A peer-reviewed version of this preprint was published in PeerJ on 17 May 2018.

View the peer-reviewed version (peerj.com/articles/4793), which is the preferred citable publication unless you specifically need to cite this preprint.

Blankenchip CL, Michels DE, Braker HE, Goffredi SK. 2018. Diet breadth and exploitation of exotic plants shift the core microbiome of *Cephaloleia*, a group of tropical herbivorous beetles. PeerJ 6:e4793 https://doi.org/10.7717/peerj.4793

PeerJ Preprints NOT PEER-REVIEWED

Diet breadth and exploitation of exotic plants shift the core microbiome of tropical herbivorous beetles

Chelsea L. Blankenchip [1] , **Dana E. Michels** [1] , **H. Elizabeth Braker** [1] , **Shana K. Goffredi** [Corresp. 1]

[1] Occidental College, United States

Corresponding Author: Shana K. Goffredi
Email address: sgoffredi@oxy.edu

The beetle genus *Cephaloleia* has evolved in association with tropical ginger plants and for many species their specific host plant associations are known. Here we show that the core microbiome of six closely-related Costa Rican *Cephaloleia* species comprises only 8 bacterial groups, including members of the *Acinetobacter*, Enterobacteriacea, *Pseudomonas*, *Lactococcus,* and *Comamonas*. The *Acinetobacter* and Enterobacteriacea together accounted for 35% of the total average 16S rRNA ribotypes recovered from all specimens. Further, microbiome diversity and community structure was significantly linked to beetle diet breadth, between those foraging on <2 plant types (specialists) versus 9+ plants (generalists). Moraxellaceae, Enterobacteriaceae, and Pseudomonadaceae were highly prevalent in specialist species, and also present in eggs, while Rickettsiaceae associated exclusively with generalist beetles. Bacteria isolated from *Cephaloleia* digestive systems had complementary capabilities and suggested a possible beneficial role in both digestion of plant-based compounds, including xylose, mannitol, and pectin, and possible detoxification, via lipases. *Cephaloleia* species are currently expanding their diets to include exotic invasive plants, yet it is unknown whether their microbial community plays a role in this transition. In this study, colonization of invasive plants was correlated with a dysbiosis of the microbiome, suggesting a possible relationship between gut bacteria and niche adaptation.

1
2
3
4
5
6 # Diet breadth and exploitation of exotic plants shift the core
7 # microbiome of tropical herbivorous beetles
8
9
10
11 Chelsea L. Blankenchip, Dana E. Michels, H. Elizabeth Braker, Shana K. Goffredi*
12
13 Department of Biology, Occidental College, Los Angeles CA, 90041
14
15
16
17
18
19
20
21
22
23
24 *Corresponding Author:
25
26 Dr. Shana K. Goffredi
27 Biology Dept.
28 Occidental College
29 1600 Campus Rd
30 Los Angeles, CA 90041
31 sgoffredi@oxy.edu
32 323-259-1470

PeerJ Preprints NOT PEER-REVIEWED

The beetle genus *Cephaloleia* has evolved in association with tropical ginger plants and for many species their specific host plant associations are known. Here we show that the core microbiome of six closely-related Costa Rican *Cephaloleia* species comprises only 8 bacterial groups, including members of the *Acinetobacter*, Enterobacteriacea, *Pseudomonas*, *Lactococcus,* and *Comamonas*. The *Acinetobacter* and Enterobacteriacea together accounted for 35% of the total average 16S rRNA ribotypes recovered from all specimens. Further, microbiome diversity and community structure was significantly linked to beetle diet breadth, between those foraging on <2 plant types (specialists) versus 9+ plants (generalists). Moraxellaceae, Enterobacteriaceae, and Pseudomonadaceae were highly prevalent in specialist species, and also present in eggs, while Rickettsiaceae associated exclusively with generalist beetles. Bacteria isolated from *Cephaloleia* digestive systems had complementary capabilities and suggested a possible beneficial role in both digestion of plant-based compounds, including xylose, mannitol, and pectin, and possible detoxification, via lipases. *Cephaloleia* species are currently expanding their diets to include exotic invasive plants, yet it is unknown whether their microbial community plays a role in this transition. In this study, colonization of invasive plants was correlated with a dysbiosis of the microbiome, suggesting a possible relationship between gut bacteria and niche adaptation.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26692v1 | CC BY 4.0 Open Access | rec: 14 Mar 2018, publ: 14 Mar 2018

49 **INTRODUCTION**

50 Mutually beneficial symbioses are the rule, rather than the exception, and the discovery and elucidation of
51 the role of symbiotic microorganisms to animal life has emerged as an important area of research. Among
52 insects, persistent bacterial partnerships are well documented and believed to play a critical role in host
53 adaptation to specific niches. Microbiological studies on aphids, stinkbugs, psyllids, white flies,
54 mealybugs, and leaf-hoppers have provided insights into the physiological, ecological, and evolutionary
55 history of bacterial symbioses with insects that primarily consume plant sap (reviewed in 1-4). It is widely
56 accepted that symbioses allow insect herbivores to exploit plants more effectively, however little specific
57 evidence has been reported for chewing phytophagous insects, particularly in the understudied tropical
58 rainforests (5-7).
59
60 With more than 200 described species, the neotropical beetle genus *Cephaloleia* (Chevrolat) has evolved
61 in specific association with gingers in the order Zingiberales (8-11). For ~50 MY, *Cephaloleia* beetles
62 have specialized on the immature rolled leaves of these gingers, which they use exclusively for nutrition,
63 development, reproduction, and as shelter (12). Host plant associations are known for most of the
64 sympatric species of *Cephaloleia* that inhabit the lowland rainforest in and around La Selva Biological
65 Station, Costa Rica (13-14). As a group, they display variability in diet breadth, ranging from
66 hypergeneralist species, such as *C. belti* which feeds on 15+ plants from three Zingiberales families, to
67 specialist species, such as *C. placida* which is only found on a single plant species (12,15). Studies
68 exploring the link between gut bacterial community and diet in non-sap sucking insects have produced
69 conflicting results (16-18), and very few have examined closely-related insects with both generalist and
70 specialist feeding strategies. Fierer and colleagues recommended that in order to resolve the effects of diet
71 on the microbiome, future studies should focus on a single group of insects with varied diets (17). Thus,
72 the contrasting dietary breadths of *Cephaloleia* beetles makes them an ideal natural group by which to
73 examine the, perhaps reciprocal, role of the microbiome on niche adaptation. Additionally, in the last two
74 decades, at least four Zingiberales from South America have invaded the tropical rainforest of La Selva,
75 including the pink velvet banana and white ginger lily (19). Interestingly, at least eight *Cephaloleia* beetle
76 species, including several specialists, are currently expanding their diets to exploit these novel
77 Zingiberales (19).

78 Understanding the presence, diversity, and pervasiveness of bacteria associated with insects, especially in
79 relation to diet breadth, is a necessary and integral component of insect nutritional ecology (20). In this
80 paper, we sought to characterize the diversity of bacteria, via 16S rRNA sequencing and bacterial
81 cultivation, associated with adults (and a small number of eggs) of six species of *Cephaloleia* beetles,
82 including two generalist and four specialist species. Initially we hypothesized that generalist species
83 would have a more diverse microbiome, as an adaptation to a wide range of plant types or as a
84 consequence of increased encounters with diverse bacteria associated with plant tissues (7,21). Major
85 beetle bacterial groups were cultured to determine their metabolic capabilities and whether they might aid
86 in plant digestion or detoxification of plant compounds by the host insect. Three *Cephaloleia* species were
87 collected from invasive white ginger and pink banana, including the generalist *C. belti* and specialists *C.*
88 *placida* and *C. dilaticollis*. Comparisons between beetles feeding on native versus invasive plants was
89 expected to reveal specific bacterial groups related to colonization of exotic plants and provide a better
90 understanding of the role of the microbiome in adaptation.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26692v1 | CC BY 4.0 Open Access | rec: 14 Mar 2018, publ: 14 Mar 2018

MATERIALS AND METHODS

Specimen Collection. Adult beetles (n = 38; Figure 1) were collected in 2014-2016 at La Selva Biological Station, a 1500-hectare ecological reserve in a lowland tropical rainforest site in northern Costa Rica (10˚26'N, 83˚59'W; current Costa Rican Ministry of the Environment and Energy permit #R-026-2015-OT-CONAGEBIO). Adults were located by searching for rolled Zingiberales leaves that were then unrolled and beetles collected with forceps. Partially dissected beetles were preserved in 70% ethanol, at 4˚C, for molecular analysis. For bacterial cultivation, the digestive systems of the beetles were dissected in sterile 1X phosphate buffered saline (PBS) and homogenized using a ground glass mortar and pestle. The resulting homogenate was spread onto Trypticase Soy Agar (TSA) and incubated at ambient temperatures. Resulting bacterial colonies were selected and stored at -80˚C in 30% glycerol (in 1X PBS). All samples were transported back to the US for further processing.

Specimen Identification. Adult beetles were identified based on diagnostic morphology and host plant (the latter mainly for specialist beetles that feed on only a single plant type; 12,15). In one cased we employed DNA analysis for identification; *Cephaloleia dilaticollis* currently encompasses two cryptic species, one of which is a specialist and the other a generalist. Total genomic DNA was extracted using the Qiagen DNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The cytochrome c oxidase I (COI) gene was amplified via the polymerase chain reaction (PCR) using the insect COI primers 1718F (5'-GGAGGATTTGGAAATTGATTAGTTCC-3') and 3661R (5'-CCACAAATTTCTGAACATTGACCA-3') according to McKenna and Farrell 2005 (12). Successful PCR reactions, determined via electrophoresis, were cleaned using MultiScreen HTS plates (Millipore Corporation, Bedford, MA) and sequenced via Laragen, Inc. (Los Angeles, CA). Beetle species identification was confirmed based on COI sequences, upon consultation with Dr. Carlos García-Robledo (University of Connecticut).

