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Article

Exploring Knockdown Resistance (*kdr*) Mutation in *Anopheles gambiae* and *Anopheles coluzzii* Malaria Vectors in the Mountainous Plains Across the Cameroonian Great-West Domain

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Simple Summary

Malaria is the most widespread endemic disease in Cameroon, especially in mountainous plain regions where environmental factors such as increased rainfall and temperature create an ideal milieu for the *Anopheles* mosquito vectors in these fertile, high-altitude areas, contributing to persistent malaria risk. This is even more problematic considering insecticide resistance in key malaria vectors like *Anopheles gambiae sensu lato* directly compromising the effectiveness of long-lasting insecticidal nets by allowing mosquitoes to survive exposure. In this study, a holistic investigation of the common knockdown resistance mechanism (*kdr* mutation) was conducted on the sibling species *An. gambiae* and *An. coluzzii* to explore the extent of *kdr* and assess how landscape features might have shaped the evolutionary profile of *kdr* alleles in these malaria vectors throughout the Cameroonian Great-west domain. Using PCR-based genotyping assays and sequencing, it was confirmed the widespread presence of the West African *L1014F-kdr* allele in both mosquito species. Meanwhile, the East African *L1014S-kdr* allele was found only in *An. gambiae* specimens collected from 125 to 800 meters above the sea level. These findings demonstrate the decisive role of continuous monitoring of insecticide resistance and vector bionomics to guide the selection of effective control tools in mountainous plains.

Abstract

Malaria control programmes across Africa and beyond are threatened by increasing insecticide resistance in the major anopheline vectors. In the malaria vectors *Anopheles gambiae sensu lato*, two point-mutations (*L1014F* and *L1014S*) in the voltage-dependent sodium channel gene that confer target-site knockdown resistance (*kdr*) to DDT and pyrethroid insecticides, have been described in several studies across the northern sudano-sahelian and the southern forested ecological zones of Cameroon. Contrarily, there is an unclear *kdr* status in anophelines of mountainous agro-ecosystems across the Cameroon Great-west domain. In order to determine the evolutionary profile of *kdr* alleles in *An. gambiae* and *An. coluzzii* sibling species both found in the Cameroon Great West domain, genotyping of the *kdr* locus on a total of 1172 individual specimen across five mountainous massifs, and sequencing on a minimum-size of 10 individuals per localities of a 510 base pairs fragment of the downstream exon-20, were performed. Knockdown resistance *1014F* allele was found to be widespread with *An. gambiae* having high frequencies compared to *An. coluzzii*. Meanwhile *1014S*-

kdr allele was confined in *An. gambiae* populations. The results suggest that *kdr* alleles may have arisen through introgression. Estimates of genetic variability provided evidence of selection acting on these alleles, particularly the 1014F which was driven to fixation. Spatial occurrence of 1014F was heterogenous, being seemingly influenced by land elevation and gene flow. This study delineates the comprehensive distribution of *kdr* mutations in *An. gambiae* and *An. coluzzii* across Cameroonian mountainous ecosystems. Taking action to limit the spread of *kdr* alleles into mountainous landscapes would be helpful for the management and sustainability of malaria vector control.

Keywords: malaria; *Anopheles gambiae*; *Anopheles coluzzii*; knockdown resistance; mountainous plains; Cameroon

1. Introduction

Vector control is a key preventive strategy for malaria [1]. Effective malaria vector control relies heavily on two core insecticidal interventions: deployment of insecticide-treated nets (ITNs) – mainly long-lasting insecticidal nets (LLINs) treated with synthetic pyrethroids – and indoor residual spraying (IRS) of insecticides [2]. Significant reductions in malaria morbidity and mortality since 2000 have mainly been due to the widespread implementation of these two insecticidal interventions [3]. However, insecticide resistance is an increasing problem faced by those who need insecticides to efficiently control medical, veterinary and agricultural pests [4–6]. In African *Anopheles* malaria vectors, the problem extends to all major group of insecticides used in public health, including: pyrethroids, organochlorines, organophosphates and carbamates [7]. Since the first case of dichloro diphenyl trichloroethane (DDT) resistance in 1947, the incidence of insecticide resistance has increased annually at an alarming rate with at least 1000 pesticide resistant arthropod species reported in the recent years [8–10]. Moreover, resistance mechanisms have also been developed by many insects to novel insecticides with different mode of action [11,12].

In recent years, many insecticide resistance mechanisms have been detected, and resistance detection methods have been developed [13–16]. These mechanisms are broadly divided into four categories including: a) increased metabolic detoxification of insecticides, b) decreased sensitivity target-site proteins, c) decreased rates of insecticide penetration, and d) behavioural modification [17,18]. Target-site resistance is designated by the modification of the insect molecule where the insecticide binds, decreasing its effects [19]. Neurotoxic insecticides have as their ultimate target different molecules from the insect nervous system: the enzyme acetylcholinesterase (for organophosphates and carbamates), the GABA-aminobutyric acid receptor (for cyclodienes), the nicotinic acetylcholine receptors (for spinosyns and neonicotinoids) and the voltage-gated sodium channel (for DDT and pyrethroids) [20]. Just like DDT, pyrethroids act very fast in the central nervous system of the insects, leading to convulsions, paralysis and eventually death, an effect known as *knockdown* [21]. However, unlike DDT, pyrethroids are not claimed to cause severe risks to the environment or to animal or human health, hence its widespread use [22–24].

The main pyrethroid resistance mechanism (the knockdown resistance phenotype, *kdr*) occurs due to point mutations in the voltage-gated sodium channel (VGSC) in the central nervous system, the target of pyrethroids and DDT [25,26]. Knockdown resistance mutations of the VGSC gene, both leucine to phenylalanine substitution (L1014F _ *kdr*-West) and leucine to serine substitution (L1014S _ *kdr*-East) are the two mutations inducing target-site resistance in insects [13,27].

In *Anopheles gambiae* and *An. coluzzii*, two major Afrotropical malaria vectors, these two mutations were identified across the African continent [28–34]. Field surveys revealed a widespread distribution of the 1014F-resistant allele in West Africa [35,36], although significant differences were found in the frequency of this allele between the two species that are considered units of incipient speciation within *A. gambiae* complex [37,38].

In Cameroon, the genotyping of the *kdr* locus showed that both the L1014F and L1014S alleles occurred in *Anopheles gambiae sensu lato* (s.l.) populations of the forested and sudano-sahelian bio-

ecological zones [32,39–45], in urban [46–48] as well as rural settings [49]. Although the L1014F allele is the most common, studies are increasingly revealing the presence of the L1014S allele in *An. gambiae sensu stricto* (s.s.) populations across the country [39,45,46]. The increased use of agro-pesticides, the expansion of agriculture and pollution of urban settings as well as the expansion of pyrethroids used as the only insecticides recommended by the World Health Organization for insecticide-treated materials, have been accompanied with a rapid selection by pyrethroids of *kdr* alleles in *An. gambiae* s.l. and other insect pest across the country in few years [50–52].

The distribution and frequency of these mutations raise serious concerns about the sustainability of insecticide-based vector control strategies. This is particular evident when one considers that previous studies revealed a potential impact of landscape especially mountainous features on malaria vectors bionomics [53] and the spread of resistance makers in *An. funestus* s.s. [54], another major malaria vector in the country and even in similar environment in the African continent [55–57].

