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Article

Spatial Distribution of *Microsporidia* MB Along Clinal Gradient and the Impact of Its Infection on Pyrethroid Resistance in *Anopheles gambiae* s.l. Mosquitoes from Nigeria and Niger Republic

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Abstract: Background: *Microsporidia* MB (MB), a promising biological control agent suppresses *Plasmodium falciparum* transmission in *Anopheles* mosquitoes. Methods: This study examined the spatial distribution of MB infection in natural populations of *An. gambiae* s.l. mosquitoes collected in Nigeria and Niger Republic, and its potential modulation of insecticide susceptibility in the *Anopheles* mosquitoes. Results: The overall prevalence of MB in F₀ mosquitoes was 12.25% (95% CI: 7.76%-16.75%); 25 mosquitoes out of 204 were positive. Geographic variation was observed, with a higher prevalence (5/15 mosquitoes) in Ebonyi State (33.33%, 95% CI: 9.48%-57.19%, Fisher's exact test, p = 0.008). Infection rates were higher in *An. coluzzii* mosquitoes (21/133 mosquitoes), estimated at 15.79% (95% CI: 9.59%-21.99%) compared to *An. gambiae* s.s. mosquitoes (4/67), approximately 5.63% (95% CI: 0.27%-11.00%), $\chi^2 = 4.44$; df = 1, p = 0.035]. Resistant mosquitoes had a significantly higher prevalence of MB infection at 28.57% (95% CI: 16.74%-40.40%) and OR of 3.33 (95% CI: 1.23-9.03, p = 0.017) compared with susceptible mosquitoes. Conclusion: MB has wide geographic distribution across Nigeria and Niger Republic. *An. coluzzii* and resistant mosquitoes are more infected with this fungi. Understanding the geographic and species-specific infection patterns and implication in insecticide resistance could guide targeted vector control strategies using this biological agent.

Keywords: *Anopheles*; malaria; *Microsporidia* MB; control; insecticide resistance; spatial distribution

1. Introduction

Malaria remains a major global public health issue, particularly in the African continent, where more than 94% of the infection and 95% of malaria-related mortalities occur, disproportionately in children under the age of 5 and pregnant women [1]. Nigeria has the highest burden of malaria in the world, accounting for ~26% of malaria cases and ~30% of malaria-related deaths globally. However, the neighboring Niger also faces significant malaria challenges, with a prevalence of approximately 237 cases per 1,000 inhabitants in 2022 [2], and contributing 5.9% of malaria-related deaths globally, ranking third, only below Nigeria and Democratic Republic of Congo [1].

The major *Anopheles* species found in Niger and Nigeria belong to the *Anopheles gambiae* complex and secondarily the *Anopheles funestus* s.s. [3–8]. These species exhibit considerable ecological diversity and wide distribution patterns across different habitats [9–11]. Their distribution is often influenced by environmental factors, such as changes in temperature, humidity, and precipitation [11,12]. Studies conducted in Niger and Nigeria have highlighted the abundance and broad distribution of species within the *Anopheles gambiae* complex and *Anopheles funestus* group along various ecological zones [4,7,13]. *Plasmodium* infection status varies across the two countries, with *P. falciparum* being the major parasite, with parasite rate of between 0.77% and 22.3% in *An. coluzzii* from several studies conducted in Nigeria [4,14]. From Niger, according to a study published in 2019 the parasite rate was ~13% in *An. coluzzii* [13]. Recent discoveries have also identified *Plasmodium vivax* from Niger [15].

Excessive use of insecticides for vector control threatens to derail progress in malaria control efforts. Resistance has now been reported to nearly all classes of insecticides used for public health, with pyrethroids, the key ingredients in insecticide-treated bed nets (ITNs) being affected disproportionately [1]. In Niger, pyrethroid resistance towards alpha-cypermethrin, deltamethrin, and permethrin has been documented [13,16–19]. Also, in Nigeria, widespread pyrethroid resistance has been reported in *Anopheles* mosquitoes. Studies have shown high resistance levels in southern and northern Nigeria, with significant operational implications for ITN effectiveness [4,20–24]. A comprehensive RNA-seq analysis, including mosquitoes from Niger and Nigeria, has identified molecular drivers of pyrethroid resistance, such as overexpression of detoxification enzymes and target-site insensitivity mutations [25].

