

Arboviruses and Blood Meal Sources in Zoophilic Mosquitoes at Human-Wildlife Interfaces in Kenya

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Abstract

Background: Zoophilic mosquitoes play an important role in the transmission of arboviruses of medical importance at human-wildlife interfaces, yet arbovirus surveillance efforts have been focused mostly on anthropophilic mosquitoes. Understanding the diversity of zoophilic mosquitoes and their associated feeding patterns and arboviruses can inform better vector control strategies.

Materials and Methods: We morphologically identified mosquitoes collected from two game reserves in Kenya, the Maasai Mara National Reserve (MMNR) and locations near the Shimba Hills National Reserve (SHNR). Representative mosquitoes were also identified by cytochrome c oxidase subunit 1 (COI) barcode sequencing. In addition, we identified the vertebrate hosts of mosquito blood meals from the contents of each mosquito's abdomen by high-resolution melting (HRM) analysis and sequencing of COI, 16S ribosomal RNA, and cytochrome b gene PCR products. Similarly, mosquito arbovirus infections were identified by HRM analysis and sequencing of *Alphavirus*- and *Flavivirus*-specific RT-PCR products.

Results: Of 2858 mosquitoes collected, 51 were engorged with blood meals from seven different vertebrate hosts, including humans, birds, domestic, and peridomestic animals and wildlife. *Culex* was the most abundant mosquito genus, with *Culex pipiens* being the most abundant species in both study regions. Among MMNR samples, we detected dengue serotype-2 virus (DENV-2) for the first time in *Aedes tarsalis* and *Aedes tricholabis*, as well as Sindbis virus in male *Cx. pipiens*. We also detected DENV-2 in *Aedes aegypti* sampled from locations near the SHNR. Human and diverse wildlife blood meals were identified, including bushbuck blood in the dengue-infected *Ae. tarsalis* and both human and hippopotamus blood in a single *Eretmapodites chrysogaster* mosquito.

Conclusions: Our findings highlight the potential risk of sylvatic dengue and Sindbis transmission to humans by zoophilic mosquitoes at human-wildlife interfaces in Africa. Of specific importance, we provide evidence of sylvatic DENV-2 in *Ae. tarsalis* and *Ae. tricholabis*, representing potential new dengue vectors.

Keywords: zoophilic mosquitoes, dengue virus, Sindbis virus, mosquito blood meal, *Aedes*, *Eretmapodites*

Introduction

ZOO PHILIC MOSQUITOES (preferring animal blood to human blood) are important vectors that transmit emerging and re-emerging viruses from animals to humans, and thus pose a serious threat to global health and security (Conway et al., 2014). In sub-Saharan Africa, diverse *Aedes* spp., including *Aedes aegypti*, which is mainly an anthropophilic

species on other continents (McBride et al., 2014), circulates in sylvatic and peridomestic cycles involving wild animals, including birds, domestic mammals, and humans (Sylla et al., 2009). Similarly, *Culex* mosquitoes mainly obtain blood meals from birds, although mammals are also involved and can also transmit arboviruses to humans (Omondi et al., 2015, Grubaugh et al., 2017). Despite playing an important role in the transmission of arboviruses of medical importance at

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human-wildlife interfaces, most zoophilic mosquitoes are understudied, with most surveillance efforts focusing on anthropophilic mosquitoes.

Over the last two decades, Kenya has been hit hard by outbreaks of arboviral diseases, including chikungunya fever (WHO, 2018), Rift Valley fever (RVF) (Baba et al., 2016a), and dengue fever (Baba et al., 2016b). Control strategies for these outbreaks are limited by a lack of vaccines, except for RVF and yellow fever, which have vaccines licensed for use in animals (Mansfield et al., 2015) and humans (Monath and Vasconcelos, 2015), respectively. Therefore, early detection of viruses in their vectors and control of vector populations remain indispensable for strategies curbing arboviral disease outbreaks.

In Kenya, as in many other resource-limited countries, systematic epidemiological surveillance of circulating arboviruses in their biological vectors is sporadic and limited in scope, especially in wildlife-livestock-human interfaces. Therefore, we focused on identifying the diversity of zoophilic mosquitoes and their vertebrate blood meals and associated flaviviruses and alphaviruses of public health importance at the human-wildlife interfaces of the Maasai Mara National Reserve (MMNR) in southwestern region of Kenya and the Shimba Hills National Reserve (SHNR) in the south coast of Kenya.

Materials and Methods

Mosquito sampling

We obtained sampling approval from the Kenya Wildlife Service (KWS) and Department of Health offices before sampling mosquitoes in the MMNR and from locations near

the SHNR (Fig. 1). The two protected wildlife reserves were selected because of the encroaching human settlements along the reserves' borders and reported cases of human arboviral infections (Eastwood et al., 2017). The MMNR is a game reserve with diverse vertebrate wildlife that covers some 1510 km² in Narok County, southwestern Kenya, and shares a border with the Serengeti National Park, Tanzania. The "Great Migration" between the Serengeti National Park and the MMNR of large populations of zebra (*Equus quagga*) and wildebeest (*Connochaetes taurinus*) was ongoing during the sampling period. The SHNR is a wildlife sanctuary located in Kwale County along Kenya's south coast. It is within the eastern African coastal forest belt and home to a variety of wildlife species, including the endangered sable antelope (*Hippotragus niger*).

