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GENOTYPING OF ISOLATED VIRUSES FROM RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) IN CROATIA

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Summary

Infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV) are important pathogens in rainbow trout aquaculture. Detection of these viruses in Croatia initiated investigation of their genetic relatedness to the worldwide IHNV and IPNV isolates. For this purpose, determination of nucleotide sequences of G and NV genes for IHNV and VP2/NS region for IPNV was performed. Phylogenetic analyses revealed that Croatian IHNV isolate was clustering within European clade most closely related to the North American M genogroup. Croatian IPNV isolate appeared in the cluster of genogroup III, together with French, English, Danish and Norwegian isolates. These results are important for further epidemiological studies of IHNV and IPNV outbreaks in Croatia.

Key words: infectious hematopoietic necrosis virus, infectious pancreatic necrosis virus, genotype, phylogenetic relatedness

INTRODUCTION

Good fish health management is essential for profitable farming. Outbreaks of viral diseases represent specific issue in fish health management due to the high mortality rate of infected fish and lack of their adequate treatment. Infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic

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necrosis virus (IPNV) are among causing agents of diseases whose survey in Croatia is regulated by »Decree on the measures of animal health protection against infectious and parasite diseases« issued yearly by Ministry of Agriculture, Forestry and Water Management (Oraić and Zrnčić, 2005). There is currently no commercially available vaccine against IHNV (Kurath et al., 2006), and vaccines based on killed virus or recombinantly produced viral peptides for IPNV do not show complete effectiveness (Allnutt et al., 2007). Movement of contaminated fish, fry and eggs in rapidly growing fisheries trade, gives rise to the worldwide spreading of IHNV and IPNV. To explain routes and mechanisms of spreading, epidemiology and evolution of these viruses, genotyping based on the nucleotide sequence analysis have to be applied.

IHNV, a member of the family *Rhabdoviridae*, genus *Novirhabdovirus*, causes infectious hematopoietic necrosis, disease which can induce over 90% of fish mortality (Bootland and Leong, 2003). The virus was first identified in western North America in 1969 (Wolf, 1988), while in Europe it was first detected in Italy and France (Baudin–Laurencin, 1987; Bovo et al., 1987). The IHNV genome is a linear, single–stranded, non–segmented (–) RNA, encoding six proteins: N (nucleocapsid protein), P (phosphoprotein), M (matrix protein), G (glycoprotein), NV (non–virion protein) and L (polymerase) (Kurath and Leong, 1985; Morzunov et al., 1995). Partial or whole nucleotide sequences of G and NV genes have been used for genotyping of worldwide IHNV isolates. Analyzing these two genes, Nichol et al. (1995) confirmed correlation between IHNV genotypes and their geographic origin. Subsequently, partial G gene sequence analysis of 323 IHNV North American isolates revealed 3 major IHNV genogroups designated U, M and L for the upper, middle and lower portions of IHNV geographical range in North America (Garver et al., 2003; Kurath et al., 2003). Enzmann et al. (2005) showed that all investigated European IHNV isolates formed one clade most closely related to the M genogroup. Recent studies (Nishizawa et al., 2006; Kim et al., 2007) indicated that several Japanese and Korean IHNV isolates constitute new JRt (Japanese Rainbow trout) genogroup.

IPNV, a member of the family *Birnaviridae*, genus *Aquabirnavirus*, causes infectious pancreatic necrosis and can produce high mortality in infected fish (Roberts and Pearson, 2005), although it is commonly associated with apparently healthy asymptotic carriers (Reno, 2003). This virus has a broad range of fish hosts and is probably outspread in all major trout–farming countries (Essbauer and Ahne, 2001). Infectious pancreatic necrosis was first described in North America in the 1950s and in France in 1965 (Wolf, 1988). IPNV genome is a bisegmented, double–stranded RNA (Dobos, 1995). Larger segment A encodes capsid proteins VP2 and VP3, non–structural protease NS and anti–apoptic protein VP5. Segment/B encodes VP1 polymerase. Problem of determining genotypes of IPNV is closely related to other

aquabirnaviruses isolates and they are usually together included in phylogenetic studies. For aquabirnaviruses genotyping, nucleotide analysis of partial or whole genome segment A, as well as segment B was performed (Hsu et al., 1995; Blake et al., 2001; Cutrín et al., 2004; Romero–Brey et al., 2004; Zhang and Suzuki, 2004). Nishizawa et al. (2005) ap plied VP2/NS joining region based genotyping approach and identified 7 different genogroups of aquabirnaviruses.

