Molecular Characterization of *Anopheles gambiae s.l.* from the Three Vegetation Zones in Rivers State, Nigeria

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Abstract
Understanding the genetic structure of mosquito populations is important for addressing important biological and public health issues such as evolution, spread of insecticide resistance alleles and epidemiology of vector-borne diseases. *Anopheles gambiae s.l.* is comprised of genetically distinct species that are morphologically indistinguishable. This study therefore aims to establish the sibling species and molecular characterization of *Anopheles gambiae s.l.* responsible for transmitting malaria parasites in the three ecological zones. Sample collection was carried out during the rainy season from May to December which coincides with the peak period of vector abundance. Adults of laboratory-reared, field-collected larvae of this test-mosquitoes were used for this study. To characterize the vectors, DNA was extracted from tissues of *An. gambiae s.l.* samples, amplified and sibling species identified by multiplex polymerase chain reaction (PCR). Restricted fragment length polymorphism (RFLP) aided to identify the M (*An. coluzzi)* and S (*An. gambiae s.s.)* forms. Neighbor-Joining method was used to infer the evolutionary history. *An. gambiae s.s.* (sibling species) is present in the entire Rivers state while *An. gambiae s.s.* (S-form) predominates the lowland forest vegetation and *An. coluzzi* predominates the mangrove and fresh water swamp vegetations. The sequenced samples from the three vegetations were closely related to *An. gambiae* with accession number KP165373.1. The study makes available baseline information on characterization and monitoring of species of malaria vectors in Rivers State, Nigeria.
Keywords: Molecular characterization, *Anopheles gambiae,* vegetation, Rivers State.
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1. Introduction
Global malaria deaths, mostly impacting young children and pregnant women, fell slightly in 2021 but remain at more than half a million every year and have still not recovered to covid-19 pre-pandemic levels. These latest findings demonstrate that – now more than ever – the need to renew momentum in the fight against one of the world's oldest and deadliest enemies (WHO, 2022).

   Anopheles gambiae sensu lato (*An. gambiae s.l.*) occurs widely throughout southern, eastern, and central Africa. The *An. gambiae s.l.* is currently comprised of eight sibling species namely *An. gambiae sensu stricto (s.s.)* (Giles 1902), *An. arabiensis* (Patton 1905), *An. quadriannulatus* (Theobald 1911), *An. amharicus* (Coetzee *et al.,* 2013). *An. bwambae* White, 1985, *An. melas* (Theobald 1903), *An. merus* (Dönitz 1902) and *An. coluzzi* (Coetzee *et al.,* 2013). Analysis has shown that five of the species in the *An. gambiae s.l.* (*An. gambiae s.s.*, *An. arabiensis*, *An. quadriannulatus, An. merus,* and *An. melas*) differ sufficiently in the intergenic spacer (IGS) regions of their ribosomal deoxyribonucleic acid (rDNA) to be distinguished from each other by species-specific

restriction fragment length polymorphisms (RFLP) (Collins *et al.,* 1988). Members of *An. gambiae s.l.* have been identified as major vectors of human malaria parasites in the African continent. They are comprised of genetically and behaviourally distinct species that are morphologically indistinguishable (Davidson *et al.,* 1967; Service, 1985; Hunt *et al.,* 1998). Thus, identification of the sibling species and molecular forms using molecular methods can have important implications in subsequent planning and implementation of the most appropriate vector control measures (Coetzee *et al.,* 2000; Fanello *et al.,* 2002; Koekemoer *et al.,* 2002). The principal Anopheles mosquito sibling species and molecular forms within the *An. gambiae s.l.* is responsible for transmission of malaria parasites (Kabbale *et al.,* 2016). Identification of species within the *An. gambiae s.l.* is essential for the correct evaluation of malaria vector ecology studies and control programs (Gale & Crampton, 1987). Understanding the genetic structure of mosquito populations is important for addressing important biological and public health issues such as evolution, spread of insecticide resistance alleles and epidemiology of vector-borne diseases (Tripet *et al.,* 2001; Fanello *et al.,* 2003).

Several methods for identifying species of mosquito complexes have been developed such as the molecular investigation involving identification of members of the *An. gambiae s.l.* using polymerase chain reaction (PCR) techniques which is based on DNA specific nucleotide differences. This has become the standard method for species identification and studying the genetic structure (Scott *et al.,* 1993; Wilkins *et al.,* 2006; Hamza *et al.,* 2014). The importance of *An. gambiae s.l.* stems from the fact that it is highly anthropophilic and has the capacity to exploit different kinds of habitats that are created either directly or indirectly by humans; ranging from temporary sunlit pools, water collected in depressed soil by livestock and in ditches resulting from construction, to permanent water bodies in rice fields and irrigation canals. This adaptive flexibility in exploiting different larval habitats is evidenced by its wide geographical distribution and its occurrence in a variety of micro and macro environmental conditions throughout tropical Africa (Lanzaro *et al.,* 1998; Budiansky, 2002). The key to successful vector control measures is knowledge of the local vector populations, the species identity, role in transmission and the susceptibility of the local mosquitoes to insecticides used to control them. Baseline surveys to collect this information need to be carried out prior to implementation of malaria vector control interventions and during on-going susceptibility surveys in order to guard against the development of increasing insecticide resistance. This is becoming more important as insecticide resistance increases and spreads across Africa (Nwankwo *et al.,* 2017; Ekerette and Ebere, 2022a; 2022b).

