# Identifying genomic changes associated with insecticide resistance in the dengue mosquito *Aedes aegypti* by deep targeted sequencing

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The capacity of mosquitoes to resist insecticides threatens the control of diseases such as dengue and malaria. Until alternative control tools are implemented, characterizing resistance mechanisms is crucial for managing resistance in natural populations. Insecticide biodegradation by detoxification enzymes is a common resistance mechanism; however, the genomic changes underlying this mechanism have rarely been identified, precluding individual resistance genotyping. In particular, the role of copy number variations (CNVs) and polymorphisms of detoxification enzymes have never been investigated at the genome level, although they can represent robust markers of metabolic resistance. In this context, we combined target enrichment with high-throughput sequencing for conducting the first comprehensive screening of gene amplifications and polymorphisms associated with insecticide resistance in mosquitoes. More than 760 candidate genes were captured and deep sequenced in several populations of the dengue mosquito *Ae. aegypti* displaying distinct genetic backgrounds and contrasted resistance levels to the insecticide deltamethrin. CNV analysis identified 41 gene amplifications associated with resistance, most affecting cytochrome P450s overtranscribed in resistant populations. Polymorphism analysis detected more than 30,000 variants and strong selection footprints in specific genomic regions. Combining Bayesian and allele frequency filtering approaches identified 55 nonsynonymous variants strongly associated with resistance. Both CNVs and polymorphisms were conserved within regions but differed across continents, confirming that genomic changes underlying metabolic resistance to insecticides are not universal. By identifying novel DNA markers of insecticide resistance, this study opens the way for tracking down metabolic changes developed by mosquitoes to resist insecticides within and among populations.

[Supplemental material is available for this article.]

Mosquitoes are vectors of numerous human diseases, representing a major threat for public health worldwide (Lounibos 2002). Dengue and Chikungunya viruses are both transmitted by the mosquito *Aedes aegypti* and represent a burden in more than 100 countries putting more than 2.5 billion people at risk (WHO 2009, 2014). Since the Second World War, chemical insecticides have been massively used for controlling vector populations and reducing disease transmission, but their efficacy is now threatened by resistance mechanisms developed by mosquitoes. Insecticide resistance is widespread in *Ae. aegypti* and affects most insecticides used for vector control (Ranson et al. 2010). Resistance to pyrethroid insecticides, the primary insecticide family used against adult mosquitoes, is particularly worrying in the context of the re-emergence of dengue and other arboviruses worldwide (Bhatt et al. 2013). Although attempts are made to develop new insecticides or alternative mosquito control strategies (Scholte et al. 2004; Lacey 2007; Hoffmann et al. 2011; Walker et al. 2011; Harris et al. 2012), their large-scale implementation in tropical

regions will require at least a decade. Until this, characterizing molecular mechanisms underlying resistance is crucial for tracking down resistance alleles and improving resistance management strategies (Corbel et al. 2013).

Resistance to pyrethroids can be the consequence of various mechanisms, such as nonsynonymous mutations affecting the voltage-gated sodium channel targeted by these insecticides, i.e., knockdown resistance (*kdr*) mutations, a lower insecticide penetration, its sequestration, or its biodegradation (metabolic resistance) (Hemingway et al. 2004; Li et al. 2007). *Kdr* mutations and metabolic resistance are known as the two primary resistance mechanisms in mosquitoes. Several *kdr* mutations have been identified in *Ae. aegypti*, and the association between the V1016G/I and the F1534C mutations and pyrethroid resistance has been confirmed (Brengues et al. 2003; Saavedra-Rodriguez et al. 2007; Yanola et al. 2011). Monitoring the frequency of these mutations in field populations is possible through simple DNA-based diagnostic assays and provides key data for resistance management strategies.

Metabolic resistance is far less understood, although this type of resistance is frequent and often accounts for a significant part of the resistance phenotype (Li et al. 2007; Nkya et al. 2013). Such a resistance mechanism is caused by an increased activity of detoxification enzymes. These detoxification enzymes include cytochrome P450 monooxygenases (P450s or *CYPs* for genes), carboxy/cholinesterases (CCEs), glutathione S-transferases (GSTs), and UDP-glycosyl-transferases (UDPGTs), although other families can be involved (Hemingway et al. 2004; David et al. 2013). Their high diversity (roughly 300 genes in *Ae. aegypti*) and the complexity of insecticide biodegradation pathways make the identification of those conferring resistance challenging. Theoretically, metabolic resistance can be the consequence of an increased expression of one or multiple detoxification enzymes capable of metabolizing the insecticide and/or the selection of variants showing a higher insecticide metabolism rate due to conformational modifications. Because overexpression is frequently associated with overtranscription, most candidate genes were identified based on their differential transcription in resistant populations compared to susceptible counterparts using transcriptomics (for review, see David et al. 2013). Although subjected to inherent difficulties associated with gene expression studies (e.g., RNA handling and degradation, uncontrolled variation across time, tissues, and populations), these approaches identified several detoxification enzymes overtranscribed in resistant populations, some of them being later validated as pyrethroid metabolizers (for review, see Vontas et al. 2012; David et al. 2013). Although these approaches proved their value for identifying metabolic resistance genes, they failed to pinpoint the genomic changes associated to their overexpression. In addition, because a limited quantity of mRNA can be extracted from a single mosquito, these studies were conducted at the population level without estimating the frequency of resistant alleles within populations. A constitutive overtranscription can be the consequence of an up-regulation controlled by *cis*/*trans* genomic regulatory elements or an increased gene copy number (Li et al. 2007). Although gene amplifications have been associated with the overtranscription of a few pyrethroid resistance genes in mosquitoes (Wondji et al. 2009; Itokawa et al. 2010, 2011; Bariami et al. 2012), no comprehensive screening of gene amplifications linked to insecticide resistance has ever been conducted. Considering the central role of copy number variation (CNV) in adaptations associated with a "gene dosage" effect (for review, see Kondrashov 2012) and the richness of the *Ae. aegypti* genome in transposable elements known to favor duplication events (Nene et al. 2007), conducting a comprehensive screening of gene amplifications associated with insecticide resistance may allow identifying reliable DNA markers of metabolic resistance. Besides, most genes linked to metabolic resistance were identified based on their differential expression while the importance of nonsynonymous changes affecting detoxification enzymes have clearly been neglected by molecular screenings. As for gene amplifications, these polymorphisms are of high interest for monitoring the dynamics of insecticide resistance mechanisms among and within mosquito populations.

In this context, the present study aimed at combining the target enrichment technology with high-throughput sequencing for identifying gene amplifications and polymorphisms linked to pyrethroid resistance in the dengue vector, *Ae. aegypti*. More than 760 genes, including those encoding for detoxification enzymes, cuticle proteins, ATP-binding cassette (ABC) transporters, neurotransmitter receptors, and voltage-gated channels, were captured and deep sequenced in several susceptible and pyrethroid-resistant populations from three geographical regions (Asia, South America, and laboratory populations). After phenotyping populations for resistance to the pyrethroid deltamethrin and segregating most resistant individuals, gene amplifications and polymorphisms associated with deltamethrin resistance were identified by deep targeted sequencing of large pools of individuals. Gene amplifications were validated on individual mosquitoes, and their impact on transcription levels were validated. Polymorphism data were first used to identify genomic regions under selection through a hierarchical Bayesian approach taking into account population structure. Then, nonsynonymous variants strongly linked to deltamethrin resistance were identified by combining those identified by the Bayesian approach and those displaying allele frequencies mostly associated with resistance. Quantitative PCR assays were used for cross validating polymorphism data and studying the association between gene amplifications and known target-site mutations. Results are discussed in regard to known insecticide resistance mechanisms and the identification of novel DNA markers of insecticide resistance.

