Field experience on the Atlantic salmon papillomatosis in Russia and first molecular characterization of the associated herpesvirus

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Abstract

Papillomatosis of Atlantic salmon (*Salmo salar*) has been reported for decades in Russia, Scandinavia and Scotland. The disease is typically benign although heavy losses have occasionally been reported. A herpesviral etiology has been suggested based on ultrastructural evidence; however, the virus has not been isolated or genetically characterized. In this study, we provide the first viral sequences detected in the papillomas from diseased Russian Atlantic salmon. Phylogenetic analyses, based on the partial sequences of the herpesviral polymerase and terminase genes, supported the virus as a novel member of the genus *Salmonivirus* within the family *Alloherpesviridae*. The sequences of the Atlantic salmon papillomatosis virus differ markedly from those of the three known salmoniviruses, therefore the authors propose the *Salmonid herpesvirus 4* species designation to be considered for approval by the International Committee on Taxonomy of Viruses.

Introduction

Atlantic salmon papillomatosis is a benign skin disease that has been reported since the 1950s in wild and farmed Atlantic salmon (*Salmo salar*) in Scandinavia, Scotland and in the northwestern part of Russia. Since 1971, Atlantic salmon papillomatosis has been known in fish hatcheries on the Russian Kola Peninsula (Wirén 1971, Chronwall 1976, Bylund et al. 1980, Wolf 1988, Shchelkunov et al. 1992). The disease mainly affects juveniles in fresh water and occasionally migrating adults returning to rivers to spawn (Vladimirskaya 1957, Carlisle & Roberts 1977). The disease begins slowly with focal hyperplasia and petechial hemorrhages of the skin progressing to large (5-15 mm) multifocal pale white papilloma-like lesions. Affected fish appear lethargic and may succumb to opportunistic microorganisms.

A viral agent, resembling a herpesvirus, has been observed within the proliferating epidermal cells of papillomatous tissues by electron microscopy (Wolf 1988, Shchelkunov et al. 1992). Large (110 nm) icosahedral nucleocapsids were seen in nuclei of the degenerating epithelial cells, while the numerous released enveloped virions were 200-250 nm in diameter. Attempts to isolate the virus from diseased fish using several fish cell lines yielded negative results (Shchelkunov et al. 1992). To date, no attempt has been made to genetically characterize the putative herpesvirus.

The herpesviruses of fish and amphibians have been classified into the family *Alloherpesviridae*, under the order *Herpesvirales* together with the herpesviruses of higher vertebrates (*Herpesviridae)* and mollusks (*Malacoherpesviridae*) (Davison et al. 2009). Presently, the family *Alloherpesviridae* contains four genera with 12 accepted virus species. The genus *Batrachovirus* contains the herpesviruses of amphibians, while the genera *Cyprinivirus* and *Ictalurivirus* comprise the herpesviruses of cyprinids, eel, catfish and sturgeons. The herpesviruses of salmonid fish are clustered into the fourth genus, the *Salmonivirus*. The genus contains three species accepted by the International Committee on Taxonomy of Viruses (ICTV) (Pellett et al. 2011): *Salmonid herpesvirus 1*, *Salmonid herpesvirus 2* and *Salmonid herpesvirus 3*. *Salmonid herpesvirus* 1 (SalHV-1) was first isolated from overtly healthy rainbow trout (*Oncorhynchus mykiss*) broodstock during spawning at a fish hatchery in Washington, USA. (Wolf & Taylor 1975). *Salmonid herpesvirus 2* (SalHV-2) was isolated in RTG-2 and CHSE-214 cell lines from masou salmon (*Oncorhynchus masou*), coho salmon (*Oncorhynchus kisutch*), sockeye salmon (*Oncorhynchus nerka*), and rainbow trout (Sano 1976, Kimura et al. 1981, Horiuchi et al. 1989, Suzuki 1993). *Salmonid herpesvirus 3* (SalHV-3) was described from lake trout (*Salvelinus namaycush*) (Bradley et al. 1989, McAllister & Herman 1989), but it has never

been isolated in cell culture. The other two salmoniviruses induce proliferative skin diseases causing either papillomas (SalHV-2) or epidermal hyperplasia (SalHV-3) in affected fish.

