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IDENTIFICATION AND DISTRIBUTION OF MOLECULAR FORMS OF *Anopheles gambiae SENSO STRICTO* (Mopti) and (Savanah). IN TURE AND BAMBAM COMMUNITIES OF GOMBE STATE.

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Abstract: The lack of information on identification and distribution of molecular forms of *Anopheles gambiae senso stricto* (mopti form) and (savanah form) Are one of the major hindrance facing malaria vector control implementation in west Africa. This work focused on identification and distribution of molecular forms of *Anopheles gambiae senso stricto* (mopti form) and (savanah form). in Ture and Bambam communities of Gombe state. Morphological identified mosquitoes were subjected to PCR analysis to further comfirm the species complex and the molecular form present in the tested sample. The result of this study showed that 25(25%) were *Anophele gambiae* s.l while 75(75%) did not amplify. From those that amplified, result shows that 25(25%) belongs to *Anopheles gambiae* s.l. with 11 (22%) and 14 (28%) recorded in Bambam and Ture communities respectively. No other sibling species of *An. gambiae* complex was recorded. A total of 4(16%) *An. Coluzzi* [M form], 15(60%) M/S Hybrid molecular forms of *An. gambiae* s.s. was recorded in both study communities with 6(24%) non-amplified (Fig. 5). No *An. gambiae* (S form) was recorded in both locations, whereas, 2 *An. coluzzi*(M form) was recorded in each locations. Fifteen 15 M/S Hybrid form was recorded from both locations with Ture community having 6(40%) while 9(60%) was recorded for Bambam community.

Keywords: *Anopheles gambiae*, Polymerase chain reaction, Mopti and Savanna, Senso stricto.

Introduction

The *Anopheles gambiae* sensu lato (S.L) species complex contains the most important mosquito vectors of malaria in sub-Saharan Africa. It comprises seven morphologically indistinguishable sibling species up to four of which may be sympatric (Coetzee, 2004). The principal malaria vectors in the complex are *Anopheles gambiae* sensu stricto (S.S.) and *Anopheles arabiensis*. Of the remaining members, *Anopheles quadriannulatus*species A, which is widespread in southern Africa, and *Anopheles quadriannulatus* species B, found in Ethiopia, are considered to be zoophilic non-malaria vectors (Coetzee, 2004; Coetzee, *et al.*, 2000). *Anopheles melas* and *An. Merus* are salt water breeding and consequently only important vectors in coastal region (Moreno, *et al.*, 2004).

In West Africa, *An. Gambiae* s.s. has been divided into five chromosomal forms designated with a non-Linean nomenclature: bamako, mopti, savanna, forest, and bissau (Coluzzi, *et al.*, 1985). *Anopheles gambiae* s.s. exists throughout most of Sub-Saharan Africa, but there are many polymorphisms including chromosomal inversions that appear to be involved in the adaptation of subpopulations to different environments (Coluzzi, *et al*., 2002). Della Torre, *et al.*, 2005 reports that substantial local variation exists in relative frequencies of Molecular forms of *An. gambiae* s.s. in the extreme west of Africa. M form predominates in most of Gambia, while farther inland in eastern Senegal, almost all members of the species are S form (Caputo, *et al.*, 2008). In Nigeria, S form predominates over the M form over the years (Okorie, *et al.*, 2011). Of particular interest are areas in which both forms exist, as the occurrence of natural hybrids may be possible. Elsewhere in Africa, areas of sympatry consistently exhibit very low frequencies of hybrid forms, but a few years ago exceptionally high proportions of M/S hybrids were reported at a few sites near the west coast, with 3% at Dielmo in Senegal (Ndiath, *et al.*,2008), 7% at Njabakunda in Gambia (Caputo, *et al.*, 2008), and 24% at Antula in GuineaBissau (Oliveira, *et al.*, 2008).

Survey in the Southern Guinea has shown mostly S form near the coast, and both M andS forms with a very low frequency of hybrids (Carnevale, *et al.*, 2010). In Northern Senegal *An. gambiae*s.s. is less common compared to *An. arabiensis* and exist mostly as M form while, in Eastern Senegal, studies shown that S form is more abundant than M form with rare M/S hybrid (Caputo, *et al.*, 2008). Hybrid was first reported in Nigeria by Okorie, *et al.*, (2015) where he reported one M/S hybrid form in Ibadan, suggesting restricted gene flow between *An. coluzzi* and *An. gambiae* providing strong support for reproductive isolation between the two species (Ndiath, *et al.*, 2012).

