

# Relative Contributions of Genetic and Non-Genetic Factors on Mosquito Microbiota

**Haikel Nasser Bogale**

University of Maryland Baltimore <https://orcid.org/0000-0002-1907-788X>

**Matthew V. Cannon**

University of Maryland Institute for Genome Sciences

**Kalil Keita**

Programme National de Lutte contre le Paludisme

**Denka Camara**

Programme National de Lutte contre le Paludisme

**Yaya Barry**

Programme National de Lutte contre le Paludisme

**Moussa Keita**

Programme National de Lutte contre le Paludisme

**Drissa Coulibaly**

Malaria Research and Training Center

**Abdoulaye K Kone**

Malaria Research and Training Center

**Ogobara K. Doumbo**

Malaria Research and Training Center

**Mahamadou A. Thera**

Malaria Research and Training Center

**Christopher V. Plowe**

Duke Global Health Institute

**Mark Travassos**

University of Maryland Center of Vaccine Development and Global Health

**Seth Irish**

Centers for Disease Control and Prevention

**David Serre** (✉ [DSerre@som.umaryland.edu](mailto:DSerre@som.umaryland.edu))

University of Maryland Institute for Genome Sciences

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Research

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# Abstract

## Background

The commensal microbiota of mosquitoes impacts their development, immunity, and competency, and could provide a target for alternative entomological control approaches. However, despite the importance of the mosquito/microbiota interactions, little is known about the relative contribution of genetic and non-genetic factors in shaping the bacterial communities of mosquitoes.

## Methods

We used a high-throughput sequencing based assay to characterize the bacterial composition and diversity of 665 individual field-caught mosquitoes, as well as their species, genotype at an insecticide resistance locus, blood meal composition, and the eukaryotic parasites and viruses they carry. We then used these data to rigorously estimate the individual effect of each parameter on the bacterial diversity as well as their relative contribution to the microbial composition.

## Results

Overall, multivariate analyses did not reveal any significant contribution of the mosquito species, insecticide resistance or blood meal to the bacterial composition of the mosquitoes surveyed. Infection with parasites and viruses only contributed very marginally and the main driver of the bacterial diversity was the location where each mosquito was collected which explained roughly 20% of the variance observed.

## Conclusions

This analysis shows that, when confounding factors are taken into account, the sites where the mosquitoes are collected is the main driver of the bacterial diversity of wild-caught mosquitoes, although further studies will be needed to determine how the specific components of the local environment affecting the bacterial composition.

## Background

*Aedes*, *Culex* and *Anopheles* mosquitoes can transmit eukaryotic parasites and viruses to humans and are responsible for devastating diseases such as malaria, lymphatic filariasis, dengue, West Nile, and Zika that affect hundreds of millions of people worldwide and cause hundreds of thousands of deaths annually [1]. Successful disease elimination campaigns have focused on interrupting transmission of these diseases by targeting their mosquito vectors. Thus, in the early twentieth century, vector control approaches primarily relied on the use of chemicals and larviciding tools (i.e., petroleum oils and

larvivorous fish) to eliminate larval and adult stages of the mosquito [2, 3]. Environmental and human health concerns brought by the persistent use of chemicals like Dichlorodiphenyltrichloroethane (DDT) over the years have partly led to the increased use of pyrethroids, which are safer alternatives, in vector control measures [3]. In the last three decades, entomological control strategies based on pyrethroid-treated bed nets and indoor residual spraying have been extensively used in the fight against vector-borne diseases with considerable success in reducing disease burden [4–6]. However, wide-spread use of these approaches, combined with exposure to agricultural pest control chemicals, have led to the emergence and rapid spread of insecticide-resistance alleles in many areas [7, 8]. In addition, several populations of mosquitoes have modified their behaviors (e.g., the host biting time [9, 10] and location [11, 12] or the host species preference [13, 14]) upon exposure to insecticides. This acquisition of chemical and behavioral resistance to insecticides threatens the advances made in control of mosquito-borne diseases and highlight the need for alternative measures.

One alternative to chemicals is to leverage biological agents to control mosquito populations (sometimes referred to as biological controls). For example, *Bacillus thuringiensis*, a spore-forming bacterium with larvicidal characteristics, is used extensively against disease-transmitting insects as well as agricultural pests [15]. In recent years, the use of *Wolbachia* as a biological control agent has also gained momentum, with studies revealing its ability to promote pathogen interference and to reduce the life span of mosquitoes [16–18]. Furthermore, recent findings have highlighted how modifications of the bacterial communities present in the midgut of mosquitoes could decrease or inhibit transmission of pathogens. For example, studies have demonstrated the importance of gut microbiota in mosquito larval development and shown that bacteria are required for *Aedes* mosquitoes to survive to the adult stages [19, 20]. Similarly, elimination of native microbiota resulted in delayed growth in *Anopheles* larvae [21]. Functional studies on adult stage mosquitoes showed that the gut microbiota can increase the resistance of mosquitoes to human pathogens by modulating the mosquito innate immune response [22, 23] or directly through production of anti-pathogen molecules from specific microbial species [24, 25]. Overall, these studies demonstrate the potential of microbiota manipulations for inhibiting pathogen transmission and/or reducing vector competence.

However, while these laboratory and field studies highlight the role of the mosquito midgut microflora in regulating the development and transmission of human pathogens, very little is known about the factors that shape the diversity of the bacterial composition in wild mosquitoes. Some studies have suggested that mosquito collection location is associated with the composition of the microbiota [26, 27] while others showed that the microbial composition differed between mosquito species, even when they are closely related and collected at the same location [28], or reared under the same conditions [20]. In addition, there is evidence that resistance to pesticides can be mediated by symbiont bacteria [29, 30], and therefore it is possible that genetic resistance to insecticides could also influence the microbial composition (e.g., resistance would allow exposure of the gut microbiota to insecticides and alter the composition of the microbiota). Lastly, the source of the blood-meal has also been shown to impact gut microbiota composition, with mammalian blood-meal source altering the gut bacterial composition of adult mosquitoes [31]. However, since these studies typically addressed only one of those factors at a

time (without correcting for confounding effects), the relative contribution of each of these parameters to shaping the midgut microflora composition of wild-caught mosquitoes remain unclear.

Here, we analyzed 665 individual *Anopheles* mosquitoes collected in Guinea and Mali. We characterized the microbial composition of these mosquitoes and screened them for a large variety of eukaryotic parasites and viruses. We also characterized the species of all mosquitoes, genotyped them at a major insecticide resistance locus and examined the source of their last blood meal. We then tested how these genetic and non-genetic factors influenced the bacterial composition, the species richness and the bacterial diversity, and simultaneously estimated the relative contribution of these factors to the mosquito microbiota.

