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Exploring the impact of the widely introduced Pacific oyster *Magallana gigas* on the dispersal of *Bonamia* (Haplosporida): a global snapshot

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ABSTRACT: *Bonamia* (Haplosporida) are oyster parasites capable of devastating oyster populations. The near-circumglobal distribution of the host generalist *B. exitiosa* has previously been associated with the natural and anthropogenic dispersal of broadly distributed non-commercial oysters in the *Ostrea stentina* species complex. Here, we took a global snapshot approach to explore the role of the widely introduced Pacific oyster *Magallana gigas*, a commercially important species that can be found on every continent except Antarctica, in transporting *Bonamia.* We screened 938 *M. gigas* individuals from 41 populations in this oyster's native and non-native geographic range for presence of *Bonamia* DNA using PCR. *B. exitiosa* was the only species detected and only within 2 of 5 populations from southern California, USA (10 and 42% PCR prevalence). Therefore, *M. gigas* could have played a role in transporting *B. exitiosa* to California (if introduced) and/or maintaining *B. exitiosa* populations within California, but morphological confirmation of infection needs to be done to better understand the host—parasite dynamics within this system. We detected no *Bonamia* DNA within any other non-native *M. gigas* populations (n = 302) nor within native *M. gigas* populations in Japan and Korea (n = 582) and thus found no evidence to support the co-dispersal of *M. gigas* and other *Bonamia* species. Lower sample sizes within some populations and the non-systematic nature of our sampling design may have led to false negatives, especially in areas where *Bonamia* are known to occur. Nevertheless, this global snapshot provides preliminary guidance for managing both natural and farmed oyster populations.

KEY WORDS: Non-native introductions · Bivalves · Haplosporidian · *Crassostrea gigas* · Bonamiasis

1. INTRODUCTION

Bonamia Pichot, Comps, Tigé, Grizel & Rabouin, 1980 (Haplosporida), are protistan parasites of oysters that cause the disease bonamiasis and have been associated with large-scale oyster mortality events (e.g. Hine et al. 2001). These parasites are presumably transmitted directly from host to host (Elston et al. 1986, Hine 1996) and therefore pose a great concern for introduction to non-native areas via the transport of their oyster hosts. There are 4 described species: *B. ostreae* Pichot, Comps, Tigé, Grizel & Rabouin, 1980; *B. exitiosa* Hine, Cochennac & Berthe, 2001; *B. perspora* Carnegie, Burreson, Hine, Stokes, Audemard, Bishop & Peterson, 2006; and *B. roughleyi* (Farley, Wolf & Elston, 1988, renamed by Cochennec-Laureau et al. [2003] and considered *nomen dubium* by Carnegie et al. [2014]). There are also 3 undescribed

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parasites: 2 *B. exitiosa*-like species (1 from California, USA, and 1 from Chile) and a more divergent species from Hawaii, USA (Hill et al. 2014). *B. ostreae* and *B. exitiosa* were described from 2 different oyster species (*Ostrea edulis* and *O. chilensis*, respectively) and were thought to have fairly restricted geographic distributions, with *B. ostreae* and *B. exitiosa* restricted to the Northern and Southern hemispheres, respectively (Hill et al. 2014). Surveys of additional oyster species and localities outside these presumed geographic ranges, however, have revealed additional susceptible host species and a broader geographic distribution of both parasites (e.g. Hill et al. 2014, Lane et al. 2016).

The spread of *B. ostreae* across the Northern Hemisphere has been attributed to the movement of infected *O. edulis* oyster seed from California to France and Spain (Elston et al. 1986, Cigarría & Elston 1997), but recently *B. ostreae* was reported from New Zealand in *O. chilensis* (Lane et al. 2016). The near-cosmopolitan distribution of *B. exitiosa*, which is known to infect at least 7 oyster species across 3 genera, has been hypothesized to have developed via co-dispersal with the non-commercial crested oyster *O. stentina*, presumably via natural (e.g. rafting on pumice) and anthropogenic mechanisms (e.g. biofouling on wooden ships during the Age of Exploration ca. 1400s–1800s; Hill-Spanik et al. 2015). It is important to note that since Hill-Spanik et al. (2015), the taxonomy of the *O. stentina* species complex has been reassessed; the validity of *O. equestris* has been reestablished; and a novel species, *O. neostentina*, has been described (Hu et al. 2019). Nevertheless, the presence of *B. exitiosa* in California, USA, likely cannot be explained by the dispersal of oysters within the *O. stentina* species complex given that these oysters have never been reported from California.

