View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by University of Dundee Online Publications

University of Dundee

The bacterial community associated with adult vine weevil (Otiorhynchus sulcatus) in UK populations growing on strawberry is dominated by Candidatus Nardonella

Morera-Margarit, P.; Bulgarelli, D.; Pope, T.; Graham, R.; Mitchell, C.; Karley, A. J.

Published in:
Entomologia Experimentalis et Applicata

DOI:
10.1111/eea.12757

Publication date:
2019

Document Version
Peer reviewed version

Link to publication in Discovery Research Portal

Citation for published version (APA):
Morera-Margarit, P., Bulgarelli, D., Pope, T., Graham, R., Mitchell, C., & Karley, A. J. (2019). The bacterial community associated with adult vine weevil (Otiorhynchus sulcatus) in UK populations growing on strawberry is dominated by Candidatus Nardonella. *Entomologia Experimentalis et Applicata*, *167*(3), 186-196. https://doi.org/10.1111/eea.12757

General rights
Copyright and moral rights for the publications made accessible in Discovery Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from Discovery Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the public portal.

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 24. Jun. 2020

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Title

The bacterial community associated with adult vine weevil, *Otiorhynchus sulcatus* Fabricius, UK populations growing on strawberry (*Fragaria x ananassa*), is dominated by *Candidatus Nardonella*

Authors

P. Morera-Margarit[1,2], D. Bulgarelli[3], T. Pope[2], R. Graham[2], C. Mitchell[1] and A. J. Karley[1*]

Addresses

1 The James Hutton Institute, Dundee, United Kingdom

2 Harper Adams University, Newport, United Kingdom

3 Plant Sciences, School of Life Sciences, University of Dundee, Dundee, United Kingdom.

Correspondence: Alison.Karley@hutton.ac.uk

Short Title:

The Vine weevil bacterial microbiota

Key words: bacterial diversity, Illumina sequencing, insect adaptation, operational taxonomic unit, proteobacteria, 16S rRNA gene.

For Peer Review

This is the peer reviewed version of the following article: "The bacterial community associated with adult vine weevil (*Otiorhynchus sulcatus*) in UK populations growing on strawberry is dominated by *Candidatus* Nardonella", *Entomologia Experimentalis et Applicata* (2019) which has been published in final form at https://doi.org/10.1111/eea.12757. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.

Abstract

Otiorhynchus sulcatus Fabricius, commonly known as black vine weevil or simply vine weevil, is an important pest of soft fruit and ornamental crops. This species is endemic to temperate areas of Europe but has spread to many other areas over the last century, including North America and Australasia. The ability of vine weevils to adapt to such different environments is difficult to reconcile with the parthenogenetic reproduction strategy, which is likely to underpin a low genetic diversity. It is therefore tempting to hypothesize that weevil adaptation to different environments is mediated, at least partly, by the microbial communities inhabiting these insects. As a first step towards testing this hypothesis we characterised the composition of the bacterial microbiota in weevils from populations feeding on strawberry plants across four geographically-separate locations in the United Kingdom. We performed 16S rRNA gene Illumina amplicon sequencing, generating 2,882,853 high-quality reads. Ecological indices, namely Chao1 and Shannon, revealed that the populations used for this study harboured a low diversity and an uneven bacterial microbiota. Furthermore, β-diversity analysis failed to identify a clear association between microbiota composition and location. Notably, a single Operational Taxonomic Unit (OTU) phylogenetically related to *Candidatus Nardonella* accounted for 81% of the total sequencing reads for all tested insects. Our results indicate that vine weevil bacterial microbiota resembles other insects as it has low diversity and it is dominated by few taxa. A prediction of this observation is that location *per se* may not be a determinant of the microbiota inhabiting weevil populations. Rather, other or additional selective pressures, such as the plant species used as a food source, ultimately shape the weevil bacterial microbiota. Our results will serve as a reference framework to investigate other or additional hypotheses aimed at elucidating vine weevil adaptation to its environment.

For Peer Review

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

24 # Introduction

25 The association between insects and bacteria has received significant interest in recent decades as
26 many studies have demonstrated the potential importance of these partnerships for insect fitness.
27 Stable associations between two or more organisms, frequently termed symbiosis, is a widespread
28 phenomenon in nature with outcomes ranging from negative to neutral to beneficial, often
29 classified as parasitism, commensalism or mutualism, respectively. These associations can be
30 categorized based on the grade of dependency as primary symbionts, which show strong
31 interdependence and have typically long co-evolutionary history with the host, and facultative
32 symbionts, which show more recent association and are not strongly interdependent. Research on
33 insect-bacteria associations have often focused on pairwise mutualist symbiotic relationships from
34 which insects acquire quantifiable benefits, although often the bacterial community harbored by
35 insects is poorly characterized. Some insects with restricted diets rely on bacteria to compensate
36 nutritional deficiencies. For instance, the pea aphid *Acyrthosiphon pisum* Harris is provided with
37 essential amino acids and the vitamin riboflavin by its obligate endosymbiotic bacterium *Buchnera*
38 *aphidicola* (Nakabachi & Ishikawa, 1999) and the tsetse fly *Glossina morsitans* Westwood is
39 provided with essential vitamins by the endosymbiotic bacterium *Wigglesworthia glossinidia*
40 (Nogge, 1981). Furthermore, bacteria can improve insect host fitness by degrading toxic secondary
41 metabolites produced by plants as a chemical defense. This is the case for the coffee berry borer
42 *Hypothenemus hampei* Ferrari which harbors *Pseudomonas* bacteria that detoxify caffeine by
43 expressing caffeine demethylase genes (Ceja-Navarro et al., 2015). Importantly, certain bacteria
44 have been shown to render their insect hosts less susceptible to predators and pathogens. This has
45 been illustrated for the pea aphid, which is protected from parasitism by the parasitoid wasp
46 *Aphidus ervi* Haliday when aphids are infected with the bacterium *Hamiltonella defensa* (Oliver

For Peer Review

~~et al., 2005; Oliver et al., 2003) and from infection by the entomopathogenic fungus *Pandora neoaphidis* Remaud & Hennebert when aphids harbor the bacterium *Regiella insecticola* (Scarborough et al., 2005), and for the fruit fly *Drosophila melanogaster* Meigen, which becomes more resistant to RNA viruses when infected with the bacterium *Wolbachia* (Hedges et al., 2008). Weevils belong to the superfamily Curculionoidea which is one of the largest insect groups with more than 60,000 described species (Lyal & Alonso-Zarazaga, 2006). Weevil-associated bacteria studies, similarly to research on other insects, have typically focused on the symbiotic association between the bacterium *Nardonella* and different weevil species. Research started at the beginning of the 1990s with the observation of intracellular microorganisms confined in specialized cells, called bacteriocytes, in the rice weevil *Calandra oryzae* Linnaeus, although it remained undetermined whether the observed bacteria constituted a "symbiotic organ" or were simply "accessory cells" (Mansour, 1927; 1930; Pierantoni, 1927). Further investigation combining molecular techniques and fitness measures showed that these bacteria were present in different weevil species and were involved in adult development (Campbell et al., 1992; Nardon & Grenier, 1988). Nonetheless, it was not until the beginning of the 21st century that Lefevre et al. (2004), based on a phylogenetic analysis of the 16S rRNA gene, identified this microorganism as a γ-proteobacterium and designated the new lineage *Candidatus Nardonella.* This bacterium has been shown to be widespread throughout the weevil superfamily and is estimated to have become associated with weevils 125 million years ago (Conord et al., 2008; Lefevre et al., 2004). Nevertheless, some studies revealed that *Nardonella* has been replaced by another bacterium in species of the genus *Curculio* and the tribe Curculionini, highlighting the dynamic nature of insect-bacteria associations (Toju et al., 2010; Toju et al., 2013). Subsequent studies focused on identifying *C. Nardonella* in other weevil species and on studying other features of its biology,~~

For Peer Review

~~such as population dynamics during different insect life stages or the location of the *Nardonella* bacteriocytes in insect tissues (Conord et al., 2008; Hosokawa & Fukatsu, 2010; Hosokawa et al., 2015; Huang et al., 2016; Mansour, 1930; Nardon et al., 2002; Toju & Fukatsu, 2011). Importantly, Anbutsu et al. (2017) working on the black hard weevil *Pachyrhynchus infernalis* Fairmaire showed that *Nardonella* is involved in cuticle formation by contributing to tyrosine synthesis as its suppression produced adults with low tyrosine titers and reddish, crumpled and/or deformed elytra.~~

Vine weevils, *Otiorhynchus sulcatus*, are parthenogenetic triploid females endemic to central Europe (Moorhouse et al., 1992). In the last two centuries, vine weevil distribution has expanded rapidly, primarily through plant trade routes, and this species is now found in most parts of Europe, and in parts of North America, South America, New Zealand and Japan (Kingsley, 1898; Masaki et al., 1984; Moorhouse et al., 1992; Prado, 1988). Vine weevils have been recorded developing successfully on 150 different host plant species (Moorhouse et al., 1992; Smith, 1932; Warner & Negley, 1976) with particular preference for strawberry (Hanula, 1988; van Tol et al., 2004; van Tol & Visser, 1998). Based on the ability of vine weevil to invade and establish in different environments despite its parthenogenetic reproduction mode, we hypothesized that the bacterial community associated with vine weevils could play an important role in insect adaptation.

