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Genetic Structure Of *Anopheles coluzzii* and *Anopheles gambiae* Populations In Northern Benin (West Africa) In The Context Of Indoor Residual Spraying

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Abstract

From 2019 to 2021, pirimiphos-methyl, clothianidin + deltamethrin, and clothianidin alone were successively used to protect communities in the Donga and Alibori departments against mosquito bites. This study aims to analyze the genetic structure of *Anopheles coluzzii* and *Anopheles gambiae* over three years of intervention.

The vector population studied comes from adult mosquitoes collected monthly from 2019 to 2021 in the study communes. Mosquitoes were collected on the night of each visit in four houses per village and two villages per commune. Molecular analysis of populations was carried out on 3,134 sampled anopheles.

The frequencies of *kdr-L995F*mutation were similar between the treated communes and the control commune, suggesting constant pressure from the pyrethroids and DDT used in agriculture. *Anopheles coluzzii* showed relatively lower frequencies than *Anopheles gambiae* for *kdr-L995F* mutation, linked to its preference for permanent breeding sites, which are less exposed to insecticides during the dry season. The *ace-1-G280S* mutation shows low frequencies in both species' populations. Genetic differentiation is high for the *ace-1* gene. Genetic differentiation varies for the voltage-gated sodium channel (*vgsc)* gene, suggesting variable gene flow between sub-populations.

The clothianidin + deltamethrin mixture could reduce the frequency of *kdr-L995F*. Genetic differentiation is very significant and similar between the *ace-1* (*G280S*) gene sub-populations.

Keywords: Genetic differentiation, *kdr-L995F*, *ace-1-G280S, Anopheles coluzzii*, *Anopheles gambiae*, IRS, clothianidin, Benin

Introduction

Background

Efforts to combat malaria continue to face a convergence of challenges, mainly in Africa, which carries the heaviest burden of this devastating disease **[1]**. The COVID-19 pandemic, along with other humanitarian crises, pressures on health systems, budgetary constraints, rising biological threats, and the declining effectiveness of crucial malaria control strategies, are severely hampering progress towards global malaria control targets **[1]**. Malaria vectors are becoming increasingly resistant to insecticides used in public health for net impregnation and indoor residual spraying (IRS) **[2]**.

In Benin, the species *Anopheles gambiae* and *Anopheles coluzzii* are the main vectors that dominate malaria transmission **[3]**. To control them in the northern region, where malaria incidence is high, the national malaria control program (NMCP) has turned to indoor residual spraying (IRS) alongside the use of long-acting insecticidal nets (LLINs) **[4]**. IRS involves spraying the inside walls of homes with residual insecticides to eliminate mosquito vectors and reduce

malaria transmission **[5]**. From 2019 to 2021, three types of insecticide were successively used for large-scale community IRS in the departments of Donga and Alibori in northern Benin **[4]**. The used insecticides included pirimiphos-methyl 300 CS, a combination of clothianidin 500g/kg with deltamethrin 62.5 g/kg, and clothianidin 50 WG on its own. This periodic change of insecticides with different modes of action was aimed at optimizing the efficacy of IRS, which can be influenced by several factors, including the genetic variability of Anopheles populations.

This study aims to examine the genetic makeup of *Anopheles coluzzii* and *Anopheles gambiae* populations in the northern Benin region, specifically investigating the potential impact of IRS on the genetic diversity of these vector populations. The findings from these analyses will offer essential insights for enhancing the efficiency of malaria vector control methods.

Methods

Study area

Bembèrèkè commune in Borgou was selected as a control in comparison to the IRS-treated communes of Donga (Djougou and Copargo) and Alibori (Kandi and Gogounou) **(Figure 1)**

The Department of Borgou is bordered to the north by the Department of Alibori and to the southeast by the Department of Donga **(Figure 1)**. Like Donga and Alibori, Borgou has a dry and rainy season, with annual rainfall between 900 and 1,300 mm. The average annual temperature in Borgou is around 26°C, and relative humidity varies between 30% and 70%. The soils are tropical ferruginous, ferritic, sandy clay, or sandy clay, and gneissic granite **[6]**. Borgou is a highly agricultural department. Around 66% of its population is involved in agriculture, covering 54% of the department's total surface area (13,962 km² / 25,856 km²).

Around 69% of households in the Alibori department are agricultural (74693/108351), corresponding to around 14% of Benin's farming population, making it the breadbasket of Benin **[7]**. Rainfall varies between 700 mm and 1,200 mm. The flat terrain, which is sometimes shaped by a Cretaceous sedimentary series or crowned by armored hillocks sloping down to the river Niger and hills of ferruginous sandstone, features sparse shrub savannah and gallery forests bordered by watercourses. The primary soils are of the ferruginous type on crystalline bedrock, very fertile alluvial soils in the Niger Valley and clayey, black silty soils in the lowlands, swamps, and very fertile gallery forests, where rice, market gardening, and yam are grown.

The average rainfall in Donga is between 1,200 mm and 1,300 mm. During the rainy season, the watercourses flood the low-lying areas, making them ideal for rice growing. The soils are crude mineral, tropical ferruginous, indurated, and hydromorphic. Gallery forests are formed by dense vegetation along watercourses. 59% of households in Donga were farmers (39,461/66,433), and 96.8% of farming households were involved in crop production and 3% in animal production **[8]**.