In comparison to the rest of the country, less information is available on the distribution of the *kdr* mutations in *An. gambiae* s.l. populations across the Great West hilly domain of Cameroon. This area holds intensive agricultural practices with a massive usage of agro-pesticides likely to enhance resistant phenotype and genotype in local vector populations. Here, the most commonly used pesticides are: (i) paraquat and glyphosate to control weeds, (ii) alpha-cypermethrin and chlorpyrifos against insects, and (iii) metalaxyl and maneb applied to control fungal diseases [58]. It could therefore be important to investigate how landscape features coupled to human practices either in agriculture or through the use of LLINs have contributed to shape the distribution of knockdown resistance profile in *An. gambiae* s.l. populations in agro-ecosystems of the mountainous plains across the Cameroonian Great West.

To support the implementation of control strategies against malaria-transmitting vectors across mountainous landscapes, we investigated the level of pyrethroid-based *kdr* resistance in *An. gambiae* and *An. coluzzii* populations of mountainous plains across the Cameroonian Great-west domain by genotyping the *kdr* locus and sequencing the downstream exon-20 region of the VGSC gene. This study ultimately aimed to characterize the genetic variability in the distribution of *kdr* alleles and relate this to signature of selection and patterns of genetic structure in ten *An. gambiae* s.l. populations.

2. Materials and Methods

2.1. Study Sites and Sampling

DNA samples from individual field collected (from the year 2010 to 2015) adult female mosquitoes morphologically identified as *An. gambiae* s.l. were obtained from thirty collections sites in ten localities across five mountainous massifs of the Cameroon Great West domain [53,54] which comprises three regions including: the South-West, the West and the North-West regions of Cameroon (

Figure 1, **Error! Reference source not found.** and **Error! Reference source not found.**). The surveyed area spans 56,602 square kilometres from 4°3' latitude north and 9°22' longitude east at 9 meters above the sea level (m a.s.l.) in the Tiko Atlantic coast to 6°17' latitude north and 11°08' longitude east ranging from 700 m a.s.l on the Mbaw plain to grassy highlands in Ndop at 1285 m a.s.l.

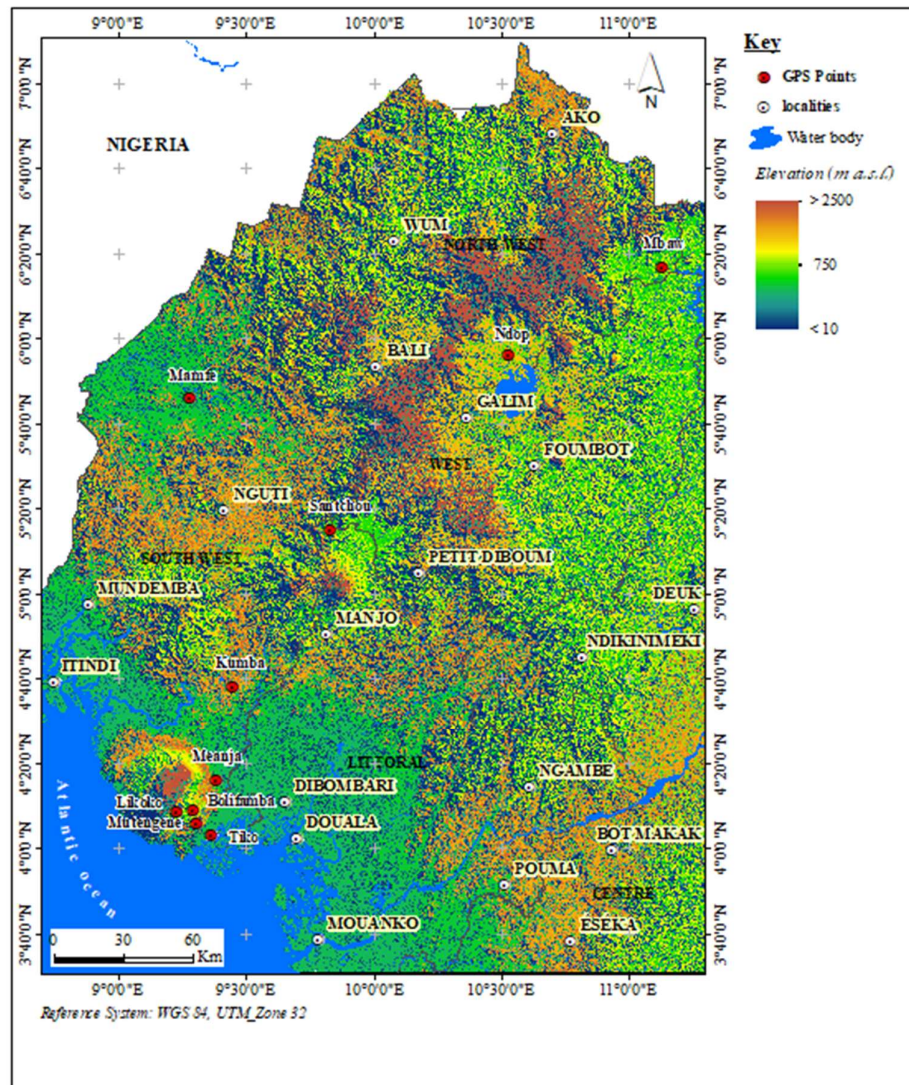


Figure 1. Map of the study area.

DNA extraction protocol and molecular identification by PCR of cryptic female mosquitoes within the *An. gambiae* complex were performed as previously described [53].

2.2. Genotyping of the *kdr* Gene Mutation

Real-time PCR (TaqMan assay) was used to quantify the *kdr* genotype at amino acid position 1014 of the VGSC gene following the protocols of Bass, Nikou [59]. *kdr*_Forward (5'-CATTTTCTTGGCCACTGTAGTGAT-3') and *kdr*_Reverse (5'-CGATCTTGGTCCATGTTAATTTGCA-3') were standard oligonucleotide's primers. Samples were genotyped for the wild-type (WT_susceptible) allele using probe 5'-CTTACGACTAAATTTC-3' labelled with HEX fluorescent quencher at the 5' end, for the 1014F and 1014S *kdr* alleles using respectively *kdr*-W (5'-ACGACAAAATTTC-3') and *kdr*-E (5'-ACGACTGAATTTC-3') probes labelled with 6-FAM fluorescence. The primers *kdr*-Forward, *kdr*-Reverse and the WT-probe were used in separated assay with either *kdr*-W probe for 1014F detection or *kdr*-E probe for 1014S detection. Real-time PCR reactions were done Agilent MX3005 machine using a 96-well format. Frequency distribution of *kdr* genotypes and alleles were compared using the Chi-square (χ^2) test, with statistical significance set at $p < 0.05$.

2.3. Amplification and Sequencing of the Downstream Exon-20 Region of the VGSC Gene

The *kdr* genotypes were confirmed in a subset of ten samples in nine (out of 10) surveyed localities (Tiko, Bolifamba, Likoko, Meanja, Kumba, Mamfe, Santchou, Ndop and Mbaw plain) by amplifying a 510 base pairs (bp) fragment spanning the downstream exon-20 region of the VGSC gene. Estimations on sample size were based on previous assumption showing that for single nucleotide polymorphism makers, the most complete and unbiased representation of genetic diversity present in an individual can be obtained by incorporating a minimum of 10 individuals into the discovery data set, thus ensuring the discovery of both common and rare polymorphisms [60]. Amplifications (by classical PCR) were performed using primers Exo20-Gamb_F (5'-AAATGTCTCGCCCAATCAG-3') and Exo20-Gamb_R (5'-GCACCTGCAAAACAATGTCA-3') as previously described [43,61,62]. Direct sequencing (using the reverse primer Exo20-Gamb_R) was applied on successful amplicons after a purification step using the Exo-SAP (Exonuclease I Shrimp Alkaline Phosphate) clean up protocol (ThermoFisher Scientific, Santa Clara, CA, USA). The sequences of the 500bp reverse fragment of the VGSC exon-20 region were aligned using the ClustalW tool [63], and a manually edited in BioEdit version 7.2.5 [64] in order to confirm polymorphic positions and sequence chromatograms.

