Title: Natural *Wolbachia* infections are common in the major malaria vectors in Central Africa

Running title: *Wolbachia* diversity in African *Anopheles*

Authors

Diego Ayala[1,2,*], Ousman Akone-Ella[2], Nil Rahola[1,2], Pierre Kengne[1], Marc F. Ngangue[2,3], Fabrice Mezeme[2], Boris K. Makanga[2], Carlo Costantini[1], Frédéric Simard[1], Franck Prugnolle[1], Benjamin Roche[1,4], Olivier Duron[1] & Christophe Paupy[1].

Affiliations

[1] MIVEGEC, IRD, CNRS, Univ. Montpellier, Montpellier, France.

[2] CIRMF, Franceville, Gabon.

[3] ANPN, Libreville, Gabon

[4] UMMISCO, IRD, Montpellier, France.

[*] **Corresponding author:**

Diego Ayala, MIVEGEC, IRD, CNRS, Univ. Montpellier, 911 av Agropolis, BP 64501, 34394 Montpellier, France; phone: +33(0)4 67 41 61 47; email: diego.ayala@ird.fr

Abstract

During the last decade, the endosymbiont bacterium *Wolbachia* has emerged as a biological tool for vector disease control. However, during long time, *Wolbachia* was thought to be absent in natural populations of *Anopheles*. The recent discovery that species within the *Anopheles gambiae* complex hosts *Wolbachia* in natural conditions has opened new opportunities for malaria control research in Africa. Here, we investigated *Wolbachia* infectious status in 25 African *Anopheles* species in Gabon (Central Africa). Our results revealed the presence of *Wolbachia* in 16 of these species, including the major malaria vectors in this area. The infection prevalence varies greatly among species, confirming that sample size is a key factor to detect the infection. Moreover, our sequencing and phylogenetic analyses showed the important diversity of *Wolbachia* strains that infect *Anopheles*. Co-evolutionary analysis unveiled patterns of *Wolbachia* transmission within *Anopheles* species, suggesting that past independent acquisition events were followed by co-cladogenesis. The large diversity of *Wolbachia* strains that infect natural populations of *Anopheles* offers a promising opportunity to select suitable phenotypes for suppressing *Plasmodium* transmission and/or manipulating *Anopheles* reproduction, which in turn could be used to reduce the malaria burden in Africa.

Key-words: *Anopheles*, *Wolbachia*, diversity, co-evolution, disease control.

Introduction

Malaria still affects millions of people and causes thousands of victims worldwide, and sub-Saharan Africa pays the highest tribute [1]. Currently, vector-control measures (e.g., insecticide-treated bed nets or indoor residual spray) are the largest contributors to malaria eradication [2]. Indeed, if these interventions are maintained or increased, malaria burden should be drastically reduced in Africa before 2030 [3]. These predictions are based on the constant effectiveness of these methods. However, the spread of insecticide resistance [4] and vector behavioural changes related to the massive use of bed nets [5] will challenge malaria eradication in the coming decades. Therefore, it is indispensable to develop alternative and environmentally friendly control strategies for the millennium development goal of malaria eradication [6].

Several methods have been proposed to accompany or replace the use of synthetic insecticides [7]. Among them, the use of the maternally inherited *Wolbachia* bacteria (α-proteobacteria, Anaplasmataceae family) has emerged as a promising alternative biological tool for fighting malaria and other vector-borne diseases [7-11]. This bacterium exhibits a large spectrum of interactions with its hosts: from mutualism and commensalism to parasitism [12]. Moreover, *Wolbachia* has proved its ability to invade mosquito populations and/or to prevent pathogen infections in some of the most important mosquito vectors [11]. Indeed, artificial *Wolbachia* infection has been successfully used to suppress dengue transmission in natural populations of *Aedes aegypti* [13,14]. Similarly, artificial infection of *Anopheles* (the vector of human malaria) with *Wolbachia* strains has a negative impact on the transmission of *Plasmodium* parasites [15-17], providing a relevant alternative for malaria control. However, during long time, *Wolbachia* was thought to be absent in natural populations of *Anopheles* [18].

69 Yet, since 2014, *An. gambiae, An. coluzzii* and *An. arabiensis* (three major malaria
70 vectors) populations from Burkina Faso and Mali (West Africa) were found naturally
71 infected by *Wolbachia* [19-21]. Noteworthy, they showed a negative correlation between
72 *Wolbachia* infection and *Plasmodium* development [20,21]. These findings support the
73 development of novel vector control strategies based on *Wolbachia-Anopheles*
74 interactions. However, although *Wolbachia* naturally infects 40% - 60% of arthropods
75 [22,23], infection was delimited to *Anopheles* species within the *gambiae* complex.
76 Indeed, during the last decade, many other malaria mosquito species worldwide
77 (n=38) were unsuccessfully screened for *Wolbachia* infection [9,18,24]. Therefore, the
78 recent discovery of *Wolbachia* infecting species within the gambiae complex across
79 West Africa couple with the development of new molecular tools, make necessary to
80 screen other natural occurring *Anopheles* populations for the presence of the
81 bacterium.
82
83 In this study, we investigated the presence of *Wolbachia* in 25 *Anopheles* species in
84 Gabon, Central Africa. We sampled mosquitoes across the country and in a variety of
85 ecological settings, from deep rainforest to urban habitats. By using a molecular
86 approach, we confirmed *Wolbachia* presence in 16 species, including all the major
87 malaria vectors in Central Africa (*An. gambiae, An. coluzzii, An. funestus, Anopheles*
88 *nili* and *Anopheles moucheti*). The prevalence of *Wolbachia* infection was particularly
89 high in *An. nili* and *An. moucheti*. Phylogenetic analysis revealed that all the infected
90 mosquito species hosted *Wolbachia* bacteria belonging to the supergroups A or B that
91 exhibit high genetic diversity. Finally, we explored the co-evolution between
92 *Wolbachia* and *Anopheles*. The results have direct implications for the development of

93 new and environmentally friendly vector control strategies and open new directions
94 for research on pathogen transmission and reproductive manipulation.
95
96 **Results**
97 ***Wolbachia naturally infects a large number of Anopheles species from Gabon.***
98 In this study, we screened 648 mosquitoes from eight sites in Gabon (Fig 1, Table 1,
99 Table S1). Based on morphological traits [25] and molecular analysis [26-30], we identified
100 25 *Anopheles* species (Text S1), although further molecular studies may be conducted
101 to confirm the species status of the new taxa. Our sample included all the species in
102 which the presence of *Wolbachia* was previously investigated in Africa (*An. gambiae,*
103 *An. coluzzii, An. funestus* and *An. coustani)*, with the exception of *An. arabiensis* that
104 is absent in Gabon (Table 1). By PCR-amplification of a *16S* rRNA fragment [20], we
105 found 70 *Wolbachia*-positive specimens that belonged to 16 different *Anopheles*
106 species, distributed throughout the country (Fig 1, Table S1). With few exceptions,
107 *Wolbachia* infection rate was lower than 15% in most species (n=11), as observed in
108 other arthropods [22,23]. The exceptions concerned the *An. moucheti* complex, *An.*
109 *jebudensis* and *An. nili,* in which more than 50% of sampled mosquitoes were infected
110 (Table 1). None of these species was previously screened for *Wolbachia* infection. We
111 also found an important correlation between the number of screened and the number
112 of infected individuals (r^2=0.36, Table S1), confirming that the screening effort is the
113 critical factor for *Wolbachia* detection in *Anopheles*. Indeed, we estimated that a
114 sample size of 60 individuals per species was needed to quantify correctly a
115 prevalence lower than 15%, with a probability of 95% (Fig 2). As our sample size
116 mostly varied from 1 to 58 individuals for each species, with few exceptions in which

117 sample size was lower than five individuals, the probability of a correct estimation of
118 *Wolbachia* prevalence ranged between 68% and 93% (Fig 2).
119
120 ***Wolbachia is maternally inherited in An. moucheti.***
121 Although *Wolbachia* is mainly maternally transmitted [12], horizontal transmission may
122 occasionally occur in natural conditions [31-33]. To confirm the vertical transmission in
123 the infected mosquito species, we focused on *An. moucheti* for logistic reasons (i.e.,
124 highest *Wolbachia* prevalence and ease of sampling). Although *An. moucheti* has
125 never reproduced in insectary conditions to date, we obtained eggs from six
126 *Wolbachia*-infected females that were placed individually in 1.5ml Eppendorf tubes
127 for egg laying. In total, we analysed the infectious status of 79 progeny by PCR
128 amplification of the same *16S* rRNA fragment [20] (Table S2) and found that 70 were
129 infected, with an average vertical transmission frequency of 97.54% (range: 90% -
130 100%).
131
132 **Naturally occurring *Wolbachia* strains in *Anopheles* reveal high genetic diversity**.
133 By sequence analysis of the *16S* rRNA fragment PCR-amplified from each *Anopheles*
134 sample (Table 1), we could assign the *Wolbachia* strains to three pre-existing
135 supergroups: A (n=5), B (n=64) and C (n=1) (Fig 3). Specifically, we detected
136 supergroup B *Wolbachia* in 64 mosquitoes belonging to all 16 infected *Anopheles*
137 species. We found supergroup A *Wolbachia* in five individuals from four species (*An.*
138 *funestus, An. coluzzii, An. vinckei* and *An. carnevalei*), thus providing examples of
139 superinfection, as previously observed in *Ae. albopictus* [34] (Fig 3). Noteworthy, none
140 of the mosquitoes examined was co-infected by *Wolbachia* strains belonging, for
141 instance, to the supergroups A and B. Moreover, we confirmed that *Wolbachia*

