

# Endosymbiotic Candidates for Parasitoid Defense in Exotic and Native New Zealand Weevils

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**Abstract** Some insects are infected with maternally inherited bacterial endosymbionts that protect them against pathogens or parasitoids. The weevil *Sitona obsoletus* (= *Sitona lepidus*) is invasive in New Zealand, and suspected to contain such defensive symbionts, because it is particularly resistant to a Moroccan strain of the parasitoid *Microctonus aethiopoidea* (which successfully attacks many other weevil species), and shows geographic variation in susceptibility to an Irish strain of the same parasitoid. Using 454 pyrosequencing, we investigated the bacterial community associated with *S. obsoletus*, two other exotic weevils (*Sitona discoideus* and *Listronotus bonariensis*) and two endemic New Zealand weevils (*Irenimus aequalis* and *Steriphys variabilis*). We found that *S. obsoletus* was infected by one strain of *Wolbachia* and two strains of *Rickettsia*, none of which were found in any other weevil species examined. Using diagnostic PCR, we found that *S. obsoletus* in the Northland region, where parasitism is highly variable, were primarily infected with *Wolbachia* and *Rickettsia* strain 2, indicating that these two symbionts should be investigated for potential defensive properties. In comparison, *S. discoideus* lacked any apparent maternally inherited

bacterial endosymbionts. In the other weevil species, we found a different strain of *Wolbachia* and two different strains of *Spiroplasma*. Two weevil species (*St. variabilis* and *L. bonariensis*) were infected with distinct strains of *Nardonella*, the ancestral endosymbiont of weevils, whereas three weevil species (*S. obsoletus*, *S. discoideus*, and *I. aequalis*) lacked evidence for *Nardonella* infection. However, *I. aequalis* was consistently infected with a novel Enterobacteriaceae strain, suggesting that a symbiont replacement may have taken place, similar to that described for other weevil clades.

**Keywords** Biological control · Facultative endosymbionts · Metagenome · Symbiotic defense

## Introduction

Bacterial endosymbionts perform a wide array of services for their eukaryotic hosts, including provision of defense against natural enemies [1]. Among insect hosts, bacteria have been shown to protect insects against viruses [2, 3], infective nematodes [4], fungal pathogens [5–7], predators [8], and parasitoid wasps [9, 10]. One of the earliest documented examples of bacterial-provided defense was in the invasive alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae). Three strains of this weevil became established in North America, only two of which were susceptible to the parasitoid, *Microctonus aethiopoidea* Loan (Hymenoptera: Braconidae), which had been released in an attempt to provide biological control for the weevil [11]. Hsiao [12] found that *M. aethiopoidea* performance in the resistant Western strain of *H. postica* improved significantly when the weevils had been pretreated with antibiotics. This strain of the weevil is

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naturally infected with the maternally inherited bacterium *Wolbachia* [13], which is best known for reproductively manipulating host insects to promote bacterial infection in host populations [14, 15]. The concomitant removal of *Wolbachia* infection and increase in parasitoid susceptibility led to the hypothesis that *Wolbachia* was the causative agent of parasitoid defense in *H. postica* [12].

However, unequivocal demonstration of *Wolbachia*-provided defense against *M. aethiopoulos* has not been verified, as other strains of bacteria may have co-infected *H. postica* along with *Wolbachia* and been simultaneously cured by the antibiotic treatment. Curculionid weevils seem particularly prone to infection by facultative bacterial symbionts, and simultaneous infection by multiple facultative symbionts is common [16–18]. It therefore remains possible that a co-infecting symbiont, rather than *Wolbachia*, might be responsible for parasitism protection in Western strains *H. postica*.

Understanding which symbiont(s) provide weevils with defensive characteristics is important, because many weevil species are invasive or have the potential to be invasive worldwide. For example, weevils such as *Otiorhynchus sulcatus* (F.) (vine weevil), *Rhynchophorus ferrugineus* Olivier (red palm weevil), *Sitona lineatus* L. (pea leaf weevil), and *H. postica* (alfalfa weevil) can cause tremendous economic damage to crops if uncontrolled and consequently have been the focus of significant investment in biological control effort (e.g., [11, 19–21]).

To maximize the probability of success against invasive insect species, classical biological control programs consider many aspects of the target insect and putative biological control agent(s), including correct taxonomic identification, geographic origin, host plants, geographic and climatic range, phenology, and intraspecific genetic variation [22, 23], as well as non-target impacts once the biocontrol agent has been released (e.g., [24]). The potential impact of entomophagous biocontrol agents against target and non-target hosts could also be influenced by the symbiont communities within these host populations. Symbiont infection therefore could potentially be used to predict the efficacy of candidate control agents, if the defensive traits of various bacteria were reliably characterized.

In New Zealand, for example, bacterial symbionts could be responsible for the variable success of biological control programs to control exotic weevil pests in pasture ecosystems. The lucerne pest, *Sitona discoideus* Gyllenhal (Coleoptera: Curculionidae) [25], has been significantly controlled by a Moroccan strain of *M. aethiopoulos* that was introduced in a classical biological control program [26, 27]. Unfortunately, this parasitoid strain was subsequently found to have a wide non-target host range and to be able to successfully parasitize and develop in many weevil species, including native and introduced beneficial weed biocontrol species [28]. Interestingly, however, this strain of *M. aethiopoulos* readily attacked but was unable to develop in the clover root weevil, *Sitona obsoletus* Gyllenhal (Coleoptera: Curculionidae), a damaging

exotic pest of white clover [29, 30]. This result was also observed by Sundaralingam et al. [31] on *S. obsoletus* collected in Pennsylvania. While genetic differences between *M. aethiopoulos* strains associated with different *Sitona* species [32, 33] may explain the poor survival of Moroccan *M. aethiopoulos*, this parasitoid strain's ability to successfully develop in a wide range of novel hosts suggested that *S. obsoletus* might possess a defensive microbe that *S. discoideus* lacks. Additionally, subsequent biological control efforts for *S. obsoletus* with a more successful strain of *M. aethiopoulos* sourced from Ireland [34] have shown geographic variation in success rates in New Zealand. In Northland (latitude S35), establishment of the parasitoid was variable across sites [35, 36] with high parasitoid egg and larval mortality in some sites but not all (P. Gerard, unpublished data). This was in contrast to observations in Canterbury (latitude S43) where there has been 100 % establishment and negligible larval mortality in the host (unpublished data). These patterns suggest that *S. obsoletus* shows intraspecific variation in defensive efficacy, which may or may not be associated with bacterial endosymbionts.

