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Profiling insecticide resistance in Anopheles gambiae s.l. and Anopheles stephensi from Kenya using in-country nanopore sequencing

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Abstract

Background

Malaria remains a threatening disease in Kenya, with over 70% of the population at risk of getting infected. Although vector control interventions such as indoor residual spraying and insecticide treated nets have significantly reduced transmission, the emergence of insecticide resistance and spread of invasive species such as *Anopheles stephensi* threaten these gains. This emphasizes the need for continuous insecticide resistance monitoring to inform NMCPs on the selection and deployment of effective vector control tools. Therefore, we developed a simple and cost-effective nanopore sequencing insecticide resistance method that targets key insecticide resistant markers, including target site *(Vgsc, Gaba receptor*) and metabolic resistance markers (*Ace1* and *Gste2*).

Results

The panel achieved a mean sequencing depth of > 1,000 for most targets and was able to define the resistance profiles of 69/70 (98%) *An. gambiae s.s*, 45/48 (94%) *An. arabiensis* and 63/80 (79%) *An. stephens*i isolates collected across Kenya. The KDR East (*Vgsc* L995S) mutation was at a high frequency in *An. gambiae s.s* (85%) but at low frequencies in both *An. stephensi* (5%) and *An. arabiensis* (13%). The KDR West mutation (*Vgsc* L995F) was at low frequencies in *An. gambiae* s.s. (2%), *An. arabiensis* (12%), and *An. stephensi* (2%); the *Gaba* A296S mutation was also present at low frequencies in the same species, at 2%, 4%, and 6%, respectively. The *Gste2* L119V mutation was exclusive to *An. gambiae s.s* at 2% frequency. In addition, several previously unreported mutations were identified.

Conclusion

We have developed and implemented a nanopore sequencing approach for insecticide resistance surveillance in Kenya. The moderate to low levels of resistance mutation frequencies identified in *An. gambiae s.l.* and *An. stephensi* suggests resistance to permethrin, DDT, deltamethrin and dieldrin insecticides. These results also provide insights into Kenyan *An. stephensi* insecticide resistance profiling that can be utilized in controlling this invasive vector.

Background

In Kenya, malaria remains a pressing health concern, with 70% of the population at risk (1). Malaria is endemic around Lake Victoria and the coastal regions, and recent increase in transmission in North-Western Kenya (2). In western and coastal parts of Kenya, the primary malaria vectors are *An. gambiae s.s*, *An. arabiensis* and *An. funestus* (3). *An. stephensi* and *An. coluzzii* have recently been reported in northern Kenya, with *An. stephensi* observed to be spreading rapidly (4, 5). However, the contribution of these species to malaria transmission and insecticide resistance status is yet to be evaluated (4–6).

Kenya's approach to vector control aligns with the UN Sustainable Development Goals and the Kenyan nation's Vision 2030 plan, using a combination of strategies that adapt to local differences in climate, mosquito species, and altitude (7). The National Malaria Control Programme (NMCP) relies on large-scale deployment of interventions such as insecticide-treated nets and indoor residual spraying to protect communities (8). Yet, challenges like insecticide resistance and unique transmission patterns make it clear that ongoing innovation and tailored strategies are needed to reduce malaria's toll, particularly in the hardest-hit areas (9).

The use of insecticide-based vector control tools in public health and agricultural applications has served as the main selection pressure driver in *Anopheles* species, leading to the development of resistance. Insecticide resistance (IR) mechanisms in *Anopheles* are grouped into four categories: target-site resistance, in which mutations directly inhibit an insecticide interacting with its target (e.g. mutations in *Vgsc, Gaba receptor*, and *Ace1* (10–15)); metabolic resistance, in which insecticide metabolism is altered (e.g. Cyp P450s, Coeae, *Gste2* (16–20)), cuticular thickening, in which insecticide absorption is reduced (e.g. CPRRs, CPLCA, CPRs (21–25)), and behavioral resistance (e.g. changes in biting time (26)). Generally, *Anopheles* employ multiple resistance mechanisms in concert (27). Importantly, the molecular markers that underpin resistance are known, enabling genetic surveillance to inform NMCP insecticide usage.

Polymerase Chain Reaction (PCR) assays targeting known point mutations associated with resistance are widely used to assess IR (28), but are unable to detect novel mutations. Whole-genome sequencing (29), and genome-wide association studies provide enormous granular data, through which many novel IR loci have been identified (14, 19, 20, 30–33), but they are costly, low-throughput and require highly specialised laboratories and personnel. In contrast targeted sequencing allows known and novel mutations in IR associated genes to be surveilled in the local setting. Nanopore sequencing in particular, facilitated by the portable and low-cost MinION sequencing device (Oxford Nanopore Technologies, ONT) is particularly accessible, requiring minimal laboratory infrastructure end users are empowered to visualise and respond to their own data in a rapid fashion. Furthermore, as a long sequencing technology, entire genes can be assessed and all novel mutations tracked. The NOMADS project has augmented this ecosystem with additional tools e.g. primer development software and realtime sequencing dashboards to support the establishment of new sequencing assays capable of generating actionable public health data. To date, only a handful of assays for targeted nanopore sequencing of *Anopheles* have been developed for species identification, blood meal detection (34) and wolbachia detection (35), while significant gaps remain for IR .

