proceedings

MDPI

Proceedings

Influence of a Major Mountainous Landscape Barrier (Mount Cameroon) on the Spread of Metabolic (*GSTe2*) and Target-Site (*Rdl*) Resistance Alleles in the African Malaria Vector *Anopheles funestus* †

Nathalie Amvongo-Adjia [1,2,3,6,*], Jacob M. Riveron [3], Flobert Njiokou [1,3], Samuel Wanji [2,4] and Charles S. Wondji [3,5,*]

[1] Department of Animal Biology and Physiology, Faculty of Science, University of Yaoundé 1, P.O. BOX 812, Yaoundé, Cameroon; njiokouf@yahoo.com
[2] Research Foundation for Tropical Diseases and Environment (REFOTDE), University of Buea, P.O. BOX 474, Buea, Cameroon; swanji@yahoo.fr
[3] Centre for Research in Infectious Diseases (CRID), LSTM Research Unit, P.O. Box 13591, Yaoundé, Cameroon; jacob.riveron_miranda@syngenta.com
[4] Department of Microbiology and Parasitology, University of Buea, P.O. BOX 63, Buea, Cameroon
[5] Department of Vector Biology, Liverpool School of Tropical Medicine, Liverpool L35QAUK, UK
[6] Medical Research Centre, Institute of Medical Research and Medicinal Plants Studies (IMPM), P.O. BOX 13033, Yaoundé, Cameroon
* Correspondence: amvongo.n@gmail.com (N.A.-A.); charles.wondji@lstmed.ac.uk (C.S.W.)
† Presented at the 1st International Electronic Conference on Genes: Theoretical and Applied Genomics, 2–30 November 2020; Available online: https://iecge.sciforum.net/.

Received: date; Accepted: date; Published: date

Abstract: Understanding the ecological factors impacting the spread of insecticide resistance makers is necessary to design suitable resistance management strategies in malaria dominant vectors' species. Here we examined the influence of the highest mountain in West Africa (Mount Cameroon; 4100 m elevation) on the spread of both metabolic and target-site resistance alleles in *An. funestus sensu stricto* (s.s) populations. Although high frequencies of $GSTe2^R$ (67%–81%) and Rdl^R (49%–90%) resistance alleles were observed in *An. funestus* s.s. populations throughout the study area, it was highlighted a reverse progression of the speed of spread of these two mutations, with increased frequencies of $GSTe2^R$ while Rdl^R decreased following the altitude increment across the mountain; observations that were further reinforced through the Bayesian and population structure analyses. Overall, high levels of polymorphisms were observed within both genes (respectively 12 and 16 haplotypes for *GSTe2* and *Rdl*). However, the reduced diversity patterns of resistance allele carriers revealed signatures of positive selection within the two genes which could hamper the efficacy of current vector control tools in such bioecological zones if not properly monitor.

Keywords: Malaria; *Anopheles funestus sensu stricto*; insecticide resistance; mount cameroon

1. Introduction

In Cameroon, malaria is the leading cause of morbidity and mortality accounting for an estimated 6.2 million clinical cases and 12,500 deaths [1]. *Anopheles funestus sensu stricto* (s.s.) is one of the four major malaria vectors in the country [2,3], being mostly prevalent in the Sudan savanna domain. However, this species had been found transmitting *Plasmodium falciparum* malaria parasite in Cameroon highlands such as the Mount Cameroon region [4] and the locality of Santchou in the western Cameroon [5]. The insecticide resistance profile of *An. funestus* s.s. has previously been explored for some populations, with multiple resistance to pyrethroids,

dichlorodiphenyltrichloroethane (DDT) and carbamates reported in the soudano-sahelian [6–8] and forested [9,10] zones of Cameroon. Two mutations, the *L119F* of the glutathione s-transferases epsilon 2 (GSTe2) gene conferring resistance to DDT and pyrethroids [11], and the *A296S* of the γ-aminobutyric acid (GABA) gene implicated in resistance to dieldrin (*Rdl*) [6], have been identified in *An. funestus* s.s. African populations and were respectively associated to separated resistance mechanisms: the increased metabolic detoxification of insecticides commonly known as metabolic resistance, and the decreased sensitivity of the target proteins on which an insecticide acts, so called target-site resistance [12]. The spread of these resistance alleles are not uniformed across the African continent, and presence of barriers to gene flow has been suggested to explain the restriction in the spread of these alleles [13,14].

Major landscape modifications such as Rift Valley have been suggested as the main continent-wide barrier to the spread of resistance alleles in *An. funestus* [13,14]. It remains to know whether other major but local landscape modifications such as major mountain chains could also restrict the spread of these resistance alleles. The Mount Cameroon chain with its highest peak at 4100 m above the sea level (m a.s.l.), is the highest mountain in West Africa and thus constitutes a major landscape variation in the region with potential to impact patterns of gene flow between populations of species across this region as. This potential influence on the spread of resistance alleles across the region remains unknown, although it has already been reported high level of resistance to pyrethroids in *An. coluzzii* and *An. gambiae* vector populations in this region [15]. Therefore, similar resistance profile could be expected in *An. funestus* vectors and understanding the influence of the high altitude of Mount Cameroon in the spread of resistance alleles will help to improve the management of related resistance.

2. Materials and Methods

2.1. Study Site, Adult Mosquitio Sampling and Identification

Entomological surveys were conducted from sea level to 800 m a.s.l. due to previous surveys where the absence of *Anopheles* mosquitoes in areas situated above 800 m elevation was reported during four consecutive seasons [16]. The localities surveyed during this study were: Tiko village (4°3′ N, 9°22′ E and elevation 9 m a.s.l.) and Likomba (4°5′ N, 9°20′ E and elevation 70 m a.s.l) considered as lowlands (Tiko village and Likomba were further considered as a single collection site: Tiko), Mutengene (4°05′57″ N, 9°18′29″ E, altitude 220 m a.s.l.) and Meanja (4°16′ N, 9°23′ E, altitude 305 m a.s.l.) considered as mid altitude areas, and Likoko (4°8′41″ N, 9°13′38″ E, elevation 800 m a.s.l.), a highland area. Localities of Tiko, Likomba, Mutengene and Likoko follow an altitudinal transect on the southwest and west edge of the mountain whereas Meanja was selected as a outside-elevated area on the eastern edge of the mountain to assess if contrasting events occur across the mountain (Supplementary Figure S1).

Prior to the entomological field activities, authorisations were seek from village's chiefs, sub-chiefs, quarter's heads and household's owners. Mosquito collectors were invited to sign a consent form before participating in night collections and could withdraw whenever wanted. In addition, presumptive malaria prophylaxes were given to them at the end of collections.

Female adult mosquitoes were collected from the year 2010 to 2014, indoor and outdoor households between 06:00 PM to 06:00 AM using the landing catch on volunteers' technique as previously reported [17]. Female *Anopheles* caught were morphologically identified [18], and for each specimen identified as belonging to either the *An. gambiae* complex or the *An. funestus* group, genomic DNA was extracted following the DNA extraction buffer protocol [19] on whole mosquitoes. Sibling species of the *An. gambiae* complex were distinguished using conventional PCR [20] and PCR-RFLP [21] of the ribosomal intergenic spacer IGS of the nuclear rDNA gene, whereas members of the *An. funestus* group were characterized by amplifications of the internal transcribed spacer ITS2 [22].

