

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

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15 Key words: Atlantic salmon papillomatosis, *Alloherpesviridae*, *Salmonivirus*, fish  
16 herpesvirus, PCR

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18 The GenBank accession numbers of the sequences reported in this paper are: JX886026-  
19 JX886029.

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35 **Abstract**

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

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69 **Introduction**

70 Atlantic salmon papillomatosis is a benign skin disease that has been reported since the  
71 1950s in wild and farmed Atlantic salmon (*Salmo salar*) in Scandinavia, Scotland and in the  
72 northwestern part of Russia. Since 1971, Atlantic salmon papillomatosis has been known in  
73 fish hatcheries on the Russian Kola Peninsula (Wirén 1971, Chronwall 1976, Bylund et al.  
74 1980, Wolf 1988, Shchelkunov et al. 1992). The disease mainly affects juveniles in fresh  
75 water and occasionally migrating adults returning to rivers to spawn (Vladimirskaya 1957,  
76 Carlisle & Roberts 1977). The disease begins slowly with focal hyperplasia and petechial  
77 hemorrhages of the skin progressing to large (5-15 mm) multifocal pale white papilloma-like  
78 lesions. Affected fish appear lethargic and may succumb to opportunistic microorganisms.

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

86 The herpesviruses of fish and amphibians have been classified into the family  
87 *Alloherpesviridae*, under the order *Herpesvirales* together with the herpesviruses of higher  
88 vertebrates (*Herpesviridae*) and mollusks (*Malacoherpesviridae*) (Davison et al. 2009).  
89 Presently, the family *Alloherpesviridae* contains four genera with 12 accepted virus species.  
90 The genus *Batrachovirus* contains the herpesviruses of amphibians, while the genera  
91 *Cyprinivirus* and *Ictalurivirus* comprise the herpesviruses of cyprinids, eel, catfish and  
92 sturgeons. The herpesviruses of salmonid fish are clustered into the fourth genus, the  
93 *Salmonivirus*. The genus contains three species accepted by the International Committee on  
94 Taxonomy of Viruses (ICTV) (Pellett et al. 2011): *Salmonid herpesvirus 1*, *Salmonid*  
95 *herpesvirus 2* and *Salmonid herpesvirus 3*. *Salmonid herpesvirus 1* (SalHV-1) was first  
96 isolated from overtly healthy rainbow trout (*Oncorhynchus mykiss*) broodstock during  
97 spawning at a fish hatchery in Washington, USA. (Wolf & Taylor 1975). *Salmonid*  
98 *herpesvirus 2* (SalHV-2) was isolated in RTG-2 and CHSE-214 cell lines from masou salmon  
99 (*Oncorhynchus masou*), coho salmon (*Oncorhynchus kisutch*), sockeye salmon  
100 (*Oncorhynchus nerka*), and rainbow trout (Sano 1976, Kimura et al. 1981, Horiuchi et al.  
101 1989, Suzuki 1993). *Salmonid herpesvirus 3* (SalHV-3) was described from lake trout  
102 (*Salvelinus namaycush*) (Bradley et al. 1989, McAllister & Herman 1989), but it has never

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

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

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## 108 **Material and methods**

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

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

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128 **Molecular study.** The molecular characterization was begun with nested PCRs using  
129 consensus primers targeting the partial DNA polymerase and terminase genes (homologous to  
130 ORF57 and ORF69 in the *Ictalurid herpesvirus 1* genome; RefSeq number: NC\_001493)  
131 (Table 2). The primers for the polymerase and terminase genes were designed by aligning the  
132 nucleotide sequences of the salmoniviruses (SalHV-1, -2, and -3; GenBank accession  
133 numbers: EU349281, EU349273, FJ641908, FJ641909, EU349284, EU349277) using the  
134 BioEdit software package (Hall 1999). The design of primers to amplify the partial  
135 glycoprotein gene (homologous to ORF46 in the *Ictalurid herpesvirus 1* genome) sequences  
136 for SalHV-3 and -4 relied on a previously published alignment of SalHV-1 and -2 (Davison

