Novel *Wolbachia* strains in *Anopheles* malaria vectors from Sub-Saharan Africa

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Abstract

Anopheles (*An.*) mosquitoes contain bacteria that can influence *Plasmodium* parasites. *Wolbachia*, a common insect endosymbiont, has historically been considered absent from *Anopheles* but has recently been found in *An. gambiae* populations. Here, we assessed a range of *Anopheles* species from five malaria-endemic countries for *Wolbachia* and *Plasmodium* infection. Strikingly, we found *Wolbachia* infections in *An. coluzzii*, *An. gambiae* s.s, *An. arabiensis*, *An. moucheti* and *An.* species 'A' increasing the number of *Anopheles* species known to be naturally infected by this endosymbiont. Molecular analysis suggests the presence of phylogenetically diverse novel strains, while qPCR and 16S rRNA sequencing indicates that *Wolbachia* is the dominant member of the microbiota in *An. moucheti* and *An.* species 'A'. We found no evidence of *Wolbachia/Asaia* co-infections, and presence of these endosymbionts did not have significant effects on malaria prevalence. We discuss the importance of novel *Wolbachia* strains in *Anopheles* and potential implications for disease control.

Introduction

Malaria is transmitted to humans through inoculation of *Plasmodium (P.)* sporozoites during the infectious bite of an infected female *Anopheles* (*An.)* mosquito. The genus *Anopheles* consists of 475 formally recognised species with ~40 vector species/species complexes responsible for the transmission of malaria at a level of public health concern [1]. During the mosquito infection cycle, *Plasmodium* parasites encounter a variety of resident microbiota both in the mosquito midgut and other tissues. Numerous studies have shown that certain species of bacteria can inhibit *Plasmodium* development [2–4]. For example, *Enterobacter* bacteria that reside in the *Anopheles* midgut can inhibit the development of *Plasmodium* parasites prior to their invasion of the midgut epithelium [5,6]. *Wolbachia* endosymbiotic bacteria are estimated to naturally infect ~40% of insect species [7] including mosquito vector species that are responsible for transmission of human diseases such as *Culex (Cx.) quinquefasciatus* [8–10] and *Aedes (Ae.) albopictus* [11,12]. Although *Wolbachia* strains have been shown to have variable effects on arboviral infections in their native mosquito hosts [13–15], transinfected *Wolbachia* strains have been considered for mosquito biocontrol strategies, due to a variety of synergistic phenotypic effects. Transinfected strains in *Ae. aegypti* and *Ae. albopictus* provide strong inhibitory effects on arboviruses, with maternal transmission and cytoplasmic incompatibility enabling introduced strains to spread through populations [16–22]. Open releases of *Wolbachia*-transinfected *Ae. aegypti* populations have demonstrated the ability of the *w*Mel *Wolbachia* strain to invade wild populations [23] and provide strong inhibitory effects on viruses from field populations [24], with releases currently occurring in arbovirus endemic countries such as Indonesia, Vietnam, Brazil and Colombia (https://www.worldmosquitoprogram.org).

The prevalence of *Wolbachia* in *Anopheles* species has not been extensively studied, with most studies focused in Asia using classical PCR-based screening, and up until 2014 there has been no evidence of resident strains in mosquitoes from this genus [25–29]. Furthermore, significant efforts to establish artificially-infected lines were, up until recently, also unsuccessful [30]. Somatic, transient infections of the *Wolbachia* strains *w*MelPop and *w*AlbB in *An. gambiae* were shown to significantly inhibit *P. falciparum* [31] but the interference phenotype is variable with other *Wolbachia* strain-parasite combinations [32–34]. A stable line was established in *An. stephensi*, a vector of malaria in southern Asia, using the *w*AlbB strain and this was also shown to confer resistance to *P. falciparum* infection [35]. One potential reason postulated for the absence of *Wolbachia* in *Anopheles* species was thought to be due to the presence of other endosymbiotic bacteria, particularly from the genus *Asaia* [36]. This acetic acid bacterium is stably associated with several *Anopheles* species and is often the dominant species in the mosquito microbiota [37]. In laboratory studies, *Asaia* has been shown to impede the vertical transmission of *Wolbachia* in *Anopheles* [36] and was shown to have a negative correlation with *Wolbachia* in mosquito reproductive tissues [38].

Recently, resident *Wolbachia* strains have been discovered in the *An. gambiae* s.l. complex, which consists of multiple morphologically indistinguishable species including several major malaria vector species. *Wolbachia* strains (collectively named *w*Anga) were found in *An. gambiae* s.l. populations in

135 Burkina Faso [39] and Mali [40], suggesting that *Wolbachia* may be more abundant in the *An.*
136 *gambiae* complex across Sub-Saharan Africa. Globally, there is a large variety of *Anopheles* vector
137 species (~70) that have the capacity to transmit malaria [41] and could potentially contain resident
138 *Wolbachia* strains. Additionally, this number of malaria vector species may be an underestimate given
139 that recent studies using molecular barcoding have also revealed a larger diversity of *Anopheles*
140 species than would have be identified using morphological identification alone [42,43].
141
142 In this study, we collected *Anopheles* mosquitoes from five malaria-endemic countries; Ghana,
143 Democratic Republic of the Congo (DRC), Guinea, Uganda and Madagascar, from 2013-2017. Wild-
144 caught adult female *Anopheles* were screened for *P. falciparum* malaria parasites, *Wolbachia* and
145 *Asaia* bacteria. In total, we analysed mosquitoes from 17 *Anopheles* species that are known malaria
146 vectors or implicated in transmission, and some unidentified species, discovering five species of
147 *Anopheles* with resident *Wolbachia* strains; *An. coluzzii* from Ghana, *An. gambiae* s.s., *An.*
148 *arabiensis, An. moucheti* and *Anopheles* species 'A' from DRC. Using *Wolbachia* gene sequencing
149 we show that the resident strains in these malaria vectors are diverse, novel strains and qPCR and
150 16S rRNA amplicon sequencing data suggests that the strains in *An. moucheti* and *An*. species 'A'
151 are higher density infections, compared to the strains found in the *An. gambiae* s.l. complex. We
152 found no evidence for either *Wolbachia-Asaia* co-infections, or for either endosymbiont having any
153 significant effect on the prevalence of malaria in wild mosquito populations.
154
155 **Results**
156
157 **Mosquito species and resident *Wolbachia* strains.** *Anopheles* species composition varied
158 depending on country and mosquito collection sites (**Table 1**). We detected *Wolbachia* in *An. coluzzii*
159 (previously named M molecular form) mosquitoes from Ghana (prevalence of 4% - termed *w*Anga-
160 Ghana) and *An. gambiae* s.s. (previously named S molecular form) from all six collection sites in DRC
161 (prevalence range of 8-24%) in addition to a single infected *An. arabiensis* from Kalemie in DRC
162 (**Figure 1, Table 1**). The molecular phylogeny of the ITS2 gene of *Anopheles gambiae* s.l. complex
163 individuals (including both *Wolbachia*-infected and uninfected individuals analysed in our study)
164 confirmed molecular species identifications made using species-specific PCR assays **(Figure 2).**
165 Novel resident *Wolbachia* infections were detected in two additional *Anopheles* species from DRC;
166 *An. moucheti* (termed *w*AnM) from Mikalayi, and *An*. species A (termed *w*AnsA) from Katana.
167 Additionally, we screened adult female mosquitoes of *An.* species A (collected as larvae and adults)
168 from Lwiro, a village near Katana in DRC, and detected *Wolbachia* in 30/33 (91%), indicating this
169 resident *w*AnsA strain has a high infection prevalence in populations in this region. The molecular
170 phylogeny of the ITS2 gene revealed *Wolbachia*-infected individuals from Lwiro and Katana are the
171 same *An.* species A (**Figure 3**) previously collected in Eastern Zambia [43] and Western Kenya [44].
172 All ITS2 sequences were deposited in GenBank (accession numbers MH598414 – MH598445)
173 **(Supplementary Table 1)**.

174 ***Wolbachia* strain typing**. Phylogenetic analysis of the 16S rRNA gene demonstrated that the 16S
175 sequences for these strains cluster with other Supergroup B strains such as *w*Pip (99-100%
176 nucleotide identity) (**Figure 4a).** When compared to the resident *Wolbachia* strains in *An. gambiae*
177 s.l. populations from Mali [40] and Burkina Faso [39], *w*Anga-Ghana is more closely related to the
178 Supergroup B strain of *w*Anga from Burkina Faso. Although a resident strain was detected in *An.*
179 *gambiae* s.s. and a single *An. arabiensis* from DRC through amplification of 16S rRNA fragments
180 using two independent PCR assays [40,45], we were unable to obtain 16S sequences of sufficient
181 quality to allow further analysis. The *Wolbachia* surface protein (wsp*)* gene has been evolving at a
182 faster rate and provides more informative strain phylogenies [46]. As expected, however, and similar
183 to *Wolbachia*-infected *An. gambiae* s.l. from Burkina Faso [39] and Mali [40], a fragment of the wsp
184 gene was not amplified from *Wolbachia*-positive samples from *An. gambiae* s.s*, An. arabiensis* and
185 *An. coluzzii*. Similarly, no wsp gene fragment amplification occurred from *w*AnM-infected *An.*
186 *moucheti*. However, wsp sequences were obtained from both *Wolbachia*-infected individuals of *An.*
187 species A from Katana. We also analysed the wsp sequences of 22 specimens of *An*. species A from
188 Lwiro (near Katana) and found identical sequences to the two individuals from Katana. Phylogenetic
189 analysis of the wsp sequences obtained for the *w*AnsA strain, for both individuals from Katana
190 (*w*AnsA wsp DRC-KAT1, *w*AnsA wsp DRC-KAT2) and three representative individuals from Lwiro
191 (*w*AnsA wsp DRC-LWI1, *w*AnsA wsp DRC-LWI2, *w*AnsA wsp DRC-LWI3) indicates *w*AnsA is most
192 closely related to *Wolbachia* strains of Supergroup B (such as *w*Pip, *w*AlbB, *w*Ma and *w*No) which is
193 consistent with 16S rRNA phylogeny. However, the improved phylogenetic resolution provided by wsp
194 indicates they cluster separately (**Figure 4b**). Typing of the *w*AnsA wsp nucleotide sequences
195 highlighted that there were no exact matches to wsp alleles currently in the *Wolbachia* MLST
196 database (https://pubmlst.org/wolbachia/) (**Table 2)**. All *Wolbachia* 16S and wsp sequences were
197 deposited into GenBank (accession numbers MH605275 – MH605285) **(Supplementary Table 2)**.

198 Multilocus sequence typing (MLST) was undertaken to provide more accurate strain phylogenies. This
199 was done for the novel *Wolbachia* strains *w*AnM and *w*AnsA in addition to the resident *w*Anga-Ghana
200 strain in *An. coluzzii* from Ghana. We were unable to amplify any of the five MLST genes from
201 *Wolbachia*-infected *An. gambiae* s.s. and *An. arabiensis* from DRC (likely due to low infection
202 densities). New alleles for all five MLST gene loci (sequences differed from those currently present in
203 the MLST database) confirm the diversity of these novel *Wolbachia* strains (**Table 2**). The phylogeny
204 of these three novel strains based on concatenated sequences of all five MLST gene loci confirms
205 they cluster within Supergroup B (**Figure 5a**). This also demonstrates the novelty as comparison with
206 a wide range of strains (including all isolates highlighted through partial matching during typing of
207 each locus) shows these strains are distinct from currently available sequences (**Figure 5a, Table 2**).
208 The concatenated phylogeny indicates that *w*AnM is most closely related to a Hemiptera strain:
209 Isolate number 1616 found in *Bemisia tabaci* in Uganda, and a Coleoptera strain: Isolate number 20
210 found in *Tribolium confusum*. Concatenation of the MLST loci also indicates *w*AnsA is closest to a
211 group containing various Lepidoptera and Hymenoptera strains from multiple countries in Asia,
212 Europe and America, as well as two mosquito strains: Isolate numbers 1830 and 1831, found in

