Journal of Invertebrate Pathology 112 (2013) 208–212

Contents lists available at SciVerse ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

The occurrence of haplosporidian parasites, *Haplosporidium nelsoni* and *Haplosporidium* sp., in oysters in Ireland

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ARTICLE INFO

Article history:
Received 31 May 2012
Accepted 29 November 2012
Available online 10 December 2012

Keywords:
Crassostrea gigas
Ostrea edulis
Haplosporidium nelsoni
Haplosporidium sp.
Haplosporidium armoricanum

ABSTRACT

The phylum Haplosporidia is a group of obligate protozoan parasites that infect a number of freshwater and marine invertebrates. Haplosporidian parasites have caused significant mortalities in commercially important shellfish species worldwide. In this study, haplosporidia were detected in Pacific oysters *Crassostrea gigas* originating in Ireland and were subsequently identified independently in laboratories both in Ireland and in Spain as *Haplosporidium nelsoni*. In Ireland, *H. nelsoni* plasmodia were also observed in the heart tissue of a single *Ostrea edulis*. A range of techniques including heart smear screening, histology, standard polymerase chain reaction (PCR), direct sequencing and *in situ* hybridisation with an *H. nelsoni* specific DNA probe were carried out to confirm diagnosis. This is the first reporting of *H. nelsoni* in oysters in Ireland and this is the first reporting of the detection of this haplosporidian in *O. edulis*. In Ireland, another haplosporidian was also observed in a single *O. edulis* during heart smear screening. PCR and DNA sequencing were carried out and indicated the presence of a *Haplosporidium* sp., most likely *Haplosporidium armoricanum*. The low prevalence and intensity of infection of both haplosporidian species in Irish *C. gigas* and in particular *O. edulis* may indicate that their presence is inconsequential.

1. Introduction

The phylum Haplosporidia contains obligate protozoan parasites of a number of fresh water and marine invertebrates including tunicates, polychaetes and molluscs (Burreson and Ford, 2004; Moiseeva et al., 2004; Siddall and Aguado, 2006). To date there are four genera allocated to this phylum; *Urosporidium*, *Minchinia*, *Haplosporidium* and *Bonamia* (Reece et al., 2004). Once introduced to a naïve host population, some haplosporidian species have had a significant impact on commercial shellfish species worldwide due to the mortalities they cause (Ford and Haskin, 1982; Burreson et al., 2000; Burreson and Reece, 2006). The haplosporidian *Haplosporidum nelsoni* is the infectious agent of the disease Multinucleate Sphere X (MSX) (Haskin et al., 1966) in the eastern oyster, *Crassostrea virginica*. Plasmodia are the most common life stage of *H. nelsoni* found in oysters, ranging in size from 5 to >50 μm in diameter, containing a variable number and size of nuclei (Ford, 2010). *H. nelsoni* spores are rarely found in adult oysters and are more common in juveniles with advanced infections (Ford, 2010).

The Pacific oyster, *Crassostrea gigas*, is a known host to this pathogen; however, *C. gigas* is not highly susceptible to *H. nelsoni*, which occurs at a low prevalence in this oyster species (Chun, 1972; Kern, 1976; Friedman et al., 1991; Friedman, 1996; Renault et al., 2000; Wang et al., 2010; Ford, 2010). *H. nelsoni* is thought to have been introduced into North America in the 1950's unintentionally with *C. gigas* consignments from Japan (Andrews, 1984; Friedman, 1996). It jumped host from *C. gigas* to a more susceptible naïve host, the eastern oyster, *C. virginica*, where it caused mass mortalities (Friedman, 1996; Burreson et al., 2000). *H. nelsoni* has been detected in the United States (Haskin and Andrews, 1988; Friedman et al., 1991; Friedman, 1996), Canada (Stephenson et al., 2003), Korea (Chun, 1972; Kern, 1976; Kang, 1980; Burreson et al., 2000), Japan (Kamaishi and Yoshinaga, 2002) and China (Wang et al., 2010). Haplosporidian plasmodia resembling *H. nelsoni* have been detected in the Olympia oyster, *Ostrea conchaphila*, from Oregon, USA, that had been imported from California, USA, a *H. nelsoni*-infected area. (Mix and Sprague, 1974). Within Europe, haplosporidian plasmodial stages were reported in *C. gigas* at a very low prevalence (0.27%) from the French Atlantic coast in 1993 and species-specific molecular diagnostics confirmed them

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0022-2011/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jip.2012.11.013

as *H. nelsoni* (Renault et al., 2000). Although unintentional introductions of *H. nelsoni* with shellfish assignments may occur, it is conceivable that *H. nelsoni* may also be dispersed by ocean circulation and in ship ballast water (Burreson and Ford, 2004). Several OIE approved diagnostic techniques including histology, standard polymerase chain reaction (PCR) and *in situ* hybridisation (ISH) are used to detect *H. nelsoni* (Ford and Figueras, 1988; Stokes et al., 1995; Renault et al., 2000; Stokes and Burreson, 2001).

