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1 **Genetic identification of two Acipenser iridovirus-European variants using high-resolution melting**  
2 **analysis**

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29 **Abstract**

30 Acipenser iridovirus-European (AcIV-E) is an important pathogen of sturgeons. Two variants differing  
31 by single-nucleotide polymorphisms (SNP) in the Major Capsid Protein gene have been described, but  
32 without any indication as to their prevalence in farms. To facilitate epidemiological studies, we  
33 developed a high-resolution melting (HRM) assay to distinguish between two alleles (var1 and var2)  
34 differing by five point substitutions. The HRM assay detected as little as 100 copies of plasmids  
35 harboring cloned sequences of var1 and var2, which have melting temperatures (T<sub>m</sub>) differing by  
36 only 1 °C. **The assay was specific of AcIV-E as demonstrated by the absence of signal when testing a  
37 related, yet distinct, virus as well as DNA from an AcIV-E-negative sturgeon sample.** Experiments with  
38 mixtures of two distinct plasmids revealed abnormal melting curve patterns, which showed dips just  
39 before the main melting peaks. These dips in the curves were interpreted as the dissociation of  
40 heteroduplexes fortuitously created during the PCR step. Screening AcIV-E-positive field samples of  
41 Russian sturgeons from three farms revealed the presence of var2, based on the T<sub>m</sub>. However, for a  
42 few samples, the melting curves showed patterns typical of var2 as the dominant viral genome,  
43 mixed with another minor variant which proved to be var1. In conclusion, HRM is a simple method to  
44 screen for AcIV-E var1 and var2 and can be used on a large scale in Europe to trace these two  
45 variants which likely represent two genetic lineages.

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49 **1. Introduction**

50 Acipenser iridovirus-European (AcIV-E) is a nucleocytoplasmic large DNA virus (NCLDV) infecting  
51 various sturgeon species in Europe (Bigarré et al., 2017; Ciulli et al., 2016). Its complete genome  
52 sequence has not been established; to date, only a 6 kb sequence encompassing the major capsid  
53 protein (MCP) gene and a replication factor C (RFC) gene is available (Pallandre et al., 2018). Based  
54 on this sequence, this virus has been shown to be related to other NCLDV sturgeon viruses found on  
55 the American continent, for instance the white sturgeon iridovirus (WSIV) and Namao virus (NV)  
56 (Bigarré et al., 2017; Clouthier et al., 2018; Pallandre et al., 2018). Since their initial observation by  
57 electron microscopy, these sturgeon NCLDVs have been classified in the *Iridoviridae* family, based on  
58 the shape of their particles (Clouthier et al., 2013; Hedrick et al., 1990). However, their MCP gene is  
59 very different from all other fish iridoviruses, and shows more genetic relationships with sequences  
60 from the *Mimiviridae* family, which are also members of the order *Megavirales*, as are NCLDVs  
61 (Colson et al., 2013). Recent genomic data confirmed the striking relationships of these fish viruses  
62 with *Mimiviridae* (Clouthier et al., 2018; Pallandre et al., 2018). Thus, it is likely that these sturgeon  
63 viruses will be reclassified and renamed in the near future.

64 To date, preliminary data indicate that AcIV-E is prevalent in European farmed sturgeons where it is  
65 associated with variable levels of mortality and morbidity, Russian sturgeon (*Acipenser  
66 gueldenstaedtii*) being particularly susceptible. The impact of the virus varies greatly, depending on  
67 the host species, the age of the fish, the presence of other pathogens and other factors still to be

68 identified. The genotype of the virus probably also influences virulence although poor data is  
69 available about the genetic diversity of AcIV-E.

70 In a previous study, two variants of AcIV-E showed single-nucleotide polymorphisms (SNPs), differing  
71 by at least five nucleotides in a particular region of the MCP gene (Bigarré et al., 2017). One variant  
72 (var1) carries the sequence *A<sub>GCCAATGT</sub>AAAC* and the second variant (var2) has the sequence  
73 *C<sub>GCCAATGT</sub>GCTT*. In a very small initial sample, the var2 allele was commonly found, whereas var1 was  
74 found in only three samples in co-infection with var2 in two host species from two countries. It is not  
75 known whether these two alleles reflect the existence of two viral lineages with many additional  
76 differences in the rest of the genomes or are simply variants of a unique lineage. However, the  
77 analysis of the RFC gene within a same viral population also strongly suggested the circulation of two  
78 lineages (Pallandre et al., 2018). In this view, the epidemiological analysis of molecular marker in the  
79 MCP is of interest to screen for a putative correlation between the lineages and virulence properties,  
80 or the lineages and geographic origin.

81 High-resolution melting (HRM) is a method commonly used for distinguishing SNPs in pathogens  
82 (Tong and Giffard, 2012). After amplification of a selected genomic region, a slow heat-driven fusion  
83 is carried out during which the fluorescence emitted by an intercalating (preferentially saturating)  
84 dye decreases as the DNA progressively switches from the double-stranded to the single-stranded  
85 state. The temperature at which half of the double-stranded DNA has melted (melting temperature,  
86 *T<sub>m</sub>*) is specific to a particular primary sequence. HRM has been used for distinguishing species within  
87 a genus or variants within a species for both RNA and DNA viruses (Gelaye et al., 2017; Lieveld et al.,  
88 2017; Tong and Giffard, 2012; Towler et al., 2010). This type of analysis requires a less laborious  
89 method than the sequencing of PCR products.

