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## RESEARCH ARTICLE

# Phylogeny of *Aedes* Mosquitoes, Dengue and Chikungunya Viruses Along the Coastline of Kenya

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### ABSTRACT

**Background and Objective:** There are arthropod-borne disease outbreaks as a result of pathogen influx including arboviruses which are transmitted by strains of *Aedes* species that occur periodically in varying spots on the globe. This study aimed to determine the phylogenetic relationship of *Aedes* mosquitoes, Dengue and Chikungunya viruses along the Coastline of Kenya. **Materials and Methods:** This was necessitated by the paucity of documented information on phylogeny of the viruses and their vectors. A sampling of adult *Aedes* mosquitoes was done by the Bio-gent Sentinel trap and aspiration. Mosquitoes were identified and sorted based on their morphological features and molecular techniques. RNA was extracted using Trizole®. Identification of *Aedes* species, Dengue and Chikungunya was done by a real-time polymerase chain reaction. Phylogeny trees were plotted by using the interactive tree of life. **Results:** *Aedes aegypti* was identified for the first time in this study along the Coastline of Kenya. Four serotypes of Dengue were identified, with Dengue-4 identified for the first time in this region. Only the East/Central/South African genotype of the Chikungunya virus was identified. *Aedes* mosquitoes, Dengue and Chikungunya had a close evolutionary relationship among themselves and to those from Africa, Asia and America. **Conclusions:** *Aedes aegypti* and Dengue virus serotype 4 were present along the Kenyan Coastline. There was a close evolutionary relationship among the identified haplotypes and to those from Africa, Asia and the American continent. Identification of new haplotypes indicates possible continuous evolution of the *Aedes* mosquito vectors and the viruses.

**Key words:** *Aedes*, dengue, chikungunya, serotype, genotype, evolutionary, phylogeny

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## INTRODUCTION

Mosquitoes are responsible for the transmission of many pathogens, including viruses, bacteria and parasites between people, domestic and wild animals all over the world<sup>1</sup>. Phylogeny refers to the analysis and description of the evolutionary history of a group of organisms<sup>2</sup>. In biological studies, the primary goal of developing an evolutionary tree is to describe the evolutionary relationships between or among organisms; based on relative recency of common ancestry<sup>3</sup>. Phylogenetic analysis using analytical tools like parsimony phylogenetics could provide information on the common genetic changes (mutations) and modifications in biochemical pathways<sup>4</sup>. Such information could be crucial in connecting omics and the study of arboviral diseases. This could be by providing seamless, predictive, dynamic and multidimensional analysis. Such analysis could be utilized for timely diagnosis, detection and prognosis; identification of biomarkers and determination of effective treatment.

An increase in gene flow among *Aedes aegypti* populations between Africa and Saudi Arabia was previously reported; with phylogenetic relationships indicating that the two genetically distinct *Aedes aegypti* present in Saudi Arabia acquired their origin from the dual African ancestors<sup>5,6</sup>. In Africa, the phylogeny of West and East African isolates of *Aedes aegypti* s.l indicated the existence of two clades of origin<sup>5</sup>. The basal and derived/ upper clade: the basal clade had isolates mainly from West Africa while the upper clade had isolates mainly from East Africa. These findings demonstrated *Aedes aegypti* mosquitoes all over the world were composed of mosquitoes originating from one of the two matrilineages (basal and derived). Similarly, that there existed an evolutionary relationship among *Aedes aegypti* s.l populations from West and East Africa.

Earlier phylogenetic analysis of the Dengue virus demonstrated that DENV-1 isolates from Mombasa were related to those from Djibouti, China and Indonesia. DENV-2 isolates from Mombasa originated from those of West Africa: Senegal and Burkina Faso and were related to those from Asia. DENV-3 isolates from Mandera, Mombasa and Wajir originated from those of Brazil, Paraguay and Peru and were related to isolates from Pakistan, China and India<sup>7</sup>.

Phylogeny of the Chikungunya virus demonstrated that the Asian genotype emerged from the ECSA sometime between 1879 and 1927<sup>8</sup>. More so, there were indications that the Asian genotype was related to strains from Asia and the Caribbean<sup>9</sup>. In Kenya, evolutionary studies on the ECSA genotype indicated that Kenyan isolates had a close evolutionary relationship among themselves and to those from China<sup>10</sup>. Therefore, this study was conducted to determine the phylogenetic relationship of *Aedes* mosquitoes, Dengue and Chikungunya viruses along the Coastline of Kenya. This was necessitated by the paucity of documented information on phylogeny of the viruses and their vectors.

