.*, 1996) and has been

found to be informative for large-scale IHNV phylogenetic analyses (Troyer *et al.*, 2000; Emmenegger *et al.*, 2000; Emmenegger & Kurath, 2002).

The first new regional study presented here included 49 IHNV isolates from the Columbia River basin, which is the largest and most productive salmon watershed in the contiguous 48 United States, extending through much of the states of Washington, Oregon and Idaho. The second new region included 38 IHNV isolates from watersheds in California and the southern coast of Oregon. We have also included single IHNV isolates from Japan, France and Italy, where it is presumed that IHNV was introduced from North American sources by shipments of infected eggs or fry (Wolf, 1988; Bootland & Leong, 1999). Data from these new isolates have been combined with all sequence data from the previous studies to produce a comprehensive phylogenetic analysis. The general features of the virus collections from different regions that comprise this analysis vary due to inherent differences in salmonid host species ranges and fish culture practices in the different geographical areas. This is summarized in Table 1.

The 323 IHNV isolates in this analysis represented 106 different collection sites throughout the geographical range of IHNV and were isolated over a period of 36 years from 1966–2001 (Figs 1 and 2). The great majority of the viruses (96%) were isolated from one of eight different salmonid

Table 1. Features of regional IHNV isolate collections that constitute this study

Region	No. of virus isolates	No. of sites	Year range	No. of different host species	Host species: comment	Host stock type: comment*
Alaska	42	23	1977–1996	5	71% Sockeye salmon	45% Hatchery 43% Wild
British Columbia	42†	20	1974–1997	6	57% Sockeye salmon 26% Atlantic salmon	31% Netpen 31% Spawning channel 38% Wild
Washington coast‡	61	19	1976–2000	6	57% Sockeye salmon	56% Hatchery 18% Wild
Idaho study site§	88†	4	1978–1998	1	100% Rainbow trout	100% Fish farm
Columbia River basin	49	29	1971–1999	5	45% Chinook salmon 37% Steelhead trout	84% Hatchery
Oregon coast and California	38	9	1966–2001	3	82% Chinook salmon 16% Steelhead trout	87% Hatchery
International	3	3	1982–1987	1	100% Rainbow trout	66% Fish farm 33% Hatchery

*Stock type describes the proportion of isolates from each region that were obtained from wild host fish or fish in various forms of aquaculture. The remaining proportion of each isolate set not specified in the table are either from unknown stock type or types representing only a small fraction of the set.

†These two studies used RNase protection assay of the full-length G gene to analyse the total number of virus isolates shown. Representative isolates from each haplotype were then analysed by nucleotide sequencing.

‡The Washington coastal region does not include the Columbia River basin.

§The Idaho study site is a localized site of four trout farms (Troyer *et al.*, 2000) rather than a large geographical area.

