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Vector control and genetic structure of *Anopheles gambiae* S.l. populations revealed by the *Kdr* mutation in some agro-ecological areas of high malaria incidence in Benin (West Africa)

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Abstract: The resistance of *An. gambiae s.l.* populations to pyrethroids led by the *L1014F Kdr* mutation is a scientific evidence in Benin. Knowledge of the genetic structure of *Anopheles* populations in high-incidence areas is becoming imperative to identify the evolutionary forces implicated. The study was conducted in 3 agro-ecological zones, each with specific characteristics. The surveyed localities are from the main eco-epidemiological areas of Benin where malaria transmission is high. Thus, larvae of *An. gambiae s.l.* were collected and reared to adulthood at the insectarium of the Centre de Recherche Entomologique de Cotonou. Species identification of the adult

female mosquitoes were performed in a morphological way and, through PCR. The genetic structure of each population was determined via the *L1014F* resistance allele of the *Kdr*.Of the 557 specimens of *An. gambiae s.l.* sampled in the study area, 381 were *An. gambiae s.s.*, 174 *An. coluzzii* and 02 *An. arabiensis*. Overall, the *L1014F Kdr* frequency was high ($p < 0,05$) for all populations of *An. gambiae s.l.* No statistical difference ($p > 0.05$) was observed for the distribution of *L1014F* which appears to be homogeneous within the different species populations analysed by agro-ecological zone. Heterozygous deficiency is general in *An. gambiae s.s.* and *An. coluzzii* populations in the different agro-ecological zones. From low genetic differentiation in all *An. gambiae s.s.* populations, it increased to moderate to significant in Bar Land Zone and Northen Cotton Zone for *An. coluzzii*, respectively.

Keywords: pyrethroids, mosquitoes, *Anopheles arabiensis*, symplesiomorphy,

INTRODUCTION

Background: Malaria is an infectious disease caused by protozoan parasites of the genus *Plasmodium* that is transmitted to humans through the bite of female *Anopheles* mosquitoes [1]. The major malaria vector in Benin is *An. gambiae* s.l., a complex whose the 3 most commonly encountered sibling species are: *Anopheles gambiae* s.s., *Anopheles coluzzii* and *Anopheles arabiensis* [2]. Analyses with DNA coding for ribosomal subunits (rDNA) have identified two molecular forms of *An. gambiae* s.l., formerly known as M and S forms, in West Africa [3, 4]. More recently, the M and S molecular forms have been taxonomically described as distinct species belonging to the *An. gambiae* s.l. complex. They were called Anopheles coluzzii and *Anopheles gambiae* sensu stricto, respectively. Insecticide-based vector control tools such as long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS), remains the most effective tools [6-9]. With the use of different classes of insecticides to control mosquitoes, insecticide resistance in mosquito vectors is becoming increasingly prevalent [10].

Thus, pyrethroid resistance is now widespread in sub-Saharan Africa, particularly in Benin [11-15]. The genetic basis identified in these cases of resistance remains the *Kdr* mutation, whose *L1014F* allele frequency is now increasingly high in cotton-growing and large-scale agriculture areas where chemicals are used intensively. Similar observations were made in Burkina Faso by Diabaté *et al.* [16]. Furthermore, Akogbéto *et al.* [17] reported that several mosquitos' species, in particular *An. gambiae* s.l., lay their eggs in waters located near crop areas. As a result, these eggs are exposed to insecticides during phytosanitary treatments. These same authors also showed that pesticide residues present at ground level during the metamorphosis of the larvae would exert a selection pressure by eliminating susceptible ones [17].

Indeed, according to Reid and McKenzie [18], the use of agricultural pesticides in Benin favored the insecticide resistance selection in *An. gambiae* s.l. From a phylogenetic point of view, this is easy to understand since the character targeted by the action of the pesticide is generally a symplesiomorphy, a trait shared by a large set of living organisms. In addition, exposure to several commonly encountered compounds including heavy metals, phytochemicals and petroleum can induce cross-resistance to insecticides in malaria vectors [19] and taxa that are close to them. Indeed, some studies have concluded

that apart from the *L1014F Kdr* mutation, the overexpression of certain genes of the cytochrome P450 family (Cyp6M2 and Cyp6P3) is involved in mosquito resistance to pyrethroids [20].

In these conditions, where pyrethroids resistance is widespread throughout sub-Saharan Africa [21], studies on the diversity of resistance genes in populations of *Anopheles* will enable the improvement of the tools used to fight malaria. Some studies of population genetics based on the analysis of molecular data have shown that pesticide resistance in insects has a genetic basis [22 - 24]. The authors formally concluded that knowledge of the diversity and genetic structure of vector populations would be an important asset for a better management of resistance to synthetic chemicals.

Kdr mutation is deeply fixed in vector populations in Benin [25]. Despite, the means used elsewhere and in Benin to control the resistance phenomenon, the malaria incidence in certain areas such as Gogounou, Kérou, Kandi, Péhunco, Kouandé, Covè and Zogbodomey remains high [26]. The hypothesis that the change of the genetic structure of the vector populations (increase in the frequency of the *Kdr* mutation in the areas mentioned above), following the misuse of insecticides or insecticide impregnated materials, has caused the fixation of the resistance allele by genetic drift - selection is plausible.

To date, few data on the population genetics of this species in relation to the *L1014F Kdr* mutation are available in Benin [27]. Thus, a deep and detailed knowledge of the genetic structure of vector populations and gene flows within and between resistant populations is necessary to fully understand their biology, and better appreciate the dynamics of resistance developed. This will allow an accurate assessment of the impact of the malaria control measures.

MATERIAL AND METHOD

Study area: The study was carried out in Benin, a West African country located in the intertropical zone between the equator and the Tropic of Cancer, more precisely between the parallels 6°30' and 12°30' latitude North and meridians 1° and 3 ° 40' east longitude.It covers a total area of approximately 114,763 km2. The relief of Benin is not very uneven with a low, sandy coastal area limited by lagoons; a plateau of ferruginous clay and a silico-limestone plateau dotted with some undergrowth [28]. In the northwest, there is the Atacora massif, while in the northeast are the very fertile plains of Niger made up siliceous clay.

