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Identification of the *Anopheles marshallii* group and vector species composition in the forest ecozone of Akwa Ibom State, Southern Nigeria

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Abstract

Background Accurate vector species identification is essential to distinguish the co-existence of primary and secondary vectors of malaria sustaining transmission in different settings. This evaluation reviews the identity, bionomics, and behaviour of malaria vectors where composition shifted in the forest ecological zone in Mkpat-Enin Local Government Area of Akwa Ibom State, Nigeria.

Methods Anopheline mosquito samples were collected from pyrethrum spray catch collections and CDC light traps from 2016 through 2022. Samples were morphologically identified and subjected to polymerase chain reaction assays and sequencing analysis to confirm the identity of unamplified and/or misidentified samples. Mosquito bionomics such as abundance, indoor and outdoor human biting rates (HHR), indoor resting density (IRD), biting time, *Plasmodium falciparum* infection and blood meal sources of the mosquito samples were determined from the 2021 and 2022 samples. Data analysis was performed using one-way ANOVA (analysis of variance) tests and chi-square tests in GraphPad Prism version 9.0.

Results A total of eight *Anopheles* species were identified: *Anopheles coustani*, *Anopheles funestus* group, *Anopheles gambiae* sensu lato (*s.l.*), *Anopheles maculipalpis*, *Anopheles marshallii* group, *Anopheles moucheti*, *Anopheles nili, and Anopheles obscurus*. The outcome of the analysed data confirmed a shift in the composition from *An. gambiae s.l.* to the *An. marshallii* group. The IRD, indoor and outdoor HBR of the *An. marshallii* group were not significantly different from that of *An. gambiae s.l.* in 2021 ($p > 0.05$) but significantly higher in 2022 ($p < 0.05$). The peak indoor and outdoor HBR of the *An. marshallii* group were observed to be higher than those of *An. gambiae s.l.* during the dry season. In both years, the *An. gambiae s.l.* and the *An. marshallii* group mosquitoes showed comparable preference for bovine blood meal. The preference for human blood meal was not significantly different ($p = 0.21$) between both species. The *An. marshallii* group was found to have been infected with *Plasmodium falciparum* sporozoites ($n = 2$) alongside *An. gambiae* sensu stricto (*s.s.*) ($n = 1$) and *Anopheles coluzzii* ($n = 2$).

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Conclusion The *An. marshallii* group was confirmed as an abundant malaria vector species in.

Mkpat-Enin Local Government Area of Akwa Ibom State. While similar indoor vector control interventions are recommended for both species; other interventions may be needed to address the early evening outdoor biting observed in the *An. marshallii* group.

Keywords *Anopheles marshallii*, Malaria vector dynamics, Scale morphological identification, Phylogeny, Species composition, Forest ecology

Background

Malaria is a major public health problem in Nigeria, and the country accounts for 31.1% of the global burden for malaria [1]. Nigeria has five major ecological zones that have been reported to support diverse *Anopheles* species. Some of these anopheline species are primary or major vectors while some are regarded as secondary and minor vector species. However, the diversity and abundance of these malaria vectors vary by ecozone. Available data have identified between 10 and 12 *Anopheles* species associated with malaria transmission across six states in the five ecological zones of Nigeria [2, 3]. The *Anopheles gambiae* sensu lato (*s.l.*) and *Anopheles funestus* group consist of several species including efficient primary vector species involved in the transmission of malaria in Africa, including Nigeria. Members of *An. gambiae s.l.* are widespread in Nigeria regardless of the ecological regions. The *An. funestus* group also is an efficient vector but is known to have limited distribution based on its eco-typic breeding site requirement [4–6]. Other vector species that have been identified in Nigeria and are considered as secondary vectors of malaria across Africa include, but are not limited to: *Anopheles coustani* complex, *Anopheles pharoensis*, *Anopheles pretoriensis, Anopheles rivulorum*, *Anopheles rufipes* and *Anopheles squamosus* [4, 6].

Each *Anopheles* species exhibits different behaviours, for example, peak time of biting, blood meal preferences and location of feeding and resting, which can influence the role in malaria transmission and the choice of vector control intervention. The co-existence and behaviours of these vector species largely influence their ability to survive and sustain malaria transmission in different localities at different times of the year, contributing to varied vectoral capacities and relative roles in transmission dynamics. As such, accurate species identification and understanding of vector behaviour, distribution and other bionomic factors have become essential to proffer effective vector control decisions.

Integrated vector management is featured prominently in the Nigeria National Malaria Strategic Plan (NMSP) 2021–2025, which aims to reduce parasite prevalence from 27 to 10% and malaria-related deaths to less than 50 per 1000 live births by 2025 [7, 8]. Such lofty goals require understanding bionomics of vector species and the impacts of vector species on malaria transmission in each ecological setting in the country. Currently, insecticide-treated nets (ITNs) are the main vector control tool in the country.

Akwa Ibom State, in the forest ecological zone of southern Nigeria, contributed an estimated 2.8% of the 68 million malaria cases in Nigeria, and microscopy showed an increase in malaria prevalence from 22.8% in 2015 to 30.1% in 2021[8]. In Akwa Ibom, over 10.6 million ITNs have been distributed since 2009, most recently in 2022, with about 3.6 million alpha-cypermethrin + Piperonyl butoxide ITNs distributed. However, the utilization rate of ITNs has decreased, from 36.8% in 2015 to 17.8% in 2021[8].

This evaluation reviews the entomological surveillance activities at the Akwa Ibom site over seven years (2016–2022) and documents a shift in vector species composition over that period. It also outlines procedures carried out in 2021–2022 to investigate the identity, bionomics and behaviour of the preponderant *An. marshallii* group, co-existing with *An. gambiae s.l.* in Akwa Ibom State.

Methods

Study area

The collections were made in Ibekwe Akpayan community, Mkpat Enin Local Government Area (LGA), Akwa Ibom State (Fig. 1). Akwa Ibom is in the mangrove/tropical rain forest ecological zone in southern Nigeria. Mkpat Enin LGA is one of 31 LGAs in Akwa Ibom; it is located at 5.25°N 7.50°E, approximately 60 kms (37 miles) south of Uyo, the capital of the state. Most of the people in Mkpat Enin LGA are farmers, fishers and traders, and the area is also rich in oil and natural gas, with a growing industrial sector.

