MALARIA VECTOR CONTROL

A cytochrome P450 allele confers pyrethroid resistance on a major African malaria vector, reducing insecticide-treated bednet efficacy

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Metabolic resistance to insecticides such as pyrethroids in mosquito vectors threatens control of malaria in Africa. Unless it is managed, recent gains in reducing malaria transmission could be lost. To improve monitoring and assess the impact of insecticide resistance on malaria control interventions, we elucidated the molecular basis of pyrethroid resistance in the major African malaria vector, *Anopheles funestus*. We showed that a single cytochrome P450 allele (*CYP6P9a*_R) in *A. funestus* reduced the efficacy of insecticide-treated bednets for preventing transmission of malaria in southern Africa. Expression of key insecticide resistance genes was detected in populations of this mosquito vector throughout Africa but varied according to the region. Signatures of selection and adaptive evolutionary traits including structural polymorphisms and cis-regulatory transcription factor binding sites were detected with evidence of selection due to the scale-up of insecticide-treated bednet use. A cis-regulatory polymorphism driving the overexpression of the major resistance gene *CYP6P9a* allowed us to design a DNA-based assay for cytochrome P450–mediated resistance to pyrethroid insecticides. Using this assay, we tracked the spread of pyrethroid resistance and found that it was almost fixed in mosquitoes from southern Africa but was absent from mosquitoes collected elsewhere in Africa. Furthermore, a field study in experimental huts in Cameroon demonstrated that mosquitoes carrying the resistance CYP6P9a_R allele survived and succeeded in blood feeding more often than did mosquitoes that lacked this allele. Our findings highlight the need to introduce a new generation of insecticide-treated bednets for malaria control that do not rely on pyrethroid insecticides.

INTRODUCTION

Prevention of malaria relies heavily on the use of insecticide-based vector control interventions, most notably pyrethroid-based long-lasting insecticidal bednets. These tools have been credited with more than 70% of the decrease in malaria mortality in the past 15 years, having helped avert more than 663 million clinical cases of malaria (*1*). However, resistance of mosquito vectors to insecticides, particularly pyrethroids, is threatening their continued effectiveness. Unless it is managed, the recent gains in reducing malaria transmission could be lost (*2*). Elucidating the genetic basis and evolution of insecticide resistance among mosquito vectors of malaria is crucial for designing resistance management strategies and preventing the resurgence of malaria (*2*).

Without genetic information on insecticide resistance genes and associated molecular markers in the mosquito vector, it is difficult to track and anticipate the course of insecticide resistance or assess its impact on malaria transmission and the effectiveness of control tools such as long-lasting insecticide-treated bednets. The current inability to track metabolic resistance in this way in all major malaria vectors in Africa including *Anopheles gambiae* and *Anopheles funestus* is a major obstacle to the design of rational, evidence-based resistance management strategies. Of the four classes of insecticides deployed in public health, pyrethroids are the most widely used and are the main class recommended for use in insecticide-treated bednets. Therefore, understanding the mechanisms conferring pyrethroid resistance on mosquitoes is of critical importance.

Two major causes of insecticide resistance are metabolic resistance and target-site insensitivity (*3*). Target-site insensitivity to pyrethroids is due to knockdown resistance (*kdr*) caused by a modification of the insecticide target (a sodium channel) in mosquitoes (*4*). In contrast, metabolic resistance remains less well characterized despite posing a greater risk to malaria control interventions (*5*). Although candidate resistance genes have been detected (*6*–*9*), it has proved difficult to dissect the molecular bases of metabolic resistance and to detect associated molecular markers because of the size of gene families involved in detoxification, redundancy among their members, and the multiple mechanisms through which metabolic resistance can arise (*10*). Cytochrome P450 monooxygenases have consistently been associated with pyrethroid resistance but, unlike *kdr* (*4*), there is no DNA-based marker to track P450-mediated resistance and to assess its impact on malaria control strategies.

Here, we elucidated the complex molecular basis and genomic evolution of metabolic resistance to pyrethroids in the major African malaria vector *A. funestus*. We detected key DNA-based markers of pyrethroid resistance and designed a field-applicable diagnostic assay. We used this assay to track pyrethroid resistance across Africa and to demonstrate that this metabolic resistance in mosquitoes reduced the efficacy of insecticide-treated bednets for preventing malaria transmission.

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RESULTS

RNA sequencing transcriptional profiling of mosquitoes identifies candidate pyrethroid resistance genes

To identify genes associated with pyrethroid resistance in the mosquito vector *A. funestus* Africa-wide, we performed RNA sequencing (RNA-seq)–based transcriptional profiling of mosquitoes from four different African regions: southern (Malawi), East (Uganda), West (Ghana), and Central (Cameroon). We compared these RNA-seq profiles to those from a laboratory colony of mosquitoes (FANG) that were susceptible to all insecticides. The quality metrics and the alignment parameters are presented in table S1. The number of differentially expressed genes between each of the four populations and the FANG susceptible strain is shown in Venn diagrams (Fig. 1A and fig. S1A); the expression profile is shown in the volcano plots for each country (fig. S1, B to E). After quality control and analyses (table S1), Gene Ontology (GO) enrichment was performed to assess the generic metabolic terms associated with resistance. Permethrin-resistant mosquitoes in Malawi showed enrichment of gene ontologies associated with cytochrome P450 genes in genes overexpressed relative to the fully susceptible FANG strain. These GO terms included heme binding, tetrapyrrole binding, oxidoreductase activity, and iron binding (fig. S2A). These GO terms among others were also enriched in the overexpressed gene set in mosquitoes from Ghana (fig. S2B). In Ugandan mosquitoes, more GO terms were enriched among the overexpressed genes but were not directly associated with detoxification activities (fig. S2C).

Pronounced differences in the expression of key candidate genes were observed among *A. funestus* from the four different African regions (Fig. 1, A to C; fig. S1, A to E; and table S1). Genes encoding cytochrome P450 were frequently overexpressed (adjusted $P < 0.05$) with the genes *CYP6P9a* (AFUN015792; 60.5-fold) and *CYP6P9b* (AFUN015889; 23.9-fold) showing increased overexpression in Malawi mosquitoes compared to those from other regions (<7-fold; Fig. 1, B and C). Other cytochrome P450s were overexpressed more in mosquitoes from one region than in mosquitoes from other African regions (Fig. 1, B and C) including *CYP9K1* (AFUN007549), which was highly overexpressed in mosquitoes from Uganda (5.2-fold), only moderately overexpressed in those from Ghana (2.9-fold), and not overexpressed in mosquitoes from Malawi or Cameroon compared to FANG mosquitoes. *CYP6P5* (AFUN015888) was overexpressed in mosquitoes from Ghana (6.3-fold), Cameroon (5.8-fold), and Uganda (4.1-fold), but not in those from Malawi compared to FANG mosquitoes. The duplicated *CYP6P4a* and *CYP6P4b* were highly overexpressed in mosquitoes from Ghana (44.8- and 23.9-fold, respectively), moderately so in those from Malawi and Uganda (<6-fold), and not overexpressed in those from Cameroon compared to FANG mosquitoes. *CYP325A* was highly overexpressed in mosquitoes from Cameroon (26.9-fold) but less so in those from other regions (<6-fold) compared to FANG mosquitoes. Other

