FINAL REPORT

Aquatic Animal Health Subprogram: development of molecular diagnostic expertise for the mollusc pathogen *Bonamia* sp.

Serge Corbeil and Mark St. J. Crane

June 2004

FRDC Project No. 2003/622

Australian Government
Department of Agriculture, Fisheries and Forestry

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NON-TECHNICAL SUMMARY

2001/624	Aquatic Animal Health Subprogram: development of molecular diagnostic expertise for the mollusc pathogen *Bonamia* sp.

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OBJECTIVES:

1. To develop and validate real-time TaqMan PCR assay for the detection of *Bonamia* species in mollusc tissues.

2. To develop strategies and primers for conventional PCR amplification and sequencing of DNA products (amplicons) that will allow the differentiation of the Australian *Bonamia* from the exotic *Bonamia* spp.

3. To establish the detection limit of the TaqMan PCR assay.

4. To establish a reference collection of material (mollusc tissues) infected with exotic *Bonamia* species.

NON-TECHNICAL SUMMARY:

OUTCOMES ACHIEVED

The major outcomes of this project are: (1) development of reagents and procedures that are capable of detecting and identifying the various known species of *Bonamia*; (2) development of a capability to distinguish between the various known species of *Bonamia*, (3) demonstration that *Bonamia* sp. (NSW isolate) in Australia is different from both the New Zealand and the Northern Hemisphere isolates of *Bonamia* spp., and (4) development of a draft Australian and New Zealand Standard Diagnostic Procedure (ANZSDP) for the detection and identification of *Bonamia* spp.

Bonamia sp. is a serious pathogen of oysters that can cause significant losses in cultured oysters. The development/establishment of an internationally recognised standard diagnostic technique for the detection and identification of *Bonamia* sp. and related pathogens will permit State/Australian Government agencies to establish accurate information on the presence or absence of these pathogens in oyster populations. Thus the use of these procedures will play an important role in the management of any disease outbreak caused by *Bonamia* sp. or other *Bonamia* species. Identification of infected and uninfected stock will allow industry

and State officers to implement a disease management plan based on accurate information. Isolation of infected and uninfected oyster populations will be possible thus enhancing our capability to control and/or eradicate the disease.

Furthermore, State diagnostic laboratories will benefit by being provided with sensitive and specific reagents and procedures that have been validated using both exotic and enzootic isolates. Based on accurate diagnoses, State officials responsible for conducting activities of the Local Disease Control Centre (LDCC) will be able to make informed decisions during implementation of disease management procedures.

Infections with *Bonamia ostrea* and *B. exitiosa* are listed by the OIE and, as a Member State, Australia is obliged to report on their presence or absence in Australia. Such reporting can only be achieved if there are sensitive and specific reagents and procedures in place that are recognised by international agencies. Using internationally accepted procedures, Australia can be confident of providing accurate information to the OIE, other international agencies and to our international trading partners.

Sequence data comparing the Australian *Bonamia* isolate with other exotic isolates will assist in determining the relationship between the Australian isolate and exotic isolates. Such data will be useful in identifying any new variants that may occur in the future as well as identifying any incursion of exotic isolates. Again, this type of information will allow appropriate management procedures to be put in place, as required. To date, it has been shown that the NSW isolates of *Bonamia* sp. are different from the exotic isolates reported from New Zealand and Northern Hemisphere countries.

KEYWORDS: ***Bonamia*; oysters; diagnostic procedures; phylogeny**

ACKNOWLEDGEMENTS

Assistance from State Government officials in NSW Fisheries, PIRSA and DPIWE Tasmania who have provided samples for analysis is gratefully acknowledged. In addition, the authors acknowledge the collaborative effort with the OIE Reference Laboratory for Mollusc Diseases (Ifremer, La Tremblade, France) and, in particular, the willing and friendly collaboration with the OIE laboratory staff (Drs Isabelle Arzul and Franck Berthe). Also, the authors acknowledge the assistance of Dr Susan Bower (Pacific Biological Station, Canada) and Dr Nancy Stokes (Virginia Institute for Marine Science, USA) who provided DNA from related haplosporidian organisms.

BACKGROUND

Some cultured and wild oysters in Australia are known to harbour *Bonamia* sp., a pathogenic protistan. Infections with *Bonamia ostrea* and *B. exitiosa* are listed by the Office Internationales des Epizooties (OIE). *Bonamia* sp. has caused mortalities in *Ostrea angasi* in Victoria, Tasmania and Western Australia (Hine, 1996). To date, Australia has limited diagnostic capability (histology and electron microscopy) for this pathogen.

With regards to control of mollusc diseases, access to diagnostic tests that are rapid, reliable and sensitive is of fundamental importance. Hence, laboratories around the world are now developing disease-monitoring programs based on DNA diagnostic techniques for the most economically important pathogens (Berthe, 2002). DNA is a useful molecule to target for diagnostic procedures because its sequence does not usually vary with the life cycle of the pathogen. Furthermore, some genes, such as the small-subunit ribosomal DNA genes are often useful targets because there are multiple copies in the genome, which ensures increased sensitivity of the developed test (Berthe *et al*., 1999).

NEED

Australian edible mollusc production has recently increased, reaching a total gross value of ca. $70,000,000 in 2000/2001. The growing world-demand for aquaculture products opens export opportunities for Australia's aquaculture sector (Australian Fisheries Statistics, 2001). However, translocation of live molluscs is often associated with pathogen spread with resulting disease outbreaks having detrimental effects on production, trade and the ability to open new overseas markets (Berthe and Boudry, 1999). A rapid and reliable diagnostic capability (expertise and molecular tools) for the detection and identification of the most economically important mollusc pathogens worldwide is not currently available in Australia. Such diagnostic capability is necessary to ascertain the presence or absence of *Bonamia* sp. a notifiable pathogen in domestically farmed oysters. It is essential to develop both expertise and modern molecular assays (e.g. PCR, gene sequencing (Berthe *et al*., 1999)) for identification and comparison of the *Bonamia* sp. present in Australia with overseas *Bonamia* spp. (Pichot *et al*., 1980; Campalas *et al*., 2000; Diggles *et al*., 2003). Without development of this expertise, including the required reagents and procedures, it will be difficult to establish a health certification and surveillance program for the mollusc aquaculture industry. Moreover, these resources are essential for development of strategies for disease management in Australia.

It is anticipated that significant outcomes of the proposed project will include development of reagents and procedures for the specific and sensitive detection and identification of *Bonamia* spp. from oyster stocks. Furthermore, the use of molecular tools such as PCR and gene sequencing will allow for the comparison of *Bonamia* sp. isolate in Australia with exotic isolates. This information will form the basis for the development of a Molecular Standard Diagnostic Technique for this aquatic animal pathogen, as part of AQUAPLAN.

OBJECTIVES

1. To develop and validate real-time TaqMan PCR assay for the detection of *Bonamia* species in mollusc tissues.

2. To develop strategies and primers for conventional PCR amplification and sequencing of DNA products (amplicons) that will allow the differentiation of the Australian *Bonamia* from the exotic *Bonamia* spp.

3. To establish the detection limit of the TaqMan PCR assay.

4. To establish a reference collection of material (mollusc tissues) infected with exotic *Bonamia* species.

METHODS

Importation of Reference Materials

All diagnostic tests established during the course of this project were applied to the exotic isolates of *Bonamia* as well as the NSW isolate.

Exotic isolates of *Bonamia* spp.
Overseas laboratories were contacted with a request to provide reference isolates of *Bonamia* spp.. Import permits (permit #200216314 & #200404064) were obtained from AQIS.

Fixed oyster tissues (*Ostrea edulis*) infected with exotic isolates of *Bonamia ostreae* isolates from France, Ireland and the Netherlands was kindly supplied by the OIE Reference Laboratory (Drs Isabelle Arzul and Franck Berthe, Ifremer, La Tremblade, France). Fixed oyster tissues (*Tiostrea chilensis*) infected with exotic isolates of *Bonamia exitiosa* were provided by Dr Ben Diggles (DigsFish Pathology Services Ltd, New Zealand) and Dr Mike Hine (MAF, New Zealand).

Purified and fixed *Bonamia ostreae* cells were supplied by the OIE Reference Laboratory (Drs Isabelle Arzul and Franck Berthe, Ifremer, La Tremblade, France).

DNAs from related Haplosporidian organisms was provided by Dr Susan Bower (Pacific Biological Station, Canada) and Dr Nancy Stokes (Virginia Institute for Marine Science, USA).

Australian isolate of *Bonamia*
Fixed tissues from *Bonamia*-infected flat oysters (*Ostreae angasi*), grown in NSW, were provided by Dr Symon Dworjanyn (NSW Fisheries, Port Stephens).

PCR Assays for the Identification and Differentiation of *Bonamia* Isolates

PCR analysis, using *Bonamia*-specific primers (Cochennec *et al*. 2000), was undertaken on each of the reference isolates. Two single-step PCR assays, targeting the 18S gene and the internal transcribed region (ITS1) of the rRNA operon were performed on the extracted DNA.