2.2. Genotyping of GSTe2 and Rdl Resistance Markers

GSTe2 mutation associated to DDT and pyrethroids' resistance in *An. funestus* was genotyped using an allele-specific PCR (AS-PCR) assay previously described by [23]. The same was done for the *Rdl* mutation conferring resistance to dieldrin as described by [24]. Frequency distribution of *GSTe2* and *Rdl* genotypes and alleles were compared using the Chi-square (χ^2) test, with statistical significance set at $p < 0.05$.

2.3. Polymorphism Analysis of Resistance Genes

2.3.1. Genetic Variability of *An. funestus* s.s. across the Mount Cameroon Region Based on *GSTe2* and *Rdl* Full Gene Sequencing

Full-length sequences (exons and introns) of *GSTe2* and *Rdl* were individually amplified for forty samples (ten samples per locality each: Tiko, Mutengene, Meanja and Likoko) according to previous protocols [11,24]. Thereafter, successful amplicons were purified using Exo-SAP clean up protocol (ThermoFisher Scientific, Santa Clara, CA, USA) and directly sequenced on both strands. The polymorphic positions were identified through a manual analysis of sequence chromatograms using BioEdit 7.2.5 [25] based on sequence differences in multiple alignments using ClustalW [26]. Genetic diversity parameters were computed using dnaSP 5.10 [27]. Haplotype networks were then built using the TCS program [28,29] to assess the connection between haplotypes. The level of pairwise genetic differentiation were estimated using the K_{ST} statistics [30] as implemented in dnaSP 5.10. In addition, the nucleotide sequences of the haplotypes were submitted to Genbank (*GSTe2* accession numbers: MN562756, MN562757, MN562760, MN562764–MN562766, MN562768–MN562771, MN562774 and MN562775; *Rdl* accession numbers: MN562780–MN562795).

2.3.2. Phylogenetic Trees of Haplotypes

Best-fit substitution model for each dataset was assessed based on the Bayesian Information Criterion (BIC) in MEGA 10.1.6 [31]; thus indicating that the Tamura 3-parameter and Hasegawa-Kishino-Yano models best described *GSTe2* and *Rdl* haplotype datasets respectively. These models were then used to build the respective ML tree using MEGA 10.1.6 with 500 bootstrap replications for the robustness of the trees. Neighbour-Joining trees were also constructed with pairwise G_{ST} genetic distances [32] between subpopulations still in MEGA 10.1.6.

3. Results

3.1. Anopheles Species Composition

A total of 4911 female mosquitoes caught across the study localities during the entire collection period. Of these, morphological identification revealed that 3194 (65%) female mosquitoes belong to the genus *Anopheles* including species of the *An. gambiae* complex (2747; 86%), *An. funestus* group (390; 12.2%) and three secondary vector species (1.6% *An. hancocki*, 0.2% *An. nili* s.l. and 0.03% *An. ziemanni*), with a significant difference noted in the frequency distributions of *Anopheles* sp. between localities ($p < 0.0001$). PCR-species identification performed from 3135 females morphologically identified as *An. gambiae* s.l. and *An. funestus* s.l. revealed that three *An. gambiae* siblings coexist in the study area: *An. coluzzii* (44.7%), *An. gambiae* (28.6%) and *An. melas* (12%), in addition to 23 hybrids *An. coluzzii* × *An. gambiae* (0.7%). Whereas, all *An. funestus* s.l. individuals were identified as *An. funestus* s.s. (Supplementary Figure S2).

3.2. Detection of Mutations Associated to DDT and Dieldrin Resistance in An. funestus s.s. Mosquitoes

The presence of *GSTe2* and *Rdl* mutations was investigated respectively in 339 and 218 *An. funestus* s.s. specimens across the study area. Genotyping results showed high frequencies of $GSTe2^R$ (66.7%–97.1%) and Rdl^R (68.4%–90%) resistance cases in *An. funestus* s.s. populations (Supplementary Figure S3), with an overall resistant case estimated at 93.5% (317/339) for *GSTe2* gene and 74.3% (162/218) for *Rdl* gene. The *GSTe2* mutation had a predominance of homozygote resistant genotypes

throughout the study area, with increased frequencies of *GSTe2* heterozygotes following the climb in altitude whereas the occurrence of susceptible mosquitoes decreases with land elevation. Meanwhile for *Rdl* mutation, although homozygotes resistant mosquitoes almost dominated across the study area, frequencies of heterozygotes and susceptible mosquitoes followed reverse tendencies than that observed in *GSTe2* (Figure 1A). Here, the frequencies of heterozygotes seemed to decrease with altitude while the occurrence of susceptible mosquitoes increased with land elevation.

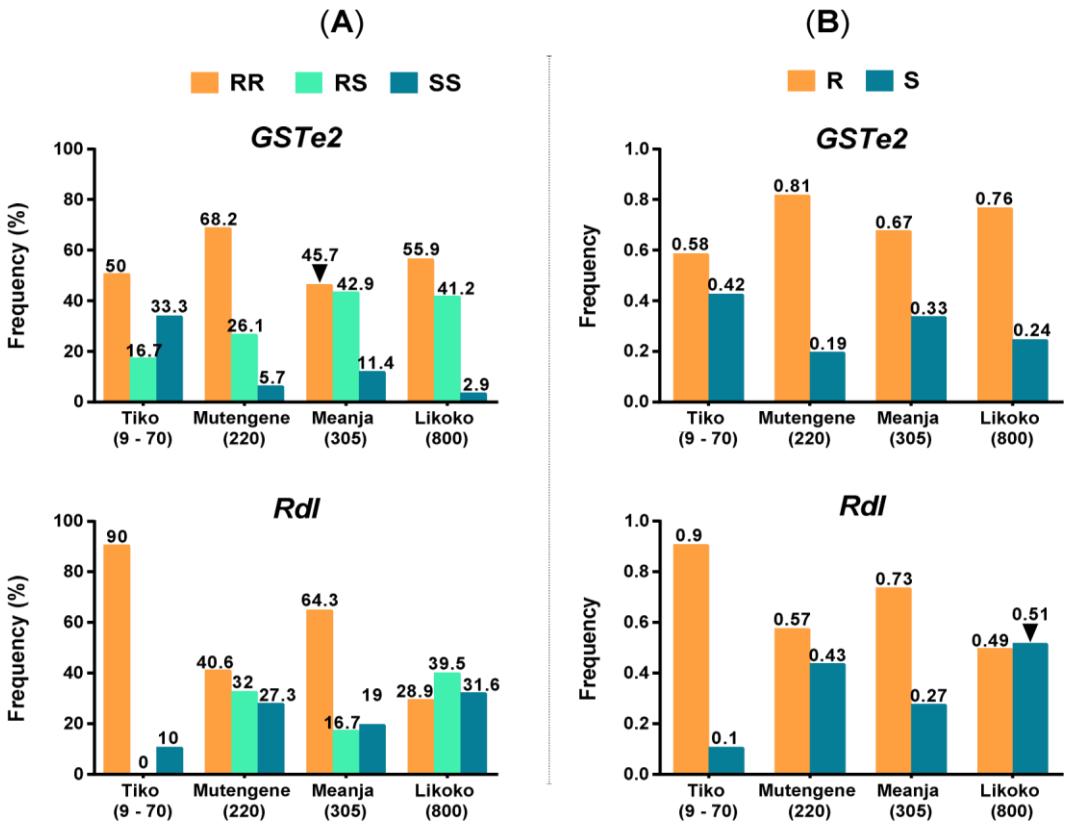

Figure 1. Genotype (A) and allele (B) frequency distributions of *GSTe2* and *Rdl* mutations in *An. funestus* s.s. populations for the different localities surveyed (*altitude in m a.s.l.*).

