

“Mid-G” Region Sequences of the Glycoprotein Gene of Austrian Infectious Hematopoietic Necrosis Virus Isolates Form Two Lineages within European Isolates and Are Distinct from American and Asian Lineages[∇]

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Infectious hematopoietic necrosis virus (IHNV) is one of the most important pathogens of salmonid fish. In this study a comprehensive phylogenetic analysis of the genetic evolution and variety of Austrian IHNV strains, as well as selected strains ensuring worldwide coverage, is presented. The phylogenetic investigation is based on sequences comprising the “mid-G” region of the G gene, and it includes all currently available IHNV sequences of the G gene with a length of at least 615 bp. Austrian IHNVs are located—together with other European IHNV isolates—in two clusters of genogroup M (M-Eur1 and M-Eur2) and are clearly separated from American and Asian lineages. The genetic clustering, however, could not be linked to certain clinical symptoms or significant differences in the mortality rates.

Infectious hematopoietic necrosis virus (IHNV) is one of the most important fish pathogens causing lethal disease in salmonid fish (37, 49, 51). The first reported epidemics of IHNV occurred in the United States at Washington and Oregon fish hatcheries during the 1950s (41). Subsequently, IHNV spread to Alaska (in 1974), throughout the Hagerman Valley to Idaho (1977 to 1980), and then was found in salmonids of the Columbia River (early 1980s), and finally was detected in the Pacific Northwest (34). In 1971 the virus spread to Japan (39, 43) and subsequently to Taiwan, China, and Korea (43). Probably due to the expanding commercial sale of infected eggs and fish, IHNV was introduced to Europe, where it was for the first time recorded in France and Italy in 1987 (5, 9), followed by detection in Belgium in 1989 and 1990 (17), in Germany in 1992 (17), and in Switzerland in 1993 (31). The source of the virus and the route of IHNV introduction to Europe are unknown; within Europe German salmonids were probably infected through fish and eggs imported from Italy and France (19). The first outbreak of IHN in Austria was recognized in 1994, reported by Weber (Office International des Epizooties) and mentioned by Bergmann et al. (6).

IHNV is the type species of the genus *Novirhabdovirus*; it belongs to the family *Rhabdoviridae* within the order *Mononegavirales* (7, 48). The virus has a linear single-stranded RNA genome of 11,137 bp in length (44). The IHNV genome encodes six structural proteins: a nucleocapsid protein (N), a

polymerase-associated phosphoprotein (P or M1), a matrix protein (M or M2), a surface glycoprotein (G), a nonvirion protein (NV), and a viral RNA polymerase (L) (26, 32, 33, 37, 44, 45). The denotation *Novirhabdovirus* was first introduced in 2000 in the seventh report of the International Committee on Taxonomy of Viruses (48). The term “novi-” stands for the “nonvirion (NV)” gene, an additional unique gene, localized between the G and L genes, which is specific for this virus family (27). The complete genomic sequences of IHNV were determined in 1995 in parallel by Schütze et al. (44) and Morzunov et al. (37). These two sequences (GenBank accession numbers X89213 and L40883, respectively) are the only complete genomic IHNV sequences available to date.

The glycoprotein (G) is an N-glycosylated class I transmembrane protein of approximately 500 amino acids and forms trimeric peplomers at the surface of the virion (11, 25, 29). It is responsible for the entry of the virus into the cell by receptor-mediated endocytosis (36). The IHNV glycoprotein induces neutralizing antibodies (8, 36). High levels of immunity were induced after intramuscular injection of plasmids mediating expression of the G proteins (4, 12, 13, 23, 24). Vaccination with plasmids encoding other viral proteins was not successful, which confirmed that anti-G neutralizing antibodies were required to protect fish against challenge infection (12).

Coinfections with other rhabdoviruses, e.g., viral hemorrhagic septicemia virus (VHSV), or with viruses of the family *Birnaviridae* such as infectious pancreatic necrosis virus (IPNV), were observed frequently (1, 2, 3, 10, 49).

Genetic analysis and phylogenetic studies of IHN viruses have previously been performed mostly on American fish samples; only a few Asian or European IHNV isolates have been investigated thus far (14, 15, 19, 22, 34, 38, 39, 46, 47). These analyses exhibited surprisingly low genetic diversity within the IHNV G genes. A 303-nucleotide long part within the IHNV G

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gene, the so-called “mid-G” region that contains putative antigenic determinants (28), has been found to be valuable for IHNV phylogenetic analyses (14, 15, 22, 34, 46, 47). In general, the phylogenetic relationship of IHN viruses was found to correlate with the geographic origin of virus isolates rather than with host species or with temporal factors (47). A limited correlation with host species was described by Kurath et al. (34), and time-related divergences between Japanese isolates before and after the 1980s were observed by Nishizawa et al. (39).

The objective of the present study was to conduct a comprehensive phylogenetic analysis of IHNV isolates from Austria and from around the world.

MATERIALS AND METHODS

Sample collection. The occurrence and distribution of virus infections in Austrian trout farms was monitored by examining healthy, as well as naturally diseased fish, mainly rainbow trout, at the Austrian Reference Laboratory for Fish Diseases at the University of Veterinary Medicine, Vienna, Austria, between 1993 and 2003. The number of farms submitting fish material ranged from 66 in 1993 (919 pieces of individual salmonid fish) to 40 in 2003 (submitting 1,531 fish).

