Diversity of bacterial symbionts associated with the tropical plant bug *Monalonion velezangeli* (Hemiptera: Miridae) revealed by high-throughput 16S-rRNA sequencing.

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Abstract: Insects and microbes have developed complex symbiotic relationships that evolutively and ecologically play beneficial roles for both, the symbiont and the host. In most Hemiptera insects, bacterial symbionts offer mainly nutritional, defense and reproductive roles and have promoted the adaptive radiation of several hemipteran phytophagous lineages. The tropical plant bug *Monalonion velezangeli* (Hemiptera: Miridae) is a polyphagous herbivore considered an important insect pest for several economically-relevant tropical crops; however, information about the composition of its bacterial microbiota was missing. In this study, we describe the diversity and structure of the bacterial microbiota in the nymph and adult life stages of *M*. *velezangeli* using Illumina high-throughput sequencing of 16S ribosomal RNA gene amplicons (meta-barcoding). We found that both insect life stages share a similar microbiota in terms of bacterial diversity and community structure. The intracellular symbiont *Wolbachia* (~92%) dominated the overall microbiome composition in these life stages, and along with bacteria genera *Romboutsia*, *Ignavibacterium*, *Clostridium*, *Allobaculum*, *Paracoccus*, *Methylobacterium*, *Faecalibacterium*, *Collinsella*, *Rothia*, *Sphingomonas* and other 4 undetermined genera, were found as members of the core microbiota. Based on PCR screening and DNA sequencing of *wsp* gene, *Wolbachia* infection was confirmed in almost 80% of samples, represented by two different isolates or strains within the supergroup B. This data opens new questions and avenues to better understand the contribution of symbiotic bacteria in the biological performance of this insect pest and provide bases to explore other insect control methods.

Key Words: bacterial microbiota, endosymbiont, plant bug, *Wolbachia*, *Monalonion*, *Coffea*.

45 **Introduction**:
46 Most insects harbor diverse microbiota inside their body that collectively perform important
47 biological roles for the insect-host in processes such as nutrition, reproduction, immunity, and
48 development. These symbiotic interactions involve microbes adapted to live inside specialized
49 host cells (intracellular symbionts); or outside cells (extracellular symbionts) mostly in the gut
50 lumen or within specialized structures in the posterior midgut. Insect gut-associated
51 microorganisms (specially bacteria) have been proposed as key players in the adaptive
52 radiation of herbivorous insects by allowing them to metabolize or assimilate recalcitrant plant
53 compounds or to exploit low-nutrient plant contents by providing additional nutritious molecules.
54 The outcomes of this symbiotic interplay in plant-feeding insects has also extended in some
55 cases to the control of host-plant defense responses for the benefit of the insect (Chung et al.
56 2013; Acevedo et al. 2017; Schausberger 2018; Li et al. 2019). Moreover, insect-associated
57 microbial symbionts have been shown conferring resistance to chemical insecticides in various
58 pest insects (Kikuchi et al. 2012; Blanton and Peterson 2020; Sato et al. 2021).
59
60 Equally important, some intracellular symbionts (e.g. *Wolbachia*) that frequently reside within
61 the reproductive tissues of most insects are well known as manipulators of insect reproduction.
62 *Wolbachia* are maternally inherited bacterial symbionts that infect at least 65% of insect species
63 (Hilgenboecker et al. 2008) and are capable of altering host reproduction and fitness in order to
64 achieve high frequency of infection in the host populations (Stouthamer et al. 1999). This
65 manipulation can involve cytoplasmic incompatibility (CI) (Sinkins 2004; Dylan Shropshire et al.
66 2020); parthenogenesis (Werren 1997; Vavre et al. 2004; Zhou et al. 2021); male-killing (Hurst
67 et al. 1999; Fukui et al. 2015); and feminization (O'Neill et al. 1997; Hiroki et al. 2002; Narita et
68 al. 2007). Additionally, several lines of evidence show that *Wolbachia* can affect behavioral
69 patterns in their hosts by altering mating; feeding; locomotion; or aggressive behavior, as well
70 as learning and memory capacity (reviewed by (Jie Bi 2020)).
71
72 Several Hemiptera plant-feeding insect species in the suborders Sternorrhyncha (e.g. aphids,
73 whiteflies, psyllids, scale insects, mealybugs); Auchenorrhyncha (e.g. planthoppers, leafhoppers
74 and cicadas), and Heteroptera (e.g. stink bugs and plant bugs) harbor a variety of insect-
75 microbial symbiosis. These insects have piercing and sucking mouthparts for stylet-sheath
76 feeding (phloem and xylem sap-suckers) as in Sternorrhyncha and Auchenorrhyncha; or
77 macerate-and-flush feeding (sucking of extraorally digested plant tissues) as seen in some
78 Heteroptera. In consequence, several of these phytophagous species are agricultural pests of
79 economic importance. Most members of Sternorrhyncha and Auchenorrhyncha harbor
80 intracellular obligate symbionts within specialized cells (bacteriocytes) that provide essential
81 amino-acids and vitamins to the insect; compounds commonly deprived from the poor nutritional
82 plant-sap diet (Moran and Telang 1998). On the other hand, most phytophagous Heteroptera
83 members lack intracellular symbionts but have developed relationships with extracellular
84 symbionts in special midgut compartments (e.g. midgut crypts and caeca), mainly within the
85 infraorder Pentatomomorpha (e.g. stink bugs, flat bugs and seed bugs). However, special
86 symbiont-harboring midgut compartments seems to be absent in most phytophagous species in
87 the infraorder Cimicomorpha (e.g. true plant bugs and lace bugs).
88

89 The plant bug *Monalonion velezangeli* (Hemiptera: Miridae: Bryocorinae) is a neotropical
90 polyphagous insect in the infraorder Cimicomorpha native to Central and South America. This
91 insect feeds on 21 plant species in 14 families (Giraldo J. and Benavides M. 2012; Rodas et al.
92 2014; Ocampo Flórez et al. 2018) and it is considered a notorious agricultural pest in cacao
93 (*Theobroma cacao*, Malvaceae); avocado (*Persea americana*, Lauraceae) (Jaimes et al. 2015;
94 Ramírez-Gil et al. 2019); guava (*Psidium guava*, Myrtaceae); and tea (*Camellia sinensis*,
95 Theaceae). *Monalonion velezangeli* is also an emerging pest for coffee crops in Colombia,
96 specially in the southern coffee-producing regions of the country (Ramirez C. et al. 2008). The
97 immatures (nymph) and adults of *M*. *velezangeli* feed on terminal shoot tips, young leaves or
98 fruits, causing cell-death at the feeding sites as the main direct damage. Severe plant damages
99 are mainly caused by nymphal stages when they inject enzyme-rich saliva into the plant tissues
100 for extraoral digestion of the cell contents. Current recommendations for pest management vary
101 according to host crops; however, common methods include cultural practices (e.g. manual
102 collection of insects in the field or flaming), biological control with fungal entomopathogens, and
103 insecticides. Despite its significance as an agricultural pest, several aspects of the biology of *M.*
104 *velezangeli* remain poorly studied, including the composition of its microbiota.
105
106 Diversity and functional characterization of symbiotic microbiota in Miridae plant bugs have
107 been poorly studied, except for the strictly phytophagous cotton fleahopper *Pseudatomoscelis*
108 *seriatus* (Fu et al. 2021) and the omnivorous *Adelphocoris suturalis* (Xue et al. 2021; Luo et al.
109 2021) to our knowledge. We consider *M. velezangeli* as a strict phytophagous insect based on
110 the reports of host plant species identified for this plant bug (Giraldo J. and Benavides M. 2012;
111 Rodas et al. 2014; Ocampo Flórez et al. 2018), the lack of reports of other feeding habits and
112 the fact that all known members of the mirid subfamily Bryocorinae are herbivorous as well
113 (Jung and Lee 2012; Namyatova and Cassis 2016). Knowledge about *M. velezangeli*
114 associated microbiota is fundamental not only to better understand insect biology but also it
115 could provide new opportunities for development of insect management methods. For example,
116 symbiont-mediated RNA interference (smRNAi) is emerging as a potential approach for control
117 of pest insects in agriculture (Dyson et al. 2022), and as an efficient tool for insect gene
118 functional analysis (Lariviere et al. 2022). However, before any study on the role of the
119 microbiota in insect biology or exploration of symbiont-based methods for pest control can be
120 carried out, it is necessary to characterize the taxonomic composition of microbes within the
121 insect body. In this study, we analyzed for the first time the diversity and structure of the
122 symbiotic microbiota within *M*. *velezangeli* nymph and adult life stages using high-throughput
123 DNA amplicon sequencing of bacterial 16SrRNA gene (DNA meta-barcoding). Here we
124 discovered a diverse microbiota stable across life stages but dominated by few bacterial genera,
125 including the presence of the endosymbiont *Wolbachia*.
126
127 **Methods**:
128
129 **Insect collection, DNA isolation and 16SrRNA sequencing**:
130 Samples of immature and adult individuals of *M*. *velezangeli* feeding on leaves of multiple coffee
131 plants (*Coffea arabica* var. Castillo) were collected from a coffee plantation in the Department of
132 Huila (Segovianas, Coordinates: 2.3784, -75.88291), Colombia. At the place of collection,

133 insects were externally sterilized by washing three times with 75% ethanol and immediately
134 conserved in 96% ethanol for DNA isolation. Three independent samples of immatures (pools of
135 5 nymphal stages, one per instar) and three independent samples of adults (pools of one female
136 and one male) of *M*. *velezangeli* were used for microbiota analysis. Total DNA was isolated from
137 whole-body insects using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany),
138 including a lysozyme treatment according to the manufacturer protocol. DNA integrity was
139 checked on agarose gel and quantified on Nanodrop (Invitrogen, Waltham, MA, USA). PCR
140 amplification of the hypervariable region V3V4 of the bacterial 16SrRNA gene was performed
141 using primers 341F (5′-CCT AYG GGR BGC ASC AG- 3′) and 806R (5′- GAC TAC NNG GGT
142 ATC TAA T- 3′) (Caporaso et al. 2011; Klindworth et al. 2012). Illumina sequencing libraries
143 were generated with NEBNext® Ultra™ DNA Library Prep Kit (New England BioLabs, Ipswich,
144 MA, USA). The 16SrRNA amplicon Illumina 250PE libraries were sequenced using NovaSeq
145 platform (Illumina, San Diego, CA, USA) at Novogene Corporation Inc. (Sacramento, CA, USA).
146

