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Ostreid herpesvirus in wild oysters from the Huelva coast (SW Spain)

M. López-Sanmartín[1,*], J. R. López-Fernández[1], M. E. Cunha[2], R. De la Herrán[3], J. I. Navas[1]

[1]IFAPA Centro *Agua del Pino,* Consejería de Agricultura, Pesca y Desarrollo Rural, Junta de Andalucía, Ctra. El Rompido-Punta Umbría Km 3.8, 21459 Cartaya, Huelva, Spain

[2]Estação Piloto de Piscicultura de Olhão, Instituto Português do Mar e Atmosfera, Av. 5 de Outubro, s/n, 8700-305 Olhão, Portugal

[3]Departamento de Genética, Facultad de Ciencias, Universidad de Granada, 18071 Granada, Spain

ABSTRACT: This is the first report of ostreid herpesvirus 1 microvariant (OsHV-1 µVar) infecting natural oyster beds located in Huelva (SW Spain). The virus was detected in 3 oyster species present in the intertidal zone: *Crassostrea gigas* (Thunberg, 1793), *C. angulata* (Lamarck, 1819) and, for the first time, in *Ostrea stentina* Payraudeau, 1826. Oysters were identified by a specific polymerase chain reaction (PCR) and posterior restriction fragment length polymorphism (RFLP) analysis based on cytochrome oxidase I (COI) mitochondrial DNA. Results confirmed that *C. angulata* still remains the dominant oyster population in SW Spain despite the introduction of *C. gigas* for cultivation in the late 1970s, and its subsequent naturalization. *C. angulata* shows a higher haplotype diversity than *C. gigas*. OsHV-1 virus was detected by PCR with C2/C6 pair primers. Posterior RFLP analyses with the restriction enzyme *Mfe*I were done in order to reveal the OsHV-1 µVar. Detections were confirmed by DNA sequencing, and infections were evidenced by *in situ* hybridization in *C. gigas*, *C. angulata* and *O. stentina* samples. The prevalence was similar among the 3 oyster species but varied between sampling locations, being higher in areas with greater harvesting activities. OsHV-1 µVar accounted for 93% of all OsHV-1 detected.

KEY WORDS: *Crassostrea gigas* · *Crassostrea angulata* · *Ostrea stentina* · Herpesvirus · Microvariant · OsHV-1 µVar · *In situ* hybridization

INTRODUCTION

It is presumed that the Portuguese oyster *Crassostrea angulata* (Lamarck, 1819) was brought into Europe from Asia by Portuguese navigators in the 16th century (Menzel 1974, Buroker et al. 1979). Naturalized oyster populations have been collected and farmed in Portugal and Spain since the late 19th century. In the early 20th century, the cultivation of *C. angulata* spread to France, replacing the traditional flat oyster (*Ostrea edulis*) farming affected by unexplained mortalities (Buestel et al. 2009). However, in the 1970s, Portuguese oyster cultures collapsed in Europe due to gill necrosis disease (Comps & Duthoit 1976). Subsequently, the Japanese oyster *C. gigas* (Thunberg, 1793) was introduced in France, and later throughout Europe, allowing recovery of the oyster farming industry (Grizel & Héral 1991). *C. gigas* farming in Europe had not been compromised until the onset of the viral diseases caused by ostreid herpes virus type 1 (Renault & Novoa 2004). Since the first description of a herpes-like virus infection in oysters was reported in the eastern oyster *Crassostrea virginica* by Farley et al. (1972), herpes viruses

*Corresponding author: monserratls@yahoo.es

have been related to mortalities of several species of cultured molluscs worldwide causing significant economic losses for aquaculture (Renault & Novoa 2004). The first purification and partial genome characterization of a herpes-like virus was carried out by Le Deuff & Renault (1999) from larval *C. gigas*. This virus, associated with the sporadic high mortalities of larval *C. gigas* in French private hatcheries since 1991 (Renault et al. 1994), was subsequently identified as ostreid herpesvirus type 1 (OsHV-1) within the genus *Ostreavirus* (Davison et al. 2009).

OsHV-1 is known to infect and cause significant mortalities of several bivalve species (EFSA AHAW 2015). Diverse primer pairs have been described for PCR diagnosis of OsHV-1, but the C2/C6 primer set is used most often (Arzul et al. 2001a) due to the amplified part, open reading frame 4 (ORF4), a highly variable part of the virus genome. In 2010, a particularly virulent strain of OsHV-1 was described and associated with a new pattern of mass mortality of *C. gigas* (Thunberg, 1793) spat in Europe (Segarra et al. 2010). This variant, known as microvariant (µVar), shows specific mutations in a microsatellite locus upstream of ORF4 and within ORF4 (Segarra et al. 2010). Aranguren et al. (2012) developed a simple method by PCR restriction fragment length polymorphism (RFLP) to enable differential diagnosis of OsHV-1 µVar from the OsHV-1 reference type.

