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Article

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

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

Keywords: Ace-1 G119S mutation, Insecticide resistance, *Anopheles gambiae*, Cameroon, malaria

1-Introduction

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

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

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

2. Methods

2.1. Mosquito sampling

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

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

2.2. Insecticide bioassays

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

2.3. Species identification and Ace-1 G119S mutation genotyping

These analyses were done using total genomic DNA extracted from 91 field-collected adult mosquitoes randomly selected (F_0) and F_1 alive and dead mosquitoes after exposure to bendiocarb (25 alive and 67 dead) and propoxur (30 alive and 38 dead). DNA was extracted from whole mosquito following the Livak protocol previously described [41]. Identification of species within An. gambiae complex was determined using the SINE PCR protocol [42]. The presence of the G119S mutation was screened with TaqMan real-time PCR assay (using Agilent Mx3005 qRT-PCR thermocycler) following the protocols established by Bass and colleagues [43]. Each reaction was conducted in a total volume of 10 µl comprise of 5 µl Sensimix (Bioline), 0.25 µl of 40x Probe Mix coupled to allelic-specific primers, 4.25 µl of dH20, and 1 µl of genomic DNA. Thermocycling conditions were an initial 10 min at 95 °C, followed by 40 cycles each of 92 °C for 15 sec, and 60 °C for 1 min. Two probes labelled with fluorochromes FAM and HEX were utilised to detect the resistant mutant and the wild type susceptible alleles, respectively. Genotypes were scored from bi-directional scatter plots of results produced by the Mx3005 v4.10 software. Thereafter, the correlation between G119S genotypes and bendiocarb resistance phenotypes was assessed by estimating the odds ratio (OR) and the statistical significance based on Fisher exact probability test.

2.4. Ace-1 gene amplification, sequencing and cloning

A region of 924-bp in a sequence of the ace-1 gene, encompassing exons 4–6 (VectorBase AgamP3 annotation; G119S position in exon 5 corresponding to the third coding exon) was amplified from 55 female *An. gambiae*: 15 from F0 (field-collected adult mosquitoes), 40 from F_1 mosquitoes after exposure to insecticide (10 alive and 10 dead after exposure to bendiocarb, 10 alive and 10 dead after exposure to propoxur). The amplification by PCR was carried out following the protocol previously described by Essandoh and collaborators [25]. Briefly, each reaction was conducted a total volume of 50 µl containing 10 picomoles of each primer Ex2Agdir1 (5'AGG TCA CGG TGA GTC CGTACG A 3') and Ex4Agrev2 (5' AGG GCG GAC AGC AGA TGC AGC GA 3'), 10 mM dNTPs, ddH2O, 5X HF Phusion buffer, and 1u of Phusion Taq polymerase (Fermentas). The cycle parameters were: 1 cycle 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 final extension at 72°C for 5 min. The PCR products were purified using the Qiaquick purification kit (QIAgen, Hilden, Germany) and 28 amplicons (12 F_0 field collected adults, 8 alive and 8 dead after exposure to bendiocarb) were sequenced directly using the primers Ex2Agdir1 and Ex4Agrev2 to

