

# Directionally selected cytochrome P450 alleles are driving the spread of pyrethroid resistance in the major malaria vector *Anopheles funestus*

Jacob M. Riveron, Helen Irving, Miranda Ndula, Kayla G. Barnes, Sulaiman S. Ibrahim, Mark J. I. Paine, and Charles S. Wondji<sup>1</sup>

Vector Biology Department, Liverpool School of Tropical Medicine, Liverpool L3 5QA, United Kingdom

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Pyrethroid insecticides are critical for malaria control in Africa. However, resistance to this insecticide class in the malaria vector *Anopheles funestus* is spreading rapidly across Africa, threatening the success of ongoing and future malaria control programs. The underlying resistance mechanisms driving the spread of this resistance in wild populations remain largely unknown. Here, we show that increased expression of two tandemly duplicated P450 genes, *CYP6P9a* and *CYP6P9b*, is the main mechanism driving pyrethroid resistance in Malawi and Mozambique, two southern African countries where this insecticide class forms the mainstay of malaria control. Genome-wide transcription analysis using microarray and quantitative RT-PCR consistently revealed that *CYP6P9a* and *CYP6P9b* are the two genes most highly overexpressed ( $>50$ -fold;  $q < 0.01$ ) in permethrin-resistant mosquitoes. Transgenic expression of *CYP6P9a* and *CYP6P9b* in *Drosophila melanogaster* demonstrated that elevated expression of either of these genes confers resistance to both type I (permethrin) and type II (deltamethrin) pyrethroids. Functional characterization of recombinant *CYP6P9b* confirmed that this protein metabolized both type I (permethrin and bifenthrin) and type II (deltamethrin and lambda-cyhalothrin) pyrethroids but not DDT. Variability analysis identified that a single allele of each of these genes is predominantly associated with pyrethroid resistance in field populations from both countries, which is suggestive of a single origin of this resistance that has since spread across the region. Urgent resistance management strategies should be implemented in this region to limit a further spread of this resistance and minimize its impact on the success of ongoing malaria control programs.

metabolic resistance | P450 allelic variation | heterologous expression | GAL4/UAS expression

Pyrethroid insecticides are critical for malaria control in Africa. They are the only insecticide class recommended by the World Health Organization (WHO) for the impregnation of long-lasting insecticide nets (LLINs) and are also the major insecticide used in indoor residual spraying (IRS). With no new public health insecticide class introduced in the past 20 y, it is crucial to maintain the efficacy of pyrethroids against malaria vectors as long as possible. This requires a good understanding of the molecular basis of resistance to this insecticide class in field populations of malaria vectors to design suitable resistance management strategies. This important need is highlighted by the WHO Global Malaria program, which, in its recent Global Plan for Insecticide Resistance Management (1), stressed the need to fill gaps in knowledge on resistance mechanisms in malaria vectors to ensure the success of ongoing and future vector control programs.

Malaria is highly endemic in southern Africa, accounting for 30–50% of all outpatient hospital visits in Mozambique and Malawi and is also the main cause of hospital admissions in children under 5 y (2, 3). To reduce this burden, vector control interventions through large-scale distribution of LLINs and implementation of IRS are being rapidly scaled up in these countries (2, 3). However, the resistance developed by malaria vectors to pyrethroids is a serious threat to the success of these control methods (4).

The mosquito *Anopheles funestus* is one of the main malaria and lymphatic filariasis vectors in these countries due to its wide geographical distribution, its anthropophilic behavior, and its high vectorial capacity in the region (with infection of  $>5\%$ ) (3–5). Resistance to pyrethroids in *An. funestus* was first reported in South Africa in 1999 (4) and has been reported in many populations both in Mozambique (4, 6, 7) and in Malawi (8) with potentially negative impact on control interventions. In contrast, only low-level resistance (75–100% mortality) has been observed in *Anopheles arabiensis* and *Anopheles gambiae* in this region (9).

Preliminary investigations of underlying resistance mechanisms of the pyrethroid resistance in field populations of *An. funestus* in southern Africa have indicated that a P450-based metabolic resistance is the main mechanism as indicated by synergist assays with piperonyl butoxide (PBO) (7, 10) with no knockdown resistance mutation identified yet in this species. However, the specific genes conferring this resistance in field populations remain unknown. Previous characterization of pyrethroid resistance mechanisms in a laboratory resistant strain of *An. funestus*, FUMOR identified three quantitative trait loci (QTL) regions named *rp1* (resistance to permethrin 1), *rp2*, and *rp3* (11–13). The potential role of these QTL in the pyrethroid resistance in field populations remains to be established. Additionally, it is not known whether the rapid spread of this pyrethroid resistance in southern Africa is under the control of a unique mechanism that could have been favorably selected by ongoing control interventions or whether the resistance occurred independently several times.

In this study, using a genome-wide transcriptional and functional analysis, we successfully demonstrate that the up-regulation of the tandemly duplicated cytochrome P450s *CYP6P9a* and *CYP6P9b* is the main mechanism responsible for pyrethroid resistance in field populations of *An. funestus* in southern Africa. Furthermore, we show that both genes are under strong directional selection with resistance driven mainly by a single allelic variant in each gene, suggesting that the resistance, spreading across southern Africa, has a single genetic origin.

