

1 **TITLE:** North American fireflies host low bacterial diversity.

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3 **AUTHORS:** Emily A. Green <sup>a</sup>, Scott R. Smedley <sup>b,c</sup>, Jonathan L. Klassen <sup>a,d</sup>

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5 <sup>a</sup> Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut,

6 USA

7 <sup>b</sup> Department of Biology, Trinity College, Hartford, Connecticut, USA

8 <sup>c</sup> Deceased

9 <sup>d</sup> To whom correspondence should be addressed: 91 N Eagleville Rd, Unit 3125, Storrs, CT,

10 06269-3125. [jonathan.klassen@uconn.edu](mailto:jonathan.klassen@uconn.edu)

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13 Six informative key words: Fireflies, microbiome, microbial ecology, insects, low-complexity

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15 **ABSTRACT**

16           Although there are numerous studies of fireflies' mating flashes, lantern  
17 bioluminescence, and anti-predation lucibufagin metabolites, almost nothing is known about  
18 their microbiome. We therefore used 16S rRNA community amplicon sequencing to characterize  
19 the gut and body microbiomes of four North American firefly species: *Ellychnia corrusca*,  
20 *Photuris* sp., *Pyractomena borealis*, and *Pyropyga* sp. These firefly microbiomes all have very  
21 low species diversity, often dominated by a single species, and each firefly species has a  
22 characteristic microbiome. Although the microbiomes of male and female fireflies did not differ,  
23 *Photuris* sp. gut and body microbiomes did, with their gut microbiomes being enriched in  
24 *Pseudomonas* and *Acinetobacter*. *E. corrusca* egg and adult microbiomes differed except for a  
25 single egg microbiome that shared a community type with *E. corrusca* adults, which could  
26 suggest microbial transmission from mother to offspring. Mollicutes that had been previously  
27 isolated from fireflies were common in our firefly microbiomes. These results set the stage for  
28 further research concerning focus on the function and transmission of these bacterial symbionts.

29

30 **INTRODUCTION:**

31           Beetles (Order: Coleoptera) are one of the most diverse insect groups, containing nearly  
32 400,000 species (1). The beetles in the family Lampyridae are commonly known by many  
33 names: fireflies, lightning bugs, glow worms, lamp-lighters, and night-travelers, all describing  
34 the bioluminescent lantern that they use to signal to their mates (2). Fireflies are found on every  
35 continent except Antarctica, living in woods, plains, and marshes. North America is home to  
36 over 125 firefly species, including *Ellychnia corrusca*, *Photuris* sp., *Pyractomena borealis* and  
37 *Pyropyga* species (2). These 4 species occur sympatrically, ranging from the eastern coast of

38 Canada, south to Florida, and west to the Great Plains (3, 4). *E. corrusca*, *P. borealis* and  
39 *Pyropyga* sp. are all closely related to each other, and both *E. corrusca* and *Pyropyga* sp. are  
40 diurnal with no lantern (2, 5). *E. corrusca*, *P. borealis*, and many other North American fireflies  
41 use lucibufagins as a chemical defense. Lucibufagins are cardioactive C-24 steroidal pyrenes and  
42 a subclass of the bufadienolides (6–11) that create a disagreeable taste to predators and thereby  
43 provide fireflies with protection from bats, birds and spiders (12, 13). However, the biosynthetic  
44 origin of lucibufagins is not known.

45 In this study, we focus especially on two of these firefly species with unique lifestyles: *E.*  
46 *corrusca* and *Photuris*. *E. corrusca* is nicknamed the winter firefly because it has a winter-spring  
47 activity cycle, in contrast to the late spring-summer activity cycles of most other fireflies (1, 3).  
48 *E. corrusca* larvae in New England (North America) extend their larval stage across two years  
49 instead of one to ensure they ingest ample calories, emerging in the late fall of the second year as  
50 adults and sexually maturing during the winter to start mating, which lasts until late spring (14).  
51 Normally, adult fireflies do not feed. However, northern (Massachusetts, USA) *E. corrusca*  
52 firefly adults ingest interstitial fluid and sap from maple trees, which is thought to help them  
53 survive during the cold winters (4, 14). The *Photuris* adult life cycle occurs during the summer  
54 months (June-August), but unlike other fireflies, *Photuris* sp. females mimic the mating light  
55 display of female *Photinus* fireflies to attract *Photinus* males, earning them the nickname  
56 “femme fatale” (15). Once a male *Photinus* firefly is near, the *Photuris* female will attack and  
57 kill the male, gaining nutrients and lucibufagins that she uses to protect herself, her eggs, and her  
58 pupae, which do not themselves produce lucibufagins (6). Although *E. corrusca* are not active  
59 during the same season as *Photuris* sp., *Photuris* sp. will attack and eat *E. corrusca* to gain

60 protective lucibufagins in the lab (2, 10). This predation in a lab setting could give insight into  
61 the selective pressures that caused *E. corrusca* to mate in the winter instead of in the summer.

62 Beetles feed on many different substrates, following herbivorous (16) omnivorous (17),  
63 xylophagous (18, 19), detritivorous (20), and predatory diets (6). Beetles belonging to similar  
64 taxonomic families but that have different diets have distinct microbial communities (21, 22).  
65 Diets lacking in nutrients often cause insects to rely on nutritional symbionts to provide vital  
66 nutrients (23), as in dung (24), carabid (17), and carrion beetles (25). Although there is a vast  
67 amount of research studying beetle microbiomes, only a handful of studies consider firefly-  
68 associated microbes. In those studies, several Mollicute bacteria were isolated from the fireflies  
69 *E. corrusca* and *Photuris* spp., but this does not give insight into the composition of their  
70 microbiome beyond these strains (26–30). To fill this gap, we used 16S rRNA community  
71 amplicon sequencing to survey the microbiomes of *Photuris* sp., *E. corrusca*, *Pyractomena*  
72 *borealis*, and *Pyropyga* sp. fireflies. Our results show that fireflies have simple, species-specific  
73 microbiomes, and generate hypotheses about how diet and seasonality may drive firefly  
74 microbiome structure and function.

75

## 76 **METHODS**

### 77 **Sample collection**

78 Live *E. corrusca* and *Photuris* sp. fireflies were collected by S. Smedley during the  
79 winter of 2016-17 and spring/summer of 2017 from residential and camping/forest areas within  
80 Vernon, Bolton and Andover, Connecticut, U.S.A. *Pyractomena borealis* larvae and adults were  
81 raised in the laboratory by S. Smedley. After collection, *E. corrusca*, *Photuris* sp., and *P.*  
82 *borealis* fireflies were stored at -80°C in air-filled vials. *Pyropyga* sp. fireflies were collected by

83 Lynn Faust in summer 2016 from Ohio and Tennessee and stored in 95% ethanol. Collection  
84 dates and sample locations are listed in Supplemental File 1.

