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# *Perkinsus* sp. infections and *in vitro* isolates from *Anadara trapezia* (mud arks) of Queensland, Australia

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ABSTRACT: *Perkinsus* sp. protists were found infecting *Anadara trapezia* mud ark cockles at 6 sites in Moreton Bay, Queensland, Australia, at prevalences of 4 to 100% during 2011 as determined by surveys using Ray's fluid thioglycollate medium. *Perkinsus* sp. lesions were found among gill and visceral connective tissues in histological samples from several cockles, where basophilic, eccentrically vacuolated *Perkinsus* sp. signet ring trophozoites and proliferating, *Perkinsus* sp. schizont cells were documented. Two *Perkinsus* sp. isolates were propagated *in vitro* during August 2013 from gill tissues of a single infected *A. trapezia* cockle from Wynnum in Moreton Bay. DNA from those isolate cells amplified universally by a *Perkinsus* genus-specific PCR assay, and rDNA-internal transcribed spacer sequences respectively grouped them with *P. olseni* and *P. chesapeaki* in phylogenetic analyses. This is the first report of *P. chesapeaki* in Australia, and the first report of a *P. chesapeaki in vitro* isolate from an Australian mollusc host. Although *P. olseni* was originally described in 1981 as a pathogen of abalone in South Australia, and has subsequently been identified as a prevalent pathogen of numerous other molluscs worldwide, this is also the first report of a *P. olseni*-like *in vitro* isolate from an Australian mollusc host.

KEY WORDS: Cockle · *Perkinsus olseni* · *Perkinsus chesapeaki* · RFTM



## INTRODUCTION

*Perkinsus* sp. pathogens are important parasites of molluscs that have caused drastic mortalities in various bivalve species throughout the world, including the eastern oyster *Crassostrea virginica* in the USA (Andrews & Hewatt 1957), the clams *Ruditapes decussatus* and *R. philippinarum* in Europe and Asia (Ruano & Cachola 1986, Azevedo 1989, Maeno et al. 1999) and abalones in Australia (Lester & Davis 1981).

Mud arks *Anadara trapezia* (also known as Sydney cockle or blood cockle) occur naturally in seagrass intertidal areas in Moreton Bay in southeastern Queensland, Australia, but are not harvested or cultured for human consumption. *Perkinsus* sp. infections have been reported among *A. trapezia* mud arks of Moreton Bay (Lester et al. 1990, Goggin & Lester 1995), based on results of assays with Ray's fluid thioglycollate medium (RFTM, Ray 1966). Replicate independent amplifications and analyses of nucleotide sequences from internal transcribed spacer (ITS), non-transcribed spacer (NTS) and other segments of rRNA gene complexes of *Perkinsus* sp. cells from Moreton Bay *A. trapezia* hosts have consistently indicated close homologies with *P. olseni* sequences (Goggin 1994). Indeed, a *Perkinsus* sp. whose rDNA-NTS sequences were amplified from Moreton Bay *A. trapezia* was included among numerous mollusc pathogens from Australia, New Zealand and Europe that were collectively synonymized as *P. olseni* (Murrell et al. 2002).

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Despite extensive use of *Perkinsus* sp. cells from Moreton Bay *A. trapezia* for DNA sequencing (Goggin & Barker 1993, Goggin 1994, Murrell et al. 2002), disease transmission experiments (Goggin et al. 1989) and pathogen physiology investigations (Goggin et al. 1990), natural prevalences for such infections are not explicitly reported beyond a statement that at least 50% showed heavy infections in 1 sample (Goggin et al. 1990). Reports on histopathological characteristics of *Perkinsus* sp. infections in *A. trapezia* are limited to a single image from an experimental infection (Lester et al. 1990). Surprisingly, there is no report to date of a *Perkinsus* sp. isolate that was propagated *in vitro* from any of the numerous affected Australian molluscs.

Here we report characteristics of Australian *Perkinsus* sp. *in vitro* isolates that were propagated from a Moreton Bay *A. trapezia* cockle. *Perkinsus* sp. infection prevalences are reported for *A. trapezia* populations at 6 sites in Moreton Bay, as are histopathological characteristics of *Perkinsus* sp. lesions among those infected *A. trapezia*.