Adult mosquitoes were collected between January and June, 2018, using Centers for Disease Control and Prevention (CDC) light traps and Biogent (BG) sentinel traps (Biogents AG, Regensburg, Germany) baited with carbon dioxide (from dry ice). To utilize 24 traps effectively, we placed them randomly within the study sites, at least 50 m from each other. Near the SHNR, traps were placed at the periphery of villages near to the reserve's border and away from artificial lights. Both the CDC light traps and BG sentinel were suspended ~2 m above the ground. The two types of traps were operated from 4 p.m. to 6:30 a.m. at dawn, and for four consecutive days. The sampled mosquitoes were immediately stored in liquid nitrogen and transported to the laboratory at icipe, Nairobi. Sampling was not done to compare the two study areas, but to have more data on novel arboviruses circulating in mosquito species found along the wildlife-human interfaces, to inform on possible disease outbreaks.

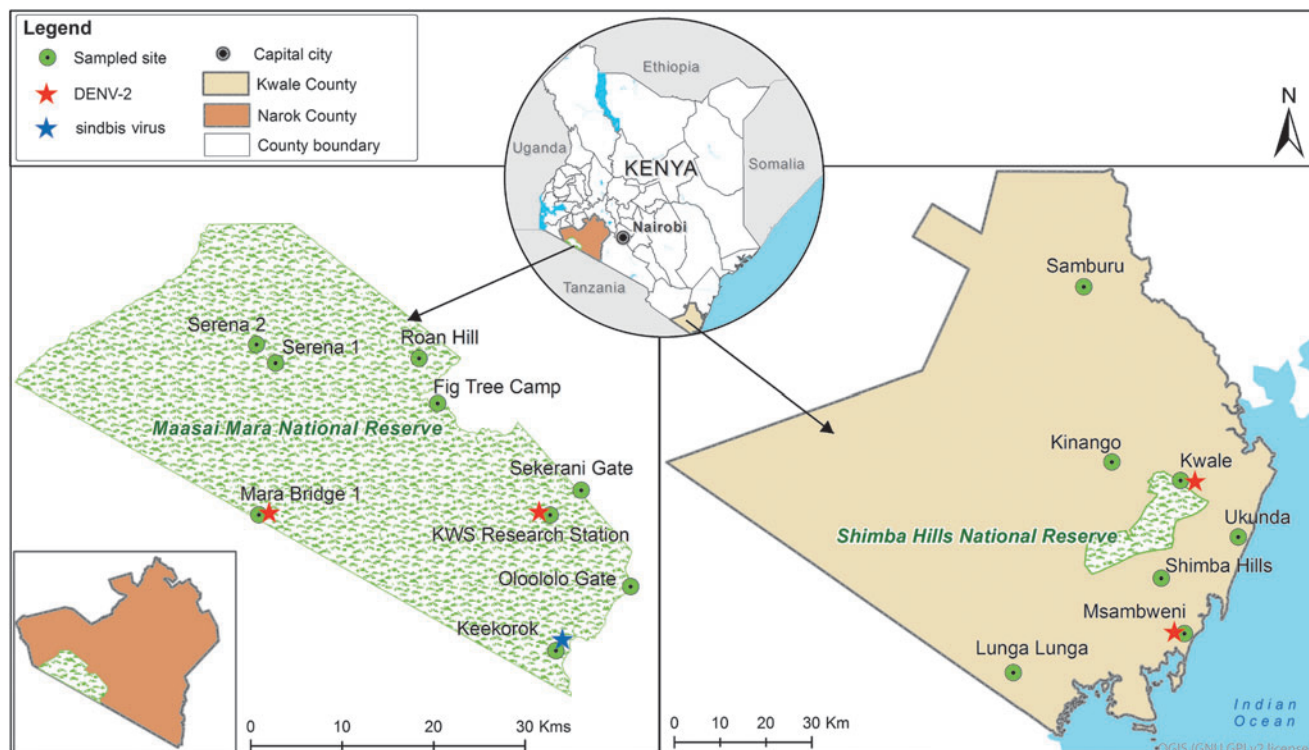


FIG. 1. Map of mosquito sampling locations and sites of arbovirus detection in the MMNR in Narok County, Kenya, and near the SHNR in Kwale County, Kenya. The map was prepared using common-license shape files in QGIS software. MMNR, Maasai Mara National Reserve; SHNR, Shimba Hills National Reserve.

Morphological and molecular identification of mosquitoes

Using morphological identification keys (Edwards, 1941, Gillies and de Meillon, 1968, Gillies and Coetzee, 1987), we identified mosquitoes on Petri dishes placed on ice-chilled blocks under a stereomicroscope. We pooled up to 25 mosquitoes into sterile 1.5-mL microcentrifuge tubes according to species, sex, site, date of collection, and blood-fed status, and stored them at -80°C until used for molecular tests. Blood-fed mosquitoes were placed individually into tubes with assigned “pool” numbers. Representative individual mosquitoes from both study sites were identified using molecular assays (Folmer et al., 1994). DNA was extracted from one leg of individual mosquitoes using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and mitochondrial cytochrome c oxidase subunit 1 (COI) genes were amplified using LCO1490 and HCO2198 primers (Table 1). The PCR conditions included a 15-min initial denaturation step at 95°C , followed by 40 cycles of 1 min at 95°C , 40 s at 50°C , and 2 min at 72°C , and a final 5-min elongation at 72°C .