147 **Processing of 16SrRNA sequence data and taxonomic classification**:
148 Demultiplexed raw 16SrRNA sequences were processed using QIIME2 v.2020.8 (Bolyen et al.
149 2019) as follows. Paired-end read sequences were quality-filtered, denoised and clustered
150 using DADA2 (Callahan et al. 2016) (*dada2 denoise-paired*) to produce Amplicon Sequence
151 Variants (ASV). The ASVs were taxonomically classified using the plugin *feature-classifier*
152 *classify-sklearn* with the GreenGenes database (version 13_8) using default confidence
153 threshold (≥ 0.7). The ASVs that could not be identified to genus with Greengenes, were blasted
154 against the NCBI Microbial Genome sequences (Bacteria and Archaea) to identify best hits and
155 also compared with the EzBioCloud Database (version 2021.07.07) for assignation of genus
156 using 97% identity threshold on both searches. When NCBI-BLAST and EzBiocloud resulted in
157 contradictory Genus best-hits at >=97% identify each, the original GreenGenes identification
158 taxon level was maintained. Contaminant sequences identified as chloroplast or mitochondria
159 were removed from processed data tables and excluded from further analyses.
160

161 **Diversity analysis and taxon abundance comparisons**:
162 The ASV tables for raw abundance and taxonomy classification were exported from QIIME2 and
163 processed through the MicrobiomeAnalyst tool (Dhariwal et al. 2017; Chong et al. 2020) using
164 the Marker Data Profiling (MDP) pipeline, as follows. The ASV abundances were brought to the
165 total sum scaling for data normalization and further analysis of diversity. Alpha-diversity was
166 estimated using the number of observed taxa (Observed), Chao1, ACE, Fisher and Shannon
167 (H’) indexes. Statistical differences between groups (Nymph vs Adult) were assessed with
168 Mann-Whitney *U* test. Beta-diversity was assessed using Bray-Curtis distance between groups
169 and their ordination visualized with Principal Coordinate Analysis (PCoA) and Non-metric
170 Multidimensional Scaling (NMDS). Statistical differences in community structure between
171 groups was tested with the permutational multivariate analysis of variance (PERMANOVA, one-
172 way) and the analysis of similarities (ANOSIM, one-way), both based on Bray-Curtis distance
173 and as implemented on Past v.4.08 (Hammer-Muntz et al. 2001). Differences in dispersion
174 within each group was tested using PERMDISP (Anderson and Walsh 2013). Bacteria taxon
175 abundance bar-plots were built with the MicrobiomeAnalyst tool and the heatmap plots using

176 Matrix2png (Pavlidis and Noble 2003). Statistical differences for taxon abundances between
177 groups were tested with the Mann-Whitney *U* test.
178
179 **Molecular screening of *Wolbachia* endosymbiont**:
180 From the *M. velazangeli* individuals collected in this study, abdomen samples were separately
181 dissected from nymphs or adults under sterile conditions in a stereoscope and individually used
182 for DNA isolation with DNeasy Kit (Qiagen) as described above. Detection and classification of
183 *Wolbachia* was performed following the *wsp* gene (*Wolbachia* surface protein) PCR-based
184 method established by Zhou et al. (Zhou et al. 1998) as follows. PCR screening was done with
185 the *wsp*-specific primers wsp81F (5′-TGG TCC AAT AAG TGA TGA AGA AAC-3′) and wsp691R
186 (5′-AAA AAT TAA ACG CTA CTC CA-3′) in 20 μL reactions containing 1x Green GoTaq®
187 reaction buffer (Promega, USA), 250 μM dNTPs, 0.5 μM of each primer, 0.5 u of GoTaq®
188 polymerase (Promega, Madison, WI, USA) and 1 μL of DNA template. PCR cycling involved
189 one step of denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 40 sec; 55°C for 30
190 sec and 72°C for 40 sec, with a final extension of 72°C for 5 min. DNA template integrity was
191 additionally tested by PCR with universal primers for arthropod 28SrRNA gene sequences
192 (28sF3633: 5′-TAC CGT GAG GGA AAG TTG AAA-3′, and 28sR4076: 5′-AGA CTC CTT GGT
193 CCG TGT TT-3′) using the same PCR reaction conditions and cycling described above. Total
194 DNA from a naturally *Wolbachia*-infested fruit fly (*Drosophila melanogaster*) laboratory strain
195 was used as positive control in the PCR screening experiments. PCR amplicons were visualized
196 with agarose gel electrophoresis.
197
198 **Sanger DNA sequencing and phylogenetic analysis of *Wolbachia wsp* amplicons**:
199 A group of 10 randomly selected *wsp* PCR amplicons (wsp81F/691R primers) derived from the
200 *M. velezangeli* DNA samples were further purified using the QIAquick PCR Purification Kit
201 (Qiagen) following the manufacturer protocol. Purified amplicons were directly submitted to ABI
202 automated bidirectional sequencing with wsp81F and wsp691R primers. DNA sequence
203 chromatograms were processed in Chromas v2.6.6 (https://technelysium.com.au/wp/chromas/)
204 for quality and primer-sequence trimming. Bidirectional sequences for each sample were
205 clustered into single DNA contigs (isolates) using GeneStudio v.2.2.0
206 (https://sourceforge.net/projects/genestudio/). The DNA contig sequences were compared with
207 available sequence data at GeneBank nt database using BLASTn search algorithm and were
208 deposited at GeneBank under accession numbers OR129441-OR129450.
209
210 A phylogenetic analysis of the *M. velezangeli*-derived *wsp* sequences was performed using the
211 web-based Phylogeny.fr platform (Dereeper et al. 2008) along with *wsp* sequences from
212 representative insect-derived *Wolbachia* endosymbiont isolates at the GeneBank database.
213 Sequences were aligned with ClustalW (v2.1) (Thompson et al. 1994). After alignment, positions
214 with gaps were removed from the alignment. The phylogenetic tree was reconstructed using the
215 maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT) (Guindon and
216 Gascuel 2003). The default substitution model was selected assuming an estimated proportion
217 of invariant sites (of 0.003) and 4 gamma-distributed rate categories to account for rate
218 heterogeneity across sites. The gamma shape parameter was estimated directly from the data

219 (gamma=0.398). Reliability for internal branches were assessed using the aLRT test (SH-Like)
220 (Anisimova and Gascuel 2006).
221
222 **Results**:
223
224 **Microbial 16SrRNA sequence data**:
225 After removing putative contaminant sequences, a total of 491,802 denoised, non-chimeric
226 merged sequences for the V3V4 variable region of the bacterial 16SrRNA gene were produced
227 among all samples (nymph and adult). From these sequences, 123 ASVs were inferred, with a
228 mean number of ASVs for adult and nymph samples of 57 and 79, respectively. The number of
229 Illumina reads and ASV sequences for each sample are detailed in Table 1. Rarefaction curves
230 showed that all samples reached richness saturation (Fig. 1A) indicating that sequencing effort
231 was enough to capture total diversity (Good's coverage > 99.99% for all samples, Table 1).
232
233 **Diversity of bacterial community**:
234 The bacterial diversity associated with the nymph and adult life stages of *M. velezangeli* was
235 analyzed through five Alpha-diversity indices (Fig. 1B and Table 2). Statistically, no differences
236 for species richness (Observed species), abundance (Chao1 and ACE) and abundance
237 distribution indices (Fisher and Shannon) were detected between nymph and adult stages
238 (Observed species: U = 0, p = 0.1; Chao1: U = 0, p = 0.1; ACE: U = 0, p = 0.1; Fisher: U = 0, p
239 = 0.1; Shannon: U = 3, p = 0.7).
240
241 Differences in microbial community structure (Beta-diversity) between nymph and adult was
242 assessed with PERMANOVA and ANOSIM analyses and their ordinal distances (Bray-Curtis
243 dissimilarity) visualized with PCoA and NMSD plotting (Fig. 1C,D). PERMANOVA tests whether
244 distance in community structure differs between groups (e.g. nymph vs adult) (Anderson 2001),
245 whereas ANOSIM tests whether distances between groups are greater than within groups
246 (Clarke 1993). Both analyses indicated no significant differences in microbial community
247 structure between nymphs and adults (PERMANOVA: F-value: 0.4774; R-squared: 0.1135; p-
248 value = 0.5016; ANOSIM: R: -0.1111; p-value = 0.7019). Since these two statistical tests are
249 sensitive to differences in dispersion (variance) within groups, we assessed this with
250 PERMDISP (Anderson and Walsh 2013). This analysis showed that there is homogeneity of
251 multivariate dispersions between nymph and adult samples (PERMDISP: F-value: 0.1958; p-
252 value: 0.681).
253
254 Since the removal of *Wolbachia*-associated sequences is a regular practice for microbiome
255 analysis in insects when they are detected in bacterial 16SrRNA libraries (Chandler et al. 2014;
256 Rudman et al. 2019), we also analyzed this procedure in our data. The removal of *Wolbachia*
257 reads did not alter the similarities in alpha diversity indices between nymphs and adults
258 (Supplementary Table S1) (Observed species: U = 0, p = 0.1; Chao1: U = 0, p = 0.1; ACE: U =
259 0, p = 0.1; Fisher: U = 0, p = 0.1; Shannon: U = 3, p = 0.1). Similarity in microbial community
260 structure between life stages also remained unchanged (PERMANOVA: F-value: 1.698; R-
261 squared: 0.5142; p-value = 0.2028; ANOSIM: R: 0.2593; p-value = 0.2992; PERMDISP: F-
262 value: 0.0993; p-value = 0.7684).

