# Genetic Diversity and Molecular Characterization of Mosquitoes (Diptera: Culicidae) In North-Central Nigeria Using Ribosomal DNA ITS2 and Mitochondrial 16S-DNA sequences

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## ABSTRACT

Mosquitoes are vectors of various life-threatening diseases like malaria, yellow fever, dengue fever etc. Their close proximity to human habitations allows ease for disease transmission. They have been identified by key morphological tools like their wings, legs, bristles etc. but closely related species are difficult to identify based on morphology. Molecular tools have, therefore, been employed to help with the more accurate identification. This study was aimed at identifying and characterizing different mosquito species in five different states in North-Central Nigeria using internal transcribed spacer 2 (ITS2) and mitochondrial 16S rDNA regions. Mosquito larvae were collected from stagnant water in breeding places at each collection site in North-central Nigeria. Morphological identification was carried out using standard keys. DNA extraction was performed using EZNA extraction kit. PCR amplification of ribosomal *ITS2* and mitochondrial 16S-rDNA gene regions were carried out. The PCR amplicons were sequenced using primers initially used for the PCR. Sequence data were aligned in MEGA 6.0 using ClustalW multiple alignment feature and then compared with GenBank databases for similarity. Phylogenetic analysis of DNA sequences from the ITS2 region was able to distinguish two mosquito subfamilies; Anophelinae and Culicinae as well as differentiate between and amongst *Culex* and *Aedes* species. However, it was unable to effectively distinguish between the two different species of *Anopheles* sequenced. Mitochondrial 16S rRNA marker was also able to distinguish the two mosquito subfamilies. It efficiently identified and differentiated *Culex*, *Aedes* and *Anopheles* mosquito species sequenced in this study. This study concludes that heterogeneity among Nigerian populations of Anopheles mosquitoes of may likely impact malaria vector control programs. We recommend the combination of nuclear and mitochondrial markers for effective and reliable phylogenetic study and determination of evolutionary relationship among mosquito species.

**Keywords:** ***Aedes, Anopheles, Culex, Internal Transcribed Spacer gene, Genetic diversity, Mitochondrial, Ribosomal DNA***

## Introduction

Mosquitoes are insects that are classified under the order Diptera and family Culicidae. They have segmented bodies, a pair of wings, and three pairs of long hairy legs, feathery antenna and long mouthparts (**1**). There are three sub-families under the family Culicidae these includes Toxorhynchitinae, Anophelinae and Culicinae. Toxorhynchitinae has only one genus, *Toxorhynchites* and is not of any medical importance because it feeds on nectar rather than blood (**1**). Malaria is a mosquito-borne tropical disease and has no doubt remain an important public health problem in some tropical and subtropical countries including Nigeria. Malaria is caused mainly by *Plasmodium falciparum* and mosquitoes in the sub-family Anophelinae

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effectively transmit plasmodium parasites. The genera under this subfamily include; *Anopheles*, *Bironella* and *Chagasia.* Species of the genus *Anopheles* can effectively transmit the six species of the genus *Plasmodium* parasites. *Anopheles gambiae* is the most popular because of its role in the transmission of *Plasmodium falciparum* **(2)**. *Anopheles* can also transmit filarial parasite *Wuchereria bancroffti* and *Brugia malayi* as well as other arboviruses in human (**3**). The subfamily Culicinae has about 30 genera which are also of medical importance. Culicine mosquitoes like *Aedes* spp. and *Culex* spp. are important carriers of human pathogens e.g. viruses and filarial worms. They are also known to transmit avian malarias (**4**). Species identification based on morphological characters and DNA sequences are the two major approaches that have been employed by scientists all over the world to confirm the identity of biological specimen. Identification, abundance and diversity studies of mosquitoes have been documented (**5, 6)**. Wing morphometry has also been used for differentiation of *Aedes aegypti* in Nigeria (**7**). Study of the population genetic structure of *Anopheles nili* has been carried out using microsatellite analysis (**8**). Over the years, there have been remarkable progress in the use of molecular techniques for the identification of species (**9-16**). Some of the gene regions that have been previously used for genotypic characterization of mosquitoes include mitochondrial cytochrome oxidase subunit I & II (COI and COII) genes (**17-20**) and Shouche and Patole (**16**) evaluated genetic relatedness of 450 bp hypervariable region of the mitochondrial 16S rRNA gene in three major genera of mosquitoes, *Aedes, Anopheles* and *Culex.* PCR-RFLP technique has been used for identification of members of the Anopheles species **(21**, **22**). Similarly, DNA sequences of different gene regions of the nucleus and mitochondria have been amplified to deduce the evolutionary relationship among species because they show high rates of nucleotide substitution (**23**). 16S rDNA, NADH dehydrogenase, ITS1 and ITS2 genes **(24, 25**). A combination of COI and ITS2 gene regions have been used to estimate genetic diversity, abundance, and distribution of mosquito populations collected from island and mainland sites of Kenya's Lakes Victoria and Baringo (**26**).

Sharma *et al.* (2009) (**27**) have previously used RAPD as molecular marker to investigate genetic variability in *Culex quinquefasciatus* populations. The study revealed that RAPD is ideal for genetic analysis of Culex mosquito populations. Similarly, Ashraf et al., (**28**) reported the use of RAPD marker for genetic analysis of *Aedes aegypti* mosquito populations collected from Dengue outbreaks in Pakistan and the study concluded that *Ae. aegypti* populations are genetically more diverse as previously reported in Pakistan. Sequence amplification by PCR and deduction of evolutionary relationship from the data have also been used to differentiate and characterize mosquito species (**29**). Taken into consideration the overwhelming evidences from the literature that mosquitoes are responsible for the transmission of medically important pathogens and parasites which cause malaria, dengue, yellow fever, encephalitis or filariasis (**2, 30-32)**, details of its biology, ecology and molecular diversity are required for sustainable, effective and integrated vector control management strategies. Therefore, there is an urgent need to deeply study and understand the population genetic structure and gene flow patterns of mosquitoes particularly the vectors of malaria and other diseases spread by mosquitoes. Also, precise differentiation of mosquito species using molecular methods is no doubt fundamental to proper and successful malaria vector integrated control strategies in Nigeria. Unfortunately, there is limited information available in the literature about the extent of genetic diversity and relatedness existing between and/or within mosquito populations especially in North-central Nigeria. Such information will no doubt be useful and assist in the development of locally-adapted malaria vector control measures and ensure success in the war against the disease in Nigeria. This study was aimed at investigating genetic diversity and phylogenetic relationships that exist between and among mosquito populations in North-central Nigeria using *ITS2* and *16S rDNA* gene in order clarify phylogenetic positions of Anopheles, Aedes and Culex mosquito. It is expected that this will provide a baseline data and evidence of their potential as molecular markers, increase our understanding of mosquito phylogeny and give a more robust support for mosquito phylogenetic hypothesis and systematics in Nigeria.

