Molecular detection of the apicomplexan parasite ‘X’ (APX) in bivalves in New Zealand

Suong Thao Nguyen

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ABSTRACT

The flat oyster (Bluff oyster, dredge oyster) *Ostrea chilensis* is an iconic seafood species in New Zealand that forms the basis of one of the oldest commercial fisheries in the country and is under development for aquaculture. However, the productivity of commercially important flat oyster populations has been severely hampered by an apicomplexan parasite called apicomplexan-X (APX) which can act alone, or together with bonamiosis. Despite the commercial significance of this disease, the biology of APX is virtually unknown, with an absence of knowledge of hosts, life cycle and taxonomic identity. There are also no specific detection or control measures available to deal with disease outbreaks. Therefore, the aim of this research was to begin to fill the gaps in our knowledge of APX to assist in understanding and combating the disease.

Two unique APX 18S rRNA gene sequences (~1.8kb) were isolated from APX in flat oysters by firstly applying generic apicomplexan primers followed by sequencing, and then further extension of the obtained sequences using specific primers. BLASTN search of the GenBank database and phylogenetic analysis confirmed the apicomplexan origin of the obtained sequences. Both sequences were confirmed to be specifically isolated from APX using their complementary *in situ* hybridisation probes, which exclusively and clearly bound to the APX zoites in histological sections of infected oysters.

Based on the generated APX 18S rRNA gene portions, this current study provides an effective PCR method for specific APX detection. A specific forward primer APX-For and reverse primer APX-Rev were designed based on the two *in situ* hybridisation probes previously identified from regions which have the highest variability, discriminating the APX 18S rRNA sequences from partial 18S rRNA sequences of flat oyster, Pacific oyster (*Crassostrea gigas*), *Bonamia ostreae* and *Bonamia exitiosa*. Several approaches, including using a dilution series method, direct comparisons with histology results, and testing on DNA

of other apicomplexan species were performed to prove that the PCR method has strong analytical and diagnostic sensitivity, as well as good analytical specificity for the reliable detection of APX.

The new PCR method was then used to confirm the close affinity of APX-like cells seen by histology in other bivalve hosts including green-lipped mussels (*Perna canaliculus*), Mediterranean mussels (*Mytilus galloprovincialis*) and hairy mussels (*Modiolus areolatus*). Amplification followed by sequencing and phylogenetic analysis indicated that APX-similar structures observed in mussels are genetical identitical (99-100%) to APX in flat oysters, thereby extending the range of hosts for disease. Samples of green-lipped, Mediterranean and hairy mussels positive with APX were collected from widely geographically distributed locations in New Zealand, indicating the wide range of the parasite. However, histology showed low APX intensity and mild host response in the examined mussels compared to APX occurring in flat oyster. The results of this study have greatly extended our knowledge of APX and delivered a useful molecular tool for reliably detecting the disease which will help with detecting and managing APX in commercially important shellfish populations in New Zealand.

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And to you: *Ostrea chilensis*!

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GLOSSARY OF TECHNICAL TERMS

Entomogenous: (Greek, *entomon,* insects + *genes*, arising in): microorganisms which arise in insects.

Gametogony: the formation of gametes during the sexual stage of the life cycle in certain protozoans; the sexual stage itself.

Gamont (Synonym: gametocyte): the generation of individuals of Protozoa which gives rise to gametes which unite in pairs each to form a zygote.

Heteroxenous: infesting more than one kind of host

Merogony: an asexual replication process which increases the number of infective cells (merozoites) by multiple fission (schizogony) which is used by some protozoan parasites.

Merozoite (Greek: *meros*, part [of a series], +*zōon*, animal): any of the individuals produced by merogony in the asexual phase of the life cycle of a sporozoan, which infects further host cells and in turn forms meronts or gamonts**.**

Monoxenous: living on only one kind of host throughout its life cycle.

Oocyst (Greek, *ōon*, egg + *kystis*, bladder): a hardy, thick-walled spore, able to survive for lengthy periods outside a host.

Sporogony (Greek, *sporos*, seed, spore + *genesis*, origin): the formation of sporozoites in a sporozoan protozoa, a process of asexual division within the sporoblast, which becomes the sporocyst within an oocyst; following fusion of gametes (gametogony) and zygote (sporont) formation.

Sporozoite (sporo, relating to spores + Greek *zoion,* animal): a motile spore-like stage in the life cycle of some parasitic sporozoans (e.g., the malaria organism) that is typically the infective agent introduced into a host.

Zygote (**Greek, *zugōtos*,yoked, from *zugoun*, to yoke):** a diploid cell resulting from the fusion of two haploid gametes, i.e., a fertilized ovum.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Overview of Apicomplexa

The phylum Apicomplexa is a large and diverse group of single-celled eukaryotes that contains almost 6000 described species of either facultative or obligate intracellular parasites (Adl et al. 2007, Rueckert et al. 2010). Traditionally, the phylum contains four distinct groups: the coccidians, the gregarines, the haemosporidians, and the piroplasms (Leander et al. 2003, Morrison 2009). Among them, malaria which belongs to the haemosporidians, is the most important parasitic disease in humans, while coccidia (*Toxoplasma gondii, Eimeria* spp.) have caused heavy losses in domestic animals and affected more than a billion people worldwide (Eissa et al. 2015). Piroplasms (*Babesia* spp. and *Theileria* spp.) kill many domestic animals in many countries, while the gregarines (*Selenidium* spp., *Lecudina* spp., *Nematopsis* spp.) contains common parasites of many vertebrate and invertebrate hosts including insects, polychaete worms and molluscs (Jones 1975, Landers 1991, Lee et al. 2000, Lord & Omoto 2012, Boucher & Bosch 2015, Tuntiwaranuruk et al. 2004).

Phenotypic traits such as morphology, host, tissue and vector specificities rather than phylogenetic characteristics were originally used to define these four groups within the phylum Apicomplexa. Consequently, the evolutionary relationships among the four groups, and their subsequent taxonomic arrangement remain unclear (Morrison 2008). Nevertheless, it is estimated that less than 1% of the diversity within Apicomplexa has been revealed (Lee et al. 2000, Morrison 2009).

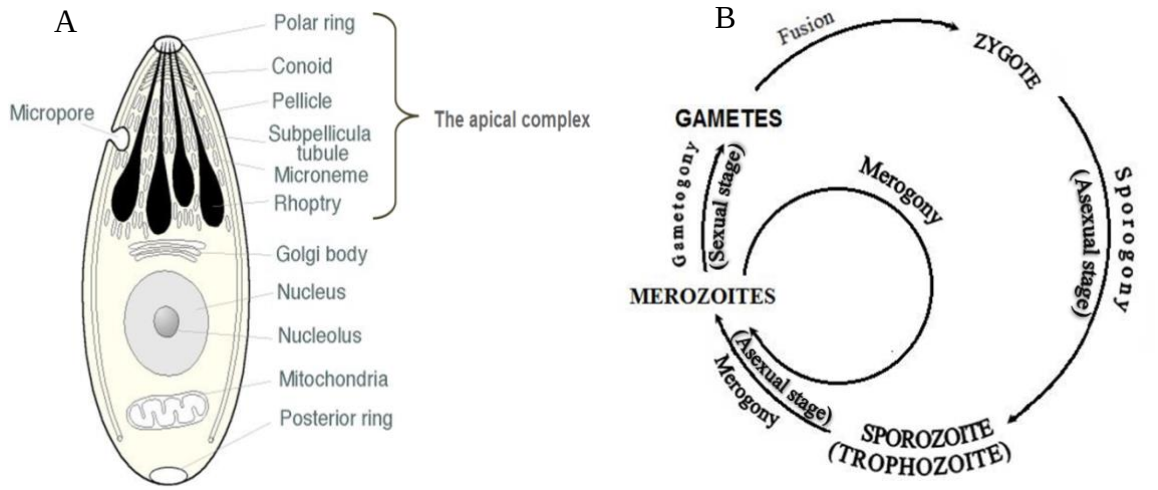


Figure 1.1 A) General apicomplexan structure, and B) life cycle (Source: adapted from Lee et al. 2000)

The defining feature of Apicomplexa is the apical complex, a group of structures located at the anterior region of the cell, which is an elaborate tool used for the host cell invasion processes (Figure 1.1A). The apical complex provides both a semi-rigid framework to these apically pointed cells, and a focal point for secretory organelles that release various complex mixtures of proteins (e.g., laminin, lectin-like molecules, SSP2-TRAP, SAG-1(P30) in *Toxoplasma gondii;* PfSUB1, PfSUB2, PfRESA, PfRIMA in *Plasmodium falciparum*) that mediate interaction with, and invasion of the host cell (Dubremetz et al. 1998, Lee et al. 2000, Blackman & Bannister 2001, Mercier et al. 2005). Most apicomplexans are monoxenous, being able to complete their life cycle (Figure 1.1B) within only a single host. A small proportion of apicomplexans are heteroxenous as they can alter the behaviour of intermediate hosts to pass to the definitive host (Lee et al. 2000, Blazejewski et al. 2015). These relationships with hosts are unique, and have prompted scientists to speculate on the long, and intertwined history (co-evolution) between early metazoans and early diverging alveolates which occurred over 600 million years ago (Wakeman 2013).

1.2 Emergence of apicomplexan-X in New Zealand shellfish

The New Zealand aquaculture industry is dominated by the culture of bivalve molluscs, notably the green-lipped mussel (*Perna canaliculus*) and the Pacific oyster (*Crassostrea gigas*) (Hine 2002a, Jeffs 2003). There is also the harvest of wild populations of several species of bivalves, for some of which there is increasing interest in developing commercial aquaculture methods, such as flat oyster (*Ostrea chilensis*), littleneck clams (*Austrovenus stutchburyi*), and scallop (*Pecten novaezelandiae*) (Hine 2002b). Since 1986, the shellfish industry has been under passive disease surveillance when epizootics caused by *Bonamia exitosa* - a haplosporidian parasite with microcells belonging to the genus *Bonamia* was first noted in flat oysters, virtually destroying the commercial fishery for flat oysters in Foveaux Strait (Doonan et al. 1994) (Figure 1.2). Bonamiosis affects both juveniles and adults of flat oysters and is a major threat to New Zealand's flat oyster stocks (Diggles et al. 2002). The second most serious disease of flat oysters in New Zealand is a parasitic disease caused by an undescribed apicomplexan labelled apicomplexan-X (APX) (Diggles et al. 2002). APX has been reported in New Zealand's flat oysters with high prevalence, occurring in 85 - 99% of 6400 *O. chilensis* individuals sampled from Foveaux Strait between 1986 and 1991 (Hine 1991). It was suggested initially that there was no relationship between the intensity of APX infection and either the intensity or distribution of concurrent *B. exitiosa* infections (Hine 1991). Subsequently, this view changed as observations indicated that APX zoites appeared to be capable of increasing the sensitivity of the flat oysters to *B. exitiosa* by occupying and destroying haemocytes, destroying vesicular connective tissue cells, and by utilising host glycogen reserves (Hine 2002a). Oysters heavily parasitized by the apicomplexan (Figure 1.3) were found to have severe damage to their vesicular connective tissue, leading to host sterility and death (Hine 2002a).

There are no data for the economic losses of commercially fished flat oyster populations caused by APX. However, the analysis of the intensity of *B. exitiosa* infection in relation to intensity of zoite infection in a large sample of flat oysters from Foveaux Strait suggested that of the total mortalities, 40.5% would be due to APX zoites only, 49.2% would be due to combined APX zoite and *B. exitiosa* infections, and 10.2% to *B. exitiosa* only (Hine 2002a). Co-infection of the flat oyster with both APX and *B. exitiosa* was thought to be capable of reducing the population of commercial size oysters in Foveaux Strait (> 58 mm diameter) by 91% in 1975 (Hine 2002a) (Figure 1.4). The high mortality of flat oysters when APX zoites act synergistically with *B. exitiosa* has been explained in two ways. Firstly, both *B. exitiosa* and APX infect haemocytes and therefore in concurrent infections haemocytes would become more rapidly depleted than if the same infection levels occurred independently (Hine 2002a). Secondly, each parasite would synergistically exacerbate the problems caused for the oyster's energy reserves, especially during and immediately after the peak spawning period when the peak prevalence and intensity of *B. exitiosa* infection occurs (Hine 2002a).

Apicomplexans with a similar structure to the APX of flat oysters in New Zealand have also been reported in green-lipped mussel from the Marlborough Sounds (Diggles et al. 2002) (Figure 1.5). These apicomplexans found in mussels are also slipper-shaped with a central nucleus and posterior amylopectin granules. The only life history stage observed in mussels was zoites, and heavy infections of the apicomplexan in mussels were associated with focal destruction of the vesicular connective tissue cells, a pattern consistent with observations of APX infections in flat oysters (Diggles et al. 2002, Hine 2002b). Recently, APX-like cells have also been observed in Mediterranean mussels (*Mytilus galloprovincialis*) and hairy mussels (*Modiolus areolatus*) (S. C. Webb unpubl. data) in New Zealand. These observed apicomplexans do not resemble any other known apicomplexan except the APX found in flat oysters and green-lipped mussels in New Zealand. These observations suggest that the APX

found in flat oysters is the same species as found in other New Zealand bivalves. If this is the case, there is a risk that APX disease outbreak could be transmitted among hosts, with the potential to create problems for New Zealand aquaculture. Despite the fact that APX can cause serious mortality in endemic New Zealand bivalves, the identity, prevalence, epidemiology, life cycle of this APX species are still largely unknown. The management of this parasite in fished and aquaculture populations of shellfish is greatly hampered by a lack of substantive scientific information.

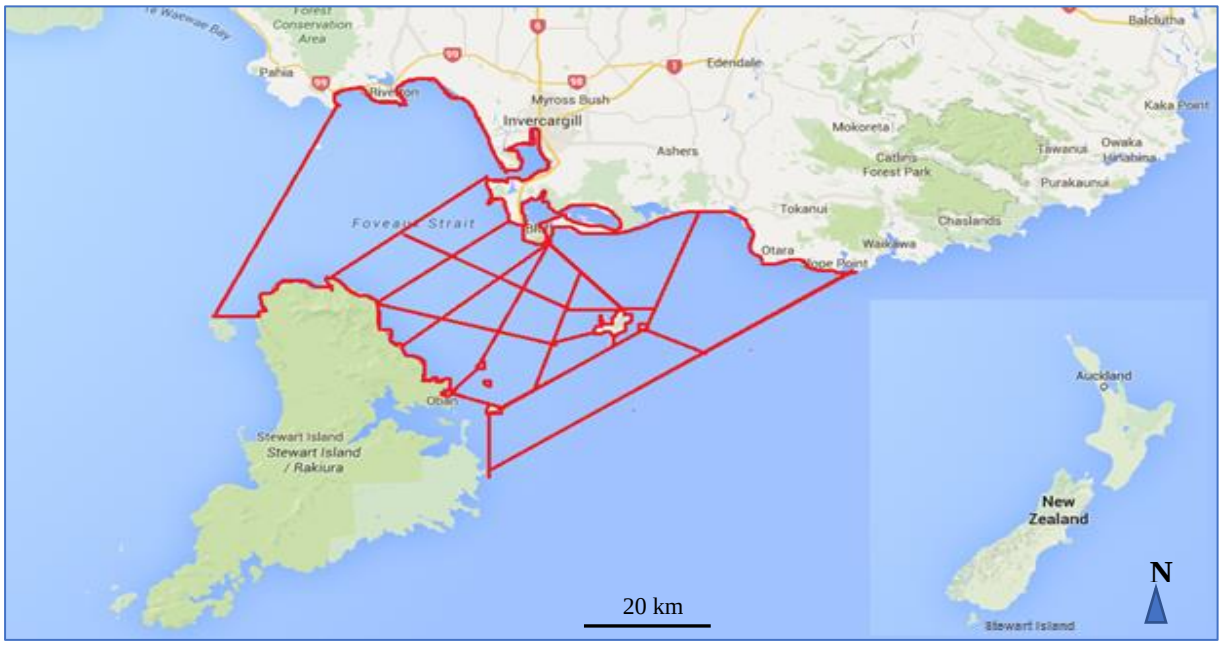


Figure 1.2 Foveaux Strait showing the flat oyster (*Ostrea chilensis*) management area which is the largest commercial fishery for flat oysters in New Zealand and was the site (46°35'09.9"S, 168°04'27.4"E) where APX was first reported (Hine 1996).

1.3 Histological descriptions of APX

General descriptions of APX pathology and some features of APX have been revealed by histological analyses of infected New Zealand flat oyster samples. The affected organs are most often kidneys and the digestive diverticulae. In addition, APX is commonly found in the haemolymph sinuses and suprabranchial sinuses (Hine 2002a). In heavy infections, extensive tissue damage is also observed in the vesicular connective (Leydig) tissue and within (i.e.,

intracellularly) the vesicular connective tissue cells. Intra-epithelial infections of the gonad have also been identified (Hine & Jones 1994).

The pathology of APX in flat oysters has been divided into five stages (Hine 2002a). The first phase of the disease is characterized by the presence of a few (<5) parasites which are difficult to observe without extensive and systematic searching of the histology section. In the second phase, a few parasites are observed in most tissue, either intercellularly between vesicular connective tissue cells (VCTC) or in small groups within haemocytes. In the third phase, APX are numerous around blood sinuses, particularly the suprabranchial sinuses and among the VCTC of the sub-epithelium, in the gills and mantle. Haemocytosis occurs and leads to excessive accumulations of haemocytes in the vesicular connective tissue and haemolymph sinuses. The infiltration of the parasite around the VCTC results in their separation into islets. Parasitic infection of the renal epithelium is often associated with epithelial hyperplasia. In phase 4, the parasites are abundant in all tissues, and many CTC lesions are evident. In the sub-epithelium and around the gonads, as well as the gut and digestive diverticulae, while the islets of VCTC become progressively smaller as zoite numbers increase. VCTC also detach from each other, leaving spaces that are empty or contain zoites in and around large numbers of haemocytes. Gonad follicles are often empty, but a few oogonia or spermatogonia are usually present on the germinal epithelium. At the same size, the body mass of oysters heavily infected with APX are typically smaller and thinner compared with uninfected oysters. In phase 5, parasites and haemocytes are abundant around the gonads, gut and digestive diverticulae, with tissues becoming congested, while VCTC are destroyed, leaving a stroma of necrotic debris (Hine 2002a). Replete haemocytes with pycnotic nuclei and destruction of gonad follicles are common. Oyster digestive diverticulae either appear spongy, with spaces between epithelial cells, or the epithelial cells are flattened rather than columnar. Gonad follicles are often empty and lack germinal epithelium. The bodies of oysters with phase 5 infection of APX are

frequently small, thin and watery, which is thought to result in their subsequent demise (Hine 2002a).

The only distinct developmental stage of the APX life cycle observed in flat oysters from Foveaux Strait were called zoites because it was not clear whether they were the result of sporogony or merogony (Hine 2002a). Using Transmission Electron Microscopy (TEM), the zoites were observed to contain all the structures typical of apicomplexan zoites including two polar rings, subpellicular microtubules, a conoid, rhoptries and micronemes. The zoites are elongated and elliptical in outline (about 8 µm long and 5 µm wide) with a round nucleus occupying almost the entire width of the cell halfway down their length (Hine 2002a).

