Detection of *Bonamia ostreae* in fixed *Ostrea edulis* tissues by use of specific PCR assays

Anna K. Flood

BSc Biomedical Science

2007
ACKNOWLEDGEMENTS

Several people have contributed for this project to be completed. First I would like to thank my supervisor Dr. Derek Neylan for the support throughout this project and for guiding me through the writing of the thesis. I would also like to thank Dr. Fergus Ryan for all the help with my PCR work.

I wish to thank the Marine Institute of Ireland and Caviston’s Restaurant in Sandycove for providing oyster tissue samples. Many thanks to Daireen Caffrey for supplying reagents and materials and for always being helpful and answering all my questions. I would also like to thank Joanna Fay and the other students in the lab for their encouragement and good advices.

Thanks to Dr. Patrick McHale at D.I.T and Pia Ek and Christina Bittkowski at Uppsala University and for making this exchange possible.

A big thank-you to my friend Louise for sharing this experience with me and for all the fun we have had during our time in Dublin. We did this together!

And finally, a special thanks to my family for always supporting me, giving me inspiration and helping me get through the difficult times. I would not be here without you.
ABSTRACT

Infection by the parasite *Bonamia ostreae* has infected and caused major mortality of the flat oyster, *Ostrea edulis*, over the last 25 years throughout the coasts of Europe and the United States of America. The conventional techniques for the diagnosis of infection with *Bonamia ostreae* are typically by histology and cytology. Both have a low sensitivity and *Bonamia ostreae* in weekly infected oysters can remain undetected when analyzed by such techniques. Molecular methods like the Polymerase Chain Reaction have recently been applied for a more reliable and sensitive detection of *Bonamia ostreae*.

The aim of this project was to optimize a PCR for the specific detection of the 18S Small Ribosomal subunit rDNA gene of *Bonamia ostreae* in formalin fixed *Ostrea edulis* tissues. While the PCR was successfully optimized for purified oyster DNA from fresh tissue it was difficult to apply on formalin fixed oyster tissues due to poor quality DNA from the fixed tissues. Ethanol fixed tissues were also tested for *Bonamia ostreae*, however, the primers were not specific for *Bonamia ostreae* and uninfected oysters also tested positive which led to the conclusion that the PCR could not be used as a reliable detection method for *Bonamia ostreae* in oysters. Despite using alternative primers which were designed to amplify other components of the *Bonamia ostreae* genome no consistent results were achieved to reliably use the PCR method for the accurate detection of *Bonamia ostreae* in oysters. The conclusion of this project is that other genomic sites in *Bonamia ostreae* must be identified as a target for PCR for this test to be specific.

**Keywords:** *Bonamia ostreae, Ostrea edulis, SSU rDNA gene, Polymerase Chain Reaction*
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1. INTRODUCTION

1.1 Ostrea edulis

The European flat oyster, *Ostrea edulis*, is a bivalve mollusc, which means ‘two shells’. Other examples of molluscs are snails, slugs, mussels, cockles and clams. *Ostrea edulis* has an oval or pear-shaped shell with a rough, scaly surface and a pearly inner surface as shown in figures 1.1 and 1.2 (Jackson 2003). The two valves of the shell are different shapes. The left valve is concave and fixed to the seabed and the right valve is flat and sitting inside the left. *Ostrea edulis* can grow up to 110mm long and the oyster cements itself to the seabed. Growth of other nearby molluscs can result in competition for space. This may have an effect on the size and shape of the oyster which can be extremely variable. Oysters are normally found where the seabed is hard. They are often observed in muddy areas attached to debris or any available hard surface. An oyster feeds naturally on suspended organic particles. Feeding is carried out by pumping water through a filter in the gill chamber to remove suspended organic particles (Jackson 2003).

![Figure 1.1](image_url)

*Figure 1.1 The shell of Ostrea edulis is slightly scalloped, beige, yellowish or cream in colour with light brown or bluish concentric bands.*
Oysters can live for as long as 15 years but the typical life span is 5-10 years. The flat oyster starts life as male and becomes mature at around 3 years of age when it starts producing sperm. Oysters are protandrous alternating hermaphrodites which mean that after spawning the male oyster becomes an egg producing female and then it switches back to a male and so on. Eggs produced during the female stage are held in the gills and mantle cavity. The eggs are fertilized by sperm from another animal and drawn in by the inhaled water flow used for feeding and respiration. The fertilized eggs are retained in the mantle cavity for 7-10 days during early development until they become free-swimming larvae. Growth is quite rapid for the first year and a half. It then remains constant at around 20 grams per year before slowing down after five years (Jackson 2003).

*Ostrea edulis* is found all around Britain and Ireland. The main stocks are now in the west coast of Scotland, the south-east of England especially around the River Thames estuary, the Solent, the River Fal, and Lough Foyle in Ireland. The oyster is also found naturally from the Norwegian Sea south through the North Sea down to the Atlantic coast of Morocco. It is also found in the Mediterranean Sea and extends into the Black Sea. The
native *Ostrea edulis* oyster has also been introduced and is cultivated in North America, Australia and Japan. There is an ecological concern with translocation of oysters and other live molluscs as it can be a dispersal mechanism for different parasites into areas where it currently does not occur (Jackson 2003). Translocation of live molluscs is generally recognised as a major underlying cause of the spread of molluscan pathogens and diseases (Berthe et al. 1999, Corbeil et al. 2006).

### 1.2 Bonamia Ostreae

#### 1.2.1 Introduction and distribution

*Bonamia ostreae* is a protozoan parasite that inhabits and multiplies in the haemocytes of the European flat oyster, *Ostrea edulis* (Fisheries research services 2007). The haemocyte disease of *Ostrea edulis* caused by *Bonamia ostreae* is also known as bonamiosis. The disease is notifiable to the Office International des Epizooties, the World Organisation for Animal Health, and is a serious threat to flat oyster aquaculture (Carnegie et al. 2003). *Bonamia ostreae* naturally occurs in *Ostrea edulis* and in some other *Ostrea* species, for example *O. puelchana, O. angasi, O. denselamellosa and O. chilensis*. The Pacific oyster, *Crassostrea Gigas*, also known as the Rock oyster, mussels and clams are not susceptible to infection. *Bonamia ostreae* is not a human health risk; it is only a health problem for the European flat oysters (Office International des Epizooties 2006). Infection by *Bonamia ostreae* has caused an extensive mortality of *Ostrea edulis* throughout European and United States coasts for at least 25 years (da Silva et al. 2005).

Results of initial ultrastructural studies suggested that this parasite was associated with the phylum Haplosporidia (Bonami et al. 1985, Brehélin et al. 1982) which was confirmed by
molecular biological analysis (Carnegie et al. 2000). The haemocyte disease caused by *B. ostreae* had been observed and reported in the 1960’s in California, USA, as an insignificant finding (Katkansky et al. 1969, Elston et al. 1986) but was later described as serious in 1979 after causing catastrophic oyster mortality in Brittany, France (Comps et al. 1980, Pichot et al. 1980, Carnegie et al. 2003).

The geographical distribution of *Bonamia Ostreae* today, as shown in figure 1.3, is primarily Europe (Denmark, France, the Republic of Ireland, Italy, the Netherlands, Spain and the United Kingdom, excluding Scotland), the United States of America (California, Maine and Washington State) and Canada (British Columbia) (Office International des Epizooties 2006).

![Geographical distribution of Bonamia Ostreae](image)

**Figure 1.3 Geographical distribution of Bonamia Ostreae**
1.2.2 The history of Bonamia ostreae

The European oyster Ostrea edulis is an important source of shellfish and has supported a traditional and commercial fishery for centuries. Oysters infected with Bonamia ostreae were first diagnosed in France in 1979 by Comps et al. (1979). The French, in an attempt to improve the genetics of their oyster industry, imported some brood stock from USA, and infections of Bonamia ostreae broke out in the oyster beds in France in 1979 which resulted in a decreased production from 1000 tonnes to almost zero per year (Corbeil et al. 2006b).

The disease had previously been observed and reported in several populations of Ostrea edulis from the southwest coast of North America in the 1960’s (Elston et al. 1986, Cochennec et al. 2000). The use of monoclonal antibodies demonstrated no antigenic differences between Bonamia ostreae isolates originating from Europe and those from the United States (Mialhe et al. 1988b, Cochennec et al. 2000). These results and available documentation of trade and transfer of oysters between California and Europe lead to the hypothesis that the disease described in California in the 1960’s was caused by Bonamia ostreae and that this disease spread from North America to Europe (Elston et al. 1986, Grizel 1997, Cigarria & Elston 1997, Corbeil et al. 2006b, Cochennec et al. 2000). Bonamia ostreae has now spread to a number of other European countries including Spain, the Netherlands, Britain and Ireland (Van Banning 1982, Howard 1994, S. A. Lynch et al. 2006).

