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

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

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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].

Yet, since 2014, *An. gambiae, An. coluzzii* and *An. arabiensis* (three major malaria vectors) populations from Burkina Faso and Mali (West Africa) were found naturally infected by *Wolbachia* [19-21]. Noteworthy, they showed a negative correlation between *Wolbachia* infection and *Plasmodium* development [20,21]. These findings support the development of novel vector control strategies based on *Wolbachia-Anopheles* interactions. However, although *Wolbachia* naturally infects 40% - 60% of arthropods [22,23], infection was delimited to *Anopheles* species within the *gambiae* complex. Indeed, during the last decade, many other malaria mosquito species worldwide (n=38) were unsuccessfully screened for *Wolbachia* infection [9,18,24]. Therefore, the recent discovery of *Wolbachia* infecting species within the gambiae complex across West Africa couple with the development of new molecular tools, make necessary to screen other natural occurring *Anopheles* populations for the presence of the bacterium.

In this study, we investigated the presence of *Wolbachia* in 25 *Anopheles* species in Gabon, Central Africa. We sampled mosquitoes across the country and in a variety of ecological settings, from deep rainforest to urban habitats. By using a molecular approach, we confirmed *Wolbachia* presence in 16 species, including all the major malaria vectors in Central Africa (*An. gambiae, An. coluzzii, An. funestus, Anopheles nili* and *Anopheles moucheti*). The prevalence of *Wolbachia* infection was particularly high in *An. nili* and *An. moucheti*. Phylogenetic analysis revealed that all the infected mosquito species hosted *Wolbachia* bacteria belonging to the supergroups A or B that exhibit high genetic diversity. Finally, we explored the co-evolution between *Wolbachia* and *Anopheles*. The results have direct implications for the development of

new and environmentally friendly vector control strategies and open new directions for research on pathogen transmission and reproductive manipulation.

Results

Wolbachia naturally infects a large number of Anopheles species from Gabon.

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

sample size was lower than five individuals, the probability of a correct estimation of *Wolbachia* prevalence ranged between 68% and 93% (Fig 2).

Wolbachia is maternally inherited in An. moucheti.

Although *Wolbachia* is mainly maternally transmitted [12], horizontal transmission may occasionally occur in natural conditions [31-33]. To confirm the vertical transmission in the infected mosquito species, we focused on *An. moucheti* for logistic reasons (i.e., highest *Wolbachia* prevalence and ease of sampling). Although *An. moucheti* has never reproduced in insectary conditions to date, we obtained eggs from six *Wolbachia*-infected females that were placed individually in 1.5ml Eppendorf tubes for egg laying. In total, we analysed the infectious status of 79 progeny by PCR amplification of the same *16S* rRNA fragment [20] (Table S2) and found that 70 were infected, with an average vertical transmission frequency of 97.54% (range: 90% - 100%).

Naturally occurring *Wolbachia* strains in *Anopheles* reveal high genetic diversity.

By sequence analysis of the *16S* rRNA fragment PCR-amplified from each *Anopheles* sample (Table 1), we could assign the *Wolbachia* strains to three pre-existing supergroups: A (n=5), B (n=64) and C (n=1) (Fig 3). Specifically, we detected supergroup B *Wolbachia* in 64 mosquitoes belonging to all 16 infected *Anopheles* species. We found supergroup A *Wolbachia* in five individuals from four species (*An. funestus, An. coluzzii, An. vinckei* and *An. carnevalei*), thus providing examples of superinfection, as previously observed in *Ae. albopictus* [34] (Fig 3). Noteworthy, none of the mosquitoes examined was co-infected by *Wolbachia* strains belonging, for instance, to the supergroups A and B. Moreover, we confirmed that *Wolbachia*

previously identified in *An. gambiae s.l.* from Burkina Faso and Mali are included in the supergroups A and B [19,21]. Finally, we found that one *An. coustani* individual was infected by a *Wolbachia* strain from the supergroup C that is strictly known to infect filarial worms. We thus investigated the presence of filarial nematode DNA in the mosquito by PCR amplification and sequencing of a fragment of the *COI* filarial gene [35], followed by phylogenetic analysis with RAxML. Our results confirmed the presence of *Dirofilaria immitis* in this specimen (Fig. S1). This canine filarial parasite hosts *Wolbachia* and is transmitted by many mosquitoes, including *Anopheles* [36]. Therefore, it is not surprising to find an *An. coustani* specimen infected by this filarial nematode. This specimen was excluded from further investigations.

