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RESEARCH

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Morphological and Molecular identification of Anopheles Mosquitoes in six selected Local Governments in Ekiti State, South-West Nigeria

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ABSTRACT

Background: Malaria poses a significant public health challenge in Nigeria. The identification of *Anopheles* mosquitoes involves both morphological and molecular approaches. Morphological identification relies on physical characteristics such as body shape and wing patterns, while molecular characterization uses genetic markers to differentiate species and understand their genetic diversity. These methods play crucial roles in studying Anopheles mosquitoes, which are vectors for diseases like malaria, aiding in their surveillance, control, and research efforts. This study aimed to identify *Anopheles* mosquito species in six selected local governments of Ekiti State, South West, Nigeria,, using both morphological and molecular methods.

Methods: Mosquito larvae were collected between April 2022 and July 2023 from eighteen locations in Ado, Oye, Ise-Orun, Ido-Osi, Efon and Ikere local governments respectively. We employed standard dipper sampling techniques to collect mosquito larvae from all types of larval habitats, including both manmade and natural sources, at each study location throughout the mosquito breeding season. The collected larvae were reared to the adult stage. The adult mosquitoes were then identified morphologically using morphological keys, and their species were confirmed through Multiplex Polymerase Chain Reaction for molecular characterisation. The data obtained were analysed using SPSS version 27.0, with a p-value set at 95%.

Results: The study identified *Anopheles gambiae* (80.5%) and *Anopheles funestus* (19.5%) as the major malaria vectors. Two sibling species of *Anopheles gambiae* s.l were identified by PCR: *Anopheles gambiae* s.s (75.4%) and *Anopheles arabiensis* (18.8%), *Anopheles coluzzii* (M form) (55.3%) and Savannah (S-form) (44.7%).

Conclusion: Integrating molecular techniques with morphological analysis can provide valuable insights into the species composition, population dynamics, and biological characteristics of malaria vectors.

Keywords: Malaria, *Anopheles* mosquitoes, larvae, morphology, polymerase chain reaction

1.0 INTRODUCTION

Malaria, a debilitating disease prevalent in Africa and Asia, is caused by Plasmodium protozoa transmitted by *Anopheles* mosquitoes [1]. In Africa, the primary malaria vectors belong to the *Anopheles gambiae* complex and *Anopheles funestus* group, with *Anopheles gambiae* s.s., *Anopheles arabiensis*, and *Anopheles coluzzii* being the main culprits [2].Malaria is the most important and life-threatening parasitic disease in Africa which accounted for about 249 million clinical cases and resulted in over 608 thousand deaths globally in the year 2022 [1]. The *Anopheles* genus, particularly the Anophelinae subfamily, plays a crucial role in spreading malaria and other significant public health pathogens such as dengue fever, Zika virus, chikungunya, yellow fever, and West Nile virus [3]. *Anopheles gambiae*, *Anopheles funestus* and *Anopheles arabiensis* are the three major vectors of malaria in Nigeria [4, 5]. Species complexes exist within the genus *Anopheles* [6].

However, identifying these species is challenging due to their similar morphological features, leading to difficulties in accurately determining sibling species and subspecies. The sub-species within the *Anopheles gambiae* complex and the *Anopheles funestus* group are morphologically indistinguishable but exhibit differences in behavior, host feeding preferences and breeding requirements [7, 8]. Traditional methods for identifying malaria vectors rely on morphological traits. However, due to the limitations of these methods, it is crucial to utilize molecular (DNA-based) techniques for accurate identification and characterization of these vectors. The *An. gambiae* sensu lato complex includes species such as *An. melas*, *An. merus, An. arabiensis*, *An. quadriannulatus* (A and B), *An. bwambe*, and *An. gambiae* sensu stricto, which has recently been divided into *An. gambiae* sensu stricto (S form) and *An. coluzzii* (M form) [9].