OsHV-1 and different variants have been described worldwide associated with mortality events in the larval and juvenile stages of *C. gigas* in France (Renault & Novoa 2004), the US (Burge et al. 2006), Ireland (Lynch et al. 2012), Spain (Roque et al. 2012), Australia (Jenkins et al. 2013) and New Zealand (Keeling et al. 2014). Other bivalve species with reported larval mortalities associated with the presence of OsHV-1 are *Ruditapes philippinarum* (Renault et al. 2001), *R. decussatus* and *Ostrea edulis* (Renault & Arzul 2001), *C. angulata* and *C. rivularis* (Arzul et al. 2001a), and *Pecten maximus* (Arzul et al. 2001b). OsHV-1 viruses have also been identified without mortalities associated in *C. gigas* from Mexico (Grijalva-Chon et al. 2013), Korea (Jee et al. 2013), Japan (Shimahara et al. 2012) and China (Bai et al. 2015).

In Spain, OsHV-1 and OsHV-1 µVar have been detected and associated with seed and adult oysters in the mouth of the Ebro River in Catalunya (Roque et al. 2012), Galicia (Villalba et al. 2015) and Asturias and Cádiz (Aranguren et al. 2012). Recently, OsHV-1 µVar was reported in association with mortality events in the adult stages of *C. angulata* (Batista et al. 2015) and *O. edulis* (López-Sanmartín et al. 2016). However, no data are available on the presence of OsHV-1 on the Huelva coast, one of the areas with the greatest potential for oyster farming in Andalusia.

The South Atlantic coast of Spain is characterized by large areas of marshes that have traditionally been exploited for salt production, although currently most of them are dedicated to aquaculture. This coastline comprises 2 provinces, Huelva in the north and Cádiz in the south. The coast of Huelva has had historically significant natural beds of the Portuguese oyster *C. angulata*. As in the rest of Europe, *C. gigas* was introduced in Andalusia for culturing in the late 1970s with the possibility of naturalized populations establishing. Unfortunately, the excellent growth prospects declined in 1987, when ostreid culture and marketing were banned in Huelva due to concentrations of heavy metals that were greater than the allowable limits (Consejería de Agricultura y Pesca 1987a,b). All cultivated oysters were removed and buried. Nevertheless, oyster farming continued in Cadiz. Corrective measures and extensive monitoring programmes were instituted in accordance with European regulations (Council of the European Communities 1991, 2006, 2011), but capture and marketing of oysters were not authorized again on the coast of Huelva until 1 September 2011. During this period (1987 to 2011), there was no oyster harvesting in the province of Huelva, and only a few trials were conducted with seed of *C. gigas* in the Carreras River. Therefore, the natural oyster beds on the Huelva coast had been recovering for 24 yr. Taking advantage of this natural experiment, in the summer of 2011, we sampled the natural oyster beds just before they were harvested again. The purposes of this study were first to identify the oyster species that are growing in both wild and farmed oyster populations, and second to determine the presence and prevalence of OsHV-1 in the main growing areas on the coastline of Huelva.

MATERIALS AND METHODS

Sampling, DNA extraction and histology

Intertidal zones of the rivers Guadiana (Ayamonte), Carreras (Isla Cristina and Pinillos) and Piedras (Nuevo Portil and Rompido) were sampled in June 2011 (Fig. 1). About 60 oysters from each location were collected randomly without distinguishing oyster species (*Crassostrea gigas, C. angulata* or the dwarf oyster *Ostrea stentina* Payraudeau, 1826). Each oyster was measured, weighed and opened in

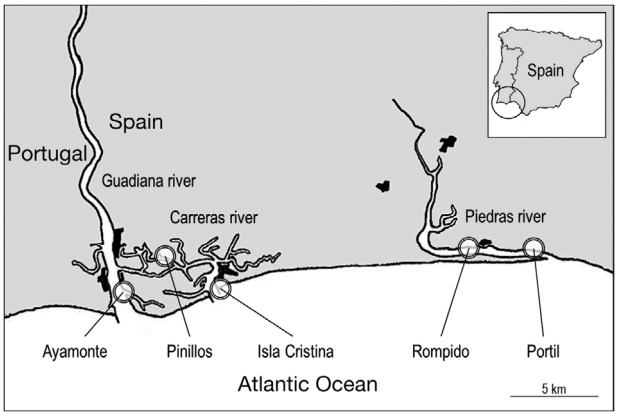

Fig. 1. Study areas on the Huelva coast (SW Spain) where oyster (*Crassostrea angulata*, *C. gigas* and *Ostrea stentina*) samples were collected (Ayamonte on the Guadiana River, Pinillos and Isla Cristina on the Carreras River, Rompido and Nuevo Portil on the Piedras River)

aseptic conditions to avoid cross-contamination. The condition index (CI) of each oyster was estimated as $ww \times 10^6/L^3$ where ww is wet meat weight (mg) and L is the dorsal–ventral shell length (mm). In order to perform DNA analysis, pieces of mantle and gill tissue were removed and preserved in an aqueous solution with 20% DMSO, 0.25M EDTA and 30 mg ml^{-1} NaCl at 4°C (Seutin et al. 1991). DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's recommendation.

Transverse portions of each specimen were fixed in Davidson's solution (Shaw & Battle 1957), dehydrated in an ethanol series and embedded in paraffin. Serial sections (4 µm thick) were used respectively for histological staining with Mayer's haematoxylin and eosin-phloxine (Luna 1968) and *in situ* hybridization (ISH).