***Bonamia* DNA extraction**
DNA from *Bonamia*-infected and uninfected oyster tissues was extracted using a QiaAmp DNA minikit (Qiagen) according to the manufacturer's instructions. DNA bound to minicolumns was eluted then resuspended in a final volume of 100µL of sterile deionised water. Extracted DNA was diluted 1 in 10 before being used in conventional PCR analysis or real-time TaqMan assay specific for *Bonamia*.

PCR amplification of the rRNA 18S gene and ITS1 region
Samples for PCR analysis were prepared from infected oyster tissues. Twelve pairs of primers were used to amplify overlapping fragments covering the full length of the 18S gene (1601 base pairs). One primer pair (Bo/Boas), used, was originally described by Cochennec *et al*. (2000). Other primers are listed in table

1. The complete ITS1 region was amplified using the primers ITS-7 for/ITS-7 rev as well as ITS-8 for/ITS8 rev (Table 1).

Ethanol-fixed tissues (mantle and gills) were rinsed in phosphate buffered saline (PBS) and then DNA was extracted by using a QiaAmp DNA minikit (Qiagen) according to the manufacturer's instructions. DNA was eluted and resuspended in a final volume of 100µL of sterile deionised water.

Taq DNA polymerase (Promega) was used for PCR. In all PCRs, the primers were used at a final concentration of 1µM. Aliquots (2µL) of DNA sample were added to 23µL of reaction mixture for the amplification. Thermo cycling conditions for the 18S gene PCR were: 95^{o}C for 2 min (1 cycle), followed by 94^{o}C for 30 sec, 55^{o}C for 45 sec and 72^{o}C for 45 sec (40 cycles), followed by 72^{o}C for 5 min (1 cycle). PCR cycling condition for the ITS region were: 95^{o}C for 2 min (1 cycle), 94^{o}C for 30 sec, 50^{o}C for 45 sec and 72^{o}C for 45 sec (40 cycles), followed by 72^{o}C for 5 min (1 cycle). The resulting amplicons were resolved by electrophoresis in 2% agarose gels in 1X TAE (Tris-acetate 0.004M, EDTA 0.001M) buffer. Gels were stained with ethidium bromide (0.5 µg mL^{-1}).

Table 1. Primer sequences used for amplifying *Bonamia* 18S gene

Name	Sequence 5'-3'
18S For-A	TGCATGTCCAAGTATAAACACG
18S Rev-A	AACGCGATCCGACAAAATA
18S For-B	TCGCGGGAGTGCATATTAG
18S Rev-B	GGATGTGGTAGCCGTTTCTC
18S For-C	TGAGAAACGGCTACCACATC
18S Rev-C	GGGGGCAGATATCCAACTAC
18S For-D	CGACTAAGCATTGGGCTACC
18S Rev-D	GTGGGGTCTCGTCCGTTAT
18S For-E	CGAGACCCCACCCATCTAAC
18S Rev-E	CAAAGGGCAGGGACGTAATC
18S For-F	GCTGTTAAAACGCTCGTAGTTG
18S Rev-F	ATCATTACTCCAGCTCAAAACCA
18S For-G	CGGGCCAGAGGTAAAATTC
18S Rev-G	CCCCCTGAGTCCGAAAAC
18S For-J	GTGCATGGCCGTTCTTAGTC
18S Rev-J	TTGCCCCAATCTTCCATCT
18S For-M	GAATGCGGGTTCGATTC
18S Rev-M	TTGGGTAATTTGCGCACC
18S For-N	CTTCGGCGCCGCCAC
18S Rev-N	GCTTTCGCATAAGTTAG
ITS For-7	ATTACGTCCCTGCCCTTTGT
ITS Rev-7	ACGAGGTTCGCGGTTTTTAT
ITS For-8	CGTAACAAGGTTTCCGTAGGT
ITS Rev-8	TGCTTTTTGCGTTTGTGTAGT

Comparison of the Nucleic Acid Sequence of the Australian Isolate with Sequences of Known Exotic Isolates of *Bonamia*

Sequence Determination

18S rRNA gene sequence and ITS region sequence were determined by direct sequencing of the PCR product. Sequencing was carried out using an ABI PRISM Ready Reaction Big dye® Termination Cycle Sequencing Kit (Perkin-Elmer) and an ABI PRISM model 377XL DNA sequencer (Sequencing Analysis 3.4.1 Software, version 2.6) (Perkin-Elmer).

Restriction Fragment Length Polymorphism

Bonamia isolates can be differentiated by digesting (with the restriction enzymes Bgl1 and HaeII) the PCR products amplified by the Bo-Boas primers (Cochennec *et al*., 2000). The mix is incubated at 37°C for 90 min (5 µL of PCR product, 1µL of restriction enzyme, 2µL buffer 10X, 12µL of water). Analysis of the digestion is facilitated by resolving the amplicons on a 2% polyacrylamide gel stained with ethidium bromide and observed under U.V. light.

Diagnostic Real Time PCR TaqMan Assay

The TaqMan technology was chosen for the development of a rapid, sensitive and reliable molecular diagnostic assay specific for *Bonamia*.

TaqMan Primers and Probe

An ABI Prism®7700 Sequence Detection System and software Sequence Detector version 1.9 (PE Applied Biosystems) were used for the analysis and storage of data. Primers and probe for the multiplex TaqMan assay were designed using Primer Express Software version 1.5 (PE Applied Biosystems). The *Bonamia* primers and probe were based on the ITS-1 of the rRNA operon, a relatively conserved genomic region among *Bonamia* isolates. Primer and probe sequences were as follows:

Forward primer (ITS-For): 5'-CCCTGCCCTTTGTACACACC-3';
Reverse primer (ITS-Rev): 5'-TCACAAAGCTTCTAAGAACGCG-3';
6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA), labelled probe (Bon ITS): 6FAM-TTAGGTGGATAAGAGCCGC-TAMRA

The ITS primers were used at a final concentration of 900nM. The 18S endogenous control primers, used to validate the DNA extraction procedure, were used at a final concentration of 113nM. The ITS FAM probe and the 18S control VIC probe were used at a final concentration of 250nM and 31nM, respectively. The reactions were carried out in a 96-well plate in a 25-µL-reaction volume containing 12.5µL Universal Master Mix (PE Applied Biosystems). Aliquots (2µL) of each DNA sample were added. The following thermal cycling conditions were used (50°C for 2 min, 95°C for 10 min, followed by 35 cycles of 95°C for 15 sec and 63.6°C for 1 min). All reactions were repeated three times independently to ensure the reproducibility of the results. A sample was considered positive when the change in fluorescence (ΔR_n) of FAM or VIC, relative to that of ROX (internal reference signal), exceeded the set threshold values of 0.05 for FAM and 0.05 for

VIC in the linear range of the amplification plots at a cycle threshold (C_T) value below 35. C_T is defined as the cycle at which a statistically significant increase in fluorescence output above background is detected.

18S ribosomal RNA Taqman assay as an internal control

The use for the Applied Biosystems 18S rRNA Taqman assay was investigated as an endogenous control. The 18S rRNA Taqman verifies the extraction procedure and confirms the integrity of the extracted DNA. The test also determines the absence of PCR inhibitors. The 18S rRNA assay is multiplexed with the *Bonamia* test and therefore must be performed under primer limiting condition.

Specificity testing

The specificity of the TaqMan assay was evaluated by assaying DNA extracted from various isolates of *Bonamia* as well as from closely related organisms such as *Haplosporidium nelsoni*, *Haplosporidium costale* and *Mikrocytos mackini*.

Sensitivity testing

The sensitivity of the TaqMan assay was assessed by assaying DNA extracted from 44 oyster tissues, previously graded by conventional histopathology (Mike Heasman, NSW Fisheries and Ben Diggles, DigsFish Pathology Services Ltd, pers. comm.) for the presence of *Bonamia* cells.

Detection limit

To determine the detection limit of the TaqMan assay, tests were performed using a dilution series of samples of DNA extracted from purified *Bonamia* cells obtained from the Ifremer Laboratory in France.

RESULTS AND DISCUSSION

Importation of Reference Materials

Fixed oyster tissues infected with exotic isolates of *Bonamia*, and purified DNA from related organisms were imported from the OIE Reference Laboratories (Drs Franck Berthe and Isabelle Arzul from Ifremer, La Tremblade, France; Dr Susan Bower from the Pacific Biological Station, Nanaimo, Canada; Dr Nancy Stokes from VIMS, Virginia, USA).

PCR Assays for the Identification and Differentiation of *Bonamia* Isolates

Following DNA extraction, PCRs were performed and the resulting amplicons were sequenced as described in the Methods section.