The Pacific oyster *Magallana gigas* (Thunberg, 1793), which was recently removed from the genus *Crassostrea* by Salvi et al. (2022), is one host that has been speculated to have transported *B. exitiosa* and *B. ostreae* outside their reported ranges (Hill-Spanik et al. 2015, Helmer et al. 2020) but is understudied in terms of its relationship with *Bonamia.* Native to Japan, Russia, Korea, and China, *M. gigas* was widely and deliberately introduced for commercial aquaculture starting at least 150 yr ago (NRC 2004). *M. gigas* has spread further via biofouling, ballast water, and/or deliberate transplantation and is now documented on every continent except Antarctica (NRC 2004, Ruesink et al. 2005). *M. gigas* appears to act as a carrier or reservoir host for *B. exitiosa* and *B. ostreae* based on natural exposure and co-habitation experiments done in Ireland and Spain (Culloty et al. 1999, Lynch et al. 2010), though its official classification as a vector/reservoir is uncertain (EFSA AHAW 2023). There is no evidence to date that *M. gigas* dies when infected by *Bonamia* (Culloty et al. 1999, Lynch et al. 2010), and as such, individuals of *M. gigas* could conceivably be considered non-susceptible carriers and disperse *Bonamia* without succumbing to disease. To explore the role of *M. gigas* in the dispersal of *Bonamia*, we used PCR and DNA sequencing to screen for *Bonamia* DNA in *M. gigas* samples that were opportunistically collected across its wide geographic range.

2. MATERIALS AND METHODS

2.1. Sample collection

In both the introduced and native range, *Magallana gigas* has escaped from oyster farms and formed self-perpetuating populations. We collected from wild or feral populations of *M. gigas* at 41 localities from 13 countries between 2008 and 2021 (Table 1, Table S1 in the Supplement at www.int-res.com/articles/suppl/d161p039_supp.pdf). Susceptible hosts of bonamiasis are known to be present in each country/region sampled, and *Bonamia* spp. have been previously detected in 9 of the 13 (Table 1, Table S2). At each locality, we collected ~20 individuals at least 1 m apart from natural and artificial substrata outside of obvious aquacultural infrastructure. The exceptions are the Chilean samples, which were a mix of aquacultured (n = 11) and feral (n = 3) samples collected from the same estuary (Estero Tongoy; Table S1). Whole bodies, or in some cases mantle tissue only, were placed into 95% ethanol and shipped to Charleston, South Carolina, USA. Approximately 1 to 2 mm^3 of mantle and gill (or mantle only) was excised and used for DNA extraction using the Macherey-Nagel Nucleospin Tissue Kit following manufacturer instructions. We confirmed that all individuals were *M. gigas* using high-throughput sequencing of mitochondrial and nuclear loci (E. Sotka et al. unpubl. data).

2.2. PCR and sequencing

All samples were initially screened for *Bonamia* with generic primers BON-319F and BON-524R (Hill et al. 2010), which amplify a 206 bp portion of the small subunit rRNA (SSU rRNA) gene. A 25 µl total reaction contained 1× Promega GoTaq buffer, 1.5 mM $MgCl_2$, 0.4 mg ml^{-1} BSA, 0.2 mM dNTPs,

Table 1. Number of localities (nLocalities) from which *Magallana gigas* was collected, total number of individuals collected (nSamples), and *Bonamia* spp. PCR prevalence by country/region in this study. The status of *Bonamia* and presence of known host species of *Bonamia* spp. in each region are also listed (see also Table S2). Present: *Bonamia* spp. have been detected; Unknown: no reports of *Bonamia* because the parasites either are absent or have not been detected

