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1 **The bacteriome of bat flies (Nycteribiidae) from the Malagasy region: a**
2 **community shaped by host ecology, bacterial transmission mode, and host-**
3 **vector specificity.**

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25 **1 Abstract**

26 The Nycteribiidae are obligate blood-sucking Diptera (Hippoboscoidea) parasitizing bats.
27 Depending on species, these wingless flies exhibit either high specialism or generalism
28 towards their hosts, which may in turn have important consequences in terms of their
29 associated microbial community structure. Bats have been hypothesized to be reservoirs of
30 numerous infectious agents, some of which have recently emerged in human populations.
31 Thus, bat flies may be important in the epidemiology and transmission of some of these bat-
32 borne infectious diseases, acting either directly as arthropod vectors or indirectly by shaping
33 pathogen communities among bats populations. In addition, bat flies commonly have
34 associations with heritable bacterial endosymbionts that inhabit insect cells and depend on
35 maternal transmission, through egg cytoplasm, to ensure their transmission. Some of these
36 heritable bacteria are likely obligate mutualists required to support bat fly development but
37 others are facultative symbionts with unknown effects. Here, we present bacterial community
38 profiles obtained from seven bat fly species, representing five genera, parasitizing bats from
39 the Malagasy region. The observed bacterial diversity includes *Rickettsia*, *Wolbachia*, several
40 *Arsenophonus*-like-organisms, as well as other members of the Enterobacteriales, and a
41 widespread association of *Bartonella* bacteria from bat flies of all five genera. Using the well-
42 described host specificity of these flies and data on community structure from selected
43 bacterial taxa with either vertical or horizontal transmission, we show that host/vector
44 specificity and transmission mode are both important drivers of bacterial community
45 structure.

46

47 **2 Introduction**

48 Bats are increasingly recognized as natural reservoirs of a large number of emerging
49 infectious agents (1-4). It is thus implicit that vectors of bat-borne disease will play important
50 roles in the epidemiology and dynamics of infectious agents that can eventually emerge in
51 human populations. Further, bats are hosts to different ectoparasites, including mites, fleas,
52 ticks, and bat flies (5, 6). Bat flies (Diptera: Hippoboscoidea) are obligate blood-sucking
53 parasites that are classically divided into two families -- the Streblidae and the Nycteribiidae
54 (7). Together, the Hippoboscidae (louse or ked flies), Streblidae, and Nycteribiidae are
55 referred to as the Pupipara *sensu stricto* due to their adenotrophic viviparity, where all
56 larval developmental stages occur within the adult female's body and are nourished by milk
57 glands until they are ready to pupate. This particularity of the Pupipara *s.s.* is thought to
58 promote vertical parasite transmission, thus influencing the epidemiological role of these
59 vectors in disease transmission.

60 To date, studies of microorganisms associated with nycteribiids have mainly focused on two
61 groups of bacteria - *Bartonella* spp. (8-11) and *Arsenophonus*-like organisms (referred to
62 hereafter as ALOs) (12-15). These bacterial genera offer contrasting model systems for
63 investigating the biotic factors driving the structures of associated microbial communities.
64 *Bartonella* are parasitic intracellular bacteria infecting vertebrate erythrocytes and many
65 species are considered to be zoonotic (16) and linked to disease in humans (17-20), including
66 the recently identified bat-associated *B. mayotimonensis* (21). Nycteribiids are known to host
67 a wide variety of *Bartonella* spp. (10), and contrasting patterns of *Bartonella* bat-host
68 associations have been described across Africa (8, 9, 22), South America (23-25), Europe
69 (26), and Asia (27).

70 In contrast, ALOs are symbiotic organisms engaged in complex interactions with a variety of
71 arthropod species (28-30). The group making up ALO is a monophyletic lineage that includes
72 different genera, such as *Arsenophonus*, *Aschnera*, *Riesia*, and other unnamed groups (ALO-1
73 to ALO-3) (12-15). In bat flies, ALOs are primary and obligate endosymbionts that are
74 vertically transmitted from infected mothers to offspring due to the presence of bacteriocytes
75 in the milk glands of all nycteribiid species (12-15). It is thought that ALOs may play a role in
76 the provisioning of B vitamins, which are deficient in vertebrate blood, the only food source

77 for these flies (13). The ubiquity of ALOs in bat flies, in which infection is at fixation,
78 corroborates the hypothesis of an obligate endosymbiont (12-15). Such patterns have been
79 found in other exclusive blood-feeding species like bedbugs (31) and tsetse flies (32), two
80 insect groups which rely on a single food source throughout their developmental cycle and
81 harbor beneficial microbes that provide nutrients absent from their restricted diets.

82 Nycteribiid species show varying levels of bat host specificity. For example, a recent study
83 reported a lack of host specificity and genetic structure in *Cyclopodia horsfieldi*, found on
84 several fruit bats of the genus *Pteropus* (Pteropodidae) in southeastern Asia (33). On islands
85 in the western Indian Ocean, it has been observed that the genera *Penicillidia* and *Nycteribia*
86 are relatively promiscuous with respect to their host-associations, exchanging freely between
87 several *Miniopterus* spp. (Miniopteridae) and *Myotis goudoti* (Vespertilionidae) (5).
88 Interestingly, these insectivorous bat species are often found living in syntopy (in physical
89 contact within day roosts), thus likely facilitating vector sharing (34). The picture is rather
90 different for fruit bat flies belonging to the genus *Eucampsipoda*, which have been observed
91 to uniquely parasitize pteropodids of the genus *Rousettus* on continental Africa or on islands
92 in the southwestern Indian Ocean (5), despite these bats often sharing caves as day roost sites
93 with other bat species (35). Similarly, *Eidolon* spp. fruit bats are infested exclusively by flies
94 from the genus *Cyclopodia*, with *C. dubia* and *C. greeffi* specifically parasitizing Malagasy *E.*
95 *dupreanum* and African *E. helvum*, respectively. Thus, bat fly communities on Madagascar,
96 neighboring islands in the Comoros Archipelago, and continental Africa are composed of
97 species that are either specialists (e.g.: *Eucampsipoda madagascarensis*, *E. theodori*, *E.*
98 *africana*, *C. dubia*, and *C. greeffi* found strictly on *R. madagascariensis*, *R. obliviosus*, *R.*
99 *aegyptiacus*, *Eidolon dupreanum*, and *E. helvum*, respectively) or parasitize a broader range
100 of hosts, such as *Penicillidia leptothrinax* and *N. stylidiopsis* found on several Malagasy
101 *Miniopterus* spp. as well as on *Myotis goudoti*. Under these interesting biological
102 circumstances, variation in the patterns of infectious agent transfer between mammalian and
103 arthropod hosts are likely determined by a number of factors including the specificity of host-
104 vector interactions, the nature of vector-microorganism interactions (ranging from strict
105 parasitism to mutualism), the transmission mode, and confounding factors of host ecology
106 (Figure 1).

