



RESEARCH ARTICLE

**REVISED** Diversity and Molecular Characterization of Mosquitoes (Diptera: Culicidae) in Selected Ecological Regions in Kenya. [version 2; peer review: 1 approved, 2 approved with reservations]

Previously titled: Diversity and Molecular Characterization of Mosquitoes (Diptera: Culicidae) in selected ecological regions in Kenya.

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**Abstract**

Mosquitoes play a predominant role as leading agents in the spread of vector-borne diseases and the consequent mortality in humans. Despite reports on increase of new and recurrent mosquito borne-disease outbreaks such as chikungunya, dengue fever and Rift Valley fever in Kenya, little is known about the genetic characteristics and diversity of the vector species that have been incriminated in transmission of disease pathogens. In this study, mosquito species were collected from Kisumu city, Kilifi town and Nairobi city and we determined their genetic diversity and phylogenetic relationships. PCR was used to amplify the partial cytochrome oxidase subunit 1 (CO1) gene of mosquito samples. Molecular-genetic and phylogenetic analysis of the partial cytochrome oxidase subunit 1 (CO1) gene were employed to identify their relationship with known mosquito species. Fourteen (14) haplotypes belonging to genus *Aedes*, nine (9) haplotypes belonging to genus *Anopheles* and twelve (12) haplotypes belonging to genus *Culex* were identified in this study. Findings from this study revealed a potentially new haplotype belonging to *Anopheles* genus and reported the first molecular characterization of *Aedes cumminsii* in Kenya. Sequence results revealed variation in mosquito species from Kilifi, Kisumu and Nairobi. Since vector competence varies greatly across species as well as species-complexes and is strongly associated with specific behavioural adaptations, proper species identification is important for vector control programs.

**Open Peer Review**

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	1	2	3
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Any reports and responses or comments on the article can be found at the end of the article.

**Keywords**

Aedes, Anopheles, Culex, chikungunya, Rift Valley fever, dengue fever

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**REVISED** Amendments from Version 1

The names of the pathogens have been rewritten based on the general rule, typos have been corrected accordingly and the conclusion has been strengthened as recommended.

**Any further responses from the reviewers can be found at the end of the article**

## Introduction

Mosquitoes are vectors responsible for transmission of numerous pathogens causing diseases such as, malaria, lymphatic filariasis, avian malaria and arboviruses including dengue virus, chikungunya virus, yellow fever virus, West Nile virus, and Zika virus (Charrel *et al.*, 2007; Semenza, 2014). Africa is one of the major hosts of mosquitoes responsible for mosquito-borne viruses (Braack *et al.*, 2018) that are of great medical importance and contribute to the current global public health threat (Enserink, 2007; Gubler, 2002; Higgs, 2014). Seasonal and environmental changes play a role in the global distribution of mosquito species and the arboviruses they transmit (Anyamba *et al.*, 2001; Hasnan *et al.*, 2016). The global spread of vector-borne diseases has resulted in multiple calls on nations to enhance surveillance of emerging arboviruses that requires understanding of the species composition and distribution of potential mosquito vectors (Grout *et al.*, 2017; Kollars *et al.*, 2016).

In the recent past, there has been an increasing spread of mosquito-borne viruses such as chikungunya virus, dengue virus and Rift Valley fever virus in Kenya, thus prompting a need for further research (Johnson *et al.*, 1982; Konongoi *et al.*, 2018). The available literature on mosquitoes in Kenya mainly addresses aspects of morphological identification of mosquito vectors and limited molecular characterization (Lutomiah *et al.*, 2013; Mwangangi *et al.*, 2013). Despite mosquitoes being a key public health challenge in Kenya, little is known about their species diversity and distribution along different ecological zones such as the Kenyan coast and Kenya's capital city. Subsequently, population genetic studies on mosquito vectors in Kenya have focused on the *Anopheles* genus because of its significance in endemic malaria transmission (Chen *et al.*, 2006; Chen *et al.*, 2004; Lukindu *et al.*, 2018). In addition, most of the studies on mosquito vector composition and diversity are based on mosquitoes confined to a single habitat or with a limited habitat range (Ajamma *et al.*, 2016a; Muturi *et al.*, 2006). The species composition and distribution of *Anopheline* mosquitoes in Kenya, particularly along the Kenyan coast, have broadly been reported over that of *Culicine* mosquitoes (Mbogo *et al.*, 2003; Midega *et al.*, 2007; Midega *et al.*, 2010). Moreover, little has been documented on the species composition and diversity of all mosquito groups by use of molecular markers. As such, understanding the species composition and diversity patterns of the suggested vectors is pivotal to the judicious deployment of existing vector control strategies and the development of new effective vector control interventions (Kraemer *et al.*, 2016).

In this study, we employed molecular genetic techniques, involving PCR and sequencing of cytochrome oxidase subunit 1 (CO1) gene to identify and characterize mosquito species in Nairobi, Kisumu and Kilifi Counties in Kenya.

## Methods

### Study sites

This study was carried out at Nairobi, Kilifi and Kisumu Counties in Kenya. Kilifi and Kisumu regions were chosen purposively due to their high abundance of mosquito vectors (WHO, 2017) and vector-borne disease burden, while Nairobi region was selected because it's a major international and domestic destination for both humans and parasites (Wesolowski *et al.*, 2012). Two sampling sites were randomly selected from each of the three regions as follows: Kisumu; Ahero and Kisumu town, Kilifi; Kilifi town and Mazingira Park and Nairobi; Nairobi city centre, and Northern Bypass (Figure 1).