142 previously identified in *An. gambiae s.l.* from Burkina Faso and Mali are included in
143 the supergroups A and B [19,21]. Finally, we found that one *An. coustani* individual was
144 infected by a *Wolbachia* strain from the supergroup C that is strictly known to infect
145 filarial worms. We thus investigated the presence of filarial nematode DNA in the
146 mosquito by PCR amplification and sequencing of a fragment of the *COI* filarial gene
147 [35], followed by phylogenetic analysis with RAxML. Our results confirmed the
148 presence of *Dirofilaria immitis* in this specimen (Fig. S1). This canine filarial parasite
149 hosts *Wolbachia* and is transmitted by many mosquitoes, including *Anopheles* [36].
150 Therefore, it is not surprising to find an *An. coustani* specimen infected by this filarial
151 nematode. This specimen was excluded from further investigations.
152
153 To expand our knowledge on the *Wolbachia* strains that infect natural *Anopheles*
154 populations, we PCR-amplified, sequenced and analysed the *16S* rRNA fragment and
155 also fragments from three other conserved *Wolbachia* genes (*coxA, fbpA* and *ftsZ*) that
156 are commonly used for strain typing and evolutionary studies [37] (Fig 3). We used a
157 new nested PCR protocol (see *Methods*) for samples that could not be genotyped
158 using the classical Multi Locus Sequence Typing (MLST) primers (Table S1). Our
159 phylogenetic analyses confirm the *16S* results, assigning most of the species to
160 supergroups A and B. Few exceptions (Fig 3, gene *coxA*) showed some incongruence
161 with regard to *16S*. They suggest signals of recent recombination between the
162 supergroups A and B, as it was previously demonstrated [37]. Detailed sequence
163 analysis revealed that mosquito species belonging to the same group or complex (i.e.,
164 *An. moucheti* and *An. gambiae*) displayed a common *Wolbachia* haplotype (Fig. 3 and
165 Fig. 4). Conversely, some species with lower prevalence displayed a variety of
166 haplotypes (i.e., *An. coluzzii, An. marshallii, An. vinckei* or *An. funestus*). The case of

167 *An. vinckei* was particularly interesting because the three infected specimens
168 displayed different haplotypes for the analysed *Wolbachia* genes. Moreover, one
169 specimen (*An. vinckei* M002, Fig 3) revealed a completely different *Wolbachia* strain.
170 Overall, none of the *Wolbachia* strains discovered in this study matched previously
171 annotated *Wolbachia* strains or the strain that infects *An. gambiae* in Burkina Faso
172 and Mali [19,21] (Fig. 3 and Fig. 4). Within the supergroup B, we could easily
173 distinguish at least two strains. The strain infecting *An. moucheti* (*wANMO*) was
174 similar to several *Anopheles* species, such as *An. gambiae* or *An. marshallii*, while the
175 strain infecting *An. nili* (*wANNI*) was more closely related to those found in other
176 mosquitoes species like *Ae. albopictus* or *Cx. quinquefasciatus* (Fig. 3 and Fig. 4).
177 Conversely, the other haplotypes were associated with one specific host.
178
179 ***Wolbachia* independently evolves in malaria-transmitting mosquitoes.**
180 As *Wolbachia* is mainly a maternally inherited bacterium, the host mitochondrial
181 DNA (mtDNA) is a suitable marker to study its evolutionary history in *Anopheles* [38].
182 Analysis of *COII* sequences from 176 specimens belonging to the 25 *Anopheles*
183 species collected in Gabon provided the most exhaustive phylogenetic tree of
184 *Anopheles* in Central Africa (Fig 4). Concerning *Wolbachia* infection, we observed
185 the independent acquisition and loss across the different *Anopheles* species clades.
186 Moreover, the genetic distances of *Anopheles* species and their *Wolbachia*
187 counterparts were not correlated (Mantel test, $p > 0.05$) (Fig. S2). Despite, mosquitoes
188 from the same complex, and therefore genetically very close, shared similar
189 *Wolbachia* haplotypes (Fig 4 and Fig. S2). Finally, we investigated how *Wolbachia*
190 evolves within each *Anopheles* species [39]. Our results revealed that *Wolbachia*-

191 infected and non-infected mosquitoes shared the same mtDNA haplotype (Fig 3),
192 indicating that infection status and host haplotypes are not associated.
193
194 **Discussion**
195 The present study provides three key conclusions. First, the genus *Anopheles* includes
196 a large number of species that are naturally infected by *Wolbachia* (16/25), with high
197 infection prevalence among major malaria vectors. Second, *Anopheles*-infecting
198 *Wolbachia* bacteria show high genetic diversity, with similar haplotypes detected in
199 different *Anopheles* species. Third, the independent evolution of *Wolbachia* and
200 *Anopheles* might be interpreted as multiple acquisition events with horizontal
201 transmission. The large diversity of *Wolbachia* strains that infect many natural
202 *Anopheles* populations opens new perspectives about their use for reducing pathogen
203 transmission and/or for reproductive manipulation in *Anopheles* with the aim of
204 decreasing malaria burden in Africa.
205
206 For long time, the scientific community has investigated how to use *Wolbachia* for
207 fighting vector-borne diseases [7-9,11]. In arthropods, *Wolbachia* is broadly spread,
208 including among *Culex* and *Aedes* mosquitoes. At the genus *Anopheles*, the infection
209 was delimited to species within the gambiae complex [19,21]. Several hypotheses can be
210 put forward to explain this underrepresentation. First, low infection prevalence or
211 local variations could have hindered the discovery of *Wolbachia* infections. In our
212 study, most *Anopheles* species exhibited a prevalence lower than 15% (Table 1). This
213 pattern is common in many other arthropods [22,23], and it is usually associated with a
214 weak manipulation of the host reproduction and/or imperfect maternal transmission [40].
215 Our statistical analysis confirmed that important sampling and screening efforts are

216 required to detect *Wolbachia* in species with low infection rates, such as *Anopheles*
217 (Fig 2). In general, our sampling effort was higher than in previous studies (n <30)
218 [9,24], which could explain why we found more species infected. Moreover, local
219 frequency variations among populations could also hinder the detection of *Wolbachia*
220 infections [41]. For instance, we sampled *An. coluzzii* in three different sites, but we
221 only found *Wolbachia*-infected mosquitoes at La Lopé (Fig 1, Table S1). Therefore,
222 sampling in different localities at seasons would improve detection rates. Second,
223 low-density W*olbachia* infections may not be detected in *Anopheles* by the routinely
224 used molecular tools, as previously reported for other arthropods [42,43] and recently in
225 *Anopheles gambiae* [21]. Our results indicate that conventional amplification
226 (conventional PCR) analysis allowed detecting *Wolbachia* infection only in 6 of 16
227 species (*An. moucheti, An m. nigeriensis, An. "GAB-3", An nili, An. jebudensis* and
228 *An. vinckei*), presumably with high *Wolbachia* density. Moreover, some *Anopheles*
229 species with high *Wolbachia* infection rates, such as *An. moucheti* or *An. nili*, were
230 never screened before this study [9].
231
232 Unexpectedly, our work revealed that *Anopheles* species are infected by different
233 *Wolbachia* species. Although three previous studies reported *Wolbachia* infection in
234 the *An. gambiae* germline [19-21], we do not know whether *Wolbachia* naturally invade
235 and is maternally inherited in the infected mosquito species from Gabon. Indeed,
236 horizontal gene transfer (resulting in the insertion of *Wolbachia* genes within the
237 mosquito genome), or parasitism (e.g., by filarial nematodes) could explain the
238 presence of *Wolbachia* in an organism without vertical transmission. However, the
239 *Wolbachia* sequences we identified were genetically close to those found in other
240 Diptera and we did not observe any signal of extensive divergence, which would be

241 expected in the case of horizontal gene transfer [44,45] (Figs 3 and 4). Alternatively,
242 mosquitoes could have been parasitized by *Wolbachia*-hosting nematodes [46,47] or
243 mites [48]. However, these *Wolbachia* strains belong to other, easily distinguishable
244 supergroups (Fig 3 and Fig. S1). Moreover, the analysis of *An. moucheti* F1 confirm,
245 at least in this species, that no other biological *Wolbachia* contamination were present
246 in our analysis. In conclusion, we found evidences to suggest that *Wolbachia* is
247 naturally present and maternally inherited in the natural populations of *Anopheles*
248 species of Central Africa analysed in our study, as in *An. moucheti* (Table S2).
249
250 In Central African *Anopheles*, *Wolbachia* acquisition seems to be independent of the
251 host phylogeny (Figs 3 and 4). Our results revealed that the genetic distances between
252 *Wolbachia* and *Anopheles* are not positively correlated (Mantel test, $p > 0.05$) (Fig.
253 S2). Therefore, *Wolbachia* evolves independently of the host lineage, suggesting that
254 lateral transfers (i.e., predation [49], parasitism [50]) are the principal ways for *Wolbachia*
255 acquisition in Gabonese *Anopheles*. For instance, it could be the situation between *An.*
256 *moucheti, An. marshallii An. gambiae* and *An. coluzzii*, which share similar
257 *Wolbachia* strains. Although the different larval ecology between those species would
258 suggest other ways of lateral transfer (i.e. during nectar feeding [32]). On the other hand,
259 we found that all the *Anopheles* species belonging to the same complex shared related
260 *Wolbachia* strains (Fig. 4). Permeable reproductive barriers among members of the
261 same complex could facilitate the intermittent movement of the bacterium [51].
262 Interestingly, although they share similar *Wolbachia* strains, sibling species showed
263 different infection prevalence. Indeed, *An. carnevalei* and *An. m. nigeriensis* exhibited
264 frequencies lower than 15%, whereas *An. nili* and *An. moucheti*, their respective
265 counterparts and the most important malaria vectors in their complex, displayed