90 To distinguish between the two known variants of AcIV-E in infected tissues, we developed an HRM-  
91 based assay.

92

## 93 **2. Materials and Methods**

### 94 2.1 Samples

95 **Most** samples were from Russian sturgeons collected between **2016** and 2017. Gills, fins and/or  
96 mucus were collected from dead or moribund fish captured in three different French farms (F1,  
97 F2, F3) (Table 1). Two samples from 2015 (15/52 and 15/56) were used only to produce  
98 plasmids containing a portion of the viral genome.

### 99 2.2 Nucleic acid extraction from tissues and diagnostics

100 For one fish sampled in 2015 (15/52), total DNA was extracted at ANSES, using a NucleoSpin  
101 Blood kit (Macherey-Nagel) (Bigarré et al., 2017). Total DNAs from fish samples from 2016 were  
102 extracted at the Laboratoires des Pyrénées et des Landes using a NucleoSpin Tissue kit (Macherey-  
103 Nagel). Briefly, about 0.1 g of pooled organs (gills and fins) was crushed in lysis buffer using ceramic  
104 beads and a tissue homogenizer (Precellys 24, Bertin). A volume of 25 µL of this homogenate was  
105 incubated for 3 h at 56 °C with proteinase K. Nucleic acids (**NA**) were eluted in 100 µL of elution  
106 buffer. For samples from 2017, extracted at ANSES, organs were ground in PBS buffer (1:10, w/v)

107 in Lysing Matrix D tubes (MP Biomedicals) and shaken for 20 sec in a FastPrep FP120  
108 homogenizer (ThermoFisher). After a centrifugation at 5000 g for 3 min, 200 µL of the  
109 supernatants were extracted using the Nucleospin Virus kit (Macherey-Nagel) according to the  
110 provided protocol, except that the incubation with proteinase K was carried out for 15 min at  
111 70°C instead of 3 min at room temperature. Elution was performed in 50 µL of RNase-free  
112 water. Extracted nucleic acids (DNA and RNA) were quantified by spectrophotometry with a  
113 Nanodrop 1000 (ThermoFisher). **The presence of viral DNA was tested by real-time PCR with a  
114 Taqman probe as previously described (Bigarré et al., 2017).**

115

### 116 2.3 Plasmid construction and sequencing

117 **To evaluate the sensitivity of the PCR and the T<sub>m</sub> of the product of each variant, two plasmids**  
118 **previously constructed in 2015 were used (Bigarré et al., 2017). pPVP130 is a PCR4-TOPO**  
119 **plasmid (Invitrogen) in which was inserted a 1344 bp-PCR product containing a large portion of**  
120 **the AciV-E MCP gene (sample 15/52, allele var1) amplified with primers oPVP339 and oPVP344**  
121 **(Bigarré et al., 2017). pPVP131 is a similar plasmid with a PCR product from another sample**  
122 **(15/56, allele var2). Both plasmids were retransformed in *Escherichia coli* in 2017 to produce**  
123 **fresh solutions using a Nucleospin Plasmid kit (Macherey-Nagel). Plasmids were quantified with**  
124 **a spectrophotometer SmartSpec™ 3000 (BioRad). Sanger sequencing revealed a total of 15**  
125 **point variations between the viral sequence inserts, of which five were within the region**  
126 **amplified by PCR HRM, while the 10 others were outside this region. Therefore, the two**  
127 **amplicons differed only by five substitutions.**

128

129 For the analysis of the intra-sample viral diversity by clone sequencing, conventional PCR (cPCR)  
130 was performed on samples with the forward oPVP339 and reverse oPVP340 primers (Bigarré et  
131 al., 2017). This pair of primers targets a 636 bp portion of the MCP gene, including the variable  
132 region of interest. In a total volume of 50 µL, 500 ng of total DNA were used with 0.4 mM of  
133 each primer, 1.5 µM of MgCl<sub>2</sub>, 200 µM of dNTP and 2.5 U of TaqGold polymerase (Applied  
134 Biosystems). The following cycles were applied: 1 step of 8 min at 94 °C followed by 40 cycles at  
135 94 °C for 30 sec, 53 °C for 30 sec, 72 °C for 30 s and a final extension at 72 °C for 10 min. A  
136 volume of 10 µL was run on a precast 2% agarose E-gel (Invitrogen) for 15 or 30 min before  
137 observation under UV light. The 40 µL left were cleaned and eluted in 30 µL using a Nucleospin  
138 Gel and PCR Clean-up kit (Macherey-Nagel). A volume of 2 µL of the eluted product was used  
139 for TA cloning (Invitrogen). Plasmids from individual clones were purified using the Nucleospin  
140 Plasmid kit (Macherey-Nagel), and then sequenced using the Sanger method and a 3130  
141 Genetic Analyzer (Applied Biosystems). Sequences were verified and edited with VectorNT111  
142 (ThermoFisher).

143

144

### 145 2.4 HRM analysis

146 The forward oPVP440 (5' GTCTGAGGCCTTTGATGCAG 3') and reverse oPVP441 (5'  
147 AGCGCTTCAACATTTCAACTT 3') primers were designed to generate a 102 bp PCR product from  
148 the MCP gene, encompassing the variable region containing the SNPs. A PCR reaction was  
149 performed in a total volume of 20 µL with 5 µL of a plasmid solution or **1 to 2** µL of total nucleic  
150 acids from fish at 100 ng/µL and 400 nM of each HRM primer were used with the EvaGreen mix

151 (Bio-Rad) as recommended. On a Bio-Rad CFX96 thermocycler or on an Applied Biosystems  
152 QuantStudio5 (QS5), the amplifications were run using the following protocol: 98 °C for 2  
153 minutes followed by 40 cycles of 98 °C for 5 seconds and 60 °C for 5 seconds. Then, the double-  
154 stranded products were dissociated by ramping up the temperature from 65°C to 95°C with a  
155 0.1°C step every 5 seconds. All assays were performed in duplicates. For each run, a negative  
156 control (water) and two distinct positive controls (plasmids pPVP130 and pPVP131) were added,  
157 each in duplicates.

