

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 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 bancrofti* 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 Eet 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) (Figure 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.

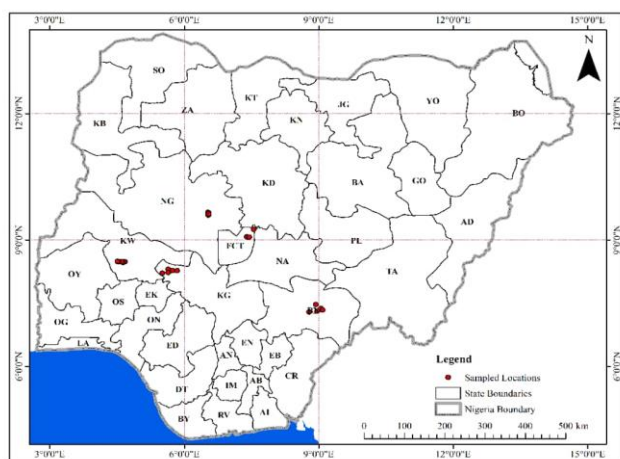


Figure 1. Map showing geographical coordinates and sample origins where mosquitoes were collected in North-central Nigeria

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 °C until further use for genotyping by polymerase chain reaction (PCR).

PCR Amplification of Ribosomal ITS2 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 °C for 30s, denaturation at 95 °C for 10s, annealing at 50 °C for 30s, elongation at 72 °C for 30s for 30 cycles and final elongation at 72 °C for 2 minutes. Five 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 14.5 µL of nuclease free water, 5 µL of 5X Hot FIREPol Blend Master Mix with 7.5 mM MgCl₂, 0.25 µL of each primer and 5 µL of the DNA template. The protocol for this amplification was initial denaturation at 95 °C for 15 minutes, denaturation at 95 °C for 30s, annealing at 55 °C

for 1 minute, elongation at 72 °C for 30s for 30 cycles and final elongation at 72 °C 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 Biotech, 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 (Figures 2 and 3).

Table 1. Mosquito populations, geographical locations and geographical coordinates

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

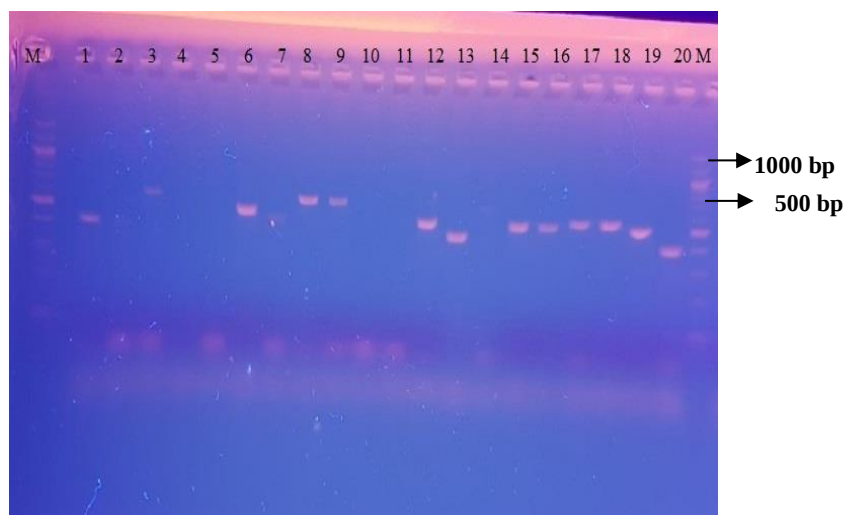


Figure 2. Amplification profile of internal transcribed spacer 2 (ITS2) region of mosquito samples 1-20. M= 100 bp marker used to estimate amplicon sizes. The extracted PCR fragments were then run on 2% agarose gel using 1X TBE running buffer at 80 volts. DNA agarose gel electrophoresis lasted for 90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