To expand our knowledge on the *Wolbachia* strains that infect natural *Anopheles* populations, we PCR-amplified, sequenced and analysed the *16S* rRNA fragment and also fragments from three other conserved *Wolbachia* genes (*coxA, fbpA* and *ftsZ*) that are commonly used for strain typing and evolutionary studies [37] (Fig 3). We used a new nested PCR protocol (see *Methods*) for samples that could not be genotyped using the classical Multi Locus Sequence Typing (MLST) primers (Table S1). Our phylogenetic analyses confirm the *16S* results, assigning most of the species to supergroups A and B. Few exceptions (Fig 3, gene *coxA*) showed some incongruence with regard to *16S*. They suggest signals of recent recombination between the supergroups A and B, as it was previously demonstrated [37]. Detailed sequence analysis revealed that mosquito species belonging to the same group or complex (i.e., *An. moucheti* and *An. gambiae*) displayed a common *Wolbachia* haplotype (Fig. 3 and Fig. 4). Conversely, some species with lower prevalence displayed a variety of 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

required to detect *Wolbachia* in species with low infection rates, such as *Anopheles* (Fig 2). In general, our sampling effort was higher than in previous studies (n <30) [9,24], which could explain why we found more species infected. Moreover, local frequency variations among populations could also hinder the detection of *Wolbachia* infections [41]. For instance, we sampled *An. coluzzii* in three different sites, but we only found *Wolbachia*-infected mosquitoes at La Lopé (Fig 1, Table S1). Therefore, sampling in different localities at seasons would improve detection rates. Second, low-density W*olbachia* infections may not be detected in *Anopheles* by the routinely used molecular tools, as previously reported for other arthropods [42,43] and recently in *Anopheles gambiae* [21]. Our results indicate that conventional amplification (conventional PCR) analysis allowed detecting *Wolbachia* infection only in 6 of 16 species (*An. moucheti, An m. nigeriensis, An. "GAB-3", An nili, An. jebudensis* and *An. vinckei*), presumably with high *Wolbachia* density. Moreover, some *Anopheles* species with high *Wolbachia* infection rates, such as *An. moucheti* or *An. nili*, were never screened before this study [9].

Unexpectedly, our work revealed that *Anopheles* species are infected by different *Wolbachia* species. Although three previous studies reported *Wolbachia* infection in the *An. gambiae* germline [19-21], we do not know whether *Wolbachia* naturally invade and is maternally inherited in the infected mosquito species from Gabon. Indeed, horizontal gene transfer (resulting in the insertion of *Wolbachia* genes within the mosquito genome), or parasitism (e.g., by filarial nematodes) could explain the presence of *Wolbachia* in an organism without vertical transmission. However, the *Wolbachia* sequences we identified were genetically close to those found in other 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.*

aegypti and *Ae. albopictus*. In conclusion, our findings are merely the "tip of the iceberg" in the research field on *Wolbachia* use to control vector-borne diseases, particularly in malaria.

Methods

Research and ethics statements

Mosquitoes were collected in Gabon under the research authorization AR0013/16/MESRS/CENAREST/CG/CST/CSAR and the national park entry authorization AE16008/PR/ANPN/SE/CS/AEPN. Mosquito sampling using the human-landing catch (HLC) method was performed under the protocol 0031/2014/SG/CNE approved by the National Research Ethics Committee of Gabon.

Mosquito sampling and DNA extraction

Mosquitoes were sampled in eight sites across Gabon, Central Africa, from 2012 to 2016 (Fig 1, Table 1, Fig. S1). These sites included sylvatic (national parks) and anthropic habitats (villages and cities). Adult females were collected using CDC light traps, BG traps and HLC. Collected specimens were taxonomically identified according to standard morphological features [25,60]. Then, they were individually stored in 1.5 mL tubes at -20°C and sent to CIRMF for molecular analysis. When possible, at least 30 mosquitoes (from 1 to 58) for each *Anopheles* species from different sites were selected for genomic analysis. Total genomic DNA was extracted from the whole body using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Genomic DNA was eluted in 100 µL of TE buffer. 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

