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7 Presence of viruses in wild eels from the Albufera Lake (Spain)

8 I. Bandín [1*], S. Souto[1], J. M. Cutrín[1], C. López-Vázquez[1], J. G. Olveira[1], C. Esteve[2], E.
9 Alcaide[2], C.P. Dopazo[1]

10 1. Unidad de Ictiopatología -Patología Viral, Departamento de Microbiología y
11 Parasitología, Instituto de Acuicultura, Universidad de Santiago de Compostela, 15782
12 Santiago de Compostela, Spain

13 2. Departamento de Microbiología y Ecología, Universidad de Valencia, E-46100
14 Burjassot, Valencia, Spain

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16 *Corresponding author
17 Tel: +34881816087
18 Fax: +34881816047
19 e- mail: isabel.bandin@usc.es
20
21 co-authors e-mail: sandra.souto@usc.es, juanm.cutrin@usc.es,
22 mdelcarmen.lopez.vazquez@usc.es, jose.olveira@usc.es, maria.esteve@uv.es,
23 elena.alcaide@uv.es, carlos.pereira@usc.es
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26 Running title: Viruses in wild eels
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Abstract

A virological analysis was conducted on wild eels from the Albufera Lake (Spain). A total of 179 individuals at different growth stages were collected in two different surveys (2004 and 2008). Presence of anguillid herpesvirus (AngHV-1), aquabirnavirus, and betanodavirus was confirmed by PCR procedures in both surveys, although the number of detections was clearly higher in 2008 (83% of the eels analyzed resulted positive for virus presence). Ang HV-1 was the viral agent most frequently detected, followed by aquabirnaviruses. Betanodavirus were detected by the first time in wild eels and although the detections were only made by nested-PCR high percentage of positives were achieved. In addition, in 2008 seven aquabirnaviruses were isolated. Phylogenetic analysis performed using partial sequences of both genomic segments of aquabirnaviruses indicated that the seven isolates could be typed as WB (genogroup I) on the basis of segment A sequences, but when segment B was used six of them clustered with C1 strain (genogroup V) and one was typed as Ab (genogroup II). These results indicate natural reassortment between different strains of aquabirnaviruses in the eels. Although betanodavirus were not isolated in cell culture the analysis of the sequence of the nested PCR product indicated that they clustered with SJNNV genotype. The diversity of viral agents and the high level of viral detections suggest that viral infections may play a more prominent role in European eel declining than initially thought.

Key words: wild eels, *Anguilla anguilla*, Anguillid herpesvirus, Aquabirnaviruses, betanodaviruses

56 **Introduction**

57 Eels are catadromous fish species, spending the majority of their life cycle in fresh
58 water or estuaries, and returning to the sea to spawn. The European (*Anguillla anguilla,*
59 L.) and American eels (*A. rostrata,* Lesuer) share the same spawning grounds and travel
60 thousands of kilometers to the Sargasso Sea (McCleave & Leckner, 1987; Tesch &
61 Wegner, 1990). At the onset of migration, adult eels undergo a number of
62 morphological changes, including skin colour change, in particular a "silvering" of the
63 belly, increase in eye size, a reduction in the gut and development of the gonads (Van
64 Ginneken & Maes, 2005).

65 Over the last three decades there has been a general decline in eel stocks worldwide,
66 with dramatic declines of abundance of European eels, and in a lesser extent Japanese
67 eels. Although it is difficult to ascribe clearly identifiable reasons for this phenomenom,
68 the decline has been attributed to a number of possible explanations such as habitat
69 reduction, climatic variation, pollution, overfishing, and more recently, infectious
70 diseases (Dekker 2003a, b; Van Ginneken *et al.* 2005; Esteve & Alcaide 2009; Haenen
71 *et al.* 2009; Jakob *et al.* 2009; Székely *et al.* 2009; Haenen *et al.* 2010; van Beurden *et*
72 *al.* 2012).

73 Eels are susceptible to infections by different bacteria, parasites and viruses.
74 Outbreaks of many of the diseases are temperature dependent and those most
75 thoroughly studied occur under aquaculture conditions (Richards, 1978). However,
76 viruses have not received much attention. A number of viruses have been isolated from
77 farmed and/or wild European eel, including aquabirnavirus, betanodavirus, the
78 rahbdoviruses eel virus America (EVA), eel virus European-X (EVEX) and viral
79 haemorraghic septicaemia virus (VHSV), and the herpesvirus *Anguillid herpesvirus*-1
80 (Ang HV-1) (Sano *et al.* 1977; Castric *et al.* 1992; Jørgensen *et al.* 1994; Davidse *et al.*

1999; Chi, Shieh & Lin, 2003; Van Ginneken *et al.* 2004, 2005; Haenen *et al*. 2009, 2010; Jakob *et al.* 2009). Reports on viral detections or isolations from wild eels have so far been restricted to western, central and northern Europe (Castric & Chastel, 1980; Jørgensen *et al.* 1994; Scheinert & Baath, 2004, 2006; Lehmann *et al.* 2005; Van Ginneken *et al.* 2005; Jakob *et al.* 2009; Haenen *et al.* 2010). This study reports the first virological analysis conducted on a wild eel stock in South Europe, from the Albufera lake (Spain), a freshwater lake separated from the Mediterranean Sea by a small littoral bar.