In Niger and Nigeria high pyrethroid resistance in *Anopheles gambiae* is associated with increased activities of metabolic enzymes (P450 + GST) and the West Africa knock down resistance (*kdr-w*) mechanism [18,19,22,25,26]. While much of research focus has been on these major resistance mechanisms, mechanisms such as the role of gut microbiome in mediating pyrethroid resistance are receiving attention. For example, Dada and colleagues have demonstrated that variations in the *Anopheles* gut microbiome can influence pyrethroid resistance, suggesting an additional layer of complexity to resistance management [27].

In the face of the resistance challenges associated with synthetic insecticides, efforts are increasingly invested towards alternative, more eco-friendly control measures. For example, *Wolbachia*, and *Bacillus thuringiensis* (*Bti*). However, the recent discovery of Microsporidia *MB*, an intracellular symbiont in *Anopheles* mosquitoes, has sparked interest due to its potential for malaria control. The presence of *MB* in *Anopheles* mosquitoes negatively impacts their ability to transmit *Plasmodium* [28]. *MB* accumulates in mosquito tissues, with sexual and vertical transmission ensuring its persistence. Its prevalence and intensity in tissues during the mosquito lifecycle suggest an adaptation for maximizing transmission while minimizing virulence [29,30]. These traits facilitate its spread within *Anopheles* populations. The presence of *MB* has been confirmed in several *Anopheles* species across Africa, including in Kenya, Ghana, Niger, and Benin [7,28,31,32]. Studies have shown that its prevalence varies by geographic region, habitat type, and climatic conditions. Unfortunately, these studies have been geographically restricted to single countries. For instance, in Kenya, *MB* prevalence in *An. gambiae* complex mosquitoes was higher in low-altitude areas with warm temperatures and high rainfall compared to high-altitude areas with cooler temperatures and lower rainfall [28]. Similarly, in Ghana, *MB* prevalence in *An. funestus* was higher in forest zones compared

to savanna zones [31]. These findings underscore the significant role of environmental conditions in shaping the geographic distribution of *MB*.

While *MB* shows promise as a biological control agent [33], its effectiveness may be influenced by fluctuating environmental conditions and inherent variability in transmission rates, complicating its use. Also, its potential to influence mosquito phenotypes, particularly insecticide resistance, remains unexplored. Understanding interspecific and intraspecific differences of *MB* within *Anopheles* populations and their potential role in resistance modulation is essential for informing malaria control strategies. This study investigated the spatial distribution of *MB* along clinal gradient of increasing aridity and assess its ability to influence insecticide resistance phenotype in *Anopheles* mosquitoes from Nigeria and Niger Republic.

2. Materials and Methods

2.1. Collection of *Anopheles* mosquitoes Along Gradient of Increasing Aridity

Mosquitoes were collected across twelve sites, spanning contrasting ecological settings: one site in Niger Republic and eleven in Nigeria (Figure 1). These sites cover mangrove swamp, tropical rainforest, Guinea savanna, Sudan savannah, and the Sahel (Table 1). Blood fed female *Anopheles* mosquitoes (*F*₀) resting indoor were collected using Prokopack electric aspirators (John W. Hook, Gainesville, FL, USA) early in the morning, between 05:00-06:00 am. The mosquitoes were transferred to Bayero University Kano, in paper cups (using cooling boxes) and maintained under standard insectarium condition (25°C and 75% relative humidity, with 12h:12h light:dark cycles), fed with 10 % sugar. Collections were conducted in Nigeria between June-September and in Niger in September 2024.