the standard primers, 2 µL of the obtained product was amplified again using internal primers specific for each gene: *coxA* (coxA_NF-2: 5'-TTTAACATGCGCGCAAAAGG-3'; coxA_NR-2: 5'-TAAGCCCAACAGTGAACATATG-3'), *ftsZ* (ftsZ_NF-2: 5'-ATGGGCGGTGGTACTGGAAC-3'; ftsZ_NR-2: 5'-AGCACTAATTGCCCTATCTTCT-3'), and *fbpA* (fbpA_NF-1: 5'-AGCTTAACTTCTGATCAAGCA-3'; fbpA_NR-1: 5'-TTCTTTTTCCTGCAAAGCAAG-3'). Cycling conditions for *coxA* and *ftsZ* were: 94°C for 5min followed by 36 cycles at 94°C for 15s, 55°C for 15s and 72°C for 30s, and a final extension step at 72°C for 10min. For *fbpA*, they were: 94°C for 5min followed by 36 cycles at 94°C for 30s, 59°C for 45s and 72°C for 90s, and a final extension step at 72°C for 10 min. The resulting fragments (*coxA*, 357bp; *fbpA*, 358-bp; and *ftsZ*, 424-bp) were sequenced bidirectionally by Genewiz (UK). The new sequences obtained in this study were submitted to GenBank (accession numbers XX to XX, Supplementary Table 1).

Phylogenetic and statistical analysis

All *Wolbachia* sequences for the *16S, coxA, fbpA* and *ftsZ* gene fragments and for *Anopheles* COII were corrected using *Geneious* R10 [64]. The resulting consensus sequences for each gene were aligned with sequences that represent the main known *Wolbachia* supergroups obtained from GenBank (see Table S1). Only unique haplotypes for each species were included in the analysis. Inference of phylogenetic trees was performed using the maximum likelihood (ML) method and RAxML [65] with a substitution model GTR + CAT [66] and 1000 bootstrapping replicates. Finally, all MLST *Wolbachi*a sequences were used to build phylogenetic trees using RAxML

(GTR+CAT model, 1000 bootstrapping replicates). Trees were visualized with iTOL v.3.4.3 [67].

To quantify the accuracy of the observed *Wolbachia* infection prevalence, the influence of sample size on its estimation was assessed. Assuming that *Wolbachia* prevalence within a host species follows a beta binomial distribution [23] yielding many species with a low or a high *Wolbachia* prevalence but few with an intermediate one, we quantified, for each sample size, the proportion of samples (over 1,000 realizations) that could yield an estimate that was not significantly different from the prevalence over the whole population with a z-test and a significance threshold at 95%. As expected, sample size can be small for very low or very high prevalence (60 individuals are enough in 95% of cases for these extreme prevalence), while it has to be much higher for intermediate prevalence values (up to 150 individuals for a prevalence close to 50%).

All statistical analyses were performed using "R" v3.2.5 (R Development Core Team, http://cran.r-project.org/), with the addition of the "ggplot2" library [68].

Author contributions

D.A., O.D. and C.P. designed the experiments. D.A., O.A., N.R. and P.K., performed the experiments. D.A., F.S., C.C., O.D and C.P. analysed the data. D.A. performed the sequencing analysis. D.A. and B.R. performed the statistical analysis. D.A., N.R., M.N., F.M., B.M., C.P. and F.P. provided samples for the analysis. D.A., O.D. and C.P. wrote the manuscript.

Competing interests

390 The authors declare no competing interests.

391

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398

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628

629

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
	An. brunnipes		0	1	0
	An. cinctus		0	2	0
moucheti	*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.

