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

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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 family Culicidae includes the these 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 previously used for been 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, Anopheles and Culex. PCR-RFLP Aedes. 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 mosquitoes that 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 Unfortunately, Nigeria. 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.

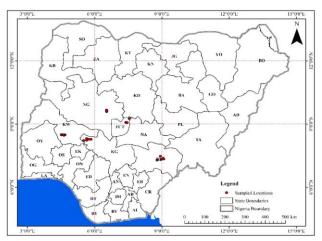


Figure 1. Map showing geographical coordinates and sample origins where mosquitoes were collected in North-central Nigeria

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° 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°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. 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 16SrRNA 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 μ L PCR mixture was prepared and it contained 14.5 μ L of nuclease free water, 5 μ L of 5X Hot FIREPol Blend Master Mix with 7.5 mM MgCl₂, 0.25 μ L of each primer and 5 μ L 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 μ L 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 16SrDNA 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 Northcentral 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)

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

 Table 1. Mosquito Populations, Geographical Locations and Geographical Coordinates

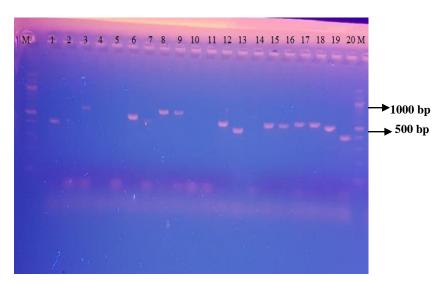


Figure 2. Amplification profile of internal transcribed spacer 2 (ITS2) region of mosquito samples 1-20. M= 100bp marker used to estimate amplicon sizes. The extracted PCR fragments were then run on 2% agarose gel using 1X TBE running buffer at 80 volts. DNA agarose gel electrophoresis lasted for 90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

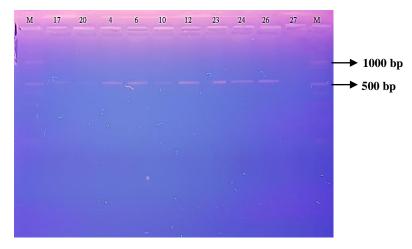


Figure 3. Amplification profile of the mitochondrial 16S rDNA region of some mosquito samples.

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.

