Vol. 127: 1–9, 2017
https://doi.org/10.3354/dao03175

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published December 19

Partial 18S rRNA sequences of apicomplexan parasite 'X' (APX), associated with flat oysters *Ostrea chilensis* in New Zealand

Nguyen Thao Suong[1,2,*], Stephen Webb[2], Jonathan Banks[2], Kevin C. Wakeman[4,5], Henry Lane[3], Andrew Jeffs[1], Cara Brosnahan[3], Brian Jones[3], Andrew Fidler[1]

[1]Institute of Marine Science, University of Auckland, Auckland 1142, New Zealand
[2]Cawthron Institute, Nelson 7010, New Zealand
[3]Ministry for Primary Industries, Wallaceville, Upper Hutt 5018, New Zealand
[4]Office of International Affairs, Hokkaido University, Sapporo 060-0810, Japan
[5]Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan

ABSTRACT: Apicomplexa is a large phylum of parasitic protists renowned for significant negative health impacts on humans and livestock worldwide. Despite the prevalence and negative impacts of apicomplexans across many animal groups, relatively little attention has been given to apicomplexan parasites of invertebrates, especially marine invertebrates. Previous work has reported an apicomplexan parasite 'X' (APX), a parasite that has been histologically and ultrastructurally identified from the commercially important flat oyster *Ostrea chilensis* in New Zealand. This apicomplexan may exacerbate host vulnerability to the infectious disease bonamiosis. In this study, we report 18S rRNA sequences amplified from APX-infected *O. chilensis* tissues. Phylogenetic analyses clearly established that the 18S sequences were of apicomplexan origin; however, their detailed relationship to known apicomplexan groups is less resolved. Two specific probes, designed from the putative APX 18S rRNA sequence, co-localised with APX cells in *in situ* hybridisations, further supporting our hypothesis that the 18S sequences were from APX. These sequences will facilitate the future development of inexpensive and sensitive molecular diagnostic tests for APX, thereby assisting research focussed on the biology and ecology of this organism and its role in morbidity and mortality of *O. chilensis*.

KEY WORDS: APX zoites · Molecular identification · Shellfish disease · *In situ* hybridisation

INTRODUCTION

The phylum Apicomplexa is a large and diverse group of parasitic protists that comprises almost 6000 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

*Corresponding author: sngu189@aucklanduni.ac.nz

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 2002).

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 2002). The only distinct APX developmental stages observed in specimens of flat oysters were identified as zoites (Hine 2002). APX zoites appear to be associated with increased susceptibility of wild flat oysters to infection with the haplosporidian parasite *Bonamia exitiosa* (see Hine 2002). 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 2002).

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 our 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.

MATERIALS AND METHODS

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 was opened with a separate sterilised knife. Each oyster was examined for any gross pathology such as weight or colour abnormalities before dissecting out 2 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× magnification.

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 the 18S rRNA gene sequence was achieved in 2 steps. Initially, a product of 0.6 kb was amplified using the forward primer F2 (5′-GGT AGY GAC AAG AAA TAA CAA C-3′) paired with reverse primer R2 (5′-GAY TAC GAC GGT ATC TGA TCG TC-3′) (Wakeman 2013). Polymerase chain reaction (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 visualisation 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′-GCG CTA CCT GGT TGA TCC TGC C-3′) (Wakeman 2013) paired with reverse primer 1906Rev1

(5′-TTT CAC CTC TGA CAG TAC AAA TAC-3′). The 3′ extension was obtained using the forward primer 3011For1 (5′-AAT GCA AGG CAT TTA CTG CTT TG-3′) paired with the reverse primer SSUR4 (5′-GAT CCT TCT GCA GGT TCA CCT AC-3′) (Wakeman 2013) 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).

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 number KX774501) and 'Isolate from oyster 2′ (KX774502) were aligned with 18S rRNA sequences (n = 43) obtained from GenBank that represented major eukaryote groups and included apicomplexans and alveolates (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 2 new 18S sequences generated in this study were aligned with 27 additional sequences downloaded from GenBank representing major apicomplexan lineages and 3 sequences from dinoflagellates and 2 sequences from colpodellids as the outgroup, 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 neighbour-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).

