

1 Article

# 2 The G119S acetylcholinesterase (Ace-1) target site 3 mutation confers carbamate resistance in the major 4 malaria vector *Anopheles gambiae* from Cameroon: A 5 challenge for the coming IRS implementation

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22 **Abstract:** Growing resistance is reported to carbamate insecticides in malaria vectors in Cameroon.  
23 However, the contribution of acetylcholinesterase (Ace-1) to this resistance remains uncharacterised.  
24 Here, we established that the Ace-1<sup>R</sup> mutation is driving resistance to carbamates in *Anopheles*  
25 *gambiae* populations from Cameroon. Insecticide bioassay on field collected mosquitoes from  
26 Bankeng, a locality in southern Cameroon, showed high resistance to the carbamates bendiocarb  
27 (64.8 ± 3.5 % mortality) and propoxur (55.71 ± 2.9 %) but a full susceptibility to the organophosphate  
28 fenithrothion. The TaqMan genotyping of the Ace-1<sup>R</sup> mutation with field-collected adults revealed  
29 the presence of this resistance allele (39%). A significant correlation was observed between the Ace-  
30 1<sup>R</sup> and carbamate resistance at allelic [(bendiocarb; OR = 75.9; P<0.0001) and (propoxur; OR= 1514;  
31 P<0.0001)] and genotypic [RR vs SS (bendiocarb; OR = 120.8; P<0.0001) and (propoxur; OR= 3277;  
32 P<0.0001) levels. Furthermore, the presence of the mutation was confirmed by sequencing an Ace-1  
33 portion flanking codon 119. The cloning of this fragment revealed a likely duplication of Ace-1 in  
34 Cameroon as mosquitoes exhibited at least three distinct haplotypes. Phylogenetic analyses showed  
35 that the predominant Ace-1<sup>R</sup> allele is identical to that from West Africa suggesting a recent  
36 introduction of this allele in Central Africa from the West. The spread of this Ace-1<sup>R</sup> represents a  
37 serious challenge to future implementation of IRS-based interventions using carbamates or  
38 organophosphates in Cameroon.

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40 Keywords: Ace-1 G119S mutation, Insecticide resistance, *Anopheles gambiae*, Cameroon, malaria  
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## 44 1-Introduction

45 During the last decades, the fight against malaria disease made significant progress, halving  
46 malaria deaths and decreasing its incidence by over a third [1, 2]. These significant outcomes have  
47 been mainly driven by the scale-up of insecticide-based vector control interventions, such as long-  
48 lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) [1, 3]. Out of the four  
49 recommended insecticide classes in public health, pyrethroids have been the insecticides of choice  
50 for both strategies [1, 4]. Unfortunately, the intense use of these chemicals for public health and  
51 agricultural purposes has led to the development of insecticide resistance in malaria vectors [4]. This  
52 rapid expansion of pyrethroid resistance could reverse progress achieved in reducing malaria burden  
53 due to the significant reduction of the efficacy of LLINs [5]. In order to sustain the efficacy of IRS and  
54 maintain or recover the efficacy of pyrethroids for Insecticide Treated nets (ITNs), the World Health  
55 Organization (WHO) recommends application of insecticides having different mode of action or  
56 temporal replacement by different insecticide classes [6].

57 Over the past few years, there has been an increasing interest in using carbamate (CMs) and  
58 organophosphates (OPs) for public health purposes as alternatives to pyrethroids [7]. Indeed,  
59 numerous studies conducted under semi field conditions in experimental huts have shown the  
60 effectiveness of CMs and OPs against pyrethroid-resistant *An. gambiae* mosquito [7-12]. Furthermore,  
61 the beneficial effects of these insecticides while used for IRS, have largely been reported in several  
62 African countries [13-17]. Encouraged by these interesting results and with financial and technical  
63 support primarily from the United States President's Malaria Initiative (PMI)/United States Agency  
64 for International Development (USAID), since 2006, several African countries started introducing the  
65 use of carbamate or organophosphate-based IRS in their national vector control strategy [13, 16, 18-  
66 21]. Unfortunately, a reduced susceptibility to CMs has been increasingly observed in *An. gambiae*  
67 populations from West Africa [22-27]. This reduced susceptibility is associated with the emergence  
68 of the Ace-1 mutation gene in *Anopheles gambiae* mosquito [22, 25, 26, 28, 29]. This mutation resulting  
69 from a single amino acid substitution at codon 119 from glycine to serine (G119S) was reported to  
70 confer cross-resistance to CMs and OPs in mosquito species [30, 31]. The spread of this mechanism of  
71 resistance represents a serious threat for the effectiveness of IRS implementation in Africa. In contrast,  
72 in Central Africa, resistance to CMs had so far only been moderate with little or no evidence that Ace-  
73 1 was playing any role [32]. This has led the President Malaria Initiative (PMI) program, which was  
74 recently implemented in Cameroon, to include the use of carbamate and organophosphate-based IRS  
75 as a core component of the malaria control strategy in Cameroon [33]. The implementation of this  
76 strategy is expected to improve vector control in this country where high pyrethroid resistance level  
77 have been reported in *Anopheles* mosquito species [34]. Nevertheless, the effectiveness of this strategy  
78 could be limited by the resistance to CMs already reported by some previous studies in *An. gambiae*  
79 populations of Cameroon [32, 34-37]. To avoid a rapid loss of effectiveness of such IRS control  
80 intervention, it is important to evaluate the current level of resistance to these insecticide classes and  
81 also to assess the potential contribution of the Ace-1<sup>R</sup> particularly as it confers cross-resistance to both  
82 CMs and OPs.