Molecular Microbiome Analysis. Adult *Cephaloleia* beetles from 6 species found on native plants (n = 29) and 3 species found on invasive plants (n = 9) were examined for microbiome composition via 16S rRNA gene barcode sequencing (Table 1). As described above, total genomic DNA was extracted using the Qiagen DNeasy Kit (Qiagen, Valencia, CA) according the manufacturer's instructions. All extractions were from individual beetles, with the exception of *C. dorsalis*, which due to its small size, 2-3 individuals were pooled to achieve positive PCR results. PCR amplification of the bacterial 16S rRNA gene was performed using the specific primers, 515F (5'-GTGCCAG-CMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'; 22). The thermal cycling profile used was as follows: an initial denaturation at 94˚ C, then 45 sec at 94˚ C, 1 minute at 50˚ C, and 90 sec at 72˚ C, for 29 cycles, followed by 10 minutes at 72˚ C. Successful PCR amplifications, assessed via electrophoresis, were pooled, in duplicate, and barcodes were added according to the Earth Microbiome Project (EMP; 22); 5 µl of the amplicon product from PCR#1 was used as template in a 5 cycle, 25 µl reconditioning reaction with the same EMP-recommended conditions and the full EMP primers (515f_barcode: AATGATACGGCGAC-CACCGAGATC-TACACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA; 806r_barcode: CAAGCAGAA-GACGGCATACGAGAT-X-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT), where X indicates a unique 12-bp barcode. Adding the barcode indices at the second step minimizes PCR bias

135 that would result from employing long primers over many cycles (23). Further, the use of the
136 'reconditioning' PCR for barcoding, as well as the pooling of duplicate amplifications ahead of barcoding,
137 was an attempt to minimize PCR errors and bias, respectively (24). Samples were mixed together in
138 equimolar amounts and purified in bulk through a Qiagen PCR Purification kit. At all PCR steps,
139 amplification success and purity was checked by gel electrophoresis. Paired-end sequences
140 (2*x* 250 basepair) were generated from barcoded amplicon products at Laragen, Inc on an Illumina
141 MiSeq platform. At Laragen, the raw data was passed through a filter which demultiplexed the library
142 into individual samples and removed any sequences which had >1 basepair mismatch on the 12-
143 basepair barcode sequence, and assigned quality scores to each basepair call on every sequence. At the
144 same time, adapter, barcode, and primer sequences were removed.
145
146 Sequence processing was performed in QIIME 1.8.0 (Quantitative Insights Into Microbial Ecology; 25).
147 Sequences were clustered at 99% similarity and a representative sequence from each cluster was assigned
148 a taxonomic identification using the Silva115 database. Via barcode amplicon sequencing, 13829 to
149 68837 sequences were recovered from each specimen (Tables 1, S1). To avoid artifacts of sequencing
150 depth, the number of sequence reads was standardized to 13829 sequences per specimen, based on the
151 lowest sequence number for specimen 'Cp_inv1' (*C. placida* on invasive white ginger; Table 1). The
152 dataset was further cut off at 1% (i.e. the number of sequence hits for a single bacterial OTU across all 38
153 specimens must have been greater than 138 to be included). After this cutoff, sequences ranged from
154 9899-13324 per specimen (Table 1). *Wolbachia* was observed in all specimens (~11% of all sequences),
155 but was removed from subsequent analysis based on its known prevalence in insects as a reproductive
156 pathogen. Sequences corresponding to chloroplasts and mitochondria were removed from the data set.
157 NMDS, ANOSIM, and SIMPER analyses were completed in Primer-E after square-root transforming the
158 dataset and calculating Bray-Curtis similarities (26). An ANOSIM R value close to "1.0" suggests
159 dissimilarity between groups. Close environmental and cultured relatives were chosen using top hits
160 based on BLAST (www.ncbi.nlm.nih.gov). RStudio was used to perform ANOVA calculations using a
161 script available at http://sites.oxy.edu/ sgoffredi/Goffredi_Lab/LabScripts.
162
163 **Cephaloleia belti *phenotypes and diagnostic PCR.*** *Cephaloleia belti* individuals (n = 45) were
164 photographed and sized (length and width at pronotum) using imageJ (27). The eight largest and eight
165 smallest beetles were dissected for molecular analysis according to the methods described above. Total
166 DNA of the body of the beetles was extracted using the Qiagen DNeasy Kit (Qiagen, Valencia, CA)
167 according the manufacturer's instructions. For these 16 beetles, a diagnostic PCR using two different sets
168 of pathogen-specific PCR primers was performed specifically for the bacterial genera *Rickettsia* (Rsp-F
169 5'- CGCAACCCTCATTCTTATTTGC-3', Rsp-R 5'-CCTCTGTAAACACCAT-TGTAGCA-3'; 28) and
170 *Spiroplasma* (Spiro_16SF 5'-GGTCTTCGGATTGTAAAGGTCTG-3', Spiro_16SR 5'-
171 GGTGTGTACAAGACCCGAGAA-3'; 29) with the following thermal protocol: an initial 5 minute
172 denaturation at 94˚C, then 1 minute at 94˚C, 1 minute at 56˚C, and 1 minute at 72˚C, for 29 cycles, and a
173 final 5 minute extension at 72˚C. Successful PCR amplification was determined via electrophoresis and
174 confirmed to be *Rickettsia* or *Spiroplasma* via direct Sanger sequencing (Laragen, Inc).
175
176 ***Bacterial cultivation***. Initial bacterial suspensions in 30% glycerol stocks were re-grown on TSA plates at
177 30˚C. Growth was checked for morphological purity before being suspended in 40 µl of alkaline PEG
178 (60g of PEG 200 with 0.93 mL of 2M KOH and 39 mL of water). This suspension was then heated to
179 96˚C for 20 minutes in order to lyse the bacterial cells and liberate the DNA. The 16S rRNA gene was

180 then amplified directly using the general PCR primers 27F and 1492R (30) and the following thermal
181 protocol: an initial 5 minute denaturation at 94˚C, followed by 94˚C for 45 seconds, 54˚C for 1 minute,
182 and 72˚C for 90 seconds, for 29 cycles, and a final 72˚C extension for 10 minutes. Successful
183 amplification were checked via electrophoresis, cleaned using MultiScreen HTS plates (Millipore
184 Corporation, Bedford, MA), and sequenced at Laragen Inc. Sequences were compared with the NCBI
185 BLAST database to determine bacterial identity. Bacteria were propagated on TSA plates to ensure
186 proper activity prior to metabolic testing. The ability to digest lactose/glucose, xylose, mannitol, and
187 pectin was determined using phenol red agar (HiMedia, with 10% of each substrate). Protein digestion
188 was determined using Litmus Milk tubes purchased from Carolina Biological Supply Company
189 (Burlington, NC USA). The ability to breakdown lipids was tested using an APIZYM analysis
190 (bioMerieux, Inc; Durham, NC USA), according to the manufacturer's instructions.

191 ***Data availability.*** The raw barcode sequence data are available from the Dryad Digital
192 Repository: doi:10.5061/dryad.5fj6t. Raw sequences were aligned and quality control for unidentified
193 base pairs and chimeras was performed according to the specifics noted at
194 http://sites.oxy.edu/sgoffredi/Goffredi_Lab/LabScripts). The QIIME processed data are also available
195 from the Dryad Digital Repository: doi:10.5061/dryad.5fj6t. 16S rRNA sequences for bacterial isolates
196 are available from GenBank under accession numbers MF776885-MF776899.

197

198 **RESULTS**

199 **The limited core microbiome of *Cephaloleia* beetles.** Using barcode 16S rRNA analysis, the
200 microbiome of adults of six species of *Cephaloleia*, foraging on native plant diets, was characterized
201 taxonomically (Table 1). Collectively, 168 bacterial OTU's ('operational taxonomic unit'; defined as
202 99% sequence similarity) were recovered from all adult specimens examined (n = 29), while individual
203 beetles generally associated with 47-152 OTUs (100 ± 31). Greater than 60% of *Cephaloleia* specimens
204 contained a core group of eight bacterial OTUs, including three members of the *Acinetobacter*, two
205 undefined Enterobacteriacea, *Pseudomonas*, *Lactococcus,* and a *Comamonas* (Figure 3, Supplemental
206 Table S1). These 8 bacterial OTUs comprised the majority of 16S rRNA sequences recovered from each
207 *Cephaloleia* individual (up to 88%).

208 A single bacterial family, Moraxellaceae dominated the microbiomes of all 29 specimens feeding on
209 native plants, combined (representing 24% of the total recovered sequences). The genus *Acinetobacter*, in
210 particular, comprised the vast majority of the Moraxellaceae sequences and accounted for 23% of the 16S
211 rRNA sequences recovered overall. Of the 19 different *Acinetobacter* OTUs, three were responsible for
212 60% of the total *Acinetobacter* diversity and were each present in >75% of beetles (OTUs-131476, 21817,
213 and 28305; Figure 3, shown in purple; Supplemental Table S1), suggesting them to be members of the
214 core *Cephaloleia* microbiome. *Acinetobacter* OTU131476 was found in 22 of 29 specimens, and was
215 11% abundant (on average, for all sequences recovered in each of 29 beetles found on native plants;
216 Figure 3). This OTU was 98% similar to bacteria associated with both leaf cutter ants and fig wasps
217 (GenBank accession #'s LN564930, HQ639556). *Acinetobacter* OTU21817 was also found in 22 of 29
218 specimens, represented 6% average abundance (Figure 3), and was 100% similar to bacteria found in the
219 midgut of a leafworm moth (GenBank accession # KU841476). *Acinetobacter* OTU28305 was found
220 within 23 of 29 specimens, totalling 6% average abundance (Figure 3), and was 100% identical to

221 *Acinetobacter baylyi* (GenBank accession # NR115042), and others found in the rhizosphere.

222 Unidentified Enterobacteriaceae were also dominant in beetles found on native plants, representing 12%
223 of the total recovered sequences. Of the 10 OTUs that comprised the Enterobacteriaceae within
224 *Cephaloleia* beetles, a single OTU was responsible for 65% of the total Enterobacteriaceae diversity. This
225 dominant Enterobacteriaceae OTU-79811 was present in >93% of beetle specimens, was 8% abundant on
226 average (for all 29 beetles found on native plants; Figure 3, shown in blue), and was 100% similar to
227 *Enterobacter*/*Klebsiella* bacteria recovered from scarab beetles, sand flies, and pill bugs (GenBank,
228 unpublished). Two additional OTUs (OTU127346 and OTU79806) each accounted for ~15% of the
229 remaining Enterobacteriaceae and were present in >15 of 29 specimens (Figure 3). These OTUs were
230 related to *Citrobacter* and *Raoultella* OTUs found in the microbiome of numerous insects, including
231 honeybees (KR269812), scarab beetles (KT956239) and fruit flies (KX997073).