In 2005 and 2006, IHNV and IPNV were detected at two different salmonid farms in Croatia (Vardić et al., 2006, 2007). The aim of this study was to perform genotyping of these viruses in order to learn more about their sources and genotypes. To accomplish this task, phylogenetic approach was applied using nucleotide sequences of G and NV genes for IHNV isolate and VP2/NS joining region for IPNV isolate.

MATERIAL AND METHODS

Rainbow trout fry infected by IHNV (average weight = 25.58 g) and IPNV (average weight = 2.16 g) were randomly caught by netting from the two different Croatian fish farms (Vardić et al., 2006, 2007). IHNV infected trouts were imported from a Slovenian fish farm with previous IHNV history, while complete rearing process of IPNV infected fry was done in Croatia (data from farm owners). Pooled tissue homogenates (spleen, heart, kidney and brain) were used for virus isolation on EPC cells (epithelioma papulosum cyprini) (Fijan et al., 1983) and their subsequent confirmation by RT–PCR (Barlič–Maganja et al., 2002).

Total RNA from infected samples was extracted using TRI reagent (MRC) and applied in a single–step RT–PCR (Promega) for amplification of the gene regions of interest: IHNV G gene (1527 bp), IHNV NV gene (337 bp) and IPNV VP2/NS region (310 bp). Conditions and compositions of reactions were described previously (Vardić et al., 2007), as well as specific primers (Enzmann et al., 2005; Nishizawa et al., 2005). PCR products were purified by a QIAquick gel extraction kit (Qiagen) and sequenced by »ABI PRISMR 3100 Avant Genetic Analyzer« (DNA Service, Ruđer Bošković Institute). Sequencing was repeated for the sequence confirmation. The European Bioinformatics Institute (EMBL–EBI) WU–Blast2 web server was used to identify similar sequences. Thereafter, sequences were compared by ClustalW (Thompson et al., 1997) to find out degree of homology. Phylogenetic analyses were performed using PAUP* 4.0 beta version (Swofford, 2002). Parsimony (heuristic search with tree–bisection–reconnection (TBR) branch swapping option) unrooted trees were constructed. The significance of the branching order was assessed by bootstrap resampling of 1000 replicates. Accession numbers of nucleotide sequences for IHNV and IPNV worldwide isolates available at GenBank were cited in previous reports (Enzmann et

al., 2005; Nishizawa et al., 2005, 2006; Kim et al., 2007). G and NV gene sequences of Croatian IHNV strain, as well as the sequence of VP2/NS junction region of Croatian IPNV were stored at the GenBank under accession numbers: EU219616, EU219617 and EU219618.

RESULTS AND DISCUSSION

Nucleotide variance of whole G gene between Croatian (Cro/05) and other worldwide IHNV isolates available at GenBank was in range of 1.5% to 5.8%. Cro/05 showed the largest similarity (98.4%–98.5%) to the European isolates, for which Enzmann et al. (2005) proposed origin in the French type IHNV. Strain Fs832, isolated in the southern Germany and Vi100, imported in Germany from France had the smallest rate of divergence to Cro/05 (1.5%). First French isolate (F1), strain K and strains Fs30/95, Fs42/95 and Fs62/95 from Germany varied 1.6% from Cro/05. This is in agreement with previous observation based on the short Mid–G region (303 bp), that also confirmed 99. 7% similarity between Croatian and French isolates (Vardić et al., 2007). These results suggest introduction of IHNV in Croatia from French IHNV isolate source through Slovenia, because infected trout fry were imported from Slovenia. European isolates were generally more similar to Cro/05 (1.5% to 2.9% difference) than North American isolates (2.7% to 4.0% difference) and Asian isolates (2.7% to 5.8% difference). South Korean IHNV isolate RtUi02 was the most divergent (94.2% identity) from Cro/05.

Phylogenetic analysis based on the whole G gene sequences of Cro/05 and representative European, North American and Asian isolates revealed that genetic clustering of IHNV isolates correlates with their geographic origin (Fig. 1), as it was described previously (Nichol et al., 1995; Kurath et al., 2003; Enzmann et al., 2005; Nishizawa et al., 2006; Kim et al., 2007).

Cro/05 belongs to the European IHNV cluster, most closely related to the North American M genogroup. As IHNV in North America pre–dates its discovery in Europe, it is most likely that European IHNV isolates originated from North American M–genotype ancestor from Columbia River Basin with Hagerman Valley (Enzmann et al., 2005; Garver et al., 2003). In European IHNV cluster there are two subgroups: M–Eur1, which probably originated from French IHNV isolates, and M–Eur2, which may have source in Italian IHNV type (Enzmann et al., 2005). Our phylogenetic analysis showed that Cro/05 isolate belongs to M–Eur1 (French) subgroup (Fig. 1).

