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PCR-RFLP and genetic diversity in *Anopheles coluzzii* populations in Benin (West Africa)

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Abstract
Knowledge of the genetic diversity of species is necessary to better develop programs for the management of living resources. Molecular polymorphism in *Anopheles coluzzii is of* interest in that the gene pool certainly offers its populations the possibility of colonizing extremely different environments in Africa and elsewhere. To better understand the mechanisms underlying the spectacular adaptation observed in this vector, it is imperative to have a perfect mastery of the biology and ecology of these organisms and to identify the genetics of its populations. To this end, several methodological and analytical approaches have been used using the *Kdr* gene. They were completed by the study of molecular diversity using PCR-RFLP of intron 1 and exon 32-33 of the same marker. Populations of this species showed a heterozygous deficiency. Enzyme digestion revealed monomorphism even though some cases of diversity were observed.

Keywords: *Anopheles coluzzii*, genetic diversity, PCR-RFLP, Benin

Introduction
In Sub-Saharan Africa, major human diseases such as malaria, dengue fever, and filariasis are transmitted by different species of mosquitoes [1, 2]. These diseases represent a serious global threat to public health and have a significant economic impact on tropical developing regions [1]. In Benin, malaria remains the leading cause of consultation in health facilities with a frequency of 37% among adults and 41% among children under 5 years of age [3]. It is caused by a protozoan of the genus *Plasmodium*, the most common species of which is *P. falciparum*. The main malaria vectors in the West African sub-region are species of the *Anopheles gambiae* complex [4].
The eradication of this infection consists of the elimination of the unicellular germ through prophylaxis, that of the vector by using different control methods (chemical, biological and integrated) as well as the sanitation of their living environments by avoiding the swarming of potentially vector populations. Vector control programs aim to eliminate this disease for the peaceful health of human populations. This control involves the use of chemicals belonging mainly to the pyrethroid class [5]. However, the resistance of mosquito populations to these synthetic chemicals is increasing at a dramatic rate, threatening the effectiveness of these programs in insecticide-treated areas where mosquito-borne diseases are still prevalent. Insect resistance to chemicals may be the result of genetic restructuring within vector populations. The maintenance of ancestral polymorphism and spontaneous mutations that may occur sporadically in genes that code for proteins targeted by the insecticide (target site insensitivity) [6], reduced penetration or sequestration, or increased biodegradation of the insecticide due to overexpression of genes resulting in overproduction of detoxification enzymes (metabolic resistance) [7] are among the mechanisms that living organisms use to circumvent control strategies.
Since the end of the 1990, research teams have applied the molecular PCR - RFLP approach to the problem of speciation in *Anopheles gambiae*. Thus, the study of the intergenic fragments of rDNA has made it possible to define restriction sites differentiating the molecular forms *Mopti on the* one hand and *Savane - Bamako* on the other. It was then possible to synthesize primers for a specific PCR to reveal two different profiles which were called M and S [8, 9].

Both molecular forms were later considered as species in their own right due to the high speciation [10]. The *M* form is now called *Anopheles coluzzii and* the *S* form is called *Anopheles gambiae*, both taxonomically related to the *Anopheles gambiae* complex. It has been demonstrated that gene flows between the *M* and *S* forms are much reduced [11, 12]. Moreover, the genome sequencing of these species of the West African *Anopheles gambiae* complex has revealed molecular polymorphisms characteristic of the mechanisms developed by these species to adapt to their environment [13]. Although the mechanisms of insecticide resistance are intensively studied in mosquitoes, dramatic adaptation is often considered to be the sole result of selection pressure caused by chemicals used in vector control [14, 15].

A significant advance in vector control must take into account the dynamics of vector populations through their genetic diversity but also their ecology and the geographies to which they are best adapted. The research work of Fassinou *et al.* (2019) has shown that variation in selection 3 pressures is at the origin of the modification of the genetic structure of *Anopheles coluzzii* populations in the different agro-ecological zones that exist in Benin [16].