Amplification and sequencing of the *Ostrea 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.

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 numbers 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, 2 probes, ISH-APX-Pr01 (5′-ATC AAA CCG GAT TCT CAC TCA AAG A-3′) and ISH-APX-Pr02 (5′-GTG AGT CGA GAA CAA AGA ACA TTC-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 3 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 2 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 3 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 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.

RESULTS

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 (Fig. 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.

Amplification and sequencing of partial APX 18S rRNA gene sequences

An amplicon of 0.6 kb was generated from flat oyster gDNA templates using the 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

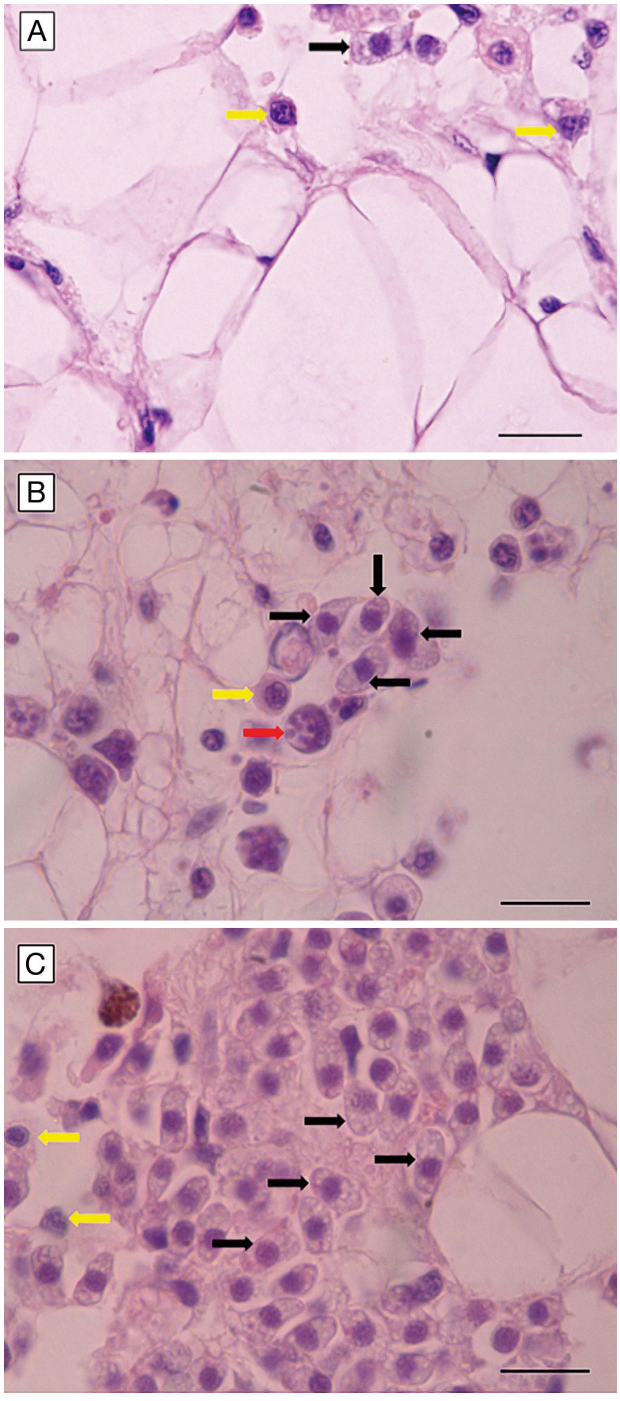

Fig. 1. Histopathological differences in the intensity of apicomplexan-X (APX) infection in the examined *Ostrea chilensis.* Black arrows: APX, red arrow: *Bonamia* microcells within haemocyte, yellow arrows: normal haemocytes. (A) Light infection: <5 parasites visible in the field of view. (B) Light to moderate infection: a few zoites are observed in most tissues between connective tissue cells. (C) Moderate to heavy infection: zoites are numerous among and within the 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

rDNA sequence from *Eimeria* sp. (Apicomplexa, accession number JF419346, 82% identity, E-value 2×10^{-143}).