	10	20 30	40 50	60 70 80 90 100
Culex quinquefascia			ACAAGTTGAACGCATATTGCA	
Culex quinquefascia Culex quinquefascia			ACAAGTTGAACGCATATTGCA ACAAGTTGAACGCATATTGCA	
Anopheles gambiae				CATCGTACAAC-AGTACGATGTACACATTTTTGAGT CATCGGACGTTTAATCCCGACCGATGCACACATTCTTGAGT
Anopheles coluzzii			ATAAGTTGAACGCATATGGCG	CATCGGACGTTTAATCCCGACCGATGCACACATTCTTGAGT
Anopheles gambiae				CATCGGACGTTTAATCCCGACCGATGCACACATTCTTGAGT
	TCATATGTGA-CT	GCAGGACACATGAACACCG	ACAAGTTGAACGCATATTGCA-	CATCGTACAAC-AGTACGATGTACACATTTTTGAGT
Culex sp.EU346656			ACAAGTTGAACGCATATTGCA-	
Aedes aegypti			ACACGTTGAACGCATATTGCA-	
Aedes aegypti KY38			ACACGTTGAACGCATATTGCA	
Aedes aegypti Aedes aegypti KF471			ACACGTTGAACGCATATTGCA- ACACGTTGAACGCATATTGCA-	
Aedes aegypti XF471 Aedes aegypti JX423			ACACGTTGAACGCATATTGCA	CATCGTACTACCAGTACGATGTACACATTTTTGAGT CATCGTACTACCAGTACGATGTACACATTTTTGAGT
Aedes acgypti 04425			ACACGTTGAACGCATATTGCA	
Aedes aegypti KF471			ACACGTTGAACGCATATTGCA	
Culex australicus	GATGTGA-CT	GCAGGACACATGAACACCG	ACAAGTTGAACGCATATTGCA	CATCGTACAAC-AGTACGATGTACACATTTTTGAGT
Culex australicus K			ACAAGTTGAACGCATATTGCA-	CATCGTACAAC-AGTACGATGTACACATTTTTGAGT
Culex australicus K			ACAAGTTGAACGCATATTGCA-	
Anopheles arabiensi				CATCGGACGTTTAATCCCGACCGATGCACACATTCTTGAGT
Anopheles arabiens Anopheles arabiens			ATAAGTTGAACGCATATGGCG	CATCGGACGTTTAATCCCGACCGATGCACACATTCTTGAGT CATCGGACGTTTAATCCCGACCGATGCACACATTCTTGAGT
Culex bitaeniorhync			ACAAGTTGAACGCATATGGCG-	
Culex bitaeniorhync			ACAAGTTGAACGCATATTGCA	
Culex bitaeniorhync			ACAAGTTGAACGCATATTGCA	
Periplaneta americ	T (G <mark>C</mark> AGGA <mark>CACAT</mark> GAA <mark>CATC</mark> G	ACATTTCGAACGCACATTGCG0	GTCCTTGGATTTCCAATCCCGGACCACGCCTGGCTGAGG
		120 130	140 150	160 170 180 190 200
				ACCCAAAATGGGGTTTTGCTGCCTTC
Culex quinquefasciaGCC Culex quinquefasciaGCC	TA-TATTTATC	TATTCAACTGTGCGTATTCAACTGTGCG-	-CAC-ACACGC	ACGCAAAATGGGGTTTTGCTGCCTTC
Culex quinquefasciaGCC	IA-TATTTATC IA-TATTTATC	TATTCAACTGTGCG-	-CAC-ACACGC	
Culex quinquefasciaGCC1 Culex quinquefasciaGCC1 Culex quinquefasciaGCC1 Anopheles gambiae GCC1	IA-TATTTATC IA-TATTTATC IA-TATTTATC IACTAATTACCAAA	TATTCAACTGTGCG TATTCAACTGTGCG TATTCAACTGTGCG GTCTCATTTAGTTAACTA-	-CAC-ACACGC -CAC-ACACGC	ACGCAAAATGGGGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTCCTGCCTTC
Culex quinquefasciaGCC Culex quinquefasciaGCC Culex quinquefasciaGCC Anopheles gambiae GCC Anopheles coluzzii GCC	IA-TATTTATC IA-TATTTATC IA-TATTTATC IACTAATTACCAAA IACTAATTACCAAA	TATTCAACTGTGCG TATTCAACTGTGCG TATTCAACTGTGCG GTCTCATTTAGTTAACTA GTCTCATTTAGTTAACTA	-CAC-ACACGC	ACGCAAAATGGGGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTGCTGCCTTC CGCGAGGTGCCCGGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGGTCATCCGACGCACTG
Culex quinquefasciaGCC Culex quinquefasciaGCC Culex quinquefasciaGCC Anopheles gambiae GCC Anopheles gambiae KGCC	IA-TATTTATC IA-TATTTATC IA-TATTTATC IACTAATTACCAAA IACTAATTACCAAA IACTAATTACCAAA		-CAC-ACACGC	ACGCAAAATGGGGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTGCTGCCTTC CGCGAAGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG
Culex quinquefasciaGCC Culex quinquefasciaGCC Culex quinquefasciaGCC Anopheles gambiae GCC Anopheles coluzzi GCC Anopheles gambiae KGCC Culex sp. GCC	TA-TATTTATC TA-TATTTATC TA-TATTTATC TACTAATTACCAAA TACTAATTACCAAA TACTAATTACCAAA TA-TATTTATC 	TATTCAACTGTGCG TATTCAACTGTGCG TATTCAACTGTGCG GTCTCATTTAGTTAACTA- GTCTCATTTAGTTAACTA- GTCTCATTTAGTTAACTA- AATTCAACTGTGCA-	CAC-ACACGC- CAC-ACACGC- CAC-ACACGC- CAGTGGCCGTC- CAGTGGCCGTC- CAGTGGCCGTC- CGC-ACACGCGCCCCCGCGTG	ACGCAAAATGGGGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTTGCTGCCTTC CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG
Culex quinquefasciaGCC Culex quinquefasciaGCC Culex quinquefasciaGCC Anopheles gambiae GCC Anopheles coluzzii GCC Anopheles gambiae KGCC Culex sp. GCC Culex sp.EU346656.1GCC	ГА ТАТТТАТС ГА ТАТТТАТС ГА ТАТТТАТС ГА ТАТТТАТС ГАСТААТТАССААА ГАСТААТТАССААА ГАСТААТТАССААА ГА ТАТТТАТС ГА ТАТТТАТС	TATTCAACTGTGCG TATTCAACTGTGCG TATTCAACTGTGCG GTCTCATTTAGTTAACTA GTCTCATTTAGTTAACTA GTCTCATTTAGTTAACTA GTCTCATTTAGTGTGCA AATTCAACTGTGCA	CAC-ACACGC CAC-ACACGC CAC-ACACGC CAGTGGCCGTC CAGTGGCCGTC CAGTGGCCGTC CGC-ACACGCGCCCCGCGTG CGC-ACACGCGCCCCGCGTG	ACGCAAAATGGGGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTGCTGCCTTC CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATTCCGACGCACTG TGGGTGTCCTGCCCGCCACGCACTG
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Culex quinquefasciaGCC Culex quinquefasciaGCC Anopheles gambiae GCC Anopheles coluzzii GCC Anopheles gambiae KGCC Culex sp. GCC Culex sp. GCC Aedes aegypti KY382GCC	ГА - ТАТТТАТС ГА - ТАТТТАТС ГАСТААТТАССААА ГАСТААТТАССААА ГАСТААТТАССААА ГА - ТАТТТАТС	TATTCAACTGTGCG TATTCAACTGTGCG TATTCAACTGTGCG GTCTCATTTAGTTAACTA GTCTCATTTAGTTAACTA GTCTCATTTAGTTAACTA AATTCAACTGTGCA AATTCAACTGTGCA CATTCAACTATACG CATTCAACTATACG	CAC-ACACGC CAC-ACACGC CAC-ACACGC CAGTGGCCGTC CAGTGGCCGTC CAGTGGCCGTC CGC-ACACGCGCCCCGCGTG CGC-ACACGCGCCCCGCGTG CGCCGCCCGC CGCCGCCCGC	ACGCAGAATGGGGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTGCTGCCTTC CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG TGGGTGTCGTGCACGCAGCATGGTGTTTTGCTGCCTTA GGGGATGATGAGAGAATGATGTTTTCCCTGCCTTC GGGGATGATGCGTAGTGATGTTTTCCCTGCCTTC
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Culex quinquefasciaGCC Culex quinquefasciaGCC Culex quinquefasciaGCC Anopheles gambiae GCC Anopheles gambiae GCC Anopheles gambiae KGCC Culex sp. GCC Culex sp. GCC Culex sp. EU346656.1GCC Aedes aegypti KY382GCC Aedes aegypti KY382GCC Aedes aegypti KF471GCC Calex australicus GCC Aedes aegypti KF471GCC Culex australicus KGCC Culex australicus KGCC Anopheles arabiensiGCC Anopheles arabiensiGCC	TATTTATC TATTTATC TATTTATC TATTTATC TACTAATTACCAAA TACTAATTACCAAA TACTAATTACCAAA TATTTATC	TATTCAACTGTGCG TATTCAACTGTGCG TATTCAACTGTGCG GTCTCATTTAGTTAACTA GTCTCATTTAGTTAACTA AATTCAACTGTGACA AATTCAACTGTGCA CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG GTCTCATTTAGTTAACTA GTCTCATTTAGTTAACTA	- CAC - ACACGC	ACGCAAAATGGGGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTGCTGCCTTC CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG TGGTGTCCTGCACGCACCATGGTGTTTTCCTGCCTTA GGGGATGATGAGAGAATGATGTTTTCCCTGCCTTA GGGGCGATGCGTAGCGGTGATGTTTTCCCGCCTTC GGCGCGTATGCGTGGTGATGTTTTCCCGCCTTC GGCGCGTATGCGTGGTGATGTTTTCCCGCCTTC GGCGCGTATGCGTGGTGATGTTTTCCCGCCTTC GGCGCGTATGCGTGGTGATGTTTTCCCGCCTTC GGCGCGTATGCGTGGTGATGTTTTCCCGCCTTC GGCGCGCTATGCGTGGTGATGTTTTCCCGCCTTC GGCGCGTATGCGTGGTGATGTTTTCCCGCCTTC CGCGCGCTATGCGTGGTGATGTTTTCCCGCCCTTC CGCGCGCTATGCGTGGTGATGTTTTCCCGCCCTTC CGCGCGCGTATGCGTGGTGATGTTTTCCCGCCCTTC CGCGCGCGTATGCGTGGTGATGTTTTCCCGCCCTTC CGCGCGCGTATGCGTGGTGATGTTTCCCGCCCTTC CGCGCGCGTGTGCTCCGGGTCATCCGACGCACTG CGCGGAAGGTGTCCCGGGTCATCCGACGCACTG CGCGAAGGTGTCCGGGTCATCCGACGCACTG
