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ORIGINAL PAPER

Detection of the bacterial endosymbiont *Neorickettsia* in a New Zealand digenean

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Abstract *Neorickettsia* are endosymbiotic bacteria that infect digeneans (Trematoda). These bacteria are of interest worldwide because of their ability to move from the parasite to its host, where they can cause serious diseases of humans and animals. While several disease-forming species of *Neorickettsia* have been well studied, and numerous *Neorickettsia* types have been identified in regions such as North America and parts of Asia, records from other locations are sparse. To date, there have been no reports of *Neorickettsia* from New Zealand. We screened ten species of digeneans infecting seven native gastropod species (both marine and freshwater) found near Dunedin, New Zealand, for the presence of neorickettsial infections. A >1300 bp long section of 16S rRNA belonging to a *Neorickettsia* bacterium was isolated from opecoelid digeneans of two individuals of the mudflat topshell snail *Diloma subrostrata*. These sequences represent the first evidence of neorickettsial infection in native New Zealand animals and are also the first *Neorickettsia* found in digeneans of the family Opecoelidae.

Keywords Parasitism · Trematodes · Endosymbionts · Anaplasmataceae · Rickettsiales

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Introduction

There is a growing recognition that microbial symbionts are playing key roles in the interaction between helminth parasites and their hosts, modulating processes ranging from host resistance to parasite virulence (Dheilly et al. 2015). Already many bacterial symbionts have been isolated from helminth parasites. For example, *Neorickettsia* is a genus of proteobacteria belonging to the Order Rickettsiales whose members form symbioses with digenean parasites. Digeneans (Platyhelminthes, Trematoda) are parasitic flatworms with complex life cycles, infecting a diverse range of vertebrates and invertebrates. Briefly, the typical digenean life cycle consists of eggs released from the definitive (vertebrate) host, which infect a mollusc (first intermediate host) and form sporocysts or rediae; these sporocysts or rediae produce cercariae which are released into the environment and enter the second intermediate host, where they develop into metacercariae which reinfect the definitive host when the latter ingests the second intermediate host. *Neorickettsia* spp. are vertically transmitted through the various life cycle stages of their digenean hosts, and can also be horizontally transmitted from parasite to host (Vaughan et al. 2012). The latter transmission strategy is responsible for several well-studied diseases of humans and animals (reviewed in Vaughan et al. 2012).

Neorickettsia have been identified worldwide, though the majority of isolates have come from locations where neorickettsial diseases of vertebrates are well known, such as salmon dog poisoning (Cordy and Gorham 1950; Philip et al. 1953) and Potomac horse fever in the USA (Rikihisa and Perry 1985; Palmer 1993), and Sennetsu fever in Japan (Fukuda et al. 1954; Misao and Kobayashi 1954; Misao and Katsuta 1956) and southeast Asia (Holland et al. 1985; Newton et al. 2009). Conversely, there is a paucity of data

on *Neorickettsia* from locations where they have not been identified as disease-causing agents, such as Europe, Africa and Australasia (Vaughan et al. 2012).

There are currently ~80 recognised digenean families (Olson et al. 2003), and neorickettsial DNA sequences have so far been detected in members of approximately half of these families (Tkach et al. 2012; Vaughan et al. 2012; Greiman et al. 2014). In this study, we screened digeneans representing ten species and seven families, which were obtained from a range of freshwater and marine gastropods (Mollusca, Gastropoda) found near Dunedin, South Island, New Zealand, for the presence of *Neorickettsia* spp. bacteria. Our results suggest that, while apparently not widespread among local digeneans, neorickettsial infections are present in this area. The neorickettsial sequence obtained here is the first to be found in an opecoelid digenean, and appears to represent a new *Neorickettsia* genotype.