Oyster species determination

Oyster identification was carried out by PCR-RFLP of the mitochondrial cytochrome oxidase C subunit I (COI) as proposed by Boudry et al. (1998) and reviewed by Lapègue et al. (2004). Briefly, amplicons obtained using primers COIF and COIR (Folmer et al. 1994) were digested by 4 endonucleases: FastDigest® *Taq*I, FastDigest® *Sau*3A, FastDigest® *Hha*I and FastDigest® *Mse*I (Fermentas) and analysed by agarose gel electrophoresis. Interpretation of the results obtained was performed according to the scheme proposed by Batista (2007) (Fig. 2).

Detection of OsHV-1 by PCR-RFLP and sequencing

OsHV-1 DNA was detected by PCR amplification with C2/C6 primers (Arzul et al. 2001c). This primer pair amplifies a fragment of ORF4 and a flanking non-coding region of virus genome. The PCR conditions followed Batista et al. (2007). Briefly, PCR was carried out in a 25 µl volume containing 10 µM of each primer, 0.625 U of MyTaq™ DNA Polymerase (Bioline) and 100 ng of DNA template in 1× MyTaq™ Reaction Buffer. Amplifications were performed using an Eppendorf Mastercycler Gradient thermocycler: 1 cycle of 95°C for 1 min; 35 cycles at 95°C for 15 s, 58°C for 15 s and 72°C for 10 s; and finally 72°C for 10 min. Each run included a negative control (deionised sterile water) and 2 positive controls consisting of pCR2.1-TOPO plasmidic DNA containing region C from OsHV-1 and OsHV-1 µVar, respectively. Both positive controls were provided by the

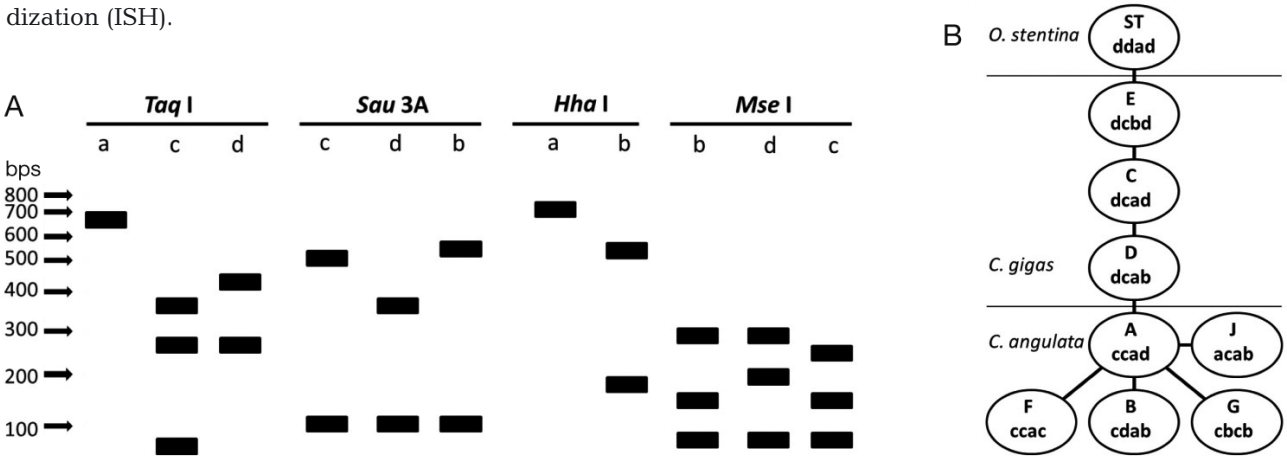

Fig. 2. (A) Restriction fragment patterns of a cytochrome oxidase C subunit I (COI) gene fragment digested with *Taq*I, *Sau*3A, *Hha*I and *Mse*I based on Boudry et al. (1998) and improved by Lapègue et al. (2004). Restriction fragments smaller than approximately 70 bp are not shown. (B) Minimum spanning tree of COI PCR-RFLP haplotypes from *Crassostrea angulata* (A, J, F, G and B), *C. gigas* (E, C and D) and *Ostrea stentina* (ST) according to Batista (2007)

Centro de Investigacións Mariñas, Consellería do Medio Rural e do Mar, Xunta de Galicia (Spain). PCR products were stained by BluXyo (Bioline), electrophoresed on a 1.5% agarose gel and visualized using a GelDoc-IT Transilluminator (Bioimaging Systems). OsHV-1 µVar was detected by RFLP analysis of PCR products with the *Mfe*I endonuclease described by Aranguren et al. (2012). The products of RFLP were also stained by BluXyo, run on a 1.5% agarose gel and visualized using a GelDoc-IT Transilluminator.

A total of 27 C2/C6 PCR products of the expected size were purified using the ExoStar Purification Kit (GE Healthcare) according to the manufacturer's instructions. The purified PCR products of the C2/C6 region were sequenced using both primers by the company SECUGEN (Madrid, Spain). The sequences obtained were compared with sequences available in GenBank using BLAST (Altschul et al. 1997).