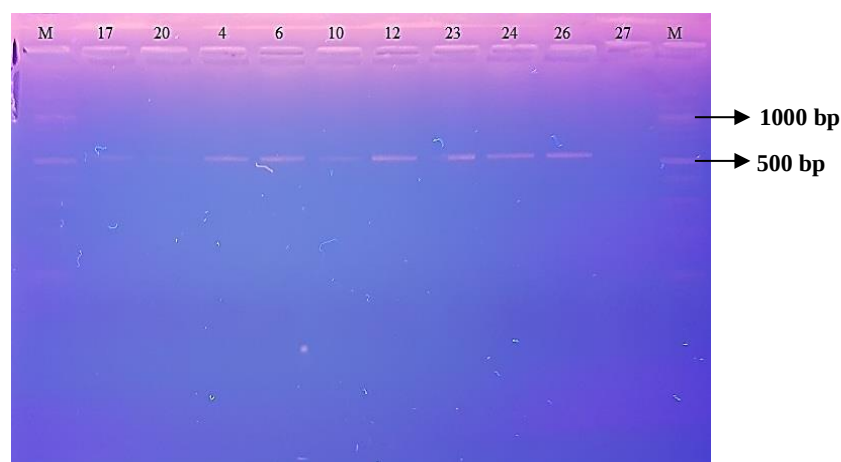


Figure 3. Amplification profile of the mitochondrial 16S rDNA region of some mosquito samples

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

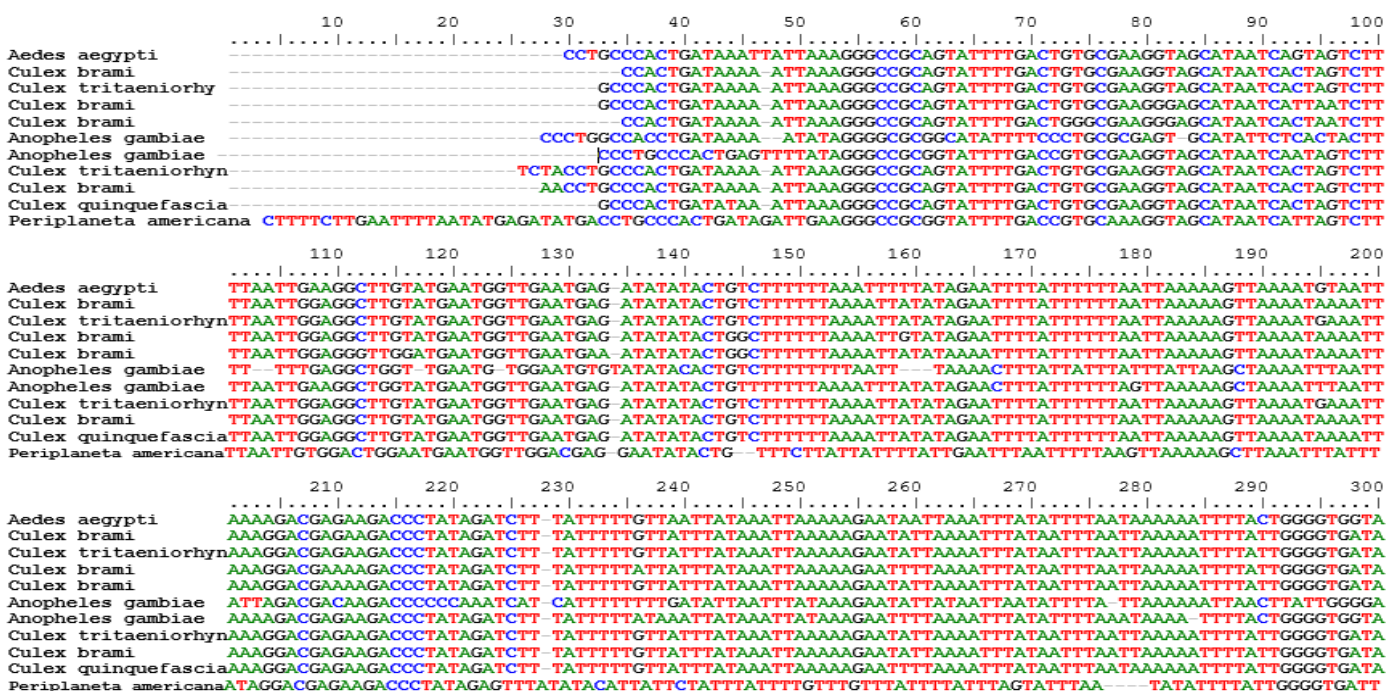
low evolutionary divergence in the ITS2 region of the *Anopheles*, *Aedes* and *Culex* species sequenced in the study and the DNA sequences of mosquitoes retrieved from the Genbank. The estimate of evolutionary divergence in the 16S rDNA region between pairs of the different mosquitoes sampled for this 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 (Figures 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.