266 frequencies higher than 60% (Table 1). Moreover, our *An. gambiae* and *An. coluzzii*
267 populations are infected by different *Wolbachia* strains than those detected in Burkina
268 Faso and Mali. Similarly, in mosquitoes [41] and ants [52], the same species is infected by
269 different *Wolbachia* strains according to the region. The availability of whole-genome
270 sequences for *Wolbachia* strains will enlighten the intricate phylogenetic relationships
271 among the different strains in *Anopheles* [53].
272
273 **Conclusions**
274 *Wolbachia* has emerged as a tangible and compelling tool for controlling vector-borne
275 diseases [13,14]. In this study, we demonstrated the natural presence of this
276 endosymbiont bacterium in a large number of *Anopheles* species, including the five
277 major malaria vectors in Central Africa. It has been shown that *Wolbachia* ability to
278 interfere with pathogen transmission depends on the bacterium strain. Therefore, our
279 results offer the opportunity to determine the roles of the different *Anopheles*-
280 infecting *Wolbachia* strains in suppressing *Plasmodium* transmission and/or
281 manipulating *Anopheles* reproduction. For instance, the three most infected species
282 (*An. moucheti*, *An. nili* and *An. vinckei*) play an important role in human (the first
283 two) and non-human malaria transmission in the deep forest of Gabon [54,55]. Therefore,
284 we could investigate *Wolbachia* positive [20] and negative [56,57] effects on the
285 susceptibility to *Plasmodium* infection in their natural hosts. Moreover, the strongest
286 phenotype impact on suppressing pathogen transmission or reproductive manipulation
287 has been observed in exogenous *Wolbachia* infections [15,17,58,59]. Therefore, the
288 availability of *Wolbachia* strains that infect natural *Anopheles* populations offers
289 promising opportunities for experimental and theoretical studies on *Anopheles*, but
290 also on other mosquito families that are vectors of other diseases, including *Ae.*

291 *aegypti* and *Ae. albopictus*. In conclusion, our findings are merely the "tip of the
292 iceberg" in the research field on *Wolbachia* use to control vector-borne diseases,
293 particularly in malaria.
294
295 **Methods**
296 **Research and ethics statements**
297 Mosquitoes were collected in Gabon under the research authorization
298 AR0013/16/MESRS/CENAREST/CG/CST/CSAR and the national park entry
299 authorization AE16008/PR/ANPN/SE/CS/AEPN. Mosquito sampling using the
300 human-landing catch (HLC) method was performed under the protocol
301 0031/2014/SG/CNE approved by the National Research Ethics Committee of Gabon.
302
303 **Mosquito sampling and DNA extraction**
304 Mosquitoes were sampled in eight sites across Gabon, Central Africa, from 2012 to
305 2016 (Fig 1, Table 1, Fig. S1). These sites included sylvatic (national parks) and
306 anthropic habitats (villages and cities). Adult females were collected using CDC light
307 traps, BG traps and HLC. Collected specimens were taxonomically identified
308 according to standard morphological features [25,60]. Then, they were individually stored
309 in 1.5 mL tubes at -20°C and sent to CIRMF for molecular analysis. When possible,
310 at least 30 mosquitoes (from 1 to 58) for each *Anopheles* species from different sites
311 were selected for genomic analysis. Total genomic DNA was extracted from the
312 whole body using the DNeasy Blood and Tissue Kit (Qiagen) according to the
313 manufacturer's instructions. Genomic DNA was eluted in 100 µL of TE buffer.
314 Specimens belonging to the *An. gambiae* complex, *An. funestus* group, *An. moucheti*

315 complex and *An. nili* complex were molecularly identified using PCR-based
316 diagnostic protocols [26-29,61].
317
318 ***Wolbachia* screening and Multi Locus Sequence Typing (MLST) analysis**
319 *Wolbachia* infection in adult females was detected by nested PCR amplification of a
320 *Wolbachia*-specific *16S* rDNA fragment (~400 bp) using 2 µL of host genomic DNA,
321 according to the protocol developed in Catteruccia's laboratory [20]. Amplification of
322 this *16S* rDNA fragment in infected *Aedes albopictus* and *Culex pipiens* genomic
323 DNA (data not shown) confirmed the performance of this nested PCR protocol to
324 detect *Wolbachia* in broad number of mosquitoes [20]. In order to avoid potential
325 contaminations, we used *Ae. albopictus* and *Cx quinquefasciatus* from Gabon as
326 positive controls. As negative controls, we used water and *Ae. aegypti*. Moreover, we
327 included species and timing control, carrying out the PCR amplifications for each
328 species independently and in different days. The amplicon size was checked on 1.5%
329 agarose gels, and amplified *16S* rDNA fragments were sent to Genewiz (UK) for
330 sequencing (forward and reverse) to confirm the presence of *Wolbachia*-specific
331 sequences. The DNA quality of all samples was confirmed by the successful
332 amplification of a fragment (~800 bp) of the mitochondrial gene *COII* in all the
333 *Anopheles* species under study [30,62]. PCR products were run on 1.5% agarose gels, and
334 *COII* fragments from 176 mosquito specimens of the 25 species were sequenced
335 (forward and reverse) by Genewiz (UK) for the *Anopheles* phylogenetic studies.
336 *Wolbachia*-positive genomic DNA samples (2 µL/sample) were then genotyped by
337 MLST using three loci, *coxA (~450 bp) ftsZ (~500 bp)* and *fbpA (~460 bp)* [63], and
338 according to standard conditions [37]. If the three fragments could not be amplified, a
339 newly-developed nested PCR protocol was used. Specifically, after the first run with

340 the standard primers, 2 µL of the obtained product was amplified again using internal
341 primers specific for each gene: *coxA* (coxA_NF-2: 5'-
342 TTTAACATGCGCGCAAAAGG-3'; coxA_NR-2: 5'-
343 TAAGCCCAACAGTGAACATATG-3'), *ftsZ* (ftsZ_NF-2: 5'-
344 ATGGGCGGTGGTACTGGAAC-3'; ftsZ_NR-2: 5'-
345 AGCACTAATTGCCCTATCTTCT-3'), and *fbpA* (fbpA_NF-1: 5'-
346 AGCTTAACTTCTGATCAAGCA-3'; fbpA_NR-1: 5'-
347 TTCTTTTTCCTGCAAAGCAAG-3'). Cycling conditions for *coxA* and *ftsZ* were:
348 94°C for 5min followed by 36 cycles at 94°C for 15s, 55°C for 15s and 72°C for 30s,
349 and a final extension step at 72°C for 10min. For *fbpA*, they were: 94°C for 5min
350 followed by 36 cycles at 94°C for 30s, 59°C for 45s and 72°C for 90s, and a final
351 extension step at 72°C for 10 min. The resulting fragments (*coxA*, 357bp; *fbpA*, 358-
352 bp; and *ftsZ*, 424-bp) were sequenced bidirectionally by Genewiz (UK). The new
353 sequences obtained in this study were submitted to GenBank (accession numbers XX
354 to XX, Supplementary Table 1).
355
356 *Phylogenetic and statistical analysis*
357 All *Wolbachia* sequences for the *16S, coxA, fbpA* and *ftsZ* gene fragments and for
358 *Anopheles* COII were corrected using *Geneious* R10 [64]. The resulting consensus
359 sequences for each gene were aligned with sequences that represent the main known
360 *Wolbachia* supergroups obtained from GenBank (see Table S1). Only unique
361 haplotypes for each species were included in the analysis. Inference of phylogenetic
362 trees was performed using the maximum likelihood (ML) method and RAxML [65] with
363 a substitution model GTR + CAT [66] and 1000 bootstrapping replicates. Finally, all
364 MLST *Wolbachi*a sequences were used to build phylogenetic trees using RAxML

365 (GTR+CAT model, 1000 bootstrapping replicates). Trees were visualized with iTOL
366 v.3.4.3 [67].
367 To quantify the accuracy of the observed *Wolbachia* infection prevalence, the
368 influence of sample size on its estimation was assessed. Assuming that *Wolbachia*
369 prevalence within a host species follows a beta binomial distribution [23] yielding many
370 species with a low or a high *Wolbachia* prevalence but few with an intermediate one,
371 we quantified, for each sample size, the proportion of samples (over 1,000
372 realizations) that could yield an estimate that was not significantly different from the
373 prevalence over the whole population with a z-test and a significance threshold at
374 95%. As expected, sample size can be small for very low or very high prevalence (60
375 individuals are enough in 95% of cases for these extreme prevalence), while it has to
376 be much higher for intermediate prevalence values (up to 150 individuals for a
377 prevalence close to 50%).
378
379 All statistical analyses were performed using "R" v3.2.5 (R Development Core Team,
380 http://cran.r-project.org/), with the addition of the "ggplot2" library [68].
381
382 **Author contributions**
383 D.A., O.D. and C.P. designed the experiments. D.A., O.A., N.R. and P.K., performed
384 the experiments. D.A., F.S., C.C., O.D and C.P. analysed the data. D.A. performed the
385 sequencing analysis. D.A. and B.R. performed the statistical analysis. D.A., N.R.,
386 M.N., F.M., B.M., C.P. and F.P. provided samples for the analysis. D.A., O.D. and
387 C.P. wrote the manuscript.
388
389 **Competing interests**

The authors declare no competing interests.