In the last decade, advances in sequencing and computational approaches have enabled the characterization of the microbial communities associated with both plant and animal eukaryotic hosts, i.e. their microbiotas, at an unprecedented depth (Hacquard et al., 2015). Perhaps not surprisingly, such advances have been exploited to gain novel insights into the ecology of weevil microbiota. For instance, Hirsch et al. (2012) revealed that parthenogenetic species tend to harbor a less diverse bacterial community in comparison with sexual species in the weevil genus

For Peer Review

Otiorhynchus. White et al. (2015) studied the bacterial community associated with exotic and endemic weevils in New Zealand and speculated that the presence of *Wolbachia* and *Rickettsia* could be involved in weevil resistance to parasitoids used in biocontrol. The influence of insect diet on shaping the bacterial microbiota composition was reported in the red palm weevil *Rhynchophorus ferrugineus* Olivier, the cotton boll weevil *Anthonomus grandis* Boheman and the pine weevil *Hylobius abietis* Linnaeus (Ben Guerrero et al., 2016; Berasategui et al., 2017; Montagna et al., 2015). Research by Berasategui et al. (2016) on the bacterial community composition in pine weevil populations across Europe revealed that despite significant variation in bacterial community composition, a core bacterial microbiota seemed to be shared by all pine weevil populations.

Many studies have shown that location can affect the bacterial microbiome of insects. For example, bacterial community richness and composition varied significantly between *D. melanogaster* populations collected from geographically separated areas of the USA (Corby-Harris et al., 2007). Furthermore, collection area was shown to clearly influence bacterial community assemblage of melon aphid, *Aphis gossypii* Glover, populations sampled across four Hawaiian Islands (Jones et al., 2011). Thus, as a first step to understand the influence of bacteria on vine weevil biology and fitness, we applied high-throughput sequencing techniques to investigate the existence of bacterial community patterns associated with location. For this purpose, we characterized the bacterial community associated with vine weevil populations infesting strawberry plants from geographically separated regions of the UK. Nevertheless, our results indicated that the sampled populations had a highly ~~conserved~~ similar bacterial community dominated by a single bacterial ~~sequence~~ phylotype, classified as *C. Nardonella*, which accounted for 81% of sequencing reads retrieved from all studied insects.

For Peer Review

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

116 ## Materials and methods

117 ### Vine weevil adult populations

118 Vine weevil adults were collected during summer 2015, 2016 and 2017 from an area of
119 approximately 50 m^2 within strawberry crops at five different sites across the UK. Insects collected
120 at different locations were considered as different populations. Exceptionally, we considered
121 insects collected at the Invergowrie site as two separated populations, despite coming from the
122 same area, as they were collected in two consecutive years and could harbour different bacterial
123 community influenced by the different environmental conditions experienced. Details of the
124 collection sites are presented in Table 1 and Figure 1. The collection sites in Stafford were only
125 separated by 766 m whereas the Shifnal and Woore collection sites were separated from these two
126 sites an average distance of 30 km. The collection site in Invergowrie was 494 km distant in
127 average from the rest of the sites. Following collection, insects were directly frozen with liquid N_2
128 and stored at -80°C until further use.

129 ### DNA extraction

130 DNA extraction was performed on eight insects from each population except for the Stafford_2
131 population in which four insects were used due to the small sample size at this site (one insect =
132 one replicate). Insects were surface sterilised in a 1% bleach (May and Baker LTD, Dagenham,
133 England) solution for one minute (Lawrence et al., 2015; Malacrinò et al., 2018). To remove the
134 remaining bleach insects were submerged in autoclaved water three times, each time the insects
135 were submerged for one minute. Surface sterilised insects were ground individually using pestle
136 and mortar sterilised by exposing to UV light for 10 minutes. Once the whole sample was ground
137 to a powder, total DNA was extracted using the NucleoSpin Kit (Macherey-Nagel, Düren,
138 Germany) following the manufacturer's instructions and the alternative step suggested in the Kit

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

139 protocol. An additional incubation at 70°C for 10 minutes was included, after the 10 minutes lysis
140 step at 65°C specified in the protocol, to lyse gram negative bacterial cell walls. Extracted DNA
141 was stored at -20°C in autoclaved Eppendorf tubes until further use.

142 **PCR amplification of the 16S rRNA gene**

143 A fragment of the V4 hypervariable region of the 16S rRNA gene was used for the current bacterial
144 community study as it has been shown to yield optimal community analysis in previous studies
145 (Caporaso et al., 2011) and it was chosen as a reference marker for the Earth Microbiome Project
146 (EMP) (Gilbert et al., 2010). The primers used, 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and
147 806R (5'-GGACTACHVGGGTWTCTAAT-3'), carry an Illumina adapter, pad and linker at the
148 5' terminus. Additionally, the reverse primer (806R) carries a unique barcode which is a 12-base
149 error correcting Golay code to allow multiplexing, i.e. sequencing different samples
150 simultaneously.

151 The Kapa HiFi HotStart PCR kit (Kapa Biosystems, Wilmington, USA) was used to amplify the
152 targeted DNA fragment in a G-Storm GS1 Thermal Cycler (Gene Technologies, Somerton, UK).
153 The PCR mixture (20 µL) consisted of 4 µL of 5X Kapa HiFi Buffer, 1 µL of a 10 ng/µL Bovine
154 Serum Albumin solution (Roche, Mannheim, Germany), 0.6 µL of a 10 mM Kapa dNTPs solution,
155 0.6 µL of a 10 µM solution of each primer, 0.25 µL of Kapa HiFi polymerase (0.02 U/µL), 8 µL
156 of sterile water and 1 µL of a 10 ng/µL solution of the template DNA. Samples in the thermocycler
157 were subjected to three minutes of DNA initial denaturation at 94°C, then 35 cycles of 30 seconds
158 of DNA denaturation at 98°C, 30 seconds of primer annealing at 50°C, and one minute of DNA
159 elongation at 72°C, followed by a final elongation step of 10 minutes at 72°C.

160 Based on the protocol described by Costello et al. (2009) and adopted by the EMP, each insect
161 replicate was PCR amplified using a specific combination of forward and reverse primers with a

For Peer Review

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

162 unique, replicate-specific, barcode. For each primer pair combination, the corresponding PCR
163 reaction was performed in simultaneous triplicates to diminish amplification biases, with an
164 additional no template control. PCR reactions were combined in a barcode-wise manner, i.e.
165 amplification replicates of the same primer pair were mixed and were tested on a 1.5% agarose gel
166 with the corresponding no template control. The simultaneous triplicate amplification procedure
167 was repeated three times for each primer pair combination. So, for each primer pair combination
168 we performed nine amplifications in total. Finally, all PCR products were mixed in a barcode-wise
169 manner (nine amplifications mixed) and kept at -20°C until further use.

170 **Illumina MiSeq library preparation and sequencing**

171 PCR products were purified with Agencourt AMPure XP kit (Beckman Coulter, Brea, USA) using
172 0.7 µL AMPure XP beads per 1 µL of sample. The DNA concentration of 3 µL of each PCR
173 reaction, mixed according to their barcode, was quantified using Picogreen (ThermoFisher, UK)
174 following the manufacturer's recommendations. Next, the amplicon library was generated by
175 mixing individual barcoded replicates in an equimolar ratio. The library was sequenced by the
176 Genome technology group at the James Hutton Institute, Dundee UK, using Illumina MiSeq
177 platform with paired-end reads of 150 bp per read.