2. Materials and methods

2.1 Study Area

Rivers State lies between latitude 4′45″N and longitude 6′50″E and is a predominantly low-lying pluvial state in southern Nigeria, located in the eastern part of the Niger Delta on the ocean ward extension of the Benue Trough. The inland part of the state consists of tropical rainforest, and towards the coast, the typical Niger Delta environment features many mangrove swamps. Rivers State has a total area of 11,077 km^2. It has a population of about 5,198,716 as of the 2006 census. The population grew to 7,303,900 with a population density of 755.4/km^2 as of 2016 estimate, with an annual population change of 3.5% from 2006-2016. Population estimate in the vegetations in 2016 were: lowland forest - 3131600, mangrove - 2,504,900, and fresh water swamp – 1,378,700 (RVSG, 2021). In the south, it is bounded by the Atlantic Ocean. Its topography ranges from flat plains, with a network of rivers and tributaries. Average temperatures are typically between 25 °C and 28 °C. Some parts of the state still receive up to 150 mm (6 in) of rainfall during the dry period. Relative humidity rarely dips below 60% and fluctuates between 90% and 100% for most of the year (RVSG, 2021). The land surface of Rivers State can be divided into three zones: freshwater swamps, mangrove swamps and coastal sand ridges. The freshwater zone extends northwards from the mangrove swamps. This land surface is generally less than 20 m above sea level. As a lower Niger floodplain, it contains a greater silt and clay foundation and is more susceptible to perennial inundation by river floods. The floodplain's total thickness rises to about 45 m in the northeast and over 9 m in the beach ridge barrier zones to the southwest. On coastal sand ridges, the soils are mostly sandy or sandy loams. Various crops are supported including coconut, oil palm, raffia palm and cocoyam. The drier upland region of Rivers State covers 61% of landmass while the riverine areas, with a relief range of 2 m to 5 m, take up 39% (RVSG, 2021). Rivers State has vast expanse of swamped areas which empty in the Bight of Bonny (ocean), hugely forested area, as well as a large area of mangrove cover, creating conducive environment for malaria vectors. There are two distinct seasons in the study area: a dry season from November to April and a rainy season which extends from May to October with a short break in August. Rivers state consist of urban and rural settlements as well as residential and industrial areas spread across the three ecological regions. The major occupation in the urban area is manufacturing and commercial industries while in the rural area, it is fishing and subsistence farming. The study area is also rich in crude oil mineral deposit, giving rise to extensive extraction and development activities cutting across the entire region. No extensive spraying or major vector control programmes have been carried out previously in this area until 2013 when LLINs were distributed by the National Malaria and Vector Control Programme and IRS was carried out in some communities in the study area

(RVSG, 2021). The major consideration in the division of the study area into three, is that they represent different ecological regions, with different environmental conditions that may have varying effect on the distribution and the population genetics of the *An. gambiae s.l.* There was ease of accessibility to the collection sites since it lies within areas of human settlement (Hamza *et al.,* 2014). Rivers State is composed of three climatic/ecological regions: lowland forest, freshwater swamp, mangrove and based on these regions the area was divided for the purpose of this study.
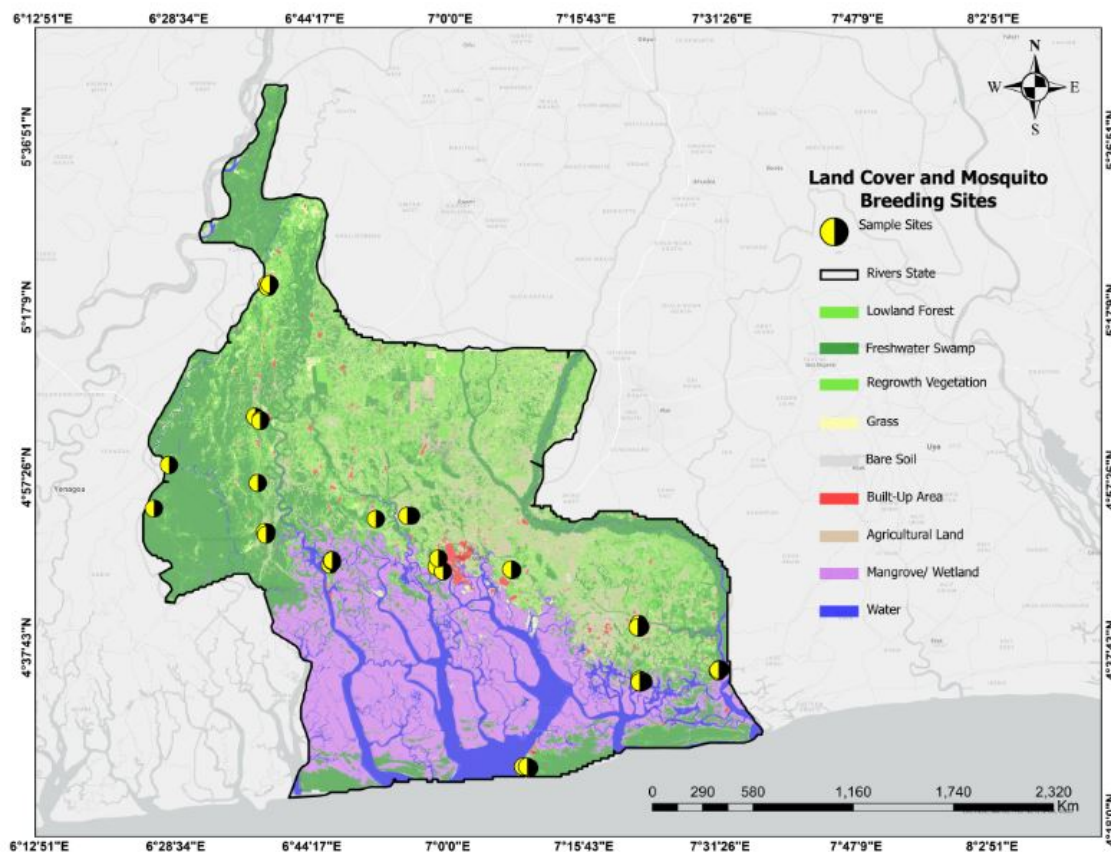

Figure 1. Map of Rivers State showing the Ecological Zones and Sampled Breeding Sites of *Anopheles gambiae s.l.*

2.2 Mosquito Sampling
Various potential sample breeding sites of Anopheles mosquitoes included borrow pits, vehicle tyre imprint, footprints, temporary sunlit pools, ditches in construction sites, puddles formed alongside lakes, drains, rain puddles, ponds and stagnant waters, among others, in the lowland forest, mangrove and fresh water swamp vegetations. The sampling was carried out from May 2019 to December 2019 and also from May 2020 to October 2020, This was to ensure that sample collection was done in the rainy season, when there was abundance of larvae, Standard dipper (400 ml) with one (1 m) handle was used for larvae collection from different breeding sites (WHO, 2015). The various breeding sites from which samples were collected are shown on Figure 1.