## Methods

### Sample Collections

Mosquitoes were collected in Guinea by Human Landing Catches (HLC) and Pyrethrum Spray Catches (PSC) in 2017. The captured mosquitoes were placed in Eppendorf tubes containing ethanol and shipped to the University of Maryland School of Medicine for analysis. Mosquitoes were collected in Mali from homes in Bandiagara using PSC in 2011. The captured mosquitoes were placed in Carnoy's solution (1 volume of acetic acid for 3 volumes of ethanol) and shipped to the molecular biology laboratory of the Malaria Research and Training Center (MRTC) in Bamako, for DNA extraction. See Table 1 for details of sites/villages mosquitoes were collected and type of collection method used.

Table 1  
Mosquito collection locations, methods and numbers

Country	Site	Village	GPS Coordinates	Collection methods used	# of mosquitoes
Guinea	Kissidougou	Tongbekoro	9.294295, -10.147953	HLC	62
		Keredou	9.208919, -10.069525	HLC	53
		Gbangbadou	9.274363, -9.998639	HLC	3
	Kankan	Dalabani	10.463692, -9.451904	HLC/PSC	79
		Makono	10.291124, -9.363358	HLC/PSC	76
		Balandou	10.407669, -9.219096	HLC	25
	Faranah	Balayani	10.1325, -10.7443	HLC	80
	Dabola	Sognessa	10.739297, -11.130771	HLC	20
		Bissikrima	10.848375, -10.941491	HLC	27
		Saourou	10.715935, -11.066280	HLC	33
	Boffa	Walia	10.219103, -14.173951	HLC	100
	Mamou	Soyah	10.291308, -11.986196	HLC	14
		Sere	10.653212, -12.043033	HLC	26
		Daressalaam	10.409101, -12.179962	HLC	1
Mali	Bandiagara	Bandiagara	14.35005, -3.61038	PSC	81

Abbreviations: GPS, Global Positioning System; HLC, Human Landing Catch; PSC, Pyrethrum Spray Catch

## Guinea Mosquito DNA Extraction

DNA was extracted from each mosquito using a modified version of the Qiagen 96-well extraction protocol. Briefly, whole individual mosquitoes were placed in each well of a 96-well plate with five 1 mm RNASE free oxide beads for homogenization. Each mosquito was homogenized using a TissueLyser for 6

minutes at 20 m/s in the lysis buffers provided by the Qiagen DNeasy 96 Blood & Tissue Kit (Qiagen N.V., Hilden, Germany). The Plates were then centrifuged at 1,500 x *g* for 3 minutes. The homogenates were then incubated for 1 hour at 55 °C, spun again, and incubated overnight at 55 °C. After a final spin, the supernatant was transferred to a 96-Well DNeasy plate to bind the DNA. The columns were washed twice before elution of the DNA. Nanodrop was used to determine DNA concentration.

## Mali Mosquito DNA Extraction

DNA was extracted from the body section of each mosquito using Chelex-100 (Bio-Rad Laboratories, Hercules, California) protocol. Briefly, dissected individual mosquito sections were placed in separate 1.5 mL tubes containing deionized water. Pipette tips were used to grind each sample in the tube. Each sample was further homogenized in PBS (1X)/1% saponin solution, shaking gently for 20 minutes. The homogenates were then incubated at room temperature (25 °C) overnight. The tubes were then centrifuged at 20,000 x *g* for 2 minutes and supernatants were discarded. After washing with PBS (1X), each pellet was resuspended in deionized water and 20% Chelex-100 resin solution. This mixture was placed on a heating block for at least 10 minutes and stirred every 5 minutes. DNA was eluted after a final spin for 1 minute.

## PCR primers

DNA extracted from each mosquito was amplified using primers targeting bacterial 16S rRNA primers [32], mosquito *kdr-west* (L1014F) [33], *cox1* and S200 × 6.1 [34] loci, mammalian mitochondrial 16S rRNA sequences, as well as eukaryotic parasite and virus primers [35] (Additional file 1: Table S1).

## PCR amplification and sequencing

DNA extracted from individual mosquitoes (and 95 water control samples) were amplified separately with each primer pair using the Promega GoTaq DNA Polymerase with the following conditions: initial denaturing step at 95 °C for 2 minutes followed by 40 cycles of 95 °C for 30 seconds, 50 °C for 30 seconds and 72 °C for 30 seconds, and a final extension of 5 minutes. Only 35 cycles were used to amplify bacterial 16S rRNA. All PCR products generated from one DNA sample (i.e., individual mosquito) were pooled together and reamplified in a second PCR to add a unique barcode and the Illumina sequencing adaptors [35]. Finally, all barcoded products from all mosquitoes were pooled and sequenced simultaneously on Illumina HiSeq 2500 to generate 300 bp paired-end reads [35].

## RNA virus detection

Viral sequences were analyzed by first synthesizing cDNA from carry-over RNA present in DNeasy extracted samples using M-MLV Reverse Transcriptase (Promega, Madison, Wisconsin). Briefly, 2 µl of mosquito DNA extract from each sample was incubated with 1 µl of random hexamers (0.5 µg) and 12 µl of RNA-free water at 70 °C for 5 minutes. After this denaturation step, 1.25 mM of each dNTP, 25 units of recombinant RNasin® Ribonuclease Inhibitor, and 200 units of M-MLV RT are added and the cDNA synthesis is carried out at 37 °C for 60 minutes. The cDNA products are then used as templates for PCR amplification and sequencing as described above.

# Bioinformatic analyses

## Microbiota assessment

After discarding reads that did not carry the 16S rRNA primers or the expected barcode sequences, each read was assigned to a given sample. The resulting demultiplexed FASTQ files were then analyzed in DADA2 [36] (v1.6.0) by first trimming low-quality bases at the end of each read pair (> 250 bp for forward, > 210 for reverse reads) using the following parameters: *maxN* = 0, *maxEE* = 2, *truncQ* = 2. Dereplication was done by combining identical reads and assigning the number of reads belonging to each unique read (*derepFastq*). Next, the dereplicated data was analyzed with the *dada* core sample inference algorithm followed by the merging of read pairs that overlap by at least 12 bases (*mergePairs*). An amplicon sequence variant table (ASV) table was constructed with *makeSequenceTable* for all samples and chimeras were removed using *removeBimeraDenovo*. For taxonomic assignment, Silva (v128) [37] was used as a training set (using *assignTaxonomy* and *addSpecies*) to create taxonomy data. Finally, the R package phyloseq (v1.25.2) was used to combine the ASV table, taxonomy data, and sample metadata for downstream microbiome data analyses. Samples with less than 5,000 reads were discarded from further analysis, as they likely represent low-level cross contamination. PCoA with Bray-Curtis and weighted UniFrac distance matrices were calculated in phyloSeq. Adonis analyses were performed to investigate the statistical significance of observed differences in PCoA plots. Adonis calculates significance via *P* value with  $R^2$  representing the variation explained by sample groupings (i.e., collection site, mosquito species, *kdr-w* genotype, blood-meal status, and infection status).