In addition, between 2001 and 2007, 85 fish organ pools (one pool consisted of approximately 10 fish samples) were sent directly to the molecular virology laboratory at the Institute of Clinical Virology for IHNV, VHSV, and IPNV routine testing by means of reverse transcription-PCR (RT-PCR).

IHNV, VHSV, and IPNV isolation and antigen demonstration. Fish, which showed under visual inspection clinical symptoms suggestive of one of the three major salmonid fish diseases—IHN, VHS, or IPN—were euthanized, necropsied, and submitted for further investigations by the following laboratory tests.

IHNV isolation attempts were carried out by using the well established *Epithelioma papulosum cyprini* (EPC) cell line (21). Monolayers grown on 24-well cell culture plates at 20°C were inoculated with suspensions of hematopoietic organs at final dilutions of 1:100 and 1:1,000, respectively, and incubated at 15°C. The supernatants of monolayers that showed a cytopathic effect after an incubation period of 2 to 4 days were passaged on EPC cells grown on microscopic slides in order to subsequently perform an IHNV-specific immunofluorescence antibody test (IFAT) (Bio-Fluo IHN kit, art.no. Bio K008; Bio-X Diagnostics, Jemelle, Belgium); the IHNV-specific monoclonal antibody was used in a dilution of 1:20. The test was performed according to the manufacturer's instructions and the diagnostic guidelines laid down in the European Commission Decision 2001/183/EC of 22 February 2001.

VHS and IPN viruses were isolated in the fibroblastoid centrarchid fish cell line *Bluegill fry* (BF-2) established by Wolf et al. in 1966 (50). Cell culture isolates, as well as original organ homogenates of clinically diseased fish, were tested thereafter by an enzyme-linked immunosorbent assay (ELISA; ISO 9001.2000 certified; TEST-LINE, Ltd., Brno, The Czech Republic).

Isolation of RNA. The collected cell culture isolates from the Laboratory for Fish Diseases were initially stored frozen at -80°C.

Fish tissue pools, including head kidney and spleen, which were sent directly to the molecular virology laboratory for routine RT-PCR testing, were homogenized using sterile sand, resuspended in distilled and diethyl pyrocarbonate-treated water, and frozen at -80°C for at least 30 min.

Subsequently, all cell culture isolates and organ suspensions were thawed and centrifuged at 1,700 × g for 5 min. A volume of 140 µl of each supernatant was used for RNA extraction, using a QIAamp viral RNA purification kit (Qiagen) according to the manufacturer's instructions.

Primer design. One primer pair amplifying a 673-bp DNA fragment within the G gene was designed on the basis of the entire IHNV sequence deposited at GenBank database under accession no. X89213. The forward and reverse primer sequences were 5'-³⁴¹⁸ACAACCGCAGCCGCTCTGTA³⁴³⁷-3' and 5'-⁴⁰⁹⁰CAGCGACCGTCATGCACATC⁴⁰⁷¹-3', respectively. The primers used for the detection of possible coinfections with VHSV had the sequences 5'-ACCTGTTCGACCAGCTTCTT-3' (positions 782 to 801 of the G gene of the VHSV isolate, GenBank accession no. X59148) for the forward primer and 5'-CACAGTCACCTCGCATGATT-3' (positions 1535 to 1516 of the same VHSV isolate) for the reverse primer, respectively; this primer pair is supposed to amplify a 754-bp segment within the G gene of VHSV. The primer pair amplifying a partial sequence of the major structural polypeptide VP2 in the samples with possible

double infections with IPNV exhibited the sequences 5'-¹⁴²CAAGGCAACCGCAACTACT¹⁶¹-3' and 5'-⁷²⁶ATKGCAGCTGTGCACCTCAT⁷⁰⁷-3', respectively, resulting in a 584-bp DNA fragment. The nucleotide positions refer to the VP2 gene of the IPNV sequence deposited under the GenBank accession no. D00701. All oligonucleotide primers were derived by using the Primer Designer Program (version 3.0; Scientific & Educational Software) and synthesized by Invitrogen (Life Technologies).

Detection of IHNV RNA by RT-PCR. cDNA synthesis and PCR were carried out in a single step by using a commercially available kit (OneStep RT-PCR kit; Qiagen) according to the manufacturer's recommendations. The annealing temperature of 60°C was used for 40 PCR cycles. Each reaction contained 0.8 µM (final concentration) of each of the primers and 2.5 µl volume of RNA extract (corresponding to 10% of the total reaction volume). Additionally, 4 U of RNasin RNase inhibitor (Promega) was used. All amplifications were performed in a GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer) or in a Mastercycler gradient (Eppendorf). After gel electrophoresis and ethidium bromide staining, DNA was extracted directly from the PCR product by using PCR Kleen Spin columns (Bio-Rad) according to the manufacturer's instructions. As positive controls for RT-PCR optimization, we used ATCC strain VR-714 and the first French IHNV isolate 32/87 (obtained from P. de Kinkelin, Jouy-en-Josas, France).

For the detection of VHSV and IPNV nucleic acid, respectively, the RT-PCR conditions were applied as described above for the detection of IHNV RNA.