South Benin has a subequatorial climate made up of 4 seasons including two rainy alternated by the two dry. The north of the country has a Sudanese climate with a rainy season and a dry season [28] whose duration varies from one area to another. According to the Ministry of Agriculture, Livestock and Fisheries, Benin has eight separate agro-ecological zones [29]. Three of them were surveyed in the present study. These are the Northern cotton zone, South Borgou Food Crop Zone (both located in the northern part of the country) and, the bar land zone which is in the southern part. They were chosen based on the relative homogeneity of their climatic and agro-pedological parameters, their cropping systems, the density of their population and, the plant cover [29]. In total, seven (07) study sites were selected in these three agro-ecological zones (Figure 1). Thus, Covè and Zogbodomey were selected in the bar land zone and, Kouandé and Péhunco in the food-producing zone of South Borgou, Kérou, Kandi and Gogounou were also investigated in the cotton zone of northern Benin. High use of insecticides was performed in all these sites [30] where malaria incidence is also high.

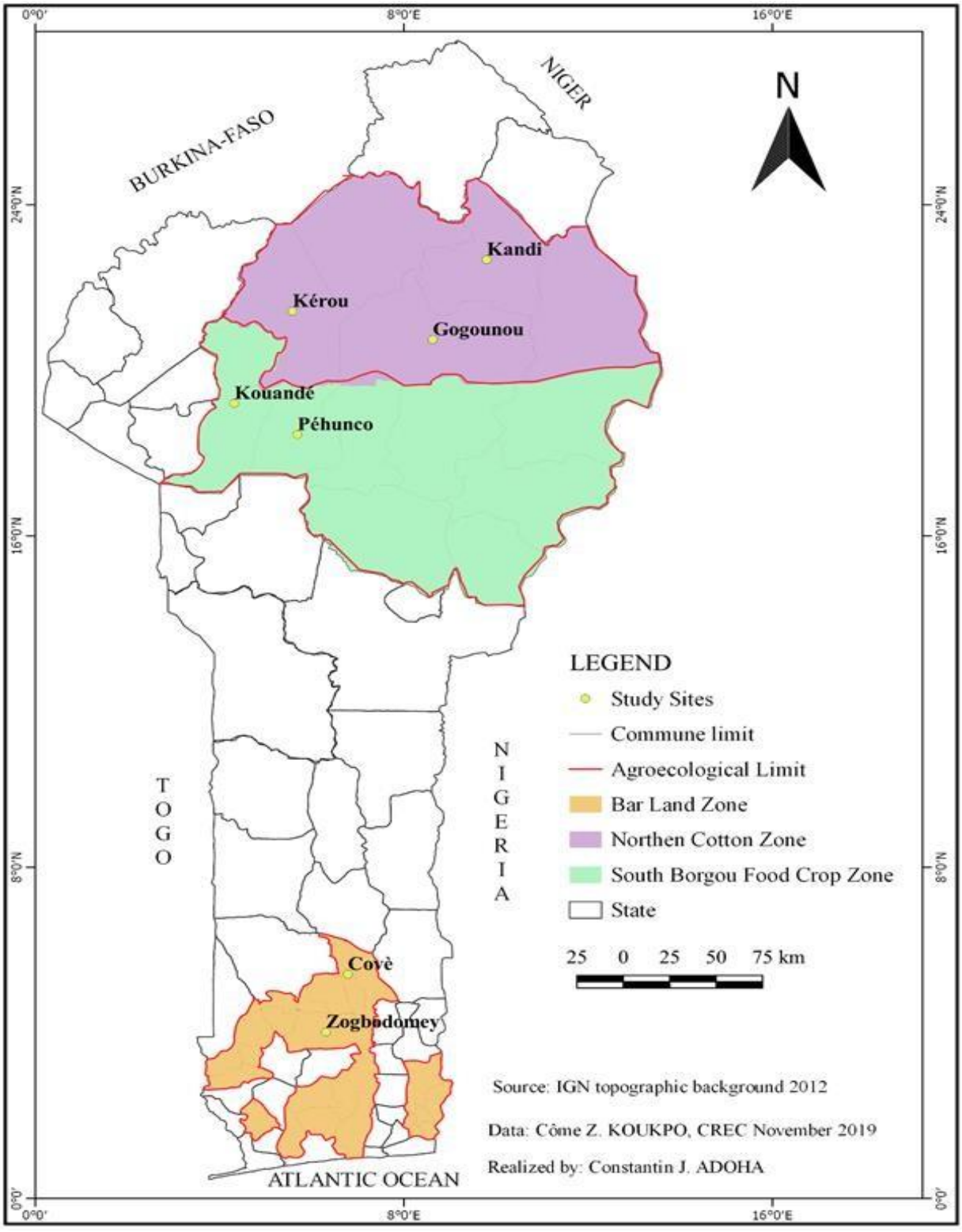

Figure 1: Map of the study sites.

Some characteristics of agro-ecological study areas

South Borgou Food Crop Zone: It covers the Department of Borgou and the Southeast part of the Atacora Department with an area of 23,444 km². In total, seven districts, N'Dali, Nikki, Kalalé, Sinendé, Pehunco, Bembèrèkè and Kouandé are covered by the area. The climate is Sudanian and presents a Sahelian tendency towards the North of the country [29]. As previously reported, the climate is characterized by two seasons, a rainy and, a dry one. The rainy season is from May to September with an annual height of 900 to 1,300 mm [29]. However, the spatio-temporal distribution of precipitation generates marked rainfall deficits in places. Agriculture is the most important economic activity in the area. The cultivation of corn, sorghum, millet and rice is widely practiced there. Besides cereals, the area has developed the cultivation of yams, cassava, cowpeas, peanuts, cotton and cashews, which are important sources of income.

Cotton zone of North Benin: This area bounded in the East by the Federal Republic of Nigeria, in the North by the districts of Malanville and Karimama, in the South by the districts of Kalalé, Bembéréké, Sinendé, Péhunco and Kouandé and, in the West by the district of Tanguiéta and part of the Republic of Burkina-Faso [29]. It covers an area of 20,930 km² that includes the districts of Banikoara, Kérou, Kandi, Ségbana and, Gogounou of which approximately 56% is cultivable land. It occupies 18% of the national territory. The climate is Sudanese.

The area also has two seasons, a dry and a rainy one. The rainy season normally spread over a six-month period (April to October). April rains are sometimes rare. Thus, in the best of cases, the rainy season does not really set in until May. Average annual rainfall varies from 800 to 1,200 mm [29]. But nowadays annual rainfall hardly exceeds 1,000 mm. The area is crossed by tributaries of the Niger River (Mékrou, Alibori and Sota), as well as rivers that drain their waters there. The predominant vegetation is a tree savannah that has been greatly degraded by human influence, gradually becoming a shrubby savannah. Agriculture is very developed there with a predominance of cotton cultivation which represents 38% of production. The attractiveness of farmers in the area for cotton is reinforced by the availability of inputs which are also used for other crops, especially cereals. The area also produces maize, sorghum, rice, yams and legumes (peanuts and cowpeas).

