Aquatic Animal Health and Biosecurity Subprogram: ***Perkinsus olseni*** **in Abalone *Haliotis* spp.– development of fit-for-purpose tools to support its management**

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May 2021

FRDC Project 2016/009

ISBN 978-0-646-82822-0
Perkinsus olseni in Abalone – development of fit-for-purpose tools to support its management

Project No. 2016/009

2020

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In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

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The authors wish to express their appreciation to Dr Matt Padula from the University of Technology Sydney, whose assistance was absolutely critical with the proteomics work; Dr Laila Abudulai and Dr Michael Clarke from the Biological and Molecular Mass Spectrometry Facility, Centre for Microscopy, Characterisation and Analysis, University of Western Australia, for their contribution with the proteomics work; Dr Melinda Niedermayer for *in vitro Perkinsus* propagation; and Jerald Yam for contributions to bioinformatic analysis. We are also very grateful to Chris Dungan from the Cooperative Oxford Laboratory, USA, and to Nick Gudkovs for providing us with some isolates of *P. olseni*.

Finally, we would like to express our sincere thanks to the abalone industry (WA abalone industry: Ocean Grown Abalone Ltd, 888 Abalone Ltd and also Nick Savva and the Australian Abalone Growers' Association) for their support throughout this project.

Abbreviations

‰	Parts per thousand
AAGA	Australian Abalone Growers Association
AbHV-1	Abalone herpes-like virus
ABTS	2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-di-ammonium salt
ACN	Acetonitrile
ANACC	Australian National Algae Culture Collection
ANI	Average Nucleotide Identity
APC	Allophycocyanin
ASW	Artificial Sea Water
ATCC	American Type Culture Collection
AVG	Abalone Viral Ganglioneuritis
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
BUSCO	Benchmarking Universal Single-Copy Orthologs
CMCA	Centre for Microscopy, Characterisation and Analysis
COG	Clusters of Orthologous Genes
dbCAN	Metaserver for automated carbohydrate-active enzyme annotation
DME	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
ECP	Extracellular products
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay

EtOH	Ethanol
FAO	Food and Agriculture Organization for the United Nations
FBS	Foetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GVP	Gross value Production
HAT	Hypoxanthine-Aminopterin-Thymidine
HCl	Hydrochloric Acid
HRP	Horseradish Peroxidase
HSFM	Hybridomas Serum-free Medium
IDAS	Intensity Dependent Acquisition Speed
IFA	Immunofluorescence Assay
IgG	Immunoglobulin G
IL6	Interleukin 6
ITS	Internal Transcribed Spacer
KLH	Keyhole Limpet Hemocyanin
LC	Liquid Chromatography
LSU	Large Subunit ribosomal
mAb	monoclonal Antibody
mOsm	milliosmoles
MS	Mass Spectrometry
Nano-LC-ESI-MS/MS	Nano Liquid Chromatography – Electrospray Ionisation Tandem Mass Spectrometry
NSW	New South Wales
OIE	Office Internationale des Épizooties (World Organisation for Animal Health)
P/S	Penicillin/Streptomycin
PBS	Phosphate Buffer Saline
PE	Phycoerythrin
PEG	Polyethylene Glycol
QTOF	Quadrupole Time-Of-Flight
RFTM	Ray's Fluid Thioglycollate Medium
RT2	Real Time Re-Think
SD	Standard Deviation
SE	Standard Error
SCO	Single Copy Orthologous
SNP	Single Nucleotide Polymorphism
SA	South Australia
tBLASTn	See page 40
TFA	Trifluoroacetic Acid
U	Unit
WA	Western Australia

Executive Summary

What the report is about

The genus *Perkinsus* includes parasites of marine molluscs, two of which are notifiable to the World Organisation for Animal Health (OIE). Consequently, *Perkinsus* present in commercially important molluscs, including abalone *Haliotis* spp. in Australia, can represent a barrier to international trade. The ability to detect this parasite quickly is critical for both the abalone industry and government agencies. Although there are existing laboratory-based diagnostic tests for *Perkinsus*, such tests are time consuming and laborious. Innovative diagnostic methods that rely on antibodies, such as flow-cytometry and rapid immune-enzymatic tests (e.g. using filter paper card where there is a colorimetric change if the parasite is present), would be a great asset as a convenient, reliable, specific and rapid test. Therefore, the aim of the project was to develop an antibody able to bind to the abalone *Haliotis* spp. parasite *P. olseni*, so a test could be developed in the future.

However, developing an antibody presented some challenging prerequisites, including the propagation of the parasite *in vitro*, access to the *P. olseni* genome sequence, and the identification of *P. olseni* surface antigens. These prerequisites were achieved in this project allowing the generation of hundreds of candidate antisera in mice that were immunised with either whole *Perkinsus* cells or specific *P. olseni* surface antigens. After screening all antisera using enzyme-linked immunosorbent assay (ELISA), immunofluorescence, and flow-cytometry against different geographical isolates of *P. olseni*, as well as another *Perkinsus* species and negative controls, four antisera were selected and two monoclonal antibodies were produced.

This collaborative project was carried out by scientists from the Western Australia Department of Primary Industries and Regional Development (WA DPIRD, Dr Cecile Dang), the New South Wales Department of Primary Industries (NSW DPI, Dr Daniel Bogema, Dr Cheryl Jenkins, Dr Jeffrey Go), The Harry Perkins Institute of Medical Sciences (Kathleen Davern), the University of Western Australia (Eliot Hanrio), and the abalone industry. This work was undertaken from 2016 to 2020.

Background

Abalone are an economically important commodity ranking fourth nationally behind rock lobsters, salmon, and prawns, with a gross value production (GVP) of $190 million in 2018 (ABARES[1]). Furthermore, the production of abalone in Western Australia and Australia has the potential to increase further with the development of land-based and sea-ranching aquaculture facilities. The parasite *Perkinsus olseni* has caused significant mortalities in commercially important mollusc species worldwide including abalone (Liggins and Upston, 2010) and was associated with mass mortality events in Australia (Goggin and Lester, 1995). Moreover, the demise of blacklip abalone fisheries in New South Wales (NSW) since the early

[1] https://www.agriculture.gov.au/abares/research-topics/fisheries/fisheries-and-aquaculture-statistics/trade-2018

1990s was associated with *Perkinsus* (Liggins and Upston, 2010). *Perkinsus olseni* is listed internationally as a notifiable disease by the World Organisation for Animal Health (OIE)[2]. It has been reported in several mollusc species in NSW, Victoria, South Australia (SA), and WA and is to date the only species of *Perkinsus* infecting abalone in Australia.

Due to its ability to cause mass mortalities in molluscs, the presence of *P. olseni* can adversely affect the translocation of live animals as well as market access and be a significant barrier to international trade. Consequently, the abalone industry has a need to detect *P. olseni* infection on a quick and efficient manner. The first step in developing a new immunological diagnostic test is to develop an antibody specific to this parasite species. Achieving this would enable new diagnostic methods to be developed in the future.

Aims/objectives

(1) Propagate *Perkinsus olseni in vitro*
(2) Sequence the genome of *P. olseni*
(3) Develop *P. olseni* specific antibodies

Methodology

To achieve these three objectives, research was undertaken at four locations: the WA DPIRD laboratory facilities in Perth, the NSW DPI Elizabeth Macarthur Agriculture Institute at Menangle, the Centre for Microscopy, Characterisation and Analysis (CMCA) at the University of Western Australia, and the Harry Perkins Institute of Medical Research in Perth.

To achieve the first aim, we used the *in vitro* culture methodologies developed by Chris Dungan from the Oxford Cooperative Research Laboratory in the USA. Abalone and other molluscs were collected from the wild in Queensland and Western Australia. Only blood cockles, *Anadara trapezia*, from Queensland yielded successful proliferations of *P. olseni*. *In vitro* cultures of the parasite isolated from South Australia were donated by Nick Gudkovs from the CSIRO Australian Animal Health Laboratory (AAHL). Overseas isolates from Spain, Japan and New Zealand were obtained from Chris Dungan.

After optimising the extraction of DNA, the genomes of the parasites were sequenced using a combination of short and long read DNA sequencing methods (MinION, PacBio and Illumina) and a reference genome was generated. NSW DPI performed the sequencing and bioinformatics analyses.

Finally, the surface proteins of different geographical isolates were extracted using two different methods and analysed at the CMCA. The results were compared to the reference genome using bioinformatics at the University of Technology, Sydney. The identified and selected peptide sequences were sent to DGpeptides Co Ltd, China, where the corresponding

[2] https://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/chapitre_perkinsus_olseni.pdf

synthetic peptides were made. Monoclonal antibodies were developed by immunising three batches of mice using two different methods: whole parasite cells and synthetic peptides.

Results/key findings

The project was able to successfully propagate a new *P. olseni* isolate from Queensland and successfully cultured the isolates from Spain, Japan, New Zealand, and South Australia as well as *P. chesapeaki*, which was used as a negative control. We were unable to culture the Western Australian (WA) isolate. However, isolates from different geographical areas such as South Australia, New Zealand, Japan, and Spain were included to account for intraspecies diversity. Subsequent data generated in this study demonstrated that the lack of inclusion of a WA isolate affected neither the results nor the outcome of this project.

A complete reference genome for *P. olseni* from South Australia was generated as well as other *P. olseni* genomes for different geographical isolates and for *P. chesapeaki* from Queensland. This was the first time that the genomes of these two parasites were sequenced and they will provide invaluable insights into the physiology and origin of this parasite. They will also boost studies investigating host/parasite interactions and pathogenicity. The genomic data categorised the isolates into two groups based on their level of heterozygosity and gene content, with the Oceanian isolates (Australia and New Zealand) having a lower level of heterozygosity than the Eurasian isolates (Japan and Spain). With respect to the diagnostic methods, the internal transcribed spacer (ITS) region that is used for diagnostic tests worldwide, is repeated between 91 and 410 times in the *P. olseni* genome, which makes it a suitable region for presence/absence testing but unsuitable for quantification using qPCR to determine infection load of the parasite.

Two surface proteins classified as moonlighting proteins: an uncharacterised yet conserved hypothetical protein and a putative 60S ribosomal subunit protein L4, were common to all the *P. olseni* geographical isolates and were retained as candidates for antibody targets. Therefore, synthetic peptides from these proteins were produced and mice were immunised with these peptides. Mice were also immunised with whole *P. olseni* cells of the South Australian isolate. The immunisation process was repeated with three batches of mice over a two-year period resulting in the production of several antibodies specific to *P. olseni*, including different life stages of the parasite.

Implications for relevant stakeholders

Important implications for stakeholders as a result of the project include:

- **The *in vitro* culture of a new isolate of *P. olseni* from Queensland blood cockles.** This new culture will be invaluable for assessing differences in pathogenicity between parasites from different regions, as this isolate will be deposited to the most relevant cell culture collection such as the American Type Culture Collection (ATCC) or the European Collection of Authenticated Cell Cultures (ECACC) or Cell Bank Australia, thereby making it available to the scientific community.

- **The availability of the *P. olseni* genome.** This will provide access to a repertoire of thousands of genes, which will be invaluable for future studies investigating pathogenicity or host-parasite interactions using gene expression, transcriptomics that compare the genomic features of different geographical isolates, or that select abalone resistant to *P. olseni* by breeding.

- **Improved knowledge of existing diagnostic targets.** This study demonstrated that the ITS region, which is currently the target of all molecular tests for diagnosing perkinsosis infection worldwide, is repeated between 91 to 410 times in the *P. olseni* genome. This makes it suitable as a target for determining pathogen presence/absence but unsuitable for quantification. This has implications not only for the scientific community, but also for governments and non-governmental organisations and for industry with respect to the interpretation of diagnostic tests results. Indeed, there is currently no recommended test suitable for quantification at the species level. One hundred and six potential pairs of probe and primers were identified in this study and are suitable for developing a quantitative diagnostic test (all single copy targets in the genome). Therefore, the development of a molecular quantitative test is now facilitated and accessible to anyone. The benefit would be invaluable for the seafood industry if an infection is detected on farm in the future as a quantitative test would enable the identification of "hotspots" where the intensity of infection is the highest.

- **Development of tools for immunological diagnostic tests.** The development of an antibody binding to *P. olseni* obtained in this project is the first step towards establishing new diagnostic methods such as flow-cytometry tests or rapid enzymatic tests that could be used in the field (e.g. using filter paper card where there is a colorimetric change if the parasite is present). Indeed, it would be essential for industry to be able to detect an infection with *P. olseni* in a timely manner and by doing so, enable quicker management decisions in order to mitigate the impact on farm. Whilst the parasite does not currently induce clinical signs in Australian aquaculture premises, its presence can severely impact trade. In addition, climate change and an increased frequency of extreme weather events such as heat waves or droughts could increase both prevalence and intensity of infection. Indeed, this parasite demonstrates a higher proliferation rate and increased virulence at warmer temperatures. Therefore, early detection on farms (followed by state and federal laboratory confirmation) would be beneficial not only in quickly managing and mitigating an infection on farm but also in wild populations.

- **Improved tools to advance disease preparedness.** The abalone industry will directly and indirectly benefit from the tools and information that have been generated by this project, which constitute the foundation for further work in order to enhance industry preparedness to *P. olseni* outbreaks. Indeed, further studies could (1) develop a qPCR test able to quantify an infection; (2) develop an antibody-based test, which would improve early detection of *P. olseni* on farm; (3) understand the mechanics behind an infection using transcriptomics, which would be invaluable to identify risk factors associated with disease development or producing *P. olseni*-resistant abalone spat as part of a selective breeding program.

Recommendations

Industry and government stakeholders have recognised the need for rapid and reliable assessment of infection status and parasite load in abalone tissues, haemolymph, and water samples, which would provide critical tools for surveillance and 'on-farm' management of *Perkinsus* infections in their stocks.

- Further validation work for the antibodies produced in this study would be necessary before commencing development of a rapid pond-side (point of care) test.
- Once the antibodies are fully validated, it is recommended that immunodiagnostic tests are developed. For instance, tests could be developed using ELISA, which would be quantitative, or a paper test (e.g. immunochromatography test) could be developed for presence/absence detection.
- If a quantitative molecular diagnostic test is deemed necessary by state or federal authorities, it could readily be developed using the list of probe/primers generated in this report.

Keywords

Perkinsus olseni, *Perkinsus chesapeaki*, *Haliotis* spp., genome, antibodies, *in vitro* culture, proteomics, diagnostic test

Introduction

Abalone *Haliotis* spp. are distributed globally across many countries, however, most of the Australian species of commercial significance are found in the southern waters of Australia, ranging from the coast of New South Wales, around Tasmania and as far north as Shark Bay (approximately 800 km north of Perth) in Western Australia.

Global abalone aquaculture production has quadrupled between 2006 and 2016, expanding from 34,867 tonnes to 162,771 tonnes (Food and Agriculture Organization for the United Nations (FAO), 2019). One of the countries that has seen a significant expansion of abalone aquaculture is China. In Australia, Tasmania, South Australia and Victoria are the major states involved in temperate abalone culture, with some operations now underway in Western Australia. Australia contributed 0.6% of the global farmed abalone production in 2016/17 (Cook, 2018) There are established farms for two temperate species, the blacklip abalone (*Haliotis rubra*) and greenlip abalone (*H. laevigata*) and their hybrid (*H. laevigata* x *rubra*) in Tasmania, South Australia, Victoria and Western Australia. The Australian production of cultured abalone is expected to grow and is projected to reach $209 million by 2023-24 (Department of Agriculture, Water and the Environment, 2019). Abalone aquaculture in Australia is important economically, supporting employment and productivity in regional areas. However, like any other aquaculture industry, an important limiting factor of increased production is disease.

In 2005-2006, abalone viral ganglioneuritis (AVG) caused mass mortalities in Victoria. AVG is caused by a virulent herpes-like-virus (AbHV-1) that affects the nervous tissue of abalone causing rapid mortality (Hooper *et al.* 2007, Savin *et al.* 2010). Outbreaks of AVG in both farmed and wild abalone populations in Australia were associated with the rapid onset of high mortality rates (up to 90%) in all age classes. The known historic Australian distribution of the virus includes Victoria and Tasmania. To date outbreaks have occurred in Victorian farmed and wild populations, while in Tasmania they have occurred in farmed populations and in processing locations, with presence of the virus also detected in healthy, wild stock.

There are other abalone diseases that are also caused by *Vibrio* spp., flavobacteria and non-specific fungal infections. The impacts of *Vibrio* spp. are usually confined to individual farms, while the consequences of flavobacteria and non-specific fungal infections are not considered as serious. *Vibrio* spp. and flavobacteria are ubiquitous in the marine environment. Vibriosis, associated with mortalities, has been reported from farms in South Australia, Tasmania, Western Australia and Victoria (Australian Abalone Growers Association (AAGA), personal communication). Outbreaks of the disease are thought to be related to water temperature and farm practices, as they are with non-specific fungal infections.

Perkinsosis or infection with *Perkinsus* spp. affects a wide range of molluscs worldwide and has caused mass mortalities of commercially important molluscs globally. Seven species of *Perkinsus* have been described to date: *P. marinus, P. olseni, P. qugwadi, P. chesapeaki, P. mediterraneus, P. honshuensis*, and *P. beihaiensis* (Soudant *et al.*, 2013). *Perkinsus marinus*

was the first described species that was reported after severe oyster (*Crassostrea virginica*) mortalities in the USA in 1946 (Ray, 1996). The second described *Perkinsus* species, *P. olseni* (Lester and Davis, 1981), was reported from the abalone *Haliotis rubra rubra* in South Australia. Both *P. marinus* and *P. olseni* are on the international list of notifiable diseases by the World Organisation for Animal Health and they are both known to cause mass mortalities in their host populations. *Perkinsus olseni* has since been reported in about 27 species of mollusc including oysters, mussels, clams and abalone over a wide range of geographical locations such as Europe, Asia-Pacific and south America (Arzul *et al.*, 2012; Azevedo, 1989; Casas *et al.*, 2002; Cremonte *et al.*, 2005; Dang *et al.*, 2010; Goggin and Lester, 1995; Pagenkopp Lohan *et al.*, 2018; Park *et al.*, 2005; Robledo *et al.*, 2000)

In Australia, Perkinsosis has affected both abalone fisheries and aquaculture stock. In greenlip abalone (*H. laevigata*) culture facilities, two outbreaks of *P. olseni* occurred, causing 30 to 40% mortality in the 3 – 4 cm shell length group (Goggin and Lester, 1995). In both cases *H. rubra* taken from an infected site had been introduced to the facility prior to the outbreaks (Goggin and Lester, 1995). While this disease has caused minor mortalities in farmed stock in Australia (Goggin and Lester, 1995), it has caused significant mortalities in wild stock. Indeed, this parasite has been suspected of causing mortalities in abalone and associated with devastated stocks of *H. laevigata* from the Gulf of St. Vincent side of the Yorke Peninsula, South Australia (Lester, 1986; O'Donoghue *et al.*, 1991). *Perkinsus olseni* has also been deemed responsible for the demise of black-lip *H. rubra* populations in NSW between 1992 to 2000s (Liggins and Upston, 2010). In some areas such as Jervis Bay and Port Stephens, stocks of abalone were depleted by 74% (Liggins and Upston, 2010).

Perkinsus olseni proliferates in tissues of the host and may produce pustules (spherical brown abscesses up to 8 mm in diameter containing a caseous creamy-brown deposit) in the foot and mantle of *H. rubra* and *H. laevigata* thereby reducing the market value. It is known to induce clinical signs in abalone in South Australia and NSW but not in Western Australia. Liggins and Upston (2010) reported that warmer temperatures are associated with greater infection intensity and that water temperature is a contributing factor in determining the disease progression and mortalities. *Perkinsus olseni* has a unique life cycle consisting of trophozoites, hypnospores and zoospores without any intermediate host (Auzoux-Bordenave *et al.*, 1995). *Perkinsus* spp. can be directly transmitted between molluscan hosts (Villalba *et al.*, 2004). Prezoosporangia that escape from rupturing pustules (abscesses) or decaying dead abalone undergo further development to zoosporangia in seawater. Within nine days at 20°C and three days at 28°C, hundreds of motile, biflagellated zoospores (approximately 3 × 5 µm) leave the zoosporangium and can infect a new host (Goggin *et al.*, 1989).

Given the potential risk that *P. olseni* poses to the abalone fishing and aquaculture industries (and other mollusc industries) in Australia, it is critical to be able to detect it quickly and reliably. Moreover, if the impact of *P. olseni* on aquaculture stock has been limited to date, it cannot be excluded that modification of the environment due to climate change, such as an increase of temperature, which is known to promote *P. olseni* infection, modulates the infection intensity, prevalence and thereby increases the risk of infection. The abalone aquaculture and

fisheries industry, as well as government stakeholders, have recognised the need for rapid and reliable assessment of infection status and intensity in abalone tissues, haemolymph, and water samples in order to provide critical tools for potential surveillance and 'on-farm' management of *Perkinsus* infections.

This collaborative project, aimed at producing an antibody able to recognise *P. olseni*, lays the platform for future development of diagnostic tests that improve the detection of this parasite. This will have benefit in the long-term, not only for the development of the aquaculture sector, but also to assist wild fisheries management.

The primary objective of this project is to obtain an antibody that can bind to *P. olseni*-specific surface antigens. However, this requires preliminary steps such as the ability to culture the parasite *in vitro*, generation of a reference genome sequence, and then immunisation of mice for diagnostic antibody production. With this as the aim, this report is divided into the three chapters as listed:

(1) Propagating *Perkinsus olseni in vitro*
(2) Sequencing the *Perkinsus olseni* genome
(3) Development of *Perkinsus* specific antibodies

Objectives

The original objective of the project was to “develop and evaluate optimised diagnostic capabilities for Australian *Perkinsus* spp. isolates for sampling and testing based on estimates of sensitivity and specificity to meet accepted standards for detecting infection and for testing for freedom”.

Given that the data generated by the sequencing of the genome revealed that the molecular tests recommended by the OIE for detecting and not quantifying the parasite are acceptable, it was possible to remove *improvement of molecular tests* as an objective of the project. Also, in accordance with the time required to perform the work, with the complexity and the difficulties in generating antibodies against *Perkinsus olseni* using animals, the objectives were revised, and three new objectives were added as follows:

(1) Propagating *Perkinsus olseni in vitro*
(2) Sequencing the *Perkinsus olseni* genome
(3) Development of *Perkinsus*-specific antibodies

Whilst the project could not deliver a new serological diagnostic test, which was originally planned, it did establish the foundation to develop this test with the production of several antibodies recognising *P. olseni*.

Part 1

Propagating *Perkinsus olseni in vitro*

1.1. Introduction

Developing an antibody binding specifically to *P. olseni* surface antigens necessitates to be able to propagate the parasite *in vitro*, so enough material can be obtained for genome sequencing.

Cell culture is an aseptic *in vitro* technique where living cells are cultured under precise and controlled environmental conditions. The medium supplies all the nutrients and growth factors required for the growth and proliferation of the cells. Cell cultures have provided the scientific community with some invaluable tools for basic research such as the investigation of cellular functions and processes. For instance, most of the molecular pathways that take place inside a cell were elucidated using cell culture techniques.

Several species of *Perkinsus* have been propagated *in vitro* such as *P. marinus* (Peyre *et al.*, 1993), *P. olseni* (Casas *et al.*, 2002), *P. chesapeaki* (Burreson *et al.*, 2005; Dang *et al.*, 2015), *P. mediterraneus* (Casas *et al.*, 2008), and *P. beihaiensis* (Moss *et al.*, 2008). *In vitro* propagation of this parasite has enabled molecular phylogenetic studies (Burreson *et al.*, 2005) and investigation of the impact of environmental conditions on the growth and proliferation of the parasite. It has also allowed to obtain enough material for sequencing the genome of the parasite or to perform trancriptomics studies. Having access to *in vitro* parasite cultures provides researchers with unlimited research opportunities. Most of these cultures of *Perkinsus* have been deposited with the American Type Culture Collection (ATCC), Manassas, Virginia, and are available to scientists worldwide. They have contributed in a better understanding of the cellular processes of this parasite.

1.2. Materials and Methods

1.2.1. Culture trials for new Australian *Perkinsus olseni* isolates

1.2.1.1. Sampling sites in Australia

In Western Australia, the first collection of mollusc occurred in 2016. Greenlip abalone, *Haliotis laevigata,* were collected from a farm (n = 30) as well as from a wild population at Augusta on the South coast of Western Australia (n = 20). Roe's abalone *Haliotis roei* were sampled from a farm on the south coast (n = 10), and also from the Western Australian Fisheries and Marine Research Laboratories at Hillarys Boat Harbour (n = 25). One species of cockle, *Anadara* sp. (n = 30), and three species of clams (n = 30; *Costallista impar*, *Circe plicatina* and another unidentified species) were collected from Shark Bay, approximately 800 km north of Perth. Animals were transported as soon as possible to the laboratory for analysis.

Those molluscs did not lead to any propagating cultures of *Perkinsus*, so greenlip abalone *Haliotis laevigata* were collected from an ocean ranching farm (n = 30) in Augusta on 6th of April 2018 and from the wild in Augusta (n=73) on 18th of March 2018. They were transported to the laboratory in Perth and thirty animals from the sea ranching site and ten animals from

the wild were processed for cell culture proliferation using the protocol detailed in Burreson et al (2005). The remaining animals were housed in an aquarium tank and were progressively used over time for cell culture proliferation attempts.

In Queensland, cockles *Anadara trapezia*, were collected from Wynnum near the mouth of the Brisbane River in Moreton Bay, a site known for its high intensity of perkinsosis. The first batch of cockles (n = 60) was sampled on 28th June 2017 and sent to the aquatic Animal Health Research Laboratory in Perth. As this first attempt failed, more cockles (n = 50) were collected from Wynnum on the 4th January 2018 and processed for culture immediately after collection at the Animal Health Laboratory of the University of Queensland in Brisbane.

For each animal, duplicate gill tissue samples were excised aseptically to serve as inocula for *in vitro* pathogen cultures in Ray's fluid thioglycollate medium (RFTM). Diagnostic assays for *Perkinsus* spp. parasites were performed on replicate gill tissue biopsies from all collected animals (Ray, 1952) in order to identify promising inocula for propagation of *in vitro* isolates. Paired gill tissue biopsies from each of the sampled animals were also preserved in 95% ethanol (EtOH) for DNA extraction to support subsequent PCR and sequencing assays.

1.2.1.2. In vitro *propagation of* P. olseni *isolates*

For *in vitro* propagation of *Perkinsus* spp. parasites, two dedicated gill tissue samples were aseptically excised to serve as culture inocula from each of the sampled molluscs. Both gill tissue samples from each mollusc were incubated in 2 mL of RFTM in 24-well culture plates at 27°C in the dark for 2 days. To identify tissues with the highest abundances of enlarged *Perkinsus* spp. cells, one member of each pair of RFTM-incubated gill tissues was stained with Lugol's iodine solution for evaluation by light microscopy. Duplicate, unstained RFTM-incubated gill tissues from animals showing the highest abundances of *Perkinsus* spp. cells, were transferred to 29‰, 1:1 Dulbecco's modified Eagle's medium (DME):Ham's F-12 culture medium containing antimicrobials (DME/F12-3, Burreson *et al.* 2005), whose osmolality was equivalent to that of slightly diluted seawater (29 ‰ = 850 milliosmoles (mOsm) kg^{-1}). Tissue samples were disrupted and suspended by pipette-trituration, and 2 ml $well^{-1}$ of the resulting suspensions were dispensed at into replicate wells of sterile, lidded, 24-well culture plates. Antimicrobials and their concentrations used in the primary culture medium included penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}), gentamicin (50 µg ml^{-1}), chloramphenicol (5 µg ml^{-1}), and nystatin (200 U ml^{-1}) (Dang, *et al.* 2015). Covered well-plates containing primary isolate inocula were incubated at 27 °C in the dark, with daily microscopic observations to detect proliferation of *Perkinsus* spp. cells. If animals presented a very low intensity of infection, they were not processed for cell proliferation as there is evidence (Chris Dungan's personal communication and PI's previous experiences) that only heavily infected animals yield successfully proliferating cells.

Once isolate proliferation was confirmed in primary cultures, proliferating cell populations were sub-cultured through several passages in which antimicrobials were sequentially reduced,

and finally eliminated, from the culture media, the aim being to obtain an axenic culture (a culture that is free from any other living organisms other than the one required). During this process, isolates were cryopreserved and preserved in ethanol for further molecular work. Monoclonal cultures were obtained using a 96-well plate by diluting the stock culture until only 1 cell per well was achieved.

For cryopreservation, early stationary phase cells were pelleted by centrifugation (5 min, 20°C, 240 × *g*) and resuspended in a freezing medium composed of the propagation medium supplemented to 8% (v/v) with cell culture grade dimethyl sulfoxide (DMSO) and 6% (v/v) foetal bovine serum (FBS). For cryopreservation, suspensions were frozen to -75°C at approximately -1°C min^{-1}, tested for adequate post-thaw viability after 24 h, and transferred to -80°C for short to medium term storage or to liquid nitrogen for long-term storage.

From this culture study, two isolates of *Perkinsus* spp. were obtained, coded as Qld 39 and Qld 46.

1.2.1.3. In vitro *morphometrics and cell cycle characteristics*

For comparison with previous morphometric data from *P. olseni* isolates, 50 live cells of several types from each flask were measured microscopically to estimate mean dimensions of trophozoites, schizonts, zoosporangia, and other cells after 72 h of proliferation. Proliferative zoosporangia and schizonts in flask cultures were also differentially enumerated to estimate their proportions among those cell populations. Means and standard deviations were calculated and reported for morphometric variables. Non-motile cell types of *P. olseni* isolates were imaged directly and live using an inverted light microscope.

1.2.1.4. Molecular-genetic analysis of DNAs from clonal in vitro *isolates and host mollusc gill tissue*

Deoxyribonucleic Acid (DNA) was extracted from cells of proliferating isolates, using the DNeasy Tissue Kit (Qiagen) following the manufacturer's protocol. Internal transcribed spacer (ITS) regions of the ribosomal RNA gene complex were amplified by PCR with the *Perkinsus* genus-specific primers PerkITS-85 and PerkITS-750 (Casas, *et al.* 2002) in 25 µl reactions containing PCR buffer that included bovine serum albumin (BSA) (0.2 mg ml^{-1}), $MgCl_2$ (1.5 mM), dNTPs (0.2 mM), primers (0.1 µM), and *Taq* polymerase (0.025 U/µl). Cycling conditions included denaturation at 95 °C for 4 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 65 C for 1 min; with a final extension at 65 °C for 5 min. Genus-specific PCR amplifications of rRNA large subunit genes (LSU) and type 1 actin genes (actin) were performed as previously described (Moss *et al.* 2008), using the primers listed in Table 1. PCR products were stained with SYBR safe, separated on 1.5% agarose gels, and visualized by UV illumination.

Table 1: PCR primers and DNA probes used in this study, including the *Perkinsus* genus-specific ITS, LSU, and actin primers, *Perkinsus* species-specific ITS primers.

Target	Primer/Probe	Sequence (5'-3')	Product length (bp)	Reference
Perkinsus genus ITS	PerkITS-85 PerkITS-750	CCG CTT TGT TTR GMT CCC CAT CAT TAG AAG GCC TGA TGT	~ 675	Casas *et al.* (2002)
Perkinsus genus actin	PerkActin1-130F PerkActin1-439R	ATG TAT GTC CAG ATY CAG GC CTC GTA CGT TTT CTC CTT CTC	330	Moss *et al.* (2008)
Perkinsus genus LSU	PerkITS2-217 LSU-B	GTG TTC CTY GAT CAC GCG ATT ACG AAC GAT TTG CAC GTC AG	1,170	Moss *et al.* (2008)
P. chesapeaki ITS	PchesITS-5 PchesITS-3	CAA GAA GGA CTG CGC TAG GTA ACG ATT CTG AGA CCA AAG C	~ 177	Reece *et al.* (2017)
P. olseni ITS	PolsITS-140F PolsITS-600R	GAC CGC CTT AAC GGG CCG TGT T GGR CTT GCG AGC ATC CAA AG	~ 480	Moss *et al.* (2006)
Universal non-metazoan	18S-EUK581-F 18S-EUK1134-R	GTGCCAGCAGCCGCG TTTAAGTTTCAGCCTTGCG	~ 600	Carnegie *et al.* (2003)

The presence of *P. olseni* and *P. chesapeaki* were tested by species-specific PCRs targeting rDNA ITS regions, as both species are present in Australia. For *P. olseni*, the primers PolsITS-140F and PolsITS-600R (Moss *et al.*, 2006), (Table 1) were used under slightly modified conditions that included increasing BSA concentration to 0.2 mg ml^{-1}, and using 40 amplification cycles of 94 °C for 1 min, 57 °C for 1 min, and 65 °C for 3 min; with a final extension at 65 °C for 10 min. Amplification of *P. chesapeaki* DNA was performed using the primers PchesITS-5 and PchesITS-3 (Table 1) in 25-µl reactions containing PCR buffer that included BSA (0.2 mg ml^{-1}), $MgCl_2$ (1.5 mM), dNTPs (0.2 mM), primers (0.1 µM), and *Taq* polymerase (0.24 U $µl^{-1}$). Cycling conditions included denaturation at 94 °C for 4 min followed by 40 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 90 s; with a final extension at 72 °C for 5 min. For amplification of *P. chesapeaki* ITS region sequences from infected host tissue DNA, an initial amplification was performed with the *Perkinsus* genus-specific primers PerkITS-85 and PerkITS-750 as described above. From genus-specific reaction mixtures containing PCR products, 0.25 µl were used as template in subsequent nested PCR reactions with the *P. chesapeaki*-specific primers, as described above.

