Aquatic Animal Health Subprogram:

Development of improved molecular diagnostic tests for *Perkinsus olseni* in Australian molluscs

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Cover Photo: *Perkinsus olseni* in the greenlip abalone (*Haliotis laevigata*) with permission of Professor Ryan Carnegie, OIE Perkinsus Reference Laboratory, Virginia Institute of Marine Science.

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Abbreviations

AAHL	Australian Animal Health Laboratory
ARFTM	Alternative Ray's Fluid Thioglycollate Medium
ASe	analytical sensitivity of assay
ASp	analytical specificity of assay
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool - http://blast.ncbi.nlm.nih.gov/
CSIRO	Commonwealth Scientific and Industrial Research Organisation
cPCR	conventional PCR
C_T	real-time PCR threshold cycle
Davidson's	Davidson's fixative (Shaw and Battle, 1957)
DMEM	Dulbecco's Modified Eagle Medium
DMEM/Ham's F12-3%	DMEM with Ham's Nutrient Mixture F12 and 3% foetal bovine serum
DMSO	Dimethyl sulfoxide - $(CH_3)_2SO$
DNA	deoxyribonucleic acid
DSe	sensitivity of diagnostic test
DSp	specificity of diagnostic test
FBS	foetal bovine serum
FRDC	Fisheries Research and Development Corporation
g	gravitational force
gDNA	genomic DNA
Ham's F12	Ham's Nutrient Mixture F12
ICES	Conseil International pour l'Exploration de la Mer
ITS	internal transcribed spacer
LSU	rRNA large subunit ribosomal RNA
MGB	minor groove binding
nt	nucleotide
NTS	non-transcribed spacer
nt	nucleotide
OIE	Office International des Epizooties – the World Organisation for Animal Health
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
qPCR	quantitative PCR (real-time PCR)
RNA	ribonucleic acid
SASW	sterile artificial seawater
SCAAH	Sub-committee on Aquatic Animal Health
SSU rRNA	small subunit ribosomal RNA
tRNA	transfer RNA
TS	transverse section
v/v	volume per volume
w/v	weight per volume

Executive Summary

Background

Perkinsus is the widespread cause of disease and lost production in mollusc fisheries world-wide. Found mostly in temperate waters, two species are listed internationally as notifiable by the OIE and also appear on Australia's National List of Reportable Diseases of Aquatic Animals. Although *Perkinsus marinus* is exotic to Australia, *Perkinsus olseni* is enzootic and well-known as the cause of serious infections in various wild abalone populations in south-eastern Australia.

The rapid identification and reliable differentiation of species is a major issue in the diagnosis and management of Perkinsosis in Australia. Traditional methods of *Perkinsus* diagnosis, such as histology and Ray's thioglycollate culture, are straightforward and practical, however they lack sensitivity and fail to differentiate specific species. The molecular methods currently recommended by the OIE are based on conventional 1-step PCR which is generally more labour intensive, slower and less sensitive than real-time PCR.

The primary aim of this project was to develop and validate a *species-specific* real-time PCR (qPCR) assay for *Perkinsus olseni*.

Aims/Objectives

The aims and objectives of the project were:

1. Undertake a targeted molecular, histological and cultural examination of known Perkinsus-infected wild abalone populations to compare existing methods of detection.

2. Establish representative axenic (single species) cultures of Perkinsus sp. from infected abalone.

3. Use established PCRs and DNA sequencing methods to confirm the presence of P. olseni and determine the genetic diversity, including other Perkinsus sp. from these populations.

4. Develop and validate qPCR methods for the detection and identification of P. olseni in infected abalone.

5. Compare and evaluate the performance of the objective 4 qPCR with existing conventional PCR methods for detection of P. olseni.

Methodology

Scientists from the CSIRO Australian Animal Health Laboratory in Geelong, have undertaken a study of *Perkinsus* in blacklip abalone to develop a new qPCR system for the diagnosis of *P. olseni*. The design of primers and probe for the *P. olseni* Taqman® assay was based on DNA sequence from infected abalone collected from South Australia over the course of the project and sequence data publically available through GenBank.

Single species (axenic) cell cultures of *P. olseni* from abalone were established using standard methods of propagation. Cell cultures were confirmed by sequencing and phylogenetic analysis of the ITS, LSU and actin genes. DNA sequence was independently confirmed by experts in Japan.

The accumulated test data were used to compare the new qPCR assay with exiting test methods. Validation of the *P. olseni* qPCR was based on a wide range of samples including mollusc field samples, cloned plasmids and axenic reference cultures of *Perkinsus*. The analytical sensitivity (ASe) of the test system was determined using serially diluted plasmid DNA and genomic DNA from cultured cells. All calculations of diagnostic sensitivity and specificity were based on testing of naturally infected animals. The diagnostic performance characteristics of the assay were also determined by ROC and Bayesian analysis to obtain estimates of diagnostic sensitivity and specificity.

Results

A species-specific Taqman® based real-time PCR for *P. olseni* was developed. The analytical specificity (ASp) of the system was 100% with respect to a range of non-target *Perkinsus* reference samples (n = 42). The system could detect 1 to 2 copies/µl of target DNA in a background of abalone genomic DNA, and plasmid dilutions between 2 and 20 copies per PCR reaction represent the 95% confidence limit of analytical sensitivity as recommended by the OIE. The C_T values from the equivalent amount of *P. olseni* genomic DNA (600 pg to 1 fg) were linear over this range.

ROC analysis provided an estimate of the relative diagnostic sensitivity (DSe) of 94.6% and diagnostic specificity (DSp) of 92.8% at a preliminary cut-off C_T of 41.29 for the new AFDL *Pols* qPCR using the OIE species-specific PCR assay as a reference. When different tissues were used our analysis shows that the DSe obtained with the AFDL *Pols* qPCR with gill and muscle were similar, 0.88 and 0.92, respectively.

While the primary focus of the project was the development of the qPCR, cell cultures were to be used as the basis of DNA sequencing. Although this strategy was modified, two cryopreserved axenic cultures of *P. olseni* were established. These are the first *in vitro* cultures of *P. olseni* propagated from abalone in Australia. The culture from Thistle Island, South Australia 2012 (12:978-11T) was obtained from a site near Memory Cove, South Australia and was accepted as the holotype culture for *P. olseni* by the ATCC.

The sequencing and phylogenetic analysis undertaken in this project confirmed the taxonomy and identification of the field samples used for test development and the identity of the *in vitro* cultures. Analysis of DNA sequence from the ITS region indicated that there had been little change in this region since 1989 and that this region remains a stable and useful target for molecular detection.

Implications for relevant stakeholders

The molecular assay developed in this project provides rapid detection and identification of species and has application for testing individuals from outbreaks or high-throughput surveillance of populations for certification and management of stocks.

The development of a highly sensitive and highly specific diagnostic test has the potential to provide state government authorities, their diagnostic laboratories, and their fisheries managers and regulators with improved diagnostic capability not only for the diagnosis of Perkinsosis (specifically, rapid identification of the causative agent) but also for the detection and identification of sub-clinical infections. The provision of a validated diagnostic test with estimates of diagnostic specificity and sensitivity allows the design of surveillance programs and sampling protocols to be based on reliable scientific data. The test can be implemented in the recently developed abalone farm accreditation program.

Recommendations

It is recommended that details of this test be provided to diagnostic laboratories for immediate implementation in state jurisdictions that have a need for *Perkinsus* diagnostics. Moreover, the method and validation should be published in a peer-reviewed scientific journal to facilitate wider adoption of the test through inclusion in the OIE Manual of Diagnostic Tests for Aquatic Animals.

Keywords

Australia, mollusc, protozoa, *Alveolata, Perkinsozoa, Perkinsea, Perkinsida, Perkinsidae, Perkinsus olseni*, *Perkinsus atlanticus*, Perkinsosis, PCR, real-time PCR, qPCR, cell-culture, *in vitro* culture, *Haliotis rubra*, blacklip abalone, PCR validation, ROC analysis, Bayesian analysis, *in vitro* culture, diagnostic validation.

Introduction

The discovery of *Perkinsus* arose out of investigations, beginning in 1946, into the cause of massive mortalities and population decline in the native American oyster *Crassostrea virginica*, in the Gulf of Mexico. At first, the activity of oil companies close to the coast was thought to be responsible, but a small spherical protozoan, first called *Dermocystidium marinum* was discovered in the diseased oysters (Mackin, Owen and Collier, 1950). We now know this organism as *Perkinsus marinus*.

Typically, early descriptions of *Perkinsus* species relied on differences in geographic and host ranges as a basis for species discrimination. Although variations in morphology were observed among *Perkinsus*, it is difficult to differentiate species based on morphology alone, and in some cases difficult to distinguish *Perkinsus* from other protists, such as *Colpodella*, which has similar morphological features (Villalba *et al*., 2004). Nevertheless morphological analysis was used to support numerous reclassifications, at times placing *Perkinsus* in: the Ascomycetales, Entomophthorales, Saprolegnales, Haplosporidia, Labyrinthuloides and the Apicomplexa (Mackin, 1951; Sprague, 1954; Mackin and Boswell, 1956; Mackin, 1962; Mackin and Ray, 1966; Perkins, 1976). In 1978 the genus *Perkinsus* was established, placing *P. marinus* in the Apicomplexa (Levine, 1978). Unfortunately considerable disagreement on the classification of *Perkinsus* remained.

The first DNA sequences for *Perkinsus* were published by Fong *et al*. (1993) and Goggin and Barker (1993). Their phylogenetic analysis of the small subunit ribosomal RNA (SSU rRNA) gene sequence suggested that *Perkinsus* species were more closely related to Dinoflagellates than the Apicomplexans. Since then, the development of DNA technology has facilitated further re-evaluation through analyses of multiple DNA loci, such as the ITS and NTS regions, and the SSU rRNA, LSU rRNA and actin genes commonly targeted. As a result, the taxonomic position of species within the genus *Perkinsus* has now been refined (Burreson *et al*., 2005; Murrell *et al*., 2002).

More generally, Zhang *et al.* (2011) have supported the affiliation of the genus *Perkinsus* with an independent lineage (*Perkinsozoa*) positioned between the phyla of Dinoflagellata and Apicomplexa, within the group of taxa referred to as the *Alveolates*, which includes the ciliates, dinoflagellates and apicomplexans. Based on these analyses, current classification places the genus *Perkinsus* within the phylum Perkinsozoa in the superphylum Alveolata.

Domain:	Eukaryota
Kingdom:	Chromalveolata
Superphylum:	Alveolata (Possessing cortical alveoli, cavities)
Phylum:	Perkinsozoa[1] (Genus *Perkinsus* and protists that do not fit existing Alveolata phyla)
Class:	Perkinsea
Order:	Perkinsida
Family:	Perkinsidae
Genus:	*Perkinsus* (Levine 1978)

Seven species of *Perkinsus* are currently recognized: *P. beihaiensis* (Moss *et al*., 2008), *P. chesapeaki (*syn. *P. andrewsi*) (McLaughlin *et al*., 2000), *P. honshuensis* (Dungan and Reece, 2006), *P. marinus* (Mackin *et al*., 1950), *P. mediterraneus (*Casas *et al.,* 2004), *P. olseni* (syn. *P. atlanticus*) (Lester and Davies, 1989) and *P. qugwadii* (Blackbourn *et al.,* 1998).

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[1] Norén, F, Moestrup, Ø, Rehnstam-Holm, Ann-Sofi. 1999. *Parvilucifera infectans norén et moestrup gen. et sp. nov.* (perkinsozoa phylum nov.): a parasitic flagellate capable of killing toxic microalgae. *European Journal of Protistology* 35(3): 233–254.

Perkinsus is the widespread cause of disease and lost production in mollusc fisheries world-wide. Found mostly in temperate waters, two species are listed internationally as notifiable by the OIE[2] and also appear on *Australia's National List of Reportable Diseases of Aquatic Animals*[3]. Although *Perkinsus marinus* is exotic to Australia, *Perkinsus olseni* is enzootic and well-known as the cause of serious infections in various wild abalone populations in south-eastern Australia.

Perkinsus proliferates in tissues and may produce brown pustules or abscesses. These may contain a caseous creamy-brown exudate. In chronically infected populations, these abscesses are found in the pedal muscle and mantle of *Haliotis rubra* and *H. laevigata* thereby significantly reducing their market value. In outbreaks of *P. olseni* infection in culture facilities in Australia, 30 to 40% mortalities have occurred among *H. laevigata*. In each case, introduced blacklip abalone (*H. rubra*) were found to be responsible for the outbreaks (Goggin and Lester, 1995). Transmission of this parasite occurs directly between individual molluscs. Prezoosporangia from rupturing abscesses or decaying abalone undergo development to zoosporangia in seawater. This occurs within nine days at 20 °C and three days at 28 °C and hundreds of motile, biflagellate zoospores are produced. The zoospores are infective to abalone as well as other molluscs (Goggin *et al*., 1989). Field studies indicated that infections of *P. olseni* in wild *H. rubra* are positively correlated with both water temperature and size of abalone, the parasite being maintained in the environment by *H. rubra* with negligible contributions from other susceptible abalone species or other molluscs (Lester et al. 2001).

According to the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE, 2015), *P. olseni* has an extremely wide host range. Known hosts include the clams *Anadara trapezia*, *Austrovenus stutchburyi*, *Ruditapes decussatus*, *R. philippinarum, Tridacna maxima, T. crocea, Protothaca jedoensis* and *Pitar rostrata* (Goggin & Lester, 1995; Villalba *et al.*, 2004; Cremonte *et al.*, 2005; Park *et al.*, 2006; Sheppard & Phillips, 2008); oysters *Crassostrea gigas*, *C. ariakensis*, and *C. sikamea* (Villalba *et al.*, 2004); pearl oysters *Pinctada margaritifera, P. martensii*, and *P. fucata* (Goggin & Lester, 1995; Sanil *et al.*, 2010); and abalone *Haliotis rubra, H. laevigata, H. scalaris*, and *H. cyclobates* (Goggin & Lester, 1995). It is likely that other sympatric bivalve and gastropod hosts exist within the geographic range.

The detection, rapid identification and reliable differentiation of species is a major issue in the diagnosis and management of Perkinsosis in Australia and elsewhere. Histology and, above all, incubation of host tissues in Ray's fluid thioglycollate medium (ARFTM) have been the classic methods for *Perkinsus* diagnosis. These traditional methods, are straightforward and practical, however they lack sensitivity and fail to differentiate specific species.

PCR has established itself as a sensitive diagnostic technique for *Perkinsus* (Villalba *et al*., 2004), with the ability to detect and differentiate species (Coss *et al*., 2001: Gauthier *et al*., 2006; Audemard *et al*., 2004; Moss *et al*., 2006; Moss *et al*., 2008). However, the molecular methods currently recommended by the OIE are based on conventional 1-step PCR which is generally more labour intensive, slower and less sensitive than the real-time PCRs now implemented for many diseases in diagnostic laboratories.

The primary aim of this project was to develop and validate a *species-specific* real-time PCR (qPCR) assay for *Perkinsus olseni*. The samples collected for initial screening would also be used to formally validate the new molecular test procedures developed, providing direct confirmation of the accuracy and reliability of both the assays and associated test procedures compared with the existing conventional PCRs. At the same time the proposed sampling would provide an opportunity to establish *in vitro* cultures of *P. olseni* from blacklip abalone (*Haliotis rubra*) for DNA sequencing and phylogenetic analysis. The cultures would also be available for further research, and will be made available through the ATCC. The targeted field sampling of known infected populations would also provide an opportunity to investigate speculation arising from the genetic analysis of *Perkinsus* samples from blacklip abalone in NSW. These data suggested that a *Perkinsus* ITS variant detected previously, possibly constitutes a new species (Reece *et al*., 2010).

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[2] http://www.oie.int/en/international-standard-setting/aquatic-code/access-online/

[3] http://www.agriculture.gov.au/animal/aquatic/reporting/reportable-diseases

Objectives

1. Undertake a targeted molecular, histological and cultural examination of known *Perkinsus*-infected wild abalone populations to compare existing methods of detection.

2. Establish representative axenic (single species) cultures of *Perkinsus* sp. from infected abalone.

3. Use established PCRs and DNA sequencing methods to confirm the presence of *P. olseni* and determine the genetic diversity, including other *Perkinsus* sp. from these populations.

4. Develop and validate qPCR methods for the detection and identification of *P. olseni* in infected abalone.

5. Compare and evaluate the performance of the objective 4 qPCR with existing conventional PCR methods for detection of *P. olseni*.

Methods

Reference and Control Samples

A range of *Perkinsus* reference samples was obtained as a result of generous donation from a number of laboratories. In particular, the authors thank the OIE *Perkinsus* Reference Laboratory, Virginia Institute of Marine Science and Dr Chris Dungan, NOAA Cooperative Oxford Laboratory.

Reference materials included formalin-fixed paraffin-embedded tissues for histology, ethanol-fixed tissues from a variety of mollusc species and ethanol-fixed axenic cultures of most *Perkinsus* species. The axenic cultures provided a source of uncontaminated *Perkinsus* genomic gDNA. The reference samples accumulated over the course of the project are summarised in Appendix 2.

Field Sampling

It was assumed that larger animals were older and were thus potentially exposed to infection over a longer period of time. Collection by divers was therefore biased towards the selection of larger animals.

Field Sampling – Victoria (*Perkinsus*-negative DNA library)

In order to establish a baseline for testing and validation of the qPCR test developed here a large *Perkinsus*-negative DNA library from blacklip abalone (*Haliotis rubra*) was prepared. Clinically normal wild abalone (n=200) were collected with the assistance of commercial divers from multiple sites from 38° 24 209 S to 142° 28 627 E along the southern Victorian coast in 2012. This area was selected because there are no reports of *Perkinsus* infection here and these populations are recognised as being free of *Perkinsus*.

Sampling was conducted in 2012 over a period of weeks from sub-tidal reefs at 5-7 m. A mean water temperature of 13.3°C was reported at this time. Tissue samples of gill and muscle from individual animals were fixed in 90% v/v ethanol and stored at 4°C prior to nucleic acid isolation.

Field Sampling – South Australia

The purpose of sample collection in South Australia was to obtain a range of *Perkinsus*-infected tissues from naturally infected populations of abalone. These tissues would provide material for qPCR test development, tissue culture propagation of *Perkinsus olseni* and PCR test validation. With the assistance of divers from South Australian Research and Development Institute, Primary Industries and Regions South Australia (PIRSA-SARDI), sampling was undertaken at 6 locations around the Port Lincoln area from April 2012 to April 2014. As with the Victorian collection, all sampling was confined to the blacklip abalone (*Haliotis rubra*).

As divers tend to avoid infected reefs, these sampling sites were selected on the basis of historical reports of *Perkinsus* in blacklip abalone at or nearby these locations. These included the east side of Taylor's Island 34° 52.606'S, 136° 0.897'E, where 50 animals of mean length 131 mm were taken. This site was the location of an earlier FRDC-funded study, FRDC Project No. 2000/151, which reported a high prevalence of chronic infection in previous years. As Memory Cove was the site from which *P. olseni* was first described (Lester and Davis, 1981)[4] animals were collected (n = 10, mean length 128 mm) from this site 34° 59.279'S 135° 59.150'E in order to obtain representative material as close as possible to that originally described. Disease

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[4] In the original publication which describes *Perkinsus olseni* the *type locality* of host abalone is reported as near Pt. Lincoln, South Australia Latitude 35°56'S, Longitude 135°59'W. This position is between New Zealand and South America. As samples from Memory Cove were also examined, this site was assumed to be the type locality.

had been observed recently by divers at Thistle Island 34° 57.173'S 136° 7.791'E (n = 22, mean length 137 mm), and 3 sites around Baird Bay (near 33° 9.462'S 134° 25.833'E) where 17 animals were taken.

The sampling at Baird Bay was undertaken on 13/4/2013 with the assistance of the Abalone Industry Association of SA. This site had recently experienced periodic outbreaks of what was thought to be Perkinsosis in the previous year. A total of 17 animals were taken from 3 sites around 33° 9.462'S 134° 25.833'E from This area was subject to commercial consideration and the precise position of sampling was not provided. Figures 1 and 2 provide a general overview of the location of sampling sites in South Australia. A further sampling of 30 animals was undertaken at the Thistle Island site in April 2014, to obtain more tissues for validation.
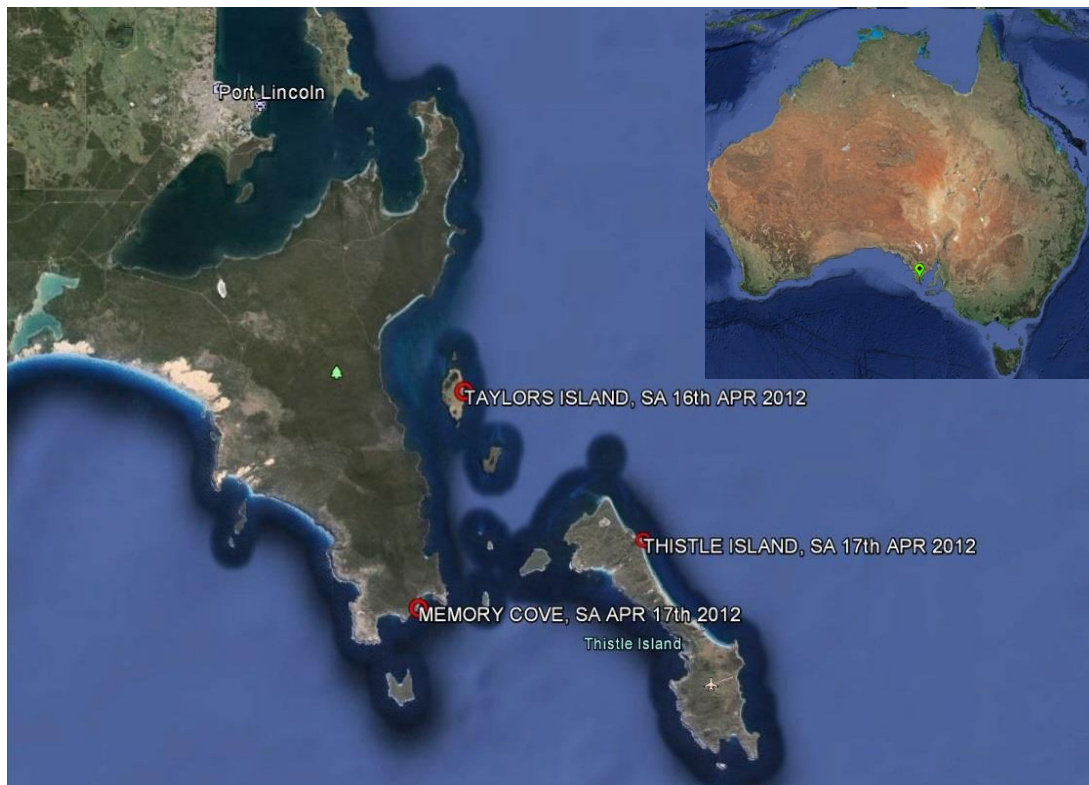
Figure 1 Location of Taylor's Is., Thistle Is. and Memory Cove sampling sites near Pt Lincoln, SA
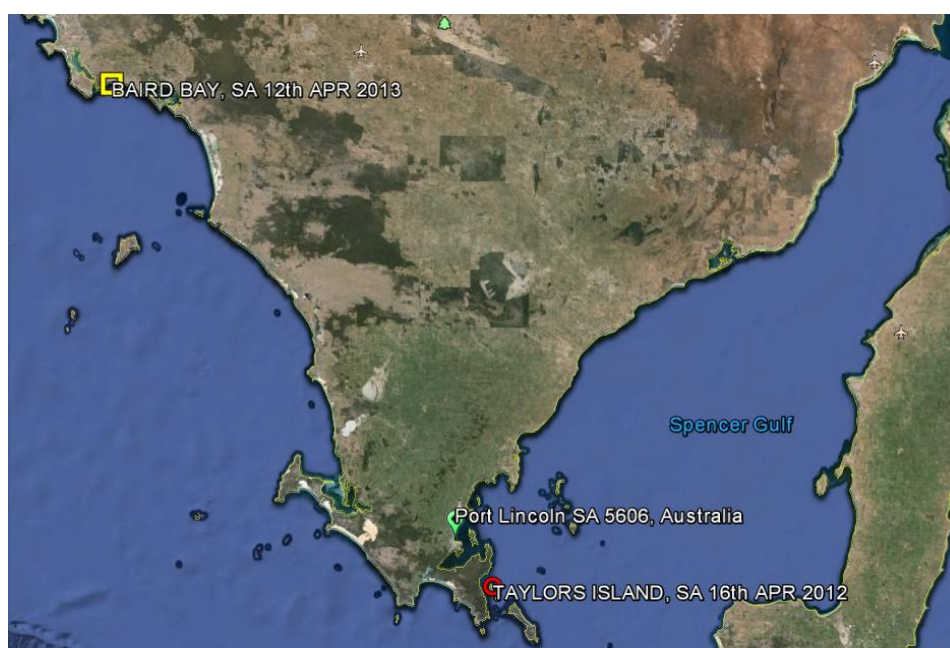
Figure 2 Location of abalone sampling sites around Pt Lincoln, including Baird Bay

Field Sampling – New South Wales

The purpose of this sampling was to obtain a wider range of geographically dispersed *P. olseni* in order to further validate the qPCR, to establish *in vitro* cultures from this area and to further characterise the genetic diversity of *P. olseni* in Australia. Of particular interest was the recently reported ITS variant from this area (Reece *et al.*, 2010).

Despite the widespread mortalities attributed to *Perkinsus* in NSW in the early 2000's (Liggins *et al.*, 2010), only passive monitoring is undertaken for *Perkinsus* in NSW. As a result there is little information on the current distribution of *Perkinsus* along the NSW coast. It is difficult to accept that the mass mortalities and infection previously observed were self-limiting and that *Perkinsus* has disappeared from the east coast of Australia. The lack of current information from commercial fishers and Fisheries NSW regarding the distribution of *Perkinsus* was a significant impediment to obtaining useful samples from NSW. As our funding was confined to the sampling of a limited number of specific sites, an area around St Georges Head, just south of Jervis Bay, was selected for targeted sampling and a permit was obtained from the NSW National Parks service. Although no sampling has taken place at this site for some years both *P. olseni* and the ITS variant (Reece *et al.* 2010) were both previously reported from this area with reasonable frequency (Liggins and Upston, 2010). Given the apparent spread of disease southwards along the coast, this area and an adjacent area just south at Sussex Inlet were selected for sampling.

With the help of a commercial diver, collection of blacklip abalone (n = 30) was undertaken in June 2013. Multiple sites around St Georges Head and Sussex Inlet were sampled. The water temperature was not recorded at the time, but the temperature was reported to be <17°C at a depth of 5 to 7 m. The GPS co-ordinates of the St Georges Head sampling sites were as follows: 1. 35°08.600' S 150°45.760' E, 2. 35°09.192' S 150°45.686' E, 3. 35°09.679' S 150°45.598' E, 4. 35°10.212' S 150°45.042' E and 5. 35°12.033' S 150°41.755' E and are shown in Figure 3.

Figure 3 Southern Jervis Bay sampling area for blacklip abalone in NSW

Laboratory Examination

All abalone were subjected to detailed laboratory examination which included, gross examination for lesions or other signs of disease, culture in ARFTM, tissue culture, histology, PCR (OIE-recommended assays) and the *genus-specific* real-time (qPCR) of Gauthier *et al.* (2006). Samples were also screened using the new real-time PCR (AFDL *Pols* qPCR) developed in this project. Together these data were analysed and used to validate the assay.

Histology

All samples for histology were cut directly into pre-labelled histology cassettes and fixed in Davidson's solution. If samples could not be processed within 48 hours, the fixed tissues were transferred to 70% (v/v) ethanol before embedding. Samples for PCR were taken from adjacent tissues at the same time. The procedure for selecting tissues was standardised to maximise the possible detection of *Perkinsus* and ensure consistency between populations. The process of dissection and sampling is summarised in Appendix 4.

Where gross lesions were observed, these were split between RFTC, PCR and histology. When lesions were too small to do this accurately the tissue was split between RFTC and PCR.

Figure 4 Typical focal lesions (1–2 mm) *Perkinsus* found in muscle adjacent to the epipodial fringe. Here a small 3 x 12 mm section of tissue was taken for histology (12:978-30).

Ray's Fluid Thioglycollate Culture (RFTC)

Since first described by Ray in 1952 there have been numerous implementations of *Perkinsus* "culture" using Ray's Fluid Thioglycollate Medium (ARFTM), including methods for quantification (IFREMER, 2011). Although RFTC was not of primary interest in this project, it was included for comparison, as an additional test for validation, and if possible as a source of viable hypnospores for *in vitro* culture. We used the Alternative Ray's fluid thioglycollate medium (ARFTM) used by Dungan (2010) which included antibiotics (Appendix 6).

ARFTM medium was prepared fresh and dispensed in 10 ml aliquots. Finely dissected fresh tissues, consisting of approximately 1 to 2 g pooled gill, mouthparts, mantle, muscle (including visible lesions) and hepatopancreas, were added to fresh medium. The tubes were then incubated in the dark at 22°C for at

least one week before microscopic examination. After the first samples from South Australia were cultured, hepatopancreas was cultured separately in a second tube.

A small amount of tissue including debris from the bottom of the tube was transferred to a glass microscope slide, a drop of Lugol's Iodine was added and the tissues teased apart. The stained material was covered with a 40 x 20 mm glass coverslip and the whole area of the coverslip was examined by light microscopy with normal illumination using the x10 objective. Intensity of infection was scored subjectively.

0	0 = no *Perkinsus* observed
1	1 = very light infection 1 to 10 hypnospores in tissue prep.
2	2 = light to moderate, 10 to 30 hypnospores in tissue prep.
3	3 = moderate, all fields x100 have several parasites
4	4 = heavy infection, half of the tissues have hypnospores staining blue-black with parasites.
5	5 = very heavy infection, majority of tissue stains blue-black. Enormous numbers of parasites.

In vitro culture

Methods for *in vitro* culture of *Perkinsus* in this project were based on routine procedures implemented at the NOAA Cooperative Oxford Laboratory, Oxford, MD. Isolation medium was supplemented with antibiotics using the standard and maximum concentrations for *P. marinus* as a guide. Medium used for culture and preservation is summarised in Appendix 7.

Various strategies for culture were used during the course of the project, including direct inoculation of lesion material into 24-well plates containing DMEM/Ham's F12-3% at different salinities and using selected ARFTM-incubated tissues with heaviest *Perkinsus sp.* hypnospore densities, as a source of inoculum. Thioglycollate cultures (ARFTM) of pooled tissues (mouth parts, mantle, muscle, gills and digestive diverticula) from all animals were incubated in parallel to provide an indication of which samples contained the highest parasite numbers and the general level of infection in the population.

In each case the inoculum was washed in several changes of either DMEM/Ham's F12-3% or artificial sea water (Sigma Cat. No. S9883) containing antibiotics. This was achieved in sterile 50 ml screw-capped tubes, where, after careful mixing by inversion, the hypnospores were allowed to settle to the bottom of the tube and the supernatant removed with a 10 ml serological pipette before transfer to 24-well culture plates. Plate wells contained 2ml of DMEM/Ham's F12-3% *Perkinsus* sp. culture medium (Dungan and Hamilton, 1995). Resulting suspensions were serially diluted at 0.5 ml/well into three additional wells containing 2ml of culture medium. Inoculated culture plates were covered, incubated both at 20°C and 25°C, and observed for *Perkinsus* sp. proliferation.

Contamination was a significant problem and wells that were overcome by bacterial or fungal growth were carefully aspirated dry and disinfected with 80% v/v ethanol. When promising cell replication was observed, these wells were passed into fresh 24-well plates or 25 cm^2 culture flasks. Cell culture-adapted cells were normally ready for sub-culture after 7–10 days at 25°C in DMEM/Ham's F12-3% culture medium. Antimicrobials were sequentially reduced or eliminated from the medium after 2 or 3 passages.

Selected isolates were cloned by limiting dilution (sequential 1:2 dilutions) in 96-well culture plates. Wells were examined closely (using x10 and x20 objectives) under phase-contrast optics using an inverted light microscope (Leica DMIL LED with DMC2900 camera). Wells containing individual cells were identified. In this way, both clonal and polyclonal axenic cultures were subsequently expanded in culture flasks and cryopreserved in vapour phase above liquid N$_2$.

The viability of all cryopreserved cultures was confirmed using the procedure outlined in Appendix 7.

PCR sampling and Isolation of Nucleic Acids

In most cases the tissues for PCR were taken at the same time as samples for histology, the tissues being taken from adjacent sites at the same time. Given the necessary constraints associated with tissue dissection, sampling and transport, all tissue samples were fixed in 80-90% (v/v) ethanol and stored at 4-6°C prior to DNA extraction.

As DNA isolation directly affects all downstream applications, it is the most critical step in molecular testing.

As *Perkinsus* infection appeared as focal lesions with uneven distribution through affected tissues we looked to homogenising larger (2-4 g) and perhaps more representative samples. Various methods of sample homogenisation were trialled. Ethanol-fixed abalone tissues, particularly muscle and mantle are very tough and rubbery and were particularly difficult to homogenise. We found that homogenisation of fixed tissues was generally impractical for processing large numbers of ethanol-fixed samples. Even mechanical processing with substrates such as, 1 mm steel microspheres, silicon shards and ceramic beads, did not produce a homogeneous sample for DNA extraction (data not shown). After homogenizing large pools of tissues many samples were inhibitory in the endogenous 18S control PCR or reacted inconsistently between replicate PCRs.

In order to facilitate the comparison of results between test runs and maintain consistency for the purposes of test validation a standardised extraction procedure was adopted and all sample processing was based on the uniform chemical lysis of samples. In this case we used the QIAGEN DNeasy Tissue kit (Cat. No. 69504).

The MagMAX™ Express 96 system was used to prepare the *Perkinsus*-negative abalone library as repetition stress was an issue with these samples. The modification was justified on the basis that previous equivalence testing has shown this system was comparable to column-based extraction for herpesvirus in mollusc tissue. The remainder of testing, including that of reference and field samples, relied on the standard Qiagen kit (above) with columns as supplied by the manufacturer.

Nucleic acid extraction of normal abalone (Perkinsus-negative DNA library)

These samples were collected over a period of time at a number of sites along the coast. When sufficient sample numbers for mass processing were accumulated, a standard weight of 20-30 mg tissue, consisting of 15 mg of gill and 15 mg of muscle tissue, from individual animals was dissected into ATL buffer with Proteinase K (180 µl ATL + 20 µl PK), as recommended. Samples were incubated overnight at 56°C or until lysed. Lysed samples were inverted or gently vortexed several times to mix during the course of digestion. Samples were then centrifuged briefly (3 min at 10,000 x *g*), after which 50 µl of supernatant was used for DNA isolation by MagMAX™ Express 96 system. Total nucleic acid was eluted with paramagnetic beads using 80 µl of MagMAX™ elution buffer for each sample. The nucleic acids (n = 200) were dispensed into duplicate 96-well PCR plates and stored at -20°C until use.

Nucleic acid extraction of reference control samples and field specimens

Control samples and field specimens were comprised of both ethanol-fixed tissues and fixed cells propagated in tissue culture. A standard weight of between 20-30 mg of tissue, or 10 mg of cells, was lysed in Qiagen ATL/PK buffer according to the manufacturer's instructions. DNA isolation was then carried out using Qiagen columns (Qiagen DNeasy Blood and Tissue Kit (250): Cat. No. 69506) with final elution of DNA in 100 µl AE buffer. DNA samples were stored at -20°C when not in use.

Taylor's Island (12:978), Memory Cove (12:979a)[5] and Thistle Island and (12:979b)[6]

The purpose of sample collection in South Australia was to obtain a range of *Perkinsus*-infected tissues from naturally infected populations of abalone. A total of 82 blacklip abalone were sampled as a source of

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[5] 12:979[a] = 12:979-1 to 10

[6] 12:979[b] = 12:979-11 to 34

Perkinsus from South Australia. These tissues provided material for DNA sequencing and qPCR test development, tissue culture propagation of *Perkinsus olseni* and PCR test validation.

Thistle Island (14:0233)

The primary purpose of this sampling was to develop an understanding of the distribution of *Perkinsus* DNA around lesions in the tissues of affected abalone and to determine how to best target abalone sampling for detection by PCR. In addition to testing visible lesions by PCR, multiple PCR samples from normal tissues immediately adjacent to lesions and samples from apparently normal animals were also screened. Multiple samples from all animals were tested in parallel using both the OIE conventional PCR and the AFDL *Pols* qPCR.

After the animals were euthanized by cooling on ice and their shells removed, the visceral mass was separated from the underlying muscle. The gills and the external surface of the viscera were closely examined for the presence of gross lesions. The muscle was sliced transversely at 1 to 2 mm intervals along the entire length of each animal and the cut surface examined for lesions. Gross lesions were only observed in muscle. For PCR examination, in addition to visible muscle lesions, one half of the gill was dissected longitudinally and fixed in ethanol. From this, 3 sub-samples were taken (30 mg) at 3 levels along the length of the gill. Normal muscle was also taken from 3 locations in the pedal muscle. If *Perkinsus* lesions were visible in the muscle, samples of "normal" muscle were taken from the same transverse section containing the lesion, but from an area which appeared normal. In this case separate disposable scalpel blades were used for each sample to eliminate possible cross-contamination from the site of the visible lesion. A small piece of digestive gland (approximately 200-300 mg) was also taken from the lower third of the left side of the visceral mass from all 30 animals. All samples for PCR were fixed in 80% ethanol. At the same time, corresponding samples were fixed in Davidson's fixative for histological examination. All fixed samples for DNA extraction and PCR were further dissected to provide a uniform sample of approximately 30 mg of tissue for digestion.

PCR Analysis

The 18S ribosomal RNA FAST qPCR was essential for confirming the suitability of our test samples for PCR and is summarised in the PCR Methods of Appendix 3. The 18S targeted assay provided confirmation of the successful extraction of nucleic acids from a sample and distinguished samples where PCR was inhibited. The qPCR plate format and the use of Taqman® Fast Universal PCR Master Mix provided rapid turnaround for convenient QC of samples. Subsequent testing with species-specific plasmid or gDNA controls provided confirmation of the performance of each mastermix.

All conventional PCR testing for *Perkinsus* was based on the OIE-recommended methods according to the standard implementation used at CSIRO-AAHL (Appendix 3). Similarly all real-time assays, including the 18S qPCR, the **genus-specific** qPCR of Gauthier *et al.* (2006) and the AFDL qPCR for *P. olseni*, developed in the course of this project were carried out using standard methods summarised in Appendix 3. The reagents used for mastermix preparation and amplification parameters are summarised.

PCR screening of *Perkinsus*-negative DNA library

The full library of 200 Victorian samples from the *Perkinsus*-free zone were screened for *P. olseni* using the OIE 85/750 cPCR (OIE-recommended 1-step **genus-specific** conventional PCR), the OIE 140/600 cPCR (OIE-recommended 1-step **species-specific** conventional PCR for *P. olseni),* the real-time **genus-specific** assay of Gauthier *et al.* (2006), sensitive to 1 fg *Perkinsus* genomic DNA (gDNA), and a host-directed 18S qPCR to verify the extraction and assess sample inhibition.

Real-time PCR Test Development

Given the focus and considerable accumulating data of DNA sequence in the ITS region of *Perkinsus* and the use of this region for other molecular assays, including the OIE-recommended PCR tests, we aimed to similarly exploit this region for a specific *P. olseni* qPCR test.

DNA sequence data were generated from the ITS region using the OIE genus-specific primers 85F-750R, from a range of *Perkinsus* samples (n = 16) collected as part of this project including from both Taylor's Island and Thistle Island. These South Australian *Perkinsus* DNA sequences were aligned with a range of *P. olseni* sequences from GenBank to provide a map of both variable and conserved sites which was used for primer/probe design. Similar consensus sequences for *P. marinus, P. chesapeaki, P. honshuensis, P. beihaiensis* and *P. mediterraneus* were also included. Various possible primer and probe combinations for *P. olseni* were generated using Primer Express 3 (Life Technologies). These were assessed for specificity using the consensus sequences for the non-target *Perkinsus* species. Two forward and 2 reverse primers specific for *P. olseni* which passed the performance criteria of Primer Express were selected. Before synthesis, the specificity of the candidate primers was checked using NCBI primer BLAST (Ye *et al*., 2012) and the GenBank NR database.

The best primer combination was selected by their performance in SYBR assay with serially diluted *P. olseni* gDNA, followed by Taqman screening of gDNA from ATCC cultures of *P. olseni, P. marinus, P. chesapeaki* and *P. honshuensis.*

Analytical Sensitivity (ASe) and Specificity (ASp)

Replicate testing of serially diluted gDNA and plasmid DNA was undertaken to determine analytical specificity (ASp) and sensitivity (ASe). The accumulated test data of field and reference samples were used to estimate diagnostic specificity (DSp) and sensitivity (DSe) by statistical methods, including ROC and Bayesian analysis.

Analytical Sensitivity

Both *P. olseni* plasmid and gDNA from reference cultures were used to obtain an estimate of the analytical sensitivity (ASe) of the prototype AFDL *Pols* qPCR system. Plasmid testing was used to determine the specific copy number detected and testing of genomic DNA provided an estimate of the quantity of gDNA that could be detected.

Plasmids (Invitrogen pPCR2.1-TOPO) containing *Perkinsus* inserts prepared from the OIE 85F-750R cPCR (OIE diagnostic region) amplicon were constructed by standard methods using axenic clonal cultures as a source of genomic DNA. Plasmid stocks of 10^8 copies/µl were prepared using a standard molar/mass calculation and the Qubit 2.0, (Invitrogen) fluorimeter to provide an initial measurement of the starting concentration. Yeast tRNA carrier (Invitrogen Cat. No. 15401-029) at a concentration of 50 ng/µl in 1 mM TE buffer (pH 7.6) was used as diluent.

For the sake of simplicity, the analytical sensitivity (ASe) was determined experimentally (ASe$_{exp}$) as suggested by Caraguel *et al.* (2011). In this case, 5 replicates of each plasmid, diluted in an appropriate non-target matrix, were tested to provide an estimate of ASe$_{exp}$ at 95% confidence, as required by the OIE. Recognising the potential for the dilution medium to influence PCR amplification, the testing was repeated with 3 different diluents; 1 mM TE buffer (pH 7.6), yeast tRNA at 50 ng/µl in 1 mM TE buffer and pooled abalone genomic DNA at 50 ng/µl. The later would serve to simulate detection in a matrix of abalone gDNA at a level found in normal field samples.

Analytical Specificity

All the *Perkinsus* samples used to assess analytical specificity (ASp) were derived from sources outside Australia and represent a wide range of mollusc host species with a wide geographic (global) distribution. The axenic cultures provided uncontaminated *Perkinsus* gDNA target, at high concentration without interfering host or other DNA, significantly favouring any cross-reaction that might occur in the candidate assay (AFDL *Pols* qPCR).

No *P. mediterraneus* or *P. qugwadii* samples were obtained and these species were not tested. While the specificity (ASp) using these 2 species could not be validated by testing, the DNA consensus alignments of *P. mediterraneus*, *P. qugwadii* and the test primers and probe show significant mismatch (Figure 17). While confirmatory testing was not undertaken, the major differences in sequence observed over the diagnostic

region are sufficient for us to expect that the analytical specificity of our *P. olseni* assay (AFDL *Pols* qPCR) would be maintained for these species.

