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Distribution and Impact of the Oyster Parasite *Bonamia Ostreae* in Maine, and its Detection Using DNA Probes

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DISTRIBUTION AND IMPACT OF THE OYSTER PARASITE *BONAMIA OSTREAE IN* MAINE, AND ITS DETECTION USING DNA PROBES

BY

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Thesis Advisor: Dr. Bruce Barber

An Abstract of the Thesis Presented
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Health management efforts in Maine related to the flat oyster (*Ostrea* edulis) parasite *Bonamia ostreae* are limited by a lack of knowledge of the parasite's distribution and impact in both wild and cultured populations. This information would be more readily obtainable with improved diagnostic tools. The objectives of this dissertation were to design sensitive and specific DNA probes for detection of B. *ostreae;* to assess the prevalence and intensity of parasite infections in wild oyster populations in Maine; and to evaluate growth and mortality of cultured oysters in the Damariscotta River, where B. *ostreae* is enzootic.

A polymerase chain reaction (PCR) assay, designed for sensitive in vitro detection of *Bonamia ostreae* and evaluated against a standard histocytological technique, was positive for *B. ostreae* in all (100%) "heavily" and "moderately"

infected oysters, most (73.3%) “lightly” and “scarcely” infected oysters, and many (37.9%) of the oysters in which the parasite was undetected. No PCR amplification occurred when control (uninfected) oysters were used. A fluorescent hybridization assay designed for in situ detection of B. osfreae in thin sections resulted in specific probe binding to B. osfreae rDNA. Controls using mismatch oligonucleotides confirmed the specificity of this assay. *B.* osfreae was regularly detected at low prevalences and intensities in one (Gun Point Creek) but not three other (Spinney Creek, Linekin Bay, Blue Hill Salt Pond) wild oyster populations. Growth and mortality of hatchery-produced oysters were greater at an upriver (Little Point) site than at a downriver (Lowes Cove) site on the Damariscotta River. These differences were related to the greater variation in temperature and salinity occurring at the Little Point site. *Bonamia osfreae* was detected in only one oyster.

The results of this study have greatly increased our ability to detect *Bonamia osfreae* in flat oysters, which will lead to improved management options for growers. There are locations where *B. osfreae* is not detectable. Under the right environmental conditions, flat oysters can be grown to market size in less than three years. The molecular techniques developed will be useful for future determination of the life cycle of *B. osfreae.*

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INTRODUCTION

The European flat oyster, *Ostrea edulis* Linné, inhabits stenohaline waters along the Atlantic coast of Europe from Norway to Morocco, and on the Mediterranean coast from France to Greece (Hèral and Deslous-Paoli 1991). It is hermaphroditic (though young oysters are predominantly male) and broods its larvae (Ahmed 1975). Gametogenesis and spawning occur partly in response to rising summertime temperatures (Medcoff and Needler 1941); temperatures near the northern extreme of 0. *edulis's* range are seldom high enough, though, for the oyster to spawn regularly (Loosanoff 1962; Johannesson et a/. 1989).

Ostrea edulis has long been the target of commercial harvesters. Overharvesting in the mid-20th century, however, changed industry practices, and the culture of hatchery-produced oyster seed arose to augment production at natural oyster beds. With the culture of 0. *edulis* came an increase in the transplantation of oysters from productive areas such as Brittany, France to depleted areas such as the Netherlands and Spain. Beginning in the 1960s, 0. *edulis* seed was transplanted to locations throughout coastal Europe (Heral and Deslous-Paoli 1991).

Ostrea edulis was also introduced to the United States. The first transplantation of oysters from the Netherlands to Maine (via Milford, Connecticut) occurred in 1949, and several others occurred in the following years in an effort to offset diminished softshell clam landings (Loosanoff 1955; Welch 1963). Recruiting populations were established, primarily around the Casco Bay

and Boothbay Harbor areas, and commercial landings of 0. *edulis* in Maine reached 14,435 bushels by 1989 (Hidu and Lavoie 1991; Lipton et a/. 1992). 0. *edulis* was also transported to the West Coast, where commercial hatcheries produced seed oysters for European and American growers (Elston et al. 1986).

The wide transplantation of *Ostrea edulis* ultimately caused the decline of the fishery for this oyster in Europe, as two pathogenic protistans appeared and were rapidly distributed with oyster shipments. The first of these, *Marteilia refringens*, appeared in France in 1967 (Comps 1970); the second, *Bonamia* ostreae, thought to have originated in California (Elston et *a/.* 1986; Friedman and Perkins 1994), appeared in France in 1979 (Pichot et *a/. 1980;* Comps et *al.* 1980).

A small protistan parasite was observed in 1965 in Ostrea *edulis* from experimental populations in California that had suffered heavy (approaching 100%) mortality (Katkansky et *al.* 1969). These "microcells", 2 to 4 µm and "cytozoic within leucocytes or free in the blood", almost certainly were *Bonamia ostreae* (Elston et *al.* 1986). *B. ostreae* was presumably transported from California during the 1970's with shipments of live 0. *edulis* to France and eastern North America (Elston et *a/. 1986;* Friedman and Perkins 1994). It caused heavy flat oyster mortality in Brittany in 1979 (Pichot et *a/. 1980;* Comps et *al. 1980)* and over the next eight years spread through much of coastal Europe, including the Netherlands, Denmark, England, Spain, and Ireland (van Banning 1991; Hudson and Hill 1991; Montes et a/. 1991; McArdle et *a/. 1991).*

Elston *et al. (1986)* observed *Bonamia ostreae* in Washington state, USA, in the descendants of broodstock from the Elkhorn Slough, California hatchery that is thought to have originally supplied infected *Ostrea edulis* to France. Friedman et *al.* (1989) observed *B. osfreae* in flat oysters from two new California locations in 1986. By 1991 *B. osfreae* had reached Maine, USA, and infected the descendants of 0. *edulis* that had originated from the Netherlands in 1949 and California in the 1970's (Friedman and Perkins 1994). *B. osfreae* was observed in Damariscotta River oysters at 34 and 45% prevalence in 1991 and 1992, respectively; 20% prevalence in 1993 (Barber and Davis 1994); and 0 to 14.6% prevalence in 1994-1995 (Zabaleta and Barber 1996). *B. osfreae* was also observed in 1993 in two of four Casco Bay oyster populations at a maximum prevalence of 14.3% (Barber and Davis 1994).

Perkins (1987, 1990) placed *Bonamia osfreae* in the Phylum Haplosporidia, a group characterized by the formation of plasmodia, which multiply in connective tissue by sporulation and irregular multiple fission (Sprague 1979). The presence of haplosporosomes indicates that *B. osfreae* deserves haplosporidian status, even though *B. osfreae* spores have not been found, and direct transmission between hosts occurs in *B. osfreae* but in no other haplosporidian studied (Perkins 1987; Bucke 1988). Two other haplosporidians, *Haplosporidium nelsoni* and *Haplosporidium costale*, are parasites of the eastern oyster, *Crassostrea virginica*. A third, *Minchinia teredinis*, is a shipworm (*Teredo* spp.) parasite. The relationship of *B. osfreae* to other "microcells" (Farley *et al.*

1988), such as *Mykrocytos mackini*, the causative agent of Denman Island disease in *Crassostrea gigas* in British Columbia, Canada; *Mikrocytos roughleyi*, which causes Australian winter disease in *Saccostrea commercialis* in New South Wales, Australia; and *Bonamia* sp., a species antigenically distinct from *B. ostreae* that has been found to cause mortality in *Tiostrea lutaria* in Foveaux Straight, New Zealand (Hine 1991), remains unresolved.

The life cycle of *Bonamia ostreae* remains uncertain as well. Three cell types have been identified using electron microscopy: “dense” and “cleat” forms, distinguishable by size (the clear form is slightly larger) and organelle content (both have mitochondria and haplosporosomes, but the clear form lacks other organelles) (Pichot et *a/.* 1980); and a “plasmodial” form, larger than the others (to 6 μm diameter) and occurring only in moribund *Ostrea edulis* (Brehelin et *al.* 1982). Each of these typically occurs intracellularly within hemocytes (Balouet et *al.* 1983), but may be found extracellularly; they also occur in the branchial epithelia, where they appear to divide rapidly (Montes et a/. 1994). All forms are capable of directly infecting nearby oysters (Bucke 1988).

Bonamia ostreae apparently enters its oyster host in either the branchial epithelium or the digestive tract, where it is quickly phagocytized by a hemocyte (Bucke 1988). Inside a host cell vacuole, the parasite interferes with host cytocidal mechanisms and proliferates rapidly. Destruction of the host cell results in the release of the parasite into the hemolymph, where it is again phagocytized by a macrophage (Montes et *al.* 1994). In this manner the infection progresses, and sinuses throughout the oyster become enlarged and crowded

with infected hemocytes (Bucke 1988). Oyster hemocytes and tissues ultimately become necrotic, and the valves begin to gape; at this stage, parasites are released into the surroundings, and presumably infect other oysters.

Bonamia ostreae is detected using standard histopathological techniques. Histocytological diagnosis using stained hemolymph or heart smears is a common alternative and is considered useful for preliminary screening of oyster populations (Culloty and Mulcahy 1996; Zabaleta and Barber 1996; O'Neill et *al.* 1998). Both of these methods detect low-intensity, subclinical infections with difficulty, and can be highly subjective. *B. ostreae* may be difficult to distinguish from routine hemocytic inclusions (Bucke and Feist 1985, Bucke 1988, McArdle et *a/.* 1991). Highly specific monoclonal antibodies developed for *B. ostreae* (Boulo et *al.* 1989, Rogier et *al.* 1991, Cochennec et a/. 1992) were less subjective and more sensitive, but they reacted weakly or not at all with *B. ostreae* from outside of Europe, suggesting that serological differences in *B. ostreae* over space and time may limit the usefulness of this tool (Zabaleta and Barber 1996). The high cost of producing monoclonal antibodies, coupled with the need to revise the antibodies as epitopes evolve, makes the wide application of these impractical (Tortora et *al. 1995).*

Molecular diagnostic assays have been developed in recent years for countless human, plant, and animal pathogens, and exist for detection of several parasites of commercially important marine bivalves, such as *Haplosporidium nelsoni*, *Haplosporidium costale*, *Minchinia teredinis, Perkinsus marinus, Martelia refringens*, and *Bonamia ostreae* (Fong et *a/. 1993;* Stokes et *al.* 1995a & b; Ko

et a/. 1995; Marsh et *a/.* 1995; Carnegie et *al.* 1999; Cochennec et *a/. 2000).* Such assays commonly assume one of two forms: polymerase chain reaction (PCR) tests or *in* situ hybridization (ISH) assays. The PCR detects parasites by enzymatically amplifying parasite DNA (when present) *in vitro* from a bulk host-parasite genomic DNA mixture. The DNA amplified from even a small number of parasites is easily observed on an agarose gel. ISH assays detect parasite DNA *in* situ, on standard histopathological slides, by hybridizing to parasite DNA or RNA oligonucleotide probes which are made visible by the labels they carry. These labels may be fluorescent, such as fluorescein or Texas Red, and visualized using an epifluorescence microscope. Alternatively, detection may be by colorimetric means (e.g., Stokes et *al.* 1995a & b, Cochennec et *a/. 2000).* Both techniques are superior to histopathology and histocytology in sensitivity and specificity.

While molecular diagnostic techniques are powerful, potential problems exist with their use. A standard PCR, in particular, offers no quantitation of parasite presence, gives no indication of host health status, and is susceptible to both false positive diagnoses and false negative diagnoses. False positive diagnoses can arise from the amplifcation of a contaminant, of non-viable, degraded parasite DNA, or of related, non-pathogenic strains. False negative diagnoses can arise as a function of error in sampling host tissue or from, in the case of viruses in particular, rapid evolution of genetically distinct strains. The use of proper controls is essential in PCR diagnostics. *In situ* hybridization assays overcome some of these drawbacks because a tissue context and

morphological validation exist for any positive signal. However, the advantage of morphological validation vanishes when very small pathogens are considered, and a tissue context is less meaningful when observing a pathogen in an abnormal host (Walker and Subasinghe 2000; McGladdery 2000). For now, molecular diagnostics are best used in conjunction with established, traditional diagnostic techniques (Burreson 2000).

"Effective disease management and risk analyses rely on accurate data and information" (McGladdery et *al.* 2000). Management of *Bonamia* osfreae in Maine is currently hindered because of limitations with standard diagnostic techniques, and by uncertainty regarding its distribution and impact. The objectives of this study were:

1) Development of a PCR assay for detection of B. *ostreae*;
2) Development of a fluorescent *in situ* hybridization assay for detection of B. *ostreae*;
3) Determination of the prevalence and intensity of *B. osfreae* infections in four wild Maine oyster populations; and
4) Evaluation of growth and mortality of cultured *Ostrea edulis* in the *B.* ostreae-enzootic Damariscotta River.

CHAPTER 1

Development of a PCR assay for detection of the oyster pathogen *Bonamia ostreae* (Pichot et *al.* 1980), and support for its inclusion in the Haplosporidia

INTRODUCTION

Bonamia osfreae (Pichot et al. 1980) is a protistan pathogen of flat oysters, *Osfrea edulis* Linné, in Europe (Pichot et *al.* 1980; Balouet et *a/. 1983;* Montes 1990; van Banning 1991; McArdle et *al.* 1991; Hudson and Hill 1991), the Pacific coast of the USA (Elston et *al.* 1986; Friedman et a/. 1989), and Maine, USA (Barber and Davis 1994; Friedman and Perkins 1994). Its phylogenetic relationship to other protists is uncertain. Although the possession of haplosporosomes suggests an affinity to the Haplosporidia (Pichot *et a/. 1980;* Perkins 1990), *B. osfreae* does not form spores (Pichot *et a/.* 1980), which are considered primary diagnostic characters of this phylum (Sprague 1979).

Rapid and accurate diagnosis of *Bonamia osfreae* is essential to the management of bonamiasis in wild and cultured oyster populations. Standard diagnosis is performed with fixed and stained tissue sections (histopathology). Detection using fixed and stained heart and hemolymph smears (histocytology) is more rapid and less expensive, and considered useful for the preliminary screening of oyster populations (Zabaleta and Barber 1996; O'Neill *ef al. 1998).* A major disadvantage of standard histopathology and cytology is low sensitivity. The small size of *B. osfreae* (2 to 3 μm) makes it difficult to recognize subclinical infections in thin sections, and several investigators have reported trouble detecting *B. osfreae* at low intensity in stained blood smears (Bucke and Feist 1985; Bucke 1988; McArdle *ef* al. 1991). In addition, the resemblance of *B. osfreae* to routine intrahemocytic inclusions (Bucke and Feist 1985) makes false *positive* microscopic diagnosis a possibility. Sensitive and specific

immunohistological diagnostic methods have been developed for B. *ostreae* (Boulo et *al.* 1989; Rogier et *al.* 1991; Cochennec et *a/. 1992).* However, the antibodies developed for *B.* ostreae in Europe react weakly or not at all with *B.* osfreae from populations outside of Europe (Zabaleta and Barber 1996), suggesting that serological differences in *B.* ostreae between locations and possibly over time may limit the usefulness of this tool.

