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

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1 **Research Article**

2

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

5

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17

18 **Abstract**

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

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

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

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

37

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

40

## 41 **Introduction**

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

54 Reports of sporadic viral infection due to infectious pancreatic necrosis virus (IPNV),  
55 betanodaviruses or spring viraemia of carp virus (SVCV) infections in *Acipenser* sp. in Europe have  
56 been reported (Nougayrede, 1988; Athanassopoulou et al., 2004; Vicenova et al., 2011). Whereas,  
57 herpesviral and iridoviral infections are among the most reported causes of mortality outbreaks in  
58 sturgeons (Hedrick et al., 1990; 1991; Watson et al., 1995; Raverty et al., 2003; Shchelkunov et al.,  
59 2009; Kurobe et al., 2011; LaPatra et al., 2014).

60 Actually, herpesviral infection in sturgeons is attributable to two different viruses, the  
61 Acipenserid herpesvirus-1 (AciHV-1) and the Acipenserid herpesvirus-2 (AciHV-2; Kurobe et al.,  
62 2008). In addition, several iridolike viruses have been detected in sturgeons. The first and most  
63 studied iridolike virus associated with mortality outbreaks of sturgeon (*A. transmontanus*) was the  
64 white sturgeon iridovirus (WSIV) that has been reported in farmed and wild white sturgeon of the  
65 Pacific Coast from California to Canada (Hedrick et al., 1990; LaPatra et al., 1994; Raverty et al.,  
66 2003). Other iridolike viruses have been detected in different sturgeon species in North America

67 such as the Missouri River sturgeon iridovirus (MRSIV) in pallid sturgeon (*Schaphirhynchus albus*)  
68 and shovelnose sturgeon (*S. platorhynchus*) and the shortnose sturgeon virus (SNSV) in shortnose  
69 sturgeon (*Acipenser brevirostrum*). A recent phylogenetic study, however, showed that the viruses  
70 WSIV, MRSIV and SNSV are only distantly related to *Iridoviridae*, and are included in a group  
71 actually referred to as sturgeon nucleo-cytoplasmic large DNA viruses (NCLDV) in the order  
72 *Megavirales*, a new term not formally adopted by the International Committee of the Taxonomy of  
73 Viruses (Clouthier et al., 2015). Further two viruses named British Columbia white sturgeon virus  
74 (BCWSV) and Namao virus (NV), detected in white sturgeon and lake sturgeon (*A. fulvescens*)  
75 respectively in North America, are also included in the sturgeon NCLDV group (Clouthier et al.,  
76 2013; 2015). Based on light and electron microscopy, iridovirus-like infection has also been  
77 reported, in Northern Europe in Russian sturgeon (*A. gueldenstaedtii*) associated with mortality  
78 (Adkison et al., 1998). Furthermore, similar outbreaks are frequently reported by sturgeon farmers,  
79 but only few thorough investigations have been conducted for these outbreaks. Sturgeon NCLDVs  
80 appear to be endemic in populations of *Acipenseridae* found throughout North America (Clouthier  
81 et al., 2015). At present, a true picture of the geographical distribution of sturgeon NCLDV  
82 infection in Europe is not available.

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

88

## 89 **Materials and methods**

### 90 *Clinical history and sampling*

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

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

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

117

118 *Histopathology*

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

125

### 126 *Bacteriological examination*

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

144



145 *RNA, DNA extraction and virus detection*

146 Fresh organs (brain, spleen, heart, kidney, gill, skin and fins) from diseased fish (AG-2, AB,  
147 AG-3) as well as recovered fish (AG-1, AG-2rec, ABrec) were collected for RNA and DNA  
148 extraction. Tissues were stored at  $-80^{\circ}\text{C}$  until processing for nucleic acid extraction. Three fish  
149 from each batch were tested individually.