Determination of RT-PCR sensitivities. One Austrian sample (19/02) positive for both IHNV and VHSV and one Italian IHNV sample (10/03) positive also for IPNV served as targets for the evaluation of the sensitivities of the three RT-PCRs. We intentionally used naturally double-infected samples for evaluation of the RT-PCR sensitivities since we comparatively evaluated monospecific and duplex RT-PCR assays (the latter results are not shown in the present study). RNA extracts of these samples were serially diluted in nuclease-free water from 10⁻¹ to 10⁻¹². The sensitivities of all reactions were evaluated.

Sequencing and sequence analysis of IHN viruses. An ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, United Kingdom) diluted in ABI Prism BigDye 5× sequencing buffer (Applied Biosystems) at a ratio of 2 to 3 was used for the sequencing PCR. The final primer concentration used for this reaction was 0.2 µM. For the removal of unincorporated dye terminators directly from sequencing reactions, the spin columns of a DyeEX 2.0 spin kit (Qiagen) were used. Clean reaction products were sequenced in both directions by using an ABI Prism 310 genetic analyzer (Perkin-Elmer) automatic sequencing system.

The obtained sequences of 20 investigated IHN viruses were aligned by using the Align Plus program (version 3.0, serial no. 43071; Scientific & Educational Software). The sequences were compared to the sequences of 2 IHNV reference strains and 45 already-published IHN viruses, mainly originating from the United States, Japan, and Germany. The identity of the compiled nucleotide sequences was verified by BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST/>), and the sequences were submitted to GenBank under accession numbers AY524107 to AY524129.

Phylogenetic analysis. In the phylogenetic analysis, all current available IHNV sequences of the G gene with a length of at least 615 bp were included. Phylogenetic investigations were performed by using the phylogeny inference program package, PHYLIP (20). The stability of the trees was tested by bootstrap resampling analysis of 1,000 replicates computed with the SEQBOOT program. Genetic distances between each pair of sequences were calculated by using the DNADIST program based on the Kimura two-parameter model with a transition/transversion ratio of 2. From these distance matrices the phylogenetic tree was generated by the neighbor-joining method of the Neighbor program and was displayed by DRAWGRAM. The bootstrap values were determined with the CONSENSE program. The tree was rooted with the five Japanese “JRT” sequences. These clearly distinct sequences were chosen as an outgroup in order to provide a better evolutionary direction for the other viruses as shown by Salemi and Van de Peer in an example for vertebrate phylogeny (42). Bootstrap values from the consensus tree were added to the nodes of the branches as described by Salemi and Van de Peer (42).

RESULTS

Clinical symptoms. The observed clinical signs of the IHNV-positive fish, as well as other characteristics of the IHNV isolates, are presented in Table 1. In 10 of 19 IHN outbreaks double infections with VHSV were diagnosed, and in one

TABLE 1. Characteristics of 19 Austrian and 1 Italian IHNV isolates investigated in this study

| Isolate no. | Time of outbreak (mo yr) | Site of outbreak (city/district/federal state) | Host ^a | Clinical signs ^b | | | | | | Double infection | GenBank accession no. |
|-------------|--------------------------|--|-------------------|-----------------------------|----|----|----|----|----|------------------|-----------------------|
| | | | | GA | VA | CD | VP | MP | SM | | |
| 49/94 | April 1994 | Kalwang/Leoben/Styria | rbt | + | - | + | - | - | - | VHSV | AY524129 |
| 55/94 | Apr 1994 | Gföhl/Krems/Lower Austria | rbt | + | - | + | - | - | + | | AY524124 |
| 95/94 | May 1994 | Kaindorf/Hartberg/Styria | rbt | - | - | + | - | - | - | VHSV | AY524127 |
| 106/94 | May 1994 | Wolfen/Steyr/Upper Austria | rbt | + | + | + | - | - | - | VHSV | AY524108 |
| 113/94 | May 1994 | Mödling/Mödling/Lower Austria | rbt | + | + | + | + | - | + | | AY524110 |
| 236/94 | Aug 1994 | Krieglach/Mürzzuschlag/Styria | rbt | + | + | + | + | - | - | | AY524117 |
| 264/94 | Oct 1994 | St. Roman/Schärding/Upper Austria | rbt | + | + | + | - | - | - | | AY524120 |
| 13/95 | Feb 1995 | St. Veit/St. Johann/Pongau/Salzburg | rbt | + | + | + | - | + | - | VHSV | AY524111 |
| 17/95 | Feb 1995 | Innermanzing/Neulengbach/Lower Austria | rbt | + | + | + | + | - | - | VHSV | AY524113 |
| 80/95 | May 1995 | Graz/Graz/Styria | rbt | + | + | + | - | - | - | | AY524126 |
| 152/97 | Sept 1997 | Rainbach/Schärding/Upper Austria | rbt | + | + | + | + | + | - | VHSV | AY524112 |
| 174/97 | Oct 1997 | Hofkirchen/Rohrbach/Upper Austria | rbt | + | + | + | - | - | + | | AY524115 |
| 4/98 | Jan 1998 | Trofaiach/Leoben/Styria | rbt | + | + | + | + | + | - | | AY524122 |
| 65/98 | May 1998 | Liebenfels/St. Veit/Glan/Carinthia | rbt | + | - | + | - | - | - | VHSV | AY524125 |
| 106/98 | June 1998 | Graz/Graz/Styria | rbt | - | - | - | - | - | - | | AY524109 |
| 173/98 | Oct 1998 | Hollenstein/Waidhofen/Ybbs/Lower Austria | grl | - | - | + | + | + | - | VHSV | AY524114 |
| 4/99 | Jan 1999 | Bad Eisenkappel/Klagenfurt/Carinthia | rbt | - | - | + | - | + | - | VHSV | AY524123 |
| 19/02 | Feb 2002 | St. Peter/Braunau/Upper Austria | rbt | + | + | + | - | - | - | VHSV | AY524116 |
| 252/03 | Dec 2003 | Mühldorf/Krems/Lower Austria | rbt | - | + | + | - | - | - | | AY524118 |
| 10/03 | Jan 2003 | Trient/Trentino/South Tyrol/Italy | rbt | - | - | - | - | - | - | IPNV | AY524128 |

