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First report of heterogeneity and sympatry of malaria vectors in Southern Gombe, Northeastern Nigeria: its implications for malaria vector control

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Abstract. A survey of the malaria vectors in an area is a critical component of an effective vector control strategy. This study aimed to investigate the malaria vectors in four communities of Southern Gombe, Northeastern Nigeria. A total of 3200 adult female *Anopheles* reared from larvae in the four communities from two Local Government Areas (LGAs) in Southern Gombe were identified. *Anopheles pretoriensis* were dominant 1662 (51.9%) followed by *An. gambiae sl* 868(27.1%), *An. maculipalpis* 267(8.3), *An. rufipes* 252(7.9) and *An. coustani* 10(0.3%), and the least were *An. pharoensis* 6(0.2%). The remaining 135(4.3%) were unidentified. Of the 262 *An. gambiae* sl identified by species-specific PCR method, 135(51.5%) were found to be *An. coluzzii*, 60(22.9%) *An. gambiae* and only 1(0.4%) was *An. arabiensis* whereas hybrid constitutes 16(6.1%). To determine significant differences in species composition, the results from the four study sites were pooled together. *Anopheles pretoriensis* was significantly different from all the other species identified (p = <0.0001 - 0.0454). *An. gambiae sl.* was significantly different from *An. coustani* and *An. pharoensis* (p = 0.0258, 0.0249 respectively). There was no significant difference between *An. maculipalpis*, *An. rufipes*, *An. coustani* and *An. pharoensis* (p = 0.9261 - >0.9999). There was a significant difference between the number of species identified as *An. coluzzii* and *An. arabiensis* (p = 0.0025). But there was no significant difference between *An. coluzzii* and *An. gambiae* (p = 0.1212). The heterogeneity and sympatry of *Anopheles* species observed is a threat to malaria control as the secondary vectors have behaviour that evades the current vector control interventions. It is expedient to re-strategize the vector control interventions in the study area.

Keywords: Malaria vector, Vector control, Gombe, *Anopheles*

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Introduction

Malaria is a life-threatening disease caused by *Plasmodium* parasites (Campos *et al.*, 2023). Globally, there were an estimated 249 malaria cases in 2022. This shows a rise of 5 million malaria cases compared to the 2021 reported cases. The 2022 cases were from 85 countries with endemic malaria, and the *Sub-Saharan Africa*n Region recorded the majority of this increase. With 233 million of the 2022 cases, the *Sub-Saharan Africa*n continent accounted for around 94% of the global total cases. Nigeria accounted for 27% of the global total cases carrying a greater percentage than any other country (WHO, 2023). In Nigeria, malaria accounts for up to 60% of all outpatient visits to healthcare facilities and 30% of all hospital hospitalizations. Malaria is thought to be responsible for 25%

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of baby fatalities, 30% of child deaths, and 11% of maternal deaths. According to estimates, this disease costs Nigeria's economy ₦132 billion annually (approximately US$167,300) in lost wages due to treatment expenses, preventative measures, wasted manpower, etc (National Malaria Elimination Programme & Federal Ministry of Health Nigeria, 2014). The bulk of malaria cases in *Sub-Saharan Africa* is a consequence of the widespread of *An. gambiae* sl across the continent (Sinka *et al.*, 2010).

Malaria is known to be spread by *Anopheles* mosquitoes. The geographic distribution of these species varies, and they can be categorized as primary vectors – those that are most efficient in transmitting the parasite in a given region or secondary vectors – those that are of secondary significance in the epidemiology of the disease. The secondary vectors are sometimes disregarded, although research has shown that they may continue to transmit malaria when primary vector populations are reduced or eradicated (Antonio-Nkondjio *et al.*, 2006; Lobo *et al.*, 2015; Braack *et al.*, 2020). *Anopheles gambiae* sl, *An. funestus* group and other secondary vectors have been widely reported as important vectors of malaria parasites in *Sub-Saharan Africa* (Sinka *et al.*, 2010; Tabue *et al.*, 2017; Epopa *et al.*, 2019; Olatunbosun-Oduola *et al.*, 2019; Braack *et al.*, 2020; Bedasso *et al.*, 2022; Nelly Armanda Kala-Chouakeu *et al.*, 2022; Nkya *et al.*, 2022; Saili *et al.*, 2023). *Anopheles gambiae, An. coluzzii and An. arabiensis* were all incriminated in malaria transmission in Nigeria, (Awolola *et al.*, 2018; Olatunbosun-Oduola *et al.*, 2019; Wahedi *et al.*, 2021). Other Anopheline species reported in Nigeria are *An. funestus*; *An. moucheti*, *An. melas*, *An. leesoni, An. coustani, An. pharoensis, An. rufipes, An. nili, An. squamosus, An. pretoriensis, and An. longipalpis* (Awolola *et al.*, 2005; Oyewole *et al.*, 2007, 2010; Ebenezer *et al.*, 2014; Garba *et al.*, 2017; National Malaria Control Programme, 2020; PMI, 2021; Adeogun *et al.*, 2023).

