A rapidly selected 4.3kb transposon-containing structural variation is driving a P450-based resistance to pyrethroids in the African malaria vector Anopheles funestus

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Abstract

Deciphering the evolutionary forces controlling insecticide resistance in malaria vectors remains a prerequisite to designing molecular tools to detect and assess resistance impact on control tools. Here, we demonstrate that a 4.3kb transposon-containing structural variation drives pyrethroid resistance in central/eastern African populations of the malaria vector Anopheles funestus. In this study, we analysed Pooled template sequencing data and direct sequencing to identify an insertion of 4.3kb containing putative transposons in the intergenic region of two P450s CYP6P5-CYP6P9b in mosquitoes of the malaria vector Anopheles funestus from Uganda. We then designed a PCR assay to track its spread temporally and regionally and decipher its role in insecticide resistance. The insertion originates in or near Uganda in East Africa, where it is fixed and has spread to high frequencies in the Central African nation of Cameroon but is still at low frequency in West Africa and absent in Southern Africa. A strong association was established between this SV and pyrethroid resistance in field populations (SV+ vs SV-; OR=29, P< 0.0001) and is reducing pyrethroid-only nets' efficacy. Genetic crosses and qRT-PCR revealed that this SV enhances the overexpression of CYP6P9a/b but not CYP6P5. A marked and rapid selection was observed with the 4.3kb-SV frequency increasing from 3% in 2014 to 98 % in 2021 in Cameroon. Our findings highlight the underexplored role and rapid spread of SVs in the evolution of insecticide resistance and provide additional tools for molecular surveillance of insecticide resistance.

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Abstract:

Deciphering the evolutionary forces controlling insecticide resistance in malaria vectors remains a prerequisite to designing molecular tools to detect and assess resistance impact on control tools. Here, we demonstrate that a 4.3kb transposon-containing structural variation drives pyrethroid resistance in central/eastern African populations of the malaria vector *Anopheles funestus* . In this study, we analysed Pooled template sequencing data and direct sequencing to identify an insertion of 4.3kb containing putative transposons in the intergenic region of two P450s *CYP6P5 -CYP6P9b* in mosquitoes of the malaria vector *Anopheles funestus* from Uganda. We then designed a PCR assay to track its spread temporally and regionally and decipher its role in insecticide resistance. The insertion originates in or near Uganda in East Africa, where it is fixed and has spread to high frequencies in the Central African nation of Cameroon but is still at low frequency in West Africa and absent in Southern Africa. A strong association was established between this SV and pyrethroid resistance in field populations (SV+ vs SV-; OR=29, $P < 0.0001$) and is reducing pyrethroid-only nets' efficacy. Genetic crosses and qRT-PCR revealed that this SV enhances the overexpression of *CYP6P9a/b*but not *CYP6P5* . A marked and rapid selection was observed with the 4.3kb-SV frequency increasing from 3% in 2014 to 98 % in 2021 in Cameroon. Our findings highlight the underexplored role and rapid spread of SVs in the evolution of insecticide resistance and provide additional tools for molecular surveillance of insecticide resistance.

Introduction

The control of vector-borne diseases, which accounts for more than 17% of all infectious diseases reported in 2020, still greatly relies on using insecticides despite a recently recommended vaccine (WHO, 2021). Insecticides have effectively reduced the number of cases and deaths due to these diseases, with over 80% of the reduction in malaria cases between 2000 and 2015 attributed to their use (Bhatt et al., 2015). All LLINs currently used, including the new generation nets, contain a pyrethroid insecticide due to their high potency and low toxicity in humans (Mosha et al., 2022). However, malaria vectors have developed resistance to pyrethroids which has spread widely in field populations (Riveron et al., 2019), severely affecting our ability to control*Anopheles* with evidence that it could be impacting malaria transmission (Mosha et al., 2022; Protopopoff et al., 2018).

Insecticide toxicity can act as intense selective pressure, leading to the rapid evolution of resistance through the overexpression or modified activity of detoxification enzymes such as cytochrome P450s (Weedall et al., 2019;), alteration of the target site (Martinez-Torres et al., 1998; Weill et al., 2004), thickening of the cuticle (Balabanidou et al., 2016) and behavioural changes (Kreppel et al., 2020). The widespread insecticide resistance is a major global challenge threatening the efficacy of current and future vector control tools (Challenger et al., 2023).

Most studies on the molecular bases of insecticide resistance have focused on single nucleotide polymorphisms

and small indels because they can be readily identified with short reads (Duneau, 2018; Weedall et al., 2020; Weetman, 2018). However, growing evidence shows that structural variants (SVs) contribute to adaptive mechanisms, including insecticide resistance (Lucas et al., 2019).

SVs represent an essential source of genetic variation and are defined as large DNA sequence variations, including duplications, deletions, insertions, inversions, mobile-element transpositions, and translocations throughout the genome (Alkan, Coe, & Eichler, 2011). Structural variants are abundant across chromosomes. They are frequently found near genes where they are often associated with expression and likely contribute to phenotypic variations (Alonge et al., 2020) and are predominantly shaped by transposons (Brookfield, 2004). Decades of research have shown that the alteration of cis-regulatory regions by SVs can lead to perturbation of gene expression and phenotype (Alonge et al., 2020; Weischenfeldt et al., 2013). In *Anopheles* mosquitoes, gene copy number variations have been identified and correlated with increased expression of insecticide resistance-associated genes (Lucas et al., 2019; Weedall et al., 2020). A 6.5kb structural variant in*An. funestus* was recently shown to be associated with the increased expression of two cytochrome P450 genes whose overexpression confers high resistance to pyrethroids (Mugenzi et al., 2020) in Southern Africa and is absent elsewhere (West, Central and East Africa) suggesting a restriction to gene flow (Weedall et al., 2020). Population studies have shown a clear demarcation between Southern and Central/Western/Eastern populations with the clustering of Ghanaian, Cameroonian and Ugandan populations (Weedall et al., 2020). This points to the possibility of resistance-associated mutations not only emerging independently within a population but also spreading from another resistant population as seen for the cytochrome P450s *CYP6P9a* and*b* in southern Africa (Barnes et al., 2017).

A previous study on the promoter region of an insecticide resistance gene *CYP6P9b* in *An. funestus* showed that this gene's 1kb upstream region failed to amplify in certain countries (Uganda and Cameroon) while it was successfully amplified in most regions (Mugenzi et al., 2019). Therefore, it was hypothesised that a structural variant in this region could prevent PCR amplification.

Following this observation, we identified a 4.3 kb insertion in the p450s loci *rp1* previously identified by QTL mapping, where two insecticide resistance genes, *CYP6P9a* and *CYP6P9b* are found (Wondji et al., 2009). This 4.3kb SV is shown to be present in Central, East and West Africa while absent in southern regions. Temporal monitoring of this structural variant in *An. funestus* populations of Cameroon revealed that it was under strong selection, showing that it might be an adaptive variation or be linked with a nearby adaptive mutation. Genetic crosses showed a strong association between this structural variant and resistance to pyrethroids, and an association was found between the presence of this structural variant and the overexpression of nearby genes.