## MATERIALS AND METHODS

### Cockle samples, RFTM assays and histopathology

*Anadara trapezia* mud arks were collected by hand in muddy intertidal seagrass beds at 6 sites in Moreton Bay during the summer (November to December) of 2011 (n = 215) and at 1 site in August 2013 (n = 10; Fig. 1, Table 1). Cockles were returned to The University of Queensland (UQ) laboratories, where they were dissected to obtain tissue samples for analytical and experimental uses. During the summer of 2011, a transverse sample through the visceral mass and 1 complete pair of gill demibranchs were processed together for histopathology from each sampled cockle. In addition to gill tissues, histological sections included the mantle, visceral mass (digestive organs and gonads) and foot. Tissues were fixed for 24 h in a 10% (v/v) formalin fixative, and were then dehydrated and embedded into paraffin blocks before sections were cut at 5 µm and stained with Mayer's haematoxylin and eosin (Howard et al. 2004).

To assess perkinsosis prevalences at the 6 sites sampled in 2011, gill tissues from 215 cockles were analysed using RFTM methods (Ray 1952) as modified by Choi et al. (1989). Gill tissues excised from 18 to 50 cockles of each sample were weighed before inoculation into tubes containing RFTM, and were incubated in the dark at room temperature for 5 d before analyses. Following RFTM incubation, gill tissues from survey cockles were lysed twice for 1 h in 2 M sodium hydroxide at 60°C, before enlarged *Perkinsus* sp. hypnospores were pelleted by centrifugation and washed twice by centrifugation with phosphate-buffered saline (PBS). Washed pellets were

Table 1. Prevalences of *Perkinsus* sp. infections among *Anadara trapezia* cockles from 6 Moreton Bay (Queensland, Australia) sites, which were tested by Ray's fluid thioglycollate medium (RFTM) assays during November and December 2011

| Sample site | Site code (Fig. 1) | No. infected/ sample size | RFTM prev. (%) |
|---|---|---|---|
| Oyster lease, N. Stradbroke Is. | A | 2/50 | 4 |
| Deanbilla N. Stradbroke Is. | B | 3/18 | 17 |
| Banana Bank, N. Stradbroke Is. | C | 3/30 | 10 |
| Sea grass, N. Stradbroke Is. | D | 7/30 | 23 |
| Manly | E | 39/40 | 98 |
| Wynnum | F | 47/47 | 100 |
| Total | | 101/215 | 47 |

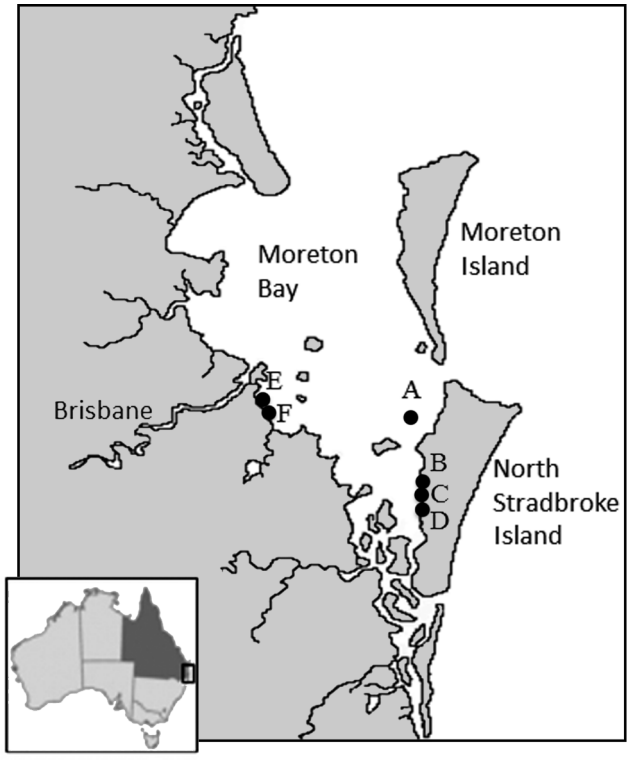



Fig. 1. Locations of Moreton Bay sampling sites A–F (see Table 1), and the location of Moreton Bay on the southeast coast of Queensland, Australia (inset)

resuspended in PBS for microscopic evaluations to detect and quantify *Perkinsus* sp. hypnospores.