	10	20	30	40	50	60	70	80	90	100
Culex quinquefascia	ATGTGA	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGTACAAC	AGTAC	GATGTACACATTTTGTAGT					
Culex quinquefascia	A	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGTACAAC	AGTAC	GATGTACACATTTTGTAGT					
Culex quinquefascia	TGTGAA	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGTACAAC	AGTAC	GATGTACACATTTTGTAGT					
Anopheles gambiae	AATGTGAAC	CTGCAGGACACATGAACATTGATAAGTTGAACGCATATTGGCG	CATCGGACGTTTAAATCCCGACCGATGCACACATTCTTGTAGT							
Anopheles coluzzii	AATGTGAAC	CTGCAGGACACATGAACATTGATAAGTTGAACGCATATTGGCG	CATCGGACGTTTAAATCCCGACCGATGCACACATTCTTGTAGT							
Anopheles gambiae	AATGTGAAC	CTGCAGGACACATGAACATTGATAAGTTGAACGCATATTGGCG	CATCGGACGTTTAAATCCCGACCGATGCACACATTCTTGTAGT							
Culex sp.	TATATCATATGTA	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGTACAAC	AGTAC	GATGTACACATTTTGTAGT					
Culex sp.EU346656	ATGTGAAC	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGTACAAC	AGTAC	GATGTACACATTTTGTAGT					
Aedes aegypti	ATGTGA	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGTACAAC	AGTAC	GATGTACACATTTTGTAGT					
Aedes aegypti KY38	ATGTGA	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGTACAAC	AGTAC	GATGTACACATTTTGTAGT					
Aedes aegypti	GTAGATGTA	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGGAC	TACCAAGTAC	GATGTACACATTTTGTAGT					
Aedes aegypti KF471	TGTGAAC	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGTACAAC	AGTAC	GATGTACACATTTTGTAGT					
Aedes aegypti JX423	ATGTGAAC	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGTACAAC	AGTAC	GATGTACACATTTTGTAGT					
Aedes aegypti	AATGTGA	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGTACAAC	AGTAC	GATGTACACATTTTGTAGT					
Aedes aegypti KF471	TGTGAAC	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGTACAAC	AGTAC	GATGTACACATTTTGTAGT					
Culex australicus	GATGTGA	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGTACAAC	AGTAC	GATGTACACATTTTGTAGT					
Culex australicus K	ATGTGAAC	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGTACAAC	AGTAC	GATGTACACATTTTGTAGT					
Culex australicus K	ATGTGAAC	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGTACAAC	AGTAC	GATGTACACATTTTGTAGT					
Anopheles arabiensis	TATGTGA	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGGACGTTTAAATCCCGACCGATGCACACATTCTTGTAGT							
Anopheles arabiensis	ATGTGAAC	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGGACGTTTAAATCCCGACCGATGCACACATTCTTGTAGT							
Anopheles arabiensis	ATGTGAAC	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGGACGTTTAAATCCCGACCGATGCACACATTCTTGTAGT							