263
264 **Taxonomic composition of bacterial community**:
265 From the 123 ASV, 107 (87%) were taxonomically assigned to at least the Phylum level.
266 Taxonomic distribution of ASVs included 10 bacteria phyla, 18 classes, 22 orders, 33 families
267 and 36 genera. Distribution of relative abundances for phylum, order and genus levels are
268 shown in Figure 2 and fully detailed for all taxonomic levels in Supplementary Tables S2 to S6.
269 Overall, the Phylum Proteobacteria (92.6%) and Firmicutes (5.2%) represented almost the full
270 microbiota detected in this study (Supplementary Table S2, Fig.2A). The orders Rickettsiales
271 (Phylum Proteobacteria: Class Alphaproteobacteria) and Clostridiales (Phylum Firmicutes:
272 Class Clostridia) with 91.9% and 4.8% abundance, respectively, dominated the bacterial
273 community. To a lesser extent, other 20 orders were present at or below 1% overall abundance
274 (Supplementary table S4, Figure 2B).
275
276 From the total 123 ASVs, 95 (77.2%) were assigned to the genus level, where 66 ASVs (53.7%)
277 were annotated using GreenGenes (≥ 0.7 confidence level) and 29 ASVs (23.6%) annotated
278 using BLASTn and BioCloud (≥ 97% identity to top-hit for both algorithms). The remaining 28
279 ASVs (22.8%) were considered as undetermined at genus level (Not Assigned). At the genus
280 level, *Wolbachia* (Rickettsiaceae) dominated the overall abundance (91.9%) across nymph and
281 adult samples, followed by *Romboutsia* (1.8%), *Ignavibacterium* (0.8%), *Clostridium* (0.70%),
282 *Mycoplasma* (0.5%), *Allobaculum* (0.4%), *Blautia* (0.4%), *Eubacterium*_g23 (0.3%),
283 *Sporobacter* (0.3%), *Paracoccus* (0.3), *Methylobacterium* (0.2%), *Dorea* (0.2%),
284 *Sediminibacterium* (0.1%), *Faecalibacterium* (0.1%), and *Ruminococcus* (0.1%) as the top 15
285 taxa. Other 34 genera were present at abundances below 0.1% across all life stages
286 (Supplementary Table S6, Figure 2C). The relative abundances for bacteria taxa in all
287 taxonomic levels (Phylum to Genus) were similar between both insect life stages (Mann-
288 Whitney *U* test, p-values > 0.05, Supplementary Tables S2 to S6). Similarly, no statistical
289 differences were found at bacterial ASV level between both life stages (Mann-Whitney *U* test, p-
290 values > 0.05).
291
292 Removal of *Wolbachia* sequences from this analysis did not alter the similarities in the overall
293 relative abundances at ASV or genus levels between life stages (Mann-Whitney *U* test, p-values
294 > 0.05) as estimated above; despite changes in the proportions of total reads counts and
295 relative taxon abundances across the individual samples. Additionally, apart from *Wolbachia*,
296 the list of the top ten most abundant genus remained unchanged; and in all cases the
297 microbiota was dominated by *Romboutsia* with few changes in the order of remaining genera
298 (Fig. 3B). However, the exclusion of *Wolbachia* resulted in Firmicutes (69.3%) as the overall
299 dominant Phylum, followed by Proteobacteria (10.4%), Chlorobi (6.2%) and other seven Phylum
300 to a lesser extent (Fig. 3A).
301
302 **Core microbiota**:
303 According to the data collected in this study, the core microbiota of *M. velezangeli* is composed
304 of 21 bacterial ASVs (17% of all ASVs) that were consistently shared between the nymph and
305 adult life stages (ASVs present in all samples in this study) (Fig. 2D). These core ASVs were
306 identified by analyzing 42 ASVs that were present in all samples of either life stage. The

307 remaining 21 ASVs were only present in one or the other life stage. Other 81 ASVs (65.9% of all
308 ASVs) were not consistently detected in all samples of each life stage and may represent
309 transient or non-resident microbes within the microbiome of *M*. *velezangeli*.
310
311 The bacterial genus assignments for the core 21 ASVs based primarily on 16S GreenGenes
312 database are shown in Table 3. These core bacterial genera, listed in decreasing order of
313 abundance, included: *Wolbachia*, *Romboutsia*, *Ignavibacterium, Clostridium*, *Allobaculum,*
314 *Paracoccus*, undetermined Anaerolineaceae, *Methylobacterium, Faecalibacterium,*
315 undetermined Lachnospiraceae, *Collinsella*, *Rothia,* undetermined Peptostreptococcaceae,
316 *Sphingomonas* and undetermined Coriobacteriaceae.
317
318 ***Wolbachia* PCR detection and profiling**:
319 Taking into account the large proportion of *Wolbachia*-associated ASVs found in our *M.*
320 *velezangeli* samples, we decided to further investigate the presence of *Wolbachia*
321 endosymbiont in *M. velezangeli* by PCR screening of the *wsp* gene. About 79% of the insect
322 individuals tested (22 out of 28) from the Segovianas collection locality resulted positive for
323 *Wolbachia* infection according to the amplification of a ~600 bp DNA band (Figure 4). A PCR
324 test for DNA template integrity showed that all 28 (100%) *M. velezangeli* DNA samples were of
325 PCR quality based on the successful amplification of a DNA band for the arthropod 28SrRNA
326 gene target. This indicates that lack of *wsp* amplification in 21% (6/28) of the samples could be
327 explained by the absence of *Wolbachia* infection and not because of a low DNA template
328 quality.
329
330 Sanger sequencing was performed for 10 randomly selected *wsp* DNA amplicons derived from
331 *M. velezangeli* samples. All Sanger sequences had clearly defined single-pick chromatograms,
332 which suggested the presence of single *Wolbachia*-strain infections in each sequenced sample.
333 A multiple sequence alignment showed that these isolates can be grouped in two distinct *wsp*
334 sequence haplotypes that share a 78% similarity between (Supplementary Figure S1).
335 Haplotype 1 (hereafter wMvel1) was represented by 80% (8/10) of the sequence isolates in this
336 study, whereas haplotype 2 (hereafter wMvel2) was represented by the remaining 20% (2/10)
337 isolates. A BLASTn search against the GeneBank database showed that wMvel1 *wsp* sequence
338 was 99.46% identical (top hit) to a *Wolbachia wsp* isolate from the butterfly *Acraea equitorialis*
339 (GenBank accession: AJ271195); whereas wMvel2 *wsp* was 98.91% identical (top hit) to a
340 *Wolbachia wsp* isolate from the planthopper *Perkinsiella saccharicida* (GenBank accession:
341 GU190768) (Hughes et al. 2011). Phylogenetic analysis clustered all wMvel *wsp* sequences
342 within the *Wolbachia wsp* B supergroup clade (Figure 5) and assigned the distinct wMvel *wsp*
343 haplotypes to two distant subclades, respectively; along with their corresponding *wsp* BLASTn
344 top-hits (see above).
345
346 **Discussion**:
347 We used 16SrRNA amplicon high-throughput sequencing to investigate for the first time the
348 diversity of the symbiotic bacteria community associated with the tropical plant bug *M.*
349 *velezangeli*. Here, we found a relatively diverse core microbiota dominated by genera
350 *Wolbachia*, *Romboutsia*, *Ignavibacterium* and *Clostridium*. Although this plant bug is a

351 polyphagous herbivore considered a pest for various tropical crops in America, here we focused
352 the bacteria screening on a population feeding on coffee plants in Colombia. In this study, we
353 found that overall bacteria diversity (Alpha diversity, Fig. 1B) was similar between the nymph
354 and the adult life stages. Based on the most abundant taxa (ASVs with overall abundance
355 >0.01%), the bacterial community composition (Beta diversity) is conserved between these two
356 developmental stages; however, there is a degree of variability relative to the presence of
357 bacteria with low abundance within and between life stages. The immature forms of *M*.
358 *velezangeli* go through 5 nymphal instars that differ among them mainly on body size (Giraldo J.
359 et al. 2010). The microbial composition we present in this work for the nymph is based on
360 pooled individuals from all instars; hence, whether the overall bacterial community diversity and
361 structure experience any changes along nymphal development needs to be addressed in future
362 analyses. The bacterial 16SrRNA gene sequence has been used historically as a gold standard
363 genetic marker to infer bacteria taxonomic identity and community diversity in high-throughput
364 microbiome studies, especially throughout the partial sequencing of some of its nine
365 hypervariable sequence regions (V1 to V9) (Van de Peer et al. 1996). In our study, we used the
366 sequences of the combined V3V4 variable regions, a 16S sequence section commonly utilized
367 in microbiome analysis; however, it must be noticed that the used of partial sequences of this
368 gene marker can result in overestimation of microbial diversity due to intragenomic
369 heterogeneity (Sun et al. 2013), and does not offer enough accuracy for bacteria identification at
370 the species or strain level (Johnson et al. 2019a). Being aware of this bias, we mainly describe
371 the microbial taxonomic diversity in this study at genus level as the deepest taxonomic rank.
372
373 We found that the intracellular symbiont *Wolbachia* dominated the full microbiota associated
374 with *M*. *velezangeli*, representing about 92% of the bacterial load within the body of nymph and
375 adult stages. The observed high abundance of *Wolbachia* in our samples may indicate a
376 proportionally elevated titer of this endosymbiont in the analyzed insects as well. Presence of
377 *Wolbachia* endosymbiont was also confirmed by PCR screening in *M. velezangeli* samples and
378 DNA sequence analysis of wMvel *wsp* isolates indicates they belong to *Wolbachia* B
379 supergroup. Insect-infecting *Wolbachia* strains with major biological effects have been mostly
380 associated with host reproductive disturbances (e.g. CI, parthenogenesis, male-killing and
381 feminization) (Serbus et al. 2008; Werren et al. 2008; Kaur et al. 2021) and recently with effects
382 on other behavioral and physiological processes, including nutrition, defense and insecticide-
383 resistance (Hosokawa et al. 2010; Nikoh et al. 2014; Zug and Hammerstein 2015; Zhang et al.
384 2020; Soh and Veera 2022). In other mirid species, the presence of *Wolbachia* has been
385 associated with reproductive alterations and nutritional roles. For example, in the predatory
386 mirid bug *Macrolophus pygmaeus*, this parasitic bacteria induces strong CI (Machtelinckx et al.
387 2009). In the hematophagous bed bugs *Cimex lectularius* and *Cimex hemipterus* (Hemiptera:
388 Miridae), *Wolbachia* infection creates a obligate mutualism that is essential for normal insect
389 growth and reproduction via provisioning of B vitamins (Hosokawa et al. 2010; Laidoudi et al.
390 2020). *Wolbachia* infection in insects have been mainly associated to the host reproductive
391 tissues, but it is also commonly found in several insect somatic organs or tissues, including
392 brain, salivary glands, gut, malpighian tubules, muscles, fat bodies (Casper-Lindley et al. 2011;
393 Pietri et al. 2016; Diouf et al. 2018) and also as habitant of bacteriocytes (Hosokawa et al.
394 2010). The presence of this parasitic endosymbiont in *M*. *velezangeli* raises new questions

about the possible biological implications, if any, for this plant bug. The detection of two distinct wMvel *wsp* haplotypes in our analysis suggest that multiple *Wolbachia* strains are present in the insect population tested here; however, insect individuals seem to be infected by single *Wolbachia* strains. Additionally, the prevalence of infection is not 100% across all insect individuals, which seems to indicate that an obligate mutualism would not be the case for *M. velezangeli* - *Wolbachia* relationship.