Histology slides of cockles from the sites with the highest infection prevalences (Wynnum and Manly; Sites F and E in Fig. 1) were examined by light microscopy for pathological alterations, and for *Perkinsus* sp. pathogen cells.

Since data from the 2011 survey revealed a high prevalence of perkinsosis in cockles from Wynnum, we returned to that site in August 2013 to collect 10 cockles from which to propagate *Perkinsus* sp. *in vitro* isolates. In this paper, the term '*in vitro* isolate' is used in the traditional microbiological sense to specify axenic *Perkinsus* sp. isolate cultures that were propagated *in vitro*. Duplicate gill tissue samples from 10 cockles were aseptically excised, separately inoculated into antimicrobial-supplemented RFTM (including 25 g $l^{-1}$ of sodium chloride) in wells of sterile 24-well plates and incubated at 27°C for 48 h. Antimicrobials and their concentrations included penicillin (500 U $ml^{-1}$), streptomycin (500 µg $ml^{-1}$), gentamicin (500 µg $ml^{-1}$), chloramphenicol (25 µg $ml^{-1}$) and nystatin (300 U $ml^{-1}$).

### *In vitro Perkinsus* spp. isolate propagation

For *in vitro Perkinsus* spp. propagation, one of each pair of duplicate samples of RFTM-incubated gill tissue was stained with Lugol's iodine and observed under a stereomicroscope to identify cockles with heavily infected gill tissues. Unstained duplicate gill tissues from 6 cockles with the highest abundances of enlarged *Perkinsus* sp. hypnospores were transferred to 960 mOsm $kg^{-1}$ DME:Ham's F-12 propagation medium (DME/F12-3, Sigma components) containing antimicrobials (Burreson et al. 2005). Tissue samples were disrupted and suspended by pipet trituration, and the resulting suspensions were dispensed at 2 ml $well^{-1}$ into wells of sterile, lidded, 24-well plates. Antimicrobials and their concentrations included penicillin (100 U $ml^{-1}$), streptomycin (100 µg $ml^{-1}$), gentamicin (50 µg $ml^{-1}$), chloramphenicol (5 µg $ml^{-1}$) and nystatin (200 U $ml^{-1}$). The lipid supplement (Sigma L5146) to the DME/F12-3 propagation medium was initially included at 0.05% (v/v), half the concentration used by Burreson et al. (2005). Covered well plates containing primary isolate inocula were incubated at 27°C for 30 d, with daily microscopic observations to detect proliferation of *Perkinsus* sp. pathogen cells.

Isolate proliferation was confirmed in homogenates of gill tissues from 1 of the cockles 10 d after inoculation into DME/F12-3 culture medium, and proliferating parasite cell populations were sub-cultured for several passages in which antimicrobials were sequentially reduced and eliminated from culture media. Two axenic isolate cultures that were propagated from gill tissues of the same cockle were subsequently expanded in culture flasks and cryopreserved. Following isolate cryopreservation in Brisbane, Australia, 3 replicate suspensions of live cells of each isolate were transferred to the Maryland Department of Natural Resources (DNR) laboratory (Oxford, MD, USA), where each isolate was separately expanded and cryopreserved. At each stage where individual isolate cell lines were expanded and cryopreserved, cell pellets of each subculture were frozen, or were preserved in sterile vials containing 1 ml of 100% ethanol, for PCR amplifications and DNA sequencing.

Cells of both polyclonal isolates were sub-cultured in T25 tissue culture flasks at similar low densities, at 27°C in DME/F12-3 nutrient medium. Morphological cell characteristics were recorded with an inverted microscope equipped with Hoffman modulation contrast optics.

### PCR assays, DNA sequencing and phylogenetic analyses of *in vitro* isolates

DNA was first extracted at UQ from cells of both *in vitro Perkinsus* sp. isolates, using the DNeasy Tissue Kit (Qiagen) following the manufacturer's protocol. ITS regions of the ribosomal RNA gene complex were amplified by PCR with the *Perkinsus* genus-specific primers PerkITS-85 and PerkITS-750 (Casas et al. 2002), using previously described amplification parameters (Burreson et al. 2005). The presence of *P. olseni* within isolate cultures was confirmed by species-specific PCR with the primers PolsITS-140F and PolsITS-600R (Moss et al. 2006). The positive PCR control for both assays was DNA extracted from the *P. olseni* isolate ATCC PRA 205 (Dungan et al. 2007).

