1 **A genomic amplification affecting a carboxylesterase gene cluster confers**

2 **organophosphate resistance in the mosquito *Aedes aegypti*: from genomic**

3 **characterization to high-throuput field detection**

4 **Running head**: genomic amplification associated with insecticide resistance

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Abstract

By altering gene expression and creating paralogs, genomic amplifications represent a key component of short-term adaptive processes. In insects, the use of insecticides can select gene amplifications causing an increased expression of detoxification enzymes, supporting their usefulness for monitoring the dynamics of resistance alleles in the field. In this context, the present study aims to characterise a genomic amplification event associated with resistance to organophosphate insecticides in the mosquito *Aedes aegypti* and developing a molecular assay to monitor the associated resistance alleles in the field. An experimental evolution experiment using a composite population from Laos confirmed the association between the over-transcription of multiple contiguous carboxylesterase genes on chromosome 2 and resistance to multiple organophosphate insecticides. Combining whole genome sequencing and qPCR on specific genes confirmed the presence of a ~100 Kb amplification spanning at least five carboxylesterase genes at this locus with the co-existence of multiple structural duplication haplotypes. Field data confirmed their circulation in South-East Asia and revealed high copy number polymorphism among and within populations suggesting a trade-off between this resistance mechanism and associated fitness costs. A dual-colour multiplex TaqMan assay allowing the rapid detection and copy number quantification of this amplification event in *Ae. aegypti* was developed and validated on field populations. The routine use of this novel assay will improve resistance tracking in this major arbovirus vector.

Key words: genomic amplification, insecticide resistance, mosquito, *Aedes aegypti*, carboxylesterase, diagnostic assay

1 | INTRODUCTION

The amplification of genomic regions overlapping genes can affect fitness by creating paralogs that can diverge to generate novel functions or by altering gene expression levels (Iskow et al., 2012). Often called genomic amplification when multiple copies are present, such mechanism has been shown to be a major driver of short-term adaptation (Kondrashov, 2012). Indeed, amplification events are frequent in natural populations (Campbell & Eichler, 2013) and can be subjected to positive selection (Chatonnet et al., 2017; Cooper et al., 2007; Zhang, 2003).

In insects, genomic amplifications have been shown to play a key role in the evolution of insecticide resistance by three distinct mechanisms. First, the duplication of genes encoding insecticide targets can allow resistant individuals to reduce the fitness costs associated with target-site mutations by allowing the co-existence of the susceptible and the resistant alleles (Assogba et al., 2015; Labbé et al., 2007). Second, the neofunctionalization of new gene copies can lead to novel adaptive functions (Zimmer et al., 2018). Third, genomic amplifications affecting genes encoding detoxification enzymes leading to their over-expression can confer the insect a higher capacity to degrade or sequester insecticides (Bass & Field, 2011). This later mechanism has been reported in all three mosquito genera of high medical importance, *Aedes*, *Anopheles* and *Culex*, affecting various detoxification enzyme families including cytochrome P450 monooxygenases (*P450*s) and carboxy/choline esterases (*CCE*s) (Cattel et al., 2019; Lucas et al., 2019; Weetman et al., 2018).

Genomic amplifications affecting *CCE*s have even been described as the most common route of enzyme over-production in mosquitoes (Bass & Field, 2011). A classic example comes from the house mosquito *Culex pipiens* in which amplified carboxylesterases genes (in conjunction with the *ace-1* G119S target-site mutation) have spread across the globe, providing high resistance to organophosphate insecticides (Raymond et al., 2001). In *Aedes* mosquitoes, the low chance of occurrence of the G119S *ace-1* mutation because of strong genetic constraints

97   (Weill et al., 2004) suggest that *CCE* amplifications play a central role in organophosphate

98   resistance and are thus of high interest for resistance monitoring.

99   In the tiger mosquito *Aedes albopictus*, the over-expression of two *CCE* genes (*CCEAE3A* and

100   *CCEAE6A*) through gene amplification was associated with resistance to the organophosphate

101   insecticide temephos (Grigoraki et al., 2017). In the yellow fever mosquito *Aedes aegypti*,

102   AAEL023844 (formerly *CCEAE3A*) and other *CCE* genes belonging to the same genomic

103   cluster were also found overexpressed through gene amplification in temephos-resistant

104   populations (Faucon et al., 2015, 2017; Poupardin et al., 2014). Further functional studies

105   confirmed that *CCEAE3A* is able to sequester and metabolize the active form of temephos in

106   both *Ae. aegypti* and *Ae. albopictus* (Grigoraki et al., 2016). Although the genomic structure

107   and polymorphism of this *CCE* amplification was studied in *Ae. albopictus* (Grigoraki et al.,

108   2017) such work has not been conducted in *Ae. aegypti*. In addition, the role of *CCEAE3A*

109   amplification in resistance to other insecticides remains unclear. Finally, no high-throughput

110   assay has yet been developed to track this resistance mechanism in natural populations although

111   such tool would significantly ease resistance monitoring and management.

112   In this context, we combined an experimental evolution experiment with RNA-seq and

113   whole genome sequencing to confirm the association between this genomic amplification, the

114   overexpression of *CCE* genes and resistance to the organophosphate insecticide malathion in

115   *Ae. aegypti*. Bioassay data also support the importance of the *CCE* amplification in resistance

116   to other organophosphates insecticides. Comparing gene Copy Number Variations (CNV)

117   between the different genes of this genomic cluster suggested the presence of at least two

118   distinct haplotypes occurring in South-East Asia (SEA), both associated with resistance.

119   Investigating their spatial dynamics in natural populations confirmed their co-occurrence in the

120   field with a high copy number polymorphism within and among populations. Based on these

121   results, we developed a novel high-throughput multiplex TaqMan assay allowing the

4

122 quantitative detection of this *CCE* amplification in hundreds of individual mosquitoes within a

123 few hours. By reducing the human power and infrastructure needs associated with bioassays,

124 this molecular assay will improve the tracking and management of organophosphate resistance

125 in natural mosquito populations.

126

127 2 | MATERIAL AND METHODS

128

129 2.1 | Field sampling and mosquito lines

130 *Ae*des *aegypti* larvae and pupae were collected in households and temples of eleven villages

131 belonging to five provinces of Laos in 2014 (Table S1). Previous work confirmed the

132 circulation of organophosphate and pyrethroid resistance alleles in these populations together

133 with the presence of amplification affecting AAEL023844 (formerly *CCEAE3A*) (Marcombe

134 et al., 2019). These populations were maintained under controlled conditions (27 ± 2 ºC and 80

135 ± 10% relative humidity) at the Institut Pasteur of Laos for 5 generations and used for

136 experimental evolution (see below).

137 A second round of larvae collection was conducted in 2017 for studying the spatial dynamics

138 of *CCE* genomic amplifications in SEA. Fourteen different populations were sampled in Laos,

139 Thailand and Cambodia (see details in Table S1) and adults were stored individually at -20°C

140 in silica gel until molecular analyses.

141

142 2.2 | Experimental selection

143 A Laos composite population was created by pooling 50 virgin males and 50 virgin females of

144 each population into a single cage (Table S1). This population was then maintained for 2

145 generations without insecticide selection to allow genetic mixing before initiating insecticide

146 selection. The Laos composite population was then split in 2 lines (N > 1000 in each line): one

147   line being maintained without insecticide selection (NS line) while the second line (Mala line)

148   was artificially selected with malathion at the adult stage for 4 consecutive generations (from

149   G1 to G5). For this, batches of thirty-three-day-old non-blood-fed adult mosquitoes (~1000

150   individuals of mixed sex) were exposed at each generation to filters papers impregnated with

151   malathion using WHO test tubes. A constant dose of 5% malathion coupled with an exposure

152   time of 10 min (leading to ~90% mortality at G1) were used through the whole selection

153   process. Surviving adults (mainly females) were collected 48h after insecticide exposure, blood

154   fed on mice and allowed to lay eggs to generate the next generation.