Bar land zone: It is so called, taking into account the type of soil (barro) made up of sandy clay in the wet state. It covers an area of 10,500 km² and includes more than twenty districts. The climate is sudano-guinean with two rainy and two dry seasons. The rainfall generally recorded in the area varies between 800 and 1,400 mm per year. The expansion of agriculture is limited by the structure of the soil that is characterized by a low water retention capacity, thus posing risks of water stress for annual crops. The hydrography is marked by a normal drainage profile and the presence of rivers and shallows. Plant production includes corn, peanuts, cowpeas, cassava, yams, taro, peppers, coffee, cotton, fruit trees (mango, citrus fruits, pineapples), oil palm and market garden crops [29]. Private irrigation initiatives from artesian boreholes or from rivers are developing for the off-season production of vegetable and rice crops.

Collection of mosquito larvae and rearing: Mosquito vector larvae were collected from all study sites during the rainy season (April - November 2017). These collections of larvae of *An. gambiae* s.l. were carried out in the natural mosquito breeding sites using a ladle. Once sampled, the larvae were filtered

and stored in labelled pots. They were then transported to the CREC insectary where they were reared until adulthood at 27-29˚C (temperature) and 60-90% (relative humidity).

Morphological and molecular identification of mosquito species: After the emergence of the collected larvae, the adult female mosquitoes were identified using the morphological identification keys of Gillies & Coetzee [31] and, Gillies & De Meillon [32]. The morphologically identified individuals went through the PCR technique for molecular species identification. Thus, Anopheles DNA were extracted by grinding the whole mosquitoes in 200 µl of 2% CTAB. After 5 minutes in a water bath at 65 °C, they were mixed with 200 µl of chloroform, then centrifuged at 14,000 rounds per minute (rpm) for the same duration.

The supernatant was gently collected in another tube containing 200 µl of isopropanol, then centrifuged at 12,000 rpm for 15 minutes. The pellet was purified with 200 µl of 70% ethanol. The whole was again centrifuged at 14000 rpm for 5 minutes. The contents of the tube were gently inverted to keep the pellet on the bench for at least 3 hours so that it dried. Finally, 20 µl of bi-distilled water was added to the pellet left in suspension on the bench for the whole night or half a day. The SINE 200 PCR protocols of Santolamazza *et al*. [33] and Scott *et al*. [34] were used for a complete identification of the molecular species of the *An. gambiae* s.l. complex.

Detection of the *L1014F* allele of the *Kdr* mutation in the molecular species: The *L1014F* allele has been identified as the resistant molecular form of the *Kdr* mutation and, has been detected in wild individuals using the PCR protocol of Martinez-Torres *et al*. [35].

It is a diagnostic test by Allelic Specific Amplification Polymerase - PCR (PASA - PCR) whichconsists in using four oligonucleotides or primers called Agd1, Agd2, Agd3, Agd4 and Taq polymerase to search by amplification, resistant or susceptible alleles from the DNA fragment coding for the voltage-gated sodium channel (vgsc) in each mosquito tested.

The Agd1 / Agd2 primer pair flanks the *Kdr* mutation by amplifying a 293 bp product as a control. The Agd3 / Agd1 primer pair only mates with the resistance portion of the *Kdr* mutation to selectively amplify a 195bp fragment. The Agd4 / Agd2 pair associates only with the susceptible portion of the mutation, by amplifying a 137 bp fragment. The nucleotide sequences of these primers described by these authors are as follows:

Agd1 : 5'-ATAGATTCCCCGACCATG-3' ;

Agd2 : 5'-ACAAGGATGATGAACC-3' ;

Agd3 : 5'-AATTTGCATTACTTACGACA-3' ;

Agd4 : 5'-CTGTAGTGATAGGAAATTTA 3'.

Analysis of the genetic structure of the *L1014F* allele of the *Kdr* mutation by agro-ecological zone for each molecular species: To better interpret the results, all individuals of a same species from an agro-ecological zone were considered to be a population while, those of a same site were considered as a sub-population. For a better understanding of the genetic structure of the populations of *An. gambiae* s.l., the following analyzes were carried out.

The *L1014F Kdr* allelic frequencies were determined and its distribution was compared within and between the populations of each species with the proportions comparison test using the R software. Also, after determining the mutation frequency with the Genepop software version 4.2, the observed heterozygosity (H_o) and that expected (H_e) were calculated for each species population sampled.

In addition, the fixation index (F_{IS}) provides information on the degree of inbreeding within populations. It quantifies the deviation from panmixis. It has been shown that, when F_{IS} <0, the population considered has an excess of heterozygous.

By contrast, if F_{IS}> 0, the population has an heterozygous deficit. In this case, several factors among which the Wahlund effect, inbreeding, genetic drift, selection in favor of homozygous genotypes or their combination, could be at stake. To have a clear idea on the evolution of the populations, F_{IS} were calculated using Genepop software version 4.2. [36].

Based on the same principle, the F_{ST} differentiation index expresses the decrease in heterozygosity linked to the divergence within the populations considered. For that, $F_{ST} = (H_T - H_S) / H_T$. So, it is therefore the parameter of genetic divergence within populations [36].

If the F_{ST} equals to or is very close to 0, this means that there are high genetic exchanges between populations (little genetic differentiation: panmictic population). Conversely, if the F_{ST} is close to 1, this results in strong genetic differentiation between populations.

This suggests very little or no gene flow between populations and reflects the existence of organisms of a higher taxonomic level which could be treated as separate species. To interpret this index, the criteria defined by Wright (1969) [37] were used. Thus, when the F_{ST}:

- is between 0 and 0.05, the differentiation is weak.

-ranges from 0.05 to 0.15, the differentiation is moderate

-varies from 0.15 to 0.25, there is a differentiation

-is beyond 0.25 the differentiation is very significant.

RESULTS

Molecular species identification: Out of the 557 specimens of *An. gambiae* s.l. analyzed, 381 *An. gambiae* s.s., 174 *An. coluzzii* and, only 02 *An. arabiensis* were found. The species were in variable proportions according to the agro-ecological zones **(Table 1).** Of the 247 specimens analyzed in the Northern Cotton Zone, 175 were *An. gambiae* s.s., 70 *An. coluzzii* and, 02 *An. arabiensis*. In the South Borgou Food Crop Zone, 114 *An. gambiae* s.s. and 48 *An. coluzzii* were detected among the 162 specimens of *An. gambiae s.l.* tested. Finally, in the Bar Land Zone, among the 148 individuals tested, 92 were *An. gambiae* s.s. and the remaining 56 were *An. coluzzii*. Overall, *An. gambiae* s.s. was the predominant species in the different agro-ecological zones.