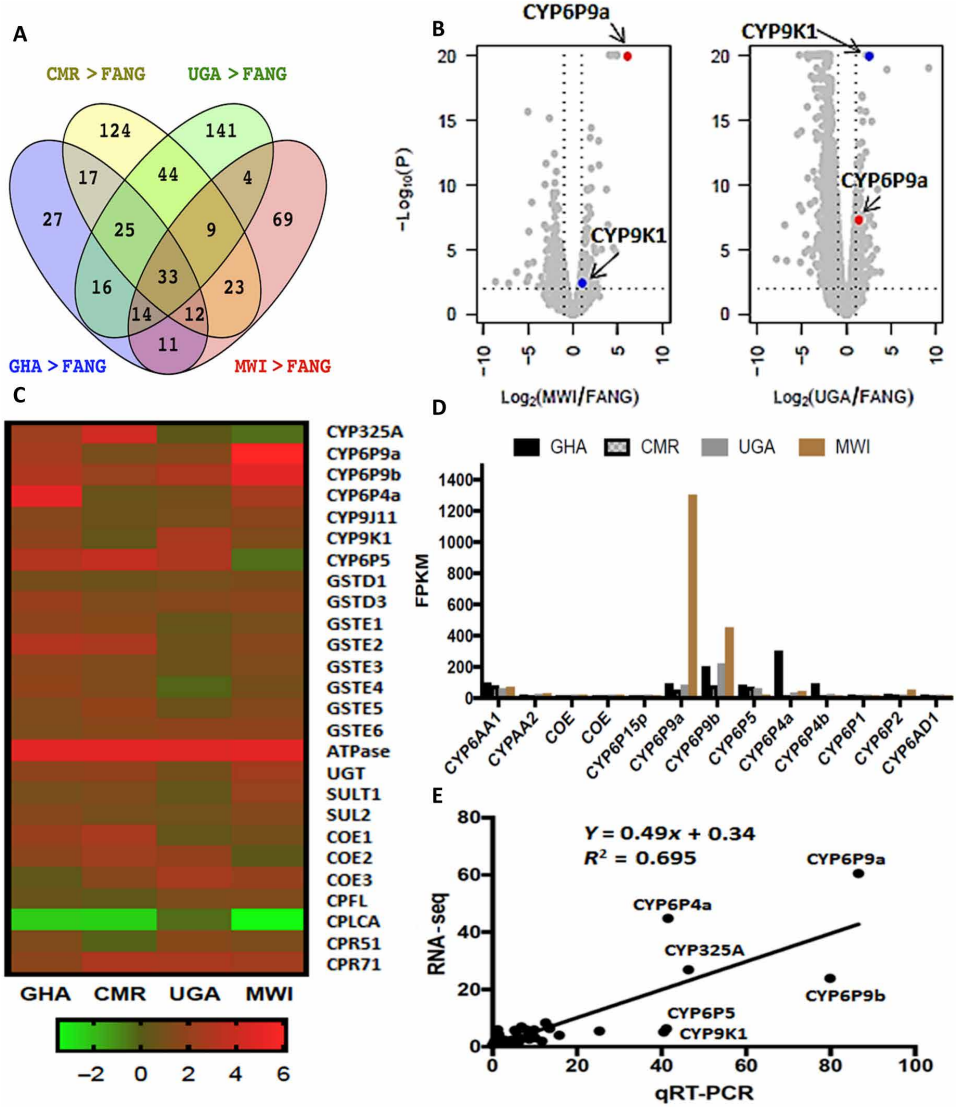

Fig. 1. RNA-seq transcriptional profiling of pyrethroid-resistant populations of *A. funestus* Africa-wide. (**A**) The Venn diagrams show the number of differentially up-regulated genes in *A. funestus* mosquitoes from different countries in Africa relative to the pyrethroid-susceptible FANG laboratory strain at a false discovery rate of <0.01 and a fold change of >2. (**B**) Volcano plots of differential gene expression between pyrethroid-exposed mosquitoes from four different countries in Africa (Cameroon, CMR; Malawi, MWI; Ghana, GHA; and Uganda, UGA) and the susceptible FANG strain. The volcano plots highlight differences in the expression of key resistance genes (e.g., *CYP6P9a* and *CYP9K1* in mosquitoes from different African countries). (**C**) Heatmap showing the differential expression of candidate detoxification genes among mosquitoes from different African countries. (**D**) Fragments per kilobase of transcript per million mapped reads (FPKM) for genes from the *rp1* pyrethroid resistance QTL. The histogram highlights differences among mosquitoes from four different African countries. (**E**) Correlation between RNA-seq and qRT-PCR data. The data shown are mean ± SEM ($n = 3$).

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cytochrome P450s were moderately overexpressed, including two paralogous genes, *CYP9J11* and *CYP6N1*, which were up-regulated in mosquitoes from southern and West Africa, whereas *CYP315A1* was overexpressed in mosquitoes from all sites but Malawi.

Other detoxification-associated gene families were also overexpressed, including a cluster of glutathione *S*-transferase epsilon genes (*GSTe1*, *GSTe3*, *GSTe4*, *GSTe5*, and *GSTe2*), known dichlorodiphenyltrichloroethane (DDT) resistance genes (*11*) that were up-regulated in mosquitoes from all regions except East Africa (table S2). The *GSTD1* gene was overexpressed in mosquitoes from Malawi, Ghana, and Uganda, suggesting a role for this gene in these regions but not in Cameroon where it was not significantly overexpressed (table S2). A carboxylesterase (AFUN002514) was also overexpressed in mosquitoes from Central and West Africa with 5.5- and 3.6-fold expression, respectively, in mosquitoes from Cameroon and Ghana (table S2). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) with 15 genes confirmed these regional differences, with a close correlation observed between qRT-PCR and RNA-seq results for the four countries when compared to FANG mosquitoes ($R^2 = 0.85$; $P < 0.001$) (Fig. 1E and fig. S3, A to C). Control mosquitoes not exposed to insecticides also showed a strong correlation ($R^2 = 0.695$, $P = 0.002$ in mosquitoes from Malawi) with permethrin-resistant samples used for RNA-seq (Fig. 1E), supporting a constitutive expression of these candidate resistance genes to confer resistance. However, differences were observed for the expression of some genes such as the *CYP6Z1* P450, which, with qRT-PCR (but not RNA-seq), exhibited an up-regulation in Malawi (FC 66) and Ghana (FC13.4) mosquitoes (fig. S3). This gene has previously been shown to be overexpressed in southern Africa using microarray and qRT-PCR (*7*), suggesting that RNA-seq could have missed or that the primers were not efficient with the susceptible FANG strain.

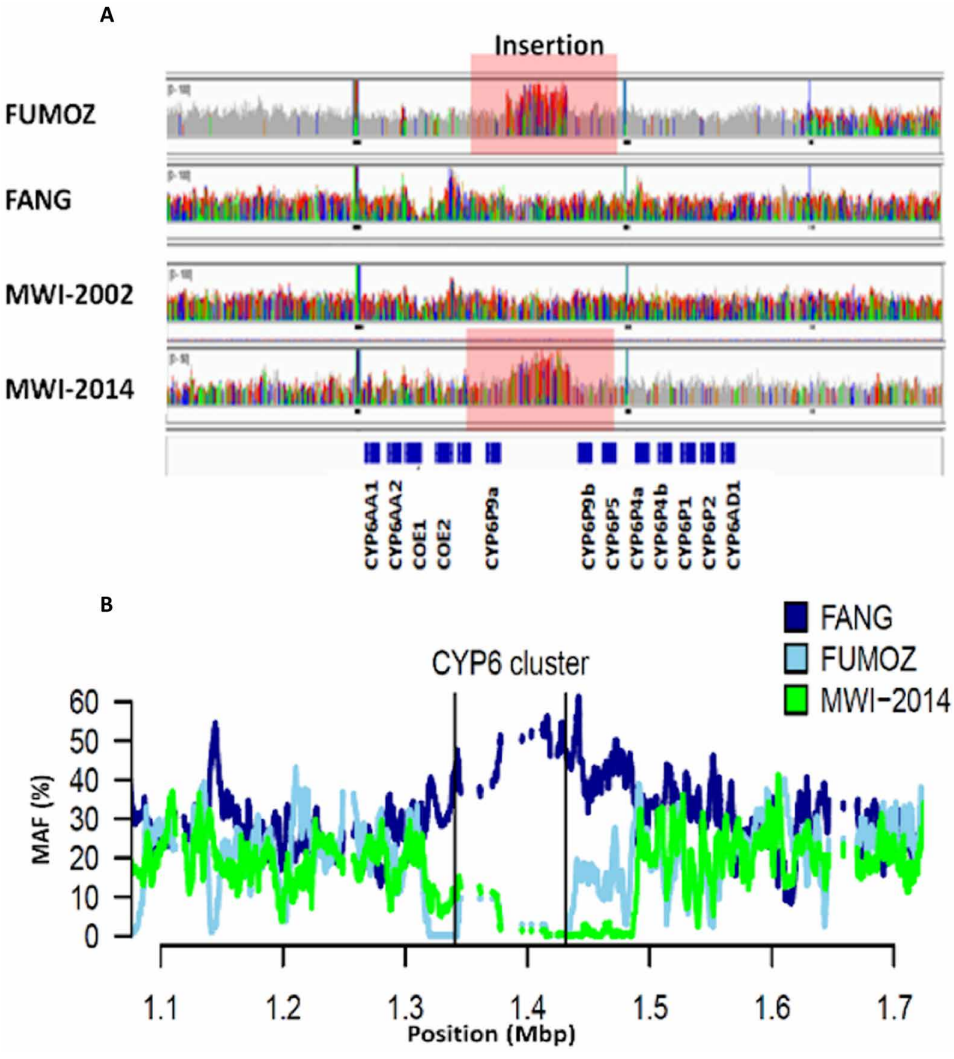

Fig. 2. Genetic signatures in the mosquito genome associated with pyrethroid resistance. (**A**) Contrasting polymorphism patterns among resistant FUMOZ and susceptible FANG mosquito strains. Also shown are contrasting polymorphism signatures between pre-bednet intervention (MWI-2002) and post-bednet intervention (MWI-2014) mosquito samples from Malawi. Data were aligned to 120 kb *rp1* bacterial artificial chromosome sequence (Integrative Genomics Viewer screenshot). Each row shows the alignment depth (on a log scale for display purposes); coverage depth is capped at >100×. Gray columns represent bases identical to the reference sequence, whereas colored columns indicate variant sites with a minor allele frequency > 10%. The genes of the cytochrome P450 cluster are indicated below the panel. An increased read coverage was observed between *CYP6P9a* and *CYP6P9b* in pyrethroid-resistant mosquito samples indicating a 6.5-kb insertion. (**B**) Shown is the major gene signature after a selective sweep through the mosquito genome detected around the *rp1* QTL pyrethroid resistance region on the 2R chromosome. This signature contained cytochrome P450 genes from the *CYP6* cluster and was found in mosquitoes from southern Africa but not in the susceptible FANG strain, after plotting minor allele frequency (MAF).

Whole-genome polymorphism analysis of mosquitoes collected in the field identifies insecticide resistance loci

Metabolic resistance could be conferred by point mutations in coding and cis-/trans-regulatory regions of the mosquito genome. Therefore, we scanned the whole genome for pyrethroid resistance–related signatures of selective sweeps (that is, a marked reduction in genetic diversity in a genomic region because of intense selection of a predominant allele) in the highly pyrethroid-resistant population of mosquitoes from Malawi to detect pyrethroid resistance loci. We performed pooled-template whole-genome sequencing on field-collected population samples from southern Africa where high expression of *CYP6P9a* and *CYP6P9b* genes has been observed. We also comparatively assessed the genomes of mosquitoes from Malawi and compared these genomes to those of the laboratory pyrethroid-resistant FUMOZ-R strain and the pyrethroid-susceptible FANG strain. We detected contrasting patterns of polymorphisms between the genomes of these strains and also between the genomes of the

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MWI-2002 and MWI-2014 mosquito samples collected in Malawi before and after bednet intervention, respectively (Fig. 2A and table S3). The major selective sweep for genomes of Malawi and FUMOZ-R mosquitoes was on scaffold KB119169, spanning the *rp1* pyrethroid resistance region on chromosome arm 2R (Fig. 2B). Plotting minor allele frequencies across the region revealed a valley of reduced genetic diversity around the cluster of cytochrome P450 genes on the *rp1* pyrethroid resistance QTL, correlating a selected *rp1* haplotype with *CYP6P9a* overexpression (Fig. 1D). This selective sweep appeared to be at or near fixation in a contemporary Malawian mosquito population as well as in the FUMOZ-R strain, with little diversity observed around the cytochrome P450 gene cluster in this highly pyrethroid-resistant mosquito population (Fig. 2B). No reduced diversity was observed in the pyrethroid-susceptible FANG strain, suggesting an association between this selective sweep and pyrethroid resistance in line with the very low *CYP6P9a* expression in FANG mosquitoes (Fig. 2, A and B). These results are consistent with previous reports of selection on highly overexpressed resistance genes (*9*).