155   Three-day-old non-blood-fed adult females (not exposed to insecticide) were sampled after four

156   generations and used for bioassays and molecular work. Mosquitoes were identified as follows.

157   G1: initial composite population, G5-NS: line maintained without insecticide pressure for four

158   generations, G5-Mala: line maintained under malathion selection for four generations. Sampled

159   mosquitoes were stored at -20°C until molecular analyses.

160

161   **2.3 | Bioassays**

162   Bioassays were used to monitor the dynamics of malathion resistance during the selection

163   process. Four replicates of 20 three-day-old non-blood-fed females not previously exposed to

164   insecticide and reared in same insectary conditions were sampled at each generation and

165   exposed to the insecticide as described above using the same dose and exposure time as for

166   artificial selection. Mortality was recorded 48h after exposure.

167   Cross resistance to other insecticides was investigated in G5 individuals (G5-Mala and G5-NS)

168   not previously exposed to insecticide. Calibrated individuals were exposed to three distinct

169   insecticides: the organophosphates fenitrothion and temephos, and the pyrethroid deltamethrin.

170   For the adulticides fenitrothion and deltamethrin, bioassays were performed on eight replicates

171   of fifteen three-day-old non-blood-fed females with the following doses and exposure times:

172 fenithrotion 1% for 30 min, deltamethrin 0.05% for 20 min. Mortality rates were recorded 48h

173 after exposure. For the larvicide temephos, bioassays were performed on eight replicates of

174 twenty calibrated third instar larvae exposed to 0.08 mg/µL temephos for 24h in 200 ml tap

175 water and mortality was recorded at the end of exposure.

176

177 **2.4 | RNA sequencing**

178 RNA-sequencing was performed to compare gene expression levels between the NS line, the

179 Mala line and the fully susceptible reference line Bora-Bora. This experiment was performed

180 on unexposed G6 individuals (progeny of the last generation of selection) in order to avoid gene

181 induction/repression effects that can be caused by insecticide exposure. For each line, four

182 RNA-seq libraries were prepared from distinct batches of 25 calibrated three-day-old non-

183 blood-fed females not exposed to insecticide. Total RNA was extracted using Trizol® (Thermo

184 Fisher Scientific) following manufacturer's instructions. RNA samples were then treated with

185 the RNase-free DNase set (Qiagen) to remove gDNA contaminants and QC checked using

186 Qubit (Thermo Fisher Scientific) and bioanalyzer (Agilent). RNA-seq libraries were prepared

187 from 500 ng total RNA using the NEBNext® Ultra^{TM} II directional RNA library Prep Kit for

188 Illumina (New England Biolabs) following manufacturer's instructions. Briefly, mRNAs were

189 captured using oligodT magnetic beads and fragmented before being reverse transcribed using

190 random primers. Double stranded cDNAs were synthesized end-repaired and adaptors were

191 incorporated at both ends. Libraries were then amplified by PCR for 10 cycles and purified

192 before QC check using Qubit fluorimeter and Bioanalyzer. Libraries were then sequenced in

193 multiplex as single 75 bp reads using a NextSeq500 sequencer (Illumina).

194 After unplexing and removing adaptors, sequenced reads from each library were loaded into

195 Strand NGS V3.2 (Strand Life Science) and mapped to the latest *Ae. aegypti* genome assembly

196 (Aaeg L5) using the following parameters: min identity = 90%, max gaps = 5%, min aligned

197 read length = 25, ignore reads with >5 matches, 3'end read trimming if quality <20, Kmer size

198 = 11, mismatch penalty = 4, gap opening penalty = 6, gap extension penalty = 1. Mapped reads

199 were then filtered based on their quality and alignment score as follows: mean read quality >

200 25, max N allowed per read = 5, mapping quality ≥120, no multiple match allowed, read length

201 ≥ 35. Quantification of transcription levels was performed on the 14626 protein-coding genes

202 using the DESeq method with 1000 iterations (Anders & Huber, 2010). Only the 11825 genes

203 showing a normalized expression level ≥ 0.5 (~0.05 RPKM) in all replicates for all lines were

204 retained for further analysis. Differential gene transcription levels between each line across all

205 replicates were then computed using a one-way ANOVA followed by a Tukey post-hoc test

206 and P values were corrected using the Benjamini and Hockberg multiple testing correction

207 (Benjamini & Hochberg, 1995). Genes showing a fold change (FC) ≥ 3 (in either direction) and

208 a corrected P value ≤ 0.001 in the G6-Mala line versus both susceptible lines (G6-NS and Bora-

209 Bora) were considered as differentially transcribed in association with insecticide resistance.

210

2.5 | Whole genome sequencing

212 The occurrence of a genomic amplification affecting the *CCE* cluster on chromosome 2 at ~174

213 Mb was investigated by sequencing the whole genome of the Nakh-R population from Thailand

214 as compared to the fully susceptible line Bora-Bora. This population was used for the genomic

215 characterization of this CCE amplification because *i*) this population was known resistant to

216 carry organophosphate resistance alleles (Faucon et al., 2015), *ii*) it showed an over-expression

217 of this CCE gene cluster likely associated with a genomic amplification (Faucon et al., 2015,

218 2017) *iii*) it was collected from the field and therefore can be used to control for genetic drift

219 effects potentially occurring in the laboratory selected line (G5-Mala). For each population,

220 genomic DNA was extracted from 2 batches of 50 adult females and gDNA extracts were then

221 pooled in equal proportion into a single sequencing library as described in Faucon et al., (2015).

222   Whole genome sequencing was performed from 200 ng gDNA. Sequencing libraries were

223   prepared according to the TruSeq DNA Nano Reference guide for Illumina Paired-end Indexed

224   sequencing (version oct 2017) with a mean insert size of 550 bp. Sequencing was performed on

225   a NextSeq 550 (Illumina) as 150 bp paired-reads. After unplexing and adaptor removal, reads

226   were mapped to the latest *Ae. aegypti* genome assembly (Aaeg L5) using BWA-MEM with

227   default parameters (version 0.7.12). Sequenced reads were then sorted using samtools sort

228   (version 1.2), annotated using Picard FixMateInformation (version 1.137) and PCR duplicates

229   were identified using Picard MarkDuplicates (version 1.137). Normalized coverage profiles

230   between the resistant and the susceptible populations were then compared using non-duplicated

231   reads with a mapping quality score above 60.

232

233   **2.6 | Quantification of Copy Number Variations**

234   Among the six genes located within the genomic amplification detected on chromosome 2,

235   three genes clearly annotated as *CCE* and distributed throughout the cluster were studied:

236   AAEL019678, AAEL023844 (formerly *CCEAE3A*) and AAEL005113 (*CCEAE1A*). For each

237   gene, specific primer pairs were designed using NCBI primer Blast (Table S2). In order to

238   quantify CNV in natural populations and in individuals, genomic DNA was extracted either

239   from seven pools of five adult females (mean CNV comparison between lines) or from single

240   adult females (estimation of amplification prevalence) using the cetyltrimethylammonium

241   bromure (CTAB) method (Collins et al., 1987) and diluted to 0.5 ng/µL prior to amplification.

242   Pooled samples were amplified in duplicates while individual mosquito samples were amplified

243   only once. Quantitative PCR reactions consisted of 3 µL gDNA template, 3.6 µL nuclease free

244   water, 0.45 µL of each primer (10µM), and 7.5 µL of iQ SYBR Green Supermix (Bio-Rad).