83 The present study characterized the mechanisms involved in the resistance to carbamate  
84 detected in *An. gambiae* population from southern Cameroon. The G119S Ace-1 mutation was  
85 detected with significant correlation with carbamate resistance whereas, evidence of duplication of  
86 the gene was found.

87

## 88 2. Methods

### 89 2.1. Mosquito sampling

90 Adult and larval stages of *An. gambiae* sl mosquitoes were collected in the locality of Bankeng (4°  
91 38' 43" N; 12° 13' 03" E), a recent irrigated rice growing village in forest area in central Cameroon.  
92 Adult female mosquitoes were collected indoor on the walls and on the roof of different houses across

93 the village between 6:00 AM and 10:00 AM using electric aspirators (Rule In-Line Blowers, Model  
94 240). Mosquitoes were kept in paper cups and transported to the insectary of the Centre for Research  
95 in Infectious Diseases (CRID) in Yaoundé where they were morphologically identified and sorted by  
96 species according to the morphological identification keys of Gillies and De Meillon [38] and Gillies  
97 and Coetzee [39]. Mosquitoes were thereafter stored at -80°C for molecular analysis. Mosquitoes were  
98 collected at the larval stage from *An. gambiae* s.l. specific breeding sites across the village using the  
99 dipping method. Larvae from stage 1 to 4 and pupae were transferred in bottles and then transported  
100 to the insectary where they were reared until the adult stage.

101

## 102 2.2. Insecticide bioassays

103 Insecticide bioassay tests were carried out using 2-5-day old female adults obtained from field  
104 collected larvae. Unfed mosquitoes were exposed to: 0.1% bendiocarb, 1.0% propoxur and 1.0%  
105 fenitrothion-treated papers for one hour following WHO standard procedures [40]. The mortality  
106 rates were recorded 24h after exposure and WHO criteria were used to determine the resistance status  
107 of mosquitoes. Alive mosquitoes after exposure were kept in -80°C whereas dead individuals were  
108 stored in silica gel and kept in -20°C.

109

## 110 2.3. Species identification and *Ace-1* G119S mutation genotyping

111 These analyses were done using total genomic DNA extracted from 91 field-collected adult  
112 mosquitoes randomly selected (F<sub>0</sub>) and F<sub>1</sub> alive and dead mosquitoes after exposure to bendiocarb  
113 (25 alive and 67 dead) and propoxur (30 alive and 38 dead). DNA was extracted from whole mosquito  
114 following the Livak protocol previously described [41]. Identification of species within *An. gambiae*  
115 complex was determined using the SINE PCR protocol [42]. The presence of the G119S mutation was  
116 screened with TaqMan real-time PCR assay (using Agilent Mx3005 qRT-PCR thermocycler) following  
117 the protocols established by Bass and colleagues [43]. Each reaction was conducted in a total volume  
118 of 10 µl comprise of 5 µl Sensimix (Bioline), 0.25 µl of 40x Probe Mix coupled to allelic-specific primers,  
119 4.25 µl of dH<sub>2</sub>O, and 1 µl of genomic DNA. Thermocycling conditions were an initial 10 min at 95 °C,  
120 followed by 40 cycles each of 92 °C for 15 sec, and 60 °C for 1 min. Two probes labelled with  
121 fluorochromes FAM and HEX were utilised to detect the resistant mutant and the wild type  
122 susceptible alleles, respectively. Genotypes were scored from bi-directional scatter plots of results  
123 produced by the Mx3005 v4.10 software. Thereafter, the correlation between G119S genotypes and  
124 bendiocarb resistance phenotypes was assessed by estimating the odds ratio (OR) and the statistical  
125 significance based on Fisher exact probability test.

126

## 127 2.4. *Ace-1* gene amplification, sequencing and cloning

128 A region of 924-bp in a sequence of the *ace-1* gene, encompassing exons 4–6 (VectorBase AgamP3  
129 annotation; G119S position in exon 5 corresponding to the third coding exon) was amplified from 55  
130 female *An. gambiae*: 15 from F<sub>0</sub> (field-collected adult mosquitoes), 40 from F<sub>1</sub> mosquitoes after  
131 exposure to insecticide (10 alive and 10 dead after exposure to bendiocarb, 10 alive and 10 dead after  
132 exposure to propoxur). The amplification by PCR was carried out following the protocol previously  
133 described by Essandoh and collaborators [25]. Briefly, each reaction was conducted a total volume of  
134 50 µl containing 10 picomoles of each primer Ex2Amdir1 (5'AGG TCA CGG TGA GTC CGTACG A  
135 3') and Ex4Agrev2 (5' AGG GCG GAC AGC AGA TGC AGC GA 3'), 10 mM dNTPs, ddH<sub>2</sub>O, 5X HF  
136 Phusion buffer, and 1u of Phusion Taq polymerase (Fermentas). The cycle parameters were: 1 cycle  
137 at 98°C for 4 min, followed by 35 cycles of 98°C for 30 sec, 64°C for 15 sec and 72°C for 30 sec, with  
138 final extension at 72°C for 5 min. The PCR products were purified using the Qiaquick purification kit  
139 (QIAGEN, Hilden, Germany) and 28 amplicons (12 F<sub>0</sub> field collected adults, 8 alive and 8 dead after  
140 exposure to bendiocarb) were sequenced directly using the primers Ex2Amdir1 and Ex4Agrev2 to

141 confirm the presence of the G119S mutation and assess signature of selection at this Ace-1 in this  
142 location.