^a rbt, rainbow trout; grl, grayling.

^b GA, gill anemia; VA, visceral anemia; CD, catarrh digestive tract; VP, visceral petechiae; MP, muscular petechiae; SM, splenomegaly.

asymptomatic case (10/03) a double infection with IPNV was demonstrated. Due to these coinfections it was not possible to unequivocally attribute the observed clinical symptoms to IHNV infection, but the symptoms may also have resulted from VHSV infection or may have been a consequence of infections with both viruses. As can be seen in Table 1, catarrh of the digestive tract, gill anemia, and visceral anemia are clinical features that were present in most of the investigated IHNV-positive fish independent of VHSV coinfections. In Fig. 1 three representative IHNV-positive rainbow trout are shown: trout 3 exhibits splenomegaly, while the spleen of trout 2 is of normal size; trout 2 shows one of the most frequently observed clinical signs seen in IHN cases, i.e., catarrh of the digestive tract. On the other hand, as demonstrated in trout 1, muscular petechiae were rarely observed in pure IHN cases, whereas they were more frequently associated with VHSV infections. The only pure IHN outbreak, in which the trout exhibited muscular petechiae (no. 4/98), actually proved to be a very severe outbreak with a high mortality rate. The mortality rates were known for approximately half of the outbreaks (not shown), and two general tendencies could be observed: (i) the mortality rates were somewhat higher in the earlier years (especially 1994 and 1995) than in more recent years, and (ii) the mortality rate was not increased in cases of IHNV-VHSV coinfections. Asymptomatic infections were also recorded (e.g., no. 10/03).

IHNV IFAT on EPC cells and VHSV ELISA. During the 10-year study period at the Clinic of Fish Diseases, samples from 18 rainbow trout farms from different areas in Austria and from one grayling facility proved positive for IHNV by IFAT (Fig. 2). In nine cases from rainbow trout farms, as well as in the case of the graylings, a double infection with VHSV was detected by ELISA.

RT-PCR results. All of the above-mentioned IHNV and VHSV infections were confirmed by RT-PCR. All of these 19

IHNV-positive samples were included in the phylogenetic investigations described here. From an additional 85 salmonid fish pools investigated at the Institute of Clinical Virology between 2001 and 2007 only by routine RT-PCR before importation to Austria, one German and three Italian samples tested positive for IHNV during the period from 2001 to 2003, while during the period from 2004 to 2007 all samples were negative for IHNV nucleic acid. One Italian sample from 2003 exhibited an IHNV-IPNV double infection. This sample was also included in the phylogenetic analysis.

All designed primer pairs exhibited highest sensitivities when used in monospecific RT-PCR assays. The IHNV primers were able to detect IHNV nucleic acid up to a dilution of 10^{-11} , the IPNV primers identified IPNV nucleic acid up to a dilution of 10^{-9} , and the VHSV primers showed a detection limit of 10^{-10} .

Nucleotide and amino acid alignments and phylogenetic analysis. Sequence analysis of a 615-bp nucleotide stretch comprising 38% of the G gene was performed on 20 field isolates (19 Austrian and 1 Italian), 2 reference strains investigated in the present study, and on a further 45 IHNV strains available in the GenBank database, a total of 67 sequences.

Phylogenetic analysis of the partial G gene indicated clearly three major genetic groups of IHNV present in salmonid fish: the North American/European (M), the North American/Asian (U+L), and the small Japanese JRt genogroup (Fig. 3).

The North American/European IHNV group M splits into one North American subgroup (M-USA) and two different European subgroups denoted M-Eur1 and M-Eur2.

The North American subgroup M-USA contains mainly IHNV strains from Idaho and single isolates from Washington and Japan.

The North American/Asian genogroup U+L comprised different North American IHNV strains mainly from California, Washington, and Oregon. The oldest North American strain,