## Results

### Deltamethrin resistance levels

Bioassays on adult females revealed a broad range of resistance to deltamethrin across populations (Table 1). Exposing mosquitoes for 1 h to various doses of insecticide showed that the lethal dose killing 50% of individuals ($LD_{50}$) varied up to 750-fold between susceptible populations and the most resistant populations from French Guiana. Resistant populations from Thailand showed an intermediate resistance level (about 250-fold), and the laboratory selected population showed a slight resistance (approximately fivefold). These different resistance levels were confirmed by WHO diagnostic assays, in which the time necessary to knock down 50% of individuals ($KDT_{50}$) varied from 11 min for susceptible populations to >8 h for the most resistant ones.

### Sequencing metrics and coverage

The use of target enrichment technology followed by multiplexed Illumina sequencing allowed obtaining an average of 4.35 million 75-bp reads per sample (Supplemental Table 1). Filtering them according to read pairing, sequence quality, and mapping quality

**Table 1.** Deltamethrin resistance levels

| | | | | | | R+/R− segregation | |
|---|---|---|---|---|---|---|---|
| Population | Group | Resistance level | $KDT_{50}^{a}$ | $KDT_{50}$ 95% CI | $LD_{50}^{b}$ | $LT_{25}$ (min)$^{c}$ | $LT_{75}$ (min)$^{c}$ |
| Livp S | Genome | Susceptible | 11.5 | 10.8–12.1 | 0.001 | — | — |
| Bora S | Laboratory | Susceptible | 12.1 | 11.6–12.7 | 0.001 | — | — |
| Delta R | Laboratory | Slightly resistant | 14.2 | 13.5–15.0 | 0.005 | 30 | 80 |
| Patg S | Thailand | Susceptible | 17.0 | 15.1–19.2 | 0.001 | — | — |
| Phet R | Thailand | Resistant | 98.2 | 91.4–106.7 | 0.25 | 40 | 120 |
| Nakh R | Thailand | Resistant | 92.9 | 87.7–99.2 | 0.25 | 40 | 120 |
| NwOr S | S. America | Susceptible | 11.9 | 11.2–12.6 | 0.001 | — | — |
| Cayn R | S. America | Highly resistant | >500 | — | 0.75 | 30 | 180 |
| StGe R | S. America | Highly resistant | >500 | — | 0.75 | 30 | 180 |

$^{a}KDT_{50}$: time (min) necessary to knock down 50% of individuals. $KDT_{50}$ was determined using papers impregnated with 0.05 g/100 mL deltamethrin following WHO insecticide testing recommendations.
$^{b}$Dose of insecticide (g/100 mL) used for paper impregnation leading to 50% mortality after 1 h exposure.
$^{c}$R−/R+ segragation of resistant populations consisted in separating individuals dead at $LT_{25}$ from those surviving at $LT_{75}$ using impregnated paper corresponding to the $LD_{50}$.

allowed successful mapping of 68% of reads on the *Ae. aegypti* genome. The majority of mapped reads consisted in perfect matches (46.6%), whereas reads matching with one and two or more mismatches represented 12.6% and 9.2% of sequenced reads, respectively. The mean coverage was homogenous across samples with a total mean coverage of 83× (Supplemental Fig. 1).

### Gene amplifications linked to deltamethrin resistance

Of the 763 targeted candidate genes, 727 showed a mean coverage greater than 90 reads per kilobase per million reads (RPKM) and were considered for further analysis. Among them, 41 genes showed an increased coverage >1.5-fold in any resistant population compared to the mean coverage of all susceptible ones, together with an increased coverage >1.0-fold in $LT_{75}$ survivors (R+ phenotype) compared to individuals dead at $LT_{25}$ (R− phenotype). These genes were considered amplified in association with deltamethrin resistance (Supplemental Table 2). These genes mostly encoded detoxification enzymes with a significant enrichment in P450s (Fig. 1). Most gene amplifications occurred in gene clusters located within a few genomic supercontigs spread across all chromosomes (Fig. 2). Gene amplification profiles were highly conserved within each region but showed marked differences between continents. Thai resistant populations (Nakh R and Phet R) showed a strong amplification of four *CCEs* located in supercontig 1.142 and another one in supercontig 1.1678. Two *CYP6s* located in supercontig 1.702 and two sulfotransferases in supercontig 1.975 were also amplified in Thai resistant populations. In contrast, resistant populations from South America (Cayn R and StGe R) showed a marked amplification of four *CYP6s* located in supercontig 1.1327. Resistant populations from both regions also shared the amplifications of several *CYP9Js* in supercontigs 1.1188 and 1.221. As expected, the less resistant laboratory population (Delta R) exhibited fewer gene amplifications associated with resistance. The amplification profile of the Delta R population shared particular gene amplifications with field resistant populations. This included a chloride channel also detected in Thailand together with two *CYP4Js* and a glycosyltransferase also detected in South America.

### Validation of gene amplifications on individual mosquitoes

Nine gene amplifications identified by deep sequencing belonging to two P450 clusters and one CCE cluster heavily impacted by gene amplifications were cross-validated by qPCR on individual mosquitoes. The presence of gene amplifications was confirmed for all targeted genes. A good correlation was observed between CNVs obtained by deep sequencing and qPCR (Supplemental Fig. 2). Individual qPCR data reveal high copy number polymorphism within resistant populations (Supplemental Fig. 3). Estimating the frequency of gene amplifications within each population revealed that amplifications are frequent in resistant populations and sometimes also present at low frequencies in susceptible populations (Supplemental Fig. 4). Comparing the amplification profile of each gene across all individuals revealed *CYP6* and *CCE* clusters are likely affected by single amplification events (Supplemental Fig. 5). In contrast, different copy numbers were frequently observed for genes belonging to the *CYP9J*
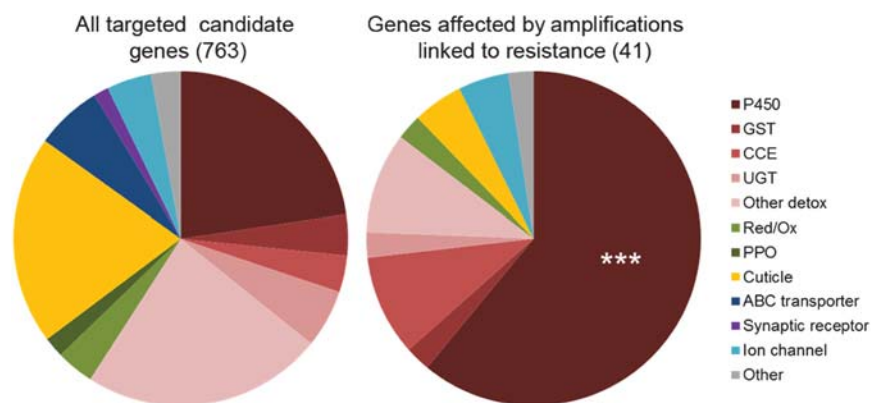