The present study was aimed at genetically characterizing a novel alloherpesvirus detected in Russian Atlantic salmon populations suffering from papillomatosis.

Material and methods

Field observations, sample collection, and DNA extraction. The observations reported in this study are derived from sampling hatcheries and natural waterways in the Kola Peninsula (Murmansk Province, Russia) over the last decade. Papilloma tissues were sampled from eighteen wild or cultured Atlantic salmon from July to September 2011 in the basins of the Kola and Tuloma rivers, which enter the Kola Bay of the Barents Sea (Table 1). Papilloma tissues were collected individually from anesthetized fish and preserved in Bouin's fixative (for histology) or in absolute ethanol (for molecular genetic study) until homogenization, which was carried out by mortar and pestle with a small amount of sterile sand. Subsequently, the samples were digested with proteinase K, treated with guanidine-hydrochloride and the DNA precipitated with ethanol (Dán et al. 2003). The extracted DNA was stored at -20°C until further examination. *Salmonid herpesvirus 3* DNA used in the study originated from infected lake trout (*Salvelinus namaycush*) skin tissues from the Bayfield hatchery in Wisconsin in 1998 (Kurobe et al. 2009).

Gross and Microscopic Pathology Samples were fixed in Bouin's fixative, embedded in paraffin, sectioned (4 - 5 μm), stained with hemotoxylin and eosin, and viewed by light microscopy according to standard procedures.

Molecular study. The molecular characterization was begun with nested PCRs using consensus primers targeting the partial DNA polymerase and terminase genes (homologous to ORF57 and ORF69 in the *Ictalurid herpesvirus 1* genome; RefSeq number: NC_001493) (Table 2). The primers for the polymerase and terminase genes were designed by aligning the nucleotide sequences of the salmoniviruses (SalHV-1, -2, and -3; GenBank accession numbers: EU349281, EU349273, FJ641908, FJ641909, EU349284, EU349277) using the BioEdit software package (Hall 1999). The design of primers to amplify the partial glycoprotein gene (homologous to ORF46 in the *Ictalurid herpesvirus 1* genome) sequences for SalHV-3 and -4 relied on a previously published alignment of SalHV-1 and -2 (Davison

1998). The 50 µl PCR cocktails consisted of 34 µl distilled water, 10 µl of 5× buffer (Phusion, Finnzymes), 0.5 µl thermo-stable DNA polymerase enzyme (Phusion, Finnzymes), 1 µl (50 µM) of each the forward and reverse primer, 1.5 µl of dNTP solution of 10 mM concentration, and 2 µl target DNA (in the second round 5 µl target DNA was applied from the first round). The reactions were performed in a T1 Thermocycler (Biometra). For PCRs targeting the DNA polymerase and terminase, the following program was used: initial denaturation at 98°C for 5 min, followed by 45 cycles of denaturation at 98°C for 30 s, annealing at 50°C for 30 s, and elongation at 72°C for 60 s. The final extension was performed at 72°C for 3 min. For the amplification of the partial glycoprotein gene an annealing temperature of 46°C and a 2 minute extension step was used.

The PCR products were visualized by electrophoresis in 1% agarose gel. For DNA sequencing, bands were gel purified with the QIAquick Gel Extraction Kit (Qiagen) and sequenced directly with the inner primers (polymerase and terminase). The larger amplification products (glycoprotein) were cloned into plasmids using the CloneJET PCR Cloning Kit (Fermentas), according to the protocol of the manufacturer. The plasmid containing the amplified target was sequenced with pJETfo and pJETre primers (Fermentas). Sequencing reactions were prepared with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and electrophoresis was carried out in an ABI 3100 Automated Capillary DNA Sequencer.

Phylogenetic analysis. The quality of the sequence reads was analyzed using BioEdit (Hall 1999) and Staden (Staden 1996) program packages. The deduced amino acid sequences of the polymerase and terminase genes were aligned using Mafft v6.935b (Katoh et al. 2005). The aligned polymerase and terminase genes were concatenated and the best fit amino acid model determined in TOPALI v2.5 program. A Bayesian phylogenetic analysis was performed using MrBayes (Huelsenback & Ronquist 2001) within the TOPALi v2.5 program package and interface (Milne et al. 2004) 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.