245   PCR amplification were performed on a CFX qPCR system (Bio-Rad) with cycles as follows:

246   95°C 3 min followed by 40 cycles of 95°C 15 secs and 30 secs for hybridization. A dilution

247 scale made from a pool of all gDNA samples was used for assessing PCR efficiency. Data were

248 analyzed using the ΔΔCt method (Pfaffl, 2001) taking into account PCR efficiency. Two control

249 genes (the *P450* AAEL007808 and the chloride channel protein AAEL005950) shown to be

250 present as single copies in multiple *Ae. aegypti* strains and populations (Faucon et al., 2015)

251 were used for normalization. For each gene, CNV were expressed as mean relative gDNA

252 quantity as compared to the fully susceptible line Bora-Bora. For assessing genomic

253 amplification frequencies, all individuals showing a CNV $\geq$ 2.5-fold as compared to the Bora-

254 Bora line were considered positive. This threshold was chosen in order to avoid false positives

255 as a consequence of qPCR technical variations ($<$ 2-fold in negative control samples). Structural

256 duplication haplotypes were assigned based on the detection of CNV for all three CCE genes

257 (haplotype A) or only for AAEL019678, and AAEL023844 (haplotype B).

258 Individual CNV levels obtained for the *CCE* gene AAEL023844 by qPCR were cross-validated

259 by digital droplet PCR (ddPCR). Briefly, each sample was partitioned into ~20,000 nanoliter-

260 sized droplets using the QX 200 droplet generator (Bio-Rad) by mixing synthetic oil with 20

261 µL PCR mix containing 2X ddPCR Evagreen supermix (Bio-Rad), 0.9 mM of each primer and

262 5 µL of template gDNA at 0.5 ng/µL. Emulsified reaction mixtures were then amplified with a

263 C1000 thermal cycler (Bio-Rad) for 40 cycles. After amplification, the number of positive and

264 negative droplets were quantified for each sample using the QX 200 droplet reader (Bio-Rad)

265 and the positive/negative ratio was used to estimate the initial DNA assuming a Poisson

266 distribution. A similar procedure was applied to the control gene AAEL007808 present as a

267 single copy. After normalizing for initial gDNA quantity, CNV were expressed as relative

268 gDNA quantity as compared to the fully susceptible line Bora-Bora.

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272 **2.7 | CNV quantification using TaqMan multiplex assay**

273 A TaqMan multiplex assay allowing the concomitant quantification of the *CCE* gene

274 AAEL023844 (present in both duplication haplotypes) and the control gene AAEL007808 from

275 single mosquitoes within the same qPCR reaction was developed. For each gene, primers and

276 probes were designed using Primer3web v 4.1.0 (Rozen & Skaletsky, 2000) with the AaegL5

277 assembly as reference genome for assessing specificity. For each gene, exonic regions were

278 targeted in order to limit amplification variations associated with natural polymorphism (Table

279 S2). The assay was then tested on all individuals detected as positive by qPCR representing 27

280 individuals belonging to seven populations from three countries. Each reaction mixture

281 contained 12.5 μL of qPCR probe Master Mix (Bio-Rad), 2.25 μL of each primer (10μM),

282 0.625 μL of each probe (10 μM), 1.25 μL of nuclease free water and 1 μL of template DNA

283 (0.5 ng/μL). PCR amplifications were performed on a CFX qPCR system (Bio-Rad) with cycles

284 set as follows: 95°C for 10 min followed by 40 cycles of 95°C for 10 secs and 60°C for 45 secs

285 followed by FAM and HEX levels reading (see Supplementary File 1 for a user guideline on

286 this TaqMan assay).

287

288 **2.8 | Statistical analysis**

289 All statistical analyses were performed with R v3.6.2 (R Core Team, 2013), using the package

290 lme4 for all mixed models (Bates et al., 2015). Mortality data were statistically compared across

291 conditions by using a Generalized Linear Model (GLM) with mixed effects (binomial family)

292 in which the replicates were included as a random factor. For comparing mean CNV obtained

293 from pools of mosquitoes, normalized gDNA levels obtained for each gene were Log_2

294 transformed and compared across conditions using a GLM with mixed-effects in which the

295 replicates were included as a random factor. For comparing CNV obtained from individual

296 mosquitoes, normalized gDNA levels were Log_2 transformed and compared between genes

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297 using a one-way ANOVA. A Pearson's product moment correlation coefficient test was used

298 to compare normalized gDNA quantities obtained from qPCR and ddPCR.

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300 **3 | RESULTS**

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302 **3.1 | Dynamics of organophosphate resistance during experimental selection**

303 Maintaining the Laos composite population under selection with malathion resulted in the rapid
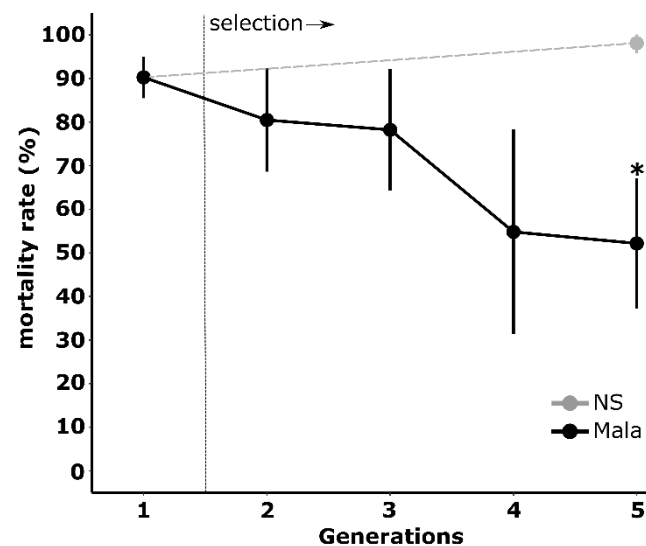
304 rise of resistance (Figure 1).

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314 **FIGURE 1.** Dynamics of malathion resistance during the selection process. Black: Laos
315 composite population selected with malathion; Grey: Laos composite population maintained
316 without selection. Stars indicate a significant mortality difference as compared to the initial
317 population (N=4, GLM mixed effects binomial family, *p<0.05).
318

319 Mortality to malathion dropped gradually from 90.3% in G1 to 52.2 % after four generations

320 of selection (GLMER test: z=2.058, P<0.05 for G5-Mala vs G1). Conversely, we observed a

321 slight (not significant) increase of mortality to 99.1% after four generations without selection

322 (GLMER test: z=-0.433, P=0.665).

323 Bioassays performed with different insecticides revealed that selection with malathion for four

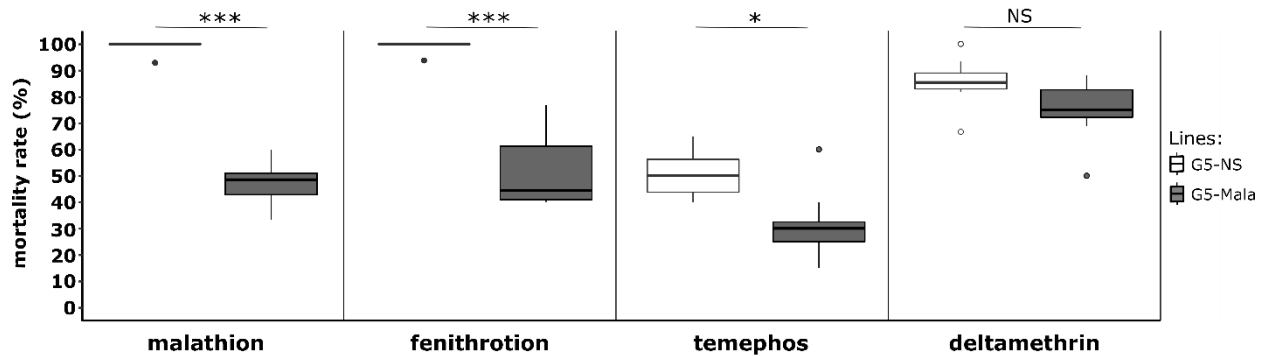324 generations also select resistance to other organophosphate insecticides (Figure 2).