Concatenation of the 2 putative 18S APX sequences extended from 5′ and 3′ direction using the primer 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 2 sequences overlapped by 429 nucleotides allowing unambiguous concatenation of the 2 sequences. Using this approach, 18S rRNA sequences were generated from 2 flat oyster gDNA samples and, after the 5′ and 3′ PCR primer sequences were removed, were deposited in GenBank (isolates from oyster 1 and 2: accession numbers KX774501 and KX774502, respectively) for a portion of the 18S rRNA gene. The 2 apicomplexan 18S rRNA gene sequences differed at 3 nucleotide positions.

Phylogeny of the apicomplexan 18S rRNA sequences

Phylogenetic analysis of the data set containing our putative APX 18S gene sequences and the 43 18S sequences representing the major eukaryote groups placed our sequences as a sister-group to the coccidian *Toxoplasma gondii* with well-supported bootstrap values (data not shown). Phylogenetic analyses of the 34-taxa data set that focussed on the relationships within the apicomplexans showed that our 2 putative APX sequences formed a sister clade to the group consisting of *T. gondii*, *Hepatozoon catesbianae* and *Adelina bambarooniae,* albeit with low bootstrap support (Fig. 2).

Amplification and sequencing of an oyster 18S rRNA gene sequence

At the time this work was undertaken, only 2 *Ostrea 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 in GenBank under accession number KX977494.

In situ hybridisation

From the alignment of APX 18S rRNA sequences (GenBank accession numbers KX774501 and KX774502) with flat oyster (KX977494), Pacific oyster (AB064942), *Bonamia ostreae* (AF262995.1) and *B. exitiosa* (JF495410.1) sequences, 2 probes (ISH-APX-Pr01 and ISH-APX-Pr02; Fig. 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 (Fig. 4). Examination of the cells to which the ISH probes bound morphologically resembled the APX cells identified in the histological specimens (Fig. 1). None of the probes bound to host tissue, and all controls were negative (Fig. 4). Hybridisation results were reproducible when 18 runs were made.

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 NZ 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