2.3 Rearing of Mosquito Larvae
Larvae from the different breeding sites in a particular ecological zone were pooled together, kept alive and placed in loosely capped plastic containers after which they were transported to the insectary of Malaria Vector Surveillance and Insecticide Resistance Monitoring Laboratory of the Department of Animal and Environmental Biology, Rivers State University, Port Harcourt, Nigeria. The processing of the materials for this study was carried out at the laboratory where the mosquitoes were reared to adulthood following the methods of Gerberg *et al.* (1994). In the laboratory the *Anopheles* larvae and the breeding water from the sampling sites, which were transported in a plastic container were gently poured into a white tray. A 3 ml-rubber pipette was used to pick the larvae from the white tray into another plastic container. This method was used to sort the *Anopheles* larvae into

plastic containers holding de-chlorinated water. The containers were covered with nets fastened with elastic bands and placed on platforms containing water below, to prevent crawling insects from invading the larvae. The larvae were fed with ground biscuits every two days and monitored till adult emergence. Newly emerged adults were separated into females and males, using aspirator (length-60cm: half of it made of glass and the other half made of rubber, diameter-1cm), to pick them individually from the adult cage. Females have non-plumose antennae with palp about as long as proboscis while the males have plumose antennae with palp about as long as proboscis and swollen at ends. Adults were kept in screen cages and fed continuously on 10% glucose solution. Cages were kept at 26°C - 29°C and 74% - 82% relative humidity.

2.4 Morphological Identification of *An. gambiae s.l*

Members of *An. gambiae s.l.* were morphologically separated from other anopheline mosquitoes using the morphological identification keys of Gillies and De-Mellion (1968) and Gillies and Coetzee (1987).

2.5 DNA Extraction from *An. gambiae s.l.* Mosquito Tissue

DNA was extracted from the tissues of *An. gambiae s.l.* samples using Quick-DNA^{TM} Tissue/Insect Miniprep Kit by Zymo Research, U.S.A., according to manufacturer's instructions. Resistant or susceptible *An. gambiae s.l.* mosquitoes, tested with a known insecticide from a known vegetational region, was transferred into a 1.5 ml Eppendoff tube. 50 µl BashingBead^{TM} Buffer was added to the Eppendoff tube and ground slowly with a grinding stick until it was completely ground. 350 µl of BashingBead^{TM} Buffer was added to the tube to complete the volume to 400 µl and vortexed the tube to permit total mixing of the solution. The ZR BashingBead^{TM} Lysis Tube (2.0 mm) was centrifuged in a micro centrifuge at 10,000 rpm for 1 minute. A Zymo-Spin^{TM} III-F Filter was placed in a Collection Tube and the supernatant was transferred onto it and centrifuged at 8,000 rpm for 1 minute. The Collection Tube with filtrate was collected and the Zymo-Spin^{TM} III-F Filter was discarded. 1,200 µl of Genomic Lysis Buffer was added to the filtrate in the Collection Tube and vortexed for 10 seconds. The Zymo-Spin^{TM} IICR Column 1 was placed in a Collection Tube and 800 µl of the mixture from the step above was transferred and centrifuged at 10,000 rpm for 1 minute. The remaining mixture in the Collection Tube was not discarded but was used later again. The flow through the filtrate from the Collection Tube was discarded and the same Zymo-Spin^{TM} IICR Column1 was placed back in the Collection Tube. The remainder of the mixture previously in the step above was transferred to the Collection Tube and centrifuged at 10,000 rpm for 1 minute. The flow was discarded through the filtrate from the Collection Tube and the same Zymo-Spin^{TM} IICR Column1 was placed in a new Collection Tube. 200 µl DNA Pre-Wash Buffer was added to the Zymo-Spin^{TM} IICR Column in the new Collection Tube and centrifuged at 10,000 rpm for 1 minute. 500 µl g-DNA Wash Buffer was added to the Zymo-Spin IICR Column in the Collection Tube and centrifuged at 10,000 rpm for 1 minute. The Zymo-Spin^{TM} IICR Column was transferred to a clean 1.5 ml Eppendoff tube and 100 µl DNA Elution Buffer was added. The Zymo-Spin^{TM} IICR Column in the Eppendoff tube was centrifuged at 10,000 rpm for 30 seconds to elute the DNA. The eluted solution of the Eppendoff tube containing the *An. gambiae s.l.* DNA was stored at -20°C for subsequent PCR analysis (Quick-DNA^{TM}, n.d.).

2.6 PCR Amplification of *An. gambiae s.l.* and Identification of the Sibling Species

The extracted DNA samples of *Anopheles gambiae s.l.* were stored in a -20°C freezer for further identification by PCR (Scott *et al.,* 1993). The following sibling species were suspected in the DNA extract, these included, *An. arabiensis, An. melas, An. quadriannulatus,* and *An. gambiae s.s.* The molecular identification of *Anopheles gambiae s.s.* was done by multiplex PCR reaction, including other subspecies of the complex, *An. arabiensis, An. melas* and *An. quadriannulatus.*