158 To test the specificity of the HRM primers, sturgeon samples producing no signal by taqman PCR  
159 were assessed. Additionally, a European sample (14/117) positive for a variant of NV was tested  
160 (Bigarré et al., 2017). Similarities with the published partial sequences of other NCLDV were  
161 searched by aligning DNA sequences: WSIV (Genbank DQ897645), NV from Canada (JX155659),  
162 NV from Europe (KU301309) and Acipenser herpesvirus type 2 from USA (FJ815289).

163 In a first step to evaluate the analytical sensitivity, serial 10-fold dilutions ( $10^8$  to 1) of each  
164 pure solutions of plasmids were assayed in duplicates using PCR-HRM in one session. In a  
165 second step, three selected concentrations ( $10^2$ , 10 and 1) of both pure solutions of plasmids  
166 replications were tested over series of sessions. The solutions of 100 and 10 copies were tested  
167 in 42 repeats over eight sessions with the QS5 while the one-copy solution was tested in 18  
168 replicates over five sessions. These same three concentrations were also used with 200 ng of  
169 total nucleic acids of a virus-negative sample in order to test the effect of host nucleic acids on  
170 the analytical sensitivity. A total of 24 replicates per solution was tested over 3 sessions.

171 In order to mimic co-infections of var1 and var2, various ratios (80:1; 40:1; 20:1; 10:1; 5:1; 1:1;  
172 1:5; 1:10; 1:20; 1:40; 1:80) of pure plasmids (diluted at  $10^4$  copies/ $\mu$ l) were mixed, with (200 ng)  
173 or without total host nucleic acids from a virus-negative sturgeon.

174 Amplification and melting curve data were generated and analyzed using CFX manager 3.1 (Bio-  
175 Rad) and QuantStudio Design & Analysis software v1.4.2 (Applied Biosystems). In addition, the  
176 High-Resolution Melt software v3.1 (Applied Biosystems) was used to obtain the normalized  
177 curves.

178

### 179 **3. Results**

#### 180 **3.1 HRM specificity**

181 A series of Russian sturgeon samples (48), previously determined as negative for AcIV-E with real-  
182 time TaqMan PCR, produced no signal in the PCR coupled with the HRM (PCR-HRM) assay (not  
183 shown). This result demonstrates that there is no cross-reaction of the HRM primers with the  
184 genome of the Russian sturgeon.

185 The HRM primers did not match the partial genome (66 kb) available for Acipenser herpesvirus 2,  
186 which is a pathogen infecting several sturgeon species (Dospoly et al., 2017; Dospoly et al., 2011).  
187 To estimate the possibility of cross-reactions of the HRM primers with homologous regions of viruses  
188 related to AcIV-E, the sequences of the oligonucleotides were aligned with the MCP sequences of NV  
189 and WSIV. The HRM primers had a total of 17 mismatches (7+10) with WSIV and therefore would not

190 amplify this portion of the genome under the stringent PCR conditions we used. Aligning with two  
191 MCP sequences of NV (from Canada and Europe), one HRM primer harbored only one mismatch, and  
192 the second had seven mismatches. The possibility of amplification was therefore very low, according  
193 to general recommendations for quantitative PCR (Lefever et al., 2013). Nevertheless, to verify non-  
194 amplification, PCR-HRM was performed on a fish sample infected with a European variant of NV. No  
195 signal was produced demonstrating the absence of cross-reactions between the HRM primers and  
196 the tested variant of NV (not shown).

### 197 3.2 Analytical sensitivity of HRM

198 Serial 10-fold dilutions ( $10^8$  to 1) of plasmids pPVP130 and pPVP131 were assayed using PCR-  
199 HRM. When one complete series was tested for each plasmid, the lower limits of detection  
200 were 10 copies for plasmids pPVP 130 and pPVP131 ( $R^2 = 0.999$  and  $0.996$ ; efficiencies =  $108.4$   
201 % and  $105.5$  %, respectively for the QS5 thermocycler;  $R^2 = 0.997$  and  $0.998$ ; efficiencies =  $98.7$  %  
202 and  $100.7$  % respectively for the CFX96). With more replicates for three selected  
203 concentrations, pPVP130 produced a positive signal for all repeats (42) at 100 copies and for 41  
204 out of 42 repeats at 10 copies (97.6%). With the plasmid pPVP131, a positive signal was  
205 produced at a frequency of 97.6% (41/42) at 100 copies and 78.5% (33/42) at 10 copies. When  
206 adding 1 to 100 copies of each plasmid to total nucleic acids from a virus-negative fish sample,  
207 similar sensitivities were obtained compared to pure plasmids: 100 copies were always  
208 detected for both plasmids (24/24), 10 copies detected at a frequency of 100 % and 71 % (24/24  
209 and 17/24 for pPVP130 and pPVP131 respectively) and 1 copy poorly detected (8 and 4 %).  
210 Therefore, the analytical sensitivity was at least 100 copies of plasmids. In addition, a series of  
211 10-fold dilutions (100 ng to 1pg) of nucleic acids from one virus-positive sample was tested by HRM,  
212 producing efficiencies of 106.2% ( $R^2 = 0.997$ ) and 108.9% ( $R^2 = 0.994$ ) with the QS5 and CFX96  
213 respectively. Therefore, the plasmids gives comparable results to those obtained with the viral  
214 genome mixed with the host NA.