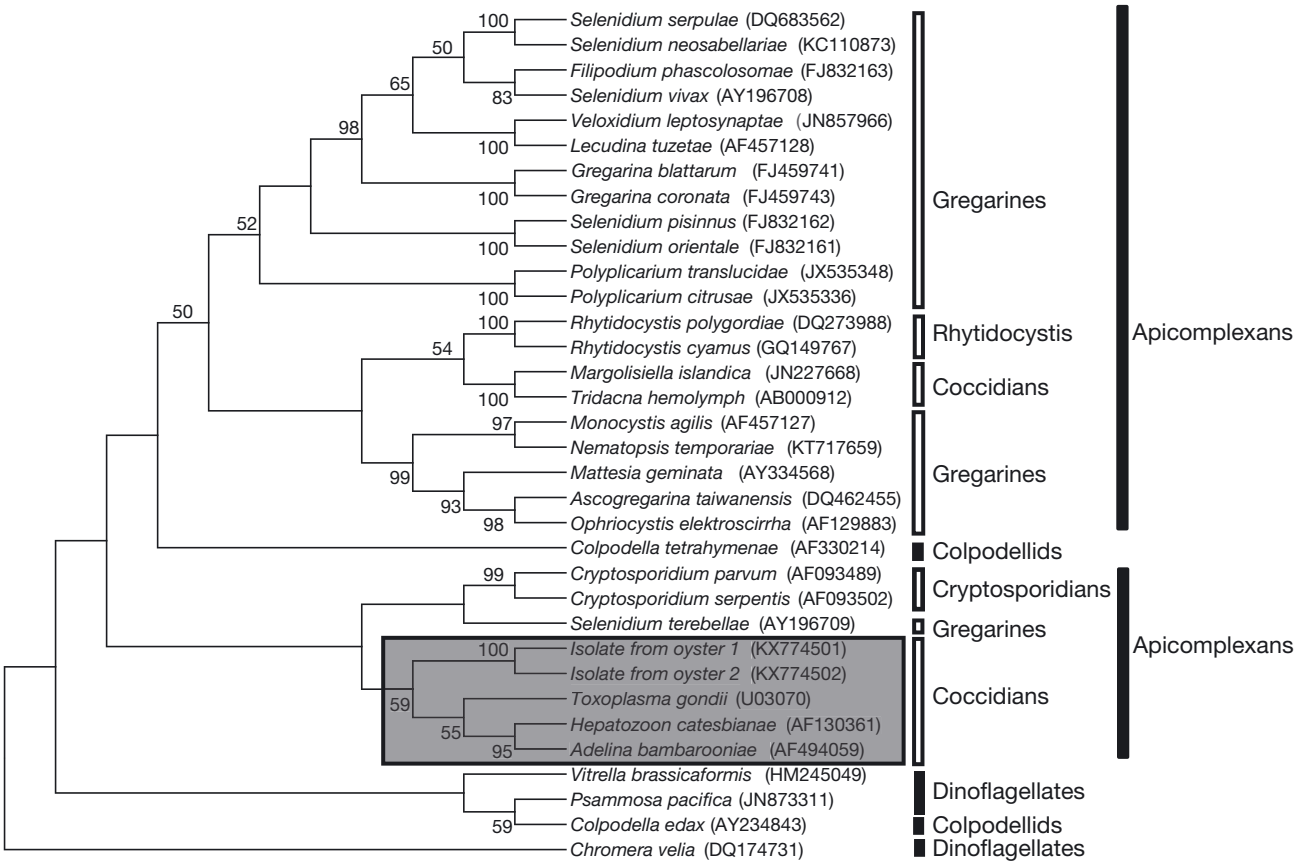

Fig. 2. Phylogenetic analyses of 2 apicomplexan-X (APX) isolates from the flat oyster *Ostrea chilensis* with representatives of known apicomplexan sequences. Colpodellids and dinoflagellates were used as outgroups. The 2 APX sequences group with the clade consisting of *Toxoplasma gondii*, *Hepatozoon 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

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 (Rueckert et al. 2010, 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 galloprovincialis* (S. Webb unpubl. data). However, the use of morphological characters to distinguish between species can be misleading, especially when variability within an individual species occurs. Our primers 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 verte-