Complex evolution of the *rp1* cluster of *CYP6* genes associated with pyrethroid resistance

As the *rp1* gene cluster was consistently associated with pyrethroid resistance, a fine-scale analysis of this locus (120 kb) was performed, revealing evidence of complex molecular evolution that was most likely under insecticide-driven selection. Inspection of pooled-template whole-genome alignments showed two anomalous features in the 8.2-kb sequence between *CYP6P9a* and *CYP6P9b* (Fig. 2A and fig. S4A). In some samples, the coverage depth was greater than for the surrounding sequence, and some samples showed read pairs in the correct relative orientation but with greater than expected insert sizes. This was indicative of a large indel, that is, either a "deletion" in the sequenced genome or an "insertion" in the reference genome (fig. S4A). This insertion corresponded to 6545 base pairs (bp) and appeared to be fixed in the FUMOZ mosquito colony sample with evidence that the inserted sequence was homologous to another region nearby (on the same assembly scaffold) in the genome (fig. S4B). In contrast, the FANG pyrethroid-susceptible mosquito strain showed evidence of the deletion form of the indel (fig. S4A). This insertion, nearly fixed across the genomes of mosquitoes from southern Africa (26 of 27), was absent from the genomes of mosquitoes collected from elsewhere on the continent in which only a 1.7-kb intergenic region was observed after PCR (table S4). RNA-seq data showed that the inserted region was a transcribed region, showing evidence of splicing and containing three microRNAs but no cytochrome P450 genes.

We assessed the composition of this 6.5-kb insert to elucidate its role by sequencing the full 8.2-kb *CYP6P9a/b* intergenic region and analyzing it using GPMiner, a program for identifying promoter regions and annotating regulatory features (*12*). This insert contained

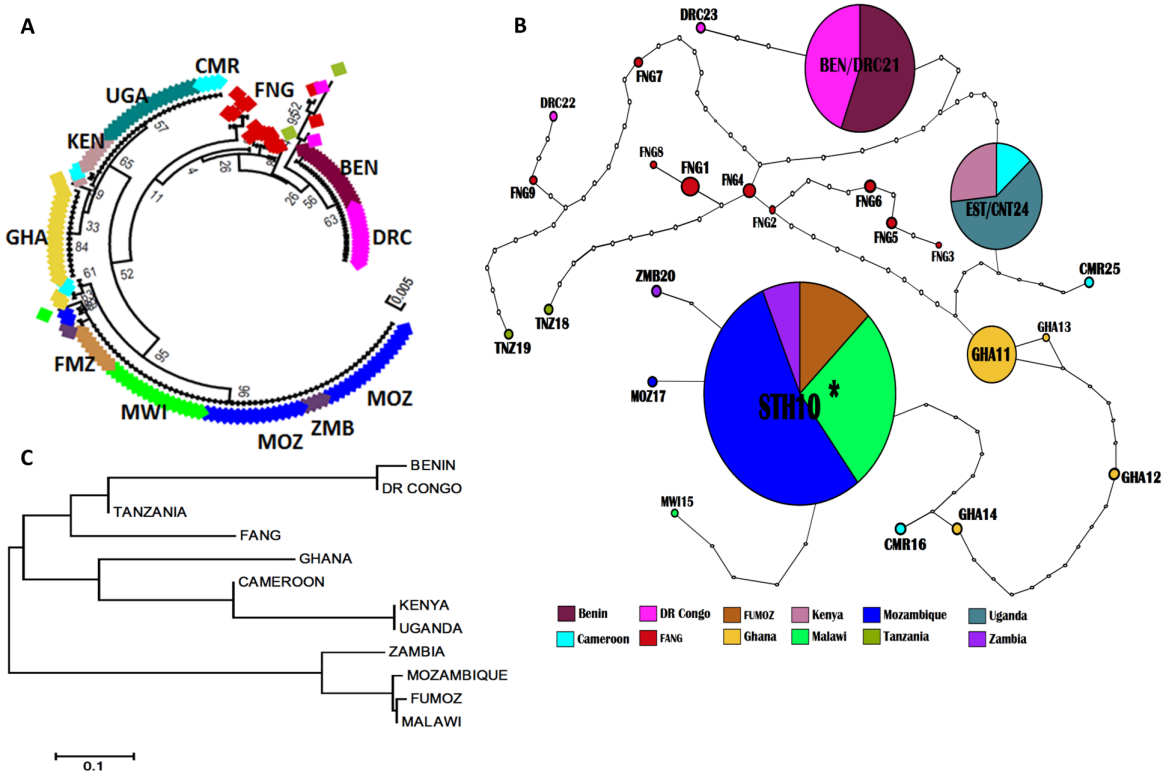

Fig. 3. Genetic diversity of the *CYP6P9a* 5UTR region in mosquitoes from different regions of Africa. (**A**) Molecular phylogenetic analysis of the *CYP6P9a* 5′UTR region in genomes of *A. funestus* mosquitoes collected across Africa, generated by the ML method. The evolutionary history of *CYP6P9a* promoter haplotypes of mosquitoes from across Africa was inferred using the ML method based on the Tamura three-parameter model. (**B**) Africa-wide TCS network for the *CYP6P9a* haplotypes showing four predominant regional haplotypes in southern Africa (STH10), West Africa (Ghana, GHA11), Central Africa (Benin/Democratic Republic of Congo, BEN/DRC21), and East/Central Africa (EST/CNT24). "*" indicates ancestral haplotype. Lines connecting haplotypes and each node represent a single mutation event (respective polymorphic positions are given on each branch). (**C**) Neighbor-joining phylogenetic tree of *CYP6P9a*-based genetic distance between 10 different populations of *A. funestus* mosquitoes from across Africa (N_{ST} estimates). MOZ, Mozambique; ZMB, Zambia; TNZ, Tanzania; DRC, Democratic Republic of Congo; FNG, FANG mosquito strain; FMZ, FUMOZ mosquito strain.

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abundant binding sites for transcription factors including a CpG island (1.3 kb); several GATA, TATA (35), CCAAT (12), and GC (11) boxes; and overrepresented oligonucleotides. It also contained several binding sites for key transcription factors associated with xenobiotic detoxification, including Cap-n-Collar-C (CnCC) (51 sites) and muscle aponeurosis fibromatosis (MafK), suggesting that this insertion may be able to drive *CYP6P9a/b* overexpression. The insertion also contained a microsatellite (FUNR) between 6082 and 6482 bp, which was only 80 bp from the 5′ untranslated region (5′UTR) of *CYP6P9a*. Previous genotyping of this marker in mosquitoes Africa-wide revealed marked differences associated with a pyrethroid resistance profile (*13*). The FUNR microsatellite marker was not present within the 1.7-kb intergenic region between *CYP6P9a* and *CYP6P9b* in the genome of the pyrethroid-susceptible FANG mosquito strain. It has been shown in other insects such as aphids that microsatellite loci are involved in up-regulation of cytochrome P450, conferring insecticide resistance (*14*).

CYP6P9a regulatory region polymorphisms associated with pyrethroid resistance

To detect cis-regulatory mutations controlling *CYP6P9a/b*-based pyrethroid resistance, we compared an 800-bp sequence immediately upstream of *CYP6P9a* in mosquito samples from across Africa. Genomes of mosquitoes from several locations exhibited low or no polymorphisms in this region (fig. S5A). The sample with the highest diversity was the fully susceptible FANG mosquito strain, with diversity indices supporting selection acting on the *CYP6P9a* gene in field populations of mosquitoes resistant to pyrethroids. Despite the low diversity in the 800-bp sequence upstream of *CYP6P9a* observed in genomes of mosquitoes from different regions, mosquito populations from southern Africa consistently exhibited a different polymorphism pattern to those from other regions, including the presence of an AA insertion 8 bp upstream of a putative CCAAT box present only in southern Africa mosquito samples through an A/C substitution (fig. S5A). The AA insertion located 359 bp from the start codon of *CYP6P9a* was tightly associated with other polymorphisms in a haplotype (STH10) that was nearly fixed in the genomes of southern Africa mosquitoes (63 of 68), reflecting the marked selective sweep observed around this gene in this African region. Analysis of the phylogenetic tree revealed four clusters of haplotypes in mosquitoes from different regions: southern Africa (Malawi, Mozambique, and Zambia plus the FUMOZ-R strain), East-Central Africa (Kenya, Uganda, and Cameroon), West Africa (Ghana), and West-Central Africa (Benin and Democratic Republic of Congo) (Fig. 3A). The FANG pyrethroid-susceptible strain formed its own cluster that was divergent from the other clusters.

Closer analysis of the haplotypes using a haplotype network confirmed the presence of four major haplotypes corresponding to these four geographical clusters: STH10 in southern Africa, EST/CNT24 in East-Central Africa, BEN/DRC21 in West-Central Africa, and GHA11 in Ghana only (West Africa) (Fig. 3B). Unexpectedly, the other three geographical regions compared to southern Africa also exhibited predominant haplotypes in the mosquito populations to near fixation, contrary to previous data where they were found to be more polymorphic (*13*). This result suggested that resistance to pyrethroid beyond southern Africa could have been selected through *CYP6P9a* or other genes in the vicinity of the *rp1* QTL region. The neighbor-joining phylogenetic tree showing the genetic distances based on the N_{ST} genetic differentiation index between different countries (Fig. 3C) correlated with the polymorphism patterns showing countries clustering according to the haplotype diversity patterns from the maximum likelihood (ML) tree (Fig. 3A) or the Templeton, Crandall, and Sing (TCS) network (Fig. 3B).