143 To investigate the presence of Ace-1 duplication, purified DNA amplified from 18 alive  
144 mosquitoes after exposure to bendiocarb (8 mosquitoes) and propoxur (10 mosquitoes) were selected  
145 for cloning using the Thermo scientific CloneJET™ PCR Cloning Kit. The colonies were screened for  
146 the presence of the inserted amplicon using the supplied pJET1.2 primers according to the  
147 manufacturer's instructions, and bands of approximately 900 bp were regarded as potential the Ace-  
148 1 clones. Thereafter, for each individual, 5 clones were amplified, purified and sequenced. All the  
149 successfully sequenced samples were aligned using ClustalW [44] as implemented in Bioedit  
150 software. The alignment was done with the consensus sequence from Kisumu strain exported from  
151 VectorBase. The polymorphism analysis was performed using Dnasp v5.10 [45], while MEGA 10.1.0  
152 [46] was used to build a maximum likelihood tree from the aligned sequences after equalization  
153 length. An haplotype network was also constructed using TCS program [47] and tcsBU [48]

154

### 155 3. Results

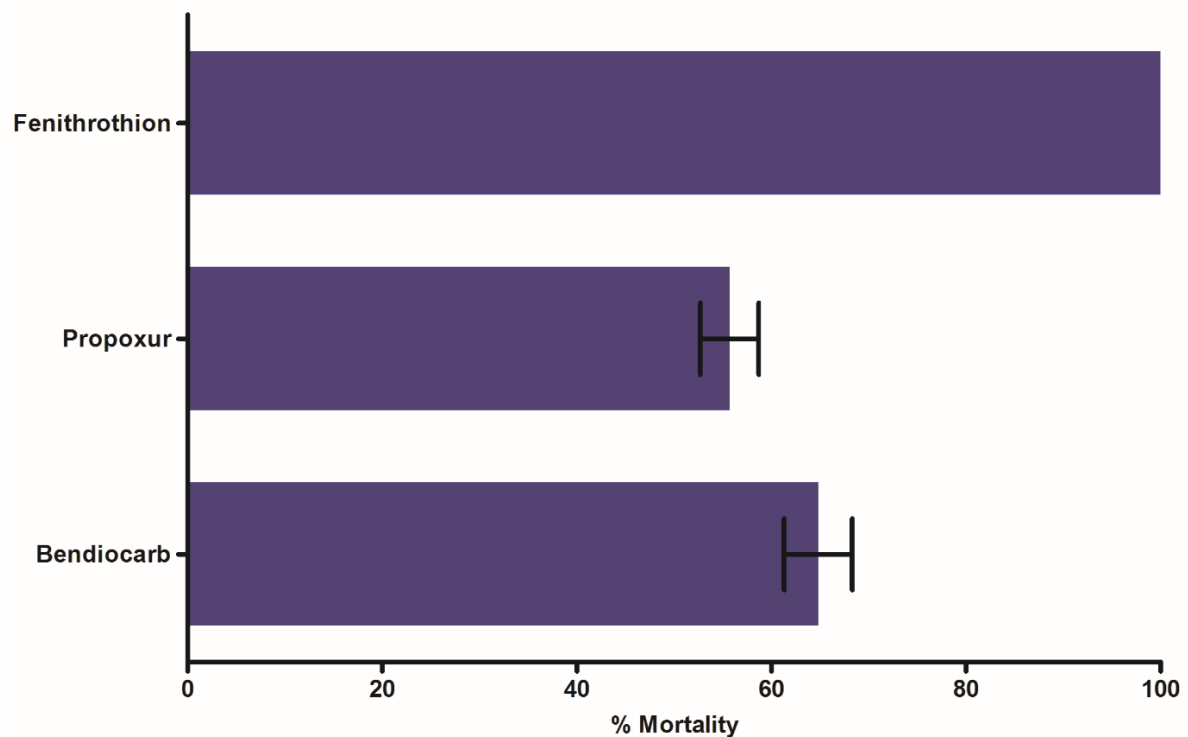
#### 156 3.1. Mosquito collection and species molecular identification

157 A total of 323 indoor resting blood-fed female ( $F_0$ ) were collected and were all morphologically  
158 identified as members of *An. gambiae* complex. Out of the 200  $F_0$  mosquitoes randomly selected and  
159 tested for molecular identification, 98.5% (198/200) were *An. gambiae*, whereas only 2 mosquitoes were  
160 identified as *An. coluzzii*.

161

#### 162 3.2. Insecticide bioassay

163 Overall, 260  $F_1$  female adults mosquitoes aged 2-5 days obtained from field collected larvae were  
164 exposed to bendiocarb, propoxur and fenitrothion. Resistance was detected for the two carbamate  
165 tested with mortality rates of  $64.8 \pm 3.5$  % and  $55.71 \pm 2.9$  % respectively for bendiocarb and propoxur.  
166 However, exposure to fenitrothion led to a 100% mortality showing a full susceptibility to this  
167 insecticide (figure 1).