Culex bitaeniorhynch	AGATGTGA	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGTACAAC	AGTAC	GATGTACACATTTTGTAGT					
Culex bitaeniorhynch	GATGTGA	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGTACAAC	AGTAC	GATGTACACATTTTGTAGT					
Culex bitaeniorhynch	TGTGAAC	CTGCAGGACACATGAACACCGACAAGTTGAACGCATATTGCA	CATCGTACAAC	AGTAC	GATGTACACATTTTGTAGT					
Periplaneta americana	TGCAGGACACATGAACATCGACATTTGCAACGCACATTCGCGT	CCTTGGATTTCCTCAATCC	CGGACACGCGCTGGCTGAGG							
	110	120	130	140	150	160	170	180	190	200
Culex quinquefascia	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Culex quinquefascia	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Culex quinquefascia	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Anopheles gambiae	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Anopheles coluzzii	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Anopheles gambiae	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Culex sp.	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Culex sp.EU346656	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Aedes aegypti	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Aedes aegypti KY38	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Aedes aegypti	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Aedes aegypti KF471	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Aedes aegypti JX423	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Aedes aegypti	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Aedes aegypti KF471	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Culex australicus	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Culex australicus K	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Culex australicus K	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Anopheles arabiensis	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Anopheles arabiensis	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Anopheles arabiensis	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Culex bitaeniorhynch	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Culex bitaeniorhynch	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Culex bitaeniorhynch	GCCTA	TATTTATC	TATTTCAACTGTGCG	CAC	ACACGC	ACGCAAAATGGGGT	TTTTGCTGCCTTC			
Periplaneta americana	GTGCGCCACATCAAAATCT	TGGGCGCGTGTGCTCGT	CATCAGACGACGAGGTC	GGGCGCGAGC	TTGGGGGACCCCTCCATCTCGCCTCTTGAAGAGA					
	210	220	230	240	250	260	270	280	290	300
Culex quinquefascia	GGGGGGTG	GCAAAACATTTCAAAACGCTCAGCGGCTCGGGGTTTTCTTTTC	GGGACGGCCA	CAC	TGGGGCGCACGCCCGGCATGAACGGAC					
