Molecular detection of a putatively novel cyprinid herpesvirus in Sichel (*Pelecus cultratus*) during a mass mortality event in Hungary

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Summary. In the early summer of 2014, mass mortality of Sichel (*Pelecus cultratus*) was observed in Lake Balaton, Hungary. Histological examination revealed degenerative changes within the tubular epithelium, mainly in the distal tubules and collecting ducts in the kidneys and multifocal vacuolisation in the brain stem and cerebellum. The routine molecular investigations showed the presence of the DNA of an unknown alloherpesvirus in some specimens. Subsequently, three genes were amplified and sequenced partially from the putative herpesviral genome (DNA polymerase, terminase, and helicase). Phylogenetic tree reconstruction, based on the concatenated sequence of these three conserved genes, implied that the virus undoubtedly belongs to the genus *Cyprinivirus* within the family *Alloherpesviridae*. The sequences of the Sichel herpesvirus differ markedly from those of the three known cypriniviruses (CyHV-1, CyHV-2 and CyHV-3); putatively representing the fourth virus species in the genus.

The family *Alloherpesviridae* includes the herpesviruses (HVs) detected or isolated from amphibian and fish species [4]. The family contains four genera, one of them the genus *Cyprinivirus* comprises the HVs of cyprinids (family Cyprinidae) and that of the European eel (*Anguilla anguilla*). The genus contains four species accepted by the International Committee on Taxonomy of Viruses (ICTV) [20]: *Cyprinid herpesvirus 1*, *Cyprinid herpesvirus 2*, *Cyprinid herpesvirus 3* and *Anguillid herpesvirus 1*. Interestingly, members of the genus *Cyprinivirus* can infect fish from two different superorders (Elopomorpha, Ostariophysi), although the HVs are considered to have narrow host range. *Cyprinid herpesvirus* 1 (CyHV-1) was first isolated from carp (*Cyprinus carpio*) in Japan [21]. *Cyprinid herpesvirus 2* (CyHV-2), originally described as goldfish haematopoietic necrosis virus, was isolated also in Japan from goldfish (*Carassius auratus*) [15]. *Cyprinid herpesvirus 3* (CyHV-3), is also known as koi herpesvirus, was described from common and koi carp [13]. The complete genome sequence of the above mentioned cypriniviruses and that of the *Anguillid herpesvirus 1* (AngHV-1) was determined [1, 5, 24].

During the early summer of 2014, mass mortality occurred among Sichel in Lake Balaton (Hungary), approximately 20.000 cadavers were collected by fishermen, other species were not affected. The present study was aimed at genetically characterizing a novel alloherpesvirus (AHV) detected in Sichel.

Cadavers of six Sichel were sent to our laboratory (64.5-149.7 g) for histopathological and molecular investigations. All cadavers were necropsied immediately after arrival with routine tissue collection from the main organ systems (gills, brain, liver, kidney, spleen, intestine and eyes) for histopathological examination. Tissues were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned at approximately 5 micrometer, mounted on glass slides, and stained with hematoxilin and eosin. The exterior mucus, gills and eyes were sampled for parasite examination through an optical microscope. For bacteria isolation, kidney tissue was inoculated onto blood agar plates.

For the molecular investigations organ samples were homogenized using the TissueLyser high-throughput disruption instrument (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. 200 µL of supernatants from organ homogenates were extracted using the Roche MagNA Pure LC automated system with a Total Nucleic Acid Isolation Kit (Roche Diagnostics, Indianapolis, IN) and eluted in 100 µL of elution buffer. Subsequently, the samples were tested for the presence of CyHV-3 with an expansively used PCR for the detection of cyprinid HVs [10].

After realizing that the samples contain a probably novel virus species, virus isolation was attempted on EPC (Epithelioma papulosum cyprini) and CCG (Common carp gill) cell lines [11, 18]. The pooled organ homogenates were diluted to a 10% (w/v) suspension in an MEM medium (Gibco) complemented with antibiotics (Penicillin 300 U/ml, Streptomycin 300 µg/ml). The suspension was centrifuged at 2000 × g for 10 min, and the supernatant was transferred into a new tube immediately. Three parallel inoculations (500 µl suspension per flask) were made in 25-cm^2 flasks of EPC and CCG monolayers at 80% confluency. The flasks were incubated at 20°C and 25°C and checked for the appearance of cytopathic effect (CPE) daily.

