**Discovery of knock-down resistance in the major African malaria vector *Anopheles funestus* .**

**Authors**

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**Abstract**

A major mechanism of insecticide resistance in arthropod pests is knock-down resistance (*kdr*) caused by mutations in the voltage-gated sodium channel (*Vgsc*) gene. Common in most malaria *Anopheles* vector species, *kdr* mutations have never been observed in *Anopheles funestus*, the principal malaria vector in Eastern and Southern Africa. From whole-genome sequencing of 333 *An. funestus* samples from a breadth of populations in Tanzania, we found 8 novel amino acid substitutions in the *Vgsc* gene, including the *kdr* variant, L1014F (L976F in *An. funestus*), in tight linkage disequilibrium with another (P1842S). The mutants were found only at high frequency in one region, with a significant decline between 2017 and 2023. When evaluating the resistance phenotype of these samples, we found a strong association between L976F and survivorship to the exposure to DDT insecticide, but no association with a pyrethroid insecticide (deltamethrin). Further study is necessary to identify the origin of *kdr* in *An. funestus*, and the extent to which it may threaten the effectiveness of insecticide-based vector control in Africa.

**Teaser**

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Emergence of a novel insecticide resistance mechanism in an African malaria mosquito threatens control of the killer disease.

**Introduction**

Chemical insecticides are central to the control of agricultural pests and disease vectors. The control of *Anopheles* mosquitoes through the distribution of over 2.9 billion insecticide-treated bed nets (ITNs)1 has helped avert an estimated 633 million cases of malaria2 - a disease that still kills 600,000 yearly1. However, the widespread use of insecticides for agricultural pest and disease vector control also has detrimental consequences, including direct lethal and sub-lethal effects on human and animal health3,4 and destabilizing effects on ecosystem structure and function. For example, insecticide exposure is a key stressor affecting the population decline of pollinators, essential for ecosystem health and food production5,6.

A key obstacle to the sustainable control of malaria is the evolutionary arms race between mosquitoes, and insecticide-based mosquito control. Strong selection pressures generated by insecticide-based agricultural pest and disease vector control activities have resulted in the independent evolution of a diverse range of mechanisms that confer insecticide resistance (IR) phenotypes in numerous insect species8. One of the earliest described IR mechanisms was the emergence of knock-down resistance (*kdr*), mediated by mutations in the target-site of pyrethroid and organochlorine insecticides, located in the voltage-gated sodium channel gene (*Vgsc*), an essential component of the nervous system9. These *kdr-*driven resistance phenotypes appeared rapidly after the introduction of the organochlorine dichloro-diphenyl-trichloroethane (DDT) spraying for insect control in the mid-20th century10 and eventually evolving to confer resistance to pyrethroids 11,12, the key ingredient in ITNs - the first line of defence against malaria. In an era of stalling gains of malaria control [**REF**], and concerted efforts both to develop a new generation of ITN and IRS products [REF], and proactively manage the deployment existing insecticides to maximise efficacy, intensified surveillance, including genomic surveillance [**REF]**, of malaria vector populations is critical for providing real-time warning of insecticide resistance emergence.

As part of phenotypic and genomic surveillance done in Tanzania to understand the evolution and spread of insecticide resistance in *Anopheles funestus -* the dominant malaria vector in Eastern and Southern Africa15, we report the first discovery of *kdr* mutations in *An. funestus.* We discover that this mutation confers resistance to DDT, but not deltamethrin, despite a complete ban on DDT use for agriculture and vector control in Tanzania since 200816. We suggest environmental contamination from extensive DDT stockpiles17, or unofficial agricultural use, as possible causes. The emergence of *kdr*, which threatens the control of major crop pests and vectors of disease, such as *An. gambiae* and *Aedes aegypti*18, highlights the potential of chemical insecticide contamination or unofficial use to exert unexpected and potentially harmful impacts on public health.