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

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

3. Results

3.1. Mosquito collection and species molecular identification

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

3.2. Insecticide bioassay

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

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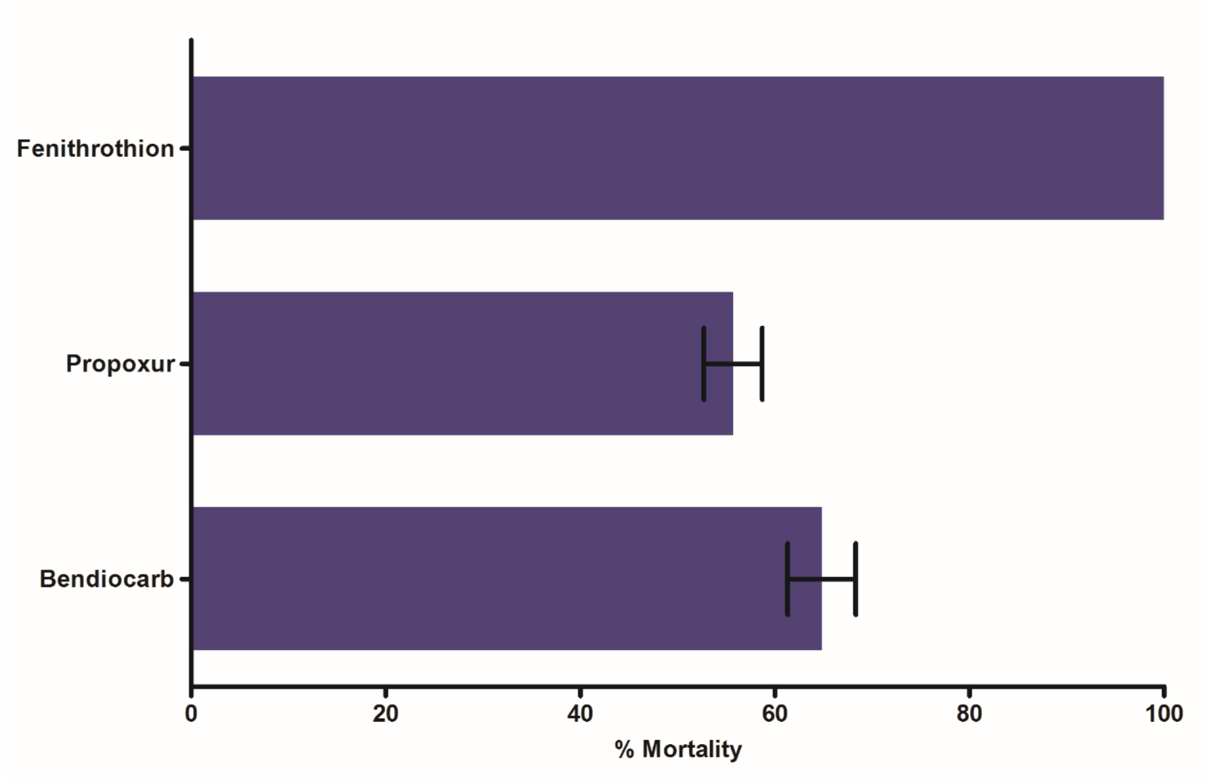

Figure 1: Susceptibility status of An. gambiae mosquito population from Bankeng, central Cameroon. Mortality rates were recorded 24h post-exposure insecticides. Data are shown as mean ± SEM (n=260).