Country/ region sampled	nLocalities *M. gigas*	nSamples *M. gigas*	*Bonamia* spp. PCR prevalence in *M. gigas* (%)	*Bonamia* status	Host species present	Reference
Argentina	2	30	0	Present	*Ostrea equestris, O. puelchana*	Hill et al. (2014)
Chile	1	14	0	Present	*Ostrea chilensis*	Hill et al. (2014)
Denmark	1	17	0	Present	*Ostrea edulis*	Madsen & Thomassen (2015), Sas et al. (2020)
France	3	36	0	Present	*Ostrea edulis, O. puelchana*	Goulletquer et al. (2002), Pascual et al. (1991), Sas et al. (2020)
Ireland	1	37	0	Present	*Ostrea edulis*	Culloty & Mulcahy (1996), Sas et al. (2020)
Japan	15	490	0	Unknown	*Crassostrea ariakensis*[a]	CABI (2022)
New Zealand	3	32	0	Present	*Ostrea chilensis, O. equestris, Saccostrea glomerata*	Cranfield et al. (2005), Hill et al. (2014), Lane et al. (2016)
Norway	1	59	0	Unknown	*Ostrea edulis*	CABI (2022), Sas et al. (2020)
Pacific Northwest (USA, Canada)	3	30	0	Present	*Ostrea edulis, O. lurida*	Elston et al. (1986), Hill et al. (2014), Marty et al. (2006)
South Korea	3	92	0	Unknown	*Crassostrea ariakensis*[a]	CABI (2022)
Spain	2	25	0	Present	*Ostrea edulis*	Abollo et al. (2008), Carrasco et al. (2012), Montes et al. (2003)
Sweden	1	22	0	Unknown	*Ostrea edulis*	CABI (2022)
Southern California (USA)	5	54	13.0	Present	*Ostrea edulis, O. lurida*	Hill et al. (2014)
TOTAL	41	938	0.7			

[a] Host species present became infected when experimentally introduced to North Carolina, USA (outside their native range) (Burreson et al. 2004)

0.25 µM of each primer, 0.4× *Red*iload (Invitrogen), 1 U of Promega GoTaq, and 3 µl of DNA template. PCR cycling was as follows: 94°C for 4 min; followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min; then 72°C for 7 min. Positive and negative controls were included for each PCR.

Because SSU rRNA gene sequences are too conserved to distinguish *B. exitiosa* and the 2 undescribed *B. exitiosa*-like spp. found in California, USA, and Chile, samples that were PCR positive using the generic primers were subsequently screened using a species-specific PCR with primers haploITSf and ITS-B (Hill-Spanik et al. 2015), which target the more variable internal transcribed spacer (ITS) region of the SSU rRNA gene and produce a ~750 bp product. Reagent concentrations were as above, except 2 mM of $MgCl_2$ was used; cycling was done as in Hill et al. (2014). Positive products were amplified again using the same conditions as above to provide sufficient DNA concentrations for sequencing.

PCR products were electrophoresed on 2% agarose I gels stained with GelRed (Biotium) and visualized under UV light. *Bonamia* PCR positive bands were faint in comparison to the positive control, so products (and their negative controls) were reamplified to obtain enough DNA for sequencing. Products were purified using Exo-SAP-IT (Affymetrix) following the manufacturer's protocol. Direct bidirectional sequencing (using the same primers as in the PCRs above) was done by Eurofins MWG Operon. Sequences were edited (i.e. complementary sequence and chromatogram comparisons) using Sequencher version 5.4 (Gene Codes). Edited sequences were compared to those in GenBank using BLAST.

Three alignments of our resulting sequences and all *B. exitiosa* ITS1–5.8S–ITS2 sequences in the GenBank database (Table S3) were performed using the auto strategy in MAFFT version 7 (Katoh & Standley 2013) as implemented online at EMBL-EBI (Madeira et al. 2022). The first alignment included 321 sequences and was optimized for retention of as much sequence data as possible (489 bp) and thus did not include shorter *B. exitiosa* sequences from *Ostrea edulis* from Italy (EU672891; 107 bp of ITS1) or from the UK (MT184259–MT184268; ITS1 data only). The second alignment contained 331 sequences (all except EU672891) containing only ITS1 and was 240 bp. The third alignment contained sequences from all localities and hosts (332 sequences) but was only 105 bp long. The alignments were then analyzed using TCS version 1.2.3 (Clement et al. 2000) to estimate the gene genealogy (i.e. statistical parsimony) with gaps treated as a fifth state and maximum connection steps at 95%. The software programs tcsBU (Múrias dos Santos et al. 2016) and Inkscape 0.92 (www.inkscape.org) were used to finalize the networks.