Analysis of the allele frequencies of *GSTe2* and *Rdl* mutations in *An. funestus* s.s. populations from the four localities revealed same pattern than those obtained with genotyping results (Figure 1B). *GSTe2*^{R} and *Rdl*^{R} resistant alleles were predominantly represented in almost all the collection sites except in Likoko where the *Rdl*^{S} susceptible allele was predominant (with a frequency of 0.51). However, both markers showed contrary evolution in their alleles' frequency distributions when considering land elevation. For resistant alleles, while the frequency of *GSTe2*^{R} seemed to increase with altitude, *Rdl*^{R} frequency decreased with altitude. Rather for susceptible alleles, as the *GSTe2*^{S} frequency is decreasing with altitude, the *Rdl*^{S} increased with land elevation. For both makers, Chi-square test statistical analysis of genotype frequency distributions between localities showed significant differences except in Tiko/Meanja (*GSTe2*: $p = 0.273$, *Rdl*: $p = 0.244$), Mutengene/Likoko (*GSTe2*: $p = 0.17$, *Rdl*: $p = 0.423$) and Meanja/Likoko (only *GSTe2*: $p = 0.354$).

3.3. Analysis of the Polymorphism of GSTe2 and Rdl Genes across Mount Cameroon An. funestus s.s. Populations

3.3.1. Sequence Analysis of Full Length *GSTe2* and *Rdl* Genes

GSTe2 and *Rdl* full length fragments were successfully amplified in 36 and 34 (respectively for both makers) *An. funestus* s.s. genomic DNA samples with specific primers for both mutations [11,24]. The PCR products were purified, sequenced and aligned. The alignment and comparison of sequences obtained with that referenced in Genbank confirmed the presence of chain A glutathione S-transferase Epsilon 2 protein of *An. funestus* (AHC31021.1) and exon 7 encoding the M2 transmembrane domain region of *An. funestus* GABA-receptor gene (AZB49494.1) respectively for *GSTe2* and *Rdl* amplicons. A point mutation (CTT to TTT) at position 92, inducing an amino acid change of leucine to phenylalanine (L92F) and which confers resistance to pyrethroids in *An. funestus* s.s. mosquitoes was observed in all *GSTe2* resistant samples of the Mount Cameroon. Likewise, the GCA (alanine) to TCA (serine) mutation at position 49 (A49S) was observed in *An. funestus* s.s. dieldrin resistant samples (Supplementary Figure S4).

3.3.2. Haplotype Distribution of the GSTe2 Gene

A total of 14 variable or polymorphic sites which led to the formation of 12 haplotypes were observed within the 729 bp fragment of *GSTe2* gene of *An. funestus* s.s. moquitoes across the Mount Cameroon region (Figure 2A and Table 1). Overall, the *GSTe2* polymorphism level was average (haplotype diversity: Hd = 0.56). The number of haplotypes (h) and its associated diversity indice (Hd) seemingly decreased (0.68–0.51) from the lowland Tiko (9–70 m a.s.l.) to Likoko situated at the highest altitude (800 m a.s.l.). Samples carrying L92 susceptible allele were found highly polymorphic (h = 9 and Hd = 0.91) as compared to 92F resistant allele carriers (h = 5 and Hd = 0.36).

Table 1. Genetic variability parameters of *GSTe2* full mutation.

Samples	N	S*	h (Hd)	Syn	NSyn	π (k)	D	F*
Per allele								
S	18	11	9 (0.91)	4	2 (D34E, L92F)	0.003 (2.69)	-0.84^{ns}	-0.70^{ns}
R	54	6	5 (0.36)	2	1 (L92F)	0.001 (0.60)	-1.36^{ns}	-0.27^{ns}
Per locality *(altitude in m a.s.l.)*								
Tiko (9–70)	12	8	6 (0.68)	4	1 (L92F)	0.003 (1.97)	-1.38^{ns}	-1.77^{ns}
Mutengene (220)	20	8	7 (0.58)	4	1 (L92F)	0.002 (1.38)	-1.31^{ns}	-2.13^{ns}
Meanja (305)	20	7	5 (0.56)	2	2 (D34E, L92F)	0.001 (1.04)	-1.55^{ns}	-2.54^{ns}
Likoko (800)	20	7	5 (0.51)	1	1 (L92F)	0.002 (1.32)	-1.40^{ns}	-0.76^{ns}
All	72	14	12 (0.56)	6	2	0.002 (1.36)	-1.62^{ns}	-1.02^{ns}

N = number of sequences (2n); S*, number of polymorphic sites; h, number of haplotypes (Hd = haplotype diversity); Syn, Synonymous mutations; Nsyn, Non-synonymous mutations; π, nucleotide diversity (k = mean number of nucleotide differences); D and F* Tajima's and Fu and Li's statistics; ns, not significant; S = susceptible; R = resistant; m a.s.l. = meters above the sea level.

Haplotype networks were built using TCS software. In one hand, haplotype network representation with respect to collection sites (Figure 2B) showed the presence of a unique major haplotype (H2: 47 sequences) distributed from lowland to highland across the study area. The ancestral haplotype (H1) appeared in lowland (Tiko) and midlands (Mutengene and Meanja) but not in highland (Likoko). Three haplotypes (H4, H9 and H11) were simultaneously identified in Tiko and Mutengene sites located between 9 and 220 m a.s.l., another one haplotype (H3) was concomitantly found in both Tiko (9–70 m a.s.l.), Meanja (305 m a.s.l.) and Likoko (800 m a.s.l.), and one other (H5) only found in Meanja and Likoko. Out of the twelve haplotypes identified five occurred as singletons and were distributed as follows: two (H10 and H12) in Mutengene (220 m a.s.l.), one (H8) in Meanja (305 m a.s.l.) and two (H6 and H7) in Likoko (800 m a.s.l.) (Figure 2A).