The extremely high abundance of ASV sequences identified as *Wolbachia* in our samples (~92% overall abundance) could be introducing a potential confounding effect in the estimation of relative abundances for the actual gut-associated bacterial taxa. This possible issue was recently analyzed by Wilches et al. (Wilches et al. 2021) using the spotted-wing drosophila (*Drosophila suzukii*) as a case of study when NGS is applied to investigate the microbiome in *Wolbachia*-infected insect samples. The authors detected large discrepancies in the measures of alpha and beta diversity, as well as in the relative abundances of several bacteria taxa in the microbiome between *Wolbachia*-infected fly samples (mean abundance of 98.8% for *Wolbachia* sequences) and non-infected. This and other research work (Wilches et al. 2021; Henry and Ayroles 2021) have shown that in some cases removing the *Wolbachia*-associated reads from the analyses could also have major impacts in the interpretation of the study results, which may be specially relevant when comparing infected samples versus non-infected. In our study we addressed the impact of removing *Wolbachia* reads in microbiota diversity and structure in *M*. *velezangeli*. Here, the exclusion of *Wolbachia*-associated sequences did not affect the similarity in microbiota composition as seen when these sequences are included. Although this comparison involves two sample groups (nymph and adult) with *Wolbachia*-infection, it is likely that this may not be the case when comparing infected versus non-infected samples.

Apart from *Wolbachia* (Proteobacteria), the remaining top 10 most abundant bacterial genera detected in *M*. *velezangeli* include members of Phylum Firmicutes (5.2% overall abundance), such as *Romboutsia*, *Clostridium*, *Allobaculum*, Blautia, *Eubacterium*_g23, Sporobacter, *Dorea* and *Faecalibacterium,* as well as the Proteobacteria genera *Paracoccus*, *Methylobacterium* and the Chlorobi genus *Ignavibacterium*. Members of these Firmicutes genera have been previously found in the alimentary canals of other arthropods (Grech-Mora et al. 1996; Husseneder et al. 2017; Li et al. 2020; Shukla and Beran 2020; Fang et al. 2020; Mejía-Alvarado et al. 2021). In our study, *Romboutsia* (1.75%) (Firmicutes: Peptostreptococcaceae) was the second most abundant bacterial genus across all samples. Members of this genus have been mainly registered in the microbiota of guts from several vertebrate animals (Gerritsen et al. 2014, 2017; Ricaboni et al. 2016; Johnson et al. 2019b) and also insects (Shukla and Beran 2020). There is no information about the functional roles of the *Romboutsia* members as gut symbionts; however they seem to be well adapted to live within animal guts (Gerritsen et al. 2017, 2019). Similarly, members of *Paracoccus*, *Methylobacterium* and *Ignavibacterium* are regular habitants of arthropod guts (Zhang et al. 2016, 2018; Sajnaga et al. 2022). We infer that most abundant bacteria genera found in this study, with the exclusion of *Wolbachia*, are likely residents of the *M. velezangeli* gut lumen and may be involved in important biological processes for this plant bug. Several of these symbionts (*Romboutsia*, *Ignavibacterium, Clostridium*, *Paracoccus*, *Allobaculum*, *Methylobacterium*, *Faecalibacterium*, *Collinsella*, *Rothia* and *Sphingomonas*) were

439 found to be consistently present in all our samples of nymph and adult stages and we consider
440 them as members of the insect gut-associated core microbiota. Most of these genera, except for
441 *Paracoccus, Methylobacterium* and *Sphingomonas*, are primarily anaerobic bacteria taxa.
442 Compared with the microbiota associated with the cotton fleahopper *P*. *seriatus* (Hemiptera:
443 Miridae) (Fu et al. 2021) and *A*. *suturalis* (Hemiptera: Miridae) (Xue et al. 2021), the composition
444 at the genus level within *M*. *velezangeli* is clearly different, being dominated within *P*. *seriatus*
445 by bacteria *Diaphorobacter*, *Lactococcus*, *Pseudomonas*, *Pantoea* and *Izhakiella*; and within *A*.
446 *suturalis* by *Erwinia*, *Acinetobacter*, *Staphylococcus*, and *Lactococcus*. These differences in
447 microbiota composition could be associated with environmental differences due to host-plant
448 species, feeding habits and geographical origins.
449
450 Several bacteria isolates found in *M. velezangeli*'s microbiota that belong to genera
451 *Paracoccus, Methylobacterium* and *Sphingomonas*, which contain mostly aerobic species; are
452 potential culturable strains and may also represent candidate symbionts for paratransgenic
453 approaches such as symbiont-mediated RNAi (Dyson et al. 2022). The use of bacteria within
454 paratransgenesis applications requires a culturable symbiont genetically manipulable (Ratcliffe
455 et al. 2022) and especially amenable under aerobic culturable conditions for greater ease in the
456 engineering and experimentation processes. Future attempts for selection of culturable bacterial
457 isolates from the insect gut tissues will reveal what microbes have these characteristics in *M.*
458 *velezangeli*'s microbiota.
459
460 **Conclusions**:
461 The tropical plant bug *M*. *velezangeli* harbors a diverse microbiota and, in some cases, it can be
462 dominated by the intracellular symbiont *Wolbachia.* The *M. velezangeli* microbiota also contain
463 potential gut-associated members of the genera *Romboutsia, Ignavibacterium, Clostridium,*
464 *Allobaculum, Paracoccus, Methylobacterium, Faecalibacterium, Collinsella, Rothia,*
465 *Sphingomonas*. The persistent detection of these bacteria genera in nymphal and adult life
466 stages indicates they seem to be part of the core microbiome and likely play important biological
467 roles in the normal development of *M*. *velezangeli*. Additionally, our observations suggest that
468 multiple *Wolbachia* strains are present in *M. velezangeli* populations but insect individuals seem
469 to harbor single-strain infections. The findings reported by this study offer new avenues to
470 improve our understanding of the microbiome contribution in the biology of Miridae plant bugs
471 such as the tropical insect pest *M*. *velezangeli*.
472
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474
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498
499 **References**:

500 Acevedo FE, Peiffer M, Tan C-W, et al (2017) Fall armyworm-associated gut bacteria modulate
501 plant defense responses. Mol Plant Microbe Interact 30:127–137

502 Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. Austral
503 Ecol 26:32–46

504 Anderson MJ, Walsh DCI (2013) PERMANOVA, ANOSIM, and the Mantel test in the face of
505 heterogeneous dispersions: What null hypothesis are you testing? Ecol Monogr 83:557–
506 574

507 Anisimova M, Gascuel O (2006) Approximate Likelihood-Ratio Test for Branches: A Fast,
508 Accurate, and Powerful Alternative. Syst Biol 55:539–552

509 Blanton AG, Peterson BF (2020) Symbiont-mediated insecticide detoxification as an emerging
510 problem in insect pests. Front Microbiol 0.: https://doi.org/10.3389/fmicb.2020.547108

511 Bolyen E, Rideout JR, Dillon MR, et al (2019) Reproducible, interactive, scalable and extensible
512 microbiome data science using QIIME 2. Nat Biotechnol 37:852–857

513 Callahan BJ, McMurdie PJ, Rosen MJ, et al (2016) DADA2: High-resolution sample inference
514 from Illumina amplicon data. Nat Methods 13:581–583

515 Caporaso JG, Lauber CL, Walters WA, et al (2011) Global patterns of 16S rRNA diversity at a
516 depth of millions of sequences per sample. Proc Natl Acad Sci U S A 108 Suppl 1:4516–
517 4522

518 Casper-Lindley C, Kimura S, Saxton DS, et al (2011) Rapid fluorescence-based screening for
519 *Wolbachia* endosymbionts in *Drosophila* germ line and somatic tissues. Appl Environ
520 Microbiol 77:4788

521 Chandler JA, James PM, Jospin G, Lang JM (2014) The bacterial communities of *Drosophila*
522 *suzukii* collected from undamaged cherries. PeerJ 2.: https://doi.org/10.7717/peerj.474

523 Chong J, Liu P, Zhou G, Xia J (2020) Using MicrobiomeAnalyst for comprehensive statistical,
524 functional, and meta-analysis of microbiome data. Nat Protoc 15.:
525 https://doi.org/10.1038/s41596-019-0264-1

526 Chung SH, Rosa C, Scully ED, et al (2013) Herbivore exploits orally secreted bacteria to
527 suppress plant defenses. Proc Natl Acad Sci U S A 110:15728–15733

528 Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure.
529 Austral Ecol. https://doi.org/10.1111/j.1442-9993.1993.tb00438.x