Nucleotide variance of NV gene between Cro/05 and available European and North American isolates was from 2.0% (German strain Fs832/94, M–Eur1 French subgroup) to 4.1% (WRAC, North American isolate, M genogroup). However, phylogenetic analysis could not confirm clustering of U, M and L genogroups, and was no informative for European IHNV isolates ancestor (Fig. 2), as it was shown by Enzmann et al. (2005).

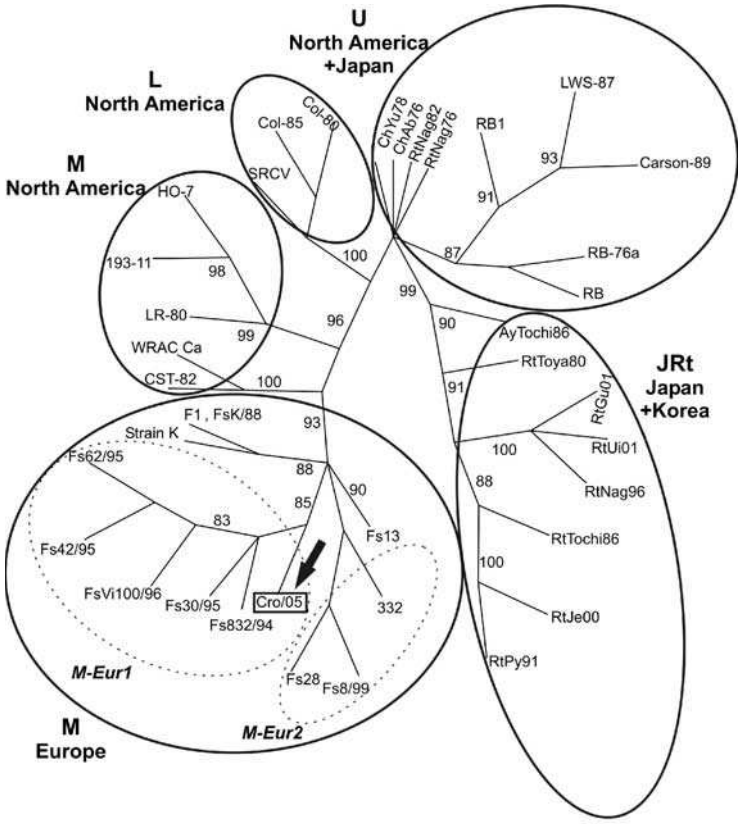

Figure 1. Unrooted phylogenetic tree constructed from the whole G gene sequences of European, North American and Asian IHNV isolates using maximum parsimony method. Croatian isolate (Cro/05) is within M–Eur1 subgroup

Slika 1. Nezakorijenjeno filogenetsko stablo izrađeno na temelju sljedova nukleotida cijelog gena G europskih, sjevernoameričkih i azijskih izolata virusa ZHN primjenom metode »maximum parsimony«. Hrvatski je izolat (Cro/05) unutar podgrupe M–Eur–1.

For genotype identification of Croatian IPNV isolate, we determined nucleotide sequence of VP2/NS region, which was successfully applied for Japanese aquabirnaviruses genotyping by N i s h i z a w a et al. (2005). Nucleotide sequence of VP2/NS region of CroIPNV isolate was the most similar (99.7% identity) to French and English isolates (Fr21, d'Honninethum and OV2) from trout and oyster. Japanese isolate Tomakomai, originated from rainbow trout, was the most divergent (71.3%) from CroIPNV (Fig. 3).

European aquabirnavirus isolates were generally the most similar to CroIPNV (0.3% to 23.2% of divergence). VP2/NS sequence similarity of