## Materials and Methods

### Sample collection and experimental set up

Larval samples were collected from April, 2018 - March, 2019. They were collected from earthen ponds, abandoned tyres, gutters, abandoned wells, containers, stagnant water etc. in different states in North Central Nigeria. The states included Kwara, Niger, Kogi, Benue and Abuja (Federal Capital Territory) (Fig. 1). Larval samples were transported to the laboratory and sorted in bowls according to genera. The bowls were covered with nets and labeled using the genera of the mosquito and the location of collection. After the adults emerged, they were collected using aspirators and put in labeled 1.5 mL collection tubes containing silica gel to help preserve the samples and prevent the body parts from breaking into pieces.

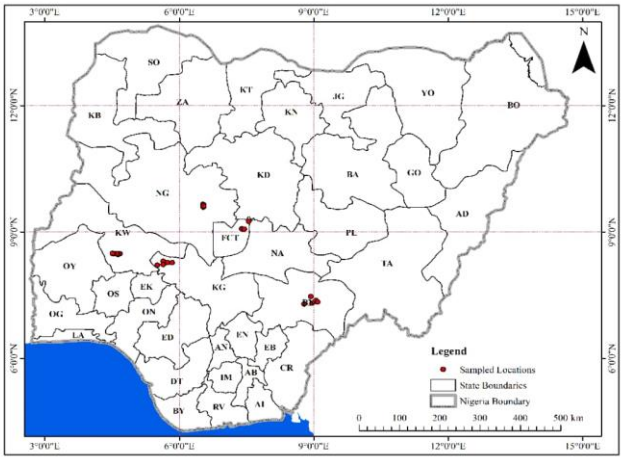



**Figure 1. Map showing geographical coordinates and sample origins where mosquitoes were collected in North-central Nigeria**

The collected mosquito samples were preserved over desiccated silica gel in 1.5Ml Eppendorf tubes. They were later examined using a dissecting microscope (Olympus SZ 40) and identified to species level using morphological identification keys previously described by Gillies and Coetzee (**33**).

### DNA Extraction

DNA was extracted from the whole body of the mosquito using Zymo Research Quick DNA Insect Miniprep Kit for 50 preps with few modifications to manufacturer's instructions. The yield and quality of the extracted DNA was checked using Nanodrop ND-1000 UV/Vis spectrophotometer (Nanodrop Technologies, Inc., DE, USA) and was later stored at -20$^0$C until further use for genotyping by polymerase chain reaction (PCR).

### PCR Amplification of Ribosomal ITS 2 Gene

The primers used for the amplification of the ITS2 region were;
Forward primer- 5′ ATC ACT CGG CTC GTG GAT CG 3′
Reverse primer- 5′ ATG CTT AAA TTT AGG GGG TAG TC 3′ (**34**).
Amplification was carried out using Q5 High Fidelity DNA polymerase from New England Biolabs. A 25 µL reaction mixture was prepared using 0.5 U Q5 Hot Start High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA), 100 µmol/L dNTPs, A 25 µL reaction mixture was prepared containing; 5µL of 5X Q5 reaction buffer, 0.5 µL of 10 mM dNTPs, 1.25 µL of each primer, 0.25 µL of Q5 High Fidelity DNA polymerase, 5 µL of 5X Q5 High GC Enhancer, 6.75 µL of nuclease free water and 5 µL of template DNA. The reaction protocol- initial denaturation at 95℃ for 30s, denaturation at 95℃ for 10s, annealing at 50℃ for 30s, elongation at 72℃ for 30s for 30 cycles and final elongation at 72℃ for 2 minutes. 5 µL of each reaction mixture was run on 2% DNA agarose gel with 1X TBE running buffer and stained with ethidium bromide stain. Electrophoresis was conducted at 90 volts for 90 minutes.

### PCR Amplification of Mitochondrial 16S-rRNA Gene

Primers for this study were adopted from Shouche and Patole (**16**) and the sequences include;
Forward primer- 5′ CGC CTG TTT ATC AAA AAC AT 3′
Reverse primer- 5′ CTC CGG TTT GAA CTC AGA TC 3′
A 25 µL PCR mixture was prepared and it contained 14.5µL of nuclease free water, 5µL of 5X Hot FIREPol Blend Master Mix with 7.5 mM $MgCl_2$, 0.25 µL of each primer and 5 µL of the DNA template. The protocol for this amplification was initial denaturation at 95℃ for 15 minutes, denaturation at 95℃ for 30s, annealing at 55℃

for 1 minute, elongation at 72℃ for 30s for 30 cycles and final elongation at 72℃ for 10 minutes. 5 µL of each reaction mixture was run on 2% agarose gel with 1X TBE running buffer. Electrophoresis was conducted at 90 volts, 150 mA for 90 minutes. Double bands were observed after the amplification so the bands of interest were excised from the gel and purified using EZNA gel extraction kit from Omega Bio-Tek, Inc, 400, USA.

## Phylogenetic Analysis

The ribosomal DNA ITS2 and mitochondrial 16S-rDNA mosquito samples were sent to Inqaba Biotec, South Africa for sequencing. Base calling and trimming of the sequences were carried out using FinchTV. The sequences were aligned with the Clustal W multiple alignment feature on BioEdit software version 7.2.5. Phylogenetic trees were constructed by the maximum likelihood (ML) method using MEGA v. 6 (Tamura *et al.*, 2013) and pairwise genetic distances were inferred using MEGA 6.0.

## Results

Mosquito genera sampled included *Culex*, *Aedes* and *Anopheles*. Figure 1 represent a map showing geographical coordinates and sample origins where mosquitoes were collected at different locations in North-central Nigeria. The genus *Culex* had the highest overall prevalence compared to other genera sampled. In FCT and Niger, all genera except *Aedes* were collected. In this study, among the states sample in North-central Nigeria, no *Anopheles* sample was collected in Kogi state but in Benue state, very high numbers of *Anopheles* samples were recorded and just one *Aedes* sample. A total of ten (10) mosquito species comprising of three genera were documented in this study (Table 1). Polymerase chain reaction (PCR) amplicons pattern following 2% agarose gel electrophoresis is indicated in Figure 2. This depicts different amplicon sizes of the ITS2 region of the mosquito samples. PCR amplicons sizes ranged from 400-700 base pairs. PCR mixture for each sample was loaded into each well and run at 90 volts for 90 minutes and bands were viewed under UV transilluminator. The 100bp marker was used to estimate the sizes of the different bands on the gel. The samples were purified because there were double bands on the gel after PCR. The fragment of interest was excised and gel extraction was carried out. PCR results were positive for ITS2 and 16S rDNA gene regions (Figure 2 and 3)