DNA fragments amplified by *Perkinsus* genus-specific PCRs from clonal cultures and host tissue or the *P. chesapeaki* species-specific PCR (ITS) from host tissue were purified for sequence analysis, using a QIAGEN QIAquick® PCR purification kit according to the manufacturer's protocol. PCR products were sequenced bidirectionally using an AB3730xl capillary sequencer at the Australian Genome Research Facility in Perth. All sequences were quality scored and visually checked for possible sequencing errors using Geneious software (https://www.geneious.com). They were then analysed using the basic local alignment search tool (BLASTn) online (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

1.2.2. Culture of existing *P. olseni* isolates

With no success in the proliferation of the WA isolate, and considering that we needed to access as many isolates of *P. olseni* from different geographical locations as we could in order to be able to develop the test, a range of isolates were imported as a live culture under the permit # 0002119934 issued by the Department of Agriculture, Water and Environment (Table 2). Those isolates of *P. olseni* originated from Europe, New Zealand, and Japan and are referred to by their American Type Culture Collection (ATCC) identification number.

Table 2: Isolates of *P. olseni* donated by Dr Chris Dungan and used in this study.

Isolate reference number	Isolate identity	Source origin	Source host	Source region	Source site	Source tissue
ATCC PRA-31/E3	*Perkinsus olseni*	Spain	*Ruditapes decussatus*	Galicia	Ria de Arousa	gill
ATCC PRA-179	*Perkinsus olseni*	Japan	*Ruditapes philippinarum*	Mie	Gokasho Bay	gill
ATCC PRA-205	*Perkinsus olseni*	New-Zealand	*Austrovenus stutchburyi*	North island	Mangemangaroa Stream	mantle& gill
ATCC PRA-207	*Perkinsus olseni*	New-Zealand	*Austrovenus stutchburyi*	North island	Mangemangaroa Stream	mantle& gill

An additional isolate, SA-00978-12T from South Australia was provided by Nick Gudkov from CSIRO AAHL and subsequently cultured in the Fish Health Laboratory in Perth. This isolate was used to build our reference genome for *P. olseni* on the basis that it had been isolated from Australian abalone and therefore was likely to be of highest relevance to *P. olseni* in Australian abalone industries.

1.2.3. Summary of *Perkinsus* sp. isolates used in this study

To summarise, we used in this project the isolates PRA-205, PRA-207, PRA-179, PRA-31/E3 that we imported from the US into Australia (Table 2) as well as the South Australian isolate (00978-12T) and 2 Queensland isolates (Qld 39 and Qld 46). We also used the Queensland isolate of *P. chesapeaki* (ATCC PRA-425) as a negative control for all the proteomic and genomic studies. Those isolates were cultured in 1:1 DME:Ham's F-12 medium containing 100 $U.ml^{-1}$ of penicillin and streptomycin as described in Burreson *et al.* (2005). No isolate from WA was used in the study because of an unsuccessful cell proliferation.

1.3. Results and Discussion

1.3.1. Culture of Australian isolates

1.3.1.1. In vitro *propagation and identification of* P. olseni *isolates*

Perkinsus spp. cells were detected with a high intensity in WA in 2015, however wild abalone sampled from the same locations in 2018 during this study had a low prevalence and intensity of infection. Almost no *Perkinsus* spp. parasites were detected in the first batch of abalone from the sea ranching site in Augusta. Only one parasite cell was observed by microscopy in three animals, highlighting the very low intensity of infection. When duplicate gill tissues of mollusc species presented a low intensity of infection, they were either not processed for cell proliferation or when they were, cell proliferation was unsuccessful. The cells observed by microscopy are referred to as *Perkinsus* sp. although we previously demonstrated the presence of *P. olseni* in greenlip abalone from this area using molecular tools. The cells observed had a morphology consistent with that of *Perkinsus*.

Some roe's abalone (*Haliotis roei*) collected at the research facility in Hillarys Boat harbor presented with a high intensity of infection and were processed for cell culture. After 10 days, those primary cultures revealed some signs of proliferation (Figure 1). Zoospores were seen swimming in the wells (isolate H4A4) while signs of division by schizogony were observed in isolates H4B4 and H4B2.

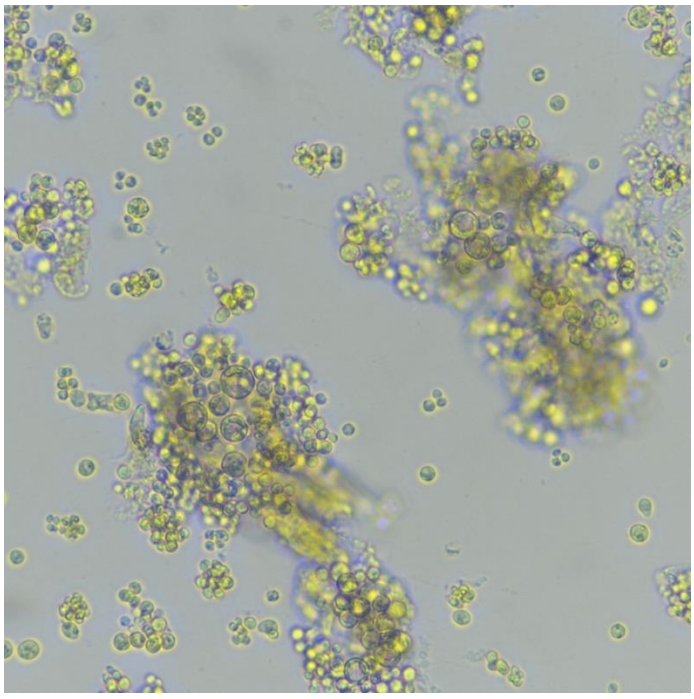

Figure 1: Proliferative *Perkinsus*-like cells from sample H4B2 isolated from *Haliotis roei*.

After subculture and confirmation of proliferation of isolates H4A4, H4B4, and H4B2, DNA was extracted and PCRs for species identification were performed. The three PCRs targeting *Perkinsus* genus (LSU, actin and ITS) as well as species specific PCRs for *P. olseni* and *P. chesapeaki* did not result in a PCR product, leading to some suspicion about the nature of those cells based on their morphology. Subsequently, the PCR from Carnegie *et al.* (2003) targeting protozoan and fungus parasitic organisms was used and produced a positive 600bp band. This product was sent for sequencing and the BLASTn analysis revealed that those cells showed 100% identity with organisms belonging to the to the Thraustochytrids group (*Thaustochytrium* sp. and *Aurantiochytrium* sp.), which are known parasites of abalone. By microscopy, *Thaustochytrium sp.* appear different from *Perkinsus* cells. Indeed, the thraustochytrid parasite cells refract light differently from *P. olseni*, with the thraustochytrid being more refractile.

The duplicate gill tissues from cockles, *A. trapezia*, collected in Moreton Bay in June 2016, yielded some proliferation from two individual cockles (cockle # 8 and 11), however we experienced problems with contamination as a fungus was also proliferating. Despite all our efforts to save the culture and eliminate the fungus-like organism, all our cultures were lost. We hypothesized that the two days the cockles spent in transportation was the source of the problem. Thus, the PI went to Brisbane in January 2018 to collect more cockles and processed them immediately post-collection at the animal health laboratory at the University of Queensland. This proved successful as several isolates of *Perkinsus*-like organism from three individuals proliferated successfully (cockles # 6, 7 and 46; Figure 2) and without any contamination by other microorganisms.

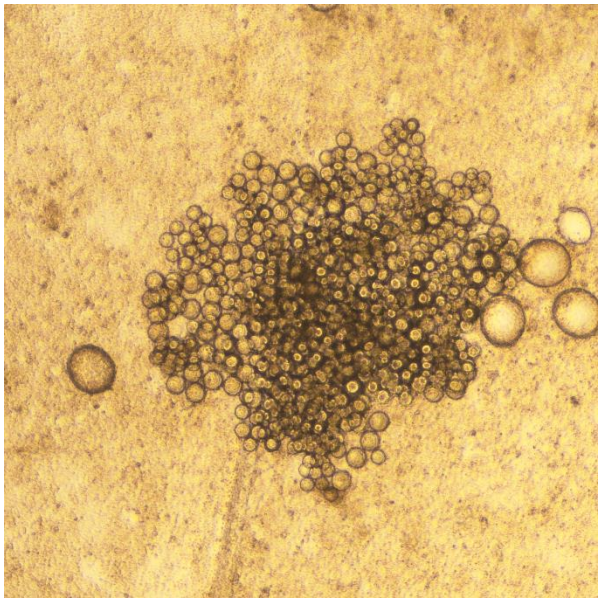

Figure 2: Proliferative *Perkinsus* cells isolated from the cockle *Anadara trapezia*.

Species-specific PCR of DNAs isolated from three clonal isolate cultures proliferating in flasks from *A. trapezia* cockles, gave strong amplifications with *P. olseni*-specific primers. Samples from cockle 6 (1/4) also yielded amplification when *P. chesapeaki* primers were used. DNAs from two clonal isolate cultures (cockle 7 and 46) amplified with the *P. olseni*-specific primers, but not with the *P. chesapeaki*-specific primers. Amplicons from the *P. chesapeaki*-specific and *P. olseni*-specific PCR of clonal isolate cultures were sequenced. BLASTn analysis of the ITS region from the genus assay of host tissue DNA indicated that the sequences were most similar to *P. olseni* and *P. chesapeaki* sequences in GenBank.

A nested PCR using species-specific primers was also performed with the ITS-genus PCR products and confirmed that the two isolates 7 and 46 are *P. olseni* and do not contain any *P. chesapeaki* DNA.

1.3.1.2. In vitro *isolate morphometrics and cell cycle characteristics*

In vitro P. olseni isolates from *A. trapezia* gill tissues had all *in vitro* cell types characteristic of other *Perkinsus* spp. isolates. Means, ranges, and standard deviations for dimensions and proportional frequencies of those *in vitro* cell types are listed in Table 3.

Table 3: Diameters of *Perkinsus olseni in vitro* cell types isolated from *A. trapezia* (± Standard Deviation (SD)) (n = 50).

		Isolate from cockle #7	Isolate from cockle #46
Trophozoite	Mean diameter (µm)	75.2	59.5
	SD	25.7	18.8
Schizont	Mean diameter (µm)	77.4	89.1
	SD	16.9	37.7
Zoosporangia	Mean diameter (µm)	63.9	58.5
	SD	12.6	8.8

Mature vacuolated, signet-ring trophozoites were most abundant, with diameters that ranged from 27.7 to 169.7 µm with means of 75.2 ± 25.7 µm and 59.5 ± 18.8 µm for isolates 7 and 46, respectively (Table 3). Trophozoites showed a large central vacuole, an eccentric nucleus, and emarginated cytoplasm that contained optically refractive spherical bodies of various sizes (Figure 3). Trophozoites predominantly proliferated by schizogony. The intracellular contents of mature trophozoites acquired an amorphous, granular appearance before showing internal cytokinetic divisions.

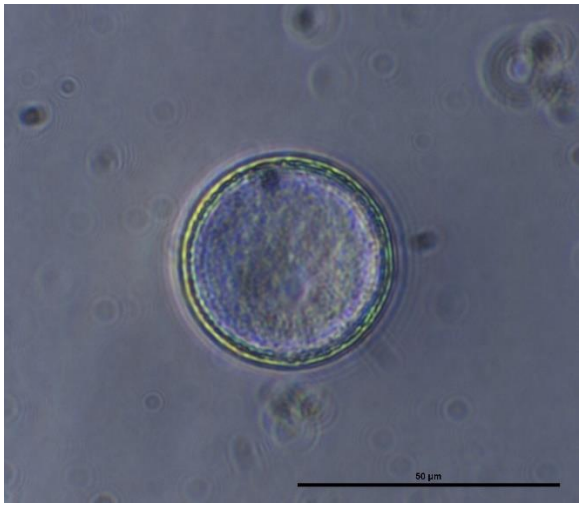

Figure 3: Trophozoite isolated from the cockle # 46.

Schizonts had diameters (± SD) ranging from 38.2 to 211.0 µm with means of 77.4 ± 16.9 µm and 89.1 ± 37.7 µm for isolates 7 and 46 respectively (Table 3). Once formed internally, sibling progeny cells enlarged, lysed the schizont cell wall, and spilled forth as masses of small (5 to 10 µm), highly adherent trophozoites that subsequently dissociated and enlarged to proliferate typically by schizogony themselves (Figure 3). Durably adherent clusters of sibling trophozoites were very abundant.

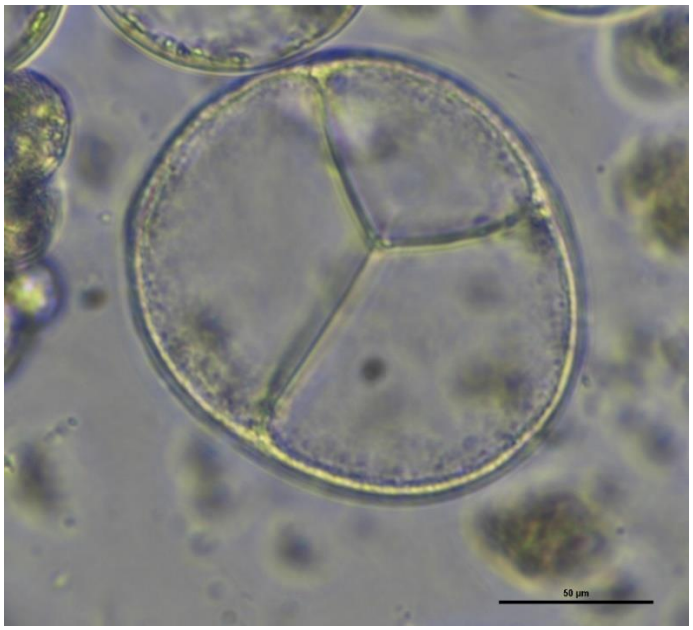

Figure 4: Trophozoite in an early stage of schizogony.

Zoosporulation occurred in trophozoites with diameters larger than 39.0 µm followed by extrusion of a discharge tube from plugged pores in their cell walls (Figure 5). Mean diameters were 63.9 ± 12.6 µm and 58.5 ± 8.8 µm for isolates 7 and 46, respectively (Table 3). Following discharge pore and tube formation, central vacuoles disappeared as the sporoplasm condensed

into a central opaque and granular mass that subsequently divided by successive bipartitions to yield zoosporonts (Figure 5).

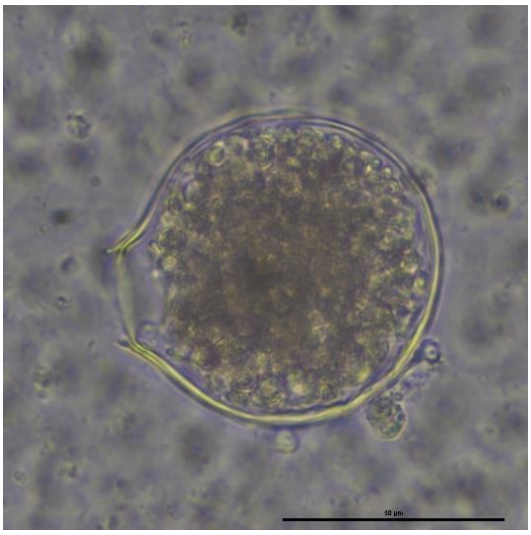

Figure 5: Zoosporangium displaying discharge tube formation (scale bar 50 µm).

Maturing zoosporonts developed paired flagella and were vigorously motile within the zoosporangium. They exited the zoosporangium via the discharge pore. Zoospores remained motile for approximately 48 h before shedding their flagella and becoming small trophozoites.

1.3.2. Culture of *P. olseni* overseas isolates

All the isolates received from the USA that were donated by Dr Chris Dungan successfully proliferated *in vitro* and were subsequently used for this project.

1.4. Conclusion

In total, eight isolates of *P. olseni* listed in Table 4 below were available and were used throughout the rest of the project. *P. olseni* isolates were not obtained from WA mollusc samples, however the need to examine diverse isolates was addressed by including *P. olseni* isolates from five different geographical areas (2 within Australia and 3 overseas). *Perkinsus chesapeaki*, which was previously isolated by the PI, was used as a negative control in all subsequent tests.

Based on our experiences, we recommend that attempts to isolate *Perkinsus* sp from molluscs should focus on samples with a high intensity of *Perkinsus* infection identified through parallel RFTM testing and that only freshly sampled mollusc tissues are used to minimise contamination of cell cultures. Cell species identification through molecular testing is also

critical to ensure that the correct *Perkinsus* target has been isolated rather than incidental parasites that may be present in the initial sample.

Table 4: Isolates of *Perkinsus* proliferating *in vitro* and available for the project.

Isolate reference number	Isolate identity	Source country	Source host	Source region	Source site	Source tissue	Antibody type
ATCC PRA-31/E3	*P. olseni*	Spain	*Ruditapes decussatus*	Galicia	Ria de Arousa	gill	monoclonal
ATCC PRA-179	*P. olseni*	Japan	*Ruditapes philippinarum*	Mie	Gokasho Bay	gill	monoclonal
ATCC PRA-205	*P. olseni*	NZ	*Austrovenus stutchburyi*	North island	Mangemangaroa Stream	mantle& gill	monoclonal
ATCC PRA-207	*P. olseni*	NZ	*Austrovenus stutchburyi*	North island	Mangemangaroa Stream	mantle& gill	monoclonal
SA-00978-12T	*P. olseni*	AUS	*Haliotis laegivata*	South Australia	Taylor Island	muscle	monoclonal
Qld-46	*P. olseni*	AUS	*Anadara trapezia*	Queensland	Moreton Bay	gill	polyclonal
Qld-39	*P. olseni*	AUS	*Anadara trapezia*	Queensland	Moreton Bay	gill	polyclonal
ATCC PRA-425	*P. chesapeaki*	AUS	*Anadara trapezia*	Queensland	Moreton Bay	gill	monoclonal

Part 2

Sequencing the *Perkinsus olseni* genome

2.1. Introduction

The genomes of five isolates of *Perkinsus olseni* and one *P. chesapeaki* was sequenced to compare the genomics with proteomics data in order to identify surface proteins that are unique to *P. olseni*. The information generated was also used to compare and evaluate the genomics differences between isolates from different geographical locations. To the best of our knowledge, this is the first comparative genomics study of species within the class Perkinsea of the phylum Perkinsozoa. This study is the first publication of the genome sequences of *P. olseni* and *P. chesapeaki*. Also, acquisition of the whole genome sequences provides invaluable information for future studies to improve the understanding of the biology, pathogenicity and epidemiology of *Perkinsus* spp. infections. To date, the lack of genomic resources has limited the understanding of *P. olseni* pathogenicity and the scope of host-parasite interaction studies. Indeed, a better understanding of the host-parasite interactions, the mechanisms driving host resistance and susceptibility, as well as the parasite's virulence and infectivity, are all critical to understand how the impact of the disease can be mitigated. Some authors have used *de novo* assembly of transcriptomes, to study host-parasite interaction in *P. olseni* (Hasanuzzaman *et al.*, 2016), however, such analyses or any other gene expression/proteomics work would be facilitated by the availability of the *P. olseni* genome.

2.2. Materials and Methods

2.2.1. Samples, genomic DNA purification, sequencing and assembly

Perkinsus isolates were generously donated by Dr Chris Dungan and Nick Gudkovs with source details provided in Table 4. Whole genome sequencing of the isolates SA-00978-12T, PRA-31/E3, PRA-179, PRA-205, and PRA-207 was achieved using either a hybrid Oxford Nanopore (ONT) MinION and Illumina NextSeq, or Illumina NextSeq only approach.

For Illumina-only sequencing, *Perkinsus* cells were cultured in 50 mL DME/F12-3 culture medium (Sigma-Aldrich components) (Burreson *et al*, 2005) and harvested by centrifugation at 5,000 × *g* for 10 min and 3 × Artificial Sea Water (ASW) washes. DNA was purified using the Genomic-Tip 100/G extraction kit (QIAGEN) as described in the manufacturer's instructions. Briefly, harvested and washed cells were disrupted by incubation in 7 mL Buffer B1 (50 mM Tris·Hydrochloric Acid (HCl), pH 8.0; 50 mM Ethylene Diamine Tetra Acetic Acid (EDTA), pH 8.0; 0.5% Tween-20; 0.5% Triton X100) containing 100 µg/mL RNase A, 1.1 mg/mL lysozyme and 2.8 mg/mL proteinase K followed by 150 rpm horizontal shaking at 37°C for 30 min. Following shaking, 1.2 mL of Buffer B2 (3 M guanidine HCl; 20% Tween-20) was added and the preparation incubated overnight at 56°C with gentle mixing. Purified genomic DNA was tagmented (Bruinsma *et al*, 2018) (random cutting of DNA by transposases to create 'tags') using the Nextera Flex kit (Illumina) and sequenced using an Illumina NextSeq utilising paired 2x150 cycles. For *P. olseni* SA-00978-12T, an additional sequencing library was generated using the Nextera XT kit (Illumina) from the same DNA extraction and sequenced using an Illumina MiSeq utilising paired 2x250 cycles. Illumina NextSeq

sequencing was performed at the Ramaciotti Centre for Genomics (UNSW Sydney, Australia), MiSeq sequencing was performed with the assistance of the Australian Centre for Genomic Epidemiological Microbiology (http://www.ausgem.net). For quality control, sequencing reads were checked using *fastqc* v0.11.8 (Wingett and Andrews, 2018). Assembly of Illumina-only data was performed using *spades* v3.13.0 (Bankevich *et al.*, 2012) and assembly statistics generated using *quast* v5.0.2 (Gurevich *et al.*, 2013). Assemblies were contaminant-screened using *blobtools* v1.0 and *blastn* searches (Altschul *et al.*, 1990; Laetsch and Blaxter, 2017). Genome completeness was assessed as previously described (Stephens *et al.*, 2018) using Benchmarking Universal Single-Copy Orthologs (BUSCO) v3.0.2b (Simão *et al.*, 2015) and *diamond blastp* v0.9.24.125 (Buchfink *et al.*, 2014) searches of the alveolate_stramenophiles_ensembl dataset (retrieved 2019-09-19 from https://busco.ezlab.org/).

For MinION ONT sequencing, *Perkinsus* genomic DNA was purified with the same method used for Illumina sequencing with additional large fragment enrichment. Large genomic DNA fragments (>1.5 kbp) were enriched using a 1X volume of AMPure XP beads (Beckman-Coulter) in 11% Polyethylene Glycol (PEG)-8000 (Sigma), 1 mM EDTA, 10 mM Tris-HCl and 1.6M NaCl and further purification using 70% ethanol. AMPure XP treated samples were further assessed with pulsed field gel electrophoresis to ensure short DNA fragments were removed. Enriched large fragment genomic DNA was then end-prepped for ONT sequencing using the Sequencing Ligation kit (LSK-108) and sequenced for 24 h on a MinION 9.4.1 Rev. C (ONT). Reads were quality checked as described above. Hybrid ONT/Illumina assemblies were generated by first assembling ONT sequencing reads with *flye* v2.4 (Kolmogorov *et al.*, 2019). Long read assemblies were polished using *nanopolish* v0.10.2 and five iterations of *pilon* v1.22 utilizing Illumina sequencing reads sourced from the same genomic DNA extraction (Loman *et al.*, 2015; Walker *et al.*, 2014).

2.2.2. Genome annotation

Whole genome annotation was achieved with *funannotate* v1.5.3 using options designed for non-fungal genomes (Palmer and Stajich, 2019). The *P. olseni* SA-00978-12T isolate ONT/Illumina hybrid genome assembly was used as the training genome. RNA evidence was collected from direct transcriptome sequencing of *P. olseni* SA-00978-12T performed at the Ramaciotti Centre for Genomics and data from a previous transcriptome study (Hasanuzzaman *et al.*, 2016). The *P. olseni* SA-00978-12T hybrid assembly was first cleaned of repetitive small contigs using the reduction step of *redundans* v0.14a (with options: --identity 0.95 --overlap 0.95 --minLength 500 --noscaffolding --norearrangements --nogapclosing) (Pryszcz and Gabaldón, 2016). Repeats were then soft-masked using *RepeatMasker* v4.0.9 (via *funannotate mask*) and a custom repeat library generated from all available genomes of *Perkinsus* spp. using *RepeatModeler* v1.0.11 with default options (Smit and Hubley, 2008-2015; Smit *et al.*, 2013-2015).

Gene models for *de novo* predictor training were generated with *funannotate train*. Briefly, RNA-seq evidence was trimmed of adapted sequences with *trimmomatic* v0.36, followed by normalisation and genome-guided assembly with *trinity* v2.8.5 and the soft-masked *P. olseni* SA-00978-12T genome assembly (Bolger *et al.*, 2014; Grabherr *et al.*, 2011). *PASA* v2.3.3 was then used to generate gene models using *trinity* RNA contigs cleaned with *seqclean* (within *PASA*), aligned to the *P. olseni* SA-00978-12T genome assembly and transcoded (Haas *et al.*, 2003). Finally, *kallisto* v0.46.0 was used to identify PASA gene models with the highest likelihood to generate final gene models used to train *de novo* predictors (Bray *et al.*, 2016).

Gene prediction was performed on all genomes with *funannotate predict* using *augustus* v3.3.2 trained by *P. olseni* SA-00978-12T PASA gene models, and *Genemark ES* v4.38 self-trained or trained by *P. olseni* SA-00978-12T, when self-training failed (Lomsadze *et al.*, 2005; Stanke *et al.*, 2008). RNA-seq *trinity* assemblies (above) were provided as transcript evidence and aligned to assemblies with *minimap2* v2.17-r941 (Li, 2018). UniProt DB v2019_06 proteins were provided as protein evidence and aligned to assemblies using *exonerate* v2.4.0 (Slater and Birney, 2005). Ribosomal and transfer RNA sequences were predicted with *barrnap* v0.9 and *tRNAscan-SE* v2.0.2, respectively (Chan and Lowe, 2019; Seemann, 2018). 5’ and 3’ UTR regions were annotated using *funannotate update* by comparing predicted genes to RNA-seq-based *PASA* alignments.

Functional annotation was achieved with *funannotate annotate* utilizing multiple databases and search strategies. Similarity searches were performed with *diamond blastp* against the UniProt DB and searches with *InterProScan5* v75.0 were performed locally using *funannotate iprscan* (Jones *et al.*, 2014). Additional functional annotations were generated by *hmmer* v3.2.1 searches of the Pfam database v32.0 (Eddy, 2009; Finn *et al.*, 2014). CAZymes function was assigned using *hmmer* searches of dbCAN v7.0 (Yin *et al.*, 2012). Putative proteases were identified with *diamond blastp* searches of the MEROPS database v12.0 (Rawlings *et al.*, 2008). Product names and Clusters of Orthologous Groups (COG; a similarity-based method of identifying protein function) protein assignments were generated by combining results of *EggNOG-mapper* v1.0.3 using Eggnog database v4.5.1 and *diamond blastp* searches of the UniProt DB with *Gene2Product* v1.44 from *funannotate* (Huerta-Cepas *et al.*, 2017; Jensen *et al.*, 2008). Transmembrane domains and signal peptides were predicted with *phobius* v1.01 and *signalp* v4.1 (Käll *et al.*, 2004; Petersen *et al.*, 2011).

2.2.3. Comparative genomics analysis

Ortholog clustering and detection of unique gene presence analysis was performed using *orthofinder* v2.3.3 (Emms and Kelly, 2019). Repetitive genome regions were identified using the custom *RepeatModeler* library (above) and repeat scans were performed on genome assemblies using *RepeatMasker* and on raw sequencing reads using *dnaPipeTE* v1.3.1_07.dec.2017 using a genome size of 40 Mbp and genome coverage of 0.4x (Goubert *et al.*, 2015). Analysis of rRNA gene copy was performed comparing the average coverage depth of reads aligned to rRNA sequences from *P. olseni* (AF509333) and *P. chesapeaki*

(AY305326), with the average coverage depth of reads aligned to single-copy-ortholog genes that were identified with *orthofinder* (Emms and Kelly, 2019).

Quantification of k-mer abundance was performed using *KMC* v3.1.1 (Kokot *et al.*, 2017). Individual heterozygosity estimation was performed for *P. olseni* isolates by variant calling using *gatk* v4.1.3.0 best practice workflow to a scaffolded, heterozygosity-reduced *P. olseni* SA-00978-12T reference genome generated by using *redundans* with default options (Poplin *et al.*, 2018). Reads were aligned to the reference using *bwa mem* v0.7.17 (Li, 2018), with the resulting alignment file sorted, and duplicates marked using *picard* v2.20.5 (Picard toolkit, Broad Institute, GitHub repository). With *gatk*, base-quality scores were then recalculated by the bootstrap method where hard-filtered variants (DP > 50, DP < 150, QD < 2.0, FS > 60.0, MQ < 40.0, MQRankSum < -12.5, ReadPosRankSum < -8.0) were used as the input for successive rounds of base-quality score recalibration until convergence was achieved (this occurred after five cycles for all samples) and resulting in final individual gVCF variants files generated using *gatk HaplotypeCaller*. Variant files were then joint genotyped with *gatk GenotypeGVCFs* and hard-filtered *gatk VariantFiltration* as above. Individual heterozygosity was calculated with the proportion of heterozygous loci method (heterozygous loci/genotyped loci) using variants genotyped across all *P. olseni* isolates. For *P. chesapeaki,* heterozygosity was estimated using a scaffolded, heterozygosity-reduced *spades* assembly of *P. olseni* isolate PRA-425 using *redundans* with default options. Variants were called as described above but without joint genotyping, ie. *gatk HaplotypeCaller* produced a single genotyped VCF file which was then hard-filtered with *gatk VariantFiltration*.

Estimation of ploidy was achieved with *nQuire* (commit version 2018-04-05) (Weib *et al.*, 2018). For all samples, sequencing reads sourced from the Illumina NextSeq libraries were mapped to the scaffolded heterozygosity-reduced *P. olseni* SA-00978-12T reference genome described above. To obtain additional coverage for sample SA-00978-12T, reads from both the NextSeq and MiSeq sequencing libraries were mapped and merged into a single bam file. Ploidy was estimated by running *nQuire create* using a minimum mapping quality of 30 and upper and lower coverage limits of 10,000x and 20x, respectively. Log-likelihood's generated from *nQuire lrdmodel* were assessed using both raw and denoised (via *nQuire denoise*) allele frequencies. Ploidy estimation was additionally performed using *nQuire* for each contig individually using the same parameters.

Pairwise average nucleotide identity was calculated with *pyani* v0.2.7 using the ANIb method described in Goris *et al.* (2007). Sequence concatenation, phylogenomic tree inference, model-testing, gene tree inference, and concordance factor calculation were performed with *iqtree* v1.7-beta18 using the edge-proportional partition model and single-copy-orthologs sequence alignments identified with *orthofinder* (Chernomor *et al.*, 2016; Kalyaanamoorthy *et al.*, 2017; Nguyen *et al.*, 2015). Branch support values were calculated by using non-parametric bootstrap with 100 replications. All bioinformatics software used in this study was installed manually or with the bioconda channel of the conda package manager (Dale *et al.*, 2018).

2.3. Results

2.3.1. Genome assembly and structure

Assembly quality metrics and statistics are summarised in Figure 6 and Table 5. For *P. olseni*, a substantial difference in assembly quality can be observed in the Illumina assemblies of isolates from New Zealand (PRA-205 and PRA-207) vs Japan/Spain (PRA-31/E3 and PRA-179) with high contig number and low N50 values observed in New Zealand isolates (Fig. 6), Illumina-only assemblies of the Australian-sourced SA-00978-12T isolate produced a similar result to the New Zealand isolates (data not shown). For simplicity, we will refer to *P. olseni* isolates SA-00978-12T, PRA-205 and PRA-207 as the Oceanian isolates and PRA-31/E3 and PRA-179 as the Eurasian isolates for the remainder of this report. *Perkinsus chesapeaki* PRA-425 produced a genome assembly similar in quality to the Eurasian *P. olseni* isolates.

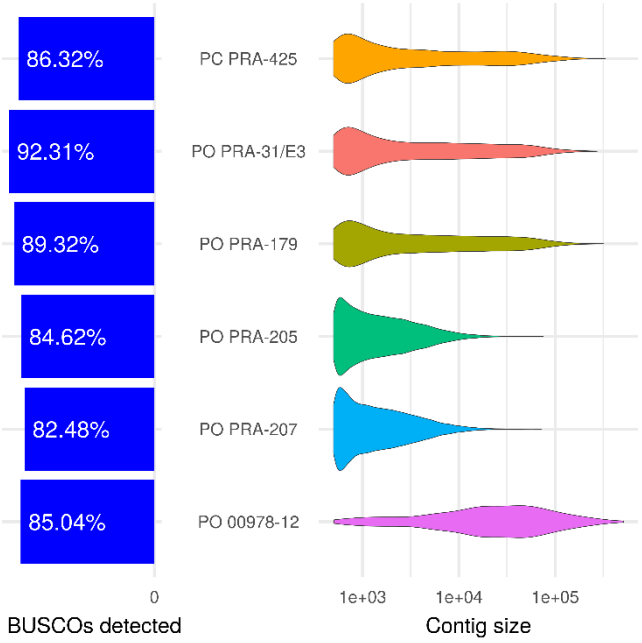

Figure 6: Assembly completeness and contiguousness. Left: Proportion of BUSCO genes detected using the alveolata stramenopiles ensemble library. Right: Violin plots describing relative number of contigs and their lengths for each isolate sequenced in this study.

