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Outbreak of mortality in Russian (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeons associated with sturgeon nucleo-cytoplasmatic 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 NCLDV. 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) (Rzepkowska 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 nucleocytoplasmic 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 m³ 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°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°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 μl of RNA. Thermal cycling conditions were adapted to
158 a suitable annealing temperature: 58°C (betanodaviruses), 55°C (SVCV) and 60°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°C until use. Five μl 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 (Doszpöly 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 MgCl₂, 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). 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

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* x *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 coinfecting 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

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;

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 NCLDV (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

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 NCLDV is 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

Batches	Clinical signs	Species	skin, fin and gill pool	Gill	Spleen	kidney
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

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 NCLDV_s 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 NCLDV. BCWSV: British Columbia white sturgeon virus (JX155660); NV: Nanao 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	87.9	87.2	86.2	73.6
	95.0	96.5	95.5	92.2	79.5

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

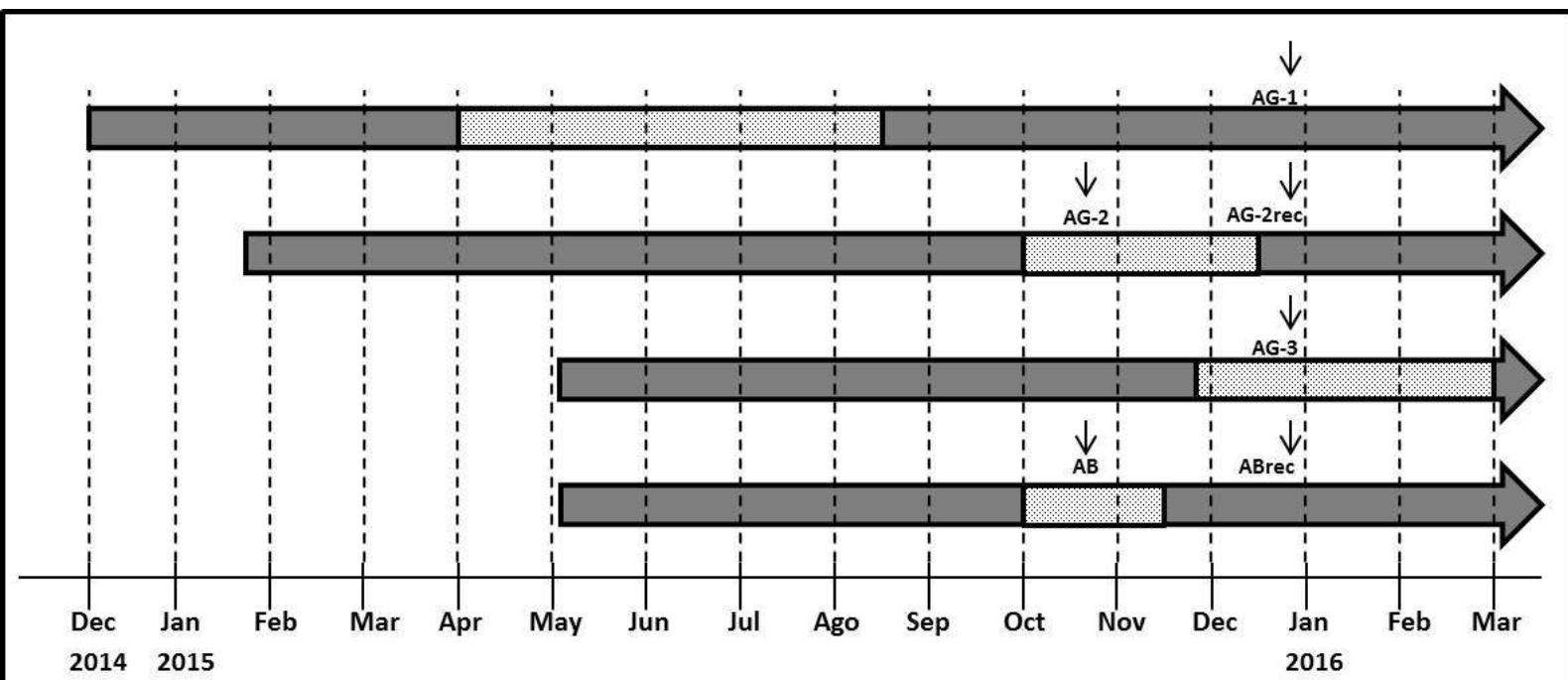
6 Fig. 2. (A) *A. gueldenstaedtii*, dorsal fin, lateral view. Multifocal ulceration with red margins are
7 present on bony plates. (B) *A. baerii*, dorsal fin, dorsal view. Exfoliating white-yellowish cutaneous
8 strands are evident on the dorsal fin. (C) *A. gueldenstaedtii*, gills. Lamellar hyperplasia 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).