We therefore developed a novel nanopore-based amplicon panel and sequencing protocol that enables IR profiling across *Anopheles gambiae s.l*. The amplicon panel targets five common insecticide-resistance associated genes and produces amplicons of between 800 and 1200bp. We deployed the assay locally in Kisumu, Kenya, sequencing 69 *An. gambiae s.s.*, 45 *An. arabiensis* and 63 *An. stephensi* samples, characterising resistance mutations for permethrin, DDT, deltamethrin and dieldrin insecticides.

Methods

Samples

Specimens, caught by UV light trap or human landing catches, of *An. gambiae s.s* and *An. arabiensis* samples were collected from Nov-Dec 2023 from Western Kenya (Siaya county) (36), and *An. stephensi* samples collected between 2022 and 2024 from Northern Kenya (Wajir, Isiolo and Marsabit counties) (Fig. 1a) (6). Genomic DNA was extracted from the individual mosquitoes using a cost-effective destructive ethanol precipitation protocol (37) and confirmatory species identification was performed using primers targeting the internal spacer transcribed spacer region2 (ITS2) of ribosomal DNA (38, 39).

Multiplex PCR panel

Multiply software (https://github.com/JasonAHendry/multiply) was used to target four insecticide resistance genes; acetylcholinesterase 1 (*Ace1*), *Gste2*, gamma-aminobutyric acid (*Gaba*)-gated chloride channel and voltage-gated sodium channel (*Vgsc*). Provided with our regions of interest, *Multiply* explored 223 candidate amplicons (median 38 per target region), and provided a final set of 8 amplicons (Table 1) arranged in three multiplex PCR combinations, optimised to minimize primer dimers and off-target binding, while covering all six target regions (Supplementary file1). Primers were pooled at equimolar concentrations of 10 µM, and multiplex PCR was conducted to ensure that all *Anopheles* species target amplified (Supplementary file 3). The 25 µl PCR reaction consisted of 14.5 µl High Fidelity Q5 Polymerase, 2.5 µl primer pool and 8 µl template DNA. The PCR cycling conditions included an initial denaturation step at 98℃ for 30s followed by 35 amplification cycles. Each amplification cycle included a denaturation step at 98℃ for 7s and extension step at 64℃ for 3 min. A final extension was performed at 64℃ for 10 min.

Purification and targeted nanopore sequencing

The PCR products were purified at 0.5X ratio using AMPure beads (A63880, Beckman) and eluted in 15uL water. Eluted DNA was quantified using a Qubit Fluorometer using Qubit dsDNA High Sensitivity Assay kit. Unique barcodes were assigned to each sample from 96-Native Barcode Ligation kit prior to pooling and adapter ligation. Sequencing libraries were prepared using the Ligation Sequencing Kit (SQL-LSK114, ONT) following the manufacturer's protocol with minor modifications (https://www.protocols.io/edit/nomads-ir-insecticide-resistance-panel-for-surveil-j6i9crch7). In the final wash, we used the Short Fragment Buffer (SFB) and eluted the final library in 15 µl of elution buffer. The library was loaded in a MinION Flow Cell (R10.4.1) and sequenced using MinION Mk1B instrument. Basecalling was performed in real-time with MinKNOW using the super-accurate (SUP) model.

Downstream analysis

Using Nomadic, a browser-based dashboard showing real-time mapping and amplicon-based analysis plots (https://jasonahendry.github.io/nomadic/), we aligned the FASTQ files to their respective reference genomes (*Anopheles gambiae PEST*, *Anopheles arabiensis* Dongola 2021, *Anopheles stephensi* UCISS 2018), downloaded from Vectorbase (40) (release 67), using Minimap2 (41) (v 2.24-r1122). We then generated a Binary Alignment Map (BAM) file by converting the mapping output Sequence Alignment Map (SAM) file using SAMtools (42) (v.1.17 http://github.com/samtools). The alignment BAM file was then sorted and indexed to the reference genome awaiting variant calling. We performed variant calling using BCFtools *mpileup* and BCFtools *call* (v.1.17, http://samtools.github.io/bcftools) to generate a variant call format (VCF) file with all SNPs and indels. Amino acid changes were annotated using BCFtools *csq* (v1.17).

SNP filtering, allele frequencies and haplotype phasing

We filtered out all SNPs that were not in our target regions and low quality SNPs (< Q10). We used WhatsHap (43) (v.2.3, https://github.com/whatshap/whatshap/) to phase all heterozygous mutations. The resulting file was used to calculate the allele and genotype frequencies using scikit-allele (44) (v.1.3.13, https://github.com/cggh/scikit-allel) in Python.

Results

Development of an IR panel for surveillance in Anopheles gambiae s.l.

We used Multiply(45) to design primers targeting four genes associated with insecticide resistance in *Anopheles gambiae s.l.*: *Vgsc, Gaba, Gste2*, and *Ace1*. Three multiplex PCR primer combinations were tested on *An. gambiae s.s.* and *An. arabiensis* genomic DNA. Individually, all amplicons generated a product of the expected size, although for some (*Vgsc-L995, Vgsc-402*) off-target amplification was also visible (Supplementary File 2A). After sequencing the three primer combinations on a R10.4 Flongle Flow Cell, we selected the set with the highest overall coverage, which we hereafter refer to as the NOMADS Insecticide Resistance (NOMADS-IR) panel. We evaluated the NOMADS-IR panel using archived samples up to 10 years old and observed consistent, strong amplification across all years, demonstrating that the panel performs reliably even with older samples (Supplementary file2 B).