530 Dereeper A, Guignon V, Blanc G, et al (2008) Phylogeny.fr: robust phylogenetic analysis for the
531 non-specialist. Nucleic Acids Res 36:W465–W469

532 Dhariwal A, Chong J, Habib S, et al (2017) MicrobiomeAnalyst: a web-based tool for
533 comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Res
534 45.: https://doi.org/10.1093/nar/gkx295

535 Diouf M, Miambi E, Mora P, et al (2018) Variations in the relative abundance of *Wolbachia* in the
536 gut of *Nasutitermes arborum* across life stages and castes. FEMS Microbiol Lett 365.:
537 https://doi.org/10.1093/femsle/fny046

538 Dylan Shropshire J, Leigh B, Bordenstein SR (2020) Symbiont-mediated cytoplasmic
539 incompatibility: What have we learned in 50 years? https://doi.org/10.7554/eLife.61989

540 Dyson P, Figueiredo M, Andongma AA, Whitten MMA (2022) Symbiont-mediated RNA
541 interference (SMR): Using symbiotic bacteria as vectors for delivering RNAi to insects.
542 Methods Mol Biol 2360:295–306

543 Fang J-X, Zhang S-F, Liu F, et al (2020) Differences in gut bacterial communities of *Ips*
544 *typographus* (Coleoptera: Curculionidae) Induced by enantiomer-specific α-pinene. Environ
545 Entomol 49:1198–1205

546 Fukui T, Kawamoto M, Shoji K, et al (2015) The endosymbiotic bacterium *Wolbachia* selectively
547 kills male hosts by targeting the masculinizing gene. PLoS Pathog 11:e1005048

548 Fu Z, Antwi JB, Sword GA, et al (2021) Geographic variation of bacterial communities
549 associated with cotton fleahopper, *Pseudatomoscelis seriatus*. swen 46:17–32

550 Gerritsen J, Fuentes S, Grievink W, et al (2014) Characterization of *Romboutsia ilealis* gen.
551 nov., sp. nov., isolated from the gastro-intestinal tract of a rat, and proposal for the
552 reclassification of five closely related members of the genus *Clostridium* into the genera
553 *Romboutsia* gen. nov., *Intestinibacter* gen. nov., *Terrisporobacter* gen. nov. and
554 *Asaccharospora* gen. nov. Int J Syst Evol Microbiol 64:1600–1616

555 Gerritsen J, Hornung B, Renckens B, et al (2017) Genomic and functional analysis of
556 *Romboutsia ilealis* CRIBT reveals adaptation to the small intestine. PeerJ 5.:
557 https://doi.org/10.7717/peerj.3698

558 Gerritsen J, Hornung B, Ritari J, et al (2019) A comparative and functional genomics analysis of
559 the genus *Romboutsia* provides insight into adaptation to an intestinal lifestyle. bioRxiv
560 845511

561 Giraldo J. M, Benavides M. P (2012) Conozca los hospedantes sitios de alimentación y

562 oviposición de la chinche de la chamusquina del café. Cenicafe

563 Giraldo J. M, Benavides M. P, Villegas G. C (2010) Aspectos morfológicos y biológicos de
564 *Monalonion velezangeli* Carvalho and Costa (Hemiptera : Miridae) en café. Cenicafe
565 6:195–205

566 Grech-Mora I, Fardeau M-L, Patel BKC, et al (1996) Isolation and characterization of
567 *Sporobacter termitidis* gen. Nov., sp. Nov., from the digestive tract of the wood-feeding
568 termite *Nasutitermes lujae*. Int J Syst Evol Microbiol 46:512–518

569 Guindon S, Gascuel O (2003) A Simple, Fast, and Accurate Algorithm to Estimate Large
570 Phylogenies by Maximum Likelihood. Syst Biol 52:696–704

571 Hammer-Muntz O, Harper D, Ryan P (2001) PAST: paleontological statistics software package
572 for education and data analysis. Palaeontologia Electronica 4:1–9

573 Henry LP, Ayroles JF (2021) Meta-analysis suggests the microbiome responds to evolve and
574 resequence experiments in *Drosophila melanogaster*. BMC Microbiol 21:1–14

575 Hilgenboecker K, Hammerstein P, Schlattmann P, et al (2008) How many species are infected
576 with *Wolbachia*? A statistical analysis of current data. FEMS Microbiol Lett 281:215–220

577 Hiroki M, Kato Y, Kamito T, Miura K (2002) Feminization of genetic males by a symbiotic
578 bacterium in a butterfly, *Eurema hecabe* (Lepidoptera: Pieridae). Naturwissenschaften 89.:
579 https://doi.org/10.1007/s00114-002-0303-5

580 Hosokawa T, Koga R, Kikuchi Y, et al (2010) *Wolbachia* as a bacteriocyte-associated nutritional
581 mutualist. Proc Natl Acad Sci U S A 107:769

582 Hughes GL, Allsopp PG, Brumbley SM, et al (2011) Variable infection frequency and high
583 diversity of multiple strains of Wolbachia pipientis in Perkinsiella Planthoppers. Appl
584 Environ Microbiol 77:2165–2168

585 Hurst GDD, Jiggins FM, von der Schulenburg JHG, et al (1999) Male-killing *Wolbachia* in two
586 species of insect. Proceedings of the Royal Society B: Biological Sciences 266:735

587 Husseneder C, Park J-S, Howells A, et al (2017) Bacteria associated with *Piezodorus guildinii*
588 (Hemiptera: Pentatomidae), with special reference to those transmitted by feeding. Environ
589 Entomol 46:159–166

590 Jaimes LFT, Valenzuela JRC, Londoño GAC, et al (2015) Relación entre la presencia y el daño
591 de *Monalonion velezangeli* Carvalho & Costa y algunos factores climáticos en cultivos de
592 aguacate cv. Hass. CTA 16:79–85

593 Jie Bi YW (2020) The effect of the endosymbiont *Wolbachia* on the behavior of insect hosts.
594 Insect Sci 27:846

595 Johnson JS, Spakowicz DJ, Hong B-Y, et al (2019a) Evaluation of 16S rRNA gene sequencing
596 for species and strain-level microbiome analysis. Nat Commun 10:1–11

597 Johnson TA, Sylte MJ, Looft T (2019b) In-feed bacitracin methylene disalicylate modulates the
598 turkey microbiota and metabolome in a dose-dependent manner. Sci Rep 9.:
599 https://doi.org/10.1038/s41598-019-44338-5

600 Jung S, Lee S (2012) Molecular phylogeny of the plant bugs (Heteroptera: Miridae) and the
601 evolution of feeding habits. Cladistics 28:50–79

602 Kaur R, Shropshire JD, Cross KL, et al (2021) Living in the endosymbiotic world of *Wolbachia*: A
603 centennial review. Cell Host Microbe 29:879–893

604 Kikuchi Y, Hayatsu M, Hosokawa T, et al (2012) Symbiont-mediated insecticide resistance. Proc
605 Natl Acad Sci U S A 109.: https://doi.org/10.1073/pnas.1200231109

606 Klindworth A, Pruesse E, Schweer T, et al (2012) Evaluation of general 16S ribosomal RNA
607 gene PCR primers for classical and next-generation sequencing-based diversity studies.
608 Nucleic Acids Res 41:e1–e1

609 Laidoudi Y, Levasseur A, Medkour H, et al (2020) An earliest endosymbiont, *Wolbachia*
610 *massiliensis* sp. nov., strain PL13 from the bed Bbg (*Cimex hemipterus*), type strain of a
611 new supergroup T. Int J Mol Sci 21:8064

612 Lariviere PJ, Leonard SP, Horak RD, et al (2022) Honey bee functional genomics using
613 symbiont-mediated RNAi. bioRxiv 2022.04.22.489157

614 Li Q, Fan J, Sun J, et al (2019) Anti-plant defense response strategies mediated by the
615 secondary symbiont *Hamiltonella defensa* in the wheat aphid *Sitobion miscanthi*. Front
616 Microbiol 0.: https://doi.org/10.3389/fmicb.2019.02419

617 Li R, Rongrong LI, Min LI, et al (2020) Composition and function of the microbiotas in the
618 different parts of the midgut of *Pyrrhocoris sibiricus* (Hemiptera: Pyrrhocoridae) revealed
619 using high-throughput sequencing of 16S rRNA. Eur J Entomol 117:352–371

620 Luo J, Cheng Y, Guo L, et al (2021) Variation of gut microbiota caused by an imbalance diet is
621 detrimental to bugs' survival. Sci Total Environ 771:144880

622 Machtelinckx T, Van Leeuwen T, Vanholme B, et al (2009) *Wolbachia* induces strong
623 cytoplasmic incompatibility in the predatory bug *Macrolophus pygmaeus*. Insect Mol Biol
624 18:373–381

625 Mejía-Alvarado FS, Ghneim-Herrera T, Góngora CE, et al (2021) Structure and dynamics of the
626 gut bacterial community across the developmental stages of the coffee berry borer,
627 *Hypothenemus hampei*. Front Microbiol 0.: https://doi.org/10.3389/fmicb.2021.639868

628 Moran NA, Telang A (1998) Bacteriocyte-associated symbionts of insects: A variety of insect
629 groups harbor ancient prokaryotic endosymbionts. Bioscience 48:295–304

630 Namyatova AA, Cassis G (2016) Systematic revision and phylogeny of the plant bug tribe
631 Monaloniini (Insecta: Heteroptera: Miridae: Bryocorinae) of the world. Zool J Linn Soc
632 176:36–136

633 Narita S, Kageyama D, Nomura M, Fukatsu T (2007) Unexpected mechanism of symbiont-
634 induced reversal of insect sex: feminizing *Wolbachia* continuously acts on the butterfly
635 *Eurema hecabe* during larval development. Appl Environ Microbiol 73:4332–4341

636 Nikoh N, Hosokawa T, Moriyama M, et al (2014) Evolutionary origin of insect-*Wolbachia*
637 nutritional mutualism. Proc Natl Acad Sci U S A 111.:
638 https://doi.org/10.1073/pnas.1409284111