Results

Field observations. On the Kola Peninsula, Atlantic salmon papillomatosis has been observed every year over the last four decades, causing significant losses in hatchery reared or wild juvenile Atlantic salmon. Young fish originating from hatcheries located on the rivers of the Barents Sea basin were affected more often and more severely than those belonging to populations inhabiting the rivers of the White Sea basin.

For instance, at hatcheries, where fertilized salmon eggs from the Umba River and other rivers entering the White Sea were incubated, papillomatosis was not observed every year. The tumors first appeared in the largest 2-summer-old fish weighing 20g or more. The proliferative lesions began to degenerate in autumn and sloughed off before the next spring. However, the lesions reappeared in 3-summer-old fish by the time of their release into rivers in June - July. The prevalence of papillomatosis in these hatcheries typically did not exceed 2.0 %.

However, in hatcheries where eggs were taken from the salmon caught in the Kola River of the Barents Sea basin, severe Atlantic salmon papillomatosis epizootics occurred every year. The disease affected 3-summer-old fish in July just before their release into the rivers. From August - September 2006, a survey was performed for papillomatosis in fish inhabiting the rivers that enter the Kola Bay. The disease was found in all the investigated rivers starting from the Kola River and tributaries down to small rivers where hatchery-reared juveniles are not released. That year, the prevalence of papillomatosis in the Kola River was 4.0 % in August and increased to 17.0 % in September in 2-summer-old to 4-summer-old fish. The 1-summer-old juveniles displayed no signs of disease.

Overall, the onset of disease was usually first detected in young captive stock at water temperatures between 10 - 16°C. Degeneration and sloughing of the growths and death of the weakened fish usually took place at water temperatures below 10°C. Mortality among the affected fish in this period can reach 1.5 % per day.

Within the last few years, to prevent papillomatosis, Kola Peninsula Atlantic salmon hatcheries have shortened the rearing period to 1 year before fish are released into the rivers. Based on this, one may speculate that the young salmon become infected and develop clinical disease due to the stress of captivity (e.g. high stocking densities) and continued retention of the smoltifying fish in freshwater captivity.

Gross and Microscopic Pathology Affected individuals had single or multiple papillomas ranging in size from 5 to 15 mm in diameter on the dorsal aspect of the body or along the lateral line, caudal peduncle, and fins (Figures 1a). The largest number of papillomas was observed on the caudal peduncle and caudal fin. Individual papillomas often coalesced and became hemorrhagic. Some fish suffering from papillomas also displayed gross internal abnormalities including: splenomegaly, mottling of the liver, and hyperemia of the liver and posterior gut (data not shown).

Papillomatous outgrowths were characterized by an exceptional amount of epithelial hyperplasia and a loss of mucous cells (Figure 1b). Affected epithelial cells often displayed karyomegaly. The hyperplastic epidermis was nourished by interdigitating dermal pegs composed of proliferating connective tissue and associated vasculature. Other cutaneous abnormalities included: loss of an identifiable basement membrane, loss or deformation of scales within the dermis, and zones of epithelial necrosis.

Molecular study. Of the 18 samples tested, the first round PCR results produced a 397 bp DNA fragment in 11 samples using the DNA polymerase primers and a 425 bp fragment was produced in 5 samples using the terminase primers. The second round DNA polymerase and terminase gene targets generated 240 bp and 185 bp amplicons for each of the 18 samples, respectively. All polymerase gene sequences from all samples were identical, as were the terminase gene sequences. From the glycoprotein gene, a 1521 bp fragment was amplified from all 18 samples. Three of them were cloned and sequenced yielding identical sequences. For SalHV-3 sample, the same glycoprotein primer pair generated a 1518 bp amplicon that was cloned and sequenced. The nucleotide sequence identities of the glycoprotein genes of SalHV-3 and Atlantic salmon papillomatosis virus (ASPV) proved to be 77%. The sequences of the polymerase, terminase and glycoprotein genes of the ASPV, as well as that of the glycoprotein gene of SalHV-3 were deposited to GenBank (Acc. Nos. JX886026-JX886029). The G+C content of the concatenated nucleotide sequences (partial polymerase, terminase and glycoprotein genes) of the ASPV proved to be 50.38%, while that of SalHV-3 was 53.49%.