Materials and Methods

Mosquito samples

This study involved field-collected mosquitoes and an insecticide-susceptible laboratory strain. Mosquito collections were carried out in 4 villages in Cameroon where *An. funestus* s.s. is the predominant vector: Gounougou (9°03'00"N, 13deg43'59"E) in 2014, 2017, 2020, 2021; Elende (3deg41'57.27"N, 11deg33'28.46"E) in 2020 (Nkemngo et al., 2020); Mibellon (6deg46'N, 11deg70'E) in 2016, 2018, 2020 (Menze et al., 2018) and Tibati in 2018 and 2021 (6deg28' N, 12deg37' E) (Tchouakui, Fossog, et al., 2019). Indoor resting mosquitoes were collected with Prokopack aspirators between 06:00 am and 9:00 am, following verbal consent from the house owner. The collected blood-fed *Anopheles*females were kept for 5 days until fully gravid before putting them in 1.5ml tubes for forced egg-laying. The F_1s were reared at the Centre for Research in Infectious Diseases (CRID) until the emergence of adults. F_1 mosquitoes from Gounougou were used for susceptibility testing and evaluating bed net efficacy.

Molecular identification followed the cocktail PCR method of Koekemoer et al. (Koekemoer et al., 2002) to discriminate members of the *An. funestus* group and confirm the species as *An. funestus* s.s was done. The *An. funestus* s.s laboratory strain FANG, maintained at the insectary of CRID, was used for the crossing and qRT-PCR as the reference strains to determine gene expression.

2. Mosquito Rearing

Insectaries were maintained under standard conditions at 26 +- 4*C with a relative humidity of 70 +- 10%. Larvae from field and laboratory strains were fed with ground fish food (TetraMin(r) tropical flakes, Tetra(r), Blacksburg, VA, USA), and adults were provided with 10% sucrose solution on cotton wool.

3. Establishing crossing between field and laboratory mosquitoes.

Field-caught mosquitoes from Elende were crossed with the FANG *An. funestus* colony to allow segregation of the resistance allele already at a very high frequency in the population. F_1 larvae from the field mosquitoes and larvae from the FANG colony were reared until the pupal stage, then individually placed in 15ml tubes with 2ml of water and stoppered with cotton wool. This was done to obtain virgin female mosquitoes for each strain. Female field-collected mosquitoes were crossed with male FANG to generate a field x FANG line. A reciprocal crossing with the female FANG and the male from the field was also generated. Only the female FANG crossed with males for the field was successfully established. This line was maintained until the F_3 generation when bioassays were performed.

4. WHO insecticide susceptibility tests

The resistance profile to public health insecticides was determined using the WHO susceptibility bioassays protocol (WHO, 2016). About 100 female mosquitoes aged 2-5 days old were exposed to the insecticide(s) for either 30 minutes or 1 hour, then transferred to holding tubes and provided with 10% sucrose solution. Fifty mosquitoes exposed to non-impregnated papers were used as a control group. The initial knockdown effect was scored immediately after exposure time, while the mortality rates were scored 24 hours post-exposure. These bioassays were conducted at 26 +- 2degC and 80 +- 10% relative humidity.

5. WHO cone assays

The bio-efficacy of several bed nets was evaluated using the WHO cone assay protocol (WHO, 2013). Cohorts of 50 mosquitoes were exposed to 25cm x 25cm pieces of the netting of PermaNet 3.0, PermaNet 2.0, Olyset, and Olyset Plus. PermaNet 3.0 had 2 pieces from the roof and the side, as the top is impregnated with insecticide and PBO while the side has only the insecticide. Insecticide-free nets were used as controls to which mosquitoes were also exposed. Mosquitoes were exposed to the nets for 3 minutes in 5 replicates (each of 10 mosquitoes) and then transferred into paper cups. The knockdown effect was recorded at 1-hour post-exposure, and the mortality rate was scored 24 hours post-exposure.

6. Screening of the structural variant near *CYP6P5/CYP6P9b* from whole-genome sequences from different regions of Africa

Based on the previous observations of the likely presence of an SV in the intergenic region between *CYP6P9b* and *CYP6P5* (Mugenzi et al., 2019) due to the lack of amplification of the 1kb fragment, SV was searched using PoolSeq data from *An. funestus* populations available under European Nucleotide bioprojects PRJEB24384, PRJEB13485, PRJEB35040, PRJEB35040, PRJEB24351 and PRJEB10294 (Weedall et al., 2019, 2020) for 12 populations of *An. funestus* sampled across Africa from 2002 to 2016. These were aligned to the *An. funestus* F3 genome (Ghurye et al., 2019) using BWA (v0.7.17-r1188) (Li et al., 2009). Reads were coordinate sorted, and duplicates were marked with Picard (v2.18.15) ("Picard Toolkit," 2019). The subsequent alignments were visually inspected for evidence of an insertion in the intergenic region of *CYP6P5* and *CYP6P9b* in IGV. In addition, non-reference transposon insertions were searched for computationally using TranSurVeyor (v1.0) (Rajaby & Sung, 2018).

Visualising nucleotide diversity across the 4.3-kb SV

Alignments were re-created with the *An. funestus* F3 genome (VectorBase release 57, Ghurye et al., 2019) for Tororo (Uganda), Mibellon (Cameroon) and Obuasi (Ghana) populations using BWA (v0.7.17-r1188) (Li et al., 2009). Reads were coordinate sorted, and duplicates were marked with picard (v2.27.2) ("Picard Toolkit," 2019). Pileup files for each population were created using Samtools (v1.6) (Li et al., 2009) with minimum phred-scaled base quality of 10 and mapping quality per read of 20. Population genetic statistics

nucleotide diversity and Tajimas' D were generated for 1000 bp non-overlapping windows across chromosome 2 using npstat (v1.0) (Ferretti et al., 2013) for each population separately. Plots of nucleotide diversity from positions 8,525,000 to 8,575,000 incorporating the *rp1* resistance locus and 4.3 kb insertion site between *CYP6P5* and *CYP6P9b*were created using the karyoploteR package (v1.16.0) in R version 4.0.3 (Gel & Serra, 2017). A ratio of nucleotide diversity between Uganda and Cameroon, Ghana as two closely-related populations (Weedall et al., 2020) and susceptible FANG populations was plotted, and an upper-bound of 100 was arbitrarily chosen for 1kb windows of very low, or zero, diversity in order to plot the pattern of diversity across the region.

PCR amplification and sequencing of the entire *CYP6P5–CYP6P9b* intergenic region

To validate the poolseq results, which indicated the presence of a structural variant in the upstream region of *CYP6P9b* , the entire intergenic region between *CYP6P5* and *CYP6P9b* was amplified. Primers were 6P9dplF CCC CCA CAG GTG GTA ACT ATC TGA A located at 19bp before the stop codon of *CYP6P5* and the 6P9Ra/b TAC ACT GCC GAC ACT ACG AAG located at 35bp after the start codon of*CYP6P9b* and the Phusion high fidelity DNA polymerase (Thermo Scientific) was used for the amplification. The Phusion Taq PCR mix consisted of 3µl of 5x HF buffer, 0.12µl of 25mM dNTPs, 10mM forward and reverse primers, 0.15µl Phusion Taq, 9.71µl of deionised water and 1µl of DNA for 15µl reaction. Thermocycler conditions were: pre-denaturation at 98°C for 1 minute; 35 cycles of denaturation at 98°C for 10 seconds, annealing at 62°C for 30 seconds, extension at 72°C for 4 minutes; a final extension at 72°C for 10 minutes. PCR amplicons were stained with Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH) and visualised and size-scored following (1%) agarose gel electrophoresis, using an ENDURO GDS (Labnet) UV transilluminator. PCR products were gel-purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), ligated into PJET1.2 blunt-end vectors and Sanger-sequenced from each end of the 5.3 kb fragment using the plasmid-specific sequencing primers pJET1.2F and pJET1.2R. To sequence the complete fragment, four additional internal sequencing primers were used (Table S1). Sequence data were analysed with BioEdit software (Hall, 1999).