Table 1
List of the target genes, amplicon sizes, and resistance mutations. bp, basepairs.

Chromosome	Start	Stop	Gene	Amplicon (bp)	Resistance mutations	Insecticides
2L	2390443	2391584	*Vgsc*	1142	402L	Type I/II pyrethroids, DDT
2L	2422485	2423463	*Vgsc*	979	995S (*KDR*-e), 995F (*KDR*-w)	Type I/II pyrethroids, DDT
2L	2428804	2429878	*Vgsc*	1075	1527T, 1570Y	Deltamethrin, permethrin, Type I/II pyrethroids, DDT
2L	25428663	25429767	*Gaba*	1105	296G, 296S	Organochlorines
2R	3491270	3492332	*Ace1*	1063	280S	Carbamates, organophosphates
3R	28597890	28598734	*Gste2*	843	114T, 119V	DDT, permethrin

Application to An. gambiae s.s. and An. arabiensis

We sequenced 70 *An. gambiae s.s* and 48 *An. arabiensis* field samples with the NOMADS-IR panel in Kenya at KEMRI, Kisian. Of the 70 *An. gambiae* s.s. samples, 69 (98%) passed quality control while in *An. arabiensis* 45 (94%) of the 48 passed quality control and proceeded to downstream analysis. Greater than 95% of sequencing reads from *An. gambiae* and *An. arabiensis* were successfully mapped to *Anopheles gambiae PEST* and *Anopheles arabiensis* Dongola 2021 reference respectively (Fig. 2a); for both species the median read mapping percentage exceeded 99% and we obtained a median coverage of > 1,000 reads for all targets (Fig. 2b).

Genetic marker allele frequencies in An. gambiae s.s and An. arabiensis

A total of 277 unique variants (63 synonymous, 19 missense, 180 intronic, and 15 indels) were identified in *An. gambiae s.s* while in *An. arabiensis* 218 variants (38 synonymous, 16 missense, 129 intronic, and 35 indels) were identified. The number of variants per target region was highly variable, with *Ace1* and *Gste2* regions having the highest number of variants (Supplementary file2, C) in both species.

Two missense mutations of interest, L995S (KDR East) and L995F (KDR West), were identified in *Vgsc*. In *An. gambiae s.s*, L995S and L995F were observed at a frequency of 85% (59/69 samples) and 2% (1/69 sample) respectively (Fig. 2c). In *An. arabiensis*, the prevalence for both KDR East and west were 13%

(6/45 samples) and 12% (5/45 samples) respectively (Fig. 2d). No V402L or N1570Y mutations were identified in either species.

In *Gaba*, A269S was identified in both species with low allele frequencies of 2% (1/69 samples) and 4% (6/45 samples) for *An. gambiae s.s* and *An. arabiensis*, respectively. No mutations of interest were identified in *Ace1* in the two species but a number of missense mutations were identified at low frequencies (Fig. 2c,d)).

In *Gste2*, L119V was observed in *An. gambiae s.s* at a frequency of 2% (1/69 samples), other identified SNPs in the regions included T154S, 18% (12/69 samples); I126F, 12% (8/69 samples); K123N; 5% (3/69 samples), S98A, 1% (1/69 samples); V91I, 1% (1/69 samples); T77S, 1% (1/69 samples); G26S, 2% (1/69 samples); T21A, 4% (3/69 samples); and N3K, 99% (68/69 samples). For the *Gste2* region in *An. arabiensis*, the reads mapped to four different genes of the Gste family (AARA21_014232 encoded as *Gste2_1*; AARA21_013962, *Gste2_2;* AARA21_007478, *Gste2_3;* AARA21_014818 ,*Gste3)*. *Gste2* is part of a large gene family, and in *An. arabiensis* these four nearly identical copies made mapping ambiguous (Supplementary file2 C).

A total of 11 missense mutations were identified in these four *Gste* regions, three of which were fixed (45/45 samples) (*Gste2_2* E183V, *Gste2_1* E176V, *Gste2_1* Q177L; Fig. 2d), suggesting the resistance mechanisms in these two species are quite different despite coming from the same geographical location.

Detection of dual KDR mutations in An. gambiae s.s. and An. arabiensis

We identified the presence of both the KDR East (*Vgsc* L995S) and the KDR West (*Vgsc* L995F) mutations simultaneously within one *An. gambiae s.s* and one *An. arabiensis* sample. Both samples carried heterozygous KDR double mutations as shown in Fig. 3a. In *An. gambiae s.s*, phasing revealed that the KDR East and KDR West mutant alleles were on different chromosomes (Fig. 3b); the same was true for *An. arabiensis*.