Morphological identification often cannot differentiate between these species, as it is less effective for distinguishing sibling species within a complex because these species often exhibit nearly identical physical characteristics, making them indistinguishable based on morphology alone. PCR is frequently employed with primers designed for all members of the Anopheles complex to determine which specific species are present in a given area [10]. However, multiple species can co-exist in the same area, each exhibiting unique behaviors and transmission dynamics, which may lead to multi-species transmission. Effective malaria control and potential elimination efforts must involve accurate identification of malaria vectors to distinguish between primary vectors, secondary vectors, and non-vectors, ensuring that resources are not misallocated to controlling less significant vectors. To overcome these challenges, this research employs morphological analysis and molecular techniques [11, 12] to identify malaria vectors in six major Local Government Areas of Ekiti State, Nigeria. By combining these approaches, we aim to accurately determine the malaria vector species and contribute to a better understanding of malaria transmission and control in the region.

2.0 METHODOLOGY

2.1 Ethical Approval

The research received ethical approval from the Faculty of Science Ethics Committee at the Federal University Oye, Ekiti State. Informed consent was obtained from local residents in the study area, and a local contact helped identify suitable study sites. The research was conducted in six local government areas in Ekiti State, Nigeria, namely Ado, Oye, Efon, Ise-Orun, Ido-Osi, and Ikere, with three communities randomly selected from each area. Larvae of Anopheles mosquitoes were collected from gutters, tyre tracks, ponds, and ditches in the selected communities. They were identified based on their horizontal positioning on the water surface [13] and the geographic coordinates of the collection sites were recorded using a GPS device.

2.1.1 Rearing of *Anopheles* mosquito larvae

Mosquito larvae collected were carefully transported in clearly labeled plastic containers to the insectaries at the Entomology unit of the Department of Animal and Environmental Biology, Federal University Oye-Ekiti where they were reared to adulthood. Larvae from different breeding sites were kept separately to ensure accurate species identification. In the laboratory, larvae were placed in individual bowls labeled accordingly and maintained under controlled conditions: room temperature of 27°C and relative humidity of 82%. The water in the bowls was regularly changed to prevent mortality from excess food. Pupae that emerged were transferred using a Pasteur pipette to labeled plastic cups covered with nets to contain the adult mosquitoes upon emergence. An opening covered with cotton wool allowed for the aspiration of emerged adults, which were closely monitored. Emerging adults were exposed to insecticides, and dead mosquitoes were stored in labeled Eppendorf tubes with desiccated silica gel for PCR analysis.

2.1.2 Morphological identification of *Anopheles* mosquitoes

Morphological identification of the mosquitoes was conducted using a stereomicroscope and morphological keys [11] to identify the species. For molecular identification, DNA was extracted from the legs of *Anopheles gambiae* specimens, and PCR amplification was performed at the Molecular Entomology and Vector Control Research Laboratory of the Nigerian Institute of Medical Research in Lagos following the protocols of [12]. ;

2.2 Statistical Analysis

The distribution of *Anopheles* species groups, identified through both morphological and molecular methods, was evaluated using a chi-square test. All statistical analyses were conducted using SPSS version 27, with a significance level set at ≤0.05.

Table 1. Distribution of *Anopheles* mosquitoes' species by Local Government areas

LGA	*Anopheles gambiae*	*Anopheles funestus*	Total (%)	Chi-square	p-value
Ado	270 (20.2)	90 (27.7)	360	18.932	0.002
Oye	300 (22.4)	60 (18.5)	360		
Efon	180 (13.5)	60 (18.5)	240		
Ikere	240 (17.9)	48 (14.8)	288		
Ise-Orun	192 (14.3)	42 (12.9)	234 (14.1)		
Ido-Osi	156 (11.7)	25 (7.7)	181 (10.9)		
Total	1338 (80.5)	325	1663		

3.0 RESULTS

Table 1 presents the distribution of two *Anopheles* species, *Anopheles gambiae* and *Anopheles funestus* identified morphologically under a binocular microscope across the six Local Government Areas (LGAs) in Ekiti State. A total of 1663 *Anopheles* mosquitoes collected from all the LGA were identified morphologically using stereo microscope. Out of the total *Anopheles* mosquitoes identified, 1338 (80%) were *Anopheles gambiae* species while 325 (20%) were *Anopheles funestus* species. Among other LGA, Ado recorded a larger proportion (90; 28%) of *Anopheles funestus* species, followed by Oye and Efon-Alaaye, which recorded 60 (19%), respectively. Significant variation was observed in the distribution of these two mosquito species among the LGAs ($p < 0.05$).