Distribution of Anopheles species Across Nigeria

PCR species specific assay conducted on *An. gambiae* in Benin Republic revealed 100%*An. gambiae*s.s. (Aizoun, *et al.*, 2013). Similar study conducted in Ghana reveals only*An. gambiae*s.s. as the only sibling species of *An. gambiae* complex (Kabula, *et al*.,2011).

Previous study conducted in Calabar, Nigeria revealed that out of 83.4% *An. gambiae*s.l collected, 65% were found to be *An. gambiae*and 11% were *An. Arabiensis* and 7.3% were unidentified species Molecular characterization of *An. gambiae* complex conducted in Niger Delta region of Nigeria shows that of 203 *An. gambiae*s.l. collected, 88% were *An. gambiae*s.s., 4.4% amplified as *An. arabiensis*while 6.9% of the population belong s to *An. Merus* Ebenezer, *et al.*, 2012).

Morphological and Molecular characterization conducted in Uyo reveals that out of the 700 mosquitoes collected in two communities, only 69(9.9%) belongs to *An. gambiae*s.l, further study reveals *An. gambiae*s.s.as the only species in the complex (Inyang and Mfonobong, 2016).

PCR-Restriction Fragment Length Polymorphism (Rflp)

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis has been used to identify closely related species of public health importance from different life stages (MalgornSpace&Coquoz, 1999). It is a fast, easy, low cost technique for routine diagnostic purposes (Litjens, *et al.*, 2001). In PCR-RFLP analysis, the detection of polymorphic patterns between individuals is based on differences in the sizes of the restriction fragments obtained from the amplified DNA region generated by a specific endonuclease or a multiple set of restriction enzymes This technique has been used in identifying the molecular forms of *An. Gambiae*s.s.(Fanello,*etal.*,2002).

Material and Methods

Identification of Anopheles Samples

Morphological identification

Morphological identification of the survivor and dead mosquitoes were carried out with the aid of identification keys (Maureen Coetzee 2020) followed by preserving individually on silica gel in Eppendorf tube for molecular identification. The following key features were considered for the identification of *Anopheles gambiae*

Wings : Have dark spots on the wing veins
Proboscis/palps :Black spot on the palps
Legs : spotted or speckled legs
Colour : Yellowish brown to brown

Molecular Identification of Anopheles gambiae Senso Lato.

The polymerase chain reaction (PCR) identification of *Anopheles gambia* the molecular identification were carried out at institute of Medical research Institute Yaba, Lagos State Nigeria. Samples of the adult mosquitoes morphologically identified as *Anopheles gambiae*s.l. were differentiated to siblings' species level and also to M and S forms using Polymerase Chain Reaction (PCR) method. Based on the following procedures, one hundred (100) mosquitoes of the *Anopheles gambiae s.l* were selected at random from a pool of resistant species with equal representation from the two different location sites with each of the samples having equal chance of being selected, which have been previously identified morphologically.

Samples from survivor and dead mosquitoes were identified to species level using specie-specific PCR assay. The PCR was performed with universal and species specific primers for the *An. gambiae* complex. Molecular identification of *An. gambiae* species complex is based on the species specific nucleotide sequences in the ribosomal DNA intergenic spacers (IGS) following the procedure of Scott *et al.,* (1993). The amplified DNA was separated on a 2.0% agarose gel stained with ethidium bromide and viewed under a Gel documentation machine.

Amplification of DNA

Polymerase Chain Reaction (PCR) is the most common DNA amplification method in molecular biology (VanRansburg, *et al.,* 1996). The method used for the PCR analysis was positive control method. Five sets of primers designed from the DNA sequences of the intergenic spacer (ITS) region of *An. gambiae*complex ribosomal DNA (rDNA) were used in PCR for the member species identification. The sequence details of these primers abbreviated Universal (UN), *gambiaes.l*(GA), *merus*and *melas*(ME), *Arabiensis*(AR) and *quadriannulatus*(QD) are given in Table 2. The UN primer anneals to the same position on the rDNA sequences of all five species, GA anneals specifically to *An. gambiae*s.s., ME anneals to both *An. merus*and *melas*, AR to *An. arabiensis* and QD to *An. quadriannulatus*.