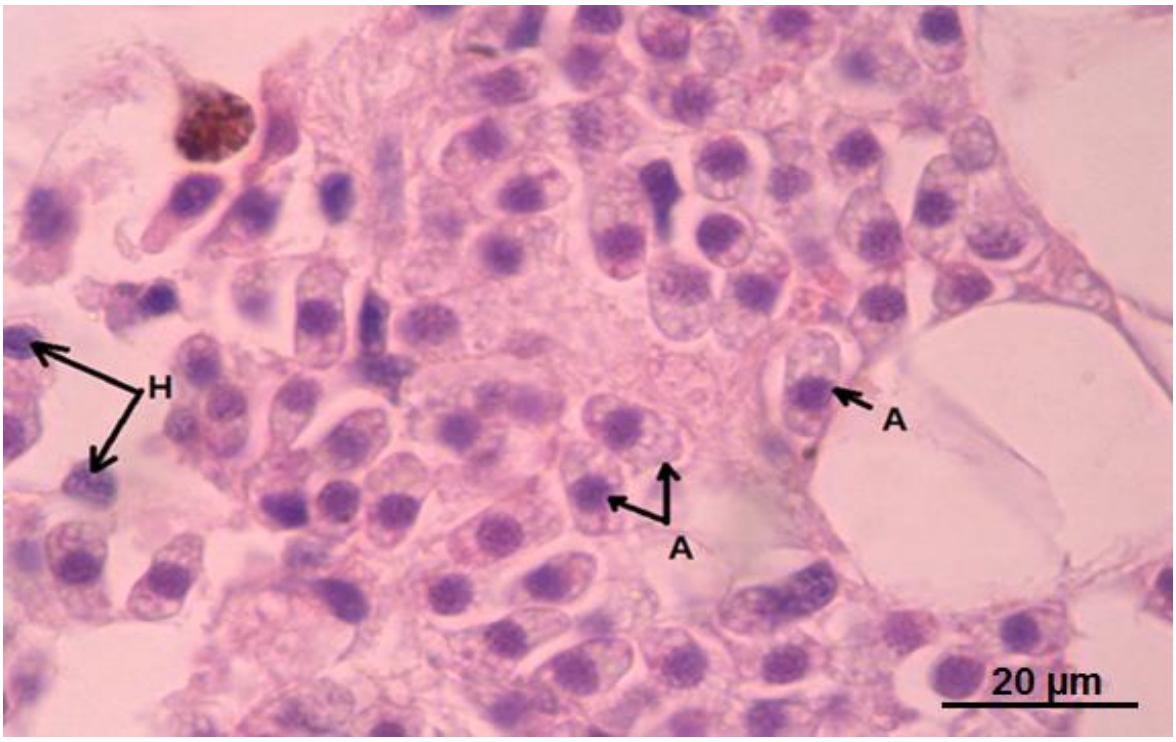


Figure 1.3 Many apicomplexan-X zoites (A) with some haemocytes (H) in the connective tissue of *Ostrea chilensis* (H&E stain)

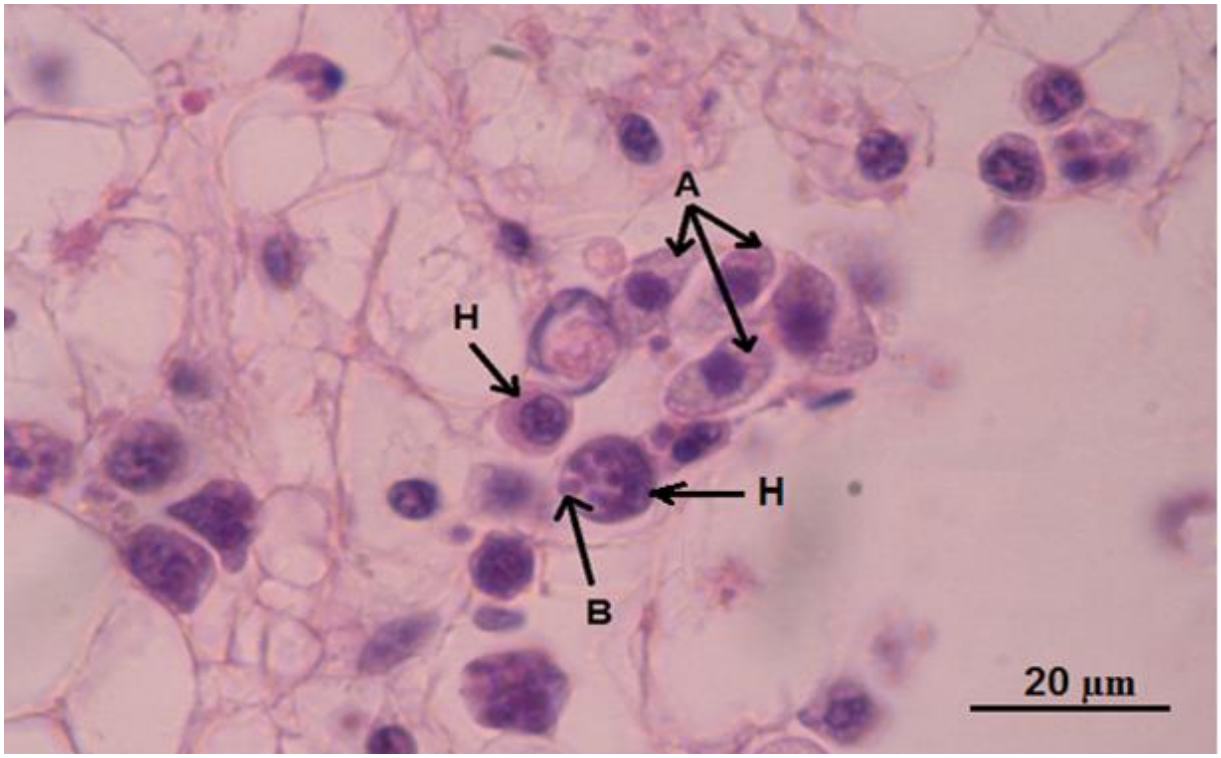


Figure 1.4 *Bonamia* microcells (B) inside haemocytes (H) and apicomplexan-X (A) in the connective tissue of *Ostrea chilensis* (H&E stain)

Histopathological examinations have provided important information of APX (e.g. overall structures, infected tissues). However, histological sections are thin, two dimensional slices, from a three dimensional piece of tissue. In the initial phase of APX infection when only a few (<5) parasites are present in a host section, the histological thin section may not represent the infected area and consequently the infected site can be easily missed. In addition, the occurrence of only one developmental stage in the tissues of *O. chilensis* suggests that the parasite is heteroxenous (Hine 2002a). Thus APX probably uses another host, possibly a species that is in close contact with oysters, for expressing its alternate life cycle stages (Diggles et al. 2002). Hine (2002a) suggested the other stages of APX may be the sexual stages of an apicomplexan detected in the gut epithelium of the terebellid polychaete, *Pseudopista rostrate*, which was commonly found in association with infected wild flat oysters. However, it was impossible to confirm by histological diagnosis whether these apicomplexan stages were those of morphologically similar coelomic gregarines or the APX zoites (Hine 2002a). Confirmation

of an intermediate host would be possible by developing a specific probe from oyster APX zoites and using the probe for *in situ* hybridization with apicomplexans in potential alternate hosts. If an intermediate host can be identified, it may be possible to break the life-cycle by separating or eliminating the host from the vicinity of cultured oysters or mussels.

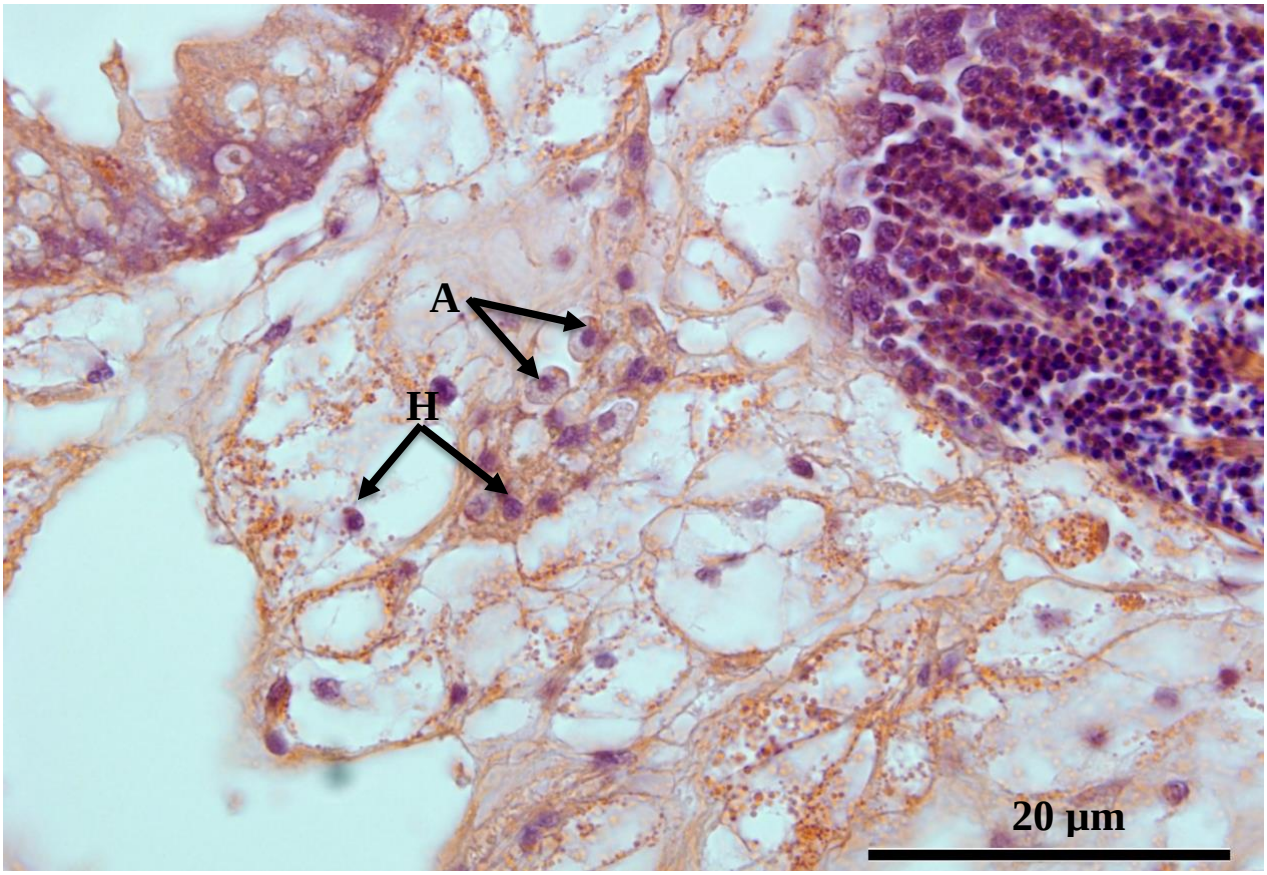


Figure 1.5 Apicomplexa-X like cells (A) and haemocytes (H) in the connective tissue of the green-lipped mussel (*Perna canaliculus*) (H&E stain)

1.4 Research aims

Information regarding APX as a pathogen of commercially important bivalves in New Zealand is limited. The detection of APX in shellfish using histological techniques is nonspecific, costly and labour intensive. Molecular genetic techniques provide the potential to more reliably confirm presence or absence of the parasite in whole samples of marine invertebrates, and to

definitively confirm the identity of APX found in different host species. Therefore, the specific aims of the research be undertaken for this thesis are:

(1) To characterise an 18S rRNA sequence from APX for: (i) facilitating the development of cost-effective PCR-based APX detection methods, and (ii) enabling associated phylogenetic investigations of APX. Based on histopathology work, flat oyster samples infected with APX will be identified and DNA extracted to amplify 18S rRNA sequences from APX. A challenge in achieving this is that APX cells cannot easily be isolated and dissected from the oyster tissue. In effect, this means that the PCR primer specificity for APX is critical for isolating and amplifying out the sequences from amongst the other genetic material present in oysters. Therefore, generic 'apicomplexan PCR primers' obtained from Kevin Wakeman (Tokyo University) and next generation sequencing of total DNA extracted from APX infected oysters were used to obtain useful APX genomic sequence data. A further complication is that apicomplexans are remotely related to dinoflagellates and consequently there are difficulties using simple BLASTN searches for distinguishing APX from dinoflagellate sequences which also may be present in the gut of oyster tissues (Figure 1.6). Hence, the phylogenetic analysis is essential.

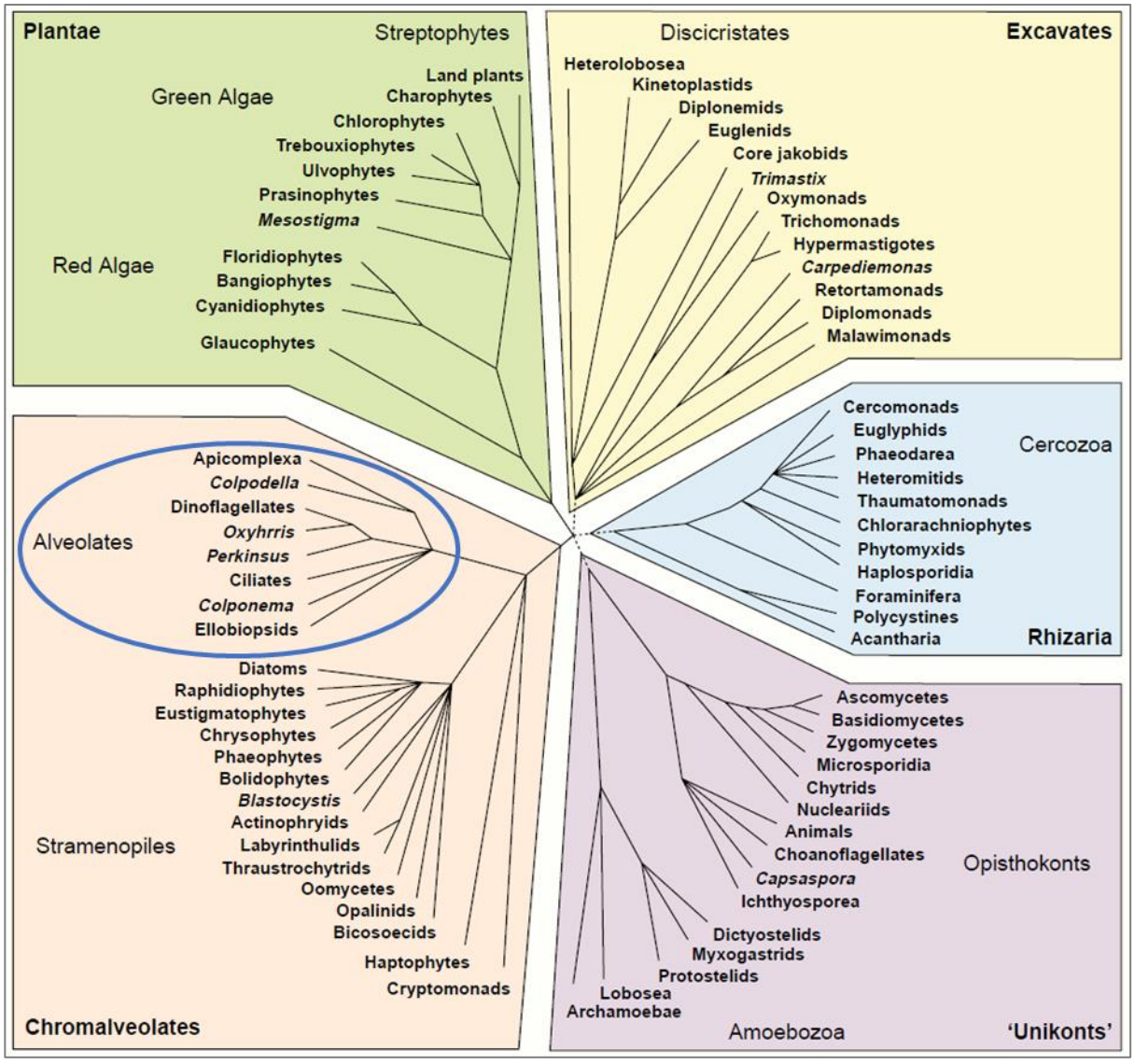


Figure 1.6 Diversity across the eukaryotic tree of life. With the help from molecular data, particularly the analysis of small subunit rRNA sequences, the Apicomplexa is positioned among the Alveolata group with the dinoflagellates and ciliates (blue circle). Within this clade, the Apicomplexan is more closely related to dinoflagellates than to the ciliates. (Source: adapted from Keeling et al. (2005).

In situ hybridisation (ISH) is the powerful final step to localize and confirm the presence of the specific APX 18S rRNA sequence in oyster tissue sections. A labelled complementary DNA probe was designed to hybridize to APX DNA sequence within infected oyster tissue section. This labelled DNA probe could then be detected by

using an antibody to detect the label on the probe. After visualizing the results under a microscope, it was possible to obtain temporal and spatial information about APX gene expression. The probes used in this process must allow discrimination of APX 18S rRNA sequence from the 18S rRNA sequences of the flat and Pacific oysters (*Crassostrea gigas*). The Pacific oyster was included in this analysis as a "negative control" tissue for the *in situ* hybridisation (Lane et al. 2016). The probes also must not hybridise with *Bonamia* spp. 18S rRNA sequences.

(2) To develop a specific molecular genetic technique for detecting APX. Primers which specifically target APX 18SrRNA were designed based on regions which showed the highest variability, discriminating the APX 18S rRNA sequences from the flat oyster, Pacific oyster, *Bonamia ostreae* and *Bonamia exitiosa* partial 18S rRNA sequences. Specificity was promoted by including C and G residues at the 3'-end of the primer to further destabilise interactions with mismatched nucleotides. Further testing of the primers on other apicomplexan species was conducted to ensure high analytical specificity of the primers.

(3) To determine whether the APX commonly found in *O. chilensis* in New Zealand is the same species as an apicomplexan found in green-lipped, hairy and Mediterranean mussels in New Zealand waters. Histological studies identified green-lipped, hairy and Mediterranean mussels infected with APX of similar structure to that found in *O. chilensis* in New Zealand. The APX-specific primers developed in Research Aim 1 were used to obtain the sequence of the 18S rRNA gene from mussels and then compared to the APX sequence obtained from flat oysters.

(4) Estimate the prevalence of APX among green-lipped, hairy and Mediterranean mussel populations collected from several locations in New Zealand. The testing used the PCR-based detection methods developed earlier in Research Aim 1. Histological

methods were also used to explore phenotypic differences in APX identified from different sources. The genetic variation in APX in different host species and from different geographical locations and informative sequences were used to determine phylogenetic relationships among APX from different host species and geographical locations.

CHAPTER 2: AMPLIFICATION OF PARTIAL 18S rRNA SEQUENCES OF APICOMPLEXAN – 'X' (APX), AN APICOMPLEXAN PARASITE ASSOCIATED WITH FLAT OYSTERS (*OSTREA CHILENSIS*) IN NEW ZEALAND

2.1 Introduction

The phylum Apicomplexa is a large and diverse group of parasitic protists that comprises almost 6,000 described species of either facultative or obligate intracellular parasites (Adl et al. 2007, Rueckert et al. 2010). While some apicomplexans are well known and serve as models for understanding apicomplexan infection and disease (e.g., *Plasmodium* and *Toxoplasma*), most apicomplexan groups have yet to be thoroughly studied. In particular, comparatively little data are available for apicomplexans parasitising molluscs, even though lineages of apicomplexans have been associated with harmful effects and mortalities in cultured and wild molluscs (Leibovitz et al. 1984, Whyte et al. 1994, Friedman et al. 1995, Winstead et al. 2004, Ceuta & Boehs 2012, Cheng 2012). For example, a mass mortality event of the Iceland scallop (*Chlamys islandica*) stock in Breidafjordur, west Iceland, is believed to have been caused by an apicomplexan, *Aggregata* sp. (Kristmundsson et al. 2015). It was estimated that during 2000-2006, the main scallop population in Iceland decreased by 84%, despite a halt in commercial fishing from 2003. By contrast, the ubiquitous coccidian, *Nematopsis* sp. is common in bivalves worldwide (Tuntiwaranuruk et al. 2004, Uddin et al. 2011, Ceuta & Boehs 2012, Sühnel et al. 2016) but is rarely reported to cause any tissue reaction (Azevedo & Cachola 1992, Azevedo & Matos 1999), unlike apicomplexan – 'X' (APX) reported in this paper which can severely damage its host (Hine 2002a).

APX reported from the flat oyster (*Ostrea chilensis*) from Foveaux Strait, New Zealand (NZ) does not morphologically resemble any other apicomplexans previously identified in

molluscs (Hine 2002a). The only distinct APX developmental stages observed in specimens of flat oysters were identified as zoites (Hine 2002a). APX zoites appear to be associated with increased susceptibility of wild flat oysters to infection with the haplosporidian parasite *Bonamia exitiosa* (Hine 2002a). The infection of flat oysters with *B. exitiosa* and APX from late 1985 to 1993 reduced the population of commercially harvestable oysters (> 58 mm shell diameter) in Foveaux Strait to 9% of its 1975 numbers (Hine 2002a).

Although APX is recognized as a major threat to the viability of the fledgling flat oyster aquaculture industry in NZ (Diggles et al. 2002), the parasite has been little studied, due in part to the lack of a cost-effective detection method. Currently, the only available method for the detection and enumeration of APX is the microscopic examination of fixed host tissues after histological staining. A cost-effective genetic test for APX would facilitate studies into the life cycle of APX, as well as aid in efficiently quantifying infection rates within host populations.

Genetic tests based on 18S rRNA gene sequences have been used to identify and to study the systematics of other apicomplexan species (Allsopp et al. 1994, Criado-Fornelio et al. 2004).Currently, there are no published sequences representing APX. Therefore, the aim of this study was to characterise an 18S rRNA sequence from APX so as to facilitate the development of cost-effective PCR-based APX detection methods in the near future.