232 Three additional OTUs were highly prevalent in *Cephaloleia* microbiomes (present in ~64% of
233 individual beetles), including a *Lactococcus* OTU-157643 representing an average abundance of ~4%
234 (Figure 3), a *Pseudomonas* OTU-126400 with an average abundance of ~2% (related to bacteria
235 recovered from mosquitoes and sand flies; KY041526; 31), and a *Comamonas* OTU-104860, also with an
236 average abundance of ~2% (most closely related to bacteria found in association with fruit flies;
237 KX994588; Figure 3, Supplemental Table S1).

238 A large number of cultured isolates recovered from the digestive systems of *Cephaloleia* (81% of 37
239 isolated bacterial colonies) were members of the Moraxellaceae, Enterobacteriaceae, and
240 Pseudomonadaceae, based on 16S rRNA gene sequencing. Several isolates had 16S rRNA sequences
241 identical to the dominant bacteria identified via barcode 16S rRNA sequencing, including Acineto3 (=
242 *Acinetobacter* OTU-28305), Entero4 (= Enterobacteriaceae OTU-127346), and Pseudo2 (= *Pseudomonas*
243 OTU-126400). The Enterobacteriaceae were found to utilize plant-based compounds, including xylose
244 and pectin (7 of 9 isolates), mannitol (8 isolates), and lactose/glucose (all 9 isolates). In contrast, none of
245 the four *Acinetobacter* isolates in this study were able to digest these compounds, but instead uniquely
246 displayed esterase C4, lipase C8, and lipase C14 capabilities (Figure 4).

247 **Diet breadth influences the microbiome of *Cephaloleia* beetles.** Overall, the microbiome of specialist
248 beetle species was significantly higher in diversity than generalists (2.6 ± 0.5 versus 1.9 ± 0.5,
249 respectively; $p = 0.0006$, one-way ANOVA, Figure 5). Measures of bacterial diversity (via the Shannon
250 diversity index) were 0.8-2.5 for *C. belti*, 1.1-1.9 for *C. reventazonica*, 2.1-3.3 *C. fenestrata*, 2.0-3.0 for *C.*
251 *dorsalis*, 2.4-2.9 for *C. dilaticollis*, and 1.5-3.0 for *C. placida* (Table 1). Further, NMDS ordination
252 revealed the microbial assemblages of *Cephaloleia* to be strongly differentiated by diet breadth (i.e.,
253 generalist versus specialist; $R = 0.74$, $p = 0.001$, analysis of similarity [ANOSIM]; Figure 6A). SIMPER
254 analysis implicated several bacterial families associated with this difference. For example, the
255 Moraxellaceae, Enterobacteriaceae, and Pseudomonadaceae comprised a significantly higher percentage
256 of the bacterial community in beetle species categorized as specialists (34%, 17%, and 6% of recovered
257 sequences on average for specialist individuals, respectively) versus generalists (4%, 3%, 0.3%
258 respectively; all $p < 0.0013$, one-way ANOVA; Figures 2, 5). In contrast, results indicate that generalist
259 *Cephaloleia* beetles were colonized by bacteria traditionally thought of as pathogens, including *Rickettsia*
260 and *Rickettsiella* (discussed in more detail in supplemental results). The Rickettsiaceae comprised a
261 significantly higher percentage of the bacterial community in beetle species categorized as generalists, *C.*

262 *belti* and *C. reventazonica* (28% of recovered sequences on average) versus specialists (only 0.8%; p <
263 0.0001, one-way ANOVA; Figure 4; Supplemental Table S1). For example, two *Rickettsia* OTUs
264 (OTU7980 and OTU153335; Rickettsiaceae) were collectively present in only 11 out of 29 specimens,
265 but comprised 11-26% abundance on average (for all 29 beetles found on native plants; Figure 3). These
266 OTU's were 99% similar to *Rickettsia* found in leafhoppers (KR709154) and ticks (MF002591), to name
267 a few. Similarly, a *Spiroplasma* OTU (Spiroplasmataceae) was present in 12 of 29 specimens,
268 represented an average abundance of ~12%, and was primarily observed in four individuals, two *C.*
269 *dorsalis* and two *C. belti* (Figures 2,3). This OTU57080 was 100% similar to the *Spiroplasma* associated
270 with *Drosophila.* The incidence of *Rickettsia* and *Spiroplasma* was determined, via diagnostic PCR, to be
271 highest in the smallest individuals of *C. belti* (81% prevalence, n = 8) versus the largest (56% prevalence,
272 n = 8). Finally, a single OTU of *Rickettsiella* (Coxielliaceae) was only found in three *C. belti* individuals,
273 but was highly abundant (~31% on average; Figure 3). This OTU43304 was 99% similar to *Rickettsiella*
274 bacteria, also found in sand flies and ticks.

275 ***Bacteria associated with the eggs of* Cephaloleia *beetles found on native plants.*** *Cephaloleia* eggs (n =
276 5) were collected adhered to plastic after mating pairs were kept for a brief time in captivity in bags.
277 Similar to the adults, eggs were examined for microbiome composition via 16S rRNA gene barcode
278 sequencing (Table 1). Four of the most common bacterial OTUs associated with adult beetles
279 (*Acinetobacter*, Enterobacteriaceae, *Comamonas*, and *Pseudomonas*) were consistently observed in eggs
280 (Figure 7, Table 1), suggesting vertical transmission from mother to offspring. NMDS analysis revealed a
281 general overlap of the microbial communities associated with eggs and adults (ANOSIM R = 0.19, p =
282 0.060; data not shown). The egg-associated microbiome of the generalist *C. belti* was significantly
283 different from the adults (ANOSIM R = 0.95, p = 0.022), based mainly on a near absence of *Rickettsia* in
284 the eggs (only 0.01% abundance; Figure 7, Table 1).

285 ***Cephaloleia* beetles foraging on invasive plants have distinct microbiomes.** Specialist *Cephaloleia*
286 species collected on invasive plants exhibited an apparent dysbiosis in their microbiome. NMDS
287 ordination revealed a distinct bacterial community structure between the specialist beetles collected from
288 native plants, compared to those on invasive plants, including *C. placida* and *C. dilaticollis* both on white
289 ginger (*Hedychium coronarium*; ANOSIM R = 0.97, p = 0.001; Figure 6B). The specialist species found
290 on invasive plants possessed a lower abundance of both Moraxellaceae (21% average 16S rRNA
291 abundance when on invasive plants compared to 41% for native feeders; P = 0.0472, one-way ANOVA)
292 and Enterobacteriaceae (6% average abundance in beetles feeding on invasive plants, as opposed to 18%
293 for those feeding on native plants; P = 0.0357; Figure 2; Supplemental Table S1). This decrease in typical
294 microbiome membership may relate directly to a concomitant increase in microbiome members such as
295 Brevinemataceae, which was significantly more abundant in both specialist species on invasive plants
296 (21% average abundance; a single OTU43892, ~99% similar to *Brevinema* found in insect larvae and
297 other invertebrates, represented 97% of all Spirochaete sequences), compared to those on native plants
298 (2%; P = 0.012; Figure 2). The four specimens of the generalist species *C. belti* collected on pink banana
299 (*Musa velutina*) also showed a slight shift in microbiome (ANOSIM R = 0.27, p = 0.05; Figure 6B;
300 Supplemental Table S1), with significant increases in Spiroplasmataceae and Enterobacteriaceae
301 abundance (ANOVA p = 0.05 for both; Figure 2).
302

303 **DISCUSSION**

304 Virtually every living organism has an associated collection of bacteria and bacteria-sourced genes (i.e.
305 microbiome), which account for more genetic and functional potential than even the host genome. Insects
306 have emerged to be important for this research due to their variable nutritional strategies and ecological
307 dominance. The speciose genus *Cephaloleia* has evolved in association with tropical ginger plants and,
308 for many species at La Selva Biological Station in northeastern Costa Rica, their specific host plant
309 associations are known. Several *Cephaloleia* species are also currently expanding their diets to include
310 exotic invasive plants, yet it is not known whether their microbial community plays a role in this
311 transition. For this reason, Costa Rican rolled-leaf beetles within the genus *Cephaloleia* present a unique
312 opportunity to distinguish the effects of host diet from host taxonomy on the associated gut bacteria, as
313 well as to explore whether movement of these specialized insects onto invasive host plants results in
314 changes to the bacterial communities. The factors that affect insect gut bacterial communities are still not
315 fully understood. In particular, diet has been shown to affect gut microbial communities in some insects
316 (16) and conversely be a poor predictor of gut bacterial community composition in others (17). In this
317 study, we show that the core microbiome of six closely related *Cephaloleia* species primarily includes the
318 Moraxellaceae, Enterobacteriaceae, and Pseudomonadaceae, and that diet breadth is significantly linked
319 to microbiome diversity and community structure.

320 Several lines of evidence suggest that the core recovered bacterial OTUs are beneficial to *Cephaloleia*
321 beetle hosts. Four of the most common bacterial OTUs associated with adult beetles were consistently
322 observed in eggs (see supplemental materials), suggesting vertical transmission from mother to offspring.
323 Sampling occurred over the course of 13 months, thus showing that these microbiome members likely
324 have a non-transient relationship with their host. Additionally, *Pseudomonas*, *Enterobacter,* and *Pantoea*
325 (also Enterobacteriaceae) have been found to play influential roles in development, nutrition, and success
326 in other herbivorous beetles and true bugs (4, 32; among others). A study by Minard et al. suggests that
327 the mosquito *Aedes albopictus* specifically associates with *Acinetobacter* to help with digestion of plant
328 nectar (33), while *Acinetobacter* and *Pseudomonas* in bark beetle digestive systems contribute to the
329 nutritional requirements of the insect via the breakdown of plant-based compounds (34).

