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Mapping the genetic relatedness of Outdoor-Biting *Anopheles* mosquitoes in Zambia.

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Simple Summary

Outdoor biting *Anopheles* mosquitoes have been gaining attention due to their potential role in sustaining malaria transmission by avoiding indoor vector control interventions. The efficacy of mitigation efforts that primarily target indoor biting and resting mosqui-toes may be undermined by these mosquitoes. The identification of these less studied mosquito taxa is challenging due to cryptic morphological features and the limited num-ber of molecular reference sequences in databases. The advancements in sequencing technologies have led to a steady increase in the generation of mitochondrial genomes (mitogenomes). Mitogenomes have proved to be robust in resolving species identification, population structure and phylogenies in metazoans when compared to commonly used molecular barcodes. Our work highlights the use of mitochondrial genomes for under-standing the genetic relatedness of the less studied outdoor-biting *Anopheles* with refer-ence to the primary vectors of malaria. The datasets generated in this study can be used to improve interventions for malaria control and employ molecular diagnostics for accurate species identification.

Abstract

The zoophilic and exophilic traits of outdoor-biting *Anopheles* has led to this group largely being overlooked for their role in malaria transmission, despite several species now rec-ognized as locally important in regions of sub-Saharan Africa. Given the current limita-tions with identification of these understudied species, it is crucial to accurately correlate morphological features to molecular data. Here, we produced high quality reference se-quence data for representative understudied anopheline species to better understand the phylogenetic relationships between under- and well-studied vectors of malaria. For mito-chondrial genome assembly, shallow shotgun sequencing was implemented on single mosquito specimens and phylogenetic analyses were performed on the concatenated pro-tein coding genes of the mitogenomes using a Bayesian approach. This study generated 10 complete mitogenomes focusing on less-studied taxa with an average length 15,383 bp and A-T content of 77.1% consistent with other anophelines containing 37 genes. Bayesian inference analysis yielded four main clades with molecular dating indicating that well-studied malaria vectors diverged from outdoor-biting species more than 65 million years ago. These findings support the taxonomic grouping of mosquitoes belonging to the *Anopheles* genus based on morphological characteristics and can provide molecular diag-nostics for species identification enabling more precise and adept interventions for ma-laria control.

Keywords: outdoor biting; mitochondrial genome; malaria; *Anopheles*; understudied

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1. Introduction

As African nations strive towards malaria control and elimination, insecticide re-sistance and residual malaria transmission challenge current vector-targeted malaria in-terventions [1–4]. While the primary vectors *Anopheles* funestus, An. coluzzii and An. gambiae are the focus of these mitigation strategies [5,6], selection pressure from indoor-focused measures such as indoor residual spraying (IRS) and insecticide treated nets (ITNs) have led to changes in mosquito behavior and populations [7–9]. One key change is the recog-nition that secondary *Anopheles* vectors such as An. rufipes [10,11], An. pharoensis [12], An. squamosus [13] and An. coustani [13–15], contribute to sustaining residual transmission in sub-Saharan Africa. These mosquito species are considered largely exophagic and exoph-ilic, behaviors that have allowed them to evade indoor vector control interventions [7,8,16,17].

Despite frequent collection alongside the primary malaria vectors, the genomics, ecology, biology, and behavior of these long-overlooked anopheline mosquito species are poorly understood [9]. Furthermore, the constraints of overlapping and cryptic morpho-logical features [18–21], together with the absence of available molecular data in genomic databases [17,20,22,23], have made robust morphological and molecular identification of these less studied anopheline species extremely challenging. Studies have also reported evidence of functional heterogeneity in anopheline genomes which influence their behav-ioral plasticity, a crucial characteristic for defining vectorial capacity and adaptability [24,25]. Therefore, the accurate identification and bionomic characterization of under-studied *Anopheles* species is now critical given their key role as local vectors in driving re-sidual malaria transmission in Zambia, Madagascar, southern Mozambique, Ethiopia and Kenya [13,15,26–29].