639 Ocampo Flórez V, Durán Prieto J, Albornoz M, Forero D (2018) New plant associations for
640 *Monalonion velezangeli* (Hemiptera: Miridae) in green urban areas of Bogotá (Colombia).
641 Acta biolColomb 23:205–208

642 O'Neill SL, Hoffmann AA, Werren JH (1997) Influential Passengers: Inherited Microorganisms
643 and Arthropod Reproduction. Oxford University Press, USA

644 Pavlidis P, Noble WS (2003) Matrix2png: a utility for visualizing matrix data. Bioinformatics 19.:
645 https://doi.org/10.1093/bioinformatics/19.2.295

646 Pietri JE, DeBruhl H, Sullivan W (2016) The rich somatic life of *Wolbachia*. Microbiologyopen
647 5:923

648 Ramirez C. HJ, Gil P. ZN, Benavides M. P, Bustillo P. AE (2008) *Monalonion velezangeli*: La
649 chinche de la chamusquina del café. Cenicafe

650 Ramírez-Gil JG, López JH, Henao-Rojas JC (2019) Causes of Hass avocado fruit rejection in
651 preharvest, harvest, and packinghouse: economic losses and associated variables.
652 Agronomy 10:8

653 Ratcliffe NA, Furtado Pacheco JP, Dyson P, et al (2022) Overview of paratransgenesis as a
654 strategy to control pathogen transmission by insect vectors. Parasit Vectors 15:112

655 Ricaboni D, Mailhe M, Khelaifia S, et al (2016) *Romboutsia timonensis*, a new species isolated
656 from human gut. New Microbes and New Infections 12:6–7

657 Rodas CA, Serna R, Hurley BP, et al (2014) Three new and important insect pests recorded for
658 the first time in Colombian plantations. Southern Forests 76:245–252

659 Rudman SM, Greenblum S, Hughes RC, et al (2019) Microbiome composition shapes rapid
660 genomic adaptation of *Drosophila melanogaster*. Proc Natl Acad Sci U S A 116.:
661 https://doi.org/10.1073/pnas.1907787116

662 Sajnaga E, Skowronek M, Kalwasińska A, et al (2022) Comparative Nanopore sequencing-
663 based evaluation of the midgut microbiota of the summer chafer (l.) associated with
664 possible resistance to entomopathogenic nematodes. Int J Environ Res Public Health 19.:
665 https://doi.org/10.3390/ijerph19063480

666 Sato Y, Jang S, Takeshita K, et al (2021) Insecticide resistance by a host-symbiont reciprocal
667 detoxification. Nat Commun 12:1–8

668 Schausberger P (2018) Herbivore-associated bacteria as potential mediators and modifiers of
669 induced plant defense against spider mites and thrips. Front Plant Sci 0.:
670 https://doi.org/10.3389/fpls.2018.01107

671 Serbus LR, Casper-Lindley C, Landmann F, Sullivan W (2008) The genetics and cell biology of
672 *Wolbachia*-host interactions. Annu Rev Genet 42:683–707

673 Shukla SP, Beran F (2020) Gut microbiota degrades toxic isothiocyanates in a flea beetle pest.
674 Molecular Ecology 29:4692–4705

675 Sinkins SP (2004) *Wolbachia* and cytoplasmic incompatibility in mosquitoes. Insect Biochem
676 Mol Biol 34:723–729

677 Soh LS, Veera SG (2022) Bacterial symbionts influence host susceptibility to fenitrothion and
678 imidacloprid in the obligate hematophagous bed bug, *Cimex hemipterus*. Sci Rep 12.:
679 https://doi.org/10.1038/s41598-022-09015-0

680 Stouthamer R, Breeuwer JA, Hurst GD (1999) *Wolbachia pipientis*: microbial manipulator of
681 arthropod reproduction. Annu Rev Microbiol 53:71–102

682 Sun D-L, Jiang X, Wu QL, Zhou N-Y (2013) Intragenomic Heterogeneity of 16S rRNA Genes
683 Causes Overestimation of Prokaryotic Diversity. Appl Environ Microbiol.
684 https://doi.org/10.1128/AEM.01282-13

685 Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of
686 progressive multiple sequence alignment through sequence weighting, position-specific gap
687 penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

688 Van de Peer Y, Chapelle S, De Wachter R (1996) A quantitative map of nucleotide substitution
689 rates in bacterial rRNA. Nucleic Acids Res 24:3381–3391

690 Vavre F, de Jong JH, Stouthamer R (2004) Cytogenetic mechanism and genetic consequences
691 of thelytoky in the wasp *Trichogramma cacoeciae*. Heredity 93:592–596

692 Werren JH (1997) Biology of *Wolbachia*. Annu Rev Entomol 42:587–609

693 Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: master manipulators of invertebrate biology.
694 Nat Rev Microbiol 6:741–751

695 Wilches D, Coghlin P, Floate K (2021) Next generation sequencing, insect microbiomes, and
696 the confounding effect of *Wolbachia*: a case study using *Drosophila suzukii Matsumura*
697 (Diptera: Drosophilidae). Canadian Journal of Zoology 99.: https://doi.org/10.1139/cjz-2020-
698 0260

699 Xue H, Zhu X, Wang L, et al (2021) Gut bacterial diversity in different life cycle stages of
700 *Adelphocoris suturalis* (Hemiptera: Miridae). Front Microbiol 0.:
701 https://doi.org/10.3389/fmicb.2021.670383

702 Zhang D, Wang Y, He K, et al (2020) *Wolbachia* limits pathogen infections through induction of
703 host innate immune responses. PLoS One 15:e0226736

704 Zhang L, Yun Y, Hu G, Peng Y (2018) Insights into the bacterial symbiont diversity in spiders.
705 Ecol Evol 8:4899–4906

706 Zhang S, Gan L, Qin Q, et al (2016) *Paracoccus acridae* sp. nov., isolated from the insect
707 *Acrida cinerea* living in deserted cropland. Int J Syst Evol Microbiol 66:3492–3497

708 Zhou J-C, Zhao Q, Liu S-M, et al (2021) Effects of thelytokous parthenogenesis-inducing
709 *Wolbachia* on the fitness of *Trichogramma dendrolimi* Matsumura (Hymenoptera:
710 Trichogrammatidae) in superparasitised and single-parasitised hosts. Front Ecol Evol 0.:
711 https://doi.org/10.3389/fevo.2021.730664

712 Zhou W, Rousset F, O'Neil S (1998) Phylogeny and PCR-based classification of Wolbachia
713 strains using wsp gene sequences. Proc Biol Sci 265:509–515

714 Zug R, Hammerstein P (2015) *Wolbachia* and the insect immune system: what reactive oxygen

species can tell us about the mechanisms of *Wolbachia*–host interactions. Front Microbiol 0.: https://doi.org/10.3389/fmicb.2015.01201

18

759 **Tables**:
760
761 **Table 1**. Overview of Illumina 16SrRNA-amplicon sequencing of the bacterial microbiota in
762 *Monalonion velezangeli*.
763

Sample	Raw PE reads	Clean PE reads	Raw merged sequences	Clean merged sequences	Total ASVs	Good's coverage
Adult 1	173,233	96,265	74,667	69,782	63	100%
Adult 2	162,775	109,344	90,923	90,168	62	100%
Adult 3	167,579	109,789	91,180	90,009	45	100%
Nymph 1	170,239	110,169	87,856	86,212	82	99.99%
Nymph 2	172,490	85,101	60,756	58,026	85	100%
Nymph 3	169,525	115,394	97,798	97,605	71	100%

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765
766 **Table 2**. Alpha diversity indices for 16SrRNA-based microbiota in *Monalonion velezangeli*.
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Sample	Observed	Chao1 (±se)	ACE (±se)	Fisher	Shannon (H')
Adult1	63	63 (±0.0)	63 (±1.69)	6.82	0.81
Adult2	62	62 (±0.0)	62 (±2.90)	6.50	0.41
Adult3	45	45 (±0.0)	45 (±1.91)	4.55	0.37
Nymph1	82	83 (±2.33)	82.5 (±3.25)	8.94	0.46
Nymph2	85	85 (±0.0)	85 (±2.97)	9.78	1.12
Nymph3	71	71 (±0.0)	71 (±2.66)	7.49	0.39

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778 **Table 3.** Bacterial genus annotations for ASVs considered as members of the core microbiota in
779 *Monalonion velezangeli*.

ID	Overall abundance	Genus (Family) rank annotation[#]
ASV01	91.7%	*Wolbachia* (Rickettsiaceae)
ASV02	0.97%	*Romboutsia* (Peptostreptococcaceae)*
ASV03	0.79%	*Romboutsia* (Peptostreptococcaceae)*
ASV04	0.73%	*Ignavibacterium* (Ignavibacteriaceae)*
ASV05	0.47%	*Clostridium* (Clostridiaceae)*
ASV06	0.26%	*Paracoccus* (Rhodobacteraceae)
ASV07	0.25%	Undetermined (Anaerolineaceae)*
ASV08	0.22%	*Allobaculum* (Erysipelotrichaceae)
ASV09	0.19%	*Methylobacterium* (Methylobacteriaceae)
ASV10	0.14%	*Sediminibacterium* (Chitinophagaceae)
ASV11	0.13%	*Allobaculum* (Erysipelotrichaceae)
ASV12	0.12%	*Faecalibacterium* (Ruminococcaceae)
ASV13	0.11%	*Clostridium* (Clostridiaceae)
ASV14	0.09%	Undetermined (Lachnospiraceae)
ASV15	0.09%	*Collinsella* (Coriobacteriaceae)
ASV16	0.09%	*Rothia* (Micrococcaceae)
ASV17	0.06%	*Clostridium* (Clostridiaceae)
ASV18	0.06%	Undetermined (Peptostreptococcaceae)
ASV19	0.06%	*Allobaculum* (Erysipelotrichaceae)
ASV20	0.05%	*Sphingomonas* (Sphingomonadaceae)
ASV21	0.04%	Undetermined (Coriobacteriaceae)