DNA fragments amplified with the *Perkinsus* genus-specific primers were purified using a Qiagen QIAquick® PCR purification kit according to the manufacturer's protocol. PCR products were sequenced bidirectionally on an AB3730xl capillary sequencer at the Australian Genome Research Facility in Brisbane. Isolate cells were then shipped to the DNR laboratory for diagnostic confirmation and *in vitro* characterization. Additional genus-specific and *P. olseni*-specific PCR fragments were cloned and sequenced at the Virginia Institute of Marine Science (VIMS) as previously described (Moss et al. 2008). At

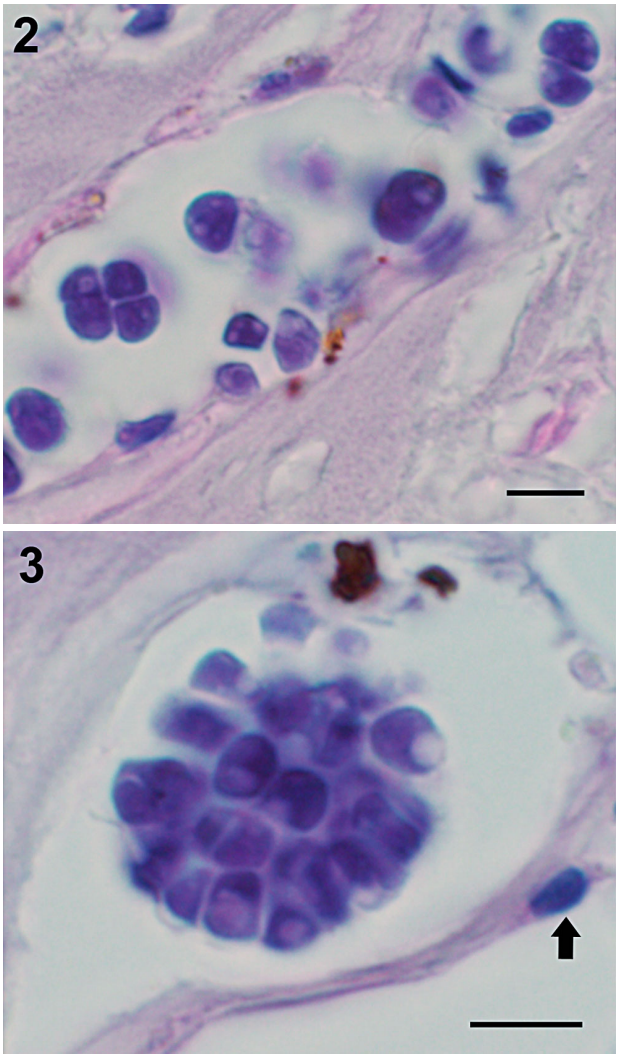
both facilities, sequences were quality scored and visually checked for possible sequencing errors before they were analysed using the basic local alignment search tool (BLAST) online (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and were then aligned with available sequences for *Perkinsus* spp. from GenBank using the MUSCLE algorithm (Edgar 2004) in MacVector 12.6 (MacVector). Parsimony jackknife analysis was done with PAUP*4.0a136 (Swofford 2002), with gaps treated as missing and 100 random additions of 1000 replicates.

## RESULTS

### RFTM and histological assays

*Perkinsus* sp. infections were detected at variable prevalences of 4 to 100%, by RFTM assays of gill tissues of *Anadara trapezia* cockles in samples collected from 6 Moreton Bay sites during November and December 2011 (Table 1). The highest prevalences of 98 to 100% were detected among cockle samples from 2 inshore sites (Wynnum and Manly) near the mouth of the Brisbane River, and lower prevalences of 4 to 23% were detected among samples from 4 sites on the western, bayside shore of North Stradbroke Island (Fig. 1). In 2013, the prevalence determined by examination of RFTM-incubated gill tissues of Wynnum cockles was also 100% (n = 10).