**Table 1. Mosquito Populations, Geographical Locations and Geographical Coordinates**

| S/No. | Mosquitoes genera | Mosquitoes species | Geographical Locations | Geographical Coordinates |
|---|---|---|---|---|
| 1 | **Culex** | Culex *quinquefasciatus* | Kwarimpa, Abuja | N9°04 ' 21.5 '', E7°23' 40.9 '' |
| | | Culex australicus | Yandev, Benue | N7°20'12.19'', E9°4'57.15'' |
| | | Culex *bitaeniorhynchu* | | |
| | | Culex brami | Gurara, Minna | N9°35' 14.1'', E6°32' 03.9'' |
| | | Culex sp | Yagba East, Kogi | N8°16' 29.0'', E5°44' 04.4'' |
| | | Culex *quinquefasciatus* | Ilorin, Nigeria | N8°27' 44.0'', E4°38' 02.5'' |
| 2 | **Anopheles** | Anopheles *arabiensis* | Kwarimpa, Abuja | N9°04' 21.5'', E7°23' 40.9'' |
| | | Anopheles *gambiae* | Mbayion, Benue | N7°28' 1.72'', E8°56' 8.59'' |
| | | | Gurara, Minna | N9°36''1.18'',E6°32' 48.77'' |
| | | | Ilorin, Nigeria | N8°29' 24.0'', E4°30' 37.2'' |
| 3 | **Aedes** | ***Aedes aegypti*** | Mbatiav, Benue | N7°17' 32.44'', E8°47' 2.59'' |
| | | | Yagba West, Kogi | N8°12' 49.5'', E5°30' 34.1'' |
| | | | Ilorin, Nigeria | N8°28' 53.7'', E4°40' 30.6'' |

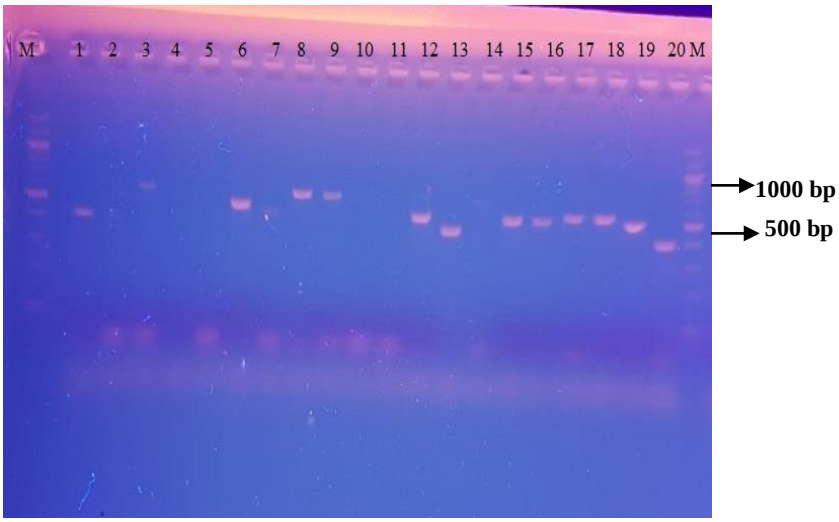



**Figure 2. Amplification profile of internal transcribed spacer 2 (ITS2) region of mosquito samples 1-20. M= 100bp marker used to estimate amplicon sizes. The extracted PCR fragments were then run on 2% agarose gel using 1X TBE running buffer at 80 volts. DNA agarose gel electrophoresis lasted for 90 minutes to achieve optimum separation of the DNA fragment on the agarose gel.**

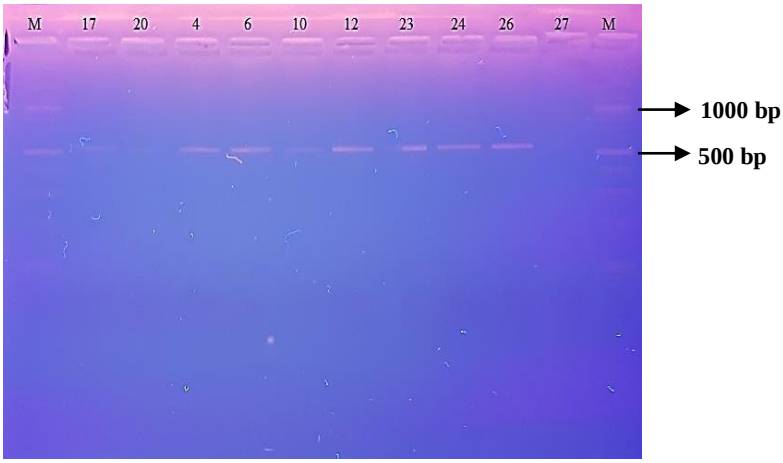
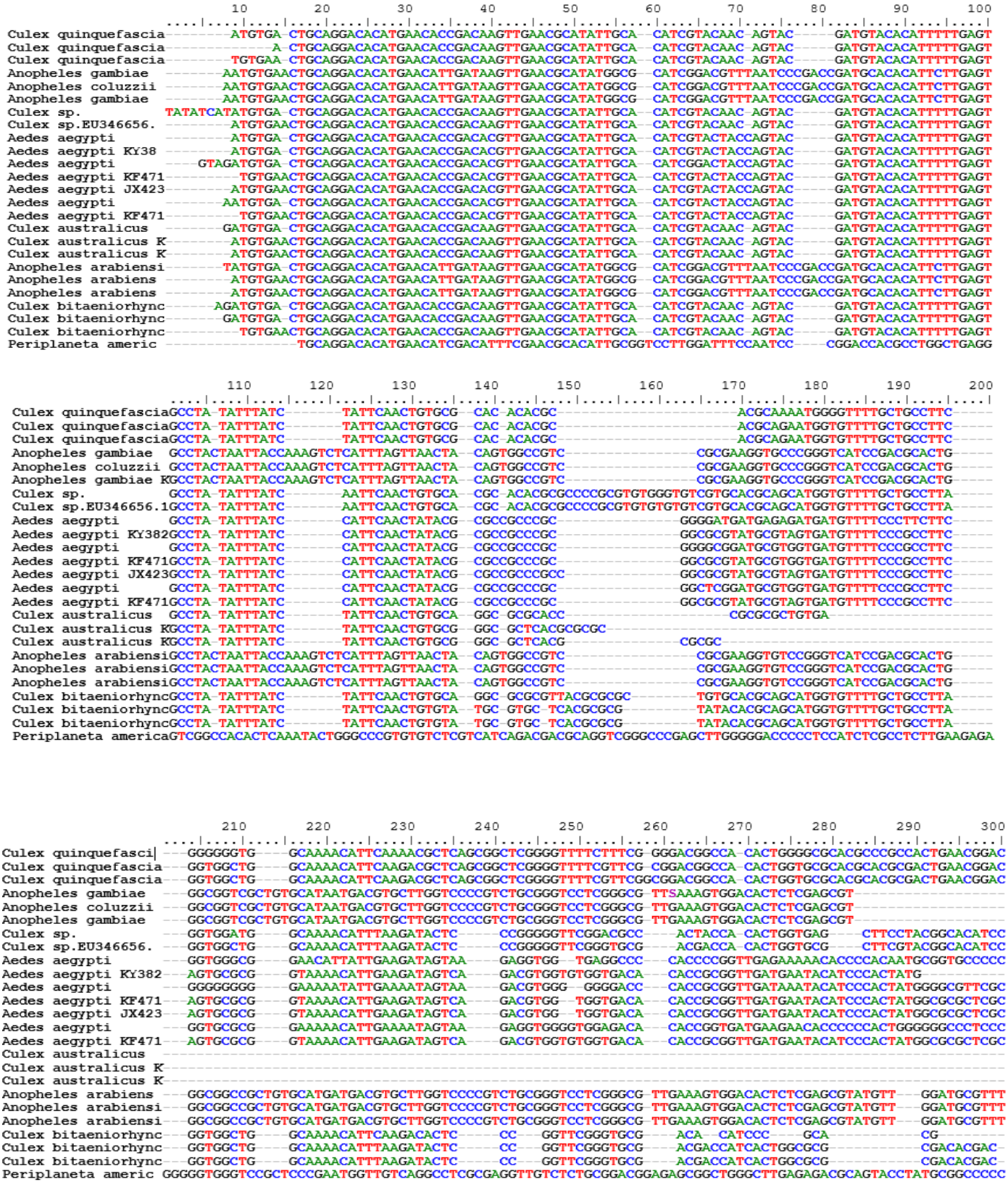