Table 2 shows the distributions of *Anopheles* species in each communities under the selected local governments in which Egbe community in Oye LG recorded the highest number 115 (38.3%) of *Anopheles gambiae*, while NTA areas of Ado LG recorded the highest number of 34 (37.8%) of *Anopheles funestus*.

Table 3 shows the result of the molecular characterization of the *Anopheles gambiae* complex sibling species across the six Local Government Areas (LGAs) in Ekiti State. Overall, of the 1,338 *Anopheles gambiae* complex sibling

Table 2. Distribution of *Anopheles* mosquitoes species by communities across selected local Government areas in Ekiti State

LGA	Location	*Anopheles gambiae*	*Anopheles funestus*	Total (%)	Chi-square	p-value
Ado	Adebayo	78 (28.9)	25 (27.8)	103 (28.6)	0.042	0.979
	NTA	100 (37.0)	34 (37.8)	134 (37.2)		
	Ilawe	92 (34.1)	31 (34.4)	123 (34.2)		
	Total	270 (75.0)	90 (25.0)	360 (100)		
Oye	Irona	98 (32.7)	18 (30.0)	116 (32.2)	0.463	0.793
	Irare	87 (29.0)	20 (33.3)	107 (29.7)		
	Egbe	115 (38.3)	22 (36.7)	137 (38.1)		
	Total	300 (83.3)	60 (16.7)	360 (100)		
Efon	Iltawure	85 (47.2)	19 (31.7)	104 (43.3)	4.623	0.099
	Orisumbare	52 (28.9)	24 (40.0)	76 (31.7)		
	Babalola	43 (23.9)	17 (28.3)	60 (25.0)		
	Total	180 (75.0)	60 (25.0)	240 (100)		
Ikere	Ajolagun	69 (28.8)	18 (37.5)	87 (30.2)	1.785	0.409
	Ikoyi 1	90 (37.5)	14 (29.2)	104 (36.1)		
	Ikoyi 2	81 (33.8)	16 (33.3)	97 (33.7)		
	Total	240 (83.3)	48 (16.7)	288 (100)		
Ise-Orun	Uso	45 (23.4)	10 (23.8)	55 (23.5)	0.493	0.781
	Olele	88 (45.8)	17 (40.5)	105 (44.9)		
	Erinwa	59 (30.7)	15 (35.7)	74 (31.6)		
	Total	192 (82.1)	42 (17.9)	234 (100)		
Ido-Osi	Sabo	60 (38.5)	7 (28.0)	67 (37.0)	1.025	0.599
	Orin	44 (28.2)	8 (32.0)	52 (28.7)		
	Oke Bakare	52 (33.3)	10 (40.0)	62 (34.3)		
	Total	156 (86.2)	25 (13.8)	181 (100)		

Table 3. Molecular characterisation of *Anopheles gambiae* complex sibling species identified across the study LGAs

LGA	Total *A. gambiae* examined	*A. gambiae* s.s. (%)	*A. arabiensis* (%)	Unidentified (%)	χ^2	p-value
Ado	270	200 (74.1)	52 (19.3)	18 (6.7)	14.52	0.150
Ikere	240	185 (77.1)	40 (16.7)	15 (6.3)	8	0
Efon	180	137 (76.1)	30 (16.7)	13 (7.2)		
Ise-Orun	192	148 (77.1)	36 (18.8)	8 (4.2)		
Ose	300	236 (78.7)	50 (16.7)	14 (4.7)		
Ido-Osi	156	102 (65.4)	44 (28.2)	10 (6.4)		
Total	1338	1008 (75.3)	252 (18.8)	78 (5.8)		

4).

