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Investigation of prevalence and phylogenetic classification of *Microsporidia MB* and insecticide target site insensitivity resistance mutations in *Anopheles gambiae* s.l. and *Anopheles funestus* mosquitoes from Busia, Kenya

Herzel Tiffany Wandera[1,2*], Godfrey Nattoh[3,4], Daniel Kiboi[1], Manase Onyango Aloo[5], Stanley Kitur[2], Sharon Nyagaka[2], Malvin Atieno Otieno[2], Eric Ochomo[6,7], Damaris Matoke-Muhia[2], Jeremy Keith Herren[5] and Luna Kamau[2]

Abstract

Background *Microsporidia MB*, a *Plasmodium*-transmission-impairing symbiont in *Anopheles arabiensis*, has malaria control potential. This study assessed its prevalence and phylogeny in *An. gambiae* s.l. and *An. funestus* in Busia, Kenya and investigated the influence of environmental factors on its occurrence. Additionally, the prevalence of key insecticide resistance mutations in these mosquito populations was determined.

Methods Mosquito larvae and adults were collected from three sub-counties in Busia County, Kenya and identified based on morphological characteristics. PCR was used to determine *Anopheles* species distribution and *Microsporidia MB* prevalence following DNA extraction from the samples. Insecticide resistance target-site mutations were identified using TaqMan genotyping in a subset of the mosquito samples. Multivariable logistic regression models were used to assess associations of *Microsporidia MB* infection and ecological factors. *Microsporidia MB*-positive samples were whole-genome sequenced and phylogenetically analysed.

Results Overall, *An. gambiae* s.l. (including *An. gambiae* s.s. and possibly *An. coluzzii*) comprised 57.3% of samples analysed while *An. funestus* comprised 25.7% and *An. arabiensis* 17% and their distribution varied significantly across the three sub-counties (Chi-square, $\chi^2 = 577.44$, df = 4, $p < 0.001$). *Microsporidia MB* prevalence was low to moderate (0 to 6.4%) and highest in *An. gambiae* s.l. *Anopheles gambiae s.l.* showed significantly higher odds of infection compared to *An. arabiensis* (aOR = 5.94, 95% CI: 1.96–26.77, $p = 0.006$). Larvae reared to adults had significantly lower odds of infection than indoor-collected adults (aOR = 0.48, 95% CI: 0.26–0.86, $p = 0.014$). Insecticide resistance genotyping revealed high frequencies of *kdr*-East (94.7%) and *kdr*-West (60%) mutations in the *Anopheles* subset analysed, while

*Correspondence:
Herzel Tiffany Wandera
twandera@kemri.go.ke

Full list of author information is available at the end of the article

Ace-1 and *GSTe2* mutations were absent. Phylogenetic analysis placed *Microsporidia MB* isolated from Busia, within Clade IV, closely related to the originally sequenced Ahero reference, but still distinct from other microsporidian clades (I and III).

Conclusion The present study highlights the occurrence of *Microsporidia MB* in multiple *Anopheles* vectors associated with malaria suggesting its broader potential as a vector control tool. The high prevalence of *kdr* mutations indicate a significant challenge to insecticide-based vector control in the region. Further investigation into the phenotypic expression of insecticide resistance in these populations is important. Results of the phylogenetic analysis suggest a common ancestry for *Microsporidia MB* isolates from Busia with the Ahero reference one, highlighting shared traits with potential for malaria control.

Keywords *Anopheles*, *Microsporidia MB*, Insecticide resistance, Ecology, Phylogenetics

Background

Anopheles mosquitoes are the primary vectors of malaria in sub-Saharan Africa, causing over half a million deaths annually in the last 24 years [1]. Busia County is a malaria-endemic region characterized by a warm and humid climate, providing favourable conditions for mosquito breeding and transmission [2]. With two rainfall patterns annually, the mosquito vector population in this region is usually high, and malaria transmission is intense due to suitable climate conditions [3].

The current primary method for controlling malaria is vector management, which involves the use of long-lasting insecticidal nets (LLINs), insecticide residual sprays (IRSs), and use of larvicides in mosquito breeding sites [4]. While the above interventions have been effective, they have also led to significant insecticide resistance challenges [5, 6]. Furthermore, synthetic insecticides can have detrimental environmental impacts and off-target effects on beneficial insects. In the search for sustainable and resistance-proof vector control strategies, microbial interventions associated with mosquitoes are gaining prominence. For instance, the intracellular endosymbiont *Wolbachia* has shown success in reducing dengue transmission by *Aedes aegypti* [7]. Beyond *Wolbachia*, other biological agents demonstrate potential for vector control. *Bacillus thuringiensis* (Bt) is a well-established larvicide with high specificity [8, 9].

Microsporidia MB has recently gained significant attention as a promising candidate for malaria control [10]. This microorganism can spread within *Anopheles* populations, impairing *Plasmodium* transmission, and has been naturally found in *Anopheles arabiensis*, as well as other *Anopheles* species [11–13]. Studies have shown that *Microsporidia MB* does not impair host fitness, development, and mating; however, unknown gaps remain [14–16]. M*icrosporidia MB* exhibits potential to be both vertically transmitted (mother to offspring) and horizontally transmitted (sexually) [17, 18]. Unlike many parasitic microsporidians of mosquitoes, *Microsporidia MB* (clade IV), appears to have minimal negative effects on its host, making it a particularly attractive candidate compared to *Wolbachia* in *Anopheles*, where low infection intensity often hinders effective spread via cytoplasmic incompatibility, which typically require higher *Wolbachia* titres [19].

Insecticide resistance, often driven by target-site mutations such as *kdr* (L1014F/S), poses a significant threat to the effectiveness of insecticide-based malaria control tools. Mutations in genes like the voltage-gated sodium channel (*kdr)*, acetylcholinesterase *(Ace-1)* and glutathione S-transferase epsilon 2 (*GSTe2)* are known to be functionally linked to phenotypic resistance to various insecticide classes [20–23]. Monitoring the prevalence and distribution of these resistance markers is crucial for understanding the local efficacy of vector control interventions and for guiding appropriate resistance management strategies. Larval habitat diversity can also be used as a proxy to identify potential hotspots for *Microsporidia MB* for the development and successful implementation of targeted vector control measures. Phylogenetic analysis aids to investigate the genetic relatedness of different *Microsporidia MB* isolates and to explore how factors such as geographic location and host species might have shaped their diversification. While *Microsporidia MB* holds significant promise for malaria control, current knowledge regarding its phylogenetic diversity, particularly within key malaria vector populations like those in Busia, Kenya, remains limited. This study used phylogenetic methods to classify *Microsporidia MB* isolates from Busia, comparing them to the Ahero, Kenya reference [24] and other microsporidia. We aimed to understand genetic divergence patterns influenced by geography and *Anopheles* host, shedding light on the symbiont's evolution.

Methods

Study sites

With a cross-sectional study design, field sampling of *Anopheles* indoor-resting adult and larval mosquitoes was carried out in three villages of three geographically dispersed sub-counties in Busia, which is a malaria endemic zone in Kenya. The sub-counties were Teso

South approximately (0° 39' 0.0" N, 34° 21' 0.0" E), Butula (0° 20' 30" N, 34° 20' 7" E) and Budalangi (0° 27' 43" N, 34° 9' 30" E). Busia County generally has a warm and humid climate. The temperatures are relatively high throughout the year, with average daily temperatures ranging from 20 °C to 30 °C. Busia County experiences a bimodal rainfall pattern, with long rains occurring from March-June and short rains occurring from August-November. The annual rainfall in Busia County varies, but it is generally high, ranging from 760 mm to 1,940 mm on average. The northern areas receive more than 1750 mm of precipitation, and the southern areas closer to Lake Victoria receive between 760 and 1,250 mm of precipitation [25] (Fig 1).

Mosquito sampling, handling and rearing

For better representation of the mosquito population and distribution, mosquito collection in Busia was carried out in June 2023 during the wet season, since the wet season has increased population densities of mosquitoes because of increased breeding and larval development. Indoor-resting gravid and engorged female *An. gambiae* s.l. and *An. funestus* were collected between 07:00 and 10:00 h using both manual aspirators and Prokopack aspiration to maximize sample collection. Similarly, larvae were collected using standard scoopers from larval sites such as swamps, sand harvests, borrow pits and rain pools that were adjacent to the homesteads where adults were collected. The larval sites sampled included accessible still water bodies with relative sunlight or shade and some organic matter that can support larval development. Larval water parameters (temperature, pH and dissolved oxygen) were determined using a Hach HQ series pH/DO/Conductivity portable meter equipped with Intellical™ probes (PHC101 for pH and Hach LDO101 for dissolved oxygen) that have an in-built temperature sensing feature. For water parameters, 50 mL water samples were collected in a glass beaker to ensure full probe immersion. Measurements were taken once daily between 12:00 PM and 3:00 PM during larval sampling. The larval samples were transported to the KEMRI/CBRD Molecular Entomology Laboratory, reared into adult insects and identified based on morphological characteristics. The field-collected adult mosquitoes were also identified based on morphological characteristics, transported in cooler boxes and stored at −80 °C. Both groups underwent DNA extraction, molecular species identification and *Microsporidia MB* detection.

