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# Diversity and molecular epidemiology of Ostreid herpesvirus 1 in farmed *Crassostrea gigas* in Australia: Geographic clusters and implications for "microvariants" in global mortality events

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1        **Diversity and molecular epidemiology of *Ostreid herpesvirus 1* in farmed *Crassostrea***  
2        ***gigas* in Australia: geographic clusters and implications for “microvariants” in global**  
3        **mortality events**

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31

## 32 **Highlights**

- 33 • A relatively low diversity of OsHV-1 genotypes was found in Australia
- 34 • Distinct *OsHV-1* genotypes were geographically clustered in estuaries
- 35 • Australian *OsHV-1* genotypes grouped in a globally distinct cluster
- 36 • Australian POMS outbreaks were not due to OsHV-1 microvariants

37

## 38 **Abstract**

39 Since 2010, mass mortality events known as Pacific oyster mortality syndrome (POMS) have  
40 occurred in *Crassostrea gigas* in Australia associated with *Ostreid herpesvirus 1*. The virus was  
41 thought to be an OsHV-1  $\mu$ Var or “microvariant”, i.e. one of the dominant variants associated  
42 with POMS in Europe, but there are few data to characterize the genotype in Australia.  
43 Consequently, the genetic identity and diversity of the virus was determined to understand the  
44 epidemiology of the disease in Australia. Samples were analysed from diseased *C. gigas* over  
45 five summer seasons between 2011 and 2016 in POMS-affected estuaries: Georges River in  
46 New South Wales (NSW), Hawkesbury River (NSW) and Pitt Water in Tasmania. Sequencing  
47 was attempted for six genomic regions. Numerous variants were identified among these regions  
48 ( $n = 100$  isolates) while twelve variants were identified from concatenated nucleotide sequences  
49 ( $n = 61$  isolates). Nucleotide diversity of the seven genotypes of C region among Australian  
50 isolates ( $P_i 0.99 \times 10^{-3}$ ) was the lowest globally. All Australian isolates grouped in a cluster  
51 distinct from other OsHV-1 isolates worldwide. This is the first report that Australian outbreaks  
52 of POMS were associated with OsHV-1 distinct from OsHV-1 reference genotype,  $\mu$ Var and  
53 other microvariants from other countries. The findings illustrate that microvariants are not the  
54 only variants of OsHV-1 associated with mass mortality events in *C. gigas*. In addition, there  
55 was mutually exclusive spatial clustering of viral genomic and amino acid sequence variants  
56 within estuaries, and a possible association between genotype/amino acid sequence and the

57 prevalence and severity of POMS, as this differed between these estuaries. The sequencing  
58 findings supported prior epidemiological evidence for environmental reservoirs of OsHV-1 for  
59 POMS outbreaks in Australia.

60

61 **Keywords: Ostreid herpesvirus, variant, diversity, polymorphism, phylogeny, *Crassostrea***  
62 ***gigas*, Australia**

63

## 64 **1. Introduction**

65 Since late last century, the Pacific oyster, *Crassostrea gigas*, has been subject to  
66 recurrent disease events (Nicolas et al., 1992; Renault et al., 2000; Renault et al., 1994; Samain  
67 et al., 2007). However, in 2008 a marked increase in mass mortality outbreaks occurred in  
68 France (Cochennec-Laureau et al., 2010; Martenot et al., 2011; Segarra et al., 2010) and later  
69 in other areas in the world (Abbadi et al., 2018; Batista et al., 2015; Gittenberger et al., 2016;  
70 Hwang et al., 2013; Keeling et al., 2014; Mortensen et al., 2016; Peeler et al., 2012; Roque et  
71 al., 2012; Segarra et al., 2010). The disease was called Pacific oyster mortality syndrome  
72 (POMS) when it first appeared in Australia in 2010 in the Georges and Parramatta rivers, which  
73 are both major estuaries in Sydney (Jenkins et al., 2013; Paul-Pont et al., 2013a). POMS causes  
74 considerable economic losses in Australia, New Zealand and Europe (Fuhrmann et al., 2019).  
75 It is a multifactorial disease influenced by environmental conditions such as water temperature  
76 and host characteristics such as age and size (Hick et al., 2018; Petton et al., 2013) and although  
77 it has a proposed polymicrobial etiology involving bacteria (de Lorgeril et al., 2018; Friedman  
78 et al., 2005; Petton et al., 2021), outbreaks are induced by infection with *Ostreid herpesvirus 1*  
79 microvariants (Batista et al., 2015; Gittenberger et al., 2016; Hwang et al., 2013; Jenkins et al.,  
80 2013; Keeling et al., 2014; Mortensen et al., 2016; Peeler et al., 2012; Renault et al., 2012;  
81 Roque et al., 2012). The disease typically occurs in summer and mortality can be reproduced

82 experimentally by controlled exposure of oysters to the virus alone in a dose-responsive and  
83 temperature-dependent manner (de Kantzow et al., 2016; Paul-Pont et al., 2015).

84 *Ostreid herpesvirus 1*, OsHV-1, which is the only species included in the genus  
85 *Ostreavirus*, is a double-stranded DNA virus (Davison et al., 2009). The presence of the gene  
86 coding for the ATPase subunit of a terminase involved in the packaging of viral DNA inside  
87 the capsid, in addition to intranuclear replication, places OsHV-1 in the *Malacoherpesviridae*  
88 under the order *Herpesvirales* (Davison et al., 2009; Davison et al., 2005). Members of this  
89 taxon are widespread, both in terms of the range of species infected and also geographically.  
90 They have been detected in many hosts besides *C. gigas* including *Crassostrea virginica* (Farley  
91 et al., 1972), *Ostrea angasi* (Hine and Thorne, 1997), *Ostrea edulis* (Comps and Cochenec,  
92 1993), *Tiostrea chilensis* (Hine et al., 1998), *Pecten maximus* (Arzul et al., 2001a), *Ruditapes*  
93 *philippinarum* (Renault et al., 2001), *Ruditapes decussatus* (Renault and Arzul, 2001),  
94 *Crassostrea angulata* (Batista et al., 2015), *Scapharca broughtonii* (Bai et al., 2016; Xia et al.,  
95 2015), *Carcinus maenas* (Bookelaar et al., 2018), *Chlamys farreri* (Bai et al., 2019), *Octopus*  
96 *vulgaris* (Prado-Alvarez et al., 2021) and in numerous sites around the world including Europe,  
97 Asia, Oceania and the Americas (Abadi et al., 2018 ; Batista et al., 2015; Burge et al., 2021;  
98 Gittenberger et al., 2016; Hwang et al., 2013; Jenkins et al., 2013; Keeling et al., 2014;  
99 Mortensen et al., 2016; Peeler et al., 2012; Roque et al., 2012; Segarra et al., 2010; Shimahara  
100 et al., 2012).

101 A specific microvariant, OsHV-1  $\mu$ Var, was described in association with the large  
102 increase in mortality in *C. gigas* on the French coast in 2008 (Segarra et al., 2010), but the  
103 detection of this virus in a specimen collected before 2008 (Martenot et al., 2012) raises the  
104 question of its actual or exclusive involvement in the increasing global phenomenon of oyster  
105 mortality. OsHV-1  $\mu$ Var was strictly characterised by mutations upstream of ORF 4 consisting  
106 mainly of 12 deletions in the microsatellite zone and a few mutations upstream and downstream