Table 5 shows the result of the molecular forms of the *Anopheles gambiae* complex sibling species across six Local Government Areas (LGAs) in Ekiti State.

A total of 470 *Anopheles gambiae* complex sibling species were examined to ascertain their morphological forms. Most of the species from Ado (56.9%) and Ikere (61.2%) were of the S forms. However, most of the mosquitoes in Efon, Ise-Orun, Oye, and Ido-Osi were of the M forms, with 100%, 58.3%, 71.4% and 51.0% prevalence, respectively. There were significant variations in the distribution of the forms across the LGAs (p=0.000) (table 5) and across the communities (Table 6)

4.0 DISCUSSION

The prevalence of *Anopheles gambiae* and *An. funestus* observed in this study is consistent with previous research highlighting their anthropophilic behavior [14]. This behavioural trait significantly enhances their ability to transmit malaria in sub-Saharan Africa, where they are

Table 4. Distribution of *Anopheles* mosquito species by communities across selected local Government areas

LGA	Location	Total *Anopheles gambiae* examined	*Anopheles gambiae* s.s (%).	*Anopheles arabiensis* (%)	Unidentified (%)	Chi-square	p-value
Ado	Adebayo	97	70 (72.2)	20 (20.6)	7 (7.2)	4.53	0.339
	NTA	100	81 (81.0)	14 (14.0)	5 (5.0)		
	Ilawe	73	49 (67.1)	18 (24.7)	6 (8.2)		
	Total	270	200 (74.1)	52 (19.3)	18 (6.7)		
Oye	Irona	123	102 (82.9)	17 (13.8)	4 (3.3)	3.582	0.466
	Irare	76	60 (78.9)	13 (17.1)	3 (3.9)		
	Egbe	101	74 (73.3)	20 (19.8)	7 (6.9)		
	Total	300	236 (78.7)	50 (16.7)	14 (4.7)		
Efon	Iltawure	64	50 (78.1)	10 (15.6)	4 (6.3)	1.531	0.821
	Orisumbare	62	47 (75.8)	9 (14.5)	6 (9.7)		
	Babalola	54	40 (74.1)	11 (20.4)	3 (5.6)		
	Total	180	137 (76.1)	30 (16.7)	13 (7.2)		
Ikere	Ajolagun	74	52 (70.3)	16 (21.6)	6 (8.1)	3.275	0.512
	Ikoyi 1	77	60 (77.9)	12 (15.6)	5 (6.5)		
	Ikoyi 2	89	73 (82.0)	12 (13.5)	4 (4.5)		
	Total	240	185 (77.1)	40 (16.7)	15 (6.3)		
Ise-Orun	Olele	66	48 (72.7)	15 (22.7)	3 (4.5)	1.513	0.824
	Uso	52	40 (76.9)	10 (19.2)	2 (3.8)		
	Erinwa	74	60 (81.1)	11 (14.9)	3(4.1)		
	Total	192	148 (77.1)	36 (18.8)	8 (4.2)		
Ido-Osi	Orin	60	43 (71.7)	14 (23.3)	3 (5.0)	2.065	0.723
	Sabo	58	37 (63.8)	17 (29.3)	4 (6.9)		
	Oke Bakare	38	22 (57.9)	13 (34.2)	3 (7.9)		
	Total	156	102 (65.4)	44 (28.2)	10 (6.4)		

species, 1008 (75.3%) *Anopheles gambiae s.s.*, 252 (18.8%) *Anopheles arabiensis*, and 78 (5.8%) were unidentified. In each LGAs, *Anopheles gambiae* s.s. was the most prevalent (ranging between 65.4% and 78.7% of all *Anopheles gambiae* complex examined). There were no significant variations in the distribution of the variants across the LGAs (p=0.150) (Table 3) and across the communities (Table

Table 5. Molecular forms of *Anopheles gambiae* complex sibling species identified across the study LGAs