Culex quinquefasciaGCCT Culex quinquefasciaGCCT Culex quinquefasciaGCCT Anopheles gambiae GCCT Anopheles gambiae KGCCT Culex sp. EU346656.1GCCT Culex sp.EU346656.1GCCT Aedes aegypti GCCT Aedes aegypti GCCT Aedes aegypti KF471GCCT Aedes aegypti KF471GCCT Aedes aegypti KF471GCCT Culex australicus GCCT Culex australicus GCCT Culex australicus KGCCT Anopheles arabiensiGCCT Anopheles arabiensiGCCT Anopheles arabiensiGCCT Culex bitaeniorhyncGCCT	TATTTATC TATTTATC TATTTATC TATTTATC TACTAATTACCAAA TACTAATTACCAAA TACTAATTACCAAA TATTTACCAAA TATTTACCAAA TATTTACCAAA TATTTACCAAA TATTTACCAAA TATTTACC TATTTATC	TATTCAACTGTGCG TATTCAACTGTGCG TATTCAACTGTGCG GTCTCATTTAGTTAACTA GTCTCATTTAGTTAACTA AATTCAACTGTGCA AATTCAACTGTGCA CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTGTGCA TATTCAACTGTGCG TATTCAACTGTGCG GTCTCATTTAGTTAACTA GTCTCATTTAGTTAACTA	CAC-ACACGC CAC-ACACGC CAC-ACACGC CAGTGGCCGTC CAGTGGCCGTC CGC-ACACGCGCCCCGCGTG CGC-ACACGCGCCCCGCGTG CGCCGCCCGC CGCCGCCCGC CGCCGCCCGC	ACGCAAAATGGGGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTGCTGCCTTC CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG TGGGTGTCGTGCACGCAGCAGCGACTG GGGGTGATGCACGCAGCATGGTGTTTTGCTGCCTTA GGGGCGGATGATGGAGGTAGTGTTTTCCCGCCTTA GGGGCGGATGCTGGTGGTGATGTTTTCCCGCCTTC GGCGCGATGCTGGTGGTGATGTTTTCCCGCCTTC GGCGCGATGCGGGTGATGGTGTTTTCCCGCCTTC GGCGCGATGCGGGGTGATGTTTTCCCGCCTTC GGCGCGATGCGGGGTGATGTTTTCCCGCCTTC GGCGCGATGCGGGGGAGGTGATGTTTTCCCGCCTTC GGCGCGATGCGTGGTGATGTTTTCCCGCCTTC GGCGCGATGCGTGGTGATGTTTTCCCGCCTTC GGCGCGATGCGTGGTGATGTTTTCCCGCCTTC CGCGCGATGCGTGGTGATGTTTTCCCGCCTTC CGCGCGATGCGTGGTGATGTTTCCCGCCTTC CGCGCGAAGGTGTCCGGGTCATCCGACGCACTG CGCGGAAGGTGTCCGGGTCATCCGACGCACTG CGCGAAGGTGTCCGGGTCATCCGACGCACTG CGCGAAGGTGTCCGGGTCATCCGACGCACTG CGCGAAGGTGTCCGGGTCATCCGACGCACTG CGCGAAGGTGTCCGGGTCATCCGACGCACTG CGCGAAGGTGTCCGGGTCATCCGACGCACTG CGCGAAGGTGTCCGGGTCATCCGACGCACTG
Culex quinquefasciaGCCT Culex quinquefasciaGCCT Culex quinquefasciaGCCT Anopheles gambiae GCCT Anopheles coluzzii GCCT Anopheles gambiae KGCCT Culex sp. GCCT Culex sp. GCCT Aedes aegypti KY382GCCT Aedes aegypti KY382GCCT Aedes aegypti KF471GCCT Aedes aegypti KF471GCCT Aedes aegypti KF471GCCT Culex australicus KGCCT Culex australicus KGCCT Culex australicus KGCCT Culex australicus KGCCT Anopheles arabiensiGCCT Anopheles arabiensiGCCT Culex bitaeniorhyncGCCT	TATTTATC TATTTATC TATTTATC TATTATC TACTAATTACCAAA TACTAATTACCAAA TACTAATTACCAAA TATTTACCAAA TATTTACCAAA TATTTACCAAA TATTTACC TATTTATC	TATTCAACTGTGCG TATTCAACTGTGCG TATTCAACTGTGCG GTCTCATTTAGTTAACTA- GTCTCATTTAGTTAACTA- AATTCAACTGTGCA- AATTCAACTGTGCA- CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG TATTCAACTGTGCG	-CAC-ACACGC	ACGCAAATGGGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTGCTGCCTTC CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG GGGGTGTCGTGCACGCAGCATGGTGTTTTGCTGCCTTA GGGATGATGAGCAGCATGGTGTTTTCCCGCCTTA GGGGCGGATGCGTAGTGATGTTTTCCCGCCTTC GGCGCGATGCGTAGTGATGTTTTCCCGCCTTC GGCGCGATGCGTGGTGATGTTTTCCCGCCTTC GGCCGCATGCGTAGTGGTGATGTTTTCCCGCCTTC GGCCGCATGCGTGGTGATGTTTTCCCGCCTTC GGCCGCATGCGTGGTGATGTTTTCCCGCCTTC GGCCGCATGCGTGGTGATGTTTTCCCGCCTTC GGCCGCATGCGTGGTGATGTTTTCCCGCCTTC GGCCGCGATGCGTGGTGATGTTTTCCCGCCTTC GGCCGCGATGCGTGGTGATGTTTTCCCGCCTTC GGCCGCGATGCGTGGTGGTGATGTTTTCCCGCCTTC CGCGCGCGTATGCGTGGTGATGTTTTCCCGCCTTC CGCGCGCGTCGGGTGGTGATCGTTTCCCGCCTTC CGCGCCGCTTCCGGGTCATCCGACGCACTG CCCGCAAGGTGTCCGGGTCATCCGACGCACTG CGCGCAAGGTGTCCGGGTCATCCGACGCACTG CGCGCAAGGTGTCCGGGTCATCCGACGCACTG TGTGCACGCAGCATGGTGTTTTGCTGCCTTA TATACACGCAGCATGGTGTTTTGCTGCCTTA
Culex quinquefasciaGCC Culex quinquefasciaGCC Culex quinquefasciaGCC Anopheles gambiae GCC Anopheles gambiae GCC Anopheles gambiae KGCC Culex sp. GCC Culex sp. GCC Culex sp. EU346656.1GCC Aedes aegypti KY382GCC Aedes aegypti KY32GCC Aedes aegypti KF471GCC Calex australicus GCC Culex australicus KGCC Culex australicus KGCC Culex australicus KGCC Anopheles arabiensiGCC Anopheles arabiensiGCC Culex bitaeniorhyncGCC Culex bitaeniorhyncGCC	TATTTATC TATTTATC TATTTATC TATTTATC TACTAATTACCAAA TACTAATTACCAAA TACTAATTACCAAA TATTTATC TATTTACCAAA TATTTACCAAA TATTTACCAAA TATTTATC	TATTCAACTGTGCG TATTCAACTGTGCG TATTCAACTGTGCG GTCTCATTTAGTTAACTA GTCTCATTTAGTTAACTA AATTCAACTGTGACA AATTCAACTGTGCA AATTCAACTGTGCA CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTATACG CATTCAACTGTGCGA TATTCAACTGTGCG TATTCAACTGTGCG GTCTCATTTAGTTAACTA GTCTCATTTAGTTAACTA TATTCAACTGTGCA TATTCAACTGTGCA TATTCAACTGTGCA TATTCAACTGTGCA TATTCAACTGTGCA TATTCAACTGTGCA TATTCAACTGTGCA TATTCAACTGTGCA TATTCAACTGTGCA TATTCAACTGTGCA	- CAC - ACACGC	ACGCAAAATGGGGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTGCTGCCTTC ACGCAGAATGGTGTTTTGCTGCCTTC CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG CGCGAAGGTGCCCGGGTCATCCGACGCACTG TGGGTGTCGTGCACGCAGCAGCGACTG GGGGTGATGCACGCAGCATGGTGTTTTGCTGCCTTA GGGGCGGATGATGGAGGTAGTGTTTTCCCGCCTTA GGGGCGGATGCTGGTGGTGATGTTTTCCCGCCTTC GGCGCGATGCTGGTGGTGATGTTTTCCCGCCTTC GGCGCGATGCGGGTGATGGTGTTTTCCCGCCTTC GGCGCGATGCGGGGTGATGTTTTCCCGCCTTC GGCGCGATGCGGGGTGATGTTTTCCCGCCTTC GGCGCGATGCGGGGGTGATGTTTTCCCGCCTTC GGCGCGATGCGTGGTGATGTTTTCCCGCCTTC GGCGCGATGCGTGGTGATGTTTTCCCGCCTTC GGCGCGATGCGTGGTGATGTTTTCCCGCCTTC CGCGCGATGCGTGGTGATGTTTTCCCGCCTTC CGCGCGATGCGTGGTGATGTTTTCCCGCCTTC CGCGCGATGCGTGGTGATGTTTCCCGCCTTC CGCGCGAAGGTGTCCGGGTCATCCGACGCACTG CGCGGAAGGTGTCCGGGTCATCCGACGCACTG CGCGAAGGTGTCCGGGTCATCCGACGCACTG CGCGAAGGTGTCCGGGTCATCCGACGCACTG CGCGAAGGTGTCCGGGTCATCCGACGCACTG CGCGAAGGTGTCCGGGTCATCCGACGCACTG CGCGAAGGTGTCCGGGTCATCCGACGCACTG CGCGAAGGTGTCCGGGTCATCCGACGCACTG