107 which impact the amino acid sequence. In addition, at ORF 42-43 OsHV-1  $\mu$ Var had a  
108 substitution of a thymine instead of a cytosine downstream of the sequence without showing any  
109 impact on the amino acid sequence (Segarra et al., 2010). Following the publication of the  
110 OsHV-1 $\mu$ Var sequence from region C, partly located at ORF 4 (Segarra et al., 2010), several  
111 isolates were analysed and this revealed a range of « microvariants » close to the OsHV-1  $\mu$ Var  
112 sequence (Burge et al., 2021; Burioli et al., 2018; Martenot et al., 2012; Morga et al., 2021).  
113 Data for numerous OsHV-1 genotypes characterised by sequencing of various coding and non-  
114 coding regions or whole genomes are available: in Europe, the reference genome isolated from  
115 *C. gigas* larvae (Davison et al., 2005), the variant OsHV-1 var sampled from *P. maximus* (Arzul  
116 et al., 2001a) and the microvariant OsHV-1  $\mu$ Var from *C. gigas* spat and juveniles (Abbadì et  
117 al., 2018; Burioli et al., 2017; Segarra et al., 2010); in China, Acute Viral Necrosis Virus (Ren  
118 et al., 2013), OsHV-1-SB isolated from *S. broughtonii* (Bai et al., 2016; Xia et al., 2015) and  
119 the variant isolated from *C. farreri* (Bai et al., 2019); in Sweden OsHV-1 SW6 found in *O.*  
120 *edulis* (Morga et al., 2021); and in many parts of the world, so called « microvariants » or  
121  $\mu$ Vars, which are similar to OsHV-1  $\mu$ Var (Burge et al., 2021; Morga et al., 2021). Double-  
122 stranded DNA viruses are known to be genetically stable; DNA viruses in general have  
123 mutation rates of  $10^{-8}$  to  $10^{-6}$  substitutions per nucleotide site per cell infection, a lower rate than  
124 RNA viruses, due the viral DNA polymerase that exhibits proofreading activity (Peck and  
125 Lauring, 2018). However, Morga et al. (2021) recently reported a faster evolutionary rate than  
126 usual for OsHV-1. The emergence of variations may be due to selection pressure and the ability  
127 of the virus to adapt to its environment and host as reported in other members of the  
128 *Herpesvirales* (Renzette et al., 2013). The available data suggest that pathogenic subtypes of  
129 OsHV-1 known as microvariants possess genetic diversity beyond that defined for a single  
130 genotype by Segarra et al. (2010), but the extent of diversity and how this relates to their global  
131 emergence is unclear.

132 The first detection of OsHV-1 associated with mortality of *C. gigas* in Australia occurred  
133 in 2010 but despite recurrent outbreaks there has been little characterisation of the genetic  
134 diversity of the viruses affecting *C. gigas* in Australia (Jenkins et al., 2013). Available data  
135 suggest that the genotype responsible for the index case in 2010 was close to OsHV-1  $\mu$ Var but  
136 had some differences (Jenkins et al., 2013). Given that OsHV-1 genetic diversity could affect  
137 the gravity of the disease (Delmotte et al., 2022; Martenot et al., 2011; Morga et al., 2021), it is  
138 important to study the genetic composition of the Australian isolates to better understand  
139 epidemiological patterns in Australia. Detailed, long term, epidemiological studies in Australia  
140 revealed that POMS outbreaks recurred at the same sites over successive years (Whittington et  
141 al., 2019). The source of OsHV-1 for these disease events between 2011 and 2016 was  
142 environmental rather than spread through oyster farming activities (Whittington et al., 2018).

143 The aim of this report is to (i) study the identity and genetic diversity of OsHV-1 in  
144 archival samples collected during mortality events in Australia using six regions sequenced by  
145 Sanger technology, (ii) compare the Australian viruses with international isolates previously  
146 published, and iii) make inferences based on diversity and phylogeny about the source and  
147 transmission of the virus to inform management decisions for disease prevention and control.

148

## 149 **2. Materials and methods**

150

### 151 **2.1. Oyster sample selection**

152

153 Oyster samples were selected over the five summer seasons between 2011 and 2016  
154 from the entire geographical distribution of POMS disease outbreaks in commercial aquaculture  
155 in Australia. Comprehensive data for each sample including location, age, size, mortality rate  
156 and viral load are provided in Supplementary data 1, linked by references to scientific

157 publications on these populations. In summary, all samples were collected on oyster farms by  
158 researchers from the University of Sydney and all oysters in the final dataset were initially  
159 recruited as spat from an OsHV-1 free hatchery in Tasmania (Table 1). All samples were tested  
160 using OsHV-1 real-time PCR (Martenot et al., 2010) and positive samples containing the  
161 highest concentration of OsHV-1 DNA from among the samples collected that day were  
162 selected for sequencing. A total of 118 samples were selected and sequence was subsequently  
163 obtained from at least one region of the genome from 107 samples to which a unique “isolate  
164 ID” was then assigned. For seven of these (all wild *C. gigas* or *Saccostrea commercialis* from  
165 the Georges River in the 2013-2014 season) sequence was obtained only for Region 3 which  
166 had no variation (see below); these isolates were excluded from further analysis, leaving 100  
167 isolates available for analysis, all being samples from farmed *C. gigas* (Supplementary data 1).  
168 Of the 100 samples of *C. gigas*, 38 were collected during periods of OsHV-1 infection in the  
169 Hawkesbury River estuary north of Sydney NSW (from Coba Bay n=2; Kimmerikong Bay  
170 n=5; Mooney Mooney n=15; Mullet Creek n=10; Porto Bay n=6), 60 were collected in the  
171 Georges River estuary south of Sydney NSW (from Limekiln Bar n=1; Neverfail Bay n=2; Site  
172 A n=13; Site B n=10; Site C n=25; Sylvania Waters n=1; The Shed n=8), while two were  
173 collected in Tasmania (from Pitt Water n=2) (Figure 1). Exact locations are illustrated in maps  
174 in Whittington et al. (2019) and in de Kantzow et al. (2017). Sampled oysters had an age range  
175 of 5.5 months to 17 months and a size range of 5mm to 110mm. They were all the subject of  
176 published studies in which details of the oyster populations are reported (de Kantzow et al.,  
177 2017; Hick et al., 2018; Paul-Pont et al., 2013a; Paul-Pont et al., 2013b; Paul-Pont et al., 2014;  
178 Whittington et al., 2015a; Whittington et al., 2015b; Whittington et al., 2019) (Supplementary  
179 data 1).

180

181 2.2. Description of the selected regions



182

183           Six regions of the OsHV-1 genome were targeted for Sanger sequencing. Firstly, four  
184 regions were selected based on their discriminatory value for distinguishing microvariant  
185 genotypes from among other OsHV-1 variants. Region 1 and Region 2 targeted ORF 4 and  
186 ORF 43, respectively. They were selected in order to distinguish OsHV-1 $\mu$ Var (Segarra et al.,  
187 2010), OsHV-1 $\mu$ Var $\Delta$ 9, OsHV-1 $\mu$ Var $\Delta$  15 described in Europe (Martenot et al., 2012;  
188 Martenot et al., 2011) and other microvariants (Bai et al., 2016). Region 3 covered ORFs 35,  
189 36, 37 and 38 and bounded a large deletion in OsHV-1 $\mu$ Var that induced the suppression of  
190 ORF 36 and 37 and disrupted ORF 38 (Burioli et al., 2017; Martenot et al., 2015; Martenot et  
191 al., 2013; Morga et al., 2021; Renault et al., 2012). Region 4 was selected for the high level of  
192 polymorphism suitable for distinguishing genotypes of OsHV-1 (Batista et al., 2015). It is  
193 located between ORF 49 and ORF 50 and is a non-coding site. Region 5 located on ORF 11,  
194 showed a large deletion of 1386bp in microvariants (Morga et al., 2021 ; Burioli et al., 2017 ;  
195 Martenot et al., 2013). Finally, Region 6, which could be involved in virulence mechanisms is  
196 a polymorphic region of interest; it targets ORF 88 coding for a transmembrane glycoprotein  
197 (Martenot et al., 2013). PCR and sequencing reaction primers used for each region are provided  
198 in Table 2 .