Culex quinquefascia	GGTGGCTG	GCAAAACATTTCAAAACGCTCAGCGGCTCGGGGTTTTCTTTTC	GGGACGGCCA	CAC	TGGGGCGCACGCCCGGCATGAACGGAC					
Culex quinquefascia	GGTGGCTG	GCAAAACATTTCAAAACGCTCAGCGGCTCGGGGTTTTCTTTTC	GGGACGGCCA	CAC	TGGGGCGCACGCCCGGCATGAACGGAC					
Anopheles gambiae	GGCGGTGCGTGTGCATATGACGTGCTTGGTCCCGCTCTCGGGTCCCTCGGGCG	TTTAAAGTGGACACTCTCGAGCGT								
Anopheles coluzzii	GGCGGTGCGTGTGCATATGACGTGCTTGGTCCCGCTCTCGGGTCCCTCGGGCG	TTTAAAGTGGACACTCTCGAGCGT								
Anopheles gambiae	GGCGGTGCGTGTGCATATGACGTGCTTGGTCCCGCTCTCGGGTCCCTCGGGCG	TTTAAAGTGGACACTCTCGAGCGT								
Culex sp.	GGTGGATG	GCAAAACATTTAAGATATCTC	CCGGGGGTTCCGGACGCC	ACTACCA	CAC	TGGTGTGAG	CTTCTACGGCACATCC			
Culex sp.EU346656	GGTGGATG	GCAAAACATTTAAGATATCTC	CCGGGGGTTCCGGACGCC	ACTACCA	CAC	TGGTGTGAG	CTTCTACGGCACATCC			
Aedes aegypti	GGTGGCGG	GCAAAACATTTAAGATATCTC	CCGGGGGTTCCGGACGCC	ACTACCA	CAC	TGGTGTGAG	CTTCTACGGCACATCC			
Aedes aegypti KY38	AGTGCGCG	GTAATAATTTGAAGATAGTCA	GACGTGGTGTGGTGACA	CACCGCGGTTGATGAATACATCCCACTATG						
Aedes aegypti	GGGGGGGG	GTAATAATTTGAAGATAGTCA	GACGTGGTGTGGTGACA	CACCGCGGTTGATGAATACATCCCACTATG						
Aedes aegypti KF471	AGTGCGCG	GTAATAATTTGAAGATAGTCA	GACGTGGTGTGGTGACA	CACCGCGGTTGATGAATACATCCCACTATG						
Aedes aegypti JX423	AGTGCGCG	GTAATAATTTGAAGATAGTCA	GACGTGGTGTGGTGACA	CACCGCGGTTGATGAATACATCCCACTATG						
Aedes aegypti	GGTGGCGG	GTAATAATTTGAAGATAGTCA	GACGTGGTGTGGTGACA	CACCGCGGTTGATGAATACATCCCACTATG						
Aedes aegypti KF471	AGTGCGCG	GTAATAATTTGAAGATAGTCA	GACGTGGTGTGGTGACA	CACCGCGGTTGATGAATACATCCCACTATG						
Culex australicus	GGTGGCTG	GCAAAACATTTAAGATATCTC	CCGGGGGTTCCGGACGCC	ACTACCA	CAC	TGGTGTGAG	CTTCTACGGCACATCC			
Culex australicus K	GGTGGCTG	GCAAAACATTTAAGATATCTC	CCGGGGGTTCCGGACGCC	ACTACCA	CAC	TGGTGTGAG	CTTCTACGGCACATCC			
Culex australicus K	GGTGGCTG	GCAAAACATTTAAGATATCTC	CCGGGGGTTCCGGACGCC	ACTACCA	CAC	TGGTGTGAG	CTTCTACGGCACATCC			
Anopheles arabiensis	GGCGGCGCGTGTGCATATGACGTGCTTGGTCCCGCTCTCGGGTCCCTCGGGCG	TTTAAAGTGGACACTCTCGAGCGTATGTT								
Anopheles arabiensis	GGCGGCGCGTGTGCATATGACGTGCTTGGTCCCGCTCTCGGGTCCCTCGGGCG	TTTAAAGTGGACACTCTCGAGCGTATGTT								
Anopheles arabiensis	GGCGGCGCGTGTGCATATGACGTGCTTGGTCCCGCTCTCGGGTCCCTCGGGCG	TTTAAAGTGGACACTCTCGAGCGTATGTT								
Culex bitaeniorhynch	GGTGGCTG	GCAAAACATTTAAGATATCTC	CCGGGGGTTCCGGACGCC	ACTACCA	CAC	TGGTGTGAG	CTTCTACGGCACATCC			
Culex bitaeniorhynch	GGTGGCTG	GCAAAACATTTAAGATATCTC	CCGGGGGTTCCGGACGCC	ACTACCA	CAC	TGGTGTGAG	CTTCTACGGCACATCC			
Culex bitaeniorhynch	GGTGGCTG	GCAAAACATTTAAGATATCTC	CCGGGGGTTCCGGACGCC	ACTACCA	CAC	TGGTGTGAG	CTTCTACGGCACATCC			
Periplaneta americana	GGGGGTGGGTCCCGTCCCGAATGGTTGTGACGGCTCGGAGGTTGTCTCTCGGACGGAGACGGCTGGGCTTGAGAGACGAGTACCTATGCGGCCCGCC									