Methods

Sample collection

Gastropod snails known to harbour digenean parasites (Leung et al. 2009; Macfarlane 1939) were collected from several locations around Dunedin, New Zealand, throughout the course of 2015 (Table 1). All sampled snail species are marine, except for *Potamopyrgus antipodarum* which inhabits freshwater. Due to varying prevalence of infection among digenean species, the total number of snails collected varied among species, but a minimum of 30 individuals infected with each parasite species were used for subsequent analysis. Snails were returned alive to the laboratory immediately following collection and maintained in aquaria until use. Digenean cercariae or sporocysts were isolated from infected snails for molecular analysis using the following methods: shedding of cercariae was induced by (1) incubating the snails in 12-well plates containing filtered seawater at 25 °C for 2.5 h (*Zeacumantus subcarinatus*), or (2) incubating in 12-well plates containing filtered seawater at room temperature for 8 h on an orbital shaker set at 80 rpm (*Austrolittorina cincta*). For snail species which could not be induced to shed cercariae, sporocysts (*Amphibola crenata*, *Diloma aethiops*, *Diloma subrostrata*, *Potamopyrgus antipodarum*) or cercariae (*Cominella glandiformis*) were removed from snails by breaking open the snails and carefully removing the parasites. In all cases, at least 30 cercariae or sporocysts were collected from each individual snail. Parasites were identified morphologically, and identities were confirmed by ITS sequencing of representative samples of each species. All digenean samples were stored in 99 % ethanol until use.

Molecular screening

Genomic DNA was extracted from digeneans using either the Qiagen DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, California) or the Invitrogen PureLink Genomic DNA kit (Thermo Fisher Scientific, Waltham, Massachusetts) following the manufacturer's instructions. Nucleic acid was eluted into a final volume of 200 μL, then concentrated to ~50 μL using an Eppendorf Concentrator Plus (Eppendorf, Hamburg, Germany).

Nested PCRs were carried out using a slightly modified version of the protocol used by Greiman et al. (2014). Primers used in the first PCR round were n16S-25F (5-TCAGAACGAACGCTAGCGGT-3) and n16S-1500R (5-

Table 1 Sampling locations (with latitude and longitude) and digenean/host species used in this study

Gastropod species	Digenean species	Family	Life cycle stage	Sampling site
Amphibola crenata	Unidentified	Unidentified	Sporocyst	Purakaunui Inlet 45.76S, 170.63E
Austrolittorina cincta	*Parorchis* sp.	Philophthalmidae	Cercaria	Lower Portobello Bay (LPB) 45.83S, 170.67E
	Unidentified Notocotylid	Notocotylidae	Cercaria	LPB
Cominella glandiformis	*Curtuteria* sp.	Echinostomatidae	Cercaria	LPB
Diloma aethiops	Opecoelid sp. A	Opecoelidae	Sporocyst	LPB
Diloma subrostrata	Opecoelid sp. A	Opecoelidae	Sporocyst	LPB
Potamopyrgus antipodarum	*Coitocaecum parvum*	Opecoelidae	Sporocyst	Tomahawk Lagoon 45.90S, 170.54E
Zeacumantus subcarinatus	*Acanthoparyphium* sp.	Echinostomatidae	Cercaria	LPB
	Galactosomum sp.	Heterophyidae	Cercaria	LPB
	Maritrema novaezealandensis	Microphallidae	Cercaria	LPB
	Philophthalmus sp.	Philophthalmidae	Cercaria	LPB

AAAGGAGGTAATCCAGCCGCAGGTTCAC-3), and in the second round were n16S-50F (5-TAGGCTTAACACAT GCAAGTCGAACG-3) and n16S-1400R-1 (5-CGGTYAGCTCACTAGCTTCGAGTAA-3). Each reaction contained 200 pM each primer, 200 μM each deoxynucleotide triphosphate, 3 mM (first round) or 1.5 mM (second round) $MgCl_2$, 1 unit FIREPol DNA polymerase (Solis BioDyne, Tartu, Estonia) and 1 x FIREPol reaction buffer in a total volume of 25 μl. Template consisted of 4 μl of extracted genomic DNA (first round) or 2 μl of first-round PCR product (second round). Both first and second round PCRs were run for 35 cycles with an annealing temperature of 56 °C. PCR products were purified using the MEGAquick-spin DNA Purification kit (iNtRON Biotechnology, Seongnam, Korea). Sequencing was carried out with the primers n16S-50F and n16S-1400R, along with internal primers 16S-n900F (5-GACTCGCACAAGCGGTGGAGTAT-3) and 16S-n900R (5-ATACTCCACCGCTTGTGCGAGTC-3) (Greiman et al. 2014) using an ABI 3730xl DNA Analyser at Genetic Analysis Services, University of Otago, New Zealand.

Contiguous sequences were assembled using Geneious version 8.0.5 (Biomatters, Auckland, New Zealand) and were searched against the GenBank nucleotide database using BLAST within the Geneious program. Newly generated sequences and BLAST matches were aligned with ClustalW (Thompson et al. 1994), and phylogenetic analysis was carried out using PHYML (Guindon and Gascuel 2003) with the HKY85 substitution model and 100 bootstrap replicates.

