Outbreak of mortality in Russian (Acipenser gueldenstaedtii) and Siberian (Acipenser baerii) sturgeons associated with sturgeon nucleo-cytoplasmatic large DNA virus

Research Article

Outbreak of mortality in Russian (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeons associated with sturgeon nucleo-cytoplasmatic large DNA virus.

S. Ciulli [a,] *, E. Volpe [a], R. Sirri [a], P.L. Passalacqua [a], F. Cesa Bianchi [b], P. Serratore [a], L. Mandrioli [a]

[a] *Department of Veterinary Medical Sciences, University of Bologna, Viale Vespucci, 2, 47042 Cesenatico, FC, Italy*

[b] *Cesena Campus, University of Bologna, Viale Vespucci, 2, 47042 Cesenatico, FC, Italy*

* Corresponding author. Tel.: +0547338959.

E-mail address: sara.ciulli@unibo.it (S. Ciulli).

Abstract

Diseased outbreaks with high mortality in farmed sturgeon are a limiting factor to the success of this emerging aquaculture sector in Europe. Thorough investigations of outbreaks can determine the aetiological agents, identify important pathological and epidemiological pathways of infections and pave the way for effective control strategies.

A thorough investigation of a mortality outbreak in Russian (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeons in Italy, demonstrated the primary involvement of a sturgeon nucleo-cytoplasmic large DNA virus (NCLDV). While, the taxonomy classification of this new virus is still uncertain, its involvement in sturgeon mortality outbreaks in Europe is, for the first time, fully investigated and described. Furthermore, the coinfection of bacteria such as motile *Aeromonas* spp. and *Acinetobacter* spp. was reported.

Genetic characterisation showed the close relationship between the European sturgeon NCLDV with North American sturgeon NCLDVs. Similarly, to the latter, the European sturgeon NCLDV persists in survivors. Furthermore, a systemic distribution of the European sturgeon NCLDV was evident in diseased *A. baerii* and *A. gueldenstaedtii* and in recovered *A. gueldenstaedtii*.

These epidemiological and pathological findings will help in the identification of effective control strategies for sturgeon NCLDV infection, which afflicts an important and emerging European aquaculture sector.

Keywords: Acinetobacter spp.; *Acipenser gueldenstaedtii; Acipenser baerii;* Mortality outbreak; Sturgeon nucleo-cytoplasmic large DNA virus.

Introduction

Sturgeon farming is increasing in several European countries, including Italy, which became the European Union's leading country for caviar production with 25 tons produced in 2013 (Marino, 2015). Several species are farmed, and Russian (otherwise known as Danube) (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeons are desirable due to their short reproductive cycles and desired products (caviar and meat) (Rzepkowska and Ostaszewska, 2014; Williot et al., 2005). However, the success of sturgeon farming is heavily restricted due to the paucity of information related to sturgeon diseases and relative control methods. Intensive culture exposes the fish to several sources of stress, such as high stock densities and manipulations that predispose animals to a number of infectious diseases associated with viral or bacterial pathogens (Georgiadis et al., 2000). Bacteria such as motile *Aeromonas* spp., in fact, have been isolated from sturgeon, generally as a consequence of severe stress and high stock densities or opportunistic, secondary to a primary viral infection (Raverty et al., 2003; Colussi et al., 2005).

Reports of sporadic viral infection due to infectious pancreatic necrosis virus (IPNV), betanodaviruses or spring viraemia of carp virus (SVCV) infections in *Acipenser* sp. in Europe have been reported (Nougayrede, 1988; Athanassopoulou et al., 2004; Vicenova et al., 2011). Whereas, herpesviral and iridoviral infections are among the most reported causes of mortality outbreaks in sturgeons (Hedrick et al., 1990; 1991; Watson et al., 1995; Raverty et al., 2003; Shchelkunov et al., 2009; Kurobe et al., 2011; LaPatra et al., 2014).

Actually, herpesviral infection in sturgeons is attributable to two different viruses, the Acipenserid herpesvirus-1 (AciHV-1) and the Acipenserid herpesvirus-2 (AciHV-2; Kurobe et al., 2008). In addition, several iridolike viruses have been detected in sturgeons. The first and most studied iridolike virus associated with mortality outbreaks of sturgeon (*A. transmontanus*) was the white sturgeon iridovirus (WSIV) that has been reported in farmed and wild white sturgeon of the Pacific Coast from California to Canada (Hedrick et al., 1990; LaPatra et al., 1994; Raverty et al., 2003). Other iridolike viruses have been detected in different sturgeon species in North America

such as the Missouri River sturgeon iridovirus (MRSIV) in pallid sturgeon (*Schaphirhynchus albus*) and shovelnose sturgeon (*S. platorhynchus*) and the shortnose sturgeon virus (SNSV) in shortnose sturgeon (*Acipenser brevirostrum*). A recent phylogenetic study, however, showed that the viruses WSIV, MRSIV and SNSV are only distantly related to *Iridoviridae*, and are included in a group actually referred to as sturgeon nucleo-cytoplasmic large DNA viruses (NCLDVs) in the order *Megavirales*, a new term not formally adopted by the International Committee of the Taxonomy of Viruses (Clouthier et al., 2015). Further two viruses named British Columbia white sturgeon virus (BCWSV) and Namao virus (NV), detected in white sturgeon and lake sturgeon (*A. fulvescens*) respectively in North America, are also included in the sturgeon NCLDV group (Clouthier et al., 2013; 2015). Based on light and electron microscopy, iridovirus-like infection has also been reported, in Northern Europe in Russian sturgeon (*A. gueldenstaedtii*) associated with mortality (Adkison et al., 1998). Furthermore, similar outbreaks are frequently reported by sturgeon farmers, but only few thorough investigations have been conducted for these outbreaks. Sturgeon NCLDVs appear to be endemic in populations of *Acipenseridae* found throughout North America (Clouthier et al., 2015). At present, a true picture of the geographical distribution of sturgeon NCLDV infection in Europe is not available.

The present work describes a disease outbreak with increased mortality in farmed juvenile Russian (*Acipenser gueldenstaedtii*) and Siberian *(Acipenser baerii)* sturgeons in Italy. Diagnostic investigation demonstrated that both an NCLDV and bacteria such as motile *Aeromonas* spp. and *Acinetobacter* spp. were involved in the outbreak. Particularly, some interesting pathological and epidemiological insights about the sturgeon NCLDV infection were achieved.