## Results

**Resistance Status of *An. funestus* in Mozambique and Malawi.** The *An. funestus* population from Malawi (Chikwawa) is resistant to both type I (0.75% permethrin) and type II pyrethroids (0.05% deltamethrin) with, respectively, a  $47.2 \pm 7.5\%$  and a  $42.3 \pm 8\%$

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Data deposition: The microarray chip design is A-MEXP-2245; microarray data reported in this paper have been deposited in Array Express (accession no. E-MTAB-1257); and the DNA sequences deposited in the GenBank database (accession nos. JX627267–JX627312).

<sup>1</sup>To whom correspondence should be addressed. E-mail: c.s.wondji@liverpool.ac.uk.

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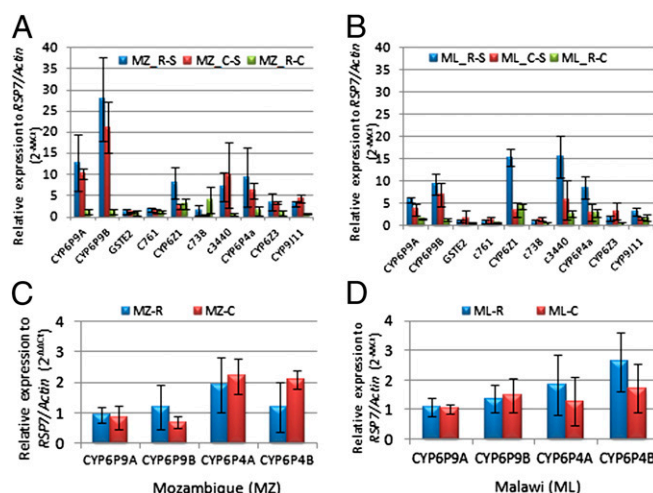
mortality rate for females after 1 h exposure (Fig. S14). A higher resistance level was observed in the population from Mozambique (Chokwe) with no mortality recorded after a 1-h, 30-min exposure and less than 50% mortality after a 3-h, 30-min exposure. Pre-exposure to PBO induced a near full susceptibility to permethrin in both populations (95% and 96% in Malawi and Mozambique, respectively), suggesting that cytochrome P450s play a major role in the observed pyrethroid resistance.

**Genome-Wide Transcriptional Analysis Using Microarray. Experimental design.** A custom microarray chip containing 44,000 probes (60mer) was used to identify the set of genes associated with pyrethroid resistance in Malawi and Mozambique. Labeled cRNA was obtained from three biological replicates for the following samples: (i) resistant (R) (mosquitoes alive after a 1-h exposure to 0.75% permethrin); (ii) control (C) (mosquitoes unexposed to insecticide and thus representative of the wild-type population); and (iii) susceptible (S) (unexposed mosquitoes from the fully susceptible laboratory strain FANG). These cRNA were reciprocally hybridized against each other in a triangle design with three types of comparisons: R-S for resistant vs. susceptible, R-C for resistant vs. control, and C-S for control vs. susceptible. This experimental design identifies candidate genes while taking into account the difference in genetic background between strains. The number of genes differentially expressed (more than twofold change,  $q < 0.01$ ) for each comparison and between comparisons are indicated in Fig. S1 B and C, respectively, for Mozambique and Malawi.

**Genes up-regulated in resistant mosquitoes.** The two P450 genes *CYP6P9a* and *CYP6P9b* were consistently the most overexpressed genes in both countries for the R-S and C-S comparisons (Table S1). However, a higher overexpression of *CYP6P9b* ( $q < 0.01$ ) was observed in Mozambique in both R-S (FC50.2) and C-S (FC64.9) than was observed in Malawi (FC30.5 and 26.6 for R-S and C-S, respectively). This pattern is opposite that of *CYP6P9a*, which is more expressed in Malawi in both R-S (FC62.7) and C-S (FC69.7) than in Mozambique (FC38.5 and FC37.9 for R-S and C-S, respectively). The consistency of the overexpression of these two genes is further supported by the fact that all of the three probes of each gene are all overexpressed in R-S and C-S (Table S2). None of the probes of these two genes was overexpressed in the R-C comparison in Mozambique, probably due to the high resistance level in these samples, meaning that both R and C are very resistant and have a similar expression pattern for resistance genes. In Malawi, where resistance is more moderate, *CYP6P9a* was overexpressed in R-C (FC7.9) as was *CYP6P9b* (FC3.2).

Other genes commonly overexpressed in both countries include other P450 genes (*CYP9J11*, *CYP6Z1*, *CYP6P4b*, *CYP6Z3*, and *CYP6P2*), ATP-binding cassette (ABC) transporters (*ABCB7* and *ABCG4*), glutathione-S-transferases (*GSTe2*), short-chain dehydrogenases, ESTs of unknown function, and other gene families. Additional microarray information is presented in SI Results.

