First detection of *Wolbachia* in the New Zealand biota

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Abstract

*Wolbachia* is one of the most widespread intracellular bacteria on earth, estimated to infect between 40 and 66% of arthropod species in most ecosystems that have been surveyed. Their significance rests not only in their vast distribution, but also in their ability to modify the reproductive biology of their hosts, which can ultimately affect genetic diversity and speciation of infected populations. *Wolbachia* has yet to be formally identified in the fauna of New Zealand which has high levels of endemic biodiversity and this represents a gap in our understanding of the global biology of *Wolbachia*. Using High Throughput Sequencing (HTS) of host DNA in conjunction with traditional molecular techniques we identified six endemic Orthoptera species that were positive for *Wolbachia* infection. In addition, short-sequence amplification with *Wolbachia* specific primers applied to New Zealand and introduced invertebrates detected a further 153 individuals positive for *Wolbachia*. From these short-range DNA amplification products sequence data was obtained for the ftsZ gene region from 86 individuals representing 10 host species. Phylogenetic analysis using the sequences obtained in this study reveals that there are two distinct *Wolbachia* bacteria lineages in New Zealand hosts belonging to recognised *Wolbachia* supergroups (A and B). These represent the first described instances of *Wolbachia* in the New Zealand native fauna, including detection in putative parasitoids of infected Orthoptera suggesting a possible transmission path. Our detection of *Wolbachia* infections of New Zealand species provides the opportunity to study local transmission of *Wolbachia* and explore their role in the evolution of New Zealand invertebrates.

Introduction

The bacterium *Wolbachia* [1,2] is estimated to infect between 40 and 66% of arthropod species worldwide [3–5] making it among the most abundant intracellular bacterial genera. *Wolbachia* is a maternally inherited endosymbiont that can induce a range of host phenotypic responses, including cytoplasmic incompatibility, male death, feminization, and parthenogenesis [6–10]. *Wolbachia* infections can therefore have long-term evolutionary effects on their host lineages, in addition to immediate reproductive modifications, by providing pathways to rapid reproductive isolation and influencing the evolution of sex-determining mechanisms [6,7,9–11].
Wolbachia is also being trialled as a biocontrol agent of invasive and disease transmitting insects including medflies [12] and mosquitoes as part of the Eliminate Dengue Program [13–15]. As Wolbachia can become an obligate parasite of parasitic worms it is also the target of research into antimicrobial drugs by the Anti-Wolbachia Consortium, with the goal of preventing the growth and reproduction of the worms and preventing the diseases they induce [16,17].

Wolbachia prevalence differs among species and among populations of the same species, ranging in infection frequency between 30 and 100% of individuals within a population [18,19]. Infection rates are a complex issue not yet well understood, but they are likely to be dynamic, and involve host dispersal and the nature of the host-parasite relationship. For example it has been suggested that the degree of infection may be a result of Wolbachia acting as a mutualistic secondary symbiont rather than an exclusive reproduction parasite [20–22].

The mechanism(s) by which Wolbachia moves between host populations has yet to be confirmed, but is unlikely to be solely via vertical transmission. Genetic similarity of Wolbachia found in parasitoids and parasitoid hosts suggest horizontal transmission [23–25]. It has been shown that microinjection of Wolbachia infected cells can facilitate transfer [26], and this indicates how Wolbachia might be transferred by ovipositing parasitoids. Should the parasitoid egg fail to develop, Wolbachia may move into the host and persist into further generations. Alternatively, the bacterium may be transferred through the digestive system of invertebrates feeding on Wolbachia infected hosts as in some other endoparasites (e.g. Gordian worms). Horizontal transmission via the digestive track has been shown to be effective in whiteflies. Wolbachia was observed to persist in leaves for up to 50 days, which if fed upon by un-infected whiteflies, resulted in Wolbachia infections in the majority of whiteflies [27].

Phylogenetic studies have identified 16 globally distributed supergroups of Wolbachia [10,28–32]. Incongruence between Wolbachia and host phylogenies suggests many episodes of horizontal transfer resulting in unrelated hosts in the same region sharing similar strains of Wolbachia [25,33]. However, inference of phylogenetic relationships is also complicated by recombination among Wolbachia strains [28,34,35], and host–parasite coevolution [36]. For this reason, a Multilocus Sequence Typing (MLST) system is now widely used as it allows differentiation between even closely related strains of Wolbachia [37].

The New Zealand invertebrate fauna has many distinctive features including high levels of species endemicity [38]. As a large continental island, physically isolated from neighbouring terrestrial ecosystems for many millions of years, the biota has had opportunities to evolve in novel ways and it is frequently posited that the biota has been strongly influenced by their ancient isolation [39,40]. If so, this predicts that distinctive species interactions could have evolved including unique strains of endosymbionts such as Wolbachia. However, to date no Wolbachia infections have been reported from any New Zealand native invertebrate species, reflecting few targeted attempts at their discovery. We tested whether Wolbachia could be detected and if so whether there was evidence of distinctive evolutionary lineages in endemic New Zealand insects.

Methods

Two different approaches were employed to survey potential hosts for Wolbachia infection; bioinformatics and molecular ecology. The first approach made use of bioinformatic tools to search for evidence of Wolbachia ‘contamination’ in High Throughput Sequencing (HTS) data (reads and assembled contigs) from various insects. These low coverage DNA sequence data-sets were produced to infer molecular phylogenies of the invertebrate species using multicopy markers (e.g. [41,42]. The second approach used the MLST primer sets [37] to search for
evidence of Wolbachia in a wide range of target templates representing multiple host species and populations.

Mining next generation DNA sequences

As part of a phylogenomic study of endemic New Zealand Orthoptera that have distinctive regional diversity, we carried out HTS of genomic DNA isolated from members of three families: Acrididae, Anostostomatidae, and Rhaphidophoridae (Table 1). The DNA libraries were sequenced on one lane of an Illumina HiSeq2000 by BGI [43]. Approximately 1–4 Gigabytes of 100bp paired end sequencing data was generated for each of the 21-sequenced species.