Detection of OsHV-1 by ISH

In order to verify the presence of OsHV-1 in oyster tissues, ISH was carried out using 2 different digoxigenin (DIG)-labelled probes designed for region C: C2C6-DIG probe (698 bp) and S1-DIG probe (50 bp). C2C6-DIG was synthesized using the PCR DIG Probe Synthesis Kit (Roche) according to the manufacturer's instructions with the C2/C6 primer pair (Arzul et al. 2001c) and OsHV-1 µVar DNA as template. S1-DIG probe (50 bp) (5'-/5DIGN/ GCC TTT CAC AGA ATT TTG CAC CTT GAC CAA AGC CAT CAC ATC AGC CAG CA-3'), synthesized by Eurogentec and located in ORF4, shares 100% similarity with the OsHV-1 reference type.

The ISH procedure followed López-Flores et al. (2008). This method differs slightly from that described by Stokes et al. (1995) and Lipart & Renault (2002). In brief, serial paraffin sections (4 µm thick) in salinized slices (SuperFrostUltra Plus; Thermo Scientific) were deparaffinized in isoparaffin H, rinsed with ethanol, gradually rehydrated and permeabilized with Proteinase K (50 µg ml^{-1}) in phosphate-buffered saline (PBS: 150 mM NaCl, 12.5 mM Na_2HPO_4, 3 mM KH_2PO_4) for 10 min at 37°C. Proteolysis was stopped with glycine 0.2% in PBS. A prehybridization step was carried out with 100 µl of prehybridization buffer (50% formamide, 4× SSC, 5× Denhardt's solution, 0.50 mg ml^{-1} yeast tRNA, 0.25 mg ml^{-1} salmon testes DNA) on each slice, covered with plastic coverslips and placed in a humid chamber for 60 min at 42°C. Afterwards, prehybridization solution was replaced with 100 µl of hybridization solution (prehybridization buffer containing 5 ng of probe $µl^{-1}$). DNA was denatured at 95°C for 15 min and then cooled at 0°C for 1 min prior to incubation overnight in a humid chamber at 42°C. After hybridization, cover slips were removed and sections were rinsed twice for 5 min at room temperature with 2× SSC and 1× SSC and twice for 10 min with 0.5× SSC at 37°C. Later, slides were washed with 100 mM Tris-HCl, 150 mM NaCl, pH 7.5 at room temperature (2 × 5 min) and immersed in blocking solution (0.2% p/v blocking reagent [Roche] in 100 mM Tris-HCl, 150 mM NaCl, 2 mM malic acid and 0.3% v/v Triton X-100) for 60 min. DIG-labelled probe detection was carried out with anti-Dig-AP (Roche) 3 mU ml^{-1} in blocking solution, using 100 µl per slide for 3 h at room temperature. Thereafter, slides were rinsed in 100 mM Tris-HCl, 150 mM NaCl, pH 7.5. Alkaline phosphatase activity was detected in each of the slides with 5 µl NBT/BCIP stock solution (Roche) in 245 µl basic buffer (100 mM Tris-base, 50 mM MgCl2, 100 mM NaCl, 24 µg levamisole ml^{-1}, pH 9.5) for 3 h in darkness at 20°C. Slides were counterstained using Bismarck brown Y (0.5% in water) for 1 min and mounted in Shandon aqueous mounting media and left to dry for 24 h. Positive reactions were detected under microscopy by a typical blue-black precipitate. Negative controls (without the DIG-labelled probe in the hybridization buffer and oyster tissue uninfected with OsHV-1) as well as a positive control (*C. gigas* tissues infected with OsHV-1 µVar) were included in the analysis. *C. gigas* tissues used as positive controls were provided by the Institut de Recerca i Tecnologia Agroalimentaries, Generalitat de Catalunya, Spain.

Statistical analysis

Data of continuous variables (size, weight and CI) were tested for normality and homoscedasticity, and transformed according to the best fit, previously analysed. Comparisons were performed by single-factor ANOVA and analysis of covariance to include a covariate that could influence the outcome. In order to investigate non-random associations between qualitative variables (herpesvirus presence/absence, locations, species or haplotypes), a chi-squared test was used. Fisher's exact test was carried out when the data set did not meet the conditions required for the calculation of the asymptotic significance. Monte Carlo simulation was performed if the requested Fisher's exact test failed for lack of memory. A Bonferroni correction was used to adjust the significance values for multiple

comparisons between proportions. Probability for hypothesis rejection was $p < 0.05$. Statistical analyses were performed using IBM SPSS Statistics (v. 21).

RESULTS

Oyster species and haplotype distribution

Table 1 shows the shell length and condition index (CI) of different oyster specimens from different sampling sites. *Crassostrea angulata* and *C. gigas* shell length did not differ significantly from each other, but both were significantly different from *Ostrea stentina*. CI did not differ among the 3 species (Table 1). The 3 oyster species were found in the 3 sampled rivers in similar proportions (Fig. 3). *C. angulata* was the most abundant (69%), followed by *C. gigas* (19%) and *O. stentina* (12%). A significant association between the presence of different haplotypes and sample locations was detected. Among the haplotypes of *C. angulata* (haplotypes A, B, F, G and J), haplotype A was the most abundant (65%), with significant differences between sampling sites. Haplotype A was significantly higher in Rompido (Piedras River upstream) than in Portil (Piedras River downstream) and Pinillos (Carreras River). The other haplotypes of *C. angulata* also showed significantly different proportions between sites (Fig. 3). Haplotype B (17%) was significantly different in the rivers Guadiana (39%), Carreras (19%) and Piedras (4%). Haplotype F (2%) was absent in the Carreras River, but had a similar presence in Guadiana (2%) and Piedras (3%). Haplotype G was not detected in the Guadiana River, and no significant differences were found in Carreras (15%) and Piedras (5%). Haplotype J (8%) was also absent in the Guadiana River, and its presence was significantly different between Piedras (20%) and Carreras (1%).