107 Here, in order to investigate the associations between bacteria and nycteribiids, we have
108 obtained bacterial 16S gene pyrosequencing data from bat flies of the genera *Eucampsipoda*,
109 *Penicillidia*, *Nycteribia*, *Cyclopodia*, and *Basilina* (*Paracyclopodia*), taken from bats sampled
110 in the western Indian Ocean region (Madagascar and the Union of the Comoros). We identify
111 a number of previously undescribed bacterial associations within the Nycteribiidae, compare
112 the phylogenetic relationships of some of these novel bacterial taxa, and propose a model for
113 how host-vector-parasite interactions may shape the bacterial communities hosted by these
114 flies.

115 **3 Materials and Methods**

116 3.1 **Taxon sampling and identification:**

117 Bats were captured using mist nets and harp traps generally placed at cave entrances, or
118 butterfly nets to obtain individuals from day roost sites in caves. This study was conducted in
119 strict accordance with the terms of research permits issued by national authorities (Direction
120 du Système des Aires Protégées, Direction environment et des Forêts, and Madagascar
121 National Parks [Madagascar]; and Centre National de Documentation et de Recherche
122 Scientifique [Union of the Comoros]), following the laws of these countries, and the
123 associated research permit numbers are listed in the acknowledgements. Upon capture, each
124 individual cataloged bat was placed in a separate clean cloth bag until the collection of
125 relevant biological data and they were examined for ectoparasites. The ectoparasites were
126 collected with forceps and stored in separate vials containing 70% ethanol. Morphological
127 identification of the ectoparasites was carried out using published keys and descriptions (36)
128 and host bat specimens are housed in the Field Museum of Natural History (FMNH),
129 Chicago, and *Département de Biologie Animale, Université d'Antananarivo (UADBA)*,
130 Antananarivo. A considerable portion of the nycteribiid specimens used herein came from a
131 previous study that evaluated the evolutionary origins of bat flies on the Comoro Islands and
132 Madagascar (5). New samples of *Cyclopodia dubia* and *Basilia (Paracyclopodia)* sp. were
133 collected on Madagascar from *Eidolon dupreanum* and *Scotophilus* spp., respectively, as part
134 of this study.

135 3.2 **Nucleic acid extraction and pyrosequencing design.**

136 Nycteribiid flies were removed from ethanol storage and dried, then crushed using 2 x 2 mm
137 tungsten beads in a Tissue Lyzer (Qiagen). Nucleic acids were extracted using a Qiagen EZ1
138 robot with the DNA Tissue Kit, according to the manufacturer's protocol and as previously
139 published (5). Nucleic acids were pooled by species for 16S gene pyrosequencing, as detailed
140 in Supplementary Table 1

141 16S gene pyrosequencing was performed as previously described (37). Briefly, 16S V3 and
142 V4 variable regions were amplified via PCR specific primers targeting the upstream and
143 downstream regions of the V3-V4 segment: the 3' end of forward (TACGGRAGGCAGCAG)
144 and reverse (GGACTACCAGGGTATCTAAT) bacterial specific primers were associated in
145 5' with MID tags, a GsFLX key and GsFLX adapters. The quantity of each PCR product was
146 then determined with Picogreen and all products were mixed together in equimolar
147 concentrations prior 454 GsFLX sequencing (Genoscreen).

148 All reads less than 250 bp in length were discarded using the Geneious pro software package
149 (38). Remaining reads were analyzed using the SILVA online NGS tool (available online at
150 www.arb-silva.de/ngs). Raw sequence reads were aligned with a gap extension penalty of 2
151 and a gap penalty of 5. Reads were filtered based on the following quality criteria: Minimum
152 length - 250 bp, minimum quality score - 30, maximum percent ambiguities - 1%, minimum
153 base pair score - 30 and maximum percent repetitive - 2%. Remaining reads were clustered
154 into operational taxonomic units (OTUs) at a threshold sequence identity of 99%. OTUs were
155 classified by BLAST score comparison against the SILVA rRNA database version 115, with a
156 classification similarity threshold of 93%. OTUs were used exclusively for initial taxonomic
157 interpretations, and all quantitative data were calculated from the numbers of original
158 sequence reads. Statistical significance of differences in proportions of sequence data was
159 calculated by Chi-squared comparisons with a Yate's correction.

160 3.3 Sequence data and genetic analyses

161 Specific gene fragments were amplified using primer pairs detailed in Table 1. Sequence data
162 were analyzed using the Geneious pro software package (38). All final sequence data were
163 deposited in GenBank, and the relevant accession numbers are listed in Table 2. Coding
164 sequences were aligned in-frame using the Geneious pro translation align tool and the
165 standard ClustalW cost matrix. Non-coding sequences were aligned using MUSCLE (39).
166 Network analyses were performed in Splitstree4 (40), using the NeighbourNet algorithm.
167 Maximum likelihood phylogenies were generated through RAxML v8.0.0 (41), using the
168 RAxML GUI (v1.3), with 1000 bootstrap replicates and the GTR+Gamma substitution model
169 as suggested by JModeltest2 (v.2.1.6) (42). Coding sequence alignments were partitioned by
170 base position, and single or multiple outgroup sequences were specified prior to analysis. Best

171 tree outputs from RAxML were used as topology inputs in the R software package, exploiting
172 the ape (43) and vegan (44) packages in order to test evolutionary congruence using the
173 ParaFit algorithm (45). Bacterial phylogenies used for ParaFit analysis contained only
174 sequences obtained in this study, and were compared against a host-nycteribiid phylogeny
175 generated based on Cytochrome oxidase I (COI) sequence data. COI sequences were taken
176 from (5), and data from *Cyclopodia dubia* and *Basilina* (*Paracyclopodia*) sp. were provided by
177 Ramasindrazana *et al.* (article in preparation).

178 4 Results

179 4.1 Bacterial community composition as revealed by 16S gene pyrosequencing:

180 Pyrosequencing data were obtained from seven independent samples, originating from seven
181 nycteribiid species: *Eucampsipoda madagascarensis*, *E. theodori*, *Penicillidia* sp. (cf.
182 *fulvida*), *P. leptothrinax*, *Nycteribia styliidiopsis*, *Cyclopodia dubia*, and *Basilina*
183 (*Paracyclopodia*) sp. Samples contained pooled DNA, extracted from different numbers of
184 nycteribiid flies. A summary of sample composition, as well as the statistics of
185 pyrosequencing data acquisition, including numbers of reads obtained and quality control
186 results are presented in Supplementary Table 1.

187 We then analyzed bacterial diversity at the class level. The bacterial communities associated
188 with all bat fly samples were dominated by three bacterial phyla, Alphaproteobacteria,
189 Betaproteobacteria, and Gammaproteobacteria, which represented 17%, 3%, and 78% of total
190 sequences, respectively. The vast majority of Betaproteobacteria were observed in *E. theodori*
191 and showed closest similarity to uncultured bacteria of the Family Neisseriaceae. The
192 majority of Neisseriaceae are common commensal bacteria that are often associated with
193 mammals, but *Neisseria gonorrhoeae* and *N. meningitides* are the known causative agents of
194 gonorrhea and meningococcal meningitis in humans, respectively.