Materials and methods

Clinical history and sampling

In the autumn of 2015, an acute disease outbreak occurred in a sturgeon farm situated in North Italy. The outbreak resulted in a mass mortality of fingerlings of two species: Russian

(*Acipenser gueldenstaedtii*) and Siberian *(Acipenser baerii)* sturgeons. In the hatchery, fish were reared in 1.5 m^3 fiberglass tanks supplied with well water at a constant temperature of about 16 °C with a stocking density of 10 g/L. Cultured fish were fed commercial diet (Protec, Skretting, Italy) at a rate of 1% wet body weight.

Fish showed lethargy and anorexia and had a lighter colour than normal. Erratic and looping swimming were also present in *A. gueldenstaedtii*. Mortality was 50 and 90% for *A. baerii* and *A. gueldenstaedtii*, respectively. Fish affected were 7-13 cm in length and weighted 5-10 g. Mortality was not observed in sturgeons of different sizes reared in the fattening unit that received water from the hatchery. Fish with clinical signs of both species (batch AG-2: *A. gueldenstaedtii*; batch AB: *A. baerii*) involved in the outbreak were collected and carried alive to the laboratories for sampling (Fig. 1). A second sampling was conducted eight weeks later to collect recovered fish (AG-2rec: A. *gueldenstaedtii*; ABrec: *A. baerii)*. In addition to the batches previously involved in the outbreak, two further batches of fish were collected (Fig. 1). Batch AG-1 consisted of a group of *A. gueldenstaedtii* of about one year old that experienced a similar disease outbreak four months earlier than batch AG-2; at the time of sampling, the fish of AG-1 batch were asymptomatic and do not show any sign of the disease by at least four months. Batch AG-3 consists in a group of younger fish (A. *gueldenstaedtii* with an average weight of 15 g) than AG-2 that entered the farm during the outbreak of AG-2. At the time of the second sampling, batch AG-3 showed similar signs to those showed by AG-2 during the previous outbreak, however the mortality was slightly lower in batch AG-3 (70%) than in batch AG-2 (90%). Fish collected for sampling were euthanized with an overdose of tricaine methanesulfonate (200 ppm) and sampled immediately for diagnostic investigations using clean, sterile dissecting instruments to prevent contamination between the outside and inside of the fish and between tissues. Specific details about preservation and processing of samples for analyses are described in following specific paragraphs.

Histopathology

Samples of operculum, fins, skin of the dorsal region, mouth and nares, gills, brain, liver, spleen, intestine and kidney of fish showing clinical signs (AG-2, AB, AG-3), as well as recovered fish (AG-1, AG-2rec) were fixed in 10% buffered formalin and processed for routine histology. The head and fins were previously decalcified in a medium strength decalcifying solution, RDM Histo-Decal (Histo-Line Laboratories, Milan, Italy), for 24 h. Sections of 3 µm were cut, stained with haematoxylin and eosin (H&E) and evaluated under a light microscope.

Bacteriological examination

Diseased (AG-2, AB, AG-3) and recovered fish (AG-1, AG-2rec, ABrec) were examined for the presence of bacteria. Kidney and brain were sampled by loop and immediately streaked onto Tryptone Soy Agar (TSA, Oxoid, UK). Plates were incubated at 20 °C for three days. The material from a few randomly chosen dominant colonies was inoculated onto TSA and incubated at 20 °C for 24 h, and pure cultures were obtained for three selected isolates from *A. gueldenstaedtii* and of two isolates from *A. baerii*. Colonies were Gram-stained for cell morphology determination and subjected to biochemical tests for preliminary identification. Oxidase (Oxidase detection strips, Oxoid) and catalase activities, reduction of nitrate to nitrite (Zen-Yoji et al., 1973), motility and indole production onto SIM Medium (Oxoid) were evaluated. Then molecular identification through 16S rDNA amplification and sequencing was conducted. Briefly, DNA was extracted from colonies through the boiling method and amplification of 16S rDNA was performed with primers P0F and P6R using 1 µl of each lysed cell suspension according to the procedure described previously (Caccamo et al., 1999). Polymerase chain reaction products were purified and sequenced to confirm the bacterial identity. Sequences were obtained through the Bio-Fab Sequencing Service (Rome, Italy), and then analysed through the online Basic Local Alignment Search Tool (BLAST) web interface provided by the National Center for Biotechnology Information (NCBI) to confirm bacterial identity (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RNA, DNA extraction and virus detection

Fresh organs (brain, spleen, heart, kidney, gill, skin and fins) from diseased fish (AG-2, AB, AG-3) as well as recovered fish (AG-1, AG-2rec, ABrec) were collected for RNA and DNA extraction. Tissues were stored at −80 °C until processing for nucleic acid extraction. Three fish from each batch were tested individually.

Ribonucleic acid was extracted from about 25 mg of tissue using NucleoSpin® RNA II (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. The RNA was used soon after the extraction or stored at −80 °C. The extracts were subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) analysis to detect betanodaviruses, spring viraemia of carp virus (SVCV) and infectious pancreatic necrosis virus (IPNV) with protocols previously described (Ciulli et al., 2006; OIE, 2012; Pinheiro et al., 2015). Briefly, a reaction mix was arranged for each target using the SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, USA) with specific primers and 3 µl of RNA. Thermal cycling conditions were adapted to a suitable annealing temperature: 58 °C (betanodaviruses), 55 °C (SVCV) and 60 °C (IPNV). For betanodaviruses and SVCV detection, a nested PCR was arranged with taq polymerase (Invitrogen, Carlsbad, USA) as previously described (Nishizawa et al., 1994; OIE, 2012). Particularly, brains from fish showing nervous signs (batches AG-2, AB) were collected to be examined with RT-PCR for betanodaviruses. Spleen, kidney and heart pools (batches AG-2, AB) were subjected to SVCV and IPNV RT-PCR.

Deoxyribonucleic acid was extracted from about 25 mg of tissue using the Purelink Genomic DNA kit (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. The DNA was used soon after the extraction or stored at −20 °C until use. Five µl of DNA extracted from skin, fin and gill pools (batches AG-2, AB) was subjected to two PCR analyses with taq polymerase (Invitrogen, Carlsbad, USA) to detect herpeviruses and iridoviruses following previously described protocols (Doszpoly et al., 2008; Bigarré et al., 2016). Particularly, for

iridovirus DNA detection, 2 fragments were amplified: fragment A (primers opPV339-oPV340) and fragment B (primers oPV341-oPV344 (Bigarré et al., 2016).