On-going oyster health surveys of *C. gigas* and *O. edulis* from Ireland revealed a low prevalence of plasmodia and microcell-like cells resembling that of haplosporidians. In this study, morphological examination and DNA-based molecular techniques were used to identify these haplosporidians as *H. nelsoni* and a second *Haplosporidium* sp. most likely *Haplosporidium armoricanum*.

2. Materials and methods

As part of a larger survey of *C. gigas* and *O. edulis* in Ireland, samples of both oyster species were screened simultaneously to determine general health status. In total, four samples consisting of 556 cultured *C. gigas* (2–3 years) from Cork Harbour and six samples consisting of 1310 cultured *O. edulis* (2–4 years) from Cork Harbour and various other Irish locations were screened (2006–2008). In Ireland, for both *C. gigas* and *O. edulis*, heart smears were carried out to screen for any pathogens in the hemocytes. Heart smears are routinely used to screen for the haplosporidian *Bonamia ostreae* (Bachére et al., 1982; OIE, 2006). Routine precautionary measures were taken to ensure that contamination did not occur when the heart was removed from the pericardial cavity. The oyster was opened carefully to avoid disruption of the pericardial cavity, excess seawater and fluids were drained away and the area around the pericardial cavity was blotted with clean tissue paper to remove any potential contaminants. The heart was then removed using clean forceps. Imprints were made on slides and the heart was frozen for subsequent DNA extraction and PCR. Transverse sections of the oyster tissues, including gills, digestive gland and gonad were fixed in Davidson's fixative for 24 h and were subsequently processed for histology and stained using Harris hematoxylin and eosin. ISH was carried out on transverse sections of the oyster using a digoxigenin (DIG) labelled DNA probe which was prepared using the PCR DIG probe synthesis kit from Roche. ISH allows for the detection of complementary genetic material in cells or in tissues thus allowing visualization of the pathogen *in situ*. One hundred µl of shell cavity fluid of each individual was also frozen for PCR analysis.

PCR assays, using similar mastermix and thermocycling conditions, including generic haplosporidian primers (HAP-F1 and HAP-R3) (Renault et al., 2000) or specific MSX primers (MSX 607F, MSX 831R) (Ko et al., 1999) and MSX-A', MSX-B primers (Renault et al., 2000) (Table 1), were carried out on DNA extracted from all of the *C. gigas* and the subsample of *O. edulis* heart tissue and shell cavity fluid samples ($n = 300$) collected during the warmer summer months. PCR negative controls consisted of double distilled water (ddH_20). As this was a preliminary screening of individuals with presumptive plasmodia and microcells and because both haplosporidians had not been screened or detected in the laboratory in Ireland or in Spain prior to this study, positive controls were not used in the initial PCRs but *H. nelsoni* and *Haplosporidium* sp. DNA that was isolated in those PCRs was used as positive controls in all subsequent screening. DNA was extracted using 10% Chelex® 100 resin (Walsh et al., 1991; Lynch et al., 2008). Any samples observed to be positive in the PCR for *H. nelsoni* or *Haplosporidium* sp. were also screened using a PCR for *B. ostreae* and *B. exitiosa* using a genus *Bonamia*-specific primer pair (Cochennec et al., 2000) which included a positive control consisting of genomic DNA from *O. edulis* heavily infected with *B. ostreae*and a negative control consisting of ddH_2O. Products that were amplified were cut from the gel and the isolated DNA was purified using Qiagen Qiaquick gel extraction kit. The DNA was sent to Eurofins MWG BIOTECH AG Martinsried, Germany, for direct sequencing.

In Ireland, *in situ* hybridisation was performed using standard procedures and all solutions and buffers were made up according to the protocol described by Sambrook and Russell (2001). Probe-target hybrids were detected with an alkaline- phosphatase-conjugated antibody by a colour reaction and were counterstained by Bismarck Brown Y solution (Lynch et al., 2008). A negative control (no probe or probe to something not in the sample) was used for comparison.