For amplifying longer regions from the DNA polymerase, terminase and helicase genes consensus primers were designed (Table 1) using the sequences of CyHV-1 and 2 and 3 and AngHV-1 (GenBank Acc. No.: NC_013668.3; NC_019491.1; NC_019495.1; NC_009127.1). The PCR mixtures for amplifying the above mentioned regions contained 34 µl distilled water, 10 µl Phusion® 5X HF buffer, 1.5 µl dNTP solution (10 mM), 1 µl of each primer (50 pM), 0.5 µl Phusion® High-Fidelity DNA polymerase enzyme (Thermo Scientific) and 2 µl target DNA. The PCR programs consisted of an initial step at 98 °C for 3 min, followed by 45 cycles with denaturation at 98 °C for 10 sec, annealing at 56 °C for 30 sec, and elongation at 72 °C for 1 min. The final extension was at 72 °C for 3 min. All PCR products were excised from agarose gel (1%), purified with the QIAquick Gel Extraction Kit (Qiagen), and sequenced directly with the forward and reverse primers. The sequencing reactions were performed with the use of the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The electrophoresis was carried out by a commercial service provider on an ABI PRISM 3100 Genetic Analyzer.

The quality of the sequence reads was analyzed using BioEdit [12] and Staden [23] program packages. Phylogenetic relations among alloherpesviruses (AHVs) were inferred from the analysis of 263 nt sequence alignment of the DNA polymerase gene (15 fish AHVs), and from the analysis of 835 aa sequence alignment consisting of the concatenated sequences of 3 conserved genes (DNA polymerase, terminase, helicase) from 6 AHVs. The nucleotide sequences of the DNA polymerase and the deduced amino acid sequences of the DNA polymerase, terminase and helicase genes were aligned using Mafft v6.935b [16]. Bayesian phylogenetic analyses were performed using MrBayes [14] within the TOPALi v2.5 program package and interface [19] with the following parameters: Markov chain was run for 10 million generations, four independent analyses were conducted, each with 1 cold and 3 heated

chains. Sampling occurred every 10 generations with the first 25% of Markov chain Monte Carlo samples discarded as burn-in. The WAG amino acid and the HKY nucleotide substitution models were found to be the best fit for the data using the TOPALI v2.5 program.

The cadavers of six Sichels found dead were in slightly emaciated condition. Macroscopically no external or internal lesions were observed except the red foci in the ventral part of the eyes (4 specimens). Microscopic lesions were more frequent in the kidneys. Degenerative changes within tubular epithelium mainly in the distal tubules and collecting ducts were observed. The dilatation of these tubules, and the exfoliation of tubular epithelial cells from the basement membrane were characteristic (Figure 1a). Caryomegalia, and necrotic changes of nucleus, picnosis, rhexis were frequent. In the brain samples congestion and multifocal vacuolisation in the brain stem and cerebellum (Figure 1b) were observed. No histological lesions occurred in the gills, liver, splen and eyes. Microscopic examination of fresh preparations from the gills revealed a minor infestation of *Ergasilus sp*. and Myxobolus spores, and in one case *Trichodina sp*. The external mucus proved to be negative for the presence of parasites, whilst in the eyes Myxobolus spores were observed. Bacterial isolation attempts gave negative results.

No CPE was observed during the virus isolation attempt. After 2 weeks a blind passage was carried out. There was not any CPE in this second passage, as well, and even the PCR for the detection of the viral DNA failed.

Of the 6 Sichel samples tested for the presence of cyprinid AHVs, the PCR [10] produced a 363 bp DNA fragment in two samples with identical nt sequences. Subsequently, the positive samples were applied for obtaining longer sequences from more genes. From the DNA polymerase, terminase and helicase genes, 1134, 1127 and 694 bp fragments were amplified, respectively (representing approximately the half of the terminase and the ¼ of the polymerase and helicase genes). The sequences were deposited to GenBank (Acc. Nos. KM357276-KM357278). The G+C content of the concatenated nucleotide sequences of the Sichel HV proved to be 60.07%. The Figure 2a shows phylogenetic relations within the family *Alloherpesviridae* (amphibian AHVs excluded); the Sichel HV undoubtedly clusters in the genus *Cyprinivirus*, as the sister species to *Cyprinid herpesvirus 2*. The phylogenetic tree reconstruction (based on 10x longer sequences) presented in Figure 2b, illustrates the clear separation of species Sichel HV from the other CyHVs. Additionally, the nucleotide comparison of the CyHV-1, -2, -3 and Sichel HV (2955 bp) was calculated (Table 2).