Diagnostic Test Validation and Real-time (qPCR) Test Performance

The design of primers and probe for the *P. olseni* Taqman® assay was based on *Perkinsus* DNA sequence generated from South Australian abalone collected at the beginning of this project and further sequence data made publically available by the National (USA) Centre for Biotechnology Information through GenBank.

Validation of the *P. olseni* qPCR was based on the testing of a wide range of samples including; mollusc field samples, cloned plasmids and axenic reference cultures of *Perkinsus*. All calculations of test sensitivity and specificity were based on testing of naturally infected animals collected in the field from wild populations. *P. mediterraneus* and *P. qugwadii* were not included in testing as few samples of these species are available and none could be sourced for use in this project.

Diagnostic Sensitivity (DSe) and specificity (DSp)

The performance characteristics of the AFDL *Pols* qPCR for detection of *Perkinsus olseni* was determined by Latent Class Analysis with Bayesian modelling to obtain estimates of diagnostic sensitivity and specificity. A Cochran statistical model was used to assess repeatability and a comparative statistical analysis of gill and muscle results was undertaken to compare the relative performance of these specimens in the qPCR assay. In order to reduce fragmentation of this analysis, the performance and validation analysis are presented together in the results with discussion. The data summary for statistical analysis is shown in Appendix 8.

Sequence and Phylogenetic Analysis

Analysis of Australian *Perkinsus olseni* from blacklip abalone field samples

Amplification of the ITS region of *Perkinsus* was achieved using the two OIE methods. For convenience, sequencing was mostly confined to those samples that were also positive by standard 1-step conventional PCR, however some data from samples subjected to prolonged cycling times (70 cycles) were also included in analysis. The prolonged cycling procedure was useful in confirming some real-time positive samples with high C_T values. Its general application was limited in this analysis. Although both the OIE *Perkinsus spp.* PCR (85-750) and the OIE *Perkinsus olseni* (140-600) PCR were sequenced, the *Perkinsus spp.* PCR (85-750) provided the longer DNA product and was used for sequence analysis and subsequent phylogenetic analysis in the majority of cases.

Analysis of *Perkinsus olseni* cultures

In addition to DNA sequence analysis of *Perkinsus* field samples, a more detailed analysis was undertaken to better characterise the four *P. olseni* axenic cultures established in this project. DNA analysis was expanded to include the ITS region, the large sub-unit of the rRNA gene (LSU) and the actin gene. These data were used to confirm the uniformity of both the polyclonal and clonal cultures established, confirm the identity of the cultures and generally provide more data on the genetic composition of *Perkinsus* in Australia. These data were also used for the submission of isolate 12:00978-12 to the ATCC and to establish it as the holotype culture of *P. olseni*. Replicating cultures were also sent to Japan where our sequence data were independently confirmed at Tokyo University by Prof Tomoyoshi Yoshinaga.

Sequence Alignments and Phylogenetic Analysis

Sequence analysis and alignments were achieved using Geneious software v7.1.7 (Biomatters) and the phylogenetic trees were constructed using both the Geneious software v7.1.7 (Biomatters) and Mega6. Sequence files were *de novo* assembled (without extrinsic comparison to existing data) and primer trimmed. Any discrepancies in sequence alignments were acknowledged by the use of ambiguous bases.

Sequence alignments and trimming were conducted using Geneious, while Mega6 was used to build sequence alignments and construct phylogenetic trees. Briefly, neighbour-joining trees were performed using the Tajima-Nei model with 10,000 bootstrap replicates.

The evolutionary history was inferred using the neighbour-Joining method of Saitou and Nei (1987). The optimal tree with sum of branch length is shown for each analysis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The evolutionary distances being computed using the Tamura-Nei method (Tamura and Nei, 1993) and are in the units of the number of base substitutions per site. The analysis involved 44 nucleotide sequences for the ITS, 30 sequences for the LSU and 41 sequences for the actin gene. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013). The 1st+2nd+3rd+non-coding codon positions were included. All sequence positions containing gaps and/or missing data were eliminated. The total number of positions in the final dataset for each analysis is indicated in the figure legends.

Results

Reference and Control Samples

The reference samples for PCR included 5 of the 7 known species of *Perkinsus*. All samples were received as ethanol-fixed specimens. *P. olseni* (syn. *P. atlanticus*)*, P. marinus, P. chesapeaki* (syn. *P. andrewsi*)*,* and *P. honshuensis* came as axenic cultures fixed in ethanol. *P. beihaiensis* was received as fixed tissues from naturally infected clams. A large stock of homogenised ethanol-fixed *Crassostrea virginica* tissues infected with *P. marinus* was also established.

The reference samples were screened by PCR using the OIE genus-specific conventional PCR (85/750), the OIE *P. olseni* species-specific conventional PCR (140/600), the *Perkinsus* genus-specific Taqman® assay of Gauthier *et al.* (2006) and the AFDL *Pols* qPCR assay. The 4 PCR systems have specificity for either *Perkinsus spp.* (genus-specific) or *P. olseni* (species-specific). The specificity of each test is listed under each test name summarised in Table 1. The results of this testing was generally straightforward and confirmed the ASp of the AFDL *Pols* qPCR.

Although not officially recognised, the Gauthier *et al.* (2006) genus-specific qPCR was found to be an excellent reference test for all samples, particularly when the level of detection of the OIE genus-specific assay was beyond limits, as was the case with some *P. beihaiensis* tissue samples (red highlight, Table 1). The Gauthier assay was designed to detect all *Perkinsus spp.* and has very similar analytical sensitivity (ASe) compared to the AFDL *Pols* qPCR system (Table 3 and 4 below).

All the *P. olseni* (syn. *P. atlanticus*) samples were positive by all tests, as expected. This testing was consistent with the specificity expected of the system and furthermore demonstrated the ability of the AFDL *Pols* qPCR system to detect *P. olseni* strains of diverse origin including a range of exotic strains from overseas.

The only anomalous results were obtained with *P. honshuensis*. Our samples of *P. honshuensis* (highlighted in blue, Table 1) originated from cultures derived from the Manila clam *Venerupis philippinarum* (Dungan, 2006). In this study, some clams were reported to be co-infected with both *P. honshuensis* and *P. olseni.* As the DNA sequence of these species appears to be well-differentiated over the diagnostic region (Figure 17) we attributed the positive *P. olseni* result with these samples to the co-infection reported by these authors. Subsequent PCR analysis of cultures derived from the same sample (Mie-3g) by Umeda and Yoshinaga (2012) further explain our results and showed that some polyclonal cultures of *P. honshuensis*, including some obtained from the ATCC, are mixed and contain both *P. honshuensis* and *P. olseni*. In this case our sample, Mie-3g-A, gave consistently positive results for both the *Perkinsus* genus-specific assays and *P. olseni* assays, whereas sample Mie-3g-B was consistently negative by the AFDL *Pols* qPCR. The isolated positive result for *P. olseni* with the OIE *P. olseni*-specific conventional PCR (140/600) was not replicated and could not be explained in isolation.

In order to unambiguously establish the specificity of the AFDL *Pols* qPCR with respect to *P. honshuensis*, we selected for a *P. honshuensis* plasmid (Invitrogen pPCR2.1-TOPO) with an insert generated by the OIE 85F-750R PCR (diagnostic region) from sample Mie-3g-A.The insert was confirmed by DNA sequencing to be from *P. honshuensis*. PCR screening of the *P. honshuensis* plasmid did not show any signal above background in the AFDL *Pols* qPCR system over the range of 2×10^1 to 2×10^7 copies (Figures 21, 22 and Table 5). Despite the anomalous results obtained in testing the unreliable *P. honshuensis* gDNA samples by conventional PCR, the clear negative result obtained with the AFDL *Pols* qPCR with high concentrations of *P. honshuensis* plasmid convincingly demonstrates the specificity of the new qPCR system with respect to *P. honshuensis* in the region targeted by the assay.

The test results obtained for *P. marinus, P. chesapeaki* and *P. beihaiensis* were clear-cut. The samples derived from culture (*P. marinus, P. chesapeaki*) had high levels of DNA target and were test-negative by all the *P. olseni*-specific tests, including the new AFDL *Pols* qPCR.

Table 1 Summary of PCR analysis of *Perkinsus* reference samples.

Species	Isolate	OIE 85/750 PCR	OIE 140/600 PCR	Gauthier GENUS qPCR	AFDL *Pols* qPCR
	Test specificity	*Perkinsus* genus	*P. olseni*	*Perkinsus* genus	*P. olseni*
Species	**Isolate**				
P. olseni	NZMSAs-5	+	+	+	+
P. olseni	Mie-5mg	+	+	+	+
P. atlanticus (=P. olseni)	PaG3F	+	+	+	+
P. olseni	NZMSAs-24	+	+	+	+
P. olseni	Mie-13v	+	+	+	+
P. olseni	Mie-4g	+	+	+	+
P. olseni	NZMSAs-21	+	+	+	+
P. marinus	PXBICv-22	+	-	+	-
P. marinus	EBPICv-15	+	-	+	-
P. marinus	LA-25	+	-	+	-
P. marinus	HFtP-14	+	-	+	-
P. marinus	PXBICv-25	+	-	+	-
P. marinus	CRTW-3he	+	-	+	-
P. marinus	DBNJ-1	+	-	+	-
P. marinus	EBPICv-18	+	-	+	-
P. marinus	LICT-1	+	-	+	-
P. marinus	PRPC/DI 9403	+	-	+	-
P. marinus	CRTW-3he	+	-	+	-
P. andrewsi (P. chesapeaki)	PAND-A8-4a	+	-	+	-
P. chesapeaki	PXSATp-6	+	-	+	-
P. chesapeaki	EBNPMb-1	+	-	+	-
P. chesapeaki	CRMA-J55	+	-	+	-
P. chesapeaki	PXDCRc-8	+	-	+	-
P. chesapeaki	CRTP-17	+	-	+	-
P. chesapeaki	YRKCMb-1	+	-	+	-
P. chesapeaki	PXDCRc-5	+	-	+	-
P. chesapeaki	EBNPMl-4	+	-	+	-
P. chesapeaki	CHBRMa-14	+	-	+	-
P. chesapeaki	EBNPMl-5	+	-	+	-
P. chesapeaki	CHBRMa-14	+	-	+	-
P. honshuensis	Mie-3g-A	+	+	+	+
P. honshuensis	Mie-3g-B	+	+	+	-
	Tissue				
P. beihaiensis	3870-5	+	-	+	-
P. beihaiensis	3870-10	+	-	+	-
P. beihaiensis	3871-1	+	-	+	-
P. beihaiensis	3871-2	-	-	+	-
P. beihaiensis	3871-3	-	-	+	-
P. beihaiensis	3871-5	-	-	+	-
P. beihaiensis	3871-6	+	-	+	-
P. beihaiensis	3872-1	-	-	+	-
P. beihaiensis	3872-2	+	-	+	-
P. beihaiensis	3872-3	-	-	+	-
P. beihaiensis	3872-7	+	-	+	-
P. beihaiensis	3872-8	+	-	+	-
P. beihaiensis	3873-4	+	-	+	-
P. beihaiensis	3873-7	+	-	+	-
P. beihaiensis	3875-2	+	-	+	-
P. beihaiensis	3875-5	+	-	+	-

The *P. beihaiensis* reference samples were in the form of naturally infected tissues from the field and, as such, the levels of target DNA was variable and, in some cases, below the threshold of detection of the OIE genus-specific conventional PCR (85/750). All the samples that were positive by the OIE genus-specific conventional assay were negative by both *P. olseni*-specific assays, including the AFDL *Pols* qPCR. Although *P. beihaiensis* samples 3871-2, 3871-3, 3871-5, 3872-1 and 3872-3 were test negative by the OIE genus-specific conventional PCR (85/750), these samples were all positive by the Gauthier genus-specific Taqman® assay. We attributed this to the greater sensitivity (ASe) of the Gauthier Taqman® T-Perk assay over the OIE one-step conventional PCR. Given that the Gauthier *genus-specific* assay and the AFDL *Pols* qPCR have very similar analytical sensitivity (shown previously), it would not be unreasonable to expect some amplification after 45 cycles if there was "cross-reaction" with *P. beihaiensis* in the AFDL *Pols* qPCR. This was not the case and all the *P. beihaiensis* field samples were unambiguously negative in the AFDL *Pols* qPCR, with no signal observed in any sample after 45 cycles. We concluded that the specificity of the AFDL *Pols* qPCR system with respect to this species was confirmed.

Laboratory Examination of Field Samples

Victorian Field Samples – PCR of *Perkinsus*-negative DNA library

The full library of 200 Victorian samples from the *Perkinsus*-free zone was screened for *P. olseni* with the OIE 85/750 cPCR (genus), the OIE 140/600 cPCR (species)*,* the real-time assay of Gauthier *et al.* (2006), which is genus-specific for *Perkinsus* and sensitive to 1 fg *Perkinsus* gDNA, and the AFDL *Pols* qPCR.

A few light bands of unexpected size were observed in the conventional OIE PCR tests. In each case these bands were sequenced and shown to be non-specific artefacts of amplification (data not shown). Duplicates of all the Victorian abalone samples were tested twice with each PCR and were test-negative. All samples were confirmed by the 18S host-directed PCR. Given that these samples were derived from geographically removed and commercially exploited abalone populations with no history of *Perkinsus*, and that all PCR testing was negative for *Perkinsus*, these samples were accepted as known negative samples for the AFDL *Pols* qPCR test validation.

South Australian Field Samples

Taylor's Island (12:978), Memory Cove (12:979a)[7] and Thistle Island (12:979b)[8]

A total of 82 blacklip abalone (mean length 133.1 mm) were examined. Of these, 31 (38%) displayed gross lesions or external signs of disease (Figure 7, 8, 9). At Taylor's Island, 16 of 50 animals (32%) were found positive by Ray's Test with 12 of 50 positive by histology (24%). At Memory Cove, only one animal tested positive, while at Thistle Island 11 of 22 (50%) animals were positive in Ray's medium with 10 histological positives (45%). A total of 27 abalone were positive by culture in Ray's medium (33%), with 23 of 82 animals examined, positive by histology (28%).

		Lesions	RFTC	Histology	85/750 cPCR	140/600 cPCR	AFDL qPCR	Total Animals
Taylor Island	12-978	20	16	12	17	18	29	50
Memory Cove	12-979-1	0	0	1	0	0	1	10
Thistle Island	12-979-11	11	11	10	10	15	16	22
		31	27	23	27	33	46	82

[7] 12:979ᵃ = 12:979-1 to 10

[8] 12:979ᵇ = 12:979-11 to 34

Baird Bay

A total of 17 blacklip abalone were examined. Of these, 12 (71%) displayed gross lesions, these same animals were found positive by Ray's Test. Histology found 11 of the animals infected (65%). Animal 13:01031-15 had lesions and was positive by Rays test but *Perkinsus* could not be identified

		Lesions	RFTC	Histology	85/750 cPCR	140/600 cPCR	AFDL qPCR	Total Animals
Baird Bay	13-01031	12	12	11	11	10	14	17

Thistle Island (14:0233)

A total of 30 blacklip abalone were collected from an area thought to have a high prevalence of infection. The aim was to obtain a range of diseased samples. From these a smaller number of animals displaying gross lesions would be selected for examination, according to severity of disease. Where previously animals from this area had a high rate of infection (50.6%), on this occasion of the 30 animals examined, only 8 animals (20%) displayed visible muscle lesions similar to those previously associated with *Perkinsus*. Of these, only 2 animals had severe gross lesions spread throughout the musculature and were obviously heavily infected. On this basis it was decided to examine all animals to maximise the data available for validation. Subsequent culture of these animals in Ray's fluid thioglycolate medium (RFTC) revealed 4 infected animals (9, 11, 12 and 24), all with gross lesions. Ten animals were suspect positives by histology (9, 11, 12, 18, 21, 24, 26, 28, 29, and 30). All animals with gross lesions were *Perkinsus*-positive by one or more PCR tests.

Table 2 Summary of testing of Thistle Island samples 14:0233

No.	Lesions	RFTC*	Histo	cPCR Gills	cPCR DG	cPCR Muscle	Muscle Lesions	qPCR Gills	qPCR DG	qPCR Muscle	Muscle Lesions	cPCR Result	qPCR Result
1	N	0	-	-	-	-		Und/Und/Und	Und	Und/Und/Und	NS	-	-
2	N	0	-	POS	-	-		37.62/36.62/Und	Und	Und/Und/39.76	NS	Positive	Positive
3	N	0	-	-	-	-		Und/Und/Und	Und	Und/Und/Und	NS	-	-
4	Y	0	-	-	-	-	POS	Und/Und/Und	Und	Und/Und/Und	38.74	Positive	Positive
5	Y?	0	-	-	-	-	-	Und/Und/Und	Und	Und/Und/Und	?	-	-
6	N	0	-	POS	POS	POS		Und/43.74/39.78	38.62	Und/39.00/38.04	NS	Positive	Positive
7	N	0	-	-	-	-		Und/Und/Und	Und	Und/Und/Und	NS	-	-
8	N	0	-	-	-	-		Und/Und/Und	Und	Und/Und/Und	NS	-	-
9	Y	2	+	-	-	-	POS	Und/36.80/Und	Und	Und/Und/Und	38.61	Positive	Positive
10	N	0	-	-	-	-		Und/Und/Und	Und	Und/43.25/Und	NS	-	Positive
11	Y	3	+	-	-	POS	-	Und/Und/Und	Und	Und/Und/Und	34.68	Positive	Positive
12	Y	2	+	-	-	-	-	39.44/Und/Und	Und	Und/Und/Und	35.46	-	Positive
13	N	0	-	-	-	-		Und/Und/Und	Und	Und/Und/Und	NS	-	-
14	N	0	-	-	-	-		39.64/37.79/Und	Und	Und/Und/Und	NS	-	Positive
15	N	0	-	-	-	-		Und/41.93/Und	Und	Und/Und/Und	NS	-	Positive
16	N	0	-	-	-	-		Und/Und/Und	Und	40.92/Und/40.57	NS	-	Positive
17	N	0	-	-	-	-		Und/Und/Und	Und	Und/Und/Und	NS	-	-
18	Y	0	+	-	-	-	-	Und/Und/42.04	Und	Und/Und/Und	35.57	-	Positive
19	N	0	-	POS	-	-		Und/39.90/Und	Und	Und/Und/Und	NS	Positive	Positive
20	N	0	-	-	-	-		Und/Und/Und	Und	40.82/Und/Und	NS	-	Positive
21	N	0	+	-	-	-		Und/Und/Und	Und	Und/Und/Und	NS	-	-
22	N	0	-	-	-	-		Und/Und/Und	Und	Und/Und/Und	NS	-	-
23	N	0	-	-	-	-		Und/Und/Und	Und	Und/Und/Und	NS	-	-
24	Y	2	+	POS	-	POS	POS	Und/Und/37.80	Und	36.57/38.18/36.31	38.72	Positive	Positive
25	N	0	-	-	-	-		Und/38.99/Und	Und	Und/Und/Und	NS	-	Positive
26	Y	0	+	POS	-	POS	POS…	Und/Und/37.77	Und	Und/38.96/38.01	34.17	Positive	Positive
27	N	0	-	POS	-	-		39.70/33.98/40.54	Und	Und/Und/Und	NS	Positive	Positive
28	N	0	+	-	-	-		Und/Und/Und	Und	38.01/Und/40.14	NS	-	Positive
29	Y	0	+	POS	-	POS	-	37.87/36.16/Und	Und	37.76/Und/35.33	33.87	Positive	Positive
30	N	0	+	NS	-	-		NS	Und	Und/Und/Und	NS	-	-
		N=26 P=4	N=20, P=10	N=22, P=7	N=29 P=1	N=25 P=5	N=4, P=4	N=69 P=18	N=29, P=1	N=74 P=16	P=8	N=20 P=10	N=11 P=19

* RFTC 0 = no spores, 1 = 1 to 5, 2 = 2 to 20, 3 = >20 spores

DG = digestive gland, NS = no lesion sample, POS = one or more replicates PCR positive, - = all replicates negative
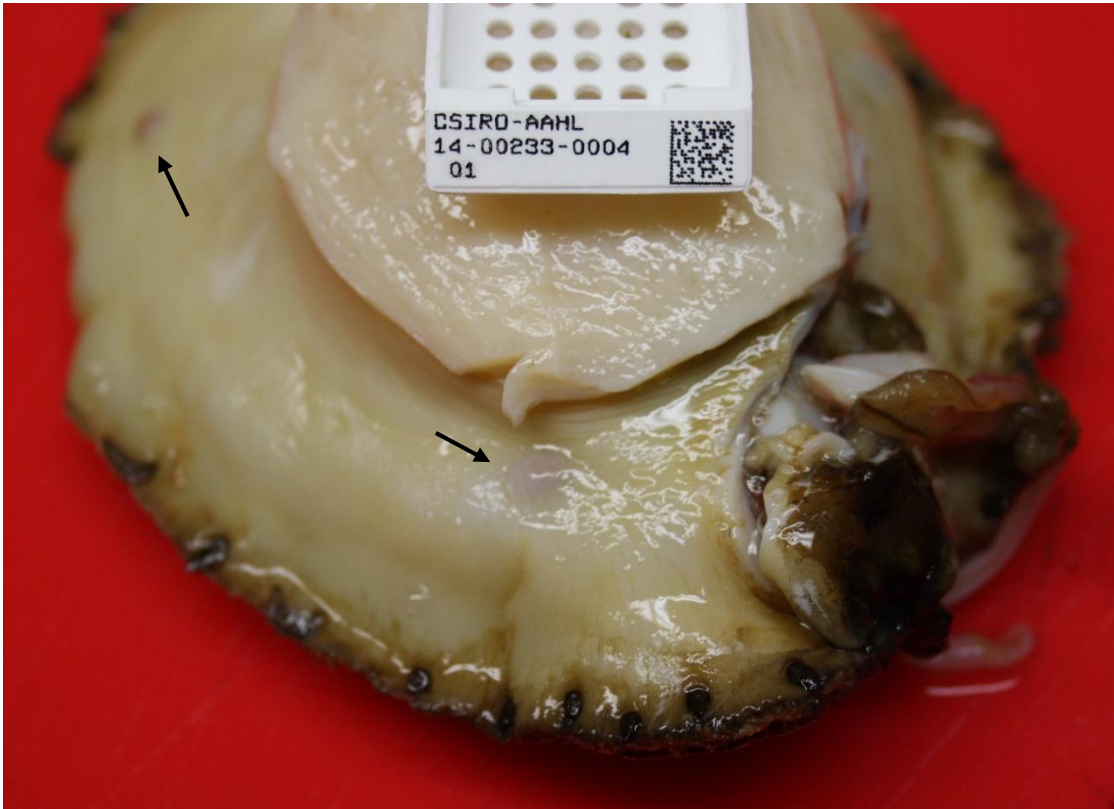
Figure 5 Anterior view of *H. rubra* showing gross lesions of *P. olseni* on the ventral surface of the pedal muscle (Thistle Island, South Australia 14-0233-4). The anterior 7–8 mm lesion is embedded in muscle, a second lesion (visible at the anterior end, arrows) is clearly visible below the surface.
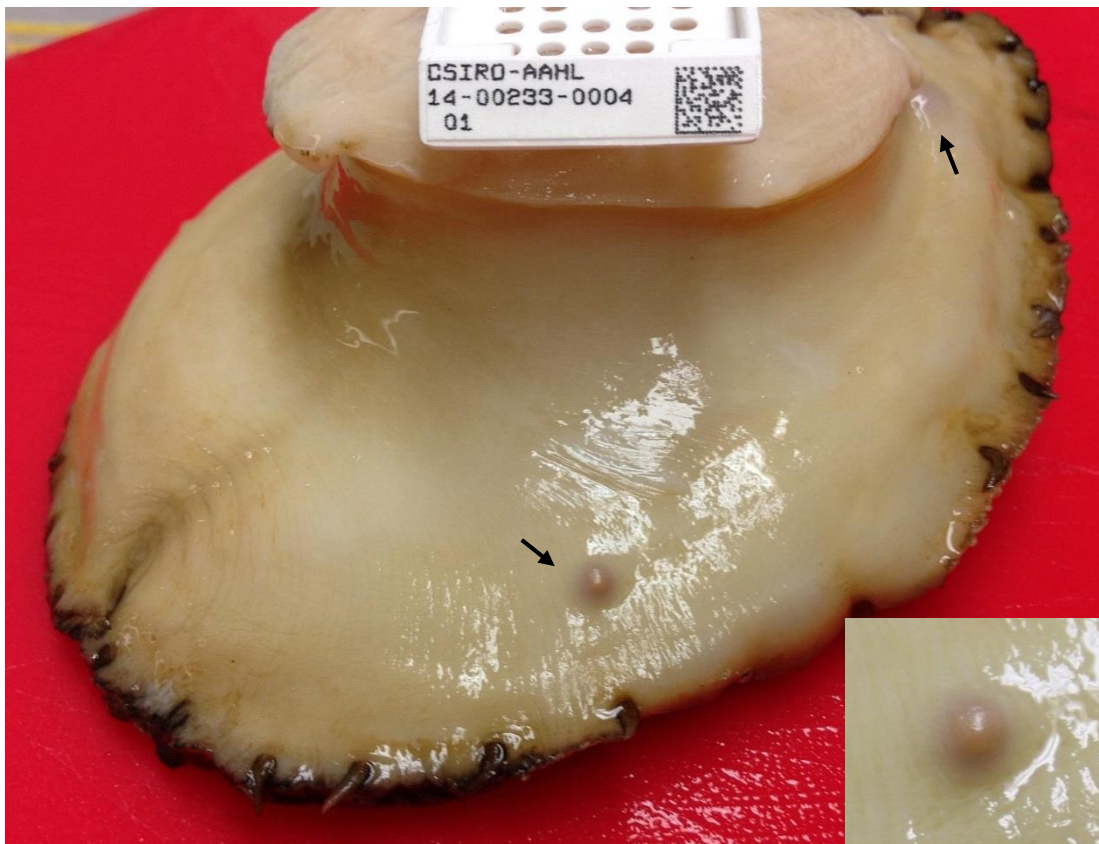
Figure 6 Posterior view of 14:0233-4 showing typical focal lesion of *P. olseni* on the ventral surface of the foot (arrows).

Figure 7 Section through large gross lesion of *Perkinsus olseni* in pedal muscle of blacklip abalone (*H. rubra*). The 3 pedal nerve chords are visible in the centre of the specimen (12:979-17).

Figure 8 Sequential 3 mm transverse sections through the pedal musccle of abalone showing focal discontinuous dispersed lesions throughout the whole musculature of animal 12:978-33.

Figure 9 Large isolated *Perkinsus* lesion observed in TS of muscle in animal 12:979-12. Inset - ARFTM culture from this lesion (x10).
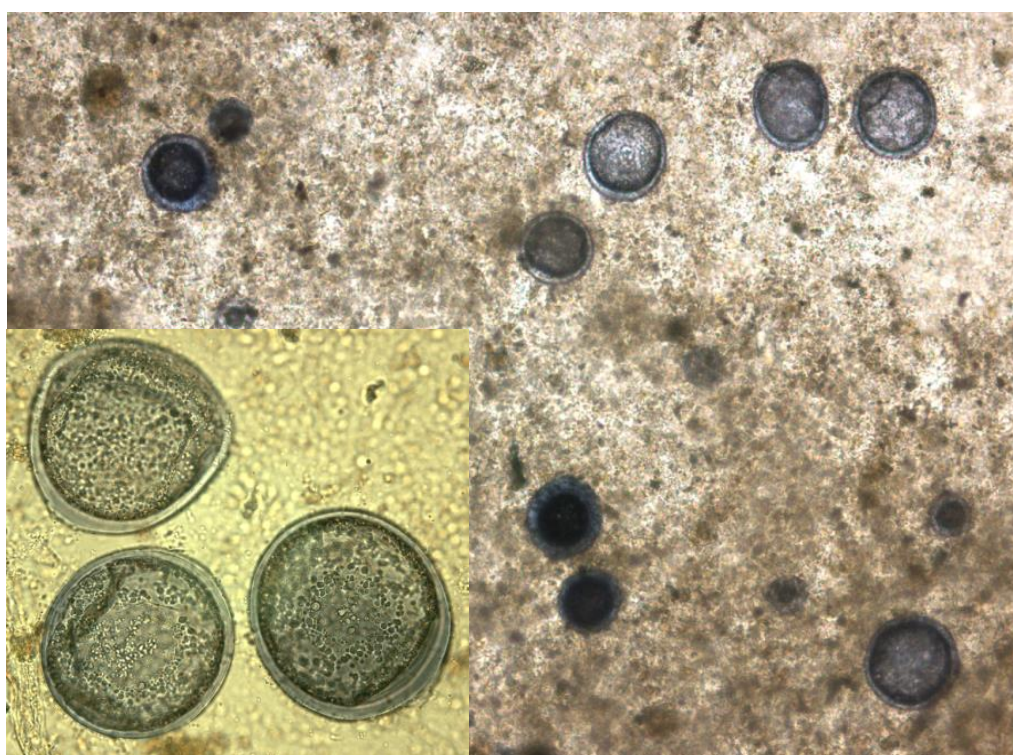
Figure 10 Characteristic blue/black *Perkinsus* hypnospores from thioglycollate medium stained with Lugol's Iodine for Ray's Test (12:979-12), 7 days post-inoculation at 25°C

NSW Field Samples

All animals collected from NSW appeared clinically normal and were test-negative for *Perkinsus* spp. by RFTC, the OIE *Perkinsus* cPCRs and the AFDL *Pols* qPCR. Although some large inflammatory lesions, areas of inflammation, and bacterial lesions were seen in histology, no *Perkinsus* was observed.

There have been no reports of suspect *Perkinsus* outbreaks to NSW Fisheries for many years. According to the NSW Aquatic Biosecurity Risk Management Group, the last evidence of *Perkinsus* in abalone was from

2005. Similarly, commercial divers operating south of Sydney are not aware of any *Perkinsus* disease, as was seen in the early 2000's. The previous field sampling in this project was based on confirmed detection in 2004/5 in the Jervis bay area. No *Perkinsus* was detected in these samples. Although an additional field collection of samples from New South Wales, just south of the previous site between Bendalong to Ulladulla was organised for early April, this was cancelled due to tropical cyclone Ita which had a severe impact on weather conditions at this time. No further testing was conducted on samples from New South Wales.

In vitro culture

While Dang *et al.* (2015) have recently established *in vitro Perkinsus* cultures from the mud ark cockle (*Anadara trapezi*) in Queensland, prior to this project *P. olseni* has not been propagated from abalone in Australia. In this project, attempts at *in vitro* culture were confined to samples from South Australia. Two strategies were used. Tissues were either dissected free and immediately transferred to tissue culture medium with antibiotics (modified by Dungan after Burreson *et al*., 2005), or the developing hypnospores from fluid thioglycollate culture were decontaminated with sterile seawater (29‰) containing antibiotics before transfer to tissue culture medium.

Sampling 1 - Taylor's Island and Thistle Island, South Australia (12:978, 12:979)

The first sampling from South Australia was undertaken at the Lincoln Science Centre at Port Lincoln. Only tissues with visible gross lesions were cultured, these being confined to specimens from Taylor's Island and Thistle Island. Fresh lesions (0.5 to 1 g) were excised and each placed directly into 5 ml of DMEM/Ham's F12 medium containing 100 U/ml penicillin/100 µg/ml streptomycin, for transport to the laboratory. These samples were kept cool at ambient temperature and in the dark prior to processing. In the laboratory, the tissues and surrounding medium were transferred to duplicate wells of 24-well tissue culture plates (NUNC) and cultured as outlined previously.
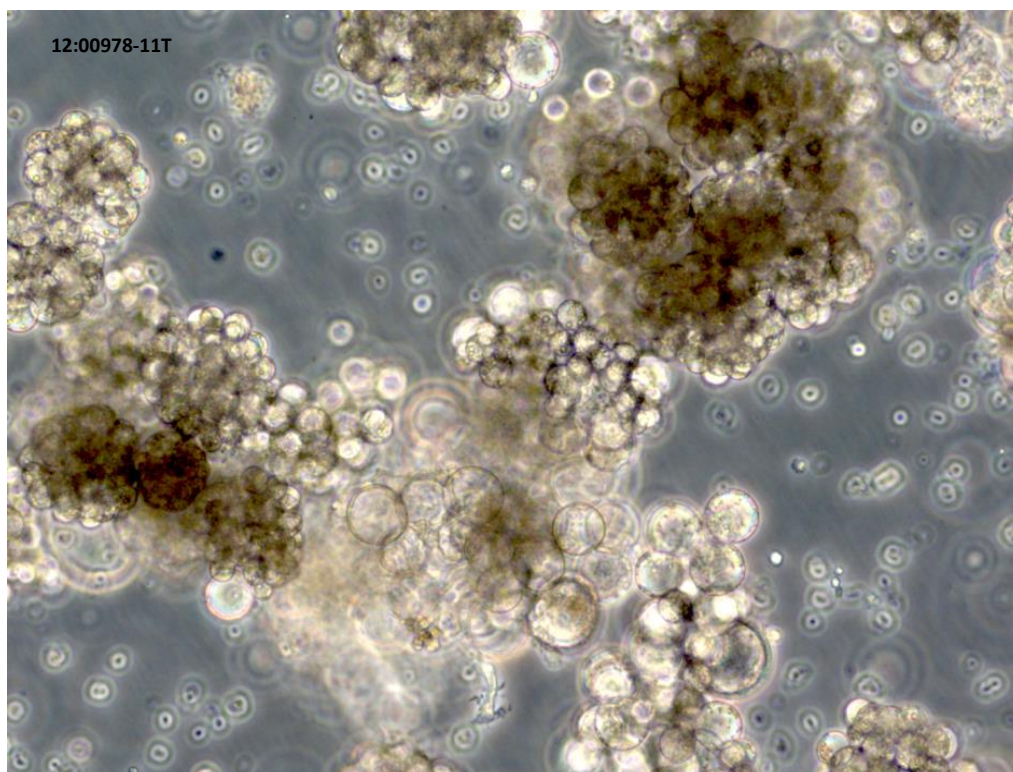
Figure 11 Tissue culture (DMEM/Ham's F12-3% of *Perkinsus*-infected tissues (12:978-11T) showing the first signs of *P. olseni* replication at day 10 at 25°C (10x).

Replicating cells from a few different samples were seen after 10 days culture. Cells appeared as grape-like clusters of varying size, with little structure floating free in the medium (Figure 6). Although no

zoosporangia were observed, these clusters were assumed to be *Perkinsus olseni* and the small refractile cells as recently released zoospores.

At the same time, there was considerable bacterial and fungal contamination of the primary cultures in the cluster dishes. In order to avoid fungal contamination of the *Perkinsus* cultures, uncontaminated wells from each of the samples were sub-cultured to fresh DMEM/Ham's F12-3% with antibiotic supplement, as soon as significant replication was observed. Media with a range of salinities (29‰ - 35‰) were used in the hope that growth would be improved at a particular salinity, increasing the chance of obtaining successful propagation.

Although sub-cultured cells could be seen floating in the media, the growth of *Perkinsus* was not sustained at any salinity, including the original salinity of 29‰. None of the sub-cultures showed the rapid growth first observed in the second week of primary culture. After approximately one month the cultures were rationalised by selecting uncontaminated wells at 29‰ from each animal and transferring these 25 cm^2 culture flasks. Fresh DMEM/Ham's F12-3% growth medium was added and the flasks were left at 20°C. The cultures were periodically supplemented (50-60 days) with additional L-glutamine but no growth was observed over the next 5 to 6 months.

Sampling 2 - Baird Bay, South Australia (13:01031)

A second attempt at *in vitro* culture was undertaken with the samples from Baird Bay. These samples were processed in the laboratory, but due to transport delays these abalone were not as fresh as those from the previous sampling. Lesions were cultured in Ray's medium and where no gross lesions were observed, a small pooled sample of mouth parts, mantle, muscle, gills and digestive diverticula was also cultured in Ray's medium (the same tissues as were routinely taken for PCR and histology). Cells in the form of developing hypnospores from fluid thioglycollate culture were decontaminated by washing the cells (3x) in sterile seawater containing antibiotics (200 U/ml penicillin and 200 µg/ml streptomycin), before transfer to 24-well tissue culture plates containing DMEM/Ham's F12-3% medium. In this case, the primary isolation medium was adjusted to a salinity of 33 ppt, in order to mimic the salinity of seawater at the sampling site.

No growth from lesion material or replication of hypnospores were observed from the Baird Bay samples in primary culture. After 4 weeks incubation, uncontaminated wells were transferred to 25 cm^2 flasks with additional medium and left at 20°C for prolonged incubation.

Sampling 3 - Thistle Island, South Australia (14:0233)

The second sampling from Thistle Island in April 2013 provided the final opportunity to sample known infected abalone and establish *Perkinsus olseni* cultures from the vicinity of the first description (Lester and Davis, 1981) from Memory Cove, SA (Figure 1).

Abalone showing obvious gross lesions (14:0233-4, 5, 9 and 11) were cultured directly into duplicate wells of 24-well plates containing DMEM/Ham's F12-3% at 3 salinities (29, 32 and 35‰). These cultures were duplicated at 2 temperatures (20 and 25°C) which reflected the range of water temperatures at the sampling site over the summer period. The actual water temperature at collection was 17.5°C. In addition, hypnospores from the ARFTM (29‰) culture, obtained from sample 14:0233-9 were concentrated by centrifugation at 300 x *g* for 3 minutes, washed twice in 29‰ artificial seawater with antibiotics and transferred to 4 replicate wells with medium at the same salinity (29‰). These wells were then serial diluted across the 24-well plate, as described previously.

In the case of cultures where multiple salinities were seeded with hypnospores taken directly from tissues, almost all wells at 29‰ were quickly overcome with fungal growth, with cultures at the higher salinities of 32 and 35‰ less affected. After 10 days, all uncontaminated wells containing hypnospores were consolidated into 7 individual 25cm^2 tissue culture flasks containing 29‰ medium, with fresh antibiotic supplement, and incubated at 20°C. These cultures were retained as before and examined every 7 to 10 days for signs of growth. No further growth was observed in these flasks.

At this time, the DMEM/Ham's F12-3% FBS, 29‰ 25cm^2 tissue culture flasks from previous testing in 2012 and 2013, were examined. These flasks were filled with masses of condensed cells which appeared dark and granular. Although there had been some significant growth previously, these cultures had not been examined for some time and their viability was uncertain. Sediment and debris from these flasks were passed into fresh media at 3 salinities (29, 32 and 35‰).

Cultures 12:978-11T and 13:1031-12T

After 3 or 4 days, the condensed cells started to show signs of replication in flasks. Growth was first observed at a salinity of 29‰, with a small number of new cells appearing in the dark clumps floating in the medium. Clusters of new cells continued to emerge, presumably through division of daughter schizonts. No zoosporangia were observed at this stage. These early cultures appeared to grow slowly and were conservatively passaged with minimal dilution at 10 to 14 day intervals. Subsequently, the cultures at 32 and 35‰ also started to grow. Because the cells grew as clusters and were variable in size, it was impossible to estimate cell number accurately. Attempts to pipette the cultures vigorously did not separate the cell aggregates sufficiently to allow cell counts. After 2 or 3 passages the cultures appeared to be better adapted to a salinity of 29‰ and 32‰ than the medium at 35‰, although this assessment is largely subjective. Sequential photographs of cells titrated across 24 well plates showed little difference in cell growth at either salinity. On this basis the cultures were rationalised to a few overlapping passages at 29‰ at 25°C. As the isolates were derived from a small number of animals and *in vitro* culture was no longer a primary focus of the project, the cultures were rationalised to 1 isolate from each site. A representative culture from Thistle Island, South Australia 2012 (12:978-11T) and another from Baird's Bay, South Australia (13:1031-12T) were propagated without selection (polyclonal) and stored in the vapour phase above liquid nitrogen. These cultures were also cloned by terminal dilution in 96-well micro-titre plates. In this case, an actively growing culture of each isolate was serially diluted in quadruplicate wells. Wells containing single spores were marked on the plate and growth from these wells used for subsequent passage. In each case, cloned cells were expanded in a 25 cm^2 flask and, after good growth was achieved, 50 μl was used to seed 200 ml of 29‰ growth medium in 1 litre bottles. The cultures were incubated in an orbital incubator with very slow rotation at 25°C for 7 days, before concentration and freezing in cryopreservation medium containing DMSO. The viability of cryopreserved cultures was confirmed after 24 hours and again after 3 months storage at -196°C. The media and procedure are summarised in Appendix 7.