Table1 Primers sequence for identifying *An. gambiae*sibling species

An. gambiaes.s.	Abbreviation	Primer sequencing
Universal	UN	GTGTGCCCCTTCCTCGATGT
An. arabiensis	AR	AAGTGTCCTTCTCCATCCTA
An. gambiaes.l	GA	CTGGTTTGGTCGGCACGTTT
An. merus/melas	ME	TGACCAACCCACTCCCTTGA
An. quadriannulatus	QD	CAGACCAAGATGGTTAGTAT

These primers in conjunction with other reagents bind to specific *Anopheles gambiae*s.l species when added to the template DNA extracted from sample species. The primers stored in a frozen state at -20^{0}C were removed from the freezer and placed on an icepack to defrost. The master mix [containing buffer, deoxynucleotidetriphosphate (dNTPs), primers, Magnesium Chloride ($MgCl_2$), sterile water and RTaq (an enzyme)] was prepared and measured according to their proportion (Table 3) using micro-titre pipette into 0.5µl eppendorf tubes and then place inside a vortex machine for it to mix.

Table 2 Proportion of master mix for identifying *An. gambiae* sibling species

Master mix constituents	X 1 sample(µl)	X 100 samples(µl)
Sterile water(dH_2O)	5.7	570
10X PCR buffer with $MgCl_2$	1	100
dNTPs	1	100
$MgCl_2$	1	100
Taq DNA polymerase (enzyme)	1	100
Primers	2.25	225
Total	11.95	1195

NB: 12µl was added to 1µl of template DNA

12µl of master mix was dispensed into the 0.5ml eppendorf tubes going into the PCR machine. 1 µl of the extracted DNA of each sample was pipetted into the 0.5ml eppendorf tubes containing the 12ml master mix. The Eppendorf tubes were placed in PCR machine. The PCR machine was programmed for *An. gambiaecomplex*

Results and Discussion

A total number of 150 mosquitoes; 72(48%) and 78(52%) from Ture and Bambam communities respectively, were randomly pooled for morphological identification; result shows 100% *Anopheles gambiae* s.l. No other species of *Anopheles* was recorded

Table 4 Morphological identification of *An. gambiae* mosquitoes in Ture and Bambam communities

Location	*Anopheles gambiae* s.l (%)	*Anopheles funestus* (%)	Total
Ture	72(48)	-	72(48)
Bambam	78(52)	-	78(52)
			150(100

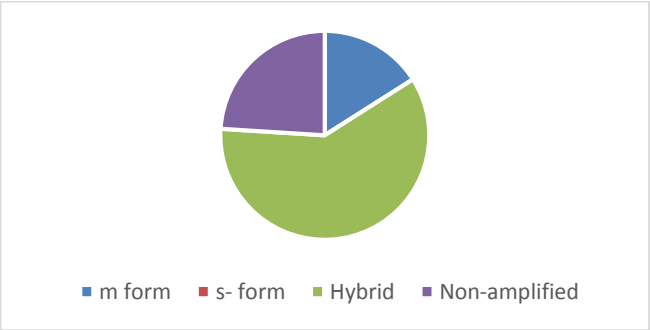

Fig. 5: Percentage of Molecular forms of *An. Gambiae s.s* in the study bcommunities

Molecular Characterization

PCR Identification of Sibling species of Anopheles gambiae complex

100 samples were randomly selected from mosquitoes morphologically identified as *Anopheles gambiae* s.l. (50 from each study communities). Species-specific PCR based assay was conducted on them, 25(25%) amplified while 75(75%) did not amplify (Table 6). From those that amplified, result shows that 25(25%) belongs to *Anopheles gambiae* s.s. with 11 (22%) and 14 (28%) recorded in Bambam and Ture communities respectively. No other sibling species of *Anopheles gambiae* complex was recorded.

Table 5 Sibling species composition of *An. gambiae*complex across the study sites.

Location	An. ***Gambiae s. s***	*An. Arabiensis*	Total amplified	Non-amplified
	14(14) Ture	-	14	36
Bambam	11(11)	-	11	39
	25(100)	-	25	75

PCR Identification of the Anopheles gambiae s.s molecular Forms

A total of 4(16%) *An. coluzzi*[M form], 15(60%) M/S Hybrid molecular forms of *An. gambiae*s.s. was recorded in both study communities with 6(24%) non-amplified (Fig. 5). No *An. gambiae*(S form) was recorded in both locations, whereas, 2 *An. coluzzi*(M form) was recorded in each locations. Fifteen 15 M/S Hybrid form was recorded from both locations with Ture community having 6(40%) while 9(60%) was recorded for Bambam community.