Design of a simple PCR assay to detect the 4.3kb SV and analysis of its distribution and association with pyrethroid resistance.

A PCR was designed to discriminate between mosquito samples with the 4.3kb structural variant and those without, consisting of 3 primers. Two primers (4.3kb_INSL_F: GGG GCG CTT TAG TTG AGA T and 4.3kb_INSR_R: CAC GTT TCA AGT GCA GGT GA) form a pair flanking the insertion but, due to the size of the insertion, amplify only for samples lacking the insertion, to produce a 281bp amplicon. A third primer (4.3kb_INS_R: CAT ACG CCT CTC CAG CAT GGA) binding within the structural variant forms a pair with 4.3kb_INSL_F to give a 780bp product only from samples containing the insertion. PCR amplification was done using the Kapa Taq PCR kit (Kapa Biosystems) with a 15µl reaction mix composed of 10x buffer A, 0.75µl of 25mM $MgCl_2$, 0.12µl of 25mM dNTPs, 0.51µl of each primer, 0.12µl of Kapa Taq enzyme, 10.49µl of deionised water and 1ul of genomic DNA. Thermocycler conditions were: pre-denaturation at 95 °C for 5 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute; a final extension at 72°C for 5 minutes. PCR products were separated by (1.5%) agarose gel electrophoresis, stained with Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH) and visualised on a UV transilluminator. After optimisation, these assays were used to investigate any possible association between this structural variant and pyrethroid resistance using field F_1 and laboratory mosquitoes by genotyping mosquitoes dead and alive after insecticide bioassays.

The spatio-temporal distribution of this structural variant in *An. funestus* s.s. populations collected in different location across Africa (Ghana, Cameroon, Kenya, Uganda, Tanzania, and Mozambique) was investigated. Genomic DNA samples from previous collections from across Africa (Weedall et al., 2020) were also used.

Assessment of the expression of genes in proximity to the 4.3kb structural variant using qRT-PCR.

To determine if the 4.3kb SV affected the expression of nearby genes, qPCR was used to compare the expression of 3 genes (*CYP6P5* ,*CYP6P9a,* and *CYP6P9b*) found near it. The Elende x Fang cross was used to generate pools of mosquitoes with different genotypes: homozygous for SV (SV+/SV+), heterozygous for SV (SV+/SV-), and homozygous no SV (SV-/SV-).

After rearing Elende x Fang mosquitoes to the F_3generation, adult females aged 3-5 days old were collected and kept at -80°C. DNA was extracted from the legs as described previously (Mugenzi et al., 2020) and used for the 4.3kb insertion genotyping. Bodies were kept in RNAlater (ThermoFisher scientific) and stored at -80°C until genotyping was completed. The bodies were grouped in triplicates of 8 mosquitoes, each according to their genotypes; SV+/SV+, SV+/SV-, and SV-/SV-. RNA was extracted by genotype using the Arcturus PicoPure RNA Isolation Kit (Life Technologies), and cDNA was synthesised using the Superscript III (Invitrogen) as previously described (Mugenzi et al., 2020). Expression levels of *CYP6P5* , *CYP6P9a,* and *CYP6P9b* were assessed relative to the susceptible FANG strain on the Agilent MX3005. Relative expression and fold-change of each target genes were calculated according to the 2^{-CT} method incorporating PCR efficiency (Schmittgen & Livak, 2008) after normalisation with the*An. funestus* housekeeping ribosomal protein S7 (RSP7) and actin 5C genes.

Identification of putative transcription factor binding sites present in 4.3kb SV.

To check the possible role of this SV in gene regulation, we analysed the DNA sequence of the 4.3kb SV using the CiiiDER software (Gearing et al., 2019). CiiiDER uses the MATCH algorithm to predict the transcription factor binding sites in a query set of DNA sequences. First, the JASPAR CORE non-redundant vertebrate transcription factors (Mathelier et al., 2016) were used as the position frequency matrix PFM transcription factor model. Then we selected transcript factors like Aryl hydrocarbon receptor (Ahr), Muscle aponeurosis fibromatosis (Maf), previously implicated in xenobiotic response (Amezian, Nauen, & Le Goff, 2021; Kalsi & Palli, 2015).

Plasmodium **infection in relation to the 4.3kb Structural variant.**

Genotyping results for Obout 2016 samples revealed the presence of the three genotypes for the 4.3kb SV. Also, previous data had shown that mosquitoes collected in Obout in May 2016 had high *Plasmodium*infection rates. The *Plasmodium* infection rate was 38.7% (72/186), 79.2% of which was *P. falciparum* , 12.5% ovale-vivax-malariae OVM + and 8.3% mix infection (Nkemngo et al., 2022). Screening for *Plasmodium* infection using TaqMan assay was done on 186 whole mosquito specimens from Obout. The real-time PCR MX 3005 (Agilent, Santa Clara, CA, USA) system was used for the amplification (Bass et al., 2008). Briefly, 2 µL of gDNA for each sample was used as a template in a 3-step program with a pre-denaturation at 95 *C for 10 mins, followed by 40 cycles of 15 sec at 95 *C and 1 min at 60 *C. The primers (Falcip+: TCT GAA TAC GAA TGT C, OVM+: CTG AAT ACA AAT GCC, Plas-F: GCT TAG TTA CGA TTA ATA GGA GTA GCT TG, Plas R: GAA AAT CTA AGA ATT TCA CCT CTG ACA) were used together with two probes tagged with fluorophores: FAM to detect *Plasmodium falciparum* , and HEX to detect *Plasmodium ovale* , *Plasmodium vivax,* and *P. malariae. P. falciparum* samples and a mix of *P. ovale* , *P. vivax,* and *P. malariae* were used as positive controls. A sub-set of positive samples was subjected to Nested PCR to confirm and discriminate the species detected by TaqMan based on the protocol of (Snounou et al., 1993). Only the *P. falciparum* positives and non-infected were used to investigate the *Plasmodium* infection rates in mosquitoes with different genotypes for the 4.3kb SV. In total 79 mosquitoes were analysed.

Data analysis

The percentage mortality for the bioassays and percentage genotypic and allelic frequencies for the dead and alive groups were computed in Excel 2016 spreadsheet (Microsoft). In addition, the odds ratio statistical test was calculated on the medcalc website (https://www.medcalc.org/calc/odds_ratio.php) to determine the strength of association between genotypes and the ability to survive insecticide exposure. Fisher exact test was used to assess the significance level for the differences observed in the bioassay results, and results were considered significant at P value less than 0.05.