As our primary interest was directed towards the development of a real-time PCR assay, not much time was devoted to characterising the development and growth of our *in vitro* cultures. New sub-cultures developed slowly at first, after passage they appeared to become better adapted to the culture conditions and their growth characteristics changed. Initially, replication appeared as division of daughter schizonts, with little differentiation among replicating cells. After 1 or 2 passages, growth appeared as a continuous cycle of expanding cell numbers by simultaneous proliferation by schizogony and zoosporulation. Figures 12 to 14 show the progression observed in early cultures with clusters of daughter trophozoites, containing eccentric nuclei and prominent vacuoles dominating the entire culture, to a point (Figure 13) where zoosporangia were found in some cultures. Figures 13 and 14 show the appearance of the zoosporangia discharge tube, which was less commonly observed, through to the release of zoospores into the medium. Flagellated zoospores within the zoosporangium were numerous and highly active.
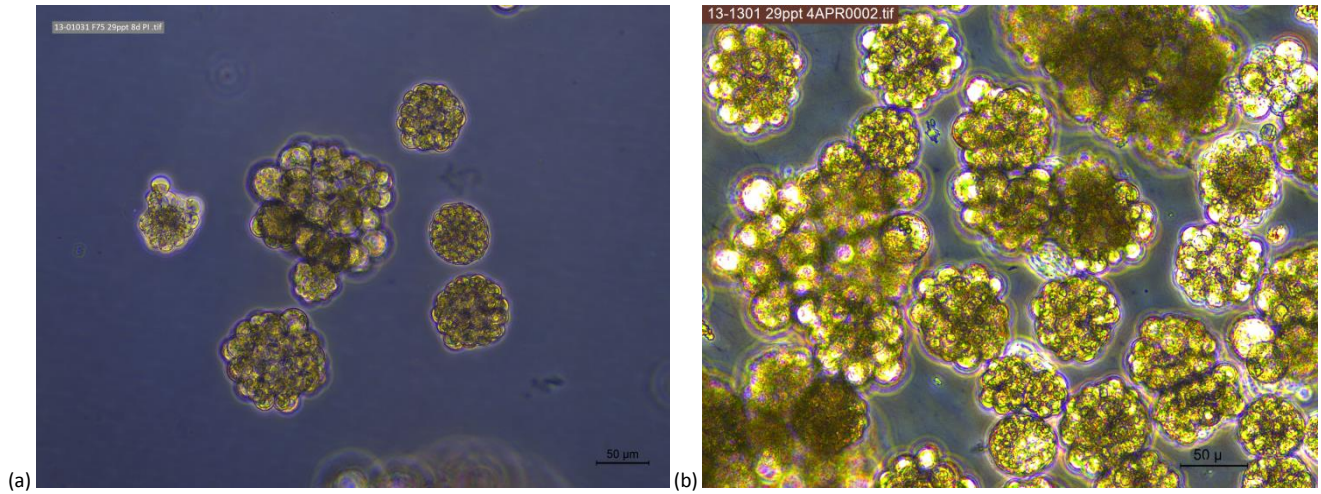
Figure 12 (a) Early culture of *P. olseni*, few if any zoosporangia were visible. (b) initial cultures were dominated by daughter schizonts.
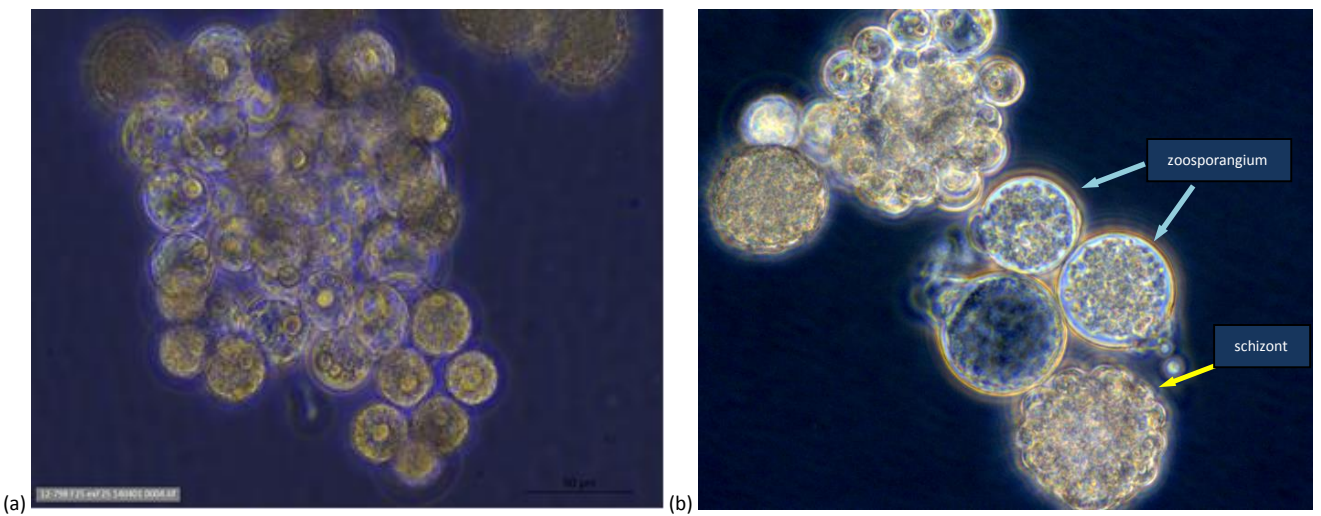
Figure 13 (a) Cluster of recently formed daughter trophozoites with eccentric nuclei, granular cytoplasm and containing numerous vacuoles. (b) *P. olseni* after passage 4 in 29‰ growth medium at 25°C. Both schizonts (yellow arrow) and zoosporangia (blue arrow) are visible in most fields (x100).
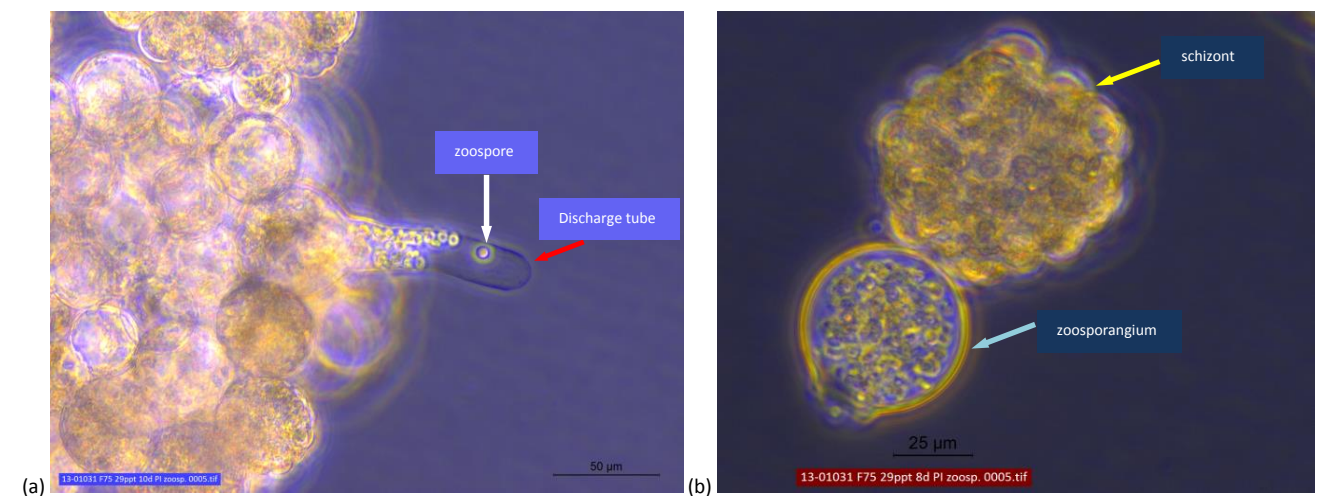
Figure 14 (a) Zoosporangia with extended discharge tube (red arrow). Motile zoospores are clearly visible in discharge tube. (b) Schizont (yellow) and adjacent zoosporangium (blue) ~60µm, highly active zoospores are contained and easily visible before release.
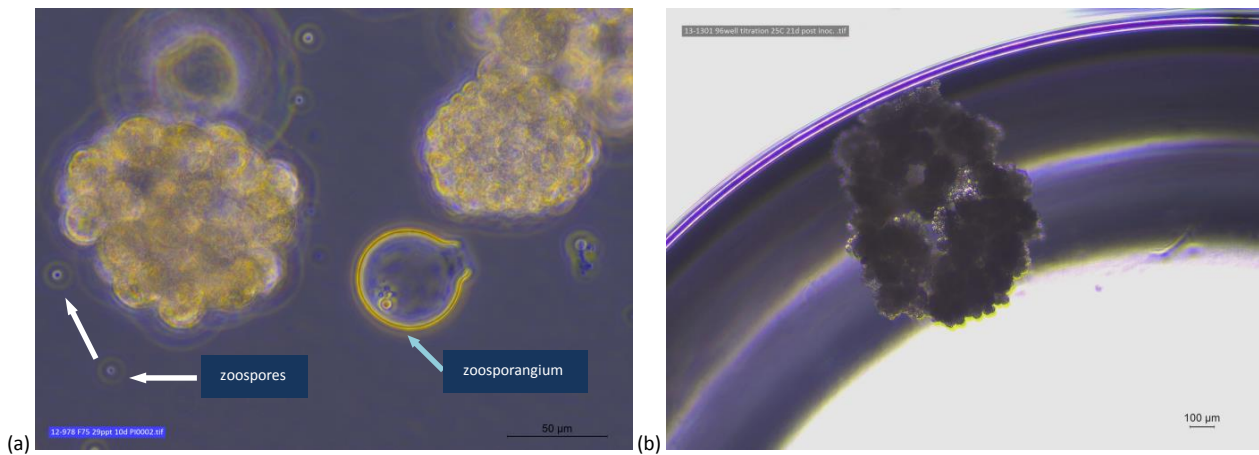
Figure 15 (a) Zoosporangium with single remaining active zoospore. The refractile structures lower left and top centre are highly motile zoospores (arrows) moving through the culture medium. (b) Cells, replicating in 96-well plate, derived from a single hypnospore, and subsequently used to establish clonal culture 13:1031-12T.

For practical reasons all the *Perkinsus* cultures were rationalised to 2 representative isolates, 12:978-11T and 13:1031-12T. These isolates were well-adapted to our 29 ppt growth medium and showed clear morphological characteristics of *Perkinsus*. Both isolates were first confirmed with the OIE 140/600 cPCR (Figure 16) and further confirmed by DNA sequencing of the ITS region (OIE 140/600 cPCR and OIE 85/750 cPCR amplicons) followed by BLAST (Altschul *et al.*, 1997) and phylogenetic analysis of the ITS, LSU and actin genes. These cultures were also sent to Professor Tomoyoshi Yoshinaga at the University of Tokyo, Japan for independent confirmation.
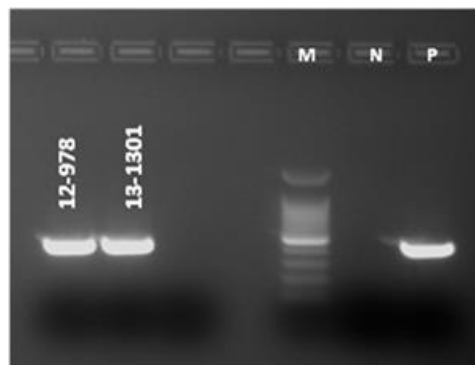
Figure 16 *Perkinsus olseni* cPCR (OIE *Pols* 140/600) of *in vitro* cultured cells from 2012 and 2013 (12:978-11T and 13:1031-12T). Expected amplicon size 460 bp. Negative and positive controls are shown for reference. (M) = Promega 100bp ladder with prominent 500bp band.

Real-time PCR Test Development

The candidate primers and probe for this assay were designed to target the internal transcribed spacer region (ITS) of the *Perkinsus* genome, the same region used for the OIE-recommended assays. In order to confer specificity, each of the candidate primers and probe were selected with at least 3 centrally placed nucleotide mismatches when aligned with other closely related *Perkinsus* species. The primers could be used in various configurations. The best combination of primers was determined by screening serially diluted *P. olseni* gDNA isolated from axenic ATCC cultures in a SYBR assay. The summary analysis of the AFDL *Pols* qPCR *P. olseni* primers and probe design is shown in Figure 17. The NCBI consensus sequences were generated from GenBank accessions data. A region of the ITS2 sequence of the South Australian (SA) *P. olseni* consensus sequence constructed from multiple alignments of *Perkinsus* positive samples sequenced over the course of this project is shown for reference. The primer/probe binding regions are aligned to demonstrate sequence variation in the probe binding regions for non-target *P. olseni* spp..

Figure 17 AFDL *Pols* qPCR Primer-Probe binding sites in *Perkinsus olseni*.

DNA consensus sequences for (A) *P. olseni*, (B) *P. marinus*, (C) *P. chesapeaki*, (D) *P. mediterraneus*, (E) *P. honshuensis*, (F) *P. beihaiensis* and (G) *P. qugwadi* aligned against the South Australian *P. olseni* consensus and the AFDL *Pols* qPCR primer-probe binding sites summarising the sequence variation and design rationale in the target regions with non-*P. olseni* sp.
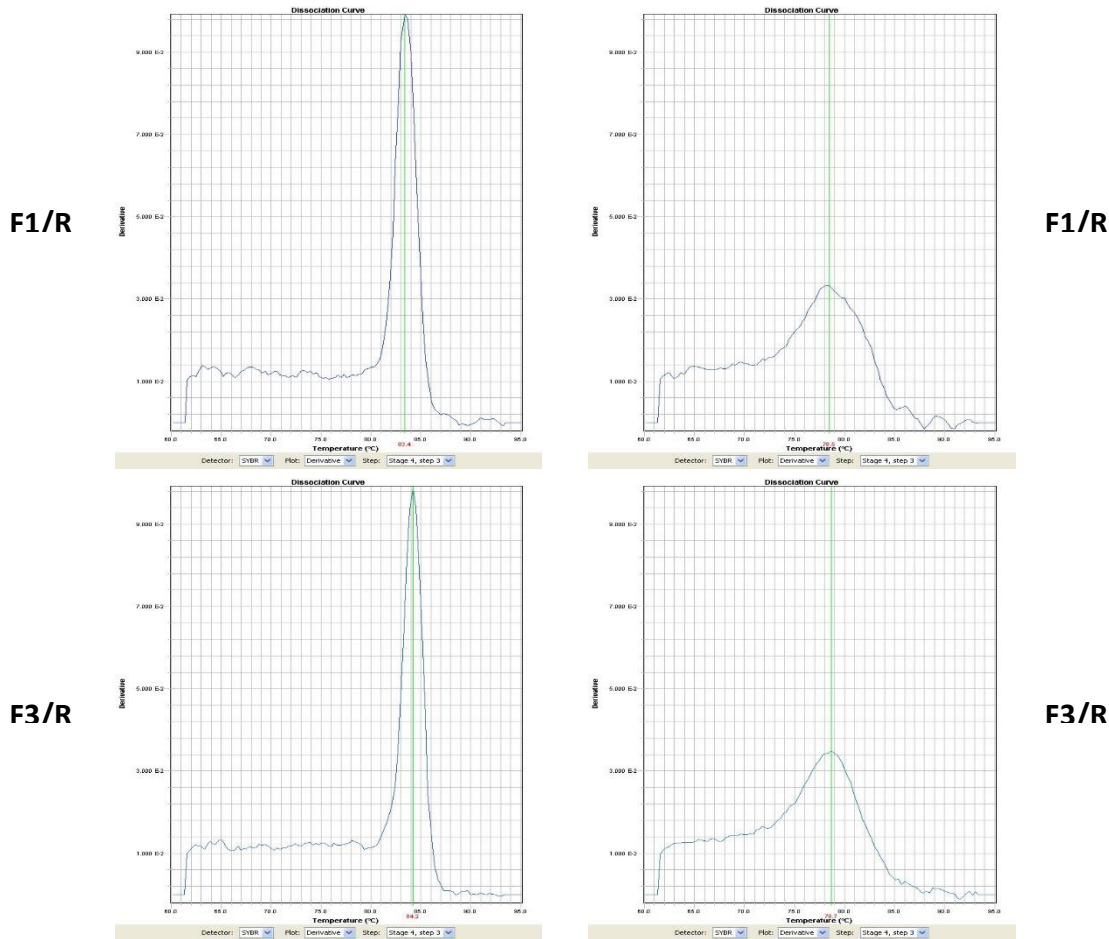
Figure 18 SYBR green assay of *P. olseni* genomic DNA with candidate primer combinations F1/R1, R1/R7, F3/R1 and F3/R7

When the amplification of *P. olseni* gDNA was assessed we found the F1/R1 and F3/R1 primer combinations produced the highest signal-to-noise ratios in the SYBR assay (Figure 18). The best combination of primers was then further assessed by screening standardised serially diluted gDNA from *P. olseni, P. marinus, P. chesapeaki,* and *P. honshuensis,* by Taqman assay. The F1/R1 primer combination produced significant amplification with high template concentrations of *P. marinus, P. chesapeaki,* and *P. honshuensis,* the F3/R1 primer combination showed the best analytical sensitivity and specificity over five 10-fold dilutions of DNA target (Table 2).

Table 3 Mean C_T values of candidate primer combinations F1/R1 and F3/R1, using serially diluted genomic DNA from axenic reference cultures of *P. olseni, P. marinus, P. chesapeaki,* and *P. honshuensis.*

	NEAT		10^{-1}		10^{-2}		10^{-3}		10^{-4}		10^{-5}	
	F1R1	F3R1	F1R1	F3R1	F1R1	F3R1	F1R1	F3R1	F1R1	F3R1	F1R1	F3R1
P olseni	4.09	3.95	7.24	6.80	10.01	9.38	14.08	14.60	16.84	17.16	18.71	19.28
P marinus	33.75	-	38.01	-	44.26	-	-	-	-	-	-	-
P chesapeaki	29.56	-	33.07	-	36.93	-	-	-	-	-	-	-
P honshuensis	32.96	-	39.98	-	37.97	-	-	-	-	-	-	-

Following selection of the F3/R1 primer combination, preliminary evaluation of the specificity of the system was undertaken using a set of plasmid samples. These *Perkinsus* plasmids were prepared by cloning the OIE 85F-750R cPCR amplicon from gDNA from axenic ATCC reference cultures into the pPCR2.1-TOPO vector

(Invitrogen). The copy number of plasmids was adjusted to provide the same concentration of DNA target for each species. Amplification plots for R3/F1 amplification of the ITS target from *P. olseni*, *P. marinus*, *P. chesapeaki*, and *P. honshuensis* are shown in Figure 19.
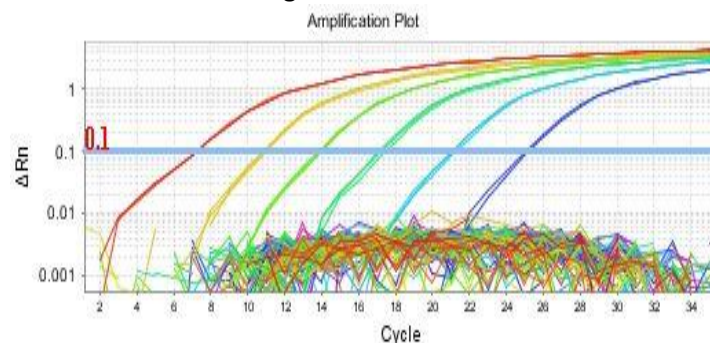
Figure 19 Amplification plots of prototype real-time assay (130722) using control plasmids prepared from *P. olseni, P. marinus, P. chesapeaki,* and *P. honshuensis* serially diluted from 10^{-1} to 10^{-8} to evaluate specificity over a range of target concentrations.

The prototype AFDL *Pols* qPCR system was then compared with the Gauthier *et al*. (2006) T-Perk genus-specific Taqman assay, where both assays showed similar performance. Both assays could reliably detect down to 20 copies of *P. olseni* target (Table 4).

Table 4 Plasmid screening: Mean C_T of serially diluted pTOPO *P. olseni* (85/750) plasmid with Gauthier T-Perk genus-specific Taqman assay and the AFDL *Pols* qPCR Taqman assay using a set threshold of 0.1.

Plasmid Copy Number	Gauthier T-Perk qPCR	AFDL *Pols* qPCR
20,000,000	14.09	12.62
2,000,000	17.51	16.31
200,000	20.99	19.97
20,000	24.56	23.78
2,000	27.94	27.25
200	31.86	31.57
20	35.32	35.28

Genomic DNA from *P. olseni* reference sample Mie-5mg (ATCC PRA-180) was quantified (Qubit v2.0, Invitrogen) and adjusted over a range of concentrations from 1 µg down to 1 fg DNA/µl using 1 mM TE buffer with 50 ng/µl tRNA as carrier. The C_T values obtained with the AFDL *Pols* qPCR and the Gauthier genus-specific assay with gDNA are presented in Table 4. The C_T values correspond to plasmid copy numbers of 2×10^6 (600 pg) down to 20 copies (1 fg). These results show that both assays have comparable analytical sensitivity with both plasmid and gDNA and are linear over this range.

Table 5 gDNA sceening: Mean C_T values of serially diluted *P. olseni* gDNA (ATCC PRA-180) with Gauthier T-Perk genus-specific Taqman assay and the AFDL *Pols* qPCR Taqman assay using a set threshold of 0.1.

gDNA (per reaction)	Gauthier T-Perk	AFDL *Pols* qPCR
1 fg	35.191	36.359
10 fg	32.105	31.815
100 fg	28.815	27.957
1 pg	25.31	24.361
10 pg	21.878	20.62
100 pg	18.398	16.947
600 pg	16.826	15.094
2000 *Pols* plasmid copies	Und	27.11
200 *Pols* plasmid copies	Und	31.45
20 *Pols* plasmid copies	Und	35.315

A standard threshold of 0.1 for both assays was applied. The values shown are mean values of duplicates.

Analytical Sensitivity (ASe)

The analytical sensitivity of the test system was determined with serially diluted *P. olseni* plasmid derived from ATCC PRA-180 gDNA. Serially diluted *P. olseni, P. marinus, P. chesapeaki* and *P. honshuensis* plasmids were also tested in parallel. Typical amplification curves for this titration is shown in Figure 20. The mean C_T values for these plasmids (triplicate samples), after 45 cycles of amplification is summarised in Table 4.

The plasmid dilutions provide a ready means of comparing and assessing the performance of the AFDL *Pols* qPCR with respect to other similar assays. The C_T values of this assay were consistent with the performance of other DNA targeted Taqman assays and are similarly comparable to the published Gauthier *Perkinsus* assay (Table 5) described previously. The standard curve (Figure 21) had a slope of -3.652, with Y intercept at 37.702 and a R2 value of 0.999 with an Eff% of 87.84.
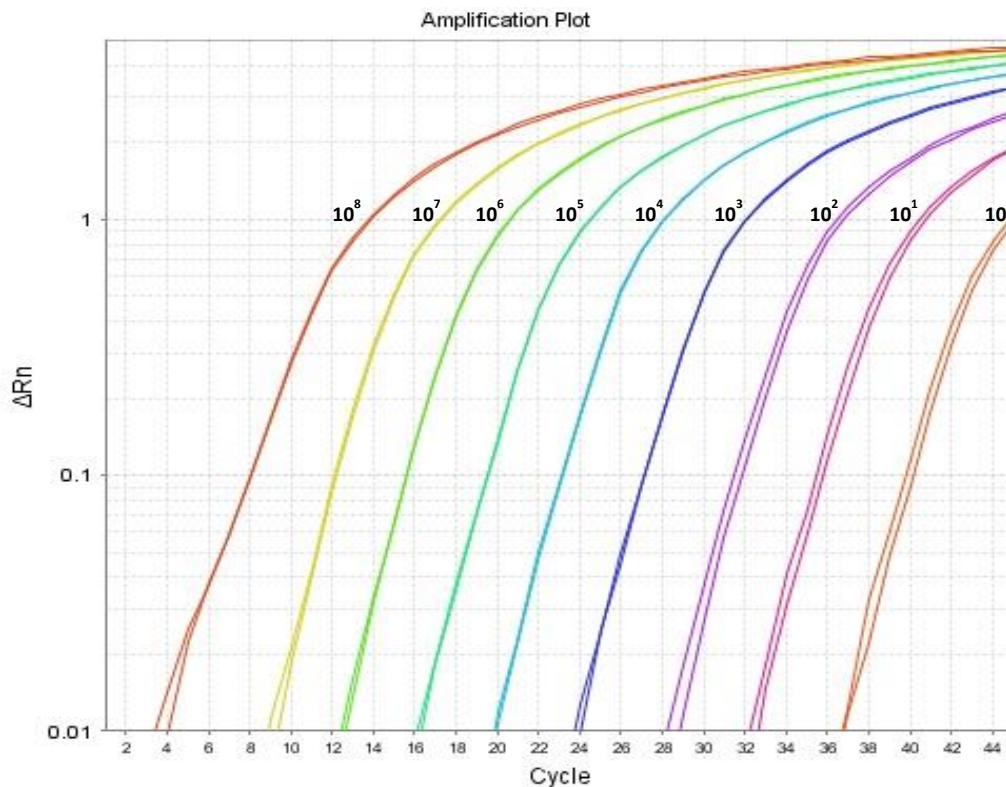
A fixed cut-off of 0.1 was used. Und = undetermined, no amplification

Figure 20 Amplification curves of AFDL *Pols* qPCR, ΔRn versus Cycle number obtained with serially diluted *P. olseni* plasmid (ITS region 85/750) and *P. marinus, P. chesapeaki* and *P. honshuensis*. Amplification was obtained with *P. olseni* only. Mean C_T values are shown in Table 5 below.

Target: Pols Slope: -3.652 Y-Inter: 37.702 R2: 0.999 Eff%: 87.848
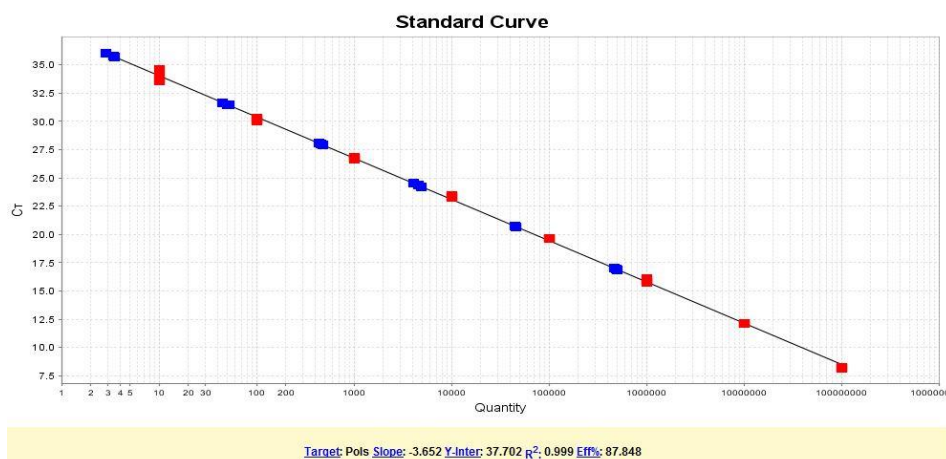
Figure 21 Standard curve of serially diluted *P. olseni* plasmid and gDNA in the AFDL *Pols* PCR (130909), Fig. 20 above.

Table 6 Mean C_T values of serially diluted cloned plasmid DNA ITS region (85/750), of *P. olseni, P. marinus, P. chesapeaki* and *P. honshuensis* in the AFDL *Pols* qPCR (fixed cut-off 0.1).

Plasmid concentration	*P. olseni*	*P. marinus*	*P. chesapeaki*	*P. honshuensis*
10^8 copies/µl	8.07	Und	Und	Und
10^7 copies/µl	12.15	Und	Und	Und
10^6 copies/µl	15.63	Und	Und	Und
10^5 copies/µl	19.52	Und	Und	Und
10^4 copies/µl	23.15	Und	Und	Und
10^3 copies/µl	27.16	Und	Und	Und
10^2 copies/µl	31.72	Und	Und	Und
10^1 copies/µl	35.64	Und	Und	Und
10^0 copies/µl	39.96	Und	Und	Und

A fixed cut-off of 0.1 was used. Und = undetermined, no amplification after 45 cycles.

Plasmid dilutions provide a useful source of quantified target for positive controls, however they do not necessarily reflect the sensitivity with field samples. In order to better understand the possible effect of the sample on sensitivity, further screening of serially diluted plasmid target using tRNA and pooled abalone DNA as diluent was undertaken. PCR negative pooled abalone genomic DNA at 50 ng/µl was used to simulate detection in abalone DNA at a concentration that is expected in normal field samples. Typical results for the *P. olseni* pPCR2.1-TOPO plasmid are summarised in Table 6 and confirm that C_T values of replicate samples are reproducible and in this case reasonably independent of the sample diluents used.

Table 7 Comparison of mean C_T values of serially diluted *P. olseni* plasmid DNA ITS (85/750), using tRNA and pooled abalone genomic DNA as diluent in the AFDL *Pols* qPCR.

P. olseni plasmid concentration	Buffer[1]	tRNA diluent (50 ng/µl)	Abalone DNA diluent (50 ng/µl)
10^8 copies/µl	8.07	8.31	8.21
10^7 copies/µl	12.15	12.62	12.13
10^6 copies/µl	15.63	16.31	15.91
10^5 copies/µl	19.52	19.97	19.66
10^4 copies/µl	23.15	23.78	23.35
10^3 copies/µl	27.16	27.25	26.77
10^2 copies/µl	31.72	31.57	30.13
10^1 copies/µl	35.64	35.28	34.00

[1] 10 mM TE, pH 7.6

Parallel testing of serially diluted *P. olseni* plasmid was also undertaken to determine the analytical sensitivity of this system and to confirm the sensitivity was comparable to other real-time PCR DNA detection systems. The results of *P. olseni* plasmid screening with the AFDL *Pols* qPCR and the Gauthier T-Perk genus-specific Taqman assay, were well-correlated and yielded consistently similar C_T values (Table 4). This comparison provided a level of confidence that the performance of our system was consistent with other real-time detection systems for *Perkinsus* and what might be expected of a species-specific real-time PCR detection system for *P. olseni* and was sufficiently sensitive for the purposes of testing. At low plasmid dilutions (1 to 2 copies/µl reaction) the results of replicate testing were inconsistent and the standard deviation was relatively high and variable. We found variable results using 2 copies per reaction and a high standard deviation (SD=1.6) in the system. Plasmid dilutions between 2 and 20 copies per reaction represent the lower limits of reliable analytical sensitivity. The plasmid titration in abalone DNA was repeated with 5 replicates at each dilution to determine the experimental analytical sensitivity ASe_{exp}

(Caraguel *et al.*, 2011). In this case, 20 copies per reaction represents the ASe$_{exp}$ at 95% confidence, as recommended by the OIE. Lower dilution factors could be used to further refine the estimate of ASe$_{exp.}$

Analytical Specificity (ASp)

The analytical specificity was confirmed using a range of *Perkinsus* cultures, fixed tissues, plasmids and 200 negative tissue samples from Victorian abalone.

The "*Perkinsus*-negative" DNA library was first tested by 18S qPCR to confirm extraction and identify samples with PCR inhibition. Four of the 200 had unusually high concentrations of DNA and inhibited PCR amplification. These samples were diluted 1:5 and confirmed by re-test. Duplicates of each sample in the "*Perkinsus*-negative" library were tested twice by both real-time assays (Gauthier et al. (2006) and AFDL *Pols* qPCR) and the OIE conventional PCRs. The negative samples in the AFDL *Pols* qPCR gave consistently low background fluorescence and a high signal to noise ratio with respect to the positive control (Figure 22).
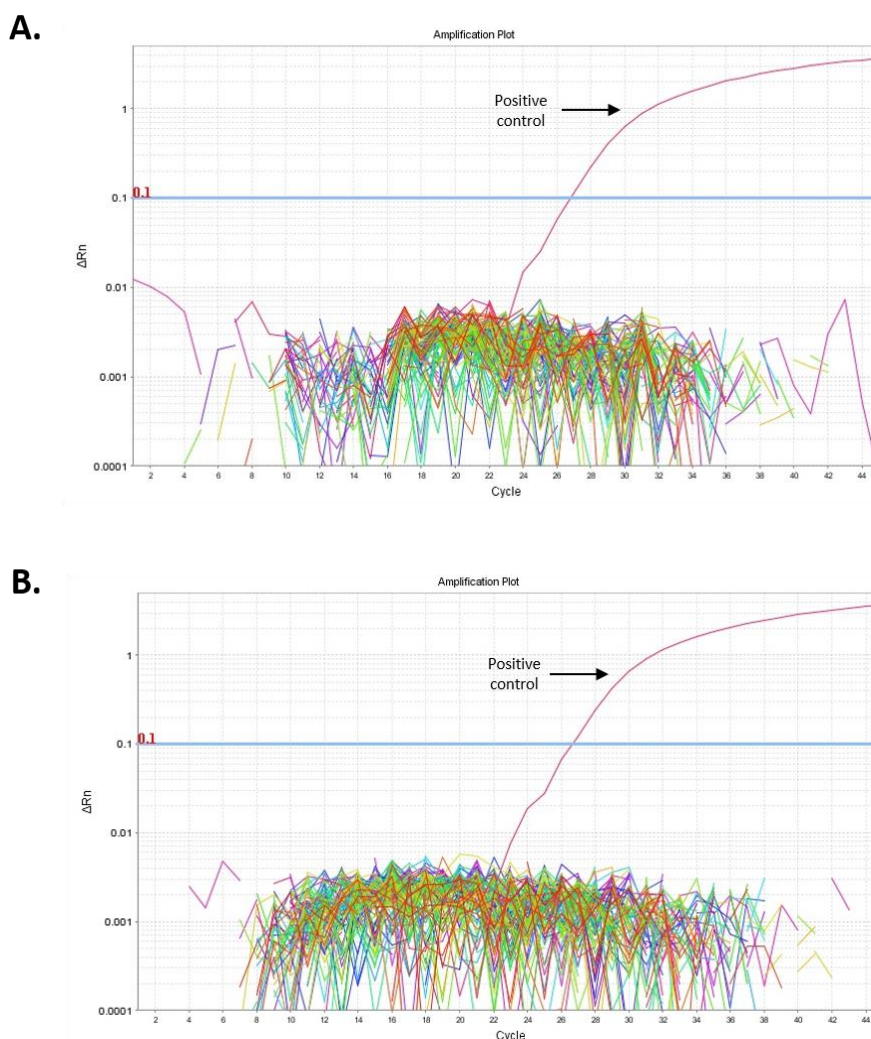
Figure 22 Typical amplification plot of AFDL *Pols* qPCR with Perkinus-negative abalone DNA. The threshold level of 0.1 is shown in blue and the positive control indicated.

All samples were test-negative (i.e. undetermined) in the AFDL *Pols* qPCR and showed no signs of amplification after 45 cycles. The consistently low background observed with the 200 "negative" abalone from different locations, reinforced our confidence in the specificity of the system, so much so that we regarded any amplification above the threshold was most likely positive and required further investigation, either by retest or resampling. This view was somewhat refined by Bayesian analysis, which served to better define the limits of the test.

All of the non-*P.olseni Perkinsus* samples used to assess analytical specificity (ASp) were derived from sources outside Australia and represent a wide range of mollusc host species with a wide geographic (global) distribution. The axenic cultures provided uncontaminated *Perkinsus* gDNA target, at high concentration without interfering host or other DNA, significantly favouring any cross-reaction that might occur in the newly developed candidate assay (AFDL *Pols* qPCR). No samples of *P. mediterraneus* and *P. qugwadii* were obtained and these species were not tested. While the specificity (ASp) of these 2 species could not be validated by testing, the DNA consensus alignments of *P. mediterraneus*, *P. qugwadii* and the test primers and probe show significant mismatch (Figure 17). While confirmatory testing could not be undertaken, the major differences in sequence observed over the diagnostic region are sufficient for us to expect that the ASp of the *P. olseni* assay would be maintained for these species.

The PCR test results obtained for *P. marinus, P. chesapeaki* and *P. beihaiensis* were clear-cut. The DNA derived from cultures (*P. marinus, P. chesapeaki*) had high levels of target and were test-negative by the AFDL *Pols* qPCR, as were the *P. beihaiensis* tissues. On this basis, in the absence of false positives and false negatives we concluded that the ASp was 100%.These results are summarised in Table 1.

Positive control plasmid and threshold

Over the course of assay development plasmid dilutions were used to provide a reproducible source of positive control for the assay and as a means of monitoring on-going performance of the Taqman chemistry. Once established, the plasmid controls could be reproduced readily. Typically 20, 200 and 2000 copies per reaction (10, 100 and 1000 copies per µl) were prepared using yeast tRNA at 50 ng/µl in 1 mM TE buffer as carrier and included on every test plate. The benefit of a nucleic acid carrier in the buffer was particularly noticeable for dilutions at low copy number.

Although the Applied Biosystems 7500 v2.3 real-time PCR analysis software on which the assay was developed provides an auto threshold feature for data analysis, a fixed cut-off value of 0.1 was used for all testing. This allows for a direct comparison of fluorescence results, including the C_T of control samples from different tests run over time. This was particularly useful in confirming test performance as new reagents were introduced. A plasmid dilution of 20 target copies was within the analytical range and provided consistently reliable positive result at low concentrations and was adopted as the low-level standard for testing. Further dilutions up to 20,000 copies per reaction were used for higher target concentrations. Typical results for tests run over a period of approximately one month are summarised in Table 7 and show the relative consistency of the mean C_T at higher concentrations with the variation in C_T increasing as the level of target DNA decreases. The standard deviation similarly increases with decreasing target concentration. These data show the consistency of plasmid control results from the AFDL *Pols* qPCR system obtained with repeated testing over a range of concentrations. Table 8 shows similar results for samples of *P. olseni* genomic DNA covering a similar range of C_T values with 2 pg to 20 fg of DNA.

Table 8 Typical C_T values and standard deviation (SD) for *P. olseni* pTOPO-plasmid controls (triplicate) over a range from 20,000 to 20 copies per reaction over multiple runs in the AFDL *Pols* qPCR test system. The mean C_T values are included for reference

	20,000	SD	2,000	SD	200	SD	20	SD
1	25.31	0.09	29.17	0.08	33.21	0.12	37.43	1.05
2	25.27	0.08	29.36	0.07	33.15	0.2	37.14	1.02
3	25.39	0.08	29.1	0.06	33.03	0.14	36.79	0.58
4	25.44	0.26	29.14	0.04	32.95	0.11	37.05	0.24
5	24.67	0.06	28.11	0.03	32.47	0.15	35.76	0.24
6	25.29	0.07	29.27	0.11	33.27	0.23	37.39	0.74
7	25.37	0.03	29.23	0.05	33.2	0.13	37.43	0.55
8	25.15	0.03	28.95	0.05	33.06	0.05	36.34	0.72
Mean	**25.24**		**29.04**		**33.04**		**36.92**	

Table 9 Typical C$_T$ values and standard deviation (SD) of *P. olseni* genomic DNA at 3 concentrations over multiple runs in the AFDL *Pols* qPCR, in the same concentration range shown in Table 6 above.

	2 pg	*SD*	**200 fg**	*SD*	**20 fg**	*SD*
1	29.26	0.04	33.57	0.2	37.68	0.58
2	29.21	0.029	33.63	0.132	37.57	0.4
3	29.14	0.13	34.07	0.1	38.36	0.52
4	29.08	0.11	33.84	0.24	37.39	0.08
5	28.58	0.11	33.74	0.4	37.29	1.11
6	29.43	0.05	34.89	0.51	38.46	0.66
7	29.45	0.05	35.03	0.1	38.52	0.62
8	29.35	0.03	34.55	0.38	39.47	1.75

A candidate Taqman® assay for *P. olseni* has been established and shown to be both specific and sensitive with purified DNA using target and non-target species.

Thistle Island PCR analysis (14:0233)

The main purpose of this sampling at Thistle Island was to develop an understanding of the distribution of *Perkinsus* DNA around lesions in the tissues of affected abalone and to determine how to best target abalone sampling for detection by PCR. Additional results would also be generated for test validation.

Animals underwent close examination of all external surfaces. The pedal muscle was sliced transversely at 1 to 2 mm intervals along the entire length of each animal (Figure 8, and Appendix 5) and the cut surface examined for lesions. Gross lesions were only observed in muscle. "Normal" muscle was taken from 2 sites 2 to 3 mm from muscle lesions, in the same transverse section. Three sub-samples of 30 mg each were taken at 3 levels along the length of the gill. A small piece of digestive gland (approximately 200-300 mg) was also taken from the lower third of the left side of the visceral mass from all 30 animals. All samples for DNA extraction and PCR were further dissected to provide a uniform amount of approximately 30 mg of tissue for DNA extraction.

All 216 DNA samples generated from this sampling were screened in duplicate by 18S qPCR to confirm the DNA extraction and identify where inhibition might be a source of subsequent testing bias. For the purpose of this analysis, we again set a threshold value of 0.1 for the qPCR and regarded any result above the threshold value as positive.

Our 20-copy plasmid control had a mean C$_T$ of 35.27. The average C$_T$ of the *Perkinsus* muscle lesions tested was 36.23 from the Thistle Island field samples (14:0233). This serves to highlight the observation that positive C$_T$ values in the AFDL *Pols* qPCR, are high, even when gross lesions are tested directly. This suggests that the spectacular lesions in abalone muscle are host-derived tissues rather than masses of proliferating parasites. The positive C$_T$ values from sub-clinically infected animals are also high, indicating low levels of DNA template. In samples from sub-clinical or clinically normal animals, replicate testing of multiple individual samples taken from the same transverse section (all processed separately) sometimes gave inconsistent results. Because these results fall at the limits of qPCR detection, the selection of a test cut-off value, if one is required, must be based on objective investigation such as the Receiver Operator Curve (ROC) and Bayesian analysis undertaken here and further summarised in Appendix 8. This analysis were based on a data set which included all the testing undertaken in project.

A complete set of results was generated for animals with gross lesions using the OIE 85/750 cPCR (genus-specific), and a full set of results for all 30 animals (216 DNA samples) was generated with the OIE 140/600 cPCR (species-specific) and newly developed AFDL *Pols* qPCR (Tables 9 and 10). In all cases the PCR was carried out in a 25 µl volume, using 2 µl of sample as a source of DNA template.

The real-time C_T result in the tables represents an individual sample, and is expressed as the mean of each sample tested in triplicate. Testing was also carried out on samples at a 1:10 dilution (1:10 results not shown) to eliminate possible concentration effects as a source of error. Conventional PCR assays were performed as single tests on the DNA sample. These results provide a direct comparison of the relative performance of each test with a common set of samples derived from a range of tissues from naturally infected animals.

Table 10 Summary of individual results for separate samples tested by the OIE *Perkinsus* genus-specific and species-specific conventional PCR assays for 14:0233.

OIE 85/750 PCR (genus-specific assay)

No.	Lesions	Gills	DG	Normal Muscle	Muscle Lesions
1	N				NS
2	N				NS
3	N				NS
4	Y	- / - / -	-	- / - / -	-
5	N?	- / - / -	-	- / - / -	-
6	N				NS
7	N				NS
8	N				NS
9	Y	- / - / -	-	- / - / -	-
10	N				NS
11	Y	- / - / -	-	- / - / **POS**	-
12	Y	- / - / -	-	- / - / -	-
13	N				NS
14	N				NS
15	N				NS
16	N?				NS
17	N				NS
18	Y?	- / - / -	-	- / - / -	-
19	N				NS
20	N				NS
21	N				NS
22	N				NS
23	N				NS
24	Y	- / - / **POS**	-	- / - / **POS**	-
25	N				NS
26	Y	- / - / **POS**	-	- / - / -	-
27	N				NS
28	N				NS
29	Y?	- / - / -	-	- / - / -	-
30	N				NS

OIE 140/600 PCR (*P. olseni* specific assay)

No.	Gills	DG	Normal Muscle	Muscle Lesions
1	- / - / -	-	- / - / -	NS
2	**POS** / **POS** / -	-	- / - / -	NS
3	- / - / -	-	- / - / -	NS
4	- / - / -	-	- / - / -	**POS**
5	- / - / -	-	- / - / -	-
6	- / - / **POS**	**POS**	- / **POS** / -	NS
7	- / - / -	-	- / - / -	NS
8	- / - / -	-	- / - / -	NS
9	- / - / -	-	- / - / -	**POS**
10	- / - / -	-	- / - / -	NS
11	- / - / -	-	- / - / **POS**	-
12	- / - / -	-	- / - / -	-
13	- / - / -	-	- / - / -	NS
14	- / - / -	-	- / - / -	NS
15	- / - / -	-	- / - / -	NS
16	- / - / -	-	- / - / -	NS
17	- / - / -	-	- / - / -	NS
18	- / - / -	-	- / - / -	-
19	- / **POS** / -	-	- / - / -	NS
20	- / - / -	-	- / - / -	NS
21	- / - / -	-	- / - / -	NS
22	- / - / -	-	- / - / -	NS
23	- / - / -	-	- / - / -	NS
24	- / - / **POS**	-	**POS** / - / **POS**	**POS**
25	- / - / -	-	- / - / -	NS
26	- / - / **POS**	-	- / **POS** / **POS**	**POS**
27	- / **POS** / -	-	- / - / -	NS
28	- / - / -	-	- / - / -	NS
29	- / **POS** / -	-	**POS** / - / **POS**	-
30	No samples	-	- / - / -	NS

DG = digestive gland, NS = no lesion sample, POS = cPCR-positive (clear amplicons of the correct size visible), - = cPCR-negative (no visible amplicon)

Table 11 Summary of corresponding results for replicate samples tested by the AFDL *Pols* qPCR assay for 14:0233.