780 # The taxonomic classification was determined using the 16S GreenGenes (GG) database with a
781 confidence level of ≥0.7. For ASVs where GG failed to assign a Genus taxon, the Genus identification
782 was performed using the BLASTn and BioCloud search algorithms with a concomitant ≥97% sequence

783 identity for their top hits (taxa denoted with asterisk [*]). Further details can be found in the Methods
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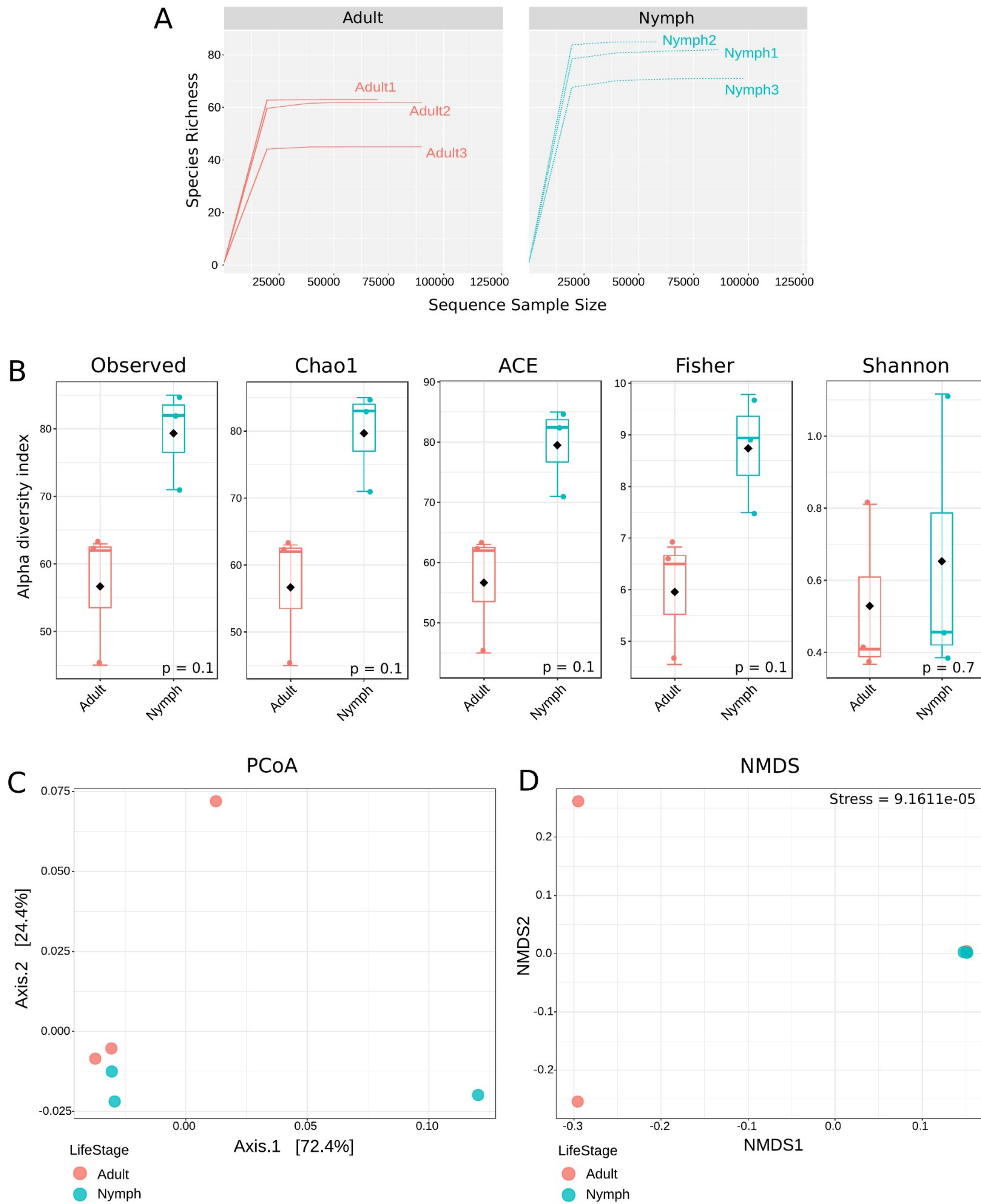

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814 **Figure 1**. Diversity and community structure of the bacterial microbiota in adult and nymph life
815 stages of *Monalonion velezangeli*. (A) Rarefaction curves. (B) Alpha diversity indices and their
816 corresponding p-value of the Mann-Whitney U test. (C) Principal coordinate analysis (PCoA)
817 plot based on Bray-Curtis dissimilarity of bacterial communities in nymphs and adults. (D) Non-
818 metric multidimensional scaling (NMDS) ordination analysis plot based on Bray-Curtis
819 dissimilarity of bacterial communities in nymphs and adults. Stress value represents the
820 goodness-of-fit for the NMDS analysis.

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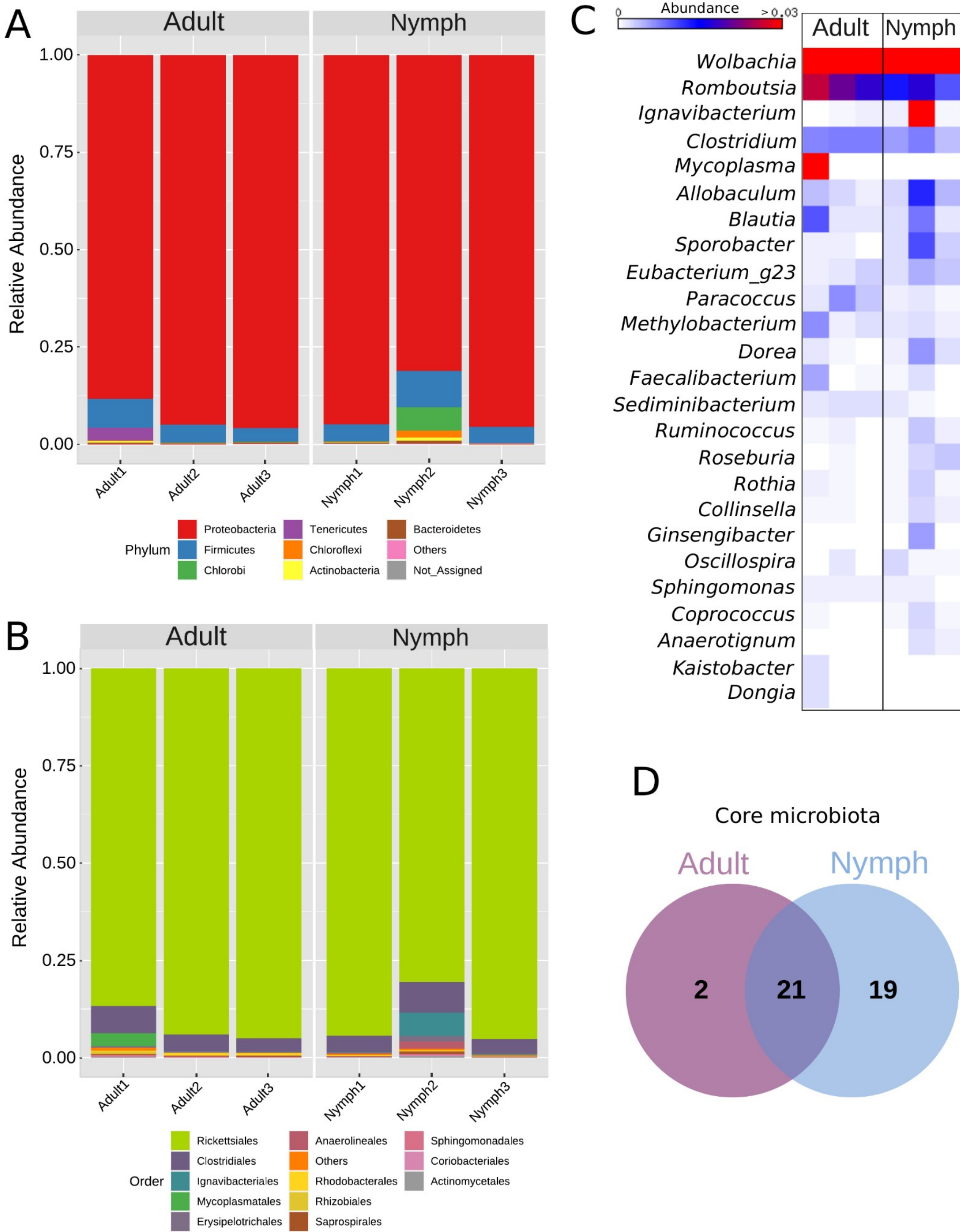

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823 **Figure 2**. Taxonomic composition of the bacterial microbiota in nymphs and adults of
824 *Monalonion velezangeli*. (A) Relative abundance at Phylum level. (B) Relative abundance at
825 Order level. (C) Heatmap for relative abundances at genus level. (D) Number of ASV
826 sequences consistently detected on either adults of nymphs and number of shared ASVs (circle
827 intersection) as members of the core microbiota.