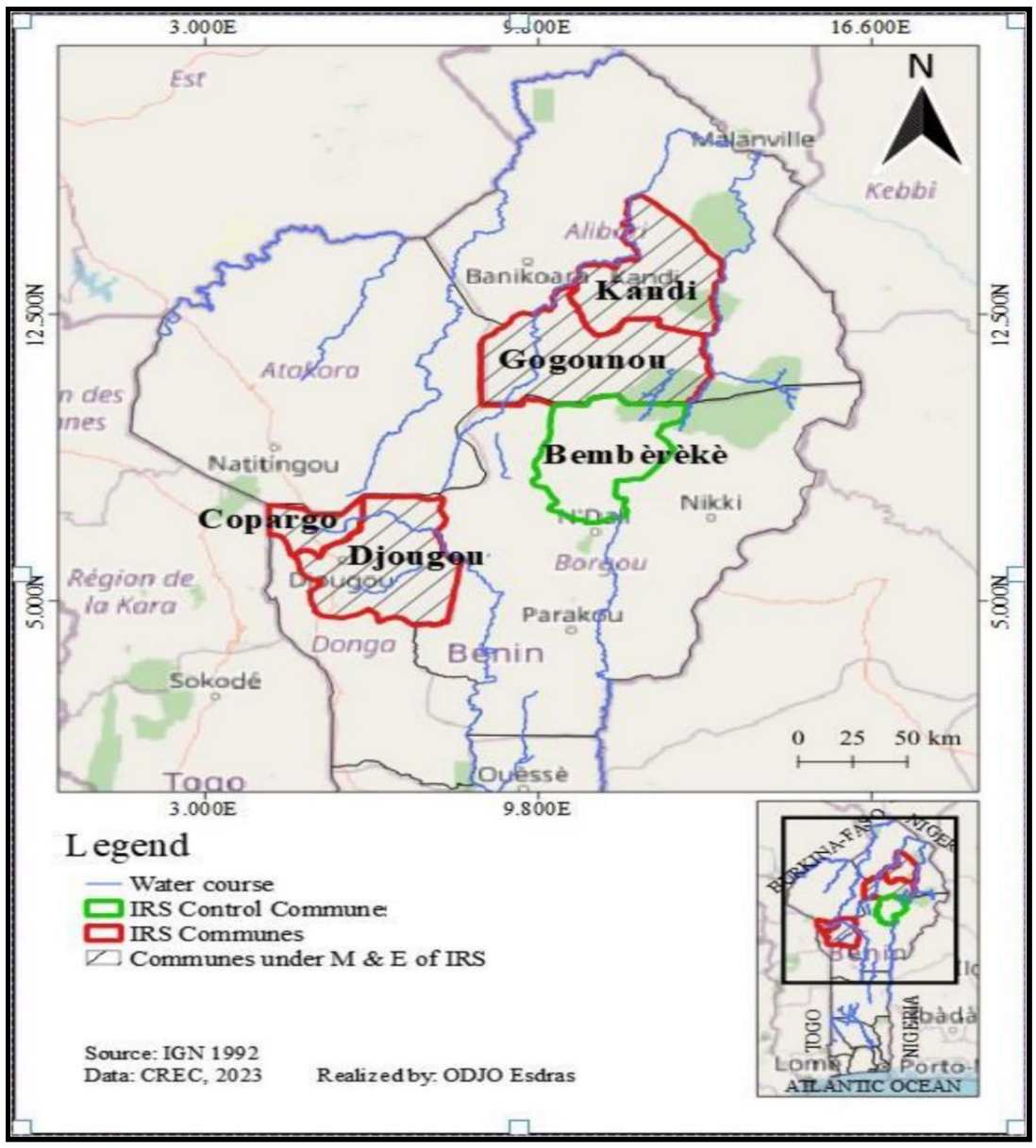


Figure 1: Map of the study communes

Collection of mosquito samples

The *Anopheles gambiae s.l.* The population studied came from adult mosquitoes collected monthly in 2019, 2020, and 2021 by capture from human volunteers in the control commune (Bembèrèkè) and the IRS communes (Gogounou and Kandi to the north of Bembèrèkè, Djougou, and Copargo to the southeast of Bembèrèkè).

Two villages were chosen in each commune: one central and one peripheral. Local volunteers captured adult mosquitoes in four houses per visit, with one collector inside and one outside each household, conducting hourly collections. However, no insecticide-resistance bioassays were performed on the collected mosquitoes **[9, 10]**.

Molecular characterization of vector

Collected mosquitoes were identified and separated at the species level based on morphological criteria according to established taxonomic keys **[11, 12]**.

3,134 *Anopheles gambiae mosquitoes* collected from the field were subjected to molecular characterization. Genomic DNA extraction was performed on the mosquito's body, including the abdomen,wings, and legs. The molecular biology protocol outlined by Myriam and Cécile **[13]** was employed for this stage.

Species identification

The genomic DNA extracted was used for molecular identification of the species within the *Anopheles gambiae* complex.

All mosquitoes were subjected to PCR using the Scott *et al.* **[14]** protocol to identify the different species of the *Anopheles gambiae* complex. The primers used below should identify the following species: *Anopheles gambiae s.s.* (AG), *Anopheles arabiensis* (AA), *Anopheles melas* (ME), *Anopheles merus* (ME), *Anopheles quadriannulatus* A and B (QD).

UN: GTGTGCCGCTTCCTCGATGT

AG: CTGGTTTGGTCGGCACGTTT

AA: AAGTGTCCTTCTCCATCCTA

ME: TGACCAACCCACTCCCTTGA

QD: CAGACCAAGATGGTTAGTAT

PCR was performed in a 30-cycle program. Denaturation at 94 °C for 30 s, hybridization at 50 °C for 30 seconds, and elongation at 72 °C for 30 seconds. The amplified DNA copies were kept at a final temperature of 4 °C before migration on a 2.5% agarose gel with ethidium bromide used as an intercalating agent.

The technique of Santolamazza *et al.* **[15]** was used to distinguish the twin species *Anopheles gambiae* and *Anopheles coluzzii*. The PCR program includes an initial denaturation at 94 C for 5 minutes, followed by 35 cycles. Each cycle involves denaturation at 94 °C for 30s, hybridization at 54 °C for 30s, and elongation at 72 C for 30 s. A final elongation at 72 °C for 10 min is performed to allow complete amplification of the sequences. The following primers were used:

200X6.1FTCGCCTTAGACCTTGCGTTA and

200X6.1R-CGCTTCAAGAATTCGAGATAC.

The PCR products are maintained at 4°C before undergoing migration through 1.5% agarose gel electrophoresis, utilizing ethidium bromide as an intercalating agent.

Identification of the *L995F* mutation within the *kdr* gene

The presence of the resistance allele (*L995F*) of the *kdr* gene in samples collected from each study site was detected by PCR following the protocol described by Martinez-Torres *et al.* **[16]**. The following primers were used.

Agd1: 5′-ATAGATTCCCCGACCATG-3′;

Agd2: 5′-AGACAAGGATGATGAACC-3′;

Agd3: 5′-AATTTGCATTACTTACGACA-3′;

Agd4: 5′-CTGTAGTGATAGGAAATTTA-3′.

The amplification protocol consists of 40 cycles, each comprising an initial denaturation at 94°C for 1 minute, hybridization at 48°C for 2 minutes, and elongation at 72°C for 2 minutes. The PCR concludes with a final elongation step at 72°C for 10 minutes **[16]**.

Identification of the *G280S* mutation within the *ace-1* gene.

The Weill *et al*. protocol **[17]** was employed to detect the *G280S* mutation in mosquitoes. The PCR was conducted using the following primers:

Moustdir1 5′CCGGGNGCSACYATGTGGAA3′ and

Moustrev1 5′ACGATMACGTTCTCYTCCGA3′. The amplification program consisted of thirty cycles, comprising denaturation at 94°C for 30 seconds, hybridization at 52°C for 30 seconds, and elongation at 72°C for 1 minute. Subsequently, PCR products underwent digestion with the AluI restriction enzyme following the manufacturer's guidelines before being migrated onto a 2% agarose gel.