**Figure 1.** Gene families affected by gene amplifications associated with deltamethrin resistance. (*Left*) Frequency of each gene family among the 763 captured genes. (*Right*) Frequency of each gene family among the 41 genes affected by genic amplifications associated with deltamethrin resistance. Proportions of each gene family between all captured genes and genes affected by genic amplifications were compared using a one-sided Fisher's exact test: (***) $P < 0.001$.
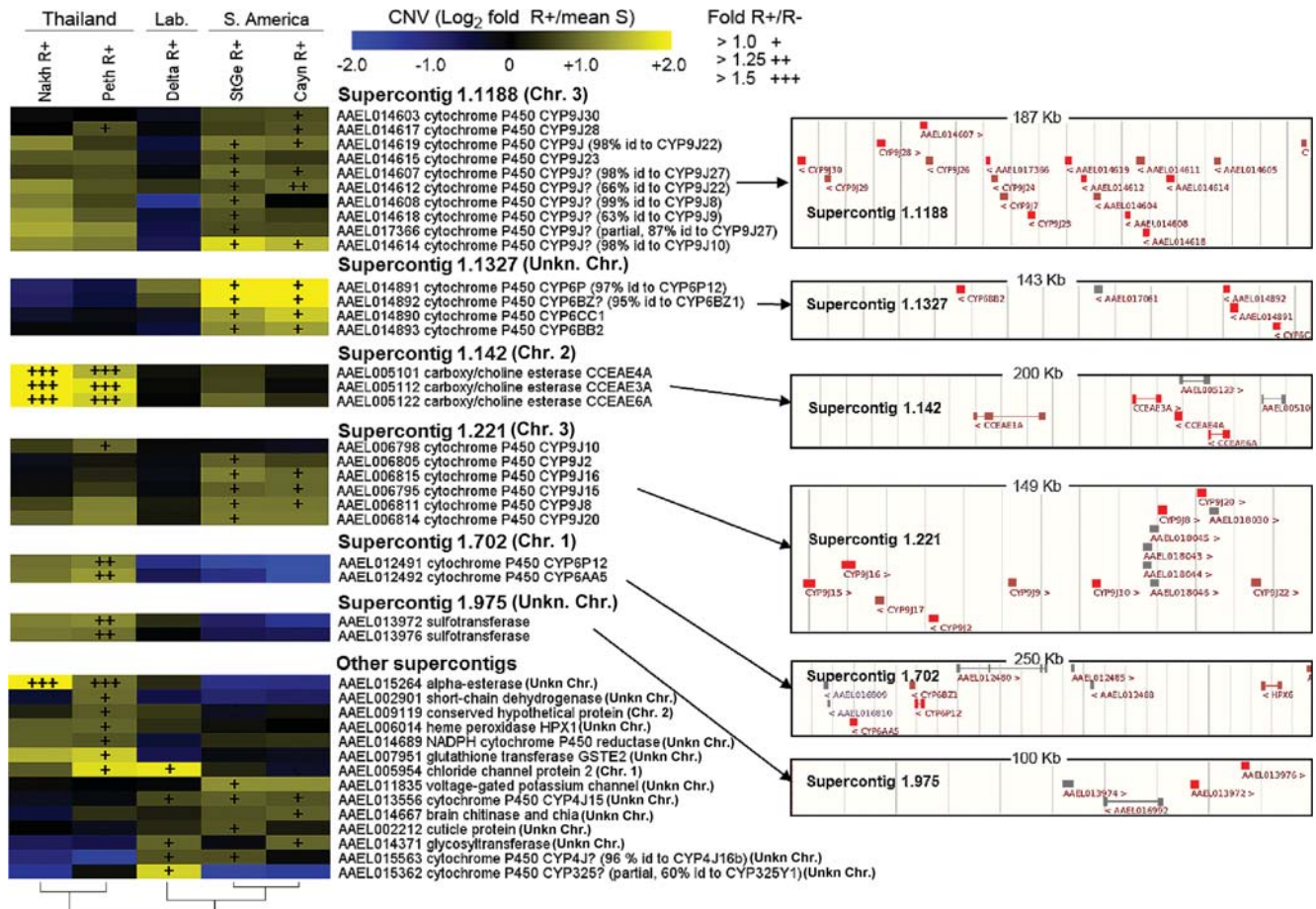
**Figure 2.** Gene amplifications associated with deltamethrin resistance. (*Left*) CNV profiles of genes affected by gene amplifications associated with deltamethrin resistance. Color scale shows (R+)/(meanS) CNV for each resistant population, and overimposed "+" marks show (R+)/(R−) CNV. (*Right*) Location of gene amplifications on genomic supercontigs. Amplified genes are shown in red. Nonamplified genes are shown in maroon. Genes not included in the capture design are shown in gray. Chromosomal locations are shown as described in Timoshevskiy et al. (2014) and Juneja et al. (2014).

cluster on supercontig 1.1188, suggesting that distinct duplication events affect this large P450 cluster.

### Impact of gene amplifications on transcription levels

Comparing gene amplification levels obtained by DNA-sequencing with transcription levels obtained by RT-qPCR for the nine candidate genes across all resistant populations confirmed that gene amplifications are often associated with increased transcription (Supplemental Fig. 6). High and significant positive correlations were observed for most genes except for two *CYP9Js* in supercontig 1.1118, where positive correlations were not significant.

### Association of gene amplifications with target-site mutations

Among the nine gene amplifications genotyped by qPCR, only one was found associated with *kdr* mutations (Supplemental Table 3). This association occurred in the Nakh population from Thailand, where a positive correlation between the presence of *CYP9J28* amplification and the presence of the V1016G mutation was found ($r = 0.61$; $P = 0.025$). As a consequence of the total segregation of V1016G and F1534C mutations in Thailand, the F1534C mutation was negatively correlated to *CYP9J28* amplification in this population ($r = -058$; $P = 0.036$).

### Polymorphisms identified by deep targeted sequencing

A total of 41,469 polymorphisms supported by an important coverage were called against the reference genome. Among them, 30,400 were polymorphic among populations. A principal component analysis (PCA) based on allele frequency variation across all populations confirmed the overall population structure and the different genetic background of Thai, South-American, and laboratory (French Polynesia) populations (Supplemental Fig. 7). The reliability of polymorphism data was confirmed by comparing the frequencies of V1016I, V1016G, and F1534C *kdr* mutations estimated by deep targeted sequencing and those obtained by qPCR on individual mosquitoes (Supplemental Fig. 8). The presence of distinct *kdr* mutations at position 1016 in Thailand and South America was confirmed, and the F1534C mutation was also detected in most populations.