	210	220 2	30 2	40	250	260	270	280	290	300
										1
Culex quinquefasciGG	GGGGTGGC	AAAACATTCAA							GCCACTGA/	CGGAC
		AAAACATTCAA								
		AAAACATTCAA								
	GGTCGCTGTGC									
	GGTCGCTGTGC									
	GGTCGCTGTGC									
		AAAACATTTAA						GAGCTT	CCTACGC2	CATCC
Culex sp. EU346656 GG		AAAACATTTAA						GCGCTT		
		ACATTATTGAA			TGAGGCCC			AACACCCCAC		
		AAAACATTGAA						TACATCCCAC		
		AAAATATTGAA			GGGGGACC			TACATCCCAC		TTCCC
541		AAAACATTGAA			TGGTGACA			TACATCCCAC		
		AAAACATTGAA			TGGTGACA			TACATCCCAC		
		AAAACATTGAA			GGTGGAGACA			CACCCCCAC		
Aedes aegypti KF471AG		AAAACATTGAA			TGTGGTGACA			TACATCCCAC		
Culex australicus	100000 01			0400100	1010010404		OTTOATOAA	IACATOCCAC	171000000	~1000
Culex australicus K										
Anopheles arabiens GG	CCCCCCTCTC	ATGATGACGTG	CTTGGTCCCC	CTCTCCCCC	TCCTCGGGCG	TTGAAAGTO	GACACTCTC	CACCGTATCT	TGGATC	CGTTT
	GGCCGCTGTGC									CGTTT
-	GGCCGCTGTGC									CGTTT
		AAAACATTCAA				ACAC				
		AAAACATTTAA			TTCGGGTGCG			GCG		
		AAAACATTTAA			TTCGGGTGCG		ATCACTGGC			CGAC-
Periplaneta americ GGGGG										

