

PARTIAL GENOME ANALYSIS OF SIBERIAN STURGEON ALLOHERPESVIRUS SUGGESTS ITS CLOSE RELATION TO ACIHV-2 – SHORT COMMUNICATION

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Partial genome sequence of a herpes-like virus, isolated from Siberian sturgeon (*Acipenser baeri*), was determined and subjected to phylogenetic analysis. The virus (SbSHV) has been shown to be the causative agent of an acute disease with high mortality in farmed juvenile sturgeons in Russia. Two fragments (of 7000 and 300 base pairs in length) encompassing 3 complete and 3 partial ORFs were amplified by PCR. Sturgeon herpesvirus strains classified into species *Acipenserid herpesvirus 2* (AciHV-2), have been isolated and partially sequenced from several regions (California, Idaho, Oregon and Canada) of North America from white (*A. transmontanus*) and shortnose sturgeons (*A. brevirostrum*). The sequence of the SbSHV strain shared highest identity with that of the Canadian strain originating from shortnose sturgeon. The phylogenetic analysis also confirmed that SbSHV is closely related to AciHV-2 and could also be classified into this virus species. This is the first report on the occurrence of AciHV-2 in Europe. Previously, only another virus species, AciHV-1 has been detected in farmed white sturgeons in Italy. The size and position of ORFs in the examined gene block confirmed that this genomic region is highly conserved in members of the genus *Ictalurivirus*.

Key words: Acipenserid herpesvirus, Siberian sturgeon, *Alloherpesviridae*, PCR

Herpes-like viruses have been described from more than 20 fish species to date (Waltzek et al., 2009). By now, genome sequence data are available from 13 herpesviruses (HVs) originating from *Anamnia*. Full genome sequences have

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been published from two ranid HVs (Davison et al., 2006), as well as from the channel catfish HV (IcHV-1) (Davison, 1992), koi HV (CyHV-3) (Aoki et al., 2007), and anguillid HV-1 (van Beurden et al., 2009).

The increasing availability of molecular data facilitated the introduction of radical changes in the taxonomy of HVs. Two new families and a new order (*Herpesvirales*) have been established recently (Davison et al., 2009). For the only known HV of invertebrate origin (oyster HV-1), the family *Malacoherpesviridae* has been created. For the HVs of fishes and amphibians (*Anamnia*), another new family, *Alloherpesviridae* has been established (Davison et al., 2009). Subdivision of the new families into subfamilies and genera is presently in progress. In the family *Alloherpesviridae*, four genera are officially approved (<http://www.ictvonline.org>). The genus *Ictalurivirus* contains HVs of both chondrosteian fish (AciHV-2) and bony fish (IcHV-1 and 2).

In a fish hatchery at Konakovo near Moscow, a serious disease outbreak has occurred causing mass mortality (close to 100%) among different sturgeon fingerlings recently. The most vulnerable species was the Siberian sturgeon (*Acipenser baeri*) (Shchelkunov et al., 2009). The pathological findings resembled those previously attributed to acipenserid herpesvirus type 2 (AciHV-2) infections in the United States (Watson et al., 1995). The causative agent has been isolated and found to be a herpes-like virus (Shchelkunov et al., 2009). In this study, partial genomic sequences of the Siberian sturgeon herpesvirus (SbSHV) were determined and compared to the corresponding sequences from other sturgeon and fish HVs.

From North American sturgeon species, including white (*A. transmontanus*) and shortnose sturgeon (*A. brevirostrum*), two serologically distinct HVs have been described previously (Watson et al., 1995). Partial sequences from the genes of the DNA-dependent DNA polymerase and the terminase have been determined and submitted to the GenBank from seven isolates (Kelley et al., 2005). In accordance with the results of serology, the existence of two acipenserid herpesvirus species, AciHV-1 and 2 (each comprising several isolates) has been confirmed (Kurobe et al., 2008). Based on the results of phylogenetic studies (Doszpoly et al., 2008), AciHV-2 has been proposed to be classified into the genus *Ictalurivirus* of the recently established family *Alloherpesviridae* (Davison et al., 2009). However, the genus classification of AciHV-1 is still pending. A longer genomic fragment (of 8000 base pairs) of AciHV-2 has also been sequenced recently, the analysis of which revealed a conserved gene block shared by the genome of HVs in the genus *Ictalurivirus* (Doszpoly et al., unpublished).

We chose to sequence the corresponding genome fragment of the SbSHV to facilitate direct comparisons. This is the first report on the molecular characterisation of a Eurasian sturgeon HV. The natural host of the virus, the Siberian sturgeon, normally does not have a contact with the North American sturgeon species, and, to the best of our knowledge, there has been no official direct trade in live sturgeons between North America and Russia within the last ten years.