1.2.3 The pathology of bonamiosis

Bonamia ostreae is a spherical parasite which measures 2-3 µm in diameter and infects the oyster haemocytes (Fisheries research services 2007). The parasite proliferates within the
haemocytes, which are the effector cells of the oyster immune system. Haemocytes fail to kill the parasite leading to haemocyte destruction and haemocytic infiltration of tissues (Comps 1983, da Silva et al. 2005). These parasites quickly become systemic with large numbers of infected haemocytes leading to the death of the oysters.

The complete life cycle of *Bonamia ostreae* is not known but the parasite can be transmitted directly from oyster to oyster. The parasite passes from one infected oyster via the water to other nearby oysters which take it in during feeding or respiration (Elston et al. 1986). Once the oyster is exposed to *Bonamia ostreae*, it takes 4-6 weeks for an infection to be observed. (Culloty and Mulcahy 1996, S. A. Lynch et al. 2006). It has also been showed that it is possible to infect healthy naïve oysters by cohabitation with infected oysters (Culloty et al. 1999). Infections probably begin when the haemocytes phagocytise *Bonamia ostreae* cells that have penetrated the gill epithelium. The parasite proliferates in the haemocytes and disperses throughout the oyster. With eventual death of the host, *Bonamia ostreae* passes via the water to nearby oysters, and the cycle begins anew (Bucke 1988, Montes et al. 1994, Carnegie et al. 2003).

Infection in oysters rarely results in clinical signs of disease and many infected oysters appear normal. Some infected oysters may show a yellow to black discolouration and extensive lesions, i.e. perforated ulcers, on the gills and mantle (Office International des Epizooties, 2006). Some oysters die with light infections, and heavily infected oysters have a tendency to be in poorer condition than uninfected oysters. In early infections, *Bonamia ostreae* is often associated with a central haemocyte infiltration in the connective tissue of the gill and mantle. Later infections will result in an increased number of infected haemocytes (Fisheries research services 2007).
The disease can occur throughout the year but there is a seasonal variation in infection by *Bonamia ostreae*. The highest prevalence in the Northern Hemisphere is in September with the highest mortality usually occurring at water temperatures of 12°-20°C. In the Southern Hemisphere, *Bonamia ostreae* shows the highest prevalence from January to April while the parasite is barely detectable in September and October (Office International des Epizooties 2006).

### 1.2.4 Prevention of bonamiosis

There is no applicable treatment for oysters infected by *Bonamia ostreae* but different strategies have been tried to minimise the effect of this disease or to eliminate it and to recover the natural oyster beds (Office International des Epizooties, 2006). In Holland, in an unsuccessful attempt to eradicate the disease, oysters were methodically removed from beds and oyster deployment in affected areas was banned (van Banning 1988 & 1991, da Silva et al. 2005).

In France, a plan combining zootechnical prophylaxis and eradication measures was performed (Grizel et al. 1986, da Silva et al. 2005) but the effect was limited and eventually, the oyster industry in France, the country that supported the highest world production of *Ostrea edulis*, replaced this species by the non-susceptible oyster *Crassostrea gigas*.

In Galicia, Spain, the oyster industry was forced to shift to a strategy involving the introduction of oysters from other countries which were grown for a short period (less than 1 year), and harvested before bonamiosis caused mortality (da Silva et al. 2005).

Present studies are involving development of Ostrea edulis lines selected for Bonamia ostreae resistance (Carnegie et al. 2004).
1.3 Diagnostic techniques for the detection of *Bonamia ostreae*

1.3.1 Current diagnostic techniques

Standard diagnosis of infection with *Bonamia ostreae* today is generally achieved by histology and cytology techniques which are currently recommended for screening purposes by the Office International des Epizooties. When mortalities occur, various presumptive diagnostic methods can be used in addition to histology. When a pathogen is encountered, electron microscopy should be used for specific identification (Office International des Epizooties 2006).

When *Bonamia ostreae* is diagnosed by histology, samples are assessed by classical histological methods such as the haematoxylin-eosin staining method. It is recommended that two sections per oyster are examined. The parasite may occur within the haemocytes or extracellularly, however for a positive diagnosis to be made the parasite must be observed within the haemocytes (see figure 1.4) (Office International des Epizooties 2006).

![Image of Bonamia ostreae in haemocytes](image.png)

*Figure 1.4* *Bonamia ostreae* (arrows) in haemocytes within an accumulation of haemocytes in the connective tissue of a heavily infected Ostrea edulis. The parasites appear as very small cells of 2-5 μm wide. Haematoxylin and eosin stain.
For cytological examination, sections (imprints) of oyster heart tissue are put on glass histological sides, air-dried and fixed in methanol. The sections are stained using a commercially available staining kit such as Hemacolor® staining. The parasite has basophilic cytoplasm and an eosinophilic nucleus. The parasite may be observed inside or outside the haemocytes (see figure 1.5). The parasites are enlarged by this method as they are spread on the slide and results can be compared with those observed with histological examination (Office International des Epizooties 2006).

![Figure 1.5 Bonamia ostreae within haemocytes (arrows) and extracellular (arrow heads) in a heart imprint from a heavily infected Ostrea edulis. They appear as very small cells (2-5 μm wide) with a basophilic cytoplasm and an eosinophilic nucleus. Hemacolor® stain.](image)

1.3.2 Molecular diagnostic techniques

Conventional techniques such as histology and cytology used for the detection of *Bonamia ostreae* are time consuming, requires highly trained staff and have a low sensitivity (Berthe et al. 1999, da Silva & Villalba 2004, Corbeil et al. 2006) The small size of *Bonamia ostreae* makes it difficult to recognize sub clinical infections in thin sections, and several investigators have reported trouble detecting *Bonamia ostreae* at low levels (Bucke & Feist
1985, Bucke 1988, McArdle et al. 1991, Carnegie et al. 2000). It has been suggested that low–level infections of *Bonamia ostreae* may well remain undetected in oyster samples tested by such techniques (Diggles et al. 2003, Corbeil et al. 2006). The sustainability of oyster farming and the management of bonamiosis in wild and cultured oyster populations depend on early and sensitive warning diagnostic methods to diagnose the presence of the parasite. The development of diagnostic assays which are more sensitive and specific than traditional histological and cytological techniques is important for the management of bonamiosis in the flat oysters *Ostrea edulis* (Carnegie et al. 2000).

Immunoassays for the detection of *Bonamia ostreae* infection of oysters held a great early promise (Mialhe et al. 1988; Boulo et al. 1989; Cochennec et al. 1992). Monoclonal antibodies were designed for *Bonamia ostreae* from Europe, however, the antibodies developed for *Bonamia ostreae* in Europe reacted weakly or not at all with *Bonamia ostreae* from populations outside of Europe (Zabaleta & Barber 1996, Carnegie et al. 2000). It was suggested that serological differences between *Bonamia ostreae* strains could limit the usefulness of antibody based techniques (Carnegie et al. 2004).

DNA probes and the Polymerase Chain Reaction (PCR) have been recently introduced for the detection of *Bonamia ostreae* and are also promising new insights into the life cycle, transmission, and diversity of *Bonamia ostreae*. The Polymerase Chain Reaction (PCR) is now a well established molecular technique used to amplify short regions of a DNA strand. This can be a single gene, just a part of a gene or a non-coding sequence. PCR copies the process of DNA replication and can amplify millions of times a sequence of DNA. The PCR reaction is carried out in small reaction tubes (0.2-0.5ml volumes), containing a reaction volume of typically 25μl and requires some basic components. The DNA template
contains the region of the DNA fragment to be amplified and primers are synthetic oligonucleotides constructed so that they are complementary to the DNA region that is to be amplified. Taq DNA polymerase is the most common enzyme used to synthesize a DNA copy of the region to be amplified and Deoxynucleotide triphosphates, dNTPs is the material from which the Taq polymerase builds the new DNA. The reaction also requires a buffer solution which provides a suitable chemical environment for optimum activity and stability of the Taq polymerase (Reed et al. 2003). Initiation of the PCR takes place when the primers are allowed to hybridize (anneal) to the single strands of the target DNA, followed by enzymatic extension of the primers by the Taq polymerase. A single PCR cycle consists of three steps, carried out at different temperatures as follows:

1. Denaturation of DNA template by heating to 94-98°C separating the individual stands of the target DNA
2. Annealing of the primers, this occurs when the temperature is reduced to 55-65°C
3. Extension of the primers by the Taq polymerase at 72°C

The temperature changes in PCR are normally achieved using a computerized thermal cycler, which is an incubator block that can be programmed to vary temperatures, incubation times and cycle numbers (Reed et al. 2003).