2.2 Materials and methods

2.2.1 Histological detection of APX infections

Live flat oysters (n = 36; shell height = 66-98 mm) collected from Foveaux Strait in August 2015 were scrubbed to remove excess debris on the external surfaces, and each opened with a separate sterilised knife. Each oyster was examined for any gross pathology such as weight or colour abnormalities before dissecting out two transverse sections (3-5 mm thick); each section contained gill, mantle, gonad and digestive gland. One section was stored in a 5 ml

microcentrifuge tube and frozen at -70°C for molecular assays. The second section was placed into a labelled histology cassette and fixed for 48 h in 4% paraformaldehyde in seawater, and then placed in 70% ethanol for histological analysis. Fixed oyster tissue was then embedded in paraffin, sectioned (5 μm), mounted on glass slides and stained with haematoxylin and eosin for histological examination. Histology slides were examined under an Olympus BX51 light microscope at 100×, 400× and 1000× total magnifications.

2.2.2 Amplification and sequencing of the APX 18S rRNA gene

Total genomic DNA was extracted from frozen flat oyster digestive gland and gill tissues (n = 12 oyster individuals) using a Zymo Genomic DNA Tissue Mini Prep Kit, (Zymo Research) following the manufacturer's instructions. Subsequent amplification of 1.8 kb of 18S rRNA gene sequence was achieved in two steps. Initially, a product of 0.6 kb was amplified using the primer pair forward primer F2 (5' – GGTAGYGACAAGAAATAACAAC – 3') paired with reverse primer R2 (5' – GAYTACGACGGTATCTGATCGTC – 3') (Wakeman 2013). Polymerase chain reactions (PCR) mixtures consisted of 10 μl 2× MyFi Mix (BioLine); 1 μl forward primer (10 pmol μl^{-1}); 1 μl reverse primer (10 pmol μl^{-1}); 1 μl extracted gDNA and nuclease-free water to a final volume of 20 μl. Thermocycling conditions were 95°C for 2 min; 5 cycles of 95°C for 20 s, 50°C for 30 s, 72°C for 2 min; followed by 35 cycles of 95°C for 20 s, 60°C for 30 s, 72°C for 2 min; final extension of 72°C for 7 min, hold at 15°C. Amplification products were separated on a 1% (w/v) agarose gel and stained with ethidium bromide (0.5 μg ml^{-1}) before visualization under UV illumination. Amplification products of the expected size were purified from the agarose gel with a Nucleospin® gel and PCR clean-up kit (Macherey-Nagel) and sequenced by an external contractor (Massey Genome Service, NZ).

In the second stage of the amplification procedure, the 18S rRNA sequences obtained from the 0.6 kb F2/R2 amplification products were used to design specific primers which were then paired with generic apicomplexan 18S rRNA primers to extend from the F2/R2 sequence

in both 5' and 3' directions. The 5' extension was obtained using the forward primer PF1 (5' – GCGCTACCTGGTTGATCCTGCC – 3') (Wakeman 2013) paired with reverse primer 1906Rev1 (5' – TTTCACCTCTGACAGTACAAATAC – 3'). The 3' extension was obtained using the forward primer 3011For1 (5' – AATGCAAGGCATTTACTGCTTTG – 3') paired with the reverse primer SSUR4 (5' – GATCCTTCTGCAGGTTCACCTAC – 3') using thermocycling conditions of 95°C for 2 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s; final extension of 72°C for 5 min; hold at 15°C.

Amplification products of the expected sizes were purified from agarose gels and ligated into a T-tailed cloning vector (pGEM-T Easy, Promega) and sequenced by an external contractor (Massey Genome Service, NZ).

2.2.3 Phylogeny of the apicomplexan 18S rRNA sequences

In the first data set, two 1.8 kb 18S rRNA sequences, 'Isolate from oyster 1' (GenBank accession numbers KX774501) and 'Isolate from oyster 2' (KX774502) were aligned with 18S rRNA sequences (n = 43) obtained from GenBank (Appendix 1) that represented major eukaryote groups and included apicomplexans and alveolates (Allsopp et al. 1994, Yubuki & Leander 2008). The 18S rRNA sequence of the coccidian *Toxoplasma gondii* (U03070) was included as a representative of the Apicomplexa.

To elucidate the relationship between the novel sequences among known apicomplexans (especially marine apicomplexans), the two new 18S sequences generated in this study were aligned with 27 additional sequences downloaded from GenBank (Figure 2.2) representing major apicomplexan lineages and three sequences from dinoflagellates and two sequences from colpodellids as the out group, forming the second data set of 34 taxa. Sequences were aligned using Clustal W and manually edited using Mesquite (Maddison & Maddison 2016) to exclude hypervariable regions for which homology could not be assigned confidently.

Phylogenies were estimated using the Neighbor-Joining method (Saitou & Nei 1987) with the K2P model (Kimura 1980), and bootstrap support for each node was estimated from 2000 pseudoreplicates in MEGA version 6.06 (Tamura et al. 2013).

2.2.4 Amplification and sequencing of the O. chilensis *18S rRNA gene sequence*

To facilitate *in situ* hybridisation probe design, the *O. chilensis* 18S rRNA gene sequence was required to help reduce the chances of any proposed probes hybridising with *O. chilensis* 18S rRNA. To obtain 1.8 kb of *O. chilensis* 18S rRNA sequence data, a PCR product of 1.8 kb was amplified from *O. chilensis* gDNA templates using the generic 18S rRNA primer pair PF1/SSUR4 (Wakeman 2013). PCR reaction mixtures consisted of 10 µl 2× MyFi Mix (BioLine), 1 µl forward primer PF1 (10 pmol μl^{-1}); 1 µl reverse primer SSUR4 (10 pmol μl^{-1}); 1 µl extracted gDNA; and nuclease-free water to a final volume of 20 µl. Thermocycling conditions were 95°C for 2 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s; final extension of 72°C for 5 min; hold for 15°C. Amplification products of the expected size were purified from the agarose gel (Nucleospin® gel and PCR clean-up kit; Macherey-Nagel) and ligated into a T-tailed cloning vector (pGEM-T Easy, Promega) and sequenced by the Massey Genome Service.

2.2.5 In situ hybridisation (ISH)

To further support the hypothesis that the 18S gene sequences amplified from this study belonged to APX, specific ISH probes were designed. Using Clustal W (Larkin et al. 2007), the APX 18S rRNA gene sequences we obtained in this study (GenBank accession number KX774501 and KX774502) were aligned with a flat oyster 18S rRNA sequence (KX977494), a Pacific oyster (*Crassostrea gigas*) 18S rRNA sequence (AB064942) and 18S rRNA sequences from *Bonamia* species, i.e. *B. ostreae* (AF262995.1) and *B. exitiosa* (JF495410.1). From the aligned 18S rRNA sequences, two probes ISH-APX-Pr01 (5'–

ATCAAACCGGATTCTCACTCAAAGA – 3') and ISH-APX-Pr02 (5' – GTGAGTCGAGAACAAAGAACATTC – 3') were designed using Oligo Calc software (Kibbe 2007) that were specific to APX, but had mismatches along the non-target 18S rRNA sequences of flat oysters, Pacific oysters and *Bonamia* species. Both probes were labelled with a digoxigenin (DIG) at the 3' end (Sigma-Aldrich).

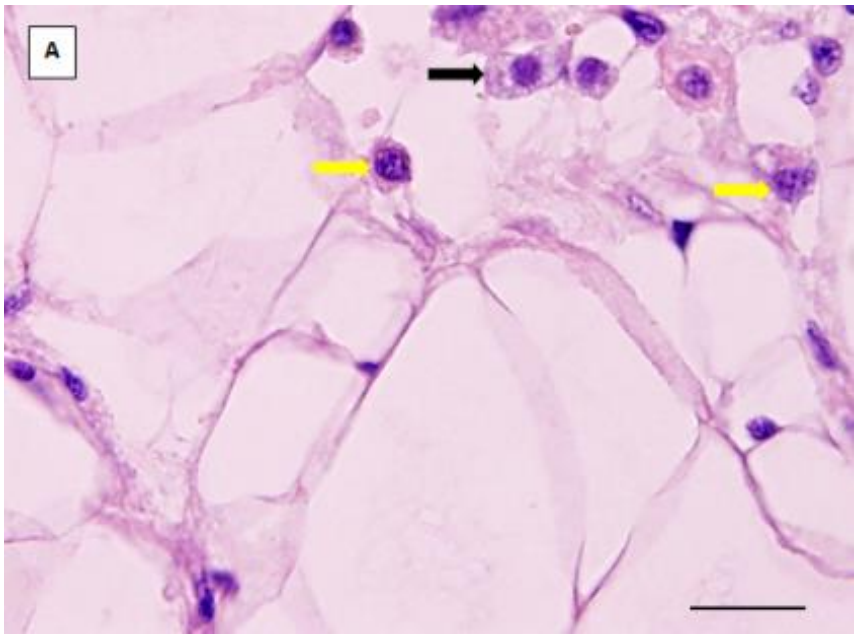
Oyster tissue sections for ISH were prepared by the Institute of Veterinary, Animal and Biomedical Sciences, Massey University, NZ. Briefly, 5 μm thick cross sections were cut from paraffin-embedded blocks of paraformaldehyde-fixed tissue taken from three flat oysters (12 sections each) that tested positive for APX by histology and PCR. The cross sections were placed on Superfrost Plus slides (Menzel Gläser) and air dried. Nine mounted tissue sections from each of two Pacific oysters (Cawthron Aquaculture Park, Nelson, NZ) which were APX-negative were prepared in the same process.

Eighteen ISH assays were carried out on a set of three slides; a negative control (Pacific oyster), a positive control (flat oyster sample infected with APX) and a no-probe control (hybridisation buffer only). Sections from each flat oyster with APX infections were tested with the ISH probes ISH-APX-Pr01 and ISH-APX-Pr02. The ISH procedure (Stokes & Burreson 1995, Bueno 2015) was modified slightly as follows. Concentration of Proteinase K was used at 100 μg ml−1. Protease digestion time was increased to 18 min. The washing step with 1.0% glycine in 1× PBS for 5 min was removed. Pre-hybridisation buffer was removed from the tissue sections and replaced with 80 μl hybridisation solution consisting of the pre-hybridisation buffer and DIG-labelled DNA probe at the final concentration of 3 ng μl^{-1}. Hybridisation temperature was 45°C for probe ISH-APX-Pr01 and 42°C for probe ISH-APX-Pr02. The ISH labelled specimens were examined under an Olympus BX51 light microscope for the presence of dark blue to black precipitates in cells.

2.3 Results

2.3.1 Histological confirmation of APX infection of flat oyster tissues

APX zoites were observed in the histological sections of all 36 flat oysters examined, albeit with varying levels of infection intensity (Figure 2.1). The zoites were elongate and elliptical in shape, with a mean ± SE length of 7.4 ± 0.2 µm (range: 5 –10.5 µm, n = 60). Examination of the tissue sections from 36 infected flat oysters found that zoites were most commonly located in the connective tissue between digestive gland tubules, proximal to the intestine, less commonly in the gill epithelium and mantle. APX was not observed in gonad tissues. From an initial histological screen of 36 flat oysters, 12 flat oysters heavily infected by APX were selected for the extraction of genomic DNA.



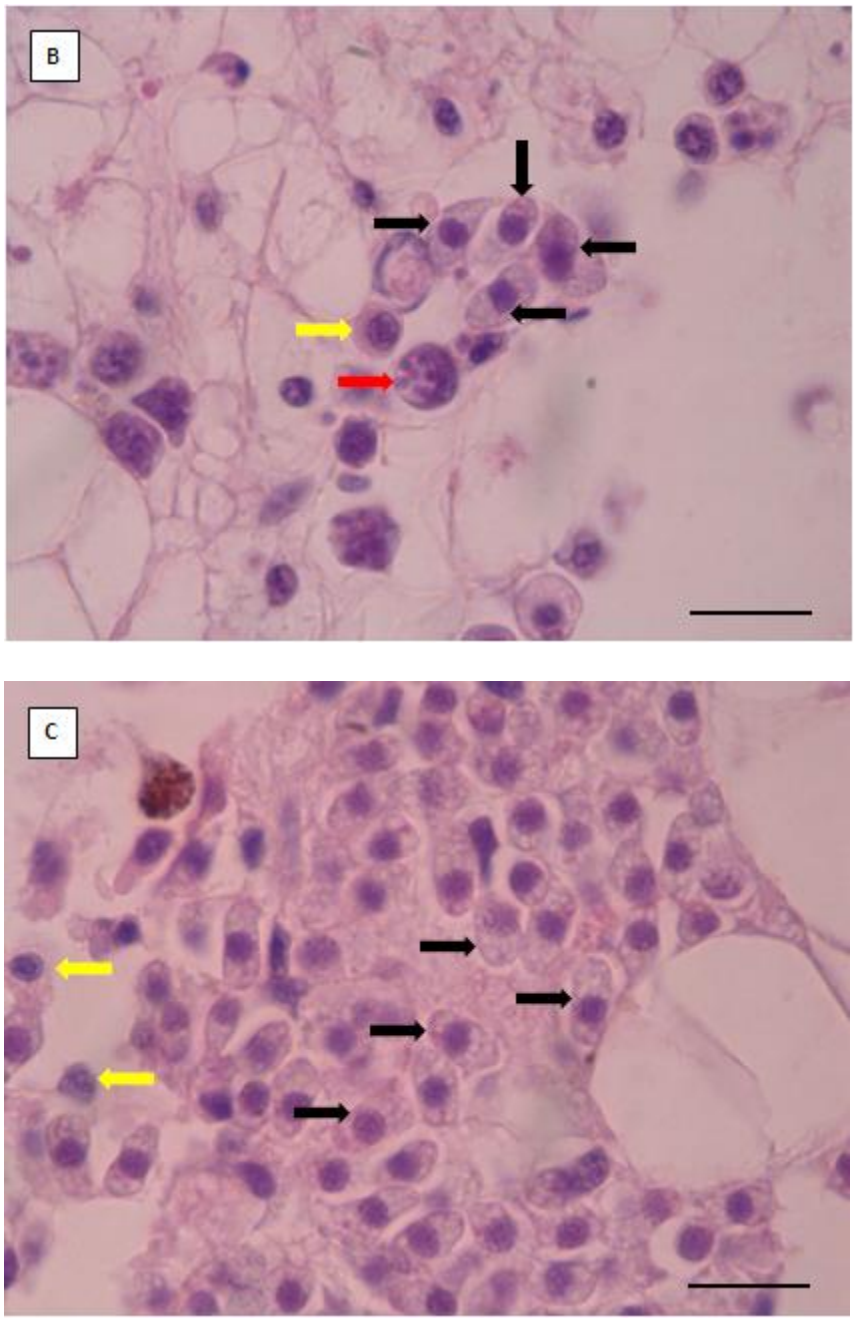


Figure 2.1 Histopathological differences in the intensity of apicomplexan-X (APX) infection in the examined *Ostrea chilensis*. Black arrows: APX, red arrow: *Bonamia* microcells within haemocyte, yellow arrows: normal haemocytes. (A) Light infection: <5 parasites visible in the field of view. (B) Light to moderate infection: a few zoites are observed in most tissues between vesicular connective tissue cells. (C) Moderate to heavy infection: zoites are numerous among and within the vesicular connective tissue cells, and in the gills and mantle (not shown). The

levels of infection intensity are defined based on the classification of Hine (2002). Scale bars = 10 μm, H&E stain.

2.3.2 Amplification and sequencing of partial APX 18S rRNA gene sequences

An amplicon of 0.6 kb was generated from flat oyster gDNA templates using a PCR primer pair F2/R2 targeting apicomplexan 18S rRNA sequences. The PCR product was directly sequenced and a BLASTN search of the GenBank database found the sequence most closely matched with 18S rDNA sequence from *Eimeria* sp. (Apicomplexa, accession number JF419346, 82% identity, E value 2 $x10^{-143}$).

Concatenation of the two putative 18S APX sequences extended from 5' and 3' direction using the primers pairs PF1/1906For1 and 3011For1/SSUR4 generated 917 and 1320 nucleotide portions of the 18S rRNA gene, producing a composite sequence of 1809 bp. The two sequences overlapped by 429 nucleotides allowing unambiguous concatenation of the two sequences. Using this approach, 18S rRNA sequences were generated from two flat oyster gDNA samples and, after the 5' and 3' PCR primer sequences were removed, were deposited on GenBank (isolate from oyster 1 and 2: accession numbers KX774501 and KX774502, respectively) for a portion of the 18S rRNA gene. The two aplicomplexan 18S rRNA gene sequences differed at three nucleotide positions.

2.3.3 Phylogeny of the apicomplexan 18S rRNA sequences

Phylogenetic analysis of the data set containing the two putative APX 18S gene sequences and the 43 18S sequences representing the major eukaryote groups placed the putative sequences as a sister-group to the coccidian *Toxoplasma gondii* with well-supported bootstrap values (Appendix 1). Phylogenetic analyses of the 34-taxa data set that focused on the relationships within the apicomplexans showed that the two putative APX sequences formed sister clade to

the group consisting of *T. gondii*, *Hepatozoon catesbianae* and *Adelina bambarooniae*, albeit with low bootstrap support (Figure 2.2).

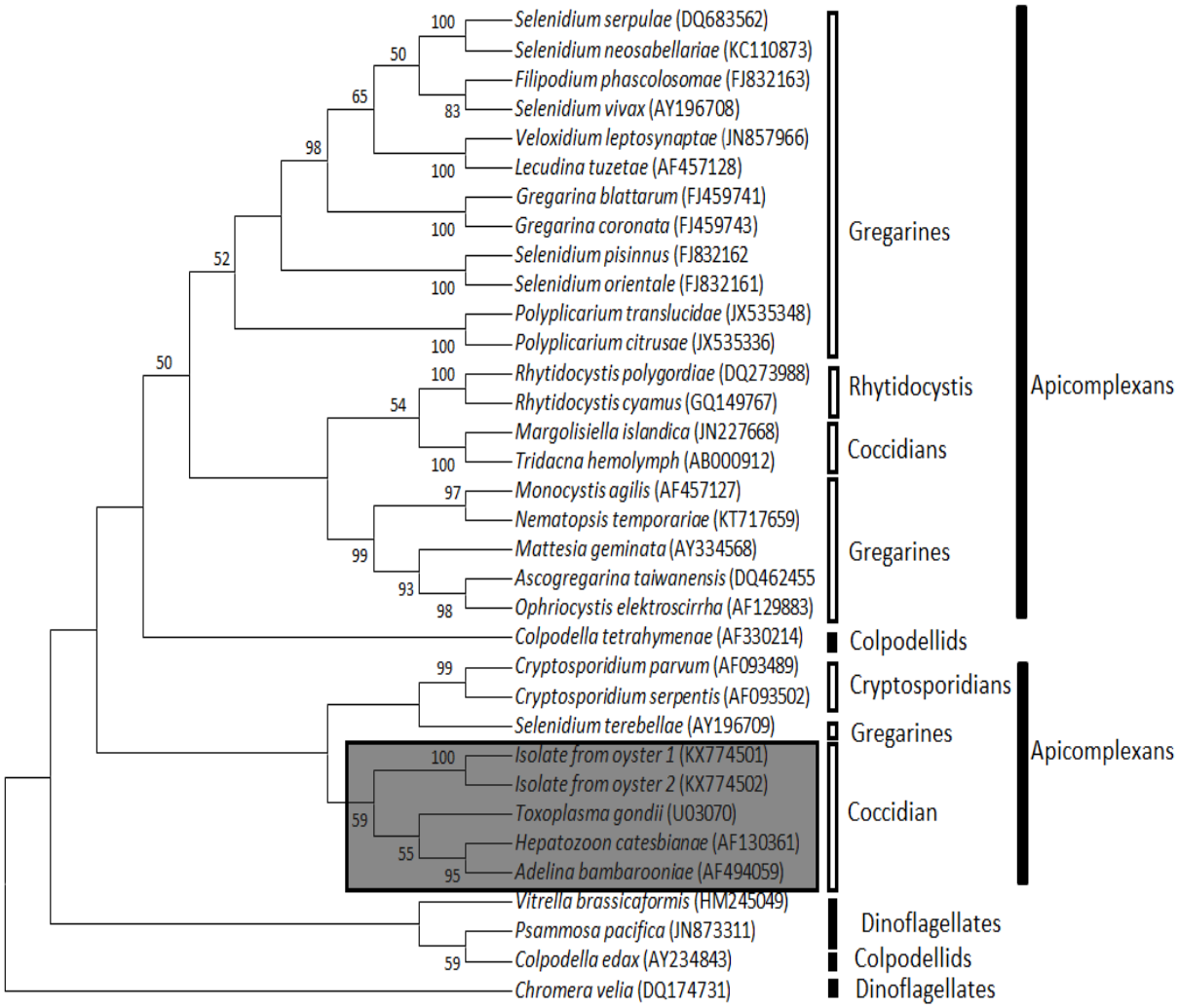


Figure 2.2 Phylogenetic analyses of 2 apicomplexan-X (APX) isolates from the flat oyster *Ostrea chilensis* with representatives of known apicomplexan sequences. Colpodellids and dinoflagellates were used as outgroups. The 2 APX sequences group with the clade consisting of *Toxoplasma gondii*, *Hepatozoon catesbianae* and *Adelina bambarooniae* and are highlighted in the box. Numbers above the nodes are bootstrap support values >50% (of 2000 replicates). GenBank accession numbers of all sequences used are shown in parentheses.