Of the *C. gigas* haplotypes (C, D and E), haplotype E was not detected in any of the sampled rivers (Fig. 3), and haplotypes C (86%) and D (14%) showed similar relative occurrences in the different localities. The distribution of *O. stentina* (haplotype ST) was similar between rivers but significantly different within sites in the Piedras River: *O. stentina*

Table 1. Location and date of sampling, species, number of specimens (N), dorsal–ventral shell length and condition index (CI) of sampled oysters (*ww*: wet meat weight; *L*: length); nd: not determined

Location and date (dd/mm/yy)	Species	N	Shell length (mm), mean ± SE	CI ($ww \times 10^6/L^3$), mean ± SE
Ayamonte 15/05/11	*C. angulata*	44	103 ± 6.6	nd
	C. gigas	12	82 ± 16.0	nd
	O. stentina	4	22 ± 2.8	nd
Pinillos 14/06/11	*C. angulata*	40	63 ± 3.1	26 ± 1.4
	C. gigas	11	51 ± 7.1	27 ± 2.7
	O. stentina	8	28 ± 1.6	24 ± 3.9
Isla Cristina 14/06/11	*C. angulata*	41	55 ± 3.8	30 ± 2.4
	C. gigas	9	47 ± 4.3	31 ± 7.3
	O. stentina	6	38 ± 7.8	25 ± 5.9
Rompido 15/06/11	*C. angulata*	48	46 ± 2.3	32 ± 3.2
	C. gigas	11	53 ± 5.6	24 ± 3.7
Portil 15/06/11	*C. angulata*	29	39 ± 2.5	32 ± 2.0
	C. gigas	13	56 ± 3.4	39 ± 2.3
	O. stentina	16	25 ± 1.3	19 ± 1.8

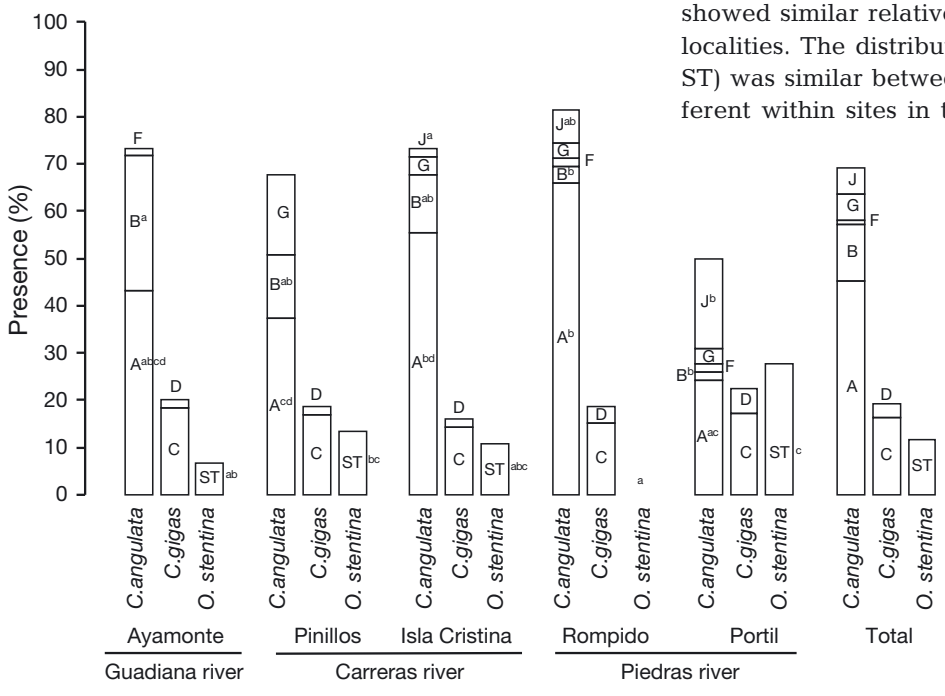

Fig. 3. Oyster haplotypes in the Guadiana, Carreras and Piedras Rivers. Letters correspond to haplotypes of *Crassostrea angulata* (A, B, F, G and J), *C. gigas* (C and D) and *Ostrea stentina* (ST). Different superscripts on the same haplotype indicate significant differences between locations