1.3.3 The ribosomal RNA gene and specific Bonamia ostreae PCR primers

The development of PCR by Cochennec et al. (2000) to detect the small subunit (SSU) ribosomal RNA gene of Bonamia ostreae provided a better and more sensitive method for the detection of Bonamia ostreae infection in flat oysters.
The ribosomal RNA gene is DNA that produces ribosomal RNAs. This is one of the few genes that do not produce a protein. The ribosomal RNAs produced by the ribosomal RNA gene are needed in great quantities by the cell so these genes are repeated genes which mean that thousands of copies are placed side by side. The ribosomal gene repeat is shown in figures 1.6 and 1.7. The 18S gene product (RNA, not a protein) combines with proteins to form the small ribosomal subunit of the ribosome. The 5.8S RNA gene product, the 5S gene product and the large subunit RNA gene product combine with proteins to form the large ribosomal subunit of the ribosome (Reed et al. 2003).

**Figure 1.6** The ribosomal gene repeat containing the 18S gene, the 5.8S gene, the large subunit RNA gene, the 5S gene and the non-coding Internal Transcribed Spacer (ITS) regions 1 and 2.
The ribosomal gene repeat. The 18S gene product combined with proteins form the small ribosomal subunit and the 5.8S gene product, the 5S gene product and the large subunit RNA gene product (27S RNA) combined with proteins form the large ribosomal subunit.

The 18S gene is conserved and changes very slowly while the Internal Transcribed Spacer 1 (ITS-1) region and the Internal Transcribed Spacer 2 (ITS-2) region are variable non-coding DNA sequences which accumulate mutations much faster than conserved genes. (Reed et al. 2003) The ITS regions are often very useful when amplified by PCR assays too confirm relation between species within a genus and to locate specific sequences for detection of a certain species.

Sequence comparison of the 18S gene and the ITS-1 region of the small subunit ribosomal gene has helped clarify the taxonomic position of Bonamia isolates and has led to the development of diagnostic methods capable of detecting and distinguishing different isolates of Bonamia. (Carnegie & Cochennec 2004, Corbeil et al. 2006b).

Cochennec et al. (2000) and Carnegie et al. (2000) presented a “Bonamia ostreae-specific” PCR assay. The specific PCR primers, BO (forward) and BOAS (reverse), used to detect Bonamia ostreae in these studies are shown in figure 1.8. The BO/BOAS primers are amplifying a 300bp sequence of the Bonamia ostreae 18S ribosomal RNA gene. To obtain
the entire ITS-1 region the primers Sbo (forward) and Ra58 (reversed), originally described by Le Roux et al. (1999) can be used (see figure 1.8). The reverse ITS-1 primer Ra58 could not be found within the sequence shown in figure 1.8 so the size of the amplified ITS-1 product is unknown.

Figure 1.8 Nearly complete small subunit ribosomal DNA sequence of Bonamia ostreae, Genebank [AF262995]. 18S ribosomal RNA gene and ITS-1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence. The primers BO (forward) and BOAS (reverse) are marked with yellow colour. Marked with blue colour is the ITS-1 primer Sbo (forward).
1.3.4 Aim of project

The aim of this project is to optimize a Polymerase Chain Reaction for the specific detection of *Bonamia Ostreae* in fixed *Ostrea edulis* tissues. DNA will be extracted from fixed *Ostrea edulis* tissues using an in-house protocol and a commercial DNA extraction kit and will be compared for the DNA extraction efficiency. Purified DNA will be optimized for PCR. Two different primer pairs will be used for the specific detection of *Bonamia Ostreae*, the BO/BOAS which amplify a sequence of the 18S ribosomal RNA gene and the Sbo/Ra58 which amplify the ITS-1 region. It is hoped that a standardized and specific PCR will be developed for the detection of *Bonamia Ostreae*. 
2. MATERIAL AND METHODS

2.1 Materials

2.1.1 DNA and tissue samples used in the study

Purified DNA and fixed tissue samples from *Ostrea edulis* infected or uninfected with *Bonamia ostreae* were provided from the Marine Institute, Blanchardstown, Ireland. The different samples used were Purified DNA, Formalin fixed DNA and Ethanol fixed DNA. Fresh flat oysters, *Ostrea edulis*, and Pacific rock oysters, *Crassostrea Gigas* were also purchased from Cavistons fish shop, Sandy Cove, County Dublin. All DNA and the reagents required were stored at -20ºC.

2.1.2 In-house DNA extraction

- 3M Sodium acetate
- Chloroform
- Digestion solution (0.05M Tris-HCl, 0.2M NaCl, 0.05M EDTA, 1% SDS and 0.05mg/ml Proteinase K at pH 8)
- Ethanol 100%
- Ethanol 70%
- PBS (Phosphate Buffered Saline)
- Phenol:Chloroform:ISOamylalcohol (49:49:2)
- TE buffer (10mM Tris and 1mM EDTA at pH 8).
2.1.3 QIAamp DNA mini kit extraction

- QIAamp DNA mini kit, QIAGEN, cat. No 51304, lot. No 127128163

2.1.4 Fresh oyster tissue preparation

- Ethanol 100%
- PBS

2.1.5 Polymerase Chain Reaction

- 10 X PCR buffer, Invitrogen, 10342-053, lot. No 1329152A, conc. 10X
- Autoclaved MilliQ water
- dNTPs, Invitrogen, cat. No 10297-018, lot. No 1220153, conc. 100nM each
- Magnesium Chloride (MgCl₂)
- MgCl₂, Invitrogen, cat. No 10342-053, lot. No 1329152A, conc. 50mM
- Taq polymerase, Invitrogen, cat. No 10342-053, lot. No 1329152A, conc. 500 units

2.1.6 PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Manufacturer</th>
<th>Nucleotide sequence</th>
<th>Tm</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>BO (forward)</td>
<td>PROLIGO</td>
<td>CATTTAATTGTCGGGCGGC</td>
<td>62°C</td>
<td>58°C</td>
</tr>
<tr>
<td>BOAS (reverse)</td>
<td>PROLIGO</td>
<td>CTGATCGCTCTCGATCCCC</td>
<td>64°C</td>
<td>58°C</td>
</tr>
<tr>
<td>Sbo (forward)</td>
<td>SIGMA</td>
<td>CAAGCTGGTTGATCCTGCC</td>
<td>64°C</td>
<td>63°C</td>
</tr>
<tr>
<td>Ra58 (reverse)</td>
<td>SIGMA</td>
<td>CGCATTTCGCTGCGTTTC</td>
<td>70°C</td>
<td>63°C</td>
</tr>
<tr>
<td>BO sequencing (forward)</td>
<td>PROLIGO</td>
<td>CACGACGTGTAAACGACATT</td>
<td>62°C</td>
<td>58°C</td>
</tr>
<tr>
<td>BOAS sequencing (reverse)</td>
<td>PROLIGO</td>
<td>GGATAACAATTTCACACAGGCT</td>
<td>64°C</td>
<td>58°C</td>
</tr>
</tbody>
</table>
2.1.7 Detection of PCR products by electrophoresis

- 1X Tris-Boarate-EDTA (TBE) buffer
- 6X Loading buffer (0.25% Xylene Cyanole, 0.25% Bromophenol blue, 30% Glycerol in water)
- Agarose D-1 LowEEO, CONDA, cat. No 8016, lot. No H091035
- DNA size ladder, Invitrogen, 100 bp
- Ethidium bromide (0.5µg/ml)
- MilliQ water
2.2 Methods

PCR was performed with two different sets of primers to detect *Bonamia ostreae* DNA in *Ostrea edulis* tissues. The specific amplification of the *Bonamia ostreae* 18S ribosomal RNA gene was optimized for purified DNA samples from infected and uninfected *Ostrea edulis* with the primer pair BO and BOAS (Originally described by Cochennec at al. (2000). The pure DNA samples were provided from the Marine Institute and the DNA had been extracted using ProteinaseK and phenol:chloroform:isoamylalcohol extraction.

To apply the optimized BO/BOAS PCR on fixed *Ostreae edulis* tissues DNA was extracted from formalin and ethanol fixed oyster tissues using an in-house method and a QIАamp DNA mini kit from QIAGEN.

A different set of primers, Sbo/Ra58 (Le Roux et al. 1999), was used to evaluate the specificity and sensitivity of the BO/BOAS primers. The Sbo/Ra58 PCR amplified the Internal Transcribed Spacer 1 (ITS-1) region of *Bonamia ostreae*. DNA isolated from fresh oysters was also used to determine the specificity and sensitivity of the primers.