DNA probes and polymerase chain reaction (PCR) assays have been developed for numerous bivalve parasites, including *Haplosporidium nelsoni* (Fong et *a/.* 1993; Stokes and Burreson *1995;* Stokes et *a/.* 1995a), *Minchinia teredinis* (Stokes *et al.* 1995b), *Marteilia sydneyi* (Anderson *et a/.* 1995), *Mikrocytos roughleyi* (Adlard and Lester 1995), and most recently *Bonamia osfreae* (Carnegie *et al.* 1999; Cochennec *et a/.* 2000). We describe here the development of a PCR assay for detection of *B. osfreae* in *Osfrea edulis*, based on 18S ribosomal DNA (rDNA) sequences conserved between *B. osfreae* populations in Europe and the USA, and provide molecular evidence supporting the affinity of *B. osfreae* to the Haplosporidia.

MATERIALS AND METHODS

Oysters were collected from Cork Harbour, Ireland in November 1997 (n = 71), Gun Point Creek, Maine in August 1999 and October 1999 (n = 47 and 37, respectively), and Ria de Arosa, Spain in January 2000 (n = 30), where *Bonamia osfreae* is enzootic (Montes 1990; Barber and Davis 1994; Culloty and Mulcahy 1996; O'Neill *et al.* 1998). *Osfrea edulis* from Spinney Creek, Maine (n = 30) and

juvenile *Crassostrea virginica* from the Great Wicomico River, Virginia, USA (n = 19) served as negative controls, as *B.* osfreae has never been detected in these locations. *Haplosporidium nelsoni* (MSX) spores were observed in 5% of juvenile Great Wicomico River C. *virginica* at the time of sampling (Nancy Stokes, Virginia Institute of Marine Sciences, pers. comm.).

The right or upper valve of each oyster was removed, and either ~ 0.1 to 0.5 g gill tissue was excised or 200 µl hemolymph drawn (or both) for genomic DNA extraction (see below). Hemocytes were then prepared for cytological diagnosis of *Bonamia osfreae.* Ventricles from Cork Harbour oysters and gill fragments from Ria de Arosa oysters were removed, blotted on tissue paper, then dabbed on a glass microscope slide. Cells were fixed in methanol and stained with Hemacolor (Merck) rapid blood stain (Culloty and Mulcahy 1996). For Gun Point Creek and Spinney Creek oysters, fluid from the pericardial cavity was withdrawn and mixed 1:5 to 1:10 with 0.45 pm-filtered ambient seawater. A drop of this suspension was placed on a microscope slide. Cells were given 15 min to adhere, then fixed and stained using Hemostat (Fisher Scientific) solutions (Zabaleta and Barber 1996). Slides were examined microscopically for 5 min at 400 to 1000X magnification. Infections were scored as "heavy" if all observed hemocytes contained *B. ostreae;* "moderate" if *B. ostreae* was visible in all microscope fields; "light" if 11 to 100 *B.* ostreae were observed within 5 min; "scarce" if 1 to 10 *B. ostreae* were observed; and "undetected" if no unambiguous *B. ostreae* were observed (Bach&e et *a/.* 1982; Culloty and Mulcahy 1996).

Genomic DNA from Cork Harbour oysters was extracted as follows. Gill fragments (~ 0.1 g) fixed in 95% ethanol were rinsed twice in ddH_2O and minced, then incubated overnight at 55° in a digestion solution of 0.05M Tris-HCl, 0.2M NaCl, 0.05M EDTA, 1% SDS, and 0.05 mg/ml Proteinase K at pH 8.0. After a single extraction with phenol and two with chloroform:isoamyl alcohol (24:1), DNA was precipitated with absolute ethanol:3M ammonium acetate (11 :1) and quantified spectrophotometrically. For Gun Point Creek, Spinney Creek, and Great Wicomico River oysters, DNA was extracted from 200 µl of hemolymph using a QIAamp DNA Mini Kit (QIAGEN Inc.) following the manufacturer's Blood and Body Fluid Spin Protocol. For Ria de Arosa oysters, DNA was extracted from ~ 0.1 g of gill tissue using a QIAamp DNA Mini Kit following the manufacturer's Tissue Protocol. To compare PCR results based on DNA extraction from gill with those based on extraction from hemolymph, an additional extraction using gill tissue of 37 Gun Point Creek *Ostrea edulis* (October 1999) was performed using a QIAamp DNA Mini Kit following the manufacturer's Tissue Protocol.

The PCR-based part of this study comprised two phases, one of PCR protocol development and one of confirmation of the specificity and sensitivity of the final PCR protocol. The development phase proceeded in a stepwise fashion, first using the PCR to amplify candidate protistan rDNA fragments from a bulk genomic DNA mixture (Protocol A), then sequencing an amplicon that appeared to correspond with *Bonamia ostreae* infections, and finally using two increasingly more specific PCR protocols (Protocols B and C) to establish the

identity of the putative *B. ostreae* amplicon. Each PCR protocol in this development phase used template DNA from Cork Harbour oysters (n = 71), but differed from the others in PCR primer sequence and annealing temperature (Table 1 .1, Fig. 1 .1). Reaction conditions were as follows: each 50µl reaction

Table 1 .1. Primer sequences, annealing temperatures, and predicted amplicon size of PCR Protocols A, B, and C.

Protocol	Primers	T_m	Product size
A	A_F: TGTGATGCCCTTAGATGTYCT A_R: GCTGCGTCCTTCATCGWT	55°C	528 bp
B	B_F: CAGCCRTCTAACTAGCTSTCGC B_R: CGGGTCAAACTCGTTGAACG	55°C	122 bp
C	C_F: CGGGGGCATAATTCAGGAAC CR: CCATCTGCTGGAGACACAG	59°C	760 bp

T_m = annealing temperature

mixture comprised 45 µl PCR cocktail (IX PCR buffer [GibcoBRL], 1.5 mM MgC12 [Perkin-Elmer], 0.2 mM dNTP mix [GibcoBRL], 0.05 µM forward and reverse primers, and 2.5 units PLATINUM@ *Taq* DNA polymerase [GibcoBRL]), and 50 ng template DNA . Thirty-five reaction cycles (94°C for 1 min, 55 to 59°C for 1 min, 72°C for 1 min) were followed by a 10 min extension at 72°C. Amplification products were stained with ethidium bromide and visualized using agarose gel electrophoresis.

The first PCR protocol (Protocol A) was designed to amplify a mixed protistan 18S-ITS 1 rDNA fragment from bulk oyster-protist genomic DNA (Cork

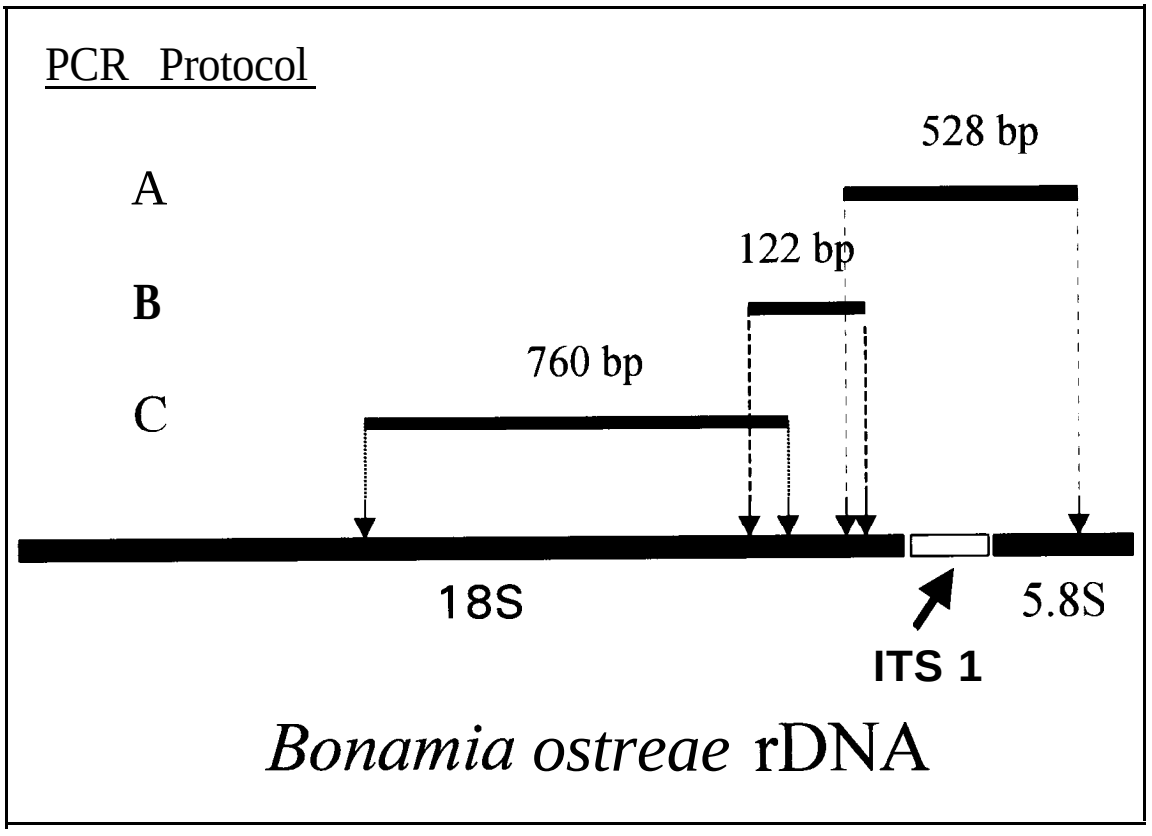

Figure 1.1. Diagrammatic representation of the PCR assay development. The PCR product of Protocol A could not be predicted a priori. This 528 bp product represents the single candidate *Bonamia ostreae* PCR amplicon resulting from this assay. The Protocol B amplicon was predicted based on alignment of the amplicon A sequence with several haplosporidian sequences (see text). The Protocol C amplicon was predicted based on the complete putative *B. osfreae* 18S-ITS 1 rDNA sequence.

Harbour) using PCR primers specific for target sites that bore substantial homology across a wide range of alveolate protists, the taxon to which the Haplosporidia (Siddall et a/. 1995; Flores et *a/.* 1996) and thus *Bonamia osfreae* (Pichot ef *al.* 1980; Perkins 1990) are thought to belong. ITS 1 length variation between *Ostrea edulis* and *B. osfreae* provided resolution of a mixed PCR product on an agarose gel. A similar approach was used in the development of a PCR assay for another microcell parasite, *Mikrocytos roughleyi* (Adlard and Lester 1995). Degenerate primers (A_F and A_R; see Table 1 .1)were designed by aligning several 18S-ITS1 rDNA sequences deposited in Genbank (*Ostrea edulis* [gb U88709], *Arion rufus* [gb X00131], *Stagnicola e/odes* [gb AF013138], *Drosophila orena* [gb Z28549], *Saccharomyces bayanus* [gb Z95945], *Amphidinium belauense* [gb L13719], *Alexandrium affine* [gb AB006995], *Alexandrium catenella* [gb AB006990], *Alexandrium insuefum* [gb AB006996], *Alexandtium minutum [g* b U27499], *Alexandrium pseudogonyaulax [g* b AB006997], *Alexandrium famarense* [gb AB006991], *Crypfhecodinium cohnii* [gb M25 116], *Gonyaulax spinifera [g* b AF05 1832], *Gymnodinium sanguineum [g* b U41085], *Perkinsus atlanticus* [gb U07697], *Perkinsus marinus* [gb X75762 and U07700], *Perkinsus olseni* [gb U07701], *Perkinsus sp.* [gb U07698], *Scrippsiella nutricula* [gb U52357], *Symbiodinium pilosum* [gb *X62650], Cryptosporidium parvum* [gb L25642 and AF040725], *Eimeria maxima* [gb AF027723], *Giardia ardeae* [gb *X58290* M38598 M73684 M73685], *Neospora caninum* [gb L49389], *Oxyfricha nova* [gb X03948 M14601], *Plasmodium falciparum* [gb U21939], *P. malariae* [gb M54890], *Prorocenfrum micans* [gb Ml 4649], *Theileria parva* [gb

L26331], Toxoplasma *gondii* [gb L37415 and L49390], *Colpidium campy/urn* [gb M35557], *Colpidium colpoda* [gb M35558], Glaucoma *chattoni* [gb M35559], *Tefrahymena corlissi* [gb U *173561, Tefrahymena leucophrys* [gb M35555], *Tefrahymena paravorax* [gb M35556], *Tefrahymena patula* [gb M35553], *Tefrahymena pyriformis* [gb X01 *533* M10752], *Tefrahymena vorax* [gb M35554], and *Haplosporidium nelsoni* [gb U19538]) using GDE (Wisconsin Package Version 9.0, Genetics Computer Group [GCG], Madison, Wisconsin) and were commercially synthesized (Operon Technologies, Inc., Alameda, CA). Although the length of a B. *ostreae* sequence generated by this protocol could not be predicted, an 0. *edulis* amplicon of -1000 bp in size was predicted. PCR products longer or shorter than this could thus be considered candidate *B. osfreae* sequences. A single 528 bp fragment amplified from 14 of 71 Cork Harbour oysters was cloned using a TOPO TA Cloning Kit (Invitrogen) and sequenced at the University of Maine DNA Sequencing Facility. This amplicon occurred coincident with heavier *B. osfreae* infections and a Genbank Blast search indicated a resemblance of this product to haplosporidian 18S rDNA, making it a strong candidate for *B. osfreae* rDNA.

The second PCR protocol (Protocol B) was specific for the putative *B. osfreae* fragment previously amplified. Primers (B_F and B_R; Table 1.1) were designed by aligning this sequence with oyster *(Osfrea edulis* [gb U88709]) and haplosporidian (*Haplosporidium nelsoni* [gb U19538], *Haplosporidium louisiana* [gb U47851], *Minchinia teredinis* [gb U20319], and *Urosporidium crescens* [gb U47852]) sequences identified by Genbank Blast search to be most similar to it.

The reverse primer targeted a sequence that diverged from known haplosporidian sequences, and the forward primer was designed to bind to a consensus haplosporidian sequence. A positive signal for putative *B.* ostreae rDNA was indicated by the presence of a 122 bp amplicon.

PCR Protocol C was also specific for putative *Bonamia* ostreae rDNA. Protocol C was designed following amplification (conditions as above; forward primer sequence WAYCTGGTTGATCCTGCCAGT [Medlin et *al.* 1988], reverse primer A_R, T_m = 55°C) and sequencing of the entire putative *B. ostreae* 18S/ITS 1 rDNA sequence (submitted to Genbank under accession # AF262995) from one Gun Point Creek and three Cork Harbour oysters. (These four sequences diverged by just 0.39% over 1945 bp of 18S and ITS 1.) Both forward and reverse (C_F and C_R; Table 1 .1) primer sequences were exact matches for the putative *B. ostreae* target sequence. A 760 bp product was predicted. A final evaluation of Cork Harbour oysters with Protocol C was performed.

To confirm the sensitivity of Protocol C, oysters from Gun Point Creek (low *Bonamia ostreae* prevalence; light infection intensity) and Ria de Arosa (higher *B. ostreae* prevalence; full range of infection intensities) were screened. To assess the specificity of Protocol C, control oysters (*Ostrea edulis* and *Crassostrea virginica*, the latter not a host for *B. ostreae)* from areas in which *B. ostreae* is not enzootic (Spinney Creek and Great Wicomico River, respectively) were screened.