2.3.4 Amplification and sequencing of an oyster 18S rRNA gene sequence

At the time this work was under taken, only two *O. chilensis* 18S rRNA sequences were available on GenBank, both of 875 bp (accession numbers EU660788, EU660789). Using the generic 18S rRNA primer pair PF1/SSUR4, a PCR product of 1.8 kb was amplified from *O. chilensis* gDNA templates. After trimming off the PCR primer sequences, the resulting 1776 bp sequence was 99.8 % identical with both previously reported *O. chilensis* 18S rRNA sequences EU660788 and EU660789. The 1776 bp *O. chilensis* 18S rRNA sequence was deposited on GenBank with accession number KX977494.

2.3.5 In situ *hybridisation*

From the alignment of APX 18S rRNA sequences (GenBank accession numbers KX774501 and KX774502) with flat oyster (KX977494), Pacific oyster (AB064942), *B. ostreae* (AF262995.1) and *B. exitiosa* (JF495410.1) sequences, two probes (ISH-APX-Pr01 and ISH-APX-Pr02; Figure 2.3) appeared promising by Oligo Calc analysis and were experimentally evaluated. ISH was performed with both ISH-APX-Pr01 and ISH-APX-Pr02 probes for the detection of APX using high-temperature denaturation on paraformaldehyde-fixed, paraffin wax sections of APX infected flat oysters, and visualised by an alkaline phosphatase/nitroblue tetrazolium detector system. Both probes ISH-APX-Pr01 and ISH-APX-Pr02 showed complete hybridisation to APX cells, which resulted in a dark, coarsely granular blue reaction product (Figure 2.4). Examination of the cells to which the ISH probes bound morphologically resembled the APX cells identified in the histological specimens (Figure 2.1). None of the probes bound to host tissue, and all controls were negative (Figure 2.4). Hybridisation results were reproducible when 18 runs were made.

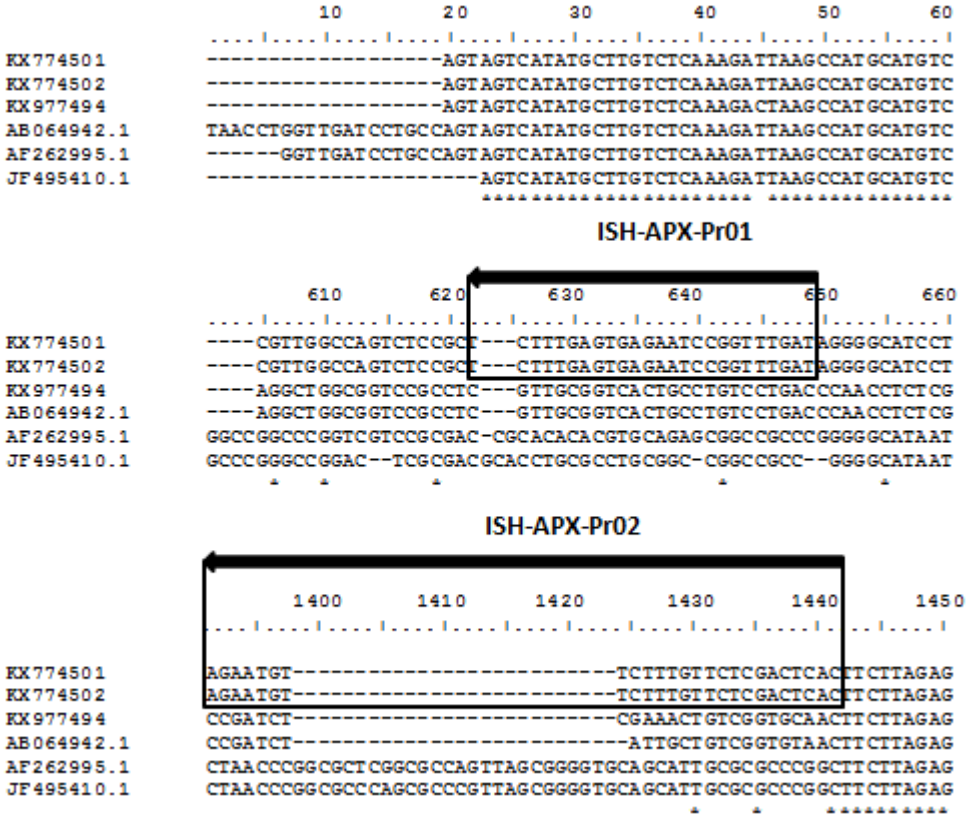


Figure 2.3 Design of probes for *in situ* hybridisation detection of apicomplexan-X (APX) 18S rRNA in infected oyster *Ostrea chilensis* tissues. Partial 18S rRNA sequences of APX (GenBank accession nos. KX774501, KX774502), flat oyster (KX977 494), Pacific oyster (AB064942), *Bonamia ostreae* (AF262995) and *B. exitiosa* (JF495410) were aligned to identify 2 regions discriminating the APX 18S rRNA sequences from the others. Two *in situ* hybridisation probes (ISH-APX-Pr01 and ISHAPX-Pr02) were designed which were reverse-complements of the 2 regions indicated.

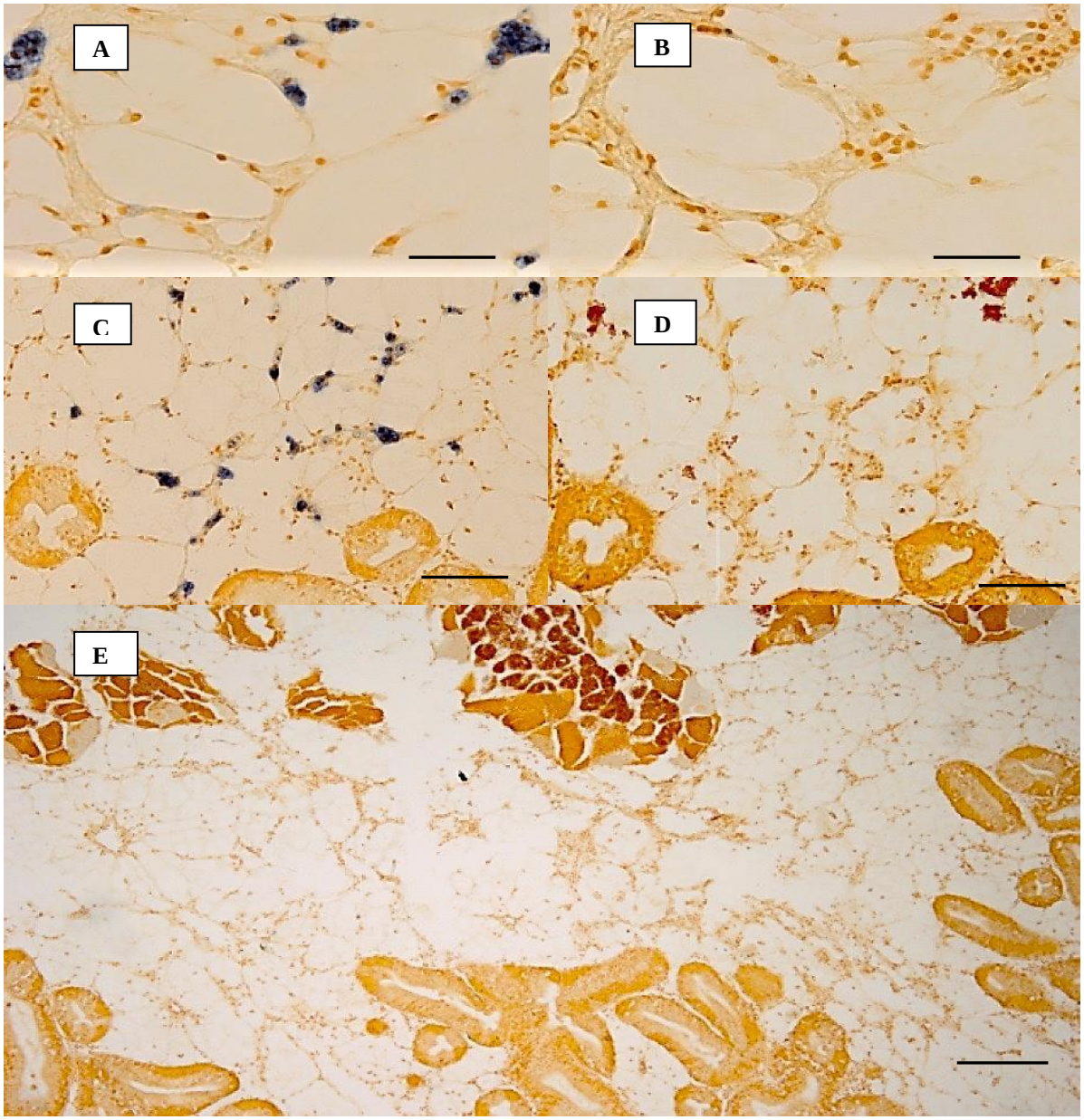


Figure 2.4 Detection of apicomplexan-X (APX) 18S rRNA sequences in APX-infected flat oyster *Ostrea chilensis* tissue sections by *in situ* hybridisation. (A,C) Clear labelling (blue dye deposition) of APX cells by both probes ISH-APX-Pr01 (A) and ISH-APXPr02 (C). No labeling of flat oyster host cells was apparent. (B,D) Negative controls consisting of APX-infected flat oyster tissue processed but omitting either probe ISH-APX-Pr01 (B) or probe ISH-APXPr02 (D). (E) Negative control consisting of non-APX-infected Pacific oyster *Crassostrea gigas* tissue hybridised with either probe ISH-APX-Pr01 or ISH-APX-Pr02. Scale bars = (A,B) 20 µm, (C,D) 50 µm, (E) 100 µm

2.4 Discussion

Apicomplexans are known to be important from both economic and human health perspectives. In addition, many lineages of apicomplexans are closely associated with marine invertebrates, including a number from commercially significant bivalve species from around the world (Morado et al. 1984, Whyte et al. 1994, Tuntiwaranuruk et al. 2004, Aranda et al. 2011, Uddin et al. 2011, Kristmundsson et al. 2015). This includes the unnamed and poorly understood APX found in New Zealand flat oysters that has been associated with mass mortality events in the largest commercial flat oyster fishery in NZ (Cranfield et al. 2005). From 1986 to 1988, APX infected 80-100% of oysters sampled in Foveaux Strait, and in association with *Bonamia exitiosa*, oyster stocks were reduced by up to 80% (Hine 1989). However, there is little information on the identity, prevalence and epidemiology of APX, and the life cycle of APX is unknown.

Using primers designed for apicomplexans from other host groups, we obtained sequences for a portion of the 18S rRNA gene from DNA extracted from flat oysters that were histologically confirmed to be infected with APX. The 18S sequences obtained were most similar to apicomplexan sequences in GenBank, and a phylogenetic analysis of 18S rRNA sequences for 43 eukaryote species downloaded from GenBank revealed that the putative APX sequences were most closely related to sequences from the apicomplexan *Toxoplasma gondii.* Confirmation of the identity of APX sequences was also supported by their corresponding ISH probes which clearly and exclusively labelled the APX zoites in histological sections of infected oysters. These results strongly support the contention that we isolated sequences for the APX infecting flat oysters.

Among marine apicomplexans, use of the small subunit (SSU) rDNA sequences for evaluating the diversity of marine gregarines (an apicomplexan subclass) has been very informative for delimiting closely related species from one another and for discovering major

clades of gregarine species (Peoples et al. 2012, Wakeman & Leander 2013). Apicomplexans with similar morphology to the APX zoites found in flat oysters in NZ have been reported from green-lipped mussel (*Perna canaliculus*) from the Marlborough Sounds (Diggles et al. 2002). The apicomplexans found in green-lipped mussel do not resemble any other known apicomplexan except the APX found in NZ flat oysters, and they have not been reported from any other host (Diggles et al. 2002). Recently, apicomplexans with similar morphology to the APX zoites have also been found in NZ blue mussels (*Mytilus galloprovinciali*) (S. C. Webb unpubl. data). However, the use of morphological characters to distinguish between species can be misleading, especially when variability within an individual species occurs. Evidences from other protists also show that morphological similarity is not necessarily correlated with genetic similarity (Wakeman 2013). The primers developed in this study may resolve ambiguities and uncertainties occurring in this issue.

The phylogeny estimated from the APX 18S sequences and 18S sequences for Apicomplexa downloaded from GenBank found APX grouped with the apicomplexans *Hepatozoon catesbianae*, *Adelina bambarooniae* and *T. gondii*. *Hepatozoon* spp. are apicomplexan parasites infecting tetrapod vertebrates and hematophagous arthropods (Boulianne et al. 2007), while coccidian parasites of the genus *Adelina* are known to be strictly entomogenous (Lange & Wittenstein 2001). *Hepatozoon catesbianae* has been described inhabiting the erythrocytes of American bullfrogs (*Rana catesbeiana*), green frogs (*Lithobates clamitans*) (Boulianne et al. 2007) and mosquitoes (*Culex territans*) *(Desser et al. 1995)*, while *A. bambarooniae* has been recorded as a parasite of the cane beetle (*Dermolepida albohirtum*) *(Harris et al. 2011)*. Although phylogenetic analyses clearly established that the 18S sequences were of apicomplexan origin; their detailed relationship to known apicomplexan groups is less resolved. Greater improvements in support, resolution, and accuracy could be achieved by extending the length of APX sequences analyzed in the future.

A key advantage of PCR is the ability to detect DNA from parasites when the tissues available are not appropriate for histology, for example when they are in a state of decomposition or have been frozen. PCR is also a sensitive method for detecting specific sequences of DNA. However, PCR does not confirm the presence of a viable pathogen, infection or disease. For this, other techniques such as histology and ISH are also required as used here, to allow visualisation of the parasite in host tissues (Burreson 2008).

In summary, the present study shows that APX in flat oysters can be detected by PCR, and the results from ISH tests and phylogenetic analyses supported the contention that I have obtained sequences for APX parasitising NZ flat oysters. The sequences I obtained and the primers I designed will increase the range of research that can be done on APX. The method has the potential to confirm the identity of APX-like cells seen previously by histology in NZ *P. canaliculus* and *M. galloprovincialis.* Additionally, despite the extensive histopathological examination of numerous flat oyster individuals, only the zoite stage of APX has ever been observed (Hine 2002a). No process of replication (either sexual or asexual) has ever been observed so far and the sister group of APX, *T. gondii,* is known for adopting several forms in its life cycle which spread through several hosts, suggesting the possibility that APX is heteroxenous. Future work should focus on validating the PCR methods developed in this current research and using the test to detect potential secondary host(s) of APX that will ultimately assist in managing APX in affected populations of flat oysters.

CHAPTER 3: PCR TEST TO SPECIFICALLY DETECT THE APICOMPLEXAN-'X' (APX) PARASITE FOUND IN FLAT OYSTERS (*OSTREA CHILENSIS*) IN NEW ZEALAND

3.1 Introduction

Shellfish aquaculture contributed 31% of all seafood consumed in 2015 and is expected to grow to meet the future demand (FAO 2017). However, microbial diseases caused by protozoans (e.g. *Marteilia* spp., *Perkinsus* spp., and *Bonamia* spp.), bacteria (e.g. *Vibrio parahaemolyticus*, *Flexibacter* sp.), and viruses (e.g. white spot syndrome virus [WSSV], ostreid herpes virus) continue to hinder industry growth (Doonan et al. 1994, Chou et al. 1995, Tajima et al. 1997, Raftos et al. 2014, Lee et al. 2015). These pathogens may infect a single species, a group of related species (e.g. *Marteilia sydneyi* in Sydney rock oysters), a broad range of species (e.g. *Perkinsus olseni*, WSSV) (Raftos et al. 2014), all shellfish life stages (e.g. *Vibrio harveyi*) (Austin & Zhang 2006, Mine & Boopathy 2011) or a specific life stage (e.g. *Vibrio tubiashii*) (Elston et al. 2008) and can cause devastating disease epizootics. As examples, pathogenic diseases result in up to 40% (>$3 billion USD) of annual production losses to global shrimp aquaculture (Stentiford et al. 2012) and have caused epizootics resulting in >90% stock depletion among oyster species such as *Crassostrea virginica* reared in Chesapeake Bay, USA (Guo & Ford 2016), *Ostrea edulis* reared in Brittany, France (Naciri-Graven et al. 1998) and *Saccostrea glomerata* reared on the east coast of Australia (Adlard & Nolan 2015).

The flat oyster (*Ostrea chilensis*) provides the basis of the oldest commercial fishery in New Zealand and is worth ~$20 million USD annually (Velvin 2015). Parasitic diseases, particularly bonamiosis caused by *Bonamia exitiosa* and *Bonamia ostrea*, represent the greatest threat to *O. chilensis* and resulted in the collapse of an emerging industry to farm this species (Doonan et al. 1994, Diggles et al. 2002, Lane et al. 2016). Bonamia infection in flat oysters is often accompanied by infection with an uncharacterised apicomplexan-'X' or APX parasite

(Hine 2002a, Lane et al. 2016). Oyster co-infection by *Bonamia exitiosa* and APX has been associated with dramatically reduced population numbers in commercial fisheries, such as occurred in Foveaux Strait between 1985 and 1993 (Diggles et al. 2002). This concurrent infection is thought to be synergistically detrimental to the host, as both *B. exitiosa* and APX are associated with depletion of the host glycogen reserve (Hine 2002a). Severe infections of APX otherwise occur in apparently healthy flat oysters and may predispose those oysters to bonamiosis (Diggles et al. 2002). Despite the pathogenic threat posed by APX, its life cycle, infection mechanisms and seasonal prevalence remain largely unknown.

Histopathology has revealed a high prevalence of APX zoites in flat oysters (Diggles et al. 2002, Hine 2002a). For example, examination of 6455 oysters sampled from Foveaux Strait between 1986 and 1991 identified >85% to be infected with APX (Hine 1991). In addition, high APX infection loads appeared to affect oyster gametogenesis, with 9% of high-load oysters having empty gonad follicles lacking germinal epithelium compared with only 2% of moderate-load and 1% of low-load oysters (Hine 2002a). Furthermore, vesicular connective tissue in oysters heavily parasitised by APX was found to be severely damaged, likely leading to host sterility and death (Hine 2002a).

In addition to APX in flat oysters, zoite life-stage apicomplexans with similar unique morphology (slipper-shaped with a central nucleus and posterior amylopectin granules) have been detected in the green-lipped mussel (*Perna canaliculus*) from the Marlborough Sounds in New Zealand (Diggles et al. 2002, Hine 2002b, Webb 2008) and more recently in the Pacific oyster (*Crassostrea gigas*) and blue mussel (*Mytilus edulis*) in New Zealand (S. C. Webb unpubl. data). These commercially important bivalve species may be hosts for APX, however, histology evidence relies primarily on apicomplexan morphology which may be non-specific, for example if those apicomplexans supposed to be APX are cryptic species. Furthermore, histology is costly and time-consuming.

To detect APX more specifically and efficiently, a 1.8 kbp region of the APX 18S rRNA gene was recently amplified from APX-infected *O. chilensis* and used to generate APX 18S rRNA-specific *in situ* hybridisation probes (Suong et al. 2017). Building on this, we describe a sensitive and specific PCR test for the APX 18S rRNA gene derived from APX-specific primer sequences designed for the *in situ* hybridisation probes. PCR analytical specificity was confirmed by testing 16 specimens of other apicomplexan DNA extracted from a range of hosts, analytical sensitivity (detection limit) was determined using DNA dilution series and diagnostic sensitivity was determined by comparing PCR data and histological data for 75 flat oysters infected with APX at varying severities estimated by histology.

3.2 Materials and methods

3.2.1 Samples

Oysters (n = 75) were collected from Foveaux Strait, New Zealand. After measuring oyster length and cleaning off excess external shell debris, each oyster was opened using a sterilised knife and a transverse section (3-5 mm thick) containing gill, mantle and visceral mass was cut and stored frozen at -70°C in a 5 ml tube. For histology, another comparable tissue section was placed into a labelled histology cassette and fixed for 48 h in 4% formaldehyde in seawater before transfer to 70% ethanol.