310 320 330 340 350 360 370 380 390 400
Culex quinquefasciaGACGACGACGAGAGAATAAATCCCTC-CCCCCCCCCCCTGGGTGGCCGATGTATTATCTTTCTCT
Culex quinquefasciaGACGACGGTGAGAATACATCCCAC-ACACCCAGCTGGGCTTGGGCGCCGATGTA
Culex quinquefasciaGACGACGGTGAGAATACATCCCAC-ACACCAACCTGGCTTGGGCGCCGATGTA
Anopheles gambiae
Anopheles coluzzii
Anopheles gambiae K
Culex sp. GCACACGGCGAGAATACGTCCCACTGTACCTTGCTGGATTGTACAACAATGTAACCTCTCTGCCGCGGGTGATGCAGCCACACACACACGTACATA-
Culex sp.EU346656.1GCACACGGCGAGAATACATCCCACTGTACCAGCCTGGCTTGGACGACGATGTAAACTCTCTGCCGGGATGATGCAGCCACGTACACGTAC
Aedes aegypti CCTCGTGGTGTGGG-TATATCATATTCTACAAAAAAAATCACAATATTTGTCTTAAAAATATGGTGTGACACCCCCCCAACACTTCATATTATAAA
Aedes aegypti KY382
Aedes aegypti TCGCCTGGTGGG-GATTCCATCTTTCACTAACTAACTCCCCTATAGAAGGCCTCAAATAAGGGTGTGACTACCCCCTAAATTAAAGAATAATA-
Aedes aegypti KF471TCGCCTTGTGTTG TATTCCATCATCACTAAC TAACTCCCTATAGTAGGCCTCAAATAATGTGTGGACTACCCCCTAAATTAAG Aedes aegypti JX423TCGCCTTGTGTTG TATTCCATCACTAAC TAACTCCCCTATAGTAGGCCTCAAATAATGTGTGGACTACCCCCTAAATTAAGCATAT
Aedes aegypti JX423TCGCCTTGTGTTG-TATTCCATCATTCACTAACTAACTCCCTATAGTAGGCCTCAAATAATGTGTGACTACCCCCTAAATTTAAGCATAT Aedes aegypti TCCTCTTGTGTTG-TATCCCATTATTCACTAACCTCCCCACCTACCTCTCGAATAGGGGTCGAATAACGCCTAAATACCGACTTTATTCCAGCAT
Aedes aegypti Kr471TCGCCTTGTGTG-TATTCCATACTACTACTACTACTACTACTCTCTGTATAGGCCTAATAC
Culex australicus K
Anopheles arabien CGTGCTGGCGGGGGGGTCTGATGAGTATGCATTGTGGGTGTGTGAGAGCCGCCATGACTCGAACTAATGCTACGTCATGCACAATGGCCGCCCAGAGCCTACTC
Anopheles arabiens CGTGTTGGTGGTGTGTGTGCGTAGGGCTTGTGGGTGTGTGT
Anopheles arabiensicGTGTTGGCGGGGGGTGTTGGGGGCTAGGGGCTTGGGGGGGG
Culex bitaeniorhyncGCGAGAATACATCCCACAGCACCAGCCTGGCTTGGGCGTCGATGTAAACTCTCTCGGCCTTTGTGCGTGTCCACTCAACG
Culex bitaeniorhyncGTGAGAATACATCCCACA-CACCAACCTGGCTTGGGCGCCGATGTAAGCTCTCCAGTCATCCGTTGTCGCGGGTGCCGCGTTGACACCCACC
Culex bitaeniorhyncGTGAGAATACATCCCACA-CACCAACCTGGCTTGGGCGCCGATGTAAGCTCTCAGTCATCCGTTGTCGCGGGTGCCGCGGTGACACCCACC
Periplaneta americ GCCCAGCACGGCTCGTCCCGCCAGCGACCTCTTTTGCGACCTGTTCTCTAAAACCCCGACCTCAGATCAGGCGAGACTACCCGCCTGAATTTAAGCATATCA