Results

1. Identification of 4.3kb structural variant in *Anopheles funestus* populations.

Inspection of the PoolSeq IGV alignments revealed a prominent coverage increase in the intergenic region in Uganda in 2014 (Figure 1A), corresponding to a transposon insertion position of 8,556,411-8,556,420 on Chromosome 2 with an insertion sequence of "CAAATGTACA". There was weak visual evidence for the insertion in Cameroon, and it was not present in other PoolSeq populations. TranSurVeyor detected an insertion at the same positions in Uganda but not in Cameroon or any other population. The Inserted fragment is present in the reference strain FUMOZ at positions 24,765,013-24,760,696 on Chromosome 3 within the intron of an uncharacterised gene (AFUN019979). Due to the mixed-template PoolSeq approach, it was not possible to accurately determine the frequency of the SV in Uganda and Cameroon in 2014, although it appears to have been of intermediate to high frequency in Uganda and very low frequency in Cameroon.

Nucleotide diversity was reduced in Uganda for the intergenic region (Figure S1) compared to Cameroon and FANG datasets and other regions . This was accompanied by low Tajima's D values in the intergenic region between *CYP6P5* and *CYP6P9b* genes (Table S2). There are multiple peaks of low diversity in Uganda across the *rp1* locus versus other populations, suggestive of complex regional patterns of evolution not restricted to the 4.3 kb insertion. When the ratio of nucleotide diversity was inverted between Uganda and the other populations, a flat profile indicating little reduced diversity at other *rp1* loci versus Uganda was observed (Figure S2).

2. Amplification and sequencing of the 4.3kb structural variant insertion site in Uganda and Cameroon populations.

The entire intergenic region between *CYP6P5* and *CYP6P9b* was amplified, cloned and sequenced to confirm the presence of this structural variant in Uganda and Cameroon *An. funestus* s.s. populations. PCR amplicons of approximately 1kb (no insertion) and 5.4kb (with the insertion) were cloned into pJET1.2/blunt cloning vectors and Sanger-sequenced. To sequence the full fragment, four additional sequencing primers were used (Table S1). Sequence reads were aligned to reconstruct the full 5.4 kb region. To improve the sequence, pooled template WGS data were aligned to the draft 5.4 kb intergenic sequence flanked by the two coding genes (accession number : OR000399). Results revealed a 4311bp fragment inserted 494bp downstream of the translation stop codon of *CYP6P5* and 494bp upstream of the translation start codon of *CYP6P9b* (Figure 1A & C). Figure 1C shows the deeper coverage across the middle portion of the sequence, suggesting that it is a genomic fragment occurring in multiple copies in the genome.

Analysis of the entire 5.4kb for the open reading frames revealed the presence of 2 open reading frames corresponding to putative transposable elements. BLASTp identified these transposons to match a gag-like protein from *Culex pipens* (AAB86424.1) and a reverse transcriptase-like protein from *Aedes aegypti* (AAA29354.1) (Figure 1C).

3. Design of a genotyping assay for detection of the 4.3kb SV and delimitation of its geographical distribution.

The availability of the 4.3kb structural variant sequence and its flanking region facilitated the design of a simple PCR for genotyping its presence or absence in laboratory and field samples. This assay consists of three primers, two flanking this SV and one within it (Figure 1D), as previously designed for a nearby 6.5kb SV between *CYP6P9a* and *b* genes (Mugenzi et al., 2020). The assay was initially tested on Uganda (Tororo, 2014) and Cameroon (Mibellon, 2021) samples containing the insertion and showed a band at 780bp. Further genotyping of Uganda (Tororo 2014 and Mayuge, 2017) mosquito samples confirmed its presence and at a high frequency approaching fixation of the 4.3kb SV in Uganda mosquitoes with 100% frequency in Tororo and 97.83% in Mayuge with no mosquito found homozygous for the wild SV- allele (Figure 2A). Similar results were obtained for the Cameroon mosquito populations collected in Elon (2021), Mibellon (2021), Gounougou (2021) and Elende (2020), with SV+ allele frequencies of 94%, 100%, 98% and 94%, respectively

(Figure 2A). Exploring its distribution in other localities across Africa revealed that it was absent from Ghana (West Africa) in 2014 and Mozambique (southern Africa) in 2015 and present at a very low frequency in East African Tanzania (3% SV+) in 2018 (Figure 2A). Genotyping of the *An. funestus*FUMOZ and FANG lab colonies showed that this structural variant was absent from those colonies.

4. Temporal monitoring of the 4.3kb SV allele frequency suggests it has evolved under strong positive selection

The high frequencies of this structural variant observed in Central (Cameroon) and Eastern Africa (Uganda) suggested that it is under a strong positive selection. To investigate this possibility, we measured the frequencies of this SV in *An. funestus* s.s. populations of Cameroon collected in 2014, 2016, 2017, 2018, 2020 and 2021. Samples were available for 3 locations: Tibati and Mibellon, both from the Adamawa region and Gounougou (Northern region).

In Tibati, a temporal comparison of the frequency of the 4.3kb structural variant in samples collected in 2018 revealed a 76% SV+ allele frequency and complete fixation of SV+ in 2021 Figure 2B. In addition, all three genotypes (SV+/SV+, SV+/SV- and SV-/SV-) were detected in 2018 at frequencies of 79%, 9% and 19%, respectively, while in 2021, only the SV+/SV+ genotype was detected (Figure 2C), indicating positive selection on the 4.3kb structural variant in this location.

A similar pattern was observed in Mibellon, with the structural variant present at a low allelic frequency (26%) in 2016, increasing to 98% in less than 2 years (2018) and then to 100% in 2020 (Figure 2D). In 2016, mosquitoes without the 4.3kb SV (SV-/SV) were more common (59%) than those with the structural variant in either the homozygous or heterozygous state (39% SV+/SV+; 11% SV+/SV-) (Figure 2E). By 2018 and 2020, the SV-/SV- homozygote was no longer detected, indicating that this 4.3kb structural variant was also driven to fixation under positive selection in this region.

Genotyping of this SV in samples collected in Gounougou in 2014, 2017, 2020 and 2021 revealed a similar selection pattern to those observed in Tibati and Mibellon, with 3% in 2014, 31% SV+ in 2017, 63% in 2020 and 98% in 2021 (Figure S3A). The SV+/SV+ homozygous genotype was at a very low frequency of 3% in 2014, reached a frequency of 26% in 2017 which doubled to 54% by 2020 in about 3 years and then almost got to fixation in 2021 (Figure 2F).

The rapid increases in allele frequency of the 4.3kb SV in these 3 regions indicate strong selection acting on this structural variant (or a tightly linked allele) in these mosquito populations.

Analysis of recent samples collected in Ghana and Benin (West Africa) in 2022 showed that this insertion is now present in these localities. In Ghana, this SV was identified at a frequency of 5% with only 3 heterozygotes (Figure S3B & S3C), while in the Benin 2022 samples, a higher frequency of 33% was observed (SV+). The genotypes were present at frequencies of 16% (SV+/SV+), 34% (SV+/SV-) and 50% (SV-/SV-) (Figure S3D & S3E).