2.4. Phylogenetic Structure Analysis of the Studied Species

Estimates of DNA polymorphism at the exon-20 including polymorphic sites S , number of haplotypes h , Haplotype diversity Hd , Synonymous mutations Syn , Non-synonymous mutations $Nsyn$, nucleotide diversity π , mean number of nucleotide differences k , Tajima D and Fu and Li F^* neutrality statistics were computed using DnaSP version 6.12.03 [65]. In addition, the level of pairwise genetic differentiation was estimated using the K_{ST} statistics which significance was assessed by permutation of subpopulation identities and re-calculating K_{ST} 10,000 times as implemented in DnaSP 6.12.03.

Genealogical relationship among haplotypes were estimated by constructing an haplotype network with a controlled level of reticulations using the integer neighbour-joining (IntNJ) technique in PopART software [66]. The IntNJ method begins by computing a matrix of Hamming distances between unique haplotype sequences which are further used to infer a tree using the neighbour-joining method [67]. The integer restriction is particularly important for low-divergence data sets which are often analysed using haplotype networks.

A maximum-likelihood (ML) phylogenetic tree of haplotypes was constructed based on the Tamura 3-parameter best-fit model with 500 bootstrap replications for the robustness of the tree, as computed using the Bayesian Information Criterion (BIC) in MEGA version X [68]. A neighbour-joining (NJ) tree was also constructed using pairwise GammaST genetic distances [69,70] between subpopulations in MEGA X.

3. Results

Throughout the surveyed area, the majority of field-caught *An. gambiae* s.l. molecularly identified were *An. gambiae* (1627/2627 _ 61.9%) and *An. coluzzii* (801/2627 _ 30.5%) (**Error! Reference source not found.**). Genotyping of the *kdr* mutations was performed in 622 *An. coluzzii* (447 from Tiko and 175 from Meanja) and 413 *An. gambiae* (46 from Mutengene, 46 from Bolifamba, 46 from Likoko, 46 from Kumba, 44 from Mamfe, 97 from Santchou, 38 from Ndop and 43 from Mbaw plain). In addition, 116 *An. melas* (from Tiko) and 19 *An. coluzzii* $\times$ *An. gambiae* hybrids (from Ndop) were genotyped for *kdr*.

3.1. Frequency and Distribution of *kdr* Mutations

The 1014F mutation of the *kdr* locus was successfully identified from a total of 1172 *An. gambiae* s.l. individuals (including *An. coluzzii*, *An. gambiae*, *An. coluzzii* $\times$ *An. gambiae* hybrids and *An. melas*), whereas no L1014S mutation was detected using TaqMan assay. An overall frequency of 40.7% of the

L1014F mutation was recorded, with 11.0% being resistant homozygous individuals (*RwRw*: *1014F/1014F*) while 29.7% were heterozygous specimens of the genotype *L1014/1014F* (*RwS*). The susceptible homozygous (*SS*: *L1014/L1014*) were the mostly represented of the specimen analysed (Figure 2A, **Figure 2B**). Thus, the frequency of *L1014* susceptible allele (0.74) was significantly (*p* value < 0.0001) more represented than that of the *1014F* resistant allele (0.26) (Figure 2C).

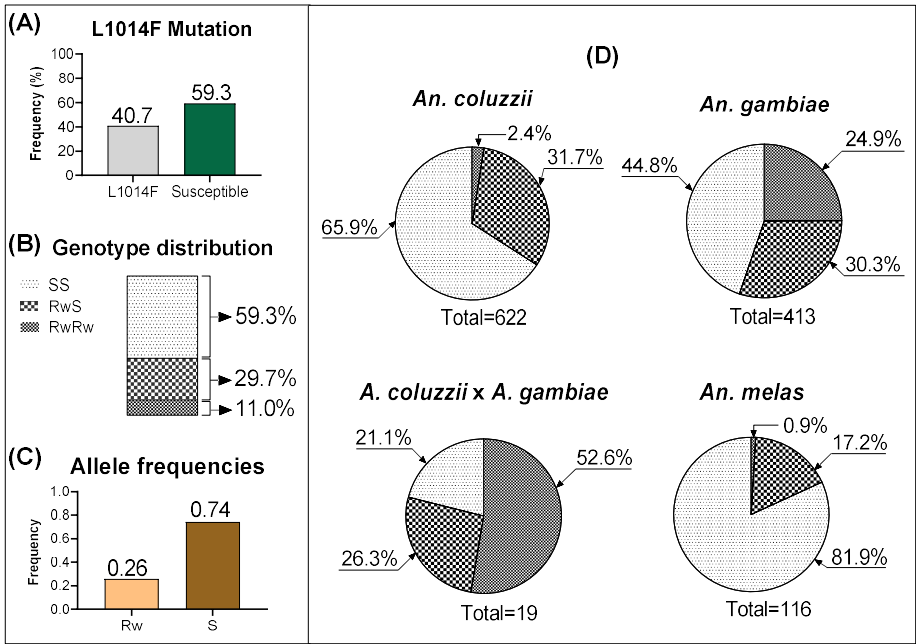


Figure 2. L1014F mutation (A), genotypic (B) and allelic (C) frequencies in *An. gambiae* s.l. subspecies analysed (D). **Legend:** S = susceptible allele (L1014); *Rw* = *kdr*-West resistant allele (1014F); SS = Susceptible (L1014/L1014); *RwS* = Heterozygous *kdr*-West (L1014/1014F); *RwRw* = Homozygous *kdr*-West (1014F/1014F).

Careful analysis considering the species within the *An. gambiae* complex revealed that the *L1014F* mutation was mostly carried by *An. gambiae* and *An. coluzzii* x *An. gambiae* hybrids specimen with the latter having the highest proportion of 1014F/1014F double resistant individuals (52.6%). *Anopheles coluzzii* and *An. melas* were predominantly harbouring the *L1014/L1014* susceptible genotype, with a frequency ranging from 65.9 to 81.9% (Figure 2D).

The spatial trends of the *L1014F* mutation in the *VGSC* gene showed a widespread distribution of this mutation across the surveyed area, although *kdr* associated genotypes were not homogeneously present in the samples analysed (and **Error! Reference source not found.**). It was noted an apparent specialization of 1014F/1014F homozygous resistant genotype carriers to higher altitude ranges, from 750 m a.s.l. in Santchou (*relative abundance*: 51.0%) to 1295 m a.s.l. in the Ndop plain (*relative abundance*: 75.0%) although these were lowly represented in Likoko (*relative abundance*: 13.0%) situated at 800 m a.s.l. in the Mount Cameroon massif. Rather, *L1014/L1014* susceptible specimen significantly abundant (*p* value < 0.001, *relative abundance* ranging from 54.5% to 84.3%) from low to mild altitude landscapes (9 – 600 m a.s.l.).