213 *Aedes cinereus* and *Coquillettidia richiardii* in Russia. This highlights the lack of concordance
214 between *Wolbachia* strain phylogeny and their insect hosts across diverse geographical regions. We
215 also found evidence of potential strain variants in *w*AnsA through variable MLST gene fragment
216 amplification and resulting closest-match allele numbers. A second *w*AnsA-infected sample, *An. sp.*
217 A/1 (W+) DRC-KAT2, only amplified hcpA and coxA gene fragments and although identical
218 sequences were obtained for wsp **(Figure 4b)** and hcpA, genetic diversity was seen in the coxA
219 sequences, with typing revealing a different, but still novel allele for the coxA sequence from this
220 individual (*w*AnsA(2) coxA DRC-KAT2) **(Figure 5b)**. MLST gene fragment amplification was also
221 variable for *w*Anga-Ghana-infected *An. coluzzii,* requiring two individuals to generate the five MLST
222 gene sequences, and for the hcpA locus, more degenerate primers (hcpA_F3/hcpA_R3) were
223 required to generate sequence of sufficient quality for analysis. This is likely due to the low density of
224 this strain potentially influencing the ability to successfully amplify all MLST genes, in addition to the
225 possibility of genetic variation in primer binding regions. Despite the sequences generated for this
226 strain producing exact matches with alleles in the database for each of the five gene loci, the resultant
227 allelic profile, and therefore strain type, did not produce a match, showing this *w*Anga-Ghana strain is
228 also a novel strain type. The closest matches to the *w*Anga-Ghana allelic profile were with strains
229 from two Lepidopteran species: Isolate number 609 found in *Fabriciana adippe* from Russia, and
230 Isolate number 658 found in *Pammene fasciana* from Greece, but each of these only produced a
231 match for 3 out of the 5 loci. The concatenated phylogeny for this strain **(Figure 5a)** indicates that
232 across the 5 MLST loci, *w*Anga-Ghana is actually most closely related to a Lepidopteran strain found
233 in *Thersamonia thersamon* in Russia (Isolate number 132). The phylogeny of *Wolbachia* strains
234 based on the coxA gene (**Figure 5b)** highlights the genetic diversity of both the *w*AnsA strain variants
235 and also *w*Anga-Ghana compared to the *w*Anga-Mali strain [40]; coxA gene sequences are not
236 available for *w*Anga strains from Burkina Faso [39]. All *Wolbachia* MLST sequences were deposited
237 into GenBank (accession numbers MH605286 – MH605305) **(Supplementary Table 3)**.
238
239 **Resident strain densities and relative abundance**. The relative densities of *Wolbachia* strains were
240 estimated using qPCR targeting the ftsZ [47] and 16S rRNA [40] genes. ftsZ and 16S rRNA qPCR
241 analysis indicated the amount of *Wolbachia* detected in *w*AnsA-infected and *w*AnM-infected females
242 was approximately 1000-fold higher (Ct values 20-22) than *Wolbachia*-infected *An. gambiae s.s., An.*
243 *arabiensis* and *w*Anga-Ghana-infected *An. coluzzii* (Ct values 30-33). To account for variation in
244 mosquito body size and DNA extraction efficiency, we compared the total amount of DNA for
245 *Wolbachia*-infected mosquito extracts and conversely, we found less total DNA in the *w*AnsA-infected
246 extract (1.36 ng/µL) and the *An. moucheti (w*AnM-infected) extract (5.85 ng/µL) compared to the
247 mean of 6.64 +/- 2.33 ng/µL for *w*Anga-Ghana-infected *An. coluzzii*. To estimate the relative
248 abundance of resident *Wolbachia* strains in comparison to other bacterial species, we sequenced the
249 bacterial microbiome using 16S rRNA amplicon sequencing on *Wolbachia*-infected individuals. We
250 found *w*AnsA, *w*AnsA(2) and *w*AnM *Wolbachia* strains were the dominant operational taxonomic units
251 (OTUs) of these mosquito species (**Figure 6**). In contrast, the lower density infection *w*Anga-Ghana
252 strain represented only ~10% of the OTUs within the microbiome.

253 ***P. falciparum, Wolbachia* and *Asaia* prevalence.** The prevalence of *P. falciparum* in female
254 mosquitoes was extremely variable across countries and collection locations (**Figure 1, Table 1**) with
255 very high prevalence recorded in *An. gambiae* s.s. from villages close to Boke (52%) and Faranah
256 (44%) in Guinea. Despite the collection of other *Anopheles* species in Guinea, *An. gambiae* s.s. was
257 the only species to have detectable malaria infections. In contrast, malaria was detected in multiple
258 major vector species from DRC, including *An. gambiae* s.s, *An. arabiensis* and *An. funestus* s.s. A
259 high prevalence of *P. falciparum* was also detected in *An. gambiae* s.s. from Uganda for both
260 collection years; 19% for 2013 and 36% for 2014. In contrast, no *P. falciparum* infections were
261 detected in any of the *An. coluzzii or An. melas* collected in Ghana. In Madagascar, *P. falciparum*
262 was detected in only two species; *An. gambiae s.s.* and *An. rufipes*. We compared the overall *P.*
263 *falciparum* infection rates in *An. gambiae* s.s. mosquitoes collected across all locations from DRC to
264 determine if there was any correlation with the presence of the low density *w*Anga-DRC *Wolbachia*
265 resident strain. Overall, of the 128 mosquitoes collected, only 1.56% (n=2) had detectable *Wolbachia*-
266 *Plasmodium* co-infections compared to 10.16% (n=13) where we only detected *Wolbachia*. A further
267 11.72% (n=15) were only PCR-positive for *P. falciparum*. As expected, for the vast majority of
268 mosquitoes (76.56%, n=98) we found no evidence of *Wolbachia* or *P. falciparum* present, resulting in
269 no correlation across all samples (Fisher's exact *post hoc* test on unnormalized data, two-tailed,
270 *P*=0.999). Interestingly, one *An.* species 'A' female from Katana was infected with *P. falciparum*.
271
272 For all *Wolbachia*-infected females collected in our study (including *An. coluzzii* from Ghana and novel
273 resident strains in *An. moucheti* and *An. species* A), we did not detect the presence of *Asaia*. No
274 resident *Wolbachia* strain infections were detected in *Anopheles* mosquitoes from Guinea, Uganda or
275 Madagascar. However, high *Asaia* and malaria prevalence rates were present in *Anopheles*
276 mosquitoes from Uganda and Guinea (including multiple species in all four sites in Guinea). We
277 compared the overall *P. falciparum* infection rates in *An. gambiae* s.s. collected across all locations
278 from Guinea, with and without *Asaia* bacteria, and found no overall correlation (Fisher's exact *post*
279 *hoc* test on unnormalized data, two-tailed, *P*=0.4902). There was also no overall correlation between
280 *Asaia* and *P. falciparum* infections in *An. gambiae* s.s. from Uganda for both 2013 (Fisher's exact *post*
281 *hoc* test on unnormalized data, two-tailed, *P*=0.601) and 2014 (Fisher's exact *post hoc* test on
282 unnormalized data, two-tailed, *P*=0.282).
283
284 *Asaia* can be environmentally acquired at all life stages but can also have the potential to be vertically
285 and horizontally transmitted between individual mosquitoes. Therefore, we performed 16S
286 microbiome analysis on a sub-sample of *Asaia*-infected *An. gambiae* s.s. from Kissidougou (Guinea),
287 a location in which high levels of *Asaia* were detected by qPCR (mean *Asaia* Ct = 17.84 +/- 2.27).
288 *Asaia* in these individuals is the dominant bacterial species present (**Figure 7a)** but in Uganda we
289 detected much lower levels of *Asaia* (qPCR mean Ct = 33.33 +/- 0.19) and this was reflected in *Asaia*
290 not being a dominant species (**Figure 7b**). The alpha and beta diversity of *An. gambiae* s.s. from
291 Kissidougou, Guinea and Butemba, Uganda shows much more overall diversity in the microbiome for
292 Uganda individuals (**supplementary figure S1**). Interestingly, 2/5 of these individuals from

293 Kissidougou (Guinea) were *P. falciparum*-infected compared to 3/5 individuals from Uganda. To
294 determine if the presence of *Asaia* had a quantifiable effect on the level of *P. falciparum* detected, we
295 normalized *P. falciparum* Ct values from qPCR (**supplementary figure S2a**) and compared gene
296 ratios for *An. gambiae s.s*. mosquitoes from Guinea, with or without *Asaia* (**supplementary figure**
297 **S2b**). Statistical analysis using student's t-tests revealed no significant difference between
298 normalized *P. falciparum* gene ratios (p= 0.51, df =59). Larger variation of Ct values was seen for
299 *Asaia* (**supplementary figure S2c**) suggesting the bacterial densities in individual mosquitoes were
300 more variable than *P. falciparum* parasite infection levels.
301
302 **Discussion**
303 Malaria transmission in Sub-Saharan Africa is highly dependent on the local *Anopheles* vector
304 species but the primary vector complexes recognised are *An. gambiae* s.l.*, An. funestus* s.l. *An. nili*
305 s.l. and *An. moucheti* s.l. [41,48]. *An. gambiae* s.s. and *An. coluzzii* sibling species are considered
306 the most important malaria vectors in Sub-Saharan Africa and recent studies indicate that *An. coluzzii*
307 extends further north, and closer to the coast than *An. gambiae s.s.* within west Africa [49]. In our
308 study, high malaria prevalence rates in *An. gambiae* s.s. across Guinea would be consistent with high
309 malaria parasite prevalence (measured by rapid diagnostic tests) in Guéckédou prefecture, and the
310 overall national malaria prevalence estimated to be 44% in 2013 [50]. However, malaria prevalence
311 has decreased in the past few years with an overall prevalence across Guinea estimated at 15% for
312 2016. Although our *P. falciparum* infection prevalence rates were also high in DRC, recent studies
313 have shown comparable levels of infection with 35% of *An. gambiae* s.l. mosquitoes infected from
314 Kinshasa [51]. We detected *P. falciparum* in *An. gambiae* s.s, *An. arabiensis*, *An. funestus* s.s. and
315 *An*. species A from DRC. Morphological differences have been widely used for identification of
316 malaria vectors but species complexes (such as *An. gambiae* s.l. and *An. funestus* s.l.) require
317 species-diagnostic PCR assays. Historically, malaria entomology studies in Africa have focused
318 predominantly on species from these complexes, likely due to the fact that mosquitoes from these
319 complexes dominate the collections [43]. In our study, we used ITS2 sequencing to confirm
320 secondary vector species that were *P. falciparum*-infected given the difficulties of morphological
321 identification and recent studies demonstrating the inaccuracy of diagnostic species PCR-based
322 molecular identification [52]. Our study is the first to report the detection of *P. falciparum* in *An.*
323 *rufipes* from Madagascar; previously this species was considered a vector of *Plasmodium* species of
324 non-human origin and has only very recently been implicated in human malaria transmission [53].
325 However, detection of *P. falciparum* parasites in whole body mosquitoes does not confirm that the
326 species plays a significant role in transmission. Detection could represent infected bloodmeal stages
327 or oocysts present in the midgut wall so further studies are warranted to determine this species ability
328 to transmit human malaria parasites.
329
330 The mosquito microbiota can modulate the mosquito immune response and bacteria present in wild
331 *Anopheles* populations can influence malaria vector competence [4,5]. Endosymbiotic *Wolbachia*
332 bacteria are particularly widespread through insect populations but they were commonly thought to be

absent from *Anopheles* mosquitoes. However, the recent discovery of *Wolbachia* strains in the *An. gambiae* s.l. complex in Burkina Faso and Mali [39,40] in addition to our study showing infection in *Anopheles* from Ghana and DRC, suggest resident strains could be widespread across Sub-Saharan Africa. The discovery of resident strains in Burkina Faso resulted from sequencing of the 16S rRNA gene identifying *Wolbachia* sequences rather than screening using *Wolbachia*-specific genes [39]. Intriguingly, *Wolbachia* infections in these mosquitoes could not be detected using conventional PCR targeting the wsp gene. As the wsp gene has often been used in previous studies to detect strains in *Anopheles* species [25,27], this could explain why resident strains in the *An. gambiae* s.l. complex have gone undetected until very recently. Recent similar methods using 16S rRNA amplicon sequencing to determine the overall microbiota in wild mosquito populations has provided evidence for *Wolbachia* infections in *An. gambiae* in additional villages in Burkina Faso [54] and *Anopheles* species collected in Illinois, USA [55]. Our study describing resident *Wolbachia* strains in numerous species of *Anopheles* malaria vectors also highlights the potential for *Wolbachia* to be influencing malaria transmission, as postulated by previous studies [39,40,56]. Although no significant correlation was present for malaria and *Wolbachia* prevalence in the 128 *An. gambiae* s.s. individuals from DRC, we only detected co-infections in two individuals compared to 13 and 15 individuals infected only with *Wolbachia* or *P. falciparum* respectively. As the majority (77%) of samples had neither detectable *Wolbachia* resident strains or *P. falciparum,* a larger sample size would be needed to determine if there is a correlation, as shown previously in both Burkina Faso [56] and Mali [40]. The infection prevalence of resident *Wolbachia* strains in *An. coluzzii* from Ghana (4%) and *An. gambiae* s.s. from the DRC was variable but low (8-24%), consistent with infection prevalence in Burkina Faso (11%) [39] but much lower than those reported in Mali (60-80%) [40] where infection was associated with reduced prevalence and intensity of sporozoite infection in field-collected females.

The discovery of a resident *Wolbachia* strain in *An. moucheti*, a highly anthropophilic and efficient malaria vector found in the forested areas of western and central Africa [41], suggests further studies are warranted that utilize large sample sizes to examine the influence of the *w*AnM *Wolbachia* strain on *Plasmodium* infection dynamics in this malaria vector. *An. moucheti* is often the most abundant vector, breeding in slow moving streams and rivers, contributing to year round malaria transmission in these regions [57,58]. This species has also been implicated as a main bridge vector species in the transmission of ape *Plasmodium* malaria in Gabon [59]. There is thought to be high genetic diversity in *An. moucheti* populations [60,61] which may either influence the prevalence of *Wolbachia* resident strains or *Wolbachia* could be contributing to genetic diversity through its effect on host reproduction. A novel *Wolbachia* strain in *An*. species 'A', present at high infection frequencies in Lwiro (close to Katana in DRC), also suggests more *Anopheles* species, including unidentified and potentially new species, could be infected with this widespread endosymbiotic bacterium. *An.* species A should be further investigated to determine if this species is a potential malaria vector given our study demonstrated *P. falciparum* infection in one of two individuals screened and ELISA-positive samples of this species were reported from the Western Highlands of Kenya [62].