## MATERIALS AND METHODS

**Study area:** The study was carried out in selected sites along the coastline of Kenya from March 2018-December 2019 (Fig. 1). An indoor and outdoor sampling of adult *Aedes* mosquitoes were by Bio-gent sentinel (BG) traps (Biogents, Regensburg, Germany) and aspiration technique. Briefly, the BG traps were baited with solid carbon dioxide (dry ice) and suspended at least 0.5 m from the ground during outdoor sampling. Prokopack aspirators (John W. Hock, Gainesville, Florida,

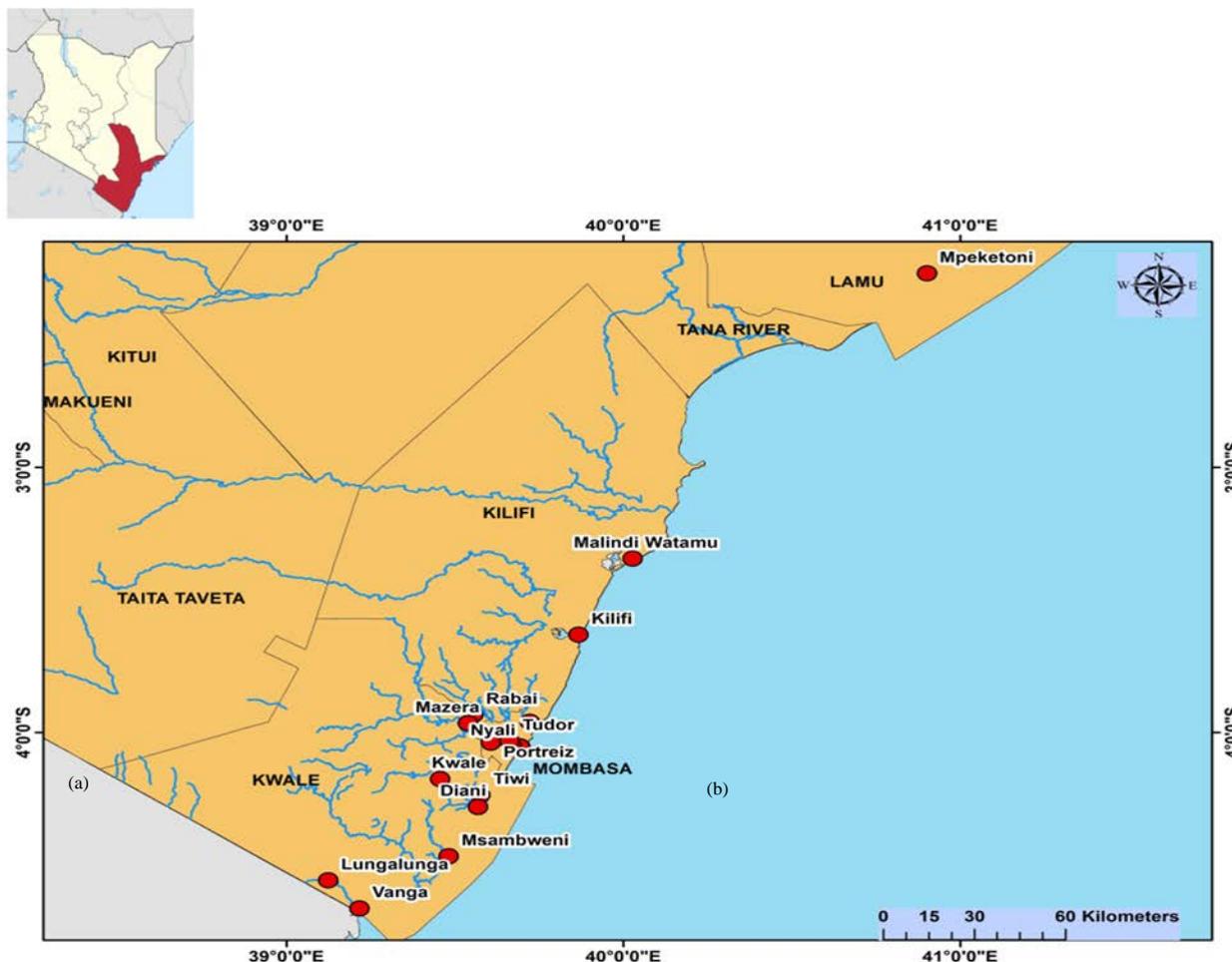


Fig. 1: Study sentinel sites, (a) A map of Kenya indicating the coastal region and (b) Sentinel study sites on the Coastline of Kenya

USA) were used for aspiration. Both BG trap and aspiration were performed twice a day; at dawn between 0500-1000 h and in the afternoon between 1500-1800 h. All captured mosquitoes were transported live in net cages to the field sorting insectary.

**Morphological identification of *Aedes* mosquitoes:** In the sorting insectary, the live *Aedes* mosquitoes were placed on a white filter paper in a Petri dish full of dry ice for 5 min. The stunning mosquitoes were identified morphologically to their species level, physiological status and sex before sorting. Morphological identification of mosquito species was by stereoscopic microscope (WITec, Germany)<sup>11</sup> and according to manual “Mosquitoes of the Ethiopian Region”<sup>12</sup>.