Material and Methods

Fish

A total of 199 wild eels were collected from the Albufera lake in two different years 2004 and 2008. In 2004 sampling was performed only in autum and winter months, whereas in 2008 sampling was performed once a month all over the year, except in July and August. Sampled individuals comprised different stages of development and were classified into three size classes as described previously (Esteve & Alcaide, 2009): young yellow (undifferentiated eels), yellow (yellow individuals and also silvering males) and silver (silvering females). Fish were killed and aseptically dissected in the laboratory of microbiology of the University of Valencia, where bacteriological analysis and determination of presence of swim-bladder nematode parasites were performed. Immediately after dissection, samples of spleen/kidney, and brain of each individual fish were frozen in separate tubes and sent to the Instituto de Acuicultura of the University of Santiago de Compostela, where they were processed for virological examination.

Cell lines

Monolayers of chinook salmon embryo (CHSE-214), bluegill fry (BF-2), rainbow trout gonad (RTG-2), epithelioma papillosum cyprini (EPC), brown bullhead (BB), eel kidney (EK-1) and E-11 cells (a clone of the cell line striped snakehead SSN-1) were used for primary detection of the virus. All cell lines were grown in Eagle's minimum essential medium (EMEM, Hyclone Laboratories Inc., Logan, UT, USA), with the exception of E-11 which was grown in L-15 (Gibco, Scotland, UK). The media were supplemented with 10% foetal calf serum (FCS, BioWhittaker, Belgium), 100 I.U. ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. The CHSE-214, RTG-2, BF-2 cells were grown at 15°C, EPC and EK-1 at 20°C and BB and E-11 at 25°C. Semiconfluent monolayers of all cell lines in 24-well plates were maintained at 15°C, and the medium substituted by EMEM (1 ml per well) with 0 or 2% FCS for viral infection.

Virus isolation

From each individual, brain samples were used for betanodavirus isolation, and pools of spleen and kidney were tested for the presence of any other viruses. Tissue samples were mixed (1:10) with Earle's balanced salt solution (Hyclone Laboratories Inc.) supplemented with antibiotics (1000 UI/ml penicillin, 1000 μg/ml streptomycin, 500 μg/ml gentamycin and 10 μg/ml amphotericin B) and homogenised. After centrifugation of the homogenates at 2000 g for 20 min, the supernatants were transferred to new tubes, incubated for 4 hours at 15 ºC and inoculated (diluted at 10^{-1} and 10^{-2}) in duplicate onto semiconfluent monolayers of the cell lines in 24-well plates. The cultures were incubated at 15°C (CHSE-214, BF-2, RTG-2, EPC), 20ºC (EK-1, BB), or 25ºC (E-11) and examined daily to detect development of cytopathic effect (CPE). After 15 d, positive and negative samples (cultures showing CPE or no-CPE, respectively) were subcultured by inoculating 0.1 ml of the scraped cell suspension onto new cultures in 24-well plates. Subcultivations were terminated after 21 d of incubation.

131 Non-infected cells were used as controls. Crude virus, i.e. cell suspensions from
132 positive samples, was maintained at –70°C until use.

133 **PCR and RT-PCR assays.**

134 Total DNA was extracted from aliquots of the tissue samples using DNeasy kit
135 (Qiagen, Hilden, Germany) following manufacturer´s instructions, and subjected to
136 PCR using the specific AngHV-1 primers described by Rijsewijk *et al.* (2005).
137 Amplification was performed in a MyCycler™ Thermal Cycler (Bio-Rad, Hercules,
138 CA, USA) using the following parameters: initial heating at 95ºC for 4 min, followed by
139 40 cycles of denaturation at 95ºC for 30 s, annealing at 55ºC for 30 s and extension at
140 72ºC for 30 s with a final extension at 72ºC for 10 min. The PCR products were then
141 subjected to electrophoresis through a 1.5% SeaKem® LE agarose gel (FMC
142 Bioproducts, Philadelphia, PA, USA)

143 Total RNA was extracted from aliquots of the tissue samples using RNeasy kit
144 (Qiagen) following manufacturer´s instructions. Primer pairs used for RT-PCR were
145 $BA4_D$ and $BA4_U$ for IPNV (Cutrín *et al.* 2005) and F2-R3 (Nishizawa *et al.* 1994) for
146 nodavirus. Complementary DNA (cDNA) synthesis was performed by mixing the viral
147 RNA with 2.5 ng/µl of random primers (Promega) heating at 95ºC for 5 min and
148 incubating at 4ºC for at least 1 min. Then a reverse transcription mixture containing
149 Superscript III RT (Invitrogen) was added and incubated at 25° C for 10 min. The RT
150 reaction was performed 50 min at 50° C, followed by a 5 min denaturation (85 ºC) of
151 the DNA/RNA duplex, and the inactivation of RT enzyme. The PCR reaction was
152 performed using 4 µl of cDNA, 1.25 U of GoTaq® Flexi DNA polymerase (Promega)
153 and 0.5 µM of the specific primer set. Following an initial 4 min denaturation step at
154 94° C, the mixture was subjected to 40 cycles of amplification (30 s at 94°C, 30 s at

