First characterization of the parasite Haplosporidium costale in France and development of a real-time PCR assay for its rapid detection in the Pacific oyster, Crassostrea gigas

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First detection of the parasite *Haplosporidium costale* in France and development of a Real-Time PCR assay to screen its presence in the Pacific oyster, *Crassostrea gigas*

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Abstract

The Pacific cupped oyster *Crassostrea gigas* is one of the most "globalized" marine invertebrates and its production is predominant in many parts of the world including Europe. However, it is threatened by mortality events associated with pathogenic microorganisms such as the virus OsHV-1 and the bacteria *Vibrio aestuarianus*. *C. gigas* is also a host for protozoan parasites including haplosporidians. In contrast with *Haplosporidium nelsoni* previously detected in Europe, *H. costale* was considered exotic although its presence in French oysters had been suggested in the 80ies based on ultrastructural examination. Here, a combination of light and transmission electron microscopy, PCR and sequencing has allowed characterizing the presence of the parasite in the context of low mortality events which occurred in 2019 in France. Histological observation revealed the presence of uninucleated, plasmodial and spore stages within the connective tissues of some oysters. Ultrastructural features were similar to *H. costale* ones in particular the presence of axe-shaped haplosporosomes in spore cytoplasms. Three fragments of the genome including partial small subunit rRNA gene, the ITS-1, 5.8S and ITS-2 array and part of the actin gene were successfully sequenced and grouped with *H. costale* homologous sequences. This is the first time that the presence of *H. costale* is confirmed in *C. gigas* in France. In addition, a TaqMan real time PCR assay has been developed and validated to enable the rapid and specific detection of the parasite. The application of the PCR assay on archived samples has revealed that the parasite is present in French oyster populations at least since 2008. Considering the little information available on this parasite, the newly developed TaqMan assay will be very helpful to investigate the temporal and geographic distribution and the life cycle of the parasite in France and more generally in *C. gigas* geographic range.

Keywords: Haplosporida; *Haplosporidium costale*; oyster; *Crassostrea gigas*; Parasite; Real-Time PCR

Introduction

Originated from the north eastern Asia, the Pacific cupped oyster *Crassostrea gigas* is currently produced in different parts of the world (Herbert et al., 2016) including France where its production is estimated around 85 000 t per year (FAO 2019). This production is based on wild spat collected in the field and on hatchery-produced spat, mainly triploids. Movements of oysters regularly occur between spat collection areas or hatcheries and oyster growing areas in France and also between France and some European countries including Ireland, Italy, Portugal, Spain or United-Kingdom.

Massive mortality events of *Crassostrea gigas* have been reported since the 90ies in France. Associated with the presence of the virus OsHV-1 or with the presence of the bacteria *Vibrio aestuarianus*, these mortalities have strongly impacted the production of oysters in France and more widely in Europe (EFSA Panel on Animal Health and Welfare (AHAW), 2015). Protozoan parasites such as *Perkinsus marinus*, *Mikrocytos mackini*, *Haplosporidium nelsoni* have been shown to infect *C. gigas*. Whereas the two first parasite species have only been detected in America and are exotic to Europe, *H. nelsoni* has also been reported in *C. gigas* with low prevalence in Northern America (Haskin & Andrews, 1988; Friedman et al., 1991; Friedman, 1996; Stephenson et al., 2003), Asia (Chun 1972; Kern 1976; Kang 1980; Burreson et al. 2000; Kamaishi and Yoshinaga 2002; Wang et al. 2010) and Europe (Lynch et al. 2013, Renault et al. 2000). Its presence does not seem associated with the occurrence of mortality in *C. gigas*.

In France, the surveillance of mollusc diseases is mainly based on the reporting of mortality events by producers. Although this passive approach might present some biases, it aims to detect emerging situations using mortality as an alert signal (Lupo et al. 2014). Investigation of *C. gigas* abnormal mortality is usually carried out not only by testing oysters by PCR for the detection of the virus OsHV-1 or the bacteria *Vibrio aestuarianus* but also by histology. Histology allows the observation of lesions and the detection of a range of pathogens including protozoan parasites (Carnegie et al. 2016).

Haplosporidium costale belongs to the phylum Cercozoa, order Haplosporida and family Haplosporidiidae (Arzul and Carnegie, 2015). Also called SSO (Sea Side Organisms), it has been reported in the context of seasonal mortality events affecting the American oyster *Crassostrea virginica* in high salinity (>25‰) coastal bays on the east coast of U.S.A (Couch and Rosenfield 1968, Andrews and Castagna 1978, Andrews 1984). Low prevalence and intensity of infection has also been reported in *C. virginica* on the Atlantic coast of Canada. Although, the presence of parasite DNA has been detected in *Crassostrea gigas* in China (Wang et al. 2010), no effect has been reported in this oyster species. In *C. virginica*, infections with *H. costale* seem to be acquired in early summer. Plasmodia are generally not observed in oyster tissues before the following spring. Plasmodia rapidly multiply and sporulate in the connective tissues of the digestive gland, mantle and gonad, sporulation coinciding with host death in May-June. In contrast with *H. costale*, the congeneric species *H. nelsoni* sporulates in epithelia of the digestive tubules. Nevertheless, in the absence of spores, histology does not allow distinguishing between both parasite species (Burreson and Reece, 2006). Specific molecular assays have been developed to detect *H. costale* in oysters including a conventional PCR targeting the 18S (Ko et al. 1995) and a multiplex PCR assay allowing the concurrent detection of *H. nelsoni*, *H. costale* and *Perkinsus marinus* (Penna et al. 1999, 2001; Russell et al. 2000, 2004).

Between December 2018 and June 2019, weak mortality has been observed in juvenile *C. gigas* in Vendée and Charente Maritime, France. Primary investigations revealed the presence of *Haplosporidium*-like parasites in oyster tissues. Molecular and ultrastructural analyses have subsequently been carried out to characterize the parasite species and pointed out *Haplosporidium costale*.

The detection of the parasite *H. costale* for the first time in France in association with low mortality has raised some questions notably regarding its origin and distribution. In order to answer these questions, a TaqMan™ Real-Time PCR assay allowing its rapid and specific detection has been developed. Its specificity and sensitivity have been determined and it has been compared with the conventional PCR assay developed by (Stokes & Burreson, 2001) and previously recommended by the

OIE (OIE, 2003). This TaqMan™ assay has finally been tested on archived samples collected in main oyster farming areas in France either in the context of mortality events or studies by the French network of surveillance of mollusc diseases (REPAMO).

Material and methods

I- Characterization of the parasite *Haplosporidium costale*

1- Oyster *Crassostrea gigas* samples

Motivated by low but recurrent mortality observed since December 2018, 17 moribund juvenile oysters *Crassostrea gigas* (coded 19-030 hereafter), weighting around 10g, were collected in March 2019 at the Ifremer nursery facilities in Bouin, Vendée. These oysters were produced in March 2018 in the Ifremer hatchery-nursery in La Tremblade, Charente Maritime, and transferred to the Ifremer nursery in Bouin in May 2018. Cumulative mortality of the oysters during their nursery period reached 7.2% in June 2019.

A second sampling of 10 moribund adult oysters (coded 19-082 hereafter) was collected in June 2019 at the Ifremer experimental farm located in the Seudre River in La Tremblade. Oysters were transferred from the Ifremer nursery in Bouin one week earlier and showed 0.004%-week mortality rate. Initially produced in the Ifremer hatchery in La Tremblade in June 2016, these oysters grew in the facilities in La Tremblade except between August and October 2017, and between July 2018 and June 2019 when they grew in the facilities in Bouin.

During their stabling in La Tremblade and Bouin nurseries, oysters were subjected to a 400 L/h seawater flow enriched with a cultured phytoplankton diet (*Isochrysis galbana*, *Tetraselmis suecica*, and *Skeletonema costatum*) provided ad libitum (50,000 cells/mL).

2- Oyster tissue processing and diagnostic approach

At reception, moribund oysters were opened, checked for the presence of macroscopic signs and processed as described in the procedure available on the EURL for mollusc diseases website (https://www.eurl-mollusc.eu/content/download/143184/file/SampleProcessing_Edition1.pdf).

Sections of organs including gills, mantle, gonad and digestive gland were prepared from each oyster for histology. In addition, about 50 mg of gills, adductor muscle, digestive gland and mantle were collected for bacteria isolation and remaining tissues were frozen (sample 19-030) or fixed in absolute ethanol for molecular analyses (sample 19-082).

Presumptive methods including Real-Time PCR for the detection of OsHV-1 from approximately 25 mg of mantle and gills tissues according to Martenot et al. (2010) and Real-Time PCR for the detection of bacteria belonging to *Vibrio Splendidus* clade or to *Vibrio aestuarianus* species from main isolated bacteria according to Saulnier et al. (2017) were carried out and did not yield positive results except the detection of bacteria belonging to the *Splendidus* clade in some individuals.

Consequently, histological examination of tissues was done in order to test the presence of parasites or lesions. Among oysters collected in June, gill and digestive gland imprints were also done from one individual showing abnormal black coloration of the soft tissues. The observation of parasites looking like parasites of the genus *Haplosporidium* in histology and on imprints has motivated the achievement of a range of molecular analyses, *in situ* hybridization and ultrastructural examination that are described below.

3- Imprints, histology and *in situ* hybridization

Gill and digestive gland imprints were fixed in absolute ethanol, stained with Hemacolor (Merck) and directly observed on a BX50 microscope (Olympus).