There have been cases where the presumed secondary malaria vectors have been shown to play a significant role in the transmission of malaria. The contribution of these vectors were majorly outdoor (Goupeyou-Youmsi *et al.*, 2020; Joseph *et al.*, 2013; Tabue *et al.*, 2017), though they were also incriminated in indoor transmission (Saili *et al.*, 2023). The outdoor feeding and resting habits of secondary malaria vectors and their frequent contact with humans infected with *Plasmodium* species might elude vector control interventions (Ciubotariu *et al.*, 2020).

Limited data on malaria vectors is available in Southern Gombe and there is paucity of up-to-date community-specific data on the vectors in the region. A crucial part of the epidemiological research of malaria is the species-level identification of individual vectors within the same morphologically identical taxon. It is the first step in developing a strategic control programme and will aid in the incrimination of the malaria vectors in Gombe state. Though there were other national entomological surveillance of malaria vectors in Nigeria as reported by (Adeogun *et al.*, 2023), however, this study is the first report of community-specific data on the co-existence of both primary and alternate vectors of malaria in Southern Gombe, Northeastern Nigeria. This has a tremendous implication in the control of malaria vectors which currently mainly targets the mosquitoes biting humans indoors.

Materials and methods

Study Area

The study was carried out in four communities of Southern Gombe. Gombe state is located in the North-eastern part of Nigeria and has a land mass of 20,265 km² area. The state experiences both dry and wet seasons commencing between November - March and April – October respectively. With wide-open grassland that dries out during the dry season, the vegetation is typical of the Sudan Savanna. Gombe State is surrounded by Yobe State to the north, Adamawa and Taraba States to the south, Borno State to the east, and Bauchi State to the west. Most of the residents of Gombe South are farmers producing both food and cash crops. Yam, tomato, cassava, sorghum, maize, millet, cowpea, and peanut are some of its food crops, while cotton is grown mostly for export.