In this paper we provide the first molecular data from the genome of a novel AHV originating from Sichel. More than a decade passed since the last cyprinid HV had been

described [13]; moreover almost all known AHVs were at least reported by EM investigation before the year of 2000, and in some cases these results were confirmed by virus isolation and/or DNA sequence data, as well [2, 3, 25]. Since then, in the new millennium, further studies were carried out on the formerly reported viruses; lots of herpesviral sequences were deposited to the GenBank confirming that the viruses previously described as HVs are really belonging to the family *Alloherpesviridae* ([1, 7, 8, 24, 25]. And in some cases, already known AHVs were reported from novel hosts [6, 9, 17, 22]. In this work, we report a novel, yet unseen AHV.

Three partial gene fragments of the viral genome (DNA polymerase, terminase and helicase genes) were amplified, sequenced and analyzed suggesting that the Sichel HV is probably a new virus species in the genus *Cyprinivirus*. The phylogenetic tree (Figure 2a) contains all the known fish AHVs with available DNA polymerase sequences, even the two recently described variants of the CyHV-3 (strain 3468/10 and 12ITT-426) [10]. The Sichel HV seems to be a sister species of the CyHV-2 on this figure. It could be seen that the phylogenetic distances between the CyHV-3 variants are remarkably less than that of between the Sichel HV and CyHV-2, suggesting that the Sichel HV is a novel virus species and not only a variant of the CyHV-2, which seems to have a narrow host range, it was described from two very closely related fish species (goldfish and Prussian carp). The DNA polymerase gene of the CyHV-1 and CyHV-3 does not contain any intron; however that of the CyHV-2 is composed of five exons [5]. The amplified region of the Sichel HV also contains an intron on this 1 kb long fragment like CyHV-2. The second phylogenetic tree contains only the officially accepted members of the genus *Cyprinivirus*, and the Sichel HV with the AciHV-2 as an outgroup (Figure 2b). This calculation based on much longer sequences concatenated from three genes (unfortunately from the different CyHV-3 variants there are no sequence data from these genes). This calculation and the nucleotide comparison (Table 2) may support our theory that the Sichel HV is not a variant of any known cypriniviruses, but a novel virus species within the genus *Cyprinivirus*. However, more sequence data (full genes or rather the whole genome) needs to confirm that the Sichel HV is undoubtedly a novel AHV species, in this case it would be the fourth virus species (CyHV-4) in the genus *Cyprinivirus*.

As for the Sichel loss in Lake Balaton, toxicological examinations were not carried out, the bacterial investigations proved to be negative, the minor infestation of different parasites do not explain the massive mortality event. The presence of the novel herpesviral DNA was detected only in one-third of the examined specimens. The histopathological abnormalities found in the samples could be a result of simple degradation, not neccesseraly due to viral

effect. Taken together these facts, a direct connection between the presence of the herpesviral DNA and the mass mortality of Sichel could not be revealed. The causative agent of the outbreak remains unknown, further virological and toxicological studies would be needed for answering this question. As for the role of the novel HV acting in Sichel mortality, examining the pathogenicity of Sichel HV in experimental infections, the isolation of the virus would be essential.