373 The variability of *Wolbachia* prevalence rates in *An. gambiae* s.l. complex from locations within DRC
374 and Ghana and previous studies in Burkina Faso [39] and Mali [40] suggest the environment is one
375 factor that influences the presence or absence of resident strains. In our study we found no evidence
376 of *Wolbachia-Asaia* co-infections across all countries, supporting laboratory studies that have shown
377 these two bacterial endosymbionts demonstrate competitive exclusion in *Anopheles* species [36,38].
378 We also found that *Asaia* infection densities (whole body mosquitoes) were variable and location
379 dependent which would correlate with this bacterium being environmentally acquired at all life stages,
380 but also having the potential for both vertical and horizontal transmission [37]. Significant variations in
381 overall *Asaia* prevalence and density across different *Anopheles* species and locations in our study
382 would also correlate with our data indicating no evidence of an association with *P. falciparum*
383 prevalence in both Guinea and Uganda populations. Further studies are needed to determine the
384 complex interaction between these two bacterial endosymbionts and malaria in diverse *Anopheles*
385 malaria vector species. Horizontal transfer of *Wolbachia* strains between species (even over large
386 phylogenetic differences) has shaped the evolutionary history of this endosymbiont in insects and
387 there is evidence for loss of infection in host lineages over evolutionary time [63]. Our results showing
388 a new strain present in *An. coluzzii* from Ghana (phylogenetically different to strains present in *An.*
389 *gambiae* s.l. mosquitoes from both Burkina Faso and Mali), strain variants observed in An. species A,
390 and the concatenated grouping of the novel *Anopheles* strains with strains found in different Orders of
391 insects, support the lack of congruence between insect host and *Wolbachia* phylogenetic trees [64].
392
393 Our qPCR and 16S microbiome analysis indicates the densities of *w*AnM and *w*AnsA strains are
394 significantly higher than resident *Wolbachia* strains in *An. gambiae* s.l. However, caution must be
395 taken as we were only able to analyse selected individuals and larger collections of wild populations
396 would be required to confirm these results. Native *Wolbachia* strains dominating the microbiome of
397 *An. species* A and *An. moucheti* is consistent with other studies of resident strains in mosquitoes
398 showing *Wolbachia* 16S rRNA gene amplicons vastly outnumber sequences from other bacteria in
399 *Ae. albopictus* and *Cx. quinquefasciatus* [65,66]. The discovery of novel *Wolbachia* strains provides
400 the rationale to undertake vector competence experiments to determine what effect these strains are
401 having on malaria transmission. The tissue tropism of novel *Wolbachia* strains in malaria vectors will
402 be particularly important to characterise given this will determine if these endosymbiotic bacteria are
403 proximal to malaria parasites within the mosquito. It would also be important to determine the
404 additional phenotypic effects novel resident *Wolbachia* strains have on their mosquito hosts. Some
405 *Wolbachia* strains induce a reproductive phenotype termed cytoplasmic incompatibility (CI) that
406 results in inviable offspring when an uninfected female mates with a *Wolbachia*-infected male. In
407 contrast, *Wolbachia*-infected females produce viable progeny when they mate with both infected and
408 uninfected male mosquitoes. This reproductive advantage over uninfected females allows *Wolbachia*
409 to spread within mosquito populations.
410
411 *Wolbachia* has been the focus of recent biocontrol strategies in which *Wolbachia* strains transferred
412 into naïve mosquito species provide strong inhibitory effects on arboviruses [19,20,67–70] and
413 malaria parasites [31,35]. The discovery of two novel *Wolbachia* strains in *Anopheles* mosquitoes,

414 potentially present at much higher density than resident strains in the *An. gambiae* s.l. complex, also
415 suggests the potential for these strains to be transinfected into other *Anopheles* species to produce
416 inhibitory effects on *Plasmodium* parasites. *Wolbachia* transinfection success is partly attributed to
417 the relatedness of donor and recipient host so the transfer of high density *Wolbachia* strains between
418 *Anopheles* species may result in stable infections (or co-infections) that have strong inhibitory effects
419 on *Plasmodium* development. Finally, if the resident strain present in *An. moucheti* is at low infection
420 frequencies in wild populations, an alternative strategy known as the incompatible insect technique
421 (IIT) could be implemented where *Wolbachia*-infected males are released to suppress the wild
422 populations through CI (reviewed by [22]). In summary, the important discovery of diverse novel
423 *Wolbachia* strains in *Anopheles* species will help our understanding of how *Wolbachia* strains can
424 potentially impact malaria transmission, through natural associations or being used as candidate
425 strains for transinfection to create stable infections in other species.
426
427 **Materials and Methods.**
428
429 ***Study sites & collection methods.*** *Anopheles* adult mosquitoes were collected from five malaria
430 endemic countries in Sub-Saharan Africa; Guinea, Democratic Republic of the Congo (DRC), Ghana,
431 Uganda and Madagascar between 2013 and 2017 (**Figure 1**). Human landing catches, CDC light
432 traps and pyrethrum spray catches were undertaken between April 2014 – February 2015 in 10
433 villages near four cities in Guinea; Foulayah (10.144633, -10.749717) and Balayani (10.1325, -
434 10.7443) near Faranah; Djoumaya (10.836317, -14.2481) and Kaboye Amaraya (10.93435, -
435 14.36995) near Boke; Tongbekoro (9.294295, -10.147953), Keredou (9.208919, -10.069525), and
436 Gbangbadou (9.274363, -9.998639) near Kissidougou; and Makonon (10.291124, -9.363358),
437 Balandou (10.407669, -9.219096), and Dalabani (10.463692, -9.451904) near Kankan. Human
438 landing catches and pyrethrum spray catches were undertaken between January – September 2015
439 in seven sites of the DRC; Kinshasa (-4.415881, 15.412188), Mikalayi (-6.024184, 22.318251),
440 Kisangani (0.516350, 25.221176), Katana (-2.225129, 28.831604), Kalemie (-5.919054, 29.186572),
441 and Kapolowe (-10.939802, 26.952970). We also analysed a subset from collections obtained from
442 Lwiro (-2.244097, 28.815232), a village near Katana, collected between in September – October
443 2015. A combination of CDC light traps, pyrethrum spray catches and human landing catches were
444 undertaken in Butemba, Kyankwanzi District in mid-western Uganda (1.1068444, 31.5910085) in
445 August and September 2013 and June 2014. CDC light trap catches were undertaken in May 2017 in
446 Dogo in Ada, Greater Accra, Ghana (5.874861111, 0.560611111). In Madagascar, sampling was
447 undertaken in June 2016 at four sites: Anivorano Nord, located in the Northern domain, (-
448 12.7645000, 49.2386944), Ambomiharina, Western domain, (-16.3672778, 46.9928889), Antafia,
449 Western domain, (-17.0271667, 46.7671389) and Ambohimarina, Central domain, (-
450 18.3329444, 47.1092500). Trapping consisted of CDC light traps and a net trap baited with Zebu
451 (local species of cattle) to attract zoophilic species [71].
452
453

454 ***DNA extraction and species identification.*** DNA was extracted from individual whole mosquitoes or
455 abdomens using QIAGEN DNeasy Blood and Tissue Kits according to manufacturer's instructions.
456 DNA extracts were eluted in a final volume of 100 µL and stored at –20°C. Species identification was
457 initially undertaken using morphological keys followed by diagnostic species-specific PCR assays to
458 distinguish between the morphologically indistinguishable sibling mosquito species of the *An.*
459 *gambiae* [72–74] and *An. funestus* complexes [75]. To determine species identification for samples of
460 interest and samples that could not be identified by species-specific PCR, Sanger sequences were
461 generated from ITS2 PCR products [76].
462
463 ***Detection of P. falciparum and Asaia.*** Detection of *P. falciparum* malaria was undertaken using
464 qPCR targeting an 120-bp sequence of the *P. falciparum* cytochrome c oxidase subunit 1 (Cox1)
465 mitochondrial gene [77] as preliminary trials revealed this was the optimal method for both sensitivity
466 and specificity. Positive controls from gDNA extracted from a cultured *P. falciparum*-infected blood
467 sample (parasitaemia of ~10%) were serially diluted to determine the threshold limit of detection, in
468 addition to the inclusion no template controls (NTCs). *Asaia* detection was undertaken targeting the
469 16S rRNA gene [78,79]. Ct values for both *P. falciparum* and *Asaia* assays in selected *An. gambiae*
470 extracts were normalized to Ct values for a single copy *An. gambiae* rps17 housekeeping gene
471 (accession no. AGAP004887 on www.vectorbase.org) [80,81]. As Ct values are inversely related to
472 the amount of amplified DNA, a higher target gene Ct: host gene Ct ratio represented a lower
473 estimated infection level. qPCR reactions were prepared using 5 µL of FastStart SYBR Green Master
474 mix (Roche Diagnostics), a final concentration of 1µM of each primer, 1 µL of PCR grade water and 2
475 µL template DNA, to a final reaction volume of 10 µL. Prepared reactions were run on a Roche
476 LightCycler® 96 System and amplification was followed by a dissociation curve (95˚C for 10 seconds,
477 65˚C for 60 seconds and 97˚C for 1 second) to ensure the correct target sequence was being
478 amplified. PCR results were analysed using the LightCycler® 96 software (Roche Diagnostics). A
479 sub-selection of PCR products from each assay was sequenced to confirm correct amplification of the
480 target gene fragment.
481
482 ***Wolbachia* detection.** *Wolbachia* detection was first undertaken targeting three conserved *Wolbachia*
483 genes previously shown to amplify a wide diversity of strains; 16S rDNA gene [40,45], *Wolbachia*
484 surface protein (wsp) gene [46] and FtsZ cell cycle gene [82]. DNA extracted from a *Drosophila*
485 *melanogaster* fly (infected with the *w*Mel strain of *Wolbachia*) was used a positive control, in addition
486 to no template controls (NTCs). 16S rDNA [45] and wsp [46] gene PCR reactions were carried out in
487 a Bio-Rad T100 Thermal Cycler using standard cycling conditions and PCR products were separated
488 and visualised using 2% E-Gel EX agarose gels (Invitrogen) with SYBR safe and an Invitrogen E-Gel
489 iBase Real-Time Transilluminator. FtsZ [47] and *16S* rDNA [40] gene real time PCR reactions were
490 prepared using 5 µL of FastStart SYBR Green Master mix (Roche Diagnostics), a final concentration
491 of 1µM of each primer, 1 µL of PCR grade water and 2 µL template DNA, to a final reaction volume of
492 10 µL. Prepared reactions were run on a Roche LightCycler® 96 System for 15 minutes at 95˚C,
493 followed by 40 cycles of 95˚C for 15 seconds and 58˚C for 30 seconds. Amplification was followed by

494 a dissociation curve (95˚C for 10 seconds, 65˚C for 60 seconds and 97˚C for 1 second) to ensure the
495 correct target sequence was being amplified. PCR results were analysed using the LightCycler® 96
496 software (Roche Diagnostics). To estimate *Wolbachia* densities across multiple *Anopheles* mosquito
497 species, *ftsZ* and 16S qPCR Ct values were compared to total dsDNA extracted measured using an
498 Invitrogen Qubit 4 fluorometer. A serial dilution series of a known *Wolbachia*-infected mosquito DNA
499 extract was used to correlate Ct values and amount of amplified target product.

500 ***Wolbachia* MLST.** Multilocus sequence typing (MLST) was undertaken to characterize *Wolbachia*
501 strains using the sequences of five conserved genes as molecular markers to genotype each strain.
502 In brief, 450-500 base pair fragments of the gatB, coxA, hcpA, ftsZ and fbpA *Wolbachia* genes were
503 amplified from individual *Wolbachia*-infected mosquitoes using previously optimised protocols [83]. A
504 *Cx. pipiens* gDNA extraction (previously shown to be infected with the *w*Pip strain of *Wolbachia*) was
505 used a positive control for each PCR run, in addition to no template controls (NTCs). If no
506 amplification was detected using standard primers, further PCR analysis was undertaken using
507 degenerate primers [83]. PCR products were separated and visualised using 2% E-Gel EX agarose
508 gels (Invitrogen) with SYBR safe and an Invitrogen E-Gel iBase Real-Time Transilluminator. PCR
509 products were submitted to Source BioScience (Source BioScience Plc, Nottingham, UK) for PCR
510 reaction clean-up, followed by Sanger sequencing to generate both forward and reverse reads.
511 Sequencing analysis was carried out in MEGA7 [84] as follows. Both chromatograms (forward and
512 reverse traces) from each sample was manually checked, edited, and trimmed as required, followed
513 by alignment by ClustalW and checking to produce consensus sequences. Consensus sequences
514 were used to perform nucleotide BLAST (NCBI) database queries, and searches against the
515 *Wolbachia* MLST database (http://pubmlst.org/wolbachia) [85]. If a sequence produced an exact
516 match in the MLST database we assigned the appropriate allele number, otherwise the closest
517 matches and number of differences were noted. The Sanger sequencing traces from the wsp gene
518 were also treated in the same way and analysed alongside the MLST gene locus scheme, as an
519 additional marker for strain typing

520 **Phylogenetic analysis.** Alignments were constructed in MEGA7 by ClustalW to include all relevant
521 and available sequences highlighted through searches on the BLAST and *Wolbachia* MLST
522 databases. Maximum Likelihood phylogenetic trees were constructed from Sanger sequences as
523 follows**.** The evolutionary history was inferred by using the Maximum Likelihood method based on the
524 Tamura-Nei model [86]. The tree with the highest log likelihood in each case is shown. The
525 percentage of trees in which the associated taxa clustered together is shown next to the branches.
526 Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and
527 BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite
528 Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The
529 trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Codon
530 positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data
531 were eliminated. The phylogeny test was by Bootstrap method with 1000 replications. Evolutionary
532 analyses were conducted in MEGA7 [84].

533 **Microbiome Analysis**. The microbiomes of selected individual *Anopheles* were analysed using
534 barcoded high-throughput amplicon sequencing of the bacterial 16S rRNA gene. Sequencing
535 libraries for each isolate were generated using universal 16S rRNA V3-V4 region primers [87] in
536 accordance with Illumina 16S rRNA metagenomic sequencing library protocols. The samples were
537 barcoded for multiplexing using Nextera XT Index Kit v2. Sequencing was performed on an Illumina
538 MiSeq instrument using a MiSeq Reagent Kit v2 (500-cycles). Quality control and taxonomical
539 assignment of the resultant reads were performed using CLC Genomics Workbench 8.0.1 Microbial
540 Genomics Module (http://www.clcbio.com). Low quality reads containing nucleotides with quality
541 threshold below 0.05 (using the modified Richard Mott algorithm), as well as reads with two or more
542 unknown nucleotides were removed from analysis. Additionally reads were trimmed to remove
543 sequenced Nextera adapters. Reference based OTU picking was performed using the SILVA SSU
544 v128 97% database [88]. Sequences present in more than one copy but not clustered to the database
545 were then placed into de novo OTUs (97% similarity) and aligned against the reference database with
546 80% similarity threshold to assign the "closest" taxonomical name where possible. Chimeras were
547 removed from the dataset if the absolute crossover cost was 3 using a k-mer size of 6. Alpha diversity
548 was measured using Shannon entropy (OTU level).