### 85 **Sample preparation & DNA extraction**

86 All fireflies were surface-sterilized using 3 rounds of a 10 second submersion in 70%  
87 ethanol, followed by a 10 second submersion in phosphate-buffered saline (PBS) (31). Surface-  
88 sterilized fireflies were dissected into two separate tissues: a gut sample and the remaining  
89 carcass “body” sample. DNA from the tissue dissections were extracted using a bead beating and  
90 chloroform-isopropanol protocol as described in (32), except using 0.7 g of 1 mm and 0.3 g of  
91 0.1 mm silica/zirconium beads and 5 cycles of bead-beating & chilling on ice. Negative controls  
92 containing only the DNA extraction reagents were processed alongside each batch of tissue  
93 samples. The DNA concentration of each extract and negative control was determined using the  
94 Qubit dsDNA high-sensitivity assay protocol and a Qubit 3.0 fluorimeter (Invitrogen, Carlsbad  
95 California).

### 96 **16S V4 rRNA PCR screen & community amplicon sequencing**

97 DNA samples were PCR amplified using primers 515F and 806R (targeting the bacterial  
98 16S rRNA gene V4 region) to determine the presence of bacterial DNA (33). Ten nanograms of  
99 template DNA was added to 5 µl Green GoTaq Reaction Mix Buffer (Promega, Madison,  
100 Wisconsin, USA), 1.25 units of GoTaq DNA Polymerase (Promega, Madison, Wisconsin, USA),  
101 10 µmol of each primer, and 300 ng/µl BSA (New England BioLabs Inc. Ipswich  
102 Massachusetts), to which nuclease free H<sub>2</sub>O was added to a volume of 25 µl. Thermocycling  
103 conditions (BioRad, Hercules, California) were: 3 min at 95°C, 30 cycles of 30 sec at 95°C, 30  
104 sec at 50°C, and 60 sec at 72°C, followed by a 5 min cycle at 72°C and then an indefinite hold at  
105 4°C. Gel electrophoresis was used to confirm the expected band size of 300–350 bp.

106 All samples that had a gel band of the expected size were prepared for community  
107 amplicon sequencing of the 16S rRNA gene V4 region using an Illumina MiSeq at the  
108 University of Connecticut Microbial Analysis, Resources and Services (MARS) facility.  
109 Approximately 30 ng of DNA from each sample was added to a 96-well plate containing 10  
110  $\mu\text{mol}$  each of the forward and reverse Illumina-barcoded versions of primers 515F and 806R, 5  
111  $\mu\text{l}$  AccuPrime buffer (Invitrogen, Carlsbad, California), 50 mM  $\text{MgSO}_4$  (Invitrogen, Carlsbad,  
112 California), 300 ng/ $\mu\text{l}$  BSA (New England BioLabs Inc. Ipswich, Massachusetts), a 1 $\mu\text{mol}$   
113 spike-in of both non-barcoded primers 515F and 806R, and 1 unit AccuPrime polymerase  
114 (Invitrogen, Carlsbad, California), to which nuclease-free  $\text{H}_2\text{O}$  was added to a volume of 50  $\mu\text{l}$ .  
115 Reaction mixes were separated in triplicate reactions (each with a volume of 16.7  $\mu\text{l}$ ) into a 384  
116 well plate using an epMotion 5075 liquid handling robot (Eppendorf, Hamburg, Germany). The  
117 resulting 384 well plate was then transferred to a thermocycler (Eppendorf, Hamburg, Germany),  
118 which used the following conditions: 2 min at 95°C, 30 cycles of 15 sec at 95°C, 60 sec at 55°C,  
119 and 60 sec at 68°C, followed by a final extension for 5 min at 68°C and then an indefinite hold at  
120 4°C. After PCR, triplicate reactions were re-pooled using the epMotion, and DNA concentrations  
121 were quantified using a QIAxcel Advanced capillary electrophoresis system (QIAgen, Hilden,  
122 Germany). Samples that had concentrations  $>0.5$  ng/ $\mu\text{l}$  were pooled using equal weights of DNA  
123 to create the final sequencing libraries. Libraries were then bead-cleaned using Mag-Bind  
124 RXNPure plus beads (OMEGA, Norcross, Georgia) in a 1:0.8 ratio of sequencing library to bead  
125 volume. Cleaned library pools were adjusted to a concentration of 1.1 ng/ $\mu\text{l} \pm 0.1$  ng/ $\mu\text{l}$ , which  
126 was confirmed using the Qubit dsDNA high-sensitivity assay on a Qubit 3.0 fluorimeter  
127 (Invitrogen, Carlsbad, California). Microbial community sequencing on an Illumina MiSeq

128 (Illumina, San Diego, California) was completed in 2 batches, the first composed of 52 samples  
129 and the second composed of 195 samples.

### 130 **Post-sequencing & bioinformatic analyses:**

131 Our DNA sequencing produced 245 16S rRNA community amplicon sequencing  
132 datasets, including 22 negative controls. Reads were analyzed using R v3.5.3 (34) and the dada2  
133 v1.11.1 (35) pipeline for amplicon sequence variants (ASVs)  
134 (<https://benjjneb.github.io/dada2/tutorial.html>, accessed: November 11, 2017). Read counts  
135 ranged from 2 to 888,868 (Suppl. File S1). Metadata files for the samples were imported into  
136 phyloseq v1.26.1 (31, 32), creating a phyloseq R object that was used for subsequent analyses.  
137 Reads that were not classified as belonging to the kingdom Bacteria using the SILVA database  
138 v128 were removed (38, 39). ASVs that matched to mitochondria were then removed separately,  
139 because SILVA included them in the kingdom Bacteria. Samples were screened for  
140 contamination using the decontam v1.2.1 (40) prevalence protocol with a default threshold value  
141 of 0.1. No reads were flagged as contamination, and 17 samples with 0 reads were removed,  
142 resulting in 1,675 unique ASVs (Supplemental File S1). Negative control samples were not  
143 considered further. All samples in the dataset were then rarefied to 10,000 reads and read counts  
144 were converted to relative abundances. The final phyloseq object contained 133 samples from 20  
145 *Photuris* adults, 4 *Pyropyga* adults, 7 *Pyractomena borealis* larvae, 1 *Pyractomena borealis*  
146 adult, 95 *Ellychnia corrusca* adults, and 6 *E. corrusca* eggs. Despite amplification during the  
147 initial PCR screen, of the initial 223 insect samples, 74 samples were removed due to low read  
148 counts (53 *E. corrusca* adult samples, 3 *E. corrusca* egg samples, 9 *Photuris* sp. samples, 2  
149 *Pyropyga* sp. samples, 4 *P. borealis* adult samples & 3 *P. borealis* larvae samples). These low  
150 read counts may have been due to: 1) high amounts of host DNA acting as a PCR inhibitor; 2)

151 there being a minimal firefly microbiome, leading to limited template concentrations; or 3) other  
152 technical issues such as inefficient PCR amplification using primers that contained the Illumina  
153 barcodes compared to our initial PCR screen using non-barcoded primers.