5. The 4.3kb SV is associated with pyrethroid resistance

We next aimed to establish if this 4.3kb contributes to pyrethroid resistance, thus explaining its swift increase in frequency in field populations of *An. funestus* from Central and East Africa.

5.1 Genotyping of field samples reveals a correlation between the 4.3kb SV and pyrethroid resistance.

Gounougou samples obtained from the deltamethrin 0.05% WHO tube bioassays in 2018 (when the 4.3kb structural variant was not yet fixed) were genotyped to determine any association between the 4.3kb SV and deltamethrin resistance phenotype. The mortality rate for deltamethrin was 49.8±11.6%. Genotyping of 77 mosquitoes exposed to deltamethrin (35 alive and 42 dead) revealed a strong association ($\chi 2$=33.8; P<0.00001) between this structural variant and the deltamethrin resistant phenotype, with 91.4% (32/35) of the survivors 24 hours post-exposure being homozygous for the structural variant (SV+/SV+), 5.7% (2/35) of the survivors being heterozygous (SV+/SV-) and only 2.9% (1/35) being homozygous without this SV (SV-/SV-) (Figure 3A; Table 1). Among the dead, the distribution of the genotypes was as follows 26.2% (SV+/SV+), 16.7% (SV+/SV-) and 57.1% (SV-/SV-). The odds ratio confirmed the positive association

between the 4.3kb SV genotype and the ability to survive exposure to deltamethrin, with SV+/SV+ having a higher association with survival than the SV+/SV- [OR: 10.2; CI: 1.8-56.5; P = 0.0080] and SV-/SV- [OR: 69.8; CI: 8.4-578.5; P = 0.0001]. The higher odds ratio obtained when comparing the SV+/SV+ genotype to the SV+/SV- genotype (10.18) indicates an additive effect of each 4.3 kb structural variant allele. Also, at the allelic frequency, we observed that 94.3% of the alive had the SV+ allele against 5.7% lacking the 4.3kb SV allele showing that this structural variant is strongly associated with the deltamethrin resistance phenotype (OR: 29; CI: 11.6-73.2 ; P< 0.0001). Of the dead, 65.5% lacked the 4.3kb SV (SV-), and 34.5% had the 4.3kb SV (SV+), revealing a lower likelihood for the individuals with the 4.3kb SV to be dead compared to those without (Figure 3B; Table 1).

5.2 The 4.3kb SV is reducing the efficacy of LLINs through genotyping of cone assays samples.

To validate the ability of the 4.3 kb SV marker to predict the impact of resistance on the efficacy of LLINs, we genotyped F_1Gounougou samples obtained from cone assay with pyrethroid-only and PBO-based nets. A mortality of 100% was obtained when the Kisumu susceptible strain was exposed to these nets. Genotyping of dead and alive mosquitoes from Gounougou 2018 showed a differential survival for mosquitoes with the 4.3kb SV depending on the bed net used. Mosquitoes were phenotypically resistant to almost all the nets tested with mortality rates below 80%, 9±3.8% for Olyset, 53±11.5% for Olyset plus, 15±3.0% for PermaNet 2.0. PermaNet 3.0 was the most effective, with mortality rates of 100% when a piece of the top was used while the side gave 33 ±5.6%. A significant difference in the distribution of genotypes of the 4.3kb SV was observed for PermaNet 2.0 between dead and alive mosquitoes (χ^2= 112.7; P< 0.00001) (Figure 3C; Table 1). No mortality was observed for mosquitoes with either 1 (SV+/SV-) or 2 (SV+/SV+) alleles for the structural variant. The alive PermaNet 2.0 samples were composed of 74.1% (17/27) SV+/SV+, 22.2% (6/27) SV+/SV- and 14.8% (4/27) SV-/SV- while all the dead were SV-/SV- (100%) (Figure 3C; Table 1). Comparing the proportion of each SV genotype between alive and dead mosquitoes revealed that SV+/SV+ homozygote mosquitoes were significantly more likely to survive exposure to PermaNet 2.0 than those completely lacking the 4.3kb SV (SV-/SV-) (OR: 823.45; CI: 148.4-14005.3; P<0.0001). Heterozygote (SV+/SV-) mosquitoes also exhibited a greater ability to survive exposure to PermaNet 2.0 than homozygotes susceptible wild genotype (SV-/SV-) (OR: 291.8; CI: 16.8-5059.6; P=0.0001). SV+/SV+ homozygote mosquitoes also significantly survived better than heterozygotes (SV+/SV-) (OR: 25.4; CI: 1.31-490.7; P=0.032) showing an additive effect of the 4.3kb SV on the resistance phenotype. When comparing at the allelic level, it was revealed that possessing a single 4.3kb SV+ allele confers a significantly higher ability to withstand exposure to PermaNet 2.0 compared to the SV- allele (OR: 565.08; CI: 33.9-9422.4; P<0.0001) (Fig. S3A; Table 1).

Genotyping of 43 samples (with 40 alive and 3 dead) exposed to Olyset, impregnated with permethrin, was inconclusive as only 3 mosquitoes were dead. Analysis of the Olyset plus exposed mosquitoes revealed a significant difference in the survival ability of 4.3kb SV genotypes (χ^2= 56.4; P< 0.00001). The distribution of the 4.3kb SV in the alive samples was 64.3% (9/14) for the SV+/SV+, 0% for the SV+/SV- and 35.7% (5/14) for the SV-/SV- (Fig. S3B). Among the dead samples, we had 45.5% (10/22) SV+/SV+, 9.1% (2/22) SV+/SV- and 45.5% (10/22) SV-/SV-. Hence there were no significant differences in surviving exposure to Olyset Plus between the SV+/SV+ and the SV-/SV- genotype (OR: 1.8; CI: 1.1 to 3.2; P = 0.04). A similar result was obtained at the allelic level, with 64.3%of the alive having the SV+ allele and 35.7% having the SV- allele, while for the dead, both alleles were equally represented at 50% each (Fig.S3C; Table 1). No significant survival likelihood was observed when comparing SV+ and SV- (OR: 1.78; CI: 1.01 to 3.13; P = 0.05).

5.3 Genetic crosses further reveal an association between the 4.3kb SV and pyrethroid resistance

Another approach was to use samples generated from crossing Elende field mosquitoes (SV+/SV+) and FANG lab susceptible colony (SV-/SV-), allowing them to interbreed and later conduct bioassays.

Results from deltamethrin (0.05%, type II) bioassay at F_3 exposed for 60 minutes revealed 80.1 ± 5% mortality rates showing that the crossing was still resistant. Genotyping of the 4.3kb SV revealed a strong association between the presence of this structural variant and the ability to survive exposure to deltamethrin.

SV+ is significantly associated with survival to deltamethrin exposure (OR: 19.4; CI: 9.5 to 39.6; P< 0.0001) (Figure 3D; Table 2). Homozygote SV+/SV+ mosquitoes had significantly higher likelihood of surviving than the heterozygotes SV+/SV- (OR: 37; CI: 2-689.9; P=0.016) and the homozygote lacking the SV SV-/SV- (OR: 1517; CI: 28.6-80383.1; P = 0.0003) genotypes. The heterozygotes also had higher chances of surviving than homozygotes SV-/SV- (OR: 41; CI: 2.2 to 761.8; P = 0.01).