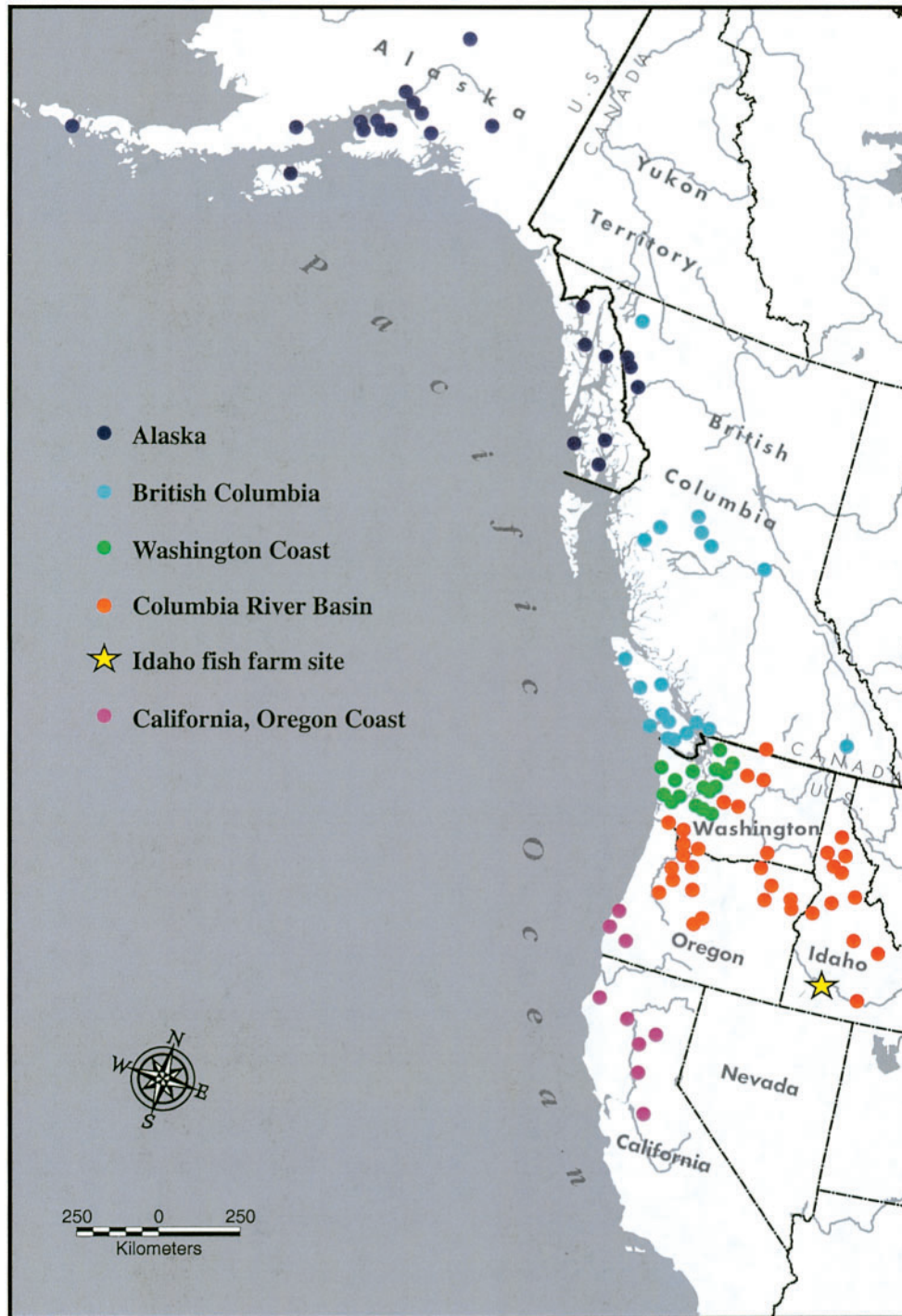


Fig. 1. Geographical distribution of IHN virus isolation sites. Coloured dots indicate virus isolation sites associated with the five regional studies that comprise this analysis. The star denotes the localized Idaho trout farm study site (Troyer *et al.*, 2000).

host types: sockeye salmon (*O. nerka*), kokanee salmon (land-locked *O. nerka*), chinook salmon (*O. tshawytscha*), chum salmon (*O. keta*), coho salmon (*O. kisutch*), Atlantic salmon (*Salmo salar*), rainbow trout (*O. mykiss*) and steelhead trout (ocean-run *O. mykiss*). Approximately 45 % of the isolates were from spawning adult hosts and 45 % were

from juveniles at various stages, with the remaining 10 % from fish of unknown age. Typically, virus isolations from adult fish are from routine surveys of asymptomatic spawning host populations, while isolates from juveniles are usually associated with disease signs or epidemics involving mortality. We have tried as far as possible to include IHN

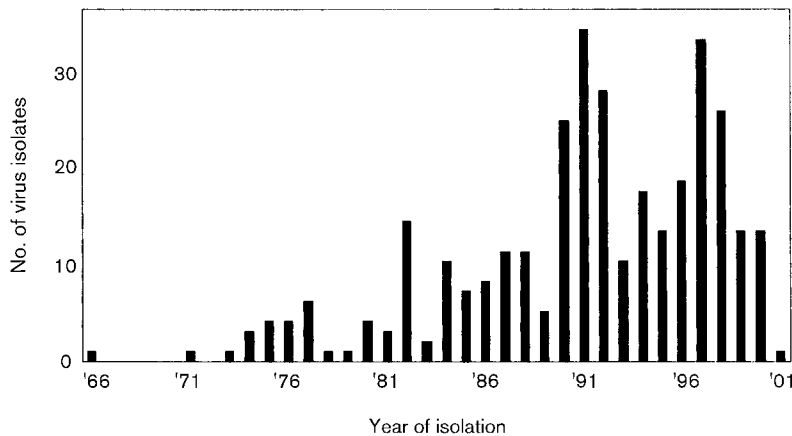


Fig. 2. Temporal distribution of the isolation dates (by year) for the 323 IHNV isolates analysed in this study.

isolates from wild fish, but due to the declines in wild populations and greater sampling effort directed at cultured populations only approximately 18% of the isolates were from wild fish and at least 76% were from fish in hatcheries, spawning channels, netpens and fish farms.

METHODS

Virus isolates. Geographical sites of origin for virus isolates analysed in this study are shown in Fig. 1. Sources and features of IHNV isolates from Alaska (Emmenegger *et al.*, 2000), coastal Washington (Emmenegger & Kurath, 2002) and the Idaho trout farm study site (Troyer *et al.*, 2000) have been described. For the current work, most virus isolates were provided by colleagues from natural resource agencies, as detailed in the Acknowledgements. The Japanese IHNV was the Shizuoka 1987 isolate (Winton *et al.*, 1988) and the French IHNV was isolate 32-87 (Arkush *et al.*, 1989). The Italian reference isolate was provided as sequence data for isolate IT-217A (G. Bovo, unpublished data). All IHNV isolates were obtained as virus in frozen cell-culture supernatants prepared using standardized fish health protocols (LaPatra, 1994). Although the exact passage history of some historical isolates could not be obtained, the great majority of the isolates would have been passed only once or twice in fish cell-culture lines, such as epithelioma papulosum cyprini cells (Fijan *et al.*, 1983) or chinook salmon embryo 214 cells (Lannan *et al.*, 1984).