154 Alpha diversity was measured using the phyloseq ‘plot\_richness’ command, and Beta  
155 diversity was measured using weighted and unweighted unifrac distance metrics. Weighted and  
156 unweighted unifrac distances were calculated, ordinated, and viewed using the ‘distance’,  
157 ‘ordinate’, and ‘plot\_ordinate’ phyloseq commands, respectively. PERMANOVA statistical tests  
158 were calculated using vegan v 2.5-4 (41). Although weighted unifrac (WUF) and unweighted  
159 unifrac (UUF) distances were used for each test, to keep the text concise only one test is listed in  
160 the text and the complimentary values are presented in Supplementary Tables S1-S4.

161 Differences in the relative abundances of taxa in firefly microbiomes were compared  
162 using DESeq2 (42), using the Parametric fitType, Wald tests, and an adjusted alpha value of  
163 0.01. A heatmap was constructed for the 26 most abundant genera in the dataset using the gplots  
164 v3.03 (<https://www.rdocumentation.org/packages/gplots>) command ‘heatmap.2’ and using the  
165 Euclidean and ward.D distance metrics. To avoid redundancy in the heatmap, ASVs were  
166 grouped by genus name using the phyloseq command ‘tax\_glom’. Covariance between the top  
167 26 taxa were calculated using SpiecEasi v1.0.7 (43), and the SpiecEasi output file was exported  
168 into Cytoscape v3.7.2 (44) using igraph v1.2.4.2 (45) for visualization.

169 The most abundant Mollicute ASVs from our dataset included four *Mesoplasma* ASVs,  
170 two *Spiroplasma* ASVs, and one *Entomoplasma* ASV. A phylogenetic tree was constructed to  
171 show the relationships between the 16S rRNA sequences of Mollicutes from this study and those  
172 that had been previously isolated from fireflies. Reference Mollicute sequences from taxa  
173 belonging to the same genera as our firefly ASVs were selected from the SILVA database,



174 especially those Mollicutes that had been isolated from fireflies and other beetles. Additional  
175 Mollicute reference sequences for fireflies that were not represented in the SILVA database were  
176 downloaded from NCBI. Sequences were aligned using MUSCLE v3.8.31 (46) and trimmed to  
177 the same length. The phylogenetic tree, rooted by the 16S rRNA genes from *Bacillus subtilis* and  
178 *Mycoplasma haemominutum*, was calculated using a GTRGAMMAI substitution model and 500  
179 bootstrap replicates in RAxML v8.2.11 (47). The topology of the tree created using these  
180 selected sequences agreed with that constructed using all Mollicute sequences in SILVA,  
181 ensuring that taxon selection did not bias our phylogenetic analysis.

182         The commands used for all analyses is attached as Suppl. File S2. All data are available  
183 on NCBI under BioProject PRJNA563849. Raw sequencing reads are deposited in SRA under  
184 BioSample numbers SAMN14678004 – SAMN14678257.

185

## 186 **RESULTS**

### 187 **Firefly microbiomes are typically dominated by single taxa**

188         We characterized the bacterial communities in 133 firefly gut and body dissections  
189 (Suppl. File S1) using community amplicon sequencing of the 16S rRNA gene. All firefly  
190 microbiomes had low  $\alpha$ -diversity (Suppl. Fig. S1). Shannon diversity scores for the firefly  
191 microbiomes ranged from 0.70 to 2.52, and alpha diversities of *Photuris* sp. and *E. corrusca*  
192 microbiomes differed from each other, as did those from *Pyropyga* sp. and *E. corrusca* (Dunn  
193 test,  $p = 0.001$  and  $p = 0.003$  respectively). A heatmap of the top 26 bacteria genera found in the  
194 microbiomes reflects these low levels of  $\alpha$ -diversity, with most firefly microbiomes dominated  
195 by a single taxon but with minute amounts of other taxa also present (Fig. 1). Unsupervised  
196 clustering of these data grouped *E. corrusca* samples tightly together at the left side of the heat

197 map, while the samples from other species clustered together on the right. Samples from  
198 different tissue dissections and sexes did not cluster together for any species of firefly.

199 Using these top 26 bacteria genera, we identified taxa whose relative abundances were  
200 correlated with each other to infer potential interactions between them (Suppl. Fig. S2). Of the 26  
201 bacteria genera, the relative abundances of 14 genera did not correlate with those of another  
202 genus in our dataset, and the relative abundances of 11 genera were positively correlated with  
203 those of another genus. The relative abundances of *Ralstonia* and *Cupriavidus*, found in the  
204 *Pyropyga* sp. samples, were strongly and positively correlated with each other, as were the  
205 relative abundances of *Tanticharoenia* and *Gluconobacter*, found in *E. corrusca* eggs, and the  
206 relative abundances of *Gordonia* and *Tsukamurella*, found in *Photuris* sp.. Only the relative  
207 abundances of *Mesoplasma* and *Acinetobacter* were negatively correlated with each other, and  
208 these taxa were not found together in any sample.

209 In the heatmap, many samples clustered together that were dominated by single taxa.  
210 Based on these clusters, we defined community types based on the genus or genera of bacteria  
211 that were present in these samples with relative abundances  $\geq 30\%$  (Fig. 2). *E. corrusca* adults  
212 were assigned to 6 distinct community types: T-1 (*Mesoplasma*), T-2 (*Salmonella*), T-3  
213 (*Mesoplasma* & *Salmonella*), T-4 (*Serratia*), T-5 (*Rickettsia*) and T-6 (*Pseudomonas*). *E.*  
214 *corrusca* egg microbiomes were all assigned to T-13 (*Gluconobacter*), except for a single egg  
215 microbiome that belonged to T-2 (*Salmonella*), the same as some *E. corrusca* adult microbiomes.  
216 *Photuris* sp. microbiomes were assigned to 3 community types: T-8 (*Acinetobacter*), T-9  
217 (*Yersinia*) and T-6 (*Pseudomonas*). *Pyractomena borealis* and *Pyropyga* sp. were assigned to T-  
218 10 (*Empedobacter*) and T-12 (*Cupriavidus*), respectively.