Mosquito collection

Centers for disease control and prevention light trap collections

From October 2016 to September 2022, every year and for three consecutive nights per month, two Centers for Disease Control and Prevention light traps (CDC LTs) (each with one human sleeper) were placed indoors and outdoors at four different houses within the Ibekwe

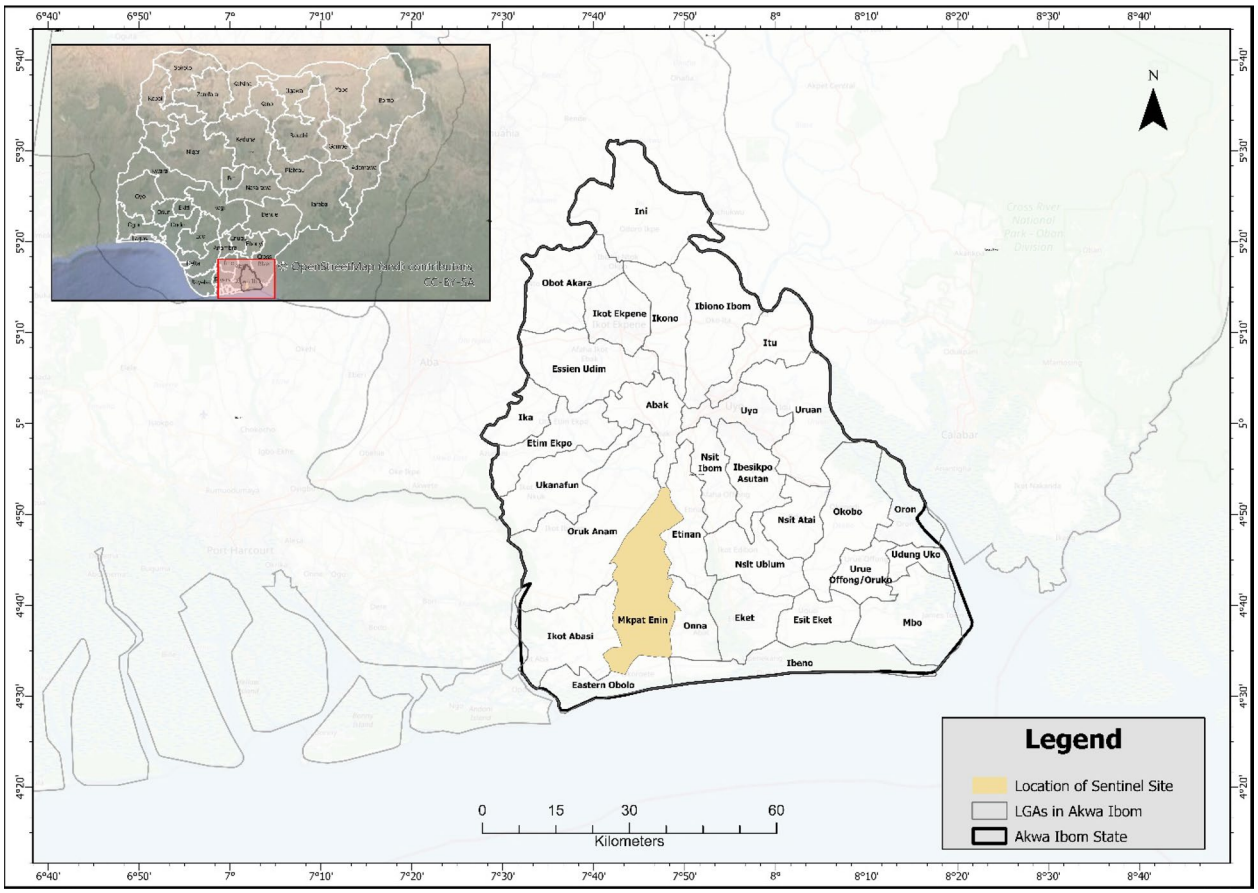

Fig. 1 Map of Akwa Ibom State, Nigeria, showing the surveillance area

Akpayan community (Fig. 1) to measure mosquito biting time indoors and outdoors. The houses selected for the CDC trap collections were maintained throughout the period of collection. The CDC LTs were hung from the ceiling at the foot of the bed occupied by a person sleeping under an untreated net [9]. CDC LTs were operated from 6 p.m. to 6 a.m. with collection cups changed hourly throughout the night. The LT collections were used as a proxy for human landing catches to determine the peak biting times and locations. The mean indoor and outdoor human biting rate (HBR) was calculated as the number of mosquitoes collected per human-baited CDC LT per night.

Pyrethrum spray catch collections

The sample size required 32 trap nights for PSC collections. This was now divided into 10 houses for day 1, and 11 houses, for day 2 and 3 respectively. The minimum number of houses allowed according to protocol is six per location. The collections were carried out each in two different areas per month within the Ibekwe Akpayan community. The PSC method [10] was carried out to monitor indoor resting mosquitoes. The PSC was conducted between 6 to 10 am. The mean indoor resting density was determined by calculating the number of mosquitoes per house per collection day over a period of 7 years.

Identification of malaria vectors

All *Anopheles* mosquito samples collected by both CDC LTs and PSCs were sorted and identified morphologically [11, 12], at the Nasarawa State University Keffi laboratory, and preserved in a labeled Eppendorf tube containing desiccated silica gel. Preserved samples were then sent to Nigeria Institute of Medical Research (NIMR) in Lagos for molecular species identification, sporozoite detection and identification of blood meal source. At NIMR, twenty percent of mosquitoes collected and initially morphologically identified as *An. gambiae s.l.* were subjected to the *An. gambiae* species-specific multiplex polymerase chain reaction (PCR) [13, 14]. Records of the number of samples from Akwa Ibom that successfully amplified or failed amplification between 2016 and 2020 were obtained from the database to provide background on the trend

of non-amplifications. The high proportion of unamplified samples from yearly database records between 2017 and 2018 ranged between 25% and 30%, thus requiring additional laboratory analysis at Wits Research Institute for Malaria (WRIM), University of Witwatersrand, Johannesburg, South Africa. Samples were labelled with unique codes prior to analysis. Confirmation of these samples was conducted at WRIM using scale morphology, followed by PCR of the internal transcribed spacer (ITS2), cytochrome oxidase subunit I (COI) and nucleotide sequencing.

Molecular identification of mosquito samples

Two batches of fresh mosquito samples were collected from Akwa Ibom and set aside for confirmation in WRIM. These were preserved in self-desiccating silica gel in microcentrifuge tubes and sent to WRIM in July and December 2021. The first batch of the unknown mosquitoes sent in July 2021 was drawn from PSC and CDC LT collections while the second batch was from CDC Light trap collections in December 2021. Out of the second batch, only twenty were morphologically identified as *Anopheles hargreavesi* (using scale morphology) and selected for molecular confirmation. They were processed for DNA extraction and amplification of the ITS2 and COI using standard protocols [15]. Amplicons were subsequently purified, sequenced and analysed.

The ITS2 sequences were initially aligned using the Seqman Pro Assembler (Lasergene v 10.1.1) with a minimum match of 95%. Assembled contigs were manually examined for insertions, deletions and repeat structures that may impact assembly, inflate divergence and decrease identity scores. These contigs were further divided into sub-contigs based on consistent single nucleotide polymorphisms. A low limit of 98% identity was used to assemble ITS2 sequences, and low-quality or contaminated sequences were not included in the analysis. The consensus sequences of these ITS2 contigs were compared (using Basic Local Alignment Search Tool, BLASTn) to the National Center for Biotechnology Information (NCBI) database for species identification.

The COI sequences were similarly sequenced and assembled and compared to the NCBI and BOLD56 (The Barcode of Life Data Systems) databases for confirmation of species identities. Sequence groups (henceforth called 'species') were merged (minimum identity of 94%) when COI BLASTn results indicated that they belonged to the same species for a final minimum match of greater than 95%. These sequences obtained from the BLASTn tool were compared to the NCBI database (https://blast.ncbi.nlm.nih.gov). Sequences were submitted to the NCBI database (ITS2: PP915781-PP915785; COI: PP919268-PP919271).