155 58°C, and 30 s at 72°C) with a final extension of 10 min at 72°C. PCR products were
156 subjected to electrophoresis as described above

157 The nested RT-PCR was performed following the same protocol as described
158 previously using 3 µl from the RT-PCR reaction and the specific primers BA4DI and
159 BA4UI for IPNV (Cutrín et al., 2005), and F21-R31 for nodavirus (Olveira *et al.* 2008).

160 **Genome sequencing**

161 Monolayers of CHSE-214 or BF-2 cells, grown in 25-cm^2 flasks were infected with
162 the corresponding viral strain. After extensive development of CPE, culture fluids were
163 harvested and centrifuged at 3,000 × g for 30 min to remove cellular debris, and the
164 supernatant ultracentrifuged at 100,000 × g for 2 h at 4°C. The pellet was resuspended
165 in 300 µL of SSC 1X (15 mM Sodium Citrate, 150 mM NaCl, pH 7.0). The viral
166 suspension was treated with 2µg µL^{-1} proteinase K (Sigma-Aldrich, St Louis, MO,
167 USA) and 0.05% SDS for 2 h at 56 °C. The viral RNA was extracted with phenol-
168 chloroform-isoamyl alcohol (pH 4.3) and ethanol precipitated overnight at -20°C, in the
169 presence of 0.3 M sodium acetate (pH 5.2). The pellet was washed with 70% ethanol,
170 centrifuged, dried in a Speedvac (Savant Instruments Inc., Holbrook, NY, USA) and
171 resuspended in 50 µL of SSC 1X. The extracted RNA was quantified by
172 spectrophotometry at 260 nm using a NanoDrop ND-1000 Spectrophotometer
173 (NanoDrop Technologies, Thermo Scientific, Wilmington, DE, USA) and stored at -20°
174 C.

175 The cDNA synthesis was performed as previously described using 10 ng µL^{-1} of
176 viral RNA. Two pairs of primers were used for PCR amplification of the VP2/NS
177 junction region of segment A of IPNV, HepF and HepR (Heppell *et al.* 1992) and P14
178 and P12 (Blake *et al.* 2001). For amplification of segment B, the primer set VP1_172F
179 and VP1_783R (5´CGGATACTTAGGCCGCGAGC3´ and 5´ TGCCGTCGTGTCTCCTTTGGT

180 3′, respectively), located between nucleotide positions 172 and 783 of the open reading
181 frame of the polymerase was used. In the case of betanodaviruses, owing to the absence
182 of viral isolates, the nested PCR products were used for sequencing.

183 Automated sequencing was performed using a CEQ™ 8000 Genetic Analysis
184 System (Beckman Coulter, Krefeld, Germany). Sequences were confirmed at least twice
185 by sequencing upstream and downstream and, to resolve any inconsistence a second set
186 of sequencing was performed. The sequences were edited using DNASTAR
187 Lasergene® v.7.1 SeqMan II and EditSeq (DNASTAR, Lasergene Inc, Madison, WI,
188 USA), and subjected to multiple sequence alignment using the DNASTAR Lasergene
189 v.7.1 MegAlign program (DNASTAR). Following alignment phylogenetic analysis of
190 nucleotide sequences was performed according to the neighbour-joining (NJ) method,
191 as implemented in TOPALi v2 software. The statistical support for each node was
192 evaluated by bootstrap analysis with 1000 replicates. The trees were constructed using
193 TreeView version 1.5 (University of Glasgow, Glasgow, U.K.).

194 For comparative purposes, nucleotide sequences of reference strains of
195 aquabirnaviruses and betanodavirus deposited in the GenBank were used (Table 1). An
196 Infectious bursal disease virus (IBDV) strain was used as outgroups in the phylogenetic
197 analysis of IPNV.

198 **Results**

199 In 2004 virological analysis was conducted on 62 eels (16 young yellow, 25 yellow
200 and 21 silver) and only 23% of the individuals were positive for any of the virus
201 assayed, as interpreted from the PCR results (Table 2). Considering the results by size-
202 classes, the lowest percentage of detection (6%) was observed in the undifferentiated
203 individuals. Only one young yellow individual harboured virus (it was positive for
204 betanodavirus). Higher percentages of virus-positive fish were observed in the older

205 individuals: 36% among yellow ones and 19% in silver stages. Most of the positive
206 yellow individuals (8 out of 9) were infected with AngHV-1, whereas IPNV was
207 detected in only one individual and betanodavirus in 2. Regarding the 8 infected
208 silvering females, 3 were positive for AngHV-1 and only 1 for betanodavirus. Finally,
209 one eel (a yellow individual) showed a mixed viral infection (AngHV-1+IPNV). No
210 virus was isolated in cell culture from any of the eels sampled after two blind passages.