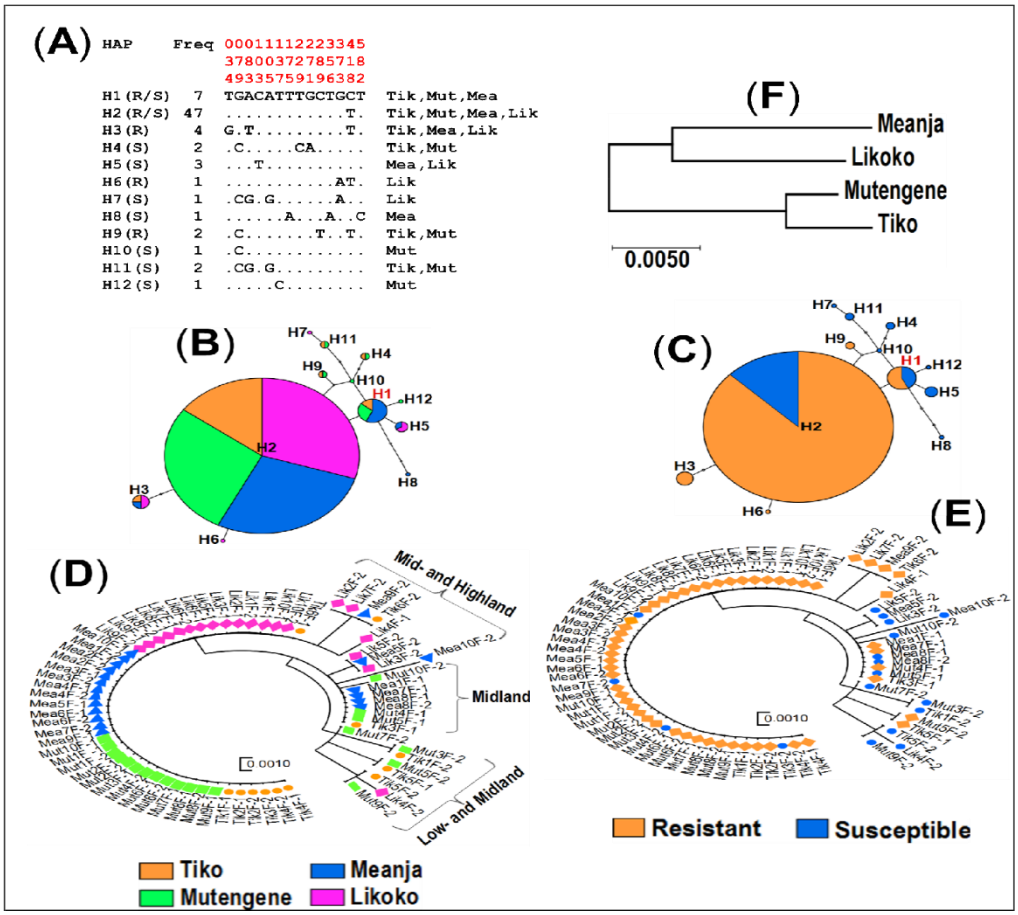

Figure 2. Genetic diversity and polymorphism patterns of *GSTe2* DNA sequences across the Mount Cameroon. (**A**) Haplotype diversity patterns of the 729bp GSTe2 fragment in Tiko (Tik), Mutengene (Mut), Meanja (Mea) and Likoko (Lik); H = haplotype; R = resistant; S = susceptible; polymorphic sites are in *red*. TCS haplotype networks showing haplotype's generation within GSTe2 gene in *An. funestus* s.s with respect to localities (**B**) and allelic profiles (**C**)**;** haplotypes are presented in circular shape scaled to reflect their respective frequencies, ancestral haplotype in *red*. Maximum likelihood phylogenetic trees of *GSTe2* DNA sequences among localities (**D**) and allelic profiles (**E**) showing an apparent separation between susceptible samples and resistant ones. (**F**) Neighbour-joining tree of the genetic distances showing a genetic relatedness to the landscape along the Mountain altitudinal transect with Tiko and Mutengene (9–220 m a.s.l., both located on the southwest edge) clustering together than Likoko (800 m a.s.l., west edge) and Meanja (305 m a.s.l., eastern edge).

Comparatively, haplotype network representation with respect to the allele type (either susceptible or resistant) carried by the sequences analysed (Figure 2C) showed that out of the twelve haplotypes recorded only two (H1 and H2) were common to both L92 and 92F carriers whereas three haplotypes (H3, H6 and H9) were strictly found in 92F carriers and the remaining seven haplotypes (H4, H5, H7, H8, and H10–H12) appeared only in L92 carriers. It was noted that common haplotypes to both L92 and 92F carriers were predominantly found in resistance sequences (H1: 4 sequences of 92F and 3 sequences of L92; H2: 41 sequences of 92F and 6 sequences of L92). Also, out of five haplotypes which appeared as singletons, four were strictly associated to L92 carriers (H7, H8, H10 and H12) whereas only one haplotype (H6) originated from a 92F carrier.

Evidence of selection acting on *GSTe2* gene could be noted as 92F-resstant allele carriers exhibited low diversity parameters (h = 5, Hd = 0.36 and π = 0.001), unless negative and non-significant values were obtained from Tajima D and Fu and Li F* neutrality tests (Table 1).

3.3.3. Haplotype Distribution of the GABA-Receptor Gene across Mount Cameroon Populations of *An. funestus* s.s.

Analysis of 68 sequences for the 1006 bp fragment of the GABA-receptor gene showed the presence of 16 polymorphic sites and the formation of an equal number of haplotypes (Figure 3A and Table 2). Genetic variability parameters with respect to allelic profiles and among the four tested *An. funestus* s.s. populations showed that the high number of haplotypes occured in A49-susceptible allele carriers (h = 10, with an equivalent high haplotype diversity Hd = 0.90) and for the localities of Mutengene (at 220 m a.s.l., h = 9 and Hd = 0.85) and Likoko (at 800 m a.s.l., h = 7 and Hd = 0.83) while the lowest number of haplotypes was found in Tiko (h = 1, Hd = 0.00) at the lowest base of the Mount Cameroon (9–70 m a.s.l.) with no polymorphic site detected.

Table 2. Genetic variability parameters of *Rdl* full mutation.

Samples	N	S*	h (Hd)	Syn	NSyn	π (k)	D	F*
Per Allele								
S	25	11	10 (0.90)	0	0	0.003 (2.53)	−0.44^{ns}	−0.53^{ns}
R	43	6	6 (0.30)	1	0	0.0005 (0.54)	−1.61^{ns}	−1.65^{ns}
Per locality *(altitude in m a.s.l.)*								
Tiko (9–70)	12	0	1 (0.00)	0	0	0 (0.00)	n.a.	n.a.
Mutengene (220)	20	9	9 (0.85)	1	1 (A49S)	0.002 (1.97)	−0.76^{ns}	−0.39^{ns}
Meanja (305)	20	10	6 (0.68)	0	1 (A49S)	0.002 (2.00)	−1.02^{ns}	0.04^{ns}
Likoko (800)	16	8	7 (0.83)	0	1 (A49S)	0.002 (2.38)	−0.05^{ns}	−0.21^{ns}
All	68	16	16 (0.71)	1	1	0.002 (1.85)	−1.32^{ns}	−1.87^{ns}

N = number of sequences (2n); S*, number of polymorphic sites; h, number of haplotypes (Hd = haplotype diversity); Syn, Synonymous mutations; Nsyn, Non-synonymous mutations; π, nucleotide diversity (k = mean number of nucleotide differences); D and F* Tajima's and Fu and Li's statistics; ns, not significant; S = susceptible; R = resistant; m a.s.l. = meters above the sea level.

The most common haplotype (H2: 36/68 sequences) was found to be carrying the 49S-resistant allele and was distributed throughout the study area (Figure 3A–C), unlike the ancestral haplotype (H4: 6/36 sequences) which carried the A49-susceptible allele and distributed at low (Tiko: 9–70 m a.s.l.) and mid (Mutengene: 220 m a.s.l.; Meanja: 305 m a.s.l.) altitude but not in highland (Likoko: 800 m a.s.l.). Approximately one third of the haplotypes (6/16) appeared as singletons shared half by A49 (H6, H12 and H13) and 49S (H7, H8 and H15) allele carries, and identified from mid (Mutengene: H12, H13 and H15; Meanja: H8) to highland (Likoko: H6 and H7).