Sequence analysis. The phylogenetic analysis shown is based on the sequence of a 303 nucleotide region in the middle of the IHNV G gene, referred to as the mid-G sequence (nt 686–988 of the full-length IHNV G gene sequence; GenBank accession no. U50401). Nucleotide sequences of cDNA fragments generated by reverse transcription and nested PCR were determined as described previously (Emmenegger *et al.*, 2000). Sequence files were edited using Sequencher 4.1 software (Gene Codes Corp.) and analysed using MacVector 6.0 and AssemblyLIGN 1.0.9 applications (Oxford Molecular Group). Virus isolates with identical sequences were combined into groups referred to as ‘sequence types’ and given names beginning with a representative isolate in the group, followed by letters to indicate all of the region(s) of origin in the group: A, Alaska; B, British Columbia; W, Washington Coast (excluding the Columbia River); FF, four farm Idaho study site (Troyer *et al.*, 2000); R, Columbia River; S, Snake River; O, Oregon coast; and C, California. For sequence types representing more than one virus isolate, an ‘n’ plus a number at the end of the name indicates the total number of virus isolates in that group (see Fig. 3). As exceptions to this general nomenclature, the names of the twelve IHNV isolates

previously published in Nichol *et al.* (1995) were retained, and isolates from Round Butte hatchery (Anderson *et al.*, 2000) retained their RB prefix nomenclature. Single virus isolates from France, Italy and Japan were designated FR, IT and JP, respectively.

All nucleotide sequences and virus isolates used in this work are available from the authors on request. Representative mid-G sequences from each major phylogenetic genogroup are available in GenBank as: U genogroup, accession nos AY102268–AY102270, nt 686–988 of accession no. U50401 and nt 639–942 of accession nos L40877 and L40880; M genogroup, accession nos AF237983–AF237992 (Troyer *et al.*, 2000; note that AF237987 represents the sequence type designated WRACFFn6 in Fig. 3) and nt 639–942 of accession nos L40871, L40875, L40876 and L40878; L genogroup, accession nos AY102271 and AY102272 and nt 639–942 of accession nos L40873, L40874 and L40881.

Phylogenetic analyses were carried out with the neighbour-joining and parsimony programs in PAUP* version 4 (Swofford, 1998) and PHYLIP version 3.5c (Felsenstein, 1993), using 1000 bootstrapped replicates of the infile data. The tree shown in Fig. 3 is a PAUP neighbour-joining phylogram with branch lengths accurately indicating genetic distance, and all branches with bootstrap values less than 70 have been collapsed to polytomies. Due to the lack of a closely related outgroup species, multiple trees were originally generated using the oldest isolates from each genogroup as outgroups. The resolution of the three major genogroups was consistent regardless of the outgroup used (data not shown). The final tree was drawn using the 12 sequence types within the L genogroup as the outgroup, because numerous analyses confirmed that the L genogroup was consistently monophyletic and phylogenetically distinct from the rest of the sequences (Emmenegger *et al.*, 2000; Troyer *et al.*, 2000; data not shown).

Maximum pairwise nucleotide diversity within sets of sequences was obtained using data from the absolute distance matrix generated in PAUP* version 4. Mean number of pairwise differences and intrapopulation nucleotide diversity (π) within each major genogroup were calculated according to the method of Nei (1987), as applied in the Arlequin version 1.1 (Schneider *et al.*, 1997) and DnaSP (Rozas & Rozas, 1999) software programs. Nucleotide substitution rates within each major genogroup were calculated in InStat version 3.01 (GraphPad Software) by plotting the year of isolation for each virus isolate in the genogroup against its genetic distance from the ancestral node of the genogroup (U, M or L). The slope of the linear regression line indicates the number of nucleotide substitutions per nucleotide site per year. The ratio of non-synonymous to synonymous nucleotide substitutions within each genogroup and the average number of nucleotide differences between isolates in different genogroups were