2.2.1 DNA extraction with in-house method

DNA was extracted from formalin and alcohol fixed oyster tissues with an in-house protocol, originally described by Carnegie et al. 2000. Approximately 0.1g of oyster gill tissue was weighed and washed with PBS. The tissue was put on a microscopic slide and two scalpel blades were used to cut the tissue into smaller fragments. The tissue was digested with 200µl of digestion solution (0.05M Tris-HCl, 0.2M NaCl, 0.05M EDTA, 1% SDS and 0.05mg/ml Proteinase K at pH 8) in an eppendorf tube overnight at 55°C and more Proteinase K was added if required.
A standard Phenol extraction protocol was used for the extraction of DNA. 200µl of Phenol:Chloroform:isoamylalcohol (49:49:2) was added to the digested tissue and the tube was vortexed and centrifuged for 1 minute. The aqueous phase containing the DNA was transferred to a fresh tube. An equal amount (200µl) of chloroform was added and the mix centrifuged for 1 minute. The aqueous phase containing the DNA was transferred to a fresh tube. 1/10 volume of 3M Sodium acetate was added and after vortexing, 2.2 volumes of 100% ethanol was added and the mixture was vortexed again. The tube was incubated at –70ºC for 30 minutes and followed by centrifugation for 10 minutes. The supernatant was aspirated and the pellet was washed twice with 70% ethanol and air dried for 5-10 minutes. The pellet was resuspended in 20µl of TE buffer and stored at –20ºC.

2.2.2 DNA extraction with QIAamp DNA mini kit

DNA was extracted from formalin and ethanol fixed oyster tissues and from fresh oyster tissues with a QIAamp DNA mini kit (QIAGEN). DNA from approximately 20-30mg of tissue was extracted using the QIAamp DNA mini kit according to the manufacturer’s instructions. The QIAamp DNA mini kit lysis buffer contained Proteinase K and a spin procedure was used where DNA was bound to minicolumns, eluted and resuspended in a final volume of 100µl of buffer. The DNA was stored at –20ºC prior to PCR amplification.

2.2.3 DNA extraction from fresh Ostrea edulis tissues

The upper valve of each oyster was removed and gill tissue was collected and prepared for analysis. At dissection the hinge was located and a knife inserted between the shells. To open the oyster a knife was worked back and forward and slide across the top of the shell to cut the adductor muscle holding the shells together. Once the oyster was opened it was
instantly put in to 100% ethanol for 5 minutes. Gill tissue was identified (see figure 2.1), collected and stored in 100% ethanol for future DNA isolation and PCR amplification.

Figure 2.1 Location of oyster gills

2.2.4 Estimation of DNA concentration by agarose gel electrophoresis

To estimate the concentration and integrity of the genomic DNA extracted from all *Ostrea edulis* tissues the DNA was visualized on a 1% agarose gel by electrophoresis. The DNA samples were prepared by adding 2µl of 6X loading buffer to 1 µl, 2µl or 10µl of extracted DNA. Samples were loaded into the wells of the agarose gel and electrophoresed at 125V, 100mA for approximately 45 minutes.

2.2.5 BO/BOAS PCR amplification

The BO/BOAS primers were used to amplify a 300bp sequence of the *Bonamia ostreae* 18S ribosomal RNA gene. The PCR assay was applied on DNA samples extracted from fixed and fresh *Ostrea edulis* tissues. To evaluate the specificity and sensitivity of the
primers, uninfected oyster DNA, weakly and strongly *Bonamia ostreae* infected oyster DNA and human DNA were used. Sterile water was used as a negative control.

The BO/BOAS PCR was performed in a 25-ul vial containing 1µl of template DNA mixed with 5µl of 5X PCR buffer containing MgCl₂ (final concentration 5,5mM), 0.5µl of 10mM dNTPs, 0.5µl of each 100ng/µl primer and 0.2µl (1 unit) of Taq DNA polymerase. The thermocycling conditions are shown in table 2.1.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
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<tbody>
<tr>
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<tr>
<td>95°C</td>
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<tr>
<td>75°C</td>
<td>5 min</td>
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</tbody>
</table>

35 cycles

*Table 2.1 Thermocycling conditions for the BO/BOAS PCR reaction.*

### 2.2.6 Sbo/Ra58 PCR amplification

The Sbo/Ra58 primers were used to amplify the Internal Transcribed Spacer 1 (ITS-1) region of *Bonamia ostreae*. The product size was unknown. The PCR assay was applied on DNA samples extracted from fixed and fresh *Ostrea edulis* tissues. To evaluate the specificity and sensitivity of the primers, uninfected oyster DNA and weakly and strongly *Bonamia ostreae* infected oyster DNA was used. Sterile water was used as a negative control.

The Sbo/Ra58 PCR was performed in a 25-ul vial containing 1µl of template DNA mixed with 5µl of 5X PCR buffer containing MgCl₂ (final concentration 1,5mM), 0.5µl of 10mM
dNTPs, 0.5µl of each 100ng/µl primer and 0.2µl (1 unit) of Taq DNA polymerase. The thermocycling conditions are shown in table 2.2.

<table>
<thead>
<tr>
<th>Temperature</th>
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<tbody>
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<tr>
<td>72°C</td>
<td>1 min</td>
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<tr>
<td>75°C</td>
<td>5 min</td>
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</tbody>
</table>

35 cycles

**Table 2.2 Thermocycling conditions for the Sbo/Ra58 PCR reaction.**

### 2.2.7 Optimisation of PCR reactions

To accomplish an effective PCR protocol with optimal conditions for the primers to bind and for the Taq DNA polymerase to synthesize DNA, an initial protocol had to be optimized by varying different conditions in the PCR reaction. Stock solutions of 5X PCR buffer containing different concentrations of MgCl₂ were prepared and used for MgCl₂ titration. Different annealing temperatures, number of cycles in the PCR reaction and different template DNA concentrations were evaluated.

### 2.2.8 Detection of PCR products by agarose gel electrophoresis

All PCR products were resolved by electrophoresis in 1.5% agarose gels in 1X TBE buffer. 1.5g of agarose powder was mixed with 100ml of 1X TBE buffer to achieve the concentration of 1.5%. Agarose gels were stained with 5µl ethidium bromide (0.5µg/ml) and photographed under UV light to initiate visualization of DNA products after electrophoresis at 125 V for approximately 45 minutes.
3. RESULTS

3.1 Optimisation of the BO/BOAS primers for purified *Ostrea edulis* DNA

3.1.1 Evaluation of the DNA quality from purified DNA samples

To evaluate the quality and intensity of the purified DNA provided by the Marine Institute, 1µl of uninfected, weakly infected and highly infected *Ostrea edulis* DNA were electrophoresed on a 1% agarose gel according to the method described in paragraph 2.2.4. The results are shown in figure 3.1.

Figure 3.1 shows that all three samples, uninfected, weakly infected and highly infected *Ostrea edulis*, contained high molecular weight DNA with no visible degradation and would therefore be suitable for PCR.
3.1.2 PCR amplification from purified DNA samples

To find out if the specific 300bp *Bonamia ostreae* sequence could be amplified from the purified DNA samples using the BO/BOAS primers, a PCR was set up according to a protocol described by Cochennec et al. (2000). A MgCl2 concentration of 2.5mM was used and the annealing temperature was 58ºC. The PCR products were electrophoresed on a 1.5% agarose gel and the results are shown in figure 3.2.

As seen in figure 3.2 no DNA products were visible on the gel. The negative control was negative (lane 5).

Figure 3.2

1 - 100 bp DNA size marker  
2 - Uninfected Ostrea edulis DNA  
3 - DNA from weakly infected Ostrea edulis  
4 - DNA from highly infected Ostrea edulis  
5 - Negative control, MilliQ water
3.1.3 MgCl₂ titration of purified DNA samples

In order to optimize the conditions for the PCR reaction in paragraph 3.1.2, a MgCl₂ titration was performed on the purified DNA samples. The MgCl₂ concentrations used for each sample were 0, 2, 4 and 6mM. The products were run on a 1.5% agarose gel and the results are shown in figure 3.3.