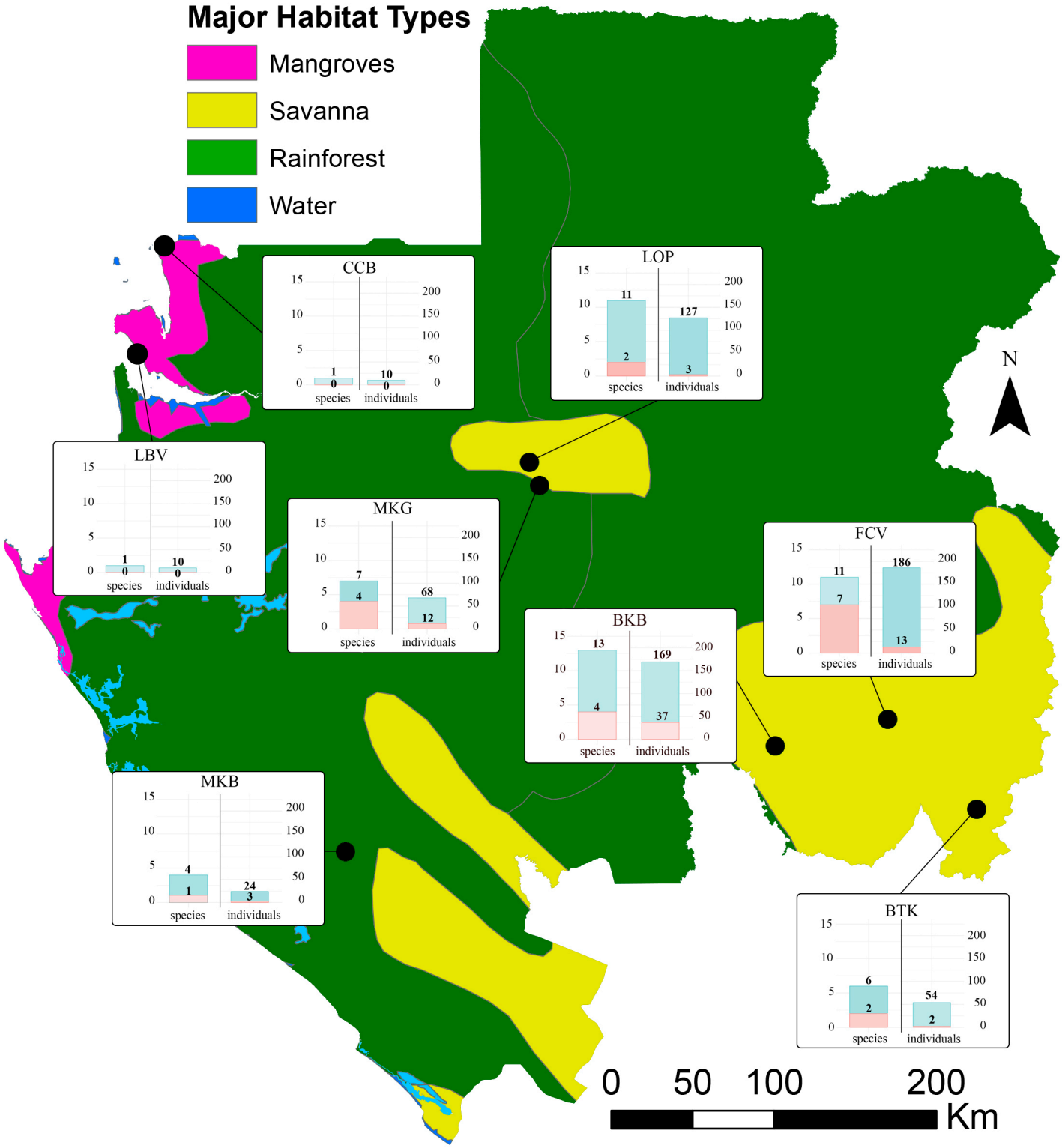
696

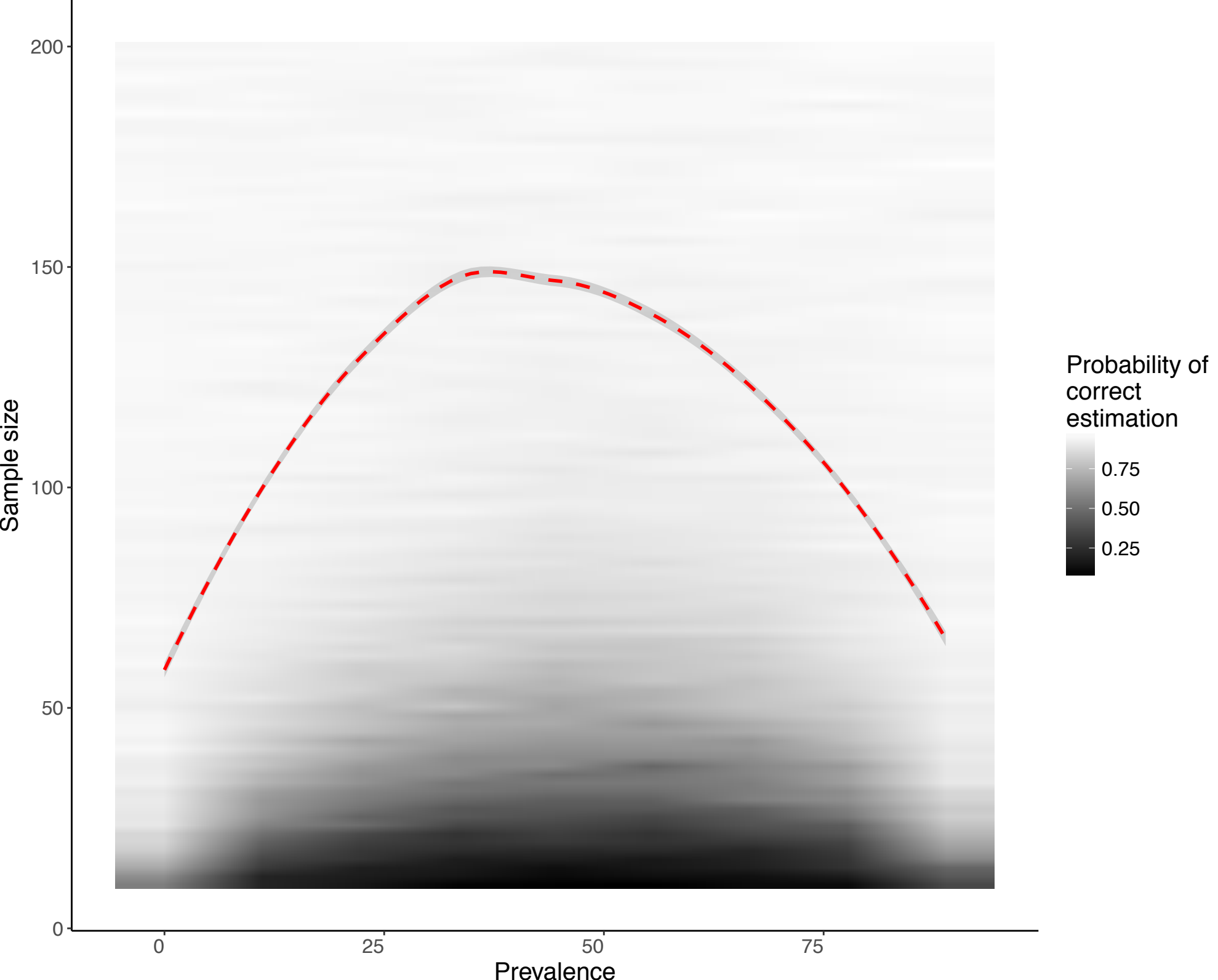
697 **Figure S1**. Rooted maximum likelihood phylogeny of the filarial *Wolbachia* sequence
698 isolated from one *An. coustani* specimen.
699 The tree was inferred with RAxML [65] using the sequence of the filarial *COII* fragment
700 amplified from the *An. coustani* specimen BNG78 (in blue) and public 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.