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332 **FIGURE 2.** Cross resistance of the Malathion-resistant line to other insecticides. G5-NS:
333 Composite population maintained without selection. G5-Mala: composite population selected
334 with malathion. For each insecticide, stars indicate a significant mortality difference between
335 G5-NS and G5-Mala individuals (N=8, GLM mixed effects binomial family, *p<0,05,
336 ***p<0,001, NS: non-significant).

337

338 As compared to the G5-NS line, the G5-Mala line showed a significant increased resistance to

339 the organophosphates fenitrothion at the adult stage (GLMER: z=-3.455, P<0.005) and

340 temephos at the larval stage (GLMER: z=-2.194, P<0.05). Conversely, no significant increased

341 resistance to the pyrethroid deltamethrin at the adult stage was observed suggesting that

342 malathion did not select for pyrethroid resistance alleles.

343

344 **3.3 | Genes associated with malathion resistance**

345 RNA-seq analysis identified 84 and 124 genes over- and under-transcribed, respectively, in G6-

346 Mala adult females (adjusted P value ≤ 0.001 and FC ≥ 3-fold) as compared to the susceptible

347 lines G6-NS and Bora-Bora (Table S3). Among them, 24 genes encoded proteins potentially

348 associated with known insecticide resistance mechanisms (target-site resistance, cuticle

349 alteration, detoxification and sequestration or altered transport) including 14 cuticle genes and

350 10 detoxification genes. Only seven candidate genes were over-transcribed in the G5-Mala line,

351   all being associated with detoxification (Figure 3A). This included a microsomal glutathione S-

352   transferase on chromosome 1 (AAEL006818, 13-fold *versus* G6-NS), the cytochrome P450

353   *CYP6N17* on chromosome 2 (AAEL010158, 8-fold *versus* G6-NS) and five contiguous *CCE*

354   genes at ~174 Mb on chromosome 2 (AAEL015304, AAEL019679, AAEL019678,

355   AAEL005123 and AAEL023844 (formerly *CCEAE3A*, up to 10-fold *versus* G6-NS). A closer

356   look at this genomic region revealed the presence of an additional *CCE* gene on the 5' side of

357   the cluster (AAEL005113 *CCEAE1A*) which was not significantly over-transcribed in the

358   resistant line. Although the *GST* AAEL006818 and the P450 *CYP6N17* were significantly over-

359   transcribed in the G6-Mala line, these two genes were also found over-transcribed in two other

360   Laos lines selected with unrelated insecticides (data not shown), invalidating their specific

361   association with malathion resistance.

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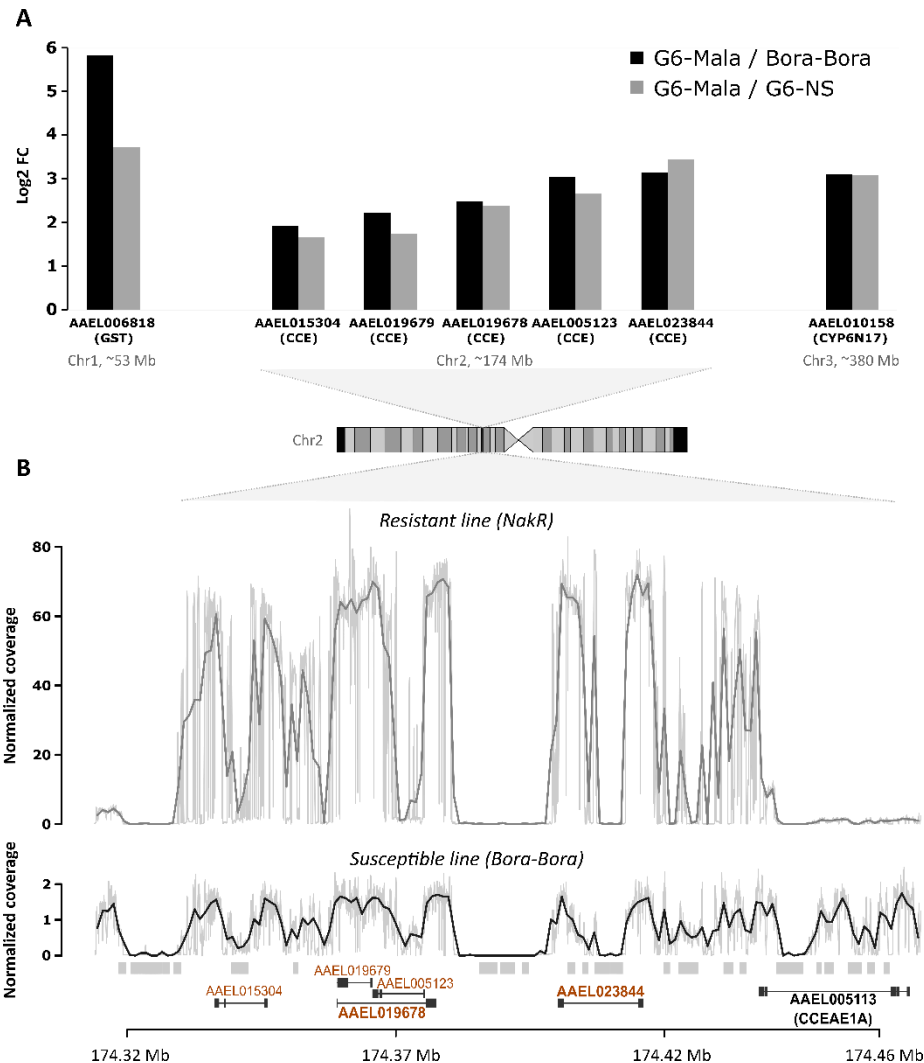
FIGURE 3. Genomic amplification associated with carboxylesterase overexpression. A: Detoxification genes over-transcribed in the malathion-resistant line. Transcription levels were quantified by RNA-seq using 4 biological replicates per line. Detoxification genes showing a 3-fold over-transcription and a corrected P value <0.001 in the Malathion-resistant line G6-Mala versus both the unselected line NS (grey) and the fully susceptible line Bora-Bora (black) are shown. The genomic location of each gene is indicated. B: Comparison of normalized read coverage profiles at the carboxylesterase locus between a Thai resistant line (Nakh-R) (grey) and the fully susceptible line Bora-Bora (black). Coverage profiles were obtained by divided the raw coverage by the average coverage found on the chromosome 1 and from whole genome DNA-seq performed on pools of 100 individuals. The genomic location of carboxylesterase genes is indicated according to Aaeg L5.1 annotation. Genes found overexpressed by RNA-seq are shown in orange. Genes targeted by qPCR are shown in bold. Repeated elements associated with coverage gaps are indicated as grey boxes.