219 **Firefly species harbor unique microbial communities**

220 The four sampled firefly species had distinct bacterial communities (Weighted Unifrac  
221 (WUF) PERMANOVA:  $R^2=0.215$ ,  $p=0.001$ ; Fig. 3A, Suppl. Fig. S3, Suppl. Table S1). In a  
222 PCoA ordination of WUF beta-diversity distances, *E. corrusca* samples clustered together,  
223 spanning from left to right (Fig. 3). *Pyropyga* sp. samples clustered together with some *E.*  
224 *corrusca* samples and those from *Photuris* sp. on the left side of Figure 3A, and *Photuris* sp.  
225 samples grouped together down the left side and at the bottom, along with the *Pyractomena*  
226 *borealis* samples. Species-specific clustering can be seen in the UUF plot, with *E. corrusca*  
227 having the most variance (Suppl. Fig. S3B). The separation between the *E. corrusca* and  
228 *Photuris* sp. microbiomes was maintained in a better-balanced comparison when the few  
229 *Pyropyga* sp. and *P. borealis* samples were excluded (WUF PERMANOVA:  $R^2=0.164$ ,  $p=0.001$ ;  
230 Suppl. Fig S4, Suppl. Table S2).

231 We identified the bacterial taxa whose relative abundance differed between *E. corrusca*  
232 and *Photuris* sp. fireflies using DESeq. Although *Pseudomonas* ASVs were found in both  
233 fireflies, one *Pseudomonas* ASV was more abundant in *Photuris* sp. and two other *Pseudomonas*  
234 ASVs were more abundant in *E. corrusca* (Fig. 3B). Two *Mesoplasma* ASVs, *Salmonella*,  
235 *Serratia*, *Rickettsia* and *Rickettsiella* were all more abundant in *E. corrusca* than in *Photuris* sp.,  
236 and *Tsukamurella*, *Gordonia*, *Curvibacter* and *Sphingobacterium* were all more abundant in  
237 *Photuris* sp. than in *E. corrusca* (Fig. 3B).

### 238 **Gut and body microbiomes are distinct, but these differences are firefly-specific**

239 The above analysis indicated that the four sampled firefly species all hosted distinct  
240 microbiomes (Fig. 3). However, further analyses using these same data also indicated that  
241 different firefly tissues might also host distinct microbiomes, in a species-specific manner (WUF  
242 PERMANOVA for the interactions between species and tissue:  $R^2=0.048$ ,  $p=0.003$ ; Suppl. Fig.

243 S3, Suppl. Table S1). In contrast, a parallel analysis using only samples for which sex was  
244 determined indicated that microbiomes did not differ between the sexes, regardless of species or  
245 tissue type (all PERMANOVAs testing for differences between sexes:  $p > 0.05$ ; Suppl. Fig. S5,  
246 Suppl. Table S3). In the analysis described above with *Pyractomena borealis* and *Pyropyga* sp.  
247 samples removed to avoid possible artifacts due to unbalanced sample sizes, firefly tissue  
248 microbiomes again differed in a species-specific manner (WUF PERMANOVA for interactions  
249 between *E. corrusca* and *Photuris* sp. gut and body microbiomes:  $R^2=0.053$ ,  $p=0.001$ ; Suppl.  
250 Fig. S4, Suppl. Table S2).

251 Because these PERMANOVAs indicated that tissue microbiomes differed in a species-  
252 specific manner, we repeated our analysis for each species separately. *E. corrusca* eggs, gut and  
253 body microbiomes differed from each other (WUF PERMANOVA:  $R^2=0.122$ ,  $p=0.001$ ), with  
254 most gut and body samples clustered together in the PCoA and most egg samples clustered  
255 separately (Fig. 4A, Suppl. Table S4, Suppl. Fig. S6). When the egg samples were removed from  
256 the analysis, *E. corrusca* gut and body microbiomes differed from each other only when using  
257 the UUF distance metric (UUF PERMANOVA:  $R^2= 0.072$ ,  $p=0.001$ ; WUF: Suppl. Table S4).  
258 *Wolbachia* had low relative abundance in all *E. corrusca* samples, but was the only bacterial  
259 genus that more abundant in *E. corrusca* eggs than in adults. *Mesoplasma*, *Pseudomonas*,  
260 *Acinetobacter* and several other genera were all more abundant in *E. corrusca* adults than in eggs  
261 (Fig. 4B). *E. corrusca* eggs and adults also had distinct community types, with *E. corrusca* egg  
262 microbiomes mainly assigned to T-13 (*Gluconobacter*), and only a single egg microbiome  
263 assigned to T-3 (*Salmonella*) like those of *E. corrusca* adults (Fig. 2). In contrast, *Photuris* sp.  
264 gut and body microbiomes more strongly differed from each other (WUF PERMANOVA:  $R^2=$   
265  $0.340$ ,  $p=0.001$ ; Fig. 4C, Suppl. Fig. S7, Suppl. Table S4), with *Pseudomonas*, *Acinetobacter*,

266 *Leucobacter*, *Elizabethkingia*, *Curvibacter* and *Achromobacter* all being more abundant in  
267 *Photuris* sp. guts than in bodies (Fig. 4D). *Pyropyga* sp. gut and body microbiomes did not differ  
268 from each other (WUF PERMANOVA:  $R^2=0.312$ ,  $p=0.667$ ; Suppl. Table S4), and *P. borealis*  
269 gut and body microbiomes differed only when using the UUF distance metric (UUF  
270 PERMANOVA:  $R^2= 0.616$ ,  $p=0.008$ ; Suppl. Table S4). These conclusions should be considered  
271 preliminary because of the small sample sizes available for both *Pyropyga* sp. and *P. borealis*.

## 272 **Fireflies host an abundance of potentially symbiotic Mollicutes**

273 Mollicutes were the most abundant bacteria in our 133 firefly samples, consistent with  
274 the handful of Mollicutes that had previously been isolated from different firefly species (27, 29,  
275 30, 48–50). We therefore created a phylogenetic tree to discover how our most abundant  
276 Mollicute ASVs were related to the sequences of these known firefly Mollicute isolates (Fig. 5).  
277 The most prevalent *Spiroplasma* ASV (ASV Spiroplasma1), found in many of the *E. corrusca*  
278 samples, was similar to the 16S rRNA sequence of *S. corruscae*, a species that was first isolated  
279 from *E. corrusca* (28). ASV Spiroplasma2 was found in one *Photuris* sp. sample, and was  
280 similar to the 16S rRNA sequence of *S. ixodetis*, a male-killing agent in butterflies (51). *S.*  
281 *ixodetis* has not been found in fireflies to date. The four *Mesoplasma* ASVs, mainly found in *E.*  
282 *corrusca*, are all similar to the 16S rRNA sequences for *M. corruscae* and *Entomoplasma*  
283 *ellyphniae*, which had both been isolated previously from *E. corrusca* (29, 48, 49). Although  
284 originally separated using cell morphology and culture media requirements, the genera  
285 *Mesoplasma* and *Entomoplasma* are paraphyletic (52). Our phylogenetic tree showing that both  
286 *M. corruscae* and *E. ellyphniae* cluster tightly together suggests that *E. ellyphniae* should be  
287 reclassified as a member of the genus *Mesoplasma*. The single *Entomoplasma* ASV, found in  
288 *Pyractomena borealis* adults, closely resembled the 16S rRNA sequence for *E. somnilux*, which

289 had been previously isolated from *Pyrrhotomena angulate* (27). This phylogenetic tree shows that  
290 the Mollicutes sequences detected in study are highly similar to strains that had been previously  
291 isolated from fireflies, except for that related to *S. ixodetis*.