Degenerate primers for the detection of sturgeon NCLDV were designed on the basis of an alignment arranged with sequences of the major capsid protein (MCP) gene available on the GenBank database (White sturgeon iridovirus: DQ897645 and AY996807; Namao virus: JX155659; Shortnose sturgeon virus: KM606973; Missouri River sturgeon iridovirus: JX155661; BC white sturgeon virus: JX155660). Genome positions of designed primers are 688-713 for primer SIfor and 922-942 for primer SIrev based on the sequence of WSIV (GenBank accession number DQ897645). Polymerase chain reaction was set up by the analysis of several reaction mixes and a gradient study for annealing optimisation. The best performances were obtained using a 25 µl reaction mix containing 5 µl of DNA, 1X PCR buffer, 2 mM $MgCl_2$, 200 µM of dNTP, 0.8 µM of each primer (SIfor: TTTACTTCBTACAAGCAAGAATATTT, SIrev: ACCTTCGCGRTTRATWGTTTT) and 1.25 U of taq polymerase (Invitrogen, Carlsbad, USA). The optimal thermal cycling conditions were 95°C for five minutes, followed by 45 cycles of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s. Final extension was prolonged for 10 min. This new PCR protocol was applied to skin, fin and gill pools and single organs (gill, spleen, kidney) of all batches to detect sturgeon NCLDV presence. The results of all RT-PCR and PCR analyses were checked by agarose gel electrophoresis of PCR products along with a 100 bp DNA molecular marker (Invitrogen, Carlsbad, USA). Positive and negative controls were run along with all reactions. In cases of positivity, one PCR product of each batch was purified and subjected to sequencing to confirm the virus identity. Sequences were obtained through the Bio-Fab Sequencing Service (Rome, Italy), and then they were analysed through the online Basic Local Alignment Search Tool (BLAST) web interface provided by NCBI to confirm virus identity (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Furthermore, deduced amino acid sequences were aligned and compared with similar sequences available in GenBank (http://www.ncbi.nlm.nih.gov) using Clustal W in BioEdit software (bioedit.software.informer.com). The percentage of similarity of

196 pairwise distances was calculated with BioEdit software. Phylogenetic analysis of the deduced
197 amino acid sequence of an MCP fragment (413 amino acid) was performed with MEGA 6 software
198 (www.megasoftware.net) using the maximum-likelihood. The infectious spleen and kidney necrosis
199 virus (ISKNV; GenBank accession number: AF371960), genus *Megalocytivirus*, family
200 *Iridoviridae*, was used as the outgroup. Bootstrap analysis was carried out on 1000 replicates.
201
202 **Results**
203
204 *Gross and histopathology*
205 At gross examination, in both *A. gueldenstaedtii* (batch AG-2) and *A. baerii* (batch AB), the
206 dorsal fin showed multifocal ulceration with red margins (Fig. 2 A). About 10 % of *A. baerii*
207 showed hyperplastic and exfoliative lesions of the dorsal fin (Fig.2 B). One specimen of *A.*
208 *gueldenstaedtii* had mild hyperaemia around the mouth and at the basis of the pectoral fins. The
209 gills of both species appeared brilliant red with abundant mucous, lamellar hyperplasia and
210 multifocal small pinpoint haemorrhages (Fig. 2 C). At the opening of the coelomic cavity the organs
211 of both species appeared moderately pale, and the liver was soft with a marbled appearance (Fig. 2
212 D). The intestine was moderately dilated with the presence of whitish mucous casts in all examined
213 animals. Other organs were unremarkable. No gross lesions were observed in recovered fish (AG-1,
214 AG-2rec) or in the AG-3 batch.
215 Histological evaluation showed gills with moderate hyperplasia of the lamellar epithelium,
216 mild infiltration of inflammatory cells, mainly lymphocytes and a less number of mast cells, and a
217 diffuse degeneration and necrosis of pillar cells lining lamellar vascular channels in both diseased
218 (AG-2) and recovered fish (AG-1, AG-2rec; Fig. 3 A). Diseased fish of batch AG-3 showed similar,
219 but milder, histological features than fish of other batches. The skin displayed a mild hyperplasia
220 with focal ulceration. In both gill and skin, there were a few hypertrophic cytomegalic epithelial
221 cells with homogeneous amphophilic cytoplasmic inclusions and an eccentric nucleus (Fig. 3 B).

These cells were more numerous in diseased fish of batch AG-2 than in recovered fish of batches AG-1 and AG-2rec. The liver of fish of all batches showed a diffuse hepatocellular degeneration and vacuolisation together with a moderate lymphoplasmacytic periductal and perivascular hepatitis. The intestine displayed a moderate amount of sloughed epithelial cells and mucous in the lumen. The remaining organs did not show pathological changes.

Bacteriological examination

A few small, white, convex, circular colonies were grown on the TSA plates seeded with brain and kidney specimens from batches AG-2 and AB. Additionally, small, convex, circular colonies with slight brown pigmentation were grown on the TSA plates seeded with kidney samples from batch AG-2. Four white colonies, two isolated from kidneys of *A. gueldenstaedtii* (AG-2/C11, AG-2/C12) and two from *A. baerii* (one from brain AB/C15 and one from kidney AB/C18), and one colony with slight brown pigmentation isolated from brain samples of *A. gueldenstaedtii* (AG-2/C4) were selected for further characterisation. No colonies were grown on the TSA plates seeded with brain and kidney samples of recovered fish (AG-1, AG-2rec, ABrec) or diseased fish of batch AG-3.

Isolates AG-2/C11, AG-2/C12, AB/C15 and AB/C18 were bipolar, Gram negative, oxidase negative, catalase positive, non-motile and nitrate negative. Comparison of a 811 bp fragment of 16S rDNA gene sequences showed a 100% nucleotide identity among isolates AG-2/C11, AG-2/C12 and AB/C15, whereas a nucleotide identity of 99.2% was revealed between AB/C18 and the other isolates. Molecular identification of these isolates after Blasting in NCBI showed 100% identity of AG-2/C11, AG-2/C12 and AB/C15 with *Acinetobacter johnsonii* sequences (GenBank accession numbers: CP010350, KP296204, LN774684) and 100% identity of AB/C18 with *Acinetobacter tjernbergiae* sequences (GenBank accession numbers: KU312797, KU337241).