Seventeen alveolate 18S rDNA sequences (*Alexandrium belauense* [gb L13719], *Ctypfhecodinium cohnii* [gb M64245 M34847], *Perkinsus marinus* [gb

X757621, Prorocentrum micans [g b M 146491, *Symbiodinium corculorum* [gb L13717], *Cryptosporidium serpentis* [gb AF1513761, *Theileria annulata* [gb M64243 M34845], *Toxoplasma gondii* [gb X75429], *Oxytricha nova* [gb X03948 M14601], *Paramecium tetraurelia* [gb X03772], *Tetrahymena pyriformis* [gb M98021], *Stylonichia pustulata* [gb M *146001, Haplosporidium costale* [gb U20858], *H. louisiana* [gb U47851], *H. nelsoni* [gb X741 31], *Minchinia teredinis* [gb U20319], and *Urospondium crescens* [gb U47852]) were aligned with the putative *Bonamia ostreae* 18S gene using CLUSTAL W (Thompson *et al. 1994)* and refined by eye, using Genedoc (Nicholas and Nicholas 1997) and taking secondary structure into account, for phylogenetic analysis. Length variable sequences of uncertain positional homology, corresponding with *Tetrahymena pyriformis* (gb M98021) bases 65-70, 121-130, 169-239, 261-273, 469-490, 626-716, 750-760, 792-798, 815-818, 1012-1023, 1306-1329, 1344-1357, 1429-1485, and 1625-1654, were excluded from the analysis. Unweighted maximum parsimony analysis was performed on 1121 aligned positions using PAUP (Swofford 1999). Taxa were added to the resulting trees by stepwise addition, with the tree-bisection-reconnection branch swapping algorithm in use. Bootstrap proportions (100 replicates) for resulting trees were calculated using PAUP's heuristic bootstrap search function.

RESULTS

PCR Protocols A, B, and C generated amplicons of the predicted sizes, presumably *Bonamia ostreae* rDNA, in 19.7%, 22.5%, and 63.4%, respectively,

of Cork Harbour oysters. Diagnosis using ventricular hemolymph smears revealed a putative *B. osfreae* prevalence of 53.5%.

Table 1.2 illustrates the frequency with which each protocol generated a

Table 1.2. Percentage of detection of a putative *Bonamia ostreae* PCR amplicon in Cork Harbour, Ria de Arosa, and Gun Point Creek (GPC) oysters (August and October 1999 samples pooled) grouped by cytological infection intensity score. Example: 72.7% under Cork Harbour, Protocol B indicates that this protocol detected *B. ostreae* in 8 of 11 or 72.7% of oysters diagnosed using cytology as harboring "moderate" *B. osfreae* infections.

		Cork Harbour			Ria de Arosa		GPC		Overall	
Intensity	n	Prot. A	Prot. B	Prof. C	n	Prot. C	n	Prot. C	n	Prot. C
Heavy	5	100	100	100	3	100	0	N/A	8	100
Moderate	11	63.6	72.7	100	4	100	1	100	16	100
Light	8	12.5	25.0	87.5	5	100	2	50.0	15	86.7
Scarce	14	0.0	0.0	50.0	8	87.5	8	75.0	30	66.7
Undetected	33	3.0	3.0	45.5	10	50.0	73	32.9	116	37.9

putative *Bonamia ostreae* amplicon in Cork Harbour oysters pooled by cytological infection intensity score. Among oysters of a given infection intensity, Protocol C was more frequently positive for putative *B. ostreae* (and thus more sensitive) than Protocol B, and similarly, Protocol B was more sensitive than Protocol A. Oysters diagnosed as heavily infected were always associated with a positive PCR signal for putative *B. osfreae* regardless of protocol used. In oysters with lighter infections, however, a positive PCR signal was less frequently present. Cork Harbour oysters in which *B. ostreae* was undetected microscopically were rarely positive (3.0%) for putative *B. osfreae* using PCR Protocols A and B, but often positive (45.5%) using Protocol C. The Protocol C results using Ria de Arosa oysters mirrored those from Cork Harbour. The putative *B. ostreae* amplicon was detected in every "heavily"-, "moderately"-, or

"lightly"-infected oyster, most "scarcely"-infected oysters, and half of those (50.0 %) in which *B. ostreae* was undetected cytologically. The Protocol C results using Gun Point Creek oysters were less illustrative, as these oysters displayed a narrow range of infection intensities. However, as in the trials using oysters from Cork Harbour and Ria de Arosa, a putative *B. ostreae* amplicon was observed in most (75.0%) "scarcely"-infected oysters, and many (32.9%) of those considered "uninfected". In total, 57 of the 69 oysters (82.6%) determined to be infected using cytology were confirmed to contain putative *B. ostreae* rDNA using Protocol C. In addition, 44 of 116 oysters (37.9%) thought to be uninfected as determined using cytology actually harbored putative *B. ostreae* rDNA.

Overall prevalence of *Bonamia ostreae* at locations examined in this study using PCR Protocol C was greater than that determined using cytology (Fig. 1.2). In addition, *B. ostreae* has never been detected microscopically in control Spinney Creek *Ostrea edulis*, and it was undetected in these and Great Wicomico River *Crassostrea virginica* using Protocol C.

Prevalence of putative *Bonamia ostreae* in thirty-seven Gun Point Creek oysters was the same using either gill DNA or hemolymph DNA as a PCR template. However, in three oysters the gill sample was positive for putative *B. ostreae* and the hemolymph sample was not, and in another three the hemolymph sample was positive and the gill was not.

Phylogenetic analysis using 366 parsimony-informative positions placed the putative *Bonamia ostreae* sequence within the Haplosporidia (Fig. 1.3) on a single most parsimonious tree (length = 1851). Bootstrap support was significant (100%) for the monophyly of the Haplosporidia and also (90%) for the placement of putative *B. ostreae* in an internal clade with *Haplosporidium costale*,

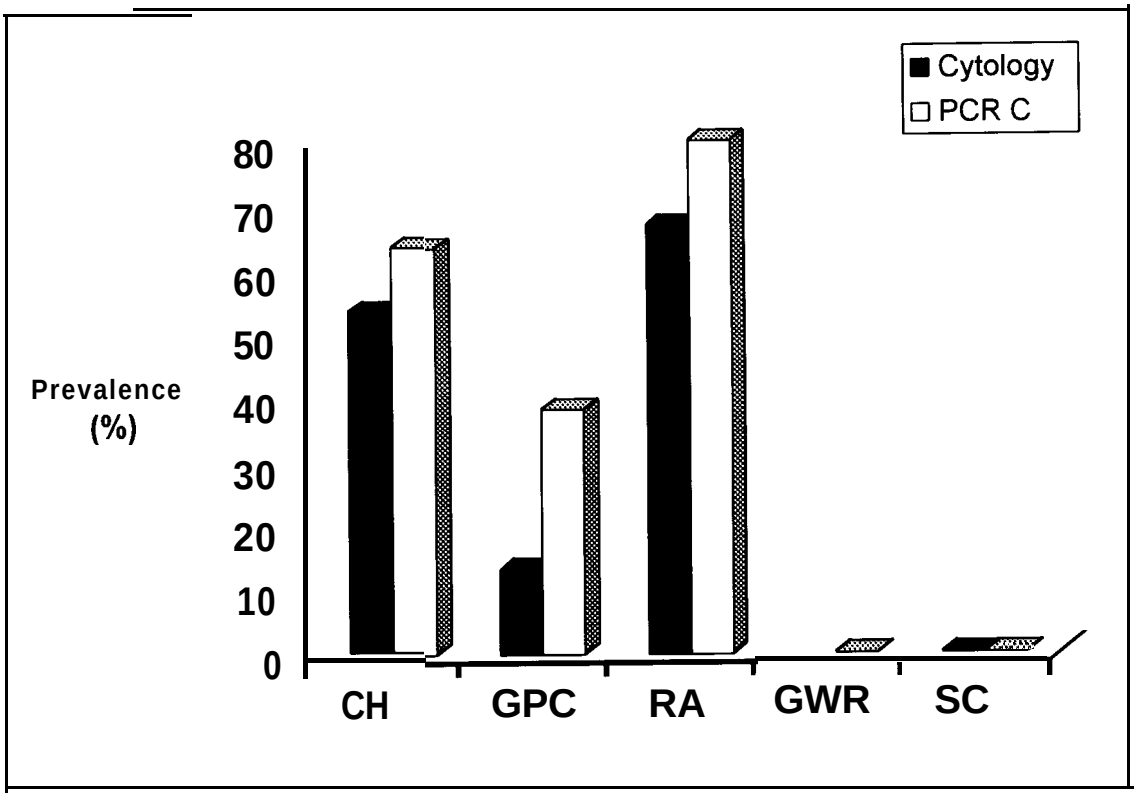

Figure 1.2. *Bonamia ostreae* prevalence in five *Ostrea edulis* samples and one *Crassostrea virginica* sample (GWR) determined using standard cytology and PCR Protocol C. Experimental (known *B. ostreae*-enzootic) groups: CH = Cork Harbour; GPC = Gun Point Creek; RA = Ria de Arosa. Control (presumed B. osfreae-free) groups: GWR = Great Wicomico River; SC = Spinney Creek.

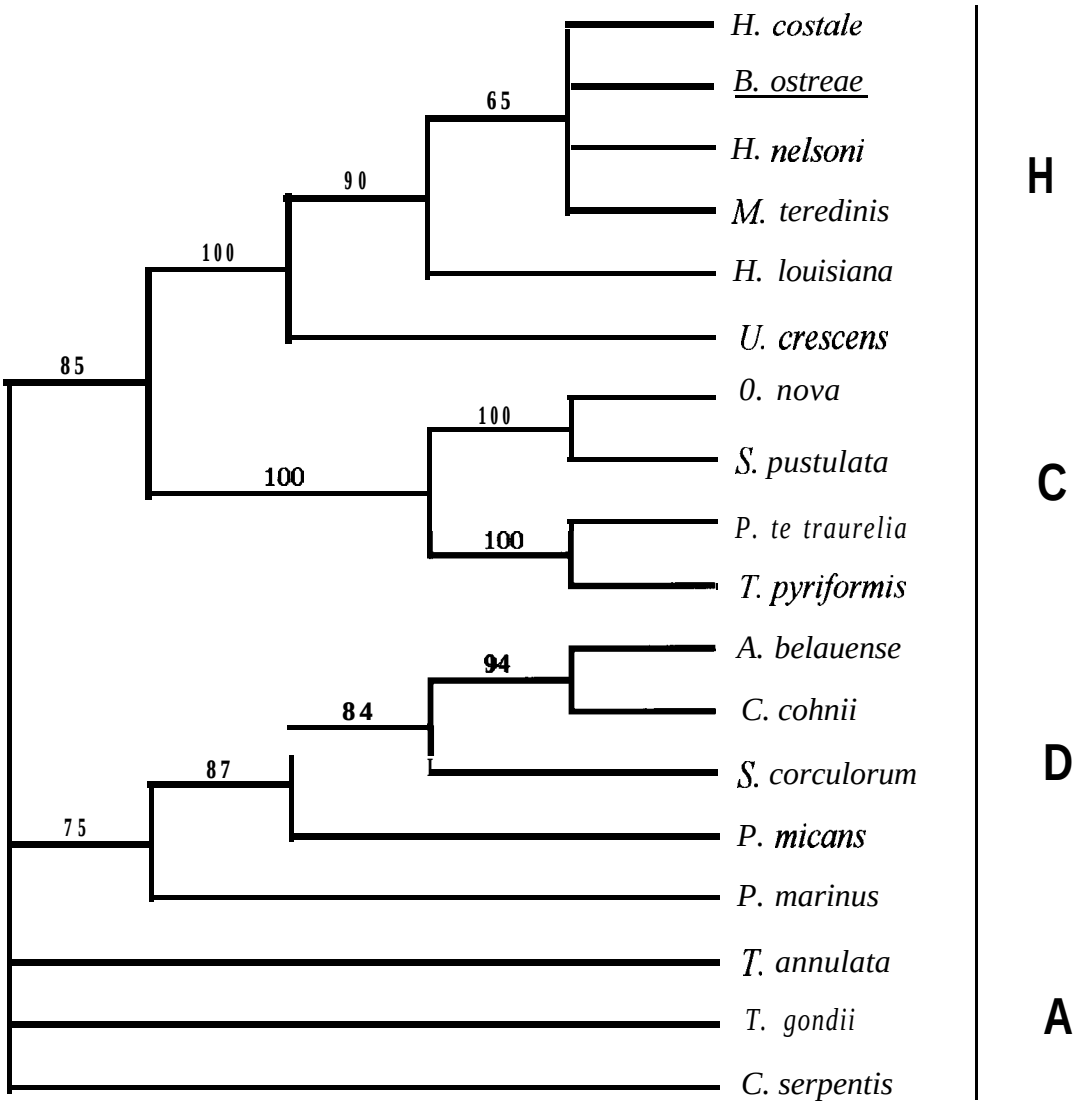

Figure 1.3. A single most parsimonious tree, rooted with representatives of the Apicomplexa, resulting from unweighted maximum parsimony analysis. Numbers represent percentages of 100 bootstrap replicates. H = Haplosporidia. C = Ciliophora. D = Dinoflagellata. A = Apicomplexa.

Haplosporidium nelsoni, *Minchinia teredinis,* and *Haplosporidium louisiana.* This analysis did not resolve the branching order among these organisms.

DISCUSSION

The strong correspondence between the presence of putative *Bonamia ostreae* PCR amplicons and *B. ostreae* infections (determined cytologically) indicates that these PCR products indeed represent *B. ostreae* rDNA. "Lightly"-, "moderately"-, or "heavily"-infected oysters comprised just 33.8% of the Cork Harbour sample, yet were associated with 92.9% of the positive PCR Protocol A signals, and 93.8% of those in Protocol B. All cytologically determined "heavy" and "moderate" *B. ostreae* infections were detected using Protocol C, and "light" infections were detected 86.7% of the time. A positive PCR signal for *B. ostreae* was never observed in control oysters from Spinney Creek and Great Wicomico River.

Although not quantitatively determined, the sensitivity of the PCR protocol increased from Protocol A through Protocol C, as the match of primer sequence to target *Bonamia ostreae* rDNA improved. Protocol A was expected to be less specific since primers A_F and A_R were designed to amplify a broad range of protistan rDNA from an oyster-protist mixture. Subsequent sequence analysis confirmed a primer-target mismatch to *B. ostreae* rDNA of 19.0 to 23.8% in the forward primer and 0.0 to 5.6% in the reverse. Accordingly, only relatively heavy *B. ostreae* infections were detected with this protocol. Protocol B was more sensitive, with a 13.7 to 22.7% forward primer-target mismatch and a perfect match of reverse primer-target. All the infections detected with Protocol A plus two additional infections were detected with Protocol B. Protocol C had a perfect primer-target match and was most sensitive, detecting all the infections detected with Protocols A and B, plus several more. A greater prevalence of *B. ostreae*

was determined in all samples using PCR Protocol C as compared to cytology because of the superior sensitivity of the PCR in detecting very light infections.

Histocytologically determined infection intensity is expected to correlate with the amount of *Bonamia* ostreae DNA in the PCR reaction mixture, *i.e.,* DNA extracted from heavily infected oysters should contain comparatively larger amounts of parasite DNA. Furthermore, as hemocytes containing *B.* ostreae in heavily infected oysters are numerous and diffuse (Balouet et *al.* 1983; Bucke and Feist 1985), any given tissue sample from such oysters is likely to contain *B.* ostreae cells. Both cytological techniques and PCR protocols of even low sensitivity (e.g., Protocol A) should detect *B.* ostreae in such samples. Lightly infected oysters, by comparison, would have relatively little parasite DNA, and because such infections are characterized by isolated, focal infiltrations, tissue samples from such oysters might not contain any parasite cells (and thus parasite DNA) at all. False negative results, attributable to sampling error, would thus be possible regardless of technique. Cytological techniques are additionally susceptible to false negative diagnoses because when present at a very low intensity, *B.* ostreae may be mistaken for "other intracytoplasmic inclusions or spermatozoa" (Bucke and Feist 1985).