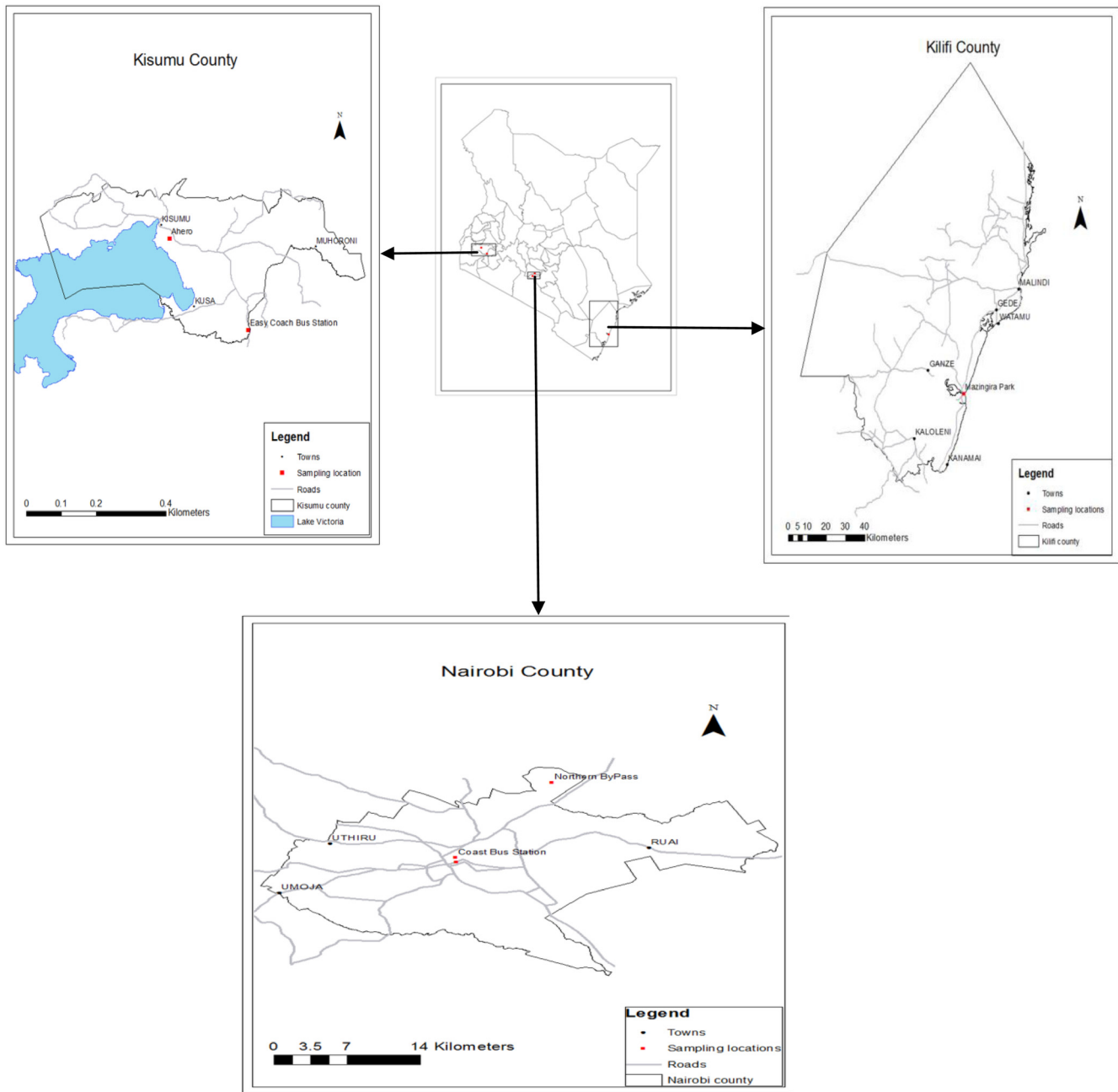
### Sampling strategy

The trapping of mosquitoes was carried out in the respective counties during the dry season (January–February 2018) and wet season (March–April 2018). The captures were conducted day and night using the Pyrethrum Spray Catch (PSC) method as used by (Ndiath *et al.*, 2011). The specimens were adult mosquitoes, which were morphologically sorted in the field into their respective genera, and transported in liquid nitrogen to the laboratory for further molecular analysis. A total of 2,438 adult mosquitoes were collected. Of these, 894, 824 and 720 adult mosquito samples were collected in Nairobi, Kisumu and Kilifi respectively. From the overall collection, 300 hundred mosquitoes per county were randomly selected for PCR. A total of 25 sequences per study region were used for phylogenetic and genetic diversity analysis as described in an earlier study (Hale *et al.*, 2012).

### PCR analysis

Total genomic DNA was extracted from whole body of individual mosquitoes using the Collins' protocol (Collins *et al.*, 1987) with minor modifications. A DNA homogenizing buffer (containing 0.1 M NaCl, 0.2 M sucrose, 0.01 M EDTA and 0.03 M Tris pH 8) was mixed with a lysis buffer (containing 0.25 M EDTA, 2.5% w/v SDS and 0.5 M Tris pH 9.2) in the ratio of 4:1 to make up the grinding buffer (GB). Each mosquito was homogenized in 100  $\mu$ L of the GB, using a hand-held pestle homogenizer and incubated for 30 min at 55°C. Into each sample, 14  $\mu$ L of 8 M potassium acetate (KAc), a deproteinating reagent was added and then incubated for 30 min at room temperature before centrifuging at 13,000 rpm for 15 min to get the supernatant that contained the nucleic acid component. 95% ethanol was used to precipitate the genomic DNA. Centrifugation at 13,000 rpm for 10 minutes was done to obtain the nucleic acid pellet. This was followed by a washing step using 70% ethanol. The DNA pellet was suspended in 100  $\mu$ L of TE buffer pH 7.2 and stored at  $-20^{\circ}\text{C}$  awaiting subsequent experimental procedures.

The primer set; Forward (LCO1490\_GGTCAACAAATCATAAA-GATATTGG) and Reverse (HCO2198 TAAACTTCAGGGTGAC-CAAAAATCA) synthesized by MacroGen (OG180803-187) and previously published by Folmer *et al.* were used in molecular identification of the mosquito species (Folmer *et al.*, 1994). In a 10  $\mu$ L PCR reaction volume, the PCR mix consisted of 2  $\mu$ L 1 $\times$  HOT FIREPol<sup>®</sup> Eva Green mix (Solis BioDyne, Tartu, Estonia) catalogue number 08-31-00008, 6  $\mu$ L of nuclease-free water, 0.5 picomoles of each primer and 1  $\mu$ L of the DNA template. The fragments were amplified using applied biosystems



**Figure 1.** A map of Kenya showing the location of sampled mosquitoes.

ProFlex SN 297802057 thermocycler under the following cycling parameters; initial denaturation for 15 min at 95°C, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 50°C (*Anopheles*, *Aedes*, *Culex*) for 30 sec, and extension at 72°C for 30 sec, and a final extension at 72°C for 7 min. The PCR products from the amplification of the mitochondrial cytochrome c oxidase 1 (CO1) region of the mosquito after purification using QIAquick® gel extraction kit catalogue number 28706, were shipped for sequencing at Macrogen Inc., South Korea.

### Sequence analysis

Resultant mitochondrial cytochrome c oxidase 1 (CO1) sequence chromatograms were edited and visualized using Chromas Lite version 2.6.5. The sequences were deposited in GenBank and accession numbers assigned accordingly. Consensus sequences were aligned using ClustalX version 2 (Thompson *et al.*, 1997), and visualized using Seaview version 4.7 (Gouy *et al.*, 2010). Unique sequences (haplotypes) were identified using DnaSP version 6 (Librado & Rozas, 2009). Sequence polymorphisms were identified using DnaSP and

visualized using Jalview version 2.10.5 (Waterhouse *et al.*, 2009). DNA sequence divergence was analysed using DnaSP. These unique sequences were compared with reference sequences from other parts of the world, selected to represent the *Aedes*, *Anopheles* and *Culex* genera previously reported and available from GenBank (Benson *et al.*, 2011). Other sequences similar to the study sequences in GenBank obtained using the Blastn algorithm were also included in the analysis. Multiple alignment and comparison of the study sequences and GenBank references were performed using ClustalX. Phylogenetic and molecular evolutionary analyses were conducted using Software for Molecular Evolutionary Genetics (MEGA7) (Kumar *et al.*, 2016). Phylogenetic trees were constructed using the maximum likelihood (ML) method rooted using *Lutzomyia longipalpis*. The phylogenetic trees were estimated using the best-fit general time-reversible (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites. Bootstrap resampling process (1000 replications) was employed to assess the robustness of individual nodes of phylogeny (only >50% were indicated). The resultant tree was visualized using Dendroscope version 3 (Huson & Scornavacca, 2012).