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

164 Deoxyribonucleic acid was extracted from about 25 mg of tissue using the Purelink  
165 Genomic DNA kit (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. The  
166 DNA was used soon after the extraction or stored at  $-20^{\circ}\text{C}$  until use. Five  $\mu\text{l}$  of DNA extracted  
167 from skin, fin and gill pools (batches AG-2, AB) was subjected to two PCR analyses with taq  
168 polymerase (Invitrogen, Carlsbad, USA) to detect herpesviruses and iridoviruses following  
169 previously described protocols (Dospoly et al., 2008; Bigarré et al., 2016). Particularly, for

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

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

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

227

#### 228 *Bacteriological examination*

229 A few small, white, convex, circular colonies were grown on the TSA plates seeded with  
230 brain and kidney specimens from batches AG-2 and AB. Additionally, small, convex, circular  
231 colonies with slight brown pigmentation were grown on the TSA plates seeded with kidney samples  
232 from batch AG-2. Four white colonies, two isolated from kidneys of *A. gueldenstaedtii* (AG-2/C11,  
233 AG-2/C12) and two from *A. baerii* (one from brain AB/C15 and one from kidney AB/C18), and  
234 one colony with slight brown pigmentation isolated from brain samples of *A. gueldenstaedtii* (AG-  
235 2/C4) were selected for further characterisation. No colonies were grown on the TSA plates seeded  
236 with brain and kidney samples of recovered fish (AG-1, AG-2rec, ABrec) or diseased fish of batch  
237 AG-3.

238 Isolates AG-2/C11, AG-2/C12, AB/C15 and AB/C18 were bipolar, Gram negative, oxidase  
239 negative, catalase positive, non-motile and nitrate negative. Comparison of a 811 bp fragment of  
240 16S rDNA gene sequences showed a 100% nucleotide identity among isolates AG-2/C11, AG-  
241 2/C12 and AB/C15, whereas a nucleotide identity of 99.2% was revealed between AB/C18 and the  
242 other isolates. Molecular identification of these isolates after Blasting in NCBI showed 100%  
243 identity of AG-2/C11, AG-2/C12 and AB/C15 with *Acinetobacter johnsonii* sequences (GenBank  
244 accession numbers: CP010350, KP296204, LN774684) and 100% identity of AB/C18 with  
245 *Acinetobacter tjernbergiae* sequences (GenBank accession numbers: KU312797, KU337241).

246 The isolate AG-2/C4 was oxidase positive, catalase negative, motile and nitrate positive.

247 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

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

281 A comparison between sequences of PCR products obtained with the two independent PCR  
282 protocols for AG-2 and AB batches showed 100% nucleotide identity in the overlapping region.  
283 Comparison among viral sequences of the 208 bp MCP gene fragment (Sifor/rev PCR products  
284 without primer sequences) of different batches showed 100% nucleotide identity except for the AG-  
285 1 sequence which showed 99.5% nucleotide identity. However, the deduced amino acid sequence  
286 comparison showed a 100% identity among all viruses detected in this study in different batches  
287 and sturgeon species (Table 2). The assembling of three neighbouring sequence fragments, the two  
288 obtained with the assay developed by Bigarrè (fragments A and B) and one obtained with the  
289 method developed in the manuscript permitted to obtain a 1240 bp sequence for the virus detected  
290 in AG-2 and AB batches that showed 100% nucleotide identity to each other. A comparison among  
291 sequences of the 1240 bp MCP gene fragment of sturgeon viruses detected in this study for AG-2  
292 and AB batches, hereafter named European sturgeon NCLDV, with North American sturgeon  
293 NCLDV sequences retrieved from the GenBank database showed the highest nucleotide identity  
294 with Namao virus and British Columbia white sturgeon virus (87.9%) and the lowest value with  
295 white sturgeon iridovirus (73.6 %). Similarly, the highest deduced amino acid identity was with  
296 Namao virus (96.5 %) and the lowest was with white sturgeon iridovirus (79.5%) (Table 3).