	410	420	430	440	450	460
Culex quinquefasciatus						
Culex guinguefasciatus KU74394						
Culex guinguefasciatus FJ41603						
Anopheles gambiae						
Anopheles coluzzii KT160244.1						
Anopheles gambiae KT160243.1						
Culex sp.						
Culex sp.EU346656.1						
Aedes aegypti	AAGATAAG					
Aedes aegypti KY382418.1						
Aedes aegypti						
Aedes aegypti KF471579.1						
Aedes aegypti JX423805.1						
Aedes aegypti						
Aedes aegypti KF471577.1						
Culex australicus						
Culex australicus KX865985.1						
Culex australicus KX865984.1						
Anopheles arabiensis	TGACCGGTACACCT	TCGTCGACGAG	CGATGCAGTT	AACTAAATGA	GACTTTCTCA	ATTA
Anopheles arabiensis DQ287771.	T					
Anopheles arabiensis DQ287752.	T					
Culex bitaeniorhynchus	GGAAGCTCTCTCCA	TATATGTA	GGCCTCAAAT	AATGTGTGAC	TACCCCCTAA	ATTTAAACATATA
Culex bitaeniorhynchus KY05348	AGAACCGTCTATAG	TATACCATGTA	GGCCTCAAAT	AATGTGTGAC	TACCCCCTAA	ATTTAAGCAT
Culex bitaeniorhynchus DQ16842	AGAACCGTCAATAG	TACCATGTA	GGCCTCAAAT	AATGTGTGAC	TACCCCCTAA	-TTTAAGCATA
Periplaneta americana (KF89983	ATAA					