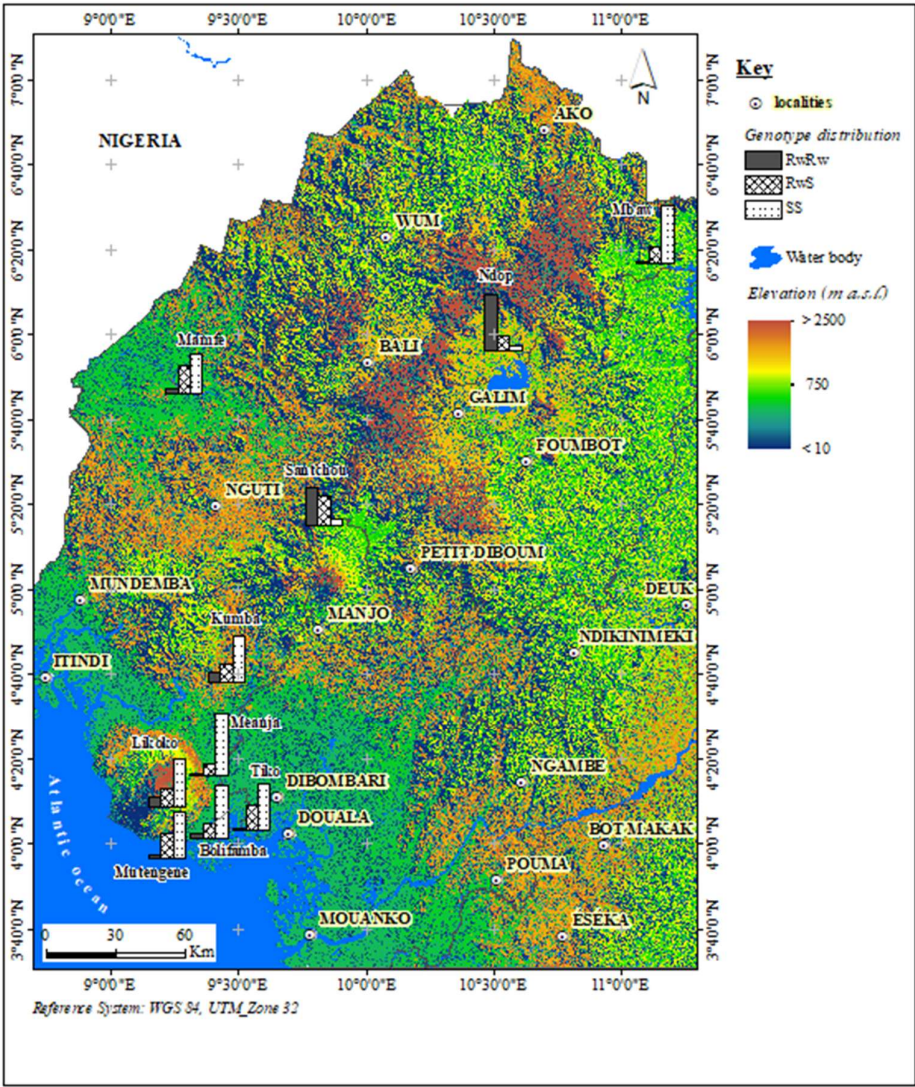


Figure 3. Sampling distribution of the *L1014F_kdr* genotypes for *An. gambiae* s.l. mosquitoes tested across space.

3.2. Nucleotide Polymorphisms in *An. gambiae* and *An. coluzzii* Exon-20 Region of the VGSC Gene

A 510 bp fragment in the exon-20 region of the VGSC gene was successfully investigated in 87 (2N = 174 sequences) out 90 randomly selected *An. gambiae* and *An. coluzzii* specimen [specifically 2N = 134 sequences of *An. gambiae* (20 in Bolifamba, 20 in Likoko, 20 in Kumba, 20 in Mamfe, 20 in Santchou, 16 in Ndop and 18 in Mbaw plain) and 2N = 40 *An. coluzzii* sequences (20 in Tiko and 20 in Meanja)]. The alignment and comparison of the sequences obtained with that referenced in Genbank (NCBI, Bethesda, USA) showed 98% identities with S₆ transmembrane segment of domain II in *An. gambiae* voltage-gated sodium channel (accession number: CAA739201).

Sequence analysis showed the presence of 9 polymorphic sites (**Error! Reference source not found.**), of which 7 nucleotide variations occurred within the intron spanning the exon-20 region. The remaining two nucleotide polymorphisms corresponded to point mutations TTA to TTT and TTA to TCA at position codon 1014 (with the coding region) respectively inducing functional amino-acid substitutions of leucine to phenylalanine (*L1014F*) and leucine to serine (*L1014S*) both involved in *kdr* phenotypes.

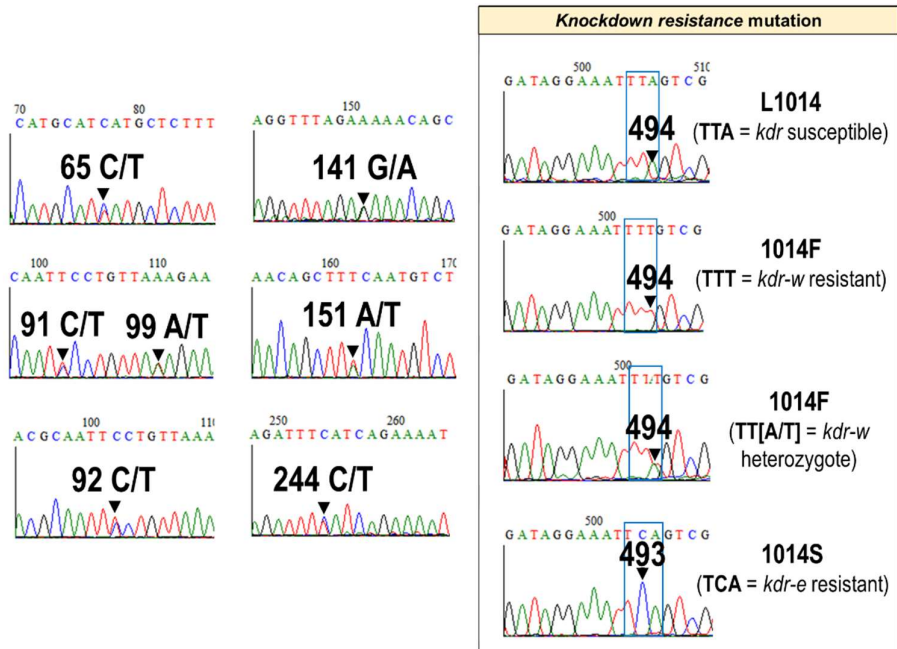


Figure 4. Sequencing traces showing nucleotide polymorphisms detected across the 510bp fragment spanning the exon-20 region of the *kdr* locus.

Interestingly, the *L1014S* mutation (*kdr*-East) was detected in *An. gambiae* specimen in six localities situated at most 800 m a.s.l. (from the least to high elevated localities: Mamfe, Kumba, Bolifamba, Mbaw, Santchou and Likoko), whereas absent at highest altitudes precisely in Ndop situated between 1168 and 1285 m a.s.l. (**Error! Reference source not found.**). No *An. coluzzii* sequence analysed was found carrying the *L1014S* mutation.

Table 1. Genetic variability parameters for exon-20 region of *kdr* locus in *An. gambiae* s.l. populations throughout the study area.