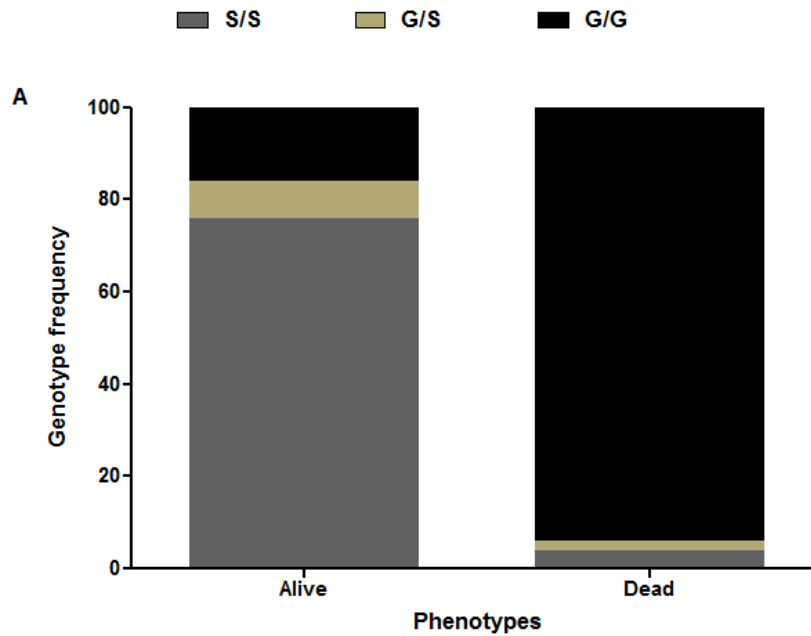


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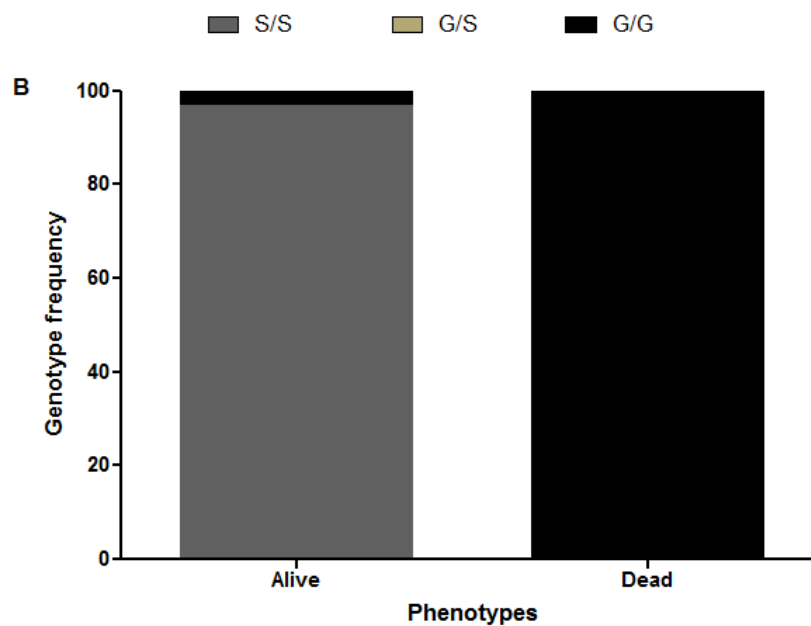
169 **Figure 1:** Susceptibility status of *An. gambiae* mosquito population from Bankeng, central Cameroon.  
 170 Mortality rates were recorded 24h post-exposure insecticides. Data are shown as mean  $\pm$  SEM (n=260).

### 171 3.3. *Ace-1* mutation genotyping and association with insecticide resistance profile

172 *Ace-1* mutation was genotyping in both  $F_0$  field collected mosquitoes and  $F_1$  female mosquitoes  
 173 exposed to insecticide. The 119S resistant allele was detected in 38.7% (34 homozygotes and 2  
 174 heterozygotes) out of the 93  $F_0$  field collected mosquitoes randomly screened. Out of the 25 alive  
 175 mosquitoes after exposure to bendiocarb, 76.0%, 8% and 16% of alive mosquitoes were genotyped  
 176 homozygotes resistant (S/S), heterozygote (G/S) and homozygote susceptible (G/G genotype),  
 177 respectively (Figure 2A). In contrast for dead mosquitoes, 4.5% were S/S, 1.5 G/S and 94% G/G. For  
 178 propoxur, 100% of dead mosquitoes were homozygote susceptible whereas 96.6% and 3.4% of alive  
 179 mosquitoes were homozygote resistant and homozygote susceptible, respectively (Figure 2B). The  
 180 *Ace-1<sup>R</sup>* mutation was strongly associated with carbamate resistance for both allelic [OR = 75.90 CI:  
 181 (18.72 - 307.8) for bendiocarb; OR= 1514 CI: (59.5 – 38560) for propoxur] and genotypic [OR = 120.8  
 182 CI: (25.0 - 583.3) and OR= 3277; CI: (130.2 – 82490) for bendiocarb and propoxur respectively] levels.