3.2.2 Histology

Fixed oyster specimens were processed and tissue sections were stained with hematoxylin and eosin at Taranaki Medlab (New Plymouth, N.Z.). As described by Gagné et al. (2015) with some modifications, each tissue section was viewed at low magnification (100 to 200×) to confirm specimen quality, at intermediate magnification (400×) to detect lesions, host responses and the presence of other pathology, and at high magnification (1000×) to detect APX. Under high magnification, 25 fields of both visceral mass (digestive gland, gut and

connective tissue) and gill tissue were examined. Each field-of-view was graded from 0 to 3 according to Hine (2002a) with some modifications, Grade 0 = no APX observed; Grade 1 = light infection (<5 parasites); Grade 2 = moderate infection (parasites easily observed, particularly around the haemolymph sinuses); Grade 3 = heavy infection (parasites abundant in all tissues especially among and within connective tissues, and with many lesions evident). An oyster was assigned at Grade 0 if there is no APX in all 25 examined fields of view, Grade 1, Grade 2 and Grade 3 if at least 19/25 (90% confidence level and 10% margin of error) fields of view were found with light infection, moderate infection and heavy infection, respectively. Tissue sections were viewed three times to ensure grading consistency.

3.2.3 PCR primer sequences

The APX 18S rRNA PCR test primers APX-For (5'-TCTTTGAGTGAGAATCCGGTTTG-3') and APX-Rev (5'-GTGAGTCGAGAACAAAGAACATTC-3') were designed to target unique sequences of specific ISH-APX-Pr01 and ISH-APX-Pr02 *in situ* hybridisation probes identified in alignments of APX 18S rRNA gene to 18S rRNA gene sequences of the flat oyster, Pacific oyster (*Crassostrea gigas*), *Bonamia ostreae* and *Bonamia exitiosa* (Figure 3.1). Each primer sequence was also subjected to an independent Basic Local Alignment Search Tool (BLAST) search to help ensure specific amplification of APX DNA.

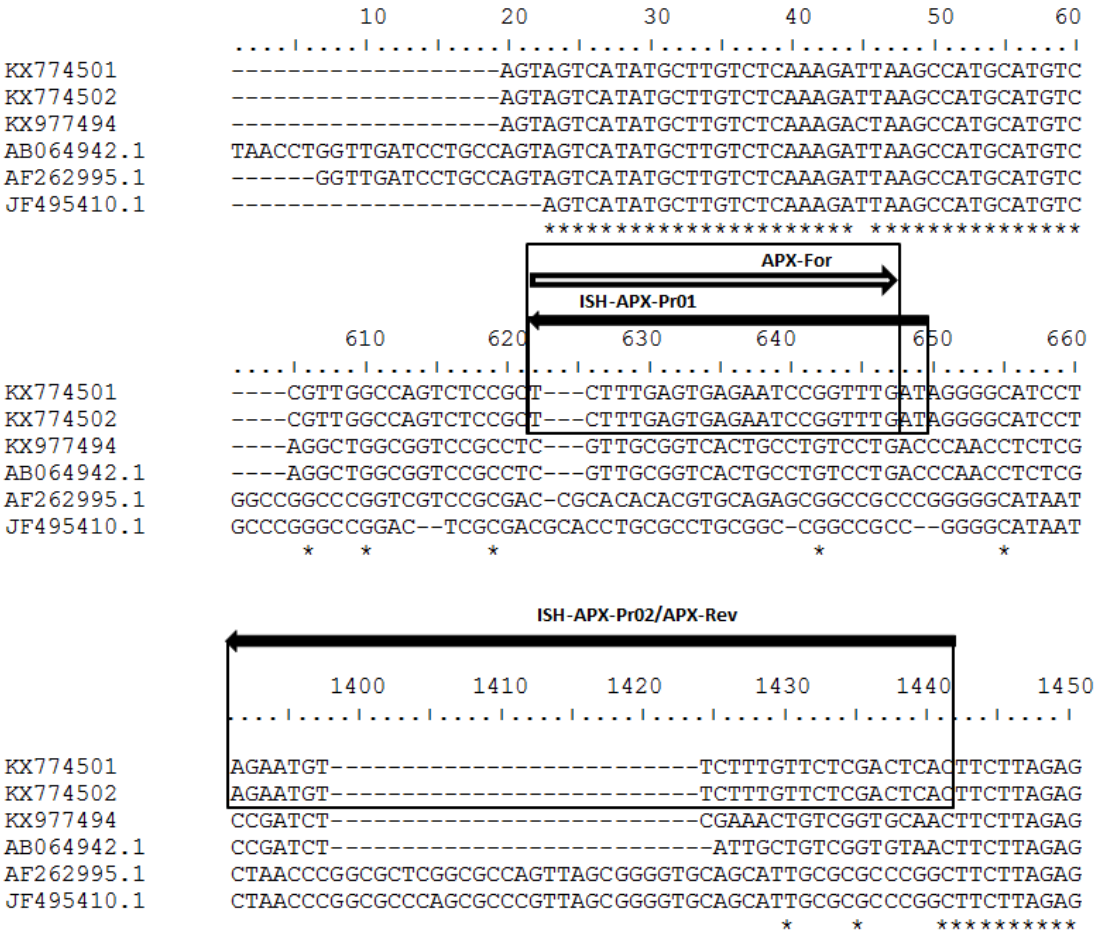


Figure 3.1 Location of PCR primers APX-For and APX-Rev based on *in situ* hybridisation probes ISH-APX-Pr01 and ISH-APX-Pr02 that specifically bound to apicomplexan-X (APX) cells within infected flat oyster tissues. Two *in situ* hybridisation probes (ISH-APX-Pr01 and ISH-APX-Pr02) were designed based on two regions discriminating the APX 18S rRNA sequences (KX774501, KX774502) from the flat oyster (KX977494), Pacific oyster (AB064942), *Bonamia ostreae* (AF262995) and *Bonamia exitiosa* (JF495410) partial 18S rRNA sequences. Note bases 61 – 599 and 661 – 1390 are omitted for clarity.

3.2.4 DNA extraction and PCR

DNA was extracted from 20 to 25 mg flat oyster gill and digestive gland tissue using a commercial kit (Zymo Genomic DNA Tissue Mini Prep Kit) following the manufacturer's instructions. Each PCR (20 µl final volume) was comprised of 10 µl 2 × MyFi Mix (BioLine), 10 pmoles of each APX-For and APX-Rev primer and either 1 µl template DNA (150 ng) or ultra-pure distilled water (Invitrogen) as a no-template control. Thermal cycling conditions were 95°C for 2 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s; 72°C for 5 min and hold at 15°C. Amplified DNA (723 bp) was resolved in a 1% (w/v) agarose gel containing 0.5 µg ml^{-1} ethidium bromide and visualised using a UV transilluminator. Any PCR product of the expected size (723 bp) was purified using the Nucleospin® gel and PCR clean-up kit (Macherey-Nagel), ligated into pGEM-T Easy vector (Promega) and pDNA clones containing inserts were sequenced by an external contractor (Massey Genome Service). All sequences were used to interrogate GenBank for similar sequences using BLAST.

To determine the analytical sensitivity of the PCR test, the primer set 3011For1 (Suong et al. 2017) SSUR4 (Wakeman 2013) was used to produce a 1296 bp APX 18S rRNA gene DNA product. The 1296 bp APX 18S rRNA gene DNA product amplified by PCR was purified as above, quantified by UV spectrophotometry and diluted to a concentration of 100 ng $µl^{-1}$ in sterile distilled water. This DNA was used to prepare serial 10-fold dilutions down to 1 ag $µl^{-1}$ that were then mixed 1:1 with DNA (150 ng $µl^{-1}$) from a flat oyster confirmed to be APX-free by both PCR and histology. DNA dilutions were then amplified in triplicate with the APX 18S rRNA PCR test primers APX-For and APX-Rev, and a 10 µl aliquot of each 20 µl PCR was analysed by gel electrophoresis. The lowest dilution 0.1 fg $µl^{-1}$ generating a visible 723 bp DNA band in all 3 replicate reactions was converted to dsDNA copy number based on the sequence length or mass using DNA/RNA copy number calculator tool (http://www.endmemo.com/bio/dnacopynum.php; accessed 28 Jan 2018). As two or more

criteria generally need to be met to diagnose a pathogen with confidence (Ramis et al. 2012), the diagnostic sensitivity and specificity of the PCR test was estimated based on histology as the reference method.

To examine PCR test analytical specificity, DNA extracted from 16 apicomplexan species spanning 6 genera were tested (Table 3.1). The generic apicomplexan PCR primers PF1 (5'-GCGCTACCTGGTTGATCCTGCC-3') and SSUR4 (5'-GATCCTTCTGCAGGTTCACCTAC-3') and the PCR reaction and cycling conditions described above was used to confirm the presence of apicomplexan DNA.

To identify PCR inhibitors in the four DNA extracts that were APX PCR negative but positive for APX infection by histology, 2 µl template DNA (150 ng μl^{-1}) was mixed with 2 µl salmon DNA (100 µg ml^{-1}). This mixture was then amplified in a 10 µl real-time quantitative PCR (qPCR) prepared using Express qPCR Super Mix (Invitrogen), 1 µl 10 mg ml^{-1} PCR primers Sketa F3 (5'-GGTTTCCGCAGCTGGG-3') and Sketa R2 (5'-CCGAGCCGTCCTGGTCTA-3'), 0.2 µl hydrolysis probe RoxIB®RQ (5'-AGTCGCAGGCGGCCACCGT-3') and 3.8 µl sterile distilled water. DNA was amplified using a Rotor-Gene Q thermal cycler (QIAGEN) and the thermal cycling conditions 50°C for 2 min, 95°C for 2 min and 40 cycles of 95°C for 15 s, 60°C for 1 min. Quantification cycle numbers were calculated by measuring fluorescence at 610 nm after excitation at 585 nm. Salmon DNA (2 µl) and water were amplified as positive and no-template controls, respectively. To see if the effect of inhibitors in the four DNA extracts could be mitigated, PCRs for APX were repeated using DNA templates (150 ng μl^{-1}) diluted to 1:10, 1:30, 1:50 and 1:100 with sterile distilled water respectively (Schrader et al. 2012), and by adding bovine serum albumin (BSA) to each PCR at the final concentration of 0.4 µg μl^{-1} (Carnegie et al. 2006).

Table 3.1 Apicomplexan species included in this study to test the specificity of PCR primers developed to detect APX. +: amplification, -: no amplification detected. The APX specific primer set APX-For/APX-Rev amplified a 723 bp portion of the APX 18S rRNA gene, the apicomplexan generic primer set PF1/SSUR4 amplified a 1795 bp portion of apicomplexan 18S r RNA gene.

Species	Source	APX-For/APX-Rev	PF1/SSUR4
Toxoplasma gondii	Patient - ADHBC	-	+
Toxoplasma gondii	Vaccine Toxovax® - MUC	-	+
Neospora caninum	Pure culture - MUC	-	+
Selenidium sp.1	Sabellidae - HKUC	-	+
Selenidium sp.2	Sabellidae - HKUC	-	+
Selenidium sp.3	Cirratulidae - HKUC	-	+
Selenidium sp.4	Terebellidae - HKUC	-	+
Cephaloidophorida sp.1	Crustacea - HKUC	-	+
Cephaloidophorida sp.2	Crustacea - HKUC	-	+
Cephaloidophorida sp.3	Crustacea - HKUC	-	+
Lecudina sp.1	Nereididae - HKUC	-	+
Lecudina sp.2	Nereididae - HKUC	-	+
Lecudina sp.3	Nereididae - HKUC	-	+
Lecudina sp.4	Nereididae - HKUC	-	+
Lecudina sp.5	Nereididae - HKUC	-	+
Thiriotia sp.	Crustacea - HKUC	-	+
APX	Fat oysters	+	+

ADHBC: Auckland District Health Board collection (New Zealand); MUC: Massey University collection (New Zealand); HKUC: Hokkaido University collection (Japan)

3.3 Results

PCR test sensitivity and specificity

As determined from gel analyses of the 723 bp DNA product generated using serial 10-fold dilutions of purified PCR product, the APX 18S rRNA gene PCR test reliably detected down to 0.1 fg DNA (Figure 3.2, lane 11), corresponding to 135 gene copies. Of the 75 DNA extracts from frozen flat oyster digestive gland and gill tissue samples, 723 bp DNA products were amplified from 41/41 Grade 1, 23/23 Grade 2, 5/9 Grade 1 and 0/2 Grade 0 (APX-negative) oysters as graded by histology (Hine 2002a). Real-time PCR testing the four DNA extracts that

were APX PCR-negative after adding salmon DNA using the salmon-specific Sketa F3:R2 primer pair indicated the presence of PCR inhibitors (data not shown). While fluorescence was clearly generated after ~25 cycles when salmon DNA was amplified alone, when mixed with any of the four PCR-negative oyster samples scored as Grade 1 for APX by histology, no fluorescence was evident up to 40 cycles. Neither further dilution of the 4 DNA templates nor adding BSA to the PCR tests resulted in detectable fluorescence.

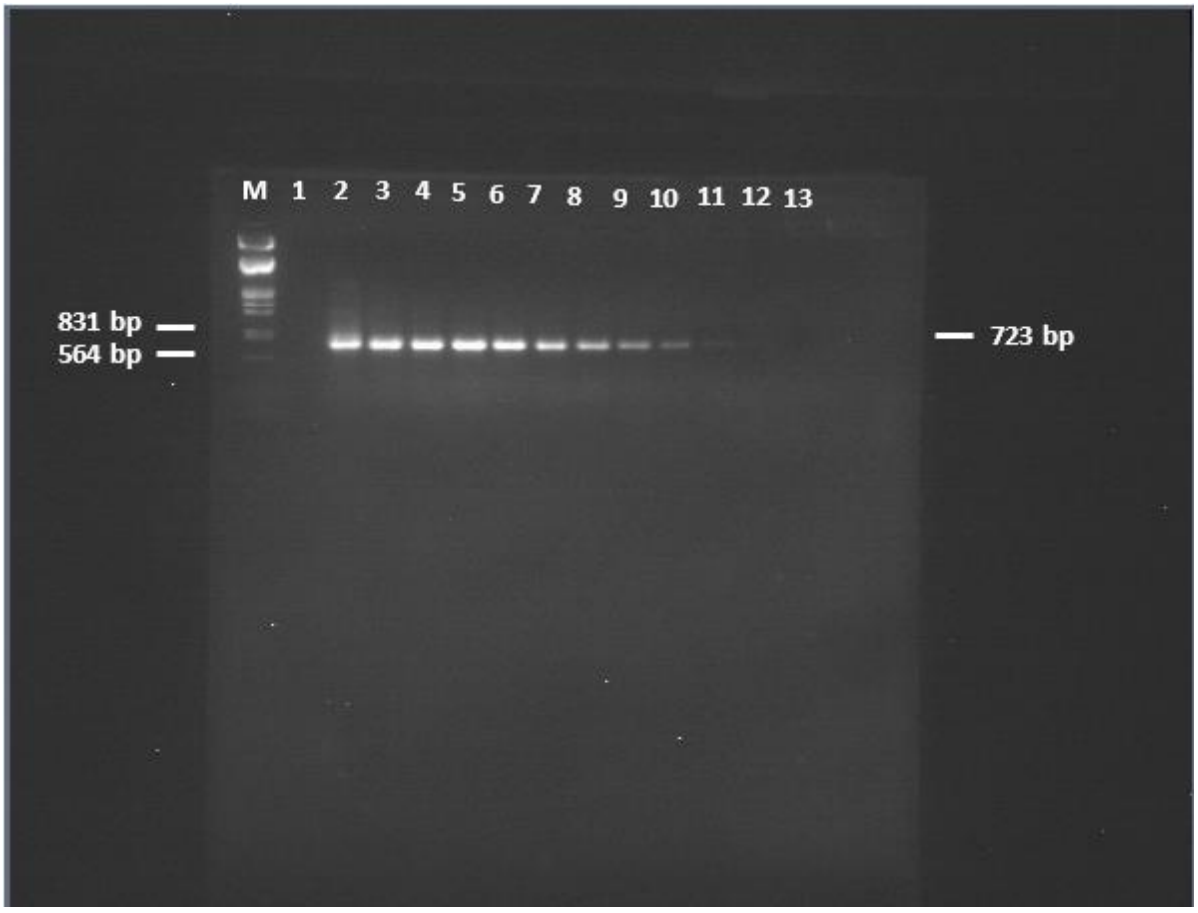


Figure 3.2 Detection limit of the PCR primer pairs APX-For/APX-Rev for detection of apicomplexan-X (APX). Lanes: M, PCR ladder (564-21226 bp, Thermo Scientific™); 1: negative control (water); 2-13: serial 10-folds dilutions of APX DNA ranging from 100 ng μl^{-1} to 1 ag μl^{-1}. Numbers on the left indicate sizes of molecular markers. The number on the right indicates the size of the amplified product.

The APX PCR test did not amplify a DNA product using DNA extracted from any of the 16 apicomplexan species tested that spanned six genera (Table 3.1, data not shown), thus supporting the specificity of the APX 18S rRNA test. In addition, purified APX PCR products from positive samples were invariant compared to the APX 18S rRNA gene sequence deposited in GenBank for which the PCR primers were designed (KX774501; Suong et al. 2017).

3.4 Discussion

To date, APX detection has relied on laborious methods such as histology using hematoxylin and eosin and periodic acid-Schiff stains, or transmission electron microscopy (Hine 2002a). Such methods are also less informative when a specimen has died and is decomposing or has been frozen. Another drawback is their potential to misidentify morphologically similar apicomplexan species such as the apicomplexans detected in green-lipped mussel (Diggles et al. 2002), Pacific oysters and blue mussels (S. C. Webb unpubl. data).

To address these diagnostic problems, a PCR test specific to the APX 18S rRNA gene was designed and assessed. Test analytical specificity was promoted by designing the PCR primers to 18S rRNA gene sequences differing substantially, particularly at their 3'-ends, from cognate sequences of both oysters and *Bonamia* spp. (Balboa et al. 2011), and by including C and G residues at the primer 3'- ends to further destabilise interactions with mismatched nucleotides (Benita et al. 2003, Balboa et al. 2011, Lorenz 2012).

The test detection limit was 135 APX gene copies per 1 µl primer, and PCR data correlated well with histological grading of APX infection loads in clinical samples (Hine 2002a). Of the 4/9 Grade 1 clinical samples that tested PCR-negative for APX, qPCR testing for salmon DNA in the presence of oyster DNA identified the co-extraction of PCR inhibitors to be a likely cause of the APX PCR test failing to detect the low concentrations of APX DNA in these samples. Such false-negatives due to PCR inhibitors in DNA extracts of shellfish such

as oysters have been associated with high concentrations of polysaccharides such as glycogen in some tissue types (Atmar et al. 1995, Schrader et al. 2012), and with bivalve molluscs may also arise through filter-feeding seston (Schrader et al. 2012). Since dilution of DNA template or addition of BSA to the PCR test did not improve the analytical sensitivity, further work to identify the inhibitory substances and exclude them during DNA extraction might help enhance the PCR test robustness in detecting APX in low-grade infections.

Described here is the first PCR test to specifically detect the apicomplexan-X (APX) parasite found in flat oysters in New Zealand. Benefits anticipated from use of the test include increased speed of detection as well as the ability to confirm histology-based diagnosis, characterise genetic variants and potentially differentiate APX zoites within and among bivalve hosts. The PCR test should also assist in finding the thus-far unrecognised non-zoite life stages of APX, if such stages exist.

CHAPTER 4: PCR AND HISTOLOGY IDENTIFIES NEW BIVALVE HOSTS OF APICOMPLEXAN- 'X' (APX): A COMMON PARASITE OF THE NEW ZEALAND FLAT OYSTER (*OSTREA CHILENSIS*)

4.1 Introduction

The phylum Apicomplexa is a group of more than 6,000 obligate protozoan parasites that cause morbidity and mortality in a wide range of vertebrates and invertebrates (Cova et al. 2018). Shellfish such as scallops, clams, cockles, mussels and oysters host a wide range of apicomplexans (Azevedo & Matos 1999, Hine 2002a, Uddin et al. 2011, Abdel-Baki et al. 2012, Kristmundsson et al. 2015). Some apicomplexan transmission stages may be found free in coastal waters and incidentally accumulate in bivalve tissues through particle-feeding processes (e.g., *Toxoplasma gondii*) (Ben-Horin et al. 2015). When introduced to a naive host population, some apicomplexan species cause little or no apparent negative effects in bivalve hosts (e.g., *Nematopsis* sp.) (Azevedo & Matos 1999), while others have had a significant impact on shellfish species, due to the morbidities and mortalities they cause (e.g., *Aggregata* sp. in the Iceland scallop *Chlamys islandica*) (Kristmundsson et al. 2015).