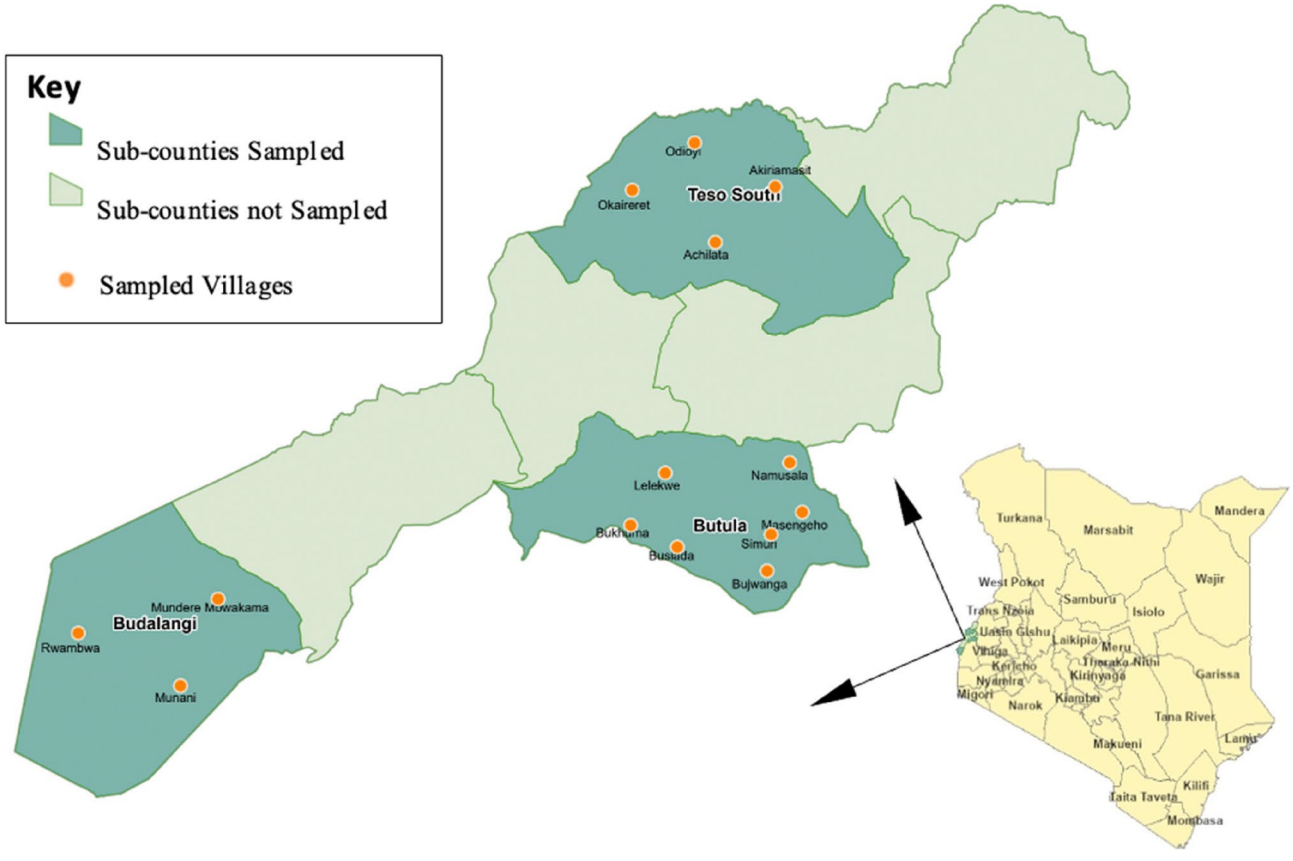

Fig. 1 Map showing the *Anopheles* sampling sites in Busia, western Kenya

Molecular species and *Microsporidia MB* identification

DNA was extracted from individual mosquitoes via the protein precipitation method (Puregene; Qiagen, the Netherlands). Members of the *An. gambiae* species complex and *An. funestus* groups were distinguished via molecular assay methods as described by Scott et al. [26] and Koekemoer and others [27] respectively. The detection and quantification of *Microsporidia MBs* were performed with specific primers (MB18SF: CGCCGGCCGT GAAAAATTTA and MB18SR: CCTTGGACGTGGGAG CTATC) previously designed to detect the 560 bp of the ribosomal RNA (rRNA) conserved gene of *Microsporidia MBs* in *An. arabiensis* [28]. The PCRs were performed in a final volume of 11 µl containing 2 µl of HOTFirepol® Blend Master mix Ready-To-Load (Solis Biodyne, Estonia; mix composition: 7.5 mM magnesium chloride, 2 mM each dNTP, HOT FIREPol® DNA polymerase), 0.5 µl of 10 µM forward and reverse primers, 2 µl of template and 6.0 µl of nuclease-free PCR water. The PCR cycling conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 62 °C for 90 s and extension at 72 °C for 60 s. The final elongation was performed at 72 °C for 5 min. The *Microsporidia MB* DNA positive control was obtained from Jeremy Herren's laboratory (icipe) and included in each analysis.

Analysis of molecular markers associated with insecticide resistance

The presence of selected molecular markers associated with insecticide resistance was determined in a subset of *An. gambiae* s.l. and *An. funestus* samples using the TaqMan assay described by Riveron et al. [29]. For *An. gambiae* s.l., assays were performed for Knockdown resistance (KDR) mutations in the voltage-gated sodium channel (Vgsc-1014 F), which are associated with resistance to pyrethroids and DDT, and Ace1-G119S28, which is associated with resistance to carbamates and organophosphates. The L114T-GSTe2 mutation in the glutathione S-transferase epsilon 2, associated with resistance to DDT and permethrin resistance [29], was assayed in both *An. funestus* and *An. gambiae* s.l. samples.

Analysis of *Microsporidia MB* infection and ecological factors across larval habitats

To determine the environmental predictors of *Microsporidia MB* infection, the associations between larval habitat type, characteristics and *Microsporidia MB* distribution were evaluated.

Whole genome sequencing and assembly of *Microsporidia MB* isolates

A total of 66 samples positive for *Microsporidia MB* by PCR were subjected to quality control measures including qubit analysis for nucleic acid abundance and qPCR to establish the melting profiles and ct values, and gel electrophoresis to assess sample integrity and base size. Of these, 22 samples (33.3%) with a relative 18 S ct range of 18–24 indicating high infection intensity, were selected and shipped for sequencing.

Paired-end short-insert libraries were prepared from these samples using the KAPA HiFi HotStart Library Amp Kit. Sequencing was performed via DNBSeq technology (2 × 150 bp reads) at the Beijing Genomics Institute (BGI) (Shenzen, China). Quality of the generated raw reads was checked using FastQC to assess GC content. Host reads were removed by mapping to *An. gambiae* s.s. (GenBank: GCA_000005575.1 and *An. arabiensis* (GenBank: GCA_000349185.1) reference genomes from the NCBI RefSeq database (release 219) via the Burrows–Wheeler Aligner (BWA) v0.7.17. SAMtools v1.3.1 was used to filter out host-mapped reads. Bacterial contaminants were then removed using Kraken2 (v2.0.8) with the minikraken_8 GB_20200312 database [30].

The remaining clean reads, enriched for *Microsporidia MB* sequences, were then de novo assembled using SPAdes v4.0. A megablast search was conducted against microsporidia proteins to remove nontarget contigs. The raw reads were then reassembled against the cleaned assembly using BWA-MEM v0.7.17 to generate a final consensus genome assembly for each isolate.

Phylogenomic analysis

The evolutionary relationships of the *Microsporidia MB* isolates, were inferred using a phylogenomic approach based on concatenated protein sequences from single-copy orthologous genes. The genome assemblies utilized for this analysis are in supplementary Table 4. Protein-coding genes were predicted from the assembled DNA sequences of all microsporidia genomes using MicroAnnot [31]. MicroAnnot was selected for its specialized capability to accurately annotate microsporidian genomes, including the identification of small genes. The analysis identified Open reading frames (ORFs) with a minimum size of 200nt. Specific parameters for MicroAnnot were set as follows: glimmer size: 300, Database model: *Encephalitozoon cuniculi*, E-value Homology: −15, E-value small: −5, E-value Te: −10. The output included.gff,.fa (predicted protein sequences converted to.faa),.embl, and.gb files.