The likelihood of obtaining false negative PCR results for any single oyster may be reduced through parallel screening of multiple tissue samples. Alternatively, a post-PCR aliquot of reaction solution may be used as the template for a second PCR. When *Bonamia ostreae* rDNA amplifies weakly, this may increase the amount of PCR product to a detectable level. However, using these approaches often would erode the cost advantage PCR holds over histopathology, and make it substantially more expensive than cytology. Going to such lengths to prevent false negative diagnoses is not always necessary The challenge for managers of bonamiasis is to determine when it is important,

that is, to define an acceptable level of false negative diagnostic results for a given set of circumstances (e.g., for transfer of hatchery-produced seed to a B. ostreae-free nursery area).

The absence of amplification in Spinney Creek (where *Bonamia ostreae* has never been detected) and Great Wicomico River oysters (a species not susceptible to *B. ostreae)* suggests a low rate of false positive results (for *B. ostreae* and *Haplosporidium nelsoni*) using Protocol C. Thus, the high proportion (37.9%) positive PCR results with oysters determined to be uninfected using cytology was most likely the result of the inherent difficulty of making cytological diagnoses at very low infection intensities.

Several authors (e.g., Pichot et *a/.* 1980; Perkins 1990) have proposed that the presence of haplosporosomes indicates a phylogenetic affinity of *Bonamia ostreae* to the Haplosporidia. Analysis of DNA sequence data indicates that *B. ostreae* is indeed a haplosporidian. In addition, the finding that the Haplosporidia forms a monophyletic sister clade to the Ciliophora supports the conclusions of Flores et a/. (1996), who described the relationship of the Haplosporidia to other alveolate taxa. The branching order of *B. ostreae* with respect to other microcell protistans, Bonamia *spp., Mikrocytos mackini* and *Mikrocytos roughleyi* (Farley et *a/.* 1988) awaits resolution. The similarity between Cork Harbour and Gun Point Creek *B. ostreae* 18S rDNA sequences indicates a single B. *ostreae* species at these locations.

The sensitive PCR assay described here, specific for *Bonamia ostreae* in Europe and North America, will be important in the management of bonamiasis for aquaculture wherever B. *ostreae* occurs. Large numbers of oysters may be rapidly and non-destructively screened for this parasite. (Forty adult oysters may be processed in as little as eight hours [including DNA extraction and PCR], a rate commensurate with cytology but faster than histopathology, which can take

days.) This will be particularly useful when the absence of *B.* ostreae from a prospective culture location needs to be confirmed, or seed and broodstock need to be certified *B.* ostreae-free. An *in* situ analog of this protocol will be very useful in illuminating the life history of *B. ostreae.*

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CHAPTER 2

A fluorescent *in situ* hybridization assay for detection of the flat oyster (*Ostrea edulis*) parasite *Bonamia ostreae*

INTRODUCTION

Protistan parasites of marine bivalve molluscs have traditionally been detected histopathologically, using microscopic examination of fixed and stained tissue sections. This approach is effective for determining the general health of shellfish and for screening non-specifically for parasites. Histopathology is expensive and time consuming, however, and often ineffective at detecting very light infections, particularly of smaller protists. Furthermore, cryptic life stages of poorly understood parasites may also escape detection. Histocytological diagnoses (using stained hemolymph smears) can be faster and less expensive, but no more sensitive than histopathology. Histopathology and histocytology are particularly inadequate for managing bonamiasis, a hemocytic parasitosis caused by *Bonamia* ostreae (Pichot et *a/.* 1980), the haplosporidian parasite of flat oysters *Ostrea edulis* Linné. *B. ostreae* cells are small enough (2 to 4 µm) to be overlooked in subclinical infections, or mistaken for routine intracytoplasmic inclusions in stained blood smears (Bucke and Feist 1985; Bucke 1988; McArdle et a/. 1991), which are considered useful for preliminary screening of oyster populations (Zabaleta and Barber 1996; Culloty and Mulcahy 1996; O'Neill et al. 1998).

Immunocytological diagnostic techniques developed for *B. ostreae* were effective when applied to oyster populations in France (Boulo et *al.* 1989), but performed poorly in the USA (Zabaleta and Barber 1996), suggesting that serological differences between *B.* ostreae at different locations limits the usefulness of such assays. Nucleic acid-based diagnostic techniques are more

promising. Polymerase chain reaction (PCR) assays provide superior specificity and sensitivity compared to traditional techniques, and have been developed for numerous bivalve parasites, including *Haplosporidium nelsoni* (Stokes et a/. 1995a; Burreson *et a/.* 2000), *Minchinia teredinis* (Stokes et *al.* 1995b), *Marteilia sydneyi* (Anderson et *a/.* 1995), *Mikrocytos roughleyi* (Adlard and Lester 1995), *Marteilia refringens* (Le Roux et *a/.* 1999), and *Bonamia ostreae* (Carnegie et *a/.* 1999; Cochennec et *al.* 2000). However, even under optimum circumstances PCR assays may generate false negative or false positive results. For example, a small tissue sample taken from a lightly-infected host simply might not contain parasite cells, which would cause the PCR to report a false negative. Alternatively, DNA from a parasite cell passing over the gill surface of a non-infected oyster might generate a false positive PCR signal. The PCR may also detect degraded, non-viable parasite DNA or related, non-pathogenic strains. For these reasons, the PCR is best used in conjunction with standard histopathological techniques that can verify any positive signal (Burreson 2000).

In situ hybridization assays capture the sensitivity and specificity of PCR assays and are less vulnerable to false positive signals because hybridization to standard histopathological sections provides a context for any positive signal. That is, the parasite may be visualized within the host tissue. (This is particularly useful for epizootiological studies and the detection and identification of potential cryptic forms). Typically, such assays rely on indirect light microscopic visualization of DNA probes labeled with digoxigenin. After a post-hybridization incubation with anti-digoxigenin-alkaline phosphatase and addition of a suitable

substrate, hybridization of the probe is indicated by deposition of a chromogenic enzyme reaction product. Such is the basis of assays for *Haplosporidium nelsoni* (Fong et *a/.* 1993; Stokes and Burreson 1995), *Minchinia teredinis* (Stokes *et al.* 1995b), *Marteilia refringens* (Le Roux et *a/.* 1999), and *Bonamia osfreae* (Cochennec et *al. 2000).*

Such calorimetry-based detection systems produce very strong signal and are powerful for detecting target DNA or RNA present at low copy numbers, such as some mRNA. The chromogenic signal produced by such assays, however, is somewhat diffuse (Schwartzacher and Heslop-Harrison 2000). This may diminishish resolution, particularly at the high magnifications at which *Bonamia ostreae* must be observed. Fluorescently-labeled DNA probes present an alternative. Fluorescent signal may be very precisely localized within a cell (Schwartzacher and Heslop-Harrison 2000), and while weaker than chromogenic signal, it may be amplified using multiple oligonucleotides in a single cocktail (Trembleau and Bloom 1995). The development of an *in situ* assay for *B. ostreae* using fluorescein-labeled DNA probes was the objective of this project.

MATERIALS AND METHODS

Sample collection and histological processing

Ostrea edulis (n = 70) were collected from a *Bonamia* ostreae-enzootic location (Gun Point Creek, Maine) on 4 June 2000. Hemolymph was non-destructively drawn from the adductor muscle of each oyster using a hypodermic needle, diluted 1:5 to 1 :10 in artificial seawater (30‰), and placed on a glass

microscope slide. Cells were given 10 min to adhere, then fixed and stained using Hemostat (Fisher Scientific) solutions (Zabaleta and Barber 1996). Slides were immediately screened for the presence of *B. ostreae* using **a** compound microscope at 400X until five moderately- to heavily-infected oysters were identified. These individuals were then sacrificed, fixed in Davidson's Solution (Shaw and Battle 1957) for 24 h, dehydrated, cleared, and embedded in paraffin. Serial sections (5 µm) were placed on slides treated with 3-aminopropyltriethoxysilane and dried at 50 to 55°C. The first slide in each series was stained with hematoxylin and eosin and coverslipped for standard histopathological evaluation. The rest were used for *in situ* hybridization.

Thin (5 µm) sections of paraffin-embedded, *Haplosporidium nelsoni*-infected *Crassostrea virginica* were provided by Nancy Stokes (Virginia Institute of Marine Sciences) for use as negative controls.

Preparation of probes

DNA oligonucleotides used in this study are listed in Table 2.1. Each was synthesized with a 5' fluorescein label (absorbance λ = 490 to 496 nm; emission λ = 514 to 521 nm) by Operon Technologies, Inc. (Alameda, CA), and then purified using thin-layer chromatography (plate: 20 cm x 20 cm x 250 µm Whatman [Kent, United Kingdom] PE SIL G/UV, polyester-backed and silica-coated; running buffer: 55 ml n-propanol, 35 ml ammonium hydroxide, IO ml H_2O). UME-OE-385 was specific for an *Ostrea edulis* ribosomal RNA sequence

Table 2.1. Fluorescein-labeled oligonucleotides used in this study: their sequences, melting temperatures (T_m), and positions relative to reference sequences. Dashes indicate identity to sequence above.

Oligonucleotide	Sequence	T_m [a]	Positions
UME-OE-385	5' TCATGCTCCCTCTCCGG 3'	56°C	385-401 [b]
UME-90-I	5' CGAGGCAGGGTTTGT 3'	48°C	169-183 [c]
UME-90-I M	5' ----c----c----- 3'	48°C	169-183 [c]
UME-BO-2	5' GGGTCAAACTCGTTGAAC 3'	54°C	1491-1508 [c]
UME-BO-2M	5' --c--------c------ 3'	54°C	1491-1508 [c]
UME-BO-3	5' CGCTCTTATCCACCTAAT 3'	52°C	1682-1699 [c]
UME-BO-3M	5' -C---------T------ 3'	52°C	1682-1699 [c]

[a] Theoretical melting temperature based on $T_m = 2(A + T) + 4(G + C)$
[b] Refers to numbering used in Genbank submission U88709 (*Ostrea edulis*)
[c] Refers to numbering used in Genbank submission AF262995 ***(Bonamia ostreae)***

similar (88.2% identity) to a corresponding *Bonamia ostreae* sequence, and served as a positive control. UME-BO-1, UME-BO-2, and UME-BO-3 were designed for B. *ostreae*-specificity after alignment with *Ostrea edulis* (Genbank accession number *[g* b] U88709), *Haplosporidium nelsoni (g* b U 19538), *Haplosporidium louisiana* (gb U47851), *Minchinia teredinis* (gb U20319), and *Urosporidium crescens* (gb U47852) using the program Se-AI (Oxford University Evolutionary Biology Group, UK). UME-90-I M, UME-BO-2M, and UME-BO-3M were mismatch probes that differed from the corresponding *B.* ostreae-specific probes in each case by two substitutions.

Fluorescent in *situ* hybridization

Four consecutive sections from **a** single flat oyster (designated OE) and two control *Crassostrea virginica* sections (CV) were deparaffinized 3 times in xylene for 10 min, and passed through a descending ethanol series (lOO%, 95%, 80%, 70% for 10 min each) into PBS (once for 10 min, once for 5 min). After a digestion in Proteinase K (100 µg/ml in PBS for 15 min at 37 ºC, followed by phosphate-buffered saline [PBS] plus 0.2% glycine for 5 min) and treatment with acetic anhydride (5% [v/v] in 0.1M triethanolamine-HCl [pH 8.0] for 10 min at room temperature, followed by PBS for 10 min [Schwarzacher and Heslop-Harrison 2000]), the slides were equilibrated in 5X SET for 10 min and incubated with prehybridization buffer (5X SET, 0.2% bovine serum albumin, 0.025% SDS) for 30 min at 45ºC. The prehybridization buffer was then drained off and replaced with 10 to 12 µl of prehybridization buffer containing 2 to 10 ng/µl of the appropriate oligonucleotide(s) (= hybridization solution). Slide OE-1 received no probe; slide OE-2, UME-OE-385; slide OE-3, UME-BO-1, 2, and 3; slide OE-4, UME-BO-IM, 2M, and 3M; and slides CV-1 and CV-2, UME-BO-IM, 2M, and 3M. The sections were coverslipped with parafilm and then placed in humid chambers and incubated overnight at 45ºC. After washing the next day three times for a total of 5 min in 0.2X SET at 42ºC, the sections were allowed to air dry and then mounted using Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA) and glass coverslips. Slides were examined at 600-1000x using a Nikon Labophot-2 epifluorescent microscope with a dual fluorescein-Texas Red filter.

A fifth section from oyster OE and a third from oyster CV were stained with hematoxylin and eosin.

RESULTS

The oligonucleotides comprising the *Bonamia* ostreae-specific probe cocktail hybridized to *B. ostreae* rRNA in thin sections (Fig. 2.1A). Staining was limited to a single population of very numerous 2 to 4 µm spherical structures that usually were located within hemocytes, but sometimes were free in connective or epithelial tissue or the hemolymph. Each of these structures contained a large, eccentric area that was not stained. Examination of sections stained with hematoxylin and eosin (Fig. 2.1 D) confirmed that the fluorescent staining was of *B. ostreae* cells, and the dark, unstained areas were *B.* ostreae nuclei.