549 ***Statistical analysis.*** Fisher's exact *post hoc* test in Graphpad Prism 7 was used to compare infection
550 rates. Normalised qPCR Ct ratios were compared using unpaired t-tests in GraphPad Prism 7.
551
552 **Authors' contributions.** MK, JO, JB, EH, MLT, FNR, KK, DC, YB, FW, EZM, YAA, ARM, TAA
553 performed field collections. CLJ, GGL, MK, JO, KS, EH & TW performed sample analysis. CLJ
554 performed sequence analysis. GG, SH, KK, MP, YF and GLH performed 16S microbiome sample
555 analysis. SRI, GLH and TW provided overall supervision. CLJ and TW wrote the initial draft.
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573
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873 **Figure legends**

874 **Figure 1**. **Locations of *Anopheles* species collections (including *Wolbachia*-infected species)**
875 **and *P. falciparum* malaria prevalence rates in mosquitoes (across all species for each**
876 **location). A)** Overall map showing the five malaria-endemic countries where mosquito collections
877 were undertaken. **B)** High malaria prevalence rates in Guinea, and *Wolbachia*-infected *An. coluzzii*
878 from Ghana (no *P. falciparum* detected). **C)** *Wolbachia* strains in *An. gambiae* s.s., *An. arabiensis*, *An.*
879 *species A* and *An. moucheti* from DRC and variable *P. falciparum* prevalence rate in DRC and
880 Uganda. **D)** Low *P. falciparum* infection rates in Madagascar and no evidence of resident *Wolbachia*
881 strains. (*W*+; *Wolbachia* detected in this species).
882

883 **Figure 2**. **Maximum Likelihood molecular phylogenetic analysis of *Anopheles gambiae***
884 ***complex* ITS2 sequences from field-collected mosquitoes**. The tree with the highest log likelihood
885 (-785.65) is shown. The tree is drawn to scale, with branch lengths measured in the number of
886 substitutions per site. The analysis involved 42 nucleotide sequences. There were a total of 475
887 positions in the final dataset. DRC = Democratic Republic of the Congo (red): KAL = Kalemie, MIK =
888 Mikalayi, KIN = Kinshasa, KAT = Katana. GHA = Ghana (blue): DOG = Dogo. GUI = Guinea (green):
889 KSK = Kissidougou. MAD = Madagascar (purple): ANT = Antafia. UGA = Uganda (maroon): BUT =
890 Butemba. (W+; individual was *Wolbachia* positive, W-; individual was *Wolbachia* negative).
891

892 **Figure 3. Maximum Likelihood molecular phylogenetic analysis of *Anopheles* ITS2 sequences**
893 **from field-collected mosquitoes outside of the *An. gambiae* s.l. complex**. The tree with the
894 highest log likelihood (-3084.12) is shown. The tree is drawn to scale, with branch lengths measured
895 in the number of substitutions per site. The analysis involved 118 nucleotide sequences. There were a
896 total of 156 positions in the final dataset. DRC = Democratic Republic of the Congo (red): KAT =
897 Katana, LWI = Lwiro, MIK = Mikalayi. GUI = Guinea (green): FAR = Faranah, KAN = Kankan, KSK =
898 Kissidougou. MAD = Madagascar (purple): AMB = Ambomiharina. (W+; individual was *Wolbachia*
899 positive, W-; individual was *Wolbachia* negative).
900

901 **Figure 4**. **Resident *Wolbachia* strain phylogenetic analysis using 16S rRNA and wsp genes. A)**
902 Maximum Likelihood molecular phylogenetic analysis of the 16S rRNA gene for resident strains in *An.*
903 *coluzzii* (*w*Anga-Ghana; blue), *An. moucheti* (*w*AnM; green) and *An. species A* (*w*AnsA; red). The tree
904 with the highest log likelihood (-660.03) is shown. The tree is drawn to scale, with branch lengths
905 measured in the number of substitutions per site. The analysis involved 17 nucleotide sequences.
906 There were a total of 333 positions in the final dataset. Accession numbers of additional sequences
907 obtained from GenBank are shown, including *w*Pip (navy blue), *w*Anga-Mali (purple) and *w*Anga-
908 Burkina Faso strains (maroon). **B)** Maximum Likelihood molecular phylogenetic analysis of the wsp
909 gene for *w*AnsA-infected representative individuals from the DRC (red). (KAT = Katana, LWI = Lwiro.)
910 The tree with the highest log likelihood (-3663.41) is shown. The tree is drawn to scale, with branch
911 lengths measured in the number of substitutions per site. The analysis involved 83 nucleotide
912 sequences. There were a total of 443 positions in the final dataset. Reference numbers of additional

913 sequences obtained from the MLST database (IsoN = Isolate number) or GenBank (accession
914 number) are shown. Strains isolated from mosquitoes are highlighted in navy blue.
915
916 **Figure 5**. ***Wolbachia* MLST phylogenetic analysis of resident *Wolbachia* strains in *An. coluzzii,***
917 ***An. moucheti* and *An.* species A. A**) Maximum Likelihood molecular phylogenetic analysis from
918 concatenation of all five MLST gene loci for resident *Wolbachia* strains from *An. coluzzii* (*w*Anga-
919 Ghana; blue), *An. moucheti* (*w*AnM; green) and *An.* species A (*w*AnsA; red). The tree with the highest
920 log likelihood (-10606.13) is shown and drawn to scale, with branch lengths measured in the number
921 of substitutions per site. The analysis involved 94 nucleotide sequences. There were a total of 2067
922 positions in the final dataset. Concatenated sequence data from *Wolbachia* strains downloaded from
923 MLST database for comparison shown with isolate numbers in brackets (IsoN). *Wolbachia* strains
924 isolated from mosquito species highlighted in navy blue, bold. Strains isolated from other Dipteran
925 species are shown in navy blue, from Coleoptera in olive green, from Hemiptera in purple, from
926 Hymenoptera in teal blue, from Lepidoptera in maroon and from other, or unknown orders in black.
927 **B)**. Maximum Likelihood molecular phylogenetic analysis for coxA gene locus for resident *Wolbachia*
928 strains from *An. coluzzii* (*w*Anga-Ghana; blue), *An. moucheti* (*w*AnM; green) and *An.* species A
929 (*w*AnsA and *w*AnsA(2); red). The tree with the highest log likelihood (-1921.11) is shown and drawn to
930 scale, with branch lengths measured in the number of substitutions per site. The analysis involved 84
931 nucleotide sequences. There were a total of 402 positions in the final dataset. Sequence data for the
932 coxA locus from *Wolbachia* strains downloaded from MLST database for comparison shown in black
933 and navy blue with isolate numbers (IsoN) from MLST database shown in brackets. *Wolbachia* strains
934 isolated from mosquito species highlighted in navy blue. GenBank sequence for *w*Anga-Mali coxA
935 shown in maroon with accession number.
936
937 **Figure 6**. **The relative abundance of resident *Wolbachia* strains in *Anopheles*.** Bacterial genus
938 level taxonomy was assigned to OTUs clustered with a 97% cut-off using the SILVA SSU v128 97%
939 database, and individual genera comprising less than 1% of total abundance was merged into
940 "Others".
941
942 **Figure 7. The relative abundance of bacteria in *An. gambiae* s.s. comparing two locations with**
943 **contrasting *Asaia* infection densities.** Bacterial genus level taxonomy was assigned to OTUs
944 clustered with a 97% cut-off using the SILVA SSU v128 97% database, and individual genera
945 comprising less than 1% of total abundance was merged into "Others".
946
947 **Supplementary Figure S1**. **Alpha and beta diversity of *An. gambiae* s.s. from Kissidougou,**
948 **Guinea and Butemba, Uganda. A)** Alpha diversity using the Shannon diversity index shows the
949 relative abundance of bacterial genera. **B)** To identify dissimilarities in the bacterial community
950 structure between the microbiome, principal coordinates analysis (PCoA) was performed based on a
951 Bray-Curtis dissimilarity matrix based on 97% clustered OTUs.

Supplementary Figure S2. Prevalence of the bacterial endosymbiont *Asaia* and malaria parasites in *An. gambiae* s.s. mosquitoes from Guinea. **A)** Normalised *P. falciparum*: *An. gambiae* gene Ct ratio for mosquitoes that are infected with malaria and +/- *Asaia* bacteria. **B)** *P. falciparum* and *Asaia* infection rates (%) in 152 *An. gambiae* s.s. females. **C)** Box and whisker plot of Ct values for detection of *Asaia* and *P. falciparum* malaria showing more variable levels of *Asaia* detected.

Table 1. *Anopheles* mosquito species collected from locations within five malaria-endemic countries and *P. falciparum*, *Wolbachia* and *Asaia* prevalence rates. Species in different locations infected with *Wolbachia* are in bold. *Adult individuals from Lwiro (Katana), DRC were collected as both larvae and adults so have been excluded from *P. falciparum* and *Asaia* prevalence analysis.

Country	Location	Species	Individuals	Infection prevalence (%)		
				P. falciparum	***Wolbachia***	***Asaia***
Guinea	Faranah	*An. gambiae* s.s.	48	43.8	0.0	50.0
		An. arabiensis	7	0.0	0.0	100.0
		An. nili	9	0.0	0.0	100.0
	Kissidougou	*An. gambiae* s.s.	44	18.2	0.0	100.0
		An. species O	1	0.0	0.0	100.0
	Boke	*An. gambiae* s.s.	21	52.4	0.0	28.6
	Kankan	*An. gambiae* s.s.	48	38.1	0.0	56.3
		An. sp. unknown	1	0.0	0.0	0.0
DRC	Mikalayi	***An. gambiae* s.s.**	16	29.4	11.8	11.8
		An. moucheti	1	0.0	100.0	0.0
		An. funestus s.s.	13	30.8	0.0	15.4
	Kisangani	*An. gambiae* s.s.	25	12.0	8.0	20.0
		An. arabiensis	4	25.0	0.0	0.0
	Katana	*An. gambiae* s.s.	23	8.7	8.7	4.4
		An. funestus s.s.	5	0.0	0.0	0.0
		***An.* species A**	2	50.0	100.0	0.0
	Lwiro (Katana)	***An.* species A***	33	N/A	90.1	N/A
	Kapolowe	*An. gambiae* s.s.	9	11.1	11.1	0.0
		An. funestus s.s.	5	20.0	0.0	0.0
	Kalemie	***An. gambiae* s.s.**	28	7.1	21.4	3.6
		An. arabiensis	2	0.0	50.0	0.0
	Kinshasa	***An. gambiae* s.s.**	27	22.2	14.8	3.7
		An. funestus s.s.	2	50.0	0.0	0.0
Ghana	Dogo	***An. coluzzii***	286	0.0	4.2	32.9
		An. melas	1	0.0	0.0	100.00
Uganda	Butemba (2013)	*An. gambiae* s.s.	57	19.3	0.0	80.7
	Butemba (2014)	*An. gambiae* s.s.	135	36.3	0.0	48.1
		An. arabiensis	1	0.0	0.0	0.0
Madagascar	Anivorano Nord	*An. funestus*	8	0.0	0.0	25.0
		An. gambiae s.s.	3	0.0	0.0	33.3
		An. arabiensis	2	0.0	0.0	100.0
		An. mascarensis	38	0.0	0.0	39.5
		An. maculipalpis	9	0.0	0.0	11.1
		An. coustani	22	0.0	0.0	27.3
		An. rufipes	11	0.0	0.0	27.3
	Ambomiharina	*An. funestus*	12	0.0	0.0	83.3
		An. pharoensis	7	0.0	0.0	42.9
		An. rufipes	19	10.5	0.0	68.4
		An. maculipalpis	9	0.0	0.0	0.0
		An. gambiae s.s.	8	0.0	0.0	0.0
		An. coustani	24	0.0	0.0	25.0
		An. squamosus	10	0.0	0.0	20.0
		An. mascarensis	2	0.0	0.0	50.0
	Antafia	*An. gambiae* s.s.	11	27.3	0.0	45.5
		An. pauliani	2	0.0	0.0	50.0
		An. rufipes	2	0.0	0.0	50.0
		An. mascarensis	2	0.0	0.0	0.0
	Ambohimarina	*An. funestus*	1	0.0	0.0	0.0
		An. gambiae s.s.	1	0.0	0.0	0.0
		An. arabiensis	2	0.0	0.0	0.0
		An. rufipes	7	0.0	0.0	42.9
		An. coustani	18	0.0	0.0	11.1
		An. maculipalpis	8	0.0	0.0	12.5
		An. squamosus	52	0.0	0.0	3.9
		An. mascarensis	11	0.0	0.0	0.0

1001 **Table 2. Novel resident *Wolbachia* strain wsp and MLST gene allelic profiles.** Exact matches to
1002 existing alleles present in the database are shown in bold, novel alleles are denoted by the allele
1003 number of the closest match and shown in red (number of single nucleotide differences to the closest
1004 match). *alternative degenerate primers (set 3) used to generate sequence.
1005