199

## 200       2.3. Sample processing

### 201           2.3.1. Extraction

202           Each sample was derived from whole animal or dissected mantle and gill tissues of one  
203 or more oysters, depending on their size (Supplementary data 1). The DNA extraction was  
204 performed according to previous studies (Evans et al., 2014; Whittington et al., 2019). Briefly,  
205 oysters were collected in the field and transported directly to the laboratory where they were  
206 frozen at  $-80^{\circ}\text{C}$  until processed. A  $400\pm 100$  mg sample was homogenised by bead beating

207 using sterile stainless steel beads (Aussie Sapphires) and 1 mL distilled water and placed into a  
208 TissueLyser II machine (Qiagen) for 2 min at frequency 30, repeated once. All samples were  
209 clarified by centrifugation at 1340 g for 2 min in a microcentrifuge and supernatants were  
210 removed and stored in sterile tubes at  $-80^{\circ}\text{C}$ . Nucleic acids were purified using a 5X  
211 MagMAX<sup>TM</sup>-96Viral RNA Isolation Kit (Ambion, Life Technologies<sup>TM</sup>,  
212 Mulgrave,Australia) and then MagMAX<sup>TM</sup> Express 96 magnetic particle processor (Applied  
213 Biosystems<sup>TM</sup>, Life Technologies<sup>TM</sup>, Mulgrave, Australia) according to manufacturers'  
214 instructions for a final volume of 50  $\mu\text{L}$  using the AM1836 deep-well standard programme  
215 (Ambion, Life Technologies<sup>TM</sup>, Mulgrave, Australia). Purified nucleic acids were stored at  
216  $-20^{\circ}\text{C}$ .

217

### 218 2.3.2. PCR

219 Various PCR were performed in order to target the 6 regions described above in part 2.2.  
220 The primers are reported in Table 2. For Region 1 and Region 2, each reaction contained 5  $\mu\text{l}$   
221 DNA extract, 5 $\mu\text{l}$  10X PCR buffer (66.6mM Tris-HCl,16.6mM  $(\text{NH}_4)_2\text{SO}_4$ , 2.5mM  $\text{MgCl}_2$ ,  
222 1.65mg/ml bovine serum albumin,10mM beta-mercaptoethanol), 10 $\mu\text{l}$  dNTP mix (1mM),  
223 250nM each forward and reverse primer, 5U DNA polymerase (taq:pfu mix) and nuclease-free  
224 water to a final volume of 50 $\mu\text{l}$ . Thermocycling was performed (Corbett Research CGI960)  
225 according to the following conditions: 1 cycle at  $94^{\circ}\text{C}$  for 2min; 35 cycles consisted in  $94^{\circ}\text{C}$   
226 for 1min,  $50^{\circ}\text{C}$  for 1 min and  $72^{\circ}\text{C}$  for 1min, and a final extension at  $72^{\circ}\text{C}$  for 5 min. For the  
227 remaining regions, each reaction contained 10 $\mu\text{l}$  5X HiFi reaction buffer (Bioline) (containing  
228 10mM  $\text{Mg}^{2+}$ ), 0.5 $\mu\text{l}$  dNTP mix (100mM), 1 $\mu\text{l}$  each forward and reverse primer (400nM), 1 $\mu\text{l}$   
229 (2U) Velocity DNA polymerase (Bioline), 1.5 $\mu\text{l}$  DMSO (BIO-21098 -Bioline Aust Pty. Ltd),  
230 5 $\mu\text{l}$  template DNA and nuclease-free water to a final volume of 50 $\mu\text{l}$ ; thermocycling was

231 performed according to the following conditions: 1 cycle at 98°C for 2min; 35 cycles consisted  
232 in 98°C for 30s, 62°C for 30s and 72°C for 30s, and a final extension at 72°C for 10 min.

233 The amplicons were visualised using 5µl of each amplification product on a 2% agarose  
234 gel, assessed against a molecular weight marker and visualised using RedSafe (iNtRON  
235 Biotechnology) on a GelDoc transilluminator (Biorad).

236

### 237 2.3.3. Sequencing

238 Amplified PCR product was purified by incubation with ExoSAP-IT/Cleansweep  
239 (ThermoFisher Scientific) then submitted for Sanger sequencing. For several samples where a  
240 single amplicon was not obtained, bands of the expected size were excised from the agarose gel  
241 and DNA was purified using the QIAquick Gel Extraction Kit (Qiagen). The amplicons were  
242 sent to the Australian Genome Research Facility or to the Monash Health Translation Precinct  
243 Medical Genomics Facility Australia for sequencing. Forward and reverse sequencing were  
244 performed in reactions with relevant PCR primers and internal primers (Table 2).  
245 Chromatograms were reviewed, analyzed and primer sequence was removed, using FinchTV  
246 (Geospiza).

247

### 248 2.4. DNA data processing

249

250 For each region targeted, nucleotide sequences were aligned with the reference genome  
251 NC\_005881.2 and the two microvariant genomes KY242788 and KY271630, using MEGA10  
252 (Kumar et al., 2018) with the MUSCLE algorithm. The sequences were trimmed and the  
253 chromatograms were again specifically checked for all mutations on forward and reverse  
254 strands. Base positions described in results are mapped to the reference genome NC\_005881.2.

255 Phylogenetic trees were constructed using MEGA 10 with the Neighbour Joining  
256 method and 1000 replications of bootstraps and the method based on Maximum Composite  
257 Likelihood was applied. Two trees were obtained: the first was based on the concatenated  
258 sequence of Regions 1, 2, 3, 4 and 6 (Region 5 was not included due to too few sequences - see  
259 results); the second tree was based on Region 1. The trees were unrooted because the ancestry  
260 of the reference genotype is uncertain. In addition, the use of another *malacoherpesviridae*,  
261 Haliotid herpesvirus, was impracticable because the ORFs had no sequence in common making  
262 alignment impossible. The unrooted tree allows the analysis of diversity but does not orient the  
263 genotypes temporally.

264 Nucleotide diversity ( $Pi$ ) for Region 1 (C region) including the microsatellite region was  
265 calculated using DnaSP v6: DNA Sequence Polymorphism Analysis of Large Datasets (Rozas  
266 et al., 2017) and compared with the results of Mineur et al. (2015), after adding six sequences  
267 (JN800089, JN800075, JN800082, JN800083, JN800088 and JN800072) to be consistent with  
268 these authors. Briefly, the calculation was performed on the alignment; the indel polymorphism  
269 module with the “Multiallelic”gap option was used to consider all InDel events. Nucleotide  
270 diversity was listed according to geographic region.  $Pi$  values reported by Mineur et al. (2015)  
271 were corrected for frequency equal to one because multiple occurrences of an identical  
272 genotype may not be reported. To make the data comparable,  $Pi$  in the current study was not  
273 corrected because only one sequence per genotype was included in the calculation.

274

275

## 276 2.5. Amino acid sequence prediction

277

278 In order to estimate whether some putative mutations could induce a modification in the  
279 phenotype of the variant, a prediction of amino acid sequence was performed on coding sites in

280 Regions 1, 2, 3, 5 and 6. The translations of the ORFs were performed with ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>) and the Bioedit sequence alignment editor. The translations  
281 were checked using Blastp (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>). These  
282 predictions were aligned with the prediction of the reference genome NC\_005881.2 and the two  
283 microvariant genomes KY242788 and KY271630 using Mega 10.  
284

285

286

### 287 **3. Results**

#### 288 3.1. DNA sequencing analysis

289

##### 290 3.1.1. Description of the variants

291 All nucleotide sequences are available on the NCBI database and accession numbers are  
292 listed in Table 3. Results and metadata for each of the 100 samples is provided in Supplementary  
293 data 1. Sequence alignments are illustrated in Supplementary data 2.  
294

294

295 For Region 1, located at ORF 4, nucleotide sequence was obtained from 87 of 100  
296 samples. None of the sequences were identical to the OsHV-1 reference sequence but they were  
297 partially similar to the microvariants, with the presence of most of the mutations except the two  
298 substitutions of adenine instead of guanine in the downstream region (base 178,696 and  
299 178,702). However, they all presented a novel substitution of an adenine instead of a guanine  
300 in a different position (base 178,705), also in the downstream region. Several mutations were  
301 observed allowing the identification of five different nucleotide sequences: G1, G2, G4, G6 and  
302 G7 (Figure 1, Supplementary data 2). Eight of the sequences that grouped in G4 (Supplementary  
303 data 2) had 15 deletions (base 178,558 - 178,572) in the microsatellite zone instead of the 12

304 deletions seen in microvariants (Burioli et al., 2017; Segarra et al., 2010), as was observed in  
305 OsHV-1 $\mu$ Var $\Delta$  15 reported in Martenot et al. (2012).

