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PCR and histology identify new bivalve hosts of Apicomplexan-X (APX), a common parasite of the New Zealand flat oyster *Ostrea chilensis*

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ABSTRACT: Apicomplexan-X (APX) is a significant pathogen of the flat oyster *Ostrea chilensis* in New Zealand. The life cycle and host range of this species are poorly known, with only the zoite stage identified. Here, we report the use of molecular approaches and histology to confirm the presence of APX in samples of green-lipped mussels *Perna canaliculus*, Mediterranean mussels *Mytilus galloprovincialis* and hairy mussels *Modiolus areolatus* collected from widely distributed locations in New Zealand. The prevalence of APX infection estimated by PCR was 22.2% (n = 99) and 50% (n = 30) in cultured green-lipped mussels from Nelson and Coromandel, respectively; 0.8% (n = 258), 3.3% (n = 150) and 35.3% (n = 17) in wild Mediterranean mussels from Nelson, Foveaux Strait and Golden Bay, respectively; and 46.7% (n = 30) in wild hairy mussels from Foveaux Strait. Histology detected all cases of PCR that were positive with APX and appeared to be more sensitive. The prevalence of APX estimated by histology in green-lipped mussels from Coromandel was 60% versus 50% by PCR, and 4.3%, 10.7% and 52.9% by histology versus 0.8%, 3.3% and 35.3% by PCR in wild Mediterranean mussels from Nelson, Foveaux Strait and Golden Bay, respectively. The specific identity of the parasite found in mussels was determined by sequencing PCR products for a portion (676 bp) of the 18S rRNA gene; the resulting sequences were 99–100% similar to APX found in flat oysters. Phylogenetic analyses also confirmed that all isolates from green-lipped, Mediterranean and hairy mussels grouped with APX isolates previously identified from flat oysters. This study indicates the wide geographical distribution of APX and highlights the potentially multi-host specific distribution of the parasite in commercially important bivalve shellfish.

KEY WORDS: *Bonamia exitiosa* · Host range · *Modiolus areolatus* · *Mytilus galloprovincialis* · Parasitic disease · *Perna canaliculus*

1. INTRODUCTION

The phylum Apicomplexa is a group of more than 6000 obligate protozoan parasites that cause morbidity and mortality in a wide range of vertebrates and invertebrates (Seeber & Steinfelder 2016). 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 apicomplexans are free-living 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

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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. The infective stages of APX in flat oyster are zoites; other stages such as oocysts or 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 2 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 crowd around the gonads, gut and digestive diverticulae, often associated with the destruction of connective tissues and gonad follicles (Hine 2002a). Oysters infected with APX appear 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 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. Webb unpubl. data) in New Zealand. In *P. canaliculus,* heavy APX infections have been associated with focal destruction of 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.

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, Wakeman 2013, Rueckert et al. 2015). In addition, evolutionary processes are much slower in the 18S rRNA gene compared with COI, making it potentially a more informative marker for delimiting closely related species (see review by Renoux et al. 2017). Recently, a specific PCR and an sequencing protocol has been established to target the APX 18S rRNA gene sequence in flat oysters (Suong et al. 2018). 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.

2. MATERIALS AND METHODS

2.1. Sample collection sites and histology

Cultured adult green-lipped mussels were sampled from the Cawthron Aquaculture Park, Nelson (n = 99, mean length ± SD = 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 (Fig. 1). One histology slide stained with hematoxylin and eosin was prepared from each mussel following the methods outlined in Howard et al. (2004), and the remaining tissues were kept frozen at –70 °C. Histology slides were screened for presence or absence of APX-like organisms under an Olympus BX51 compound light microscope at 100× magnification.