183



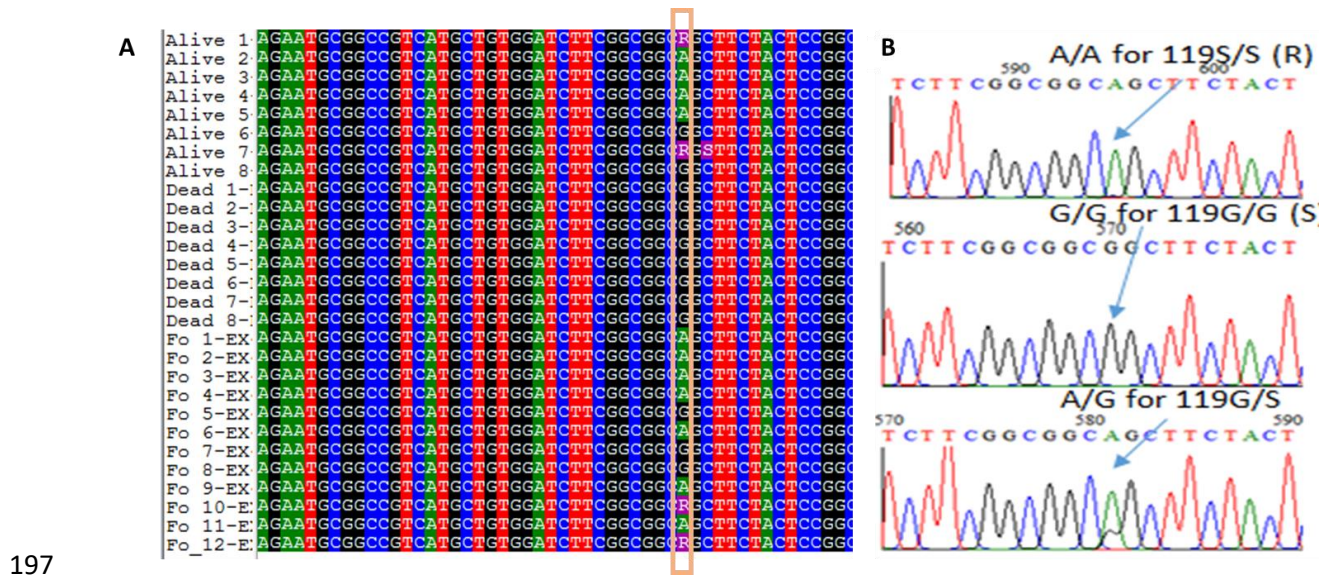
184

185 **Figure 2:** Distribution of Ace-1 G119S genotypes and association with bendiocarb (A) and propoxur  
 186 (B) resistant phenotype.

### 187 3.4. Genetic diversity of Ace-1 in Bankeng

188 A region of 924 bp including the 119 codon of the ace-1 gene was amplified from 28 mosquitoes  
 189 (12 F<sub>0</sub>, 8 dead and 8 alive after exposure to bendiocarb) in order to confirm the presence of the 119S  
 190 allele and to assess the genetic diversity of this gene. A 705 bp sequence was commonly aligned for  
 191 the 28 samples (Additional file 1). A G-to-A substitution at position 397, corresponding to the 119  
 192 codon, was observed in 11 sequences (7 F<sub>0</sub> and 4 F<sub>1</sub> alive) in comparison with the reference sequence  
 193 from susceptible Kisumu strain, (Figure 3). Heterozygote mosquitoes were detected (2 F<sub>0</sub> and 2 F<sub>1</sub>  
 194 alive mosquitoes) with overlapping peaks for G and A at the same position (represented by the

195 ambiguity code R, Figure 3). Interestingly, no substitution was detected in all the sequences from the  
196 8 dead F<sub>1</sub> mosquitoes (Figure 3).



198 **Figure 3:** Sequencing of the portion of the Ace-1 gene spanning the G119S mutation. A) Sequence  
199 alignment of the Ace-1 gene at the G119S point mutation in field collected adult mosquitoes (F<sub>0</sub>), F<sub>1</sub>  
200 alive and dead mosquitoes 24h after exposure to bendiocarb. R represents the heterozygote  
201 genotype A/G. B) Chromatogram traces showing the three genotypes at the 119 coding position.

202 Analysis of the polymorphism patterns of the Ace-1 portion resulted in the alignment of a  
203 common 705 bp detecting overall 35 polymorphic sites with a higher value of 25 and 29 in alive and  
204 F<sub>0</sub> populations respectively and lower value in dead (3) individuals (table 2). The number of  
205 haplotypes, the haplotype diversity and the genetic diversity were higher for F<sub>0</sub> and F<sub>1</sub> alive  
206 mosquitoes than for F<sub>1</sub> dead mosquitoes. Most substitutions were synonymous with only the G119S  
207 as the single non-synonymous substitution (Table 1).

208

209 Table 1: Summary statistics for polymorphism in Ace-1 gene including the G119S mutation in *An.*  
210 *gambiae* mosquito population from Bankeng, Central Cameroon.