**RNA extraction and cDNA synthesis:** Pools of *Aedes* mosquitoes (20 mosquitoes per pool) were homogenized by a mortar and pestle and RNA extracted using 1 mL of Trizol® as earlier described<sup>5</sup>. Only the unfed and gravid mosquitoes were utilized in this analysis. The female blood-fed were excluded to avoid contamination of the virus which could be in the blood meal. cDNA synthesis was done on 10 µL of the

Table 1: The primers for identification of Aedes mosquitoes

Target	Primer name	Nucleotide sequences (5' to 3')	Polarity	Product (bp)
Mosquito RNA marker	Act-2F	ATGGTCGGYATGGGNCAGAAGGACTC	Forward	683
	Act-8R	GATTCCATACCCAGGAAGGADGG	Reverse	
<i>Ae. aegypti</i> s.l	18SFHIN	GTAAGCTTCCTTTGTACACACCGCCCGT	Forward	550
	Aeg.r1	TAACGGACACCGTTCTAGGCCCT	Reverse	
<i>Ae. tricholabis</i>	UV	TGTGAACTGCAGGACACAT	Forward	
<i>Ae. pembraensis</i>	PEM	GCATCGATGGGTTAATCATG	Reverse	405
<i>Ae. ochraceous</i>	OCH	CAAGCCGTTCCAGCCCTGATT	Reverse	501
<i>Ae. albicosta</i>	ALB		Reverse	
<i>Ae. fulgens</i>	FUL		Reverse	
<i>Ae. mcintoshi</i>	MCN		Reverse	
<i>Ae. fryeri</i>	FRR		Reverse	
ND4	ND4sb+	TGATTGCCTAAGGCTCATGT	Forward	344
	ND4sb-	TTCGGCTCCTAGTCGTTTCAT	Reverse	

Table 2: Primers and probes for the identification of flaviviruses and alphaviruses

Primers and Probes	Sequence	Tm °
<b>Flavivirus</b>		
Flavi allS (Forward Primer)	5'-TACAACATgATggggAARAgAgARAA-3'	53.8
Flavi all AS2 (Reverse Primer)	5'-gTgTCCCAgCCNgCKgTgTCATCWgC-3'	
Flavi all AS4 (Reserve Primer)	5'-gTgTCCAGCCNgCKgTRTCRTC-3'	80.4
3P <sub>i</sub> (Probe)	FAM-Tg+gTWYATgT+ggYTNg+gRgC-NFQ-MGB	50.3
3P <sub>ii</sub> (Probe)	FAM-CCgTgCCATATggTATATgTggCTgggAgC-NFQ-MGB	
3P <sub>iii</sub> (Probe)	FAM-TTTCTggAATTTgAAgCCCTgggTTT-NFQ-MGB	
<b>Pan-alphavirus</b>		
F2A (Forward Primer)	5'- ATGATGAARTCIGGIATGTTYT-3'	
R2A (Reverse Primer)	5'-ATYTTIACTTCCATGTTTCATCCA-3'	
R3A (Reverse Primer)	5'-ATYTTIACTTCCATRTTCARCCA-3'	
R4A (Reverse Primer)	5'-ATYTTIACTTCCATGTTGACCCA-3'	
ATTO425 (Probe)	- AT+GTT+GTC+GT+CIC+CIAT-BHQ1/LNA	

extracted RNA to generate cDNA by using EcoDry Premix (Random hexamers) (Clontech Laboratories, Inc., Mountain View, CA, USA) in a procedure described earlier<sup>6,13</sup>.

**Molecular Identification and sequencing of Aedes mosquitoes and Viruses:** The mtNAD4 gene was utilized for the identification of Aedes mosquitoes using primers listed (Table 1) as earlier described<sup>5,6</sup>. Identification of serotypes of Dengue virus in the Aedes mosquitoes was based on the amplification of the target viral genes (E/NS1/NS5) in RNA using multiplex PCR with a panel of general flavivirus family primers (Table 2) as earlier described<sup>14</sup>. Samples tested positive for flavivirus were further tested with consensus primers for the Dengue virus. These primers were D1 and D2 and they target the E/NS1 junction of the virus genome. Only samples tested positive with Dengue consensus primers were further tested for the four Dengue serotypes using appropriate primers as described (Table 3)<sup>7,13</sup>. Identification of genotypes of the Chikungunya virus was based on the amplification of the target viral gene E1 in the RNA using multiplex PCR with the AgPath-ID One-step RT-PCR kit (Applied Biosystems, Carlsbad, California, USA) using a similar method<sup>8,10</sup> using a panel of general alphaviruses primers (Table 2). Samples tested positive for the alphavirus were further tested with conventional primers for the Chikungunya virus (Table 4). The amplified gene products were