330 The *in vitro* metabolic capabilities of bacteria isolated from *Cephaloleia* digestive systems provide
331 further evidence for their possible beneficial role. Representative isolates within the Enterobacteriaceae
332 and Moraxellaceae revealed complementary capabilities with regard to the break down plant-related
333 compounds (ex. xylose, mannitol, and pectin) versus lipids, respectively. Previously, Enterobacteriaceae
334 were also found in *Bombyx mori* larvae (Lepidoptera) to similarly utilize mannitol and pectin, suggesting
335 a role in the digestion of the mulberry leaf diet of the host (35). Interestingly, esterases in insects have
336 been shown to detoxify defensive plant compounds (36), thus esterase-producing bacteria like
337 *Acinetobacter* could also provide this service to *Cephaloleia*. Preliminary experiments suggest that
338 *Acinetobacter* isolates grow better in the presence of *Calathea* (Zingiberales) extract as the only source of
339 nutrients (data not shown). Tolerance to, and metabolism of, plant extracts hints at a possible role of
340 either detoxification or direct nutrient acquisition by the dominant *Cephaloleia* microbiome, although
341 further research with cultured bacterial isolates is necessary to examine this more fully.

342 *Cephaloleia* beetles in this study exhibited contrasting dietary breadths; the two generalist species *C. belti*
343 and *C. reventazonica* feed on 9-15 plants from many Zingiberales families, while the four specialist
344 species, including *C. dilaticollis, C. dorsalis, C. fenestrata* and *C. placida,* each feed on only 1-2 plant
345 species (12,15). This comparison of six congeneric *Cephaloleia* species with varying diet breadth satisfies
346 the recommendation of Jones et al. (17) to essentially remove taxonomy as a factor confounding the

347 influence of diet on the gut microbiome. Overall, the microbiome of specialists was significantly higher in
348 diversity than generalists, and was comparatively dominated by the Moraxellaceae, Enterobacteriaceae,
349 and Pseudomonadaceae. The shift shown in Figure 6A perhaps suggests that part of Axis 1 could be due
350 to intrinsic species differences (ex. *C. belti* and *C. reventazonica*, the generalist species, appear separated),
351 but that axis 2 is likely driven by diet breadth (all specialists cluster tightly together). It is worth noting
352 here that *C. dilaticollis* encompasses two cryptic species, with opposing diet breadths, and that the
353 individuals in this study possessed a microbiome community shared with other specialists. Thus, it was
354 possible to infer the limited diet breath of this particular *C. dilaticollis* sub-species, based solely on a
355 distinctive microbiome structure and diversity. The specialist sub-species of *C. dilaticollis* was
356 subsequently confirmed via insect COI sequencing, in consultation with Dr. Carlos Garcia-Robledo
357 (University of Connecticut).
358
359 Generalist *Cephaloleia* beetles were, by contrast, colonized by bacteria traditionally thought of as
360 pathogens, including *Rickettsia* and *Rickettsiella*, and the pattern of occurrence in these beetles (Figure 3,
361 shown in green) is consistent with a pathogen-like relationship (37) in that they infect only a few
362 individuals (and thus exhibit low prevalence), but when present, they achieve high numbers (and thus
363 high abundance). Individuals that were colonized by these groups demonstrated a striking paucity of
364 several of the most prevalent 'core' microbiome members observed in all other beetles, including
365 *Acinetobacter* and Enterobacteriaceae (Figures 2, 5). Sakurai et al. 2005 similarly showed that *Rickettsia*
366 presence in aphids reduced the population of the beneficial bacterial symbiont *Buchnera* to 50-60% of its
367 density in *Rickettsia*-free individuals. If these interloper microbial groups are detrimental, it would follow
368 that beetles demonstrating dysbiosis would suffer fitness deficits, including weight loss and poor survival.
369 Indirect observations support this assertion, in that the smallest *C. belti* individuals appeared to have a
370 higher incidence of *Rickettsia* colonization (75%, as compared with 37% for the largest individuals, n = 8
371 in both groups). In other studies, *Rickettsia* has had a positive effect on insects, including higher fecundity,
372 faster development, and fungal resistance (38-40). Whether beneficial or pathogenic, it will be interesting
373 to further examine the possible antagonistic relationships among members of the *Cephaloleia* microbiome.
374
375
376 Over the past several years, at least eight *Cephaloleia* species at La Selva Biological Station have been
377 found foraging on invasive crêpe ginger, false bird-of-paradise, pink velvet banana, and white ginger (27).
378 In this study, specialist *Cephaloleia* species collected on invasive plants exhibited an apparent dysbiosis
379 in the membership of both core groups, the Moraxellaceae and Enterobacteriaceae, and non-core groups
380 Brevinemataceae and Spiroplasmataceae It is not known if these differences represent a change along a
381 continuum as beetles adapt to exotic plants, or whether the changes in microbiome facilitate movement
382 onto new plants, or neither. Determining whether an elastic microbial repertoire can be a form of direct,
383 and rapid, environmental adaptation by the host is a next critical step given that the colonization of
384 invasive plants is an inevitable new reality for all generalist and specialist herbivores. A 2013 NSF-
385 sponsored report urged the scientific community to better understand phenotypic plasticity and sensitivity
386 of animals to future changing environments (41), yet none of the statements considered animal-associated
387 microbiomes, or the immense potential of this metabolic reservoir for maintaining function in the face of
388 changing ecosystems.
389

390 **Conclusion.** The tremendous diversity of insect herbivores, particularly in tropical rainforests, is due in
391 part to the relative specificity of their diets (42-43). In this study, Costa Rican beetle species within the
392 genus *Cephaloleia*, with known diet breadths ranging from generalist (foraging on 9-15+ plants) to
393 specialist (foraging on < 2 plant species), were analyzed for their associated gut microbial community.
394 The core microbiome of six closely-related species of Costa Rican *Cephaloleia* beetles was limited and
395 mainly included members of the *Acinetobacter*, Enterobacteriacea, *Pseudomonas*, *Lactococcus,* and
396 *Comamonas*. Contrary to expectations, the microbiome diversity was significantly higher in specialist
397 species, compared to generalists, and was dominated by these core groups (as were the eggs). Generalist
398 beetles had lower diversity, primarily due to the exclusive dominance of bacteria thought to be pathogens,
399 including the Rickettsiaceae. Bacteria isolated from *Cephaloleia* digestive systems had complementary
400 capabilities and suggested a possible beneficial role in both digestion of plant-based compounds,
401 including xylose, mannitol, and pectin, and possible detoxification, via lipases. Additionally, changes in
402 abundance of rare plants may significantly influence the balance between nutritional specificity and
403 dietary breadth of herbivores (44-45), and in this study, *Cephaloleia* specimens collected from exotic
404 invasive plants revealed a dysbiosis of the microbiome. Additional experiments are necessary in order to
405 fully determine whether the microbiome differences observed in this study are the product of intrinsic
406 differences among species or result from shifts to novel plant diets. The possible relationship between gut
407 bacteria and niche adaptation, however, remains an important and urgent research question as organisms
408 respond to future altered landscapes.
409
410
411 **ACKNOWLEDGEMENTS**
412
413 The authors thank Dr. Erin Brinton and Natalie Gonzalez for assisting in sample collection while in Costa
414 Rica, Dr. Gretchen North for providing the intellectual support to identify plants, Dr. Carlos Garcia-
415 Robledo for sharing his knowledge of *Cephaloleia*, and the members of the Occidental College Microbial
416 Symbiosis Laboratory. Funding for this project is provided by the Fletcher Jones Science Scholars Award,
417 the Occidental College Undergraduate Research Center Academic Student Project Grant, and the
418 Occidental College International Programs Office.
419

420 **References**
421

422 1. Moran, N.A. & Telang, A. Bacteriocyte–associated symbionts of insects. *BioScience* **48**, 295–304.
423 (1998).

424 2. Wernegreen, J.J. Genome evolution in bacterial endosymbionts of insects. *Nat. Rev. Genet.* **3**, 850–861
425 (2002).

426 3. Douglas, A.E. The microbial dimension in insect nutritional ecology. *Funct. Ecol.* **23**, 38–47 (2009).

427 4. Bistolas, K.S., Sakamoto R.I., Fernandes J.A. & Goffredi S.K. Symbiont polyphyly, co-evolution, and
428 necessity in pentatomid stinkbugs from Costa Rica. *Front Microbiol.* **5**, 349 (2014).

429 5. Genta F.A., Dillon R.J., Terra W. & Ferreira C. Potential role for gut microbiota in cell wall digestion
430 and glucoside detoxification in *Tenebrio molitor* larvae. *J Insect Physiol* **52**, 593–601 (2006).

431 6. Kuriwada T., Hosokawa T., Kumano N., Shiromoto K., Haraguchi D., et al. Biological role
432 of *Nardonella* endosymbiont in its weevil host. *PloS ONE* **5**, e13101 (2010).

433 7. Hansen, A.K. & Moran, N.A. The impact of microbial symbionts on host plant utilization by
434 herbivorous insects. *Molecular Ecology* **23**, 1473–1496 (2014).

435 8. Staines, C.L. The genus *Cephaloleia* (Coleoptera: Chrysomelidae) in Central America and the West
436 Indies. *Rev. Biol. Trop. Special Publication* **3**, 3–87 (1996).

437 9. Wilf, P., Labandeira, C.C., Staines, C.L., Windsor, D.M., Allen, A.L. & Johnson, K.R. Timing the
438 radiations of leaf beetles: hispines on gingers from latest Cretaceous to recent. *Science* **289**, 291–294
439 (2000).

440 10. McKenna D.D. & Farrell, B.D. Tropical forests are both evolutionary cradles and museums of leaf
441 beetle diversity. *Proc Natl Acad Sci USA* **103**, 1047–1051 (2006).

442 11. García-Robledo C. & Staines C.L. Herbivory in gingers from latest Cretaceous to present: is the
443 ichnogenus *Cephaloleichnites* (Hispinae, Coleoptera) a rolled-leaf beetle? *Journal of Paleontology* **82**,
444 1035–1037 (2008).

445 12. McKenna D.D. & Farrell B.D. Molecular phylogenetics and evolution of host plant use in the
446 neotropical rolled leaf 'hispine' beetle genus *Cephaloleia* (chevrolat) (Chrysomelidae: Cassidinae).
447 *Mol Phylogenet Evol*. **37**, 117-131 (2005).

448 13. Staines C.L. Hispines (Chrysomelidae: Cassidinae) of La Selva Biological Station, Costa Rica. In:
449 Jolivet P, Santiago-Blay J, Schmitt M, editors. Research on Chrysomelidae 3. *Zookeys* **157**, 45–65
450 (2011).