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

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

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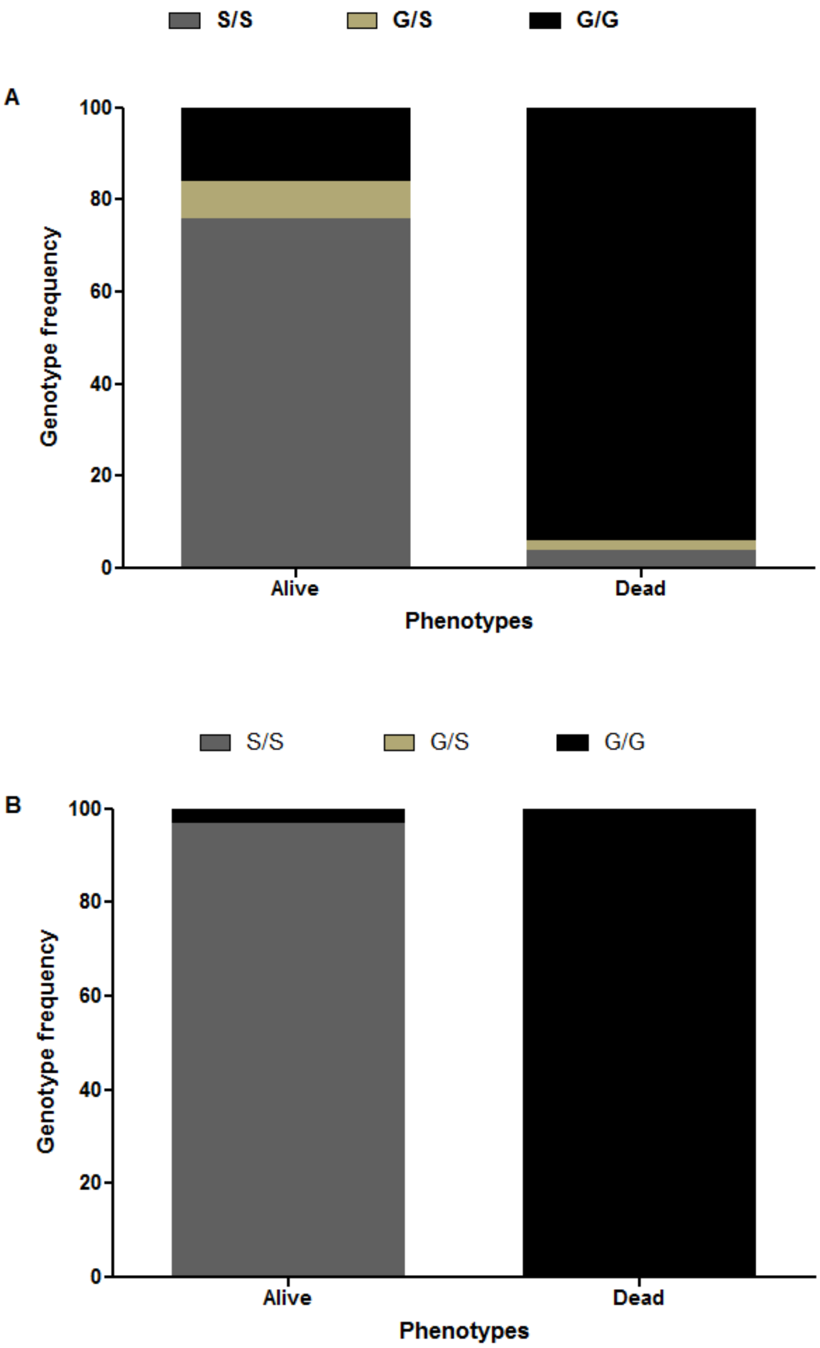

Figure 2: Distribution of Ace-1 G119S genotypes and association with bendiocarb (A) and propoxur (B) resistant phenotype.

3.4. Genetic diversity of Ace-1 in Bankeng

A region of 924 bp including the 119 codon of the ace-1 gene was amplified from 28 mosquitoes (12 F_0, 8 dead and 8 alive after exposure to bendiocarb) in order to confirm the presence of the 119S allele and to assess the genetic diversity of this gene. A 705 bp sequence was commonly aligned for the 28 samples (Additional file 1). A G-to-A substitution at position 397, corresponding to the 119 codon, was observed in 11 sequences (7 F_0 and 4 F_1 alive) in comparison with the reference sequence from susceptible Kisumu strain, (Figure 3). Heterozygote mosquitoes were detected (2 F_0 and 2 F_1 alive mosquitoes) with overlapping peaks for G and A at the same position (represented by the ambiguity code R, Figure 3). Interestingly, no substitution was detected in all the sequences from the 8 dead F_1 mosquitoes (Figure 3).