Statistical analysis

To calculate the allelic frequencies of the *kdr-L995F* and *ace-1 G280S* genes in each commune and by species, stratified 3×3×3 and 2×3×3 contingency tables were done in IBM-SPSS Statistics Subscription® (Build 1.0.01406). The Pearson chi-square ($\chi 2$) test was conducted to compare proportions. The allele frequencies of *kdr-L995F* and *ace-1*R *G280S* were calculated using the formula:

$$F(R) = \frac{2nRR + nRS}{2(nRR + nRS + nSS)}$$

Where F® is the frequency of resistance, n is the number of mosquitoes of a given genotype, RR is the homozygous resistant genotype, RS is the heterozygous resistant genotype, and SS is the susceptible genotype **[18]**.

F_{IS} Fixation Index

This parameter quantifies the variance between the population of individuals identified in the H_O heterozygous state and the expected heterozygous (H_E) rate. It is also called the pan mixing deviation and is calculated by the formula: $F_{IS} = 1 - (H_O / H_E)$ at the specified locus within a population of diploid individuals. This index represents the departure from the Hardy-Weinberg equilibrium. It varies from -1 to

+1 and indicates the heterozygote deficit per population, per locus, and for all loci. F_{IS} is positive when the population has a deficit of heterozygotes compared with the panmictic equilibrium and negative in the opposite case. If the assumptions of the Hardy-Weinberg model are met, *it will* be equal to H_E. When the observed heterozygosity falls below the expected heterozygosity ($H_O < H_E$), it indicates an overrepresentation of homozygotes compared to anticipated in the studied populations. This excess of homozygotes can then be assimilated to a risk of inbreeding, drift, selection, and differentiation within populations.

Genetic differentiation (F_{ST}) of populations

The Hardy-Weinberg Equilibrium was assessed for each population using Genetics software version 4.7.5. The fixation index (F_{IS}) was computed using Genepop software, following the method described by Weir and Cockerham **[19]**.

An F_{ST} *value* nearing 0 indicates substantial genetic interchange among populations, implying minimal genetic differentiation and a panmictic population. Conversely, an F_{ST} value approaching 1 signifies significant genetic divergence between populations, indicating limited or no gene flow. According to Wright, an F_{ST} between 0 and 0.05 indicates little differentiation; an F_{ST} *between* 0.05 and 0.15 indicates moderate differentiation; an F_{ST} *between* 0.15 and 0.25 suggests significant differentiation, and above 0.25, the F_{ST} *illustrates* very significant differentiation **[20]**.

The various communes concerned were considered sub-populations, and the genetic differentiation of the population (F_{ST}) was evaluated between the control commune and each IRS commune by year and by commune according to the years of the IRS campaign. The indices of genetic differentiation within populations (F_{ST}) were calculated using Genepop version 4.7.5 software.

Results

Allele frequencies of *kdr-L995F* mutation

Overall, analysis of these data reveals that the allelic frequency of the *kdr-L995F* mutation is relatively high in the whole population and varies according to the anopheline populations and the insecticide used. Although the insecticides used for spraying are not directly responsible for the *kdr-L995F* mutation, an increase in the frequency of this mutation has been observed in specific populations. **(Figure 2)**.

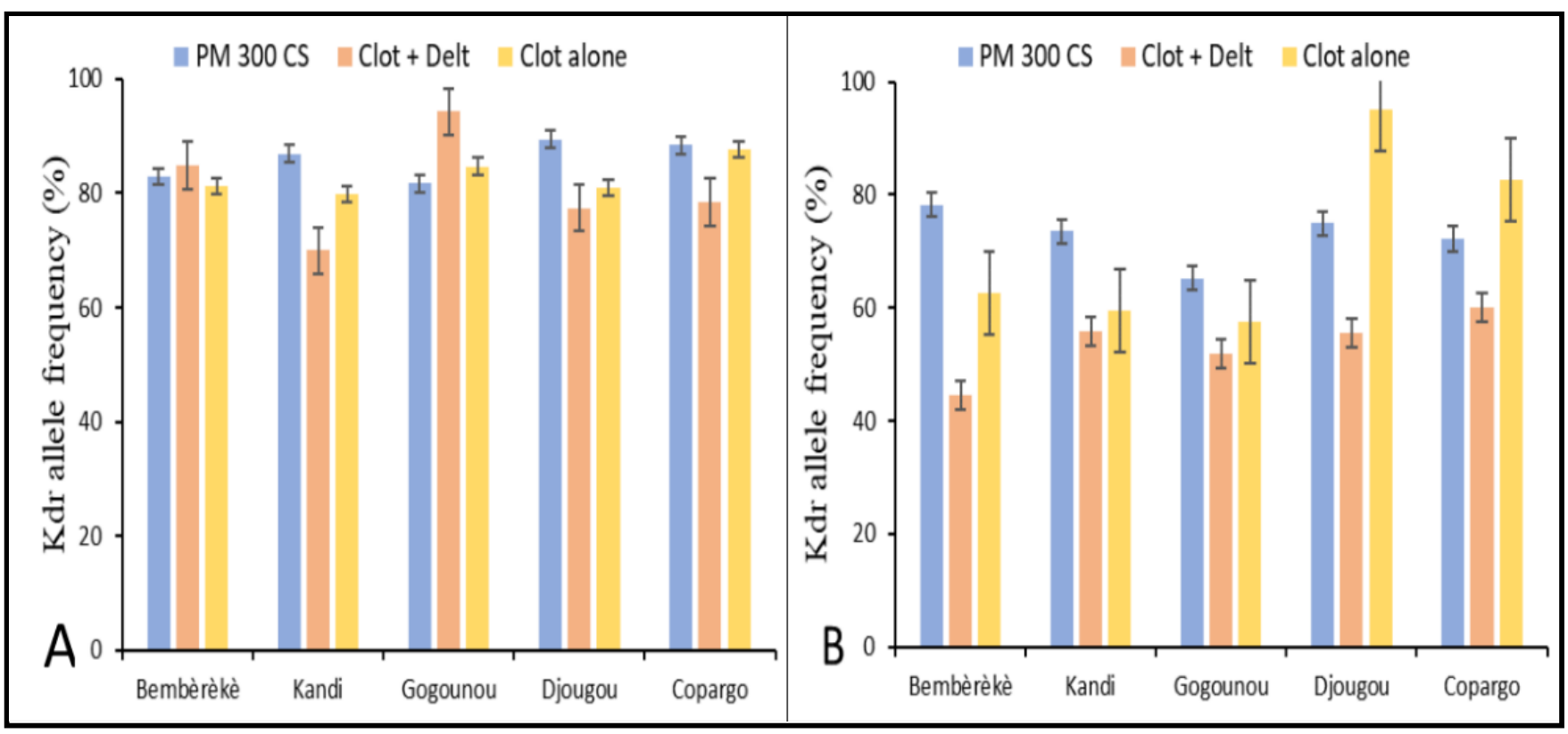


Figure 2: Bar chart showing the *kdr* allele frequency (percentage with standard error bars in the first panel) in *An. gambiae* (A) and *An. coluzzii* (B) in the communes of the Alibori and Donga departments of Benin by species and IRS period PM: pirimiphos-methyl; CS: capsule suspensions; Clot + Del: mixture clothianidin 500 g/kg + deltamethrin 62.5 g/kg; Clo: clothianidin 50 WG; WG: Water dispersible granules