## Eukaryotic parasites, viruses, and blood meal composition assessment

First, reads that did not contain the exact barcode and primer sequences were discarded. The rest of the reads were assigned to a given sample according to their unique barcode sequence. In order to remove low-quality bases and sequences that were likely primer dimers, each sequence was searched for the forward and reverse primers and trimmed after the reverse primer (if both primers are found). Sequences where the forward primer was found but the reverse primer was missing were left untrimmed. Untrimmed sequences that belong to a primer with an expected amplicon length of < 300 bp were trimmed from 50 bp from the 5' end for further quality filtering. Afterwards, filtered paired-end sequences from each read pair were merged using FLASH to generate a consensus sequence of the overlapping region. Each correctly merged sequence was trimmed of both amplification primers (forward and reverse) and kept only if it was longer than 90 bp. Then all concatenated sequences amplified with the same primer pair (from all samples) were compared to each other and a single copy of each unique sequence was kept (while the number of times it is observed in each sample was recorded). Unique sequences that were observed less than 10 times across all samples were removed as they likely represent instances of sequences carrying errors [35]. The remaining unique sequences were compared against all DNA sequences annotated on the NCBI nr database using BLAST [35]. We then retrieved the taxonomic information of the most similar sequence(s) if it had at least 70% identity over the entire sequence length.

Finally, we summarized the parasite and virus species identified, the percent identity (i.e., similarity to the most similar sequence(s) on NCBI), and the number of reads observed in each sample.

When evaluating the blood meal composition of each mosquito, the same procedure was applied but only samples with at least 1,000 reads were considered (to avoid including possible cross-contamination or sequences mis-assigned to one sample due sequencing errors in the barcode) [38].

*kdr* genotype (L1014F) and species determination (S200 × 6.1)

Reads amplified from the *kdr* and S200 × 6.1 primer pairs were processed as described above, with sequences assigned to their specific sample, filtered for quality, and merged with FLASH.

For the L1014F locus, the top two most abundant unique sequences in each sample (Seq1 and Seq2) were considered for further analysis. If the abundance of Seq2 was greater than 35% of the sum of Seq1 and Seq2 abundance, the sample was considered heterozygous Seq1/Seq2 while if this ratio was smaller than 35%, the sample was deemed homozygous for Seq1. Only samples with at least 1,000 reads were analyzed.

For the S200 × 6.1 locus, the most abundant sequence for each sample was compared against DNA sequences on NCBI as described above. For samples with greater than or equal to 1,000 reads, the species level taxonomy was retrieved and the *Anopheles* species as well as the total read count were summarized. A primer targeting the COX1 gene (CulicCox1) was used to identify *An. nili* species in samples as it was not able to be determined by the S200 × 6.1 locus [34, 39]

## Results

### Bacterial composition of *Anopheles* mosquitoes from West Africa

We extracted DNA from 665 individual *Anopheles* mosquitoes collected in Guinea (N = 584) and Mali (N = 81). To characterize the microflora of each mosquito, we amplified and sequenced the V2 variable region of the bacterial 16S ribosomal RNA genes (see Methods for details). We obtained a total of 8,467,703 sequences derived from 760 samples (665 mosquitoes and 95 extraction controls). On average, each mosquito samples yielded 11,730 sequences compared to 5,984 sequences on average per extraction control. We assigned these sequences to 21,527 amplicon sequence variants (ASVs, analogue of operational taxonomic units [36]), representing 37 phyla including *Proteobacteria* (6,692 ASVs accounting for 64% of all reads), *Firmicutes* (26%), *Actinobacteria* (6%) and *Bacteroidetes* (2%) (Fig. 1).

To investigate differences in bacterial composition among mosquitoes, we calculated  $\beta$ -diversity estimates using weighted UniFrac and Bray-Curtis dissimilarity matrices. Principal coordinate analyses (PCoA) conducted using Bray-Curtis dissimilarity or weighted UniFrac distances showed that the microbial composition separates mosquitoes into distinct clusters (Fig. 2A and Additional file 2: Figure S1A, respectively). These clusters appeared to group mosquitoes collected in the same sites (Fig. 2), and this observation held true when we restricted our analyses to mosquitoes only collected from sites in

Guinea (Fig. 2B and Additional file 2: Figure S1B). The details of all the ASVs identified and their taxonomy are provided in Additional file 3: Table S2.

### Assessment of mosquito species and *kdr* mutation

We simultaneously genotyped the same mosquitoes at loci informative of their species and insecticide-resistance status by high-throughput sequencing (see Methods).

Out of 665 mosquitoes, 551 (82.9%) were successfully genotyped for the S200 × 6.1[34] and *cox1*[39] loci. We primarily used the S200 × 6.1 locus to identify the species of each mosquito as i) this locus was more robustly amplified and sequenced than the *cox1* locus (with an average read count of 2,917 and 1,181 per mosquito, respectively) and ii) provided clearer taxonomic resolution (with, for example, 233–234 (mean of 233.67) nucleotides differentiating the sequences from *Anopheles gambiae s.s.* from those of *Anopheles coluzzii*, compared to 0–4 (mean of 1.71) nucleotide differences using the *cox1* locus) (Additional file 4: Table S3). However, the S200 × 6.1. locus systematically failed to yield sequences for some mosquitoes that were identified at *Anopheles nili* using the *cox1* sequences. Overall, we identified that the mosquitoes analyzed belonged to five *Anopheles* species. 404 mosquitoes (74.5%) were identified as *Anopheles gambiae s.s.*, while the remaining mosquitoes consisted of *Anopheles coluzzii* (61 mosquitoes or 11.3%), *Anopheles melas* (with 57 mosquitoes or 10.5%), *Anopheles arabiensis* (8 mosquitoes or 1.5%), and *Anopheles nili* (7 mosquitoes or 1.3%) (Fig. 3). We also identified 5 mosquitoes that were heterozygous for the S200 × 6.1 locus and likely represented F1 hybrids of *An. gambiae s.s.* and *An. coluzzii* species. The species distribution varied extensively between locations, with *An. gambiae s.s.* accounting for more than 90.00% of all mosquitoes collected in five out of six locations in Guinea, while *An. melas* was the most abundant species (79.2%, 57/72) in Boffa, a coastal region in western Guinea, and *An. coluzzii* predominated in Bandiagara, Mali (86.3%, 44/51) (Fig. 3).

Pyrethroid resistance is often due to a point mutation in the voltage gated sodium channel gene, described as knockdown resistance (*kdr*)[33]. 550 (82.7%) of the mosquitoes were successfully genotyped at this locus (with an average coverage of 2,436 reads per mosquito). In Guinea, with the exception of mosquitoes collected in Boffa, most mosquitoes (> 92.6%) were homozygous for the *kdr-w* (L1014F) alleles that is associated with resistance to pyrethroids [33] (Fig. 4). In Boffa, where *An. melas* is the predominant species, most mosquitoes were homozygous for the wild-type allele (L1014L). In Mali, the distribution was more heterogeneous, with roughly equal proportions of mosquitoes homozygous for the wild-type, resistant allele or heterozygous. Across mosquitoes, the genotype at the *kdr-w* locus correlated almost perfectly with the mosquito species, with *An. gambiae* carrying primarily L1014F alleles while *An. arabiensis* and *An. melas* were essentially wild-type. Only *An. coluzzii* showed high proportion of both alleles (Additional file 5: Figure S2). The details of all the genotypes and sequences amplified from each mosquito are provided in Additional file 6: Table S4.