451 14. García-Robledo, C. & Staines, C.L. Monograph: The genus *Cephaloleia* in the Americas (Coleoptera:
452 Chrysomelidae: Cassidinae). *Zookeys.* **436**, 1–355 (2014).

453 15. García-Robledo C., Erickson D.L., Staines C.L., Erwin T.L. & Kress W.J. Tropical plant-herbivore
454 networks: reconstructing species interactions using DNA barcodes. *PLoS ONE*. **8**, e52967 (2013).

455 16. Colman D.R., Toolson E.C. & Takacs-Vesbach C.D. Do diet and taxonomy influence insect gut
456 bacterial communities? *Mol Ecol.* **21** 5124-5137 (2012).

457 17. Jones R.T., Sanchez L.G. & Fierer N. A cross-taxon analysis of insect-associated bacterial diversity.
458 *PLoS ONE*. **8**, e61218 (2013).

459 18. Rahman, N.A., D.H. Parks, D.L. Willner, A.L. Engelbrektson, S.K. Goffredi, F. Warnecke, R.H.
460 Scheffrahn & P. Hugenholtz. A molecular survey of Australian and North American termite genera
461 indicates that co-evolution is the primary force shaping termite gut microbiomes. *Microbiome* ***3***, 5
462 (2015).

463 19. García-Robledo C. & Horvitz, C.C. Experimental demography and the vital rates of generalist and
464 specialist insect herbivores on native and novel host plants. *J Anim Ecol*. **80**, 976-989 (2011).

465 20. Douglas, A.E. Microbial brokers of insect-plant interactions revisited. *J. Chem. Ecol.* **39**, 952–961
466 (2013).

467 21. Engel, P. & Moran, N. A. The gut microbiota of insects–diversity in structure and function. *FEMS*
468 *Microbiology Reviews*, **37**, 699-735 (2013).

469 22. Caporaso J.G., Lauber C.L., Walters W.A., Berg-lyons D., Lozupone C.A., Turnbaugh P.J., Pierer N.
470 & Knight R. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample.
471 *Proc Natl Acad Sci USA*. **108**, 4516-4522 (2011).

472 23. Berry D., Ben Mahfoudh K., Wagner M. & Loy A. Barcoded primers used in multiplex amplicon
473 pyrosequencing bias amplification. *Applied Environmental Microbiology*. **77**, 7846–7849 (2011).

474 24. Kennedy K., Hall M.W., Lynch M.D., Moreno-Hagelsieb G. & Neufeld J.D. Evaluating bias of
475 Illumina-based bacterial 16S rRNA gene profiles. *Applied Environmental Microbiology* **80**, 5717-22
476 (2014).

477 25. Caporaso J.G., Kuczynski J., Stombaugh J, *et al.* QIIME allows analysis of high-throughput
478 community sequencing data. *Nature Methods*. **7**, 335-336 (2010).

479 26. Clarke K.R. & Warwick R.M. Change in marine communities, 2nd edn. PRIMER-E Ltd.. Plymouth
480 (2001).

481 27. Schneider C.A., Rasband W.S. & Eliceiri K. NIH image to imageJ: 25 years of image analysis. *Nat*
482 *Methods* **9**, 671-675 (2012).

483 28. Giulieri S., Jaton K., Cometta A., Trellu L.T. & Greub G. Development of a duplex real-time PCR for
484 the detection of Rickettsia spp. and typhus group rickettsia in clinical samples. *FEMS Immunol Med*
485 *Microbiol* **64**, 92-97 (2012).

486 29. Haselkorn, T. S., Markow, T. A. & Moran, N. A. Multiple introductions of the *Spiroplasma* bacterial
487 endosymbiont into Drosophila. *Molecular Ecology* **18**, 1294-1305 (2009).

488 30. Lane D.J. 16S and 23S rRNA sequencing, p 115–148. *In* Stackenbrandt E., & Goodfellow M. (ed),
489 Nucleic acid techniques in bacterial systematics. John Wiley and Sons, New York, NY (1991).

490 31. Li K., Chen H., Jiang J., Li X., Xu J. & Ma Y. Diversity of bacteriome associated with *Phlebotomus*
491 *chinensis* (Diptera: Psychodidae) sand flies in two wild populations from China. *Sci Rep.* **6**, 36406
492 (2016).

493 32. Wang J., Chung S.H., Peiffer M., Rosa C., Hoover K., Zeng R. & Felton G.W. Herbivore oral
494 secreted bacteria trigger distinct defense responses in preferred and non-preferred host plants. *J Chem*
495 *Ecol.* **6**, 463-474 (2016).

496 33. Minard G., Tran F.H., Raharimalala F.N., Hellard E., Ravelonandro P., Mavingui P. & Moro C.V.
497 Prevalence, genomic and metabolic profiles of *Acinetobacter* and *Asaia* associated with field-caught
498 *Aedes albopictus* from Madagascar. *FEMS Microbiol Ecol.* **83**, 63-73 (2013).

499 34. Briones-Roblero C.I., Rodriguez-Diaz R., Santiago-Cruz J.A., Zuniga G. & Rivera-Orduna F.N.
500 Degredation capacities of bacteria and yeasts isolated from the gut of *Dendroctonus rhyzophagus*
501 (Curculionidae: Scolytinae). *Folia Micobiol.* **62**, 1-9 (2017).

502 35. Anand A.A., Vennison S.J., Sankar S.G., Prabhu D.I., Vasan P.T., Raghuraman T., Geoffrey C.J. &
503 Vendan S.E. Isolation and characterization of bacteria from the gut of *Bombyx mori* that degrade
504 cellulose, xylan, pectin and starch and their impact on digestion. *J Insect Sci.* **10**, 107 (2010).

505 36. Snyder M.J., Champagne D.E., Cohen M.B. & Howard J.J. Effects of plant diet on detoxification
506 enzyme activities of two grasshoppers, *Melanoplus differentialis* and *Taenipoda eques*. *J Chem Ecol.*
507 **24**, 2151-2165 (1998).

508 37. Azad A.F. & Beard C. Rickettsial pathogens and their arthropod vectors. *Emerg Infect Dis.* **4**, 179-186
509 (1998).

510 38. Sakurai M., Koga R., Tsuchida T., Meng X.Y. & Fukatsu T. *Rickettsia* symbiont in the pea aphid
511 *Acrythosiphon pisum*: novel cellular tropism, effect on host fitness, and interaction with the essential
512 symbiont *Buchnera*. *Appl Environ Microbiol.* **71**, 4069-4075 (2005).

513 39. Himler A.G., Adachi-Hagimori T., Bergen J.E., Kozuch A., Kelly S.E., Tabashnik B.E., Chiel E.,
514 Duckworth V.E., Dennehy T.J., Zchori-Fein E. & Hunter M.S. Rapid spread of a bacterial symbiont
515 in an invasive whitefly is driven by fitness benefits and female bias. *Science*. **332**, 254-256 (2011).

516 40. Łukasik P., van Asch M., Guo H., Ferrari J. & Godfray H.C.J. Unrelated facultative endosymbionts
517 protect aphids against a fungal pathogen. *Ecol. Lett*. **16**, 214 –218 (2013).

518 41. Padilla, D. K., Daniel, T. L., Dickinson, P. S., Grünbaum, D., Hayashi, C., Manahan, D. T., ... &
519 Tsukimura, B. Addressing grand challenges In organismal biology: the need for synthesis. *BioScience*
520 **64**, 1178-1187 (2014).

521 42. Novotny V., Drozd P., Miller S.E., Kulfan M., Janda M., et al. Why are there so many species of
522 herbivorous insects in tropical rainforests? *Science* **313**,1115–18 (2006).

523 43. Forister M.L., Novotny V., Panorska A.K., Baje L., Basset Y., Butterill P.T., Cizek L, …., Dyer L.A.
524 The global distribution of diet breadth in insect herbivores. *Proc Natl Acad Sci USA*. **112,** 442-447
525 (2015).

526 44. Norton, D. A. & Didham, R. K. Comment on "Why are there so many species of herbivorous insects
527 in tropical rainforests?". *Science* **315**, 1666-1666 (2007).
528
529 45. Novotny, V., Drozd P., Miller S.E. *et al*. Response to comment on "Why are there so many species of
530 herbivorous insects in tropical rainforests? Science **315**, 1666c (2007a).

PeerJ Preprints NOT PEER-REVIEWED

531 **Figure Legends**

532 **Figure 1.** Costa Rican *Cephaloleia* species in this study include, from left to right, (top) *C. dilaticollis, C.*
533 *placida, C. reventazonica* and (bottom) *C. dorsalis, C. fenestrata,* and *C. belti*. At far right - a mating pair
534 of *C. erichsonii* on a young rolled leaf of *Calathea lutea*. All life stages and most behavior, including
535 mating, take place on plants within the order Zingiberales. Scale bars = 1 mm for all images, except far
536 right where the scale bar is 1 cm. Photo credits: S. Goffredi

537 **Figure 2.** Relative abundance of bacterial families from (A) beetles foraging on native plants, including
538 *Cephaloleia placida, C. dilaticollis*, *C. fenestrata*, *C. doralis, C. belti*, and *C. reventazonica*, and (B)
539 beetles foraging on invasive plants *C. placida*, *C. dilaticollis*, and *C. belti* compared to those species
540 foraging on native plants. Each color group on the graph represents a distinct genus-level OTU or lowest
541 level available. Families that constituted <1% of sequences from individual specimens were grouped as
542 'Other'.

543 **Figure 3.** Prevalence versus relative abundance of bacterial OTUs associated with 6 *Cephaloleia* species
544 (n = 29 specimens, collected on native diets). Certain dominant OTU groups are indicated separately by
545 color (ex. *Acinetobacter*), with twelve shown by OTU# based on ≥ 60% prevalence or ≥ 10% relative
546 abundance (including 4 noted in green that matched bacterial groups typically thought of as pathogens).