For *Anopheles gambiae*

The frequency of the *kdr-L995F* mutation varied across different years and insecticide treatments. In 2019, when pirimiphos-methyl 300 CS was used, the frequency ranged from 82% to 89%. For the mixture of clothianidin 500 g/kg and deltamethrin 62.5 g/kg in 2020, the frequency ranged from 70% to 94%. In 2021, when clothianidin 50 WG was used alone, the frequency ranged from 80% to 88% (Figure 2A). For each insecticide, no remarkable difference was observed between the *permutation* frequencies of the treated communes and those of the control commune. *Anopheles gambiae* showed a *kdr-L995F* mutation frequency of 89% with pirimiphos-methyl 300 CS in Djougou, compared with 77% with the Clothianidin + Deltamethrin mixture. Apart from Gogounou, the clothianidin 500 g/kg + deltamethrin 62.5 g/kg mixture appears to have reduced the frequency of the *permutation* in the communes treated.

For *Anopheles coluzzii*

The magnitude of the *kdr-L995F* mutation varied across different insecticide treatments. For pirimiphos-methyl 300 CS, the frequency ranged from 65% to 78%, while for the mixture of clothianidin 500 g/kg and deltamethrin 62.5 g/kg, it ranged from 45% to 60%. Clothianidin 50 WG alone exhibited frequencies ranging from 58% to 95%. Notably, *Anopheles coluzzii* generally displayed a lower prevalence of the *kdr-L995F* mutation than *Anopheles gambiae* across most regions **(Figure 2)**.

Allele frequencies of *ace-1 G280S* mutation

The data in Figure 3 shows the allelic frequencies of the *G280S* mutation of the *ace-1* gene in *Anopheles gambiae and Anopheles coluzzii* in the control commune (Bembèrèkè) and in the IRS communes (Kandi, Gogounou, Djougou and Copargo) exposed to various insecticides. The mutation occurred infrequently across all the study communes, with a maximum value of 5%.

For *Anopheles gambiae*

Frequencies varied from one commune to another. The allelic frequency was 2% to 3% with pirimiphos-methyl 300 CS, 1% to 5% with the clothianidin + deltamethrin mixture, and 3% to 4% with Clothianidin alone. No notable difference ($p > 0.05$) was detected between the average allele frequency of the IRS communes and that of the control commune **(Figure 3A)**.

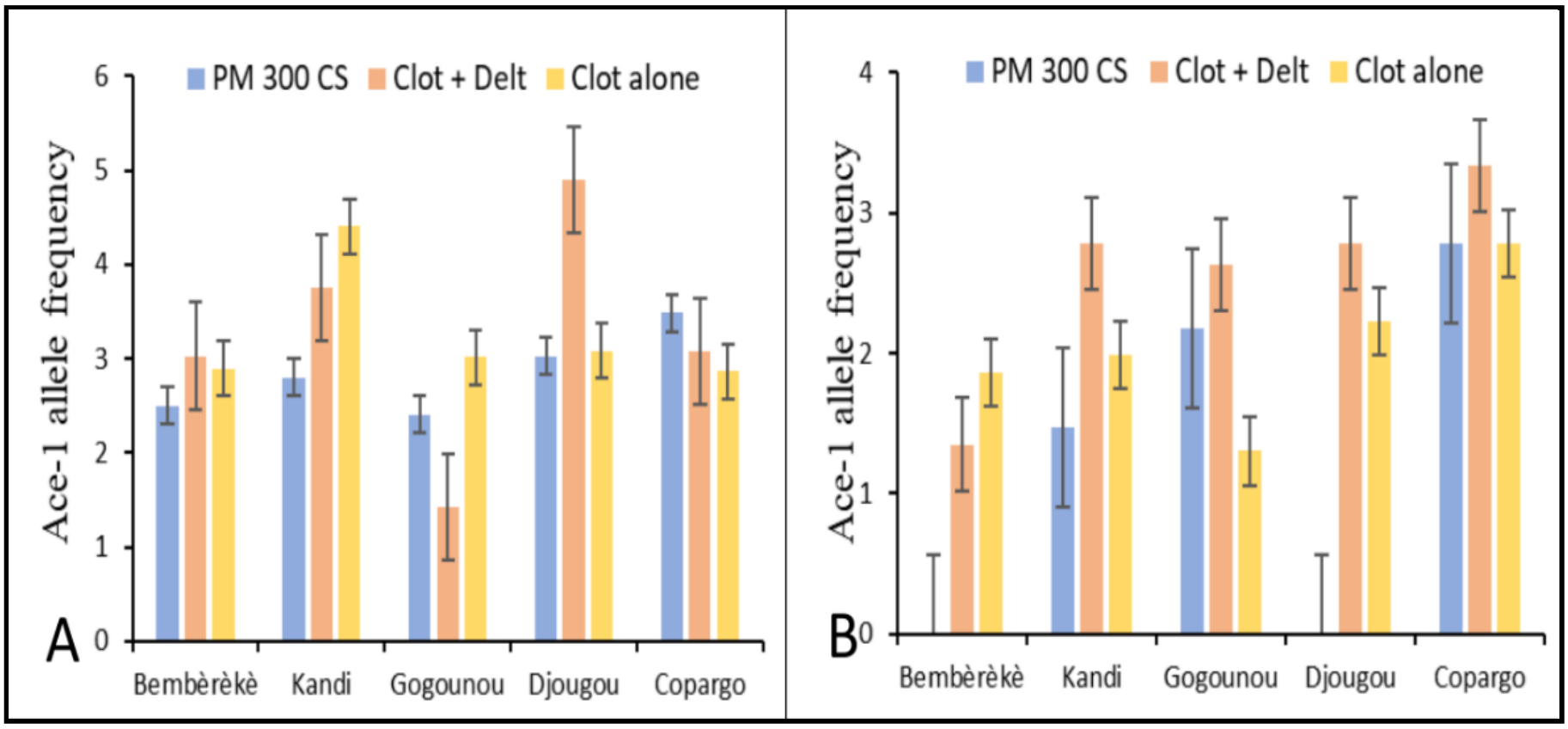


Figure 3: Bar chart showing the *ace-1* allele frequency (percentage with standard error bars in the first panel) in *An. gambiae* (A) and *An. coluzzii* (B) in the communes of the Alibori and Donga departments of Benin by species and IRS period PM: pirimiphos-methyl; CS: capsule suspensions; Clot + Del: mixture clothianidin 500 g/kg + deltamethrin 62.5 g/kg; Clo: clothianidin 50 WG; WG: Water dispersible granules

For *Anopheles coluzzii*

The mutation frequency was 0% to 3% with pirimiphos-methyl 300 CS, 1% to 3% for the clothianidin and deltamethrin mixture, and for clothianidin 50 WG alone.