Cross-comparing RNA-seq data with normalized coverage profiles obtained from whole

genome sequencing of the Thai resistant population Nakh-R known to over-express these CCE

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416 genes (Faucon et al., 2015, 2017) revealed a ~50-fold increased coverage in this genomic region

417 as compared to the susceptible line (Figure 3B). In addition, this region included multiple low-

418 coverage sections associated with the presence of repeated elements (mostly due to unresolved

419 read assembly). The pattern of genomic amplification observed in the Thai resistant population

420 Nakh-R was in agreement with the expression pattern observed in the Laos resistant line G6-

421 Mala with the same five first CCE genes being amplified, but not *CCEAE1A*.

422 The occurrence of this genomic amplification in Laos was then confirmed by qPCR (Figure

423 4A). Before selection (G1), a slight non-significant elevation of gene copy number was

424 observed for three *CCE* genes belonging to this genomic cluster as compared to the fully

425 susceptible line Bora-Bora with important variations suggesting a high inter-individual

426 heterogeneity in the initial line. Although not significant, gene copy number were even lower

427 after four generations without selection (G5-NS) with less variations observed. Conversely,

428 four generations of selection with malathion lead to a strong increase in gene copy number for

429 the *CCE* genes AAEL019678 and AAE023844 in G5-Mala individuals (up to 32-fold). A lower

430 increase (~8-fold) associated with a higher variance was observed for the gene *CCEAE1A*,

431 suggesting that not all G5-Mala individuals carry multiple copies of this gene.

432 Quantification of *CCE* genes copy number in individual mosquitoes by qPCR confirmed the

433 presence of at least two distinct structural duplication haplotypes in Laos with haplotype A

434 including the three *CCE* genes and haplotype B not including *CCEAE1A* (Figure 4B). While

435 the prevalence of these two *CCE* haplotypes was low in the initial composite population (7%

436 for the haplotype A and 0% for the haplotype B) and in the G5-NS (<4% for each haplotype)

437 their cumulated frequency reached 84% in G5-Mala individuals with haplotype B being more

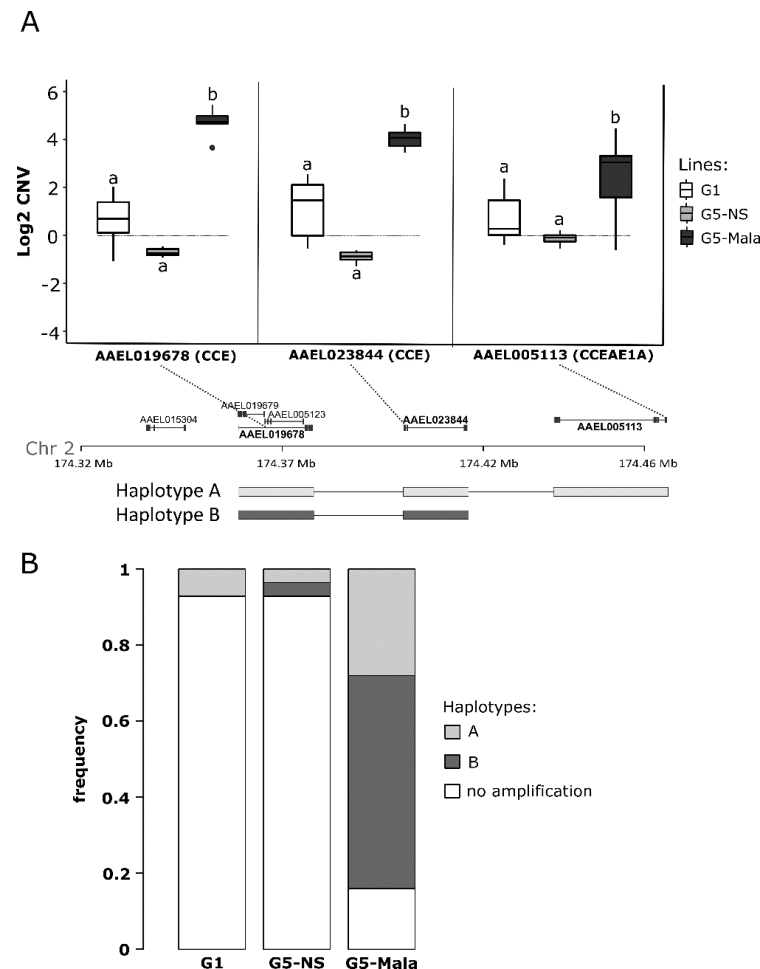438 frequent (67%) than haplotype A (33%).

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440

FIGURE 4. Duplication haplotypes at the carboxylesterase locus. A: CNV of three selected CCE genes located in different positions of the carboxylesterase locus. For each gene, the position of the qPCR amplification product is indicated (dashed lines). Mean CNV were estimated by qPCR on pools of mosquitoes and are expressed as gDNA quantity relative to the fully susceptible line Bora-Bora (horizontal grey line). G1: unselected line at generation 1, G5-NS: unselected line at generation 5, G5-Mala: malathion-resistant line after 4 generations of selection. Distinct letters indicate significant mean CNV variations between populations (GLM mixed effects, N=7, p≤ 0.05). The two structural duplication haplotypes deduced from CNV data are represented. B: Frequencies of each haplotype in the different lines. Haplotypes frequencies were deduced from individual CNV data obtained by qPCR from 28 mosquitoes per line for the three genes AAEL019678, AAEL023844 (formerly *CCEAE3A*) and AAEL005113 (*CCEAE1A*).

3.4 | Prevalence and copy number polymorphism in SEA

The spatial dynamics of this genomic amplification event was investigated in field populations from Laos, Cambodia and Thailand. A total of 302 mosquitoes belonging to 14 field populations were genotyped for the presence of *CCE* amplifications using qPCR. Seven populations distributed across the three countries were found positive with at least one individual carrying

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476 the duplication haplotype A or B (Figure 5). The prevalence of *CCE* amplifications was low in

477 most studied populations, high in two populations from Cambodia (26% and 29%) and very

478 high in the Nakh-R Thai population known to be resistant to organophosphates (79 %) (Faucon

479 et al., 2015). Although both haplotypes were detected through the study area, all positive

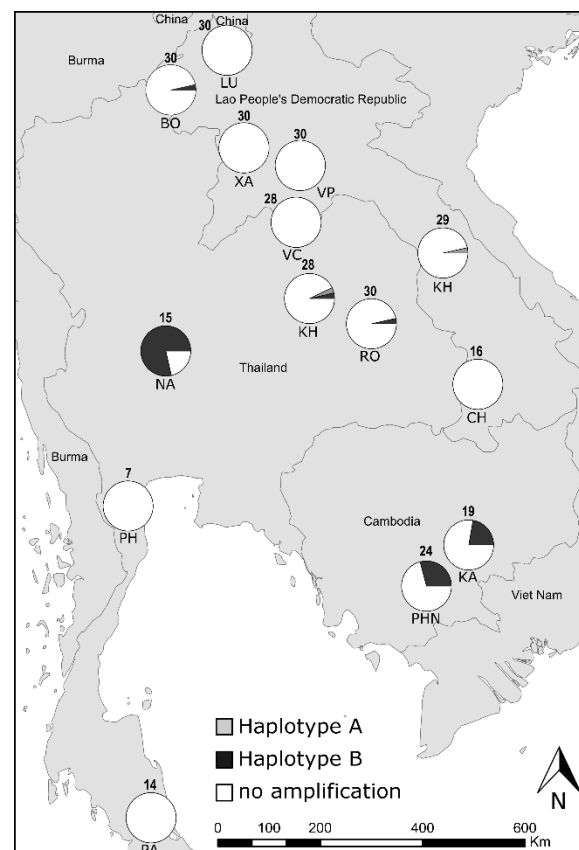480 individuals from populations showing a high prevalence only carried haplotype B.

FIGURE 5. Prevalence of the carboxylesterase gene amplification in South-East Asia. For each population, the frequency of each haplotype is shown. The number of individuals genotyped is shown above each pie chart. Haplotypes frequencies were deduced from CNV data obtained by qPCR on individual mosquitoes for the three genes AAEL019678, AAEL023844 and AAEL005113 (*CCEAE1A*). Only individuals showing a CNV $\geq$ 2.5-fold (as compared to the susceptible line Bora-Bora) for any gene were considered positive. The description of the studied populations is presented in Table S1.