**Results**

Resistance to all major classes of insecticide is common in *An. funestus* and is primarilymediated through the increased activity of enzymes that bind to and metabolise insecticides (metabolic resistance)19,20. This contrasts with *An. gambiae* where resistance is mostly conferred by a combination of metabolic and target-site resistance8. As part of an insecticide resistance surveillance study (**REF**), we investigated phenotypic resistance (as measured by mosquito survival 24hours following insecticide exposure) in *An. funestus* to the discriminating doses of deltamethrin (type II pyrethroid) and DDT and tested whether the metabolic enzyme synergist piperonyl butoxide (PBO), which is increasingly used on ITNs21, could restore susceptibility in pyrethroid-resistant populations in Tanzania. The mosquitoes were phenotypically resistant to deltamethrin; lowest in south-eastern Tanzania, Lindi (7%, CI 3.4 – 15.7), and highest in the west Katavi (55%, CI 43.9 – 65.5) but PBO restored susceptibility in all locations. Resistance to DDT was recorded only in Morogoro region (68%, CI 57.8 - 77.9) (**Figure 1B**). The DDT binding site in mosquitoes is on the *Vgsc*, and mutations on this gene are strongly linked to resistance phonotypes in *An. gambiae* (**REF**).

We analysed whole-genome-sequencing (WGS) data from 333 mosquitoes sampled from 10 sites across Tanzania (**Figure 1A**). We performed genome-wide selection scans (GWSS) with the G123 statistic26 to test for evidence of selective sweeps in the *An. funestus* genome associated with known or novel IR loci (**Figure 1D**); grouping samples by administrative region (see **Supp. Table 1** for per-group sample numbers). We detected a clear signal of elevated G123 in the region containing the *Vgsc* gene in samples from the Morogoro region in the southeastern part of the country (**Figure 1D**). In Kagera, Katavi, and Mwanza regions, there was no visible sign of a selective sweep at or near the *Vgsc* region. In Dodoma, Lindi, Ruvuma, and Tanga, there were peaks of elevated G123 near to *Vgsc*, but these appeared within the context of relatively high G123 across the chromosome **(Figure 1D).**

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**Figure 1: (A)** Map of *An. funestus* collection locations. Points indicate sample collection locations. Point colour indicates the administrative region from which samples were collected. **(B)**: Phenotypic insecticide resistance profile of *An. funestus* to DDT using bioassay data adopted from our recent surveillance (**REF**). The colours represent the various regions where the bioassays were conducted, and error bars are 95% confidence interval. The black and red dotted lines on the y-axis represent the 98 and 90% mortality threshold. (**C)** L976F and 1842S frequencies, in Morogoro region, over time. Y axis indicates allele frequency, X axis indicates date. Line and point colour refer to mutation, specified in the legend. Bars indicate 95% confidence intervals. (**D**) G123 selection scans of *An. funestus* chromosome 3RL, coloured and windowed by sample collection region (where n>20 – see **Supp Table 2**). X axis indicates position (in base-pairs (bp)), Y axis indicates the selection statistic G123. Grey dotted line indicates the location of the *Vgsc* gene. Note Mwanza region is absent from panel **D** as there were too few samples (n<20) to perform a selection scan.

Mutations in *Vgsc* confer *kdr* in numerous pest and insect taxa22. While not previously reported in *An. funestus*23, *kdr* mutations in major malaria vectors within the *An. gambiae* complex are subject to intense selection24,25 and confer resistance to pyrethroid and organochlorine insecticides used in ITNs and insecticide sprays12,22. We searched our data for mutations in the *Vgsc* gene and found 8 amino acid substitutions occurring at frequencies greater than 5% (**Figure 2A**). Of these, two alleles, L976F and P1842S occurred at the highest frequency (**Figure 2A**). The frequencies of P1824S and L976F were highest in samples collected from Morogoro in 2017 (0.75 and 0.90 respectively) **(Figure 2A)** anddeclined yearly, reaching their lowest frequency in samples collected in 2023 (0.48 and 0.56 respectively; 𝜒2 = 12.15, p=0.0005; **Figure 2B).** These mutations occurred at very low frequencies or were absent in all other locations (**Figure 2A).** We aligned the *An. funestus* *Vgsc* sequence (Gene ID: AFUN2\_008728.R15290) with that of *Musca domestica* (Gene ID: X96668) and *An. gambiae* (AGAP004707-RD AgamP4.12 gene set)25. The amino acid change at *An. funestus* L976F corresponded to L1014F in *M. domestica* and L995F in *An. gambiae* in domain II subunit 6 (IIS6) of the *Vgsc* gene (**Table 1**), which in *An. gambiae* species complex drastically increases IR to DDT and pyrethroids12,26. The second variant P1842S corresponded to P1874S in *An. gambiae* and P1879 in *M. domestica* and were all in the C-terminal domain (**Table 1**).