Figure 3.3

1 - 100 bp DNA size marker 8 - DNA from weakly infected Ostrea edulis (4mM MgCl₂)
2 - Uninfected Ostrea edulis DNA (0mM MgCl₂) 9 - DNA from weakly infected Ostrea edulis (6mM MgCl₂)
3 - Uninfected Ostrea edulis DNA (2mM MgCl₂) 10 – DNA from highly infected Ostrea edulis (0mM MgCl₂)
4 - Uninfected Ostrea edulis DNA (4mM MgCl₂) 11 - DNA from highly infected Ostrea edulis (2mM MgCl₂)
5 - Uninfected Ostrea edulis DNA (6mM MgCl₂) 12 - DNA from highly infected Ostrea edulis (4mM MgCl₂)
6 - DNA from weakly infected Ostrea edulis (0mM MgCl₂) 13 - DNA from highly infected Ostrea edulis (6mM MgCl₂)
7 - DNA from weakly infected Ostrea edulis (2mM MgCl₂) 14 - 100 bp DNA size marker

Figure 3.3 is showing very strong bands of the expected size for the highly positive sample at all MgCl₂ concentrations. As seen in lane 8, 4mM MgCl₂ gave the best result for the weakly infected sample. An unexpected weak band could be seen for the uninfected sample at a MgCl₂ concentration of 6mM in lane 5. However this band is not visible on the photograph of the gel. All negative controls containing 0mM MgCl₂ were negative.
3.1.4 MgCl₂ titration of weakly positive purified DNA sample

To further investigate the best MgCl₂ concentration for the weakly positive sample a broader range of MgCl₂ concentrations were used in a titration. The concentrations went from 0mM to 6mM with an interval of 0.5. The products were electrophoresed on a 1.5% agarose gel and the result is shown in figure 3.4.

Figure 3.4 shows that a 300bp product was amplified at MgCl₂ concentrations from 3mM up to 6mM. The MgCl₂ concentration that gave the best result for the weakly infected sample was 5.5mM, see lane 13.
3.1.5 MgCl$_2$ titration of uninfected purified DNA sample

A weak band was observed for the uninfected sample at a concentration of 6mM MgCl$_2$ in figure 3.4. To determine whether that was due to contamination a MgCl$_2$ titration was performed. The range of MgCl$_2$ went from 4mM to 10mM. Weakly and highly positive purified DNA samples were used as positive controls at 5.5mM MgCl$_2$. Results are shown in figure 3.5.

![Figure 3.5](image)

**Figure 3.5**

1 - 100 bp DNA size marker  
2 - Negative control, MilliQ water  
3 - DNA from uninfected Ostrea edulis (4mM MgCl$_2$)  
4 - DNA from uninfected Ostrea edulis (5mM MgCl$_2$)  
5 - DNA from uninfected Ostrea edulis (6mM MgCl$_2$)  
6 - DNA from uninfected Ostrea edulis (7mM MgCl$_2$)  
7 - DNA from uninfected Ostrea edulis (8mM MgCl$_2$)  
8 - DNA from uninfected Ostrea edulis (9mM MgCl$_2$)  
9 - DNA from uninfected Ostrea edulis (10mM MgCl$_2$)  
10 - DNA from weakly infected Ostrea edulis (5.5mM MgCl$_2$)  
11 - DNA from highly infected Ostrea edulis (5.5mM MgCl$_2$)  
12 - 100 bp DNA size marker

Figure 3.5 confirms that the negative sample provided from the Marine Institute was positive with MgCl$_2$ concentrations between 5mM and 10mM (lanes 4-9). Results for positive and negative controls were as expected.
3.1.6 PCR amplification from purified DNA samples with optimized protocol

Figure 3.3 and 3.4 show that a MgCl$_2$ concentration of 5.5mM is suitable for weakly and highly infected samples to be amplified with the BO/BOAS PCR. These conditions were used to establish the optimized PCR protocol described in paragraph 2.2.5. Uninfected, weakly infected and highly infected *Ostrea edulis* DNA was analysed in according to this protocol. Products were electrophoresed on a 1.5% agarose gel and the results are shown in figure 3.6.

The results in figure 3.6 show the optimized conditions for the PCR assay using the primers BO/BOAS to amplify the specific 300bp *Bonamia ostreae* sequence. A strong band was observed for the highly infected sample (lane 5) and a slightly weaker band for the weakly infected sample (lane 4). The uninfected sample and the negative control were both negative.
3.2 BO/BOAS PCR amplification from DNA extracted with in-house method from formalin fixed Ostrea edulis tissues

3.2.1 Evaluation of the DNA quality from formalin fixed DNA extracted with the in-house method.

DNA was extracted from formalin fixed Ostrea edulis tissues using an in-house protocol as described in paragraph 2.2.4 in the methods section. To evaluate the quality and intensity of the DNA extracted from formalin fixed Ostrea edulis tissues with this method, 4µl of uninfected and infected samples were electrophoresed on a 1% agarose gel. The results are shown in figure 3.7.

![Figure 3.7](image)

**Figure 3.7**

1 - 100 bp DNA size marker
2 - DNA extracted from uninfected Ostrea edulis tissue
3 - DNA extracted from infected Ostrea edulis tissue

Figure 3.7 shows highly degraded DNA for both samples.
3.2.2 PCR amplification from formalin fixed DNA samples using the optimized BO/BOAS protocol

Despite the highly degraded DNA shown in figure 3.7 the uninfected and infected sample were analysed according to the optimized protocol for the BO/BOAS PCR described in paragraph 2.2.5. A positive purified DNA sample was used as a positive control. The products were electrophoresed on a 1.5% agarose gel and the result is shown in figure 3.8.

![Image of gel with band at 300bp for positive control in lane 3, and no products in lanes 4 and 5.](image)

**Figure 3.8**

1 - 100 bp DNA size marker  
2 – Negative control, MilliQ water  
3 – Positive control, DNA from weakly infected Ostrea edulis  
4 - DNA isolated from uninfected Ostrea edulis tissue  
5 - DNA isolated from infected Ostrea edulis tissue

Figure 3.8 shows a very weak band at 300bp for the positive control in lane 3, again poorly visible in the photograph of the gel. No products were observed for the uninfected and infected DNA samples extracted from formalin tissues (lanes 4 and 5). The negative control was negative.
3.2.3 Optimisation of PCR amplification for formalin fixed DNA samples

In an attempt to optimize the conditions for the formalin fixed DNA samples in the BO/BOAS PCR the template DNA was diluted 1:2, 1:5 and 1:10 and then analysed according to the standard protocol described in paragraph 2.2.5. A positive purified DNA sample was used as a positive control. The products were electrophoresed on a 1.5% agarose gel and the results are shown in figure 3.9.

As shown in figure 3.9 a 300 bp product was amplified for the positive control in lane 9. No bands could be seen for any of the diluted DNA samples. The negative control was negative.
3.2.4 Optimisation of PCR amplification from formalin fixed DNA samples

In another attempt to optimize the PCR conditions for the formalin fixed DNA samples, the amplicons from the PCR assay in paragraph 3.2.3 were used as template DNA in a new PCR according to the method described in paragraph 2.2.5. A positive purified DNA sample was used as a positive control. The products were electrophoresed on a 1.5% agarose gel and the results are shown in figure 3.10.

![Figure 3.10](image)

**Figure 3.10**

1 - 100 bp DNA size marker  
2 - Negative control, MilliQ water  
3 – Amplicon from uninfected Ostrea edulis tissue (1:2)  
4 – Amplicon from uninfected Ostrea edulis tissue (1:5)  
5 – Amplicon from uninfected Ostrea edulis tissue (1:10)  
6 – Amplicon from infected Ostrea edulis tissue (1:2)  
7 – Amplicon from infected Ostrea edulis tissue (1:5)  
8 – Amplicon from infected Ostrea edulis tissue (1:10)  
9 – Positive control, DNA from weakly infected Ostrea edulis  
10 - 100 bp DNA size marker

As shown in figure 3.10 the PCR amplification generated a weak DNA fragment of the expected size 300bp for the positive control in lane 9 but no results were obtained for any of the PCR amplicons. The negative control was negative.
3.3 BO/BOAS PCR amplification of DNA extracted with QIAamp DNA mini kit from formalin fixed *Ostrea edulis* tissues

3.3.1 Evaluation of the DNA quality from formalin fixed DNA extracted with QIAamp DNA mini kit

DNA was extracted from formalin fixed tissues with a QIAamp DNA mini kit to examine if less degraded and higher quality DNA could be provided which would be more suitable for PCR. 10µl of uninfected and infected samples was electrophoresed on a 1% agarose gel according to the method described in paragraph 2.2.4 and the results are shown in figure 3.11.

![Figure 3.11](image)

**Figure 3.11**

1 - 100 bp DNA size marker  
2 - DNA isolated from uninfected *Ostrea edulis* tissue  
3 - DNA isolated from infected *Ostrea edulis* tissue

Figure 3.11 shows no visible DNA for either of the samples.
3.3.2 MgCl₂ titration of formalin fixed samples extracted with QIAamp DNA mini kit

Despite that no visible DNA was observed in 3.3.1 a MgCl₂ titration was performed to establish whether PCR products could be amplified from the DNA extracted with the QIAamp DNA mini kit. Uninfected and infected DNA samples were analyzed according to paragraph 2.2.5. The range of MgCl₂ went from 3mM to 10mM for each sample. Weakly and highly positive purified DNA samples were included as positive controls at 5.5mM MgCl₂. The products were electrophoresed on a 1.5% agarose gel and the results are shown in figure 3.12.