After at least 48 h in Davidson's fixative, tissue sections were maintained in 70% ethanol until they were dehydrated and embedded in paraffin for histology according to standard procedures

(Howard et al. 1983). Paraffin blocks were cut in 2–3 µm sections and stained with haematoxylin and eosin. Slide examination was done using a BX50 (Olympus) microscope.

The protocol followed for ISH was adapted from (Stokes and Burreson, 2001). The *Haplosporidium costale* specific oligoprobe (SSO1318) was purchased with 3' digoxigenin-label. Three µm thick tissue sections on silane-prep™slides (Sigma, France) were dewaxed, rehydrated, and treated with proteinase K (100 µgml−1 in TE buffer [Tris 50 mM, EDTA 10 mM]) at 37 °C for 10 min. Slides were dehydrated by immersion in an absolute ethanol bath and air-dried. Sections were then incubated with 100 µl of hybridization buffer (50% formamide, 10% dextran sulfate, 4×SSC [0·06M Na3 citrate, 0·6M NaCl, pH 7], 250 µgml−1 yeast tRNA and 10% Denhardt's solution) containing 5 ng µl−1 of digoxigenin-labelled probes (Eurogentec).

Target DNA and digoxigenin-labelled probe were denatured at 95 °C for 5 min and the hybridization was carried out overnight at 42 °C. Sections were washed in 2×SSC at room temperature (RT) (2×5 min), in 0·4×SSC at 42 °C (10 min) and in solution I (100mM maleic acid, 0·15M NaCl, pH 7·5) for 5 min. Tissues were then blocked for 30 min at room temperature with blocking reagent (Roche) (1% w/v) in solution I. Specifically bound probe was detected using an alkaline phosphatase-conjugated mouse IgG antibody against digoxigenin diluted at 1·5Uml−1 in solution I (1 h, RT). Excess of antibody was removed by 2 washes in solution I (1min) and equilibrated in solution II (0·1 M Tris pH 8, 0·1MNaCl, 0·05M MgCl2, pH 9·5). Slides were incubated in NBT/ BCIP, a chromogenic substrate for alkaline phosphatase, diluted in solution II (20 µl ml−1) in the dark. Slides were subsequently observed using a BX 50 microscope (Olympus). Negative controls included samples without digoxigenin-labelled probe in the hybridization mixture or without antibodies during the revelation step. Positive control consisted of *Crassostrea virginica* infected with *Haplosporidium costale* originating from U.S.A. (kindly provided by R. Carnegie).

4- Molecular analyses for the detection and characterization of *Haplosporidium costale*

DNA Extraction

Total DNA was extracted from approximately 25 mg of -20°C frozen gill, digestive gland and mantle tissues using the Wizard ® Genomic DNA Purification Kit (Promega, Inc.) according to the manufacturer's protocol. Extracted samples were stored at 4°C until being tested by PCR.

DNA amplification

Different combinations of primers were used to test the presence of the parasite *Haplosporidium costale* and to amplify parts of its genome (Table 1). PCR tests were carried out using a T100 thermal cycler (BioRad). For primers previously described, PCR analyses were done following authors' recommendations (Table 1). For primers designed in the present study, reactions of 20µl contained 1X buffer (Promega), 1.5 mM MgCl2, each primer at 0.5 µM, each dNTP at 0.125 µM, 2 U goTaq polymerase (Promega) and 200-250 ng template DNA. Cycling parameters were 94°C for 5 min; 40 cycles of amplification at 94°C for 1 min; annealing temperature as described in Table 1 for 1 min. Final elongation was carried out at 72°C for 10 minutes.

Positive and negative controls were included in each PCR run. Positive controls consisted of DNA extracted from known infected samples. Negative controls consisted of bi-distilled water used in the extraction and real-time PCR steps.

Amplification products were resolved on 1% agarose gels stained with ethidium bromide and visualized using UV illuminator. Expected amplicon size is provided for each primer pair in Table 1.

Correct size products were excised from the gels, purified using Amicor Kit (Millipore) and sequenced.

Sequencing

Purified PCR products were sent to LightRun Sequencing to the company Eurofins genomics, DE. Chromatograms were analysed using FinchTV 1.4 (Geospiza).

Some purified PCR products were home sequenced using the ABI Prism Big Dye Terminator v3.1 sequencing kit following manufacturer's instructions. DNAs were sequenced using the ABI 3130xl

Avant Genetic Analyses (Applied Biosystems). Analyses of the sequences were completed using Finch TV (Biospiza).

Phylogenetic analyses

Sequences obtained were compared with those in the GenBank database using the BLAST algorithm (Altschul et al. 1997).

18S sequences

Available *18S* gene sequences from Haplosporidian organisms and the Paramyxida *Marteilia refringens* were downloaded from GenBank and included in the phylogenetic analyses together with the sequences longer than 1000 bp obtained in the present study. Alignments were performed using ClustalW (Thompson et al. 1994) in MEGA 7 (Kumar et al. 2016) with default parameters. The analysis involved 46 nucleotide sequences and a total of 2071 characters.

Prior to the phylogenetic analysis of sequences, the program jModelTest 0.1.1 (Posada, 2008) was used to select the best fitting substitution model according to the corrected Akaike information criterion (AIC) (Hurvich & Tsai, 1993). A total of 88 candidate models, including models with equal/unequal base frequencies, with/without a proportion of invariable sites (+I), and with/without rate variation among sites (+G) were tested.

The best-fit model of nucleotide substitution was the GTR+I+G model. Tree topology was therefore inferred based on a Bayesian approach using MrBayes v 3.1.2 (Huelsenbeck & Ronquist, 2001) and implementing the GTR + I + G model of nucleotide substitution.

Internal transcribed spacer (ITS) regions, including the ITS 1, 5.8S rRNA gene, and ITS 2

Available ITS1-5.8S - ITS2 sequences from *Carnegie et al. 2014* were downloaded from GenBank and aligned with sequences obtained in this study using ClustalW (Thompson et al., 1994) in MEGA 7 (Kumar et al., 2016) with default parameters.

Evolutionary divergence was estimated between sequences obtained in this study and closest sequences found in Genbank using the Maximum Composite Likelihood model (Tamura et al., 2004). The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). Differences in the composition bias among sequences were considered in evolutionary comparisons (Tamura & Kumar, 2002). Evolutionary analyses were conducted in MEGA 7 (Kumar et al. 2016).

The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated, resulting in a total 409 positions.

5- Ultrastructural examination (19-082)

Pieces (1 mm^3) of mantle, gills and digestive gland of one oyster showing an abnormal black coloration and for which examination of imprints revealed the presence of parasites looking like parasites of the genus *Haplosporidium* were immediately fixed with 4% glutaraldehyde for 24 hours at 4°C. The samples were then washed in 0.2 M cacodylate buffer (3x30 min) and post- fixed for 1 h in 1% osmium tetroxide (OsO_4) in 0.2 M cacodylate buffer, cleared in propylene oxide, and embedded in epon resin. Ultrathin sections were obtained using copper grids and double-stained with 5% uranyl acetate and 5% lead citrate and then examined at 80 kV on a JEOL 1110 transmission electron microscope equipped with a Morada digital camera and iTEM imaging software (Soft Imaging System, Olympus).

II- Development, validation of a Real Time TaqMan™ PCR assay for the detection of Haplosporidium costale and its application on the field

1- Oyster samples

Oyster samples used to assess diagnostic performances of the new PCR assay were collected from three populations displaying presumably different levels of prevalence (488 oysters in total): (i) a population supposed to be free of parasite of oysters from a high biosecurity level area of Ifremer experimental facilities in Bouin (ii) a population supposed to present a moderate prevalence (10-30%) of the parasite including oysters from contaminated facilities at Bouin in which the parasite has been

initially described (iii) a population supposed to present a low prevalence (<10%) of the parasite of wild oysters from Vendée (Supplementary Material Table S1).

In addition, the presence of the parasite in previous years was checked using the new PCR assay on archived *Crassostrea gigas* DNA material collected in different contexts: mortality, macroscopic abnormalities or field study (263 individuals from 17 batches, information available in Table 9).

2- Real Time TaqMan™ PCR development

Oyster DNA Extraction

DNA extraction was performed on fresh oyster tissues or archived material frozen or conserved in ethanol as described above.

Primers and probe design

We used the primer set targeting the *18s* gene (1358F 1507R) initially designed for conventional PCR (Ko et al. 1995). In addition, two probes were designed using Primer Express® Software v3.0 (Applied Biosystems, Foster City, CA, USA) after comparing *Haplosporidium costale* sequence with closely related ones: *Haplosporidium lusitanicum* (AY449713.1), *H. pickfordi* (AY452724.1), *H. edule* (DQ458793.1), *H. raabei* (HQ176468.1), *H. montforti* (DQ219484.1) and *H. nelsoni* (U19538.2). The set was validated *in silico* using NCBI primerBLAST (Basic Local Alignment Search Tool; www.ncbi.nlm.nih.gov/blast/). The sequences were as follows: 1358F (forward primer): 5'-TACTGCTAGCGCTTGTTCGCAAGAT-3'; 1507R (reverse primer): 5'- TCGGGTCGGCCCGCTGACTGGGT-3'; Probe1 (probe): 5'-FAM- GAAGGTCTGGGCTGCACGCG-3'-TAMRA; Probe2: 5'-FAM-AGGGACAATCTGTGCTCAGCAGATGG-3'-TAMRA. The amplicon size was 149 bases. The oligonucleotides were synthesized and purchased from Eurogentec.