**Validation of the Microarray Up-Regulation with Quantitative RT-PCR.** Quantitative real-time PCR (qRT-PCR) was used to validate the microarray results for 10 genes among the most up-regulated in Mozambique and Malawi including six P450 genes, one GST (*GSTe2*), a short-chain dehydrogenase, and two genes with unknown function. qRT-PCR results confirm the up-regulation of eight genes in Mozambique (Fig. 1A) and seven in Malawi (Fig. 1B). A significant correlation between qRT-PCR results and microarray results was observed in Mozambique ( $R^2 = 0.748$ ;  $P = 0.002$ ) (Fig. S1D) but not in Malawi ( $R^2 = 0.085$ ) ( $P = 0.8$ ) (Fig. S1E) partly due to a more marked difference in expression for both *CYP6P9a* and *CYP6P9b* between the two methods in Malawi. A significant correlation was observed between qRT-PCR results of R-S and C-S in both countries ( $R^2 = 0.94$ ,  $P = 0.0$  in Mozambique;  $R^2 = 0.71$ ,  $P = 0.021$  in Malawi). *CYP6P9a* and *CYP6P9b* were the top up-regulated genes in Mozambique with a fold-change of 12.8 and 27.9, respectively, for the R-S comparison. Lower expression levels for both genes were obtained in



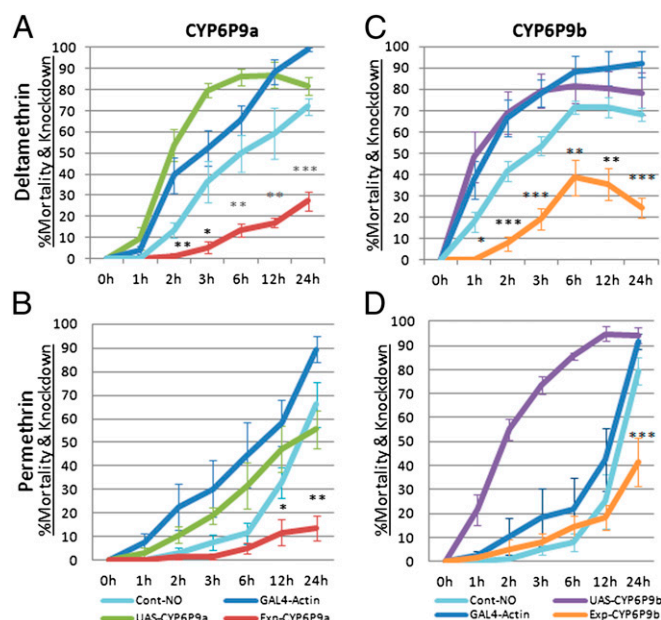
**Fig. 1.** Quantitative PCR results: Differential expression by qRT-PCR of 10 genes up-regulated in microarray assays in Mozambique (A) and in Malawi (B). Relative fold-change in gene copy number for *CYP6P9a*, *CYP6P9b*, *CYP6P4a*, and *CYP6P4b* in Mozambique (C) and in Malawi (D). Error bars represent SD ( $n = 3$ ).

Malawi with fold change (FC) of 4.8 and 9.4, respectively, for *CYP6P9a* and *CYP6P9b*. Four other P450 genes had significant up-regulation in Mozambique and Malawi, including *CYP6Z1*, *CYP6P4a*, *CYP6Z3*, and *CYP9J11* (Fig. 1A and B).

**Analysis of Copy-Number Polymorphism of Duplicated P450 Using Quantitative PCR.** Quantitative PCR (qPCR) was used to assess whether a potential copy-number variation or gene amplification could be associated with the significant up-regulation in resistant mosquitoes of the duplicated P450 genes *CYP6P9a* and *CYP6P9b* and also for *CYP6P4a* and *CYP6P4b*. A comparison of pooled genomic DNA of resistant mosquitoes (R) or of control mosquitoes (C) to susceptible mosquitoes (S) (dead after insecticide exposure) revealed that there was no difference in copy number between resistance phenotypes for *CYP6P9a* and *CYP6P9b* both in Mozambique (Fig. 1C) and in Malawi (Fig. 1D). No significant difference was also observed for *CYP6P4a* and *CYP6P4b* between resistant and susceptible samples in both countries.

**Transgenic Expression of *CYP6P9a* and *CYP6P9b* in *Drosophila melanogaster*.** To establish whether *CYP6P9a* and *CYP6P9b* overexpression can independently confer resistance to pyrethroid insecticides using the GAL4/UAS expression system, transgenic *Drosophila melanogaster* strains expressing each gene were successfully generated under the control of either the 6g1-HR-GAL4 driver (6g1-HR-*CYP6P9a* and 6g1-HR-*CYP6P9b*) or the Act5C-GAL4 driver (Act5C-*CYP6P9a* and Act5C-*CYP6P9b*). Each transgene was also confirmed to be expressed only in the F<sub>1</sub> progeny of the GAL4/UAS crosses after RT-PCR. For both genes, qRT-PCR revealed a higher expression in the progeny generated with the ubiquitous Act5C-GAL4 driver than with the tissue-specific 6g1-HR-GAL4 driver (Fig. S2 A and B).