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

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

303

## 304 **Discussion**

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

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

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

326 genus that has gained increasing attention in recent years as a result of its potential to cause severe  
327 infections in a wide range of hosts including fish (Kozínska et al., 2014). However, members of the  
328 genus *Acinetobacter* are also commonly reported in soil, healthy fish, and water (Kozínska et al.,  
329 2014), and are usually perceived as normal saprophytic microorganisms. In this respect, their  
330 isolation from altered skin and diseased fish could be the result of microorganism penetration into  
331 internal organs after skin damages (Kozínska et al., 2014). Nevertheless, the role of some  
332 *Acinetobacter* species such as *A. baumannii* and *A. johnsonii* as pathogens for freshwater fish has  
333 been well documented in the last years (Xia et al., 2008; Kozínska et al., 2014). In our study, the  
334 involvement of *Acinetobacter johnsonii* in *A. gueldenstaedtii* (batch AG-2) and *A. baerii* (batch  
335 AB) and *Acinetobacter tjernbergiae* in *A. baerii* was shown by molecular characterisation. Some  
336 lesions observed in the outbreak described in this study, such as multifocal ulceration with red  
337 margins (*A. gueldenstaedtii* and *A. baerii*) and mild hyperaemia around the mouth and at the base of  
338 the pectoral fins (*A. gueldenstaedtii*), have been previously described associated with natural and  
339 experimental infections with *Acinetobacter johnsonii* and *A. baumannii* (Kozínska et al., 2014).  
340 *Acinetobacter* infected fish were also infected with the European sturgeon NCLDV, however, due  
341 to the considerable presence of these bacteria in tested specimens and the type of observed lesions,  
342 we hypothesise a contributory role of *Acinetobacter* in the type and severity of lesions in this  
343 outbreak. This hypothesis is supported by the observation that *Acinetobacter* infection was not  
344 detected in a different batch of *A. gueldenstaedtii* (AG-3) involved in a disease outbreak, with lower  
345 mortality, observed in the same farm.

346         Viral screening conducted through molecular methods permitted us to exclude the  
347 involvement of viruses previously detected in sturgeons and occasionally associated with mortality  
348 outbreaks such as betanodaviruses, IPNV and SVCV (Nougayrede, 1988; Athanassopoulou et al.,  
349 2004; Vicenova et al., 2011). We also excluded the presence of herpes-like viruses, which have  
350 been frequently involved in mortality outbreaks in several sturgeon species both in North America  
351 and in Europe, including Italy (Hedrick et al., 1991; Watson et al., 1995; Shchelkunov et al., 2009;



352 LaPatra et al., 2014). Two independent molecular analyses confirmed the presence of viral DNA  
353 related to sturgeon iridoviruses in analysed specimens, actually best defined as sturgeon NCLDVs  
354 (Clouthier et al., 2015). While histology can not distinguish among etiological agents, it suggested a  
355 viral-induced lesion, in fact, the morphology and the location of hypertrophic epithelial cells and  
356 necrosis of pillar cells are similar to those described by Clouthier et al. (2013), Raverty et al. (2003)  
357 and Hedrick et al. (1990) associated with viral infections. The developed PCR-based assay was  
358 successfully used to track the viral infection in diseased and surviving fish. The analysis of several  
359 tissues from a wide range of fish on the farm involved in the outbreaks provided data that may lead  
360 to some interesting pathological and epidemiological insights about this infection. *Acipenser*  
361 *gueldenstaedtii* and *A. baerii* were similarly affected, according to the number of positive animals  
362 out of those tested (3/3) and the tissue dissemination of viral DNA (skin, fins, gill, spleen and  
363 kidney). However, the infection had different impact on *A. gueldenstaedtii* and *A. baerii* according  
364 to total mortality and duration of the outbreak, with *A. gueldenstaedtii* being the more susceptible  
365 species. Specific viral DNA was easily detected in internal organs (spleen and kidney) of all  
366 diseased animals of both sturgeon species involved in the outbreak, consistent with a systemic  
367 infection, even though no viral-induced histopathological lesions were observed in these internal  
368 organs. White sturgeon iridovirus was reported as an epitheliotropic virus that does not result in  
369 systemic infection, but localises in epithelial cells of the integument (Hedrick et al., 1990; LaPatra  
370 et al., 2014). While consistent or specific histopathological lesions of WSIV infection were not  
371 detected in the liver or other internal viscera of challenged fish, a previous study showed that WSIV  
372 can be isolated from internal organs such as the kidney and spleen during an experimental infection  
373 (Watson et al., 1998). Furthermore, the ability of WSIV to replicate in splenic cells was previously  
374 proven due to its replication in a splenic cell line (Hedrick et al. 1991) even if WSIV showed better  
375 replicating ability in cell lines of different tissue origins (Watson et al., 1998). A complex lifecycle  
376 has been identified for North American sturgeon NCLDVs (Watson et al., 1998; Kurobe et al.,  
377 2011; Clouthier et al., 2015); similarly, for European sturgeon NCLDV detected in this study, we