Identification of blood meal sources

The individual abdomens of engorged mosquitoes were separated from the rest of the body using sterile dissecting pins and sharp-pointed forceps and placed into individual sterile 1.5-mL microcentrifuge tubes (Omondi et al., 2015). The other body parts of individual mosquitoes were stored in correspondingly labeled sterile 1.5-mL microcentrifuge tubes. Genomic DNA was extracted from the engorged abdomens using an in-house sodium dodecyl sulfate optimized method. Briefly, the abdomens of blood-fed mosquitoes were squashed in $300\ \mu\text{L}$ cell lysis buffer using a handheld battery-operated homogenizer and then incubated at 65°C for 15 min. Thereafter, $100\ \mu\text{L}$ of protein precipitation buffer was added, followed by vortexing for 30 s at room temperature before placing on ice for 5 min, and then centrifuging at 25,000 relative centrifugal force (rcf) for 5 min. The pellets were discarded, and the supernatants were placed in fresh tubes with $300\ \mu\text{L}$ isopropanol, then mixed by inverting 100 times, and centrifuged at 25,000 rcf for 30 min. The supernatants were then removed and $300\ \mu\text{L}$ of cold 70% ethanol was added to each tube. Tubes were inverted 50 times, and then centrifuged for 15 min. The ethanol was pipetted off and the tubes were inverted on paper towels overnight to air dry. The DNA pellets were then eluted with $50\ \mu\text{L}$ of nuclease-free water and stored in the fridge at 4°C for 3 h before use.

Three sets of PCR primers (Table 1) targeting vertebrate COI, cytochrome b (*cyt b*), 16S ribosomal (r)RNA genes were used to identify blood meal sources in separate $10\text{-}\mu\text{L}$ PCR reactions containing $6\ \mu\text{L}$ of nuclease-free PCR water, $0.5\ \mu\text{M}$ concentration of each primer, $2\ \mu\text{L}$ of $5\times$ Hot FIREPol EvaGreen HRM Mix (Solis Biodyne, Tartu, Estonia), and $1\ \mu\text{L}$ of DNA as a template, using the same conditions as described by Omondi et al. (2015). Briefly, the thermal cycling conditions used for the *cyt b* primers were as follows: initial denaturation for 1 min at 95°C , followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 20 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 7 min. Cycling conditions for the 16S rDNA fragment were similar to those of *cyt b*, except for an annealing temperature of 56°C . The thermal cycling

TABLE 1. PRIMERS USED

Target gene	Primer name	Primer sequence (5' to 3')	Amplicon size (bp)	Citation
Vertebrate COI (HRM)	Uni-Minibar F1	TCCAATAATCACAAARGATATTGGTAC	~205	Meusnier et al. (2008)
	Ronping R	TATCAGGGGCTCCGATTAT		Lee et al. (2015)
Vertebrate <i>cyt b</i> (HRM)	Cyt b For	CCCCTCAGAATGATATTTGTCCTCA	~383	Boakye et al. (1999)
	Cyt b Rev	CCATCCAACATCTCAGCATGATGAAA		
Vertebrate 16S rRNA (HRM)	Vert 16S For	GAGAAAGACCCTRTGGARCTT	~200	Omondi et al. (2015)
	Vert 16S Rev	CGCTGTTATCCCTAGGGTA		
<i>Alphavirus</i> nsP4	Vir 2052 F	TGGCGCTATGATGAAATCTGGAATGTT	~90	Eshoo et al. (2007)
	Vir 2052 R	TACGATGTTGTCGTCGCCGATGAA		
<i>Alphavirus</i> nsP1	Alpha M2W	YAGAGCDTTTTCCGAYSTRGCHW	~435	Pfeffer et al. (1997)
	Alpha cM3W	ACATRAAIKIGITGTRCRAACCDAYCC		
<i>Alphavirus</i> nsP1	Alpha M2W2	TGYCNCVTGMDNWSYVNCARGAYCC	~310	Pfeffer et al. (1997)
	Alpha cM3W	ACATRAAIKIGITGTRCRAACCDAYCC		
<i>Flavivirus</i> NsP5	Flavi JV2a F	AGYMGHCATHHTGGTWCATGTGG	~185	Villinger et al. (2017)
	Flavi JV2a R	GTRTCCCADCDDGGCDGTRTCATC		
Vertebrate COI barcoding	VF1d_t1	TGTAAAACGACGGCCAGTCTCAACCAACCACCAARGAYATYGG	~750	Ivanova et al. (2012)
	VR1d_dt	CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCRAARAAYCA		
Insect COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	~710	Folmer et al. (1994)
	HCO2198	TAAACTTCAGGTTGACCAAAAAATCA		

cyt b, cytochrome b; COI, cytochrome c oxidase subunit 1; HRM, high-resolution melting; rRNA, ribosomal RNA.

conditions for PCRs utilizing the COI primers included an initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 60 s, followed by a final extension at 72°C for 7 min.

Normalized high-resolution melting (HRM) profiles were generated between ramping temperatures 75°C and 88°C, and compared to those from positive control DNA extracts from *Homo sapiens* (human), *Felis catus* (cat), *Kobus ellipsiprymnus* (waterbuck), *Gallus gallus* (chicken), *Capra hircus* (goat), *Bos taurus* (cow), *Sus scrofa* (pig), *Oryctolagus* sp. (rabbit), *Canis familiaris* (dog), *Nanger granti* (gazelle), and *Syncerus caffer* (buffalo) tissues obtained from previous studies (Omondi et al., 2015, Ogola et al., 2017). Each set of PCRs included a sample of DNA extracted from male mosquitoes as a negative control. We sequenced representative samples to confirm blood meal sources identified by their HRM profiles.

Samples with HRM profiles that did not match any of the control HRM profiles were reamplified using COI barcoding primers (Table 1) in a SimpliAmp thermocycler (Thermo Fisher Scientific, MA). Thermal cyclic conditions included initial denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 20 s, 57°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 7 min.