**Table 1**: Comparative non-synonymous nucleotide variation in the voltage-gated sodium channel gene. Position is relative to the *Anopheles funestus* strain FUMOZ reference, chromosome arm 3RL.Codon numbering according to *Anopheles funestus* *Vgsc* transcript AFUN2\_008728.R15290**,** *Anopheles gambiae* transcript AGAP004707-RD in gene set AgamP4.12, and *Musca domestica* EMBL accession X96668 Williamson *et al.*27.

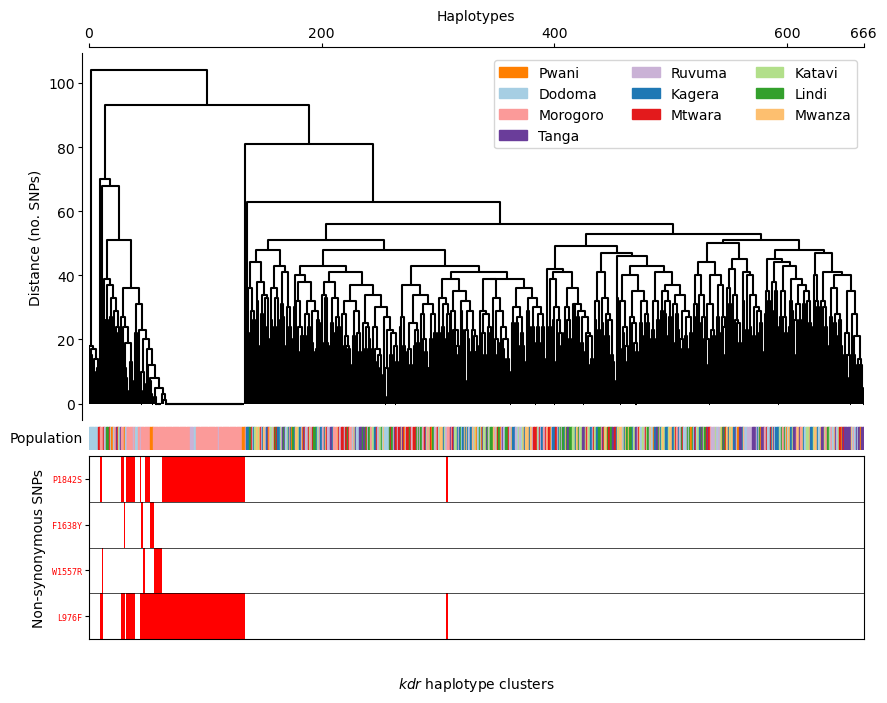
|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Position** | ***An. funestus*** | ***An. gambiae*** | ***M. domestica*** | **Domain** |
| 3RL:44,115,564 T>C | I2030V | I2061 | P2063 | COOH |
| 3RL:44,115,768 C>A | G1962C | A1994 | P1997 | COOH |
| 3RL:44,116,128 G>A | P1842S | P1874S | P1879 | COOH |
| 3RL:44,116,334 T>C | N1773S | N1805 | N1810 |  |
| 3RL:44,116,923 A>T | F1638Y | F1670 | V1675 |  |
| 3RL:44,117,167 A>T | W1557R | W1589 | W1594 |  |
| 3RL:44,122,391 C>A | G1144C | G1173 | G1180 |  |
| 3RL:44,125,475 T>A | L976F | L995F | L1014 | IIS6 |