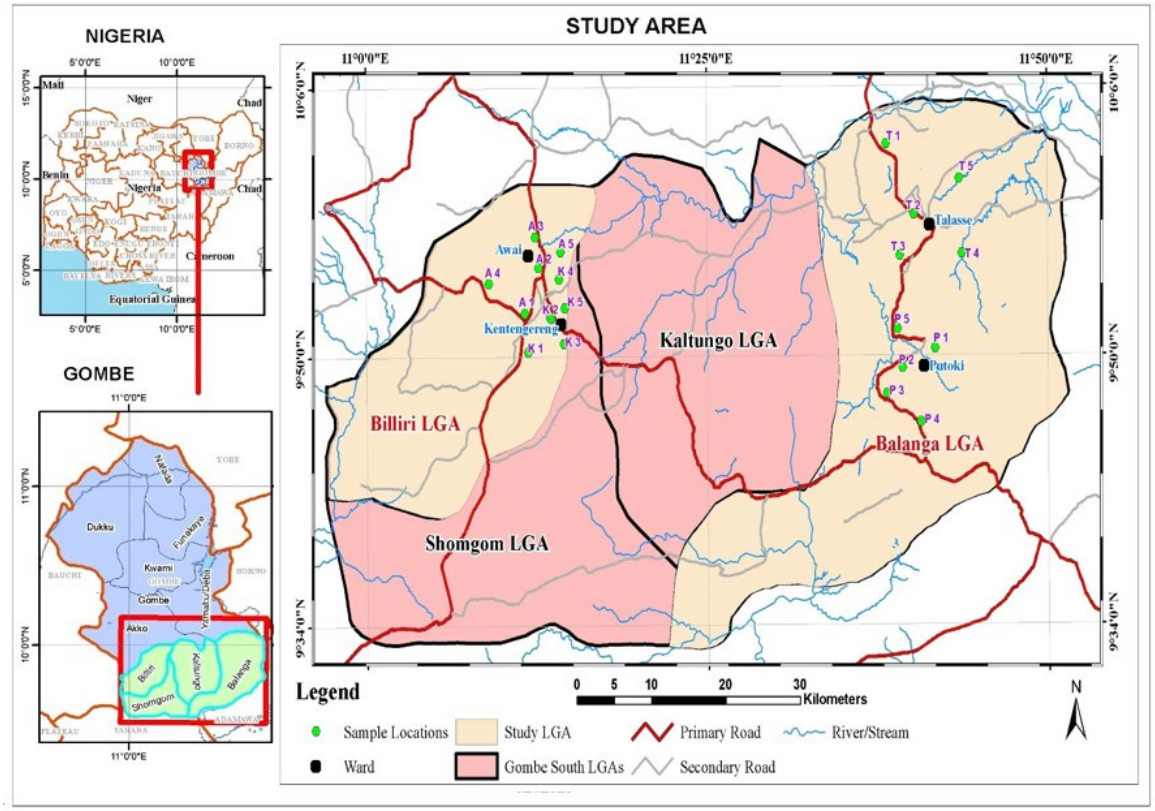

Fig. 1. Map of Study Area Showing Selected LGAs and Sample Locations in Southern Gombe

They also keep goats, cattle, sheep, and horses and practice the traditional crafts of weaving and dyeing cotton. Along with keeping cattle, goats, sheep, and horses, they also engage in traditional crafts like weaving and cotton dying. Gombe State has a malaria prevalence of 30% (National Malaria Control Programme, 2020). The GPS coordinates of the study communities are Awai (Lat. 9.902760^0, Long. 11.214893^0), Kentengereng (Lat. 9.883385^0, Long. 11.228432^0), Talasse (Lat. 9.973737^0, Long. 11.669845^0), Putoki (Lat. 9.839087^0, Long. 11.677787^0) (Fig. 1).

Study design

The study LGAs and communities were chosen based on ease in finding *Anopheles* mosquito breeding sites and preliminary studies conducted (unpublished data) using the Randomized sampling method. Breeding sites were sampled based on specific peculiarities/characteristics that *Anopheles* mosquito breeding sites possess as well as a good coverage of the community.

Collection and rearing of Anopheles mosquitoes

Between April and December 2022, mosquito larvae were collected simultaneously from natural development sites such as pools, ditches, streams, rice fields, and brick pits using dippers in each of the four study localities. The coordinates of the study communities and breeding sites were established using Global Positioning System (GPS). Larvae were transferred to the Gombe state sentinel insectary at the Gombe State University in small, clear containers. The larvae were then transferred to plastic trays (5cm x 27cm x 36cm) to an approximate depth of 2cm and each tray was then covered with nets labelled to reflect and labelled appropriately according to (WHO, 2016) protocol. The larvae were kept in trays at 25-28ºC and 76±5% relative humidity and fed with 200mg of ground fish food and yeast powder once every morning until their emergence into adults. The adults were then transferred to adult cages.

Morphological Identification of Anopheles mosquitoes

The morphological identification key (Coetzee, 2020) focusing on the Abdomen, legs, maxillary palps and wings was used in the identification. In all, 800 female *Anopheles* mosquitoes were randomly selected from the adults that emerged from each community and were morphologically identified.

The primer sequences are as follows:

IMP-UN	5’ GCTGCGAGTTGTAGAGATGCG 3’
AR-3T	5’ GTGTTAAGTGTCCTTCTCCGTC 3’
GA-3T	5’ GCTTACTGGTTTGGTCGGCATGT 3’

ME-3T	5' CAACCCACTCCCTTGACGATG 3'
QD-3T	5' GCATGTCCACCAACGTAAATCC 3'
IMP-S1	5' CCAGACCAAGATGGTTCGCTG 3'
IMP-M1	5' TAGCCAGCTCTTGTCCACTAGTTTT 3'

Molecular Identification of Sibling Species of An. gambiae s.l Using Standard Polymerase Chain Reaction (PCR).