Acknowledgements

We thank the "Agence Nationale de la Preservation de la Nature" (ANPN) and the "Centre National de la Recherche Scientifique et Technologique of Gabon" (CENAREST) that authorized this study and facilitated the access to the national parks of La Lopé, Moukalaba-Doudou and Plateaux Batékés. We specially thank Sonia Lekana for helping with the preliminary sequencing analysis.

References

1 WHO. World Malaria Report 2015. (World Health Organization, 2015).

2 Bhatt, S. *et al.* The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. *Nature* **526**, 207-+, doi:10.1038/nature15535 (2015).

3 Griffi, J. T. *et al.* Potential for reduction of burden and local elimination of malaria by reducing Plasmodium falciparum malaria transmission: a mathematical modelling study. *Lancet Infect. Dis.* **16**, 465-472, doi:10.1016/s1473-3099(15)00423-5 (2016).

4 Ranson, H. & Lissenden, N. Insecticide Resistance in African Anopheles Mosquitoes: A Worsening Situation that Needs Urgent Action to Maintain Malaria Control. *Trends in Parasitology* **32**, 187-196, doi:10.1016/j.pt.2015.11.010 (2016).

5 Pates, H. & Curtis, C. in *Annual Review of Entomology* Vol. 50 *Annual Review of Entomology* 53-70 (Annual Reviews, 2005).

6 Newby, G. *et al.* The path to eradication: a progress report on the malaria-eliminating countries. *Lancet* **387**, 1775-1784 (2016).

7 McGraw, E. A. & O'Neill, S. L. Beyond insecticides: new thinking on an ancient problem. *Nat Rev Micro* **11**, 181-193 (2013).

8 Hoffmann, A. A., Ross, P. A. & Rasic, G. Wolbachia strains for disease control: ecological and evolutionary considerations. *Evolutionary Applications* **8**, 751-768, doi:10.1111/eva.12286 (2015).

9 Bourtzis, K. *et al.* Harnessing mosquito-Wolbachia symbiosis for vector and disease control. *Acta Tropica* **132**, S150-S163, doi:10.1016/j.actatropica.2013.11.004 (2014).

10 Kambris, Z., Cook, P. E., Phuc, H. K. & Sinkins, S. P. Immune Activation by Life-Shortening Wolbachia and Reduced Filarial Competence in Mosquitoes. *Science* **326**, 134-136, doi:10.1126/science.1177531 (2009).

11 Iturbe-Ormaetxe, I., Walker, T. & Neill, S. L. O. Wolbachia and the biological control of mosquito-borne disease. *Embo Reports* **12**, 508-518, doi:10.1038/embor.2011.84 (2011).

12 Werren, J. H., Baldo, L. & Clark, M. E. Wolbachia: master manipulators of invertebrate biology. *Nature Reviews Microbiology* **6**, 741-751, doi:10.1038/nrmicro1969 (2008).

13 Hoffmann, A. A. *et al.* Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission. *Nature* **476**, 454-U107, doi:10.1038/nature10356 (2011).

14 Schmidt, T. L. *et al.* Local introduction and heterogeneous spatial spread of dengue-suppressing Wolbachia through an urban population of Aedes aegypti. *PLOS Biology* **15**, e2001894, doi:10.1371/journal.pbio.2001894 (2017).

15 Hughes, G. L., Koga, R., Xue, P., Fukatsu, T. & Rasgon, J. L. Wolbachia Infections Are Virulent and Inhibit the Human Malaria Parasite Plasmodium Falciparum in Anopheles Gambiae. *Plos Pathogens* **7**, doi:10.1371/journal.ppat.1002043 (2011).

16 Kambris, Z. *et al.* Wolbachia Stimulates Immune Gene Expression and Inhibits Plasmodium Development in Anopheles gambiae. *Plos Pathogens* **6**, doi:10.1371/journal.ppat.1001143 (2010).

17 Bian, G. *et al.* Wolbachia Invades Anopheles stephensi Populations and Induces Refractoriness to Plasmodium Infection. *Science* **340**, 748-751, doi:10.1126/science.1236192 (2013).

18 Hughes, G. L. *et al.* Native microbiome impedes vertical transmission of Wolbachia in Anopheles mosquitoes. *Proceedings of the National Academy of Sciences* **111**, 12498-12503, doi:10.1073/pnas.1408888111 (2014).

19 Baldini, F. *et al.* Evidence of natural Wolbachia infections in field populations of Anopheles gambiae. *Nature Communications* **5**, doi:10.1038/ncomms4985 (2014).

20 Shaw, W. R. *et al.* Wolbachia infections in natural Anopheles populations affect egg laying and negatively correlate with Plasmodium development. *Nature Communications* **7**, doi:10.1038/ncomms11772 (2016).

21 Gomes, F. M. *et al.* Effect of naturally occurring *Wolbachia* in A*nopheles gambiae s.l.* mosquitoes from Mali on *Plasmodium falciparum* malaria transmission. *PNAS* **114**, 12566-12571, doi:10.1073/pnas.1716181114 (2017).

22 Duron, O. *et al.* The diversity of reproductive parasites among arthropods: Wolbachia do not walk alone. *Bmc Biology* **6**, doi:10.1186/1741-7007-6-27 (2008).

23 Zug, R. & Hammerstein, P. Still a Host of Hosts for Wolbachia: Analysis of Recent Data Suggests That 40% of Terrestrial Arthropod Species Are Infected. *Plos One* **7**, doi:10.1371/journal.pone.0038544 (2012).

24 Osei-Poku, J., Han, C., Mbogo, C. M. & Jiggins, F. M. Identification of Wolbachia Strains in Mosquito Disease Vectors. *PLOS ONE* **7**, e49922, doi:10.1371/journal.pone.0049922 (2012).

25 Gillies, M. T. & Coetzee, M. C. *A Supplement to the Anophelinae of Africa South of the Sahara (Afrotropical region)*. (The South African Institute for Medical Research, 1987).

475 26 Santolamazza, F. *et al.* Insertion polymorphisms of SINE200
476 retrotransposons within speciation islands of Anopheles gambiae
477 molecular forms. *Malar J* **7**, 163, doi:1475-2875-7-163 [pii]
478 10.1186/1475-2875-7-163 (2008).
479 27 Cohuet, A. *et al.* Species identification within the *Anopheles funestus* group
480 of malaria vectors in Cameroon and evidence for a new species. *American*
481 *Journal of Tropical Medicine and Hygiene* **69**, 200-205 (2003).
482 28 Kengne, P. *et al.* Molecular differentiation of three closely related
483 members of the mosquito species complex, *Anopheles moucheti*, by
484 mitochondrial and ribosomal DNA polymorphism. *Medical and Veterinary*
485 *Entomology* **21**, 177-182 (2007).
486 29 Kengne, P., Awono-Ambene, P., Nkondjio, C. A., Simard, F. & Fontenille, D.
487 Molecular identification of the *Anopheles nili* group of African malaria
488 vectors. *Medical and Veterinary Entomology* **17**, 67-74,
489 doi:doi:10.1046/j.1365-2915.2003.00411.x (2003).
490 30 Rahola, N. *et al.* Description of Anopheles gabonensis, a new species
491 potentially involved in rodent malaria transmission in Gabon, Central
492 Africa. *Infection Genetics and Evolution* **28**, 628-634,
493 doi:10.1016/j.meegid.2014.05.012 (2014).
494 31 Ahmed, M. Z., De Barro, P. J., Ren, S. X., Greeff, J. M. & Qiu, B. L. Evidence for
495 horizontal transmission of secondary endosymbionts in the Bemisia
496 tabaci cryptic species complex. *PLoS One* **8**, e53084,
497 doi:10.1371/journal.pone.0053084 (2013).
498 32 Li, S. J. *et al.* Plant-mediated horizontal transmission of Wolbachia
499 between whiteflies. *The ISME journal* **11**, 1019-1028,
500 doi:10.1038/ismej.2016.164 (2017).
501 33 Werren, J. H., Zhang, W. & Guo, L. R. Evolution and phylogeny of
502 Wolbachia: reproductive parasites of arthropods. *Proc Biol Sci* **261**, 55-63,
503 doi:10.1098/rspb.1995.0117 (1995).
504 34 Sinkins, S. P., Braig, H. R. & Oneill, S. L. WOLBACHIA SUPERINFECTIONS
505 AND THE EXPRESSION OF CYTOPLASMIC INCOMPATIBILITY. *Proceedings*
506 *of the Royal Society B-Biological Sciences* **261**, 325-330,
507 doi:10.1098/rspb.1995.0154 (1995).
508 35 Casiraghi, M., Anderson, T. J. C., Bandi, C., Bazzocchi, C. & Genchi, C. A
509 phylogenetic analysis of filarial nematodes: comparison with the
510 phylogeny of Wolbachia endosymbionts. *Parasitology* **122**, 93-103,
511 doi:10.1017/s0031182000007149 (2001).
512 36 Simon, F. *et al.* Human and Animal Dirofilariasis: the Emergence of a
513 Zoonotic Mosaic. *Clinical Microbiology Reviews* **25**, 507-+,
514 doi:10.1128/cmr.00012-12 (2012).
515 37 Baldo, L. *et al.* Multilocus sequence typing system for the endosymbiont
516 Wolbachia pipientis. *Appl Environ Microbiol* **72**, doi:10.1128/aem.00731-
517 06 (2006).
518 38 Richardson, M. F. *et al.* Population Genomics of the Wolbachia
519 Endosymbiont in Drosophila melanogaster. *PLOS Genetics* **8**, e1003129,
520 doi:10.1371/journal.pgen.1003129 (2012).
521 39 Charlat, S. *et al.* The joint evolutionary histories of Wolbachia and
522 mitochondria in Hypolimnas bolina. *BMC Evolutionary Biology* **9**, 64,
523 doi:10.1186/1471-2148-9-64 (2009).