Apicomplexan - 'X' (APX) is an endemic parasite of the flat oyster (*Ostrea chilensis*) in New Zealand (NZ). The infective stages of APX in flat oyster are zoites and other stages such as oocysts, sporocysts have never been observed (Hine 2002a). The zoites are elongated and elliptical in outline (about 8 µm long and 5 µm wide) with a round nucleus halfway down their length that occupies almost the entire width of the cell. Transmission electron microscopic examination of APX zoites has revealed the typical features of an apicomplexan including two polar rings, subpellicular microtubules, a conoid, rhoptries and micronemes (Hine 2002a).

In *O. chilensis*, the mode of infection with APX-zoites is thought to be via division and growth within the haemocytes, until the haemocyte energy reserves are depleted and haemocyte

lysis occurs (Hine & Jones 1994, Hine 2002a). Histopathological changes have been associated with different APX infection intensities, causing varying degrees of pathology in oyster organs. In heavy infections of APX, parasites and haemocytes crowded around the gonads, gut and digestive diverticulae, often associated with the destruction of connective tissues and gonad follicles (Hine 2002a). Oysters infected with APX appeared smaller, thinner and watery, compared with uninfected oysters of the same shell size (Hine 2002a). APX is also strongly suspected of increasing the susceptibility of oysters to *Bonamia exitiosa* by destroying haemocytes and vesicular connective tissue cells, and depleting host glycogen reserves (Hine 2002a). Mass mortality of commercially important populations of *O. chilensis* of up to 91% has been reported in dual infections with *B. exitiosa* and APX (Hine 2002a).

Zoite stages of an APX-like organism have been reported from green-lipped mussels (*Perna canaliculus*), a commercially-important species (Diggles et al. 2002), and observed in Mediterranean mussels (*Mytilus galloprovincialis*) and hairy mussels (*Modiolus areolatus*) (S. C. Webb unpubl. data) in New Zealand. In *P. canaliculus*, heavy APX infections have been associated with focal destruction of vesicular connective tissue cells (Diggles et al. 2002, Hine 2002b). If APX in flat oysters and APX-like zoites found in green-lipped, Mediterranean and hairy mussels are the same species, shared susceptibility could complicate any disease control measures. Since morphological traits can be inadequate for distinguishing many apicomplexan species, genotypic comparison is an essential requirement for delimiting parasite ranges (Sabat et al. 2013). Additionally, information regarding the prevalence of APX in other bivalve hosts is limited.

The 18S rDNA has been reported as a reliable marker gene for apicomplexan identification as it contains both highly variable and conserved regions (Leander et al. 2003, Rueckert et al. 2015). In addition, evolution progresses are much slower in the 18S rRNA gene in compared with Cytochrome c Oxidase Subunit I (COI) gene, making the 18S rRNA gene

potentially a more informative marker for delimiting closely related species (reviewed by Renoux et al. 2017). Recently, a specific PCR and sequencing protocol has been established to target APX 18S rRNA gene sequence in flat oysters (Suong et al. 2018). Therefore, the aim of this study is to confirm the identity of APX and estimate prevalence of infection in green-lipped, Mediterranean and hairy mussels, by applying both histological and molecular methods., by applying both histological and molecular methods.

4.2 Materials and methods

4.2.1 Sample collection sites and histology

Cultured adult green-lipped mussels were sampled from the Cawthron Aquaculture Park, Nelson (n= 99, mean length = 136.1 ± 7.9 mm) in 2016 and from a mussel farm site near Coromandel Peninsula (n=30, mean length = 100.2 ± 7.2 mm) in 2017. Adult wild Mediterranean mussels were sampled from the Cawthron Aquaculture Park, Nelson (n=258, mean length = 69 ± 8.5mm) in 2014; Foveaux Strait (n=150, mean length = 34.3 ± 9.2 mm) in 2015; and Golden Bay (n=17, mean length = 77 ± 22 mm) in 2017. Adult wild hairy mussels were sampled from Foveaux Strait (n=30, mean length = 51.7 ± 5.5 mm) in 2018 (Figure 4.1). One histology slide contained gill, mantle, gonad and digestive gland was prepared from each mussel and stained with hematoxylin and eosin following the methods outlined in Howard et al. (2004). The remaining tissues were kept frozen at -70 ^{o}C. Histology slides were screened for presence or absence of APX-like organisms under an Olympus BX51 compound light microscope at 100× magnification.

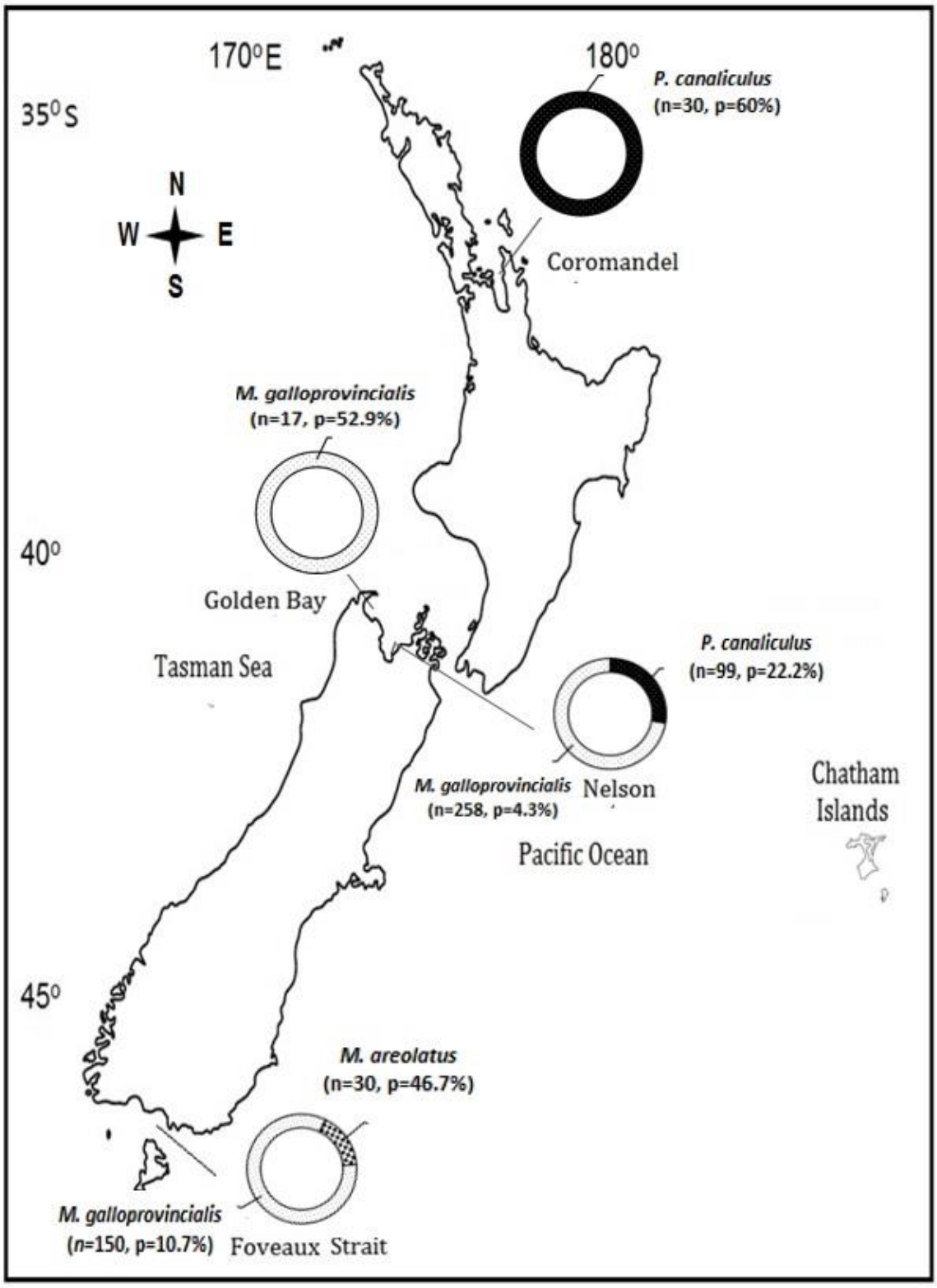


Figure 4.1 Map of New Zealand showing the sample collection sites Coromandel, Golden Bay, Nelson and Foveaux Strait. The circles represent the proportion of each mussel species examined for Apicomplexan-X collected at each site.

4.2.2 DNA extraction and PCR amplification

Total genomic DNA was extracted from a 25 mg composite of frozen digestive gland and mantle tissue of each bivalve using a Zymo Genomic DNA Tissue Mini Prep Kit, (Zymo Research) following the manufacturer's instructions. For detecting the APX DNA in the host-tissues, a nested PCR approach was used. The apicomplexan primers 3011For1 (Suong et al. 2017) and SSUR4 were first employed and the amplified products were then used as a template in a second PCR reaction to amplify a 723 bp APX-specific DNA fragment with APX-For and APX-Rev primers (Suong et al. 2018). The 18S rRNA gene sequences (723bp) spanned the variable regions V2 to V7 as described by Renoux et al. (2017). Each PCR reaction (20 µl final volume) contained 10 µl 2 x MyFi Mix (BioLine), 10 pmoles of each primer, 1 µl (150 ng) template DNA and ultra-pure distilled water (Invitrogen). Thermal cycling conditions used were 95°C for 2 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s; 72°C for 5 min and hold at 15°C. Ultra-pure distilled water and DNA of flat oyster infected with APX were used as template in the negative and positive controls, respectively.

Following PCR, 10 µl of the PCR products were visualized on 1% agarose gel stained with Redsafe (iNtRON Biotechnology) at concentration of 0.02% under UV light. The presence of a single band of the appropriate size on the gels was considered a positive result.

4.2.3 Sequencing

To confirm that the nested-PCR products were APX, positive samples from each bivalve species from each sampling site were selected for direct DNA sequencing as recommended for a parasite in a new host and/or a new location (OIE 2011). All PCR products were purified using the Nucleospin® gel and PCR clean-up kit (Macherey-Nagel) before being sequenced directly in both forward and reverse directions using Massey Genome Service, NZ. The sequences were deposited on GenBank after the 3' and 5' primer sequences had been removed.

Sequences obtained were compared with sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990).

4.2.4 Phylogenetic analyses

The sequences generated from samples infected with APX-like zoites from cultured green-lipped mussels from Nelson and Coromandel, wild Mediterranean mussels from Nelson, Foveaux Strait, and Golden Bay, and wild hairy mussels from Foveaux Strait were aligned with four APX sequences previously isolated from flat oysters (GenBank accession numbers KX774501, KX774502, MH375571, MH375569) using ClustalW incorporated in MEGA version 6.06 (Tamura et al. 2013), forming the first data set of 26 sequences. These sequences were distributed into four groups based on host species (green-lipped mussels, flat oysters, Mediterranean mussels, hairy mussels) using the Select taxa and group function of MEGA version 6.06 (Tamura et al. 2013). Overall mean genetic distance of 26 APX isolates and intraspecific convergence of APX isolates within each host species, and interspecific APX distances among different host species, were calculated in Mega using a Kimura 2 parameter (K2P) model (Kimura 1980). Species identifications using genetic data are dependent on genetic distances among specimens. Kimura's two-parameter model (K2P) (Kimura 1980) is the de facto standard metric for computing these distances. Collins et al. (2012) have shown that differences in distance calculated by the best fitting model and the K2P model estimates were usually minimal, and importantly, identification success rates were largely unaffected by model choice even when interspecific threshold values were reassessed.

To determine the evolutionary relationship of the novel sequences generated in the present study, the first data set of 26 sequences were aligned with 25 additional sequences downloaded from GenBank from an array of aquatic apicomplexans (*Eimeria tenella*, *Colpodella tetrahymenae*, *Margolisiella islandica*, *Tridacna* hemolymph apicomplexan, *Colpodella pontica*, *Besnoitia besnoiti*, *Toxoplasma gondii*, *Cryptosporidium serpentis*,

Cryptosporidium parvum, *Colpodella edax*), Cercozoa (*Chlorarachnion* sp.), aveolates (*Alveolata* sp., *Stentor coeruleus*, *Chromera velia*), fungi (*Phytophthora megasperma*, *Hyphochytrium catenoides*), ciliates (*Paramecium tetraurelia*, *Sterkiella histriomuscorum*), Perkinsozoa (*Perkinsus atlanticus*), diatoms (*Cylindrotheca closterium*, *Chrysolepidomonas dendrolepidota*), and dinoflagellates (*Alexandrium catenella*, *Gonyaulax polyedra*, *Akashiwo sanguinea*, *Pfiesteria piscicida*), forming the second data set of 51 sequences. Phylogenetic trees for both data sets were estimated using the Maximum likelihood method with the Tamura-Nei model (Tamura & Nei 1993), and bootstrap support for each node was estimated from 1,000 pseudoreplicates in MEGA version 6.06 (Tamura et al. 2013). The tree was rooted with the diatom group.

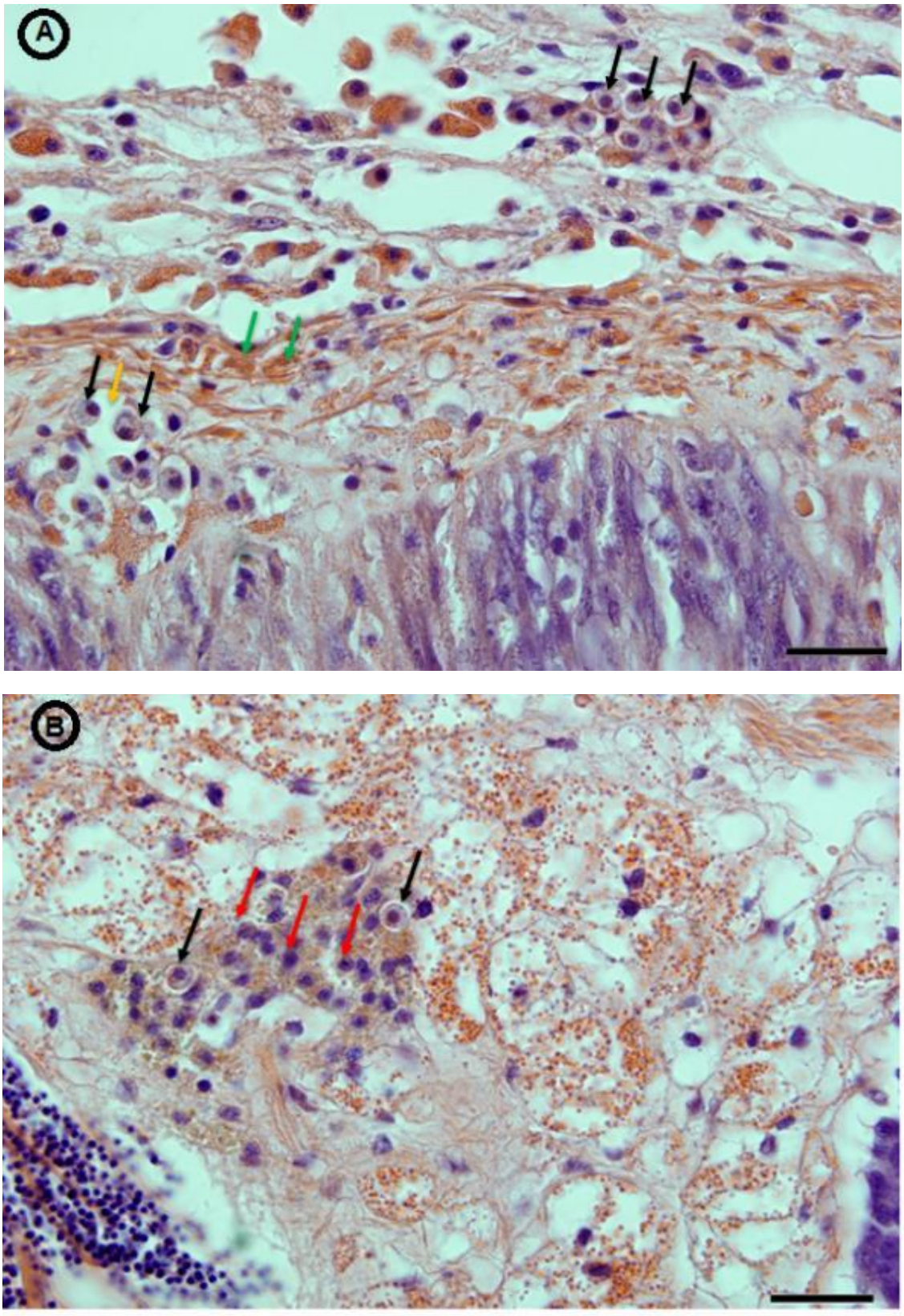
4.3 Results

4.3.1 Histology

Histological observations showed typical characteristics of APX-like zoites (e.g., indistinct nucleus and scattered glycogen granules (Hine 2002a)) in the connective tissues of all three bivalve species examined (Figure 4.2 A-D). The zoites were oval in shape with a width and length of 3.9-5.2 × 5.2-9.2 μm (n=70) in green-lipped mussels; 4.0-5.9 × 5.7-8.0 μm in Mediterranean mussels (n=30) and 3.9-5.7 × 5.2-8.4 μm in hairy mussels (n=30).

The prevalence of APX-like zoites estimated by histopathology was 22.2% (22/99) in cultured green-lipped mussels from Nelson, and 60% (18/30) from Coromandel. APX-like zoites occurred in wild Mediterranean mussels from Nelson, Foveaux Strait and Golden Bay at 4.3% (11/258), 10.7% (16/150) and 52.9% (9/17) respectively. In wild hairy mussels from Foveaux Strait, APX-like zoites occurred in 46.7% (14/30) of individuals. All histology-positive samples had only a very light level of infection with only a few APX-like zoites scattered or aggregated in small groups of 3-5 zoites, mostly in the connective tissue of the

digestive gland and mantle tissue. Histopathological changes were restricted to the presence of brown cells, the accumulation of haemocytes and detached connective tissues leaving voids in the tissue structure (Figure 4.2 A-B-D).


A
B

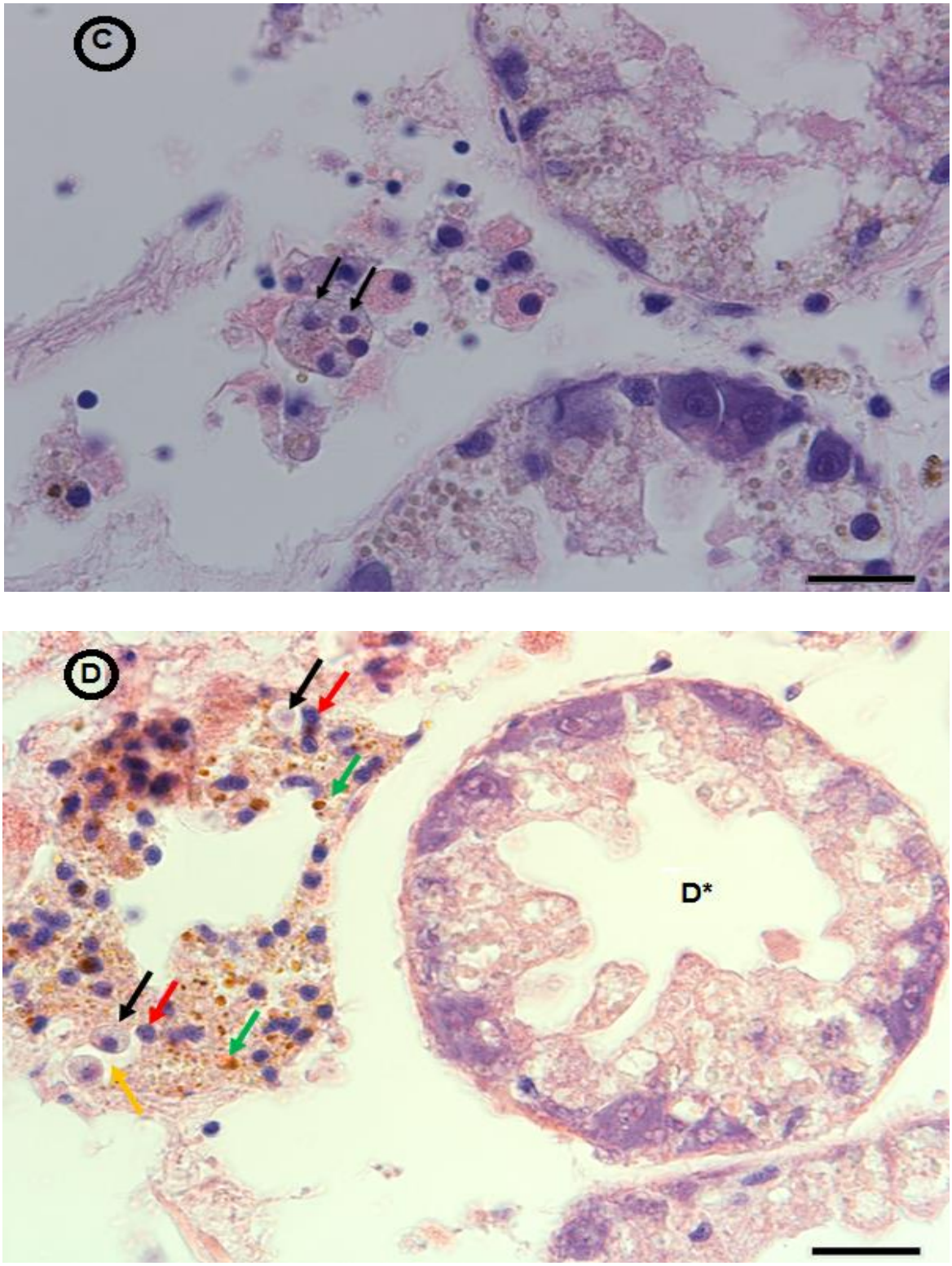


Figure 4.2 Histological sections of *P. canaliculus* (A-B), *M. areolatus* (C), and *M. galloprovincialis* (D). Black arrow: APX-like zoites, red arrow: the accumulation of haemocytes around APX-like cells, green arrow: brown cells, yellow arrow: empty space in the connective tissue, (D*): normal digestive tubule, scale bar: 10µm

4.3.2 PCR screening

PCR screening of the tissues using APX specific primers (APX-For and APX-Rev) amplified a product specific to APX (ca. 723bp) in specimens of green-lipped, Mediterranean and hairy mussels. The prevalence of APX infection estimated by PCR was at 22.2% (22/99) in cultured green-lipped mussels from Nelson, and 50% (15/30) from Coromandel. APX occurred as indicated by PCR in wild Mediterranean mussels from Nelson, Foveaux Strait and Golden Bay at 0.8% (2/258), 3.3% (5/150) and 35.3% (6/17) respectively. In wild hairy mussels from Foveaux Strait, amplification of APX DNA was achieved in samples from 46.7% (14/30) of individuals. All the PCR positive samples had positive histology results.