As a first step toward understanding the nature of *S. obsoletus* resistance to parasitism, the objective of this paper was to investigate bacterial diversity associated with this pest species versus other co-occurring exotic and native weevils in New Zealand, and to identify potential endosymbiotic associates of *S. obsoletus* and other weevil species that might confer resistance to parasitism.

## Methods

### Specimen Collection and Preparation

We investigated two populations of *S. obsoletus*: the Canterbury population (S43.70, E172.33), which typically is subject to high levels of parasitism from the introduced Irish parasitoid *M. aethiopoulos*, and the Northland population (S35.00, E173.49), which typically exhibits lower mean levels of parasitism [36]. We collected adult specimens from each population and larval specimens from the Canterbury population, as indicated in Table S1. We also collected specimens from four other Curculionidae weevil species; two that have been accidentally introduced to New Zealand (*S. discoideus* and Argentine stem weevil *Listronotus bonariensis* Kuschel) and two native New Zealand weevil species (*Irenimus aequalis* Broun and *Steriphus variabilis* Broun). Adults of *S. obsoletus*, *L. bonariensis*, *I. aequalis*, and *St. variabilis* were collected from ryegrass (*Lolium perenne*)/white clover (*Trifolium repens*) pasture while *S. discoideus* was collected from a lucerne (*Medicago sativa*) crop. Weevils were collected using a modified blower vac to suck the weevils into a collecting net fitted to the inlet that was then hand sorted to extract the

weevils [37]. Larvae were collected from soil around the roots of white clover plants growing in pasture. Adult weevils were examined under a stereomicroscope ( $\times 10$  magnification) and male and female weevils identified by the shape of the distal ventrite (e.g., [38–40]).

All specimens were preserved in 95 % ethanol. Between 8 and 18 specimens (as indicated in Table S1) were selected from each population, and individually surface sterilized in 5 % sodium hypochlorite solution (1 min), followed by three rinses in 95 % ethanol (1 min each), and a final rinse in sterile PCR grade water (1 min). For the two larger weevil species, *S. obsoletus* and *S. discoideus*, elytra were removed prior to homogenization. DNA was extracted from individual specimens using DNeasy blood and tissue kits (Qiagen, Valencia, CA) following manufacturer's specifications. Proteinase K digestion was performed for 3 h at 56 °C. All specimens were re-eluted in two washes of 100  $\mu$ l elution buffer, except for *L. bonariensis* specimens, which due to their smaller size were resuspended in two washes of 50  $\mu$ l elution buffer.

#### 454 Pyrosequencing

To characterize overall bacterial community diversity per species, eight pooled samples of DNA, representing three introduced and two native New Zealand weevil species (Table S1), were analyzed for bacterial diversity. For each sample, DNA from eight to ten specimens of each species was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, Delaware), a subsample of DNA per specimen was standardized to a concentration of 20 ng DNA/ $\mu$ l, and then pooled into a single sample. The samples were sent to Research and Testing Laboratory (Lubbock, TX) where they were multiplexed in bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) using a 454 FLX instrument (Roche, Nutley, New Jersey) and analyzed as described in Brady and White [41]. Samples were run in two batches on different dates (Table S1). All samples were evaluated with 28 F (5'-GAGTTT GATCNTGGCTCAG-3') and 519R (5'-GWNNTACNGCGG CKGCTG-3') primers, which amplified approximately 500 bp of the 5' end of bacterial 16S rRNA [42–44]. To determine whether results were sensitive to primer set selection, two of the *S. obsoletus* samples and one *St. variabilis* sample (identified in Table S1) were additionally evaluated with 939F (5'-TTGACGGGGCCCCGAC-3') and 1492R (5'-TACCTTGT TACGACTT-3') primers, which amplified approximately 550 bp of the 3' end of bacterial 16S rRNA [45].

#### Diagnostic Screening and Strain Typing

Based on the pyrosequencing results, DNA from individual specimens was diagnostically screened for four bacterial genera (*Wolbachia*, *Rickettsia*, *Spiroplasma*, and *Nardonella*), all of which have been associated with weevils as

endosymbionts. Diagnostic PCR reactions were conducted using published bacterial primer sets, each specific to one of the endosymbiotic genera and performed as described by the original paper (Table S2). For *Nardonella*, we designed an alternate reverse primer that was better matched to bacterial strains discovered in this study (Table S2). PCR products were run on a 1 % agarose gel stained with either Gel-Red (Biotium, Hayward, CA) or RedSafe™ (iNtRON Biotechnology, Korea). All PCR sets included DNA from specimens of known infection status as positive controls, as well as a template-free reaction as a negative control. For populations in which any of these bacterial genera were detected, the PCR product from at least one specimen per population was sequenced at the University of Kentucky Advanced Genetic Technologies Center (UK-AGTC) using an ABI 3730 DNA Analyzer (Applied Biosystems) to verify bacterial identity.

Additional Sanger (BigDye®v 3.1, Applied Biosystems) sequencing was conducted at the UK-AGTC or at MacroGen (Korea) to (1) determine strain type distribution of *Rickettsia* symbionts across populations of *S. obsoletus*, (2) determine the multilocus sequence type (MLST) for *Wolbachia* strains detected in *S. obsoletus* and *St. variabilis* and (3) investigate a dominant but unusual symbiont detected in the native weevil *I. aequalis*.