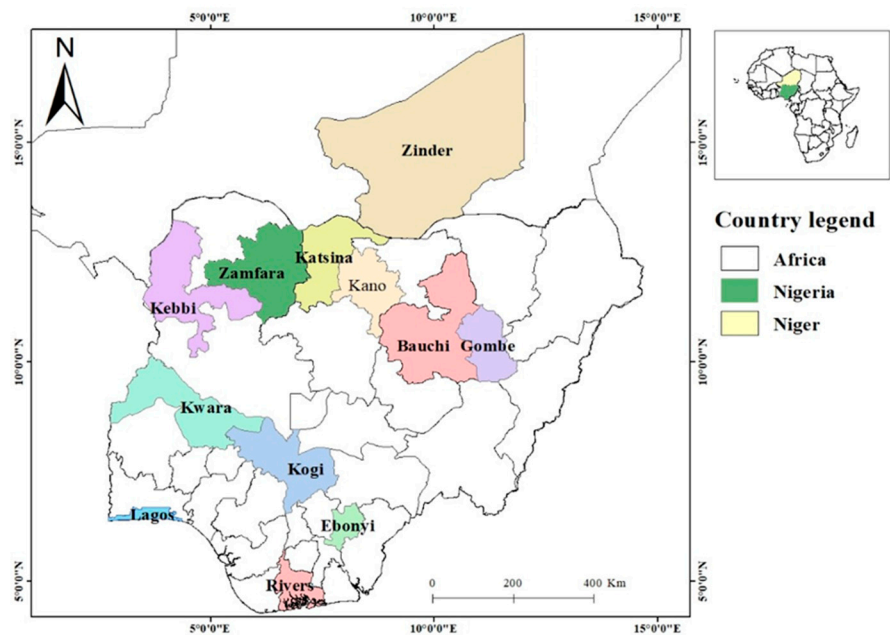


Figure 1. Map of sampling locations in Nigeria and Niger Republic.

Table 1. Sampling design and localities for *Anopheles* mosquito collection and bioassays.

Locality	n	Species	Bioassay	Ecological setting
F₀ Parents caught indoor using Prokopack aspirators				
Gayi Niger, Magaria	32	<i>An. coluzzii</i>	-	Sahel
Gajerar Giwa, Katsina State	32	<i>An. coluzzii</i>		Sahel
Gaa-Bolorunduro, Kwara State	15	<i>An. gambiae</i> s.s.	-	Guinea Savanna
Zariagi, Lokoja, Kogi State	32	<i>An. gambiae</i> s.s.	-	Guinea Savanna
Gotomo, Kebbi State	15	<i>An. coluzzii</i>	-	Sudan Savanna
Tsunami, Gusau, Zamfara State	15	<i>An. coluzzii</i>	-	Sudan Savanna
Ugalaba, Ezza North, Ebonyi State	15	<i>An. coluzzii</i>	-	Tropical Rainforest
	24	<i>An. coluzzii</i>	-	Mangrove
Obio/Akpor LGA, Rivers State				Swamp
	24	<i>An. gambiae</i> s.s.	-	Mangrove
Badagry, Lagos State				Swamp
F₁ progenies used for bioassays				
Gajerar Giwa, Katsina State	16	<i>An. coluzzii</i>	Permethrin	Sahel
Dadin Kowa, Gombe State	16	<i>An. coluzzii</i>	Deltamethrin	Guinea Savanna
Zariagi, Lokoja, Kogi State	24	<i>An. gambiae</i> s.s.	Deltamethrin	Guinea Savanna
Gamjin Bappa, Karaye, Kano State	16	<i>An. coluzzii</i>	Deltamethrin	Guinea Savanna
Gadau, Bauchi State	16	<i>An. coluzzii</i>	Deltamethrin	Sudan Savanna
Obio/Akpor LGA, Rivers State	24	<i>An. coluzzii</i>	Permethrin	Mangrove Swamp