Application to Anopheles stephensi

The primers designed for our multiplex PCR were developed for *Anopheles gambiae s.l.*. Despite this, we attempted sequencing a set of 80 *Anopheles stephensi* samples due to the urgent public threat posed by the vector in East Africa. Of the 80 *An. stephensi* samples, 63 (79%) passed quality control and were used in downstream analysis. The *An. stephensi* samples derived from North-eastern Kenya and were collected from 2022 through 2024, and previously Sanger sequenced to confirm species (46) (Fig. 1a). The 63 samples passing quality control showed robust amplification on an agarose gel (Supplementary File 3). We mapped reads to the *An. stephensi* UCISS 2018 reference genome. We observed a lower

median mapping rate of 75% and some imbalance across amplicons compared to *An. gambiae s.s* (Fig. 4a and Fig. 4b); however, we still had a median coverage of > 1,000 across most targets.

Genetic markers allele frequencies in Anopheles stephensi

We identified 663 variants (262 synonymous, 33 missense, 331 intronic and 37 indels) in *Anopheles stephensi* across all 63 samples. In the *Vgsc* gene, three missense mutations of interest were identified at low frequencies: L995S, 5% (3/63 samples); L995F, 2% (1/63 samples); and T1515M, 1% (1/63 samples) (Fig. 4c). No V402L and N1570Y mutations were identified in *An. stephensi*. In the *Gaba* gene, A269S and T312M were identified at 6% (4/63 samples) and 2% (1/63 samples) (Fig. 4c). At the *Ace* region, the 280S was not identified but the novel mutations A312S and R254L were identified at 1% (1/63 samples) frequency (Fig. 4c). At the *Gste2* region, no targeted mutation (L119V) was identified but new mutations were identified (D125G, 1% (1/63 samples); V91I, 2% (1/63 samples); G26S, 10% (6/63 samples); and T21A, 56% (35/63 samples)) (Fig. 4c).

Discussion

We designed a field-deployable assay targeting key insecticide resistance (IR) markers in three *Anopheles* species to enable rapid and routine IR surveillance. Most methods for genomic profiling of *Anopheles* vectors rely on shipping samples abroad for sequencing and detection of mutations. However, this approach is expensive, slow to generate key findings i.e. resurgence of *An. coluzzii* in Turkana where samples had to be shipped abroad (5), and disenfranchises local researchers by disconnecting them from data generation. In addition, delayed government and Nagoya Protocol approvals for shipment of samples can further increase the turnaround time. In-country sequencing accelerates this process, enables early detection of novel mutations, builds country ownership by empowering local scientists and enables NMCPs to rapidly respond to threats as they are identified.

We deployed the NOMADS-IR assay in Kisumu, Kenya and trained the laboratory technicians and researchers on nanopore sequencing. Starting with DNA, genomic data can be generated within 2 days, with results displayed in a real-time dashboard using Nomadic software. We significantly reduced DNA extraction cost to less than $1 USD per sample by utilising a crude extraction method where all reagents are sourced locally rather than relying on expensive DNA extraction kits (~$5). Following optimization, the overall cost of the workflow decreased from an estimated $25 per sample to approximately $20 per sample.

To design our panel, we focused on key genes associated with insecticide resistance i.e. voltage-gated sodium channel (*Vgsc*), gamma-aminobutyric acid (*Gaba*)-gated chloride channel, acetylcholinesterase 1(*Ace1*) and Glutathione S-transferase epsilon 2 *(Gste2)*(26). Mutations in some of these genes have been observed in Kenyan *Anopheline* mosquitoes, including the pyrethroid knockdown resistance (*KDR*) mutations L995S-KDR East and L995F-KDR West in the *Vgsc* gene. The former was initially identified at low frequencies in *An. gambiae* populations in the early 2000s, but may now be fixed (47, 48), while the latter was reported more recently in 2013 at very low frequencies in western Kenya (0.008–0.05) (49).

Our findings confirmed KDR East in *An. gambiae s.s* is nearing fixation (85%) while it is at a low frequency in *An. arabiensis* (13%). KDR West was found at low frequencies in both species (2% and 12% respectively). These mutations have been the primary drivers of pyrethroid resistance in *Anopheles* populations in Kenya, in a 2009–2010 trial, regions of higher deltamethrin-based LLINs coverage showed increased frequencies of the KDR East resistance allele (48).

The impact of co-circulating KDR East and KDR West mutations on control efforts is unknown, although it has been documented in West (Gabon and Cote D'voire) and East African (Tanzania, Kenya and Uganda) mosquito populations (50–54). We present here the first evidence of individual *An. gambiae s.s* and *An. arabiensis* mosquitoes carrying dual KDR East and West mutations. As expected, the two mutations were found on separate chromosomes, consistent with the mosquitoes being progeny of mating between individual KDR East and KDR West carriers. Although KDR is the main mechanism of resistance for *Vgsc*, other mutations such as V402L and N1570Y have also been validated to confer resistance. V402L only confers resistance in the absence of any other mutations while N1570Y has been found to be concurrent with KDR West to confer pyrethroid resistance (55, 56). In this study, no V402L and N1570Y mutations were identified in *An. gambiae s.s* or *An. arabiensis*.

The A296S mutation in the *Gaba*-gated chloride channel is the key molecular mechanism conferring resistance to dieldrin in *Anopheles* mosquitoes (13, 57, 58). Despite dieldrin not currently being used for vector control, resistance has been reported mostly in west and central Africa at high frequencies (58) but was recently reported in Kenyan *An. coluzzii* at very low frequency (~ < 5%)(5). We identified A296S mutation at low frequencies in both *An. gambiae s.s* (2%) and *An. arabiensis* (4%) which confirms the emergence of this variant in the East African population.