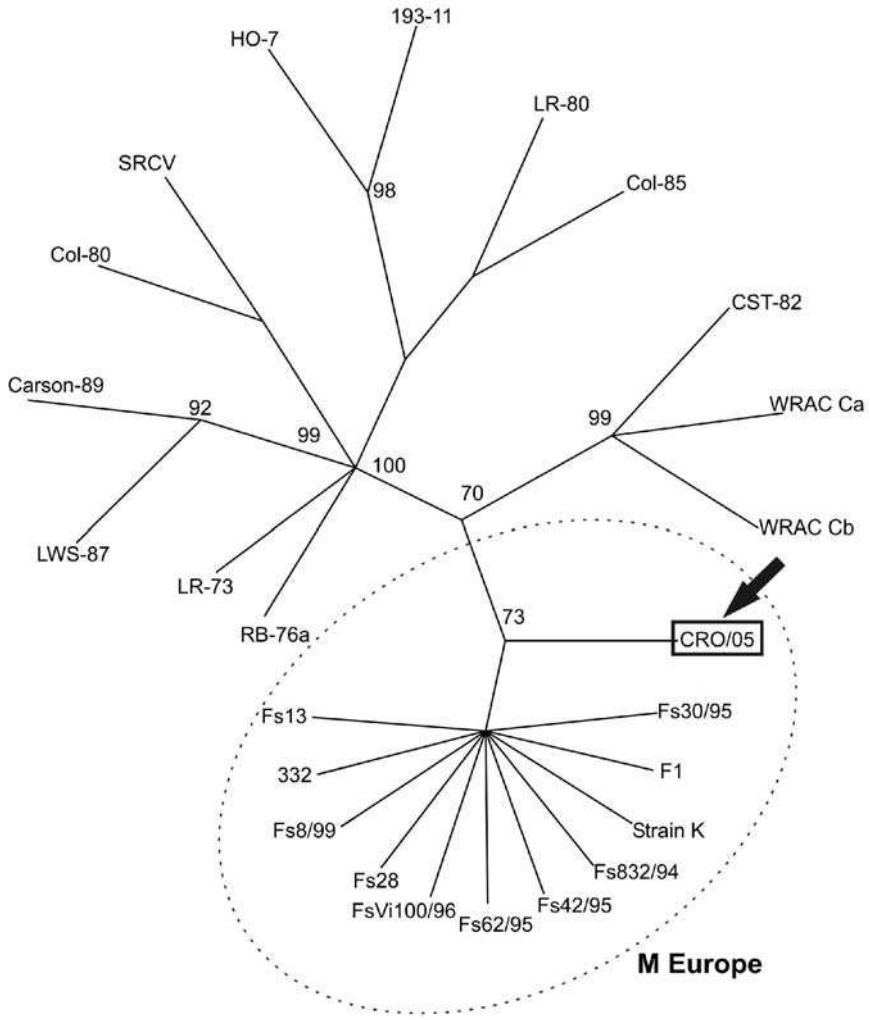

Figure 2. Unrooted phylogenetic tree constructed from the NV gene sequence of European and North American IHNV isolates using maximum parsimony method. Croatian IHNV isolate is in the box, marked with arrow
Slika 2. Nezakorijenjeno filogenetsko stablo izrađeno na temelju sljedova nukleotida gena NV europskih i sjevernoameričkih izolata virusa ZHN primjenom metode »maximum parsimony«. Hrvatski je izolat (Cro/05) označen strelicom.

CroIPNV and North American aquabirnavirus isolates ranged between 72.3% and 75.5%, while for Canadian isolates similarity was between 71.9% and 86.0%. The largest variation was observed in relation to Japanese aquabirnavirus isolates (71.3% to 74.8% identity).

Figure 3. Comparison of VP2/NS joining region sequences between Croatian — CRO, French — Fr21 (most similar) and Japanese — Tomakomai (most divergent) isolates
Slika 3. Usporedba sljedova nukleotida vezne regije VP2/NS hrvatskog — CRO, francuskog — Fr21 (najsličnijeg) i japanskog — Tomakomai (najrazličitijeg) izolata

Phylogenetic analysis based on the nucleotide sequence of VP2/NS region revealed that CroIPNV belongs to genogroup III, together with several European isolates (Fig. 4).

However, there is more complex correlation pattern between aquabirnavirus genotypes and their geographic origin. IPNV and other aquabirnaviruses included in phylogenetic analysis present various group of isolates from different aquatic organisms. It seems that these viruses had a global distribution prior to the widespread dissemination of infected fish between countries (R e n o, 2003; N i s h i z a w a et al., 2005). Therefore, although the rate of VP2/NS nucleotide similarity between CroIPNV and European isolates (Fr21, OV2) was high (99.7%), for deduction of possible IPNVCro epidemiological source, larger parts of virus genome as well as its serotype have to be analyzed.