As part of another study, 100 *C. gigas* were sent from Cork Harbour (Ireland) to the laboratory of the Centro deInvestigacións Mariñas, Spain, in March 2006, where they were kept in quarantine conditions at 17–18 °C and 30 ppt. The water in the tanks had been filtered (0.2 µm) and UV treated. In Spain, the 43 *C. gigas* surviving after 3 months in quarantine conditions were processed for diagnosis. A gill piece from each oyster was preserved in 95% ethanol for PCR analysis. Transverse sections of the oyster meat, including gills, digestive gland and gonad were fixed in Davidson's solution and processed with standard procedures to produce histological sections that were stained with Harris haematoxylin and eosin. After histological examination, the preserved gill piece of one of the oysters that had shown haplosporidian-like plasmodia was used for PCR analysis using HAP-F2 and HAP-R1 primers (Renault et al., 2000). PCR products were ligated into cloning vector pCR2.1 at 14 °C overnight and transformed into *E. coli* One Shot Top 10F' Chemically Competent (Invitrogen Life Technologies™). Transformed cells were screened by PCR using the vector's M13 forward (5′ GTA AAA CGA CGG CCA G 3′) and M13 reverse (5′ CAG GAA ACA GCT ATG AC 3′) primers. Positive clones were cleaned for sequencing using the ExoSAP-IT reagent (USB Corporation) according to the manufacturer's instructions. Sequencing was performed by Secugen (Madrid, Spain) and the chromatograms were analysed using ChromasPro v.1.41 Technelysium Pty Ltd. The paraffin block corresponding to that oyster was sent to Virginia

Table 1
Diagnostic methods used to screen oysters in Ireland and in Spain and results for each method.

Oyster species	*n*	Heart smear	Histology	PCR (tissue)				PCR (Shell cavity fluid)			ISH	Sequencing & BLAST analysis
				HAP-F1 + HAP-R3	HAP-F2 + HAP-R1	MSX-A' + MSX-B	MSX 607F + MSX 831R	HAP-F1 + HAP-R3	MSX-A' + MSX-B	MSX 607F + MSX 831R		
Ireland												
C. gigas	556	(1/556)	(0/2)	(1/556)	–	(1/556)	–	(1[a]/556)	(1[a]/556)	(1[a]/556)	(0/2)	*H. nelsoni*
O. edulis	1310	(1/1310)	(0/1)	(1/300)	–	(0/300)	–	(0/300)	(0/300)	–	(0/1)	*H. nelsoni*
		(1/1310)	(0/1)	(1/300)	–	–	–	(0/300)	(0/300)	–	(0/1)	*Haplosporidium* sp.
Spain												
C. gigas	43	–	(2/43)	(2/43)	(2/43)	–	–	–	–	–	(2/43)	*H. nelsoni*

[a] An additional *C. gigas*.

Institute of Marine Science for ISH using a *H. nelsoni* specific DNA probesynthesized 5′ end-labeled oligonucleotide DIG probe made by Sigma Genosys, MSX1347, as previously described (Stokes and Burreson, 2001). A negative control (no probe or probe to something not in the sample) was used for comparison.

3. Results

In the samples screened in Ireland, presumptive haplosporidian plasmodia were detected in the heart smear of one *C. gigas* sample (0.64% (1/156)) in June 2007 (Fig. 1) and in one *O. edulis* sample (0.69% (1/143)) in June 2007 (Fig. 2), both sampled from Cork Harbour. The plasmodia had a size range of 11 μm–18 μm and contained 5–17 nuclei which were 1–2 μm in size. Fewer than 10 *H. nelsoni*-like plasmodia were visualised in each of the two heart smears and all were extracellular. Presumptive microcells, unlike the *H. nelsoni* plasmodia, were observed in one other *O. edulis* heart smear in June 2007. All three oysters were negative for *B. ostreae*and *B. exitiosa* by microscopic examination of heart smears and by PCR of corresponding DNA using genus *Bonamia*-specific primers (Cochennec et al., 2000).