2.2. Morphological and Molecular Identification of *Anopheles* Mosquitoes to Species Level

Gravid F₀ female mosquitoes (4-5 d old after blood meal) were forced to lay eggs inside 1.5 mL Eppendorf tubes. They were morphologically identified [34]. Molecular identification to species level were done using SINE200 PCR [35]. Genomic DNA was extracted using the LIVAK protocol [36]. For each gDNA premix of 14 µl comprised of 1.5 µl of 10x Taq A buffer, 0.75 µl of MgCl₂, 0.12 µl of dNTP mixes, 0.51 µl each of the SINE200 F and R primers, 0.12 µl of KapaTaq Polymerase (Kapa Biosciences, Wilmington, MA, USA), and 10.49 µl of ddH₂O were constituted. 1 µl each of gDNA were added to the premix. Thermal cycling conditions were an initial denaturation at 95°C for 5 min, followed by 35 cycles each of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 1 min. A final extension step was performed at 72°C for 5 minutes. PCR products were separated on 1.5% agarose gel stained with pEqGREEN, and visualized using GelDoc, with expected band sizes of 479 bp, 245 bp and 220 bp respectively for *An. coluzzii*, *An. gambiae* s.s. and *An. arabiensis*.

2.3. Investigation of Spatial Distribution of MB Infection in *Anopheles* Mosquitoes

Subsets of gDNA extracted from the F₀ females above (Table 1) were used for PCR identification of MB infection. PCR amplification of the 18S rRNA gene was performed using MB18SF and MB18SR primers according to previously published protocol [28]. Briefly, each 12 µl reaction mix contained 3 µl of 5x master mix (Solis Biodyne, Estonia), comprise of 7.5 mM of MgCl₂, 2 mM of each dNTP mixes and HOT FIREPol® DNA polymerase. This was mixed with 0.75 µl each of F and R primer, 3 µl of DNA template, and 7.5 µl of nuclease-free water. Thermal cycling conditions included an initial denaturation at 95°C for 15 mins, followed by 35 cycles each of denaturation at 95°C for 30 s,

annealing at 59°C for 45 s, and extension at 72°C for 45 s, PCR products were separated on 1.5% agarose gel stained with pEqGREEN, and visualized using GelDoc for the expected band size of approximately 500 bp characteristic of MB.

2.4. Investigation of the Modulation of Insecticide Resistance by MB

The F₁ mosquitoes from six sites in Nigeria, which were phenotyped with pyrethroid insecticides were selected to screen MB infection (Table 1). Subsets each of alive and dead mosquitoes were screened for infection, attempting to even the number of resistant (R) and susceptible (S) mosquitoes. Bioassays were conducted using impregnated papers in WHO tubes according to [37]. Mosquitoes were exposed to 0.05% deltamethrin or 0.75% permethrin for 1 h, transferred to holding tubes and supplied with 10 % sugar. Mortalities were recorded 24 h post-exposure. Details of the insecticide test profiles will be provided in a separate publication (in preparation).

2.5. Data Analysis

Data were analysed using XLSTAT software [38], to investigate the relationships between MB infection status, *Anopheles* species, locality and insecticide resistance. To evaluate the relationships between variables, a Chi-square test or Fisher's Exact test of independence was applied to determine whether MB infection status is associated with species, locality and insecticide susceptibility. The odds ratio (OR) was determined to measure the strength of association between variables. *P* values of $p < 0.05$ were considered statistically significant.

Multiple Correspondence Analysis (MCA) was done using XLSTAT's "Factor Analysis" package, a multivariate technique designed for categorical data. The MCA was applied to explore key relationships in the study, including the association between MB infection status and *Anopheles* species, the relationship between infection status and mosquito susceptibility (resistant vs. susceptible), and the spatial distribution of MB across sampled localities.