## 292 **DISCUSSION:**

293 Our results show that all of our sampled species of firefly have low-complexity  
294 microbiomes. The alpha diversity of our firefly microbiomes was very low, and all samples had  
295 only a few taxa present in high abundance (Fig 1). The low number of correlations between the  
296 relative abundances of firefly taxa (Suppl Fig. S2) is consistent with this predominance of simple  
297 microbiomes, because (by definition) few interactions can occur when only a single bacterium is  
298 abundant in a microbiome, and correlations will inevitably be weak when there is high variability  
299 between microbial communities. This variance and low diversity could be explained by  
300 microbiomes being acquired via neutral community assembly. Fireflies may be exposed to  
301 environmental bacteria by chance, and these bacteria may then multiply and form a stable  
302 microbiome. This would explain why there is high variance between the microbiomes from  
303 individual insects of the same firefly species.

304 Samples could be assigned to one of thirteen community types, defined by the taxa in  
305 these samples with >30% abundance. Different firefly species rarely shared the same community  
306 type, and most community types were defined by a single taxon, with only one community type  
307 having two taxa that were each >30% abundant (Fig. 2). The presence of multiple community  
308 types in each firefly species creates questions about whether the bacteria are either: 1) resident  
309 members replicating within the firefly, or 2) transient members that replicate more slowly than  
310 the rate of expulsion from the firefly (53). Although such transient microbiomes are not likely to  
311 be conserved between fireflies, it is possible that they do have some effect on their hosts

312 (positive or negative). Community types vary within the same firefly species, suggesting that  
313 these microbiomes are transient, some bacteria were found in extremely high relative abundances  
314 (Fig. 1) that could suggest a stable resident microbiome of unknown function. It is possible that  
315 each community type provides a different function for the firefly. It is also possible that the  
316 multiple, highly abundant bacteria in different community types, such as *Mesoplasma*,  
317 *Salmonella*, and *Pseudomonas*, could provide similar functions for their hosts, although this may  
318 be unlikely for Mollicute bacteria due to their reduced genomes.

319 Fireflies from different species also had distinct microbiomes (Fig. 3A). *E. corrusca* and  
320 *Photuris* sp. had distinct bacterial communities, and samples from the same species clustered  
321 together in PCoA plots of beta-diversity (Suppl. Fig. 4). Differences between the microbiomes of  
322 these two species might be due to their different lifestyles. North American *E. corrusca* are  
323 active in winter and may feed on tree sap (4, 14), and so *E. corrusca* microbial communities may  
324 therefore be acquired via the ingestion of these fluids. Unlike *E. corrusca*, *Photuris* sp. are  
325 predatory and active in the summer. Insect gut microbiomes often vary based on differences in  
326 their host's habitat and diet (e.g., omnivory vs. carnivory) (22), and such differences might  
327 underlie the differences that we observed between *E. corrusca* and *Photuris* sp. microbiomes.  
328 Both *E. corrusca* and *Photuris* sp. hosted *Pseudomonas* ASVs that were similar to those  
329 previously found on trees and in soils, which could be explained by *E. corrusca* and *Photuris* sp.  
330 living in soil, on tree bark, and on plant leaves (2), where *Pseudomonas* is common (54). All of  
331 the *E. corrusca* and *Photuris* sp. samples used in this study were collected from Connecticut,  
332 USA, and whether these microbiomes vary in other geographic locations needs future research.

333 For some firefly species, eggs, guts, and bodies had distinct microbiomes. *E. corrusca*  
334 adult and egg microbiomes differed from each other, with samples clustering separately in



335 PCoAs (Fig. 4A, Suppl. Fig. S6), but an *E. corrusca* egg also had the same community type (T-  
336 2; *Salmonella*) as that of some adults. Although it is unknown which firefly laid this egg, these  
337 results suggest that it might be possible for *Salmonella* to be transmitted vertically between *E.*  
338 *corrusca* adults and eggs. The mechanisms that *E. corrusca* may use to acquire these microbes  
339 from the environment are unknown, but there are other insects that rely on horizontal  
340 transmission for their symbionts, such as the bean bug *Riptortus pedestris*, which acquires its  
341 *Burkholderia* symbiont from soil each generation using a mucus-filled organ that the  
342 *Burkholderia* specifically penetrates and colonizes (55–57). Although *E. corrusca* is not known  
343 to have an organ that selects for a specific bacterial symbiont, *Mesoplasma* was found in many  
344 samples of *E. corrusca*. It is possible that *E. corrusca* may select this bacterium from the  
345 environment, or that *Mesoplasma* selects *E. corrusca* as a preferred host. *E. corrusca* is the most  
346 sampled firefly species in our study, and hosts microbiomes belonging to the most community  
347 types. This increased sampling may therefore have increased the number of community types  
348 detected in this organism relative to the other species in our study.

349 *Photuris* sp. gut and body microbiomes also differed from each other, with gut  
350 microbiomes having higher amounts of *Pseudomonas* and *Acinetobacter* than body microbiomes  
351 (Figs. 4C and 4D). These ASVs may be acquired from the firefly's environment, because both  
352 ASVs were similar to other strains of bacteria that are common in soil and on plants. The  
353 bacteria present in the *Photuris* sp. gut may be derived from eating other fireflies, and thus the  
354 *Pseudomonas* ASVs that are common in *Photuris* sp. guts may originate from their last meal.  
355 The *Photuris* microbiome may therefore be transient and composed of the prey's microbiome.  
356 Alternatively, it is possible that the *Photuris* sp. gut microbiome is not transient, and the gut  
357 provides a niche that is favorable for the growth of *Pseudomonas* and *Acinetobacter* microbes



358 that might originate from soil, plants, or the prey's microbiome and subsequently colonize the  
359 *Photuris* sp. gut. The origin of the body microbiome remains unclear. Body dissections include  
360 all other parts of the firefly except for the gut, meaning that other non-gut tissues and  
361 hemolymph from inside of the firefly could have been colonized with bacteria. Alternatively,  
362 adult firefly abdomens are surrounded with sclerotized segments, called tergites, on their ventral  
363 and dorsal sides (2). These tergites have small gaps between them that allow for movement.  
364 Although our firefly samples were ethanol-washed before dissection, this sterilization may not be  
365 absolutely perfect, and the body microbiome may therefore include bacteria that are trapped  
366 within these segments (58). The bacteria trapped between these tergites may be acquired during  
367 constant movement on tree bark or in the leaf litter, where many bacteria are found.