Identification of arboviruses

Pools of 1–25 mosquitoes were homogenized in a Mini-BeadBeater-16 homogenizer (BioSpec, Bartlesville, OK). Remains of the blood-fed mosquitoes after removal of the engorged abdomens were individually homogenized. Briefly, the pools of the mosquitoes were added to microcentrifuge tubes containing 750 mg of 2-mm yttria-stabilized zirconium oxide beads (Glen Mills, Clifton, NJ), 150 mg of 0.1-mm

zirconia/yttria beads, and 450 μ L of phosphate-buffered saline. The supernatants obtained were loaded into 96-well plates for viral RNA extraction.

Viral RNA was extracted using the MagNA 96 Pure DNA and Viral NA Small Volume Kit (Roche Applied Science, Penzberg, Germany) in a MagNA Pure 96 (Roche Applied Science) automated extractor. RNA extracts were immediately reverse transcribed to cDNA using 10 μ L of the viral RNA as a template in 20- μ L reactions using the High-Capacity cDNA Synthesis Kit (Life Technologies, Carlsbad, CA). Non-ribosomal hexamers (0.6 mM) were used during the reverse transcription (Endoh et al., 2005). We screened for flaviviruses and alphaviruses by PCR-HRM using cycling conditions as published by Villinger et al. (2017) in a Rotor-Gene Q HRM capable thermocycler (Qiagen, Redwood City, CA).

Flaviviruses were screened using the Flavi-JV2a-F and Flavi-JV2a-R primers and alphaviruses were screened using the Vir-2052-F and Vir2052-R primers (Table 1) in 10- μ L PCR-HRM assays using 2 μ L cDNA template, 5 μ L MyTaq HS master mix (Bioline Reagents Limited, London, UK), 1 μ L of 50 μ M SYTO-9 saturating intercalating dye (Life technologies), 1 μ L of the primer mix, and 1 μ L of nuclease-free water. Dengue virus (DENV), sindbis virus (SINV), chikungunya virus, and Semliki Forest virus culture stocks were used as positive controls and nuclease-free water was used as a negative control (Villinger et al., 2017). To further characterize alphaviruses identified by PCR-HRM, cDNA of *Alphavirus* positive samples was re-amplified using Alpha-M2W and Alpha-cM3W primers. These PCR products were diluted 10-fold and 1- μ L volumes of these PCR product dilutions were used as templates in hemi-nested PCRs using the same reverse primer (Alpha-cM3W) with a nested forward primer (Alpha-M2W2) (Table 1) in a SimpliAmp thermocycler (Thermo Fisher Scientific). We determined the minimum infection rates (MIRs; number of

TABLE 2. MOSQUITO ABUNDANCE AND DIVERSITY IN THE MMNR AND NEAR THE SHNR

Species	Narok County (in MMNR)				Kwale County (near SHNR)			
	Female	Male	Total	% Total	Female	Male	Total	% Total
<i>Aedes aegypti</i>	3	1	4	0.55	158	108	266	12.45
<i>Aedes metallicus</i>	1	0	1	0.14	—	—	—	—
<i>Aedes simpsoni</i>	3	0	3	0.42	—	—	—	—
<i>Aedes tarsalis</i>	4	0	4	0.55	—	—	—	—
<i>Aedes tricholabis</i>	17	0	17	2.35	—	—	—	—
<i>Aedes</i> sp	1	0	1	0.14	—	—	—	—
<i>Anopheles coustani</i>	61	0	61	8.45	—	—	—	—
<i>Anopheles funestus</i>	17	1	18	2.49	—	—	—	—
<i>Anopheles gambiae</i>	2	2	4	0.55	—	—	—	—
<i>Anopheles</i> spp.	2	0	2	0.28	—	—	—	—
<i>Anopheles squamosis</i>	2	0	2	0.28	—	—	—	—
<i>Culex aethiopicus</i>	2	0	2	0.28	—	—	—	—
<i>Culex annulorhis</i>	7	0	7	0.97	1	0	1	0.045
<i>Culex cinereus</i>	22	10	32	4.43	27	20	47	2
<i>Culex pipiens</i>	242	36	278	38.5	436	888	1324	61.99
<i>Culex poicilipes</i>	12	2	14	1.94	—	—	—	—
<i>Culex tigripes</i>	8	12	20	2.77	—	—	—	—
<i>Culex univittatus</i>	158	29	187	25.9	1	1	2	0.094
<i>Culex vansomerani</i>	14	1	15	2.1	—	—	—	—
<i>Culex zombaensis</i>	21	24	45	6.23	113	384	497	23.27
<i>Eretmapodites chrysogaster</i>	2	0	2	0.28	—	—	—	—
<i>Mansonia africana</i>	5	2	7	0.97	—	—	—	—

MMNR, Maasai Mara National Reserve; SHNR, Shimba Hills National Reserve.

positive pools/total specimens tested \times 1000) of specific arboviruses detected in either sex of specific mosquito species sampled in either the MMNR or from locations near the SHNR.

Sequencing and phylogenetic analyses

Mosquito, vertebrate blood meal, and arbovirus PCR products were electrophoresed alongside 1 kb DNA ladders (Solis Biodyne) for 31 min at 300 A/125 V in 1.5% ethidium bromide-stained agarose gel in Tris-borate ethylenediaminetetraacetic acid (EDTA) buffer. DNA bands of interest were excised from agarose gels with clean, sharp scalpels over a blue-light transilluminator for visualization and purified using GenScript QuickClean II Gel Extraction Kit

(GenScript) following the manufacturer's protocol. The blood meal 16S rRNA and *cyt b* HRM amplicons were purified using the ExoSAP-IT kit (USB Corporation, Cleveland, OH). The purified amplicons were then sent for sequencing in both directions at Macrogen (Netherlands).