Table 5: Assembly quality metrics and statistics for *Perkinsus* spp. genomes.

Assembly	**NCBI Accession**	**No. contigs**	**Largest contig**	**Total length**	**GC (%)**	**N50**	**N75**	**L50**	**L75**	**# N's per 100 kbp**	**% Repeats (RM)**	**Heterozygosity**	**BUSCO genes detected**
P. olseni SA-00978-12T	JABANP000000000	1358	512101	63100281	51.54	97262	50101	174	405	0.46	25.1%	5.47%	85.47%
P. olseni PRA-179	JABAHT000000000	3286	319780	39988496	51.72	46099	20585	257	571	58.59	22.2%	0.20%	84.19%
P. olseni PRA-205	JABANM000000000	38356	75311	87426342	51.87	3813	1771	5995	14419	45.86	21.0%	7.49%	82.05%
P. olseni PRA-207	JABANO000000000	41491	72221	90451874	51.86	3586	1670	6531	15803	34	20.9%	7.71%	83.76%
P. olseni PRA-31-e3H	JABANN000000000	3355	272596	39908507	51.72	46304	20498	260	576	62.73	22.3%	0.19%	88.46%
P. chesapeaki PRA-425	JAAPAO000000000	3544	332528	41684223	46.59	45858	22420	261	582	131.92	11.1%	0.10%	91.03%
P. marinus ATCC 50983	GCF_000006405.1	17897	1800699	86567544	47.41	157560	3077	124	1533	646.2	25.5%	n.c.	98.72%

2.3.2. Mitochondrial genomes

Sequencing reads generated from all isolates passed quality control checks using FastQC, excepting per-sequence GC content, which was observed in two peaks at ~16% and ~52% GC. Closer investigation of the 16% GC sequences revealed these reads represented mitochondrial sequences consistent with previously reported *Perkinsus* mitochondrial GC proportions (Masuda *et al.*, 2010). These reads were observed at higher quantities in Oceanian sourced *P. olseni* isolates (6.9-14.3% vs 0.52-1.2% of total reads < 25% GC) and with higher coverage observed in blobplots (Figure 7). Assemblies of mitochondrial sequences were also observed as multiple diverse contigs consistent with those found in dinoflagellates (Masuda *et al.*, 2010).

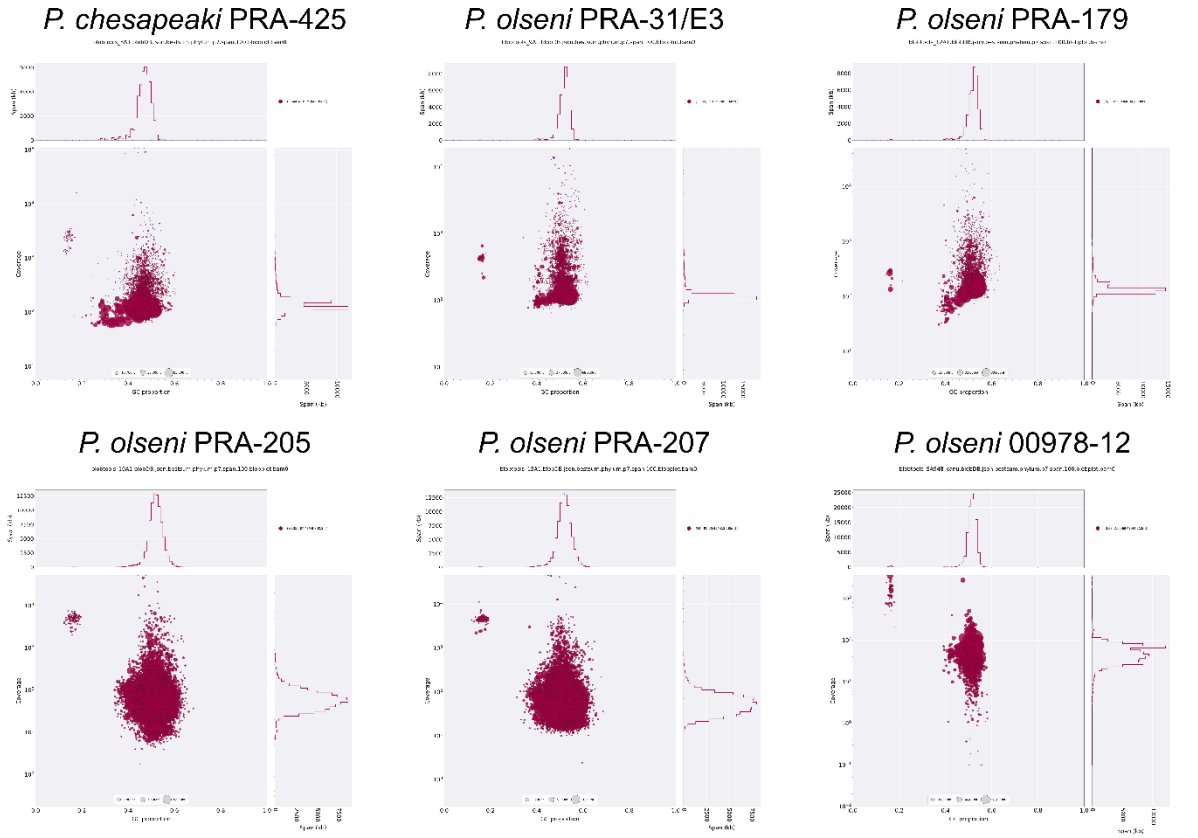

Figure 7: Blobplot representing the proportion of *P. olseni* mitochondrial GC sequences.

Perkinsus mitochondrial genomes have been described as similar to those found in *Dinoflagellata* and exist as mixtures of haplotypes within each cell, the sum of which only contain genes for cytochrome oxidase b (*cob*) and cytochrome oxidase c subunits I (*cox1*) and III (*cox3*). To examine mitochondrial gene content, we queried all *Perkinsus* isolates sequenced in this study with *cox1* and *cob* genes identified previously (Masuda *et al.*, 2010). We found evidence for these genes in all isolates. Additionally, we identified that previously defined transcriptional frameshift sites associated with AGG and CCC codons were conserved and unchanged in all isolates (Masuda *et al.*, 2010; Zhang *et al.*, 2011). Previous studies have also

indicated difficulty in identifying homologs to cytochrome oxidase subunit III (*cox3*) (Jackson *et al.*, 2012) within *Perkinsus* isolates. We queried all assemblies in this study and the *P. marinus* ATCC 50983 genome with tBLASTn and a suite of 11 COX1 and 17 COX3 protein sequences, sourced from alveolates (COX1: AHA41651, ATY40856, AOX48574, BAM68226, AAP57984, QEQ55538, AXQ37355, ATD12931, ATD12880, ATD12877, AOF41416; COX3: AHA41680, ATY40857, AOX48575, AHA41639, BAM68227, AFJ68049, AGF95348, CCE53572, ABR15113, AAP57986, QEQ55539, AXQ37353, ATD12930, AOF41415, ATD12879, ATD12876, YP_002650843).

All COX1 protein sequences produced short matches against all *Perkinsus* genome assemblies consistent with previous studies (Masuda *et al.*, 2010; Zhang *et al.*, 2011). However, tBLASTn searches with COX3 protein sequences produced no hits in any *Perkinsus* isolate. Finally, we searched for *cox3* homologs using *Dinoflagellata cox3* sequences sourced from a previous study (Jackson and Waller, 2013). We found no hits using several BLASTn and tBLASTx search strategies with these sequences.

2.3.3. Heterozygosity

To investigate the intrasample diversity of *P. olseni* and *P. chesapeaki* we examined the six isolates using k-mer coverage counts and alignment to the *P. olseni* SA-00978-12T reference sequence with heterozygosity reduced with *redundans* and variant calling using the Genome Analysis Toolkit (McKenna *et al*, 2010) best-practices workflow. Using variants called from reference alignments, we found a substantial difference in the numbers of heterozygous Single Nucleotide Polymorphisms (SNPs) and indels between the Eurasian and Oceanian *P. olseni* isolates (Figure 8A). The heterozygosity of the Eurasian isolates ranged from 0.19-0.2%, while the heterozygosity of the Oceanian isolates ranged from 5.47-7.71% (Table 5). Heterozygosity in *P. chesapeaki* PRA-425 was estimated to be 0.1%.

Table 6: Features of homozygous peaks for *Perkinsus* spp. isolates.

Isolate	Homozygous peak (kmer abundance)	Bases sequenced	Peak/depth	Normalised depth/peak kmer abundance
P. chesapeaki	96	7404886188	1.2964E-08	1.06
P. olseni PRA-31/E3	91	6253616411	1.4552E-08	0.95
P. olseni PRA-205	22	7017534060	3.135E-09	4.39
P. olseni PRA-179	102	7404326191	1.3776E-08	1.00
P. olseni PRA-207	19	6631609807	2.8651E-09	4.80
P. olseni SA-00978-12T	21	5915637744	3.5499E-09	3.88

Analysis with *KMC* and *GenomeScope* also showed substantial differences in k-mer coverage frequencies with multimodal peaks observed in the Oceanian isolates but single peaks observed

for the Eurasian isolates (Figure 8B). Graphs of k-mer coverage frequency in diploid organisms are usually bi-modal with the peaks representing heterozygous and homozygous regions with the former peak's height found to be proportional to the degree of heterozygosity of a genome sequence (Kajitani *et al.*, 2014). The observation of lone homozygous peaks in the k-mer coverage graphs of the Eurasian isolates and *P. chesapeaki* PRA-425 is consistent with the observation of heterozygosity at 0.1-0.2% (Kajitani *et al.*, 2014).

In contrast, the *P. olseni* isolates sourced from New Zealand and Australia showed higher heterozygosity in reference alignments and multimodal peaks. The modes of the homozygous peaks observed in the Eurasian isolates were 4-5x higher than the initial (heterozygous) peaks of the Oceanian isolates (Figure 8B and Table 6). In diploid organisms the difference between the initial heterozygous peak and the following homozygous peak is 2x. This larger than expected difference between modes in monoclonal samples is indicative of polyploidy.

2.3.4. Polyploidy

To investigate polyploidy further, we examined the shapes of heterozygous allele frequency histograms generated from variant-calling. While few heterozygous SNPs were present in the Eurasian *P. olseni* isolates and *P. chesapeaki* PRA-425, a very broad range of allele frequencies were observed with a peak at allele frequency of 50% (Figure 8C). For the Oceanian *P. olseni* isolates, SA-00978-12T shows strong peaks at allele frequencies of 25% and 75% with a smaller peak at 50%, indicative of tetraploidy, while PRA-207 shows strong peaks at allele frequencies of 20% and 80%, with smaller peaks at 40 and 60% and indicative of pentaploidy. PRA-205 shows strong peaks at 20-25% and 75-80% and may represent a mixture of tetraploidy and pentaploid cells.

Analysis of ploidy was also performed using *nQuire* (Weib *et al.*, 2018). Importantly, *nQuire* only permits estimation of ploidies ranging from 2-4 and was therefore used to assess the likelihood of ploidies greater than or equal to four in *P. olseni* and *P. chesapeaki*. Using denoised data, delta-log-likelihoods were lowest when the tetraploid fixed model was compared to the free model for all isolates, indicating that all *Perkinsus* isolates were most likely of ploidy four or greater (Table 7).

Table 7: *nQuire* likelihoods for *Perkinsus* isolates generated on raw, repeat filtered on a per-contig basis.

Reference sequence SA-00978-12T was used for *P. olseni* isolates and PRA-425 for *P. chesapeaki*; dip=diploid, tri=triploid, tet=tetraploid, bad=not enough variants to call ploidy, d_(ploidy)=likelihood difference from free model employed by *nQuire* to estimate ploidy, smallest difference is bolded and used to estimate ploidy.

Denoised

Strain	ploidy call	free	dip	tri	tet	d_dip	d_tri	d_tet
PRA425	Tetraploid	1.30E+04	7.81E+03	4.17E+03	9.69E+03	5.20E+03	8.85E+03	**3.33E+03**
PRA31-e3H	Tetraploid	2.41E+04	3.13E+03	6.79E+03	2.31E+04	2.10E+04	1.73E+04	**1.01E+03**
PRA205	Tetraploid	1.23E+06	-6.31E+04	2.62E+05	1.01E+06	1.29E+06	9.67E+05	**2.23E+05**
PRA179	Tetraploid	2.16E+04	3.76E+03	6.81E+03	2.12E+04	1.79E+04	1.48E+04	**3.94E+02**
PRA207	Tetraploid	1.16E+06	-6.00E+04	2.07E+05	8.02E+05	1.22E+06	9.54E+05	**3.59E+05**
SA948	Tetraploid	9.55E+05	-1.89E+04	2.65E+05	8.37E+05	9.74E+05	6.90E+05	**1.18E+05**

Denoised and repeat filtered

Strain	ploidy call	free	dip	tri	tet	d_dip	d_tri	d_tet
PRA425	Tetraploid	1.16E+04	7.38E+03	3.67E+03	8.38E+03	4.24E+03	7.95E+03	**3.25E+03**
PRA31-e3H	Tetraploid	1.32E+04	4.02E+03	4.34E+03	1.26E+04	9.14E+03	8.82E+03	**5.42E+02**
PRA205	Tetraploid	9.76E+05	-5.17E+04	2.00E+05	7.92E+05	1.03E+06	7.76E+05	**1.84E+05**
PRA179	Tetraploid	1.34E+04	3.51E+03	4.23E+03	1.31E+04	9.91E+03	9.18E+03	**3.15E+02**
PRA207	Tetraploid	9.12E+05	-4.66E+04	1.61E+05	6.18E+05	9.59E+05	7.51E+05	**2.95E+05**
SA948	Tetraploid	7.40E+05	-1.34E+04	2.04E+05	6.51E+05	7.53E+05	5.36E+05	**8.94E+04**

Denoised by contig – Number of contigs and total number of bases with ploidy call (percentage total)

Strain	# dip contigs	# tri contigs	# tet contigs	# bad contigs	dip (Mbp)	tri (Mbp)	tet (Mbp)	bad (Mbp)
PRA425	641	611	**844**	1448	6.7	8.6	**17**	9.4
PRA31-e3H	158	179	**366**	152	10.7	11.4	**32.7**	1.1
PRA205	137	150	**539**	29	6.8	6.6	**42.4**	0.096
PRA179	159	176	**379**	141	11.8	10.5	**32.6**	0.971
PRA207	125	141	**558**	31	6	5.9	**43.9**	0.968
SA948	142	193	**470**	50	6.8	10.8	**38.2**	0.167

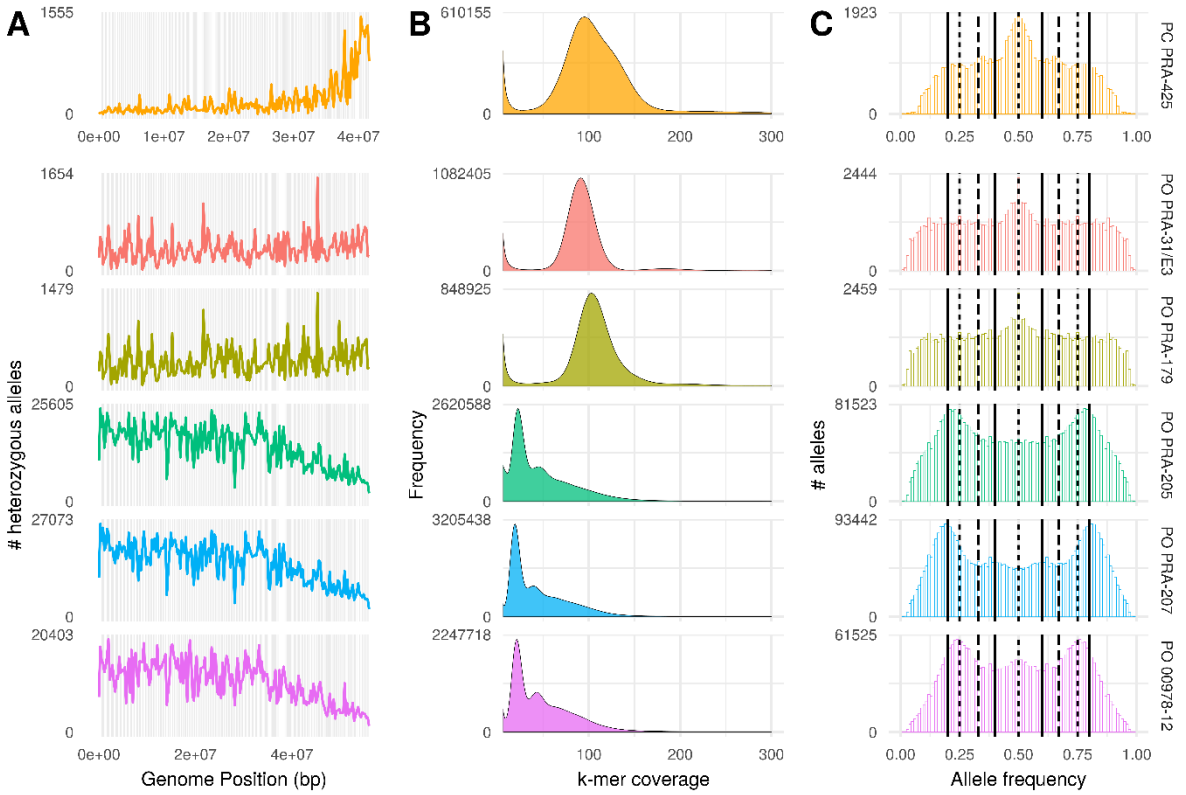

Figure 8: Heterozygous variation in *Perkinsus*. A. Polygrams (total bins = 200) showing the total frequency of heterozygous alleles vs genome position, where scaffolds have been sequentially concatenated from largest to smallest, for *Perkinsus* isolates sequenced in this study. Scaffold length is denoted by alternating white/grey background shading. B. Filled frequency polygrams of k-mer coverage (number of times k-mers of a particular coverage appear; bin size = 1). C. Histograms depicting the abundance of allele frequencies (binsize = 0.01) for heterozygous variants. Solid, dotted, and dashed lines denote locations of expected peaks for pentaploid, tetraploid, and triploid organisms, respectively.

2.3.5. Repetitive sequences

To examine repetitive content in *Perkinsus* spp. we examined *RepeatMasker* output for the published *P. marinus* sequence as well as *P. olseni* and *P. chesapeaki* genome assemblies. For *P. olseni*, the highest proportion of repetitive DNA was the hybrid assembly of *P. olseni* SA-00978-12T, which was comparable in size to the repetitive genome of *P. marinus* (Figure 9). Short-read assemblies of the remaining *P. olseni* isolates ranged from 20.9%-22.3% repetitive content. *Perkinsus chesapeaki* was found to have considerably less repetitive genome content than other *Perkinsus* spp. To investigate if genome assembly quality was affecting estimation, we also examined a short-read assembly of *P. olseni* isolate SA-00978-12T, which was found to have a repetitive genome proportion of 21.2% and indicating that repetitive genome size was underestimated in short-read assemblies

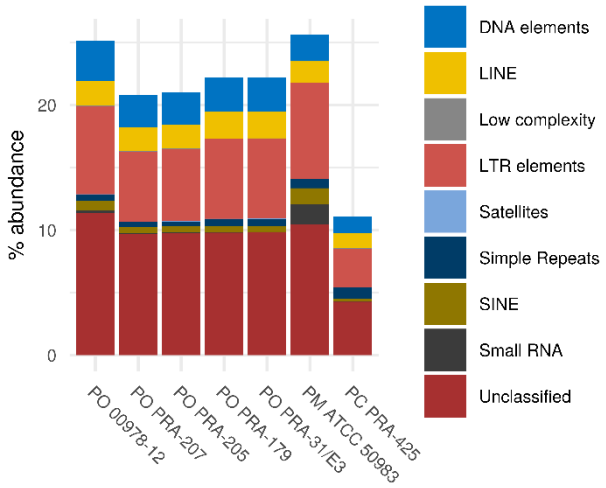

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Figure 9: Abundance of repetitive and transposable elements in *Perkinsus* genome assemblies.

In addition to repetitive sequences, we also examined copy number variation of the ribosomal RNA transcriptional unit within *Perkinsus* isolates, as we had observed instances of tandem gene duplication of this unit within long read sequences. To assess copy number in each isolate, we used read-depth of the rRNA transcriptional unit compared with the read-depth of 362 single copy ortholog groups sequenced as an estimator. Average sequencing coverage of the rRNA transcriptional unit region ranged from 127 to 691 times higher than the average coverage depth of single copy gene sequences (Table 8). *Perkinsus chesapeaki* had the highest copy number of rRNA, which was considerably elevated compared to the *P. olseni* isolates, with 9.5% of sequencing reads aligning to *P. chesapeaki* rRNA.

Table 8: ribosomal rRNA (45S) metrics for *Perkinsus* spp. isolates. SCO: Single Copy Orthologous.

Isolate	% nuclear reads mapped to 45S	Average 45S sequence coverage	Average SCO coverage	Inferred gene copy (coverage)
PRA-425	9.52%	87053	125.97	691
PRA-31-E/3	1.66%	13379	105.1	127
PRA-205	5.70%	51159	132.54	386
PRA-179	4.89%	46192	121.65	380
PRA-207	4.38%	35226	116.16	303
SA-00978-12T	2.74%	21291	105.86	201

2.3.6. Gene content

The characterised gene content of *P. olseni*, *P. marinus* and *P. chesapeaki* is highly conserved, with generally similar proportions of COG categorised genes in each genome sequence (Fig. 10). Variations include higher proportions of cytoskeletal, nuclear structure, intracellular trafficking, secretion and vesicular transport genes in *P. olseni* PRA-205 and PRA-207, elevated proportions of energy production and conversion, translation, ribosomal structure and biogenesis genes in *P. marinus*, and relatively increased quantities of post-translational modification, protein turnover, and chaperones in *P. chesapeaki* (Figure 10A).

Orthologue clustering of *Perkinsus* genes identified a high proportion of unique genes with only 10,328 of 28,196 total orthologous gene groups containing genes shared between at least two species (Fig. 10B). Analysis of *P. olseni*-containing orthogroups revealed a higher proportion of orthogroups unique to the Oceanian isolates (12,312 of 22,148) (Fig. 10C). This number is inflated by a higher number of duplicated and/or fragmented genes (Fig. 10D) but still likely remains high even when this is considered.

2.3.7. Taxonomy and phylogenetics

To further explore the taxonomy of *Perkinsus* species, we examined whole genome sequences of *P. marinus*, *P. chesapeaki* and *P. olseni* with Average Nucleotide Identity (ANI) and phylogenetic methodologies (Figure 11). For phylogenetic analysis, very high bootstrap branch support was observed and gene and site concordance factors for this tree configuration were also high with 20-24% gene discordance and 20-30% site discordance factors determined for alternative quartets. The ANI of *P. olseni* isolates ranged from 90.9%, which was observed between isolates PRA-207 and PRA-31/E3, to 99.7%, which was observed between isolates PRA-31/E3 and PRA-179. A clear separation can be observed between the Eurasian and Oceanian isolates with a maximum similarity of 95.5% ANI between these two groups. This separation is less than the ANI that is observed between species of the Apicomplexa phylum (containing parasitic alevolates) where species delineation is associated with ANIs at less than 85% (Bogema *et al.*, 2018).

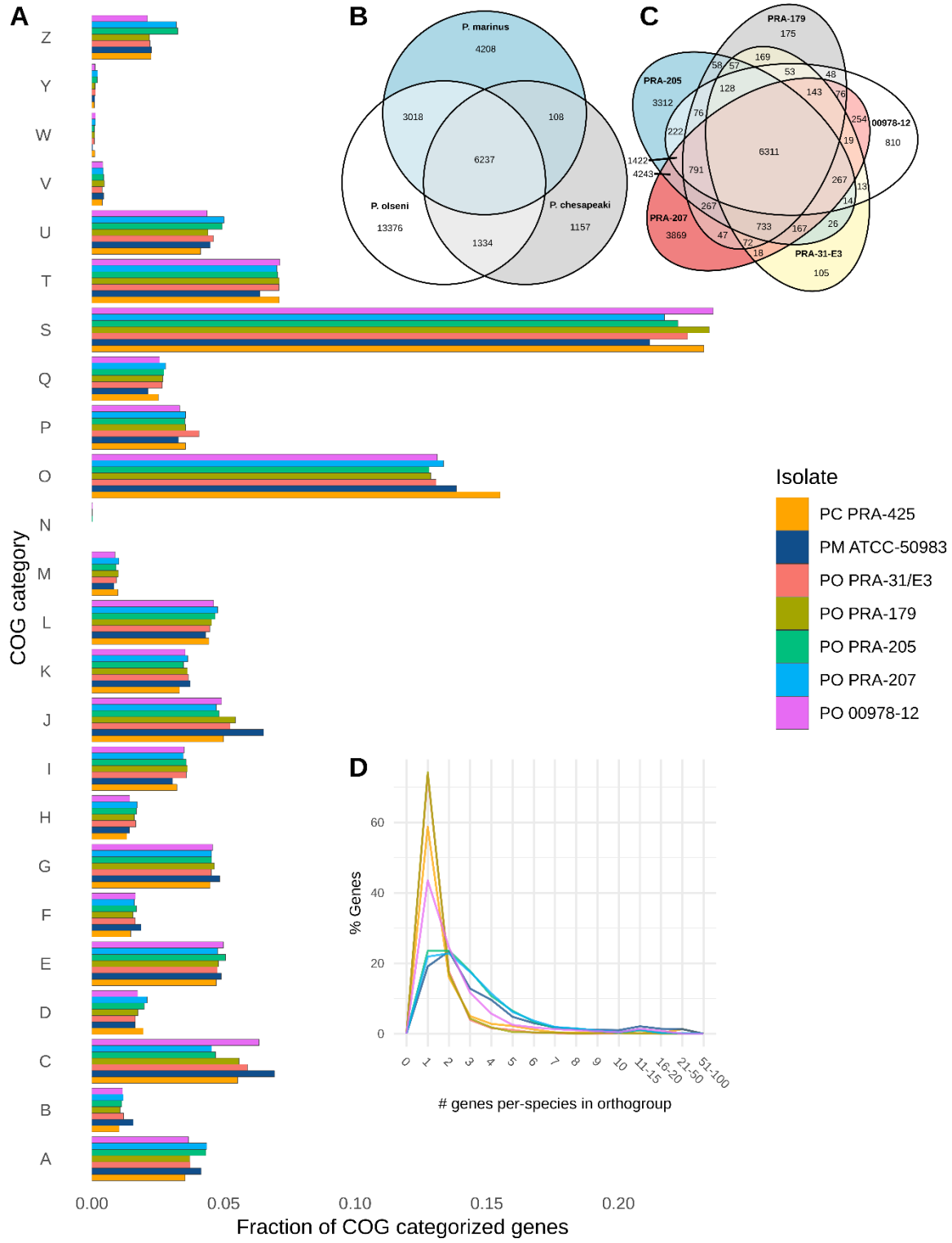


Figure 10: Gene content in *Perkinsus* spp. A. Proportions of proteins matching Clusters of Orthologous Groups (COGs) categories for *P. olseni*, *P. marinus* and *P. chesapeaki* as identified by eggNOG mapper. Y-axis represents the following COG categories (A) RNA processing and modification, (B) Chromatin Structure and dynamics, (C) Energy production and conversion, (D) Cell cycle control and mitosis, (E) Amino Acid metabolism and transport, (F) Nucleotide metabolism and transport, (G) Carbohydrate metabolism and transport, (H) Coenzyme metabolism, (I) Lipid metabolism, (J) Translation, (K) Transcription, (L) Replication and repair, (M) Cell wall/membrane/envelop biogenesis, (N) Cell motility, (O) Post-translational

modification, protein turnover, chaperone functions, (P) Inorganic ion transport and metabolism, (Q) Secondary Structure, (T) Signal Transduction, (U) Intracellular trafficking and secretion, (Y) Nuclear structure, (Z) Cytoskeleton, (R) General Functional Prediction only, (S) Function Unknown BC. Venn diagrams of orthologous groups identified when comparing *P. olseni, P. marinus* and *P. chesapeaki* (B) and the five *P. olseni* isolates sequenced in this study (C). D. The proportion of genes for each species vs the number of genes (per species) in orthogroups. Higher numbers of per-species genes/orthogroup in *P. marinus* and *P. olseni* PRA-205 & PRA-207 indicates greater gene duplication or fragmentation in these annotated genomes.

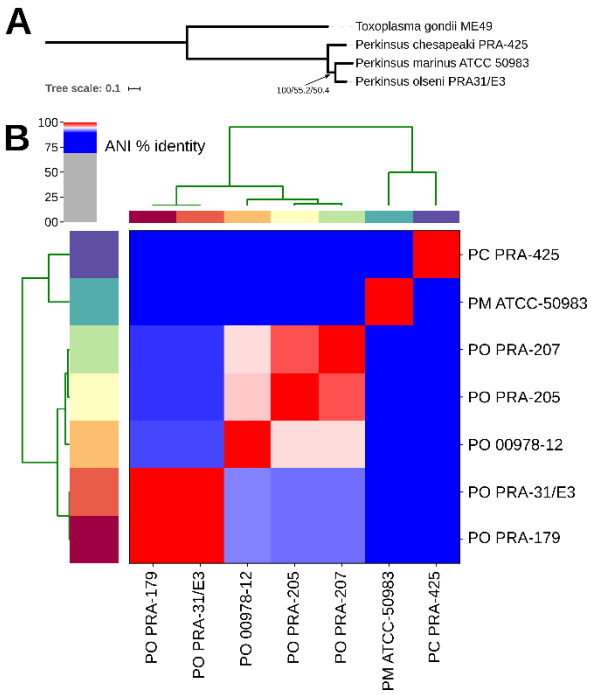


Figure 11: Similarity of *Perkinsus* genome sequences. A. Species-level multilocus phylogeny of *Perkinsus* whole genome sequences. The tree was inferred by maximum-likelihood using an alignment of 362 concatenated protein sequences from single copy orthologues present in all species. *Toxoplasma gondii* str. ME49 was included as an outgroup. Labels denote nonparametric bootstrap support, gene concordance factor and site concordance factor, respectively. B. Average nucleotide identity heatmap of *Perkinsus* genomes.

2.4. Discussion

In this study we have generated the first annotated genome assemblies for *P. olseni* and *P. chesapeaki*. Overall, the proportion of detected BUSCO genes were above 80% in all sequenced genomes, which demonstrates the high level of completion of these genome assemblies. Nevertheless, there was a lower N50 value (a metric that represents the fragmentation level of a genome assembly) and a higher number of contigs for the *P. olseni* Oceanian isolates compared to the *P. olseni* Eurasian isolates and *P. chesapeaki*, indicating fragmented genome sequences. Despite this, assembly quality metrics for *P. olseni* Oceanian isolates compare well with the *P. marinus* genome assembly (Figure 7). *Perkinsus olseni* Oceanian isolates also displayed a high heterozygosity of 5.47-7.71% when compared to *P. olseni* Eurasian and *P. chesapeaki* isolates (0.1 to 0.2%). Ploidy estimation revealed that all *Perkinsus* isolates are likely tetraploid or greater. Additionally, the *P. olseni* Eurasian genomes had a similar proportion of repetitive content to the *P. olseni* Oceanic isolates. The characterized gene content appears to be highly conserved in *P. olseni*, *P. marinus* and *P. chesapeaki*. However, gene numbers often differed substantially and the *P. olseni* Oceanic isolates shared a high proportion of unique orthogroups. Importantly, average nucleotide identities suggest that all *P. olseni* isolates sequenced here belong to the same species.

Assembly quality was greater for the *P. olseni* Eurasian and *P. chesapeaki* isolates when compared with the *P. olseni* Oceanian isolates where more fragmented genomes were obtained. Similarly, the Sanger-sequenced *P. marinus* genome (GCF_000006405.1, ATCC 50983) is highly repetitive and fragmented. The *P. marinus* assembly appears to consist of several highly contiguous scaffolds and numerous very small fragmented ones, which is demonstrated by higher N50 and lower N75 values for *P. marinus* when compared to the Eurasian *P. olseni* and *P. chesapeaki* assemblies (Figure S7. Sanger sequencing has been observed to produce higher quality assemblies than 2nd generation short-read methods and also for heterozygous sample types (Henson *et al.*, 2012; Kajitani *et al.*, 2014). Regardless, the higher fragmentation observed in the *P. olseni* Oceanian assemblies could be explained by the following hypotheses: (1) high genetic variation between individuals in the population that was sequenced; (2) incomplete sequencing coverage of the genome; (3) high number of sequence repetition events like tandem gene duplication, which is a common trait of the dinoflagellate group; and (4) high heterozygosity; and/or (5) polyploidy. The first and second hypotheses are unlikely as monoclonal isolates of *P. olseni* were sequenced in this study and BUSCO searches revealed high genome completeness. The third hypothesis does not explain this observation as *P. olseni* Eurasian isolates showed a slightly lower proportion of repetitive elements than the *P. olseni* Oceanian isolates. This is the opposite of what would be expected if repetition were the cause of increased assembly fragmentation, which makes this hypothesis unlikely.