**Figure 3. Amplification profile of the mitochondrial 16S rDNA region of some mosquito samples.**

Multiple sequence alignment results in Figures 4 and 5 showed lack of apparent nucleotide sequence variations in ITS2 rDNA and mt16S rRNA sequences of the collected mosquito population may probably explain why Culex, Aedes and Anopheles formed separate distinct clades in the constructed phylogenetic trees shown in Figure 6 and 7. Molecular phylogenetic analysis of the sequences from the ITS2 region of the mosquitoes inferred by maximum likelihood method is presented in Fig. 6. Table 2 represents the pairwise distance between each species of mosquito against themselves and against other species to infer the evolutionary divergence amongst them. As revealed in this table, there was low evolutionary divergence in the ITS2 region of the Anopheles, Aedes and Culex species sequenced in the study and the DNA sequences of mosquitoes retrieved from the Genbank. The estimate of evolutionary divergence in the 16S rDNA region between pairs of the different mosquitoes sampled for this study is shown in Table 3. Unlike the ITS2 region, there was considerable difference in the 16S rDNA regions of the two samples of *Anopheles gambiae* sequenced. There were also distances amongst and between all the other genera sequenced.

The phylogenetic tree showed the branching out of the two subfamilies Culicinae and Anophelinae. It is evidenced from this phylogenetic tree that

Culex, Aedes and Anopheles species formed three distinct clades and are clearly separated while Culex, Aedes and Anopheles spp clustered together. *Periplaneta americana* was used as an outgroup when constructing the two phylogenetic trees (Figure 6 and 7). The green squares represent sequences retrieved from the GenBank NCBI database. The black circles represent the sequences from this study and the red diamond represents the out-group. The values on the nodes of the tree are the bootstrap values after 1000 bootstrap replications.



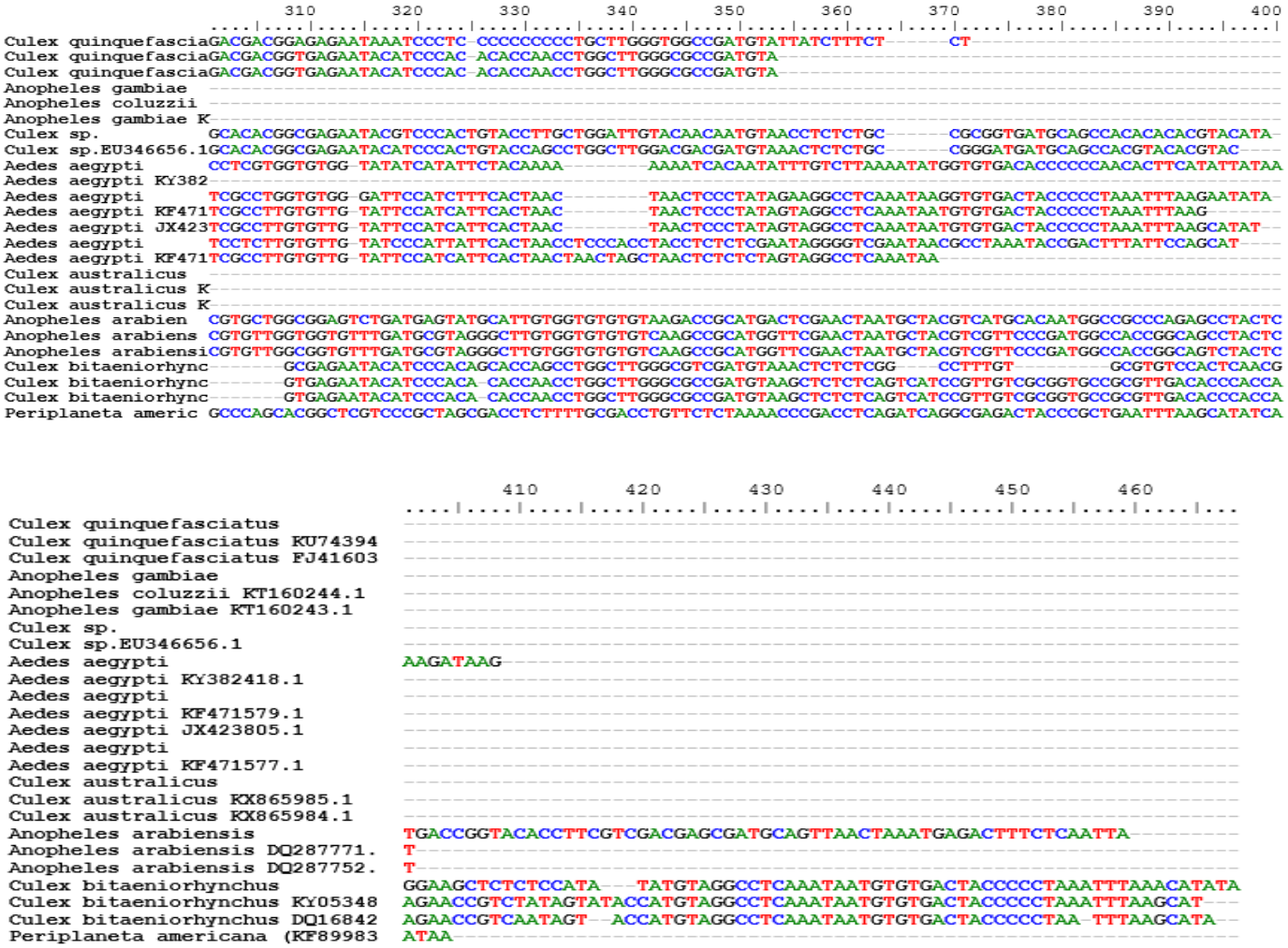
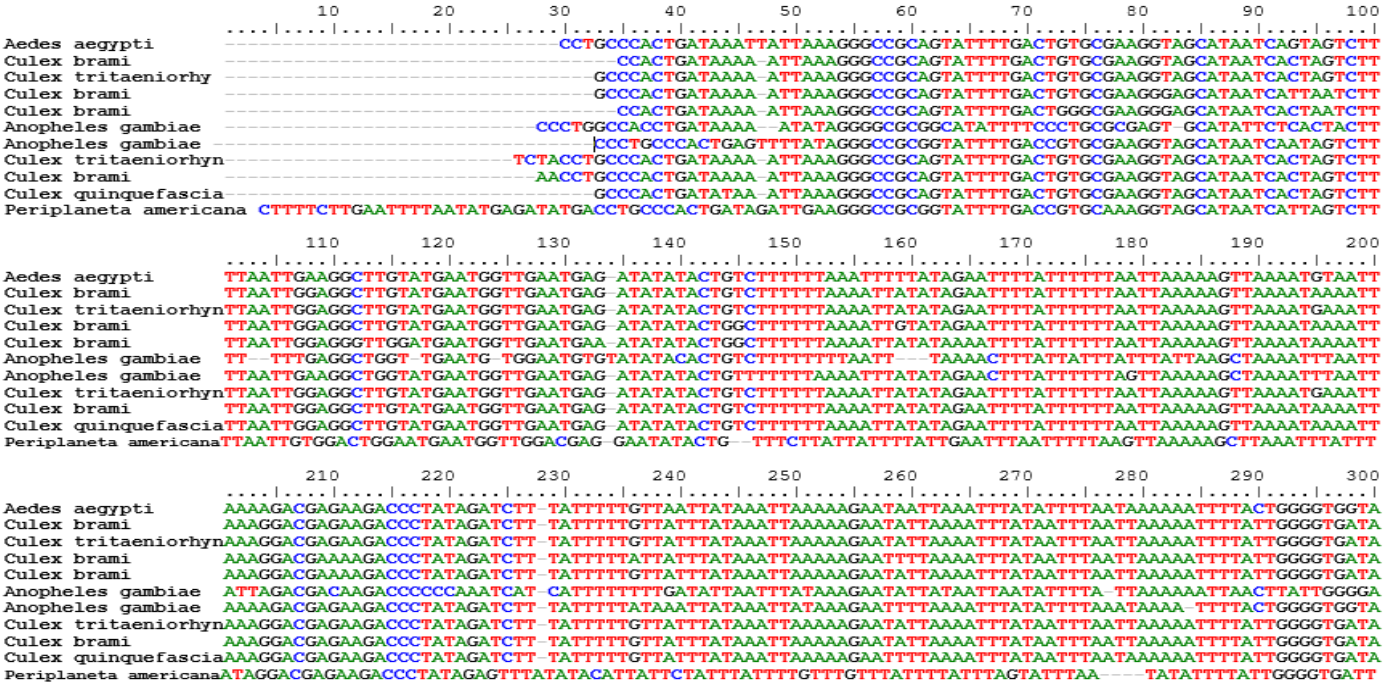