When the primers were newly purchased, the primer tubes were centrifuged for about 10 seconds before opening the tube and reconstituting it with nuclease free water to prevent the lyophilized primer from dispersing. To prepare 100 µM of each primer stock solution, corresponding volume of nuclease free water was added as stated in the primer manual. To prepare 20 µM of primer, 20 µl of the 100 µM primer stock solution was collected and 80 µl of nuclease free water added. PCR tubes were labeled according to the samples and placed on the ice pack. The different settings of the micropipette were used to add the following reagents in the order: One Tag Master Mix-14 µl, Universal Forward Primer-2 µl, Reverse Primer for *An. gambiae s.s.*-2 µl, Reverse Primer for *An. merus*-2 µl, Reverse Primer for *An. arabiensis*-2 µl, Reverse Primer for *An. quadriannulatus*-2 µl, *An. gambiae s.l.* DNA extract-5 µl, Nuclease free water-21 µl, Total reaction volume-50 µl. This Amplification protocol was adopted and modified from the work by Fanello *et al.* (2002)

The tubes were inserted into the thermal cycler and 35 cycles of amplification was run using the following conditions: Pre-denaturation- (94°C, 5 minutes), Denaturation- (68°C, 30 seconds), Annealing- (68°C, 30 seconds), Elongation- (72°C, 60 seconds), Final elongation- (72°C, 5 minutes) (Higa *et al.,* 2010). Primer sequence used include:

UN---- Universal forward primer…… 5'-GTG TGC CCC TTC CTC GAT GT-3'

GA---- reverse primer for *An. gambiae s.s.* …5'-CTG GTT TGG TCG GCA CGT TT-3'
ME---- reverse primer for *An. melas* ……5'-TGA CCA ACC CAC TCC CTT GA -3'
AR---- reverse primer for *An. arabiensis* ...5'- AAG TGT CCT TCT CCA TCC TA -3'
QD---- reverse primer for *An. quadriannulatus* ……5'- CAG ACC AAG ATG GTT AGT AT -3'
Expected Band sizes
An. gambiae s.s. - 390bp
An. arabiensis - 315bp
An. melas - 464bp
An. quadriannulatus - 153bp

Samples of *An. gambiae s.l.* that were resistant and susceptible to different kinds of insecticides, were used in the PCR gene amplification for the identification of the sibling species. DNA extract from each of the set of mosquito samples tested with a particular insecticide, were put into each well of the agarose gel. When the population of mosquitoes (of a particular tested insecticide) during grinding was much and could not be contained in one micro tube they were split into more than one micro tube, this resulted in samples of mosquito DNA extracts of deltamethrin resistant in wells 2 and 3, DDT resistant in wells 5-7 and primiphos-methyl resistant in wells 9 and 10, in the lowland forest vegetation as shown on Table 1.

Table 1. Samples of DNA Extract of *An. gambiae s.l.* in each well of the Agarose Gel for PCR Analysis

Agarose gel well number	Samples of *An. gambiae s.l.* in the agarose gel wells		
	Lowland forest vegetation	**Fresh water swamp vegetation**	**Mangrove Vegetation**
1	Permethrin resistant	Lambda-cyhalothrin resistant	Lambda-cyhalothrin resistant
2	Deltamethrin resistant	DDT resistant	Permethrin resistant
3	Deltamethrin resistant	Primiphos-methyl resistant	DDT resistant
4	Lambda-cyhalothrin resistant	Deltamethrin resistant	Primiphos-methyl resistant
5	DDT resistant	Propoxur resistant	Deltamethrin resistant
6	DDT resistant	Permethrin resistant	Alpha-cypermethrin resistant
7	DDT resistant	Bendiocarb susceptible	Bendiocarb susceptible
8	Propoxur resistant		
9	Primiphos-methyl resistant		
10	Primiphos-methyl resistant		
11	Alpha-cypermethrin resistant		
12	Primiphos- methyl susceptible		

2.7 Restricted Fragment Length Polymorphism (RFLP) Differentiation of *An. gambiae s.s.* to *An. colluzzi* and *An. gambiae s.s.* (M and S Forms)
After identifying the *An. gambiae s.s* using molecular technique, samples of *An. gambiae s.l.* that were resistant and susceptible to various insecticides (during susceptibility testing), were used in the RFLP to distinguish between *An. gambiae s.s.* and *An. colluzzi*. DNA extract from each of the sets of mosquito samples tested with a particular insecticide, were put into each well of the agarose gel. It was subjected to RFLP to identify the M and S form. Products of the samples identified as *An. gambiae s.s.* were further digested with *Hha* I enzyme to separate between *An. gambiae s.s* and *An. colluzzi* species (Fanello *et al.,* 2002). To carry out M and S form of the *An. gambiae s.s.,* 16 µl of PCR product was placed in a sterile 0.2 µl PCR reaction tube, 2 µl of 10X restriction buffer was added to the same tube. After which 1 µl of *Hha* 1 restriction enzyme was then added to the tube. The content of the tube was well mixed to ensure homogeneous mixture and incubated at 37ºC for 3 hours. Amplified fragments of digested products were analysed by electrophoresis on a 2% ethidium bromide agarose gel and were visualised under ultraviolet light to verify different size pattern.
Expected Fragment Lengths (Band sizes)
An. gambiae s.s. (S-form) - 257bp (presence of the *Hha* 1 restriction site)
An. colluzzi (M-form) - 367bp (absence of the *Hha* 1 restriction site)
Samples of *An. gambiae s.l.* that were resistant and susceptible in the knockdown test, were used in the RFLP to distinguish between *An. gambiae s.s.* and *An. colluzzi*. DNA extract from each of the set of mosquito samples tested with a particular insecticide, were put into each well of the agarose gel. From Table 1, in the lowland forest vegetation, only sample 5 was used, samples 6 and 7 were not used, to avoid excessive repetition of DDT.

2.8 Sequencing of the PCR Amplified Products
PCR products were purified prior to sequencing. The evolutionary distances were computed using the Kimura 2-parameter method (Tamura *et al.,* 2004). Evolutionary analysis was carried out on purified products, using MEGA X (Kumar *et al.,* 2018) and evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987).