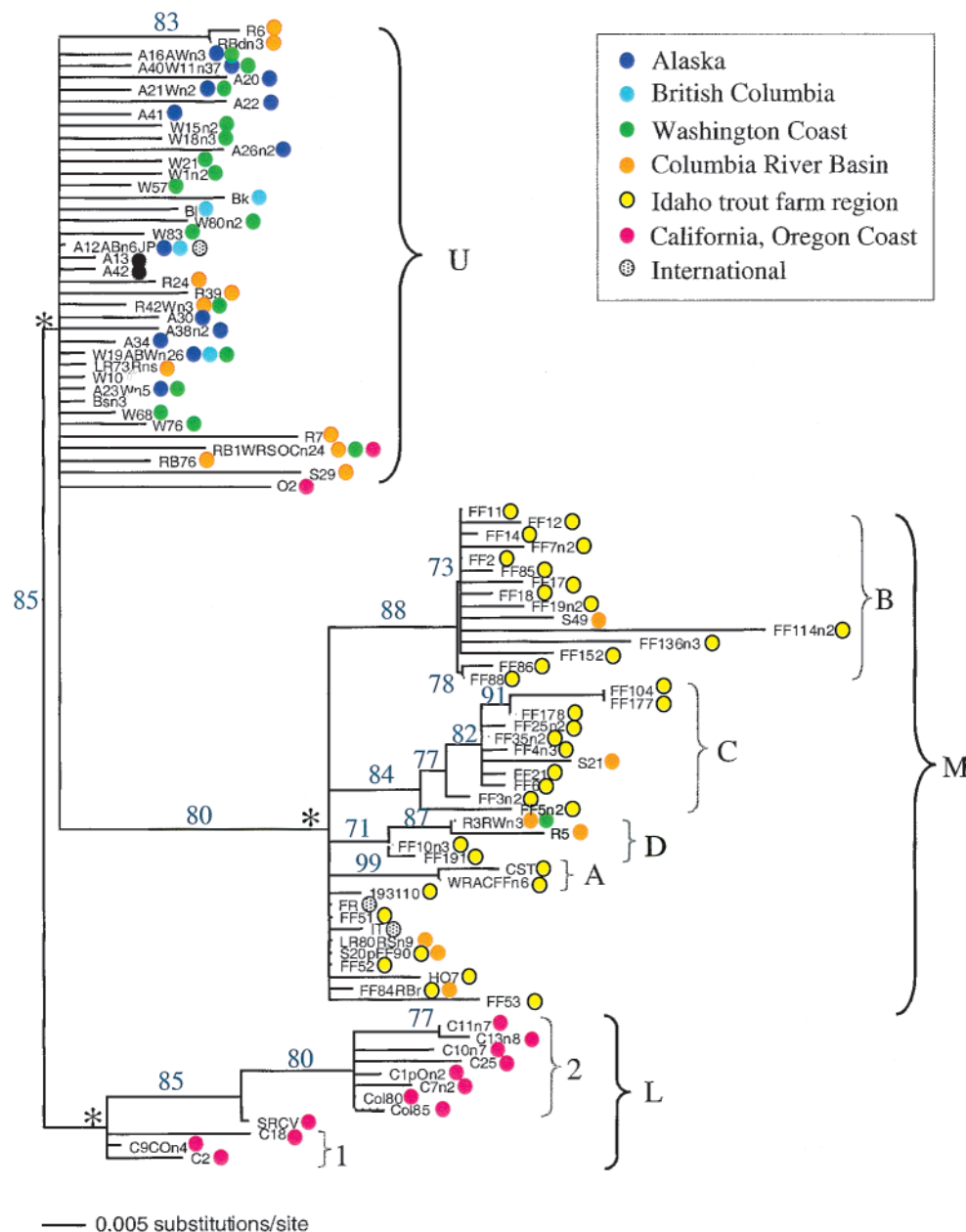


Fig. 3. Representative unrooted phylogenetic tree indicating evolutionary relationships between 93 mid-G (303 nucleotides) sequence types derived from 323 IHN virus isolates. Individual sequence types shown at terminal nodes are named as described in the Methods, with an 'n' plus a number suffix to indicate the total number of IHN virus isolates when there is more than one isolate within a sequence type. The tree shown is a neighbour-joining distance tree in which horizontal branch lengths accurately reflect genetic distance. Bootstrap values from 1000 resampled data sets are indicated at major nodes and the tree has been collapsed at nodes with bootstrap values less than 70. Coloured dots indicate the geographical source(s) of the isolate(s) within each sequence type, as shown in the boxed legend and in Fig. 1. The Idaho trout farm region isolates shown in yellow include both the four farm study site isolates (Troyer *et al.*, 2000) and Hagerman Valley isolates from Nichol *et al.* (1995). Black brackets indicate the three major genogroups, U, M and L, and grey brackets indicate subgroups A–D within the M genogroup (Troyer *et al.*, 2000) and subgroups 1–2 within the L genogroup. Asterisks denote the individual genogroup ancestral nodes used to calculate evolutionary rates (substitutions per nucleotide per year) within the U, M and L genogroups.

calculated using the methods of Nei & Gojibori (1986), as applied in the DnaSP software program (Rozas & Rozas, 1999). Due to the high level of redundancy within the U genogroup, the non-synonymous/

synonymous substitution rate was calculated both with redundancy included in the input data (value shown in Table 2) and with no redundancy in the input data (value 0.0774).

Table 2. Features of IHNV genogroups in the mid-G sequence phylogeny

(a) Biological

Genogroup	No. of virus isolates	No. of different mid-G nt sequence types	No. of different mid-G aa sequence types	Isolation year range	General host type(s)
U	180	39	16	1971–2000	Sockeye, chinook, steelhead
M	107	42	31	1978–1998	Mostly rainbow trout
L	36	12	11	1966–2001	Mostly chinook

(b) Genetic

Genogroup	Genogroup topography	nt differences		Intra-genogroup nt diversity ($\pi \pm SD$) \ddagger	Rate of evolution \S	Non-synonymous nt substitution rate	Synonymous nt substitution rate	Non-syn./syn. nt substitution ratio
		Max. no.*	Mean no. \dagger					
U	No internal structure	9	2.7 \pm 1.4	0.0088 \pm 0.0005	0.2 $\times 10^{-3}$	0.00141	0.03576	0.039411
M	Four internal subgroups	23	10.4 \pm 4.8	0.0323 \pm 0.0015	1.2 $\times 10^{-3}$	0.02616	0.05869	0.4457
L	Two internal subgroups	10	3.5 \pm 1.8	0.0115 \pm 0.0016	0.6 $\times 10^{-3}$	0.01096	0.01418	0.7729

*Maximum number of nucleotide differences between mid-G sequences of virus isolates within each genogroup.

