

Updating Anopheles species and symbiont associations from the Sud Kivu Region of Eastern Democratic Republic of Congo

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Abstract

Background

The Democratic Republic of Congo (DRC) contributes the second highest number of malaria cases worldwide, with 11.7% of all global malaria deaths in 2024. Continual political instability, particularly in Eastern DRC, has resulted in difficulties implementing effective entomological surveillance systems to determine the extent of malaria vector species diversity. The DRC is thought to harbour more than 60 *Anopheles* (An.) species, predominantly based on morphological identification. Entomological surveys that involve molecular identification are key to implementing effective vector control strategies and identifying symbionts such as *Wolbachia* bacteria and *Microsporidia* MB that have the potential to inhibit malaria transmission.

Methods

We collected adult *Anopheles* mosquitoes from the Sud Kivu region of eastern DRC during two entomological surveys (November 2021–October 2022 and August 2023). Mosquitoes from the first survey were morphologically identified as *An. gambiae* complex or unidentified species and from the second survey *An. demeilloni* or unidentified species. Molecular identification using PCR analysis and Sanger sequencing was undertaken in addition to screening for the presence of *Wolbachia* and *Microsporidia* MB symbionts.

Results

An entomological survey at six sites (November 2021–October 2022) resulted in the collection of predominantly *An. gambiae sensu lato* (s.l.) (83%, n = 708). DNA was extracted from monospecific pools of *An. gambiae* s.l. and PCR analysis revealed evidence for only *An. gambiae sensu stricto* (s.s.) and all pools were negative for both *Wolbachia* and *Microsporidia* MB. A second entomological survey (August 2023) collected predominantly *An. demeilloni* (87%, n = 131). Additional species were molecularly identified as *An. maculipalpis* (n = 2), and *An. coustani* s.l. (n = 8). We observed wing vein variation in *An. coustani* s.l. highlighting difficulties with morphological identification. PCR analysis revealed *Wolbachia* prevalence rates of 71% (n = 83) and 32% (n = 31) in Maziba and Lwiro for *An. demeilloni* respectively and no other *Anopheles* species were infected.

Conclusions

Our findings indicate that seasonality influences the prevalence of *Anopheles* species and molecular confirmation of the presence of *An. maculipalpis* and *An. coustani* s.l. adds to the *Anopheles* species diversity present in the Sud Kivu region of eastern DRC. We show that all pools of *An. gambiae* are

negative for *Wolbachia* and *Microsporidia MB*, while *An. demeilloni* are positive for *Wolbachia*, consistent with our previous studies.

Introduction

Malaria is a mosquito-borne disease transmitted by mosquito species within the *Anopheles* (*An.*) genera. Malaria continues to pose a significant global health burden, with an estimated 282 million cases and 610,000 deaths in 2024 [1]. The World Health Organisation (WHO) African Region accounts for 94% of cases and 95% of deaths globally [1]. The central African country of the Democratic Republic of Congo (DRC) accounted for the second-highest number of malaria cases in the WHO African Region (~40 million in 2024), contributing to 11.7% of malaria-related deaths in the region [1]. Continual political instability, particularly in Eastern DRC, has resulted in difficulties implementing effective entomological surveillance systems to map the distribution of *Anopheles* species and to monitor malaria vector dynamics.

It is estimated that the DRC harbours 64 *Anopheles* species, but this is mostly based on morphological identification only [2]. In the high altitude (> 1600 m above sea level) Sud Kivu Region of Eastern DRC, the village of Lwiro has been documented to harbour *An. funestus*, *An. marshalli*, *An. gibbinsi* (formerly *An. marshalli gibbinsi*), *An. demeilloni*, *An. coustani* and *An. christyi* [3, 4]. More recent studies have seen temporal variation in species in Lwiro with *An. funestus* and *An. marshalli* declining over time while *An. gambiae sensu lato* (s.l.) has now become established at higher altitudes [5, 6]. To date we do not have clear knowledge of the factors governing this dynamic, apart from population growth and anthropophilic factors related to climate change [1, 7]. The few entomological studies in the Sud Kivu region focus on surveillance of the main malaria vectors *An.s gambiae* s.l. and *A. funestus* s.l., mentioning non-vectors only incidentally [8]. Few studies in the region have used molecular biology to classify or update the *Anopheles* fauna, making the current list of species less precisely known [4].