Samples	N	S	h (Hd)	Syn	NSyn	π (k)	D	F*	
<i>Anopheles</i> species ID									
<i>An. Coluzzii</i>	40	6	9 (0.61)	0	1 (kdr-W)	0.002 (0.85)	- 1.07 ^{ns}	- 0.58 ^{ns}	
<i>An. Gambiae</i>	134	5	6 (0.53)	0	2 (kdr-W, kdr-E)	0.002 (1.09)	0.41 ^{ns}	- 0.78 ^{ns}	
<i>kdr</i> Allelic profile									
L1014	44	5	6 (0.61)	0	0	0.001(0.73)	- 0.91 ^{ns}	0.57 ^{ns}	
1014F	94	2	3 (0.06)	0	0	0.0002 (0.08)	- 1.22 ^{ns}	- 1.29 ^{ns}	
1014S	36	2	3 (0.54)	0	0	0.001 (0.57)	0.35 ^{ns}	- 0.54 ^{ns}	
<i>Mountain massif</i>	<i>localities</i>								
Mount Cameroon	Tiko	20	5	7 (0.64)	0	1 (kdr-W)	0.002 (0.93)	- 1.05 ^{ns}	- 0.68 ^{ns}
	Kumba	20	3	3 (0.64)	1	1 (kdr-E)	0.003 (1.53)	2.17*	1.52 ^{ns}
	Meanja	20	4	6 (0.58)	0	1 (kdr-W)	0.001 (0.75)	- 0.97 ^{ns}	- 0.17 ^{ns}
	Bolifamba	20	3	4 (0.60)	0	2 (kdr-W, kdr-E)	0.002 (1.05)	0.63 ^{ns}	0.09 ^{ns}

	Likoko	20	3	3 (0.49)	1	1 (kdr-E)	0.002 (1.15)	0.97 ^{ns}	1.15 ^{ns}
	ALL	100	8	11 (0.77)	0	2	0.003 (1.70)	0.24 ^{ns}	1.08 ^{ns}
Western Highlands	Mamfe	20	4	5 (0.73)	0	2 (kdr-W, kdr-E)	0.003 (1.48)	0.92 ^{ns}	0.44 ^{ns}
Mount Oku	Mbaw plain	18	4	4 (0.31)	0	2 (kdr-W, kdr-E)	0.001 (0.73)	- 1.13 ^{ns}	- 0.94 ^{ns}
Kupe Manengoumba	Santchou	20	3	4 (0.43)	0	2 (kdr-W, kdr-E)	0.001 (0.68)	- 0.51 ^{ns}	- 0.26 ^{ns}
Mount Bamboutos	Ndop	16	1	2 (0.23)	0	1 (kdr-W)	0.0005 (0.23)	- 0.45 ^{ns}	0.45 ^{ns}
ALL		174	9	12 (0.68)	0	2	0.003 (1.48)	- 0.14 ^{ns}	0.30 ^{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; L1014 = susceptible allele; 1014F = *kdr* West-type resistant allele; 1014S = *kdr* East-type resistant allele; ns. Not significant; *, p < 0.05.

Overall, the number of nucleotide polymorphisms detected was broadly similar in *An. coluzzii* (6 polymorphic sites out of 9) and *An. gambiae* (5/9). Moreover, mutational steps seemed to significantly occur more in L1014 susceptible individuals (5/9) as compared to 1014F and 1014S carriers (2 polymorphic sites each) (**Error! Reference source not found.**).

3.3. Genetic Variability and Haplotype Network of *An. gambiae* and *An. coluzzii* VGSC Gene

A total of twelve exon-20 haplotypes originated from the identified nucleotide polymorphisms (Genbank accession IDs: MW375948-MW375958, MW375960 and MW375962) (**Error! Reference source not found.** and Appendix A.1). Nine haplotypes out of twelve were detected in *An. coluzzii* populations while *An. gambiae* populations presented six haplotypes. The L1014 susceptible allele carriers were found more genetically diverse based on their number of haplotypes detected and its associated diversity ($h = 6$, $Hd = 0.61$). Meanwhile 1014F and 1014S resistant allele carriers recorded a reduced number of haplotypes ($h = 3$ haplotypes per allele), with 1014F individuals having a very low haplotype diversity ($Hd = 0.06$).

Spatial differences were also evident in the number of haplotypes detected per mountainous massif, with the Mount Cameroon harbouring 91.6% (11/12) of the total number of haplotypes detected; whereas the number of haplotypes progressively decreased with climb in altitude in the remaining mountain massifs from five haplotypes in Mamfe_Western Highlands (90 – 300 m a.s.l.) to two haplotypes in the Ndop area situated between 1168 and 1285 m a.s.l. within the Mount Bamboutos domain.

Constructing a Leigh-Bryant integer neighbour-joining (IntNJ) network tree (Figure 5), it was found four major haplotypes deriving in one hand from single mutational step (specifically C/T haplotype H1 contained within the L1014 susceptible cluster, A/T haplotype H2 in the 1014F resistant cluster and T/C haplotype H8 within the 1014S resistant cluster), and from a double T/C amino-acid substitution in the other hand (haplotype H9 still within the 1014S resistant cluster). The ancestral haplotype H7 (within the L1014 susceptible cluster) was detected in four mountainous massifs out the five investigated, including Mount Cameroon, Western Highlands, Kupe Manengoumba and Mount Bamboutos. Two haplotypes appeared as singletons including haplotype H11 and H12 which were respectively associated to the 1014F and 1014S clusters. It was important to note that the IntNJ haplotype network highlighted six haplotypes (H3 – H6, H10 and H11) specific to *An. coluzzii* populations, three specific haplotypes for *An. gambiae* populations (H8, H9 and H12 belonging to the 1014S resistant cluster) and another three haplotypes common to the two vector species (H7 ancestral haplotype, H1 susceptible haplotype and H2 1014F- resistant haplotype).

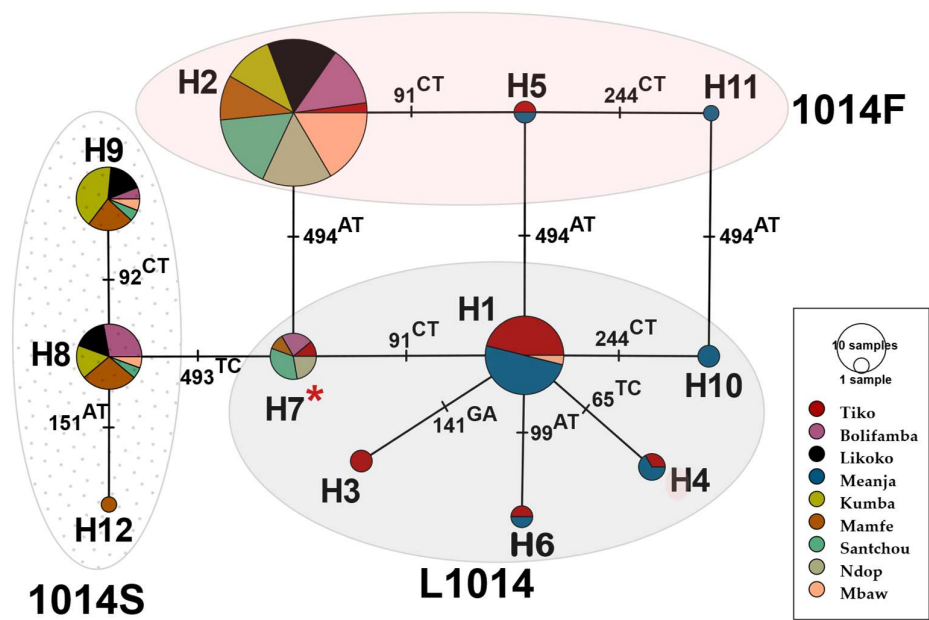


Figure 5. IntNJ network tree showing genealogical relationship among *kdr* haplotypes in *An. gambiae* and *An. coluzzii*. Haplotypes are presented in circular shape scaled to reflect their respective frequencies; (*) ancestral haplotype.