547 **Figure 4.** Metabolic capabilities of bacteria isolated from the digestive system of *Cephaloleia* beetles,
548 including the ability to use lactose/glucose, pectin, xylose, and mannitol, as well as the presence of
549 proteases, esterases and lipases. White shading and the number '1' indicate the ability to digest the
550 specified compound. Dark grey shading and the number '0' indicate an inability to digest the specified
551 compound. At left, a phylogenetic tree, based on 767 bp 16S rRNA sequences, built with Tamura-Nei
552 distance model and UPGMA method, of beetle digestive system isolates shown to the left. Scale bar, 0.1
553 divergence.

554 **Figure 5.** Box plots of beetle-associated average diversity (Shannon index) and relative percent
555 abundance of 4 key bacterial families (identified by SIMPER to be responsible for up to 46% of the
556 cumulative (dis)similarity among six *Cephaloleia* species examined in this study (n = 29 specimens,
557 collected on native diets)). Any data points outside of the 25-75% range are identified by open symbols.
558 Species abbreviations are as follows: plac = *C. placida*, dilat = *C. dilaticollis*, fen = *C. fenestrata*, dor = *C.*
559 *dorsalis*, belti = *C. belti*, rev = *C. reventazonica*.

560 **Figure 6.** Non-metric Multidimensional Scaling (NMDS) ordination of microbial communities associated
561 with *Cephaloleia* beetles. Each point represents all 16S rRNA sequences recovered from a single
562 specimen. Displayed data was square root transformed, which minimizes errors in the ordination due to
563 PCR bias while also not sacrificing genuine differences between samples. Samples with similar microbial
564 communities plot closer together. ANOSIM p values are shown. Lower stress values indicate better
565 representation of the intersample (dis)similarities in two dimensions. (A) Ordination comparing 4
566 specialist species and 2 generalist species, the latter designated by triangles. p = 0.001, suggesting a
567 distinct difference between the two feeding strategies. (B) Ordination comparing 3 species, found on both
568 native (filled symbols) and invasive plant species (open symbols). p = 0.001 for the two specialist species
569 combined; p = 0.05 for *C. belti*, suggesting a significant difference in both cases.

570

571 **Figure 7.** Relative abundance of the 6 most dominant bacterial genera, based on 16S rRNA sequences,
572 recovered from eggs (left) and adults (right) of three *Cephaloleia* species. Species abbreviations are as
573 follows (n egg, n adult, respectively): Cb = *C. belti* (2,8) Cdil = *C. dilaticollis* (2,3), Cdor = *C. dorsalis*
574 (1,3). Photo credits: S. Goffredi

Table 1 (on next page)

Beetle specimens analyzed in this study

Specimens analyzed in this study, showing the # of 16S rRNA sequences generated from barcoding, along with corresponding measures of diversity.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26692v1 | CC BY 4.0 Open Access | rec: 14 Mar 2018, publ: 14 Mar 2018

1 **Table 1:** Adult beetle specimens analyzed in this study, showing the # of 16S rRNA sequences generated
2 from barcoding, along with corresponding measures of diversity.

Species	Plant Diet [a]	Sample ID	16S rRNA sequences (initial #)	16S rRNA sequences (normalized)[b]	Shannon Diversity Value [c]
NATIVE PLANTS					
C. belti	*H. latisplatha*	Cb1	35380	12958	2.50
	H. latisplatha	Cb2	41041	13039	2.29
	H. latisplatha	Cb3	40375	12939	2.14
	H. wagneriana	Cb4	35682	13276	1.80
	Calathea sp.	Cb5	23717	13158	1.73
	H. latisplatha	Cb6	21909	13186	0.82
	unknown	Cb7	43649	13018	1.94
	H. latisplatha	Cb8	20413	12959	2.26
C. reventazonica	*H. imbracata*	Cr1	20451	13177	1.89
	H. imbracata	Cr2	42274	13237	1.16
	H. imbracata	Cr3	32029	13324	1.81
C. fenestrata	*Calathea sp.*	Cf1	67826	12572	3.00
	Calathea sp.	Cf2	49366	12887	3.12
	Calathea sp.	Cf3	15568	13151	2.21
	Calathea sp.	Cf4	26983	12894	2.29
	Calathea sp.	Cf5	32891	12758	2.46
	H. imbracata	Cf6	31801	12637	2.11
	H. imbracata	Cf7	48964	12969	3.29
C. dorsalis	*Co. malortiensus*	Cdor1	41144	12755	2.97
	Co. malortiensus	Cdor2	54265	9899	2.91
	Co. malortiensus	Cdor3	51930	12711	2.05
C. dilaticollis	*R. alpinia*	Cdil1	45418	13084	2.38
	R. alpinia	Cdil2	47713	13096	2.47
	unknown	Cdil3	46765	12948	2.92
C. placida	*R. alpinia*	Cp1	40199	12979	2.79
	R. alpinia	Cp2	45794	11664	1.49
	R. alpinia	Cp3	41131	12782	2.16

	R. alpinia	Cp4	48511	13158	2.52
	R. alpinia	Cp5	37048	12770	3.08
INVASIVE PLANTS					
C. belti	*M. velutina*	Cb_inv1	49334	13005	2.08
	M. velutina	Cb_inv2	33427	13238	1.66
	M. velutina	Cb_inv3	26965	12479	1.97
	M. velutina	Cb_inv4	30173	12983	2.19
C. dilaticollis	*He. coronarium*	Cdil_inv1	15895	12268	2.32
	H. coronarium	Cdil_inv2	15679	12650	2.27
	H. coronarium	Cdil_inv3	23419	12473	3.02
C. placida	*H. coronarium*	Cp_inv1	13829	13128	2.39
	H. coronarium	Cp_inv2	26564	12733	3.15
EGGS					
C. belti	*n/a*	Cb_egg1	68837	4410	2.90
	n/a	Cb_egg2	55636	9715	3.36
C. dorsalis	*n/a*	Cdor_egg1	45536	13217	2.84
C. dilaticollis	*n/a*	Cdil_egg1	35848	6931	2.88
	n/a	Cdil_egg2	18648	7305	3.55

3

4 [a]*H. = Heliconia, He. = Hedychium, M. = Musa, R. = Renealmia, Co. = Costas*

5 [b]define 'normalized' – without mitochondria and chloroplasts; with *Wolbachia*

6 [c]diversity values without *Wolbachia*

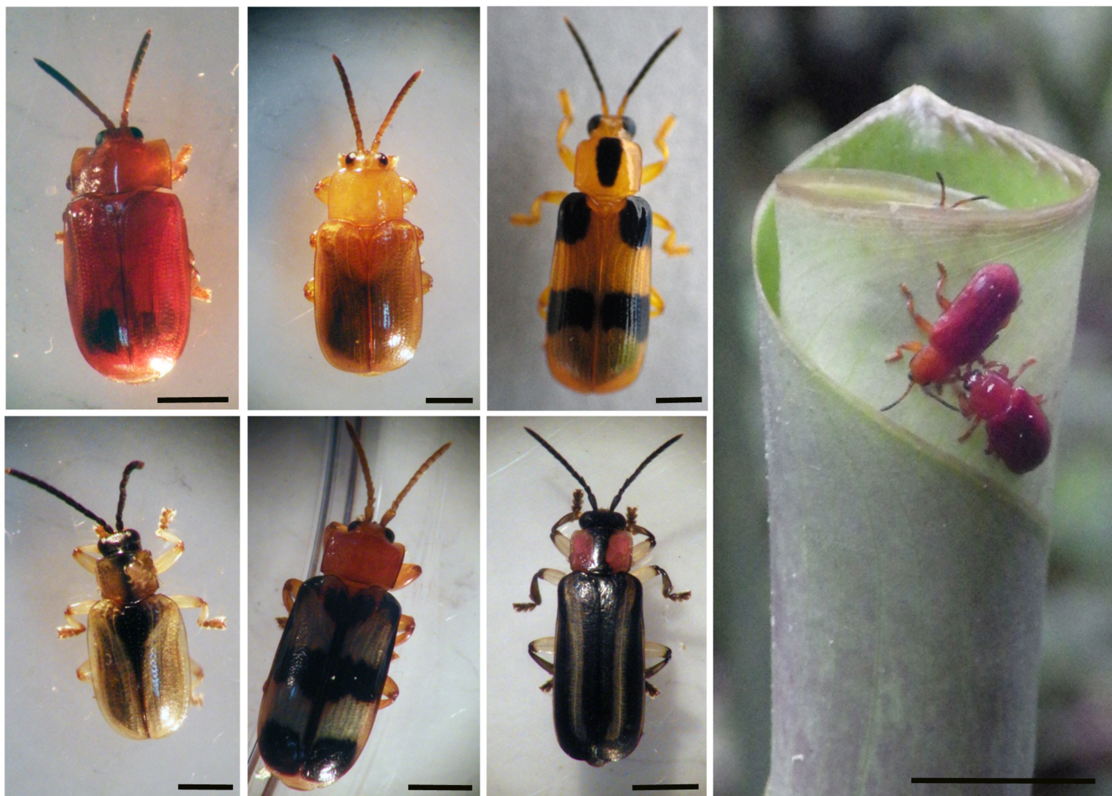
7

Figure 1(on next page)

Costa Rican *Cephaloleia* species in this study

Costa Rican *Cephaloleia* species in this study include, from left to right, (top) *C. dilaticollis, C. placida, C. reventazonica* and (bottom) *C. dorsalis, C. fenestrata,* and *C. belti*. At far right - a mating pair of *C. erichsonii* on a young rolled leaf of *Calathea lutea*. All life stages and most behavior, including mating, take place on plants within the order Zingiberales. Scale bars = 1 mm for all images, except far right where the scale bar is 1 cm. Photo credits: S. Goffredi