Entomological surveillance and planning of vector control strategies are dependent on detailed knowledge of local sympatric *Anopheles* species. Recently *An. demeilloni* has emerged as an important species in the Sud Kivu region (along with *An. moucheti* in Central/Western Africa) as a species within the *Anopheles* genera to have stable endosymbiosis with *Wolbachia* bacteria [9, 10]. The *Wolbachia* strain wAnD in *An. demeilloni* has been shown to be a prevalent, high-density infection that is maternally transmitted and dominates the mosquito microbiome [9]. As wAnD contains homologs of cytoplasmic incompatibility factor (*cif*) genes, this strain is likely to induce cytoplasmic incompatibility and spread through host mosquito populations [9]. The wAnD strain in *An. demeilloni* is being further investigated for use in *Wolbachia* biocontrol strategies in *Anopheles*, aiming to reduce the transmission of malaria. Vector competence experiments are needed but analysis of wild mosquitoes show that *Wolbachia*-positive abdomens were associated with a lower malaria sporozoites rate compared with *Wolbachia*-negative abdomens [11]. *Microsporidia MB* is another promising candidate for symbiont-based malaria control as it disrupts the capacity of *An. arabiensis* to transmit malaria [12, 13]. Like *Wolbachia*, *Microsporidia MB* is transmitted vertically from mother to offspring and has the potential to spread through vector

populations and become established at high prevalence [12]. In this study, we undertook multiple entomological surveys in the Sud Kivu Region (2021–2023) with seasonal variation and used molecular species identification to update the confirmed *Anopheles* species. In addition, we screened mosquitoes for the presence of *Wolbachia* and *Microsporidia* MB symbionts but only detected the wAnD strain of *Wolbachia* in individual *An. demeilloni* mosquitoes collected in 2023.

Materials and Methods

Specimen collection and morphological identification. *Anopheles* mosquitoes were collected in the Sud Kivu region (Eastern DRC) during two entomological surveys. From November 2021 to October 2022, adult mosquitoes were collected using CDC light traps on a vertical transect at altitudes between 1400–1800m from Maziba (-2.241816; 28.800961), Lwiro (-2.238611; 28.807527), Katana (-2.218083; 28.842861), Muganzo (-2.248277; 28.823055), Kabamba (-2.199278; 28.852208), and Formulac (-2.228861; 28.843555). A second survey was undertaken in August 2023 in Maziba (-2.241816; 28.800961) and Lwiro (-2.238611; 28.807527) with mosquitoes collected inside houses using mouth aspirators. Collection sites are shown in Fig. 1. *Anopheles* mosquitoes were transported to the Medical Entomology and Parasitology laboratory of Centre de Recherche en Sciences Naturelles (CRSN) for morphological identification using standard dichotomous keys [14, 15]. For the 2021–2022 collection, mosquitoes were identified as *An. gambiae* s.l. or other *An.* species and for the 2023 collection; *An. demeilloni* or other *Anopheles* species. Specimens were preserved in 1.5 mL Eppendorf (Eppendorf, Germany) tubes using silica gel and sent to the University of Warwick for molecular analysis. Specific morphological wing vein patterns and the sex of each specimen were recorded prior to DNA extraction.

DNA extraction and PCR based identification of *Anopheles gambiae* complex (2021–2022) and *An. demeilloni* (2023). DNA was extracted from mono-complex pools of *An. gambiae* s.l. (n = 3 adult females per pool from same collection location in 2021/2022) and from individual *An. demeilloni* specimens using DNeasy Blood and Tissue extraction kit (QIAGEN, Germany) as previously described [9]. Extracted DNA was eluted in a volume of 50 µL and concentration quantified using Qubit DNA High Sensitivity Assay kit (Invitrogen, Thermo Fisher Scientific, USA) with a Qubit 4 Fluorometer kit (Invitrogen, Thermo Fisher Scientific, USA). gDNA extracts were normalised to 5 ng/µL for downstream polymerase chain reaction (PCR) analysis. *An. gambiae* s.l. mono-complex pools were subject to PCR analysis to confirm morphological identification [16]. PCR was undertaken on n = 40 pools targeted the SINE200 insertion, a highly repetitive ~ 200 base pair (bp) element [17]. Samples were prepared with forward (5'-TCGCCTTAGACCTTGCGTTA-3') and reverse (5'-CGCTTCAAGAATTTCGAGATAC-3') primers, and amplifications performed in 20 µL reactions containing 2 µL cDNA, 2 µL of each primer (10 µM), 4 µL H₂O, and 10 µL 2× Hot Start Taq PCR Master Mix (New England Biolabs, USA) [17]. The cycling conditions in the T100 Thermal Cycler (Bio-Rad Laboratories, USA) were: 10 min at 94°C; 35 cycles of 30 sec at 94°C, 30 sec at 54°C, and 60 sec at 72°C; then 10 min at 72°C. Amplification products of 479 bp or 249 bp were indicative of *An. coluzzii* or *An. gambiae sensu stricto* (s.s.) respectively [17]. For 2023 collections, samples were extracted individually. Molecular identification was performed using a *An. demeilloni* specific quantitative polymerase chain reaction (qPCR) assay targeting internal transcribed

spacer (*ITS2*) sequence with forward (5'-GCTTAAGGCAGGTAAGGCGA-3') and reverse (5'-CGGTGTTAGAAGGCTCCGTT-3') primers [9]. qPCR reactions were performed using a LightCycler® (Roche Diagnostics, Switzerland) in a final reaction volume of 10 µL containing 5 µL of LightCycler® 480 SYBR Green Master, 2x concentration (Roche Diagnostics, Switzerland), 1 µL of 10X for each of the forward and reverse primers, 1 µL of water and 2 µL of the extracted DNA. Reactions were performed at 50°C for 2 minutes, 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds, 59°C for 22 seconds and 72°C for 1 minute. A melting cycle of 95°C for 10 seconds, 65°C for 60 seconds and 97°C for 1 second was performed to ensure the correct target was amplified.