The.faa files containing the predicted protein sequences from MicroAnnot were each concatenated into a single FASTA file per genome, which served as input for OrthoFinder v. 2.5.5 [32]. This software was used to identify orthogroups, infer orthologs and paralogs, infer gene trees, and root a species tree, as well as detect gene duplication and loss events. Orthologous protein sequences across the different microsporidia species were therefore

identified. For each single-copy gene identified by OrthoFinder, Multiple alignment via fast Fourier transform (MAFFT v 7.487) [33], was used to align protein sequences within that orthogroup. MAFFT used various algorithms, including FFT and iterative refinement, to optimize alignment by introducing gaps to account for evolutionary insertions and deletions, thus aligning homologous residues. The output was a multiple sequence alignment file for each specific orthogroup. For the subsequent species tree inference, the aligned protein sequences from all single-copy orthogroups were then concatenated into a supermatrix.

A species tree was inferred from the concatenated protein supermatrix using the Maximum Likelihood method in IQ-TREE 2 (v 2.4.0) [34]. The optimal model of protein evolution was determined using ModelFinder within IQ-TREE 2 (LG + I + G). Node support was assessed using ultrafast bootstrap replicates (1000 replicates). The resulting species trees, from both OrthoFinder and IQ-TREE 2 [34], were visualized using Dendroscope v. 3.8.1 [35].

Statistical analysis

Statistical analyses were performed using R program (v.4.4.1) in R studio (version 2024.04.2 + 764), and GraphPad Prism Software (v.10.3.1). Chi-square test was used to perform initial descriptive analyses of *Microsporidia MB* prevalence across different categories (*Anopheles* species, sub-county, development stage and sex). To assess the independent associations of demographic factors (mosquito species, sub-county, stage, and sex) with *Microsporidia MB* infection (dependent variable, coded 0 = no infection, 1 = infection), a multivariable logistic regression model was employed using the glm() function (family = "binomial") argument in R. Independent variables included molecular species (levels: *An. arabiensis*, *An. funestus*, *An. gambiae* s.l.), sub-county (levels: Budalangi, Butula, Teso South), development stage (levels: Adult, Larvae), and sex (levels: Female, Male). These were set as factors with reference levels: *An. arabiensis*, Budalangi, Adult, and Female, respectively.

Two primary models were constructed: Main Effects Model (an additive model that assessed the independent contribution of each predictor on *Microsporidia MB* infection using the formula mb_status_binary ~ *Anopheles*_species + sub_county + stage + sex). Secondly, the Interaction Model (to investigate if the effect of mosquito species on infection varied across sub-counties, and an interaction term was included using the formula mb_status_binary ~ *Anopheles*_species * sub_county + stage + sex). In this model, *Anopheles*_species * sub_county represented both main effects and their interaction term. Model selection was guided by the Akaike Information Criterion (AIC). Odds Ratios (ORs) and their 95% confidence intervals were calculated from model coefficients, and its overall significance was assessed by the Likelihood Ratio Test.

To investigate the relationship between *Microsporidia MB* infection (dependent variable, represented numerically as 0 = Negative, 1 = Positive) and the ecological factors (water temperature, water pH, dissolved oxygen and habitat type), a Firth's binary logistic regression model was employed using the logistf() function in R. This model was chosen to address potential issues of complete or quasi-complete separation that can arise in binary logistic regression with sparse data. The model assessed the combined association of these ecological factors using the formula: mb_status_num ~ water_temperature + water_pH + dissolved_oxygen + habitat_type (categorical variable). Odds Ratios (ORs) and their 95% confidence intervals were also calculated for this model, and its overall significance was assessed by the Likelihood Ratio Test.

Results

Distribution of *Anopheles species*

In total, 1,229 *Anopheles* mosquitoes were collected from the three sub-counties (Budalangi, Butula, and Teso South) and identified on the basis of their morphological characteristics. Among these, 1030 were molecularly identified via PCR techniques, comprising of 203 *Anopheles* mosquitoes from Budalangi, 239 from Butula and 588 from Teso South.

Overall, An. *gambiae* s.l. (comprising *An. gambiae* s.s. and possibly *An. coluzzii*) was the most prevalent species at 57.3% (590/1030), followed by *Anopheles funestus* 25.7% (265/1030), and *An. arabiensis* constituted 17%, (175/1030). The distribution of *Anopheles* species varied significantly across the three sub-counties (Chi-square, χ^2 = 577.44, df = 4, $p < 0.001$). For example, Teso South was predominantly *An. gambiae* s.l. (433/588), while Budalangi showed a higher proportion of *An. funestus* (174/203). Butula exhibited a mixed composition, with *An. gambiae* s.l. (156/239) being most common. Detailed proportions of *Anopheles* species by sub-county and developmental stage are presented in Fig. 2.

Mosquitoes were collected both as indoor-resting adults (62.7%, 646/1030) and as larvae successfully reared to adults (37.3% (384/1030). No larvae collected from Budalangi sub-county developed into adult mosquitoes. The proportions of *Anopheles* species identified differed significantly between indoor-collected adults and adults emerging from larval collections, in both Butula (Chi-square, χ^2 = 54.4, df = 2, $p < 0.001$) and Teso South (Chi-square, χ^2 = 117.7, df = 2, $p < 0.001$).

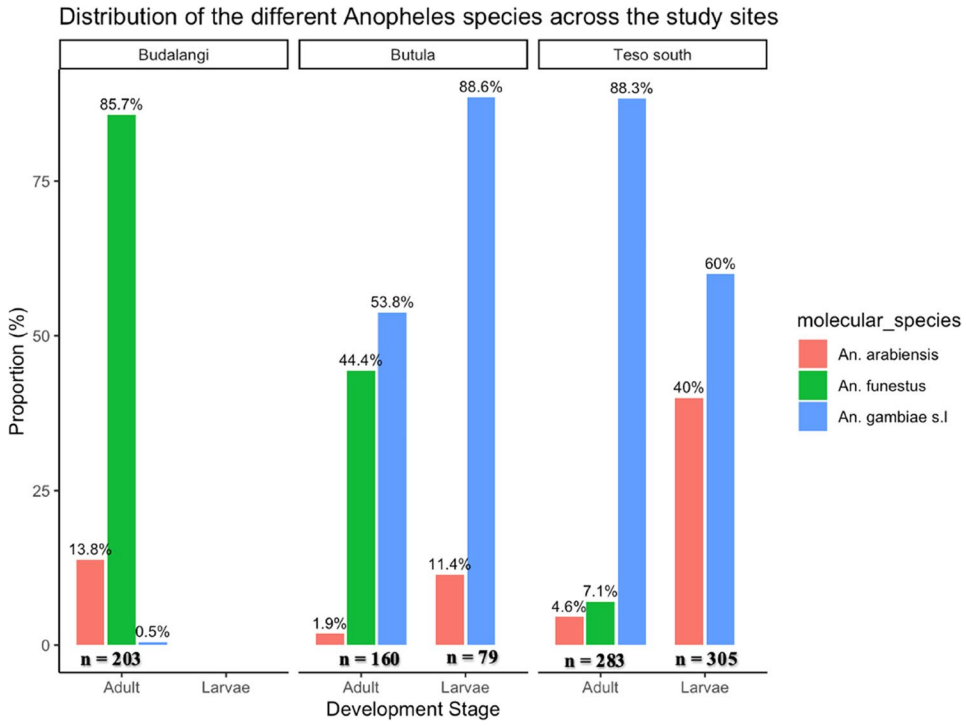

Fig. 2 The proportions of *Anopheles* species across different developmental stages from three sub-counties of Busia, Kenya