Although there is an extensive list of *Anopheles* sequences generated using molecular barcodes based on the cytochrome oxidase I (COI) and internal transcribed region 2 (ITS2) genes [12,17,18,20,26–28,30–35], there still remain limitations in available sequence for these understudied *Anopheles* species to produce robust differentiation between members of closely related taxa [17,18,31,36]. This includes cryptic species which may be incrimi-nated in residual malaria transmission but have been allotted placeholder names such as An. species 11 [17,18,20], An. species 15 [18] and An. species unknown group 1 [20], par-ticularly in the absence of comprehensive morphological identification to compliment the generated molecular barcode sequences. Furthermore, the use of the single COI gene to validate identification for less studied *Anopheles* has produced matches with low similari-ties (less than 80%) and weakly supported phylogenies. For instance, this has led to in-conclusive identities for members of the An. coustani group in earlier studies from Zambia [17,31] and Mozambique [26]. Recently, mitochondrial genomes were used to provide conclusive identities and differentiate the cryptic taxa of the An. coustani group into phy-logenetically well-supported taxonomic clades [37].

The acquisition of genomic datasets has become more accessible due to the expan-sion of sequencing and computational technologies, including mitochondrial genomes (mitogenomes) which have shown to be useful in the identification and resolution of phy-logenies for several mosquito species and species groups [38–40]. These circular, double stranded DNA molecules encode 37 genes, including 13 protein coding genes (PCGs), 22 transfer RNA (tRNA), 2 ribosomal RNA (rRNA) and a non-coding control region [40,41]. In addition to the 13 PCGs, key characteristics such as low incidence of recombination, high copy number, and maternal inheritance make the mitogenome a more effective tax-onomic tool compared to single barcodes [41–44]. Expanding mitochondrial genome re-sources to include less-studied mosquito species is essential for accurate species delinea-tion and gaining insights into mosquito ecology and systematics for public health inter-ventions. In this study, we aimed to generate high quality reference sequence data for rep-resentative understudied mosquito species and demonstrate the strength of mitogenomes compared to prior studies that were limited to the COI gene in attempts to resolve phy-logenies.

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2. Materials and Methods

2.1. Mosquito collections

Specimens were collected during routine entomological surveillance in Nchelenge, Zambia in 2023-2024 as part of the Southern and Central Africa International Centers of Excellence for Malaria Research (ICEMR) investigations. Miniature CDC Light Traps (John W. Hock Co., Gainesville, Florida), were positioned both indoors and outdoors where people gather in the evening and near to animal pens.

2.2. DNA extraction and sequencing

DNA extractions using a modified extraction method [45] were performed on single mosquito specimens morphologically identified as An. rufipes, An. maculipalpis, An. preto-riensis, An. squamosus and An. pharoensis [21]. The extracted DNA and previously extracted specimens identified using the COI gene as species 11, species 15, unknown group 1, un-known group 2 and unknown group 3 from a previous study [20] were quantified using the Qubit dsDNA assay kit (Thermo Fisher Scientific, Waltham, MA) and shipped to Se-qCenter (Pittsburgh, USA) for library construction and Illumina sequencing. The libraries were 150 bp paired end sequenced to a count of 13.3 million reads per sample.

2.3. Mitochondrial genome assembly and annotation

The mitochondrial genomes were assembled using NOVOPlasty [46] (RRID:SCR_017335) version 4.3.5 with k-mer set at 39 base pairs and An. squamosus (OP_77691) as the seed sequence. Using the MITOchondrial genome annotation (MITOS) [47] galaxy tool, generated contigs were automatically annotated using the invertebrate genetic code under default settings. The start and stop codon positions of the annotated contigs were manually adjusted in Geneious Prime (RRID:SCR_010519) version 2025.1.2 (Biomatters, Auckland, New Zealand) using reference anopheline mitochondrial genomes as a guideline. The generated contigs with corresponding annotations were submitted to the GenBank database for the assignment of accession numbers

2.4. Phylogenetic analysis and tree construction

Using jModelTest (v2.1.10) [48], the best fit base pair substitution model based on the Akaike information criterion (AIC) and the Bayesian information criterion (BIC) was de-termined under default settings using an aligned sequence matrix. This alignment was generated using the MAFFT alignment tool implemented in the Geneious Prime (RRID:SCR_010519) version 2025.1.2 (Biomatters, Auckland, New Zealand from the 13 concatenated PCGs of mitogenomes generated in this study, available mitochondrial ge-nomes of understudied African anopheline species; An. marshallii (NC_064607), An. mou-cheti (NC_064608), An. gibbinsi (OR_569715), An. nili (NC_064610), An. coustani group (PQ_585798, PQ_587039, PQ_587041, PQ_587036, PP_375116), and reference mitoge-nome sequences for the well-studied species An. gambiae (MG_930894) and the An. funestus group (MG_742172, MG_742194, MT_917162, MT_917137, MT_91714, MT_917157, MT_917163). Using Bayesian Evolutionary Analysis by Sampling Trees (BEAST) 2 soft-ware [49], inference analysis was performed on the aligned sequence matrix using tree independent runs and a 20% burn-in rate for tree building purposes under default set-tings. Bayesian analysis was also performed on an alignment generated from COI se-quences available from GenBank complementary to representative species in the mito-chondrial genome tree. Trees were visualized and annotated using FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