F_{IS} fixation index

The deviation from panmixis within the different populations was assessed **(Tables 1 and 2)**. For the *kdr L995F* mutation of the *vgsc* gene, the *Anopheles gambiae* and *Anopheles coluzzii* populations showed a positive fixation index (F_{IS}) ($F_{IS} > 0$). Expected heterozygosity (H_E) was higher than observed heterozygosity (H_O). Fixation index values varied within populations between IRS campaigns from 2019 to 2021 with different insecticides. The minimum and maximum *values* were found in Kandi ($0.35 < F_{IS} < 0.65$) for *Anopheles gambiae* and in Kandi ($F_{IS} = 0.11$) and Djougou ($F_{IS}= 0.57$) for *Anopheles coluzzii*. These positive fixation indices reflected a heterozygosity deficit within the populations **(Tables 1 and 2)**.

For the *G280S* mutation in the *ace-1* gene, the fixation index (*FIS*) was negative overall in *Anopheles gambiae* and *Anopheles coluzzii* populations. In 2020, the *Anopheles gambiae* populations of Gogounou and Copargo tended to return to equilibrium under the Hardy-Weinberg hypothesis ($F_{IS} \approx 0$). The same is true for the different populations of *Anopheles coluzzii* from 2019 to 2020. The F_{IS} values were low, showing an incipient excess of heterozygotes in the populations. **(Tables 1 and 2)**.

Table 1. Observed heterozygosity (H_O), expected heterozygosity (H_E), fixation index (F_{IS}) and number of individuals sampled of *An. gambiae* per population and per insecticide.

Population	Period (insecticide)	N	Mutations					
			kdr-L995F			*ace-1 G280S*		
			H_E	H_O	F_{IS}	H_E	H_O	F_{IS}
Bembèrèkè								
	2019 (Control)	**120**	0.284 ± 0.081	0.125 ± 0.059	0.5616	0.049 ± 0.039	0.050 ± 0.039	-0.0215
	2020 (Control)	**33**	0.261 ± 0.150	0.182 ± 0.132	0.3069	0.059 ± 0.081	0.061 ± 0.081	-0.0159
	2021 (Control)	**190**	0.305 ± 0.065	0.226 ± 0.060	0.2577	0.056 ± 0.033	0.058 ± 0.033	-0.0272
Kandi								
	2019 (PM 300 CS)	**107**	0.229 ± 0.080	0.150 ± 0.068	0.3467	0.055 ± 0.043	0.056 ± 0.044	-0.0242
	2020 (clot + delt)	**80**	0.423 ± 0.108	0.150 ± 0.078	0.6465	0.073 ± 0.057	0.075 ± 0.058	-0.0327
	2021 (clot alone)	**159**	0.319 ± 0.072	0.182 ± 0.060	0.4285	0.084 ± 0.043	0.088 ± 0.044	-0.0429
Gogounou								
	2019 (PM 300 CS)	**104**	0.300 ± 0.088	0.135 ± 0.066	0.5526	0.047 ± 0.041	0.048 ± 0.041	-0.0198
	2020 (clot + delt)	**35**	0.109 ± 0.103	0.057 ± 0.077	0.4809	0.029 ± 0.055	0.029 ± 0.055	0.0000
	2021 (clot alone)	**232**	0.260 ± 0.056	0.159 ± 0.047	0.3866	0.059 ± 0.030	0.060 ± 0.031	-0.0290
Djougou								
	2019 (PM 300 CS)	**66**	0.191 ± 0.095	0.121 ± 0.079	0.3674	0.059 ± 0.057	0.061 ± 0.058	-0.0236
	2020 (clot + delt)	**51**	0.353 ± 0.131	0.176 ± 0.105	0.5022	0.094 ± 0.080	0.098 ± 0.082	-0.0421
	2021 (clot alone)	**243**	0.310 ± 0.058	0.144 ± 0.044	0.5361	0.060 ± 0.030	0.062 ± 0.030	-0.0298
Copargo								
	2019 (PM 300 CS)	**129**	0.206 ± 0.070	0.124 ± 0.057	0.3998	0.068 ± 0.043	0.070 ± 0.044	-0.0323
	2020 (clot + delt)	**65**	0.340 ± 0.115	0.185 ± 0.094	0.4599	0.060 ± 0.058	0.062 ± 0.058	0.0240
	2021 (clot alone)	**157**	0.218 ± 0.065	0.134 ± 0.053	0.3879	0.056 ± 0.036	0.057 ± 0.036	-0.0263

N: number of sampled individuals; H_O: observed heterozygosity; H_E: expected heterozygosity; F_{IS}: fixation index; PM: pirimiphos-methyl; CS: capsule suspensions; Clot + Del: mixture clothianidin 500 g/kg + deltamethrin 62.5 g/kg; Clo: clothianidin 50 WG; WG: Water dispersible granules.

Table 2. Observed heterozygosity (H_O), expected heterozygosity (H_E), fixation index (F_{IS}) and number of individuals sampled of *An. coluzzii* per population and per insecticide.