1. Because 16S pyrosequencing results suggested the presence of two different *Rickettsia* strains in *S. obsoletus*, we designed *Rickettsia*-specific primers to amplify two overlapping segments of 16S to yield almost the entire 1500 bp 16S sequence (Rick16S480R used with universal 16SA1F, Rick16S460F, and Rick16S1530R; Table S2). The majority of the resulting Rick16S460F and Rick16S1530R PCR products were sequenced to determine the *Rickettsia* strain type per individual; based on chromatogram inspection, we saw no evidence of multiple *Rickettsia* infections within a single individual.
2. For the two *Wolbachia* positive weevil species (*S. obsoletus* and *St. variabilis*), we amplified and sequenced five multilocus sequence type (MLST) housekeeping genes from a single individual per species as described by Baldo et al. [46] (see also Table S2).
3. On finding a dominant and novel Enterobacteriaceae sequence in *I. aequalis*, we designed specific primers to amplify the whole 16S gene of this potential endosymbiont by first aligning 16S gene sequences from a range of closely related endosymbiont bacteria from the same family and targeting the variable regions among genera. Forward primers specific for the novel sequence were combined with reverse primers that target Enterobacteriaceae in general (IaqEnteroF2, IaqEnteroF6, Entero16S760R, and Entero16S1500R; Table S2). Reactions contained Taq DNA polymerase (iNtRON Biotechnology *i-StarTaq*™; at approx. 1 unit/ 10  $\mu$ l PCR), 2 mM MgCl<sub>2</sub>, 1 mM dNTPs,

0.5  $\mu$ M primers and template DNA at approx.4 ng per  $\mu$ l of PCR reaction. Cycling conditions started at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s; the final step was 5 min at 72 °C. All *I. aequalis* specimens were diagnostically screened for the presence of this bacterial strain, and the PCR products from a single individual were sequenced.

### Phylogenetic Analysis

16S sequences from this study were compared to previously published bacterial symbionts of weevils [18, 47], or their closest 16S rRNA sequences found in the GenBank® nucleotide database [48] following use of the Basic Local Alignment Search Tool (BLAST; <http://blast.ncbi.nlm.nih.gov/Blast>). The individual and concatenated *Wolbachia* MLST genes were trimmed and compared to data provided on the MLST website (<http://pubmlst.org/wolbachia/>) developed by Keith Jolley and sited at the University of Oxford [49]. The development and maintenance of this site has been funded by the Wellcome Trust.

Multiple alignments of the nucleotide sequences were generated using the program Muscle [50]. The program Seaview [51] was used to visualize and edit the alignments when needed. Phylogenetic analyses were performed using maximum likelihood (ML) inference with Phylml v3.0.1 [52]. The model selected was GTR+I+G for *Rickettsia* (1476 sites), *Spiroplasma* (2426 sites), *Wolbachia* (2079 sites) and for the

main Enterobacteriaceae tree (2679 sites). The robustness of the nodes was assessed with 500–1000 bootstrap replicates. This process was repeated using the model HKY85+I but produced the same topologies, hence only the results for the GTR models are presented. Finally, because *Wolbachia* strains can undergo intergenic recombination [53], we also conducted individual analyses on each of the five *Wolbachia* housekeeping genes, using the same protocols. All nexus files are available in the supplemental material.

### Results

#### Archived Sequences

Sequences resulting from this study have been deposited in the NCBI sequence read archive under project accession number SRP041582 (pyrosequences) and in GenBank under accession numbers KJ494864–8, and KJ522437–49 (Sanger sequences).

#### *Sitona obsoletus*

Pyrosequencing of the bacterial community across populations of the clover root weevil, *S. obsoletus*, indicated that the maternally inherited bacterial endosymbionts *Wolbachia* and *Rickettsia* were present in all pooled samples (Table 1). Reads from these two bacterial genera dominated all of the

**Table 1** Bacterial diversity in *Sitona obsoletus* as determined by bTEFAP of 16S rRNA

Bacterial taxon	Percentage of bacterial community composition for <i>Sitona obsoletus</i> (and number of reads)					
	Canterbury population				Northland population	
Primers	Adult sample 1 28F/519R	Adult sample 2 28F/519R	Adult sample 2 939F/1492R	Larval sample 28F/519R	Adult sample 1 28F/519R	Adult sample 1 939F/1492R
<i>Wolbachia</i> <sup>a,b</sup>	34.5 % (360)	72.8 % (5457)	71.6 % (3857)	59.2 % (2225)	45.3 % (1669)	31.8 % (6236)
<i>Rickettsia</i> <sup>b</sup>	51.7 % (539)	24.8 % (1857)	25.0 % (1346)	10.3 % (389)	4.0 % (146)	3.3 % (647)
Strain 1	50.9 % (531)	24.2 % (1812)	24.5 % (1320)	7.6 % (288)	2.9 % (106)	2.1 % (418)
Strain 2	0.8 % (8)	0.6 % (45)	0.5 % (26)	2.7 % (101)	1.1 % (40)	1.2 % (229)
(Obligate)	0	0	0	4.5 % (170)	0	0
Enterobacteriales						
<i>Pantoea</i>	3.5 % (36)	0	<0.1 % (1)	0	49.0 % (1807)	63.6 % (12,490)
<i>Lactococcus</i>	7.7 % (80)	0	0	0	0.1 % (3)	0.1 % (15)
<i>Rhizobium</i>	0	0	0	25.3 % (952)	0.3 % (10)	0
<i>Enterobacter</i>	1.3 % (14)	0	0	0	0.2 % (8)	0.1 % (10)
<i>Rahnella</i>	0	1.8 % (135)	3.2 % (177)	0	0.2 % (6)	0.9 % (179)
Others <sup>c</sup>	1.3 % (14)	0.6 % (45)	0.1 % (8)	0.6 % (23)	1.0 % (36)	0.2 % (46)
Total reads	1043	7494	5415	3759	3685	19,623

<sup>a</sup> Generic classifications include both genus (95–97 % identity) and species (>97 % identity) matches

<sup>b</sup> Taxa that are known bacterial endosymbionts of arthropods

<sup>c</sup> Composed of taxa with less than 1 % representation in the sample

adult samples from Canterbury, composing 85–97 % of reads. *S. obsoletus* larvae from Canterbury additionally had strong representation from bacteria in the genus *Rhizobium* (25 %), presumably from consuming nodulated legume roots that were colonized by these bacteria. In contrast, the majority (49–64 %) of reads from the Northland sample aligned with bacteria in the genus *Pantoea* (Family Enterobacteriaceae), bacteria that are frequently associated with both plant and insect samples. *Wolbachia* and *Rickettsia* were also both present in the Northland sample, but *Rickettsia*, in particular, contributed a relatively minor proportion of reads (3–4 %) when compared to the two adult Canterbury samples, which had 25 and 52 % *Rickettsia*. The two adult Canterbury samples, which were collected on different dates and analyzed in different pyrosequencing runs, yielded a similar range of bacterial species, but the percentage composition was variable. For the two *S. obsoletus* samples that were analyzed using two different primer sets (28F–519R and 939F–1492R), the community composition appeared to be largely congruent.