40 Engelstadter, J. & Hurst, G. D. D. The Ecology and Evolution of Microbes that Manipulate Host Reproduction. *Annual Review of Ecology Evolution and Systematics* **40**, 127-149, doi:10.1146/annurev.ecolsys.110308.120206 (2009).

41 Dumas, E. *et al.* Population structure of Wolbachia and cytoplasmic introgression in a complex of mosquito species. *Bmc Evolutionary Biology* **13**, doi:10.1186/1471-2148-13-181 (2013).

42 Arthofer, W., Riegler, M., Avtzis, D. N. & Stauffer, C. Evidence for low-titre infections in insect symbiosis: Wolbachia in the bark beetle Pityogenes chalcographus (Coleoptera, Scolytinae). *Environmental Microbiology* **11**, 1923-1933, doi:10.1111/j.1462-2920.2009.01914.x (2009).

43 Augustinos, A. A. *et al.* Detection and Characterization of Wolbachia Infections in Natural Populations of Aphids: Is the Hidden Diversity Fully Unraveled? *Plos One* **6**, doi:10.1371/journal.pone.0028695 (2011).

44 Woolfit, M., Iturbe-Ormaetxe, I., McGraw, E. A. & O'Neill, S. L. An Ancient Horizontal Gene Transfer between Mosquito and the Endosymbiotic Bacterium Wolbachia pipientis. *Mol. Biol. Evol.* **26**, 367-374, doi:10.1093/molbev/msn253 (2009).

45 Dunning Hotopp, J. C. *et al.* Widespread lateral gene transfer from intracellular bacteria to multicellular eukaryotes. *Science* **317**, 1753-1756, doi:10.1126/science.1142490 (2007).

46 Robert, V., Ayala, D. & Simard, F. in *Entomologie médicale et vétérinaire* (eds G. Duvallet, D. Fontenille, & V. Robert) (IRD Editions, 2017).

47 Clements, A. N. *The Biology of Mosquitoes: Development, Nutrition and Reproduction v. 1*. (CABI Publishing, 1999).

48 Simmons, T. W. & Hutchinson, M. L. A Critical Review of All Known Published Records for Water Mite (Acari: Hydrachnidiae) and Mosquito (Diptera: Culicidae) Parasitic Associations From 1975 to Present. *Journal of Medical Entomology* **53**, 737-752, doi:10.1093/jme/tjw052 (2016).

49 Le Clec'h, W. *et al.* Cannibalism and Predation as Paths for Horizontal Passage of Wolbachia between Terrestrial Isopods. *Plos One* **8**, doi:10.1371/journal.pone.0060232 (2013).

50 Plantard, O. *et al.* Detection of Wolbachia in the Tick Ixodes ricinus is Due to the Presence of the Hymenoptera Endoparasitoid Ixodiphagus hookeri. *Plos One* **7**, doi:10.1371/journal.pone.0030692 (2012).

51 Pombi, M. *et al.* Dissecting functional components of reproductive isolation among closely related sympatric species of the Anopheles gambiae complex. *Evolutionary Applications* **10**, 1102-1120, doi:10.1111/eva.12517 (2017).

52 Tsutsui, N. D., Kauppinen, S. N., Oyafuso, A. F. & Grosberg, R. K. The distribution and evolutionary history of Wolbachia infection in native and introduced populations of the invasive argentine ant (Linepithema humile). *Molecular Ecology* **12**, 3057-3068, doi:10.1046/j.1365-294X.2003.01979.x (2003).

53 Gerth, M., Gansauge, M. T., Weigert, A. & Bleidorn, C. Phylogenomic analyses uncover origin and spread of the Wolbachia pandemic. *Nature Communications* **5**, doi:10.1038/ncomms6117 (2014).

571 54 Makanga, B. *et al.* Ape malaria transmission and potential for ape-to-
572 human transfers in Africa. *PNAS* **113**, 5329-5334,
573 doi:10.1073/pnas.1603008113 (2016).
574 55 Paupy, C. *et al.* Anopheles moucheti and Anopheles vinckei Are Candidate
575 Vectors of Ape Plasmodium Parasites, Including Plasmodium
576 praefalciparum in Gabon. *PLoS ONE* **8**, doi:e57294
577 10.1371/journal.pone.0057294 (2013).
578 56 Hughes, G. L., Rivero, A. & Rasgon, J. L. Wolbachia Can Enhance
579 Plasmodium Infection in Mosquitoes: Implications for Malaria Control?
580 *Plos Pathogens* **10**, doi:10.1371/journal.ppat.1004182 (2014).
581 57 Zele, F. *et al.* Wolbachia increases susceptibility to Plasmodium infection
582 in a natural system. *Proceedings of the Royal Society B-Biological Sciences*
583 **281**, doi:10.1098/rspb.2013.2837 (2014).
584 58 Blagrove, M. S. C., Arias-Goeta, C., Failloux, A. B. & Sinkins, S. P. Wolbachia
585 strain wMel induces cytoplasmic incompatibility and blocks dengue
586 transmission in Aedes albopictus. *Proceedings of the National Academy of*
587 *Sciences of the United States of America* **109**, 255-260,
588 doi:10.1073/pnas.1112021108 (2012).
589 59 Moreira, L. A. *et al.* A Wolbachia Symbiont in Aedes aegypti Limits
590 Infection with Dengue, Chikungunya, and Plasmodium. *Cell* **139**, 1268-
591 1278, doi:10.1016/j.cell.2009.11.042 (2009).
592 60 Gillies, M. T. & de Meillon, B. *The anophelinae of Africa, south of the Sahara*.
593 Vol. 54 (The South African Institute for Medical Research, 1968).
594 61 Fanello, C., Santolamazza, F. & della Torre, A. Simultaneous identification
595 of species and molecular forms of the *Anopheles gambiae* complex by PCR-
596 RFLP. *Medical and Veterinary Entomology* **16**, 461-464 (2002).
597 62 Ndo, C. *et al.* Population genetic structure of the malaria vector *Anopheles*
598 *nili* in sub-Saharan Africa. *Malaria Journal* **9**, doi:161
599 10.1186/1475-2875-9-161 (2010).
600 63 Baldo, L. *et al.* Multilocus sequence typing system for the endosymbiont
601 Wolbachia pipientis. *Applied and Environmental Microbiology* **72**, 7098-
602 7110, doi:10.1128/aem.00731-06 (2006).
603 64 Kearse, M. *et al.* Geneious Basic: An integrated and extendable desktop
604 software platform for the organization and analysis of sequence data.
605 *Bioinformatics* **28**, 1647-1649, doi:10.1093/bioinformatics/bts199
606 (2012).
607 65 Stamatakis, A. RAxML version 8: a tool for phylogenetic analysis and post-
608 analysis of large phylogenies. *Bioinformatics* **30**, 1312-1313,
609 doi:10.1093/bioinformatics/btu033 (2014).
610 66 Stamatakis, A. in *International Parallel and Distributed Processing*
611 *Symposium.* (IEEE/ACM).
612 67 Letunic, I. & Bork, P. Interactive Tree Of Life (iTOL): an online tool for
613 phylogenetic tree display and annotation. *Bioinformatics* **23**, 127-128,
614 doi:10.1093/bioinformatics/btl529 (2007).
615 68 Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*. (Springer
616 Publishing Company, Incorporated, 2009).
617 69 Boundenga, L. *et al.* Haemosporidian Parasites of Antelopes and Other
618 Vertebrates from Gabon, Central Africa. *PLoS One* **11**, e0148958,
619 doi:10.1371/journal.pone.0148958 (2016).

70 Hamon, J. & Mouchet, J. Secondary vectors of human malaria in Africa. *Medecine tropicale : revue du Corps de sante colonial* **21**, 643-660 (1961).

71 Olson, D. M. *et al.* Terrestrial ecoregions of the worlds: A new map of life on Earth. *Bioscience* **51**, 933-938, doi:10.1641/0006-3568(2001)051[0933:teotwa]2.0.co;2 (2001).

72 Neafsey, D. E. *et al.* Highly evolvable malaria vectors: The genomes of 16 Anopheles mosquitoes. *Science* **347**, 43-+, doi:10.1126/science.1258522 (2015).

630 **Figures and Tables**

631

632 **Table 1**. Summary of the *Anopheles* species screened in this study.