All nucleotide sequences were edited and aligned using the MAFFT plugin in Geneious software version 8.1.9 (created by Biomatters) (Kearse et al., 2012). Closely related sequences were identified by querying the GenBank nucleotide database using the Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/BLAST/). Maximum likelihood phylogenetic trees of SINV RNA sequences and mosquito COI sequences were constructed using PHyML v. 3.0 (Guindon et al., 2010). The phylogenies employed the Akaike information

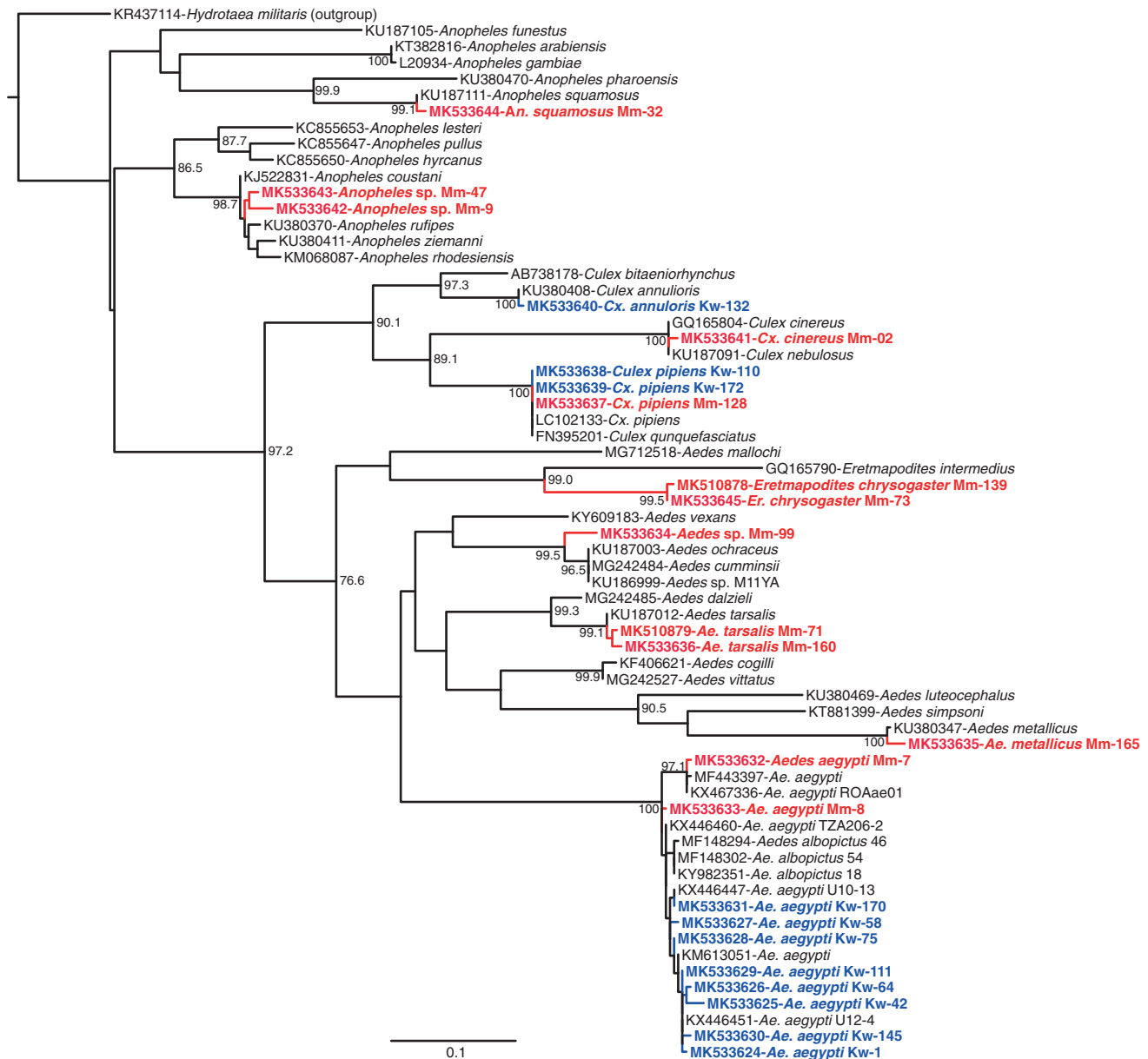


FIG. 2. Maximum likelihood phylogenetic tree of 658-nucleotide mosquito COI sequences. GenBank accessions, species, and isolates are indicated. The sequences obtained in this study are indicated in red (from the MMNR) and in blue (from locations near the SHNR). *Hydrataea militaris* was used as an outgroup. Bootstrap values at the major nodes are of percentage agreement among 1000 replicates. The branch length scale represents substitutions per site. COI, cytochrome c oxidase subunit 1.