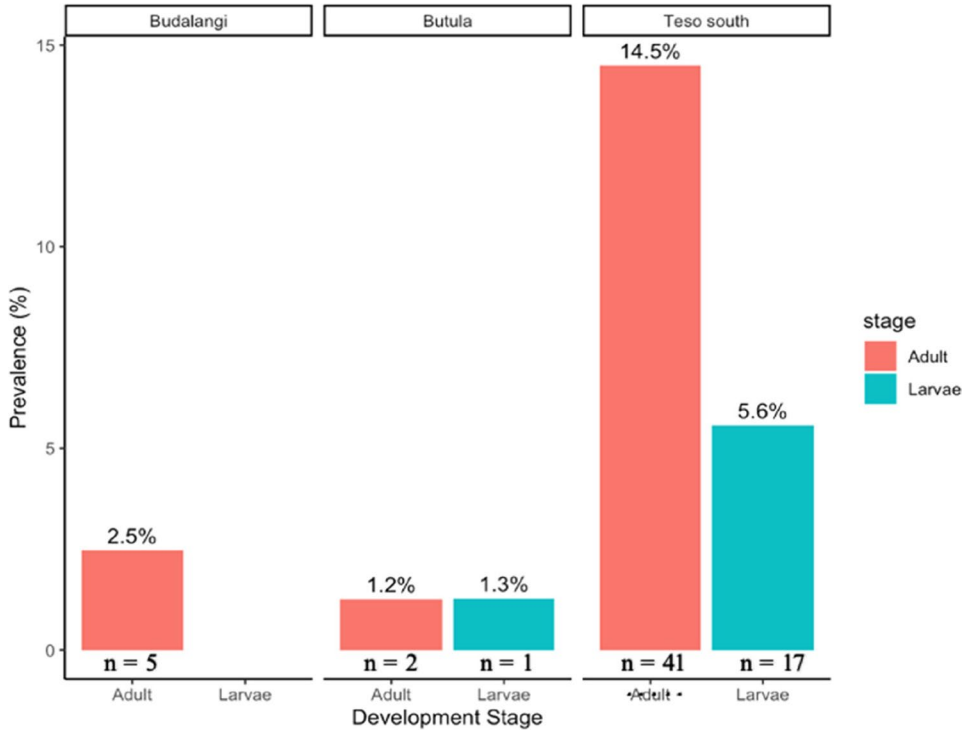

Fig. 3 Prevalence of *Microsporidia MB* by sub-county and development stage

Microsporidia MB prevalence and demographic factors

Overall, *Microsporidia MB* was detected at a prevalence of 6.4% (66/1030) in Busia County. In bivariate analyses (Supplementary Table 1), *Microsporidia MB* prevalence varied significantly across the three *Anopheles* species (Chi-square test, $\chi^2 = 26.985$, df = 2, $p < 0.001$), with *An. gambiae* s.l. showing the highest crude odds of infection (OR = 6.25, 95% CI: 1.93–20.2, $p = 0.002$) compared to *An.*

arabiensis. Prevalence of *Microsporidia MB* also varied significantly among the three sub-counties (Chi-square test, $\chi^2 = 35.97$, df = 2, $p < 0.001$), with Teso South showing significantly higher crude odds of infection (OR = 4.33, 95% CI: 1.71–10.96, $p = 0.002$) compared to Budalangi. No significant differences in crude odds of infection were observed by developmental stage (OR = 0.61, 95% CI: 0.35–1.06, $p = 0.082$) or sex (OR = 1.52, 95% CI: 0.92–2.51, $p = 0.089$).

A multivariable logistic regression model performed and chosen for its optimal fit (AIC = 442.61) showed significant improvement over a null model (Likelihood Ratio Test, Chi-square test, $\chi^2 = 61.76$, df = 6, $p < 0.001$). After adjustment, *Anopheles gambiae* s.l. showed significantly higher odds of *Microsporidia MB* infection compared to *Anopheles arabiensis* (Adjusted Odds ratio, OR = 5.94, 95% CI: 1.96–26.77, $p = 0.006$). *Anopheles funestus* did not show significant difference in odds of infection compared to *Anopheles arabiensis* (Adjusted Odds ratio, OR = 0.96, 95% CI: 0.17–6.44, $p = 0.967$).

Regarding sub-county, *Anopheles* mosquitoes from Butula sub-county compared to Budalangi showed no significant difference in the adjusted odds of infection (Odds ratio, OR = 0.14, 95% CI: 0.02–0.93, $p = 0.054$). Similarly, Teso South sub-county did not show significantly different odds of infection compared to Budalangi (Adjusted Odds ratio, OR = 1.18, 95% CI: 0.20–6.47, $p = 0.851$). Larvae had significantly lower adjusted odds of infection compared to adults (Odds ratio, OR = 0.48, 95% CI: 0.26–0.85, $p = 0.014$). No significant difference in adjusted infection odds was found between male and female mosquitoes (OR = 1.41, 95% CI: 0.84–2.38, $p = 0.195$).

An interaction model between *Anopheles* species and sub-county was also tested but was not statistically significant (Chi-square test, $\chi^2 = 1.2436$, df = 4, $p = 0.871$) and exhibited estimation instability, suggesting that the independent effect of species on *Microsporidia MB* infection does not significantly vary across the sub-counties. The estimated odds ratios and their 95% confidence intervals are presented in supplementary Table 1 (Fig. 3)

Relationships between *Microsporidia MB* infection and ecological factors across larval habitats

The mean water temperature across the five habitats sampled, namely, the sand harvest, borrow pit, rainpool, hoof print and swamp habitats, was 31 °C (± 2.76), while the mean water pH was 7.3, and the mean dissolved oxygen content was 6.6 mg/l ± 0.79. (Supplementary Table 3).

An additive multivariate Firth's binary logistic regression model, employed to investigate the relationship between *Microsporidia MB* and ecological factors (water temperature, water pH, dissolved oxygen and habitat type) within the larvae reared to adults mosquito population, was not statistically significant (Likelihood Ratio Test, Chi-square test, $\chi^2 = 7.7$, df = 7, $p = 0.360$). Furthermore, no individual predictor showed a statistically significant association with *Microsporidia MB* infection (all $p > 0.05$). The estimated odds ratios and their 95% confidence intervals and prevalence of *Microsporidia MB* by habitat are presented in supplementary Tables 2 and 6 respectively.

Prevalence of insecticide target site insensitivity mutations

A total of 134 samples were genotyped for the presence of the *kdr-e*, *kdr-w*, *Ace-1*, and *GSTe2* molecular markers associated with insecticide resistance. Of these, only the *kdr-e* and *kdr-w* mutations were detected, whereas the *Ace-1* and *GSTe2* mutations were absent in the tested samples. The *kdr-e* mutation was observed at the high prevalence of 94.7% ($n = 58$) while the *kdr-w* mutation was observed at 60% ($n = 10$) of the genotyped samples.

Phylogenetic classification of *Microsporidia MB*

The genomic content (GC%) content of the Teso08 and Teso98 samples were 44.01% and 46.06%, respectively. The Budalangi sample had a GC content of 44.77%. Both samples from Teso South mapped to *An. gambiae* s.s. host. After mapping, host and other bacterial reads were filtered to remain with *Microsporidia MB.* It was noted that the Budalangi sample mapped to *An. arabiensis* host. Given that the Budalangi sample had degraded *Microsporidia MB*, (observed during assembly) it was deemed unsuitable to include in phylogenomic analysis. Another *Anopheles arabiensis* sample from Busia that was earlier sequenced was included in the phylogenetic analysis. This sample yielded 360,389,934 reads with a GC of 44.52%.

Three *Microsporidia MB*-positive samples from Busia (Teso94, Teso8 and Busia) were successfully assembled into contig-level genomes. MicroAnnot Predicted Protein Sequences (per genome) from these assemblies, along with eight reference microsporidia genomes (supplementary file 2). OrthoFinder assigned 19,595 genes (78.8% of total) to 3,265 orthogroups. Of these, 727 orthogroups contained all species, and 445 consisted entirely of single-copy genes. These 727 single-copy gene trees (those with all species present) were used by STAG (Species Tree from All Genes) method to infer the final species tree from OrthoFinder (Fig. 4). *Edhazardia aedes* and *Anncaliia algerae* were identified as the best outgroups for this species tree inference by OrthoFinder.

A subsequent species tree was inferred from the concatenated protein supermatrix sequences (supplementary file 4), generated from MAFFT alignment of the 445 orthogroups consisting of single copy genes that were present in all species (supplementary file 3) using the

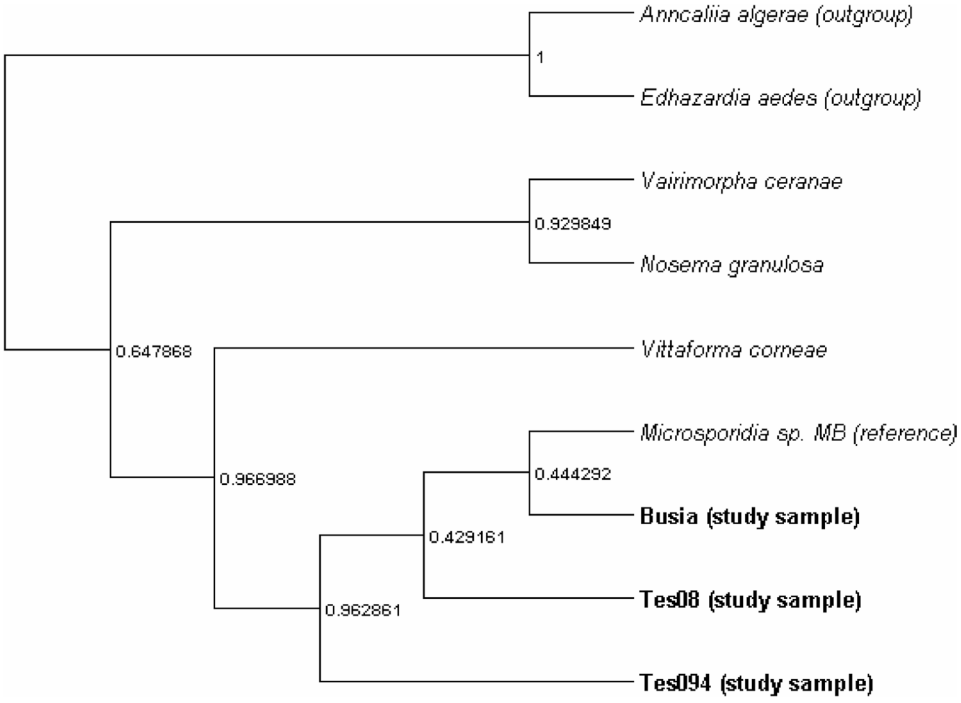

Fig. 4 Phylogenomics Species tree of microsporidians generated by OrthoFinder and visualized using Dendroscope v. 3.8.1. Numbers at the nodes represent support values on a scale from 0 to 1, with values closer to 1 indicating stronger support of gene concordance