Real Time TaqMan™ assay

DNA (5 µL) were transferred into 15 µL of PCR mix, consisting of TaqMan® Supermix (Biorad, 10 µL), primers 1358F (0.3 µL, 300 nM f.c.), 1507R (0.3 µL, 300 nM f.c.), and Probe2 (0.3 µL, 300 nM f.c.). Ultrafast QPCR Mastermix® (Agilent) was also tested in similar conditions. Assays were run on Mx3000 and CFX Connect machines (Agilent, Biorad). The reaction was run at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15s and 60 °C for 30s.

To confirm our amplification, PCR products were sequenced. Briefly, PCR products were purified by ExoStar (GE Healthcare-Life Sciences). Sequence reactions were performed with BigDye® Terminator V3.1 (Applied Biosystems), followed by dye purification with BigDye X-terminator™ purification kit and sequencing with ABI3003xl (Applied Biosystems). All sequences were identical and corresponded to the targeted region, as confirmed by Blast analyses.

3- Validation

Inclusivity and exclusivity tests

Inclusivity and exclusivity were evaluated by testing a panel of samples from the collection of the National and European Union Reference Laboratories for Mollusc Diseases previously characterized (information available in Table 6).

Analytical characteristics

The Real-Time PCR limit of detection (LDpcr) was determined using a synthetic plasmid including 691 bp [from position 817 to 1507] of *H. costale* 18S gene in pUC57 (Eurogentec) and oyster samples naturally infected (samples 19-030 and 19-082 detailed above).

Three independent trials were performed on three independent two-fold serial dilutions (2 - 0.075 DNA copies per µl of template), with eight replicates of each dilution level. LDpcr was determined by the smallest number of nucleic acid targets given 95% of positive results. Standard curves were generated by linear regression analysis of the threshold cycle (CT) measured for each amplification vs. the log10 copy number for each standard dilution. The measured quantity for each dilution level was determined in retrospect by using the formula: $\log x = [CT - b]/a$, where a is the

slope, b is the Y-intercept, and x is the quantity. Using Probit norm (NF U47-600-2, february 2015), the quantification limit of the assay was then determined.

Repeatability, preliminary reproducibility and the influence of matrix DNA concentration on amplification were also estimated (Supplementary Material S1).

Diagnostic characteristics

Diagnostic performances were assessed by comparing the conventional PCR assay developed by (Stokes and Burreson 2001) and the new real-time PCR. The diagnostic sensitivity (DSe) and specificity (DSp) of the PCR assays were assessed through latent class analysis. A Bayesian approach was used, where parameters were estimated by Markov Chain Monte Carlo (MCMC) methods via Gibbs sampling. Analysis was performed as described by Joseph et al. (1995) by adapting the model to 2 methods and 3 populations. To consider the possible dependence between PCR assays, conditional dependence was modelled using the covariance between methods within the diseased class (Vacek, 1985; Dendukuri and Joseph, 2001) (Supplementary Material S2). Firstly, analysis was performed with non-informative priors (model 1); additional models were made by restricting the prevalence of the negative group to 0 (model 2) and by the determination of the DSe for conventional PCR (model 3). As few information is available on the *H. costale* conventional PCR, DSe estimation was based on data available for a conventional PCR targeting a closely related parasite, *H. nelsoni* (Gagné et al., 2015). So, the beta priors (152.8758, 27.8016) were used for DSe, reflecting 95% confidence that the DSe of the conventional PCR is above 80% with a mode set at 85%. The corresponding beta distribution was generated using the betabuster software (downloaded from http://www.epi.ucdavis.edu/diagnostictests/betabuster.html). The models were implemented in JAGS within the R statistical software environment using packages rjags. For all models, four Monte–Carlo Markov chains (MCMCs) were run with 50°000 iterations with the first 10°000 iterations discarded as a "burn-in". Convergence of the MCMC chain was assessed by confirming that the history plots ran stably and did not switch to different regions.

Results

I- Characterization of the parasite *Haplosporidium costale*

1- Imprint examination, histology and *in situ* hybridization

In sample 19-030, 11 out of 13 tested oysters showed *Haplosporidium* parasites (Table 2) including uninucleated (Figure 1a), plasmodial stages (Figure 1b) and sporonts in connective tissues of the digestive gland, gills, gonadal follicles, labial palps, kidney and muscle. Plasmodial stages appeared binucleated or with few nuclei. Some of them had an irregular shape. Some "foamy" forms were also noticed in the connective tissues of the digestive gland (Figure 1c). Infection level was light to high and the presence of the parasite appeared associated with hemocyte infiltration (Figure 1d), necrosis, abnormal nuclear pictures and the presence of undetermined bacteria.

In sample 19-082, *Haplosporidium* parasites including spores were observed on gill and digestive gland imprints prepared from the oyster (Table 2; Figure 1e) showing an abnormal black color of the soft tissues.

Out of the 10 tested oysters in histology, only this oyster appeared infected with parasites, mostly spores, invading the connective tissues of the digestive gland, gills and around the gonad (Figure 1f). Some spores could be seen crossing the digestive epithelia (Figure 1g). No hemocyte infiltration was observed in this infected oyster. The other tested individuals showed hemocyte infiltration or necrosis but no parasite was detected.

In situ hybridization revealed positive labeling in all the oysters found infected with the parasite in histology. Although all parasite stages appeared recognized by the probe, spores showed less or sometimes low labeling compared to other parasite stages (not shown).

2- Molecular characterization

Amplification at the expected size was obtained for 12 to 17 oysters from sample 19-030 and for 1 to 5 oysters for sample 19-082 depending on the primer pair (Table 2).

Some PCR products were sequenced (Table 3). For a same primer pair, sequences were identical. Whatever the primer pair used, these sequences showed 99% to 100% identity with *Haplosporidium costale* (KC578010.1; AF387122.1) (Table 3).

In order to extend sequences obtained on the 18S fragment and to amplify the internal transcribed spacer (ITS) region and actin gene, different primer pairs were tested and combined (Table 1).

Two sequences, 1445 and 1354 bp in size, were obtained on the 18S fragment for samples 19-030 and 19-082, respectively (GenBank references: MZ666334 and MZ666335). These sequences displayed between 98,31 and 99,45 % identities with *H. costale* (AF387122.1) and *Haplosporidium* sp. *Saccostrea glomerata* (JX977120.1)

Three 453 bp sequences were obtained for the ITS region including partial small subunit ribosomal RNA gene, the ITS 1, 5.8S rRNA gene, ITS 2, and partial sequence of the large subunit ribosomal RNA gene. Two sequences obtained from oysters from sample 19-030 were 100% identical (GenBank reference: MZ666374) and the third one from 19-082 (GenBank reference: MZ666375) showed 2 transversions (G instead of C and A instead of C in position 267 and 272 respectively in 19-082). These sequences appeared 99,56 and 100% identical with *H. costale* KF790901.1.

Finally, 2 identical sequences (598nt), one from each oyster sample, were obtained for the actin gene (GenBank reference: MZ673037). These sequences were 100% identical with *H. costale* AY450407.1.

Phylogenetic analysis carried out on 18S long sequences from Haplosporidian organisms and using *Marteilia refringens* as outgroup showed that Haplospdoridian species were included in a paraphyletic group (Figure 2). Species of the genus *Haplosporidium* clustered in a main clade separated from species of the genera *Bonamia* and *Minchinia.* Whereas *Bonamia* species formed a monophyletic group well supported by Bayesian posterior probabilities, the relationships between *Minchinia* species were not clear. Urosporidium species clustered together within the *Haplosporidium* clade. Among *Haplosporidium* species, *H. nelsoni*, *H. diporeirae*, *H. pinnae* and *H. carcini* grouped together whereas apart from *H. paragon* which grouped with *Haplosporidium* sp. in *Syllis nipponica* and *Urosporidium* species, other species were distributed in two clusters, one including *H. edule*, *H. raabei*, *H. pickfordi*, *H. tuxtlensis*, *H. lusitanicum*, *H. montforti* and a second one with *Haplosporidium* sp. MYE from *Ostrea edulis* characterized as *H. armoricanum* (Engelsma pers comm) and the group of very closely related *H. costale* and *Haplosporidium* sp. from *Saccostrea glomerata*. Both sequences obtained from *Crassostrea gigas* from France were included in this monophyletic group well supported by Bayesian posterior probability (0.92).

The alignment of 18S-ITS1-5.8S-ITS2-28S sequences from *Haplosporidium costale* (KF790901 -2) and *Haplosporidium* sp. from *Saccostrea glomerata* (KF790894-900) allowed identifying two groups: the first one including two sequences obtained from *Crassostrea virginica* in North America (Nova Scotia-Canada and Maine and Virginia- U.S.A) and the second one including the 7 sequences from *S. glomerata* from New South Wales, Australia. Both sequences obtained from *Crassostrea gigas* from France appeared closer to North American (0,12%) than Australian ones (5,74%) (Table 4).

Among the Northern American and French sequences, polymorphism consisted of 2 transversions for one of the French sequences and a deletion of 29 nucleotides in one Northern American.

The alignment of French, Northern American and Australian sequences showed one variable region framed by two conserved ones. This variable region which probably contains the ITS-1 appears distinct between French-Northern American and Australian sequences.

3- Ultrastructural description

Both plasmodia and sporonts were observed in the connective tissue of gills and digestive gland (Figure 3a). Dimensions and numbers of organelles are reported for each parasite stage in Table 5.