3. RESULTS

A total of 938 *Magallana gigas* samples were screened using the *Bonamia* generic primers, and *Bonamia* was detected in only 7 samples and only from southern California, USA (San Diego Bay [n = 6] and Newport Bay [n = 1]). SSU rDNA sequences were 100% similar to sequences of *Bonamia* sp. (e.g. KC578009) and *B. exitiosa* (e.g. JF831802). All except 1 sample from San Diego Bay, California, amplified using the species-specific ITS rDNA primers, and resulting sequences (n = 5) were identical to one another (GenBank accession number PP092477) and differed from the *B. exitiosa* ITS rDNA sequence from Newport Bay, California (n = 1; PP092476) by 8 indels in a 489 bp alignment. These ITS sequences were 99 to 100% similar to sequences of *B. exitiosa* sensu stricto (e.g. JF831669 and JF831720). The 1 sample from San Diego Bay that was only successfully sequenced using the generic SSU rDNA primers was identified as a *B. exitiosa*-like species rather than *B. exitiosa* sensu stricto, as the 160 bp sequence was 99.2 to 100% similar to sequences from these parasites in GenBank (e.g. JF831804, JF831802, GQ366703) and differed from all other *Bonamia* spp. sequences by 1.8 to 5.7%. Therefore, *Bonamia* DNA was detected in only 0.7% of all samples worldwide (7 of 938 samples; Table 1), but PCR prevalence was relatively high where it was detected: 41.7% (5 of 12 screened) prevalence of *B. exitiosa* in San Diego Bay and 10.0% (1 of 10 screened) in Newport Bay, California (Table S1).

The network topologies resulting from the ITS1 and full ITS region data were similar (Fig. 1 & Fig. S1, respectively), but unsurprisingly, most resolution was lost when using the shortest alignment containing only partial ITS1 data (network not shown). For each alignment, there were 173, 107, and 43 haplotypes, respectively. As in Hill-Spanik et al. (2015), the network resulted in 4 reasonably well-defined clusters (Fig. 1). The San Diego Bay haplotype was the same as the dominant haplotype in the California group found in *Ostrea lurida* in Elkhorn Slough, California. The Newport Bay haplotype was shared with one that was not only found in *O. lurida* from Elkhorn Slough, California, but also *O. equestris* and *O. chilensis* from New Zealand, *O. angasi* from Australia, and *O. puelchana* and *O. equestris* from Argentina in the Southern Hemisphere group (Fig. 1). *B. exitiosa* ITS sequences found in *O. edulis* from the UK (added to GenBank since 2015) were found in all groups except the California group.

4. DISCUSSION

Bonamia exitiosa was the only *Bonamia* species detected in our PCR survey of *Magallana gigas* across 938 oysters collected from 41 localities in 13 countries. *B. exitiosa* was detected in 2 localities in California, USA, and at relatively high PCR prevalence (6 of 54 oysters from southern California, or 11.1%). The *B. exitiosa* ITS haplotypes from *M. gigas* were identical to those previously reported from California in native oyster *Ostrea lurida* that were collected from Elkhorn Slough in 2004 and 2009 (Hill et al. 2014), and the network analysis suggested some connectivity with the haplotypes mostly found in the Southern Hemisphere. Finding the California lineage of *B. exitiosa* in non-native *M. gigas* in California may suggest that *M. gigas* had a role in the dispersal and/or maintenance of *B. exitiosa.* Because our tissues were only preserved for molecular analysis (and not for any morphological analyses), we cannot rule out that the detections could also simply be traces of *Bonamia* DNA from the environment, especially as the band intensities for all *Bonamia* PCR products were faint.