### Polymorphisms associated with deltamethrin resistance

Searching for selection footprints using a Bayesian approach taking into account population structure (Foll et al. 2014) identified 156 outlier loci significantly associated with deltamethrin resistance. These outlier loci included 31 nonsynonymous variants and affected multiple supercontigs spread over all chromosomes.
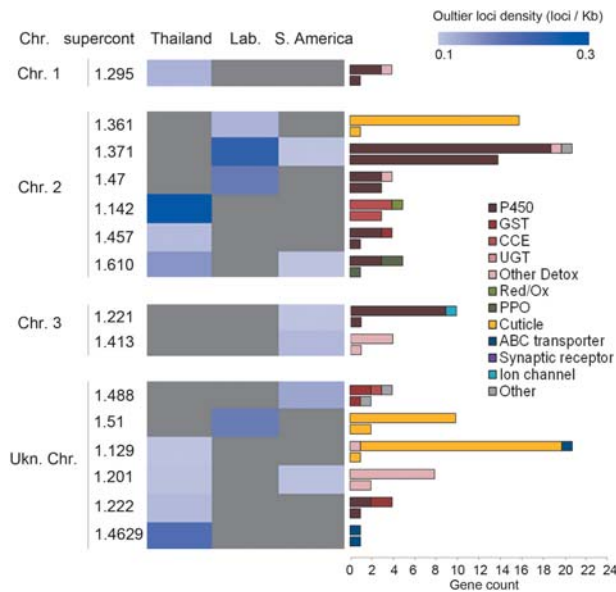
**Figure 3.** Supercontigs showing selection footprint. For each region, loci displaying a significant BayeScan3 $F_{sc}$ $Q$-value <0.05 and both f(R+)-f (S) and f(R+)-f(R−) allele frequency variation occurring in the same direction were considered as outliers. For each supercontig, outlier loci densities were obtained by dividing the number of outlier loci by the total length of all captured genes (outlier loci per kb of captured region). Outlier loci densities are shown as a blue color scale. Gray stands for an absence of outlier loci. For each supercontig, horizontal bars show the number of captured genes from each gene family (*upper* bar) compared to those affected by outlier loci (*lower* bar). Supercontigs are clustered according to their putative chromosomal location as described in Timoshevskiy et al. (2014) and Juneja et al. (2014).

Selection footprints varied between geographical regions with outlier loci densities ranging from 0 to 4.1 per kb of captured sequence (Fig. 3). In Thailand, supercontigs 1.142 and 1.4629 showed high outlier loci densities with three CCE and one ABC transporter being affected. Six other supercontigs also displayed selection footprints impacting two P450s, two other detox enzymes, one PPO, and one cuticle protein. High outlier loci densities were identified in laboratory populations in supercontigs 1.371, 1.47, and 1.51 affecting 17 P450s and two cuticle proteins. Selection footprints were less pronounced in South America but showed overlap with laboratory and Thai populations.

Of the 30,400 polymorphic loci, 3054 loci showed an allele frequency variation between R+ phenotypes and their respective susceptible population ≥40% together with an allele frequency variation between R+ and R− phenotypes ≥5%. These loci included 465 nonsynonymous variants (Supplemental Table 4). Comparing the Bayesian and the frequency filtering approaches revealed that the Bayesian approach was overall more stringent (Supplemental Fig. 9). However, the stringency of the Bayesian approach was high in South America, medium in Thailand, and low in laboratory populations following the decreasing diversity of these groups. The frequency filtering approach showed an opposite stringency with more variants identified in South America than Thailand and even less in laboratory populations. Frequency filtering identified 16 nonsynonymous variants shared by distinct geographic regions, whereas the Bayesian approach only identified variants being specific to each region.

Combining the best candidates identified by each approach identified 55 nonsynonymous variants strongly associated with deltamethrin resistance (Fig. 4; Supplemental Table 4). These variants affected 39 distinct genes mainly belonging to P450s, UGTs, CCEs, and cuticle proteins. As for gene amplifications, allele frequency patterns were conserved within regions but often differed among continents. Most nonsynonymous variants affecting P450s were identified from South American or laboratory populations. These P450s mostly belong to CYP6, CYP9, and CYP12 families. Conversely, most variants affecting CCEs were identified from Thai populations. Two were both affected by gene amplifications and nonsynonymous variants with *CCEAE4A* bearing height distinct nonsynonymous variants associated with resistance. Several UGTs were also affected by nonsynonymous variants, with three showing an increased frequency across multiple regions. ABC transporters and cuticle proteins were most frequently affected in South America. Several other enzymes potentially involved in insecticide detoxification were also affected by nonsynonymous variants, including two aldehyde oxidases, one alcohol dehydrogenase, and one sulfotransferase. Finally, the nicotinic acetylcholine receptor (nAchR) AAEL004935 targeted by neonicotinoid insecticides and the voltage-gated sodium channel (VSGC) AAEL006019 targeted by pyrethroid insecticides were also affected. Variants detected in the VGSC corresponded to the S989P and F1534C *kdr* mutations previously described in this species. None of the V1016I/G *kdr* mutations previously associated with pyrethroid resistance was retained as best candidates. Neither of them was retained by the Bayesian approach, and the V1016G mutation only passed the allele frequency filtering in Thailand (Supplemental Table 4).

## Discussion

### Deep targeted sequencing for studying insecticide resistance

Screening for genomic changes associated with insecticide resistance remained challenging until the sequencing of mosquito genomes (Holt et al. 2002; Nene et al. 2007; Arensburger et al. 2010). Afterward, the development of mosquito DNA microarrays allowed screening for resistance genes based on their differential transcription, leading to the identification of detoxification genes conferring resistance (David et al. 2005, 2013; Strode et al. 2008; Vontas et al. 2010; Edi et al. 2014). However, these screenings focused on differential transcription while genomic changes such as CNVs and polymorphisms were neglected. Then, the development of high-throughput DNA sequencing allowed screening for genomic variants across whole genomes. However, such an approach required a huge amount of sequences to reach the minimum genome coverage necessary for accurately quantifying CNV and allele frequencies, making it unaffordable when several samples have to be compared. Although RNA-seq is more affordable and can generate gene expression and variant data concomitantly, this approach is not suitable for identifying CNVs and does not provide reliable polymorphism data from poorly expressed genes. In addition, allele frequencies inferred from RNA-seq are to be taken with caution because they can be altered by allele-specific expression events (Gaur et al. 2013).