**Tests with transgenic *CYP6P9a* flies.** Bioassays carried out with deltamethrin (type II pyrethroid) on the transgenic Act5C-*CYP6P9a* indicated an increased resistance in these flies (Fig. 2A) with a significantly lower mortality rate (22% after 24 h;  $P < 0.001$ ) compared with the two parental lines (82% and 98% mortality, respectively, for UAS-*CYP6P9a* and Act5C-GAL4) and the control progeny with no *CYP6P9a* expression (Control-NO) (generated from crossing the driver line and the line without the UAS vector) (74% mortality). Similar results were obtained with permethrin (type I pyrethroid) (Fig. 2B) with a significantly reduced mortality (13.7% after 24 h;  $P < 0.01$ ) in the transgenic Act5C-*CYP6P9a* compared with the parental lines (55.6% and 89.6%,



**Fig. 2.** Bioassays results with transgenic strains for *CYP6P9a* and *CYP6P9b*. (A) Test with deltamethrin on transgenic Act5C-*CYP6P9a* flies (Exp-*CYP6P9a*) and three control strains [two parental (UAS-*CYP6P9a* and GAL4-Actin) and the progeny from the cross between the Gal4-Act5C females and the attP40 males (which do not overexpress the P450 transgene) (Cont-NO)]. (B) Test with permethrin on transgenic Act5C-*CYP6P9a*. (C) Test with deltamethrin on transgenic Act5C-*CYP6P9b*. (D) Test with permethrin on transgenic Act5C-*CYP6P9b*.

respectively, for UAS-*CYP6P9a* and Act5C-GAL4) and the Control-No (66.1% mortality).

A similar trend was observed for deltamethrin bioassays of the transgenic 6g1-HR-*CYP6P9a* flies, in which expression is under the control of a tissue-specific promoter, but the difference in mortality rate was less marked ( $P < 0.05$ ) either compared with the parental lines (Fig. S2C) or the Control-NO (Fig. S2D). No significant difference of mortality rate was observed for the experiment with permethrin on 6g1-HR-*CYP6P9a* flies (Fig. S2E). **Tests with transgenic *CYP6P9b* flies.** Bioassays carried out with deltamethrin on the transgenic Act5C-*CYP6P9b* also indicated an increased resistance in these flies (24.4% mortality;  $P < 0.001$ ) (Fig. 2C) compared with the two parental lines (78% and 92% mortality, respectively, for UAS-*CYP6P9a* and Act5C-GAL4) and the progeny with no *CYP6P9b* expression (Cont-No; 68% mortality). Similar results were obtained with permethrin (Fig. 2D) with lower mortality (41% mortality;  $P < 0.01$ ) observed in the transgenic Act5C-*CYP6P9b* flies compared with the two parental lines (94% and 92%, respectively, for UAS-*CYP6P9a* and Act5C-GAL4) and the progeny with no *CYP6P9b* expression (79.5% mortality). The 6g1-HR-*CYP6P9b* flies showed only a significantly reduced mortality ( $P < 0.05$ ) for deltamethrin tests compared with the parental lines (Fig. S2F) but not compared with the Control-No (Fig. S2G). No significant difference in mortality rate was observed for the permethrin experiment with these 6g1-HR-*CYP6P9b* transgenic flies (Fig. S2H). Altogether, these results indicate that both *CYP6P9a* and *CYP6P9b* genes are able to independently confer deltamethrin and permethrin resistance.

#### Heterologous Expression of *CYP6P9a* and *CYP6P9b* in *Escherichia coli*.

To confirm that both *CYP6P9a* and *CYP6P9b* alleles from Malawi and Mozambique can metabolize pyrethroid insecticides, recombinant enzymes of these genes were tested using in vitro metabolism assays. No expression was detected for *CYP6P9a* cotransformed with cytochrome P450 reductase (CPR) into *Escherichia coli* JM109 cells. Subsequent use of other *E. coli* cells such as DH5 $\alpha$  and Rosetta

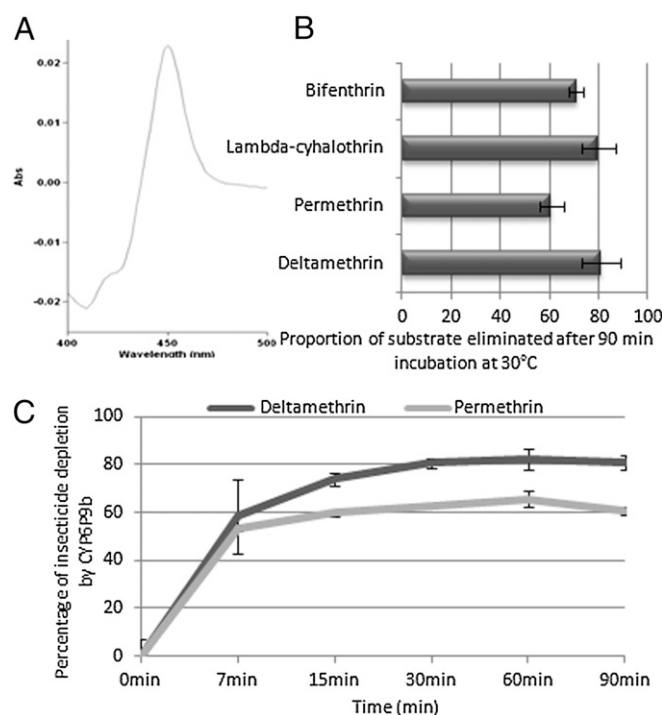
cells did not improve the results. A successful expression was detected for *CYP6P9b* at 21 °C after a 72-h incubation.