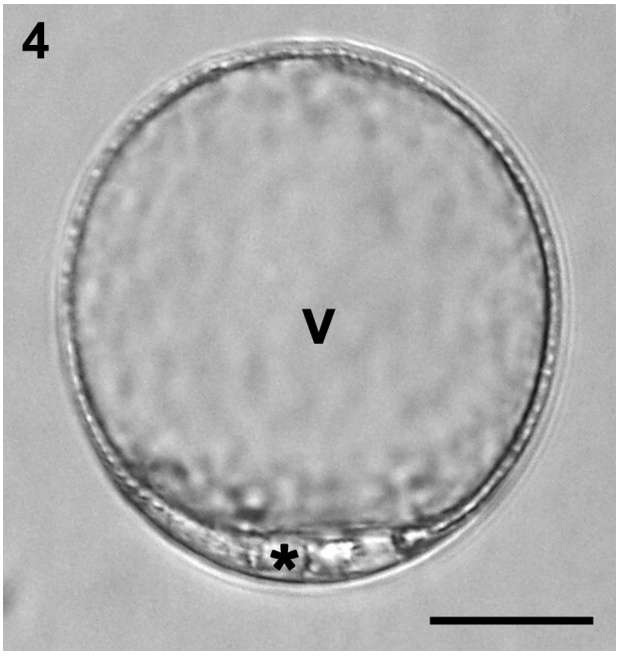
Histological lesions were confirmed among gill or visceral connective tissues of a subsample of 2/19 cockles that were preserved for histological analyses from the December 2011 sample from Wynnum (Table 1). Active proliferation at the time of fixation was evident among *Perkinsus* sp. cells in all histological lesions, where basophilic pathogen cells with diameters of 2 to 5 µm occurred in groups of 2 to 4 clustered sibling trophozoites (Fig. 2), or in schizonts containing more than 20 sibling progeny cells (Fig. 3). Trophozoites showed both the large eccentric vacuoles and eccentric nuclei with prominent nucleoli that characterize *Perkinsus* spp. Haemocytes infiltrated *A. trapezia* host tissues surrounding *Perkinsus* sp. lesions at low or moderate densities, but phagocytosis of *Perkinsus* sp. cells was not observed. Haemocytes infiltrating infected tissues were amoebocytes, rather than the nucleated erythrocytes that also circulate in *A. trapezia* haemolymph (Dang et al. 2013). Amoebocyte abundances were relatively low in *A. trapezia* connective tissues, and increased moderately in proximity to colonizing *Perkinsus* sp. cells.