378 observed two stages of infection: an acute, lethal disease (AG-2 batch) and a persistent, subclinical  
379 infection (AG-1 batch). Analysis of different sturgeon batches present in the farm showed the  
380 persistence of viral DNA in tissues of survivor *A. gueldenstaedtii* up to four months after the end of  
381 an outbreak with a different presence in tested tissues. These surviving fish also showed viral-  
382 induced lesions such as hypertrophic cytomegalic epithelial cells in the skin. Previous studies  
383 showed inconsistent results about the ability to detect WSIV infection in survivor fish (Kwak et al.,  
384 2006; Drennan et al., 2007). However, the persistence of viral DNA and the histologic lesions were  
385 shown in an experimental infection of *A. transmontanus* with WSIV up to nine months after  
386 exposure (Kwak et al., 2006). Similarly, MRSIV DNA was detected in recovered pallid sturgeon  
387 (*Scaphirhynchus albus*) about five months following the last mortality (Kurobe et al., 2011).  
388 Moreover, the authors demonstrated that the survivor fish were able to transmit the infection to  
389 juvenile pallid sturgeon by cohabitation (Kurobe et al., 2011). While a wide variability was  
390 observed among tissue samples, all studies mainly considered epithelial tissues (Kwak et al., 2006;  
391 Drennan et al., 2007; Kurobe et al., 2011), whereas viral persistence at the level of the internal  
392 organs has not been investigated so far for sturgeon NCLDV. In our study, viral DNA persistence  
393 time was dependant on tissue analysed, with a constant finding in skin, fin and gill pools of  
394 recovered *A. gueldenstaedtii* and poor findings in internal organs four months after the end of the  
395 outbreak. This finding is in accordance with a pathogenesis study on WSIV, in which histologic  
396 analysis, actually showed a prolonged presence of lesions in olfactory organs, gill, skin and  
397 esophagus compared to kidney, liver and spleen (Watson et al., 1998). Furthermore, the results of  
398 the investigation of viral DNA presence in the organs of recovered fish of the two analysed species  
399 argue for a different epidemiological role of *A. gueldenstaedtii* and *A. baerii* in maintaining the  
400 infection at the population level. Even though the *A. baerii* were collected a few weeks after the end  
401 of the outbreak, only one of them was positive for the viral DNA, while in contrast all *A.*  
402 *gueldenstaedtii* fish were positive up to four months after the end of the outbreak. However, further  
403 studies, based on large, statistically representative samples of the sturgeon fish population should be

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

406 Sequence analysis of an MCP gene fragment obtained from tested specimens showed a 99.5-  
407 100% nucleotide identity between viruses detected in *A. gueldenstaedtii* and *A. baerii*, proving the  
408 ability of the European sturgeon NCLDV to infect more than one species. Also, North American  
409 sturgeon NCLDVs are not species-specific, such as MRSIV that was found in both shovelnose  
410 (*Scaphirhynchus albus*) and pallid sturgeon (*S. platyrhynchus*) (Kurobe et al., 2010). Genetic  
411 comparison with North American sturgeon NCLDVs showed that the virus detected in this study  
412 can be completely included in this group. On the basis of the percentages of nucleotide and amino  
413 acid identities and the phylogenetic tree topology we could assume that WSIV may have evolved  
414 from a common ancestor of the European sturgeon NCLDV, detected in this study, and other  
415 sturgeon NCLDVs detected in North America (MRSIV, SNSV, BCWSV, NV). No relationship  
416 between genetic clustering and geographical distribution was revealed, in accordance with that  
417 previously reported for North American sturgeon NCLDVs (Clouthier et al., 2015).