The isolate AG-2/C4 was oxidase positive, catalase negative, motile and nitrate positive. The bacterial morphology under light microscopy showed Gram negative coccobacilli. An 837 bp

248 fragment of a 16S rRNA gene was obtained for the isolate. Molecular identification of this isolate
249 after Blasting in NCBI showed 100% identity with *Aeromonas sobria* sequences (GenBank
250 accession numbers: JX164206, GU187060). Further comparison of the *A. sobria* isolate in this
251 study with an *A. sobria* sequence isolated from a sturgeon (GenBank accession number: KR010958)
252 showed a 96.7% nucleotide identity. The 16S sequences obtained in this study have been deposited
253 in GenBank (NCBI; GenBank accession numbers: KX237751-KX237755).
254
255 *Virus detection and characterisation*
256 Negative results were obtained for betanodaviruses, SVCV and IPNV via RT-PCR and
257 nested PCR analyses of batches AG-2 and AB. Similarly, no PCR products were obtained for
258 herpesvirus from batches AG-2 and AB.
259 Samples of both species *A. gueldenstaedtii* (AG-2) and *A. baerii* (AB) collected during the
260 outbreak were positive for iridovirus with the protocol developed by Bigarré et al. (2016).
261 Polymerase chain reaction product specificity was confirmed by sequencing PCR products.
262 Primers designed in this study for sturgeon NCLDV detection amplified a specific 255 bp
263 fragment from skin, fin and gill pools from AG-2 (*A. gueldenstaedtii*) and AB (*A. baerii*) batches.
264 Sturgeon NCLDV DNA was also detected in all skin, fin and gill pools of AG-3 (animals with
265 signs) as well as in recovered *A. gueldenstaedtii* (AG-2rec, AG-1, asymptomatic animals). On the
266 other hand, in recovered *A. baerii* (ABrec), sturgeon NCLDV DNA was detected only in 1/3 of the
267 skin, fin and gill pools (Table 1). The PCR product-specificity was confirmed by sequencing the
268 MCP gene fragment obtained for each batch (AB, AG-1, AG-2, AG-3). Viral sequences obtained in
269 this study have been deposited in GenBank (NCBI; GenBank accession numbers: KX244954-
270 KX244957).
271 The analysis of internal organs showed the presence of sturgeon NCLDV DNA in both
272 spleen and kidney samples of all fish collected during the outbreaks (AB, AG-2, AG-3) (Table 1).
273 Regarding recovered fish, different results were obtained among batches and species. In recovered

A. gueldenstaedtii collected two weeks after the end of the outbreak (batch AG-2rec), specific DNA was present in both the spleen and kidney of all tested fish (Table 1). On the other hand, in batch AG-1, sturgeon NCLDV DNA was found in the spleen of one out of three tested fish, whereas all three tested kidneys resulted positive for sturgeon NCLDV. Batch AG-1 consisted of asymptomatic *A. gueldenstaedtii*, but with a history of a mortality outbreak dating back four months earlier than the sampling time (Fig. 1; Table 1). Lastly, sturgeon NCLDV DNA was not detected in any internal organs in recovered *A. baerii,* collected six weeks after the end of the outbreak (Table 1).

A comparison between sequences of PCR products obtained with the two independent PCR protocols for AG-2 and AB batches showed 100% nucleotide identity in the overlapping region. Comparison among viral sequences of the 208 bp MCP gene fragment (SIfor/rev PCR products without primer sequences) of different batches showed 100% nucleotide identity except for the AG-1 sequence which showed 99.5% nucleotide identity. However, the deduced amino acid sequence comparison showed a 100% identity among all viruses detected in this study in different batches and sturgeon species (Table 2). The assembling of three neighbouring sequence fragments, the two obtained with the assay developed by Bigarrè (fragments A and B) and one obtained with the method developed in the manuscript permitted to obtain a 1240 bp sequence for the virus detected in AG-2 and AB batches that showed 100% nucleotide identity to each other. A comparison among sequences of the 1240 bp MCP gene fragment of sturgeon viruses detected in this study for AG-2 and AB batches, hereafter named European sturgeon NCLDV, with North American sturgeon NCLDV sequences retrieved from the GenBank database showed the highest nucleotide identity with Namao virus and British Columbia white sturgeon virus (87.9%) and the lowest value with white sturgeon iridovirus (73.6 %). Similarly, the highest deduced amino acid identity was with Namao virus (96.5 %) and the lowest was with white sturgeon iridovirus (79.5%) (Table 3).

On the basis of the phylogenetic analysis of the deduced amino acid sequence of an MCP fragment (413 amino acid), the sturgeon virus detected in this study was included in the cluster of sturgeon NCLDVs; particularly, the phylogenetic analysis divided sturgeon NCLDVs into two

sister groups, one represented by the WSIV alone and the other consisting of the European sturgeon NCLDV detected in this study and all the other North American sturgeon NCLDVs (MRSIV, BCWSV, SNSV, NV) (Fig. 4).

Discussion

Mortality outbreaks are frequently reported in sturgeon farming worldwide. As a result of a long history in sturgeon breeding and, consequently, in investigations related to sturgeon health problems, disease outbreaks have been more frequently reported in North America. However, sturgeon farming is increasing in several European countries, resulting in a need to investigate and define the aetiological causes of mortality outbreaks that affect sturgeon species in Europe.

Few mortality outbreaks have been reported in sturgeon associated with primary bacterial pathogens such as the case of lactococcosis in hybrid sturgeon, Bester (*Huso huso* x *Acipenser ruthenus*) (Chen et al., 2012); however, the isolation of other bacteria from sturgeon was reported especially as superinfections secondary to viral infections or as a consequence of severe stress and high stock densities (Raverty et al., 2003; Colussi et al., 2005). Bacteria isolated most frequently from sturgeon were *Aeromonas hydrophila* and other motile *Aeromonas* species that have been reported in *A. transmontanus*, *A. baerii*, *A. ruthenus*, *Huso dauricus* and hybrid sturgeon (*Huso huso* × *Acipenser ruthenus*) (Raverty et al., 2003; Colussi et al., 2005; Cao et al., 2007). Based on genetic and biochemical screening, a motile *Aeromonas* isolate, detected from a single batch of *A. gueldenstaedtii* (AG-2) collected during the mortality outbreak was identified as *Aeromonas sobria*. This bacterium has not been detected in others batches. Fish of AG-2 batch were also coinfected by the European sturgeon NCLDV. Similarly, to what reported in other sturgeon disease outbreaks (Raverty et al., 2003), we hypothesise that *A. sobria* was a superinfection that emerged in fish weakened by the viral infection.