In *An. gambiae*, multiple *kdr* haplotypes have evolved independently28,29. To elucidate *Vgsc* haplotype structure in *An. funestus,* we computed pairwise linkage disequilibrium (LD) using the Rogers and Huff method30, between nonsynonymous variants occurring at a frequency of > 5% in Tanzanian *An. funestus* **[Figure 2C**]. We found that P1824S occurred in tight LD with L976F (*D’*=0.95) **[Figure 2C].** Of other non-synonymous polymorphisms, F1638Y and W1557R exhibited only weak LD with L976F [**Figure 2C]**. We constructed a haplotype clustering dendrogram from haplotypes in all 333 individuals, from the *Vgsc* gene **[Figure 3].** The clustering dendrogram disclosed three major cladesand three main combinations of the four most prevalent *Vgsc* alleles **[Figure 3].** The most striking signal was a subclade of identical, or near-identical haplotypes containing both L976F and P1842S [**Figure 3],** indicating a selective sweep on a combined L976F/P1842S haplotype. This combined haplotype was present at higher frequencies in the Morogoro region relative to the neighbouring regions of Pwani, Ruvuma, and Dodoma [**Figure 3**].Most amino acid substitutions were present in a single clade in samples from Pwani, Dodoma, Ruvuma, and especially Morogoro [**Figure 3**]. This extremely restricted geographic hotspot of *kdr* is in stark contrast to its distribution in *An. gambiae* where *kdr* mutationsspread rapidly across vast spatial scales in response to strong selection pressure24,31.

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**Figure 2: (A)** Heatmap of *Vgsc* allele frequencies. Y axis labels indicate mutation effect, chromosome position, and nucleotide change. X axis labels indicate collection date, heatmap intensity indicates frequency where darker = higher, with frequency labelled in each heatmap facet. Heatmap is panelled by sample collection region. (**B)** L976F and 1842S frequencies, in Morogoro region, over time. Y axis indicates allele frequency, X axis indicates date. Line and point colour refer to mutation, specified in the legend. Bars indicate 95% confidence intervals. (**C)** Heatmap of linkage disequilibrium (LD) (Rogers and Huff R) between nonsynonymous variants in the *Vgsc* gene at frequency > 5%. LD is indicated by fill colour. SNP effects and positions are labelled on X and Y axes. (**D**) Denotes the association of L976F and P1842S with resistance to Deltamethrin and DDT. Colour and panelling are by mutation, x axis indicates genotype, y axis indicates mortality, point shape indicates mean for each insecticide and line indicates the 95% CI based on generalised mixed model prediction.

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**Figure 3:** Clustering of haplotypes at the *Vgsc* gene (LOC125769886, 3RL:44105643-44156624). Dendrogram branch length corresponds to no. SNPs difference (y axis). Tips correspond to individual haplotypes (x axis). The coloured Population bar denotes the administrative region of origin (as described by the legend). Red blocks at the bottom indicate the presence of the labelled non-synonymous SNPs in the *Vgsc* gene.

To explore the possible association between L976F and P1842S alleles with DDT and deltamethrin resistance, we genotyped surviving (resistant) and dead (susceptible) mosquitoes from IR bioassays for both L976Fand P1842S loci). Neither locus was associated with deltamethrin resistance: L976F (𝜒2 = 0.04, p = 0.84) and P1842S (𝜒2 = 0.59, p = 0.44). (**Figure 1C**). We found a strong association with DDT resistance in mosquitoes carrying the mutant allele of L976F (𝜒2 = 9.23, odds ratio = 11.0, p = 0.0024) and a marginally non-significant positive association for P1842S (𝜒2 = 3.75, p = 0.0528) (**Figure 1C**). DDT is a largely obsolete, banned, pesticide that is no longer widely used for vector control in Tanzania, or in Africa as a whole, due to its bio-accumulative and toxic properties. However, the presence of DDT resistance phenotypes in *An. funestus* in Morogoro in 201532 suggests *kdr*-mediated resistance to DDT has been present at least since then, and the emergence of *kdr* resistance to DDT suggests that future use of DDT for IRS may become even less favoured.The lack of association of *kdr* with pyrethroid resistance might be due to the strong metabolic resistance shown to pyrethroids in *An. funestus,* reducing the benefit of *kdr* 33**.** However, since our testing included only type II pyrethroids, additional data are required to assess potential impacts of *kdr* on other pyrethroids and on commercial products. The association of *kdr* with resistance to DDT but not pyrethroids, combined with selection signals and recently declining *kdr* allele frequencies where we have time series, suggests recent-past, rather than contemporary selection, perhaps due to factors other than the current use of public health pesticides.