Reduced values of genetic variability estimates of the 1014F allele ($h = 3$, $Hd = 0.06$, $\pi = 0.0002$ and $k = 0.08$) are evidence of a strong selection driving to fixation of the *kdr* West type mutation, although non-significant and negative values of neutrality estimates (Tajima D and Fu and Li F^*). Likewise, the 1014S allele seemed to be under selection as well ($h = 3$, $Hd = 0.54$, $\pi = 0.001$, $k = 0.57$ and positive but not significant neutrality estimates Tajima $D = 0.35$) but no right to a genetic fixation. Deep observations genetic diversity parameters showed that selection processes of *kdr* mutations are increasingly occurring from mid-altitude areas in Bolifamba (~ 500 m a.s.l. in the Mount Cameroon massif) to highland in Ndop (more than 1000 m a.s.l., Mount Bamboutos massif) where the greatest loss of diversity is more evident ($h = 2$, $Hd = 0.23$, $\pi = 0.0005$, $k = 0.23$).

3.4. Phylogenetic Relationship Between Exon-20 *kdr* Haplotypes

Haplotype clustering based on a maximum-likelihood (ML) phylogenetic tree (**Error! Reference source not found.**) further confirmed profiles obtained with the IntNJ haplotype network. The marked low diversity of *kdr* mutations was illustrated, specifically for the 1014F west-type *kdr* which is potentially driving near genetic fixation. The ML tree of the 510bp exon-20 illustrates a high diversity of haplotypes in L1014 susceptible allele carriers. Overall, L1014 susceptible samples formed their own cluster (with high mutational steps) situated in between separated resistant population's clusters which contained reduced genetic diversities.

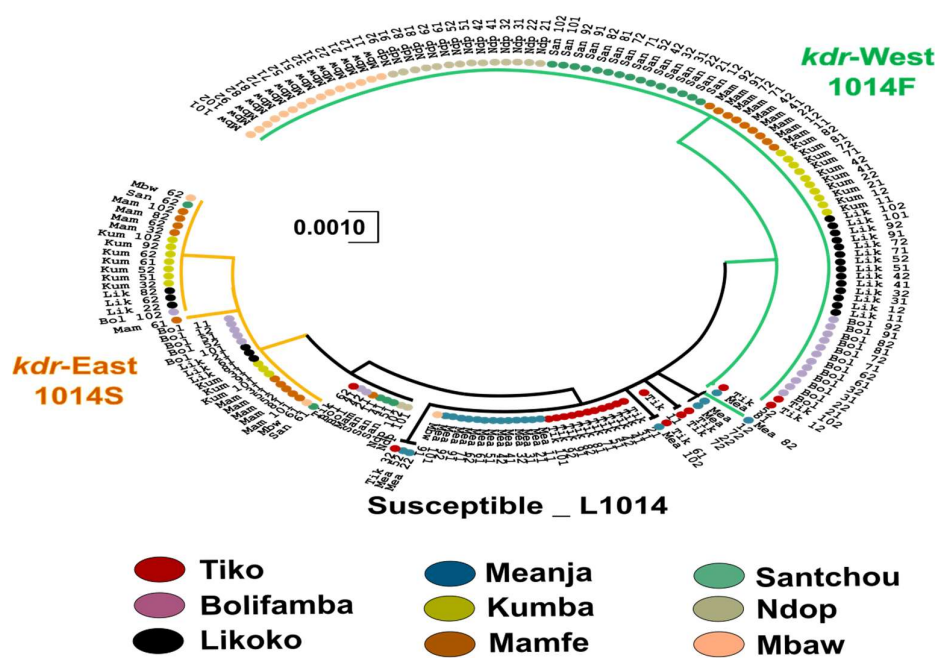


Figure 6. Maximum-likelihood phylogenetic tree of *kdr* haplotypes based on exon-20 sequences among analysed samples.

Likewise, the patterns of clustering according to the neighbour-joining (NJ) genetic distances between surveyed localities illustrated an overall segregation between the *An. coluzzii* block and the *An. gambiae* block (Figure 7). Observation that was further supported by values of K_{ST} genetic differentiation estimates and index of gene flow N_m (**Error! Reference source not found.**) showing a marked closeness between Tiko/Meanja *An. coluzzii* populations (reduced K_{ST} = 0.011, p value > 0.05 and moderately high N_m = 6.63). These later were significantly isolated (p value < 0.0001) from all remaining *An. gambiae* populations ($0.31 \leq K_{ST} \leq 0.61$ and $0.15 \leq N_m \leq 0.51$).

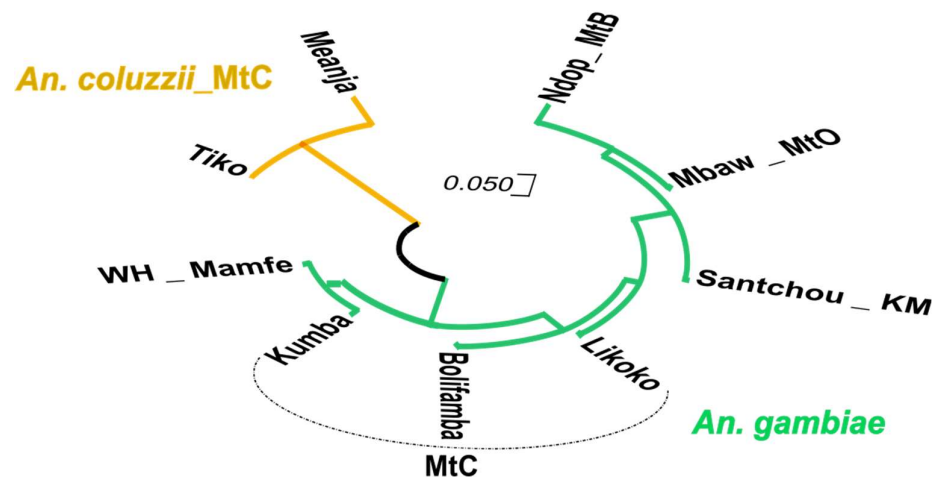


Figure 7. Neighbour-joining tree of exon-20 region of the VGSC gene between localities investigated. Mount Cameroon (MtC), Western Highlands (WH), Kupe Manengoumba (KM), Mount Oku (MtO), Mount Bamboutos (MtB).

Moreover, although an overall relatedness of *An. gambiae* populations was highlighted, deep observations of K_{ST} estimates and N_m index (**Error! Reference source not found.**) led to the identification of three sub-blocks represented by Bolifamba/Likoko_MtC sub-block (K_{ST} = - 0.02, p

value > 0.05 and $N_m = 26.13$), Kumba_MtC/Mamfe_WH sub-block ($K_{ST} = -0.02$, p value > 0.05 and $N_m = 26.05$) and finally Santchou_KM/Mbaw_MtO/Ndop-MtB sub-block ($-0.008 \leq K_{ST} \leq 0.004$, p value > 0.05 and N_m ranging from 8.25 to 32.82). Interestingly, genetic similarities and moderate gene flow were also identified between Bolifamba/Likoko_MtC sub-block and Santchou in the Kupe Manengoumba mountainous massif ($0.004 \leq K_{ST} \leq 0.01$, p value > 0.05, N_m ranging from 6.58 to 8.31).