195 Of the sequences identified as Alphaproteobacteria, 55% came from *Wolbachia*, 26% from
196 *Bartonella*, and 17% from *Rickettsia*. Nearly all Gammaproteobacteria (> 99%) came from
197 the order Enterobacteriales, and, for all bat fly species but one (*Cyclopodia dubia*), they were
198 mostly distributed between two genera, *Arsenophonus* and *Aschnera*, which clearly belong to

199 the ALO group (12-15). The case of *C. dubia* is subtly different: using BLAST, most of the
200 bacterial sequences from this bat fly species have identity to other Enterobacteriales members
201 than ALOs, such as *Providencia*, *Erwinia*, and *Dickeya*. Interestingly, the bacteria originating
202 from *C. dubia* may actually form a new Enterobacteriales group (see below).

203 Bacterial community data are summarized in Supplementary Figure 1.

204 We then further investigated bacterial taxa by sequencing additional loci. Taxa were selected
205 because of their possible medical importance, such as the genera *Rickettsia* and *Bartonella*,
206 with their relation to pathogenic strains causing rickettsioses and bartonelloses, respectively,
207 or because they were common to multiple species of nycteribiid fly and represented a
208 significant proportion of the total number of reads obtained by pyrosequencing, such as
209 *Wolbachia* and Enterobacteriales (threshold retrospectively set at 2.5% of the total number of
210 reads).

211 All accession numbers for the PCR-generated sequences in this study are listed in Table 2.

212 4.2 *Rickettsia*

213 During pyrosequencing, 16S sequences closely related to *Rickettsia* were obtained from
214 *Eucampsipoda madagascarensis* and *Penicillidia leptothrinax*. These sequences represented
215 5.5% and 15.3% of reads in *E. madagascarensis* and *P. leptothrinax*, respectively. The *gltA*
216 locus of *Rickettsia* was amplified from both *E. madagascarensis* and *P. leptothrinax* samples.
217 Classification using BLASTx showed that the obtained *gltA* sequences showed little
218 homology to known species of *Rickettsia*, the strongest match showing 90% identity to *gltA*
219 from *R. raoultii*, a bacterium identified in hard ticks from China (46). In addition, a 1,350 bp
220 fragment of the 16S gene of *Rickettsia* was amplified from *E. madagascarensis*. Although the
221 phylogeny of *Rickettsia* could only be poorly resolved based on these data (not shown),
222 sequence similarity was strongest with *R. gravesii*, *R. barbariae*, and *R. tarasevichiae*,
223 bacteria previously shown to be associated with hard ticks belonging to the genera
224 *Amblyomma* and *Ixodes*.

225 4.3 *Wolbachia*

226 Pyrosequencing of the 16S locus revealed that *Wolbachia* was present in all sampled fly
227 species except *Eucampsipoda madagascariensis* and *E. theodori*. The percentage of reads
228 matching with *Wolbachia* ranged from less than 1% in *Nycteribia stylidiopsis* to nearly 40%
229 in *Basilina (Paracyclopodia)* sp. PCR-based screening using the *wsp* system (47) was then
230 used to test individual nycteribiid samples for the presence of *Wolbachia*. In accordance with
231 the pyrosequencing results, *Wolbachia* was identified in *Penicillidia leptothrinax* (14/23;
232 61%), *P. sp. (cf. fulvida)* (1/1; 100%), *Cyclopodia dubia* (6/9; 67%), and *Basilina*
233 (*Paracyclopodia*) sp. (5/5; 100%).

234 A selection of DNA fragments from the *Wolbachia wsp*, *fbpA*, *ftsZ*, and *hcpA* genes (48) were
235 amplified from *C. dubia*, *Basilina (Paracyclopodia)* sp., and *P. leptothrinax*. Sequence data
236 were additionally obtained from the *wsp* gene from *Wolbachia* of *Penicillidia sp. (cf. fulvida)*,
237 showing 100% identity to those sequences obtained from *Wolbachia* infecting *P. leptothrinax*,
238 however attempts to amplify other loci were unsuccessful for these samples. As has been
239 observed elsewhere (49, 50), network analysis of the *wsp* gene data against reference
240 sequences from the *Wolbachia* MLST database (<http://pubmlst.org/wolbachia/>) suggested
241 high levels of diversification and recombination between *Wolbachia* spp. from well-defined
242 phylogenetic groups (Supplementary Figure 2), and, thus, the *wsp* gene data were abandoned
243 for phylogenetic characterization of the sequences obtained from our bat fly samples.

244 Network and phylogenetic analyses of concatenated sequences (*fbpA*, *ftsZ*, and *hcpA*)
245 suggested that all identified bat fly *Wolbachia* belong to the F subgroup (Figure 2). Sequence-
246 types from different nycteribiid species were grouped by host species and were paraphyletic,
247 sharing identity with *Wolbachia* strains from multiple host types and geographical origins,
248 suggestive of independent evolutionary histories and multiple introductions of *Wolbachia* into
249 bat fly populations.

250 4.4 *Arsenophonus*-like organisms (ALOs)

251 The Enterobacteriales were by far the most predominant bacterial taxa identified in
252 pyrosequencing analyses, with 73% of all sequencing data identified as either ALOs or a new

253 and unnamed Enterobacteriales group. Further 16S data were obtained from multiple samples
254 of each nycteribiid species. Network analysis was performed using reference strains listed in
255 Supplementary Table 2. Nycteribiid endosymbionts belonging to the Enterobacteriales fell in
256 three distinct groups (Figure 3). Insectivorous bat associated *Nycteribia* and *Penicillidia*
257 possessed ALOs associated with the *Aschnera* subgroup, whereas fruit bat associated
258 *Eucampsipoda* and insectivorous bat associated *Basilina* (*Paracyclopodia*) sp. possessed ALOs
259 belonging to the *Arsenophonus* subgroup. Interestingly, network analysis suggested that the
260 endosymbionts from *Cyclopodia dubia* form their own, new group within the
261 Enterobacteriales family, which is closely related to the known ALO members (*Cyclopodia*
262 group in Figure 3).

263 4.5 *Bartonella*

264 *Bartonella* sequences were detected by pyrosequencing in all bat fly species. *Eucampsipoda*
265 *theodori*, the only bat fly in this study from the Union of the Comoros and parasitizing
266 *Rousettus obliviosus*, showed significantly higher proportions of sequences from *Bartonella*
267 ($p < 0.0001$) than its sister species *E. madagascarensis* from Madagascar occurring on *R.*
268 *madagascariensis*. The percentage of reads derived from *Bartonella* ranged from $< 1\%$ in
269 *Basilina* (*Paracyclopodia*) sp., *Penicillidia leptothrinax*, and *Nycteribia stylidiopsis* to 17.4%
270 in *E. theodori*.

271 The *gltA* locus was amplified from these nycteribiid samples. Only 15 sequences that
272 demonstrated clear identity to unambiguously annotated *Bartonella* strains in BLAST
273 searches, and were monophyletic (with *Bartonella* reference sequences in preliminary
274 phylogenetic studies) were retained for analysis. *Bartonella* sequences were obtained from all
275 bat fly species except *E. madagascarensis*. Additional *Bartonella*-specific amplifications
276 based on the 16S and *rpoB* loci were also attempted, but were largely unsuccessful.