Within the *M. gigas* from the rest of the oyster's native and non-native range, we detected no *Bonamia.*

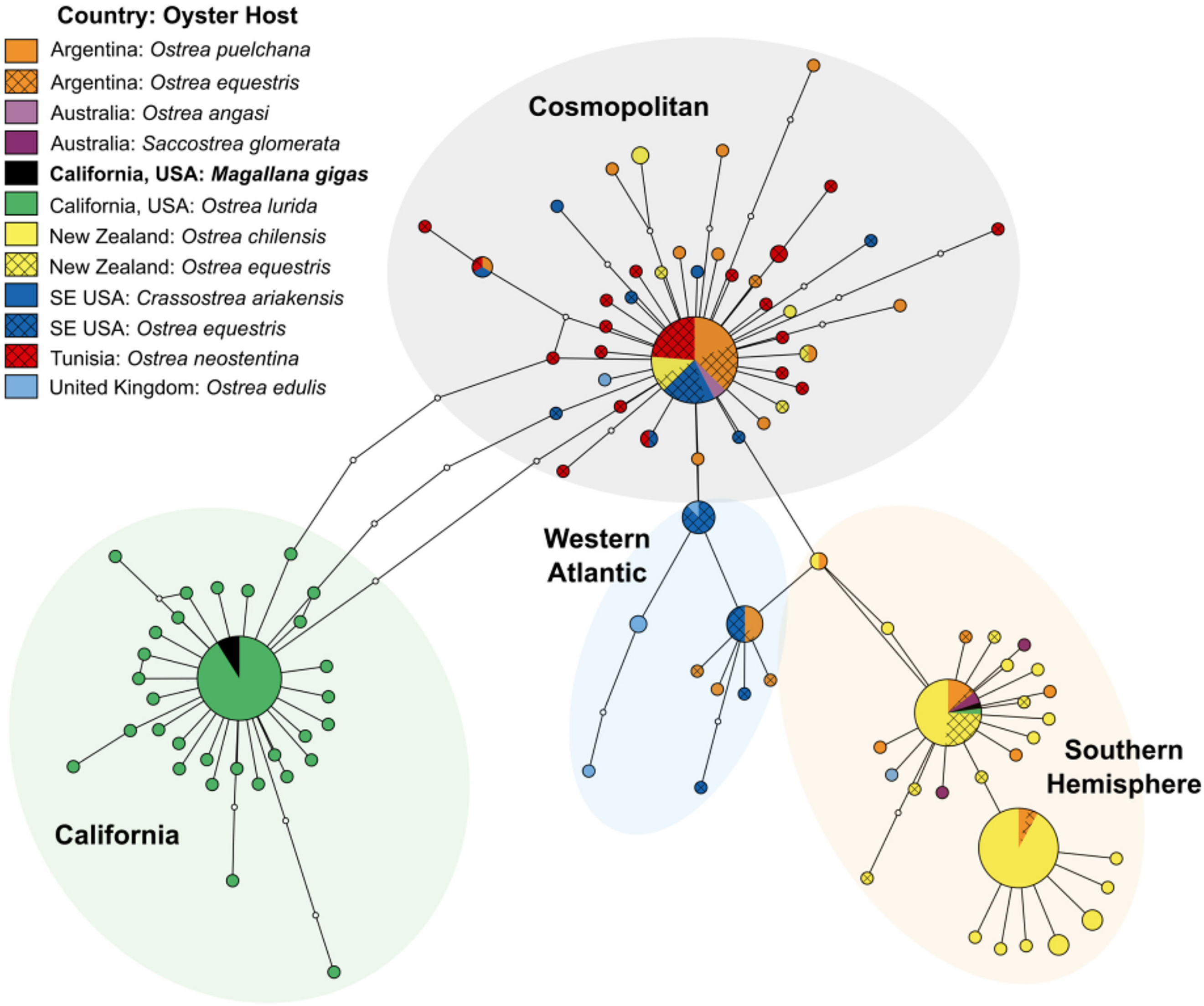

Fig. 1. TCS network based on a 240 bp alignment of 331 *Bonamia exitiosa* first internal transcribed spacer region rRNA gene sequences, which resulted in 107 haplotypes. Geographical groupings after Hill-Spanik et al. (2015). Cross-patterns indicate host species within the *Ostrea stentina* species complex across its broad geographical range