One highly duplicated sequence we observed was the ribosomal RNA transcription unit. Currently, ITS regions 1 and 2 are the most commonly used targets for diagnosing *P. olseni* infections worldwide and recommended by the World Organisation for Animal Health. We found extensive and variable duplication of this region, meaning that diagnostic tests that target rRNA transcription unit sequences are suitable for presence/absence screening but not suitable

for quantifying parasite load of infection (Appendix 3). A previous study also observed multiple polymorphic sites within the *Perkinsus* ITS locus, multiple ITS loci and high intra-isolate sequence variation, which was comparable to inter-isolate variation (Brown *et al.*, 2004).

A higher heterozygosity can explain the lower genome quality of the *P. olseni* Oceanian isolates and indeed, they presented a higher heterozygosity (5.47-7.71%) than the *P. olseni* Eurasian isolates (0.19-0.2 %) and *P. chesapeaki* (0.1%), which were characterised by a heterozygosity deficiency. The cause of the various levels of heterozygosity observed in the study will be discussed later in the section comparing the different geographical isolates that were sequenced. A higher ploidy can also be associated with some difficulties in the assembly and a fragmented genome. Our results suggested that *Perkinsus* is tetraploid or greater and a combination of a higher heterozygosity and polyploidy can explain the difficulty of the assembly and the more fragmented genome of the Australian isolates.

Polyploidy or whole-genome duplication results in heritable occurrence of more than two sets of chromosomes, which enlarges and diversifies an organism's genome and is thought to influence phenotypes and fitness (Otto and Whitton, 2000; Wei *et al.*, 2019). Polyploidy could confer some evolutionary advantages to *Perkinsus* parasites. Indeed, polyploid organisms can potentially employ alternative copies of duplicated genes to respond to novel environments and therefore drive the ecological and evolutionary success (Wei *et al.*, 2019). The higher number of alleles in polyploid organisms may mean each individual has a better chance of carrying a new beneficial mutation (Otto and Whitton, 2000), with beneficial alleles tending to be at a higher frequency than deleterious alleles. Polyploidy is relatively common in plants but only occurs occasionally in animals (Otto and Whitton, 2000). However, a sort of polyploidy called mosaic aneuploidy has been described in some Apicomplexa parasites when the ploidy differs between chromosomes (Sterkers *et al.*, 2012). Some studies have revealed that populations of some Kinetoplastida parasites such as *Leishmania* or Apicomplexa parasites such as *Trypanosoma* are actually an ensemble of different ploidy states, which includes individuals that are monosomic, disomic or trisomic for different chromosomes (Calo *et al.*, 2013; Mannaert *et al.*, 2012). Although, the Dinoflagellates, which are the closest phylogenetic group to Perkinsozoa are known to be haploid with ephemeral diploid stages (Nuismer and Otto, 2004).

Some authors have previously pointed out the uncertainty of *Perkinsus* ploidy (Fernández Robledo *et al.*, 2011) whereas others using microsatellite loci have suggested the diploidy of *Perkinsus* cells (Pardo *et al.*, 2011; Reece *et al.*, 2001; Reece *et al.*, 1997; Robledo *et al.*, 1999; Thompson *et al.*, 2011; Vilas *et al.*, 2011). Several of these studies inferred diploidy from PCR-amplified sequence data analysis or microsatellite data (Pardo *et al.*, 2011; Reece *et al.*, 2001; Robledo *et al.*, 1999; Vilas *et al.*, 2011) and may have only observed bi-allelic loci. Whereas others have acknowledged the possibility of polyploidy in *Perkinsus* but did not consider it, likely due to the complexity of required analyses and a lack of observed polyploidy in the closely-related dinoflagellates (Thompson *et al.*, 2011). However, ploidy is yet to be confirmed in the laboratory. Our observations of polyploidy in genome sequences are interesting but the

additional observation of low heterozygosity in the *P. olseni* Eurasian and *P. chesapeaki* isolates implies that the organism likely also has a haploid life-stage. The ploidy of an organism is a critical factor to population genetic inferences and should be considered in the interpretation of published data where the assumption of diploidy was made (Thompson *et al.*, 2011).

While the number of isolates examined in this study was very small, two distinct geographical sub-groups of *P. olseni* were observed. The Eurasian isolates, comprising those sourced from Spain and Japan, and the Oceanian isolates, comprising those sourced from Australia and New Zealand. These two sub-groups differ primarily by heterozygosity and gene content but also by differences in genome identity, which were observed using ANI. Examination of the *P. olseni* Eurasian isolates showed that they are less genetically diverse with a lower gene content and a lower heterozygosity than the *P. olseni* Oceanian isolates. The low heterozygosity and gene content could be explained by the Wahlund effect caused by a genetic drift after the introduction of the parasite to those areas. Indeed, the level of heterozygosity in a population is modulated and linked to the way individuals disperse between populations and sub-populations and mate within populations (Biollaz *et al.*, 2010; Montarry *et al.*, 2015). Previous study of *P. olseni* sourced from Spain has shown very low polymorphism in microsatellites, which was hypothesized to be attributed to founder effects caused by the introduction of *P. olseni* through imported clams from the Asia-Pacific (Vilas *et al.*, 2011). The hypothesis that *P. olseni* originated from the Pacific region and introduced into Europe and South America with the transfer of Manila clam *Ruditapes philippinarum* has previously been put forward (Vilas *et al.*, 2011; Villalba *et al.*, 2004). The conclusions from our dataset are limited by low sample numbers but the data indicates that *P. olseni* may have originated from the Oceanian region and was introduced to Europe and Asia, causing those introduced subpopulations to experience a decrease in their genetic diversity and heterozygosity. The data showing the distinction between Oceanian and Eurasian isolates revealed that the absence of a WA isolate did not impact the results of this project. Further study of *P. olseni* genomics using larger numbers of Asia-Pacific sourced isolates would provide improved insights into the biological history of *P. olseni*.

The genetic content of *Perkinsus* spp. is poorly understood compared to members of the Apicomplexa but a single genome sequence for *P. marinus* has been available for over a decade which has allowed comparative genome and gene-content studies across species of the infrakingdom Alveolata (Templeton and Pain, 2015). For *P. olseni*, genes important to multiple processes have been reported in a previous transcriptome study (Hasanuzzaman *et al.*, 2016). Despite the presence of this sequence data, and several studies on *Perkinsus* mitochondrial sequences, no *cox3* homolog has currently been identified (Jackson *et al.*, 2012; Masuda *et al.*, 2010; Zhang *et al.*, 2011). Here we were unable to find evidence of *cox3* homolog presence using extensive tBLASTn searches of three *Perkinsus* species, whereas similar search strategies produced multiple hits for *cox1* in all isolates. Dinoflagellate *cox3* genes often have peculiar configurations which include the widespread requirement for mRNA trans-splicing (Jackson and Waller, 2013) and a *cob-cox3* gene fusion found in *Oxyrrhis marina* (Slamovits *et al.*, 2007). While the *cox3* gene is a feature of both Apicomplexan and Dinoflagellate

mitochondrial genomes it is absent from the mitochondria of several ciliates (Brunk *et al.*, 2003; Burger *et al.*, 2000; Pritchard *et al.*, 1990). This is also consistent with several extracellular domains that have not been found in *Ciliophora* or *Perkinzoa* but present in both Apicomplexa and Dinoflagellata sequences (Templeton and Pain, 2015), although we were able to identify some of these domains in sequences from this study including the Myxo disulfo repeat, ChtBD1, PAN-APPLE, RICIN, Laminin G3/ Pentraxin, CCP (Sushi) domains. The availability of several new *Perkinsus* genomes may aid future gene comparison studies and further understanding of Alveolate evolution.

In addition to five novel *P. olseni* genomes, we have produced the first genome assembly of the related parasite *P. chesapeaki*. Sequence studies of this type will provide improved resources for future population genetics studies and diagnostic test design. Like the *P. olseni* Eurasian isolates, the *P. chesapeaki* isolate showed extremely low heterozygosity, which provides supporting evidence to the hypothesis that *P. chesapeaki* is an introduced species to Australia. Indeed, *P. chesapeaki* has previously been postulated to originate from North America (Arzul *et al.*, 2012). Furthermore, observations of reduced gene content in the *P. olseni* Eurasian isolates may also explain the lower number of genes unique to *P. chesapeaki* observed in this study (Fig. 10b). Examination of the species-level phylogeny of the *Perkinsus* genus revealed *P. chesapeaki* on a separate branch to the monophyletic *P. olseni* and *P. marinus* group (Fig. 11a), a structure that is consistently observed in previous phylogenetic analyses (Dang *et al.*, 2015; Elandaloussi *et al.*, 2009; Ramilo *et al.*, 2016; Villalba *et al.*, 2004).

Part 3

Development of *Perkinsus olseni*-specific antibodies

3.1. Introduction

It is important to be aware of the different life stages of *Perkinsus* when developing an antibody test as different proteins can be expressed in different life stages. The life cycle of *Perkinsus olseni* consists of three main life stages: trophozoite, zoosporangium and zoospore (Azevedo, 1989; Sagristà *et al.*, 1996) (Figure 12). Trophozoites are the vegetative form that proliferate in the tissues of the host. Enlarged trophozoites dividing into 32 daughter cells are called schizonts. After rupture of the cell wall, smaller cells or immature trophozoites are released into the host tissue. They enlarge and a vacuole forms inside the cell walls, leading the cells to become mature trophozoites (Blackbourn *et al.*, 1998; Goggin and Lester, 1995; Perkins, 1976). While some cells can be released in the environment through diapedesis and in faeces (Bushek *et al.*, 2002b), the cycle is most likely to continue inside the host until its death, when trophozoites are then expelled in the environment (Ragone Calvo *et al.*, 2003). When the host dies and the environment becomes deprived of oxygen, trophozoites expand and form a thick cell wall, becoming prezoosporangia (Azevedo, 1989; Perkins, 1976; Sagristà *et al.*, 1996). This also occurs in other anaerobic environments such as within faeces or pseudofaeces (Bushek *et al.*, 2002a; Ragone Calvo *et al.*, 2003) or when trophozoites are incubated in fluid thioglycollate medium. Prezoosporangia are then released into the water column and develop into zoosporangia. Then, zoosporangia undergo vegetative proliferation, leading to the formation of hundreds of zoospores. These mobile bi-flagellated ellipsoidal zoospores are then released through a discharge tubule into the water column in a process known as zoosporulation (Azevedo, 1989; Casas *et al.*, 2002; Lester and Davis, 1981; McLaughlin *et al.*, 2000; Villalba *et al.*, 2004).

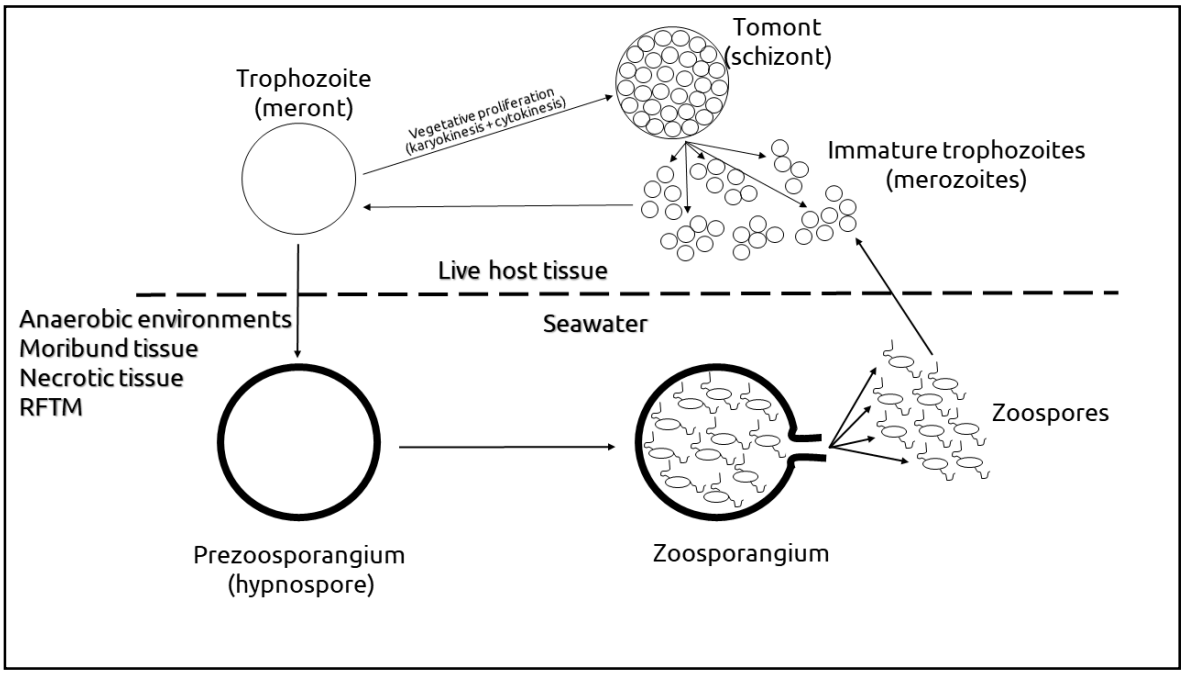


Figure 12: *P. olseni life cycle, adapted from* Liggins & Upston, 2010

The molecular diagnostic methods currently used and recommended to detect infection with *P. olseni* are time-consuming and their specificity and sensitivity remains questionable. Therefore, the intention of the current project is to develop an antibody that cross-reacts with the parasite cells. It would allow the development of an immunological test using any sample, be it water, haemolymph or mollusc tissue in the future. Indeed, to detect infection with *P. olseni*, the OIE - World Organisation for Animal Health recommends as a gold standard the Ray's Fluid Thioglycollate Medium (RFTM) method with final confirmatory species-diagnosis using PCR and DNA sequencing. The OIE recommends conventional PCR assays for routine surveillance using molecular tools, such as the *Perkinsus* genus PCR, which has to be followed by species-specific assay if positive results are detected (OIE, 2019). The RFTM method is not species-specific and non-*Perkinsus* cells may survive the RFTM culture method, which upon microscopic analysis may lead to false detection of the parasite or over-estimates its true infection intensity. Also, the relatively small tissue sample and coverage required for PCR analyses, relative to the whole-animal or whole-gill RFTM processing, increases the probability of a false-negative result given the heterogeneous distribution of *P. olseni* in the host tissues.

An antibody test would considerably reduce both the time and costs necessary to detect infections with this parasite on farmed or wild populations of molluscs. The aim is to develop a monoclonal antibody that could bind to any life stages of *P. olseni*, hypothesising that some common *P. olseni* antigenic determinant would be expressed in all life stages. Montes *et al.* (2001) showed that the protein PWP-1 was expressed by all walled development stages of the parasite, making the hypothesis that common antigenic determinants exist across all life stages of this parasite plausible. A monoclonal antibody would provide researchers with unlimited quantities of standardised immunodetection reagent which could be used subsequently to develop immunoassays.

The potential value of immunological techniques for detecting marine pathogens has been established already, as the use of an antibody is the basis of a fast and specific test. Researchers have tried to develop polyclonal and monoclonal antibodies against *Perkinsu*s spp. since 1991 (Choi *et al.*, 1991), but the resulting antibodies failed to bind either to every life stage of *Perkinsus* spp. or cross-reacted with closely-related groups such as dinoflagellates or with oyster tissues (Bushek *et al.*, 2002a; Choi *et al.*, 1991; Montes *et al.*, 1995). More recently, Kaewsalabnil *et al.* (2015b) produced and characterised a monoclonal antibody that reacted with two life stages of the parasite, but was not tested for cross-reactivity with other closely related organisms. Conversely to previous studies, the current project adopted an innovative approach using genomics and proteomics in order to identify unique proteins from the parasite cell wall and this approach has never been tried before.

3.2. Materials and Methods

3.2.1. Development of antibodies using whole *Perkinsus* cells

The aim of the project was to produce antibodies specific to *Perkinsus olseni* by immunising mice with peptides present at the surface of the parasite. Those surface peptides needed to be present in all the geographical isolates of *P. olseni* used in the study. As it was a complex and long process, mice were also immunised with whole parasite cells (originated from an *in vitro* cell culture).

3.2.1.1. Immunisation and hybridoma production

Antibodies were obtained by injecting four A/J inbred strain female mice (8 weeks of age) intra-peritoneally with 20 µL of washed whole *P. olseni* cultured cells mixed with 20 µL Advax™ adjuvant (Vaxine). Advax was used as this adjuvant affords high antibody responses in mice with no adverse effects on animals, and is approved by the Harry Perkins Animal Ethics Committee (AEC). Administration of the antigen was with the approval 009 from the AEC of the Harry Perkins Institute of Medical Research. The culture used for immunisations was mainly composed of trophozoites and schizonts with a lesser proportion of zoosporangia. We chose to immunise the mice with the SA-00978-12T isolate of *P. olseni* as the representative of an Australian isolate. SA-00978-12T was maintained in exponential phase in culture and was brought to the Harry Perkins Institute of Medical Research where the inoculum was prepared. The inoculum contained 50 µg of total live *P. olseni* cells weight per mouse.

Following injection, the animal's immune system is expected to produce high levels of antibodies specific for some antigens present at the surface of the cells. Animals were injected three times on days 1, 22 and 43 to boost the immune response. The second injection activates memory cells that makes Immunoglobulin G (IgG) antibodies against the antigen and those cells undergo affinity maturation, which result in a pool of antibodies with higher affinity. On the third injection, those B cells producing antibodies with higher affinity to antigen-binding sites will proliferate and produce more antibody than their lower affinity peers. Blood was taken 11 days after the third injection and antisera stored at -20°C. This first phase lasted 3 months.

Antisera were screened to evaluate if polyclonal antibodies bound to specific antigens at the surface of the parasite cells (Figure 13) using Enzyme-Linked Immunosorbent Assay (ELISA) and immunofluorescence assays (IFA). Mice received a final boost 7 weeks after the third one and were culled 4 days after this final boost. The spleen was dissected and cryopreserved for future fusions. The spleen from the two mice showing the highest response were selected for the cell fusion step of hybridoma production.

Sp2/0-Ag 14 cells (mouse spleen myeloma/B cell, Sigma 85072401) were expanded in Hybridoma-Serum-free Medium (HSFM) containing 5% FBS (Sigma F8192), Penicillin-Streptomycin (P/S; 100 U/mL, Sigma P7539), Interleukin 6 (IL6) (0.05 ng/mL; made in-house), and one confluent vented T180 flask (Greiner 690175) cultured at 10% CO_2, 37 degrees

and were ready the day before the fusion. The confluent T180 flasks were split 1:3 in order to have a ratio of 10^8 cells per spleen. The HAT (Sodium Hypoxanthine 100 µM, Aminopterin 400 µM, Thymidine 16 µM final concentrations; Sigma H0262) medium was tested with a small aliquot of Sp2/0 (myeloma cells) and all cells died in it.

On the day of the fusion, spleens were collected in cold Dulbecco's modified Eagle's medium (DME) + P/S (100 U/mL) on ice, and single cell suspensions of those spleens were prepared in cold DME + P/S (100 U/mL). In order to extract the antiserum, blood was also collected. Cell suspensions were prepared, washed once (2 spins at 1500 x g for 5 minutes), and left on ice until ready to be mixed with the Sp2/0 cells for the final wash. In the meantime, Sp2/0 cells were harvested into 50 mL Falcon tubes. These Sp2/0 suspensions were centrifuged at 1500 × g for 5 minutes, had their supernatant removed and were resuspended in DME + P/S prewarmed to 37°C. These tubes were then pooled into a single 50 mL volume for each lot of three T180 flask prepared the previous day. An aliquot of Sp2/0 cells was taken for a cell count and viability using eosin dye exclusion and microscopy, and cells were shown to be > 95% viable. 10^8 Sp2/0 cells were washed in 50 mL of warm (37°C) DMEM two times, and mixed with cell suspensions from one spleen for a third wash. Tubes were then spun at 2100 × g for 5 minutes to pellet the cells and the supernatant was removed. 1 mL of pre-warmed PEG 1500 was added directly to the pellet drop by drop while gently stirring. Stirring continued for another minute. A volume of 20 mL of DME + P/S was then added over 5 minutes while gently stirring. Tubes were then centrifuged at 1500 × g for 5 minutes and the supernatant removed. Pellets were then resuspended in 60 mL warm HFSM/5% FBS, P/S, IL6/ Hypoxanthine-Aminopterin-Thymidine (HAT) medium. Three 96-well flat-bottom plates were plated per spleen, with 200 µL of cell suspensions per well. After 2 days of incubation at 10% CO_2, 37°C, the plates were checked to see if the 1X HAT medium (Sigma H0262) properly killed the unfused Sp2/0 cells. The cultures were then fed every 3 days by removing 100 µL of medium from the top of each well before adding 100 µL of fresh HFSM/5% FBS, P/S, IL6 medium. After 7 days, clumps of hybridomas were visible in most wells. It was then decided to proceed with antibody screening. Three days before the ELISA screening, a last feed of HFSM was done. Antibody-positive cultures were identified by ELISA (see Section 3.2.1.2) and cells of interest were transferred into 24-well plates and were fed with HAT medium (Sigma H0262) until Day 14. Following that, cells were fed with medium containing HAT only for a further 7 days, then with medium without HAT.

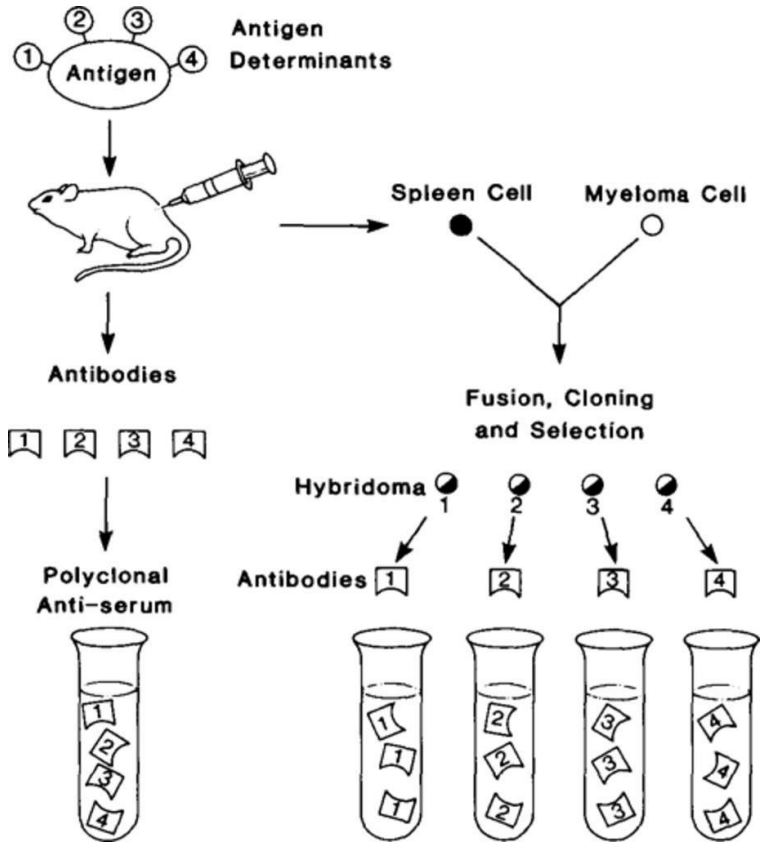


Figure 13: Schematic drawing of polyclonal and monoclonal antibody production following immunisation of a mouse.

Wells with cultures that returned OD_{405} values above background were kept, and re-screened to check for genuine positives. Those genuine positives were expanded to 6-well plates (1 mL of each were frozen at -20ºC as a back-up, while 1 mL was used to keep the expansion going). After two weeks, the HAT medium was changed to HFSM/5% FBS, P/S, IL6 medium, and cultures were allowed to grow for another fortnight. At least three vials of each positive culture were frozen, and supernatants were collected and stored at 4°C for subsequent use. This whole process was repeated twice using whole *P. olseni* cells.

Selected hybridomas that were positive by ELISA were cultured for large-scale production of purified antibodies. Hybridoma cells were grown in Bioreactors in HSFM containing 1% FBS until confluent. Culture supernatant was harvested, cells and debris were removed by centrifugation and final filtration of the supernatants. Monoclonal antibody (mAb) was purified from supernatants by affinity chromatography on protein G-Sepharose 4B Fast Flow. Supernatants were run over a protein G-Sepharose column at 4°C to allow mAb to bind to the beads. The column was then washed extensively with Phosphate Buffered Saline (PBS), pH 7.3. Bound mAb was eluted by running a solution of 0.1 M glycine/HCl pH 2.8 over the column. Eluted mAb was immediately neutralised using 1 M TRIS/HCl pH 8. The protein peak measured by OD_{280} was pooled, then dialysed against PBS. After dialysis the final protein concentration was determined and the mAb concentrated or diluted to 1 mg/ml. Purified mAb was stored in sterile filtered PBS, pH 7.3 at 1 mg/mL.

3.2.1.2. Screening

Mouse antisera were screened against *P. olseni* antigens using ELISA and IFA. The antisera were screened by ELISA to determine the titre (strength) of the immune response to *P. olseni* antigens. Generally, only mice with high titres are used for fusion, to ensure that positive hybridomas are obtained. Antisera were also screened by IFA on parasites to attempt to elucidate the life cycle stages and parasites that the antisera reacted with, as well as the location of the target antigens, e.g. surface of the parasites. Supernatants following fusion were initially screened first using ELISA due to the large number of wells to be screened (almost 300 per fusion). Once positive wells were identified by ELISA these were then assayed by IFA, again to visualise the location of binding and specificity on different parasite types. The wells of interest were then screened again using flow-cytometry as a quantitative method.

Development of ELISA test

P. olseni cells were harvested (300 × *g* for 10 minutes) from an *in vitro* culture and resuspended in PBS, before being lysed with FastPrep™ Lysing Matrix D (MPBio) at 30 Hz for 1 minute in a TissueLyser II (Qiagen). The content of each tube was then resuspended in 5 mL of 1:20 carbonate buffer (pH 9.6). 50 µL of each cell suspension was then adsorbed on an ELISA plate (Costar Serocluster 96-Well U bottom plates, non-treated vinyl) overnight at 4°C.

The coated ELISA plate was washed three times with 0.05% Tween 20/PBS. A volume of 50 µL of 0.1% BSA/PBS was added to each well, followed by 1 µL of test mouse serum, and incubated at room temperature for 1 hour. Then, the plate was washed three times with 0.1% BSA/PBS. 50 µL of 1:1000 Goat Anti-Mouse IgG γ chain Antibody, Horseradish Peroxidase (HRP) conjugated, Species Adsorbed (MerckMillipore AP503P) in 0.1% BSA/PBS was then added to each well, and incubated at room temperature for 1 hour. The plate was washed twice with 0.05% Tween 20/PBS, and once with tap water. Separately, 5 mL of ELISA developing buffer was prepared as follow: 500 µL citric acid (0.1 M, pH 4.2), 100 µL 2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-di-ammonium salt (ABTS, 9.1 mM; Sigma 10102946001), 4.4 mL filtered sterile distilled water, 5 µL of hydrogen peroxide from a 30% stock solution. A volume of 50 µL of that buffer was added to each well, and absorbance at OD_{405} was measured after 15 minutes on a plate reader (Wallace Victor 3) after addition of the substrate (ABTS in citrate buffer and hydrogen peroxide). The serum from a non-injected mouse was used as a reference.

To evaluate the affinity of the hybridoma antisera, the antisera were titrated in 0.1% BSA. PBS was added to the wells and incubated for 1 hour at room temperature. Antisera were titrated at the following dilutions: 10^{-2}, 5×10^{-3}, 2.5×10^{-3}, 1.25×10^{-3}, 6.25×10^{-4}, 3.13×10^{-4}, 1.56×10^{-4}, 7.81×10^{-5}, 3.91×10^{-5}, 1.95×10^{-5}, and 9.77×10^{-6}. Supernatants from the hybridoma culture wells were removed and were aliquoted in ELISA plates (Costar Serocluster 96-Well U bottom plates, non-treated vinyl). and analysed following the protocol described above.

Development of immunofluorescence assays

P. olseni cell cultures were centrifuged (300 × *g* for 10 minutes) and washed twice with PBS. After the last wash, the pellets were resuspended in 10 mL of 0.1% of BSA/PBS and divided in 1.5 mL tubes, with 500 µL of *P. olseni* cells suspension per tube.

Each purified hybridoma supernatant was diluted 1:500 (final) by addition of 1 µL to the tube containing 500 µl of *P. olseni* cells. Supernatants for testing were diluted 1:2. The tubes were then vortexed and incubated on ice for 30 minutes. Each tube was then centrifuged and the pellet washed with 0.1% BSA/PBS three times. After the last wash, 5 µL of 1:500 dilution of goat anti-mouse IgG in 0.1% BSA/PBS in 0.1% BSA/PBS was added to each tube, and then incubated for 30 minutes on ice in the dark. The secondary antibodies used were the following: Fluorescein Isothiocyanate (FITC) goat anti-mouse IgG (Thermofisher, F-2761) or Texas red-goat anti-Mouse IgG (Thermofisher, T-6390). Each tube was then centrifuged and the pellet washed three times with 0.1% BSA/PBS. The content of each tube was then put in a 96-well plate, and observed under a fluorescence inverted microscope (Olympus IX71) at an excitation wavelength of 490 nm for the FITC secondary antibody, or 596 nm for the Texas Red secondary antibody.

Development of flow-cytometry assays

Flow-cytometry is a powerful method that can separate single-cells based on fluorescence, cell size and complexity and allows the calculation of accurate proportions of fluorescent antibody-labelled cells. Three life stages of *Perkinsus olseni* were analysed using this method: (1) trophozoites, which are easily identifiable, represent the main cell population in *in vitro* cultures. Each geographical isolate of *P. olseni* was propagated *in vitro* in T25 flasks and washed twice in 0.1% BSA/PBS (1000 × *g* for 5 min); (2) hypnospores or prezoosporangia, which were induced by placing cells from *in vitro* cultures in RFTM for 5 days in the dark; and (3) zoospores, which can be obtained after placing RTFM-incubated cells in aerated sea water for 3 days. Cells were observed under an inverted microscope before being processed for flow-cytometry. Cells were resuspended in 0.1% BSA/PBS and aliquoted in 1.5 mL tubes. 2 µL of mAb purified antibody or 250 µL of hybridoma supernatant or 2 µl antiserum as positive control was added per tube and incubated for 30 minutes on ice. The antiserum was either the supernatant of hybridoma cells after the fusion (identified by the well number) or purified antibodies. After the incubation, cells were washed twice with 0.1% BSA/ PBS (1000 × *g* for 5 min). 5 µL of goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody coupled with the fluorophore Alexa Fluor 647 (ThermoFisher Scientific A21235) diluted 1:1000 in 0.1% BSA/PBS was added to each tube and incubated for 30 minutes on ice in the dark. After the tubes were centrifuged and the pellets washed twice with 0.1% BSA/PBS, 120 µL of the samples was added to a non-binding black U-bottom plate (Greiner, reference 650209) and 80 µL was analysed on a Becton Dickinson Accurri C6 Plus flow-cytometer using a threshold of 20,000 on FSC-H and a medium flow rate. The laser used for this experiment produced an excitation wavelength of 640 nm and the emission was captured using the filter 675/25. The data generated were analysed using FlowJo software version 10.

Negative and positive controls used in screening tests

Two types of negative controls were used:

(1) Two technical negative controls, which consisted of *P. olseni* cells going through the same process as the rest of the samples and incubated in the medium control. The technical negative control 1 consisted of HFSM/ 5% FBS, P/S, and IL6 medium and contained the secondary antibody. Conversely, the technical negative control 2 or medium control did not contain the secondary antibody and was used when some issues were experienced with autofluorescence.

(2) Four negative control cells that were cells other than *P. olseni*, so the specificity of the antisera could be evaluated. Those cells consisted of:
   a. *Perkinsus chesapeaki*, isolate PRA-425 from ATCC (Table 4);
   b. *Thraustochytrium* sp., isolate H4B2, which was cultured in this project (see chapter 1);
   c. The dinoflagellate *Amphidinium carterae*, isolate CS-383 from the Australian National Algae Culture Collection (ANACC);
   d. The dinoflagellate *Symbiodinium tridacnidarum*, isolate CS-73 from ANACC.

3.2.2. Development of antibodies using *P. olseni* specific antigens

3.2.2.1. Identification of surface peptides common to all* P. olseni *isolates

Extraction of surface peptides, method 1: Proteolytic shaving

A proteolytic shaving was performed in order to isolate/digest the surface proteins from the different geographical isolates that were grown *in vitro* and that are listed in Table 3. The protocol to isolate surface proteins has been adapted from Wei *et al.* (2012).