## Determination of the blood meal composition

To characterize the composition of the last blood meal of each these mosquitoes, we used the same DNA extract to amplify and sequence a short fragment of the mammalian mitochondrial 16S rRNA gene. 133 mosquitoes yielded > 1,000 reads and were considered blood fed in later analyses. 126 mosquitoes carried human DNA, 14 mosquitoes cow DNA, and 2 sheep DNA (Fig. 5 and Additional file 7: Table S5). Nine mosquitoes fed on more than one mammalian host species (Fig. 5). The blood meal composition differed between sites with, for example, 12 mosquitoes (20.1%) from Kankan that fed, at least partially, on cow while mosquitoes from all other sites, in Mali and Guinea, fed almost exclusively on human.

## **Identification of eukaryotic parasites and viruses from individual mosquitoes**

Finally, we determined whether each mosquito carried a eukaryotic parasite and/or arbovirus using a sequencing-based assay recently developed in our laboratory [35]. After PCR amplification, and sequencing of DNA extracted from individual mosquitoes, we identified DNA sequences from eukaryotes and viruses from 127 mosquitoes, with on average, 1,876 reads supporting each identification in each mosquito. Nine out of 95 extraction controls (water samples that have been processed simultaneously) were also positive for one or more parasites, but with an average of 54 reads per parasite (Additional file 8: Table S6). The low read counts in those water controls could be explained by low-level cross-contamination during extraction, or sequence mis-assignment due to sequencing errors in the Illumina index sequences [35].

Table 2  
Eukaryotic parasites and viruses identified from screening mosquito samples

Taxon targeted	Species identified (# positive)	% Identity
Apicomplexa A	<i>Theileria</i> sp. (24)	100
Apicomplexa B	<i>Plasmodium falciparum</i> (8)	100
Apicomplexa C	<i>Theileria</i> sp. (3)	100
Microsporidia	<i>Parathelohania anopheles</i> (38)	92.47
	<i>Hazardia milleri</i> (1)	97.38
	<i>Culicospora magna</i> (6)	99.7
	<i>Microsporidium</i> sp. 3 NR-2013 (34)	97.01
Nematoda A	<i>Acanthocheilonema viteae</i> (12)	100
	<i>Loa loa</i> / <i>Dipetolenma</i> sp. (7)	99.64
	<i>Setaria labiatopapillosa</i> (11)	100
	<i>Auanema rhodensis</i> (1)	98.21
Nematoda B	<i>Setaria yehi</i> / <i>Setaria digitate</i> (4)	99.72
	<i>Acanthocheilonema viteae</i> (1)	99.72
	<i>Dipetolenma</i> sp./ <i>Filariodea</i> sp. (3)	98.94
Flavivirus	<i>Anopheles flavivirus</i> variant 2 (2)	99.06
	<i>Anopheles flavivirus</i> variant 2/ variant 1 (1)	88.26
	<i>Culex flavivirus</i> (1)	99.06

Table shows the parasite and viral taxon targeted by each primer, the species identified and the number of mosquitoes positive for that species, and the percent match of the sequences amplified to that of the NCBI database.

Eight mosquitoes (1.2%), from 3 sites (6 of them identified as *An. gambiae*), yielded DNA sequences identical to *Plasmodium falciparum*, the primary cause of human malaria in Africa. We detect DNA belonging to *Theileria* species in a relatively high number of mosquitoes (27/665 or 4.1%). *Theileria* species are protozoan parasites that can be infective to domestic (i.e. cattle) and wild (buffalo) animals and transmitted by ticks [40]. Interestingly, from the fourteen samples for which *Bos indicus* (cow) was identified with greater than 500 reads, we detect *Theileria* species in twelve (86%), suggesting the tick-transmitted parasite may have been ingested by these mosquitoes during their last blood-meal. Seven mosquitoes yielded a DNA sequence identical to *Loa loa* and to several other filarial worms (and were further characterized as deriving from *Mansonella perstans* after sequencing of longer amplification products, M. Cannon. personal communication). Several DNA sequences were closely related to known

parasites of mosquitoes themselves, belonging to microsporidia [41] (e.g., *Parathelohania sp.*), mosquito-transmitted nematodes (e.g., *Setaria sp.* [42, 43]), as well as two recently discovered *Anopheles* flaviviruses (e.g., *Anopheles flavivirus variant 1* and *variant 2*) [44] (Table 2). The details of all parasite sequences amplified and their taxonomic information for each mosquito are provided in Additional file 8: Table S6.

## Evaluation of the factors influencing microbial composition of wild mosquitoes

The characterization of the mosquito species, insecticide-resistance genotype, blood meal status and infection from the same mosquitoes that have been examined for their microbial diversity enables a rigorous assessment of the relative contribution of these factors to the microbial composition. Note that to avoid possible biases introduced by sample storage or DNA extraction, we restricted this analysis to mosquitoes collected in Guinea that were all processed identically and simultaneously. We implemented an analysis of variance [45] to simultaneously test the contribution of each factor, while accounting for the others (multivariate analysis). The geographical location of the samples explained most of the variation in microbial composition (Adonis test,  $R^2 = 0.200$ ,  $p = 0.001$ ), whereas the mosquito species (Adonis test,  $R^2 = 0.004$ ,  $p = 0.208$ ), *kdr-w* genotype (Adonis test,  $R^2 = 0.006$ ,  $p = 0.438$ ), and feeding status (Adonis test,  $R^2 = 0.003$ ,  $p = 0.173$ ) were not statistically associated with the bacterial composition. The mosquito infection status was significantly associated with the microbial composition but had a very marginal effect (Adonis test,  $R^2 = 0.015$ ,  $p = 0.001$ ) (Table 3). To further investigate the relative roles of geography and species that are confounded in this dataset, we examined PCoAs of the bacteria composition, restricting the analysis to i) all *An. gambiae* collected in seven sites (Additional file 9: Figure S3A) and ii) mosquitoes from all *Anopheles* species collected in Boffa (Additional file 9: Figure S3B). Together, these analyses validated the results of the statistical testing and confirmed that geographical location of the mosquitoes had a much greater influence on the bacterial composition than the species of the mosquitoes.