**Discussion**

In a genomic surveillance study in Tanzanian *An. funestus*, we discovered eight novel *Vgsc* mutations. Two of these, L976F and P1842S, confer *knockdown resistance* (*kdr*), occurring in tight linkage disequilibrium and at high frequencies (up to 90%) in the Morogoro region over 4 years, with limited spread to neighbouring regions. The mutation L976F showed an association with resistance to DDT, but not to pyrethroid insecticides. The role of *kdr* in pyrethroid resistance phenotypes in other *Aedes, Culux* and *Anopheles* vectors, make the discovery of *kdr* in *An. funestus* asignificant and unwelcome development that has the potential to pose a new threat to vector control in the region. Reassuringly, a lack of association between *kdr* and deltmethrin resistance indicates that the emergence of *kdr* is not linked to, nor is presently likely to threaten, the mass rollout of PBO-pyrethroid bed nets currently underway in Tanzania as a response to IR. This does not preclude a role for *kdr* in the *An. funestus* IR armamentarium in the future, and urgent follow-up study is required to determine they confer *kdr* resistance phenotypes to other widely used pyrethroids, such as permethrin, and alpha-cypermethrin, as well as other insecticide families, especially PBO- and pyrrole formulations currently being rolled out in new ITN products across the African continent [REF WHO recommendations].

This discovery raises intriguing questions over the conditions that have enabled emergence of *kdr* in *An. funestus*. It has always been a mystery why target-site resistance exists in *An. gambie* and not in *An. funestus*. Our data suggesting that *Vgsc* mutation in *An. funestus* don’t confer target-site resistance to pyrethroids, indicate a possible answer to this question, and explain why, despite extreme selection pressures imposed by pyrethroid control that have facilitated widespread propagation of resistant *Vgsc* haplotypes across the African continent in *An. gambiae* [ref Clarkson, 21], the emergence of *kdr* in Tanzanian *An. funestus* remains relatively localised. Mechanistic studies, including *in silico* modelling of pyrethroid binding to *Vgsc,* [**REF**], and expression studies of mutant *Vgsc* proteins in *Xenopus* oocytes [**REF],** will enable comparisons between taxa that will elucidate this further.

If the ubiquitous use of pyrethroids in vector control did not select for the emergence of *kdr*, from whence came *kdr?* Even more curiously, tsuggest that the selection pressure causing the emergence of *kdr* has eased (although). Given the role of *kdr* in conferring resistance to DDT, three plausible origin scenarios seem most likely: vector control, agriculture, or stockpiling of organic pollutants. There is no record of DDT use in the last decade for agriculture or vector control in the Morogoro region, or Tanzania as a whole. Tanzania, as a signatory to the 2009 Stockholm Convention on Persistent Organic Pollutants (SCPOP), banned the production, importation, and usage of DDT16, except for limited use in malaria vector control. In 2008, Tanzania rolled out an ambitious malaria vector control strategy relying on large scale use of DDT for indoor residual spraying (IRS), implemented in 60 districts across the country (**REF**), and later discontinued in 201034. Morogoro, where we detected *kdr,* was not part of this expanded campaign [**Figure 4A**]. Prior to the SCPOP, Tanzania imported large stockpiles of DDT mostly for agricultural pest control [**Figure 4B**]. However, following the ban, there has been anecdotal reports of continued illegal use of DDT amongst farmers to date (**REF**). The Africa Stockpiles Programme (ASP) was launched in 2005 to eliminate stockpiles of obsolete pesticides, including DDT. At this time, it was estimated that Tanzania still possessed approx. 1,500 tonnes of obsolete pesticides 35. Intriguingly, Mahenge-Ulanga in the Morogoro region held a DDT stockpile of 4.5 tons (as of 2004) in 36, approximately 100 km away fromwhere DDT-resistant *An. funestus* were detected in this study. Additionally, a large stockpile of 19 tons of DDT was also held in Morogoro town (**Figure 4B**), where over 300 tonnes of DDT-contaminated soil were removed for disposal in 2012 17. The ASP and the Tanzanian Government eliminated 100% of inventoried publicly held DDT stockpiles and conducted extensive environmental remediation by programme close in 2013 37. However, extensive DDT contamination remains 38. For example, 1040 tonnes of DDT contaminated soil were removed in 2014/2015 36, and DDT remains in widespread use by private individuals . The coincident proximal location of high levels of *kdr* in *An. funestus* with large past DDT stockpiles as well as the presence of widespread DDT contamination and private usage, leads us to hypothesise that the two most likely scenarios of *kdr* emergence in *An. funestus* are contamination of local larval breeding sites from agricultural or stockpiled DDT. The removal of DDT stockpiles by the ASP, and ongoing environmental remediation, may have contributed to reduced selection pressure on *kdr*, evident from declining frequency in Morogoro. Continued monitoring of allele frequency and future studies of *kdr* frequencies targeted towards sites of known DDT contamination will establish whether this hypothesis is correct.