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

	10	20	30	40	50	60	70	80	90	100
Aedes aegypti Culex brami									CATAATCAGT/ CATAATCACT/	
Culex brami Culex tritaeniorhy									CATAATCACT	
Culex brami									CATAATCATT	
Culex brami									CATAATCACT/	
Anopheles gambiae			CCCTGGG	CACCTGATAA	AA-ATATAC	GGGCGCGGCA	TATTTTCCCT	GCGCGAGT G	CATATTCTCAC	TACTT
Anopheles gambiae									CATAATCAAT/	
Culex tritaeniorhy	n								CATAATCACT/	
Culex brami									CATAATCACT/	
Culex quinquefasci									CATAATCACT/	
Periplaneta americ	ana CTTTTCTTGAA	TTTTAATATG	AGATATGACC	TGCCCACTGA	TAGATTGAAG	GGCCGCGGTA	TTTTGACCGT	GCAAAGGTAG	CATAATCATT/	GTCTT
	110	120	130	140	150	160	170	180	190	200
Aedes aegypti	TTAATTGAAGGCTT									
Culex brami	TTAATTGGAGGCTT									
Culex tritaeniorhy	nTTAATTGGAGGCTT	GTATGAATGG	TTGAATGAG	ATATATACTG	TCTTTTTAA	AATTATATAG	AATTTTATTT	TTTAATTAAA	AAGTTAAAAT(GAAATT
Culex brami	TTAATTGGAGGCTT	GTATGAATGG	TTGAATGAG	ATATATACTG	GCTTTTTTAA	AATTGTATAG	AATTTTATTT	TTTAATTAAA	AAGTTAAAAT/	AAATT
Culex brami	TTAATTGGAGGGTT									
Anopheles gambiae	TTTTTGAGGCTG									
Anopheles gambiae	TTAATTGAAGGCTG									
Culex tritaeniorhy Culex brami	TTAATTGGAGGCT1 TTAATTGGAGGCT1									
Culex guinguefasci										
Periplaneta american										
iciipidicoli dilettodi										
	210	220	230	240	250	260	270	280	290	300
Aedes aegypti	AAAAGACGAGAAGA									
Culex brami	AAAGGACGAGAAGA									
Culex tritaeniorhy										
Culex brami Culex brami	AAAGGACGAAAAGA AAAGGACGAAAAAGA								TTTTATTGGGG	
Anopheles gambiae	ATTAGACGACAAGA									
Anopheles gambiae	AAAAGACGACAAGA									
Culex tritaeniorhy										
Culex brami	AAAGGACGAGAAGA									
Culex quinquefasci										
Periplaneta american										