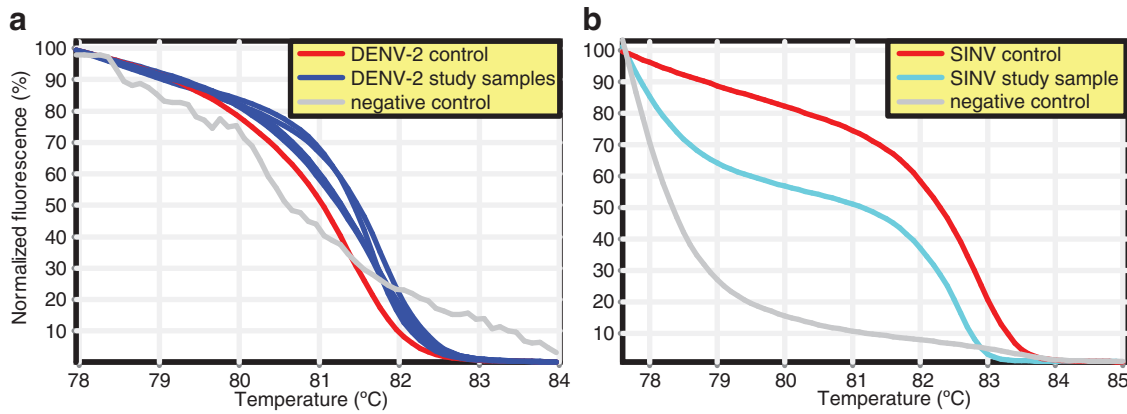


FIG. 3. Normalized HRM profiles of RT-PCR of (a) dengue serotype-2 virus (*Flavivirus*) and (b) SINV (*Alphavirus*) represented as percent fluorescence. HRM, high-resolution melting; SINV, Sindbis virus.

criterion (Lefort et al., 2017) for automatic model selection and tree topologies were estimated using nearest neighbor interchange improvements over 1000 bootstrap replicates. The phylogenetic trees were visualized using FigTree v.1.4.2 (Rambaut, 2014).

Results

Mosquito diversity and abundance

From the MMNR, 722 mosquitoes, comprising 20 species from five genera, were sampled (Table 2). *Culex* was the most

abundant genus, with *Culex pipiens* being the most abundant species (35.8%). The *Culex* genus also had the greatest diversity with nine species identified, followed by five *Aedes* spp. (GenBank acc. nos. MK533632–MK533636) and four *Anopheles* spp. (Table 2, Fig. 2). Among the *Anopheles*, two *Anopheles* specimens (GenBank acc. nos. MK533642–MK533643) with sequences clustering among *Anopheles coustani* complex sequences (Fig. 2) were obtained. We also sampled two *Eretmapodites chrysogaster* mosquitoes (GenBank acc. nos. MK533645 and MK510878) and a single *Aedes metallicus* mosquito (GenBank acc. no. MK533635).

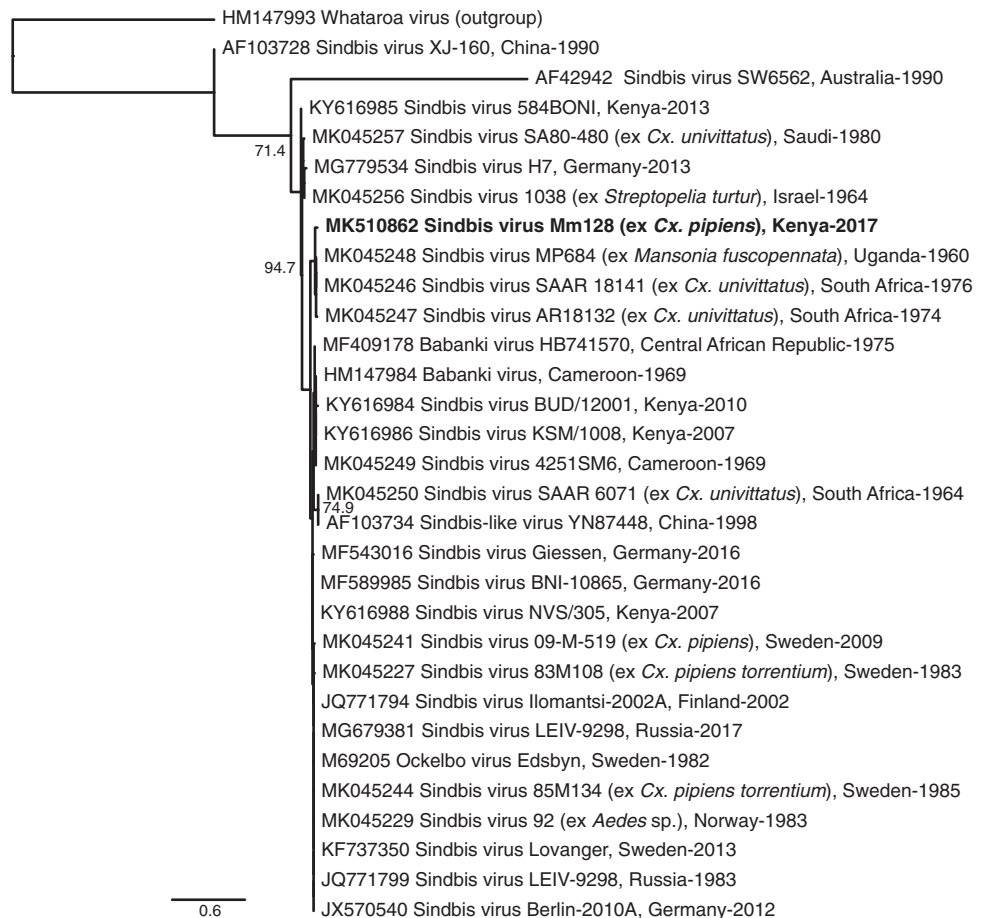


FIG. 4. Maximum likelihood phylogenetic tree of 254-nucleotide SINV sequences. GenBank accessions, strain names, country, and year of isolates are indicated. The sequence obtained in this study is red. Whataroa virus was used as an outgroup. Bootstrap values at the major nodes are of percentage agreement among 1000 replicates. The branch length scale represents substitutions per site.