\dagger Mean number of nucleotide differences between all possible pairs of isolates within each genogroup.

\ddagger Intrapopulational nucleotide diversity equal to the average number of nucleotide differences per site between all possible pairs of isolates within a genogroup.

\S Estimated rate of evolution within each genogroup, in number of nucleotide substitutions per site per year.

||Due to the high level of redundancy within the U genogroup the non-synonymous/synonymous substitution rate was calculated both with redundancy included in the input data (value shown in table) and alternatively with no redundancy in the input data (value 0.0774).

RESULTS

Mid-G sequences and phylogenetic analyses

Comparisons of the mid-G sequences (303 nucleotides) of these 323 IHNV isolates revealed a total of 93 different sequence types, with an overall maximum pairwise nucleotide diversity of 8.6%. Among the 93 sequence types identified, there were 54 that were unique to single virus isolates, 18 that were identical in two isolates and nine that each represented three virus isolates. Another eight sequence types represented groups of four to nine isolates. All other virus isolates fell into one of three large, highly prevalent sequence types. Two sequence types (W19ABWn26 and RB1WRSOCn24; Fig. 3) represented 26 and 24 different virus isolates, respectively, and the most common sequence type (A40W11n37) was found in 37 isolates. The A40W11n37 and W19ABWn26 sequences had been identified previously as the highly prevalent 'Skagit' and 'Lake Washington' sequence types in the coastal Washington IHNV study (Emmenegger & Kurath, 2002).

Phylogenetic analyses of these 93 sequence types by both distance and parsimony methods consistently produced trees in which all IHNV isolates fell into one of three distinct

major genogroups that were supported by significant bootstrap values. A representative tree is shown in Fig. 3, in which the branch lengths accurately reflect genetic distance. The three major genogroups have been designated U, M and L, to indicate their general correlation with the upper, middle and lower portions of the IHNV geographical range in North America, as will be described below. These three groups differed in their topography and overall levels of diversity (Table 2). The U genogroup had almost no discernible internal structure, but instead suggested radial evolution of relatively short branches from a common ancestor. Despite this simple structure, the U genogroup contained 39 sequence types representing 180 isolates, making it the largest of the three groups. In contrast, the M genogroup contained 42 sequence types representing 107 IHNV isolates. This genogroup had a complex internal structure, including four subgroups supported by high bootstrap confidence values. These corresponded to the four subgroups, A–D, identified previously as co-circulating within individual facilities in the Idaho trout farm study site (Troyer *et al.*, 2000). Although these subgroups might alternatively be interpreted as independent major groups, their close geographical and biological features (described below) made it reasonable to consider them subgroups

within the major M genogroup. The L genogroup, which had 12 sequence types representing 36 isolates, had an internal branching structure indicating at least two well-supported subgroups.

In addition to different topographical structures, the three genogroups differed in their overall levels of intragenogroup genetic diversity (Table 2b). As a simple measure of this phenomenon, the maximum pairwise nucleotide diversity within the M genogroup was 7.6% (23 nucleotide differences out of 303 nucleotides), which was more than double the maximum diversity found within either the U or the L genogroups. A more comprehensive measure is the average intrapopulational nucleotide diversity, π (Nei, 1987), which takes into account the number of isolates represented by each sequence type (Rozas & Rozas, 1999). The π value for the U, M and L genogroups was 0.0088, 0.0323 and 0.0115, respectively. This is equivalent to a mean of 2.7, 10.4 and 3.5 nucleotide differences between isolates within the U, M and L genogroups, respectively. Thus, the genetic diversity within the IHNV M genogroup is three- to fourfold higher than that of the L and U genogroups. In intergenogroup comparisons, the average number of nucleotide differences between mid-G sequences of virus isolates from the different genogroups was 11.2 (3.7%) for U versus M, 11.0 (3.6%) for U versus L and 18.0 (5.9%) for M versus L.

Although we do not assume a molecular clock applies to IHNV evolution, a simple estimate of evolutionary rates within each major genogroup was made by plotting the year of isolation for each virus isolate against its genetic distance from the ancestral node of the genogroup (nodes indicated by asterisks in Fig. 3). Although there is potential for bias in this method because the sequence tips are not independent data points, it does provide a useful approximation of evolutionary rates. Linear regressions were significant with $P < 0.001$ for all three genogroups, indicating significant positive correlations between year of isolation and distance from the base of the tree. The different slopes of the lines indicated different evolutionary rates (Table 2b). The highest rate occurred in the M genogroup, with 1.2×10^{-3} nucleotide substitutions per site per year. This is twice the rate observed for the L genogroup and sixfold the rate of U genogroup IHNV. Despite their differences, all three rates fall within the range of rates previously reported for molecular evolution of RNA viruses (reviewed in Domingo *et al.*, 2001; Jenkins *et al.*, 2002). Ratios of non-synonymous to synonymous nucleotide substitutions also varied, with the U genogroup being notably lower than the other genogroups, but they were all less than 1.0, indicating a lack of discernible positive selection pressure (Table 2b).