LGA	Total *A. gambiae* examined	M- forms (%)	S- forms (%)	Chi-square	p-value
Ado	102	44 (43.1)	58 (56.9)	64.31	0.000
Ikere	98	38 (38.8)	60 (61.2)		
Efon	51	51 (100)	0		
Ise-Orun	72	42 (58.5)	30 (41.7)		
Oye	49	35 (71.4)	14 (28.6)		
Ido-Osi	98	50 (51.0)	48 (49.0)		
Total	470	260 (55.3)	210 (44.7)		

Table 6. Distribution of *Anopheles* mosquito species by communities across selected local Government areas in Ekiti State

LGA	Location	Total *Anopheles gambiae* examined	M- forms (%)	S- forms (%)	Chi-square	p-value
Ado	Adebayo	38	18 (47.4)	20 (52.6)	0.946	0.623
	NTA	31	14 (45.2)	17 (54.8)		
	Ilawe	33	12 (36.4)	21 (63.6)		
	Total	102	44 (43.1)	58 (56.9)		
Oye	Irare	18	10 (55.6)	8 (44.4)	4.134	0.127
	Egbe	16	14 (87.5)	2 (12.5)		
	Irona	16	11 (68.8)	5 (31.3)		
	Total	50	35 (70.0)	15 (30.0)		
Efon	iltawure	21	21 (100)	0	-	-
	Babalola	17	17 (100)	0		
	Orisumibare	13	13 (100)	0		
	Total	51	51 (100)	0		
Ikere	Ajolagun	26	8(30.8)	18 (69.2)	0.962	0.618
	Ikoyi 1	34	14 (41.2)	20 (58.8)		
	Ikoyi 2	38	16 (42.1)	22 (57.9)		
	Total	98	38 (38.8)	60 (61.2)		
Ise-Orun	Uso	23	13 (56.5)	10 (43.5)	0.067	0.967
	Olele	30	18 (60.0)	12 (40.0)		
	Erinwa	19	11 (57.9)	8 (42.1)		
	Total	72	42 (58.3)	30 (41.7)		
Ido-Osi	Orin	36	20 (55.6)	16 (44.4)	3.605	0.165
	Oke Bakare	30	18 (60)	12 (40.0)		
	Sabo	32	12 (37.5)	20 (62.5)		
	Total	98	50 (51.0)	48 (49.0)		

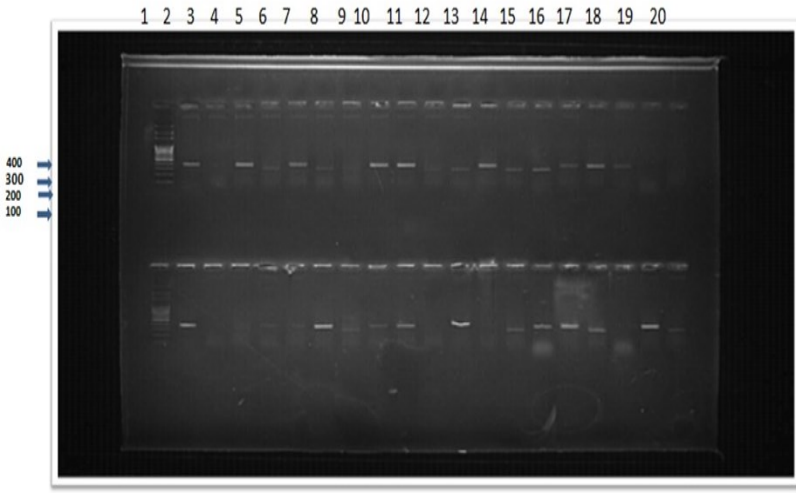

Plate 1. PCR Characterization of members of the *Anopheles gambiae* group
Lane 1: 100 bp DNA ladder, Lane 2: Positive control for *Anopheles gambiae s.s.* Lane 3: Negative control, Lane 4 – 20: Sample wells