2.5. Dating time estimation

Divergence time estimations were calculated using the previously mentioned pa-rameters used for Bayesian inference in BEAST2. The frequently referenced Ae-des-*Anopheles* divergence time

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of approximately 154.7 million years ago (MYA) [50] was used as the calibration point set to normal distribution.

3. Results

The 10 mitochondrial genomes produced in this study were consistent with other Af-rican anopheline mitogenomes represented in NCBI's GenBank database as comprised of 13 PCGs, 22 tRNAs and 2 rRNAs, with lengths ranging from 15,534 bp (Unknown group 2) to 15,346 bp (An. pharoensis) and a mean AT content of 77.1 % (Table 1).

Bayesian inference for the mitochondrial genomes resulted in a phylogenetic tree which separated specimen sequences (Figure 1) compared to COI tree which resulted in 4 weakly supported main clades (Figure S1). The most recent common ancestor (MRCA) for An. funestus and An. gambiae with the outdoor biting *Anopheles* included in this study was 66.45 and 69.65 MYA respectively (Figure 2).

Table 1. Genome characteristics for mitochondrial genomes of 8 understudied anopheline mos-quito species generated in this study.

Identification	Contig Size	GC %	AT %	GenBank Accession #
Morphological				
An. pretoriensis	15348	23.0	77.0	PP_068257
An. pharoensis	15346	23.7	76.3	PP_068256
An. rufipes	15362	22.9	77.1	PP_068259
An. squamosus	15349	23.1	76.9	PP_068255
An. maculipalpis	15361	23.4	76.6	PP_093765
Molecular				
Species 11	15350	23.1	76.9	PV_943469
Species 15	15354	22.8	77.2	PV_943468
Unknown group 1	15398	21.1	78.9	PV_943467
Unknown group 2	15534	23.1	76.9	PX_257875
Unknown group 3	15436	20.3	79.7	PX_240906

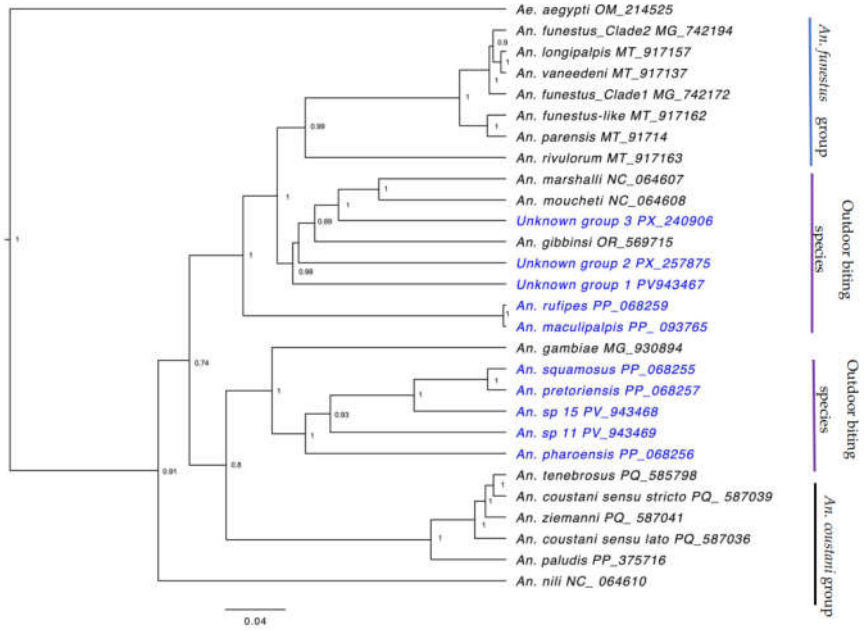

Figure 1. Bayesian tree showing the phylogenetic relationship of 10 new mitochondrial genomes (highlighted in blue) of understudied *Anopheles* mosquito species with other available anophe-line sequences. The tree includes

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assigned accession numbers and was constructed using BEAST v2.7.6. The posterior probabilities supporting the tree topology are represented by the values at the nodes.