Mosquito species	*Wolbachia* strain	*Wolbachia* gene allele					
		wsp	*gatB*	*coxA*	*hcpA*	*ftsZ*	*fbpA*
An. species A	*w*AnsA	152 (34)	140 (4)	122 (16)	6 (7)	7 (1)	10 (1)
An. species A	*w*AnsA(2)	152 (34)	-	36 (1)	6 (7)	-	-
An. moucheti	*w*AnM	-	9 (2)	11 (1)	74 (3)	7 (2)	7 (12)
An. coluzzii	*w*Anga-Ghana	-	**9**	**64**	**3***	**177**	**4**

1006

1007 **Supplementary Table 1. Additional sample details and ITS2 GenBank accession numbers.**

Location	**Species**	**Sample ID**	**Wolbachia status**	**ITS2 accession number**
Guinea: Kissidougou	*Anopheles* sp. O/15	GUI-KSK1	W-	MH598414
Guinea: Kissidougou	*Anopheles gambiae s.s.*	GUI-KSK2	W-	MH598415
Guinea: Faranah	*Anopheles nili*	GUI-FAR1	W-	MH598416
Guinea: Faranah	*Anopheles nili*	GUI-FAR2	W-	MH598417
Guinea: Kankan	*Anopheles* sp. unknown	GUI-KAN1	W-	MH598418
DRC: Lwiro	*Anopheles* sp. A/1	DRC-LWI1	W+	MH598419
DRC: Lwiro	*Anopheles* sp. A/1	DRC-LWI2	W+	MH598420
DRC: Lwiro	*Anopheles* sp. A/1	DRC-LWI3	W+	MH598421
DRC: Katana	*Anopheles* sp. A/1	DRC-KAT1	W+	MH598422
DRC: Katana	*Anopheles* sp. A/1	DRC-KAT2	W+	MH598423
DRC: Mikalayi	*Anopheles moucheti*	DRC-MIK1	W+	MH598424
DRC: Kinshasa	*Anopheles gambiae s.s.*	DRC-KIN1	W+	MH598425
DRC: Mikalayi	*Anopheles gambiae s.s.*	DRC-MIK2	W+	MH598426
DRC: Kalemie	*Anopheles gambiae s.s.*	DRC-KAL1	W+	MH598427
DRC: Kalemie	*Anopheles arabiensis*	DRC-KAL2	W+	MH598428
DRC: Katana	*Anopheles gambiae s.s.*	DRC-KAT3	W-	MH598429
Ghana: Dogo	*Anopheles coluzzii*	GHA-DOG1	W+	MH598430
Ghana: Dogo	*Anopheles coluzzii*	GHA-DOG2	W+	MH598431
Ghana: Dogo	*Anopheles coluzzii*	GHA-DOG3	W+	MH598432
Ghana: Dogo	*Anopheles coluzzii*	GHA-DOG4	W-	MH598433
Ghana: Dogo	*Anopheles coluzzii*	GHA-DOG5	W-	MH598434
Ghana: Dogo	*Anopheles melas*	GHA-DOG6	W-	MH598435

Uganda: Butemba	*Anopheles gambiae s.s.*	UGA-BUT1	W-	MH598436
Uganda: Butemba	*Anopheles gambiae s.s.*	UGA-BUT2	W-	MH598437
Uganda: Butemba	*Anopheles gambiae s.s.*	UGA-BUT3	W-	MH598438
Uganda: Butemba	*Anopheles gambiae s.s.*	UGA-BUT4	W-	MH598439
Uganda: Butemba	*Anopheles arabiensis*	UGA-BUT5	W-	MH598440
Madagascar: Antafia	*Anopheles gambiae s.s.*	MAD-ANT1	W-	MH598441
Madagascar: Antafia	*Anopheles gambiae s.s.*	MAD-ANT2	W-	MH598442
Madagascar: Antafia	*Anopheles gambiae s.s.*	MAD-ANT3	W-	MH598443
Madagascar: Ambomiharina	*Anopheles rufipes*	MAD-AMB1	W-	MH598444
Madagascar: Ambomiharina	*Anopheles rufipes*	MAD-AMB2	W-	MH598445

1008

1009 **Supplementary Table 2. *Wolbachia* 16S and wsp GenBank accession numbers**

Sample ID	Strain	16S	wsp
DRC-LWI1	*w*AnsA	MH605275	MH605281
DRC-LWI2	*w*AnsA	MH605276	MH605282
DRC-LWI3	*w*AnsA	MH605277	MH605283
DRC-KAT1	*w*AnsA	-	MH605284
DRC-KAT2	*w*AnsA(2)	-	MH605285
DRC-MIK1	*w*AnM	MH605278	-
GHA-DOG1	*w*Anga-Ghana	MH605279	-
GHA-DOG2	*w*Anga-Ghana	MH605280	-

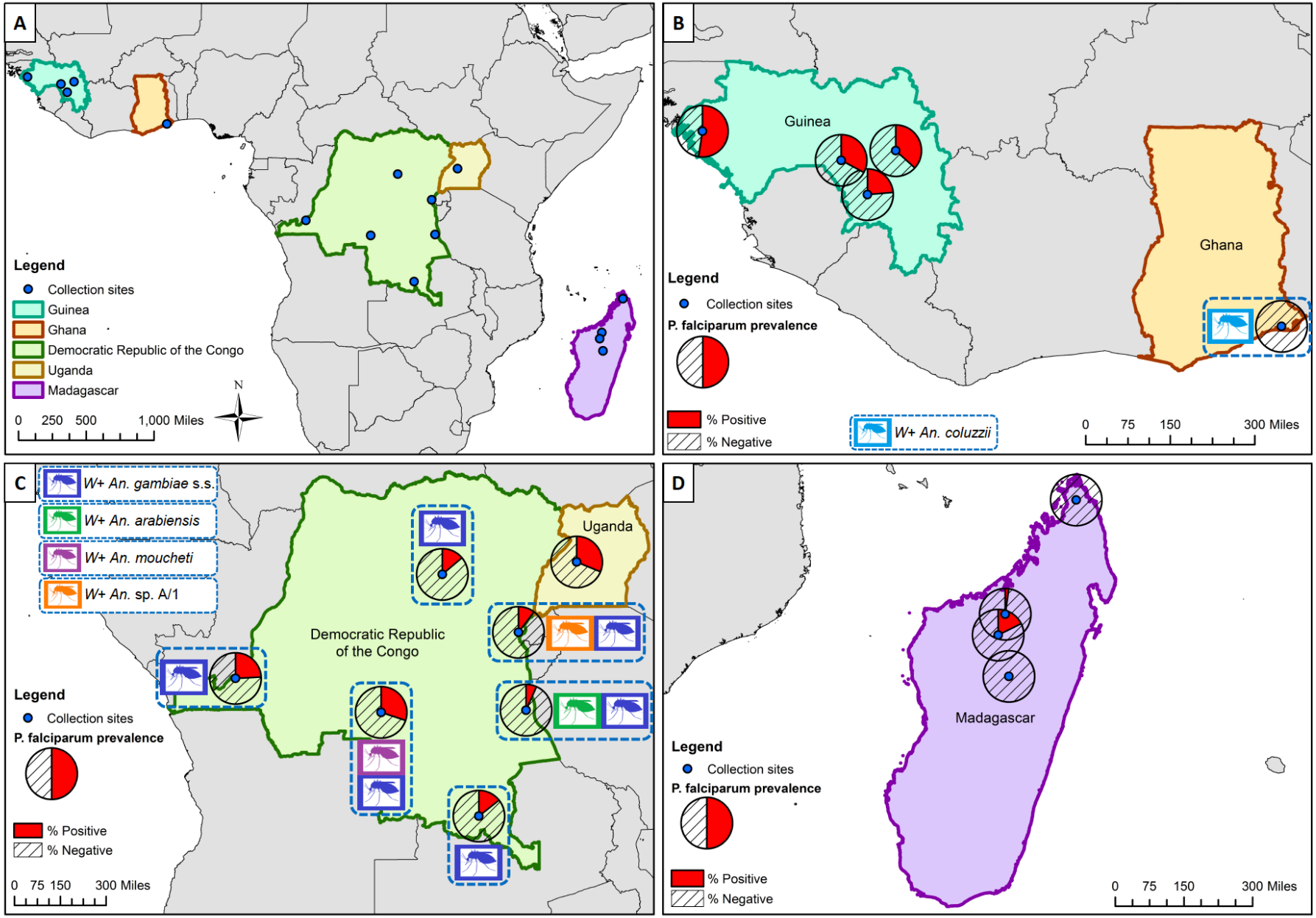
1010

1011 **Supplementary Table 3. *Wolbachia* MLST gene GenBank accession numbers**

Sample ID	Strain	gatB	coxA	hcpA	ftsZ	fbpA
DRC-LWI1	*w*AnsA	MH605286	MH605290	MH605295	MH605299	MH605302
DRC-KAT1	*w*AnsA	MH605287	MH605291	-	-	MH605303
DRC-KAT2	*w*AnsA(2)	-	MH605292	MH605296	-	-
DRC-MIK1	*w*AnM	MH605288	MH605293	MH605297	MH605300	MH605304
GHA-DOG1	*w*Anga-Ghana	MH605289	MH605294	-	MH605301	MH605305
GHA-DOG2	*w*Anga-Ghana	-	-	MH605298	-	-

1012

Figure 1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Legend
Collection sites
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Ghana
Democratic Republic of the Congo
Uganda
Madagascar
N
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B
Guinea
Ghana
Legend
Collection sites
P. falciparum prevalence
% Positive
% Negative
W+ An. coluzzii
0 75 150 300 Miles
C
W+ An. gambiae s.s.
W+ An. arabiensis
W+ An. moucheti
W+ An. sp. A/1
Uganda
Democratic Republic of the Congo
Legend
Collection sites
P. falciparum prevalence
% Positive
% Negative
0 75 150 300 Miles
D
Madagascar
Legend
Collection sites
P. falciparum prevalence
% Positive
% Negative
0 75 150 300 Miles