PeerJ Preprints NOT PEER-REVIEWED



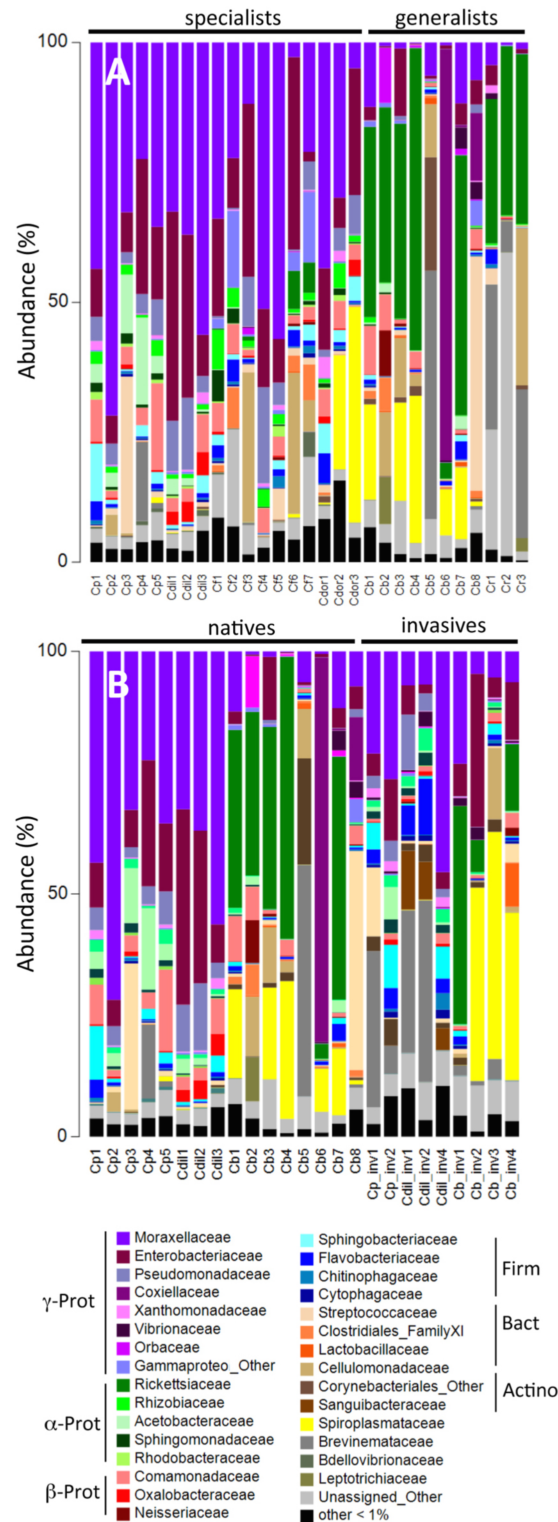
PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26692v1 | CC BY 4.0 Open Access | rec: 14 Mar 2018, publ: 14 Mar 2018

Figure 2 (on next page)

Relative abundance of bacterial families

Relative abundance of bacterial families from (A) beetles foraging on native plants, including *Cephaloleia placida, C. dilaticollis*, *C. fenestrata*, *C. doralis, C. belti*, and *C. reventazonica*, and (B) beetles foraging on invasive plants *C. placida*, *C. dilaticollis*, and *C. belti* compared to those species foraging on native plants. Each color group on the graph represents a distinct genus-level OTU or lowest level available. Families that constituted <1% of sequences from individual specimens were grouped as ‘Other’.

PeerJ Preprints NOT PEER-REVIEWED

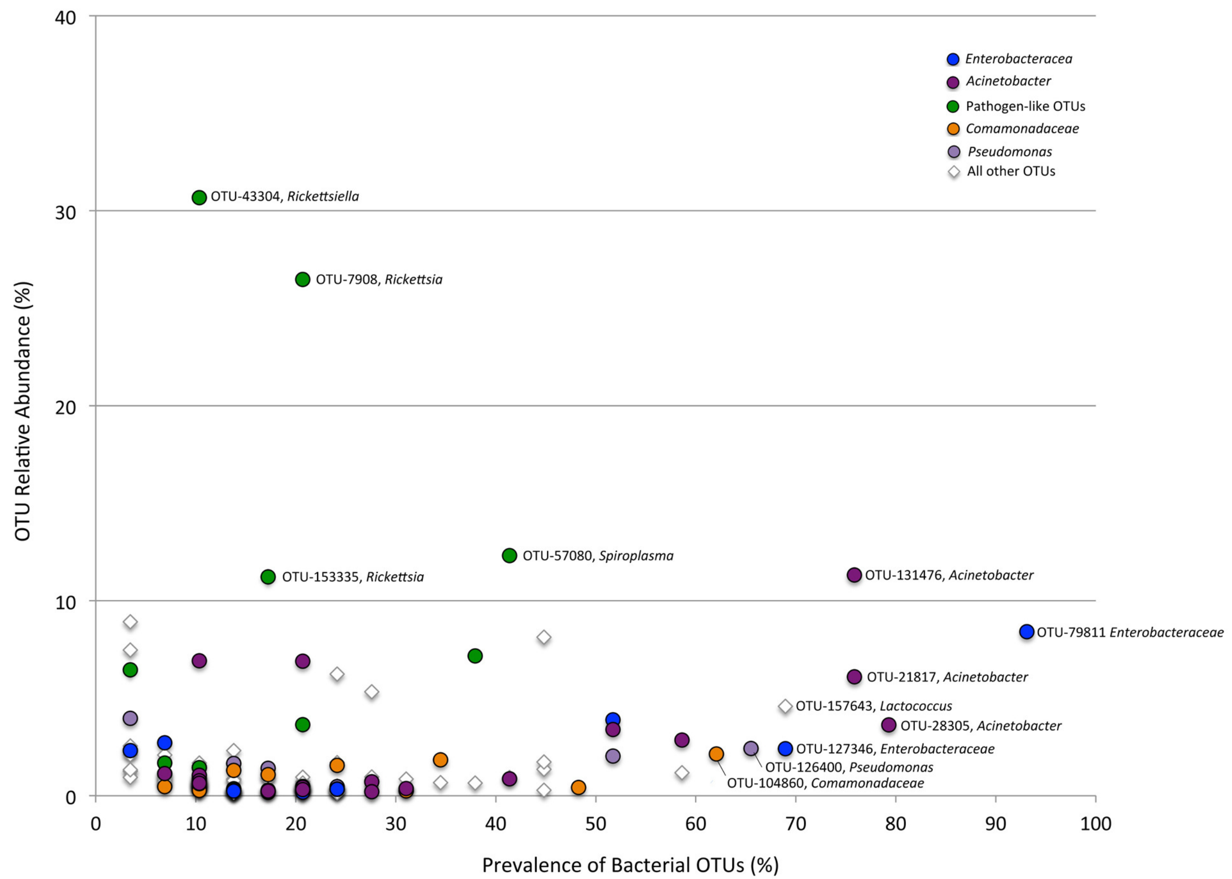


PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26692v1 | CC BY 4.0 Open Access | rec: 14 Mar 2018, publ: 14 Mar 2018

Figure 3 (on next page)

Prevalence versus relative abundance of bacterial OTUs

Prevalence versus relative abundance of bacterial OTUs associated with 6 *Cephaloleia* species (n = 29 specimens, collected on native diets). Certain dominant OTU groups are indicated separately by color (ex. *Acinetobacter*), with twelve shown by OTU# based on ≥ 60% prevalence or ≥ 10% relative abundance (including 4 noted in green that matched bacterial groups typically thought of as pathogens).


40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100
OTU Relative Abundance (%)
Prevalence of Bacterial OTUs (%)
Enterobacteracea
Acinetobacter
Pathogen-like OTUs
Comamonadaceae
Pseudomonas
All other OTUs
OTU-43304, Rickettsiella
OTU-7908, Rickettsia
OTU-57080, Spiroplasma
OTU-153335, Rickettsia
OTU-131476, Acinetobacter
OTU-79811 Enterobacteraceae
OTU-21817, Acinetobacter
OTU-157643, Lactococcus
OTU-28305, Acinetobacter
OTU-127346, Enterobacteraceae
OTU-126400, Pseudomonas
OTU-104860, Comamonadaceae

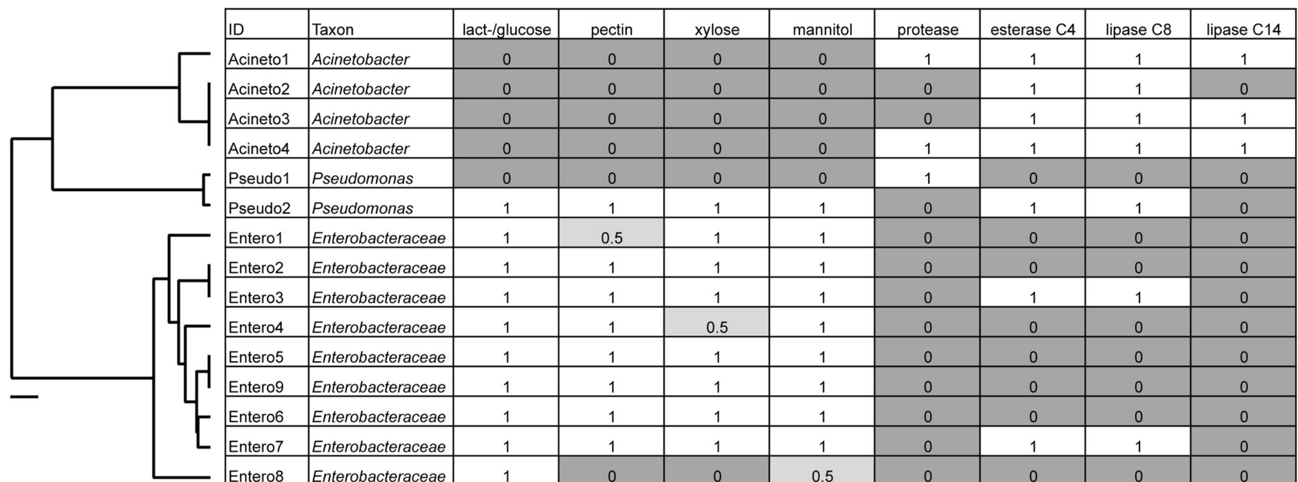
PeerJ Preprints NOT PEER-REVIEWED

Figure 4(on next page)

Metabolic capabilities of bacteria isolated from the digestive system of *Cephaloleia* beetles

Metabolic capabilities of bacteria isolated from the digestive system of *Cephaloleia* beetles, including the ability to use lactose/glucose, pectin, xylose, and mannitol, as well as the presence of proteases, esterases and lipases. White shading and the number ‘1’ indicate the ability to digest the specified compound. Dark grey shading and the number ‘0’ indicate an inability to digest the specified compound. At left, a phylogenetic tree, based on 767 bp 16S rRNA sequences, built with Tamura-Nei distance model and UPGMA method, of beetle digestive system isolates shown to the left. Scale bar, 0.1 divergence.