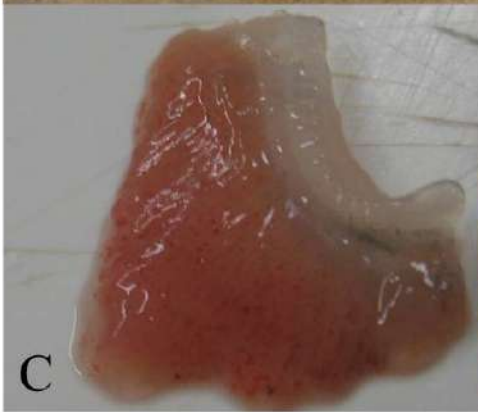
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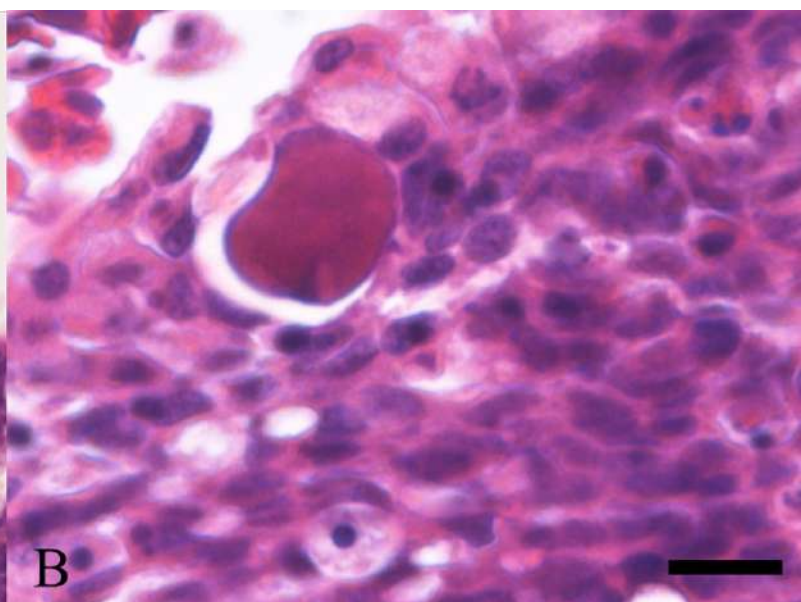
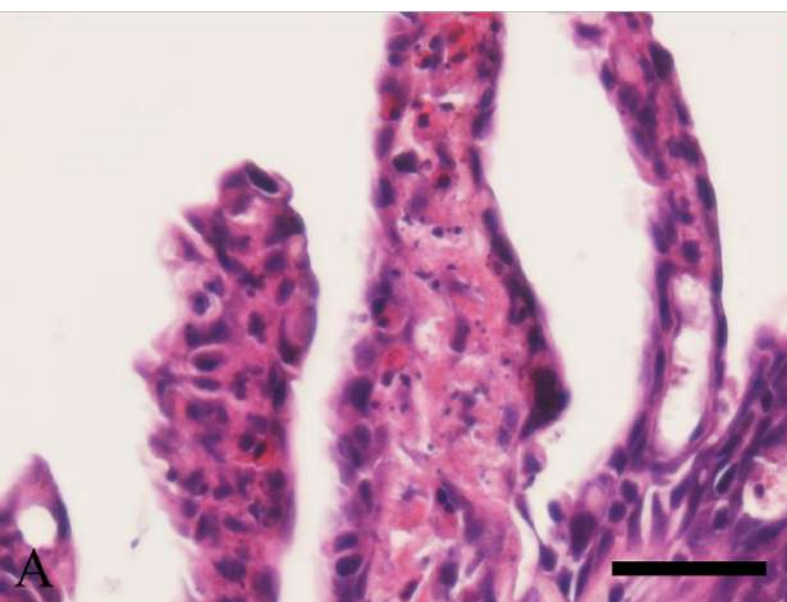
13 Fig. 3. (A) *A. gueldenstaedtii*, gills. Severe and diffuse degeneration and necrosis of pillar cells
14 lining lamellar vascular channels; numerous karyorrhectic debris are evident (scale bar=50µm). (B)
15 *A. gueldenstaedtii*, gills. An hypertrophic cytomegalic epithelial cell with homogeneous
16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