215  
216 For plasmid copy numbers between  $10^8$  and 10, the mean  $T_m$  values obtained for the var1 and  
217 var2 were  $75.3 (\pm 0.19)$  and  $76.3 (\pm 0.35)$  respectively, using CFX96, and were  $76.2 (\pm 0.13)$  and  
218  $77.2 (\pm 0.09)$  using QS5 (Fig. 1). Thus, for a same thermocycler, the  $T_m$  values of the two distinct  
219 amplicons differed by 1 °C. For a same amplicon, the  $T_m$  values differed by 0.9 °C depending on  
220 the used thermocycler. Therefore, the two alleles were distinguishable by HRM. The  $T_m$  values  
221 obtained for different quantities of a given plasmid were almost identical at  $10^3$  copies and  
222 above, but dropped slightly at lower copy numbers.

223

### 224 3.3 HRM with plasmids mixed in different ratios

225 To simulate possible co-infections of viral variants within a same fish sample, controlled  
226 mixtures of plasmids were assayed using the HRM protocol. Different ratios of the two plasmids  
227 were analyzed using PCR-HRM. At all ratios, only one peak was observed on the melting curves  
228 between 73 and 80 °C, instead of the two peaks expected considering the presence of two  
229 distinct plasmid amplicons (Fig. 2a and 2b). Most likely, the resolution of the method was not  
230 high enough to separate the two peaks differing by only by 1 °C. Noticeably, at a 1:1 ratio, the  
231 unique peak was wider than those of the pure plasmids used as controls and showed a  $T_m$  value  
232 of 76.8 °C (QS5 platform), intermediate to the two expected individual  $T_m$  values (Fig. 2b). At

233 other (asymmetric) ratios (1:80 to 1:5), the observed  $T_m$  values were nearly identical to those  
234 of the most abundant plasmid (Fig. 2a). Therefore, the observation of a unique melting peak did  
235 not always indicate the presence of a single amplicon.

236 However, for ratios of 1:40 to 1:1, the melting curves showed an unusual pattern at  
237 temperatures lower than 73°C. Between about 70 and 73°C, thus at temperatures lower than  
238 the melting point of the dominant amplicon, a sudden dip occurred in the curves (Fig. 2a, 2c).  
239 **The different shapes of the curves obtained with these ratios and those of the positive controls**  
240 **were particularly visible after normalization using var1 as a reference (fig. 2d).** We interpreted  
241 this dip as the dissociation of a population of double-stranded (ds) DNA, which was likely  
242 composed of unstable heteroduplexes fortuitously created during the PCR. If this interpretation  
243 is correct, this dip in the curve simply indicates the presence of a mixture of distinct sequences  
244 in the DNA before amplification. Furthermore, there is no visible dip in the curve at plasmid  
245 ratios of 1:80, which is probably below the detection limit of the heteroduplexes. To  
246 demonstrate a direct link between the dip in the curve and the presence of heteroduplexes,  
247 further experimental assays were performed. Equal quantities of each plasmid were amplified  
248 by PCR and we tested their products in different conditions. When the two individual products  
249 were mixed after the PCR phase and subsequently dissociated by HRM, there was no dip in the  
250 melting curve at values less than 71°C (Fig. 3c), exactly as if the products had not been mixed at  
251 all (Fig. 3a). However, when the two individual products were mixed after the PCR, melted at  
252 98°C for 5 minutes, subsequently re-associated at 20°C for 15 minutes and then finally assayed  
253 using HRM, the characteristic dip was observed (Fig. 3d), exactly as when the plasmids were  
254 mixed before the PCR (Fig. 3b). Therefore, the artificial creation of heteroduplexes by heat-  
255 denaturing dsDNA, mixing and re-associating single-stranded DNA of two distinct amplicons  
256 induced the appearance of a marked dip in the melting curve plot before the main fusion peak.  
257 It was therefore concluded that this dip was associated with of a mixture of distinct sequences  
258 present before amplification.

259 The same plasmids ratios (1:80 to 1:1) were also tested with the presence of host NA. The  
260 **obtained**  $T_m$  values were similar compared to those from mixes of pure plasmids, varying  
261 **between 0.1 and -0.25 (data not shown).** Compared to the positive controls (pure plasmids +  
262 **NA), the  $T_m$  of the 1:1 ratios were intermediate as expected. For ratios 1:40 to 1:1, the curves**  
263 **showed the same dips than those observed with the plasmids without additional NA. Therefore,**  
264 **in our conditions, the presence of host NA did not prevent the detection of hybrids.**

### 265 266 3.4 HRM with field samples

267 When testing field samples, the two plasmids were used at  $10^4$  copies in separate wells as  
268 positive controls in order to check the efficiency of the PCR and as a reference for the  $T_m$  of  
269 each variant. A series of field samples from 2016 and 2017, determined as positive for AcIV-E  
270 using TaqMan PCR, were tested using PCR-HRM. In the 128 samples, all produced  $T_m$  values  
271 indicating the presence of the var2 allele (Fig. 4). None of the samples produced the  $T_m$   
272 expected for var1 alone. However, for seven samples from 2017, their melting curve exhibited a  
273 dip just before the fusion of the main product (Fig. 4a and b). **The normalization of the curves**  
274 **before the main peak confirmed major differences of curve shapes between distinct samples,**

275 with two groups observed (fig. 4c). One sample from 2016 also exhibited this dip (not shown).  
276 These observations strongly suggest the presence of another variant co-infecting these eight  
277 samples infected with var2. To verify this assumption, a cPCR (primers oPVP339 and oPVP340)  
278 was carried out on each of the eight fish samples, targeting a portion of the viral MCP gene. The  
279 products were cloned and several randomly chosen clones were sequenced. For each suspected  
280 co-infection, some clones corresponding to var1 and var2 were identified at various ratios, with  
281 var2 being the most frequently found (Table 2). In total, 21 clones harboring var1 and 94 clones  
282 with var2 were identified. Of the 21 clones corresponding to var1, 20 were strictly identical to the  
283 HRM region of pPVP130, whereas one exhibited a single mutation. Similarly, of the 94 clones of var2,  
284 93 were identical to the HRM region of pPVP131, and one showed one substitution. However,  
285 regarding these two single changes, it was not possible to determine whether they were actual  
286 mutations persisting within the viral population or simply artefacts produced during the PCR  
287 step. Consistent with the findings in the eight samples from 2016 and 2017, a typical dip in the  
288 melting curve was also observed for sample 15/52 from 2015 for which the presence of var1  
289 and var2 had previously been demonstrated (not shown).