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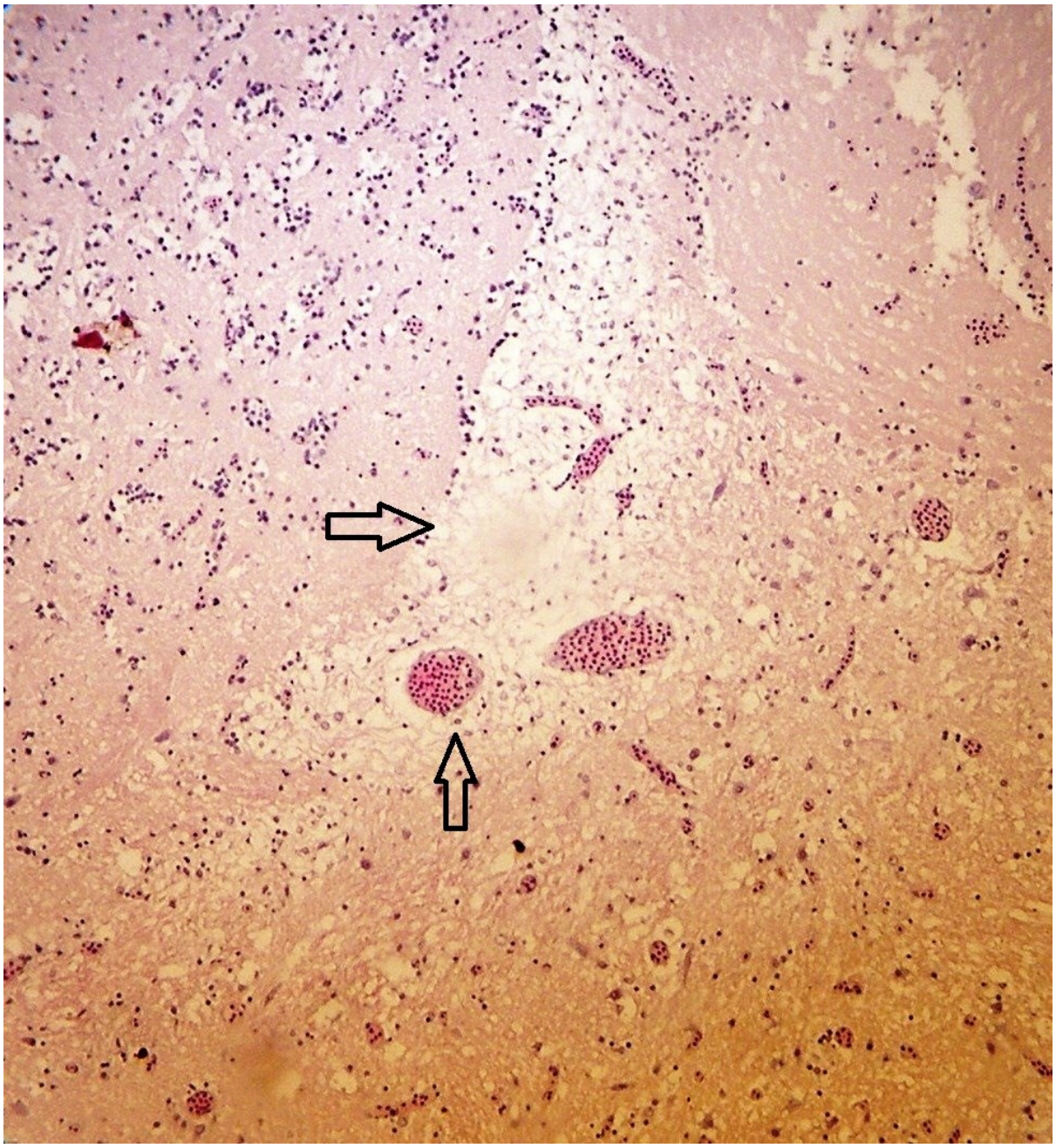
Figures and Legends

Figure 1a. Exfoliation of degenerated tubular epithelial cells from the basement membrane, karyomegalia (upward arrow) and necrotic changes of nucleus (downward arrow). Haematoxilin – eosin, 400X. **2a.** Congestion and focal vacuolization (arrows) in the cerebellum. Haematoxilin – eosin, 200X.