Table 3  
Relative contribution of mosquito factors on microbial variation

Factor	Df	R <sup>2</sup>	P-Value
Location	7	0.2	0.001
Mosquito species	4	0.004	0.208
<i>kdr-w</i> genotype	2	0.006	0.438
Blood-meal	1	0.003	0.173
Infection status	1	0.015	0.001
Residuals	440	0.772	

Table shows, for each factor, the Df, R<sup>2</sup> (percent of variation explained), and P-value (significance value) calculated by Adonis. Abbreviations: Df, Degrees of freedom

## Discussion

The importance of the mosquito microbiota on vector biology and pathogen transmission has been well recognized, with several studies demonstrating the role of endogenous bacteria on the vector's development [19, 20], immunity [22, 23], and competency [46]. However, few studies have examined the factors that shape the bacterial composition of mosquitoes and most of those used laboratory-reared mosquitoes to assess influences on bacterial communities [20, 31, 47]. This latter limitation could be especially problematic since bacterial diversity of wild-caught *Anopheles* mosquitoes has been shown to be greater than that of mosquitoes reared in the laboratory [48]. In addition, these studies typically focus on testing the influence of a single factor on the bacterial composition without accounting for confounding factors. In this study, we examined the microbial diversity of 665 individual wild-caught *Anopheles* mosquitoes collected in six sites in Guinea and one site in Mali. For each mosquito, we characterized their species, *kdr-w* genotype, blood-meal status, and infection with various eukaryotic parasites and viruses. We then simultaneously estimated the relative contribution of each of those genetic and non-genetic factors on the microbial composition of the mosquitoes. In this analysis, the mosquito collection site accounted for ~ 20% of the variation in bacterial composition, whereas the other factors showed marginal or non-significant contribution (Table 3).

Our findings are consistent with previous studies that showed that collection site was a major contributor to the microbial diversity of field-caught *Anopheles* mosquitoes [26, 49–51]. For example, Muturi *et al.* found that sampling site has a strong effect on microbial composition and diversity, even when examining nine different mosquito species [27]. Note however that “collection site” in our study summarizes many parameters. Thus, the influence of the “collection site” on the microbial composition could reflect the effect of differences in larval habitats, flora the mosquitoes rely on for nectar feeding, and/or local population differences. In this regard, it is worth noting that the adult mosquito bacterial

composition has been shown to vary depending on the larval breeding sites and the bacterial composition of these aquatic habitats [52]. In addition, sugar source appears to have a pronounced influence on the vectoral capacity of *An. sergentii* mosquitoes [53] and impacts the microbial composition of laboratory-reared adult mosquitoes [54]. Future studies using a denser, more local sampling of wild-caught mosquitoes, will be required to better understand the individual contribution of these local parameters.

On the other hand, our analyses provided new insights on the role of other factors on the microbial composition of mosquitoes. We did not observe any significant contribution of feeding status on microbial variation of the wild mosquitoes. This finding contrasts with a previous study that described that the bacterial diversity of *Aedes aegypti* mosquitoes fed on human, chicken, or rabbit blood was significantly lower than that of newly emerged unfed mosquitoes [31]. This discrepancy could reflect differences between mosquito species/genera, or more likely, differences between wild-caught mosquitoes (that might have had prior blood meals) and laboratory-reared mosquitoes with less variable microbial composition. Similarly, our study did not reveal any significant contribution of genetic factors (i.e. mosquito species, *kdr-w* genotype) on mosquito microbial variation. These observations contrast with a previous study that described distinct bacterial compositions in two species of *Culex* mosquitoes collected from the same site and with identical larval aquatic environment [28], possibly due to differences among *Culex* species in their larval feeding habits [55]. The L1014F *kdr* allele frequency was reported high or near fixation in Kankan and Kissidougou sites of Guinea and low in Boffa in a previous study [56], consistent with our findings here. In theory, selection of insecticide-resistant mosquitoes could also select for bacterial species with adverse effects (i.e. influence on disease transmission). Here, we did not see evidence of L1014F resistant allele influence on adult mosquito microbial composition. Note however that resistance allele genotypes and mosquito species only represent a small fraction of the genetic factors that could impact the mosquito microbiota and that, given our observation that the collection site is strongly associated with the bacterial composition, it would be interesting to further investigate whether genetic diversity is associated with the microbiota of mosquitoes [57].

Interestingly, we observed a marginal but statistically significant association between infection status (infected n = 127 vs. non-infected n = 513) and the mosquito microbial composition. Modification of insect gut microbiota by parasitic [58] or viral [59] infections has been demonstrated in few studies. Pathogenic or non-pathogenic (e.g., insect-specific viruses) species could be involved in crosstalk with insect metabolism pathways or immune system to influence the microbiota [60]. It is worth noting that, due to the low infection rate with parasitic and viral species we found in the mosquitoes (< 5.0%), we assessed the influence infection has on the microbiota using an aggregate of all the parasite and viruses we detected (as opposed to individual parasite and virus species) and it is possible that the effect of one organism on the microflora might be diluted down and undetected once analyzed together with other parasites and viruses that have no influence on the bacterial communities. For example, *Theileria sp.* are transmitted by ticks and unlikely to be viable in mosquitoes and therefore, have probably little to no contribution on the influence of infection on the mosquito microbiota. Future studies assessing the direct influence of some of the parasites found in abundance in this study (e.g., *Parathelohania sp.*,

*Microsporidium sp.*) and the recently discovered virus (*Anopheles flavivirus*) could further elucidate the tripartite relationship between the mosquito, microbiota, and mosquito infecting agents.

## Conclusions

In summary, this study provides a comprehensive assessment of the microbial composition and diversity of 665 wild mosquitoes and a simultaneous examination of the relative contribution of five different mosquito factors on microbial variation. This approach enables rigorous estimation of the importance of these factors to shaping the bacterial composition, while correcting for their often confounding effect. Our results highlight the prominent role of the mosquito collection site and, to a lesser extent, the parasitic and viral infection, on shaping the bacterial composition of wild-caught mosquitoes. These findings provide a solid foundation to implement further investigations and examine the specific components of the environment (e.g., bacterial communities of the larval habitats, source of nectar, genetic diversity) shaping the microbial composition of wild mosquitoes and the mechanisms mediating these effects.

## Abbreviations

### PCoA

Principal coordinates analysis

### *kdr-w*

Knockdown resistance west

### DDT

Dichlorodiphenyltrichloroethane

### ASV

Amplicon sequence variant

## Declarations

## Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

## Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files.

## Competing interests

The authors declare that they have no competing interests. The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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## Author's contributions

HNB, MVC and DS conceptualized and designed experiments. KK, DC, YB, MK and SI provided mosquito specimen. DC, AKK, OKD, MAT, CVP, MT provided mosquito DNA samples. HNB performed the experiments, analyzed data and wrote the original draft of the manuscript. MVC, SI and DS critically revised the manuscript. All authors read and approved the final manuscript.