**Figure 3.12**

1 - 100 bp DNA size marker  
2 - Negative control, MilliQ water  
3 - Uninfected Ostrea edulis DNA (3mM MgCl₂)  
4 - Uninfected Ostrea edulis DNA (4mM MgCl₂)  
5 - Uninfected Ostrea edulis DNA (5mM MgCl₂)  
6 - Uninfected Ostrea edulis DNA (6mM MgCl₂)  
7 - Uninfected Ostrea edulis DNA (7mM MgCl₂)  
8 - Uninfected Ostrea edulis DNA (8mM MgCl₂)  
9 - Uninfected Ostrea edulis DNA (9mM MgCl₂)  
10 - Uninfected Ostrea edulis DNA (10mM MgCl₂)  
11 – Infected Ostrea edulis DNA (3mM MgCl₂)  
12 – Infected Ostrea edulis DNA (4mM MgCl₂)  
13 – Infected Ostrea edulis DNA (5mM MgCl₂)  
14 – Infected Ostrea edulis DNA (6mM MgCl₂)  
15 – Infected Ostrea edulis DNA (7mM MgCl₂)  
16 – Infected Ostrea edulis DNA (8mM MgCl₂)  
17 – Infected Ostrea edulis DNA (9mM MgCl₂)  
18 - Infected Ostrea edulis DNA (10mM MgCl₂)  
19 - DNA from weakly infected Ostrea edulis (5.5mM MgCl₂)  
20 - DNA from highly infected Ostrea edulis (5.5mM MgCl₂)
As seen in figure 3.12 a product of 300bp was amplified and observed for the weakly and the highly infected positive controls (lanes 19 and 20). Visible products of the right size were seen for the infected samples (lanes 13-18). The concentration of MgCl$_2$ that gave the best result for the infected sample was 5mM as seen in lane 13. Some unspecific bands could be seen on the gel. Surprisingly, very weak bands were also observed for the uninfected sample (lanes 5 and 6) but the negative control was negative.
3.3.3 MgCl₂ titration of formalin fixed samples extracted with QIAamp DNA mini kit

A new MgCl₂ titration was performed to further investigate if the best MgCl₂ concentration for the formalin fixed DNA samples extracted with the QIAamp DNA mini kit was 5mM as seen in figure 3.12. MgCl₂ concentrations of 5mM and 5.5mM were used for uninfected and infected samples according to the method described in paragraph 2.2.5. Weakly and highly positive purified DNA samples were used as positive controls at 5.5mM MgCl₂. The products were electrophoresed on a 1.5% agarose gel and the result is shown in figure 3.13.

![Figure 3.13](image)

**Figure 3.13**

1 - 100 bp DNA size marker
2 - Negative control, MilliQ water
3 - Uninfected Ostrea edulis DNA (5mM MgCl₂)
4 - Uninfected Ostrea edulis DNA (5.5mM MgCl₂)
5 – Infected Ostrea edulis DNA (5mM MgCl₂)
6 - Infected Ostrea edulis DNA (5.5mM MgCl₂)
7 - DNA from weakly infected Ostrea edulis (5.5mM MgCl₂)
8 - DNA from strongly infected Ostrea edulis (5.5mM MgCl₂)

Figure 3.13 is showing expected results, 300bp products, for both positive controls (lanes 7 and 8). A barely visible product was observed for the infected formalin fixed DNA sample at a MgCl₂ concentration of 5.5mM in lane 6. The negative control was negative.
3.3.4 PCR amplification from formalin fixed DNA samples using the optimized protocol

In one last attempt to amplify the specific *Bonamia ostreae* sequence with the BO/BOAS primers from formalin fixed tissues, four different positive samples and four different negative samples were analyzed according to the standard protocol described in paragraph 2.2.5. Weakly and highly positive purified DNA samples were used as positive controls. The products were electrophoresed on a 1.5% agarose gel and the result is shown in figure 3.14.

![Image of gel](image_url)

**Figure 3.14**

1 - 100 bp DNA size marker  
2 - Negative control, MilliQ water  
3 - Uninfected Ostrea edulis DNA  
4 - Uninfected Ostrea edulis DNA  
5 - Uninfected Ostrea edulis DNA  
6 - Uninfected Ostrea edulis DNA  
7 - Infected Ostrea edulis DNA  
8 – Infected Ostrea edulis DNA  
9 – Infected Ostrea edulis DNA  
10 – Infected Ostrea edulis DNA  
11 - DNA from weakly infected Ostrea edulis  
12 - DNA from highly infected Ostrea edulis  
13 - 100 bp DNA size marker

Figure 3.14 shows strong bands for both positive controls (lanes 11 and 12). Only one infected sample out of four was positive on the gel, a weak band was observed for the infected sample in lane 9. The negative control was negative.
3.4 BO/BOAS PCR amplification from DNA extracted with QIAamp DNA mini kit from ethanol fixed and fresh *Ostrea edulis* tissues.

3.4.1 Evaluation of the DNA quality from ethanol fixed DNA extracted with QIAamp DNA mini kit

To see if ethanol fixed tissues could give a better PCR than formalin fixed tissues DNA was extracted from ethanol fixed tissues with a QIAamp DNA mini kit. 1μl of two uninfected samples, two weakly infected samples and one highly infected sample were electrophoresed on a 1% agarose gel according to the method described in paragraph 2.2.4 and the results are shown in figure 3.15.

![Figure 3.15](image)

1 - 100 bp DNA size marker
2 – Highly infected *Ostrea edulis* DNA
3 – Weakly infected *Ostrea edulis* DNA
4 - Weakly infected *Ostrea edulis* DNA
5 - Uninfected *Ostrea edulis* DNA
6 - Uninfected *Ostrea edulis* DNA

Figure 3.15 shows highly degraded DNA for all ethanol fixed samples. The gel was also overloaded with DNA.
3.4.2 PCR amplification from ethanol fixed DNA samples using the optimized protocol

Despite having degraded DNA from the ethanol fixed tissues two uninfected samples, two weakly infected samples and one highly infected sample were analyzed for PCR using the same method as used for formalin fixed tissue (see paragraph 2.2.5). Weakly and highly positive purified DNA samples were used as positive controls as well as a negative control. The products were electrophoresed on a 1.5% agarose gel and the result is shown in figure 3.16.

As shown in figure 3.16 a strong band at 300bp was observed for the highly infected sample in lane 3. Strong bands were observed for the weakly positive samples (lanes 4 and 6) and surprisingly, strong bands were also seen for the two uninfected samples (lanes 5 and 7). The negative control was negative. Both positive controls gave strong expected bands at 300bp (not shown in figure).
3.4.3 PCR amplification from ethanol fixed DNA samples using the optimized protocol

Surprisingly, the results in figure 3.16 show that a 300bp sequence was amplified for all ethanol fixed samples, even those that were supposedly negative samples. To confirm this result, four additional negative samples were provided by the Marine Institute and were analysed according to the method described in paragraph 2.2.5. The products were electrophoresed on a 1.5% agarose gel and the result is shown in figure 3.17.

Figure 3.17 shows that a 300bp sequence was amplified and observed for all four negative samples. Some non specific bands were also observed for sample number 4.
3.4.4 Evaluation of the DNA quality from fresh oyster DNA extracted with QIAamp DNA mini kit

To estimate the sensitivity and specificity of the BO/BOAS PCR and find out why all alcohol fixed samples came out positive, a negative oyster control had to be established. Therefore, flat oysters, *Ostrea edulis*, and Pacific rock oysters, *Crassostrea Gigas* were purchased from a local restaurant and DNA was extracted according to paragraph 2.2.2 and 2.2.3. 1µl of two flat oyster DNA samples and two rock oyster DNA samples were electrophoresed on a 1% agarose gel according to paragraph 2.2.4 and the results are shown in figure 3.18.

As seen in figure 3.18 degraded DNA was observed for all fresh oyster samples.
3.4.5 PCR amplification from ethanol fixed DNA samples and fresh oyster samples using the optimized protocol.

The DNA extracted from fresh flat- and rock oyster tissues was amplified in the BO/BOAS PCR according to paragraph 2.2.5 and the results are shown in figure 3.18. The ethanol fixed DNA samples shown in figure 3.15 were also analysed in the same reaction. Weakly and highly positive purified DNA samples were used as positive controls. The products were electrophoresed on a 1.5% agarose gel and the results are shown in figure 3.19.