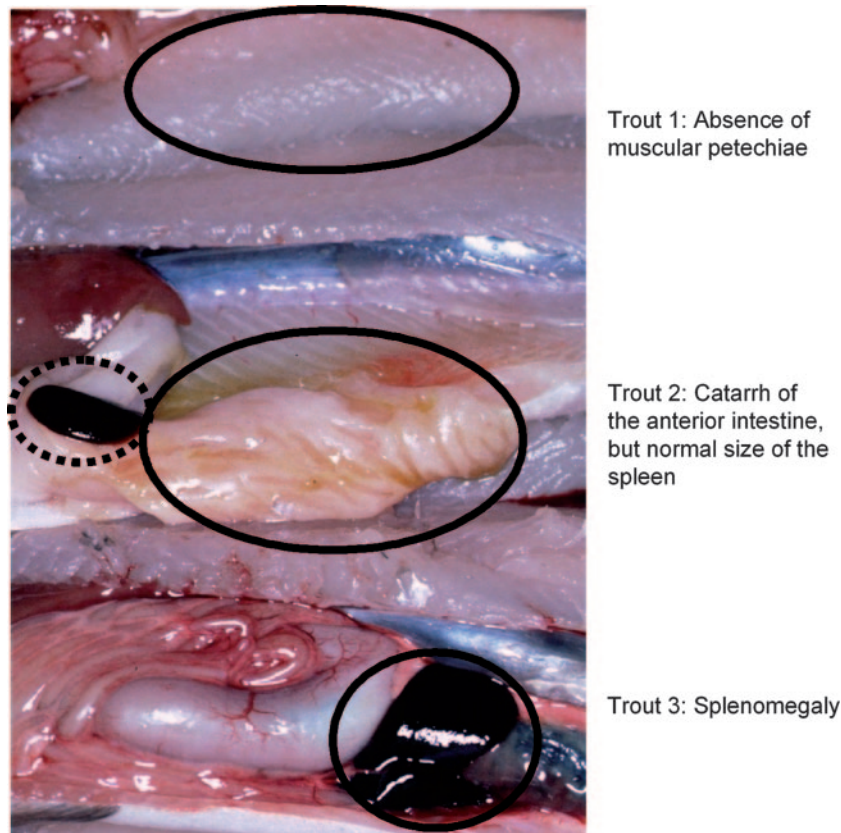


FIG. 1. Examples of clinical signs observed in IHNV-infected rainbow trout.

SRCV, isolated in 1966 in California, falls into this genogroup, too. This group also contains five Japanese IHNV strains, most of which were isolated before 1980.

The JRt genogroup consists of only five IHNV strains isolated in Japan in or after 1980.

The major clustering was well supported with bootstrap values of 894 (M/U+L) and 857 (JRt), respectively. The divisions into two separate European subclusters M-Eur1 and M-Eur2 received bootstrap support values of 594 and 179, respectively.

All 67 sequences compared in the present study revealed a maximum nucleotide and amino acid diversity of 4.40 and 5.88%, respectively. Nucleotide sequences within the major

genogroups exhibited identities of not less than 97%. When comparing amino acid sequences the above-mentioned genetic clusters were evident as well. The IHNV isolates and strains in the M genogroup differed at a maximum of 6 of 204 amino acids (97% identity rate), whereas the North American/Asian isolates and strains were different at a maximum of 8 locations (96% identity rate). The five Japanese isolates of the JRt genogroup demonstrated a maximum of 13 amino acid differences (94% identity rate) (Table 2).

Of the 14 Austrian isolates within the first M-Eur1 subgroup, 12 proved to be 100% identical to each other independent of year (mainly from 1994 and 1995) and area (five different Austrian federal states) of isolation.

The second European M-Eur2 subgroup, consisting of five Austrian, three German, and two Italian IHN viruses, is closely related to the first European subgroup, and three Austrian and one Italian IHN viruses within this subgroup exhibited the same nucleotide sequence. Most of the IHN viruses in this subgroup were isolated between 1997 and 2003 (Fig. 3).

Finally, our analysis revealed that all Austrian IHN viruses showed only six different nucleotide sequences. With the notable exception of the unique 65/98 isolate in the M-Eur1 group, the maximal nucleotide diversity between the Austrian isolates was only 1.67% (6 to 10 mismatches out of 615 nucleotides). On the amino acid level the diversity was comparably low (1,96%). The divergences of the above-mentioned Austrian IHN virus 65/98 (GenBank accession no. AY524125) were 2.44 and 3.92% nucleotide and amino acid sequences

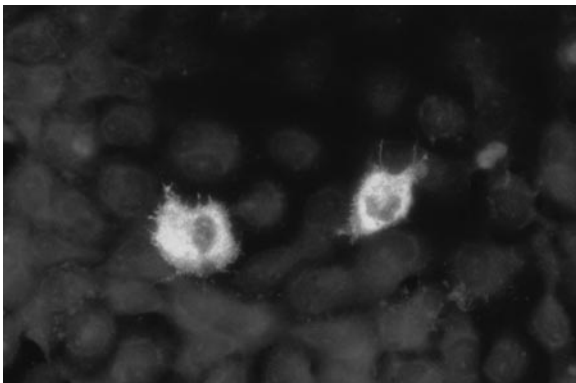


FIG. 2. IHNV immunofluorescence pattern in EPC cells.