Correlation of phylogeny with virus isolate features

The resolution of these IHNV isolates into three major genogroups correlated very strongly with their geographical sites of isolation as shown by coloured symbols in Fig. 3. The U genogroup included all the Alaska and British Columbia

isolates and 60/61 Washington coastal watershed isolates. It also included 67% (33/49) of the Columbia River basin isolates and single isolates from Oregon, California and Japan. In contrast, the M genogroup contained all isolates from the Idaho trout farm study site and 33% (16 out of 49) of the Columbia River basin isolates. It also contained a single Washington coastal isolate (#22 in Emmenegger & Kurath, 2002) and the European isolates from France and Italy. The L genogroup contained all but one of the California isolates and all but one of the Oregon coast isolates. This strong geographical correlation indicated distinct geographical ranges of the three major IHNV genogroups, with an overlap between the U and M genogroup ranges in the Columbia River basin, as shown in Fig. 4.

There was some general correlation of the three genogroups with host species, but it was not as clear as the geographical

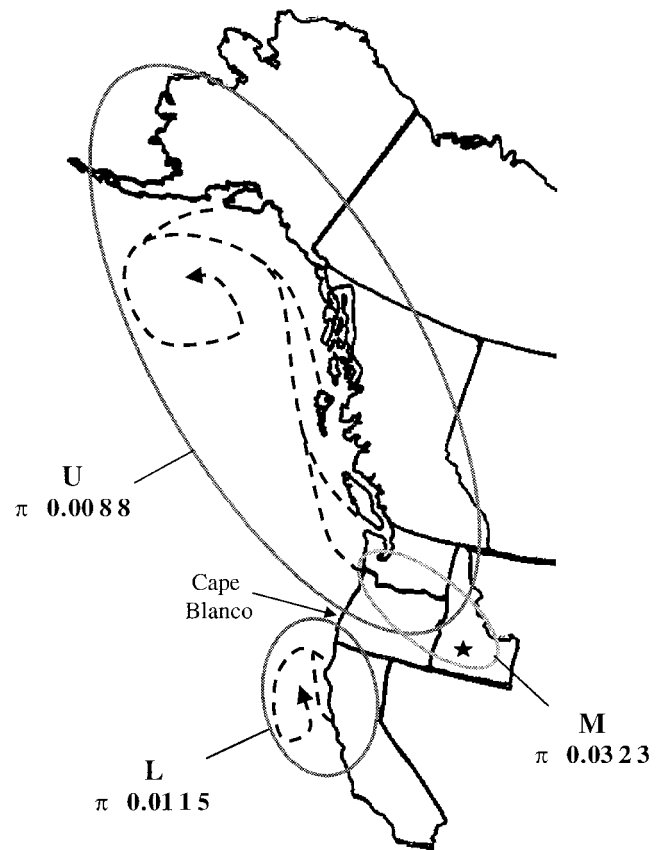


Fig. 4. Geographical ranges of the three major IHNV genogroups in western North America. The π values are the intrapopulational nucleotide diversity levels observed within each of the three genogroups. The star denotes the localized Idaho trout farm study site. The arrow shows the location of a known biogeographical transition zone for aquatic organisms at Cape Blanco on the Oregon coast. Dotted arrows indicate generalized salmon migration routes during the ocean phase of their anadromous life cycles.

correlation. The U genogroup was mostly virus isolated from sockeye salmon in the northern portion of the genogroup range, but it also included large numbers of IHNV isolates from chinook salmon and steelhead trout in the Columbia River basin. The M genogroup IHNV was mostly from rainbow trout and the L genogroup IHNV was mostly from chinook salmon, although there were also several steelhead isolates from California in the L genogroup. As a general observation, in cases where more than one host species occurred in close proximity, we typically found that they had IHNV types that were identical or nearly identical. Similarly, wild and cultured fish in close geographical proximity tended to have identical or very similar virus types. This is similar to patterns observed in phylogenetic analyses of natural rabies virus isolates (Holmes *et al.*, 2002).

There was no discernible correlation of the major IHNV genogroups with year of isolation of the viruses, as each genogroup covered at least a 20-year period and they all persisted into the present. The only temporal difference was that the earliest available U and L genogroup IHNV isolates were from 1971 and 1966 respectively, while the first M genogroup isolate was from 1978.