Figure 3.19

1 - 100 bp DNA size marker
2 – Negative control, MilliQ water
3 – Fresh flat oyster DNA
4 - Fresh flat oyster DNA
5 - Fresh rock oyster DNA
6 - Fresh rock oyster DNA
7 - Highly infected Ostrea edulis DNA
8 - Weakly infected Ostrea edulis DNA
9 - Uninfected Ostrea edulis DNA
10 - Weakly infected Ostrea edulis DNA
11 – Uninfected Ostrea edulis DNA
12 – DNA from weakly infected Ostrea edulis
13 - DNA from highly infected Ostrea edulis
Figure 3.19 shows that the negative control was negative and bands of expected size and intensity were seen for both positive controls. A 300bp sequence was amplified and observed for the 5 alcohol fixed samples (lanes 7-11), which was the same result as shown in figure 3.16. Surprisingly, all fresh oyster DNA samples did also generate positive results. A 300bp product was visible for the DNA extracted from the flat oysters (lanes 3 and 4) while a smaller size product of approximately 200bp was observed for the DNA extracted from the rock oyster (lanes 5 and 6). Some unspecific products were also observed. The negative control was negative.
3.4.6 Optimisation of PCR amplification used for ethanol fixed DNA samples and fresh oyster samples

In an attempt to evaluate the specificity and quality of the BO/BOAS primers a different set of primers were used for comparison. These primers were BO/BOAS sequencing primers. A human DNA control was also added to the reaction and the same PCR as used in figure 3.19 was performed. The products were electrophoresed on a 1.5% agarose gel and the results are shown in figure 3.20.

**Figure 3.20**

1 - 100 bp DNA size marker
2 – Negative control MilliQ water
3 – Fresh flat oyster DNA
4 - Fresh flat oyster DNA
5 - Fresh rock oyster DNA
6 - Fresh rock oyster DNA
7 - Highly infected Ostrea edulis DNA
8 - Weakly infected Ostrea edulis DNA
9 - Uninfected Ostrea edulis DNA
10 – Human DNA control
11 – Negative control MilliQ water (seq.primers)
12 – Fresh flat oyster DNA (seq.primers)
13 - Fresh flat oyster DNA (seq.primers)
14 – Fresh rock oyster DNA (seq.primers)
15 – Fresh rock oyster DNA (seq.primers)
16 – Highly infected Ostrea edulis DNA (seq.primers)
17 – Weakly infected Ostrea edulis DNA (seq.primers)
18 – Uninfected Ostrea edulis DNA (seq.primers)
19 – Human DNA control (seq.primers)
20 - 100 bp DNA size marker (seq.primers)
Figure 3.20 shows the results for the normal BO/BOAS primers (lanes 1-10) and for the BO/BOAS sequencing primers (lanes 11-20). The results for the different primer pairs corresponded with each other; however the bands were slightly weaker for the sequencing primers. Both ethanol fixed and fresh oyster DNA generated positive results and the product for the fresh rock oysters had a different size, approximately 200bp instead of 300bp. No result was seen for the human DNA control (lanes 10 and 19) and the negative controls were negative.
3.5 Optimisation of the Sbo/Ra58 PCR for DNA extracted with QIAamp DNA mini kit from ethanol fixed and fresh Ostrea edulis tissues.

3.5.1 MgCl₂ titration of strongly positive ethanol fixed DNA sample

Since the specificity of the two sets of primers indicated non specific amplification of Bonamia ostreae another set of primers was selected for PCR. The primer pair, Sbo/Ra58, which amplifies the ITS-1 region of Bonamia ostreae was used to optimize a PCR assay. The size of the Sbo/Ra58 product was unknown. The aim was to compare the results from the ITS-1 PCR with the results from the BO/BOAS PCR assays. In order to optimize the conditions for the ITS-1 PCR a MgCl₂ titration was performed on a highly positive ethanol fixed DNA sample according to the method described in paragraph 2.2.6. The MgCl₂ concentrations used were 0, 1, 1.5, 2, 2.5, 3 and 4mM and the annealing temperature was 61 ºC. The products were run on a 1.5% agarose gel and the results are shown in figure 3.21.

![Figure 3.21](image)

**Figure 3.21**

1 - 100 bp DNA size marker
2 - DNA from highly infected Ostrea edulis (0mM MgCl₂)
3 - DNA from highly infected Ostrea edulis (1mM MgCl₂)
4 - DNA from highly infected Ostrea edulis (1.5mM MgCl₂)
5 - DNA from highly infected Ostrea edulis (2mM MgCl₂)
6 - DNA from highly infected Ostrea edulis (2.5mM MgCl₂)
7 - DNA from highly infected Ostrea edulis (3mM MgCl₂)
8 - DNA from highly infected Ostrea edulis (4mM MgCl₂)
As shown in figure 3.21 two strong DNA fragments were observed at approximately 250bp which presumable could be the ITS-1 region of *Bonamia ostreae*. Strong bands were also seen at higher molecular weights. The optimal MgCl₂ concentration which generated less unspecific products was at 1mM and 1.5mM (lanes 3 and 4). The negative control containing 0mM MgCl₂ was negative (see lane 2).
3.5.2 PCR amplification from ethanol fixed DNA samples

To evaluate the optimal annealing temperature for the ITS-1 primers and to try and remove the double band observed in figure 3.21, the annealing temperature was increased and 63°C and 65°C were tested. The reactions were performed on highly positive and weakly positive ethanol fixed DNA samples according to paragraph 2.2.6. The products were run on a 1.5% agarose gels. Results are shown in figures 3.22 and 3.23.

No results were obtained for 65°C annealing temperature as shown in figure 3.22. Figure 3.23 shows that a single strong band was amplified for the highly infected sample in lane 3 at 63°C but no band was seen for the weakly positive sample. The negative controls in figure 3.22 and 3.23 were negative. These results show that doing the PCR at 63°C resulted in a single band for the highly infected sample at approximately 250bp. The double band as shown in figure 3.21 was eliminated.
3.5.3 PCR amplification from ethanol fixed DNA samples and fresh oyster samples

The DNA extracted from uninfected fresh oyster tissues that surprisingly gave positive results when analysed with the BO/BOAS primers, see figure 3.19, were analysed using the ITS primers according to the method described in paragraph 2.2.6. Four different negative ethanol fixed DNA samples also generated positive results in the BO/BOAS PCR, see figure 3.17, and were included in the PCR. The products were run on a 1.5% agarose gel and the results are shown in figure 3.24.

Figure 3.24 shows that no products were amplified for the fresh oyster DNA samples (lanes 7-10). A weak product of approximately 250bp was observed for two negative ethanol fixed DNA samples out of four (lanes 5 and 6). The negative control was negative.
3.5.4 MgCl₂ titration of weakly positive ethanol fixed DNA sample

To investigate why no band was seen for the weakly positive sample in figure 3.23 a MgCl₂ titration was performed according to paragraph 2.2.6. The MgCl₂ concentrations used were 0, 1, 1.5, 2, 2.5, 3, 4 and 5mM and the annealing temperature was 63 °C. The products were run on a 1.5% agarose gel and the results are shown in figure 3.21.

In contrast to the previous PCR results, figure 3.25 shows that two bands of high molecular weight were seen for the MgCl₂ concentrations 2-5mM (lanes 5-9). The size of the products differs significantly when compared with the ones obtained for the highly positive sample in figure 3.21. The negative control was negative.
4. DISCUSSION

The aim of this project was to optimize a Polymerase Chain Reaction for the specific detection of *Bonamia Ostreae* in fixed *Ostrea edulis* tissues. Two extraction methods were evaluated for DNA extraction efficiency for fixed and fresh oyster tissues. The BO/BOAS primer pair was used to amplify the 18S small subunit ribosomal gene sequence of *Bonamia ostreae*.

4.1 Optimisation of the BO/BOAS amplification

The optimal conditions for the BO/BOAS reaction were achieved when the initial protocol described by Cochennec et al. (2000) was modified. Purified infected and uninfected *Ostreae edulis* DNA samples were used for this purpose. A range of different MgCl₂ concentrations were evaluated for uninfected and highly and weakly infected DNA samples. Instead of adding PCR buffer and MgCl₂ separately into the premix, stock solutions of 5x PCR buffer containing different concentrations of MgCl₂ were made. The consequence of this was more invariable results due to a reduced amount of pipetting errors. The protocol was also easier to reproduce since the same buffer could be used for different reactions.

Figures 3.3 and 3.4 show that the optimum MgCl₂ concentration for both highly and weakly infected oyster DNA was approximately 5.5mM. At this concentration the PCR amplification generated a strong DNA fragment of the expected size, 300bp.