Three virus strains of SbSHV (SK1/0406, SK2/0506 and BK/0506) were isolated and propagated on SSO-2 cell line that had been established from Siberian sturgeon pooled kidney, liver and spleen tissues (Shchelkunov et al., 2009). Using a commercial kit (Central Research Institute of Epidemiology, Moscow), the DNA was extracted from 100 or 200 µl cell culture supernatant of the 6th passage of strain SK1/0406, or of the 5th passage of the other two strains, respectively. The extracted DNA was spotted on Whatman filter paper and thus transported to the molecular biology laboratory where it was eluted with 250 µl nuclease-free water (after having been soaked at 4 °C for 3 h). Subsequently, the samples were concentrated to a volume of 50 µl in a vacuum centrifuge (Speed-Vac) and stored at –20 °C until use.

The molecular study was started with a PCR and sequencing approach. The primers used in this study are listed in Table 1. PCRs were carried out in 50-µl reaction volume. The reaction mixture consisted of 34 µl distilled water, 10 µl of 5× buffer (Phusion, Finnzymes), 0.5 µl thermostable DNA polymerase enzyme (Phusion, Finnzymes), 1 µl (50 µM) of each (forward and reverse) primer, 1.5 µl of dNTP solution of 10 mM concentration, and 2 µl target DNA. The reactions were performed in a T1 Thermocycler (Biometra). For PCRs with consensus degenerate primers, the following program was used: initial denaturing at 98 °C for 5 min, followed by 45 cycles of denaturing at 98 °C for 30 s, annealing at 46 °C for 30 s, and elongation at 72 °C for 60 s. The final elongation was performed at 72 °C for 3 min. For PCRs with specific primers, a similar cycling profile was used except for the annealing temperature (which was increased to 56 °C) and the elongation times (6 min during the cycles and 10 min in the final extension step). The PCR products were visualised by electrophoresis in 1% agarose gels. For DNA sequencing, the DNA bands were cut out from the gels, purified with the QIAquick Gel Extraction Kit (Qiagen) and sequenced directly with the inner primers. Larger amplification products were molecularly cloned by the use of the GeneJet kit (Fermentas). The positive plasmid clones were sequenced with pJETfo and pJETre primers (Fermentas) and by the primer walking method. The sequencing reactions were performed by the use of the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem). The electrophoresis was carried out in an ABI 3100 Automated Capillary DNA Sequencer. The sequences were analysed with the BioEdit program (Hall, 1999) and the readings were joined using the Staden program package (Staden, 1996). Multiple alignments were prepared with the ClustalW in the BioEdit program with default settings. Phylogenetic calculations were carried out with a 449-nucleotide-long fragment from the DNA polymerase gene. The maximum likelihood (PhyML) program, with HKY85 nucleotide (nt) substitution model (with 1000 bootstraps set), was used online at the Mobyli portal (<http://mobyli.pasteur.fr/cgi-bin/portal.py>) of the Pasteur Institute, Paris. When visualising the tree, we selected the CyHV-3 as outgroup.

Table 1

The primers used in the PCRs. Ambiguities are marked with the code recommended by IUPAC

Target	Primers
ORF57+58 (277 bp)	outer forward: 5'-AAG GSV CAG ATT YTC ATG-3' outer reverse: 5'-TCA CRW ASC CBC CYC TCC-3' inner forward: 5'-GAY ATG ATG TAY GTG TGY AA-3' inner reverse: 5'-ACM CCY CGK GTG TGY GCG GC-3'
ORF62 (258 bp)	forward: 5'-TTY CAR BTN GAR YTN ATG MGN GG -3' reverse: 5'-TG NGC YTG NAC NAC DAT NTC DAT -3'
ORF69 (231 bp)	forward: 5'-CCN GGN ATG ATG TGY CCN TG-3' reverse: 5'-IGN GTN CCR TTN GCR TAN GTN GG-3'
ORF57-62 (6607 bp)	outer forward: 5'-AAG ACG TGA CCT ACG ACA AGC TCG AT-3' outer reverse: 5'-TTG ACG GAC ACA TTG GAT TAA ACG AT-3' inner forward: 5'-CTT GAT TGA TTC TCA GCT GGT TGT GC-3' inner reverse: 5'-TTT AAA CAA GTC ACT TCC AAA TTG GT-3'

The sequences were deposited in the GenBank database (accession numbers EU883666–EU883668 and GU253908–GU253911). The nested PCR, targeting the DNA polymerase gene, resulted in a specific fragment of 277 base pairs (bp) in length from all the three strains. From the first exon of the terminase gene, which is open reading frame 62 (ORF) in the genome of ictalurid (Ic) HV-1, a 258-bp-long part was amplified also from all the three strains. The respective nt sequences of the strains were identical, therefore only one isolate (strain SK1/0406) was studied further. The nested PCR, designed to amplify a fragment between the genes of the DNA polymerase and terminase, resulted in a 6607-bp-long product. An additional PCR product of 231 bp was gained from the SK1/0406 strain from the second exon of the terminase gene (the homologue of ORF 69 of IcHV-1).