4. Discussion

The Cameroon Great West area characterised with a moderate to high malaria endemicity level [71–76], in addition to a widespread distribution of major Afrotropical malaria vectors (*An. gambiae*, *An. coluzzii* and *An. funestus*) [53,77–80] offers an exceptional opportunity for implementing control strategies (such as insecticide-based control interventions) across mountainous agroecological landscapes [81]. This study represents a comprehensive investigation of the *kdr*-related mutations in *An. gambiae* s.l. mosquitoes across mountainous plains of the Cameroon Great West domain.

- **Distribution of target-site insensitivity mutations**

This study shows that *L1014F* target-site mutation is widespread in *An. gambiae* s.l. across the Cameroon Great West domain. The *1014F* allele was constantly present from the Mount Cameroon south-western limits to grassy highlands in the North-west region of Cameroon, in agreement with previous reports which stated *L1014F* target-site resistance as a major mechanism driving *An. gambiae* s.l. insecticide resistance across the country [32,39–45]. The *L1014S* *kdr*-type mutation was not detected by TaqMan high-throughput approach in analysed samples. Bass, Nikou [59] have identified the method to deliver the greatest specificity and sensitivity in *kdr* genotyping, although increase in failed reactions could occurred. The *1014S* allele had previously been detected throughout all ecological zones of Cameroon, from the forest to highlands similar to those explored in this study [39,41,44,45]. Therefore, further survey will be required to ascertain the contribution of *1014S* allele in target-site mechanism of insecticide resistance in *An. gambiae* s.l. populations across mountainous plains of the Cameroon Great West domain.

The *L1014F* mutation was evidenced in all four sub-species analysed within the *An. gambiae* s.l. complex, with much higher frequencies obtained in *An. gambiae* and *An. coluzzii* × *An. gambiae* hybrid populations than in *An. coluzzii* and *An. melas*. Previous studies have shown that frequencies of *L1014F* mutation are higher in *An. gambiae* compared to *An. coluzzii* in Cameroon [32], Benin [31], Democratic Republic of Congo [28] and Senegal [30] among others; except for some urban peri-urban settings [45]. Furthermore, the high abundance of *L1014F* mutation in *An. coluzzii* × *An. gambiae* hybrid populations (15/19: 78.9%, especially from the Ndop _ Mount Bamboutos area) than in other subspecies of the Gambiae complex may result from an apparent replacement of *An. coluzzii* [80] by introgressed *An. gambiae* [53] carrying the *L1014F* *kdr* mutation. Similar scenarios were mentioned in Burkina-Faso [82] and Mali [83]. However, additional investigations are needed to further confirm whether this pattern of resistance in hybrid populations is consistent with a collapse of local *An. coluzzii* populations by *1014F* resistant *An. gambiae* carriers.

Although the *L1014F* mutation had a moderate occurrence in *An. coluzzii* (34.1%), comparison with results from surveys conducted in 2005 [41] showed 6 times increment of *L1014F* frequency in *An. coluzzii* populations in the study area (precisely in the locality of Tiko). Moreover, prior to this study no *kdr* alleles have been reported in *An. melas* from Cameroon, whereas both alleles had previously been identified in Senegal [84] though at low frequencies (ranging from 0.40 to 1.12%). Yet, there is still no record on *An. melas* implication in malaria parasite transmission in Cameroon [32,85]. However, with an overall relative occurrence of *L1014F* mutation estimated at 18.1%, insecticide resistance monitoring activities need to be applied as well for this species which is considered as a good vector in the neighbouring Equatorial Guinea [86].

This study reveals also spatial heterogeneity of *L1014F*-*kdr* mutation across the Cameroon Great West domain. The marked abundance of the *L1014F*-*kdr* mutation with a dominance of *1014F/1014F* resistant genotypes in highlands such as Santchou (750 – 800 m a.s.l., relative frequency of *kdr*-

L1014F: 91.8%) and Ndop (1168 – 1285 m a.s.l., relative frequency of *kdr*-L1014F: 94.6%) compared to low-mild elevated areas (9 – 600 m a.s.l., relative frequencies ranged from 23.2 to 45.4%), could suggest fitness processes conferring 1014F resistant allele carriers greater adaptability to low temperatures compared to susceptible populations. Similar observations were made in Kenya [87,88] where less than 30% mortality rates of *kdr*-L1014S homozygous resistant *An. gambiae sensu stricto* (ss) mosquitoes against deltamethrin treated nets (2.5 – 16.7 mg/m²) were found under low temperature conditions (< 25.3°C).

Nonetheless, with the rise in use of LLINs or IRS in Cameroon since 2011 [32,89] added to a history of rice agriculture (in both areas in contrast to Likoko also situated at 800 m a.s.l.) where agro-pesticides use is often intense, strong selection of insecticide resistance in insect pests might have occurred if larvae breeding sites located in cultivated fields were exposed to insecticides applied to control agricultural pests. In cotton-growing regions of the northern Cameroon [90] or the "cotton-belt" of Burkina-Faso [91], investigators observed loss of susceptibility to dichlorodiphenyltrichloroethane (DDT) and permethrin confirmed by a rise in frequencies (up to 97%) of *kdr*-L1014F associated resistance.

Even though the overall frequency of the *L1014F-kdr* was estimated at 40.7%, the increasingly widespread distribution of this mutation in vector populations could significantly hamper the effectiveness of vector control tools such as LLINs and IRS across mountainous landscapes [74,81]. Unfortunately, since no parallel susceptibility bioassays were performed on the samples used for genotyping, we could not establish a precise association of current vector control interventions with specific *kdr* allele. Moreover, since observations made in this study are based on entomological surveys conducted from 2010 to 2015, more recent investigations should be performed to assess the current *kdr* trends across the region.

- **Sequence analysis and Genetic variability patterns of the 510bp fragment in exon-20 region of *kdr* locus**

Sequence analysis of the downstream exon-20 *VGSC* locus suggests four possible origins of the *kdr* alleles in *An. coluzzii* and *An. gambiae* populations across the Cameroon Great West domain as represented in the IntNJ haplotype network. Three of these events originate from the common amino acid change TTA to TTT at *kdr* locus from three different *L1014* susceptible progenitor haplotypes, H7 (the ancestral haplotype), H1 and H10 all resulting in 1014F west-type *kdr* allele. Whereas the fourth event originates from an independent single-step polymorphism resulting in the TTA to TCA amino acid substitution associated to the *L1014S kdr*-East mutation. This was strictly found in subsets of *An. gambiae* analysed samples (six populations out of nine), although *L1014S kdr*-East mutation remains undetectable in *An. gambiae* and *An. coluzzii* populations using Bass, Nikou [59] TaqMan *kdr* assay in contrast to Ibrahim, Mukhtar [92] who with sequencing confirmed the absence of *L1014S kdr* mutation in *An. coluzzii* Nigerian populations as genotyped using the same protocol. Whether the presence of *L1014S kdr*-East mutation in six *An. gambiae* populations out of seven analysed in this study (1014F was the only *kdr* allele found in Ndop highland) resulted from a *de novo* mutation or if it is identical by introgressed descent to that found in the rest of the country [46,93] and neighbouring countries [61,94] remains to be clearly understood.