Phylogenetic analysis. The phylogenetic calculations were based on the concatenated deduced amino acid sequences of the DNA polymerase and terminase genes (142 total amino acid characters) from 15 alloherpesviruses. The WAG amino acid substitution model was found to be the best fit for the data using the TOPALI v2.5 program. The separation of four main groups (genera) was supported by the high posterior probabilities of the Bayesian

analysis. The analysis supported the classification of ASPV (labeled SalHV-4 in figure 2) as the sister species to *Salmonid herpesvirus 3* within the genus *Salmonivirus*.

Discussion

In this paper we have provided the first molecular data from the genome of ASPV. Three partial gene fragments (DNA polymerase, terminase and glycoprotein genes) supported the classification of the virus as a novel salmon alloherpesvirus. These data are consistent with previous ultrastructural evidence (Shchelkunov et al. 1992). Short sequences from the genomes of SalHV-1, -2 and -3 (Bernard & Mercier 1993, Davison 1998, Waltzek et al. 2009) revealed these viruses cluster together as the genus *Salmonivirus* within the family *Alloherpesviridae* (Waltzek et al. 2009). The phylogenetic analysis demonstrated that ASPV represents the newest member of the genus *Salmonivirus* as the sister species to SalHV-3.

The close genetic relationship of SalHV-3 and the ASPV is evident from the high nucleotide sequence identities of the conserved polymerase (92%) and terminase (94%) genes. These percentages are higher than that of the SalHV-1 and -2, which are sister species to each other (76% and 85% respectively). However, comparison of the SalHV-3 and ASPV partial glycoprotein gene nucleotide sequences revealed a greater genetic distance (77% identity) suggesting these viruses are distinct species (figure 3).

According to a hypothesis for adenoviruses (Wellehan et al. 2004), feline immunodeficiency virus, (Poss et al. 2006) and canine parvovirus (Shackelton et al. 2006), decreasing G+C content in a viral genome might reflect adaptation to a new host following a host jump. The partial sequences of both SalHV-3 and ASPV display balanced G+C content, suggesting they have co-evolved with their hosts over time as distinct viral species. Furthermore, Atlantic salmon have been shown to be refractory to SalHV-3 (Epizootic Epitheliotropic Disease Virus, EEDV) upon experimental challenge (Bradley et al. 1989; McAllister & Herman 1989). Finally, the diseases caused by the two agents are notably different as ASPV causes papillomas and EEDV results in hyperplastic lesions that appear as gray patches on the body and fins (McAllister & Herman 1989).

In this investigation we genetically characterized a novel alloherpesvirus from Russian Atlantic salmon suffering from papillomatosis. Given the sequences of the ASPV differ markedly from those of the three known salmoniviruses, the authors propose the *Salmonid herpesvirus 4* (SalHV-4) species designation to be considered for approval by the International Committee on Taxonomy of Viruses. Future studies are needed to verify

whether this novel alloherpesvirus is the same as those previously detected by electron microscopy in Atlantic salmon suffering from papillomatosis (Shchelkunov et al. 1992). Furthermore, isolation of the alloherpesvirus and subsequent controlled challenged studies will be required to elucidate the role the virus plays in oncogenesis.

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TABLES

Table. 1. Collected Atlantic salmon papilloma tissue samples, their origin and stage of the host sampled.

No.	Geographical location (river)	Data of collection (2011)	Stage
1	Pecha	August	parr, wild
2	Pak	July	parr, hatchery
3	Pak	July	parr, hatchery
4	Tuloma	July-August	adult, hatchery
5	Tuloma	July-August	adult, hatchery
6	Tuloma	July-August	adult, hatchery

7	Tuloma	July-August	adult, hatchery
8	Tuloma	July-August	adult, hatchery
10	Tuloma	July-August	adult, hatchery
11	Kulanga	September	parr, wild
12	Kulanga	September	parr, wild
13	Kulanga	September	parr, wild
14	Kulanga	September	parr, wild
15	Kulanga	September	parr, wild
16	Kulanga	September	parr, wild
17	Kola	August	parr, hatchery
18	Kola	August	parr, hatchery

363

364 **Table. 2.** The primers used in the PCRs.