3. Results

3.1 PCR Products of *Anopheles gambiae s.l.* Gene Amplification in Lowland Forest Vegetation

The electrophoretic patterns of the PCR products revealed in Figure 2 that samples 1-12 in the lowland forest vegetation, were positive for *An. gambiae s.s.* with bands at 390 base pairs (bp). The lane L is the DNA molecular weight ladder (molecular marker).
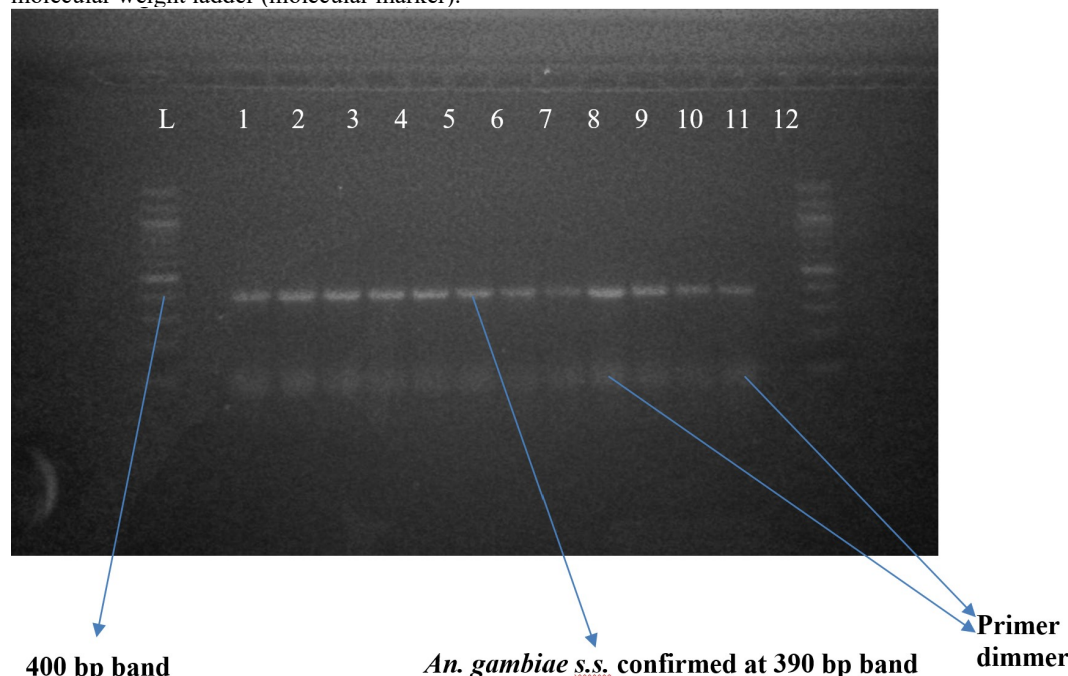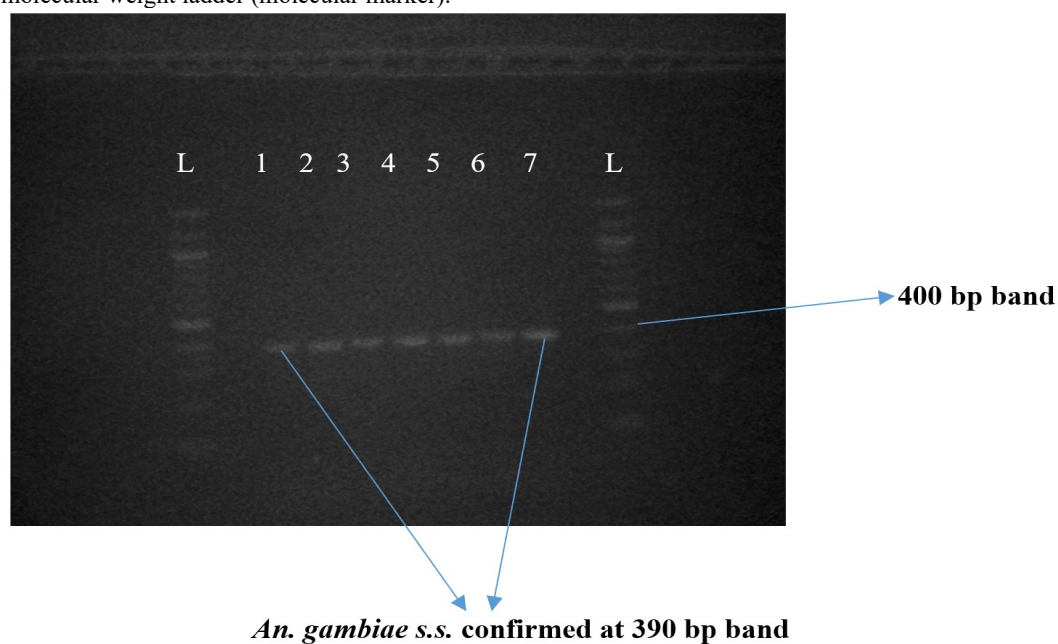

Figure 2. PCR Products of *Anopheles gambiae s.l.* Gene Amplification in Lowland Forest Vegetation

3.2 PCR Products of *Anopheles gambiae s.l.* Gene Amplification in Mangrove Vegetation

The electrophoretic patterns of the PCR products revealed in Figure 3 that samples 1-7 in the mangrove vegetation, were positive for *An. gambiae s.s.* with bands at 390 base pairs (bp). The lane L is the DNA molecular weight ladder (molecular marker).