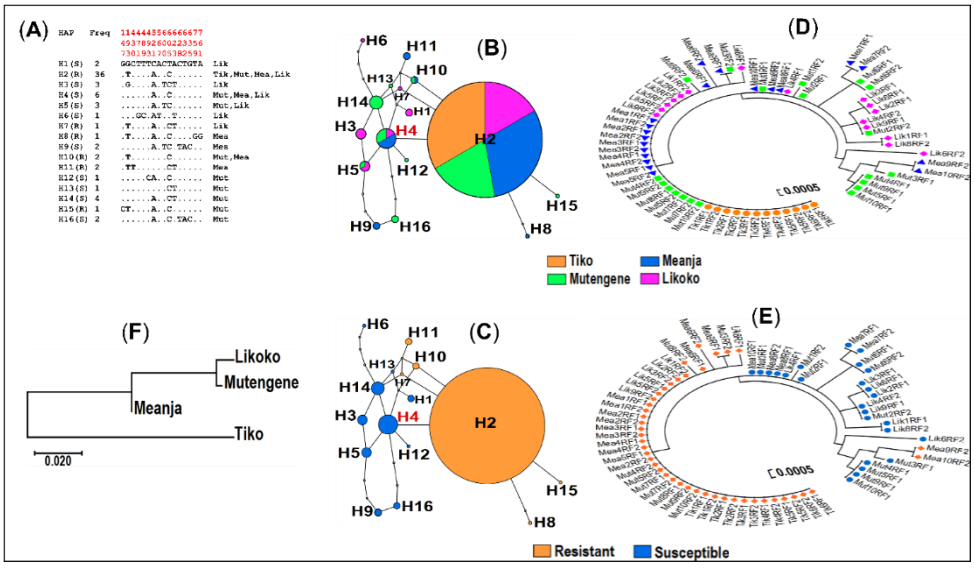

Figure 3. Genetic diversity and polymorphism patterns of the GABA-receptor gene across the Mount. Cameroon area. (**A**) Haplotype diversity patterns of a 1006bp fragment of the GABA-receptor gene in

Tiko (Tik), Mutengene (Mut), Meanja (Mea) and Likoko (Lik); H = haplotype; R = resistant; S = susceptible; polymorphic sites are in *red*. TCS haplotype networks showing a high polymorphism of the GSTe2 gene in *An. funestus* s.s with reduced number of mutational steps between haplotypes with respect to localities (**B**) and allelic profiles (**C**); haplotypes are presented in circular shape scaled to reflect their respective frequencies; Ancestral haplotype in *red*. Maximum likelihood phylogenetic trees of *Rdl*-DNA sequences among localities (**D**) and allelic profiles (**E**) showing a marked separation between A49 and 49S allele carriers. (**F**) Neighbour-joining tree of the genetic distances showing an apparent genetic relatedness associated with the altitude along the Mount Cameroon with Tiko being genetically differentiated from other localities.

Overall, there were negative and non-significant values of the selection test from Tajima D and Fu and Li F* (Table 2). However, the positive but not significant F* in Meanja (F*: 0.04), in addition to low values of haplotype and nucleotide diversity recorded in 49S-resitant allele carriers (Hd = 0.30 and $\pi = 0.0005$ respectively) could be indicators of an ongoing selection acting on the GABA receptor gene within the Mount Cameroon *An. funestus* s.s. populations.

3.4. Population Structure at GSTe2 and Rdl Mutations in An. funestus s.s. across Mount Cameroon

Construction of the maximum likelihood (ML) phylogenetic tree based on *GSTe2* gene showed the presence of major consensus cluster across the Mount Cameroon, in addition to three apparent separated clusters formed by haplotypes of low- to midland, midland and mid- to highland (Figure 2D). This was further supported by reduced values of genetic differentiation estimates ($-0.0006 \leq K_{ST} \leq 0.010$, all not significant; Supplementary Table 1) obtained between the four localities tested and the Nm gene flow index which showed a marked genetic closeness between Tiko/Mutengene (low- and midland) and Meanja/Likoko (mid- and highland) (Figure 2F). Meanwhile, the ML tree with respect to allelic profile highlighted the reduced diversity of 92F resistant allele carriers across the study area (Figure 2E).

Similarly, analysis of the ML phylogenetic tree at the GABA –receptor for Rdl mutation based on localities highlighted the presence of a main consensus cluster across the Mount Cameroon and three other apparent clusters generated by midland, mid-/highland and highland haplotypes (Figure 3D); whereas the ML tree with respect to allelic profiles highlighted the marked reduced diversity of 49S resistant allele carriers (Figure 3E). The construction of a NJ tree of genetic distance (Figure 3F) revealed that the lowland Tiko clusters separately from other localities aligned following the same altitudinal transect (Mutengene and Likoko) or situated eastward of the mountain (Meanja), all at higher elevation level (200–800 m a.s.l.). This latter observation was further supported by the consistency of high and significant values of genetic differentiation estimates observed between Tiko and the other three localities ($0.037 < K_{ST} < 0.158$) (Supplementary Table S2).

4. Discussion

The implementation of effective insecticide resistance management strategies relies on the good understanding of the direction and speed of spread of resistance alleles among mosquito populations. This study assessed the influence of the Mount Cameroon on the spread of both GST-mediated metabolic resistance and Rdl-based target-site resistance among population of *An. funestus* s.s. malaria vector.

Results from this study correlates with observations from previous reports [17,33] indicating the predominance of *Anopheles* mosquitoes within the overall mosquito fauna found in the Mount Cameroon region. *Anopheles* mosquitoes were collected throughout the study area (from lowland to highland), and a total of seven *Anopheles* species were identified; these included: sibling species (*An. coluzzii*, *An. gambiae* and *An. melas*) of the *An. gambiae* sensu lato (s.l.) complex, *An. funestus* s.s., *An hancocki*, *An. nili* and *An. ziemanni*. The same species have already been found in other elevated areas such as those of the western [5] and north-western Cameroon [34].

The genotyping of GSTe2-based DDT resistance maker showed the presence at high frequencies of $GSTe2^R$ genotypes and alleles in all *An. funestus* s.s. populations tested throughout the Mount

Cameroon altitudinal gradient (7–800 m a.s.l.). This presence of GST-based metabolic resistance was further confirmed after sequencing by the detection of a single substitution of nucleotide at position 92 inducing an amino acid change of leucine (CTT) to phenylalanine (TTT) [11]. GST-based metabolic resistance is common in a number of anopheline species including *An. gambiae* s.l. [35,36] and *An. funestus* s.s. [7,23,37], reflecting the heavy use of DDT and pyrethroids for malaria control over several decades [38,39]. In Cameroon, the intensive use of this class of insecticides to control agriculture pests especially in agroecosystems such as those found in the Mount Cameroon region (especially Tiko, Mutengene and Meanja in this study), may have contaminated mosquitoes breeding sites, thus exerting significant and constant selection pressure on *Anopheles* populations [33].

Similarly, *An. funestus* s.s. populations from the Mount Cameroon exhibited high levels of mutations in the *Rdl* gene which encoded for the GABA-receptor subunit. Dieldrin resistance have already been documented in insect species [40] including the malaria vectors *An. stephensi* [41], *An. gambiae* s.l. [42] and *An. funestus* [6]. In Cameroon, high frequencies of *Rdl* mutation have also been reported in areas other than the Mount Cameroon region [7,24,43,44] despite the fact that cyclodienes are no longer used for control programs. Moreover, observations made in this study raise concerns on the use of agrochemicals targeting the GABA-receptor in the agricultural environment. Thus, understanding the factors which could possibly explain the persistence of this resistance in *An. funestus* populations are greatly needed in order to get insights on resistance management.

Our results showed substantial variation in GSTe2-based and dieldrin resistance trends within the Mount Cameroon domain. Interestingly, $GSTe2^{R}$ allelic frequencies increased with land elevation whereas Rdl^{R} frequencies decreased with climb in altitude. These observations suggest that altitude could positively favour the establishment of *An. funestus* GSTe2-resistant populations from mid- (Mutengene and Meanja) to highland (Likoko) areas, unlike to dieldrin-resistant populations which seemed to be more adapted to Tiko, the lowest elevated site of the Mount Cameroon region as investigated in this study. Land elevation had previously been reported as one important influential predictor of increase in pyrethroid resistance in the *An. gambiae* species complex in West Africa though not in East Africa [45]. However, this study highlights some uncertainties of the potential influence of altitude on the maintenance of insecticide resistances in malaria vector populations under specific environmental conditions similar to that of the study area. Therefore, field sampling to measure resistance is the only means of informing resistance management decisions alongside an assessment of the historical and contemporary role of pesticide usage and the role of public health insecticide use in the development of insecticide resistance in malaria vectors as previously reported [46].