Table 1: Number of *An. gambiae s.s.*, *An. coluzzii* and *An. arabiensis* found in the surveyed agro-ecological zones

Agroecological Zones	Localities	Geographical coordinates	Number tested	*Anopheles gambiae s.s.*	*Anopheles coluzzii*	*Anopheles arabiensis*
Northen Cotton Zone	Kérou	10°58'19.614N ; 1°59'54.034E	72	48	24	0
	Kandi	11°5'10.759N ; 2°45'33.401E	78	58	20	0
	Gogounou	10°50'21.643N ; 2°50'6.172E	97	69	26	2
	Total		**247**	**175**	**70**	**2**
South Borgou Food Crop Zone	Kouandé	10°25'55.873N ; 1°46'58.065E	78	58	20	0
	Péhunco	10°13'51.221N ; 2°0'1.541E	84	56	28	0
	Total		**162**	**114**	**48**	**0**
Bar Land Zone	Covè	6°21'53.381N ; 2°27'51.926E	88	56	32	0
	Zogbodomey	7°1'2.245N ; 2°12'11.036E	60	36	24	0
	Total		**148**	**92**	**56**	**0**
Total			**557**	**381**	**174**	**2**

The very low number of *An. arabiensis*, statistically insignificant and unrepresentative caused that the species was no longer considered in further analyses.

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Frequency of the *L1014F Kdr* mutation in each molecular species

Tables 2a and 2b show frequencies of the *L1014F Kdr* mutation in *An. gambiae* s.s. and *An. coluzzii* in the vector populations of the different agro-ecological zones. Overall, the values obtained are very significant ($P < 10^{-3}$).

In *An. gambiae* s.s., the frequency varied from 86.86% to 89.13% in the different agro-ecological zones **(Table 2a).** It was 86.86%, 87.29% and 89.13% respectively in the Northen Cotton Zone, the South Borgou Food Crop Zone and, the Bar land Zone.

Also, for *An. coluzzii* vectors populations, it varies from 82.14 to 88.54% between the different agro-ecological zones **(Table 2b).** They are respectively 82.14%, 88.54% and 87.50% in the Northen Cotton Zone, South Borgou Food Crop Zone and Bar land Zone.

Finally, no significant difference was observed in general in the distribution of the *L1014F* allele, which remained almost homogeneous across the Northen Cotton Zone, South Borgou Food Crop Zone and Bar Land Zone populations ($P > 0.05$) for all species **(Table 2c).**

Genetic structure of *Anopheles gambiae s.l.* via the *L1014F* allele of the *Kdr* gene by agro-ecological zone in each species studied: Heterozygosis deficiency, fixation index and Hardy-Weinberg balance: Table 3 shows the variation in heterozygosity observed and that expected in the different populations of the different agro-ecological zones. In addition, it expresses the variations in the fixation index within different vector populations.

In the populations of *An. gambiae* s.s., the observed heterozygosity (H_o) varied from 0.1140 to 0.1739. On the other hand, the expected heterozygosity varied from 0.1948 to 0.2290 within the same populations. The analysis of the data collected within shows a highly significant deficit in heterozygosity in some vector populations. These expected heterozygosity values (H_e) show that certain populations, in this case those of the Northern Cotton Zone and the South Borgou Food Crop Zone have deviated from the Hardy - Weinberg equilibrium ($p < 0.05$) . On the other hand, the vector population of *An. gambiae s.s.* of the Bar Land Zone presented a certain panmixy ($p > 0.05$) **(Table 3).**

The F_{IS} (fixation index) estimate provides additional information on the degree of inbreeding of individuals from crosses between directly related individuals within populations in agro-ecological zones. This index varied from 0.1078 to 0.4898 in *An. gambiae s.s.* It is precisely 0.3518, 0.4898 and 0.1078 respectively in the Northern Cotton Zone, South Borgou Food Crop Zone and Bar Land Zone for the same populations of *An. gambiae s.s.* These positive and high F_{IS} values confirm the deficit in observed heterozygous as above.

The same analyzes carried out in the populations of *An. coluzzii* show that the observed heterozygosity (H_o) varied from 0.1042 to 0.2143. In addition, the expected heterozygosity varied from 0.1544 to 0.2955 in the same populations. A comparison of the observed and expected heterozygoties by agro-ecological zone reveals a deficit of highly significant heterozygosity in most of these populations. Also, the expected heterozygosity values (H_e) obtained showed that all these populations of *An. coluzzii* deviated from the Hardy - Weinberg equilibrium ($p < 0.05$) **(Table 3).** The F_{IS} (fixation index) provides information on the degree of inbreeding of individuals from crosses between directly related individuals within agro-ecological zones. It varied from 0.2762 to 0.5168 for the populations of *An. coluzzii*. It is precisely 0.2762, 0.4629 and 0.5168 respectively in the Northern Cotton Zone, South Borgou Food Crop Zone and Bar Land Zone for the same *An. coluzzii* populations. Positive and high F_{IS} confirm the deficit in observed heterozygous as above.

Table 2a: Frequency of the *L1014F Kdr* mutation in *Anophles gambiae s.s.* species in the three agro-ecological zones

Agroecological Zones / Localities	N	*Anopheles gambiae s.s*			F*(L1014F)%*	p-value
		N_1 *L1014F/L1014F*	N_2 *L1014F/L1014L*	N_3 *L1014L/L1014L*		
Northern Cotton Zone						
Kérou	48	35	10	3	83.33	< 0,001
Kandi	58	50	6	2	91.38	< 0,001
Gogounou	69	54	10	5	85.51	< 0,001
Total	**175**	**139**	**26**	**10**	**86.86**	< 0,001
South Borgou Food Crop Zone						
Péhounco	56	47	5	4	88.39	< 0,001
Kouandé	58	46	8	4	86.21	< 0,001
Total	**114**	**93**	**13**	**8**	**87.28**	< 0,001
Bar Land Zone						
Covè	56	48	8	0	92.86	< 0,001
Zogbodomey	36	26	8	2	83.33	< 0,001
Total	**92**	**74**	**16**	**2**	**89.13**	< 0,001

F(*L1014F*): percentage frequency of the *L1014F* allele of the *Kdr* gene within each population of *Anopheles gambiae s.s.* N represents the total number of each population; N1 is the number of the homozygous *L1014F/L1014F* resistant genotype within each population; N2 is the number of the heterozygous *L1014F/L1014L* genotype within each population; N3 represents the membership of the homozygous susceptible genotype *L1014L/L1014L* within each population; Genotypes *L1014F/L1014F* and *L1014F/L1014L* are of resistant phenotype while genotype *L1014L/L1014L* are of susceptible phenotype.