3. Results

3.1. MB Infects *Anopheles* Mosquitoes from Contrasting Ecological Settings Along Clinal Gradient

A total of 204 adult female F₀ mosquitoes were initially analysed for MB infection, out of which 25 were infected (gel pictures in File S1) 12.25% prevalence (95% CI: 7.76%-16.75%). In addition F₁ samples (from sites where F₀ females were not screened for MB infection), which were used for bioassays and screened for MB infection (see below) were lumped in this analysis. MB is widespread across sites with only 2 out of the 12 sites negative (Figure 2). The prevalence of infection varied, ranging from 0% to 33% (File S2). For F₀ females the infection rate was highest in Ebonyi State, where 5 out of 15 mosquitoes were infected (33.33%, 95% CI: 9.48%-57.19%); followed by Rivers State, where 7 out of 24 mosquitoes were positive (29.17%, 95% CI: 10.98%-47.35%). However, mosquitoes from Kwara and Gombe States were not infected. A Fisher's test confirmed a statistically significant association between MB infection status and localities ($p = 0.008$).

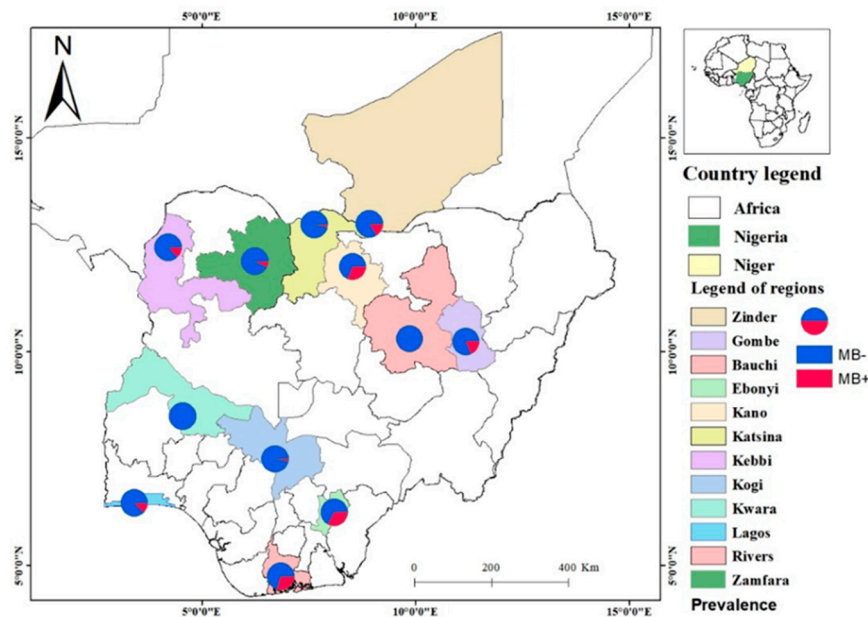


Figure 2. Spatial distribution of MB along clinal gradient of aridity spanning Nigeria and Niger Republic.

Infection varied across *Anopheles* species with *An. coluzzii* exhibiting a higher infection rate. A total of 21 out of 133 *An. coluzzii* mosquitoes were infected, translating into prevalence of 15.79% (95% CI: 9.59%-21.99%), compared to only 4 out of 67 for *An. gambiae* s.s. (prevalence = 5.63%, 95% CI: 0.27%-11.00%). A statistically significant association was observed between MB infection status and mosquito species ($\chi^2 = 4.44$; $df = 1$, $p = 0.035$). Furthermore, the odds ratio of infection with MB was 3.14 (95% CI: 1.03-9.54, $p = 0.035$) for *An. coluzzii*, suggesting a positive association, when compared to odds ratio of 0.32 (95% CI: 0.11-0.91) for *An. gambiae* s.s. Relationship between MB infection status and *Anopheles* species was also analysed using multiple correspondence analysis (Figure 3).