Patterns of genetic differentiation based on *GSTe2* mutation revealed that Tiko (9–70 m a.s.l.) and Mutengene (220 m a.s.l.) populations of *An. funestus* s.s. are genetically differentiated to that of Meanja (305 m a.s.l.) and Likoko (800 m a.s.l.) as they formed a unique cluster compared to others on the neighbour-joining tree of distance. Out of twelve haplotypes identified, *An. funestus* s.s. populations from Tiko and Mutengene appeared to share six haplotypes of which three are exclusively found in these localities. The causes of this clustering could be associated with the similar geographical position of both populations around the Mount Cameroon (southwestern edge) or the presence of the mountain itself which affects the population genetic structure and speed of spread of $GSTe2^{R}$ allele between *An. funestus* s.s. populations. Patterns of *GSTe2* population genetic support the contrast in resistance patterns between *An. funestus* s.s. populations and further suggest the presence of barriers to gene flow between these populations. Similar geographical barriers to the spread of resistance alleles has been mentioned for other resistance makers such as P450-based metabolic resistance in *An. funestus* [47] or knock-down resistance (*kdr*) mutations in *An. gambiae* [48].

Comparatively, genetic variability patterns within the GABA-receptor based *Rdl* mutation showed that *An. funestus* s.s. population from Tiko at the base of the Mount Cameroon is more genetically differentiated to mid- and highland vector populations as it separately clusters to other localities. This strong differentiation observed on *Rdl* mutation in Tiko was confirmed by the high and significant values of K_{ST} statistics of genetic differentiation obtained for *An. funestus* s.s. population from Tiko (Supplementary Table S2). Once more, it can be hypothesized that the presence

of the Mount Cameroon (mountain) influences the contrast in *Rdl* resistance patterns between populations of *An. funestus* s.s. in the study area thus suggesting the presence of barriers of gene flow between *An. funestus* s.s. populations. This is further supported by the reduced genetic diversities parameters (h and Hd), positive value of Fu and Li F* index and significant K_{ST} statistics obtained in Meanja midland (eastern edge of the Mount Cameroon) compared to mid- (Mutengene) and highland (Likoko) localities of the Great West (Table 2 and Supplementary Table S2). Nevertheless, investigations of more vector populations from both sides of the Mount Cameroon are needed to validate such hypothesis.

A strong selection process was observed on both 92F and 49S resistance allele carriers. This is seen by the reduced genetic diversity parameters (h, Hd, π and k) with limited number of mutational steps (polymorphic sites) between haplotypes in resistance allele carriers compared to susceptible allele carriers which maintained high diversity parameters (Tables 1 and 2). Analysis of ML tree based on allelic profiles (Figures 2 and 3) further illustrated the reduced diversity of resistance allele carriers in both cases. Furthermore, the most predominant haplotype is found in 85% (GSTe2-based mutation) to 100% (*Rdl* mutation) resistance allele carriers which is indicative of ongoing selection on 92F and 49S alleles contrasting with L92 and A49 susceptible alleles which maintained high number of singletons. Similar selection patterns have been observed in P450 [47] and GSTe2 [11] genes in *An. funestus* populations from other African regions. The selection process could be due to intensive use of insecticides through routine integrated control carried out by the National Malaria Control Program in the Mount Cameroon region, particularly in the localities surveyed. In addition, positive selection could also be associated with adaptation of mosquito larval stages to agricultural pesticides and other adverse conditions, such as temperature and landscape [49,50].

5. Conclusions

Broadly, this study highlights for the first time the presence of GSTe2-based metabolic resistance and the GABA-receptor target-site mutation associated to dieldrin resistance in the malaria vectors *An. funestus* s.s. across the Mount Cameroon domain. Both $GSTe2^R$ and Rdl^R alleles were found with high frequencies in almost all the localities surveyed; however, the speed of spread of these two molecular mechanisms appears to be influenced by the presence of a major mountainous barrier, the Mount Cameroon which contrasts the resistance and diversity patterns of the two genes between populations of *An. funestus* s.s. More alarming, we provide evidence of positive selection occurring on $GSTe2^R$ and Rdl^R throughout the Mount Cameroon region, which if not adequately monitor could drive to fixation in response to a greater selection of resistance in the future. This emphasizes the need of molecular studies of multiple collections sites throughout such mountainous landscapes to fully elucidate the role of environmental changes on the acquisition of insecticide resistance in *Anopheles* vector populations and to mitigate against further spread of resistance through the development of new vector management strategies.

Supplementary Materials: The following are available online at http://www.xxxxx, Figure S1: Map of the study site, Figure S2: Female's anophelines species composition across the study area, Figure S3: Frequency distribution of *GSTe2* (A) and *Rdl* (B) mutations in *An. funestus* s.s. populations from the localities surveyed, Figure S4: Sequence chromatograms of a 729 bp fragment in *GSTe2* gene (A) and a 1006 bp fragment of the GABA-receptor gene (B) for susceptible (top), homozygote resistant (middle) and heterozygote (bottom) *An. funestus* s.s., Table S1: Patterns of genetic differentiation between *An. funestus* s.s. populations based on KST estimates from *GSTe2* mutation with (Nm), Table S2: Patterns of genetic differentiation between *An. funestus* s.s. populations based on KST estimates from *Rdl* mutation with (Nm).

Author Contributions: Conceptualization, S.W., F.N. and C.S.W.; methodology, N.A.-A.; validation, S.W., F.N., J.M.R. and C.S.W.; formal analysis, N.A.-A. and C.S.W.; writing—original draft preparation, N.A.-A., J.M.R. and C.S.W. All authors have read and agreed to the published version of the manuscript.