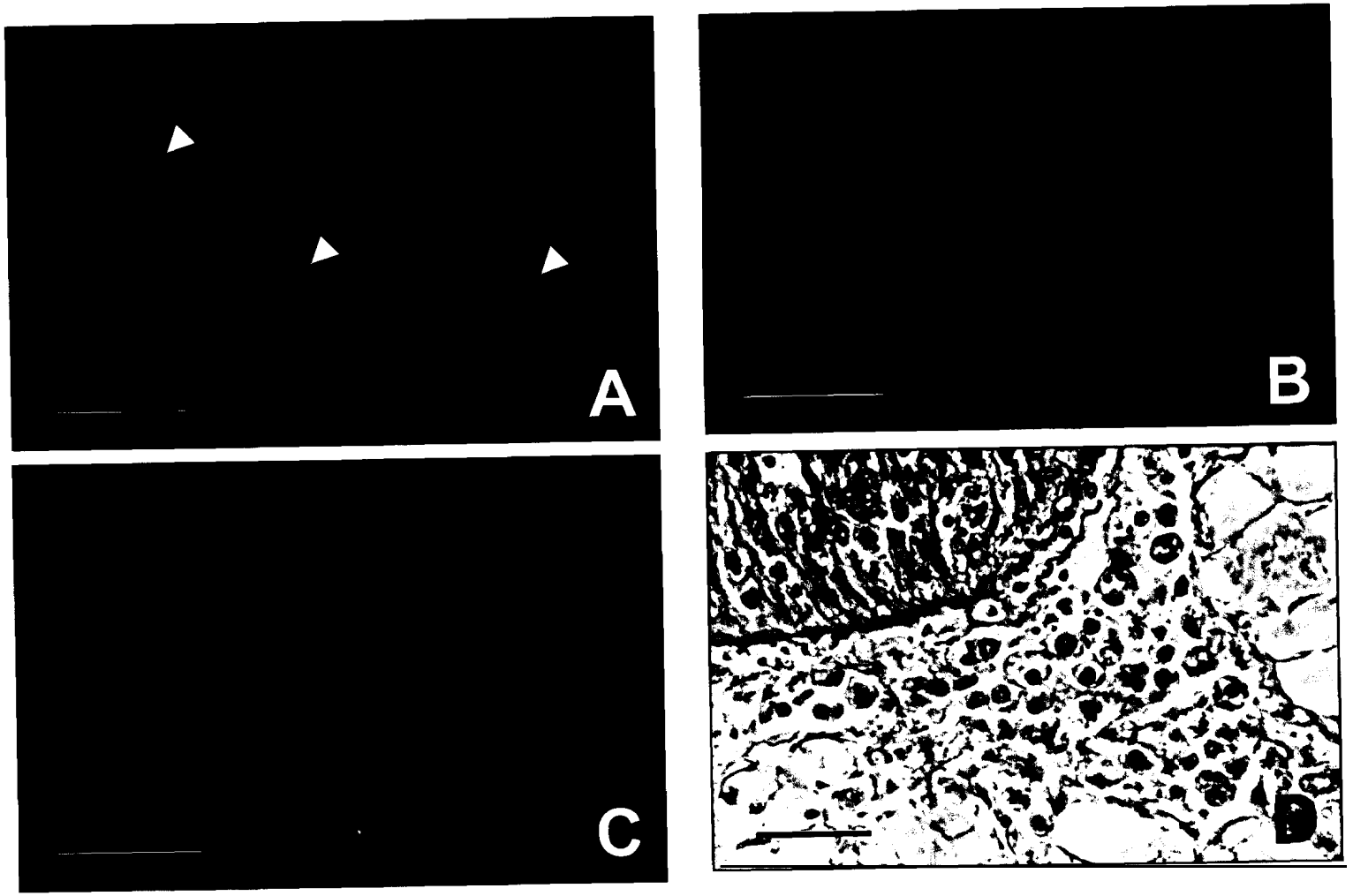
While hybridization of the *Bonamia ostreae* probe cocktail to *B. ostreae* cells was strong, the mismatch probe cocktail did not hybridize to *B. ostreae* ceils (Fig. 2.1B). This slide was indistinguishable from the no-probe control (Fig. 2.1C), indicating that binding of the *B. ostreae* probes in Fig. 2.1A was sequence-specific.

The *Bonamia* ostreae-specific probe cocktail did not hybridize to sections of *Crassostrea virginica* infected with *Haplosporidium nelsoni*.

DISCUSSION

The strong signal produced by hybridization with the *Bonamia ostreae*-specific oligonucleotide cocktail combined with the complete absence of

Figure 2.1. Fluorescent *in situ* hybridization of *Bonamia* ostreae-specific and control oligonucleotide cocktails to serial *Ostrea edulis* sections. Note intestinal wall at top of each photomicrograph. Arrowheads indicate *B. ostreae* cells. A. Slide OE-3: hybridization of the B. *ostreae* cocktail to an 0. *edulis* section. B. Slide OE-4: hybridization of the mismatch probe cocktail. C. Slide OE-3: no probe control. D. photomicrograph of section stained with hematoxylin and eosin. Scale bars = 50 μm (A-C) or 25 μm (D).


A
B
C
D

hybridization with the mismatch oligonucleotide cocktail demonstrated the sequence specificity of the *B. ostreae*-specific oligonucleotides. This specificity was reinforced by the absence of hybridization of *B.* ostreae specific probes to *Haplosporidium nelsoni*. Furthermore, the observed staining pattern closely matched expectations of a bright, green halo of 2 to 4 µm outside diameter (caused by oligonucleotides hybridizing to the relatively abundant cytoplasmic *B. osfreae* rRNA) surrounding a darker central area (the nucleus, excluding ri bosomes).

The sensitivity of this *in situ* hybridization was not quantified; however, individual *Bonamia ostreae* cells were easily detectable above background. What is not clear is whether or not these *B. ostreae* cells represented a single population of cell types (e.g., all "dense" or all "clear" forms [Pichot et al. 1980]) or a mixed population of two cell types that stained equally intensely. No multinucleate "plasmodial" forms (Brehelin et *a/. 1982)* were observed. Stokes et *a/.* (1995b) found that strongest hybridization of *Minchinia teredinis-specific* probes was to the least mature spores, which they attributed to differences in spore wall penetrability. While spore penetrability may not be applicable to 5. *ostreae,* which appears to have no spores, differential detectability with oligonucleotide probes is. If the number of ribosomes and thus rRNA present in a cell is directly proportional to the cell's activity, then inactive or resting stages of a cell should contain less rRNA relative to actively proliferating cells. As rRNA is the primary probe target, inactive or resting stages may be unnoticed using oligonucleotide probes because target rRNA is present a low quantity in these

cells. The problem of low target rRNA abundance may have some bearing on the identification of any cryptic life stages of *B. ostreae* or the detection of *B. ostreae* at low intensity in prepatent infections using an *in situ* hybridization assay.

The use of "multi-oligoprobes", a cocktail of specific oligonucleotide probes, amplifies fluorescent signal in roughly additive fashion, depending on the number of oligonucleotides in the cocktail. It is thus an effective way to increase sensitivity without sacrificing sensitivity (Trembleau and Bloom 1995). The use of multiple digoxigenin-labeled oligonucleotides with colorimetric visualization should give the same result. Alternatively, an increase in sensitivity can be effected by incorporating multiple digoxigenin-labeled oligonucleotides into a single PCR product, then using this product as a probe, with subsequent colorimetric visualization. However, this will reduce specificity. The sequence of a PCR product used as a probe may bear strong sequence identity to a corresponding sequence in related species. Indeed, this was the observation of Cochennec *et a/.* (2000), who found that their 300 bp probe for *Bonamia ostreae* cross-hybridized strongly with *Haplosporidium nelsoni*. Multiple oligonucleotides can provide higher specificity than a single polynucleotide, and thus are more useful in a diagnostic assay.

Fluorescent microscopic detection of parasites is faster and less expensive than colorimetric detection, as no post-hybridization antibody-enzyme and substrate treatments are needed. It also permits the use of multiple stains, so different parasites may be detected simultaneously on a single slide.

However, the epifluorescent microscopes required for fluorescent detection are substantially more expensive than standard light microscopes. For this reason, detection of *Bonamia ostreae* using an assay analogous to that described here, with colorimetric detection of hybridization, may be practical for detection of *B. ostreae* in laboratories not equipped with fluorescence microscopy. This assay, used in conjunction with the PCR (Carnegie et *al.* 1999; Cochennec et *al. 2000)* and traditional histopathological techniques, will do much to illuminate the epizootiology of this organism.

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Dawna Beane prepared the slides for histopathology and *in situ* hybridization. The work reported in this manuscript was supported in part by the Maine/New Hampshire Sea Grant Program and by the Northeastern Regional Aquaculture Center at the University of Massachusetts Dartmouth, through Grant No. 96-38500-3032 from the Cooperative State Research, Education, and Extension Service (CSREES) of the United States Department of Agriculture (USDA).

CHAPTER 3

Prevalence and intensity of *Bonamia ostreae* infections in flat oysters from Maine, USA

INTRODUCTION

The haplosporidian protist *Bonamia* ostreae (Pichot et a/. 1980) is the causative agent of bonamiasis, a haemocytic parasitosis of the flat oyster, Ostrea *edulis* (Balouet et *al.* 1983; Bucke 1988; Perkins 1990; Carnegie et *a/. 2000).* It is transmitted directly among neighboring oysters (Elston et a/. 1987; Bucke 1988) and so is a major threat to 0. *edulis* in commercial beds and aquaculture settings.

The origin of *Bonamia ostreae* has been traced to California, where Katkansky et *a/.* (1969) first observed a protistan "microcell" parasite that was likely B. *ostreae* (Elston et *al.* 1986; Elston and Holsinger 1988). It is believed to have been transported with *Ostrea edulis* seed from California to Europe in the late 1970's and to Washington state by the 1980's (Elston et *a/. 1986). B. ostreae* has caused significant (>60%) mortality in 0. *edulis* populations throughout Europe (Comps et a/. 1980; van Banning 1991; Hudson and Hill 1991; Montes et *a/.* 1991; McArdle et a/. 1991) and on the west coast of North America (Elston et *a/.* 1986; Friedman et *a/.* 1989). It has been enzootic in the Damariscotta River, Maine, USA since at least 1991 (Friedman and Perkins 1994; Barber and Davis 1994; Zabaleta and Barber 1996), and in Casco Bay, Maine since 1993 (Barber and Davis 1994). Its impact on oysters in Maine, however, is uncertain.

Ostrea edulis was transplanted to Maine from the Netherlands six times between 1949 and 1961 to establish a nearshore oyster fishery, several times from the Netherlands and California in the 1970's for aquaculture, and once from France in 1978 for research (Loosanoff 1955; Welch 1963; Hidu and Lavoie

1991). Flat oyster populations now occur from Blue Hill Bay west to the Piscataqua River, with dense populations occurring in Casco Bay (Heinig and Tarbox 1984; Hidu and Lavoie 1991). Commercial harvests peaked in the late 1980's but subsequently declined, presumably due to overharvesting, lack of spawning in cool summers, substrate limited recruitment, winter mortality, or (perhaps) bonamiasis (Loosanoff 1962; Heinig and Tarbox 1984; Hidu and Lavoie 1991; Barber and Davis 1994). One or more of the introductions in the 1970's probably carried *Bonamia* ostreae to Maine (Friedman and Perkins 1994).

Efforts to culture *Ostrea edulis* in Maine preceded the establishment of harvestable populations and continue today, primarily in the Damariscotta River and Spinney Creek, a tributary of the Piscataqua River and a location where *Bonamia* ostreae has never been observed (Hidu *et a/.* 1981; Hidu and Lavoie 1991). The expansion of flat oyster aquaculture into new areas and the export of seed and broodstock, however, are limited by a lack of information on the distribution of *B.* ostreae in Maine. The objective of this study was to determine the seasonal prevalence and intensity of B. ostreae infections in four oyster populations.

MATERIALS AND METHODS

Flat oysters *Ostrea edulis* (n = 20 to 72) were hand-collected by divers from four locations, Spinney Creek (43°5'50" N, 70°45'55" W; samples provided by Spinney Creek Shellfish), Gun Point Creek (43°46'28" N, 69°56'50" W), Linekin Bay (43°51'33" N, 69°35'25" W), and the Blue Hill Salt Pond (44°21'30"

N, 68°34'20" W) (Fig. 3.1) between September 1995 and June 2000. Only the Spinney Creek population was commercially harvested during this study.

Oyster population density was estimated at Gun Point Creek, Linekin Bay, and the Blue Hill Salt Pond, but not at Spinney Creek. The area of each oyster population was initially defined by visual survey, and a position that could be returned to on subsequent trips was selected. At each sampling date, a line was extended from that position at a randomly selected angle, and oysters (live and dead) in a 1-m^2 quadrat at 10 to 20 randomly selected positions along the transect were counted. For the purposes of this study, a dead oyster was defined as empty valves with the hinge ligament intact. The mean number of oysters m^{-2} for the date was calculated, and the population density of live and dead oysters for each site calculated as the average of these. Confidence intervals (95%) were calculated for live and dead oyster densities and for the frequencies of dead oysters (Zar 1996).

Surface water temperature (±0.1°C) was recorded at each sampling date using a bucket thermometer, and a water sample was collected for subsequent salinity estimation (±1 ‰) using a hand-held refractometer.

Prevalences and intensities of *Bonamia ostreae* were determined using either standard histopathology or histocytology, following measurement of oyster shell height with calipers (±0.1 mm) and a check for gross signs of pathology. Histopathology and histocytology are equally effective at detecting moderate and heavy *B. ostreae* infections, but less so at detecting light, local infections (Zabaleta and Barber 1996; Culloty and Mulcahy 1996). Samples processed for

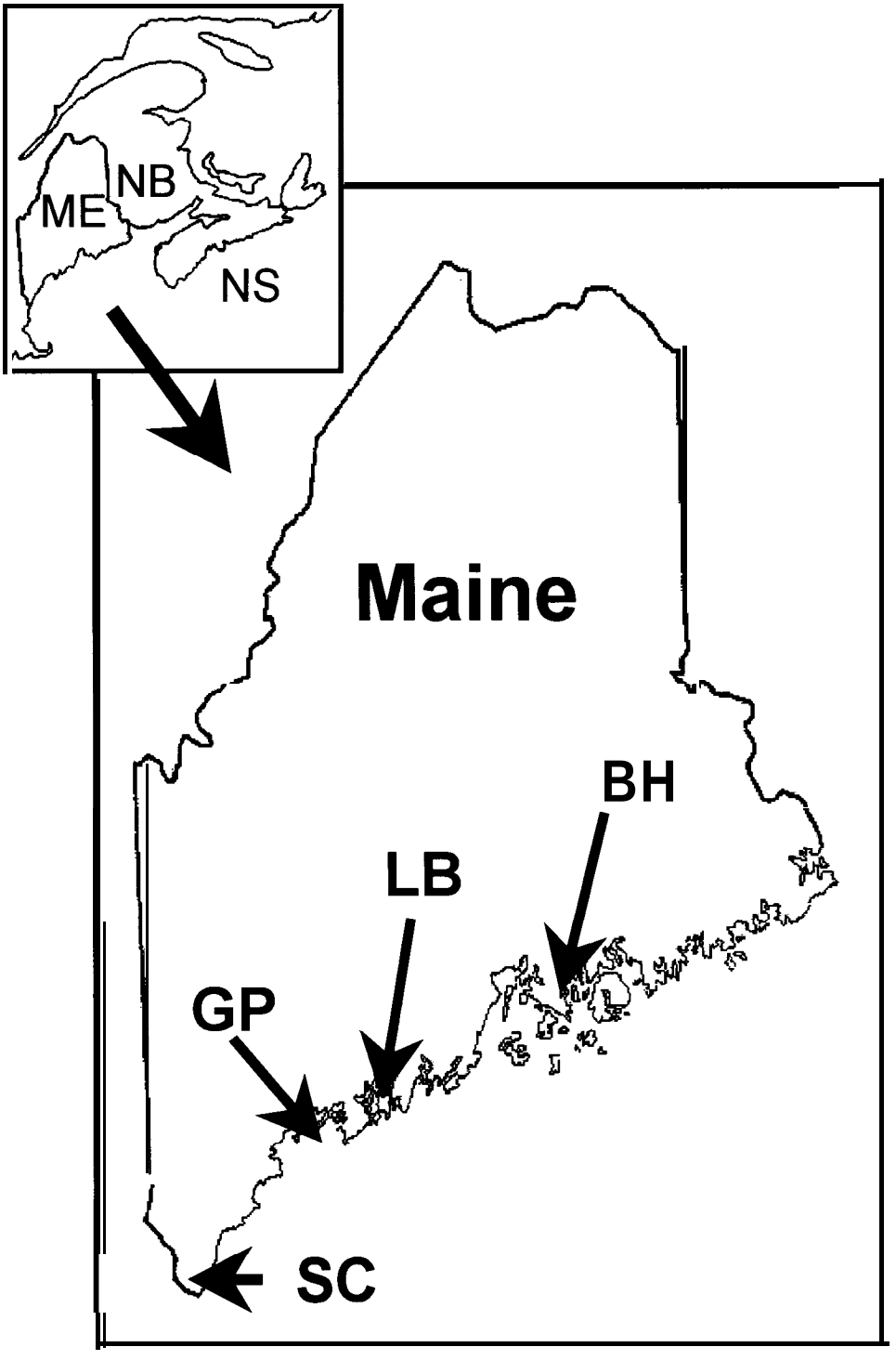

Figure 3.1. Approximate study locations. SC = Spinney Creek, GP = Gun Point Creek, LB = Linekin Bay, and BH = Blue Hill Salt Pond. Inset: ME = Maine, NB = New Brunswick, Canada, and NS = Nova Scotia, Canada.

histopathology were fixed for 24 h in Dietrich's Fixative, dehydrated through an ascending ethanol series, and embedded in paraffin; sectioned at 5 µm; then deparaffinized in xylene, rehydrated through a descending alcohol series, and stained with hematoxylin and eosin (Zabaleta and Barber 1996). Slides were then coverslipped and screened using a light microscope at 1000X magnification following cursory examination at 100X.