	2n	S	Ka	Ks	h	hd	$\pi$	D	D*	Fs
Alive	16	25	1	8	10	0.825	0.01	-0.384ns	-0.801ns	0.561ns
Dead	16	3	0	1	4	0.650	0.001	0.467ns	-0.038ns	-0.151ns
F <sub>0</sub>	24	29	1	12	10	0.757	0.009	-0.755ns	-1.721ns	0.588ns
Total	56	35	1	14	23	0.853	0.01	-0.507ns	-2ns	-3.695*

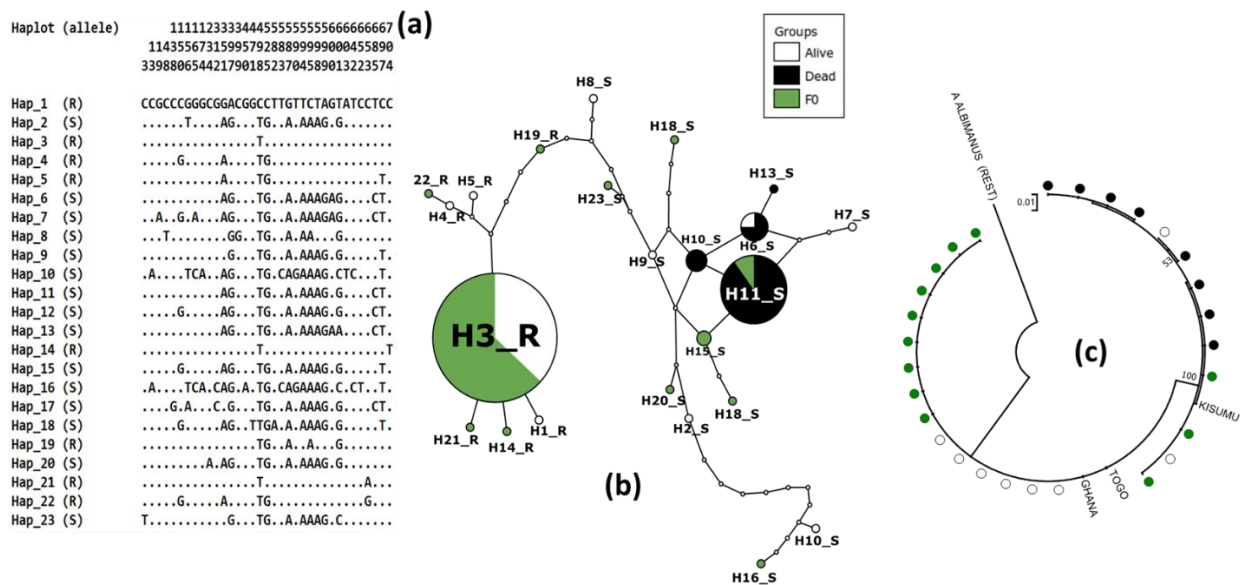
211 2n: number of sequences; D: Tajima's statistics; D\*: Fu and Li's statistics; h: number of haplotypes;

212 hd: haplotype diversity; ns: not significant;  $\pi$ : nucleotide diversity; S: number of polymorphic sites;

213 Ka: synonymous substitution; Ks: non-synonymous substitution

214

215 A total of 23 different haplotypes were identified including 4, 8 and 10 specific to dead, alive and  
 216 F<sub>0</sub> mosquitoes respectively, while 1 haplotype (H13) is shared by dead and alive mosquitoes, one  
 217 (H11) by dead and F<sub>0</sub> and another one (H3) by alive and F<sub>0</sub> mosquitoes (Figure 4). The analysis of the  
 218 haplotype network showed two ancestral haplotypes (H3 and H11). Furthermore, it was observed a  
 219 trend of clustering according to phenotype, with all susceptible grouped in one cluster and the  
 220 resistant to another cluster (Figure 4-b). The phylogenetic tree emphasized this observation by clearly  
 221 showing specific cluster between resistant (F<sub>0</sub> and F<sub>1</sub> alive individuals genotyped as RR by TaqMan  
 222 assay) and susceptible (F<sub>1</sub> dead individuals genotyped as SS) mosquitoes (Fig 3-C). Interestingly, the  
 223 predominant resistant haplotype from F<sub>0</sub> and F<sub>1</sub> alive mosquitoes was identical to resistant alleles  
 224 previously detected in Ghana and Togo, in West African region.  
 225