368 Mollicutes have been isolated from several different firefly species, but their prevalence  
369 and function is unclear (26, 27, 50, 59, 60). We detected 4 *Mesoplasma* ASVs in North  
370 American fireflies, mainly in *E. corrusca*, that all closely resembled *M. corruscae* and  
371 *Entomoplasma ellychniae*, which were both isolated previously from *E. corrusca*. As mentioned  
372 in the results, *M. corruscae* and *E. ellychniae* very likely both belong to genus *Mesoplasma* (52).  
373 *Mesoplasma* frequently occur on plants without causing disease, and therefore it could be  
374 horizontally acquired by *E. corrusca* when they feed on nutrient-poor tree sap to aid in survival  
375 during cold winter months (4, 14).. Future work will be required to determine the function, if  
376 any, of these Mollicutes in their firefly hosts.

377 Our results provide the first description of the microbiomes found in *E. corrusca*,  
378 *Photuris* sp., *Pyropyga* sp. and *Pyractomena borealis*. These microbiomes have low alpha  
379 diversities and are species-specific. Some firefly species also have distinct egg, gut, and body

380 microbiomes. Future research will determine the function of these microbiomes and how they are  
381 acquired from their environment or transmitted between hosts.

382

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387 for their thoughtful feedback on this manuscript before submission, and the UConn Microbial  
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392

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563 the firefly beetle *Ellychnia corrusca*. Int J Syst Bacteriol 39:284–289.
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- 565

566 **FIGURE LEGENDS**

567 Figure 1) Heat map of the 26 most abundant bacterial genera found in the studied fireflies. To the  
568 left, a dendrogram represents the Euclidian distances between the relative abundances of reads  
569 assigned to each bacterial genus, which are labeled on the right. The top dendrogram clusters the  
570 relative abundances of these genera in each firefly sample using ward.D distances. On the  
571 bottom, 3 different rows indicate the host taxon, tissue type, and sex type for each sample,  
572 indicated by the different colors in the key to the left of the figure. The topmost color key  
573 represents the relative abundance of reads in each sample that were assigned to each bacterial  
574 genus, with white and dark blue representing 0 and 100% relative abundance, respectively. n =  
575 133

576 Figure 2) Firefly microbial community types. The X axis indicates the number of samples that  
577 were assigned to each community type. Colors indicate firefly species, as indicated by the key.  
578 Note that some gut and body samples originate from the same individual firefly and so are  
579 counted twice.

580 Figure 3) A: PCoA of Weighted Unifrac distances between microbial communities for all four  
581 firefly species. Species are differentiated using colors. n = 133. B: Over- and underrepresented  
582 ASV sequences in *E. corrusca* and *Photuris* sp. adults, including both gut and body samples. The  
583 X-axis indicates the genus of bacteria whose relative abundances differed between firefly  
584 species, and the Y-axis indicates the log<sub>2</sub> fold change in relative abundance between samples,  
585 where the higher numbers indicate overrepresentation in *Photuris* and negative numbers indicate  
586 overrepresentation in *E. corrusca*. Colors indicate phyla. n = 114