CYP6P9a regulatory region changes selected for by insecticide-treated bednet scale-up

We next assessed whether the differences observed in the 5′UTR and upstream region of the *CYP6P9a* gene in mosquitoes from southern Africa compared to those from other regions were a result of selective pressure from insecticides. We compared mosquito samples from southern Africa before the scale-up of insecticide-based interventions, such as use of long-lasting insecticide-treated bednets, and mosquito samples after scale-up of bednet use. Thirty-nine clones of the 800-bp fragment upstream of *CYP6P9a* were obtained and sequenced from mosquitoes from Mozambique and Malawi before the bednet intervention and were compared to 52 clones obtained after the bednet intervention. Preintervention mosquito samples showed highly polymorphic regions in the 800-bp fragment (table S5 and fig. S5B). Before bednet intervention, there were many segregating sites (61 in Malawi mosquitoes and 25 in Mozambique mosquitoes), many haplotypes (19 of 30 and 12 of 30, respectively), and high nucleotide diversity ($\pi = 0.027$ and 0.012) (table S5 and fig. S5B). By contrast, in the mosquito samples from Malawi and Mozambique obtained after the bednet intervention, there was low diversity as revealed by a number

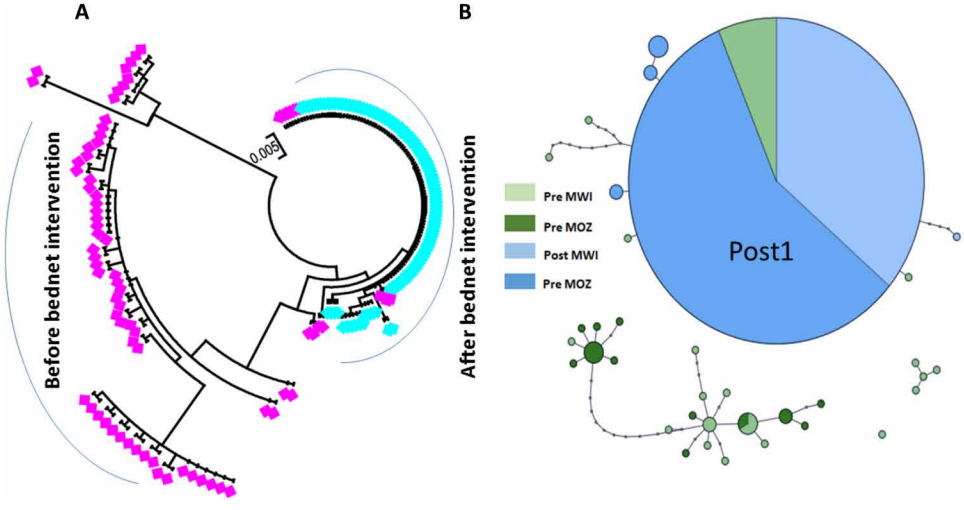

Fig. 4. Impact of bednet usage on the genetic diversity of the *CYP6P9a* promoter in mosquitoes. (**A**) Shown is the ML phylogenetic tree of *CYP6P9a* in *A. funestus* mosquitoes from Malawi collected before and after introduction of pyrethroid-treated bednets. This tree shows a cluster of highly diverse haplotypes (pink) before bednet intervention but a nearly fixed haplotype (blue) after bednet intervention. (**B**) TCS haplotype network in mosquitoes collected in Malawi (MWI) and Mozambique (MOZ) before bednet intervention (Pre) and after bednet intervention (Post). The network reveals a major resistant haplotype after intervention (Post1), but a very diverse set of haplotypes before bednet intervention.

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of polymorphic sites (S) of 4 and 3 and a haplotype number of 2 and 4, respectively, with extremely low nucleotide diversity (π = 0.00066 and 0.0008). These differences were reflected on the ML phylogenetic tree, showing that mosquito samples taken before bednet intervention not only clustered together but also were more diverse and showed several haplotypes (Fig. 4A). In contrast, mosquito samples obtained after bednet intervention showed a markedly reduced haplotype number (Fig. 4A). A haplotype network confirmed that the major haplotype associated with pyrethroid resistance and now nearly fixed in all southern Africa mosquito populations was present in the pre-bednet intervention samples but at a much lower frequency of only 4 of 39 (10.2%) (Fig. 4B). This was compared to a much higher frequency of 44 of 52 (84.6%) in post-bednet intervention samples, with other haplotypes being only one or two mutational steps away from the predominant one (Fig. 4B). The pre-bednet intervention haplotypes were polymorphic and were separated by a number of mutational steps, whereas the post-bednet intervention mosquito samples showed a markedly reduced diversity (Fig. 4B). A detailed analysis of the polymorphisms between pre- and post-bednet intervention mosquito samples revealed that the AA insertion as well as the CCAAT box were now fixed (52 of 52) in all southern Africa mosquito populations after bednet intervention. The CCAAT box was also present in pre-bednet intervention samples but only at a very low frequency (7 of 24 and 0 of 15 for Malawi and Mozambique mosquito populations, respectively). Furthermore, a second binding site for the Nrf2:MafK xenobiotic transcription factor was found only in post-bednet intervention samples and was linked to both the AA insertion and the CCAAT box. These major modifications suggest that scale-up of use of insecticide-treated bednets is likely to have been a major factor driving evolution in *A. funestus* mosquito populations in southern Africa.

A molecular marker associated with overexpression of a pyrethroid resistance gene

Having confirmed that genomic changes upstream of *CYP6P9a* were associated with pyrethroid resistance in mosquitoes, we next searched for the mutations responsible for the overexpression of *CYP6P9a* in pyrethroid-resistant mosquitoes. We used a luciferase assay to assess the role of polymorphisms found in the 800-bp sequence upstream of the translation start site of *CYP6P9a* (including the 5′UTR). The 800-bp sequence upstream of the *CYP6P9a* translation start site in both FUMOZ and FANG mosquito strains was successfully cloned and sequenced. To narrow down the region containing the regulatory motifs, four different-sized fragments (800, 500, 300, and 150 bp) immediately upstream of the translation start codon were cloned upstream of a reporter gene in a pGL3 vector. These constructs were used in luciferase reporter gene assays. The assays demonstrated that although 800-bp insertions from both FUMOZ and FANG mosquito strains drove reporter gene expression, expression driven by the FUMOZ strain insertion was three times higher than that driven by the FANG strain insertion (fig. S6A). This finding supported a role for this region in the differential expression of *CYP6P9a* between pyrethroid-resistant and pyrethroid-susceptible mosquitoes. Progressive deletion of the 800-bp pGL3-FZ-*CYP6P9a* was performed to identify the major regulatory elements driving this differential gene expression. The first deletion from 800 to 500 bp did not affect the activity of the fragment. However, cutting the fragment from 500 to 300 bp (removing the AA insert and the CCAAT box) resulted in a 33% reduction of *CYP6P9a* activity in the FUMOZ strain ($P < 0.001$) (Fig. 5A). Subsequent deletion from 300 to 150 bp, which removed the AA insert, the CCAAT box, and the pyrethroid resistance–specific CnCC/MafK binding site, led to an 89% reduction in *CYP6P9a* activity ($P < 0.001$). This showed that

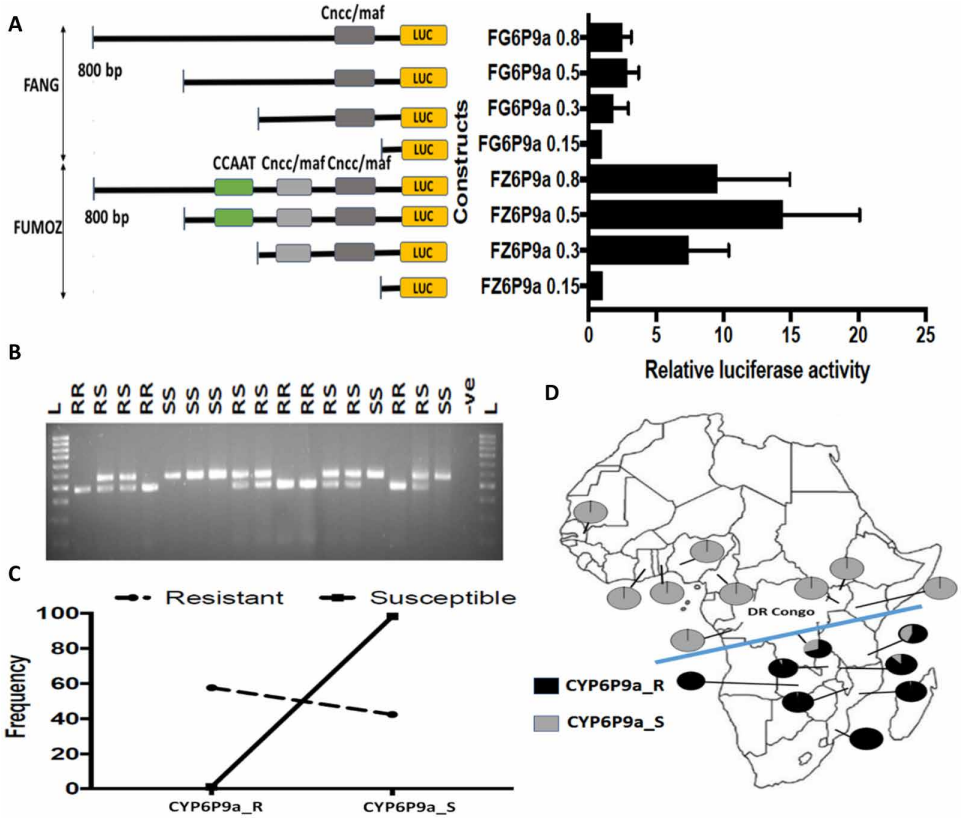

Fig. 5. A DNA-based assay to detect cytochrome P450–mediated metabolic-based pyrethroid resistance in mosquitoes. (**A**) Luciferase promoter assay for detecting the *CYP6P9a* 5′ flanking region. Shown are progressive serial deletions in an 800-bp fragment of the promoter region of *CYP6P9a* enabling detection of the variants causing pyrethroid resistance (mean ± SD; $n = 6$). (**B**) Agarose gel showing *CYP6P9a* fragments from the Taq I PCR-RFLP assay distinguishing the three genotypes of pyrethroid resistance: RR (homozygous resistance), RS (heterozygous resistance), and SS (susceptible). (**C**) Significant correlation ($P < 0.0001$) between the *CYP6P9a* resistance allele (*CYP6P9a*_R) and pyrethroid (permethrin) resistance. (**D**) Africa-wide distribution of the *CYP6P9a* resistance allele (*CYP6P9a*_R) in *A. funestus* mosquitoes showing near fixation in mosquitoes from southern Africa. The blue line represents the geographical limit of the spread of the *CYP6P9a*_R allele in Africa. Circles represent the frequency distribution of alleles in each location.