Comparison of the Nucleic Acid Sequence of the Australian Isolate with Sequences of Known Exotic Isolates of *Bonamia*

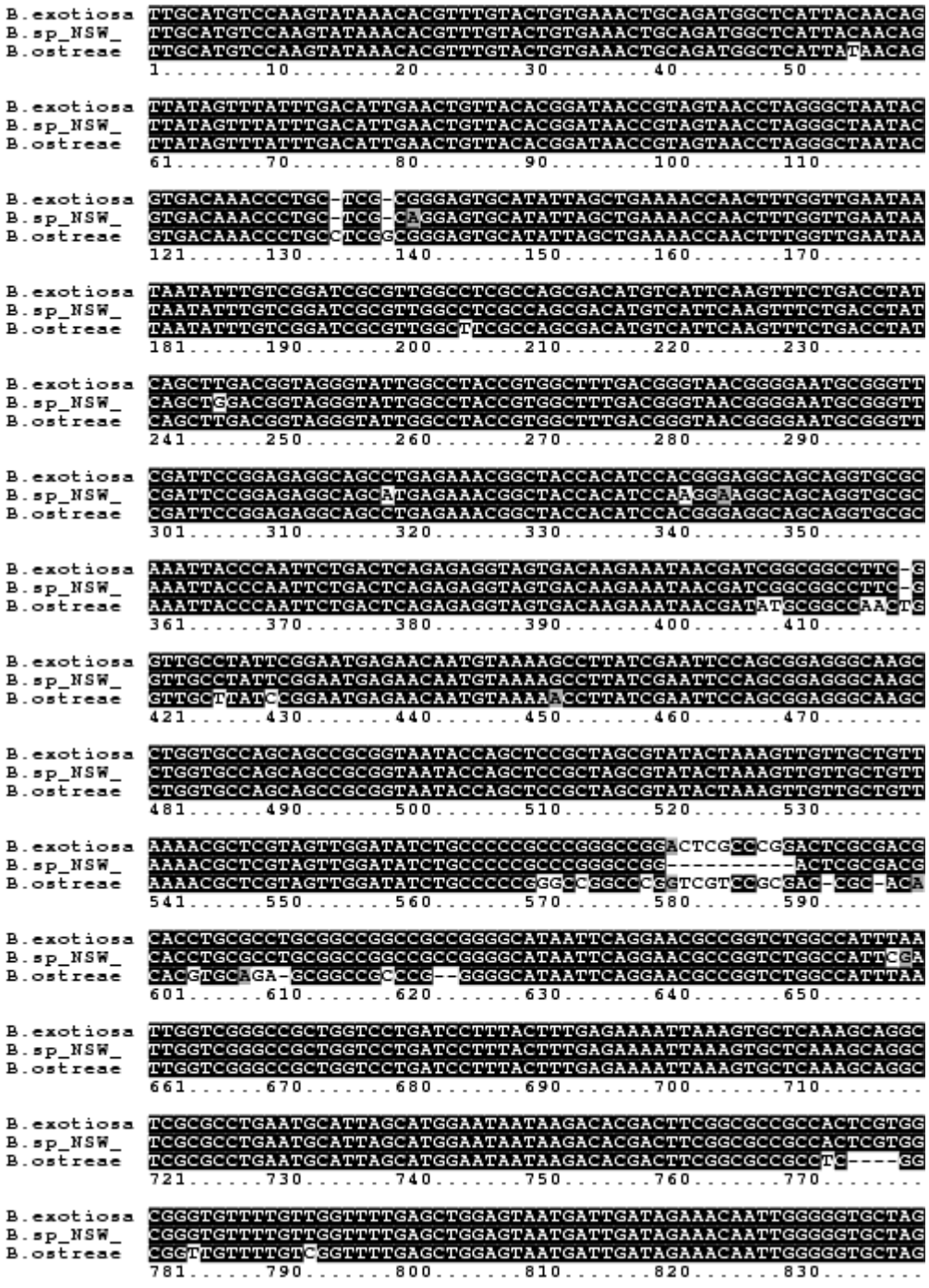
In order to evaluate the relatedness of the Australian *Bonamia* isolate to exotic isolates, sequencing of the 18S gene of the rRNA operon of *Bonamia* sp. was performed. A nucleotide sequence comparison between the Australian and exotic isolates of *Bonamia* was performed. Results indicated that a higher level of nucleotide homology exists between the *Bonamia* sp. and *B. exitiosa* (New Zealand) than between *Bonamia* sp. and *B. ostreae* (figure 1). The 18S gene of the Australian isolate presents seven base-pair mismatches and a deletion of ten consecutive base pairs when compared with the New Zealand isolate. On the other hand, slightly more differences exist between the *Bonamia* sp. and *B. ostreae* (Europe), with 42 base-pair mismatches, 9 base-pair insertions and 13 base-pair deletions, including a deletion of 10 consecutive base pairs.

The alignment of the ITS1 rRNA sequences from *Bonamia* sp. and *B. ostreae* presents further evidence of the genetic differences between the Australian *Bonamia* and the European *Bonamia* (figure 2).

Phylogenetic analyses were performed using 1603 base pairs from the 18S region and 156 bases from the ITS region including insertions and deletions. The Australian *Bonamia* isolate sequences will be deposited in GenBank in the near future.

Restriction Fragment Length Polymorphism Assay

The RFLP assay is a quick and useful alternative to gene sequencing for the differentiation of genomic sequences. Originally developed by Cochennec (2001), the digestion of the 300 bp amplicon (obtained with the Bo-Boas PCR) by the Bgl1 restriction enzyme, produces two smaller fragments if the DNA originates from European *Bonamia* isolates (figure 3). Laboratories that do not have ready access to sequencing facilities could establish which isolate of *Bonamia* is present in their oysters by using the RFLP assay.


B.exotiosa
B.sp_NSW_
B.ostreae

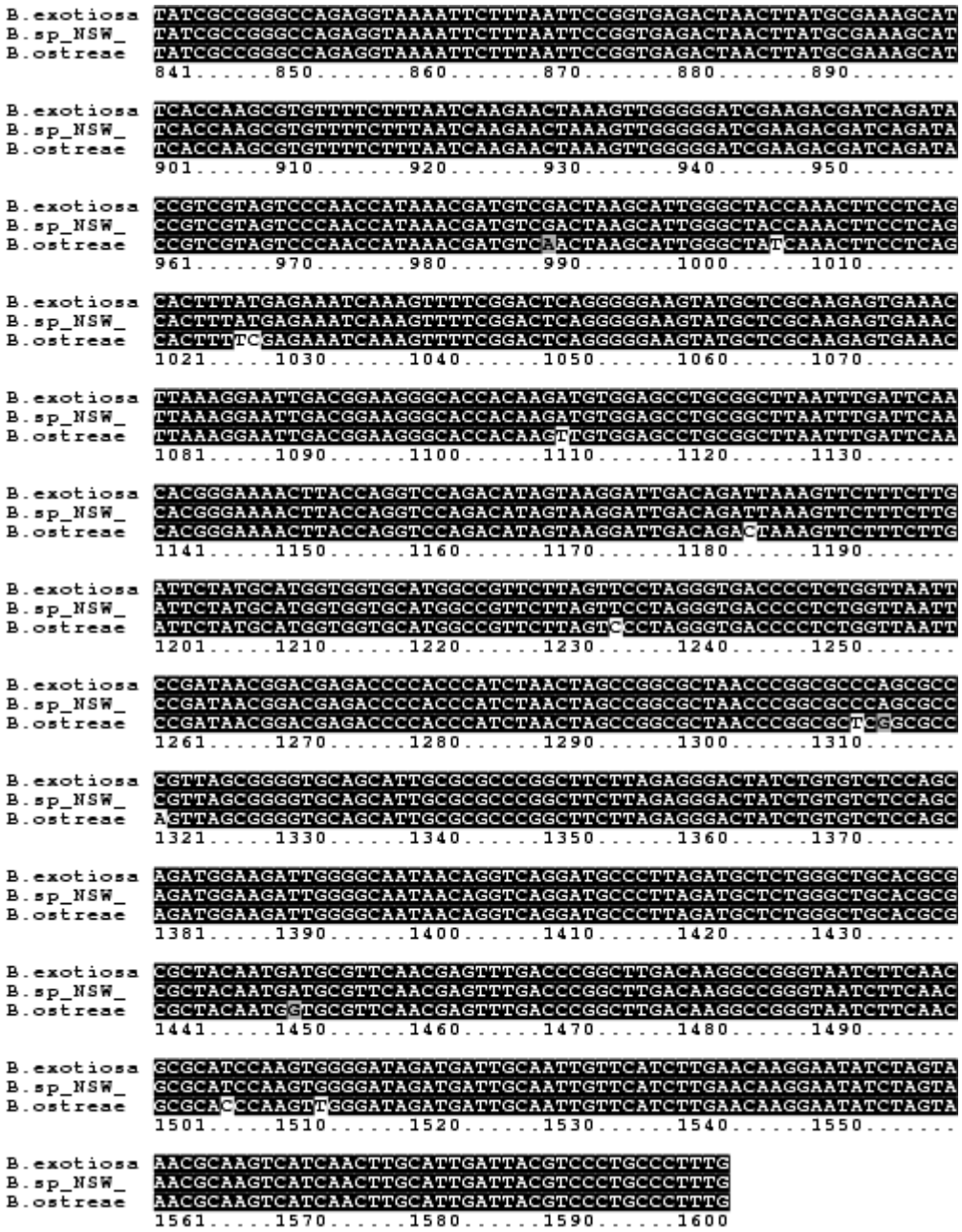


Figure 1. Alignment of *Bonamia* 18S rRNA gene sequences (1603 bp). 18S rRNA gene sequences from New Zealand (*B. exitiosa*), Australia (*B.* sp.), and Europe (*B. ostreae*) were aligned and nucleotide differences were highlighted. Grey areas indicate nucleotide mismatches with regards to the Australian isolate sequence. Dashes represent nucleotide deletions.