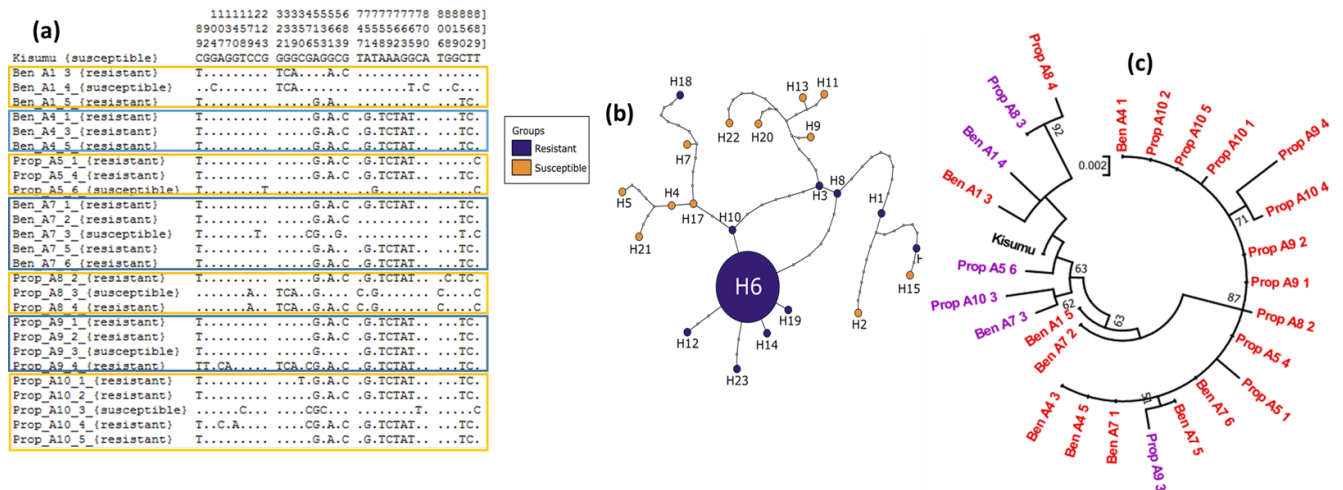
226

227 **Figure 4:** Polymorphism patterns of Ace-1 gene from direct sequencing. A) Polymorphic sites and  
 228 haplotypes detected. Haplotypes are labeled with S (susceptible) or R (resistant). b) TCS haplotype  
 229 network showing the resistant and susceptible haplotype clusters. Lines connecting haplotypes and  
 230 each node represent a single mutation event; (c) Maximum likelihood phylogenetic tree of Ace-1 gene  
 231 supporting the clustering of haplotypes according to mosquito resistance status  
 232

### 233 3.5. Investigation of duplication of Ace-1 in Bankeng

234 In order to investigate the presence of the Ace-1 duplication, the same Ace-1 portion from F<sub>1</sub>  
 235 mosquitoes alive after exposure to insecticide was cloned. Out of the 10 samples successfully cloned  
 236 and sent for sequencing, 7 (3 exposed to bendiocarb: BenA1, BenA4, BenA7 and 4 exposed to  
 237 propoxur: PropA5, PropA8, PropA9, PropA10) were successfully sequenced and analyzed (Figure 5,  
 238 additional file 2). Overall, each of these samples provided a minimum of three cloned haplotypes  
 239 useful to investigate the presence of duplications. Except for sample BenA4 which contained only a  
 240 single resistant haplotype, most mosquitoes carried at least three different haplotypes. A single  
 241 glycine allele (susceptible) was observed for each sample, whereas, 2 and 3 different serine allele  
 242 (resistant) were detected in 4 (BenA1, PropA5, PropA8, PropA9) and two (BenA7 and PropA10)  
 243 different mosquitoes (Figure 5-a and 5-c). The haplotype network shows two different clusters: one  
 244 composed by resistant alleles and another by mostly susceptible allele. (Figure 5b) The allele H6 was  
 245 the major resistant haplotype whereas there is no dominant allele among susceptible alleles.





246

247 **Figure 5:** Polymorphism patterns of Ace-1 gene from cloning. A) Polymorphic sites and haplotypes  
 248 detected. b) TCS haplotype network showing the resistant and susceptible haplotype clusters. Lines  
 249 connecting haplotypes and each node represent a single mutation event; (c) Maximum likelihood  
 250 phylogenetic tree of Ace-1 gene supporting the clustering of haplotypes according to mosquito  
 251 resistance status. .

252

#### 253 4. Discussion

254 Encouraged by interesting results observed in the reduction of malaria transmission in countries  
 255 where non-pyrethroid-based IRS have been intensively implemented during the last decade,  
 256 several other countries in Africa are planning to start using this strategy to control malaria.  
 257 Carbamate (CMs) and organophosphates (OP) are the two insecticide classes mostly currently used  
 258 for IRS in areas of high pyrethroids resistance. Unfortunately, resistance to these insecticides is now  
 259 being reported in malaria vectors across the African continent. To preserve the efficacy of IRS it is  
 260 essential to understand the mechanisms underlying this resistance. In Cameroon, where IRS is  
 261 planned to be implemented shortly through PMI activities, resistance to carbamate has already been  
 262 reported in *An. gambiae* mosquitoes [32, 34, 49]. However, up to now, the molecular mechanisms  
 263 involved in this resistance has not been characterized. The present study showed the evidence of  
 264 ace-1 mutation in *An. gambiae* mosquito population from Cameroon and its association with  
 265 carbamate resistance. Moreover, the analysis of the sequence bearing the G119S, mutation led to the  
 266 detection of the duplication of this mutation in carbamate-resistant mosquitoes.

267 High level of carbamate resistance was observed in *An. gambiae* population tested in the present  
 268 study and is consistent with other previous studies across the country [32, 36, 37, 49, 50]. As the use  
 269 of carbamate and organophosphate insecticides for public health has not been effective to date or is  
 270 very limited in Cameroon, it could be assumed that the primary source of selection must be from  
 271 agricultural usage. This hypothesis could be supported by previous results of Antonio-Nkondjio  
 272 and collaborators showing that mosquitoes originating from cultivated sites were more resistant to  
 273 bendiocarb than those collected elsewhere [32]. This can be reinforced by the presence of an  
 274 important watermelon field using important quantity of pesticide in the village where mosquito  
 275 collection was carried out. Furthermore, agriculture-driven selection of resistance to carbamates in  
 276 *An. gambiae* mosquitoes was abundantly reported in West Africa [24-26, 51].