Plasmodia

Plasmodia including up to three nuclei appeared amoeboid in shape measured 7.1 ± 1.9 µm in the long dimension (Figure 3b). A fibrillar ribbed layer was observed around some plasmodial stages (Figure 3c).

Nuclei, 2.3 µm in mean diameter, were often paired suggesting division by fission and usually showed large nucleolus. Intranuclear microtubules were noticed in one plasmod (Figure 3d).

The cytoplasm included up to 15 mitochondria with tubular cristae generally located around the nucleus as well as few lipid droplets (up to 5) (Figure 3b). Smooth and anastomosing endoplasmic reticulum could be observed but not Golgi apparatus.

Sporonts

Sporonts, 11.5±2.2 µm in length and 8.6±1.5 µm in width, with up to 13 uninucleated sporoblasts were observed close to plasmodial stages in the connective tissue of gills and digestive gland (Figure 4a). Young sporonts with sporoblasts showing dotted walls suggesting spore wall formation could be seen (Figure 4b). Sporoblasts measuring 2.3±0.4 µm by 2.8±0.6 µm consisted of endosporoplasms surrounded with a dense episporoplasm which included sometimes one lipid droplet, dense bodies and up to 5 mitochondria (Figure 4c).

Endosporoblasm length ranged between 1.1 and 4.6 µm and contained one nucleus (1.35 µm in diameter), sometimes one lipid droplet, dense bodies and up to 5 mitochondria. Lipid droplets could be seen in young sporoblasts and rarely in mature ones. On the contrary, numerous haplosporomes (up to 19) located in the endosporoplasm occurred in mature sporoblasts and less in young ones (Figure 4d). Nuclear Membrane Bound Golgi (NM-BG) (Figure 4e) could be observed. The spherule, consisting of long cisternae of sER was observed more frequently below the operculum in mature spores (Figure 4e). Haplosporosomes appeared spherical or axehead-shaped (Figure 4f).

The mature spore wall, 82.7±13.4 µm across, included a thick external electron dense layer, a lighter layer and finally an inner denser layer (Figure 4f).

In addition, spores showed an operculum, 0.9 to 2.1 µm long, attached by a hinge to the spore wall (Figure 4f). The operculum was more often observed in mature spores but sometimes in less mature ones (Figure 4d). No structure looking like filament was clearly observed although some pictures suggest the presence of epispore cytoplasmic extensions (Figure 4g).

II- Development, validation of a Real Time TaqMan PCR assay for the detection of *Haplosporidium costale* and its application on the field

1- Method development and validation

To allow a large evaluation of *Haplosporidium costale* DNA presence in French Pacific oyster populations, a Real Time TaqMan PCR method was developed. Two DNA probes were designed and tested at different concentrations in a TaqMan assay amplifying 149 bp of the 18S region (Figure 5). Earlier amplifications and a better efficiency (estimated at 98.5%) were observed with probe 2 at a final concentration of 300 nM. This mix was thus selected.

Inclusivity and exclusivity tests yielded expected results with only *H. costale* DNA detected positive whereas DNA from closely related parasites were negative (Table 6). Real-Time PCR detection limit (LD_{PCR}) was evaluated on both infected oyster DNA and plasmidic DNA. LD_{PCR} 95% was estimated at 4.25 copies/µl. (Supplementary material Table S2). Moreover, repeatability and preliminary reproducibility of the assay were evaluated and considered as excellent (variations < 5%) (Supplementary material Table S3)**.** The influence of *Crassostrea gigas* DNA matrix was also tested and no inhibition of the real time PCR was observed for DNA concentration ranging between 5 and 250 ng/µl (Supplementary material Table S4).

Finally, the real time PCR assay was compared with the conventional PCR using a panel of field samples (Table 7). Both conventional and real-time PCR did not yield positive results in oyster samples from the *Haplosporidium costale* free population. However, the real-time PCR detected more *H. costale* positive samples in the population with low and moderate prevalence than the conventional PCR.

Diagnostic sensitivity (DSe) and specificity (DSp) were estimated using Bayesian latent class analysis with conditional dependence and different models were evaluated (Table 8). In all Bayesian models, the Markov Chain Monte Carlo appeared to converge. All history plots and quantile plots were stable. DSp of the real time PCR and conventional PCR in these 3 models were very high, ranging from 95.5 to 99.5% whereas DSe strongly differed: for models 1 (without priors) and 2 (prevalence of free population=0), no real difference appeared for the DSe but it was especially low for the conventional PCR (38.5% for model 1 and 44.8% for model 2). Using prior (model 3), the DSe clearly increased to reach 83.6 % for conventional PCR and 92.6% for real time PCR. The use of priors in conditional dependence improved the estimation of parameters specially the DSe.

Similarly, DSe and DSp were influenced by the prevalence. The negative population was close to zero in the models 1 and 3, the low prevalence group ranged to 8.2 to 27.5% and the moderate prevalence population to 27.2 to 54.5. The lowest values in the different groups were obtained with model 3 using prior (Table 8).

2- Application on the field

Using this molecular method, *Haplosporidium costale* DNA presence was screened on 263 samples collected from two main French farming areas located in the Atlantic coast (Marennes Oléron and Arcachon bay). On average, 44% and 27% of oysters sampled were positive in Arcachon bay and in Marennes Oléron bay, respectively. Detection frequency ranged from 0% to 75% in Atlantic samples, depending on the year. Notably, first detection corresponded to oysters sampled in 2008 in Arcachon (Table 9). In this sample collection, *H. costale* DNA was detected in oysters quasi-continuously from 2008 to 2017 in France. Indeed, despite the low number of individuals available some years and the lack of samples in 2010 and 2015, positive amplifications were noticed in 2008, 2009, 2012, 2013, 2014, 2016, 2017 (Table 9).

Finally, parasite DNA could be amplified in oysters sampled during signaled mortality events (8 batches), but also in the context of field studies outside any mortality event (6 batches).

Discussion

Crassostrea gigas is native in Asia and has been introduced to 66 countries outside its native range (Herbert et al. 2016). Sometimes considered as invasive, it is currently one of the most "globalized" marine invertebrates and its production is predominant in many parts of the world including in Europe (Herbert et al. 2016). However, the Pacific oyster can be affected by mortality events associated with pathogenic microorganisms including the virus OsHV-1 and the bacteria *Vibrio aestuarianus* (EFSA 2015). *C. gigas* is also susceptible to protozoan parasites such as *Haplosporidium nelsoni* or *H. costale* (Arzul and Carnegie, 2014). Interestingly, on the eastern coast of U.S.A, *C. gigas* appears less susceptible than the congeneric species *C. virginica* to these parasite species (Burreson and Ford 2004; Arzul and Carnegie 2014).

In contrast with *Haplosporidium nelsoni* previously detected in Europe (Lynch et al. 2013, Renault et al. 2000), *H. costale* was considered exotic. However, a parasite looking like *H. costale* was described in transmission electron microscopy in *Crassostrea gigas* in France in 1988/1989 but the lack of molecular information did not allow concluding about its formal identification (Comps and Pichot, 1991).

In the present study, a combination of microscopy and molecular tools has allowed characterizing the presence of the parasite *Haplosporidium costale* in the context of low mortality events in France.

In histology, plasmodia and spore stages were observed in the connective tissues of the digestive gland, mantle and gonad. A diversity of plasmodial stage shapes were noticed and notably the presence of foamy forms generally found during infection with *Haplosporidium costale* (Ryan Carnegie,

pers com). Haemocytic infiltration was also reported in infected oysters. The parasite distribution in oyster tissues and the association with intense hemocyte infiltration are concordant with *H. costale* tropism and impact in *C. virginica* (Burreson and Stokes 2006).

Ultrastructural features of the parasite detected in France resemble Haplosporidian ones (Rosenfield et al. 1969; Hine et al. 2009) and are closer to *Haplospordidium costale* and *H. armoricanum* (Perkins, 1969; Rosenfield et al. 1969; Hine et al. 2009) than other members of the genus. Plasmodia usually show paired nuclei surrounded by mitochondria as described in *H. costale* in *C. virginica* (Perkins 1969). Sporulation appears similar to the spore maturation described for *H. costale* including the formation of spore wall with an operculum, the presence of a complex membranous organelle, the spherule, as well as the appearance of axe-headed haplosporosomes (Perkins 1969). Similarly, to *H. costale*, no filament has been observed on spores in our study (Perkins 1969; Rosenfield et al. 1969). However, some pictures could suggest the presence of epispore cytoplasmic extensions (Figure 4g) as it has been reported for *H. armoricanum* in *Ostrea edulis* in Galicia (Azevedo et al. 1999).

The presence of Haplosporidian parasites looking like *Haplosporidium costale* in transmission electron microscopy has earlier been reported in three oysters *Crassostrea gigas* collected from Thau lagoon, South of France (Comps and Pichot, 1991). Interestingly, these parasitized oysters appeared grey coloured macroscopically as observed for one oyster in our study collected in June 2019. Sporocyst and spore size as well as the number of spores in sporocysts appear smaller in our study compared to the data available in Comps and Pichot (1991) and compared to data available for *H. armoricanum* (Hine et al. 2007). These features could vary with host species and sites as previously suggested (Perkins and Van Banning 1981). The resemblance between *H. armoricanum* and *H. costale* spores has already been highlighted based on ultrastructural investigations (Hine et al. 2007, 2009, 2020). *H. armoricanum* has been characterized based on several examinations of flat oysters from France, Spain and The Netherlands (van Banning 1977; Cahour et al. 1980; Perkins and van Banning, 1981; Bachère and Grizel, 1983; Azevedo et al. 1999; Hine et al. 2007). The similarity of spores from *H. costale* and *H. armoricanum* raises questions regarding their relative taxonomic position. Although no molecular information is currently available for *H. armoricanum*, Hine et al. (2007) have previously reported that their molecular phylogenies are closely related.