418

## 419 **Conclusions**

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

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

436

#### 437 **Conflict of interest statement**

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

440

441

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445

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1 **Table 1**

2 NCLDV's presence in tested specimens of different sturgeon species and batches.

3  
4

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

5

6 nd: not determined



1 **Table 2**

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

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		<b>Nucleotide identity</b>			
		<b>AB</b>	<b>AG-1</b>	<b>AG-2</b>	<b>AG-3</b>
<b>Amino acid</b>	<b>AB</b>		99.5	100.0	100.0
	<b>AG-1</b>	100.0		99.5	100.0
	<b>AG-2</b>	100.0	100.0		100.0
	<b>AG-3</b>	100.0	100.0	100.0	

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1 **Table 3**

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

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

1 **Figure captions**

2

3 Fig. 1. Timing diagram of outbreaks and sampling. The thick arrows represent sturgeon batches.

4 Dotted area represent outbreaks' time. Thin arrows represent sampling time.

5

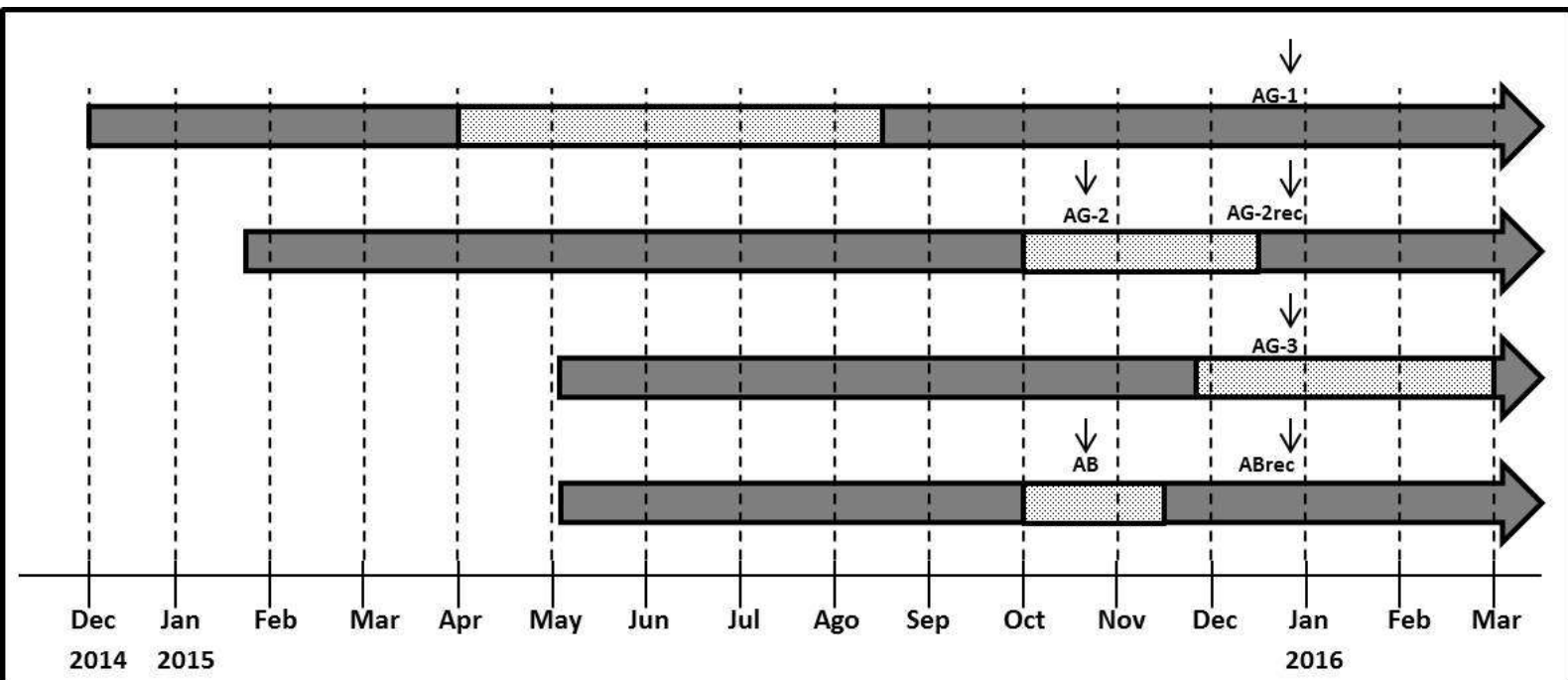
6 Fig. 2. (A) *A. gueldenstaedtii*, dorsal fin, lateral view. Multifocal ulceration with red margins are  
7 present on bony plates. (B) *A. baerii*, dorsal fin, dorsal view. Exfoliating white-yellowish cutaneous  
8 strands are evident on the dorsal fin. (C) *A. gueldenstaedtii*, gills. Lamellar hyperplasia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