It is known that the polymerase chain reaction (PCR) initially produced a flowering of nucleotide markers that directly access the existing polymorphism at the DNA level, but very few of these are shown to be of real value for population genetics studies. Moreover, most of the molecular markers are under the strong influence of selection. Added to this are the dominance effects of certain alleles without obscuring the epistasis that can mask the actual expression of a gene. The reliability of the marker results to be reproducible from one laboratory to another is an indispensable condition. Based on these findings and except nucleotide sequencing, the two molecular markers that are best suited to microevolution studies are RFLP (Restriction Fragment Length Polymorphism) and microsatellites. The present study aims to analyze the genetic diversity of *Anopheles coluzzii* populations in the agro-ecological zones of Benin using PCR-RFLP of certain key fragments of *Kdr* gene to assess the molecular variation they present within them and the gene flows they maintain between them.

Materials and Methods

Study area

The study was conducted in Benin between April 2017 and June 2019. Larval surveys were carried out in the eight agro-ecological zones of the country (National Report on Human Development 2015) [17]. They are the extreme northern zone of Benin (Zone I), the cotton zone of northern Benin (Zone II), the food-producing zone of South Borgou (Zone III), the West Atacora zone (Zone IV), the central cotton zone (Zone V), the Terre de barre zone (Zone VI), the depression zone (Zone VII) and the fisheries zone (Zone VIII). Larvae were collected in these zones and then reared for completion of their biological development in the laboratory under specific conditions. Figure 1 shows the agro-ecological zones with localities where the breeding sites have prospected.

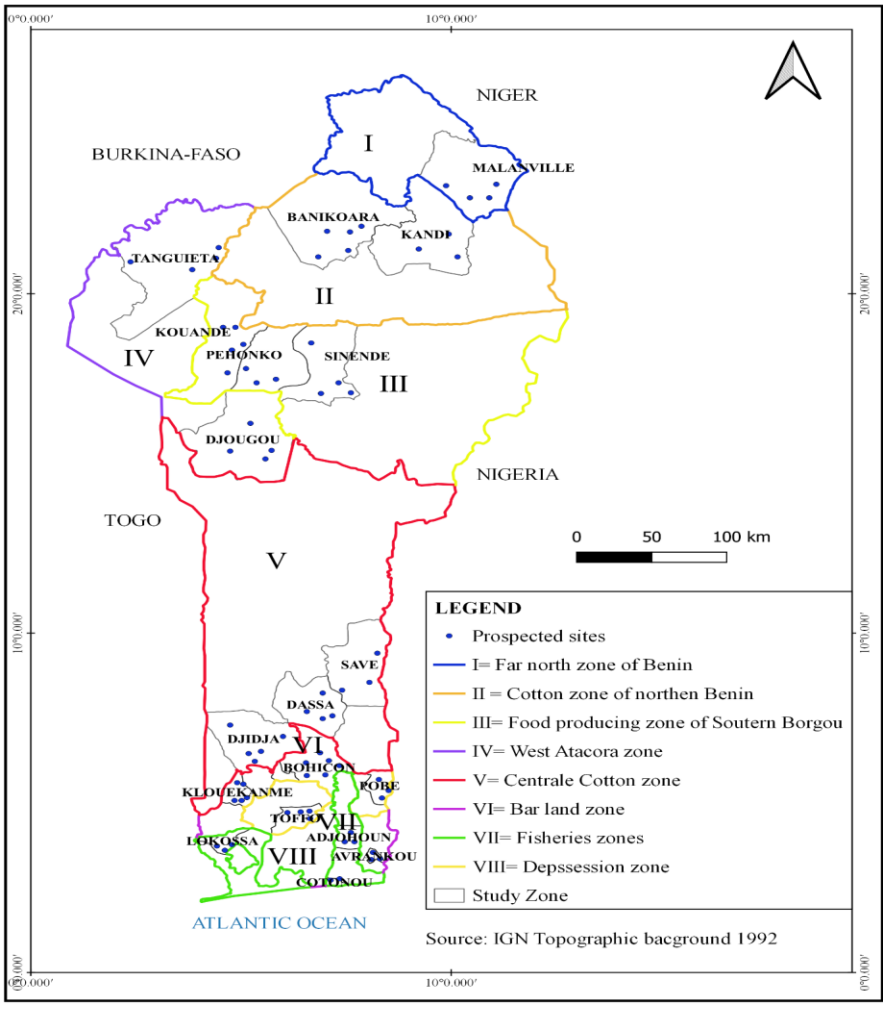

Fig 1: Map showing agro-ecological zones with prospected localities

Collection of samples

Through successive survey missions, Anopheles larvae are collected in previously defined areas using the dipping technique [18]. It consists of collecting mosquito larvae from the surface of the breeding sites using a ladle. The larvae of *Anopheles gambiae s.l.* are collected, filtered and then poured into various containers before being transported to the insectarium of the Centre de Recherche Entomologique de Cotonou (CREC) for rearing.