211 No relationship was observed between the detection of virus and the presence of
212 bacteria and/or parasites in the eels. Only four of the 14 virus positive fish showed
213 bacterial infection and nematodes were observed in only two (data not shown).

214 The survey performed in 2008 included 117 individuals and, in this case, a much
215 higher percentage of eels (79%, corresponding to 93 individuals), resulted positive for
216 the presence of virus by PCR (Table 3). In addition, almost 60% of the virus positive
217 eels showed a concomitant bacteriological infection and/or parasitological infestation
218 (data not shown). Analyzing by size-classes, there was in fact a similar percentage of
219 detection observed in the three size classes, around 80%, although a slightly lower value
220 was obtained among the young yellow, as occurred in the first survey (Table 3).
221 AngHV-1 was the viral agent most frequently detected in all size classes, with
222 percentages of detection over 50% (Table 3). IPNV showed also a high level of
223 prevalence, with the highest values observed in the silvering females (43% by PCR and
224 57% by nested PCR), and decreased in younger ages, especially if only the RT-PCR
225 results are considered. Prevalences of betanodavirus were higher at the youngest stages,
226 and it is remarkable that virus was only detectable after nested-PCR amplification. It
227 was observed that the highest number of detections of AngHV-1 and nodavirus was
228 obtained from May to November when water temperatures ranged from 18 to 22.7ºC.

229 However, IPNV detections were distributed over the whole sampling period (water
230 temperatures ranging from 10 to 22.7ºC).

231 None of the samples developed CPE in first passage on any of the cell lines used 15
232 days after inoculation. After a blind passage there was evidence of CPE produced by 4
233 samples in CHSE-214 and by 1 in BF-2. In addition, 2 samples developed CPE in both
234 CHSE-214 and BF-2. Viral isolates were identified by PCR procedures. All 7 isolates
235 obtained from CHSE-214 and/or BF-2 were identified as IPNV. IPNV isolates were
236 obtained mainly from undifferentiated individuals (5), two of which were also infested
237 with the nematode *Anguillicoloides crassus*. The other two IPNV isolates were obtained
238 from healthy individuals, a yellow eel and a silvering female.

239 Partial nucleotide sequences of both genomic segments of the seven IPNV isolates
240 were analyzed and compared with those from reference strains available in GenBank.
241 For segment A, a 538 nt fragment corresponding to the VP2/NS junction region was
242 analyzed (GenBank accession nos. JF734364-JF734370) revealing that the seven IPNV
243 isolates showed a sequence identity of 99.8% with WB (accession no. AF342727),
244 reference strain of genogroup I, whereas the similarity with strains of the remaining
245 genogroups was lower (from 77.9 to 78.8 %) (Table 4). Comparison of a fragment of
246 498 nt corresponding to the VP1 region (segment B) showed differences among the
247 isolates. Thus, one (SpAa-IAusc1748.08, accession no. JF734360) had a total identity
248 (100%) with Ab strain (accession no. JF734350), the reference strain of Genogroup II,
249 whereas the remaining six isolates (accession nos. JF34357-34359 and JF34361-34363)
250 showed the highest similarity with the Genogroup IV (95.2 ± 0.2%).

251 Phylogenetic analysis based on partial sequences of segment A is shown in Fig 1A.
252 The tree constructed using an IBDV strain as an outgroup showed the seven genogroups
253 of aquabirnaviruses and revealed that the 7 eel IPNV sequences clustered with

254 Genogroup I (serotype A1). The phylogenetic analysis based on segment B (Fig. 1b)
255 also confirmed the 7 genogroups, but depicted different relationships among the eel
256 viral sequences. Thus, six were grouped with reference strain C1 (Genogroup IV) and
257 one (SpAA_IAusc1748.08) with genogroup II (reference strain Ab).

258 Betanodavirus fragments amplified from a selection of 10 fish were analyzed
259 and compared with those available in GenBank belonging to the four genotypes of
260 betanodavirus: striped jack nervous necrosis virus (SJNNV), red grouper nervous
261 necrosis virus (RGNNV), barfin flounder nervous necrosis virus (BFNNV) and tiger
262 puffer nervous necrosis virus (TPNNV) (Table 1). The sequences corresponded to a
263 RNA2 fragment of 179 nt within the T4 region obtained after nested PCR amplification.
264 The results obtained indicated that the ten eel sequences showed an identity of
265 99.6 ± 0.5% with the SJNNV genotype, whereas the similarity with the remaining
266 genotypes was clearly lower (65.2–71.7%).

267 **Discussion**

268 In recent years the assessment of the health status of the different European eel
269 populations has become a matter of concern as a potential factor involved in the decline
270 of eel stocks (Esteve & Alcaide 2009; Haenen et al., 2009; 2010; Jakob et al., 2009;
271 Székely et al., 2009, van Beurden et al 2012).

272 In a previous study, Esteve & Alcaide (2009) analyzed the impact of bacterial
273 diseases and parasites on the wild eel stock of the Albufera lake, and in the present
274 report we provide the first data on viral presence in that stock.