In *Silent Spring* (1962), Rachel Carson brought for the first time into the public eye the unpredictable and often remote impacts of anti-insect chemical agents on human health and on nature “on one hand delicate and destructible, on the other miraculously tough and resilient, and capable of striking back in unexpected ways” 39. Further study of *kdr* in *An. funestus* will enable identification of the origin of this mutation, and make clear the full implications of its presence in the population for vector control. Whether the emergence of *kdr* in *An. funestus* is caused by vector control, unlicensed DDT usage in agriculture, or by exposure to stockpiled DDT, our findings underscore the legacy of *Silent Spring* by reinforcing the potential for pesticides and organic pollutants to exert inadvertent influences on animal biology that may have profound and unfortunate consequences for public health.

**Materials and Methods**

All scripts and Jupyter Notebooks used to analyse genotype and haplotype data, and produce figures and tables are available from the GitHub repository: <https://github.com/tristanpwdennis/kdr_funestus_report_2023>

**Mosquito collection**

*Anopheles funestus* samples analyzed in this study were collected from ten administrative regions in Tanzania: Dodoma, Kagera, Katavi, Lindi, Morogoro, Mtwara, Mwanza, Pwani, Ruvuma, and Tanga (**Figure. 1A**). The collections were done as part of a countrywide *Anopheles funestus* surveillance project in Tanzania and were subsequently incorporated into the MalariaGEN *Anopheles funestus* genomic surveillance project database (<https://www.malariagen.net/projects/anopheles-funestus-genomic-surveillance-project>). Mosquitoes were collected in households between 2017 and 2023 using CDC light traps and mechanical aspirators. They were sorted by sex and taxa and *An. funestus* group mosquitoes preserved individually in 96-well plates containing 80% ethanol.

**Whole genome sequencing and analysis**

The samples were processed as part of the *Anopheles funestus* genomics surveillance MalariaGEN Vector Observatory (VObs) project (<https://www.malariagen.net/mosquito>).Briefly, the mosquitoes were individually whole-genome-sequenced on an Illumina NovaSeq 6000s instrument. Reads were aligned to the *An. funestus* reference genome AfunGA1 40 with Burrows-Wheeler Aligner (BWA) version v0.7.15. Indel realignment was performed using Genome Analysis Toolkit (GATK) version 3.7-0 RealignerTargetCreator and IndelRealigner. Single nucleotide polymorphisms were called using GATK version 3.7-0 UnifiedGenotyper. Genotypes were called for each sample independently, in genotyping mode, given all possible alleles at all genomic sites where the reference base was not “N”.

Complete specifications of the alignment and genotyping pipelines are available from the malariagen/pipelines GitHub repository (<https://github.com/malariagen/pipelines/> ). The aligned sequences in BAM format were stored in the European Nucleotide Archive (ENA).

The identification of high-quality SNPs and haplotypes were conducted using BWA version 0.7.15 and GATK version 3.7-0. Quality control involved removal of samples with low mean coverage, removing cross-contaminated samples, running PCA to identify and remove population outliers, and sex confirmation by calling the sex of all samples based on the modal coverage ratio between the X chromosome and the autosomal chromosome arm 3R. Full quality control methods are available on the MalariaGEN vector data user guide (<https://malariagen.github.io/vector-data/ag3/methods.html>).

We used decision-tree filters that identify genomic sites where SNP calling and genotyping is likely to be less reliable. More information on site filters can be found on the MalariaGEN vector data user guide.

Genotypes at biallelic SNPs that passed the decision-tree site filtering process were phased into haplotypes using a combination of read-backed and statistical phasing. Read-backed phasing was performed for each sample using WhatsHap version 1.0 [https://whatshap.readthedocs.io/]. Statistical phasing was then performed using SHAPEIT4 version 4.2.1 [https://odelaneau.github.io/shapeit4/].

