

# Development of a PCR assay for detection of the oyster pathogen *Bonamia ostreae* and support for its inclusion in the Haplosporidia

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**ABSTRACT:** The development of diagnostic assays more sensitive and specific than traditional histological techniques is important for the management of bonamiasis in flat oysters *Ostrea edulis*. A specific polymerase chain reaction (PCR) protocol was developed for the detection of very small amounts of *Bonamia ostreae* (Pichot et al. 1980) ribosomal DNA (rDNA) in bulk DNA from oyster gill and hemolymph. The presence of a 760 bp PCR amplification product corresponded with *B. ostreae* infections determined cytologically in 185 oysters from Ireland, Spain, and the USA. All (100%) 'heavily' and 'moderately' infected oysters, 86.7% of the 'lightly' infected oysters, and 66.7% of the 'scarcely' infected oysters were confirmed to be infected using the PCR. In addition, 37.9% of the oysters in which *B. ostreae* was not detected using cytology were positive using the PCR. Sampling error and the subjectivity of cytological diagnoses are the likely sources of disagreement between diagnostic methods in oysters with very light infections. The PCR assay developed here is more sensitive and less ambiguous than standard histological and cytological techniques. Phylogenetic analysis of DNA sequence data confirmed *B. ostreae* to be a member of the Haplosporidia.

**KEY WORDS:** *Bonamia ostreae* · PCR · Haplosporidia · *Ostrea edulis*

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## INTRODUCTION

*Bonamia ostreae* (Pichot et al. 1980) is a protistan pathogen of flat oysters *Ostrea edulis* Linné in Europe (Pichot et al. 1980, Balouet et al. 1983, Montes 1990, McArdle et al. 1991, Hudson & Hill 1991, van Banning 1991), on the Pacific coast of the USA (Elston et al. 1986, Friedman et al. 1989), and in Maine, USA (Barber & Davis 1994, Friedman & Perkins 1994). Its phylogenetic relationship to other protists is uncertain. Although the possession of haplosporosomes suggests an affinity to the Haplosporidia (Pichot et al. 1980,

Perkins 1990), *B. ostreae* does not form spores (Pichot et al. 1980), which are considered primary diagnostic characters of this phylum (Sprague 1979).

Rapid and accurate diagnosis of *Bonamia ostreae* 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 (cytology) is more rapid and less expensive, and considered useful for the preliminary screening of oyster populations (Zabaleta & Barber 1996, O'Neill et al. 1998). A major disadvantage of standard histopathology and cytology is low sensitivity. The small size of *B. ostreae* (2 to 3 µm) makes it difficult to recognize subclinical infections in thin sec-

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tions, and several investigators have reported trouble detecting *B. ostreae* at low intensity in stained blood smears (Bucke & Feist 1985, Bucke 1988, McArdle et al. 1991). In addition, the resemblance of *B. ostreae* to routine intrahemocytic inclusions (Bucke & 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, Cochenec et al. 1992). However, the antibodies developed for *B. ostreae* in Europe react weakly or not at all with *B. ostreae* from populations outside of Europe (Zabaleta & 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 al. 1993, Stokes & Burrenson 1995, Stokes et al. 1995a), *Minchinia teredinis* (Stokes et al. 1995b), *Marteilia sydneyi* (Anderson et al. 1995), *Mikrocytos roughleyi* (Adlard & Lester 1995), and most recently *Bonamia ostreae* (Carnegie et al. 1999). We describe here the development of a PCR assay for detection of *B. ostreae* in *Ostrea edulis*, based on 18S ribosomal DNA (rDNA) sequences conserved between *B. ostreae* populations in Europe and the USA, and provide molecular evidence supporting the affinity of *B. ostreae* 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 ostreae* is enzootic (Montes 1990, Barber & Davis 1994, Culloty & Mulcahy 1996, O'Neill et al. 1998). *Ostrea edulis* from Spinney Creek, Maine (n = 30), and juvenile *Crassostrea virginica* from the Great Wicomico River, Virginia (n = 19), served as negative controls, as *B. ostreae* has never been detected at 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 ~0.1–0.5 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 ostreae*. 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 & 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 µm 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 & Barber 1996). Slides were examined microscopically for 5 min at 400–1000× 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 (Bachère et al. 1982, Culloty & 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<sub>2</sub>O and minced, then incubated overnight at 55°C in a digestion solution of 0.05 M Tris-HCl, 0.2 M NaCl, 0.05 M EDTA, 1% SDS, and 0.05 mg ml<sup>-1</sup> Proteinase K at pH 8.0. After a single extraction with phenol and 2 with chloroform:isoamyl alcohol (24:1), DNA was precipitated with absolute ethanol:3 M 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 2 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 step-wise 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 2 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, Fig. 1). Reaction conditions were

Table 1. Primer sequences, annealing temperatures, and predicted amplicon sizes of PCR Protocols A, B, and C.  $T_m$  = annealing temperature