The G119S mutation in *Ace1* is associated with organophosphate resistance. In Kenya, targeted indoor residual spraying has been implemented for many years by the ministry of health in collaboration with other non-governmental organizations i.e. PMI Kinga Malaria. Since 2017, the organophosphate primiphos methyl (Actellic 300CS) has been used which could select for *Ace1* mutations (59). In this study, no *Ace1* G119S mutations were identified in *An. gambiae s.s* or *An. arabiensis*, even though phenotypic resistance has been reported in *An. arabiensis* samples from Siaya, Busia, Migori and Turkana counties (22, 60). Lastly, we assessed the mutation frequency of *Gste2*-119V, which is associated with increased permethrin resistance in Burkina Faso (16). The mutation was only identified in *An. gambiae s.s* samples at very low frequency (2%) which shows emergence of the mutation into the population.

Anopheles stephensi has been spreading rapidly across Kenya and has been confirmed in Marsabit, Mandera, Turkana, Wajir, Isiolo, Samburu, and Elgeyo Marakwet counties (46). The distribution pattern, together with modelling studies, suggests it is spreading along major transportation routes. Even with the extensive spread to different regions, the capture rate has been low with a total of 114 larvae and 33 adults collected between 2022–2024, an insufficient number for phenotypic resistance assays. Therefore, molecular genotyping is the only way to assess insecticide resistance in *An. stephensi.* Our

results revealed low frequencies of target site mutations i,e KDR East L995S (5%), KDR West L995F (2%) and *Gaba* A296S (6%) mutations commonly known to be responsible for DDT, Dieldrin, alphacypermethrin and deltamethrin resistance in other primary malaria vectors *An. gambiae sl* and *An. funestus* (47, 48, 58). The frequencies might be very low due to minimal exposure of insecticides in these regions since no Long-lasting insecticidal net (LLIN) have ever been distributed in these regions in Kenya. Additionally, these are urban and arid regions with little to no agricultural activities, therefore limited exposure of mosquito populations to agricultural pesticides.

Finding emergence of KDR East, KDR West and *Gaba* mutation frequencies in areas with minimal insecticide exposure is concerning, although no metabolic resistance markers were identified. Based on our NOMADS-IR data presented here, pyrethroids with PBO combination, i.e the new LLINs with combined insecticide or use of other new insecticides, would be an appropriate vector control approach(61–63). This example of IR surveillance demonstrates how this approach could be used to directly inform the adoption of the most appropriate vector control tool(s) to be used in different regions.

The NOMADS-IR assay has a number of limitations. As with any targeted sequencing panel, it is limited to the genes selected for the panel. Entomological surveillance is most powerful when additional metrics are known e.g. species ID, bloodmeal source, infectivity status etc. Combining these targets into a holistic assay that provides a wide range of information would be even more advantageous. Nevertheless, on balance this relatively simple and scalable protocol is amenable to further modifications to remain fit for purpose in providing actionable vector-based surveillance data.

Conclusion

We have developed a novel protocol for insecticide resistance surveillance using nanopore sequencing, called NOMADS-IR, and implemented it locally in Kisumu, Kenya. Using our approach, we identified moderate to low levels of resistance mutation frequencies on *An. gambiae s.l.* and *An. stephensi* suggesting resistance to permethrin, DDT, deltamethrin and Dieldrin insecticides. Furthermore, we described, to the best of our knowledge, the first dual KDR East and KDR West mutation in a single vector for both *An. gambiae s.s.* and *An. arabiensis*. Overall, these results demonstrate that local nanopore sequencing of *Anopheles* vectors is feasible and can generate insecticide resistance data of public health importance without delays associated with international sample shipment. Future work should expand target panels and applications, including more species and insecticide resistance markers, as well as enabling species identification and sporozoite detection markers for better exploration of population structure and resistance mechanisms to inform targeted vector control strategies.

Declarations

Ethics declarations

Ethics approval and consent to participate

Approval for this study was granted by the Kenya Medical Research Institute (KEMRI) Scientific Ethics Review Unit Steering Committee (SERU).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Declaration

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Author Contribution

JAH,CAO,EO,DJB, MM, KG,SM,DO, CA,BP,BA,BM,SP and JK: conceptualization of the study, methodology design, sample collection and revising the manuscript. JAH, CAO and DJB : data curation and software. JAH, CAO, DJB and EO: data analysis, interpretation, andvalidation as well as drafting and revising the manuscript. All authors reviewed the final manuscript version.

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Data Availability

Sequence data used by this study is available at Sequence Read Archive (SRA) under the Bio Project accession number PRJNA1517867. Custom scripts used for all the analysis are available from the primary author on request. Email:cynthiaawuor18@gmail.com.