137 1998). The 50  $\mu$ l PCR cocktails consisted of 34  $\mu$ l distilled water, 10  $\mu$ l of 5 $\times$  buffer (Phusion,  
138 Finnzymes), 0.5  $\mu$ l thermo-stable DNA polymerase enzyme (Phusion, Finnzymes), 1  $\mu$ l (50  
139  $\mu$ M) of each the forward and reverse primer, 1.5  $\mu$ l of dNTP solution of 10 mM  
140 concentration, and 2  $\mu$ l target DNA (in the second round 5  $\mu$ l target DNA was applied from  
141 the first round). The reactions were performed in a T1 Thermocycler (Biometra). For PCRs  
142 targeting the DNA polymerase and terminase, the following program was used: initial  
143 denaturation at 98°C for 5 min, followed by 45 cycles of denaturation at 98°C for 30 s,  
144 annealing at 50°C for 30 s, and elongation at 72°C for 60 s. The final extension was  
145 performed at 72°C for 3 min. For the amplification of the partial glycoprotein gene an  
146 annealing temperature of 46°C and a 2 minute extension step was used.

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

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157 **Phylogenetic analysis.** The quality of the sequence reads was analyzed using BioEdit (Hall  
158 1999) and Staden (Staden 1996) program packages. The deduced amino acid sequences of the  
159 polymerase and terminase genes were aligned using Mafft v6.935b (Kato et al. 2005). The  
160 aligned polymerase and terminase genes were concatenated and the best fit amino acid model  
161 determined in TOPALI v2.5 program. A Bayesian phylogenetic analysis was performed using  
162 MrBayes (Huelsenback & Ronquist 2001) within the TOPALi v2.5 program package and  
163 interface (Milne et al. 2004) with the following parameters: Markov chain was run for 10  
164 million generations, four independent analyses were conducted, each with 1 cold and 3 heated  
165 chains. Sampling occurred every 10 generations with the first 25% of Markov chain Monte  
166 Carlo samples discarded as burn-in.

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## 168 **Results**

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

175           For instance, at hatcheries, where fertilized salmon eggs from the Uмба River and other  
176 rivers entering the White Sea were incubated, papillomatosis was not observed every year.  
177 The tumors first appeared in the largest 2-summer-old fish weighing 20g or more. The  
178 proliferative lesions began to degenerate in autumn and sloughed off before the next spring.  
179 However, the lesions reappeared in 3-summer-old fish by the time of their release into rivers  
180 in June - July. The prevalence of papillomatosis in these hatcheries typically did not exceed  
181 2.0 %.

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

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

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

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

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

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

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

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

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## 239 **Discussion**

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

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

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

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



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

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275

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359 TABLES

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

362

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

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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'

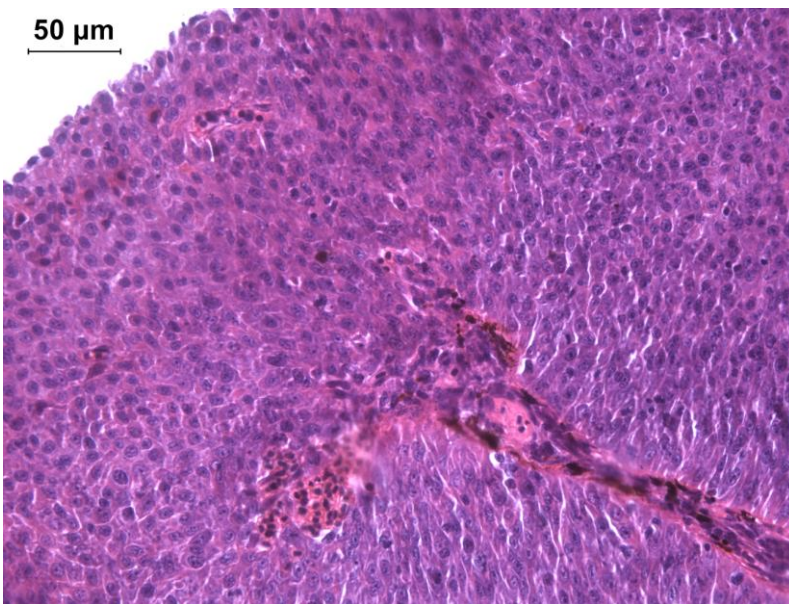
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367 **FIGURE LEGENDS**