Adults in a lodge are likely to have a high chance of being offspring of the same female. After the larvae have been reared, the adults obtained have certainly, due to genetic transmission, inherited several common traits from their parents and will therefore be individuals with a very close genetic background. For this reason, only a few individuals have been taken into account in the lodges in order to constitute for each area a population reflecting as much as possible the genetic diversity existing within it. The adults resulting from the emergence of larvae were kept in 1.5ml plastic tubes (Eppendorf) at -20 °C.

Molecular characterization of collected samples

Extraction of DNA from mosquitoes

Whole mosquitoes were ground in 200µl of 2% CTAB with a chemical composition of 20g cétyltriméthyl ammonium bromide; 100ml 1M Tris (Trishydromethylaminomethane) HCL; 20ml 0.5M EDTA (Ethylene Diamine Tetraacetic Acid) and 81.8g NaCl made up to 1L with bi-distilled water. After 5 min in a water bath at 65 °C, the grind was mixed with 200µl chloroform and then centrifuged at 12,000 rpm for 5 min. The supernatant was gently recovered in another tube and supplemented with 200µl of isopropanol. The whole was mixed well by inversion and then centrifuged again at 12,000 rpm for 15 minutes in order to carry out a first DNA precipitation. The liquid contained in the tube was skilfully inverted so as not to lose the pellet at the bottom. 200µl 70% ethanol was added to this pellet for a final washing of the waste and a final precipitation of the genetic material. After 5 minutes of final centrifugation, the contents of the tube were again finely inverted. The pellet was then drained for at least 3 hours on the benchtop. The extracted native DNA was reconstituted with 20µl sterile water and left in suspension on the bench for an overnight.

Identification of the different species of *Anopheles gambiae s.l.* collected

The strong morphological resemblance between the species of the Anopheles complex is the origin of confusion made during the identification of the taxa constituting it. To overcome this difficulty, it is necessary to supplement morphological studies with molecular analyses. For a clear separation of the taxa that were the subject of the present population genetics study, the diagnostic PCR approach was used in addition to morphological approaches to identify the species. It is based on the specific and irreversible insertion of a 230 bp transposable element (SINE200) on the X chromosome of *Anopheles coluzzii* (*M* form) while it is absent in its twin Anopheles gambiae (*S* form). This genetically inherited characteristic allows simple, unambiguous and direct recognition of the *M* and *S* molecular forms [19].

The PCR program used includes an initial denaturation at 94 °C for 5 minutes followed by 35 cycles. Each cycle comprises a denaturation phase at 94 °C for 30 seconds, primer hybridization at 54 °C for 30 seconds and elongation by sequential additions of nucleotides to the strands in synthesis at 72 °C for 30 seconds. At the end of all the cycles, a final elongation at 72 °C for 10 minutes is performed to allow a completely complete amplification of the nucleotide sequences during synthesis. In addition to the classical constituents of a polymerase chain reaction, primers of nucleotide composition 200X6.1F 5'-TCGCCTTAGACCTTGCGTTA-3' and 200X6.1R 5'-CGCTTCAAGAATTCGAGATAC-3' were used [19]. The amplifias obtained are stored at a final temperature of 4 °C before migration on 1.5% agarose gel with ethidium bromide used as intercalating agent and migration front. Figure 2, developed by Santolamazza *et al.* (2008), illustrates the characteristic electrophoretic profile expected for each species of the complex [19].