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Table 2b: Frequency of the *L1014F* Kdr mutation in *Anopheles coluzzii* species in the three agro-ecological zones

Agroecological Zones / Localities	N	*Anopheles coluzzii*			F*(L1014F)%*	p-value
		N_1 *L1014F/L1014F*	N_2 *L1014F/L1014L*	N_3 *L1014L/L1014L*		
Northern Cotton Zone						
Kérou	24	14	6	4	70.83	0,0639
Kandi	20	18	2	0	95.00	< 0,001
Gogounou	26	18	7	1	82.69	< 0,001
Total	**70**	**50**	**15**	**5**	**82.14**	**< 0,001**
South Borgou Food Crop Zone						
Péhounco	28	22	4	2	85.71	< 0,001
Kouandé	20	18	1	1	92.50	< 0,001
Total	**48**	**40**	**5**	**3**	**88.54**	**< 0,001**
Bar Land Zone						
Covè	32	30	**2**	0	96.87	< 0,001
Zogbodomey	24	16	4	4	75.00	< 0,001
Total	**56**	**46**	**6**	**4**	**87.50**	**< 0,001**

F(*L1014F*): percentage frequency of the *L1014F* allele of the *Kdr* gene within each population of *Anopheles coluzzii*. N represents the total number of each population; N1 is the number of the homozygous *L1014F/L1014F* resistant genotype within each population; N2 is the number of the heterozygous *L1014F/L1014L* genotype within each population; N3 represents the membership of the homozygous susceptible genotype *L1014L/L1014L* within each population; Genotypes *L1014F/L1014F* and *L1014F/L1014L* are of resistant phenotype while genotype *L1014L/L1014L* are of susceptible phenotype

Tableau 2c : Comparison of the *L1014F Kdr* mutation between *Anopheles gambiae s.s.* et *Anopheles coluzzii* in each agro-ecological zone.

Agro-ecological zones	*An. gambiae s.s.* F*(L1014F)%*	*An.coluzzii* F*(L1014F)%*	P-value
Northern Cotton Zone	86.86 ± 3.35	82.14 ± 5.96	0.2312
South Borgou Food Crop Zone	87.28 ± 4.03	88.54 ± 5.60	0.8965
Bar land Zone	89.13 ± 4.10	87.50 ± 5.49	0.8113

Table 3: Number of observed (H_o) and expected (H_e) heterozygous, the inbreeding index F_{IS} (F_{IS} next [36]) and the Hardy-Weinberg balance P-value (P-value (HWE)) by agro-ecological zone

Agro-ecological Zones	*An. gambiae s.s.*			*An. coluzzii*		
	H_o (H_e)	F_{IS} (W&C)	P-value (HWE)	H_o (H_e)	F_{IS} (W&C)	P-value (HWE)
Northern Cotton Zone	0.1486 (0.2290)	0.3518	0.0000	0.2143 (0.2955)	0.2762	0.0326
South Borgou Food Crop Zone	0.1140 (0.2230)	0.4898	0.0000	0.1042 (0.1544)	0.4629	0.0240
Bar land Zone	0.1739 (0.1948)	0.1078	0.2798	0.1071 (0.2207)	0.5168	0.0025

Genetic differentiation: The F_{ST} genetic differentiation index is calculated for each population at each hierarchical level (Table 4). It provides information on the degree of gene flow. For the populations of *An. gambiae s.s.*, it varied from -0.011 to 0.041, precisely at 0.041 in the Northen Cotton Zone, -0.011 in the South Borgou Food Crop Zone and 0.034 in the Bar Land Zone for the populations of *An gambiae s.s*. Low differentiation is noted for the populations of *An. gambiae s.s.*

In the populations of *An. coluzzii*, it varied from -0.010 to 0.175, specifically at 0.066 in the Northen Cotton Zone, -0.010 in the South Borgou Food Crop Zone and, 0.175 in the Bar Land Zone. Thus, moderate and significant genetic differentiations were observed respectively in the populations from the Northen Cotton Zone and the Bar Land Zone. In contrast, a weak differentiation was observed in the population South Borgou Food Crop Zone.

Table 4: F_{ST} by agro-ecological zone population

Agro-ecological zones	*An. gambiae s.s.*	*An. coluzzii*
	F_{ST}	F_{ST}
Northen Cotton Zone	0,041	0,066
South Borgou Food Crop Zone	-0.011	-0.010
Bar land Zone	0,034	0,175

DISCUSSION

The results of this study show the presence of three sibling species of the *An. gambiae s.l.* complex, distributed in variable proportions depending on the study areas. These are *An. gambiae s.s.*, *An. coluzzii* which are strongly represented and An. arabiensis which has only been found in the Northen Cotton Zone, mainly in the Gogounou site. Of the 2 highly distributed species, *An. gambiae s.s*. was in majority regardless of the agro-ecological zone. This heterogeneous distribution of both populations and species might be due to climatic conditions which are quite variable within the same agro-ecological zone but also between them. The ecological characteristics of the different agro-ecological zones, the physico-chemical properties of the mosquito breeding sites, as well as the different sampling periods could also be significant factors justifying the observed differences.

Larvae collections were carried out over the rain season during which several temporary breeding sites were subservient to *An. gambiae s.s.* [38]. The very low number of *An. arabiensis* found in Gogounou in the Northen Cotton Zone is linked to the less and less favorable living conditions for this species in the northern part of the country. A recognized savannah species which is nowadays highly threatened due to the extensive development of cotton cultivation and dizzying urbanization. These would favor the destruction of its habitat and, its likely displacement towards the southern part as it was previously noted by Gnanguènon *et al*. [39] and Koukpo *et al*. [40].

The *L1014F* resistance allele of the *Kdr* mutation is geographically distributed in significantly high proportions ($p < 0.05$) in all populations vector species. The distribution of this allele is similar ($p > 0.05$) in both species per agro-ecological zone. Recent works in Benin [40-42] have clearly mentioned the expansion and tendency of the *L1014F* allele to bind the *Kd*r mutation in the *Anopheles* populations. This is certainly comparable to the direct consequence of the control strategies developed both in

agriculture and in public health. Nkya *et al.* [19], Akogbéto *et al.* [12] and Yadouléton *et al.* [14, 43] have demonstrated in their various works that agricultural practices exert a selection pressure on populations of malaria vectors by promoting the expansion of resistant alleles associated. From these results, it appears that the universal coverage of pyrethroid treated nets is a major factor in the selection of resistance alleles. Although the use of these products in agriculture cannot be avoided, it can be severely restricted in certain cases in order to limit their impact on the biology of organisms. To replace these insecticides, many alternatives (spinosad, indoxacarb, growth regulators) usable in agriculture as well as, other protection strategies (organic cotton, genetically modified varieties) could have been introduced for a much more effective fight against pests.