DISCUSSION

In terms of virus evolution, independent evolution of separate viral lineages is generally facilitated by mechanisms of genetic isolation. It is most interesting to consider the possible mechanism(s) responsible for the evolution of IHNV into three distinct genogroups. This involves identifying factors that could account for the differences between the genogroups in genetic diversity and geographical range. Among the three genogroups identified here, the U genogroup covered by far the largest range (3700 km between the two farthest collection sites) and contained the largest number of virus isolates. Despite this, the U genogroup had the lowest intragenogroup divergence, suggesting overall genetic homogeneity and stasis, as has been recognized in previous regional studies (Emmenegger *et al.*, 2000; Emmenegger & Kurath, 2002). Historical observations suggest that IHNV within the U genogroup range 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 (Amend & Wood, 1972; Grischkowsky & Amend, 1976; Mulcahy *et al.*, 1980). Therefore, we consider the U genogroup to represent direct descendants of an original long-term association of IHNV with sockeye salmon. IHNV may have been inadvertently spread throughout the current U genogroup range by the historically common practice of salmon transplantations (Roppel, 1982; Wolf, 1988; Burgner, 1991) and the widespread use of raw, unpasteurized salmon viscera, originating mostly from Alaska and British Columbia canneries, in feed for salmon fry in hatcheries

throughout the western USA during the 1950s to 1960s (Watson *et al.*, 1954; Guenther *et al.*, 1959; Wolf, 1988).

In addition to the broad geographical range of the U genogroup, the unusually low level of genetic diversity requires some explanation. It is possible that the U genogroup represents IHNV genotypes on a high fitness peak that successfully out-compete variants that arise. This would be supported by the low evolutionary rate and exceptionally low non-synonymous substitution frequency observed for U genogroup IHNV. It is also possible that some mechanism(s) actively maintains the genetic homogeneity within the U genogroup. One unifying feature of the U genogroup isolates is that the salmonid host populations in this geographical range all share overlapping oceanic ranges during the saltwater migration phases of their anadromous life cycles (Fig. 4). It is known that salmonids from the Columbia River and all rivers northward migrate north on entering seawater and spend 1–3 years with the potential for intermingling in the nutrient-rich oceanic gyres off the coasts of Alaska and British Columbia (Thorpe, 1988; Burgner, 1991; Healey, 1991; Heard, 1991; Salo, 1991; Sandercock, 1991; Busby *et al.*, 1996; Gustafson *et al.*, 1997). This could effectively constitute one very large host population throughout this region, resulting in genetic homogeneity in the virus populations. Additional factors including salmonid straying (Quinn, 1984, 1993), or unrecognized alternative IHNV hosts or reservoirs (Traxler *et al.*, 1997; Kent *et al.*, 1998) may also contribute to maintenance of the genetic homogeneity within the U genogroup IHNV.

The observation that IHNV isolates from California differ from the IHNV isolates in the Columbia River basin and farther north has been reported by many researchers (Mulcahy *et al.*, 1984; Hsu *et al.*, 1986; Winton *et al.*, 1988; Nichol *et al.*, 1995). This is confirmed here in the existence of the L genogroup. Sockeye salmon do not occur south of the Columbia River basin and the predominant salmon species in California is chinook. Although we do not know how long IHNV has been in California, it is possible that the L genogroup originated from a U genogroup ancestor by a host jump from sockeye into chinook. This could have occurred in the California chinook hatchery system due to the feeding of chinook fry with raw sockeye viscera contaminated with U genogroup IHNV. Although the evolutionary divergence of the L and U genogroups may have involved host-specific adaptation to chinook salmon, this is not sufficient to explain their continued separation because there are several L genogroup isolates from steelhead and other host species in California and there are also many U genogroup isolates from chinook salmon in the Columbia River basin. Instead, the hypothesized influence of host oceanic migration ranges may also explain the separation of the L genogroup from the other two major IHNV genogroups. The northern boundary of the L genogroup range coincides with a recognized biogeographical transition zone for all aquatic organisms at Cape Blanco on

the Oregon coast (Fig. 4) (Busby *et al.*, 1996). One feature of this transition zone is that salmonid stocks from rivers north of Cape Blanco migrate northward toward Alaska when they enter the ocean and stocks from rivers south of Cape Blanco migrate south and west, spending their oceanic life stage in mostly coastal waters south of the mouth of the Columbia River (Fig. 4) (Nicholas & Hankin, 1988; Healey, 1991; Busby *et al.*, 1996; Myers *et al.*, 1998). Thus, the L genogroup host populations do not intermingle with the U genogroup host populations in the ocean. This may constitute distinct 'epizootiological compartments' as described for rabies virus field isolates (Holmes *et al.*, 2002). In the IHN scenario these non-overlapping ocean migration ranges may serve to maintain the separate ranges and genetic isolation of the U and L genogroups.