Wolbachia and Microsporidia MB detection. *Wolbachia* surface protein (*wsp*) gene PCR analysis were carried out using primers *wsp*81F: forward (5'-TGGTCCAATAAGTGATGAAGAAAC-3') and *wsp*691R: reverse 5'-AAAAATTAAACGCTACTCCA-3' in a 100 Thermal Cycler (Bio-Rad Laboratories, USA) as previously described [10, 18]. The PCR reactions were in a final volume of 20 µL containing 10 µL of 2X Phire Hot Start II PCR Master Mix (Thermo Scientific, USA), 2 µL of 10X for each primer, 4 µL of water and 2 µL of DNA. Forward and reverse primers targeting the *Microsporidia* MB RNA polymerase beta subunit gene (*rpoB*) gene were RPOBMBF: forward (5'-ACAGTAGGTCACCTTGATTGAATGTC-3') and RPOBMBR: reverse (5'-TACCATGTGCTTAAGTCTTTGGT-3') respectively [13]. DNA extracted from an *An. gambiae* *Microsporidia* MB-infected sample obtained from the University of Glasgow was used to confirm successful amplification of the *rpoB* gene. PCR reactions were optimized in a final volume of 20 µL containing 10 µL of 2X Phire Hot Start II PCR Master Mix (Thermo Scientific, USA), 2 µL of 10X for each primer, 4 µL of water and 2 µL of DNA.

Sanger sequencing for molecular species identification of other *An. species* (2023). *ITS2* primers (*ITS2A*: forward (5'-TGTGAACTTGCAGGACACAT-3') and *ITS2B*: reverse (5'-TATGCTTAAATTYAGGGGGT-3')) were used as previously described amplify *ITS2* gene from other *Anopheles* species [19]. PCR reactions were optimized in a final volume of 20 µL containing 10 µL of 2X Phire Hot Start II PCR Master Mix (Thermo Scientific, USA), 2 µL of 10X for each primer, 4 µL of water and 2 µL of DNA and performed in a Bio-Rad T100 Thermal Cycler. *ITS2* PCR cycling included an initial denaturation at 98°C for 30 seconds; 34 cycles of 98°C for 5 seconds, 59°C for 5 seconds, and 72°C for 30 seconds; followed by a final extension at 72°C for 5 minutes. PCR products were separated and visualised using precast 2% E-Gel EX agarose gels with SYBR safe (Invitrogen, Thermo Fisher Scientific, USA) and an E-Gel iBase Real-Time Transilluminator (Invitrogen, Thermo Fisher Scientific, USA). *ITS2* PCR products were cleaned using a Monarch Spin PCR and DNA Clean Up Kit (New England Biolabs, USA) and sent to GATC Biotech (Eurofins Genomics, UK) for Sanger sequencing. Sequencing analysis was carried out in 4peaks (<https://nucleobytes.com/4peaks/>). Sequences were manually checked, edited, and trimmed as required. Sequences were used to perform National Centre for Biotechnology Information (NCBI) basic local alignment search tool (BLAST) database queries. Sequence alignments were constructed in molecular evolutionary genetics analysis (MEGA) 12 (<https://www.megasoftware.net>) using the Muscle algorithm to include relevant sequences from the BLAST analysis [20]. The cladogram was generated using the Neighbour-joining method based on the Maximum Composite Likelihood method of nucleotide substitutions and visualised with interactive tree of life (ITOL) [21]. The tree with the highest log

likelihood after 1000 bootstrap replicates was shown. Bootstrap support values are presented on the tree. Only values ≥ 0.7 are shown.

Results

The first entomological survey (November 2021–February 2022) resulted in the collection of mosquitoes morphologically identified as belonging to the *An. gambiae* complex ($n = 708$, 83% of total) (Fig. 2A). Remaining samples were not identified to species level by morphological ID due to the possibility of misidentification prior to pooling for molecular analysis. SINE200 PCR analysis of mono-complex *An. gambiae* pools ($n = 40$) containing 3/species/collection location confirmed the presence of *An. gambiae* s.s. in all collection locations with a PCR product size of ~ 250 base pairs (Fig. 2B). There was no evidence of *An. coluzzii* present in pools from any of the six collection sites and only a single pool was inconclusive with no SINE200 PCR amplification on repeat PCRs. All mono-complex *An. gambiae* s.l. pools ($n = 236$) were negative when screened for both *Wolbachia* and *Microsporidia* MB symbionts.