The deficit in heterozygosity observed in most populations of *An. gambiae s.s.* and *An. coluzzii* from our agro-ecological zones for the *L1014F* locus would mean that there are more resistant homozygous than expected in the studied populations. This is due to the fact that the *L1014F* allele is highly selected in Benin because of the pressures due to the insecticide based tools used for the control of vector and crop pests. This would mean that susceptible homozygous are the most disadvantaged of these environments. Only bar land zone population of *An. gambiae s.s.* was in Hardy-Weinberg equilibrium ($p > 0.05$), whereas those in other agro-ecologicals zones for both species are not. This observed balance may also be linked to the size of the population used for data analysis and the marker used or, the migration of individuals promoting gene flow and random crossings.

In addition, the significant and greater than zero F_{IS} values obtained in the *Anopheles* populations of the two species confirm the general deficit of heterozygosity in the populations of the selected agro-ecological zones. That suggests a strong influence of inbreeding within the studied populations. This phenomenon is also due to the widespread selection of this locus in the different species [40, 43] by the action of pyrethroids on mosquitoes which eliminate susceptible individuals while leaving the resistant ones. The resistant ones, by preferential matings linked to the limited size of the population and inbreeding, will transmit almost exclusively the resistance allele to their offsprings, thus ensuring the expansion of this genetic mutation within the populations.

The physico-chemical parameters of the environment could be justifying arguments because they are also well correlated with the capacity for bioaccumulation of organisms [19, 38] by giving thempopulation dynamics and good adaptability. Besides chance, three main factors could explain this situation. These are genetic factors, the existence of null alleles and the Wahlund effect [45]. With regard to genetic causes, it is well known that inbreeding (mating between an individual and his ancestors, his collaterals and / or his offspring) modifies the genotypic frequencies. The consequence is a loss of genetic variability over several generations. The second factor could be inherent to the existence of null alleles [46]. A mutation in the flanking sequences of the gene could lead to the presence of null alleles. Finally, the last factor refers to the presence of sub-populations within each population which can induce the Wahlund effect.

The values of the differentiation index obtained in our study clearly express the level of genetic differentiation within subpopulations of the different populations of *An. coluzzii* and *An. gambiae s.s.* for the *L1014F* allele in the different agro-ecological zones. Poor gene differentiation has been noted in the populations of *An. gambiae s.s.* irrespective of the zone.

This suggests there is no difference between the vector populations of *An. gambiae s.s.*, probably due to the many gene flows between them as there are no ecological barriers between the populations of our agro-ecological zones. Similar results were obtained by Gelin *et al.* [47] and Fassinou *et al.* [27]. It would therefore not be delusive to think that the vector control tools put in place in Benin had relatively no impact on the genetic structure of the populations of *An. gambiae s.s.* However, we have not lost sight

of their impact in the general selection of *L1014F Kdr* mutation. Wondji *et al*. [48], by studying the impact of the use of impregnated mosquito nets on the genetic structure in *An. arabiensis* made a similar observation. One possible explanation for this result is that the residual population (after the effectiveness period of the tools put in place) is quite large.

Even if vector control tools induce high mortality in mosquitoes, the fairly large size of the residual population and the relatively long time that has elapsed between the implementation of interventions and sampling would allow to capture almost all the genetic diversity of this population. If this lack of structure is confirmed on a larger scale (the whole Benin for example), then the total and rapid expansion of the genes of interest (resistance genes for example) is possible within these vectors. It is therefore important to check this absence of structuration on a larger scale via microsatellites and to monitor resistance in this mosquito.

Furthermore, with regard to the populations of *An. coluzzii*, low, moderate and high gene differentiations, were observed in the South Borgou Food Crop Zone, the Northern Cotton Zone and the Bar Land Zone respectively. The moderate differentiation observed in the Northern Cotton Zone would be due to gene flows which obviously that differed among subpopulations in this area. They are perhaps a little more isolated from each other. However, the genetic structure observed in the population of *An. coluzzii* of the Bar Land Zone can be explained by a certain tendency of presence of barrier between the subpopulations (localities) of this kind or, linked to an error at the start of structuring. The strong selection of the marker used may not provide sufficient information on the genetic structure of the populations at different levels.

The marker used in the study can also influence the types of differentiation obtained. It is reasonable to consider exploring other tools such as microsatellites and sequencing to better study the genetic structure of these species. Now, the question raised, is now to know what is the level of expression of the other mechanisms of resistance of vectors to insecticides and what is the structure of its genes? To better clarify these fundamental concerns, ecological studies linking demography and population dynamics prove necessary or even essential for a better appropriation of the mechanisms of resistance. This will help considering more adequate strategies for a reasoned integrated fight against vectors based on biological, ecological and geographic characteristics.

CONCLUSIONS

The results of this study show the low frequency or the absence of *An. arabiensis* in areas which in the meantime were favorable. *An. gambiae s.s.* was the major species of the *An. gambiae s.l.* complex. Also, we note exchanges of *Kdr* gene flows within each subpopulation of *An. gambiae s.s.* in the different agro-ecological zones. Overall, there is no structure for the populations of *An. gambiae s.s.* in most agro-ecological zones, while moderate or even significant differentiation is noted in those of the Northern Cotton Zone and Bar Land Zone for *An. coluzzii*. To confirm these initial results, genetic monitoring of the studied populations over several years is necessary, to study their dynamics. This will enable to prioritize more effective vector control strategies. Subsequent studies will be carried out using molecular tools such as microsatellites to deepen the knowledge of the population structure of these malaria vectors in Benin.