277 The *gltA* sequences obtained from nycteribiids of Madagascar and Comoros Archipelago
278 could be seen to cluster into five distinct groups (Figure 4). Potential limitations of the *gltA*
279 locus have been discussed elsewhere (10).

280 4.6 Evolutionary Congruence

281 The global-fit method, ParaFit, was used to test the hypothesis of evolutionary congruence
282 between nycteribiids and the bacteria detected in this study, comparing phylogenetic
283 structures to those obtained using COI data from bat flies. ALOs demonstrated a significant
284 level of congruence ($p < 0.001$), whereas congruence was not significant for *Bartonella*
285 (Table 3). As the topological structure may result from a difference in levels of polymorphism
286 of the chosen markers (16S for ALOs and *gltA* for *Bartonella*), we compared their nucleotide
287 diversities (Pi value) using DnaSP v.5 (available at <http://www.ub.edu/dnasp/>). This revealed
288 that 16S was actually less polymorphic than *gltA* (Pi: 0.056 and Pi: 0.117 for 16S and *gltA*,
289 respectively), thus allowing us to reject the hypothesis of an artifactual topology of ALOs
290 resulting from higher resolution of the 16S locus. It was not possible to test *Wolbachia* or
291 *Rickettsia* lineages using this methodology due to the limited number of relevant bacterial
292 taxa identified in nycteribiid hosts.

293

294 **5 Conclusion and Discussion**

295 Here, we provide characterization of the bacterial microbiota associated with numerous
296 species of Nycteribiidae bat flies occurring on Madagascar and the nearby Comoros
297 Archipelago, and provide more detailed taxonomic information for some bacteria taxa of
298 particular interest.

299 Bacterial communities associated with the nycteribiid bat fly genera *Eucampsipoda*,
300 *Penicillidia*, *Nycteribia*, *Cyclopodia*, and *Basilia* (*Paracyclopodia*) were described by
301 pyrosequencing of the 16S locus. Variation was found in community composition in different
302 species of Nycteribiidae; however, these may be due to stochastic infection effects,
303 differences in sample pooling between species, and/or limits in sequencing repeatability. It
304 should also be noted that taxa sampling was subject to amplification bias due to the primers
305 used to amplify the 16S locus (Supplementary Table 1). Although we cannot quantify the
306 significance of these effects, our data suggest that the vast majority of bacterial diversity in all
307 samples could be described by the presence of not more than two bacterial classes – the
308 alphaproteobacteria and gammaproteobacteria. While the ALOs (gammaproteobacteria) have
309 been previously described as obligate, primary symbionts in bat flies (12-15), the nature of the
310 interactions between other bacteria and bat flies remains unknown. The overall homogeneity
311 of the bacterial community structure across nycteribiid species, independent of bat host-
312 species, suggests that infection is not merely opportunistic but selected and commonly
313 occurring taxa likely form beneficial, positively-selected interactions with their arthropod
314 hosts. On the other hand, strongly significant differences between samples from Madagascar
315 and the Comoros Archipelago (Betaproteobacteria in *E. theodori*) suggest that environmental
316 variables will also determine which interactions can be sustained.

317 Our results along with previous studies (12-15) corroborate the widespread presence of ALOs
318 in bat flies, whilst identifying a likely new genus of endosymbiont of the Enterobacteriales
319 that infects the fruit bat (*Eidolon dupreanum*) associated nycteribiid *C. dubia*. While ALOs,
320 and especially *Arsenophonus*, are globally common symbionts estimated to infect ca. 5% of
321 insect species (28, 51), we further confirm that bat flies harbor the highest diversity of
322 Enterobacteriales strains reported to date (*Arsenophonus*, *Aschnera*, and the *Cyclopodia*-
323 associated group). We also found congruent patterns of codivergence between bat flies and

324 ALOs, a result likely due to the association between members of the Nycteribiinae subfamily
325 (that includes *P. leptothrinax*, *P. sp. (cf. fulvida)*, and *N. stylidiopsis*) and *Aschnera*, which are
326 known to have codiverged over a long-evolutionary period (8, 26). However, the evolutionary
327 history of these endosymbionts in bat flies is complex: all strains found herein do not cluster
328 within a specific bat fly clade, but rather exhibit distinct evolutionary origins showing that
329 they underwent repeated horizontal transfer between distantly related host species. This
330 pattern is well illustrated by the presence, in some bat fly species, of *Arsenophonus* strains,
331 which are closely related to strains from very diverse insect species, such as aphids and
332 parasitoid wasps. Two distinct evolutionary strategies are thus acting on ALO endosymbiosis:
333 *Aschnera* is highly specialized with regards to its hosts, with ancient acquisition followed by
334 codiversification, while *Arsenophonus* is more generalist and acquired through recent
335 horizontal transfers. The presence of a previously unidentified group of bacteria in *C. dubia*
336 testifies to the diverse nature of symbiotic interactions, and further work will be required to
337 better understand how these findings modify our understanding of the evolutionary history of
338 nycteribiid-endosymbiont interactions.

339 The genus *Wolbachia* was observed to infect *Penicillidia* spp., *C. dubia*, and *Basilia*
340 (*Paracyclopodia*) sp. This common reproductive manipulator has previously been identified
341 in bat flies (13); however it is of note that we observe *Wolbachia* in multiple nycteribiid
342 species. Interestingly, all *Wolbachia* identified in bat flies belonged to the F supergroup, an
343 emerging supergroup of *Wolbachia*, which have been associated with a broad spectrum of
344 arthropod hosts including the orders Scorpiones, Blattodea, Coleoptera, Hemiptera, Isoptera,
345 Neuroptera, Orthoptera, Phthiraptera, Thysanoptera (52), and Diptera (the order containing
346 the Nycteribiidae), which are more commonly associated with supergroups A and B (51, 53).
347 *Eucampsipoda* did not harbor *Wolbachia*, and only a subset of bat flies infesting insectivorous
348 bats tested *Wolbachia* positive, showing that, unlike the ALOs, *Wolbachia* is not an obligate
349 endosymbiont of nycteribiid flies. Although the number of samples that could be sequenced in
350 greater detail was relatively limited, nycteribiid-*Wolbachia* sequences appeared species-
351 specific but paraphyletic through MLST analyses, suggesting multiple introductions of
352 independent lineages to different nycteribiid species. However, a common lineage of
353 *Wolbachia* was observed in *P. leptothrinax* and *P. sp. (cf. fulvida)*. Facultative association
354 between nycteribiid *Wolbachia* suggests that their interaction over evolutionary history is
355 more recent than that of the ALOs, despite the fact that fluorescence *in situ* hybridization-
356 based physiological studies suggest a common mechanism of vertical transmission for all

357 nycteribiid bacterial endosymbionts (13). This is in keeping with observations in other
358 arthropod species, where *Wolbachia* strains are seen to have complex phylogenetic histories
359 due to their dual capacity to form stable co-existing mixed populations within arthropod
360 communities (54, 55) and to invade entire populations by manipulating host reproduction
361 (56). While the potential of *Wolbachia* to manipulate reproduction of bat flies is unknown, it
362 is interesting to note that a recent study observed biased sex-ratios in bat fly populations
363 (*Trichobius frequens*) in Puerto Rico (57).