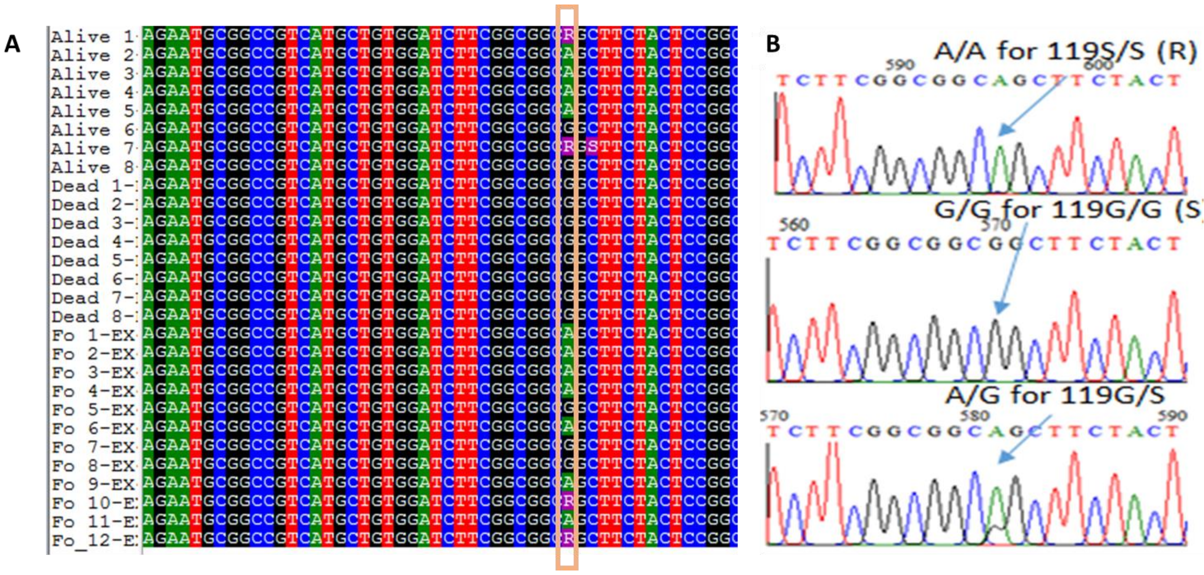

Figure 3: Sequencing of the portion of the Ace-1 gene spanning the G119S mutation**.** A) Sequence alignment of the Ace-1 gene at the G119S point mutation in field collected adult mosquitoes (F0), F1 alive and dead mosquitoes 24h after exposure to bendiocarb. R represents the heterozygote genotype A/G. B) Chromatogram traces showing the three genotypes at the 119 coding position.

Analysis of the polymorphism patterns of the Ace-1 portion resulted in the alignment of a common 705 bp detecting overall 35 polymorphic sites with a higher value of 25 and 29 in alive and F_0 populations respectively and lower value in dead (3) individuals (table 2). The number of haplotypes, the haplotype diversity and the genetic diversity were higher for F_0 and F_1 alive mosquitoes than for F_1 dead mosquitoes. Most substitutions were synonymous with only the G119S as the single non-synonymous substitution (Table 1).

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

	2n	S	Ka	Ks	h	hd	π	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
F0	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*

2n: number of sequences; D: Tajima's statistics; D*: Fu and Li's statistics; h: number of haplotypes; hd: haplotype diversity; ns: not significant; π: nucleotide diversity; S: number of polymorphic sites; Ka: synonymous substitution; Ks: non-synonymous substitution

A total of 23 different haplotypes were identified including 4, 8 and 10 specific to dead, alive and F_0 mosquitoes respectively, while 1 haplotype (H13) is shared by dead and alive mosquitoes, one (H11) by dead and F_0 and another one (H3) by alive and F_0 mosquitoes (Figure 4). The analysis of the haplotype network showed two ancestral haplotypes (H3 and H11). Furthermore, it was observed a trend of clustering according to phenotype, with all susceptible grouped in one cluster and the resistant to another cluster (Figure 4-b). The phylogenetic tree emphasized this observation by clearly showing specific cluster between resistant (F_0 and F_1 alive individuals genotyped as RR by TaqMan assay) and susceptible (F_1 dead individuals genotyped as SS) mosquitoes (Fig 3-C). Interestingly, the predominant resistant haplotype from F_0 and F_1 alive mosquitoes was identical to resistant alleles previously detected in Ghana and Togo, in West African region.