The second entomology survey in August 2023 focused on Maziba and Lwiro collection locations. In contrast, 95% (83/87) and 71% (31/44) of mosquitoes collected were morphologically identified as *An. demeilloni* from these two locations respectively (Fig. 3A). Only *An. demeilloni* samples from 2023 were positive for *Wolbachia*. The prevalence of *Wolbachia* was 71% (59/83) and 32% (10/31) within *An. demeilloni* in Maziba and Lwiro respectively (Fig. 3B). 10 *Anopheles* samples collected from Lwiro had morphological characteristics inconsistent with *An. demeilloni* (images can be found in the underlying data: <https://osf.io/qz3ra>) and were initially morphologically identified as *An. maculipalpis* ($n = 1$) or *An. coustani* s.l. ($n = 8$). *ITS2* Sanger sequencing was undertaken to confirm species identity (Table 1) including four *An. demeilloni* as representative samples from the 131 specimens morphologically identified (GenBank accession numbers PZ165618–PZ165631). Morphological identification was confirmed by *ITS2* Sanger sequencing. *ITS2* phylogenetic analysis confirmed expected evolutionary associations between *Anopheles* species (Fig. 3C) but revealed evidence of genetic variation within *An. coustani* samples. Our *ITS2* sequences grouped with a single *An. coustani* accession number (MK592070.1) and we also saw variation in the morphology of wing veins in our *An. coustani* samples (Fig. 3D). For these *ITS2* sanger confirmed *An. coustani* individuals, two males had two pale bands on vein 1/6, two females had two pale bands on vein 6 and three females had a single pale band on vein 6.

Table 1

Morphological and molecular identification of selected individuals collected from Lwiro in August 2023.

Specimen	Morphological ID	Wing vein 6 pale bands	GenBank accession number	Wolbachia (+/-)
Female	<i>An. maculipalpis</i>	-	<i>An.maculipalpis</i> .1: PZ165618	-
X3 legs	unknown	-	<i>An.maculipalpis</i> .2: PZ165619	-
Female	<i>An. coustani</i>	1	<i>An.coustani</i> .1: PZ165620	-
Female	<i>An. coustani</i>	1	<i>An.coustani</i> .2: PZ165621	-
Female	<i>An. coustani</i>	1	<i>An.coustani</i> .3: PZ165622	-
Female	<i>An. coustani</i>	2	<i>An.coustani</i> .4: PZ165623	-
Female thorax	<i>An. coustani</i>	2	<i>An.coustani</i> .5: PZ165624	-
Male	<i>An. coustani</i>	2	<i>An.coustani</i> .6: PZ165625	-
Male	<i>An. coustani</i>	2	<i>An.coustani</i> .7: PZ165626	-
Female	<i>An. coustani</i>	-	<i>An.coustani</i> .8: PZ165627	-
Female	<i>An. demeilloni</i>	-	<i>An.demeilloni</i> .1: PZ165628	+
Female	<i>An. demeilloni</i>	-	<i>An.demeilloni</i> .2: PZ165629	+
Female	<i>An.demeilloni</i>	-	<i>An.demeilloni</i> .3: PZ165630	+
Female	<i>An. demeilloni</i>	-	<i>An.demeilloni</i> .4: PZ165631	+

Discussion

Our results highlight the dominance of *An. gambiae* s.s. in October 2021 to November 2022, previously described as an important malaria vector, and the dominance of *An. demeilloni* in August 2023 [22, 23]. Reasons for this stark difference in prevalence of species across collections are likely seasonal. August is one of the dryer months of the year [24]. *An. demeilloni* has shown preference towards breeding sites in semi-permanent water sources such as streams and pools, these water sources may be the only remaining habitats during the dry months of the year resulting in a higher proportion of *An. demeilloni* [25]. In contrast, *An. gambiae* s.s. larvae are typically found in temporary, sunlit freshwater habitats such as puddles and ditches containing short plants and grass shoots [22, 23]. More of these habitats are generated during the wetter months of the year such as October and November, facilitating the expansion of the *An. gambiae* s.s. population [24]. Most samples collected from our 2021/2022 study,

were morphologically identified as *An. gambiae* and PCR targeting the SINE200 insertion element confirmed the presence of *An. gambiae* s.s. and no evidence of *An. coluzzii*. *An. coluzzii* has been detected in Kinshasa DRC and in the Kwilu Province in Western DRC but was detected at very low frequency in Northern DRC [26–29]. A wider entomological survey across the DRC revealed no evidence for the presence of *An. coluzzii* in Eastern DRC [30].