Phylogenetic analysis was used to determine the evolutionary relationships among the different species. The size of sequenced ITS2 and COI regions was compared to reference sequences from GenBank and used for analysis and phylogenetic tree construction using Molecular Evolutionary Genetics Analysis (MEGA XI) software [16]. Phylogenetic trees were generated using the Neighbor-Joining and Maximum Likelihood methods. The Neighbour-Joining method constructs a tree by using the matrix of genetic distances between the samples analysed and those downloaded [17], whereas Maximum Likelihood uses evolutionary models to identify the evolutionary trees with the highest probability of explaining the sequence relationships [18]. Bootstrap support was computed for both trees using 1,000 replications (see Additional File).

Assays for blood meal source determination

To determine the blood meal sources of the anopheles' mosquitoes, twenty percent of PCR identified mosquitoes at different abdominal stages of blood meal were analysed. The number of samples making up this 20% was calculated from the individual mosquito proportion randomly selected from the engorged mosquitoes from representative months, year and different sampling methods. While significant proportion of blood fed mosquitoes was observed in mosquitoes collected by PSC (174 in 2021, and 196 in 2022), very limited proportion of CDCLT collected mosquitoes were blood fed. The blood meal source from the abdominal cuttings of the mosquitoes was determined by enzyme-linked immunosorbent assays (ELISA) testing for human, bovine and goat blood meal sources of *Anopheles* mosquitoes [19]. The blood meal index was thereafter calculated.

Plasmodium sporozoite determination

ELISA assays, which detect the *Plasmodium falciparum* sporozoite antigen according to methods described by Burkot et al. [20] were carried out on randomly selected mosquitoes collected from PSC samples. Positive samples were boiled and retested according to Durnez et al. [21].

Data analysis

The proportion of each identified mosquito species was calculated as a percentage of each species out of the total *Anopheles* collected. The mean indoor resting density of each *Anopheles* species collected using PSC was calculated monthly by dividing the total number of mosquitoes collected by the total number of houses visited. The sporozoite infection rate, measured as the proportion of mosquitoes found with circumsporozoite antigen by ELISA after boiling, was calculated by dividing the

number of positive mosquitoes by the total number of mosquitoes tested. The mean sporozoite rate represents the average of all monthly infection rates per site and per species tested. The human blood index was calculated as the proportion of mosquitoes found to contain human IgG by ELISA out of the total mosquitoes tested. Data analysis was performed using one-way ANOVA (analysis of variance) tests and chi-square tests in GraphPad Prism version 9.0.

Results

Vector species composition

A total of eight different species of *Anopheles* mosquitoes were identified from human-baited CDC LT (indoors/outdoors) and PSC collections made in Akwa Ibom LGA over a period of seven years (2016–2022). The mosquito species identified belong to *An. coustani, An. funestus* group, *An. gambiae s.l., Anopheles maculipalpis*, *Anopheles marshallii* group, *Anopheles moucheti*, *Anopheles nili,* and *Anopheles obscurus* (Table 1). Results show that some of the samples were conclusively identified as *An. hargreavesi* (n = 8, 16%) based on morphological identification using scales, while most could not be identified due to poor sample condition and were therefore categorized as *An. marshallii* group (n = 42, 84%). Evidence from molecular tools employed to analyse samples wrongly identified *An. gambiae s.l.* as the predominant vector species in the first five (2016–2020) of the seven years of collection. The identification of the *An. marshallii* group was in the study sites; beginning in 2021, buttresses the relevance of molecular tools periodic checks and refresher training in mosquito identification (Table 1). In 2021, there was no significant difference between *An. gambiae s.l.* and the *An. marshallii* group in the monthly indoor ($p = 0.06$) and outdoor collections ($p = 0.1$). However, in 2022, a significant increase in *An. marshallii* group preponderance was recorded monthly, both indoors ($p = 0.001$) and outdoors ($p = 0.001$). Overall numbers of *An. gambiae s.l.* and *An. marshallii* group for 2021 and 2022 showed significantly greater preponderance ($p = < 0.00001$) of the *An. marshallii* group over *An. gambiae s.l.*

Molecular characterization of *Anopheles gambiae s.l.* collected from Akwa Ibom

Anopheles gambiae sensu stricto (*s.s.*), *Anopheles coluzzii*, *Anopheles arabiensis* and hybrid *An. gambiae* s.s./*An. coluzzii* were identified in varying proportions as members of the *An. gambiae* complex from the collections in Akwa Ibom. Within the Gambiae complex, *An. gambiae s.s.* was found to be the predominant species in 2016 (72.5%), 2017 (57.1%), 2018 (37.5%), 2019 (44.6%), 2020 (57.8%) and 2021 (50.6%). The proportion of *An. coluzzii* increased from 9.3% in 2016 to 68.7% in 2022, and it was the predominant species in 2022 (Fig. 2).

Confirmation of the *An. marshallii* group collected from Akwa Ibom

Morphological identification of unamplified samples

A retrospective analysis of PCR assayed samples over a 7-year period (2016–2022) showed proportions of unamplified samples were consistently greater than 5% of the samples, and as high as nearly 30% (during the 2017 reporting period) (Fig. 2). In 2022, the 50 samples sent to WRIM for confirmatory species identification, 8 (16%) were conclusively identified as *An. hargreavesi* based on thorax scale morphology, a key characteristic [12] The remaining 42 (84%) samples were generally categorized as *An. marshallii* group because of the loss of scales and were subjected to additional molecular analysis (Table 2).

Table 1 Species composition of the *Anopheles* mosquitoes by collection method, CDC LT and PSC, collected from Akwa Ibom State, Nigeria, 2016–2022

Species	2016			2017			2018			2019			2020			2021			2022		
	CDC LT		PSC	CDC LT		PSC	CDC LT		PSC	CDC LT		PSC	CDC LT		PSC	CDC LT		PSC	CDC LT		PSC
	In	Out	In	In	Out	In	In	Out	In	In	Out	In	In	Out	In	In	Out	In	In	Out	In
An. gambiae s.l	1175	576	210	1020	426	278	405	75	178	1063	332	475	1059	358	460	122	27	178	83	6	192
An. marshallii	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	885	201	132	1292	270	190
An. moucheti	257	85	12	205	102	90	250	22	119	–	–	–	25	6	11	9	0	0	–	–	–
An. maculipalpis	–	–	–	–	–	–	–	–	–	18	16	0	13	0	1	8	0	0	–	–	–
An. nili	–	–	–	–	–	–	–	–	–	–	–	–	1	0	0	–	–	–	–	–	–
An. coustani	1	0	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
An. funestus	1	1	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
An. obscurus	0	1	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Total	2319			2121			1049			1904			1934			1562			2033		