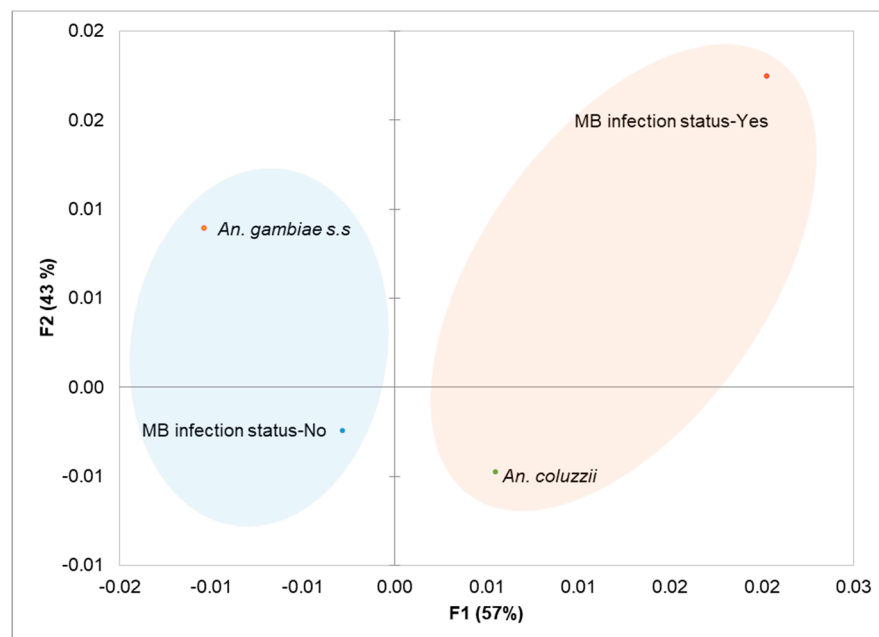


Figure 3. Spatial association between Microsporidia MB Infection Status and *Anopheles* species.

The MCA plot illustrates the association between MB infection status and *Anopheles* species by clustering variable categories with similar profiles on the factor map. The two dimensions (F1 and F2) together explain 100% of the variability in the dataset, with F1 accounting for 57% and F2

explaining 43% of the variance. On the map, *An. coluzzii* and MB+ variables clustered together in the positive quadrant of F1. This clustering indicates that these categories share similar profiles, consistent with the significantly higher prevalence of MB infection observed in *An. coluzzii*. The strong association along F1 suggests that the dimension captures the variability driven by the high infection rates in *An. coluzzii* mosquitoes. Conversely, MB- (negative infection status-No) and *An. gambiae* s.s. cluster in the negative and positive quadrants of F2, respectively. This separation reflects the lower prevalence of infection in *An. gambiae* s.s., highlighting a distinct profile compared to *An. coluzzii*. This clear spatial differentiation on the MCA plot corroborates the statistical findings, where *An. coluzzii* exhibited a significantly higher infection with MB compared to *An. gambiae* s.s.

3.2. MB Infection Probably Correlates with Pyrethroid Resistance

To test the hypothesis that MB modulate insecticide resistance a subset of F1 female mosquitoes previously exposed to pyrethroids, were PCR-screened to detect MB infection. Out of the 112 females tested with pyrethroid, an equal proportion of 50% susceptible (S) and 50% resistant (R) mosquitoes were utilized. Overall, 16 of 56 resistant (R) mosquitoes (28.57%, 95% CI: 16.74%-40.40%) were infected with MB, compared to 6 of 56 susceptible (S) mosquitoes (10.71%, 95% CI: 2.61%-18.82%). These differences in infection were statistically significant ($\chi^2 = 5.65$, $df = 1$, $p = 0.017$). The odds ratio of likelihood of surviving pyrethroid exposure (R phenotype) when MB infected was significant (OR = 3.33, 95% CI: 1.23-9.03, $p = 0.017$), compared with the non-significant odds ratio of 0.30 (95% CI: 0.11-0.81) observed in susceptible mosquitoes. The MCA plot in Figure 4 depicts the association between MB infection status and phenotypes (resistant vs. susceptible) by clustering variable categories with similar profiles. The two dimensions (F1 and F2) explain 100% of the variability, with F1 accounting for 61% and F2 explaining 39% of the variance.