Our results using molecular identification confirm the presence of *An. coustani* s.l. and *An. maculipalpis* in 2023 that earlier studies have indicated are present in Lwiro [3]. This species is considered a secondary malaria vector in other parts of the WHO African Region [31, 32]. Our morphological identification of *An. coustani* s.l. focused on the wing vein patterns which exhibited diversity on the banding of wing vein 6 (Fig. 3D). These morphological differences could be indicative of the presence of different members of the *Anopheles coustani* group, which have previously been detected in the DRC [33]. Despite these morphological differences between members of the *An. coustani* group, our ITS2 sequences did not show sequence variation based on morphology. This suggests that the ITS2 region may not contain sufficient sequence variation to distinguish between members of the *An. coustani* group. This has been seen for other *Anopheles* species complexes such as *An. gambiae* s.l. and *An. namibiensis* which share similar morphological characteristics with *An. coustani* including wing coloration [15]. *An. coustani* is frequently found in cattle enclosures, and all our 2023 specimens were collection from two houses with cattle. To our knowledge, there is only a single GenBank accession number for *An. namibiensis* (PX307523.1) and this sequence was from the cytochrome c oxidase subunit I (COX1) gene [34]. BLAST analysis to *An. coustani* GenBank sequences resulted in 655/658 (> 99%) nucleotide similarity indicating these species appear to be genetically very similar, albeit with only one genetic sequence present for analysis.

Our study also revealed the presence of *An. maculipalpis* from Lwiro in 2023. Previously only a single specimen has been recorded, suggesting that *An. maculipalpis* is not present in high densities or methods of collections used to date are not effective at collecting this species [4]. *An. maculipalpis* has a wide distribution across the WHO African Region but has historically been collected at locations up to 1000 metres above sea level in central Africa [2, 35]. Generally considered zoophilic, exophage and exophilic, *An. maculipalpis* has no proven medical importance as a malaria vector.

An. demeilloni was genetically identified in our study, again confirming the presence of this species in Eastern DRC [4, 9, 11]. Although studies have detected *Wolbachia* within a range of *Anopheles* species, many reports are based primarily on molecular detection through PCR [36]. In contrast, the wAnD and wAnM strains have strong evidence for stable endosymbiotic associations through a combination of molecular detection, high prevalence in natural populations, and intracellular visualisation using Fluorescence In Situ Hybridisation (FISH) [9]. Consistent with these findings, our data indicate that *An. demeilloni* is infected with *Wolbachia*, we found no evidence of *Wolbachia* infection in the other *Anopheles* species [9, 11].

There were higher prevalence rates of *Wolbachia* in *An. demeilloni* samples from Maziba (71%) when compared with those from Lwiro (32%). A previous published study of collections from Maziba (November 2021, n = 553) and Lwiro (January/February 2024, n = 41) reported *Wolbachia* prevalence rates of 83% and 51%, respectively [11]. The lower prevalence estimate in Lwiro should be interpreted cautiously because substantially fewer *An. demeilloni* mosquitoes were collected than in Maziba in both studies, resulting in greater uncertainty around the estimated prevalence. The high prevalence of wAnD in *An. demeilloni* from Maziba is consistent with the hypothesis that cytoplasmic incompatibility may contribute to its maintenance within host populations [9]. Supporting this hypothesis, two pairs of *cif* gene homologs have previously been identified in the wAnD genome [9].

We found no evidence for the presence of any other *Wolbachia* strains or *Microsporidia* MB in any of the *An. gambiae* s.l. mono-complex pools from the 2021/2022 survey. *Microsporidia* MB has been detected within the *Anopheles gambiae* complex with studies in Benin showing that that *An. gambiae* s.l. mosquitoes bearing the L1014F knockdown resistance (*kdr*) allele was associated with a higher prevalence and load of *Microsporidia* MB than their susceptible counterparts [37]. However, that study found the infection prevalence of *Microsporidia* MB was only 1.6% (95% CI: 0.7–3.3) in *An. coluzzii* and 2.2% (95% CI: 0.1–13.2) in *An. gambiae* s.s. [37]. This suggests *Microsporidia* MB does not result in high prevalence rates and is highly influenced by environmental conditions. In *An. arabiensis*, *Microsporidia* MB was also shown to be associated with permethrin insecticide resistance in Kenya, but populations showed variable *Microsporidia* MB infection rates with some areas having no infection [37, 38].

Conclusion

Eastern DRC including the Sud Kivu Region lacks comprehensive malaria entomological data due to logistical difficulties implementing surveys to capture the diversity of both *Anopheles* species and associated symbionts. This study demonstrates the seasonal change in *Anopheles* species composition and confirms the presence of three less well document species (*An. demeilloni*, *An. coustani* s.s. and *An. maculipalpis*) which may have a role in residual malaria transmission. We highlight the difficulties of accurate species identification of closely related sibling species even using molecular methods targeting conserved regions. Finally, we show no evidence for the presence of *Microsporidia* MB in wild *Anopheles* populations and no evidence for *Wolbachia* strains other than the wAnD strain in symbiosis with *An. demeilloni*.