TABLE 3. ARBOVIRUSES IDENTIFIED

Site	Species	Sex	Fed	Arbovirus	No. of pools	MIR
MMNR	<i>Aedes aegypti</i>	Female	Unfed	DENV-2	1	333.3
	<i>Aedes tarsalis</i>	Female	Bushbuck	DENV-2	1	250.0
	<i>Aedes tricholabis</i>	Female	Unfed	DENV-2	1	58.8
	<i>Culex pipiens</i>	Male	Unfed	Sindbis virus	1	27.8
Near SHNR	<i>Aedes aegypti</i>	Female	Unfed	DENV-2	2	12.7

MIR, minimum infection rate.

From locations near the SHNR, 2136 mosquitoes were sampled with *Cx. pipiens* being the most abundant species (61.99%). A single specimen of *Culex annulorhis* (GenBank acc. no. MK533640) was sampled. Phylogenetic analysis revealed that the *Ae. aegypti* COI gene sequences (GenBank acc. nos. MK533624–MK533631) are highly polymorphic (Fig. 2). Overall, sequencing of COI genes confirmed the identity of 14 out of 15 mosquito species and resolved one species, *Cx. annulorhis* (GenBank acc. no. MK533640), which was morphologically misidentified as *Culex ethiopicus*.

Arboviruses identified

Out of 170 mosquito pools from the MMNR, three female pools—*Aedes tarsalis* (MIR = 250.0), *Aedes tricholabis* (MIR = 58.8), and *Ae. aegypti* (MIR = 333.3)—were positive for DENV serotype-2 (DENV-2) (Fig. 3a; Supplementary Fig. S1) and one male *Cx. pipiens* (GenBank acc. no. MK533637) pool was positive for Sindbis virus (SINV) (GenBank acc. no. MK510862) (MIR = 27.8) (Figs. 3b and 4). From locations near the SHNR, out of 216 mosquito pools, 2 (0.9%) female *Ae. aegypti* pools were positive for DENV-2 (MIR = 12.7) (Table 3).

Blood meal analysis

Out of the 722 mosquitoes collected in the MMNR, 3 (0.4%) were blood engorged (Table 4). One *Er. chrysogaster* (GenBank acc. no. MK533645) had fed on both human (*cyt b* GenBank acc. no. MK510869) and hippopotamus (*cyt b* GenBank acc. no. MK510870) blood (Supplementary Fig. S2) and a *Culex zombaensis* had fed on human blood (*cyt b* GenBank acc. no. MK510866) (Fig. 5a). The DENV-2-positive *Ae. tarsalis* mosquito had a blood meal from a bushbuck (COI GenBank acc. no. MK510865; *cyt b* GenBank acc. no. MK510872), although we cannot conclude that this was the source of infection.

Of 2136 mosquitoes sampled from locations near the SHNR, 48 (2.25%) were blood fed (Table 4). The blood meal

sources included humans (COI GenBank acc. nos. MK510863–MK510864), domestic cat (*cyt b* GenBank acc. no. MK510873), house crow (*Corvus splendens*; *cyt b* GenBank acc. nos. MK510874–MK510876), house sparrow (*Passer domesticus*; Supplementary Fig. S3), chicken (*cyt b* GenBank acc. no. MK510871), and dog (*Canis lupus*; *cyt b* GenBank acc. no. MK510877) (Table 4; Fig. 5b).

Discussion

The arbovirus infections identified in zoophilic mosquitoes from the predominantly wildlife habitats of the MMNR (DENV-2 & SINV) and locations near the SHNR (DENV-2) highlight the importance of their sylvatic transmission in these ecologies, with their potential to also infect humans, as mosquitoes fed both on humans and wildlife. Many of the *Aedes*, *Culex*, *Anopheles*, and *Mansonia* mosquitoes identified are well known for their role in the transmission of arboviruses in Kenya (Ochieng et al., 2013).

However, to our knowledge, this is the first time DENV has been detected in *Ae. tarsalis* and *Ae. tricholabis*. Nonetheless, Ndumu virus (*Alphavirus*) has been isolated from *Ae. tricholabis* from North Eastern Province, Kenya (Ochieng et al., 2013), and the orthobunyaviruses, Pongola virus (Mossel et al., 2017), and Belief virus (Metselaar et al., 1974) have been isolated from *Ae. tarsalis* in Uganda. We also detected DENV-2 RNA sequences in *Ae. aegypti*, the known principal vector of dengue, Zika, chikungunya, and yellow fever viruses to humans (Patterson et al., 2016). The detection of DENV-2 in sympatric *Ae. tarsalis* and *Ae. tricholabis* gives insight into the possibility of sylvatic vectors involved in the transmission of dengue that could spill over to urban to human settlements around wildlife reserves leading to outbreaks. We were not able to determine whether the observed DENV-2 sequences in mosquitoes stemmed from established infections in the mosquito or from infected blood meals. Further studies are needed to determine the vector competency of *Ae. tarsalis* and *Ae. tricholabis* mosquitoes to

TABLE 4. BLOOD-FED MOSQUITOES AND THEIR BLOOD MEAL SOURCES IN THE MMNR AND NEAR THE SHNR

Study area	Mosquito species	Human	House sparrow	House crow	Cat	Chicken	Dog	Bushbuck	Hippopotamus
MMNR	<i>Aedes tarsalis</i>	—	—	—	—	—	—	1	—
	<i>Eretmapodites chrysogaster</i>	1*	—	—	—	—	—	—	1*
	<i>Culex zombaensis</i>	1	—	—	—	—	—	—	—
Near SHNR	<i>Aedes aegypti</i>	9	—	—	1	—	—	—	—
	<i>Culex pipiens</i>	29	2	3	—	2	1	—	—
	<i>Culex zombaensis</i>	1	—	—	—	—	—	—	—

*Mixed blood meal.