No.	Lesions	Gills	DG	Normal Muscle	Muscle Lesions
1	N	U / U / U	U	U / U / U	NS
2	N	37.62 / 36.62 / U	U	U / U / 39.76	NS
3	N	U / U / U	U	U / U / U	NS
4	Y	U / U / U	U	U / U / U	38.74
5	N?	U / U / U	U	U / U / U	Und
6	N	U / 43.74 / 39.78	38.62	U / 39.00 / 38.04	NS
7	N	U / U / U	U	U / U / U	NS
8	N	U / U / U	U	U / U / U	NS
9	Y	U / 36.80 / U	U	U / U / U	38.61
10	N	U / U / U	U	U / 43.25 / U	NS
11	Y	U / U / U	U	U / U / U	34.68
12	Y	39.44 / U / U	U	U / U / U	35.46
13	N	U / U / U	U	U / U / U	NS
14	N	39.64 / 37.79 / U	U	U / U / U	NS
15	N	U / 41.93 / U	U	U / U / U	NS
16	N?	U / U / U	U	40.92 / U / 40.57	NS
17	N	U / U / U	U	U / U / U	NS
18	Y?	U / U / 42.04	U	U / U / U	35.57
19	N	U / 39.90 / U	U	U / U / U	NS
20	N	U / U / U	U	40.82 / U / U	NS
21	N	U / U / U	U	U / U / U	NS
22	N	U / U / U	U	U / U / U	NS
23	N	U / U / U	U	U / U / U	NS
24	Y	U / U / 37.80	U	36.57 / 38.18 / 36.31	38.72
25	N	U / 38.99 / U	U	U / U / U	NS
26	Y	U / U / 37.77	U	U / 38.96 / 38.01	34.17
27	N	39.70 / 33.98 / 40.54	U	U / U / U	NS
28	N	U / U / U	U	38.01 / U / 40.14	NS
29	Y?	37.87 / 36.16 / U	U	37.76 / U / 35.33	33.87
30	N	NS	U	U / U / U	NS

DG = digestive gland, NS = no sample, U = Undetermined (negative), Number = mean of triplicate, Animals with gross lesions are indicated by dark shading, light shading indicates no gross lesions were observed.

The results of parallel testing with the 2 OIE-recommended conventional assays are shown in Table 9. Of the 63 individual samples screened using the OIE 85/750 cPCR (digestive gland samples excluded) only 4 samples (6.35%) were found positive. All these samples were derived from tissues from animals with presumptive gross lesions in the muscle. In contrast, using the same sub-set of 63 samples, the OIE 140/600 cPCR species-specific assay found 14 (22.22%) of samples positive. The observation that the OIE conventional species-specific PCR test yielded a higher number of positive results (n=14) than the genus-specific conventional PCR 85/750 (n=4), suggested that the genus-specific test possibly had lower analytical sensitivity than the species-specific assay with these samples. We recognise this comparative testing was limited and served only as a guide to the usefulness of the tests with abalone field samples.

The results of AFDL *Pols* qPCR are summarised in Table 10. In this case 22 of 63 (34.9%) samples had amplification and crossed the threshold (0.1). Of the 3 molecular assays used, the new qPCR detected the highest proportion of positive samples.

With regard to the testing undertaken with which to compare the AFDL *Pols* qPCR, there was no test which could be used as a "gold standard" for comparison. Therefore a Bayesian analysis was undertaken to determine the DSe and DSp of this assay. This analysis is presented in the next section covering diagnostic test validation and qPCR test performance. The OIE 140/600 cPCR was used as the reference test for the AFDL *Pols* qPCR (candidate test). Both tests are species-specific for *P. olseni* and had an adequate number of samples tested to compare results from different populations. In this case, the results from 3 populations with different prevalence of infection were used for ROC analysis with MedCalc and Bayesian analysis. The assumption that both tests were conditionally independent was substantiated by the fact that the reverse primers of the AFDL *Pols* qPCR assay were outside the region of the OIE 140/600 cPCR.

PCR testing of animals with *Perkinsus* lesions

Of the 8 animals with presumptive gross lesions of *Perkinsus* all the muscle lesions were test-positive by qPCR. Animal #5 had a single focal lesion which although slightly atypical attracted extra attention. Closer examination suggested that it was perhaps an encysted nematode and was scored as "Y?" in the dissection notes. This animal subsequently tested negative for *Perkinsus* by all tests.

We expected the DNA extracted from muscle including gross lesions would yield strong positive results in all PCR tests. Surprisingly this was not the case. Although the lesions were prominent, the amount of *Perkinsus* target DNA was relatively low. This observation is supported by the consistency of test results across all the assays including the very low numbers of hypnospores observed in ARFTM. In this case, none of the 8 samples were positive by the OIE conventional 85/750 PCR (genus-specific assay) and only 4 of the 8 samples (50%) were positive by the OIE conventional 140/600 PCR (*P. olseni*-specific assay). Although all the real-time PCR results were positive for these samples the C_T values from the muscle lesions were high and ranged from 33.87 to 38.74 with a mean of 36.23. It is likely that the caseous debris associated with these lesions contains few parasites and that any *Perkinsus* that may be present are lost from the liquefied debris that forms the centre of the lesion, and are lost when the tissue is processed for DNA extraction. The low number or complete absence of hypnospores in Ray's medium also suggests that spore numbers were low in the chronic lesions from these animals.

PCR testing of animals without *Perkinsus* lesions

Of the 22 animals which were free of gross lesions and appeared to be free of *Perkinsus* on close examination, 4 animals (18.2%) were positive by the OIE 140/600 species-specific cPCR in one or more of the multiple samples tested (no gill sample was taken from animal 30). When the same samples were screened by AFDL *Pols* qPCR 11 animals (50%) gave one or more positive results.

Although we did not see *Perkinsus* lesions in the gills of infected abalone, PCR results over the course of the project suggested that gills may provide a useful or even better sample for PCR detection of *Perkinsus* in abalone.

	OIE 140/600 cPCR	qPCR		OIE 140/600 cPCR	qPCR
	Gills	Gills		Normal Muscle	Normal Muscle
1	- / - / -	U / U / U	1	- / - / -	U / U / U
2	POS / POS / -	37.62 / 36.62 / U	2	- / - / -	U / U / 39.76
3	- / - / -	U / U / U	3	- / - / -	U / U / U
4	- / - / -	U / U / U	4	- / - / -	U / U / U
5	- / - / -	U / U / U	5	- / - / -	U / U / U
6	- / - / POS	U / 43.74 / 39.78	6	- / POS / -	U / 39.00 / 38.04
7	- / - / -	U / U / U	7	- / - / -	U / U / U
8	- / - / -	U / U / U	8	- / - / -	U / U / U
9	- / - / -	U / 36.80 / U	9	- / - / -	U / U / U
10	- / - / -	U / U / U	10	- / - / -	U / 43.25 / U
11	- / - / -	U / U / U	11	- / - / POS	U / U / U
12	- / - / -	39.44 / U / U	12	- / - / -	U / U / U
13	- / - / -	U / U / U	13	- / - / -	U / U / U
14	- / - / -	39.64 / 37.79 / U	14	- / - / -	U / U / U
15	- / - / -	U / 41.93 / U	15	- / - / -	U / U / U
16	- / - / -	U / U / U	16	- / - / -	40.92 / U / 40.57
17	- / - / -	U / U / U	17	- / - / -	U / U / U
18	- / - / -	U / U / 42.04	18	- / - / -	U / U / U
19	- / POS / -	U / 39.90 / U	19	- / - / -	U / U / U
20	- / - / -	U / U / U	20	- / - / -	40.82 / U / U
21	- / - / -	U / U / U	21	- / - / -	U / U / U
22	- / - / -	U / U / U	22	- / - / -	U / U / U
23	- / - / -	U / U / U	23	- / - / -	U / U / U
24	- / - / POS	U / U / 37.80	24	POS / - / POS	36.57 / 38.18 / 36.31
25	- / - / -	U / 38.99 / U	25	- / - / -	U / U / U
26	- / - / POS	U / U / 37.77	26	- / POS / POS	U / 38.96 / 38.01
27	- / POS / -	39.70 / 33.98 / 40.54	27	- / - / -	U / U / U
28	- / - / -	U / U / U	28	- / - / -	38.01 / U / 40.14
29	- / POS / -	37.87 / 36.16 / U	29	POS / - / POS	37.76 / U / 35.33
30	NS	NS	30	- / - / -	U / U / U

NS = no sample, U = Undetermined (negative) Number = mean of triplicate, Animals with gross lesions are indicated by dark shading, light shading indicates no gross lesions were observed.

PCR testing of Digestive Gland

The column-extracted DNA sample from digestive gland (DG) was found to be inhibitory in the 18S-directed PCR and although the inhibition could be eliminated by dilution, this reduced the amount of DNA target by the same proportion (data not shown). For this reason the digestive gland was not considered a useful sample for PCR detection using the conventional column-based extraction process for DNA isolation.

During preliminary *Perkinsus* OIE cPCR screening of the animals with gross lesions (4, 9, 11, 12, 18, 24, 26 and 29), a small number of animals without lesions were also tested as background negative controls. Despite the negative 18S pre-screening, the digestive gland from animal 6 was found to be positive with the OIE 140/600 *P. olseni*-specific cPCR. This PCR result was reproducible and was also confirmed with the AFDL *Pols* qPCR test (C_T 38.62). As a result we tested all the digestive gland DNA samples by both the OIE 140/600 cPCR and the qPCR in this analysis. No other PCR positive samples from digestive gland were detected by either OIE cPCR or the AFDL *Pols* qPCR. We conclude that the result from DG of animal #6 was atypical and

perhaps the result of finding a concentration of parasites in the small section of digestive gland tested. These data serve to confirm that column-extracted DNA from digestive gland is not a useful sample for *Perkinsus* PCR detection.

PCR testing of Gills

All the positive conventional PCR results were from gill, with the exception of animal 6 where gill, digestive gland and muscle were all positive by conventional PCR. The results of cPCR testing and qPCR from gill were well-correlated. In every case where the gill sample was cPCR positive the AFDL *Pols* qPCR result was also positive (Table 11). This was true of both animals with and without gross lesions in the muscle. Of the 63 gill samples from animals without gross lesions 5 samples (7.8%) were cPCR-positive and 12 samples (19.1%) were positive by qPCR. Of the 24 gill samples from animals with gross lesions 3 samples (12.5%) were positive by cPCR and 7 samples (29.2%) were positive by qPCR.

PCR testing of Muscle

Of the 66 muscle samples from 22 animals without gross lesions, only 1 sample (1.5%) was positive by cPCR and 9 samples (13.6%) were positive by qPCR (Table 3, light shading). Of the 24 muscle samples (normal muscle) from animals with gross lesions 7 samples (29.2%) were positive by cPCR, with an equal number of 7 samples (29.2%) also positive by qPCR.

PCR Sampling and Test Summary

As a general observation, based on the testing of multiple samples of each tissue type, including digestive gland, gill and muscle from all the animals, more positive samples could be detected with the AFDL *Pols* qPCR system than with the existing OIE species-specific cPCR. With a few minor exceptions the results from the OIE recommended assays were in agreement with the qPCR. We attributed any minor differences to the low copy number of *Perkinsus* target DNA in the samples. In sub-clinically infected abalone, the real-time assay provided a more sensitive method for *Perkinsus* detection over culture in ARFTM or the existing conventional PCRs.

The qPCR provides a consistently positive result when the edge of the lesion is included in the DNA extraction. Where animals have gross lesions these should be targeted and tested directly. In the case of this sampling at Thistle Island (14:0233), the low levels of parasite meant that both Ray's Test and conventional PCR did not prove reliable, even when affected tissues were included in culture. The normal muscle surrounding lesions is not necessarily a reliable sample for PCR, even with advanced lesions, where parasites might be expected in the surrounding tissue. In this case, multiple samples surrounding lesions were qPCR-negative in 5 of the 8 animals investigated.

PCR of Sub-Clinically Infected Animals

One aim of this collection was to use naturally infected animals with a range of severity of infection to optimise PCR sampling from abalone. The sampling of infected animals with obvious lesions in muscle is straightforward, however large sub-clinically infected animals without lesions are challenging.

Our work suggests that DNA samples from the digestive gland of abalone are inhibitory and not recommended for PCR. Of the gill samples from animals without gross lesions, 7.8% were positive by cPCR and 11.1% were positive by qPCR. With muscle samples from the same animals, 1.5% were positive by cPCR and 13.6% were positive by qPCR. Although no gross lesions have been observed in the gills of abalone, the gills proved to be a useful sample for detection of *P. olseni* in sub-clinically infected animals. Based on this testing, where no lesions are apparent, multiple samples from different sites including both gills and muscle are recommended to increase the rate of detection.

High C_T values

In the course of testing field samples from known infected populations, we found C_T values between 40 and 45 in the AFDL *Pols* qPCR were not uncommon. Typically 1 or 2 replicates gave a high C_T value with a limited

amplification curve possibly indicating specific amplification, with the remaining replicates "undetermined" after analysis.

In order to determine if the high C_T values in the AFDL *Pols* qPCR from Thistle Island, 14:0233 were true positives derived from *Perkinsus* DNA or were as a result of "noise" in the Taqman system, a selection of DNA samples with a high C_T were subjected to "enhanced" conventional amplification conditions to produce a amplicon that could be confirmed by DNA sequencing. The low levels of target or inhibition meant that the standard methods of conventional PCR and sequencing did not work with these samples. In order to determine if *Perkinsus* DNA was present a selection of these high C_T samples from various populations were subjected to Mohr's "enhanced" amplification procedure (Mohr *et al*., 2015) to produce a conventional PCR product which could then be confirmed by DNA sequencing. Figure 20 shows some results of this approach to amplification. In this case, the OIE conventional primers for *P. olseni* 140F/600R were used in a large volume reaction with 70 cycles of amplification using the same thermal profile recommended for the diagnostic PCR.

Figure 23 OIE 140-600 cPCR (*P. olseni*) using modified cycling conditions and template volume to provide DNA for sequence confirmation

M: 100bp ladder		Ct
1: 12-00978-31A	1.	40.45
2: 12-00979-66B	2.	42.24
3: 12-00979-78	3.	39.35
4: 13-00239-79	4.	41.94
5: 14-00233-6m2	5.	41.03
6: 14-00233-19g2	6.	40.25
7: 14-00233-24L	7.	40.24
8: 14-00233-26m2	8.	42.74
9: 14-00233-29m1	9.	41.31
10: NTC		
11: Perk POS		
12: Perk POS		
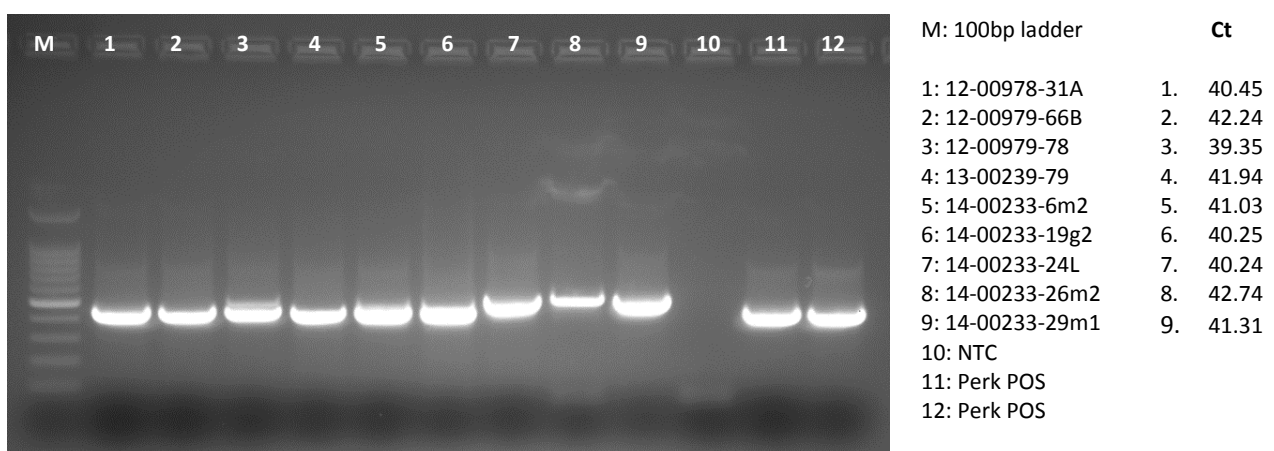
Of the 9 select test samples subjected to amplification, 6 samples with C_T values ranging from 39.35 to 42.24 produced sequence of sufficient quality for BLAST analysis (Lanes 1-6). The BLAST analysis of these samples showed 100% homology with *P. olseni* over approximately 400 nt and confirmed the presence of specific target DNA in these samples. These data along with the previous conventional PCR data for these samples indicate that the high C_T values obtained in the real-time qPCR system were derived from *Perkinsus* in the sample and that this was probably present at very low levels. Although *P. olseni* DNA samples with high C_T values could not be quantified, it was possible to confirm that at least some of these were true positives by DNA sequencing. These samples represent the limits of *P. olseni* detection of the AFDL *Pols* qPCR system.

There is a possibility that the enhanced amplification product is a result of cross-contamination, however, the documented consistency of negative samples re-tested with this and other assays under the quality system in our laboratory strong suggests this is not the case.

Although clear bands of similar intensity were observed in lanes 7, 8 and 9 (Figure 23), where samples with similar real-time C_T values were used (40.24 to 42.74), these samples failed to produce usable sequence, even with repeated testing of varying amounts of template in the sequencing reaction. While the enhanced amplification strategy can provide sufficient DNA for sequencing and has certain obvious advantages where issues of possible contamination are minimised over multiple rounds of PCR amplification using the same primers. Some awareness is required where this approach to obtaining PCR amplicons for DNA sequencing is applied. While it appears to provide a convenient way to enhance the performance of existing conventional PCR systems, amplification is not as reliable as the results in Figure 23 might suggest and the significance of the bands obtained can only be determined after confirmation by DNA sequencing, making the procedure both time-consuming and costly. In addition, the same factors influencing amplification, such as the limited availability of template are still present.

Comparison of Tests

Methods of *Perkinsus* diagnosis include Ray's thioglycollate culture, histology, PCR and DNA sequence analysis. While RFTC and histology are widely used, it is likely that with appropriate validation PCR will take over as the method of choice for rapid detection and the identification of species (Villalba *et al.*, 2004).

In this study of *Perkinsus* in abalone, individual test results of RFTC, histology and PCR were very difficult to correlate. This is in part due to focal nature of *Perkinsus* infection in abalone and the distribution of the parasite within the host. Histological lesions were often evident, with no visible parasites. Similarly PCR testing from sequential samples of muscle immediately adjacent to advanced lesions, had variable results. Given that different tissues and different amounts of tissue are used for each test meaningful comparison of individual test results is problematic.

Looking at the summary of total test results from corresponding individuals within infected populations (Table 13) we found that a similar number of infections were found by RFTC (27%, n = 43) and histology (28%, n = 44). The OIE species-specific conventional PCR found 33% were PCR positive and the AFDL *Pols* qPCR found 50% of the animals tested were PCR positive.

A summary of test results from *Perkinsus* infected population is given in table 13. Data from these populations and a small number of diagnostic submissions (data not shown) formed the basis of the ROC and Bayesian analysis for diagnostic test validation.

Table 13 Summary of corresponding test results for *Perkinsus* infected abalone populations.

		Lesions	RFTC	Histology	85/750 cPCR	140/600 cPCR	AFDL qPCR	Total Animals
Taylor Island	12-978	20	16	12	17	18	29	50
Memory Cove	12-979-1	0	0	1	0	0	1	10
Thistle Island	12-979-11	11	11	10	10	15	16	22
Baird Bay	13-1031	12	12	11	11	10	14	17
Sussex Inlet	13-1571	0	0	0	0	0	1	30
Thistle Island	14-233	8	4	10	nr	10	19	30
		51	43	44	38	53	80	159

Diagnostic Test Validation and Real-time (qPCR) Test Performance

Background

The OIE 140/600 conventional PCR (OIE test) was used as the reference test for the AFDL *Pols* qPCR (candidate test). Both tests are species-specific for *P. olseni* and had an adequate number of samples tested to compare results from different populations. In this case, the results from 3 populations with different prevalences of infection were used for ROC analysis using MedCalc (https://www.medcalc.org/) and Bayesian analysis. The assumption that both tests were conditionally independent was substantiated by the fact that reverse primers of the AFDL *Pols* qPCR were outside the region of the *Perkinsus* genome covered by the 140/600 cPCR.

ROC analysis was performed on a total of 377 samples (56 positive and 321 negative samples by the OIE 140/600 cPCR.

Bayesian analysis was performed using the cut-off generated through the ROC analysis (C_T **41.29**) and an arbitrary lower cut-off (C_T 39) was also used to allow analysis for those who cannot accept the validity of high C_T values ≥ 40.

Populations with different prevalence of Perkinsus infection
Analysis was performed using 3 populations with different apparent infection prevalences: (a) population with known history of infection (n=155, P ~ 0.3%), (b) population(s) from an area with no history of disease, disease free (n=200, P <0.01%) and (c) a population with unknown history of infection (n=22, P ~ 1%).

Reference populations
Reference samples, obtained from the OIE *Perkinsus* Reference Laboratory, were derived from naturally infected abalone populations infected with *Perkinsus olseni* (n = 8 samples) and other species of *Perkinsus*, e.g. *P. marinus* (n=11) and *P. chesapeaki* (n=12).

Bayesian analysis

Diagnostic sensitivity (DSe) and specificity (DSp) of the OIE species-specific PCR (OIE 140/600 cPCR) was estimated by testing 9 positive cultures of *P. olseni*, 40 cultures of other species of *Perkinsus* and samples collected from a *Perkinsus*-free population of abalone (n=200). The cross-classified data are presented as 2x2 tables (Tables 1 and 2) and prior values were estimated using the SISA (Simple Interactive Statistical Analysis) package found at http://www.quantitativeskills.com/sisa/statistics/diagnos.htm.

The DSe and DSp values of the OIE 140/600 cPCR thus calculated were used for the comparison of AFDL *Pols* qPCR test. The parameters for beta distribution were estimated using Beta Buster. The information from the *Perkinsus*-free population was taken as a prior for the specificity estimate (beta (201, 1)). The cut-off value for AFDL *Pols* qPCR was determined based on a Receiver Operator Curve (ROC) analysis using MedCalc. A cut-off C_T value of 41.29 was determined by the ROC analysis to balance DSe and DSp of the AFDL *Pols* qPCR. Based on this cut-off (**C_T = 41.29**), the AFDL *Pols* qPCR results were classified as either positive or negative and compared to the OIE-recommended PCR to obtain estimates of diagnostic specificity and diagnostic sensitivity using Latent Class Analysis with Bayesian modelling. The Latent class model was built under the assumption that the two tests were independent with two populations with no perfect reference standard (Branscum *et al.*, 2005). The cross-classified data for population 1 (n1=155) and population 2 (n2=22), with two different prevalence estimates (π1 = 34% & π2 <0.05%), were represented as y1 = (52, 3, 21, 79) and y2 = (1, 0, 2, 19) as given in Tables 3 and 4, respectively. Uniform distributions were used for the prevalence in the two populations (pi1 & pi2). The convergence estimates were derived using 1,000,000 iterations of simulation with sampling at every 1,000th iteration until the MC error value was <5% of the standard deviation of the node estimate. Relative prevalence, Se, Sp, SeDIFF, SpDIFF, SeSIGN and SpSIGN, the lower and upper endpoints of a 95% probability interval represented by posterior distributions for the 2.5th and 97.5th percentiles of the Monte Carlo sample were estimated. The model used for Bayesian analysis was for conditional independence of tests, provided for by the non-overlap of the PCR test primers.

Priors estimated using reference samples with a cut-off value of C_T = 41.29:

Table 14 Cross-classified data for *Perkinsus* and negative abalone DNA samples used in the Bayesian analysis

		Reference samples	
		+	-
OIE 140/600 cPCR	+	9	0
	-	0	240

Table 15 Cross-classified data for *Perkinsus* and negative abalone DNA samples used in the Bayesian analysis

	Estimate	95% MIN	95% MAX
Sensitivity	1	1	1
Specificity	1	1	1

Table 16 Cross-classified data for *Perkinsus*-infected population (Prevalence pi1~34%)

		AFDL *Pols* qPCR		
		+	-	
OIE 140/600 cPCR	+	52	3	55
	-	21	79	100
		73	82	155

Table 17 Cross-classified data for population with no history of *Perkinsus* infection (Prevalence pi1~0.05%)

		AFDL *Pols* qPCR		
		+	-	
OIE 140/600 cPCR	+	1	0	1
	-	2	19	21
		3	19	22

Priors estimated using reference samples with a cut-off value of $C_T = 39$:

A second Bayesian analysis using the same priors as given in Tables 1 and 2 was performed using an alternative **cut-off of $< C_T$ 39.** The AFDL *Pols* qPCR results were classified as either positive or negative and compared with OIE PCR for specificity and sensitivity estimates. Cross-classified data for population 1 (n1=155) and population 2 (n2=22) with two different prevalence estimates ($\pi1$ = 34% & $\pi2$ <0.05%) were represented as y1 = (50, 5, 13, 87) and y2 = (1, 0, 1, 20) and are given in Tables 5 and 6, respectively.

Table 18 Infected population (Prevalence pi1~34%)

		AFDL *Pols* qPCR		
		+	-	
OIE 140/600 cPCR	+	50	5	55
	-	13	87	100
		63	92	155

Table 19 Population with no history of infection (Prevalence pi1~0.05%)

		AFDL *Pols* qPCR		
		+	-	
OIE 140/600 cPCR	+	1	0	1
	-	1	20	21
		2	20	22

ROC analysis

The AFDL *Pols* qPCR had a relative diagnostic DSe and DSp of 94.64% (95%CI 85.1%-98.9%) and 92.83% (95%CI 89.4%-95.4%) at a cut-off of $C_T \leq 41.29$.

At a cut-off 39 C_T the AFDL *Pols* qPCR had a DSe of 91.07% (95% CI 80.4%-97.0%) and DSp of 95.64% (95%CI 92.8%-97.6%). Results for 3 populations with different prevalences are presented.

Bayesian analysis

AFDL Pols qPCR with cut-off $C_T \leq 41.29$

The AFDL *Pols* qPCR had DSe and DSp of 93.6% (95% CI 85.6-98.8) and 99.5% (95% CI 98-100), respectively, compared with the 140/600 cPCR (OIE) which had DSe and DSp of 69.4% (95% CI 58.6-79.4) and 99.6% (95% CI 98.4-100), respectively.

Table 20 Results for Bayesian analysis for AFDL *Pols* qPCR using 41.29 C_T cut-off

C_T < 41.29 = positive (AAHL test)			Posterior Distribution			
	mean	SD	MC_error	2.50%	median	97.50%
Prevalence Population 1	0.495	0.042	0.000	0.414	0.495	0.578
Prevalence Population 2	0.160	0.077	0.000	0.039	0.151	0.335
Sensitivity AHHL Test	0.936	0.034	0.000	0.856	0.941	0.988
Specificity AAHL Test	0.995	0.005	0.000	0.980	0.996	1.000
Sensitivity OIE Test	0.694	0.053	0.000	0.586	0.696	0.794
Specificity of OIE Test	0.996	0.004	0.000	0.984	0.997	1.000
Sensitivity difference	0.242	0.059	0.000	0.126	0.241	0.359
Specificity difference	-0.001	0.007	0.000	-0.017	-0.001	0.013
Sensitivity significance	1.000	0.006	0.000	1.000	1.000	1.000
Specificity significance	0.451	0.498	0.001	0.000	0.000	1.000

AFDL Pols qPCR with cut-off C_T < 39

The AFDL *Pols* qPCR had DSe and DSp of 90.2% (95% CI 80.9-96.9) and 99.5% (95% CI 98.4-100) compared with the 140/600 cPCR (OIE) which had DSe and DSp of 78.1% (95% CI 67.1-87.5) and 99.5% (95%CI 98.3-100), respectively.

Table 21 Results for Bayesian analysis for AFDL *Pols* qPCR using < 39 C_T cut-off

C_T < 39 = positive (AAHL test)			Posterior Distribution			
	mean	SD	MC_error	2.50%	median	97.50%
Prevalence Population 1	0.444	0.042	4.39E-05	0.363	0.443	0.526
Prevalence Population 2	0.119	0.068	8.32E-05	0.021	0.109	0.279
Sensitivity AHHL Test	0.902	0.041	4.93E-05	0.809	0.906	0.969
Specificity AAHL Test	0.995	0.005	8.77E-06	0.984	0.996	1.000
Sensitivity OIE Test	0.781	0.052	5.81E-05	0.671	0.783	0.875
Specificity of OIE Test	0.995	0.005	8.09E-06	0.983	0.997	1.000
0Sensitivity difference	0.121	0.062	6.25E-05	-3.49E-04	0.120	0.244
Specificity difference	-0.001	0.007	1.17E-05	-0.016	-4.71E-04	0.013
Sensitivity significance	0.975	0.157	1.76E-04	0.000	1.000	1.000
Specificity significance	0.455	0.498	7.23E-04	0.000	0.000	1.000

Repeatability

Repeatability was assessed using 37 field samples of different analyte (DNA target) concentrations in three different runs using a C_T cut-off of <39. Cochran Q statistics concluded that there was no significant difference between runs indicating satisfactory repeatability on random field samples after 3 runs at the 95% CI (P value = 0.066).

Most of the 37 samples were weak-to-strong positives. Only one sample was consistently negative yielding C_T values of 41.29, 42.74 and 40.17 over the 3 runs. As expected, the closer a sample was to the cut-off the

more likely it was to produce either a positive or negative result. There were seven samples which produced inconsistent positive and negative results. The majority, i.e., 29 samples, produced consistently positive results all the time.

Comparison of tissues for detection of *Perkinsus olseni* by AFDL *Pols* qPCR and OIE PCR

Corresponding samples of gill and muscle tissues were tested by AFDL *Pols* qPCR and OIE 140/600 conventional cPCR (reference test) to compare sensitivity and specificity.

Overlapping confidence intervals at the 95% CI indicated that there was no significant difference between DSe and DSp when gill or muscle samples from infected and non-infected abalone were used. It is recommended that parallel testing (AFDL *Pols* qPCR and OIE PCR together) of muscle and gill will achieve the highest DSe for the detection of infected abalone.

Relative DSe and DSp of the AFDL Pols qPCR for different tissues (OIE PCR reference test)

	Se (95% CI)	Sp (95% CI)
Gill	88 (81-94)	91 (85-97)
Muscle	92 (86-97)	86 (79-93)
Combined Gill & muscle	92 (86-97)	83 (75-91)

Results from the Table Gill vs Muscle indicate that muscle and gill samples do complement mutually for DSe and DSp, e.g., in infected abalone, out of 15 positive muscle samples only 3 gill specimens were positive and out to 14 positive gill specimens only 3 muscle specimens were positive. In non-infected abalone, out of 72 negative muscle specimens 61 were also negative in gill specimens and out of 73 negative gill specimens only 61 muscle specimens were negative.

Gill vs Muscle

		Muscle		
		P	N	
Gill	P	3	11	14
	N	12	61	73
		15	72	87

The data summary for statistical analysis are shown in Appendix 8

A) ROC analysis
B) Bayesian analysis (C_T 41.29 and 39 cut off)
C) Repeatability analysis
D) Comparison of specimens (Muscle vs Gill)

Discussion

In order to maximise the number of data points the results used in this analysis were derived from all tests conducted over the course of the project. As a result this performance analysis necessarily had to account for missing data where results were missing for some samples. This resulted in a relatively small number of matching results over all tests, for statistical analysis. In addition, the lack of specificity of many tests had the potential to miss-classify samples as positive for other pathogens beyond *Perkinsus olseni*. In this respect, the OIE 85/750 PCR and the Gauthier *genus-specific* qPCR were not used for analysis because they are genus and not species-specific. The RFTC and Histology were not used for analysis because they were

regarded as too non-specific. Sequencing was used for test confirmation but the number of results was limited in terms of the statistical analysis of the PCR assays. Consequently, a sub-set of data consisting of the OIE PCR (reference test) and the AFDL *Pols* qPCR was the primary source of data for establishing the performance characteristics of the candidate AFDL *Pols* qPCR test.

ROC analysis

ROC analysis was performed using 56 positive and 321 negative samples for *P. olseni* as determined by the OIE cPCR reference test. The Area under the Curve was 0.957. A significance level of <0.0001 indicates that the AFDL *Pols* qPCR is able to distinguish between the infected and non-infected group. ROC analysis was used to estimate the relative DSe (94.6) and DSp (92.8) at a preliminary cut-off of ≤ C_T 41.29 for the AFDL *Pols* qPCR.

Bayesian analysis

Bayesian analysis revealed the OIE cPCR reference test had lower DSe (78.1%) than the candidate test AFDL *Pols* qPCR (90.2%) with the abalone samples tested in our system. (Table 8, cut-off C_T of 39). Under these circumstances ROC analysis may be misleading because the reference test is likely to have missed infected abalone and produced negative results for samples which in reality were true-positive, in that the AFDL *Pols* qPCR indicated 21 false positive results in Table 3. This in turn had the effect of depressing the DSp of the candidate test. Nevertheless, the AFDL *Pols* qPCR and OIE cPCR reference tests had 100% agreement of test results in the population of 200 individuals with no history of *Perkinsus* infection or historic freedom (Table A3). These results substantiate the high DSp estimates close to 100% for both tests as confirmed in the Bayesian analysis.

This Bayesian analysis was performed using a fixed threshold of 0.1 and 2 different C_T cut-off values. A C_T cut-off of ≤41.29 for positive samples was obtained from the ROC analysis of our data. A lower C_T cut-off ≤ 39 was arbitrarily chosen to provide estimates of DSe and DSp for those laboratories that consider cut-offs beyond a C_T of 40 as unreliable for qPCR. In this case, lowering the cut-off causes a slight drop in DSe and an increased DSp due to the reaction dynamics of real-time PCR. These data clearly show that C_T values ≤41.29 in the AFDL *Pols* qPCR system are positive. C_T values ≤39 are unambiguously positive if a C_T of >40 is not accepted. In which case the range of >39-42 might be considered inconclusive and samples with a C_T > 42-45 are negative. Our experience has been that abalone DNA samples from uninfected populations produce little if any background in the AFDL *Pols* qPCR system and that C_T values between 40 and 45 are often observed in testing infected populations. Depending on the purpose of testing, inconclusive results may require re-test and/or resampling to reliably establish the infection status of a population.

Repeatability

Cochran Q statistics indicated satisfactory repeatability between runs using random samples.

Sample selection in abalone (muscle vs gills)

Disease prevalence, required probability of detection and test sensitivity are the key factors for a sampling frame and the moderate DSe of the AFDL *Pols* qPCR must be taken into consideration. Parallel testing (OIE PCR and AFDL *Pols* qPCR together) of muscle and gill specimens is likely to increase DSe of detecting *Perkinsus olseni*-infected abalone. Using the AFDL *Pols* qPCR as a screening "population test", e.g. on 10-15 abalone, at the same time would help to minimize false-negative results in low prevalence scenarios and would also be helpful to minimize false-negative results due to localised infections. Our data show that the DSe obtained with the AFDL *Pols* qPCR with gill and muscle are similar, 0.88 and 0.92, respectively. The slightly higher sensitivity in muscle

may be due in part to the natural bias of the operator towards sampling visible lesions or areas of tissue with abnormal appearance which may be indicative of infection. Should the highest level of DSe be required the parallel testing of both muscle and gill is recommended.

The relevant validation data for ROC analysis, Bayesian analysis (with C_T 41.29 and 39 cut-offs), repeatability and comparison of muscle and gill specimens are shown in Appendix 8.

Sequence and Phylogenetic analysis

Only minor variations in DNA sequence were found in alignments of the ITS region (Figures 24, 26, 28, 30 and 32) in the area adjacent the diagnostic probe. These were mostly confined to single nucleotides at specific locations, nt positions 284 and 321 in the alignments of 12-00978, 12-00979 and 13-01031. The only other variations observed were single nucleotide changes from sample 12:00979-25 (Figure 13). This included an ambiguous nucleotide W (A or T) within the area targeted by the probe which could not be differentiated in the chromatogram as the AT peaks were superimposed. As we consistently applied the most conservative interpretation to all DNA sequencing undertaken, the ambiguity was retained and is reported here for the sake of completeness. The possible sequence mismatch in the probe-binding region of 12:00979-25 did not affect detection of this sample in the real-time assay. No other sequence variations were observed in the areas targeted by the primers.

All the *P. olseni* sequences from Australian field samples collected over 3 years grouped with the other *P. olseni* sequences from GenBank in the phylogenetic analysis of individual samples (Figures 25, 27, 29, 31 and 32). The topologies of the trees generated were similar in the analyses with *P. marinus, P. honshuensis* and *P. mediterraneus* being most closely related to *P. olseni* in all the phylogenetic trees generated. These DNA sequence alignments and the phylogenetic trees on which they are based provide evidence of the taxonomic affiliation of the individual samples with respect to other *Perkinsus* species and show there is little variation in the DNA sequence in the region targeted by the real-time assay and further serve to confirm the identity of *Perkinsus olseni* and the specificity of the AFDL *Pols* qPCR system.

Figure 24 Sequence alignment of SAN 12-00978 *Perkinsus olseni* isolates.
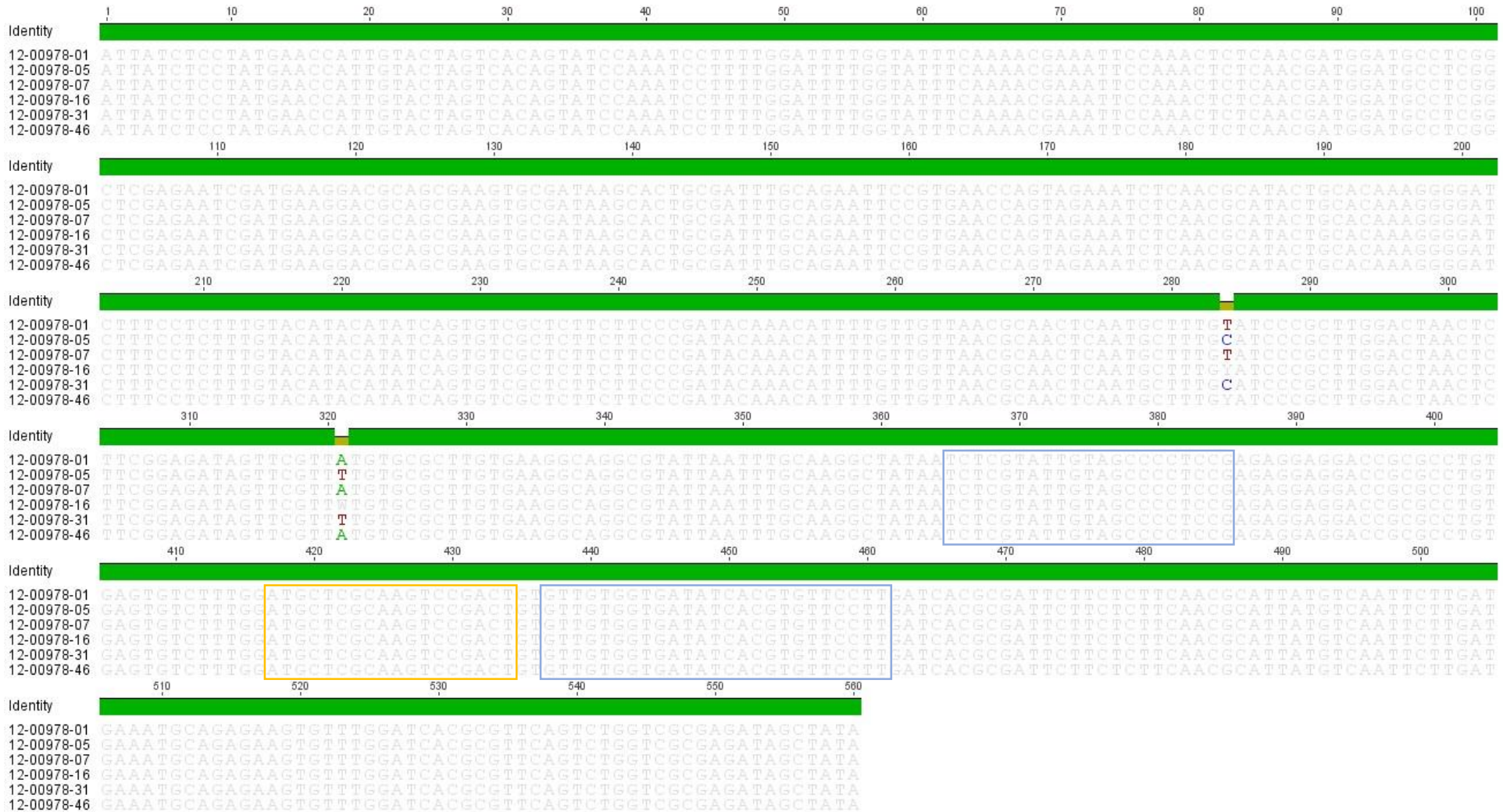
Sequence analysis of OIE *Perkinsus spp.* PCR (85-750) amplicons from *Perkinsus*-infected animals collected from Taylor's Island, South Australia (SAN 12-00978). Sequence alignment of the internal transcribed spacer (ITS) region shows the sequence variability (highlighted bases) within the site of collection. (Ambiguous bases: W= A or T, Y=T or C). The position of the AFDL *Pols* qPCR primers (blue boxes) and probe (orange box) are indicated.