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Figures

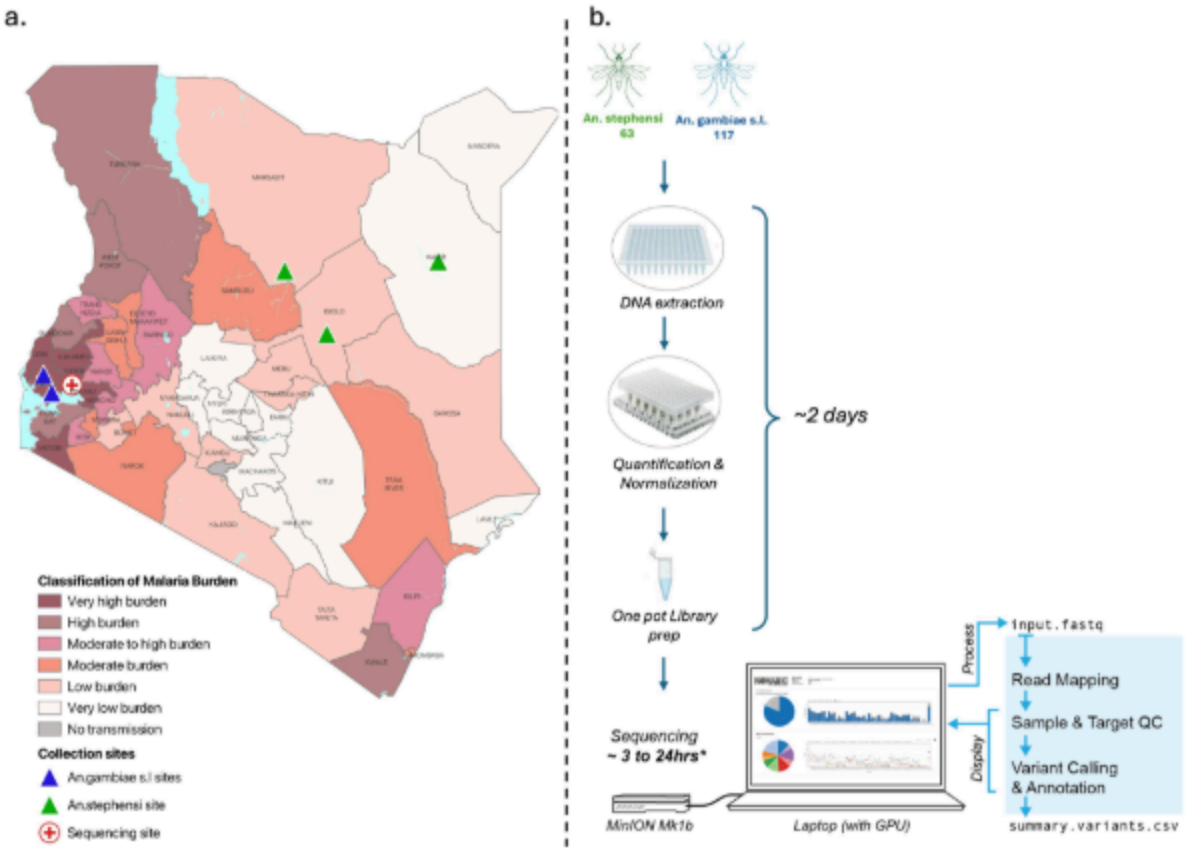


Figure 1

Graphical representation of the methodology. a. Map of Kenya showing all counties, their malaria burden classification and sample collection sites. **b.** The workflow from sample through multiplex PCR, bead cleanup, library preparation, sequencing and real-time analysis using Nomadic software.