Results

Approximately 330 snails, containing 10 species of digenean (Table 1), were screened for the presence of neorickettsial symbionts. Nucleic acid amplification was successful for all digeneans sampled, and samples from several digenean species produced PCR products of the correct size, but sequencing revealed that most of these products belonged to the host digenean or non-*Neorickettsia* spp. bacteria. Two individuals of the mudflat topshell snail, *Diloma subrostrata*, however, were positive for neorickettsial infection. The digenean parasite from which those sequences were obtained is Opecoelid sp. A (Leung et al. 2009).

The two sequences obtained from *D. subrostrata* were 1344 and 1352 bp long, and appear to represent a new genotype of *Neorickettsia*. The nearest BLAST match, with a pairwise identity of >98 %, belonged to a *Neorickettsia* sp. bacterium isolated from *Bacciger sprenti*, a fish-infecting digenean belonging to the family Faustulidae (Fig. 1). Together, these three sequences form a distinct and well-supported clade (the *B. sprenti* symbiont previously being the only member of *Neorickettsia* Clade I (Greiman et al. 2014)). This clade also contains the only *Neorickettsia* sequences found so far in Australasia. The two new neorickettsial sequences have been deposited in GenBank (accession numbers KX379496-KX379497).

Whether all digeneans within a single host were infected is unknown. Individual cercariae from several other *D. subrostrata* snails were isolated and screened, but despite successful DNA extraction no *Neorickettsia* sequences were obtained.

Discussion

We have provided here the first report of *Neorickettsia* sp. bacteria in New Zealand. The two 16S sequences obtained are also the first *Neorickettsia* sequences to be found in members of the digenean family Opecoelidae. Interestingly, the closest relative of these bacteria was isolated from *Bacciger sprenti*, a member of a different family (Faustulidae); both species are members of the suborder Xiphidiata however (Olson et al. 2003). The high level of identity between our sequences and the *B. sprenti*-derived sequence, which was isolated in Australia but infects a genetically distant digenean, suggests a lack of coevolution between bacterium and digenean host, and that the digenean-bacterium association is determined in part by availability of hosts in a given geographical region. This trend was also seen in other *Neorickettsia*-digenean associations in the phylogeny produced here.

Most of the host species examined did not appear to be infected, and the prevalence of infection in *Diloma subrostrata* was very low (~7 % of individuals screened). It is possible that we have underestimated the prevalence of *Neorickettsia* spp. in these snails, as the PCR assays may have not detected all potential *Neorickettsia* sequences. The primers used here amplified all existing *Neorickettsia* 16S sequences in silico, but given the relatively small number of these sequences that currently exist, further *Neorickettsia* species may be detected with the use of more generic 16S primers and next-generation sequencing, or by taking a metagenomics approach. Additionally, given the low infection rate seen in *D. subrostrata*, increased sampling effort may reveal *Neorickettsia* infections in the other species studied here. Furthermore, our sampling was limited to the area within and around Otago Harbour, and additional sampling from different regions of New Zealand may well identify further *Neorickettsia*-digenean associations.

The life cycle of the opecoelid parasite is currently unknown (Miller and Poulin 2001); thus the other animal species potentially infected by the neorickettsial symbiont cannot yet be identified. The other hosts involved in this parasite's life cycle probably include a crustacean or other invertebrate as second intermediate host, and a fish as definitive host, as is the common pattern among opecoelids. Given that *D. subrostrata*