Table 3: The primers for identification of serotypes of Dengue virus

Primer	Gene/protein target	Primer sequence (5' to 3')	Position
FU1	NS5	TACAACATGATGGGAAAGAGAGAGAA	9007-9032
CFD3	NS5	GTGTCCCAGCCGGCGGTGTCATCAGC	9308-9283
D1	E/NS1	TCAATATGCTGAAACGCGGAGAAACCG	38-65
D2	E/NS1	TTGCACCAACAGTCAATGTCTTCAGGTTC	455-483
TS1		CGTCTCAGTGATCCGGGGG (D1and TS1)	
TS2		CGCCACAAGGGCCATGAACAG (D1 and TS2)	
TS3		TAACATCATCATGAGACAGAGC(D1and TS3)	
TS4		CTCTGTTGTCTTAAACAAGAGA(D1 and TS4)	
D5-F	NS5	TCAATATGCTGAAACGCGHGAG	132-153
D5-R	NS5	GCGCCTTCNGNNGACATCCA	764-783

Table 4: The primers for identification of Chikungunya virus

Primer	Gene/protein target	Primer sequence (5' to 3')	Position
VIR 2052 F	NSP4	TGGCGCTATGATGAAATCTGGAATGTT	6971-6997
VIR 2052R	NSP4	TACGATGTTGTCGTCGCCGATGAA	7086-7109
CHIKV-F	E1	CGTGGTGTACAAAGGTGACG	10524
CHIKV-R	E1	ACG CCG GGTAGTTGACTATG	11170

cleaned from the gel by the MinElute PCR purification kit (Qiagen, Valencia, CA) and sequenced using Sanger high-throughput technique<sup>15</sup>.

**Data analysis:** Data were entered, cleaned and saved as comma-separated values (csv) extension in STATA 13 and transported to R. Proportions of *Aedes* species, sex orientation and their viral infection rates were determined. Generated nucleotide sequences were used for phylogenetic analysis. DNAbaser v.3.0 (<http://www.dnabaser.com/articles/SNP>) was used for editing bad calls in the raw chromatogram file generated from sequencing the forward and reverse strands. The deletion of the generated sequences of primers was done from the 5' and 3' ends. The sequences were subjected to the Basic Local Alignment Tool (BLAST) and GenBank database to compare them with available sequences and confirm the identity of the isolates. Formatting of the retrieved sequences compatible with alignment programs and identification of the correct reading frame for each sequence was done using the translation program at <http://us.expasy.org/tools/dna.html>. The sequences were aligned for identification and removal of duplicate sequences using Clustal Omega v1.2.1, scored in T-coffee (<http://tcoffee.crg.cat/>) and viewed in jalview <http://www.jalview.org/><sup>16</sup>. The sequences were manually adjusted in Se-AL software according to DNA sequence alignments for the preservation of codon homology<sup>17</sup>. Columns with more than one percent of gaps were removed from the alignment using trimAl v1.4.rev6<sup>18</sup>. Using p-model test v1.4, Maximum likelihood trees were inferred using Randomised Accelerated Maximum Likelihood (RAxML) version 8.1.20 ran with model GTR-GAMMA-I for selecting the best- t model for the maximum likelihood analyses and plotting of phylogenetic trees using an interactive tree of life<sup>19</sup>. Branch supports were computed out of 1000 bootstrap replicates based on the Tamura Nei model<sup>20</sup>.

## RESULTS

**Phylogeny of *Aedes* mosquitoes along the coastline of Kenya:** *Aedes aegypti* was identified for the first time in this study along the Coastline of Kenya. A total of 21

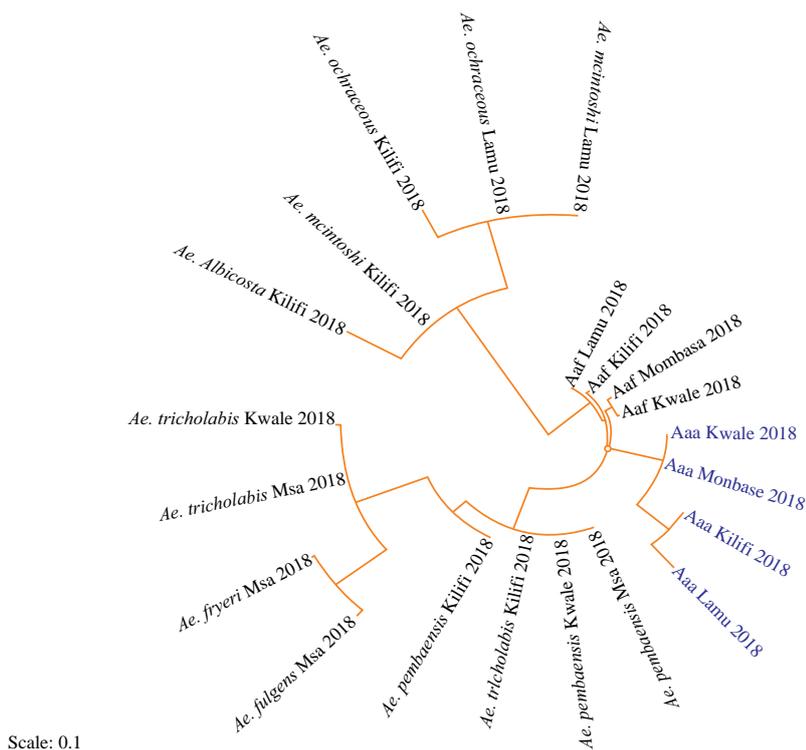


Fig. 2: Phylogeny of *Aedes* mosquitoes based on mtDNA NAD4 gene along the Coastline of Kenya