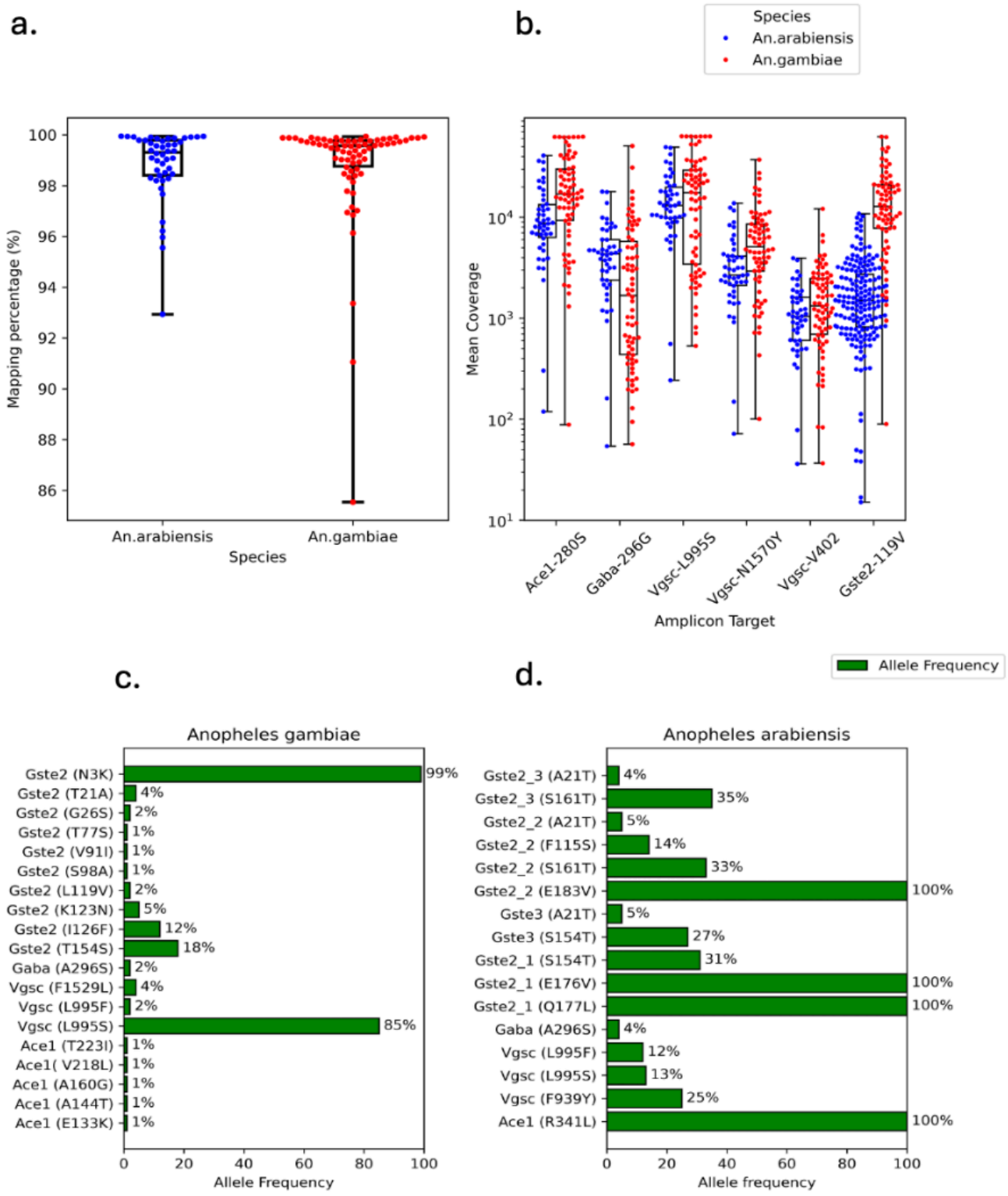


Figure 2

Analysis of *An. gambiae s.s (N=69)* and *An. arabiensis (N=45)* coverage, mapping and allele frequency plots. a. *An. arabiensis* and *An. gambiae s.s* mapping percentages to their respective reference genomes. **b.** Plot of mean coverage per target for all samples. Allele frequency of IR missense mutations for *An. gambiae s.s* **c**. and *An. arabiensis* **d**. respectively.