364 The data generated in this study also add to the knowledge of the increasing diversity of
365 *Bartonella* subgroups that are associated with both bat flies and their bat hosts. It is generally
366 thought that *Bartonella* demonstrate specificity to mammalian hosts; however, the exact role
367 of vector-host specificity in establishing host specific interactions is not fully understood (58).
368 Here, the detection of *Bartonella* in all tested bat fly species adds credence to the theory that
369 *Bartonella* and bat flies may form mutually beneficial, positively-selected interactions, which
370 may drive host specificity. However, the lack of evolutionary congruence between *Bartonella*
371 and the Nycteribiidae suggests that any selective benefit is insufficient to generate stable
372 symbiosis despite their likely ability to be transferred vertically from mother to pupa. In the
373 case of insectivorous bat-infesting Nycteribiidae of Madagascar and the Comoros,
374 transmission through blood-feeding is likely to play a direct role in the horizontal transfer of
375 *Bartonella*, as multiple nycteribiid flies (i.e. *Nycteribia* spp. and *Penicillidia* spp.) feed on the
376 same bat hosts (5). This was seen to be the case here as, for example, a single clade in our
377 phylogenetic analysis (colored orange in Figure 4) contained *Bartonella* variants from both
378 *Nycteribia* and *Penicillidia*, as well as five different bat species. However, the same
379 phylogenetic analysis also suggested exchange between *Cyclopodia dubia* and *Basilia*
380 (*Paracyclopodia*) sp. (group colored blue in Figure 4), taxa which are not known to interact
381 directly or with the same bat-host species. This suggests that other direct or indirect
382 mechanisms may promote exchange between hosts, and thus drive the intraspecific diversity
383 of *Bartonella* observed within the Nycteribiidae. This is in contrast with other members of the
384 Pupipara *s.s.*; ked flies, blood-sucking vectors of ungulates, are thought to form strict
385 interactions with *Bartonella*, which have resulted in host-specific associations (10). The
386 difference between these two situations is likely not related to vector host-specificity, as
387 members of the family Hippoboscidae are highly adapted to their respective hosts (59).
388 Instead, we can imagine that the ecology of different bat hosts may promote the diversity of
389 *Bartonella* within the Nycteribiidae, where host populations that more frequently come into

390 contact with other hosts undergo more frequent horizontal exchange. This may help explain
391 global *Bartonella* diversity as well as the previously reported regional differences in host-
392 specificity between *Bartonella* and bats (see introduction).

393 Overall, a relatively low diversity of bacteria were seen to be associated with the studied bat
394 flies from Madagascar and the Comoros, with the principal infecting taxa being the
395 Enterobacteriales, *Bartonella*, and *Wolbachia*. Varying patterns of intraspecific genetic
396 diversity were observed between these models, which we explain by differences in the nature
397 of the bacterial-nycteribiid endosymbiotic relationship, transmission mode, host-vector
398 specificity, and variations in the associated bat-host ecology (Figure 5). The transmission
399 modes of these three bacterial taxa vary, while all three are thought to be transferred vertically
400 from mother to pupa during adenotrophic viviparity, only *Bartonella* may be transferred in the
401 blood of the bat hosts. *Bartonella*, where the highest levels of intraspecific genetic diversity
402 were observed, thus have the opportunity for direct horizontal transfer due to bat fly host
403 promiscuity and may also be transferred by bat-host interactions that are independent of these
404 ectoparasites, such as with other blood-feeding vectors (e.g. Streblidae, mites or fleas) or
405 habitat overlap with other hosts. The frequency of horizontal transfer of *Bartonella* is thus
406 likely to be also influenced by ecological factors such as breeding seasonality of bats, as well
407 as their migration patterns, geographical distribution, and colony population size and
408 structure. Contrastingly the Enterobacteriales form strict, primary endosymbiotic relationships
409 with their nycteribiid hosts, but evidence of horizontal transfer between hosts that infest the
410 same bat species could be observed, suggesting that proximity likely drives the frequency of
411 horizontal endosymbiont exchange.

412 In conclusion, while the global bacterial community structure associated with the
413 Nycteribiidae is likely primarily driven by the establishment of mutually beneficial
414 relationships between microorganism and host, the origins and evolution of these associations
415 are complex. Additionally, host-associated microorganisms are known to interact (60) and
416 bacterial community structure in insects may be influenced positively by cooperation, or
417 negatively by competition or exclusion, and in turn affect the biology of their hosts (reviewed
418 in (61) and (12)). *Bartonella* appear to be the only bacteria with known pathogenic potential
419 that form strict relationships with the tested Nycteribiidae bat flies, suggesting that these
420 arthropods may be a true reservoir of *Bartonella* infection. The presence of other likely

421 pathogenic bacteria such as *Rickettsia* is only anecdotic, and may even remain detrimental to
422 the health of these arthropods. However, these unique vectors remain fascinating from an
423 epidemiological point of view, due to the diverse nature of the interactions that they form,
424 especially in tropical settings.

425

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- 634

635 Figure and Table Legends:

636 Figure 1: Host specificity between bats and bat flies (Nycteribiidae) on the Madagascar and
637 the nearby Comoros Archipelago (5).

638 Figure 2: *Wolbachia* Network analysis and group F phylogeny. The presented analyses are
639 based on concatenation of three gene loci (*fbpA:ftsZ:hcpA*), reference data was acquired from
640 the *Wolbachia* MLST database (<http://pubmlst.org/wolbachia/>). The presented network
641 structure was generated from aligned sequence data in SplitsTree4 using the NeighborNet
642 algorithm. The phylogenetic tree was produced using RaxML, bootstrap support from 1000
643 replicates is indicated by dots of different sizes on each internal node. Sequences from this
644 study are highlighted in red, orange, and blue, representing host origins from *Cyclopodia*
645 *dubia*, *Basilisa* (*Paracyclopodia*) sp., and *Penicillidia leptothrinax*, respectively. Asterisks
646 indicate that the *hcpA* region was missing from the sequence data originating from *P.*
647 *leptothrinax*. However, it should be noted that identical, but less well supported, topologies
648 were generated when using only *fbpA* and *ftsZ* (data not shown).

649 Figure 3: Enterobacteriales Network Diagram generated from 16S gene sequence data. ALO
650 = *Arsenophonus*-like organisms. The presented network structure was generated from aligned
651 sequence data in SplitsTree4 using the NeighborNet algorithm. The “*Cyclopodia* group”
652 contained only sequences from this study and refers a newly identified taxon of the
653 Enterobacteriales, all originating from *C. dubia*. Sequences used to generate this
654 representation can be found in Supplementary Tables 3 and 4. All sequences from bat flies in
655 this study are indicated by colored circles, as presented in the key.