Coexpression of *CYP6P9b* enzyme in *E. coli* with CPR produced 1 mL of bacterial membrane preparations. The carbon monoxide (CO)-difference spectrum indicated that *CYP6P9b* was expressed predominately as P450 with a low level of P420 (Fig. 3A), indicative of a good-quality functional enzyme. In vitro metabolism assays demonstrated that recombinant *CYP6P9b* metabolized both type I (nonyano) and type II (cyano) pyrethroid insecticides. The respective proportions of substrate depletion [disappearance of substrate (20  $\mu$ M) with time] were, respectively, 80.9% and 80% for deltamethrin ( $P = 0.008$ ) and lambda-cyhalothrin ( $P = 0.0023$ ), both type II; 60.7% and 71%, respectively, for permethrin ( $P = 0.01$ ) and bifenthrin ( $P = 0.001$ ), both type I (Fig. 3B). A turnover rate of  $7.0 \pm 0.5 \text{ min}^{-1}$  was achieved for deltamethrin (1:1 *cis:trans*) and  $6.35 \pm 0.8 \text{ min}^{-1}$  for permethrin (Fig. 3C). However, DDT was not metabolized by *CYP6P9b* with no significant difference ( $P = 0.13$ ) observed between the proportion of DDT depletion in the presence or in the absence of the NADPH with the recombinant *CYP6P9b*.

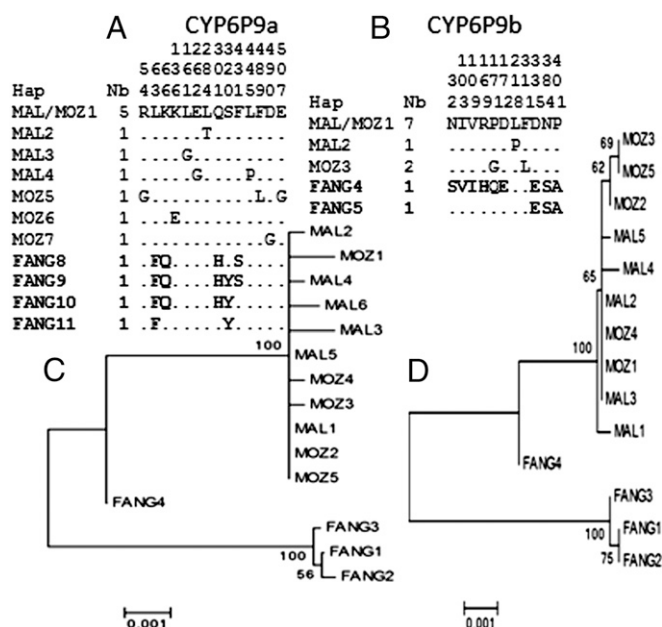
#### Patterns of Genetic Variability of *CYP6P9a* and *CYP6P9b*. Analysis of cDNA polymorphism.

Analysis of the variability of full-length cDNA sequences of *CYP6P9a* and *CYP6P9b* (1,527 bp for both) generated from total RNA indicated a significantly lower polymorphism in the two resistant field populations of Mozambique and Malawi compared with the susceptible laboratory strain FANG (Table S3) with far fewer nucleotide substitutions in Malawi and Mozambique populations (2–7) than in FANG (23 and 35, respectively, for *CYP6P9a* and *CYP6P9b*).

*CYP6P9a* is more polymorphic than *CYP6P9b* with a greater number of DNA haplotypes (12 vs. 9) (Fig. S3A and B) and amino acid sequences (11 vs. 5) (Fig. 4A and B). However, *CYP6P9a* is more similar than *CYP6P9b* between Malawi and Mozambique (Fig. S3C and D). Phylogenetic analysis of the haplotypes



**Fig. 3.** Metabolic activity of *CYP6P9b*. (A) CO-difference spectrum of *E. coli* membranes expressing *CYP6P9b*. (B) The proportion of 20  $\mu$ M insecticide cleared by 0.1  $\mu$ M P450 with 0.8  $\mu$ M cyt b5 in the presence of NADPH is indicated by bar height. Error bars represent SD ( $n = 3$ ). (C) Time course of deltamethrin and permethrin metabolism by *CYP6P9b*.



**Fig. 4.** Schematic representation of haplotypes of *CYP6P9a* and *CYP6P9b* genes between the resistant mosquitoes from Mozambique and Malawi and the susceptible FANG strain. (A and B) The polymorphic amino acid positions for both *CYP6P9a* and *CYP6P9b*, respectively. (C) Neighbor-joining tree of *CYP6P9a* and (D) *CYP6P9b* showing two clades specific to each phenotype. A number has been given to each haplotype preceded by MAL, MOZ, or FANG if it is unique to Malawi, Mozambique, or FANG strains, respectively. The column Nb indicates the number of individuals sharing the haplotype.

consistently indicated a genetic similarity between Malawi and Mozambique as their haplotypes always belong to the same cluster whereas FANG haplotypes belong to the second cluster (Fig. 4 C and D).