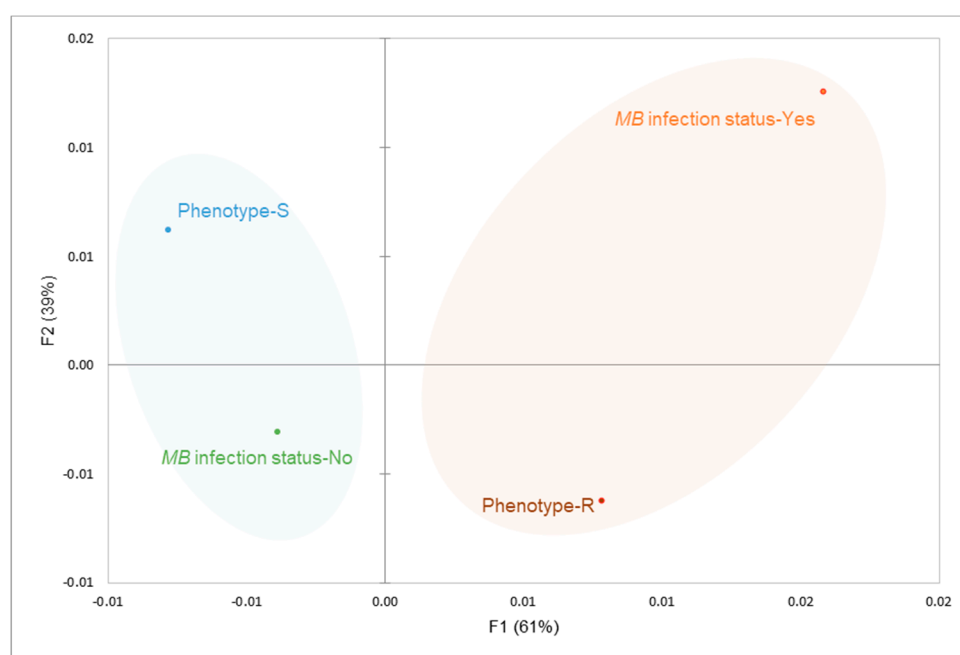


Figure 4. Spatial depiction of potential correlation between MB infection and pyrethroid resistance.

Variables Resistant (R) and MB+ (positive infection status) cluster closely in the positive quadrant of F1. This indicates association between the resistance phenotype and MB infection status. In contrast, susceptible (S) phenotype and MB- (negative infection status) cluster in the negative and positive quadrants of F2, respectively. This suggests an association between low MB infection and pyrethroid susceptibility.

4. Discussion

The development and spread of insecticide resistance which is threatening the progress so far made in malaria control targeting mosquito vectors spurs efforts for alternative control measures, including using biological agents such as *Wolbachia* and *B. thuringiensis*. The discovery of *MB* and the recent description of its ability to reduce *Plasmodium* infection in malaria mosquitoes [28] has catalyzed new efforts to understand the bionomics of this promising fungus.

This primary study generates information about the distribution of *MB* in *Anopheles* mosquitoes from contrasting ecological settings and its influence on insecticide resistance. *MB* is relatively well established in mosquito populations from Nigeria and Niger Republic, with only two mosquito populations out of 12 screened free of its infection. Indeed, previous studies have suggested that local and ecological variations influence presence of *MB* [30,31,39]. To the best of our knowledge this is the first study to report the presence of *MB* in *Anopheles* mosquitoes from Nigeria, and the second study to describe it in mosquitoes from Niger.

Across Africa *MB* appears to be well established in the main malaria vectors. The 12.25% *MB* infection prevalence observed in this study was double the 6.8% (17/251) prevalence reported in *An. coluzzii*, in a study in which no infection was observed in *An. gambiae* from Zinder city in Niger Republic [7]. However, it is lower than 53.4% prevalence previously reported in Benin Republic [32]. The *MB* prevalence observed in Ghana (1.8%) [31] and Kenya (1.7%) [39] were much lower than our findings (though fewer mosquito samples were screened in our study).