Sequences aligned using clustal omega and the phylogenetic tree constructed using RAxML version 8.1.20. Newly identified strains are highlighted in blue and abbreviated with the site of collection and the year of isolation

strains of *Aedes* species was identified. Four strains were identified for the first time in this study (highlighted in blue). Strains of *Aedes aegypti* s.l from Kilifi were closely related to those from Lamu. Similarly, strains from Kwale were closely related to those from Mombasa (Fig. 2). Phylogeny of the identified strains of *Aedes* mosquitoes along the Coastline of Kenya was compared with those previously identified in Kenya and other parts of the world (Fig. 3). Strains from West Africa (West Africa\_KF939213) formed an out-group to the identified strains. All the new strains were in the in-group. All *Aedes aegypti* were in the same cluster and were closely related to strains from Mexico\_JX297259, Uganda\_DQ176838 and Brazil\_AY906853. *Aedes aegypti formosus* strains from Lamu and Kilifi were closely related to EU446271.

**Phylogeny of Dengue and chikungunya viruses along the coastline of Kenya:**

Four serotypes of Dengue virus were identified, that is, DENV-1, DENV-2, DENV-3 and DENV-4 serotypes as shown in Fig. 4. Sixteen new strains were identified with four identified for the first time (highlighted in blue). The phylogeny of these new strains was estimated as shown in Fig. 4: Strains from Kwale were closely related to those from Mombasa. Similarly, strains from Kilifi were closely related to those from Lamu. The phylogenetic relationship of the identified strains and those identified earlier in Kenya and all over the world was estimated (Fig. 5). DENV-1 strains were closely related to those previously isolated in Mombasa (KX812500), India (JN903581), France (DQ672556) and China (EF025110). Strains of DENV-2 were closely related to those previously reported in Mombasa (KX812508),