Moreover, several *Acinetobacter* isolates were detected in both *A. gueldenstaedtii* (batch AG-2) and *A. baerii* (batch AB) involved in the mortality outbreaks. *Acinetobacter* is a bacterial

genus that has gained increasing attention in recent years as a result of its potential to cause severe infections in a wide range of hosts including fish (Kozińska et al., 2014). However, members of the genus *Acinetobacter* are also commonly reported in soil, healthy fish, and water (Kozińska et al., 2014), and are usually perceived as normal saprophytic microorganisms. In this respect, their isolation from altered skin and diseased fish could be the result of microorganism penetration into internal organs after skin damages (Kozińska et al., 2014). Nevertheless, the role of some *Acinetobacter* species such as *A. baumanii* and *A. johnsonii* as pathogens for freshwater fish has been well documented in the last years (Xia et al., 2008; Kozińska et al., 2014). In our study, the involvement of *Acinetobacter johnsonii* in *A. gueldenstaedtii* (batch AG-2) and *A. baerii* (batch AB) and *Acinetobacter tjernbergiae* in *A. baerii* was shown by molecular characterisation. Some lesions observed in the outbreak described in this study, such as multifocal ulceration with red margins (*A. gueldenstaedtii* and *A. baerii*) and mild hyperaemia around the mouth and at the base of the pectoral fins (*A. gueldenstaedtii*), have been previously described associated with natural and experimental infections with *Acinetobacter johnsonii* and *A. baumanii* (Kozińska et al., 2014). *Acinetobacter* infected fish were also infected with the European sturgeon NCLDV, however, due to the considerable presence of these bacteria in tested specimens and the type of observed lesions, we hypothesise a contributory role of *Acinetobacter* in the type and severity of lesions in this outbreak. This hypothesis is supported by the observation that *Acinetobacter* infection was not detected in a different batch of *A. gueldenstaedtii* (AG-3) involved in a disease outbreak, with lower mortality, observed in the same farm.

Viral screening conducted through molecular methods permitted us to exclude the involvement of viruses previously detected in sturgeons and occasionally associated with mortality outbreaks such as betanodaviruses, IPNV and SVCV (Nougayrede, 1988; Athanassopoulou et al., 2004; Vicenova et al., 2011). We also excluded the presence of herpes-like viruses, which have been frequently involved in mortality outbreaks in several sturgeon species both in North America and in Europe, including Italy (Hedrick et al., 1991; Watson et al., 1995; Shchelkunov et al., 2009;

LaPatra et al., 2014). Two independent molecular analyses confirmed the presence of viral DNA related to sturgeon iridoviruses in analysed specimens, actually best defined as sturgeon NCLDVs (Clouthier et al., 2015). While histology can not distinguish among etiological agents, it suggested a viral-induced lesion, in fact, the morphology and the location of hypertrophic epithelial cells and necrosis of pillar cells are similar to those described by Clouthier et al. (2013), Raverty et al. (2003) and Hedrick et al. (1990) associated with viral infections. The developed PCR-based assay was successfully used to track the viral infection in diseased and surviving fish. The analysis of several tissues from a wide range of fish on the farm involved in the outbreaks provided data that may lead to some interesting pathological and epidemiological insights about this infection. *Acipenser gueldenstaedtii* and *A. baerii* were similarly affected, according to the number of positive animals out of those tested (3/3) and the tissue dissemination of viral DNA (skin, fins, gill, spleen and kidney). However, the infection had different impact on *A. gueldenstaedtii* and *A. baerii* according to total mortality and duration of the outbreak, with *A. gueldenstaedtii* being the more susceptible species. Specific viral DNA was easily detected in internal organs (spleen and kidney) of all diseased animals of both sturgeon species involved in the outbreak, consistent with a systemic infection, even though no viral-induced histopathological lesions were observed in these internal organs. White sturgeon iridovirus was reported as an epitheliotropic virus that does not result in systemic infection, but localises in epithelial cells of the integument (Hedrick et al., 1990; LaPatra et al., 2014). While consistent or specific histopathological lesions of WSIV infection were not detected in the liver or other internal viscera of challenged fish, a previous study showed that WSIV can be isolated from internal organs such as the kidney and spleen during an experimental infection (Watson et al., 1998). Furthermore, the ability of WSIV to replicate in splenic cells was previously proven due to its replication in a splenic cell line (Hedrick et al. 1991) even if WSIV showed better replicating ability in cell lines of different tissue origins (Watson et al., 1998). A complex lifecycle has been identified for North American sturgeon NCLDVs (Watson et al., 1998; Kurobe et al., 2011; Clouthier et al., 2015); similarly, for European sturgeon NCLDV detected in this study, we

observed two stages of infection: an acute, lethal disease (AG-2 batch) and a persistent, subclinical infection (AG-1 batch). Analysis of different sturgeon batches present in the farm showed the persistence of viral DNA in tissues of survivor *A. gueldenstaedtii* up to four months after the end of an outbreak with a different presence in tested tissues. These surviving fish also showed viral-induced lesions such as hypertrophic cytomegalic epithelial cells in the skin. Previous studies showed inconsistent results about the ability to detect WSIV infection in survivor fish (Kwak et al., 2006; Drennan et al., 2007). However, the persistence of viral DNA and the histologic lesions were shown in an experimental infection of *A. transmontanus* with WSIV up to nine months after exposure (Kwak et al., 2006). Similarly, MRSIV DNA was detected in recovered pallid sturgeon (*Scaphirhynchus albus*) about five months following the last mortality (Kurobe et al., 2011). Moreover, the authors demonstrated that the survivor fish were able to transmit the infection to juvenile pallid sturgeon by cohabitation (Kurobe et al., 2011). While a wide variability was observed among tissue samples, all studies mainly considered epithelial tissues (Kwak et al., 2006; Drennan et al., 2007; Kurobe et al., 2011), whereas viral persistence at the level of the internal organs has not been investigated so far for sturgeon NCLDVs. In our study, viral DNA persistence time was dependant on tissue analysed, with a constant finding in skin, fin and gill pools of recovered *A. gueldenstaedtii* and poor findings in internal organs four months after the end of the outbreak. This finding is in accordance with a pathogenesis study on WSIV, in which histologic analysis, actually showed a prolonged presence of lesions in olfactory organs, gill, skin and esophagus compared to kidney, liver and spleen (Watson et al., 1998). Furthermore, the results of the investigation of viral DNA presence in the organs of recovered fish of the two analysed species argue for a different epidemiological role of *A. gueldenstaedtii* and *A. baerii* in maintaining the infection at the population level. Even though the *A. baerii* were collected a few weeks after the end of the outbreak, only one of them was positive for the viral DNA, while in contrast all *A. gueldenstaedtii* fish were positive up to four months after the end of the outbreak. However, further studies, based on large, statistically representative samples of the sturgeon fish population should be

carried out in the future to better define the role of the different fish species and the survivor fish in the epidemiology of this emerging sturgeon infection.