Funding: This work and the cost to publish in open access was supported by a Wellcome Trust Research Career Development Fellowship (083515/Z/07/Z) and a Wellcome Trust Senior Research Fellowship in Biomedical Sciences (101893/Z/13/Z) to CSW.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. WHO. *World Malaria Report 2019*; World Health Organisation: Geneva, Switzerland, 2019.
2. Sinka, M.; Bangs, M.; Manguin, S.; Coetzee, M.; Mbogo, C.; Hemingway, J.; Patil, A.; Temperley, W.; Gething, P.; Kabaria, C.; et al. The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: Occurrence data, distribution maps and bionomic précis. *Parasit Vectors* **2010**, *3*, 117, doi:10.1186/1756-3305-3-117.
3. Coetzee, M.; Hunt, R.; Wilkerson, R.; Della Torre, A.; Coulibaly, M.; Besansky, N. *Anopheles coluzzii* and *Anopheles amharicus*, new members of the *Anopheles gambiae* complex. *Zootaxa* **2013**, *3619*, 246–274, doi:10.11646/zootaxa.3619.3.2.
4. Wanji, S.; Tanke, T.; Atanga, S.N.; Ajonina, C.; Tendongfor, N.; Fontenille, D. *Anopheles* species of the mount Cameroon region: Biting habits, feeding behaviour and entomological inoculation rates. *Trop. Med. Int. Health* **2003**, *8*, 643–649, doi:10.1046/j.1365-3156.2003.01070.x.
5. Tchuinkam, T.; Simard, F.; Lélé-Defo, E.; Téné-Fossog, B.; Tateng-Ngouateu, A.; Antonio-Nkondjio, C.; Mpoame, M.; Toto, J.; Njiné, T.; Fontenille, D.; et al. Bionomics of Anopheline species and malaria transmission dynamics along an altitudinal transect in Western Cameroon. *BMC Infect. Dis.* **2010**, *10*, 119, doi:10.1186/1471-2334-10-119.
6. Wondji, C.; Dabire, R.; Tukur, Z.; Irving, H.; Djouaka, R.; Morgan, J. Identification and distribution of a GABA receptor mutation conferring dieldrin the malaria vector *Anopheles funestus* in Africa. *Insect. Biochem. Mol. Biol.* **2011**, *41*, 484–491, doi:10.1016/j.ibmb.2011.03.012.
7. Menze, B.; Riveron, J.; Ibrahim, S.; Irving, H.; Antonio-Nkondjio, C.; Awono-Ambene, P.; Wondji, C. Multiple insecticide resistance in the malaria vector *Anopheles funestus* from northern Cameroon is mediated by metabolic resistance alongside potential target site insensitivity. *PLoS ONE* **2016**, *11*, e0163261, doi:10.1371/journal.pone.0163261.
8. Irving, H.; Wondji, C., Investigating knockdown resistance (*kdr*) mechanism against pyrethroids/DDT in the malaria vector *Anopheles funestus* across Africa. *BMC Genet.* **2017**, *18*, 76, doi:10.1186/s12863-017-0539-x.
9. Ndo, C.; Kopya, E.; Donbou, M.A.; Njiokou, F.; Awono-Ambene, P.; Wondji, C. Elevated *Plasmodium* infection rates and high pyrethroid resistance in major malaria vectors in a forested area of Cameroon highlight challenges of malaria control. *Parasit Vectors* **2018**, *11*, 157, doi:10.1186/s13071-018-2759-y.
10. Ndo, C.; Kopya, E.; Irving, H.; Wondji, C. Exploring the impact of glutathione S-transferase (GST)-based metabolic resistance to insecticide on vector competence of *Anopheles funestus* for *Plasmodium falciparum* [version 2; peer review: 2 approved, 1 approved with reservations]. *Wellcome Open Res.* **2019**, *4*, doi:10.12688/wellcomeopenres.15061.2.
11. Riveron, J.; Yunta, C.; Ibrahim, S.; Djouaka, R.; Irving, H.; Menze, B.; Ismail, H.; Hemingway, J.; Ranson, H.; Albert, A.; et al. A single mutation in the GSTe2 gene allows tracking of metabolically based insecticide resistance in a major malaria vector. *Genome Biol.* **2014**, *12*, R27, doi:10.1186/gb-2014-15-2-r27.
12. Lui, N. Insecticides resistance in mosquitoes: Impact, mechanisms, and research directions. *Annu. Rev. Entomol.* **2015**, *60*, 537–59, doi:10.1146/annurev-ento-010814-020828.
13. Barnes, K.G.; Irving, H.; Chiumia, M.; Mzilahowa, T.; Coleman, M.; Hemingway, J.; Wondji, C.S. Restriction to gene flow is associated with changes in the molecular basis of pyrethroid resistance in the malaria vector *Anopheles funestus*. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 286–291, doi:10.1073/pnas.1615458114.
14. Weedall, G.D.; Riveron, J.M.; Hearn, J.; Irving, H.; Kamdem, C.; Fouet, C.; White, B.J.; Wondji, C.S. An Africa-wide genomic evolution of insecticide resistance in the malaria vector *Anopheles funestus* involves selective sweeps, copy number variations, gene conversion and transposons. *PLoS Genet.* **2020**, *16*, e1008822, doi:10.1371/journal.pgen.1008822.
15. Boussougou-Sambe, S.T.; Eyisap, W.E.; Tasse, G.C.T.; Mandeng, S.E.; Mbakop, L.R.; Enyong, P.; Etang, J.; Fokam, E.B.; Awono-Ambene, P.H. Insecticide susceptibility status of *Anopheles gambiae* (s.l.) in South-West Cameroon four years after long-lasting insecticidal net mass distribution. *Parasit Vectors* **2018**, *11*, 391, doi:10.1186/s13071-018-2979-1.
16. Wanji, S.; Enyong, P.; Tendongfor, N.; Ndamukong, J.; Mafo, F.; Kengne-Ouafo, A. *Biodiversity of Anopheles mosquito Species across the Mount Cameroon Region*; Research Foundation for Tropical Diseases and Environment: Buea, Cameroon, 2006.