275 The viral agent showing higher prevalence was AngHV-1, although the percentages
276 of detection were quite different between the two surveys performed (18 and 53%, in
277 2004 and 2008, respectively). Two explanations could account for such a large
278 difference. First of all, the sampling period: in 2004 the fish were caught only from

279 October to December, whereas in 2008 the samples were collected throughout the year
280 (except July and August). In addition, samples from the 2004 survey were kept frozen
281 until the end of 2007, when virological analysis was performed, and this long storage
282 time could have had a negative effect on viral detection.

283 The presence of AngHV-1 in eels from natural habitats has been reported in
284 Germany (Scheinert & Baath 2004, 2006; Lehmann *et al.* 2005; Jakob *et al*. 2009) and
285 in The Netherlands (Haenen *et al.* 2010, van Beurden *et al.* 2012). The prevalence of
286 infection observed in the 2008 survey is a little bit higher than the values given by
287 Scheinert & Baath (2006) in Southern Germany (up to 48%) and by Haenen *et al.*
288 (2010) in the Rhine watershed in The Netherlands (40%), and is remarkably higher than
289 that observed by Jakob *et al.* (2009) in Northern Germany (2%). However, virus
290 infection was only detected by PCR and no virus isolation was achieved after two blind
291 passages in EK-1. PCR is commonly used and considered a good tool for the diagnosis
292 of AngHV-1 (Hangalapura *et al.* 2007; Jakob *et al.*, 2009; Haenen *et al.*, 2010) and it is
293 regarded as more sensitive than virus isolation (Haenen *et al.,* 2010). The high
294 sensitivity of the PCR technique coupled with the absence of viral isolation suggest that
295 the eels from the Albufera lake should be considered carriers of AngHV-1.

296 It is well known that herpesviruses are able to persist for long periods of time in
297 their hosts and to recrudesce if the carrier host is stressed. It has thus been proposed that
298 AngHV-1 can establish a latent infection in eels and might easily be reactivated under
299 stressful conditions (Van Nieuwstadt, Dijkstra & Haenen, 2001). Therefore, the
300 existence of such high levels of carrier eels in the Albufera lake in 2008 (overall
301 infection 54% and up to 58% in yellow individuals and silvering females) should be a
302 matter of concern. A temperature-dependent reactivation of latent AngHV-1 infection
303 has been previously discussed by different authors (Scheinert & Baath 2004; 2006;

304 Haenen et al., 2009; Jakob et al., 2009). In our study, we have detected by PCR a higher
305 number of carrier eels among fish collected when water temperature ranged from 18 to
306 22.7ºC (59%), compared to those collected at temperatures between 10 and 15ºC (41%),
307 which seems to support a temperature–dependent reactivation of AngHV-1. However,
308 the fact that the virus could not be isolated may suggest that it was not sufficiently
309 activated to replicate in cell culture.

310 The second most frequently detected viral agent was IPNV, with a prevalence of
311 22% by PCR and increased to 45% when re-amplification by nested PCR was applied
312 (data from the 2008 survey). In addition, virus was isolated from 7 individuals and
313 subjected to phylogenetic analysis using a fragment of both genomic segments. The
314 results obtained indicated that the seven isolates exhibited a different genotyping
315 depending on which segment was analyzed. Segment A-derived phylogenies
316 indicated that the seven strains clustered with Genogroup I, closely related to the
317 reference strain WB. However, analysis of segment B depicted completely different
318 phylogenetic relationships, and thus six isolates clustered with reference strains C1
319 (within Genogroup IV) whereas one isolate was related to reference strain Ab
320 (Genogroup II). These results indicate a natural reassortment between different
321 genogroups in the IPNV strains analyzed. It is interesting that most of the strains
322 harboured a C1-type segment B. The fact that European eel and American eel (*A.*
323 *rostrata*) share spawning grounds in the Sargasso Sea could account for the presence of
324 a Canadian-type segment in the IPNV strains isolated from the European eels caught in
325 the Albufera Lake. However, most of the IPNV isolates were obtained from yellow
326 individuals that have not initiated their migration to the Sargasso Sea. A possible
327 explanation for the presence of such strains in younger individuals could be the

328 transmission from infected silver eels to their progeny, as vertical transmission of IPNV
329 has been shown in other fish species (Reno, 1999).

330 Reassortment in IPNV has been previously described in an analysis of IPNV-like
331 strains isolated from different species of wild fish captured in the Flemish Cap,
332 Newfoundland (Romero-Brey *et al.* 2009). In that study, the existence of reassortant
333 strains harbouring a WB-type segment A and Ab-type segment B (WB/Ab) was
334 reported. Subsequent studies on aquatic birnaviruses isolated from wild fish in Galician
335 coastal waters (NW Spain) confirmed the presence of natural reassortants of the same
336 type in a larger proportion of the population than in the Flemish Cap (Cutrín *et al.*
337 2010).