**Figure 4. Multiple Sequence Alignment of ITS2 region sequences of mosquitoes in this study using the ClustalW multiple alignment feature on BioEdit software.**



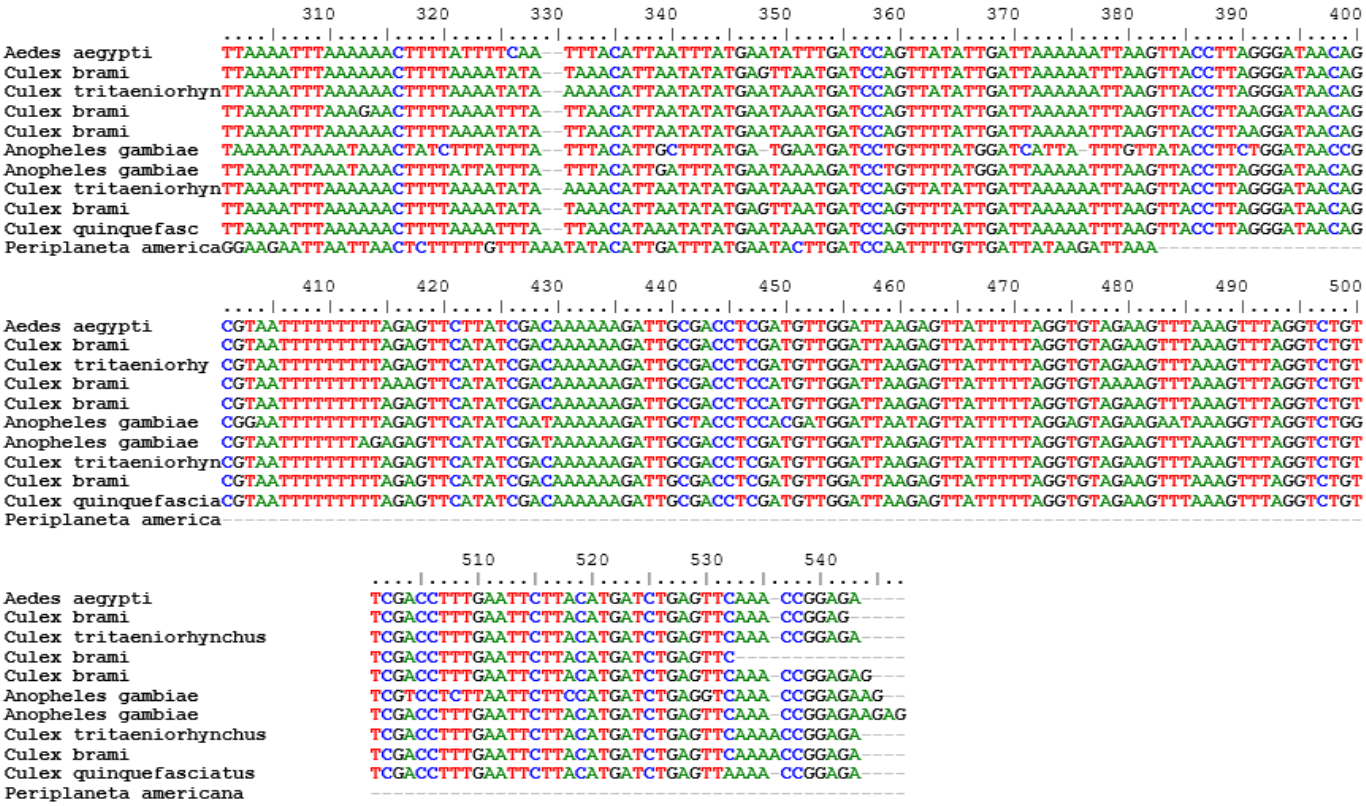



**Figure 5. Multiple Sequence Alignment of mitochondrial 16S rRNA sequences of mosquito samples used for this study using the ClustalW multiple alignment feature on BioEdit**