Group/complex	Species	Malaria role	Infected	Tested	Infection (%)
gambiae	*An. gambiae*	H	5	44	11
	An. coluzzii	H	2	58	3
moucheti	*An. brunnipes*		0	1	0
	An. cinctus		0	2	0
	An. moucheti	H, P, A	30	42	71
	An. nigeriensis	h	1	27	4
	An. "GAB-2"		5	8	63
	An. "GAB-3"		1	1	100
funestus	*An. gabonensis*	A	0	29	0
	An. funestus	H	2	37	5
	An. implexus		1	26	4
	An. jebudensis		1	2	50
	An. maculipalpis		0	29	0
nili	*An. nili*	H, A	11	19	58
	An. carnevalei	h, A	2	29	7
	An. "GAB-1"		0	19	0
coustani	*An. hancocki*	h	1	41	2
	An. theileri	h	0	24	0
	An. rodhesiensis		0	4	0
	An. coustani	h, A	2	35	6
	An. paludis	h, A	1	16	6
	An. gr coustani	h	0	51	0
	An. squamosus		0	32	0
	An. marshallii	h, P, A	2	42	5
	An. vinckei	P, A	3	30	10
			70	**648**	

633

634 Malaria role: known role for each species in malaria transmission [46,54,69,70] in humans

635 (H: major, h: secondary), primates (P), other animals (A), or unknown (blank).

636

637 **Figure 1**. Sampling sites and *Wolbachia* infection prevalence.

638 Map of Gabon showing the main African habitat types ([71], freely available at
639 http://maps.tnc.org/gis_data.html) and the sampled villages (black dots) was done
640 using ArcGIS Basic v.10. The prevalence of *Wolbachia* infection (number of infected
641 *Anopheles* species and individuals) per site is presented in bar charts. The pink colour
642 indicates positive species/individuals and blue the total number of species/individuals
643 screened for *Wolbachia* infection at that site. CCB: Cocobeach; LOP: Lopé; MKG:
644 Mikongo; BTK: National Park of Plateaux Batékés; FCV: Franceville; LBV:
645 Libreville; MKB: National Park of Moukalaba-Doudou; BKB: Bakoumba.

646

647 **Figure 2**. Probability of detecting *Wolbachia* infection.

648 The probability was estimated for each sample size an infection prevalence value. The
649 probabilityof correct estimation follows a black-blue gradient. Dashed-line delimits
650 the probability of infection detection at 90%.

651

652 **Figure 3.** Circular phylograms for the 16 *Anopheles* species that were infected with
653 *Wolbachia*.

654 The phylogenetic trees were built with RAxML [65]. The names of the *Anopheles*
655 species from which the *Wolbachia*-specific sequences were isolated in this study are
656 shown in blue (positive for *Wolbachia* supergroup B), red (positive for supergroup A)
657 and brown (positive for supergroup C), while the names of mosquitoes species
658 (*Diptera*) from which the previously published *Wolbachia* sequences were isolated
659 are in green. Red dots show branches supporting a bootstrap >70% from 1000
660 replicates.. (A) Circular phylogenetic tree using the *Wolbachia*-specific *16S* rRNA
661 fragment and *Anaplasma marginale* as outgroup. Different *Wolbachia* strains found in

662 the same *Anopheles* species are connected by pink lines. Pink bar charts indicate the
663 number of identical *Wolbachia* haplotypes found in each species. Scale bar
664 corresponds to nucleotide substitutions per site. (B) Circular phylogenetic trees based
665 on *coxA, fbpA and ftsZ* fragment sequences using *Dirofilaria immitis* (supergroup C)
666 as outgroup. Specimens with a different supergroup assignation than *16S* are marked
667 with asterisks.
668
669 **Figure 4**. Maximum likelihood phylogeny of the 25 *Anopheles* species under study
670 and *Wolbachia* haplotypes.
671 The tree was inferred with RAxML [65] using the sequences of *COII* fragments from
672 176 *Anopheles* specimens belonging to the 25 species under study and rooted with
673 *Anopheles darlingi* as outgroup (New World mosquito, diverged 100 Myr ago [72]). Red
674 dots in branches represent bootstrap values >70% from 1000 replicates. The shape of
675 each field column represent the *16S* (rectangle), *coxA* (rhombus), *fbpA* (triangle) and
676 *ftsZ* (hexagon) genes. The different *Wolbachia* gene haplotypes are indicated with
677 colour codes (all pink = the newly identified wANMO strain). The bar chart size
678 indicates the number of specimens for each haplotype and the colour their infection
679 status: grey, non-infected; blue, infected by the *Wolbachia* supergroup B; red, infected
680 by supergroup A; brown, infected by supergroup C.
681
682
683

684 **Supplementary Materials**

685 **Table S1.** Mosquitoes screened in this study and their accession numbers.

686 ID: Specimen identification name; Sites: Collection locations; Species: Morphological
687 and molecular identification; COII_hap: COII haplotype for each species; COII:
688 accession number for cytochrome oxidase subunit II gene; 16S: accession number for
689 16S rRNA gene; ftsZ: accession number for filamenting temperature-sensitive mutant
690 Z protein; fbpA: accession number fructose-bisphosphate putative aldolase protein;
691 coxA: accession number for cytochrome c oxidase subunit I.

692

693 **Text S1.** Mosquito taxonomic and molecular identification.

694

695 **Table S2**. *Anopheles moucheti* F1 used to estimate vertical transmission.

696

697 **Figure S1**. Rooted maximum likelihood phylogeny of the filarial *Wolbachia* sequence
698 isolated from one *An. coustani* specimen.

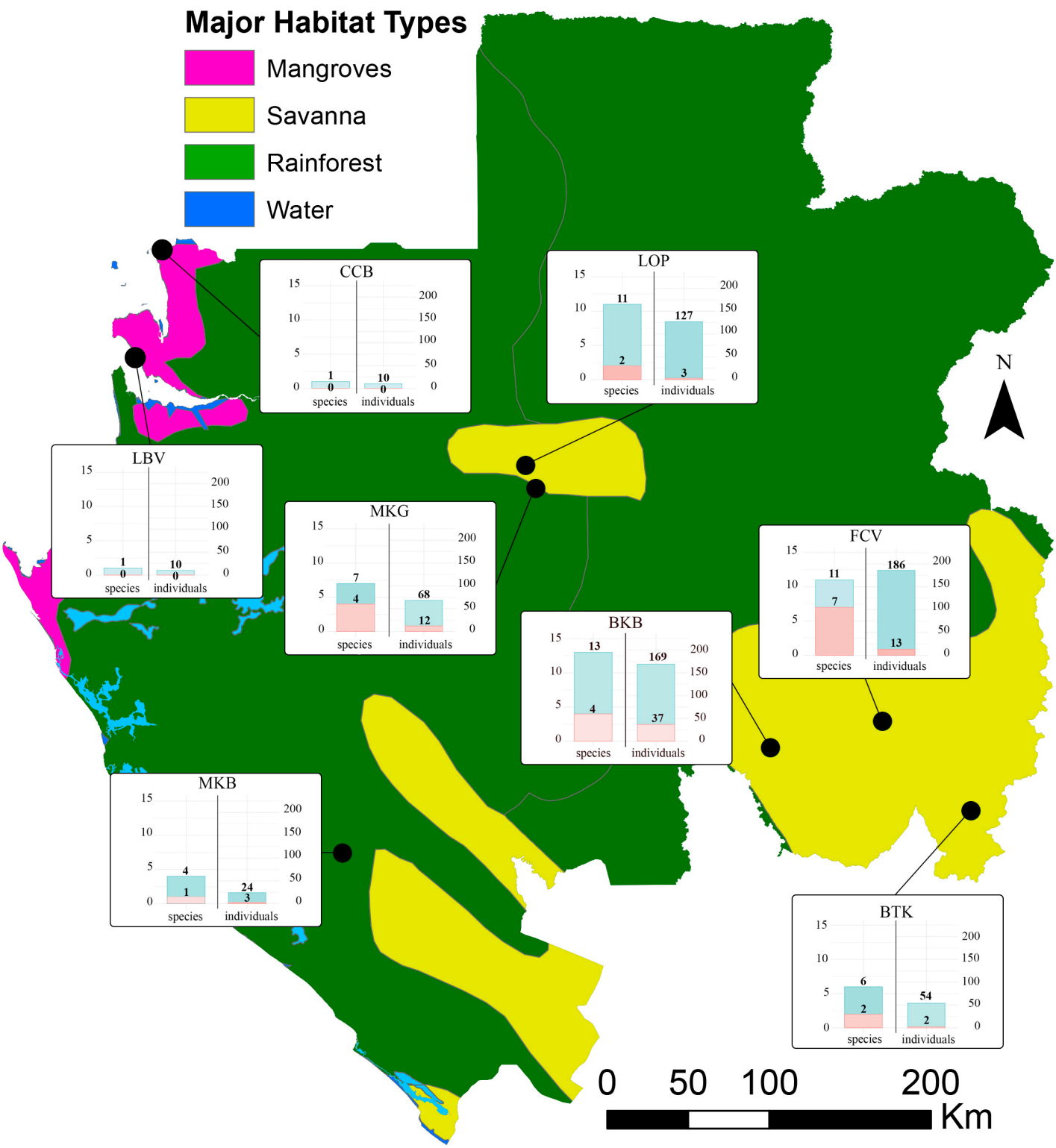
699 The tree was inferred with RAxML [65] using the sequence of the filarial *COII* fragment
700 amplified from the *An. coustani* specimen BNG78 (in blue) and public sequences
701 (NCBI) and rooted with *Brugia malayi* as outgroup. The black dot on the branch
702 indicate a bootstrap value >70% from 1000 replicates.