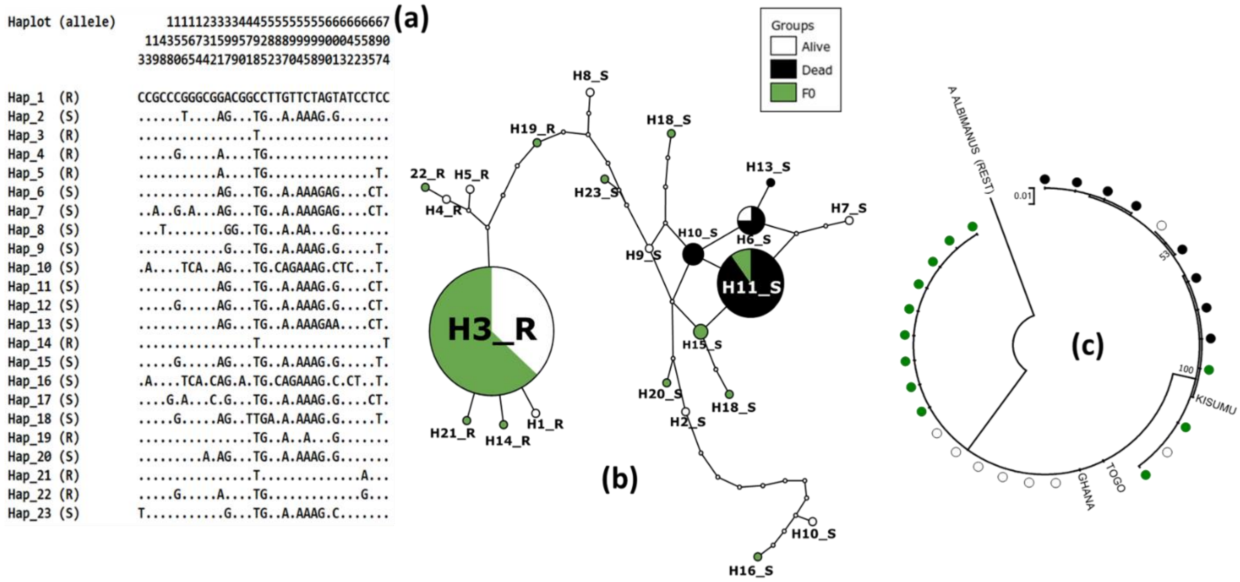

Figure 4: Polymorphism patterns of Ace-1 gene from direct sequencing. A) Polymorphic sites and haplotypes detected. Haplotypes are labeled with S (susceptible) or R (resistant). b) TCS haplotype network showing the resistant and susceptible haplotype clusters. Lines connecting haplotypes and each node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

3.5. Investigation of duplication of Ace-1 in Bankeng

In order to investigate the presence of the Ace-1 duplication, the same Ace-1 portion from F_1 mosquitoes alive after exposure to insecticide was cloned. Out of the 10 samples successfully cloned and sent for sequencing, 7 (3 exposed to bendiocarb: BenA1, BenA4, BenA7 and 4 exposed to propoxur: PropA5, PropA8, PropA9, PropA10) were successfully sequenced and analyzed (Figure 5, additional file 2). Overall, each of these samples provided a minimum of three cloned haplotypes useful to investigate the presence of duplications. Except for sample BenA4 which contained only a single resistant haplotype, most mosquitoes carried at least three different haplotypes. A single glycine allele (susceptible) was observed for each sample, whereas, 2 and 3 different serine allele (resistant) were detected in 4 (BenA1, PropA5, PropA8, PropA9) and two (BenA7 and PropA10) different mosquitoes (Figure 5-a and 5-c). The haplotype network shows two different clusters: one composed by resistant alleles and another by mostly susceptible allele. (Figure 5b) The allele H6 was the major resistant haplotype whereas there is no dominant allele among susceptible alleles.