Figure 25 Phylogenetic tree showing SAN 12:00978 *P. olseni* sequences
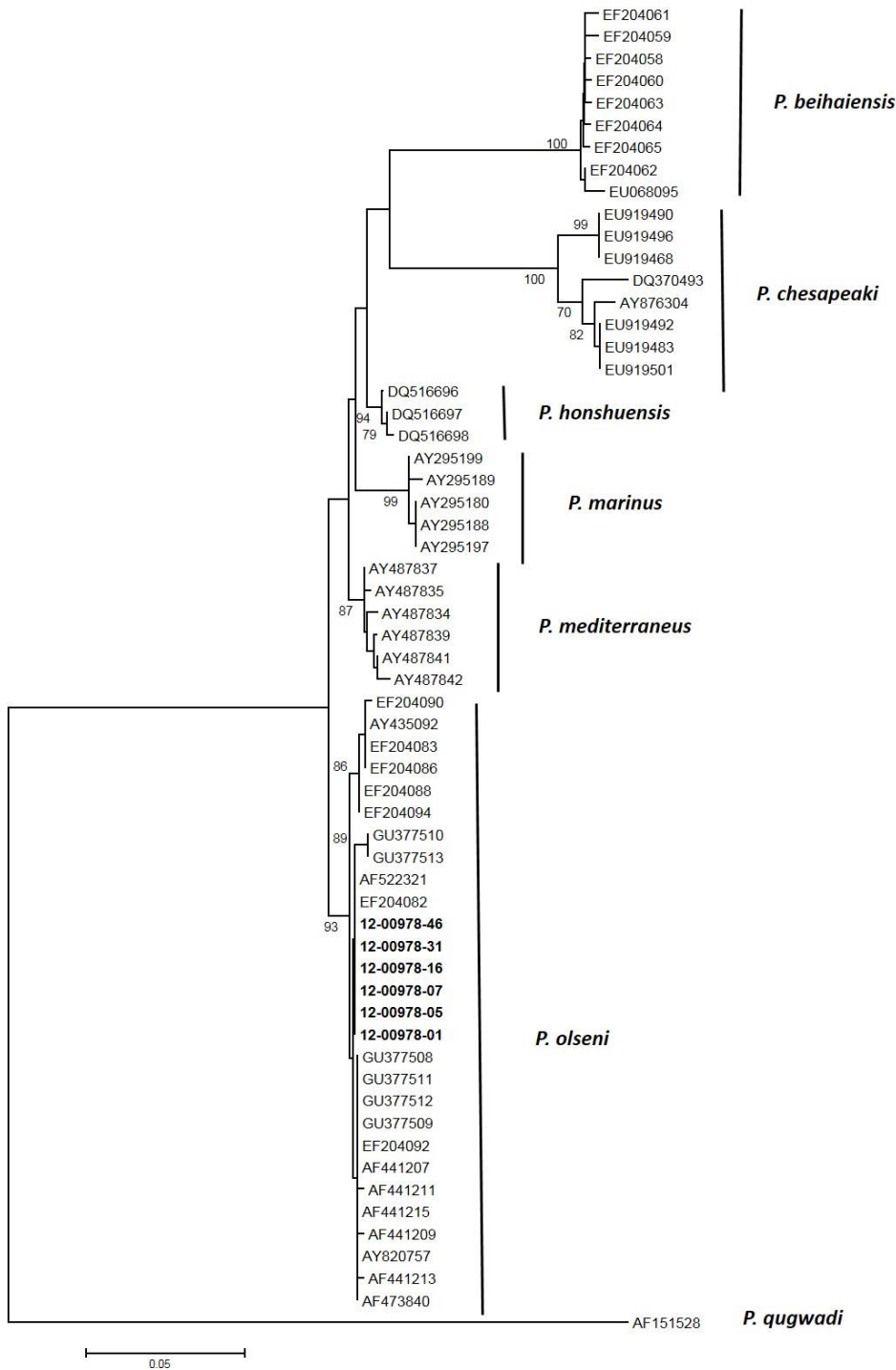
An unrooted neighbour-joining distance tree based on the ITS region of *Perkinsus* spp.. The OIE *Perkinsus spp.* PCR (85-750) products for SAN 12-00978 were sequenced (shown in bold). The phylogenetic analysis was based on 10,000 boot-strap replicates and values of >70% are shown on the tree. The scale bar represents substitutions per nucleotide site.

Figure 26 Sequence alignment of SAN 12-00979 *Perkinsus olseni* isolates.

Sequence analysis of OIE *Perkinsus* spp. PCR (85-750) amplicons from *Perkinsus*-infected animals collected from Taylor's Island, South Australia (SAN 12-00979). Sequence alignment of the internal transcribed spacer (ITS) region shows the sequence variability (highlighted bases) within the site of collection. (Ambiguous bases: W= A or T, Y=T or C, K= G or T). The position of the AFDL *Pols* qPCR primers (blue boxes) and probe (orange box) are indicated.

Figure 27 Phylogenetic tree showing SAN 12:00979 *P. olseni* sequences
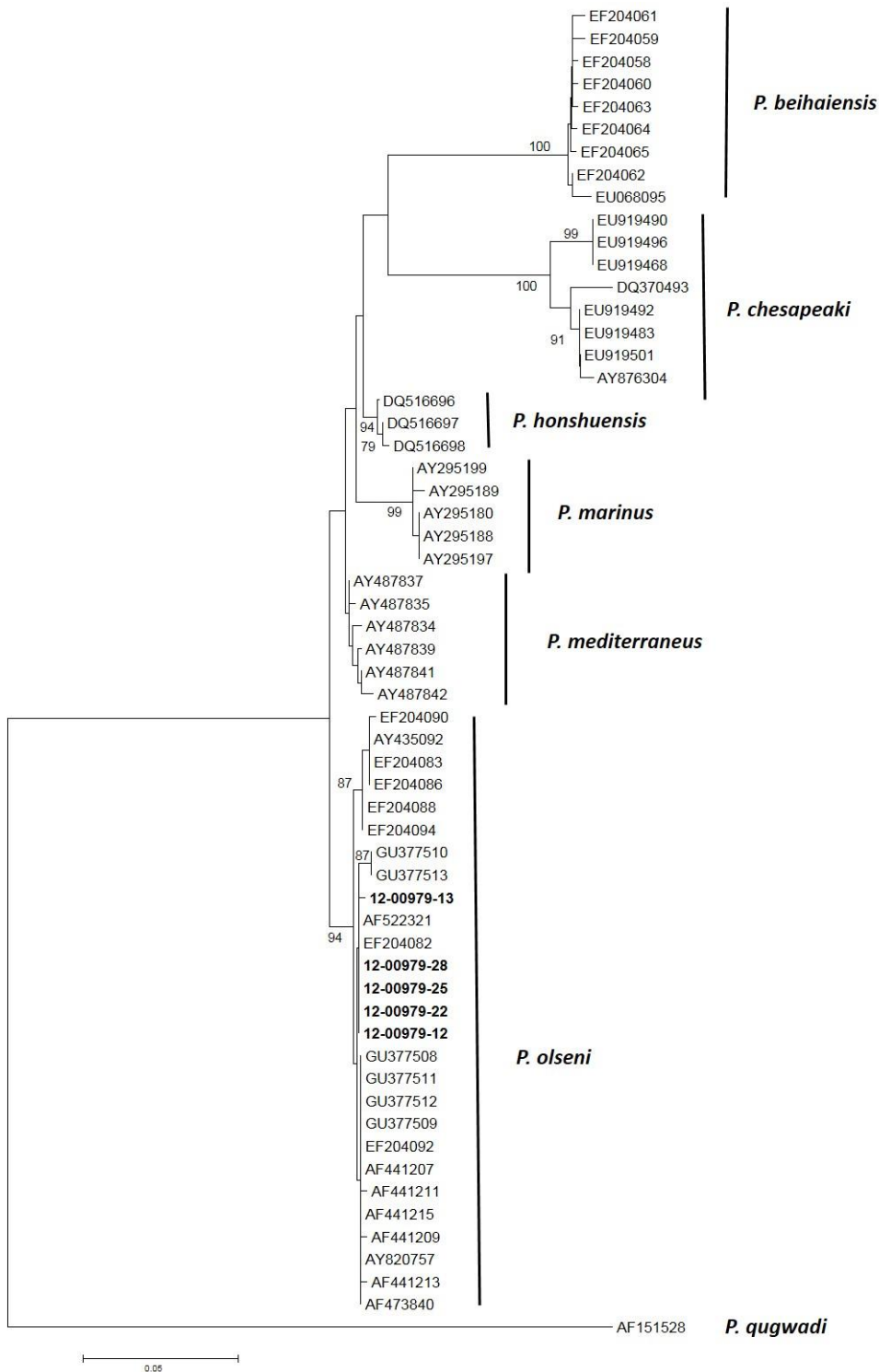
An unrooted neighbour-joining distance tree based on the ITS region of *Perkinsus* spp.. The OIE *Perkinsus spp.* PCR (85-750) products for SAN 12-00979 were sequenced (shown in bold). The phylogenetic analysis was based on 10,000 boot-strap replicates and values of >70% are shown on the tree. The scale bar represents substitutions per nucleotide site.

Figure 28 Sequence alignment of SAN 13-01031 *Perkinsus olseni*
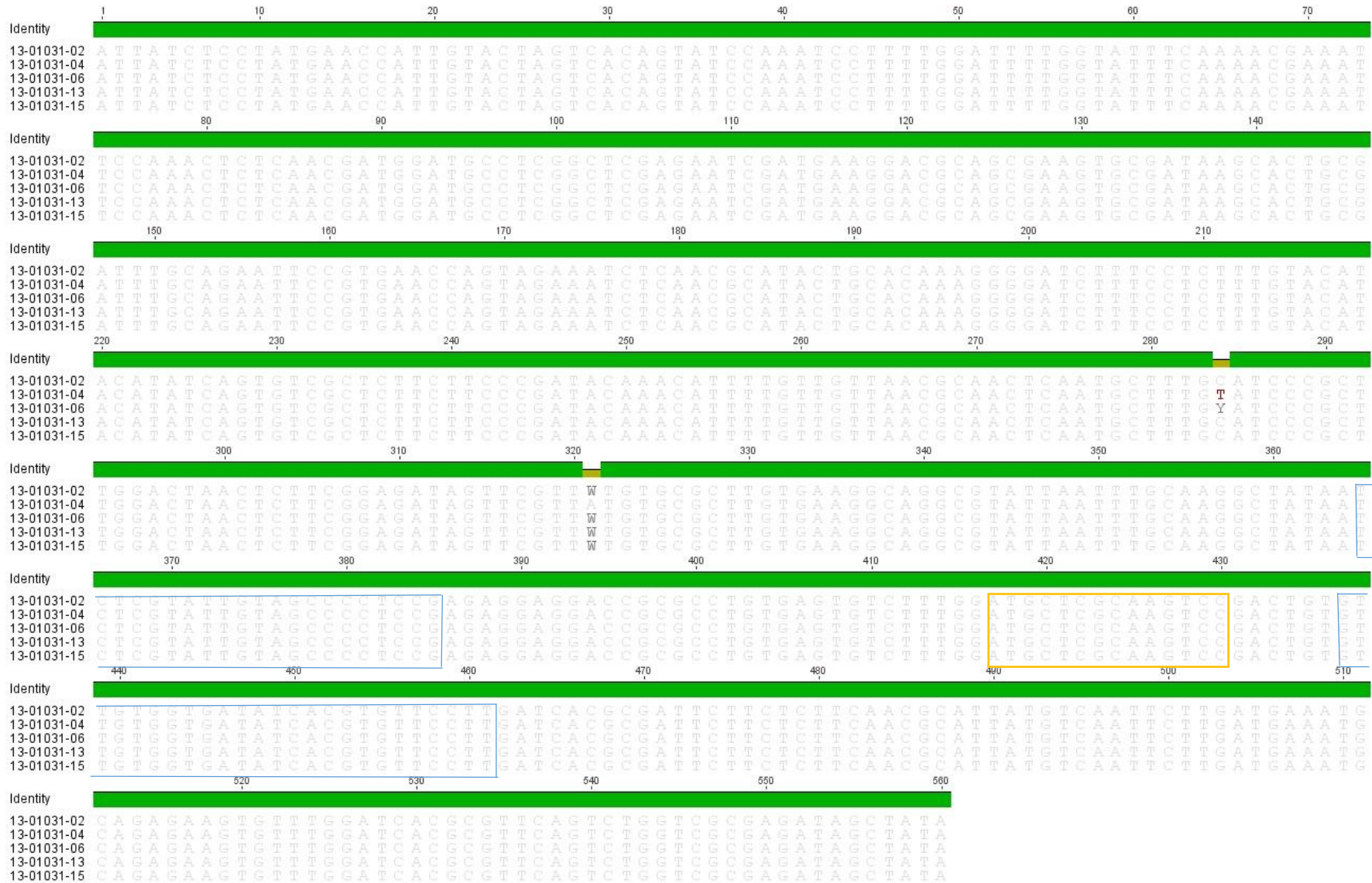
Sequence analysis of OIE *Perkinsus spp.* PCR (85-750) amplicons from *Perkinsus*-infected animals collected from Baird Bay, South Australia (SAN 13-01031). Sequence alignment of the internal transcribed spacer (ITS) region shows the sequence variability (highlighted bases) within the site of collection. (Ambiguous bases: W= A or T, Y=T or C). The position of the AFDL *Pols* qPCR primers (blue boxes) and probe (orange box) are indicated.

Figure 29 Phylogenetic tree showing SAN 13:01031 *P. olseni* sequences
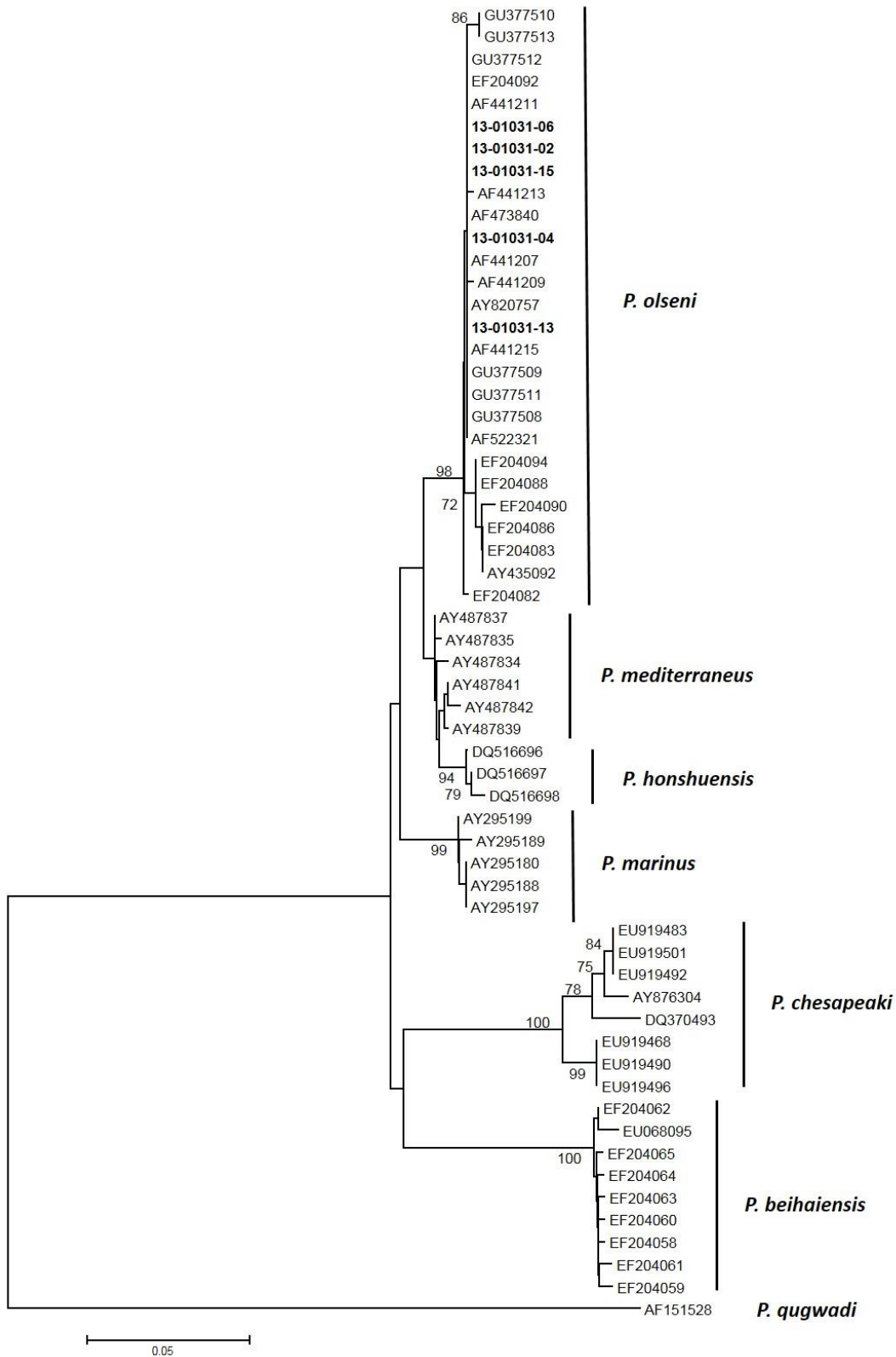
An unrooted neighbour-joining distance tree based on the ITS region of *Perkinsus* spp.. The OIE *Perkinsus spp.* PCR (85-750) products for SAN 13-01031 were sequenced (shown in bold). The phylogenetic analysis was based on 10,000 boot-strap replicates and values of >70% are shown on the tree. The scale bar represents substitutions per nucleotide site.

Figure 30 Sequence alignment of SAN 14-00233 *Perkinsus olseni* isolates
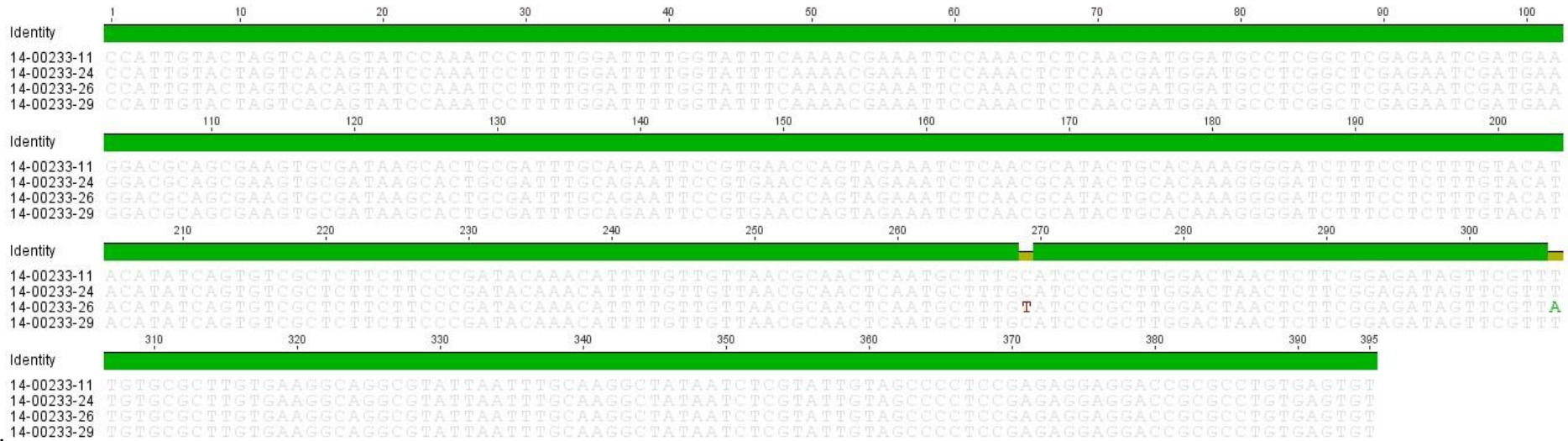
Sequence analysis of OIE *Perkinsus olseni* PCR (140-600) amplicons from *Perkinsus*-infected animals collected from Thistle Island in South Australia (SAN 14-00233). Sequence alignment of the internal transcribed spacer (ITS) region shows the sequence variability (highlighted bases) within the site of collection.

Figure 31 Phylogenetic tree showing SAN 14:00233 *P. olseni* sequences
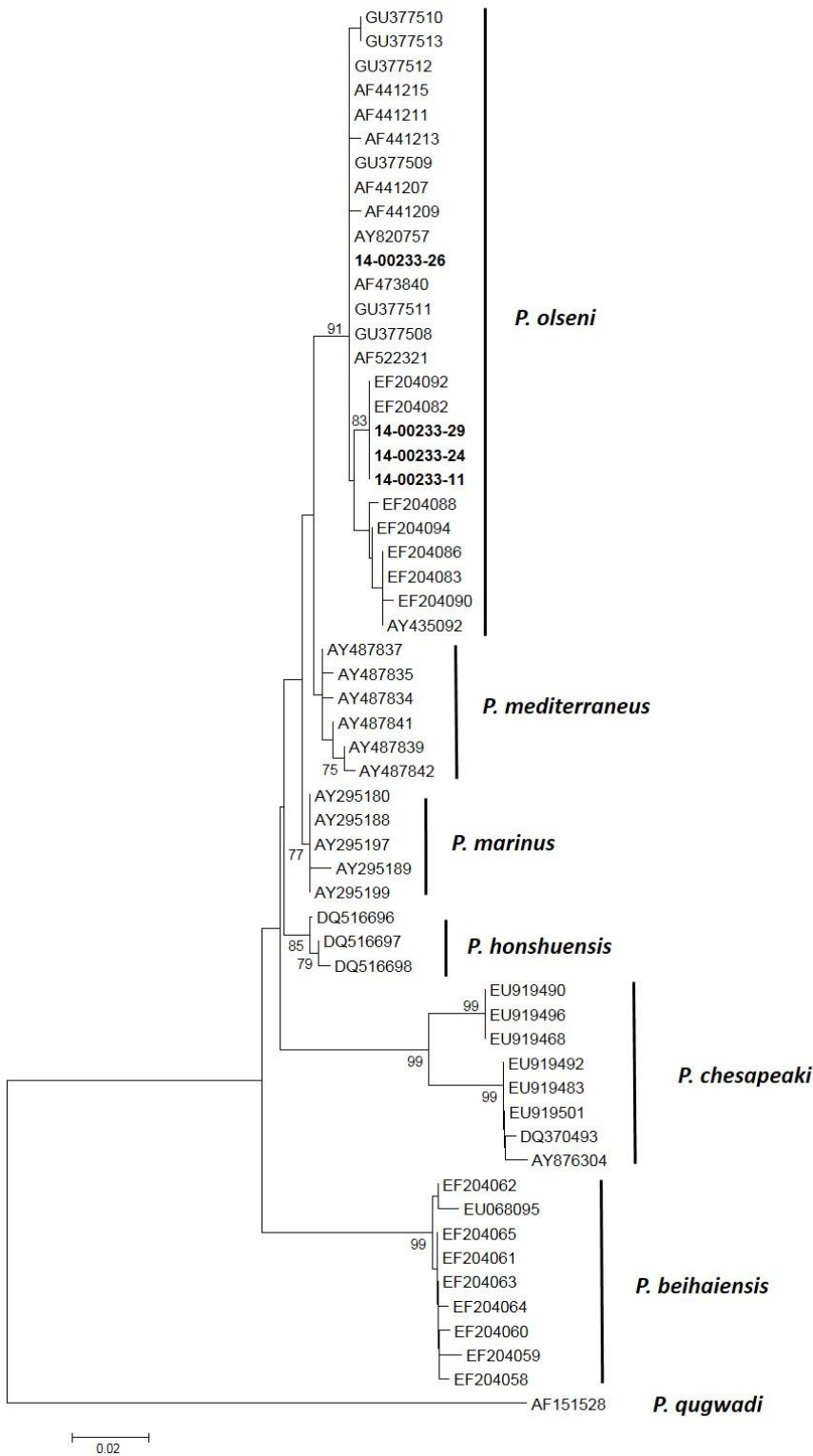
Unrooted neighbour-joining distance tree based on the ITS region of *Perkinsus* spp.. The OIE *Perkinsus olseni* PCR (140-600) products for SAN 14-00233 were sequenced (shown in bold). The phylogenetic analysis was based on 10,000 boot-strap replicates and values of >70% are shown on the tree. The scale bar represents substitutions per nucleotide site.

Figure 32 Sequence alignment of all *Perkinsus olseni* isolates
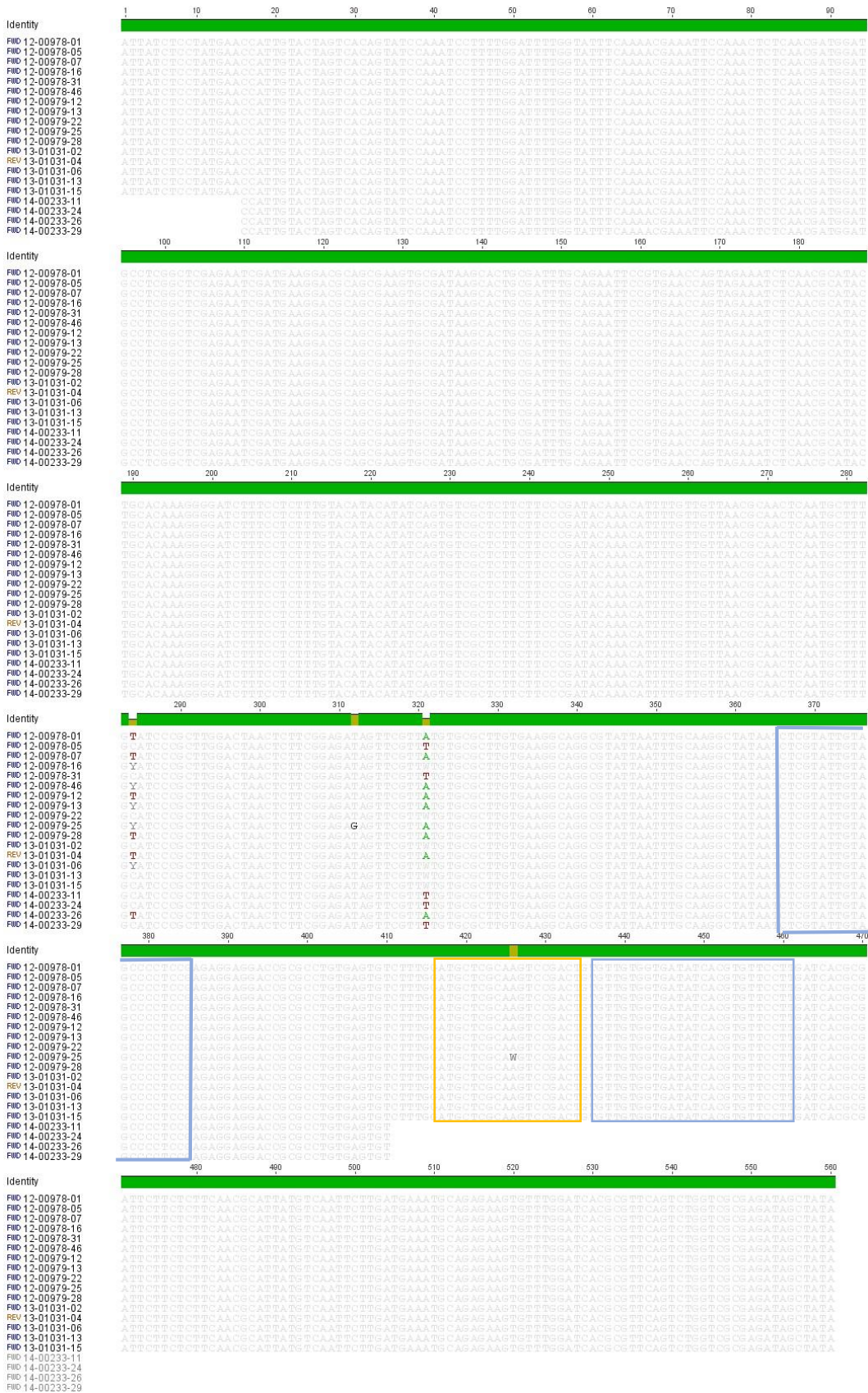
Sequence analysis of ITS region from *Perkinsus*-infected animals collected from all locations in South Australia. Sequence alignment of the internal transcribed spacer (ITS) region shows the sequence variability (highlighted bases) within South Australia. The position of the AFDL *Pols* qPCR primers (blue boxes) and probe (orange box) are indicated.

Figure 33 Phylogenetic tree based on ITS region of *Perkinsus spp.* isolates from South Australia
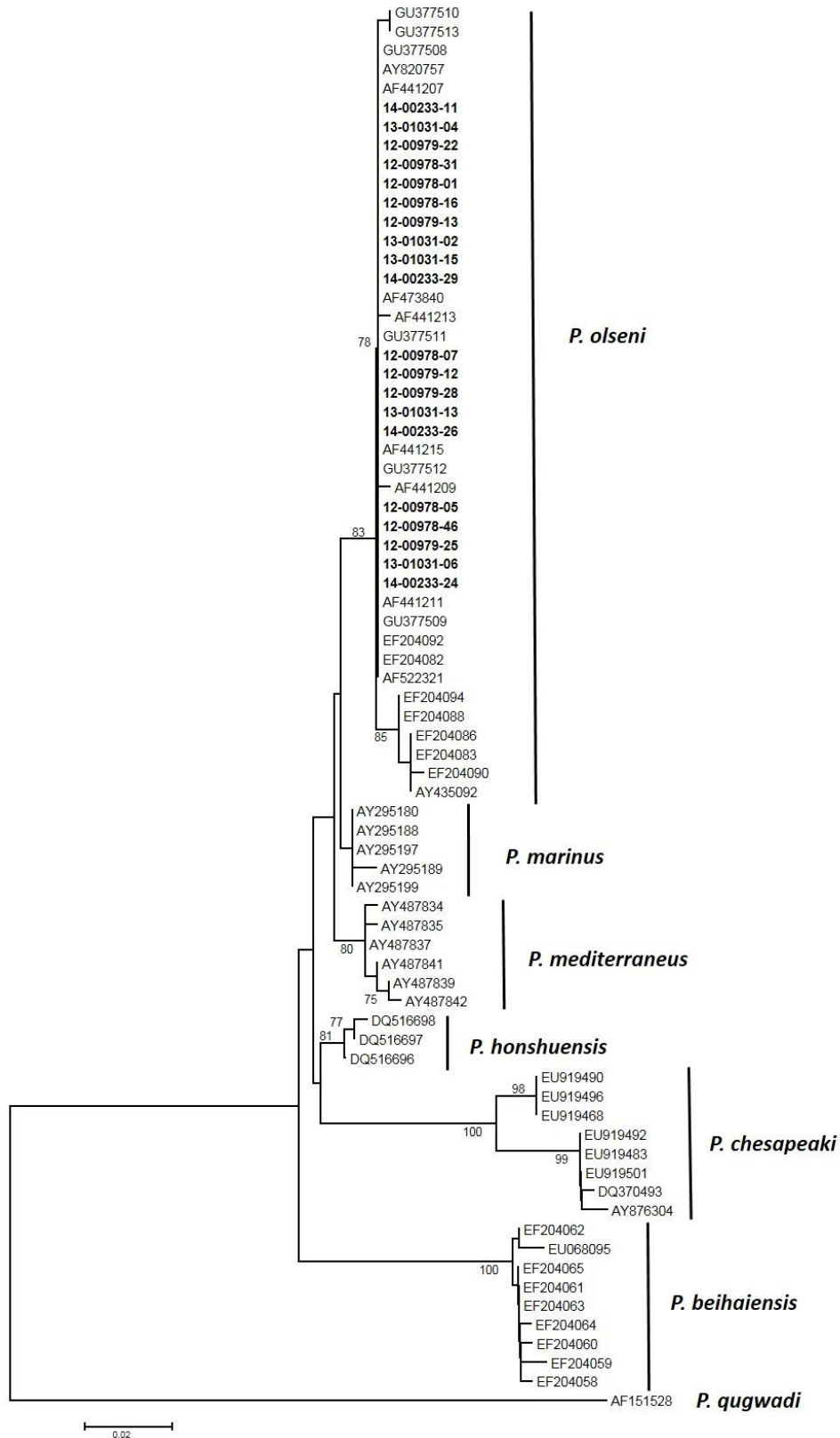
Unrooted neighbour-joining distance tree based on the ITS region of *Perkinsus* spp. isolates detected in South Australia are shown in bold. The phylogenetic analysis was based on 10,000 boot-strap replicates and values of >70% are shown on the tree. The scale bar represents substitutions per nucleotide site.

Phylogenetic analysis of Australian *Perkinsus olseni* cultures

In addition to DNA sequence analysis of abalone field samples from *Perkinsus*-infected populations sampled, sequencing and phylogenetic analysis of the ITS region, the large sub-unit of the rRNA gene (LSU) and the actin gene was undertaken on the 4 *P. olseni* axenic cultures established in this project. This analysis was undertaken to confirm the uniformity of both the polyclonal and clonal cultures established and to further characterise and confirm the taxonomy of the cultures and was required for the submission to the ATCC of 12:00978-12 as the type culture of *P. olseni*.

Phylogenetic analysis

Here we provide a detailed phylogenetic analyses of nucleotide sequences of the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene complex, of the large subunit (LSU) rRNA gene, and of the actin gene for *P. olseni* isolate 12:00978-12 from Taylor's Island, South Australia (34°52'36.36"S, 136° 0'53.82"E). The cultures were established from *Perkinsus* lesions in pedal muscle collected from *H. rubra* (blacklip abalone) in April 2012 and 2013. DNA sequence of *P. olseni* isolate 12:00978-12 is compared with GenBank sequence data representative of each of the 7 known species of *Perkinsus*. These data are consistent with and representative of the 4 cultures established in this project and are summarised as a phylogenetic tree of each gene loci (Figures 34 to 36). The primers and thermal cycling conditions are modified after Moss *et al*. (2008) and are summarised in Appendix 2. The GenBank accession numbers for sequences used in the phylogenetic analysis are summarised in Appendix 3.

Sequence analysis of the ITS region and LSU rRNA gene fragments of the rRNA gene complex showed that in all cases the amplified fragment was the targeted DNA and consistently placed this as a member of the genus *Perkinsus* and the sequences grouped in clades represented by *P. olseni*. The topologies of the trees generated, based on each of the three loci (Figures 33 to 35) were similar in the analyses with the SA *Perkinsus* sequence grouping with other *P. olseni* sequences, and *P. marinus, P. honshuensis* and *P. mediterraneus* being most closely related in all the phylogenetic trees generated. These sequence data and phylogenetic analysis provide clear evidence of the taxonomic affiliation of the SA *Perkinsus* cultures with respect to other *Perkinsus* species and serves to further confirm the identity of the *Perkinsus olseni* cultures and the specificity of the AFDL *Pols* qPCR system.

Figure 34 Phylogeny of South Australian isolate 12:00978-12 based on the ITS region
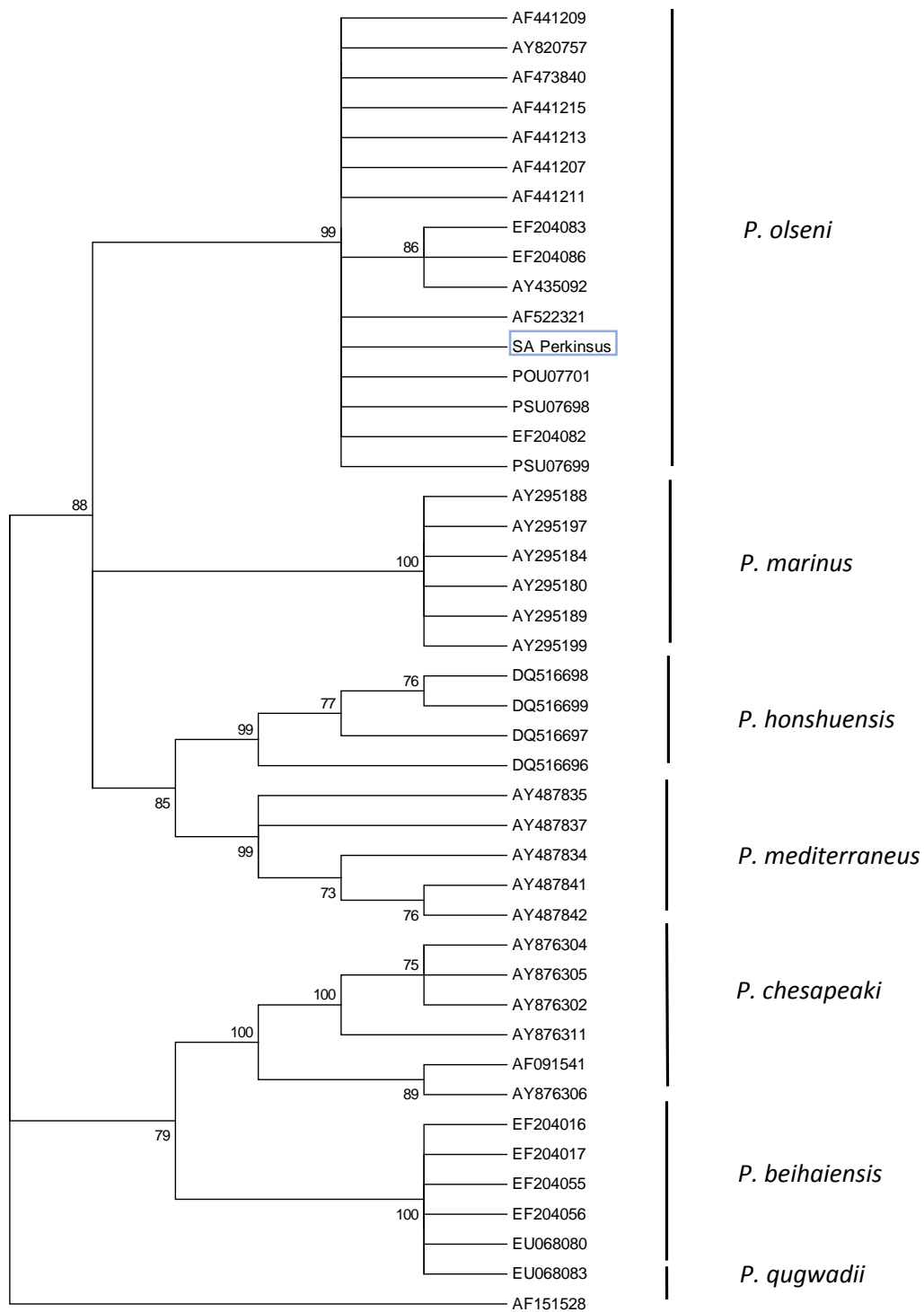
Evolutionary relationship of 7 *Perkinsus* species and South Australian isolate 12:00978-12 based on the DNA sequence of the ITS region. The optimal tree with the sum of branch length = 0.83619900 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Evolutionary distances are shown as the number of base substitutions per site. The analysis involved 44 nucleotide sequences. A total of 508 positions are represented in the final ITS dataset.

Figure 35 Phylogeny of South Australian isolate 12:00978-12 based on the LSU sequence
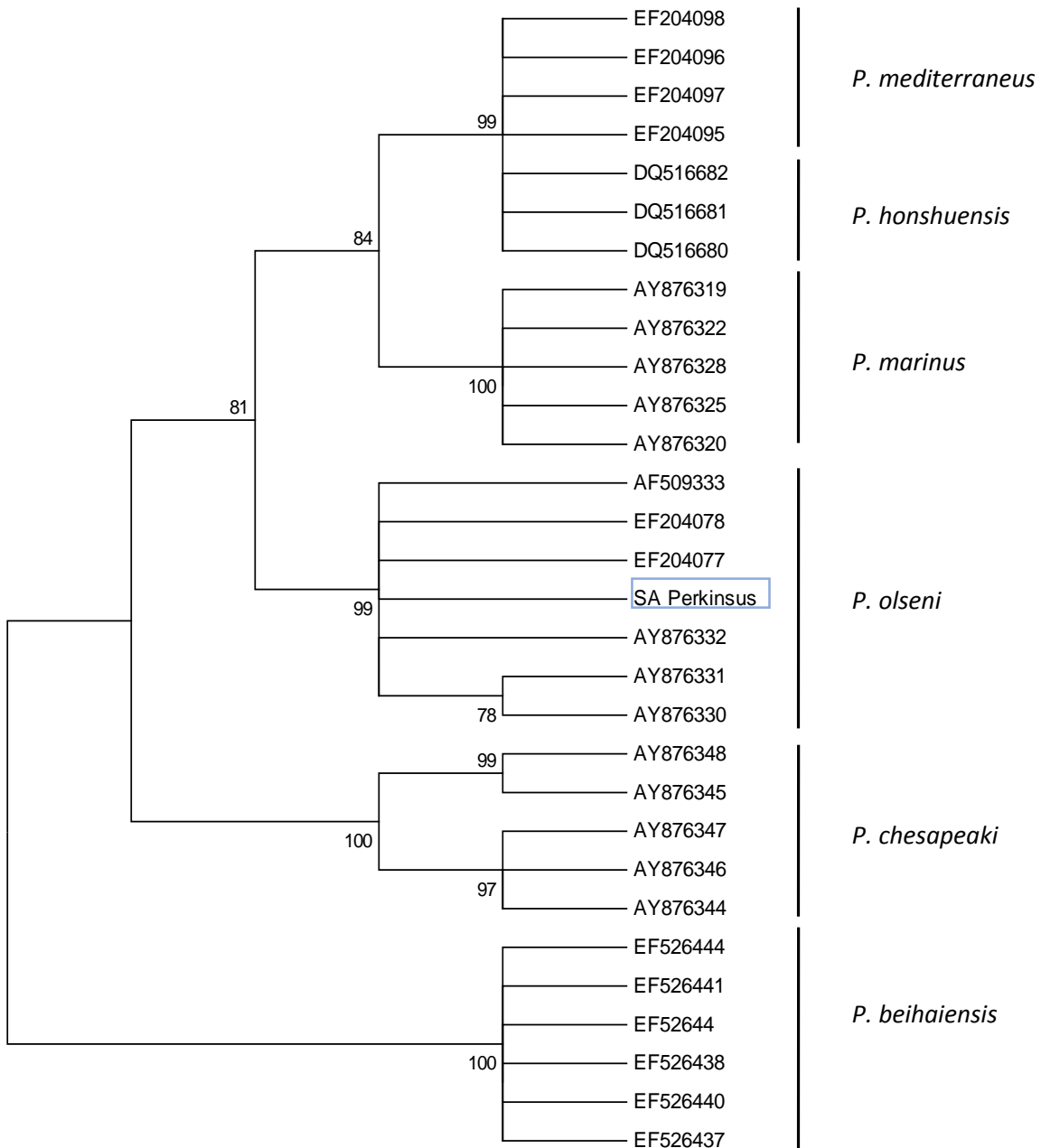
Evolutionary relationship of 6 *Perkinsus* species and South Australian isolate 12:00978-12 based on the DNA sequence of the LSU region. The optimal tree with the sum of branch length = 0.11475750 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Evolutionary distances are shown as the number of base substitutions per site. The analysis involved 30 nucleotide sequences. A total of 927 positions are represented in the final LSU dataset.