656 Figure 4: *Bartonella* phylogeny based on the *gltA* locus, generated using 1000 bootstrap
657 replicates in RaxML. Dots on internal nodes represent bootstrap support > 0.75. Sequences
658 from this study, and similar bat and bat fly associated sequences from other studies are
659 grouped into monophyletic groups by color. Bats drawn on leaves indicate sequences that
660 originated from bats or bat fly specimens. The key, underneath, details bat fly and host bat
661 origins of the associated sequences. Abbreviations used for bat flies are: *Cy.* = *Cyclopodia*,
662 *Eu.* = *Eucampsipoda*, *P* = *Penicillidia*, *N* = *Nycteribia*, and for bats are: *Ei.* = *Eidolon*, *Mi.* =
663 *Miniopterus*, *My.* = *Myotis*, *R.* = *Rousettus*, *Sc.* = *Scotophilus*. Numbers in parentheses
664 indicate identical sequences which were grouped into operational taxonomic units in RaxML.

665 Figure 5: Factors affecting speciation patterns and mono-specific microorganism diversity for
666 different host-associated microorganisms.

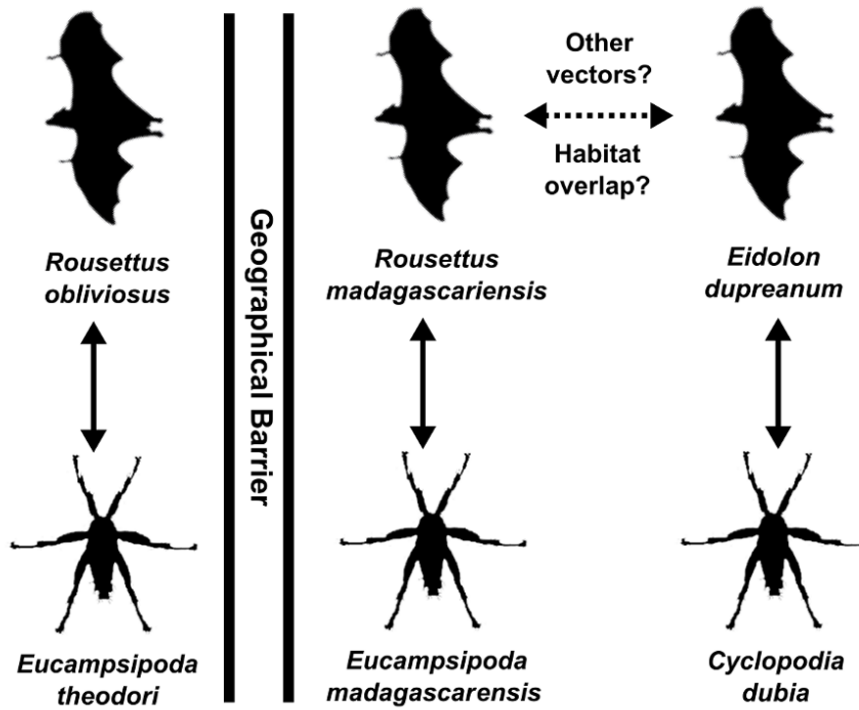
667 Table 1: Primers used in this study.

668 Table 2: Genbank accession numbers for sequences generated as part of this study.
669 Geographical, arthropod, and bat host information is also presented, along with laboratory
670 and museum identifier codes for the host mammal.

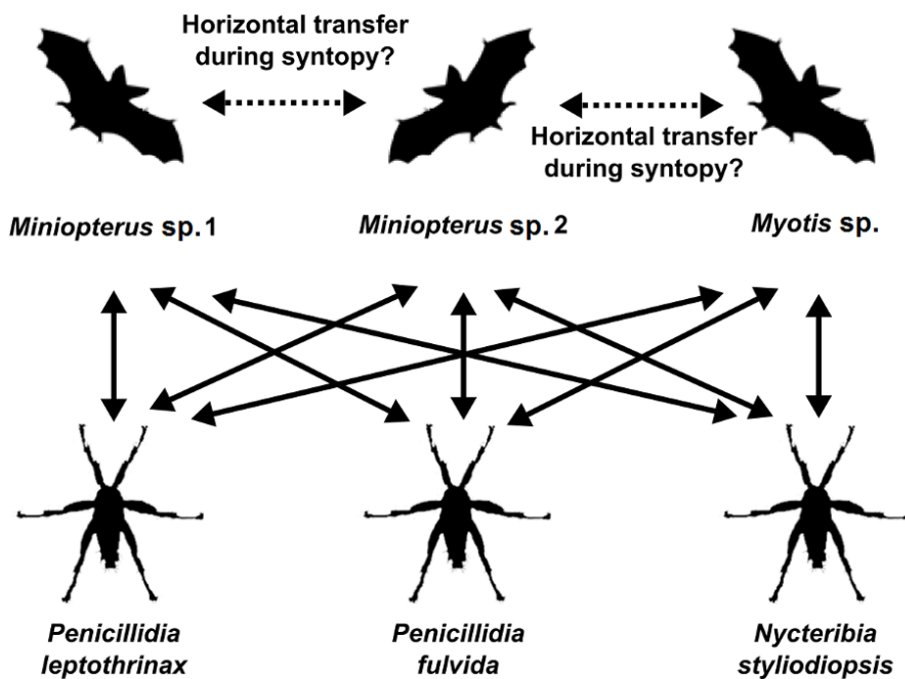
671 Table 3: Evolutionary congruence between Nycteribiidae and associated bacteria.
672 Congruence testing for *Wolbachia* was not possible due to the presence of only three
673 independent taxa.