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target	primers
DNA polymerase	outer forward: 5'- GCA ACA TGT GYG AYC TCA AYA T -3' outer reverse: 5'- AAK AGA CCR TGK KYM CCR AAT TG -3' inner forward: 5'- GAY TGG TCY GGW CTS GAG GG -3' inner reverse: 5'- CAT CAG KGA RCA DGT GTT GGG -3'
terminase	outer forward: 5'- TTT CAT MCT CGT CGA RAG GCY GCC -3' outer reverse: 5'- GGR TCR ATG GCR ATG TAR AAT CC -3' inner forward: 5'- ATG CTS GTC GCY GGB CGR AAG C -3' inner reverse: 5'- CAG RGC CTG HGT WGC VGG GTT C -3'
glycoprotein	forward: 5'-GGN CAN RCN TAY WSN TGY ATH ATG-3' reverse: 5'-TCN GTN GTN GGN ARR TAN GTR TT-3'

366

367 FIGURE LEGENDS

368

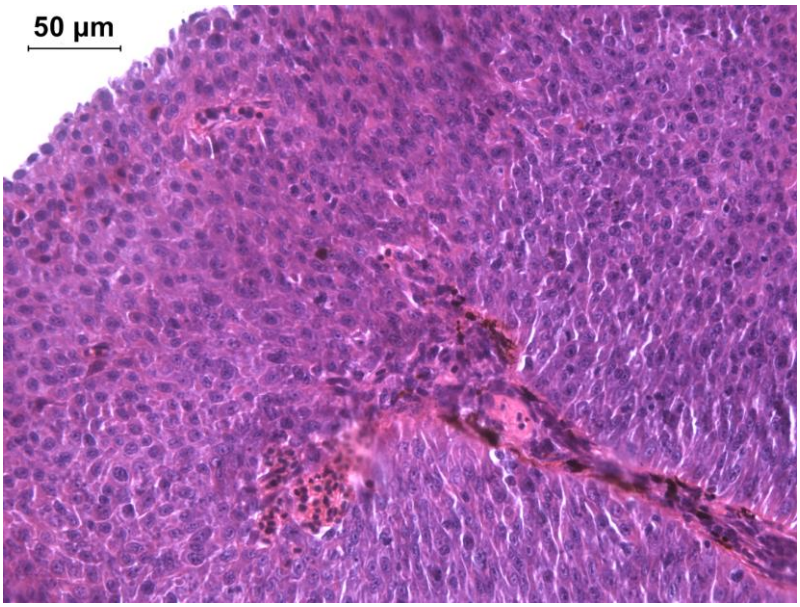

Figure. 1. (a) Papillomatosis in wild young Atlantic salmon captured at 10°C. Total length is 9.5-11.1 cm. (b) Histopathology of a mature papilloma from a wild Atlantic salmon parr reveals epithelial hyperplasia, disorganization, and a loss of mucous cells.