HTS data was analysed with PAUDA [44], and MEGAN5 [45] in order to identify Wolbachia sequences found within each HTS dataset. MEGAN5 [45] visually displays what organisms are detected in the HTS datasets and indicates the number of sequences associated with each species. If Wolbachia matches were among the 16 most frequently recorded organisms detected at the level genus, the respective invertebrate host sample was treated as positive for Wolbachia infection.

Wolbachia sequences from the positive samples were extracted and mapped against the genome of the Wolbachia endosymbiont of Drosophila melanogaster (accession number NC002978) using Geneious. v. 6 (http://www.geneious.com) [46]. Mapping was performed using the medium sensitivity setting, which equates to a minimum overlap of 25bp, at least 80% overlap identity, with a maximum of 30% mismatches allowed per read. Mapping was iterated five times using the consensus sequence of the reads and repeating the mapping

Table 1. Abundance of Wolbachia-like sequence reads in HTS from endemic New Zealand Orthoptera.

| Order  | Family             | HTS Specimen | Location       | Reads |
|--------|--------------------|--------------|----------------|-------|
| Orthoptera | Rhaphidophoridae | Macropathus sp. | Waitomo | 30817 |
| Orthoptera | Anostostomatidae  | Hemiandrus brucei | South Island | 17220 |
| Orthoptera | Rhaphidophoridae  | Talitrops sedilloti | Mohi Bush, Hawkes Bay | 2486 |
| Orthoptera | Rhaphidophoridae  | Miotopus diversus | Waioka Gorge, Gisborne | 2384 |
| Orthoptera | Rhaphidophoridae  | Neonetus sp.1 | Mohi Bush, Hawkes Bay | 1363 |
| Orthoptera | Rhaphidophoridae  | Neonetus sp.1 | Hongi’s Track, Rotorua | 1346 |
| Orthoptera | Rhaphidophoridae  | Isoplectron sp. | Canterbury | 154 |
| Orthoptera | Rhaphidophoridae  | Cave weta | Denniston | 126 |
| Orthoptera | Rhaphidophoridae  | Cave weta | Kapiti Island | 47 |
| Orthoptera | Rhaphidophoridae  | Macropathus sp. | Westport | 25 |
| Orthoptera | Anostostomatidae  | Hemiandrus focialis | Lake Taupo | 21 |
| Orthoptera | Anostostomatidae  | Hemideina crassidens | South Island | 20 |
| Orthoptera | Rhaphidophoridae  | Pharmacus chapmani | Old Man Range, Otago | 17 |
| Orthoptera | Acrididae          | Sigaus australis | Lindis Pass, South Island | 0 |
| Orthoptera | Rhaphidophoridae  | Novoplectron serratum | Chatham Island | 0 |
| Orthoptera | Rhaphidophoridae  | Pachyrhamma sp. | Balls Clearing, Hawkes Bay | 0 |
| Orthoptera | Anostostomatidae  | Hemideina thoracica | Manawatu | 0 |
| Orthoptera | Anostostomatidae  | Hemiandrus sp. | New Zealand | 0 |
| Orthoptera | Anostostomatidae  | Hemideina crassidens | North Island | 0 |
| Orthoptera | Anostostomatidae  | Motuweta riparia | North Island | 0 |
| Orthoptera | Anostostomatidae  | Hemiandrus pallilarius | Manawatu | 0 |

High Throughput Sequence samples used with location, number of sequences matching Wolbachia, and the relative abundance of Wolbachia-like sequences detected (Rank).

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process. This allows more reads to be mapped to variable regions or regions that differ from the reference sequence and reduces the likelihood of reads mapping incorrectly.

Once sequences were aligned to the reference genome, coverage at the five core gene loci of the MLST system (ftsZ, coxA, fpbA, hpcA, gatB [37]) was determined, by identifying the conserved PCR primer binding sites on the reference gene. Targeting primer binding sites allowed direct comparison between the HTS sequences and those obtained via PCR. Where there was sufficient HTS coverage the consensus sequence of mapped reads at MLST loci was included in subsequent phylogenetic analysis.

Extraction and amplification of Wolbachia from invertebrate DNA

We focused our host sampling on multiple individuals of the Orthopteran genus *Hemiandrus* (Family Anostostomatidae) that had yielded positive results in HTS analysis and for which we had suitable material[47]. DNA was extracted from leg or abdomen tissue of 204 individual *Hemiandrus* ground weta. We used a modified salting-out method incorporating an ice-cold ethanol washing step before addition of room temperature ethanol and allowing the ethanol to evaporate, leaving the DNA to be eluted in 50 μl water[48]. Extracted DNA was tested for the presence of *Wolbachia* DNA using the MLST primer combinations (Table 2). The 204 individuals represented 16 taxa (*H.* *nox*, *H.* *bilobatus*, *H.* 'disparalis', *H.* *electra*, *H.* 'elegans', *H.* *focalis*, *H.* 'furoviarius', *H.* 'horomaka', *H.* maculifrons, *H.* brucei, *H.* *luna*, *H.* nitaweta, *H.* 'onokis', *H.* 'promontorius', *H.* *subantarcticus*, and *H.* 'vicinus'), with *H.* *nox* represented by individuals from North Island and South Island [49].

*Wolbachia* infection rates in species and populations was tested using PCR targeting 5 MLST loci [37], and the variable WSP locus that has potential for distinguishing *Wolbachia* lineages [11,50]. In addition to the absence of *Wolbachia* in a sample, several technical issues could explain false negatives where amplification failed. Therefore, we used positive PCR controls with universal insect mitochondria primers LCO1490 and HCO2198 [51] to target host DNA. DNA from *Nasonia vitripennis* wasps known to be infected with *Wolbachia* was used to verify the specificity of the MLST primers used in this study.