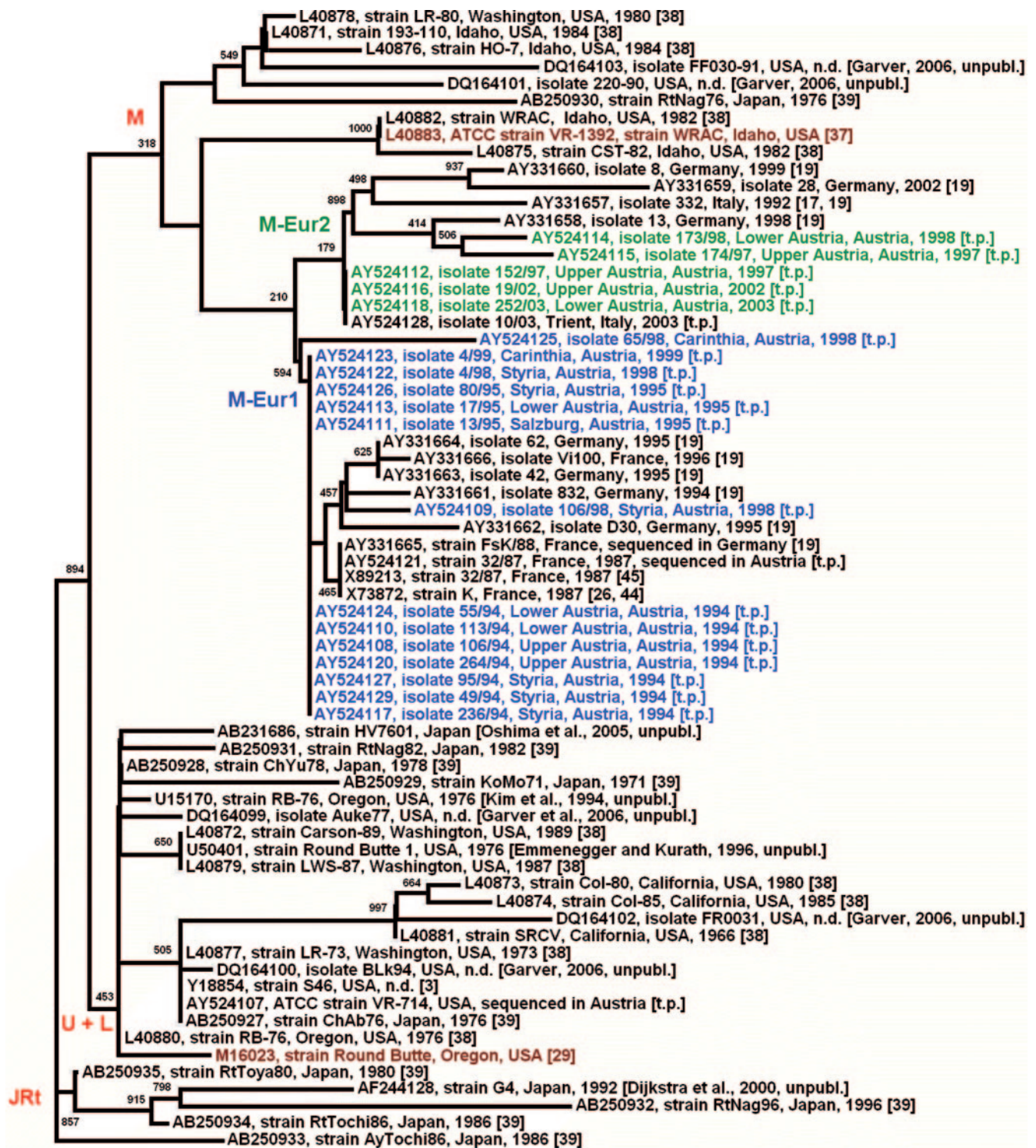


FIG. 3. Phylogenetic tree of 615-bp long nucleic acid sequences within the G gene (corresponding to nucleotide positions 3446 to 4061 of the reference strain [GenBank accession no. X89213]) (42) of 19 IHNVs from naturally infected salmonid fish from Austria, one IHNV from Italy, two IHNV reference strains from the United States and from France investigated in the present study, as well as 45 previously published IHNV sequences, mainly from the United States, Germany, and Japan. Analyses were performed by using the PHYLIP phylogeny program (version 3.57c package). The tree was rooted with the five Japanese (JRt) sequences. Bootstrap values are shown on the nodes of the clades that they are supporting. The IHNV strains used for vaccine development are indicated in dark red. The indicated years are the years in which the strains were isolated; if marked “n.d.” these data were not available. Abbreviations: t.p., this paper; unpubl., unpublished. The phylogenetic tree demonstrates three major groups: M, U+L, and JRt. European isolates cluster only within the M group and form two genetic subgroups: M-Eur1 and M-Eur2. Austrian isolates cluster within both European subgroups (in the M-Eur1 subgroup [indicated in blue] and in the M-Eur2 subgroup [indicated in green]). The M-Eur2 subgroup comprises more recent isolates indicative of a genetic shift toward the M-Eur2 subgroup since the late 1990s.

TABLE 2. Nucleotide and amino acid sequence identities within the geno- and subgroups based on multiway nucleotide and amino acid pairwise alignments

| Geno- or subgroup | Sequence identities (%) | |
|-------------------------|-------------------------|-------------|
| | Nucleotides | Amino acids |
| JRt | 97–99 | 96–98 |
| U+L | 97–99 | 97–100 |
| M | 97–100 | 97–100 |
| M-Eur | 97–100 | 97–100 |
| M-Eur1, without 65/98 | 99–100 | 99–100 |
| M-Eur1, including 65/98 | 98–100 | 97–100 |
| M-Eur2 | 97–99 | 98–100 |

respectively, compared to the other Austrian IHN viruses. The nucleotide sequence of this isolate was unique and different from all other Austrian IHNV isolates and strains. It showed unique nucleotides at positions 3754 (G instead of A), 3772 to 3774 (GGG instead of AAA), 3815 (T instead of A), and 3864 (T instead of G), which corresponded to amino acid changes of M to V (position 250), K to G (position 256), E to V (position 270), and K to N (position 286) within the G gene. Nucleotide and amino acid positions refer to the IHNV sequence deposited under GenBank accession no. X89213 (44). The same unique nucleotides at positions 3772 to 3774 and a consequential amino acid change at position 256 were found in the five Japanese strains of the JRt genogroup and in two strains from the United States (GenBank accession numbers DQ164101 and DQ164103) within the M-USA subgroup.