As with many non-cultivable micro eukaryotes, *Haplosporidium costale* genome is poorly represented in public databases. However, three fragments of the genome including part of the small subunit rRNA gene, the ITS-1, 5.8S and ITS-2 array and part of the actin gene were successfully sequenced. The actin gene sequence showed 100% identities with the sequence obtained from *Crassostrea virginica* from Virginia (USA). Two long 18S sequences (>1360 bp) were obtained and were between 98.31 and 99.45 % similar to *H. costale* from *Crassostrea virginica* from Virginia (USA) and *Haplosporidium* sp. from *Saccostrea glomerata* from New South Wales (Australia). Interestingly, the ITS1, 5.8S and ITS-2 sequence appeared more discriminating and revealed a higher distance between the French and the Australian isolates than with the North American ones. Our results confirm that the parasite observed in histology and transmission electron microscopy in juvenile and adult *C. gigas* in France is *H. costale*. However, more sequence data would be required in order to better evaluate the diversity between these different isolates and between *H. costale* and the closely related species *H. armoricanum*.

Disease control requires different types of diagnostic tools, some generic and others more specific, the choice depending on the surveillance objective. For targeted surveillance, the availability of a rapid and specific diagnostic tool is an advantage because it makes possible to diagnose a large number of individuals in a short time for a given infectious agent. A real time PCR assay was thus developed allowing the specific detection of *Haplosporidium costale* and no other closely related mollusc parasites belonging to the genera *Haplosporidium*, *Minchinia* and *Bonamia*. Primers developed by Ko et al 1995 have been shown to be specific and this specificity was reinforced by the use of a probe. The probe showed no sequence homology with other *Haplosporidium* species except with *H. orchestiae* but the primers did not present homology with this species. Several conventional (Ko et al. 1995, Stokes and Burreson 2001) and multiplex (Penna et al. 2001; Russell et al. 2004) PCR detecting this parasite exist but the development of a real time PCR provides several benefits such as a reduced

time of analysis, an increased sensitivity and a decreased cross-contamination risk. This real time PCR presented a detection limit between 2.5 and 5 copies/µL which is similar to other real time assays developed for other pathogens of molluscs (Polinski et al. 2015, Canier et al. 2020). Furthermore, the sensitivity of this real-time PCR was not affected by the presence of host genomic DNA even with high DNA load (250ng/µL host gDNA), contrary to the sensitivity of the real time PCR detecting *Mikrocytos mackini* which is reduced by 20% in presence of 80 ng/µL host gDNA (Polinski et al. 2015). The robustness of this method is particularly interesting when speaking about method transfer to routine diagnostic laboratories.

This new real-time PCR was compared with OIE-recommended conventional PCR (Stokes and Burreson 2001) on a panel of field samples categorized in three prevalence groups. Diagnostic sensitivity and specificity were estimated by Bayesian analyses in dependence conditions using prior or not. Whatever the used model, the specificity was high both for real time and conventional PCR. Meanwhile, the sensitivity varied according to the used model and could present very low results specially if no prior was added in the analyses: the PCR sensitivity barely reached 30% and 60% for the real time PCR, which is not in agreement with data available in the literature for PCR assays (Caraguel et. 2012, Ramilo et al. 2013, Aranguren and Figueras 2016). The addition of prior in the model gave sensitivity values comparable to other PCR or real time PCR targeting mollusc pathogens (Gagné et al. 2015, Canier et al. 2020, Polinski et al. 2021). The use of prior is generally recommended in Bayesian methods if prior determination is based on independent data of current data and/or based on expert scientific knowledge (Johnson et al. 2019, Cheung et al. 2021). Then, this new real time PCR assay presented interesting performances for the surveillance of *Haplosporidium costale* and was used in a survey to screen *H. costale* DNA presence in France on archived samples. Samples collected between 2008 and 2017, partly in the context of mortality events, in two main French oyster producing areas were analysed with this real time PCR and parasite DNA has been detected in France at least since 2008. Although no parasite has been observed in histology, parasite DNA could be detected in 15 to 75% of tested animals. Detection frequency of the parasite DNA was not higher in samples collected in the context of mortality events suggesting that the presence of the parasite may not have an impact on the oysters health.

As far as we know, this is the first time that the presence of *H. costale* is confirmed in *Crassostrea gigas* in France and a TaqMan real time PCR assay is developed to enable the rapid and specific detection of the parasite. The application of the PCR assay on archived samples has revealed that the parasite is present in French oyster populations at least since 2008. The lack of impact of the parasite on *C. gigas* which seems less susceptible than other species such as *C. virginica* certainly explains why it has not been previously reported. The parasite may have been present in France for many years as suggested by the description of *Haplosporidium* like parasites similar to *H. costale* in Pacific oysters collected in 1988 and 1989 in Thau lagoon in France (Comps and Pichot, 1991).

Our results raise questions regarding the factors having contributed to the development of the infection and the occurrence of low mortality in experimental batches of *Crassostrea gigas* (Lupo et al. in preparation). Considering the little information available on this parasite, the newly developed TaqMan assay will be very helpful to investigate the temporal and geographic distribution and the life cycle of the parasite in France and more generally in *C. gigas* geographic range.

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Conflict of interest

The authors declare no conflicts of interests in relation to this work.

Ethical approval

No ethical approval was required.

Data availability statement

The sequence data have been submitted to the GenBank databases under accession numbers MZ666334, MZ666335, MZ666374, MZ673037

FIGURE 1 Histological haematoxylin eosin tissue sections (a, b, c, d, f and g) and Hemacolor® tissue imprints (e) showing *Haplosporidium costale* parasites in different tissues of *Crassostrea gigas*. a Uninucleated parasites (arrows) in a venous sinus of the mantle. b Plasmodial stages with an irregular form (arrows) in the connective tissue of the digestive gland. c Foamy form (arrow) and plasmodial stage (arrowhead) in the connective tissue of the digestive gland. d Hemocytic infiltration (HI) in the connective tissue of the digestive gland associated with parasite plasmodial stages (arrows). e Plasmodial cell (arrow) and spores (arrowhead) on digestive gland imprint. f Sporonts (arrows) and free spores (arrowhead) in the connective tissue around gonad follicles. G Sporonts and spores in different positions around the intestine: sporonts (arrowhead) in the connective tissue of intestine, sporonts (arrow) inside the intestine epithelium (IE) and spores (*) into the intestine lumen (IL). Scale-bars: a, c and e =10 µm; b, f and g = 20µm; d =50µm.