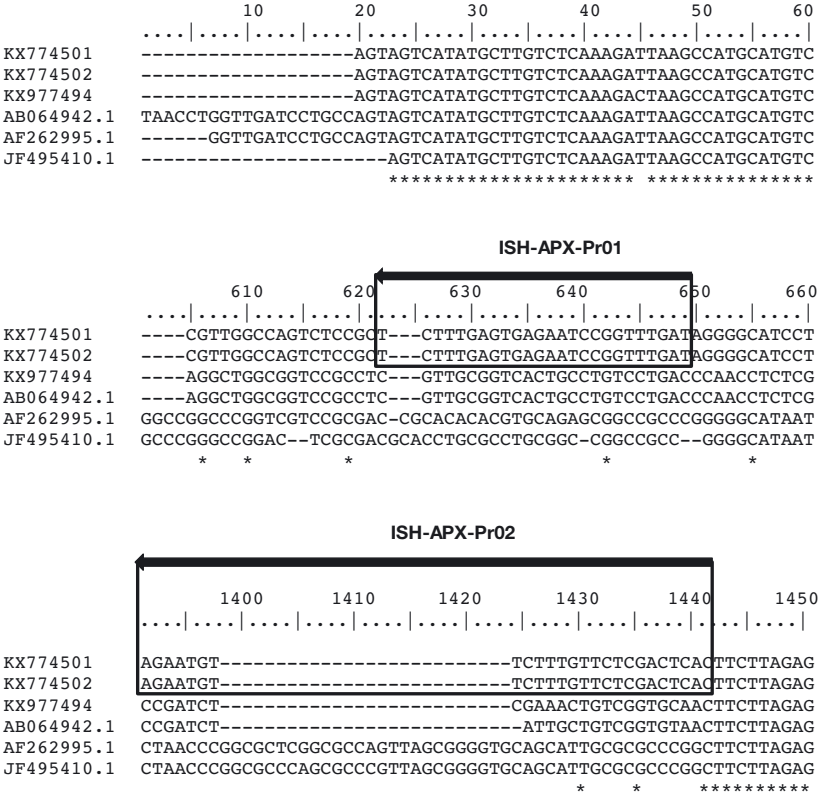

Fig. 3. Design of probes for *in situ* hybridisation detection of apicomplexan-X (APX) 18S rRNA in infected oyster *Ostrea chilensis* tissues. Partial 18S rRNA sequences of APX (GenBank accession nos. KX774501, KX774502), flat oyster (KX977494), Pacific oyster (AB064942), *Bonamia ostreae* (AF262995) 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 ISH-APX-Pr02) were designed which were reverse-complements of the 2 regions indicated

brates and hematophagous arthropods (Boulianne et al. 2007), while coccidian parasites of the genus *Adelina* are known to be strictly entomogenous (Lange & Wittenstein 2001). *H. 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).

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 also permits detection of low-intensity infections. However, while

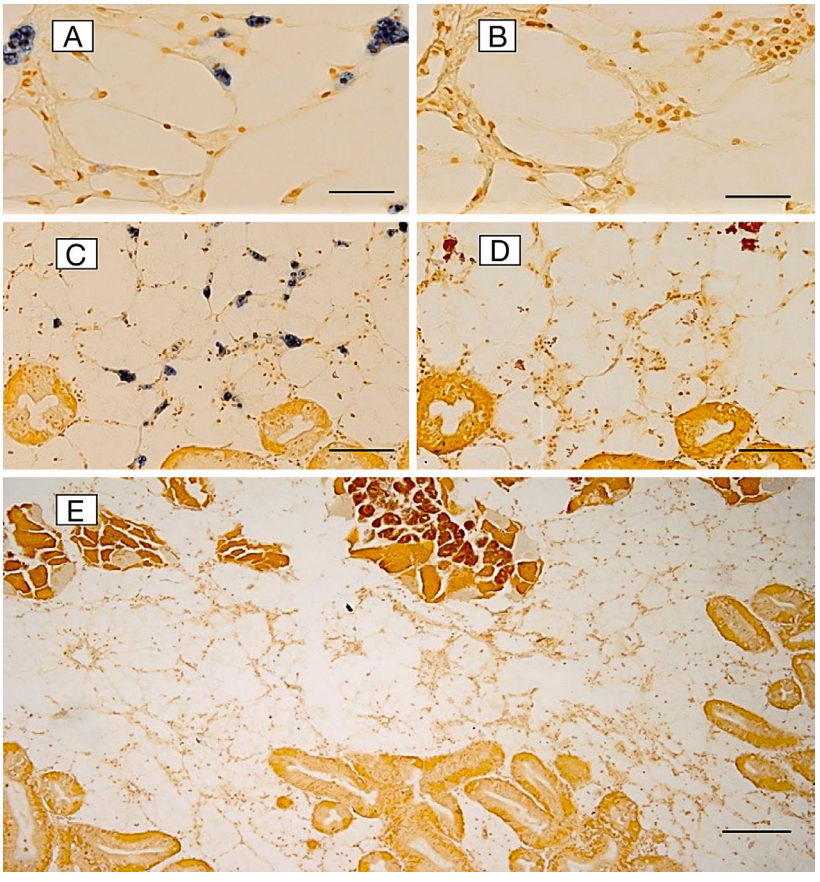

Fig. 4. Detection of apicomplexan-X (APX) 18S rRNA sequences in APX-infected flat oyster *Ostrea chilensis* tissue sections by *in situ* hybridisation. (A,C) Clear labelling (blue dye deposition) of APX cells by both probes ISH-APX-Pr01 (A) and ISH-APX-Pr02 (C). No labeling of flat oyster host cells 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-APX-Pr02 (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