504 Cross comparing CNV data obtained for the *CCE* gene AAEL023844 between standard

505 Sybrgreen qPCR and digital droplet PCR (ddPCR) indicated a good correlation between the

506 two techniques (r=0.85, P<0.001, Figure S1), suggesting that despite the technical variations

507 inherent to qPCR on single mosquitoes this approach provides a relatively good estimation of

18

508   gene copy numbers. Comparing the number of copies of each *CCE* gene between all positive

509   individuals revealed an important copy number polymorphism in the SEA with estimated copy

510   numbers ranging from 3 to ~80 copies (Figure 6). No significant differences were observed

511   between field populations (P=0.578 for AAEL019678; P=0.721 for CCEAE3A) and the

512   different lines (G1, G5-NS, G5-Mala) (P=0.827 for AAEL019678; P=0.845 for CCEAE3A),

513   suggesting that insecticide selection rather select for positive individuals than for individuals

514   carrying a higher number of copies. Overall, the mean copy number observed for the *CCE* gene

515   AAEL023844 (present in both haplotypes) was significantly higher than for the two other *CCE*

516   genes AAEL019678 and *CCEAE1A* (P<0.001 and P<0.01 respectively) possibly reflecting

517   additional structural haplotypes affecting this gene.

518
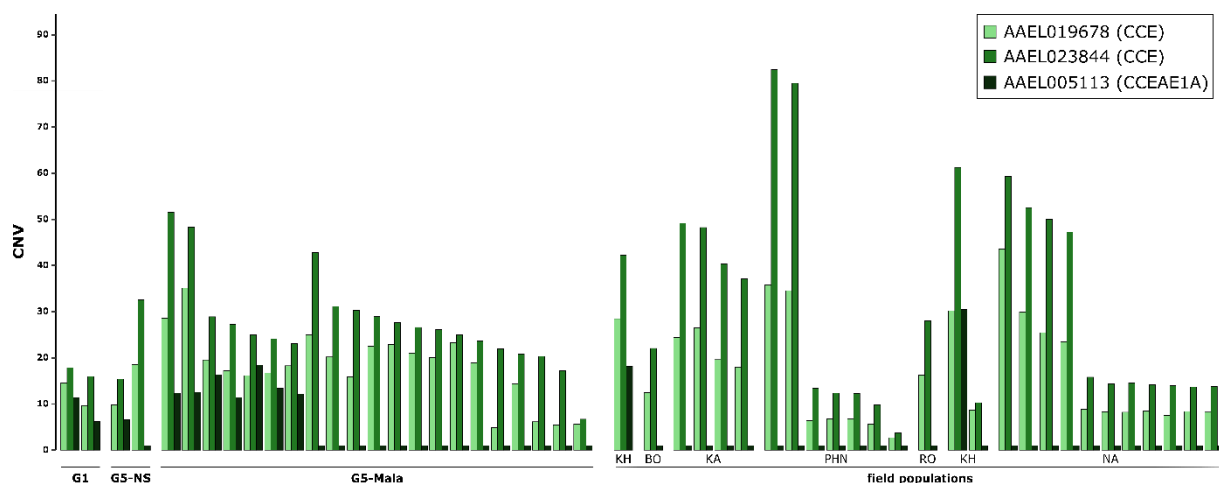
531   **FIGURE 6.** Genes copy number variations in experimental lines and field populations. For
532   each gene, CNV were estimated by qPCR on individual mosquitoes and are expressed as gDNA
533   quantity relative to the fully susceptible line Bora-Bora. Only positive individuals showing a
534   CNV ≥ 2.5-fold for any gene are shown. Names of laboratory lines and field populations are
535   indicated.

3.5 | A novel TaqMan assay to track *CCE* gene amplification in *Ae. aegypti*

A multiplex TaqMan qPCR assay allowing the concomitant amplification of the *CCE* gene AAEL023844 (included in both haplotypes) and a control gene within a single reaction was developed and tested against 27 positive individuals and 7 negative individuals belonging to all populations from which *CCE* amplification were detected (Figure 7). This assay showed a good specificity and sensitivity for detecting *CCE* amplifications in *Ae. aegypti*. All samples identified as positive by qPCR (i.e. showing a CNV higher than 2.5-fold) were also identified as positive using the TaqMan assay and no false negative was observed. A good amplification specificity was observed for both the *CCE* gene AAEL023844 and the control gene. A similar PCR efficiency of ~ 95% was observed for both the *CCE* gene AAEL023844 and the control gene leading to a C_q of ~30 cycles for both genes in absence of amplification with 0.5 ng/µL template gDNA (see Supplementary File 1 for a user guideline on this TaqMan assay).
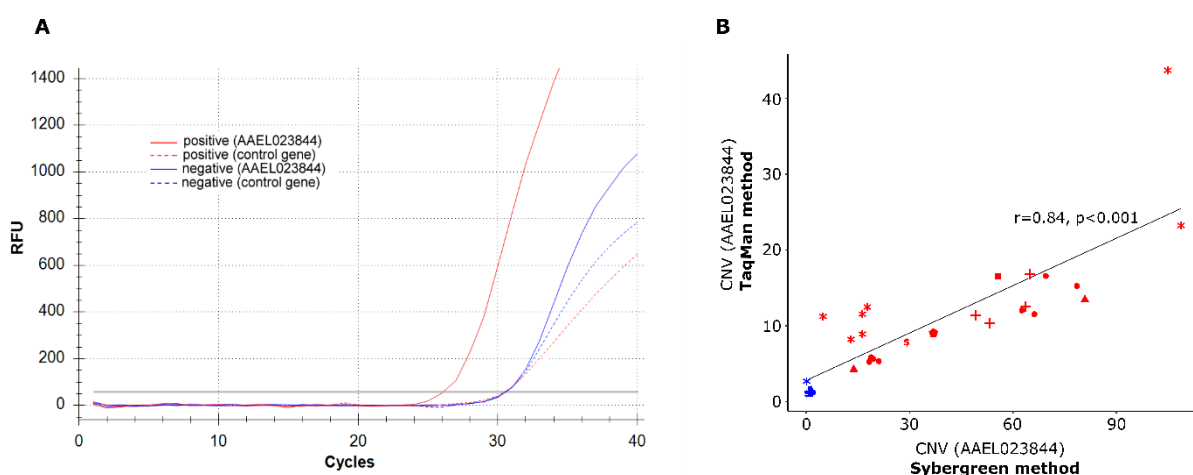

FIGURE 7. Overview of the TaqMan multiplex assay. A: Amplification profiles obtained for a positive individual (red) and a negative individual (blue). Solid line: amplification profile of the target gene AAEL023844 (formerly *CCEAE3A*, FAM probe), Dashed line: amplification profile of the control gene (AAEL007808, HEX probe). B: Comparison of CNV obtained with standard qPCR assay (SybrGreen, dual reactions) and TaqMan assay (FAM/Hex probes, single multiplex reaction). For both methods, CNV were estimated using the ΔΔCt method and are expressed as normalized gDNA quantity relative to the fully susceptible line Bora-Bora. Blue: negative individuals, Red: positive individuals. Each dot type stands for a different population.

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571 Comparing gene copy numbers estimated from standard qPCR and TaqMan assays revealed a

572 good correlation between the two techniques (r=0.84, P<0.001) (Figure 7B) although CNV

573 levels obtained with the TaqMan assay were lower as compared to those obtained with qPCR

574 and dd qPCR using amplification primers targeting a different fragment.