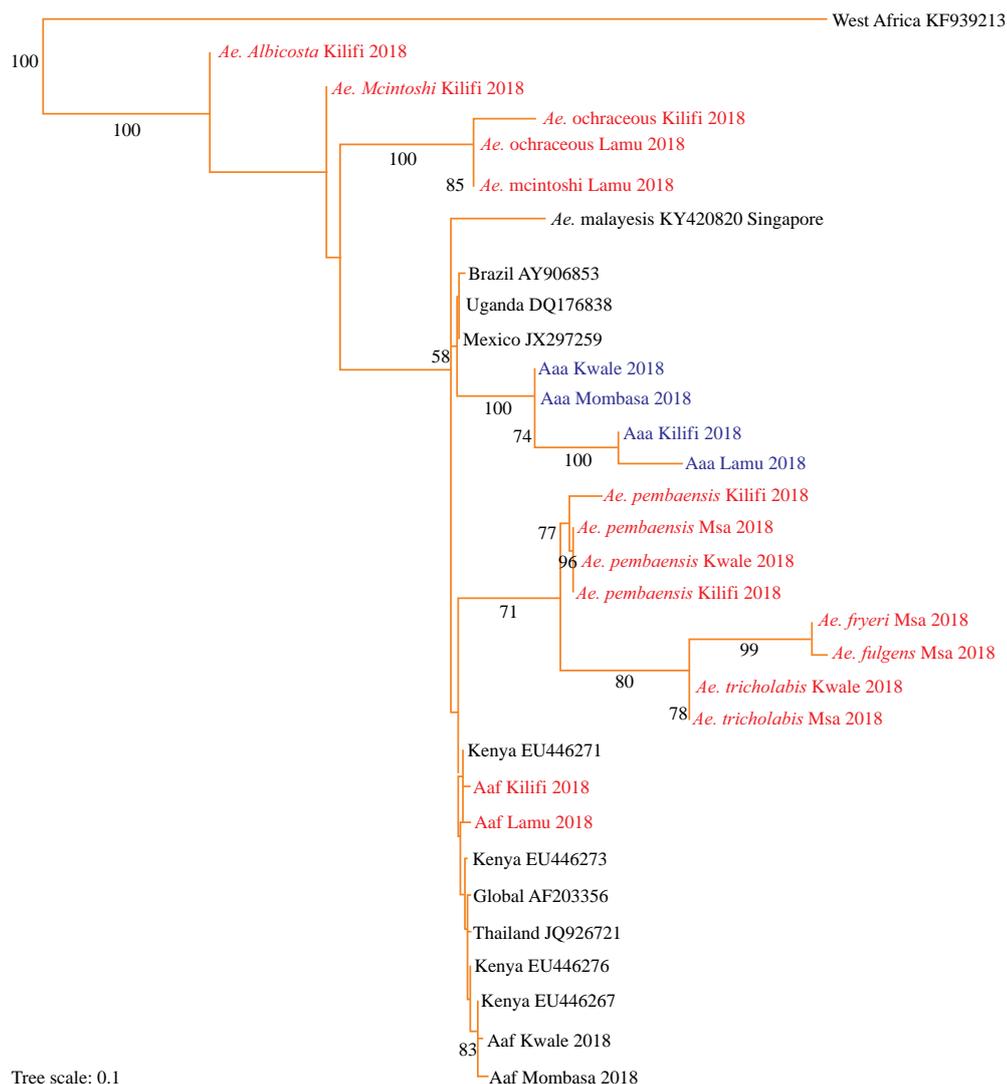


Fig. 3: Maximum likelihood tree of the mtDNA NAD4 of *Aedes* mosquitoes sampled along the Coastline of Kenya and other parts of the world

Sequences aligned using clustal omega and the phylogenetic tree constructed using RAxML version 8.1.20. Bootstrap values for 1000 replicates are indicated at the major branch points. Newly identified strains are highlighted in red while strains of *Aedes* species identified for the first time are highlighted in blue

Pakistan (KF041236) and China (KT187558). New strains of DENV-3 were closely related to those previously isolated in Mombasa (KX812504; KX812511), Brazil (AY679147), India (AY770511) and China (KF954949). The strains of DENV-4 were closely related to those from Haiti (KP140942; JF262782).

**In this study, only the ECSA genotype of the Chikungunya virus was identified (Fig. 6):** Strains from Kwale and Mombasa formed a sister cluster. Similarly, strains from Kilifi formed a cluster with those from Lamu. Also, the phylogeny between newly identified strains and previous strains was estimated (Fig. 7). Identified strains were closely related to previous strains Mombasa\_Chikv\_HQ458254 and Lamu\_Chikv\_HQ456255. The ECSA strains were closely related to the Asian strains.

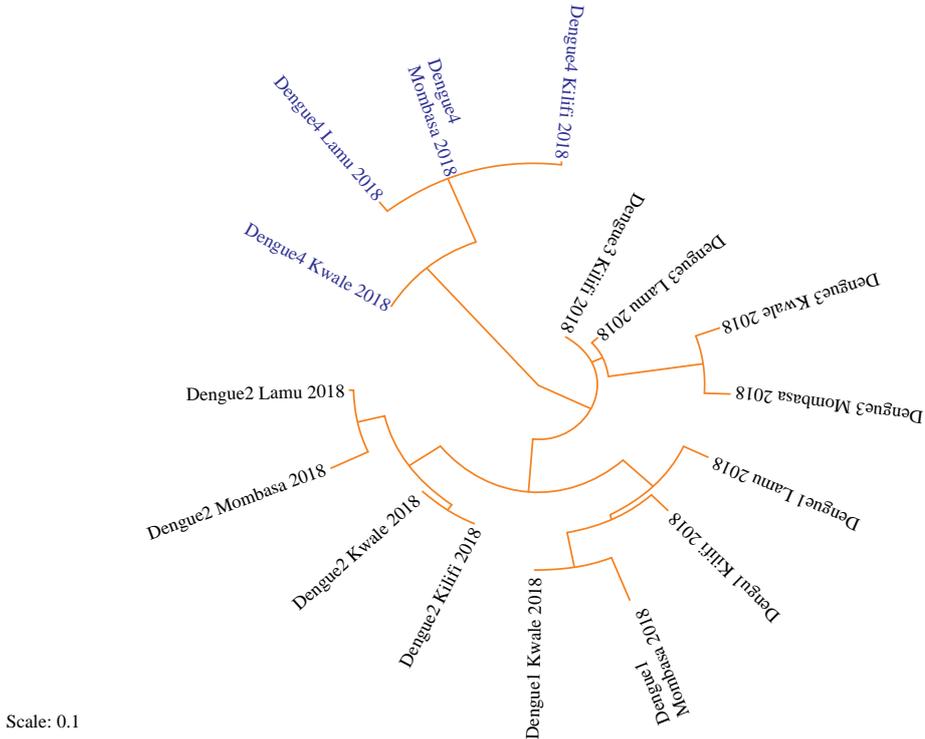


Fig. 4: Phylogeny of strains of Dengue serotypes based on the NSP5 coding regions along the Coastline of Kenya  
Sequences aligned using clustal omega and the phylogenetic tree constructed using RAxML version 8.1.20. Strains identified for the first time are highlighted in blue