For histocytological analysis, hemocytes from the pericardial cavity were removed using a syringe and mixed 1:5 to 1 :10 with 0.45 pm-filtered ambient seawater. A drop of this suspension was placed on a microscope slide. Cells were given 15 min to settle and adhere, then fixed and stained using Hemostat (Fisher Scientific) solutions (Culloty and Mulcahy 1996; Zabaleta and Barber 1996). Slides were examined microscopically at 400-I 000× magnification.

Infections were scored as "heavy" if all observed hemocytes contained *Bonamia ostreae;* "moderate" if *B. ostreae* was visible in all microscope fields; "light" if 11 to 100 *B. ostreae* cells were observed within 5 min; "scarce" if 1 to 10 *B. ostreae* cells were observed; and "undetected" if no unambiguous *B. ostreae* cells were observed within 5 min (Bachere et *a/.* 1982; Culloty and Mulcahy 1996; Zabaleta and Barber 1996).

RESULTS

Bonamia ostreae was detected in every sample at Gun Point Creek (Table 3.1). Overall prevalence of *B. ostreae* there was 8.3% (n = 555), with

Table 3.1. Collection dates, sample sizes, oyster size data, diagnostic method, prevalences, and intensities of *Bonamia ostreae* infections. Method: HP = histopathology, HC = histocytology. Intensity: H = high, M = moderate, L = low, S = scarce, U = B. *ostreae* undetected. nd: no data.

Date	n	Mean Size (mm ± 1 SD)	Method	Prevalence (%)	Intensity H	M	L	S	U
Gun Point Creek									
14 July '98	50	54.2 ± 26.2	HP	4.0	0	0	1	1	48
12 Sep. '98	72	79.2 ± 10.6	HC	5.6	0	1	1	2	68
6 Oct '98	60	53.1 ± 17.5	HC	5.0	0	1	2	0	57
1 Dec. '98	30	74.2 ± 14.4	HC	16.7	0	1	3	1	25
21 Feb. '99	57	60.3 ± 20.5	HC	7.0	0	0	2	2	53
17 Mar, '99	57	80.0 ± 10.7	HC	1.8	0	0	1	0	56
2 May '99	30	53.0 ± 15.1	HC	13.3	0	1	1	2	26
19 May '99	49	81.3 ± 11.3	HC	4.1	0	1	1	0	47
14 Aug '99	53	75.5 ± 15.6	HC	11.3	0	1	2	3	47
6 Oct '99	37	74.8 ± 11.8	HC	2.7	0	0	1	0	36
24 Oct 99	30	68.3 ± 13.5	HC	20.0	0	3	1	2	24
4 June '00	30	71.3 ± 14.7	HC	26.7	1	6	0	1	23
Spinnev Creek									
Sep. '95	25	nd	HP	0					
25 Sep. '96	25	82.4 ± 7.6	HP	0					
Dec. '96	25	nd	HP	0					
Linekin Bav									
9 Sep. '96	25	60.7 ± 7.5	HP	0					
18 Dec. '96	25	nd	HP	0					
7 Mar. '97	25	nd	HP	0					
30 Aug. '97	27	nd	HP	0					
20 July '98	48	63.6 ± 19.0	HP	0					
10 Oct. '98	60	71.6 ± 14.6	HC	0					
10 Dec. '98	53	66.2 ± 19.7	HC	0					
8 April '99	47	70.6 ± 20.8	HC	0					
21 Jun. '99	41	64.2 ± 16.4	HC	0					
Blue Hill Salt Pond									
26 July '98	28	78.4 ± 31.2	HP	0					
8 Oct. '98	54	79.1 ± 18.5	HC	0					
7 Dec. '98	42	73.5 ± 21.3	HC	0					
21 May '99	55	77.4 ± 21.4	HC	0					
27 Oct. '99	55	90.3 ± 17.6	HC	0					
6 Dec. '99	69	83.4 ± 9.2	HC	0					

sample prevalences ranging from 1.8 to 26.7%. Infected oysters ranged in size from 28.9 to 92.9 mm. No other parasites were observed, and no seasonal cycle was apparent. Infection intensities were typically low, with only 1 of 46 infections categorized as “heavy” and 15 of 46 as “moderate”. Ten of these heavier infections were observed in the two final samples (October 1999 and June 2000) and corresponded to the two highest prevalence estimates. B. ostreae was never detected at Spinney Creek (n = 75), Linekin Bay (n = 351), or the Blue Hill Salt Pond (n = 303), and no oysters from these areas displayed histopathological signs of bonamiasis.

Gross signs of pathology were never observed; histopathological evaluations of *Bonamia* ostreae-infected oysters from Gun Point Creek, however, revealed modest signs of disease. The two infected oysters from Gun Point Creek in July 1998 each displayed a single, small, focal lesion in the digestive gland where *B. ostreae* was observed. In June 2000, five “heavily” and “moderately” infected oysters (determined histocytologically) were also evaluated using histopathology. Two of these displayed heavy, systemic infections, with infiltration of hemocytes into all tissues and diapedesis of infected hemocytes across the stomach lining. One was characterized by moderate inflammation and infection of the gills only. In two others, *B. ostreae* was observed only at single, focal gill lesions.

Densities of live *Ostrea edulis* were highest at Gun Point Creek (6.5 oysters m^{-2}), intermediate at Linekin Bay (3.4 m^{-2}), and lowest at the Blue Hill Salt Pond (1 .0 m^{-2}) (Table 3.2). However, 95% confidence intervals indicated that

Table 3.2. Confidence intervals (95%) for the densities of live and dead oysters, and frequencies of dead oysters, by sample site.

Location	n	Live Oysters (m^{-2})	Dead Oysters (m^{-2})	Dead Oyster Frequency
Gun Point Creek	5	6.5 ± 4.6	3.2 ± 2.2	0.34 ± 0.14
Linekin Bay	4	3.4 ± 3.1	2.0 ± 0.7	0.46 ± 0.20
Blue Hill Salt Pond	4	0.9 ± 0.6	0.4 ± 0.6	0.24 ± 0.29

only the difference between Gun Point Creek and Blue Hill Salt Pond was significant. Densities and relative frequencies of dead oysters were similar among populations.

Temperature ranged from near freezing to at least 20ºC at Gun Point Creek, Linekin Bay, and the Blue Hill Salt Pond (Table 3.3). Minimal water

Table 3.3. Temperature (°C) and salinity (‰) data.

Gun Point Creek			Linekin Bay			Blue Hill Salt Pond		
Date	Temp	Sal	Date	Temp	Sal	Date	Temp	Sal
8/31/97	20	32	7/20/98	21	30	7/26/98	22	32
3/25/98		31	10/10/98	12		10/8/98	12	33
7/14/98	20	27	12/9/98	6	34	12/7/98	8	33
10/6/98	12	33	3/9/99	1	32	5/21/99	19	31
12/1/98	8	31	4/8/99	7	27	10/27/99	12	31
2/21/99	2	32	6/21/99	23	32			
3/17/99	8	18						
5/2/99	9	32						
5/19/99	16	31						
6/15/99	20	32						
8/14/99		31						
10/6/99		32						
10/20/99		33						
10/24/99	13	21						
11/1/99		32						
12/31/99		33						
6/4/00	13							

temperature recorded at Gun Point Creek was 2ºC, and Linekin Bay and Blue Hill Salt Pond populations were ice-covered in January and February. Mean salinity was similar at all sites.

DISCUSSION

Bonamia osfreae is enzootic not only in the Damariscotta River estuary, where it has been detected consistently since 1991 (Friedman and Perkins 1994; Barber and Davis 1994; Zabaleta and Barber 1996), but also at Gun Point Creek. *B. ostreae* was not detected at Spinney Creek, Linekin Bay, or the Blue Hill Salt Pond, and thus may not have been introduced to these areas. Alternatively, *B. ostreae* may have been introduced but persists, if at all, at prevalences and intensities below the level of detection using the methods employed here.

Spatial and temporal patchiness of *Bonamia ostreae* within oyster populations in Maine is well documented, and complicates the extrapolation of findings of this study to adjacent oyster populations. Barber and Davis (1994) found *B. ostreae* in just two of four Casco Bay sites in 1993. In the Damariscotta River estuary, Zabaleta and Barber (1996) found *B. ostreae* at all three sites in 1994 and 1995, but not at each sampling date. *B. ostreae* was observed at one site on two of four sampling dates and at two sites just once. On two sampling dates *B. ostreae* was not observed in any location. Thus, oyster populations near Gun Point Creek may be free of *B. osfreae.* On the other hand, populations in Linekin Bay may well harbor the parasite, particularly since Linekin Bay is adjacent to the *B.* ostreae-enzootic Damariscotta River estuary.

Water temperature and salinity do not explain the absence of detectable *Bonamia* ostreae infections from Spinney Creek, Linekin Bay, or Blue Hill Salt Pond. Linekin Bay and the Blue Hill Salt Pond have temperature (≥20ºC in summer, near freezing in winter) and salinity (~30‰) characteristics similar to the Damariscotta River (Zabaleta and Barber 1996; Barber and Davis 1997; Davis and Barber 1999), where *B. ostreae* is enzootic (Friedman and Perkins 1994; Barber and Davis 1994; Zabaleta and Barber 1996).

Density differences among oyster populations may explain differences in *Bonamia ostreae* prevalence. Greater host density at Gun Point Creek may have facilitated *B. ostreae* transmission. However, support is not strong for live oyster density differences among sites in this study. Furthermore, the Damariscotta River estuary populations in which Zabaleta and Barber (1996) observed *B. ostreae* were less dense (<0.5 oysters m^{-2}) than the Blue Hill Salt Pond population. A complex interaction of local physical and biological factors could explain the absence of detectable *B. ostreae* at Linekin Bay and the Blue Hill Salt Pond. It is more likely, however, that *B. ostreae* was never introduced to these locations. After the discovery of *B. ostreae* in the Damariscotta River, movement of *Ostrea edulis* between estuaries in Maine was prohibited.

The low intensities of *Bonamia ostreae* infections at Gun Point Creek despite moderate prevalences of infection (1.8 to 23.3%) and the modest signs of pathology there suggest a possible chronic persistence of the parasite in this oyster population and an associated development of tolerance to infection. Elston et *al.* (1987) found that a “carrier” population (≥30% infection) from

Washington state exhibited lower mortality (26%) than a presumably naive population which showed 99% mortality during a 46 wk cohabitation trial. Frequencies of dead oysters (and mortalities, if dead oyster frequency may be an indicator) at Gun Point Creek were not significantly different from those at Linekin Bay and the Blue Hill Salt Pond. Thus, mortality at Gun Point Creek caused by B. ostreae appears to be low, reinforcing the suggestion that the oysters there are managing the parasitic challenge successfully. Alternatively, low mortality, prevalence, and intensity of *B.* ostreae may suggest a weakly virulent parasite or inhibition of pathogenicity by environmental factors such as prolonged low winter temperatures. If indeed B. ostreae is only weakly affecting oysters at Gun Point Creek, it may be due to a combination of these factors.

Much of the distributional range of *Ostrea edulis* in Maine may be free of *Bonamia ostreae.* However, flat oyster culture in all Maine waters should be approached with caution. Sampling limitations preclude a definitive resolution of the range of *B. ostreae,* which may grow with the expansion of wild and cultured oyster populations and possible unregulated transfers. Additionally, under the right conditions, *B. ostreae* may have no less of an impact in Maine than when it first appeared in Europe. The catastrophic mortality characteristic of *B. ostreae*'s arrival in naïve European oyster populations (Comps et *al.* 1980; van Banning 1991; Hudson and Hill 1991; Montes et *al.* 1991; McArdle et *al.* 1991) may have passed unnoticed in Maine before 1983, when extensive flat oyster populations were discovered. The situation in Cork Harbour, Ireland may be instructive. Production in local oyster populations has risen to 80% of pre-B. ostreae levels,

with the parasite persisting at low prevalences and intensities (Hugh-Jones 1999). However, oysters relaid from B. ostreae-free areas suffer very high mortality (Culloty et *a/. 1999).*

ACKNOWLEDGEMENTS

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CHAPTER 4

Growth and mortality of *Ostrea edulis* at two sites on the Damariscotta River estuary, Maine USA

INTRODUCTION

The European flat oyster Ostrea *edulis* Linné was transplanted to Maine, USA six times between 1949 and 1961 to establish an alternative to the declining softshell clam (*Mya arenaria*) fishery (Loosanoff 1955; Welch 1963; Hidu and Lavoie 1991). Substantial populations developed from these introductions in Casco Bay and near Boothbay Harbor and were immediately exploited following their discovery in 1983. Landings rose to 306.7 metric tons in 1989, but fell to 0.2 metric tons five years later (National Marine Fisheries Service data). Sporadic recruitment supports a modest industry today.

Prior to the establishment of these wild populations, a culture-based industry was developed (Hidu *et a/.* 1981). *Ostrea edulis* were produced in floating trays, lantern nets, or stacked trays in Casco Bay, the Damariscotta River, and the Blue Hill Salt Pond from the early 1970's until 1983. Seed was imported initially from Europe and California, then produced locally. The oysters grew well across a wide range of water temperature (e.g., 15 to 23°C in summer in the Damariscotta River) (Packie et *al.* 1976). The estuarine sites with warm summer temperatures promoted more rapid growth (market size in 2 to 3 yrs); however, the same sites were often characterized by sub-zero winter water temperatures. High winter mortalities thus necessitated an expensive strategy of dual site culture, with estuarine sites used in summer and marine sites in winter. Flat oyster culture became economically unviable when prices fell with the discovery of the wild beds (Hidu and Lavoie 1991).

Flat oyster culture in Maine was further impacted by the discovery of *Bonamia ostreae* (Pichot et al. 1980) in wild oysters in the Damariscotta River in 1991 (Friedman and Perkins 1994; Barber and Davis 1994; Zabaleta and Barber 1996). This haplosporidian parasite (Perkins 1987, 1990) was a major factor contributing to the decline of Ostrea *edulis* populations in Europe (Comps et *al.* 1980; van Banning 1991; Hudson and Hill 1991; Montes et a/. 1991; McArdle et a/. 1991). Its current impact on cultured 0. *edulis* in Maine, however, is unclear.

The decline of European flat oyster populations and the resultant strong markets have renewed interest in the culture of this product in Maine. The objective of this project was to evaluate the growth and mortality of Ostrea *edulis* at two locations in the Damariscotta River estuary: an estuarine site characterized by high summer water temperatures and fluctuating salinity, and a marine site with cooler summer water temperatures and more stable salinity.