The uninfected *Ostrea edulis* sample showed a negative result at all MgCl₂ concentrations except for 6mM where a very weak band at 300bp was observed, see figure 3.3. The same sample was confirmed positive in figure 3.5 which eliminated that contamination could be
the cause. This sample had previously been confirmed as negative by the Marine Institute when it was analysed by histological techniques. These issues raised the question whether the BO/BOAS PCR was amplifying unspecific 18S ribosomal gene sequences or if the PCR was superior to histological techniques when it came to detecting low-level infections of *Bonamia ostreae*.

The results in figure 3.6 show the optimized conditions for the BO/BOAS PCR assay. A sequence of expected size, 300bp, was amplified and observed as a strong band for the highly infected sample and as a slightly weaker band for the weakly infected sample. No band was seen for the uninfected sample. Even if no band was observed for the uninfected sample, an internal *Ostrea edulis* control would have confirmed the accuracy of the PCR. Such control is desirable for future studies.

### 4.2 BO/BOAS amplification from formalin fixed *Ostrea edulis* tissues

One goal of this project was to establish the optimized BO/BOAS PCR protocol for formalin fixed oyster tissues. DNA was extracted from formalin fixed tissues using an in-house method and a DNA extraction kit in order to evaluate for DNA extraction efficiency for different methods.

Figure 3.7 shows highly degraded DNA for the samples extracted with the in-house method. DNA was probably damaged and degraded during formalin fixation and that is the explanation of the poor quality DNA obtained. No PCR products were visible when these samples were amplified with the BO/BOAS PCR (see figure 3.8) even though different concentrations of the template DNA were tested (see figures 3.9 and 3.10). This again
illustrates the importance of an internal *Ostrea edulis* control to confirm that oyster DNA was amplified even though no *Bonamia ostreae* DNA was amplified.

The quality of the DNA extracted from formalin fixed tissues with the QIAamp DNA extraction kit is shown in figure 3.11. The figure shows no visible DNA for either of the samples but the reason for that was probably that the DNA was diluted too much when it was eluted in the kit. A MgCl$_2$ titration was performed to evaluate the best MgCl$_2$ concentration for the formalin fixed DNA samples extracted with the DNA extraction kit and the optimum concentration as shown in figure 3.13 was 5.5mM, the same as in the optimized BO/BOAS protocol. All results from BO/BOAS PCR amplification of the formalin fixed DNA samples extracted with the DNA extraction kit were very inconsistent. For example, in figure 3.14, only one infected sample out of four came out positive and the band was very weak.

The DNA extraction kit demonstrated a higher DNA extraction efficiency than the in-house method since no in-house DNA products were amplified in PCR. However, inconsistent and poor results were obtained from the PCR amplification of DNA extracted with the kit as well.

The BO/BOAS sequence of 300bp could be difficult to amplify from highly degraded DNA extracted from formalin fixed tissues. To establish a *Bonamia ostreae* specific PCR for formalin fixed *Ostrea edulis* tissues, primers which amplify shorter PCR products might be required. A recommendation from this project is to try and develop smaller and more specific primers when trying to detect *Bonamia ostreae* in formalin fixed tissues.
Another concern about formalin fixed tissues, apart from DNA degradation, is that the extracted DNA may contain a very small amount of *Bonamia ostreae* DNA, especially at low-level infections. This can be the reason why false negative results are obtained in PCR amplification. It is also important to extract DNA from the most infected parts of the oyster tissues. Different parts of tissue from the same oyster were examined in this study to evaluate the importance of extracting DNA from the right section but no difference was observed (results not shown).

### 4.3 BO/BOAS amplification from ethanol fixed *Ostrea edulis* tissues

Due to the difficulties of using DNA extracted from formalin fixed tissues for PCR amplification, the same protocol was applied on ethanol fixed *Ostrea edulis* tissues. DNA was extracted from ethanol fixed tissues using the DNA extraction kit as it was proved to give the highest DNA extraction efficiency. The results in figure 3.15 show degraded DNA for all samples although the smear of degraded DNA on the gel presumable indicated some high molecular weight DNA.

Surprisingly, all ethanol DNA samples including the uninfected samples came out positive for *Bonamia ostreae* in the BO/BOAS PCR amplification as shown in figures 3.16 and 3.17. The samples had previously been analysed by histological techniques by the Marine Institute. The Marine Institute confirmed that some of the alcohol fixed samples had been infected with other Haplosporidian species before and some had been collected from an oyster bed near *Bonamia ostreae* infected areas.

Based on these facts, low-level or sub-clinical *Bonamia ostreae* infections could have been present in the ethanol fixed samples that were confirmed as uninfected by histological
techniques. The explanation why all ethanol samples are coming up positive could be that the BO/BOAS PCR was able to detect *Bonamia ostreae* more sensitively than the techniques used by the Marine Institute. 

Another possible explanation is that the BO/BOAS primers amplified unspecific products. Comparisons of the BO/BOAS 300bp sequence with public databases using BLAST confirmed that the sequence showed high similarity to other species and was not specific for the *Bonamia ostreae* 18S ribosomal gene. Other oyster parasites, mollusc diseases or bacteria could have caused unspecific amplification. Some of the alcohol fixed samples had been infected with other Haplosporidian species before. This could have promoted unspecific amplification of the 18S ribosomal gene from other species apart from *Bonamia ostreae*.

To estimate the sensitivity and specificity of the BO/BOAS primers and find out why all alcohol fixed samples came out positive, a negative oyster control had to be established. Therefore, flat oysters, *Ostrea edulis*, and Pacific rock oysters, *Crassostrea Gigas* were purchased from a local restaurant and DNA was extracted using the DNA extraction kit.

The unexpected PCR result from the fresh oyster DNA samples is shown in figure 3.19 where all samples were positive. The fresh oysters should not be infected since they are sold in a fish restaurant. Either is the disease of *Bonamia ostreae* more wide-spread than thought or the BO/BOAS primers are very unspecific. Contamination during DNA extraction could also be an explanation but is unlikely as high precautions were taken during tissue preparation for this project.
The size of the product amplified from the fresh rock oyster was smaller, approximately 250bp (see figure 3.19), compared with the normal BO/BOAS product of 300bp which was seen for the fresh flat oyster. The explanation could be that the primers are not specific for the *Bonamia ostreae* 18S ribosomal gene.

To further investigate the specificity and quality of the primers, a human DNA control was added to the PCR reaction and the normal BO/BOAS primers were compared with the BO/BOAS sequencing primers which would also amplify a product of 300bp. The results in figure 3.20 show that the human DNA control was negative and the results for the different primer pairs corresponded with each other. All ethanol fixed samples were still positive.

In future studies the BO/BOAS specificity has to be evaluated for other species, for example other *Bonamia* species and other molluscs. All products obtained in this project should be sequenced to confirm if the 18S ribosomal gene of *Bonamia ostreae* was amplified.

### 4.4 Sbo/Ra58 amplification from ethanol fixed and fresh *Ostrea edulis* tissues

In another attempt to evaluate the specificity and sensitivity of the BO/BOAS primers, the primer pair, Sbo/Ra58, which amplifies the ITS-1 region of *Bonamia ostreae* was used. The reverse ITS-1 primer Ra58 could not be found within the sequence shown in figure 1.8 so the size of the amplified ITS-1 product was unknown.
When a MgCl₂ titration was performed for a highly infected sample two strong DNA fragments were observed at approximately 250bp as shown in figure 3.21. The amplified sequence is probably the ITS-1 region of *Bonamia ostreae*.

The results from the BO/BOAS PCR amplification could not be compared with the results from the Sbo/Ra58 amplification due to the unknown product size of the latter sample and to limited time for optimisation of the Sbo/Ra58 PCR protocol.

### 4.5 Conclusion

The conclusion of this project is that:

- The QIAamp DNA extraction kit demonstrated a higher DNA extraction efficiency compared with the in-house method.
- The BO/BOAS primers could successfully be optimized for purified *Ostrea edulis* DNA samples.
- The BO/BOAS protocol could not be applied on formalin fixed *Ostrea edulis* tissues due to poor DNA quality and unpredictable amplification.
- Ethanol fixed *Ostrea edulis* tissues and fresh oyster tissues were successfully amplified in the BO/BOAS PCR, however all samples including uninfected samples were positive for *Bonamia ostreae* due to unspecific amplification.
- The BO/BOAS primers are not specific for the *Bonamia ostreae* 18S ribosomal gene and can not be used as an individual detection method for *Bonamia ostreae* infected oysters, *Ostrea edulis*.
- The ITS-1 primers Sbo/Ra58 need to be further established for the detection of *Bonamia ostreae*. 
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