Proto-col	Primers	$T_m$ (°C)	Product size (bp)
A	A <sub>F</sub> : TGTGATGCCCTTAGATGTYCT A <sub>R</sub> : GCTGCGTCCTTCATCGWT	55	528
B	B <sub>F</sub> : CAGCCRTCTAACTAGCTSTCGC B <sub>R</sub> : CGGGTCAAACCTCGTTGAACG	55	122
C	C <sub>F</sub> : CGGGGGCATAATTCAGGAAC C <sub>R</sub> : CCATCTGCTGGAGACACAG	59	760

as follows: each 50 µl reaction mixture comprised 45 µl PCR cocktail (1× PCR buffer [GibcoBRL], 1.5 mM MgCl<sub>2</sub> [Perkin-Elmer], 0.2 mM dNTP mix [GibcoBRL], 0.05 µM forward and reverse primers, and 2.5 U 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 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 al. 1995, Flores et al. 1996) and thus *Bonamia ostreae* (Pichot et al. 1980, Perkins 1990) are thought to belong. ITS 1 length variation between *Ostrea edulis* and *B. ostreae* 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

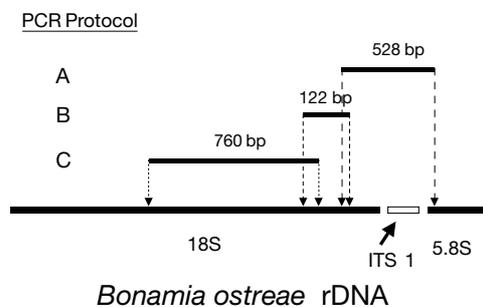


Fig. 1. Diagrammatic representation of the PCR assay development. The PCR product of Protocol A could not be predicted *a priori*. This 528 bp product represents the single candidate *Bonamia ostreae* PCR amplicon resulting from this assay. The Protocol B amplicon was predicted based on alignment of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

parasite, *Mikrocytos roughleyi* (Adlard & Lester 1995). Degenerate primers (A<sub>F</sub> and A<sub>R</sub>; see Table 1) were designed by aligning several 18S-ITS 1 rDNA sequences deposited in Genbank (*Ostrea edulis* [gb U88709], *Arion rufus* [gb X00131], *Stagnicola elodes* [gb AF013138], *Drosophila orena* [gb Z28549], *Saccharomyces bayanus* [gb Z95945], *Amphidinium belauense* [gb L13719], *Alexandrium affine* [gb AB006995], *Alexandrium catenella* [gb AB006990], *Alexandrium insuetum* [gb AB006996], *Alexandrium minutum* [gb U27499], *Alexandrium pseudogonyaulax* [gb AB006997], *Alexandrium tamarense* [gb AB006991], *Cryp-*

*thecodinium cohnii* [gb M25116], *Gonyaulax spinifera* [gb AF051832], *Gymnodinium sanguineum* [gb 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], *Oxytricha nova* [gb X03948 M14601], *Plasmodium falciparum* [gb U21939], *Plasmodium malariae* [gb M54890], *Prorocentrum micans* [gb M14649], *Theileria parva* [gb L26331], *Toxoplasma gondii* [gb L37415 and L49390], *Colpidium campylum* [gb M35557], *Colpidium colpoda* [gb M35558], *Glaucoma chattoni* [gb M35559], *Tetrahymena corlissi* [gb U17356], *Tetrahymena leucophrys* [gb M35555], *Tetrahymena paravorax* [gb M35556], *Tetrahymena patula* [gb M35553], *Tetrahymena pyriformis* [gb X01533 M10752], *Tetrahymena vorax* [gb M35554], and *Haplosporidium nelsoni* [gb U19538]) using GDE (Wisconsin Package Version 9.0, Genetics Computer Group [GCG], Madison, WI) 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 *O. edulis* amplicon of ~1000 bp in size was predicted. PCR products longer or shorter than this could thus be considered candidate *B. ostreae* 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. ostreae* infections, and a Genbank Blast search indicated a resemblance of this product to haplosporidian 18S rDNA, making it a strong candidate for *B. ostreae* rDNA.

The second PCR protocol (Protocol B) was specific for the putative *Bonamia ostreae* fragment previously amplified. Primers (B<sub>F</sub> and B<sub>R</sub>; Table 1) were designed by aligning this sequence with oyster (*Ostrea 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<sub>R</sub>, annealing temperature = 55°C) and sequencing of the entire putative *B. ostreae* 18S-ITS 1 rDNA sequence (submitted to Genbank under accession no. AF262995) from 1 Gun Point Creek and 3 Cork Harbour oysters. (These 4 sequences diverged by just 0.39% over 1945 bp of 18S and ITS 1.) Both forward and reverse (C<sub>F</sub> and C<sub>R</sub>; Table 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], *Cryptothecodinium cohnii* [gb M64245 M34847], *Perkinsus marinus* [gb X75762], *Prorocentrum micans* [gb M14649], *Symbiodinium corcolorum* [gb L13717], *Cryptosporidium serpentis* [gb AF151376], *Theileria annulata* [gb M64243 M-

34845], *Toxoplasma gondii* [gb X75429], *Oxytricha nova* [gb X03948 M14601], *Paramecium tetraurelia* [gb X03772], *Tetrahymena pyriformis* [gb M98021], *Stylonichia pustulata* [gb M14600], *Haplosporidium costale* [gb U20858], *H. louisiana* [gb U47851], *H. nelsoni* [gb X74131], *Minchinia teredinis* [gb U20319], and *Urosporidium 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 & Nicholas 1997) and taking secondary structure into account, for phylogenetic analysis. Length variable sequences of uncertain positional homology, corresponding with *T. 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 (phylogenetic analysis using parsimony) (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. ostreae* prevalence of 53.5%.