306 For Region 2, situated at ORF 43, nucleotide sequence was obtained from 77 of 100  
307 samples. They were all different from the reference sequence and the microvariants. They were  
308 separated into two groups, classified by one deletion of a thymine in the downstream region  
309 (base 60,504) (G8) for 36 samples and two deletions of two adenine in the upstream region  
310 (base 60,059 - 60,060) for 41 samples (G9) (Figure 2, Supplementary data 2).

311 In the third region coding for ORF 35 to ORF 38, nucleotide sequences were obtained  
312 from 96 of 100 samples and all were identical to the two microvariant sequences. The features  
313 included the large deletion zone of 606bp (base 52,251 - 52,856) common to microvariants and  
314 the substitution of a cytosine instead of a thymine (base 52,885). They were classified into G10  
315 (Figure 3, Supplementary data 2).

316 Eighty-seven of 100 samples presented an amplification of the fourth region located on  
317 the non-coding site between ORF 49 and ORF 50. All the nucleotide sequences presented a  
318 common substitution of an adenine instead of a guanine in the upstream zone (base 72,489) and  
319 two deletions of two thymine next to the middle zone (base 75,592 - 75,593). However there  
320 were many differences compared to the reference genotype and microvariants consisting of 19  
321 mutations over 408 nucleotides which were not observed in the microvariant genotypes. These  
322 mutations allowed the characterization of 14 groups (Figure 4, Supplementary data 2),  
323 confirming the prior observations of high variability in this region (Batista et al., 2015). Four  
324 samples of a nucleotide sequence named G12 presented a thymine as well as a cytosine (Y) at  
325 the same position (base 72,536). The chromatograms of this and all the other regions of these  
326 four samples comprising G12 were unambiguous. All samples which contained the G12 variant  
327 were pools of 5 spat (Supplementary data 1).

328 For Region 5 sitting on ORF 11, nucleotide sequence was obtained from 42 of 100  
329 samples. None had the large deletion observed in the microvariants. Even though the nucleotide  
330 sequences were close to the reference sequence, a few mutations were present allowing their  
331 classification into four groups (G31, G32, G33, G34) (Figure 5, Supplementary data 2). All had  
332 a common substitution of a thymine instead of a guanine (base 18,621).

333 Of the 91 samples which amplified from Region 6 in the first part of ORF 88, 54  
334 nucleotide sequences were similar to the reference sequence (G37). The others clustered into  
335 two groups, comprising the substitution of a cytosine instead of a guanine (base 133,574) in 36  
336 samples (G36) and a substitution of a guanine instead of an adenine (base 133,574) in one  
337 sample (G35) (Figure 6, Supplementary data 2).

338 Nucleotide sequences for Regions 1, 2, 3, 4 and 6 were obtained from 61 samples, which  
339 when concatenated led to the identification of 12 DNA variants (Table 4). V1 and V8 were the  
340 most numerous of these variants.

341

### 342 3.1.2. Phylogenetic analyses

343 Firstly, a phylogenetic tree using Regions 1, 2, 3, 4 and 6 was constructed with the  
344 concatenated nucleotide sequences of the 12 variants and the 29 whole genomes present in  
345 NCBI. All Australian genotypes clustered in the same clade, distinct from isolates from Europe,  
346 China and the reference genotype and with a distinctly shorter branch length (Figure 2).  
347 Secondly, a phylogenetic tree was constructed based only on the C region (Region 1), which  
348 has been well-studied internationally, in order to incorporate a wider range of isolates (Figure  
349 3). A single cluster distinguished all Australian genotypes from those from Europe, New  
350 Zealand, Japan and the Americas.

351

### 352 3.1.3 Diversity

353           The number of genotypes available for analysis had almost doubled from 48 to 95 since  
354 the study of Mineur et al in 2015 (Table 5). The Australian isolates had the lowest genetic  
355 diversity overall ( $Pi$   $0.99 \times 10^{-3}$ ) when compared to viruses from other regions of the world  
356 where diversity ranged from  $5.31 \times 10^{-3}$  to  $7.61 \times 10^{-3}$  (Table 5).

357

358

### 359           3.1.3. Influencing factors

360           The distribution among the sites over time of the 12 DNA variants identified from  
361 concatenated nucleotide sequences is illustrated in Table 6 while similar data for all genomic  
362 regions is presented in Table 1, Supplementary data 2.

363           Considering the concatenated nucleotide sequences, up to four variants were found in  
364 one summer season in the Georges River, compared to only one or two variants in the  
365 Hawkesbury River. Nine of the 12 variants occurred at only one site and in only one summer  
366 season. The variants V1 and V6 were present only in the Hawkesbury River; V1 was present at  
367 most sites and in each of the four summers, while V6 was found only at Kimmerikong Bay and  
368 Mooney Mooney in summer 2013-2014. The other variants were confined to the Georges River  
369 where V8 was identified at various sites within Woollooware Bay in three summer seasons. The  
370 other variants in the Georges River were detected only at a single site and a single sampling  
371 date.

372           More than one variant was detected over time at some sites; for example, five were seen  
373 at Site B in the Georges River while two were seen at both Kimmerikong Bay and Mooney  
374 Mooney in the Hawkesbury river (Table 7). More than one variant was found in the same  
375 summer season at some sites; for example, in 2014-2015 V8, V11 and V12 were found at Site  
376 B, while in 2013-2014 both V1 and V6 were found at Kimmerikong Bay and at Mooney  
377 Mooney. Furthermore, there were also several instances where different variants were detected



378 in the same population of oysters at the same time: V11 and V12 were both present at Site B  
379 on 27/1/15; V1 and V6 were both present at Kimmerong Bay and Mooney Mooney on 13/12/13  
380 and also on 25/1/14.

381 There were similar observations for Region 4 (Figure 4, Supplementary data 2), which  
382 had the most variability; 12 of the 14 variants were found in only one season and at only one  
383 site. Three of five variants in Region 1 and two of four variants in Region 5 were also seen in  
384 only one season and at only one site.

385 Two isolates from Tasmania were not incorporated into the phylogenetic analysis  
386 because of incomplete sequencing of regions from those samples. However one of these  
387 samples was the only isolate classified as G6 in Region 1 (Table 1 and Figure 1, Supplementary  
388 data 2); there were no sequencing data from this region of the other sample from Tasmania. The  
389 oysters sampled in New South Wales had been supplied from at least nine different production  
390 batches (i.e. different broodstock spawnings) from one hatchery in Tasmania. Oysters grown  
391 from five of these batches yielded more than one variant, and in one case, five different OsHV-  
392 1 variants, which has important implications when assessing the sources of the virus (see  
393 discussion).

394

### 395 3.2. Amino acid sequence prediction of variants

#### 396 3.2.1. Description of the predicted amino acid sequences

397 Predicted amino acid sequences were aligned with the reference and microvariant  
398 sequences. All alignments are illustrated in Supplementary data 2. Regions 3 and 4 were not  
399 included in this analysis because Region 3 had a large deletion of ORF 36 and 37, inducing  
400 overlap of ORF 35 and 38 and creating uncertainty about whether a longer ORF could be  
401 translated, while Region 4 was a non-coding area.

402 For Region 1, two amino acid sequences were identified for ORF 4 among the five  
403 DNA sequences described above in part 3.1.1. They were both different to the reference  
404 genotype and the microvariants. The first group was named <<A>> and contained G1, G2, G4  
405 and G7; it had an asparagine (N) instead of an aspartic acid (D). The second group named  
406 <<B>> contained G6 and had in addition a leucine (L) instead of a serine (S) (Figure 7,  
407 Supplementary data 2).

408 For Region 2, the two DNA groups characterised for ORF 43 presented two different  
409 amino acid sequences. The first was named <<C>> and contained G8. It had a similar amino  
410 acid sequence to the reference genotype and the microvariant. The second was named <<D>>.  
411 It contained G9 and was characterised by three successive mutations of a serine (S), lysine (K)  
412 and a deletion instead of a phenylalanine (F), a glutamic acid (E) and an arginine (R) (Figure  
413 8, Supplementary data 2).