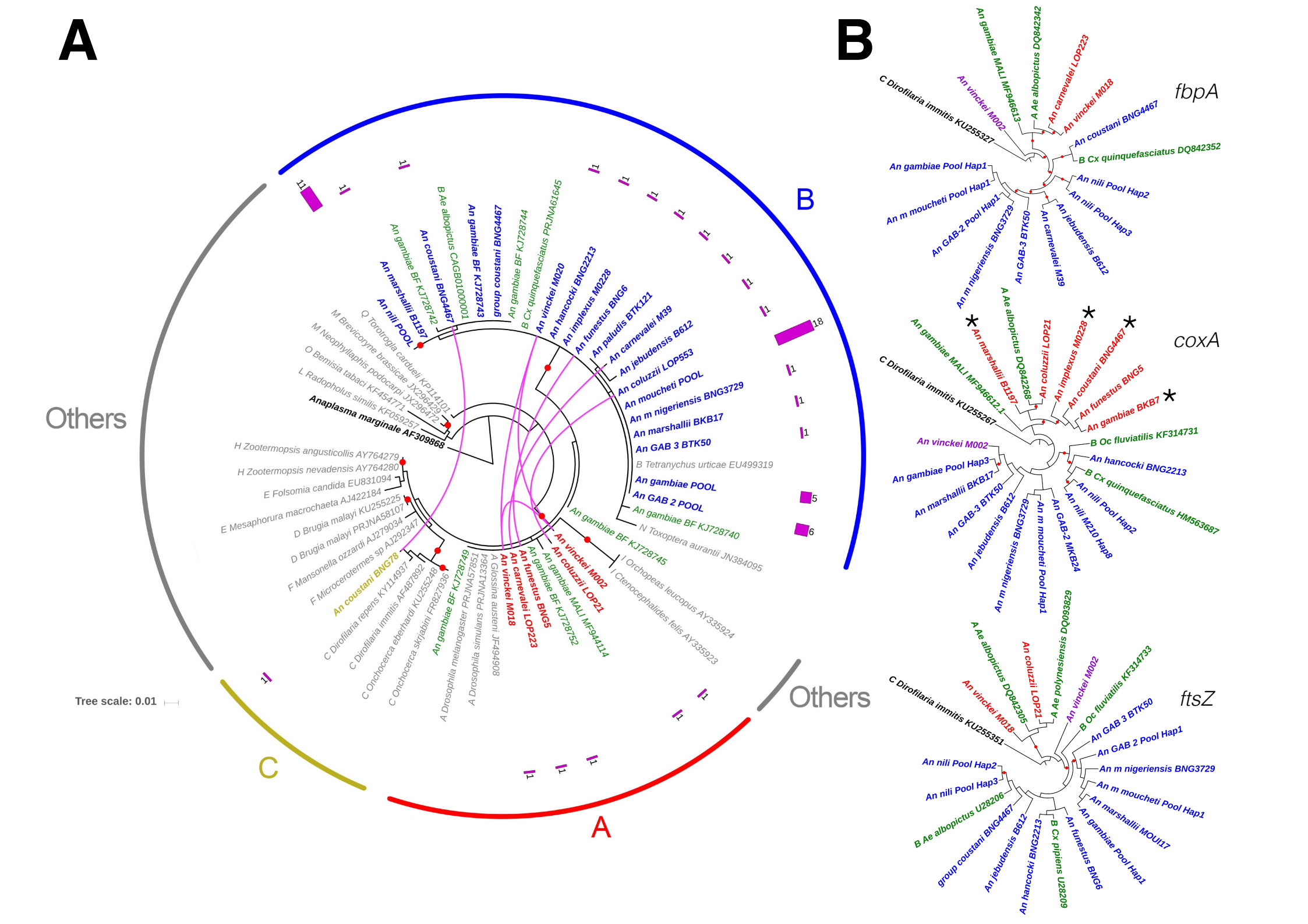
703

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].