2	0	2	0
	U		U

	310	320	330	340	350	360	370	380	390	400
Aedes aegypti	TTAAAATTTAAA									
Culex brami	TTAAAATTTAAA									
Culex tritaeniorhy										
Culex brami Culex brami	TTAAAATTTAAA									
Anopheles gambiae	TTAAAATTTAAA TAAAAATAAAAT									
Anopheles gambiae	TTAAAAATAAAAT									
Culex tritaeniorhy										
Culex brami	TTAAAATTTAAA									
Culex guinguefasc										
Periplaneta americ										
-										
	410	420	430	440	450	460	470	480	490	500
Aedes aegypti	CGTAATTTTTTT									
Culex brami	CGTAATTTTTTT									
Culex tritaeniorhy										
Culex brami	CGTAATTTTTTT									
Culex brami Anopheles gambiae	CGTAATTTTTTT CGGAATTTTTTT									
Anopheles gambiae	CGTAATTTTTTT									
Culex tritaeniorhy										
Culex brami	CGTAATTTTTTT									
Culex guinguefasci										
Periplaneta americ										
-										
		510	520) 53(D 540	0				
			.	.	.					
Aedes aegypti		TCGACCTTTG/	ATTCTTACAT	GATCTGAGT	CAAA-CCGG	AGA				
Culex brami		TCGACCTTTG/								
Culex tritaeniorhy	nchus	TCGACCTTTG/				AGA				
Culex brami		TCGACCTTTG/								
Culex brami		TCGACCTTTG/								
Anopheles gambiae		TCGTCCTCTT/								
Anopheles gambiae		TCGACCTTTG/								
Culex tritaeniorhy	ncnus	TCGACCTTTG/								
Culex brami	-+	TCGACCTTTG/ TCGACCTTTG/								
Culex quinquefasci										
Periplaneta americ	ana									

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

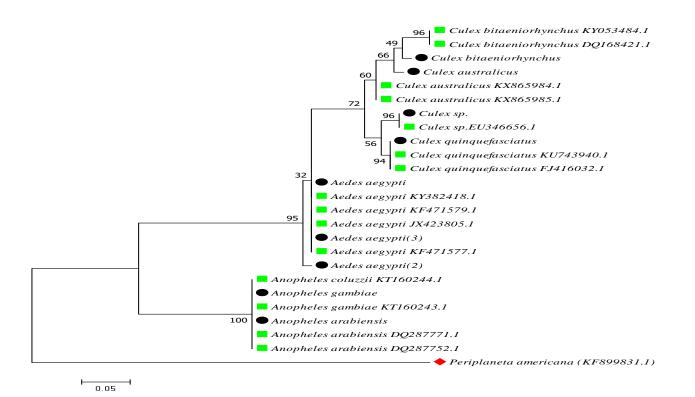


Figure 6. Molecular Phylogenetic analysis of the internal transcribed spacer 2 (ITS2) region of mosquitoes by Maximum Likelihood method inferred by Tamura-Nei method after 1000 replications.

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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	

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

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.

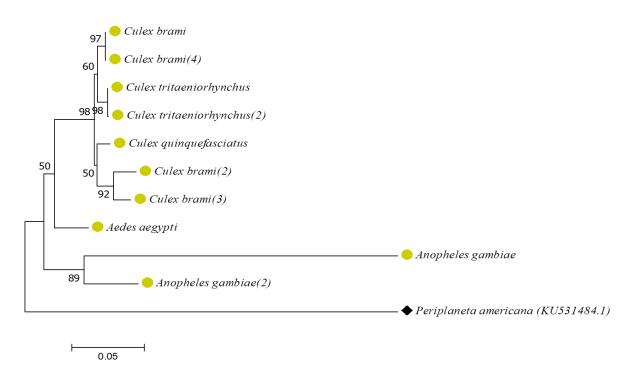


Figure 7. Evolutionary relationship in the mt16S rRNA region of mosquitoes by neighbour joining method after 1000 replications

S/NO	0	Accession	1	2	2	4	E	(7	0	0	10	11	10	12
S/NO	Organism	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	

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

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

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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 abundance over Anopheles higher species 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 (Fig 6). Similar trends were observed in the ITS2 DNA sequences of An. gambiae, An. arabiensis, Cx quinquefasciatus and Culex SD. 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

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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 anv 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 differentiating members of in 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 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 diverge quinquefasciatus from Cx. 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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Not applicable.

Consent for publication

Not applicable.

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