For the discrimination of *An. coluzzii* and *An. gambiae* (formerly M and S molecular forms respectively), atleast 80 samples were randomly picked from the morphologically identified *An. gambiae* complex, but in other communities that have less than 80 in total, the available samples were used. The combined *An. gambiae* complex and ribosomal DNA type assay was used (Wilkins *et al.*, 2006). This technique for differentiating the *Anopheles gambiae* complex is based on species-specific single nucleotide polymorphisms (SNPs) in the intergenic spacer region (IGS). The technique is supplemented with primers to simultaneously reveal the type of ribosomal DNA. To boost specificity, these additional primers also include intentional mismatches (Intentional Mismatch Primers (IMPs).

Genomic DNA (gDNA) was extracted from the whole body of each mosquito using spin column DNA extraction kit (Jena Bioscience). Molecular identification of these mosquito samples was carried out using multiplex PCR as described by (Wilkins *et al.*, 2006). Seven primers including IMP-UN (Universal), AR-3T (*Anopheles arabiensis*), GA-3T (*Anopheles gambiae* s.s), ME-3T (*Anopheles merus*), QD-3T (*Anopheles quadriannulatus*), IMP-S1 (*Anopheles gambiae*) and IMP-M1 (*Anopheles coluzziii*) were used for molecular characterization of *Anopheles gambiae* s.l sibling species.

A reaction mixture of 12.5 µl PCR reaction was prepared for each sample. The mixture contains 5.5 µl of double distilled water, 2.5µl of mastermix (5X; Solis Biodyne), 0.5 µl of each of IMP-UN, ME-3T, AR-3T, QD-3T, GA-3T, IMP-S1 and IMP-M1 (0.4 µM) as well as 1.0µl of DNA template. PCR cycle conditions was carried out in a BIO-RAD T100 thermocycler included: initial denaturation at 95 °C for 5 mins, 35 cycles of 95 °C for 30 sec, 59.2 °C for 30 sec, 72 °C for 30sec and final extension at 72°C for 5 mins with 4°C hold. Thereafter, a 1.5% agarose gel stained with ethidium bromide was used to run 10 µl of the amplified samples. Primers created fragments of 463 *An. gambiae s.l*, 387 *An. arabiensis,* 333 *An. coluzzii* (M-form) and 221 *An. gambiae* (S-form) when viewed in the gel documentation machine (Fig. 3).

Data Analysis

The number of mosquitoes collected (mosquito genera and *Anopheles gambiae* s.l. sibling species) were compared across the four study communities and two LGAs using Analysis of Variance (ANOVA) to determine the level of significance ($p<0.05$) using Statistical Package for Social Sciences (SPSS) version 22 software (Chicago, IL, USA).

Results and Discussion

A total of 3200 female *Anopheles* mosquitoes were identified from the four different communities in Southern Gombe (Kentengereng, Awai, Putoki and Talasse), Northeastern Nigeria. Overall, *An. pretoriensis* was significantly different compared to the other *Anopheles* species identified ($p = <0.0001 - 0.0454$) and more abundant with 1662 (51.9%) followed by *An. gambiae s.l.* 868 (27.1%) which was significantly different compared to *An. coustani* and *An. pharoensis* ($p = 0.0258, 0.0249$ respectively). The number of other *Anopheles* species encountered as well as their respective percentages were *An. maculipalpis* 267 (8.3%), *An. rufipes* 252 (7.9%), *An. coustani* 10(0.3%) and *An. pharoensis* 6 (0.2%) and there was no significant difference between their abundance ($p = 0.9261$ to >0.9999). However, a total of 135 (4.3%) of the mosquitoes collected were unidentified. *An. pretoriensis* was the dominant species in Kentengereng (46.9%); Putoki (47.1%) and Talasse (81.8%). Out of 1600 mosquitoes identified morphologically from Billiri LGA, *An. pretoriensis* dominates with 631(39.4%) while the least was *An. pharoensis* 4(0.3%). Again, *An. pretoriensis* was dominant in Balanga LGA constituting 1031(64.4%) and *An. pharoensis* was the least 2(0.0%) (Fig. 2).