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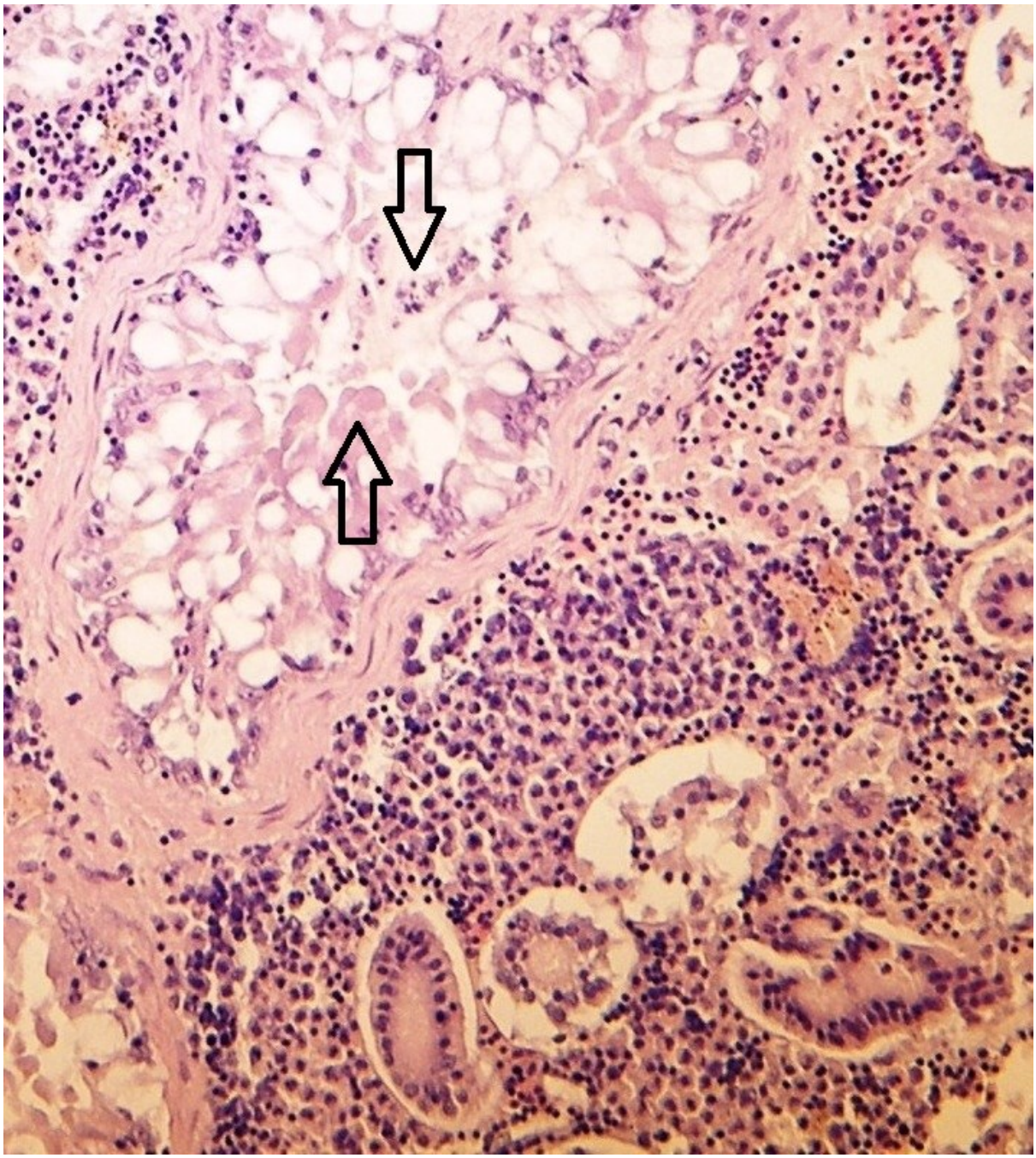
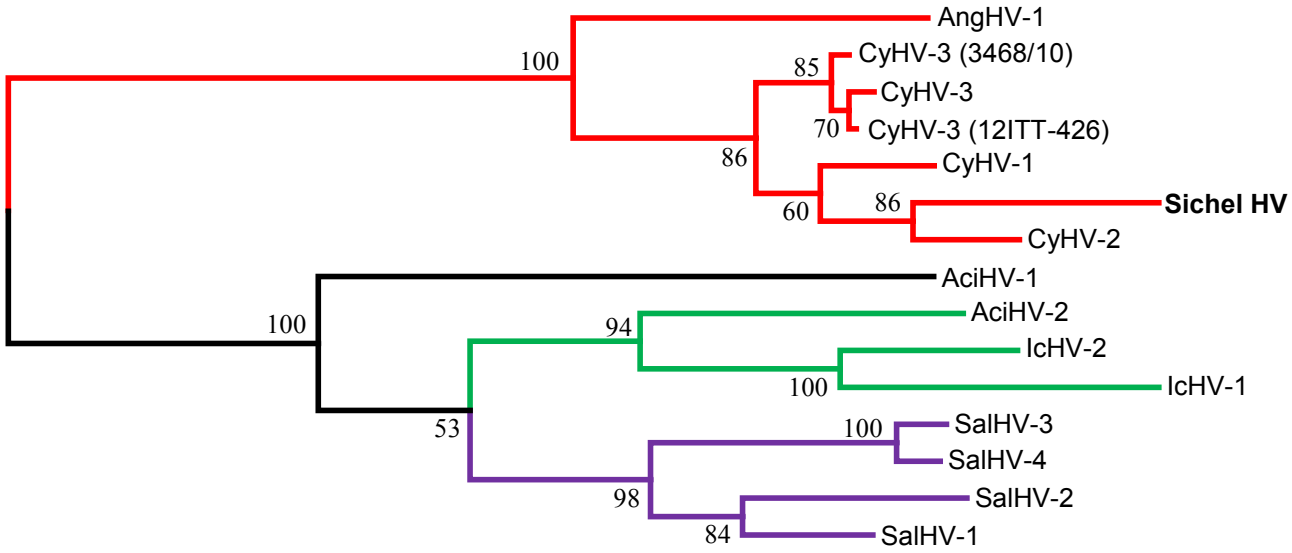