)13

Figure 2

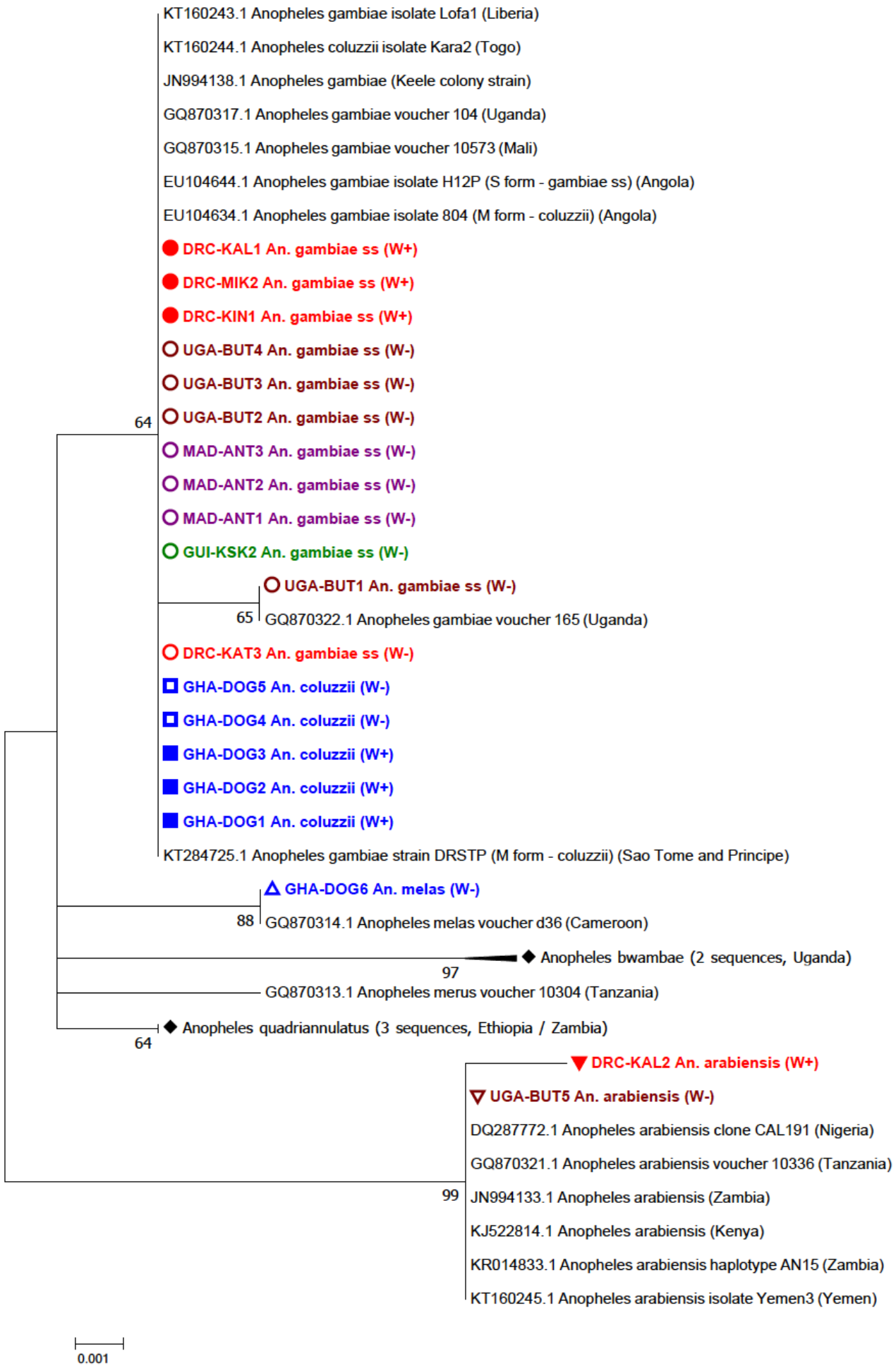
KT160243.1 Anopheles gambiae isolate Lofa1 (Liberia)
KT160244.1 Anopheles coluzzii isolate Kara2 (Togo)
JN994138.1 Anopheles gambiae (Keele colony strain)
GQ870317.1 Anopheles gambiae voucher 104 (Uganda)
GQ870315.1 Anopheles gambiae voucher 10573 (Mali)
EU104644.1 Anopheles gambiae isolate H12P (S form - gambiae ss) (Angola)
EU104634.1 Anopheles gambiae isolate 804 (M form - coluzzii) (Angola)
DRC-KAL1 An. gambiae ss (W+)
DRC-MIK2 An. gambiae ss (W+)
DRC-KIN1 An. gambiae ss (W+)
UGA-BUT4 An. gambiae ss (W-)
UGA-BUT3 An. gambiae ss (W-)
64
UGA-BUT2 An. gambiae ss (W-)
MAD-ANT3 An. gambiae ss (W-)
MAD-ANT2 An. gambiae ss (W-)
MAD-ANT1 An. gambiae ss (W-)
GUI-KSK2 An. gambiae ss (W-)
UGA-BUT1 An. gambiae ss (W-)
65
GQ870322.1 Anopheles gambiae voucher 165 (Uganda)
DRC-KAT3 An. gambiae ss (W-)
GHA-DOG5 An. coluzzii (W-)
GHA-DOG4 An. coluzzii (W-)
GHA-DOG3 An. coluzzii (W+)
GHA-DOG2 An. coluzzii (W+)
GHA-DOG1 An. coluzzii (W+)
KT284725.1 Anopheles gambiae strain DRSTP (M form - coluzzii) (Sao Tome and Principe)
GHA-DOG6 An. melas (W-)
88
GQ870314.1 Anopheles melas voucher d36 (Cameroon)
Anopheles bwambae (2 sequences, Uganda)
97
GQ870313.1 Anopheles merus voucher 10304 (Tanzania)
Anopheles quadriannulatus (3 sequences, Ethiopia / Zambia)
64
DRC-KAL2 An. arabiensis (W+)
UGA-BUT5 An. arabiensis (W-)
DQ287772.1 Anopheles arabiensis clone CAL191 (Nigeria)
GQ870321.1 Anopheles arabiensis voucher 10336 (Tanzania)
99
JN994133.1 Anopheles arabiensis (Zambia)
KJ522814.1 Anopheles arabiensis (Kenya)
KR014833.1 Anopheles arabiensis haplotype AN15 (Zambia)
KT160245.1 Anopheles arabiensis isolate Yemen3 (Yemen)
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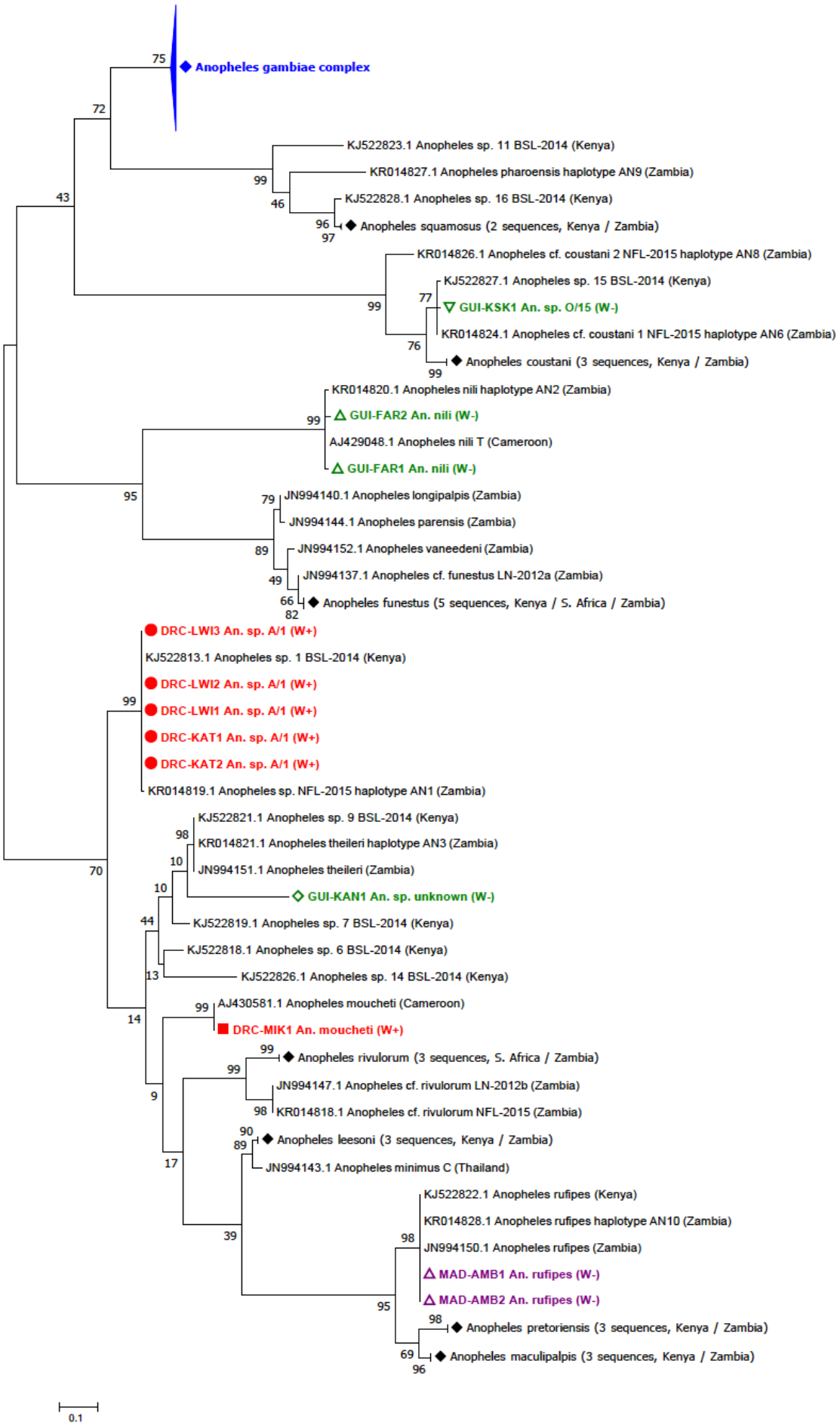
Figure 3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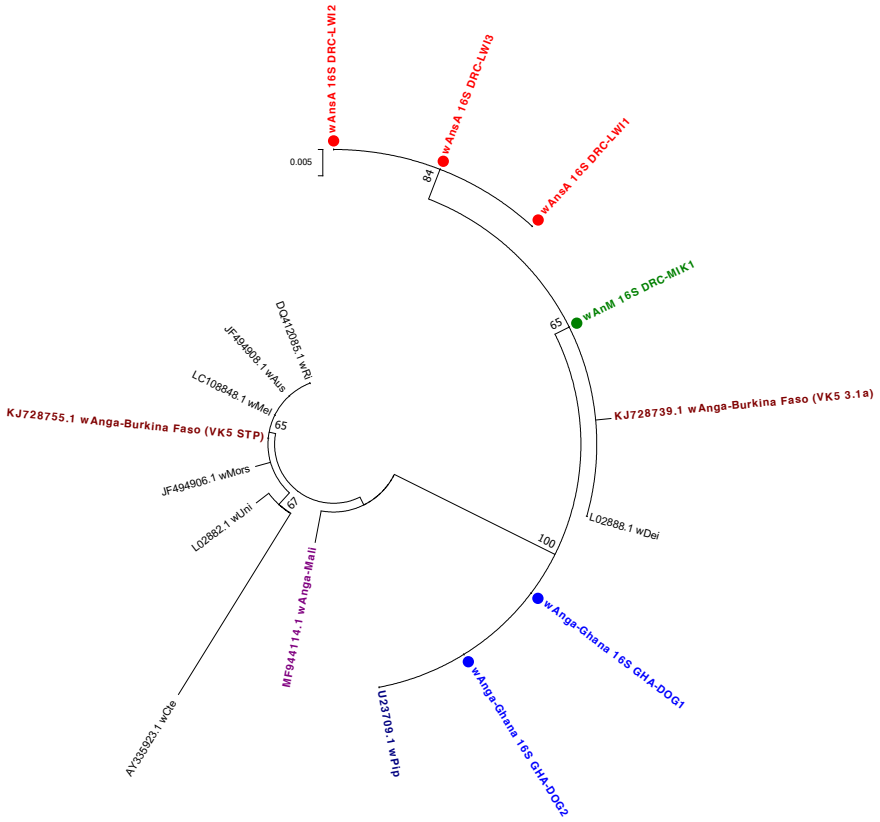
KJ522823.1 Anopheles sp. 11 BSL-2014 (Kenya)
KR014827.1 Anopheles pharoensis haplotype AN9 (Zambia)
KJ522828.1 Anopheles sp. 16 BSL-2014 (Kenya)
Anopheles squamosus (2 sequences, Kenya / Zambia)
KR014826.1 Anopheles cf. coustani 2 NFL-2015 haplotype AN8 (Zambia)
KJ522827.1 Anopheles sp. 15 BSL-2014 (Kenya)
GUI-KSK1 An. sp. O/15 (W-)
KR014824.1 Anopheles cf. coustani 1 NFL-2015 haplotype AN6 (Zambia)
Anopheles coustani (3 sequences, Kenya / Zambia)
KR014820.1 Anopheles nili haplotype AN2 (Zambia)
GUI-FAR2 An. nili (W-)
AJ429048.1 Anopheles nili T (Cameroon)
GUI-FAR1 An. nili (W-)
JN994140.1 Anopheles longipalpis (Zambia)
JN994144.1 Anopheles parensis (Zambia)
JN994152.1 Anopheles vaneedeni (Zambia)
JN994137.1 Anopheles cf. funestus LN-2012a (Zambia)
Anopheles funestus (5 sequences, Kenya / S. Africa / Zambia)
DRC-LWI3 An. sp. A/1 (W+)
KJ522813.1 Anopheles sp. 1 BSL-2014 (Kenya)
DRC-LWI2 An. sp. A/1 (W+)
DRC-LWI1 An. sp. A/1 (W+)
DRC-KAT1 An. sp. A/1 (W+)
DRC-KAT2 An. sp. A/1 (W+)
KR014819.1 Anopheles sp. NFL-2015 haplotype AN1 (Zambia)
KJ522821.1 Anopheles sp. 9 BSL-2014 (Kenya)
KR014821.1 Anopheles theileri haplotype AN3 (Zambia)
JN994151.1 Anopheles theileri (Zambia)
GUI-KAN1 An. sp. unknown (W-)
KJ522819.1 Anopheles sp. 7 BSL-2014 (Kenya)
KJ522818.1 Anopheles sp. 6 BSL-2014 (Kenya)
KJ522826.1 Anopheles sp. 14 BSL-2014 (Kenya)
AJ430581.1 Anopheles moucheti (Cameroon)
DRC-MIK1 An. moucheti (W+)
Anopheles rivulorum (3 sequences, S. Africa / Zambia)
JN994147.1 Anopheles cf. rivulorum LN-2012b (Zambia)
KR014818.1 Anopheles cf. rivulorum NFL-2015 (Zambia)
Anopheles leesoni (3 sequences, Kenya / Zambia)
JN994143.1 Anopheles minimus C (Thailand)
KJ522822.1 Anopheles rufipes (Kenya)
KR014828.1 Anopheles rufipes haplotype AN10 (Zambia)
JN994150.1 Anopheles rufipes (Zambia)
MAD-AMB1 An. rufipes (W-)
MAD-AMB2 An. rufipes (W-)
Anopheles pretoriensis (3 sequences, Kenya / Zambia)
Anopheles maculipalpis (3 sequences, Kenya / Zambia)
0.1

Figure 4

A



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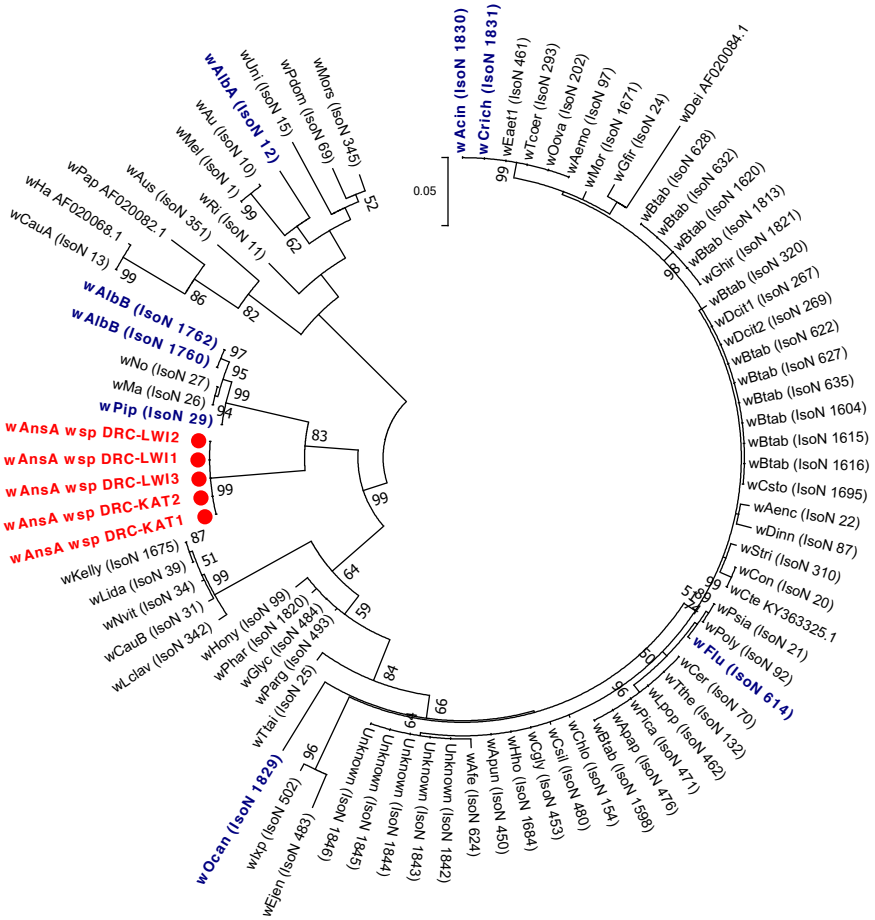
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wTcoer (IsoN 293)
wOova (IsoN 202)
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wNtor (IsoN 1671)
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wDei AF020084.1
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wDinn (IsoN 87)
wStri (IsoN 310)
wCon (IsoN 20)
wCle KY363325.1
wPsia (IsoN 21)
wPoly (IsoN 92)
wFlu (IsoN 614)
wCer (IsoN 70)
wTthe (IsoN 132)
wLpop (IsoN 462)
wPica (IsoN 471)
wApap (IsoN 476)
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Unknown (IsoN 1846)
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wParg (IsoN 493)
wGlyc (IsoN 484)
wPhar (IsoN 1820)
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wLclav (IsoN 342)
wCauB (IsoN 31)
wNvit (IsoN 34)
wLida (IsoN 39)
wKelly (IsoN 1675)
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wAnsA wsp DRC-KAT2
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wAnsA wsp DRC-LWI1
wAnsA wsp DRC-LWI2
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wMa (IsoN 26)
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wMel (IsoN 1)
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wUni (IsoN 15)
wPdom (IsoN 69)
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Figure 5