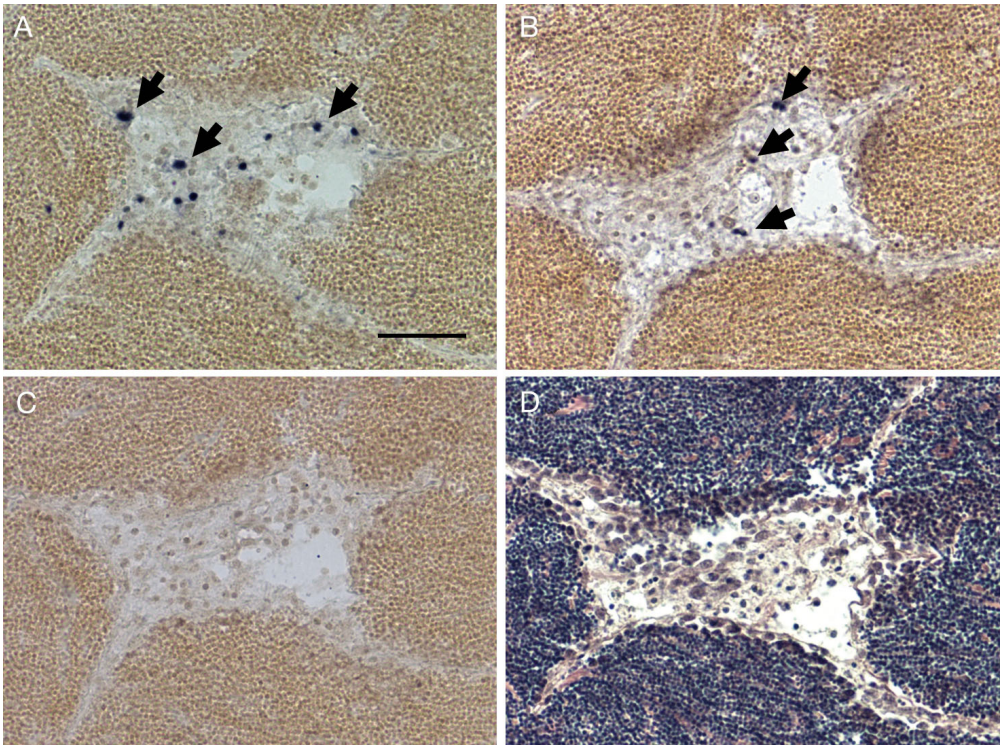

Fig. 4. Ostreid herpes- virus type 1 (OsHV-1) detection by *in situ* hybridization on serial sections from the male gonadal tissue of *Crassostrea angulata.* (A) C2C6-DIG probe showing strong positive signals (arrows) in connective tissue cells. (B) S1-DIG probe showing weak positive signals (arrows). (C) No probe. (D) Histological section stained with haematoxylin and eosin. Scalebar = 50 µm

was not detected upstream (Rompido), but was most abundant downstream (28% in Portil).

OsHV-1 detection, sequence analysis and ISH

OsHV-1 and OsHV-1 µVar were identified by PCR-RFLP analysis and DNA sequencing of several PCR positive results. Sequences of the 26 amplicons (21 *C. angulata*, 2 *C. gigas* and 2 *O. stentina*) obtained with the C2/C6 primer pair (475 bp after editing), putative OsHV-1 µVar by digestion with *Mfe*I endonuclease (Aranguren et al. 2012) was 100% identical to the sequence of OsHV-1 µVar reported by Segarra et al. (2010) (accession number HQ842610). The sequence of an amplicon of putative OsHV-1 not digested by *Mfe*I was 100% identical with the sequence of an OsHV-1 isolate collected in France in 2003 from *C. gigas* reported by Renault et al. (2012) (accession number JN800075).

In addition, the presence of viral DNA in the tissues of oysters was demonstrated by ISH on positive C2/C6 PCR specimens of the 3 oyster species from the different rivers. Large numbers of labelled cells were observed, and viral DNA was detected by the typical blue-black precipitate, mainly in the nuclei of cells. The positive reaction was found in haemocytes and connective tissue cells of the various organs, viz. mantle, gills, labial palp and gonads, as well as in heart and nerve cells. Both probes, C2C6-DIG (698 bp) and S1-DIG (50 bp), were useful to detect viral DNA; however, the signal obtained with C2C6-DIG was clearly more intense due to its larger size (Fig. 4).

OsHV-1 prevalence in oyster species, haplotypes and sampling sites

Fig. 5 shows the prevalence of OsHV-1 by species and location. According to PCR results using the C2/C6 primer pair, more than half of the sampled oysters (54%) tested positive for OsHV-1. They showed mainly (93%) the target sequence of *Mfe*I endonuclease (CAATTG), present in OsHV-1 µVar. The percentage of oysters with the OsHV-1 C2/C6 target amplicon that was not digestible by the enzyme *Mfe*I was 7%. Size and CI did not differ significantly between infected and uninfected oysters (data not shown).