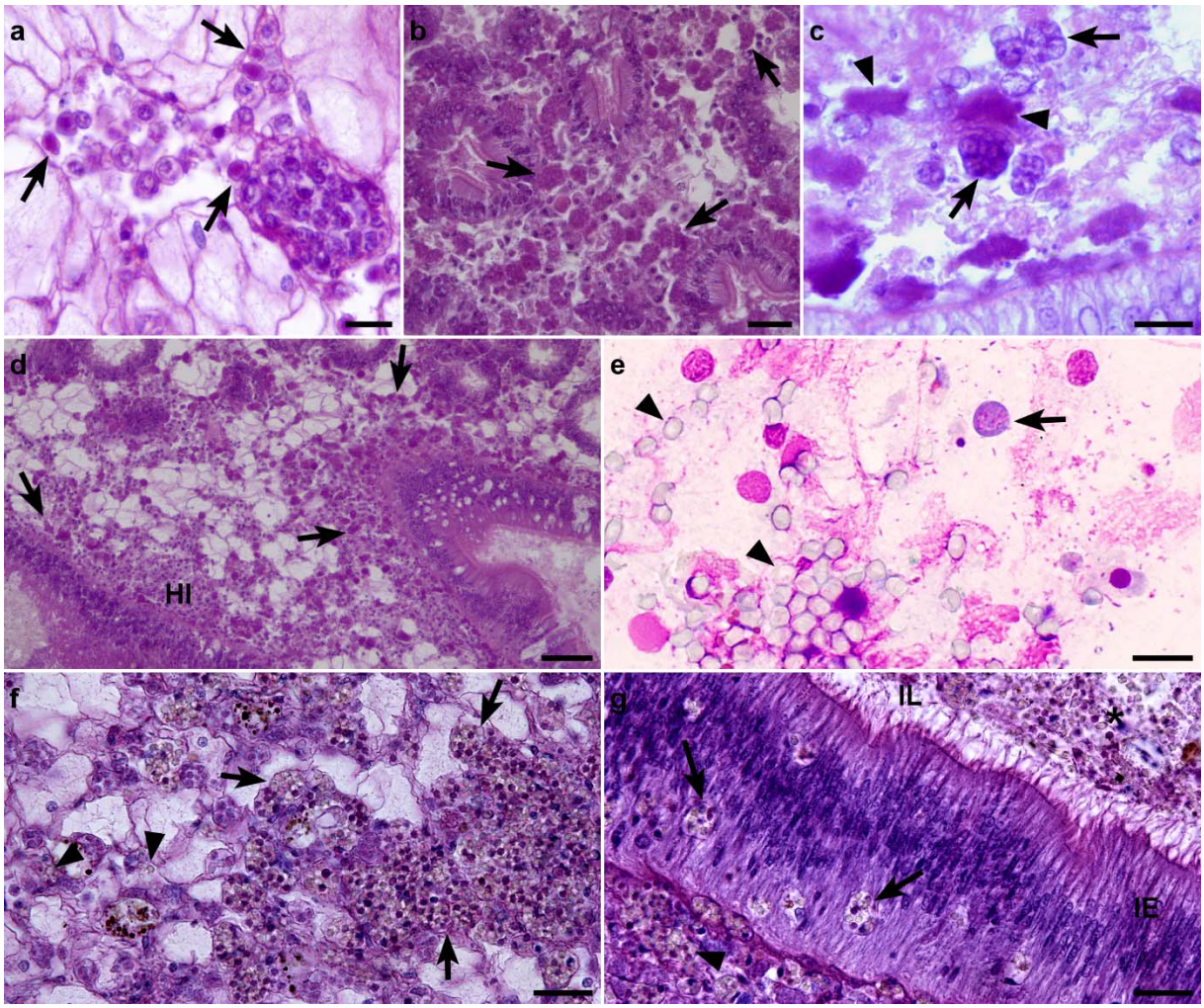

FIGURE 2 Phylogenetic tree (50% majority-rule consensus) using Bayesian Inference (MrBayes 3.1.2) based on the small subunit ribosomal gene of Haplosporidian. Circles at the node correspond to Bayesian probabilities superior to 50%. *Marteilia refringens* was used as the outgroup. Sequences obtained in this study are indicated in bold. Affiliations at a genus level are specified using colored ranges.

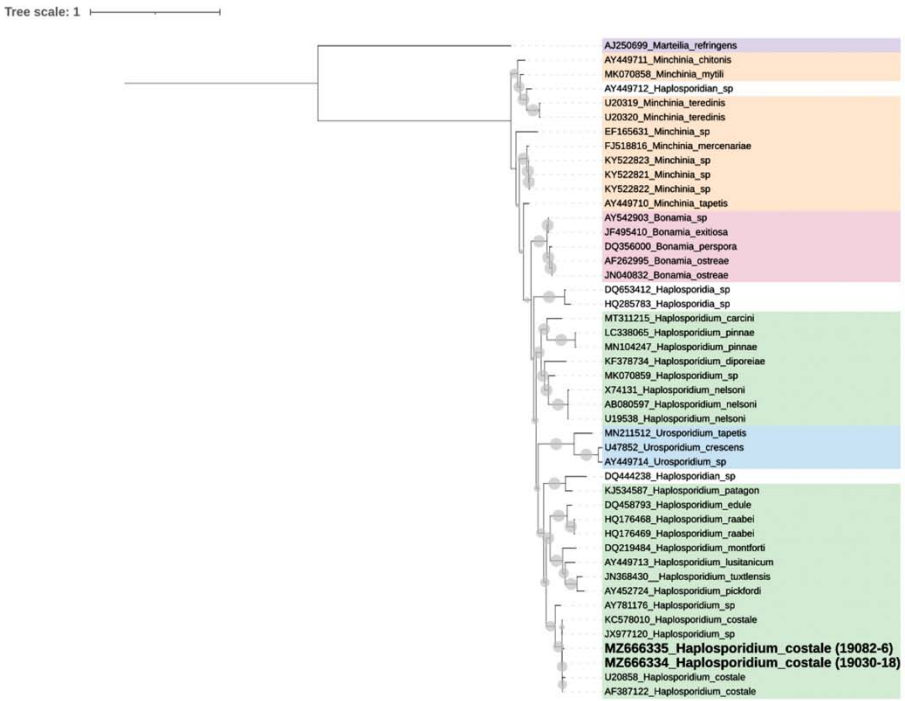

FIGURE 3 Semithin section (a) and ultrathin sections (b to d) of *Haplosporidium costale* plamodia infecting different tissues of *Crassostrea gigas*. a Plasmodial (arrow) and sporont (arrowhead) stages in the connective tissue of gills. Note the presence of intermediary stages (*). b Plasmodial stage with two nuclei (N). Note the presence of several mitochondria (arrowheads) and some lipid droplets (*) inside the cytoplasm. c Plasmodial stage with two nuclei (N) presenting a fibrillar ribbed layer (arrowheads). d Nucleus in division presenting intranuclear microtubules (arrow) and centrosomes (arrowheads). Scale-bars: a = 10µm; b, c and d= 1 µm.

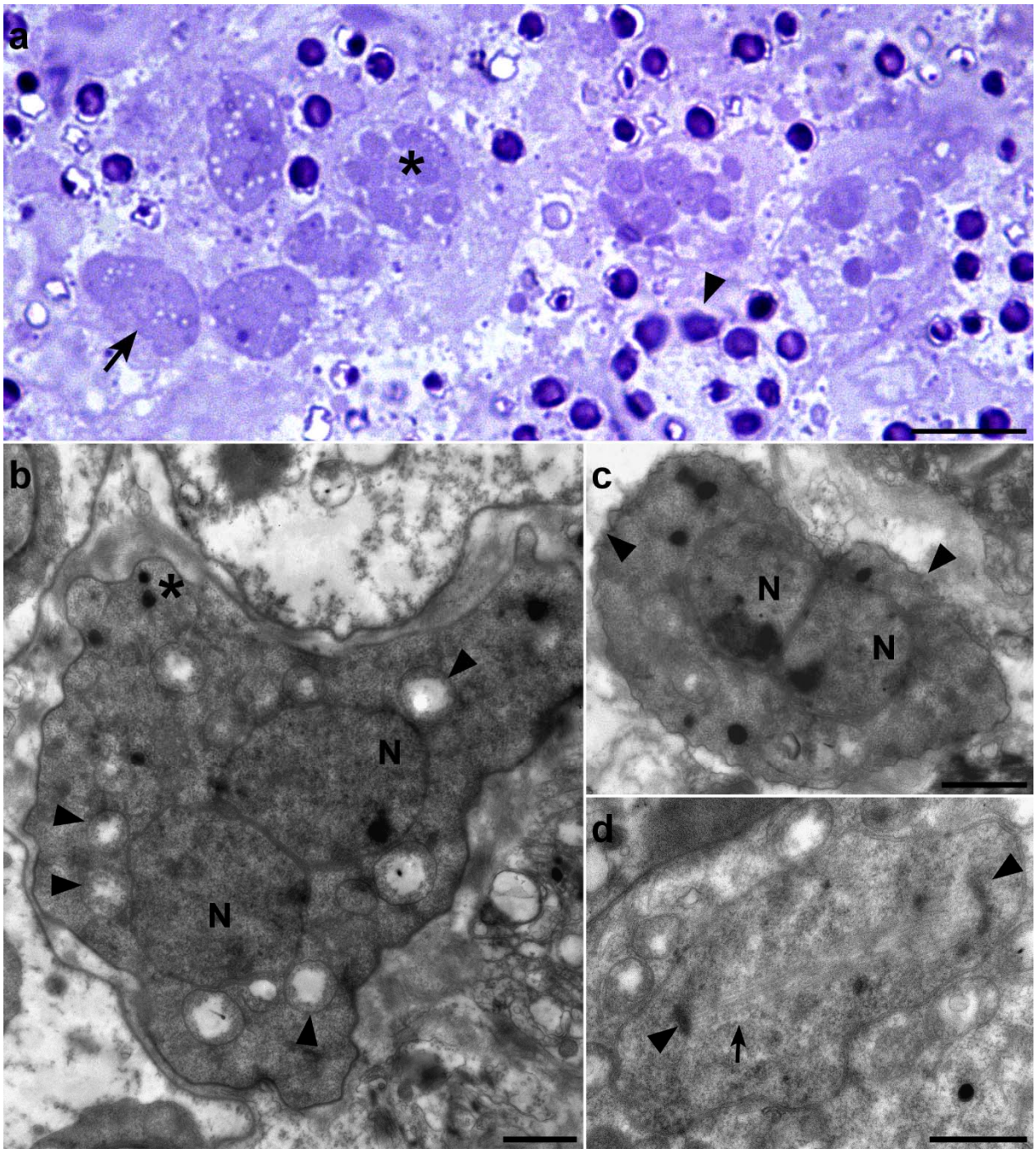

FIGURE 4 Utrathin sections of *Haplosporidium costale* sporonts infecting different tissues of *Crassostrea gigas*. a Early sporont showing different endosporoplasms (arrows) including nucleus,mitochondria (*) but no haplosporosome or spore wall. b Sporont with spore wall formation in progress which delimits sporoblast (arrows). Note the presence of an operculum (arrowhead) in a young spore. c Sporont with sporoblasts composed of endosporoplasms surrounded with an episporoplasm (arrows). Some mitochondria and lipid droplets can be seen in the episporoplasm. Endosporoplasms include nucleus, mitochondria, lipid droplets (arrowheads) and haplosporosomes. d Sporont with two spores containing haplosporosomes (*) and mitochondria (arrows). e Spore with smooth endoplasmic reticulum (arrowhead) beneath the operculum (white arrow) and Golgi apparatus (black arrow) near the nucleus (N). f Mature spore with a wall consisting in three layers (black arrow) and with an operculum attached to the spore wall by a hinge (white arrow). Note the presence of spherical (white arrowhead) or axehead-shaped (black arrowhead) haplosporosomes near nucleus (N). g Spore presenting epispore cytoplasmic extensions (arrow).

Scale-bars: a, b and c = 2µm; d= 1 µm; e, f and g=0.5µm.