Perkinsus olseni cells (Table 9) were harvested by centrifugation at 240 × *g* for 5 min. This low speed was used to avoid rupturing the cells. Cell pellets were washed three times with 1× PBS/0.3 M NaCl, pH 7.4. After the last wash, cells in the pellets had their surface peptides gently digested by the addition of 200 µL of trypsin (10 µg/mL in PBS/ 0.3 M NaCl) at 30°C for 15 minutes under constant gentle agitation. Samples were centrifugated at 240 × *g* for 5 minutes and supernatants were transferred to new tubes. Subsequently, more trypsin was added (1.7 µL at 1 µg/µL) and the preparation was incubated at 37°C for 2 hours. Samples were then cleaned up using C-18 Spin Columns (Thermo Scientific Pierce #89870) as per the manufacturer's recommendations and peptide concentrations were determined using a Nanodrop spectrophotometer.

The controls used in this experiment consisted of homogenised cells that released their intracellular content. Cell pellets from two isolates of *P. olseni* were used. 170 µL of PBS/0.3 M NaCl was added to each pellet and the tubes were placed for 10 minutes in a Bioruptor Plus sonication device (Diagenode) following the manufacturer's recommendations,

and then digested with trypsin (1.7 µL at 1 µg/µL) to a final concentration of 10 µg/mL. They were incubated for 2 hours at 37 °C, and cleaned using C18 Spin Column (Thermo Scientific Pierce #89870). The peptide concentration was measured using a Nanodrop spectrophotometer and approximately 300 ng of the sample was loaded on the Liquid Chromatography (LC) system.

Table 9: Isolates of *P. olseni* used for proteomic studies.

ATCC or ID	Species	Origin	Host	Clonality
PRA-31/E3	*P. olseni*	Spain	*Ruditapes decassatus*	monoclonal
PRA-179	*P. olseni*	Japan	*Ruditapes philippinarum*	monoclonal
PRA-207	*P. olseni*	New-Zealand	*Austrovenus stutchburyi*	monoclonal
PRA-205	*P. olseni*	New-Zealand	*Austrovenus stutchburyi*	monoclonal
Qld-46	*P. olseni*	Queensland	*Anadara trapezia*	polyclonal
Qld-39	*P. olseni*	Queensland	*Anadara trapezia*	polyclonal
SA-00978-12T	*P. olseni*	South Australia	*Haliotis laevigata*	monoclonal
PRA-425	*P. chesapeaki*	Queensland	*Anadara trapezia*	monoclonal

Extraction of surface peptides, method 2: Biotinylation

The other method used to isolate surface peptides was undertaken using Pierce Cell Surface Protein Isolation Kit (Thermo Scientific Pierce #89881), following manufacturer's protocol.

Nano Liquid chromatography – electrospray ionisation tandem mass spectrometry

Nano-Liquid chromatography-electrospray ionisation tandem mass spectrometry (Nano-LC-ESI-MS/MS) was performed using an Ultimate 3000 RSLC system (ThermoFisher Scientific, Waltham, USA) coupled to an Impact HD™ Quadrupole Time Of Flight (QTOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) via an Advance CaptiveSpray source (Bruker Daltonics).

Peptidases were reconstituted in water/ Acetonitrile (ACN, 98:2, v/v), and 0.1% (v/v) formic acid, and then injected into the LC-nESI-MS/MS system. The peptide sample was pre-concentrated onto a C18 trapping column (Acclaim™ PepMap™ 100 C18 LC Columns 75 µm × 20 mm, ThermoFisher Scientific), and washed for 10 min in 2% (v/v) ACN 0.1% (v/v) Trifluoroacetic Acid (TFA) at a flow rate of 5 µL/min. Peptide separation was performed using a 75 µm ID C18 column (Acclaim™ PepMap™ 100 C18 LC Columns 75 µm × 15 cm, ThermoFisher Scientific) at a flow rate of 0.3 µL/min using a linear gradient from 5 to 45% over 60 min, followed by a 10 min wash with 90% solvent B (solvent B: 80% (v/v) CAN, 0.1% (v/v) formic acid, and a 10 min equilibration with 5% solvent A (solvent A: 5% (v/v) ACN 0.1% (v/v) formic acid).

Mass Spectrometry (MS) scans were acquired in the mass range of 300 to 2,200 m/z in a data-dependent fashion using Bruker's protein identification Instant Expertise™ method. This method uses IDAS (intensity dependent acquisition speed) to adapt the speed of acquisition depending on the intensity of precursor ions (fixed cycle time), and RT2 (RealTime Re-Think) to exclude previously selected precursor ions from undergoing re-fragmentation unless the chromatographic peak intensity of the ion has increased by a factor of 5. Singly charged precursor ions were excluded from acquisition. Collision energy ranged from 23% to 65% as determined by the m/z of the precursor ion.

3.2.2.2. Data analyses – identification of peptides

To investigate which peptides/proteins were common across all isolates of *P. olseni*, the proteomic data were compared with translated protein sequences from the genomic data. LC-MS/MS peak raw data were converted to mgf format with msconvert (Holman *et al.*, 2014) and analysed using PEAKS Studio v8.5 by searching predicted peptides against a database of predicted proteins generated from the *P. olseni* SA-00978-12T reference genome sequence. Deamidation and oxidation post-translational modifications were included in the search parameters. Peptides were filtered using PEAKS Studio by adjusting matching parameters to produce a False Discovery Rate of < 2%. Proteins were identified if they produced a quality score of ≥ 30 and contained at least one unique peptide. Finally, protein identifications were compared to determine proteins common to all isolates. Proteins that were identified in all isolates bar one were considered commonly expressed proteins.

3.2.2.3. Expression of protein genes in different life stages of Perkinsus olseni

To ensure that identified proteins are expressed for several life stages of the parasite, the South Australian isolate of *P. olseni* SA-00978-12T was cultured *in vitro* and the two main life stages present in the suspension, trophozoites and schizonts, were sorted using a cell picker instrument at the *Centre of Microscopy, Characterisation and Analysis* at The University of Western Australia (UWA). One hundred cells of each type were sorted, the RNA was extracted using the Qiagen® RNeasy® Mini kit (Catalog number: 74104) following the manufacturer's protocol for purification of total RNA from yeast. Cell disruption was done using high-speed agitation in a TissueLyser II at 30Hz for one minute, with TeenPrep™ matrix D (1.5 mm ceramic spheres, 64% ZrO_2, 33% SiO_2; MP Biomedicals). RNA quantification was performed on a Qubit® (1.0) fluorometer using the Quant-iT RNA BR assay kit (Invitrogen™ Q10213).

Reverse transcription was performed using SuperScript™ IV First-Strand Synthesis System (Invitrogen™ 18091200) following the manufacturer's protocol, and cDNA quantification was done using the Qubit™ ssDNA Assay Kit (ThermoFisher Scientific, Q10212).

The qPCR was performed using PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific, A25742). Each reaction well contained: 10 µL of mastermix, 0.1 µL of forward

primer, 0.1 µL of reverse primer, and 5 ng of cDNA sample. Forward and reverse primers were designed manually using the genomic data we generated in part 2 of this report (Table 10). The genomic data allowed us to evaluate that gene POLS_08089 of *P. olseni* is present as a single copy and is a single exon gene of 1,179 bp and that POLS_15916 is present as a 3 copy tandem replication with a single exon gene of 1,569 bp. The gene expression data of the proteins were compared to the expression of the amydohydrolase gene, which is the housekeeping gene used in the present study and used by Leite *et al.*, 2008 (Table 10).

Table 10: Sequences of primers targeting the two proteins selected for qPCR and *Perkinsus olseni* housekeeping genes. All primers are orientated from 5' to 3'.

Name primer	Gene target	Sequence	Source
15916_R	POLS_15916	GCCGAGAAGAAGCCAGAG	Current study
15916_F	POLS_15916	AAAAATGTTGAACGAAGCACAGA	
08089_R	POLS_08089	CTCCTCGTCCTCAGACTCG	
08089_F	POLS_08089	ATGTCTACCGCTCGGCCC	
PoRT_AHFW	Amydohydrolase	CTTCAAACAGCATGGCGAGTCTGCAATT	Leite *et al.* 2008
PoRT_AHREV	Amydohydrolase	ACATGACTGACGAGGAGATGACCCGGTT	

3.2.2.4. Synthesis of peptides

The amino acid sequences of the peptides POLS_15916 were common to all isolates using both extraction methods: proteolytic shaving and biotinylation. The extracted peptides were analysed and shorter sequences were selected based on their immunogenicity, charge and hydrophilicity. The BLASTp results indicated that peptide POLS_15916 corresponds to a conserved hypothetical protein that seems to be involved in the NAD(P)+ transhydrogenase activity of the cytoplasmic membrane. The sequences were sent for further analysis by DGpeptides Co, Ltd, China (www.dgpeptides.com/en) . From the sequences provided to them, three categories of peptides were synthesised:

- conjugated to keyhole limpet haemocyanin (KLH) for immunizations;
- conjugated to BSA for ELISA screening;
- free peptides.

3.2.2.5. Immunisation and screening

The same immunisation and screening strategies were used as those previously described in the sections *3.2.1.2. Immunisation and hybridoma production* and *3.2.1.2. Screening*. Mice were immunised with peptides conjugated to KLH and in addition to being screened against *P. olseni* antigens, they were also screened against the free peptides as well as peptides conjugated to BSA.

3.3. Results

3.3.1. Development of antibodies using whole *Perkinsus* cells

Two batches of four mice were immunised with whole *P. olseni* cells and data are presented in chronological order for the first batch of mice followed by the second batch of mice.

3.3.1.1. First batch of mice

The initial screening of antisera was performed using a combination of ELISA and IFA, which are presented below.

Mouse selection for the hybridoma fusion

The antiserum from the four immunised mice revealed an OD_{405} against *P. olseni* isolate SA-00978-12T that was above the background of normal mouse serum (Figure 14). The most reactive mouse was mouse 3 with an even higher response at a dilution of 1.56E-06. The second most reactive antiserum was from mouse 2, followed by mouse 4 and mouse 1. However, the IFA revealed the brightest cells occurred with antiserum from mouse 1. Therefore, mouse 1 and mouse 3 were selected to proceed with making monoclonal antibodies by hybridoma.

Selection of hybridomas

The fusion resulted in hybridomas proliferating in 69 wells across 3 × 24-well plates labelled from 1A1 to 3D3. Once the cells showed clear signs of proliferation and visible clumps, the supernatants were screened via ELISA and IFA using *P. olseni* SA-00978-12T antigens but also using other species as negative cells controls and organisms such as *P. chesapeaki* PRA-425, the Thraustochrytrid isolate H4B2 and 2 dinoflagellates (*Amphidinium carterae* and *Symbiodinium tridacnidarum*) (Figures 15 & 16).

All the supernatants contained antisera that bound in various degrees to the *P. olseni* SA-00978-12T antigens. Some hybridoma supernatants, labelled by their plate and well numbers, are shown in Figure 15. Although the standard error was high, it seemed that the supernatant of 1A4 was the most reactive against *P. olseni* SA-00978-12T antigens in comparison to the other antigens such as those from *P. chesapeaki*, *Thraustochytrium* sp., and two algae.

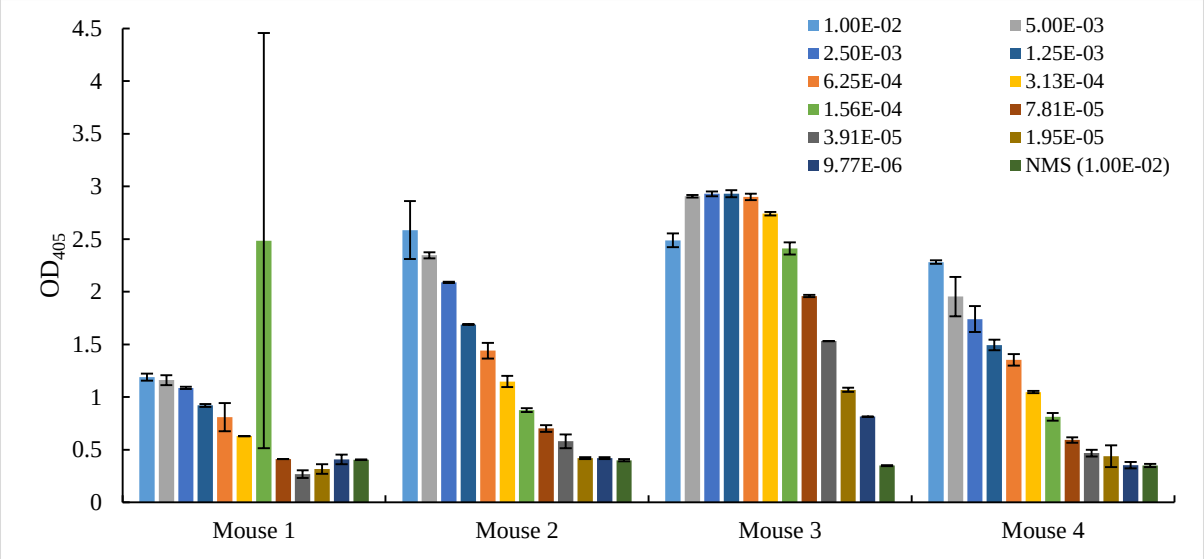


Figure 14: ELISA plot of mice antisera OD_{405} at different concentrations against *Perkinsus olseni* SA-00978-12T antigens. Data are mean ± Standard Error (SE), bar colours represent different serum concentration. NMS = normal mouse serum.

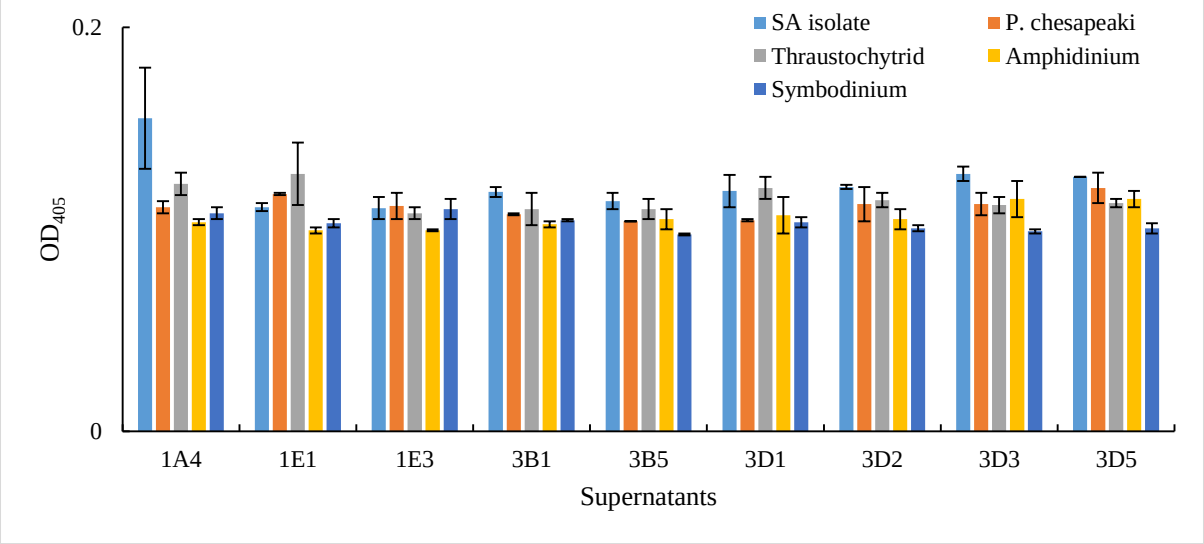


Figure 15: ELISA plot of selected supernatants against different SA-00978-12T *Perkinsus olseni* antigens (lysate) and other cells (negative cells controls). Data are mean ± SE, bar colours represent different antigen samples.

The IFA results were congruous with the ELISA results and gave further insights into where the antiserum bound (Figure 16). The best binding, as subjectively attributed to the level of fluorescence observed by epi-fluorescence microscopy, was seen for the supernatants 1A4, 1E3, 3B1 and 3D5. The technical negative control type 1 did not show any fluorescence while the positive controls (antisera from mouse 1 and 3) showed a high level of fluorescence. The IFA was repeated twice to confirm the results.

The two antibody assessment methodologies highlighted that the best candidates for further antibody development were 1A1, 1A4, 1E3, 3B1 and 3D5.

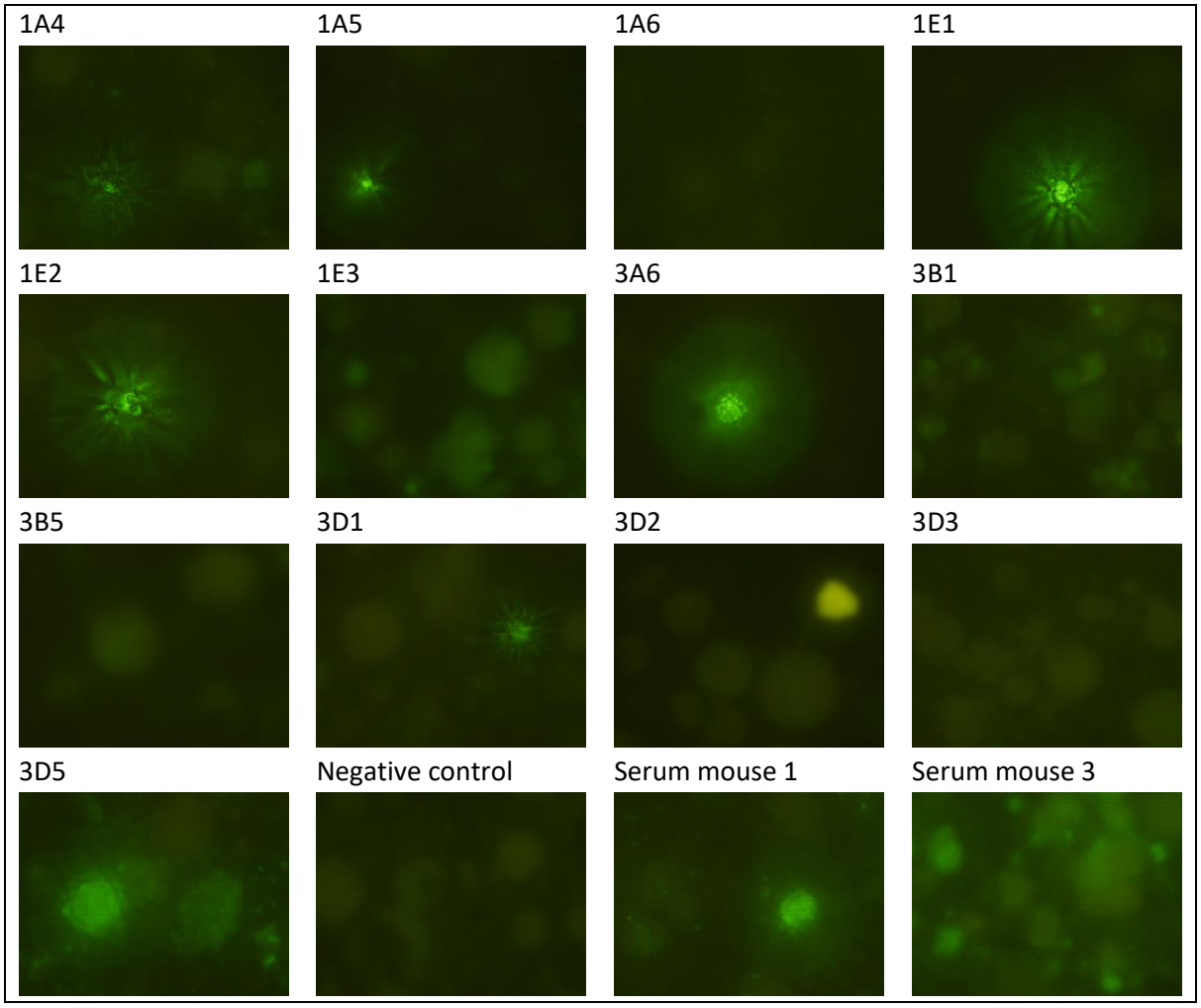


Figure 16: IFA results of selected supernatant candidates against *Perkinsus olseni* isolate ATCC PRA-179. Photos were taken at ISO 200, 2.5 s, objective x 20, the fluorophore used was FITC.

Specificity tests of the selected hybridoma supernatants

IFA were undertaken to evaluate if the selected supernatants that bound to *P. olseni* cells would cross-react with closely related organisms such as *P. chesapeaki*, *Amphidinium carterae* and *Symbiodinium tridacnidarum* or organisms that are also present in abalone tissues such as *Thraustochytrium* sp. (Figure 17), as preliminary screening test results using ELISA revealed a higher affinity of the antisera with *P. olseni* antigens rather than with cells from other organisms than *P. olseni*. All selected supernatants seemed to recognise *Thraustochytrium* sp. and *P. chesapeaki*. A red fluorescence was observed for both algal species (*A. carterae* and *S. tridacnidarum*), which was attributed to autofluorescence; however, no fluorescence was observed in the FITC channel, showing that the antisera did not cross-react with the two algal species.

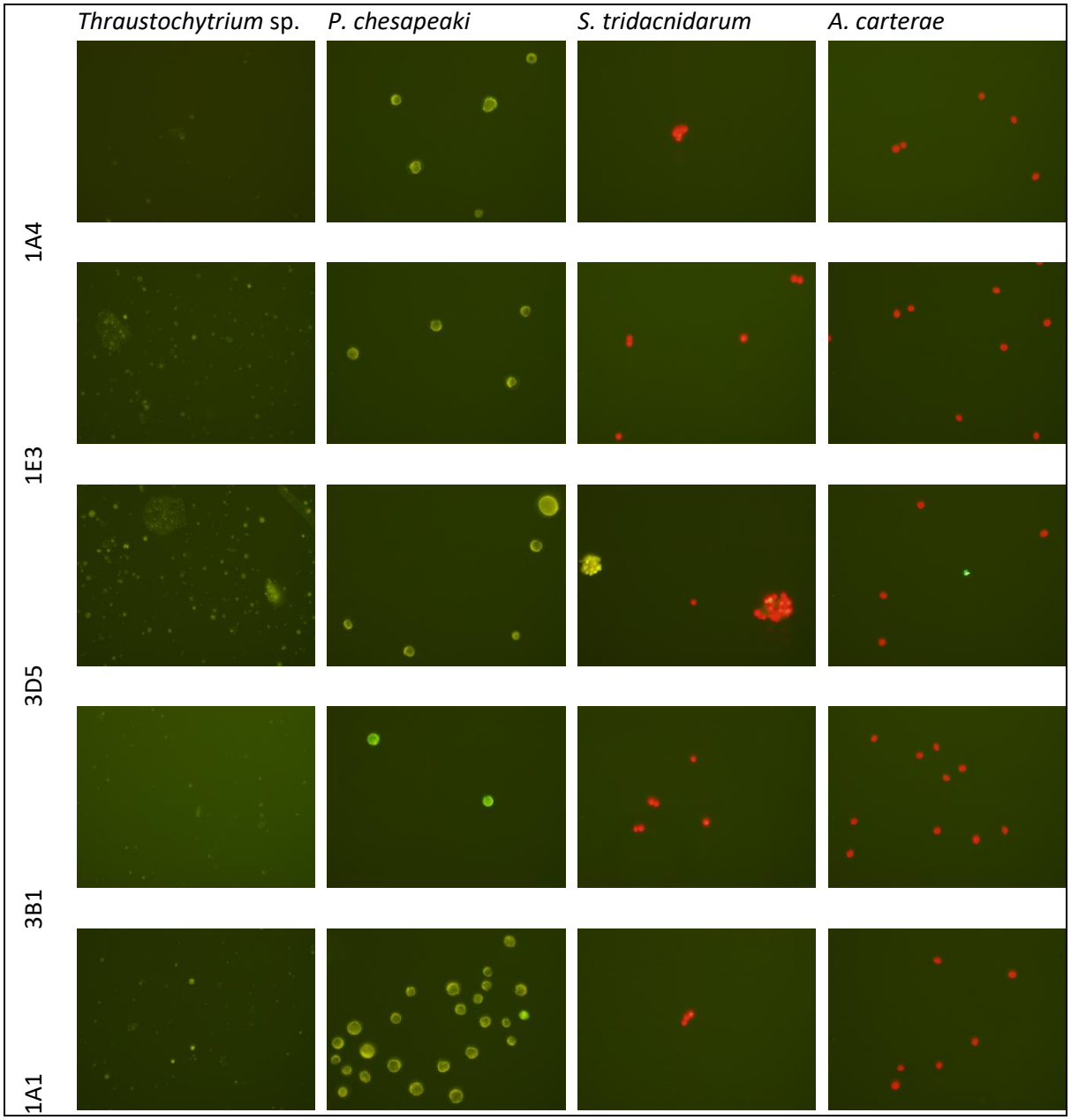


Figure 17: IFA results of selected supernatant candidates against different organisms: *Thraustochytrium* sp., *Perkinsus chesapeaki*, *Amphidinium carterae* and *Symbiodinium tridacnidarum*. The fluorophore used was FITC. Photos were taken at ISO 200, 5 s, objective x 20.

Autofluorescence of some of the isolates and controls

The IFA tests were repeated several times using the FITC-goat anti-mouse secondary antibody and some inconclusive results were obtained, such as when the same intensity of green fluorescence was detected in all samples including the negative controls. Therefore, the autofluorescence of the *Perkinsus* spp. and *Thraustochytrium* sp. isolates was assessed and the parasites were observed under the 3 channels of a fluorescent microscope (green (FITC), blue (Phycoerythrin (PE)), red (Allophycocyanin (APC)). *Perkinsus olseni*, *P. chesapeaki*, and *Thraustochytrium* sp. displayed some level of autofluorescence in the FITC-channel. *Perkinsus*

olseni was not autofluorescent in the red channel whereas *P. chesapeaki* presented a very low level of red autofluorescence. *Thraustochytrium* sp. displayed a high level of autofluorescence in the red channel as did the algae.

As the antigens from the 2 algae species seemed to not be recognised by IFA, it was decided to use a secondary antibody coupled with a far-red emitting fluorophore in the Texas Red channel. All previous IFAs were repeated with this new secondary antibody (Figure 18).

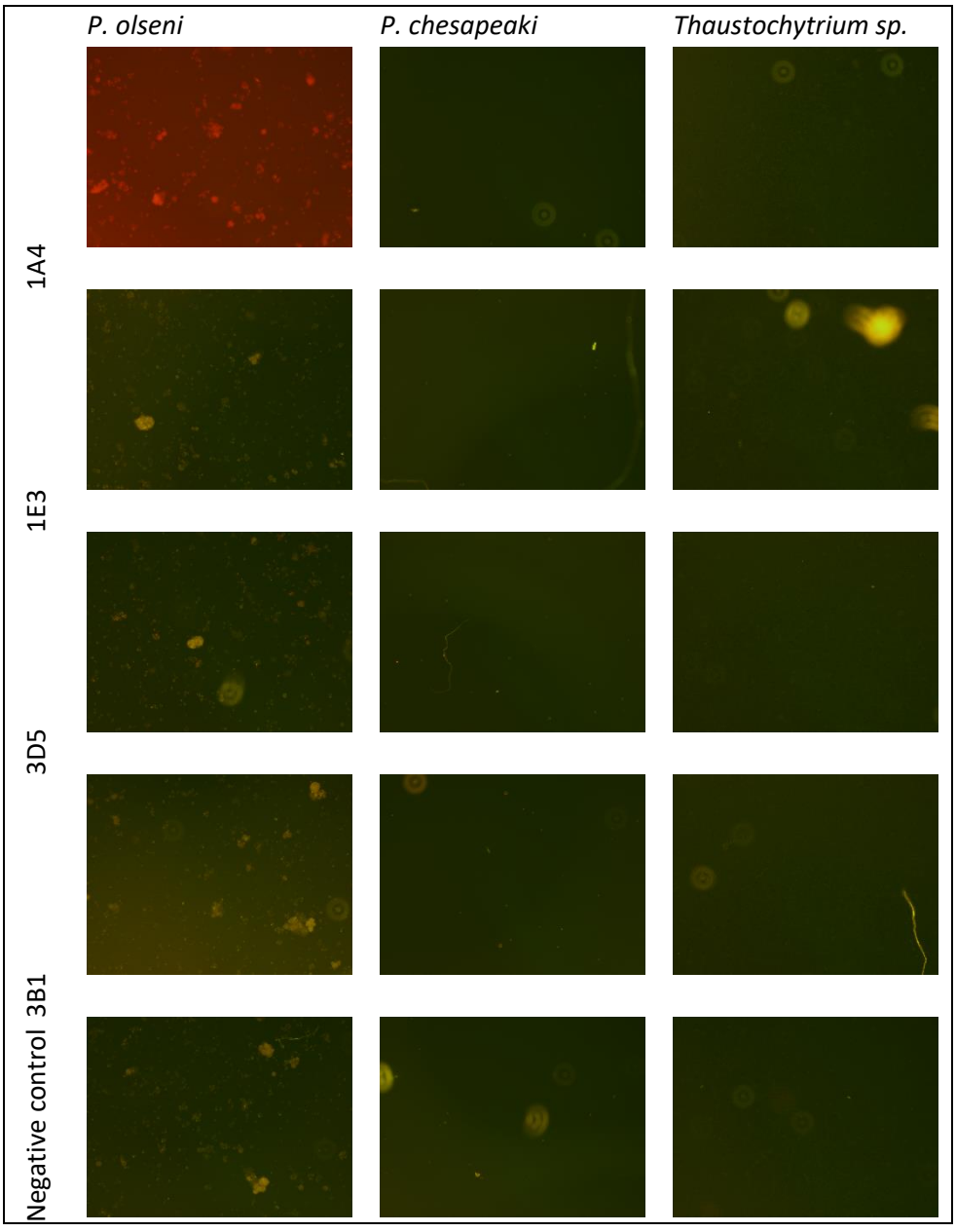


Figure 18: IFA results of selected supernatant candidates against *Perkinsus olseni*, *Perkinsus chesapeaki*, and *Thraustochytrium* sp. Photos were taken at ISO 200, 3 s, objective x 10. The fluorophore used was Texas Red. The negative control was negative control 1.

Those tests confirmed that the supernatant of 1A4 presented a positive red fluorescence against *P. olseni* antigens (SA-00978-12T). All other supernatants did not show a fluorescence above

the background level. From this, the focus of effort was on monoclonal antibody 1A4 to confirm by ELISA and IFA that it can recognise *P. olseni* isolates from any geographical location.

Supernatant 1A4

The 1A4 hybridoma supernatant reacted above background levels with all *P. olseni* geographical isolates, with the highest response displayed for the South Australian isolate of *P. olseni* SA-00978-12T (Figure 19). However, the 1A4 supernatant seemed to not recognise either *P. chesapeaki* or *hraustochytrium* sp.

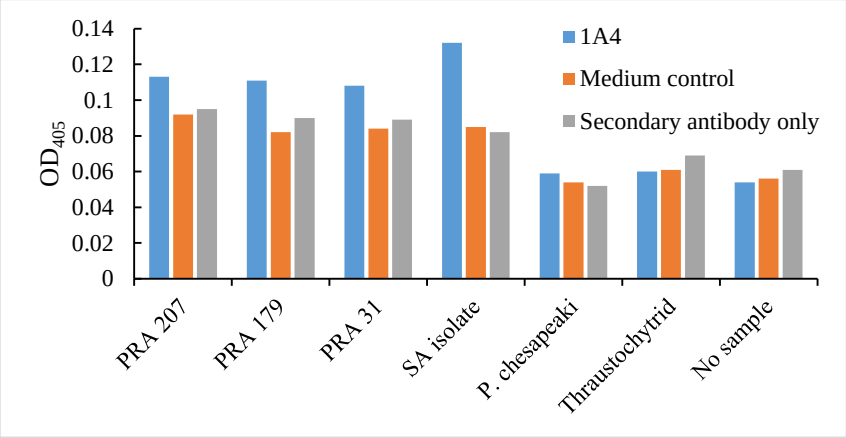


Figure 19: ELISA plot representing the response of supernatant 1A4 against antigens of *P. olseni* different geographical isolates (SA-00978-12T, PRA-207, PRA-179, PRA-31), *Perkinsus chesapeaki*, and *Thraustochytrium* sp. (Thaustochytrid), the medium control or technical negative control 2, and the technical negative control 1 containing a secondary but no primary antibody (antiserum).

Following those results, the 1A4 hybridoma cells were revived and cultured to start the antibody purification process in order to obtain a monoclonal antibody (mAb). 83 viable clones were obtained and were tested using ELISA against the lysate of *P. olseni* PRA-207 (Figure 20). However, a low reactivity was observed for most clones with only 13 clones showing an OD_{405} above background levels.

The ELISA and IFA tests were repeated using all the positive and also some negative clones shown in Figure 20 against *P. olseni* SA-0978-12T antigens; however, they led to negative results. The *P. olseni* SA-00978-12T isolate used was cultured for several weeks, so these tests were repeated using a freshly revived isolate of *P. olseni* SA-00978-12T, which had a limited number of passages in culture. This isolate gave clear results and enabled the selection of the best clone to proceed to the purification step. It also highlighted the difficulty of working with a parasite and the limitations of those methods (see discussion).