414 Region 5 located on ORF 11 had four DNA groups which translated into 3 amino acid  
415 sequences. One was similar to OsHV-1 reference genotype, and was named « E ». The second  
416 was named « F » and had a mutation of an asparagine (N) instead of a lysine (K). The third was  
417 named « G » and had 4 substitutions of a lysine (K) (Figure 9, Supplementary data 2).

418 Region 6 coding for the first part of ORF 88 presented two amino acid sequences named  
419 « H » containing G36 and G37 and « I » containing G35. <<H>> had a similar sequence to the  
420 reference genotype and one of the microvariants, KY242785, while <<I>> had one mutation of  
421 a glutamic acid (E) instead of lysine (K) (Figure 10, Supplementary data 2).

422 The amino acid prediction of the 12 DNA variants identified among 61 samples resulted  
423 in two profiles based on ORF 4, ORF 43 and ORF88: ACH (n= 34) and ADH (n=27) (Table  
424 8). Region 5 (ORF11) was not included because there were only 42 sequences available for it.

425

426 3.2.2. Influencing factors

427           The amino acid profile of isolates was different between the two estuaries in NSW  
428 (Tables 6 and 7). The ACH profile was found only in the Hawkesbury River, an estuary located  
429 40 km north of Sydney while the ADH profile was found only in the Georges River, an estuary  
430 located 20 km south of Sydney.

431

#### 432 **4. Discussion**

433

434 *Australian OsHV-1 isolates have lower diversity than OsHV-1 isolates globally*

435

436           It is difficult to compare genomic diversity between published studies of OsHV-1 due  
437 to differences in the genomic regions sequenced, sample sizes, time span, geographic extent,  
438 host species and disease state (healthy, sick, or dead). However, in this study we report diversity  
439 of *Ostreid herpes virus-1* in estuaries near Sydney Australia that may be lower than in other  
440 geographic regions of the world.

441           Regions of the OsHV-1 genome under selection pressure and other regions which  
442 accumulate a high number of substitutions have been reported (Morga et al., 2021). Selection  
443 pressure is less on a non-coding area and mutations acquired over time are less likely to be  
444 eliminated. The diversity revealed in this study is mainly explained by inclusion of non-coding  
445 Region 4 between ORF 49 and ORF 50, which is a polymorphic area (Batista et al., 2015). In  
446 this region alone, 14 groups were identified among the 87 samples (one variant for every 6.2  
447 isolates). Similarly, Batista et al. (2015) identified one variant for every 6.0 isolates examined  
448 in a study of 18 isolates from mostly diseased *C. gigas* in Portugal.

449           C region, corresponding to Region 1 in the present study, is the most commonly  
450 analysed part of the OsHV-1 genome. With the typical primers C2/C6 used for amplification  
451 there is representation of part of a non-coding region and part of ORF 4 (Batista et al., 2015).

452 Nucleotide diversity in the C region was calculated formally and compared to data published in  
453 2015 (Mineur et al., 2015). There were more sequences in public databases, but the nucleotide  
454 diversity had not changed markedly except for Europe where it appeared to have decreased  
455 (from  $15.3 \times 10^{-3}$  to  $7.6 \times 10^{-3}$ ). The Australian isolates had the lowest genetic diversity ( $Pi$   $0.99$   
456  $\times 10^{-3}$ ) overall. Globally, 48 different genotypes had been identified in C region (including the  
457 microsatellite region) by 2015 (Mineur et al., 2015). Expressed in simple terms of the frequency  
458 of genotypes among isolates examined, in Australia seven genotypes have been identified  
459 among 89 samples in diseased *C. gigas* (five from 87 isolates in the present study and two  
460 existing in NCBI), which equates to one variant for every 12.7 isolates. In contrast, in a study  
461 covering a wide area of coastal Japan, 23 genotypes were found among 123 samples from  
462 healthy *Crassostrea* species (one variant for every 5.4 isolates) (Shimahara et al., 2012); in both  
463 countries samples were collected over about 5 years. The genetic diversity among diseased  
464 oysters in Australia was clearly much less than that in healthy oysters in Japan. The data from  
465 Japan in the north west Pacific region likely represent the diverse environmental reservoir of  
466 viral types detectable in healthy shellfish, while the low diversity in Australia may represent  
467 selected environmental strains of OsHV-1 capable of causing disease in farmed oysters under  
468 certain environmental conditions, that then became locally dominant. Relatively low diversity  
469 was seen in Europe after 2008 when OsHV-1 *uVar* became the most common strain in diseased  
470 oysters (Table 5). In Europe there had been spread of the virus with movements of live oysters  
471 for farming, for example from France to Ireland and Jersey (Peeler et al., 2012). Within France,  
472 OsHV-1 *uVar* was dispersed widely with commercial movements of live oysters from  
473 Marennes-Oléron to farming areas on both the Atlantic and Mediterranean coasts (Delmotte et  
474 al., 2022). Thus, in addition to the health/disease condition of oysters and their geographic  
475 locations, the observed regional differences in the diversity of OsHV-1 genotypes depends on

476 epidemiological differences between the sources and modes of spread of the viruses. All these  
477 factors will need to be considered in future studies of the diversity of OsHV-1.

478

479         In Region 4 we observed a double nucleotide (thymine and cytosine) in four individual  
480 samples for which remaining nucleotide sequences were unambiguous (G12 – Figure 4, and  
481 Supplementary data 2) and otherwise matched the nucleotide sequence of G18 which had a  
482 cytosine at this position (Figure 4, Supplementary data 2). Our first hypothesis is that there has  
483 been a recent mutation at this position to explain why we found two different copies of this part  
484 of the genome, one being the ancestral form and the other being the mutated one. This is feasible  
485 due to the persistence of the herpesvirus in surviving oyster hosts (Evans et al., 2017b), because  
486 gene exchange in herpesviruses can occur by recombination (Thiry et al., 2005) and because  
487 Rosani and Venier (2017) showed the presence of DNA recombination-initiating promoter  
488 binding in *Malacoherpesviridae*. The four samples with this mutation were from oysters in  
489 close proximity to one another and they could have been infected almost simultaneously from  
490 a host in which the mutation first occurred, resulting in a pool of two variants in each of the four  
491 samples. This is consistent with Martenot et al (2011) who reported various genotypes in the  
492 same batch of oysters and Morga et al (2021) who reported various genotypes in the same  
493 individual. A second possibility would be that there were two otherwise identical but long-  
494 standing OsHV-1 variants in each of the four samples, due to different individuals containing a  
495 different genotype of OsHV-1, because each sample came from a pool of five individual  
496 oysters. While this does increase the chance of mixing several variants into one DNA extract,  
497 in no other sample did we find a group with a thymine at this position. The least likely  
498 explanation because the non-coding area between ORF 49 and ORF 50 is present as a single  
499 copy in the complete OsHV-1 genomes available on NCBI, is the existence of a double copy  
500 of this region.

501

502           The microsatellite zone of the C region of OsHV-1 is polymorphic because of repeated  
503 trinucleotides which allow replication mistakes, as observed by many authors (Martenot et al.,  
504 2012; Martenot et al., 2011; Mineur et al., 2015; Renault et al., 2014). The range in the number  
505 of ACT trinucleotide repeats across all genotypes globally was reported to be 3 - 13 (Mineur et  
506 al., 2015), corresponding to a range of 3 - 10 trinucleotide deletions (9 - 30 base deletions).  
507 However, we observed only two profiles among a large number of isolates, either 12 or 15 base  
508 deletions. This is consistent with the only other Australian sequences deposited in NCBI; these  
509 came from the first episode of mortality in 2010 and had 12 base deletions.