This study demonstrated a significantly higher prevalence of *MB* in *An. coluzzii*, compared to *An. gambiae* s.s. A similar pattern has been described in a previous study from Benin Republic, with a prevalence of 41.0% in *An. gambiae* s.s. versus 57.0% in *An. coluzzii* [32].

Most studies in East Africa indicate that *MB* infects *An. arabiensis* [28–30,39], while in West Africa it primarily infects *An. coluzzii* [7,31,32].

The *MB* infection rates varied significantly across the study sites, reflecting a likely influence of geographic and ecological factors. The highest infection rates were observed in Ebonyi State (a Tropical Rainforest site) and Rivers State (Mangrove Swamp) in the wild-caught mosquitoes, possibly because these localities offer particularly favourable conditions for optimal *MB* growth, including high humidity, moderate temperatures, and habitats for mosquito vectors. These findings are consistent with a previous study that reported that *MB* prevalence is influenced by species-specific and ecological factors [28]. Similarly, other studies [39,40] have documented geographic variation in *MB* prevalence, with higher rates in humid environments.

To the best of our knowledge, this is the first study to explore the hypothesis of possible association between insecticide resistance and *MB* infection. The higher prevalence of *MB* in pyrethroid resistant mosquitoes raises important questions about its potential role in modulating insecticide resistance. Several studies have linked microbiome with insecticide resistance showing that pyrethroid resistant mosquitoes harbour distinct *microbiota* compared to the pyrethroid-susceptible strains [27,41]. For example, in the major malaria vector *An. coluzzii*, an abundance of *Asaia* and *Serratia bacteria* has been associated with deltamethrin resistance [42]. Resistant mosquitoes have been found to be enriched with bacteria and enzymes that can degrade insecticides, suggesting that the *microbiota* may play a role in resistance [27]. Also, it has been shown that *MB* infection can influence the phenotype of *Anopheles* such as adaptation to varying dietary conditions [43], faster larval development and higher adult emergence rates [44], all which suggests modulation of various phenotypes. Our finding of possible association between pyrethroid resistance and *MB* infection highlights the need for further research into its roles in insecticide resistance, particularly towards the key malaria control tools, such as the long-lasting insecticidal bed nets, and the ingredients in use for indoor residual spraying. Understanding these may strengthen managing resistance and improving malaria control efforts and could lead to novel strategies to use the link with insecticide resistance to facilitate the spread of *MB* through mosquito populations for sustainable malaria control.

5. Conclusions

MB is ubiquitous in *Anopheles* mosquitoes across Nigeria, and predominantly in *An. coluzzii* in both Nigeria and Niger Republic. Its ability to infect and establish in *Anopheles* mosquitoes is probably influenced by ecological and biological factors. Understanding the geographic and species-specific infection patterns could guide targeted vector control strategies, particularly in regions with high infection prevalence.

These findings highlight the need to better understand the complexities of *MB* – mosquito interactions. A more comprehensive understanding of the impact of *MB* infection on resistance towards the major public health insecticides and malaria control tools will be key to the development of appropriate *MB* based malaria control strategies. These insights can potentially inform innovative strategies for non-synthetic insecticide approaches for malaria vector control in Africa.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. File S1: Agarose gel of *MB* and File S2: File S2. Prevalence of *MB* infection across geographical site.

Author Contributions: Conceptualization, S.S.I., J.K.H., and M.L.M.; methodology, M.L.M., A.H.S., M.M.M., Y.Y.A., S.A., U.C.N., P.C.O., D.E.N., A.O., M.U.M. and H.K.E.; software, S.S.I. and M.L.M.; validation, S.S.I. and J.K.H.; formal analysis, M.L.M., S.S.I. and M.M.M.; resources, S.S.I.; data curation, S.S.I. and M.L.M.; writing—original draft preparation, M.L.M. and S.S.I.; writing—review and editing, J.K.H., P.C.O., D.E.N. and U.C.N.; visualization, M.L.M. and S.S.I.; supervision, S.S.I.; project administration, S.S.I. and M.M.M.; funding acquisition, S.S.I. All authors have read and agreed to the published version of the manuscript.

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