PCR is a sensitive method for detecting specific sequences of DNA, 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 our contention that we have obtained sequences for APX parasitising NZ flat oysters. The sequences we obtained and the primers we 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 2002), 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.

Acknowledgements. This work was supported by the New Zealand and Viet Nam governments through a New Zealand-ASEAN Scholarship awarded to N.T.S. and in part by a NZ Ministry of Business, Innovation and Employment grant (no. CAWX1315). Our thanks go to Associate Professor Wendi Roe (Institute of Veterinary, Animal and Biomedical Sciences, Massey University) for her generous support for the ISH experiments; and to Paul McMillan (Solander Group) and Graeme Wright (Barnes Wild Bluff Oyster Company) for helping source flat oysters for this work. The article also benefited from reviewers' comments, which greatly improved the manuscript.

LITERATURE CITED

Adl SM, Leander BS, Simpson AGB, Archibald JM and others (2007) Diversity, nomenclature, and taxonomy of protists. Syst Biol 56:684–689

Allsopp MTEP, Cavalier-Smith T, Waal DTD, Allsopp BA (1994) Phylogeny and evolution of the piroplasms. Parasitology 108:147–152

Aranda DA, Frenkiel L, Brulé T, Montero J, Cárdenas EB (2011) Occurrence of Apicomplexa-like structures in the digestive gland of *Strombus gigas* throughout the Caribbean. J Invertebr Pathol 106:174–178

Azevedo C, Cachola R (1992) Fine structure of the apicomplexa oocyst of *Nematopsis* sp. of two marine bivalve molluscs. Dis Aquat Org 14:69–73

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

Boulianne B, Evans RC, Smith TG (2007) Phylogenetic analysis of *Hepatozoon* species (Apicomplexa: Adeleorina) infecting frogs of Nova Scotia, Canada, determined by ITS-1 sequences. J Parasitol 93:1435–1441

Bueno R (2015) Ostreid herpesvirus-1 infection in Pacific Oysters (*Crassostrea gigas*) - New Zealand. MSc thesis, Massey University, Palmerston North

Burreson EM (2008) Misuse of PCR assay for diagnosis of mollusc protistan infections. Dis Aquat Org 80:81–83

Ceuta LO, Boehs G (2012) Parasites of the mangrove mussel *Mytella guyanensis* (Bivalvia: Mytilidae) in Camamu Bay, Bahia, Brazil. Braz J Biol 72:421–427

Cheng TC (2012) General parasitology, 2nd edn. Elsevier, Charleston, SC

Cranfield HJ, Dunn A, Doonan IJ, Michael KP (2005) *Bonamia exitiosa* epizootic in *Ostrea chilensis* from Foveaux Strait, southern New Zealand between 1986 and 1992. ICES J Mar Sci 62:3–13

Criado-Fornelio A, Gónzalez del Río MA, Buling-Saraña A, Barba-Carretero JC (2004) The 'expanding universe' of piroplasms. Vet Parasitol 119:337–345

Desser SS, Hong H, Martin DS (1995) The life history, ultrastructure, and experimental transmission of *Hepatozoon catesbianae* n. comb., an apicomplexan parasite of the bullfrog, *Rana catesbeiana* and the mosquito, *Culex territans* in Algonquin Park, Ontario. J Parasitol 81:212–222

Diggles B, Hine P, Handley S, Boustead N (2002) A handbook of diseases of importance to aquaculture in New Zealand. Sci Technol Ser 49. NIWA, Wellington