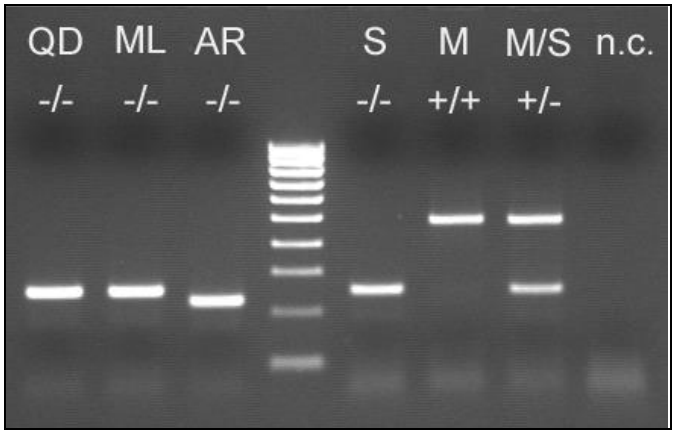

Fig 2: Diagnostic PCR based on *S200* X6.1 in *Anopheles gambiae s.l*

PCR results from locus *S200* X6.1 indicating the presence (+) or absence (-) of the insertion in females of *Anopheles gambiae* species complex. QD = *A. quadriannulatus A*; ML = *A. melas*; AR = *A. arabiensis*; S = *A. gambiae* S-form; M = *A. gambiae* M-form; M/S = hybrids from laboratory crosses; n.c. = negative control. Ladder = 100 bp (BIOLINE HyperLadder IV) [19].

Detection of the *L1014F* mutation of the *Kdr* gene in populations of the species studied

The presence of the resistance allele *(L1014F)* of the *Kdr* gene in the samples collected at each study site was demonstrated by PCR, whose protocol and amplification program are described by Martinez - Torres *et al.* (1998) [20]. PCR - PASA consists of using specific primers called Agd1, Agd2, Agd3, Agd4 and Taq polymerase to selectively amplify resistant or sensitive alleles on a fragment coding for *Vgsc* in each mosquito tested. The Agd1/Agd2 primer pair flanks the *Kdr* gene by amplifying a 293bp product as a control. The Agd1/Agd3 primer pair matches only the resistance allele of the *Kdr* gene to amplify a 195bp fragment. Finally, the Agd2/Agd4 primer pair matches only with the sensitive allele of the gene to amplify a 137bp fragment. The nucleotide sequences of these primers are Agd1: 5'-ATAGATTCCCCGACCATG-3'; Agd2: 5'-AGACAAGGATGATGAACC-3'; Agd3: 5'-AATTTGCATTACTTACGACGACA-3'; Agd4: 5'-CTGTAGTGATAGGAAATTTA-3' [18].

Genetic structure of populations

Several analytical approaches have been used to establish the genetic structure of populations. The genetic make-up of each

population is determined by calculating the gene and genotypic frequencies from the *Kdr* gene. For this purpose, the Genepop version 4.2 software was used to estimate the frequencies at each hierarchical level considered. The p-value associated with each gene frequency was evaluated according to the binomial law of software R version 3.3.3. The Hardy-Weinberg Equilibrium (EHW) test, carried out with the Genetics software version 1.3.8.1, made it possible to check whether there is pan mixing in the different populations considered.

Other indices allowing the identification of the probable causes of a possible panmixia deviation were calculated using the formulas of Weir & Cockerham (1984) as implemented in the same software Genepop version 4.2 [21]. These are the observed heterozygosity (H_o), the expected heterozygosity (H_e), the fixation index (F_{IS}), which provides information on the degree of inbreeding in the populations, and the fixation index (F_{IS}), which quantifies the panmixia difference.

Enzymatic amplification and digestion of the intron1 of the *Kdr* gene

Intron 1 of the *Kdr* gene is a nucleotide fragment with an amplified size of 1.115 bp. Its *in-vitro* amplification is performed using the protocol of Weil *et al.* (2000) [22]. The amplification program is described as follows: initial denaturation at 94 °C for 2 minutes followed by 40 cycles. Each of these cycles includes a denaturation phase at 94 °C for 30 seconds, primer hybridization at 60 °C for 30 seconds, and elongation by sequential additions of nucleotides at 72 °C for 54 seconds.