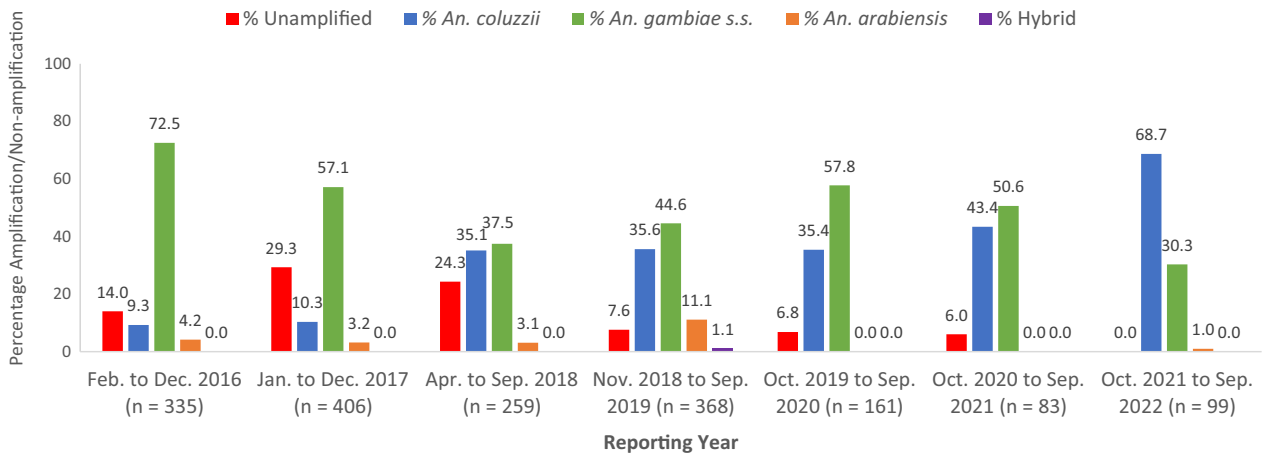

Fig. 2 Composition of members of the Anopheles gambiae complex and unamplified mosquito samples collected from Akwa Ibom State, Nigeria, 2016–2022

Sequence and phylogenetic analysis of unamplified samples

COI alignment of samples that were unamplified after the confirmatory morphological identification step showed a 93% similarity with *An. marshallii* mitochondrion, complete genome (Sequence ID: NC064607.1). However, the NCBI BLASTn showed 80% similarity in the ITS2 sequence alignment between the presumed *An. hargreavesi* and *Anopheles gibbinsi* (previously referred to as *An. species* 6 and also a member of the *An. marshallii* group). The phylogenetic analysis using ITS2 (Fig. 3) and COI (Fig. 4) showed the closest relationship of the unknown samples (labelled in Fig. 3 and Fig. 4 as the "Nigeria _Akwa Ibom_ [ITS2/COI] consensus sequence") with members of the *An. marshallii* group. Importantly, *An. hargreavesi* was absent from the nucleotide database and therefore sequence and phylogenetic alignments to this species were not possible.

Comparison of indoor resting density of *Anopheles gambiae s.l.* and *Anopheles marshallii* group

The indoor resting density of *An. gambiae s.l.* and *An. marshallii* group mosquitoes varied across the months. In 2021, *An. gambiae s.l.* had a peak indoor resting density of 2.5 mosquitoes/room/day in July (Fig. 5). During the same period, May–July, the *An. marshallii* group had lower peak (0.9 mosquitoes/room/day) steadily increasing towards August peak. In 2022, the *An. marshallii* group peaked in August at 2.7 mosquitoes/room/day. During the May and August period *An. gambiae s.l.* had low peaks (0.3 mosquitoes/room/day) (Fig. 5). The average overall indoor resting densities of *An. gambiae s.l.* and the *An. marshallii* group were not significantly different in 2021 ($p=0.6$), but in 2022, the indoor resting density of the *An. marshallii* group was significantly higher than that of *An. gambiae s.l.* ($p=0.01$).

Comparison of human biting ates of *Anopheles gambiae s.l.* and *Anopheles marshallii* group

Trends in the monthly mean bites/person/night (b/p/n) of *An. gambiae s.l.* and the *An. marshallii* group over time differed in the rainy season (June–October) and dry season (November–May). The mean indoor biting rates of *An. gambiae s.l.* was generally low throughout the study period with the highest HBR of 4.4 b/p/n in July 2022 (Fig. 6). The *An. marshallii* group predominated toward the time when the rains were receding and in the dry season, with peak mean HBRs recorded in November 2020 (23.9 b/p/n), September 2021 (26.6 b/p/n), January 2022 (21.8 b/p/n) and August 2022 (18.3 b/p/n) The overall average indoor HBR of the *An. marshallii* group mosquitoes was not significantly higher than *An. gambiae s.l.* in 2021 ($p=0.07$), but it was significantly higher in 2022 ($p=0.001$) (Fig. 6).

Similar to the patterns of the indoor HBRs, the outdoor HBRs were lower overall for both *An. gambiae s.l.* and the *An. marshallii* group. *Anopheles gambiae s.l.* had the highest HBR, recorded in September 2021 at 0.8 b/p/n (Fig. 7), while for the *An. marshallii* group it was highest during the drier seasons of November 2020 (6.4 b/p/n), September 2021 (6.3 b/p/n) and December 2021 (5.3 b/p/n) (Fig. 7). Outdoor HBR indicated a similar pattern to indoor HBR with no significant difference between the two species complexes in 2021 ($p=0.09$) and significant differences observed in 2022 ($p=0.02$).

Table 2 Sporozoite positivity rates of Anopheles mosquitoes collected by CDC LT in Akwa Ibom State, Nigeria, 2021–2022

		An. gambiae s.s						*An. coluzzii*						*An. arabiensis*							*An. marshallii* group					
		Number identified (%)		No. positive for sporozoites		SPR (%)		Number identified (%)		No. positive for sporozoites		SPR (%)		Number identified (%)		No. positive for sporozoites		SPR (%)			Number identified (%)		No. positive for sporozoites		SPR (%)	
		In	Out	In	Out	In	Out	In	Out	In	Out	In	Out	In	Out	In	Out	In	Out		In	Out	In	Out	In	Out
2021	30	13 (43.3)	6 (20)	0	0	0	0	11 (36.7)	0 (0)	0	0	0	0	0 (0)	0 (0)	0	0	0	0	50	50 (100)	0 (0)	0	0	0	0
2022	39	6 (15.4)	2 (5.1)	0	0	0	0	28 (71.8)	3 (10.7)	0	0	0	0	0 (0)	0 (0)	0	0	0	0	635	507 (100)	128 (100)	0	0	0	0

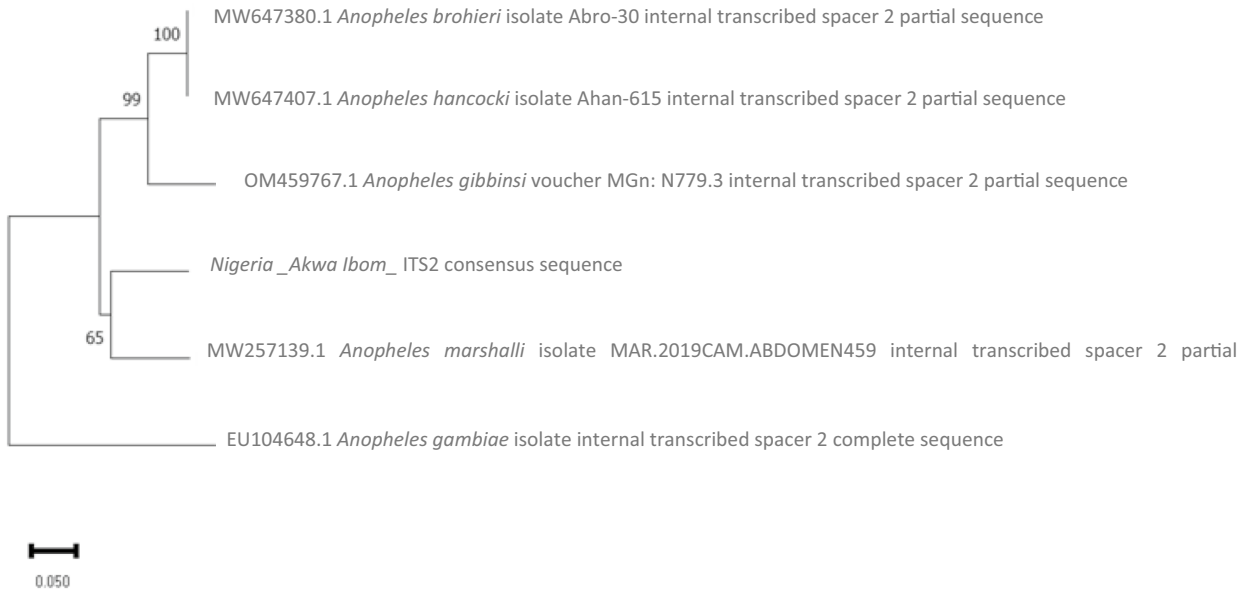

Fig. 3 Phylogenetic tree of unknown samples (presumed *Anopheles marshallii* group) from Akwa Ibom State, Nigeria, based on rDNA ITS2 sequence data