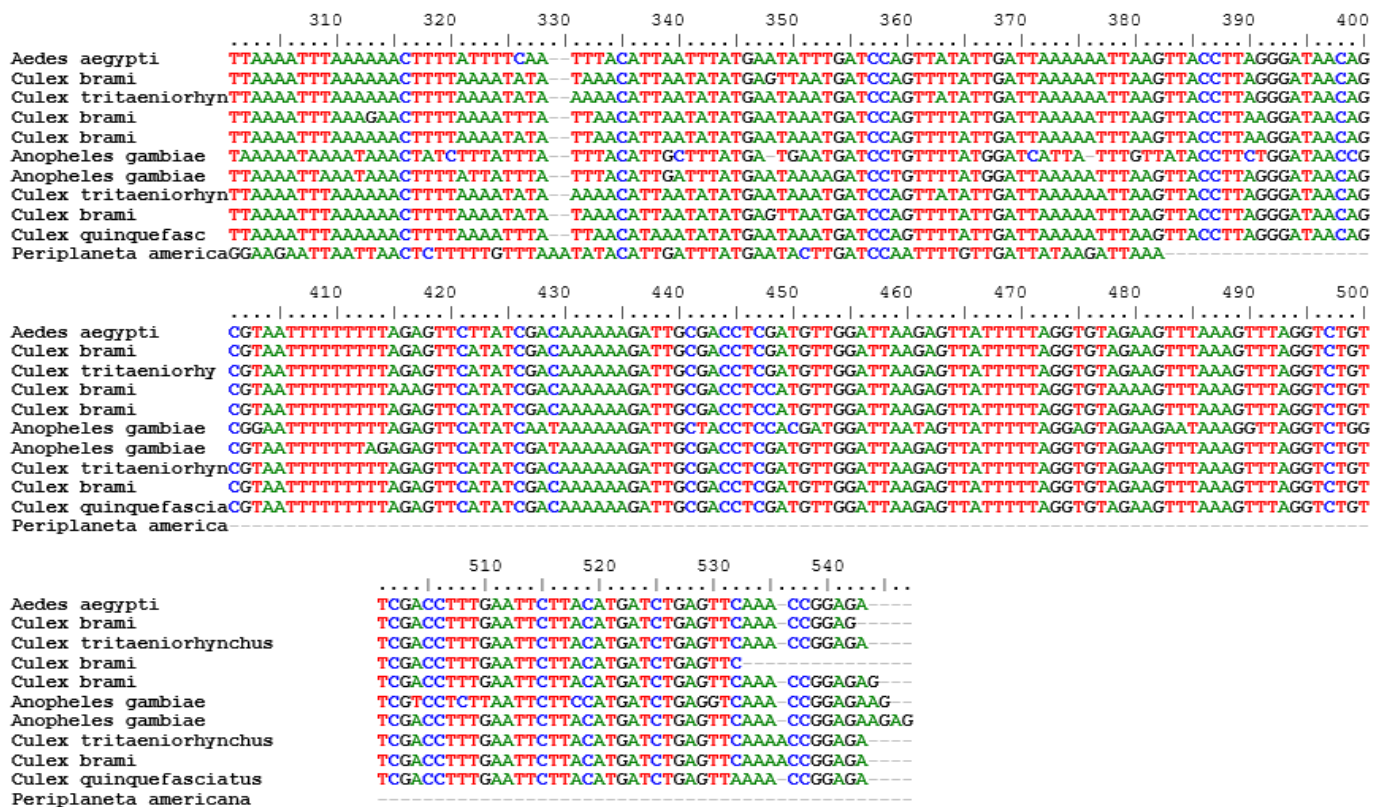


Figure 5. Multiple Sequence Alignment of mitochondrial 16S rRNA sequences of mosquito samples used for this study using the ClustalW multiple alignment feature on BioEdit