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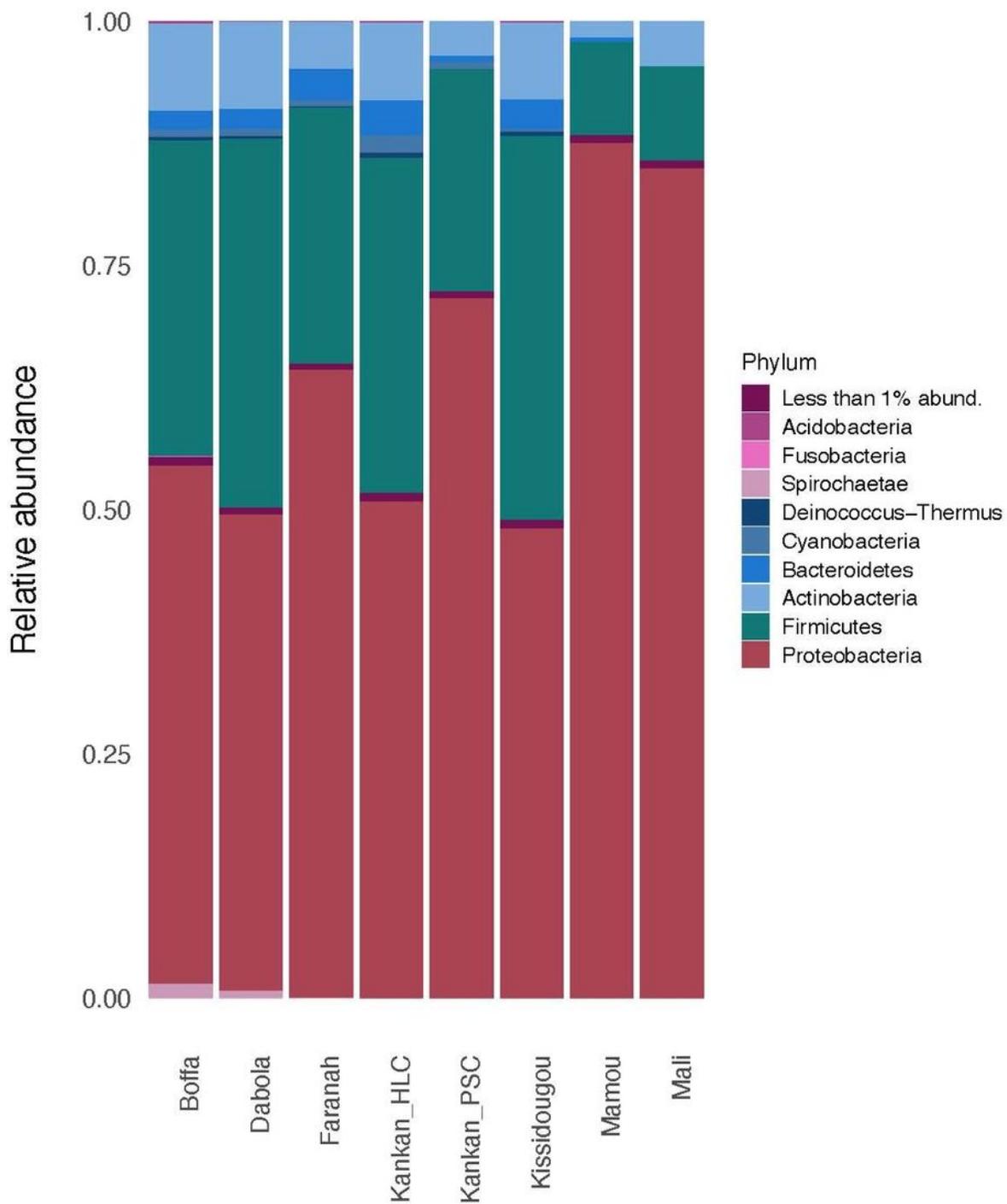
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## Figures

# Figure 1

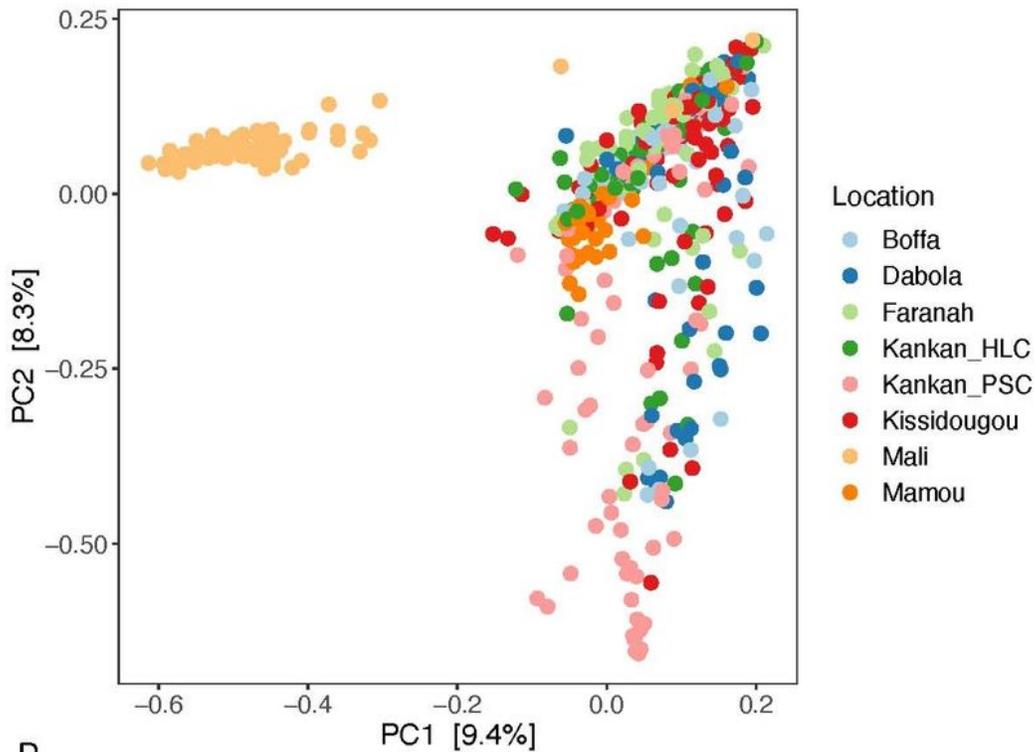


**Figure 1**

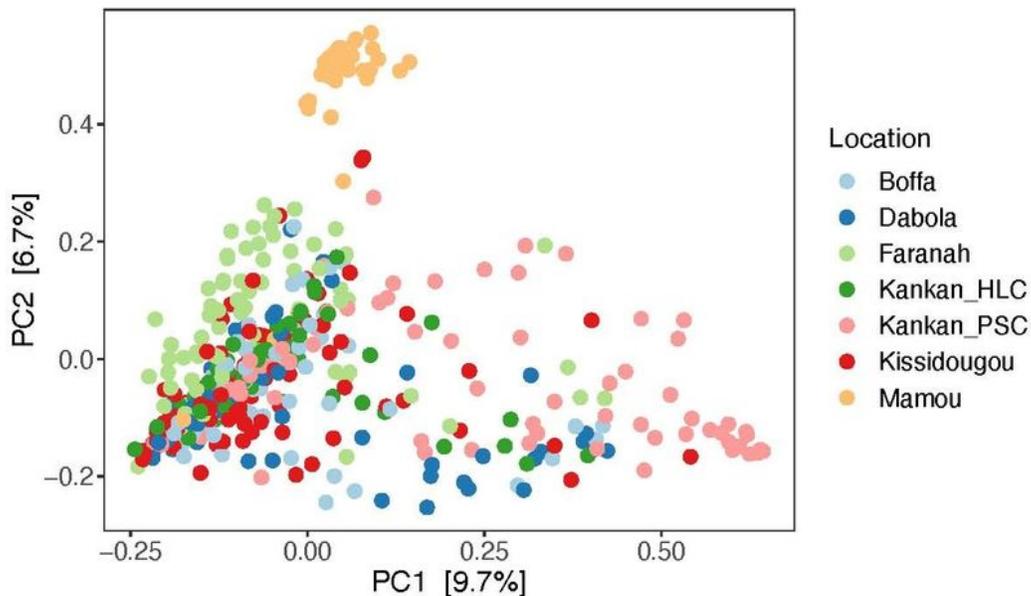
Average relative abundance of bacterial phyla from each mosquito collection site in Guinea and Mali. Bacterial species from the Proteobacteria phylum are the most abundant followed by Firmicutes and Actinobacteria. Less than 1% abund. represents phyla that make up <1% of all bacteria.