575

576 **4 | DISCUSSION**

577 Chemical insecticides remain a key component of integrated strategies aiming at preventing the

578 transmission of mosquito-borne diseases worldwide but the selection and spread of resistance

579 threatens their efficacy (Moyes et al., 2017). Controlling resistance by alternating selection

580 pressures is theoretically possible but requires an efficient monitoring of the dynamics of

581 resistance alleles in the field (Dusfour et al., 2019). Resistance to organophosphate insecticides

582 is common in the mosquito *Ae. aegypti* and particularly frequent in SEA following their massive

583 use for decades (Boyer et al., 2018; Marcombe et al., 2019; Pethuan et al., 2007; Ranson et al.,

584 2008; Vontas et al., 2012). Although several *CCE* genes are known to be involved, the genomic

585 changes underlying resistance are not fully understood. As a result, no rapid diagnostic assay is

586 available to monitor the frequency of resistance alleles in the field. By combining experimental

587 evolution and multiple sequencing approaches, we confirmed the key role of *CCE* amplification

588 in resistance to organophosphate insecticides in *Ae. aegypti* and further characterize the

589 associated genomic event. The prevalence of individuals carrying this amplification was then

590 investigated in SEA and a TaqMan multiplex qPCR assay allowing its rapid detection in single

591 mosquito specimens was developed.

592

593 **4.1 | *CCE* amplifications play a key role in organophosphate resistance in *Ae. aegypti***

594 Our experimental selection approach confirmed the presence of organophosphate resistance

595 alleles in *Ae. aegypti* populations in Laos and their rapid selection with malathion. These results

596 are consistent with the continuous use of organophosphates for vector control for 30 years in

597 Laos and the detection of resistance throughout the country (Marcombe et al., 2019). Bioassays

598 with other insecticides revealed that resistance alleles selected by malathion also confer cross-

599 resistance to other organophosphates at both larval and adult stage but not to the pyrethroid

600 deltamethrin suggesting a resistance spectrum restricted to the organophosphate family. This

601 confirms previous findings suggesting that the over-production of non-specific

602 carboxylesterases is a common adaptive response to organophosphates in mosquitoes (Cuany

603 et al., 1993; Hemingway et al., 2004; Naqqash et al., 2016).

604 RNA-seq analysis identified seven detoxification genes over-transcribed in association with

605 malathion resistance including five consecutive *CCE* genes on chromosome 2, one microsomal

606 *GST* on chromosome 1, and one P450 (*CYP6N17*) on chromosome 3. Though their role in

607 insecticide resistance cannot be excluded, the over-transcription of this *P450* and this *GST* in

608 two other sister lines selected with insecticides from different families and showing no

609 increased resistance to malathion does not support their key role in resistance to this insecticide

610 (data not shown). Conversely, the over-transcription of *CCE* genes was expected as *CCE*s have

611 often been associated with organophosphate resistance in mosquitoes. In *Cx pipiens* their

612 overproduction in response to organophosphate selection is well documented with distinct loci

613 having spread worldwide (Raymond et al., 1998). In this species, high resistance levels were

614 associated to the co-occurrence of carboxylesterases over-production through genomic

615 amplification and the presence of the *ace-1* G119S target-site mutation affecting the

616 acetylcholinesterase (Raymond et al., 2001). In *Aedes* mosquitoes, the G119S *ace1* mutation is

617 submitted to a strong genetic constraint (Weill et al., 2004) and has thus not been reported,

618 suggesting the central role of carboxylesterases over-production in resistance.

619 Whole genome sequencing and quantification of gene copy number confirmed the role of

620 genomic amplifications in the over-production of carboxylesterases associated with

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621 organophosphate resistance in *Ae. aegypti*, as previously suggested (Faucon et al., 2015, 2017;

622 Poupardin et al., 2014). In addition, our data revealed the co-existence of at least two distinct

623 structural duplication haplotypes, one including the three *CCE* genes AAEL019678,

624 AAEL023844 (formerly *CCEAE3A*) and AAEL005113 (haplotype A) and the other one not

625 including the *CCE* gene AAEL005113 located at 5' side of the cluster (haplotype B). Structural

626 polymorphism of genomic amplifications in clustered detoxification genes has been recently

627 reported in *Anopheles gambiae,* with twelve different alleles identified in a cluster of *P450*s

628 and eleven in a cluster of *GST*s (Lucas et al., 2019). In the tiger mosquito *Ae*. *albopictus,* a

629 structural polymorphism affecting a similar *CCE* cluster amplification was identified with at

630 least two distinct haplotypes: one including two *CCE* genes and the second one with only the

631 gene AALF007796, the best orthologue of *Ae. aegypti* AAEL023844 (Grigoraki et al., 2017).

632 These striking similarities between *Ae. albopictus* and *Ae. aegypti*, likely resulting from a

633 convergent adaptation, further supports the key role of *CCE* amplifications in the adaptation of

634 *Aedes* mosquitoes to organophosphate insecticides.

635 The genetic mechanism underlying the amplification of these orthologous loci has not been

636 characterized yet. Previous studies suggested the existence of "hot spots" of recombination

637 favoring structural polymorphisms (Bass & Field, 2011). In insects, the presence of

638 transposable elements is also known to favor duplication events associated with their rapid

639 adaptation to insecticides (Bass & Field, 2011; Grigoraki et al., 2017; Schmidt et al., 2010).

640 Our genomic data confirm the presence of multiple repeated elements in the vicinity of this

641 locus though further genomic analyses are required to decipher their relative involvement in

642 this genomic event.

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4.2 | Evolutionary dynamics of *CCE* amplifications

The screening of this *CCE* gene amplification by qPCR on field-collected mosquitoes confirmed its occurrence in *Ae. aegypti* populations from SEA. Its prevalence in natural populations was globally low except in Cambodia and in one Thai population from which high organophosphate resistance was previously described (Paeporn et al., 2013; Pethuan et al., 2007; Poupardin et al., 2014; Saelim et al., 2005). Although our sampling campaign was restricted to a few populations in Thailand, Laos and Cambodia, the frequent elevated esterase activities detected in association with temephos resistance in SEA suggests that this *CCE* amplification is widely spread in the region (Paeporn et al., 2013; Pethuan et al., 2007). Previous studies also support the occurrence of this *CCE* amplification in the Caribbean region with high expression levels detected for AAEL023844 from multiple islands and the presence of gene amplification validated in Guadeloupe and Saint-Martin (Goindin et al., 2017; Marcombe et al., 2009, 2012). Although this needs to be confirmed, the frequent association between elevated esterase activities and organophosphate resistance in South-America (i.e. French Guiana, Brazil, Colombia and Costa-Rica) (Bisset et al., 2013; Gambarra et al., 2013; Melo-Santos et al., 2010; Paiva et al., 2016) and New Caledonia (Dusfour et al., 2015), suggests that this *CCE* amplification is distributed worldwide.

Despite the low frequency of *CCE* amplifications in most field populations, our experimental insecticide selection showed that the frequency of these resistance alleles increases rapidly in populations submitted to insecticide selection pressure.. These findings support the highly beneficial effect of these *CCE* amplifications in the presence of insecticides but also raises the question of their fitness costs in the absence of selective pressure. Fitness costs associated with the over-production of detoxification enzymes have been previously described in various insect species (ffrench-Constant & Bass, 2017; Kliot & Ghanim, 2012). Direct measurement of energetic resources (e.g. lipids, glycogen and glucose) in *Cx. pipiens* mosquitoes over-

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671   expressing carboxylesterases suggested that resistant individuals carry up to 30% less energetic

672   reserves than their susceptible counterparts (Rivero et al., 2011). Such high metabolic cost may

673   explain the favored selection of the shorter (less costly) haplotype B in field populations and

674   laboratory lines showing a high *CCE* amplification prevalence. Although further studies are

675   required to quantify the relative importance of the different *CCE* genes included in this genomic

676   amplification in insecticide resistance, the frequent over-expression of AAEL023844 in

677   resistant populations, its inclusion in both structural duplication haplotypes and its ability to

678   sequester and metabolize temephos (Grigoraki et al., 2016) support its central role in

679   organophosphate resistance.