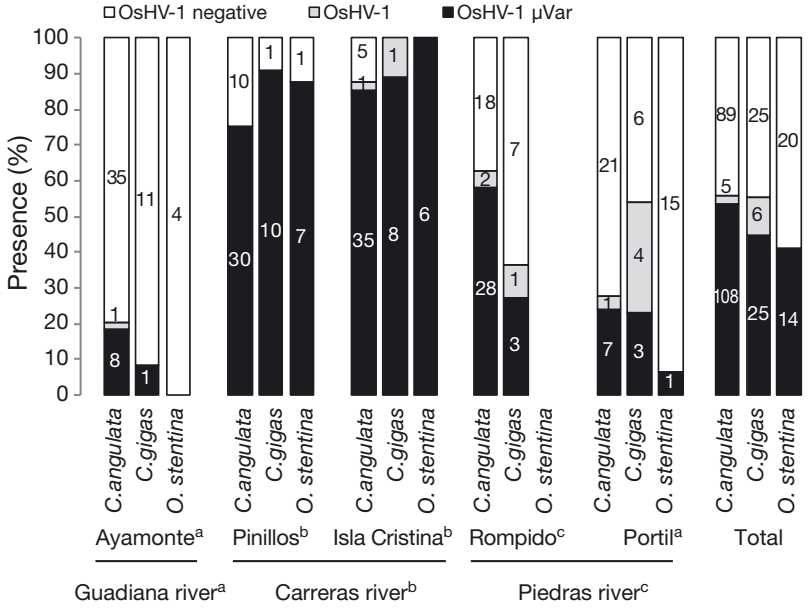

Fig. 5. Prevalence of ostreid herpes virus type 1 (OsHV-1) in oyster species (*Crassostrea angulata*, *C. gigas* and *Ostrea stentina*) from the Guadiana, Carreras and Piedras Rivers. Different superscripts above locations or rivers indicate significant differences in OsHV-1 prevalence

No significant differences were detected in OsHV-1 (OsHV-1 and OsHV-1 µVar) prevalence among different oyster species: *C. angulata*, 56% (53.5% OsHV-1 µVar); *C. gigas*, 55% (45% OsHV-1 µVar); and *O. stentina*, 41% (41% OsHV-1 µVar). However, the occurrence of OsHV-1 without the C2/C6 target amplicon that was digestible by *Mfe*I was significantly higher in *C. gigas* (11%) than in *C. angulata* (2.5%) and *O. stentina* (0%), and was particularly high in *C. gigas* from Portil (31%; Fig. 5).

Regarding sampling sites, prevalence of OsHV-1 µVar was significantly higher in the Carreras River (85%: Pinillos, 80% and Isla Cristina, 91%) than in the Piedras River (43%: Rompido, 58% and El Portil, 28%) and the Guadiana River (Ayamonte, 17%). Pinillos (Carreras River) was the only sampling point where all positive samples for herpesvirus were OsHV-1 µVar positive (Fig. 5).

No significant associations between oyster haplotypes and OsHV-1 or OsHV-1 µVar prevalence were detected in a global analysis (Fig. 6). Haplotype G (*C. angulata*) had the highest prevalence of OsHV-1 µVar (75%). None of the 3 oysters identified as haplotype F (*C. angulata*) were found to be infected. OsHV-1 prevalence was higher in haplotype C (12.5%) than in haplotypes A (2%) and B (6%), and was absent in the other haplotypes. However, none of these trends was statistically significant.

DISCUSSION

Oyster species and haplotype distribution

The South Atlantic coast of Spain is an area of historical production of *Crassostrea angulata*. In the late 1960s, the production was more than 800 t yr^{-1} (Pascual 1973). As far as we know, *C. gigas* was introduced for harvesting in the Piedras and Carreras Rivers in the late 1970s. In 1987, all farmed oysters were removed and their marketing was prohibited until September 2011. We have presented the first data on the distribution of ostreid species in intertidal natural beds on the Huelva coast after 24 yr without ostreid harvesting. Results confirmed that the Portuguese oyster *C. angulata* is the predominant species (69%), followed by the Japanese oyster *C. gigas* (19%) and the dwarf oyster *Ostrea stentina* (12%). The ratio of *C. angulata* to *C. gigas* is similar to that found by Fabioux et al. (2002) in Tavira (Portugal), just 20 km from the sampling point on the Guadiana River. Previously, Boudry et al. (1998) found higher percentages of *C. angulata* in other nearby areas: 100% in Cádiz (Spain) and 82 to 91% in Ría Formosa (Portugal). *O. stentina* is present on the Huelva coast as well as

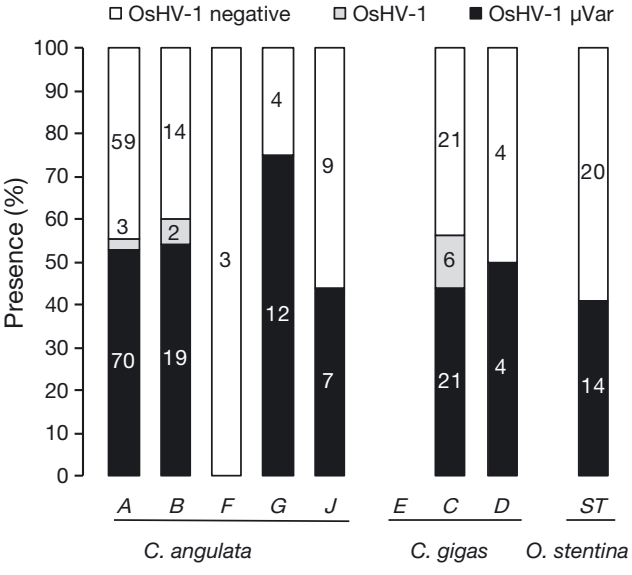

Fig. 6. Prevalence of ostreid herpes virus type 1 (OsHV-1) in oyster (*Crassostrea angulata*, *C. gigas* and *Ostrea stentina*) haplotypes from the Huelva coast (SW Spain)

elsewhere along the European and African, Atlantic and Mediterranean coasts (Lapègue et al. 2006). In contrast, the flat oyster *O. edulis* was not found in this study, suggesting that *O. edulis* may have been extirpated from the sampled intertidal natural beds on the Huelva coast.