Recently, the development of target enrichment techniques allowed conducting DNA-seq focused on genomic regions of interest, hence, drastically increasing the sequencing depth while maintaining costs at a reasonable level (Altmüller et al. 2014). By targeting more than 760 genes from all protein families potentially involved in insecticide resistance in the dengue mosquito, our
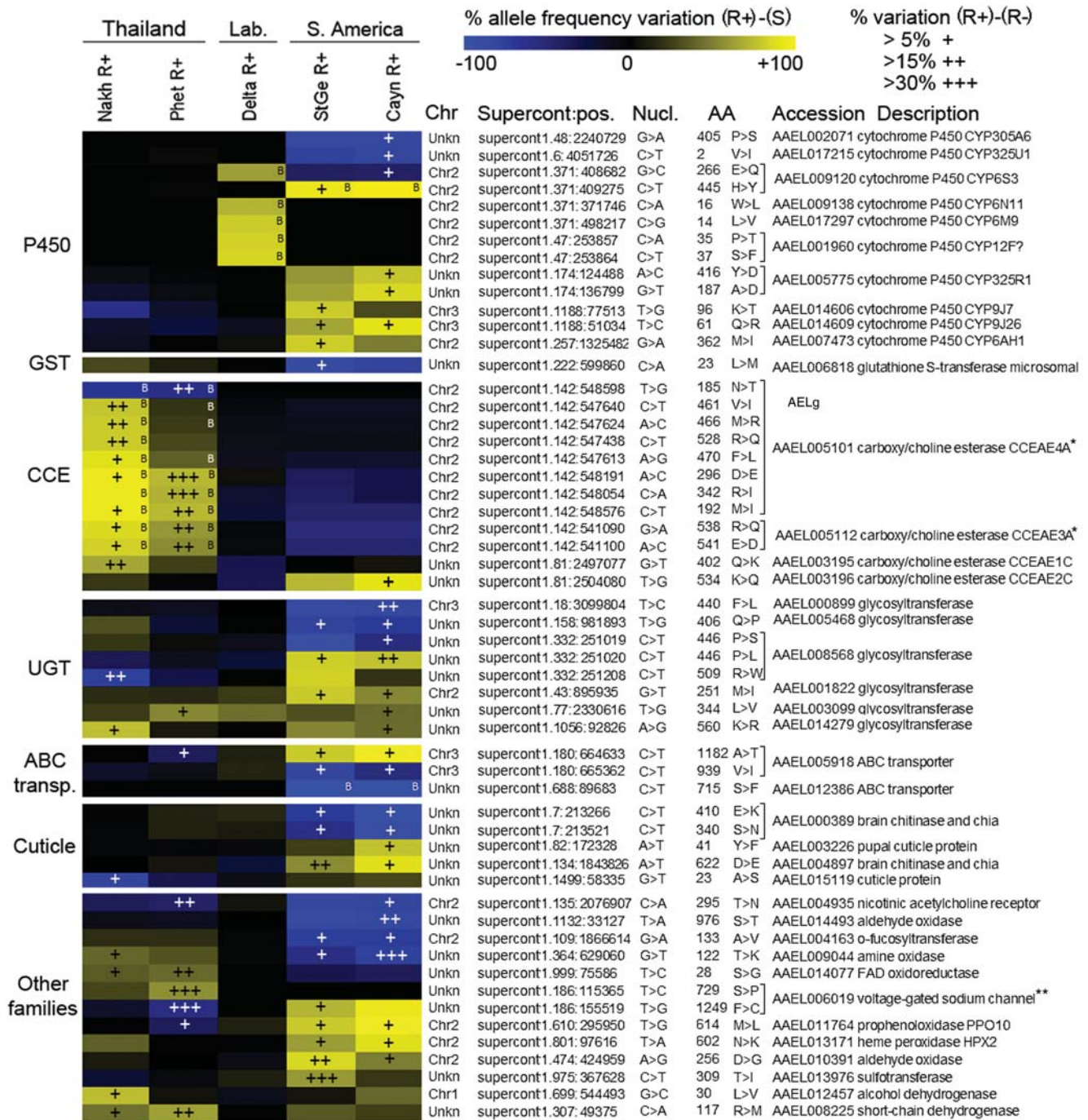
**Figure 4.** Best nonsynonymous polymorphisms associated with deltamethrin resistance. Only the 55 best differential nonsynonymous variants identified from frequency-based filtering and the Bayesian approach are shown (see Methods). For each region, allele frequency variation between each resistant population (R+ phenotypes) and their susceptible counterpart (S) are shown as a blue-yellow color scale. Blue indicates an enrichment in the reference allele, whereas yellow indicates an enrichment in the variant allele. Variants identified by the Bayesian approach are indicated by "B" marks. For variants passing frequency-based filtering or BayeScan3 filtering, allele frequency variation between R+ and R− phenotypes are shown as overimposed "+" marks. Variants are grouped by gene families and are described by the following annotations: chromosomal location (according to Juneja et al. 2014; Timoshevskiy et al. 2014), supercontig position, nucleotide change, amino acid position, amino acid change, gene accession number, and gene description. (*) Genes also found affected by CNVs linked to deltamethrin resistance. (**) The sodium channel S729P and F1249C variants correspond to the S989P and F1534C kdr mutations described in the literature due to changes in AAEL006019-RD transcript annotation.

study screened for both CNVs and polymorphisms across several samples. Our experimental design allowed reaching a very deep coverage (>80×) across multiple resistant and susceptible popula-

tions originating from different geographical areas, providing the first comprehensive screening of CNVs and polymorphisms associated with insecticide resistance. Targeted DNA-seq data were

consistent with qPCR data obtained from individual mosquitoes validating the robustness of the approach. Altogether, this study demonstrates that target enrichment coupled with high-throughput sequencing is a powerful tool for pinpointing genomic changes associated with adaptive traits from which candidate gene families are known.

## Gene amplifications associated with pyrethroid resistance

Only a few CNVs were previously associated with insecticide resistance, and no comprehensive screening has ever been conducted in mosquitoes (for review, see Bass and Field 2011). The present study identified 41 genes affected by gene amplifications linked to deltamethrin resistance. Most were previously found significantly overtranscribed in pyrethroid resistant *Ae. aegypti* populations according to data publicly available in the VectorBase expression browser (https://vectorbase.org, data sets from Strode et al. 2008; Marcombe et al. 2009; Poupardin et al. 2012; David et al. 2014). The majority of CNVs observed between resistant and susceptible populations were <2.5-fold, suggesting a predominance of duplications. However several genes displayed CNV >fivefold in particular resistant populations, suggesting that copy number may vary within continents. This was confirmed by qPCR on individual mosquitoes showing an important copy number polymorphism within and across resistant populations. Such polymorphism suggests that most amplifications are not fixed in resistant populations. The strong relationship between gene amplification and overtranscription was confirmed by RT-qPCR. Although, overexpression can also be triggered by *cis*/*trans* regulatory elements or post-translational events, the present study suggests that gene amplification is a common adaptive mechanism allowing mosquitoes to overexpress detoxification genes conferring resistance to insecticides. The importance of such mechanism may even be greater in species highly infected by transposable elements like *Ae. aegypti* because their presence is known to favor duplication events (Nene et al. 2007).

The present study revealed distinct gene amplification patterns among resistant populations according to their geographical origin. This supports that the selection of gene amplifications conferring resistance depends on the genetic background of populations, their population dynamics, and on the selection pressures they undergo. Most amplified genes encoded detoxification enzymes, suggesting that gene amplification is rather linked to metabolic resistance than resistance conferred by an altered transport/ penetration of the insecticide.