**Results**

**Phylogenetic analysis**

From each study site, 25 CO1 gene amplicons were sequenced for phylogenetic analysis. In total, 14 haplotypes belonging to genera *Aedes*, 9 haplotypes belonging to genera *Anopheles* and 12 haplotypes belonging to genera *Culex* were identified through CO1 sequence analysis. These sequences were deposited in GenBank and assigned accession numbers (Table 1). Sequence analysis revealed a unique *Anopheles* haplotype (GenBank accession number, MK300230) (Figure 3). Subsequently, haplotypes of *Anopheles gambiae*, *Anopheles funestus*, *Aedes cumminsii*, *Aedes aegypti*, *Culex pipiens* and *Culex sitiens* were found to be distributed across Kilifi, Kisumu and Nairobi mosquito populations (Table 1).

Diversity indices for the three populations, based on sequenced results were calculated as shown in (Table 2). Average number nucleotide differences (k), nucleotide diversity Pi (π) and haplotype diversity (Hd) varied among the species (Table 2).

Phylogenetic analysis of fourteen (14) *Aedes* haplotypes from Kilifi and Nairobi with similar sequences based on Blastn (NCBI)

**Table 1. Distribution of *Aedes*, *Anopheles* and *Culex* species across Kisumu, Kilifi and Nairobi.**

Region	Sample Size	Species	Number of Haplotypes	Accession Number
Nairobi	25	<i>Aedes aegypti</i>	4	MK300226, MK300227, MK300228, MK300229
		<i>Anopheles gambiae</i>	1	MK300238
		<i>Culex pipiens</i>	3	MK300248, MK300249, MK300250
Kisumu	25	<i>Anopheles gambiae</i>	5	MK300233, MK300234, MK300235, MK300236, MK300237
		<i>Anopheles funestus</i>	2	MK300231, MK300232
		<i>Culex pipiens</i>	2	MK300242, MK300247
Kilifi	25	<i>Aedes aegypti</i>	9	MK300216, MK300217, MK300218, MK300219, MK300220, MK300221, MK300222, MK300223, MK300224
		<i>Aedes cumminsii</i>	1	MK300225
		<i>Anopheles species</i>	1	MK300230
		<i>Culex pipiens</i>	3	MK300239, MK300242, MK300246
		<i>Culex sitiens</i>	5	MK300240, MK30024, MK300243, MK300244, MK300245

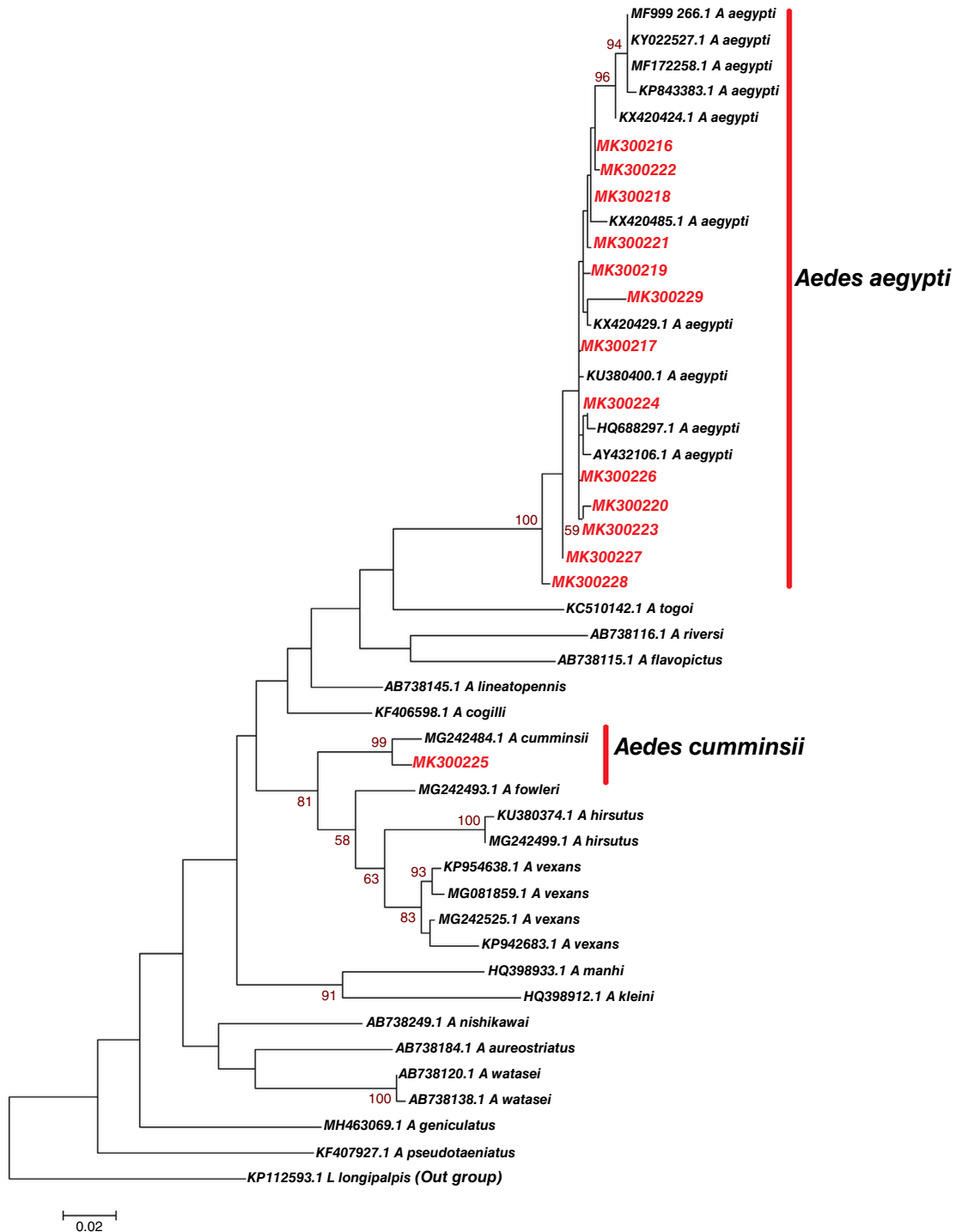
**Table 2. Genetic diversity indices in the mitochondrial cytochrome oxidase 1 (CO1) sequences of mosquito species from Nairobi, Kisumu and Kilifi.**

Region	Species	Hap	S	k	Pi (π)	Hd
Nairobi	<i>Aedes aegypti</i>	4	21	11.333	0.0160	1.000
	<i>Culex pipiens</i>	3	5	3.333	0.0047	1.000
Kisumu	<i>Anopheles gambiae</i>	5	7	3.200	0.0045	1.000
	<i>Anopheles funestus</i>	2	1	1.000	0.0236	1.000
	<i>Culex pipiens</i>	2	6	6.000	0.0085	1.000
Kilifi	<i>Aedes aegypti</i>	9	12	4.222	0.0060	1.000
	<i>Culex pipiens</i>	3	51	34.000	0.0480	1.000
	<i>Culex sitiens</i>	5	53	22.000	0.0311	1.000