178 **Illumina MiSeq data processing with QIIME**

179 The Illumina MiSeq platform generated three FASTQ files with the forward, reverse and barcode
180 sequences. The FASTQ files and the metadata information, organised in a mapping file, were
181 processed with the open source software Quantitative Insights Into Microbial Ecology (QIIME)
182 version 1.9.0 (Caporaso et al., 2010) using the default parameters unless otherwise specified.
183 Forward and reverse FASTQ files were decompressed and merged specifying a minimum
184 sequence overlap of 5 bp between pairs of reads using the command 'join_paired_ends.py' The

For Peer Review

1
2
3 185 reads were quality filtered and demultiplexed with the command 'split_libraries_fastq.py'
4
5
6 186 specifying a minimum Phred quality score of 20. The remaining high-quality reads were clustered
7
8 187 into Operational Taxonomic Units (OTUs) at 97% sequence similarity using SortMeRNA and
9
10 188 sumaclust algorithms. OTUs were defined using a subsampled open-reference OTU picking
11
12 189 approach with the command 'pick_open_reference_otus.py' against the chimera checked
13
14 190 Greengenes database version 13_5 (DeSantis et al., 2006). The output was an OTU table with the
15
16 191 identified OTUs as rows and the samples as columns, containing the abundance of each OTU per
17
18 192 sample. The OTUs that did not match by 97% similarity any bacterial sequence on the database
19
20 193 were classified as Unassigned.
21
22 194 **Identification of the Unassigned OTU_0**
23
24 195 The proportion of different Unassigned OTUs revealed that the dominant OTU was the OTU_0,
25
26 196 which accounted for 99% (2,347,616 reads) of the total reads for Unassigned OTUs (2,364,356
27
28 197 reads). This OTU matched bacterial sequences found in different members of the Curculionidae
29
30 198 family on the NCBI database. The highest matching percentage revealed similarity with bacterial
31
32 199 sequences found in *Otiorhynchus sulcatus* Fabricius (vine weevil) by 100% (GenBank: Accession
33
34 200 No. JN563788.1 and JN563787.1) and in *O. salicicola* Heyden (GenBank: Accession No.
35
36 201 JN394467.1), *O. armadillo* Rossi (GenBank: Accession No. JN394466.1) and *O. rugostriatus*
37
38 202 Goeze (GenBank: Accession No. JN394465.1) by 98% (Hirsch et al., 2012). Furthermore, it
39
40 203 matched bacterial sequences found in *Listronotus bonariensis* Kuschel by 96% (GenBank:
41
42 204 Accession No. KJ522448.1) (White et al., 2015), in *Steriphus variabilis* Broun by 93% (GenBank:
43
44 205 Accession No. KJ522449.1) (White et al., 2015) and a bacterial sequence classified as *Candidatus*
45
46 206 *Nardonella* (γ-proteobacteria) found in *Pachyrhynchus infernalis* by 92% (GenBank: Accession
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

No. AP018160.1) (Anbutsu et al., 2017). Hence, we have provisionally classified the OTU_0 as *C. Nardonella*.

Data analysis with R

To analyse the data with R software version 3.3.3 the packages phyloseq version 1.19.1 (McMurdie & Holmes, 2013) and PMCMR version 4.3 were installed from Bioconductor using the code 'source ("http://bioconductor.org/biocLite.R")' and the function 'biocLite()'. The packages dendextend version 1.8.0, vegan version 2.4-5, ape version 5.0 and ggplot2 version 3.0.0 were installed with the function 'install.packages'. The function ancom was installed using the code 'source("ancom_functions.R")' and 'source("plot_ancom.R")'.

First, a new OTU table was generated after filtering the initial OTU table obtained with QIIME ~~using the function 'prune' to remove~~ for OTUs classified as mitochondria or chloroplast, likely representing a contamination from host tissues and/or the food source. Next, we removed from the remaining OTUs list, instances matching OTUs identified as environmental contaminants of the laboratory where we generated our sequencing library (Pietrangelo et al., 2018) ~~likely representing insect and plant contamination,~~ . After this initial filtering *in silico*, we identified the most abundant OTU in the phylum Bacteroidetes was used as an outgroup to root the phylogenetic tree generated by QIIME. Third, the phyloseq package was used to create the phyloseq object combining the new OTU table, the taxonomy matrix, the phylogenetic tree and the mapping file using the command 'merge_phyloseq'. Fourth, the dataset was filtered to discard OTUs with less than five reads in at least ~~one of the populations~~ 10% of the studied insects with the function 'filter_taxa'.

To study the α-diversity, replicates were rarefied (Gotelli & Chao, 2013; Gotelli & Colwell, 2001; 2011) to a similar sequencing depth of 11,207 reads with the function 'rarefy_even_depth' from the package phyloseq. The Chao1 and Shannon indices were then calculated with the function

For Peer Review

'estimate_richness' from the package phyloseq. Normality was tested by applying a Shapiro-Wilk test with the function 'shapiro.test' which revealed that only Shannon index values were not normally distributed. Therefore, data for Observed OTUs and Chao1 index were analysed with the parametric ANOVA test paired with Tukey test for multiple comparisons with the functions 'aov' and 'TukeyHSD' from the R stats package 3.3.3. Shannon index values were analysed with the non-parametric Kruskal-Wallis test using the functions 'Kruskal.test' and 'posthoc.kruskal.dunn.test' from the package PMCMR.

To study the β-diversity, the dataset was transformed into relative abundances, i.e. sample reads/total amount of reads. A distance matrix was calculated using Bray-Curtis metrics, which considers OTU relative abundance, with the function 'ordinate' from the package phyloseq. A hierarchical cluster analysis was performed with the function 'hclust' and the generated Cluster dendrogram was modified with the function 'set' within the package dendextend before plotting. Statistical differences in microbial composition among populations were tested using a permutational multivariate analysis of variance with the function 'adonis' from the package vegan (Dixon, 2003). OTUs showing significant differences in abundance between populations were revealed by applying an analysis of composition of microbiomes with the function 'ANCOM' from the package ANCOM using the multiple correction option '1'(Weiss et al., 2017).

Results

Vine weevil bacterial microbiota is composed of 85 different bacterial taxa

We characterized the bacterial community of six vine weevil populations collected from strawberry crops grown at different locations in the UK (Table 1 and Figure 1) using an Illumina MiSeq 16S rRNA gene sequencing approach. The sequencing library yielded 3,153,991 high-quality reads which clustered in 994 Operational Taxonomic Units (OTUs) at 97% similarity.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

253 OTUs classified as chloroplast and mitochondria, as well as predicted contaminant OTUs, were
254 removed from the original file, which reduced the number of high-quality reads to 2,882,853 (per
255 sample mean 65,519; max 199,121; and min 11,224) and the number of OTUs to 931. As a result,
256 91% and 93% of the original reads and OTUs, respectively, were kept for further analysis. To
257 discard low abundance OTUs, which have low reproducibility, ~~only those~~ OTUs that had less ~~more~~
258 than five reads in at least 10% of the studied insects were removed ~~retained~~ for subsequent analysis.
259 This further reduced the number of reads to 2,871,373 and the number of OTUs to 85. Although
260 this step reduced the number of OTUs by over 90%, we retained more than 99% of the total number
261 of high-quality reads. This suggested that the bacterial microbiota of the populations tested in this
262 study comprised a relatively low number of highly abundant bacterial taxa.

263 **Vine weevil bacterial microbiota is dominated by γ-proteobacteria and α-proteobacteria**

264 To investigate the taxonomic distribution at genus level, we manually annotated the OTU_0 as *C.*
265 *Nardonella* and imposed a threshold of 1% abundance on the whole dataset for plotting
266 purposes.~~We investigated the taxonomic distribution, focusing on bacterial genera classes with a~~
267 ~~relative abundance greater than 1% on the whole dataset~~. As a result, only two bacterial genera
268 ~~classes~~ and one family, that could not be classified at genus level, were considered: *Candidatus*
269 *Nardonella* (γ-proteobacteria) and *Rickettsia* and Rickettsiaceae (α-proteobacteria) with average
270 relative abundance of 85%, 5.8% and 6.9%, respectively (Figure 2). ~~These two bacterial genus~~
271 ~~classes and family, accounted for 97.7% of the total reads generated for each of the studied insects~~
272 ~~across the 6 vine weevil populations.~~ This further supports the idea that vine weevil bacterial
273 microbiota in the sampled insects was dominated by a small number of taxa.

274 **Vine weevil populations harbor a low diversity bacterial microbiota**

For Peer Review

1
2
3 275 Within population diversity, or α-diversity, computed at OTU level, revealed low diversity in the
4
5 276 bacterial communities across vine weevil populations. On average, populations harbored a
6
7 277 bacterial community comprising 36 OTUs, a richness value (Chao1 index) of 43 and an evenness
8
9 278 value (Shannon index) of 0.5 (Figure 3). Invergowrie populations tended to harbor a less diverse
10
11 279 and more uneven bacterial community compared to the other populations. Statistical analysis of
12
13 280 the observed OTUs revealed that Invergowrie populations tended to harbor a lower number of
14
15 281 OTUs (Figure 3A, ANOVA, F =20.16, df= 5, P< 0.05)-and lower richness index values (Figure
16
17 282 3B, ANOVA, F= 16.89, df=5, P< 0.05) compared to the rest of the populations, although
18
19 283 Stafford_2 and Invergowrie_2 populations were not significantly different (Figure 2A, ANOVA,
20
21 284 H=34.13, df=5, P< 0.05). Statistical analysis of richness values revealed the existence of three
22
23 285 groups with high (Stafford_1 and Woore populations), intermediate (Stafford_2 and Shifnal
24
25 286 populations) and low (Invergowrie_1 and Invergowrie_2 populations) diversity (Figure 2B,
26
27 287 Kruskal-Wallis test, H= 25.28, df=5, P< 0.05). However, . Statistical analysis of Shannon index
28
29 288 values revealed that evenness was significantly lower only for Stafford_2 and Invergowrie_1
30
31 289 populations, compared to the rest of the populations (Figure 3C, Kruskal-Wallis test, H= 19.88,
32
33 290 df=5, P< 0.05).
34
35 291 **Vine weevil bacterial microbiota composition is dominated by *Candidatus Nardonella*.**
36
37 292 Vine weevil bacterial community diversity between populations, or β-diversity, was calculated
38
39 293 using a Bray Curtis approach, which considers OTU relative abundance. This analysis failed to
40
41 294 reveal a clear pattern associated with location as the maximum level of variation between samples
42
43 295 was only 30% (Figure 4). Nevertheless, statistical analysis revealed that despite the high similarity
44
45 296 between samples, there were significant differences in the bacterial community composition
46
47 297 between populations (Adonis test, df=5, P<0.05). We performed a rank-abundance evaluation of
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

Closer inspection of the individual OTUs identified in our library ~~to detect the microbiological basis underpinning the apparent lack of variation in OTU composition across sites. This analysis~~ revealed that samples were dominated by the OTU_0, classified as *C. Nardonella*, which represented 81% of the total sequencing reads and 84%, on average, of the sequencing reads assigned to each individual insect ~~(Figure 4)~~. Thus, the high incidence of a single bacterial phylotype classified as *C. Nardonella* governed the bacterial community assembly of the populations studied here.