DISCUSSION

Since phylogenetic research on IHNVs from Austrian fish populations in relation to international isolates had not yet been described we investigated IHNVs obtained from specimens of local rainbow trout and graylings collected during the period from 1993 to 2007. Austrian IHNV sequences were compared to international sequence data available at GenBank.

The Austrian IHN viruses are located together with other European IHNV strains and isolates within both European subgroups, named M-Eur1 and M-Eur2, of the major genogroup M (Fig. 3).

North American IHNV isolates and strains show higher divergence rates than the European viruses. Within those strains, area- and (rarely) time-dependent nucleotide sequence homologies were observed. Previously, three genogroups within the North American IHN viruses correlating with their geographic origin were identified: genogroup U (from Alaska, the British Columbia coastal watershed, and the Columbia River), genogroup M (from the Columbia River and Idaho), and genogroup L (from California and the southern Oregon coast) (34). In our phylogenetic tree both genogroups L and U were condensed to one major North American/Asian group (Fig. 3). The reason for this is probably the limited number of investigated non-European sequences. In contrast to the study of Kurath et al. (34), which included 323 sequences, our phylogenetic tree incorporated only 23 representatives of American IHNV strains and isolates. Moreover, the outcome of a phylogenetic analysis is also dependent on the length of the analyzed sequences. While in the majority of North American

studies the mid-G region of the G gene (303 bp) was investigated, we analyzed a nucleotide sequence stretch from the same region that was, however, twice as long (615 bp). This was already acknowledged in other investigations (18, 19). While in a previous study German IHNV strains were clearly segregated from North American strains (18), a subsequent analysis of full-length G gene sequences indicated that nine German IHNV isolates were most closely related to North American isolates of genogroup M (19). A recent phylogenetic study of nine Japanese IHNV isolates obtained from 1971 to 1996 and of IHNV isolates from North America and Europe revealed five major clusters including the three known genogroups (U, M, and L) for North American isolates, one genogroup for European isolates, and one genogroup for Japanese isolates. Interestingly, five Japanese IHN viruses isolated mainly in the 1970s appeared in the cluster of the American genogroup U, while the remaining Japanese IHNVs isolated in the 1980s and 1990s formed a new, specific Japanese genogroup named JRt (39). The clustering of the early Japanese IHNVs together with American IHNVs is well in accordance with the reported introduction of IHNV to Japan in 1971 via a shipment of contaminated fish eggs from Alaska (39, 43), and subsequent independent evolution may have led to a separate Japanese IHNV lineage since the 1980s.

The phylogenetic analysis of the partial G gene of IHNV presented here, which includes for the first time a significant number of Austrian IHNVs, supports results previously obtained by Enzmann et al. (19) and Nishizawa et al. (39). These findings emphasize the genetic relatedness of Austrian IHNVs to the other European members of this virus species (Fig. 3). With only a few exceptions, time-dependent division of European IHNV strains and isolates similar to Japanese IHNVs could be observed. Moreover, the Austrian IHNV isolates exhibited very short genetic distances to each other: only six different nucleotide sequences were detected in 19 Austrian IHNVs; one Austrian IHNV isolate, however, was exceptionally unique and showed four unique nucleotide exchanges which led to four unique amino acid changes. Interestingly, one of these unique amino acid changes is also found in the five Japanese strains of the JRt genogroup and in two strains from the United States (GenBank accession numbers DQ164101 and DQ164103) within the M-USA subgroup. This observation points toward worldwide independently occurring evolution of IHNVs in nature, which may more reflect the results of similar local environmental or other pressures rather than a stable evolutionary configuration. The few genetic differences identified in the isolates 173/98, 174/97, 65/98, and 106/98 could not be linked to certain clinical symptoms, a higher or lower mortality rate, or VHSV coinfections. Also, the genetic differences responsible for subclustering of the Austrian isolates in M-Eur1 and M-Eur2, respectively, did not result in clinical differences.

Our sequence analysis demonstrated a maximum nucleotide diversity of 4.40%, which confirms the suggestion that the IHNV genome has undergone only a few variations or recombinations. Previous sequence comparisons carried out by Garver et al. (22) revealed only 30 different sequence types with a maximum nucleotide diversity of 7.3% among 120 virus isolates from infected fish from the Columbia River. The max-

imum genetic diversity found throughout the geographic range of IHNV was 8.6% (34). A later study by Enzmann et al. showed that the European isolates varied from each other in the G gene by only 0 to 2.3% (19), whereas the maximum nucleotide diversity among the Japanese isolates was 4.5% (39).

Thus, the average mutation frequency of IHNV in nature is consistently lower than that of other RNA viruses (16) but is similar to the one of Borna disease virus, another virus within the order *Mononegavirales* (30, 40).