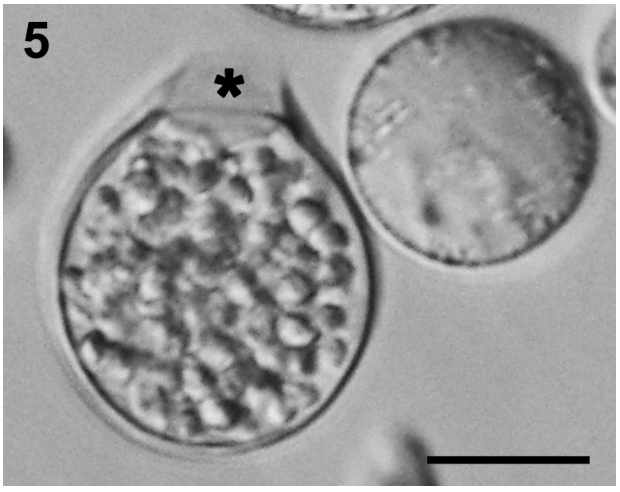


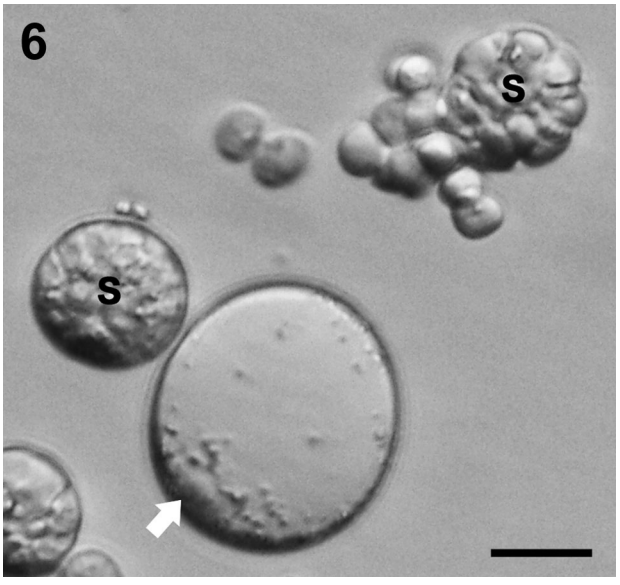

Figs. 2 & 3. Proliferating *Perkinsus* sp. cells in gill lesions of a Wynnum (Moreton Bay) *Anadara trapezia* cockle. Fig. 2. *Perkinsus* sp. signet ring trophozoites and proliferating 4-cell and 2-cell schizonts in gill arch connective tissue. Scale bar = 5 µm. Fig. 3. Multicellular *Perkinsus* sp. schizont in connective tissue of a gill arch with an infiltrating host amoebocyte (arrow). Scale bar = 10 µm

### *In vitro* isolates

Two isolates were separately propagated *in vitro* from *Perkinsus* sp. cells that enlarged among RFTM-incubated gill tissues from a single *A. trapezia* cockle. Both isolates showed *in vitro* cell types similar to those described for other *Perkinsus* sp. *in vitro* isolates, including large trophozoites that may represent prezoosporangia (Fig. 4), proliferative zoosporangia containing motile zoospores and zoospore discharge structures (Fig. 5), trophozoites with signet ring morphologies, and proliferative schizonts (Fig. 6).




Figs. 4 to 6. Cell types among proliferating *in vitro Perkinsus* sp. polyclonal cultures from a Moreton Bay *Anadara trapezia* cockle. Fig. 4. Large signet ring prezoosporangium cell with an eccentric nucleus (*) and a large central vacuole (V). Fig. 5. Zoosporangium cell containing motile zoospores and showing an extended zoospore discharge tube (*). Fig. 6. Large trophozoite showing an eccentric nucleus (arrow), and 2 adjacent schizonts (S). The younger schizont at left shows proliferative subdivisions internally and a smooth margin, and the mature schizont at upper right is releasing adherent sibling progeny cells. Scale bars = 20 µm

### Molecular-genetic characteristics of *in vitro* isolates

DNAs from cells of both *in vitro* isolate cultures were successfully amplified by the *Perkinsus* genus-specific PerkITS-85/PerkITS-750 PCR assay in the Australian (UQ) and US (VIMS) laboratories. Sequences of those amplicons obtained in Australia from early isolate DNAs indicated that isolate 1 contained primarily *P. chesapeaki* cells (Fig. 7, 1-AUS-KM983400) and that isolate 2 was predominantly *P. olseni* (Fig. 7, 2-AUS-KM983404), while the amplicons obtained at VIMS from DNA isolated from later subcultures of both isolates were primarily *P. chesapeaki*. Species-specific PCR and sequencing also confirmed *P. olseni* in early preserves of isolate 2, and also in subcultures of isolate 1. Parsimony jackknife analysis grouped rDNA-ITS region sequences from the current *Perkinsus* spp. isolates with those of *P. chesapeaki* and *P. olseni* deposited in GenBank, with jackknife support values of 100 and 98, respectively (Fig. 7). Sequences were deposited in GenBank under accession numbers KM983400–KM983419.

## DISCUSSION

### *Perkinsus* sp. infections among Moreton Bay *Anadara trapezia*

The high prevalences of *Perkinsus* sp. infections that were detected by RFTM assays of mud ark cockles sampled at Wynnum and Manly are consistent with numerous historic reports of such infections among Moreton Bay *A. trapezia* collected at those sites (Goggin et al. 1989, 1990, Goggin & Barker 1993, Goggin 1994, Murrell et al. 2002). This is the first study to document the prevalences of *Perkinsus* sp. infections in *A. trapezia* at different sites in Moreton Bay, Queensland, where the highest prevalences of 98 to 100% were detected among *A. trapezia* samples from mainland sites in western Moreton Bay near the mouth of the Brisbane River (Fig. 1, Sites E and F).