277 Cross-resistance to carbamates and organophosphates have been reported to be conferred by the  
 278 ace-1 mutation (G119S) due to a substitution of glycine by the serine in codon 119 of the gene [30,  
 279 31]. Results of the present study demonstrated the evidence of a strong association between

280 resistance to carbamates and the presence of G119S mutation in *An. gambiae* mosquito population  
281 from southern Cameroon. Indeed, almost all alive mosquitoes after exposure to both bendiocarb  
282 and propoxur were either homozygote serine or heterozygote TaqMan genotyped. Furthermore,  
283 the replacement of the G by the A nucleotide leading to substitution of the glycine by the serine,  
284 was identified in the sequences of ace-1 gene from alive mosquitoes but not in the sequence the  
285 dead mosquitoes. These results clearly demonstrate that the Ace-1 mutation is significantly  
286 involved in the occurrence of resistance to carbamates in *An. gambiae* population from Bankeng. In  
287 our knowledge, this is the first time the G119S Ace-1 mutation is clearly shown to be associated  
288 with carbamate resistance in Central African *An. gambiae* mosquito populations. Previous studies  
289 reporting the resistance to carbamates in *An. gambiae* mosquito populations from Central African  
290 countries did not detect the presence of Ace-1 G119S mutation in this region or did not establish  
291 such association [32, 52-56].

292 The Ace-1 G119S mutation have been largely reported in West Africa but not in Central Africa. Its  
293 recent emergence in Cameroon could be explained by either a de novo occurrence in local  
294 populations of *An. gambiae* or could result from a spread of this mutation from West African  
295 populations. The result of the present seems to favour the hypothesis of a migration, as the resistant  
296 allele detected here was found identical to those previously detected in Ghana and Togo [25] and in  
297 other West African countries [30, 51]. Further studies are needed to fully establish the origin of this  
298 mutation in Cameroon. However, the high frequency of the resistance allele (119S) and high ratio of  
299 mutant homozygotes in all the screened individuals is largely surprising knowing that the mutation  
300 seems to be recent in *An. gambiae* population from Cameroon. Such high allelic frequency and  
301 heterozygous deficit was reported to be resulting from a deviation from Hardy-Weinberg  
302 equilibrium in previous studies in West Africa [24, 29].

303 In the present study, the detection of at least three different alleles in some individuals after cloning  
304 of the portion of the gene provides the evidence of an ace-1 gene duplication occurrence in a field  
305 population of *An. gambiae* from Cameroon. This is interesting as it seems to indicate that the  
306 selection of the Ace-1 G119S mutation and the occurrence of the duplication are two events taken  
307 place at the same time under the same selective pressure. However, further genetic studies would  
308 be more informative for the understanding of this phenomenon. The presence of three or more Ace-  
309 1 alleles in *An. gambiae* mosquito was previously documented in several countries in West Africa  
310 [25, 57, 58]. In the present study, each sequenced individual specimen possessed at least two  
311 distinct resistant alleles and one susceptible allele. This could also explain why most mosquitoes  
312 alive after carbamate exposure were genotyped as homozygote resistant by TaqMan with a lack of  
313 heterozygotes as mosquitoes with two copies of the gene seem to have 3 resistant alleles of vs only 1  
314 susceptible allele. This is also consistent with the result of Essandoh and collaborators in Ghana, but  
315 is in contrast to previous findings in Burkina-Faso and Côte-d'Ivoire, where only one resistant and  
316 two susceptible allele were detected in *An. gambiae* mosquito [57]. It was reported that the presence  
317 of this duplication allows individuals to have both susceptible and resistant copies of the gene,  
318 which likely decreases fitness costs associated with the resistant genotype [59]. Thus, the presence  
319 of such mutation represents an important threat for carbamate-based vector control strategy  
320 because it could not only allow mosquito to survive in the presence of insecticide, but also to reduce  
321 the impact of fitness cost in absence of insecticide pressure.

## 322 5. Conclusion:

323 This study demonstrates the presence of G119S Ace-1 mutation associated with resistance to  
324 carbamate insecticides in a field population of *An. gambiae* in Cameroon. Furthermore, it also detected  
325 a duplication of the ace-1 mutation that potentially maintain the carbamate resistance in field  
326 populations by reducing associated fitness cost. The emergence and the spread of this mutation could  
327 widely impact the effectiveness of all strategy based on the use of carbamate insecticides. To insure

328 the effectiveness of the planned IRS in Cameroon, there is an urgent need to conduct further studies  
329 to assess the distribution of the Ace-1 G119S mutation and its association with resistance nationwide.

330 **Supplementary materials: Additional file 1:** Alignment of *Ace-1* sequences from direct sequencing  
331 of field collected adult mosquitoes (F0) and of dead and alive mosquitoes 24h after exposure to  
332 bendiocarb and from F0 mosquitoes. **Additional file 2:** Alignment of cloned *Ace-1* sequences from  
333 alive mosquitoes 24h after exposure to bendiocarb and propoxur in comparison of the sequence  
334 from the susceptible lab strain (Kisumu).

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336 A.B. and T.A. carried out the sample collection; L.N.; A.B. and T.A. reared and maintained the  
337 strain in the insectary; L.N., A.B., T.A. performed insecticide bioassays; L.N, T.A, D.D and H.I.  
338 performed the molecular analyses, cloning and sequencing; E.E.N, C.N., D.N.-N. and C.S.W.  
339 analyzed the data; E.E.N and C.S.W. wrote the manuscript with contributions from C.N., B.T.-F.  
340 and D.N.-N. All authors approved the manuscript.

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346

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