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both the CCAAT box and the CnCC/MafK binding sites were key regulatory enhancer elements driving the overexpression of *CYP6P9a*.

A DNA-based diagnostic assay to detect *CYP6P9a*-mediated pyrethroid resistance

To design a DNA-based diagnostic assay to detect *CYP6P9a*-mediated pyrethroid resistance, we screened the most active portion (500 bp) for the presence of restriction site polymorphisms that could be used to design a simple restriction fragment length polymorphism PCR (PCR-RFLP) assay. We found a restriction site for the Taq I enzyme (cut site, 5′-TCGA-3′) spanning an A/G mutation located 18 bp upstream of the AA insertion (fig. S6B) and completely linked with the CCAAT box and other regulatory elements on the resistance haplotype. The Taq I enzyme cut the 450-bp fragment from the putative pyrethroid resistance haplotype into two fragments of 350 and 100 bp; the putative pyrethroid-susceptible haplotype remained uncut (Fig. 5B), allowing us to genotype the resistance allele (CYP6P9a_R) in single mosquitoes. To validate the robustness of this PCR-RFLP assay to detect pyrethroid resistance, we used F_8 progeny from a cross between highly resistant (FUMOZ) and highly susceptible (FANG) mosquito strains. The genotyping of 46 mosquitoes that were highly resistant to permethrin (alive after 180 min of exposure) (*15*) revealed 9 RR, 35 RS, and only 2 SS genotypes. By contrast, 42 highly pyrethroid-susceptible mosquitoes (dead after 30 min of permethrin exposure) had 0 RR, 1 RS, and 41 SS genotypes. Therefore, the odds ratio (OR) of the likelihood of surviving exposure to permethrin when homozygous for the resistance allele (RR) of the *CYP6P9a* gene (with the CCAAT box and CnCC/MafK binding sites) increased to 922 ($P < 0.0001$) compared to mosquitoes homozygous for the susceptible allele (SS) (Fig. 5C), thus demonstrating the reliability of this DNA-based diagnostic assay.

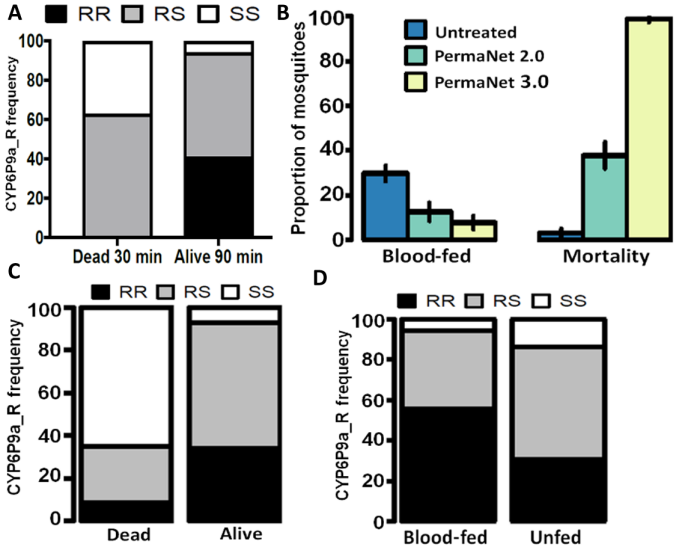

Fig. 6. Impact of *CYP6P9a*-mediated metabolic-based pyrethroid resistance on the efficacy of insecticide-treated bednets. (**A**) *CYP6P9a* genotypes correlate with pyrethroid resistance in the hybrid mosquito strain FUMOZ (pyrethroid resistant)/FANG (pyrethroid susceptible). This suggests that the FANG/FUMOZ hybrid mosquito strain can be used to assess the impact of *CYP6P9a*-mediated pyrethroid resistance on bednet efficacy. Mosquitoes were exposed to insecticide-treated bednets for 30 or 90 min to define pyrethroid-resistant and susceptible individuals. (**B**) Shown are blood feeding and mortality rates (mean ± SD; $n = 4$) for the FANG/FUMOZ hybrid mosquito strain after release-recapture studies in experimental field huts in Cameroon. Huts had untreated control bednets (blue), PermaNet 2.0 bednets (green), or PermaNet 3.0 bednets (yellow). (**C**) Shown is the *CYP6P9a* genotype (RR, RS, and SS) frequency in dead and alive mosquitoes after exposure to PermaNet 2.0 bednets. The *CYP6P9a*_R allele was associated with the ability of mosquitoes to survive exposure to pyrethroid-treated PermaNet 2.0 bednets ($P < 0.0001$). (**D**) Shown is the *CYP6P9a* genotype (RR, RS, and SS) frequency in mosquitoes that did or did not take a blood meal after exposure to PBO-treated PermaNet 3.0 bednets. The *CYP6P9a*_R allele was associated with the ability of pyrethroid-resistant mosquitoes to take a blood meal from individuals lying under PBO-treated PermaNet 3.0 bednets.

Geographical distribution of the *CYP6P9a* resistance allele across Africa

Genotyping of the CYP6P9a_R allele across Africa revealed that it was nearly fixed in mosquitoes from southern Africa and was present at an intermediate frequency (55.7%) in mosquitoes from Tanzania (East Africa) (Fig. 5D). However, CYP6P9a_R was absent from mosquitoes from Central/West Africa (Fig. 5D and fig. S6C). In the Democratic Republic of Congo, a geographical contrast was observed with the CYP6P9a_R allele present in mosquitoes from the eastern part of the country but absent from mosquitoes from the western part of the country including the capital Kinshasa (Fig. 5D). This pattern suggested a new allele/haplotype that arose in mosquitoes in southern Africa and that spread northward. Regional differences in CYP6P9a_R distribution were similar to those reported for previous markers (*7*, *11*).

Impact of *CYP6P9a*-mediated pyrethroid resistance on bednet efficacy in an experimental field hut trial

To assess the impact of the *CYP6P9a*-R haplotype on the effectiveness of long-lasting insecticide-treated bednets, we opted to use lab strains as this mutation is nearly fixed in the field in southern Africa. We crossed the highly resistant laboratory strain FUMOZ-R (where *CYP6P9a*_R is fixed) with the fully susceptible laboratory strain FANG (where *CYP6P9a*_R is completely absent). Using reciprocal crosses between the two strains, we generated a hybrid strain at the F_4 generation that we used for semi-field studies in experimental huts.

The bioassays performed with the reciprocal FANG/FUMOZ strains revealed that both hybrid strains were resistant to pyrethroids and carbamates and moderately resistant to DDT (93% mortality) (fig. S7A). As expected, resistance was lower than in the fully resistant strain FUMOZ_R, with a mortality rate of 76.1 to 80.7% when exposed to permethrin. However, a difference was observed for deltamethrin with a higher mortality rate recorded for the strain generated from crossing female FUMOZ_R to male FANG (48.5%) than in the strain from female FANG and male FUMOZ_R (77.3%). This difference could indicate the role of some candidate genes in the X chromosome for deltamethrin resistance (*CYP9K1*, for instance). The resistance pattern was similar for the carbamate bendiocarb in both reciprocal strains.

Before any field studies were conducted with the hybrid FANG/FUMOZ strain, the role of the *CYP6P9a*_R allele in observed pyrethroid resistance was confirmed. World Health Organization (WHO) bioassays showed a mortality of 39.0 and 42.3% after 30 min of exposure and mortality rates of 81.3 and 86.3% after 90 min of exposure, respectively, to permethrin and deltamethrin (Fig. 6A and fig. S7B). The OR of surviving exposure to permethrin when homozygous for

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Table 1. Experimental field hut study with the FANG/FUMOZ mosquito strain. For each comparison, estimates not sharing the same symbols (§ or ‡) are statistically different at $P < 0.05$. ns, not significant.

	Untreated nets	PermaNet 2.0	PermaNet 3.0
Total mosquitoes	356	270	322
% Exophily (CI)	11.8 (8.45–15.15)	16.7 (12.2–21.1), ns	15.8 (11.85–19.8), ns
% Blood feeding (CI)	29.5 (24.7–34.2)	14.8 (10.6–19.05)§	6.8 (4.1–9.6)§‡
% Blood feeding inhibition	–	49.8§	76.84§‡
% Personal protection (total blood-fed)	– (105)	61.9 (40)§	79.04 (22)§‡
% Mortality after blood feeding (no. dead)	1 (1)	40.0 (16)§	95.5 (21)§‡
% Mortality corrected (CI)		33.3 (27.7–38.9)§	98.7 (97.5–99.9)§‡

the resistant *CYP6P9a*_R allele (RR) was high at 693 [confidence interval (CI), 88 to 5421; $P < 0.0001$] compared to the homozygous susceptible allele (SS) (fig. S7, C and D). The OR was 131 (CI, 27 to 978; $P < 0.0001$) when comparing RR to RS, indicating that the resistance conferred by *CYP6P9a* was additive.