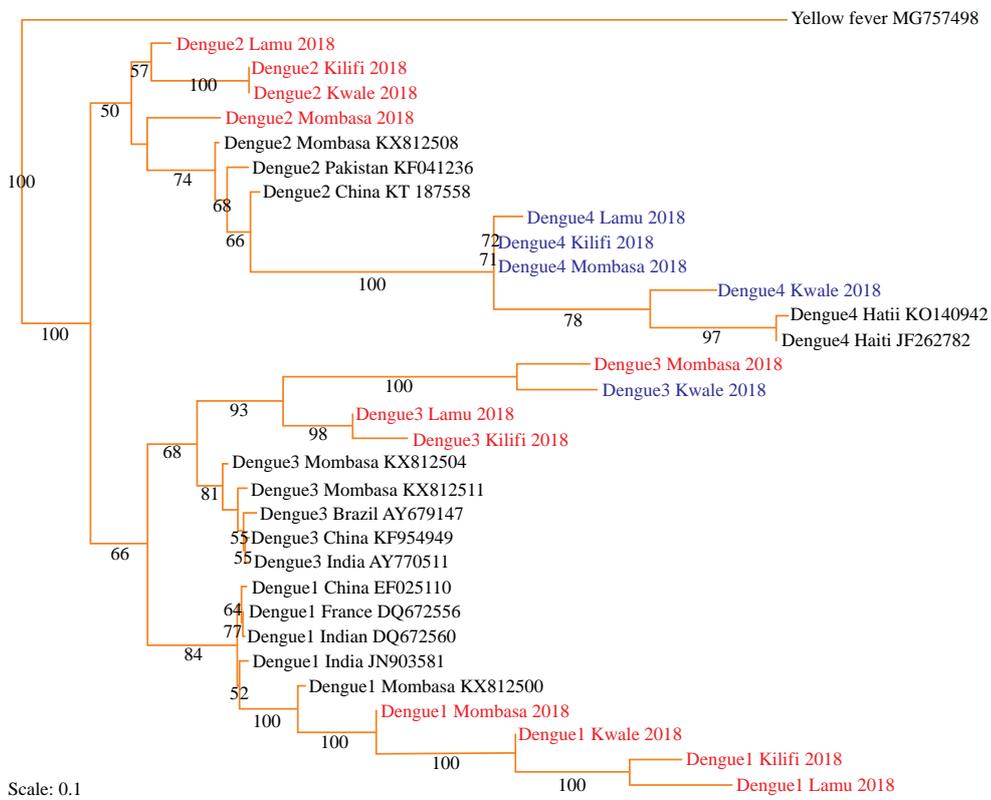


Fig. 5: Maximum likelihood tree of strains of Dengue serotypes based on the NSP5 coding regions along the Coastline of Kenya

Sequences aligned using clustal omega and the phylogenetic tree constructed using RAxML version 8.1.20. Bootstrap values for 1000 replicates are indicated at the major branch points. New strains are highlighted in red. Strains identified for the first time are highlighted in blue

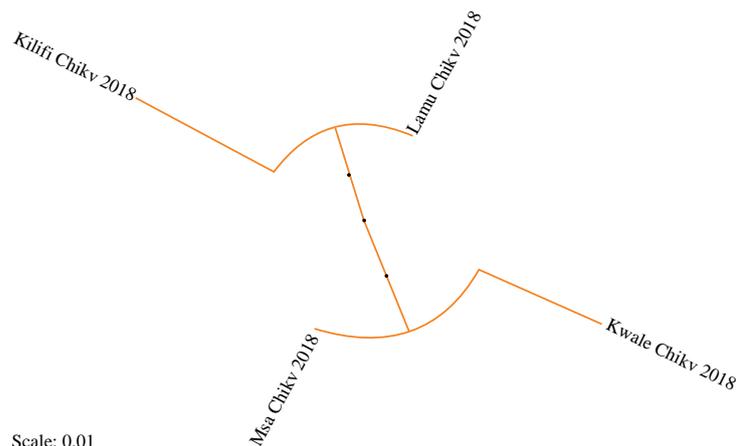


Fig. 6: Phylogeny of Chikungunya strains based on nucleotide sequence of the partial E1 gene along the Coastline of Kenya. Sequences aligned using clustal omega and the phylogenetic tree constructed using RAXML version 8.1.20.