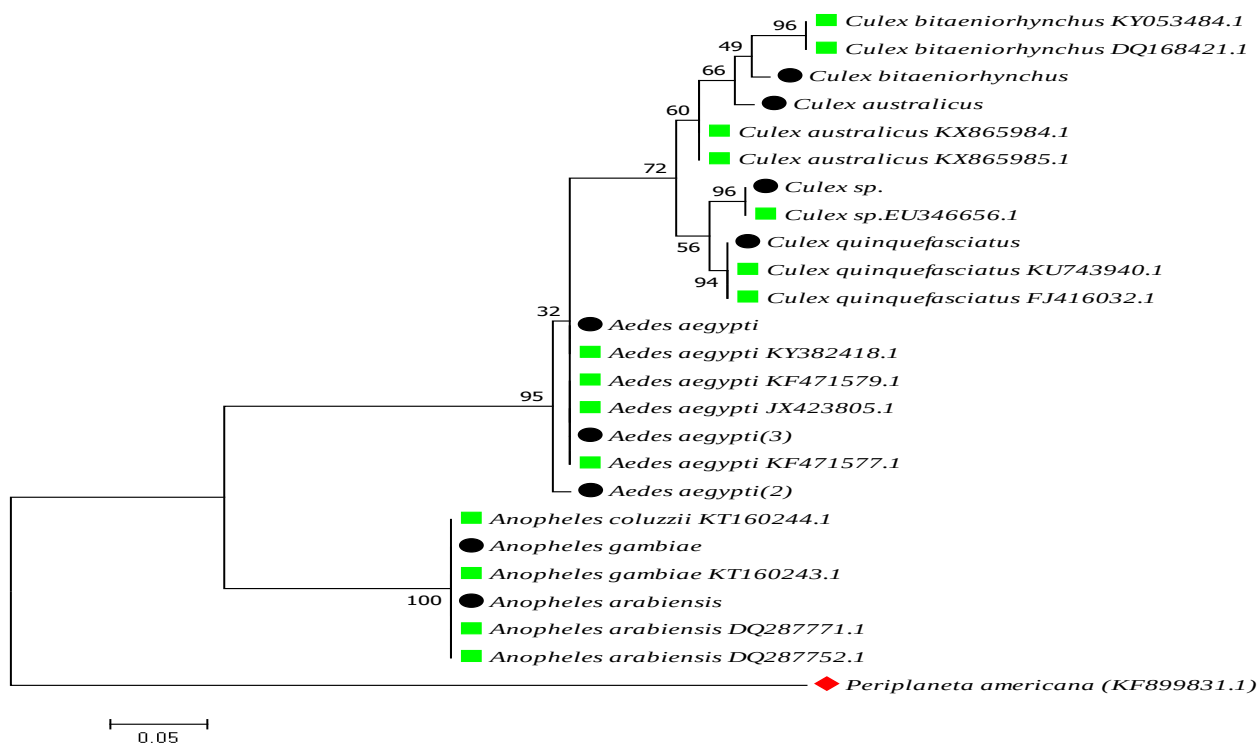


Figure 6. Molecular Phylogenetic analysis of the internal transcribed spacer 2 (ITS2) region of mosquitoes by Maximum Likelihood method inferred by Tamura-Nei method after 1000 replications

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	<i>Cx. quin</i>	This study	--																								
2	<i>Cx. quin</i>	KU743940.1	0.08	--																							
3	<i>Cx. quin</i>	FJ416032.1	0.10	0.00	--																						
4	<i>An. gam</i>	This study	0.57	0.59	0.62	--																					
5	<i>An. col</i>	KT160244.1	0.57	0.59	0.61	0.00	--																				
6	<i>An. gam</i>	KT160243.1	0.57	0.59	0.61	0.00	0.00	--																			
7	<i>Cx. sp.</i>	This study	0.26	0.19	0.21	0.54	0.54	0.54	--																		
8	<i>Cx. sp.</i>	EU346656.1	0.23	0.14	0.16	0.53	0.53	0.53	0.07	--																	
9	<i>Ae. aeg</i>	This study	0.52	0.46	0.49	0.65	0.66	0.66	0.50	0.53	--																
10	<i>Ae. aeg</i>	KY382418.1	0.31	0.30	0.33	0.61	0.62	0.62	0.29	0.30	0.13	--															
11	<i>Ae. aeg</i>	This study	0.50	0.46	0.48	0.59	0.60	0.60	0.52	0.51	0.20	0.07	--														
12	<i>Ae. aeg</i>	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	<i>Ae. aeg</i>	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	<i>Ae. aeg</i>	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	<i>Ae. aeg</i>	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	<i>Cx. aust</i>	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	<i>Cx. austr</i>	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	<i>Cx. austr</i>	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	<i>An. arab</i>	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	<i>An. arab</i>	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	<i>An. arab</i>	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	<i>Cx. bita</i>	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	<i>Cx. bita</i>	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	<i>Cx. bita</i>	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	<i>P. amer</i>	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.