Another crossing generated with the field Mibellon males (fixed for the SV+) crossed with FANG females (without this SV) at F_3revealed a similar association with permethrin (0.75%, type I) and alpha-cypermethrin (0.05%, type II). This SV was found to be linked with the ability to survive permethrin exposure (OR: 5.6; CI: 3.1 to 10.4; P< 0.0001) (Figure 3F; Table 2). At the genotypic level, the SV+/SV+ genotype had increased chances of being alive after exposure to permethrin compared to the SV+/SV- (OR: 6.4; CI: 2.0-20.8; P=0.0018) and the SV-/SV- (OR: 17.7; CI: 4.3-72.3; P = 0.0001) genotypes. Heterozygotes still had higher odds of surviving than the SV-/SV- (OR: 2.8; CI: 0.7 to 10.4; P = 0.0135) (Figure 3G; Table 2).

Furthermore, examining the frequency of the 4.3kb SV in the alpha-cypermethrin 60 minutes alive and 10 minutes dead. Among the alive, 59.09% had the SV+ while 40.91% lacked the SV- (Figure 3H; Table 2), showing that this SV is also associated with survival to alpha-cypermethrin exposure (OR: 116.4; CI: 5.5 to 24.5; P< 0.0001). Calculating the odds of having the SV and surviving revealed a positive association between both. The SV+/SV+ genotype is associated with survival to alpha-cypermethrin more than the SV+/SV- (OR: 3.05; CI: 0.8-11.2; P=0.009) and the SV-/SV- (OR: 72.7; CI: 18.9-278.8; P < 0.0001) genotypes. The SV+/SV- genotype also had more chances of surviving compared to the SV-/SV- (OR: 24; CI: 10.5 to 54.2; P < 0.0001) (Figure 3I).

6. Impact of 4.3kb structural variant insertion on the expression of nearby genes.

To assess potential effect of this structural variant on the expression of nearby genes (*CYP6P5, CYP6P9a,* and *CYP6P9b*), crosses between field samples (Elende, fully homozygous for the 4.3kb SV) and the fully susceptible FANG lab strain (4.3kb SV completely absent) were intercrossed to the F_3 generation. Quantitative real-time PCR performed on pools of each of the 3 genotypes (SV+/SV+, SV+/SV- and SV-/SV-) relative to FANG revealed increased expression of*CYP6P9a* (downstream) and *CYP6P9b* (immediately downstream) but not of *CYP6P5* (upstream) in the SV+/SV+ pool only (Figure 4A). *CYP6P9a* was most expressed in SV+/SV+ with fold change (FC) of 18.7 (*P-value* = 0.008), while SV+/SV- and SV-/SV- genotypes showed no differential expression. Similarly, CYP6P9b's expression was higher in the SV+/SV+ genotype (FC=16.0, *P value* = 0.002) and low in the SV+/SV- and SV-/SV- genotypes. This could indicate that possessing 2 copies of this 4.3kb SV further enhances the expression of these genes. For *CYP6P5* , there was no difference in the expression level for the different genotypes. Screening for transcription factors known to regulate detoxification genes in the 4.3kb SV using CiiiDER software identified Ahr, ARNT and MAF binding sites. For Ahr/Arnt, 4 binding sites were identified at positions 183-188, 3449-3454 and 4283-4288, while for 14 MAF binding sites were determined with 3 for MAFG, 2 for MAFF and 9 for MAFb.

7. Assessing the association between the 4.3kb structural variant and *Plasmodium* infection.

Genomic DNA samples extracted from whole mosquitoes collected from Obout in 2016 were screened to detect *Plasmodium* infection and grouped into 'infected' (n=37) and 'non-infected' (n=42). These were genotyped for the 4.3kb structural variant, showing a significant association between SV+/SV+, SV+/SV- and SV-/SV- and infection status ($\chi 2$= 7.0; P = 0.031). The *Plasmodium* -infected group comprised 16.2% SV+/SV+, 43.2% SV+/SV- and 40.5%, SV-/SV-showing that the homozygous SV+/SV+ were less infected than the SV+/SV- and SV-/SV- genotypes (Figure 4B). Hence the SV+/SV+ were less likely to be infected than SV-/SV- (OR: 4.05; CI: 2.2-7.3; P <0.001) while no significant difference was observed between SV+/SV+ and SV+/SV- genotypes (OR: 1; CI: 1.5-474.6; P = 0.05). Comparison of the allelic distribution of the 4.3kb SV among infected and non-infected further supported the reduced*Plasmodium* infection in mosquitoes, with 57.8% SV+ being non-infected against 45.2% SV-. The majority of Plasmodium-infected mosquitoes had SV- alleles without the structural variant (62.1%), while only 37.8% had SV+ alleles.

Discussion

This study assessed transposon-based structural variations' role in cytochrome P450-mediated metabolic resistance to insecticides in malaria mosquitoes by characterising and studying the effect of a 4.3kb intergenic insertion in a P450 cluster previously associated with resistance in the malaria vector *An. funestus.* A 4.3kb structural variant insertion first detected in East Africa in 2014 was shown to have spread rapidly in Central Africa, notably throughout Cameroon and continuing to move westward on the continent. Analysis of patterns and selection speed of this locus showed its association with strong resistance to pyrethroids, reduced bed net efficacy and*Plasmodium* infection, providing evidence that transposon-based resistance mechanism could be an important driver of metabolic resistance in malaria vectors.

Rapid selection of transposon-based resistance in malaria vectors with 4.3kb fixation in less than 5 years.

PoolSeq data analysis identified this 4.3kb insertion between the*CYP6P5* and *CYP6P9b* loci on Chromosome 2 found only in Uganda and Cameroon samples of 2014 out of 8 countries assessed. This 4.3bk SV contains 2 open reading frames (a gag-like and reverse transcriptase-like proteins), which correspond to 2 (*gag* and*pol*) out of the 3 open reading frames that characterised LTR- retrotransposons. The *gag*encodes capsid proteins, *pol* encodes enzymes regulating the transposition of a mobile element, while the missing *env* encodes a product responsible for the recognition of cell receptors and the penetration of a virus into a cell (L. N. Nefedova, Kuzmin, Makhnovskii, & Kim, 2014). The *Drosophila melanogaster*gag-related gene (gagr), a homolog to the Gypsy group of LTR retroelements, is possibly associated with the origin of new functions and the involvement in stress response in Drosophila species (L. Nefedova, Gigin, & Kim, 2022). This SV was at a high frequency in Uganda and observed at a low frequency in Cameroon in 2014. Therefore, we hypothesised that this SV spread from Uganda to Cameroon as it is inserted in an identical position but does not rule out a *de novo*origin in Cameroonian populations.