<sup>2</sup>Hap: number of haplotypes; S: number of polymorphic segregating sites; k: the average number of nucleotide differences; Pi (π): nucleotide diversity; Hd: haplotype gene diversity.

search and sequences of known *Aedes* identity revealed that study *Aedes* haplotype Accession number MK300225 clustered with *Aedes cumminsii* (Figure 2). Study haplotypes Accession number; MK300216, MK300217, MK300218, MK300219, MK300220, MK300221, MK300222, MK300223, MK300224,

MK300226, MK300227, MK300228 and MK300229 clustered with *Aedes aegypti* that has been previously identified in France (Accession number HQ688297.1). Significantly, they also clustered with *Aedes aegypti* (Accession number KX420485.1, KX420429.1 and KU380400.1) previously reported in



**Figure 2.** Maximum likelihood phylogenetic tree of partial cytochrome oxidase subunit 1 (CO1) nucleotide sequences of *Aedes* species haplotypes in Red and GenBank references in Black. The scale represents the number of differences between sequences (0.02=2%). The gamma correction for rate heterogeneity was 0.1963. The analysis involved 46 nucleotide sequences. There were a total of 657 positions in the final dataset.

Nyanza-Kisumu, Kenya (Figure 2). Genetic divergence between study *Aedes* haplotypes identified in Kilifi and Nairobi and *Aedes* species they clustered with (sequences of known species obtained from GenBank) was variable (Table 3). There was limited divergence between *Aedes aegypti* (Accession number KX420485) that has previously been identified in Nyanza-Kisumu, Kenya and study haplotypes MK300216, MK300222, MK300218 and MK300221. *Aedes aegypti* (Accession number KU380400.1) that has been reported in Nyanza-Kisumu, Kenya before showed limited divergence with study haplotype MK300217. Limited divergence was also identified between haplotype MK300224 and *Aedes aegypti* (Accession number HQ688297.1) that has been characterized in France. Greater divergence and heterogeneity was observed between *Aedes aegypti* and study haplotypes MK300225, MK300219 and MK300229. Study haplotypes MK300216, MK300220, MK300223, MK300227 and MK300228 formed a distinct clade with other *Aedes aegypti* of known identity (Figure 2).

Phylogenetic analysis of haplotypes with similar sequences to those of known identity showed a clustering of study *Anopheles* haplotype MK300231 and MK300232 with *Anopheles funestus*. Notably, they also clustered with *Anopheles funestus* (Accession number MH299888.1 and KU380404.1) that has been reported in Kilifi and Baringo counties in Kenya respectively (Figure 3). Study haplotype MK300233, MK300234, MK300235, MK300236, MK300237 and MK300238 clustered with *Anopheles gambiae* previously isolated in Uganda (Accession number MG753695.1, MG753730.1 and MG753745.1) (Figure 3). *Anopheles* haplotype MK300230 formed its own distinct clade. This study haplotype MK300230 may be a new species or novel haplotype not yet described (Figure 3). Genetic divergence between *Anopheles* haplotypes identified in Kisumu, Kilifi, Nairobi and *Anopheles* species from GenBank they clustered with was variable in some haplotypes while others were not variable (Table 4). There was very limited divergence and heterogeneity between *Anopheles funestus* and study haplotype MK300231 and MK300232. There was no divergence between *Anopheles gambiae* (Accession number DQ792577.1 and MG753695.1) and study haplotype MK300234. *Anopheles gambiae* (Accession number MG753695.1) has been identified in Uganda before. Study haplotypes MK300235, MK300233, MK300238, MK300236 and MK300237 showed limited divergence with *Anopheles gambiae*.

From the phylogenetic analysis, we further established that 12 *Culex* haplotypes from Kilifi, Kisumu and Nairobi, and similar sequences of known identity based on Blastn (NCBI) showed a clustering of study haplotype MK300240, MK300242, MK300246, MK300247, MK300248, MK300249 and MK300250 with *Culex pipiens* that have been identified in different regions of the world. Importantly, they clustered with *Culex pipiens* that has previously been identified in Nyanza-Kisumu, Kenya (Accession number KU380381.1, KU380372.1) (Figure 4). Study haplotypes MK300239, MK300241, MK300243, MK300244, MK300245 clustered with *Culex sitiens* that was earlier identified in Australia (Accession number MG712559.1) (Figure 4). Genetic divergence between *Culex* haplotypes identified in Kisumu, Kilifi, Nairobi and reference *Culex* species was slightly variable in some species, while other species showed no divergence (Table 5).