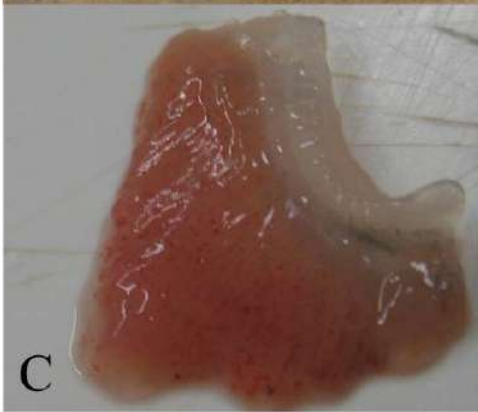
12

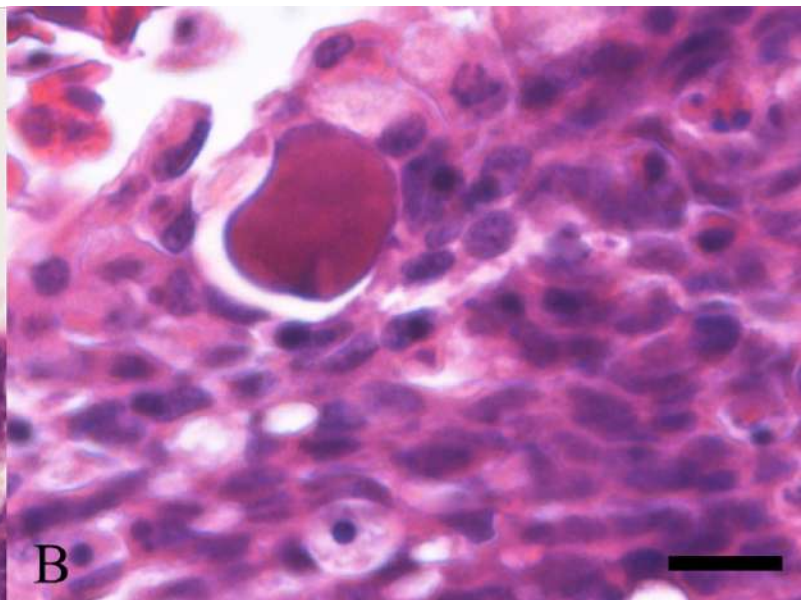
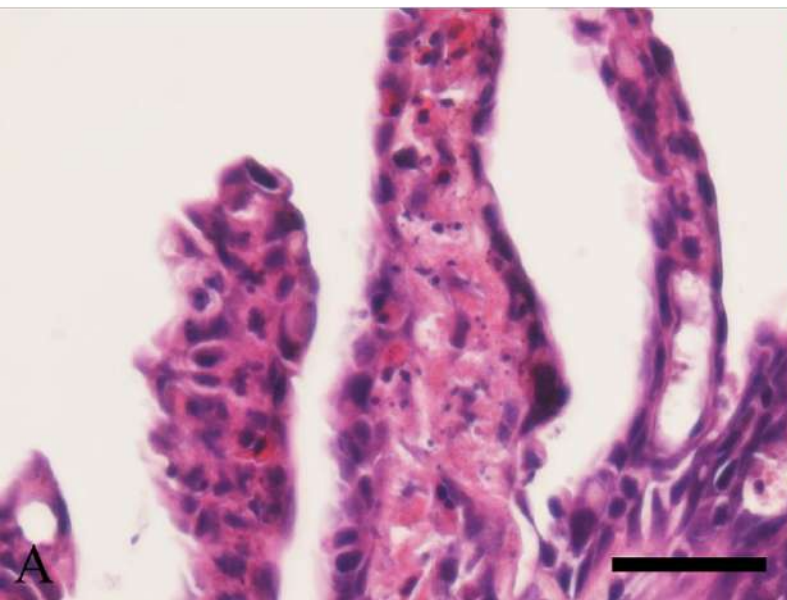
13 Fig. 3. (A) *A. gueldenstaedtii*, gills. Severe and diffuse degeneration and necrosis of pillar cells  
14 lining lamellar vascular channels; numerous karyorrhectic debris are evident (scale bar=50µm). (B)  
15 *A. gueldenstaedtii*, gills. An hypertrophic cytomegalic epithelial cell with homogeneous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