The majority of detoxification enzymes affected by gene amplifications encoded P450s belonging to *CYP9J* and *CYP6* families that were frequently associated with insecticide resistance in mosquitoes (Vontas et al. 2012; David et al. 2013). The overrepresentation of P450s was expected because their overexpression is known to play a key role in pyrethroid resistance, and they are frequently affected by copy number variants (Feyereisen 2006, 2011). Several amplified *CYP9Js* were previously found overtranscribed in resistant populations and some of them (*CYP9J24*, *CYP9J28*, *CYP9J32*) have been validated as able to metabolize pyrethroids (Stevenson et al. 2012), supporting the importance of their amplification in resistance. Among the *CYP6s* amplified, most were previously found overtranscribed in resistant populations, and *CYP6BB2* was recently involved in pyrethroid metabolism (Kasai et al. 2014). In addition, some of their close orthologs in *Anopheles* (*CYP6P3*, *CYP6P7*, *CYP6P9*, *CYP6M2*, and *CYP6AA5*) are also capable of degrading pyrethroids (Boonsuepsakul et al. 2008; Müller

et al. 2008; Duangkaew et al. 2011; Stevenson et al. 2011; Riveron et al. 2013). Altogether, these results confirm the key role of P450 in the resistance of mosquitoes to pyrethroids and their propensity to increase their expression level through gene amplification when undergoing a strong selection pressure from insecticides.

Three CCEs (*CCEAE3A*, *CCEAE4A*, and *CCEAE6A*) belonging to a single genomic cluster were highly amplified in Thai resistant populations leading to their strong overtranscription. Highest amplification levels were found in the Nakh R population which is also resistant to organophosphates (Poupardin et al. 2014). In this previous study, the overtranscription of *CCEAE3A* and *CCEAE6A* through gene amplification was associated with temephos resistance at the larval stage, although resistance to pyrethroids was also observed. Other studies performed on South American and Caribbean populations confirmed the association of some of these CCEs with temephos resistance (Marcombe et al. 2009, 2012; Saavedra-Rodriguez et al. 2014). Our data revealed higher CCE copy number in mosquitoes surviving deltamethrin exposure (R+ phenotype), supporting their role in pyrethroid resistance. Although the association between pyrethroid resistance and these CCE genes in Thailand might be due to multiresistant individuals carrying both pyrethroid- and organophosphate-resistance genes, the role of CCEs in pyrethroid metabolism has been demonstrated in mammals (Hodgson 2003; Nakamura et al. 2007) and mosquitoes (Somwang et al. 2011; Chandor-Proust et al. 2013). However, no individual mosquito CCE has yet been validated as a pyrethroid metabolizer, and insecticide sequestration may also confer resistance. Further functional studies are required for validating the precise role of these amplified CCEs in pyrethroid resistance.

Two sulfotransferases were amplified in Thai resistant populations. The overtranscription of these enzymes has been frequently described in insecticide resistant mosquitoes and deserve further attention (Poupardin et al. 2012; Marcombe et al. 2013; Nkya et al. 2013). Indeed, their involvement in insecticide degradation pathways has been validated in mammals (Lee et al. 2007).

Among GSTs, only *GSTE2* was found amplified in Thai resistant populations. This GST is known for its ability to metabolize DDT in various mosquito species (Ortelli et al. 2003; Lumjuan et al. 2005), and recent studies confirmed its role in pyrethroid resistance although pyrethroid metabolism has not been observed (Lumjuan et al. 2011; Riveron et al. 2014b).

A recent study suggested that the duplication of the gene encoding the *Ae. aegypti* voltage-gated sodium channel (VGSC) targeted by pyrethroids contributed to resistance by maintaining together wild-type allele and *kdr* mutations and reducing mutant allele deleterious effects (Martins et al. 2013). A similar role was suggested for the duplication of the acetylcholinesterase gene (carrying the *Ace1* mutation) in the resistance of *An. gambiae* to carbamates (Edi et al. 2014). Although such a mechanism is likely contributing to resistance in mosquitoes, no amplification of genes encoding proteins targeted by insecticides were detected in our study. In addition, a poor association was observed between the presence of *kdr* mutations and gene amplifications affecting detoxification enzymes in individual mosquitoes. Indeed, recombination events are probably countering such association, especially as the *VGSC* gene and amplified detoxification genes are located on distant genomic regions. In addition, fitness costs associated with both *kdr* mutations and gene amplifications have been identified (Kondrashov 2012; Brito et al. 2013; Katju and Bergthorsson 2013; Rinkevich et al. 2013).Therefore, although *kdr* mutations and gene amplifications of detoxification enzymes

co-occur in resistant populations, additive fitness costs may contribute to segregate them at the individual level.

## Polymorphisms associated with pyrethroid resistance

Apart from mutations affecting the proteins targeted by insecticides, very few nonsynonymous variants have been associated with insecticide resistance. In this context, the present study represents the first DNA-seq screening of variants associated with pyrethroid resistance in mosquitoes. Searching for selection footprints identified multiple genomic regions under selection, suggesting that pyrethroid resistance is polygenic. Several nonsynonymous variants were associated with deltamethrin resistance, but few were found in multiple geographic regions, confirming the strong influence of genetic background and population history on the selection of resistant alleles.

Although several nonsynonymous variants affecting P450s were identified from each region, those mostly associated with resistance were mainly detected in South American and laboratory populations. Some were previously identified in a permethrin-resistant strain by RNA-seq (David et al. 2014). In *Drosophila melanogaster*, functional studies demonstrated that nonsynonymous mutations in the P450 *CYP6A2* identified in the RDDT[R] resistant strain have a prominent role in resistance by enhancing DDT metabolism (Amichot et al. 2004). In the mosquito, *Anopheles funestus*, directional selection footprints were found in the coding sequence of two *CYP6Ps* capable of metabolizing pyrethroids (Riveron et al. 2014a). Most nonsynonymous variants impacting CCEs were identified in Thailand with *CCEAE4A* and *CCEAE3A* impacted by multiple variants strongly associated with deltamethrin resistance. Elevated copy numbers of these genes were also identified in Thailand, suggesting that their amplification may have been followed by their neofunctionalization under insecticide selection pressure (Katju and Bergthorsson 2013). Interestingly, the best orthologous gene of *CCEAE4A* in the sheep blowfly, *Lucilia cuprina* (LcαE7), encodes an esterase associated with organophosphate resistance through a single point mutation enhancing its activity toward these chemicals (Newcomb et al. 1997). Later, it was shown that another mutation of this gene allowed this enzyme to metabolize synthetic pyrethroids (Heidari et al. 2005; Devonshire et al. 2007). More recently, multiple randomly generated nonsynonymous mutations of this gene were shown to enhance its activity toward various pyrethroids including deltamethrin (Coppin et al. 2012). Although additional work is necessary for validating the role of these nonsynonymous variants, our data support the selection of detoxification enzyme variants as being a key process in the adaptation of mosquitoes to chemical insecticides.

Finally, multiple nonsynonymous variants were detected in the gene encoding the voltage-gated sodium channel targeted by pyrethroids (*kdr* mutations). Their frequencies were consistent with those previously reported in Thailand and South America (Stenhouse et al. 2013; Linss et al. 2014). However, neither of the two mutations extensively used as resistance markers (V1016I/G) were retained as best candidates. Although the V1016I mutation showed high frequencies in South American resistant populations, it showed a poor association with deltamethrin survival phenotype. The V1016G mutation was associated with resistance in one Thai population but was not retained among the best candidates according to our criteria. Instead, two other less-known mutations (S989P and F1534C) showed a stronger association with deltamethrin resistance. Such a result supports the

substantial contribution of other mechanisms in the resistance phenotype.