The performance of the parental and purified mAb 1A4 was investigated by IFA using *P. olseni* isolates from different geographical locations: PRA-31, PRA-179, PRA-207, and SA-00978-12T. Both the parental and the purified 1A4 recognised all the isolates tested.

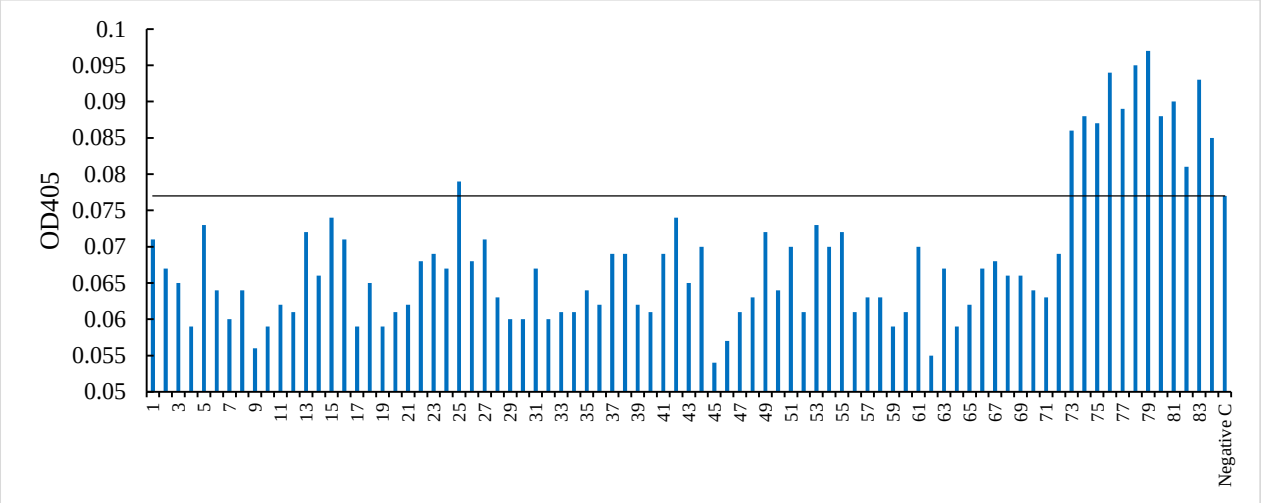
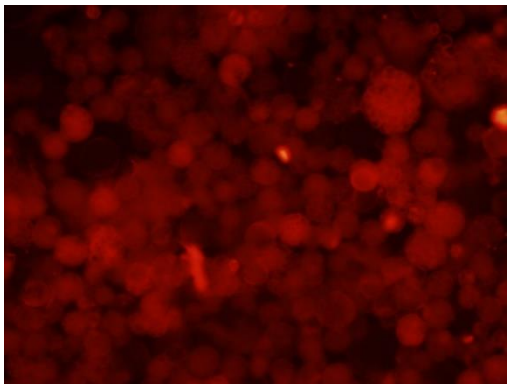
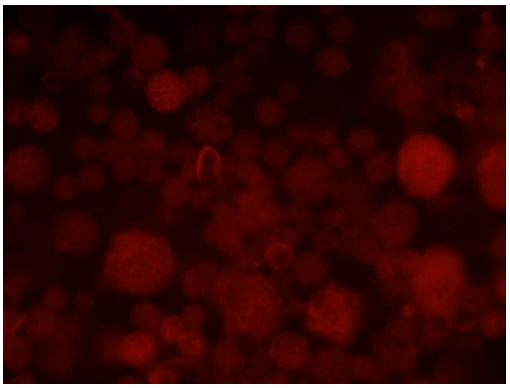
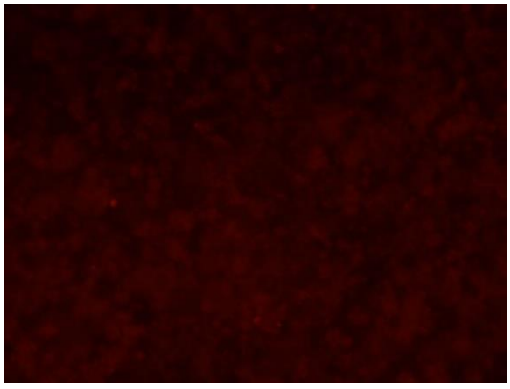
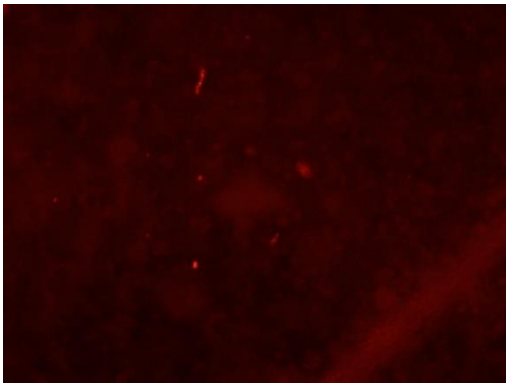
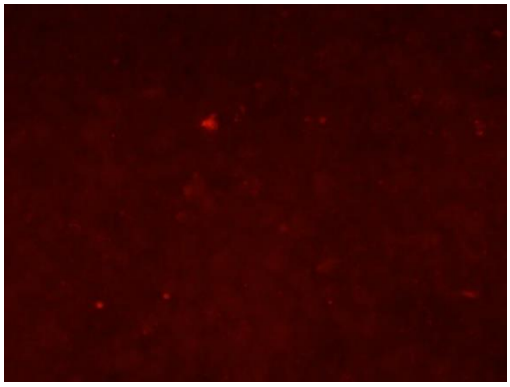
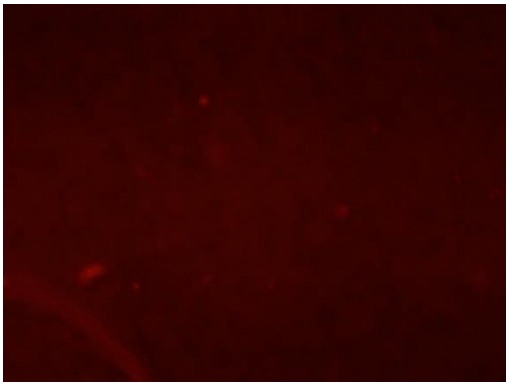
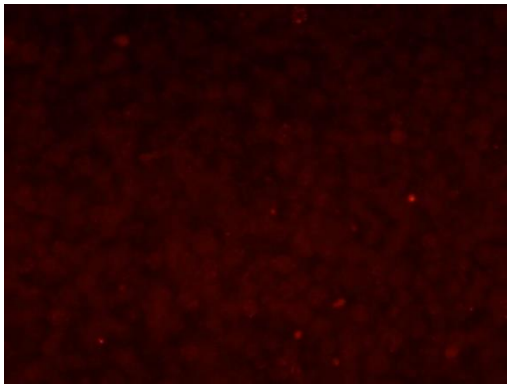
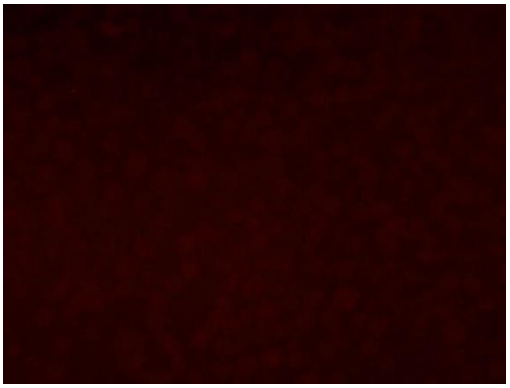


Figure 20: ELISA plot of different clones originating from the parental 1A4 hybridoma against lysate of *P. olseni* PRA-207.

Supernatant 3D5

The initial IFAs also revealed that the supernatant 3D5 seemed to be a good candidate for recognising *P. olseni* isolates. Therefore, IFAs were repeated using Texas Red as the fluorophore of the secondary antibody and the following isolates SA-00978-12T, PRA-31, PRA-179 and PRA-207, as well as the *Thaustochytrium sp.* (Figure 21). The isolates PRA-179 and PRA-207 showed a low level of fluorescence while SA-00978-12T displayed some level of fluorescence compared to the technical negative control 1. *Perkinsus olseni* isolate PRA-31 showed a very faint fluorescence, while the *Thaustochytrium sp.* had a strong fluorescence.

The supernatants selected after the initial tests (1A4, 1E3, 3B1 and 3D5) including 3D5 were used to perform an ELISA with a newly thawed *P. olseni* SA-00978-12T lysate (Figure 22). The 3D5 supernatant presented the highest OD_{450} in comparison with the other supernatants including 1A4, which highlighted some issues in working with a parasite; see discussion.

P. olseni SA-00978-12T 	*P. olseni* Sa-00978-12T negative control 1 
P. olseni PRA-179 	*P. olseni* PRA-179 negative control 1 
P. olseni PRA-207 	*P. olseni* PRA-207 negative control 1 
P. olseni PRA-31 	*P. olseni* PRA-31 negative control 1 

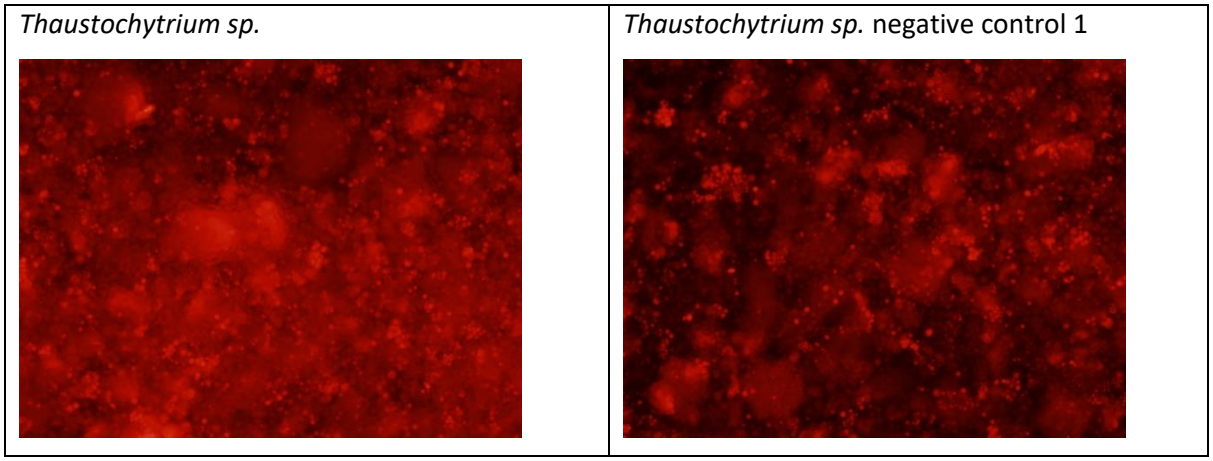


Figure 21: IFA results of different *Perkinsus olseni* isolates (SA-00978-12T, PRA-179, PRA-207, PRA-31) as well as *Thraustochytrium* sp.against the 3D5 supernatant. The fluorophore used was Texas red. The technical negative control 1 contained the secondary antibody only. Photos were taken at ISO 200, exposure time of 1.2 s, and at x 10.

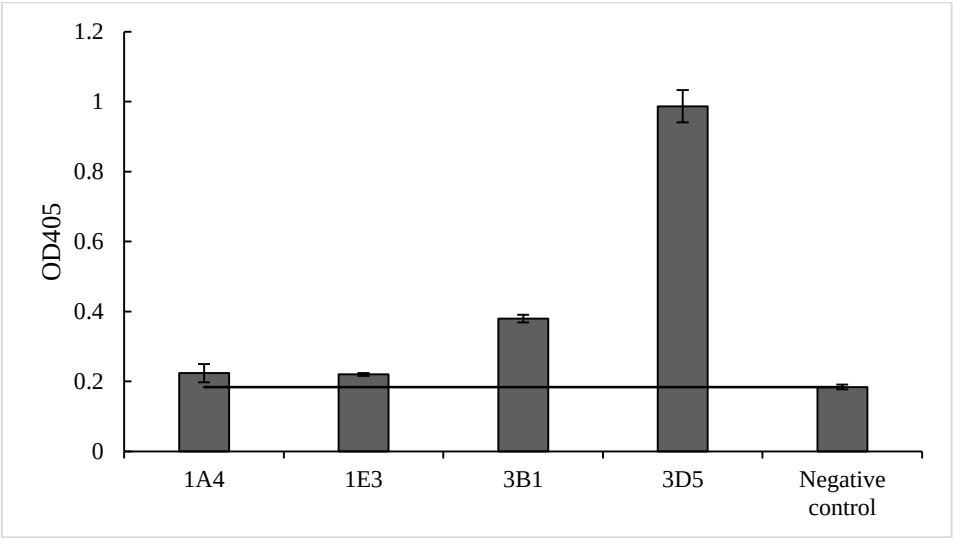


Figure 22: OD_{405} of different antisera binding to the freshly thawed South Australian *Perkinsus olseni* isolate SA-00978-12T. Horizontal line represents the threshold set by the negative control (technical negative control 1). Data are mean ± SE.

As the results were variable, the ELISA was repeated with supernatant 3D5 against all the geographical isolates of *P. olseni* (1405-01-0978 or SA-00978-12T, PRA-31, PRA-179, PRA-207) as well as *Thaustochytrium sp.* (Figure 23). The OD_{405} values obtained were relatively low but still revealed a higher response of 3D5 antiserum for all the isolates in comparison to the technical negative control 1; Figure 23. It also demonstrated that the 3D5 antiserum did not recognise the Thraustochytrid, which was used as a negative cell control to test for the specificity of the antibodies.

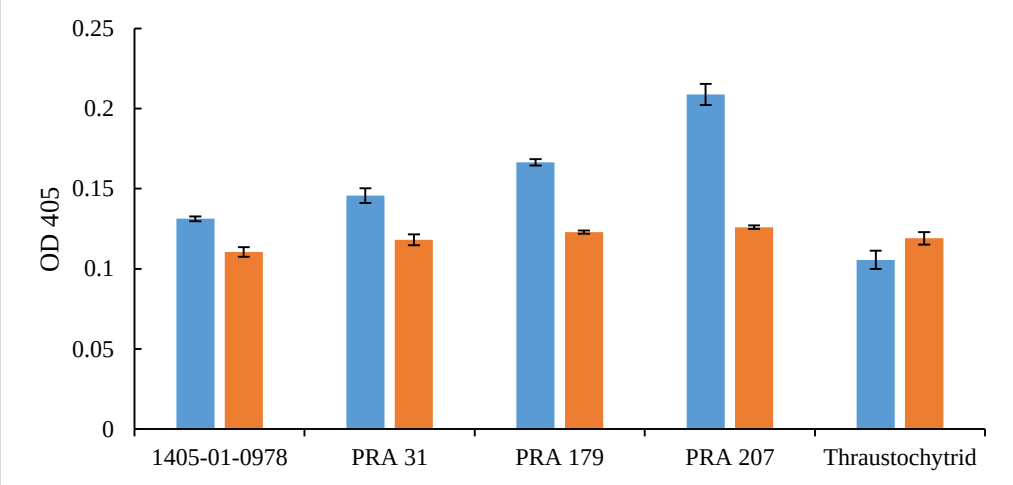


Figure 23: ELISA plot of supernatanjt 3D5 against cell lysatesof *Perkinsus olseni* (SA-00978-12T or 1405-01-00978, PRA-31, PRA-179, PRA-207) and Thraustochytrid cells. Results are presented as mean ± standard deviation. Blue columns represent the absorbance of samples; orange columns represent the absorbance of the technical negative controls 1 of those samples.

However, and in accordance with some confounding results generated by ELISA and IFA, the screening was repeated using another method: flow-cytometry (see section *3.3.3. Flow-Cytometry screening*). Indeed, IFA is a technique that is subjective to the operator.

3.3.1.2. Second batch of mice

In parallel to the tests and analysis of the results performed for the first batch of mice, the immunisation process using whole *Perkinsus* cells (lysate of *P. olseni* 1405-01-0978 or SA-00978-12T from South Australia) was repeated with a second batch of mice as a back-up.

Mouse selection for the fusion

The antisera from the second batch of mice were tested and against the antigens of *P. olseni* PRA-207, PRA-31 using ELISA (Figures 24) and against the antigens of *P. olseni* SA-00978-12T using IFA (Figure 25). In addition, the supernatants 1A4, 1E3, 3B1 and 3D5 from the first batch of mice were added to the IFA test as reference samples (Figure 25). The antisera from mice 2 and 4 exhibited the highest response for both tests and were therefore selected for the hybridoma fusion.

Among the supernatants selected from the first batch of mice, the 1A4 and 3D5 antisera showed a high fluorescence with antigen compared to background (negative control) in the IFA (Figure 25).

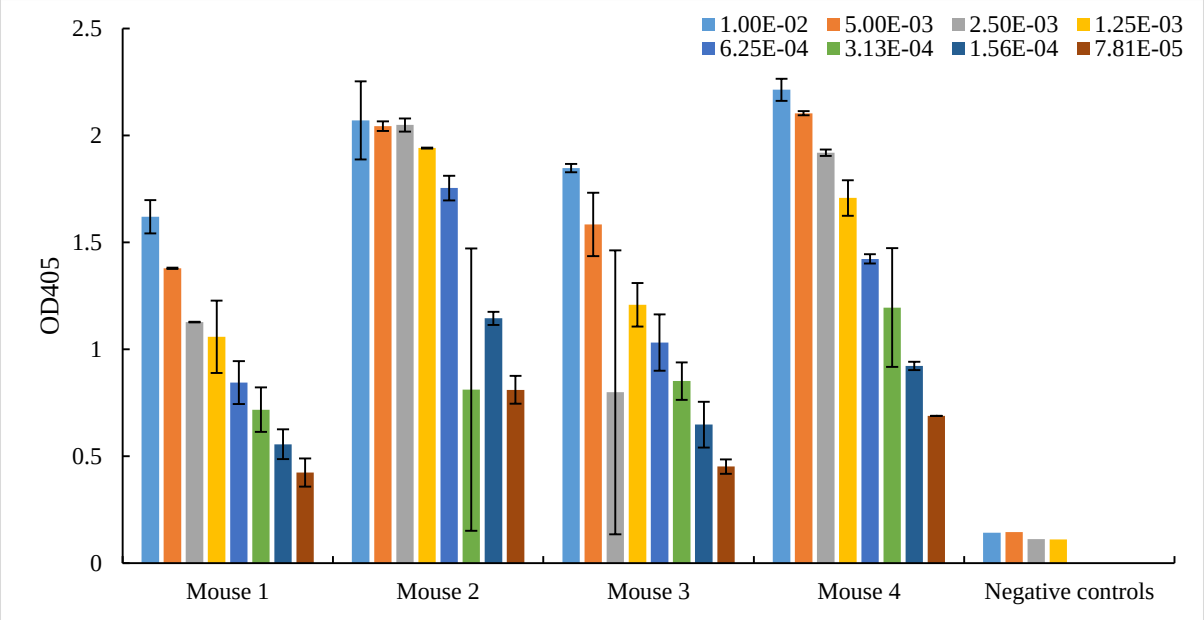


Figure 24: ELISA plot of different antisera from the 4 mice, positive controls and technical negative controls 1 against a mix of antigens (mix of PRA-207 and PRA-31 lysate). Bar colours represent decreasing concentration of antiserum. Data are mean ± SE.

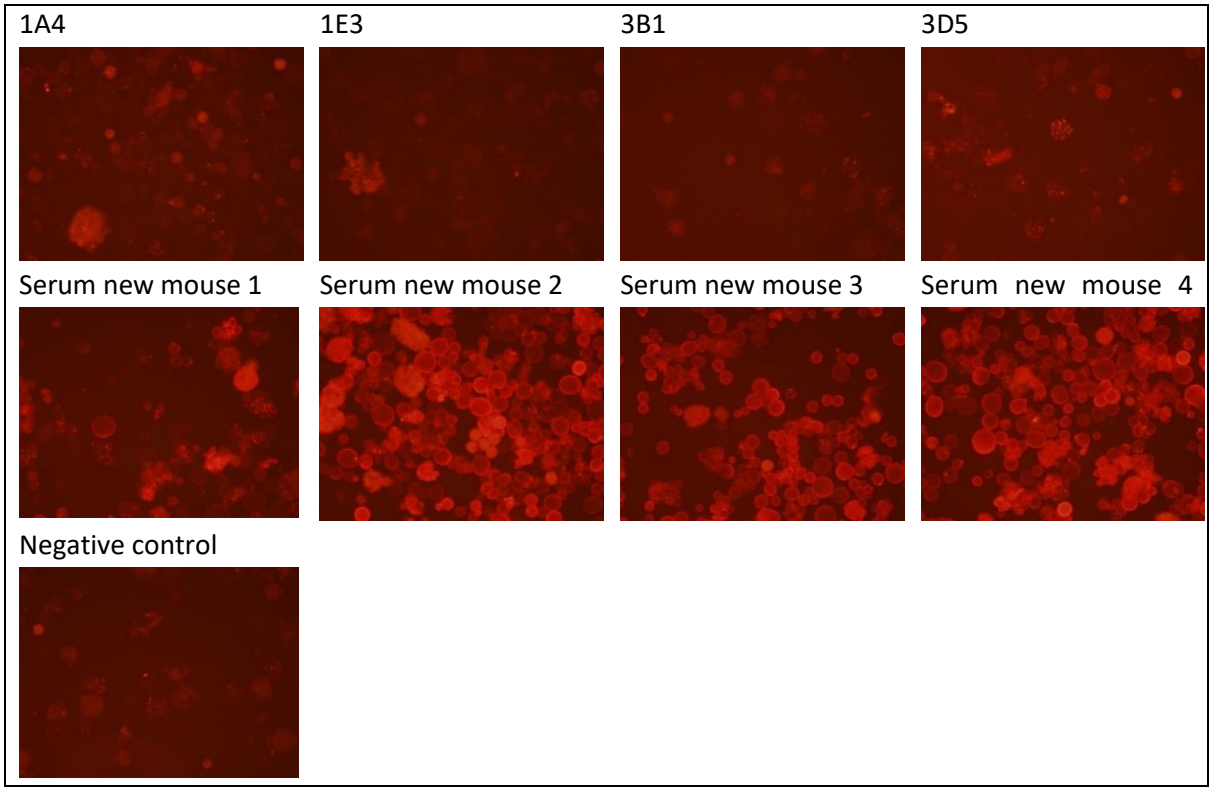


Figure 25: IFA results of different antisera from the second batch of mice against *Perkinsus olseni* SA-00978-12T isolate and the technical negative control 1. Photos were taken at ISO 200, 1.1 s, objective x 10. The fluorophore used was Texas Red.

Selection of hybridomas

The fusion resulted in hybridomas successfully proliferating in 136 wells, which were screened using first ELISA and then flow-cytometry. Out of 136 wells screened, two slightly positive clones (7E1, 7H12) and one higher positive clone (10E2) were identified using ELISA against *P. olseni* SA-0978-12T antigens. Only one of the supernatants was positive (7E1) using some freshly prepared surface antigens following the proteolytic shaving method described in the material and method section. Similarly, flow-cytometry results, which are presented in section 3.3.3. below revealed only one positive supernatant (7E1).

3.3.2. Development of antibodies using *Perkinsus olseni* specific antigens

Identification of common peptides using proteomics

The proteolytic shaving extraction method resulted in LC-Ms/MS detecting more than 500 peptides. Data from the five isolates that originated from different geographical locations (SA-00978-12T, PRA-31, PRA-179, PRA-207, Qld-39) were compared with the predicted proteins generated from the genomic data. Data from *P. chesapeaki* were used as a negative control. This resulted in the identification of 34 proteins that were common across all *P. olseni* isolates (Table 11). All 34 are moonlighting proteins, i.e. proteins that can perform different functions depending on their location within the cell.

Table 11: 34 moonlighting proteins common to all *Perkinsus olseni* isolates.

***P. olseni* proteins**	**Best protein match**	**Homology with *P. marinus***	**Description of protein match**	**Function**	**Link**
POLS_00109	XP_002788182	99.3%	heat shock protein	ATP binding	https://www.uniprot.org/uniprot/C5K6Q8
POLS_00394	XP_002782646	99.2%	chaperone protein DNAK	Protein folding	https://www.uniprot.org/uniprot/C5K4R1
POLS_01873	XP_002788841	60.5%	aldehyde dehydrogenase	Oxidoreductase activity	https://www.uniprot.org/uniprot/C5K4E4
POLS_01889	XP_002788244	84.6%	60S ribosomal protein L3	Structural constituent of ribosome	https://www.uniprot.org/uniprot/C5K610
POLS_02995	XP_002780577	96.2%	malate dehydrogenase	L-malate dehydrogenase activity	https://www.uniprot.org/uniprot/C5KT22
POLS_04688	XP_002785337	99.7%	vacuolar ATP synthase subunit b	ATP binding	https://www.uniprot.org/uniprot/C5KEJ5
POLS_05273	XP_002783797	98.6%	60S ribosomal protein L19	Structural constituent of ribosome	https://www.uniprot.org/uniprot/C5KBV0
POLS_05635	XP_002784462	96.9%	60S ribosomal protein L5	Structural constituent of ribosome	https://www.uniprot.org/uniprot/C5KI46
POLS_05636	XP_002784461	97.6%	40S ribosomal protein S7	Structural constituent of ribosome	https://www.uniprot.org/uniprot/C5KB01
POLS_06012	XP_002780651	95.1%	peroxidoxin 2	Peroxiredoxin activity	https://www.uniprot.org/uniprot/C5KT96
POLS_07171	XP_002775922	98.3%	succinate dehydrogenase	Heme binding	https://www.uniprot.org/uniprot/C5L6B1
POLS_07505	XP_002772476	99.3%	60S ribosomal protein L27	Structural constituent of ribosome	https://www.uniprot.org/uniprot/C5K735
POLS_08062	XP_002787708	99.0%	fructose-1,6-biphosphate aldolase	Involved in glycolysis	https://www.uniprot.org/uniprot/C5K7G9
POLS_08089	XP_002769741	98.7%	60S ribosomal subunit protein L4	Structural constituent of ribosome	https://www.uniprot.org/uniprot/C5KY49
POLS_08238	XP_002783667	84.2%	enolase 2	Involved in glycolysis	https://www.uniprot.org/uniprot/C5KJ81
POLS_08386	XP_002781757	93.0%	phosphoacetylglucosamine mutase	Involved in synthesis of UDP-N-acetyl-α-D-glucosamine	https://www.uniprot.org/uniprot/C5KQ05
POLS_08693	XP_002771639	100.0%	40S ribosomal protein S14	Structural constituent of ribosome	https://www.uniprot.org/uniprot/C5K7W5
POLS_08844	XP_002774012	90.0%	conserved hypothetical protein	Uncharacterized protein	https://www.uniprot.org/uniprot/C5LBK7
POLS_08890	XP_002780357	95.1%	ATP synthase subunit α	ATP synthesis coupled proton transport	https://www.uniprot.org/uniprot/C5KTP2
POLS_09133	XP_002782646	94.9%	chaperone protein DNAK	Protein folding	https://www.uniprot.org/uniprot/C5K4R1
POLS_09274	XP_002769153	97.8%	RNA helicase	Helicase activity, ATP binding, nucleic acid binding	https://www.uniprot.org/uniprot/C5LN72

POLS_09534	XP_002786917	99.0%	elongation factor 1-α S	GTPase activity, GTP binding, translation elongation factor activity	https://www.uniprot.org/uniprot/C5K9Y4
POLS_09964	XP_002769741	90.5%	60S ribosomal subunit protein L4	Structural constituent of ribosome	https://www.uniprot.org/uniprot/C5KY49
POLS_10146	XP_002771835	48.1%	NADH-cytochrome b5 reductase	Oxidoreductase activity	https://www.uniprot.org/uniprot/C5LHP7
POLS_10271	XP_002788268	98.0%	translongation elongation factor EF-1, subunit α	GTPase activity, GTP binding, translongation elongaction factor activity	https://www.uniprot.org/uniprot/C5K634
POLS_11197	XP_002780357	95.2%	ATP synthase subunit α	ATP synthesis coupled proton transport	https://www.uniprot.org/uniprot/C5KTP2
POLS_11817	XP_002786310	96.6%	eukaryotic initiation factor-2 α-subunit	Translation initiation factor activity	https://www.uniprot.org/uniprot/C5KBN6
POLS_12394	XP_002774487	75.9%	glutathione s-transferase	Regulation of pH through transferase and sodium:proton antiporter	https://www.uniprot.org/uniprot/C5LA47
POLS_12726	XP_002788110	94.6%	glutamine synthetase	Nitrogen compound metabolic process through glutamate-ammonia ligase activity	https://www.uniprot.org/uniprot/C5K6I6
POLS_14359	XP_002784650	97.6%	heat shock protein	ATP binding	https://www.uniprot.org/uniprot/C5KG90
POLS_14548	XP_002767284	59.0%	cysteine desulfurase	Catalytic activity	https://www.uniprot.org/uniprot/C5LT34
POLS_15032	XP_002774552	98.8%	cell division cycle protein	Cell division through ATP binding and hydrolase activity	https://www.uniprot.org/uniprot/C5LAB2
POLS_15404	XP_002775922	92.2%	succinate dehydrogenase	Heme binding	https://www.uniprot.org/uniprot/C5L6B1
POLS_15916	XP_002780398	96.6%	conserved hypothetical protein	Proton transmembrane transport through NAD(P)+ transhydrogenase activity	https://www.uniprot.org/uniprot/C5KTL4

The second extraction method using biotinylation led to the identification of several hundred peptides, however only two of these, POLS-08089 and POLS-15916, were common to all the isolates of *P. olseni* with both extraction methods. Respectively, the two peptides were identified as the 60S ribosomal subunit protein L4 and a conserved protein of unknown function. Molecular weights were 42.7 kDa for POLS_08089 and 55.6 kDa for POLS_15916.

Expression of peptide genes in different life stages of *P. olseni*

The expression of POLS_08089 and POLS_15916 genes was first assessed in a mix of *P. olseni* cells representing different life stages such as trophozoites, schizonts, prezoosporangia and zoospores, and secondly, in 100 trophozoites and 100 schizonts that were previously sorted using a manual cell picker (Table 12).

Table 12: ΔCt (Ct gene of interest – Ct housekeeping gene) results for the genes of interest in *Perkinsus olseni* mix (Mix of cell types from SA-0978-12T) and individual cells (trophozoites, schizonts, prezoosporangia). All samples were run in triplicate. *Perkinsus chesapeaki* and *Thraustochytrium* sp. cells were also used as negative controls.

Sample	Δ Ct 15916	Δ Ct 08089
PRA-31	0.378	-2.638
PRA-207	3.852	3.730
PRA-179	-0.585	-1.349
P. chesapeaki	0.983	1.212
Thraustochytrium sp.	-7.845	2.439
Trophozoites SA 0978	-5.66	-12.40
Schizonts SA 0978	-0.54	-10.74
Mix SA 0978	-6.70	-14.59

The results demonstrated that both peptide genes are up-regulated in trophozoites, schizonts, and the mix of cells of the South Australian isolate (SA-00978-12T), with POLS_08089 transcripts found in higher amounts than POLS_15916 (Table 12). A similar trend was observed for PRA-179 (Japan) whereas the opposite was true for PRA-31 (Spain) and PRA-207 (New Zealand) (Table 12).

PLOS_08089 corresponds to protein L4 of the 60S ribosomal unit and even if, as a moonlighting protein, it may have other functions and simultaneously be located in the cytoplasmic membrane, it is probable that its location is predominantly intracellular. Therefore, as PLOS_15916 was also up-regulated and corresponded to a protein of unknown function, two immunogenic amino acid sequences were determined from PLOS_15916 sequence and were sent to DGpeptides Co Ltd in China, which generated two synthetic peptides labelled 3335 (CAEKKPEPQKVKEYPPA) and 3336 (CKINTLETEEDIKSLRKGH). Those two peptides were subsequently injected into a third batch of mice.

Third batch of mice and selection of hybridomas

The third batch of four mice were immunised using a mix of the two peptides 3335 and 3336 conjugated to KLH. Mice were boosted twice, however, two of the mice died after this process and the spleens from the two remaining mice (mouse 1 and mouse 4) were used to proceed to the hybridoma fusion.

The antisera from mice 1 and 4 were tested using ELISA against *P. olseni* SA-00978-12T antigens but also against each of the two peptides 3335 and 3336 conjugated to BSA (Figure 26). ELISA results revealed both mouse antisera reacted with the *P. olseni* antigens but not with the peptides (Figure 26).