Location specific OTUs are dominated by members of the Proteobacteria phylum

Statistical analysis revealed that despite the lack of location-associated pattern in the microbiota composition, ~~the high similarity in bacterial community composition~~, ~~there~~ we identified ~~were~~ significant differences between populations (Adonis test, df=5, $P<0.05$, $R2$ Location= 0.37). ~~We further investigated the presence of significantly different OTUs among populations.~~ A total number of 16 OTUs was shown to vary significantly in abundance between vine weevil populations with 11, 2 and 1 of the OTUs belonging to Proteobacteria, Bacteroidetes and Actinobacteria phyla, respectively, and 2 Unassigned OTUs (ANCOM test, $P<0.01$, multiple test correction). OTUs assigned to Proteobacteria phylum belonged to Sphingomonadales and Rickettsiales orders within α-proteobacteria and to Enterobacteriales, Pseudomonadales and Xanthomonadales orders within γ-proteobacteria. OTUs assigned to Bacteroidetes phylum belonged to Sphingobacteriales and Flavobacteriales orders, and OTUs assigned to Actinobacteria phylum belonged to Actinomycetales order. The average abundance for these OTUs per population was: 0.05% for Stafford_1, 0.02% for Stafford_2, 0.08% for Shifnal, 0.12% for Woore, 0.02% for Invergowrie_1 and 0.02% for Invergowrie_2. Thus, OTUs that varied in abundance between locations represented a small fraction of the total number of reads and, despite belonging to

For Peer Review

different phyla, they were biased towards members of the Proteobacteria phylum. This observation suggests that the 37% of the variance attributed to location in the analysis, is associated, at least partially, to the fluctuation of *C. Nardonella* across populations.

Discussion

The current study characterized for the first time the bacterial community of vine weevil adults from five different UK geographic areas. Our results showed that the bacterial microbiota composition did not follow a pattern governed by location, as only a small fraction of the Operational Taxonomic Units (OTUs) varied in abundance between populations. Furthermore, the bacterial community was dominated by members of the Proteobacteria phylum, with remarkably high abundance of a single bacterium belonging to the γ-proteobacteria and classified as *Candidatus Nardonella.* These findings are consistent with those reported previously in insect bacterial community studies, which revealed a similarly low diversity of bacterial microbiota dominated by members of the Proteobacteria phylum, compared with analogous studies on vertebrates or soil (Bansal et al., 2014; Bili et al., 2016; Broderick et al., 2004; Chandler et al., 2011; Colman et al., 2012; Corby-Harris et al., 2007; Douglas, 2011; Fierer & Jackson, 2006; Gauthier et al., 2015; Ishak et al., 2011; Jones et al., 2013; Robertson-Albertyn et al., 2017; Vasanthakumar et al., 2006; Wong et al., 2011; Yun et al., 2014). This bacterial microbiota pattern seems to be common across insect clades even when targeting different 16S rRNA gene hypervariable regions (Baker et al., 2003; Guo et al., 2013; Suzuki & Giovannoni, 1996; Yang et al., 2016) or applying different DNA extraction procedures (Martin-Laurent et al., 2001). The reasons underlying such an intriguing pattern remain undetermined, although a number of hypotheses have been proposed to explain low microbial diversity in insects. One hypothesis suggests that the insect immune system fine tunes the bacterial microbiota composition in order to

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

344 tolerate only beneficial bacteria as has been seen in *D. melanogaster* and the red palm weevil
345 (Chandler et al., 2011; Dawadi et al., 2018; Lhocine et al., 2008; Login et al., 2011; Ryu et al.,
346 2008). Another hypothesis, although not exclusive, suggests that low microbial diversity results
347 from negative interactions between co-inhabiting bacteria as has been seen between *Buchnera* and
348 *Rickettsia* in the pea aphid (Sakurai et al., 2005), between *Spiroplasma* and *Wolbachia* in *D.*
349 *melanogaster* (Goto et al., 2006) and between *Bartonella* and *Rickettsia* in fleas from the genus
350 *Oropsylla* (Jones et al., 2012). Nonetheless, the biological factors shaping insect bacterial
351 microbiota in this characteristic manner remain speculative and open to future investigation.
352 The findings presented here show that vine weevil bacterial community is mainly composed of
353 members of the α and γ-proteobacteria classes with noteworthy high abundance of the OTU
354 classified as *C. Nardonella*. Conversely, a previous sequencing attempt to characterize vine weevil
355 bacterial microbiota showed that it was composed entirely of members of the α-proteobacteria
356 order and, surprisingly, *C. Nardonella* abundance was very low as it could only be detected by
357 diagnostic PCR with specific primers (Hirsch et al., 2012). Differences between the previous and
358 the current vine weevil bacterial microbiota characterization could be attributed to insect ontogeny
359 as Hirsch et al. (2012) examined 24-72h old vine weevil larvae, whereas we used vine weevil
360 adults close to maturity. Insect life stage has been shown to influence microbial community
361 composition in several insects, for example the Hessian fly *Mayetiola destructor* Say (Bansal et
362 al., 2014), species of the parasitoid wasp genus *Nasonia* (Brucker & Bordenstein, 2012), the rice
363 water weevil *Lissorhoptrus oryzophilus* Kuschel (Huang et al., 2016), the southern pine beetle
364 *Dendroctonus frontalis* Zimmermann (Vasanthakumar et al., 2006), the house fly *Musca*
365 *domestica* Linnaeus (Wei et al., 2013), *D. melanogaster* (Wong et al., 2011) and the neotropical
366 butterfly *Heliconius erato* Linnaeus (Hammer et al., 2014). Furthermore, *Nardonella* in rice water

For Peer Review

weevil was present at low titer in larvae and pupae whereas its abundance increased substantially upon adult emergence (Huang et al., 2016). The mechanisms triggering such developmental changes in microbial composition are unclear, although it has been proposed that adaptation to utilize different resources at different life stages could influence bacterial community composition (Hammer et al., 2014). An additional factor to consider is that Hirsch et al. (2012) used larvae hatched from surface sterilized eggs for bacterial community characterization. Although bacterial transmission to progeny through the egg surface has not been studied in vine weevil, egg surface sterilization could potentially eliminate an important source of bacteria for the developing insect as has been described in other members of the Coleoptera order, such as the reed beetle genus *Macroplea* (Kleinschmidt & Kölsch, 2011; Kölsch et al., 2009) and the rove beetle *Paederus sabaeus* Erichson (Kellner, 2001; 2002). Therefore, to clarify the differences between the two studies, further research should aim to characterize vine weevil larvae bacterial microbiota in comparison with egg and adult life stages.

~~Interestingly, the vine weevil populations considered in our study harbored highly conserved bacterial communities despite belonging to geographically separate areas. This could indicate that vine weevil diet plays a major role in shaping bacterial community composition, as all individuals were collected from the same host plant species. Insect diet has been proposed as an important factor influencing bacterial community composition for many insect species (Broderick et al., 2004; Chandler et al., 2011; Colman et al., 2012; Violetta et al., 2017; Yun et al., 2014). Furthermore, diet influence on bacterial community composition has been acknowledged in closely related members of the weevil superfamily Curculionoidea: the red palm weevil experienced a dramatic change in bacterial community composition after 30 days of feeding on apple, compared with the original population from which these insects were sampled (Montagna~~

For Peer Review

~~et al., 2015); the pine weevil possesses a bacterial microbiota composition resembling that of other bark beetles exploiting the same food source, whereas it differs from closely related weevils exploiting different food sources (Berasategui et al., 2016); populations of the chestnut weevil *Curculio sikkimensis* Hell collected from different *Quercus* species harbored different bacterial microbiota (Toju & Fukatsu, 2011); and the bacterial community of cotton boll weevil *Anthonomus grandis* Boheman changed significantly when fed with different artificial diets (Ben Guerrero et al., 2016). Thus, to confirm that diet is a dominant factor affecting microbial composition in vine weevils, future research should consider characterizing the bacterial community of populations from the same location infesting different host plant species.~~

Perhaps unexpectedly, location specific bacteria detected in our study constituted a small fraction of the total number of reads suggesting that location has a limited role in sculpting the composition of vine weevil bacterial microbiota. However, caution should be exerted when interpreting these data. For instance, our study could be limited by considering a relatively narrow sampling area. Furthermore, Shifnal and Woore populations lacked sampling replicates as we only analyzed one population at those locations. Hence, the greater proportion of location specific OTUs on Woore population, compared with the rest of the populations, may be derived from the sampling design rather than the intrinsic biology of the populations. Thus, future studies should aim to collect insects from a wider geographic area, including different populations from the same area, to determine if location has an influence in bacterial community composition in vine weevil.