In the single *C. gigas* and single *O. edulis* where *H. nelsoni*-like plasmodia were observed, PCR analysis of DNA of heart tissue using generic haplosporidian primers (Renault et al., 2000) resulted in a product of 348 bp (Fig. 3), the expected product size for *H. nelsoni*. Sequencing confirmed the PCR product in the Irish *C. gigas* (GenBank Accession # JX073255) and in the Irish *O. edulis* (GenBank Accession # JX073256) to be *H. nelsoni* SSU rRNA (GenBank Accession # U19538.2, AB080597.1, X74131.1). Sequences obtained from the generic PCR of *C. gigas* had 94% Query Coverage and 100% Maximum Identity and of *O. edulis* had 96% Query Coverage and 99% Maximum Identity to the *H. nelsoni* SSU rDNA sequences in GenBank using BLAST analysis (www.ncbi.nlm.nih.gov/BLAST/). In the same two samples, *H. nelsoni*-specific PCR was carried out using the MSX-A' and MSX-B primers (Renault et al., 2000). The expected 573 bp product was amplified from the *C. gigas* but not amplified from the *O. edulis*, even though both had plasmodia present in the heart smears. In addition, PCR of DNA isolated from the shell cavity fluid of another *C. gigas*, that had not demonstrated plasmodia in the heart smear or histology, using all three primer pairs (Ko et al., 1999; Renault et al., 2000) resulted in amplification of the expected product sizes. The 225 bp product amplified using the Ko et al. (1999) PCR was confirmed to be *H. nelsoni* SSU rDNA by sequencing (Accession # U19538.2, AB080597.1, X74131.1) (Fig. 3).

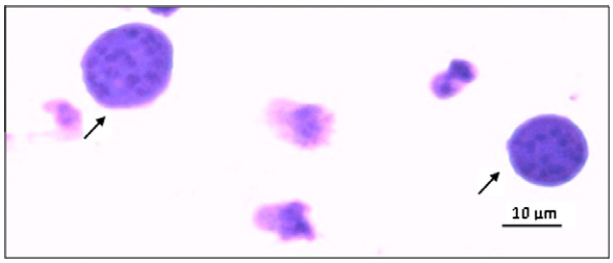

Fig. 1. *Haplosporidium nelsoni* plasmodia (arrows) (confirmed by DNA sequencing) in the heart smear of *Crassostrea gigas* (×40).

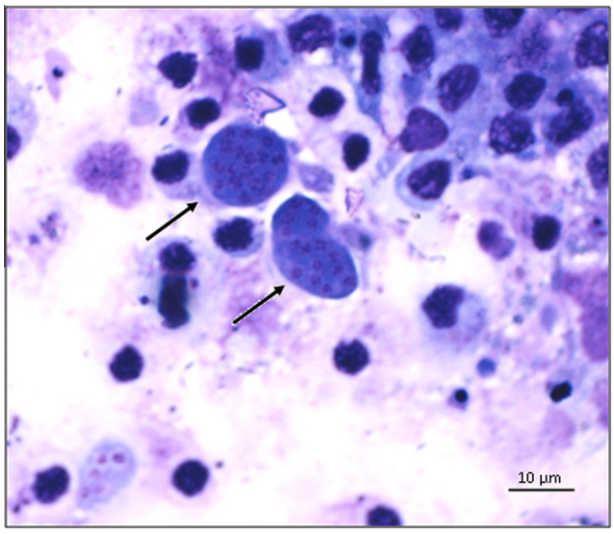

Fig. 2. *Haplosporidium nelsoni* plasmodia (arrows) (confirmed by DNA sequencing) in the heart smear of *Ostrea edulis* (×40).

In the *O. edulis* individual with presumptive microcells, multiple products of different sizes were amplified using the generic HAP-F1 and HAP-R3 primers (Fig. 3). A product was cut from the agarose gel at ~348 bp from three replicates of that individual and the DNA was isolated and purified using the Qiagen Qiaquick gel extraction kit. The Irish DNA sequence (GenBank Accession # JX073257) was confirmed to be *Haplosporidium* sp. (Accession # AY781176). Obtained sequences had 100% Query Coverage and 99% Maximum Identity to the *Haplosporidium* sp. SSU rDNA sequence in GenBank using BLAST analysis (www.ncbi.nlm.nih.gov/BLAST/).

No haplosporidian cells were detected in the tissue sections of any of the *C. gigas* and *O. edulis* deemed positive for *H. nelsoni* in Ireland. *H. nelsoni* spores were not observed in any oysters in Ireland or in Spain. *Haplosporidium* sp. plasmodia and spores were not observed in the tissues of the *O. edulis* that demonstrated presumptive microcells in the heart smear.

In Irish oysters examined in Spain, spherical to elongated eosiniphilic haplosporidian-like plasmodia, up to 20 μm in length, with numerous nuclei (up to 20) around 2 μm in diameter, were observed in the histological sections of two *C. gigas* ($n = 43$) (Fig. 4A). Those plasmodia occurred in the connective tissue of every organ represented in the sections (gills, mantle, gonad, digestive gland, adductor muscle). ISH with the *H. nelsoni*-specific DNA probe showed hybridisation to cells consistent with shape and location of the plasmodia observed by standard histology (Fig. 4B). Generic haplosporidian PCR, using primers HAP-F2 and HAP-R1, of DNA extracted from gill tissues of *C. gigas* that had shown plasmodia through histological examination yielded the expected 95 bp product from both. After cloning and sequencing of the PCR products, BLAST analysis showed that the most similar sequence in GenBank was the SSU rDNA of *H. nelsoni* (Accession # X74131) with an identity value of 99%.