Abbreviations

An.

Anopheles

BLAST

basic local alignment search tool

bp

base pair

cif
cytoplasmic incompatibility factor
COX1
cytochrome c oxidase subunit I
CRSN
Centre de Recherche en Sciences Naturelles
DNA
deoxyribonucleic acid
DRC
Democratic Republic of Congo
FISH
Fluorescence In Situ Hybridisation
ITOL
interactive tree of life
ITS2
internal transcribed spacer
kdr
knockdown resistance
MEGA
molecular evolutionary genetics analysis
NCBI
National Centre for Biotechnology Information
PCR
polymerase chain reaction
qPCR
quantitative polymerase chain reaction
rpoB
RNA polymerase beta subunit gene
s.l.
sensu lato
s.s.
sensu stricto
WHO
World Health Organisation
wsp
Wolbachia surface protein.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

JB conducted mosquito collections, laboratory work, and drafted the manuscript. IH conducted laboratory work, analysis, and drafted the manuscript. CB contributed to the selection of the sampling sites in 2021 and reviewed a draft of the manuscript. SS conducted laboratory work and reviewed the final manuscript. LC conducted laboratory work and reviewed the final manuscript. CMT conducted laboratory work and reviewed the final manuscript. TW obtained funding for the study, analysed data, drafted the manuscript, and provided overall supervision.

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Data Availability

The datasets supporting the conclusions of this article are available in the Open Science Framework (OSF) repository, <https://osf.io/qz3ra>.

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Figures

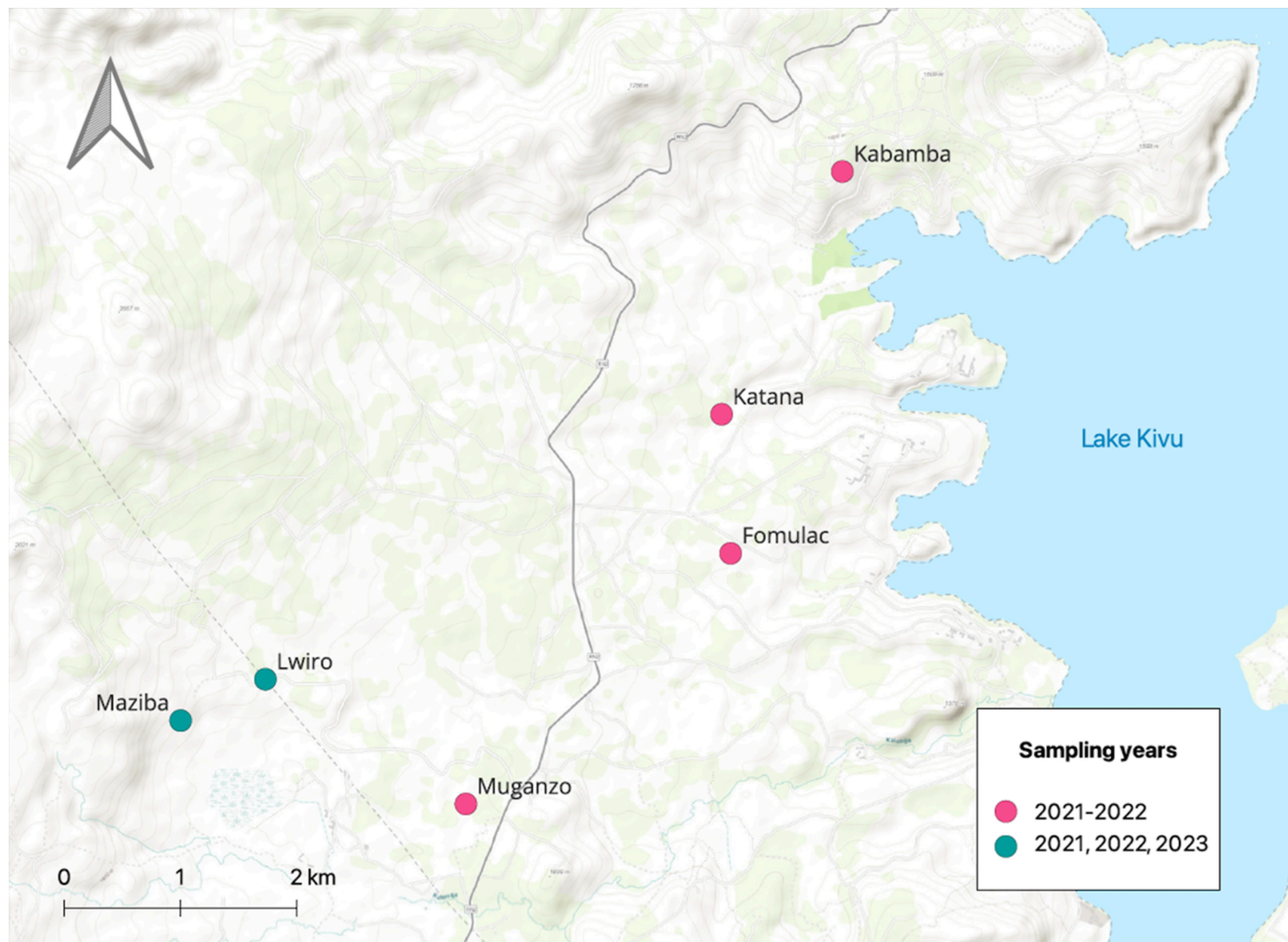


Figure 1

Map of collection sites in the DRC. Map created in QGIS 3.30.