Figure 3. PCR Products of *Anopheles gambiae s.l.* Gene Amplification in Mangrove Vegetation

3.3 PCR Products of *Anopheles gambiae s.l.* Gene Amplification in Fresh Water Swamp Vegetation

The electrophoretic patterns of the PCR products revealed in Figure 4 that samples 1-7 in the fresh water swamp vegetation, were positive for *An. gambiae s.s.* with bands at 390 base pairs (bp). The lane L is the DNA molecular weight ladder (molecular marker).
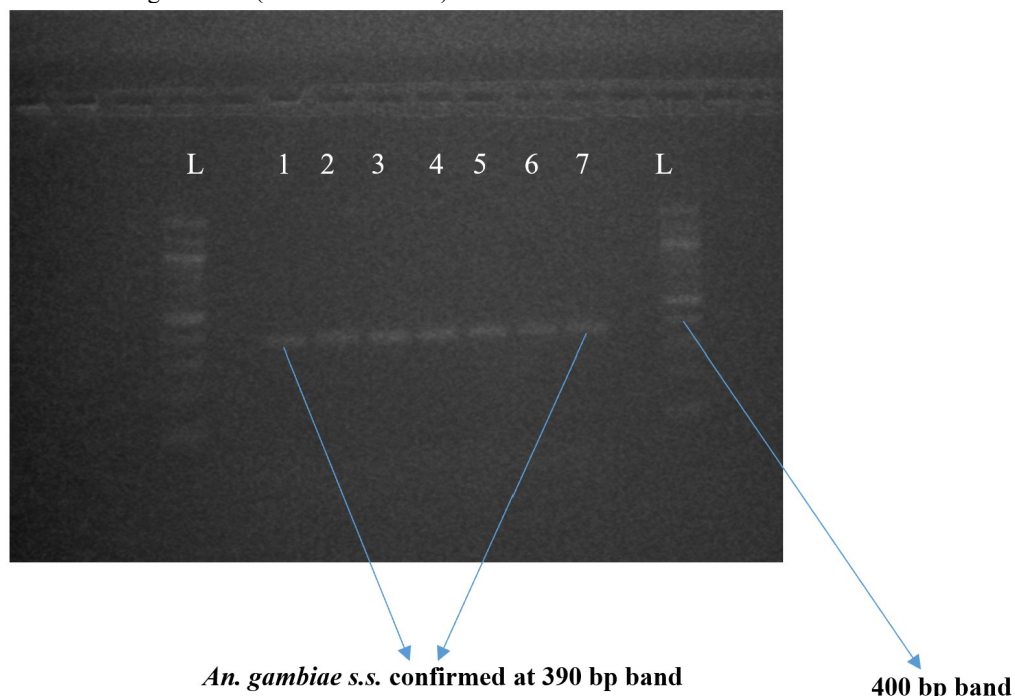

An. gambiae s.s. confirmed at 390 bp band

400 bp band

Figure 4. PCR Products of *Anopheles gambiae s.l.* Gene Amplification in Fresh Water Swamp Vegetation

3.4 Restricted Fragment Length Polymorphism (RFLP) Products of *Anopheles gambiae s.s.* in Lowland Forest Vegetation

The electrophoretic patterns of the PCR products revealed in Figure 5 that samples 1-10 in the lowland forest vegetation, were positive for *An. gambiae s.s.* which has S-form bands with a restricted fragment length of 257 at 390 base pairs (bp).
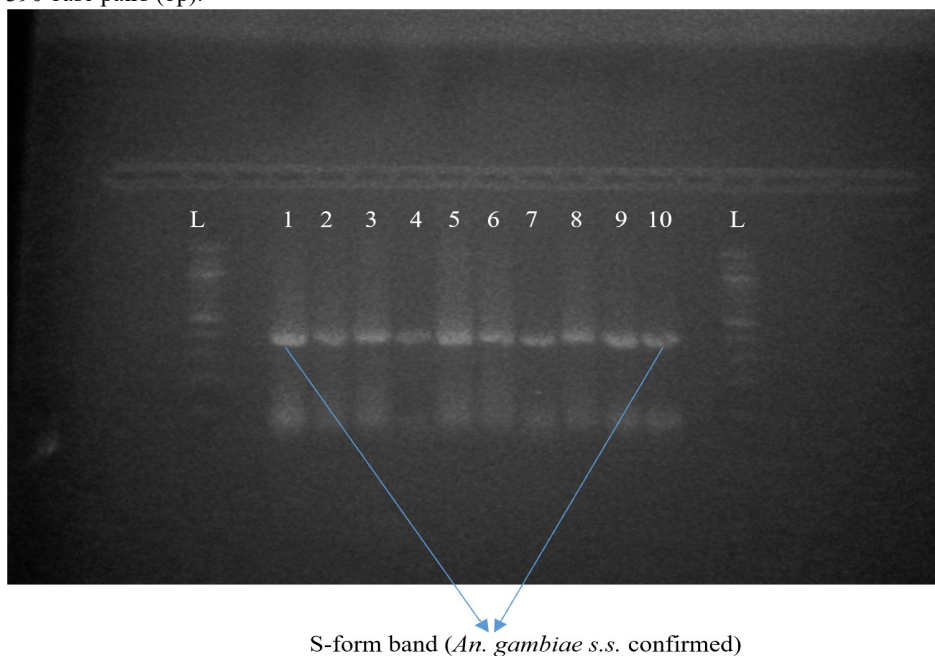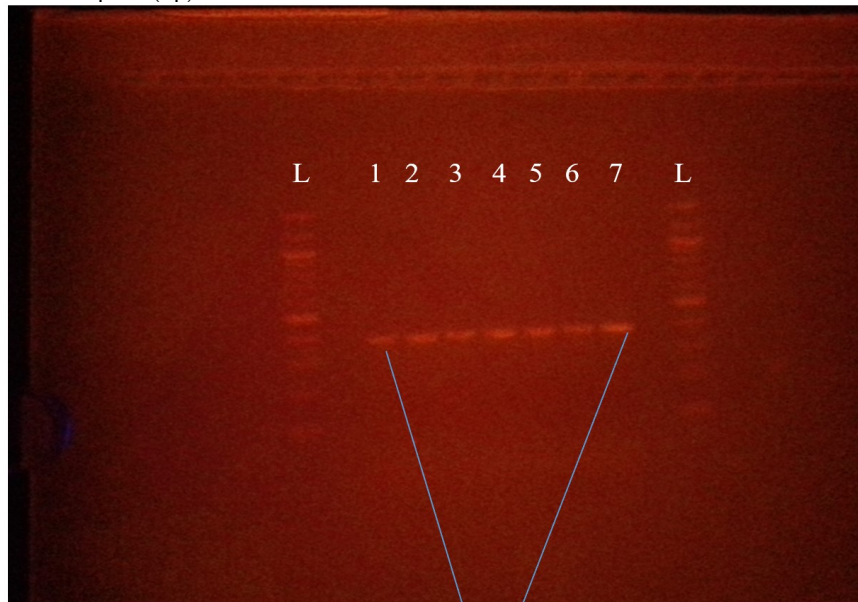