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

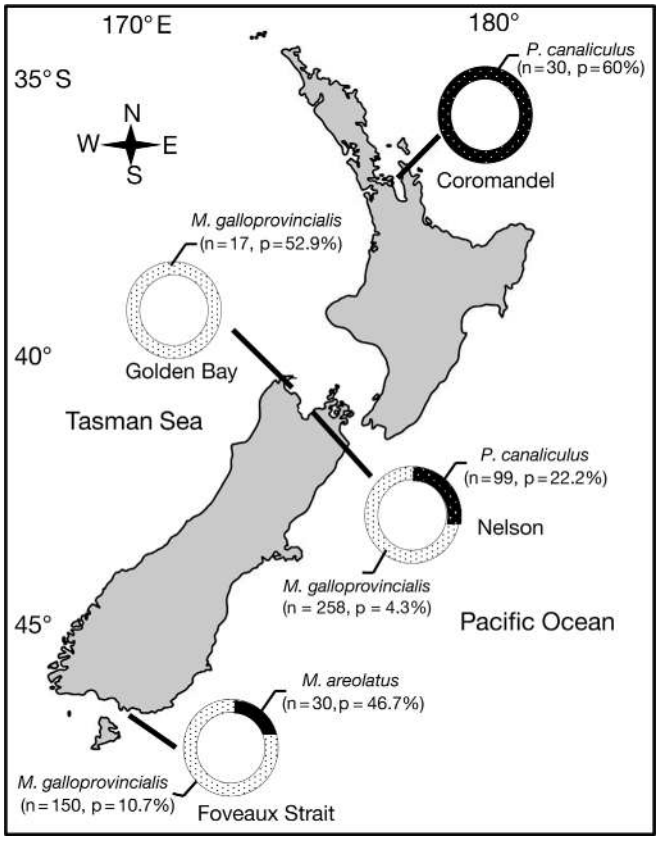

Fig. 1. Sample collection sites Coromandel, Golden Bay, Nelson and Foveaux Strait. Pie charts represent sample size (n) and histological prevalence of APX (p) for each mussel species collected at each site

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 (Wakeman 2013) 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 (723 bp) 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 × MyFi Mix (BioLine), 10 pmol 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 a 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.

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 to 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, New Zealand. 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).

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 4 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 4 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 identification using genetic data are dependent on genetic distances among specimens. Kimura's 2-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 1000 pseudoreplicates in MEGA version 6.06 (Tamura et al. 2013). The tree was rooted with the diatom group.

3. RESULTS

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 3 bivalve species examined (Fig. 2A–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 of 99) in cultured green-lipped mussels from Nelson, and 60% (18 of 30) from Coromandel. APX-like zoites occurred in wild Mediterranean mussels from Nelson, Foveaux Strait and Golden Bay at 4.3% (11 of 258), 10.7% (16 of 150) and 52.9% (9 of 17) respectively. In wild hairy mussels from Foveaux Strait, APX-like zoites occurred in 46.7% (14 of 30) of individuals. All histology-positive samples had only a very light level of infection with only a few of 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 (Fig. 2A,B,D).

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

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 6 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 number MH375573), 2 sequences from Foveaux Strait (GenBank accession numbers MH375572 and MH375570), 3 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 se-