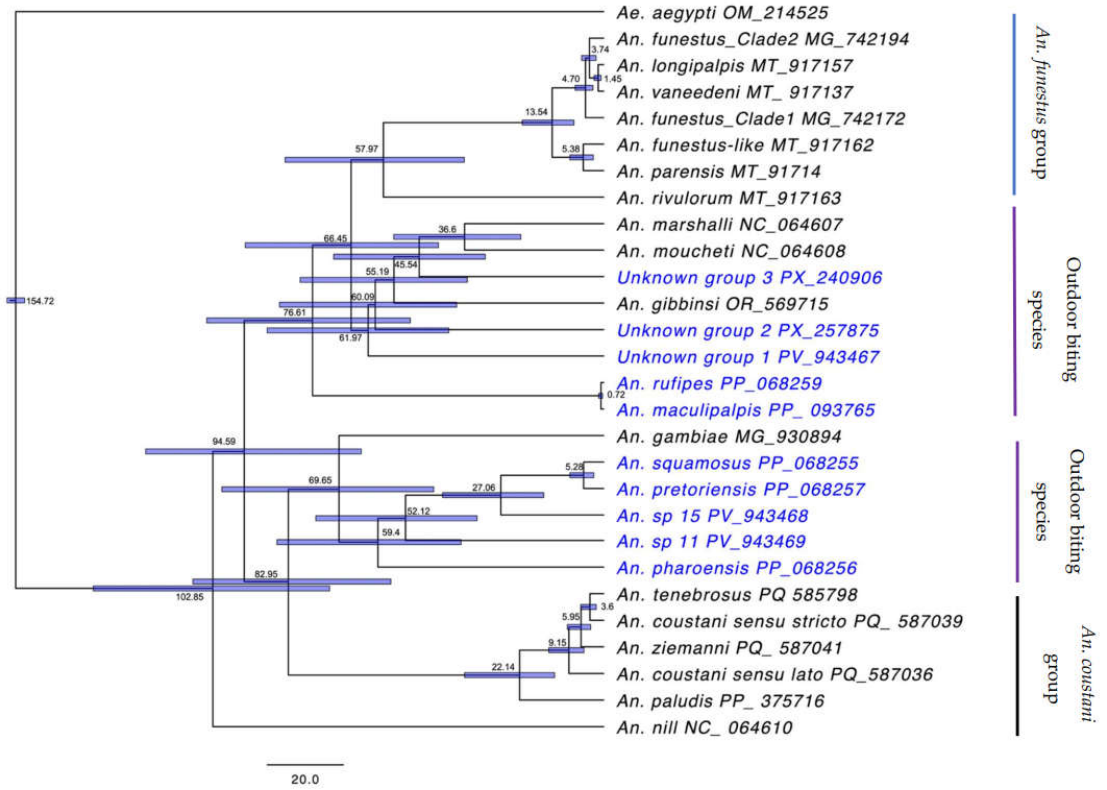

Figure 2. Phylogenetic tree showing inferred molecular divergence estimates (MYA) for outdoor biting *Anopheles* using the concatenated PCGs from mitogenomes generated in this study. The mean divergence time (MYA) predicted for each event is represented by the values at the tree nodes. The bars show the values at tree nodes, 95% confidence intervals.

4. Discussion

Bayesian phylogenetic analysis based on the concatenated PCGs from the mitoge-nomes was able to clearly delineate less studied African anopheline taxa compared to phylogenetic analyses using exclusively the COI gene as in earlier studies [17,20]. Several of these species are often misidentified during morphological examination or matched to an unassigned species following molecular barcoding [20,26,33]. This may be attributed at least in part to the unknown diversity of outdoor biting anophelines and relative inexpe-rience in morphological identification of these numerous taxa, some of which may yet to be fully described due to the bias placed on understanding highly anthropophilic, endophagic and endophilic species [6,9,17,28,31,31]. The uncertainty of vector richness of the exophagic anophelines together with their perceived adaptability and undetermined foraging times, habitats, and opportunistic feeding patterns have led to ambiguous spe-cies assignments [9,14,28,33,51]. This combination of characteristics emphasizes the need for the expansion of outdoor surveillance and investigation of the anophelines found in this space, as well as innovative strategies to overcome the shortcomings of in-door-focused interventions.