Among limited histological samples that were available from the cockles surveyed during 2011 by the current investigation, histological lesions containing proliferating *Perkinsus* sp. cells were documented here for the first time in *A. trapezia*. *Perkinsus* sp. cells in histological lesions showed the eccentric vacuoles and nuclei that are distinctive and typical among members of the genus, and they occurred as large groups of sibling progeny cells within schizonts, and also as pairs and quadrads of dividing sibling trophozoites. Although host amoebocytes infiltrated infected tissues at densities that increased moderately with proximity to *Perkinsus* sp. pathogen cells, the relatively low numbers of infiltrating amoebocytes suggested only a modest defensive response to *Perkinsus* sp. infections by *A. trapezia* hosts in our samples.

### *Perkinsus* sp. isolates

It was surprising to find that 1 *A. trapezia* from Wynnum was co-infected by 2 pathogen species: *P. olseni* and *P. chesapeaki*. This is the first report of *P. chesapeaki* infecting an Australian mollusc and is the first report of *in vitro Perkinsus* sp. isolate cultures from Australia.

Cell types that occurred among *Perkinsus* sp. isolate cultures that were propagated *in vitro* from Moreton Bay *A. trapezia* cockles were generally similar to cell types reported for other *in vitro Perkinsus* sp. isolates from diverse mollusc hosts worldwide.