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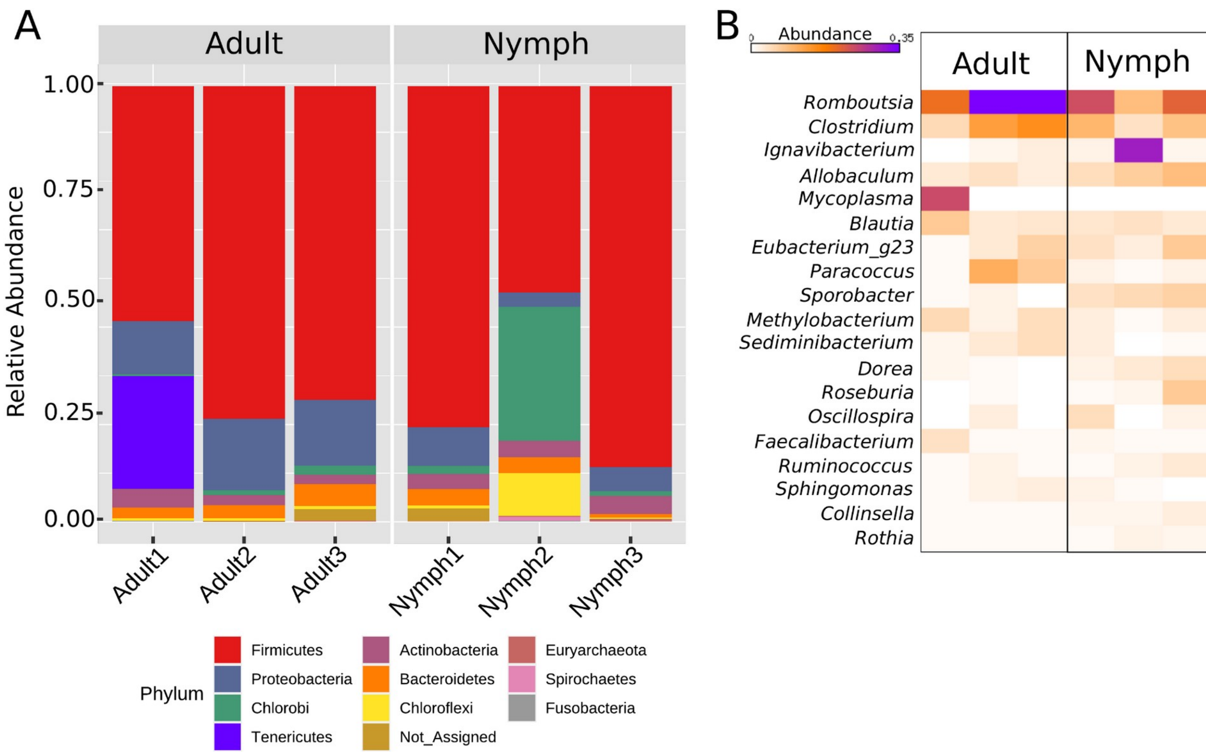

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830 **Figure 3**. Relative abundance of the bacterial microbiota, with the exclusion of *Wolbachia*-
831 associated sequences, for nymphs and adults of *Monalonion velezangeli*. (A) Relative
832 abundances at Phylum level. (B) Heatmap of relative abundances at genus level.
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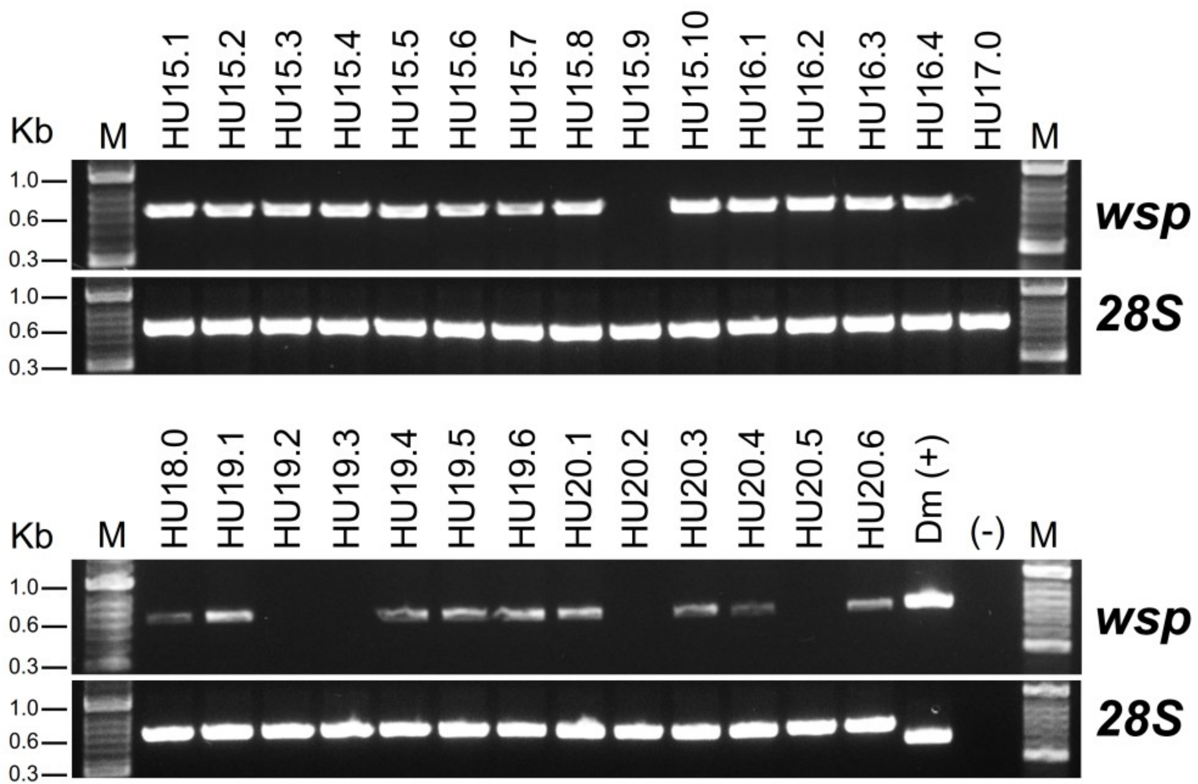

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835 **Figure 4.** Molecular screening for presence of *Wolbachia* endosymbiont in *Monalonion*
836 *velezangeli* samples. DNA samples from single insects (HU15.1 to HU20.6) were tested for
837 PCR amplification of the *Wolbachia wsp* gene using wsp81F and wsp691R primers. Quality of
838 DNA was tested by amplification of the 28SrRNA (28S) gene fragment (~700 bp). DNA from a
839 *Drosophila melanogaster* (Dm) population was used as positive control for *Wolbachia* infection,
840 and water (-) as negative control.
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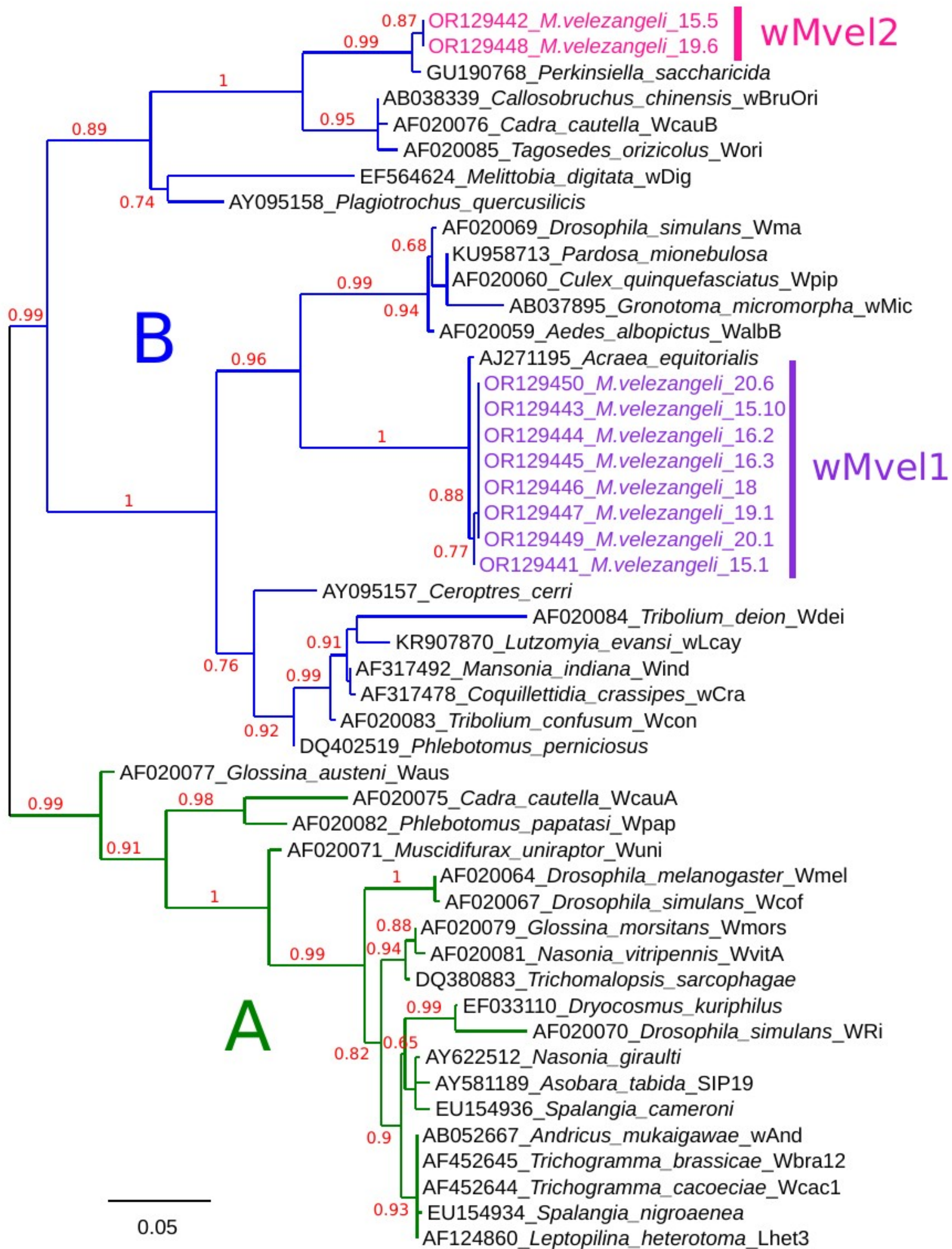

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845 **Figure 5.** Maximum Likelihood phylogenetic tree of *Wolbachia wsp* sequences from *Monalonion*
846 *velezangeli* and representative *Wolbachia* strains from other host insects at the GenBank
847 database. *Wolbachia* supergroups A (green branches) and B (blue branches) clusters based on
848 *wsp* sequences are shown. Sequence haplotypes clustering of the *M. velezangeli* wsp isolates,
849 wMvel1 and wMvel2, are shown in purple and pink colors respectively. The aLRT branch
850 supports are indicated as red numbers. Genbank accession numbers precede each sequence
851 name.