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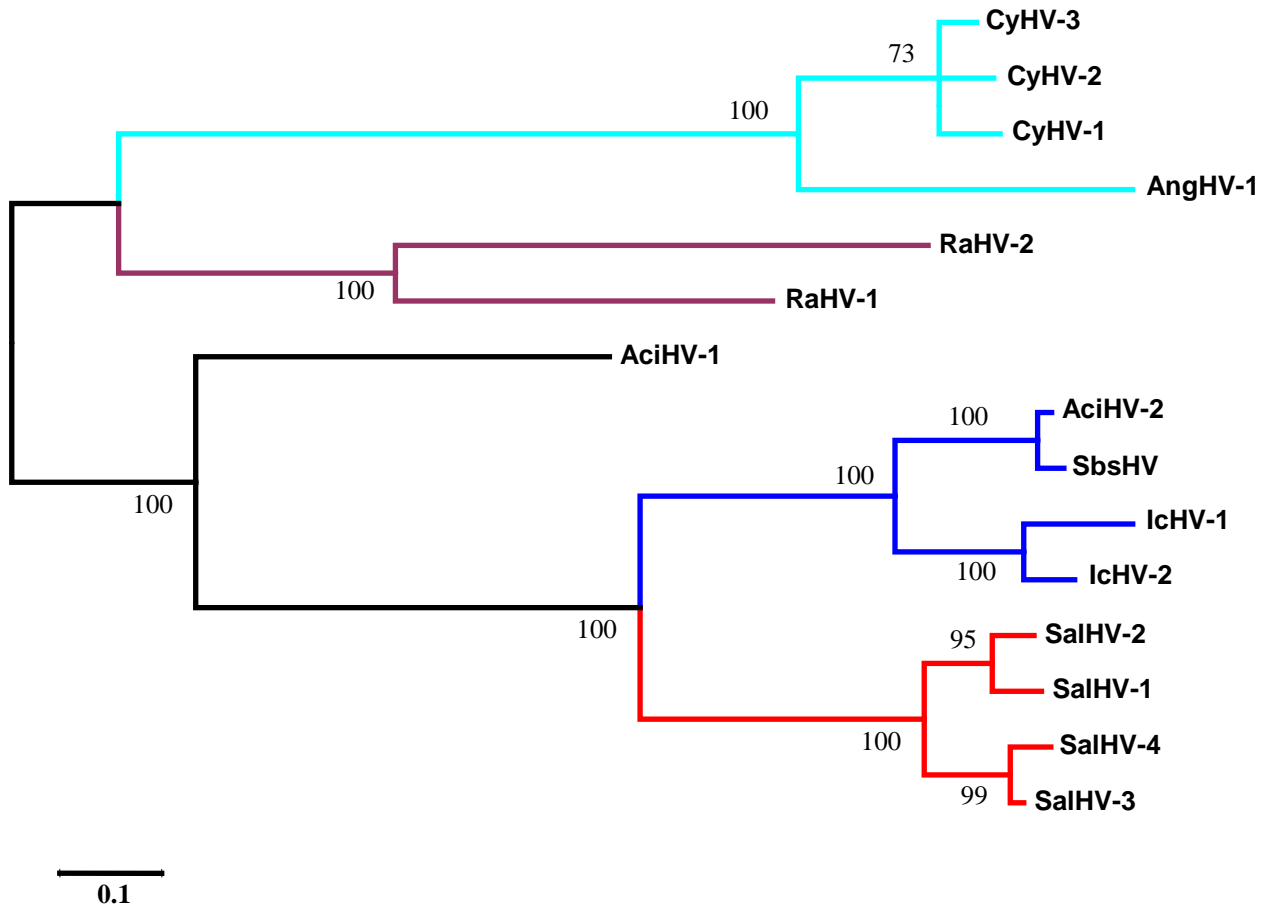
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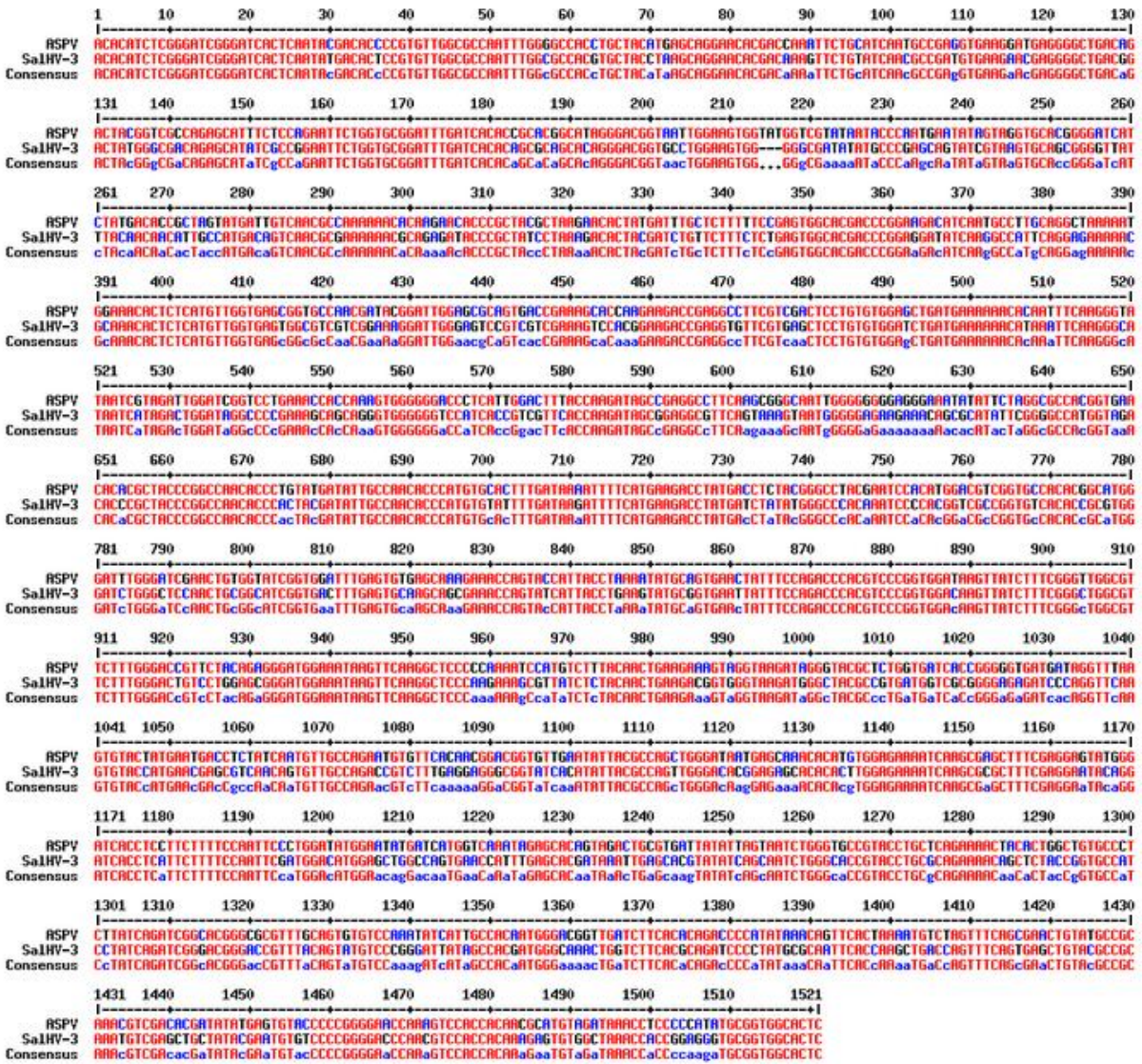
372 **Figure. 1.** (a) Papillomatosis in wild young Atlantic salmon captured at 10°C. Total length is  
373 9.5-11.1 cm. (b) Histopathology of a mature papilloma from a wild Atlantic salmon parr  
374 reveals epithelial hyperplasia, disorganization, and a loss of mucous cells.



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**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.





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 392 **Figure. 3.** Alignment of the nucleotide sequences of the glycoprotein gene sequences for  
 393 SaIHV-3 and ASPV.  
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