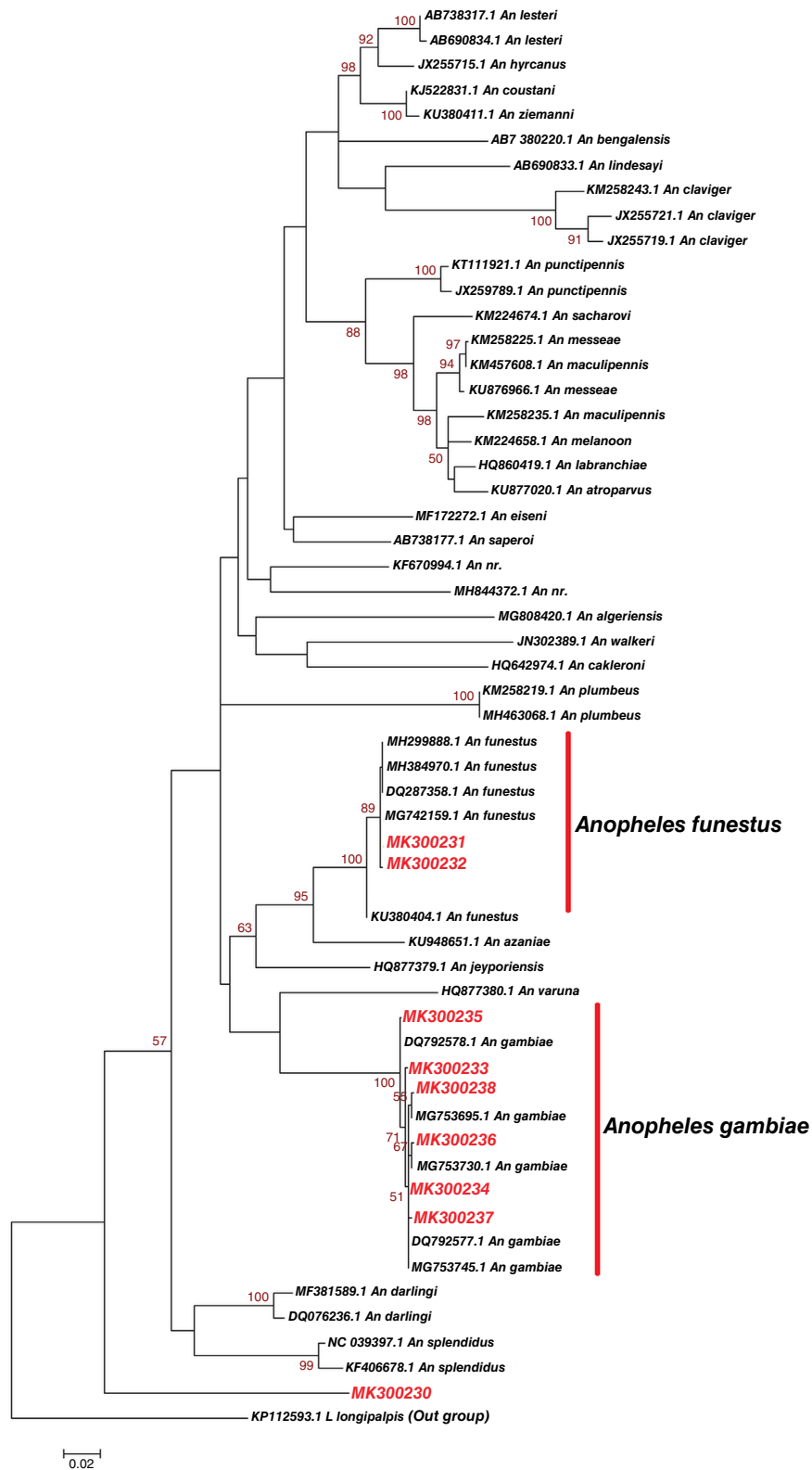
**Discussion**

This study identified *Aedes aegypti* in both Kilifi and Nairobi populations and *Aedes cumminsii* in the Kilifi population only. *Anopheles gambiae* was identified in both Kisumu and Nairobi population whereas *Anopheles funestus* was identified in Kisumu population only. A potentially novel *Anopheles* haplotype MK300230 was identified in Kilifi population. *Culex pipiens* was identified in all the three populations; Kisumu, Nairobi and Kilifi while *Culex sitiens* was only identified in the Kilifi population. The greatest diversity was in the genus *Aedes* that has 14 haplotypes, followed by *Culex* 12 and *Anopheles* 9, this is consistent with other studies looking at mosquito diversity in different ecological regions in Kenya (Mwangangi et al., 2012). Similarly, out of the 35 mosquitoes haplotypes identified in Kilifi, Nairobi and Kisumu regions, one *Culex* haplotype MK300242 from this study has been previously reported in Kisumu-Nyanza in Kenya and in Portugal (Ajamma et al., 2016b; Mixão et al., 2016), and one *Anopheles* haplotype MK300234 in Uganda (Lukindu et al., 2018). The Kilifi mosquito population had the greatest diversity and abundance of mosquito species, possibly due to its geographical position, human activities, and natural climatic conditions.

*Aedes cumminsii* has been morphologically identified in Kenya before (Mwangangi et al., 2006), however, this study reports the first molecular characterization of *Aedes cumminsii* in Kenya. *Aedes* haplotypes between Kilifi and Nairobi populations were

**Table 3. Sequence divergence between study *Aedes* species haplotypes and closely associated sequences from GenBank.**

	MK 300225	MK 300216	MK 300222	MK 300218	MK 300221	MK 300219	MK 300229	MK 300224	MK 300219
MG242484.1 <i>Ae.aegypti</i>	0.017								
KX420485.1 <i>Ae.aegypti</i>		0.008	0.009	0.006	0.009	0.013			
KX420429.1 <i>Ae.aegypti</i>							0.017		
KU380400.1 <i>Ae.aegypti</i>								0.003	
HQ688297.1 <i>Ae.aegypti</i>									0.003



**Figure 3.** Maximum likelihood phylogenetic tree of partial cytochrome oxidase subunit 1 (CO1) nucleotide sequences of *Anopheles* species haplotypes in Red and GenBank references in Black. The gamma correction for rate heterogeneity was 0.1647. The analysis involved 57 nucleotide sequences. There were a total of 658 positions in the final dataset.



**Table 4. Sequence divergence between study *Anopheles* haplotypes and known *Anopheles* species obtained from GenBank.**

	MK 300231	MK 300232	MK 300235	MK 300233	MK 300238	MK 300236	MK 300234	MK 300238
MG742159.1 <i>An.funestus</i>	0.000	0.002						
MH299888.1 <i>An.funestus</i>		0.003						
MH384970.1 <i>An.funestus</i>		0.003						
DQ287358.1 <i>An.funestus</i>		0.003						
DQ792578.1 <i>An.gambiae</i>			0.002					
MG753695.1 <i>An.gambiae</i>				0.005	0.002			
MG753730.1 <i>An.gambiae</i>						0.002		
DQ792577.1 <i>An.gambiae</i>							0.000	0.002
MG753695.1 <i>An.gambiae</i>							0.000	0.002

divergent based on nucleotide diversity tests; this could be due to different climatic zones. Thus, diversity in vector haplotypes plays an important role in vector control and management practices and epidemiology of vector borne diseases (Murugan *et al.*, 2016). Phylogenetic analysis showed presence of two *Aedes* species that is *Aedes cumminsii* and *Aedes aegypti*, in Kilifi, while Nairobi had only *Aedes aegypti* (Figure 2 and Table 1). This study has identified 4 different *Aedes aegypti* haplotypes in Nairobi. Previous studies on survey of mosquito composition in Nairobi have indicated low percentage of *Aedes* mosquito (Kinuthia *et al.*, 2017). There is therefore increased diversity in *Aedes aegypti* species from Nairobi; diversity and spread of *Aedes aegypti* has been attributed to the increase in arboviral infections (Woolhouse *et al.*, 1997). The diversity of *Aedes aegypti* in Nairobi could be the result of high population density (Gubler & Clark, 1995), poor sanitation and waste disposal as well as water management (Monath, 1994). The Kilifi population had genetically diverse forms of *Aedes aegypti* (Table 2). *Aedes aegypti* is widespread on the Kenyan coast (McDonald, 1977; Teesdale, 1955). It is the principal vector of dengue virus, chikungunya, and urban yellow fever virus (Reiter, 2010), and it was predominant in the Kilifi samples. This may contribute to their high susceptibility to dengue-outbreak reported in the region (Baba *et al.*, 2016; Chepkorir *et al.*, 2014). Secondly, factors relating to availability of breeding sites, temperature or altitudinal differences may have influenced the diversity patterns of *Aedes aegypti* in Kilifi (Barrera *et al.*, 2011). Evidence of high diversity of *Aedes aegypti* in Kilifi also means that the Kenyan coast is consistently at higher risk of yellow fever transmission (Agha *et al.*, 2017). Kilifi lies in between Malindi and Mombasa cities which are popular destinations for international tourism as well as maritime industry, and where *Aedes aegypti* is widespread (Ngugi *et al.*, 2017). Human trade and travel may bolster movement of *Aedes aegypti* (Powell & Tabachnick, 2013) and contribute to diversity of the species. In addition, invasion risk related to human travel has become far more severe (Egizi *et al.*, 2016; Wilder-Smith & Gubler, 2008). Phylogenetic

relationship between *Aedes* species from this study and other *Aedes* species of known identity from GenBank showed clustering with *Aedes cumminsii* and *Aedes aegypti* at a high bootstrap value (>90%) at the defining node on the phylogenetic tree (Figure 2). However, genetic diversity between *Aedes* species from this study and those of known identity from GenBank was variable (Table 3).