510

#### 511 *Australian isolates differed from OsHV-1 isolates globally*

512           Concatenated nucleotide sequences showed a distinct cluster including all the Australian  
513 isolates, different from all other genotypes reported worldwide and supported by the distinct  
514 distribution with a shorter branch size for the Australian genotypes (Figure 2). Although the  
515 bootstrap value was only 63, this could be explained by the presence of Region 3 located  
516 between ORF 35 and 38 including a large deletion area and region 5 located on ORF 11, with  
517 a 462bp deletion only in the microvariant. These differences are calculated as multiple mutation  
518 events when it is more likely that each corresponds to a single mutation event. Burioli et al.  
519 (2016) studied the same disturbed region and discussed the need for “sophisticated algorithms  
520 for alignment and phylogenetical analysis, adapted to take into account DNA-virus evolution  
521 analysis, in order to obtain accurate results with the correct phylogenetic distance”. The  
522 Australian clade was also confirmed by the inclusion of the only two NCBI sequences from  
523 Australia, KC685525.1 and MT157286.1 (Burge et al., 2020; Jenkins et al., 2013) in the C  
524 region tree (Figure 3). This is consistent with the phylogeny reported in Mineur et al. (2015),  
525 which was performed on the same locus with another algorithm, median joining genotype, and

526 showed three closely related genotypes from temperate Australasia. In contrast, the  
527 phylogenetic tree presented in Burge et al. (2021) with the neighbour joining method showed  
528 that the Australian genotype was close to the Japanese, American, Korean and French  
529 microvariants. However, unlike the current phylogeny, the tree described in Burge et al. (2021)  
530 is not based on evolutionary distance and contains too few samples - a single genotype from  
531 Australia - which is not sufficient to construct a cluster. The Australian viruses appear to be  
532 genetically different across the genomic regions analyzed and this raises the question about  
533 their true phylogenic origins. Sanger sequencing of a limited number of genomic regions may  
534 bias analyses compared to whole genome analysis, which will be indispensable for determining  
535 clades of OsHV-1 and should be the subject of future studies.

536

537 *Australian isolates were not OsHV-1 reference strain, OsHV-1  $\mu$ Var or microvariants*

538         Neither OsHV-1 reference genotype nor OsHV-1  $\mu$ Var were identified among the  
539 Australian samples. Currently OsHV-1 “microvariants” are described as being close to OsHV-  
540 1  $\mu$ Var as defined by Segarra et al. (2010) but not identical to it (Burge et al., 2021; Morga et  
541 al., 2021). In all of the Australian isolates the two regions which characterise OsHV-1  $\mu$ Var  
542 according to Segarra et al. (2010), ORF 4 and ORF 43, had dissimilar nucleotide sequences.  
543 None of these sequences had the large deletion in ORF 11 reported in the microvariant  
544 sequences (Burioli et al., 2017; Martenot et al., 2013; Morga et al., 2021) and presented a similar  
545 nucleotide sequence to the OsHV-1 reference virus on ORF 11 except for one point mutation.  
546 Moreover, half the samples from Australia did not present an amplification product from this  
547 region. Since there was enough biological material in the same samples to perform sequencing  
548 of the other regions, we suggest that there were changes at the primer binding sites, which others  
549 have also mentioned to be a likely reason for this problem (Arzul et al., 2001b; Martenot et al.,

550 2011); PCR-sequencing of the other regions was not uniformly successful either, consistent  
551 with many other studies of OsHV-1.

552

553 The variant of OsHV-1 known as  $\mu$ Var is thought to be responsible for the sudden  
554 escalation of mass mortality events in *C. gigas* in France from 2008, and then later in other  
555 European countries. However,  $\mu$ Var and the more recently described “microvariants” are  
556 clearly not the only variants of OsHV-1 associated with mass mortality events in *C. gigas*. The  
557 disease outbreaks in both Australia since 2010 have not been caused by these types of OsHV-1  
558 but have been just as sudden in onset, recurrent and economically devastating for the oyster  
559 industry as those in France (Fuhrmann et al., 2019). Therefore further research is required to  
560 understand the virulence determinants of OsHV-1 and how these are coded in the genome.

561

#### 562 *Internal comparison of Australian isolates*

563

564 The two estuaries studied in New South Wales had different OsHV-1 profiles. At the  
565 Georges River sites, ten variants were identified over five summer seasons while at the  
566 Hawkesbury River sites only two variants were found. Delmotte et al. (2022) showed a great  
567 diversity of variants in areas with high oyster farming activity. However, the causes of the  
568 different patterns of distribution of variants here are unknown since these two estuaries in  
569 Australia had equivalent, intense oyster farming activity using similar genetic stock of *C. gigas*  
570 from the same OsHV-1 free hatchery in Tasmania. As a consequence of the two distributions  
571 of DNA variants, the amino acid sequences of the viruses present in the Hawkesbury and  
572 Georges river estuaries were different: the ACH profile in the Hawkesbury River and ADH in  
573 the Georges River. This distinct pattern has a precedent in Europe, but with far greater  
574 geographic separation than the 60 km that exists between these two estuaries. Based on RNA-



575 Seq SNP analysis, Delmotte et al. (2020) reported two genetically different OsHV-1  
576 populations in POMS outbreaks in *C. gigas* on the Atlantic and Mediterranean coasts of France;  
577 they had amino acid sequence differences in viral membrane proteins, which may affect  
578 virulence (Delmotte et al., 2020).

579

580         Due to the selection of samples based on high viral load, which in general is correlated  
581 with mortality (Oden et al., 2011), it was not appropriate to compare the OsHV-1 isolates in  
582 this study according to their degree of replication fitness or observed mortalities. However, in  
583 the oyster populations from which the samples were collected there were differences in the  
584 patterns of mortality: spat in the Georges River estuary were 6.4 times more likely to have  
585 mortality due to OsHV-1 than those in the Hawkesbury River estuary (Whittington et al., 2019).  
586 Subclinical infection of oysters with OsHV-1 was also more likely in the Georges River (Evans  
587 et al., 2017b; Whittington et al., 2019). It is possible that the mutually exclusive genotypic  
588 variants of OsHV-1 identified between the two estuaries differed in virulence. However, other  
589 factors could also be involved. While oyster genotype was similar between the two rivers  
590 because of the common origin of spat, there could be a wide range of environmental differences.  
591 These could include differences in the co-occurrence of reservoirs/hosts, particulate vectors,  
592 filter feeding species and seaweeds that could all influence the severity of POMS (Dugeny et  
593 al., 2022; Evans et al., 2017c; Pernet et al., 2021; Whittington et al., 2018).

594

595         To our knowledge there were no transfers of commercial oysters between the Georges  
596 and Hawkesbury Rivers after first detection of OsHV-1 in the Georges River late in 2010 due  
597 to government biosecurity directives (Paul-Pont et al., 2014). The only common factor between  
598 the two oyster production locations was the recruitment site of all batches of farmed *C. gigas*  
599 in this study: Tasmania. However, the current data indicate that the virus contamination did not

600 come from this recruitment step but from the culture environment. There are three reasons for  
601 this : i) consistent pre-shipment PCR testing of all batches of spat from the hatchery by the  
602 government laboratory in Tasmania and again upon arrival in NSW by the researchers (all  
603 negative results) means that spat were specific-pathogen free (SPF); ii) different genotypes of  
604 OsHV-1 were detected in the same batch of spat after deployment in NSW; iii) a unique  
605 genotype (G6) of OsHV-1 was detected in Tasmania; G6 was not found among 85 samples  
606 from the Hawkesbury River or Georges River. Epidemiological observations alone had  
607 suggested that recurrent POMS outbreaks in Australia were derived from the environment and  
608 were not due to disease transfer with oysters during farming (Whittington et al., 2018), a feature  
609 which is now strongly supported by DNA sequence evidence. This scenario is in direct contrast  
610 to France where evidence points to a radiation of disease associated with commercial spat  
611 distribution (Delmotte et al., 2022). The time of first appearance of OsHV-1 in a putative  
612 environmental host in Australia cannot be determined, but it could have been a point source  
613 introduction to a naive population, then adaptation to local conditions. In fact there are historical  
614 records of a virus like Ostreid herpesvirus from *Ostrea angasi* in Western Australia (Hine and  
615 Thorne, 1997). These authors demonstrated herpes-like viral particles by electron microscopy,  
616 but did not have the molecular tools to identify the virus. In any case, this observation raises  
617 the possibility that OsHV-1 could be either indigenous to Australia or could have been  
618 introduced many decades ago and become endemic, and it could have mutated to form its own  
619 lineage over time, as is supported by the current phylogenetic analysis. Knowing that OsHV-1  
620  $\mu$ Var emerged around 2008 in Europe and then dispersed rapidly, the absence of OsHV-1  $\mu$ Var  
621 in Australia suggests that there has been effective compliance with the strict quarantine laws  
622 that aim to prevent introduction of exotic pathogens into Australia.