S-form band (*An. gambiae s.s.* confirmed)

Figure 5. RFLP Products of *Anopheles gambiae s.s.* in Lowland Forest Vegetation

3.5 Restricted Fragment Length Polymorphism (RFLP) Products of *Anopheles gambiae s.s.* in Mangrove Vegetation

The electrophoretic patterns of the PCR products in Figure 6 revealed that samples 1-7 in the mangrove vegetation, were positive for *An. colluzzi* which has M-form bands with an unrestricted fragment length of 367 at 390 base pairs (bp).
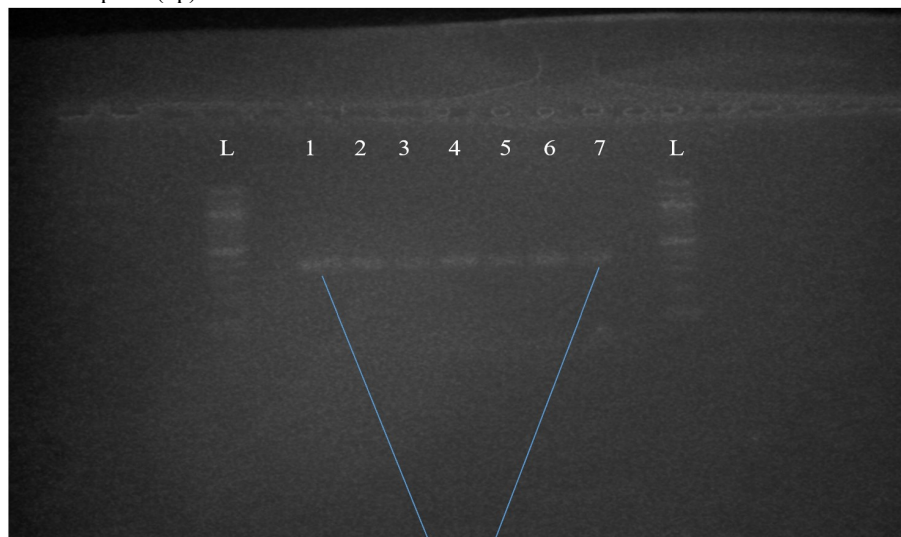

M-form band (*An. colluzzi* confirmed)

Figure 6. RFLP Products of *Anopheles gambiae s.s.* in Mangrove Vegetation

3.6 Restricted Fragment Length Polymorphism (RFLP) Products of *Anopheles gambiae s.s.* in Fresh Water Swamp Vegetation

The electrophoretic patterns of the PCR products in Figure 7 revealed that samples 1-7 in the fresh water swamp vegetation, were positive for *An. colluzzi* which has M-form bands with an unrestricted fragment length of 367 at 390 base pairs (bp).

M-form band (*An. colluzzi* confirmed)

Figure 7. RFLP Products of *Anopheles gambiae s.s.* in Fresh Water Swamp Vegetation

3.7 Phylogenetic Analysis

The phylogenetic tree was constructed from the *An. gambiae s.l.* genes amplified in this study, from fresh water swamp, lowland forest and mangrove vegetations. The closest evolutionary relative of a gene on a phylogenetic tree are formed on one branch (Figure 8). The *An. gambiae* genome from this study is closely related to *An. gambiae* (Ace-1) gene with accession number KP165373.1.
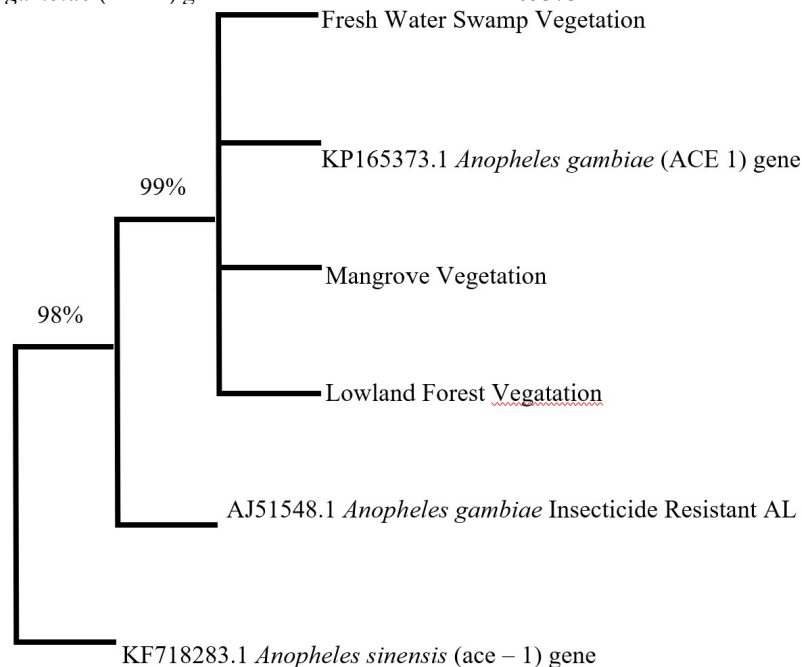

Figure 8. Phylogenetic Analysis of the *Anopheles gambiae s.l.* Samples from Fresh Water Swamp, Lowland Forest and Mangrove Vegetations