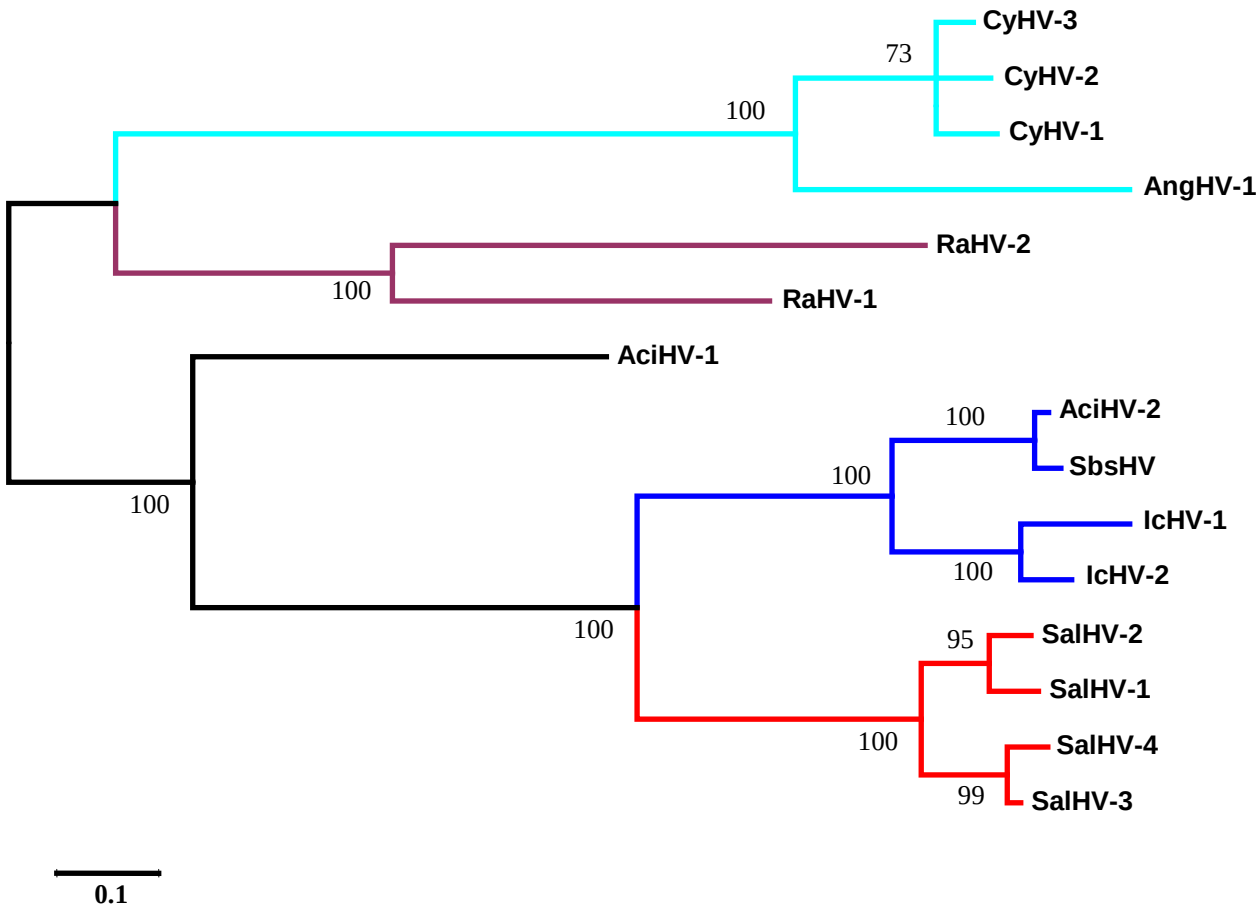

Figure. 2. Phylogenetic tree for the family *Alloherpesviridae*. The analysis was based on the Bayesian analysis (WAG amino acid model) of the concatenated amino acid sequences of DNA polymerase and terminase genes (142 amino acid characters). High statistical values confirm the topology of the tree. The four main lineages within the family (genera) are designated by different colored lines on the tree. The Atlantic salmon papillomatosis virus is marked as SalHV-4. Abbreviations: AciHV: acipenserid herpesvirus; AngHV: anguillid herpesvirus; CyHV cyprinid herpesvirus; IcHV: ictalurid herpesvirus; RaHV: ranid herpesvirus; SalHV: salmonid herpesvirus; SbsHV: Siberian sturgeon herpesvirus. GenBank and RefSeq accession numbers are: AciHV-1: EF685903, EF535573; AciHV-2: FJ815289; AngHV-1: NC_013668; CyHV-1: NC_019491; CyHV-2: NC_019495; CyHV-3: NC_009127; IcHV-1: NC_001493; IcHV-2: FJ827489, FJ815290; RaHV-1: NC_008211; RaHV-2: NC_008210; SalHV-1: EU349281, EU349273; SalHV-2: FJ641908, FJ641909; SalHV-3: EU349284, EU349277; SbSHV: GU253908, GU253910.

Figure. 3. Alignment of the nucleotide sequences of the glycoprotein gene sequences for SalHV-3 and ASPV.