# Figure 2

A



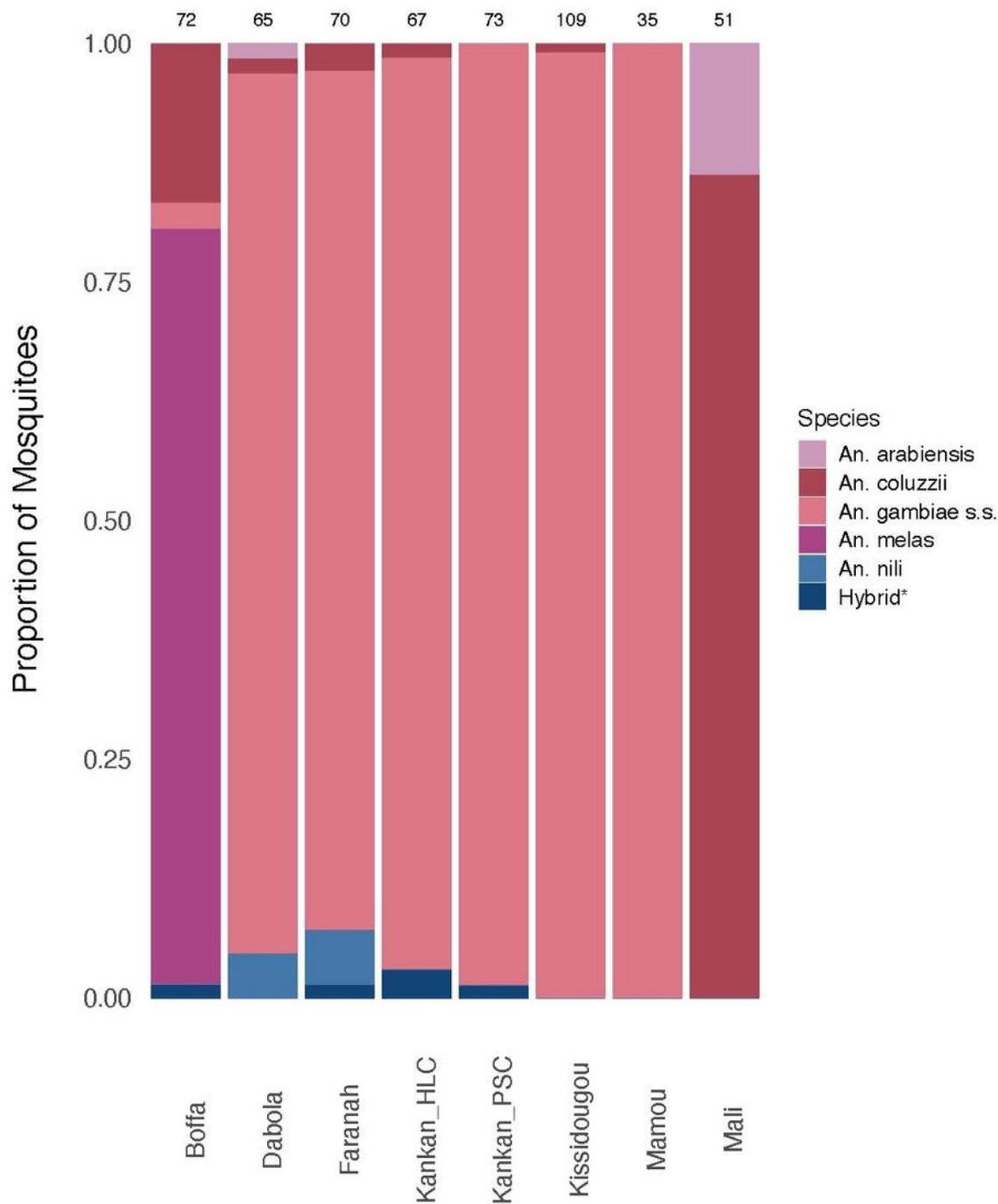
B



**Figure 2**

PCoA plot showing the dissimilarity between the microbial composition of individual mosquitoes based on Bray-Curtis metric for sites in Guinea and Mali (A) and Guinea only (B). Each dot represents the bacterial composition of a single mosquito. The numbers in brackets near the axes indicate the proportion of the variance explained by the components 1 and 2.