This study evidences the haplotypic variability of *C. angulata*, in contrast to the homogeneity of the populations of *C. gigas*. Lower levels of variability for the COI sequence of *C. gigas* with respect to *C. angulata* were already reported by Lapègue et al. (2004), comparing samples from different countries. In our study, the highest variability of haplotypes in *C. angulata* was confirmed by significant differences between samples from very close sources. Haplotypes A and B were most abundant in the Portuguese oyster. Haplotypes G, J and F, which were absent in Cádiz (Boudry et al. 1998, Huvet et al. 2000, Fabioux et al. 2002) and had a low prevalence in Portugal (Batista 2007), were detected in Huelva. Regarding Japanese oyster *C. gigas*, haplotype C (86%) was predominant according to previous studies in neighbouring areas (Boudry et al. 1998, Fabioux et al. 2002). The absence of haplotype E in our samples is noteworthy, given that it is present in France, Wales (UK), Taiwan (Boudry et al. 1998), Asturias (Spain) and Morocco (Fabioux et al. 2002). *C. angulata* diversity seems typical of wild populations, and the homogeneity of *C. gigas* could be explained by the common origin from farmed stocks. The importance of conservation of *C. angulata* variability not only as a natural resource but as a useful genetic resource for *C. gigas* aquaculture was already pointed out by Lapègue et al. (2004).

OsHV-1 prevalence in oyster species, haplotypes and sampling sites

This study confirms the presence of OsHV-1 and OsHV-1 µVar in wild populations of oysters from the Guadiana, Piedras and Carreras Rivers (Huelva coast, SW Spain). Ostreid herpesvirus does not seem to have a preference for a particular oyster species. No significant differences were detected among prevalences in *C. gigas*, *C. angulata* and *O. stentina*. OsHV-1 µVar accounts for 93% of all OsHV-1 detected, and the percentage of the OsHV-1 reference type was only 7%. The analytical procedure used in this study does not distinguish the presence of the OsHV-1 reference type when OsHV-1 µVar is present. Therefore, it was not possible to determine probable mixed infections with the type 1 reference strain and the variant µVar. It is known that the OsHV-1 reference type was already present in *C. gigas* (11%), *C. angulata* (8%) and F1 hybrids (1.7%) cultured in the Ría Formosa in 2004 (Batista et al. 2016). In the present study, oysters infected only with the OsHV-1 reference type were detected in *C. gigas* (11%) and *C. angulata* (2.5%), but not in *O. stentina* (Fig. 5). These results suggest that the reference type was originally introduced on the coast of Huelva via *C. gigas* intended for cultivation.

Geographical differences were found regarding virus prevalence. The Carreras River had the highest prevalence (85%), followed by the Piedras (35%) and the Guadiana River (15%). These different prevalences among rivers are consistent with their aquaculture activity over the years. Thus, virus prevalence was significantly higher in the Carreras River, where there has historically been more aquaculture activity. The Carreras is the only river which currently supports oyster culture in Huelva, especially in Pinillos, where *C. gigas* farming has occurred. Interestingly, at this location, all positive samples were OsHV-1 µVar (Fig. 5). The apparent disappearance of the OsHV-1 reference type and replacement with OsHV-1 µVar genotype has been observed since the emergence of the latter in 2008 (EFSA AHAW 2015). These data support the hypothesis that OsHV-1 µVar was subsequently introduced with *C. gigas.*

No significant association was detected among all different haplotypes and virus presence. Although the results are inconclusive, OsHV-1 µVar prevalence seems to be higher in haplotype G than haplotype F in *C. angulata*. Similarly, OsHV-1 prevalence was higher in haplotype C than D in *C. gigas* but was not detected in *O. stentina*. This is consistent with the description of resistant genotypes in oysters by Dégremont et al. (2013, 2015), but a greater number of samples are needed to confirm this hypothesis. The virulence of oyster herpesvirus, particularly in seeds, increases with temperatures above 16°C (Petton et al. 2013). On our coast, temperatures exceed this limit from early spring to late autumn. However, no information is available about mortalities in these oyster populations that could be associated with OsHV-1 µVar infection. Sampling was done in spring, so seeds could not be assessed given their small size. In addition, it is very difficult to detect seed oyster mortalities in natural beds, as they are quickly hidden by the development of the surviving oysters or other epibionts. Therefore, the sampled oysters were at least 1 yr old. Nevertheless, it is notable that no mortalities were detected among the sampled specimens. Recently, Batista et al. (2015) related

mortalities in spat (April 2011) and wild adults (June 2013) of *C. angulata* in the Ría Formosa, Portugal, close to Huelva.

In conclusion, oysters on the Huelva coast present mixed populations of *C. angulata, C. gigas* and *O. stentina*. This could be of great interest as a genetic resource for breeding programmes of *C. gigas*/*C. angulata*. All 3 species appear to be infected by OsHV-1, confirming the abundance of this microvariant. This is the first description of OsHV-1 µVar in *O. stentina*.

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