4. Discussion

H. nelsoni plasmodia were visualised extracellularly in heart smears and in connective tissue of *C. gigas* originating from Ireland. *H. nelsoni* DNA was detected in the heart tissue and shell cavity fluid of *C. gigas* in Ireland and in the gill tissue of Irish *C. gigas* in Spain. Furthermore *H. nelsoni* plasmodia were observed in the heart tissue smear and *H. nelsoni* DNA was detected in the heart tissue of *O. edulis* in Ireland. The lack of detection of *H. nelsoni* in the single *O. edulis* using the specific primers MSX-A' and MSX-B may be due to the "dilution effect" of combined host and pathogen DNA. The ratio of pathogen to host DNA may be quite low and the probability of positive results being attained each time may actually be quite low. Also, as the PCR screening using the MSX-A' and MSX-B primers was carried out several years after the initial

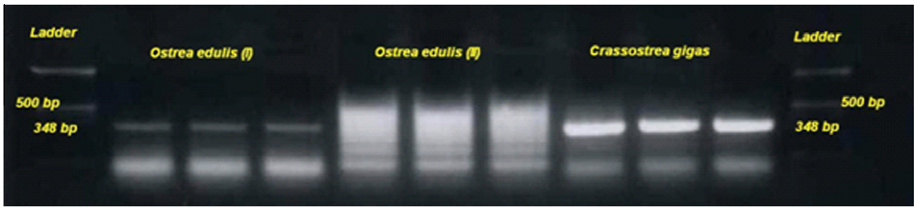

Fig. 3. Agarose gel picture showing the products amplified in triplicate reactions from the same individual using the generic HapF1/HapR3 primers (Renault et al., 2000). **(I)** *Ostrea edulis* (*H. nelsoni* plasmodia visualised in heart tissue) 348 bp product, **(II)** *Ostrea edulis* (haplosporidian-like cells visualised in heart tissue) multiple bands, **(III)** *Crassostrea gigas* (*H. nelsoni* plasmodia observed in heart tissue) 348 bp product, **Ladder** 100 bp ladder.

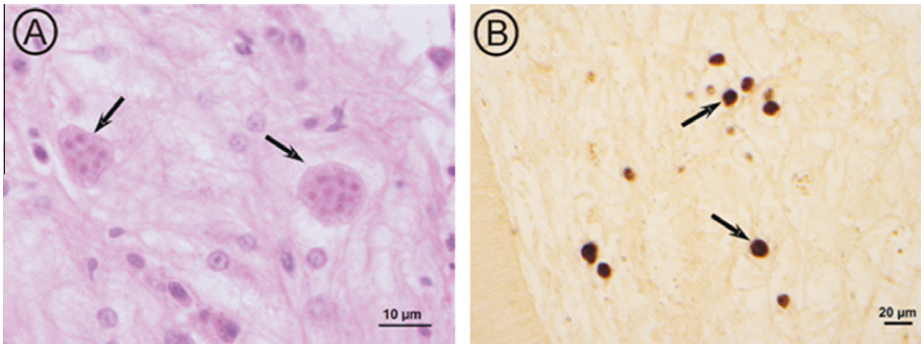

Fig. 4. Micrographs showing histological sections of an oyster *Crassostrea gigas* infected with *Haplosporidium nelsoni* plasmodia (arrows). A: Standard histological section (HHE). B: ISH with *H. nelsoni* specific DNA probe.

DNA extraction, DNA degradation may have occurred. A comparison between the *H. nelsoni* sequences obtained in this study (Accession # JX073255, JX073256) and those recorded in GenBank from North America (Accession # U19538.2, X74131.1) and Japan (Accession # AB0805971) using available genetic markers show no variation and indicate that they are the same organism. Presumptive microcells identified by sequencing as *Haplosporidium* sp. were observed extracellularly in the heart tissue of a single *O. edulis*. *H. armoricanum* has been detected in *O. edulis* in several European countries (Van Banning, 1977; Cahour et al., 1980; da Silva et al., 2005) and the ultra structure of the haplosporidian whose DNA sequence was submitted to GenBank as *Haplosporidium* sp. (AY781176) was identified as *H. armoricanum* (Hine et al., 2007). The *Haplosporidium* sp. sequence generated in this study was the same as that portion of the *Haplosporidium* sp. rDNA identified as *H. armoricanum*, therefore we conclude that the haplosporidian from Irish *O. edulis* is most likely *H. armoricanum*.