In conclusion, this work describes genotyping of Croatian IHNV and IPNV isolates. We confirmed that both viruses share their origin with other European isolates, as they were clustering together in phylogenetic analyses (Fig. 1 and Fig. 4). Based on the nucleotide sequencing of the whole G gene

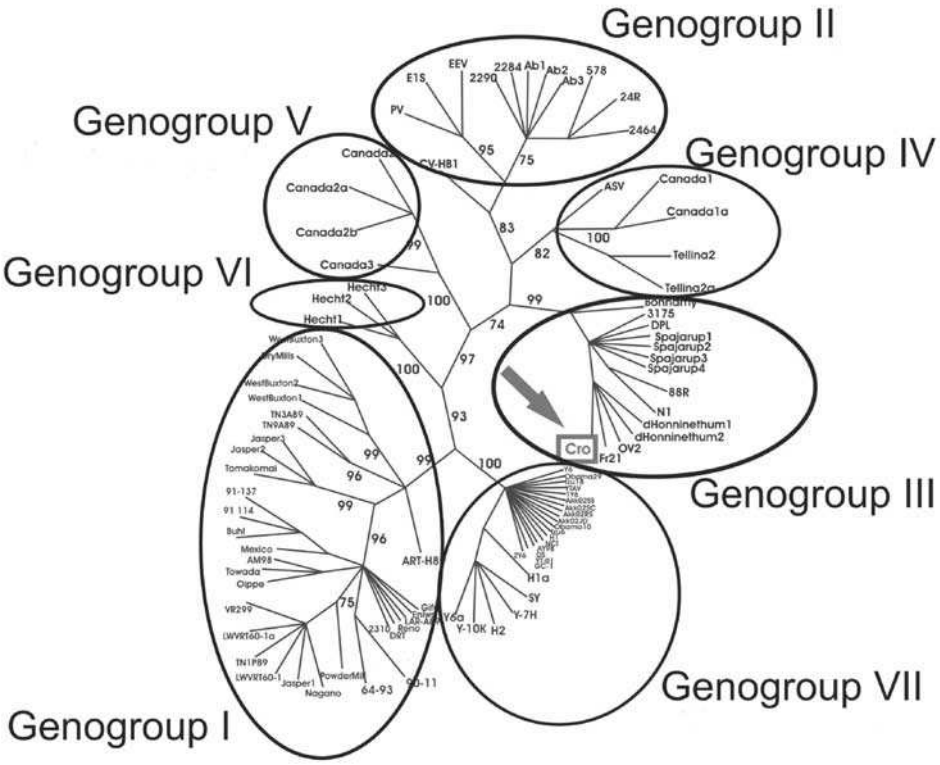

Figure 4. Unrooted phylogenetic tree constructed from VP2/NS region sequences of European, North American and Asian IPNV isolates using maximum parsimony method. Croatian isolate (Cro) is in the box, marked with arrow
Slika 4. Nezakorijenjeno filogenetsko stablo izrađeno na temelju sljedova nukleotida regije VP2/NS europskih, sjevernoameričkih i azijskih izolata virusa ZNG primjenom metode »maximum parsimony«. Hrvatski je izolat (Cro) označen strelicom.

(1527 bp) we could make hypothesis that Croatian IHNV isolate originated from the French type of IHNV. French isolates may present the origin for the several German IHNV isolates (E n z m a n n et al., 2005), and »French« IHNV genotype is outspread through the European countries mainly by infected fish, fry and eggs trade.

Determination of genetic types of fish viruses can help us to learn more about their evolution and complex circulation in the environment.

Sažetak

GENOTIPIZACIJA VIRUSA IZOLIRANIH IZ KALIFORNIJSKIH PASTRVA (*ONCORHYNCHUS MYKISS*) U HRVATSKOJ

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Virusi zarazne hematopoezne nekroze (ZHN) i zarazne nekroze gušterače (ZNG) važni su patogeni u uzgoju kalifornijskih pastrva. Nalaz tih virusa u Hrvatskoj potaknuo nas je na istraživanje suodnosa hrvatskih izolata virusa ZHN i ZNG sa svjetskim izolatima. U tu svrhu određeni su nukleotidni sljedovi gena G i NV virusa ZHN, te vezne regije VP2/NS virusa ZNG. Filogenetske su analize pokazale da se hrvatski ZHN izolat grupirao u europsku skupinu ZHN izolata, srodnih sjevernoameričkoj genogrupi M. Hrvatski ZNG izolat pripadao je genogrupi III, zajedno s francuskim, engleskim, danskim i norveškim izolatima akvabirnavirusa. Rezultati su značajni za daljnja epidemiološka istraživanja virusa ZHN i ZNG u Hrvatskoj.

Ključne riječi: virus zarazne hematopoezne nekroze, virus zarazne nekroze gušterače, genotip, filogenetski suodnos

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