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REFERENCES

1. M.Panis M. Mouchet, T.Giacomini, Paludisme autochtone et introduit en Europe. *Med Mal Infect*. 1996, 26: 393-6.
2. I.Djegbe, A.A.Missihoun, R.Djouaka, M.Akogbéto, Surveillance Entomologique : Dynamique de la population et de la résistance aux insecticides chez *Anopheles gambiae s.l* en milieu de riziculture irriguée au Sud Bénin. *J Appl Biosci*. 2017, 111: 10934-943.
3. G.Favia , A.Della Torre , M. Bagayoko , A. Lanfrancotti , N. Sagnon, Y.T.Toure, M.Coluzzi, Molecular identification of sympatric chromosomal forms of *Anopheles gambiae* and further evidence of their reproductive isolation. *Insect Mol Biol*. 1997, 6(4):377–83.
4. G.Favia, A.Lanfrancotti, L. Spanos L, Sidén-Kiamos, C. Louis, Molecular characterization of ribosomal DNA polymorphisms discriminating among chromosomal forms of *Anopheles gambiae s.s*. *Insect Mol Biol*. 2001, 10(1):19–23
5. M. Coetzee, R.H.Hunt, R. Wilkerson, A. Della Torre, M.B. Coulibaly, N.J.Besansky, *Anopheles coluzzii* and *Anopheles amharicus*, new members of the *Anopheles gambiae* complex. *Zootaxa*. 2013, 3619(3):246–74
6. WHO. World Malaria Report 2015. Geneva: World Health Organization, 2015.
7. L. Kelly-Hope, H. Ranson, J. Hemingway, Lessons from the past: managing insecticide resistance in malaria control and eradication programmes. *Lancet Infect Dis*. 2008, 8:387-9.
8. WHO. World Malaria Report 2012. Geneva, Switzerland: World Health Organization, Global Malaria Programme; pp 259.
9. P. Nwane, J. Etang, M. Chouaibou, J.C.Toto, C. Kerah-Hinzoumbé , R. Mimpfoundi, H.P. Awono-Ambene, F. Simard, Trends in DDT and pyrethroid resistance in *Anopheles gambiae ss* populations from urban and agro-industrial settings in southern Cameroon. *BMC Infect Dis*. 2009, 9: 163.
10. J. Hemingway, N.J.Hawkes, L. Mccarroll, H.Ranson, The molecular basis of insecticide resistance in mosquitoes. *Insect Bioch Mol Biol*. 2004, 34:653-65.
11. J.M.Vulule, R.F.Beach, F.K. Atieli, J.M. Roberts, D.L. Mount, R.W.Mwangi, Reduced susceptibility of *Anopheles gambiae* to permethrin associated with the use of permethrin-impregnated bednets and curtains in Kenya. *Med Vet Entomol*. 1994, 8:71-5.
12. M.Akogbéto, S.Yakoubou , [Resistance of malaria vectors to pyrethroids used for impregnated bednets, Benin, West Africa] (in French). *Bull Soc Pathol Exot*. 1999, 92:123-30.
13. V. Corbel, R. N'Guessan, C. Brengues, F. Chandre, L. Djogbenou, T. Martin, M.C. Akogbeto,J.M. Hougard , M. Rowland, Multiple insecticide mechanisms in *Anopheles gambiae* and *Culex quinquefasciatus* from Benin, West Africa. *Acta Trop*. 2007, 101:207-16.
14. A.W.Yadouleton, A. Asidi, R.F. Djouaka, J. Braima, C.D. Agossou, M.C.Akogbéto, Development of vegetable farming: a cause of the emergence of insecticide resistance in populations of *Anopheles gambiae* in urban areas of Benin. *Malar J*. 2009, 8:103.
15. F. Chandre, F. Darrier, L. Manga , M. Akogbeto, O. Faye , J. Mouchet, P. Guillet, Status of pyrethroid resistance in *Anopheles gambiae sensu lato*. *Bull World Health Org*.1999, 77: 230–34.

16. A. Diabaté, T. Baldet, F. Chandre, M. Akogbéto, T.R.Guiguemba, F. Darriet, C. Brengues, P. Guillet, J. Hemingway , G.J. Small, J.M. Hougard, The role of agricultural use of insecticides in resistance to pyrethroids in *Anopheles gambiae s.l.* in Burkina Faso. *Am J Trop Med Hyg*. 2002, 67: 617-22.

17. M.C.Akogbeto, R. Djouaka, H. Noupko, Utilisation des insecticides agricole au Bénin. *Bull Soc Pathol Exot*. 2005, 98(5):400–5.

18. M.C.Reid, and F.E. McKenzie, The contribution of agricultural insecticide use to increasing insecticide resistance in African malaria vectors. *Malar J*. 2016, 15:107.

19. T.E. Nkya, I. Akhouayri, R. Poupardin, B. Batengana, F. Mosha, S. Magesa, W. Kisinza,J.P. David,. Insecticide resistance mechanisms associated with different environments in the malaria vector *Anopheles gambiae*: a case study in Tanzania. *Malar J*. 2014, 13: 28.

20. R.F. Djouaka, A.A. Bakare, O.N.Coulibaly, M.C.Akogbeto, H. Ranson , J. Hemingway, C.Strode, Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of *Anopheles gambiae s.s.* from Southern Benin and Nigeria. *BMC Genomics*. 2008, 9:538.

21. H. Ranson , R. N'Guessan , J. Lines , N. Moiroux ,Z. Nkuni, V. Corbel, Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? *Trends Parasitol*. 2011, 27:91–8.

22. C. Ndo , C. Antonio-Nkondjio, A. Cohuet, D. Ayala,P. Kengne, I. Morlais, P.H.Awono-Ambene, D. Couret, P. Ngassam , D. Fontenille and F. Simard , Population genetic structure of the malaria vector *Anopheles nili* in sub-Saharan Africa. *Malar J*. 2010, 9:161

23. W.T.Tan , Z.M.Wang, C.X.Li , H.L.Chu, Y. Xu , Y.D.Dong, Z.C.Wang, D.Y.Chen, H. Liu, D.P. Liu , N. Liu, J. Sun and T. Zhao, First Report on Co-Occurrence Knockdown Resistance Mutations and Susceptibility to Beta-Cypermethrin in *Anopheles sinensis* from Jiangsu Province, China. *PloS One*. 2012, 7 (1): 29292. doi: 10.1371

24. C. Yang, X. Feng , Z. Huang, M. Li and X.Qiu, Diversity and frequency of kdr mutations within *Anopheles sinensis* populations from Guangxi. *Malar J*. 2016, 15:411.

25. T.G.E.Davies, L.M. Field, P.N.Usherwood, M.S.Williamson, A comparative study of voltage-gated sodium channels in the Insecta: implications for pyrethroid resistance in Anopheline and other Neopteran species. *Insect Mol Biol*. 2007, 16(3) : 361–75.