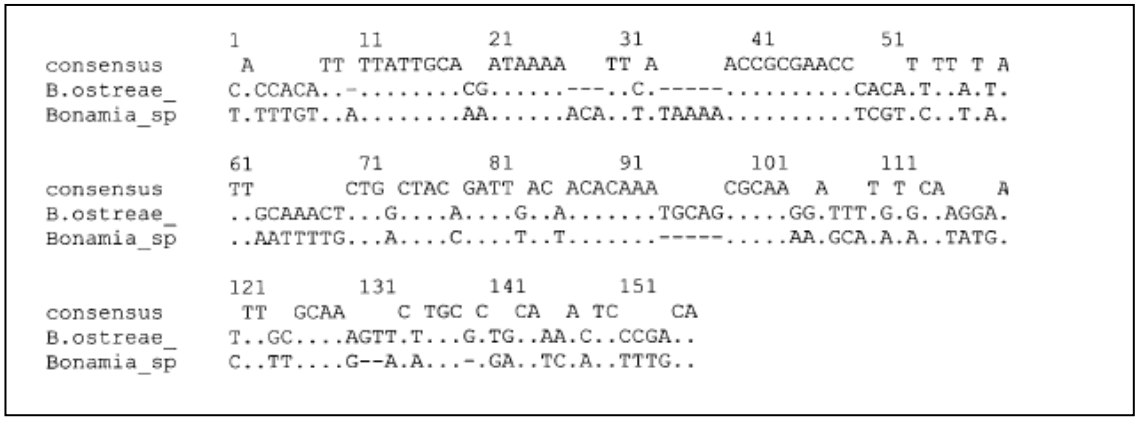


Figure 2. Alignment of *Bonamia* ITS1 rRNA sequence (156 bp). ITS1 rRNA sequences from the Australian *Bonamia* sp. and the European *B. ostreae* were aligned, and nucleotide differences were highlighted. Dashes represent nucleotide deletions. The Australian isolate presents 53 base-pair mismatches, 9 base-pair insertions and 8 base-pair deletions with the European isolate.

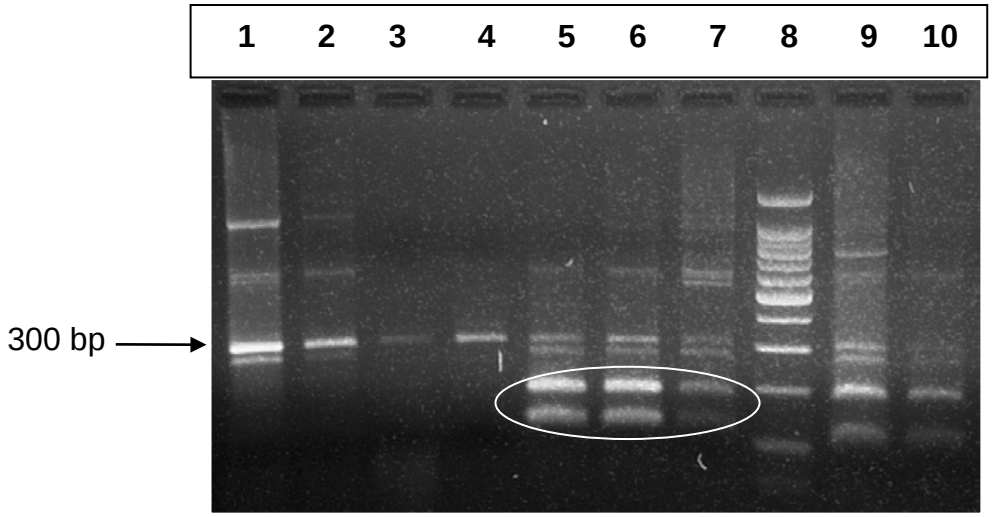


Figure 3. Restriction Fragment Length Polymorphism of the 18S rDNA Bo-Boas amplicon. Photograph of an agarose gel containing ethidium bromide-stained, *Bonamia*-specific amplicons that were digested by the restriction enzyme Bgl1. The primers used for the amplification were Bo and Boas (Cochennec *et al*., 2000), producing a 300 bp amplicon. DNA amplicons that were resistant to the enzymatic digestion are from Australian (lanes 1 and 2) and New Zealand isolates (lanes 3 and 4). DNA from European isolates, from France (lane 5), Netherlands (lane 6), and Ireland (lane 7), was susceptible to enzymatic digestion as shown by the presence of two smaller fragments (approximately 120 and 180 bp in size). Lanes 9 and 10 contain the 300 bp amplicon from NSW and NZ respectively, digested by the restriction enzyme HaeII. Lane 8 contains 100 bp ladder.

Diagnostic Real Time PCR TaqMan Assay

TaqMan Assay Specificity

In order to demonstrate that the TaqMan assay is specific to the genus *Bonamia*, DNA from related organisms were used in the assay. Results (table 2) showed that these related oyster pathogens are not detected by the TaqMan assay. In addition, all isolates of *Bonamia* available to this study were detected indicating that the TaqMan assay is pan-specific.

TaqMan Assay Sensitivity

All oyster samples judged positive by histopathology for the presence of *Bonamia* cells were found positive by TaqMan assay. Out of 21 oyster samples judged negative by histopathology, 19 were positive and 2 were negative by TaqMan assay (table 3). These results are not surprising as it is now well-established that the sensitivity of PCR assays is greater than conventional histopathology techniques. For example, similar results were obtained in a comparative study of diagnostic techniques for *B. exitiosa* from flat oysters in New Zealand (Diggles *et al.,* 2003).

In order to further validate the specificity of the diagnostic TaqMan assay, DNA samples that were weakly positive by the TaqMan assay were amplified twice by

conventional PCR using the primers Bo-Boas. The resulting amplicons were sequenced and confirmed to be specific for *Bonamia* (figure 4).

Table 2. TaqMan assay specificity

Sample	Exp. 1 Average C_T value*	Exp. 2 Average C_T value*	Exp. 3 Average C_T value*	Mean	Std Dev
Uninfected tissue (negative control)	35.00	35.00	35.00	35.00	-
Mikrocytos mackini	35.00	35.00	35.00	35.00	-
Haplosporidium nelsoni	35.00	35.00	35.00	35.00	-
Haplosporidium costale	35.00	35.00	35.00	35.00	-
Bonamia sp. (NSW)	21.69	22.44	21.74	21.96	0.42
Bonamia exitiosa (NZ)	23.60	23.96	23.53	23.70	0.23
Bonamia ostreae (France)	16.20	16.26	16.91	16.46	0.39
Bonamia ostreae (Netherlands)	16.12	16.28	16.32	16.24	0.10

N.B. C_T values ≥ 35 indicate the absence of specific amplification.
*Since, for each experiment, the TaqMan assay is performed in triplicate wells, the average C_T value is given.

Detection Limit of the *Bonamia* TaqMan assay
In order to quantify the detection limit of the *Bonamia* TaqMan assay, a dilution series of DNA extracted from the purified *Bonamia* cells. Results showed that the TaqMan PCR assay can detect approximately 10 000 cells (figure 5).

The TaqMan assay has the advantages that it is rapid, quantitative, and less prone to cross-contamination that can occur with conventional nested-PCR assays.

Conclusions

The molecular assays developed here provide specific and sensitive diagnostic procedures for the detection and identification of the Australian *Bonamia* as well as exotic *Bonamia* isolates. Sequence analysis of the PCR amplicons as well as a RFLP analysis allows differentiation between the various *Bonamia* isolates. Based on the data collected to date, it is likely that these assays form the basis for a pan-specific procedure for the detection and identification of *Bonamia* species. When available, *Bonamia* DNA samples originating from other Australian States, Chile, Argentina and Spain will be tested to confirm this hypothesis.