## New tools for monitoring resistance genes in mosquito populations

Overall, the present study confirms that gene amplifications affecting detoxification enzymes are good markers of metabolic resistance in mosquitoes. Novel nonsynonymous variants potentially enhancing their ability to detoxify insecticides were also identified. Despite significant research efforts, developing molecular tests to track the overexpression of these enzymes in field populations has proved challenging in terms of affordability, throughput, and reliability; and current vector population monitoring tools do not include metabolic resistance markers (Bass et al. 2010). Detecting their increased copy number or the presence of specific variants conferring resistance through simple PCR-based DNA assays opens up new perspectives for managing insecticide resistance. Indeed, such novel resistance markers will be accessible to developing countries in terms of technology and affordability and will allow tracking down both target-site and metabolic-resistance alleles in individual mosquitoes and monitoring their frequencies through time and space among and within natural populations.

# Methods

## Mosquitoes

Nine *Ae. aegypti* populations of distinct genetic backgrounds were used. These included four populations susceptible to insecticides and five populations showing elevated resistance to deltamethrin. Two distant geographic areas where pyrethroid resistance is threatening mosquito control were studied: South East Asia (Thailand) and South America (French Guiana). Two resistant and one susceptible population having a close genetic background were studied in each area (see Table 2 for populations' information). In addition, one laboratory population selected with deltamethrin for five generations was used together in comparison to its susceptible "parental" population. The laboratory Liverpool population used for public genome sequencing (Liverpool population) (Nene et al. 2007) was used as an additional susceptible population. Resistant populations from Thailand and French Guiana were colonized for two generations in the laboratory without insecticide selection pressure before assessing resistance levels and sample collection.

## Deltamethrin resistance levels

For each population, the exposure time necessary to knock down 50% of individuals ($KDT_{50}$) was determined on 2- to 4-d-old females using test tubes equipped with Whatman filter paper impregnated with 0.05% deltamethrin in silicone oil according to standard WHO procedure (WHO 2006). Then, a collection of Whatman filter papers impregnated with different concentrations of deltamethrin (from 0.005% to 0.75%) was used for evaluating the lethal dose for 50% of individuals ($LD_{50}$) of each population. Bioassays were conducted in duplicates of 40 2- to 4-d-old females, and mortality was recorded after 1 h exposure to deltamethrin and 24 h recovery without insecticide. For each resistant population, the impregnated paper killing 40%–60% mosquitoes after 1 h exposure was then used to segregate the most susceptible individuals (dead at $LT_{25}$: R– phenotype) from the most resistant ones

**Table 2.** Sample descriptions

| Population | Group | Full name | Origin | History (year colonized) | Deltamethrin resistance |
|---|---|---|---|---|---|
| Livp S | Genome | Liverpool (genome) | West Africa | Laboratory colony (1930s) | Susceptible |
| Bora S | Laboratory | Bora-Bora | French Polynesia | Laboratory colony (1990s) | Susceptible |
| Delta R | Laboratory | Deltamethrin R | French Polynesia | Bora S selected | Slightly resistant |
| Patg S | Thailand | Patthalung | South Thailand | Laboratory colony (2013) | Susceptible |
| Phet R | Thailand | Phetchaburi | SW of Bangkok | Field collected | Resistant |
| Nakh R | Thailand | Nakhon Sawan | Center Thailand | Field collected | Resistant |
| NwOr S | S. America | New Orleans | Louisiana | Laboratory colony (1980s) | Susceptible |
| Cayn R | S. America | Cayenne | French Guiana | Field collected | Highly resistant |
| StGe R | S. America | St-Georges de l'Oyapock | French Guiana | Field collected | Highly resistant |

(survivors at $LT_{75}$: R+ phenotype) by adjusting the exposure time from 10 min to 3 h.

## Sample preparation

All sampled mosquitoes consisted of 2- to 4-d-old non-blood-fed *Ae. aegypti* females grown in standardized laboratory conditions. For each population/phenotype, 200 individuals were collected and stored individually in silica gel. For each population, three pools of 30 females not exposed to insecticide were also collected and stored in RNAlater (Life Technologies) for gene expression analyses. Gene amplification screening by exon capture and mass sequencing was performed on gDNA extracted from two pools of 65 individuals per population/phenotype. Genomic DNA was extracted using the PureGene Core Kit A (Qiagen) following the manufacturer's instructions. For each population/phenotype, gDNA from each pool were then combined in equal quantity in order to be representative of a total of 130 individuals. Quantitative PCR validation of gene amplifications and genotyping of *kdr* mutations was performed on gDNA extracted from 12 single adult females per population/phenotype using the method described in Collins et al. (1987) and resuspended in 20 μL nuclease-free water. Gene expression analyses were performed on RNA extracted from three pools of 30 adult females per population (R and S populations) using the RNAqueous4PCR kit (Life Technologies) according to the manufacturer's instructions and resuspended in 50 μL nuclease-free water.

## Capture of target regions and sequencing

Gene capture was performed by Hybrigenics-Helixio (Clermont-Ferrand, France) using the SureSelect target enrichment system (Agilent). Capture library consisted in 51,073 overlapping RNA probes of 120 bp (baits) targeting 3458 exons belonging to 789 genes. The mean coverage of target regions was 4×. These genes were chosen according to their putative role in insecticide resistance. These genes include all detoxification enzymes sensu lato, all cuticle proteins, all ABC transporters together with several ion channels, redox enzymes, and synaptic proteins (list of captured genes in Supplemental Table 5). Capture of target genes was performed according to "SureSelect Target Enrichment System for Illumina Paired-end Sequencing Library version 1.5" protocol. Briefly, 3 μg gDNA were fragmented using a Bioruptor (Diagenode), ligated to adaptors, and amplified by PCR using Herculase II DNA polymerase (Agilent). After QC analysis, libraries were hybridized to biotinylated baits and purified using Dynabeads MyOne Streptavidin T1 beads (Life Technologies). Captured DNA fragments were amplified, purified, and multiplexed before sequencing. Paired-end sequencing was performed on a Genome Analyzer IIx (Illumina) producing 76 bp reads. An average sequencing coverage of greater than 60× was expected for each sample.

## Reads filtering and mapping

Sequenced reads were assigned to each sample (unplexing), and adaptors were removed. Overall read quality was checked for each sample using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Reads were then filtered based on their length, pairing, and quality using Trimmomatic (Bolger et al. 2014). Parameters were set as follows: read length ≥76 bp; mean Phred quality score greater than 30; and Phred quality score greater than 30 across all reads (10-bp sliding window). Only paired reads were kept for mapping. Reads were mapped to the *Ae. Aegypti* genome (AaegL2 assembly) using BWA (Li and Durbin 2009) with default parameters implemented into a Galaxy pipeline (http://galaxyproject.org). Mapped reads were loaded into Genespring NGS version 12.5 (Agilent) and further filtered according to their mapping quality (alignment score of 95 or above). In case of multiple mapped reads, only primary hits were conserved.