The 7048-bp-long genome contig of strain SK1/0406 (assembled from the nt sequences of the products of the first three PCRs, described above) contained two partial (the DNA polymerase and terminase) genes and three complete ORFs, the homologues of which are ORF59, 60 and 61 in IcHV-1 (Davison, 1992). ORF59 codes for a membrane protein, however, the function of the other two ORFs is as of yet unknown (Davison, 1992). Nonetheless, this gene block seems to be conserved in members of the genus *Ictalurivirus*, including IcHV-1 and 2, as well as AciHV-2. Indeed, the position, orientation and size of the ORFs, contained in the examined genome fragment of SbSHV, were comparable. The size of the predicted protein product of the three completely sequenced ORFs of SbSHV was 341, 393, and 322 amino acids (aa), respectively, whereas their counterparts in the Idaho strain of AciHV-2 are 358 (ORF59), 348 (ORF60) and 324 (ORF61) aa (Doszpoly et al., unpublished). The overall identity/similarity between the deduced aa sequences of the examined ORFs of SbSHV

and of the Idaho strain was 89/93%, respectively. The deduced aa sequences of the DNA polymerase and terminase gene fragments showed 100% identity with the Canadian strain (SSHV) of AciHV-2 originating from shortnose sturgeon (from which these are the only known sequences).

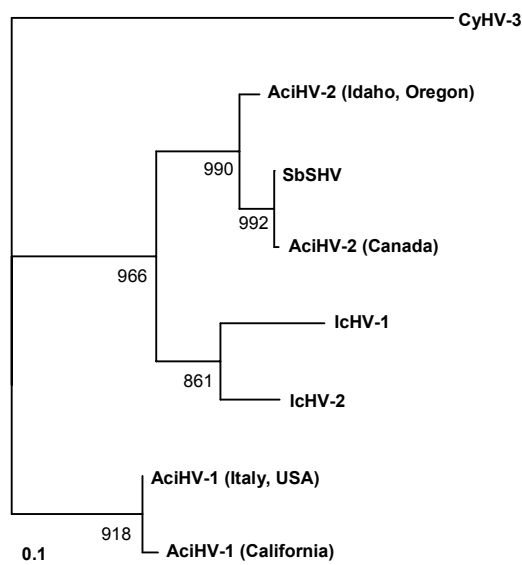


Fig. 1. Phylogenetic tree reconstruction based on maximum likelihood analysis of nt sequences of the DNA polymerase gene available from ictaluriviruses, CyHV-3 and AciHV-1. The analysis was performed on an alignment of 449 nt with HKY85 nt substitution model. High bootstrap values confirm the branching topology. Abbreviations: AciHV-1: acipenserid herpesvirus 1; AciHV-2: acipenserid herpesvirus 2; CyHV-3: cyprinid herpesvirus 3; IcHV-1: ictalurid herpesvirus 1; IcHV-2: ictalurid herpesvirus 2

The results of the phylogenetic calculations were in good agreement with the observations on the gene organisation. The phylogenetic tree reconstruction, presented in Fig. 1, illustrates the clear separation of species AciHV-1 from AciHV-2 and also from the other members of the genus *Ictalurivirus*. Previously, the distinctness of IcHV-1 and 2 on species level has been proven by serological tests (Hedrick et al., 2003), and also by sequence comparisons (Doszpoly et al., 2008). AciHV-2 is an additional species in this lineage. The nt sequences of the DNA polymerase gene of the California, Idaho and Oregon isolates (of AciHV-2) share 99–100% identity. The Canadian and Russian isolates seemed to be closely related to each other (99%). Their divergence from the other AciHV-2 isolates exceeded 1%; nonetheless, SbSHV most likely belongs to the species AciHV-2. For a final decision, the results of serological comparison would be essential.

A considerable number of fish HVs are yet unclassified, mainly because of the lack of appropriate sequence data. It is well known that many HVs might

cause severe diseases with high mortality in different piscine hosts. Due to the expansive and improperly controlled international trade in live fish and fertilised eggs, these diseases can spread rapidly between different regions of the world.

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