Diagnostic PCR showed that most (97 %) of individual specimens of *S. obsoletus* from Canterbury and all from Northland were infected with *Wolbachia* (Table 2). This *Wolbachia* strain has not previously been described: three of the five MLST genes had novel alleles that were new accessions to the *Wolbachia* PubMLST database (*coxA*, *gatB*, *hcpA*; Figs S1–S5). A reconstructed phylogeny using the concatenated MLST sequences from five genes placed this *Wolbachia* strain in supergroup A, not too distant from *Wolbachia* strains in *Ceutorhynchus* weevils (Fig. 1). Neither the pyrosequencing nor Sanger sequencing of a subset of individuals gave any indication that multiple strains of *Wolbachia* might be present in *S. obsoletus*. *Rickettsia* was present in the majority of specimens from both populations; 90 % of Canterbury specimens and 66 % of Northland specimens were infected by *Rickettsia*. Furthermore, both pyrosequencing and Sanger sequencing

indicated that two different strains of *Rickettsia* were present in both populations. The 16S sequence from these two strains diverged by 11/1294 bases (0.9 %), and both were strongly supported within a Rickettsial clade that has only been recovered from beetles, including many weevil species (Fig. 2). Both strains of *Rickettsia* were present in both populations, but their frequency of occurrence differed. Whereas the majority of *Rickettsia*-infected weevils in Canterbury had strain 1 (25/32=78 %), only 2/6 (33 %) *Rickettsia*-infected weevils from Northland had strain 1 ( $P=0.047$ , Fisher's exact test). The pyrosequencing results similarly showed a higher ratio of strain 1 relative to strain 2 in Canterbury (40–60× more strain 1 than strain 2 reads) than Northland (1.75–2.6× more strain 1 than strain 2 reads). Inspection of the Sanger sequencing chromatograms did not show evidence of multiple *Rickettsia* strains within an individual, but the possibility of multiple *Rickettsia* infection cannot be entirely excluded.

#### Other Invasive Weevils

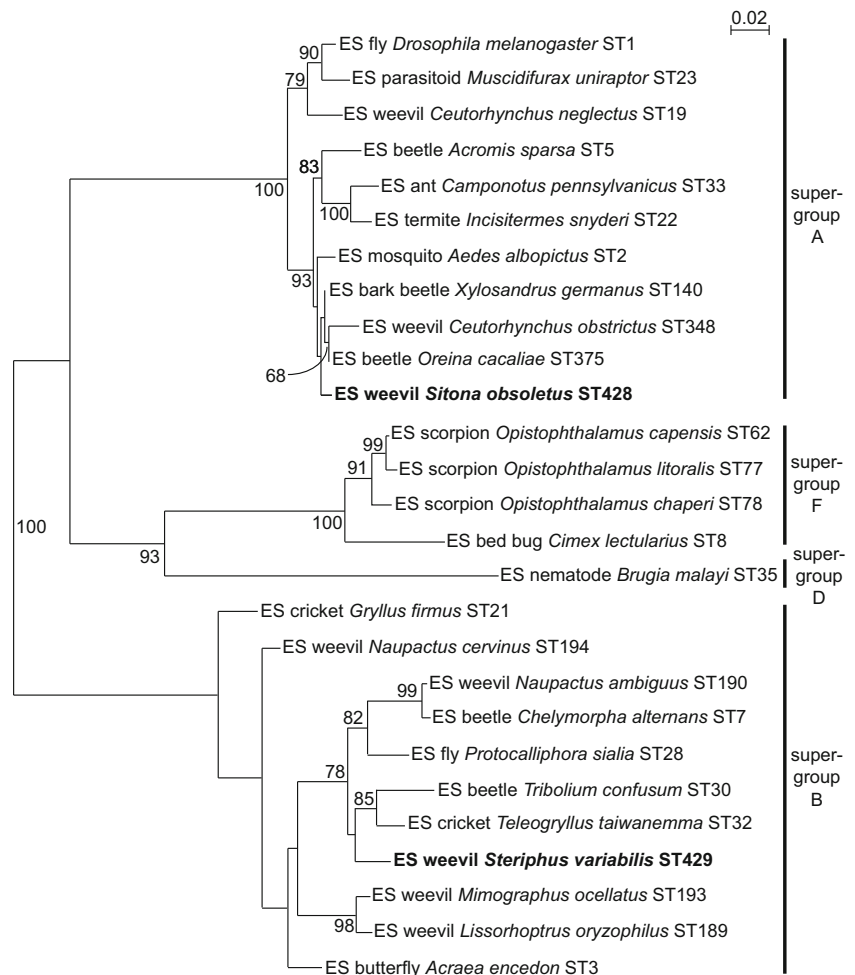
In contrast with *S. obsoletus*, the invasive congener *S. discoideus* lacked any apparent maternally inherited bacterial endosymbionts. This weevil was characterized by a diverse community of environmental bacteria (Table 3). The highest prevalence bacterial strain fell within the *Acidovorax*/*Diaphorobacter* clade, of the family Comamonadaceae, but almost 50 other bacterial genera were represented, including 30 genera at <1 % prevalence of reads within the pyrosequenced sample. The pyrosequenced bacterial community from the other invasive weevil, *L. bonariensis*, was much less diverse, with 89 % of reads belonging to a single bacterial taxon in the Enterobacteriaceae, corresponding to Candidatus *Nardonella*, the predominant bacterial associate of some weevil species (Fig. 3) [54]. We also detected 170 (4.5 %) reads of this same sequence associated with only one of the six *S. obsoletus* samples, which

**Table 2** Diagnostic screening of individual exotic and native New Zealand weevil specimens for selected bacterial genera

Bacterial taxon	<i>S. obsoletus</i>				<i>L. bonariensis</i>	<i>S. discoideus</i>	<i>I. aequalis</i>	<i>St. variabilis</i>
	Canterbury adult 1	Canterbury adult 2	Canterbury larvae	Northland adult				
<i>Nardonella</i>	0/18	0/13	0/8	0/12	11/11	0/18	0/8	17/18
Novel Enterobacteriaceae	0/18	0/13	0/8	0/12	0/11	0/18	8/8	0/18
<i>Rickettsia</i>	16/18	13 <sup>a</sup> /13	6/8	8 <sup>a</sup> /12	0/18	0/18	0/8	0/18
Strain 1	13/16	8/10	4/6	2/6				
Strain 2	3/16	2/10	2/6	4/6				
<i>Spiroplasma</i>	0/18	0/13	0/8	0/12	0/18	0/18	2/8	0/18
Strain 1							1/2	
Strain 2							1/2	
<i>Wolbachia</i>	18/18	13/13	7/8	12/12	0/18	0/18	0/8	18/18