The high incidence of the OTU classified as *C. Nardonella* in all tested insects could indicate the importance of its contribution to adult development and cuticle integrity as has been demonstrated in studies of other weevil species (Anbutsu et al., 2017; Kuriwada et al., 2010). *C. Nardonella* is a bacterial symbiont widespread throughout the weevil superfamily located in bacteriocytes

For Peer Review

forming a specialized organ, the bacteriome, which localizes at the foregut/midgut junction of larvae and at the apex of the ovarioles in adults (Conord et al., 2008; Hosokawa & Fukatsu, 2010; Hosokawa et al., 2015; Huang et al., 2016; Mansour, 1930; Nardon et al., 2002). In a recent study, the *Nardonella* genome was sequenced from the black hard weevil *Pachyrhynchus infernalis* revealing that it possesses an extremely small genome (0.20 to 0.23 Mb) with reduced metabolic capacity (Anbutsu et al., 2017), a characteristic feature for primary obligate symbionts (Moya et al., 2008). Results from the same study revealed that this bacterium could influence adult development through its involvement in tyrosine production. Therefore, based on the contribution of *Nardonella* to adult development in other weevil species, it would be of great interest to investigate the dynamics of this bacterium at all vine weevil life stages.

The findings of the present study contribute to the field of research on insect bacterial microbiota as we have comprehensively characterized vine weevil bacterial community of several insect populations by amplifying a region of the V4 hypervariable region of the prokaryotic 16S rRNA gene, paired with Illumina MiSeq sequencing technology. Moreover, our results showed that vine weevil bacterial community of the populations sampled from strawberry plants did not follow a location specific pattern and was dominated by a single bacterium identified as *C. Nardonella*. This study forms the basis for future research to understand the role of ~~diet and other~~ location-specific ~~factors such as~~ biotic and abiotic factors ~~climatic conditions~~ ~~and natural enemy pressures~~ in shaping vine weevil bacterial community. An additional interesting line of research would be to study the importance of *C. Nardonella* for vine weevil development and or reproduction. Likewise, as innovations in sequencing technology are becoming available for experimentation, it will be interesting to accurately identify and quantify the dominance of *C. Nardonella* in the vine weevil microbiota with additional methodologies. This will provide valuable insights for the field of

For Peer Review

agroecology to devise new strategies for management and biocontrol of this damaging and polyphagous insect pest.

Data Availability

The sequences generated in this study are deposited in the European Nucleotide Archive (ENA) under the study accession number PRJEB28361. The script used to analyze the data and generate the figures in this study is available on GitHub athttps://github.com/BulgarelliD-Lab/

Acknowledgments

PMM was funded by the James Hutton Institute and Harper Adams University through a joint PhD studentship. AJK and CM were funded through the strategic research program funded by the Scottish Government's Rural and Environment Science and Analytical Services Division. DB was funded by a Royal Society of Edinburgh personal research fellowship. TP and RB were supported by Harper Adams University. At the James Hutton Institute (Dundee, UK), we thank Dr Pete Hedley and Jenny Morris in the Genome Technology Centre for excellent support in preparing and generating the MiSeq sequencing library and Dr Maddy Giles for helpful comments on the manuscript.

References

Anbutsu H, Moriyama M, Nikoh N, Hosokawa T, Futahashi R, Tanahashi M, Meng X-Y, Kuriwada T, Mori N, Oshima K, Hattori M, Fujie M, Satoh N, Maeda T, Shigenobu S, Koga R & Fukatsu T (2017) Small genome symbiont underlies cuticle hardness in beetles. Proceedings of the National Academy of Sciences of the United States of America 114: E8382-E8391. doi:10.1073/pnas.1712857114.

For Peer Review

457 Baker G, Smith JJ & Cowan DA (2003) Review and re-analysis of domain-specific 16S primers.
458 Journal of Microbiological Methods 55: 541-555.

459 Bansal R, Hulbert SH, Reese JC, Whitworth RJ, Stuart JJ & Chen M-S (2014) Pyrosequencing
460 reveals the predominance of pseudomonadaceae in gut microbiome of a gall midge. Pathogens 3:
461 459-472.

462 Ben Guerrero E, Soria M, Salvador R, Ceja-Navarro JA, Campos E, Brodie EL & Talia P (2016)
463 Effect of different lignocellulosic diets on bacterial microbiota and hydrolytic enzyme activities
464 in the gut of the cotton boll weevil (Anthonomus grandis). Frontiers in Microbiology 7: 2093.

465 Berasategui A, Axelsson K, Nordlander G, Schmidt A, Borg-Karlson AK, Gershenzon J, Terenius
466 O & Kaltenpoth M (2016) The gut microbiota of the pine weevil is similar across Europe and
467 resembles that of other conifer-feeding beetles. Molecular Ecology 25: 4014-4031.

468 Berasategui A, Salem H, Paetz C, Santoro M, Gershenzon J, Kaltenpoth M & Schmidt A (2017)
469 Gut microbiota of the pine weevil degrades conifer diterpenes and increases insect fitness.
470 Molecular Ecology 26: 4099-4110.

471 Bili M, Cortesero AM, Mougel C, Gauthier JP, Ermel G, Simon JC, Outreman Y, Terrat S, Mahéo
472 F & Poinsot D (2016) Bacterial Community Diversity Harboured by Interacting Species. PLoS
473 One 11: e0155392.

474 Broderick NA, Raffa KF, Goodman RM & Handelsman J (2004) Census of the bacterial
475 community of the gypsy moth larval midgut by using culturing and culture-independent methods.
476 Applied and Environmental Microbiology 70: 293-300.

477 Brucker RM & Bordenstein SR (2012) The roles of host evolutionary relationships (genus:
478 Nasonia) and development in structuring microbial communities. Evolution 66: 349-362.

For Peer Review

Campbell BC, Bragg TS & Turner CE (1992) Phylogeny of symbiotic bacteria of four weevil species (Coleoptera: Curculionidae) based on analysis of 16S ribosomal DNA. Insect biochemistry and molecular biology 22: 415-421.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK & Gordon JI (2010) QIIME allows analysis of high-throughput community sequencing data. Nature methods 7: 335-336.

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N & Knight R (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Academy of Sciences 108: 4516-4522.

Ceja-Navarro JA, Vega FE, Karaoz U, Hao Z, Jenkins S, Lim HC, Kosina P, Infante F, Northen TR & Brodie EL (2015) Gut microbiota mediate caffeine detoxification in the primary insect pest of coffee. Nature Communications 6: 7618. doi:10.1038/ncomms8618 https://www.nature.com/articles/ncomms8618#supplementary-information.

Chandler JA, Lang JM, Bhatnagar S, Eisen JA & Kopp A (2011) Bacterial communities of diverse Drosophila species: ecological context of a host–microbe model system. Plos Genetics 7: e1002272.

Colman DR, Toolson EC & Takacs-Vesbach C (2012) Do diet and taxonomy influence insect gut bacterial communities? Molecular Ecology 21: 5124-5137. doi:doi:10.1111/j.1365-294X.2012.05752.x.

Conord C, Despres L, Vallier A, Balmand S, Miquel C, Zundel S, Lemperiere G & Heddi A (2008) Long-term evolutionary stability of bacterial endosymbiosis in Curculionoidea: additional evidence of symbiont replacement in the Dryophthoridae family. Molecular Biology and Evolution 25: 859-868.

For Peer Review

502 Corby-Harris V, Pontaroli AC, Shimkets LJ, Bennetzen JL, Habel KE & Promislow DE (2007) Geographical distribution and diversity of bacteria associated with natural populations of Drosophila melanogaster. Applied and Environmental Microbiology 73: 3470-3479.

505 Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI & Knight R (2009) Bacterial community variation in human body habitats across space and time. Science 326: 1694-1697.

507 Dawadi B, Wang X, Xiao R, Muhammad A, Hou Y & Shi Z (2018) PGRP-LB homolog acts as a negative modulator of immunity in maintaining the gut-microbe symbiosis of red palm weevil, Rhynchophorus ferrugineus Olivier. Developmental & Comparative Immunology 86: 65-77. doi:https://doi.org/10.1016/j.dci.2018.04.021.