Table 3. TaqMan Assay Sensitivity

Oyster sample #	Exp. 1 Average C_T value*	Exp. 2 Average C_T value*	Exp. 3 Average C_T value*	Mean	Standard Deviation	Histology grading Neg/Low/Medium
1	28.66	28.82	30.10	**29.19**	0.78	L
2	27.71	28.22	29.22	**28.38**	0.76	N
3	27.75	28.26	29.38	**28.46**	0.83	L
4	24.70	25.09	26.18	**25.32**	0.76	L
5	26.08	26.77	27.75	**26.86**	0.83	N
6	30.35	30.38	32.76	**31.16**	1.38	N
7	25.19	25.11	25.87	**25.39**	0.41	L
8	25.65	25.75	26.81	**26.07**	0.64	N
9	23.12	22.85	23.94	**23.30**	0.56	L
10	22.75	22.52	23.18	**22.81**	0.33	N
11	21.22	20.89	21.84	**21.31**	0.48	L
12	28.70	28.35	29.29	**28.78**	0.47	N
13	27.70	27.06	27.88	**27.54**	0.43	L
14	27.75	28.20	28.88	**28.27**	0.56	N
15	26.04	25.80	27.00	**26.28**	0.63	M
16	28.28	27.52	28.50	**28.10**	0.51	L
17	27.40	26.67	27.66	**27.24**	0.51	L
18	34.85	34.50	34.71	**34.68**	0.17	N
19	34.91	34.01	34.93	**34.61**	0.52	N
20	32.88	31.75	32.87	**32.50**	0.64	N
21	34.81	34.17	35.00	**34.66**	0.43	N
22	33.48	33.01	34.08	**33.52**	0.53	N
23	32.45	32.25	33.49	**32.73**	0.66	L
24	32.95	32.31	33.49	**32.91**	0.59	N
25	32.50	31.88	33.03	**32.47**	0.57	N
26	30.27	30.49	31.45	**30.73**	0.62	N
27	27.97	27.81	28.95	**28.24**	0.61	L
28	21.13	20.94	21.57	**21.21**	0.32	L
29	28.55	28.56	29.19	**28.76**	0.36	N
30	21.09	20.76	22.14	**21.33**	0.72	M
31	35.00	35.00	35.00	**35.00**	0.0	N
32	28.55	29.39	30.28	**29.40**	0.86	M
33	28.58	29.22	30.29	**29.36**	0.86	L
34	35.00	35.00	35.00	**35.00**	0	N
35	29.53	30.27	30.88	**30.22**	0.67	M
36	31.70	32.16	33.33	**32.39**	0.84	L
37	30.81	30.96	32.32	**31.36**	0.83	L
38	32.42	32.60	33.48	**32.83**	0.56	N
39	32.22	32.97	33.17	**32.78**	0.50	N
40	24.50	25.14	25.79	**25.14**	0.64	L
41	24.34	25.59	26.47	**25.46**	1.07	M
42	30.27	31.23	31.77	**31.09**	0.75	N
43	25.66	26.60	27.64	**26.63**	0.99	N
44	22.33	22.46	23.66	**22.81**	0.73	M
Neg ctrl	35.00	35.00	35.00	**35.00**	0	--
Pos ctrl	20.95	21.14	22.36	**21.48**	0.76	--

N.B. C_T values ≥ 35 indicate the absence of specific amplification.
*Since, for each experiment, the TaqMan assay is performed in duplicate wells, the average C_T value is given.

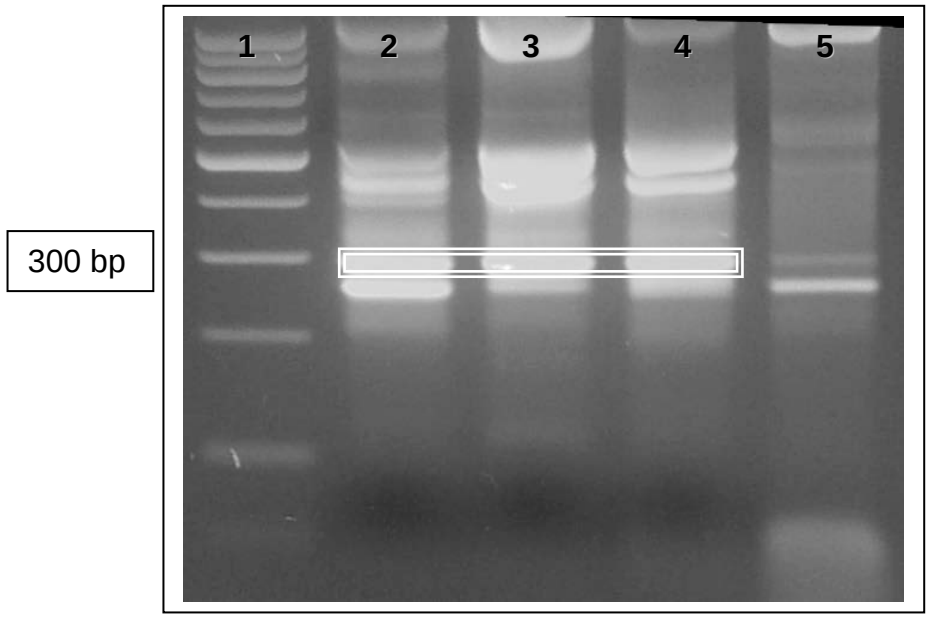


Figure 4. Conventional PCR Assay using the primers Bo-Boas for the identification of *Bonamia* DNA previously detected by TaqMan assay. Photograph of an agarose gel containing ethidium bromide stained *Bonamia* specific amplicons that were produced by performing two consecutive amplifications (equivalent to a "nested" PCR). The primers used for the amplifications were Bo and Boas (Cochennec *et al*., 2000), producing a 300 bp amplicon (boxed). Lane 1 contains 100 bp ladder, lanes 2, 3 and 4 are samples of *Bonamia*-infected oyster tissues, and lane 5 contains uninfected control tissues. The faint band in lane 5, based on sequence, is non-specific.

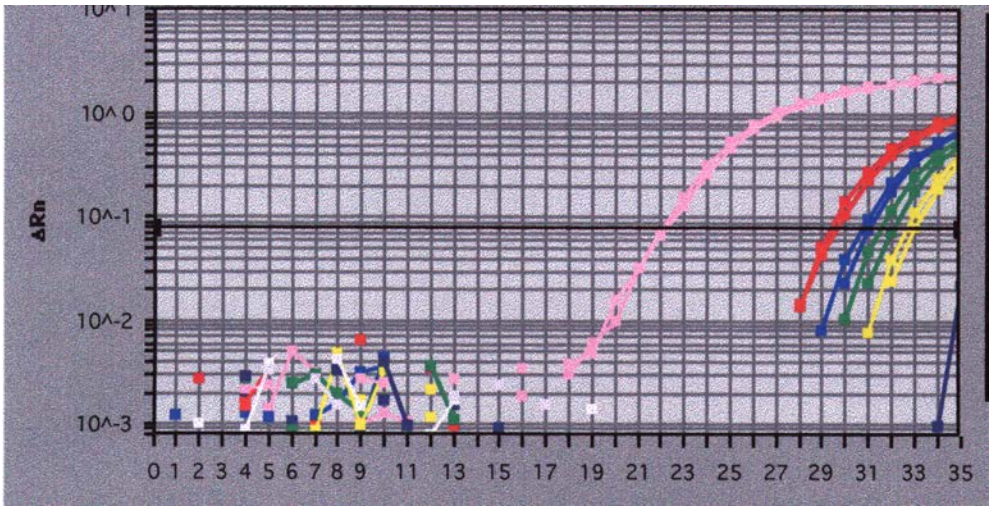


Figure 5. *Bonamia* TaqMan assay. The graph shows the log of the change in fluorescence intensity (y-axis) versus the number of threshold cycles (x-axis) for each of several different target DNA concentrations equivalent to the following *Bonamia* purified cells. Red curve: 250,000 cells; blue curve: 100,000 cells; green curve: 50,000 cells; yellow curve: 25,000 cells; black curve: 5,000 cells (undetected); pink curve: DNA extracted from a *Bonamia* infected oyster (positive control). Note that for each sample preparation, the TaqMan assay is performed in duplicate (there are two curves for each target concentration).

BENEFITS

Industry sectors that will benefit from these outcomes include oyster aquaculture in Tasmania, New South Wales, Victoria, Western Australia and Queensland. Identification of infected and uninfected stock will allow industry and State officers to implement a disease management plan based on accurate information. Isolation of infected and uninfected oyster populations will be possible thus enhancing our capability to control and/or eradicate the disease.

State diagnostic laboratories will benefit by being provided with sensitive and specific reagents and procedures that have been validated using both exotic and enzootic isolates. Based on accurate diagnoses, State officials responsible for conducting activities of the Local Disease Control Centre (LDCC) will be able to make informed decisions during implementation of disease management procedures.

Infections with *Bonamia ostrea* and *B. exitiosa* are listed by the OIE and, as a Member State, Australia is obliged to report on their presence or absence in Australia. Such reporting can only be achieved if there are sensitive and specific reagents and procedures in place that are recognised by international agencies. Using internationally accepted procedures, Australian authorities can be confident of providing accurate information to the OIE, other international agencies and to our international trading partners.

FURTHER DEVELOPMENT

The reagents and procedures developed during the course of this project have been tested for specificity and sensitivity, using a limited number of samples – from New South Wales, New Zealand, France and The Netherlands. Further work on a greater number of samples is required for these reagents and procedures to be fully validated as required by the OIE (OIE, 2003). Validation along these lines is beyond the scope of the current project. For example, during the course of this project oyster tissues were obtained from NSW only. Thus the conclusions can only refer to *Bonamia* sp. isolated from oysters grown in NSW. It is not known whether the *Bonamia* in NSW is the same as, or different from, that in Tasmania, South Australia and Western Australia. Larger numbers of samples from domestic oysters located in Tasmanian, NSW, Victorian and Western Australian waters are required, as well as further samples of exotic isolates from Europe and North and South America. Such a research effort would require an international collaboration.

PLANNED OUTCOMES

Based on the outputs of this project, diagnostic tests are now available to Australian diagnostic laboratories for the detection and identification of *Bonamia* spp. in their hosts: *Ostrea* and *Tiostrea* spp..