<sup>a</sup> Not all positive specimens were sequenced to determine strain type

**Fig. 1** Phylogenetic placement of *Wolbachia* strains found in exotic and native weevils of New Zealand, based on five concatenated *Wolbachia* multilocus sequence typing (MLST) sequences. Supergroups and sequence types (ST) are as identified by the *Wolbachia* MLST website (<http://pubmlst.org/wolbachia/>). Endosymbionts are represented by ES before the general and Latin names of the host insect. Sequences obtained from this study are in *bold type*. Bootstrap values for the maximum likelihood analysis ( $\times 1000$  replicates) are shown above or below nodes only where greater than 50



we suspect was the result of contamination during the pyrosequencing process or sample handling. Aside from *Nardonella*, 16 bacterial genera were detected in *L. bonariensis*, but none were from known endosymbiotic clades. Diagnostic screens corroborated the presence of *Nardonella* in all *L. bonariensis* specimens (Table 2), and the absence of *Rickettsia*, *Spiroplasma*, and *Wolbachia* in *L. bonariensis* and *S. discoideus* specimens. *Nardonella* was not detected in any *S. obsoletus* or *S. discoideus* specimens (Table 2).

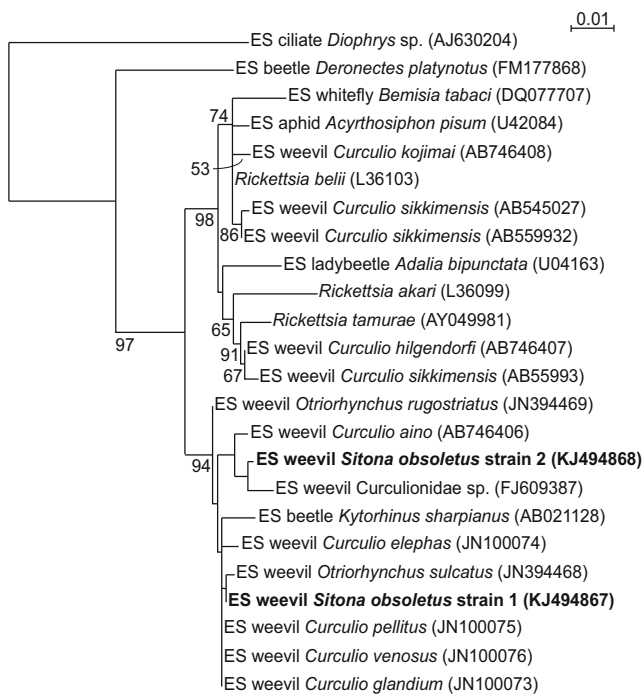
#### Native Weevils

We detected different facultative and apparently obligate endosymbiotic bacteria from both native weevils. *St. variabilis* showed a similar range of bacterial diversity regardless of the primer set used, but in this case the percentage composition was markedly different between primer sets. For 28F–519R, the total read count was small (426 reads), and most came from environmental bacteria (Table 3). However, both *Wolbachia* and *Nardonella* were detected, albeit at relatively low prevalence. These same bacteria were also detected using the 939F–1492R primer set, but here *Nardonella* was the

dominant bacterial strain, comprising 76 % of the 3146 reads. Diagnostic screening showed this bacterium to be present in all but one *St. variabilis* specimen (Table 2). Phylogenetic analysis of a 1294 bp segment of 16S rRNA strongly supported both this and the dominant *L. bonariensis* Enterobacteriaceae strains as members of the *Nardonella* clade.

The *Wolbachia* in *St. variabilis* was found in every individual (Table 2) and a reconstructed phylogeny using the concatenated MLST sequences from five genes placed it in supergroup B, revealing that this strain was quite distinct from the *Wolbachia* found in *S. obsoletus* (Fig. 1). Four of the five MLST genes had novel alleles that were new accessions to the *Wolbachia* PubMLST database (*coxA*, *ftsZ*, *gatB*, *hcpA*; Figs S1–S5). Phylogenies based on the individual MLST genes all supported placement of the *St. variabilis* *Wolbachia* in supergroup B and the *S. obsoletus* *Wolbachia* in supergroup A (Figs. S1–S5).

In *I. aequalis*, the most highly represented bacterial clade was *Spiroplasma*, constituting 42 % of the 23,000 reads (Table 3). These reads could clearly be broken into two distinct strains, differing from one another by 82/486 bp (17 %). Using *Spiroplasma*-specific diagnostic PCR of individual



**Fig. 2** Phylogenetic placement of the two *Rickettsia* strains found in the exotic New Zealand weevil, *Sitona obsoletus* based on 16S ribosomal RNA. Genbank accession numbers are shown in brackets. Endosymbionts are designated by ES, followed by the general and Latin names of the host insect. Free-living bacteria appear on their own. Sequences obtained from this study are in *bold type*. Bootstrap values for the maximum likelihood analysis ( $\times 1000$  replicates) are shown above or below nodes only where greater than 50

specimens, we found that two individuals out of eight, 1 male and 1 female, were infected with *Spiroplasma*. Sanger sequencing indicated that each of these individuals was infected with a different strain of *Spiroplasma*. The *Spiroplasma* in the female was very similar (99.5 %) to endosymbionts recovered from other weevil species in the genus *Curculio* (accession numbers AB545038, JQ692307, JN100091 [16, 18]). The endosymbiotic nature of this strain from *I. aequalis* was strongly corroborated by its clustering with sequences from a clade composed entirely of endosymbiotic *Spiroplasma* strains (Fig. 4). The *Spiroplasma* recovered from the male *I. aequalis* specimen, on the other hand, fell within a clade that contains both endosymbiotic associates of insects and an insect-vectored plant pathogen (*Spiroplasma citri*) (Fig. 4).