680   In addition to structural polymorphism, our study revealed extensive copy number variations

681   between resistant individuals in both field populations and laboratory lines with *CCE* gene

682   copies varying from 3 to ~80 as measured by our TaqMan assay. In addition, no significant

683   increase of the frequency of individuals carrying high gene copy number was observed after

684   insecticide selection supporting the existence of a trade-off between insecticide survival and

685   metabolic costs associated with the over-production of these enzymes. Such high copy number

686   polymorphism also supports the occurrence of a single duplication event followed by multiple

687   amplification events in *Ae. aegypti* as suggested in *Cx. pipiens* and *Ae*. *albopictus* (Grigoraki et

688   al., 2017; Guillemaud et al., 1999; Qiao & Raymond, 1995).

689   Though our data confirmed the role of this CCE amplification in organophosphate resistance,

690   the involvement of others mechanisms cannot be excluded. Allelic variations of

691   carboxylesterases have also been associated with organophosphate resistance in mosquitoes

692   (Callaghan et al., 1998; Mouchès et al., 1987). Indeed, both allelic variations and genes

693   amplifications coexist in natural populations and can be captured by selection depending on

694   fitness-to-environment relationships (Milesi et al., 2016). In *Ae. aegypti*, a few non-

695   synonymous variations affecting the AAEL023844 gene were associated with temephos

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696 resistance in a Thai population (Poupardin et al., 2014). However, subsequent functional studies

697 did not support the role of these variations in insecticide sequestration and metabolism

698 (Grigoraki et al., 2016). More recently, we combined controlled crosses with pool-sequencing

699 to segregate organophosphate resistance alleles in a multi-resistant population from French

700 Guiana (Cattel et al., 2019). Such approach identified a strong selection signature associated

701 with organophosphate resistance at this *CCE* locus. Interestingly, several non-synonymous

702 variations affecting *CCE* genes were positively associated with insecticide survival while no

703 CNV were detected, suggesting that the selection of particular variants at this locus may also

704 contribute to resistance. Further work is required to clarify the interplay between *CCE*

705 amplifications, sequence polymorphism and their respective roles in insecticide resistance in

706 *Ae. aegypti*.

707

4.3 | A novel TaqMan assay to track organophosphate resistance in *Ae. aegypti*

709 The present study confirmed the importance of *CCE* amplifications in insecticide resistance in

710 *Ae. aegypti*, confirming the usefulness of CNV markers for tracking resistance in the field. Our

711 cross-resistance data together with previous findings (Faucon et al., 2015, 2017; Grigoraki et

712 al., 2016; Marcombe et al., 2019; Poupardin et al., 2014) support the routine use of this marker

713 for the monitoring of resistance to various organophosphate insecticides. In terms of

714 applicability, such a CNV marker is highly superior to RNA markers classically used to detect

715 *CCE* genes overexpression because i) genomic DNA can be extracted from dead specimens of

716 any life stage stored at room temperature, ii) either pools or single individuals can be used if

717 allele frequency data are required and iii) CNV quantification by qPCR is fast, easy, affordable

718 and data are not affected by insect physiological state.

719 Genomic amplifications can be detected by PCR in two different ways as illustrated in Weetman

720 et al., 2018. The first one consists of amplifying the junction between two copies by designing

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721   specific primers toward both sides of the duplicated region. Such presence/absence assay is

722   cheap and low tech but i) does not quantify copy number, ii) requires the precise identification

723   of duplication breakpoints, which can be impaired by the high density of repeated elements in

724   flanking regions, and iii) may generate false negatives if duplication breakpoints vary in

725   position or sequence. The alternative approach adopted herein consists of comparing the copy

726   numbers between a target gene and a control gene only present as a single copy. Although this

727   approach is slightly more expensive and requires the use of a qPCR machine, time-to-result is

728   even shorter (no gel migration required) and results are not affected by structural

729   polymorphisms provided an appropriate target is defined. Though multiple *CCE* genes are

730   located within the amplified region, we selected AAEL023844 as target gene because of its

731   central position in the genomic amplification, its inclusion in both structural haplotypes, its

732   over-expression in several resistant populations worldwide (Dusfour et al., 2015; Goindin et

733   al., 2017; Marcombe et al., 2019; Moyes et al., 2017) and its ability to sequester and metabolize

734   temephos (Grigoraki et al., 2016). By targeting a coding region showing no homology with

735   other genomic regions, we ensured a good assay specificity while limiting detrimental effects

736   potentially caused by polymorphisms variations. Though this approach was successfully used

737   for CNV detection with standard SybrGreen qPCR, it still required performing two distinct

738   qPCR amplifications. Time-to-results and specificity were then further improved by the

739   development of a dual-color TaqMan assay allowing the concomitant quantification of both

740   target and control genes. This assay still proved to be highly specific and allowed reducing

741   time-to-results to ~2h for less than 1.5€ /sample including gDNA extraction, qPCR

742   consumables/reagents, amplification primers and Taqman probes.

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747 **5 | CONCLUSION**

748 While an increasing number of alternatives to chemical insecticides are being developed for

749 mosquito control (Achee et al., 2019) their optimization and deployment at a worldwide scale

750 will take at least a decade. Until then, preserving the efficacy of the few insecticides authorized

751 in public health by managing resistance is crucial to limit the impact of vector-borne diseases.

752 However, resistance management is often hampered by insufficient resistance monitoring

753 capacities, often leading to late or inappropriate implementations of management actions. In

754 this context, the deep comprehension of the genetic bases of resistance and the development of

755 molecular tools to track resistance alleles in the field still represent a significant capital gain for

756 public health. By combining experimental selection and deep sequencing, the present study

757 confirmed the key role of a genomic amplification of a carboxylesterase gene cluster in

758 organophosphate resistance in the mosquito *Ae. aegypti*. The spatial dynamics of this resistance

759 locus was investigated in SEA and a novel TaqMan assay was developed enabling its high-

760 throughput monitoring in field mosquito populations. The routine use of this assay in SEA, and

761 possibly in other tropical areas, should improve the monitoring of organophosphate resistance

762 in the arbovirus vector *Ae. aegypti*. From an evolutionary perspective, deciphering the

763 evolutionary history of the genetic events underlying this recent adaptation undoubtedly

764 deserves further attention.

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CONFLICT OF INTEREST DISCLOSURE

791   The authors of this preprint declare that they have no financial conflict of interest with the
792   content of this article.

793

ETHICAL APPROVAL

795   Blood feeding of adult mosquitoes was performed on mice. Mice were maintained in the animal

796   house of the federative structure Environmental and Systems Biology (BEeSy) of Grenoble-

797   Alpes University agreed by the French Ministry of animal welfare (agreement n° B 38 421 10

798 001) and used in accordance to European Union laws (directive 2010/63/UE). The use of

799 animals for this study was approved by the ethic committee ComEth Grenoble-C2EA-12

800 mandated by the French Ministry of higher Education and Research (MENESR).

801

DATA AVAILABILITY STATEMENT

803 The sequence data from this study have been deposited to the European Nucleotide Archive

804 (ENA; http://www.ebi.ac.uk/ena) under the accession numbers PRJEB37991 (RNA-seq data)

805 and PRJEB37993 (whole genome pool-seq data). Data and scripts have been deposited to

806 Zenodo (http://doi.org/10.5281/zenodo.3895225). All supplemental data are available in this

807 link:

808 https://drive.google.com/drive/folders/1hfgTbDI_KvlCUxuldKGScq7Psx7hKUbE?usp=shari

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