Sequence analysis of an MCP gene fragment obtained from tested specimens showed a 99.5-100% nucleotide identity between viruses detected in *A. gueldenstaedtii* and *A. baerii*, proving the ability of the European sturgeon NCLDV to infect more than one species. Also, North American sturgeon NCLDVs are not species-specific, such as MRSIV that was found in both shovelnose (*Scaphirhynchus albus*) and pallid sturgeon (*S. platorynchus*) (Kurobe et al., 2010). Genetic comparison with North American sturgeon NCLDVs showed that the virus detected in this study can be completely included in this group. On the basis of the percentages of nucleotide and amino acid identities and the phylogenetic tree topology we could assume that WSIV may have evolved from a common ancestor of the European sturgeon NCLDV, detected in this study, and other sturgeon NCLDVs detected in North America (MRSIV, SNSV, BCWSV, NV). No relationship between genetic clustering and geographical distribution was revealed, in accordance with that previously reported for North American sturgeon NCLDVs (Clouthier et al., 2015).

Conclusions

The thorough investigation of a mortality outbreak in Russian (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeons in Italy has demonstrated the primary involvement of a sturgeon NCLDV with concurrent bacterial infections such as motile *Aeromonas* spp. and *Acinetobacter* spp. Furthermore, interesting pathological and epidemiological insights about the sturgeon NCLDV infection were achieved. Genetic characterisation showed the close relationship of the European sturgeon NCLDV with North American NCLDVs. Similar to the latter, the European sturgeon NCLDV was detected in fish during the mortality outbreaks and also in survivors suggesting a possible persistent infection. Furthermore, a systemic distribution of the European sturgeon NCLDV was evidenced in diseased *A. baerii* and *A. gueldenstaedtii* and in recovered *A. gueldenstaedtii*.

Definitely, the observation of this natural outbreak appears to raise some questions in addition to some new knowledge on NCLDV infection in sturgeons, but surely paves the way to indispensable insights of this infection in Europe. Further research completing the epidemiological and pathological pictures of this infection will be critical to the identification of an effective control strategy for sturgeon NCLDV infection which afflicts an important and emerging European aquaculture sector.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table 1

NCLDVs presence in tested specimens of different sturgeon species and batches.

Batchs	Clinical signs	Species	skin, fin and gill pool	Gill	Spleen	kidney
AB	With signs	*A. baerii*	3/3	3/3	3/3	3/3
AG-2	With signs	*A. gueldenstaedtii*	3/3	3/3	3/3	3/3
AG-3	With signs	*A. gueldenstaedtii*	3/3	nd	3/3	3/3
AG-2rec	recovered	*A. gueldenstaedtii*	3/3	nd	3/3	3/3
ABrec	recovered	*A. baerii*	1/3	0/3	0/3	0/3
AG-1	recovered	*A. gueldenstaedtii*	3/3	nd	1/3	3/3

nd: not determined

Table 2

Comparisons of nucleotide and deduced amino acid sequences of the 208 bp major capsid protein (MCP) gene fragment (SIfor/rev PCR products without primer sequences) among NCLDVs detected in different batches and sturgeon species during a series of linked outbreaks (AG-2, AG, 3 AB) and surviving fish (AG-1). Percentages of similarity of pairwise distances are shown.

		Nucleotide identity			
		AB	**AG-1**	**AG-2**	**AG-3**
Amino acid	**AB**		99.5	100.0	100.0
	AG-1	100.0		99.5	100.0
	AG-2	100.0	100.0		100.0
	AG-3	100.0	100.0	100.0	

Table 3

Comparisons of nucleotide and deduced amino acid (bold) sequences of the 1240 bp major capsid protein (MCP) gene fragment among the European sturgeon NCLDV detected in this study and North American sturgeon NCLDVs. BCWSV: British Columbia white sturgeon virus (JX155660); NV: Namao virus (JX155659); SNSV: Shortnose sturgeon virus (KM606973); MRSIV: Missouri River sturgeon iridovirus (JX155661); WSIV: White sturgeon iridovirus (DQ897645). Percentages of similarity of pairwise distances are shown.

	North American sturgeon NCLDVs				
	BCWSV	**NV**	**SNSV**	**MRSIV**	**WSIV**
European sturgeon NCLDV	87.9 **95.0**	87.9 **96.5**	87.2 **95.5**	86.2 **92.2**	73.6 **79.5**