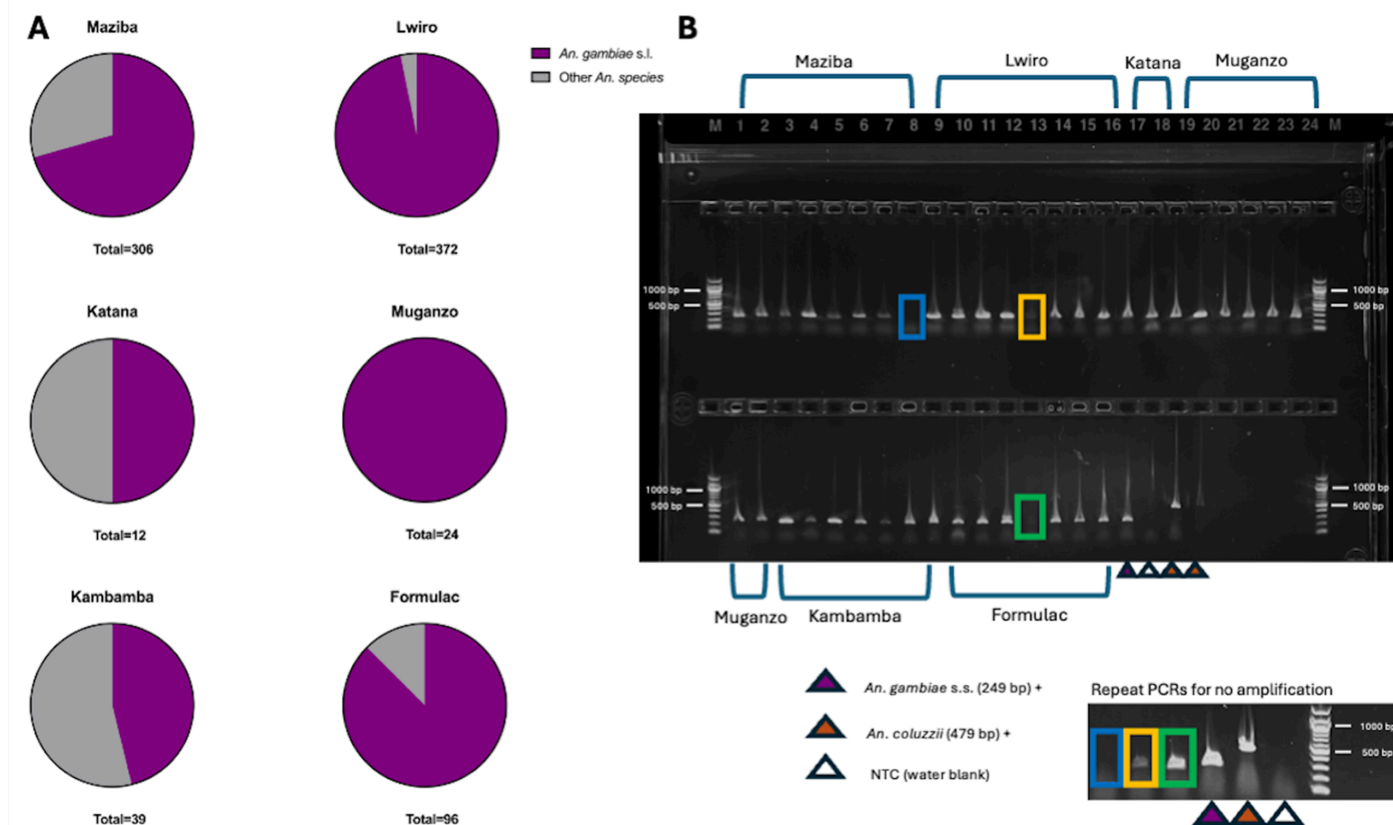


Figure 2

***Anopheles* species collected during 2021-2022 entomological surveys in the Sud Kivu region of Eastern DRC.** **A.** Prevalence of the *An. gambiae* s.l. complex (purple) based on morphologically identification in field locations; **B.** SINE200 PCR analysis of mono complex *An. gambiae* s.l. pools indicating only *An. gambiae* s.s. was present in all collection locations. Coloured boxes indicate repeat amplification for those samples which did not amplify in the first instance.