We also searched for *Wolbachia* infection in DNA from 45 Rhaphidophoridae (cave weta) from the genera *Pachyrhamma* (n = 39) and *Isopleuron* (n = 6). To increase taxonomic range, we screened 40 individuals of 24 other invertebrate species (Table 2). Sixteen of these were exotic species and eight were New Zealand native or endemic panarthropoda species. We included nine samples of the parasitoid wasp *Archaeoteleia* because Rhaphidophoridae are their hosts [52], and this parasitic interaction is a potential means of horizontal transmission of *Wolbachia*. A subset of individuals that produced a DNA fragment for the *Wolbachia* ftsZ region were sequenced using the forward ftsZ [37] primer (Macrogen Inc., Korea). DNA sequences were checked for quality and aligned to published *Wolbachia* sequences (Table 3) and our sequences extracted from the HTS samples (Table 1) using Geneious v. 6 [46].

Eighty-six *Wolbachia* DNA sequences were aligned and trimmed to produce an alignment of 211–438bp of the ftsZ locus. Phyllogenetic relationships were inferred using Bayesian phylogenetic analysis (MrBayes; HKY, chain length 1100000, subsampling frequency 200, burn-in length 100000, random 22500) [53,54](Fig 1). Incorporating a published dataset [28] with a subset of data from the present survey (n = 11) allowed us to determine which supergroup the New Zealand *Wolbachia* sequences were most similar to (Fig 2). Minimum spanning network [55] (epsilon 0) analysis was performed using PopART [56] (Fig 3).

To determine the spatial distribution of the newly discovered *Wolbachia* infections in New Zealand, ground weta and cave weta collection locations were mapped using QGIS (QGIS Development Team, 2015). Individual locations were coloured according to whether the
| Order    | Identification     | Total n | Positive n | Infected % | Sequenced n |
|----------|-------------------|---------|------------|------------|-------------|
| Orthoptera | Hemiandrus brucei | 89      | 65         | 73         | 53          |
| Orthoptera | Hemiandrus luna  | 29      | 27         | 93         | 18          |
| Orthoptera | Hemiandrus maculifrons | 63      | 36         | 57         | 6           |
| Orthoptera | Hemiandrus nox   | 10      | 3          | 30         | 2           |
| Orthoptera | Isolecetron sp.  | 6       | 3          | 50         | 2           |
| Orthoptera | Pachyrhamma sp.  | 39      | 13         | 33         | 1           |
| Orthoptera | Hemiandrus 'paturau' | 1       | 0          | 0          | 0           |
| Orthoptera | Hemiandrus bilobatus | 1      | 0          | 0          | 0           |
| Orthoptera | Hemiandrus 'disparalis' | 1      | 0          | 0          | 0           |
| Orthoptera | Hemiandrus electra | 1      | 0          | 0          | 0           |
| Orthoptera | Hemiandrus 'elegans' | 1       | 0          | 0          | 0           |
| Orthoptera | Hemiandrus focalis | 1       | 0          | 0          | 0           |
| Orthoptera | Hemiandrus 'furoviarius' | 1      | 0          | 0          | 0           |
| Orthoptera | Hemiandrus 'horomaka' | 1      | 0          | 0          | 0           |
| Orthoptera | Hemiandrus nitaweta | 1      | 1          | 100        | 0           |
| Orthoptera | Hemiandrus 'onokis' | 1      | 0          | 0          | 0           |
| Orthoptera | Hemiandrus 'promontorius' | 1      | 0          | 0          | 0           |
| Orthoptera | Hemiandrus subantarcticus | 1    | 0          | 0          | 0           |
| Orthoptera | Hemiandrus 'vicinus' | 1      | 0          | 0          | 0           |
| Hymenoptera | Archaeotelea gilbertae | 3  | 2          | 66         | 2           |
| Hymenoptera | Archaeotelea onamata | 3     | 0          | 0          | 0           |
| Hymenoptera | Archaeotelea kareere | 3     | 2          | 66         | 1           |
| Psocoptera | Ectopsocus sp.    | 2       | 2          | 100        | 1           |
| Diptera    | Chlorops sp.      | 1       | 1          | 100        | 0           |
| Lepidoptera | Aenetus virensens | 1       | 0          | 0          | 0           |
| Hymenoptera | Vespula vulgaris | 2       | 0          | 0          | 0           |
| Hymenoptera | Vespula germanica | 2       | 0          | 0          | 0           |
| Hemiptera  | Scolypopa australis | 2     | 0          | 0          | 0           |
| Plecoptera | Stenoperla sp.    | 1       | 0          | 0          | 0           |
| Coleoptera | Helimus chalybus  | 2       | 0          | 0          | 0           |
| Hymenoptera | Proctotrupoidea sp. | 1  | 0          | 0          | 0           |
| Diptera    | Musca domestica   | 1       | 0          | 0          | 0           |
| Lepidoptera | Danaus plexippus | 1       | 0          | 0          | 0           |
| Diptera    | Tipulidae         | 1       | 0          | 0          | 0           |
| Diptera    | Chrysomya rufifacies | 1     | 0          | 0          | 0           |
| Diptera    | Fannia canicularis | 1      | 0          | 0          | 0           |
| Diptera    | Drosophila sp.    | 1       | 0          | 0          | 0           |
| Diptera    | Leptotarsus sp.   | 4       | 0          | 0          | 0           |
| Hymenoptera | Apsi mellifera   | 2       | 0          | 0          | 0           |
| Diptera    | Trigonospila brevipaucies | 1  | 0          | 0          | 0           |
| Ephemeroptera | Coloburiscus numeralis | 2  | 0          | 0          | 0           |
| Trichoptera | Asteapsycha sp.  | 2       | 0          | 0          | 0           |
| Hemiptera  | Siphanta acuta    | 2       | 0          | 0          | 0           |
| Megaloptera | Archichauliodes sp. | 2  | 0          | 0          | 0           |
| Isopoda    | Ligia novaezealandiae | 1     | 0          | 0          | 0           |
| Euonymophora | Peripatoides morgani  | 3  | 0          | 0          | 0           |

Number of individuals of each species tested, number positive, and number successfully sequenced

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insects collected there were infected with *Wolbachia* or not (Fig 4). Isolate information was processed through the QGIS dataset to display the distribution of the hosts found carrying each isolate and determine if hosts of differing isolates were likely to be found in sympatry or allopatry.