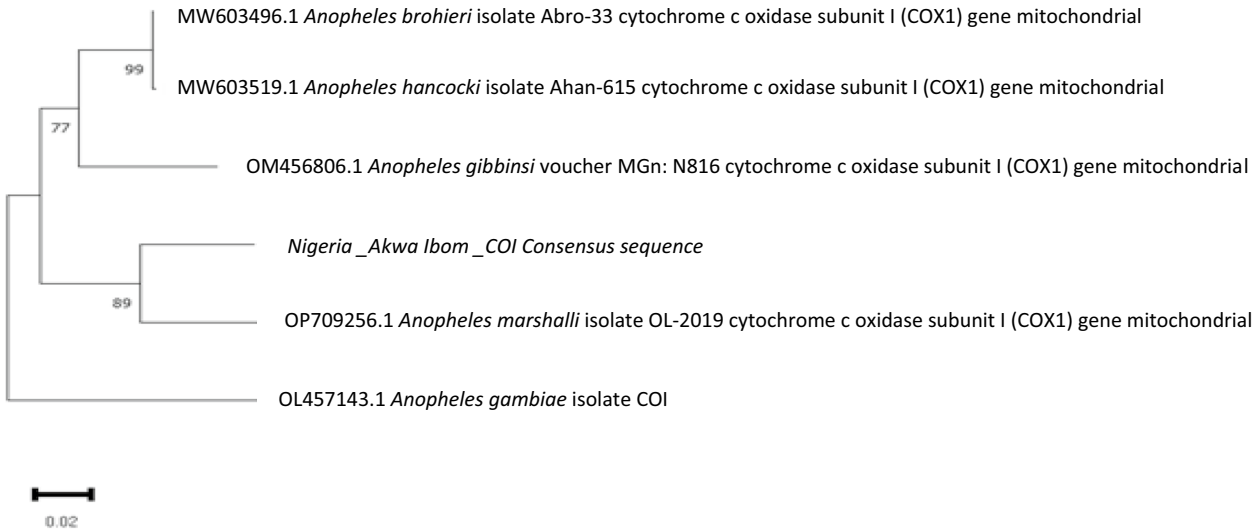

Fig. 4 Phylogenetic tree of unknown samples (presumed *An. marshallii* group) from Akwa Ibom State, Nigeria based on mtDNA COI sequence data. Figure 3 and Fig. 4: The evolutionary history was inferred using the Neighbor-Joining method [22]. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [17]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [23] and are in the units of the number of base substitutions per site. The consensus sequence is based on six nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 672 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [24]

Comparison of biting time of *Anopheles gambiae s.l.* and *Anopheles marshallii* group

Overall, *An. gambiae s.l.* had consistently low levels of biting throughout the night with a small peak 0.13 (± 0.08) mosquitoes/person/hour) between 1 a.m. and 2 a.m. in 2020. In contrast, the *An. marshallii* group had overall much higher biting, 1.5 (± 0.08) mosquitoes/person/hour) in 2021 between 2 a.m. and 3 a.m. (Fig. 8). The proportion of the *An. marshallii* group collected between 6 p.m. and 6 a.m. was significantly higher than that of *An.*

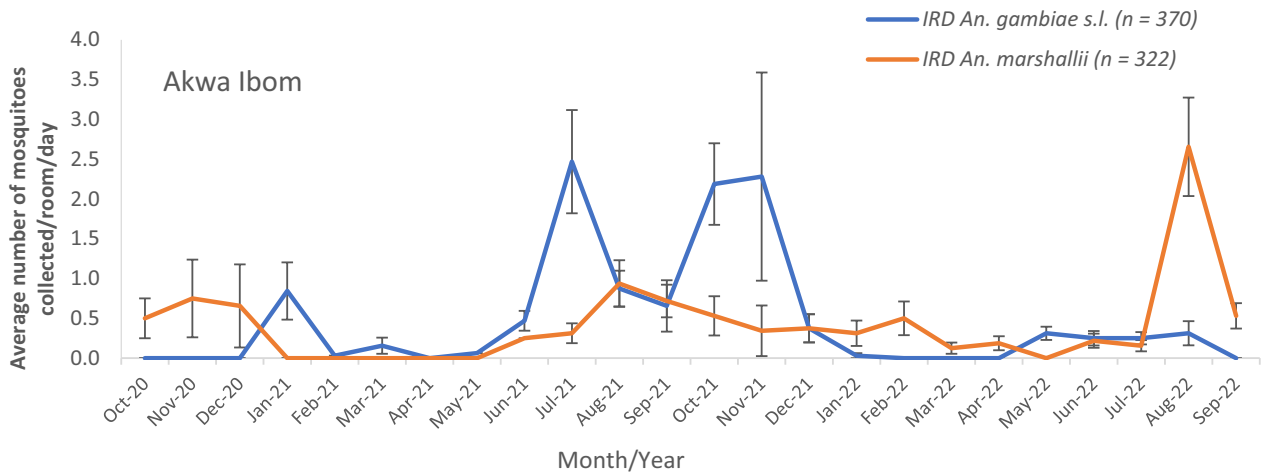

Fig. 5 Monthly indoor resting density of *Anopheles gambiae s.l.* and *Anopheles marshallii* group in Akwa Ibom State

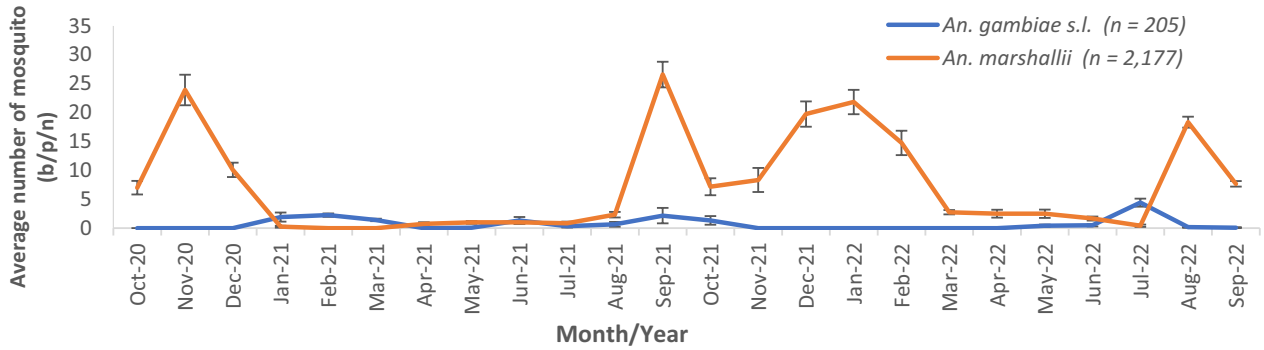

Fig. 6 Monthly mean indoor human biting rates of *Anopheles gambiae s.l.* and *Anopheles marshallii* group in Akwa Ibom State, Nigeria, 2020–2022