## Copy number variation analysis

Coverage of target genes was quantified for each sample and normalized according to the total number of filtered reads per library. Only genes showing more than 90 reads per kilobase exon model per million sequenced reads (RPKM) in all samples were considered for further analysis. Detection of copy number variation (CNV) was based on differential coverage between samples obtained from resistant and susceptible populations. Fold changes (FC) between resistant samples (R+ and R− phenotypes) and all susceptible populations (meanS) were computed for each gene. Genes satisfying the following criteria in any resistant population were considered affected by gene amplifications in association with deltamethrin resistance: [(R+)/(meanS) FC ≥ 1.5] and [(R+)/(R−) FC] > 1 (i.e., 1.5-fold more copies in resistant samples and more copies in $LD_{75}$ survivors compared to $LD_{25}$ dead).

## Validation of gene amplifications on individual mosquitoes

Gene amplifications detected by previous CNV analysis were further studied by qPCR on 156 individual mosquitoes (12 from each population/phenotype). Nine candidate genes located in three different genomic clusters affected by gene amplifications were selected: Supercontig 1.1188: AAEL014614 (*CYP9J?*), AAEL014615 (*CYP9J23*), and AAEL014617 (*CYP9J28*); Supercontig 1.1327: AAEL014890 (*CYP6CC1*), AAEL014891 (*CYP6P?*), and AAEL014893 (*CYP6BB2*); Supercontig 1.142: AAEL05112 (*CCEAE3A*), AAEL005101(*CCEAE4A*), and AAEL005122

(*CCEAE6A*). PCR primers targeting exonic regions were designed using NCBI Primer BLAST and checked for specificity against the whole *Ae. aegypti* genome (Supplemental Table 6). Two target genes showing a constant copy number across all samples from sequencing data (see above) were used for normalization (AAEL005950 and AAEL007808). Real-time quantitative PCR was performed on an iQ5 cycler (Bio-Rad). PCR reactions consisted of 3 μL gDNA template (see above), 3.6 μL nuclease free water, 0.45 μL of each primer (10 mM), and 7.5 μL of SYBR Green Supermix 2× (Bio-Rad). A dilution scale made from a pool of all gDNA samples was used for assessing PCR efficiency and quantification. All samples were amplified in triplicates (14 populations × 12 individuals × 3 replicates). After normalization, CNVs were expressed as mean relative gDNA quantity compared to one individual of the Livp S population.

### Impact of gene amplifications on transcription levels

The link between gene amplifications and increased transcription levels was investigated on nine genes (see above) by RT-qPCR. Total RNA samples from each population were treated with DNase I (Invitrogen) to remove genomic DNA following the manufacturer's instructions. Reverse transcription and qPCR reactions were performed as described in Nkya et al. (2014). Data analysis was performed according to the $\Delta\Delta_{Ct}$ method taking into account PCR efficiency (Pfaffl 2001) and using the housekeeping genes encoding the ribosomal proteins L8 (AGAP005802) and S7 (AGAP010592) for normalization. Three technical replicates were performed per population, and results were expressed as mean transcription ratio in each resistant population ± SD relative to the mean transcription ratio of all susceptible populations.

### Polymorphism calling and population structure

Polymorphisms (SNPs, multiple nucleotide polymorphisms [MNPs], and indels) were called against the reference genome using filtered reads by using Genespring NGS version 12.5 (Agilent). Calling parameters were as follows for each sample: calling score of 50 or above ($P$-value $< 10^{-5}$); ignore homopolymer 10 or higher and surrounding positions; locus coverage of 30 or more. Variants identified from each sample were further filtered based on their coverage and strand bias across all samples (coverage 30 or more; strand bias 140 or less). For each variant, allele frequencies were estimated for each population based on the number of reads supporting each allele, and genic effects were computed against the reference genome. A principal component analysis (PCA) based on allele frequencies of all polymorphic variants across all samples was used for inferring the genetic structure of the studied populations and confirming the relevance of the geographical groups.

### Searching for selection footprint using a hierarchical Bayesian approach

BayeScan3 was used for identifying loci under natural selection through a hierarchical Bayesian model, taking into account population structure and usable on pools of individuals (Foll et al. 2014). This approach estimates the probability that each locus is subject to selection by discriminating population-specific and locus-specific components of the fixation index using a logistic regression. The posterior probability of a given locus being under selection is assessed by defining two alternative models, one including the locus-specific effect and the other excluding it.

Departure from neutrality is supposed when the locus-specific component is necessary to explain the observed pattern of diversity using a reversible jump Markov chain Monte Carlo (MCMC). Because the high genetic proximity between R+ and R− phenotypes can bias the estimation of the neutral model, only S populations and R+ phenotypes from resistant populations were used. For the same reasons, loci showing allele frequency <5% or >95% in all samples were not considered. BayeScan3 was run with default parameters and the following hierarchical structure: Thailand (Patg S, Nakh R+, and Phet R+), South America (NwOr S, Cayn R+, StGe R+), and Laboratory (Bora S, Delta R+). Loci showing a $F_{sc}$ Q-value $\leq 0.05$ in any geographical group were considered under selection (i.e., outlier locus). These loci were further filtered based on their association with the resistance phenotype by retaining only those showing both [f(R+)-f(S)] and [f(R+)-f(R−)] metrics being positive (enrichment of variant allele) or negative (enrichment of reference allele). Because the *Ae. aegypti* genome is not assembled, selection footprints were looked for at the supercontigs scale. For each supercontig, the density of outlier loci was computed by dividing the number of outlier loci by the total length of captured regions within the supercontig. In order to avoid inferring selection footprint from limited genomic coverage, only supercontigs represented by not more than four genes or from which the captured region represented >10% of supercontig length were considered.

### Identifying polymorphisms mostly associated with deltamethrin resistance

Allele frequency-based filtering was combined with the Bayesian approach for identifying polymorphisms most strongly associated with deltamethrin resistance. Frequency-based filtering consisted in retaining alleles satisfying the following conditions in any resistant/susceptible population pair from each geographical group: [f(R+)-f(S) $\geq$ 40%] and [f(R+)-f(R−) $\geq$ 5%] and both metrics being positive or negative. Alleles passing both the frequency-based filtering and the Bayesian approach were retained. In addition, the best candidates obtained by each approach were also retained. For the Bayesian approach, this included loci showing $F_{sc}$ Q-values within the best 10th percentile from each region. For frequency-based filtering, this includes alleles passing frequency-based filtering and showing the five highest Abs{[f(R+)-f(S)]+[f(R+)-f(R−)]} metric from each region. Variants most associated with deltamethrin resistance were then filtered according to their genic effects and those being nonsynonymous (i.e., affecting protein sequence) were retained.

### Validation of polymorphism data and association between *kdr* mutations and gene amplifications

Allele frequencies obtained by high-throughput sequencing were validated on *kdr* mutations (V1016I, V1016G, and F1534C) on 156 individuals (12 individual mosquitoes from each population/phenotype). Genomic DNA was extracted as described above, and *kdr* mutations were detected by qPCR following the method described in Saavedra-Rodriguez et al. (2007). Associations between the presence of *kdr* mutations and gene amplifications were tested across the five resistance populations for the three *kdr* mutations and the nine genotyped gene amplifications by testing linear correlations between the *kdr* gentoype (0: homozygote wildtype, 0.5: heterozygote, 1: homozygote resistant) and gene amplification levels.

## Data access

The sequence data from this study have been submitted to the European Nucleotide Archive (ENA; http://www.ebi.ac.uk/ena) under accession number PRJEB7976.

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