Other interesting features of the phylogeny that require explanation are the origin of the high-diversity M genogroup and the sympatric overlap of the U and M genogroup ranges in the Columbia River basin. The M genogroup accounts for approximately one-third of the IHN isolates in the Columbia River basin and contains all 84 of the IHN isolates characterized from the trout farm study site in Idaho (Troyer *et al.*, 2000). It also contains all IHN isolates from 17 additional fish farms upstream and downstream of the four farms originally characterized (R. M. Troyer & G. Kurath, unpublished data). These farms are part of the Hagerman Valley trout aquaculture region, which produces 75 % of the food-sized rainbow trout in the USA (USDA Economic Research Service, 2001). Rainbow trout have no ocean migration phase in their natural life cycle and farmed fish are held captive for their entire life span (1–2 years). IHN epidemics were first observed in the Hagerman Valley in 1977 and the virus subsequently spread to become endemic throughout the valley by 1980 (Busch, 1983; Groberg, 1983). The earliest M genogroup viruses in our study were isolated in 1978 from trout farm epidemics at two sites in the Hagerman Valley. Thus, the appearance of the M genogroup seems to coincide with the emergence of IHN in the Hagerman Valley trout farming industry. There are several features of trout farming practices that may alter the selection pressures acting on IHN. These are reviewed by Troyer *et al.* (2000) and include temporal modifications of the annual spawning cycle of the host to provide a continuous year-round supply of fry, continual introduction of highly susceptible host populations into virus endemic environments, culture of fish at a relatively high constant temperature of 15 °C, culture of fish at high densities and stress levels, fragmentation of the host populations into multiple separate ponds within multiple separate facilities and use of geographically separate virus-free broodstock. The combined effects of these practices may be to increase greatly the number of rounds of virus replication per year and to reduce natural transmission bottlenecks, temporal bottlenecks and virus competition, thus facilitating virus diversification at a rate more rapid than would occur in other host populations.

The source of the original M genogroup ancestor is unknown, but it most likely involved a host jump from sockeye to rainbow trout and also adaptation to replication at the higher water temperature of 15 °C (Amend & Smith, 1975). Rate estimates suggest that this adaptation of IHN to rainbow trout in the Hagerman Valley was followed by a sixfold increase in the rate of virus evolution (Table 2). The extension of the M genogroup range into the rest of the Columbia River basin may have resulted from the occasional spread of virus from the Hagerman Valley, as previously suggested (Groberg, 1983; Hsu *et al.*, 1986). With regard to the ocean migration range hypothesis, the absence of M genogroup virus isolates from salmon north of the Columbia River may be due to the sporadic and relatively transient nature of M genogroup IHN in the lower Columbia River basin (K. A. Garver & G. Kurath, unpublished data), or it may indicate that the adaptation of M genogroup viruses to environmental conditions in the Hagerman Valley renders them less fit in northern portions of the IHN range.

The placement of the three international isolates within this phylogeny supports previous speculations regarding the spread of IHN from North America to Europe and Japan. The isolate from Japan had a mid-G sequence identical to six other U genogroup isolates from Alaska and British Columbia (sequence type A12ABn6JP; Fig. 3). This is consistent with the reported introduction of IHN to Japan in 1967 via a shipment of contaminated sockeye salmon eggs from Alaska (Sano *et al.*, 1977). The French and Italian isolates fell into the M genogroup outside the derived subgenogroups, suggesting a source common with the M genogroup ancestor.

At present very few aquatic virus species have been investigated from a comprehensive phylogeographical approach. The best example to date is the European salmonid rhabdovirus viral haemorrhagic septicaemia virus (VHSV), which has diverged into three or four genogroups that correlate with geographical origin (Benmansour *et al.*, 1997; Stone *et al.*, 1997; Snow *et al.*, 1999). A maximum of 15–18 % nucleotide diversity was observed between European and North American VHSV isolates using full-length (Benmansour *et al.*, 1997) G gene sequences. For comparison, the maximum nucleotide diversity reported for G gene sequences of rabies virus (genotype 1) and vesicular stomatitis virus isolates is 16–20 % (Tordo *et al.*, 1993; Nichol *et al.*, 1989; Bilsel & Nichol, 1990). Thus, with a maximum of 8.6 % nucleotide diversity in the variable mid-G region, the overall genetic diversity of IHN is low compared with other rhabdoviruses. This has been noted previously by Nichol *et al.* (1995), who reported a maximum of 3.4 % nucleotide diversity within the complete G gene sequences of twelve IHN isolates.

In conclusion, this analysis confirms that all IHN isolates examined to date exhibit low genetic diversity and fall into one of three major genogroups, designated U, M and L. The genogroups correlate with geographical origin and to a

limited extent with host species, but not with temporal factors. These genogroups vary in complexity and overall genetic diversity, with the M genogroup having significantly higher diversity than the other two genogroups. This phylogeny is based on a partial G gene sequence of only 303 nucleotides. However, a parallel phylogeny of over 60 of the IHNV isolates using partial N gene sequences (412 nucleotides) generated trees of extremely similar topography, with identical differentiation into the three major genogroups (K. A. Garver, R. M. Troyer & G. Kurath, unpublished data). In addition, it has been pointed out that due to the absence of intergenomic recombination in rhabdoviruses and other members of the superfamily *Mononegavirales*, results of molecular epidemiological studies should be essentially independent of the genomic site employed, provided the data are sufficiently robust (Nadin-Davies, 2000). Despite the overall low genetic diversity observed among IHNV field isolates in this study, the robust nature of the phylogeny and the high level of redundancy (i.e. number of isolates within individual sequence types) leads us to predict that any IHNV isolates from North America characterized in the future will fall within this general three genogroup phylogenetic structure. Finally, from this analysis we speculate that possible factors in IHNV evolution include host oceanic migration ranges and anthropogenic factors related to fish culture practices.

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