Complete specifications of the haplotype phasing pipeline are available from the malariagen/pipelines GitHub repository (<https://github.com/malariagen/pipelines/tree/master/pipelines/phasing-vector>).

**Identification of SNPs on *Vgsc***

To identify the *An. funestus* *Vgsc* gene and the variant that confers target-site resistance we performed alignments between the *An. gambiae* VGSC transcript AGAP004707-RD in AgamP4.12 geneset from the Ag1000 phase 3 data resource (<https://www.malariagen.net/data/ag1000g-phase3-snp> ) and AFUN2\_008728 from the *An. funestus* AfunF1.3 dataset. We extracted single nucleotide polymorphism (SNPs) altering the amino acid of VGSC protein from the *An. funestus* dataset and computed the allele frequency on the mosquito cohorts defined by the region and year of collection ((See **Supp. Table 1** for per region/year sample numbers)). Under selection pressure various alleles are expected to increase in frequency; we therefore filtered out variant alleles with a frequency lower than 5% resulting in a list of 8 variant alleles. Multiple sequence alignments of *An. funestus Vgsc* against *An. gambaie* and *M. domestica* were performed using MEGA v11.013.

**Population genetic analyses**

We searched for signatures of selective sweeps on the *Vgsc* gene using the *G123* selection statistic 41**.** G123 selection scans were performed on *An. funestus* genotypes by collection region where sample *n>*20 **[**see **Figure 1A** and **Supp Table 2**] **]** using the *g123\_gwss* function in the malariagen\_data python API (<https://malariagen.github.io/malariagen-data-python/latest/Af1.html> ). Linkage disequilibrium (Rogers and Huff’s R-squared) 30 between the 8 *Vgsc* alleles was calculated using the *rogers\_huff\_r\_between* in scikit-allel (<https://zenodo.org/record/4759368> ). Haplotype clustering was performed by performing hierarchical clustering on a Hamming distance matrix, inferred from phased *An. funestus* haplotypes, using the Scipylibrary (<https://scipy.org/citing-scipy/> ). Clustering dendrogram, and bar plot of amino acid substitutions, was plotted using the seaborn library 42.

**Association of L976F and P1842S alleles with insecticide resistance**

To test for associations between the identified mutations with IR, we exposed wild non-blood-fed *An. funestus* mosquitoes of unknown ages to standard doses of deltamethrin and DDT insecticides following the WHO tube assays. For each insecticide, we randomly separated phenotypically resistant mosquitoes (i.e., alive 24 hours post-exposure) and susceptible (i.e., dead 24 hours post-exposure) and extracted DNA from individual mosquitoes using DNeasy Blood and Tissue kit (Qiagen, Germany). The mosquitoes were identified at the species level using species-specific primers that can distinguish *An. funestus* from the other members of the group 43. To establish if the two *kdr* variants are associated with insecticide resistance, we designed PCR primers from *An. funestus* *Vgsc* (Gene ID: LOC125769886) to amplify domain IIS6 (L976F) and C-terminal (P1842S) (see **Supp Table 3** for primer and thermocycler conditions). The DNA fragments were separated on a 1% agarose gel, cut, purified using PureLink™ Quick Gel Extraction Kit (Invitrogen), and commercially Sanger sequenced. Collectively, we sequenced 76 individual mosquitoes: 56 from deltamethrin and the rest from the DDT bioassays. The frequencies of the wild type and mutant alleles were determined and correlated with phenotypes using generalised linear models in R-software v4.1.1.

**Data availability**

The sequencing data generated in this study have been deposited in the European Nucleotide Archive (<https://www.ebi.ac.uk/ena/browser/home> ) under study number PRJEB2141.

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**Contributions**

The project was conceived and supervised by FOO, FB, and DW. Field collection was performed by JOO, IHN, HB, and GM. Laboratory analysis, data acquisition and management, and preparing samples for whole genome sequencing were performed by JOO. Sequence QC, alignments, SNP calling, and haplotype phasing were performed by AHK, JN, CSC, and AM. JOO, BP, and TPWD analyzed the data and generated all figures and tables. The manuscript was drafted by JOO and TPWD and revised by all authors. Throughout the project, all authors have contributed key ideas that have shaped the work and the final paper.

**Competing interests**

The authors declare no competing interests.

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