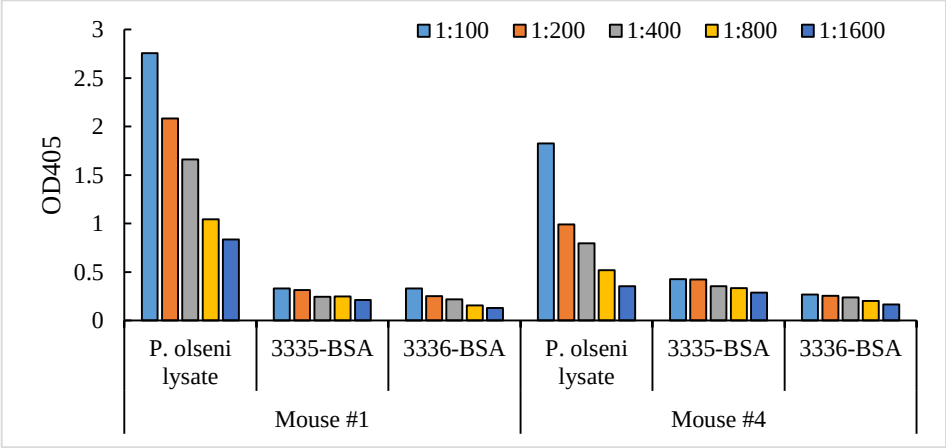


Figure 26: ELISA plot of mice 1 and 4 antisera against *Perkinsus olseni* SA-00978-12T antigens, peptide 3335 conjugated to BSA, and peptide 3336 conjugated to BSA. Barcolours represent decreasing concentration of antiserum.

The fusion resulted in hybridomas proliferating in 48 wells, which were screened using ELISA against *P. olseni* SA-0978-12T antigens, but also against the two peptides both free and conjugated to BSA.

Despite some good ELISA results from the mouse antisera, only a few hybridomas were positive when screened by ELISA. The plate coated with free peptides did not show any positive reactions whereas some positive reactions were observed in the two plates coated with the two peptides conjugated to BSA and with *P. olseni* lysate SA-00978-12T antigens. Subsequent screening using flow-cytometry confirmed five positive hybridoma supernatants, which included three slightly positive hybridomas (11A6, 11C3, 12B6) and two good positive hybridomas (11D3, 11D5) (see section below, *3.3.3. Screening using flow cytometry*).

Second batch of mice screened against the peptides

The four mouse antisera were previously screened using ELISA against *P. olseni* lysate antigens (*P. olseni* isolates PRA-31 and PRA-207; Figure 24). They were subsequently screened against *P. olseni* SA-0978-12T (Figure 27A), a mix of *P. olseni* isolates (PRA-31 and

PRA-207; Figure 27B) as well as free peptides (Figures 27C & D) and peptides conjugated with BSA (Figures 27E & F).

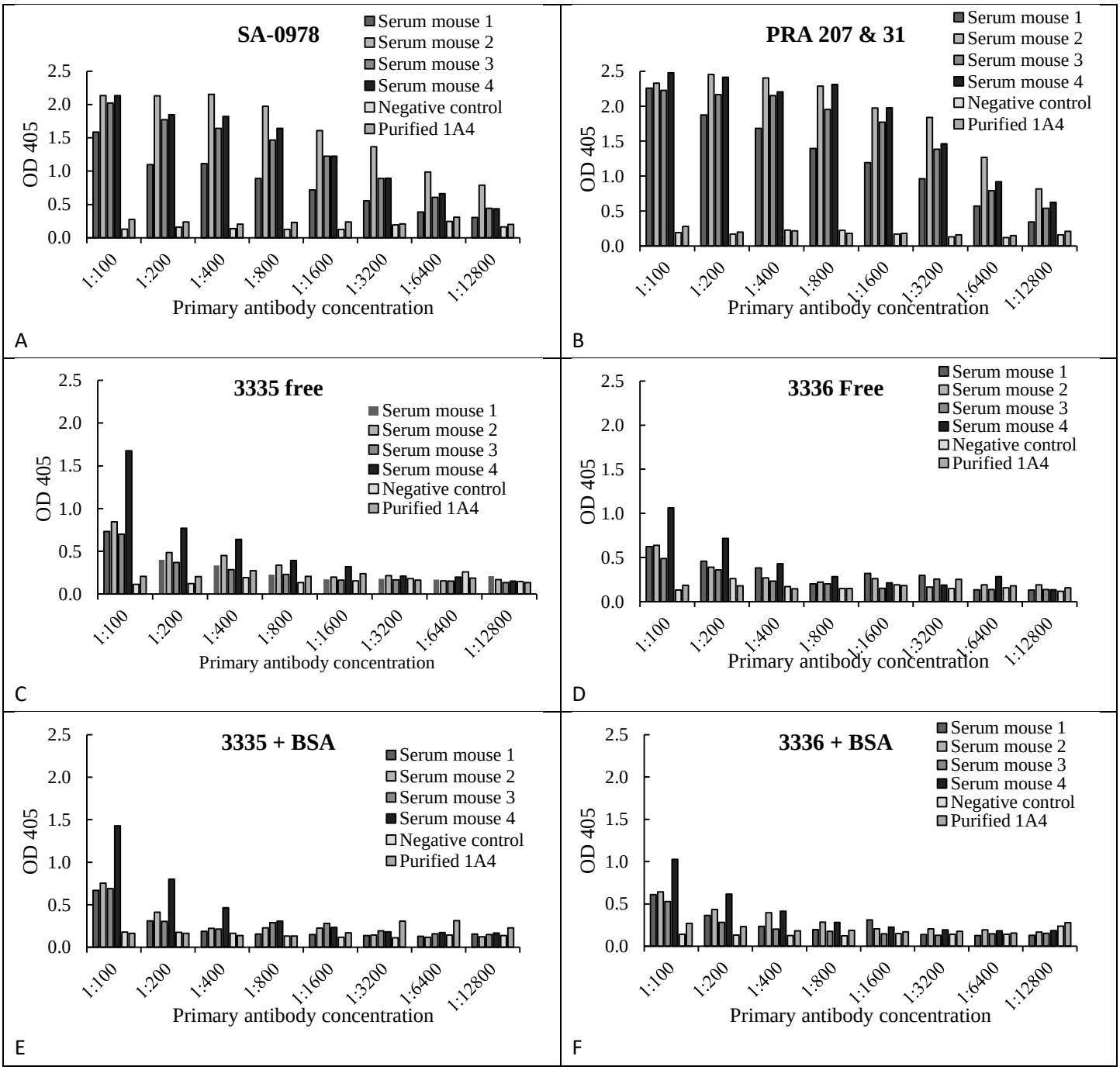


Figure 27: ELISA plots of 4 mice antisera from batch 2 against *Perkinsus olseni* SA-00978 antigens (A), a mix of PRA-207 and PRA-31 antigens (B), peptide 3335, free (C), peptide 3336, free (D), peptide 3335 conjugated to BSA (E), and peptide 3336 conjugated to BSA (F). Gray scales represent different concentration of antiserum. The purified 1A4 mAb antibody was also included in the test. The negative control used was the technical control type 1.

Those data confirmed that the purified hybridoma antibody 1A4 from mouse batch 1, which gave good results using IFA, produced poor results when screened by ELISA. It also confirmed

that the selected peptides 3335 and 3336 (POLS_15916) met expectations as it produced positive results, especially with the antisera of mouse batch 4 (Figures 27 C, D, E, and F). There was no difference in the intensity of OD_{405} whether peptides were free or conjugated to BSA. This revealed that free peptides stick to the plastic of the plate, which sometimes only occurs when peptides are conjugated with. Mouse 4 antiserum could recognise both peptides, with a higher affinity for peptide 3335, and this result was further investigated using the hybridomas obtained after the fusion.

Overall, the antisera from mice 2 and 4, of which the spleen was used for the fusion, produced the best response for all *P. olseni* isolates from different geographical locations (Figure 24 and 25) and after the fusion, three hybridoma supernatants appeared to recognise *P. olseni* antigens SA-00978-12T. Further ELISA were conducted to evaluate if the hybridoma supernatants could recognise the peptides. All hybridoma supernatants were screened against peptide 3335 and peptide 3336. Two weak positives were observed when the plate was coated with peptide 3335 (hybridoma 7H12 and 8H12) and one good positive was revealed when the plate was coated with peptide 3336 (hybridoma 10E2). These positive reactions, however, were not confirmed when screened using flow-cytometry as the final confirmation method.

First batch of mice, mAb and supernatant of 1A4 and 3D5 screened against peptides 3335 and 3336

The purified mAb 1A4 and the supernatant of 3D5 were tested using ELISA against peptide 3335, peptide 3336, *P. olseni* antigens, and shaved antigens (obtained by repeating the proteolytic shaving method). No positive result was obtained with products from these two hybridomas.

3.3.3. Screening using flow-cytometry

All hybridoma wells from the three batches of immunised mice were screened using flow-cytometry as a final confirmatory method. Isolates SA-00978-12T, PRA-31, PRA-179, and PRA-207 of *P. olseni* were assessed and displayed a wide range of size and complexity (Figure 28A). The cells used with this method and for general screening were cells proliferating *in vitro*, which were composed of a mix of trophozoites, schizonts, prezoosporangia and sometimes zoospores. The dominant cell population of *in vitro* cultures of *P. olseni* was trophozoites, and constituted the main population observed, see Figure 28A.

The secondary antibody used to perform this test was conjugated to the fluorophore Alexa Fluor 647, which is excited by the red laser and fluoresces in the far red or FL4 channel of the BD Accuri C6 Plus flow-cytometer used in the study. Figure 28B represents the technical negative control 2 (medium control containing the secondary antibody) with the fluorescence in the FL4 channel on the y-axis and the size of the cells on the x-axis for the same population of cells as shown in Figure 28A.

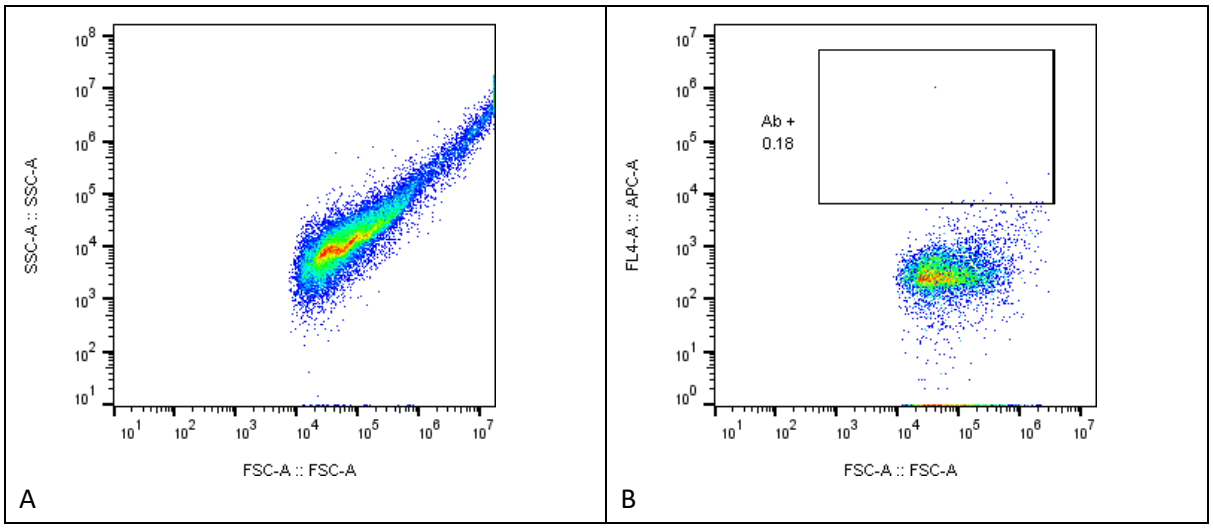


Figure 28: Cytograms representing unlabelled *Perkinsus olseni* (PRA-207) cells from an *in vitro* culture. SSC: Side Scatter; FSC: Forward Scatter; FL4: Fluorescent channel where excitation occurs with a red laser (640 nm) and the emission in the far red (>670 nm).

All supernatants from hybridomas obtained from the three batches of mice were screened and many of them produced weak positives with approximately 2% of positive trophozoites. The only supernatants that produced positives above 2% are described in Table 13 and the results are shown for the four geographical isolates of *P. olseni*. An example of a positive sample using the supernatant of hybridoma 3D5 against *P. olseni* PRA-207 is shown in Figure 29B with a reference to its technical negative control 2 (Figure 29A).

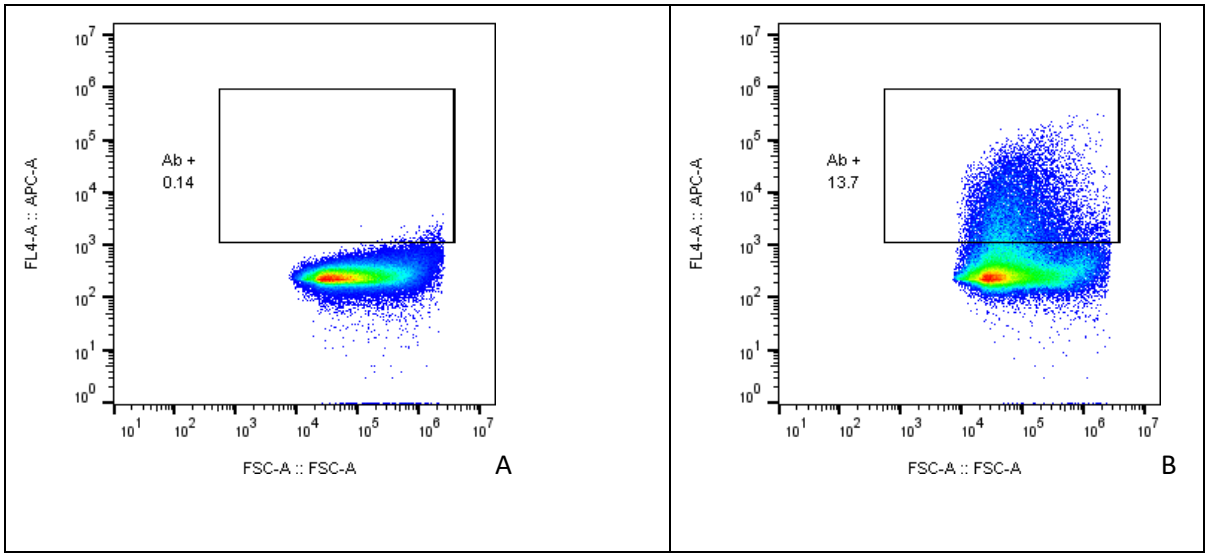


Figure 29: Cytogram showing the negative control (A) and positive cells to hybridoma 3D5 supernatant located inside the gate Ab+ for the *Perkinsus olseni* isolate PRA-207 (B).

Table 13: Percentage of positive cells of *Perkinsus olseni* isolates from different geographical locations that are recognised by different hybridoma supernatants or mAb. Supernatants 1A1, 1A4, 1E3, 1F3, 1F4, 3D1, 3D5 correspond to mice batch 1; 7E1 is from mouse batch 2; 11D3 and 11D5 to mouse batch 3. Pa: parental 1A4. The highest percentage of positive cells is highlighted in yellow.

Supernatant	**SA- 0978-12T**	**PRA-31**	**PRA-179**	**PRA-207**
Country	Australia	Spain	Japan	New Zealand
Region	Oceania	Eurasia	Eurasia	Oceania
1A1	1.080	0.230	0.110	1.540
1A4Pa	3.060	0.340	0.480	3.580
1A4 mAb	8.740	0.880	2.160	7.410
1E3	0.860	0.027	0.031	0.580
1F3	15.300	0.032	0.059	8.890
1F4	0.350	0.015	0.061	0.150
3D1	6.230	1.400	1.000	7.310
3D5	13.900	4.290	3.340	13.700
7E1	1.250	0.046	2.660	2.200
11D3	2.500	0.530	1.120	2.070
11D5	0.350	0.023	0.050	0.028
Technical negative control 2	0.140	0.029	0.014	0.008
Technical negative control 1	0.350	0.027	0.023	0.061

The highest percentage of positive cells for the four geographical isolates of *P. olseni* was obtained for the 1A4 mAb and the supernatants of hybridomas 1F3, 3D1, and 3D5; these are highlighted in yellow in Table 13. It is interesting to note that the percentage of positive trophozoites is similar based on the region of origin of the isolate. Indeed, there is a similarity between the Eurasian isolates and between the Oceanian isolates but a dissimilarity between isolates from Oceania and Eurasia (Table 13, Figure 30). The observed similarities and dissimilarities are consistent with that based on genomic analysis. For instance, the supernatant of 3D5 recognised 13.9% and 13.7% of trophozoites for the two Oceanian isolates SA-0978 and PRA-207; and 4.3% and 3.3% of trophozoites for the two Eurasian isolates PRA-31 and PRA-179 respectively (Table 13, Figure 30).

The cross-reactivity of the antisera with specific life stages of *P. olseni* was investigated using flow-cytometry on different cell types such as hypnospores, which were obtained after incubation of cultured cells in RFTM medium for 5 days. However, this did not yield any positive results as the hypnospores were too large for the flow-cytometer and seemed to present a higher level of autofluorescence than the trophozoites. We analysed the hypnospores using IFA, however this method lacked precision and whereas the hypnospores were highly fluorescent, some level of fluorescence (albeit lower) were also observed in the negative control, and thus confirming the autofluorescence observed with flow-cytometry. After

numerous attempts, obtaining zoospores by placing hypnospores in filtered sterile aerated seawater have failed.

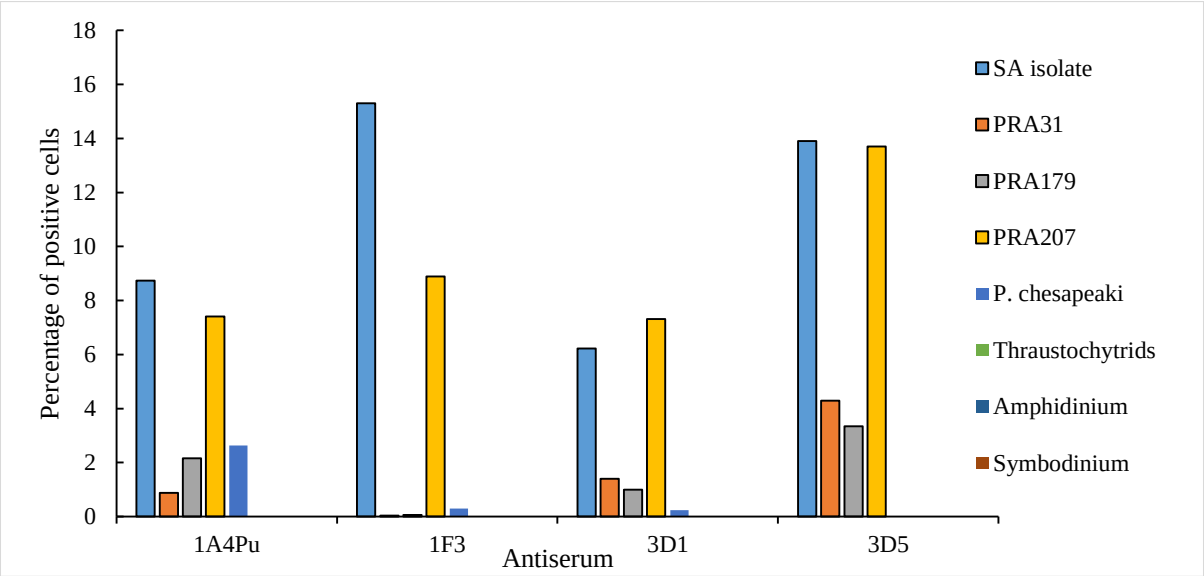


Figure 30: Percentage of positive cells for different geographical isolates of *Perkinsus olseni* (SA isolate SA-00978-12T, PRA-31, PRA-179, PRA-207) but also for the negative control cells (*Perkinsus chesapeaki*, *Thraustochytrium* sp, *Amphidinium carterae*, *Symbiodinium tridacnidarum*) for the four best performing hybridoma supernatants. 1A4Pu means 1A4 purified (mAb); the three remaining antisera are hybridoma supernatants.

3.4. Discussion

We are currently the only group to have characterised and identified the surface antigens of trophozoites and schizonts of *Perkinsus olseni* and to have used them to immunise mice and produce antibodies. The process of immunising animals such as mice to produce anti-*Perkinsus* antibodies is not an easy task; it needs to be repeated several times and often does not lead to the expected outcome. Earnhart and Kaattari (2005) experienced this when they failed to produce antibodies against extracellular products (ECPs) of *P. marinus*. Indeed, immunisation of mice against *P. marinus* can induce poor titres due to failure of the animals to mount an adequate immune response. Also, ECPs can be associated with significant toxicity for the mice (Earnhart and Kaattari, 2005). In our study, two of the third batch of mice died after receiving an injection containing a mix of the two peptides, however the cause of their death was unknown.

Several antibodies recognising *P. olseni* cells were obtained in the present study and the monoclonal antibodies reacted with a variety of antigen preparations. Whereas the IgG from 1A4 was purified, the final confirmatory test using flow-cytometry revealed that other antibodies, such as the 3D5, 3D1 and 1F3, seemed to give as good or better results than the mAb of 1A4. Indeed, the hybridoma supernatant that produced the best result was 3D5. Further work is in progress to purify the mAb and to assess its performance in detecting *P. olseni*. Screening tests such as ELISA and flow-cytometry will be performed. The supernatant of hybridoma 10E2 obtained with the third batch of mice seemed to give good results as it bound not only to *P. olseni* cells but also to the two peptides which had been generated. Those positive cross-reactions also validated the choice of the two peptides we selected to immunise the third batch of mice.

The IFA test revealed that the antibodies present in the hybridoma supernatants seemed to recognise all the life stages of *P. olseni* cells present in the culture. *In vitro* cultures of *P. olseni* are dominated by trophozoites and also present in a decreasing proportion: schizonts, hypnospores, and sometimes zoospores. The cross-reaction of the hybridomas supernatants with zoospores could not be tested in this study as we were unable to produce them. Further tests performed on 1A4 and 3D5 supernatants revealed that those antibodies did not recognise other organisms such as the two dinoflagellates *Amphidinium carterae* and *Symbiodinium tridacnidarum*, as well as *Thaustochytrium sp.*, and *P. chesapeaki*. This is the first time that an antibody that has been developed for *P. olseni* recognised exclusively *P. olseni* antigens. What is more, most of the published papers did not test their monoclonal or polyclonal antibodies against other *Perkinsus* species or when they did, the antibody was not specific and recognised other species. This is an important result from our study, which enable the development of an immunoassay against *P. olseni* in the future. Having such test available would greatly assist the abalone and other mollusc industries in their effort of preparing their businesses against potential *P. olseni* outbreaks. Indeed, the most important component of an effective disease preparedness is early detection of the infectious agent. If detected early, a rapid response can

be mounted by implementing contingency plans, which would minimise the impact of the parasite on businesses.

Developing an antibody against *Perkinsus* has always been a complicated task with limited chances of success. Table 14 summarised all the information available in the literature about the production of antibodies against *Perkinsus* spp. obtained by immunising animals with whole parasite cells or lysates. While some authors highlighted the antigenic differences between the different life stages of the parasite (Earnhart and Kaattari, 2005), the studies cited in Table 14 revealed that some antigenic determinants are conserved between different life stages of the parasite. Indeed, an animal immunised with only one life stage can produce antibodies that recognised other life stages of the parasite. Montes *et al.* (1995) obtained polyclonal antisera after immunising mice with peptide p225 secreted by the haemocytes. They identified this peptide in the capsule that surrounds the trophozoites and the remnants of the capsule on the isolated prezoosporangia. However, the antisera failed to recognise different life stages of the parasite.

Table 14: Summary of findings from studies that immunised animals with whole *Perkinsus* spp. cells to develop antibodies against *Perkinsus* spp. NT: not tested

Study	***Perkinsus* species targeted**	**Life stages recognised**	**Cross reaction with other *Perkinsus* species**	**Cross reaction with other organisms**	**Comments**
Choi *et al*. 1991	*P. marinus*	hypnospores	yes	Host tissues	polyclonal
Dungan and Robertson 1993	*P. marinus*	hypnospores, trophozoites, zoospores	yes	NT	polyclonal and monoclonal
Blackbourne *et al*. 1998	Used Dungan and Robertson (1993) polyclonal Ab		*Yes P. qugwadi*		
Bushek *et al*. 2002a	Used Dungan and Robertson (1993) mAb		Yes all species tested	dinoflagellates	
Montes *et al*. 2005	*P. olseni*	trophozoites prezoosporangium	NT		polyclonal
Park *et al*. 2010	*P. olseni*	all	NT	NT	polyclonal
Kaewsalabnil *et al.* 2015a	*P. olseni*	Zoospores hypnospores	NT	NT	monoclonal
This study	*P. olseni*	trophozoites hypnospores schizonts	No (tested *P. chesapeaki*)	No	polyclonal monoclonal

Instead of using whole parasite cells or cell lysates, some authors used extracellular products (ECPs) to immunise mice and produce anti-ECPs antibodies. In 2005, Earnhart *et al.* (2005) developed anti-ECPs *P. marinus* monoclonal antibodies, with some recognising different epitopes of the parasite wall while others recognising various structures within the cell. One of their antibodies, mAb 5A2, successfully labelled the trophozoites and schizonts of *P. marinus* but not the hypnospores (Earnhart and Kaattari, 2005). Another was specific to *P. marinus* while another was not species-specific. Other authors noticed that some antibodies bound to cells *in vitro* but not *in vivo* and vice versa (Ottinger *et al.*, 2001).

The present study also demonstrated the difficulty in getting concordance between tests such as ELISA and IFA, despite running each of the assays several times. With respect to ELISA, the conclusion was drawn that when the plate was coated with whole parasite cells antigens (whole cell lysates), there was some disparity in the distribution of the different surface antigens in each well with the majority of the antigens presented being intracellular instead of extracellular. Initially, the IFA assays performed well but at some stage, we observed confounding results where samples that were previously positive, were negative on subsequent testing. For the IFA we used the same isolate as positive control, which had undergone several passages during *in vitro* culture. After a freshly thawed isolate of the same parasite was used, the test worked well once more. This pointed out some of the issues associated with parasites. Indeed, parasites are well known to modulate the expression of their surface antigens following the environmental conditions in which they are situated (Schmid-Hempel, 2009). This, in turn can impact the ability of antibodies to recognise a specific epitope from the outer membrane of the parasite. Parasites can evade the host immune system by shielding surface components when they are opsonised by host cells or they can change their surface identity to avoid being recognised by the immune cells of the host (Sacks and Sher, 2002; Schmid-Hempel, 2009). Also, parasites can express or supress different antigens in different life stages. Numerous examples exist within the Apicomplexa, some of which are responsible for a number of serious human diseases. The Apicomplexa group, as described in Chapter 2 is closely related to Perkinsozoa and some researchers use *Perkinsus* spp. to investigate and develop remedies against human diseases (Alemán Resto and Fernández Robledo, 2014). As an example, the malaria parasite *Plasmodium falciparum* alters cell surface proteins during maturation, which results in a loss of antigens into the culture medium (Braun-Breton and Da Silva, 1988; Rosenthal *et al.*, 1987).

3.5. Conclusion

In summary, this study is the first of its kind to characterise and identify the surface antigens of *Perkinsus olseni* cells. This resulted in the identification of two proteins that contain unique epitopes to *P. olseni* and that are present in all different geographical isolates of the parasite. Two peptides corresponding to immunogenic sequences of a hypothetical protein having a transhydrogenase function were determined, synthesised and used to immunise mice. Antibodies were also obtained by immunising mice with whole *P. olseni* cells. However, obtaining antibodies that can recognise all life stages of only one *Perkinsus* species present several challenges because of the strategies that are developed by parasites to circumvent the host immune system. Indeed, parasites can control the expression of their genes and different proteins are expressed in different life stages. Moreover, the binding to the epitope can be modulated not only by the protein conformation but also by the environmental conditions in which the parasites were grown, as the expression of those proteins can be up- or down-regulated (Earnhart *et al.* 2005).

The antibodies that were developed in this project are very promising diagnostically as they seem to be specific to *P. olseni* cells and recognise several life stages. However further studies need to be conducted to confirm the cross-reactivity of the developed mAb with isolates of *P. olseni* other than the ones used in the study, with different life stages of *P. olseni*, as well as with other organisms present in seawater.

This study represents an important step toward the development of an immunoassay to diagnose infection with *P. olseni,* which would enable a rapid detection of the parasite. Being able to rapidly detect this parasite would greatly benefit the abalone industry. Indeed, early detection of infectious agents is the most important step to enable the implementation of management strategies in order to mitigate the impact of such agents on farm and therefore minimise their impact on businesses.

Main Conclusion

Perkinsus olseni is a mollusc parasite on the international list of notifiable diseases by the OIE, causing mortalities in mollusc species globally, and can be a significant barrier for trade, thereby adversely affecting market access. Furthermore, it can pose a risk for translocation of live animals for aquaculture and also impact production on farms. The aim of the project was to develop an antibody recognising specifically *P. olseni* to enable the development of an immunoassay in the future. This assay could be a rapid detection test and be used as a *Point of Care* diagnostic test.

This study represents an important step toward the development of an immunoassay to diagnose infection with *P. olseni* as some antibodies were generated that are specific for *P. olseni*. The development of this immunoassay involved the incorporation of some preliminary steps, including the *in vitro* culture of *P. olseni* to generate enough material to sequence its genome, and the identification of surface epitopes unique to *P. olseni*. Indeed, different geographical isolates of *P. olseni* from various parts of the world (Spain, Japan, New-Zealand, South Australia) were obtained and successfully cultured *in vitro*. In addition, two new isolates of *P. olseni* were propagated from blood cockles (*Anadara trapezia*) in Queensland. The proliferation of the isolates *in vitro* enabled enough nucleic acid to be obtained so that the genome could be sequenced. We have generated the first annotated genome assemblies for *P. olseni* and *P. chesapeaki*; the latter was used as a negative control for the subsequent development of diagnostic antibodies for *P. olseni*. The genome data confirmed that all the study isolates were *P. olseni*. The genome analysis provided information about the differences between the geographical location of isolates, with the two Oceanian isolates being distinct from the two Eurasian isolates. Indeed, a higher heterozygosity and a higher number of genes were observed for the Oceanian isolates in comparison to the Eurasian ones. The genome generated an invaluable amount of data and information, which were used later in the project to identify the external peptides/proteins on the surface of the parasite cells. The results generated in this study with respect to genomics and immunological characteristics of the Oceanian and Eurasian isolates showed that the absence of a WA isolate did not impact the results and outcome of the project.

This is also the first project to identify the surface proteins on *P. olseni*. Two surface proteins were identified and deemed unique to all *P. olseni* isolates used in the study: the 60S ribosomal subunit protein L4 and a conserved hypothetical protein. Two peptides were designed and synthesised from the conserved hypothetical proteins and were used, together with whole parasite cells, to generate antibody candidates recognising *P. olseni*. The development of antibodies was successful with the production of several monoclonal and polyclonal antisera that recognised only *P. olseni*. Large scale production of monoclonal antibodies is still ongoing. In summary, whilst this project did not deliver a ready to use new serological test, it did establish the foundation to develop this test with the production of several antibodies specific to *P. olseni* that could be used for a point of care test.

Implications

This project has several implications not only for abalone *Haliotis* spp. and mollusc aquaculture but also for the community of researchers all over the world with regard to the provision of new knowledge. The implications for abalone and mollusc aquaculture have been detailed at the end of this section whereas the implications for the scientific community have been explained into the first three paragraphs corresponding to the three chapters of this report. It is important to mention that this project provided two immediate outputs to industry and other stakeholders:

(1) It identified an immediate gap in existing diagnostic tests, which is a lack of quantification capability of *P. olseni* infection; and
(2) This project provided tools for the development of improved diagnostic tests, both molecular and serological.

Firstly, the availability of the *P. olseni* genome will provide an invaluable source of information for researchers worldwide as it is now available to anyone on GenBank. As a consequence, it will now be possible to study the pathogenicity of *P. olseni* and the host-parasite interaction at the molecular level. The genome analysis also provided insights into the origin of the parasite and the distinction between the Oceanian and the Eurasian isolates.

The isolation of *P. olseni* cultures from Queensland molluscs will enable other researchers to compare the pathobiology of different isolates of *P. olseni*. Once a monoclonal isolate is generated, it could be deposited to accessible culture collections such as the European Collection of Authenticated Cell Cultures (ECACC), Cell Bank Australia, or the American Type Culture Collection (ATCC) to make it accessible to the scientific community.

Finally, the latest section of this report generated proteomics information and identified unique surface proteins of *P. olseni*, which are present in all isolates from different geographical locations. By immunising mice, several good antibody candidates were developed that recognised not only *P. olseni* cells but also all its intermediate stages present in *in vitro* cultures. Monoclonal antibody hybridomas could be deposited with internationally accessible culture collections such as the European Collection of Authenticated Cell Cultures (ECACC), Cell Bank Australia, or the American Type Culture Collection (ATCC), so it would be available to the international community. This would have indirect long-term benefits for abalone and mollusc aquaculture as researchers can use the antibody hybridomas to increase the knowledge about the pathogenicity of *P. olseni*. A better understanding of the mechanisms of infection as well as the host-parasite interaction is the first step toward identifying disease mitigation strategies.

The results generated in this project will benefit the abalone and mollusc aquaculture in the short to medium and long-term. Some benefits are direct and others are indirect, i.e. when the knowledge generated enables other studies to occur. Benefits are listed in the timeline order from shortest to longer terms.

Short-term benefits for abalone and mollusc aquaculture (0-2years)

(1) *Increased awareness of current molecular tests results*. Indeed, this project revealed that the current molecular tests in use in Australia, which are recommended by the OIE, target a region of the genome that is repeated between 91 to 460 times. Consequently, the interpretation must be limited to presence/ absence and must not be used to quantify *P. olseni* infection. This is an important finding for the abalone fishery and aquaculture industries. Also, this project highlights that there is as yet no test available that quantifies the infection of *P. olseni* in tissues of animals.