At the end of all the cycles, a final elongation for 2min was performed to allow full amplification of the sequences being synthesized. In addition to the classical constituents of a polymerase chain reaction, primers of nucleotide composition I1dir: 5'-GGCAGAACGATGGGTGCGTTAG-3' and I1rev: 5'-GCATAGCACACGGMCACAATC-3' were used to carry out the selective amplification of this gene fragment. The amplifias obtained are stored at a final temperature of 4 °C before being digested by *Hind*III, *Nde*II, and *Bfa*I endonucleases. The restriction enzyme *Hind*III recognizes a specific sequence of six (6) nucleotides while *Nde*II and *Bfa*I each recognize a sequence of four (4) nucleotides. Table 1 provides information on the enzymes used in the digestion of intron 1 of the *Kdr* gene, the nucleotide sequence recognized by each of them, and the theoretical positions of the cut-off sites for each endonuclease, the number and size of bands obtained.

Table 1: Restriction enzymes used for intron 1 digestion

Intron1	Enzymes of restriction	Sequence Recognized	Positions Theoretical outage sites	Number Theoretical striped	Theorique size Fragments expected (pb)
1,115pb	*Hind*III	A/AGCTT	381, 833	3	282, 381, 452.
	*Nde*II	/GATC	239, 483,878, 1050	5	65, 136, 172, 244, 395
	*Bfa*I	C/TAG	403, 898, 980	4	82, 135, 403, 495

Amplification and enzymatic digestion of exon 32-33 of the *Kdr* gene

Exemption 32-34 is also a gene fragment with a size of 1329 bp. Its amplification by PCR is performed using the program described by Amy Lynd *et al.* (2010) and available on the link (http://www.mbe.oxfordjournals.org/) [13]. The amplification program is described as follows: initial denaturation at 94 °C for 15 minutes followed by 40 cycles. Each cycle includes denaturation at 94 °C for 60 seconds, primer hybridization at 65 °C and elongation for 60 seconds. At the end of all cycles, a final elongation was performed for 10 minutes to allow full amplification of the nucleotide sequences. Primers of nucleotide composition dir: 5'-TTCTACGAGTCGTGCGAGTGET-3' and rev: 5'-GCGATGTTATGCTGGGAGAT-3' were used during this reaction. The amplifias obtained are stored at a final temperature of 4 °C before being cut by the only restriction enzyme *Xba*I which recognizes a specific sequence of six (6) nucleotides. Table 2 provides information on this endonuclease as part of the digestion of exon 32 - 33, the nucleotide sequence it recognizes, the theoretical position of its unique cleavage site on this gene fragment, and the number and size of bands obtained.

Table 2: Restriction enzymes chosen to digest exon 32-33

Exon 32-33	Enzymes of restriction	Sequence recognized	Positions Theoretical outage Sites	Number Theoretical Striped	Theorique size Fragments expected (pb)
1,329pb	*Xba*I	T/CTAGA	478	2	478, 851

Results

Molecular identification of *Anopheles coluzzii*

Molecular identification of the species of the *Anopheles gambiae* complex collected in the different agro-ecological zones made it possible to obtain the species *Anopheles coluzzii* in the populations. Table 3 presents the numbers of each population sampled for this species.

Table 3: Numbers of *Anopheles coluzzii* in each agro-ecological zone

Agro-ecological zones	Localities	*Anopheles coluzzii*	Agro-ecological zones	Localities	*Anopheles coluzzii*
Far North Zone	Malanville	83	Area	Klouékanmè	18
	Total	83	of bar land	Avrankou	37
				Bohicon	21
Cotton zone	Kandi	78		Total	76
of North Bénin	Banikoara	53			
	Total	131	Fisheries zone	Lokossa	72
				Cotonou	91
Food producing zone of the	Péhunco	43		Adjohoun	42
South Borgou	Sinendé	47		Total	205
	Total	90			
			Depression zone	Pobè	52
West zone	Kouandé	52		Toffo	63
Atacora	Tanguiéta	46		Total	115
	Djougou	62			
	Total	160			
Cotton zone	Savè	71			
From the Centre	Dassa	66			
	Djidja	78			
	Total	215			

Analysis of this table shows that *Anopheles coluzzii* was found in all localities in each agroecological zone, although Klouékanmè and Bohicon seem to have low numbers compared to other localities.