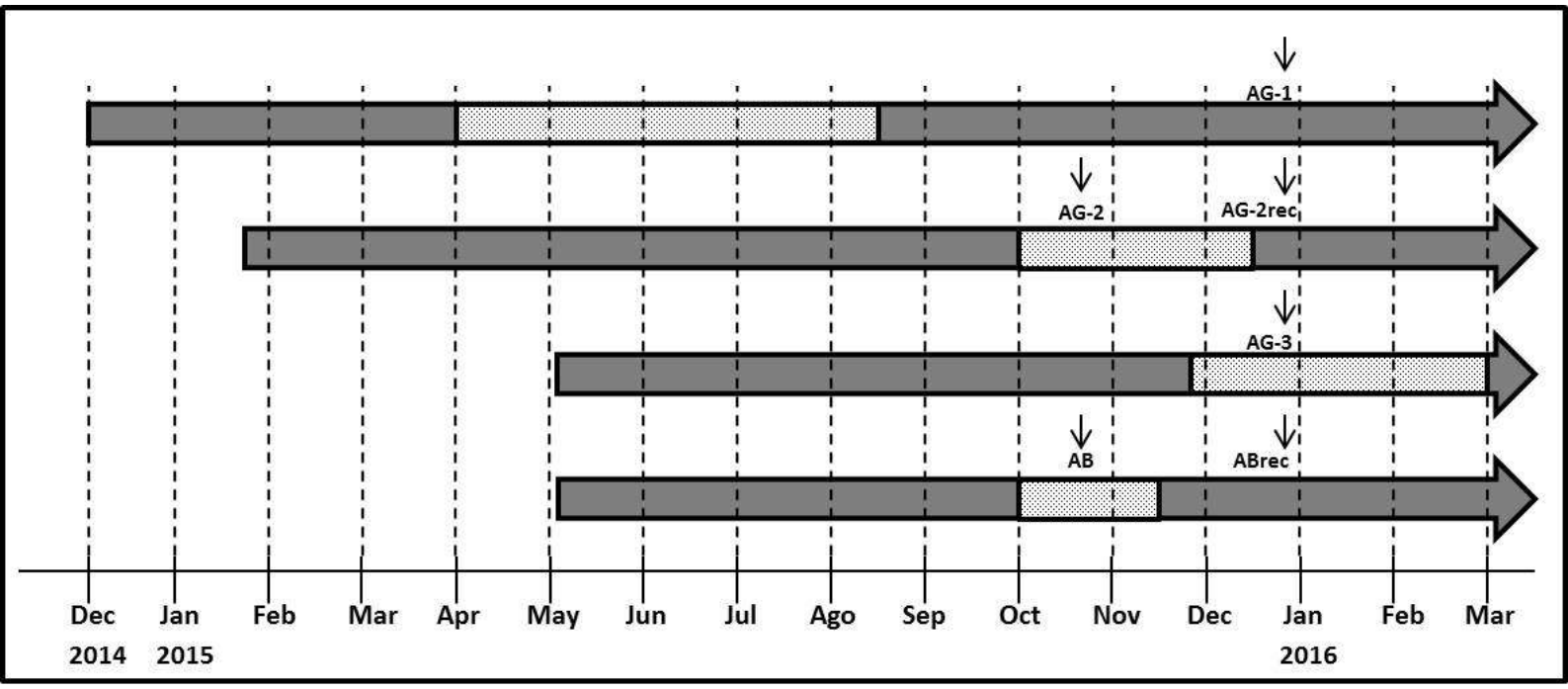
Figure captions

Fig. 1. Timing diagram of outbreaks and sampling. The thick arrows represent sturgeon batches. Dotted area represent outbreaks' time. Thin arrows represent sampling time.

Fig. 2. (A) *A. gueldenstaedtii,* dorsal fin, lateral view. Multifocal ulceration with red margins are present on bony plates. (B) *A. baerii*, dorsal fin, dorsal view. Exfoliating white-yellowish cutaneous strands are evident on the dorsal fin. (C) *A. gueldenstaedtii*, gills. Lamellar hyperplasia and multifocal small pinpoint haemorrhages are evident diffusely. (D) *A. gueldenstaedtii*, coelomic cavity. The liver displays a marbled appearance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Fig. 3. (A) *A. gueldenstaedtii*, gills. Severe and diffuse degeneration and necrosis of pillar cells lining lamellar vascular channels; numerous karyorrhectic debris are evident (scale bar=50µm). (B) *A. gueldenstaedtii,* gills. An hypertrophic cytomegalic epithelial cell with homogeneous amphophilic cytoplasmic inclusions is evident among epithelial cells (scale bar=10µm).

Fig. 4. Phylogenetic tree constructed with the deduced amino acid sequences of an MCP gene fragment (413 amino acid) of the European sturgeon NCLDV detected in this study (bold) and other North American sturgeon NCLDVs. Genbank accession number MRSIV: JX155661; NV: JX155659; BCWSV: JX155659; SNSV: KM606973; WSIV: DQ897645. The infectious spleen and kidney necrosis virus (ISKNV; Genbank accession number: AF371960), genus *Megalocytivirus*, family *Iridoviridae,* was used as the outgroup.