We also detected an unknown bacterium within *I. aequalis*, constituting 13 % of reads (Table 3). Diagnostic PCR using newly designed primers (Table S2) found that this bacterial strain was present in all *I. aequalis* specimens. There were no close matches for this bacterium in Genbank, with the closest matches for a 1486-bp segment of 16S rRNA being an unidentified bacteria at ~93 % similarity, and bacteria within the *Xenorhabdus* genus (Class Gammaproteobacteria, Order Enterobacteriales Family Enterobacteriaceae) at 89.6 % similarity. The reconstructed phylogeny, using known

endosymbionts from Enterobacteriaceae, placed this novel bacterium quite distantly from the *Nardonella* clade of weevil endosymbionts [54] (Fig. 3). However, the presence of the novel bacteria in all specimens, as well as the absence of *Nardonella* (Table 2), suggest that these bacteria might play a similar nutritional role [17, 55].

## Discussion

### Maternally Inherited Facultative Symbionts with Defensive Potential

We found three maternally inherited endosymbiotic bacterial taxa in *S. obsoletus* that plausibly might provide defense against parasitoids: one strain of *Wolbachia*, and two different strains of *Rickettsia*. Bacteria in the genus *Wolbachia* are obligate inhabitants of arthropod hosts and are often reproductive parasites [15]. However, multiple recent studies have found that *Wolbachia* can protect insect hosts against viruses [2, 3, 56], bolstering the possibility that strains of this versatile bacterium might be capable of protecting weevils such as *S. obsoletus* and *H. postica* from parasitism as originally postulated by Hsiao [12]. Bacteria in the genus *Rickettsia* are nearly as diverse in their documented effects on arthropod hosts as *Wolbachia* (reviewed in [57, 58]), including a strain that has recently been shown to protect an aphid from a fungal pathogen [7]. Moreover, both *Wolbachia* and *Rickettsia* can be found in host hemolymph [59, 60], where parasitoids such as *M. aethiopoulos* insert their eggs (e.g., [61]). Bacteria that are restricted to only a small portion of the host's anatomy (e.g., gut bacteria such as *Pantoea*, or bacteria that are restricted to bacteriomes) are likely of limited utility in defending the host against parasitoids that insert their eggs elsewhere in the host's body.

It is not yet clear if any of these symbiont strains are involved with parasitoid defense of *S. obsoletus*. These strains represent prospects for further investigation. To attribute symbiont causality, mechanistic tests of parasitoid success in differentially infected hosts will be necessary. In the meantime, correlative data from the present study suggests that two of the three symbiont strains may be particularly worth investigating for defensive properties against parasitoids. *S. obsoletus* throughout New Zealand is highly resistant to a Moroccan strain of the parasitoid *M. aethiopoulos* [30, 40], but shows geographic variation in susceptibility to the Irish strain of the same parasitoid [62]. The latter effect is not driven by parasitoid genetic variation, as parasitoid populations throughout New Zealand are derived from the same genetically homogeneous source colony [63, 64]. *Wolbachia* is the best candidate for a protective role against the Moroccan *M. aethiopoulos* strain, given its nearly universal presence in tested individuals.

**Table 3** Bacterial diversity of exotic and native New Zealand weevils as determined by bTEFAP of 16S rRNA

Bacterial taxon	Percentage of bacterial community composition per weevil species (and number of reads):					
	Primers	<i>L. bonariensis</i> 28F/519R	<i>S. discoideus</i> 28F/519R	<i>I. aequalis</i> 28F/519R	<i>St. variabilis</i> 28F/519R	<i>St. variabilis</i> 939F/1492R
<i>Spiroplasma</i> <sup>a,b</sup> (2 strains)		0	0	42.3 % (9724)	0	0
Strain 1				30.7 % (7067)		
Strain 2				11.6 % (2657)		
<i>Wolbachia</i> <sup>b</sup>		0	0	0	8.5 % (36)	12.8 % (404)
<i>Nardonella</i> <sup>b</sup>		89.2 % (3596)	0	0	3.8 % (16)	75.9 % (2389)
(Obligate) Enterobacteriales		0	0	12.9 % (2974)	0	0
<i>Acidobacterium</i>		0	2.6 % (46)	0	0	0
<i>Acinetobacter</i>		0	2.0 % (36)	0.2 % (54)	0	0
<i>Bacillus</i>		0	1.2 % (22)	0.1 % (22)	0	0
<i>Burkholderia</i>		0	0.9 % (16)	0	28.6 % (122)	6.5 % (203)
<i>Chryseobacterium</i>		0	1.4 % (25)	0.1 % (18)	0	0
<i>Diaphorobacter/Acidovorax</i> <sup>c</sup> (Family Comamonadaceae)		0	39.1 % (688)	14.4 % (3304)		0
<i>Lactobacillus</i>		0	2.8 % (50)	0	0	0
<i>Lactococcus</i>		0	0.7 % (12)	0.2 % (44)	0	1.4 % (44)
<i>Leptospira</i>		0.1 % (3)	0	0	47.7 % (203)	0.2 % (8)
<i>Novosphingobium</i>		0	2.3 % (40)	0.3 % (59)	0	0
<i>Pantoea</i>		5.4 % (217)	13.1 % (231)	24.0 % (5526)	0	0.3 % (11)
<i>Propionibacterium</i>		0	1.7 % (30)	0.5 % (124)	0	0
<i>Pseudomonas</i>		0.4 % (17)	1.0 % (18)	0.1 % (22)	2.3 % (10)	0
<i>Ralstonia</i>		0	0	0	6.1 % (26)	2.4 % (76)
<i>Rheinheimera</i>		3.4 % (137)	0	0	0	0
<i>Riemerella</i>		0	3.1 % (55)	2.3 % (521)	0	0
<i>Staphylococcus</i>		0	7.1 % (125)	0.3 % (59)	0	0
Unknown Acidobacteriaceae		0	1.1 % (20)	0	0	0
Unknown Kofleriaceae		0	1.3 % (23)	0	0	0
Unknown Patulibacteraceae		0	5.1 % (89)	0	0	0
Others <sup>d</sup>		1.5 % (61)	14.1 % (236)	2.4 % (549)	3.1 % (13)	0.3 % (11)
Total reads		4031	1762	23,000	426	3146

<sup>a</sup> Generic classifications include both genus (95–97 % identity) and species (>97 % identity) matches