4.3.3 Sequencing

Sequencing representative samples from each bivalve species from each sampling site that were infected with APX-like organisms resulted in 22 sequences. Five sequences were obtained from cultured green-lipped mussels from Nelson (GenBank accession numbers MH375556, MH375557, MH375558, MH375559 and MH375560), and six sequences from Coromandel (GenBank accession numbers MH375551, MH375552, MH375553, MH375554, MH375555 and MH375564). One sequence was obtained from wild Mediterranean mussels from Nelson (GenBank accession numbers MH375573), two sequences from Foveaux Strait (GenBank accession numbers MH375572 and MH375570), three sequences from Golden Bay (GenBank accession numbers MH375550, MH375562 and MH375563). Five sequences were obtained from wild hairy mussels from Foveaux Strait (GenBank accession numbers MH375561, MH375565, MH375566, MH375567 and MH375568). A BLAST analysis found the sequences we obtained were 99%-100% similar to APX 18S rRNA gene sequences previously isolated from flat oysters (KX774501, KX774502, MH375571 and MH375569) with 99% to 100% query coverage (E value = 0).

4.3.4 Phylogenetic analyses

Analyzing the larger data set affirmed that all APX isolates from green-lipped mussels, Mediterranean mussels, hairy mussels and flat oysters belonged to the same group that formed sister relationships with three apicomplexan clades consisting of *Cryptosporidium serpentis* and *Cryptosporidium parvum*, *Margolisiella islandica* and Tridacna hemolymph, *Besnoitia besnoiti*, *Toxoplasma gondii* and *Eimeria tenella*, respectively (Figure 4.3). The overall mean genetic distance between the 26 APX isolates was 0.28%. By contrast, BLASTN search showed 18S rRNA sequences from APX and its closest sequenced relative *Colpodella edax* differed by 10% for the 1764 bp portion of the 18S rRNA gene.

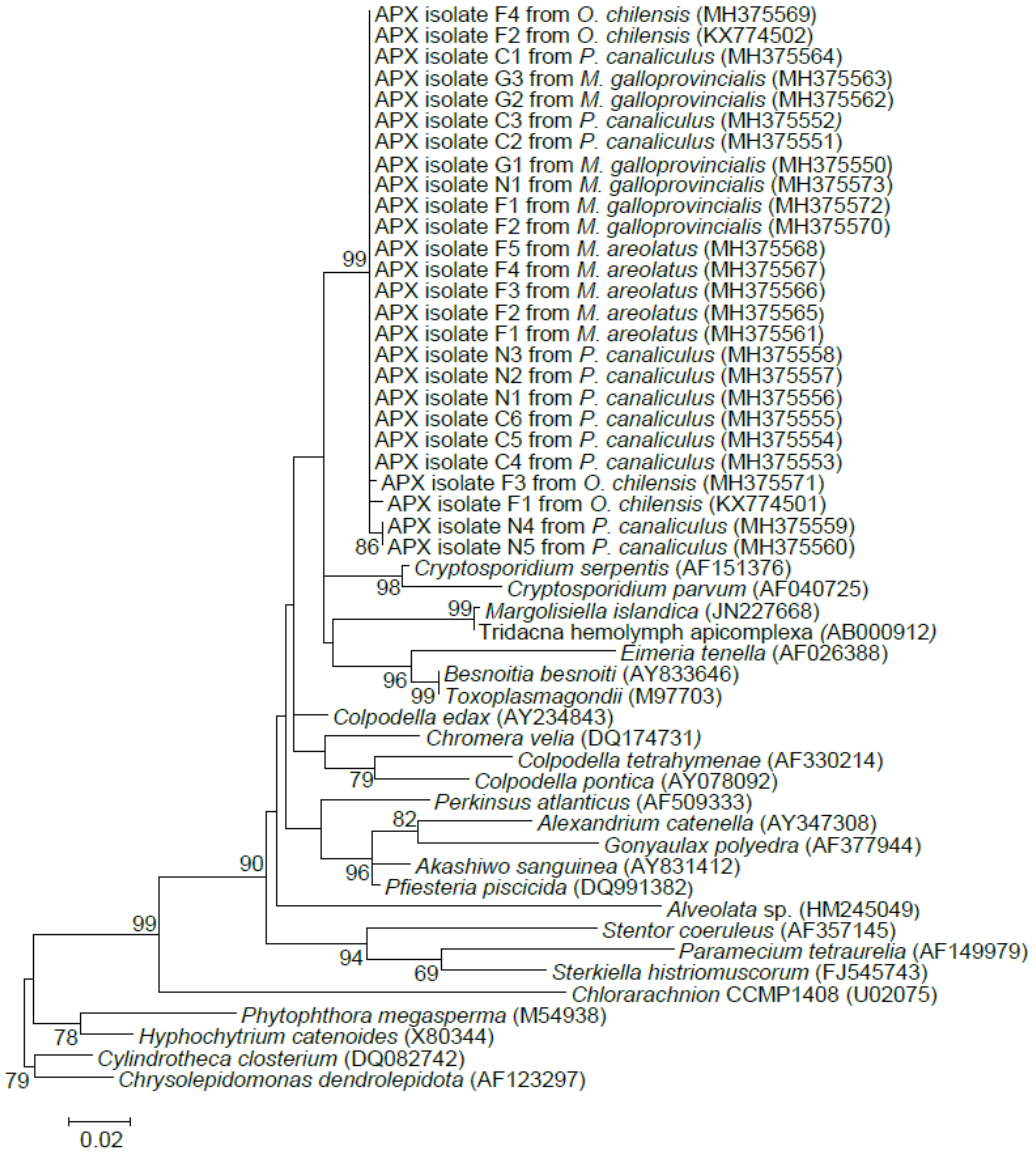


Figure 4.3 A phylogenetic tree showing the relationship of 26 parasitic Apicomplexan-X isolates to an array of aquatic apicomplexans, Cercozoa, Perkinsozoa, aveolates, fungi, ciliates, diatoms and dinoflagellates. The tree was inferred using the Maximum Likelihood method based on the Tamura-Nei model. Bootstrap support values > 50% (of 1000 replicates) are shown above the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach. The tree is drawn to scale, with branch lengths proportional to the number of substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6.

The maximum intraspecific divergence observed in APX isolates from Mediterranean mussels collected from Nelson, Foveaux Strait, and Golden Bay was 0.24% (Table 4.1).

Table 4.1 Pairwise genetic distances within each host group of APX isolates

APX host group	Mean APX pairwise 18S genetic distances within host species (%)
O. chilensis	0.22
M. galloprovincialis	0.24
P. canaliculus	0.23
M. areolatus	0.00

The maximum interspecific divergence was 0.6% between APX isolates from flat oysters collected from Foveaux Strait and APX isolates from green-lipped mussels collected from Nelson and Coromandel (Table 4.2). Phylogenetic analysis of the APX sequences found two clades, however, these clades were not correlated with geographic distributions and did not group according to host-species (Appendix 2).

Table 4.2 Pairwise genetic distances between APX isolates among host species (%)

APX host groups	APX host groups	Mean APX 18S pairwise genetic distances between host species (%)
O. chilensis	*M. galloprovincialis*	0.41
O. chilensis	*P. canaliculus*	0.60
M. galloprovincialis	*P. canaliculus*	0.24
O. chilensis	*M. areolatus*	0.56
M. galloprovincialis	*M. areolatus*	0.15
P. canaliculus	*M. areolatus*	0.12

4.4 Discussion

Apicomplexan – 'X' (APX) has significant negative impacts on the health of flat oysters (*Ostrea chilensis*) in New Zealand (Diggles et al. 2002, Hine 2002a). However, very little information is available concerning the host range of this parasite. The diagnosis of APX infection usually relies on histology, but molecular genetic-based tests hasten and improve the accuracy (i.e., lower the occurrence of false negatives caused by cryptic parasites) of the testing, and aid in determining the diversity of this parasite's host range. The findings of this study confirm the presence of an apicomplexan identified from histological examination in three important bivalve species; green-lipped mussels (*Perna canaliculus*), Mediterranean mussels (*Mytilus galloprovincialis*) and hairy mussels (*Modiolus areolatus*) that is genetically very similar to the APX of flat oysters. The mussel samples came from a range of geographic locations indicating a widespread distribution of APX.

The highest prevalence of APX was found in green-lipped mussels from Coromandel (50% by PCR and 60% by histology), and the lowest was in wild Mediterranean mussels from Golden Bay (0.8% by PCR and 4.3% by histology). Prevalence of APX also differed between mussel populations and varied with the year of collection. Higher prevalence of APX was observed in cultured green-lipped mussels in Nelson in 2016, while much lower number of APX was found in wild Mediterranean mussels in 2014, at the same place. Infection in wild populations might be a reservoir of infection for livestock (Guenther et al. 2011, Ward et al. 2013, Ruiz-Fons et al. 2014). Therefore, a higher prevalence might have been seen in wild green-lipped mussels in 2016 if the samples were collected. Direct comparisons between APX infection of wild versus reared green-lipped mussels are recommended in the future, as understanding the epidemiology of infection in wild populations might allow decision-making process to control such a hazard for cultured production (Ward et al. 2013). The lower prevalence of APX detected by PCR compared with histology can be explained by the very

low intensity of APX, in which a few cells were scattered in the host tissues and consequently could have been absent in the small amount (25mg) of tissue used for DNA extraction. Additionally, the analytical sensitivity of the PCR test was reported at 95% compared with histology while detecting APX in flat oysters (Suong et al. 2018) which could also contribute to the lower prevalence of APX detected by PCR in green-lipped, Mediterranean and hairy mussels that were examined. Traditional PCR offers the end products for DNA sequencing. However, an adaptation of the conventional PCR to quantitative real-time PCR (qPCR) which is expected to be more sensitive for the identification of APX DNA is necessary in the future. It has been reported that qPCR allows quantification of very low numbers of target genes, with detection limits as low as two copies of a gene (Smith & Osborn 2008).

To establish the species-level identity of the parasite, PCR products were sequenced and analyzed using a BLASTN search which showed 99%-100% similarity to APX isolates from flat oysters. In addition, the mean uncorrected pairwise genetic distances between the 26 sequences isolated from green-lipped mussels, Mediterranean mussels, hairy mussels and flat oysters was 0.28% suggesting that the APX sequences were closely related. Phylogenetic analysis also showed low divergence of 26 APX isolates with an average intraspecific sequence divergence range from 0.00% to 0.24 % (Table 4.2). The low divergence rate of APX isolates could be due to the conservative structure of the target region of the 18S rRNA gene sequences amplified with the specific primers. Identification of genetic regions such as the ITS regions on the ribosomal operon with more variation might be useful to rigorously test for APX differentiation across different hosts. However, it is also possible that this species has naturally low variability, as has been observed in some other Apicomplexan species that are less than 1% different at the gene sequence level (e.g. *Gregarina polymorpha* and *Gregarina niphandrodes*) (Leander et al. 2003). Low intraspecific sequence divergence (0.1–0.4 %) was also recorded in another apicomplexan, *Lankesteria hesperidii formis* (Rueckert et al. 2015).

The maximum-likelihood analysis grouped the 26 APX isolates into two distinct clusters that did not correspond with host species or geographic location. However, it is clear that all APX sequences isolated from flat oysters, green-lipped, Mediterranean and hairy mussels clustered in the same group with 99% bootstrap support (Figure 4.3).

APX zoites occurring in green-lipped mussels, Mediterranean mussels and hairy mussels were consistently at low intensity and associated with a mild host response, and there were no individuals with signs of more serious negative consequences from infection with APX. This contrasts with APX in flat oysters where APX zoites can be abundant and intensively affect their host (e.g., severe tissue damage, empty gonad follicles, Leydig cells dissociation and lysis, haemocytosis) (Hine 2002a). The low intensity of APX infection and mild host response in the three mussel species may be the outcome of intrinsic host factors such as differences in flat oyster and mussel innate immunity and physiology, parasite factors such as virulence, and the interaction of these two factors with environmental conditions, such as temperature and salinity (Soudant et al. 2013, Ben-Horin et al. 2015). Other apicomplexan species also have contrasting effects in different host species. For example, an unnamed apicomplexan in the genus *Aggregata*, has a high prevalence in the mature Iceland scallop (*Chlamys islandica*) and at high intensity causes severe damage to its host (e.g., necrosis of muscular and connective tissues, destroying adductor muscle, digestive gland and gonads), and triggers mass mortality (Kristmundsson et al. 2015). However, when found in king scallop (*Pecten maximus*) and queen scallop (*Aequipecten opercularis*) from UK waters, the same parasite has been found only at low intensity and did not cause any sign of disease to the hosts (Kristmundsson et al. 2011). It has been reported that APX zoites most often caused severe disease particularly in summer/autumn (January to April), during the peak spawning period, and peak prevalence and intensity of *Bonamia exitiosa* infection (Hine 2002a). This implies that mass mortalities are only triggered under some specific circumstances, and in the case of APX in flat oysters, may

be that a simultaneous infection with a second pathogen contributes to the morbidity caused by APX.

It is not known how APX has infected green-lipped, Mediterranean and hairy mussels. Parasite transmission rarely occurs through direct contact between bivalve hosts. In most cases, the transmission of bivalve parasites occurs by exposure to water-borne parasite stages through suspension feeding processes (Ben-Horin et al. 2015), and most protozoan parasites do not reproduce in the water column (Chu 1996). However, despite the extensive histopathological examination of numerous flat oyster and green-lipped mussel, Mediterranean mussel and hairy mussel individuals, only the zoite stage of APX has ever been observed, suggesting the potential secondary hosts of APX could be another taxon, such as polychaetes, as previously suggested by Hine (2002a).

That green-lipped, Mediterranean and hairy mussels are carrying APX is of concern as APX could be associated with morbidity and mortality in these species under certain conditions. Further research on biotic (e.g. host density, common pathogens, host developmental stages) and abiotic factors (e.g. temperature, pH, salinity) that could cause the parasite to proliferate and provoke an epidemic will help clarify the disease ecology and provide insights for establishing epidemic control and monitoring strategies.

This report confirms APX infection in New Zealand green-lipped mussels, Mediterranean mussels and hairy mussels highlighting its range of hosts and geographic distribution across New Zealand. In addition to seeking further bivalve hosts of APX, future work should also focus on the detection of other hosts supporting other stages in the APX life cycle. This will help elucidate its transmission mode and will ultimately assist in managing APX in affected host populations.

CHAPTER 5: GENERAL DISCUSSION

5.1 Summary

The main aim of this thesis was to greatly improve the knowledge of Apicomplexan-X (APX), a harmful apicomplexan parasite of the flat oyster (*Ostrea chilensis*) in New Zealand. Flat oyster samples that were confirmed by histology to be infected with APX were used for DNA extraction, followed by PCR amplification using generic apicomplexan primers and sequencing (0.6 kb). The sequence (0.6 kb) was used to develop primers that were specific for APX and thus allowed further extension for the 18S rRNA gene. The resulting sequences (~1.8 kb - GenBank accession numbers KX774501, KX774502) were confirmed to be of apicomplexan origin by assessing their taxonomic affinities with other sequences obtained from GenBank that represented major apicomplexan lineages as well as other major eukaryote groups. Two DNA-based *in situ* hybridisation probes ISH-APX-Pr01 and ISH-APX-Pr02 developed by aligning KX774501 and KX774502 with sequences of flat oysters, Pacific oysters, *Bonamia ostreae* and *Bonamia exitiosa* were then used to confirm that the sequences KX774501 and KX774502 were localised only in the APX cells visible using light microscopy (Chapter 2).

The highly conserved sequences of two *in situ* hybridisation probes also made them suitable for designing PCR primers for APX detection. Using Geneious software (version 11.0), two *in situ* hybridisation probes were adjusted to fit into a primer set for APX detection without common primer design constraints such as the GC content, primer length, melting temperature and hairpin structure. Ultimately this led to the development of a primer set for the specific detection of APX which was demonstrated to have high analytical and diagnostic sensitivity and specificity (Chapter 3). This newly developed PCR test was applied to confirm the presence and prevalence of APX in green-lipped mussels (*Perna canaliculus*), Mediterranean mussels (*Mytilus galloprovincialis*) and hairy mussels (*Modiolus areolatus*)

sampled from four locations around New Zealand (Chapter 4). In addition to providing an overview of the research results presented in this thesis, this current chapter discusses the limitations and practical implications of the results of the research and suggests possible future research directions.

5.2 Implications, limitations and future directions arising from the research

The most important discovery from the research presented in this thesis was the isolation and confirmation of Apicomplexan-X 18S rRNA gene sequences (accession numbers KX774501 and KX774502) (Chapter 2). Prior to these two 18S rRNA gene sequences being obtained, APX was commonly referred to as a coccidian based solely on the morphology of the zoite stage, so named because it was unclear whether they are sporozoites or merozoites (Hine 2002a). Analysis of the sequences confirmed the coccidian affinity of APX and revealed its broader taxonomic relationships. The APX sequences were unique, having not been previously submitted to GenBank, providing further hard evidence in support of APX from flat oysters in New Zealand being a unique taxonomic entity. In addition, a problem that can occur in parasite taxonomy is identifying the species to which morphologically differing life stages belong and this problem has often resulted in the misclassification of the various life stages of some parasites.

Support for the sequences being from APX was obtained using several independent approaches. Two *in situ* hybridisation probes ISH-APX-Pr01 and ISH-APX-Pr02 were designed from two regions discriminating the obtained sequences from those of flat oyster, Pacific oyster, *B. exitiosa* and *B. ostreae.* Microscopy showed that the *in situ* probes bound only to APX cells. However, as the APX 18S rRNA gene sequences isolated from flat oysters were ~1.8 kb and the APX 18S rRNA gene sequences isolated from green-lipped mussels, Mediterranean mussels and hairy mussels were ~ 0.7 kb, extension of these gene sequences

is recommended to understand structural variants in the APX genome (e.g., segmental duplications, gene loss and fusion events).

Future sequencing of the APX genome will allow a better understanding of the genetic variability of APX in the different host species. A useful endeavour would be to sequence the entire APX genome to allow researchers to gain deeper insights, such as identifying the types of proteins produced by APX, the link between APX phenotype and host disease, identifying the effect of changes in genes, and possibly helping to expedite the identification of potential drugs to treat infected hosts. Robust technology such as CRISPR/Cas9 system, a natural defence system of bacterial organisms that has been successfully used to alter other apicomplexan parasite genomes, such as *Plasmodium falciparum*, *Toxoplasma gondii* and *Cryptosporidium parvum*, may improve our understanding of pathogenesis and help in the development of effective drugs and treatment strategies against the parasite.