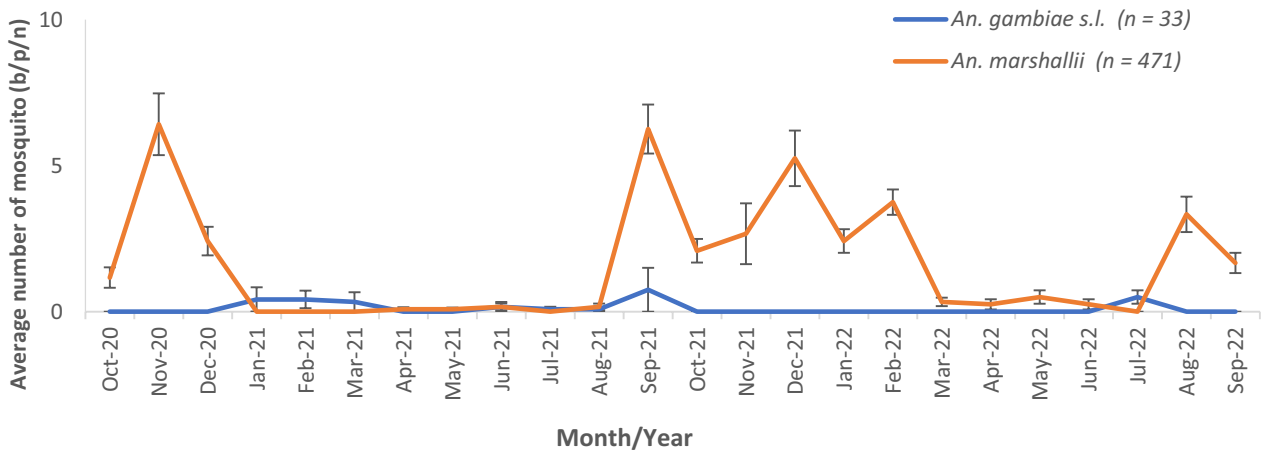

Fig. 7 Monthly mean outdoor human biting rates of *Anopheles gambiae s.l.* and *Anopheles marshallii* group in Akwa Ibom State, Nigeria, 2020–2022

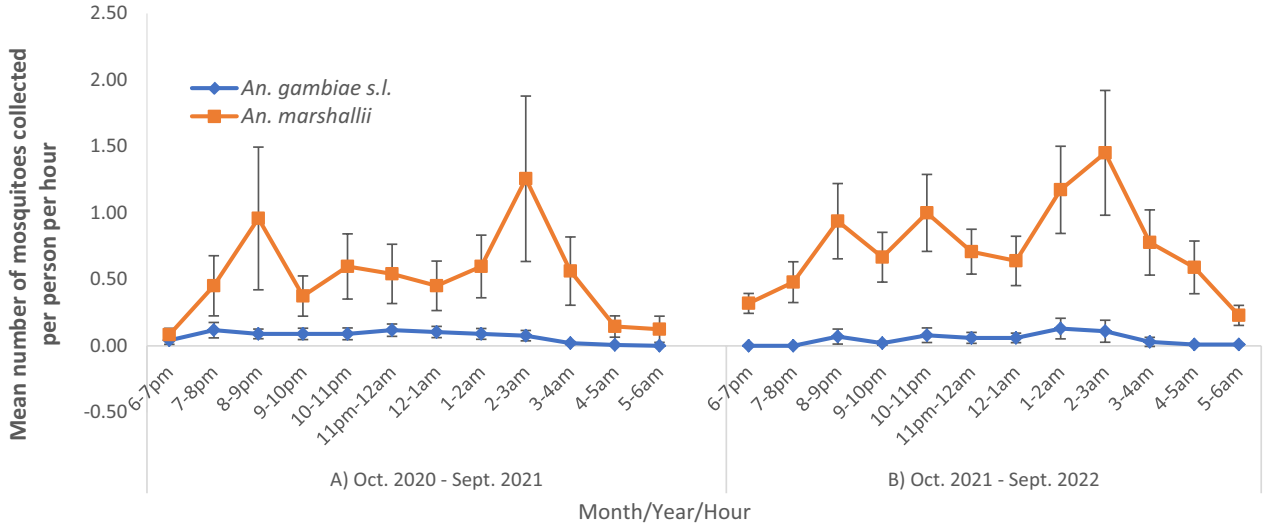

Fig. 8 Mean indoor biting rates of *Anopheles gambiae s.l.* and *Anopheles marshallii* group in Akwa Ibom State, Nigeria, 2020–2022

gambiae s.l. both indoor and outdoor in 2021 and 2022 ($p < 0.05$) (Fig. 8 and Fig. 9).

Similar to indoor biting, the average outdoor biting for *An. gambiae s.l.* was low with a peak of 0.11(± 0.06) mosquitoes/person/hour between 8 p.m. and 9 p.m. (Fig. 9) compared to the *An. marshallii* group, with 0.5 (± 0.02) mosquitoes/person/hour caught biting outdoors between 9 p.m. and 10 p.m. in 2021 (Fig. 9).

Comparison of blood meal sources and human blood index determination of *Anopheles gambiae s.l.* and *Anopheles marshallii* group

Three blood meal sources were identified in the blood meal analysis: human, goat and bovine. The analysis found that human blood was by far the largest type of blood meal for *An. gambiae s.l.* and the *An. marshallii* group collected by the PSC method. There was no significant difference ($\chi^2 = 1.7012$, $p = 0.21$) between the two mosquito species groups (Fig. 10). The proportions of mosquitoes that fed on goats and bovines were much lower. The *An. marshallii* group showed comparable preferences for these alternative blood meal sources (bovine

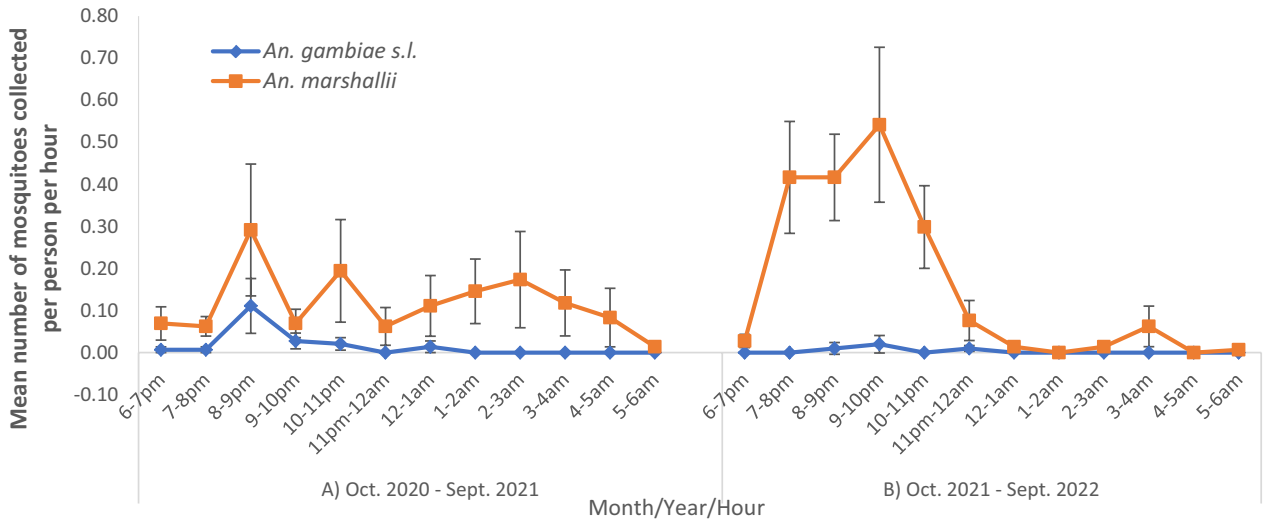

Fig. 9 Mean outdoor biting rates of *Anopheles gambiae s.l.* and *Anopheles marshallii* group in Akwa Ibom State, Nigeria, 2020–2022