In the 510bp fragment spanning exon-20 region of *kdr* locus in *An. gambiae* s.l. populations (including here *An. coluzzii* and *An. gambiae*) of the Cameroon Great West domain, 9 sites in total were found polymorphic (all constituted by nucleotide substitutions) of which 7 occurred within the intronic region spanning exon-20. Similar type and number of mutational events were previously detected by Etang, Vicente [93] in Cameroon and Pinto, Lynd [61], Africa-wide when assessing the origin of *kdr* phenotype in *An. gambiae* s.l. populations based on analysis of the upstream intron-1 of the *VGSC* gene, hence confirming that nucleotide variations occurred more frequently within intronic than exonic regions [95–97]. It could be interesting to establish the association between the number and frequencies of detected intronic polymorphisms and the occurrence of *L1014F/S kdr* mutations in *An. gambiae* s.l. populations similarly to that performed with field populations of *Aedes albopictus* by Zhou, Yang [98].

Within the coding exon-20, apart from *kdr* mutations no other polymorphisms (being synonymous or non-synonymous) were found. This is not surprising as no genetic variation has previously been found in *An. gambiae* and *An. coluzzii* *kdr* exon-20 [36,94,99] in contrast to *An. funestus* where various non-*kdr* amino acid changes were detected in the VGSC gene exon-20 [62]. Being that conserve, exon-20 of the VGSC gene therefore represents a candidate genomic region to assess whether in situ additional nucleotide polymorphisms could result in an exacerbation of target-site based *kdr* response in *An. gambiae* s.l. vector populations [92,100–102].

Analysis of genetic variability of the downstream exon-20 region of *kdr* locus indicated moderate to low variability patterns (h , H_d , π and k) of the analysed sequence in *An. gambiae* populations with respect to land elevation. These showed that *kdr* alleles (especially 1014F allele) are driven towards fixation in areas located from 700 m a.s.l. and more (Mbaw plain, Likoko, Santchou and Ndop). It was even more appreciable regarding at the single consensus and most dominant (91/174 sequences) 1014F resistant haplotype (H2) detected in all *An. gambiae* populations analysed across the Cameroon Great Western domain. Such observations were previously made in other Cameroonian grass-highlands [93] and in western Kenyan highlands [103] with respectively 1014F and 1014S *kdr* alleles being highly frequent that values of genetic variability were found reduced. Landscape barriers such as highlands had been suggested to act as genetic bottleneck in addition of contributing to the fitness decline of susceptible specimen with either *kdr*-based mutations in *An. gambiae* [87], glutathione S-transferase *epsilon* 2 metabolic-based resistance in *An. funestus* [54], or even other animal populations [104,105]. Nevertheless, more investigations are needed in other to complement the genetic barriers effect of land elevation environmental components on the spread of resistance phenotypes.

On another note, moderate to high genetic variability patterns were perceived in *An. coluzzii* populations with majority of the haplotypes detected (6/9 haplotypes) found within the L1014 susceptible cluster while remainders were associated to 1014F resistant cluster (one shared with *An. gambiae* populations and the two others specific to *An. coluzzii*). Therefore, between 2012 and 2014 progressive selection of 1014F-*kdr* allele occurred in *An. coluzzii* populations across the study area. Observations which need to be updated as recent findings had shown fixation of 1014F resistant *kdr* allele in *An. coluzzii* populations from Gounougou in the Northern Cameroon [43].

- **Genetic differentiation based on exon-20 region of the VGSC gene in *An. coluzzii* and *An. gambiae* populations**

Evolutionary analysis of exon-20 DNA sequences helped further trace the origin of *kdr* mutations in *An. coluzzii* and *An. gambiae* populations across the Cameroon Great West domain. Considering the ML phylogenetic tree 1014F and 1014S *kdr* alleles evolved from mutations affecting separated L1014 susceptible haplotypes which were predominantly found in *An. coluzzii* and *An. gambiae* for respective mutant allele. This observation could indicate a type-sensitivity to mutation of codon 1014 with respect to speciation within the Gambiae complex, further illustrated with an NJ tree of genetic distance which revealed a marked separation between *An. coluzzii* and *An. gambiae* populations. The large difference in genomic sequence between *An. coluzzii* and *An. gambiae* sibling species is approved [106]. Similarly, examination of an increased number of samples from a broad geographical range is required to fully uncover the origins of L1014F/S mutations in Gambiae populations.

Regarding population structure patterns of exon-20 in *An. gambiae* populations, an obvious *kdr* spatial differentiation was revealed. Either localities situated within the same mountainous massifs or localities situated in neighbouring massifs showed a significant genetic relatedness with occurrence of gene flow between analysed *An. gambiae* populations. This suggest that genetic flow between vector populations is not completely restricted by factors such as the mountainous massifs and land elevation which are being known to act as genetic barriers in various settings [55].

5. Conclusions

This work focused on the status of target-site based *kdr* mutations in *An. coluzzii* and *An. gambiae* populations across the Cameroon Great West domain between 2010 and 2015. A cross-distribution of

L1014F kdr-West mutation was observed in all genotyped Gambiae specimen, meanwhile the *L1014S kdr*-East mutation was revealed after DNA sequence analysis of a 510bp fragment in exon-20 of the VGSC gene. Estimates of genetic variability showed signs of fixation for *1014F-kdr* allele, whereas progressive selection of *1014S-kdr* allele was ongoing selection and could have probably driven to fixation in recent years. Such evidences are to be prioritized giving the current trend of reduced susceptibility to insecticide-based control tools in *Anopheles* vectors. Outputs can be used to optimize malaria vector control and design appropriate insecticide resistance management throughout mountainous landscapes.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: Details on the mosquito sampling; Table S2: Overall mosquito surveys; Table S3: Frequency distribution of L1014F-kdr mutation across the study area; Table S4: Population structure patterns on K_{ST} estimates from *kdr* mutations with (Nm).

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

Abbreviations

The following abbreviations are used in this manuscript:

DDT	Dichloro Diphenyl Trichloroethane
DNA	Deoxyribonucleic Acid
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
<i>kdr</i>	Knockdown resistance
LLINs	Long Lasting Insecticidal Nets
PCR	Polymerase Chain Reaction
VGSC	Voltage Gated Sodium Channel

Appendix A

Appendix A.1. Haplotype Diversity Patterns of the 510bp Fragment in Exon-20 Region of the VGSC Gene

Hap	Freq	000011244 699945499 512911434	Location
H1 (S)	26	TCCAGACTA	Tk, Me, Mb
H2 (Rw)	91	.T.....T	Tk, Bo, Li, Ku, Ma, Sa, Nd, Mb
H3 (S)	2	A....	Tk
H4 (S)	3	C.....	Tk, Me
H5 (Rw)	2	T	Tk, Me
H6 (S)	2	...T.....	Tk, Me
H7* (S)	9	.T.....	Tk, Bo, Ma, Sa, Nd
H8 (Re)	18	.T.....C.	Bo, Li, Ku, Ma, Sa, Mb
H9 (Re)	17	.TT....C.	Bo, Li, Ku, Ma, Sa, Mb
H10 (S)	2	T..	Me
H11 (Rw)	1	T.T	Me
H12 (Re)	1	.T...T.C.	Ma

Figure A1. H1 – H12 are haplotypes; Freq = frequency as number of individuals carrying an haplotype; Tiko (Tk), Meanja (Mea), Mbaw plain (Mb), Bolifamba (Bo), Likoko (Li), Kumba (Ku), Mamfe (Ma), Santchou (Sa) and Ndop (Nd); S = susceptible allele carriers; Rw = *kdr* West-type resistant allele carriers; Re = *kdr* East-type resistant allele carriers; (*) = ancestral haplotype; polymorphic sites are in red.

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