Our results from the native range of *M. gigas* are consistent with the 2 studies that have examined oysters in Asia (1980 *M. gigas* from the south coast of Korea by Shin et al. [2022], 40 *O. denselamellosa* from Japan by Hill et al. [2014]). Because of the opportunistic nature of our sampling, it is possible that we (and others) may not have detected *Bonamia* due to sampling artifacts, especially in areas where *Bonamia* appears to be prevalent in other hosts (e.g. *B. ostreae* in *O. edulis* in Ireland). No detection (false negative results) could have been the result of low sample sizes at some localities compared to previous studies (Lynch et al. 2010) or the result of seasonal variation in prevalence and transmission. While seasonality data are available for some *Bonamia* species in a particular host from a few localities (e.g. *B. ostreae* in *O. edulis* from France, Ireland, and the Netherlands; Culloty & Mulcahy 1996, Arzul et al. 2006, Engelsma et al. 2010), data are sparse for many of the hosts and localities where *Bonamia* has been detected. Our methods also may have missed localized infections, and/or infections in tissue types not sampled, as only 1 to 2 mm^3 of gill and/or mantle tissue was used. Therefore, our results should be considered as a snapshot of the distribution of *Bonamia* in *M. gigas* across its wide geographic range, and future studies should employ a more targeted sampling design with these confounding factors in mind.

The lack of connectivity of the *B. exitiosa* haplotypes from California with those from across the globe is surprising given the number of host oyster species that have been introduced to this area with little to no regulation. The European oyster *O. edulis* was introduced to California from the US east coast in the 1960s (Davis & Calabrese 1969, Wilson & Simons 1985) and is a known host of *B. exitiosa*, a *B. exitiosa*-like species (which has only been found in California *O. edulis* to date), and *B. ostreae*. The eastern oyster *Crassostrea virginica* was transplanted from the US east coast to California from 1867 to 1935 (NRC 2004, Ruesink et al. 2005), and *B. exitiosa* has also been reported to infect *C. virginica* in North Carolina and Massachusetts, USA (ICES 2013, 2014, Engelsma et al. 2014). Oysters *M. gigas* were introduced to the US west coast directly from Japan starting in the late 1800s, increased in the early to mid-1900s, and continued into the 1970s (Barrett 1963, NRC 2004, Ruesink et al. 2005, E. Sotka et al. unpubl. data). On the other hand, little to no sequencing of *B. exitiosa* has been done from these hosts. For example, the only *B. exitiosa* ITS sequences from *O. edulis* currently in GenBank are from the UK (Helmer et al. 2020) and Italy (Narcisi et al. 2010). In the network analysis, the sequences from the UK were found in all groups except the California group, thus providing no evidence for connectivity to California, and unfortunately, the alignment that included the short sequences from Italy produced a network with no resolution; therefore, we were unable to incorporate yet another locality. More sampling of *B. exitiosa* should be conducted focusing on populations of *O. edulis* and *C. virginica* to ensure the lack of connectivity with California we have seen is not due to artifacts.

In this study, we were able to incorporate an additional 75 *B. exitiosa* ITS sequences found in *O. edulis* from the UK (Helmer et al. 2020) as well as additional sequences found in *O. chilensis* from New Zealand (Lane 2018) and thus present the most updated *B. exitiosa* genealogical network herein. We could not support the hypothesis that *M. gigas* has aided in the dispersal of *Bonamia* globally, but *M. gigas* could be important in understanding *B. exitiosa* populations in California. An extensive literature review done by the European Food Safety Authority Panel on Animal Health and Welfare (EFSA AHAW 2023) found that the support for *M. gigas* as a vector or reservoir species for *B. exitiosa* and *B. ostreae* was too uncertain to officially classify it as such. This uncertainty highlights the need for more studies examining the dispersal, hosts, and distribution of important oyster pathogens, which is essential for managers of both farmed and wild oyster populations.

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