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


A
B
Others
Others
C
A
B
Tree scale: 0.01
An gambiae MALI MF946613
An vinckei M002
A Ae albopictus DQ842342
An carnevalei LOP223
An vinckei M018
C Dirofilaria immitis KU255327
An coustani BNG4467
B Cx quinquefasciatus DQ842352
An gambiae Pool Hap1
An nili Pool Hap2
An m moucheti Pool Hap1
An nili Pool Hap3
An GAB-2 Pool Hap1
An m nigeriensis BNG3729
An GAB-3 BTK50
An carnevalei M39
An jebudensis B612
fbpA
An gambiae MALI MF946612.1
An marshallii B1197
A Ae albopictus DQ842268
An coluzzii LOP21
An implexus M0228
An coustani BNG4467
An funestus BNG5
An gambiae BKB7
C Dirofilaria immitis KU255267
B Oc fluviatilis KF314731
An vinckei M002
An hancocki BNG2213
An gambiae Pool Hap3
B Cx quinquefasciatus HM563687
An marshallii BKB17
An nili Pool Hap2
An GAB-3 BTK50
An nili M210 Hap8
An jebudensis B612
An m nigeriensis BNG3729
An GAB-2 MKB24
An m moucheti Pool Hap1
coxA
A Ae albopictus DQ842305
An coluzzii LOP21
A Ae polynesiensis DQ093829
An vinckei M018
An vinckei M002
B Oc fluviatilis KF314733
C Dirofilaria immitis KU255351
An GAB 3 BTK50
An GAB 2 Pool Hap1
An nili Pool Hap2
An m nigeriensis BNG3729
An nili Pool Hap3
An m moucheti Pool Hap1
B Ae albopictus U28206
An marshallii MOU17
group coustani BNG4467
An gambiae Pool Hap1
An jebudensis B612
An hancocki BNG2213
B Cx pipiens U28209
An funestus BNG6
ftsZ
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 gambiae BF KJ728752
An gambiae MALI MF944114
An vinckei M002
An coluzzii LOP21
An funestus BNG5
An gambiae BF KJ728749
An carnevalei LOP223
An vinckei M018
A Glossina austeni JF494908
A Drosophila simulans PRJNA13364
A Drosophila melanogaster PRJNA57851
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
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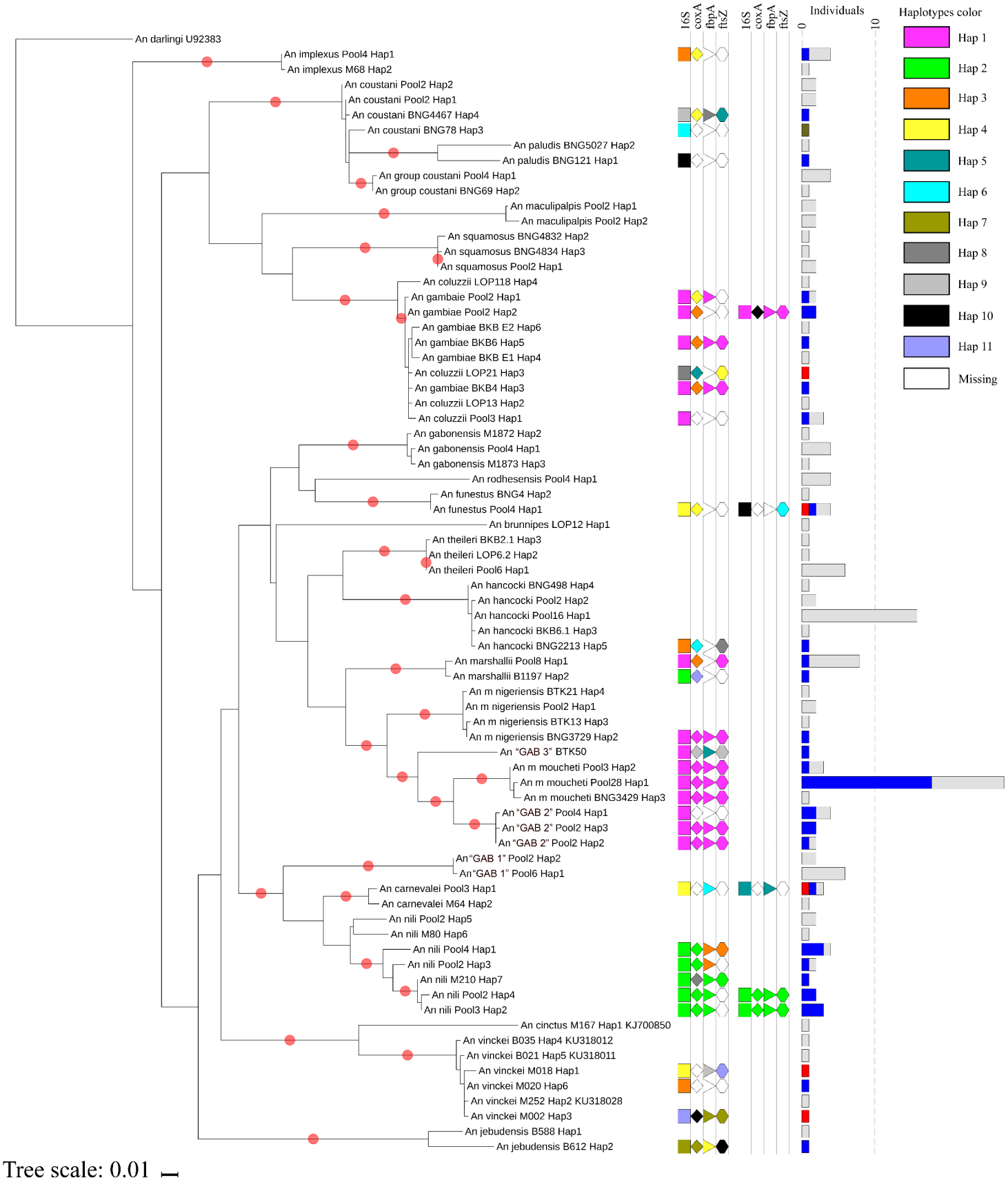
H Zootermopsis angusticollis AY764279
Anaplasma marginale AF309868
L Radopholus similis KF059257
O Bemisia tabaci KF454771
M Neophyllaphis podocarpi JX296472
M Brevicoryne brassicae JX296429
Q Torotrogla carduell KP114101
An marshallii B1197
An nili POOL
An gambiae BF KJ728742
An coustani BNG4467
B Ae albopictus CAGB01000001
An gambiae BF KJ728743
group coustani BNG4467



Tree scale: 0.01