Mosquito genotyping and population genetic structure

Table 4 shows the genetic structure of the populations and the Hardy-Weinberg Equilibrium test.

Table 4: Gene and genotypic frequencies and Hardy - Weinberg Equilibrium test in populations

Agro - ecological zones	Numbers	Genotypes (Genotypic frequencies)			F(*L1014F*)	p - value	p - value
		1014 F/F	*1014 L/F*	*1014 L/L*	%	F(*L1014F*)	(H-W)
Far North zone	83	73(87.952)	7(8.434)	3(3.614)	92.169	0.0001	0.0061
Cotton zone of the North Bénin	131	127(96.946)	2(1.527)	2(1.527)	97.709	0.0001	0.0006
Food producing of the South Borgou	90	87(96.666)	1(1.111)	2(2.222)	97.222	0.0001	0.0005
West zone Atacora	160	145(90.625)	11(6.875)	4(2.500)	94.062	0.0001	0.0009
Cotton zone of Centre	215	202(93.956)	10(4.651)	3(1.395)	96.279	0.0001	0.0019
Bar land zone	76	69(90.789)	5(6.579)	2(2.632)	94.079	0.0001	0.0171
Fisheries zone	205	187(91.219)	12(5.854)	6(2.927)	94.146	0.0001	0.0000
Depression zone	115	99(88.087)	9(7.826)	7(6.087)	89.139	0.0001	0.0000

1014F/F: homozygote resistant, *1014L/F*: heterozygote, *1014 L/L*: susceptible homozygote, p - value *F(L1014F):* p - value of the frequency of the resistance allele, p - value EHW: p - value of Hardy - Weinberg Equilibrium.

Analysis of Table 4 revealed that the frequency of the *L1014F* allele was very high in all populations. It ranged from 89.139% in the population in the depression zone to 97.709% in the population in the central cotton zone. Similarly, the frequencies of homozygote resistant individuals were high in the populations (86.275% in the Bar land zone population to 96.946% in the North Benin cotton zone population) while heterozygote and homozygote susceptible individuals had relatively low frequencies in all populations. It is noted that none of the populations studied respected the Hardy-Weinberg Equilibrium (p-value < 0.05).

Panmixia deviation

Table 5 presented the observed heterozygosity (H_o), the expected heterozygosity (H_e) and the fixation index (F_{IS} W&C) in the different populations.

Table 5: Difference in panmixia gap in *Anopheles coluzzii* populations

Agro-ecological zones	H_o	H_e	F_{IS}(W&C)
Far zone of the North	0.0843	0.1452	0.4208
Cotton zone of the	0.0153	0.0449	0.6610
Food producing zone of the South of Borgou	0.0111	0.0543	0.7963
West zone Atacora	0.0675	0.1120	0.3872
Cotton zone from the Centre	0.0465	0.0718	0.3529
Bar land zones	0.0658	0.1121	0.4150
Fisheries zones	0.0585	0.1104	0.4708
Depression zone	0.0783	0.1807	0.5682

The analysis in Table 5 revealed that in each population, the expected heterozygosity is much higher than the observed heterozygosity. There is therefore a deficit of heterozygotes in the populations analysed across the agro-ecological zones.

Molecular diversity of populations

The molecular diversity of *Anopheles coluzzii* populations was analysed with a sampling of 12 individuals per agro-ecological zone. The sample is composed of resistant homozygous individuals, heterozygous, and sensitive homozygous individuals. Figure 3 shows the image obtained following enzymatic digestion of intron 1 with the three different restriction enzymes initially selected. Numbers 1, 2, 3, 4, 5, and 6 in this picture correspond to individuals of *Anopheles coluzzii* from different populations.