511 DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P & Andersen GL (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and Environmental Microbiology 72: 5069-5072.

514 Dixon P (2003) VEGAN, a package of R functions for community ecology. Journal of Vegetation Science 14: 927-930.

516 Douglas AE (2011) Lessons from studying insect symbioses. Cell Host & Microbe 10: 359-367.

517 Fierer N & Jackson RB (2006) The diversity and biogeography of soil bacterial communities. Proceedings of the National Academy of Sciences of the United States of America 103: 626-631. doi:10.1073/pnas.0507535103.

520 Gauthier J-P, Outreman Y, Mieuzet L & Simon J-C (2015) Bacterial Communities Associated with Host-Adapted Populations of Pea Aphids Revealed by Deep Sequencing of 16S Ribosomal DNA. PLoS One 10: e0120664. doi:10.1371/journal.pone.0120664.

For Peer Review

523 Gilbert JA, Meyer F, Antonopoulos D, Balaji P, Brown CT, Brown CT, Desai N, Eisen JA, Evers
524 D & Field D (2010) Meeting report: the terabase metagenomics workshop and the vision of an
525 Earth microbiome project. Standards in genomic sciences 3: 243.
526 Gotelli NJ & Chao A (2013) Measuring and estimating species richness, species diversity, and
527 biotic similarity from sampling data.
528 Gotelli NJ & Colwell RK (2001) Quantifying biodiversity: procedures and pitfalls in the
529 measurement and comparison of species richness. Ecology letters 4: 379-391.
530 Gotelli NJ & Colwell RK (2011) Estimating species richness. Biological diversity: frontiers in
531 measurement and assessment 12: 39-54.
532 Goto S, Anbutsu H & Fukatsu T (2006) Asymmetrical interactions between Wolbachia and
533 Spiroplasma endosymbionts coexisting in the same insect host. Applied and Environmental
534 Microbiology 72: 4805-4810.
535 Guo F, Ju F, Cai L & Zhang T (2013) Taxonomic Precision of Different Hypervariable Regions
536 of 16S rRNA Gene and Annotation Methods for Functional Bacterial Groups in Biological
537 Wastewater Treatment. PLoS One 8: e76185. doi:10.1371/journal.pone.0076185.
538 Hacquard S, Garrido-Oter R, González A, Spaepen S, Ackermann G, Lebeis S, McHardy AC,
539 Dangl JL, Knight R & Ley R (2015) Microbiota and host nutrition across plant and animal
540 kingdoms. Cell Host & Microbe 17: 603-616.
541 Hammer TJ, McMillan WO & Fierer N (2014) Metamorphosis of a Butterfly-Associated Bacterial
542 Community. PLoS One 9: e86995. doi:10.1371/journal.pone.0086995.
543 Hanula JL (1988) Oviposition preference and host recognition by the black vine weevil,
544 *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). Environmental Entomology 17: 694-698.

For Peer Review

545 Hedges LM, Brownlie JC, O'Neill SL & Johnson KN (2008) *Wolbachia* and Virus Protection in
546 Insects. Science 322: 702-702. doi:10.1126/science.1162418.
547 Hirsch J, Strohmeier S, Pfannkuchen M & Reineke A (2012) Assessment of bacterial
548 endosymbiont diversity in Otiorhynchus spp.(Coleoptera: Curculionidae) larvae using a multitag
549 454 pyrosequencing approach. BMC microbiology 12: S6.
550 Hosokawa T & Fukatsu T (2010) Nardonella endosymbiont in the West Indian sweet potato weevil
551 Euscepes postfasciatus (Coleoptera: Curculionidae). Applied Entomology and Zoology 45: 115-
552 120.
553 Hosokawa T, Koga R, Tanaka K, Moriyama M, Anbutsu H & Fukatsu T (2015) Nardonella
554 endosymbionts of Japanese pest and non-pest weevils (Coleoptera: Curculionidae). Applied
555 Entomology and Zoology 50: 223-229.
556 Huang X, Huang Y, Zhang J, Lu F, Wei J & Jiang M (2016) The Symbiotic Bacteria Nardonella
557 in Rice Water Weevil (Coleoptera: Curculionidae): Diversity, Density, and Associations With
558 Host Reproduction. Annals of the Entomological Society of America 109: 415-423.
559 doi:10.1093/aesa/saw015.
560 Ishak HD, Plowes R, Sen R, Kellner K, Meyer E, Estrada DA, Dowd SE & Mueller UG (2011)
561 Bacterial diversity in Solenopsis invicta and Solenopsis geminata ant colonies characterized by
562 16S amplicon 454 pyrosequencing. Microbial ecology 61: 821-831.
563 Jones RT, Bernhardt SA, Martin AP & Gage KL (2012) Interactions Among Symbionts of
564 Oropsylla spp. (Siphonoptera: Ceratophyllidae). Journal of Medical Entomology 49: 492-496.
565 doi:10.1603/ME11244.

For Peer Review

566 Jones RT, Bressan A, Greenwell AM & Fierer N (2011) Bacterial communities of two
567 parthenogenetic aphid species cocolonizing two host plants across the Hawaiian Islands. Applied
568 and Environmental Microbiology 77: 8345-8349.
569 Jones RT, Sanchez LG & Fierer N (2013) A cross-taxon analysis of insect-associated bacterial
570 diversity. PLoS One 8: e61218.
571 Kellner RL (2001) Suppression of pederin biosynthesis through antibiotic elimination of
572 endosymbionts in Paederus sabaeus. Journal of Insect Physiology 47: 475-483.
573 Kellner RL (2002) Molecular identification of an endosymbiotic bacterium associated with pederin
574 biosynthesis in Paederus sabaeus (Coleoptera: Staphylinidae). Insect biochemistry and molecular
575 biology 32: 389-395.
576 Kingsley R (1898) On the occurrence of the black vine weevil (*Otiorhynchus sulcatus*) in Nelson.
577 Transactions and Proceedings of the New Zealnd Institute 22: 338-340.
578 Kleinschmidt B & Kölsch G (2011) Adopting bacteria in order to adapt to water—how reed beetles
579 colonized the wetlands (Coleoptera, Chrysomelidae, Donaciinae). Insects 2: 540-554.
580 Kölsch G, Matz-Grund C & Pedersen BV (2009) Ultrastructural and molecular characterization of
581 endosymbionts of the reed beetle genus Macroplea (Chrysomelidae, Donaciinae), and proposal of
582 "Candidatus Macropleicola appendiculatae" and "Candidatus Macropleicola muticae". Canadian
583 journal of microbiology 55: 1250-1260.
584 Kuriwada T, Hosokawa T, Kumano N, Shiromoto K, Haraguchi D & Fukatsu T (2010) Biological
585 role of Nardonella endosymbiont in its weevil host. PLoS One 5: e13101.
586 Lawrence AL, Hii S-F, Chong R, Webb CE, Traub R, Brown G & Šlapeta J (2015) Evaluation of
587 the bacterial microbiome of two flea species using different DNA-isolation techniques provides

For Peer Review

insights into flea host ecology. FEMS Microbiology Ecology 91: fiv134-fiv134. doi:10.1093/femsec/fiv134.

Lefevre C, Charles H, Vallier A, Delobel B, Farrell B & Heddi A (2004) Endosymbiont phylogenesis in the Dryophthoridae weevils: evidence for bacterial replacement. Molecular Biology and Evolution 21: 965-973.

Lhocine N, Ribeiro PS, Buchon N, Wepf A, Wilson R, Tenev T, Lemaitre B, Gstaiger M, Meier P & Leulier F (2008) PIMS Modulates Immune Tolerance by Negatively Regulating Drosophila Innate Immune Signaling. Cell Host & Microbe 4: 147-158. doi:https://doi.org/10.1016/j.chom.2008.07.004.

Login FH, Balmand S, Vallier A, Vincent-Monégat C, Vigneron A, Weiss-Gayet M, Rochat D & Heddi A (2011) Antimicrobial peptides keep insect endosymbionts under control. Science 334: 362-365.

Lyal CH & Alonso-Zarazaga MA (2006) Addenda and corrigenda to A World Catalogue of Families and Genera of Curculionoidea (Insecta: Coleoptera). 2. Zootaxa 1202: 21-31.

Malacrinò A, Campolo O, Medina RF & Palmeri V (2018) Instar- and host-associated differentiation of bacterial communities in the Mediterranean fruit fly Ceratitis capitata. PLoS One 13: e0194131. doi:10.1371/journal.pone.0194131.

Mansour K (1927) The Development of the Larval and Adult Mid-gut of Calandra Oryzae, Linn., the Rice Weevil. Journal Of Microscopy Science Oxford.

Mansour K (1930) Memoirs: Preliminary Studies on the Bacterial Cell-mass (Accessory Cell-mass) of Calandra Oryzae (Linn.): The Rice Weevil. Journal of Cell Science 2: 421-435.