The result is at variance with previous studies, where they all showed the dominance of *An. gambiae* sl over other *Anopheles* mosquitoes (Yorio *et al.*, 2014; Garba *et al.*, 2017; Olatunbosun-Oduola *et al.*, 2019; Wahedi *et al.*, 2020). Olatunbosun-Oduola *et al.* (2019) collected samples in Gombe state from the same LGAs with this study and found a varying result. Though their sampling locations are not the same with this study, it is thought that the species diversity seen in this study might be a result of the larger coverage of this study. In this study, a total of 20 sampling locations were used for the sampling. The habit of most previous studies is that once the expected

number of sample sizes is reached (even if it is within a single point) collections are usually discontinued and analyzed. However, as a result of the design of this work, more breeding sites were sampled and pooled together to have an average of several breeding sites within the community. Secondly, the difference might be a result of differences in communities. Therefore, it could be rightly established that *An. pretoriensis* can be found in abundance in these communities. There was a report of the emergence of secondary malaria vectors such as *An. coustani, An. pharoensis and An. rufipes* in Northeastern Nigeria as reported by (Garba *et al.*, 2017). However, these species were not found to be dominant over the primary vectors as reported in the present study. Even though the scope of this study does not include the incrimination of the vectors for malaria transmission, however, these secondary vectors were incriminated for malaria transmission previously (Antonio-Nkondjio *et al.*, 2006; Joseph *et al.*, 2013; Tabue *et al.*, 2017; Goupeyou-Youmsi *et al.*, 2020; PMI, 2021; Bedasso *et al.*, 2022; Saili *et al.*, 2023; Assa *et al.*, 2023). This opens up a new conversation in policy making especially in Gombe state, as these secondary species mainly rest and feed outdoors (Bedasso *et al.*, 2022). In other parts of *Sub-Saharan Africa*, *Anopheles rufipes, An. coustani,* and *An. pretoriensis* have all been discovered to harbour *P. falciparum* sporozoites and all of these species were detected in Southern Gombe during our investigations, often as with *An. pretoriensis* and *An. rufipes.* Malaria transmission has been attributed to secondary malaria vectors as being significant. This is because they frequently exhibit outdoor resting and feeding habits which could lengthen the period that malaria transmission continues after primary vectors have been decreased by indoor control methods (Afrane *et al.*, 2016).