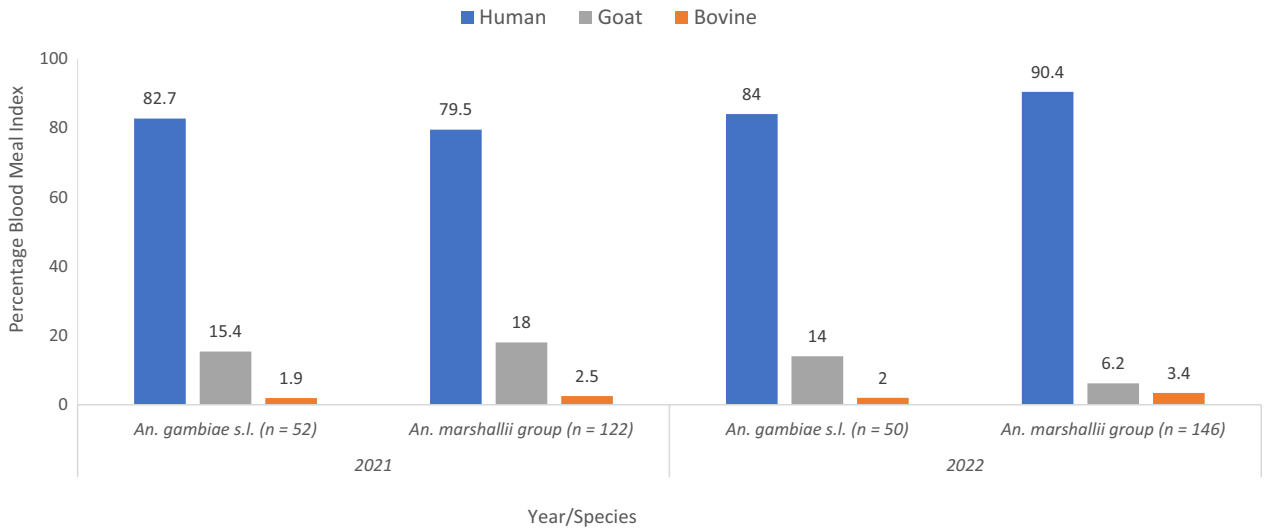

Fig. 10 Percentage of blood meal index of *Anopheles gambiae s.l.* and *Anopheles marshallii* group from pyrethrum spray collections in Akwa Ibom State, Nigeria, 2020–2022. *Please note that the mosquitoes analysed for blood meal reflected more of PSC because the sampling method had more mosquitoes with abdomen engorged with blood. Only a few of the CDCLT collected mosquitoes were blood fed and an attempt to present this gave a false high percentage of blood meal source

2.5%–3.4% and goat 6.2–18%) as did *An. gambiae s.l.* (bovine 1.9%–2.0% and goat 14.0%–33.3%) (Fig. 10). The variance in the preferences for each of the three blood meal sources were not significantly different (p = 0.28) between the two mosquito species groups.

Comparison of *Plasmodium falciparum* infectivity rates of *Anopheles gambiae s.l.* and *Anopheles marshallii* group

Plasmodium falciparum sporozoite rates were detected in *Anopheles* mosquitoes from PSC collections only (Table 2, Table 3). In 2021, sporozoite infectivity was detected in one *An. gambiae s.s.* (4.3%), and one *An. marshallii* group (0.6%) (Table 3). In 2022, one *An. marhallii* group (0.7%) and two *An. coluzzii* (5.4%) samples were sporozoite positive (Table 3).

Discussion

There is a wide array of anopheline mosquitoes capable of transmitting malaria in Africa, and each species has varied behaviours, distribution, and seasonality that influences their roles in malaria transmission [25]. For vector control to be most effective, precise data on target mosquito species are essential for selecting the best intervention and measuring impact [26]. During the 7-year surveillance period (2016–2022) eight anopheline species were collected from the mangrove /forest ecological zone of Mpat-Enim LGA in Akwa Ibom State, Nigeria, including members of *An. coustani, An. funestus* group, *An. gambiae s.l., An. maculipalpis, An. marshallii* group *An. moucheti An. nili*.and *An. obscurus* The identification of *An. marshallii* group is new within the vector species composition of mosquitoes identified in Akwa Ibom, starting in 2021.

According to Gilles and Coetzee [11], the *An. marshallii* group consists of 16 species in the Marshallii-Hancocki section (series Myzomyia). The species are *Anopheles austenii, Anopheles berghei*, *Anopheles brohieri*, *Anopheles gibbinsi*, *Anopheles hancocki*, *An. hargreavesi*, *Anopheles harperi*, *Anopheles hughi*, *Anopheles kosiensis*, *Anopheles letabensis, An. marshallii s.s., Anopheles mortiauxi*, *Anopheles mousinhoi*, *Anopheles njobiensis*, *Anopheles seydeli* and *Anopheles upemba* [12]. As with other species groups/complexes, these species have different biting/feeding behaviours, larval breeding habitats, host preferences, vectorial capacity, and other characteristics. However, the absence of accurate molecular identification methods hinders studies on this species group [12]. In agreement Harbach, [27] referred to *An. marshallii* as a complex comprising 16 known species inclusive of four identical morphological species: *An. kosiensis*, *An. letabensis, An. marshallii s.s.* and *An. hughi.* However, since some of these 16 species can be separated or distinguished by pupa and egg morphology, they are better referred to as a group and not a complex.

The challenge in the morphological identification of the *An. marshallii* group was due to the damage done by the suction of the CDC LT collection method on mosquito samples including loss of vital parts such as legs, wings and the scales on the thorax. The PSC samples also did not fare better as similar damage was done on the vital

Table 3 Sporozoite positivity rates of Anopheles mosquitoes collected by PSC in Akwa Ibom State, Nigeria, 2021–2022

Year	Total analysed *An. gambiae* s.l	*An. gambiae* s.s			*An. coluzzii*			*An. arabiensis*			Total analysed	*An. marshallii* group		
		Number identified (%)	No. positive for sporozoites	SPR (%)	Number identified (%)	No. positive for sporozoites	SPR (%)	Number identified (%)	No. positive for sporozoites	SPR (%)		Number identified (%)	No. positive for sporozoites	SPR (%)
2021	48	23 (47.9)	1	4.3	25 (52.1)	0	0.0	0 (0)	0	0	162	162 (100)	1	0.6
2022	60	22 (36.7)	0	0	37 (61.7)	2	5.4	1 (1.7)	0	0	142	142 (100)	1	0.7

parts on account of the tremors caused by exposure to pyrethroids. This study was able to confirm some of these samples by morphological identification via scale morphology, but most required molecular analysis via genetic fingerprinting of ITS2 and CO1.