(2) *Improved industry responses to* P. olsen*i outbreaks with the development of quantitative molecular diagnostic tests*. The outcome of this project will provide for the development of a quantitative molecular test that could be invaluable for abalone and mollusc aquaculture companies, in the event of *P. olseni* infection occurring on farms. It will also be invaluable for the fishing industry as well as government regulators to identify hot spots and manage translocation in wild populations. Indeed, this project designed 106 sets of probes/primers for qPCR targeting regions that are present only once in the genome and would be candidates for a quantitative PCR test.

Medium to long-term benefits for abalone and mollusc aquaculture (>2 years)

(3) *Increased preparedness by developing an immunoassay to rapidly detect* P. olseni *infections*. This project is the first step towards developing an antibody-based test, which would be a rapid and quantitative test for diagnostic laboratories and eventually a pool-side (point of care) test for farmers. Having such a test available would greatly assist the abalone and other mollusc aquaculture companies to respond to and mitigate potential *P. olseni* outbreaks. Indeed, the most important component of effective disease preparedness is early detection of infectious agents. If *P. olseni* is detected early, a rapid response can be mounted by implementing contingency plans, which would minimise the impact of the parasite on businesses.

(4) *Increased preparedness by reviewing* P. olseni *mitigation strategies and biosecurity plans on farm*. Indeed, the *P. olseni* genome generated in this project, together with the genome of the abalone species of interest, will enable transcriptomics studies in order to develop a better knowledge of the host-parasite interactions. Conducting *in vivo* challenge experiments in mesocosms using transcriptomics would allow the identification of risk factors that lead a sub-clinical infection to become clinical. Applying mitigation strategies on these risk factors on farm enables change in dynamics of an infection

(5) *Producing abalone (or other molluscs) resistant to* P. olseni. The information obtained from the genome of *P. olseni* and the abalone species of interest will allow for a better understanding of the interactions between host and parasites at a molecular level, not only highlighting the mechanisms and genes involved in the resistance of abalone to *P. olseni*, but also the susceptibility of *P. olseni* to abalone's immune defences. This molecular information can be used in the future for farm breeding programs and

restocking breeding programs to select brood stocks, which are *P. olseni*- resistant abalone.

Recommendations

The authors recommend that further studies are implemented to continue this work including:

(1) The development of a qPCR test for quantification by refining and then evaluating the sets of probes/primers identified from this study;

(2) The sequencing of *P. olseni* genome from Queensland and the evaluation of how it compares to other geographical isolates from Australia and overseas;

(3) The continuation of the antibody work in order to identify the best monoclonal antibody (mAb) and develop an immunoassay. It is recommended that a colorimetric enzyme approach is used instead of fluorescence as many non-target organisms exhibit green and red auto-fluorescence that could decrease the sensitivity and specificity of the test. ELISA tests could be developed as a laboratory-based test and also a paper-based colorimetric assay as a point-of-care (POC) diagnostic test.

Extension and Adoption

The genome of *Perkinsus olseni* has been assembled for the first time and this data was made available to the scientific community through depositing the sequences at the NCBI GenBank. This data will be a valuable contribution to the literature for the scientific community. A peer-reviewed publication has been submitted to a journal and will allow the dissemination of the data publicly.

The most appropriate way of long-term maintenance of isolates and ensuring accessibility to other researchers will be discussed with industry, FRDC and any relevant body. It is proposed that the new isolate of *P. olseni* from Queensland generated in this project will be deposited at the cell culture collection deemed most appropriate. Access to cultures would boost research about the pathogenicity of Australian isolates, which in turn will provide information and knowledge to Australian researchers and regulators.

The hybridomas generated in this project will also be deposited at an appropriate cell culture collection so that they will be available to the scientific community, thereby facilitating further research, which would assist with the development of a diagnostic immunoassay in the future.

Overall, this project generated knowledge and information that will enable the development of specific projects aiming to develop an immunoassay test with a direct benefit to industry, which could later inform management strategies of this parasite and biosecurity plans.

The authors communicated throughout this project with the abalone aquaculture sector. The authors gave oral presentations to inform the abalone industry about the project's objectives and progress at two occasions in 2017 and 2018 at the Australian Abalone Growers' Association (AAGA) Research and Development workshop and Annual General meeting in Queenscliff. The authors will also attend the next AAGA R&D workshop and present the final results and outcomes of this project, which will provide the authors with an opportunity to discuss the outcome of this project with abalone farmers broadly and obtain their feedback. In the first instance, it would provide for an evaluation of whether there are any further developments arising from this project's findings that they would like to pursue.

The results from this project were presented at scientific conferences as listed below.

DANG C., DUNGAN, C., HANRIO H., BOGEMA D. Perkinsosis in Western Australia: Status of Current Research. *International Symposium on Perkinsosis*, November 2019, Busan, South Korea.

BOGEMA D.R., YAM J., MICALLEF M., ALEX S., FELL S., GO J., JENKINS C., DANG C. Parasite of land and seas: Comparative genomics of microbial eukaryotes *Perkinsus olseni* and *Theileria orientalis. The Australian Society for Microbiology Conference*, July 2019, Adelaide, Australia.

HANRIO E., BOGEMA D., MICALLEF M., DAVERN K., ABUDULAI L., JENKINS C., GO J., BATLEY J., DANG C. Developing a diagnostic test for the protozoan parasite

Perkinsus olseni. *5th Australasian Scientific Conference on Aquatic Animal Health & Biosecurity. July 2019,* Cairns, Australia.

DANG C., JENKINS C., MILLER T., SNOW M., CARAGUEL C., GO J., BOGEMA D. *Perkinsus olseni* in molluscs from Western Australia. *111th meeting of the National Shellfisheries Association*. March 2018, Seattle, USA.

DANG C., MILLER T., GROUNDS G., BANNISTER J., STEPHENS F., NOLAN D., HILLIER P., CARAGUEL C., GO J., BOGEMA D., JENKINS C. (2017). Perkinsosis in molluscs from Western Australia. 4th Australasian Scientific Conference on Aquatic Animal Health, July 2017, Cairns, Australia.

Project Coverage

Scientific papers

Specific manuscripts corresponding to parts 2, 3, and 4 of this report will be submitted for publication by international, peer-reviewed journals. The manuscript related to part 2 is in revision while the two others are intended to be submitted within the next six months as they are included in the thesis of the PhD candidate Eliot Hanrio from The University of Western Australia.

Project materials developed

The materials that were developed during the project include:

- *In vitro* cultures of *Perkinsus olseni* from Queensland;

- Hybridomas producing antibodies against *P. olseni*

- Polyclonal and monoclonal antibodies

- Genome sequence of *P. olseni* and *P. chesapeaki*

- A list of 106 sets of probe/primers for developing qPCR

Appendix I: List of Researchers and Project Staff

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Appendix 3: Reflection on ITS region of *Perkinsus olseni* currently used in diagnostic tests

In this study sequencing of the genome demonstrated that the ITS region of *P. olseni* is repeated a number of times (from 91 to 460) in the genome, with the number of repeats varying according to the different geographical location of isolates (Table 15), The OIE recommended approach for diagnosing infection is to use primers for the ITS in a PCR test. While the ITS region is fine to be used for presence/absence screening it is not suitable for quantification of *P. olseni* using qPCR because of the large number of repeats of the ITS region.

Table 15: Coverage of the genome and ITS region of the 6 isolates sequenced.

Isolate	Origin	Average sequencing depth		Approximate copy numbers of ITS region
		ITS PCR product	Whole genome	
P. chesapeaki PRA-425	Queensland	63992.5	180.449	355
P. olseni PRA-31-E3	Spain	13955.3	153.305	91
P. olseni PRA-205	New Zealand	54891.9	119.317	460
P. olseni PRA-179	Japan	51379.4	180.615	284
P. olseni PRA-207	New Zealand	45773.2	111.603	410
P. olseni SA-00978-12T	South Australia	21479.8	103.342	208

After determining that the ITS region was repeated a number of times in *Perkinsus* spp. genomes, new sets of probe/ primers were designed to target single copy regions using the method described below.

Primer/probe sets specific to *P. olseni* were generated using the RUCS pipeline following the method developed by Thomsen *et al* (2017). The *P. olseni* SA-00978-12T isolate, long-read generated genome sequence, was used as a reference with the four *P. olseni* international isolates mapped to the reference as "positive" genomes. For the purpose of designing *P. olseni*-specific primers and probes with RUCS, *P. olseni* genomes were considered positives and other genomes (Table 16) were considered negatives. Negative genomes included 24 sequences of protists previously detected in marine environments, including the *P. chesapeaki* sequence generated by this study, *P. marinus,* and several other dinoflagellate sequences, were included as "negative" genomes (Table 16). A minimum of 10000 primer/probe sets generated by RUCs were filtered by virtual PCR analysis. Primer and probe sets were excluded if they produced multiple PCR products in any *P. olseni* isolate. Primer and probe sets were also excluded if they targeted intergenic regions, introns or if PCR products spanned multiple exons.

Table 16: Genome sequences and groups (positive/negative) used for PCR primer design with the RUCS pipeline.

Species	Strain	PCR design group
Perkinsus olseni	SA-00978-12T	Reference/Positive
Perkinsus olseni	PRA-3/E3	Positive
Perkinsus olseni	PRA-205	Positive
Perkinsus olseni	PRA-179	Positive
Perkinsus olseni	PRA-207	Positive
Breviolum minutum	Mf 1.05b.01	Negative
Chromera velia	J2	Negative
Euplotes focardii	TN1	Negative
Ichthyophthirius multifiliis	G5	Negative
Laurentiella sp.	PUJRC_G5	Negative
Moneuplotes crassus	CT5	Negative
Oxytricha trifallax	JRB310	Negative
Paramecium caudatum	43c3d	Negative
Paraurostyla sp.	PUJRC_G6	Negative
Perkinsus chesapeaki	PRA-425	Negative
Perkinsus marinus	ATCC 50983	Negative
Prorocentrum minimum	D-127	Negative
Pseudocohnilembus persalinus	36N120E	Negative
Stentor coeruleus	WM001	Negative
Sterkiella histriomuscorum	BA	Negative
Stylonychia lemnae	130c	Negative
Symbiodinium microadriaticum	CCMP2467	Negative
Symbiodinium sp. clade A	Y106	Negative
Symbiodinium sp. clade C	Y103	Negative
Tetmemena sp.	SeJ-2015	Negative
Toxoplasma gondii	ME49	Negative
Uroleptopsis citrina	UcQD2011	Negative
Urostyla sp.	PUJRC_G1	Negative
Vitrella brassicaformis	CCMP3155	Negative

A list of 106 primers/probe sets was identified by RUCS and have been included in Table 17.

Table 17: Primers/ probe sets targeting regions present only once in *Perkinsus olseni* genome.

Primer name	forward_primer	reverse primer	probe	function (blastp swissprot)	E-value
pols_rdes_0068	AGGCCTCGATAGCACCACTAT	TGATGAGCCCCATAGTGATGC	ACAGCACGAGTTCCGCGGATGAGC	L-asparaginase 1	6.00E-77
pols_rdes_0127	AGGCCTCGATAGCACCACTAT	CGGACTATGATGGCTTCGTCA	ACAGCACGAGTTCCGCGGATGAGC	L-asparaginase 1	6.00E-77
pols_rdes_0231	GGGATGACTCTGATGGGAAGG	TGATGAGCCCCATAGTGATGC	GCGAAGGGACCAGTGGGAGCAGGA	L-asparaginase 1	6.00E-77
pols_rdes_0363	GAGAGAGACTCGAAGCTGGTG	AACTCGTGCTGTCAAGATCGA	GCGAAGGGACCAGTGGGAGCAGGA	L-asparaginase 1	6.00E-77
pols_rdes_0491	GCCTCTTCCCTTGTAGTGCTT	AGACGCTTCTGTAACGAGCTG	GCAGGAGGCGGAATGGTCCTTTGT	Fumarate reductase	5.00E-83
pols_rdes_0506	CGGCTACTCCCGTCAAGTATG	CACTCTCAAGTCGTTGTCGGT	CCCTATTTCACATGGGCCCTCATGACCA	No significant hit	>1e-5
pols_rdes_0538	GCATCACTATGGGGCTCATCA	ACGACCACTATGCGGACTATG	AGCACTGGAGGAGTAGGCCATGGT	L-asparaginase 1	6.00E-77
pols_rdes_0748	GCATCACTATGGGGCTCATCA	AATAACCTTCCCGAGCTCCAG	AGCACTGGAGGAGTAGGCCATGGT	L-asparaginase 1	6.00E-77
pols_rdes_0789	AAGTGCTTGGCAGTGTAAAGC	TTCACGTCGCTCTCAAGTACC	ATTTGTCGCCCTTCTCGCCGCTCA	No significant hit	>1e-5
pols_rdes_0969	AGGATTTCGTCTGGTCGACAC	CCAGCCACCAGTAATCTCTCG	ATCACTCTCACACGGCAAGGCGGC	No significant hit	>1e-5
pols_rdes_0972	CGCCCAGAAGTATCGTGTTGA	CTGCTCGACATAACGACCCAT	TGATGCCGCTGCTAACGCCATCCG	Elongation factor 2	0
pols_rdes_1014	TCGGTGTCCCTAAGTCCAAGA	TCATGATAGCGCGGATCATCA	CCCTCCCTCGTGCCTTCTGCCAGT	Elongation factor 2	0
pols_rdes_1027	CCGAACTTCACAGTGGAGGAG	ATGAGGTAACCGTGCTCCTTG	AAGGCCGCTGGTGATGCCCGTTTC	Elongation factor 2	3.00E-57
pols_rdes_1052	GTCGTTATGTCGAGCAGGTCT	TGGTGCAGACAACAAGAGGAT	CGGTAACACCTGCGCCTTGGTCGG	Elongation factor 2	0
pols_rdes_1093	TTCCTGGACTAACGTCTCCGA	GGGACTTGAGCATCTTCTCGT	CCCTCCCTCGTGCCTTCTGCCAGT	Elongation factor 2	0
pols_rdes_1199	CCTTGGTCGGTGTTGATCAGT	GCAGATCTCAACGTGCAACTC	CGTGTCTCCTGTCGTGCGTGTTGCC	Elongation factor 2	0
pols_rdes_1312	ATCCGCGCTATCATGAACGAG	TAGCAGCGGCATCATCCATAG	GCCCAGTCCCGTCTTCGCCCAGAA	Elongation factor 2	0
pols_rdes_1626	CGGTTGATACTACTGGTGGCT	GCTTGCGTACGTTGTCGAATT	GGCTGGAGCACGCGAACTCGGTTT	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_1631	CGCTGACTATATTGGGCGACT	CCTCGGTATTGCGAAATCTGC	GAAGGCAGACGTTGTGGCGTGGGC	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_1642	GACAGCATAAGGTCAGCCTCA	GACTCATCCCTATCGGAAGGC	ACGGAAAGACATGCTGCGGGAGTGC	ATP-dependent RNA helicase dbp9	5.00E-99
pols_rdes_1657	CCGGGTCGATCGGTATAAACA	AGCCTAGTGTTGTGACCTGTG	GGCTCCGTTGGCTCTGTCGGCTGA	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_1670	GGTTCCGTGGTCTAGTTTGGT	TCCAGCCCTTGTGTTTCTTCA	TGGCAACGTGGTGAAGCTGCGGAA	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_1677	TATGATGCATCTTCGCCGGAT	CATTGTAGAGTCGGCCCCTTT	GGCTGGAGCACGCGAACTCGGTTT	Phospholipid-transporting ATPase IB	1.00E-157

pols_rdes_1679	ACTCTCACCGAGAACTGCATG	GATACCTCGCGTGGGGAATTT	TGCAGCCGTGTGATACTCATGTGAGCCA	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_1686	TGGTAAGCCGACTATGCTCAC	ACCAAACTAGACCACGGAACC	GCTTTGGTGCTGGGTGTAACGGCGT	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_1694	CCGGTTTATGCAAGCATAGGC	ATGCACACTACGACTCATCCC	ACGGAAAGACATGCTGCGGGAGTGC	ATP-dependent RNA helicase dbp9	5.00E-99
pols_rdes_1727	CGAAGATTCGCGAGGTGATTG	CATGCAGTTCTCGGTGAGAGT	GGCCGATGATCTGGGGTGCGTGAC	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_1730	CGCCTCCTCGGTTGATACTAC	CCCAGTCGTTGGTGAACTTCA	GGCTGGAGCACGCGAACTCGGTTT	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_1735	CAAACTGCGTGATGGGATTGG	TGCTGCGAACCGATACAACTA	CGGGGTCATGGCAGCAACTCGTGG	ATP-dependent RNA helicase dbp2	0
pols_rdes_1743	CGGTACTTGGTAGGGGAGACT	CGGTCGTAAATGGAGTCTCGT	CACAATCAGCTGCCAGGCCGCACT	Sugar transporter ERD6-like 6	1.00E-86
pols_rdes_1745	GGGCTGGATGTTCGGAAGATT	AGCCTAGTGTTGTGACCTGTG	CGCAGCTTCATCGGCTCGCGTTGA	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_1746	GAGATGCTCCAACGAGACTCC	TAACTACCCCCGGTGGAGATC	AGAGGACCCCGCGCCGATTTCGAT	Sugar transporter ERD6-like 6	1.00E-86
pols_rdes_1751	TCAAGAACGTCGCCTTTGGTA	CCACTTGTACAGGAAGGAGCA	CGGGCCGTTCTGCATGTTCTCCGG	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_1773	GTGTAACGGCGTTGAAGGATG	TCCAGCCCTTGTGTTTCTTCA	TGGCAACGTGGTGAAGCTGCGGAA	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_1774	CTTCAGCATCGCTCTTAGCCT	GGCGCCTGCTAAGTTTCATTT	GCCGCCAGTTCAGCCTTGACCTCC	Sugar transporter ERD6-like 6	1.00E-86
pols_rdes_1776	TGGAGTCCATCGCGAAAATGA	TTGGTAATGAGCCGTCTTCGT	GGCTGGAGCGTGCGCCTGTCTTAC	Mitochondrial substrate carrier family protein B	3.00E-49
pols_rdes_1778	TGGTCCAGGATCGAGTGAAGA	TTGGTAATGAGCCGTCTTCGT	GGCTGGAGCGTGCGCCTGTCTTAC	Mitochondrial substrate carrier family protein B	3.00E-49
pols_rdes_1860	TATGTGCGGTGAATGTACCCA	TTGGAGAAGACGTGGAACTGG	GCTCTGCAGTAAGTGTGTTCAGGGCTGG	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_1874	CGCCCACAACATTGTCATGAA	GCTTGCGTACGTTGTCGAATT	TGGGCTAGGAACTTAATTGGTCATGACGGT	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_1919	GCGACACTCCCCATAGAGAAC	GCTTGCATAAACCGGTCATCG	TGGCAAAGATCAACGTCTTCCCCACCA	ATP-dependent RNA helicase dbp9	5.00E-99
pols_rdes_1927	TATGATGCATCTTCGCCGGAT	ACACCACCGTCATGACCAATT	GGCTGGAGCACGCGAACTCGGTTT	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_1957	CGCCCACAACATTGTCATGAA	GCCGAGCAAGTAGGTAGTCAG	TCATGGTGCGCAGTCCCACTACGG	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_2000	GCGCCAGTTACAGAAGGTAGT	GAGCATCTCCACTCTTCGTGT	CACAATCAGCTGCCAGGCCGCACT	Sugar transporter ERD6-like 6	1.00E-86
pols_rdes_2040	GCCCAAGTGTTTATGTCGCAA	GTGAGCATAGTCGGCTTACCA	TGCGGTGAATGTACCCAAGCTCTGCA	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_2045	ACTCACGTGTTCTCGGACAAG	CAGAATGACAGAGCCCCAGAA	CCCCACGCGAGGTATCAAGGCAGT	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_2046	GCTGTTGCGTCTCCAAATCAT	GCTTTCCCGATAACGTTGGTG	CGAGGCATCTCACTGGACTGGACTGC	Intraflagellar transport protein 122 homolog	0

pols_rdes_2088	GGCAAAGATCAACGTCTTCCC	CACCCTCAACGATGATGTGGA	TGACCGGTTTATGCAAGCATAGGCCC	ATP-dependent RNA helicase dbp9	5.00E-99
pols_rdes_2105	TTGGTCATGACGGTGGTGTTT	CATTGTAGAGTCGGCCCCTTT	TCATGGTGCGCAGTCCCACTACGG	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_2120	AAAGGGGCCGACTCTACAATG	GCACGTGATCCTGGAGCTTAT	CGCAGCTGGAGATACCGATGCCCT	Phospholipid-transporting ATPase IB	1.00E-157
pols_rdes_2139	GTAGTAGAACTGGTGCTGCCA	TCTGTCCCGCTAGTCTACCTT	AGGGATTGAGTTCACTCGGCAAGGGA	Transmembrane 9 superfamily member 7	4.00E-170
pols_rdes_2163	GGGGAATAACTCGGCCAAGAT	GCAGCTGGCATCAACAATAGG	GCCCGTGGCTGCCGAAATCAGTAG	Sugar transporter ERD6-like 6	1.00E-86
pols_rdes_2243	GATCGCCAACCATCCAACATG	TCATGCTTCAAGTGCTTCAGC	GCCCGTGGCTGCCGAAATCAGTAG	Sugar transporter ERD6-like 6	1.00E-86
pols_rdes_2245	GCCTCAGCAACATAAGAAGGC	CGGTACTAGGTTTCGGGTAGC	GAGACGTGCTCCGCCCCAACAGGA	No significant hit	>1e-5
pols_rdes_2282	GTCCGGGGATTCAGTTCTACG	TACCGGTCAGTGGGATATCCT	GGTGGTGGGGAGGGGATAGTGCCA	Phosphatidylinositol 3-kinase, nodule isoform	3.00E-88
pols_rdes_2287	GTCCGGGGATTCAGTTCTACG	CGTTAGTGGCGGACTATGAGG	GGTGGTGGGGAGGGGATAGTGCCA	Phosphatidylinositol 3-kinase, nodule isoform	3.00E-88
pols_rdes_2330	TCCCGACGGTATGACTGAAGA	GCCATTAGAGAGGACGCTTCA	GCCCACATGGATGTATGGCTCCGACG	No significant hit	>1e-5
pols_rdes_2373	GTTGGGGATTTAGAGCCAGCA	GATCTGTAGCTGCTCGTCAGG	GAGACGTGCTCCGCCCCAACAGGA	No significant hit	>1e-5
pols_rdes_2388	GCTAGAATCCAAGGCACGTTG	CGTTAGTGGCGGACTATGAGG	GGGCCTCCGTCCGGGGATTCAGTT	Phosphatidylinositol 3-kinase, nodule isoform	3.00E-88
pols_rdes_2500	TCCTCCTTGATGGCATGGTAC	CACTCCGCCTGTACTGGATTT	CGAGGTGTTTCCCCACGAAGTCCTGC	Transmembrane protein 65	6.00E-18
pols_rdes_2576	CGCCCTTAGGGTAAGATAGGC	GTGTTCGCCACCCCTATATCC	GCCAATCACTCCAGCCCATCCCGT	Battenin	1.00E-26
pols_rdes_2604	GCGATTCCGAAGAGAAACTCG	CCTCAGCCACTCGATTGTCAT	GCACGGATGCGGGCCTGGAGAACT	Transmembrane protein 65	6.00E-18
pols_rdes_2633	AACTTCCGGACGTAACAGAGG	ATCGGAAGTGCATCAGTAGCA	ACGCGGATATAGGGGTGGCGAACA	Battenin	1.00E-26
pols_rdes_2832	GCCAAGAGTGAGAGGTTGTGA	GATGGTGTGGAGCCTAAGACG	GGCATAAAGTGGACGAAGGAGGCACCA	Phosphatidylinositol 3-kinase, nodule isoform	3.00E-88
pols_rdes_2875	TGCTGATGCATGCAGTACCTA	GGATCCAGCTGCTACGATTCA	CCCTGTATTCCCTTCCTATCAGTCCCTGGG	Amino acid transporter AVT1E	3.00E-20
pols_rdes_2927	CCCGTGTCATGGTTACCATTG	CGAGGCTCAGTGAACTTAGGG	TGCCACTGCCGTTGCTGGACGTTG	40S ribosomal protein SA	3.00E-111
pols_rdes_3360	GGTTTCGTTACAGCGTCGAAG	GTAGTTGGATCATGCGTACGC	GGCTTCGCCGCTGTACCGCATAGT	No significant hit	>1e-5
pols_rdes_3380	GCTGGACGCTAAATCGATTCC	GTGGCTCGTAGGCTAACATGA	TGGGCCACCATTGAGCGGCAAGTC	No significant hit	>1e-5
pols_rdes_3389	ACGGACGATGTACGCATGATA	TCCGCACCGAATACTAGAACG	GCCGAATGTCGCTTTGGAGGCCGT	No significant hit	>1e-5
pols_rdes_3429	AATCGGAGCTCTTGTACGCTT	ATGAGTACACGACCCGCTAAC	CCGCGTTCAAGCTGGTCCGCAAGT	No significant hit	>1e-5
pols_rdes_3442	CGCTTTTCACGGACGATGTAC	CGATGTTTAACAACGGCACCA	GCCGAATGTCGCTTTGGAGGCCGT	No significant hit	>1e-5
pols_rdes_3451	CCGGTGATTTCGAGATCGGTA	CGATGTTTAACAACGGCACCA	GCCGAATGTCGCTTTGGAGGCCGT	No significant hit	>1e-5
pols_rdes_3466	TTCGCGACTGTAGAGTTTCGA	TGAGAATCGCCCTCTCTGAGA	TGGGCCACCATTGAGCGGCAAGTC	No significant hit	>1e-5

pols_rdes_3484	TCACCGCATTCGTAGGAATCC	CGCGAAGGAGATCTCGTCAAA	TGGTAGTCCCGGCAGAACACCCCG	No significant hit	>1e-5
pols_rdes_3755	AGGTCGCATTTGACGAGATCA	GCCAGCTTCGATAGGATTCCA	CCACCTTTGTCGAACTGGCGTCGC	No significant hit	>1e-5
pols_rdes_3763	CGGGCCTGTCTCGATAAAGAC	CTGCAGTTGCTTCCAAACGAA	CCACCTTTGTCGAACTGGCGTCGC	No significant hit	>1e-5
pols_rdes_4011	TGCACATCACTCGTGATCCTC	CGCTCTGATGTGACAGACGAG	ACCTCAGAGGATGCCCGACCGAGC	No significant hit	>1e-5
pols_rdes_4189	GCTTCATCCCGTCACTCTTCA	TCAAGAAGACCAAGGCCATGA	CAACCCCATCTTCCGCCACAGCCG	Pre-rRNA-processing protein ESF1	7.00E-09
pols_rdes_4480	CATGCCTTTGAAGCCGAAAGT	CACGAGTGATGTGCAGCAAAG	CCAAGTCTCGAGATCCACAACGTAGGGT	No significant hit	>1e-5
pols_rdes_5058	GAGACCGAAGATAACGTCCGT	TCGTCTGTCATCCCGAACCTA	AGCCTTCCAATCCAGCCGAGCCCA	No significant hit	>1e-5
pols_rdes_5103	CGCGCTCAATGATACCCTCAA	GCTCAGAGGCAAGTGAGGATT	TCTTCCCGCTCGGTGTCCCATGGC	No significant hit	>1e-5
pols_rdes_5118	AGAGCGAAATCTAGGCTGGTG	CGACATCAGAACTGTGCAAGC	TCGCTCGGACTCCTCCTTGCACGA	No significant hit	>1e-5
pols_rdes_5489	AGCACTATAAGGGTCGTGGTG	CATCGTGTAGTGGACCAGAGG	ACCGGTCGGGATCTGGCGAAGGAG	Fumarate reductase	7.00E-139
pols_rdes_5498	TCAGTGCAGCTAACACCATCC	GCACCGGAATTCTCGACCATA	AAGGCCACGTCGGGTATCAACGGC	Fumarate reductase	7.00E-139
pols_rdes_5516	AGCACTATAAGGGTCGTGGTG	CCCGTTCGGATCAGCAATTTG	ACCGGTCGGGATCTGGCGAAGGAG	Fumarate reductase	7.00E-139
pols_rdes_5597	CCTCTGTCGACTGGTTGATGG	TGGTAACCCTGCACTTGTTCA	CCGCCTCGGTGGTCACTCTGTCGA	Fumarate reductase	7.00E-139
pols_rdes_5706	GGTATCAGTCCCTCCGTTCTG	CTGGAAAGTATCGTCGACGGA	GCCTACCCCTCTGGCAAGACCCAT	Fumarate reductase	7.00E-139
pols_rdes_6986	TTCACCATCTCCGTCAGCATC	CGCATTCAGAGTCTTCGACGT	CCTGCAGGATCTCATCGAGGTGGCC	Calmodulin/Calcium dependent protein kinase family	3.00E-25
pols_rdes_7537	CATGTAATCCCTGCCGTAGCT	CGTGAGCCATTCGTTCTATGG	AAGGGTCGACCGGTAGAGGCTGCC	No significant hit	>1e-5
pols_rdes_7680	CTGTCGTGAAGTGTTGGCAAC	TATGGTTCCGATACCGAGCCT	TGGATTCCCAAACGTCGGACAGCC	No significant hit	>1e-5
pols_rdes_7686	CTGACCGCCCATGATATCCAT	ACCCCTCGTGATATCGGTCTA	TCCTTCTCCGACGACTTGGGCAGT	No significant hit	>1e-5
pols_rdes_7701	ACAGGAGTAGAAGCATTGGCA	GGCGATTGATGGAGTCGGTAT	TGAAGATGTACATACCACCAATATGGACGCTCA	Acyl-lipid (8-3)-desaturase	9.00E-97
pols_rdes_7732	ACTCCCAAGATAGCACAACCG	TGCACTCAGGGATTTCAAGTCA	GCAGAGGGCGAGATAGAATCCCAATGCA	Acyl-lipid (8-3)-desaturase	1.00E-59
pols_rdes_7735	TTGAAATCCCTGAGTGCAGCT	CGTTTCGGGAGTTTCATGCTC	GCGTCATATTCGCGCGATGGCAGA	Acyl-lipid (8-3)-desaturase	1.00E-59
pols_rdes_7737	GTGCGTTGAACTCTAGCGTAG	GCACCTACTGGGAGAGTAAGC	AGGTAGATTATGACGAGGCATTGTAGGGAAGAGA	Acyl-lipid 8-desaturase	2.00E-20
pols_rdes_7745	TGACTTGAAATCCCTGAGTGCA	ATGTCATCAGCGTCGTCAACT	GCGTCATATTCGCGCGATGGCAGA	Acyl-lipid (8-3)-desaturase	1.00E-59
pols_rdes_7751	AAGACCAATGAGGCGTCTCG	GCGACTGCTACCGTCATCTTA	CGGGATGAGCTCCTCCATTAGCGCC	No significant hit	>1e-5
pols_rdes_7773	ATGCAGCCGTCTTACCCAAAT	GGGTTACATCCCATCGACGAT	TGCTGTTGTTGCCAAGAAGAGAAGGAGG	No significant hit	>1e-5
pols_rdes_7822	CTCAACGCTGCTTCATGACTC	CTCTTCCCTACAATGCCTCGT	GCGTTGAACTCTAGCGTAGATACAGCACCC	Acyl-lipid 8-desaturase	2.00E-20

pols_rdes_7886	GCAGACATACCACAACCAACAC	AGCTGCACTCAGGGATTTCAA	CCAGCACGTCCTTGAGCTACTCCCA	Acyl-lipid (8-3)-desaturase	1.00E-59
pols_rdes_8508	GCCAAGGGTGAAGTTCATCGA	AACGGCGATATGTCCTTCCAC	GGTCGGCCACAGGGTCTCCCATGA	No significant hit	>1e-5
pols_rdes_8914	CACCACGCTTGTAGGAGGATC	CTATGGTGCTAGTTGGTCGCA	CGTCGTCGTCTGCGGCATGACCTT	No significant hit	>1e-5
pols_rdes_8969	ACAGTCTCGTCATCCTGTTCG	CGAGAACATGACCCTCACTCA	CGACGACGACCCCTCTGAGCCTCG	60S ribosomal protein L17	4.00E-65
pols_rdes_8975	ATCGATAAGACAGCTGCACCT	CTCCCTATCCCTGACGTGGTA	AGTCCAAACAGACGAGGCTTGGTTGCC	No significant hit	>1e-5
pols_rdes_8979	GCGACCAACTAGCACCATAGA	ATGAAAAGAGGTCGGGCATCG	ACCCTAATCCATCGCTGTCTCCGGCA	No significant hit	>1e-5
pols_rdes_9042	ACCATTGAGCGAACCTTCTGT	GCGATGGATTAGGGTTCGTGA	ACCAGCGACACAACCAGTAGCCCT	No significant hit	>1e-5
pols_rdes_9814	GTACCGCAATAGAGTGTTGGC	CTTAGGCCCAGTAAGCTCCAG	GCGTCTGGAGAGCGCCCATGTGG	ATP-binding cassette sub-family F member 1	1.00E-135