Interestingly population structure analyses using ddRADseq, Poolseq, and microsatellites have revealed a low level of divergence between Cameroon and Ugandan populations of *An. funestus* indicating that there is likely little barrier to gene flow and increased introgression of alleles between them (Barnes et al., 2017; Weedall et al., 2020). Temporal analysis of the changes in the allelic frequency of the 4.3kb SV in Cameroon collected across the years (2014-2021) revealed a rapid selection of this marker, with its frequency reaching fixation in less than 5 years. The such rapid selection indicates that this insertion will likely provide mosquitoes with an essential adaptive advantage. This is supported by the low nucleotide diversity and negative Tajima's D values in the *CYP6P5* to *CYP6P9b* intergenic region (Figure S1, Table S2). These high frequencies observed show a similar pattern to the 6.5 kb structural variant previously identified in*An. funestus* populations from southern Africa in Malawi and Mozambique (Mugenzi et al., 2020) and was correlated with an increase in deltamethrin/permetrhin resistance observed in field populations. This 6.5kb SV was shown to increase in frequency from 5% in 2002 to about 90% in 2016 in Mozambique samples (Mugenzi et al., 2020). Similarly, in*An. gambiae* , an upstream insertion of a partial Zanzibar-like transposable element (TE), was identified in association with two other mutations (nonsynonymous point mutation in *CYP6P4* (I236M) and a duplication of *the CYP6AA1* gene) in Uganda populations at high frequency and shown to have spread to Kenya, the Democratic Republic of Congo and Tanzania (Njoroge et al., 2022).

Genotyping of recently collected samples for the 4.3kb SV revealed its presence in West Africa in Ghana and Benin at low frequencies, suggesting that this resistance allele is migrating westward. Future works with up-to-date genomic data are needed to understand the origin of this structural variant which could be through adaptive gene flow from East to Central to West, or it could be occurring de novo.

4.3kb structural variant is associated with pyrethroid resistance

This structural variant was shown to contain two putative transposons. Several transposable elements have been identified in close proximity to metabolic resistance genes (Chen & Li, 2007), with the best characterised being the Accord insertion upstream of the *CYP6G1*in *Drosophila melanogaster,* which confers resistance to DDT has spread worldwide in this species (Catania et al., 2004). The possible role of this 4.3kb transposon-containing structural variant in pyrethroid resistance was investigated using a simple PCR assay to determine

its association with the ability to survive pyrethroid insecticides exposure. A strong association was found when using field-collected samples from Gounougou and genetic crosses generated in the insectary, with most of the survivors being homozygous SV+/SV+ while heterozygous SV+/SV- and homozygous SV-/SV- were mostly dead. The association was stronger for type II pyrethroids (deltamethrin and alpha-cypermethrin) compared to type I (permethrin), indicating that this SV could be responsible for the higher resistance to type II pyrethroids observed in the Uganda and Cameroon populations (Tchouakui et al., 2021). In addition, mosquitoes with the SV+ allele were more likely to survive bed net exposure using cone assays to the standard pyrethroids-only nets (PermaNet 2.0), while PBO-containing bed nets are more effective against these mosquitoes. The results are similar to the association found with the 6.5kb SV even though the association was lower. The odds of surviving exposure to permethrin with the 6.5kb SV was 242.4 (OR) while for the 4.3kb SV it was 5.63 (OR). Further studies are needed in semi-field conditions using experimental huts as previously done for the other metabolic markers (Mugenzi et al., 2020; Weedall et al., 2019) to better understand the impact of this SV on bed net efficacy.

Transposable elements as genetic drivers of over-expression of detoxification genes in malaria vectors as the presence of 4.3kb increased expression of nearby P450s

Transposable elements are over-represented near P450s clusters in insects (Chen & Li, 2007). They can affect the expression of the gene(s) by bringing regulatory elements that can either repress or enhance the expression of nearby genes (Guio et al., 2014). Genetic crosses were used to compare the expression of three genes located near this 4.3kb SV between the 3 genotypes SV+/SV+, SV+/SV- and SV-/SV- as previously done for the 6.5kb SV (Mugenzi et al., 2020). Increased expression of 2 genes (*CYP6P9a* and *CYP6P9b*) was found only in the homozygote SV+/SV+. This higher expression of nearby P450s in the homozygote SV+/SV+ suggests that the transposons contained in this 4.3kb region may act as enhancers. Increased expression was also seen in wildtype RNASeq for Ugandan versus susceptible FANG mosquitoes sampled in 2014 (Weedall et al., 2019) but was less clear cut than the crossing data due to complex expression of *CYP6P9b* in particular across Africa. In *D. melanogaster,* the up-regulation of *CYP6G1*conferring resistance to a variety of insecticide classes (Daborn et al., 2001) correlates with the presence of an Accord retrotransposon in the 5' UTR region and this retro-transposable element contains regulatory sequences capable of increasing the expression of*CYP6G1* in detoxification organs (Chung et al., 2007). The 4.3kb SV identified here could regulate the expression of *CYP6P9a/b* by providing regulatory elements. However, further studies are needed to functionally validate this hypothesis using luciferase promoter assay or promoter assays in transgenic mosquitoes.

Reduced *Plasmodium* infection in mosquitoes with 4.3kb SV

A negative association was found between the 4.3kb SV and the malaria parasite infection, with mosquitoes lacking the 4.3kb SV being more commonly infected than those with the 4.3kb SV. We might speculate that this is linked to 4.3kb SV-driven overexpression of *CYP6P9a/b*since P450s could play a role in the mosquito response to*Plasmodium* invasion by participating in the production of nitric oxide and other reactive oxygen radicals (Han et al., 2000; Luckhart et al., 1998) helping to eliminate the parasite. Previous studies have shown specific cytochrome P450s implicated in insecticide resistance are also differentially expressed during malaria parasite invasion in mosquitoes (Félix et al., 2010). For example, *CYP6M2* and*CYP6Z1* both highly expressed in pyrethroid resistant *An. gambiae* mosquitoes (David et al., 2005; Müller et al., 2007) were also highly overexpressed in response to *Plasmodium* infection (Félix et al., 2010), suggesting an interaction between insecticide detoxification and infection. The results obtained are the opposite of what had been previously obtained with the *GSTe2* 119F resistant markers, where an association was observed with the homozygote 119F/F genotype being more associated with the *Plasmodium* infection at the oocyst and sporozoite stage (Tchouakui, Chiang, et al., 2019). These preliminary results need further validation using experimental infection studies as previously done with the *kdr* resistance mutations (Ndiath et al., 2014) to decipher the impact of P450-based resistance on malaria transmission.

Conclusion

This study identified a 4.3kb transposon-containing structural variant on chromosome 2 within a cluster of cytochrome P450 genes in Ugandan and Cameroonian populations of *Anopheles funestus* and showed it to be: associated with pyrethroid resistance, associated with enhanced expression of nearby P450 genes; rapidly evolving under strong selection in Cameroon and is spreading westward in Africa. This study shows how genetic variation, such as transposable elements, can contribute to adaptive changes and be rapidly selected in the mosquitoes carrying them. The molecular assay designed here will facilitate the detection and tracking of the spread of this transposon-based resistance and help assess its impact on control intervention and malaria transmission.

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Author contributions

C.S.W. conceived the study; C.S.W and L.M.J.M. designed the study; L.M.J.M, T.A.T, S.T.N., A.D.C., M.G.,B.D.M.,M.T., H.I M.J.W, performed the research; L.M.J.M, G.D.W., J.H.,C.S.W analyzed the data; L.M.J.M, AND C.S.W wrote the paper with input from the other authors.