290 These results showed that, for the studied period (2016-2017), var2 was the most frequently  
291 found allele in field samples and a few samples harbored a mixture of variants. Viral DNA with  
292 the var1 was found in the three studied farms.

293

#### 294 4. Discussion

295 To date, the genetic diversity of sturgeon NCLDV in Europe has been poorly studied. Two viruses  
296 have been found: AcIV-E found only in Europe and a second virus related to the Canadian NV. AcIV-E  
297 infects various sturgeon species in Europe and has been found in three countries. Whether this virus  
298 is endemic to Europe or has been imported *via* fish trade from another continent remains unknown.  
299 Regardless of its origin and considering its impact on fish production, it is important to study the  
300 evolution and dissemination of AcIV-E within Europe, ideally with the use of molecular markers. The  
301 AcIV-E MCP gene harbors a convenient molecular marker with two possible alleles, which may reflect  
302 the existence of two genetic lineages (Bigarré et al., 2017). If there are two lineages, this molecular  
303 marker will be useful to study their prevalence, providing that a simple genotyping method is  
304 available and routinely applicable on field samples. The proposed PCR-HRM assay can identify the  
305 presence of one or more MCP alleles at a reduced cost, without laborious sequencing.

306 Tests with plasmids harboring the var1 or var2 allele demonstrated the high sensitivity of the PCR-  
307 HRM, detecting as few as 10 copies, which is roughly equivalent to a specific TaqMan-based real-time  
308 PCR assay that we previously developed. For eight selected samples, the cycle threshold (Ct) values  
309 obtained by TaqMan PCR and PCR-HRM were in the same range. Therefore, the HRM can potentially  
310 be used as a diagnostic method, although this was not the initial goal. However, the method should  
311 be also tested on host species other than Russian sturgeon to ensure the absence of non-specific  
312 products in different genetic backgrounds. Furthermore, a slight shift in the T<sub>m</sub> value was observed  
313 at low plasmid loads. This shift observed for low loads of DNA had been previously reported (Yu et  
314 al., 2015). General recommendations of both thermocycler manufacturers include testing samples at  
315 Ct<30. PCR-HRM is therefore better adapted to genotyping samples with moderate to high viral  
316 loads, for instance a collection of samples previously designated positive with TaqMan PCR.

317 A variation of 1 °C was observed between the T<sub>m</sub> values of the two alleles differing by five  
318 substitutions. It was therefore possible to distinguish different amplicons and theoretically both viral  
319 variants of AcIV-E. Comparatively, a difference of 1 °C was also observed in two human  
320 herpesviruses, HSV-1 and HSV-2, differing by eight mutations in the amplified region (Lieveld et al.,  
321 2017). However, for the HSV and AcIV-E models, it is not possible to separate the melting peaks of  
322 the two genotypes in mixture. In AcIV-E, a unique peak with an intermediate T<sub>m</sub> was observed when  
323 using equal quantities of plasmids. The impossibility of obtaining two distinct peaks is likely due to  
324 the weak difference (1 °C) between the T<sub>m</sub> values.

325 In our study, the T<sub>m</sub> values varied with the thermocycler used (CFX96 or QS5), although a difference  
326 of 1 °C between var1 and var2 was consistently obtained. We have no explanation for this  
327 thermocycler effect, but it has already been observed in another study using four different HRM  
328 thermocyclers targeting human poxviruses (Gelaye et al., 2017). The influence of the thermocycler  
329 on T<sub>m</sub> further stresses the importance of using reference controls (recombinant plasmids or  
330 ultramers, for instance) in each assay.

331 Using recombinant plasmids in order to mimic viral genomes, we evaluated the efficiency of the  
332 assay to identify both variants in a single reaction. At ratios favoring one variant (1:5 to 1:80), the T<sub>m</sub>  
333 was similar to that of the dominant allele and was therefore uninformative as to the presence of the  
334 minor allele. However, for allele ratios of between 1:5 and 1:40, we attributed the presence of a dip  
335 in the melting curve to the presence of heteroduplexes melting prematurely, thus indirectly  
336 indicating the presence of the minor variant. The detection of heteroduplexes has been used in other  
337 studies to differentiate homozygote and heterozygote genomes in eukaryotes or to screen for  
338 atypical viral strains (Sabot et al., 2009; Tajiri-Utagawa et al., 2009; Wittwer, 2009). For instance,  
339 distinct viral subtypes of the influenza virus were identified by mixing PCR products of an unknown  
340 isolate with PCR products of a reference strain (Lin et al., 2008).