Export certification services will be available to industries that wish to develop export markets as well as translocate farmed stock between regions without transmitting disease, especially for those associated with producing different genetic strains in a common centralised hatchery. In addition, should pathogenic agents be detected during health surveys, industries and State officers will be able to make informed decisions with regards to brood stock translocation, stock destruction etc. Specifically, mollusc aquaculture industries and State agencies will be able to develop health surveillance programs in collaboration with AFDL. In addition, Australia will be better prepared to negotiate with international trading partners, such as France, on issues concerned with the importation of disease free molluscs from Australian sources.

The sequence data demonstrating differences between Australian *Bonamia* (NSW) isolates with other exotic isolates will assist in determining the relationship between these isolates and isolates from other States in Australia. Such data will be useful in identifying any new variants that may occur in the future as well as identifying any introduction of exotic isolates. Again, this type of information will allow appropriate management procedures to be put in place, as required.

CONCLUSION

The principal aim of this project was to establish an Australian and New Zealand Standard Diagnostic Procedure for *Bonamia* spp. based on molecular techniques.

Thus a real-time TaqMan PCR assay was developed for the detection of the Australian enzootic isolate (from NSW) as well as exotic isolates of *Bonamia*. This newer technology has several advantages over conventional PCR including a greatly reduced risk of cross-contamination, a capability to run in a microplate format as well as being quantitative. Determination of sensitivity is thus facilitated.

Furthermore, a single-step PCR assay (18S rRNA) was established and optimised for the identification and differentiation of *Bonamia* isolates (enzootic and exotic) present in oyster tissues (figure 1). The specificity of the PCR assays was demonstrated by using closely related oyster pathogens as negative controls. None of the negative controls were detected by the TaqMan assay. Conventional PCR amplification and sequencing analyses of the Australian *Bonamia* sp. demonstrated that the *Bonamia* sp. and *B. exitiosa,* from New Zealand, had nearly identical nucleic acid sequence in their small rRNA 18S sub-unit. The European isolates, while having very high levels of sequence homology, were clearly more distantly related, based on this genetic analysis. It could be suggested that the Australian *Bonamia* isolate and the New Zealand *Bonamia* isolate form a subgroup, with the caveat that, to date, only NSW isolates have been analysed.

A RFLP assay was also established to provide a rapid means of differentiating European from Oceanian *Bonamia* isolates. This assay provides an alternative to gene sequencing.

With respect to the diagnosis of Bonamiosis, the following procedures have been established at AAHL:

1. A real-time TaqMan PCR assay
2. A single-step PCR
3. A RFLP assay

The single single-step PCR assay can be used to detect *Bonamia* providing sequencing of the amplicons of the expected sizes is performed for confirmation. Sequencing also allows distinction between isolates.

An additional single-step PCR assay (targeting the ITS1 region of the rRNA operon) could be used to obtain additional sequence information for the comparison of different isolates; further differences in sequence between the NSW isolate and European isolates were demonstrated (figure 2).

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Appendix 1. Intellectual Property

All information arising from this project has been used for the development and/or establishment of Standard Diagnostic Procedures. No intellectual property has been identified.

APPENDIX 2: STAFF AND COLLABORATORS

NAME	POSITION	ADDRESS
Dr Serge Corbeil	Experimental Scientist, AAHL Fish Diseases Laboratory	CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, VIC
Dr Mark Crane	Project Leader, AAHL Fish Diseases Laboratory	
Mr Tony Pye	Senior Technical Officer, Molecular Virology Section	
Dr Stephen Pyecroft	Veterinary Pathologist	Department of Primary Industries, Water & Environment, Tasmania
Dr Symon Dworjanyn	Abalone Hatchery Manager	NSW Fisheries, Port Stephens, NSW
Dr Mike Heasman	Fisheries Biologist	
Dr Ben Diggles	Aquatic Animal Pathologist	DigsFish Pathology Services Ltd
Dr Mike Hine	Aquatic Animal Pathologist	Ministry of Agriculture and, Forestry, New Zealand
Dr Colin Johnston	Veterinary Pathologist	PIRSA, Adelaide, SA
Dr Isabelle Arzul	OIE Mollusc Diseases Expert	OIE Reference Laboratory for Mollusc Diseases, Ifremer, La Tremblade, France
Dr Franck Berthe	OIE Mollusc Diseases Expert	
Dr Susan Bower	OIE Mollusc Diseases Expert	Pacific Biological station, Nanaimo, Canada
Dr Nancy Stokes	OIE Mollusc Diseases Expert	Virginia Institute of Marine Science, USA

Draft Australian and New Zealand Standard Diagnostic Procedure

Molecular Diagnostic Procedures for the Detection and Identification of *Bonamia* spp.

Molecular Diagnostic Procedures for the Detection and Identification of *Bonamia* spp.

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SUMMARY

Some cultured and wild oysters in Australia are known to harbour Bonamia *sp., a pathogenic protistan causing bonamiosis. Infections with* Bonamia ostrea *and* B. exitiosa *are listed by the Office Internationales des Epizooties (O.I.E.).* Bonamia *sp. has caused mortalities in* Ostrea angasi *in Victoria, Tasmania and Western Australia (Hine, 1996). Until recently, Australia has had limited diagnostic capability (histopathology and electron microscopy) for this pathogen and it has been difficult, if not impossible, to distinguish enzootic and exotic isolates.*

Identification of the agent: *Diagnosis of Bonamiosis is based on a range of procedures. Presumptive diagnosis is made following clinical and pathological observations.* Bonamia *spp. is confirmed following tissue imprint and histopathological examination as described in the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE 2003). In addition, a real-time polymerase chain reaction is available for the rapid identification of* Bonamia *spp. in infected oysters.*

Status of Australia: Bonamia *sp. has been identified in flat oysters* (Ostrea angasi) *in NSW, Tasmania, Victoria and WA, It has been shown that this* Bonamia *species is different from the exotic* Bonamia ostreae *found in Europe at the genomic level. On the other hand, while different, the Australian and New Zealand isolates share a high level of similarity at the genomic level.*

Introduction

Bonamia ostreae has been described as the causative agent of substantial mortalities of the European flat oyster, *Ostrea edulis*, in France in 1979 (Pichot et al., 1980; Comps et al., 1980). Bonamiosis, the haemocyte disease caused by this pathogen is also found in oyster farming areas of Spain, the Netherlands, Ireland and Southern parts of the United Kingdom (Van Banning, 1982; Banister and Key, 1982; Polanco et al., 1984; Grizel, 1985; McArdle et al., 1991). A similar disease has been observed in populations of *O. edulis* in the USA (Barber and Davis, 1994). Another species of the genus *Bonamia*, *B. exitiosa*, was also reported in the New Zealand dredge oyster *Tiostrea lutaria* (Dinamani et al., 1987) and in the Australian flat oyster, *Ostrea angasi* (Hine, 1996). From an epidemiological perspective, it is of utmost importance to determine the relationship between the causative agents of these multiple microcell infections. The sustainability of oyster farming depends on the development of rapid diagnostic methods for the management of these pathogens and prevention of transfer of infected stocks.

The development of molecular methods allows the detection and differentiation of *Bonamia* species in oysters or in other host species and will be valuable diagnostic tools for use in life cycle studies.

Pathology

Bonamiosis is caused by intracellular protistan parasites of the genus *Bonamia* that infect haemocytes of flat oysters and is sometimes accompanied by yellow discoloration and extensive lesions on the gills and mantle (Pichot et al., 1980). However, most of the infected oysters appear normal. Pathology correlates with haemocyte destruction. Lesions occur in the connective tissue of the gills, mantle, and digestive gland. These intrahaemocytic protistans quickly become systemic with overwhelming numbers of parasites coinciding with the death of the oysters. Some evidence suggests that the pathology caused by *Bonamia* spp. depends on which host species and population is infected (OIE, 2003). Highly susceptible hosts may die with only a light infection.

Diagnostic Tests

Case definition

This molecular Australian and New Zealand Standard Diagnostic Procedure (ANZSDP) documents the methods currently used at the AAHL Fish Diseases Laboratory (AFDL) for the identification of *Bonamia* spp. The PCR methods are based on those outlined in Cochennec *et al.* (2000).

Storage of samples

Samples must be fixed in 95% ethanol to maintain integrity of the *Bonamia* DNA.

Tissues to be examined
Tissue samples suitable for examination include haemocyte-rich tissues such cardiac cavity, digestive gland and gills.

Tests available

The presumptive diagnostic methods used for Bonamiosis are cytological examination (tissue imprints) and histopathology. Transmission electron microscopy examination is performed for confirmatory identification of the pathogen. This ANZSDP documents those details relevant to *Bonamia* identification using molecular diagnostics (real-time PCR, conventional PCR and sequencing, as well as Restriction Fragment Length Polymorphism (RFLP)).