C. gigas is susceptible to *H. nelsoni*, however, infection is usually light when it occurs and significant mortalities have not been associated with its detection in this oyster species (Elston, 1999; Burreson et al., 2000). In previous studies, the prevalence of *H. nelsoni* in *C. gigas* has been found to be relatively low (Chun, 1972; Kern, 1976; Friedman et al., 1991; Friedman, 1996; Renault et al., 2000; Ford, 2010; Wang et al., 2010) compared to a significant prevalence (30–100%) in *C. virginica* in Delaware and Chesapeake Bays, USA (Andrews and Wood, 1967; Ford and Haskin, 1982; Haskin and Andrews, 1988; Ragone Calvo and Burreson, 1999). In France, between 1990 and 2004, detection of *H. nelsoni* in *C. gigas* was sporadic with an average detection frequency of 0.1% per year and a prevalence of 1% in certain areas. In 2005, *H. nelsoni* detection frequency in France had increased from 0.1% to 1% per year with an increase in prevalence of 7–10% in certain areas (Garcia et al., 2006). Sporulation of *H. nelsoni* in French *C. gigas* was first recorded in 2005 (Garcia et al., 2006). In the present study, the prevalence of *H. nelsoni* in Irish *C. gigas* is low (<0.7%), much less than 4% prevalence that has been recorded in Japan, which is within the native range of *C. gigas* (Kamaishi and Yoshinaga, 2002). To date, *H. nelsoni* has not been detected in *C. gigas* cultured in Galicia, Spain (Iglesias-Estepa et al., 2007). The environmental factors, temperature and salinity, favoured by *H. nelsoni* are present in both Ireland and Spain (Myhre and Haskin, 1970; Ford and Haskin, 1982; Ford, 1985).

It is possible that some light infections may have gone undetected in this study using the different diagnostic techniques due to low prevalence and intensity observed (Lynch et al., 2008). In Ireland, *H. nelsoni* was not detected in the tissue sections using histology or ISH of the individuals even though it was observed in the heart smear. It has been suggested that histology does not become a reliable diagnostic tool for *H. nelsoni* until parasite density is approximately 10^3–10^4 parasites g^{-1} wet tissue (Ford, 2010). The small amount of target pathogen DNA combined with the high amount of host DNA can have a "dilution effect" resulting in the PCR sensitivity being reduced as the primer-template contact and binding is reduced (Stokes et al., 1995; Lynch et al., 2008).

It is unknown what effect *H. nelsoni* has on *O. edulis*; however, *H. nelsoni*-like plasmodia have been detected in another species of the same genus, *O. lurida* (Mix and Sprague, 1974) and no mortalities were associated with the *H. nelsoni*-like plasmodia presence in *O. lurida* in Canada. Overall these results, while reporting the first incidence of *H. nelsoni* in Irish *C. gigas* and in *O. edulis* and a *Haplosporidium* sp., possibly *H. armoricanum*, in *O. edulis*, respectively in Ireland, would indicate that these oysters may be demonstrating incidental infections which are not having a significant impact on these species. However, determining how long these haplosporidians have been in Irish waters, how they might have been introduced and how they maintain themselves would be of interest.

Acknowledgments

The project was part funded by the European Regional Development Fund (ERDF) through the Ireland Wales programme (INTERREG 4A) SUSFISH. Elena Penas and María Isabel Meléndez provided technical assistance atCentro deInvestigacións Mariñas, Spain. This

is VIMS contribution #3258. The authors would like to thank the Irish shellfish growers who contributed material for the study.

References

Andrews, J.D., 1984. Epizootiology of diseases of oysters (*Crassostrea virginica*), and parasites of associated organisms in eastern North America. Helgoländer Meeresuntersuchungen 27, 149–166.

Andrews, J.D., Wood, J.L., 1967. Oyster mortality studies in Virginia. VI. History and distribution of *Minchinia nelsoni*, a pathogen of oysters in Virginia. Chesapeake Sci. 8 (1), 1–13.

Bachére, E., Durand, J., Tigé, G., 1982. *Bonamia ostreae* (Pichot et al., 1979) parasite de l'huître plate comparison de deux methods de diagnostic. ICES CM F 28, 1–11.

Burreson, E.M., Ford, S., 2004. A review of recent information on the Haplosporidia, with a special reference to *Haplosporidium nelsoni* (MSX disease). Aquat. Living Resour. 17, 499–517.