Figure 36 Phylogeny of South Australian isolate 12:00978-12 based on the actin gene
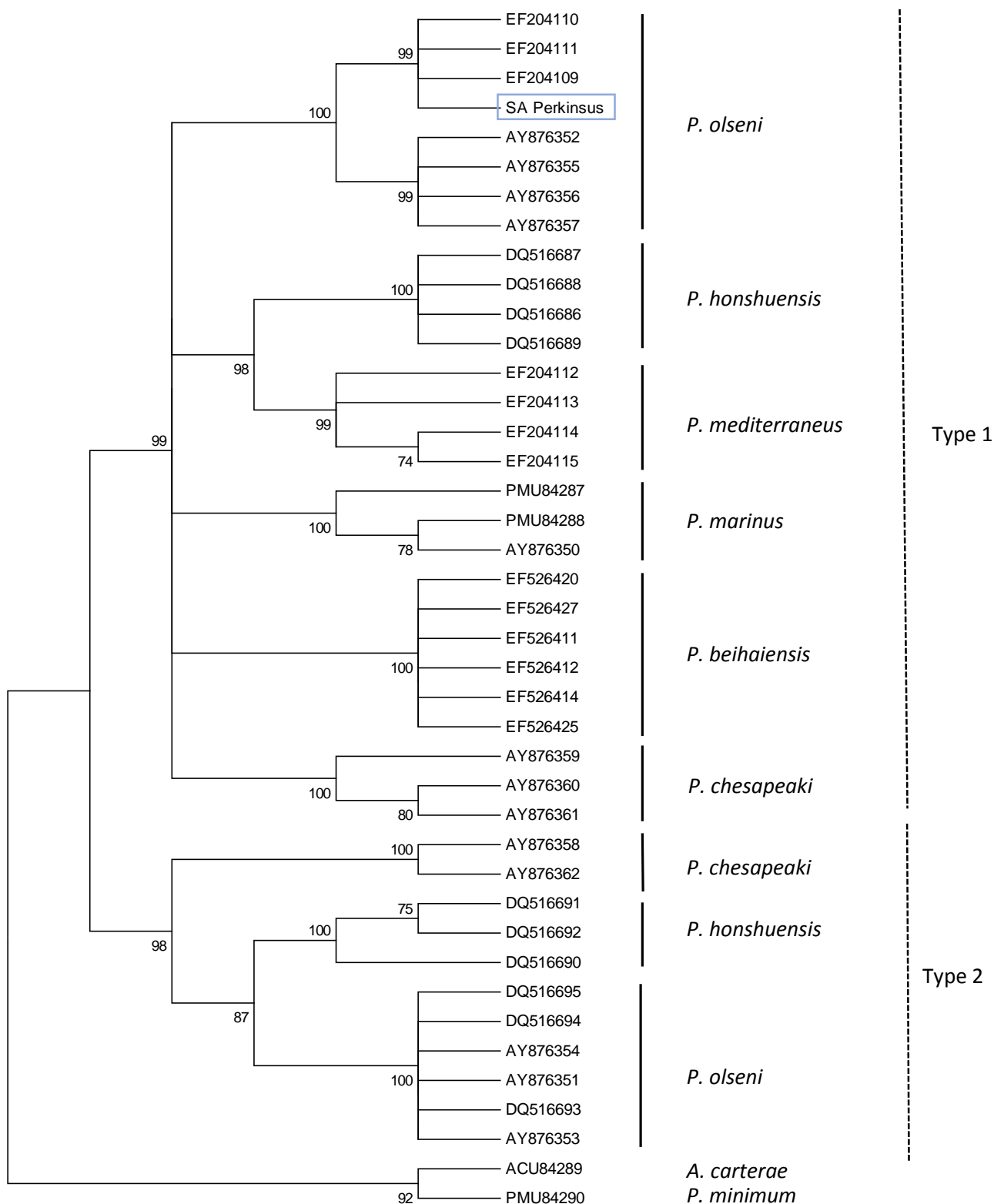
Evolutionary relationship of the 7 *Perkinsus* species and South Australian isolate 12:00978-12 based on the DNA sequence of the actin gene region. The optimal tree with the sum of branch length = 1.30579629 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Evolutionary distances are shown as the number of base substitutions per site. The analysis involved 41 nucleotide sequences. A total of 286 positions are represented in the final actin dataset.

Discussion and Conclusion

Sampling of Infected Populations and Comparison of Methods

The targeted sampling of known *Perkinsus*-infected wild abalone populations provided an opportunity to compare existing methods of detection, including RFTC, histology and PCR-based molecular methods. A summary for results from *Perkinsus*-infected populations is given in table 13. Data from these populations and some diagnostic submissions (data not shown) formed the basis of the ROC and Bayesian analysis for diagnostic test validation.

While histology and RFTC provide a cheap and convenient means of *Perkinsus* detection neither method can be used for definitive diagnosis of specific species. Novoa *et al.* (2002) reported the lack of specificity of the RFTC by DNA sequence analysis of hypnospores obtained through incubation of clam gills in RFTM. The results showed that the assay was not specific t*o members of the genus Perkinsus, and that other organisms including a Perkinsus-like organism (PseudoPerkinsus tapetis, Mesomycetozoa), also enlarged hypnospores when incubated in thioglycollate medium.*

The method for Ray's thioglycollate culture used in this project was based on the ICES standard assay (ICES, 2011). A pool of target tissues were dissected and incubated in ARFTM before examination by microscopy. A further refinement of the procedure was the total body burden assay, individual tissues or the entire animal were incubated in ARFTM then washed with NaOH to dissolve the host tissue. This method is more sensitive and accurate than the standard RFTC (Bushek *et al.,* 1994; Rodríguez and Navas, 1995) but also more expensive and time-consuming than the standard assay. The total body burden assay would be difficult to implement for screening of populations of large abalone. Comparison of the two methods in oysters, shows that the standard method does not consistently detect infections until the parasite burden is $\geq 10^3$ to 10^4 per gram wet weight of tissue (ICES, 2011). Histology and ARFTM incubation of hemolymph samples are considered less sensitive diagnostic techniques than standard RFTC incubation in the ICES (2011) report.

In the course of testing individual tissues we were surprised to find that gill samples were often positive by PCR, despite the absence of lesions. In order to determine the relative value of sampling gill and muscle in abalone we analysed and compared the OIE PCR and AFDL *Pols* qPCR results. Our data showed that parallel testing (OIE PCR and AFDL *Pols* qPCR together) of muscle and gill specimens is likely to increase DSe of detecting *Perkinsus olseni*-infected abalone. Using the AFDL *Pols* qPCR as a screening "population test", e.g. on 10-15 abalone, at the same time would help to minimize false-negative results in low prevalence scenarios and would also be helpful to minimize false-negative results due to localised infections. Our data show that the DSe obtained with the AFDL *Pols* qPCR with gill and muscle are similar, 0.88 and 0.92, respectively. The slightly higher sensitivity in muscle may be due in part to the natural bias of the operator towards sampling visible lesions or areas of tissue with abnormal appearance which may be indicative of infection. Should the highest level of DSe be required the parallel testing of both muscle and gill is recommended.

Establishment of *In Vitro Perkinsus olseni* Cultures

Replicating *in vitro* cultures of *Perkinsus olseni* were successfully established from each of the infected areas examined. This includes a sample from Thistle Island, South Australia which is near the location from which *P. olseni* was first described.

It is important to clearly distinguish between culture of *Perkinsus* in Ray's Thioglycollate Medium (ARFTM) and the *in vitro* culture undertaken here. Culture in ARFTM does not result in significant replication or increase of parasite numbers but merely allows *Perkinsus* hypnospores to increase in size making them easier to visualise by light microscopy when stained with dilute Lugol's Iodine solution (Figure 10). This is the basis of Ray's test which provides a cheap and convenient means of screening for *Perkinsus* spp*.* While it appears that *Perkinsus* spp. are preferentially selected in the medium and iodine-staining is generally

confined to *Perkinsus* hypnospores, Ray's test does not provide definitive identification. In contrast, *in vitro* tissue culture of *Perkinsus* provides a way of both amplifying cell numbers and storing the live parasite providing a ready source of *Perkinsus* for biological study. A stock of *P. olseni* cells has been established for this purpose and is available from CSIRO-AAHL for distribution to Australian laboratories. A stock of *Perkinsus olseni* gDNA has also been prepared.

Four representative axenic cultures were cryopreserved successfully. Two isolates from 2012 and 2013 were cloned by terminal dilution and the viability of the cryopreserved stocks was demonstrated by recovery and passage of cells in tissue culture. The identification of all *P. olseni* cultures was confirmed by DNA sequencing and BLAST (Altschul *et al.*, 1997) and phylogenetic analysis of the ITS intergenic region, the LSU rRNA gene and the actin gene.

A clonal culture from this location was accepted by ATCC as the holotype culture for *P. olseni.*

DNA Sequencing and Phylogenetic Analysis

The results of DNA sequencing and phylogenetic analysis provided a useful framework for the real-time PCR development. We saw little variation in DNA sequence in the diagnostic region over the course of the study and these data correlated with the other *P. olseni* sequences from GenBank, demonstrating stability in this area of the *Perkinsus* genome extending over some decades. Only minor variations in DNA sequence were found in alignments of the ITS region (Figures 23, 25, 27, 29 and 31). These were mostly confined to single nucleotides changes outside the region targeted by the real-time assay.

All the *P. olseni* sequences from Australian field samples collected over 3 years grouped with the other *P. olseni* sequences from GenBank in the phylogenetic analysis of individual samples (Figures 24, 26, 28, 30 and 32). The topologies of the trees generated were similar in the analyses with *P. marinus, P. honshuensis* and *P. mediterraneus* being most closely related to *P. olseni* in all the phylogenetic trees generated.

These DNA sequence alignments and the phylogenetic trees on which they are based provide evidence of the taxonomic affiliation of the individual samples with respect to other *Perkinsus* species and show there is little variation in the DNA sequence in the region targeted by the real-time assay and further serve to confirm the identity of the *Perkinsus olseni* cultures developed in this project and the specificity of the AFDL *Pols* qPCR system.

The sequencing and phylogenetic analysis in this project was also intended to confirm the taxonomy and identification of a possible new *Perkinsus* species found in NSW abalone samples in 2005 and also reported from Vietnamese clams imported into the USA (Liggins & Upston, 2010; Reece *et al*., 2010).

The *Perkinsus*-related mortalities which severely depleted stocks of blacklip abalone along approximately 500 km of NSW coastline since the early 1990's appears to have dropped to the point where disease due to *Perkinsus* is no longer reported. The reasons for this are not known. Areas of disease were still active and identified in a number of surveys conducted in the early 2000's where *Perkinsus* infections were identified by Ray's Test and confirmed by histology. The last reports of *Perkinsus* appear to have come from samples collected in 2005, in this case *Perkinsus olseni* was confirmed by PCR and DNA sequencing. In the course of this testing an "ITS-variant" was detected (Reece *et al*., 2010), however no further samples appear to have been collected from these areas. From that time to the beginning of this project in 2011, Perkinsosis in NSW abalone appears to have declined and no sampling for surveillance testing for *Perkinsus* was undertaken.

As a result of the original mortality events in NSW, Liggins and Upston (2010) reported that in some areas abalone stocks may have been depleted to less than 10% of virgin abundance and that the recovery of stocks would be a slow process. It is possible that populations were so severely depleted that infection was self-limiting, however it is equally possible that some unknown environmental factor or a combination of factors is responsible for the disappearance of the disease. Whatever the case may be any explanation for the disappearance of *Perkinsus* from previously infected areas in NSW abalone is speculative at best. Despite the best advice from NSW authorities, including NSW DPI and NSW Fisheries and professional

abalone divers who are responsible for the regulation of this fishery, no *Perkinsus*-infected populations could be identified at the time of this project.

As research funding for the collection of field samples in this project was based on targeted sampling of known infected populations, field collection in NSW was limited to the area were the ITS-variant was last detected. Limited sampling was conducted at five sites south of Bowen Island down to the St Georges Head in the Jervis Bay area (Site 1 (35° 8.600'S, 150° 45.760'E), Site 2 (35° 9.192'S, 150° 45.686'E), Site 3 (35° 9.679'S, 150° 45.598'E), Site 4 (35° 10.212'S, 150° 45.042'E), Site 5 (35° 12.033'S, 150° 41.755'E)). No *Perkinsus* was detected in any samples from this area and no DNA sequence analysis of either *P. olseni* or the ITS-variant from NSW was possible.

Given our inability to obtain samples of *Perkinsus* from NSW, requests were made to obtain the original DNA sequence data from the NSW blacklip abalone samples collected in 2005 for comparison. Unfortunately these data are not available.

Perkinsus was recently described from Anadara trapezia (mud arks) in Queensland (Dang *et al.* 2015). Only limited sequence of the *P. olseni* detected there was available through NCBI. Our alignments (Figure 45) only include the region of the AFDL *Pols* qPCR forward primer. However given the level of validation undertaken with other *Perkinsus* samples derived from multiple hosts and locations, we remain confident that our assay has specificity for *P. olseni.*

Development of AFDL *Pols* qPCR

The currently recommended OIE conventional PCRs for both *P. marinus* and *P. olseni* target the ITS region of the *Perkinsus* genome. PCR primers that target the internal transcribed spacer region (ITS) are recommended for PCR detection as more is known about inter- and intra-specific sequence variation of the ITS region than other possible diagnostic regions such as the non-transcribed spacer (NTS) region of the *Perkinsus sp.* rRNA gene complex (OIE, 2015a). Like the 16S rRNA gene commonly used to identify prokaryotes, this area has sufficient DNA sequence variation to allow differentiation of species while at the same time is sufficiently conserved within species to provide reliable identification.

Multiple sequence alignments were generated and used as the basis for design and then further sequencing of positive field samples was undertaken to determine if there were underlying regions of variability which might undermine the sensitivity and specificity of the real-time assay. DNA sequence data for *Perkinsus olseni* field samples from various sites around the Port Lincoln area (12-00978, 12-00979, 14-00233) and Baird Bay in South Australia (13-01031) were consistent with GenBank data on *P. olseni* from other species and geographic areas. A consensus DNA sequence of the ITS region from select samples representing all South Australian sites at which *Perkinsus* was found was constructed (Appendix 9) and aligned with both the GenBank derived consensus and the primer and probe sequences to further confirm the selection of primer and probe binding sites shown in Figure 17. Within each non-target alignment the primers and probe contain at least 3 centrally placed DNA sequence mismatches when compared to non-target species. These differences confer specificity to the AFDL *Pols* qPCR assay with respect to the other *Perkinsus* species and guided the selection of primers and probe on which the system was based.

An MGB probe was favoured for the AFDL *Pols* qPCR as these probes form an extremely stable duplex with single-stranded DNA target. The higher T_m of the duplex allows the design of shorter probes in hybridization based assays (Kutyavin *et al.* 2000). This was a particular issue when it came to locating a probe in the limited space available in the DNA alignment of *Perkinsus* ITS. While the Primer 3[®] software provided primers and probe that would theoretically work in the Taqman[®] system, the selection of these was empirically determined.

High C_T values in the AFDL Pols qPCR

In the course of testing field samples from known infected populations, we found C_T values between 40 and 45 in the AFDL *Pols* qPCR were not uncommon. Typically 1 or 2 replicates gave a high C_T value with a limited amplification curve possibly indicating specific amplification, with the remaining replicates "undetermined"

after analysis. The late amplification observed is not confined to this *P. olseni* assay and is observed in other real-time assays where a cut-off value might be applied.

These results are problematic as the C_T values may fall outside the limit of detection (LOD) suggested by extrapolation of the quantified plasmid titration and often appear inconsistently on repeat testing. Given the consistency observed with the screening of *P. olseni* plasmid and gDNA diluted in pooled abalone DNA, the reasons for late amplification are not readily explained. We assume it is the result of late amplification of low levels of DNA template in a high background of host DNA or other constituents co-purified in the samples. While our experience, based on the replicate testing and analysis of hundreds of known negative abalone samples, has been that our probe-based system is unambiguously negative after 45 cycles of amplification, the possible heteroscedasticity associated with C_T values (Caraguel *et al.*, 2011) may obscure the true nature of these samples. Bustin *et al*. (2009) observed (citing Burns and Valdivia, 2008) "Cq values >40 are suspect because of the implied low efficiency and generally should not be reported; however, the use of such arbitrary Cq cut-offs is not ideal, because they may be either too low (eliminating valid results) or too high (increasing false-positive results)". This issue is currently under review by SCAAH who may provide some perspective on this.

Within the context of this study, in order to better understand and determine the significance of high C_T results in the system, we wanted to establish if these results were indeed true positives derived from *Perkinsus* DNA or were merely a result of noise in the system derived from non-specific interactions with the Taqman chemistry. The late amplification seen in these samples precluded any chance of reliably assessing other indicators of specific amplification such as a characteristic amplification curve.

If the high C_T result could be independently confirmed by sequencing, it is reasonable to accept that the observed C_T was derived from amplification of specific target template in the system. In this case the OIE conventional primers were used in a large volume reaction with 70 cycles of amplification using the same temperatures recommended for the diagnostic PCR. Of the 9 samples subjected to amplification, 7 samples produced DNA sequence of sufficient quality for BLAST (Altschul *et al.*, 1997) analysis. This analysis confirmed the presence of *Perkinsus* and that the high C_T values obtained in the AFDL *Pols* qPCR system were derived from *P. olseni* DNA in the sample.

On this basis we could not reliably define the absolute limits of analytical sensitivity. Given the large number (n = 200) of known negative abalone samples screened (repeat testing in triplicate), and further samples from populations with unknown disease status which were subsequently shown to be uninfected were unambiguously negative in the test with the absence of any background signal, we regard any positive value as a significant result indicating a presumptive positive result for *P. olseni*, which depending on the purpose of the assay might require further sampling and testing for confirmation. On this basis a cut-off value for the system was not defined.

Along with field samples from wild populations collected over the course of the project we used a large library of 200 negative samples and some fixed reference tissues to validate the system. Final ROC analysis was performed using 56 positive and 321 negative samples for *P. olseni* using the OIE 140/600 cPCR as the reference test. The area under the curve was 0.957 with a significance level of <0.0001, indicating that the AFDL *Pols* qPCR is able to distinguish between the infected and non-infected groups. Bayesian analysis was then used to estimate the relative DSe (94.6) and DSp (92.8) at a preliminary cut-off of ≤ C_T 41.29.

Repeatability was assessed using 37 field samples of different analyte (DNA target) concentrations in three different runs using a C_T cut-off of <39. Cochran Q statistics concluded that there was no significant difference between runs indicating satisfactory repeatability on random field samples after 3 runs at the 95% CI (P value = 0.066).

Sampling Abalone for PCR

As opposed to most bivalves, blacklip abalone are large animals. This in itself possess a challenge to the application of PCR. Where small samples taken from bivalves may be representative, abalone require close inspection and considered selection of tissues for PCR detection. The current OIE Manual of Diagnostic

Tests for Aquatic Animals (OIE, 2015) recommends gill or mantle tissue for PCR in both bivalves and abalone. Our data show that the DSe obtained with the AFDL *Pols* qPCR with gill and muscle (sampled from around the epipodial fringe) are similar, 0.88 and 0.92, respectively. The slightly higher sensitivity in muscle may be due in part to the natural bias of the operator towards sampling visible lesions or areas of tissue with abnormal appearance which may be indicative of infection. Should the highest level of DSe be required the parallel testing of both muscle and gill is recommended. It is important to note that these estimates of DSe of the AFDL *Pols* qPCR with gills and muscle are based on the close examination of animals, including examination of serial slices at 2 to 3 mm intervals through the whole body after the visceral mass is removed. In the absence of lesions we found that multiple samples of gill and muscle increased the rate of detection.

Comparison of the AFDL *Pols* qPCR and the OIE 140/600 cPCR for *P. olseni*

Of the 159 animals examined from *Perkinsus*-infected populations, the OIE species-specific conventional PCR found 33% were PCR-positive and the AFDL *Pols* qPCR found 50% of the animals were PCR positive.

Bayesian analysis was used to compare the performance of the new AFDL *Pols* qPCR assay with the current OIE-recommended 140/600 cPCR for *P. olseni.* The DSe and DSp of the AFDL *Pols* qPCR was dependant on the cut-off used. Given the unresolved issues surrounding the acceptable end-point for qPCR. We calculated the DSe and DSp of each assay using 2 cut-off values. Bayesian analysis was performed using the cut-off generated through the ROC analysis (**C_T 41.29**) and an arbitrary lower cut-off (**C_T 39.00**) was also used to allow analysis for those laboratories that cannot accept the validity of high C_T values ($\geq$ 40).

AFDL Pols qPCR with cut-off $C_T \leq 41.29$

When a cut-off $C_T < 39.00$ is applied, the AFDL *Pols* qPCR had DSe and DSp of 93.6% (95% CI 85.6-98.8) and 99.5% (95% CI 98-100), respectively, compared with the 140/600 cPCR (OIE) which had DSe and DSp of 69.4% (95% CI 58.6-79.4) and 99.6% (95% CI 98.4-100), respectively.

AFDL Pols qPCR with cut-off $C_T < 39.00$

Similarly, when a cut-off $C_T < 39.00$ is applied, the AFDL *Pols* qPCR had a DSe and DSp of 90.2% (95% CI 80.9-96.9) and 99.5% (95% CI 98.4-100) compared with the 140/600 cPCR (OIE) which had DSe and DSp of 78.1% (95% CI 67.1-87.5) and 99.5% (95%CI 98.3-100), respectively.

It is concluded that, no matter which C_T cut-off value is used, the AFDL *Pols* qPCR provides a highly sensitive and specific test for the detection and identification of *P. olseni*.

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Implications

The molecular assay developed in this project provides rapid detection and identification of *Perkinsus olseni* and has application for (1) testing individuals from disease outbreaks and (2) high-throughput surveillance testing of overtly healthy abalone populations for certification and management of stocks.

The development of a highly sensitive and highly specific diagnostic test has the potential to provide state government authorities, their diagnostic laboratories, and their fisheries managers and regulators with improved diagnostic capability not only for the diagnosis of Perkinsosis (specifically, rapid identification of the causative agent) but also for the detection and identification of sub-clinical infections. The provision of a validated diagnostic test with estimates of diagnostic specificity and sensitivity allows the design of surveillance programs and sampling protocols to be based on reliable scientific data. The test can be implemented in the recently developed abalone farm accreditation program.

In addition to providing a validated qPCR test for *P. olseni*, this study has provided performance characteristics of other tests commonly used for diagnosis of *Perkinsus*. This comprehensive study has demonstrated the strengths and weaknesses of each of the tests and provides a better understanding of the issues with detection of sub-clinical infections in individual animals versus populations.

Recommendations

It is recommended that details of this test be provided to diagnostic laboratories for immediate implementation in state jurisdictions that have a need for *Perkinsus* diagnostics. Moreover, the method should be published in a peer-reviewed scientific journal with subsequent inclusion in the OIE Manual of Diagnostic Tests for Aquatic Animals. Copies of this project report will be provided to alert industry of the further options now available for the screening and subsequent management of stock.

Further development

As discussed above, through this study, a highly specific and sensitive test has been developed for the specific detection and identification of *Perkinsus olseni*. It will be through its use in other diagnostic laboratories that its utility will be tried and tested. In addition to implementation, part of the validation process includes "reproducibility", i.e., "*the ability of a test method to provide consistent results, as determined by estimates of precision, when applied to aliquots of the same samples tested in different laboratories, preferably located in distinct or different regions or countries using the identical assay (protocol, reagents and controls)*" (OIE 2015 Manual of Diagnostic Tests for Aquatic Animals, Chapter 1.1.2, http://www.oie.int/index.php?id=2439&L=0&htmfile=chapitre_validation_diagnostics_assays.htm). While desirable, determination of "reproducibility", as defined by the OIE, was beyond the scope of this project. Nevertheless, if it should be considered a priority, this further level of validation could be addressed. In this way, the test would become fully validated, according to OIE recommendations, and would then only require "monitoring and maintenance of validation status" (OIE 2015 Manual of Diagnostic Tests for Aquatic Animals, Chapter 1.1.2, http://www.oie.int/index.php?id=2439&L=0&htmfile=chapitre_validation_diagnostics_assays.htm).

Extension and Adoption

Development of the real-time PCR assay was communicated to the wider scientific community internationally and domestically through a series of three conference presentations (below).

David M. Cummins, Brian J. Jones, Mark St. J. Crane and Nicholas Gudkovs (2014) Development of a Taqman® real-time PCR assay for the detection of *Perkinsus olseni* in Australian abalone. 7th International Symposium on Aquatic Animal Heath, Portland, Oregon USA. 31st August - 5th September 2014

David M. Cummins, Brian J. Jones, Mark St. J. Crane and Nicholas Gudkovs (2014) Development of a Taqman® real-time PCR assay for the detection of *Perkinsus olseni* in Australian abalone. 10th Annual Meeting of the Australian Association of Veterinary Laboratory Diagnosticians, Adelaide, South Australia. 27th - 28th November

David M. Cummins, Nicholas Moody, Nicholas Gudkovs, Brian Jones, Mike Snow, Mark Crane (2015) A tale of two projects: The development of a Taqman® PCR assay for the detection of *Perkinsus olseni* & Positive control strategies to reduce the reliance on genomic material. 3rd Australasian Scientific Conference on Aquatic Animal Health, Cairns, Queensland Australia. 6th - 11th July.

The real-time assay developed as the primary focus of this project has been adopted for diagnostic screening by the CSIRO Australian Animal Health Laboratory.

The clonal culture of *Perkinsus olseni* 12:00978-12T from blacklip abalone at Taylor Island, near Pt Lincoln, South Australia, established during the course of the project, was accepted by the American Type Culture Collection (ATCC) as the type culture for the species. Within Australia, cultures of this isolate may be available from the CSIRO Australian Animal Health Laboratory, Geelong. This isolate will be more widely available to the scientific community through the ATCC.

Appendices

Appendix 1 - List of researchers and project staff

CSIRO Australian Animal Health Laboratory

Nicholas Gudkovs
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Appendix 2 - *Perkinsus* Reference and Control Samples

Type = C, ethanol-fixed cells propagated in tissue culture;   T, fixed tissues infected with *Perkinsus*.

Species	Isolate	Type	Source
P. olseni	NZMSAs-5	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. olseni	Mie-5mg	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. atlanticus (=*P. olseni*)	PaG3F	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. olseni	NZMSAs-24	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. olseni	Mie-13v	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. olseni	Mie-4g	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. olseni	NZMSAs-21	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. olseni	Mie-5mg	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. marinus	PXBICv-22	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. marinus	EBPICv-15	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. marinus	LA-25	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. marinus	HFtP-14	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. marinus	PXBICv-25	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. marinus	CRTW-3he	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. marinus	DBNJ-1	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. marinus	EBPICv-18	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. marinus	LICT-1	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. marinus	PRPC/DI 9403	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. marinus	CRTW-3he	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. marinus	A-1	T	Haskin Shellfish Research Laboratory, NJ, USA
P. andrewsi (=*P. chesapeaki*)	PAND-A8-4a	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. chesapeaki	PXSATp-6	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. chesapeaki	EBNPMb-1	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. chesapeaki	CRMA-J55	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. chesapeaki	PXDCRc-8	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. chesapeaki	CRTP-17	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. chesapeaki	YRKCMb-1	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. chesapeaki	PXDCRc-5	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. chesapeaki	EBNPMl-4	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. chesapeaki	CHBRMa-14	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. chesapeaki	EBNPMl-5	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. chesapeaki	CHBRMa-14	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. honshuensis[*]	Mie-3g	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. honshuensis	Mie-3g	C	Oxford Laboratory, Maryland DNR, Maryland, USA
P. beihaiensis	3870-5	T	OIE Reference Centre, VIMS, Virginia, USA
P. beihaiensis	3870-10	T	OIE Reference Centre, VIMS, Virginia, USA
P. beihaiensis	3871-1	T	OIE Reference Centre, VIMS, Virginia, USA
P. beihaiensis	3871-2	T	OIE Reference Centre, VIMS, Virginia, USA
P. beihaiensis	3871-3	T	OIE Reference Centre, VIMS, Virginia, USA
P. beihaiensis	3871-5	T	OIE Reference Centre, VIMS, Virginia, USA
P. beihaiensis	3871-6	T	OIE Reference Centre, VIMS, Virginia, USA
P. beihaiensis	3872-1	T	OIE Reference Centre, VIMS, Virginia, USA
P. beihaiensis	3872-2	T	OIE Reference Centre, VIMS, Virginia, USA
P. beihaiensis	3872-3	T	OIE Reference Centre, VIMS, Virginia, USA
P. beihaiensis	3872-7	T	OIE Reference Centre, VIMS, Virginia, USA
P. beihaiensis	3872-8	T	OIE Reference Centre, VIMS, Virginia, USA
P. beihaiensis	3873-4	T	OIE Reference Centre, VIMS, Virginia, USA
P. beihaiensis	3873-7	T	OIE Reference Centre, VIMS, Virginia, USA
P. beihaiensis	3875-2	T	OIE Reference Centre, VIMS, Virginia, USA
P. beihaiensis	3875-5	T	OIE Reference Centre, VIMS, Virginia, USA

[*]ATCC culture contaminated with *P. olseni* at source

Appendix 3 – PCR Methods

Conventional PCR (cPCR)

All conventional PCR amplification (cPCR) was carried out in a standard volume of 25 μl where DNA template was added as a 2 μl volume to 23 μl of mastermix. Thermal cycling was carried out in 0.2 ml Eppendorf PCR tubes (8-TUBE STRIP PCR CLEAN THIN WALL PK/120 STRIPS Order No. 0030 124.359).

Mastermix was based on HotStarTaq Master Mix (Qiagen, Cat. No. 203445). All primers for cPCR were synthesised by Geneworks. PCR primers were stored frozen at a working stock concentration of 18μM.

Thermal cycling was carried out in the Eppendorf MasterCycler *EP* using fast ramp rates for all reactions.

Gel electrophoresis was performed in 1.5% (w/v) TAE (Promega) gels at 90V according to standard methods. PCR bands were visualised using SYBR Safe DNA Gel Stain (Invitrogen, Cat. No. 33102) and a Safe Imager™ 2.0 Blue Light Transilluminator with a 100bp DNA Ladder (Promega Cat. No. G2101 and Invitrogen Cat. No. 15628) as standard.

Real-Time (qPCR)

All real-time PCR (qPCR) testing was undertaken in a standard volume of 25 μl in Fast Optical 96-well Reaction plates (Applied Biosystems Cat. No. 4346906), sealed with MicroAmp Optical Adhesive film (Applied Biosystems Cat. No. 4311971). DNA template was added as a 2 μl volume.

Master Mix was based on Taqman® Universal PCR Master Mix (Applied Biosystems Cat. No. 4304437)

All primers and probes used for qPCR were synthesised and HPLC purified by Life Technologies. PCR primers were stored frozen at a working stock concentration of 18μM, yielding a final concentration of 0.9μM (22.5 pmol per reaction). The MGB Taqman® probe was prepared as a 5μM working stock, yielding a final concentration of 0.25μM (6.25 pmol per reaction).

Real-time amplification was performed using Applied Biosystems® 7500 Real-Time PCR Systems.

Plasmid Preparation and Use

Plasmid controls were developed for P. *olseni* (NZMSAs-5, ATCC PRA-204), P. *marinus* (LA-25, ATCC 50775), P. honshuensis (Mie-3g, ATCC PRA-176) and P. chesapeaki (CRTP-17, ATCC 50865) using a pPCR2.1-TOPO vector with methods supplied by the manufacturer (TOPO® TA Cloning® Kit with PCR®2.1 TOPO® Invitrogen Cat: K4500-01). Briefly, genomic DNA for each of the *Perkinsus* isolates were PCR amplified using the OIE 85/750 genus-specific primers. Amplicons were gel purified (QIAgen gel purification kit) and cloned into the pCR®II-TOPO TA vector (Invitrogen). Purified plasmids were isolated and sequenced confirmed.

To establish analytical sensitivity (ASe) plasmid stock solutions were quantified using the Qubit® 2.0 Fluorometer (Invitrogen Cat. No. Q32866), with subsequent plasmid dilutions prepared using 50 ng/μl of yeast tRNA carrier (Invitrogen Cat. No. 15401-029) in 10 mM TE buffer diluent. The *P. olseni* control plasmid was also serially diluted as above in abalone host gDNA at 50 ng/μl.

PCR Primer and Probe Sequences

Primer/Probe name	Sequence (5'-3')
OIE 85 F	CCGCTTTGTTTGGATCCC
OIE 750 R	ACATCAGGCCTTCTAATGATG
OIE 140 F	GACCGCCTTAACGGGCCGTGTT
OIE 600 R	GGRCTTGCGAGCATCCAAAG
AFDL *Pols* F	TCTCGTATTGTAGCCCCTCCG
AFDL *Pols* R	AAGGAACACGTGATATCACCACAAC
AFDL *Pols* probe	FAM-ATGCTCGCAAGTCCGACT-MGB
T-perk F	TCCGTGAACCAGTAGAAATCTCAAC
T-perk R	GGAAGAAGAGCGACACTGATATGTA
T-perk probe	FAM-GCATACTGCACAAAGGG-MGB
18S F	CGGCTACCACATCCAAGGAA
18S R	GCTGGAATTACCGCGGCT
18S probe	VIC-TGCTGGCACCAGACTTGCCCTC-TAMRA

OIE 140/600 Conventional PCR for *Perkinsus olseni* (*P. olseni* ITS rRNA PCR)

Master Mix

Perkinsus olseni ITS rRNA gene PCR – 450 bp amplicon	
Reagent	**Volume for 1 rxn**
Water	9.5 µl
HotStarTaq Master Mix	12.5 µl
Primer name: **PolsITS 140F (18µM)**	0.5 µl
Primer Name: **PolsITS 600R (18µM)**	0.5 µl
Total volume	**23 µl**

Thermal Cycling Conditions

Cycles	Conditions
1	95°C for 5 minutes
40	95°C for 60 seconds 64°C for 60 seconds 72°C for 2 min
1	72°C for 5 min
Hold	4°C

Primer sequences:

Primer	Sequence
PolsITS-140	5'-GAC-CGC-CTT-AAC-GGG-CCG-TGT-T-3'
PolsITS-600	5'-GGR-CTT-GCG-AGC-ATC-CAA-AG-3'

References

OIE, 2014 Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.4.7 Infection with *Perkinsus olseni*. [http://www.oie.int/index.php?id=2439&L=0&htmfile=chapitre_*Perkinsus_olseni*.htm]

Audemard C, Reece KS, Burreson EM. 2004. Real-time PCR for the detection and quantification of the protistan parasite *Perkinsus marinus* in environmental waters. *Appl. Environ. Microbiol*. 70: 6611–6618.

OIE 85/750 Conventional PCR for *Perkinsus sp.* (*Perkinsus sp.* ITS rRNA PCR)

Master Mix

Perkinsus sp. ITS rRNA gene PCR – 703 bp amplicon	
Reagent	Volume for 1 rxn
Water	9.5 μl
HotStarTaq Master Mix	12.5 μl
Primer name: **PerkITS 85F (18μM)**	0.5 μl
Primer Name: **PerkITS 750R (18μM)**	0.5 μl
Total volume	**23 μl**

Thermal Cycling Conditions

Cycles	Conditions
1	95°C for 5 minutes
40	95°C for 30 seconds 58°C for 30 seconds 72°C for 60 seconds
1	72°C for 5 minutes
Hold	4°C

Primer sequences:

Primer	Sequence
PerkITS-85	5'- CCG CTT TGT TTG GAT CCC -3'
PerkITS-750	5'- ACA TCA GGC CTT CTA ATG ATG -3'

References

OIE Manual of Diagnostic Tests for Aquatic Animals, 2014. Chapter 2.4.7 Infection with *Perkinsus olseni*. [http://www.oie.int/index.php?id=2439&L=0&htmfile=chapitre_*Perkinsus_olseni*.htm]

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Primers and Cycling Conditions used for Sequencing

Perkinsus genus-specific ITS ribosomal RNA complex primers

PerkITS-85: CCG CTT TGT TTR GMT CCC
PerkITS-750: ACA TCA GGC CTT CTA ATG ATG

Primers = PerkITS-85 **and** PerkITS-750 **(~702 bp)**		
Profile:	**Temp**	**Time**
Initial denaturation	95	10 min
# Cycles		40
Denaturation	95	30 sec
Annealing	55	30 sec
Extension	65	2 min
Final Extension	65	5 min

Large subunit rRNA gene amplification

PerkITS2-217: GTG TTC CTY GAT CAC GCG ATT
LSU-B: ACG AAC GAT TTG CAC GTC AG

Primers = **PerkITS2-217/ LSU-B (1,170 bp fragment)**		
Profile:	**Temp**	**Time**
Initial denaturation	94	4 min
# Cycles		35
Denaturation	94	30 sec
Annealing	58	1 min
Extension	65	2 min
Final Extension	65	5 min

Type 1 actin gene amplification

PerkActin1-130F: ATG TAT GTC CAG ATY CAG GC
PerkActin1-439R: CTC GTA CGT TTT CTC CTT CTC

Primers = **PerkActin1-130F / PerkActin1-439R (330 bp)**		
Profile:	**Temp**	**Time**
Initial denaturation	94	5 min
# Cycles		40
Denaturation	94	1 min
Annealing	58.5	45 sec
Extension	68	1 min
Final Extension	68	5 min

AFDL *Perkinsus olseni* Taqman® Assay (AFDL Pols qPCR)

Perkinsus olseni specific Taqman® assay based on a MGB probe targeting ITS rRNA.

Master Mix

AFDL *Perkinsus olseni* qPCR	
Reagent	**Volumes for 1 rxn**
Water	6.75 µl
Taqman® Universal PCR Master Mix	12.5 µl
Forward Primer: *Pols* **F3** (18µM)	1.25 µl
Reverse Primer: *Pols* **R1** (18µM)	1.25 µl
Taqman® probe: *P. olseni* **probe** (5µM)	1.25 µl
DNA template (sample)	2 µl
Total volume	**25 µl**

Thermal Cycling Conditions

Cycles	Conditions
1	50°C for 2 minutes
1	95°C for 10 minutes
45	95°C for 15 seconds, 60°C for 60 seconds
Hold	4°C

Primer/Probe Sequences

Primer	Sequence
Pols-F3	5'-TCTCGTATTGTAGCCCCTCCG -3'
Pols-R1	5'-AAGGAACACGTGATATCACCACAAC -3'
Probe	
P. olseni probe	5'- **6FAM**-ATGCTCGCAAGTCCGACT-**MGB** -3'

All real-time PCR testing was carried out in a standard volume of 25 µl in Fast Optical 96-well Reaction plates.

Master Mix was based on Taqman® Universal PCR Master Mix (Applied Biosystems Cat. No. 4304437 – Life Technologies)

All primers and probes used in the project were synthesised by Life Technologies.
PCR primers were prepared at a working concentration of 18µM stock, yielding a final concentration of 0.9µM (22.5 pmol per reaction).

The MGB Taqman® probe was prepared as a 5µM working stock, yielding a final concentration of 0.25µM (6.25 pmol per reaction).

Gauthier *Perkinsus sp.* Taqman® Assay (Gauthier *genus-specific* qPCR)

Master Mix

Gauthier *Perkinsus sp.* qPCR (86 bp)	
Reagent	**Volume for 1 rxn**
Water	6.75 µl
Taqman® Universal PCR Master Mix	12.5 µl
Primer name: **T-PERK-f** (18 µM)	1.25 µl
Primer Name: **T-PERK-r** (18 µM)	1.25 µl
Taqman® probe: **T-PERK-probe** (5 µM)	1.25 µl
Total volume	**23 µl**

Thermal Cycling Conditions

Cycles	Conditions
1	50°C for 2 minutes
1	95°C for 10 minutes
45	95°C for 15 seconds, 60°C for 60 seconds
Hold	4°C

Primer/Probe Sequences

Primer	Sequence
T-PERK-f	5'- TCCGTGAACCAGTAGAAATCTCAAC -3'
T-PERK-r	5'- GGAAGAAGAGCGACACTGATATGTA -3'
Probe	
T-PERK-probe	5'- **6FAM** GCATACTGCACAAAGGG **MGBNFQ** -3'

Reference

Gauthier JD, Miller CR, Wilbure AE. 2006. Taqman® MBG real-time PCR approach to quantification of *Perkinsus marinus* and *Perkinsus spp.* in oysters. *J Shellfish Res* **25(2):** 619-624.

18S Ribosomal RNA FAST qPCR

Master Mix

18S rRNA FAST qPCR	
Reagent	**Volume for 1 rxn**
Water	6.75 µl
Taqman® Fast Universal PCR Master Mix	12.5 µl
Primer name: **18S Forward** (20µM)	1.25 µl
Primer Name: **18S Reverse** (20µM)	1.25 µl
Taqman® probe: **18S VIC probe** (5µM)	1.25 µl
Total volume	**23 µl**

Thermal Cycling Conditions

Cycles	Conditions
1	95°C for 20 seconds minutes
45	95°C for 3 seconds, 60°C for 60 seconds
Hold	4°C

Primer/Probe Sequences

Primer	Sequence
18S Forward	5'-CGG CTA CCA CAT CCA AGG AA-3'
18S Reverse	5'-GCT GGA ATT ACC GCG GCT -3'
Probe	
18S VIC probe	5'-**VIC** TGC TGG CAC CAG ACT TGC CCT C **TAMRA**-3'

Reference

TaqMan® Ribosomal RNA Control Reagents (Applied Biosystems Cat # 4308329)

Appendix 4 - Dissection Guide for Abalone

Mollusc tissues degenerate quickly. Dissected tissues for molecular analysis or histology should be taken quickly and placed directly into fixative. Dense tissues such as the foot muscle, should be thinly sliced (no more than 2mm) to allow proper fixation.

Remove the body mass from the shell by carefully sliding the mantle and epipodial fringe aside and cutting through the adductor muscle close to the contour of the shell. Place body ventral surface down with the anterior end uppermost (normal orientation).

Cut 1: Longitudinal section of head and mouth.
Cut a transverse section across the whole body between 1 and 3 cm (depending on the size of the animal) posterior to the level of the mouth. With a longitudinal cut at the mid-line, divide the small anterior section into 2 equal pieces. Cut a 2mm slice from the exposed longitudinal surface for histology. This tissue can be trimmed to fit an embedding cassette, but should include the anterior tissues (buccal cavity, radula and surrounding odontophore muscle).

Cut 2: Transverse section of gills and mantle.
The gills are visible on the left side of the body. About half way down the gills, make a transverse cut about 1cm through the gills and adjoining tissue. Cut a 2mm slice from the exposed surface, taking a section of gills and mantle.

Cut 3: Transverse section of digestive gland and stomach.
About one third down from the TS of gill, make a transverse cut through the digestive gland and stomach (this may include some gonad). Again, cut a 1cm X 2mm slice from the exposed tissue surface, taking a section of digestive gland and surrounding tissue.

Cut 4: Section of muscle.
Take a 2mm slice of muscle from the lateral margin of the foot. In order to inspect the foot, transverse cuts should be made through the entire foot muscle across the dorsal surface to expose encysted parasites or gross lesions in the muscle. The tissues from Cuts 1 and 2 may be arranged in one cassette, with the remaining tissues from Cuts 3 and 4 in a second. Additional cassettes should be used for other gross lesions.
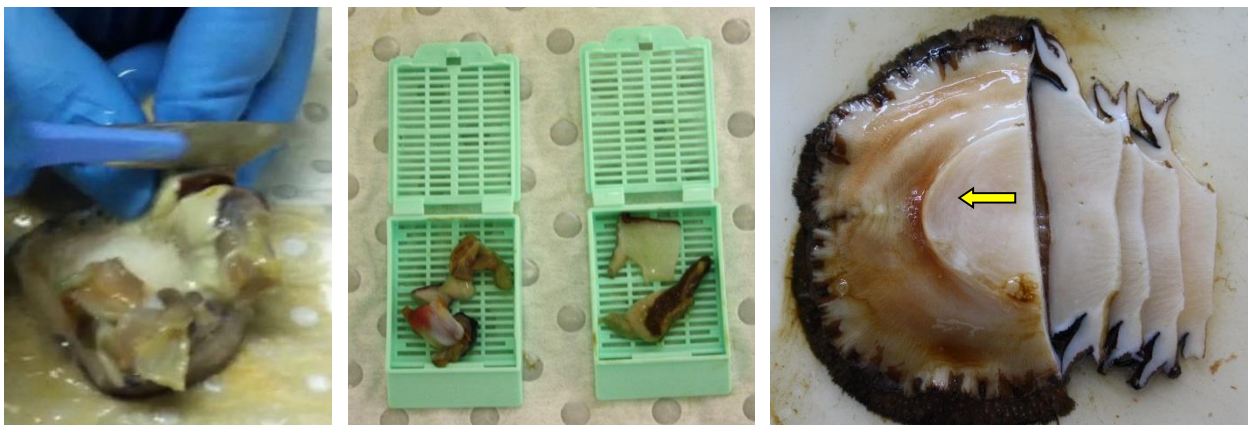
Gross lesions
Gross lesions or unusual structures should be excised, sliced thinly and fixed whole or cut into an embedding cassette.