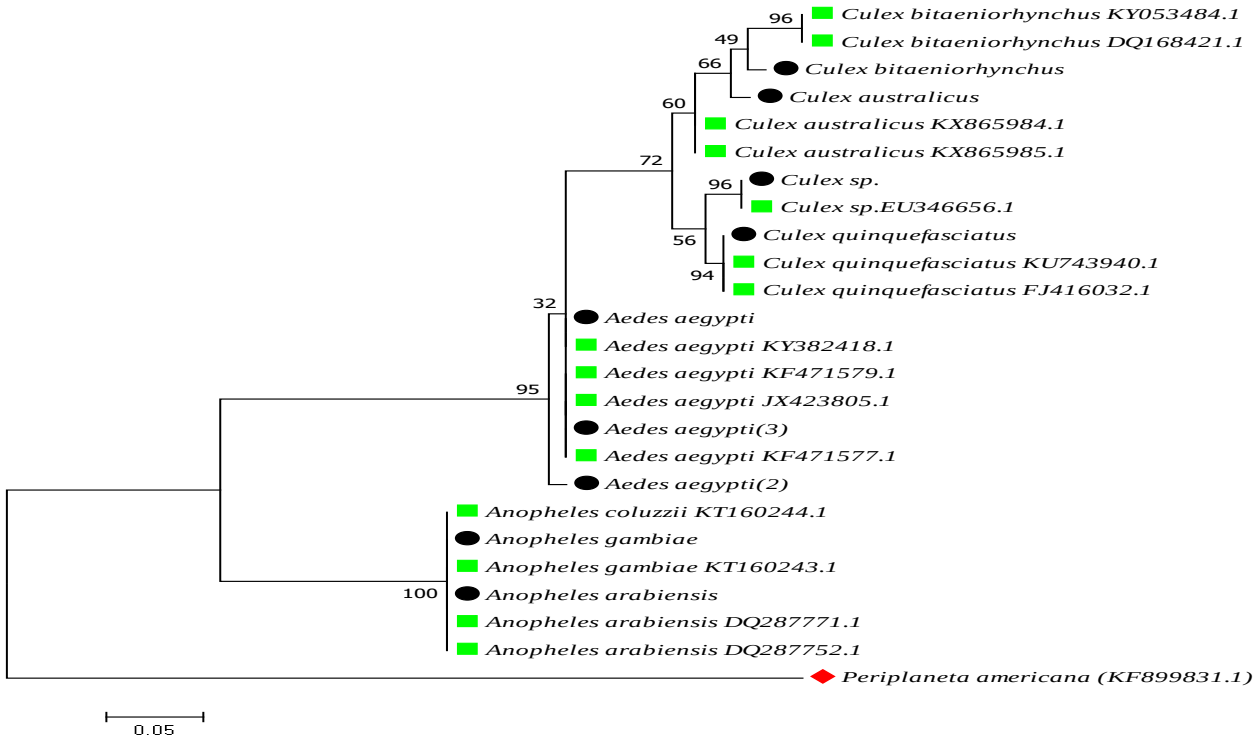



**Figure 6. Molecular Phylogenetic analysis of the internal transcribed spacer 2 (ITS2) region of mosquitoes by Maximum Likelihood method inferred by Tamura-Nei method after 1000 replications.**

**Table 2. Evolutionary Divergence of the ITS2 sequences of some mosquito species sequenced.**

| S/NO | Organism | Accession Number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | ***Cx. quin*** | This study | -- | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | ***Cx. quin*** | KU743940.1 | 0.08 | -- | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | ***Cx. quin*** | FJ416032.1 | 0.10 | 0.00 | -- | | | | | | | | | | | | | | | | | | | | | | |
| 4 | ***An. gam*** | This study | 0.57 | 0.59 | 0.62 | -- | | | | | | | | | | | | | | | | | | | | | |
| 5 | ***An. col*** | KT160244.1 | 0.57 | 0.59 | 0.61 | 0.00 | -- | | | | | | | | | | | | | | | | | | | | |
| 6 | ***An. gam*** | KT160243.1 | 0.57 | 0.59 | 0.61 | 0.00 | 0.00 | -- | | | | | | | | | | | | | | | | | | | |
| 7 | ***Cx. sp.*** | This study | 0.26 | 0.19 | 0.21 | 0.54 | 0.54 | 0.54 | -- | | | | | | | | | | | | | | | | | | |
| 8 | ***Cx. sp.*** | EU346656.1 | 0.23 | 0.14 | 0.16 | 0.53 | 0.53 | 0.53 | 0.07 | -- | | | | | | | | | | | | | | | | | |
| 9 | ***Ae. aeg*** | This study | 0.52 | 0.46 | 0.49 | 0.65 | 0.66 | 0.66 | 0.50 | 0.53 | -- | | | | | | | | | | | | | | | | |
| 10 | ***Ae. aeg*** | KY382418.1 | 0.31 | 0.30 | 0.33 | 0.61 | 0.62 | 0.62 | 0.29 | 0.30 | 0.13 | -- | | | | | | | | | | | | | | | |
| 11 | ***Ae. aeg*** | This study | 0.50 | 0.46 | 0.48 | 0.59 | 0.60 | 0.60 | 0.52 | 0.51 | 0.20 | 0.07 | -- | | | | | | | | | | | | | | |
| 12 | ***Ae. aeg*** | KF471579.1 | 0.48 | 0.43 | 0.45 | 0.61 | 0.62 | 0.62 | 0.50 | 0.50 | 0.23 | 0.00 | 0.07 | -- | | | | | | | | | | | | | |
| 13 | ***Ae. aeg*** | JX423805.1 | 0.47 | 0.43 | 0.45 | 0.60 | 0.61 | 0.61 | 0.50 | 0.50 | 0.24 | 0.00 | 0.08 | 0.00 | -- | | | | | | | | | | | | |
| 14 | ***Ae. aeg*** | This study | 0.52 | 0.46 | 0.49 | 0.62 | 0.62 | 0.62 | 0.55 | 0.54 | 0.29 | 0.09 | 0.18 | 0.15 | 0.15 | -- | | | | | | | | | | | |
| 15 | ***Ae. aeg*** | KF471577.1 | 0.49 | 0.46 | 0.48 | 0.61 | 0.62 | 0.62 | 0.48 | 0.48 | 0.22 | 0.00 | 0.09 | 0.01 | 0.01 | 0.13 | -- | | | | | | | | | | |
| 16 | ***Cx. aust*** | This study | 0.11 | 0.11 | 0.15 | 0.39 | 0.39 | 0.39 | 0.11 | 0.10 | 0.14 | 0.12 | 0.12 | 0.12 | 0.12 | 0.13 | 0.12 | -- | | | | | | | | | |
| 17 | ***Cx austr*** | KX865985.1 | 0.04 | 0.05 | 0.09 | 0.35 | 0.35 | 0.35 | 0.08 | 0.08 | 0.07 | 0.07 | 0.08 | 0.07 | 0.08 | 0.07 | 0.07 | 0.03 | -- | | | | | | | | |
| 18 | ***Cx. austr*** | KX865984.1 | 0.04 | 0.05 | 0.09 | 0.33 | 0.33 | 0.33 | 0.08 | 0.08 | 0.09 | 0.08 | 0.09 | 0.08 | 0.08 | 0.09 | 0.08 | 0.03 | 0.00 | -- | | | | | | | |
| 19 | ***An. arab*** | This study | 0.80 | 0.79 | 0.81 | 0.02 | 0.02 | 0.02 | 0.81 | 0.83 | 0.93 | 0.64 | 0.87 | 0.88 | 0.86 | 0.91 | 0.83 | 0.41 | 0.35 | 0.34 | -- | | | | | | |
| 20 | ***An. arab*** | DQ287771.1 | 0.77 | 0.77 | 0.79 | 0.01 | 0.01 | 0.01 | 0.83 | 0.81 | 0.88 | 0.64 | 0.91 | 0.88 | 0.87 | 0.92 | 0.84 | 0.40 | 0.35 | 0.33 | 0.06 | -- | | | | | |
| 21 | ***An. arab*** | DQ287752.1 | 0.77 | 0.78 | 0.81 | 0.01 | 0.01 | 0.01 | 0.80 | 0.78 | 0.90 | 0.64 | 0.92 | 0.90 | 0.90 | 0.96 | 0.86 | 0.40 | 0.35 | 0.33 | 0.06 | 0.01 | -- | | | | |
| 22 | ***Cx. bita*** | This study | 0.18 | 0.10 | 0.12 | 0.55 | 0.55 | 0.55 | 0.19 | 0.12 | 0.50 | 0.27 | 0.45 | 0.44 | 0.45 | 0.48 | 0.44 | 0.07 | 0.08 | 0.06 | 0.83 | 0.75 | 0.75 | -- | | | |
| 23 | ***Cx. bita*** | KY053484.1 | 0.20 | 0.11 | 0.13 | 0.54 | 0.54 | 0.54 | 0.21 | 0.14 | 0.57 | 0.31 | 0.54 | 0.51 | 0.51 | 0.57 | 0.48 | 0.08 | 0.10 | 0.09 | 0.82 | 0.72 | 0.74 | 0.11 | -- | | |
| 24 | ***Cx. bita*** | DQ168421.1 | 0.21 | 0.11 | 0.12 | 0.53 | 0.53 | 0.53 | 0.21 | 0.14 | 0.57 | 0.31 | 0.54 | 0.50 | 0.51 | 0.56 | 0.48 | 0.08 | 0.10 | 0.09 | 0.81 | 0.72 | 0.74 | 0.11 | 0.00 | -- | |
| 25 | ***P. amer*** | KF899831.1 | 1.22 | 1.15 | 1.15 | 0.94 | 0.95 | 0.95 | 1.18 | 1.22 | 1.11 | 1.09 | 0.96 | 1.04 | 1.00 | 1.10 | 1.21 | 0.85 | 0.83 | 0.81 | 1.40 | **1.51** | **1.51** | 1.31 | 1.22 | 1.22 | -- |