Burreson, E.M., Reece, K.S., 2006. Spore ornamentation of *Haplosporidium nelsoni* and *Haplosporidium costale* (Haplosporidia) and incongruence of molecular phylogeny and spore morphology in the Haplosporidia. J. Parasitol. 92, 1295–1301.

Burreson, E.M., Stokes, N.A., Friedman, C.S., 2000. Increased virulence in an introduced pathogen: *Haplosporidium nelsoni* (MSX) in the eastern oyster *Crassostrea virginica*. J. Aquat. Anim. Health. 12, 1–8.

Cahour, A., Poder, M., Balouet, G., 1980. Présence de *Minchinia armoricana* (Haplosporea, Haplosporida) chez *Ostrea edulius* d'origine française. C. R. Soc. Biol. 174, 359–368.

Chun, S.K., 1972. Preliminary studies on the sporozoan parasites in oysters on the southern coast of Korea. B. Korean Fish. Soc. 5, 76–80.

Cochennec, N., LeRoux, F., Berthe, F., Gerard, A., 2000. Detection of *Bonamia ostreae* based on small subunit ribosomal probe. J. Inverteb. Pathol. 76, 26–32.

da Silva, P.M., Fuentes, J., Villalba, A., 2005. Growth, mortality and disease susceptibility of oyster *Ostrea edulis* families obtained from brood stocks of different geographical origins, through on-growing in the Ría de Arousa (Galicia, NW Spain). Mar. Biol. 147, 965–977.

Elston, R.A., 1999. Health management, development and histology of seed oysters. In: Haplosporidiosis of juvenile oysters. World Aquaculture Society, Baton Rouge, Louisiana, USA, pp. 99–102 (Chapter 19, pp. 110).

Ford, S.E., 1985. Effects of salinity on survival of the MSX parasite *Haplosporidium nelsoni* (Haskin, Stauber and Mackin) in oysters. J. Shellfish Res. 5 (2), 85–90.

Ford, S.E., 2010. ICES. 2010. MSX disease of oysters caused by *Haplosporidium nelsoni*. Revised and updated by Susan E. Ford. ICES Identification Leaflets for Diseases and Parasites of Fish and Shellfish Leaflet No. 38,f102 pp. 4, <http://www.ices.dk/products/fiche/disease/2006/Sheet%20no%2038.pdf>.

Ford, S.E., Figueras, A.J., 1988. Effects of sublethal infection by the parasite *Haplosporidium nelsoni* (MSX) on gametogenesis, spawning and sex ratios of the oysters of Delaware Bay USA. Dis. Aquat. Org. 41, 121–133.

Ford, S.E., Haskin, H.H., 1982. History and epizootiology of *Haplosporidium nelsoni* (MSX), an oyster pathogen in Delaware Bay 1957–1980. J. Invertebr. Pathol. 40, 118–141.

Friedman, C.S., 1996. Haplosporidian infection of the Pacific oyster, *Crassostrea gigas* (Thunberg), in California and Japan. J. Shellfish Res. 15, 597–600.

Friedman, C.S., Cloney, D.F., Manzer, D., Hedrick, R.P., 1991. Haplosporidiosis of the Pacific oyster, *Crassostrea gigas*. J. Invertebr. Pathol. 58, 367–372.

Garcia, C., Robert, M., Arzul, A., Chollet, B., Joly, J-P., Miossec, L., Comtet, T., Berthe, F., 2006. Viral gametocytic hypertrophy of Crassostreagigas in France: from occasional records to disease emergence? Dis. Aquat. Org. 70, 193–199.

Haskin, H.H., Andrews, J.D., 1988. Uncertainties and speculations about the life cycle of the eastern oyster pathogen *Haplosporidium nelsoni* (MSX). Am. Fisher. Soc. Spec. Publica. 18, 5–22.

Haskin, H.H., Stauber, L.A., Mackin, J.A., 1966. *Minchinia nelsoni* n. sp. (Haplosporida, Haplosporidiidae): causative agent of the Delaware Bay oyster epizootic. Science (Washington D C) 153, 1414–1416.

Hine, P.M., Engelsma, M.Y., Wakefield, S.J., 2007. Ultrastructure of sporulation in *Haplosporidium armoricanum*. Dis. Aquat. Org. 77, 225–233.

Iglesias-Estepa, D., Rodríguez-González, L., Montes-Pérez J., 2007. Estudio histopatológico de Crassostrea gigas (Thunberg 1793) cultivada en las rías gallegas. In: Cerviño-Eiroa, A., Guerra-Díaz, A., Pérez-Acosta, C. (Eds.), XI Congreso Nacional de Acuicultura, Centro de Investigacións Mariñas, Consellería de Pesca e Asuntos Marítimos, Xunta de Galicia, Vilanova de Arousa (Spain), pp: 1009–1012.