Davidson's fixative (Shaw and Battle 1957)

Use

Recommended fixative for general crustacean and mollusc pathology. Fix tissues for 24-48 h, then process OR transfer to 50% ethanol followed by 70% ethanol for long term storage.

Can omit acetic acid if tissues are to be used for nucleic acid analysis.

Preparation

Davidson's stock is made by adding components in the order shown below, mix well after each addition. Add glacial acetic acid just before use, either use immediately or store at 4°C.

STOCK

Glycerine   400 ml
Formaldehyde (37-40% v/v) 800 ml
95% v/v ethanol   1200 ml
Sea water (filtered or artificial) 1200 ml
Total: 3600 ml

FINAL SOLUTION

Davidson's stock (above): 9 parts
Glacial acetic acid: 1 part

Notes:

Fixation time: Optimum fixation 24-48 h.
For cytological preps (use 1% acetic acid), 5-15 min.; refrigerate (4°C); pH acid
Thickness: optimum 4-5 mm
Processing: change to 50% ethanol for 2 h minimum, change to 70% ethanol 2 h minimum.
Long term storage: 70% ethanol or Davidson's stock without acetic acid.
Acetic acid aids in disintegration of small shells
Use acetic acid at 1% for cytological preparations.

References

Howard DW, Lewis EJ, Keller BJ, Smith CS. 2004. Histological Techniques for Marine Bivalve Mollusks and Crustaceans. Chapter 5 Fixation. NOAA Technical Memorandum NOS NCCOS 5, August 2004.

Shaw BL, Battle HI. 1957. The gross and microscopic anatomy of the digestive tract of the oyster *Crassostrea virginica* (Gmelin). Can. J. Zool. 35:325-347.

Appendix 5 – Gross appearance of abalone and *Perkinsus* lesions.

Figure 37 Normal appearance of uninfected blacklip ablaone (H. *rubra*) removed from shell for dissection.

Figure 38 Nornal abalone seen in sequential TS of the pedal muscle at 2-3 mm slices for examination for *Perkinsus* lesions. The 3 neural ganglia can be seen at the midline.
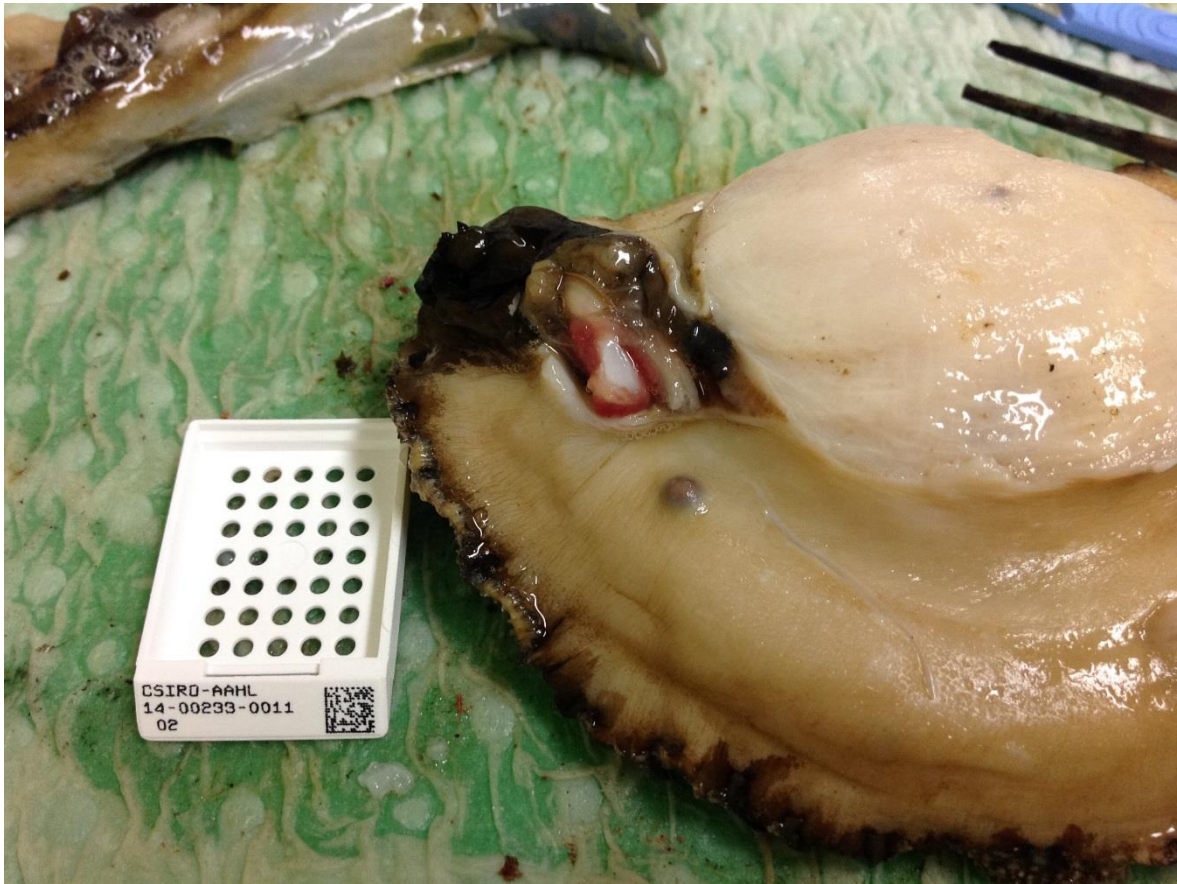
Figure 39 Prominant *Perkinsus* lesion on dorsal surface of muscle in animal 14:0233-11.

Figure 40 Large active *Perkinsus* lesion smaller dark lesions dispersed throughout the musculature. In this case, although lesions are widespread internally, no lesions were observed during external examination.

Figure 41 Lesions commonly appear scattered over the surface of the pedal muscle in infected aniamls. Encysted nematodes may produce a similar lesion. These lesions are not pathognomic for *Perkinsus*, and further laboratory examination is required for reliable diagnosis of smaller lesions.
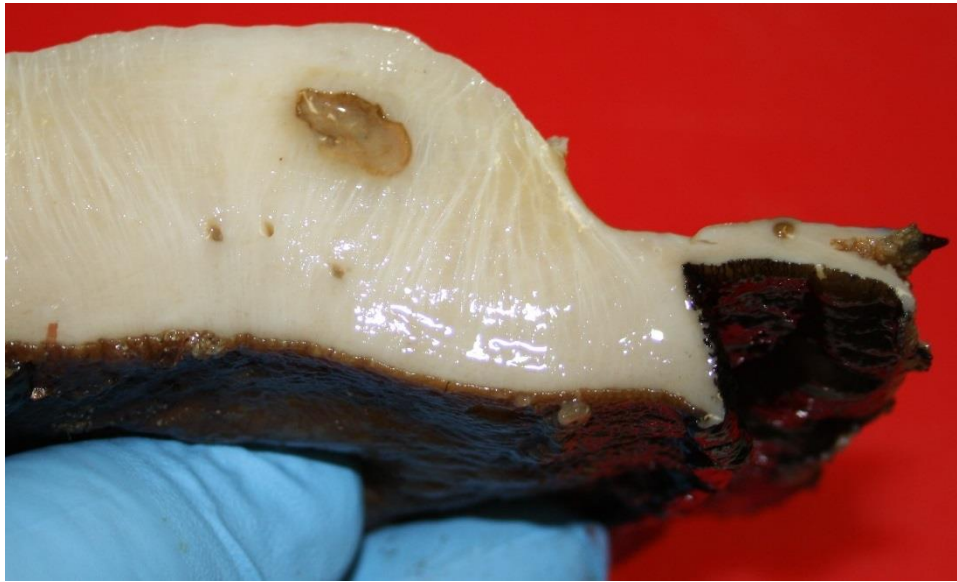
(a)

(b)
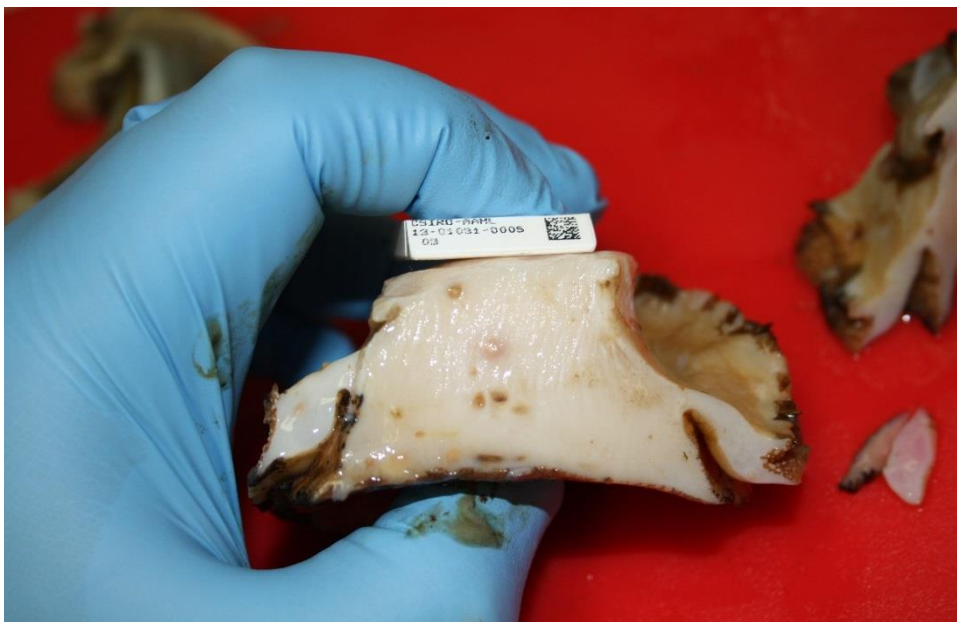
(c)

Figure 42 (a)-(c) TS through pedal muscle of various blacklip abalone (Haliotis *rubra*) infected with *P. olseni*. A vareity of lesions in severely affected harvest size animals are shown. Presumably some of these infections are chronic and persist from years to year.

Appendix 6 - Ray's Fluid Thioglycollate Medium (ARFTM)

Source: Dungan, June 1 2010 Maryland DNR, Cooperative Oxford Laboratory

Sigma A0465 was unavailable at the time of this project. Although the formulations of commercial thioglycollate may vary, a comparison of the thioglycollate media from Sigma A0465 and Oxoid CM0391 showed they were very similar and the Oxoid medium was substituted.

Alternative Ray's fluid thioglycollate medium (ARFTM)
Chris Dungan, June 1, 2010.

Alternative thioglycollate medium (Sigma A0465) 29 g
Sea salts (Sigma S9883)   16-20 g *
Distilled water   989 ml

Combine, and heat with stirring to 95-100 °C to dissolve.
Aliquot to bottles, cap loosely, and autoclave for 15-20 min. at 121 °C.
Cool to ≤ 80 °C.
Supplement with sterile components (below), cap tightly, and store at 4 °C.

Lipid mixture (Sigma L5146)   5ml (209 µg/ml)
Chloramphenicol **(mutagen)** (Sigma C0378) 1ml (50µg/ml)
Nystatin ** (Sigma N1638)   5 ml (50U/ml)
TOTAL Volume: 1000 ml

* A final ARFTM osmolality equivalent to 26 ppt seawater is achieved with 16 g/litre of artificial sea salts. For a 30 ppt salinity-equivalent in the resulting ARFTM, use 20 g/litre of artificial sea salts.

** Antimicrobials are optional, and are only for use in assays of mollusc tissues for *Perkinsus sp.* pathogens. Other antimicrobials can also be substituted (Dungan & Hamilton. 1995. *J. Eukaryot. Microbiol.* 42:379-388).

For chloramphenicol, prepare a 50 mg/ml stock solution in 100% ethanol (5 g/100 ml), store at –20 °C, and handle with care as specified above.

For stimulation of *in vitro* zoosporulation by axenic *Perkinsus sp.* cultures, omit antimicrobials, or use low-hazard 100 U/ml penicillin/100 µg/ml streptomycin.

Appendix 7 - Media for *In vitro* Culture

DMEM/Ham's F12-3% *Perkinsus* Growth Medium

Based on Dungan and Hamilton. 1995. J. Eukaryot. Microbiol. **42**:379-388, modified with NaCl from Burreson *et al.*. 2005. *J. Eukaryot. Microbiol.* **52**:258-270.

DMEM/Ham's F12 (3% FBS) growth medium, 29 ppt (850 mOsm kg^{-1})

	500 ml	1000 ml
DMEM powder (Sigma D5030)	2.1 g	4.2 g
Ham's F12 nutrient mix (Sigma N6760)	2.7 g	5.4 g
200 mM L-glutamine [2 mM final, total]	2.5 ml	5.0 ml
1.0M HEPES [25 mM final]	12.5 ml	25.0 ml
7.5% NaHCO$_3$ [7 mM final]	3.2 ml	6.4 ml
JLP carbohydrates[1]	5.0 ml	10.0 ml
Yeast extract ultra-filtrate (Sigma Y4375)	1.0 ml	2.0 ml
0.5% phenol red [0.00037% final]	0.24 ml	0.48 ml
15 ‰ SASW[2]	455.0 ml	910.0 ml
1.0 N HCl (for final pH 7.0)	≈1.5 ml	≈3.0 ml

Mix and dissolve, add HCl drop wise to adjust pH to 7.0, then filter sterilise at 0.2 µm into sterile bottles. *Just before use, the following sterile components are added aseptically at the required ratio.*

	500 ml	1000 ml
Foetal Bovine Serum (3% v/v final)	15.0 ml	30.0 ml
Lipid Concentrate (Sigma L5146, or equivalent)	0.5 ml	1.0 ml

[1] JLP carbohydrates: 5 g glucose, 1 g galactose, 1 g trehalose in 100 ml *TC grade water*. Filter 0.2 µm
[2] 15 ‰ SASW: 8.5 g sea salts (Sigma S9883) in 455 ml *TC grade water* (18 MΩ, or equivalent). Autoclave at 121°C for 30 min.

The 29 ppt medium with 850 mOsm kg-1 is the standard medium used for *in vitro* propagation of *Perkinsus sp.*. If required, NaCl can be added to mimic full strength seawater 35 ppt (1,100 mOsm kg^{-1}) – see below

DMEM/Ham's F12 (3% FBS) growth medium, 35 ppt (1,100 mOsm kg-1)

Add 1.7 ml of 350 ppt NaCl stock solution per 100 ml medium

350 ppt NaCl Stock solution (35 g NaCl in 100 ml tissue culture grade water)

- Store medium at 4°C. Re-supplement to 2mM L-glutamine every 60 days.
- Media containing 3% FBS has a shelf life of approximately 60 days.

Ordering information
Penicillin-Streptomycin = Sigma P4333
FBS = foetal bovine serum (Hyclone SH30070)
200 mM L-glutamine = Sigma G7513
1M HEPES = Sigma 80887
7.5% NaHCO$_3$ = Sigma S-8761
Chloramphenicol – C0378-5G
Amphotericin B – A2411-250MG
Cyclohexamide – C7698-1G
Nystatin – N6261-500KU

Antibiotic Supplements (antibiotics are optional)

Standard and maximum concentrations for *P. marinus*

	Standard conc. (Dungan)	Standard conc. (Burreson)	Maximum, *P. marinus*
Penicillin-Streptomycin	100 U/ml - 100 µg/ml	200 U/ml - 200 µg/ml	1000 U-µg/ml
Gentamicin	50 µg/ml	200 µg/ml	5,000 µg/ml
Chloramphenicol	5 µg/ml	50 µg/ml	50 µg/ml
Amphotericin B	0.25 µg/ml	Not used	0.25 µg/ml
Cyclohexamide	0.05 µg/ml	Not used	0.05 µg/ml
Nystatin	200 U/ml (~40 µg/ml)	50 U/ml (~10 µg/ml)	400 U/ml (~80 µg/ml)

Do not exceed recommended levels of amphotericin B and cyclohexamide

- Store medium at 4°C. Re-supplement to 2mM L-glutamine every 60 days.
- Media containing 3% FBS has a shelf life of approximately 60 days.

References

Burreson EM, Reece KS, Dungan CF. 2005. Molecular, morphological, and experimental evidence support the synonymy of *Perkinsus chesapeaki* and *Perkinsus andrewsi*. *Journal of Eukaryotic Microbiology* 52:258-270.
To selectively inhibit bacterial and fungal growth the parasite enlargement medium was supplemented with penicillin (200 U/ml), streptomycin (200 µg/ml), gentamicin (200 µg/ml), chloramphenicol (50 µg/ml), and nystatin (50 U/ml).

Dungan CF, Hamilton RM. 1995. Use of a tetrazolium-based cell proliferation assay to measure effects of *in vitro* conditions on *Perkinsus marinus* (Apicomplexa) proliferation. *J. Eukaryot. Microbiol*. 42:379–388.

Medium for Cryopreservation of Cultured *Perkinsus*

Source: Dr. Chris Dungan, Oxford Laboratory, MD, USA. Pers. comm. Nicholas Gudkovs June 1st 2010

The freezing medium used for *Perkinsus* is the normal growth medium, with approximately double the concentration of foetal bovine serum (FBS) and 8-10% (v/v) cell culture quality DMSO (Sigma D2650).

Starting with DMEM/F12 (basic *Perkinsus* medium without FBS or Sigma lipid supplement)

Component	~15 ml	~50 ml	~100 ml	Final conc. % (v/v)
DMS/ Ham's F12	12.0	42.0	84.0	
FBS	1.2	4.0	8.0	8%
DMSO	1.2	4.0	8.0	8%

Starting with DMEM/F12-3 (growth medium with 3% FBS)

Component	~15 ml	~50 ml	~100 ml	Final conc. % (v/v)
DMS/ Ham's F12	12.0	42.0	84.0	
FBS	0.7	2.3	4.6	8%
DMSO	1.1	3.9	7.8	8%

Note: This medium was successfully applied with minor modification with cultures frozen after June 2014. In this case the freezing medium contained slightly more than 10% v/v FBS with slightly less than 8% DMSO.
DMEM/Ham's F12 68 ml, 6 ml FBS and 6 ml DMSO.

Cryopreservation Procedure

To minimize ice crystal lysis/damage to cryopreserved cells, freeze slowly (-1°C /min) and thaw rapidly.

> **DMSO is toxic and may be toxic to thawed cells. Minimize the time cells are exposed to DMSO containing medium before and after freezing.**

1. Harvest cells by centrifugation at 300 X *g* for 5 minutes in sterile 50 ml tubes.

2. Aseptically aspirate supernatant medium using a sterile serological pipette. *Optional: Retain batches of used (conditioned) medium. After the Perkinsus are processed, centrifuge the used medium at 3,000 X g for 15 minutes and tip supernatant into sterile bottles (store at -20°C).*

3. Working quickly, using a serological pipette, gently suspend the cell pellets in freezing medium. Pool cells, adjust to the final concentration and volume required and mix gently before dispensing 1.0 ml volumes into pre-labelled cryovials (NUNC 2ml). Seal tubes carefully.

4. Insert cryovials into a polystyrene rack (scrap polystyrene sheet can be used for this). The vials should fit snugly; otherwise insulate the rack to facilitate slow cooling of vials.

5. Freeze vials overnight at -80°C.

6. The cells remain at -80°C for a minimum of 24 hours after which one tube is cultured to check post thaw viability.

7. The post thaw viability check should confirm the frozen culture is free of bacterial or fungal contamination, and should have >60% survival. As the cells can be highly clumped, the assessment of survival can be difficult and may be somewhat subjective.

8. The tubes are then transferred to vapour phase liquid N_2 storage (-196°C).

Freezing medium can be stored at 4°C or -20°C depending on frequency of use. If stored at -20°C the DMSO separates from the medium, thawed medium must be mixed thoroughly before use.

Thawing Cells

1. Place 5 to 10 ml of growth medium into a sterile 15 ml conical centrifuge tube.

2. Thaw the selected frozen vials **quickly** at the normal temperature used for incubation, preferably in a circulating water bath.

3. Immediately transfer the thawed cell suspension to the tube of growth medium, to dilute the DMSO.

4. Pellet cells for 5 minutes at 300 x *g*.

5. Aspirate the supernatant (diluted freezing medium) to waste.

6. Gently re-suspend the washed cell pellet in 5 ml sterile growth medium and transfer to a 25 cm^2 flask.

7. Incubate the flask at the appropriate temperature and atmosphere. We have used 25°C in normal atmosphere for *Perkinsus olseni* in this project.

8. Check cultures daily for signs of replication and record the purity and viability of the culture.

Appendix 8 – Data Summary of Bayesian Analysis for qPCR Validation

(A) ROC analysis

(A) ROC analysis **https://www.medcalc.org/**

Summary Statistics of ROC Analysis

Variable	AFDL *Pols* qPCR
Classification variable	OIE 140/600 cPCR
Sample size	377
Positive group [a]	56 (14.85%)
- ative group [b]	321 (85.15%)

[a] PCR Sp = 1   [b] PCR Sp = 0

Disease prevalence (%)	unknown

Area under the ROC curve (AUC)

Area under the ROC curve (AUC)	0.957
Standard Error [a]	0.0167
95% Confidence interval [b]	0.932 to 0.975
z statistic	27.462
Significance level P (Area=0.5)	<0.0001

[a] DeLong *et al.*, 1988   [b] Binomial exact

Youden index

Youden index J	0.8748
Associated criterion	≤41.29
Sensitivity	94.64
Specificity	92.83

Figure 43 ROC curve of AFDL *Pols* qPCR data used to generate the cut-off for Bayesian analysis
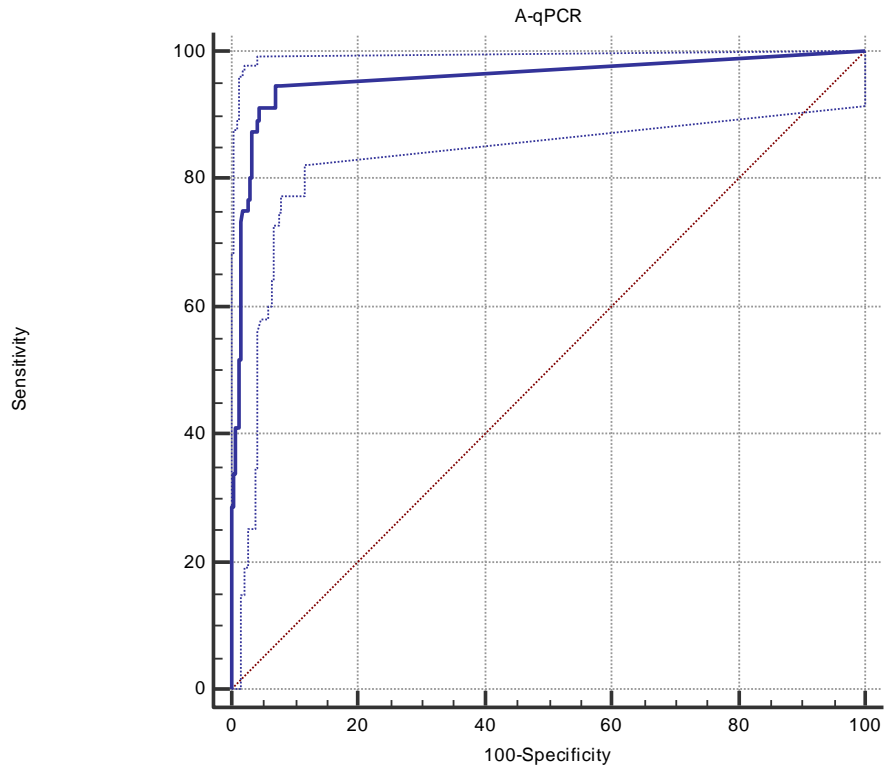
Figure 44 Interactive dot plot of AFDL *Pols* qPCR data from the ROC analysis shown in Figure 42.
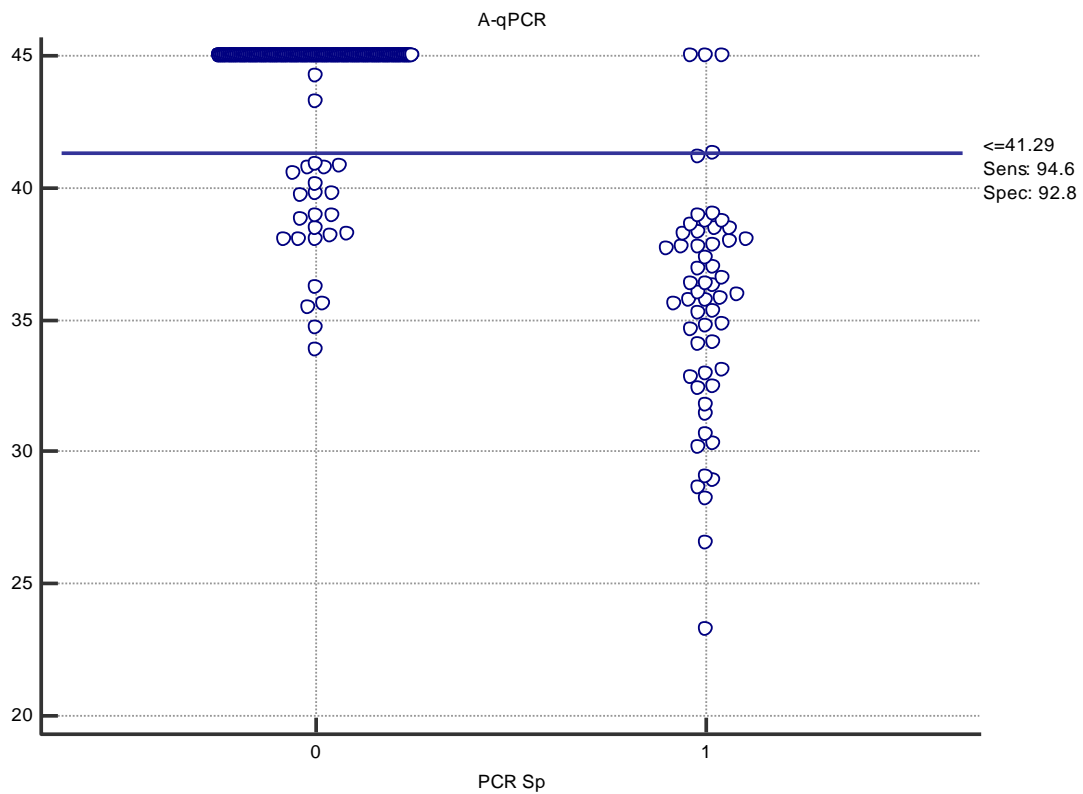
Figure 45 Modified ROC curve of AFDL *Pols* qPCR data showing the DSe and DSe for all cut-off values at the 95% CI.

Classification: PCR Sp

A-qPCR
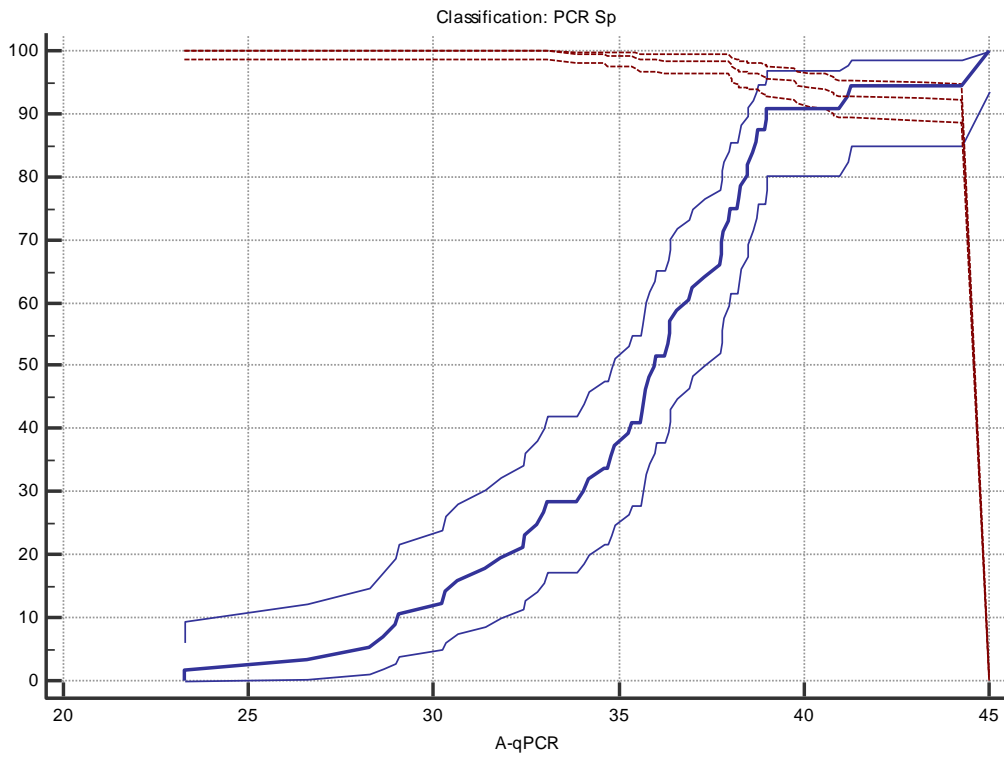
Criterion values and coordinates of the ROC curve

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	95% CI	-LR	95% CI
<23.27	0.00	0.0 - 6.4	100.00	98.9 - 100.0			1.00	1.0 - 1.0
≤23.27	1.79	0.05 - 9.6	100.00	98.9 - 100.0			0.98	0.9 - 1.0
≤26.56	3.57	0.4 - 12.3	100.00	98.9 - 100.0			0.96	0.9 - 1.0
≤28.24	5.36	1.1 - 14.9	100.00	98.9 - 100.0			0.95	0.9 - 1.0
≤28.62	7.14	2.0 - 17.3	100.00	98.9 - 100.0			0.93	0.9 - 1.0
≤28.94	8.93	3.0 - 19.6	100.00	98.9 - 100.0			0.91	0.8 - 1.0
≤29.06	10.71	4.0 - 21.9	100.00	98.9 - 100.0			0.89	0.8 - 1.0
≤30.2	12.50	5.2 - 24.1	100.00	98.9 - 100.0			0.88	0.8 - 1.0
≤30.29	14.29	6.4 - 26.2	100.00	98.9 - 100.0			0.86	0.8 - 1.0
≤30.65	16.07	7.6 - 28.3	100.00	98.9 - 100.0			0.84	0.7 - 0.9
≤31.4	17.86	8.9 - 30.4	100.00	98.9 - 100.0			0.82	0.7 - 0.9
≤31.79	19.64	10.2 - 32.4	100.00	98.9 - 100.0			0.80	0.7 - 0.9
≤32.43	21.43	11.6 - 34.4	100.00	98.9 - 100.0			0.79	0.7 - 0.9
≤32.46	23.21	13.0 - 36.4	100.00	98.9 - 100.0			0.77	0.7 - 0.9
≤32.78	25.00	14.4 - 38.4	100.00	98.9 - 100.0			0.75	0.6 - 0.9
≤32.98	26.79	15.8 - 40.3	100.00	98.9 - 100.0			0.73	0.6 - 0.9
≤33.08	28.57	17.3 - 42.2	100.00	98.9 - 100.0			0.71	0.6 - 0.8
≤33.87	28.57	17.3 - 42.2	99.69	98.3 - 100.0	91.71	12.4 - 677.9	0.72	0.6 - 0.8
≤34.05	30.36	18.8 - 44.1	99.69	98.3 - 100.0	97.45	13.2 - 717.7	0.70	0.6 - 0.8
≤34.17	32.14	20.3 - 46.0	99.69	98.3 - 100.0	103.18	14.1 - 757.5	0.68	0.6 - 0.8
≤34.62	33.93	21.8 - 47.8	99.69	98.3 - 100.0	108.91	14.9 - 797.4	0.66	0.5 - 0.8
≤34.68	33.93	21.8 - 47.8	99.38	97.8 - 99.9	54.46	13.0 - 227.4	0.66	0.6 - 0.8
≤34.79	35.71	23.4 - 49.6	99.38	97.8 - 99.9	57.32	13.8 - 238.5	0.65	0.5 - 0.8
≤34.86	37.50	24.9 - 51.5	99.38	97.8 - 99.9	60.19	14.5 - 249.6	0.63	0.5 - 0.8
≤35.25	39.29	26.5 - 53.2	99.38	97.8 - 99.9	63.05	15.2 - 260.7	0.61	0.5 - 0.8
≤35.33	41.07	28.1 - 55.0	99.38	97.8 - 99.9	65.92	16.0 - 271.8	0.59	0.5 - 0.7
≤35.46	41.07	28.1 - 55.0	99.07	97.3 - 99.8	43.95	13.7 - 141.5	0.59	0.5 - 0.7
≤35.57	41.07	28.1 - 55.0	98.75	96.8 - 99.7	32.96	11.8 - 91.7	0.60	0.5 - 0.7
≤35.62	42.86	29.7 - 56.8	98.75	96.8 - 99.7	34.39	12.4 - 95.4	0.58	0.5 - 0.7
≤35.71	46.43	33.0 - 60.3	98.75	96.8 - 99.7	37.26	13.5 - 102.7	0.54	0.4 - 0.7
≤35.83	48.21	34.7 - 62.0	98.75	96.8 - 99.7	38.69	14.1 - 106.3	0.52	0.4 - 0.7
≤35.95	50.00	36.3 - 63.7	98.75	96.8 - 99.7	40.12	14.6 - 110.0	0.51	0.4 - 0.7
≤36.02	51.79	38.0 - 65.3	98.75	96.8 - 99.7	41.56	15.2 - 113.7	0.49	0.4 - 0.6

≤36.22	51.79	38.0 - 65.3	98.44	96.4 - 99.5	33.25	13.4 - 82.2	0.49	0.4 - 0.6
≤36.33	53.57	39.7 - 67.0	98.44	96.4 - 99.5	34.39	13.9 - 84.9	0.47	0.4 - 0.6
≤36.36	55.36	41.5 - 68.7	98.44	96.4 - 99.5	35.54	14.4 - 87.5	0.45	0.3 - 0.6
≤36.37	57.14	43.2 - 70.3	98.44	96.4 - 99.5	36.69	14.9 - 90.1	0.44	0.3 - 0.6
≤36.57	58.93	45.0 - 71.9	98.44	96.4 - 99.5	37.83	15.4 - 92.8	0.42	0.3 - 0.6
≤36.895	60.71	46.8 - 73.5	98.44	96.4 - 99.5	38.98	15.9 - 95.4	0.40	0.3 - 0.6
≤36.99	62.50	48.5 - 75.1	98.44	96.4 - 99.5	40.12	16.4 - 98.0	0.38	0.3 - 0.5
≤37.31	64.29	50.4 - 76.6	98.44	96.4 - 99.5	41.27	16.9 - 100.6	0.36	0.3 - 0.5
≤37.72	66.07	52.2 - 78.2	98.44	96.4 - 99.5	42.42	17.4 - 103.3	0.34	0.2 - 0.5
≤37.76	67.86	54.0 - 79.7	98.44	96.4 - 99.5	43.56	17.9 - 105.9	0.33	0.2 - 0.5
≤37.79	69.64	55.9 - 81.2	98.44	96.4 - 99.5	44.71	18.4 - 108.5	0.31	0.2 - 0.5
≤37.83	71.43	57.8 - 82.7	98.44	96.4 - 99.5	45.86	18.9 - 111.1	0.29	0.2 - 0.4
≤37.96	73.21	59.7 - 84.2	98.44	96.4 - 99.5	47.00	19.4 - 113.8	0.27	0.2 - 0.4
≤38.01	75.00	61.6 - 85.6	98.13	96.0 - 99.3	40.12	17.9 - 89.9	0.25	0.2 - 0.4
≤38.04	75.00	61.6 - 85.6	97.82	95.6 - 99.1	34.39	16.3 - 72.7	0.26	0.2 - 0.4
≤38.06	75.00	61.6 - 85.6	97.51	95.1 - 98.9	30.09	14.9 - 60.6	0.26	0.2 - 0.4
≤38.18	75.00	61.6 - 85.6	97.20	94.7 - 98.7	26.75	13.8 - 51.8	0.26	0.2 - 0.4
≤38.22	76.79	63.6 - 87.0	97.20	94.7 - 98.7	27.39	14.2 - 53.0	0.24	0.1 - 0.4
≤38.25	76.79	63.6 - 87.0	96.88	94.3 - 98.5	24.65	13.2 - 46.1	0.24	0.1 - 0.4
≤38.3	78.57	65.6 - 88.4	96.88	94.3 - 98.5	25.22	13.5 - 47.1	0.22	0.1 - 0.4
≤38.47	80.36	67.6 - 89.8	96.88	94.3 - 98.5	25.79	13.8 - 48.1	0.20	0.1 - 0.3
≤38.475	80.36	67.6 - 89.8	96.57	94.0 - 98.3	23.45	12.9 - 42.5	0.20	0.1 - 0.3
≤38.48	82.14	69.6 - 91.1	96.57	94.0 - 98.3	23.97	13.2 - 43.4	0.18	0.1 - 0.3
≤38.61	83.93	71.7 - 92.4	96.57	94.0 - 98.3	24.49	13.6 - 44.3	0.17	0.09 - 0.3
≤38.72	85.71	73.8 - 93.6	96.57	94.0 - 98.3	25.01	13.9 - 45.1	0.15	0.08 - 0.3
≤38.74	87.50	75.9 - 94.8	96.57	94.0 - 98.3	25.53	14.2 - 46.0	0.13	0.06 - 0.3
≤38.82	87.50	75.9 - 94.8	96.26	93.6 - 98.1	23.41	13.3 - 41.1	0.13	0.06 - 0.3
≤38.95	87.50	75.9 - 94.8	95.95	93.2 - 97.8	21.61	12.6 - 37.1	0.13	0.07 - 0.3
≤38.96	89.29	78.1 - 96.0	95.95	93.2 - 97.8	22.05	12.8 - 37.8	0.11	0.05 - 0.2
≤38.97	89.29	78.1 - 96.0	95.64	92.8 - 97.6	20.47	12.2 - 34.4	0.11	0.05 - 0.2
≤39	91.07	80.4 - 97.0	95.64	92.8 - 97.6	20.88	12.4 - 35.1	0.093	0.04 - 0.2
≤39.72	91.07	80.4 - 97.0	95.33	92.4 - 97.4	19.49	11.8 - 32.2	0.094	0.04 - 0.2
≤39.76	91.07	80.4 - 97.0	95.02	92.0 - 97.1	18.27	11.3 - 29.7	0.094	0.04 - 0.2
≤39.8	91.07	80.4 - 97.0	94.70	91.7 - 96.9	17.20	10.7 - 27.5	0.094	0.04 - 0.2
≤40.14	91.07	80.4 - 97.0	94.39	91.3 - 96.6	16.24	10.3 - 25.6	0.095	0.04 - 0.2
≤40.57	91.07	80.4 - 97.0	94.08	90.9 - 96.4	15.39	9.9 - 24.0	0.095	0.04 - 0.2

≤40.78	91.07	80.4 - 97.0	93.46	90.2 - 95.9	13.92	9.1 - 21.2	0.096	0.04 - 0.2
≤40.82	91.07	80.4 - 97.0	93.15	89.8 - 95.7	13.29	8.8 - 20.1	0.096	0.04 - 0.2
≤40.92	91.07	80.4 - 97.0	92.83	89.4 - 95.4	12.71	8.5 - 19.0	0.096	0.04 - 0.2
≤41.19	92.86	82.7 - 98.0	92.83	89.4 - 95.4	12.96	8.7 - 19.3	0.077	0.03 - 0.2
≤41.29	94.64	85.1 - 98.9	92.83	89.4 - 95.4	13.21	8.9 - 19.7	0.058	0.02 - 0.2
≤43.25	94.64	85.1 - 98.9	92.52	89.1 - 95.2	12.66	8.6 - 18.7	0.058	0.02 - 0.2
≤44.24	94.64	85.1 - 98.9	92.21	88.7 - 94.9	12.15	8.3 - 17.8	0.058	0.02 - 0.2
≤45	100.00	93.6 - 100.0	0.00	0.0 - 1.1	1.00	1.0 - 1.0		

2 x 2 tables for 3 abalone populations with different Perkinsus infection prevalence.

The OIE 140/600 cPCR molecular test was used as a reference test for the AFDL *Pols* qPCR.

Table 22 2 x 2 table with results from both molecular tests and 3 populations (mixed P)

All		140/600 cPCR	
		(+)	(-)
AFDL *Pols* qPCR	(+)	53	23
	(-)	3	298

Table 23 Table of results from both molecular tests and unknown history of infection (low P)

Historical Reports with No History		140/600 cPCR	
		(+)	(-)
AFDL *Pols* qPCR	(+)	1	2
	(-)	0	19

Table 24 Table of results from both molecular tests for disease free population (very low P~<0.01)

Disease Free		140/600 cPCR	
		(+)	(-)
AFDL *Pols* qPCR	(+)	0	0
	(-)	0	200

Table 25 Table of results from both molecular tests for known infected population (high P ~ 0.3)

Known Infected		140/600 cPCR	
		(+)	(-)
AFDL *Pols* qPCR	(+)	52	21
	(-)	3	79

Receiver Operator Analysis

Of 56 positive samples as determined by Test 1, the OIE 140/600 cPCR reference test, the candidate Test 2 (AFDL *Pols* qPCR) produced 53 positive and 3 false-negative results.

Of 321 negative samples as determined by Test 1, Test 2 produced 298 negative and 23 *false-positive* results. The 200 samples from the non-infected population were negative in both tests.

Based on the comparison of results of testing these abalone DNA samples the AFDL *Pols* qPCR had a relative diagnostic DSe of 94.64% (95% CI 85.1-98.9) and a DSp of 92.83% (95% CI 89.4-95.4) at a cut-off CT of ≤ 41.29.

(B) Bayesian analysis (C$_T$ 41.29 and 39 cut-offs)

(B.1) Bayesian analysis of P. olseni AFDL Pols qPCR using a C$_T$ cut-off of 41.29

Sensitivity (DSe) and specificity (DSp) of the OIE species-specific PCR (OIE-s-PCR) for *P. olseni* was estimated by testing 9 positive cultures of *P. olseni*, 40 cultures of other species of *Perkinsus* and samples collected from a *Perkinsus*-free population of oysters (n=200). The cross-classified data are presented as a 2x2 table, and prior values were estimated using SISA (Simple Interactive Statistical Analysis online (http://www.quantitativeskills.com/sisa/statistics/diagnos.htm).