**Polymorphism analysis between susceptible and resistant field mosquitoes.** A comparative analysis of the polymorphism pattern of both genes was additionally carried out between sets of mosquitoes alive after permethrin exposure (resistant) and set of mosquitoes dead (susceptible) both in Malawi and in Mozambique. A genomic fragment including the 5' UTR region and the full-length coding sequence and the intron was analyzed for both genes, totaling 2,050 bp for *CYP6P9a* and 1,812 bp for *CYP6P9b*. The *CYP6P9a* again exhibited a higher genetic diversity than *CYP6P9b* as indicated by the higher number of DNA haplotypes (17 vs. 5) (Fig. S3 E and F) or number of alleles for amino acid sequences

(9 vs. 4) (Fig. S3 G and H; Table S3). In both countries and for both genes, the resistant mosquito samples are significantly less polymorphic than the susceptible samples (58 nucleotide substitutions in susceptible vs. 5 in resistant for *CYP6P9a*; 51 vs. 1 for *CYP6P9b*). However, the polymorphism in susceptible samples is contributed by few mosquitoes with sequence similar to the susceptible FANG strain whereas the remaining mosquitoes are similar to the resistant mosquitoes. This is confirmed by the presence of a dominant haplotype in both genes (72% for H2 in *CYP6P9a* and 89% for H1 in *CYP6P9b*) (Fig. 5 A and B).

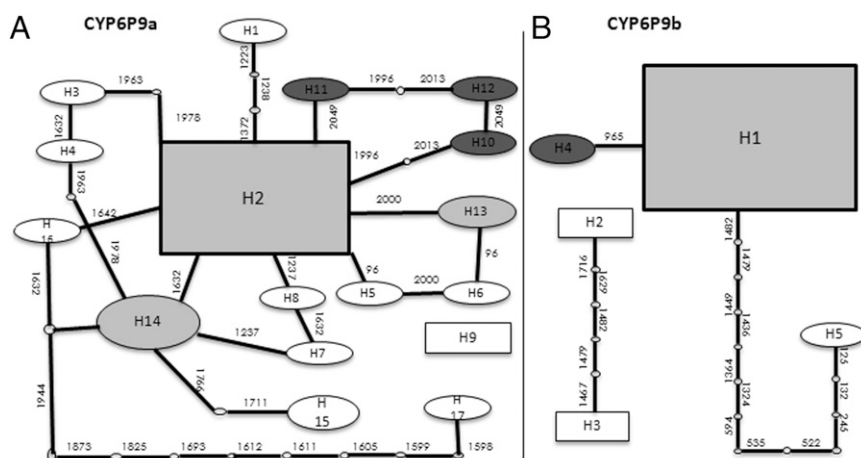
Analysis of the amino acid changes in both genes identified specific mutations predominant in resistant mosquitoes from Malawi and Mozambique compared with field and laboratory (FANG) susceptible mosquitoes. For *CYP6P9a*, F63L and Q66K mutations are common to resistant mosquitoes whereas, in *CYP6P9b*, V103I and S384N are found. The S384N mutation in *CYP6P9b* is located within the substrate recognition site 5 and eight residues downstream of the ExxR motif that stabilizes the core of the P450s and could impact the binding to pyrethroids.

## Discussion

Pyrethroid resistance in *An. funestus* is spreading across Africa but little is known about the mechanisms driving it in natural populations. In this study, we have dissected the molecular basis of this resistance in the two southern African countries of Mozambique and Malawi.

**Transcriptional Analysis Revealed the Main Resistance Genes.** Microarray and qRT-PCR consistently revealed that the two P450 genes *CYP6P9a* and *CYP6P9b*, located within the *rp1* QTL on the 2R chromosome (11), were the most up-regulated genes in both countries, suggesting that they are closely associated with pyrethroid resistance. The expression level of *CYP6P9b* was consistently higher in Mozambique than in Malawi whereas *CYP6P9a* was higher in Malawi than in Mozambique, indicating that *CYP6P9b* may have a more preeminent role in pyrethroid resistance in Mozambique whereas *CYP6P9a* may play a bigger role in Malawi.

The P450 gene amplification mechanism recently associated with insecticide resistance in the aphid *Myzus persicae* (14) and the mosquitoes *Aedes aegypti* (15) and *Culex quinquefasciatus* (16) was not observed for *CYP6P9a* and *CYP6P9b*. Indeed, their up-regulation does not seem to be caused by gene amplification as no significant copy-number variation was observed between the resistant and the susceptible mosquitoes. This suggests that *CYP6P9a*- and *CYP6P9b*-mediated pyrethroid resistance operates mainly through an over-transcription potentially controlled by *cis*-acting regulatory elements as indicated by previous QTL mapping (11). Constitutive up-regulation of cytochrome P450 has been associated with insecticide resistance in other insect species such as the malaria vector *An. gambiae* in which overexpression of



**Fig. 5.** A 95% parsimony network of *CYP6P9a* (A) and *CYP6P9b* (B) haplotypes between susceptible and resistant mosquitoes from both Mozambique and Malawi. Haplotypes are represented as an oval or a rectangular shape, scaled to reflect their frequencies. Lines connecting haplotypes and each node represent a single mutation event (respective polymorphic positions are given above branches). White shapes represent haplotypes unique in susceptible mosquitoes; grey shapes represent haplotypes predominantly found in resistant mosquitoes but also in some dead mosquitoes; dark grey shapes represent haplotypes unique to resistant mosquitoes. For both genes, some susceptible haplotypes with >20 mutation differences from others could not be linked to the major network.

*CYP6P3* (ortholog of both *CYP6P9* genes) and *CYP6M2* confers pyrethroid resistance in field populations (17, 18). Similar cases are reported in *D. melanogaster* with *CYP6G1* (19) or in the red flour beetle *Tribolium castaneum* with the *CYP6BQ9* P450 gene (20).