Table 2 illustrates the frequency with which each protocol generated a 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

Table 2. Percentage of detection of a putative *Bonamia ostreae* PCR amplicon in Cork Harbour, Ria de Arosa, and Gun Point Creek 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. ostreae* infections. na: not applicable

Intensity	n	Cork Harbour			Ria de Arosa		Gun Point Creek		Overall	
		Protocol A	Protocol B	Protocol C	n	Protocol C	n	Protocol C	n	Protocol C
Heavy	5	100	100	100	3	100	0	na	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

more sensitive than Protocol A. Oysters diagnosed as heavily infected were always associated with a positive PCR signal for putative *B. ostreae* 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 not detected microscopically were rarely positive (3.0%) for putative *B. ostreae* 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 (Table 2). The putative *B. ostreae* amplicon was detected in every 'heavily', 'moderately', or 'lightly' infected oyster, most (87.5%) 'scarcely' infected oysters, and half of those (50.0%) in which *B. ostreae* was not detected 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 (Table 2).

Overall prevalence of *Bonamia ostreae* at locations examined in this study using PCR Protocol C was greater than that determined using cytology (Fig. 2). In addition, *B. ostreae* has never been detected microscopically in control Spinney Creek *Ostrea edulis*, and it was not detected in these and Great Wicomico River *Crassostrea virginica* using Protocol C (Fig. 2).

Prevalence of putative *Bonamia ostreae* in 37 Gun Point Creek oysters was the same using either gill

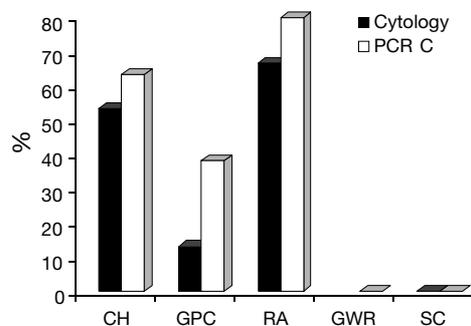


Fig. 2. *Bonamia ostreae* prevalence in 5 *Ostrea edulis* samples and 1 *Crassostrea virginica* sample (GWR) determined using standard cytology and PCR Protocol C. Experimental (known *B. ostreae* enzootic) groups: CH = Cork Harbour; GPC = Gun Point Creek; RA = Ria de Arosa. Control (presumed *B. ostreae*-free) groups: GWR = Great Wicomico River; SC = Spinney Creek

DNA or hemolymph DNA as a PCR template. However, in 3 oysters the gill sample was positive for putative *B. ostreae* and the hemolymph sample was not, and in another 3 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. 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*, *H. nelsoni*, *Minchinia teredinis*, and *H. 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,

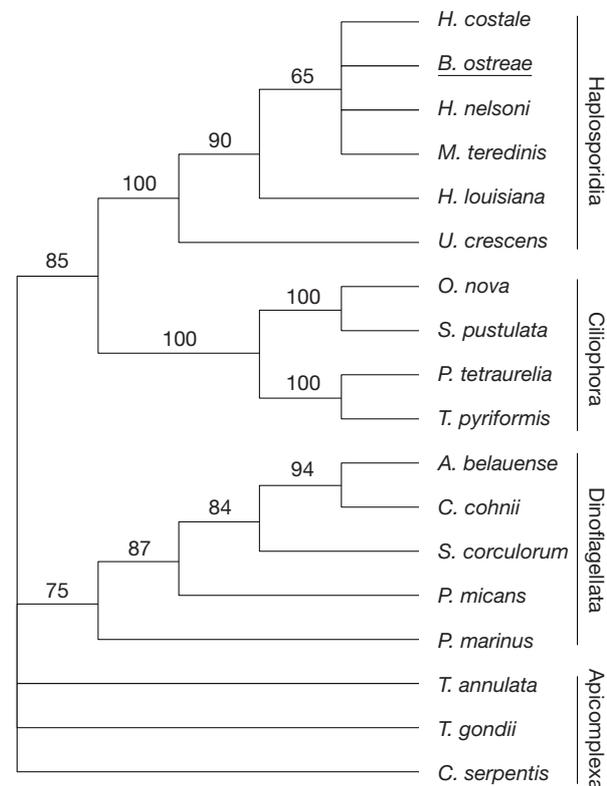


Fig. 3. A single most parsimonious tree, rooted with representatives of the Apicomplexa, resulting from unweighted maximum parsimony analysis. Numbers represent percentages of 100 bootstrap replicates

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<sub>F</sub> and A<sub>R</sub> 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 2 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.

Cytologically 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 & 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 & 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 initially 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%) of 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 al. 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 al. (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 al. 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 8 h [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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