17. Amvongo-Adjia, N.; Wirsiy, E.L.; Riveron, J.M.; Chounna Ndongmo, W.P.; Enyong, P.A.; Njiokou, F.; Wondji, C.S.; Wanji, S., Bionomics and vectorial role of anophelines in wetlands along the volcanic chain of Cameroon. *Parasit Vectors* **2018**, *11*, 471–471, doi:10.1186/s13071-018-3041-z.
18. Gillies, M.; de Meillon, B., *The Anophelinae of Africa South of the Sahara (Ethiopian Zoogeographical Region)*; South African Institute for Medical Research: Johannesburg, South Africa, 1968; Volume 2nd.
19. Cornel, A.; Collins, F. PCR of the ribosomal DNA intergenic spacer regions as a method for identifying mosquitoes in the *Anopheles gambiae* complex. *Methods Mol. Biol.* **1996**, *50*, 321–332, doi:10.1385/0-89603-323-6:321.
20. Scott, J.A.; Brogdon, W.G.; Collins, F.H. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. *Am. J. Trop. Med. Hyg.* **1993**, *49*, 520–529, doi:10.4269/ajtmh.1993.49.520.
21. Fanello, C.; Santolamazza, F.; della Torre, A. Simultaneous identification of species and molecular forms of *Anopheles gambiae* complex by PCR-RFLP. *Med. Vet. Entomol.* **2002**, *16*, 461–464, doi:10.1046/j.1365-2915.2002.00393.x.
22. Koekemoer, L.; Kamau, L.; Hunt, R.; Coetzee, M. A cocktail polymerase chain reaction assay to identify members of the *Anopheles funestus* (Diptera: Culicidae) group. *Am. J. Trop. Med. Hyg.* **2002**, *66*, 804–811, doi:10.4269/ajtmh.2002.66.804.
23. Tchouakui, M.; Chiang, M.-C.; Ndo, C.; Kuicheu, C.; Amvongo-Adjia, N.; Wondji, M.; Tchoupo, M.; Kusimo, M.; Riveron, J.; Wondji, C. A marker of glutathione S-transferase-mediated resistance to insecticides is associated with higher *Plasmodium* infection in the African malaria vector *Anopheles funestus*. *Sci. Rep.* **2019**, *9*, 5772, doi:10.1038/s41598-019-42015-1.
24. Riveron, J.; Chiumia, M.; Menze, B.; Barnes, K.; Irving, H.; Ibrahim, S.; Weedall, G.; Mzilahowa, T.; Wondji, C. Rise of multiple insecticide resistance in *Anopheles funestus* in Malawi: A major concern for malaria vector control. *Malar. J.* **2015**, *14*, 344, doi:10.1186/s12936-015-0877-y.
25. Hall, T. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **1999**, *41*, 95–98, doi:10.14601/Phytopathol_Mediterr-14998u1.29.
26. Thompson, J.; Higgins, D.; Gibson, T. CLUSTALW: Improving the sensitivity of progressive miltiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **1994**, *22*, 4673–80, doi:10.1093/nar/22.22.4673.
27. Librado, P.; Rozas, J. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **2009**, *25*, 1451–1452, doi:10.1093/bioinformatics/btp187.
28. Clement, M.; Posada, D.; Crandall, K. TCS: A computer program to estimate gene genealogies. *Mol. Ecol.* **2000**, *9*, 1657–1660, doi:10.1046/j.1365-294x.2000.01020.x.
29. Santos, A.; Cabezas, M.; Tavares, A.; Xavier, R.; Branco, M. tcsBU: A tool to extend TCS network layout and visualization. *Bioinformatics* **2015**, *32*, 627–628, doi:10.1093/bioinformatics/btv636.
30. Hudson, R.R.; Slatkin, M.; Maddison, W.P. Estimation of levels of gene flow from DNA sequence data. *Genetics* **1992**, *132*, 583–589. Available online: https://pubmed.ncbi.nlm.nih.gov/1427045 (accessed on).
31. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549, doi:10.1093/molbev/msy096.
32. Nei, M. Analysis of gene diversity in subdivided populations. *Proc. Natl. Acad. Sci. USA* **1973**, *70*, 3321–3323, doi:10.1073/pnas.70.12.3321.
33. Antonio-Nkondjio, C.; Ndo, C.; Njiokou, F.; Bigoga, J.; Awono-Ambene, H.; Etang, J.; Ekobo, A.S.; Wondji, C. Review of malaria situation in Cameroon: Technical viewpoint on challenges and prospects for disease elimination. *Parasit Vectors* **2019**, *12*, 501, doi:10.1186/s13071-019-3753-8.
34. Tabue, R.; Nem, T.; Atangana, J.; Bigoga, J.; Patchoké, S.; Tchouine, F.; Fodjo, B.; Leke, R.; Fondjo, E. *Anopheles ziemanni* a locally important malaria vector in Ndop health district, north west region of Cameroon. *Parasit Vectors* **2014**, *7*, 262, doi:10.1186/1756-3305-7-262.
35. Prapanthadara, L.; Ketterman, A. Qualitative and quantitative changes in glutathione S-transferases in the mosquito *Anopheles gambiae* confer DDT-resistance. *Biochem. Soc. Trans.* **1993**, *21 Pt 3*, 304S, doi:10.1042/bst021304s.
36. Ranson, H.; Rossiter, L.; Ortelli, F.; Jensen, B.; Wang, X.; Roth, C.; Collins, F.; Hemingway, J. Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector *Anopheles gambiae*. *Biochem. J.* **2001**, *359 Pt 2*, 295–304, doi:10.1042/0264-6021:3590295.
37. Tchakounte, A.; Tchouakui, M.; Mu-Chun, C.; Tchapga, W.; Kopia, E.; Soh, P.; Njiokou, F.; Riveron, J.; Wondji, C. Exposure to the insecticide-treated bednet PermaNet 2.0 reduces the longevity of the wild

African malaria vector *Anopheles funestus* but GSTe2-resistant mosquitoes live longer. *PLoS ONE* **2019**, *14*, e0213949, doi:10.1371/journal.pone.0213949.
38. Hemingway, J.; Ranson, H. Insecticide resistance in insect vectors of human disease. *Annu. Rev. Entomol.* **2000**, *45*, 371–391, doi:10.1146/annurev.ento.45.1.371.
39. Hemingway, J.; Hawkes, N.; Carroll, L.; Ranson, H. The molecular basis of insecticide resistance in mosquitoes. *Insect. Biochem. Mol. Biol.* **2004**, *34*, 653–665, doi:10.1016/j.ibmb.2004.03.018.
40. Ffrench-Constant, R. The molecular and population genetics of cyclodiene insecticide resistance. *Insect. Biochem. Mol. Biol.* **1994**, *24*, 335–345, doi:10.1016/0965-1748(94)90026-4.
41. Andreasen, M.; Ffrench-Constant, R. In situ hybridization to the Rdl locus on polytene chromosome 3L of *Anopheles stephensi*. *Med. Vet. Entomol.* **2002**, *16*, 452–455, doi:10.1046/j.1365-2915.2002.00390.x.
42. Du, W.; Awolola, T.S.; Howell, P.; Koekemoer, L.L.; Brooke, B.D.; Benedict, M.Q.; Coetzee, M.; Zheng, L. Independent mutations in the Rdl locus confer dieldrin resistance to *Anopheles gambiae* and *An. arabiensis*. *Insect. Mol. Biol.* **2005**, *14*, 179–183, doi:10.1111/j.1365-2583.2005.00544.x.
43. Etang, J.; Manga, L.; Chandre, F.; Guillet, P.; Fondjo, E.; Mimpfoundi, R.; Toto, J.-c.; Fontenille, D. Insecticide Susceptibility Status of Anopheles gambiae s.l. (Diptera : Culicidae) in the Republic of Cameroon. *J. Med. Entomol.* **2003**, *40*, 491–497, doi:10.1603/0022-2585-40.4.491.
44. Nwane, P.; Etang, J.; Chouaïbou, M.; Toto, J.C.; Koffi, A.; Mimpfoundi, R.; Simard, F., Multiple insecticide resistance mechanisms in *Anopheles gambiae* s.l. populations from Cameroon, Central Africa. *Parasit Vectors* **2013**, *6*, 41–41, doi:10.1186/1756-3305-6-41.
45. Hancock, P.; Hendriks, C.; Tangena, J.-A.; Gibson, H.; Hemingway, J.; Coleman, M.; Gething, P.; Cameron, E.; Bhatt, S.; Moyes, C. Mapping trends in insecticide resistance phenotypes in African malaria vectors. *PLOS Biol.* **2020**, *18*, e3000633, doi:10.1371/journal.pbio.3000633.
46. Matiya, D.; Philbert, A.; Kidima, W.; Matowo, J. Dynamics and monitoring of insecticide resistance in malaria vectors across mainland Tanzania from 1997 to 2017: A systematic review. *Malar. J.* **2019**, *18*, 102, doi:10.1186/s12936-019-2738-6.
47. Barnes, K.; Weedall, G.; Ndula, M.; Irving, H.; Mzihalowa, T.; Hemingway, J.; Wondji, C. Genomic footprints of selective sweeps from metabolic mesistance to pyrethroids in African malaria vectors are driven by scale up of insecticide-based vector control. *PLoS Genet.* **2017**, *13*, e1006539, doi:10.1371/journal.pgen.1006539.
48. Ranson, H.; N'Guessan, R.; Lines, J.; Moiroux, N.; Nkuni, Z.; Corbel, V. Pyrethroid resistance in African anopheline mosquitoes: What are the implications for malaria control? *Trends Parasitol.* **2010**, *xx*, 1–8, doi:10.1016/j.pt.2010.08.004.
49. Simard, F.; Lehmann, T.; Lemasson, J.J.; Diatta, M.; Fontenille, D. Persistence of *Anopheles arabiensis* during the severe dry season conditions in Senegal: An indirect approach using microsatellite loci. *Insect. Mol. Biol.* **2000**, *9*, 467–479, doi:10.1046/j.1365-2583.2000.00210.x.
50. Yang, M.; Ma, Y.; Wu, J. Mitochondrial genetic differentiation across populations of the malaria vector *Anopheles lesteri* from China (Diptera: Culicidae). *Malar. J.* **2011**, *10*, 216, doi:10.1186/1475-2875-10-216.

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© 2020 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).