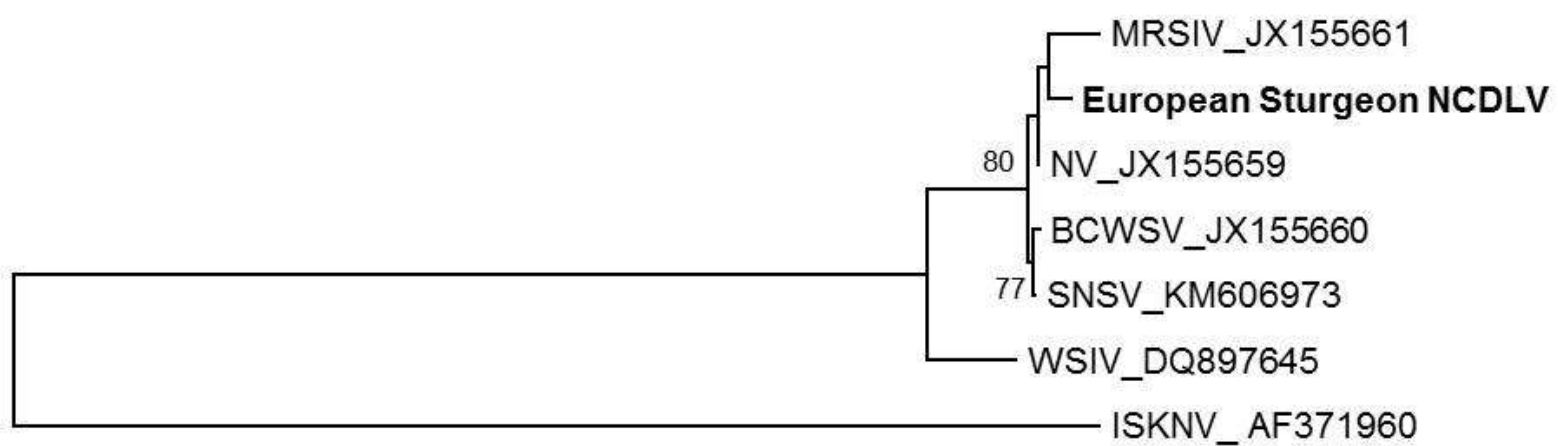
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18 Fig. 4. Phylogenetic tree constructed with the deduced amino acid sequences of an MCP gene
19 fragment (413 amino acid) of the European sturgeon NCLDV detected in this study (bold) and
20 other North American sturgeon NCLDV. Genbank accession number 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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