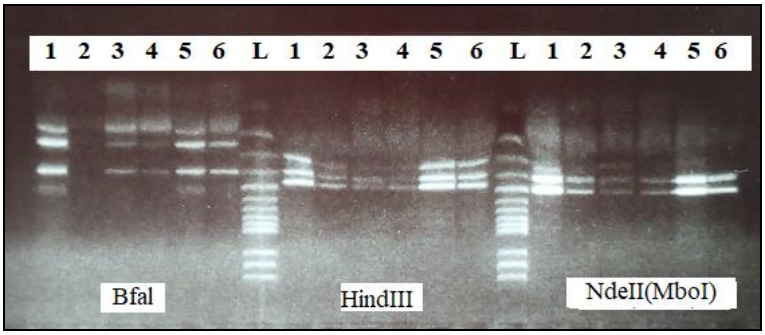

Fig 3: Enzymatic digestion of intron 1 of the *Kdr* gene

Legend: L=ladder (100bp), 1= *Anopheles coluzzii* (depression zone), 2 = *Anopheles coluzzii* (fisheries zone), 3 = *Anopheles coluzzii* (central Benin cotton zone), 4 = *Anopheles coluzzii* (bar land zone), 5 = *Anopheles coluzzii* (extreme north zone), 6 = *Anopheles coluzzii (West Atacora zone), Bfa*I, *Hind*III *and Nde*II = restriction enzymes. The direction of migration is from bottom to top.

The analysis of electrophoretic profiles obtained after amplification and enzymatic digestion of the sequences revealed a monomorphism of the gene in almost all the individuals sampled. This monomorphic was characterized by the migration to the same size as the different bands on the agarose gel. Referring to the theoretical fragment sizes expected after digestion of the intron by the *Hind*III endonuclease, the 281, 381, and 452 bp bands were observed on the gel in all populations. On the other hand, diversity seems to be observed during the digestion of intron 1 by the *Bfa*I endonuclease when comparing the populations of 1 and 5. Contrary to most populations where digestion with this enzyme produced three bands (200bp, 250bp, 400bp), the fourth band of about 500bp was observed in the populations of the depression and northern cotton-growing areas. The same is true for the digestion of intron 1 with *Nde*II, which produced two bands (400bp and 500bp) in most populations except for those in zones 3 and 5 where the third band of about 244bp is present. The theoretical bands of 65, 136, and 172 bp are not revealed by the electrophoretic profile.

Figure 4 shows the results of the enzymatic digestion of exon 32-33 of the *Kdr* gene by *Xba*I. The numbers 1, 2, 3, 4, and 5 also correspond to the individuals sampled in the different agro-ecological zones.

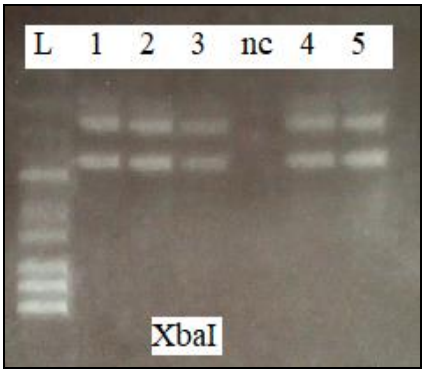

Fig 4: Enzymatic digestion of exon 32 - 33 of the *Kdr* gene

Legend: L=ladder (100bp), 1= *Anopheles coluzzii* (fisheries zone), 2 = *Anopheles coluzzii* (cotton zone of central Benin), 3 = *Anopheles coluzzii* (depression zone), nc = negative control, 4 = *Anopheles coluzzii* (food-producing zone of South Borgou), 5 = *Anopheles coluzzii* (cotton zone of North Benin), *Xba*I = restriction enzymes. The direction of migration is from bottom to top.

In all populations of *Anopheles coluzzii*, the enzymatic digestion of Exon 32-33 with *Xba*I endonuclease generated two bands of size approximately to 350 and 450 bp, indicating monomorphism.

Discussion

Molecular identification of the different species indicated that *Anopheles coluzzii* was found in all agro-ecological zones. Several studies have shown that this species is one of the most widespread vectors in Sub-Saharan Africa [23, 24]. The presence of *Anopheles coluzzii* in all breeding sites was not surprising as surveys were carried out during the dry season. In addition to this, there are a few permanent breeding sites which are certainly due to the ongoing climate change. Studies conducted in Ghana have shown that this species prefers to lay its eggs in beds that are permanent [25, 26]. On the other hand, the peculiarities observed in Bohicon and Klouékanmè in the Bar Lands zone where this vector is weakly represented could be explained by inappropriate ecological conditions that characterize these environments.