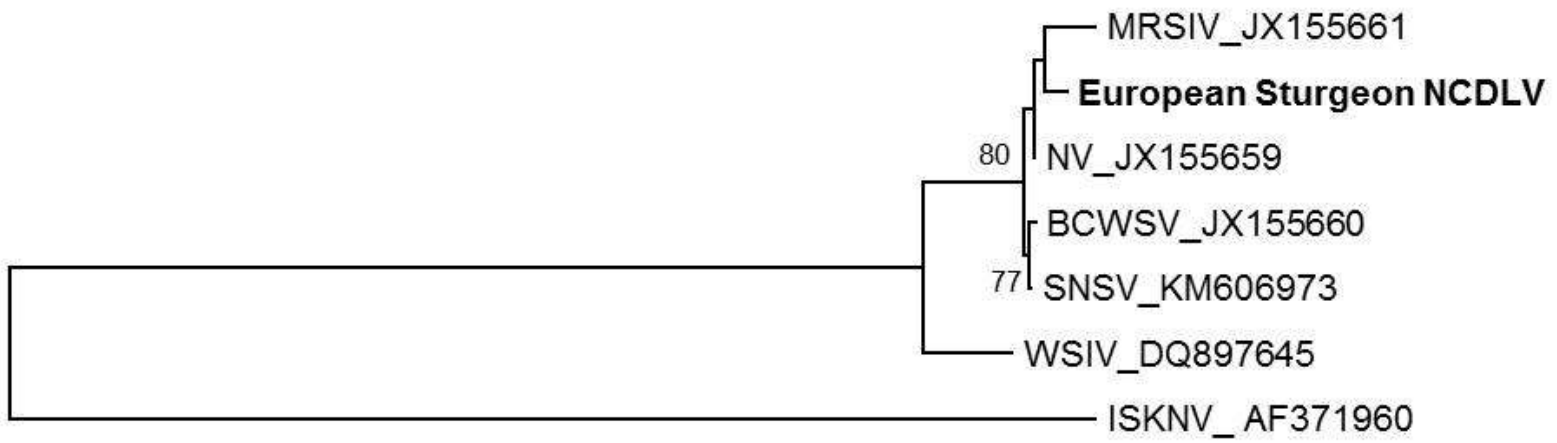
17

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









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