623         Arzul et al. (2001a) speculated 20 years ago that pathogenic forms of OsHV-1 arose  
624 from possibly benign bivalve herpesviruses which could be transmitted between bivalve species

625 and which would be favoured by modern shellfish farming practices (Arzul et al., 2001a; Arzul  
626 et al., 2001b). In Australia, there is evidence that invertebrate species in the Georges River  
627 besides *C. gigas* are exposed to OsHV-1 and may become infected, but their role in the  
628 epidemiology of POMS remains to be determined (Evans et al., 2017c). The environment is an  
629 important factor in the transmission cycle because OsHV-1 can be found in seawater and  
630 sediments (Evans et al., 2014 ;2017) and is likely transported on suspended particles over 5µm  
631 (Evans et al., 2017a; Evans et al., 2017c; Paul-Pont et al., 2013b; Whittington et al., 2015b).  
632 Depending on conditions such as temperature, the virus is expected to persist in seawater and  
633 remain infectious for several days according to *in vitro* experimentation (Hick et al., 2016;  
634 Martenot et al., 2015). This gives the virus time to transit between hosts through the  
635 environment. *Ostreid herpesvirus 1* can infect several different host species (Arzul et al., 2001a;  
636 Bai et al., 2016; Batista et al., 2015; Bookelaar et al., 2018; Comps and Cochenec, 1993; Evans  
637 et al., 2017c; Farley et al., 1972; Hine and Thorne, 1997; Hine et al., 1998; Prado-Alvarez et  
638 al., 2021; Renault and Arzul, 2001; Renault et al., 2001; Xia et al., 2015) and may persist in  
639 survivors of outbreaks or remain latent (Arzul et al., 2002; Evans et al., 2017b), which may  
640 explain why each environment has its own assemblage of viruses. While the molecular evidence  
641 strongly suggested that the virus contamination came from reservoirs in the local culture  
642 environment rather than a point source such as an oyster hatchery, the distribution of the  
643 variants across sites and time suggests that some reservoirs were persistent and widespread,  
644 while others were highly site and time specific and therefore possibly short-lived, or that  
645 transmission was infrequent. The reservoirs may be subject to local environmental influences  
646 which affect their distribution and abundance. Further field research is required to discover the  
647 environmental reservoir of OsHV-1 and the specific environmental factors that facilitate  
648 emergence of the virus. Climate change, short term climatic and water quality variations and  
649 intensification of farming are among the many factors to be considered.

650

651

## 652 **5. Conclusion**

653

654 Sequencing analyses of OsHV-1 isolates from estuaries near Sydney, Australia,  
655 revealed a low diversity of genotypes relative to other geographic regions, reflecting differences  
656 in sources and modes of spread of the viruses and the types of oyster populations (healthy, or  
657 diseased) that have been examined so far. Importantly, Australian POMS outbreaks were  
658 associated with a type of OsHV-1 distinct from others globally and were not a “microvariant”  
659 genotype. Elsewhere, since 2008 OsHV-1 microvariants have been the only variants associated  
660 with mass mortality events in *C. gigas*. Future whole genome sequencing investigations will  
661 allow more detailed classification of Australian isolates of OsHV-1. Based on both DNA and  
662 amino acid sequences, OsHV-1 variants were absolutely correlated to estuary. The *C. gigas*  
663 populations in the two estuaries near Sydney had different prevalence and severity of POMS,  
664 which suggests that the geographically mutually exclusive genotypes differed in virulence. The  
665 molecular evidence supports field epidemiological evidence that the disease-causing virus came  
666 from reservoirs in the local environment. The sequencing findings in this study help inform  
667 understanding of the various sources and modes of spread of OsHV-1 that have led to mass  
668 mortality events in farmed *C. gigas* globally.

669

## 670 **Author contributions**

671 Suzanne Trancart: Investigation, Data curation, Methodology, Visualization , Formal analysis,  
672 Writing - Original Draft, Writing - Review & Editing. Alison Tweedie: Investigation, Data  
673 curation, Writing - Review & Editing. Olivia Liu: Investigation, Writing - Review & Editing.  
674 Ika Paul-Pont: Conceptualization, Investigation, Writing - Review & Editing. Paul Hick:  
675 Investigation, Writing - Review & Editing. Maryline Houssin: Resources, Investigation,  
676 Supervision, Writing - Review & Editing. Richard Whittington: Funding acquisition, Project

677 administration, Supervision, Resources, Conceptualization, Investigation, Data curation,  
678 Formal analysis, Writing - Original Draft, Writing - Review & Editing.

679

680

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687 assistance with field work. Shellfish Culture Tasmania kindly provided all batches of spat that  
688 were deployed and sampled in cited research trials. Vickie Patten and Ann-Michele Whittington  
689 provided skilled laboratory assistance with oyster processing.

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696 **Captions to figures**

697 Figure 1 : Sampling sites. George river estuary sites are located on the bottom map  
698 corresponding to the south of Sydney and Hawkesbury river estuary sites are located on the  
699 upper map corresponding to the north of Sydney.

700

701 Figure 2: Distribution of the DNA variants according to the NCBI deposited nucleotide  
702 sequences. The unrooted phylogenetic tree was performed using the neighbour joining  
703 method and 1000 bootstrap replications were applied based on maximum composite  
704 likelihood. Regions 1,2,3,4 and 6 were concatenated on 61 Australian isolates showing 12  
705 variants (V1, V2, V3, V4, V5, V6, V7, V8, V9, V10, V11, V12). Yellow rectangle is the  
706 reference nucleotide sequence of OsHV-1. Metadata for the nucleotide sequences of the  
707 current study that are included in the tree are in Supplementary data 1 while metadata for the  
708 nucleotide sequences that are already available in NCBI are in Table 1, Supplementary data  
709 3.

710 Figure 3: Distribution of the DNA variants according to nucleotide sequences deposited in  
711 NCBI. The unrooted phylogenetic tree was performed using the neighbour joining method  
712 and 1000 bootstrap replications were applied based on maximum composite likelihood.  
713 Region 1 from 87 Australian isolates was used, showing 5 groups (G1, G2, G4, G6, G7).  
714 Crosses indicate Australian nucleotide sequences extant in NCBI; asterisks indicate the New  
715 Zealand nucleotide sequences. Yellow rectangle is the reference nucleotide sequence of  
716 OsHV-1. Metadata for the nucleotide sequences of the current study that are included in the  
717 tree are in Supplementary data 1 while metadata for the nucleotide sequences that are already  
718 available in NCBI are in Table 2, Supplementary data 3.

719

720 **List of supplemental data**

721 *Supplemental data 1*

722 Table 1 Metadata for all samples

723 Table 2 Citations of scientific publications describing the populations of oysters from which  
724 samples were collected

725

726 *Supplemental data 2*

727 Table 1 Variants by genomic region and geographic location

728 Figures 1 to 10 DNA and amino acid alignments

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730 *Supplemental data 3*

731 Genbank accession numbers of isolates included in phylogenetic trees

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*Table 1 : Number of samples of OsHV-1 PCR-positive oysters from which nucleotide sequences were analysed according to the estuary and the season of collection. The number of sites sampled are in parentheses; sites differed between seasons.*

<b>Estuary</b>	<b>2011-2012</b>	<b>2012-2013</b>	<b>2013-2014</b>	<b>2014-2015</b>	<b>2015-2016</b>	<b>Total</b>
Georges River NSW	19 (3)	9 (3)	14 (4)	13 (3)	5 (3)	60
Hawkesbury River NSW		11 (3)	15 (2)	9 (2)	3 (2)	38
Pitt Water TAS					2 (1)	2
<b>Total</b>	<b>19</b>	<b>20</b>	<b>29</b>	<b>22</b>	<b>10</b>	<b>100</b>

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Table 2 : Regions of the OsHV-1 genome targeted for PCR amplification.