Friedman CS, Gardner GR, Hedrick RP, Stephenson M, Cawthorn RJ, Upton SJ (1995) *Pseudoklossia haliotis* sp. n. (Apicomplexa) from the kidney of California abalone, *Haliotis* spp. (Mollusca). J Invertebr Pathol 66:33–38

Harris DJ, Maia JPMC, Perera A (2011) Molecular characterization of *Hepatozoon* species in reptiles from the Seychelles. J Parasitol 97:106–110

Hine PM (1989) Parasites and diseases of commercially important molluscs in New Zealand. Advances in Tropical Aquaculture. Actes Colloq Ifremer 9:199–206

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

Kibbe WA (2007) OligoCalc: an online oligonucleotide properties calculator. Nucleic Acids Res 35:W43–W46

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

Lange CE, Wittenstein E (2001) An *Adelina* sp. (Apicomplexa: Coccidia) found in *Anurogryllus muticus* (De Geer) (Orthoptera: Gryllidae). J Invertebr Pathol 77:83–84

Larkin MA, Blackshields G, Brown NP, Chenna R and others (2007) ClustalW and ClustalX version 2. Bioinformatics 23:2947–2948

Leibovitz L, Schott EF, Karney RC (1984) Diseases of wild captive and cultured scallops. J World Maricult Soc 14: 269–283

Maddison WP, Maddison DR (2016) Mesquite: a modular system for evolutionary analysis. Version 3.10. http://mesquiteproject.org (accessed 20 Oct 2016)

Morado JF, Sparks AK, Reed SK (1984) A coccidian infection of the kidney of the native littleneck clam, *Protothaca staminea.* J Invertebr Pathol 43:207–217

Rueckert S, Chantangsi C, Leander BS (2010) Molecular systematics of marine gregarines (Apicomplexa) from North-eastern Pacific polychaetes and nemerteans, with descriptions of three novel species: *Lecudina phyllochaetopteri* sp. nov., *Difficilina tubulani* sp. nov. and *Difficilina paranemertis* sp. nov. Int J Syst Evol Microbiol 60: 2681–2690

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Stokes N, Burreson E (1995) A sensitive and specific DNA probe for the oyster pathogen *Haplosporidium nelsoni.* J Eukaryot Microbiol 42:350–357

Sühnel S, Johnson SC, Gurney-Smith HJ, Da S. Ivachuk C and others (2016) A status assessment of perkinsiosis, bonamiosis, and mateiliosis in commercial marine bivalves from Southern Brazil. J Shellfish Res 35: 143–156

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

Tuntiwaranuruk C, Chalermwat K, Upatham ES, Kruatrachue M, Azevedo C (2004) Investigation of *Nematopsis* spp. oocysts in 7 species of bivalves from Chonburi Province, Gulf of Thailand. Dis Aquat Org 58:47–53

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 evolutionary history of marine gregarine apicomplexans. PhD dissertation, The University of British Columbia, Vancouver, BC

Wakeman KC, Leander BS (2013) Identity of environmental DNA sequences using descriptions of four novel gregarine parasites, *Polyplicarium* n. gen. (Apicomplexa), from capitellid polychaetes. Mar Biol 43:133–147

Whyte SK, Cawthorn RJ, McGladdery SE (1994) Co-infection of bay scallops *Argopecten irradians* with *Perkinsus karlssoni* (Apicomplexa, Perkinsea) and an unidentified coccidian parasite. Dis Aquat Org 18:53–62

Winstead JT, Volety AK, Tolley SG (2004) Parasitic and symbiotic fauna in oysters (*Crassostrea virginica*) collected from the Caloosahatchee River and Estuary in Florida. J Shellfish Res 23:831–840

Yubuki N, Leander BS (2008) Ultrastructure and molecular phylogeny of *Stephanopogon minuta*: an enigmatic microeukaryote from marine interstitial environments. Eur J Protistol 44:241–253

Editorial responsibility: Mike Hine, Fouras, France

Submitted: January 2, 2017; Accepted: September 6, 2017
Proofs received from author(s): November 6, 2017