*Anopheles* species were distributed across the three study populations Kisumu, Nairobi and Kilifi (Table 1). *Anopheles* species between Kilifi, Kisumu and Nairobi populations were highly divergent as analyzed using molecular markers. Nairobi had only one haplotype of *Anopheles gambiae* (Table 1). *Anopheles* mosquitoes have also been reported in places where malaria has been eradicated and also in malaria non endemic regions thus increasing the risk of reintroduction of malaria as well as spreading of malaria to new areas (Martens & Hall, 2000). Other than transmitting malaria, *Anopheles* mosquitoes have been indicated as carriers of arboviruses including West Nile virus and Japanese encephalitis (Thenmozhi *et al.*, 2006), as well as viruses that cause o'nyong-nyong and chikungunya fevers (Vanlandingham *et al.*, 2005). This study has indicated higher diversity of *Anopheles* haplotypes in the Kisumu population, having detected *Anopheles gambiae* and *Anopheles funestus* (Table 2). High diversity of *Anopheles* vector is a key feature for consideration in *Anopheles* management and has been associated with the rise in malaria transmission (Loaiza *et al.*, 2012). The low diversity of *Anopheles* species in Kilifi and Nairobi may be attributed to the Great Rift Valley and, high-elevation mountains in western Kenya. The vast arid area in the east of the Great Rift Valley inhibits human settlement, thus restricting *Anopheles funestus* gene flow between coastal and western Kenya (Lukindu *et al.*, 2018). *Anopheles funestus* is closely associated with human dwellings and therefore plays an important role in the transmission of malaria (Kweka *et al.*, 2013). *Anopheles gambiae* haplotypes in Kisumu were diverse, this is consistent with other studies that have reported a high genetic



**Figure 4.** Maximum likelihood phylogenetic tree of partial cytochrome oxidase subunit 1 (CO1) nucleotide sequences of *Culex* species haplotypes in Red and GenBank references in Black. The gamma correction for rate heterogeneity was 0.1790. The analysis involved 62 nucleotide sequences. There were a total of 658 positions in the final dataset.

**Table 5. Sequence divergence between study *Culex* species and known *Culex* species obtained from GenBank.**

	MK 300242	MK 300246	MK 300239	MK 300241	MK 300243	MK 300244	MK 300245
LC102132.1 <i>Culex pipiens</i>	0.000	0.002					
KU380381.1 <i>Culex pipiens</i>	0.000	0.002					
KU380372.1 <i>Culex pipiens</i>	0.000	0.002					
MG712559.1 <i>Culex sitiens</i>			0.009	0.006	0.009	0.001	0.009

diversity of *Anopheles gambiae* in Kisumu Kenya (Chen *et al.*, 2004). Phylogenetic analysis (Figure 3) and nucleotide diversity tests (Table 4) showed no divergence between Kisumu *Anopheles gambiae* haplotype MK300234 with *Anopheles gambiae* MG753695.1, used as reference that was previously isolated in Uganda (Lukindu *et al.*, 2018). This indicates the presence of genetically identical *Anopheles gambiae* between Kenya and Uganda which could be attributed to cross-border migration, or retention of shared ancestral polymorphism. Therefore, this could suggest that, these species share the same ecological niche or ancestral divergence. *Anopheles gambiae* (s.s.) (formerly *Anopheles gambiae* S-form) is a main vector of malaria in sub-Saharan Africa, where 90% of an estimated 445,000 malaria deaths worldwide occurred in 2016 (CDC - Malaria - About Malaria - Disease). Presence of both *Anopheles gambiae* and *Anopheles funestus* in Kisumu suggest that the area is still at high risk of malaria transmission. This study has identified a potentially new haplotype of *Anopheles* species MK300230 in Kilifi (Figure 3). Through molecular techniques new haplotypes of *Anopheles* species are continually being identified; for instance, new species of *Anopheles nuneztovari* have been identified in Brazil (Scarpassa *et al.*, 2016).

*Culex pipiens* was distributed across Kilifi, Kisumu and Nairobi population while *Culex sitiens* was only identified in Kilifi population (Table 1). *Culicidae* is a large and abundant group that occurs throughout temperate and tropical regions of the world, as well as the peri Arctic Circle (Schäfer & Lundström, 2001). *Culex* mosquitoes are an important vector of the zoonotic infection filariasis. Human filariasis infection is a major public health concern. Approximately 57% of those at risk of infection is in the South-East Asia Region and 37% in the African Region (“WHO | Epidemiology,” 2018). Although *Culex pipiens* is ornithophilic it can also feed on humans and mammals (Reisen *et al.*, 1990) and thus capable to transmit West Nile virus to humans. *Culex pipiens* (Linnaeus) has been identified as the primary vector of West Nile virus (Turell *et al.*, 2000). Kenyan strain of *Culex pipiens* has been confirmed to be capable of transmitting West Nile virus and its circulation among humans in Kenya has been detected (Lutomiah *et al.*, 2011; Morrill *et al.*, 1991).

Therefore, the distribution of *Culex pipiens* across Kilifi, Nairobi and Kisumu could increase the risk of West Nile virus transmissions/outbreaks in most parts of Kenya. *Culex pipiens* haplotype MK300242 was identified in both Kilifi and Kisumu population (Figure 4). This study reports distribution of identical mosquito vector species between populations. Phylogenetic analysis revealed *Culex pipiens* haplotype MK300242 from this study showed no divergence to the *Culex pipiens* sequences LC102132.1 from Portugal and KU380381.1, KU380372.1 from Nyanza Kenya (Table 5). This study identified *Culex sitiens* in the Kilifi population only, *Culex sitiens* has been found to tolerate saline waters, in Oman it has been successfully isolated from brackish water (Roberts, 1996). Consequently, parasites such as *Microsporidium*, *Amblyospora* have been isolated from *Culex sitiens* mosquito in Coastal Kenya (Sabwa *et al.*, 1984).

## Conclusion

Results from this study demonstrate that mosquito vectors that have been associated to arboviral pathogens are distributed across Kilifi, Nairobi and Kisumu counties. 35 haplotypes belonging to genus *Anopheles*, *Culex* and *Aedes* have been identified, genetic diversity of this haplotypes varies with some genus recording high diversity where's others had low diversity. A potentially new haplotype belonging to *Anopheles* genus has been identified. This implies further research on genetic characterization of mosquitoes in Kenya for an appropriate vector control and management program across the whole country.

## Data availability

### Underlying data

Culicidae cytochrome c oxidase subunit 1 (COI) gene, partial cds; mitochondrial. PopSet 1573759763: <https://www.ncbi.nlm.nih.gov/popset/1573759763?report=genbank>. Accession numbers MK300216 – MK300250

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## Version 2

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**Kentaro Itokawa** 

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The study described in the manuscript authored by Makanda et al. describes the genetic diversity of mosquitoes captured in three regions in Kenya using the mitochondrial CO1 gene. They amplified a part of CO1 by PCR and sequenced the amplicon. Their analysis includes phylogenetic clustering of each CO1 haplotype with already registered sequence entries in Genbank and calculation of some basic population genetic parameters such as nucleotide diversity (Pi) and haplotype gene diversity (Hd). The main discovery and conclusions they have drawn out from the results were

1. “A potentially novel *Anopheles* haplotype MK300230 was identified in Kilifi population.”
2. “The first molecular characterization of *Aedes cumminsii* in Kenya”
3. “higher diversity of *Anopheles* haplotypes in the Kisumu population, having detected *Anopheles gambiae* and *Anopheles funestus*”

However, there are doubtful points at each finding they are claiming in this manuscript. Many of those problems seem to stem from species identification merely by sequence without leaving insect specimens for retrospective morphological assessment (they used the whole body of insects for DNA extraction, according to the manuscript). Because of this, it would be extremely difficult to address the concerns I will describe below.