**Key:** *Cx. quin = Culex quinquefasciatus; An. col = Anopheles coluzzii ; An. gam = Anopheles gambiae; Cx sp= Culex specie; Ae. aeg = Aedes aegypti; Cx. austr = Culex australicus; An. arab =Anopheles arabiensis*; *Cx. bita= Culex bitaeniorhynchus*; *P. amer = Periplaneta americana*
The numbers 1-25 on the horizontal bar correspond with the samples listed.

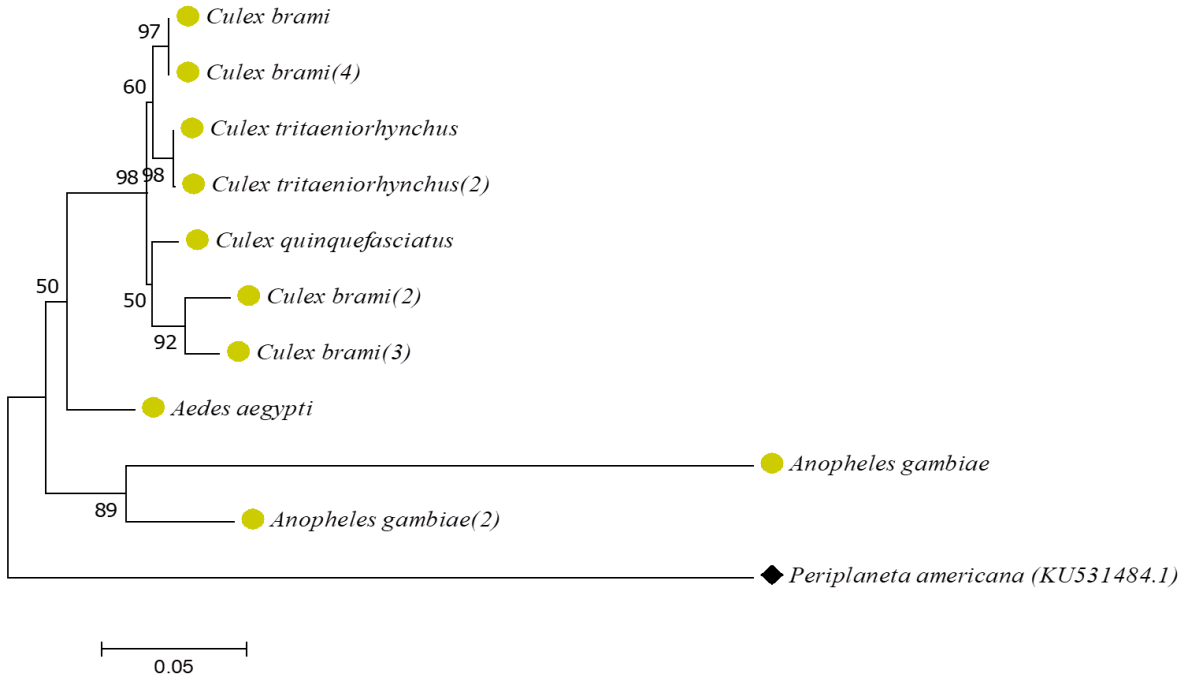



**Figure 7. Evolutionary relationship in the mt16S rRNA region of mosquitoes by neighbour joining method after 1000 replications**

**Table 3. Evolutionary Divergence of the 16s-RNA sequences of some mosquito samples sequenced for this study**

| S/NO | Organism | Accession Number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *Ae. Aeg* | This study | -- | | | | | | | | | | | | |
| 2 | *Ae. aeg* | EU352212.1 | 1.30 | -- | | | | | | | | | | | |
| 3 | *Ae. aeg* | DQ397917.1 | 0.00 | 1.30 | -- | | | | | | | | | | |
| 4 | *Cx brami* | This study | 0.06 | 1.20 | 0.06 | -- | | | | | | | | | |
| 5 | *Cx. trita* | This study | 0.05 | 1.23 | 0.05 | 0.01 | -- | | | | | | | | |
| 6 | *Cx. brami* | This study | 0.08 | 1.12 | 0.08 | 0.04 | 0.04 | -- | | | | | | | |
| 7 | *Cx. brami* | This study | 0.08 | 1.21 | 0.08 | 0.03 | 0.03 | 0.03 | -- | | | | | | |
| 8 | *An. gam* | This study | 0.30 | 1.20 | 0.30 | 0.28 | 0.29 | 0.30 | 0.29 | -- | | | | | |
| 9 | *An. gam* | This study | 0.10 | 1.31 | 0.10 | 0.11 | 0.11 | 0.12 | 0.13 | 0.27 | -- | | | | |
| 10 | *Cx. trita* | This study | 0.05 | 1.22 | 0.05 | 0.01 | 0.00 | 0.04 | 0.03 | 0.30 | 0.11 | -- | | | |
| 11 | *Cx. brami* | This study | 0.06 | 1.20 | 0.06 | 0.00 | 0.01 | 0.04 | 0.03 | 0.29 | 0.11 | 0.01 | -- | | |
| 12 | *Cx. quin* | This study | 0.06 | 1.13 | 0.06 | 0.02 | 0.02 | 0.03 | 0.03 | 0.29 | 0.11 | 0.02 | 0.02 | -- | |
| 13 | *P. amer* | KU531484.1 | 0.28 | 1.37 | 0.28 | 0.29 | 0.28 | 0.28 | 0.32 | 0.54 | 0.30 | 0.29 | 0.29 | 0.29 | -- |