674

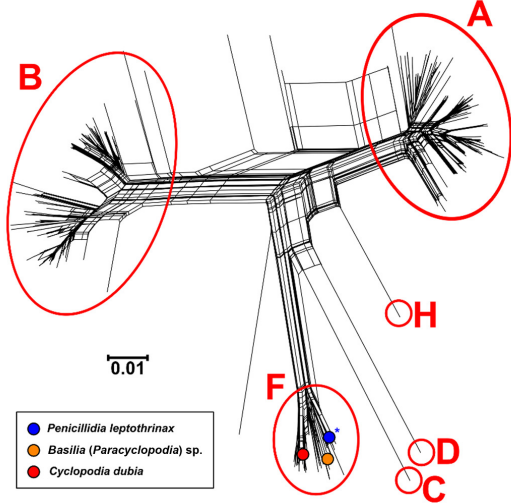
Case 1: Bat:Nycteribiid specificity



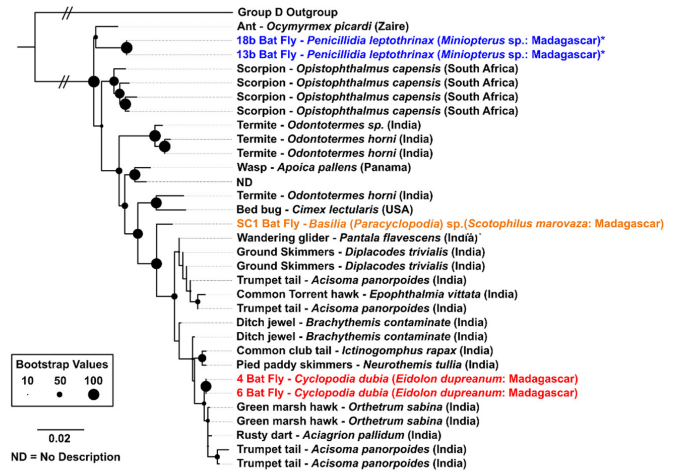
Case 2: Bat:Nycteribiid promiscuity

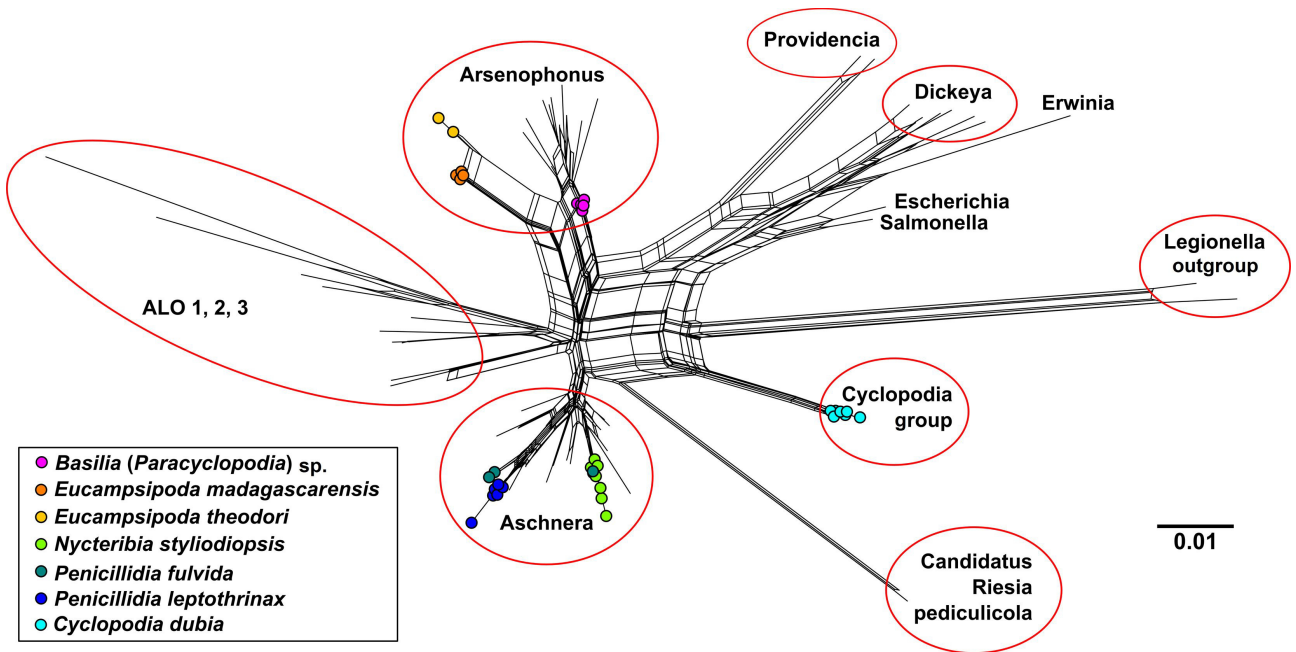


Wolbachia genus network diagram (1308 bp *fbpA:ftsZ:hcpA*)

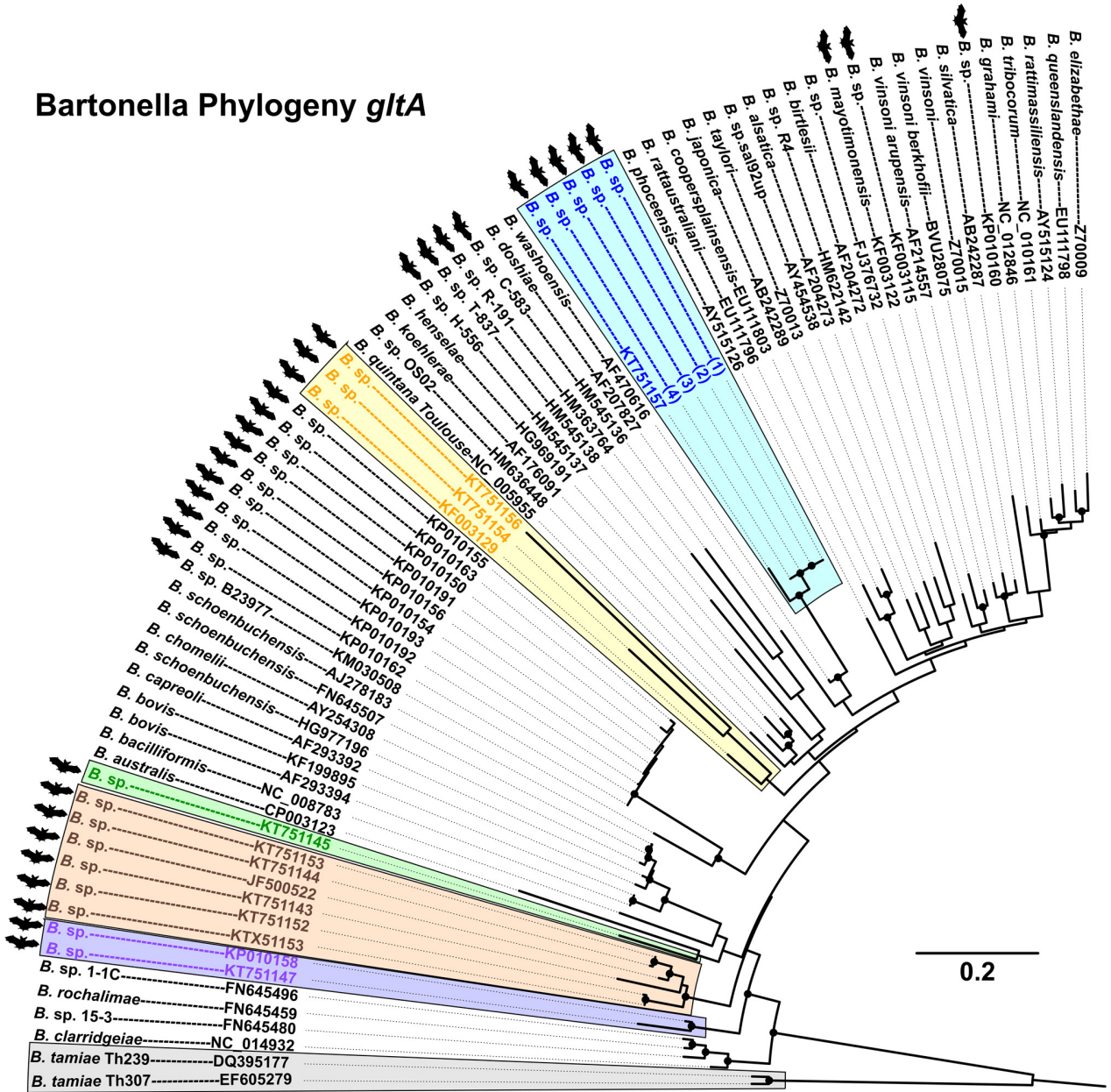


Wolbachia Group F Phylogeny (1308 bp *fbpA:ftsZ:hcpA*)