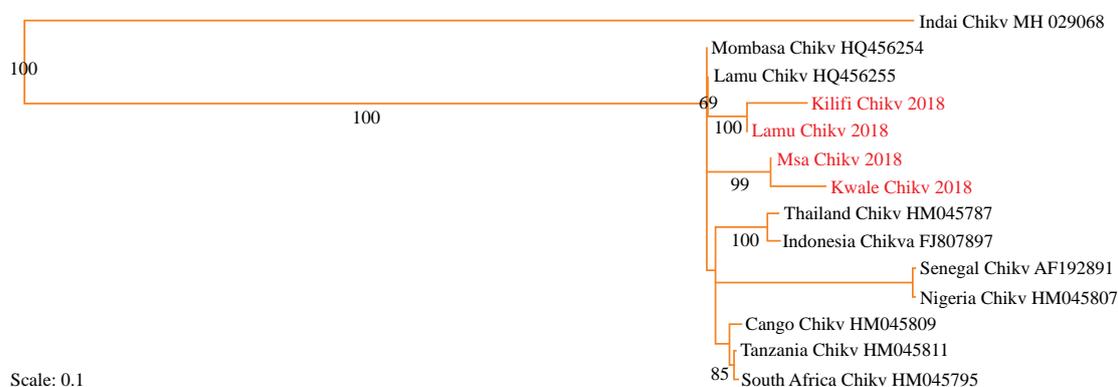


Fig. 7: Maximum likelihood phylogenetic tree among Chikungunya virus strains based on nucleotide sequence of the partial E1 gene along the Coastline of Kenya. Sequences aligned using clustal omega and the phylogenetic tree constructed using RAXML version 8.1.20. Bootstrap values for 100 replicates are indicated at the major branch points. New strains are highlighted in red

### DISCUSSION

The observation that strains of *Aedes* mosquitoes from West Africa formed an out-group implied they were related to members of in-group as they shared an orthologous gene group and could have given rise to the East African strains. Isolation of new strains in this study could be due to sampling of the entire Coastline for consecutive two years. More so, the evolution of these *Aedes* species may have occurred with time and space due to environmental selection pressure: challenges by insecticides, decrease in breeding sites and climate change especially increase in temperature.

The finding that both new and previously identified strains of *Aedes* mosquitoes from East Africa were in the in-group of the phylogenetic tree indicated a close evolutionary relationship among them and this concur with the previous reports<sup>5</sup>. The observation that all strains from Kenya and Asian continent were in the in-group implied they originated from *Aedes* mosquitoes from West Africa and this may be attributed to the fact that there exist a lot of movement of goods and people between East Africa, West Africa and the Asian continent thus a possibility of trafficking these mosquitoes between these regions.

This study reported for the first time the presence of serotype DENV-4 in *Aedes* mosquitoes along the Coastline of Kenya. This may be due to the expansive geographical area of sampling and the fact that entomological assays were done contrary to earlier serological studies<sup>7,21</sup>. Identification of sixteen more new strains of Dengue could have been due to the expansion of the study area and duration of the study. The finding that all strains of Dengue virus from Kwale and Mombasa; and Kilifi and Lamu were forming distinct sister clades indicated a close phylogenetic relationship between them. This could be attributed to the proximity between Kwale and Mombasa and between Kilifi and Lamu with improved traffic between these respective Counties with travelers carrying strains of these viruses in their bodies or *Aedes* mosquitoes in automobiles. These viruses may end up in the same host cells resulting in the related progeny of recombinant strains thus their close relationship. All identified and previous strains of Dengue were in the in-group and related to those from the Asian continent. This may be because strains of the Dengue virus had a common ancestry therefore a higher possibility of similarity in their evolutionary path<sup>22</sup>. The presence of DENV-4 in Kenya could be due to the increased number of people traveling between China, India, Haiti and Kenya.

The identification of only one genotype of the Chikungunya virus was in line with reports from recent studies<sup>10,23</sup>. This, therefore, means the other genotypes of Chikungunya virus: West Africa and Asian genotypes have not yet been introduced in the region. The strains of Chikungunya shared an orthologous gene group meaning they originated from a common ancestor. Strains from Lamu were related to those from Kilifi; and those from Mombasa with those from Kwale; this may be due to proximity of these Counties and frequency of people traveling across borders of these regions.

Strains of Chikungunya were closely related to those from Asia (Thailand and Indonesia), suggesting a close phylogeny among them. In addition, the Asian genotypes originated from the East African genotype and this conquers with the previous reports<sup>8</sup>. This could be attributed to the historical relationships between African and Asian continents since the 17<sup>th</sup> and 18<sup>th</sup> centuries. The observation that Indian genotype formed an out-group could mean it gave rise to the West African, East, Central and Southern Africa genotypes.

## CONCLUSION

*Aedes aegypti* was presented alongside the earlier identified *Aedes species*. Identification of new haplotypes indicates possible evolution of the *Aedes* mosquito vectors, Dengue and Chikungunya viruses along the Coastline of Kenya. There was a close phylogenetic relationship among the newly identified *Aedes* subspecies and haplotypes; and were related to those from West Africa, Asia and South America. This could be aided by enhanced traffic of goods and human beings among these parts of the continents carrying alongside with them *Aedes* mosquitoes infected with the viruses.

## SIGNIFICANCE STATEMENT

This study discovers the existence of evolutionary relationships among *Aedes* mosquitoes, Dengue and Chikungunya virus from coastal Kenya and those from other parts of the world. The study also discovers the presence of *Aedes aegypti* and Dengue virus serotype-4 which had not been identified in the region. This knowledge will help researchers with information on the transmission patterns of the vectors and viruses and forecast future epidemics of Dengue and Chikungunya infections.

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