<sup>b</sup> Taxa that match known bacterial endosymbionts of arthropods

<sup>c</sup> Genera indistinguishable from amplified sequence—both 100 % matches

<sup>d</sup> Composed of taxa with less than 1 % representation in the sample

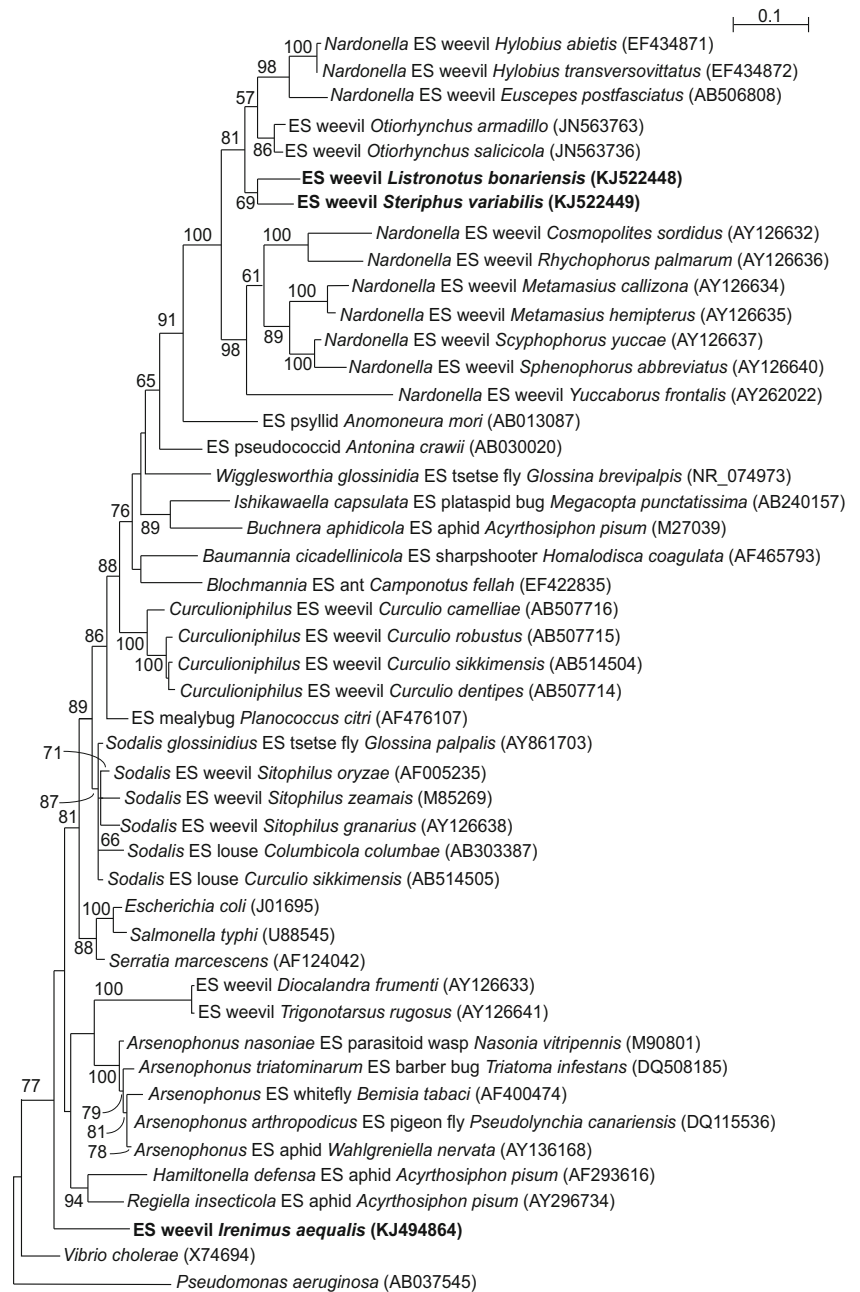
In contrast, *Rickettsia* strain 2 is the best candidate for protection against the Irish parasitoid strain, as this symbiont was found at higher prevalence in Northland, where parasitism rates are lower. Other studies have documented that symbiont strains can vary in their efficacy against different parasitoid strains [65, 66], corroborating the possibility that multiple symbiont strains may be involved in defense in *S. obsoletus*. Furthermore, the invasive congener of this species, *S. discoideus*, lacks *Rickettsia* and *Wolbachia* and is highly susceptible to both Moroccan and Irish strains of the parasitoid (M. McNeill, unpublished data).

Should both symbionts prove to be defensive, *S. obsoletus* would be a useful study system for examining complementary defensive roles of multiple symbionts in an economically important host.

Neither *Wolbachia* nor *Rickettsia* were widespread in other weevils examined in this study, despite their relatively high prevalence in weevils overall [16–18], suggesting that horizontal transmission among co-occurring weevils is not frequent within this system. No other weevil species in this study had *Rickettsia*, and only one other species had *Wolbachia*. The *Wolbachia* in *St. variabilis* was clearly distinct from that of *S. obsoletus*, however, belonging to a different clade



**Fig. 3** Phylogenetic placement of potential nutritional endosymbionts found in exotic and native New Zealand weevils based on 16S ribosomal RNA. Genbank accession numbers are shown in brackets. Latin names are given where the bacteria has been identified; endosymbiotic strains are designated by ES, followed by the general and Latin names of the host insect. Free-living bacteria appear on their own. Sequences obtained from this study are in *bold type*. Bootstrap values for the maximum likelihood analysis ( $\times 500$  replicates) are shown above or below nodes only where greater than or equal to 50

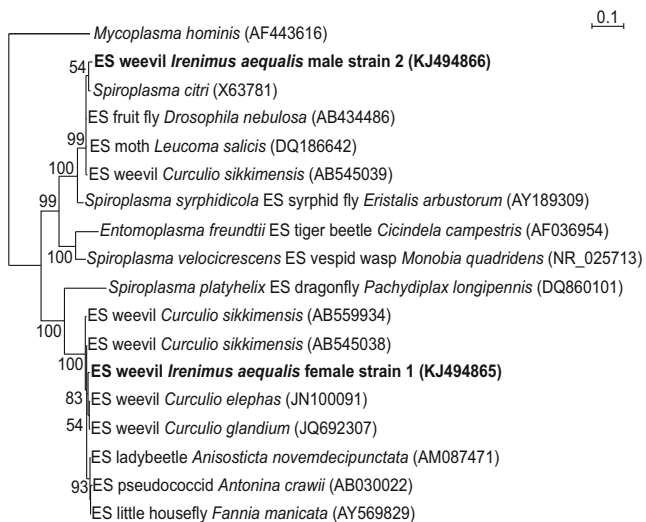


(supergroup B). Across weevils, both supergroup A and B *Wolbachia* strains have previously been reported [16, 18], but functional roles have not been attributed to any sequenced strains from weevils. The early work on potentially defensive *Wolbachia* from *H. postica* [12] was conducted before easy sequencing and strain-typing methodologies were developed, thus sequence information from the *Wolbachia* from *H. postica* is not available as a benchmark for comparison.