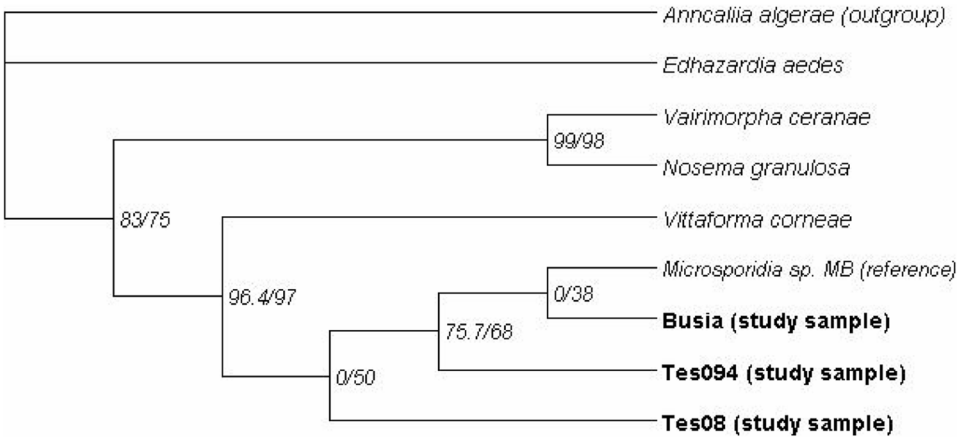

Fig. 5 Phylogenomics Species tree of microsporidians generated using Maximum Likelihood method in IQTree2 and visualized on Dendroscope v. 3.8. Numbers at the nodes represent ultrafast bootstrap (UFBoot)/SH-aLRT support values, respectively

Maximum Likelihood method in IQ-TREE 2 (v 2.4.0) [34] and visualized on Dendroscope v. 3.8.1 [35](Fig. 5).

Phylogenetic analysis of the microsporidians in both Orthofinder and IQ-TREE2 revealed a highly supported clade comprising of *Vairimorpha ceranae* and *Nosema granulosa* (OrthoFinder support: 0.929849; IQ-TREE2 support: 99/98). *Microsporidia MB* isolates and the *Microsporidia sp. MB* reference consistently clustered together in a distinct clade. This clustering was strongly supported, with *Vittaforma corneae* identified as a sister lineage to this group (OrthoFinder support: 0.966988; IQ-TREE2 support: 96.4/97). While the overall topology of major clades was consistent, the precise internal relationships and support values within the *Microsporidia MB* clade (including the reference and study samples) exhibited differences between the two phylogenomic analyses, and generally showed lower support.

In the OrthoFinder species tree (Fig. 4), the overall monophyly of the *Microsporidia MB* clade was very strongly supported (0.962861). Within this clade, Tes094 was inferred as the earliest diverging lineage. The subsequent branching placed Tes08 as sister to a clade containing the *Microsporidia sp. MB* reference and the Busia isolate, which formed a sister pair. However, the support

values for these fine-scale internal relationships were notably lower: 0.429161 for the divergence of Tes08 and 0.444292 for the reference/Busia pairing.

Conversely, the IQ-TREE2 species tree (Fig. 5) showed moderate support for the overall monophyly of the *Microsporidia MB* clade (75.7/68 ultrafast bootstrap/SH-aLRT support). Within this tree, Tes094 also appeared as an early diverging lineage. However, the subsequent branching inferred the *Microsporidia sp. MB* reference as sister to a clade containing Busia and Tes08, which themselves were sister taxa. The bootstrap support values for these internal relationships in the IQ-TREE2 tree were very low or absent (e.g., 0/38 for reference/Busia clustering, 0/50 for Tes094/Tes08 clustering), indicating very limited statistical confidence in these specific branches.

Despite the differences in internal resolution, both phylogenomic analyses consistently supported the presence of a distinct *Microsporidia MB* group in Busia that shares a close evolutionary history with the Ahero reference strain, classifying them within the Clade IV. microsporidia lineage.

Discussion

Understanding the factors that influence the infection of mosquitoes with *Microsporidia MB* in nature and how infection might influence factors that affect malaria transmission is key in designing effective malaria vector control tools. The present study revealed a low to moderate prevalence of *Microsporidia MB* in naturally occurring *An. gambiae* s.l. (which included *An. gambiae* s.s. and possibly *An. coluzzii*), *An. arabiensis* and *An. funestus* mosquito populations from Busia, Kenya, sampled during the rainy season. *Microsporidia MB* were detected both in indoor-resting adult mosquitoes and in mosquitoes collected as larvae and then reared to adults.

The results of the present study revealed varying patterns of *Anopheles* species distributions across the three sub-counties of Busia. *Anopheles gambiae* s.l. emerged as the dominant species in Teso South and Butula, which is consistent with previous studies assessing the distribution of *Anopheles* species in Busia during the rainy season [36, 37]. The high prevalence of *An. gambiae* s.l. and *An. arabiensis*, an efficient vector of malaria in sub-Saharan Africa, coupled with findings related to insecticide resistance are concerning, as this may compromise the success of conventional vector control.

In contrast, *An. funestus* was the most abundant species in Budalangi. This could be attributed to Budalangi being in close proximity to Lake Victoria compared with the other two sub-counties sampled, as studies have shown that a reduction in Lake Victoria water levels has created more breeding habitats for *An. funestus* [38]. The failure to successfully rear adults from the larvae collected from Budalangi in the insectary could be attributed to the fact that *An. funestus* was the prevalent species in this area (over 85% in the specimen collected as adults), and the general difficulty in rearing this species due to poor larval survival under laboratory conditions [39] since *An. funestus* may require a natural setup, which may be difficult to mimic in the laboratory.

The detection of *Microsporidia MB* in both *An. gambiae s.l.* and *An. funestus* highlights the natural occurrence of this potential biological control agent in Busia. This is advantageous because both species are key malaria vectors and *Microsporidia MB* can also spread naturally in infected hosts. Bivariate analysis revealed that the prevalence of *Microsporidia MB* varied significantly across the three species with the highest prevalence observed in *An. gambiae* s.l. (9.8%) compared to *Anopheles funestus* (1.9%) and *Anopheles arabiensis* (1.7%). This finding is in accordance with similar studies that have shown that *Microsporidia MB* can naturally occur in other *Anopheles* species, including *An. gambiae* s.s [12, 13, 40, 41]. ' other than the predominant *An. arabiensis*, where it was originally found [28].

Multivariate logistic regression analysis revealed that *Anopheles gambiae* s.l. independently exhibited significantly higher odds of *Microsporidia MB* infection compared to *Anopheles arabiensis* (OR = 5.94, 95% CI: 1.96–26.77, $p = 0.006$), even after controlling for sub-county, mosquito stage, and sex. This finding supports the notion that *An. gambiae* s.l. may indeed be a more susceptible host or a more efficient vector for this parasite, independent of its geographical distribution. The multivariate model also provided critical insights into the role of geographical location. While bivariate results showed Teso South sub-county with the highest overall M*icrosporidia MB* prevalence (9.9%), the logistic regression revealed that Teso South did not have significantly different odds of infection compared to Budalangi sub-county (Odds ratio, OR = 1.18, 95% CI: 0.20–6.47, $p = 0.851$), once molecular species, stage, and sex were accounted for. This strongly suggests that the higher overall prevalence in Teso South observed in simple comparisons was primarily driven by the dominant presence of the more susceptible *Anopheles gambiae* s.l. in that sub-county, rather than Teso South being an independent risk factor for infection. Further supporting this, an interaction term between *Anopheles* species and sub-county in the multivariate model was not statistically significant ($p = 0.871$), indicating that the independent effect of species on *Microsporidia MB* infection does not significantly vary across the sub-counties.