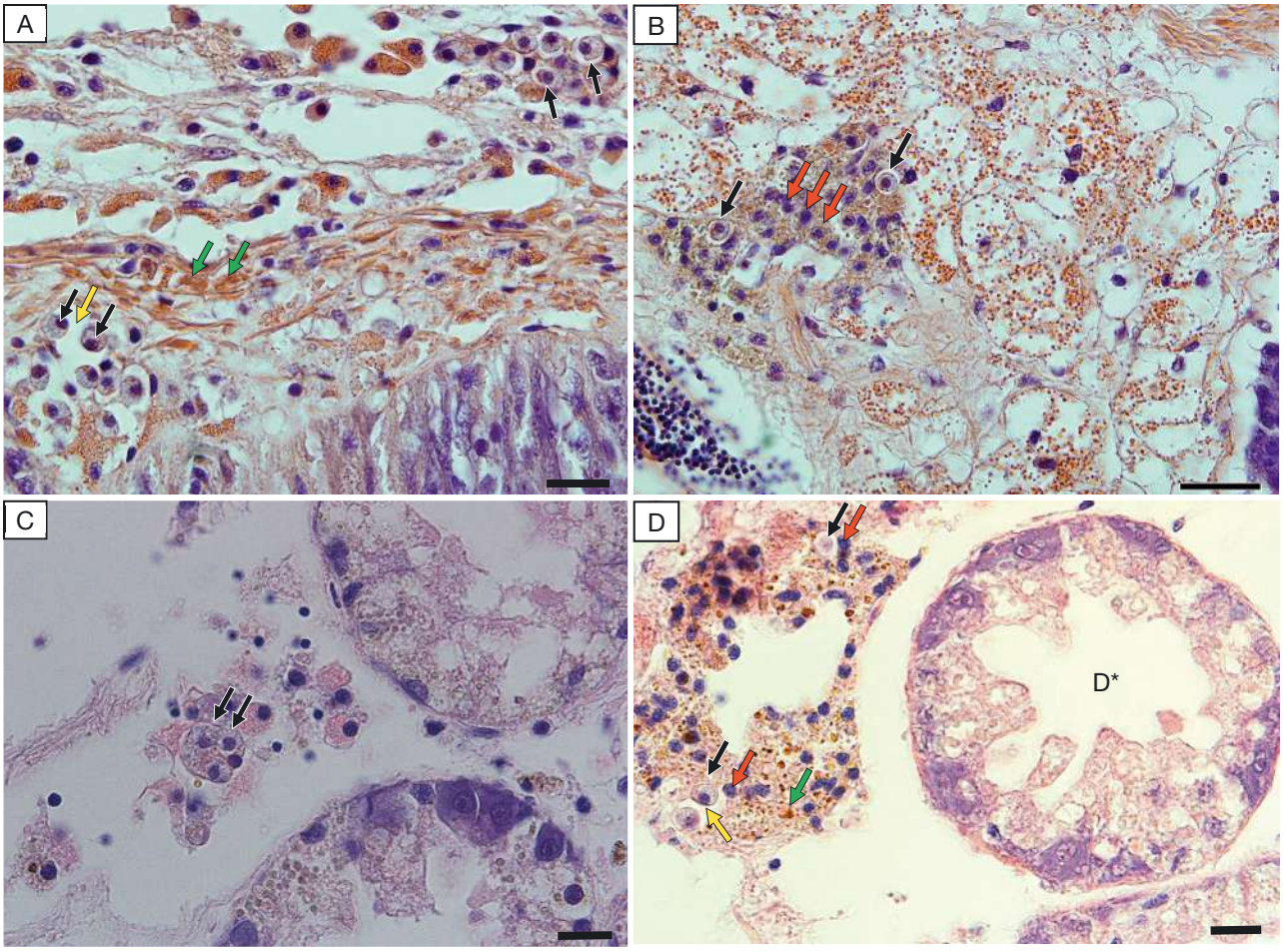

Fig. 2. Histological sections of (A–B) *Perna canaliculus*, (C) *Modiolus areolatus*, and (D) *Mytilus galloprovincialis*. Black arrows: APX-like zoites; red arrows: accumulation of haemocytes around APX-like cells; green arrows: brown cells; yellow arrows: empty space in the connective tissue due to the APX-like zoites. D*: normal digestive tubule. Scale bars: 10 µm

quences previously isolated from flat oysters (KX774501, KX774502, MH375571 and MH375569) with 99% to 100% query coverage (E value = 0).

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 3 apicomplexan clades consisting of *Cryptosporidium serpentis* and *Cryptosporidium parvum*, *Margolisiella islandica* and Tridacna hemolymph, *Besnoitia besnoiti*, *Toxoplasma gondii* and *Eimeria tenella*, respectively (Fig. 3). The overall mean genetic distance between the 26 APX isolates was 0.28%. By contrast, a 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. The maximum intraspecific divergence observed in APX isolates from Mediterranean mussels collected from Nelson, Foveaux Strait, and Golden Bay was 0.24% (Table 1). 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 2). Phylogenetic analysis of the APX sequences found 2 clades. One clade was formed with APX sequences isolated from flat oysters (KX774501, KX774502, MH375569, MH375571), 2 APX sequences from Mediterranean mussels (MH375562, MH375563) and one APX sequence from green-lipped mussel (MH375564) with 96% bootstrap support. The remaining 19 APX sequences clustered in another clade albeit with low bootstrap