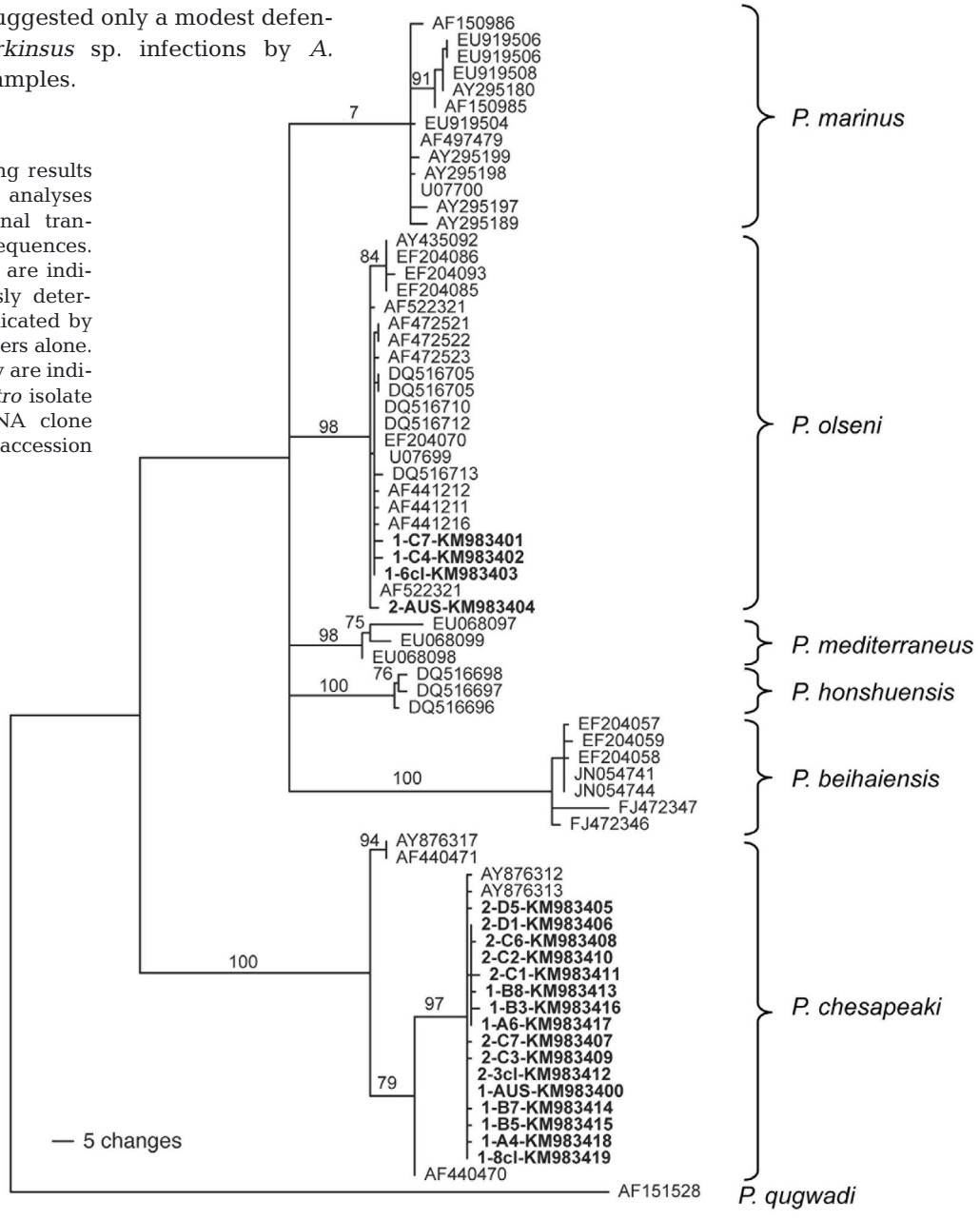



Fig. 7. Phylogram showing results of maximum parsimony analyses on *Perkinsus* spp. internal transcribed spacer region sequences. Jackknife support values are indicated at nodes. Previously determined sequences are indicated by GenBank accession numbers alone. Sequences from this study are indicated in **bold**, with *in vitro* isolate number followed by DNA clone number and GenBank accession number

These included large apparent prezoosporangia with eccentric nuclei and large central vacuoles, zoosporangia containing numerous motile zoospores and bearing plugged discharge pores, and trophozoites that proliferated by internal schizogony to yield clusters of numerous sibling progeny trophozoites (Perkins 1966, Sunila et al. 2001, Burreson et al. 2005, Dungan et al. 2007).

### *P. chesapeaki* and *P. olseni* in *A. trapezia*

We expected to find *P. olseni* in the current investigation, since it was previously reported extensively in *A. trapezia* from Moreton Bay (Goggin & Barker 1993) and is also reported to infect numerous other Australian molluscs, including *Tridacna gigas* (see Goggin 1996), *Haliotis ruber* (see Lester & Davis 1981) and *Chama pacificus* (see Murrell et al. 2002). *P. olseni* also infects numerous other bivalves around the world, including *R. philippinarum* in Korea, Japan and Europe (Navas et al. 1992, Choi & Park 1997, Park & Choi 2001, Elandaloussi et al. 2009, Dang et al. 2010), *R. decussatus* in Europe (Leite et al. 2004) and *Pitar rostrata* in Uruguay (Cremonte et al. 2005). However, this is the first report of *P. chesapeaki* in Australia.

*P. chesapeaki* was originally described in the clam *Mya arenaria* from Chesapeake Bay in North America (McLaughlin & Faisal 2000, McLaughlin et al. 2000) and from the razor clam *Tagelus plebeius* in Delaware Bay and Chesapeake Bay (Dungan et al. 2002, Bushek et al. 2008). When *P. andrewsi* and *P. chesapeaki* were synonymized (Burreson et al. 2005), that extended the *P. chesapeaki* host range to 5 other clams in Chesapeake Bay, including *Macoma balthica*, *M. mitchelli*, *Mercenaria mercenaria*, *Mulinia lateralis*, *Rangia cuneata* and *Cyrtopleura costata* (Burreson et al. 2005, Reece et al. 2008). More recently, *P. chesapeaki* was reported in *R. philippinarum* and *R. decussatus* clams in France (Arzul et al. 2012) and in *Cerastoderma edule* cockles in Spain (Carrasco et al. 2014), extending its geographic distribution to Europe and its host range to 3 new mollusc species. The current report extends its distribution to the southern hemisphere in Australia. Arzul et al. (2012) hypothesised that *P. chesapeaki* may have been introduced to France through importation of infected *M. mercenaria* from the USA. Our report begs a similar question of whether and how *P. chesapeaki* may have been introduced to Australia, especially since a relatively recent Australian survey found no such evidence (Murrell et al. 2002).

Co-infections by *P. olseni* and *P. chesapeaki* were also reported in *R. philippinarum* clams from France (Arzul et al. 2012), and co-infections by other *Perkinsus* species have also been described. Co-infections by *P. marinus* and *P. chesapeaki* occasionally occur in *Mya arenaria* clams from Chesapeake Bay (Reece et al. 2008), co-infections by *P. olseni* and *P. honshuensis* occur in Japanese *R. philippinarum* clams (Takahashi et al. 2009), and *P. marinus* and *P. olseni* co-infect *Crassostrea gasar* oysters in Brazil (da Silva et al. 2014).

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