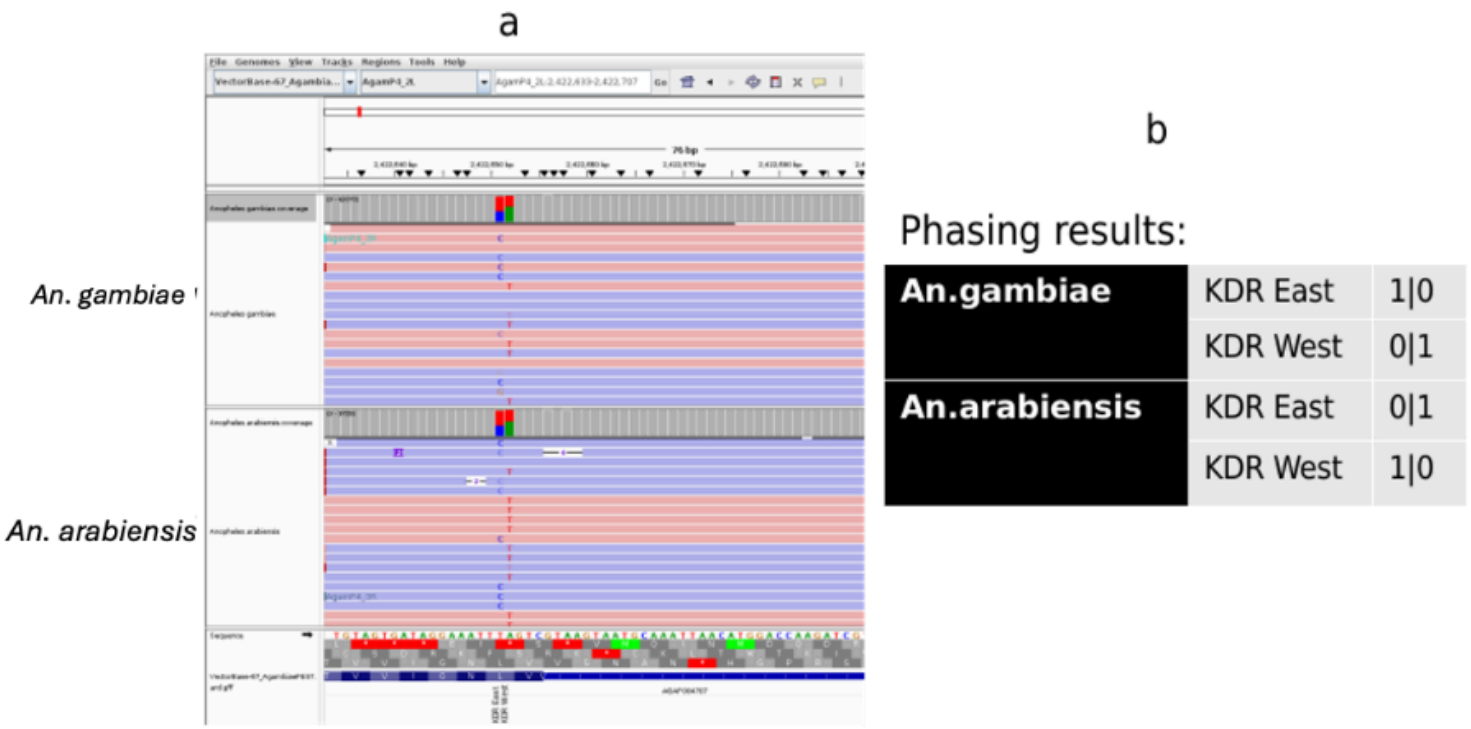


Figure 3

Dual KDR Mutation in *An. gambiae s.s* and *An. arabiensis*. a. Screenshot from Integrative Genomic Viewer (IGV) illustrating presence of both KDR East and KDR West mutations in two individual *An. gambiae s.s* and *An. arabiensis* samples. Individual reads are coloured by strand (red, forward; blue, reverse). The KDR East mutation is caused by a nucleotide change to C; KDR West to T. Note how for both samples, the KDR East and KDR West mutations are always on different reads. **b**. Phasing results for both species illustrating the mutant alleles are from different chromosomes.

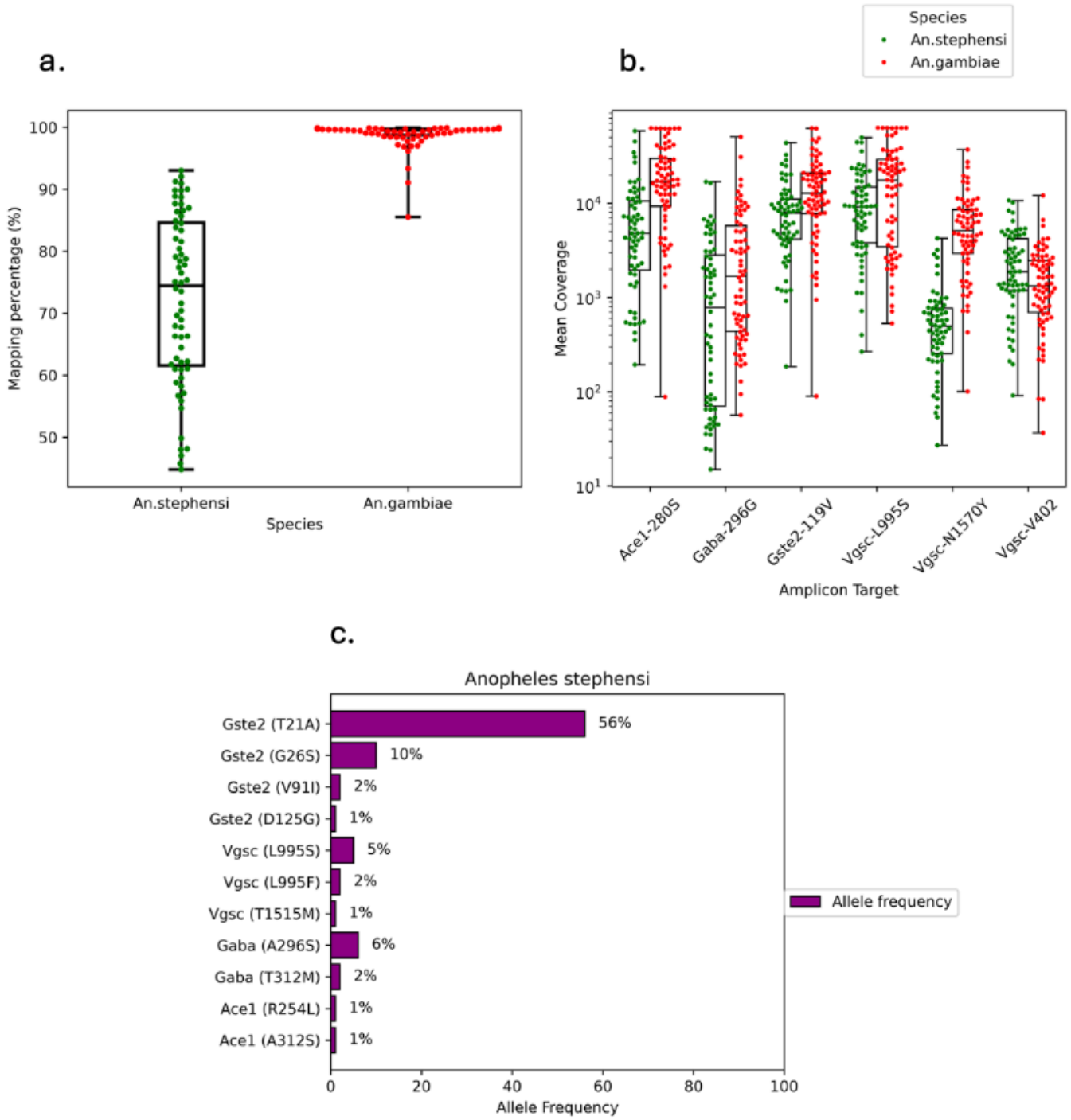


Figure 4

Analysis of *An. stephensi (N=63)* coverage, mapping and genotype frequency. Comparison of *An. stephensi*and *An. gambiae s.s* samples reference genome read mapping percentage (**a**) and mean target coverage per amplicon (**b**). *An. stephensi* allele frequencies of all IR missense mutations (**c**).

Supplementary Files

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