Females from the F_4 generation of the FANG/FUMOZ hybrid strain were used in a release-recapture experiment in huts in Cameroon with PermaNet 2.0 bednets treated with deltamethrin or PermaNet 3.0 bednets treated with deltamethrin and piperonyl butoxide (PBO), a cytochrome P450 inhibitor, or control nets that were untreated. After four consecutive nights of mosquito release and then recapture, analysis of the mosquitoes collected showed no induced exophily (mosquitoes exiting the room) with either PermaNet 2.0 or PermaNet 3.0 bednets compared to the control ($P > 0.05$) (Table 1). The percentage of females that had taken a blood meal was lower for both pyrethroid-treated bednets compared to untreated control bednets. PermaNet 3.0 bednets showed a lower number of female mosquitoes taking a blood meal than did PermaNet 2.0 bednets ($P < 0.001$) (Fig. 6B). This was reflected in the percentage inhibition of blood feeding, which was higher for PermaNet 3.0 (76.8%) than for PermaNet 2.0 (49.8%) bednets ($P < 0.0001$). Treated bednets provided greater personal protection from blood feeding than did the untreated bednets, with greater protection from PermaNet 3.0 (79.0%) than from PermaNet 2.0 (61.9%) bednets (Table 1). Analysis of mosquito mortality rates revealed higher mortality of the hybrid FANG/FUMOZ strain exposed to PermaNet 3.0 (98.7%) compared to PermaNet 2.0 (33.3%) bednets ($P < 0.001$) (Fig. 6B); low mortality was observed for mosquitoes exposed to control untreated bednets (6.2%). Mosquitoes that had taken a blood meal through the bednets showed a higher mortality rate after exposure to PermaNet 3.0 (95.5%) compared to PermaNet 2.0 (40%) bednets (Table 1).

The role of *CYP6P9a* in the loss of PermaNet 2.0 bednet efficacy

Genotyping of the *CYP6P9a* marker allowed us to assess the impact of cytochrome P450–based resistance in mosquitoes on the loss of efficacy of the pyrethroid-only treated PermaNet 2.0 bednet. Most of the mosquitoes released in the huts containing PermaNet 3.0 bednets died because of PBO inhibition of cytochrome P450s including *CYP6P9a*. Therefore, the impact of the *CYP6P9a* genotypes on the ability of mosquitoes to survive exposure to long-lasting insecticide-treated bednets was studied using the pyrethroid-only PermaNet 2.0 bednet. To avoid confounding effects from blood feeding, bednet entry, or exophily status, the distribution of the *CYP6P9a* genotypes was assessed first only among unfed mosquitoes collected in the huts. We observed significant differences in the frequency of the three genotypes between dead and alive mosquitoes ($\chi^2 = 375$; $P < 0.0001$) (Fig. 6B). Analysis of the correlation between each genotype and mortality revealed that *CYP6P9a* homozygous resistant mosquitoes (RR) were better able to survive exposure to PermaNet 2.0 bednets than were homozygous susceptible mosquitoes (SS) (OR, 34.9; CI, 15.8 to 77.1; $P < 0.0001$) (table S6). Similarly, possessing the heterozygous (RS) *CYP6P9a* genotype also conferred a significant survival advantage compared to the homozygous SS genotype (OR, 19.9; CI, 9.7 to 40.9; $P < 0.0001$). Although a higher frequency of alive mosquitoes with the RR genotype was observed compared to the RS genotype, this difference was not significant (OR, 1.75; CI, 0.82 to 3.7; $P = 0.26$). Overall, a single *CYP6P9a* resistance allele (R) conferred a greater ability to survive than did a susceptible allele (OR, 6.25; CI, 3.3 to 11.7; $P < 0.0001$) (Fig. 6C). The same association between *CYP6P9a*_R and the ability to survive exposure to PermaNet 2.0 bednets was observed when analyzing all collected samples although with lower ORs (e.g., for RR versus SS; OR, 10.8; CI, 5.6 to 20.8; $P < 0.0001$) (table S6). The impact of the *CYP6P9a* resistance gene on the ability of mosquitoes to blood feed was also assessed. We found that the distribution of the three genotypes was significantly different among blood-feeding and unfed mosquitoes for both PermaNet 2.0 ($\chi^2 = 16.9$; $P < 0.0001$) and PermaNet 3.0 bednets ($\chi^2 = 30.5$; $P < 0.0001$) (Fig. 6D). Homozygous RR mosquitoes were significantly more likely to blood feed when exposed to PermaNet 3.0 bednets than either susceptible SS mosquitoes (OR, 4.54; $P < 0.0001$) or heterozygous RS mosquitoes (OR, 2.6; $P = 0.0012$) (Fig. 6D). A similar trend was observed for PermaNet 2.0 bednets, although this was not significant (fig. S7, E and F).

DISCUSSION

This study investigated the genetic basis of metabolic resistance to pyrethroids in the mosquito *A. funestus*, a major malaria vector in Africa. We detected a DNA-based resistance marker for cytochrome P450–mediated metabolic resistance to pyrethroids and designed a field-applicable diagnostic assay to detect and track the spread of insecticide resistance across Africa. We established a direct impact of metabolic-mediated pyrethroid resistance on the efficacy of insecticide-treated bednets in an experimental field hut trial. Gene expression analyses underlined the importance of cytochrome P450 monooxygenases for pyrethroid resistance in the *A. funestus* vector of malaria in Africa, as has been previously reported for other *A. funestus* populations (*9*, *15*) and other mosquito species (*16*–*18*). Important heterogeneities in gene expression were observed among mosquito populations from different geographical regions of Africa.

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A cluster of *CYP6* genes in the *rp1* pyrethroid resistance QTL showed the greatest differences in expression in mosquitoes from different locations. The southern Africa mosquitoes showed marked overexpression of *CYP6P9a* and *CYP6P9b*, whereas West African mosquitoes from Ghana showed overexpression of *CYP6P5* and *CYP6P4a/b*. Such differences showed that the molecular basis of pyrethroid resistance in mosquitoes varied across the African continent, as previously suggested for this species (*19*) and for other vectors of malaria such as *A. gambiae* (*8*, *20*), possibly due to a combination of variation in selective pressures and restricted gene flow among regions (*13*, *21*).

The association of the *rp1* QTL locus with pyrethroid resistance was further supported by the detection of several adaptive evolutionary features across this locus including signatures of selective sweep, large structural variations in the 6.5-kb insertion, and cis-regulatory polymorphisms. The selective sweep detected in the *rp1* QTL locus in mosquitoes from southern Africa coincided with increased expression of the duplicated *CYP6P9a* and *CYP6P9b* genes encoding cytochrome P450s in mosquitoes from Malawi. This supported a key role for these genes in pyrethroid resistance (*9*). However, the presence of a predominant haplotype for the *CYP6P9a* 5′UTR in the other three African regions suggests that resistance to pyrethroid beyond southern Africa could have been selected through *CYP6P9a* or other genes in the vicinity of the *rp1* QTL region. The hypothesis of a possible hitchhiking effect here rather than the direct involvement of *CYP6P9a* was supported by the important differences observed between the four major haplotypes with more than 20 mutational steps of difference between them. Therefore, it is likely that resistance conferred by the *rp1* locus occurred through independent selective events with different genes. This is supported by RNA-seq data showing that although *rp1* genes are overexpressed in mosquitoes Africa-wide, the main genes are different. *CYP6P4a* is predominant in mosquitoes from Ghana but not in those from other geographical regions; *CYP6P5* is predominant in mosquitoes from Cameroon and Uganda, whereas *CYP6P9a* is the major gene overexpressed in mosquitoes from southern Africa. Selective sweeps associated with insecticide resistance have been reported recently in *A. gambiae* mosquitoes from across Africa (*22*).

The cis-regulatory changes that we detected in *CYP6P9a* included binding sites for transcription factors such as CnCC and MafK, which have recently been shown to be involved in insecticide resistance in *A. gambiae* (*23*) and other insects (*24*). This suggested that cis-regulatory modifications may be important drivers of resistance to insecticides. However, because cis-regulatory elements and enhancers are able to drive expression of genes from distant locations and can be upstream or downstream of these genes, future studies will need to establish the role of the 6.5-kb insertion in the overexpression of *CYP6P9a* and *CYP6P9b*. The strong association of these regulatory variations with pyrethroid resistance enabled us to design a DNA-based diagnostic tool for detecting metabolic-mediated pyrethroid resistance in mosquitoes, which should facilitate the detection and management of this major resistance mechanism in the field. This molecular assay for cytochrome P450–based metabolic resistance to pyrethroids in a malaria vector comes two decades after a DNA-based diagnostic test was designed for target-site resistance (*kdr*) (*4*) and should facilitate the study of the impact of metabolic resistance on pyrethroid resistance and malaria control interventions. Although SNPs associated with pyrethroid resistance have been detected in the dengue vector *Aedes aegypti*, no markers have been reported (*25*). Another DNA-based diagnostic test has been designed for the glutathione *S*-transferase gene (*GSTe2*) in *A. funestus* to demonstrate resistance to both pyrethroids and DDT in West/Central Africa, but it used a single amino acid change (L119F) in the coding region of the gene and not the putative causative variant regulating gene expression as done here for *CYP6P9a* (*11*).