## Results

### High throughput DNA sequencing of New Zealand Orthoptera

High throughput DNA sequence data was generated for 21 species of New Zealand Orthoptera. Using the metagenomics tools PAUDA and MEGAN to search for evidence of *Wolbachia* sequences within the unassembled insect shotgun sequencing data, six species were found to have *Wolbachia* infections (Table 1). We found similar high levels of *Wolbachia* DNA sequence in a cave weta (*Macropathus* sp.) and a ground weta (*Hemiandrus brucei*). To ascertain the level of genome coverage represented by *Wolbachia* reads in these two species, the short reads were mapped to the complete genome of the *Wolbachia* endosymbiont of *Drosophila melanogaster*. This revealed that the *Macropathus* sp. reads covered 30% of this reference genome, whilst the reads from *Hemiandrus brucei* mapped over 33.6% of the *Wolbachia* reference. Average pairwise nucleotide similarities between the reference *Wolbachia* genome and reads from *Macropathus* sp. and *Hemiandrus brucei* were 90.2% and 92%, respectively. However, due to uneven coverage across the genome only one of the MLST genes (ftsZ) had sufficient sequence information to recover its complete consensus sequence from the HTS data.

| GenBank *Wolbachia* host | GenBank ID |
|---------------------------|------------|
| Diabrotica barberi clone  | KC578107   |
| Altica lythri isolate     | KF163343.1 |
| Pheidole vallicola        | EU127749   |
| Altica helianthemi        | KF163366.1 |
| Altica palustris          | KF163363.1 |
| Altica impressicollis     | KF163368.1 |
| Altica impressicollis     | KF163367.1 |
| Drosophila innubila       | EU126333   |
| Polistes dominulus        | EU126335   |
| Precis iphila             | FJ92398.1  |
| Jalmenus evagoras         | FJ92417.1  |
| Lissorhoptrus oryzophilus | DQ256473.1 |
| Wolbachia sp.             | AJ130717.1 |
| Bombyx mandarina          | KJ69910.1  |
| Cydia fagiglandana        | KJ140034   |
| Bryobia kissiphila        | JN572863.1 |
| Bryobia praetiosa         | EU499322.1 |
| Wolbachia pipiens          | JN316217.1 |
| Mesaphorura italicana     | AJ575103.1 |
| Altica oleracea           | KF163332.1 |
| Melittobia digitata       | EU170117.1 |
| Altica oleracea           | KF163325.1 |
| Altica oleracea           | KF163324.1 |
| Serritermes serrifer      | DQ837193.1 |
| Cubitermes sp.            | DQ127295.1 |

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Four other host species samples that contained significant levels of *Wolbachia* DNA were all endemic Rhaphidophoridae (cave weta), but read numbers were lower compared to those isolated from the *Macropathus* sp. and *Hemiandrus brucei* specimens (<2500 DNA sequences) (Table 1). Low read number corresponded with lower coverage of the *Wolbachia* genome (<5%) when mapped against the reference.

**Wolbachia** infections using specific primers for DNA amplification

To further explore the degree of *Wolbachia* infection in New Zealand insects we carried out a PCR screen using primers that target *Wolbachia* MLST or WSP loci [37]. Positive results for *Wolbachia* infections from six independent Orthoptera lineages were detected through PCR. *Wolbachia* was detected in a clade of Anostostomatidae ground weta (*Hemiandrus brucei*,
Infection rates varied from 30%–93% of individuals per species where > individual were tested (Table 2). For Orthoptera species with the largest sample size infection rates were 73% (H. brucei, n = 89), 93% (H. luna, n = 29), and 57% (H. maculifrons, n = 63) positive for amplification using at least one of the MLST primer pairs. Wolbachia was detected in three of four individuals of North Island Hemiandrus nox but absent from the six individuals from the South Island Hemiandrus nox (n = 10) (Table 2). In addition, of a further 12 Hemiandrus species tested only a single Hemiandrus nitaweta individual (Table 2) gave a positive result.

A total of 45 individual cave weta were tested for the presence of Wolbachia from the genera Pachyrhamma and Isoplectron. Three of the six (50%) Isoplectron, and 13 of the 39

Hemiandrus luna, Hemiandrus maculifrons and Hemiandrus nox) and in the rhaphidophorid genera Pachyrhamma and Isoplectron (Table 2).
Pachyrhamma (33%) samples tested positive for *Wolbachia* at one or more of the *Wolbachia* specific primers (Table 2).

As a potential vector for *Wolbachia* horizontal transmission in Rhaphidophoridae and possibly other insects, the parasitoid wasp *Archaeoteleia* was tested for infection. Of nine specimens available for testing, four individuals were positive for an infection; two *A. gilbertae* and an individual each of *A. onamata* and *A. kawere* (Table 2). Of the further 40 invertebrate individuals representing twenty-four species collected from New Zealand and tested for *Wolbachia* using MLST primers few were positive. *Wolbachia* infection was identified in two species; a native tree-living booklouse *Ectopsocus* sp. and an exotic grass fly *Chlorops* sp.

*Wolbachia* infections in New Zealand Orthoptera are geographically widespread (Fig 4). At many locations individuals with *Wolbachia* infections were collected alongside individuals that were not infected with *Wolbachia*, suggesting that where *Wolbachia* is present it is not at saturation. *Wolbachia* was detected throughout the North Island and northern South Island (Fig 4), however, *Wolbachia* was detected at a lower frequency in *Hemiandrus* ground weta.
from the southern half of South Island, where only two individuals were positive for a *Wolbachia* infection (Fig 4). The *Wolbachia* infection rate in Rhaphidophoridae was highest in samples from central and northern North Island although fewer southern samples were examined (Fig 4).