A growing number of understudied anophelines have been confirmed or implicated in human malaria transmission. Despite historic consideration as a secondary vector [12,17,27,52], An. coustani is now regarded as a primary local vector in regions of Mada-gascar [15,29,53]. Additionally, previous studies have identified An. pretoriensis [34], An. pharoensis [52,54], and cryptic anopheline species

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[17,18,20] demonstrating opportunistic feeding patterns on humans, some infected with sporozoites of human malaria parasite species. *Anopheles* squamosus is another species strongly implicated as a malaria vector, with a wide geographical range, and has demonstrated variable foraging behavior towards human blood meals [13,55,56]. Related to this are a number of 'molecular taxa'. Examples include An. species 11 and An. species 15 which are often morphologically keyed as An. squamosus but are differentiated by the COI barcode and even more strongly by the mitogenome sequence (Fig. 1)[17,20]. Others include An. unknown groups 1 – 3 for which morphology and molecular barcoding was inconclusive [20]. Here the mitogenome data provide the most comprehensive insight into the identification of these 'molecular taxa', but as with prior studies, without a more extensive sequence database of recognized species, these specimens remain taxonomically unresolved. The fact that many of these exophagic taxa cluster together in the phylogenetic analysis may be an artifact of their shared ancestry and that these share behavioral adaptations may have been reinforced over millennia.

It is clear that full mitogenomes offer much more discriminatory power for a phylo-genetic approach to inquire about shared biological traits and possibly ascertain whether behaviors such as biting preference are due to recent adaptations or reflect the existence of genetically distinct lineages which may have been overlooked when restricted to morpho-logical identification. Dating time estimations from well-recognized malaria vectors fur-ther corroborate the presence of these outdoor biting *Anopheles* as cryptic lineages with distinct ecological niches, suggestive of understudied species that may maintain trans-mission outdoors, perhaps under certain conditions such as relative absence of non-human hosts, or human behavior that promotes high opportunistic human biting rates. Furthermore, the accurate taxonomic placement of these mosquitoes highlights the relationships between known vectors and putative vector species which may provide fur-ther insights into understanding the differences in biting, foraging, and vectorial capacity of these less studied species. Linking morphological reference specimens to genomic data is key for the accurate identification given the status of unassigned *Anopheles* species with sporozoites collected in the field [18,19,26,52].

5. Conclusions

Although reference sequences are available for many commonly encountered outdoor biting anopheline species, there remains a paucity of data to accurately identify and taxo-nomically place these species in the wider *Anopheles* genus. This study contributes valu-able genetic datasets representing exophagic species collected in Zambia and present across the African continent. The generation of mitochondrial genomes for cryptic unas-signed species that are commonly collected has given priority to the use integrative tax-onomy in future research. The linking of molecular data with morphological and type specimens can further strengthen the credibility of species delimitation for the assigned zoological nomenclature of these cryptic taxa. The analyses from this study identified the phylogenetic relationships between the primary malaria vectors and understudied species implicated in malaria transmission, assisting to close the genetic gap of what we know about these anophelines of public health importance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/doi/s1, Figure S1: Bayesian tree showing the phylogenetic rela-tionship of less studied *Anopheles* mosquito species with main vectors on malaria using the cy-tochrome oxidase I gene (COI) using. The posterior probabilities supporting the tree topology are represented by the values at the nodes.

Author Contributions: R.L.M.N.A. and D.E.N. conceived and designed the study. M.E.G., L.S., K.S., W.H., H.C. and M.M. performed field collections and morphological identification of mosquito specimens. R.L.M.N.A worked on laboratory extractions and bioinformatic pipelines for generated datasets. D.E.N. and R.L.M.N.A. drafted the manuscript. C.J.M and A.C.M reviewed and approved the manuscript with all authors. W.J.M. and D.E.N. attained funding, read and approved the manuscript with all authors.

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Data Availability Statement: The mitochondrial genomes are available with accession numbers PP068255 – PP068257, PP068259, PP093765, PV943467 – PV943469, PX240906 and PX257875.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

COI Cytochrome Oxidase I

ITS2 Internal transcribed spacer 2 region

PCGs Protein coding genes

tRNA Transfer RNA

rRNA Ribosomal RNA

ICEMR International Centers of Excellence for Malaria Research

AIC Akaike information criterion

BIC Bayesian information criterion

BEAST Bayesian Evolutionary Analysis by Sampling Trees

MYA Million years ago

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