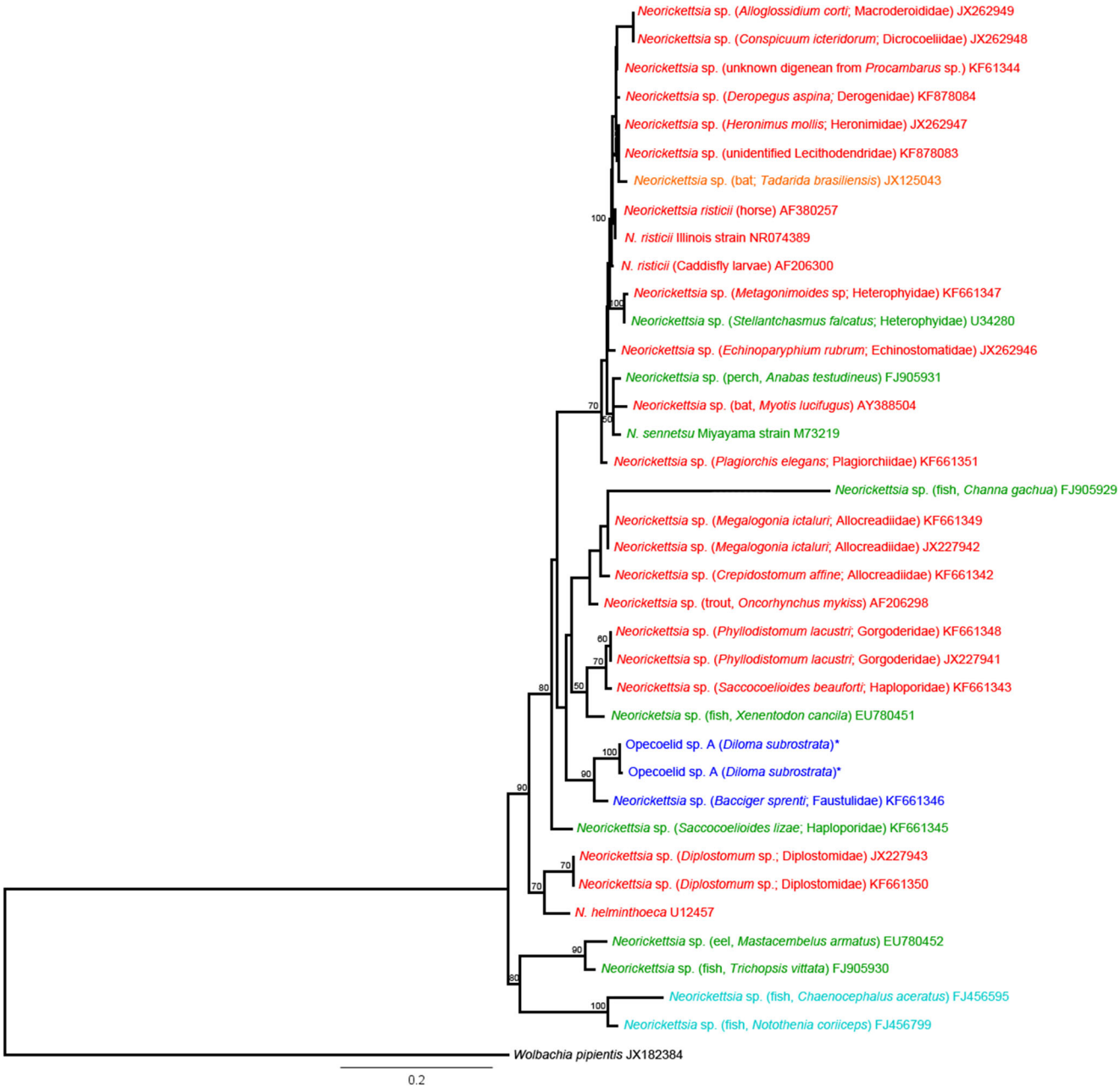

Fig. 1 Maximum likelihood tree of *Neorickettsia* sp. 16S sequences from the opecoelid digenean infecting *Diloma subrostrata* (marked with *asterisks*) and nearest BLAST matches. *Colours* represent the geographical region from which the *Neorickettsia* were isolated: North America (*red*), South America (*orange*), Asia (*green*), Australasia (*blue*), Southern Ocean (*turquoise*). Species names in parentheses are the host digenean where known, or the animal from which the bacterium was isolated. *Wolbachia pipentis* was chosen as the outgroup. *Branch numbers* represent bootstrap support (100 replicates); bootstrap values <50 are not shown. *Scale bar* represents number of nucleotide substitutions per site

is, numerically speaking, one of the dominant gastropods in the soft sediment habitat of Otago Harbour (Logan 1976; Mitchell 1980), it seems likely, even with the low infection rate seen here, that other local marine species have the potential to acquire the infection.

As this was a preliminary study aiming to determine the presence and prevalence of neorickettsial infections, we were unable to determine what physiological or behavioural responses the bacteria may cause in the digenean or gastropod hosts. Given that *Neorickettsia* spp. are the aetiological agents of several serious diseases, such as salmon dog poisoning (Philip et al. 1953, 1954), Potomac horse fever (Rikihisa and Perry 1985) and Sennetsu fever (Fukuda et al. 1954), investigating the potential effects of this newly discoverd *Neorickettsia* sp., along with a more expansive survey across New Zealand, will be the next important steps in this research.

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