The DSe and DSp values of OIE-PCR thus calculated was used for the comparison of AFDL *Pols* qPCR test. The parameters for beta distribution were estimated using Beta Buster. The information from the *Perkinsus*-free population was taken as a prior for specificity estimate (beta (201, 1)). The cut-off value for AFDL *Pols* qPCR was determined based on a Receiver Operator Curve (ROC) analysis using MedCalc. A cut-off value of C$_T$=41.29 was determined by the ROC analysis to balance DSe and DSp of the AFDL *Pols* qPCR. Based on the cut-off value of C$_T$ = 41.29, the AFDL *Pols* qPCR results were classified as positive or negative and compared with OIE PCR for specificity and sensitivity estimates using Latent Class Analysis with Bayesian modelling. The model was built under the assumptions of two independent tests, two populations and no perfect reference standard (Branscum *et al*., 2005). The cross-classified data for population 1 (n1=155) and population 2 (n2=22) with two different prevalence estimates (π1=34% & π2<0.05%) were represented as y1= (52, 3, 21, 79) and y2= (1, 0, 2, 19). Uniform distributions were used for the prevalence in the two population (pi1 & pi2). The convergence estimates were derived using 1000000 iterations of simulation with sampling done every 1000th iteration until the MC error value was <5% of the standard deviation of the node estimate. Relative prevalence, Se, Sp, SeDIFF, SpDIFF, SeSIGN and SpSIGN, the lower and upper endpoints of a 95% probability interval represented by posterior distributions for the 2.5[th] and 97.5[th] percentiles of the Monte Carlo sample were estimated.

Priors estimate using reference samples:

		Reference samples	
		+	-
OIE-s-PCR	+	9	0
(OIE 140/600 cPCR)	-	0	240

	Estimate	95% MIN	95% MAX
Sensitivity	1	1	1
Specificity	1	1	1

Infected population (Prevalence pi1~34%)

		AFDL *Pols* qPCR		
		+	-	
OIE-s-PCR	+	52	3	55
(OIE 140/600 cPCR)	-	21	79	100
		73	82	155

Population with no history of infection (Prevalence pi1~0.05%)

		AFDL *Pols* qPCR		
		+	-	
OIE-s-PCR	+	1	0	1
(OIE 140/600 cPCR)	-	2	19	21
		3	19	22

Latent class analysis with Bayesian analysis model with conditional independence of tests model;

```
{
y1[1:Q, 1:Q] ~ dmulti(p1[1:Q, 1:Q], n1)
y2[1:Q, 1:Q] ~ dmulti(p2[1:Q, 1:Q], n2)
p1[1,1] <- pi1*SeOIE*SeAAHL + (1-pi1)*(1-SpOIE)*(1-SpAAHL)
p1[1,2] <- pi1*SeOIE*(1-SeAAHL) + (1-pi1)*(1-SpOIE)*SpAAHL
p1[2,1] <- pi1*(1-SeOIE)*SeAAHL + (1-pi1)*SpOIE*(1-SpAAHL)
p1[2,2] <- pi1*(1-SeOIE)*(1-SeAAHL) + (1-pi1)*SpOIE*SpAAHL
p2[1,1] <- pi2*SeOIE*SeAAHL + (1-pi2)*(1-SpOIE)*(1-SpAAHL)
p2[1,2] <- pi2*SeOIE*(1-SeAAHL) + (1-pi2)*(1-SpOIE)*SpAAHL
p2[2,1] <- pi2*(1-SeOIE)*SeAAHL + (1-pi2)*SpOIE*(1-SpAAHL)
p2[2,2] <- pi2*(1-SeOIE)*(1-SeAAHL) + (1-pi2)*SpOIE*SpAAHL
SeOIE ~ dbeta(1,1)
SpOIE ~ dbeta(201,1)  # based on data with 0 positive out of 200 TN
SeAAHL ~ dbeta(1,1)
SpAAHL ~ dbeta(201,1) # based on data with 0 positive out of 200 TN
pi1~ dbeta(1,1)
pi2 ~ dbeta(1,1)
SeDIFF <-SeAAHL-SeOIE
SeSIGN<-step(SeDIFF)
SpDIFF <-SpAAHL-SpOIE
SpSIGN<-step(SpDIFF)
}
list(n1=155, n2=22, y1=structure(.Data=c(52,3,21,79),.Dim=c(2,2)),
y2=structure(.Data=c(1,0,2,19),.Dim=c(2,2)),Q=2)
list(pi1=0.34, pi2=0.05, SeOIE=0.80, SpOIE=0.995, SeAAHL=0.90, SpAAHL=0.995)
```

Relative prevalence, sensitivity, specificity and other estimates of AFDL *Pols* qPCR compared with OIE-s-PCR with mean, standard deviation, MC error, and posterior distributions (2.5th, Median & 97.5th percentile)

	Posterior Distribution					
	mean	SD	MC_error	2.50%	median	97.50%
Prevalence Population 1	0.495	0.042	0.000	0.414	0.495	0.578
Prevalence Population 2	0.160	0.077	0.000	0.039	0.151	0.335
Sensitivity AHHL Test	0.936	0.034	0.000	0.856	0.941	0.988
Specificity AAHL Test	0.995	0.005	0.000	0.980	0.996	1.000
Sensitivity OIE Test	0.694	0.053	0.000	0.586	0.696	0.794
Specificity of OIE Test	0.996	0.004	0.000	0.984	0.997	1.000
Sensitivity difference	0.242	0.059	0.000	0.126	0.241	0.359
Specificity difference	-0.001	0.007	0.000	-0.017	-0.001	0.013
Sensitivity significance	1.000	0.006	0.000	1.000	1.000	1.000
Specificity significance	0.451	0.498	0.001	0.000	0.000	1.000

(B.2) Bayesian analysis of P. olseni AFDL Pols qPCR using a C_T cut-off of 39

Sensitivity (DSe) and specificity (DSp) of the OIE species-specific PCR (OIE-s-PCR) for *P. olseni* was estimated by testing 9 positive cultures of *P. olseni*, 40 cultures of other species of *Perkinsus* and samples collected from a *Perkinsus*-free population of oysters (n=200). The cross-classified data are presented as a 2x2 table, and prior values were estimated using SISA (Simple Interactive Statistical Analysis online (http://www.quantitativeskills.com/sisa/statistics/diagnos.htm).

The DSe and DSp values of OIE-PCR thus calculated was used for the comparison of AFDL *Pols* qPCR test. The parameters for beta distribution were estimated using Beta Buster. The information from the *Perkinsus*-free population was taken as a prior for specificity estimate (beta (201, 1). The cut-off value for AFDL *Pols* qPCR was determined based on a Receiver Operator Curve (ROC) analysis using MedCalc. A cut-off value of C_T = 41.29 was determined by the ROC analysis to balance DSe and DSp of the AFDL *Pols* qPCR. Based on the cut-off value of C_T = 39, the AFDL *Pols* qPCR results were classified as positive or negative and compared with OIE PCR for specificity and sensitivity estimates using Latent Class Analysis with Bayesian modelling. The model was built under the assumptions of two independent tests, two populations and no perfect reference standard (Branscum *et al*., 2005). The cross-classified data for population 1 (n1=155) and population 2 (n2=22) with two different prevalence estimates (π1=34% & π2<0.05%) were represented as y1 = (50, 5, 13, 87) and y2 = (1, 0, 1, 20). Uniform distributions were used for the prevalence in the two population (pi1 & pi2). The convergence estimates were derived using 1000000 iterations of simulation with sampling done every 1000th iteration until the MC error value was <5% of the standard deviation of the node estimate. Relative prevalence, Se, Sp, SeDIFF, SpDIFF, SeSIGN and SpSIGN, the lower and upper endpoints of a 95% probability interval represented by posterior distributions for the 2.5th and 97.5th percentiles of the Monte Carlo sample were estimated.

Priors estimate using reference samples:

		Reference samples		
			+	-
OIE 140/600 cPCR		+	9	0
		-	0	240
Estimate		95% MIN		95% MAX
Sensitivity	1	1		1

Specificity	1	1	1

Infected population (Prevalence pi1~34%)

		AFDL *Pols* qPCR		
		+	-	
OIE 140/600 cPCR	+	50	5	55
	-	13	87	100
		63	92	155

Population with no history of infection (Prevalence pi1~0.05%)

		AFDL *Pols* qPCR		
		+	-	
OIE 140/600 cPCR	+	1	0	1
	-	1	20	21
		2	20	22

<u>Latent class analysis of Bayesian model with conditional independence of tests</u>:

```
{
y1[1:Q, 1:Q] ~ dmulti(p1[1:Q, 1:Q], n1)
y2[1:Q, 1:Q] ~ dmulti(p2[1:Q, 1:Q], n2)
p1[1,1] <- pi1*SeOIE*SeAAHL + (1-pi1)*(1-SpOIE)*(1-SpAAHL)
p1[1,2] <- pi1*SeOIE*(1-SeAAHL) + (1-pi1)*(1-SpOIE)*SpAAHL
p1[2,1] <- pi1*(1-SeOIE)*SeAAHL + (1-pi1)*SpOIE*(1-SpAAHL)
p1[2,2] <- pi1*(1-SeOIE)*(1-SeAAHL) + (1-pi1)*SpOIE*SpAAHL
p2[1,1] <- pi2*SeOIE*SeAAHL + (1-pi2)*(1-SpOIE)*(1-SpAAHL)
p2[1,2] <- pi2*SeOIE*(1-SeAAHL) + (1-pi2)*(1-SpOIE)*SpAAHL
p2[2,1] <- pi2*(1-SeOIE)*SeAAHL + (1-pi2)*SpOIE*(1-SpAAHL)
p2[2,2] <- pi2*(1-SeOIE)*(1-SeAAHL) + (1-pi2)*SpOIE*SpAAHL
SeOIE ~ dbeta(1,1)
SpOIE ~ dbeta(201,1)  # based on data with 0 positive out of 200 TN
SeAAHL ~ dbeta(1,1)
SpAAHL ~ dbeta(201,1) # based on data with 0 positive out of 200 TN
pi1~ dbeta(1,1)
pi2 ~ dbeta(1,1)
SeDIFF <-SeAAHL-SeOIE
SeSIGN<-step(SeDIFF)
SpDIFF <-SpAAHL-SpOIE
SpSIGN<-step(SpDIFF)
}
list(n1=155, n2=22, y1=structure(.Data=c(50,5,13,87),.Dim=c(2,2)),
y2=structure(.Data=c(1,0,1,20),.Dim=c(2,2)),Q=2)
list(pi1=0.34, pi2=0.05, SeOIE=0.80, SpOIE=0.995, SeAAHL=0.90, SpAAHL=0.995)
```

Relative prevalence, sensitivity, specificity and other estimates of AFDL *Pols* qPCR compared with OIE-s-PCR with mean, standard deviation, MC error, and posterior distributions (2.5th, Median & 97.5th percentile)

	mean	SD	MC_error	Posterior Distribution		
				2.50%	median	97.50%
Prevalence Population 1	0.444	0.042	4.39E-05	0.363	0.443	0.526
Prevalence Population 2	0.119	0.068	8.32E-05	0.021	0.109	0.279
Sensitivity AHHL Test	0.902	0.041	4.93E-05	0.809	0.906	0.969
Specificity AAHL Test	0.995	0.005	8.77E-06	0.984	0.996	1.000
Sensitivity OIE Test	0.781	0.052	5.81E-05	0.671	0.783	0.875
Specificity of OIE Test	0.995	0.005	8.09E-06	0.983	0.997	1.000
0Sensitivity difference	0.121	0.062	6.25E-05	-3.49E-04	0.120	0.244
Specificity difference	-0.001	0.007	1.17E-05	-0.016	-4.71E-04	0.013
Sensitivity significance	0.975	0.157	1.76E-04	0.000	1.000	1.000
Specificity significance	0.455	0.498	7.23E-04	0.000	0.000	1.000

(C) Repeatability analysis

The Cochran statistical model used is summarised below. The Cochran Q analysis concluded that there was no significant difference between runs indicating satisfactory repeatability on random independent extracted DNA samples.

	Animal	A-qPCR	A-qPCR	A-qPCR	Run1	Run2	Run3	
Baird Bay	13-1031-2	31.79	33.24	33.01	1	1	1	3
	13-1031-3	34.62	33.47	34.82	1	1	1	3
	13-1031-4	32.43	33.75	33.69	1	1	1	3
	13-1031-5	36.22	37.31	37.54	1	1	1	3
	13-1031-6	26.56	27.30	27.71	1	1	1	3
	13-1031-7	31.40	32.28	32.04	1	1	1	3
	13-1031-10	32.78	34.00	33.99	1	1	1	3
	13-1031-12	34.05	34.81	34.98	1	1	1	3
	13-1031-13	28.24	28.91	29.05	1	1	1	3
	13-1031-15	32.46	32.93	33.06	1	1	1	3
	13-1031-16	37.15	37.54	37.31	1	1	1	3
	13-1031-17	37.31	37.80	38.34	1	1	1	3
Taylor's Island	12-978-01	38.48	40.14	43.10	1	0	0	1
	12-978-05	41.29	42.74	40.17	0	0	0	0
	12-978-07	37.79	38.47	42.32	1	1	0	2
	12-978-08	37.83	38.21	37.57	1	1	1	3
	12-978-14	35.25	37.17	35.88	1	1	1	3
	12-978-15	33.08	34.10	33.09	1	1	1	3
	12-978-16	30.29	31.39	30.23	1	1	1	3
	12-978-18	39.72	36.49	35.61	0	1	1	2
	12-978-25	35.71	35.84	35.27	1	1	1	3
	12-978-29	34.79	35.55	34.29	1	1	1	3

	12-978-31	38.06	40.45	39.54	1	0	0	1
Thistle Island	12-979-12	29.06	30.08	29.48	1	1	1	3
	12-979-13	23.27	23.61	23.91	1	1	1	3
	12-979-14	38.47	37.01	37.32	1	1	1	3
	12-979-16	38.30	42.24	41.87	1	0	0	1
	12-979-17	35.62	35.94	36.43	1	1	1	3
	12-979-20	37.96	35.27	35.90	1	1	1	3
	12-979-22	30.20	30.87	31.35	1	1	1	3
	12-979-23	36.36	33.00	33.20	1	1	1	3
	12-979-24	32.98	33.32	33.46	1	1	1	3
	12-979-25	35.71	36.12	36.74	1	1	1	3
	12-979-26	37.72	38.96	39.38	1	1	0	2
	12-979-27	36.99	36.44	37.57	1	1	1	3
	12-979-28	36.37	37.58	38.20	1	1	1	3
	12-979-29	38.22	41.94	41.79	1	0	0	1
					35	32	30	97

Cochran Model

cut-off for positive	<39	
k	3	=countA(i5:k5)
n	37	=count(i5:i41)
df	2	=P5-1
Q	5.428571	=P7*(P5*SUMSQ(I42:K42)-L42^2)/(P5*L42-SUMSQ(L5:L41))
alpha	0.05	95% CI
p-value	0.066252	=CHIDIST(P8,P7)
Q-critical	5.991465	=CHIINV(P9, P7)
Significance	No	=IF(P10<P9,"Yes","No")

(D) Gill Tissue (OIE 140/600 cPCR vs AFDL *Pols* qPCR)

		A-qPCR		
		P	N	
OIE	P	7	1	8
	N	7	72	79
		14	73	87

Prevalence	0.09	0.03	0.15	95% CI
Sensitivity	0.88	0.81	0.94	95% CI
Specificity	0.91	0.85	0.97	95% CI
Accuracy	0.91	0.85	0.97	95% CI
Positive Predictive Value	0.50	0.39	0.61	95% CI
Negative Predictive Value	0.99	0.96	1.01	95% CI

Muscle Tissue (OIE 140/600 cPCR vs AFDL *Pols* qPCR)

		A-qPCR		
		P	N	
OIE	P	11	1	
	N	12	74	
				98

Prevalence	0.12	0.06	0.19	95% CI
Sensitivity	0.92	0.86	0.97	95% CI
Specificity	0.86	0.79	0.93	95% CI
Accuracy	0.87	0.80	0.93	95% CI
Positive Predictive Value	0.48	0.38	0.58	95% CI
Negative Predictive Value	0.99	0.96	1.01	95% CI

Gill vs Muscle

		Muscle		
		P	N	
Gill	P	3	11	
	N	12	61	
				87

Prevalence	0.16	0.08	0.24	95% CI
Sensitivity	0.21	0.13	0.30	95% CI
Specificity	0.84	0.76	0.91	95% CI
Accuracy	0.74	0.64	0.83	95% CI
Positive Predictive Value	0.20	0.12	0.28	95% CI
Negative Predictive Value	0.85	0.77	0.92	95% CI
Kappa value	0.05	-0.29	0.38	95% CI

Combined Gill & Muscle (OIE 140/600 cPCR vs AFDL *Pols* qPCR)

		AFDL *Pols* qPCR		
		P	N	
OIE	P	11	1	
	N	13	62	
				87

Prevalence	0.14	0.07	0.21	95% CI
Sensitivity	0.92	0.86	0.97	95% CI
Specificity	0.83	0.75	0.91	95% CI
Accuracy	0.84	0.76	0.92	95% CI
Positive Predictive Value	0.46	0.35	0.56	95% CI
Negative Predictive Value	0.98	0.96	1.01	95% CI

Kappa		0.52	0.29	0.75	95% CI

Our data show that the DSe obtained with the AFDL *Pols* qPCR with gill and muscle are similar, 0.88 and 0.92, respectively. The slightly higher sensitivity in muscle may be due in part to the natural bias of the operator towards sampling visible lesions or areas of tissue with abnormal appearance. Which may be indicative of infection. Should the highest level of DSe be required the parallel testing of both muscle and gill is recommended.

Gill Tissue (OIE 140/600 cPCR vs AFDL *Pols* qPCR)

		AFDL *Pols* qPCR		
		P	N	
OIE	P	7	1	8
	N	7	72	79
		14	73	87

Prevalence	0.09	0.03	0.15	95% CI
Sensitivity	0.88	0.81	0.94	95% CI
Specificity	0.91	0.85	0.97	95% CI
Accuracy	0.91	0.85	0.97	95% CI
Positive Predictive Value	0.50	0.39	0.61	95% CI
Negative Predictive Value	0.99	0.96	1.01	95% CI

Muscle Tissue (OIE 140/600 cPCR vs AFDL *Pols* qPCR)

		AFDL *Pols* qPCR		
		P	N	
OIE	P	11	1	
	N	12	74	
				98

Prevalence	0.12	0.06	0.19	95% CI
Sensitivity	0.92	0.86	0.97	95% CI
Specificity	0.86	0.79	0.93	95% CI
Accuracy	0.87	0.80	0.93	95% CI
Positive Predictive Value	0.48	0.38	0.58	95% CI
Negative Predictive Value	0.99	0.96	1.01	95% CI

Gill vs Muscle

		Muscle		
		P	N	
Gill	P	3	11	
	N	12	61	
				87

Prevalence	0.16	0.08	0.24	95% CI
Sensitivity	0.21	0.13	0.30	95% CI
Specificity	0.84	0.76	0.91	95% CI
Accuracy	0.74	0.64	0.83	95% CI
Positive Predictive Value	0.20	0.12	0.28	95% CI
Negative Predictive Value	0.85	0.77	0.92	95% CI
Kappa value	0.05	-0.29	0.38	95% CI

Combined Gill & Muscle (OIE 140/600 cPCR vs AFDL *Pols* qPCR)

		AFDL *Pols* qPCR		
		P	N	
OIE	P	11	1	
	N	13	62	
				87

Prevalence	0.14	0.07	0.21	95% CI
Sensitivity	0.92	0.86	0.97	95% CI
Specificity	0.83	0.75	0.91	95% CI
Accuracy	0.84	0.76	0.92	95% CI
Positive Predictive Value	0.46	0.35	0.56	95% CI
Negative Predictive Value	0.98	0.96	1.01	95% CI
Kappa	0.52	0.29	0.75	95% CI

Our data show that the DSe obtained with the AFDL *Pols* qPCR with gill and muscle are similar, 0.88 and 0.92, respectively. The slightly higher sensitivity in muscle may be due in part to the natural bias of the operator towards sampling visible lesions or areas of tissue with abnormal appearance. Which may be indicative of infection. Should the highest level of DSe be required the parallel testing of both muscle and gill is recommended.

Appendix 9 - DNA sequences and sequences used for phylogenetic analysis

GenBank sequences

GenBank sequences included in the ITS region analyses were the following:

1x *Perkinsus qugwadi* AF15128 (outgroup taxon)
6x *P. marinus* AY295180, AY295188, AY295189, AY295194, AY295197, AY295199;
6x *P. chesapeaki* (= *P. andrewsi*) AF091541, AY876302, AY876304, AY876305, AY876306, AY876311
15x *P. olseni* (= *P. atlanticus*) AF441207, AF441209, AF441211, AF441213, AF441215, AY435092, AF473840, AY820757, AF522321, POU07701, PSU07698, PSU07699, EF204082, EF204083, EF204086
6x *P. mediterraneus* AY487834, AY487835, AY487837, AY487839, AY487841, AY487842
4x *P. honshuensis* DQ516696, DQ516697, DQ516698, DQ516699;
Perkinsus sp. EF204015–EF204068, EF526428–EF526436, EU068080–EU068095

GenBank sequences included in analyses of the LSU rRNA gene were the following:

P. micans X16180 (outgroup taxon)
P. marinus AY876319, AY876320, AY876322, AY876325, AY876328, AY876329;
P. chesapeaki (= *P. andrewsi*) AY876344–AY876349;
P. olseni (= *P. atlanticus*) AF509333, AY876330, AY876331, AY876332, EF204077–EF204079;
P. mediterraneus EF204095–EF204098, EF204100;
P. honshuensis DQ516680–DQ516682, DQ516684;
Perkinsus sp. EF526433, EF526437–EF526441, EF526443–EF526452.

GenBank sequences included in the analyses of the actin genes were the following:

A. carterae U84289
Prorocentrum minimum U84290 (outgroup taxa)
Type 1 *P. marinus* AY876350, U84287, U84288
Type 1 *P. chesapeaki* (= *P. andrewsi*), AY876359–AY876361
Type 1 *P. olseni* (= *P. atlanticus*), AY876352, AY876355, AY876356, AY876357, EF204109, EF204110, EF204111
Type 1 *P. mediterraneus* EF204112–EF204115
Type 1 *P. honshuensis*, DQ516686, DQ516687, DQ516688, and DQ516689
Type 1 *Perkinsus sp.* EF526411, EF526412, EF526414, EFF526415, EF516418, EF526420, EF526425, EF526427

Type 2 *P. marinus* TIGR4286, TIGR5138
Type 2 *P. chesapeaki* (= *P. andrewsi*) AY876358, AY876362
Type 2 *P. olseni* (= *P. atlanticus*), AY876351, AY876353, AY876354, DQ516693, DQ516694, DQ516695, EF204108;
Type 2 *P. honshuensis*, DQ516690–DQ516692.

Consensus of South Australian *Perkinsus olseni* ITS2 region

The SA consensus sequence was generated from SAN's 12-00978, 12-00979, 13-01031 and 14-00233:

ATTATCTCCTATGAACCATTGTACTAGTCACAGTATCCAAATCCTTTTGGATTTTGGTATTTCAA
AACGAAATTCCAAACTCTCAACGATGGATGCCTCGGCTCGAGAATCGATGAAGGACGCAGCG
AAGTGCGATAAGCACTGCGATTTGCAGAATTCCGTGAACCAGTAGAAATCTCAACGCATACTG
CACAAAGGGGATCTTTCCTCTTTGTACATACATATCAGTGTCGCTCTTCTTCCCGATACAAACA
TTTTGTTGTTAACGCAACTCAATGCTTTGYATCCCGCTTGGACTAACTCTTCGGAGATAGTTCG
TTWTGTGCGCTTGTGAAGGCAGGCGTATTAATTTGCAAGGCTATAATCTCGTATTGTAGCCCC
TCCGAGAGGAGGACCGCGCCTGTGAGTGTCTTTGGATGCTCGCAAGTCCGACTGTGTTGTGG
TGATATCACGTGTTCCTTGATCACGCGATTCTTCTCTTCAACGCATTATGTCAATTCTTGATGA
AATGCAGAGAAGTGTTTGGATCACGCGTTCAGTCTGGTCGCGAGATAGCTATA

Figure 46 Alignment and comparison of *P. olseni* consensus sequence from South Australian abalone (*H. rubra*) and Queensland mud arks (*Anadara trapezia*) with *P. olseni* sequence from NCBI.
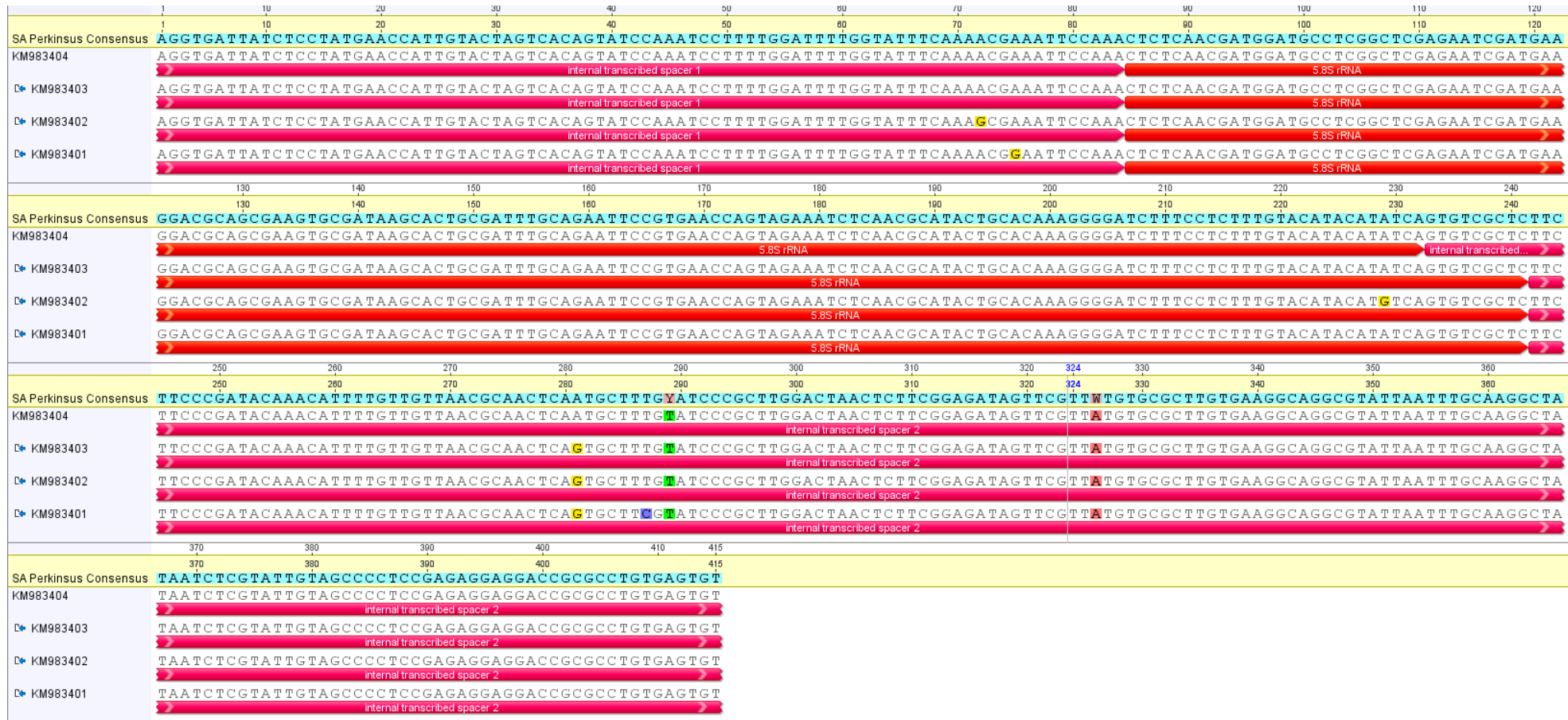
From Dang *et al.* 2015.

Confirmation of *P. olseni* isolates 12:978-11T and 13:1031-12T (University of Tokyo)

ITS DNA sequence analysis of *Perkinsus* 12:978-11T and 13:1031-12T consensus from OIE (85-750) cPCR genus-specific primers with additional DNA sequence data from NCBI.

Table 26 *Perkinsus* isolates used for DNA aligment and construction of phylogenetic tree

Species ID	Host	Location	Source
AUS_ITS	*Haliotis rubra*	South Australia	Gudkovs *et al.* (2016) this project
POU07701_*Perkinsus_olseni*	*Haliotis laevigata*	South Australia	Goggin (1994)
DQ516715_*Perkinsus_olseni*	*Ruditapes philippinarum*	Japan	Dungan and Reece (2006)
PAU07697_*Perkinsus*_atlanticus	*Ruditapes decussatus*	Portugal	Goggin (1994)
AF369974_*Perkinsus*_atlanticus	*Tapes decussatus*	Spain	Casas *et al* (2002)
AY305326_*Perkinsus*_andrewsi	*Macoma balthica*	USA	Pecher *et al.* (2004)
AY876302_*Perkinsus*_chesapeaki	*Mya arenaria*	USA	Burreson *et al.* (2005)
DQ370474_*Perkinsus_marinus*	*Crassostrea virginica*	USA	Abolo *et al.* @006)
AY295199_*Perkinsus_marinus*	*Crassostrea virginica*	USA	Brown *et al.* (2004)
AY295180_*Perkinsus_marinus*	not known	USA	Brown 1999 unpublished
AF149876_*Perkinsus_marinus*	not known	USA	Brown 1999 unpublished
ATCC PRA-181_*Perkinsus_olseni*	*Venerupis philippinarum*	Japan	Dungan and Reece (2006)

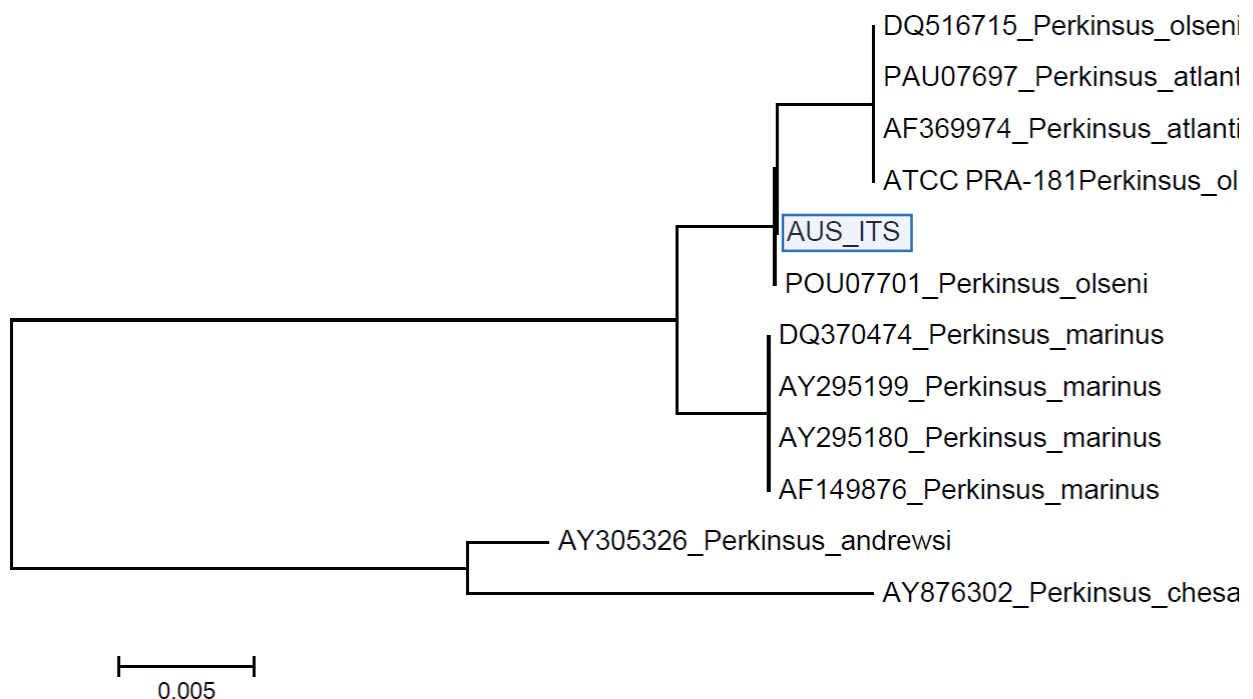**Figure 47** Phylogenetic tree of *Perkinsus* isolates, including AUS_ITS consensus.

Figure 48 Alignment of *Perkinsus* isolates, including AUS_ITS consensus sequence generated using *in vitro* cultures 12:978-11T and 13:1031-12T

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Alignment: C:\Users\YOSHINAGA114\Documents\My Dropbox\‹ ›•ašwŒ¤‹†Ž°‹¤—Lƒtƒ
comparison between P. olseni in Japan and Australia\tree\ITS\Untitled.fas

            ....|....| ....|....| ....|....| ....|....| ....|....|
                  10         20         30         40         50
AUS_ITS     GTTAGGTGAT TATCTCCTAT GAACCA--TT GTACTAGTC- ACAGTATCCA
POU07701_P  --------AT TATCTCCTAT GAACCA--TT GTACTAGTC- ACAGTATCCA
DQ516715_P  ---AGGTGAT TATCTCCTAT GAACCA--TT GTACTAGTC- ACAGTATCCA
PAU07697_P  ---------- -ATCTCCTAT GAACCA--TT GTACTAGTC- ACAGTATCCA
AF369974_P  -TTAGGTGAT TATCTCCTAT GAACCA--TT GTACTAGTC- ACAGTATCCA
AY305326_P  ---------- -------TAT GAACCATATT GTACTAGTCT AAAGTATCCA
AY876302_P  ---------- ---------- --------TT GTACTAGTCT AAAGTATCCA
DQ370474_P  -----GTGAT TAATTCCTAT GAACCA--TT GTACTAGTC- ATAGTATCCA
AY295199_P  --------AT TAATTCCTAT GAACCA--TT GTACTAGTC- ATAGTATCCA
AY295180_P  ---------- TAATTCCTAT GAACCA--TT GTACTAGTC- ATAGTATCCA
AF149876_P  ----GGTGAT TAATTCCTAT GAACCA--TT GTACTAGTC- ATAGTATCCA
ATCC PRA-1  GTTAGGTGAT TATCTCCTAT GAACCA--TT GTACTAGTC- ACAGTATCCA


            ....|....| ....|....| ....|....| ....|....| ....|....|
                  60         70         80         90        100
AUS_ITS     A-ATCC--TT TTGGATTTTG GTATTTCAAA ACGAAATTCC AAACTCTCAA
POU07701_P  A-ATCC--TT TTGGATTTTG GTATTTCAAA ACGAAATTCC AAACTCTCAA
DQ516715_P  A-ATCC--TT TTGGATTTTG GTATTTCAAA ACGAAATTCC AAACTCTCAA
PAU07697_P  A-ATCC--TT TTGGATTTTG GTATTTCAAA ACGAAATTCC AAACTCTCAA
AF369974_P  A-ATCC--TT TTGGATTTTG GTATTTCAAA ACGAAATTCC AAACTCTCAA
AY305326_P  ATATCC--TT TTGGATTTTG GTATTTCAAA ACGAAATTCC AAACTCTCAA
AY876302_P  ATATCC--TT TTGGATTTTG GTATTTCAAA ACGAAATTCC AAACTCTCAA
DQ370474_P  A-ATCCAATT TTGGATTTTG GTATTTCAAA ACGAAATTCC AAACTCTCAA
AY295199_P  A-ATCCAATT TTGGATTTTG GTATTTCAAA ACGAAATTCC AAACTCTCAA
AY295180_P  A-ATCCAATT TTGGATTTTG GTATTTCAAA ACGAAATTCC AAACTCTCAA
AF149876_P  A-ATCCAATT TTGGATTTTG GTATTTCAAA ACGAAATTCC AAACTCTCAA
ATCC PRA-1  A-ATCC--TT TTGGATTTTG GTATTTCAAA ACGAAATTCC AAACTCTCAA


            ....|....| ....|....| ....|....| ....|....| ....|....|
                 110        120        130        140        150
AUS_ITS     CGATGGATGC CTCGGCTCGA GAATCGATGA AGGACGCAGC GAAGTGCGAT
POU07701_P  CGATGGATGC CTCGGCTCGA GAATCGATGA AGGACGCAGC GAAGTGCGAT
DQ516715_P  CGATGGATGC CTCGGCTCGA GAATCGATGA AGGACGCAGC GAAGTGCGAT
PAU07697_P  CGATGGATGC CTCGGCTCGA GAATCGATGA AGGACGCAGC GAAGTGCGAT
AF369974_P  CGATGGATGC CTCGGCTCGA GAATCGATGA AGGACGCAGC GAAGTGCGAT
AY305326_P  CGATGGATGC CTCGGCTCGA GAATCGATGA AGGACGCAGC GAAGTGCGAT
AY876302_P  CGATGGATGC CTCGGCTCGA GAATCGATGA AGGACGCAGC GAAGTGCGAT
DQ370474_P  CGATGGATGC CTCGGCTCGA GAATCGATGA AGGACGCAGC GAAGTGCGAT
AY295199_P  CGATGGATGC CTCGGCTCGA GAATCGATGA AGGACGCAGC GAAGTGCGAT
AY295180_P  CGATGGATGC CTCGGCTCGA GAATCGATGA AGGACGCAGC GAAGTGCGAT
AF149876_P  CGATGGATGC CTCGGCTCGA GAATCGATGA AGGACGCAGC GAAGTGCGAT
ATCC PRA-1  CGATGGATGC CTCGGCTCGA GAATCGATGA AGGACGCAGC GAAGTGCGAT


            ....|....| ....|....| ....|....| ....|....| ....|....|
                 160        170        180        190        200
AUS_ITS     AAGCACTGCG ATTTGCAGAA TTCCGTGAAC CAGTAGAAAT CTCAACGCAT
POU07701_P  AAGCACTGCG ATTTGCAGAA TTCCGTGAAC CAGTAGAAAT CTCAACGCAT
DQ516715_P  AAGCACTGCG ATTTGCAGAA TTCCGTGAAC CAGTAGAAAT CTCAACGCAT
PAU07697_P  AAGCACTGCG ATTTGCAGAA TTCCGTGAAC CAGTAGAAAT CTCAACGCAT
AF369974_P  AAGCACTGCG ATTTGCAGAA TTCCGTGAAC CAGTAGAAAT CTCAACGCAT
AY305326_P  AAGCACTGCG ATTTGCAGAA TTCCGTGAAC CAGTAGAAAT CTCAACGCAT
AY876302_P  AAGCACTGCG ATTTGCAGAA TTCCGTGAAC CAGTAGAAAT CTCAACGCAT
DQ370474_P  AAGCACTGCG ATTTGCAGAA TTCCGTGAAC CAGTAGAAAT CTCAACGCAT
AY295199_P  AAGCACTGCG ATTTGCAGAA TTCCGTGAAC CAGTAGAAAT CTCAACGCAT
AY295180_P  AAGCACTGCG ATTTGCAGAA TTCCGTGAAC CAGTAGAAAT CTCAACGCAT
AF149876_P  AAGCACTGCG ATTTGCAGAA TTCCGTGAAC CAGTAGAAAT CTCAACGCAT
ATCC PRA-1  AAGCACTGCG ATTTGCAGAA TTCCGTGAAC CAGTAGAAAT CTCAACGCAT
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AUS_ITS      ACTGCACAAA GGGGATCTTT CCTCTTTGTA CATACATATC AGTGTCGCTC
POU07701_P   ACTGCACAAA GGGGATCTTT CCTCTTTGTA CATACATATC AGTGTCGCTC
DQ516715_P   ACTGCACAAA GGGGATCTTT CCTCTTTGTA CATACATATC AGTGTCGCTC
PAU07697_P   ACTGCACAAA GGGGATCTTT CCTCTTTGTA CATACATATC AGTGTCGCTC
AF369974_P   ACTGCACAAA GGGGATCTTT CCTCTTTGTA CATACATATC AGTGTCGCTC
AY305326_P   ACTGCACAAA GGGGATTTAT CCTCTTTGTA CATACATATC AGTGTCGCTC
AY876302_P   ACTGCACAAA GGGGATTTAT CCTCTTTGTA CATACATATC AGTGTCGCTC
DQ370474_P   ACTGCACAAA GGGGATCTTT CCTCTTTGTA CATACATATC AGTGTCGCTC
AY295199_P   ACTGCACAAA GGGGATCTTT CCTCTTTGTA CATACATATC AGTGTCGCTC
AY295180_P   ACTGCACAAA GGGGATCTTT CCTCTTTGTA CATACATATC AGTGTCGCTC
AF149876_P   ACTGCACAAA GGGGATCTTT CCTCTTTGTA CATACATATC AGTGTCGCTC
ATCC PRA-1   ACTGCACAAA GGGGATCTTT CCTCTTTGTA CATACATATC AGTGTCGCTC


             ....|....| ....|....| ....|....| ....|....| ....|....|
                 260        270        280        290        300
AUS_ITS      TTCTTCCCGA TACAAACATT TTGTTG---T TAACGCAACT CAATGCTTTG
POU07701_P   TTCTTCCCGA TACAAACATT TTGTTG---T TAACGCAACT CAATGCTTTG
DQ516715_P   TTCTTCCCGA TACAAACATT TTGTTG---T TAACGCAACT CAGTGCTTTG
PAU07697_P   TTCTTCCCGA TACAAACATT TTGTTG---T TAACGCAACT CAGTGCTTTG
AF369974_P   TTCTTCCCGA TACAAACATT TTGTTG---T TAACGCAACT CAGTGCTTTG
AY305326_P   TTCTTCCCGA TACAAACATT TTGTTGGTTT TTAATCAACT CTATGCTTTG
AY876302_P   TTCTTCCCGA TACAAACATT TTGTTGATTT ACAATCAACA TTATGCTTTG
DQ370474_P   TTCTTCCCGA TACAAACATT TTGTTG---T TAACGCAACT CAATGCTTTG
AY295199_P   TTCTTCCCGA TACAAACATT TTGTTG---T TAACGCAACT CAATGCTTTG
AY295180_P   TTCTTCCCGA TACAAACATT TTGTTG---T TAACGCAACT CAATGCTTTG
AF149876_P   TTCTTCCCGA TACAAACATT TTGTTG---T TAACGCAACT CAATGCTTTG
ATCC PRA-1   TTCTTCCCGA TACAAACATT TTGTTG---T TAACGCAACT CAGTGCTTTG


             ....|....| ....|....| ....|....
                 310        320
AUS_ITS      TATCCCGCTT GGACTA---A CTCTTCG--
POU07701_P   TATCCCGCTT GGACTA---A CTCTTCG--
DQ516715_P   TATCCCGCTT GGACTA---A CTCTTCG--
PAU07697_P   TATCCCGCTT GGACTA---A CTCTTCG--
AF369974_P   TATCCCGCTT GGACTA---A CTCTTCG--
AY305326_P   TATCCCGCTT GAATT-CCGA TTTATTGG-
AY876302_P   TATCCCGCTT GGATT-CC-- TTTATTGG-
DQ370474_P   TATCCCGCTT GAACTA---A CTCTTCG--
AY295199_P   TATCCCGCTT GAACTA---A CTCTTCGG-
AY295180_P   TATCCCGCTT GAACTA---A CTCTTCGG-
AF149876_P   TATCCCGCTT GAACTA---A CTCTTCGG-
ATCC PRA-1   TATCCCGCTT GGACTA---A CTCTTCGG-
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