A possible explanation for the highly conserved genome may lie in the acute nature of IHNV infection. Most of the viral samples analyzed were collected at the endpoint of acute-phase infections; therefore, the quasispecies of the virus population could be characterized as homogenous. It is possible that IHNV populations at chronic stages of infection may be more diverse (16). On the other hand, strong, thus-far-unknown constraints on cellular level or infection cycle could be another explanation for genome conservation.

IHNV is enzootic to western North America, and inadvertent transport of the virus with contaminated eggs and infected fish has resulted in the spread of the virus to several parts of the world. Phylogenetically, the European IHNV population is closely related to viruses of the North American genogroup M, and although the ancestor of the European IHNVs could not be identified, it most probably originated from a North American source in kind of a M genogroup ancestor (19). A new formation of two clusters of European IHN viruses within the M group has been described; the existence and differentiation of these two European IHNV clusters may be explained by the different geographic origin of the viruses: M-Eur1 comprises isolates that originally stem from France, whereas M-Eur2 includes more isolates of Italian origin (19). In 1987 the first European outbreaks of IHN were described in France and Italy (5, 9). IHNV was probably introduced to Europe only once and evolved independently in France and Italy since then (19). This notion is supported by our investigations of Austrian IHNV isolates, because the Austrian viruses do not differ remarkably from the other European IHN viruses described thus far and cluster within both the M-Eur1 and M-Eur2 subgroups of the M genogroup, which confirms the apparently monophyletic nature of European IHNVs (Fig. 3). Viruses of both European subgroups M-Eur1 and M-Eur2 were coexisting in the late 1990s in Austria. There was an interesting time-dependent shift in the late 1990s that separated Austrian and other European isolates of the mid-1990s, which are mainly represented in the subgroup M-Eur1, from those of the late 1990s and the new millennium, which are mainly represented in subgroup M-Eur2. This could indicate that an event may have taken place in the late 1990s that put a higher evolutionary pressure on European IHNVs to the advantage of viruses of the M-Eur2 group. It is difficult, however, to identify a possible reason for such an event. The epidemiological pattern of fish farming is very complex. There is a process of rapid distribution of fish and eggs between different fish farms. There is some evidence that at that time fishes infected with VHSV and IHNV were imported from Italy to Carinthia (Austria). Already in 1992 a virus of the M-Eur2 group was isolated in Germany after the importation of rainbow trout from Italy (19). Furthermore, it is known that fish eggs from Canada and

France were imported into Germany and from there to Austria and then distributed to several fish farms. In recent years, hygienic management and control have improved rapidly, in part due to the implementation of European Union (EU) legislation, especially in the larger fish farms. Most of the investigated fish originated from such large farms. In contrast, the owners of smaller fish farms are still rather hesitant to investigate their fish. This makes it difficult to carry out comprehensive epidemiological analyses. The question remains unanswered as to whether evolutionary pressure led to the observed shift from the M-Eur1 toward the M-Eur2 subgroup or whether a pattern of preferred importation of fish and fish eggs from Italy led to this current dominance of IHNV isolates of the M-Eur2 subgroup in Austria and Europe.

The evolution and further epidemiological analysis of European IHNVs may also be important for the establishment of new European vaccines against IHNV. The first reported IHNV DNA vaccine contained the glycoprotein (G) gene of the American Round Butte strain (strain RB) of IHNV (4). Three years later Corbeil et al. (12) constructed a modified version of this vaccine by cloning the G gene from the second American reference strain of IHNV designated the Western Regional Aquaculture Consortium strain (WRAC), which originated from rainbow trout from Idaho. Previous investigations have already shown that the glycoprotein of one specific IHNV isolate was able to induce protective immunity in fish against various strains of the virus (35). The DNA vaccine developed by Corbeil et al. proved to protect fish against a broad range of viral strains from different geographic locations, including isolates from the United States, France, and Japan (13), and it has been suggested that this vaccine could be used worldwide. Therefore, in subsequent studies the WRAC strain, also referred to as isolate 039-82 (ATCC VR-1392), was used again for vaccine constructs (23, 24).

Our phylogenetic investigations demonstrated that the most recent German and Austrian IHNV isolates moved molecularly more and more away from previously isolated North American IHNV strains which are used as vaccine strains (divergence of ca. 5% at both the nucleotide and the amino acid sequence levels). Therefore, we suggest either the inclusion of one of the new European IHNV strains in the development of a modern IHNV vaccine for worldwide use or that at least the older North American IHNV vaccine strains (RB1 from Oregon from 1976, WRAC from Idaho from 1982) also be checked to determine whether they provide effective protection against the currently circulating European IHNVs.

Finally, the investigations presented here showed that the IHNVs that are circulating in the Austrian fish populations are closely related to German, Italian, and French IHNVs. The spread of the agent between neighboring countries is often associated with the importation of infected eggs or fish. Also, within Austria, trade and commerce of fish without appropriate investigations led to the spread of the infection. More and more (larger) fish farms receive and maintain an EU certification which includes a specific pathogen-free status. Fish and eggs moved and introduced to new areas should originate from such EU-certified farms or should be tested before exportation. The monospecific RT-PCR assays described here proved to be specific, highly sensitive, time-saving, and reliable diag-

nostic tools, which could be used for the export and import investigations suggested above.

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