Population	Period (insecticide)	N	Mutations					
			kdr-L995F			*ace-1 G280S*		
			H_E	H_O	F_{IS}	H_E	H_O	F_{IS}
Bembèrèkè								
	2019 (Control)	**23**	0.348 ± 0.195	0.174 ± 0.155	0.5056	0.000 ± 0.000	0.000 ± 0.000	NA
	2020 (Control)	**37**	0.501 ± 0.161	0.243 ± 0.138	0.5179	0.027 ± 0.052	0.027 ± 0.052	0.0000
	2021 (Control)	**134**	0.470 ± 0.085	0.358 ± 0.081	0.2378	0.037 ± 0.032	0.037 ± 0.032	-0.0153
Kandi								
	2019 (PM 300 CS)	**34**	0.395 ± 0.164	0.353 ± 0.161	0.1081	0.029 ± 0.057	0.029 ± 0.057	0.0000
	2020 (clot + delt)	**144**	0.495 ± 0.082	0.215 ± 0.067	0.5657	0.054 ± 0.037	0.056 ± 0.037	-0.0251
	2021 (clot alone)	**201**	0.483 ± 0.069	0.363 ± 0.066	0.2490	0.039 ± 0.027	0.040 ± 0.027	-0.0178
Gogounou								
	2019 (PM 300 CS)	**23**	0.463 ± 0.204	0.348 ± 0.195	0.2542	0.043 ± 0.083	0.043 ± 0.083	0.0000
	2020 (clot + delt)	**76**	0.503 ± 0.112	0.250 ± 0.097	0.5042	0.052 ± 0.050	0.053 ± 0.050	0.0204
	2021 (clot alone)	**192**	0.490 ± 0.071	0.401 ± 0.069	0.1817	0.026 ± 0.022	0.026 ± 0.023	-0.0106
Djougou								
	2019 (PM 300 CS)	**12**	0.391 ± 0.276	0.333 ± 0.267	0.1538	0.000 ± 0.000	0.000 ± 0.000	NA
	2020 (clot + delt)	**18**	0.508 ± 0.231	0.222 ± 0.192	0.5696	0.056 ± 0.106	0.056 ± 0.106	0.0000
	2021 (clot alone)	**292**	0.095 ± 0.034	0.065 ± 0.028	0.3121	0.044 ± 0.023	0.045 ± 0.024	-0.0211
Copargo								
	2019 (PM 300 CS)	**18**	0.412 ± 0.227	0.222 ± 0.192	0.4688	0.056 ± 0.106	0.056 ± 0.106	0.0000
	2020 (clot + delt)	**15**	0.496 ± 0.253	0.267 ± 0.224	0.4717	0.067 ± 0.126	0.067 ± 0.126	0.0000
	2021 (clot alone)	**144**	0.288 ± 0.074	0.139 ± 0.056	0.5185	0.054 ± 0.037	0.056 ± 0.037	-0.0251

N: number of sampled individuals; H_O: observed heterozygosity; H_E: expected heterozygosity; F_{IS}: fixation index; PM: pirimiphos-methyl; CS: capsule suspensions; Clot + Del: mixture clothianidin 500 g/kg + deltamethrin 62.5 g/kg; Clo: clothianidin 50 WG; WG: Water dispersible granules; NA: Not Applicable.

Genetic differentiation F_{ST}

Pairwise F_{ST} was calculated for both loci, and the results were compared between the control commune and each IRS commune for each insecticide (Table 3) and each commune according to insecticides **(Table 4)**.

The F_{ST} values in Tables 3 and 4 indicate the degree of genetic differentiation between population pairs for two different loci (*kdr-L995F* and *ace-1 G280S*) over three different years (2019, 2020, 2021).

Anopheles gambiae showed significant genetic differentiation at the *kdr-L995F* locus **(Tables 3 and 4)** in 2019 and 2021 (F_{ST} = 0.24 and 0.70), followed by weak genetic differentiation in 2020 (F_{ST} = 0.02) between Bembèrèkè (control population) and Kandi (IRSpopulation). Between Bembèrèkè and Gogounou, genetic differentiation was very high in 2019 (F_{ST} = 0.80) and moderate in 2020 and 2021 (F_{ST} = 0.09 and 0.20). Bembèrèkè, along with Djougou and Copargo, showed moderate genetic differentiation in 2019 (F_{ST} = 0.10 and 0.10), high differentiation in 2020 (F_{ST} = 0.32 and 0.35), then very high differentiation in 2021 for Djougou (F_{ST} = 0.93) and low differentiation for Copargo (F_{ST} = 0.03) **(Table 3)**.

The F_{ST} values of *Anopheles gambiae for* the *ace-1 G280S* locus between Bembèrèkè and each IRS commune were globally between (F_{ST} = 0.6 and 1.0), indicating a significant genetic differentiation between the control commune and each IRS commune.

Overall, for *Anopheles gambiae*, genetic differentiation is more significant for the *ace-1 G280S* locus than for their L995F locus.

Anopheles coluzzii for the *kdr-L995F* locus **(Tables 3 and 4)** shows variations in genetic differentiation coefficients from one year to the next and between pairs of populations. Overall, the differentiation coefficients decreased from 2019 to 2021, resulting in very high genetic differentiation (F_{ST} > 0. 31) to significant genetic differentiation between Bembèrèkè and Gogounou with pirimiphos-methyl and Clothianidin alone (F_{ST} = 0.24, F_{ST} = 0.19) on the one hand, and between Bembèrèkè and Copargo (F_{ST} = 0.19) with the clothianidin + deltamethrin mixture on the other. Moderate genetic differentiation (F_{ST} = 0.09) was observed between Kandi and Bembèrèkè with the clothianidin + deltamethrin mixture. Weak genetic differentiation (F_{ST} < 0.0001) was observed with Clothianidin alone in 2021 between Bembèrèkè and Djougou and Bembèrèkè and Copargo.

For the *ace-1 G280S* locus, the genetic differentiation data between different pairs of populations show generally significant differentiation between populations and treatments.

Table 3. Genetic differentiation between *An. gambiae* and *An. coluzzii* populations in IRS communes and the control commune for the *kdr-L995F* and *ace-1 G280S* mutations