We sequenced the ftsZ region of *Wolbachia* infections from 82 Orthoptera hosts, three parasitoid wasps (*Archaeoteleia* sp.) and one booklouse (*Ectopsocus* sp.; Table 2). Incorporating representatives of all major global *Wolbachia* supergroups into a phylogenetic analysis with the New Zealand *Wolbachia*, DNA sequences revealed that the New Zealand diversity nested within supergroups A and B, based on the ftsZ gene (Fig 2). The New Zealand *Wolbachia* sequences from *Macropathus* and *Pachyrhamma* cave weta, *Ectopsocus* booklouse and some *Hemiandrus* ground weta fell within supergroup (clade) B, while the *Wolbachia* sequences from *Isoplectron* cave weta and other *Hemiandrus* ground weta fell within supergroup (clade) A. We refer tentatively to New Zealand *Wolbachia* samples that are part of supergroup A [28] as isolate A as we currently have DNA sequence from one locus. Isolate A samples included 43 sequences from New Zealand *Wolbachia*, but the New Zealand representatives did not form a monophyletic group within clade A. However, New Zealand clade B sequences differed from
all available published (GenBank) Wolbachia (Table 3) (Fig 1) by a minimum of five substitutions (Fig 3) and formed a monophyletic cluster. The closest match was the sister clade consisting of infections from China, India, and Europe [28] and we tentatively referred to as isolate B. Six host individuals had DNA sequences from both isolates, suggesting that they were infected with two different Wolbachia lineages.

Minimum spanning networks of ftsZ for isolate A (360 bp) and isolate B (228 bp) reveal the diversity within New Zealand Wolbachia (Fig 3). Fourteen distinct sequences were identified within isolate A, differing by 1–3 nucleotides. The parasitoid wasp Archaeoteleia was infected with Wolbachia having the same sequence as that obtained from three different New Zealand orthopteran host species. Seven distinct sequences were identified within isolate B Wolbachia, and these differed by a minimum of five mutations from published Wolbachia sequences (2.2%; Fig 3).

**Discussion**

Wolbachia was detected in HTS DNA sequence datasets from six orthopteran individuals that are endemic to New Zealand, representing two families and five genera (Macropathus sp., Hemiandrus brucei, Talitropsis sedilloti, Miotopus diversus, and two Neonetus specimens). Orthoptera elsewhere in the world are known to be hosts of Wolbachia [57,58], but we present the first documented cases of Wolbachia infection of any endemic New Zealand invertebrate. The samples from cave weta Macropathus sp. (Rhaphidophoridae) and ground weta Hemiandrus brucei (Anostostomatidae) provided ~30% coverage of the Wolbachia genome which was the largest in our sample. These sequences were unambiguously identified as part of the Wolbachia global diversity.

The Macropathus sp, and Hemiandrus brucei samples yielded approximately twice the number of total DNA reads compared to the other HTS datasets analysed, however, Wolbachia was also detected in a number of other Rhaphidophoridae (cave weta) samples. The level of detection in four samples was lower (<4%), but Wolbachia represented the majority of prokaryote reads detected in the analysis and DNA sequences were close matches to published Wolbachia (Pairwise % Identity and identical sites of the samples of ≥85% in M. diversus and both Neonetus specimens). In contrast, the sample from Talitropsis sedilloti had bacterial sequences with less similarity to Wolbachia (pairwise 54% and identical 27.1%) which might represent different bacteria.

**Wolbachia and the Hemiandrus maculifrons-complex**

The genomic DNA sequence datasets provided evidence of infection from single representatives of five different species (Fig 4). To investigate infection rates, we amplified DNA from numerous individuals of the same species using specific primers, targeting host species within the same genus. Wolbachia was detected in all three species of the ground weta species H. brucei, H. luna, and H. maculifrons through the MLST protocol. Infections were detected in the majority of individuals tested, with 73% of H. brucei, 93% of H. luna, and 57% of H. maculifrons. Hemiandrus brucei and H. luna showed the high-level pattern of infection as suggested by Hilgenboecker, et al. [3]. Their metaanalysis indicates that intraspecific Wolbachia infections rates tend to show a ‘most or few’ infection pattern, as very high or very low infection frequencies were more likely to occur than intermediate rates. Hemiandrus maculifrons had a lower infection rate, well below the high (>90%) infection level but much higher than in low level (<10%) infections. Within the same host, Wolbachia infections can vary among tissue types, tending to be at higher density in female reproductive tissue. Our weta DNA extractions were mostly from femur muscle and as numbers of intracellular bacteria tend to be limited in
somatic tissue this may have reduced detectability in our sample [10,34]. Small host sample sizes also make estimates of infection rates less precise, and this can be rectified by expanded sampling now that the Wolbachia target has been recognised. The same Wolbachia strain can produce various reproductive modifications (pathogenensis, male killing, cytoplasmic incompatibility) in different host lineages [10], that result in dissimilar infection frequency [7]. In the morphologically cryptic Hemiandrus species we studied their genetic similarity suggests it unlikely that Wolbachia has caused different reproductive modifications in each species, but further research will reveal if Wolbachia was a contributing factor in their speciation.

**Wolbachia and Rhaphidophoridae**

New Zealand has a high diversity of Rhaphidophoridae (cave crickets or cave weta) with at least 19 endemic genera. The orthopteran family is found worldwide and typically cave-dwelling, but several New Zealand species are unusual in that they inhabit forests and the sub-alpine zones. Wolbachia was detected infecting six of these genera; Pachyrhamma, Isoplectron, Neontetus, Talitropsis, Miotopus and Macropathus, with the highest infection rate 33% in Pachyrhamma. As we did not target specific tissue known to have high Wolbachia densities (ovarian follicles) this might underestimate the true infection rate. At least one species of Isoplectron was host to Wolbachia with an infection rate detected of 50%. Further samples will need to be tested to determine the level of infection at both the population and species level. Wolbachia was detected in Macropathus through HTS. Inclusion of this genus in further surveys would be informative.