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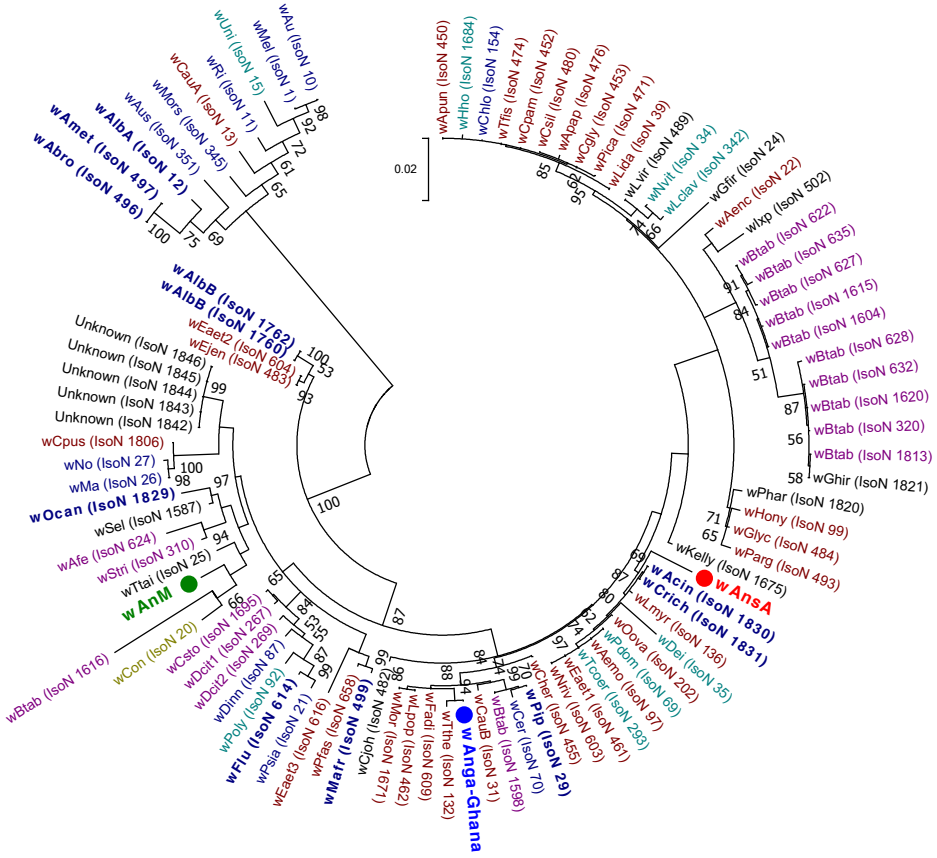
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wLvir (IsoN 489)
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wBtab (IsoN 622)
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wBtab (IsoN 1813)
wGhir (IsoN 1821)
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wHony (IsoN 99)
wGlyc (IsoN 484)
wParg (IsoN 493)
wKelly (IsoN 1675)
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wAnsA
wCrich (IsoN 1831)
wLmyr (IsoN 136)
wDei (IsoN 35)
wOva (IsoN 202)
wPtom (IsoN 69)
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wTcoer (IsoN 293)
wEaet1 (IsoN 461)
wNriv (IsoN 603)
wCher (IsoN 455)
wPip (IsoN 29)
wCer (IsoN 70)
wBtab (IsoN 1598)
wCauB (IsoN 31)
wAnga-Ghana
wThe (IsoN 132)
wFadi (IsoN 609)
wLpop (IsoN 462)
wMor (IsoN 1671)
wCjoh (IsoN 482)
wMatr (IsoN 499)
wPfas (IsoN 658)
wEaet3 (IsoN 616)
wPsia (IsoN 21)
wFlu (IsoN 614)
wPoly (IsoN 92)
wDinn (IsoN 87)
wDcit2 (IsoN 269)
wDcit1 (IsoN 267)
wCsto (IsoN 1695)
wCon (IsoN 20)
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wStri (IsoN 310)
wAfe (IsoN 624)
wSel (IsoN 1587)
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wNo (IsoN 27)
wCpus (IsoN 1806)
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Unknown (IsoN 1843)
Unknown (IsoN 1844)
Unknown (IsoN 1845)
Unknown (IsoN 1846)
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wEaet2 (IsoN 604)
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wAmet (IsoN 497)
wAlbA (IsoN 12)
wAus (IsoN 351)
wMors (IsoN 345)
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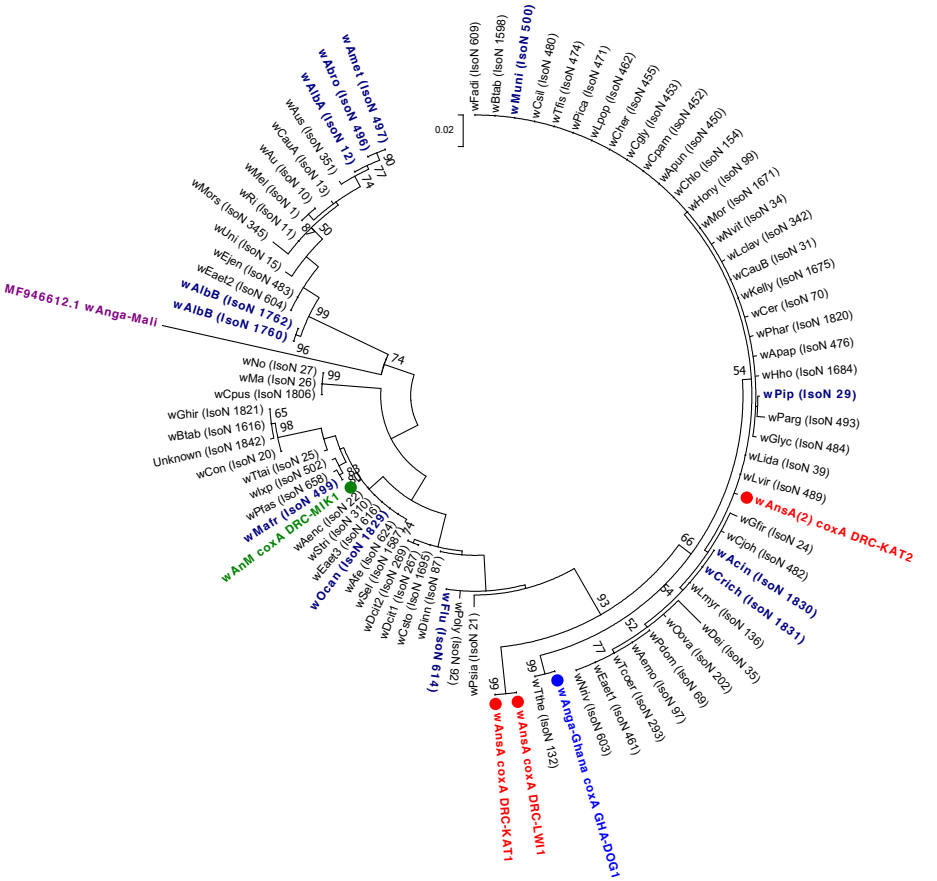
wUni (IsoN 15)
wMel (IsoN 1)
wAu (IsoN 10)

B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wPica (IsoN 471)
wLpop (IsoN 462)
wCher (IsoN 455)
wCgly (IsoN 453)
wCpam (IsoN 452)
wApun (IsoN 450)
wChlo (IsoN 154)
wHony (IsoN 99)
wMor (IsoN 1671)
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wLclav (IsoN 342)
wCauB (IsoN 31)
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wPhar (IsoN 1820)
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wLida (IsoN 39)
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wAcin (IsoN 1830)
wCrich (IsoN 1831)
wLmyr (IsoN 136)
wDei (IsoN 35)
wOva (IsoN 202)
wPtom (IsoN 69)
wAemo (IsoN 97)
wTcoer (IsoN 293)
wEaet1 (IsoN 461)
wNriv (IsoN 603)
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wThe (IsoN 132)
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wAnsA coxA DRC-KAT1
wPsia (IsoN 21)
wPoly (IsoN 92)
wFlu (IsoN 614)
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wDcit2 (IsoN 269)
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wCon (IsoN 20)
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wCpus (IsoN 1806)
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wAlbA (IsoN 12)
wAus (IsoN 351)
wCauA (IsoN 13)
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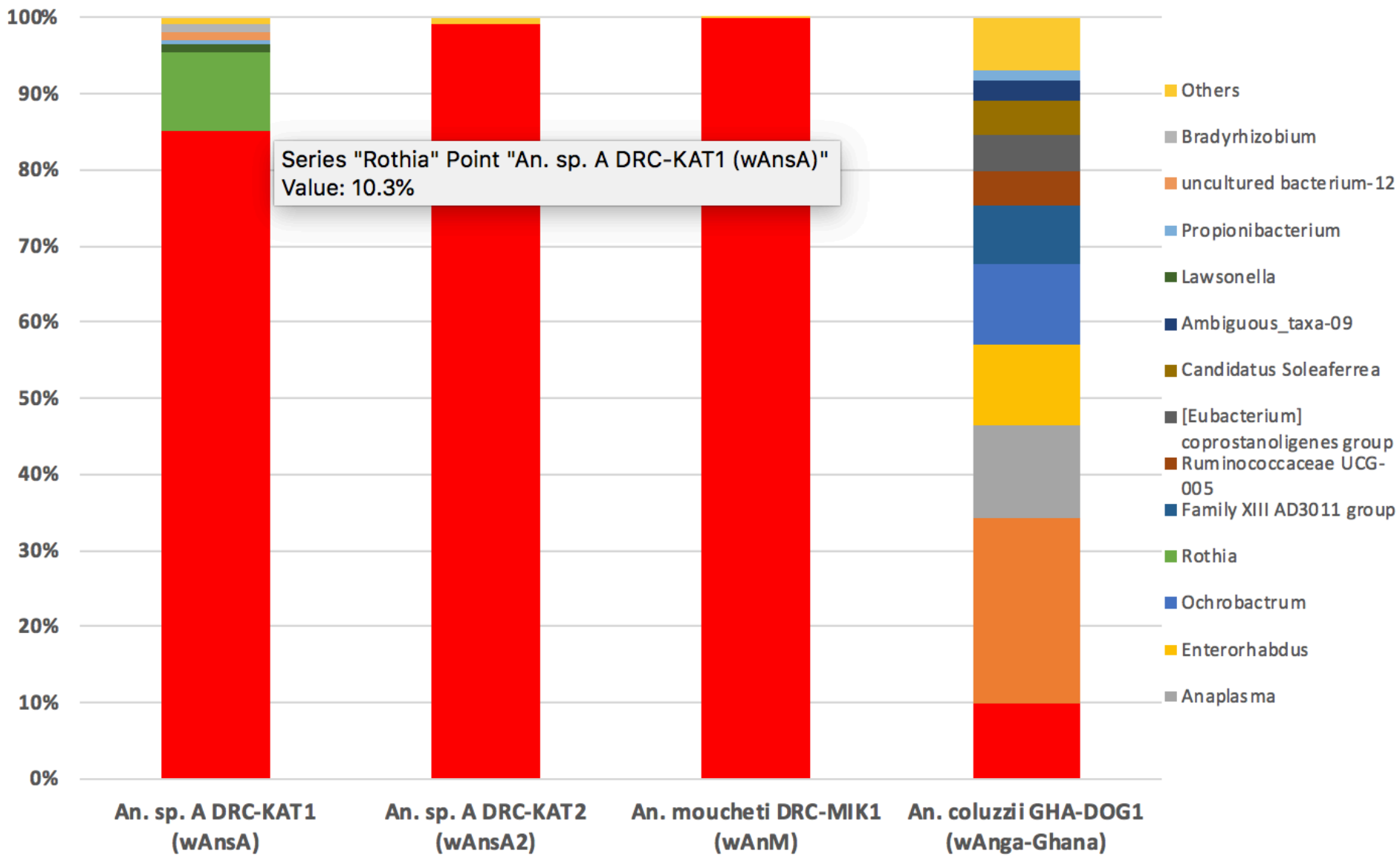
wMel (IsoN 1)
wRi (IsoN 11)
wMors (IsoN 345)
wUni (IsoN 15)