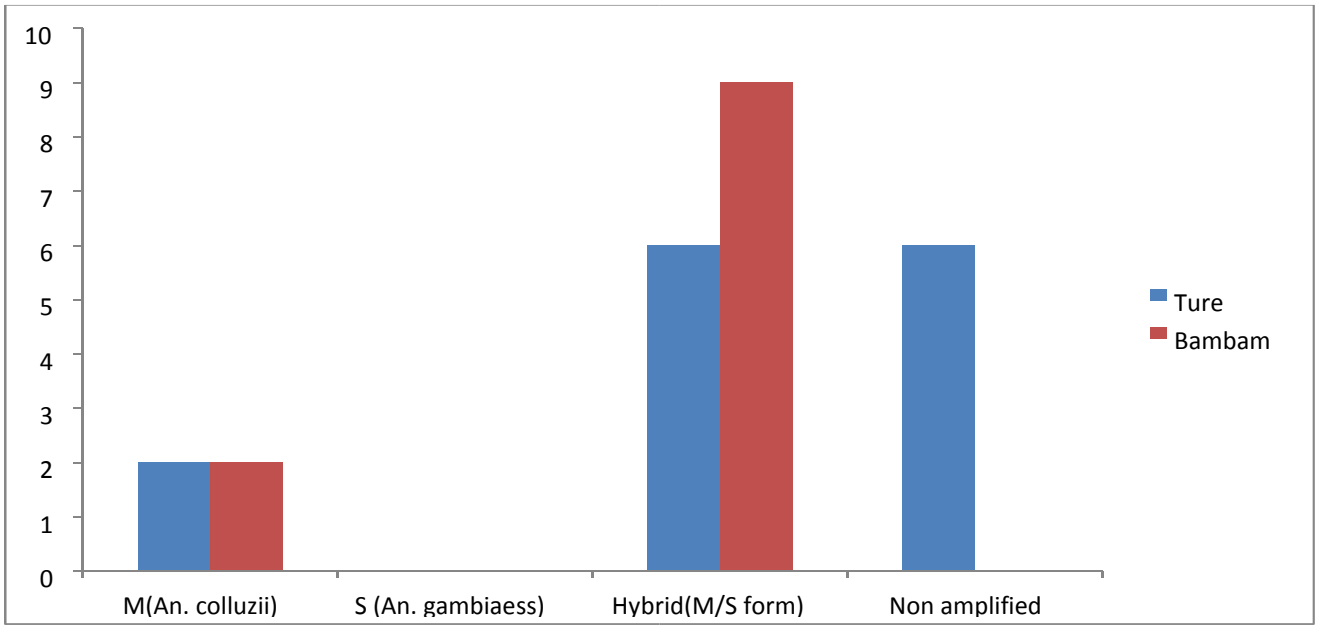

Fig. 6: Distribution of molecular forms of An. Gambiae in Ture & Bambam community

There is No S form (*Anopheles coluzii*) record in both studied communities whereas (*An. gambiae*) was recorded in both communities. This is similar to the findings of Oduola, *et al.,* 2012 where no S form (*An. coluzzi*) was recorded from 12 different communities in Lagos. The result is also in consonant with the findings of Ibrahim, *et al.,* (2014) who reported absence of S form in Sudan savannah part of the country. The absence of S form in the study area could be explained by the observation previously made that the M form is predominant in this type of ecological setting with a more permanent breeding site from the irrigation system (Coluzzi, 1984), or as a result of increased predation pressure. The M forms recorded could be due to suggestions that M forms dominated drier areas. High proportion of molecular Hybrid form recorded in this study tallies with the studies from Senegal (Ndiath, *et al.,* 2008), Gambia (Caputo, *et al.,* 2008), Antula in Guinea-Bissau (Oliveira, *et al.,* 2008). Hybrid fitness traits are subject to heterosis i.e. hybrids have increased values for fitness traits than the parental population (David, *et al.,* 2005). Hence, the implication of high M/S hybrid form on public health cannot be overemphasized. *Anopheles gambiae* s.s. is undergoing speciation, being split into two"molecular forms", currently named *An. Coluzzi* (Mopti form) and *An. Gambiae* (Savanah form). Speciation is the main process promoting biological diversity and in the context of public health it increases epidemiological complexity and these molecular forms (M and S) have been identified to be reproductively isolated (della Torre, *et al.,* 2001). The S form (now *An. gambiae*) is distributed widely throughout the *An. gambiae* species range, whereas the M form (now *An. coluzzi*) is common but restricted to western parts of Africa, and hybridization between them is rare in most areas of sympatry (della Torre, *et al.,* 2005). It is vital to understand such differentiation and genetic subdivision and its importance in vector evolution, as this complexity can affect malaria control, including resistance to insecticides (Lynd, *et al.,* 2010) susceptibility to malaria parasites and other infection disease between the M and S molecular forms appear to be minimal but evidence is accumulating that the larval stages are differentially adapted to particular features of

breeding sites, with the M form (now *An. coluzzi*) being generally more common in large areas of irrigation for crop cultivation (Diabate, *et al.*,2008).

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