### **About the “A potentially novel *Anopheles* haplotype MK300230”**

In the phylogenetic tree in Fig 3, the haplotype MK300230 locates outside of the known *Anopheles* group clade. This is very strange and interesting if that insect individual actually possessed morphological characters that look like *Anopheles*. Then I queried the sequence of MK300230 to GenBank database by blastn search and I found two notable hits, KY831299.1 (99% identical for 588 bp) and JN298693.1 (97% identical for 658 bp) both of which were registered as sequences obtained from some species of Tipuloidea. Unfortunately, MK300230 would not be a sequence for *Anopheles* or even not for a mosquito species. Probably, what the authors have actually captured was a kind of crane flies, though no clue to confirm it remains because the whole specimens have gone for DNA extraction.

### **“The first molecular characterization of *Aedes cumminsii* in Kenya”**

The claim that the insect represented by MK300225 belongs *Aedes cumminsii* is difficult to accept from the phylogenetic tree in Fig 2; there is only one reference sequence for *Aedes cumminsii* (MG242484). Even if MG242484 was the closest sequence entry to MK300225 in current database, it is a dangerous leap to conclude those two sequences are “haplotypes of same species”. With a similar reason, assigning MK300227 and MK300228 to *Aedes aegypti* in the Fig 2 and MK300247 to *Culex pipiens* in Fig 4 are also problematic because they are not clustered with reference sequences of each corresponding species at least in those figures.

Because of those issues with ambiguous species assignment of each haplotype sequences, I can not consider the calculated population genetic parameters as valid.

Utilizing DNA sequence for taxonomical identification requires extreme care for the selection of reference sequences to be used. Sadly, the description of species associated with DNA sequences existing in GenBank database is not always correct, but they are based on various levels of evidence. If one classifies his/her sequence merely based on similarity to a sequence that has a wrong species description and registered this new sequence with a wrong species assignment, this entry can be another source of another false finding by another researcher. At least, the authors should correct the ORGANISM section of MK300230 entry in GenBank which is now saying “*Anopheles splendidus*”.

Preserving specimens is important to avoid many of those pitfalls. For mosquito, there are some useful method like NaOH crude extraction (e.g. Lars Rudbeck & Jørgen Dissing, 1998, *BioTechniques* 25 (4)), which enable preparing DNA template for PCR from a single leg with only cheap cost. The study described in the manuscript authored by Makanda et al described genetic diversity of mosquitoes captured in three regions in Kenya using mitochondrial CO1 gene. They amplified a part of CO1 by PCR and sequenced the amplicon. Their analysis includes phylogenetic clustering of each CO1 haplotype with already registered sequence entries in genbank and calculation of some basic population genetic parameters such as nucleotide diversity (Pi) and haplotype gene diversity (Hd). The main discovery and conclusions they have drawn out from the results were...

1. “A potentially novel *Anopheles* haplotype MK300230 was identified in Kilifi population.”
2. “The first molecular characterization of *Aedes cumminsii* in Kenya”
3. “higher diversity of *Anopheles* haplotypes in the Kisumu population, having detected *Anopheles gambiae* and *Anopheles funestus*”

However, there are doubtful points at each finding they are claiming in this manuscript. Many of those problems seem to stem on identification of species merely by sequence without leaving insect specimens for retrospective morphological assessment (they used whole body for DNA extraction according to the manuscript). Because of this, it would be extremely difficult to address the concerns I will describe below.

- About the “A potentially novel *Anopheles* haplotype MK300230”

In the phylogenetic tree in Fig 3, the haplotype MK300230 locates outside of the known *Anopheles* group clade. This is very strange and interesting if that insect individual actually possessed morphological characters which looks like *Anopheles*. Then I queried the sequence of MK300230 to genbank database by blastn search, I found two notable hits, KY831299.1 (99% identical for 588 bp) and JN298693.1 (97% identical for 658 bp) both of which were registered as sequences obtained from some species of Tipuloidea. Unfortunately, MK300230 would not be a sequence for *Anopheles* or even not for any mosquito species. Probably, what the authors have actually captured was a kind of crane flies, though no clue to confirm it remains if the whole specimens had been used for DNA extraction.

- “The first molecular characterization of *Aedes cumminsii* in Kenya”

The claim that the insect represented by MK300225 belong *Aedes cumminsii* is difficult to accept from the phylogenetic tree in Fig 2; there is only one reference sequence for *Aedes cumminsii* (MG242484). Even of MG242484 was the closest sequence entry to MK30025 in the current database, it is a dangerous leap to conclude those two sequences are “haplotypes of same species”. For a similar reason, assigning MK300227 and MK300228 to *Aedes aegypti* in Fig 2 and MK300247 to *Culex pipiens* in Fig 4 is also problematic because they are not clustered with reference sequences of each corresponding species at least in those figures.

Because of those problems,

Utilizing DNA sequence for taxonomical identification requires extreme care for selection of reference sequences to be used. Sadly, the description of species associated with DNA sequences exiting in GenBank database is not always correct, but they are based on various levels of evidence. If one classifies his/her sequence merely based on similarity to a sequence that has a wrong species description and registered this new sequence with a wrong species assignment, this entry can be another source of another false finding by another researcher. At least, the authors should correct the ORGANISM section of MK300230 entry in GenBank which is now saying “*Anopheles splendidus*” which is obviously incorrect.

Preserving specimens is important to avoid many of those pitfalls. For mosquitoes, there are some useful methods like NaOH crude extraction (e.g. Lars Rudbeck & Jørgen Dissing, 1998, BioTechniques 25 (4)), which enable preparing DNA template for PCR from only a single leg with just a cheap cost.

### **Suggestion**

The author should revise the species assignment for each sequence more carefully and consider using ambiguous descriptions for less confident sequences (e.g. *Aedes* sp.).

### **Is the work clearly and accurately presented and does it cite the current literature?**

Partly

### **Is the study design appropriate and is the work technically sound?**

Partly

### **Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

### **If applicable, is the statistical analysis and its interpretation appropriate?**

Partly

### **Are all the source data underlying the results available to ensure full reproducibility?**

Yes

### **Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Medical entomology; Genetics



**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Reviewer Report 03 October 2019

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**Nicholas Johnson** 

Animal and Plant Health Agency, Addlestone, UK

The authors have addressed the significant comments of the review. The Conclusions section is improved although replace "where's" with 'whereas'.

No further comments.