The samples that were identified as *An. hargreavesi* using the key characteristic of scale morphology were subject to further molecular identification confirmation analysis. Using phylogenetic analysis, the closest relationship between the morphologically identified *An. hargreavesi* was to an *An. marshallii* isolate (ITS2: PP915781-PP915785; COI: PP919268-PP919271), compared to other members of the *An. marshallii* group. Those samples that were too damaged for morphological identification or that otherwise were unamplified and aligned by phylogenetic analysis with the same *An. marshallii* isolate may be *An. hargreavsi*, but they could be other members of the *An. marshallii* group. Unfortunately, this is a limitation because *An, hargreavsi* sequences are not in the nucleotide database. As such the identification remains tentative and these mosquitoes were recorded as *An. marshallii* group.

In response to these data findings, a refresher training was organized for the Akwa Ibom site technicians to strengthen their capacity on morphological identification skills, focusing on characteristic features of the *An. marshallii* group, resulting in the reduction of unamplified samples. Apart from taxonomy skills demonstrated, the usefulness of molecular tools in confirming the identity of damaged, unamplified and/or cryptic species, enhanced vector surveillance outcomes [28, 29]. There is a need to further develop specific PCR assays and/or submission of nucleotide sequences of species members to provide more accurate identification and understanding of this group.

Several secondary vectors have been recognized across Africa, including *An. coustani, An. moucheti, An. pharoensis, An. pretoriensis, An. rivulorum*, *An. rufipes, An. squamosus,* as well as the *An. marshallii* group [30–32]. Some of these have been found across various entomological monitoring sites in Nigeria and have been considered of negligible importance because of their strong zoophilic tendencies [3]. Secondary vectors have been recognized for their importance in sustaining malaria transmission during periods when the population of the primary vectors reduces, most often due to lack of rainfall in the dry season [33, 34]. Understanding the role of these secondary vectors relative to the primary vectors will help design sustainable vector control strategies [35].

Ecological variables such as quality of larval habitats, vegetation, topography, climate change and flow speed of rivers are known to have a strong influence on mosquito abundance and distribution [36]. In this study, the co-existence of three major vector species, *An. gambiae s.l., An. coluzzii* and *An. marshallii* group (alongside *An. moucheti* and *An. nili*) were observed in coconut plantations within the mangrove ecozone in Akwa Ibom. Similar reports from southern Cameroon have shown the co-existence of *An. marshallii* alongside *An. moucheti* and *An. nili* in one sentinel site (Nyabessang) out of the five sites surveyed. The ecology of Nyabessang is dense, moist forest [32], similar to the mangrove forest in Akwa Ibom. These findings provide additional information on the possible and peculiar ecology that can be exploited by the *An. marshallii* group, *An. moucheti* and *An. nili*. This flora should be considered when describing *An. marshallii* group habitat and when planning vector surveillance and control.

The data on indoor resting density indicated the co-existence of both *An. gambiae* s.l. and *An. marshallii* group mosquitoes with either species having potential to dominate the other indoors during specific periods of the year. It is apparent that both species have strong indoor resting tendencies, which are influenced seasonally. This means that vector control interventions targeting indoor resting may protect household members year-round.

Differences in HBR between *Anopheles* species have previously been observed to vary seasonally and in different eco-geographical locations [32]. The indoor and outdoor biting rate of the *An. marshallii* group in Akwa Ibom peaked when rains waned. This biting activity suggests the *An. marshallii* group could perpetuate malaria transmission during the dry season, followed by transmission largely by *An. gambiae s.l.* at the onset of the rains. Similar variation in the biting patterns at different periods of the rainy season have been reported in Mendong in Cameroon. A sharp increase in the biting rates of *An. funestus* was recorded at the beginning of the rainy periods compared to *An. gambiae s.l.,* which was present year-round but peaked during the rainy season [37].

In Akwa Ibom, the *An. marshallii* group were found biting both indoors and outdoors in the early evenings. Residual malaria transmission due to early evening and outdoor biting vectors could pose a challenge to core malaria control interventions that focus on indoor vector behaviours. Supplemental vector control tools targeting early evening and outdoor biting behaviours may need to be considered.

The sporozoite rate reported here should be carefully interpreted as limited samples were positive for *P. falciparum*. This evaluation has confirmed the *An. marshallii* group to be infected with *P. falciparum* and to demonstrate human blood feeding preferences (determined from PSC collections) similar to members of the *An. gambiae s.l.* This is consonant with the findings of

Fondjo et al. [32], WHO reported an infection rate of 0.11% in *An. marshallii* mosquitoes in Cameroon.

Conclusion

Findings from this study in Akwa Ibom, Nigeria, confirm the importance of training, supervision and access to morphological and molecular tools in ensuring accurate vector species identification. The study identified *An. marshallii* group as the most dominant during the final 2 years of the 7-year monitoring period (2016–2022). The inability to properly identify the species morphologically or due to limited molecular (PCR) tools to confirm the species suggest that the species may have existed already but was not identified in the area.

The *An. marshallii* group has the potential to extend malaria transmission or maintain malaria parasite persistence during the dry season by serving as a reservoir, as *P. falciparum* sporozoites were detected. Its distinct anthropophilic and endophagic behaviours make standard indoor vector control interventions appropriate, though complementary vector control interventions may also be needed since the mosquito species also exhibits outdoor biting in the early evening hours when people are still socially active. Understanding primary and secondary malaria vectors, shifts in vector species compositions, and species-specific bionomics and behavioural characteristics is fundamental for understanding malaria transmission in each locality and the effectiveness of vector control interventions; paramount to understanding these components is accurate species identification.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-025-05578-1.

Supplementary material 1. The sequences generated during this study were submitted to GenBank and the accession numbers COI: accession number PP919271; ITS2: Accession number: PP915785 generated.

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Author contributions

AOO, MY, AS conceived and designed the study. KA,AOO and AS oversaw programmatic activities, PUI, LS, IO, GY and IAA coordinated and supervised field implementation, entomological data collection, sample identification and data entry. GN, ME, AAI, UB, coordinated the community engagement and stakeholder's sensitization. LK, ZL, EE and MC coordinated the confirmatory morphological identification and the sequencing of samples. AA and TAO coordinated the PCR and ELISA tests conducted on the mosquito samples. AOO and PUI drafted the manuscript with input from AH, MY, LK and AS. PUI, IO, AOO and LS prepared the figures and conducted the statistical analyses and interpretation of the results. AOO, PUI, LS, IO, GY, AA, GN, ME, AAI, BU, KA, AH, JM, MY, LK, ZL, EE, MC and AS reviewed the manuscript. All authors read and approved the final manuscript.

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Data availability

The sequences generated during this study were submitted to GenBank and the accession numbers COI: accession number PP919271; ITS2: Accession number: PP915785 generated.

Declarations

Ethics approval and consent to participate

The work presented was discussed at the integrated vector subcommittee and received approval from the National Malaria Elimination Programme and the Akwa -Ibom State Malaria Elimination Programme.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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