For Peer Review

Martin-Laurent F, Philippot L, Hallet S, Chaussod R, Germon J, Soulas G & Catroux G (2001) DNA extraction from soils: old bias for new microbial diversity analysis methods. Applied and Environmental Microbiology 67: 2354-2359.

Masaki M, Ohmura K & Ichinohe F (1984) Host range studies of the black vine weevil, *Otiorhynchus sulcatus* (Fabricius)(Coleoptera: Curculionidae). Applied Entomology and Zoology 19: 95-106.

McMurdie PJ & Holmes S (2013) phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8: e61217.

Montagna M, Chouaia B, Mazza G, Prosdocimi EM, Crotti E, Mereghetti V, Vacchini V, Giorgi A, De Biase A, Longo S, Cervo R, Lozzia GC, Alma A, Bandi C & Daffonchio D (2015) Effects of the Diet on the Microbiota of the Red Palm Weevil (Coleoptera: Dryophthoridae). PLoS One 10: e0117439. doi:10.1371/journal.pone.0117439.

Moorhouse E, Charnley A & Gillespie A (1992) A review of the biology and control of the vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). Annals of Applied Biology 121: 431-454.

Moya A, Pereto J, Gil R & Latorre A (2008) Learning how to live together: genomic insights into prokaryote-animal symbioses. Nature Reviews Genetics 9: 218-229. doi:http://www.nature.com/nrg/journal/v9/n3/suppinfo/nrg2319_S1.html.

Nakabachi A & Ishikawa H (1999) Provision of riboflavin to the host aphid, *Acyrthosiphon pisum*, by endosymbiotic bacteria, *Buchnera*. Journal of Insect Physiology 45: 1-6. doi:http://dx.doi.org/10.1016/S0022-1910(98)00104-8.

For Peer Review

630 Nardon P & Grenier A (1988) Genetical and biochemical interactions between the host and its
631 endocytobiotes in the weevils Sitophilus (Coleoptere, Curculionidae) and other related species:
632 Cell to cell signals in plant, animal and microbial symbiosis (ed. Springer, pp. 255-270.
633 Nardon P, Lefevre C, Delobel B, Charles H & Heddi A (2002) Occurrence of endosymbiosis in
634 Dryophthoridae weevils: cytological insights into bacterial symbiotic structures. Symbiosis 33:
635 227-241.
636 Nogge G (1981) Significance of symbionts for the maintenance of an optimal nutritional state for
637 successful reproduction in hematophagous arthropods, Vol. 82: Parasitology (ed. CAMBRIDGE
638 UNIV PRESS 40 WEST 20TH STREET, NEW YORK, NY 10011-4211, pp. 101-104.
639 Oliver KM, Moran NA & Hunter MS (2005) Variation in resistance to parasitism in aphids is due
640 to symbionts not host genotype. Proceedings of the National Academy of Sciences of the United
641 States of America 102: 12795-12800. doi:10.1073/pnas.0506131102.
642 Oliver KM, Russell JA, Moran NA & Hunter MS (2003) Facultative bacterial symbionts in aphids
643 confer resistance to parasitic wasps. Proceedings of the National Academy of Sciences 100: 1803-
644 1807. doi:10.1073/pnas.0335320100.
645 Pierantoni U (1927) L'organo simbiotico nello sviluppo di Calandra oryzae. Rendiconto della
646 Accademia delle scienze fisiche e matematiche Napoli 35: 244-250.
647 Pietrangelo L, Bucci A, Maiuro L, Bulgarelli D & Naclerio G (2018) Unraveling the composition
648 of the root-associated bacterial microbiota of Phragmites australis and Typha latifolia. Frontiers
649 in Microbiology 9.
650 Prado E (1988) Notas sobre insectos de importancia agrícola en Chile. Agricultura Técnica. Chile
651 48: 51-54.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

652 Robertson-Albertyn S, Alegria Terrazas R, Balbirnie K, Blank M, Janiak A, Szarejko I,
653 Chmielewska B, Karcz J, Morris J & Hedley PE (2017) Root hair mutations displace the barley
654 rhizosphere microbiota. Frontiers in plant science 8: 1094.
655 Ryu J-H, Kim S-H, Lee H-Y, Bai JY, Nam Y-D, Bae J-W, Lee DG, Shin SC, Ha E-M & Lee W-
656 J (2008) Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism
657 in Drosophila. Science 319: 777-782.
658 Sakurai M, Koga R, Tsuchida T, Meng XY & Fukatsu T (2005) Rickettsia symbiont in the pea
659 aphid Acyrthosiphon pisum: Novel cellular tropism, effect on host fitness, and interaction with
660 the essential symbiont Buchnera. Applied and Environmental Microbiology 71.
661 Scarborough CL, Ferrari J & Godfray HCJ (2005) Aphid Protected from Pathogen by
662 Endosymbiont. Science 310: 1781-1781. doi:10.1126/science.1120180.
663 Smith FF (1932) Biology and control of the black vine weevil. US Department of Agriculture.
664 Suzuki MT & Giovannoni SJ (1996) Bias caused by template annealing in the amplification of
665 mixtures of 16S rRNA genes by PCR. Applied and Environmental Microbiology 62: 625-630.
666 Toju H & Fukatsu T (2011) Diversity and infection prevalence of endosymbionts in natural
667 populations of the chestnut weevil: relevance of local climate and host plants. Molecular Ecology
668 20: 853-868. doi:10.1111/j.1365-294X.2010.04980.x.
669 Toju H, Hosokawa T, Koga R, Nikoh N, Meng XY, Kimura N & Fukatsu T (2010) “Candidatus
670 Curculioniphilus buchneri,” a novel clade of bacterial endocellular symbionts from weevils of the
671 genus Curculio. Applied and Environmental Microbiology 76: 275-282.
672 Toju H, Tanabe AS, Notsu Y, Sota T & Fukatsu T (2013) Diversification of endosymbiosis:
673 replacements, co-speciation and promiscuity of bacteriocyte symbionts in weevils. The ISME
674 journal 7: 1378.

For Peer Review

van Tol R, van Dijk N & Sabelis M (2004) Host plant preference and performance of the vine weevil *Otiorhynchus sulcatus*. Agricultural and Forest Entomology 6: 267-278.

van Tol R & Visser J (1998) Host-plant preference and antennal responses of the black vine weevil (*Otiorhynchus sulcatus*) to plant volatiles. Entomologia Experimentalis et Applicata 9: 35-40.

Vasanthakumar A, Delalibera I, Handelsman J, Klepzig KD, Schloss PD & Raffa KF (2006) Characterization of gut-associated bacteria in larvae and adults of the southern pine beetle, Dendroctonus frontalis Zimmermann. Environmental Entomology 35: 1710-1717.

Violetta V, Elena G, Elena C, M. PE, Fabio M, Bessem C, Matteo C, Francesca M, Mauro M, Alberto A & Daniele D (2017) Bacterial diversity shift determined by different diets in the gut of the spotted wing fly Drosophila suzukii is primarily reflected on acetic acid bacteria. Environmental Microbiology Reports 9: 91-103. doi:doi:10.1111/1758-2229.12505.

Warner R & Negley F (1976) The genus *Otiorhynchus* in America north of Mexico (Coleoptera: Curculionidae)[Insects]. Proceedings Entomological Society of Washington.

Wei T, Hu J, Miyanaga K & Tanji Y (2013) Comparative analysis of bacterial community and antibiotic-resistant strains in different developmental stages of the housefly (Musca domestica). Applied Microbiology and Biotechnology 97: 1775-1783.

Weiss S, Xu ZZ, Peddada S, Amir A, Bittinger K, Gonzalez A, Lozupone C, Zaneveld JR, Vázquez-Baeza Y & Birmingham A (2017) Normalization and microbial differential abundance strategies depend upon data characteristics. Microbiome 5: 27.

White JA, Richards NK, Laugraud A, Saeed A, Curry MM & McNeill MR (2015) Endosymbiotic Candidates for Parasitoid Defense in Exotic and Native New Zealand Weevils. Microbial ecology 70: 274-286. doi:10.1007/s00248-014-0561-8.

For Peer Review

Wong CNA, Ng P & Douglas AE (2011) Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. Environmental Microbiology 13: 1889-1900.

Yang B, Wang Y & Qian P-Y (2016) Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. BMC Bioinformatics 17: 135. doi:10.1186/s12859-016-0992-y.

Yun J-H, Roh SW, Whon TW, Jung M-J, Kim M-S, Park D-S, Yoon C, Nam Y-D, Kim Y-J & Choi J-H (2014) Insects gut bacterial diversity determined by host environmental habitat, diet, developmental stage and phylogeny. Applied and Environmental Microbiology: AEM. 01226-01214.

For Peer Review

Figure legends

Figure 1. Location of vine weevil sampling areas across the UK. Each shape represents a population collection site.