Another important outcome from the research presented in Chapter 2 is the augmentation of the genetic information of the flat oyster *O. chilensis*. Prior to this research, there were only two DNA sequences, each of ~ 0.9 kb (accession numbers EU660788, EU660789) of the oyster available in GenBank. The deposition of the 1.8kb sequence of *O. chilensis* from this work onto GenBank (accession number KX977494) will be useful for comparisons of *O. chilensis* genomes with those of closely-related species. In addition, the *in situ* hybridisation probes and protocol for APX that were developed in this study are powerful tools for future studies of APX pathogenesis. Applications of *in situ* hybridisation may include localising APX infection in whole animals during natural and experimental infections, identifying sites of APX gene expression, analysing mRNA transcription, and mapping gene sequences in chromosomes. *In situ* hybridisation can also be applied to archival materials, including frozen tissues, and can be combined with immunohistochemistry to detect APX protein and mRNA of interest.

As the morphological features of the zoite stage and the genetic identity of APX are now described, the next crucial step for describing the parasite formally and beginning to control it effectively, is to close its life cycle. However, achieving this goal is likely to be challenging because APX is likely to have a complex life cycle, with alternate stages manifesting as other forms and through a number of different host taxa, rendering morphological identification difficult (Hine 2002a). The use of a specific PCR test followed by sequencing can help to overcome this confusion by reliably identifying the presence of APX in other potential hosts, even when presenting as an alternate stage of development, most likely also with different morphology.

The PCR assay developed in this study based on unique sequences of two specific *in situ* hybridisation probes ISH-APX-Pr01 and ISH-APX-Pr02 has been demonstrated to have high specificity for detecting APX regardless of the life stage or morphology by which it presents (Chapter 3). Based on the APX 18S rRNA sequences, the conventional PCR could also be adapted to other PCR methods such as nested-PCR, quantitative real-time PCR and droplet digital PCR (dd PCR) which would be expected to be more sensitive for the identification of APX DNA and quantification of APX infection (nested-PCR, ddPCR). However, the specific PCR test developed in this current study has not been fully validated. Future research can focus on fully validating the test by running the test on a wider range of flat oyster samples including populations that have no history of APX infection (based on morphological diagnosis by microscopy).

The test does have some limitations. The diagnostic sensitivity of the test was 95%, as 5% of samples that were APX positive by histology were found to be PCR negative, most likely due to the presence of PCR inhibitors. While histology avoids the inhibitor problem, PCR enhances the ease and speed of diagnosis. Given that less than 1% of extant apicomplexans have been formally described (Lee et al. 2000, Morrison 2009), it is likely

many more species will be discovered. The molecular data published here potentially allow the differentiation of taxa that may be discovered and are morphologically similar to APX. Genetic information from APX will also shed light onto patterns of biogeography, host affinity and phylogenetic position of other species that are closely related to APX.

The small variation detected in APX 18S rRNA sequences showed no corresponding variation with host species or geographic origin (Chapter 4). This tentatively suggests that these APX are from a single reproductive pool and that APX is indeed a single species. Further molecular characterisation, life cycle studies and transmission experiments would be needed to confirm this. That APX has been seen only recently in the Mediterranean mussel and the hairy mussel is not surprising since they, being of low commercial importance, have been subject to much lower scrutiny. Indeed, if APX were not being actively sought, it is likely that it would have been overlooked in these mussels, as APX zoites are difficult to discern by light microscopy in low intensity infections.

It is not known whether APX can be transmitted between hosts of different taxa. In pathology terms, transmission of a pathogen takes place only if three factors occur simultaneously: (1) sufficient quantity of infectious pathogen with an effective mechanism for transport of the pathogen to the host; (2) transmission of the pathogen to the correct portal of entry of the host; and (3) susceptibility of the host to infection by the pathogen (Mast et al. 1995, Aiello et al. 2016, Shields et al. 2017). It is recommended here that more detailed investigations should be conducted on APX to understand its mode of infection. Laboratory and field investigations may help ascertain whether APX is transmitted horizontally, vertically or both. Further, it is necessary to ascertain how the transmission stage is released from the oyster and how long it can remain viable in the environment. Using the PCR method that we have developed in this study, a wider range of taxa should be screened to identify definitive, intermediate and other hosts. Host life histories (age, size, breeding condition,

geographical distribution) should also be integrated in modelling exercises to identify key abiotic and biotic factors that influence APX transmission stage viability.

High local diversity often decreases disease 'pressure' for focal host species, across a range of types of parasites and hosts (Keesing et al. 2006, Lagrue & Poulin 2015, Costello 2016). The wide range of APX hosts, similarly, could mitigate the effect of APX in flat oysters. However, the 'diluting' effect of species diversity is not ubiquitous and depends on many factors such as the nature of the other species in the community, study scale, or other idiosyncrasies of particular host-parasite relationships (reviewed by Lagrue & Poulin 2015). It has been proposed that a parasite can only have high host specificity if its host is abundant, widespread, or can easily be infected, otherwise, the parasite risks extinction (Costello 2016). Given the limited distribution of highly abundant populations of the APX type host (i.e., *O. chilensis*), the use of alternative hosts by APX may be a strategy to avoid extinction given the patchy abundance of flat oysters in the wild in both New Zealand and Chile.

Apicomplexans are also well-known for deploying sophisticated mechanisms to invade a wide range of hosts and cell types (Hakimi & Cannella 2011, Reid et al. 2012, Kemp et al. 2013). Some apicomplexans have been shown to have virulence that varies over time and for different host species (Weatherhead & Bennett 1992, Gupta et al. 2011). A typical example is *T. gondii* which is capable of infecting a number of rodents, terrestrial mammals (including humans), marine mammals and birds (Tenter et al. 2000, Miller et al. 2008, English et al. 2015, Palos Ladeiro et al. 2015). In animals such as wallabies, kangaroos, goats, chickens or pigs, *T. gondii* causes acute or chronic infection. In contrast, groups such as horses are highly resistant to toxoplasmosis (Jacobs & Melton 1966, Dubey & Jones 2008, Gisbert Algaba et al. 2018). Changes in levels of pathogenicity for different host species have also been observed for *Merocystis kathae*. This apicomplexan is apathogenic in the common whelk

Buccinum undatum, but highly pathogenic when infecting various scallop species (i.e. *Chlamys islandica, Placopecten magellanicus*) (Kristmundsson & Freeman 2018).

The variation of intrinsic factors (age or sex of host, host size, host reproductive effort, host condition, host genotype), extrinsic factors (geographical region, season, year), or the complex relation between them can often account for variation in parasite prevalence or intensity (Gupta et al. 2011, Kristmundsson et al. 2015). There is no clear evidence that the external environment or other host factors might favour the high prevalence and severity of APX infection in flat oyster. However, it is speculated that both *B. exitiosa* and APX were depleting the host glycogen reserves during an observed mortality event of flat oyster population (Hine 2002a). A recent report has showed a high infection prevalence of APX (76.5%) in a sample of 149 adult *O. chilensis* in New Zealand accompanied by infections of both *B. exitiosa* (2.7%) and *B. ostrea* (40.3%) (Lane et al. 2016). Given the relationship of *Bonamia* spp. in APX disease development and severity in flat oysters are unclear, closer analysis of the existing literature and further experiments are required to understand how APX interacts with bonamiosis as it may be crucial for providing a deeper understanding of its biology and epidemiology, as well as providing a possible route to more effective disease control.

The modest intensity of APX in hairy mussels, green-lipped mussels and Mediterranean mussels compared with flat oyster is intriguing. The recent advances in genomics and transcriptomics could offer powerful new tools for development of genetic markers associated with APX resistance which could be used in a breeding selection programme for *O. chilensis* through Marker Assisted Selection (MAS). Genomic data could also elucidate the host defence mechanisms of mussels against APX to provide some novel strategies for management of diseases in bivalve aquaculture.

Two other New Zealand hosts for APX-like zoites have recently been noted in histological samples: geoducks (*Panopea zelandica*) and Pacific oysters (*Crassostrea gigas*) (S. C. Webb unpubl. data). In the latter, if APX-like zoites are indeed APX, this could be problematic as the presence of APX may exacerbate the pathogenicity of *Bonamia* spp. in *C. gigas*, as it does in *O. chilensis* (Hine 2002a). *Bonamia ostreae* has been reported in Pacific oyster haemocytes (Hervio et al. 1989, Mourton et al. 1992), tissues and shell cavity fluid (Lynch et al. 2010) of this oyster which may be a potential carrier of both *B. exitiosa* and *B. ostreae* (Lynch et al. 2010). Clearly, further work is required to ascertain the genetic affinities of this 'APX' and to investigate its interactions with *C. gigas* and *Bonamia* spp.

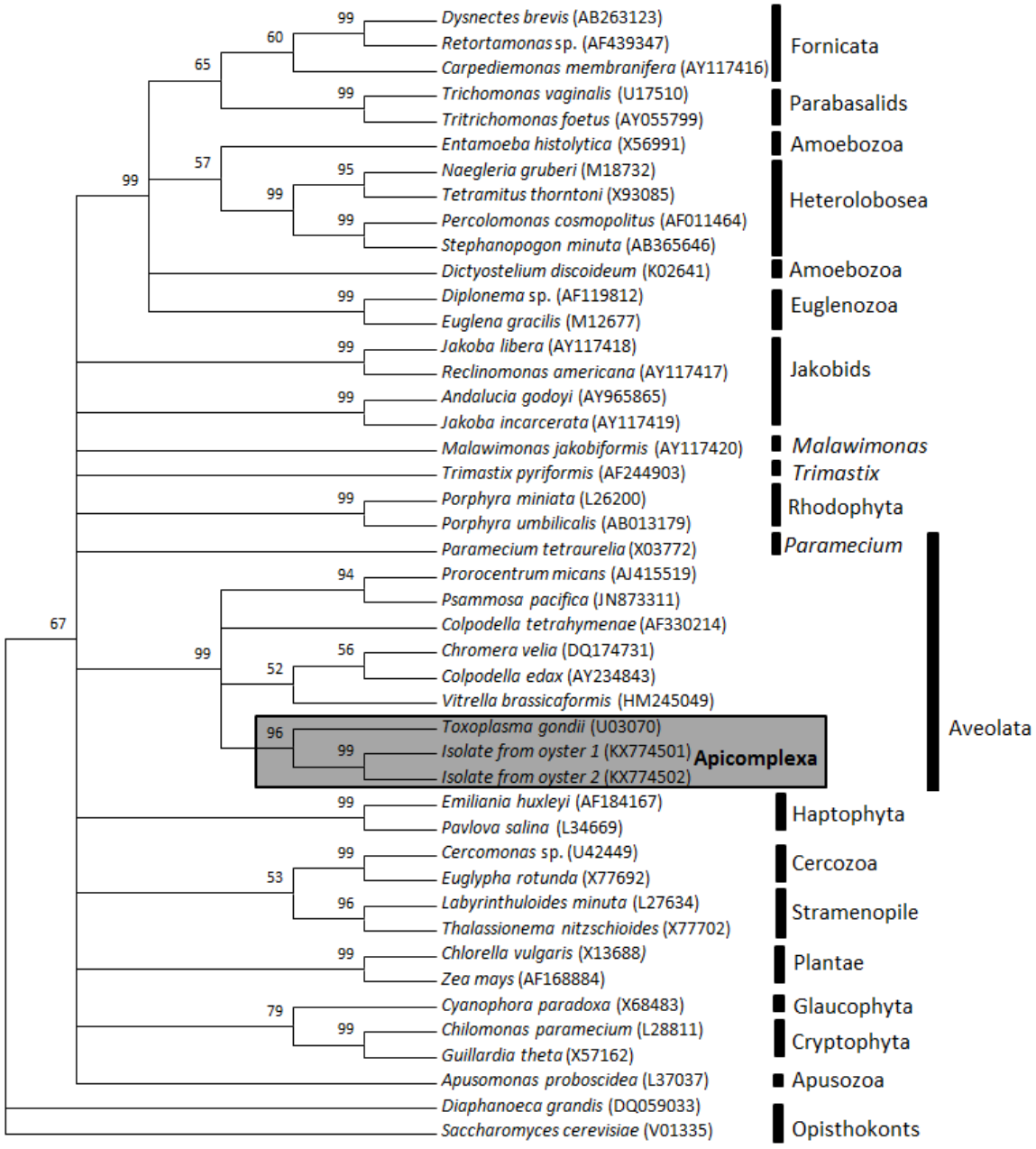
In summary, this study provides the molecular information and tools for detecting the parasite APX. The study results also revealed a greater geographic extent of APX and its presence in a much wider range of host species than previously anticipated. Existing evidence indicates that APX can have a significant contribution to mass mortality events in flat oyster stocks. However, it seems that APX infections remain at low density without causing harm or loss to other hosts including green-lipped, hairy and Mediterranean mussels. These recent findings add to our knowledge of the epidemiology of APX and reveal similarities to that of the apicomplexan *Aggregata* spp. described from Iceland scallops (Kristmundsson et al. 2015). *Aggregata* spp. appear to have episodic outbreaks that can be devastating at the population level (Kristmundsson et al. 2015). Furthermore, there is an indication that these apicomplexans are found across wide geographic areas, infecting a range of species including king scallop (*Pecten maximus*) and queen scallop (*Aequipecten opercularis*) but often present at modest levels of infection (Kristmundsson et al. 2011). It is unclear which factors could trigger these episodic outbreaks in Iceland scallops (Kristmundsson et al. 2011). A paucity of data is a major impediment to understanding the complicated interactions between bivalve hosts, the infectious apicomplexan and biotic and abiotic stresses in the aquatic environment.

Similar to the case of APX, further studies of the biology of APX should be conducted to manage a potential outbreak in any of these mollusc species in New Zealand. The molecular methods that have been developed in this study (PCRs and ISH to detect APX) will assist investigations into such issues.

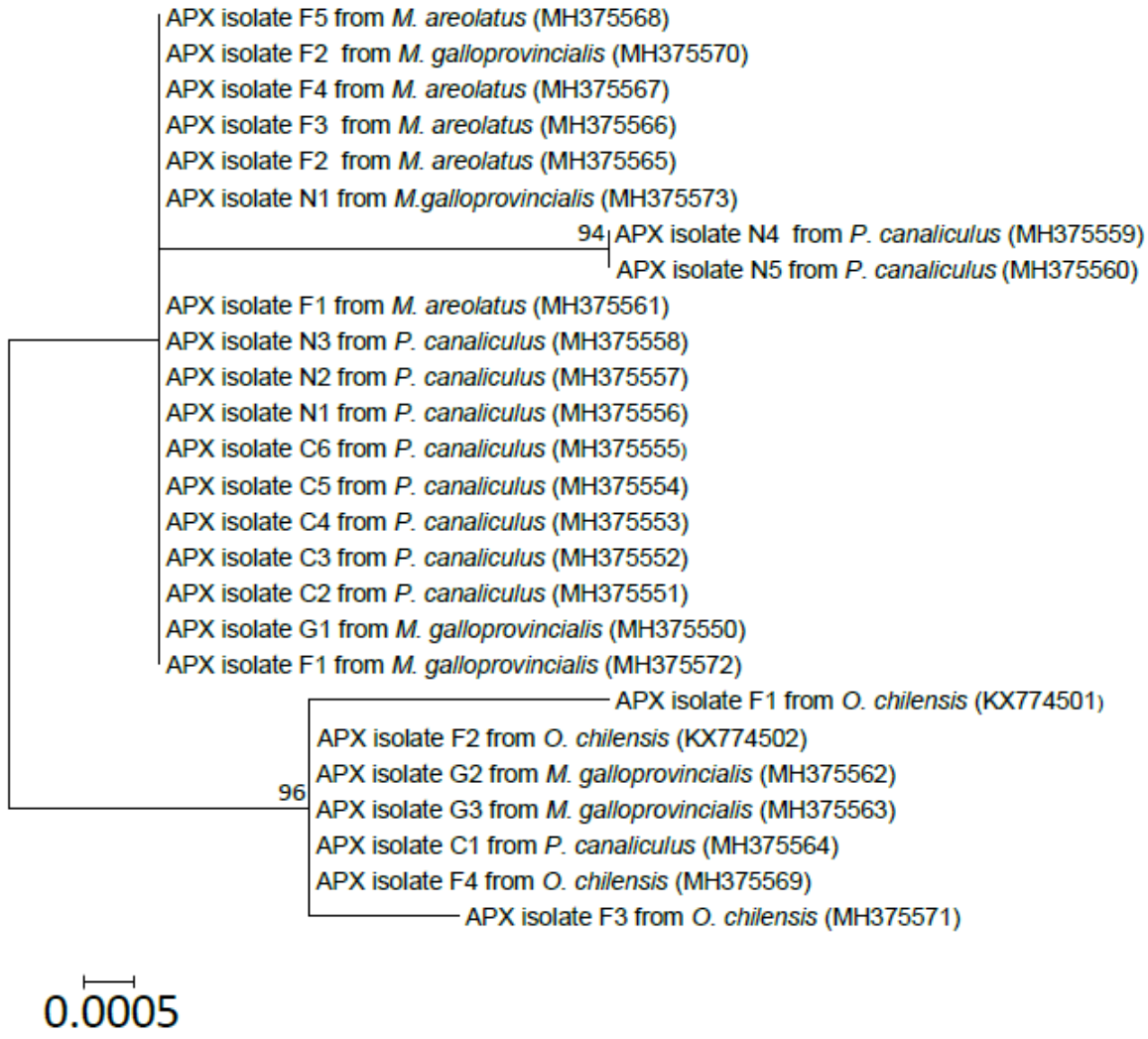
5.3 Conclusion

This thesis greatly advances our knowledge of APX by using molecular methods to confirm its taxonomic identity and its presence in a range of host bivalves. The molecular characterization of APX has created a novel and effective PCR tool to specifically and reliably confirm the presence of the parasite which has the potential to greatly improve the detection and management of the disease. This tool can also be used by researchers to advance understanding of the disease, such as identifying secondary hosts for APX, delineating the geographic range of the disease, and revealing its relationship with the etiology of bonamiosis in flat oysters. Future research, such as whole genome sequencing or determining the transmission route of the APX is recommended to further assist in managing APX in affected shellfish populations.

APPENDICES



Appendix 1. Phylogenetic position of two APX isolates from the flat oyster among diverse eukaryotes estimated from sequences for a portion of the 18S rRNA gene. The two sequences generated in this study from APX group with *Toxoplasma gondii* and are highlighted in the box. Numbers above the nodes are bootstrap support values > 50% (of 2000 replicates). GenBank accession numbers of all sequences used are shown in parentheses



Appendix 2. Phylogenetic relationships of 26 APX strains: F (Foveaux Strait), C(Coromandel), N(Nelson), G (Golden Bay) isolated from *O. chilensis*, *P. canaliculus*, *M. galloprovincialis* and *M. areolatus*. The tree was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model. Bootstrap support values are shown above the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach. Branch lengths are proportional to the number of substitutions per site.

LIST OF OUTPUTS

Published articles

- Suong TN, Banks JC, Webb SC, Jeffs A, Wakeman KC, Fidler A (2019) PCR and histology identify new bivalve hosts of Apicomplexan-X (APX): a common parasite of the New Zealand flat oyster *Ostrea chilensis*. Diseases of Aquatic Organisms 132:181-189
- Suong TN, Banks JC, Webb SC, Jeffs A, Wakeman KC, Fidler A (2018) PCR test to specifically detect the apicomplexan 'X' (APX) parasite found in flat oysters *Ostrea chilensis* in New Zealand. Diseases of Aquatic Organisms 129:199-205
- Suong TN, Webb S, Banks J, Wakeman K, Lane H, Jeffs A, Brosnahan C, Jones B, Fidler A (2017) Partial 18S rRNA sequences of apicomplexan parasite 'X' (APX), associated with flat oysters *Ostrea chilensis* in New Zealand. Diseases of Aquatic Organisms 127(1):1-9

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- Suong TN, Banks JC, Webb SC, Jeffs A, Wakeman KC, Fidler A (2018) Rapid and specific PCR assay for diagnosis of apicomplexan- 'X' (APX) associated with the flat oysters (*Ostrea chilensis*) in New Zealand. Proceedings of the 8th International Symposium on Aquatic Animal Health (p. 361), Charlottetown, PEI, Canada
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Awards

- Grant for project "Exploring Apicomplexan-X (APX) and *Bonamia* parasites in economically important shellfish fisheries" (2017-2018) New Zealand-Japan Joint Research Program (number: JSP-CAW1601-JR).
- NZMSS First Overseas Conference Travel Award (2018) for attending the 8th International Symposium on Aquatic Animal Health, Charlottetown, PEI, Canada.
- Student Travel Award (2016) for attending the 19th New Zealand Molecular Ecology Conference, Auckland, New Zealand.

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