341 Out of 128 **virus-positive** field samples analyzed using PCR-HRM, var2 was the most frequently found,  
342 alone or occasionally mixed with var1 as confirmed by sequencing. Interestingly, var1 was never  
343 found alone. The apparent absence of samples with var1 alone or dominant, may be due to the  
344 limited sampling. In the present work, samples were only from one host species (Russian sturgeon)  
345 and from three farms in one country (France). Other fish species and other regions in Europe should  
346 be screened. For example, the var1 allele has previously been detected, in co-infection with var2, in  
347 2015 in another European country and in another sturgeon species (*Huso huso*) in 2012 (Bigarré et  
348 al., 2017). Alternatively, the low occurrence of var1 may arise from low replication efficiency of this  
349 variant or from near-elimination by the fish immune system when this variant is present alone.  
350 Possibly, var1 may replicate at detectable levels only in mixed infections due to stimulating factors  
351 provided by var2.

352 The PCR-HRM assay proved to be useful for screening large numbers of sturgeon samples at a  
353 reduced cost compared with sequencing. The method is specific to AcIV-E and does not target other  
354 NCLDVs. Therefore, to diagnosis any European sample of unknown status, we recommend first using  
355 the TaqMan real-time PCR protocols available for AcIV-E and NV (Bigarré et al., 2017; Clouthier,  
356 VanWalleghem, and Anderson, 2015). Subsequently, PCR-HRM can be used specifically on samples  
357 that prove to be AcIV-E-positive. PCR-HRM can nevertheless be used in first-intention diagnosis, but  
358 it is important to keep in mind that the T<sub>m</sub> deviates slightly at low concentrations. For genotyping,

359 PCR-HRM can be used on a large scale in Europe to screen AcIV-E-positive Russian sturgeon – a  
360 species which is very sensitive to the virus – but also other sturgeon species of different geographic  
361 origins. PCR-HRM will be useful to evaluate the prevalence of the two AcIV-E variants in Europe. In  
362 particular, it is of interest to collate data on var1 epidemiology, including its biological impact on its  
363 host. Wide screening should shed light on the origins of var1 and var2, and determine if they have  
364 both been imported into Europe *via* commercial trade or if they diverged in Europe from an endemic  
365 virus or from a recent imported ancestor.

366

## 367 **Compliance with ethical standards**

### 368 **Funding**

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### 371 **Conflict of interest**

372 B. Debeuf and V. Chesneau work for private companies cited in the authors' list. The other authors  
373 declare that they have no competing interests regarding the publication of this paper.

### 374 **Ethical approval**

375 The samples originated from moribund or dead farmed fish that had not undergone  
376 experimentation and that had been euthanized in accordance with animal welfare ethics.