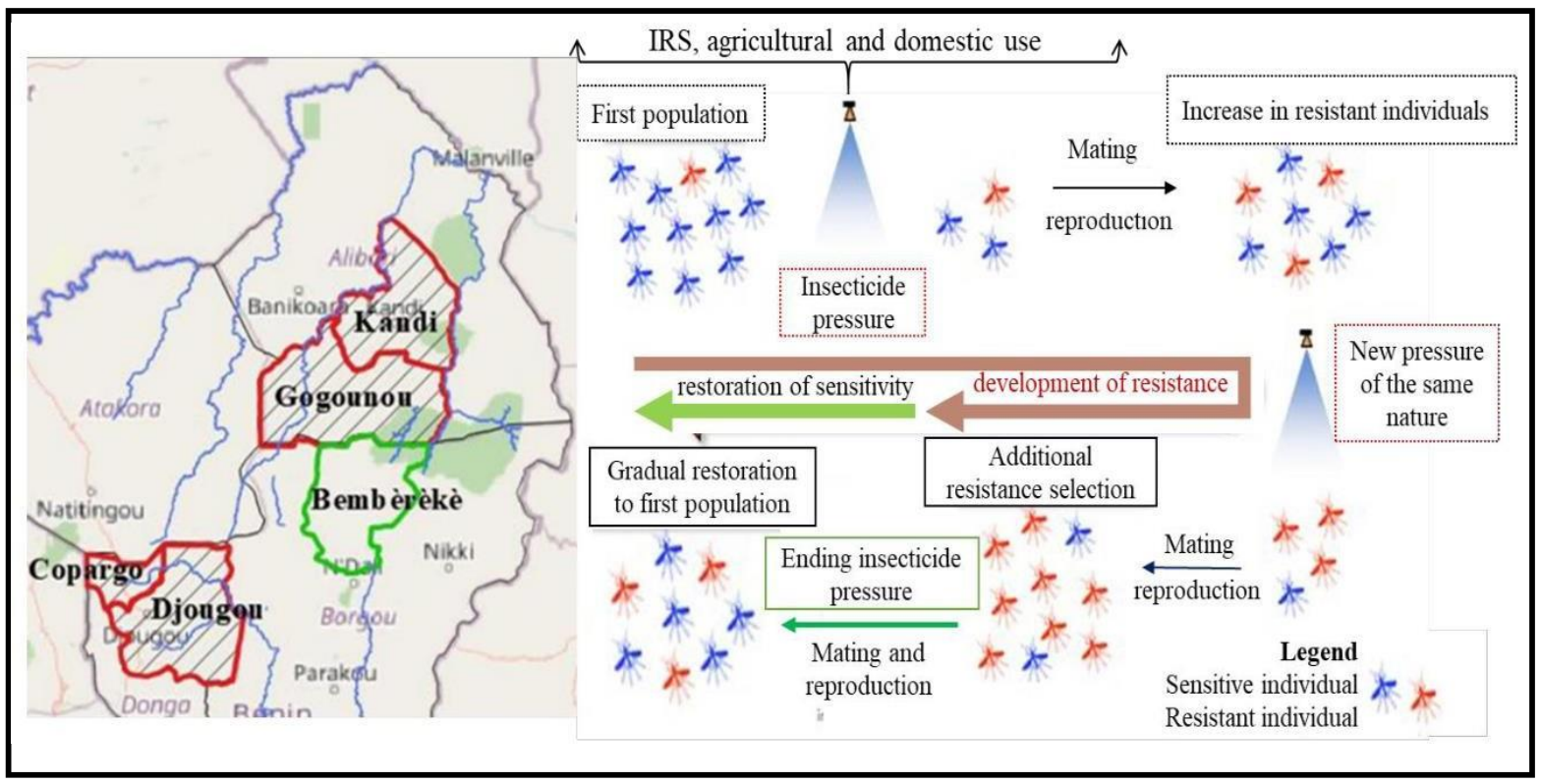
Species	Populations compared	*kdr-L995F*			*Ace-1 G280S*		
		PM (2019)	Clot + Delt (2020)	Clot alone (2021)	PM (2019)	Clot + Delt (2020)	Clot alone (2021)
An. gambiae	Kan & Bem	0.2403	**0.0194**	0.6983	1.0000	1.0000	0.3100
	Gog & Bem	0.8054	0.0921	0.1992	1.0000	0.6119	1.0000
	Djo & Bem	0.0985	0.3223	0.9311	1.0000	0.7059	1.0000
	Cop & Bem	0.0958	0.3418	**0.0265**	0.6054	1.0000	1.0000
An. coluzzii	Kan & Bem	0.6601	0.0921	0.4131	1.0000	0.6916	1.0000
	Gog & Bem	0.2478	0.3224	0.1983	1.0000	0.6703	0.7470
	Djo & Bem	0.7707	0.3142	**<0.0001**	NA	1.0000	0.8036
	Cop & Bem	0.6094	0.1969	**<0.0001**	0.4384	1.0000	0.5819

Bem : Bembèrèkè ; Kan : Kandi ; Gog : Gogounou ; Djo : Djougou ; Cop : Copargo ; PM : pirimiphos-methyl; CS: capsule suspensions; Clot + Del: mixture clothianidin 500 g/kg + deltamethrin 62.5 g/kg; Clo: clothianidin 50 WG; WG: Water dispersible granules; NA: Not Applicable.

Table 4. Genetic differentiation between *An. coluzzii* and *An. gambiae* populations in IRS and control communes for the *kdr-L995F* and *ace-1 G280S* mutations

Mutations	Species	Insecticides (years) compared	Alibori (Under IRS)		Donga (Under IRS)		Control
			Kandi	Gogounou	Djougou	Copargo	Bembèrèkè
Kdr-L995F	*An. gambiae*	P-methy (2019) & Clot + Delt (2020)	**0.0001**	**0.0081**	**0.0180**	**0.0145**	0.7189
		P-methy (2019) & Clot alone (2021)	**0.0337**	0.3701	**0.0185**	0.7989	0.6682
		Clot + Delt (2020) & Clot alone (2021)	**0.0226**	**0.0279**	0.4945	**0.0186**	0.4977
	An. coluzzii	P-methy (2019) & Clot + Delt (2020)	**0.0082**	0.1259	0.1752	0.4321	**0.0003**
		P-methy (2019) & Clot alone (2021)	**0.0320**	0.3468	0.0016	0.1693	**0.0467**
		Clot + Delt (2020) & Clot alone (2021)	0.3868	0.2523	**<0.0001**	**0.0155**	**0.0071**
Ace-1 G280S	*An. gambiae*	P-methy (2019) & Clot + Delt (2020)	0.7686	0.6994	0.5111	1.0000	1.0000
		P-methy (2019) & Clot alone (2021)	0.3664	0.8049	1.0000	0.8103	0.8080
		Clot + Delt (2020) & Clot alone (2021)	0.8147	0.5151	0.5527	1.0000	1.0000
	An. coluzzii	P-methy (2019) & Clot + Delt (2020)	0.6980	1.0000	1.0000	1.0000	1.0000
		P-methy (2019) & Clot alone (2021)	1.0000	1.0000	0.6791	1.0000	0.6070
		Clot + Delt (2020) & Clot alone (2021)	0.6098	0.4571	1.0000	1.0000	1.0000

P-methyl: pirimiphos-methyl; CS: capsule suspensions; Clot + Del: mixture clothianidin 500 g/kg + deltamethrin 62.5 g/kg; Clo: clothianidin 50 WG; WG: Water dispersible granules.



Discussion

The frequencies of the *kdr-L995F* mutation calculated in all the *Anopheles gambiae* and *Anopheles coluzzii* populations showed no remarkable difference between the treated communes and the control commune. This observation is thought to be due to the use of pyrethroid and organochlorine (DDT) insecticides in agriculture in these study areas **[21–23]**. These classes of insecticides target the voltage-gated sodium channel (*vgsc*) gene, which contains the *kdr-L995F* mutation **[24, 25]**. This heavy use of insecticides is thought to be at the origin of the selection of resistant individuals, thus increasing the frequency of the mutation in the various populations **[26, 27]**. Several studies have shown similar results in Benin and in several other countries in Africa and elsewhere **[25, 27–31]**.