The up-regulation of several other genes in resistant mosquitoes (Tables S4 and S5 and Fig. S4) suggests that, in addition to *CYP6P9a* and *CYP6P9b*, other genes with minor roles or involved in subsequent phases of pyrethroid detoxification also contribute to this resistance. Future studies are planned to assess the role of these genes in pyrethroid resistance. Among these genes is the P450 *CYP9J11*, up-regulated in both countries and located on chromosome 3L within the boundaries of the previously described pyrethroid resistance QTL *rp3* in the FUM0Z-R laboratory strain (11). The consistent up-regulation of this gene suggests that it could be the candidate gene conferring the resistance in this *rp3* QTL.

**Comparative Functional Analysis of *CYP6P9a* and *CYP6P9b*.** The major role likely played by *CYP6P9a* and *CYP6P9b* in pyrethroid resistance in field populations of *An. funestus* was well supported by the transgenic expression of each gene in *D. melanogaster*. This study confirmed that both genes are able to confer resistance independently to both type I (permethrin) and type II (deltamethrin) pyrethroids. Progeny obtained with the ubiquitous driver Act5C consistently showed a higher resistance to both pyrethroids than progeny generated using the tissue-specific driver 6g1-HR, which drives expression in the Malpighian tubules, fat body, and midgut. This differs from previous studies with *D. melanogaster* P450s for which driving the expression in a tissue-specific manner generated the best results compared with using a ubiquitous driver (21, 22). The higher level of expression of both *CYP6P9a* and *CYP6P9b* observed by qRT-PCR in progeny from the Act5C driver than in genes from 6g1-HR could explain the higher resistance level conferred by the ubiquitous driver. Additionally, the expression of the transgene in other tissues in addition to the Malpighian tubules, fat body, and midgut in progeny from the Act5C (23) may also enhance the activity of these P450s with the Act5C driver as shown recently with resistance to deltamethrin conferred in the red flour beetle *T. castaneum* by *CYP6BQ9*, a P450 specifically expressed in the brain (20).

This study has also established that *CYP6P9b* is able to metabolize in vitro both type I (permethrin and bifenthrin) and type II (deltamethrin and lambda-cyhalothrin) pyrethroids, further confirming the in vivo result obtained with transgenic expression in *D. melanogaster*. Unfortunately, *CYP6P9a* could not be expressed by using the OMPA+2 primer. Future efforts will attempt this expression with other expression systems such as the baculovirus system. In this study, *CYP6P9b* was shown not to metabolize DDT, confirming the bioassays data indicating a complete susceptibility to DDT in both countries (7, 8). This indicates that DDT should be considered as an alternative insecticide for future efforts to implement a resistance management strategy of *CYP6P9*-mediated pyrethroid resistance, notably for IRS programs.

**Patterns of Genetic Variability of *CYP6P9a* and *CYP6P9b*.** Analysis of the polymorphism pattern of both *CYP6P9a* and *CYP6P9b* in field populations revealed the predominance of one resistance allele for each gene, H2-*CYP6P9a* and H1-*CYP6P9b*. However, these two resistant haplotypes are also found in some of the dead mosquitoes after 1 h of permethrin exposure. This could be due to a misclassification in the resistance phenotype as the 1-h exposure cutoff time used to define resistance may not be the most appropriate to accurately define the resistance phenotype. The use of two time points to select the most resistant (such as LT<sub>90</sub>) and more susceptible (such as LT<sub>10</sub>) as done previously for QTL mapping in this species (11) could help to minimize such phenotype misclassification in future studies.

The very high frequency of the H2-*CYP6P9a* (72%) and H1-*CYP6P9b* (89%) haplotypes in both Malawi and Mozambique and the significantly reduced diversity in resistant mosquitoes suggest that both genes are under a strong directional selection favoring

these two alleles. This selection is comparable to the *CYP6G1* P450 gene in *D. melanogaster* where the *CYP6G1*/Accord haplotypes are highly selected in DDT-resistant field samples (24). The predominance of H2-*CYP6P9a* and H1-*CYP6P9b* haplotypes also suggests a single origin for this pyrethroid resistance in southern Africa, which subsequently spread due to selection pressure on *An. funestus* populations caused by the implementation of control interventions (LLINs and IRS) and/or agricultural use of pyrethroids. Further population genetics analysis of these genes will help to establish the patterns of genetic diversity in natural populations in this region and the extent of the selective sweep due to this resistance.

The potential association between some amino acid changes such as F63L and Q66K for *CYP6P9a* and V103I and S384N for *CYP6P9b* and the resistance phenotype is similar to the case observed for the *CYP6AB3* gene in the insect *Depressaria pastinacella* (parsnip webworm) in which five amino acid changes enhance metabolism of plant allelochemicals (25). Similarly, in humans, allelic variations in P450 (*CYP2D6*, *CYP2C9*, *CYP2C19*, and *CYP2A6*) are known to affect drug metabolism with some alleles conferring a low metabolism to certain drugs and others conferring a fast rate of metabolism (26). Further investigation of the impact of the allelic variation of *CYP6P9a* and *CYP6P9b* on the ability to metabolize pyrethroids is planned to detect potential causative mutations that could be used to design a diagnostic tool to easily detect and map this resistance in the field.