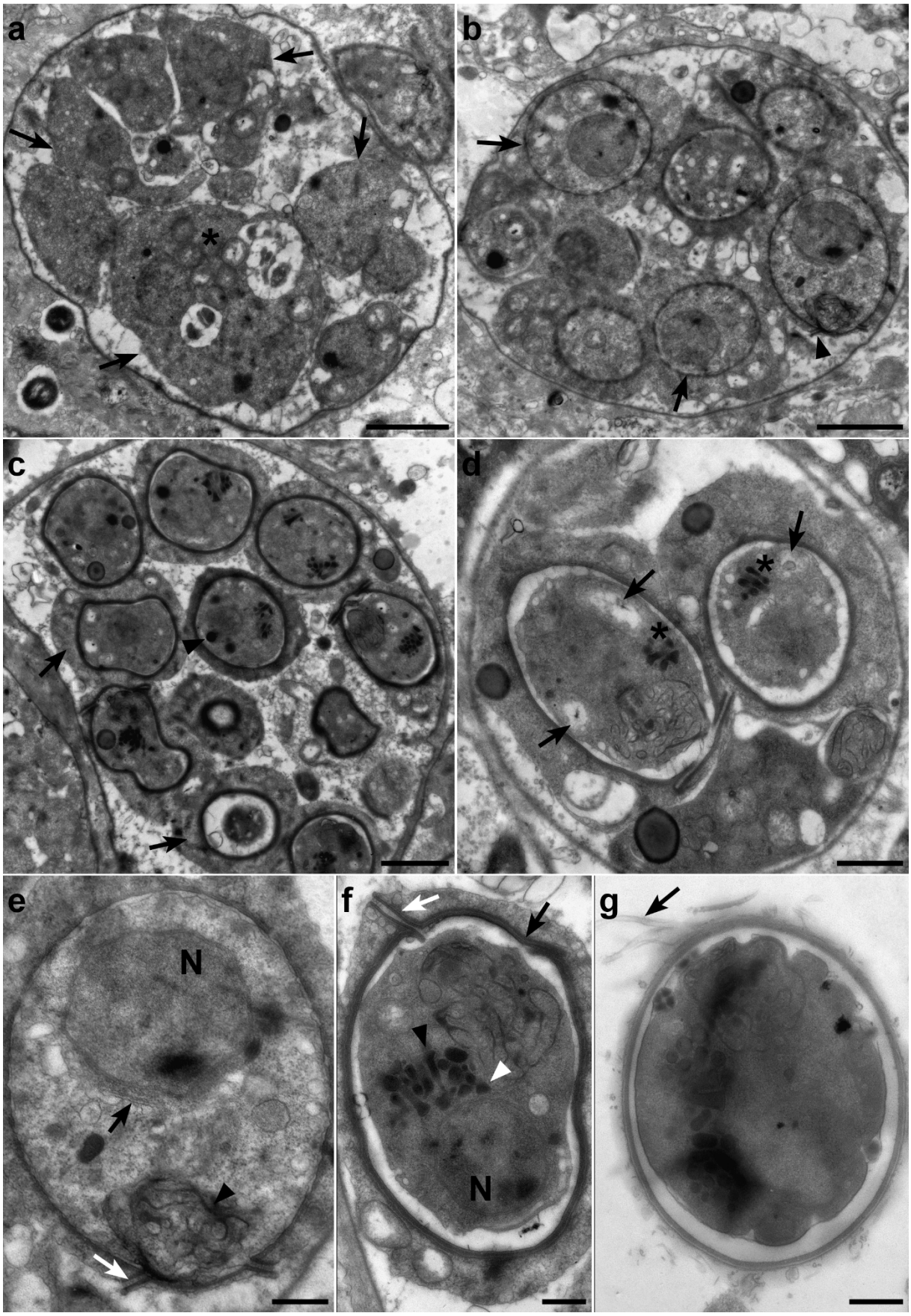
a
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FIGURE 5 Alignment of 18S sequences of *Haplosporidium costale* and closely related parasites. Position of primer and probes is represented by boxes.

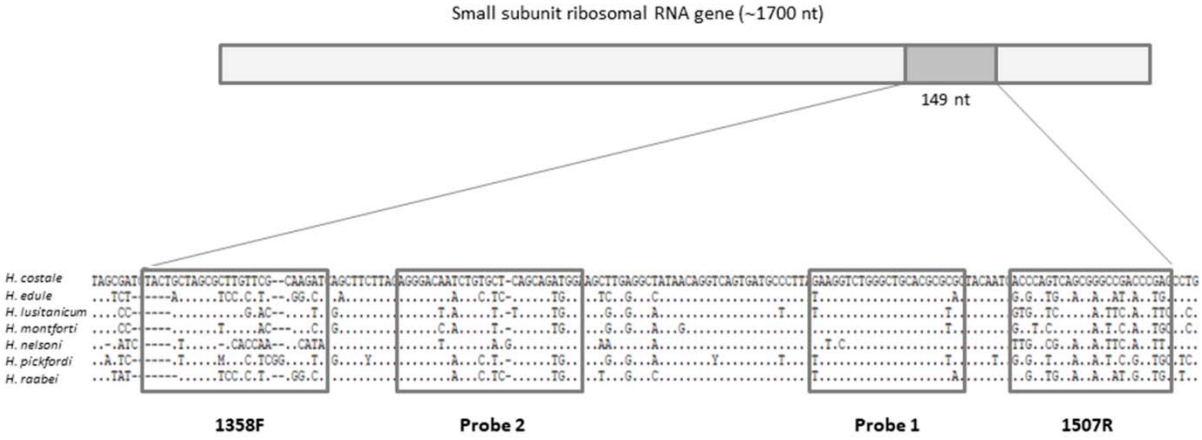

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TABLE 1: Primers used in this study to test the presence of parasites of the genus *Haplosporidium* and to sequence parts of its genome.

Primer pair	Sequences	m	Amplicon expected size	Region amplified	Reference
HapF1	GTTCTTTCWTGATTCTATGMA	9	330 bp	18S SSU rDNA	Renault et al. 2000
HapR2	GATGAAYAATTGCAATCAYCT	9			
HPNF3	CATTAGCATGGAATAATAAAACACGAC	5	600 bp	18S SSU rDNA	Catanese et al. 2018
HPNR3	GCGACGGCTATTTAGATGGCTGA	5			
SSO-A	CACGACTTTGGCAGTTAGTTTTG	5	550 bp	18S rRNA gene	Stokes and Burreson, 2001
SSO-B	CGAACAAGCGCTAGCAGTACAT	5			
16SA	AACCTGGTTGATCCTGCCAGT	5	~1400 bp	18S rRNA gene	Medlin et al. 1988
16SB	TGATCCTTCTGCAGGTTCACCTAC	5			
Haplo18Sl_int_1F	GAAACGGCTACCACATCCAC	0	630 bp	18S rRNA gene	This study
Haplo18Sl_int_1R	CCCCGGCTTTAGTTCTTGAT	0			
ITSf	GGGATAGATGATTGCAATTRTTC	5		ITS	Hill et al. 2010
ITS-B	TATGCTTAAATTCAGCGGGT	5	~800 bp	ITS	Carnegie et al. 2014
ITS 2.2	CCTGGTTAGTTTCTTTTCCTCCGC	5	~600 bp	ITS	
Actin_Hapl1_F	ACTGGCATTGTGCTTGACAG	2	~400 bp	Actin gene	This study
Actin_Hapl1_R	TTCACAGTGATCGTGGAAGG	2			
Actin_Hapl2_F	GATGTACGTCGGGATTCAGG	2	~300 bp		
Actin_Hapl2_R	TGTGCGGGTACATCGTAGTG	2			

TABLE 2 Summary of the results (Number of positive individuals/ Number of tested individuals) obtained on the two samples of oysters tested by histology, *in situ* hybridization and PCR regarding the presence of *Haplosporidium costale*

Oyster samples	Detection of *H. costale* by				
	Histology	ISH	PCR HAP F1/R2	PCR HPNF3/R3	PCR SSOA/B
19-030	11/13	11/13	12/17	10/17	17/17
19-082	1/10	1/10	2/10	1/10	5/10

TABLE 3 Number of sequences obtained by fragment and sample and results of the Blastn analysis

Gene	Primers (expected size)	Number of obtained sequences 19-030	Number of obtained sequences 19-082	Blastn results
18S	SSOA-SSOB (557 nt)	7	2	100% identities (532 nt) with *H. costale* (KC578010.1)
	HapF1-HapR1 (330 nt)	7	-	100% identities (295 nt) with *H. costale* (AF387122.1)
	HPNF3-HPNR3 (600 nt)	6	-	99,62% identities (527 nt) with *H. costale* (KC578010.1 and JX977120.1)
	Long fragment (combination of primers)	1 =a	1 =b	a= 99,45% identities (1445 nt) with *H. costale* (AF387122.1) and *Haplosporidium* sp. *Saccostrea glomerata* (JX977120.1) b= 98,31% identities (1354 nt) with *H. costale* (AF387122.1) and 98,23% with *Haplosporidium* sp. *Saccostrea glomerata* (JX977120.1)
ITS region	Combination of primers	2 (100% identical) = a	1= b	a= 100% identities (453 nt) with *H. costale* KF790901.1 b= 99,56% identities (453 nt) with *H. costale* KF790901.1
Actin	Combination of primers	1	1	100% identities (597 nt) with *H. costale* AY450407.1

TABLE 4 18S-ITS1-5.8S-ITS2-28S sequences: Estimates of Evolutionary Divergence over Sequence Pairs between Groups- The number of base substitutions per site from averaging over all sequence pairs between groups are shown. Standard error estimate(s) are shown in the last column. French Seq correspond to sequences obtained in the present study. Austr seq correspond to sequences of *Haplosporidium* sp. from *Saccostrea glomerata* (KF790894-900). Am seq correspond to sequences of *H. costale* from Nova Scotia-Canada and Maine and Virginia- U.S.A (KF790901 -2).