AG-1
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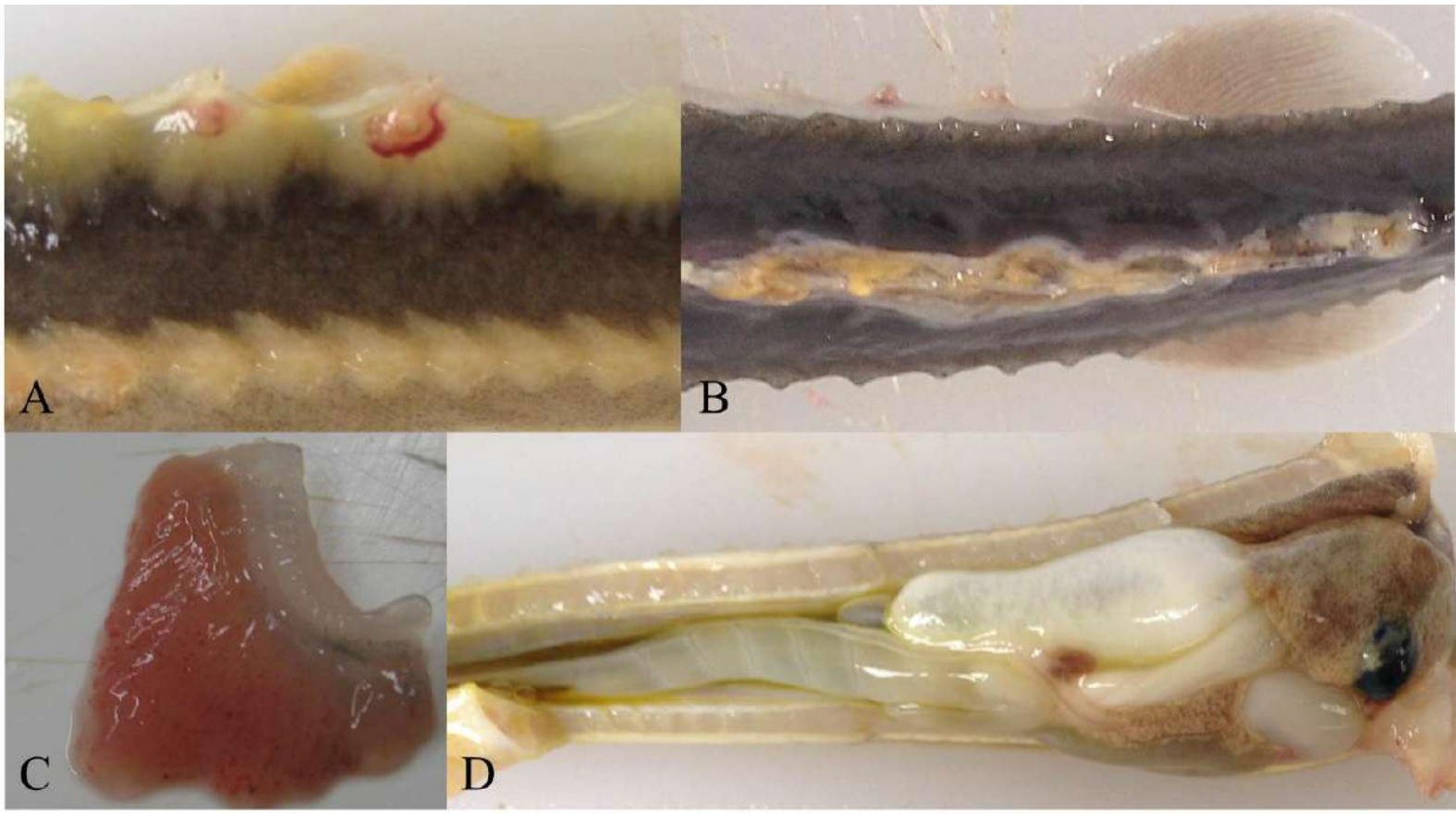
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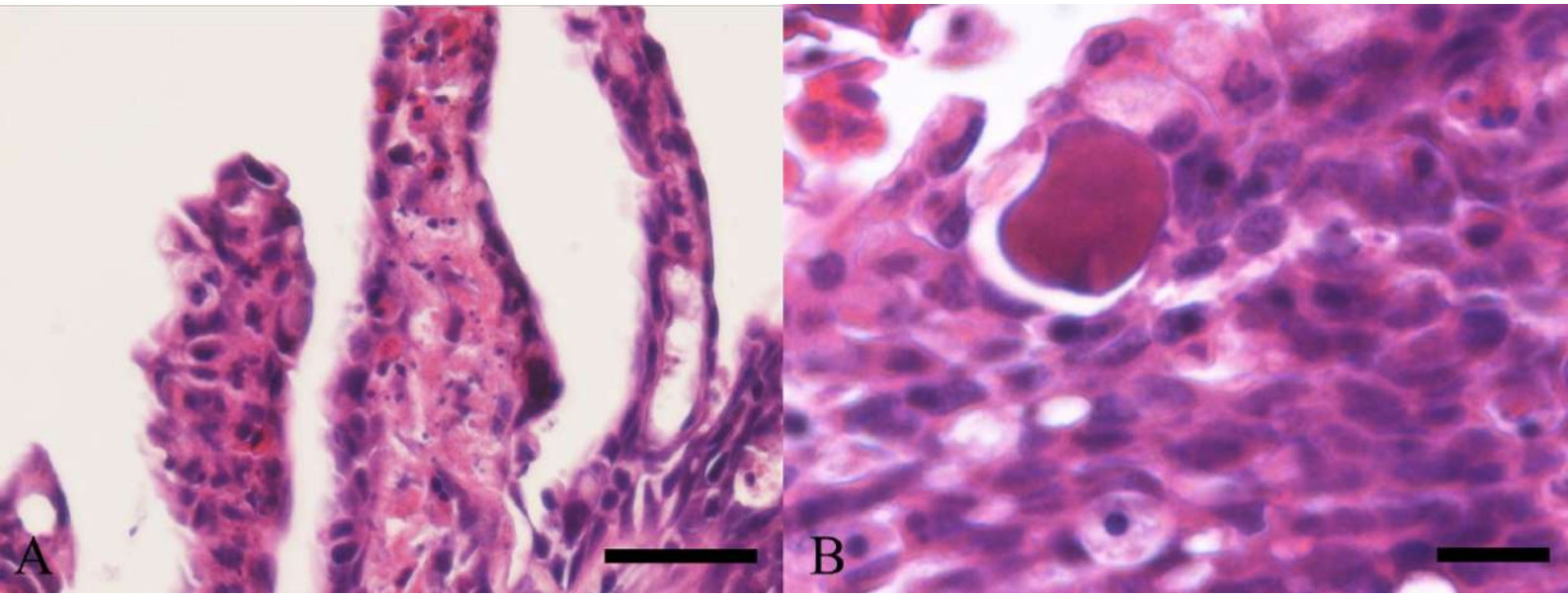
D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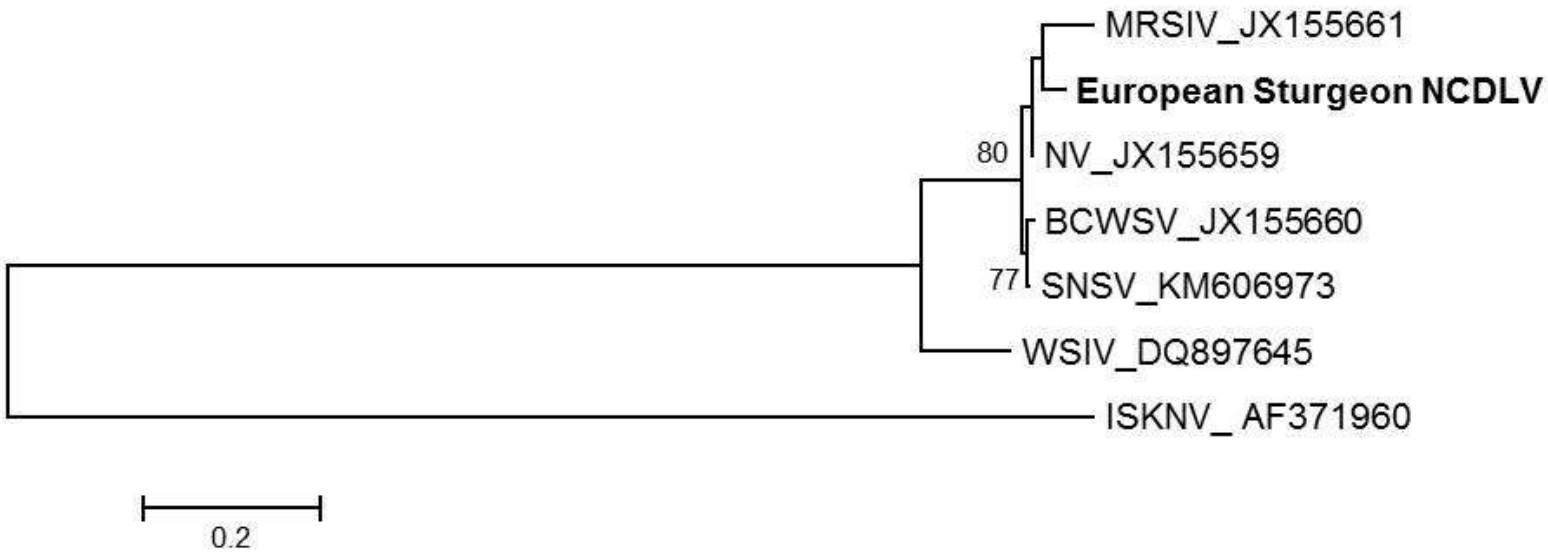
MRSIV_JX155661
European Sturgeon NCDLV
80
NV_JX155659
BCWSV_JX155660
77
SNSV_KM606973
WSIV_DQ897645
ISKNV_ AF371960
0.2