Data accessibility

All genomic data sets are available from the European Nucleotide Archive. Pooled template whole-genome sequencing data are available under study accessions PRJEB13485 (Malawi 2002 and Malawi 2014), PRJEB24384 (Ghana, Benin, Cameroon and Uganda) and PRJEB35040 (Mozambique 2002, 2016; DRC-Kinshasa and Mikalayi). The sequences have been submitted to GenBank accession number: OR000399

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Conflicts of interest

The authors declare that they have no competing interests.

Figures legends

Figure 1: Identification and genotyping of a 4.3kb structural variant. A)Transposon insertion position detected by visually inspecting the intergenic region between *CYP6P5* and*CYP6P9b* in IGV. B) PCR amplification of the *CYP6P9b* and*CYP6P5* intergenic region. Lane 1 and 19 are the 1-kilobasepair DNA size marker;2 to 16 are field samples from Cameroon-Mibelong (2016); 17 is the negative control and 18 is a positive control. C) Schematic representation of *CYP6P9b* and *CYP6P5* intergenic region with the structural variant and without the structural variant and Coverage summary of pooled WGS data from Uganda aligned to CYP6P5, CYP6P9b and the 5.5 kb intergenic region. The plot shows that the coverage depth is approximately 40x across the genes and part of the intergenic region, but increases to >100x across the middle portion, indicating that it is a multi-copy transposon. D) Schematic representation of the 4.3kb SV diagnostic assay, consisting of 2 primers flanking the insertion site and 1 in the 4.3kb SV and electropherogram showing the different genotypes. +/+= SV+/SV+, +/- =SV+/SV- and -/- =SV-/SV-.

Figure 2: Spatial and temporal distribution of the 4.3kb SV across Africa. A) Geographical distribution of the 4.3kb SV in*An. funestus* population collected across Africa showing elevated frequencies in Cameroon and Uganda and absence in Ghana and Mozambique. Allelic (B) and genotypic (C) frequencies of 4.3kb SV in Tibati showing a decrease in SV- allele and increase in SV+ over the time. Allelic (D) and genotypic (E) frequencies of 4.3kb SV in Mibellon showing a decrease in SV- allele and increase in SV+ over the time. Genotypic frequency of the 4.3kb SV in Gounougou from 2014 to 2021 (F).

Figure 3: Association of 4.3kb SV with pyrethroid resistance. A & B Genotyping results of the 4.3kb SV among the Gounougou 2018 alive and dead deltamethrin post exposure reveal a strong association between the 4.3kb SV and ability to survive. A) shows the genotype distribution and B) allelic distribution. C) Genotype frequencies of the 4.3kb SV in alive and dead mosquitoes exposed to PermaNet2 .0 bed nets showing a positive association between the 4.3kb SV and resistance. D & E illustrate the strong association between 4.3kb SV and the ability to survive exposure to deltamethrin by looking at its genotypic and allelic distribution among dead and alive F3 Elende-Fang crossing mosquitoes. F & G illustrate the strong association between 4.3kb SV and the ability to survive exposure to Permethrin by looking at its genotypic and allelic distribution among dead and alive F3 mibellon-Fang crossing mosquitoes. H & I illustrate the

strong association between 4.3kb SV and the ability to survive exposure to ⍺-cypermethrin by looking at its genotypic and allelic distribution among dead and alive F3 mibellon-Fang crossing mosquitoes.

Figure 4: Impact of 4.3kb structural variant on expression of nearby genes and *Plasmodium* infection. A) Differential qRT-PCR expression for different structural variant genotypes of three cytochrome P450 genes in the immediate vicinity of the 4.3kb SV. Error bars represent standard deviation (n = 3). ns= not statistically significant; *= significantly different at $p < 0.05$. 4.3kb SV genotypes (B) and alele (C) distribution among *Plasmodium*infected and non-infected samples collected from Obout-Cameroon 2016 showing that samples with the 4.3kb SV are less infected than those without the 4.3kb SV.

Table 1: Association between insecticide susceptibility as determined by WHO tube bioassay or WHO cone bioassay and 4.3kb SV genotype in wild-caught, female *Anopheles funestus* from Gounougou Cameroon in 2018.

Insecticide/Bednet	Comparison	OR[a]	P value	CI[b]
WHO BIOASSAY	WHO BIOASSAY	WHO BIOASSAY	WHO BIOASSAY	WHO BIOASSAY
Deltamethrin	SV+/SV+ vs SV-/SV-	69.8	0.0001	8.4 to 578.5
	SV+/SV- vs SV-/SV-	6.9	0.1380	0.5 to 87.3
	SV+/SV+ vs SV+/SV-	10.2	0.008	1.8-56.5
	SV+ vs SV-	29	< 0.0001	11.6-73.2
WHO Cone assays	WHO Cone assays	WHO Cone assays	WHO Cone assays	WHO Cone assays
PermaNet 2.0	SV+/SV+ vs SV-/SV-	823.45	0.0001	148.4-14005.3
	SV+/SV- vs SV-/SV-	291.77	0.0001	16.8-5059.6
	SV+/SV+ vs SV+/SV-	25.4	0.032	1.3-490.7
	SV+ vs SV-	565.08	0.0001	33.9-9422.4
Olyset plus	SV+/SV+ vs SV+/SV-	4.5238	0.3493	0.2 to 106.7
	SV+/SV+ vs SV-/SV-	1.8	0.04	1.1 to 3.2
	SV+/SV- vs SV-/SV-	0.3818	0.5562	0.01 to 9.4
	SV+ vs SV-	1.78	0.05	1.0 to 3.1

Table 2: Association between insecticide susceptibility as determined by WHO tube bioassay and 4.3kb SV genotype in *Anopheles funestus* crossing between field and FANG lab colony.

Insecticide	**Comparison**	**OR[a]**	**P value**	**CI[b]**
Elende X Fang crossing	Elende X Fang crossing	Elende X Fang crossing	Elende X Fang crossing	Elende X
Deltamethrin	SV+/SV+ vs SV-/SV-	1517	0.0003	28.6-8038
	SV+/SV- vs SV-/SV-	41	0.01	2.2-761.8
	SV+/SV+ vs SV+/SV-	37	0.016	2.0-689.9
	SV+ vs SV-	19.4	0.0001	9.5-39.7
Mibelong X Fang crossing	Mibelong X Fang crossing	Mibelong X Fang crossing	Mibelong X Fang crossing	Mibelong
Permethrin	SV+/SV+ vs SV-/SV-	17.71	0.0001	4.3-72.3
	SV+/SV- vs SV-/SV-	2.75	0.0135	0.7-10.4
	SV+/SV+ vs SV+/SV-	6.44	0.0018	2.0-20.8
	SV+ vs SV-	5.63	0.0001	3.1-10.4
alpha-cypermethrin	SV+/SV+ vs SV-/SV-	72.72	< 0.0001	18.9-278.
	SV+/SV- vs SV-/SV-	24	< 0.0001	10.5-54.2
	SV+/SV+ vs SV+/SV-	3.05	0.009	0.8-11.2
	SV+ vs SV-	116.43	0.0001	5.5-24.5

[a] Odds ratios, [b] confidence interval.