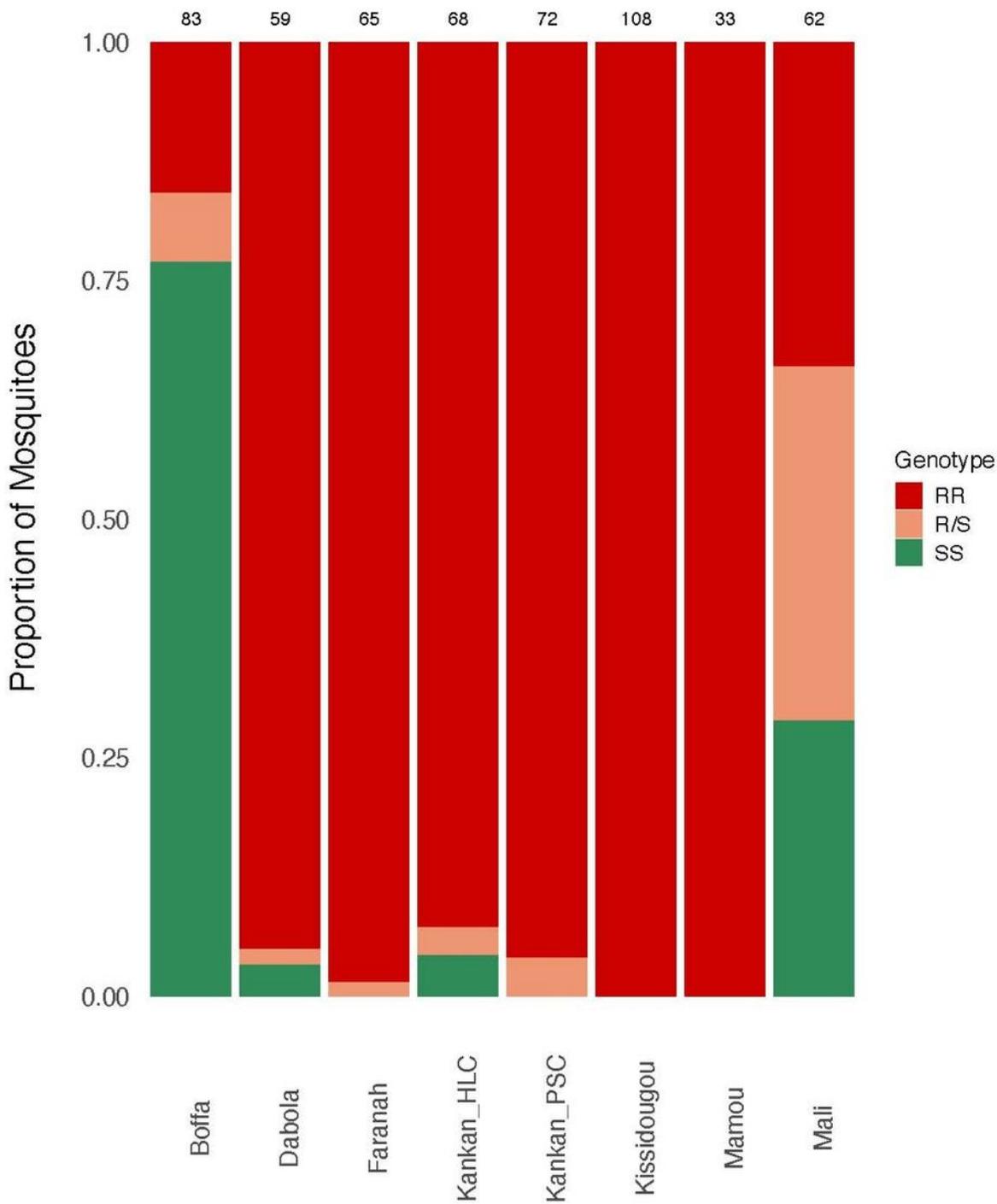
# Figure 3



**Figure 3**

Mosquito species diversity across collection sites in Guinea and Mali. Hybrid represents samples identified as heterozygous for *An. gambiae* and *An. coluzzii* at the S200X6.1 locus. Numbers above each bar represent the total number of mosquitoes that species is determined, per site.

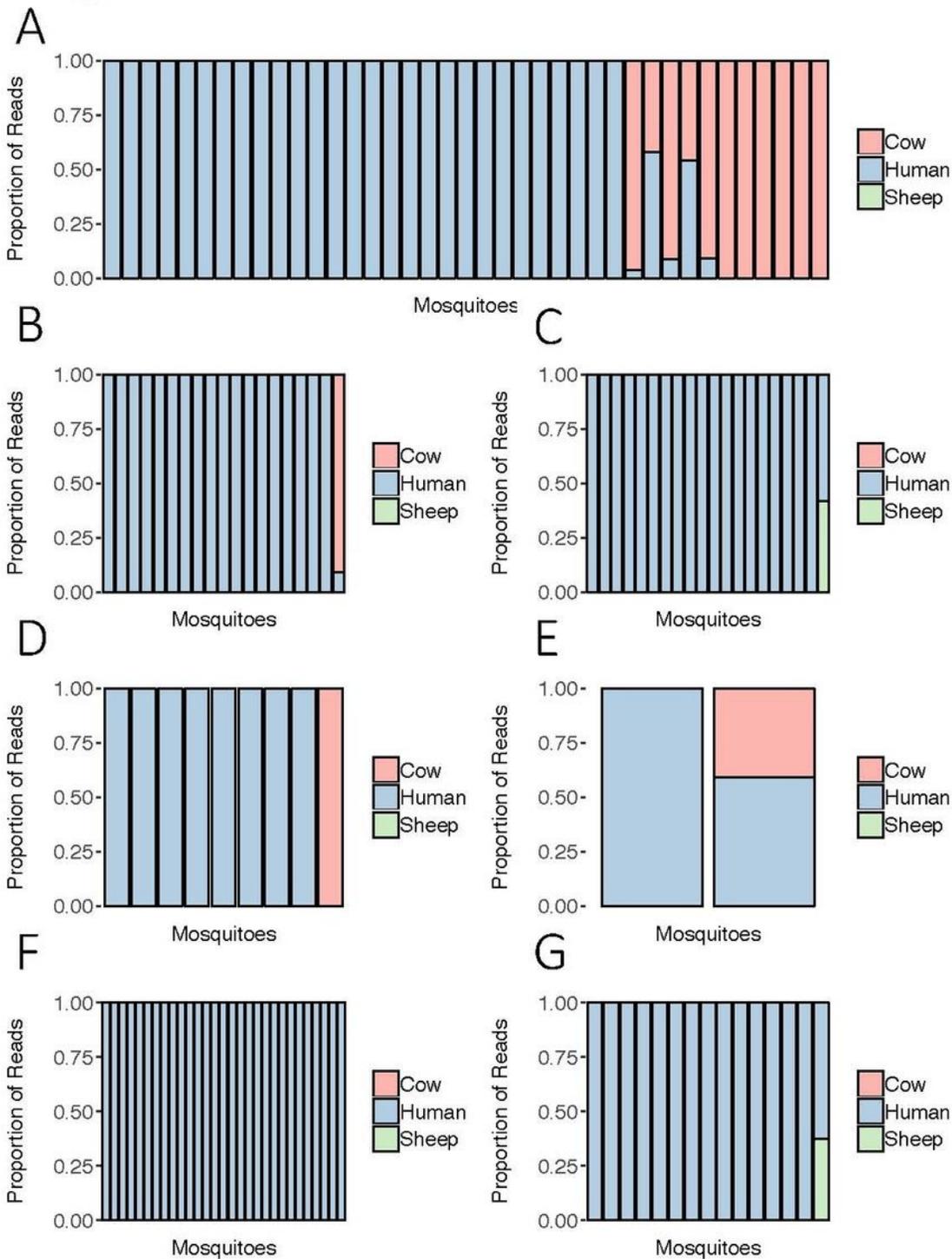
# Figure 4



**Figure 4**

Distribution of L1014F mutation (*kdr-w*) in mosquitoes collected across Guinea and Mali. Numbers above each bar represent the total number of mosquitoes that *kdr-w* genotype is identified, per site. Abbreviations: RR, homozygous resistant; SS, homozygous sensitive; R/S, heterozygous.

# Figure 5



**Figure 5**

Host blood-meal composition of individual mosquitoes collected from Kankan with PSC (A), Kankan with HLC (B), Kissidougou (C), Dabola (D), Faranah (E), Boffa (F), Mali (G). Each bar represents an individual mosquito.

## Supplementary Files

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