Bartonella Phylogeny *gltA*



KT751146 (1)	<i>Cy. dubia</i>	<i>Ei. dupreanum</i>
KT751148 (1)	<i>Cy. dubia</i>	<i>Ei. dupreanum</i>
KT751149 (1)	<i>Cy. dubia</i>	<i>Ei. dupreanum</i>
KT751150 (2)	<i>Cy. dubia</i>	<i>Ei. dupreanum</i>
KT751151 (2)	<i>Cy. dubia</i>	<i>Ei. dupreanum</i>
KP010152 (3)	<i>Cy. dubia</i>	<i>Ei. dupreanum</i>
KP010153 (3)	<i>Cy. dubia</i>	<i>Ei. dupreanum</i>
KP010164 (3)	<i>Cy. dubia</i>	<i>Ei. dupreanum</i>
KP010159 (4)	<i>Cy. dubia</i>	<i>Ei. dupreanum</i>
KP010151 (4)	<i>Cy. dubia</i>	<i>Ei. dupreanum</i>
KP010160 (3)	<i>Cy. dubia</i>	<i>Ei. dupreanum</i>
KT751157 (1)	<i>Basilia</i> sp.	<i>Sc. marovaza</i>

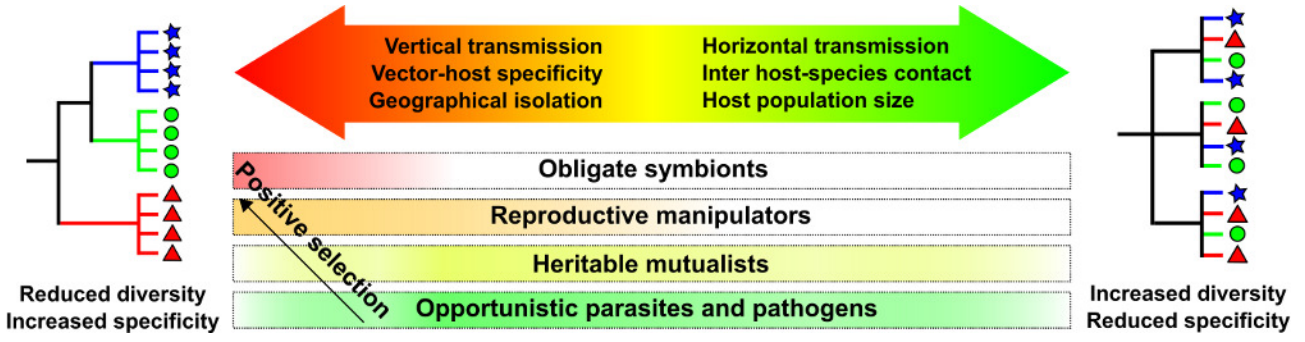
KF003129	-	<i>My. daubentonii</i>
KT751156	<i>Eu. theodori</i>	<i>R. obliviosus</i>
KT751154	<i>P. cf. fulvida</i>	<i>Mi. griveaudi</i>

KT751145	<i>P. leptothrinax</i>	<i>Mi. manavi</i>
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Outgroup		
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KT751153	<i>P. leptothrinax</i>	<i>Mi. griveaudi</i>
KT751144	<i>P. leptothrinax</i>	<i>Mi. sp.</i>
JF500522	-	<i>Mi. schreibersii</i>
KT751143	<i>P. leptothrinax</i>	<i>Mi. aelleni</i>
KT751152	<i>N. styliidiopsis</i>	<i>Mi. gleni</i>
KTX51153	<i>N. styliidiopsis</i>	<i>Mi. griveaudi</i>

KT751147	<i>Basilia</i> sp.	<i>Sc. robustus</i>
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Description	Forward Primer	Reverse Primer	Reference
16S gene Pyrosequencing	TAC GGR AGG CAG CAG	GGA CTA CCA GGG TAT CTA AT	Genoscreen
16S gene 1350bp	AGA GTT TGA TCM TGG CTC AG	TAC GGY TAC CTT GTT ACG ACT T	Personal communication
<i>Bartonella gltA</i>	GGG GAC CAG CTC ATG GTG G	AAT GCA AAA AGA ACA GTA AAC A	(23)
<i>Bartonella</i> 16S	AGA GTT TGA TCM TGG CTC AG	TAC GGY TAC CTT GTT ACG ACT T	(15)
<i>Bartonella rpoB</i>	CGC ATT GGC TTA CTT CGT ATG	GTA GAC TGA TTA GAA CGC TG	(62)
Enterobacteriales 16S	GGG TTG TAA AGT ACT TTC AGT CGT	CCT YTA TCT CTA AAG GMT TCG CTG GAT G	(51)
<i>Rickettsia gltA</i>	GGT TTT ATG ICT ACT GCT TCK TG	CAT TTC TTT CCA TIG TGC CAT C	(63)
<i>Wolbachia wsp</i>	GTC CAA TAR STG ATG ARG AAA C	CYG CAC CAA YAG YRC TRT AAA	(49)
<i>Wolbachia hcpA</i>	GAA ATA RCA GTT GCT GCA AA	GAA AGT YRA GCA AGY TCT G	(48)
<i>Wolbachia fhpA</i>	GCT GCT CCR CTT GGY WTG AT	CCR CCA GAR AAA AYY ACT ATT C	(48)
<i>Wolbachia fisZ</i>	ATY ATG GAR CAT ATA AAR GAT AG	TCR AGY AAT GGA TTR GAT AT	(48)

Table 1: Primers used in this study.

13	<i>Penicillidium leptothrix</i>	<i>Miniopterus cf. ambositrensis</i>	SMG17867/UADBA 33034	Madagascar	-	KT1751095	-	-	-	-	-	-
13b	<i>Penicillidium leptothrix</i>	<i>Miniopterus</i> sp.	SMG 17866/UADBA 33033	Madagascar	KT1751145	-	-	-	KT1751169	KT1751136	KT1751141	-
18b	<i>Penicillidium leptothrix</i>	<i>Miniopterus</i> sp.	SMG 17884/FMNH 221429	Madagascar	-	-	-	-	KT1751170	KT1751137	KT1751142	-
GR2 1	<i>Penicillidium</i> sp. (cf. <i>fulvum</i>)	<i>Miniopterus griveaudi</i>	SMG 17753/FMNH 221337	Madagascar	KT1751154	KT1751098 KT1751099	-	-	-	-	-	-
9b	<i>Penicillidium</i> sp. (cf. <i>fulvum</i>)	<i>Miniopterus</i> sp.	SMG 17878/FMNH 221423	Madagascar	-	KT1751100	-	-	-	-	-	-

Table 2: Genbank accession numbers for sequences generated as part of this study. Geographical, arthropod, and bat host information is also presented, along with museum identifier codes for the host mammal.

	<i>Wolbachia</i>	<i>Arsenophonus</i> -like-organisms	<i>Bartonella</i>
ParaFitGlobal	NA	0.087	0.060
p-value	NA	0.001	0.172

Table 3: Evolutionary congruence between Nycteribiidae and associated bacteria. Congruence testing for *Wolbachia* was not possible due to the presence of only three independent taxa.