338 Considering only segment A, the IPNV isolates obtained in the present study (typed
339 as WB) were completely different from the eel aquabirnaviruses reported previously.
340 Thus, Jorgensen *et al*. (1994) isolated a high number of aquabirnaviruses antigenically
341 related to A2 (Sp) and A3 (Ab) serotypes from wild European eels. In addition, eel virus
342 European (EVE), the etiological agent of branchionephritis in farmed eels, belongs to
343 A3 serotype of aquatic birnaviruses (Okamoto *et al.* 1983). EVE has been isolated
344 several times from farmed eels in Europe (Van Ginneken *et al.* 2004, Haenen *et al.*
345 2009, van Beurden *et al.* 2012) but there are no reports from wild eels (van Beurden *et*
346 *al.* 2012).

347 As expected most of the detections of betanodavirus were made in young individuals
348 because this viral agent usually affects larvae and juveniles (Munday, Kwang & Moody,
349 2002), and the prevalence values were considerably higher (40%) in the second survey
350 (2008). Nevertheless, it must be noted that this virus was only detected by nested-PCR,
351 which indicates that the viral load was very low. Betanodaviruses have been isolated
352 from cultured European eel by Chi et al. (2003) in Taiwan. These authors reported

353 typical symptoms of viral nervous necrosis (VNN) and mortalities in farmed eels in
354 both marine and freshwater environments, and the isolates were phylogenetically
355 characterized as RGNNV type. In that report, an increase in the water temperature was
356 considered an important factor in the outbreak of the disease.

357 In the present study the phylogenetic analysis of the viral genome sequences
358 obtained from wild European eels clustered with SJNNV type. Betanodaviruses
359 belonging to SJNNV type have been previously reported in Senegalese sole (Thiéry *et*
360 *al.* 2004; Cutrín *et al.* 2007) as well as gilthead sea bream cultured in the Iberian
361 Peninsula (Cutrín *et al*. 2007). However, subsequently some of these isolates were
362 demonstrated to be reassortant strains exhibiting a RGNNV-type RNA1 and a SJNNV-
363 type RNA2 (Olveira *et al*. 2009). Unfortunately, owing to the very low viral load, it was
364 not possible to sequence the RNA1 of the betanodaviruses detected in the present study

365 It has been reported that rearing water temperatures influence the development of
366 VNN (caused either by SJNNV or RGNNV genotypes) and that high temperatures (24-
367 28ºC) are probable predisposing factors of the disease (Arimoto, Maruyama &
368 Furusawa, 1994; Fukuda *et al*. 1996; Tanaka, Aoki & Nakai, 1998; Breuil *et al.* 2001).
369 In the case of the Albufera lake, the water temperatures when eels were caught ranged
370 from 10 to 22.7ºC, too low to favour the development of the disease. However, the high
371 percentages of nested PCR detection, especially in young individuals, means a risk of
372 epizootic outbreak if water temperature rises over 24ºC, which is not unusual in summer
373 time, or eels are subjected to other additional stressful conditions.

374 In the present study, a number of viral pathogens have been demonstrated to be
375 present in wild eels from the Albufera lake. The highest prevalence of the infection
376 corresponded to AngHV-1, as observed in other parts of Europe, but there were also a
377 high number of detections of aquabirnaviruses and betanodaviruses. These detections

378 suggest that viruses other than AngHV-1 may also be involved in the declining of the
379 European eel population, at least in South Europe.
380
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Table 1. Reference strains used in phylogenetic analyses.

Isolate	Genogroup/Genotype	Host	Accesion no.
IPNV			
WB ATCC	I	Trout	AF342727[†]/JF734348[‡]
JA ATCC	I	Trout	AF342735[†]/JF734356[‡]
Sp ATCC	III	Trout	AF342728[†]/JF734349[‡]
Ab ATCC	II	Trout	AF342729[†]/JF734350[‡]
HE ATCC	VI	Pike	AF342730[†]/JF734351[‡]
TE2 ATCC	IV	Tellina	AF342731[†]/ JF734352[‡]
C1 ATCC	IV	Trout	AF342732[†]/ JF734353[‡]
C2 ATCC	V	Trout	AF342733[†]/ JF734354[‡]
C3 ATCC	V	Arctic char	AF342734[†]/ JF734355[‡]
Y6	VII	Yellowtail	AY283781[†]/AY129662[‡]
IBDV			
OH		Chicken	430818[†]/420950[‖]
Betanodavirus			
SJ93Nag	SJNNV	Striped jack	AB056572[§]
SJ-G91	SJNNV	Striped jack	D30814[§]
SGWak97	RGNNV	Red-spotted grouper	NC_008041[§]
G9508KS	RGNNV	Red-spotted grouper	AY690597[§]
AH99NorA	BFNNV	Atlantic halibut	EF617329[§]
BF93Hok	BFNNV	Barfin flounder	EU826138[§]
TPKag93	TPNNV	Tiger puffer	D38637[§]

†Segment A sequence; ‡segment B partial sequence; ‖ segment B sequence
*Complete genome sequence, § RNA 2 sequence