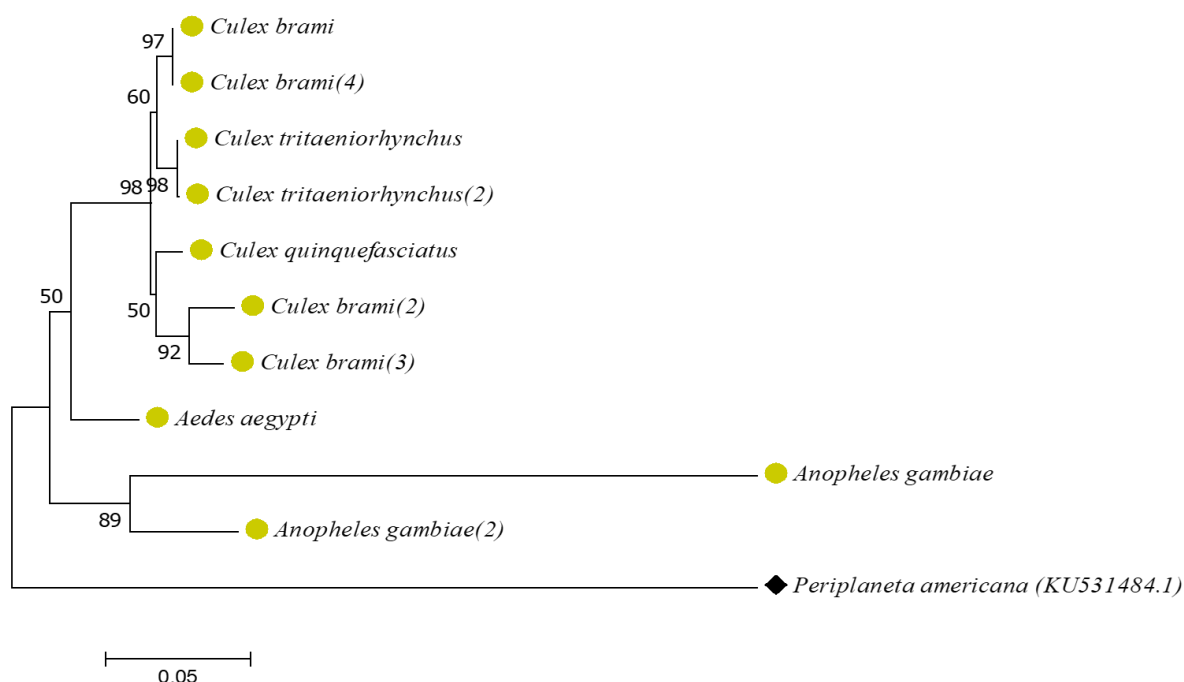


Figure 7. Evolutionary relationship in the mt16S rRNA region of mosquitoes by neighbour joining method after 1000 replications

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, overbearing 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 (Figure 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 (Figure 6). *Ae. aegypti* and *An. gambiae*. 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 *Mimomyia*. 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) (24) 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. The 16S 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 environmental 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. tritaeniorhynchus* which is similar to a study reported by Shouche and Patole (16).

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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