Figure 2a. Phylogenetic tree for the fish alloherpesviruses, the analysis was based on the Bayesian analysis (HKY nucleotide substitution model) of the partial DNA polymerase sequences (263 nt). High statistical values confirm the topology of the tree. The main lineages within the family (genera) are signed with different colored lines on the tree. **2a.** Phylogeny reconstruction for the genus *Cyprinivirus* inferred by Bayesian analysis (WAG amino acid model) using the concatenated amino acid sequences of DNA polymerase, terminase and helicase genes (835 amino acid characters). High statistical values confirm the branching topology. Abbreviations: AciHV: acipenserid herpesvirus; AngHV: anguillid herpesvirus; CyHV cyprinid herpesvirus; IcHV: ictalurid herpesvirus; SalHV: salmonid herpesvirus.



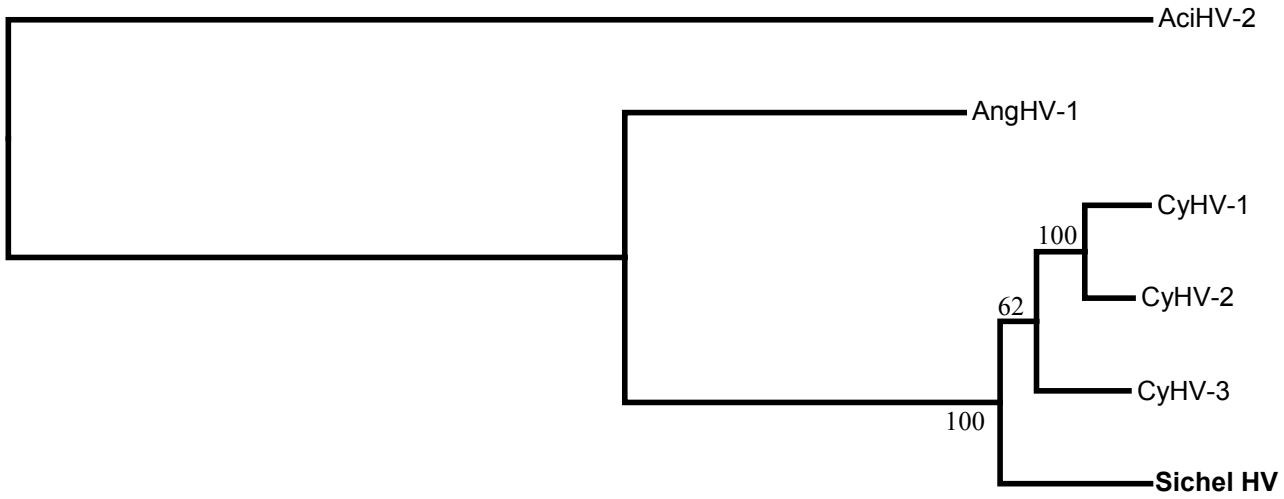


Table 1. Consensus primers used in the PCRs to amplify well-conserved regions of the DNA polymerase, terminase, and helicase genes

target	primers
DNA polymerase	forward: 5'- GGN GCN ATG GTN CAR WSN ACN AA -3' reverse: 5'- ACN GTN GCN GTR TTY TCR TAN GC -3'
terminase	forward: 5'- GCG CTG AGK ATG TCG TCY TTG -3' reverse: 5'- YGA CAT CTA CAA GCC CGA CCA -3'
helicase	forward: 5'- GTN GGN WSN GTN ACN CAR YT -3' reverse: 5'- CCY TGR CAR AAR TAN GTR TTC AT -3'

Table 2. Comparison of the nt sequences of the different CyHVs (DNA polymerase, helicase and terminase). The values in the body of the table are percentages of nucleotide similarities

	CyHV-1	CyHV-2	CyHV-3	Sichel HV
CyHV-1	-	73%	75%	72%
CyHV-2	73%	-	74%	73%
CyHV-3	75%	74%	-	74%
Sichel HV	72%	73%	74%	-