Figure 2. Taxonomic classification of bacterial community members at genus ~~class~~ level. ~~α-proteobacteria (filled area) and γ-proteobacteria (unfilled area) are shown.~~ Y-axis represents average relative abundance in percentage of reads. Bars represent each insect from the ~~a~~ population specified on the x-axis. Populations are St1: Stafford_1, St2: Stafford_2, Shf: Shifnal, W: Woore, I1: Invergowrie_1 and I2: Invergowrie_2.

Figure 3. Observed OTUs, richness and evenness of bacterial communities. A) Average number of observed OTUs per population, B) average Chao1 index values of richness per population and C) average Shannon index values of evenness per population. Plotted values sharing the same letter were not significantly different.

Figure 4. Bray-Curtis cluster dendrogram based on dissimilarity of the bacterial community associated with each insect. Each dendrogram leaf represents a single insect and different shapes represent different populations.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Tables

Table 1. Vine weevil population location and year of collection.

POPULATION	LOCATION	YEAR
Stafford_1	Stafford, Staffordshire	2017
Stafford_2	Stafford, Staffordshire	2017
Shifnal	Shifnal, Shropshire	2015
Woore	Woore, Staffordshire	2015
Invergowrie_1	Invergowrie, Dundee	2017
Invergowrie_2	Invergowrie, Dundee	2016

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30

31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 1. Location of vine weevil sampling areas across the UK. Each shape represents a population collection site

933x724mm (72 x 72 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

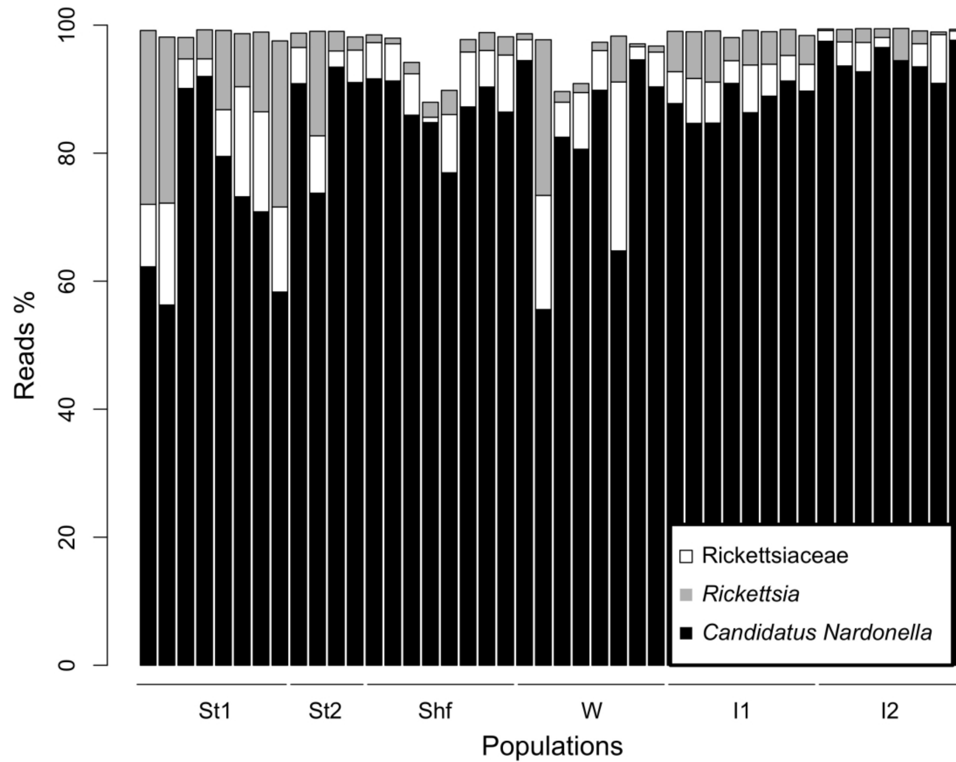

Figure 2. Taxonomic classification of bacterial community members at genus level. Y-axis represents average relative abundance in percentage of reads. Bars represent each insect from the population specified on the x-axis. Populations are St1: Stafford_1, St2: Stafford_2, Shf: Shifnal, W: Woore, I1: Invergowrie_1 and I2: Invergowrie_2.

933x724mm (72 x 72 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30

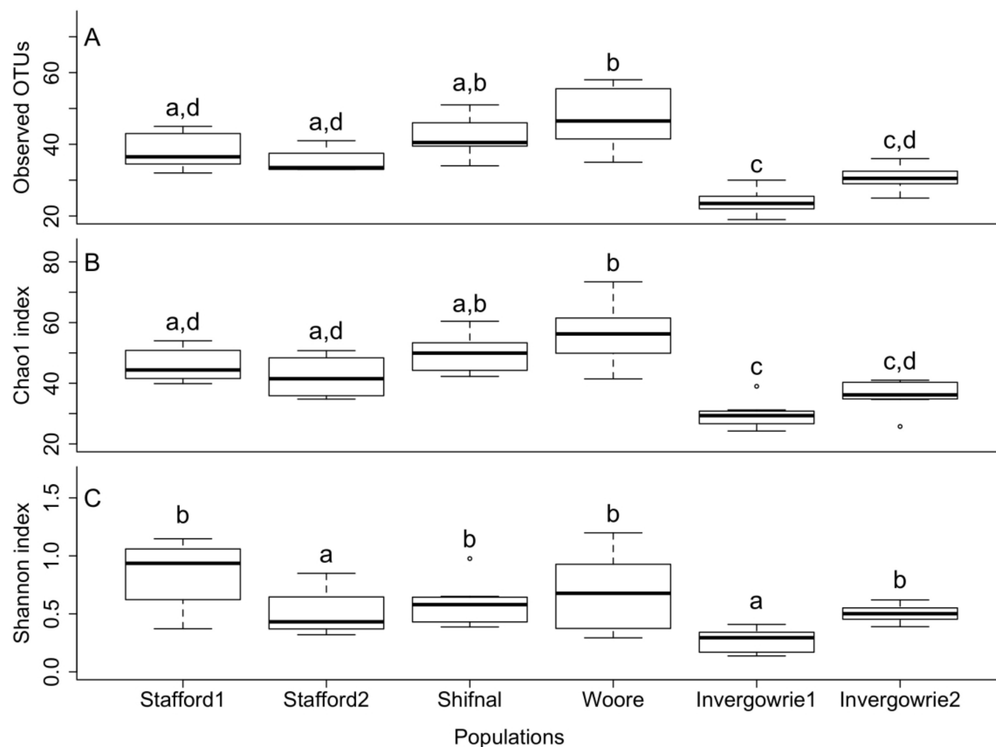

Figure 3. Observed OTUs, richness and evenness of bacterial communities. A) Average number of observed OTUs per population, B) average Chao1 index values of richness per population and C) average Shannon index values of evenness per population. Plotted values sharing the same letter were not significantly different.

933x724mm (72 x 72 DPI)

31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

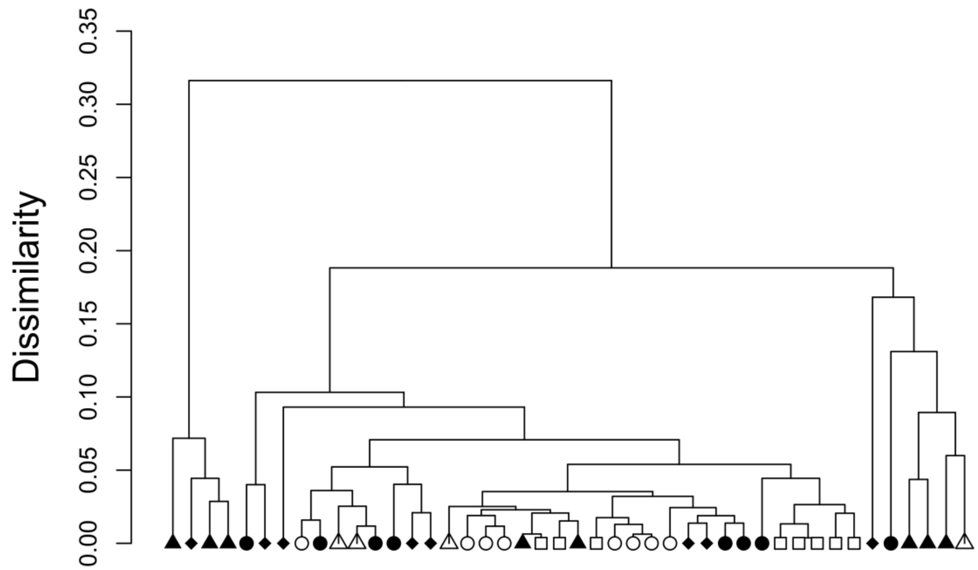

Figure 4. Bray-Curtis cluster dendrogram based on dissimilarity of the bacterial community associated with each insect. Each dendrogram leaf represents a single insect and different shapes represent different populations.

1458x833mm (72 x 72 DPI)