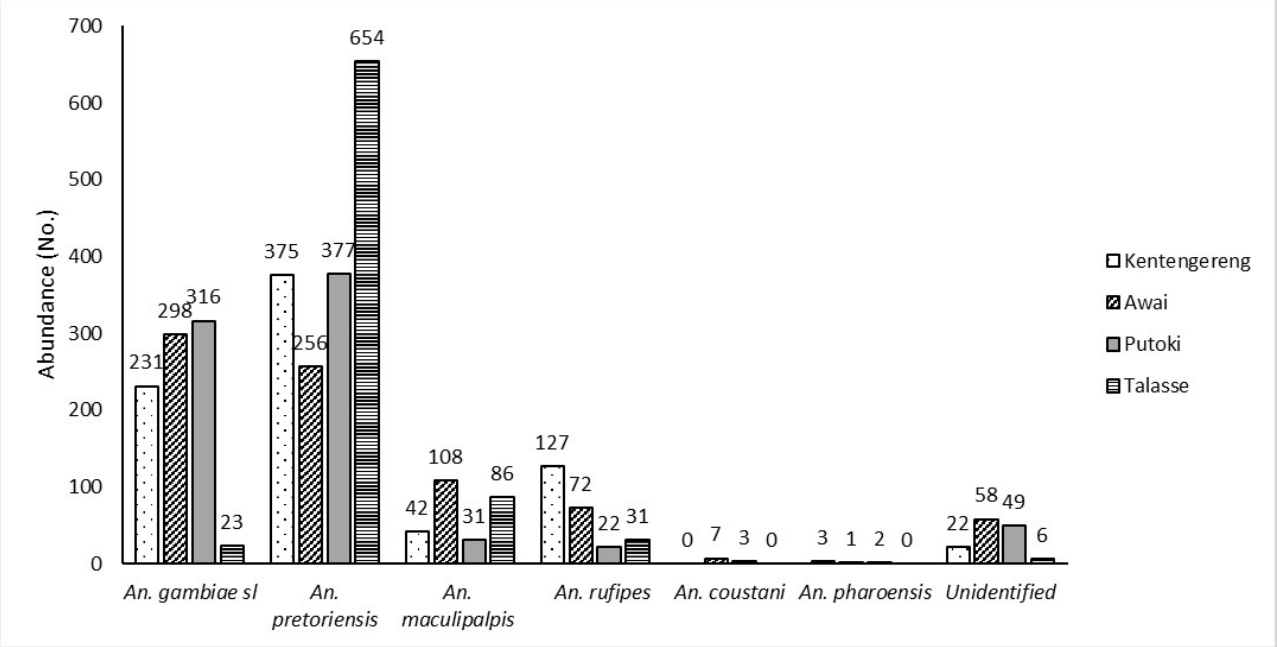

Fig. 2. Morphological Identification of female *Anopheles* mosquitoes in four communities of Southern Gombe

Table 1: PCR Molecular Identification of Sibling species of *Anopheles gambiae* sl. in Southern Gombe

LGA's	Communities	Number Assayed (N)	Species composition				
			An. gambiae	*An. coluzzii*	*An. arabiensis*	Hybrid	No Amplification
Billiri	Kentengereng	82	27(32.9)	35(42.7)	0(0.0)	1(1.2)	19(23.2)
	Awai	84	19(22.6)	52(61.9)	1(1.2)	1(1.2)	11(13.1)
Balanga	Putoki	81	13(16.0)	38(46.9)	0(0.0)	11(13.6)	19(23.5)
	Talasse	15	1(6.7)	10(66.6)	0(0.0)	3(20)	1(6.7)
	Total	262	60(22.9)	135(51.5)	1(0.4)	16(6.1)	50(19.1)

N= Total number of *Anopheles gambiae sl*, Numbers in brackets represent percentages

Since the majority of secondary vectors rest outdoor, existing management strategies do not address outdoor biting. Some of the variables that could affect the conversion of secondary vectors to primary vectors include the high rate of insecticide usage in vector control, climate change, and extraordinary changes in land use (Afrane *et al.*, 2016; Sinka, 2013). For instance, (Lafferty & Mordecai, 2016) reported that climate changes could lead to modification of vector behavior thereby affecting their distribution and eventually their ability to transmit diseases. Insecticide resistance is thought to impact greatly on malaria vector competence (Suh *et al.*, 2023), hence giving

the secondary vectors the advantage of being competent vectors when the primary vector populations are depleted. Many environmental conditions can directly impact vector behavior or pathogen transmission, which in turn can affect the occurrence and transmission of vector-borne diseases. By changing the life cycles of vectors or reservoirs, these factors may also indirectly affect vector-borne diseases, ultimately affecting their distribution and abundance (Caminade *et al.*, 2019). Surprisingly, in only in Awai, one of the four study communities, *An. gambiae* sl. were found to be dominant. This agrees with the findings of Olatunbosun-Oduola *et al.* (2019).