4. Discussion

PCR amplification of the *An. gambiae s.l.* genes were used for the specific detection of the sibling species present in lowland forest, mangrove and fresh water swamp vegetations. The amplification of the *An. gambiae s.l.* gene, used a set of primer that corresponded to highly conserved regions, which produced a band that showed that the sibling species of the *An. gambiae s.l.* was *An. gambiae s.s.* The band of DNA amplified was 390 bp, which corresponded to the gene of *An. gambiae s.s.* The primer dimmer was a potential by-product in the PCR. It is only one sibling species, *An. gambiae s.s.* that predominates the entire study area of Rivers state consisting the three vegetations. Akpan and Ebere (2015), discovered *An. gambiae s.l.* and *An. nili* in Rivers State University of Science and Technology, Port Harcourt, south-south Nigeria. In Uyo, Akwa Ibom state, southeastern Nigeria, *An. gambiae s.s.* and *An. nili* were reported (Atting and Akpan, 2016). In Kenya (East Africa), Omondi *et al.* (2017) found *An. arabiensis* in majority of sites and *An. gambiae s.s.* in one site. In Tanzania, *An. arabiensis* and *An. gambiae s.s.* were characterized by Kisinza *et al.* (2017) and in northwest Tanzania, *An. funestus, An. gambiae s.s.* and *An. arabiensis* were characterized (Protopopoff *et al.,* 2018). In north Africa, Sudan and South Sudan, Hamza *et al.* (2014) found *An. arabiensis* and *An. gambiae s.s.,* respectively. It is observed from the reports of other findings that in addition to *An. gambiae s.s.,* another predominating species was *An. arabiensis* in most of the central and eastern countries of Africa, beside *An. funestus* found in northwest Tanzania. It is noteworthy that *An. nili* was discovered in Port Harcourt, and in Uyo, Akwa Ibom state, southeastern Nigeria, as it was rarely observed and reported from that region of Africa.

PCR-RFLP was further used to specify the *An. gambiae s.s.* of the PCR gene amplification into its molecular forms of *An. gambiae s.s.* (S-form) and *An. colluzzi* (M-form). The differences in sizes between the PCR-amplified fragments and the fragments obtained after digestion of *An. gambiae s.l.* sample with Hha I enzyme, are due to the presence of a restriction site. This restriction site is present in the *An. gambiae s.s.* of the lowland forest vegetation. After digestion with the Hha I enzymes, two fragment lengths resulted: 257 bp and 110 bp long. Restriction sites are found in S-form, as was revealed in the lowland forest vegetation. The *An. gambiae* (M-form) does not have this restriction site and so does possess a single long 367 bp fragment (Fanello *et al.,* 2002). This M-form of *An. gambiae s.s.* is known as *An. colluzzi* and results revealed that it predominates the mangrove and the fresh water swamp vegetations. *An. gambiae s.l.* sibling species which include *An. gambiae s.s., An. arabiensis, An. merus/An. melas* and *An. quadriannulatus,* had primer sequences which were

used in the PCR master mix, after which DNA extracts from the *An. gambiae s.l.* samples were added. The genes of *An. gambiae s.s.* got bound to the *An. gambiae s.s.* primer sequence and they were identified on the agarose gel electrophoresis as the gene is located at 390 bp band. The *An. gambiae s.s.* identified from the PCR was further tested using RFLP for identification to *An. gambiae s.s.* (S-form) as revealed in the lowland forest vegetation of Rivers state and *An. colluzzi* (M-form) as revealed in the mangrove vegetation and fresh water swamp vegetation of Rivers state. *An. gambiae s.s., An. colluzzi* and *An. arabiensis* were reported in northern Nigeria (Abdu *et al.,* 2017), Burkina Faso (Namountougou *et al.,* 2012), Pitoa in northern Cameroon (Etang *et al.* 2016) and Mali (Cisse *et al.,* 2015). The occurrence of *An. arabiensis* in these report can be related to the fact that they fall within the grassland savanna vegetation, which cut across West African, Burkina Faso to Central African, Cameroon. Their domination of parts of these countries could be as a result of favourable climatic condition that results in the kind of vegetation that is found across that belt.

    An. gambiae s.s. and *An. colluzzi* were reported in Ibadan, southwest Nigeria (Okorie *et al.,* 2015), Taraba state, northeast Nigeria (Lamidi *et al.,* 2017) and Cameroon (Boussougou-Sambe *et al.,* 2018). In West Africa, Togo (Ketoh *et al.,* 2018), Cote d'Ivoire (Camara *et al.,* 2018), southeast Benin Republic (Yahouedo *et al.,* 2016). In East Africa, Uganda (Kabbale *et al.,* 2016), in addition to *An. gambiae s.s., An. funestus* was also reported alongside with *An. colluzzi* in West African, Kpome, in southern Benin Republic. From the reports in other places, it is observed that *An. gambiae s.s.* and *An. colluzzi*, characterized in this current study have a good widespread from the north through the west to the east of Africa and some exist in sympatry with other species such as *An. arabiensis* and *An. funestus*. From the present study *An. colluzzi* was predominant in mangrove vegetation and fresh water swamp vegetation. These are two kinds of vegetations that are thick and highly, not easily accessible because of the dense swampy nature of the fresh water swamp vegetation and the thick entangling nature of the mangrove vegetation. The *An. gambiae s.s.* was predominant in the lowland forest vegetation which is more accessible and less entangling in contrast with the mangrove and fresh water swamp vegetations. This agrees with the findings of Camara *et al.* (2018), in which *An. gambiae s.s.* was dominant in the sub-Sudanian area while *An. colluzzi* was dominant in the forest area. The observation also agrees with the statement that, the two molecular forms, *An. gambiae s.s.* and *An. colluzzi* differ in ecological preference (Lehmann & Diabate, 2008).

5. Conclusion

The PCR revealed that *An. gambiae s.s.* is present and predominates in the three vegetations in Rivers state. The RFLP revealed that *An. gambiae s.s.* (S-form) is present and predominates in the lowland forest vegetation of Rivers state while *An. colluzzi* (M-form) is present and predominates in the mangrove vegetation and fresh water swamp vegetation of Rivers state. This study has provided the first baseline information on the characterization of the sibling species of the malaria vector, *An. gambiae s.l.* across the three vegetations that make up Rivers state.

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