Table 2. Viral detections in the 2004 survey

Stages of eel development[a]	No. samples	Virus detection [no positives (percentage)]				
		AngHV-1[b]	IPNV[c]	Betanodavirus	Fish positive for virus[d]	Co-infection[e]
Young Yellow	16	0	0	1 (6%)	1 (6%)	0
Yellow	25	8 (32%)	1(4%)	2 (8%)	9 (36%)	1 (4%)
Silver	21	3 (14%)	0	1 (5%)	4 (19%)	0
	62	11 (18%)[f]	1 (2%)	4 (6%)	14 (23%)	2 (3%)

AngHV-1, Anguillid herpesvirus; IPNV, Infectious Pancreatic Necrosis Virus

[a] Eels were classified as previously described (Esteve and Alcaide, 2009) in: Young Yellow (undifferentiated eels), Yellow (yellow individuals, both males and females, and also silvering males) and Silver (silvering females)

[b] results obtained by PCR

[c] results obtained by nested PCR

[d] fish harbouring any of the virus tested

[e] fish harbouring more than one of the virus tested

[f] percentage of positives over the total of samples analyzed

Table 3. Viral detections in the 2008 survey

Stages of eel development [a]	No. samples	Virus detection [no. positives (percentage)]				
		AngHV-1[b]	IPNV[c]	Betanodavirus[c]	Fish positive for virus[d]	Co-infection[e]
Young yellow	70	36 (51%)	14(25%)/31 (44%)	0 /33 (47%)	54 (77%)	33 (47%)
Yellow	40	23 (57%)	9 (22%)/18 (45%)	0/13 (32%)	33 (82%)	18 (45%)
Silver	7	4 (57%)	3 (43%)/4 (57%)	0/1 (14%)	6 (86%)	3 (43%)
	117	62 (53%)[f]	26(22%)/53(45%)	0/47 (40%)	93 (79%)	54 (46%)

AngHV-1, Anguillid herpesvirus; IPNV, Infectious Pancreatic Necrosis Virus

[a] Eels were classified as previously described (Esteve and Alcaide, 2009) in: Young Yellow (undifferentiated eels), Yellow (yellow individuals, both males and females, and also silvering males) and Silver (silvering females)

[b] results from PCR

[c] results obtained by RT-PCR/nested PCR

[d] fish harbouring any of the virus tested

[e] fish harbouring more than one of the virus tested

[f] percentage of positives over the total of samples analyzed

Table 4. Sequence similarities between partial sequences of both genomic segments of the seven IPNV isolates and reference strains of the seven genogroups of aquabirnavirus according to Nishizawa et al (2005)

Genogroup (strain*)	Segment A	Segment B	
	Seven IPNV isolates	Six IPNV isolates	SpAa_IAusc1748_08
I (WB)	99.8	81.5	80.9
II (Ab)	78.8	91.0	100
III (Sp)	78.1	83.1	81.5
IV (C1)	77.9	95.0	92.2
V (C2 & C3)	78.5±0.1	84.5	84.1±0.3
VI (HE)	78.4	80.1	79.7
VII (Y6)	84.6	82.9	81.9