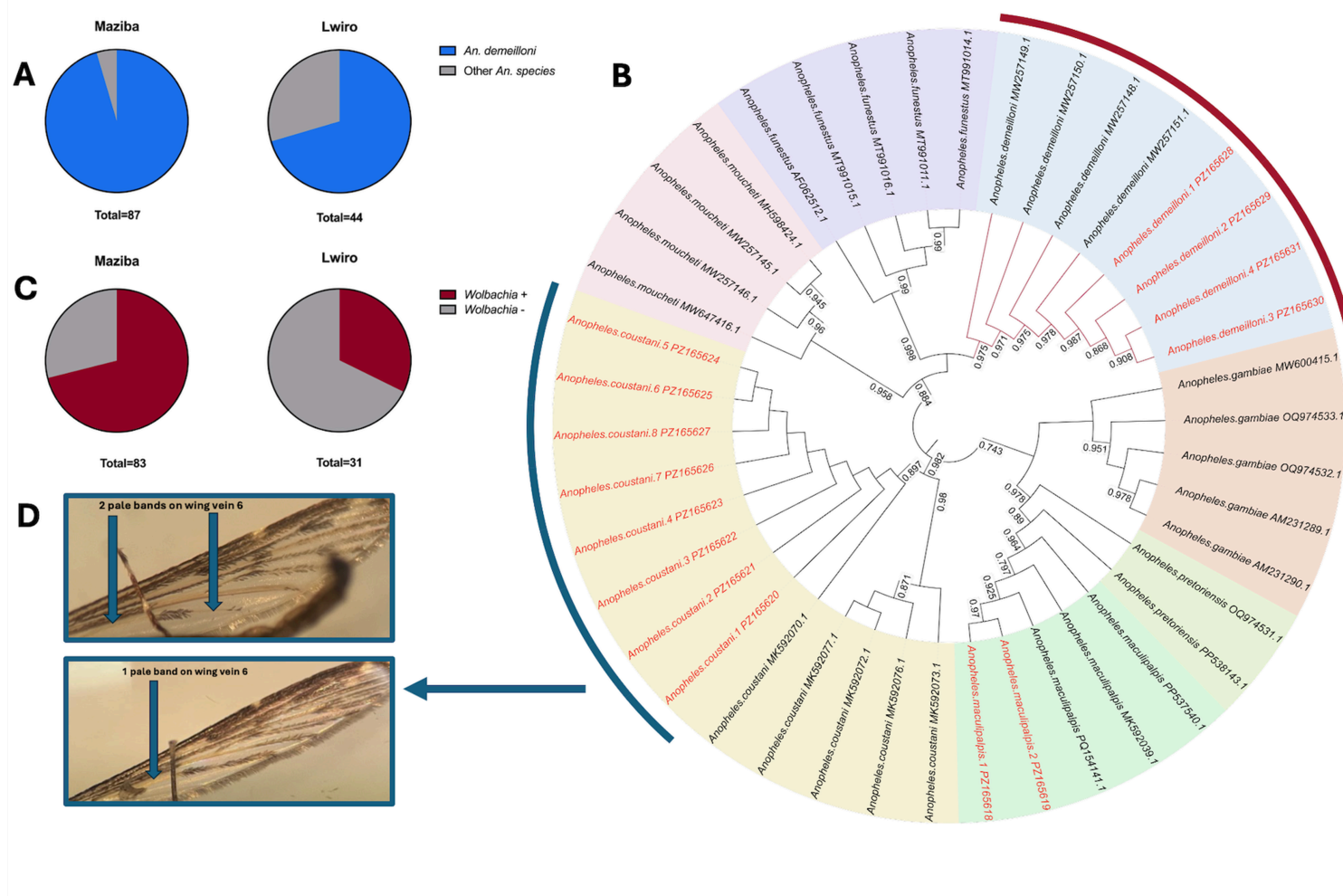


Figure 3

Anopheles species collected at Lwiro and Maziba in August 2023. **A.** Prevalence of *An. demeilloni* (blue) based on molecular identification by field location; **B.** Prevalence of Wolbachia (wsp+ shown in red) within *An. demeilloni* population based on PCR analysis by field location; **C.** ITS2 cladogram of a subset of samples collected in Lwiro, DRC (2023). Sequences with labels in red have been generated in this study (full sequences can be found in **supplementary Table S7**) and contain assigned GenBank accession numbers, whilst all other sequences are references from GenBank. Sequences were aligned using ClustalW in MEGA12 (<https://www.megasoftware.net>) and the cladogram was generated using the Neighbour-joining method based on the Maximum Composite Likelihood method of nucleotide substitutions [20]. Bootstrap support values of $\geq 70\%$ are shown on the tree. *An.* samples which are positive for Wolbachia are annotated with red; **D.** Morphological wing vein banding patterns for two representative females molecularly identified as *An. coustani* s.l. by ITS2 sanger sequencing.