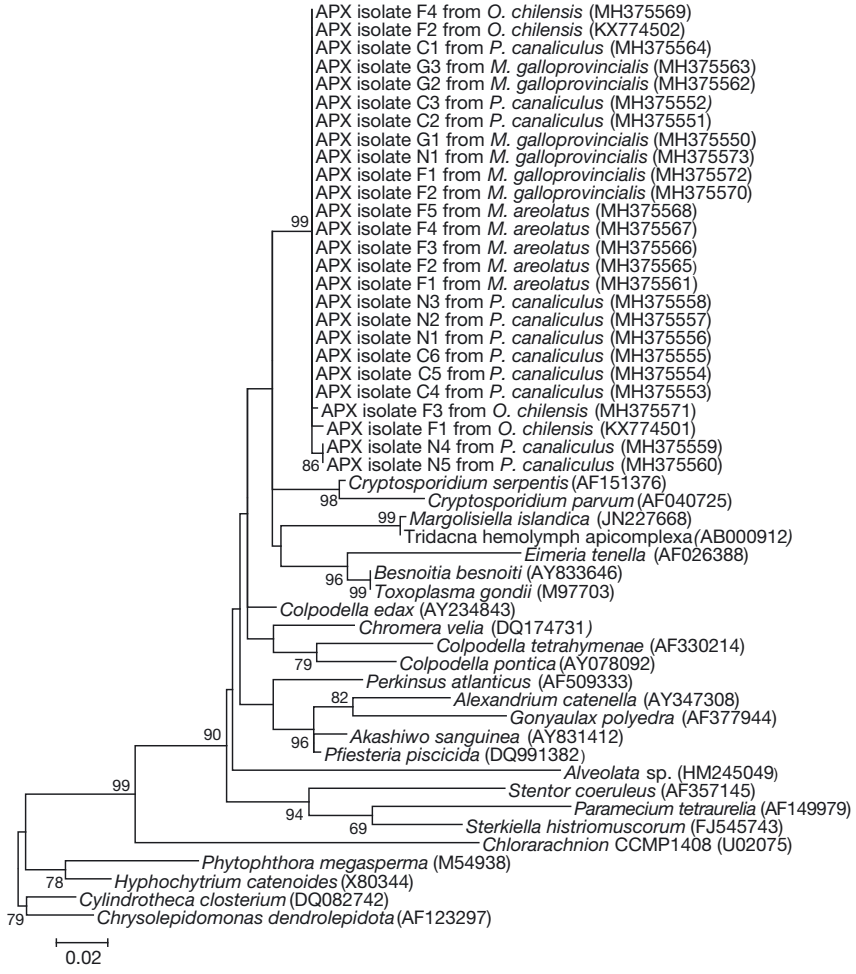

Fig. 3. Phylogenetic tree showing the relationship of 26 parasitic Apicomplexan-X isolates to an array of aquatic apicomplexans, Cercozoa, Perkinsozoa, aveolates, fungi, ciliates, diatoms and dinoflagellates. The tree was inferred using the maximum likelihood method based on the Tamura-Nei model. Bootstrap 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 the BioNJ algorithm to a matrix of pairwise distances estimated using the maximum composite likelihood approach. The tree is drawn to scale; branch lengths are proportional to the number of substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6

Table 1. Mean pairwise 18S genetic distances within each host group of APX isolates

APX host group	Genetic distance (%)
Ostrea chilensis	0.22
Mytilus galloprovincialis	0.24
Perna canaliculus	0.23
Modiolus areolatus	0.00

Table 2. Mean pairwise 18S genetic distances between APX isolates among host species

APX host group	APX host group	Genetic distance (%)
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

support. Clearly, these clades were not correlated with geographic distributions and did not group according to host-species (data not shown).

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. Here we confirm the presence of an apicomplexan identified from histological examination in 3 important bivalve species — green-lipped mussels *Perna canaliculus*, Mediterranean mussels My*tilus*

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 mussels populations and varied with the year of collection. 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, 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 and vice versa (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 (25 mg) 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 be attributed 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 2 copies of a gene (Smith & Osborn 2009).

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 2). The low divergence rate of APX isolates could be due 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 (*Gregarina polymorpha* and *Gregarina niphandrodes*) (Leander et al. 2003). Low intraspecific sequence divergence (0.1–0.4%) was also recorded in another apicomplexan, *Lankesteria hesperidiiformis* (Rueckert et al. 2015). The maximum-likelihood analysis grouped the 26 APX isolates into 2 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 (Fig. 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 3 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 2 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, 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 signs of disease in the hosts (Kristmundsson et al. 2011). It has been reported that APX zoites

most often cause severe disease in austral summer/autumn (January to April), during the peak spawning period as well as 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, a simultaneous infection with a second pathogen may contribute to the morbidity caused by APX.

It is not known how APX infects 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 that the potential secondary hosts of APX could be other taxa, such as polychaetes, as previously proposed by Hine (2002a).

The fact that green-lipped, Mediterranean and hairy mussels carry 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.

The present study 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.

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LITERATURE CITED

Abdel-Baki AAS, Al-Quraishy S, Dkhil MA, Al Nasr I, Oliveira E, Casal G, Azevedo C (2012) Ultrastructural characteristics of *Nematopsis* sp. oocysts (Apicomplexa: Porosporidae), a parasite of the clam *Meretrix meretrix* (Veneridae) from the Arabian Gulf, Saudi Arabia. Folia Parasitol (Praha) 59:81–86

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Azevedo C, Matos E (1999) Description of *Nematopsis mytellan*. n. sp. (Apicomplexa), parasite of the mussel *Mytella guyanensis* (Mytelidae) from the Amazon estuary and description of its oocysts. Eur J Protistol 35: 427–433

Ben-Horin T, Bidegain G, Huey L, Narvaez DA, Bushek D (2015) Parasite transmission through suspension feeding. J Invertebr Pathol 131:155–176