		Distance	St. Error
French seq	Austr seq	0.0574	0.0128
French seq	N. Am seq	0.0012	0.0012
Austr seq	N. Am seq	0.0559	0.0126

TABLE 5 *Haplosporidium costale* observed in *Crassostrea gigas* samples in the current study- Main ultrastructural features: dimensions and numbers of organelles for plasmodia, sporonts and spores.

Feature	Mean ± SD	Range	n
Plasmodia			
Length (µm)	7.1 ± 1.9	5 - 10.2	7
Width (µm)	5.0 ± 1.6	3.1 - 7.1	7
Number of nuclei	1.8 ± 0.9	0 - 3	7
Nucleus diameter (µm)	2.3 ± 0.9	1.6 - 4.7	13
Number of lipid droplets	2.9 ± 1.6	1 - 5	7
Number of mitochondria	5.1 ± 4.3	1 - 15	7
Sporont			
Length (µm)	11.5 ± 2.2	7.3 - 15.0	13
Width (µm)	8.6 ± 1.5	6.4 - 11.9	13
Number of spores	6.8 ± 3	2 - 13	13
Spore			
Length (µm)	2.8 ± 0.6	1.6 - 4.9	38
Width (µm)	2.3 ± 0.4	1.4 - 2.9	38
Length endosporoplasm (µm)	2.6 ± 0.6	1.1 - 4.6	38
Width endosporoplasm (µm)	2.2 ± 0.4	1.0 - 2.9	38
Nucleus diameter (µm)	1.36 ± 0.2	0.85 - 1.63	13
Number of endospore lipid droplets	0.1 ± 0.3	0 - 1	38
Number of epispore lipid droplets	0.1 ± 0.3	0 - 1	38
Number of endospore mitochondria	1.4 ± 1.3	0 - 5	38
Number of epispore mitochondria	1.1 ± 1.3	0 - 5	38
Number of endospore dense bodies	0.4 ± 0.7	0 - 2	38
Number of epispore dense bodies	0.4 ± 0.7	0 - 2	38
Number of haplosporosomes	2.5 ± 4.2	0 - 19	38
Number of spherical haplosporosomes	1.7 ± 3.4	0 - 17	38
Haplosporosome spherical diameter (nm)	120.5 ± 35	75 - 277	65

Number of axe-shaped haplosporosomes	0.8 ± 1.3	0 - 4	38
Axe haplosporosome length (nm)	343.3 ± 135.3	169.3 - 708.2	31
Axe haplosporosome shaft width (nm)	106.5 ± 20.8	67.9 - 153.3	30
Axe haplosporosome head width (nm)	176.4 ± 37.9	100.8 - 247.9	16
Operculum width (µm)	1.51 ± 04	0.9 - 2.1	8
Spore wall thickness (nm)	82.7 ± 13.4	59.5 - 100.8	12

TABLE 6 Panel of tested samples and results of inclusivity and exclusivity tests

	Parasite species	**Isolation source**	**Identification method**	**Taqman Real Time PCR result (+ / -)**
Inclusivity	*Haplosporidium costale* (n= 3)	Oyster *Crassostrea virginica* (USA provided by Ryan Carnegie, VIMS), *Crassostrea gigas (*France: Vendée)	Histology, ISH, PCR and gene sequencing	+ (100%)
Exclusivity	*Haplosporidium nelsoni (n=1)*	Oyster *Crassostrea virginica* (USA, provided by Ryan Carnegie, VIMS)	Histology, PCR and gene sequencing	- (0%)
	Haplosporidium nelsoni-(n=3)	Oyster, *Crassostrea gigas*, (France: Thau, Cancale, Brest)	Histology, PCR and gene sequencing	
	Haplosporidium pinnae- (n=2)	*Pinna nobilis,* (France: Corsica and Calanques)	Histology, PCR and gene sequencing	
	Haplosporidium armoricanum (n=1)	Flat oyster, *Ostrea edulis*, (The Netherlands provided by Marc Engeslma, WUR)	Histology, PCR and gene sequencing	
	Minchinia tapetis (n=2)	Cockle, *Cerastoderma edule* (France: Arcachon)	Histology, PCR and gene sequencing	
	Minchinia mytili (n=2)	Mussels *Mytilus edulis* (France: Baie des Veys) and *Mytilus galloprovincialis* (France: Thau)	Histology, PCR and gene sequencing	
	Bonamia ostreae (n=1)	Flat oyster, *Ostrea edulis*, (France: Baie de Quiberon)	Histology, PCR and gene sequencing	

TABLE 7 Results obtained with the new real-time PCR and the conventional PCR (Stokes and Burreson 2001) on oysters collected from three populations of *Crassostrea gigas* displaying different prevalence of *Haplosporidium costale*.

	Conventional PCR	Real time PCR	Number of individuals
Parasite free population	-	-	170
	-	+	0
	+	-	0
	+	+	0
Population with low parasite prevalence	-	-	152
	-	+	19
	+	-	1
	+	+	9
Population with moderate parasite prevalence	-	-	98
	-	+	7
	+	-	0
	+	+	32

TABLE 8 Prevalence (P), diagnostic sensitivity (DSe) and specificity (DSp) of the new real-time PCR (qPCR) and the conventional PCR (PCR) (Stokes and Burreson 2001) assays estimated by Bayesian latent class analysis in conditional dependence with the 95% probability intervals (in parentheses). Three populations of *Crassostrea gigas* displaying different prevalence of *Haplosporidium costale* were used in this analysis: parasite free population= negative(N), population with low parasite prevalence = low (L) and population with moderate parasite prevalence = moderate (M). Models include model with non-informative priors (1), model with an estimation of the prevalence of the free population (model 2) and model with prior for the DSe_{PCR} (model 3).

Parameters	Model 1	Model 2	Model 3
DSeqPCR	57.7 (27-97.9)	67.4 (30-98.6)	92.6 (78.2-99.8)
DSpqPCR	98.6 (94.8-100)	98.7 (95.1-100)	95.5 (92.3-98.6)
DSePCR	38.5 (17-71.1)	44.8 (18.9-73.7)	83.6 (77.8-88.7)
DSpPCR	99.4 (98.1-100)	99.4 (98.2-100)	99.5 (98.5-100)
PN	1.2 (0-5.1)	0	0.6 (0-2.3)
PL	27.5 (9.5-59.1)	23.3 (8.6-52.4)	8.2 (3.7-14.7)
PM	54.5 (25-96.4)	46.1 (24.2-92.2)	27.2 (19.6-35.7)

TABLE 9 Detection frequency of *Haplosporidium costale* DNA in samples of farmed oysters collected in two main French oyster farming areas (Bassin de Marennes Oléron and Bassin d'Arcachon) since 2009.

Geographical area (Total number of batches)	Year	Sampling dates	Site / GPS coordinates	Oyster age	Context of sampling: notified mortalities, macroscopic anomalies or field study	Detection frequency (Nb of positive / Nb of tested individuals)
Bassin de Marennes Oléron (12)						***29% (59/205)***
	2009	20/08/2009	Agnas; 45.873056, -1.170833	1-2 years	field study	25% (3/12)
	2011	03/05/2011	Agnas; 45.873056, -1.170833	1-2 years	field study	0% (0/12)
		18/08/2011	Barat; 45.808806, -1.159970	>2 years	macroscopic anomalies (black gills)	0% (0/5)
	2012	16/10/2012	La Baudissière; 45.924259, -1.233623	>2 years	mortality	25% (3/12)
		14/11/2012	Agnas; 45.873056, -1.170833	1-2 years	field study	10% (1/10)
	2013	16/05/2013	Artouan; 45.762818, -1.088398	1-2 years	field study	67% (8/12)
		07/08/2013	Lamouroux; 45.903214, -1.144970	>2 years	mortality	10% (2/20)
	2014	08/04/2014	La Floride; 45.803243, -1.153969	>2 years	field study	10% (2/20)
		04/09/2014	Agnas; 45.873056, -1.170833	>2 years	mortality	42% (5/12)
	2016	01/09/2016	La Floride; 45.803243, -1.153969	>2 years	field study	0% (0/30)
		01/09/2016	La Floride; 45.803243, -1.153969	>2 years	field study	53% (16/30)

	2017	28/08/2017	La Floride; 45.803243, -1.153969	>2 years	field study	19/30
Bassin Arcachon (5)						**31% (18/58)**
	2008	17/07/2008	Le Tès; 44.665, -1.13829	>2 years	mortality	60% (6/10)
	2013	04/07/2013	Grand Banc; 44.680440, -1.201571	>2 years	mortality	17% (2/12)
		25/07/2013	Marens ; 44.704443, -1.204934	1-2 years	mortality	33% (4/12)
	2014	02/04/2014	Gujan; 44.655431, -1.067254	>2 years	mortality	15% (3/20)
	2016	21/11/2016	Courbey; 44.690785, -1.230244	>2 years	macroscopic anomalies (adductor muscle calcification)	75% (3/4)