26. Ministère de la Santé. Annuaire des statistiques sanitaires 2016. Cotonou: Direction de la Programmation et de la Prospective, 2017

27. A.J.Y.H. Fassinou, Z.C. Koukpo, R.A. Ossè, F.R. Agossa, B.S Assogba, A. Sidick, W.T. Sèwadé, M.C. Akogbéto, M. Sèzonlin, Genetic structure of *Anopheles gambiae s.s* populations following the use of insecticides on several consecutive years in southern Benin. *Trop Med Health*. 2019, 47:23.

28. K.S.Adam, M. Boko, Le Bénin-Nouvelle édition mise à jour. Cotonou : Editions du Flamboyant, Paris : EDICEF, 1993, 95.

29. Rapport national sur le développement humain. Agriculture, Sécurité alimentaire et Développement Humain au Bénin, 2015. p. 54-8.

30. Ton P. La production du coton au Bénin. 2004. http://www.slire.net/download/1889/la_production_du_coton_au_b_nin.pdf. Accessed 13 Oct 2018.

31. M.T.Gillies, M.A.Coetzee, Supplement to the Anophelinae of Africa South of the Sahara (Afrotropical Region). *Publ South Afr Inst Med Res*. 1987, 1987:55.

32. M.T.Gillies, D. De Meillon, the Anophelinae of Africa South of the Sahara (Ethiopian Zoogeographical Region). *Publ South Afr Inst Med Res*.1968, 54:1-343.

33. F. Santolamazza, E. Mancini, F. Simard, Y. Qi, Z. Tu , A. Della Torre, Insertion polymorphisms of SINE200 retrotransposons within speciation islands of *Anopheles gambiae* molecular forms. *Malar J*. 2008, 7: 163.

34. J.A.Scott, G.W.Broodon, H.F.Collins, Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. *Am J Trop Med Hyg*. 1993, 49(4): 520–29.

35. D. Martinez-Torres, F. Chandre, M.S.Williamson, F. Darriet, J.B.Berge, A.L. Devonshire, P. Guillet , N. Pasteur, D. Pauron, Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae s.s*. *Insect Mol Biol*. 1998, 7(2):179–84.

36. B.S.Weir and C.C.Cockerham, Estimating F-statistics for the analysis of population structure. *Evolution*. 1984, 38(6):1358–70.

37. S.Wright, the Theory of Gene Frequencies, Evolution and the Genetics of Populations. *Publ University of Chicago Press*. 1969, 2.

38. A. Mbida Mbida, J. Etang, P. Akono Ntonga, C. Eboumbou Moukoko, P. Awono-Ambene, D. Tagne, A. Talipouo , W. Ekoko, J. Binyang, R. Tchoffo, G. Lehman, R. Mimpfoundi, New Insight into *Anopheles coluzzii* Coetzee & Wilkerson, 2013 larval ecology in the Wouri estuary, Littoral-Cameroon. *Bull Soc de Pathol Exot*. 2017, 110:92-101.

39. V. Gnanguenon, R. Govoetchan, R.F. Agossa, R. Ossè , F. Oke-Agbo , R. Azondekon , A.Arthur Sovi, R. Attolou, K. Badirou, F.T.Tokponnon, G.G. Padonou, M.C. Akogbeto, Transmission patterns of *Plasmodium falciparum* by *Anopheles gambiae* in Benin. *Malaria Journal*. 2014, 13:444.

40. C.Z. Koukpo, A.J.Y.H. Fassinou , R.A. Ossè , F.R. Agossa, A. Sovi , W.T. Sèwadé,M.C. Akogbéto, M. Sèzonlin, The current distribution and characterization of the *L1014F* resistance allele of the *kdr* gene in three malaria vectors (*Anopheles gambiae, Anopheles coluzzii, Anopheles arabiensis*) in Benin (West Africa). *Malar J*. 2019, 18:175.

41. S.A. Salako, I. Ahogni, R. Aïkpon, A.Sidick, F. Dagnon , A. Sovi, A.A. Sominahouin , F. Agossa , I. Iyikirenga , M.C.Akogbeto, Insecticide resistance status, frequency of L1014F *Kdr* and G119S *Ace-1* mutations, and expression of detoxification enzymes in *Anopheles gambiae* (*s.l.*) in two regions of northern Benin in preparation for indoor residual spraying. Parasit & Vectors. 2018, 11: 618.

42. V. Gnanguenon, F.R. Agossa , K. Badirou, R. Govoetchan, R. Anagonou , F. Oke-Agbo,R. Roseric Azondekon, R. AgbanrinYoussouf, R. Attolou, F.T.Tokponnon, R. Aïkpon, R. Ossè, M.C. Akogbeto, Malaria vectors resistance to insecticides in Benin: current trends and mechanisms involved. *Parasit & Vectors*. 2015, 8: 223

43. A.W. Yadouléton, R. N'Guessan, H. Allagbé , A. Asidi, M. Boko, R. Ossè, G. Padonou, K. Gazard, M.C.Akogbéto, The impact of the expansion of urban vegetable farming on malaria transmission in major cities of Benin. *Parasit Vectors*. 2010, 3:118.

44. P. Ntonga Akono, A. Mbouangoro, A. Mbida Mbida, C. Ndo , M.F. Peka Nsangou, S. Kekeunou, The *Anopheles gambiae* Species Complex and *kdr* Resistance Gene at the Periphery of Douala, Cameroon. *Bull Soc de Pathol Exot*. 2017; 110:122-29.

45. J. Jordana , P. Alexandrino, A. Beja-Pereira,I. Bessa, J. Canon, Y. Carretero, S.Dunner, D.Laloe , K. Moazami-Goudarzi, A.Sanchez, N.Ferrand, Genetic structure of eighteen local South European beef cattle breeds by comparative F-statistics analysis. J Anim Breed Genet. 2003, 120: 73-87.

46. A.Berthomieu, P. Kengne, H.P.Awono-Ambene , M.Raymond, D. Fontenille, M. Weill, Isolation and characterization of microsatellite DNA markers in the malaria vector *Anopheles nili*. *Mol Ecol Notes*. 2003, 3:392-93.

47. P. Gélin , H. Magalon , C. Drakeley, C.Maxwell, S. Magesa, W.Takken, C.Boëte, The fine scale genetic structure of the malaria vectors *Anopheles funestus* and *Anopheles gambiae* (Diptera: Culicidae) in the north-eastern part of Tanzania. *HAL*. 2016, Id: hal-01359593.

48. C. Wondji, F.Simard , T.Lehmann, E. Fondjo, D.Fontenille, Impact of insecticide-treated bed nets implementation on the genetic structure of *Anopheles arabiensis* in an area of irrigated rice fields in the Sahelian region of Cameroon. Molecular Ecology. 2005, 14(12):3683-93.

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