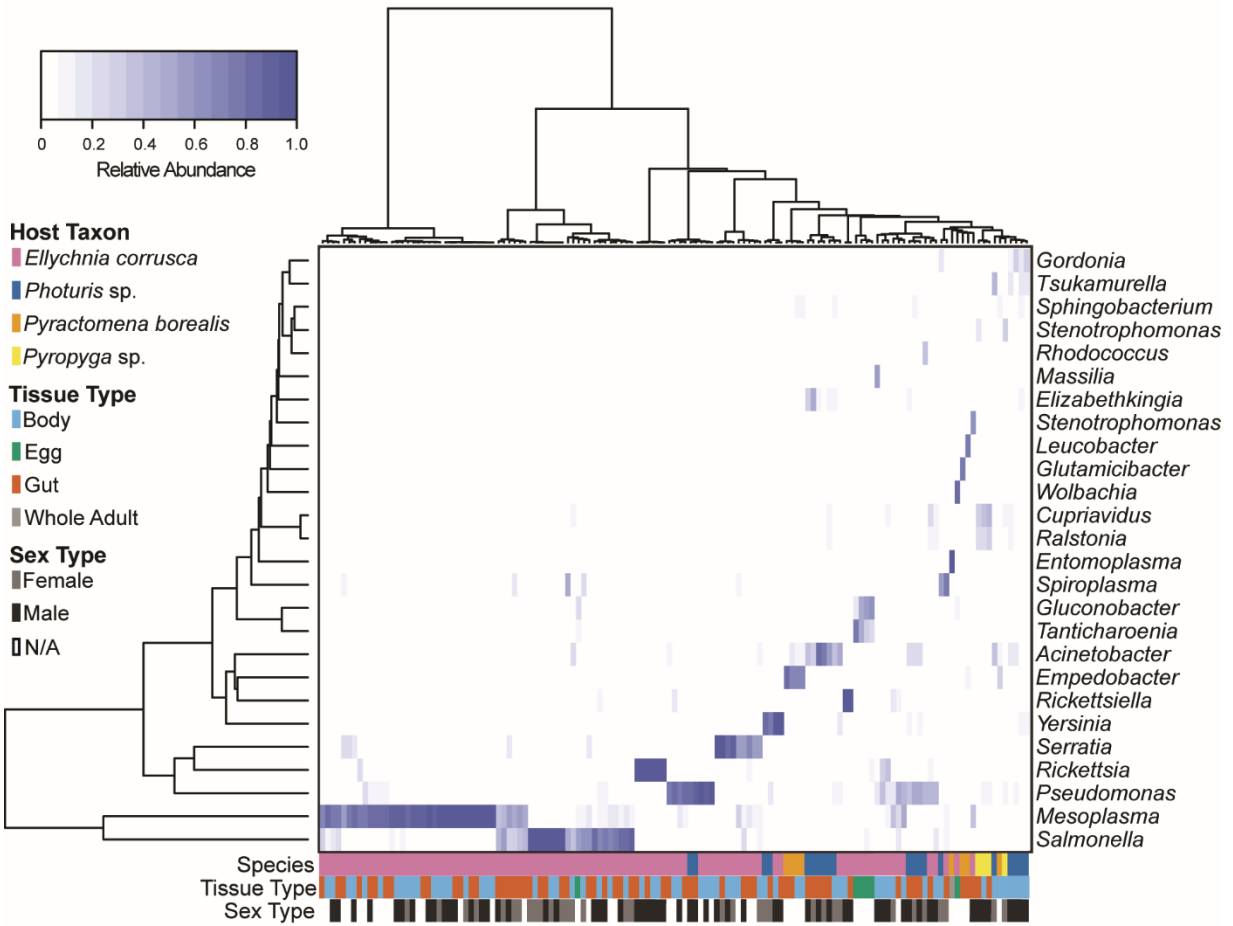
587

588 Figure 4) A) PCoA of Weighted Unifrac distances between microbiomes of *E. corrusca* eggs and  
589 adults. Egg, gut and body samples are differentiated using colors. n = 100. B) Over- and  
590 underrepresented ASV sequences in *E. corrusca* egg and adult samples. The X-axis indicates the  
591 genera of bacteria whose relative abundance differed between *E. corrusca* eggs and adults, and  
592 the Y-axis indicates the log<sub>2</sub> fold change in these relative abundances between tissues, where the  
593 higher numbers indicate overrepresentation in eggs and negative numbers indicate  
594 overrepresentation in adults. Colors indicate phyla. C) PCoA of Weighted Unifrac distances  
595 between microbiomes of *Photuris* sp. gut and body samples. Gut and body samples are  
596 differentiated using colors. D) Over- and underrepresented ASV sequences of *Photuris* sp. gut  
597 and body samples. The X-axis indicates the genus of bacteria whose relative abundance differed  
598 between *Photuris* sp. gut and body samples, and the Y-axis indicates the log<sub>2</sub> fold change in  
599 these relative abundances between tissues, where the higher numbers indicate an  
600 overrepresentation in the body samples. Colors indicate phyla. n = 20

601 Figure 5) Phylogenetic tree of the Mollicute 16S rRNA gene ASVs in our dataset (in bold) and  
602 reference 16S rRNA gene sequences, particularly those isolated from fireflies and beetles  
603 (indicated by a ^). The tree was constructed using RAxML with 500 bootstraps and rooted using  
604 *Bacillus subtilis* and *Mycoplasma haemominutum*. Bootstrap values of 60–79 and 80–100 are  
605 indicated by \* and \*\*, respectively. Numbers in parentheses indicate the number of samples in  
606 which that ASV was found, and colors group sequences from the same genera. NCBI accession  
607 numbers are shown to the right of each reference sequence. *Me* = *Mesoplasma*, *My* =  
608 *Mycoplasma*.

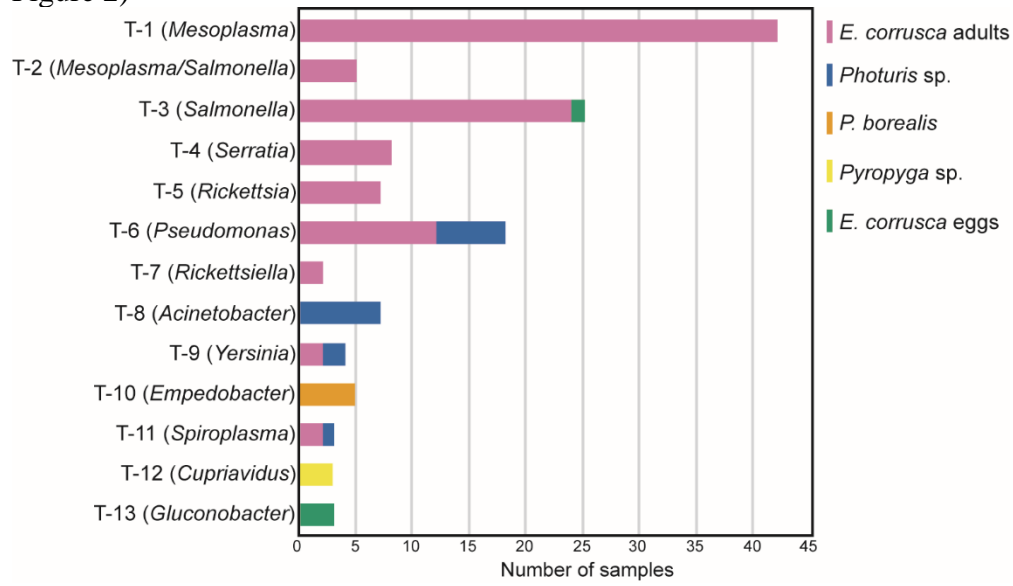
609

610 Figure 1)



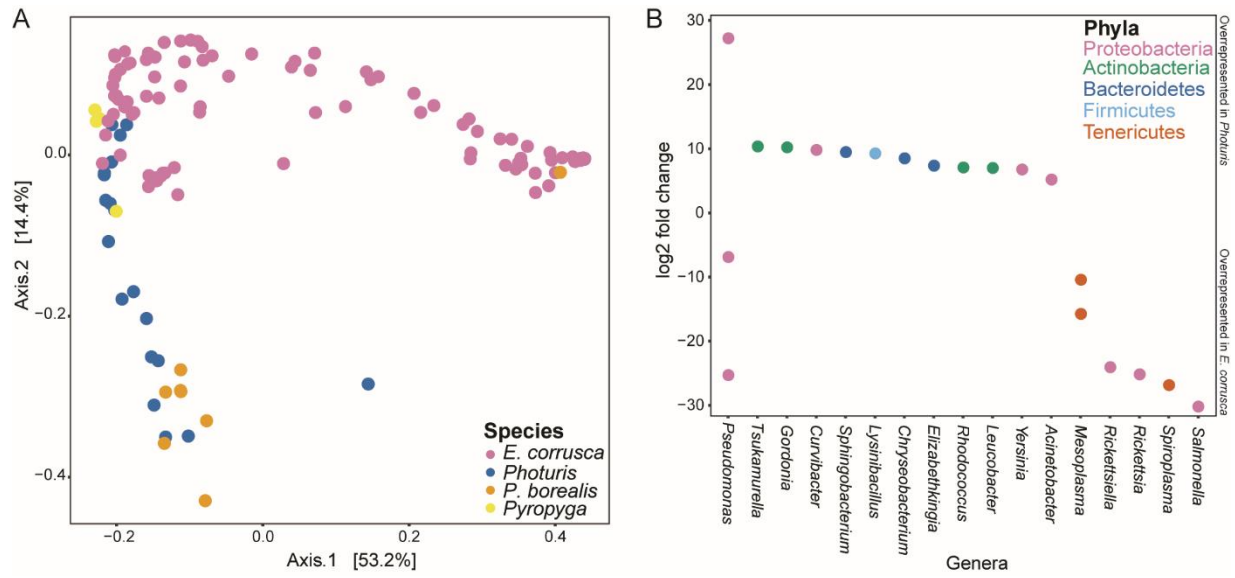
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612 Figure 2)