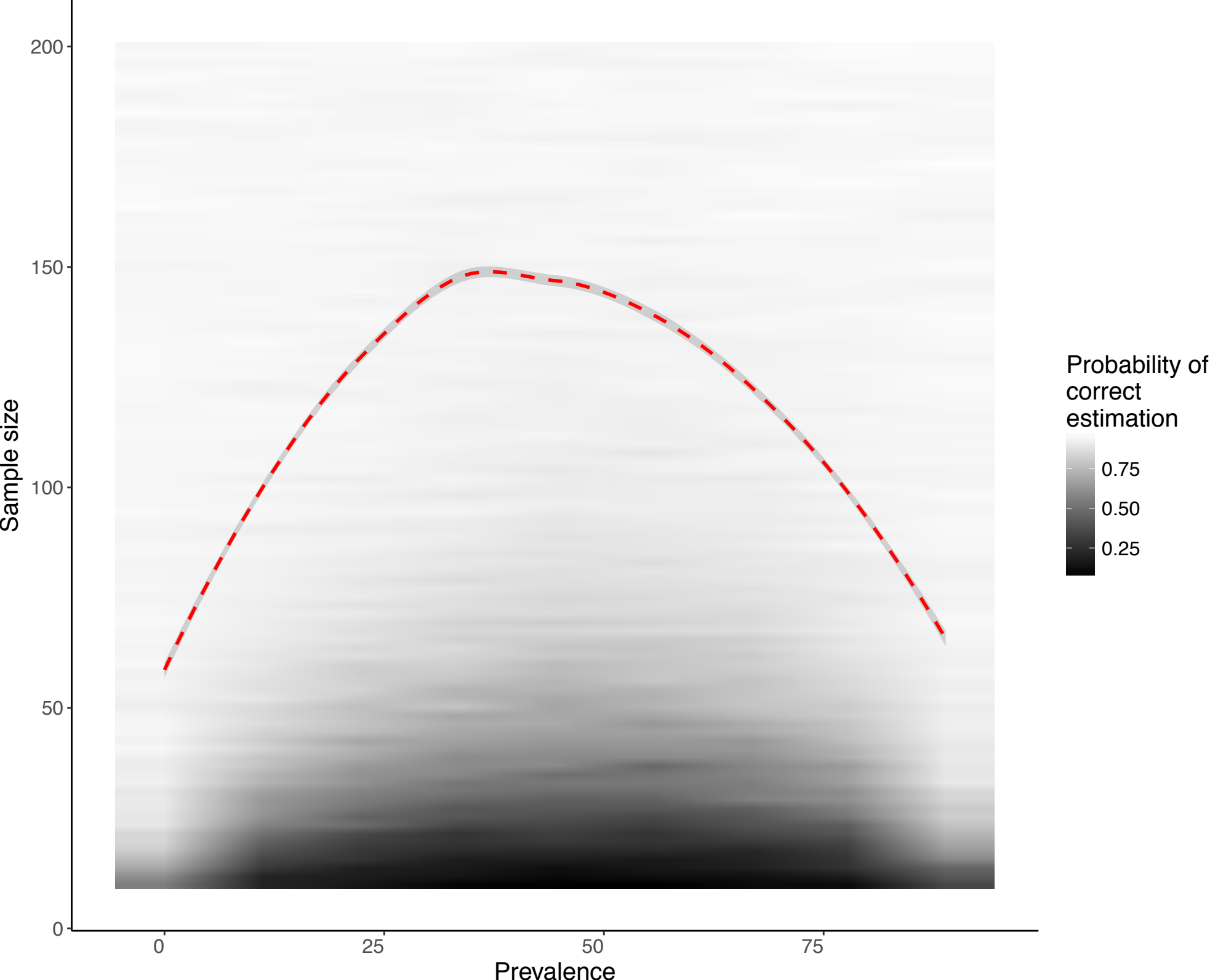
703

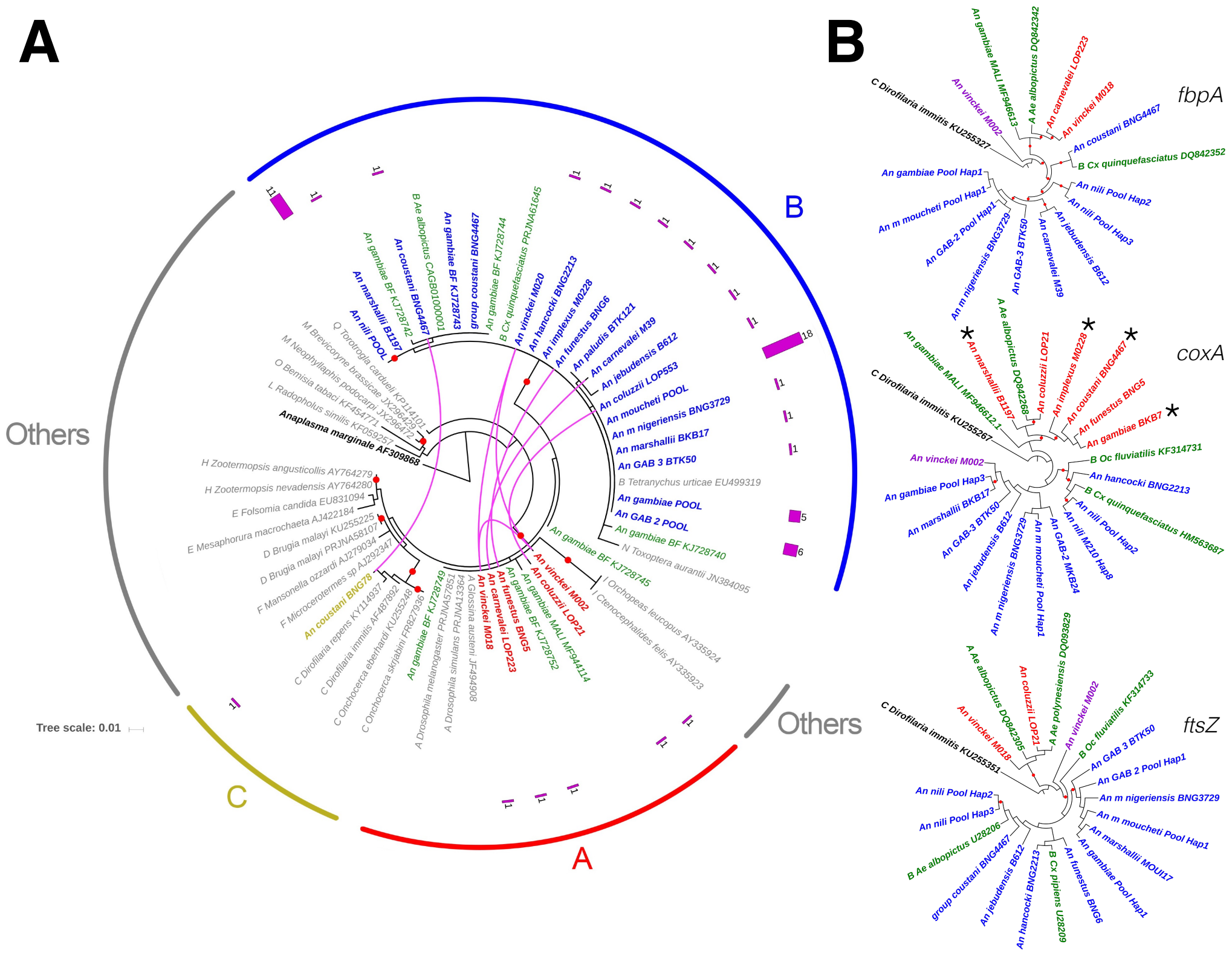
704 **Figure S2**. Scatterplot showing the genetic distances between *Wolbachia* strains (*16S*)
705 and infected *Anopheles* species *(COII)*.

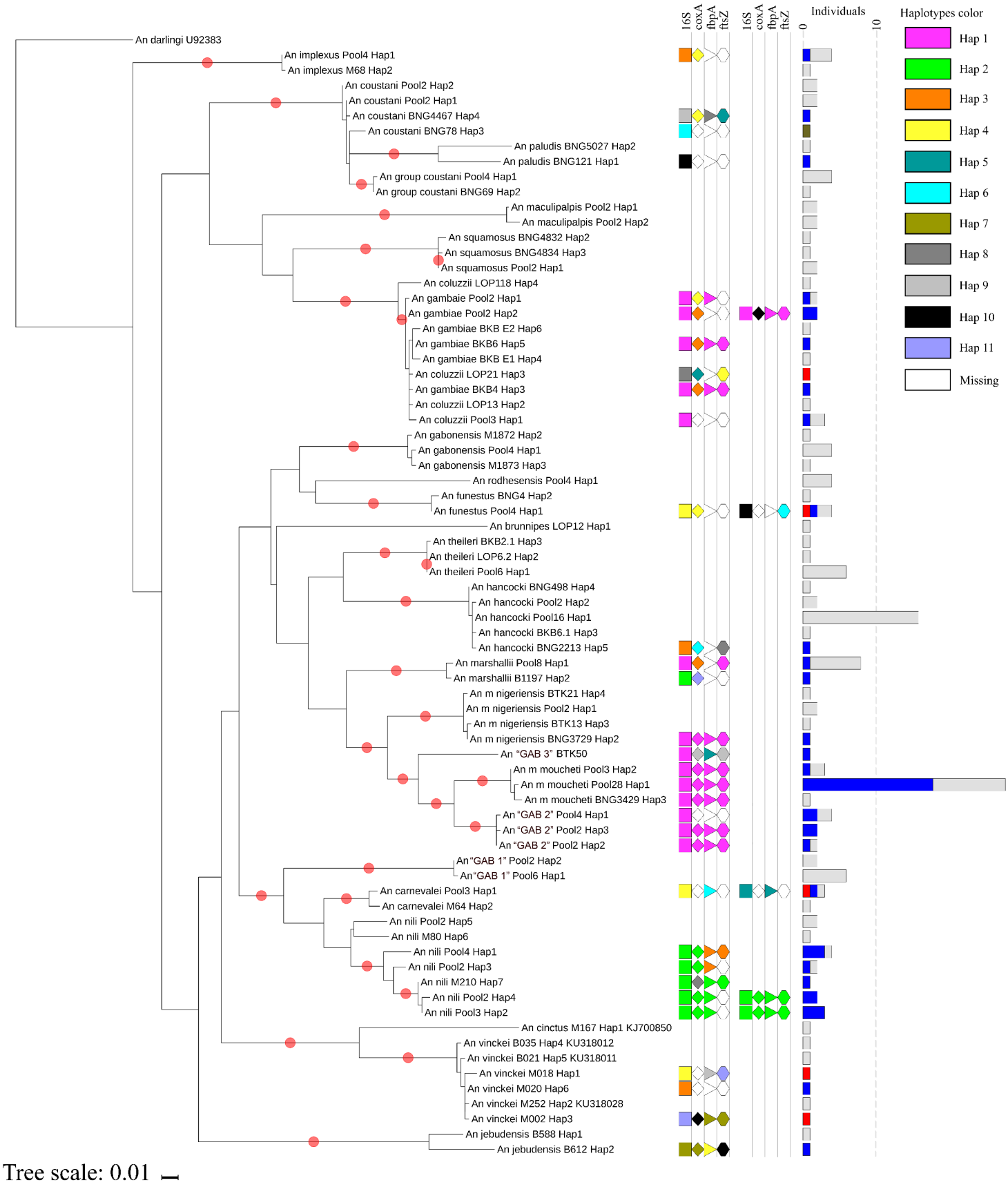
706 Genetic distances were estimated as the number of different bases between the
707 sequences of each pair of infected *Anopheles* specimens. The smoothed conditional