***Key:*** *Ae. aeg = Aedes aegypti; Cx. brami = Culex brami; Cx. trita = Culex tritaeniorhynchus; An. gam = Anopheles gambiae; Cx. quin = Culex quinquefasciatus; P. amer = Periplaneta americana*
The numbers 1-13 on the horizontal bar correspond with the samples listed.

## Discussion

In this study, diversity and distribution of mosquito species potentially involved in malaria transmission cycles in north-central regions of Nigeria with reported incidences of malaria were investigated. Genetic diversity and population genetic structure of mosquito populations have

been less studied in north-central regions of Nigeria. This is very important to be taken into great consideration when planning vector control and management strategies in the war against malaria in Nigeria and sub-Saharan Africa. There was a higher species abundance of *Culex* over other genera. Since *Culex* is known to breed in polluted areas, this high abundance could be as a result of improper waste and sewage disposal which provides a breeding habitat for these *Culex* mosquitoes. We retrieved available *ITS2* and *16sRNA* sequence data of Anopheles, Aedes and Culex members from GenBank in order to compare and match them with the DNA sequences of the Nigerian counterparts obtained from this study. This result is consistent with previous study carried out in Benin City by Aigbodion and Uyi **(35)** which showed that *Culex* and *Aedes* had higher species abundance over *Anopheles* mosquitoes. This species abundance can be positively co-related with urbanization, over-bearing effects of human activities on the environment as well as other anthropogenic activities that have led to poor waste disposal, poor sanitary levels, uncontrolled run-offs etc.

The ribosomal ITS2 gene region was able to successfully separate each genera as they seem to cluster apart in the phylogenetic tree. The tree showed the point of branching of Anophelinae from Culicinae with the subfamily Anophelinae placed in the basal position. This is consistent with a study carried out in Northwestern Iran that used ITS2 to characterize mosquito samples and reported that ITS2 successfully differentiated between mosquito subfamilies- Culicinae and Anophelinae (**34**). In this study, phylogenetic analysis revealed that the *ITS2* DNA sequences of *Ae. aegypti* mosquitoes collected from Nigeria were similar in identity with previously published data available in the GenBank database (Fig 6). Similar trends were observed in the *ITS2* DNA sequences of *An. gambiae*, *An. arabiensis*, *Cx quinquefasciatus* and *Culex sp*. *Cx. quinquefasciatus* also clustered separately from *Cx. australicus* which is a part of the *Cx. pipens* complex usually found in Australia. Although *Cx. quinquefasciatus*, from this study has similar DNA sequences with the one retrieved from the Genbank (Fig 6). *Ae. aegypti* and *An, gambiens*. This confirmed that *Cx. quinquefasciatus* and *Cx. pipens* are not monophyletic as suggested by Kohli *et al.*, (**13**). *ITS2* sequence values successfully distinguished the different *Ae. aegypti* mosquitoes sequenced in this study. This result is consistent with a study conducted in Sri Lanka by Weeraratne *et al.* (**36**) where ITS2 and COI DNA sequences distinctly differentiated *Aedes aegypti* samples from themselves, other species of *Aedes* and four other mosquito genera including *Armigeres*, *Culex*, *Mansonia* and *Mimoyia*. However, it could not give any sequence dissimilarities between *Anopheles arabiensis* and *An. gambiae*. ITS2 sequences could only detect four variable sites between the two species of *Anopheles* sequenced for this study. This result could be due to its inability to carry out intraspecific variation as suggested by Walton *et al.*, 2007 (**34**) who couldn't successfully differentiate sequences of *An. pseudowillmori* collected in China. Khoshdel-Nezamiha *et al.* (**34**) also reported this shortcoming of ITS2 sequences in differentiating members of the *An. maculipennis* complex collected from different locations in Northwestern Iran. Wilkerson, et al., (**37**) has previously used rDNA *ITS2* sequence to differentiate six species in the *Anopheles crucians* complex from mosquito samples collected from central Florida, USA. Phylogenetic analysis of the mitochondrial 16S-rDNA region also split three mosquito genera analyzed based on their subfamilies, placing Anophelinae at the basal region, thereby supporting the results of the nucleotide sequences of the ITS2 region. The 16S rDNA marker differentiated significantly the two *Anopheles* samples sequenced for this study as there were 105 variable sites observed between them. Shouche and Patole **(16)** reported that *Anopheles* species showed significant variations in their mitochondrial region even though there was no significant difference in their morphological divergence. The16S rDNA also differentiated all the *Culex* (*Cx.*) samples analyzed for this region during the study. Different clustering patterns were observed for four *Cx. brami* samples used in the study. These clustering patterns observed seem to be based on the geographic distance between these *Cx brami* samples. One of the clusters contained *Cx. brami* samples collected from Abuja, Nigeria and the other contained *Cx. brami* samples collected from Kogi state, Nigeria. In this study, it is not surprising that we observed disparity in the phylogenetic trees between

mitochondrial (COI) and nuclear (ITS2) genes. One plausible reason for this observation between mitochondrial and nuclear gene regions could possibly be due to variations in evolutionary rates. It has been reported that mitochondrial DNA mutates at higher rate than nuclear DNA sequences (**11**, **38)**. It is therefore reasonable to suggest that different ecological and enviro-nmental factors of these regions may have played a significant role in the observed nucleotide substitutions in the 16S region that made these *Cx. brami* samples cluster apart. It also showed that *Cx. quinquefasciatus* diverge from *Cx. tritaenorhynchus* which is similar to a study reported by Shouche and Patole **(16)**.

## Conclusions

This study concludes that ITS2 and 16S-rDNA are ideal tools that can be utilized for systematics and phylogenetics studies of mosquitoes and a wide variety of other organisms. We therefore recommend that further studies should be done with larger sample sizes in order to deeply understand and re-evaluate the phylogenetic relationship among the mosquito species with the use of other markers with higher discrimination power such as DNA barcoding and microsatellite.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Authors' contributions

OAI conceived and designed this study; RDS and ATK-I collected and preserved the samples; OAI, TOF and RDS performed the molecular laboratory experiments; All authors provided technical support, gave advise on the study design and contributed to the implementation of activities. OAI and RDS wrote the draft of the manuscript with very significant contributions from other authors. All authors read and approved the final version of the manuscript.

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## Availability of data and materials

All data generated and analyzed during this study are included in this published article.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

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