377

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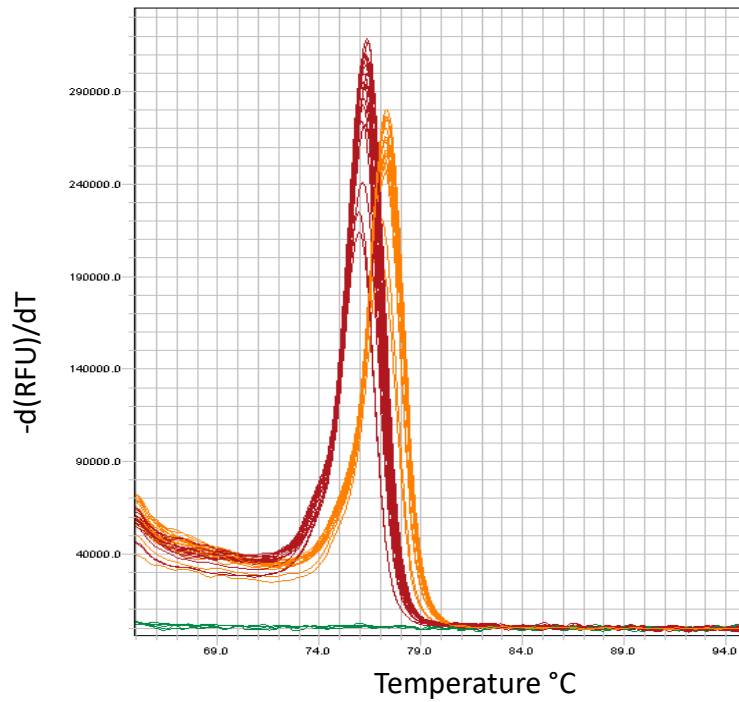
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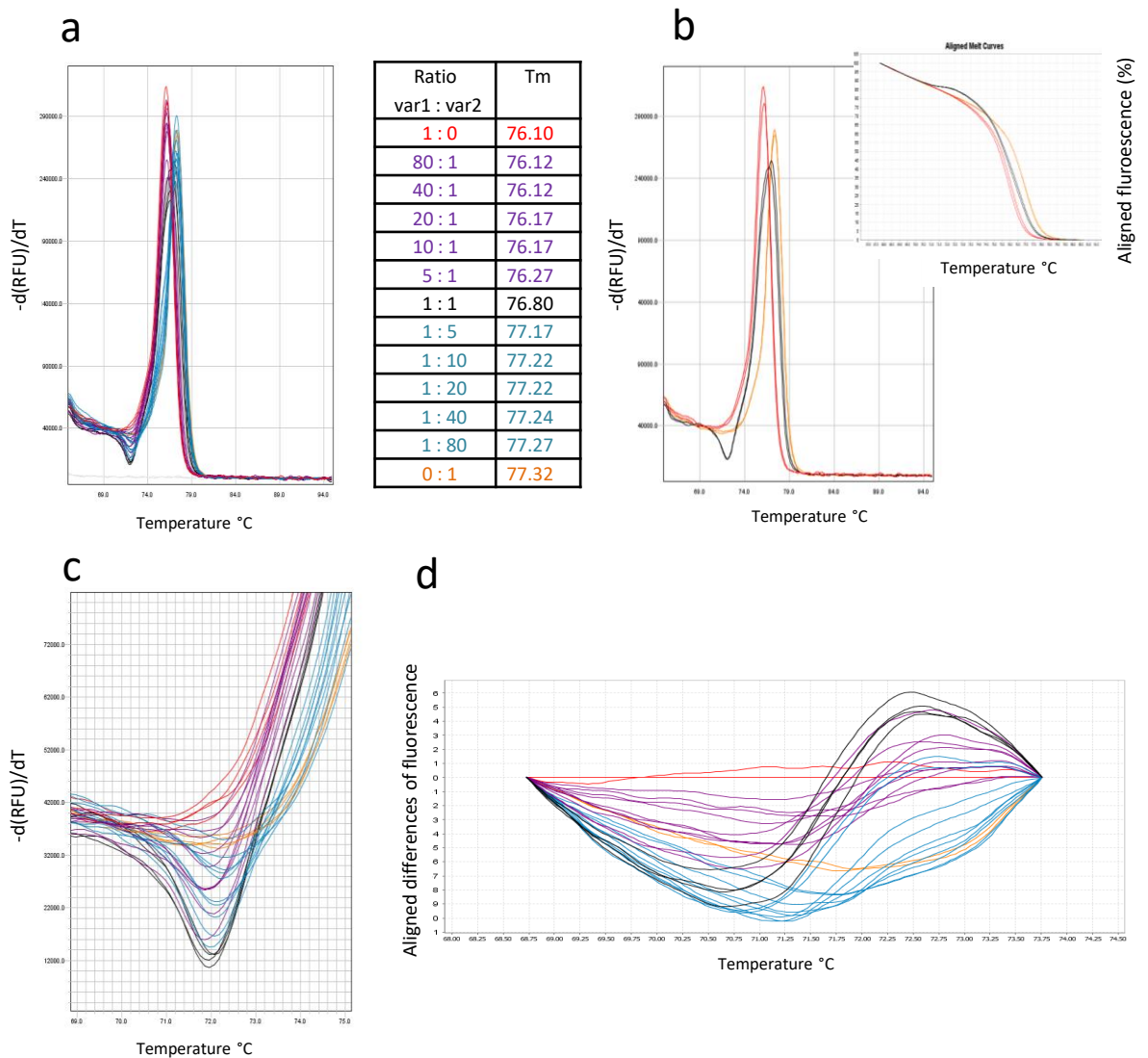
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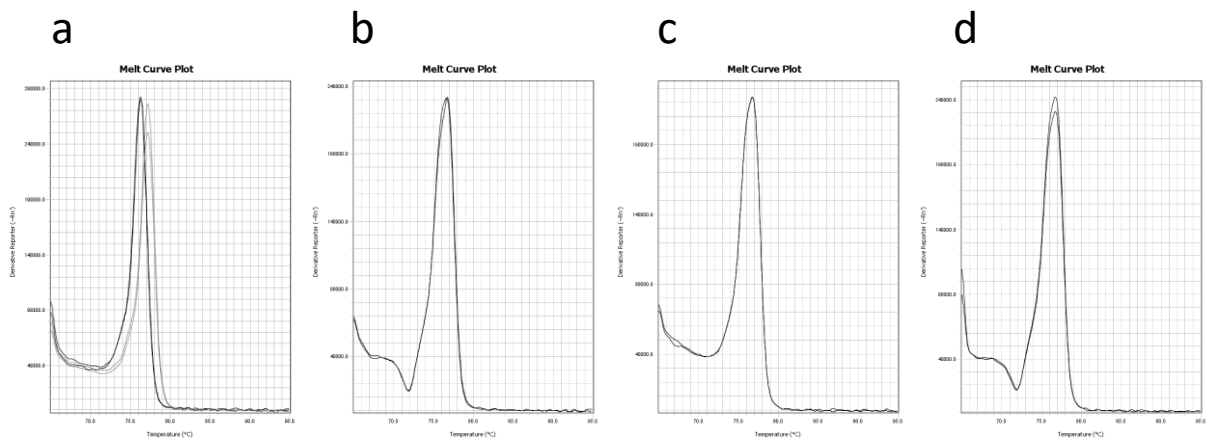
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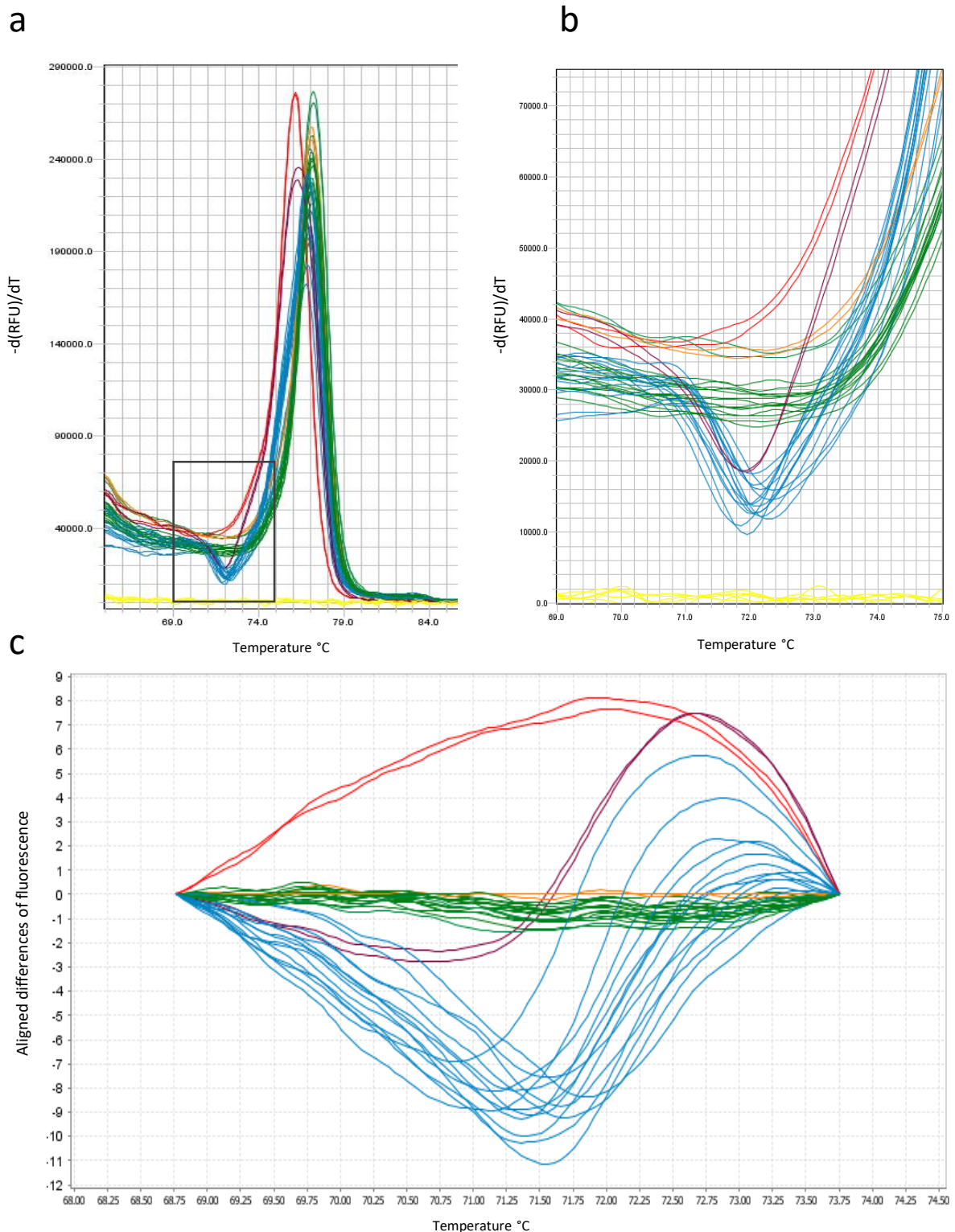
**Fig. 1.** Derivatives of the HRM assays using two plasmids carrying viral sequences of var1 and var2. Dilutions ( $10$  to  $10^8$ ) of plasmid were assayed. Orange curves: var2; red curves: var1; green: negative control (water).