We also found two strains of *Spiroplasma* in *I. aequalis*. As a symbiont, *Spiroplasma* can induce reproductive manipulations in some insect hosts [67], but has been associated with parasitoid and nematode defense in others [4, 10]. However, some

*Spiroplasma* strains are horizontally transmitted insect pathogens, insect-vectored plant pathogens or free-living in the environment [68]. Phylogenetic placement supports the likelihood that *Spiroplasma* strain 1 is a maternally transmitted facultative symbiont, given that it falls with high support in a clade entirely composed of symbiotic associates of beetles. The clade for *Spiroplasma* strain 2 was more diverse, containing both free-living and symbiotic *Spiroplasma* strains.

While this survey has focused on bacteria that are obligate and heritable associates of insects, the particularly high prevalence of *Pantoea* within the Northland *S. obsoletus* sample is also worth noting. *Pantoea* strains are frequent gut associates



**Fig. 4** Phylogenetic placement of the two *Spiroplasma* strains found in the native New Zealand weevil *Irenimus aequalis* based on 16S ribosomal RNA. Genbank accession numbers are shown in brackets. Latin names are given where the bacteria has been classified; Endosymbiotic strains are designated by ES, followed by the general and Latin names of the host insect. Free-living bacteria appear on their own. Sequences obtained from this study are in *bold type*. Bootstrap values for the maximum likelihood analysis ( $\times 1000$  replicates) are shown above or below nodes only where greater than 50

of insects, but typically are not maternally inherited: they are cultivable bacteria that are also common in plant-associated samples, and are sometimes pathogenic [69–72]. We found strains of *Pantoea* associated with all of the weevil species we examined, and all strains were very similar to environmental isolates, suggesting that all were environmentally acquired. Nevertheless, some environmentally acquired gut bacteria have been shown to have major phenotypic effects on their insect hosts [73], and *Pantoea* has been shown to influence the fitness of the insects whose guts they inhabit [74]. While relatively unlikely to play a defensive role, *Pantoea* may be worth further investigation as a potentially important associate of *S. obsoletus*.

#### Potential Nutritional Symbionts

Many weevil species are infected with maternally inherited symbionts that aid nutrition and promote normal growth and development [17, 55, 75]. These bacteria are typically housed in specialized organs, bacteriomes, that are associated with the larval gut (reviewed in [76]). The ancestral weevil endosymbiont, Candidatus *Nardonella*, has co-evolved with its weevil hosts for an estimated 125 million years [53, 75]. However, various weevil clades lack *Nardonella*. Some of these weevils are instead infected with bacteria from alternate clades [47, 77, 78], and others apparently lack nutritional endosymbionts entirely [17, 54].

In the present study, we found evidence of *Nardonella* in some of the weevil species examined, but not others. Both

*L. bonariensis* and *St. variabilis* were infected with bacterial strains that fell firmly within the *Nardonella* clade. These strains were distinct from one another (90 % similarity), as would be expected for vertically transmitted symbionts that have codiversified along with their hosts [79]. In contrast, *Nardonella* was not detected at all from *S. discoideus* or *I. aequalis*, and the few pyrosequencing reads detected from one *S. obsoletus* sample were exactly identical to those from *L. bonariensis*, suggesting that they resulted from sample contamination or sequencing barcode errors, rather than a true infection. Diagnostic tests using *Nardonella*-specific primers were consistently negative for all specimens from all three of these species.

In the case of the endemic weevil *I. aequalis*, it is possible that a nutritional symbiont replacement has taken place. An unclassified strain of Enterobacteriaceae was substantially represented among the sequences (~13 %), and was diagnostically found to be represented in all specimens tested. The 16S sequence of this bacterium was distinct from any Genbank accessions (<93 % similarity), corroborating the hypothesis that this is a specialized insect associate, rather than a general environmental bacterium. This bacterium is dissimilar to any previously described obligate weevil symbionts (Fig. 3), and thus may represent an independent symbiont replacement event at some point within the evolutionary history of *I. aequalis*, similar to the replacements that have taken place in *Curculio* and *Sitophilus* weevil genera [17, 75, 77]. However, validation of the symbiont replacement hypothesis for *I. aequalis* awaits physiological studies of bacterial localization, and/or functional tests of bacterial function within the host.

For the two *Sitona* species, it remains to be determined if any maternally inherited bacteria play a nutritional role. Lefevre et al. [54] found that nutritional endosymbionts had been lost from *Sitophilus linearis*, a weevil species that had shifted hosts from an ancestral cereal to a more nutritious legume seed. As *Sitona* weevils also feed on legumes [80], this clade of weevils may not need nutritional assistance, and may have lost their ancestral symbiont as a consequence.

#### Conclusions

The present work reinforces that weevils are host to a diverse array of bacterial associates, some of which may play a role in parasitoid defense, and others in host nutrition. To date, research on symbiont-derived defense among insect hosts has largely focused on aphids [1] and *Drosophila* [10]. Despite the fact that a weevil was the earliest suggested example of symbiont-provided defense against parasitoids [12], it remains unclear exactly which bacterial strains might provide such defense, which weevil species might benefit from it, and what

mechanism provides the defense. Here, we identify three bacterial strains that infect *S. obsoletus*, as candidate defensive symbionts. Based on their sequences, none of these bacteria are particularly unusual, belonging to clades that are widespread endosymbionts of insect in general, and weevils in particular. Should any of these symbionts prove to be defensive, the implications would be far-reaching, given that weevils are one of the most diverse clades of animals on earth, with numerous species of economic import as either pests or agents of biological control [81].

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