MATERIALS AND METHODS

Four thousand juvenile Ostrea *edulis* (-2.5 mm), produced from wild broodstock, were obtained from Mook Sea Farms, Inc. (Damariscotta, ME) in July 1997. They were deployed among eight floating trays (~ 250/tray) at each of two sites on the Damariscotta River: Little Point (44°1'00" N, 69°32'45"W, and approximately 23 km from the river mouth) and Lowes Cove (43" 55' 47"N, 69" 34' 23"W, and approximately 12 km from the river mouth) (Fig. 4.1). The oysters were initially held in envelopes of l-mm fiberglass widowscreen inserted

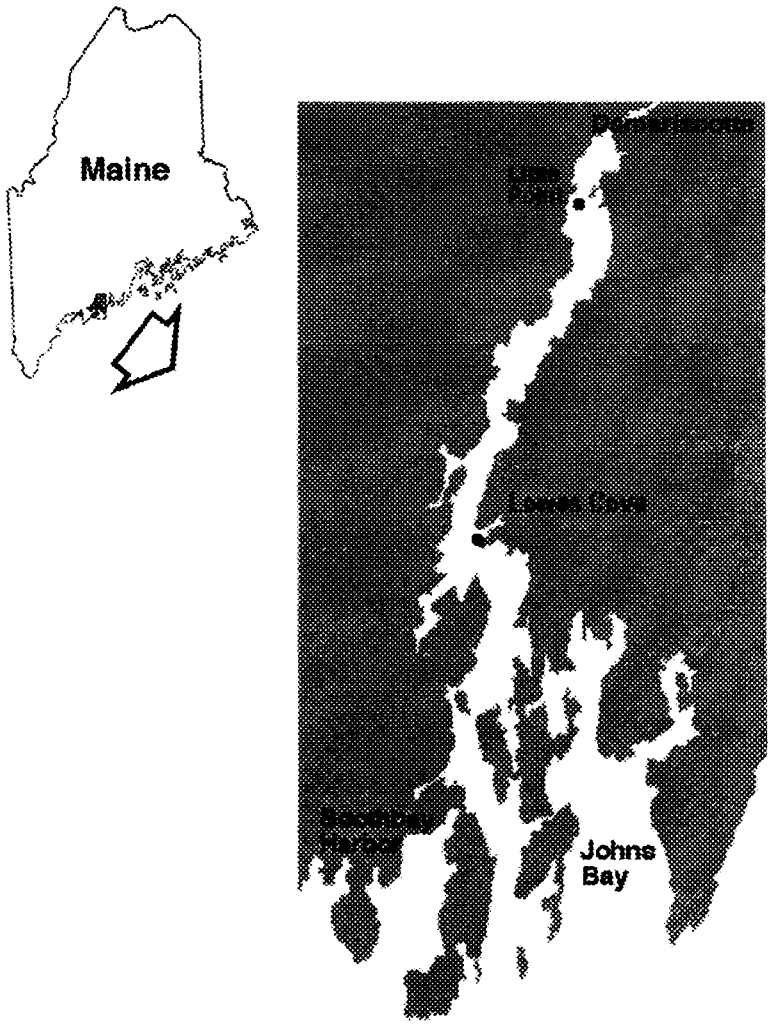

Figure 4.1. Study locations (Little Point and Lowes Cove) on the Damariscotta River estuary. Modified from Zabaleta and Barber (1996).

into extruded polyethylene cages (ADPI, 12.7 mm mesh; see Davis and Barber 1994, Barber et *al.* 1996, Davis and Barber 1999). They were transferred to trays having larger mesh sizes as they grew. Trays from both sites were held in tanks receiving flowing, ambient seawater during colder months (10 December 1997 to 15 May 1998; 26 January 1999 to 20 May 1999) at the Darling Marine Center (at Lowes Cove).

Growth, mortality, and prevalence of *Bonamia ostreae* were monitored between September 1997 and December 1999. Shell heights of twelve oysters from each tray were measured (with replacement, fO.l mm) and counts of live and dead oysters were recorded. Cumulative mortality was calculated using a life table (Krebs 1972). The null hypotheses of no between-site and -date differences in shell height and counts of dead oysters were tested using repeated measures analyses of variance, and multiple comparisons were performed using Tukey's Test.

Four oysters per tray were removed bimonthly for diagnosis of *B. ostreae* using standard histopathological analysis (September 1997, May 1998, and July 1998 only) or stained hemolymph smears (Zabaleta and Barber 1996; Culloty and Mulcahy 1996).

Temperature (±0.1°C) was recorded at both sites four times daily with dataloggers (Ryan Instruments, Redmond, WA). Water samples were collected during visits to each site for salinity estimation (±1‰) using a hand refractometer.

RESULTS

Growth was significantly faster at Little Point than at Lowes Cove (repeated measures ANOVA, $P = 0.008$) (Fig. 4.2). Oysters at both sites, however, were market size in <3 full growing seasons. Oysters at Little Point reached market size (-75 mm) in May 1999, after less than two years of growth, and were 93.5 ± 4.1 mm by late December 1999. Oysters at Lowes Cove reached market size in just over two years, in autumn 1999, and were 83.0 ± 1.7 mm in size in December 1999.

Between-site size differences were largely due to higher late summer (1997 and 1998) growth rates at Little Point (Table 4.1). Growth ceased during winter and spring, when all oysters were stored in tanks at the Darling Marine Center. The reduced growth rate at Little Point in late summer-autumn 1999 may

Table 4.1. Mean interval increase in shell height at Little Point and Lowes Cove. Negative values represent sampling error combined with little absolute growth. Multiple comparisons were performed using Tukey's Test. Significant ($\alpha = 0.05$) between-site size changes are in bold. Asterisks indicate that the within-site size change was significantly different ($\alpha = 0.05$) from that of the previous interval.

Interval ending	Size increase (mm ± 1 SD)	
	Little Point	Lowes Cove
September 1997	**22.3 ± 1.1**	**13.4 ± 2.6**
February 1998	**10.5 ± 1.9***	**7.0 ± 2.9***
May 1998	**0.0 ± 2.4***	**-0.8 ± 2.8***
July 1998	**4.5 ± 2.3**	5.1 ± 3.0*
September 1998	**30.4 ± 1.9***	**21.7 ± 2.3***
January 1999	**3.5 ± 2.5***	**5.3 ± 3.4***
March 1999	**0.3 ± 2.3**	1.1 **k2.9**
May 1999	**0.8 ± 3.0**	**-0.8** ± 1.4
Auaust 1999	**11.3 ± 2.7***	**15.4 ± 2.0***

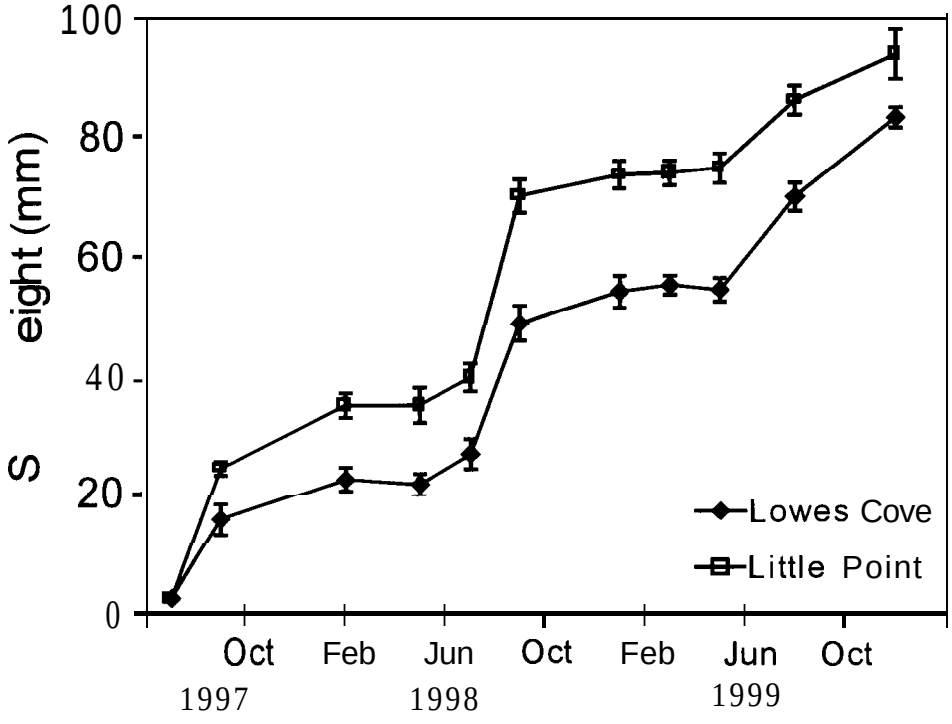

Figure 4.2. Mean shell height (mm ± 1 SD) of *Ostrea edulis* at Little Point (LP) and Lowes Cove (LC) between July 1997 and December 1999. Three of 8 Lowes Cove trays were lost prior to the December 1999 sampling.

have been due to crowding. The number of oysters in trays at both sites was similar, after thinning of Lowes Cove trays in April 1999 to compensate for higher mortality in Lowes Cove trays the previous summer, but the oysters at Little Point were larger.

Mortality also was significantly greater at Little Point than at Lowes Cove (repeated measures ANOVA, $P < 0.001$) (Fig. 4.3). Cumulative mortality at Little Point was 45.8%, as opposed to 26.7% at Lowes Cove. Mortality at both locations was low initially, rose sharply in summer and fall 1998, then leveled off through the remainder of the experiment.

Interval mortality was similar between sites except during summer and fall 1998, when counts of dead oysters at Little Point first exceeded counts at Lowes Cove by a factor of 5 (72.6 *vs.* 14.0 per tray), and then were lower by a factor of 2 (21.3 vs 42.0 per tray) (Table 4.2). Mortality at both sites was low (<5.8%) except during these intervals.

Table 4.2. Mean counts of dead oysters at Little Point and Lowes Cove. Significant ($\alpha = 0.05$) between-site counts are in bold. Asterisks indicate that a within-site count was significantly different ($\alpha = 0.05$) from that of the previous sampling date.

Date	Mean number of dead oysters per tray (mm ± 1SD)	
	Little Point	Lowes Cove
February 1998	**0.1 ± 0.4**	**1.8 ± 2.5**
May 1998	**0.4 ± 0.5**	**0.9 ± 0.6**
July 1998	**72.6 ± 23.7***	**14.0 ± 14.5***
September 1998	**21.3 ± 8.1***	**42.0 ± 9.7***
January 1999	**0.9 ± 0.4"**	**2.9 ± 3.6***
March 1999	0	0.3± **0.5**
May 1999	**2.0** ± 1.5	1.6 ± 1.3
August 1999	**5.6 ± 3.0**	**5.8** ± 5.1

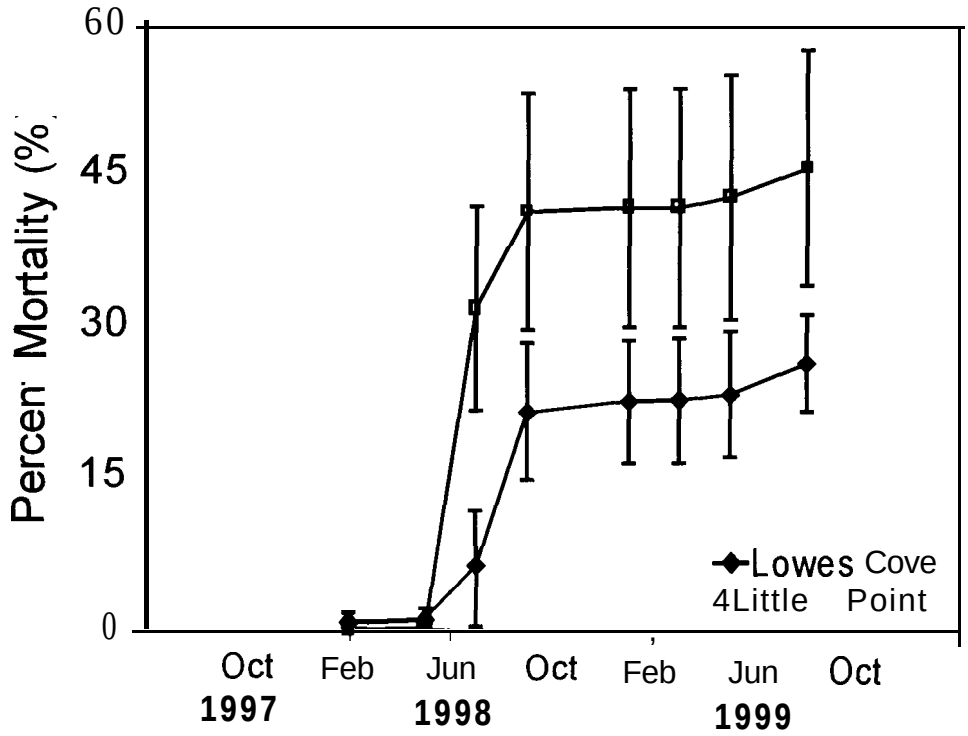

Figure 4.3. Cumulative mortality (% ± 1 SD) of *Ostrea edulis* at Lowes Cove and Little Point between February 1998 and August 1999.

Bonamia ostreae was observed only in one oyster from Little Point in May 1998, and the infection was low in intensity (<10 *B. ostreae cells* observed in hemocytes at a single focus at the digestive gland).

Weekly mean surface water temperatures exceeded 20°C for several weeks each summer at Little Point, yet rarely reached 18°C at Lowes Cove. Summer 1999 temperature data at Little Point were lost due to a datalogger malfunction. Winter temperatures at the Darling Marine Center fell below 2.0°C. Salinity was slightly higher at Lowes Cove than at Little Point (32 ± 2‰ vs. 30 ± 3‰; Mann-Whitney Test $P = 0.027$), but the range was narrower (28 to 34‰ vs. 22 to 33‰). The lowest Little Point salinity estimate (22‰) was made on 17 June 1998, after 5.7" of rain fell in 5 d on the upper Damariscotta River.

DISCUSSION

The Damariscotta River provides an excellent growth environment for *Ostrea edulis*, as previously noted by Hidu et *a/.* (1981). In this study, oysters reached market size at both sites in less than two full growing seasons, and may have grown faster had they been deployed earlier in 1997. The growth rate of oysters in this study matched or exceeded that of oysters grown in suspended culture in Ireland (60 to 70 mm in two growing seasons [Wilson 1987]) and on trestles in Atlantic France (to <60 mm in 2 yrs; Robert et *a/.* 1991), which suggests that the Damariscotta River is indeed suitable for an economically viable flat oyster culture industry. Further, similar performance may be expected at other coastal Maine sites. Packie et a/. (1976) deployed *Ostrea edulis* and

Crassostrea virginica at several sites in growth trials that lasted from 2 to 3 months. In that study, initial size at deployment varied between species and sites (11.8 to 34.5 mm), and deployment dates varied from mid-July to early September, so the results are difficult to compare. Still, 0. *edulis* grew well at all sites, adding 0.29 ± 0.05 (mean ± 1 SD) mm/day in size (0.26 mm/day in the Damariscotta River). Growth of C. *virginica*, 0.22 ± 0.05 mm/day, was significantly slower (t-test, $P = 0.0190$). Packie et *a/.* (1976) provided a further comparison of 0. *edulis* with C. *virginica,* deploying cultchless seed (0. *edulis*: 8mm; C. *virginica:* 4mm) at three locations in the Damariscotta River in a growth trial that lasted from 12 July to 11 November, 1971. Growth of 0. *edulis* in the Salt Bay (furthest upriver), at Dodge Cove (near Little Point) and at Wentworth Point (furthest downriver, and near Lowes Cove) averaged 60, 53, and 50 mm, respectively. For C. *virginica,* the corresponding means were 64, 45, and 20 mm. 0. *edulis* performed much better than C. *virginica* at Wentworth Point, in an environment more representative of that typically observed in southwestern Maine (stenohaline, with temperatures rarely reaching 20°C) than Dodge Cove/Little Point (euryhaline, with temperatures often exceeding 20°C in summer). Thus, many environments unsuitable for C. *virginica* culture may be capable of sustaining a viable flat oyster culture industry in Maine.