**Transmission**

Intracellular bacteria such as Wolbachia are regularly transmitted in egg cells from mother to offspring (vertical transmission [34]). However, Wolbachia is also suspected to be transmitted between species horizontally [25,28,34,59], potentially by an uninfected insect eating an infected one or by multiple species being host to the same parasitoid wasps [60,61]. Archaeoteleia is a genus of parasitoid wasp known for its parasitism of eggs of New Zealand Pachyrhamma cave weta species. However, the typical hosts of two species (A. gilbertae, A. karere) that were positive for Wolbachia is not known. Wolbachia was found in four individuals representing two parasitoid wasp species. The congruence between the Wolbachia infecting weta and the Wolbachia infecting Archaeoteleia may indicate an avenue for further research into a potential interspecies transmission route of Wolbachia in weta. Notably, the Wolbachia DNA sequences from both Archaeoteleia gilbertae and A. karere were identical to Wolbachia sequences from the cave weta Isoplectron (not the Pachyrhamma examined) and two ground weta species (Anostostomatidae: Hemiandrus). The presence of matching Wolbachia in Isoplectron and ground weta rather than Pachyrhamma is interesting because if it is determined that Wolbachia can be transmitted via Archaeoteleia this may be the first indication of new hosts for these parasitoids.

Within the New Zealand insect hosts examined, two distinct clades of Wolbachia were detected. Both isolates of Wolbachia have managed to infect the New Zealand Rhaphidophoridae. The New Zealand Wolbachia lineage that is part of A supergroup clustered with identical Wolbachia DNA sequences from hosts sampled outside of New Zealand (Fig 1). In contrast, other New Zealand Wolbachia ftsZ gene sequences formed a monophyletic group within supergroup B (Fig 1). This might represent a distinct New Zealand lineage of Wolbachia. Further testing of the MLST regions is required because recombination of MLST fragments between strains of Wolbachia occurs. The distribution of 'isolate B’ through Hemiandrus sister species was extensive with at least 11 confirmed H. luna hosts and three confirmed
H. maculifrons hosts in addition to the 14 confirmed H. brucei hosts. We also detected that some insect hosts were infected with both A and B isolates of Wolbachia.

To our knowledge, this work documents the first cases of Wolbachia infection in endemic New Zealand insects. We detected infection by Wolbachia in endemic species of two families of Orthoptera and in endemic parasitic wasps that attack these Orthoptera. Relatively high observed infections rates, considering our sampling of somatic tissue, in more than one Hemiandrus lineage suggest that Wolbachia is not involved in formation of reproductive barriers between ground weta species, and no definitive pattern of Wolbachia distribution has yet been determined in New Zealand. It was present in all the Hemiandrus species tested spanning both main islands. Further study including analysis of female reproductive tissue will inform on the prevalence of infections across the country and among related species, and reveal what, if any, effect, infections have on reproductive capabilities of the endemic New Zealand insect fauna.

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References

1. Hertig M, Wolbach SB (1924) Studies on Rickettsia-Like Micro-Organisms in Insects. J Med Res 44: 329–374. PMID: 19972605
2. Hertig M (1936) The rickettsia, Wolbachia pipiensis (gen. et sp. n.) and associated inclusions of the mosquito, Culex pipiens. Parasitology 28: 453–486.

3. Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH (2008) How many species are infected with Wolbachia?—A statistical analysis of current data. FEMS Microbiol Lett 281: 215–220. https://doi.org/10.1111/j.1574-6968.2008.01110.x PMID: 18312577

4. Sazama EJ, Bosch MJ, Shoudis CS, Ouellette SP, Wesner JS (2017) Incidence of Wolbachia in aquatic insects. Ecol Evol 7: 1165–1169. https://doi.org/10.1002/ece3.2742 PMID: 28303186

5. Zug R, Hammerstein P (2012) Still a Host of Hosts for Wolbachia: Analysis of Recent Data Suggests That 40% of Terrestrial Arthropod Species Are Infected. PLoS ONE 7:

6. Hoffmann AA, Clancy D, Duncan J (1996) Naturally-occurring Wolbachia infection in Drosophila simuli-ans that does not cause cytoplasmic incompatibility. Heredity 76: 1–8. PMID: 8575931

7. Hurst GD, Jiggins FM (2000) Male-killing bacteria in insects: mechanisms, incidence, and implications. Emerg Infect Dis 6: 329–336. https://doi.org/10.3201/eid0604.000402 PMID: 10905965

8. Breeuwer JAJ, Werren JH (1993) Cytoplasmic Incompatibility and Bacterial Density in Nasonia vitri-ppenni, Genetics 135: 565–574. PMID: 8244014

9. Werren JH, Windsor DM (2000) Wolbachia infection frequencies in insects: evidence of a global equilib-rium? Proc Biol Sci 267: 1277–1285. https://doi.org/10.1098/rspb.2000.1139 PMID: 10972121

10. Werren JH, Baldo L, Clark ME (2008) Wolbachia: master manipulators of invertebrate biology. Nat Rev Microbiol 6: 741–751. https://doi.org/10.1038/nrmicro1969 PMID: 18794912

11. Rokas A, Atkinson RJ, Nieves-Aldrey JL, West SA, Stone GN (2002) The incidence and diversity of Wolbachia in gall wasps (Hymenoptera: Cynipidae) on oak. Mol Ecol 11: 1815–1829. PMID: 12207731

12. Zabalou S, Riegler M, Theodorakopoulou M, Stauffer C, Savakis C, et al. (2004) Wolbachia-induced cytoplasmic incompatibility as a means for insect pest population control. Proc Natl Acad Sci U S A 101: 15042–15045. https://doi.org/10.1073/pnas.0403853101 PMID: 15469918