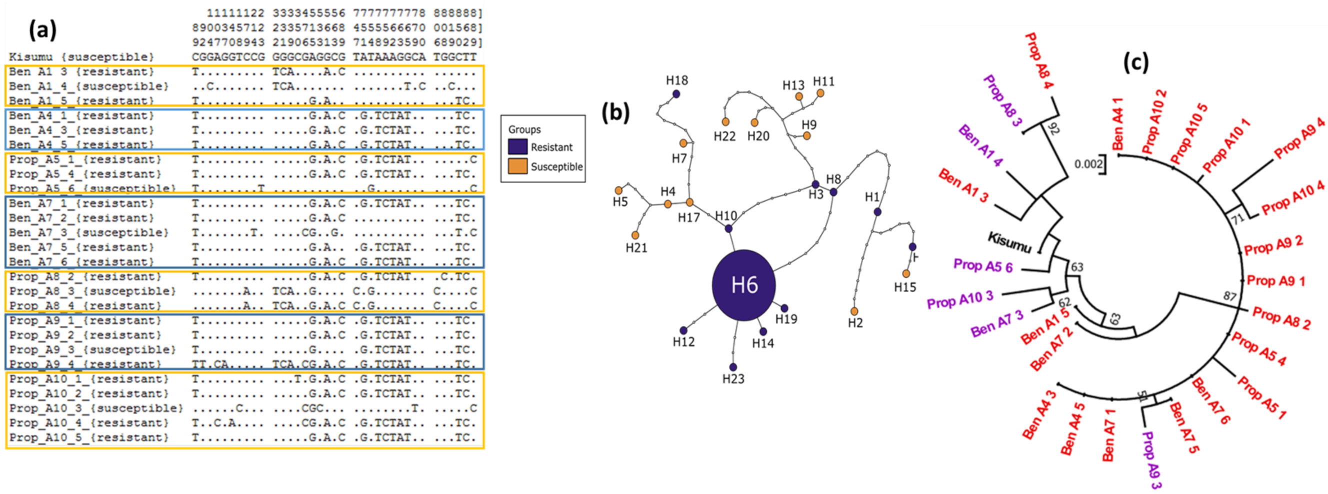

Figure 5: Polymorphism patterns of Ace-1 gene from cloning. A) Polymorphic sites and haplotypes detected. b) TCS haplotype network showing the resistant and susceptible haplotype clusters. Lines connecting haplotypes and each node represent a single mutation event; (c) Maximum likelihood phylogenetic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

4. Discussion

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

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

Cross-resistance to carbamates and organophosphates have been reported to be conferred by the ace-1 mutation (G119S) due to a substitution of glycine by the serine in codon 119 of the gene [30, 31]. Results of the present study demonstrated the evidence of a strong association between resistance to carbamates and the presence of G119S mutation in *An. gambiae* mosquito population from southern Cameroon. Indeed, almost all alive mosquitoes after exposure to both bendiocarb and propoxur were either homozygote serine or heterozygote TaqMan genotyped. Furthermore, the replacement of the G by the A nucleotide leading to substitution of the glycine by the serine, was identified in the sequences of ace-1 gene from alive mosquitoes but not in the sequence the dead mosquitoes. These results clearly demonstrate that the Ace-1 mutation is significantly involved in the occurrence of resistance to carbamates in *An. gambiae* population from Bankeng. In our knowledge, this is the first time the G119S Ace-1 mutation is clearly shown to be associated with carbamate resistance in Central African *An. gambiae* mosquito populations. Previous studies reporting the resistance to carbamates in *An. gambiae* mosquito populations from Central African countries did not detect the presence of Ace-1 G119S mutation in this region or did not establish such association [32, 52-56].

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

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

5. Conclusion:

This study demonstrates the presence of G119S Ace-1 mutation associated with resistance to carbamate insecticides in a field population of *An. gambiae* in Cameroon. Furthermore, it also detected a duplication of the ace-1 mutation that potentially maintain the carbamate resistance in field populations by reducing associated fitness cost. The emergence and the spread of this mutation could widely impact the effectiveness of all strategy based on the use of carbamate insecticides. To insure the effectiveness of the planned IRS in Cameroon, there is an urgent need to conduct further studies to assess the distribution of the Ace-1 G119S mutation and it association with resistance nationwide.

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

Author Contributions: E.E.N and C.S.W. conceived and designed the study; E.E.N.; B.T.-F.; L.N.; A.B. And T.A carried out the sample collection; L.N.; A.B. and T.A. reared and maintained the strain in the insectary; L.N., A.B., T.A. performed insecticides bioassays; L.N, T.A, D.D and H.I. performed the molecular analyses, cloning and sequencing; E.E.N, C.N., D.N.-N. and C.S.W. analyzed the data; E.E.N and C.S.W. wrote the manuscript with contributions from C.N., B.T.-F. and D.N.-N All authors approved the manuscript.

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