Detection by real-time TaqMan PCR assay and identification by PCR, sequencing and RFLP

Introduction

Polymerase chain reaction (PCR) has become an important diagnostic tool both in human and veterinary medicine. As with many infectious diseases, especially where the aetiologic agent cannot be easily propagated *in vitro*, development of procedures for the specific and sensitive detection and identification of the infectious agent by PCR has provided some diagnostic capability. PCR provides a powerful tool for the rapid identification of the agent. Furthermore, subsequent sequencing of any PCR product may allow a molecular epidemiological analysis, and, for exotic diseases in particular, this may be very important in control, prevention and eradication.

Real-time PCR is an enhanced implementation of PCR technology providing (1) a real-time monitoring of PCR amplification, and (2) quantification of target nucleic acid in the sample. The ability to include internal controls in each reaction tube by multiplexing methods provides a greater degree of confidence in the results than those obtained by conventional PCR. The PCR reaction and analysis is conducted in a closed tube system substantially improving contamination control.

The use of real-time and conventional PCR as diagnostic tools requires a large investment in human and physical resources; specialised equipment for exclusive molecular diagnosis by qualified and experienced operators is essential. All equipment and procedures need to be strictly controlled to ensure validity of results. It is recommended that diagnostic PCR activities be carried out in a dedicated suite of laboratories with strict control on the transfer of materials and personnel in and out of the suite. Diagnostic laboratories are likely to have designed their PCR facilities and generic procedures according to their own specific requirements and therefore only general direction can be given here.

Real-time PCR reagents

At AAHL an ABI Prism®7700 Sequence Detection System and software Sequence Detector version 1.9 (PE Applied Biosystems) is used for the analysis and storage of data. Primers and probe for the multiplex TaqMan assay are designed using Primer Express Software version 1.5 (PE Applied Biosystems). Thus reagents used are those that are compatible with the ABI system.

Reagents stored at room temperature
QIAamp DNA Mini Kit (QIAGEN Cat # 51304 or equivalent) that includes all buffers and Proteinase K for DNA extraction.

Reagents stored at –20°C
Taq Master Mix (Applied Biosystems cat # 4318157 or equivalent)

Reagents stored at -70°C
18S endogenous control primers and VIC probe (Applied Biosystems cat # 4308329 or equivalent)

List of equipment for the TaqMan assay
Applied Biosystems 96 well Optical reaction plates (cat # 4306737)
Applied Biosystems Adhesive cover kit (cat # 4311971)
Applied Biosystems Sequence detection system Model 7700
Macintosh Power Mac G4 computer and operating system
Applied Biosystems developed software SDS *v1.7*
Bench top centrifuge IEC model Centra and appropriate plate holders

Reagents for conventional PCR and RFLP

Reagents stored at –20°C
Taq polymerase (Promega cat # M1861)
dNTPs (1.25mM) (Promega cat # 12957510, 12957611, 13466203, 13466310)
Mg^{2+}-free buffer 10X (Promega cat # 15913012)
$MgCl_2$ (25mM) (Promega cat # 15246902)
100% Ethanol AR grade
70% Ethanol
Primers (18μM)
HotstarTaq Mastermix (QIAGEN Cat # 203443)
100bp DNA ladder & loading dye (Promega cat # G2101)
Restriction enzymes Bgl1 (Promega cat # R6071) and HaeII (Promega cat # R6661)

Reagents stored at room temperature
QIAamp DNA Mini Kit (QIAGEN Cat # 51304) that contains:
- DNA columns
- Buffer ATL
- Buffer AL
- Buffer AW1
- Buffer AW2
- Buffer AE
- Proteinase K

Agarose (BIORAD cat#162-0134)
Ethidium bromide (BIORAD cat #161-0430)
40 x TAE Buffer (Promega cat # 428A)

List of equipment

Apart from the normal range of equipment required in the standard diagnostic laboratory (e.g. refrigerators, freezers, vortex mixers, micropipettes, biological safety cabinets, centrifuges, balances, microwave oven, thermometers), specialised equipment required to undertake diagnostic PCR may include dry-heat blocks, thermocycler, gel electrophoresis equipment, UV transilluminator, camera system and nucleic acid sequencer.

Quality control

Molecular diagnosis should be operated under an ISO 17025 accredited and audited quality assurance program. Thus, such a program would include initial evaluation of kits and validation of performance; ongoing internal evaluation through mandatory use of appropriate quality control samples where available; and performance monitoring through quality assessment or proficiency programs.

External quality control samples over the appropriate range of testing must be obtained or manufactured wherever possible. Wherever possible, quality control samples should be included in every assay performed and the data presented so that run-to-run performance can be monitored. Positive, negative and reagent controls should be included as specified in the protocol. As a norm, formalin-fixed controls would be included with formalin-fixed test samples and appropriate unfixed controls would be conducted with fresh tissue, culture supernatants or other test samples. Stocks of controls should be established. These controls should be evaluated prior to storage and used in a check-testing regimen and as controls for the conduct of disease investigations.

Nucleic Acid Storage Conditions

Positive control samples should be stored at/below –70^{o}C. Stocks and controls should be aliquoted to minimise damage due to freezing and thawing.

Procedures

Sample preparation

Due to the sensitivity of PCR tests, care at every step of sample preparation must be taken to ensure that cross-contamination of diagnostic samples does not occur. Thus all instruments and sample containers must be clean and uncontaminated i.e. not pre-exposed to aquatic animal pathogens. Wherever possible it is recommended that single-use, disposable containers are used.

At AAHL, samples would be handled and processed using sterile disposable single-use containers, instruments and reagents to minimise the risks of contamination of the samples. As a general principle, samples to be used in the PCR suite for molecular diagnosis will be inactivated by an approved method prior to movement to the PCR suite.

Inactivation will be carried out by the following procedures by staff approved to work with the categories of agents.

For each aquatic animal sample, approximately 20µg of tissues are harvested using sterile scissors and tweezers and put in a sterile 1.5mL vial (conical bottom). Tissues are mashed using a disposable plastic green pestle and each sample receives 180µL of ATL buffer (provided in the commercial kit). All labelled vials are then put in a jar.

Nucleic acids are extracted from submitted samples in the Biological Safety Cabinet Class II in the PCR suite.

Nucleic acid extraction

Nucleic acid (including *Bonamia* DNA) is obtained from tissue samples using the QIAamp DNA extraction kit following manufacturer's instructions.

Diagnostic Real Time PCR TaqMan assay

The TaqMan technology was chosen for the development of a rapid, sensitive and reliable molecular diagnostic assay specific for *Bonamia*.

An ABI Prism®7700 Sequence Detection System and software Sequence Detector version 1.9 (PE Applied Biosystems) is used for the analysis and storage of data. Primers and probe for the multiplex TaqMan assay were designed using Primer Express Software version 1.5 (PE Applied Biosystems). The *Bonamia* primers and probe were based on the ITS-1 of the rRNA operon, a relatively conserved genomic region among *Bonamia* isolates. Primer and probe sequences are as follows:

Forward primer (ITS-For): 5'-CCCTGCCCTTTGTACACACC-3'
Reverse primer (ITS-Rev): 5'-TCACAAAGCTTCTAAGAACGCG-3'
6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA), labelled probe (Bon ITS): 6FAM-TTAGGTGGATAAGAGCCGC-TAMRA

The ITS primers are used at a final concentration of 900nM. The 18S endogenous control primers, used to validate the DNA extraction procedure and the absence of PCR inhibition, are used at a final concentration of 113nM. The ITS FAM probe and the 18S control VIC probe are used at a final concentration of 250nM and 31nM, respectively. The reactions are carried out in a 96-well plate in a 25µL reaction volume containing 12.5µL Universal Master Mix (PE Applied Biosystems). Aliquots (2µL) of each DNA sample are added. The following thermal cycling conditions are used (50°C for 2 min, 95°C for 10 min, followed by 35 cycles of 95°C for 15 sec and 63.6°C for 1 min). All reactions are repeated three times independently to ensure the reproducibility of the results. A sample is considered positive when the change in fluorescence (ΔR_n) of FAM or VIC, relative to that of ROX (internal reference signal), exceeds the set threshold values of 0.05 for FAM and 0.05 for VIC in the linear range of the amplification plots at a cycle threshold (C_T) value below 35. C_T is defined as the cycle at which a statistically significant increase in fluorescence output above background is detected.

Bonamia-*specific PCR (Cochennec et al., 2000)*

The PCR mixture for a single sample consists of the following reagents: 9.5µL of deionised sterile water; 12.5µL of HotStar Taq Master mix, 0.5µL of the *Bonamia* 18S rDNA forward primer Bo: CATTTAATTGGTCGGGCCG (18µM); 0.5µL of the reverse primer Boas: CTGATCGTCTTCGATCCCCC (18µM); and 2µL of extracted DNA. For multiple samples, the volumes are increased stoichiometrically. The mixture is incubated in an automatic thermal cycler (Perkin Elmer GeneAmp 2400) that is programmed for: one cycle at 94°C for 15 minutes (activation of the Hotstar Taq polymerase); 35 cycles at 94°C for 1 minute (denaturation), 55°C for 1 minute (annealing) and 72°C for 1 minute (extension); and, finally, one cycle at 72°C for 7 minutes (final extension). Amplified DNA (size: 300 bp) is detected by agarose gel electrophoresis (figure 2).