*Strains used for the pairwise identity analysis

Figure

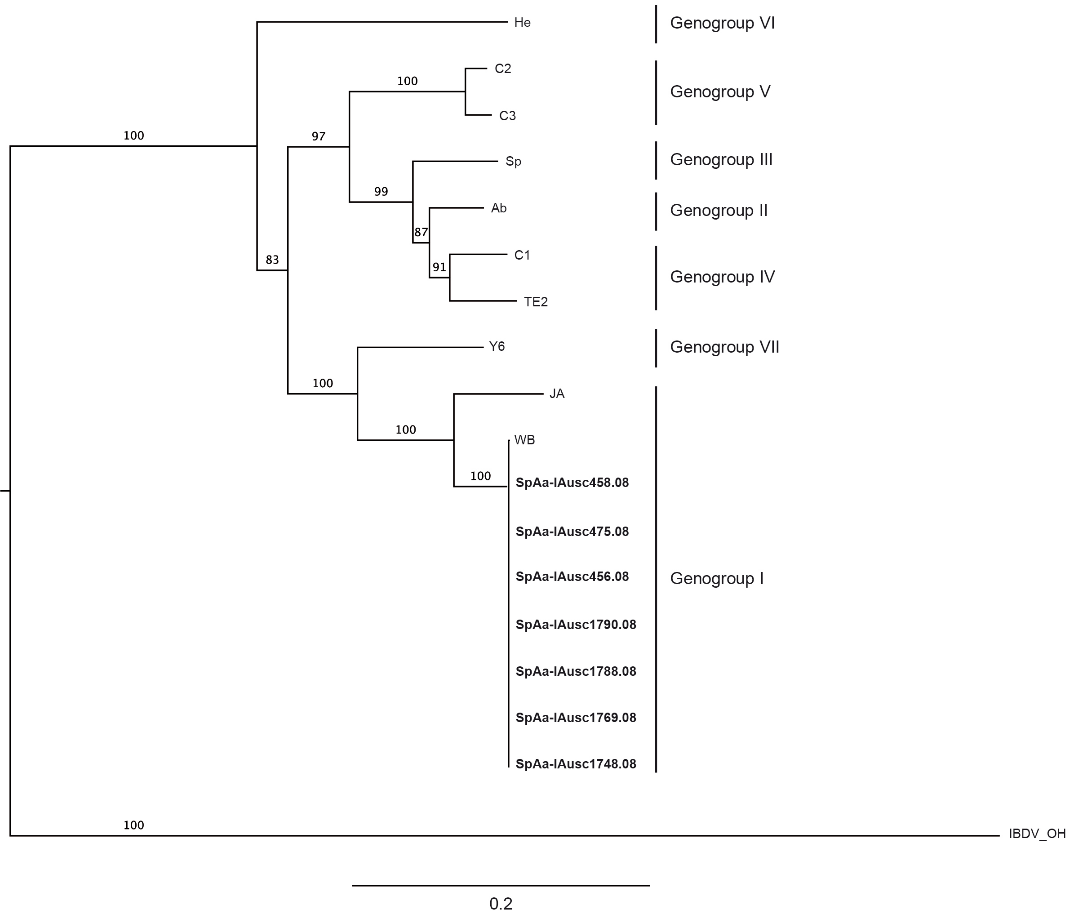

Fig 1A

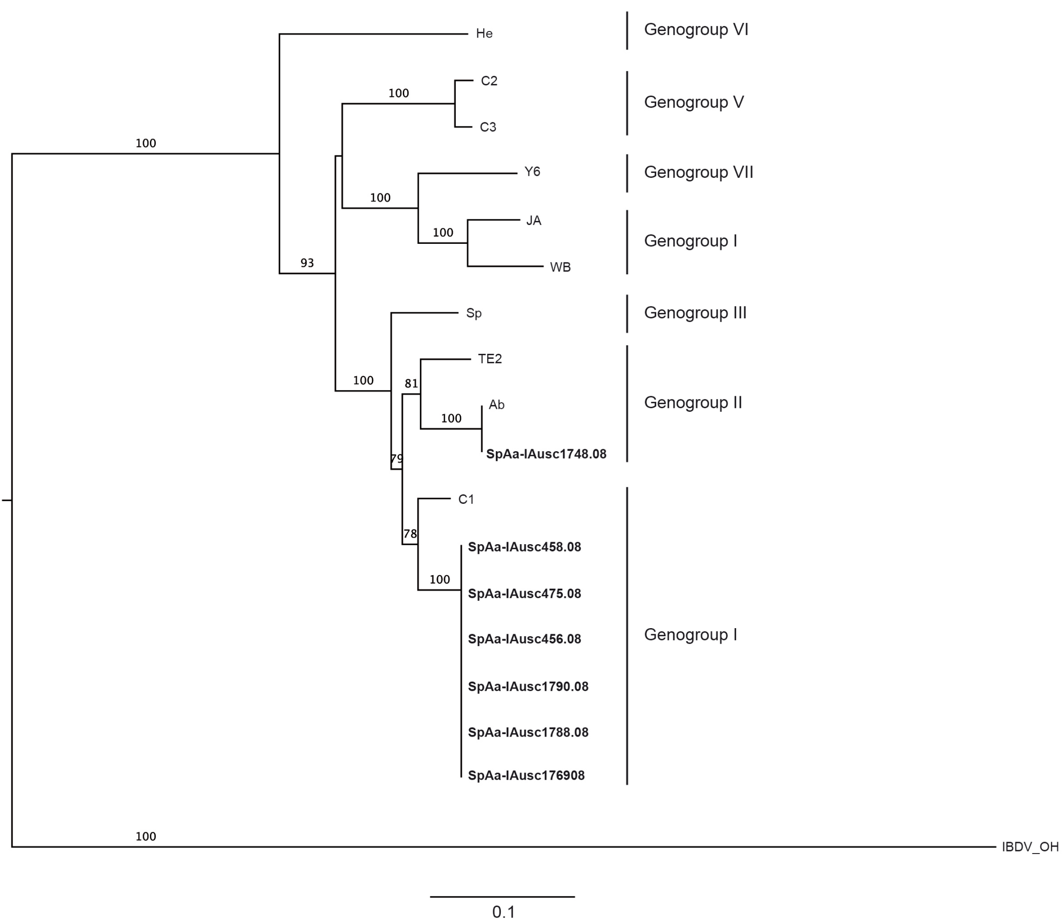

Fig 1B

Figure 1. Phylogenetic analysis performed on the eel aquabirnavirus iolates. (A) Segment A-based phylogeny (538 nt). (B) Segment B-based phylogeny (498 nt). Phylogeny was inferred by the neighbour-joining method included in TOPALi v2. and one IBDV strain and were used as the outgroup. Bootstrap values are presented as percentages of 1000 resamplings. Bars (A) 0.2 nucleotide substitutions per site. (B) 0.1 nucleotide substitutions per site. Genogroups established according to Nishizawa, Kinoshita and Yoshimizu (2005).