Kamaishi, T., Yoshinaga, T., 2002. Detection of *Haplosporidium nelsoni* in Pacific oyster *Crassostrea gigas* in Japan. Fish Pathol. (Tokyo) 37, 193–195.

Kang, P.A., 1980. On the *Minchinia* sp. infection in the oysters from Chungmu area. Bull. Natl. Fish. Res. Dev. Agency 25, 25–28 (In Korean with English Abstract).

Kern, F.G., 1976. Sporulation of *Minchinia* sp. (Haplosporida, Haplosporidiidae) in the Pacific oyster *Crassostrea gigas* (Thunberg) from the Republic of Korea. J. Protozool. 23 (4), 498–500.

Ko, Y.T., Chan, M.M.Y., Ford, S.E., Fong, D., 1999. A PCR-ELISAmethod for direct detection of the oyster pathogen *Haplosporidiumnelsoni*. Mar. Biotechnol. 1, 147–154.

Lynch, S., Mulcahy, M., Culloty, S.C., 2008. Efficiency of diagnostic techniques for the parasite *Bonamia ostreae*, in the flat oyster *Ostrea edulis*. Aquaculture 281, 17–21.

Mix, M.C., Sprague, V., 1974. Occurrence of a haplosporidian in native oysters (*Ostrea lurida*) from Yaquina Bay and Alsea Bay. Oregon. J. Invertebr. Pathol. 23, 252–254.

Moiseeva, E., Rabinowitz, C., Yankelevich, I., Rinkevich, B., 2004. "Cup cell disease" in the colonial tunicate *Botryllus schlosseri*. Dis. Aquat. Org. 60, 77–84.

Myhre, J.L., Haskin, H.H., 1970. MSX infections in resistant and susceptible oyster stocks. Proc. Nat.l Shellfish Assoc. 60, 9.

Ragone Calvo, L., Burreson, E.M., 1999. Status of the major oyster diseases in Virginia, 1998. Virginia Institute of Marine Science, Marine Resource Report 99–3, Gloucester Point.

Reece, K.S., Siddall, M.E., Stokes, N.A., Burreson, E.M., 2004. Molecular phylogeny of the Haplosporidia based on two independent gene sequences. J. Parasitol. 90, 1111–1122.

Renault, T., Stokes, N.A., Chollet, B., Cochennec, N., Berthe, F., Gérard, A., Burreson, E.M., 2000. Haplosporidiosis in the Pacific oyster *Crassostrea gigas* from the French Atlantic coast. Dis. Aquat. Org. 42, 207–214.

Sambrook, J., Russell, D., 2001. Molecular cloning: A Laboratory Manual, third ed., 2(1–3), pp. 344.

Siddall, M.E., Aguado, M.T., 2006. Molecular phylogenetic evidence of a haplosporidian parasite infecting the polychaete *Syllis nipponica* (Imajima, 1966). Parasitol. Res. 99, 309–312.

Stephenson, M.F., McGladdery, S.E., Maillet, M., Veniot, A., 2003. First reported occurrence of MSX in Canada. J. Shellfish Res. 22, 355 (Abstract).

Stokes, N.A., Burreson, E.M., 2001. Differential diagnosis of mixed *Haplosporidium costale* and *Haplosporidium nelsoni* infections in the eastern oyster, *Crassostrea virginica*, using DNA probes. J. Shellfish Res. 20, 207–213.

Stokes, N.A., Siddall, M.E., Burreson, E.M., 1995. Detection of *Haplosporidium nelsoni* (Haplosporidia: Haplosporidiidae) in oysters by PCR amplification. Dis. Aquat. Org. 23, 145–152.

Van Banning, P., 1977. *Minchinia armoricana* sp. nov. (Haplosporida), a parasite of the European flat oyster *Ostrea edulis*. J. Invertebr. Pathol. 30, 199–206.

Walsh, P.S., Metzger, D.A., Higuchi, R., 1991. Chelex 100 asa medium for simple extraction of DNA for PCR-based typingfrom forensic material. Biotechniques 10, 506–513.

Wang, Z., Lu, X., Liang, Y., 2010. A 1-year investigation of the parasite *Haplosporidium nelsoni* (MSX) in the Pacific oyster *Crassostrea gigas* from Dayaowan Bay. China. Parasitol. Res. 107, 189–193.