Interpretation

At the completion of the PCR, specific amplicons of the correct size are identified by agarose gel electrophoresis and removed using a scalpel blade. DNA extraction from the gel is performed using a Qiaquick Gel Extraction (Qiagen cat # 28704) according to the manufacturer's instructions.

DNA sequence is determined (by using the PCR primers Bo and Boas as sequencing primers). Sequence identity and genotype are determined by a Blast search of the Genbank database.

The negative control sample may have a non-specific, faint band of the same molecular size as the positive samples.

Fragment Length Polymorphism

As an alternative method to DNA sequencing, *Bonamia* isolates can be differentiated by digesting PCR products, amplified by the Bo-Boas primers (Cochennec *et al*., 2000), with Bgl1 and HaeII restriction enzymes. The mixture (5µL of PCR product, 1µL of restriction enzyme, 2µL buffer 10X, 12µL of water) is incubated at 37ºC for 90 min. Analysis of the digestion is performed by electrophoresis on a 2% polyacrylamide gel that is subsequently stained with ethidium bromide and observed under U.V. light.

Interpretation

The digestion of the 300 bp amplicon (obtained with the Bo-Boas PCR) by the Bgl1 restriction enzyme, produces two smaller fragments if the DNA originates from European *Bonamia* isolates (figure 3). Laboratories that do not have ready access to sequencing facilities could establish which isolate of *Bonamia* is present in their oysters by using the RFLP assay.

Acknowledgements

Oyster tissues infected with the Australian *Bonamia* isolate were obtained from Dr Symon Dworjanyn (NSW Fisheries). Oyster tissues infected with the European *Bonamia* isolates were obtained from Drs Isabelle Arzul and Franck Berthe (Ifremer, La Tremblade, France). The New Zealand *Bonamia* isolate was obtained from Drs Mike Hine and Ben Diggles (NAWI, New Zealand). DNAs from *Mikrocytos mackini*, *Haplosporidium costale* and *Haplosporidium nelsoni* were obtained from Drs Susan Bower (Pacific Biological Station, Canada) and Nancy Stokes (VIMS, USA) respectively. Gene sequencing was carried out by Mr Tony Pye (CSIRO Livestock Industries, AAHL).

Figures

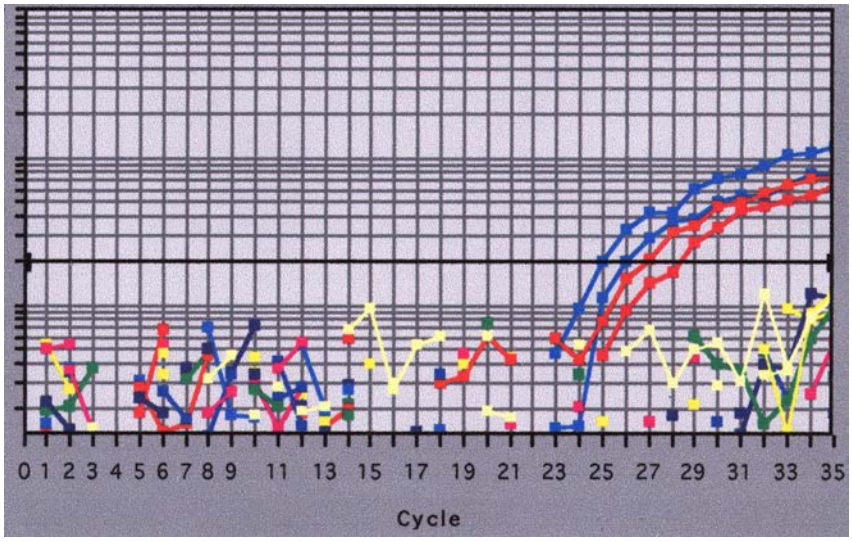

Figure 1. Real-time PCR amplification of *Bonamia* rDNA. The graph shows the log of the change in fluorescence intensity (y-axis) versus the number of threshold cycles (C_t value) (x-axis) for each DNA sample. The difference in C_t value for each sample is indicative of different levels of *Bonamia* infection in oyster samples. Lines (e.g. yellow, green) that do not reach the threshold (horizontal) bar, represent negative control oyster samples. Note that for each sample preparation, the TaqMan assay is performed in duplicate (there are two curves for each target concentration).

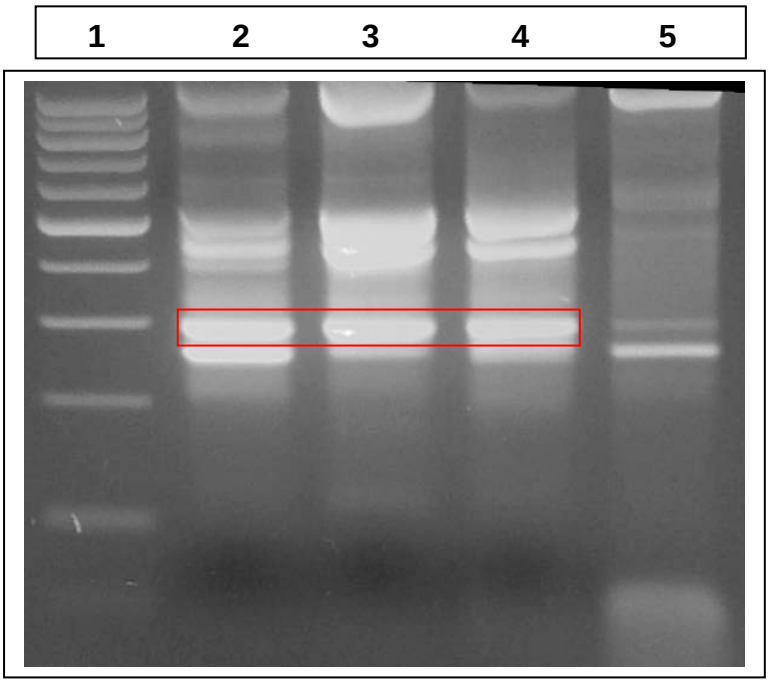

Figure 2. Conventional PCR Assay using the primers Bo-Boas for the identification of *Bonamia* DNA. Photograph of an agarose gel containing ethidium bromide-stained, *Bonamia*-specific amplicon. The primers used for the amplifications were Bo and Boas (Cochennec *et al.*, 2000), producing a 300 bp amplicon (boxed). Lane 1 contains 100 bp ladder, lanes 2, 3 and 4 are samples of *Bonamia*-infected oyster tissues, and lane 5 contains uninfected control tissues. The faint band in lane 5, based on sequence, is non-specific.

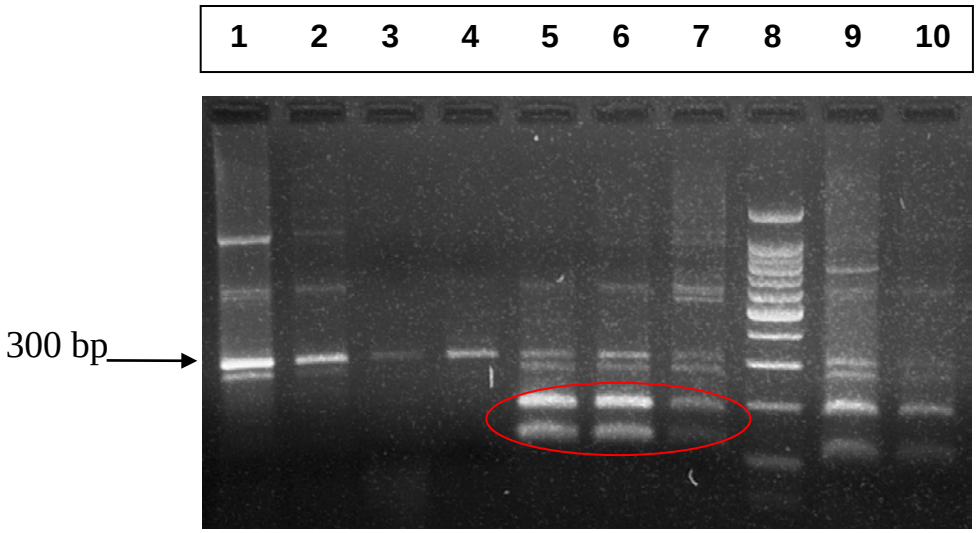

Figure 3. Restriction Fragment Length Polymorphism of the Bonamia 18S rDNA Bo-Boas amplicon. Photograph of an agarose gel containing ethidium bromide-stained, *Bonamia*-specific amplicons that were digested by the restriction enzyme Bgl1. The primers used for the amplification were Bo and Boas (Cochennec *et al*., 2000), producing a 300 bp amplicon. DNA amplicons that were resistant to the enzymatic digestion are from Australian (lanes 1 and 2) and New Zealand isolates (lanes 3 and 4). DNA from European isolates, France (lane 5), Netherlands (lane 6), and Ireland (lane 7) was susceptible to enzymatic digestion as shown by the presence of two smaller fragments (approximately 120 and 180 bp in size). Lanes 9 and 10 contain the 300 bp amplicon from NSW and NZ respectively, digested by the restriction enzyme HaeII. Lane 8 contains 100bp ladder.

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