Cumulative mortality was relatively high, approaching 50% in Little Point oysters, and 30% at Lowes Cove. Most mortality was associated with the June 1998 rain event. Chanley (1958) found 15‰ to be the minimum salinity tolerated by *Ostrea edulis* juveniles. Héral and Deslous-Paoli (1991), reviewing the

physical tolerances of 0. *edulis*, noted that the optimum salinity range for 0. *edulis* in Europe is 32 to 37‰, and that below 20‰ the oyster may die. Welch (1963), discussing the suitability of Maine waters for 0. *edulis* culture, noted that Maine waters to which 0. *edulis* was successfully introduced have a similar salinity (25 to 33‰) to the geographic source of these early transplantations, the Oosterschelde of the Netherlands. Surface salinity immediately after the June 1998 rain event was 22‰ at Little Point, and it was still low (24‰) two weeks later. This is below 0. *edulis*'s range of tolerance, and the oysters were probably weakened or killed by this stress. The salinity fluctuations at Lowes Cove were less extreme, as salinity below 28‰ was never recorded.

Surprisingly, *Bonamia ostreae* was virtually absent in this study. This may reflect a continuation of the general decline in prevalence of this parasite in flat oysters from the Damariscotta River occurring over the last 10 years. Friedman and Perkins (1994) reported initial prevalences of 34% and 45% in 1991 and 1992, respectively. Barber and Davis (1994) found a prevalence of 20% in 1993, and Zabaleta and Barber (1996) observed prevalences ranging from 0 to 16% in 1994 and 1995. This decline in parasite prevalence may be the result of declining oyster densities, unfavorable environmental conditions, or in increase in the oyster's capability to "manage" the parasite (see Elston et *al.* 1987) It is possible that the sudden decrease in salinity that was responsible for much of the oyster mortality during this study also reduced parasite populations. Another possible explanation for the low prevalence of 5. *ostreae* is that the oysters in this study did not receive an adequate disease challenge because they were not

in close proximity to infected natural populations. The fact that oysters used in this study were held in floating trays (as opposed to on-bottom trays), probably did not diminish the opportunity for infection, as flat oysters held in floating trays at Little Point between 1994 and 1995 had a 50% prevalence of *B. ostreae* infection (Barber, unpublished data).

The future of flat oyster aquaculture in Maine may indeed be bright. Growth was rapid in temperature and salinity regimes typical of Maine coastal waters, mortality rates were low except following a single serious storm. Mortality caused by such perturbations may be reduced (as at Lowes Cove) by selecting more stable, marine sites for culture. The threat of *Bonamia ostreae* remains, but selection of sites distant from dense wild oyster beds and at which time to market size is <3 years may reduce its impact. As resistance to *B. ostreae* mortality is possible (Elston et a/. 1987), a breeding program may be undertaken using oysters that have likely survived exposure to *B. ostreae.* Such a program has met some success in France (Martin et al. 1993; Hervio et *al.* 1995; Baud et a/. 1997; Naciri-Graven et *a/.* 1998,1999). In Maine, broodstock could be develop from oysters at Gun Point Creek in Casco Bay, where *B. ostreae* is enzootic (see Chapter 3).

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CHAPTER 5

Heterogeneity in *Bonamia ostreae* internal transcribed spacer (ITS 1) sequences

INTRODUCTION

Population genetic analysis of protistan parasites of marine bivalves is very much in its infancy. The inability to separate parasite tissue completely from host tissue and the difficulty in establishing axenic cultures of many parasites has precluded the development of the powerful anonymous molecular markers (e.g., microsatellites and amplified fragment length polymorphisms, or AFLP's) useful in population studies of larger organisms. Strong evidence for population or strain differentiation of protists does exist, however. Bushek (1994) found differences among geographic isolates of *Perkinsus marinus* (a dinoflagellate parasite of eastern oysters *Crassostrea virginica*) in enlargement in Ray's Fluid Thioglycollate Medium (RFTM), and Bushek and Allen (1996) found that oysters challenged with different isolates of *P. marinus* (derived from axenic cultures) developed significantly different body burdens of the parasite. Reece et *al.* (1997) found four loci to be polymorphic within and among populations of *P. marinus,* suggesting a genetic basis for the observed physiological races.

The intraspecific genetic structure of *Bonamia ostreae,* the haplosporidian parasite of the European flat oyster *(Ostrea edulis*), remains unknown. Elston et *al.* (1986) and Friedman et *al.* (1989) supported a California origin for *Bonamia ostreae,* which implies that the establishment of 5. *ostreae* in Europe and Maine was relatively recent (since 1970) and clonal. Thus, it may be hypothesized that there should be little 5. ostreae population or strain differentiation within Europe and Maine. Such a hypothesis may be directly tested using genetic data.

Elston et *a/.* (1987) concluded that low mortality in an experimental

cohabitation trial of a “carrier” population from Washington State relative to presumably naïve *Ostrea edulis* from Maine was evidence for the development of resistance to clinical 5. ostreae infections. A French flat oyster breeding program has been founded on the assumption (based on empirical data) that 0. *edulis* in France is developing resistance to 5. ostreae (Martin et *a/. 1993;* Hervio et *al.* 1995; Baud et *a/.* 1997; Naciri-Graven et *al.* 1998,1999). Such resistance is certainly a function of the interaction between parasite physiology and genetics, host physiology and genetics, and the environment. The parasite contribution to this dynamic may be assessed in part using a genetic approach.

Internal transcribed spacers reside between the 18S and 5.8S (ITS 1) and 5.8S and 28S (ITS 2) rDNA genes, and so are easily amplified with specific or universal eukaryotic rDNA primers (Goggin 1994; Reece et *a/.* 1997). Under weaker selection constraints than the 18S, 5.8S, and 28S, they accumulate substitutions more rapidly than the surrounding genes and thus are useful sources of intraspecific genetic information. The objective of this study was to compare ITS 1 sequences of *Bonamia ostreae* from oyster populations in Maine and Europe.

MATERIALS AND METHODS

Bulk *Ostrea edulis-Bonamia ostreae* genomic DNA was extracted from oysters collected from Cork Harbour, Ireland, in November 1997 (n = 71); Gun Point Creek, Maine, USA, in December 1998, August 1999, and October 1999 (n = 1, n = 37, and n = 47, respectively); Ria de Arosa, Spain, in January 2000 (n =

30); and Lake Grevelingen, the Netherlands in January 2000 (n = 10). DNA from Cork Harbour and Gun Point Creek (December 1998 specimen) oysters was extracted as follows: gill fragments (~ 0.1 g) fixed in 95% ethanol were rinsed twice in ddH_20 and minced, then incubated overnight at 55°C in a digestion solution of 0.05M Tris-HCl, 0.2M NaCl, 0.05M EDTA, 1% SDS, and 0.05-o. 10 mg/ml Proteinase K at pH 8.0. After a single extraction with phenol and two with chloroform:isoamyl alcohol (24:1), DNA was precipitated with absolute ethanol:3M ammonium acetate (11:1) and quantified spectrophotometrically. For Gun Point Creek (August and October 1999 samples), Ria de Arosa, and Lake Grevelingen oysters, DNA was extracted from ~ 0.1 g of gill tissue using a QIAamp DNA Mini Kit (QIAGEN, Inc., Valencia, CA, USA) following the manufacturer's instructions, then quantified spectrophotometrically.

DNA sequence analysis

Seven DNA samples were selected for DNA sequencing from those which showed strong *Bonamia ostreae* rDNA amplification: LF-3, TRA-21, and ROS-4 from Cork Harbour, GP-12/98-36 and GP-8199-I 6 from Gun Point Creek, RA-49 from Ria de Arosa, and LG-12 from Lake Grevelingen. A 528 bp sequence fragment of 5. *ostreae* 18S-ITS 1 was PCR amplified from each sample using primers A_F and A_R (Chapter 1: A_F: 5'-TGTGATGCCCTTAGATGTYCT-3'; A_R: 5'-GCTGCGTCCTTCATCGWT-3'), cloned using a TOPO TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA, USA), and re-amplified from single (LF-3, TRA-21, ROS-4, and GP-12/98-36) or pools of four (LF-3 [repeated], GP-8/99-16, RA-49,

and LG-12) insert-containing colonies using A_F and A_R. These PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN, Inc.) and submitted to the University of Maine DNA Sequencing Facility for sequencing using an ABI 377 Automated Sequencer. The PCR primers were used as sequencing primers, and bidirectional sequences were obtained in all cases except LG-12 and ROS-4, for which only reverse sequences were obtained.

Allele-specific PCR

An allele-specific PCR was used to estimate the frequency of two presumptive ITS 1 alleles among known *Bonamia ostreae*-infected oysters from Cork Harbour (n = 16), Gun Point Creek (n = 13), Ria de Arosa (n = IO), and Lake Grevelingen (n = 10). A single forward primer (HAPX-231 F: GAGGTGAATTAGGTGGATAAGAG) was used in conjunction with each of two reverse primers (BO-ITS-A: GCGCAAATTCCTTGCACAAAATC; BO-ITS-B: GCGCAAAGTCATTGCGCAAATTC; all primers synthesized by Operon Technologies, Alameda, CA, USA) in parallel 15 µl reactions, comprising 2.5 mM $MgCl_2$ (Perkin Elmer, Wellesley, MA, USA), 0.2 mM dNTPs (Gibco BRL, Life Technologies, Inc., Rockville, MD, USA), 0.05 µM primers, 0.75 units of Platinum Taq @DNA polymerase (Gibco BRL), and 1 .O ng/µl template DNA in IX PCR buffer (Gibco BRL). Following a 5 min denaturation at 95°C, the reaction comprised 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 65.2°C, and extension at 72°C, followed by a final IO-min extension at 72°C. Amplification products were observed on 1% agarose in IX TAE gel containing

0.4 μg/ml ethidium bromide. A no-template DNA negative control was included in each of these trials, but there were no true positive controls. Individual samples that showed no amplification with either primer pair were retested. If amplification failed a second time, the samples were tested with *B. ostreae*-specific primers C_F and C_R (Chapter 1) to determine if the template DNA had degraded.

RESULTS

DNA sequencing revealed two unambiguous *Bonamia ostreae* ITS 1 sequences, both 147 bp, with one characteristic of the European B. *ostreae* samples (genotype B), and another observed only in a Maine (GP-12/98-36) oyster (genotype A) (Table 5.1). The four base differences between sequences were restricted to a single 14 bp region. Two ambiguous sequences were also obtained. Specimen LG-12 was ambiguous at precisely those positions that were characterized by a different base in specimen GP-12/98-36 than in the unambiguous European specimens, and chromatogram data suggested that both bases were present as sequencing reaction templates. Specimen GP-8/99-16 was ambiguous at two of these positions, in a similar fashion.

Allele-specific PCR results indicated that while *Bonamia ostreae* ITS 1 allele A was widespread among European and Maine oysters, allele B was more prevalent among European samples (Table 5.2). A presumptive allele A product was present every time amplification was successful. Presumptive allele B was

Table 5.1. Sequence of the *Bonamia ostreae* ITS 1 variable region (positions 98-l 11). Dashes signify identity to the first sequence (GP-12/98-36).

Specimen	Location	Source PCR #	# of Colonies	Sequence[a]	Genotype
Unambiauous					
GP-12/98-36	Maine, USA	436(2)	1	TTT TGT GCA AGG AA	A
LF-3	Ireland	435(3)	1	A-- --C --- -T- -C	B
		458(3)	4 pooled	A-- --C --- -T- -C	B
TRA-21	Ireland	427(23)	0[b]	A-- --C --- -T- -C	B
		435(4)	1	A-- --C --- -T- -C	B
ROS-4	Ireland	435(5)	1	A-- --C --- -T- -C	B
RA-49	Spain	457(3)	4 pooled	A-- --C --- -T- -C	B
Ambiauous					
GP-8/99-16	Maine, USA	458(2)	4 pooled	--- --Y --- --- -M	
LG-12	Holland	459(4)	4 pooled	W-- --Y --- -K- -M	

[a] W = A or T; Y = C or T; K = G or T; M = A or C.
[b] Purified PCR product was directly sequenced, without a cloning step.

present much less frequently, and always in conjunction with presumptive allele A. However, repeated trials with LF-4 and LF-9 produced inconsistent results,

Table 5.2. Genotypic frequencies.

Location	n	Frequency			
		A	B	AB	No product
Gun Point Creek	13	11	0	1	1
Cork Harbour	16	9	0	4	3
Ria de Arosa	10	6	0	4	0
Lake Grevelingen	10	3	0	7	0

making these data suspect. LF-4 amplified first with BO-ITS-B, then with BO-ITS-A twice. LF-9 amplified first with both primer pairs, and then only with the pair including BO-ITS-B.

DISCUSSION

The DNA sequencing results indicate that two *Bonamia ostreae* ITS 1 sequences exist. The sequencing results for GP-8/99-16 and LG-12 were generated from a pool of clones from a single oyster, suggesting that both sequences are present in the same host. The significance of this, however, is unclear. It may represent multiple infections of a single oyster by multiple *B. ostreae* clones with different ITS 1 genotypes. It may represent infection by a single *B. ostreae* clone heterozygous at the ITS 1 locus (no data exist, however, to describe the ploidy of any haplosporidian). Finally, it may indicate that *B. ostreae* ITS 1 genes may not be evolving in concert, and so exist in multiple forms among different loci.

The allele-specific PCR results suggest that multiple ITS 1 forms do exist at the locations sampled, and even within individuals. However, the inconsistent results in consecutive trials with a single template suggest that technical problems are clouding the results. False negative results for allele B were likely. The allele-specific primers (BO-ITS-A and especially BO-ITS-B) may have formed primer dimers in the reaction mixture, which would have resulted in suboptimal PCR performance, particularly of the allele B-specific reaction. Degradation of template DNA may also have contributed to PCR inefficiency.

The four samples that did not amplify with either allele-specific primer pair amplified only weakly with the *Bonamia* ostreae 18S-specific C_F and C_R. Previous amplification with this primer pair was strong (Chapter 1). Additionally, the sequences of individual *B.* ostreae cells may have been intermediate between allele A and allele B. Primer-target mismatches would further have reduced the efficiency of the PCR. Alternatively, either reverse primer may have primed an amplification of the incorrect allele despite mismatches, generating false positives.

No evidence exists as yet for genetic structure to *Bonamia* ostreae populations. Indeed, little population genetic data exist for any shellfish parasite. Much has been made of the physiological races of *Perkinsus marinus* (reviewed in Bushek and Allen 1996), but the genetic underpinnings of this variability have only just begun to be characterized (Reece et *a/.* 1997). The introns discovered in haplosporidian actin sequencing by Reece et *al.* (2000) may harbor a genetic signal, as may intergenic spacer regions used as population markers in at least one other eukaryote, the crab *Charybdis japonica* (Ryu et *al. 1999).* These markers, therefore, show much potential for further investigation. In the case of shellfish parasites, discrimination of parasite strains or populations will greatly advance our understanding of the epizootiology of these organisms and their global transplantation.

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