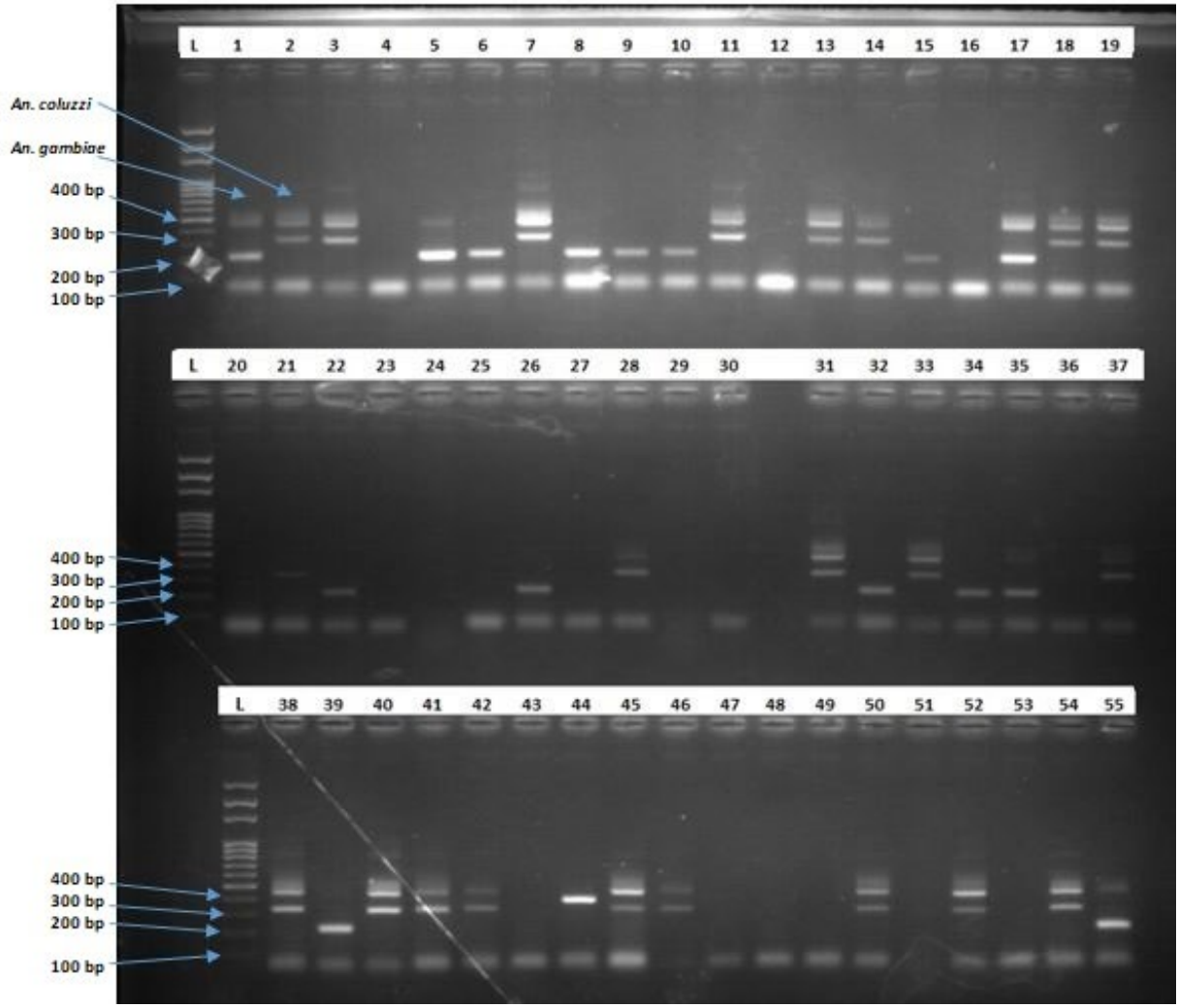

Fig. 3. PCR Gel Picture for the molecular identification of *Anopheles gambiae sl* sibling species. Fragments of 463 = *An. gambiae s.l.*; 333 = *An. coluzzii* (M-form) (Lane 2) and 221 *An. gambiae* (S-form) (Lane 1).

Overall, 262 *An. gambiae* s.l. were identified by the species-specific PCR method, where 135(51.1%) were found to be *An. coluzzii*, 60(22.9%) *An. gambiae* and only 1(0.4%) was *An. arabiensis*. There was a significant difference between the number of species identified as *An. coluzzii* and *An. arabiensis* ($p = 0.0025$). But there was no significant difference between *An. coluzzii* and *An. gambiae* ($p = 0.1212$). Hybrid constitute 16(6.1%) while 50(19.1%) samples did not amplify. Out of 166 mosquitoes identified from Billiri LGA, *An. coluzzii* constitutes the majority 87(52.4%) followed by *An. gambiae* 46(27.7%). Hybrid was only 2(1.2%) while the least was *An. arabiensis*. *An. coluzzii* was also the dominant species in Balanga LGA constituting 50% of the total collection while *An. gambiae* and Hybrid each numbered 14(14.6%). No *An. arabiensis* was found in Balanga LGA. (Table 1). The predominance of *An. coluzzii* is noted in this study. All these species were noted as the principal malaria vectors in Nigeria (Adeogun *et al.*, 2019; PMI, 2021; Adeogun *et al.*, 2023). The findings of (Ebenezer *et al.*, 2014; Ebenezer *et al.*, 2012; Olatunbosun-Oduola *et al.*, 2019) were contrary where they reported the predominance of *An. gambiae* against the findings of this study.