Figure 6


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Series "Rothia" Point "An. sp. A DRC-KAT1 (wAnsA)"
Value: 10.3%
An. sp. A DRC-KAT1 (wAnsA)
An. sp. A DRC-KAT2 (wAnsA2)
An. moucheti DRC-MIK1 (wAnM)
An. coluzzii GHA-DOG1 (wAnga-Ghana)
Others
Bradyrhizobium
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Propionibacterium
Lawsonella
Ambiguous_taxa-09
Candidatus Soleaferrea
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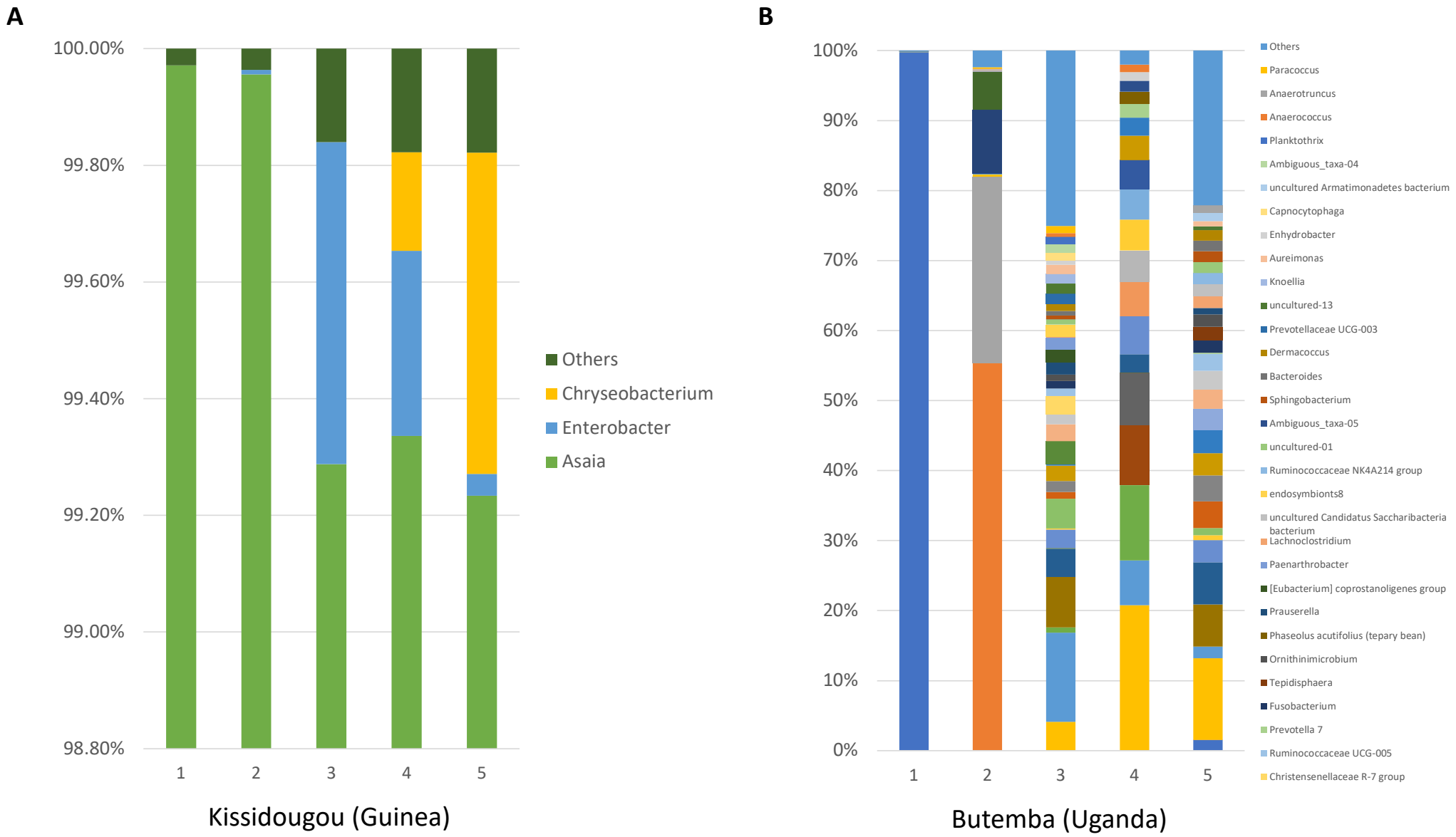
Ruminococcaceae UCG-005
Family XIII AD3011 group
Rothia
Ochrobactrum
Enterorhabdus
Anaplasma

Figure 7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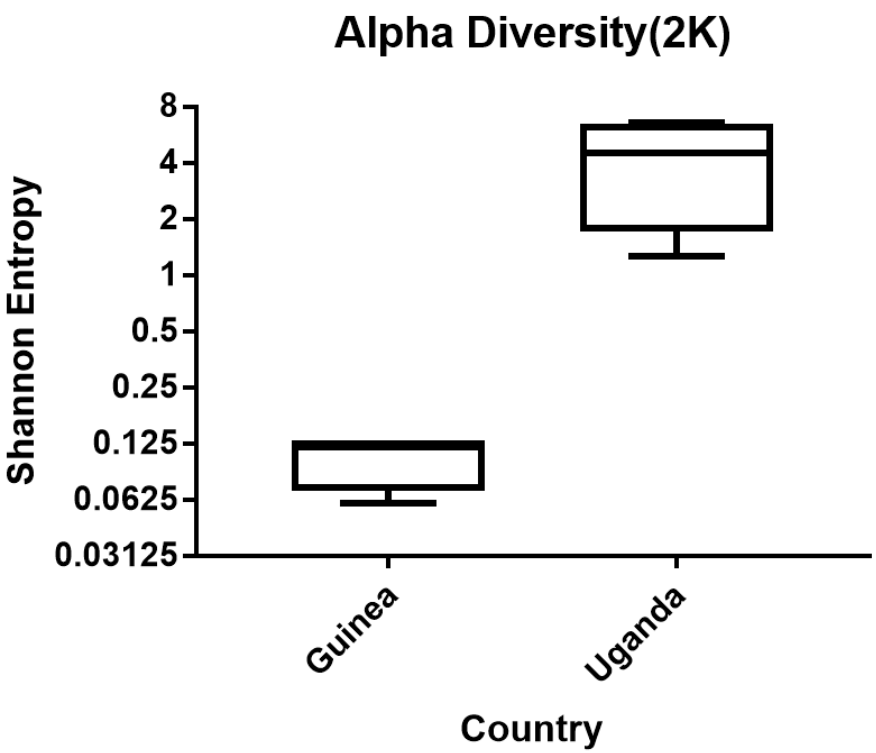
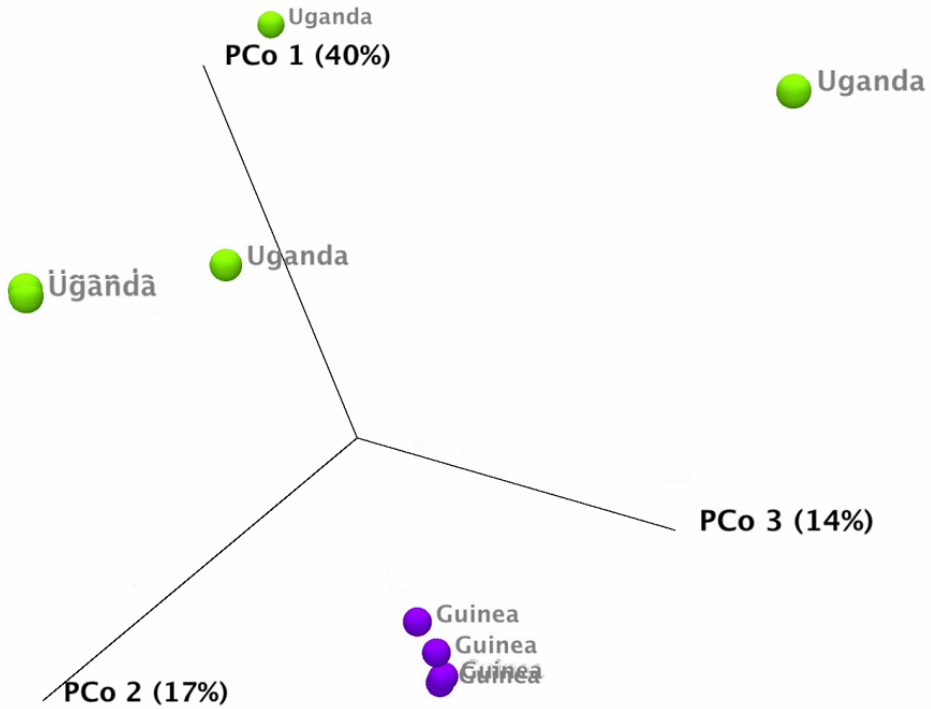
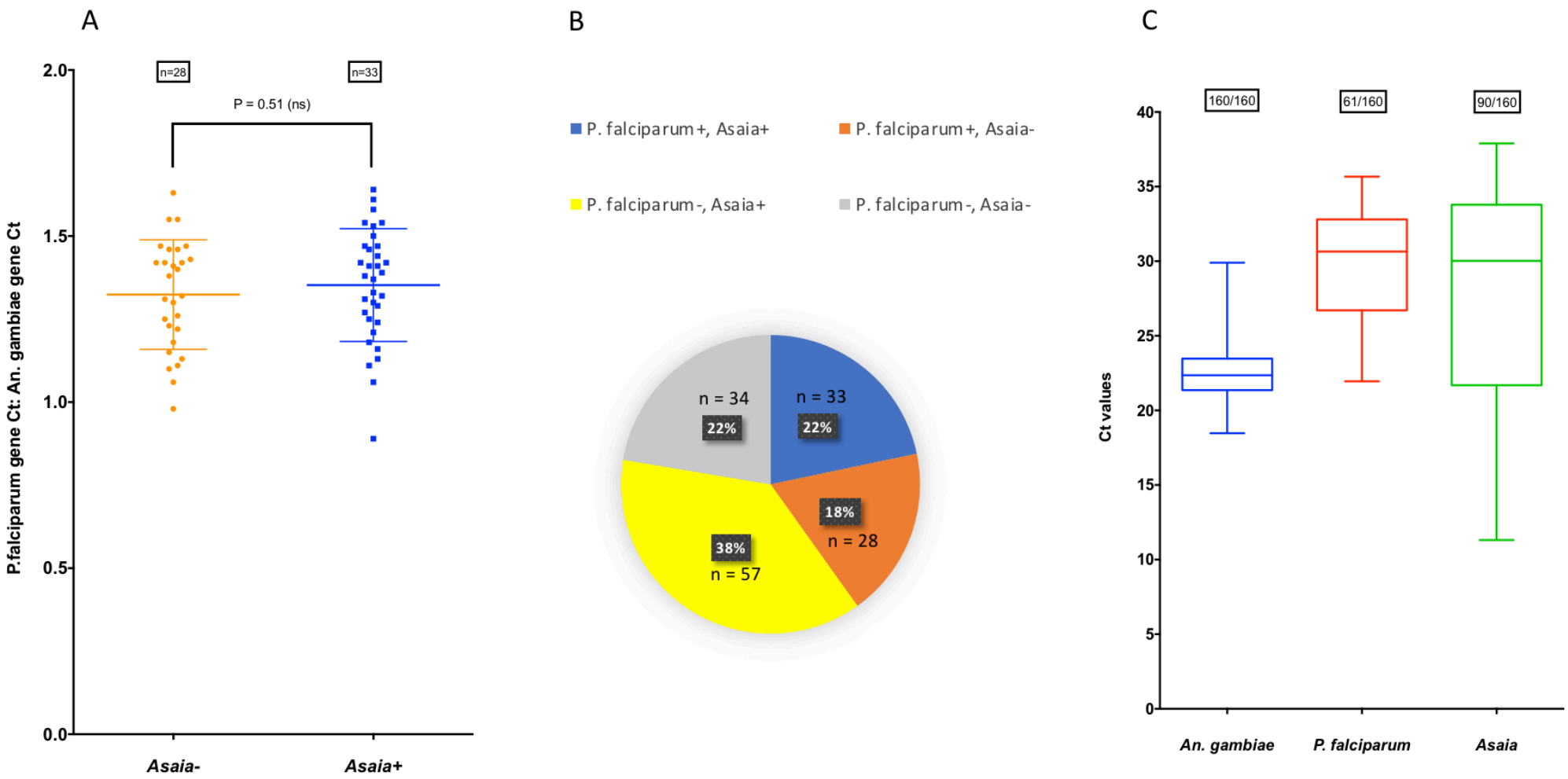
100.00%
99.80%
99.60%
99.40%
99.20%
99.00%
98.80%
1
2
3
4
5
Kissidougou (Guinea)
Others
Chryseobacterium
Enterobacter
Asaia
B
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1
2
3
4
5
Butemba (Uganda)
Others
Paracoccus
Anaerotruncus
Anaerococcus
Planktothrix
Ambiguous_taxa-04
uncultured Armatimonadetes bacterium
Capnocytophaga
Enhydrobacter
Aureimonas
Knoellia
uncultured-13
Prevotellaceae UCG-003
Dermacoccus
Bacteroides
Sphingobacterium
Ambiguous_taxa-05
uncultured-01
Ruminococcaceae NK4A214 group
endosymbionts8
uncultured Candidatus Saccharibacteria bacterium
Lachnoclostridium
Paenarthrobacter
[Eubacterium] coprostanoligenes group
Prauserella
Phaseolus acutifolius (tepary bean)
Ornithinimicrobium
Tepidisphaera
Fusobacterium
Prevotella 7
Ruminococcaceae UCG-005
Christensenellaceae R-7 group

Supplementary Figure S1

A



B



Supplementary Figure S2