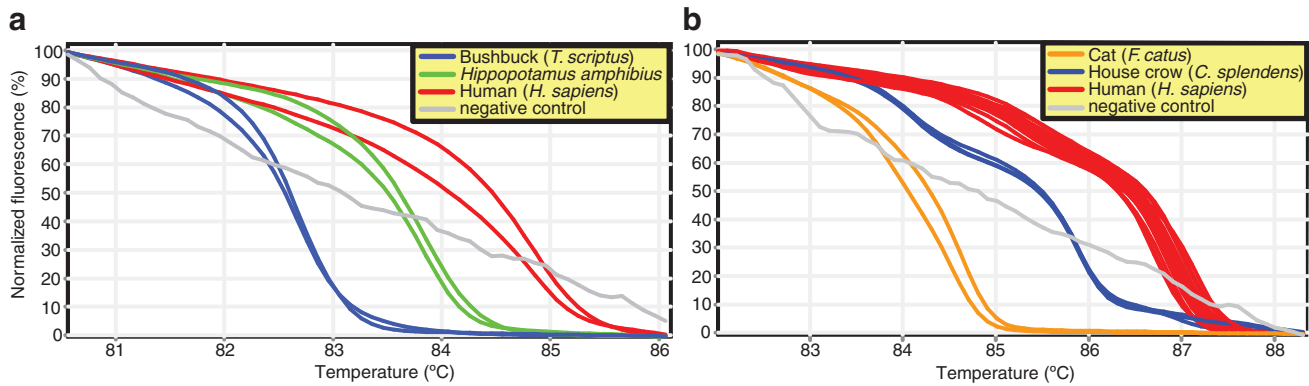


FIG. 5. Normalized HRM profiles represented as percent fluorescence of mosquito blood meal sources using (a) 16S ribosomal RNA on samples from the MMNR and (b) cytochrome b (*cyt b*) on samples from locations near the SHNR.

transmit DENV-2. Whether DENV-2 viruses in the region stem from sylvatic strains endemic to East Africa, or from more recent introductions from southeast Asia, also requires further investigation (Weaver and Vasilakis, 2009).

As male mosquitoes do not feed on blood, the SINV infection in one pool of male *Cx. pipiens* in the MMNR is likely due to vertical or environmental transmission. SINV has been isolated from *Culex* and *Aedes* mosquito species in Kenya (Sigei et al., 2018) and has been found in a *Cx. pipiens* mosquito that had fed on human blood in Baringo County, Kenya (Omondi et al., 2015). The SINV sequence identified in this study clustered most closely with recently deposited sequences of historical isolates from Uganda and South Africa isolated from *Mansonia fuscopennata* and *Culex univittatus* mosquitoes, respectively (Ling et al., 2019) (Fig. 4). This suggests that the SINV circulation is relatively stable and endemic in African wildlife and zoophilic mosquito populations with potential risk of future disease outbreaks. Birds are thought to serve as the main reservoir hosts (Hesson et al., 2016) with migratory birds having been associated with the spread of SINV across the continents (Hubálek, 2004). In Kenya, migratory birds converge along the coastal beaches, reefs, mangroves trees, and lakes in the Rift Valley during their season migrations (Bennun et al., 2000).

Most blood meals identified from both study areas were from humans. Although the MMNR is comparatively less encroached by humans than the SHNR, two out of three MMNR blood-fed mosquitoes identified had blood from humans. This could be because the traps were set in areas with human activities in the reserve. *Er. chrysogaster* had a mixed blood meal from human and hippopotamus in the MMNR. This opportunistic blood-feeding behavior (multiple-host feeding) is typically demonstrated by mosquito vectors when suitable vertebrate hosts are unavailable (Lardeux et al., 2007) and is of medical importance since the mosquito could act as bridge vectors for arbovirus transmission between wildlife animals and humans.

Aedes aegypti and *Cx. pipiens* in locations near the SHNR demonstrated a high hematophagic tendency toward humans. Our finding that *Cx. pipiens* had many blood meals from humans concurs with those of Omondi et al. (2015). However, house crow, house sparrow, chicken, and dog were also identified as *Cx. pipiens* blood meal sources. This feeding pattern makes *Cx. pipiens* a potential vector for transmitting arboviruses from avian hosts to humans. Although most

Ae. aegypti mosquitoes had fed on humans, the DENV-2 positive *Ae. tarsalis* mosquito sampled in the MMNR had a blood meal from a bushbuck. However, we cannot directly incriminate bushbuck as a reservoir for DENV-2. More serological studies need to be done from these wildlife species for reactivity against *Flavivirus* antigens.

Conclusions

Although we were not able to determine whether the zoophilic mosquitoes obtained the DENV-2 and SINV infections from wildlife, these findings highlight the potential importance of its sylvatic transmission among wildlife and zoophilic mosquitoes in maintaining the virus in enzootic circulation, which may be playing an important role in sporadic dengue outbreaks in Kenya. Eastwood et al. (2017) found evidence of DENV in olive baboons that were closer to human habitation in south coast, but not those baboons from the interior of SHNR. This suggests that spill-back from humans into wildlife may equally be occurring. Nonetheless, this study highlights that DENV-2 is circulating in an East African wildlife ecology with *Ae. tarsalis* and *Ae. tricholabis* as possible vectors and that SINV may be maintained in zoophilic mosquitoes by vertical or environmental transmission.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3

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