Sequencing region	Amplification target				Amplicon size (base pairs)	Reference	
	Position <sup>a</sup>	Gene	Primer name and purpose <sup>b</sup>	Primer sequence (5'→3')			
1  (C region)	178211 178919	ORF 4	C2 (A, S)	CTCTTTACCATGAAGATACCCACC	709	(Renault and Arzul, 2001)	
			C6 (A, S)	GTGCACGGCTTACCATTTTT			
			C1585 (S)	GTATAAATAGGCGCG			This paper
			C1586 (S)	CGCGCCTATTTATAC			This paper
			C1587 (S)	CAGACGAGGTTAAC			This paper
			C1588 (S)	GTTAACCTCGTCTG			This paper
			C1593 (S)	GGAGCTGCGGCGCTATG			This paper
2  (A region)	59950 60557	ORF 42-43	IA1 (A, S)	CGCGGTTTCATATCCAAAGTT	607	Segarra et al. (2010)	
			IA2 (A, S)	AATCCCCATGTTTCTTGCTG			
			C1589 (S)	CTTGCTCATCGTATTC			This paper
3  (605bpDel)	51979 52968	ORF 35 -36 - 37 and 38	Del 36-37F2 (A, S)	ATACGATGCGTCGGTAGAGC	989, 384 or no amplification	(Renault et al., 2012)	
			Del 36-37R (A, S)	CGAGAACCCCCATTCCTGTAA			
			C1590 (S)	CATGGTGATGAATGAAG			This paper
			C1591 (S)	CTTCATTCATCACCATG			This paper
			C1592 (S)	CATTCCTGTAAACAC			This paper
4  (NC1NC2 region)	72414 72919	Between ORF49 and 50	NC1 (A, S)	ACACCTAATGACCCCAAAGG	506	(Batista et al., 2015)	
			NC2 (A, S)	GACCAATCACCAGCTCAACA			
5	17402 19518	ORF 11	ORF11For (A, S)	ACCACCGCGCCAAAATCTG	2116 or 731	(Martenot et al., 2013)	
			ORF11Rev (A, S)	CGCTTCCTATCACCTTGTGG			
			C1872 (S)	CTAGTCGTGCTCGTTCCTCTGC			This paper
			C1873 (S)	GGCAGAGATAGAACAATG			This paper
			C1874 (S)	CATTGTGTTCTATCTCTGCC			This paper
6	133088 134107	ORF 88a	ORF88aFor (A,S)	CCCAGTCTATTATCCAGGTAC	1020	(Martenot et al., 2013)	
			ORF88aRev (A,S)	ACCGTTCCTCAATCAGTCCC			

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741 <sup>a</sup> Position on the OsHV-1 genome relative to the OsHV-1 reference genome, GenBank: AY509253

742 <sup>b</sup> Purpose: A amplification, S sequencing

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Table 3: GenBank accession numbers of the 29 DNA groups of the 6 regions analysed.

Region	Gene	Sequence Group	GenBank Accession No.				
1	C region	ORF4	G1	ON953753			
			G2	ON953754			
			G4	ON953755			
			G6	ON953752			
			G7	ON953756			
2	A region	ORF 42-43	G8	ON953757			
			G9	ON953758			
3	605 bpDel	ORF 35 to 38	G10	ON953759			
			G11	ON954001			
			G12	ON954014			
			G13	ON954007			
			G14	ON954004			
			G15	ON954003			
			G17	ON954002			
			4	NC2 region	Non-coding, between ORF 49 and ORF 50	G18	ON954013
						G19	ON954012
						G20	ON954006
						G21	ON954010
						G22	ON954009
						G23	ON954008
G24	ON954005						
5	ORF 11 gene	ORF 11	G29	ON954011			
			G31	ON953762			
			G32	ON953760			
			G33	ON953763			
			G34	ON953761			
6		ORF 88	G35	ON953765			
			G36	ON953764			
			G37	ON953766			

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*Table 4: Description of the 12 genotypic variants based on concatenated nucleotide sequences of Regions 1,2,3,4 and 6 for 61 isolates according to their group identification numbers for these regions.*

<b>Variant</b>	<b>n</b>	<b>R1</b>	<b>R2</b>	<b>R3</b>	<b>R4</b>	<b>R6</b>
		<b>ORF 4</b>	<b>ORF 43</b>	<b>ORFs 35, 36, 37, 38</b>	<b>uncoded site between ORF49 and 50</b>	<b>ORF 88</b>
V1	32	G7	G8	G10	G11	G36
V2	3	G1	G9	G10	G11	G37
V3	1	G7	G9	G10	G20	G37
V4	1	G7	G9	G10	G21	G37
V5	1	G7	G9	G10	G29	G37
V6	2	G7	G8	G10	G11	G37
V7	5	G7	G9	G10	G13	G37
V8	10	G7	G9	G10	G11	G37
V9	2	G4	G9	G10	G12	G37
V10	2	G7	G9	G10	G14	G37
V11	1	G7	G9	G10	G15	G37
V12	1	G7	G9	G10	G17	G37

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754 *Table 5: Nucleotide diversity in the C region (including the microsatellite region) of OsHV-1 from different geographical*  
755 *regions. The data are based on sequences listed in Table 2 of Supplementary data 3 as well as sequences from the current*  
756 *study. Six sequences were added to be consistent with Mineur et al (2015) (see materials and methods). NW Pacific includes*  
757 *isolates from China, Japan and Korea; NE Pacific includes Mexico and USA; Europe includes France, Ireland, Italy,*  
758 *Portugal and Spain; Australasia includes Australia and New Zealand.*

Geographic region	Current study		Mineur et al (2015)	
	No. of genotypes	Nucleotide diversity ( $P_i$ )	No. of genotypes	Nucleotide diversity ( $P_i$ )**
All	95	$7.46 \times 10^{-3}$	48	$14.97 \times 10^{-3}$
NW Pacific	45	$6.45 \times 10^{-3}$	27	$9.34 \times 10^{-3}$
Australasia	10	$1.49 \times 10^{-3}$	3	$2.09 \times 10^{-3}$
Australia	7	$0.99 \times 10^{-3}$	nr	nr
NE Pacific	5	$6.24 \times 10^{-3}$	2	$6.27 \times 10^{-3}$
Europe all years	41	$7.61 \times 10^{-3}$	16	$15.30 \times 10^{-3}$
*Europe < 2008	9	$3.43 \times 10^{-3}$	10	$4.67 \times 10^{-3}$
Europe $\geq$ 2008	32	$2.07 \times 10^{-3}$	6	$3.45 \times 10^{-3}$
France	27	$5.31 \times 10^{-3}$	nr	nr

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760 \*One sequence (AY459363) with a high frequency of undetermined nucleotides (“N”) was  
761 excluded from the current study

762 \*\*corrected for  $H_d=1$ , see materials and methods

763 nr, no result

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Table 7: Distribution of 12 DNA variants and protein prediction profiles among 61 samples from ten different sites in the Georges and Hawkesbury river estuaries between 2011 and 2016

Estuary	Site	No. samples	DNA variant	Predicted protein
Hawkesbury river (n=34)	Coba Bay	1	V1	ACH
	Kimmerikong Bay	4	V1 (n=3) V6 (n=1)	ACH
	Mooney Mooney	15	V1 (n=14) V6 (n=1)	ACH
	Mullet Creek	8	V1	ACH
	Porto Bay	6	V1	ACH
George river (n=27)	Neverfail	2	V10	ADH
	site A	2	V4 V8	ADH
	site B	5	V3 V5 V8 V11 V12	ADH
	site C	13	V8 (n=8) V9 (n=2) V2 (n=3)	ADH
	The shed	5	V7	ADH

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Table 8: Protein profiles of the 12 DNA variants identified among 61 samples from ten different sites in the Georges and Hawkesbury river estuaries between 2011 and 2016

<b>Variant</b>	<b>n</b>	<b>ORF 4</b>	<b>ORF 43</b>	<b>ORF 88</b>
V1	32	A	C	H
V2	3	A	D	H
V3	1	A	D	H
V4	1	A	D	H
V5	1	A	D	H
V6	2	A	C	H
V7	5	A	D	H
V8	10	A	D	H
V9	2	A	D	H
V10	2	A	D	H
V11	1	A	D	H
V12	1	A	D	H

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