Although the larval habitat characteristics assayed varied significantly across habitats, multivariable Firth's logistic regression model did not find a statistically significant influence of water temperature, dissolved oxygen, or habitat type on the odds of *Microsporidia MB*

infection. This suggests that within the range of parameters observed in the study, these factors may not be primary determinants of *Microsporidia MB* infection. However, the mean water temperature in the sand-harvesting larval habitat, with most abundant *Microsporidia MB*-infected larvae reared to adults, was 32 °C. This finding is in accordance with the findings of Herren et al., who reported that 32 °C was the best temperature for rearing *Microsporidia MB*-infected larvae because of the relatively short development time and high infection rate [42]. Studies have shown that high temperatures can lead to a reduction in *Wolbachia* loads, but the degree to which temperature affects *Wolbachia* intensity can vary depending on the specific *Wolbachia* strain. Some strains are more tolerant of heat stress than others are [43, 44].

Long-lasting insecticidal nets (LLINs) have been used in Busia for malaria control for over two decades, with widespread distribution beginning in the late 1990 s and continuing through the 2000 s and 2010 s [46]. This prolonged and widespread use of pyrethroids in LLINs likely explains the high frequencies of the *kdr-e* (Vgsc-L1014F) and *kdr-w* (Vgsc-L1014S) mutations observed [6]. The high prevalence of these *kdr* mutations strongly suggest that insecticide resistance may compromise the effectiveness of pyrethroid-based interventions in the area. In contrast, *Ace-1* G119S and *GSTe2* L114F mutations were not detected in any of the tested mosquito samples from the study area. The absence of the *Ace-1* mutation, which are associated with resistance to organophosphates and carbamates, is likely due to the limited or no use of these insecticide classes in the study area for vector management or agricultural pest control. It is important to acknowledge that this study did not include insecticide bioassays to determine the phenotypic resistance profile of the mosquito populations. While genotypic data, particularly for well-characterized mutations like *kdr*, are widely used as proxies for insecticide resistance, especially where functional links have been demonstrated [20, 21], direct phenotypic correlation is optimal for a comprehensive understanding.

Phylogenetic analysis supported *Vittaforma corneae* as a sister lineage to the *Microsporidia MB* group, aligning with established microsporidia phylogenetic relationships [47, 48]. The analysis revealed a close evolutionary relationship between Busia *Microsporidia MB* isolates and *Microsporidia sp. MB* reference from Ahero (Clade IV). This consistent finding, supported by high concordance factors in OrthoFinder (0.962861) and moderate to strong bootstrap support in IQ-TREE2 (75.7/68), reinforcing previous suggestions of a specific genetic lineage for *Microsporidia MB* associated with *Anopheles* mosquitoes [28]. This is significant given the high malaria prevalence in Busia [49]Within Clade IV, the isolates from Teso (Teso94 and Teso8) formed a subclade, with low support values for these specific nodes (e.g., 0.429161 and 0.444292 in OrthoFinder; 0/38 and 0/50 in IQ-TREE2), indicating a very recent divergence and over a short evolutionary timescale, where insufficient phylogenetic signal has accumulated across the genomic data to definitively resolve their exact relationship [34]. The methodological differences between gene tree reconciliation (which explicitly models gene tree discordance) and concatenation (which assumes a single underlying tree) can also contribute to these observed topological discrepancies [50].

Notably, the *Microsporidia MB* isolates from our study were distinctly separated from other microsporidian clades (Clade I and Clade III), highlighting their unique phylogenetic position. These findings indicate that the *Microsporidia MB* populations circulating in Busia share a common ancestry with the reference strain from Ahero, while exhibiting some level of genetic divergence. The close relationship, supported by the clustering of Busia isolates, suggests potential for utilizing *Microsporidia MB* for vector control. This highlights the potential for exploiting shared traits for malaria control strategies in the region.

Limitations

This study focused on the wet season, yet there could be seasonal variation in *Microsporidia MB* across the wet and dry seasons. The low to moderate prevalence of *Microsporidia MB* resulted in sparse data points for positive infection outcomes in certain analytical subsets. The estimated effects for environmental variables, such as water pH, exhibited very wide 95% confidence intervals, indicating substantial uncertainty around their true associations with *Microsporidia MB* infection. Study findings offer an initial phylogenetic classification of *Microsporidia MB* isolated from Busia since small number of samples were sequenced due to rapid degradation of *Microsporidia MB* and may limit capturing proper symbiont's phylogenetic associations. This study did not include insecticide resistance phenotype bioassays to directly assess phenotypic resistance in the mosquito populations.

Conclusion

The study revealed varying distributions of species with *An. funestus* being most prevalent in Budalangi, which may be linked to its closer proximity to Lake Victoria. Multivariate analysis confirmed *An. gambiae* s.l. as a significant independent predictor of *Microsporidia MB* infection, even when controlling for sub-county and other factors. The finding of *Microsporidia MB* in *An. gambiae* s.l. and *An. funestus* rather than predominantly *An. arabiensis*, as previously reported suggests its potential as a vector control tool. Furthermore, while Teso

South showed high overall prevalence in bivariate analyses, the multivariate results indicated this was likely due to the higher proportion of *An. gambiae* s.l. in that area, rather than Teso South being an independent risk factor for infection. Despite ecological factors being broadly important for sustaining the development of *Microsporidia MB*, the multivariable Firth's logistic regression did not identify a statistically significant association between the measured water parameters (temperature, pH, dissolved oxygen) or habitat types and the odds of infection. The high prevalence of *kdr* mutations observed in the *Anopheles* mosquito populations underscores the continued challenge of insecticide resistance in the region and highlights the need for resistance management strategies. Future studies should integrate insecticide resistance bioassays to directly assess phenotypic resistance. Phylogenomic analyses consistently establish the distinct evolutionary placement of the *Microsporidia MB* isolates from Busia, Kenya, within a well-supported clade closely related to the *Microsporidia sp. MB* reference and sister to *Vittaforma corneae*. The phylogenetic relationships findings of this study contribute to foundational understanding of the evolutionary dynamics of these *Anopheles*-associated microsporidia, which is crucial for ongoing vector control strategies.

Abbreviations

PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
ANOVA	Analysis of variance
LLIN	Long-lasting insecticidal nets
IRS	Insecticide residual sprays
KEMRI	Kenya Medical Research Institute
CBRD	Centre for Biotechnology Research and Development
DNA	Deoxyribonucleic Acid
DDT	Dichloro-diphenyl-trichloroethane
s.l.	Sensu Lato
s.s.	Sensu Stricto
NCBI	National Center for Biotechnology Institute
FFT	Fast Fourier Transform

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

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Authors' contributions

LK, DMM, EO, JKH conceptualized and designed the study.HTW, SK, SN, MA, EO, DMM, JKH, LK conducted sample collection, processing, and data collection.HTW and LK conducted the data analysis and interpretation and drafted the manuscriptGN, DN, MOA, JKH conducted the data analysis and interpretation and assisted in drafting the manuscript. All the authors read, reviewed and approved this manuscript.

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Data availability

All figures and tables supporting the conclusions of this article are included within the manuscript and supplementary files. The raw reads and genomic isolates for the study samples have been deposited at NCBI under the BioProject accession [PRJNA1242000](https:/www.ncbi.nlm.nih.gov/bioproject/PRJNA1242000). Raw reads have been deposited to the Sequence Read Archive (SRA) under accession number [SRS24495769](https:/www.ncbi.nlm.nih.gov/biosample/SAMN47576098), [SRS24495770](https:/www.ncbi.nlm.nih.gov/biosample/?term=SAMN47576099) and [SRS24585630](https:/www.ncbi.nlm.nih.gov/biosample/?term=SAMN47626518). The Whole Genome Shotgun (WGS) project for the *Microsporidia MB* genomic isolates has been deposited at DDBJ/ENA/GenBank under the accession [JBMNCT000000000.1](https:/www.ncbi.nlm.nih.gov/nuccore/JBMNCT000000000.1), [JBMPGQ000000000.1](https:/www.ncbi.nlm.nih.gov/nuccore/JBMPGQ000000000.1) and [JBMNCU000000000.1](https:/www.ncbi.nlm.nih.gov/nuccore/JBMNCU000000000.1). The versions described in this paper are version [JBMNCT01](https:/www.ncbi.nlm.nih.gov/nuccore/JBMNCT000000000.1), [JBMPGQ01](https:/www.ncbi.nlm.nih.gov/nuccore/JBMPGQ000000000.1) and [JBMNCU01](https:/www.ncbi.nlm.nih.gov/nuccore/JBMNCU000000000.1).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]Jomo Kenyatta University of Agriculture and Technology (JKUAT), Juja, Kenya
[2]Centre for Biotechnology Research and Development (CBRD), Kenya Medical Research Institute, Nairobi, Kenya
[3]Centre for Global Health Research (CGHR), Kenya Medical Research Institute, Kisumu, Kenya
[4]Kaimosi Friends University (KAFU), Kaimosi, Kenya
[5]International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya
[6]Centre for Infectious and Parasitic Disease Control Research (CIPDCR), , Kenya Medical Research Institute, Busia, Kenya
[7]Vector Biology Department, Liverpool School of Tropical Medicine, Liverpool, UK