**Is the work clearly and accurately presented and does it cite the current literature?**

Partly

**Is the study design appropriate and is the work technically sound?**

Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**

Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**

Partly

**Are all the source data underlying the results available to ensure full reproducibility?**

Partly

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Zoonotic viruses

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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Version 1

Reviewer Report 28 May 2019

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## Laban Njoroge

Invertebrate Zoology Section, The National Museums of Kenya, Nairobi, Kenya

### Abstract and Introduction:

There are a number of grammatical errors in the manuscript. The following are examples in the abstract: 'In this study, we identified mosquito species across Kisumu, Kilifi and Nairobi Counties' - I believe mosquitoes were collected from only few places in the three counties and therefore should read were collected from and not across.

There should be a word 'the' before consequent mortality in line 1 of the abstract.

Commas are missing in some areas where they should be e.g. in the abstract.

'.....Despite reports on increase of new and recurrent mosquito borne-disease outbreaks such as chikungunya, dengue fever and Rift valley fever in Kenya little is known about the genetic characteristics.' A comma after Kenya.

'PCR was used to amplify and sequence the partial cytochrome oxidase subunit 1 (CO1) gene.' This sentence creates an impression that PCR was used for both amplification and sequencing. I believe PCR was used to amplify the genes but sequencing was done using another method. This should be clarified. It is needless to repeat the word haplotypes three times.

In the introduction: '.....pathogens causing diseases such as; malaria, lymphatic filariases, avian malaria' - That semi colon after such as should not be there.

There is lack of standardization in writing names. e.g. disease names such as Chikungunya, Dengue are started with a capital letter in the main body but with small letters in the Keywords

Some scientific names of some species are wrongly written e.g. *Aedes cummnisii* should be *Aedes cumminsii*. Culicine mosquitoes not Culicine mosquitoes in some areas and others mosquitos.

### Methods:

There aren't sufficient details of the methods to allow replication by others. For instance, there is no mention of where the Pyrethrum Spray Catches were done. Was in inside houses, Bus waiting lounges, garages?

The reference for PSC collection method should clearly indicate that it is as used by Ndiath et al. 2011.

I have rated the study design study as partly appropriate as being a study that targeted diversity, one sampling method that targets only indoor resting mosquitoes was not the best. There is probably a need to justify why only PSC was used and point one method as the reason for the low diversity collected.

Again, it is not explained why bus stops in the three counties were preferred as sampling sites. If the idea was to see the contribution of transportation to the mixing of populations, then that didn't come out clearly.

'Only a few *Aedes aegypti* in Nairobi (Kinuthia *et al.*, 2017)'. The authors do not tell us it is few of what.

Few haplotypes or individuals?

diversity and spread of *Aedes aegypti* has been associated with expansion on arboviral infection.....' -

This statement needs to be rephrased. The word on can be replaced by of. Alternatively, it can be Diversity and spread of *Ae. aegypti* has been attributed to the increase in arboviral infections.

'and was predominated in the Kilifi samples.....' Should read it was predominant in Kilifi samples.

'This may contribute to the high susceptibility to dengue-outbreak reported in the region (Baba *et al*' - Should read this may contribute to their high susceptibility

'The similarities in the genetic composition between the *An. gambiae* in Kenya and Uganda is most likely due to the proximity of the two countries to one another and the exchanges is more likely over land as opposed to across lake Victoria as claimed in the discussion.

This study has indicated high diversity of *Anopheles* haplotypes in the Kisumu population' - I do not think two species only can be regarded as high diversity. Probably you should use the word higher in comparison with Nairobi and Kilifi.

'The low diversity of *Anopheles* species in Kilifi and Nairobi may be attributed to the Great Rift Valley,' - there is an abundance of *Anopheles* especially in Kilifi (see Mwangangi *et al* 2012 which you have in the references). The problem is the choice of sampling method employed. PSC targets indoor resting mosquitoes only while the highest diversity are found outdoors

#### Conclusion:

The conclusion is sounding a bit weak and it is more of a discussion than a conclusion. There isn't a strong conclusion about the findings on diversity and molecular characterization of mosquitoes encountered.

#### **References**

1. Lutomiah J, Bast J, Clark J, Richardson J, *et al.*: Abundance, diversity, and distribution of mosquito vectors in selected ecological regions of Kenya: public health implications. *J Vector Ecol.* 2013; **38** (1): 134-42 [PubMed Abstract](#) | [Publisher Full Text](#)

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

I cannot comment. A qualified statistician is required.

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** I am a mosquito taxonomist and ecologist. I am not an expert in sequencing and phylogenetic analyses and therefore that bit may require another expert.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 11 Sep 2019

**Moni Makanda**, Pan African University, Nairobi, Kenya

First and foremost I would like to thank you for taking your time to review this article. Your views were most welcome and addressed accordingly.

1. Grammatical errors were corrected throughout the article as highlighted.
2. Effect of transportation on mosquito diversity was not a focus in this study. This study looked in to the diversity of mosquitoes in the study sites being town areas.
3. The conclusion was strengthened as recommended.

**Competing Interests:** No competing interests were disclosed.

Reviewer Report 13 May 2019

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**Nicholas Johnson**

Animal and Plant Health Agency, Addlestone, UK

The article is a focused investigation of the haplotype variation observed in mosquito populations trapped at three locations in Kenya. The authors noted variation in all the 11 species reported including a number of novel observations. However, the authors do not include basic data on the actual distribution and species assemblage at the collection sites. By arbitrarily selecting 25 samples for extensive genetic analysis and ignoring the remaining samples that apparently included 894, 824 and 720 mosquitoes appears to be a fundamental omission. It is difficult to see how the authors can conclude that “The distribution varies in density” when the dataset has not been analysed. Whilst it may be beyond the resources of the team to molecularly type all 2,438 samples, without some attempt to include morphological identification of a significant proportion of these samples the manuscript is considerable diminished.

The authors should check the capitalisation of pathogens throughout. As a general rule, names derived from a place are capitalised e.g. Rift Valley fever virus, whilst those that are not are in lower case e.g. yellow fever virus, malaria.

The authors must revise the conclusions section to reflect the findings of the paper stating precisely what they have derived from their observations. At the moment the two sentences' provide a revision of the manuscripts aim and a vague statement that is unsupported by the results.

The reference for Morrill et al is incorrect. It should be Morrill et al., 1991, J Trop Med Hyg, 94, 166.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Not applicable

**Are all the source data underlying the results available to ensure full reproducibility?**

Partly

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Zoonotic viruses

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 11 Sep 2019

**Moni Makanda**, Pan African University, Nairobi, Kenya

First and foremost thank you for taking time to review the paper, your views were most welcomed and addressed.

1. It was beyond our financial capability to genetically analyse 2,438 samples. Earlier study by (Hale, 2012) support my study as adequate in phylogenetic analysis. However, morphological identification was done and further analysis by use of PCR-HRM. This data has been capture in my MSc. thesis, moreover a second publication on the same is underway. As this paper was focused on genetic diversity we focused on molecular analysis.
2. Names of pathogens throughout the article have been corrected based on the general rule.
3. The conclusion was strengthened as proposed.

4. Reference Morrill et al. 1991 was revised as advised

**Competing Interests:** No competing interests were disclosed.

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