The CYP6P9a_R allele is present principally in mosquitoes from southern Africa where it is nearly fixed in the mosquito population, but it is completely absent from mosquitoes from other regions. This difference among African populations of *A. funestus* has previously been observed, most notably for the distribution of other resistance markers such as the *L119F-GSTe2* (*11*) and the *A296S-RDL* alleles conferring resistance to the insecticide dieldrin (*26*), which are present in mosquitoes from West/Central and East Africa but are completely absent from those in southern Africa. In contrast, the *N485I-ace-1* carbamate resistance allele is present only in mosquitoes from southern Africa (*7*). Furthermore, patterns of F_{ST}-based genetic differentiation among populations of this species indicate a restriction of gene flow and increased genetic divergence in mosquitoes from southern Africa compared to those from other regions (*13*, *21*). However, there seems to be a gradual increase of CYP6P9a_R frequency from south to north in the southern Africa region as seen in Malawi, with 98% in the south (Chikwawa), 90% in the center (Salima), and 78% in the north of the country (Fulirwa) (fig. S6C). This correlates with previous observations that *CYP6P9a* overexpression was lower in the north of southern Africa and that the spread of pyrethroid resistance likely originated from far south and is spreading northward (*27*). It will be important to monitor the spread of such alleles across the African continent as there is the risk that super-resistant mosquitoes could be generated if, for instance, *CYP6P9a*-mediated pyrethroid resistance becomes combined with the *GSTe2*-based DDT resistance seen in West/Central Africa. The Democratic Republic of Congo will be particularly important to monitor as both insecticide resistance mechanisms are present in this country (*28*).

Using a new DNA-based diagnostic CYP6P9a_R assay, we assessed the direct impact of metabolic-mediated pyrethroid resistance in mosquito vectors of malaria on the efficacy of insecticide-treated bednets. We showed that cytochrome P450–mediated pyrethroid resistance reduced the efficacy of insecticide-treated bednets. Our findings help to clarify the debate about whether pyrethroid resistance directly affects the efficacy of insecticide-treated bednets (*29*). Bednets containing the insecticide synergist PBO (such as PermaNet 3.0 bednets in this study), which inhibits the activity of cytochrome P450 enzymes, provided better efficacy than did bednets treated with pyrethroid alone (PermaNet 2.0 bednets). Despite the high mosquito mortality observed here with the PBO-treated PermaNet 3.0 bednets, the CYP6P9_R allele resulted in pyrethroid-resistant mosquitoes being more likely than susceptible mosquitoes to bite individuals under the bednet and potentially transmit malaria. This suggests that efforts to eliminate malaria in Africa will be impeded unless the overreliance on pyrethroid-treated bednets to control transmission is addressed.

There are several limitations to our study. It is important to highlight that the DNA-based assay designed in this study detects pyrethroid resistance principally in mosquitoes collected in southern Africa and only applies to *A. funestus*. Therefore, further studies are needed to detect similar markers in mosquitoes from other regions of Africa and in other mosquito species such as *A. gambiae* s.l. to track cytochrome P450–mediated resistance to pyrethroids across

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Africa. Furthermore, even though *CYP6P9a* exhibited a strong correlation with the pyrethroid resistance phenotype, it might not be the only gene involved and further studies should be performed to establish the contribution of other genes, notably *CYP6P9b*. Last, the impact of metabolic-mediated resistance has been assessed in this study in a hybrid mosquito lab strain, which might not be identical to mosquitoes in the field; thus, future studies will need to use field populations of mosquitoes.

MATERIALS AND METHODS

Study design

The objectives of this study were to detect key genetic variants conferring metabolic-mediated pyrethroid resistance on the mosquito *A. funestus*, a major vector of malaria in Africa, and to design a simple DNA-based assay to monitor such resistance in field populations of mosquitoes from different African regions. Transcriptome profiling of *A. funestus* populations from four African regions—southern (Malawi), East (Uganda), West (Ghana), and Central (Cameroon)—was analyzed to detect key candidate resistance genes. Because metabolic-based pyrethroid resistance could also be conferred by point mutations in coding and cis-/trans-regulatory regions, we performed comparative whole-genome sequencing of pyrethroid (permethrin)–resistant and –susceptible mosquitoes collected in the field to screen for genomic resistance regions and polymorphisms. To comprehensively detect resistance loci, we also scanned the whole genome for pyrethroid resistance–related signatures of selective sweeps in mosquitoes from southern Africa. Because the *rp1* QTL was consistently associated with pyrethroid resistance, a fine-scale analysis of this locus was performed to detect potential structural variants associated with resistance such as indels and copy number variations. To detect cis-regulatory mutations potentially controlling *CYP6P9a/b*-mediated pyrethroid resistance, we compared an 800-bp sequence immediately upstream of *CYP6P9a* in resistant and susceptible mosquitoes. The role of insecticide-based interventions in the selection of *CYP6P9a* cis-regulatory changes was assessed by sequencing the genomes of mosquitoes collected from before and after the introduction of widespread insecticide-treated bednet usage. To establish the specific mutations controlling *CYP6P9a* overexpression, a comparative luciferase assay based on pyrethroid-resistant and pyrethroid-susceptible *CYP6P9a* promoter sequences was performed. To design a DNA-based assay to detect *CYP6P9a*-mediated pyrethroid resistance, we screened the *CYP6P9a* promoter for restriction sites and used this information in a PCR-RFLP assay to assess pyrethroid resistance in mosquitoes collected from across Africa.

Last, we assessed the impact of *CYP6P9a*-mediated metabolic-based pyrethroid resistance on the effectiveness of long-lasting insecticide-treated bednets using experimental field huts in Cameroon, Central Africa. The following three bednet treatments were compared in the experimental huts: untreated control polyethylene bednets, PermaNet 2.0-treated polyethylene bednets (deltamethrin), and PermaNet 3.0-treated polyethylene bednets (PBO + deltamethrin). To simulate a worn bednet, six holes (4 cm by 4 cm) were made in each bednet, according to WHO guidelines (*30*). The hybrid FANG/FUMOZ mosquito strain was released in each hut for six nights (80 mosquitoes per hut). Three adult volunteers were recruited from the Mibellon village in Cameroon to sleep under the bednets and to collect mosquitoes in the morning. They were provided with a written consent form and given chemoprophylaxis during the trial to prevent malaria. Ethical approval was obtained from the National Ethics Committee of the Ministry of Health in Cameroon.

Collection and rearing of mosquitoes

Two *A. funestus* laboratory colonies were used in the study. The FANG colony is a fully insecticide-susceptible colony derived from Angola (*31*). The FUMOZ colony is a multi-insecticide–resistant colony derived from southern Mozambique. Mosquitoes were collected from four primary locations across the continental range of *A. funestus*. Mosquitoes were collected in March 2014 from Obuasi (5°56′N, 1°37′W) in Ghana (*32*), in February 2015 from Mibellon (6°46′N, 11°70′E) in Cameroon, in March 2014 from Tororo (0°45′N, 34°5′E) in Uganda (*33*), and in January 2014 from Chikwawa (16°1′S, 34°47′E) in southern Malawi (*34*).

Collected mosquitoes were kept until fully gravid and forced to lay eggs using the forced-egg laying method (*35*). All F_0 females that laid eggs were morphologically identified as belonging to the *A. funestus* group according to a morphological key (*36*). Parents (F_0) and egg batches were transported to the Liverpool School of Tropical Medicine under a Department for Environment, Food and Rural Affairs (DEFRA) license (PATH/125/2012). Eggs were allowed to hatch in cups and mosquitoes were reared to adulthood in the insectaries under conditions described previously (*35*). Insecticide resistance bioassays on these samples have been previously described (*32*–*34*).

Transcription profiling of pyrethroid resistance using RNA-seq

Total RNA was extracted from pools of 10 female mosquitoes (alive after 1 hour of permethrin exposure) using the Arcturus PicoPure RNA Isolation Kit (Life Technologies), according to the manufacturer's instructions (Supplementary Materials and Methods). Pools of libraries were sequenced, eight per lane of the HiSeq 2500 (Illumina, San Diego, CA, USA) at 2 × 125-bp paired-end sequencing. Sequence library preparation and sequencing were done at the Centre for Genomic Research (CGR), University of Liverpool. RNA-seq data were analyzed as described previously (*37*). This involved an initial processing, quality assessment of sequences, and alignment to the reference sequence using the AfunF1.4 annotation. Differential gene expression analysis was performed using edgeR and the Strand NGS program (Strand Life Sciences, version 3.0).

Whole-genome sequencing of mosquito samples from southern Africa

Genomic DNA was extracted from whole mosquitoes from F_0 Malawi samples (2002 and 2014), the pyrethroid-resistant FUMOZ-R laboratory strain, and the fully susceptible FANG strain using the DNeasy kit (Qiagen, Hilden, Germany). For each sample, genomic DNA was extracted from individuals and pooled in equal amounts to form pools of DNA. These were sequenced on an Illumina HiSeq 2500 (2 × 150 bp, paired-end). Initial processing and quality assessment of the sequence data were performed as for RNA-seq data. Alignment of POOLseq R1/R2 read pairs and R0 reads to the reference sequence (the same as that used for RNA-seq alignment) as well as variant calling were performed as described previously (*13*).

Analysis of polymorphisms in the *CYP6P9a* promoter region

To detect potential causative mutations conferring pyrethroid resistance on *A. funestus*, the polymorphism of the cis-regulatory region of the pyrethroid resistance gene *CYP6P9a* was analyzed.

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Detection of the causative mutations driving up-regulation of CYP6P9a

An 800-bp region upstream of the start codon of *CYP6P9a* was amplified and directly sequenced in 15 field-collected mosquitoes each from 10 countries across different regions of Africa including southern (Mozambique, Malawi, and Zambia), East (Uganda, Kenya, and Tanzania), Central (Democratic Republic of Congo and Cameroon), and West (Benin and Ghana). Primers are listed in table S7. Amplification and purification of PCR products were performed as previously described (*13*). Sequences were aligned using ClustalW (*38*), whereas haplotype reconstruction and polymorphism analysis were done using DnaSP version 5.10 (*39*), MEGA (Molecular Evolutionary Genetics Analysis) (*40*), and TCS (*41*).

Investigation of the content of the CYP6P9a and CYP6P9b intergenic region

The entire 8.2-kb intergenic region between both genes was amplified for the FUMOZ and FANG strains in two to three fragments using primers listed in table S7. PCR was performed using the Phusion polymerase following the manufacturer's instructions. PCR products were purified and cloned into pJET1.2 plasmid.