PeerJ Preprints NOT PEER-REVIEWED



ID	Taxon	lact-/glucose	pectin	xylose	mannitol	protease	esterase C4	lipase C8	lipase C14
Acineto1	*Acinetobacter*	0	0	0	0	1	1	1	1
Acineto2	*Acinetobacter*	0	0	0	0	0	1	1	0
Acineto3	*Acinetobacter*	0	0	0	0	0	1	1	1
Acineto4	*Acinetobacter*	0	0	0	0	1	1	1	1
Pseudo1	*Pseudomonas*	0	0	0	0	1	0	0	0
Pseudo2	*Pseudomonas*	1	1	1	1	0	1	1	0
Entero1	*Enterobacteraceae*	1	0.5	1	1	0	0	0	0
Entero2	*Enterobacteraceae*	1	1	1	1	0	0	0	0
Entero3	*Enterobacteraceae*	1	1	1	1	0	1	1	0
Entero4	*Enterobacteraceae*	1	1	0.5	1	0	0	0	0
Entero5	*Enterobacteraceae*	1	1	1	1	0	0	0	0
Entero9	*Enterobacteraceae*	1	1	1	1	0	0	0	0
Entero6	*Enterobacteraceae*	1	1	1	1	0	0	0	0
Entero7	*Enterobacteraceae*	1	1	1	1	0	1	1	0
Entero8	*Enterobacteraceae*	1	0	0	0.5	0	0	0	0

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26692v1 | CC BY 4.0 Open Access | rec: 14 Mar 2018, publ: 14 Mar 2018

PeerJ Preprints NOT PEER-REVIEWED

Figure 5(on next page)

Box plots of beetle-associated average diversity (Shannon index) and relative percent abundance of 4 key bacterial families

Box plots of beetle-associated average diversity (Shannon index) and relative percent abundance of 4 key bacterial families (identified by SIMPER to be responsible for up to 46% of the cumulative (dis)similarity among six *Cephaloleia* species examined in this study (n = 29 specimens, collected on native diets)). Any data points outside of the 25-75% range are identified by open symbols. Species abbreviations are as follows: plac = *C. placida*, dilat = *C. dilaticollis*, fen = *C. fenestrata*, dor = *C. dorsalis*, belti = *C. belti*, rev = *C. reventazonica*.

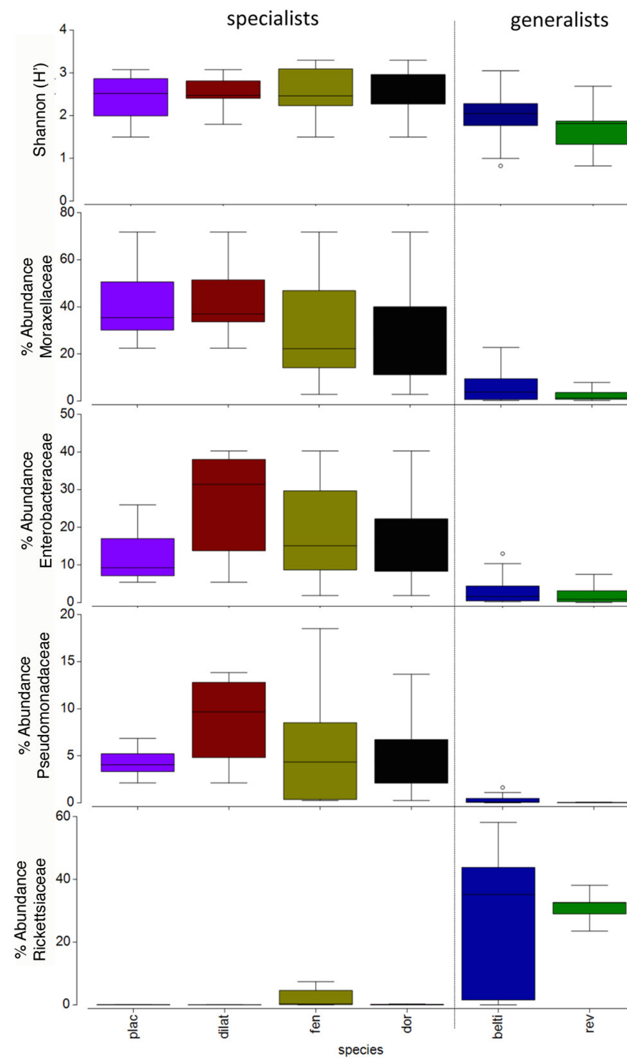
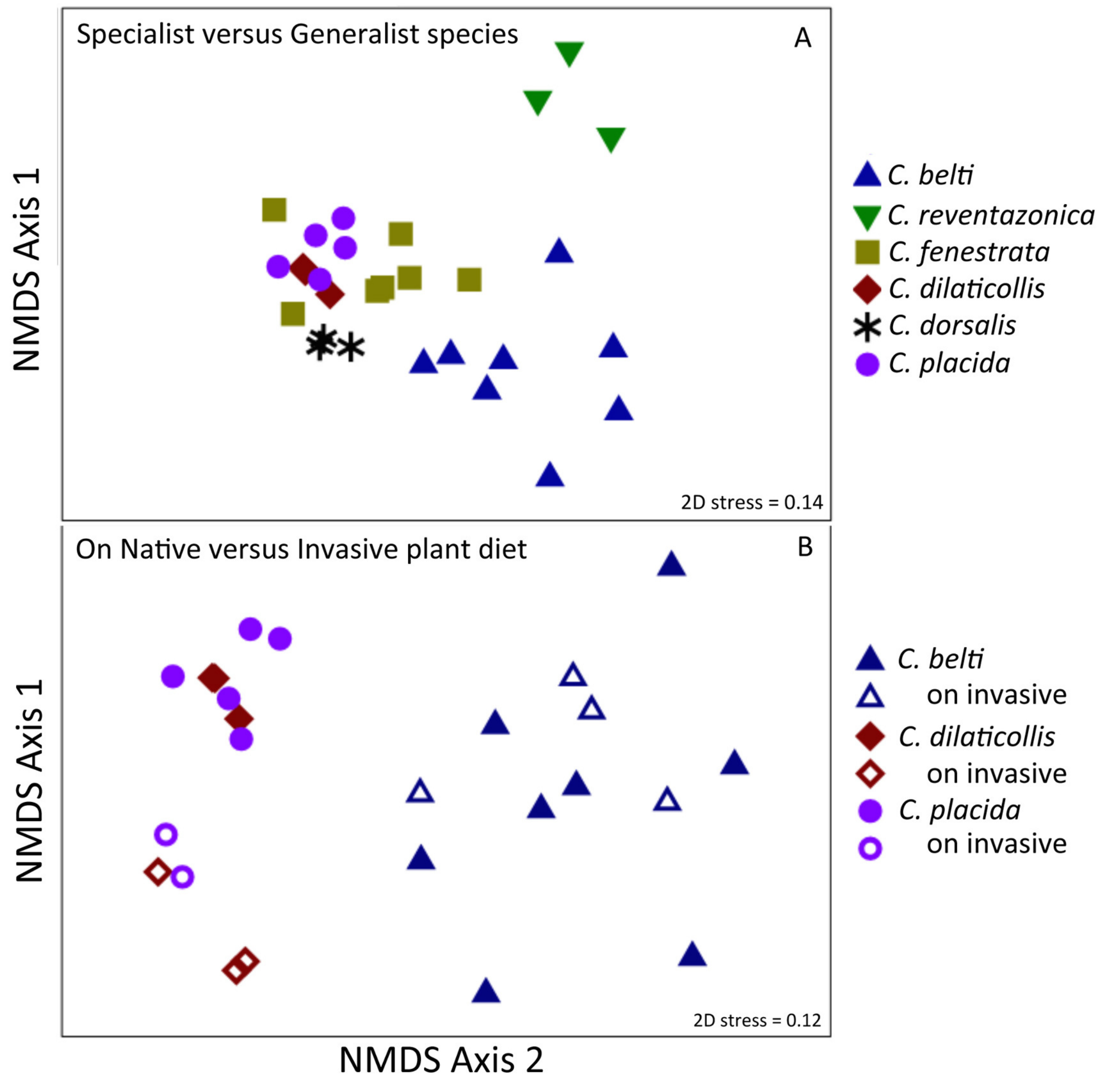

specialists
generalists
Shannon (H')
% Abundance Moraxellaceae
% Abundance Enterobacteraceae
% Abundance Pseudomonadaceae
% Abundance Rickettsiaceae
plac
dilat
fen
dor
belti
rev
species

Table 2(on next page)

Non-metric Multidimensional Scaling (NMDS) ordination of microbial communities associated with *Cephaloleia* beetles

Non-metric Multidimensional Scaling (NMDS) ordination of microbial communities associated with *Cephaloleia* beetles. Each point represents all 16S rRNA sequences recovered from a single specimen. Displayed data was square root transformed, which minimizes errors in the ordination due to PCR bias while also not sacrificing genuine differences between samples. Samples with similar microbial communities plot closer together. ANOSIM p values are shown. Lower stress values indicate better representation of the intersample (dis)similarities in two dimensions. (A) Ordination comparing 4 specialist species and 2 generalist species, the latter designated by triangles. $p = 0.001$, suggesting a distinct difference between the two feeding strategies. (B) Ordination comparing 3 species, found on both native (filled symbols) and invasive plant species (open symbols). $p = 0.001$ for the two specialist species combined; $p = 0.05$ for *C. belti*, suggesting a significant difference in both cases.



PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26692v1 | CC BY 4.0 Open Access | rec: 14 Mar 2018, publ: 14 Mar 2018

Figure 6 (on next page)

Dominant bacterial 16S rRNA sequences, recovered from eggs and adults of *Cephaloleia* species

Relative abundance of the 6 most dominant bacterial genera, based on 16S rRNA sequences, recovered from eggs (left) and adults (right) of three *Cephaloleia* species. Species abbreviations are as follows (n egg, n adult, respectively): Cb = *C. belti* (2,8) Cdil = *C. dilaticollis* (2,3), Cdor = *C. dorsalis* (1,3). Photo credits: S. Goffredi

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26692v1 | CC BY 4.0 Open Access | rec: 14 Mar 2018, publ: 14 Mar 2018

PeerJ Preprints NOT PEER-REVIEWED