recognized as important malaria vectors [15-19]. The higher relative abundance of An. gambiae s.s compared to *An. arabiensis* and their co-occurrence in this study aligns with findings from [20-23]. The prevalence of these two mosquito species can be attributed to the presence of suitable breeding sites, such as stagnant water pools located near human settlements [24]. This correlation is consistent with other studies' findings indicating that households close to breeding sites typically experience higher mosquito densities [24, 25]. In rural areas, the proximity of communities to permanent water bodies likely plays a crucial role in influencing the abundance and distribution of *An. funestus*. On the other hand, *An. gambiae* tends to prefer temporary breeding sites formed by human activities such as irrigation farming, water retention trenches, abandoned vehicle tracks, potholes, and ground pools from domestic water sources [26].

The use of molecular markers has been crucial in identifying both malaria vector and non-vector species, as well as in understanding their distribution in rural and urban environments. In this study, both *An. gambiae* s.s and *An. arabiensis* were identified within the An. gambiae group. This finding is consistent with previous research that identifies *An. gambiae* s.s and *An. arabiensis* as primary malaria vectors in Nigeria [25, 26]. *An. gambiae* s.s was found to be widely distributed and co-existed with *An. arabiensis* across all local government areas were surveyed in Ekiti state.

However, the study noted a significantly higher proportion of *An. gambiae s.s* compared to *An. arabiensis* in the study areas. . The lower occurrence of *An. arabiensis* may be due to its preference for drier ecological conditions [28-30]. This study confirms the presence of *An. arabiensis* as a major malaria vector in Ekiti state, consistent with other studies that emphasise its role in malaria transmission in the forested regions of southwestern Nigeria [14, 25].

The application of molecular techniques in this study has facilitated the precise characterization of vector species, surpassing traditional morphological identification methods used for malaria vectors in Nigeria [31]. This research has provided detailed insights into the identity of malaria vectors in specific locations within the surveyed local government areas. Such information is critical for guiding interventions and assessing their effectiveness.

Current hypotheses suggest that changes in land use patterns in African highlands may create more favourable habitats for malaria vectors, leading to increased vector populations and raising the risk of malaria transmission to humans [32, 34].

In all the local government areas surveyed, both genetic variants of *An. gambiae s.s*., known as the molecular 'M' and 'S' forms, were identified except in Efon-Alaaye LGA, where only the 'M' form was found. The exclusive presence of the 'M' form in Efon suggests the need for further investigation, potentially designating it as a prospective site for future studies on transgenic *Anopheles gambiae*. However, additional research is essential to confirm the presence of the molecular 'M' form specifically within Efon LGA. This study also revealed significant variation in the distribution of these genetic forms across the local government areas.

This study identified the predominant sibling species within the Anopheles gambiae complex across Ado, Ise-Orun, Oye, Ido-Osi, Ikere-Ekiti, and Efon-Alaaye LGAs in Ekiti State, Nigeria. The findings highlight the anthropophilic (preferring humans), endophagic (feeding indoors), and endophilic (resting indoors) behaviors of *Anopheles gambiae* sensu stricto. This suggests that interventions like long-lasting insecticide-treated nets and indoor residual spraying could be effective for controlling this species in the region. In contrast, *Anopheles arabiensis* exhibits exophagic (feeding outdoors) and exophilic (resting outdoors) behaviors, which may reduce the effectiveness of indoor residual spraying alone [35]. This underscores the importance of adopting integrated vector management strategies that address both indoor and outdoor mosquito habitats. Regular surveillance of vector species composition, distribution, and behavior is crucial for making informed decisions when implementing and adjusting effective vector control strategies in the future.

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Conflicts of Interest

The authors declare that there is no conflict of interests.

Authors' Contributions

AAA conceived and designed the study, contributed to data collection, data analysis tools, analysis of data and manuscript writing. **AAA** contributed to data collection, data analysis and manuscript writing. **HIO** contributed to the study design and manuscript writing. **SIO** performed molecular analysis. All authors approved the final copy of the manuscript.

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