**Fig. 2.** Melting curves obtained with various ratios of two recombinant plasmids. a. derivatives curves obtained for 13 ratios of 2 plasmids. The table provides the correspondances between the colors and the curves, as well as the Tm values. b. same as a, with the pure plasmids and only one mix showed (1:1). The melting curves are shown in the frame. c. magnification of a) in the pre-melting region. d. Normalized difference fluorescence curves of the step (69-73 °C) before the main fusion peak; the var1 is used as a reference.



**Fig. 3.** Melting curves obtained with recombinant plasmids. a. var1 (black dot) and var2 (grey) amplified and melted in independent wells. b. var1 and var2 mixed before PCR and melting. c. var1 and var2 in independent wells for PCR; then amplicons were mixed before melting. d. var1 and var2 amplified in independent wells; then amplicons were mixed, heated, cooled and melted.



**Fig. 4.** Melting curves obtained with field samples. a. For the clarity of the figure, only 15 samples from 2017 are shown (of 128 positive samples from 2016 and 2017). Seven samples exhibit a typical drop before the peak (blue) and eighth do not show a drop (green). As positive controls, pure plasmids var1, var2 and a mix (1:1) were included in the experiment (red, orange and purple respectively). The number of plasmid copies for this experiment was 400 for both var1 and var2. b. Magnification of a) showing the dips for some samples. c. Normalized difference fluorescence curves of the step (68.75-73.75°C) before the main fusion peak; the var2 is used as a reference.

Year	Farm	Number of assessed samples	Number of positive samples	Number of co-infected samples
2016	Farm 1	17	17	1
	Farm 2	15	15	0
2017	Farm 1	78	30	3
	Farm 2	83	53	3
	Farm 3	157	13	1
	<b>Total</b>	350	128	8

Table 1. Origin, number and virus-positive (Taqman PCR) samples of this study.

Sample	Ct (PCR Taqman)	Ct (PCR HRM)	var1	var2	var1/ var1+var2 (%)
15/052 <sup>g</sup> (F2)	nd	22.91	2	1	67
16/85 <sup>gf</sup> (F1)	28.08	26.18	1	7	13
17/113-2 <sup>f</sup> (F3)	29.8	31,14	1	18	5
17/284-5 <sup>f</sup> (F2)	24.4	24,51	6	9	40
17/285-1 <sup>g</sup> (F1)	32.1	33.08	4	7	36
17/286-1 <sup>g</sup> (F1)	28.5	29.85	1	10	9
17/286-2 <sup>f</sup> (F1)	26.5	27.42	1	8	11
17/302-5 <sup>f</sup> (F2)	29.7	30.39	4	20	17
17/381-6 <sup>f</sup> (F2)	29.2	29.73	1	14	7

Table 2. Ct values and ratios of variants found in co-infected fields samples of *A. gueldenstaedtii*. The first number in the name of the sample indicates the year (*i.e.* 15/XX is from 2015). The tissues sampled are indicated in superscript: g, gills; f, fins; gf, pool of gills and fins. The farm (Fx) of origin is indicated in brackets. The columns var1 and var2 indicate the numbers of clones obtained after cloning and sequencing of the PCR products.