mean (blue line) and the 95% confidence intervals (grey area) were plotted using the smoothing "gam" function of the ggplo2 library [68].


Major Habitat Types
Mangroves
Savanna
Rainforest
Water
CCB
species 1 0
individuals 10 0
LOP
species 11 2
individuals 127 3
LBV
species 1 0
individuals 10 0
MKG
species 7 4
individuals 68 12
FCV
species 11 7
individuals 186 13
BKB
species 13 4
individuals 169 37
MKB
species 4 1
individuals 24 3
BTK
species 6 2
individuals 54 2
N
0 50 100 200
Km


Sample size
200
150
100
50
0
0
25
50
75
Prevalence
Probability of correct estimation
0.75
0.50
0.25


A
B
Others
Others
C
A
Tree scale: 0.01
An gambiae BF KJ728743
group coustani BNG4467
An gambiae BF KJ728744
B Cx quinquefasciatus PRJNA61645
An vinckei M020
An hancocki BNG2213
An implexus M0228
An funestus BNG6
An paludis BTK121
An carnevalei M39
An jebudensis B612
An coluzzii LOP553
An moucheti POOL
An m nigeriensis BNG3729
An marshallii BKB17
An GAB 3 BTK50
B Tetranychus urticae EU499319
An gambiae POOL
An GAB 2 POOL
An gambiae BF KJ728740
N Toxoptera aurantii JN384095
An gambiae BF KJ728745
I Orchopeas leucopus AY335924
I Ctenocephalides felis AY335923
An vinckei M002
An coluzzii LOP21
An gambiae MALI MF944114
An gambiae BF KJ728752
An funestus BNG5
An carnevalei LOP223
An vinckei M018
A Glossina austeni JF494908
A Drosophila simulans PRJNA13364
A Drosophila melanogaster PRJNA57851
An gambiae BF KJ728749
C Onchocerca skrjabini FR827936
C Onchocerca eberhardi KU255248
C Dirofilaria immitis AF487892
C Dirofilaria repens KY114937
An coustani BNG78
F Microcerotermes sp AJ292347
F Mansonella ozzardi AJ279034
D Brugia malayi PRJNA58107
D Brugia malayi KU255225
E Mesaphorura macrochaeta AJ422184
E Folsomia candida EU831094
H Zootermopsis nevadensis AY764280
H Zootermopsis angusticollis AY764279
Anaplasma marginale AF309868
L Radopholus similis KF059257
O Bemisia tabaci KF454771
M Neophyllaphis podocarpi JX296472
M Brevicoryne brassicae JX296429
Q Torotrogla cardueli KP114101
An nili POOL
An marshallii B1197
An gambiae BF KJ728742
An coustani BNG4467
B Ae albopictus CAGB01000001
fbpA
An gambiae MALI MF946613
A Ae albopictus DQ842342
An carnevalei LOP223
An vinckei M018
An coustani BNG4467
B Cx quinquefasciatus DQ842352
An nili Pool Hap2
An nili Pool Hap3
An jebudensis B612
An carnevalei M39
An GAB-3 BTK50
An m nigeriensis BNG3729
An GAB-2 Pool Hap1
An m moucheti Pool Hap1
An gambiae Pool Hap1
C Dirofilaria immitis KU255327
An vinckei M002
coxA
A Ae albopictus DQ842268
An coluzzii LOP21
An implexus M0228
An coustani BNG4467
An funestus BNG5
An gambiae BKB7
An marshallii B1197
An gambiae MALI MF946612.1
C Dirofilaria immitis KU255267
B Oc fluviatilis KF314731
An hancocki BNG2213
B Cx quinquefasciatus HM563687
An nili Pool Hap2
An nili M210 Hap8
An GAB-2 MKB24
An m moucheti Pool Hap1
An m nigeriensis BNG3729
An jebudensis B612
An GAB-3 BTK50
An marshallii BKB17
An gambiae Pool Hap3
An vinckei M002
ftsZ
A Ae albopictus DQ842305
An coluzzii LOP21
A Ae polynesiensis DQ093829
An vinckei M002
B Oc fluviatilis KF314733
An GAB 3 BTK50
An GAB 2 Pool Hap1
An m nigeriensis BNG3729
An m moucheti Pool Hap1
An marshallii MOUI17
An gambiae Pool Hap1
An funestus BNG6
B Cx pipiens U28209
An hancocki BNG2213
An jebudensis B612
group coustani BNG4467
B Ae albopictus U28206
An nili Pool Hap3
An nili Pool Hap2
C Dirofilaria immitis KU255351
An vinckei M018


16S
coxA
fbpA
ftsZ
16S
coxA
fbpA
ftsZ
Individuals
0
10
Haplotypes color
Hap 1
Hap 2
Hap 3
Hap 4
Hap 5
Hap 6
Hap 7
Hap 8
Hap 9
Hap 10
Hap 11
Missing
An darlingi U92383
An implexus Pool4 Hap1
An implexus M68 Hap2
An coustani Pool2 Hap2
An coustani Pool2 Hap1
An coustani BNG4467 Hap4
An coustani BNG78 Hap3
An paludis BNG5027 Hap2
An paludis BNG121 Hap1
An group coustani Pool4 Hap1
An group coustani BNG69 Hap2
An maculipalpis Pool2 Hap1
An maculipalpis Pool2 Hap2
An squamosus BNG4832 Hap2
An squamosus BNG4834 Hap3
An squamosus Pool2 Hap1
An coluzzii LOP118 Hap4
An gambaie Pool2 Hap1
An gambiae Pool2 Hap2
An gambiae BKB E2 Hap6
An gambiae BKB6 Hap5
An gambiae BKB E1 Hap4
An coluzzii LOP21 Hap3
An gambiae BKB4 Hap3
An coluzzii LOP13 Hap2
An coluzzii Pool3 Hap1
An gabonensis M1872 Hap2
An gabonensis Pool4 Hap1
An gabonensis M1873 Hap3
An rodhesensis Pool4 Hap1
An funestus BNG4 Hap2
An funestus Pool4 Hap1
An brunnipes LOP12 Hap1
An theileri BKB2.1 Hap3
An theileri LOP6.2 Hap2
An theileri Pool6 Hap1
An hancocki BNG498 Hap4
An hancocki Pool2 Hap2
An hancocki Pool16 Hap1
An hancocki BKB6.1 Hap3
An hancocki BNG2213 Hap5
An marshallii Pool8 Hap1
An marshallii B1197 Hap2
An m nigeriensis BTK21 Hap4
An m nigeriensis Pool2 Hap1
An m nigeriensis BTK13 Hap3
An m nigeriensis BNG3729 Hap2
An "GAB 3" BTK50
An m moucheti Pool3 Hap2
An m moucheti Pool28 Hap1
An m moucheti BNG3429 Hap3
An "GAB 2" Pool4 Hap1
An "GAB 2" Pool2 Hap3
An "GAB 2" Pool2 Hap2
An "GAB 1" Pool2 Hap2
An "GAB 1" Pool6 Hap1
An carnevalei Pool3 Hap1
An carnevalei M64 Hap2
An nili Pool2 Hap5
An nili M80 Hap6
An nili Pool4 Hap1
An nili Pool2 Hap3
An nili M210 Hap7
An nili Pool2 Hap4
An nili Pool3 Hap2
An cinctus M167 Hap1 KJ700850
An vinckei B035 Hap4 KU318012
An vinckei B021 Hap5 KU318011
An vinckei M018 Hap1
An vinckei M020 Hap6
An vinckei M252 Hap2 KU318028
An vinckei M002 Hap3
An jebudensis B588 Hap1
An jebudensis B612 Hap2
Tree scale: 0.01