13. Atyame CM, Labbe P, Leibl C, Weill M, Moretti R, et al. (2016) Comparison of Irradiation and Wol-bachia Based Approaches for Sterile-Male Strategies Targeting Aedes albopictus. PLoS One 11: e0146834. https://doi.org/10.1371/journal.pone.0146834 PMID: 26765951

14. Aliota MT, Peinado SA, Velez ID, Osorio JE (2016) The wMel strain of Wolbachia Reduces Transmis-sion of Zika virus by Aedes aegypti. Sci Rep 6: 28792. https://doi.org/10.1038/srep28792 PMID: 27364935

15. Fraser JE, De Bruyne JT, Iturbe-Ormaetxe I, Stepnell J, Burns RL, et al. (2017) Novel Wolbachia-tansinfected Aedes aegypti mosquitoes possess diverse fitness and vector competence phenotypes. PLoS Pathog 13: e1006751. https://doi.org/10.1371/journal.ppat.1006751 PMID: 29216317

16. Slato BE, Taylor MJ, Foster JM (2010) The Wolbachia endosymbiont as an anti-filarial nematode tar-get. Symbiosis 51: 55–65. https://doi.org/10.1007/s13199-010-0067-1 PMID: 20730111

17. Turner JD, Sharma R, Al Jayoussi G, Tyer HE, Gamble J, et al. (2017) Albendazole and antibiotics syner-gize to deliver short-course anti-Wolbachia curative treatments in preclinical models of filariasis. Proc Natl Acad Sci U S A 114: E9712–E9721. https://doi.org/10.1073/pnas.1710845114 PMID: 29078351

18. Zhang YK, Zhang KJ, Sun JT, Yang XM, Ge C, et al. (2013) Diversity of Wolbachia in natural popula-tions of spider mites (genus Tetranychus): evidence for complex infection history and disequilibrium distri-bution. Microb Ecol 65: 731–739. https://doi.org/10.1007/s00248-013-0198-z PMID: 23429887

19. Hernandez M, Quesada T, Munoz C, Espinoza AM (2004) Genetic diversity of Costa Rican populations of the rice planthopper Tagosodes orizicolus (Homoptera: Delphacidae). Rev Biol Trop 52: 795–806. PMID: 17361572

20. Hughes GL, Allsopp PG, Brumley SM, Woolfit M, McGraw EA, et al. (2011) Variable infection frequency and high diversity of multiple strains of Wolbachia pipiensis in Perkinsiella Plant hoppers. Appl Environ Microbiol 77: 2165–2168. https://doi.org/10.1128/AEM.02878-10 PMID: 21278277

21. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN (2008) Wolbachia and virus protection in insects. Science 322: 702. https://doi.org/10.1126/science.1162418 PMID: 18974344

22. Kambris Z, Cook PE, Phuc HK, Sinkins SP (2009) Immune activation by life-shortening Wolbachia and reduced filarial competence in mosquitoes. Science 326: 134–136. https://doi.org/10.1126/science.1177531 PMID: 19797680

23. Heath BD, Butcher RDJ, Whitfield WGF, Hubbard SF (1999) Horizontal transfer of Wolbachia between phylogenetically distant insect species by a naturally occurring mechanism. Current Biology 9: 313–316. PMID: 10290997
24. Vavre F, Fleury F, Lepeit D, Fouillet P, Bouletreau M (1999) Phylogenetic evidence for horizontal transmission of Wolbachia in host-parasitoid associations. Mol Biol Evol 16: 1711–1723. https://doi.org/10.1093/oxfordjournals.molbev.a026084 PMID: 10605113

25. Werren JH, Zhang W, Guo LR (1995) Evolution and phylogeny of Wolbachia: reproductive parasites of arthropods. Proc Biol Sci 258: 55–63. https://doi.org/10.1098/rspb.1995.0117 PMID: 7644549

26. Watanabe M, Kageyama D, Miura K (2013) Transfer of a parthenogenesis-inducing Wolbachia endosymbiont derived from Trichogramma dendrolimi into Trichogramma evanescens. J Invertebr Pathol 112: 83–87. https://doi.org/10.1016/j.jip.2012.09.006 PMID: 23063745

27. Li SJ, Ahmed MZ, Lv N, Shi PQ, Wang XM, et al. (2017) Plant-mediated horizontal transmission of Wolbachia between whiteflies. ISME J 11: 1019–1028. https://doi.org/10.1038/ismej.2016.164 PMID: 27935594

28. Lo N, Casiraghi M, Salati E, Bazzocchi C, Bandi C (2002) How many Wolbachia supergroups exist? Mol Biol Evol 19: 341–346. https://doi.org/10.1093/oxfordjournals.molbev.a004087 PMID: 11861893

29. Ros VI, Fleming VM, Feil EJ, Breeuwer JA (2009) How diverse is the genus Wolbachia? Multiple-gene sequencing reveals a putatively new Wolbachia supergroup recovered from spider mites (Acari: Tarsonychidae). Appl Environ Microbiol 75: 1036–1043. https://doi.org/10.1128/AEM.01109-08 PMID: 19098217

30. Haegeman A, Vanholme B, Jacob J, Vandekerckhove TT, Claeys M, et al. (2009) An endosymbiotic bacterium in a plant-parasitic nematode: member of a new Wolbachia supergroup. Int J Parasitol 39: 1045–1054. https://doi.org/10.1016/j.ijpara.2009.01.006 PMID: 19504759

31. Glowska E, Dragun-Damian A, Dabert M, Gerth M (2015) New Wolbachia supergroups detected in quill mites (Acari: Syringophilidae). Infect Genet Evol 30: 140–146. https://doi.org/10.1016/j.meegid.2014.12.019 PMID: 25541519