Assessing the impact of insecticide-treated bednet intervention scale-up on changes in polymorphisms of the CYP6P9a promoter region

The same 800-bp region upstream of the *CYP6P9a* was amplified in mosquitoes collected before (pre-intervention) the scale-up of insecticide-treated nets and also after the scale-up (post-intervention) in Malawi (2002 and 2014) and Mozambique (2000 and 2016). The PCR products were cloned and sequenced, and sequencing data were analyzed as described above.

Genotyping of the *CYP6P9a* resistance allele using a PCR-RFLP assay

A restriction site (5′-TCGA-3′) for the Taq I enzyme at the A/G mutation located 18 bp of the AA insertion and completely tight with the CCAAT box on the resistance haplotype was used to design a PCR-RFLP assay to genotype the CYP6P9a_R allele. The RFLP6P9aF forward primer, 5′-TCCCGAAATACAGCCTTTCAG-3′, and the RFLP6P9aR reverse primer, 5′-ATTGGTGCCATCGCTAGAAG-3′, were used to amplify a partial *CYP6P9a* upstream region containing the restriction site. Ten microliters of the digestion mix made of 1 μl of CutSmart buffer, 0.2 μl of 2 U of Taq I restriction enzyme (New England Biolabs, Ipswich, MA, USA), 5 μl of PCR product, and 3.8 μl of dH_2O was incubated at 65°C for 2 hours. Restriction digest was separated on 2.0% agarose gel (Fig. 3D).

Validation of the DNA-based diagnostic test

To validate the robustness of the PCR-RFLP to detect the pyrethroid resistance in field population, F_8 progeny from a cross between highly resistant (FUMOZ) and highly susceptible (FANG) strains previously used for QTL mapping (*15*) were genotyped, and correlation with resistance phenotype was established using OR.

Geographical distribution of the resistance *CYP6P9a* allele across Africa

The geographical distribution of the resistant *CYP6P9a* allele across Africa was established by genotyping the CYP6P9a_R in 30 to 50 field-collected females of *A. funestus* from several countries in Africa using the PCR-RFLP.

Luciferase reporter assay for the *CYP6P9a* core promoter region

The region immediately 5′ of *CYP6P9a* from both FUMOZ and FANG strains was amplified using primers 6P9a1F and 6P9R a/b. These primers gave an 817-bp product for both FUMOZ and FANG, which were cloned into pJET1.2 (Thermo Fisher Scientific) and sequenced. Primers were designed (table S7) to obtain constructs of progressive serial 5′ deletions of the *CYP6P9a* promoter of 800, 500, 300, and 150 bp for the different primers. The primers incorporated either the Sac I or Mlu I (for the FUMOZ) and Kpn I or Hind III (for the FANG) to facilitate cloning in the pGL3 basic vector. Products were amplified with Phusion polymerase (Thermo Fisher Scientific) and cloned into pJET 1.2. The product was then excised from pJET1.2, ligated to pGL 3 basic (Promega), and sequenced. Plasmids were then extracted using Midiprep kit (Qiagen) to obtain high concentrations for the transfection. Dual luciferase assay was undertaken using *A. gambiae* 4a-2 cell line (MRA-917 MR4; American Type Culture Collection, Manassas, VA). Approximately 5×10^5 cells (600 μl) were subcultured from a T75 culture and seeded in each well of a 24-well plate 1 day before transfection and allowed to reach 60 to 70% confluency.

Transfection of the construct was carried out using the Qiagen Effectene transfection reagent, and the promoter activity was measured using the Dual Luciferase Reporter Assay (Promega, Madison, WI, USA). Reporter constructs (600 ng; *CYP6P9a* upstream sequences in pGL3-Basic), pGL3 without insert, and LRIM promoter in pGL3 basic were cotransfected with 1 ng of actin-*Renilla* internal control in 60 ml of DNA condensation buffer, 4.8 ml of enhancer, and 6 ml of Effectene in triplicate. After 48 hours of incubation at 25°C, the cells were washed with phosphate-buffered saline and harvested in 100 ml of passive lysis buffer (Promega), and luciferase activity was measured on a luminometer (EG & G Berthold, Wildbad, Germany). *Renilla* luciferase activity was used to normalize the construct luciferase activity. The values obtained after measuring the firefly (LAR II) luciferase activity, which represent the activity of the promoter, were divided by the corresponding *Renilla* luciferase activity values and the ratio used to compare different promoters.

Evaluating the impact of *CYP6P9a*-based pyrethroid resistance on the efficacy of insecticide-treated bednets using experimental field huts

Study area and field hut description

The study was performed in Mibellon (6°4′60″N and 11°30′0″E), a village in the Adamawa region of Cameroon where we recently built 12 experimental huts of concrete bricks, following the specific design for experimental huts from the West Africa region (*42*).

Mosquito strains

The study was carried out with a hybrid strain generated from reciprocal crossing between the highly pyrethroid-resistant strain FUMOZ-R (CYP6P9a_R) and the fully susceptible FANG strain (CYP6P9a_S) (*43*). After the initial F_1 generation obtained from the reciprocal crosses of 50 males and 50 females of both strains, the hybrid strain was reared to F_5 and F_6 generations, which were used for the release in the huts.

Susceptibility of the hybrid FANG/FUMOZ mosquito strain

WHO bioassays were carried out to assess the susceptibility profile of the two reciprocal hybrid strains for the pyrethroids (0.75% permethrin and 0.05% deltamethrin), DDT (4%), and the carbamate bendiocarb (0.1%). The bioassays were performed according to WHO protocol (*42*).

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Mosquito collection in field huts

Early in the morning, mosquitoes were collected using glass tubes from the room (the floor, walls, and roof of the hut), inside the bednet, and from the exit traps on the veranda. Each compartment of the hut had its own bag to avoid mixture between samples. Surviving mosquitoes were provided with sugar solution and held for 24 hours in paper cups after which delayed mortality was assessed. Samples were recorded in observation sheets as dead/blood-fed, alive/blood-fed, dead/unfed, and alive/unfed.

The effect of each treatment was expressed relative to the control (untreated bednets) by assessing induced exophily (the proportion of mosquitoes that exited early through the exit traps because of the treatment); the mortality rate, an indicator of the potential killing effect of the insecticide-treated bednets; and the rate of blood feeding, an indicator of insecticide resistance and personal protection.

Genotyping of the CYP6P9a metabolic resistance marker

To establish the impact of the *CYP6P9a*-mediated metabolic resistance to pyrethroids on the effectiveness of the insecticide-treated nets, the PCR-RFLP diagnostic assay was used to genotype a subset of each treatment including the dead, alive, blood-fed, and unfed mosquitoes on the veranda, in the net, and in the room.

Statistical analysis

Genes that were differentially expressed in each country and between different countries (generated from Venn diagrams) were detected using DESeq normalization with a fold change of >2. A multiple test correction was then performed using the method of Benjamini and Hochberg, at a false discovery rate of 5% (adjusted $P < 0.05$). Statistical significance of the luciferase assays was assessed after an unpaired Student's *t* test with respective replicates between the FUMOZ and FANG mosquito strains. Correlation between the *CYP6P9a*_R allele and the pyrethroid resistance phenotype was established using OR and Fisher's exact test. The effect of pyrethroid-treated bednets was established by direct comparison to untreated control bednets. The statistical significance of the difference was estimated by a logistic regression model using Wald statistic that follows a χ^2 distribution (with df = 1). OR and Fisher's exact test were used to assess the impact of *CYP6P9a*_R on the ability of mosquitoes to survive and blood feed after exposure to insecticide-treated bednets.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. Differential gene expression among four permethrin-exposed mosquito populations in Africa and the FANG strain.
Fig. S2. GO enrichment of up-regulated genes using BLAST2GO.
Fig. S3. qRT-PCR validation of the expression profile of the main detoxification genes differentially expressed between resistant and susceptible mosquito samples using RNA-seq.
Fig. S4. Insertion of a 6.5-kb intergenic fragment between *CYP6P9a* and *CYP6P9b* in mosquitoes from southern Africa.
Fig. S5. Genetic diversity patterns of an 800-bp cis-regulatory genomic fragment of *CYP6P9a* before and after scale-up of insecticide-treated bednet use.
Fig. S6. Design of a DNA-based diagnostic assay to detect and track pyrethroid resistance in mosquitoes across Africa.
Fig. S7. Impact of *CYP6P9a*-mediated metabolic-based pyrethroid resistance on the efficacy of insecticide-treated bednets using experimental field huts.
Table S1. Descriptive statistics of RNA-seq sequence read data and alignments for different samples.
Table S2. Detoxification-associated genes differentially expressed among four pyrethroid-resistant mosquito populations and the FANG susceptible mosquito strain.
Table S3. Descriptive statistics of whole-genome POOLseq sequence read data.
Table S4. Counts of reads aligned at the left and right breakpoints of the 6.5-kb insertion supporting different haplotypes.
Table S5. Population genetic parameters of the 800-bp fragment upstream of *CYP6P9a*.
Table S6. Correlation between *CYP6P9a* genotypes and mosquito mortality (PermaNet 2.0 bednets) and blood feeding after the experimental field hut trial with the FANG/FUMOZ mosquito strain.
Table S7. Primers used for characterization of the *CYP6P9a* promoter.
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A cytochrome P450 allele confers pyrethroid resistance on a major African malaria vector, reducing insecticide-treated bednet efficacy

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Tracking insecticide resistance

Malaria prevention relies extensively on mosquito control using insecticide-treated bednets. However, insecticide resistance in mosquito vectors of malaria threatens control of this disease. In a new study, Weedall *et al.* detected the major genes conferring insecticide resistance on the *Anopheles* mosquito vector of malaria in Africa. They found a DNA marker in a gene (cytochrome P450) encoding an enzyme that breaks down the insecticides used for treating bednets. The authors then designed a simple test allowing this resistance to be tracked and showed that mosquitoes carrying this resistance marker were better able to survive and to take a blood meal after exposure to insecticide-treated bednets in a field hut study in Cameroon.

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