The resistant *L1014F* allele of the *Kdr* gene is highly selected in all agroecological zones. The high allelic frequency observed in these zones is thought to have several origins. Agricultural practices requiring the heavy use of several categories of pesticides to effectively control insect pests [27], the widespread use of LLIN, and indoor residual spraying (IRS) [28] are noteworthy. The products available to public health to control mosquitoes are the same products that have been used for decades in agriculture against crop pests. This massive and continuous use of pyrethroids in public health and agriculture would also be the reason for the strong selection of the *L1014F* allele found in Anopheles coluzzii, as the modes of action are similar. Akogbéto *et al* (2005) [29] have shown that pesticide residues can exert selection pressure on certain mosquito populations as early as during larval life by eliminating susceptible individuals already at this stage of development.

The genetic structure of all *Anopheles coluzzii* populations analysed showed a pan mixing deviation resulting in a heterozygous deficit. It has been shown in population genetics that the causes of such a deviation from Hardy-Weinberg equilibrium may be the Wahlund effect, inbreeding, genetic drift, selection against heterozygotes, or their combination. Based on the cultural practices in Benin, it is tempting to think of selecting for the resistant allele in an insecticide-intensive environment, even if other factors should not be neglected. It is tempting to think that directional selection in favor of dominant homozygotes is taking place in populations of this vector species.

The results obtained for the study of molecular diversity represent the first conclusions of the analysis on the length polymorphism of restriction fragments within the different populations of the species concerned. This apparent molecular diversity observed within *Anopheles coluzzii* populations and revealed by the *Bfa*I and *Nde*II endonucleases during PCR-RFLP analyses is thought to have several origins. Species adaptation to their environment is an evolutionary mechanism that can be detected by mutations observable at the genome level [30]. The agro-ecological areas where this species lives are not subject to the same selection pressures. Such a remark can in no way be considered as a surprise in the sense that the life of organisms is governed by the permanent interactions between ecology and genetics. Nevertheless, it should be pointed out that the low genetic diversity observed in the populations of this species could depend on the resolving power of the *Kdr* gene, which is highly selective. Contrary to what one might think, the increasingly tangible evidence from molecular genetics indicates that introns of eukaryotic genes do not always have a neutral molecular evolution because of the exons that surround them and their role in the splicing process.

Although the *Kdr* gene has a low resolution for a microevolution study, it has the advantage of laying the first foundations for a more in-depth study. It has been shown that genetic diversity is underestimated with the appearance of bands with similar nucleotide sizes. Since the *Kdr* gene is under great pressure in most ecological situations, the results obtained could well diverge from the real history of this species. For that, it is quite conceivable to think about the nucleotide sequencing of this gene and to look for other molecular markers much more adapted to these kinds of themes to appreciate the molecular diversity existing within the populations of *Anopheles coluzzii*. Microsatellites are already recognized as good candidates to better address such issues. Nucleotide sequencing of a candidate gene would provide more information on the molecular polymorphism and genetic structure of populations. Molecular databases on species of the *Anopheles gambiae* complex, such as the results of the 1000 Genomes Project, are already available to better direct studies in this direction. The *L1014F* resistance allele can be expressed in several nucleotides forms which are haplotypes but which are masked in the polypeptide chain due to the degeneracy of the genetic code and the existence of null alleles [31].

Conclusions

This work has made it possible to highlight some draft results on the genetic diversity of *Anopheles coluzzii* populations in Benin. Populations of this vector present a heterozygosity deficit which justifies the strong selection of the resistant allele *L1014F*. The analysis of gene fragments using RFLP-PCR revealed the first molecular variations, even if they are apparent. It is becoming imperative to deepen this study by favoring other more resolving molecular markers to have reliable results that can be used to better define the best strategies for controlling these disease vectors.

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Authors' contributions

MS and MCA designed the study. MS and AJYHF performed the experiments. MS and LBM analyses the data. MS wrote the manuscript. MS, AJYHF, CZK, LBM, and MCA criticized and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data supporting the conclusions of this article are included within the article. The raw data used and/or analyzed in this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

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Not applicable.

Competing interests

The authors declare that they have no competing interests.

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