Furthermore, the frequencies of this mutation observed in *Anopheles coluzzii* populations are relatively lower than those of *Anopheles gambiae* over the years. This finding may be linked to the permanent roosting preferred by *Anopheles coluzzii*, which is dominant during the dry season **[32, 33]**. During this season, insecticide pressure is low without agricultural activities. However, the high allele frequency in *Anopheles gambiae* populations is linked to the use of insecticides, particularly pyrethroids **[34, 35]**, which provide crop protection and, in turn, encourage the selection of resistance in these *Anopheles gambiae* populations **[23, 30, 36]**. Concerning the *ace-1*gene, the frequency of the *G280S* mutation was low in all *Anopheles coluzzii* and *Anopheles gambiae* populations during the three years of the

study. These low allelic frequencies obtained in these populations are thought to be linked to the momentary use of these insecticides only during IRS, which does not favor the selection of resistant individuals. This low frequency of the resistant allele could also be linked to the high adaptation cost of the *ace-1* mutation **[37–40]**. Previous studies have also shown the low frequency of this mutation in Benin **[18, 41]**. Several factors could explain the differences observed in panmixia. For the *vast L995F* gene, all *Anopheles gambiae* and *Anopheles coluzzii* populations showed positive F_{IS} values, indicating a heterozygosity deficit ($F_{IS} > 0$). On the one hand, selection favors homozygous resistant individuals, and on the other, inbreeding is due to preferential mating between individuals of the same genotype. Heterozygous individuals have a lower probability of survival or reproduction than homozygous individuals due to insecticidepressure. These observations were confirmed by the decrease in the frequency of heterozygotes observed in the population **[42]**.Concerning the *ace-1 G280S* gene, the fixation index was globally negative ($F_{IS} < 0$) in *Anopheles gambiae* populations and mostly in *Anopheles coluzzii* populations, indicating an excess of heterozygosity. This excess of heterozygosity could be explained by the high genetic cost resulting in a drop in the frequency of the resistant allele. On the one hand, this could be due to natural selection,which is unfavourable to individuals carrying the mutation, resultingin a reduction in the transmission of the resistant allele to future generations. On the other hand, the low frequency of this mutation inpopulations may also be associated with this phenomenon despite themany years pirimiphos-methyl has been used in IRS. Other environmental factors and the dynamics of selection cannot be ruled out **[43–45]**. In some *Anopheles coluzzii* populations, the fixation index was substantially equal to zero, indicating a population at Hardy-Weinberg Equilibrium for the *ace-1* locus despite the use of pirimiphos-methyl. This result could be due to the higher genetic costin *Anopheles coluzzii* populations compared with *Anopheles gambiae* populations. These results could also be associated with a second- species risk of error.

Genetic differentiation is extreme in all populations of *Anopheles gambiae* and *Anopheles coluzzii* for the *ace-1* gene. This result can be explained by the high genetic cost of this gene, which limits genetic flow between the different populations. This strong differentiation could also be explained by the distance separating the various sub-populations. Other ecological or behavioral factors that have yet to be elucidated could also explain these results. With regard to genetic differentiation at the level of the *vast* gene, there was weak, moderate, and strong differentiation depending on the sub-population pairs considered. The low genetic differentiation observed in 2020 between Kandi and Bembèrèkè ($F_{ST} = 0.019$) and in 2021 between Copargo and Bembèrèkè ($F_{ST} = 0.026$) for *Anopheles gambiae* suggests high gene flow between the sub-populations. It would also be the result of the absence of a genetic barrier, which would lead to random mixing of alleles.

The same findings were observed in 2021 for the *Anopheles coluzzii* sub-populations of Djougou-Bembèrèkè and Copargo-Bembèrèkè ($F_{ST} < 0.001$). After the IRS campaigns with the clothianidin + deltamethrin mixture, there was an overall decrease in the allelic frequency of the *kdr-L995F* mutation for the *Anopheles coluzzii* and *Anopheles gambiae* populations. This decrease in *kdr-L995F* frequency is accompanied by little genetic differentiation in the *Anopheles gambiae* and *Anopheles coluzzii* populations. Thissuggests that the use of the clothianidin + deltamethrin mixture in IRSwould limit the spread of the resistant allele of the *vgsc* gene in *Anopheles gambiae* and *Anopheles coluzzii*. Additional research willenhance our comprehension of how the clothianidin + deltamethrin mixture influences the development of the *kdr-L995F* mutation in various subspecies of the *Anopheles gambiae* complex.

Conclusion

The lower frequency of the *kdr-L995F* mutation in *Anopheles coluzzii*, compared with *Anopheles gambiae*, is attributed to the preference of *Anopheles coluzzii* for permanent breeding sites, dominant during the dry season, characterized by low insecticide pressure in the absence of agricultural activities. Conversely, the elevated frequency observed in *Anopheles gambiae* is linked to the extensive application of pyrethroids and DDT, which are used for crop protection, thus promoting the emergence of resistance. The low frequency of the *G280S* mutation in populations of *Anopheles coluzzii* and *Anopheles gambiae* is explained by the momentary use of carbamates and organophosphates and by the high cost of adaptation of the *G280S* mutation. This high genetic cost could be behind the excess heterozygosity observed in specific populations, reducing the transmission of the resistant allele to future generations. The fixation index reveals a heterozygosity deficit for the *vgsc-L995F* gene, suggesting selection favoring homozygous resistant individuals and inbreeding. The high genetic differentiation for the *ace-1* gene indicates limited gene flow between populations, probably due to the high genetic cost of this gene. The clothianidin + deltamethrin mixture could have a reducing impact (yet to be elucidated) on the frequency of the *kdr-L995F* mutation in *Anopheles coluzzii* and *Anopheles gambiae* populations.

Conflict of interest: The authors state that they do not have any conflicts of interest.

Data archiving

The data on the genotypes of the various individuals by species and by population and their analysis are available and can be consulted by the public. If you have any questions, please write to the corresponding author.

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Authors' contributions

EMO, MA, AD, and CA designed the study. EMO, AD, CA, AJF, RA, BAF, CJA, BY, AAM, AS, RO, ASS, GGP and MA critically revised the manuscript. EMO, GGP, ASS, ASi, AAM, AJF, BAF, CJA BY, RO, ZCK, CSTA, CDK, LT and CA carried out the field activities and the laboratory analysis. EMO, RA, ZCK and AJF analyzed the data. EMO drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval

The study protocol was reviewed and approved by the full membership of the IECC (Grant No. IORG005698).

Abbreviations

ace-1: Acetylcholinesterase-1

CREC : Centre de Recherche Entomologique de Cotonou

IRS: indoor residual spraying

ITNs: Insecticide-treated bed nets

LLINs: Long-lasting insecticidal nets

kdr: knockdown resistance

NMCP: National Malaria Control Program

PCR: Polymerase chain reaction

PMI: U.S. President's Malaria Initiative

s.l.: Sensu lato

USAID: United States Agency for International Development

WHO: World Health Organization

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