Chu FL (1996) Laboratory investigations of susceptibility, infectivity, and transmission of *Perkinsus marinus* in oysters. J Shellfish Res 15:57–66

Collins RA, Boykin LM, Cruickshank RH, Armstrong KF (2012) Barcoding's next top model: an evaluation of nucleotide substitution models for specimen identification. Methods Ecol Evol 3:457–465

Diggles BK, Hine P, Handley S, Boustead NC (2002) A handbook of diseases of importance to aquaculture in New Zealand. NIWA Sci Tech Ser, Vol 49. NIWA, Wellington

Guenther S, Ewers C, Wieler LH (2011) Extended-spectrum beta-lactamases producing *E. coli* in wildlife, yet another form of environmental pollution? Front Microbiol 2:246

Hine PM (2002a) Severe apicomplexan infection in the oyster *Ostrea chilensis*: a possible predisposing factor in bonamiosis. Dis Aquat Org 51:49–60

Hine PM (2002b) Results of a survey on shellfish health in New Zealand in 2000. Surveillance 29:3–7

Hine PM, Jones JB (1994) Bonamia and other aquatic parasites of importance to New Zealand. N Z J Zool 21:49–56

Howard DW, Lewis EJ, Keller BJ, Smith CS (2004) Histological techniques for marine bivalve mollusks and crustaceans, 2nd edn. NOAA Tech Memo NOS NCCOS 5. http://aquaticcommons.org/14949/1/nos_nccos_5.pdf

Kimura M (1980) A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kristmundsson Á, Helgason S, Bambir SH, Eydal M, Freeman MA (2011) Previously unknown apicomplexan species infecting Iceland scallop, *Chlamys islandica* (Müller, 1776), queen scallop, *Aequipecten opercularis* L., and king scallop, *Pecten maximus* L. J Invertebr Pathol 108: 147–155

Kristmundsson Á, Erlingsdóttir Á, Freeman MA (2015) Is an apicomplexan parasite responsible for the collapse of the Iceland scallop (*Chlamys islandica*) stock? PLOS ONE 10: e0144685

Leander BS, Clopton RE, Keeling PJ (2003) Phylogeny of gregarines (Apicomplexa) as inferred from small-subunit rDNA and -tubulin. Int J Syst Evol Microbiol 53:345–354

OIE (Office International des Epizooties) (2011). Aquatic animal health code, 97th edn. Office International des Epizooties, Paris

Renoux LP, Dolan MC, Cook CA, Smit NJ, Sikkel PC (2017) Developing an apicomplexan DNA barcoding system to detect blood parasites of small coral reef fishes. J Parasitol 103:366–376

Rueckert S, Wakeman KC, Jenke-Kodama H, Leander BS (2015) Molecular systematics of marine gregarine apicomplexans from Pacific tunicates, with descriptions of five novel species of *Lankesteria*. Int J Syst Evol Microbiol 65:2598–2614

Ruiz-Fons F, Sánchez-Matamoros A, Gortázar C, Sánchez-Vizcaíno JM (2014) The role of wildlife in bluetongue virus maintenance in Europe: lessons learned after the natural infection in Spain. Virus Res 182:50–58

Sabat AJ, Budimir A, Nashev D, Sá-Leão R and others (2013) Overview of molecular typing methods for outbreak detection and epidemiological surveillance. Euro Surveill 18:20380

Seeber F, Steinfelder S (2016) Recent advances in understanding apicomplexan parasites. F1000 Res 2016:1369

Smith CJ, Osborn AM (2009) Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS Microbiol Ecol 67:6–20

Soudant P, Chu FLE, Volety A (2013) Host–parasite interactions: marine bivalve molluscs and protozoan parasites, *Perkinsus* species. J Invertebr Pathol 114:196–216

Suong NT, Webb S, Banks J, Wakeman KC and others (2017) Partial 18S rRNA sequences of apicomplexan parasite 'X' (APX), associated with flat oysters *Ostrea chilensis* in New Zealand. Dis Aquat Org 127:1–9

Suong NT, 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. Dis Aquat Org 129:199–205

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10: 512–526

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30:2725–2729

Uddin MJ, Yasin Z, Khalil M, Shau-Hwai AT (2011) Parasites of blood cockle (*Anadara granosa* Linnaeus, 1758) from the Straits of Malacca. J Shellfish Res 30: 875–880

Wakeman KC (2013) Species discovery and evolution history of marine gregarine apicomplexans. Doctoral thesis, University of British Columbia, Vancouver, BC

Ward MP, Cowled BD, Galea F, Garner MG and others (2013) Salmonella infection in a remote, isolated wild pig population. Vet Microbiol 162:921–929

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