## Conclusion

This study has demonstrated that two major alleles of the P450 genes *CYP6P9a* and *CYP6P9b* are driving resistance to pyrethroids in field populations of *An. funestus* in the southern African countries of Mozambique and Malawi. Both genes are highly expressed in resistant populations and able to efficiently metabolize both type I and II pyrethroids. The likely unique origin of this resistance suggests that, unless barriers to gene flow exist in natural populations of *An. funestus*, this resistance is likely to spread beyond these two countries, possibly impacting current and future control programs against this vector. Therefore, urgent actions should be taken to implement suitable resistance management strategies in this region. One of these actions could be the incorporation of P450 inhibitors such as PBO into pyrethroid formulations to improve the efficacy of control interventions.

## Materials and Methods

**Bioassays.** Insecticide susceptibility assays with 0.75% permethrin, other insecticides (Fig. S1A), and a synergist assay with PBO were carried out using 2- to 5-d-old F<sub>1</sub> adults from pooled families collected in Mozambique and Malawi as described previously (7).

**Microarray.** A new 4 × 44 k Agilent microarray chip (A-MEXP-2245), designed using eArray program (Agilent), was used to detect the set of genes associated with pyrethroid resistance in Malawi and Mozambique. Each array contains 60mer probes designed from *An. funestus* 454 transcriptome sequencing ESTs (27) and all transcripts of *An. gambiae*. The RNA extraction and the microarray experiment were carried out following the protocol described in SI Materials and Methods. Briefly, RNA was extracted from three batches of 10 *An. funestus* females that were 2–5 d old from the following sample sets: alive after exposure to 0.75% permethrin (R), unexposed to insecticides (C), and from the fully susceptible laboratory strain FANG (S). cRNA of each sample was amplified using the Agilent Quick Amp labeling Kit (two-color) following the manufacturer's protocol. Labeled cRNAs were hybridized to the arrays for 17 h at 65 °C according to the manufacturer's protocol.

Microarray data were analyzed using Genespring GX 12.0 software. To identify differentially expressed genes, a cutoff of twofold change and a statistical significance of  $q < 0.01$  with Storey with bootstrapping test were applied. The microarray data from this study is deposited in the Array Express under accession no. E-MTAB-1257.

**qRT-PCR.** Some of the genes most associated with resistance from the microarray analysis were assessed by qRT-PCR to validate their expression pattern using the three biological replicates for resistant, control, and FANG

(susceptible) for both countries. A detailed protocol is described in *SI Materials and Methods*.

**Copy-Number Variation of the Duplicated *CYP6P9* and *CYP6P4* Genes.** qPCR was carried out as described in *SI Materials and Methods* to assess the copy-number variation of the duplicated P450s *CYP6P9a*, *CYP6P9b*, *CYP6P4a*, and *CYP6P4b* in field populations of *An. funestus* in Malawi and Mozambique.

**Transgenic Expression of Candidate Genes in *Drosophila* Strains and Bioassays.** The predominant alleles of *CYP6P9a* and *CYP6P9b* in both Malawi and Mozambique were cloned and inserted into the pUASattB40 vector. Using the PhIC31 system, the clones were injected into the germ line of the *D. melanogaster* w<sup>1118</sup> strain. Two transgenic lines, UAS-*CYP6P9a* and UAS-*CYP6P9b*, were obtained and balanced. The expression of the transgenes in transgenic flies was confirmed using qRT-PCR. Bioassays were carried out to assess the susceptibility pattern of the transgenic flies to type I (permethrin) and type II (deltamethrin) pyrethroids. For each test, six replicates of 25 flies were placed in each vial, and the mortality plus knockdown was scored after 1, 2, 3, 6, 12, and 24 h exposure to the insecticide. Further details are provided in *SI Materials and Methods*.

**Heterologous Expression of *CYP6P9a* and *CYP6P9b* in *E. coli*.** The same alleles of *CYP6P9a* and *CYP6P9b* used to construct transgenic flies were fused to a bacterial ompA+2 leader sequence, cloned to the pCW-ori vector, and transformed into *E. coli* JM109 cells. For both genes, *E. coli* JM109 cells were cotransformed with pB13::ompA+2-*CYP6P9a* or -b and pACYC-AgCPR. Metabolism assays in the presence of membranes expressing the P450 and CPR

with Cyt-b5 were carried out using a final working concentration of 0.2 mM of permethrin, deltamethrin, lambda-cyhalothrin, bifenthrin, and DDT as detailed in *SI Materials and Methods*. The quantity of insecticide remaining in each metabolism assay was determined by reverse-phase HPLC with a monitoring absorbance wavelength of 232 nm (Chromeleon, Dionex). More details are provided in *SI Materials and Methods*.

**Analysis of Polymorphism Patterns of Candidate Genes.** The full-length cDNA of *CYP6P9a* and *CYP6P9b* of resistant mosquitoes from Malawi and Mozambique was compared with that of the susceptible FANG strain to assess their allelic variation in relation to the resistance phenotype. Additionally, a further assessment of the correlation of the polymorphism of this gene and resistance was carried out by amplifying and direct-sequencing a larger region of each gene (a portion of 5' UTR, full-length coding region, and intron) in five susceptible (dead after 1 h of exposure) and five resistant (alive after 1 h of exposure) mosquitoes in Malawi and Mozambique. Further details of the protocol and analysis are presented in *SI Materials and Methods*. Sequences have been deposited in the GenBank database (accession nos. JX627267–JX627312).

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