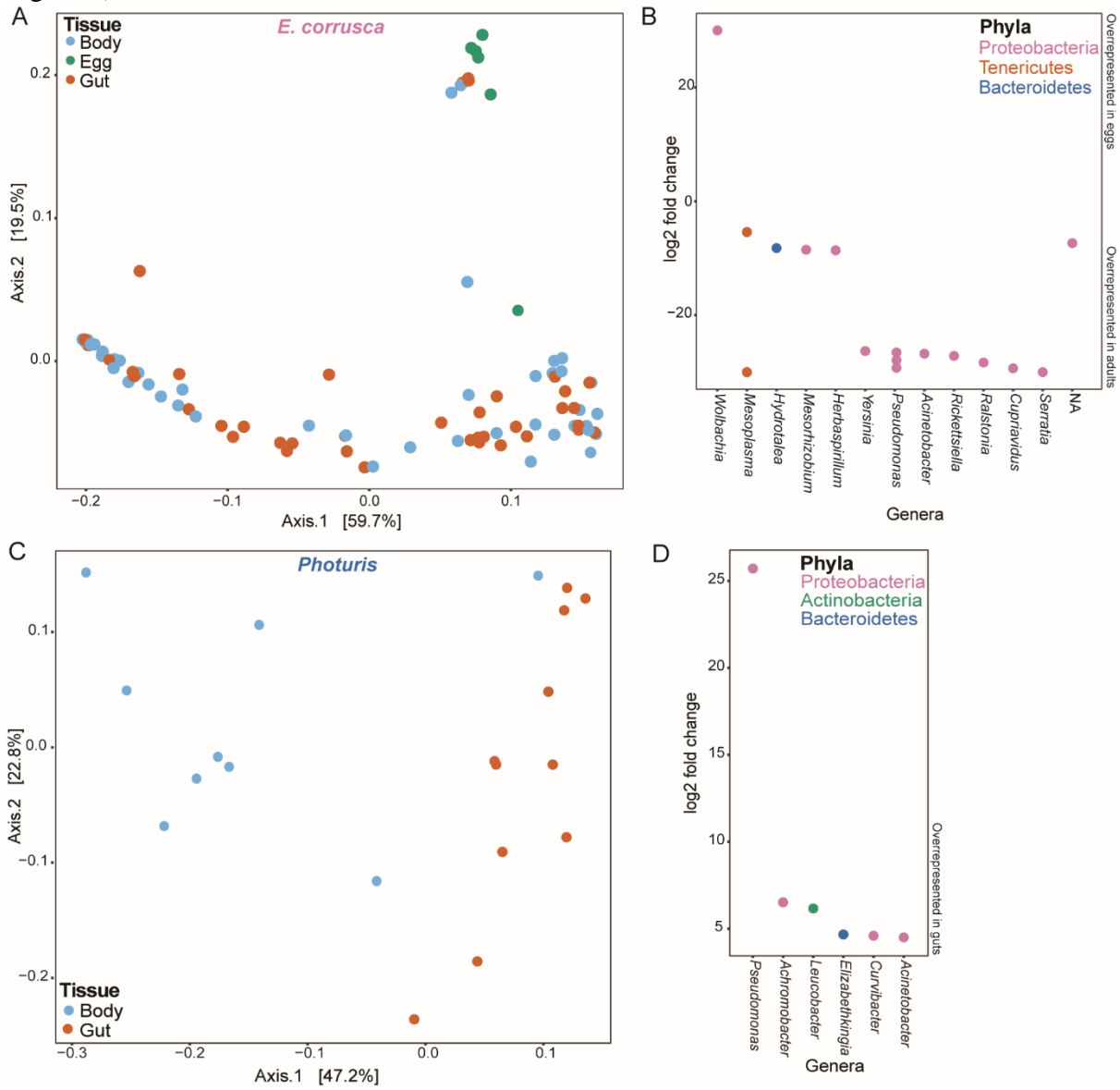
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614 Figure 3)



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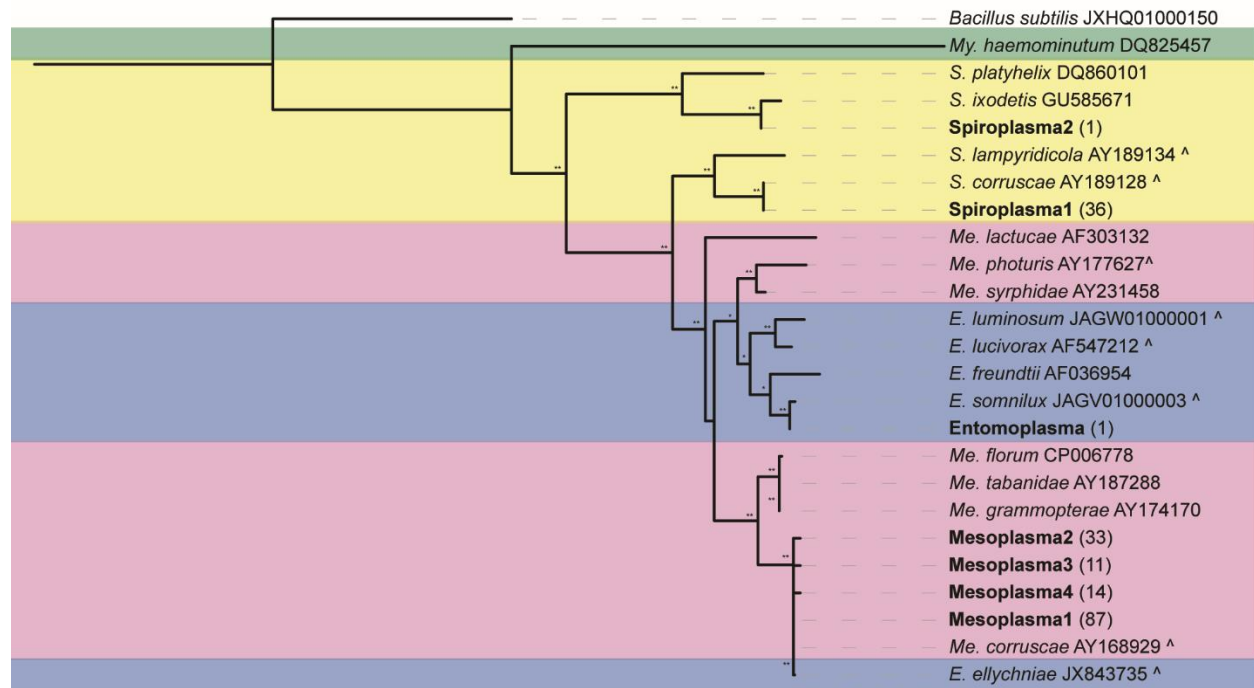
616 Figure 4)



617



618 Figure 5)  
Tree scale: 0.01 ⇐



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