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References

1. World malaria report. 2024 [Internet]. [cited 2024 Dec 13]. Available from: https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2024.
2. CHWs are Instrumental in Managing Malaria Cases at the Household Level. | Living Goods [Internet]. [cited 2024 Dec 13]. Available from: https://livinggoods.org/media/chws-are-instrumental-in-managing-malaria-cases-at-the-hous

ehold-level/#:~:text=Situated%20in%20western%20Kenya%20at,mosquito%20breeding%20and%20waterborne%20diseases.
3. Bashir IM, Nyakoe N, Van Der Sande M. Targeting remaining pockets of malaria transmission in Kenya to hasten progress towards national elimination goals: an assessment of prevalence and risk factors in children from the Lake endemic region. Malar J [Internet]. 2019 Jul 12 [cited 2024 Dec 13];18(1):233. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC6624951/.
4. Nalinya S, Musoke D, Deane K. Malaria prevention interventions beyond long-lasting insecticidal nets and indoor residual spraying in low- and middle-income countries: a scoping review. Malar J [Internet]. 2022 Dec 1 [cited 2024 Dec 13];21(1). Available from: https://pubmed.ncbi.nlm.nih.gov/35109848/.
5. Pryce J, Medley N, Choi L. Indoor residual spraying for preventing malaria in communities using insecticide-treated nets. Cochrane Database Syst Rev [Internet]. 2022 Jan 17 [cited 2024 Dec 13];2022(1):CD012688. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC8763033/.
6. Kawada H, Ohashi K, Dida GO, Sonye G, Njenga SM, Mwandawiro C et al. Insecticidal and repellent activities of pyrethroids to the three major pyrethroid-resistant malaria vectors in western Kenya. Parasit Vectors [Internet]. 2014 May 2 [cited 2024 Dec 13];7(1):208. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC4017225/.
7. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM et al. A Wolbachia symbiont in Aedes aegypti limits infection with dengue, Chikungunya, and Plasmodium. Cell [Internet]. 2009 Dec 24 [cited 2024 Dec 13];139(7):1268–78. Available from: https://pubmed.ncbi.nlm.nih.gov/20064373/.
8. Pelloquin B, Kristan M, Edi C, Meiwald A, Clark E, Jeffries CL et al. Over-abundance of Asaia and Serratia Bacteria Is Associated with Deltamethrin Insecticide Susceptibility in Anopheles coluzzii from Agboville, Côte d'Ivoire. Microbiol Spectr [Internet]. 2021 Oct 31 [cited 2025 May 12];9(2):e00157-21. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC8528120/.
9. Munyakanage D, Niyituma E, Mutabazi A, Misago X, Musanabaganwa C, Remera E et al. The impact of Bacillus thuringiensis var. israelensis (Vectobac® WDG) larvicide sprayed with drones on the bio-control of malaria vectors in rice fields of sub-urban Kigali, Rwanda. Malaria J. 2024 23:1 [Internet]. 2024 Sep 17 [cited 2025 May 12];23(1):1–13. Available from: https://malariajournal.biomedcentral.com/articles/https://doi.org/10.1186/s12936-024-05104-9.
10. Bukhari T, Pevsner R, Herren JK. Microsporidia: a promising vector control tool for residual malaria transmission. Front Trop Dis. 2022;3:957109.
11. Nattoh G, Maina T, Makhulu EE, Mbaisi L, Mararo E, Otieno FG, et al. Horizontal transmission of the symbiont microsporidia MB in Anopheles arabiensis. Front Microbiol. 2021;12:647183.
12. Akorli J, Akorli EA, Tetteh SNA, Amlalo GK, Opoku M, Pwalia R et al. Microsporidia MB is found predominantly associated with Anopheles gambiae s.s and Anopheles coluzzii in Ghana. Sci Rep [Internet]. 2021 Dec 1 [cited 2024 Apr 11];11(1):18658. Available from: https://pmc/articles/PMC8452686/.
13. Ahouandjinou MJ, Sovi A, Sidick A, Sewadé W, Koukpo CZ, Chitou S et al. First report of natural infection of Anopheles gambiae s.s. and Anopheles coluzzii by Wolbachia and Microsporidia in Benin: a cross-sectional study. Malar J [Internet]. 2024 Dec 1 [cited 2024 Apr 11];23(1):1–9. Available from: https://malariajournal.biomedcentral.com/articles/https://doi.org/10.1186/s12936-024-04906-1.
14. Nattoh G, Onyango B, Makhulu EE, Omoke D, Ang'ang'o LM, Kamau L et al. Microsporidia MB in the primary malaria vector Anopheles gambiae sensu stricto is avirulent and undergoes maternal and horizontal transmission. Parasites & Vectors 2023 16:1 [Internet]. 2023 Sep 25 [cited 2023 Nov 9];16(1):1–12. Available from: https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-023-05933-8.
15. Boanyah GY, Koekemoer LL, Herren JK, Bukhari T. Effect of Microsporidia MB infection on the development and fitness of Anopheles arabiensis under different diet regimes. Parasit Vectors [Internet]. 2024 Dec 1 [cited 2025 May 11];17(1):1–12. Available from: https://parasitesandvectors.biomedcentral.com/articles/https://doi.org/10.1186/s13071-024-06365-8.
16. Maina T, Shisia A, Gichuhi J, Bargul JL, Herren JK, Bukhari T. Maximizing horizontal transmission through mating: increased mating frequency and mating competitiveness associated with Microsporidia MB-infected Anopheles arabiensis males. Malar J [Internet]. 2025 Apr 9 [cited 2025 Apr 25];24(1):114. Available from: https://pubmed.ncbi.nlm.nih.gov/40205501/.
17. Nattoh G, Maina T, Makhulu EE, Mbaisi L, Mararo E, Otieno FG, et al. Horizontal transmission of the symbiont microsporidia MB in Anopheles arabiensis. Front Microbiol. 2021;12:1995.
18. Onchuru TO, Makhulu EE, Ronnie PC, Mandere S, Otieno FG, Gichuhi J et al. The Plasmodium transmission-blocking symbiont, Microsporidia MB, is vertically transmitted through Anopheles arabiensis germline stem cells. PLoS Pathog [Internet]. 2024 Nov 1 [cited 2024 Dec 13];20(11):e1012340. Available from: https://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1012340.
19. Jeffries CL, Lawrence GG, Golovko G, Kristan M, Orsborne J, Spence K et al. Novel wolbachia strains in anopheles malaria vectors from sub-saharan africa [version 2; referees: 3 approved]. Wellcome Open Res [Internet]. 2018 [cited 2025 May 7];3. Available from: https://pubmed.ncbi.nlm.nih.gov/30483601/.
20. Donnelly MJ, Corbel V, Weetman D, Wilding CS, Williamson MS, Black IVWC. Does kdr genotype predict insecticide-resistance phenotype in mosquitoes? Trends Parasitol [Internet]. 2009 May [cited 2025 May 21];25(5):213–9. Available from: https://pubmed.ncbi.nlm.nih.gov/19369117/.
21. Grigoraki L, Cowlishaw R, Nolan T, Donnelly M, Lycett G, Ransoni H. CRISPR/Cas9 modified An. gambiae carrying kdr mutation L1014F functionally validate its contribution in insecticide resistance and combined effect with metabolic enzymes. PLoS Genet [Internet]. 2021 Jul 6 [cited 2025 May 21];17(7):e1009556. Available from: https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1009556.
22. Elanga-Ndille E, Nouage L, Ndo C, Binyang A, Assatse T, Nguiffo-Nguete D et al. The g119s acetylcholinesterase (Ace-1) target site mutation confers carbamate resistance in the major malaria vector anopheles gambiae from cameroon: A challenge for the coming irs implementation. Genes (Basel) [Internet]. 2019 Oct 1 [cited 2025 May 26];10(10). Available from: https://pubmed.ncbi.nlm.nih.gov/31614683/.
23. Riveron JM, Yunta C, Ibrahim SS, Djouaka R, Irving H, Menze BD et al. A single mutation in the GSTe2 gene allows tracking of metabolically based insecticide resistance in a major malaria vector. Genome Biol [Internet]. 2014 Feb 25 [cited 2025 May 26];15(2):1–20. Available from:https://genomebiology.biomedcentral.com/articles/https://doi.org/10.1186/gb-2014-15-2-r27.
24. Ang'ang'o LM. Molecular characterisation of microsporidia mb species and correlation with plasmodium presence in anopheles mosquitoes in Mwea and Mbita, Kenya. 2018 [cited 2021 Nov 14]; Available from: http://erepository.uonbi.ac.ke/handle/11295/104416.
25. History of Busia County [Internet]. [cited 2025 Feb 23]. Available from: https://www.busiacounty.go.ke/about/history-of-busia-county
26. Scott JA, Brogdon WG, Collins FH. Identification of single specimens of the Anopheles gambiae complex by the polymerase chain reaction. Am J Trop Med Hyg [Internet]. 1993 [cited 2024 Oct 17];49(4):520–9. Available from: https://pubmed.ncbi.nlm.nih.gov/8214283/.
27. Koekemoer LL, Kamau L, Hunt RH, Coetzee M. A cocktail polymerase chain reaction assay to identify members of the Anopheles funestus (Diptera: Culicidae) group. Am J Trop Med Hyg [Internet]. 2002 [cited 2024 Oct 17];66(6):804–11. Available from: https://pubmed.ncbi.nlm.nih.gov/12224596/.
28. Herren JK, Mbaisi L, Mararo E, Makhulu EE, Mobegi VA, Butungi H et al. A microsporidian impairs Plasmodium falciparum transmission in Anopheles arabiensis mosquitoes. Nat Commun [Internet]. 2020 Dec 1 [cited 2024 Jul 26];11(1). Available from: https://pubmed.ncbi.nlm.nih.gov/32366903/.
29. Riveron JM, Yunta C, Ibrahim SS, Djouaka R, Irving H, Menze BD et al. A single mutation in the GSTe2 gene allows tracking of metabolically based insecticide resistance in a major malaria vector. Genome Biol [Internet]. 2014 Feb 25 [cited 2024 Oct 17];15(2):1–20. Available from: https://genomebiology.biomedcentral.com/articles/https://doi.org/10.1186/gb-2014-15-2-r27.
30. Ang'ang'o LM, Waweru JW, Makhulu EE, Wairimu A, Otieno FG, Onchuru T et al. Draft genome of Microsporidia sp. MB—a malaria-blocking microsporidian symbiont of the Anopheles arabiensis. Microbiol Resour Announc [Internet]. 2024 Apr 11 [cited 2025 Jul 2];13(4). Available from: https://doi.org/10.1128/MRA.00903-23.
31. Tournayre J, Polonais V, Wawrzyniak I, Akossi RF, Parisot N, Lerat E et al. MicroAnnot: A Dedicated Workflow for Accurate Microsporidian Genome Annotation. Int J Mol Sci [Internet]. 2024 Jan 1 [cited 2025 Jul 1];25(2):880. Available from: https://www.mdpi.com/1422-0067/25/2/880/htm.
32. Emms DM, Kelly S, OrthoFinder. Phylogenetic orthology inference for comparative genomics. Genome Biol [Internet]. 2019 Nov 14 [cited 2025 Jul 2];20(1):1–14. Available from: https://genomebiology.biomedcentral.com/articles/https://doi.org/10.1186/s13059-019-1832-y.
33. Katoh K, Rozewicki J, Yamada KD. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief Bioinform [Internet]. 2019 Jul 19 [cited 2025 Jul 4];20(4):1160–6. Available from: https://doi.org/10.1093/bib/bbx108.

34. Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, Von Haeseler A et al. IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. Mol Biol Evol [Internet]. 2020 May 1 [cited 2025 Jul 2];37(5):1530–4. Available from: https://doi.org/10.1093/molbev/msaa015.
35. Huson DH, Richter DC, Rausch C, Dezulian T, Franz M, Rupp R, Dendroscope. An interactive viewer for large phylogenetic trees. BMC Bioinformatics [Internet]. 2007 Nov 22 [cited 2025 Jul 2];8(1):1–6. Available from: https://bmcbioinformatics.biomedcentral.com/articles/https://doi.org/10.1186/1471-2105-8-460
36. Ndenga BA, Simbauni JA, Mbugi JP, Githeko AK, Fillinger U. Productivity of Malaria Vectors from Different Habitat Types in the Western Kenya Highlands. PLoS One [Internet]. 2011 [cited 2024 Dec 4];6(4):e19473. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC3085476/.
37. Odero JI, Abong'o B, Moshi V, Ekodir S, Harvey SA, Ochomo E et al. Early morning anopheline mosquito biting, a potential driver of malaria transmission in Busia County, western Kenya. Malar J [Internet]. 2024 Dec 1 [cited 2024 Apr 11];23(1):1–13. Available from: https://malariajournal.biomedcentral.com/articles;https://doi.org/10.1186/s12936-024-04893-3 .
38. Minakawa N, Sonye G, Dida GO, Futami K, Kaneko S. Recent reduction in the water level of lake Victoria has created more habitats for Anopheles funestus. Malar J. 2008;7,(119) . https://doi.org/10.1186/1475-2875-7-119
39. Ngowo HS, Hape EE, Matthiopoulos J, Ferguson HM, Okumu FO. Fitness characteristics of the malaria vector Anopheles funestus during an attempted laboratory colonization. Malar J [Internet]. 2021 Dec 1 [cited 2024 Dec 5];20(1):1–13. Available from: https://malariajournal.biomedcentral.com/articles; https://doi.org/10.1186/s12936-021-03677-3.
40. Kamau L, Bennett KL, Ochomo E, Herren J, Agumba S, Otieno S et al. The Anopheles coluzzii [internet]ange [internet]xtends into kenya: detection, insecticide [internet]esistance profiles and population genetic structure in [internet]elation [internet]o conspecific populations in West and central africa. Res Sq [Internet]. 2024 Feb 15 [cited 2024 Jul 29]; Available from: https://pmc/articles/PMC10896386/.
41. Nattoh G, Onyango B, Makhulu EE, Omoke D, Ang'ang'o LM, Kamau L et al. Microsporidia MB in the primary malaria vector Anopheles gambiae sensu stricto is avirulent and undergoes maternal and horizontal transmission. Parasit Vectors [Internet]. 2023 Dec 1 [cited 2024 Jul 29];16(1):1–12. Available from: https://parasitesandvectors.biomedcentral.com/articles/https://doi.org/10.1186/s13071-023-05933-8.
42. Otieno FG, Barreaux P, Belvinos AS, Makhulu EE, Onchuru TO, Wairimu AW et al. Temperature modulates the dissemination potential of Microsporidia MB, a malaria-blocking endosymbiont of Anopheles mosquitoes. bioRxiv [Internet]. 2024 Nov 15 [cited 2024 Dec 12];2024.11.15.623820. Available from: https://www.biorxiv.org/content;https://doi.org/10.1101/2024.11.15.623820v1.
43. High temperature results in reduced Wolbachia densities and maternal. Download Scientific Diagram [Internet]. [cited 2025 Feb 23]. Available from: https://www.researchgate.net/figure/High-temperature-results-in-reduced-Wolbachia-densities-and-maternal-leakage-A-Larvae_fig3_322703454.
44. Ross PA, Axford JK, Yang Q, Staunton KM, Ritchie SA, Richardson KM et al. Heatwaves cause fluctuations in wMel Wolbachia densities and frequencies in Aedes aegypti. PLoS Negl Trop Dis [Internet]. 2020 Jan 1 [cited 2025 Feb 23];14(1):e0007958. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC6977724/.
45. St. Leger RJ, Joshi L, Roberts D. Ambient pH Is a Major Determinant in the Expression of Cuticle-Degrading Enzymes and Hydrophobin by Metarhizium anisopliae. Appl Environ Microbiol [Internet]. 1998 [cited 2025 Feb 23];64(2):709. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC106106/.=
46. Ng'ang'a PN, Aduogo P, Mutero CM. Long lasting insecticidal mosquito nets (LLINs) ownership, use and coverage following mass distribution campaign in Lake Victoria basin, Western Kenya. BMC Public Health [Internet]. 2021 Dec 1 [cited 2025 Mar 20];21(1):1–13. Available from: https://bmcpublichealth.biomedcentral.com/articles/https://doi.org/10.1186/s12889-021-11062-7.
47. Park E, Poulin R. Revisiting the phylogeny of microsporidia. Int J Parasitol [Internet]. 2021 Sep 1 [cited 2025 Jul 4];51(10):855–64. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0020751921001417?via%3Dihub.
48. Ang'ang'o LM, Herren JK, Tastan Bishop Ö. Bioinformatics analysis of the Microsporidia sp. MB genome: a malaria transmission-blocking symbiont of the Anopheles arabiensis mosquito. BMC Genomics [Internet]. 2024 Dec 1 [cited 2025 Jul 4];25(1):1132. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC11585130/.
49. Herren JK, Mbaisi L, Mararo E, Makhulu EE, Mobegi VA, Butungi H et al. A microsporidian impairs Plasmodium falciparum transmission in Anopheles arabiensis mosquitoes. Nature Communications 2020 11:1 [Internet]. 2020 May 4 [cited 2021 Nov 14];11(1):1–10. Available from: https://www.nature.com/articles/s41467-020-16121-y.
50. Liu L, Anderson C, Pearl D, Edwards SV. Modern phylogenomics: Building phylogenetic trees using the multispecies coalescent model. Methods in Molecular Biology [Internet]. 2019 [cited 2025 Jul 4];1910:211–39. Available from: https://link.springer.com/protocol/https://doi.org/10.1007/978-1-4939-9074-0-7.

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