Conclusion

For the first time, both primary (*An. gambiae, An. coluzzii* and *An. arabiensis*) and secondary malaria vectors (*An. pretoriensis. An. rufipes, An. maculipalpis, An. coustani* and *An. pharoensis*) were found in sympatry in communities of

Southern Gombe, Northeastern Nigeria. These secondary vectors are known mainly to be exophilic and exophagic. However, the current malaria vector control strategies in Nigeria target endophilic and endophagic mosquitoes. This development poses a big threat to the control of malaria vectors and may reverse gains made so far. Therefore, it is necessary that the National Malaria Elimination Program re-strategizes its policy to meet the current challenge. Larviciding among other alternatives could be considered to target multiple mosquito species irrespective of the adult's feeding and resting behaviour.

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اولین گزارش از هتروژنیتی و شباهت ناقلان مالاریا در گومبه جنوبی از شمال شرقی نیجریه و پیامدهای آن بر کنترل ناقلان مالاریا

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چکیده

مطالعه ناقلان مالاریا در یک منطقه بخشی ضروری از یک برنامه موفق کنترل ناقلان مالاریا است. این مطالعه با هدف بررسی در چهار جمعیت از ناقلین مالاریا در جنوب گومبه، شمال شرقی نیجریه انجام شد. ۳۲۰۰ آنوفل ماده بالغ که از لاروها در چهار جمیت از دو منطقه دولتی محلی (LGA) در گومبه جنوبی پرورش یافته بودند، جمع آوری و شناسایی شدند. *Anopheles pretoriensis* با تعداد ۱۶۶۲ (۵۱/۹ درصد) گونه غالب بود و بعد از آن گونه‌های *An. gambiae* s.l. ۸۶۸ عدد (۲۷/۱ درصد)، گونه *An. maculipalpis* ۲۶۷ عدد (۸/۳ درصد)، *An. rufipes* ۲۵۲ عدد (۷/۹ درصد) و *An. coustani* ۱۰ عدد (۰/۳ درصد) و کمترین آنها *An. pharoensis* ۶ عدد (۰/۲ درصد) بود و باقیمانده آنها ۱۳۵ عدد (۴/۳ درصد) شناسایی نشدند. از ۲۶۲ *An. gambiae s. l.* با استفاده از روش PCR اختصاصی گونه شناسایی شدند، ۱۳۵ (۵۱/۵ درصد) به عنوان *An. coluzzii* ۶۰ (۲۲/۹ درصد) *An. gambiae s.l.* و تنها ۱ (۰/۴ درصد) *An. arabiensis* بود، در حالی که هیبرید ۱۶ (۶/۱ درصد) را تشکیل می‌دهد. برای تعیین تفاوت‌های قابل توجه در ترکیب گونه‌ها، نتایج حاصل از چهار محل مورد مطالعه با هم ترکیب شدند. *Anopheles pretoriensis* با تمام گونه‌های دیگر شناسایی‌شده تفاوت معنی‌داری داشت (p≤۰/۰۰۰۱–۰/۰۴۵۴). *An. gambiae* s.l. تفاوت معنی‌داری با *An. coustani* و *An. pharoensis* داشت (به ترتیب ۰/۰۲۴۹ و ۰/۰۲۵۸ =p). تفاوت معنی‌داری بین *An. maculipalpis* ، *An. Rufipes* و *Coustani An. pharoensis* وجود نداشت (به ترتیب ۰/۹۹۹۹≤ – ۰/۹۲۱ =p). بین تعداد گونه‌های شناسایی شده به عنوان *An. coluzzii* و *An. arabiensis* تفاوت معنی‌داری وجود داشت (۰/۰۰۲۵ =p). اما تفاوت معنی‌داری بین *An. coluzzii* و *An. gambiae* s.l. وجود نداشت (۰/۱۲۱۲ =p). هتروژنیتی و شباهت گونه های آنوفل مشاهده شده تهدیدی برای کنترل مالاریا است زیرا ناقلان ثانویه رفتاری دارند که از روشهای کنترل ناقلان فعلی فرار می کنند و بنابراین راهبردهایی نو جهت کنترل ناقل در منطقه مورد مطالعه به مصلحت است.

کلمات کلیدی: ناقل مالاریا، کنترل ناقل، گمبه، آنوفل

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