32. Hennig W (1981) Insect Phylogeny. Chichester, NY: Wiley.

33. Warren JH (1997) Biology of Wolbachia. Annu Rev Entomol 42: 587–609. https://doi.org/10.1146/annurev.ento.42.1.587 PMID: 15012323

34. Jiggins FM, von Der Schulenburg JH, Hurst GD, Majerus ME (2001) Recombination confounds interpretations of Wolbachia evolution. Proc Biol Sci 268: 1423–1427. https://doi.org/10.1098/rspb.2001.1656 PMID: 11429144

35. Casiraghi M, Bordenstein SR, Baldo L, Lo N, Beninati T, et al. (2005) Phylogeny of Wolbachia pipientis based on gltA, groEL and ftsZ gene sequences: clustering of arthropod and nematode symbionts in the F supergroup, and evidence for further diversity in the Wolbachia tree. Microbiology 151: 4015–4022. https://doi.org/10.1099/mic.0.28313-0 PMID: 16339946

36. Baldo L, Dunning Hotopp JC, Jolley KA, Bordenstein SR, Biber SA, et al. (2006) Multilocus sequence typing system for the endosymbiont Wolbachia pipientis. Appl Environ Microbiol 72: 7098–7110. https://doi.org/10.1128/AEM.00731-06 PMID: 16936055

37. Trewick S, Morgan-Richards M (2009) New Zealand Biology. Encyclopedia of Islands. Berkeley.: University of California Press.

38. Goldberg J, Trewick SA, Paterson AM (2008) Evolution of New Zealand’s terrestrial fauna: a review of molecular evidence. Philos Trans R Soc Lond B Biol Sci 363: 3319–3334. https://doi.org/10.1098/rstb.2008.0114 PMID: 18782728

39. Trewick SA, Paterson AM, Campbell HJ (2006) GUEST EDITORIAL: Hello New Zealand. Journal of Biogeography 34: 1–6.

40. Vaux F, Hills SFK, Marshall BA, Trewick SA, Morgan-Richards M (2017) A phylogeny of Southern Hemisphere wheelies (Gastropoda: Buccinulidae) and concordance with the fossil record. Mol Phylogenet Evol 114: 367–381. https://doi.org/10.1016/j.ympev.2017.06.018 PMID: 28669812

41. Siwyer L, Morgan-Richards M, Kool E, Trewick S (2018) Anthropogenic cause of range shifts and gene flow between two grasshopper species revealed by environmental modelling, geometric morphometrics and population genetics. Insect Conserv Divers.

42. (2014) Illumina HiSeq2000. BGI.

43. Huson DH, Xie C (2014) A poor man’s BLASTX—high-throughput metagenomic protein database search using PAUDA. Bioinformatics 30: 38–39. https://doi.org/10.1093/bioinformatics/btt254 PMID: 23658416

44. Huson DH, Mitra S, Ruscheweyh HJ, Weber N, Schuster SC (2011) Integrative analysis of environmental sequences using MEGAN4. Genome Res 21: 1552–1560. https://doi.org/10.1101/gr.120618.111 PMID: 21690186
46. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, et al. (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28: 1647–1649. https://doi.org/10.1093/bioinformatics/bts199 PMID: 22543367

47. Taylor-Smith B (2016) Evolution of diversity: analysis of species and speciation in Hemiandrus ground wehâ [PhD Thesis]. Palmerston North: Massey University.

48. Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16: 1215. PMID: 3344216

49. Taylor-Smith BL, Trewick SA, Morgan-Richards M (2016) Three new ground wehâ species and a re-description of Hemiandrus maculifrons. New Zealand Journal of Zoology 43: 363–383.

50. Zhou W, Rousset F, O’Neil S (1998) Phylogeny and PCR-based classification of Wolbachia strains using wsp gene sequences. Proc Biol Sci 265: 509–515. https://doi.org/10.1098/rspb.1998.0324 PMID: 9569669

51. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Marine Biol Biotechnol 3: 294–299. PMID: 7881515

52. Early JW, Masner L, Johnson NF (2007) Revision of Archaeoteleia Masner (Hymenoptera: Platygastridae, Scelionidae). Zootaxa 1655: 1–48.

53. Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754–755. PMID: 11524383

54. Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574. PMID: 12912839

55. Bandelt HJ, Forster P, Rohlf A (1999) Median-joining networks for inferring intra-specific phylogenies. Mol Biol Evol 16: 37–48. https://doi.org/10.1093/oxfordjournals.molbev.a026036 PMID: 10331250

56. Leigh JW, Bryant D, Nakagawa R (2015) popart: full-feature software for haplotype network construction. Methods in Ecology and Evolution 6: 1110–1116.

57. Sarasa J, Bernal A, Fernandez-Calvin B, Bella JL (2013) Wolbachia induced cytogenetical effects as evidenced in Chorthippus parallelus (Orthoptera). Cytogenet Genome Res 139: 36–43. https://doi.org/10.1159/000341572 PMID: 22907174

58. Jeong G, Ahn J, Jang Y, Choe JC, Choi H (2012) Wolbachia infection in the Loxoblemmus complex (Orthoptera: Gryllidae) in Korea. Journal of Asia-Pacific Entomology 15: 563–566.

59. Sintupachee S, Milne JR, Poonchaisri S, Baimai V, Kittayapong P (2006) Closely related Wolbachia strains within the pumpkin arthropod community and the potential for horizontal transmission via the plant. Microb Ecol 51: 294–301. https://doi.org/10.1007/s00248-006-9036-x PMID: 16598632

60. Ahmed MZ, Breinholt JW, Kawahara AY (2016) Evidence for common horizontal transmission of Wolbachia among butterflies and moths. BMC Evol Biol 16.

61. Le Clec’h W, Chevalier FD, Genty L, Bertaux J, Bouchon D, et al. (2013) Cannibalism and predation as paths for horizontal passage of Wolbachia between terrestrial isopods. PLoS One 8: e60232. https://doi.org/10.1371/journal.pone.0060232 PMID: 23593179