Polymorphism and Geographical Distribution of *vgsc* and *ace-1* Gene in *Anopheles Sinensis* Field Populations in Guizhou Province, China

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Abstract

Background: Vector control has been a key strategy in malaria prevention and elimination for decades. However, insecticide resistance is becoming a serious threat to vector control. *Anopheles sinensis* is one of the important transmission vectors for malaria in Guizhou Province, China. However, little is known on insecticide resistance status and related mechanism. In this study, the diversity and frequency of the major insecticide resistance associated genes such as voltage-gated sodium channel (*vgsc*) and acetylcholinesterase-1 (*ace-1*) genes that encoded the target proteins of Pyrethroids and OPs were investigated in field populations.

Methods: Adult mosquitoes were collected from 12 sampling sites across Guizhou by lamp trapping. Female *An. sinensis* were identified by morphological and molecular identification. Genomic DNA was extracted to amplify *vgsc* and *ace-1* gene fragments. PCR products were sequenced bi-directly. Mutations of *vgsc* gene at locus 1014 and that of *ace-1* at locus 119 were analyzed using MEGA 7.1 software, and the frequencies of mutations were calculated respectively.

Results: 5 *kdr* mutation alleles at the locus 1014 of *vgsc* gene as a result of three amino acid replacements (namely 1014F/C/S) in 548 samples of 12 *An. sinensis* populations. The total frequency of *kdr* mutation alleles was 27.4%, of which the TTT/C (F) allele had a highest mutation frequency of 22.5%. The top three mutation genotypes were from XiShui, TongZhi and DeJiang populations collected in north Guizhou. There were three alleles at locus 119 in *ace-1* gene with 49.47% of GGC/G, 0.17% of GGT/G and 50.36% of AGC/S. The 100% frequency of mutation genotypes (GS, SS) was found in CeHeng, LuoDian and SanDu populations gathered in southwest Guizhou.

Conclusion: A diverse genetic mutations of *vgsc* and *ace-1* genes are found in *An. sinensis* in Guizhou. There are a significant geographical heterogeneities of allele frequency among different populations in Guizhou. A high frequency of *kdr* mutation (>44 %) in north Guizhou. The 119S mutation of *ace-1* gene is present at a high frequency in most *An. sinensis* populations in Guizhou, especially in the previously highly endemic malaria regions. These findings suggest continued monitoring of the genotypic diversity of insecticides resistance genes may assist to formulate a region-customized resistance management strategies.

Introduction

Malaria remains an important public health problem in tropical and sub-tropical countries. According to the report from World Health Organization (WHO), there were 229 million clinical cases of and approximately 409,000 deaths from malaria infection in 2019, and nearly 92% of them occurred in Africa[1]. It also ranked the first among top five important parasitosis in China in the past. In mainland China, no indigenous malaria cases have been reported since 2017 because ‘an action plan for malaria elimination’ with a goal of eliminating malaria throughout the country by the end of 2020 was launched in 2010 by the National Ministry of Health [2, 3]. However, imported malaria cases are posing a serious challenge to malaria control for China.

Due to the lack of effective vaccine for malaria, vector control is still one of the effective measures to control and eliminate malaria transmission in the world. Insecticides remain the most important vector control method. Currently, four groups of insecticides such as DDT, pyrethroids, organophosphates (OPs) and carbamates (CBs) are recommended to control mosquito by WHO[4]. Massive sprayings of insecticides greatly limited mosquito-borne diseases and even eradicated malaria in a few areas in the past[5]. However, the widespread development of resistance in mosquitoes to insecticides is now causing serious problems in many areas[6].

As we well known, target-site insensitivity has been verified to be one of two major mechanisms that are involved in insecticide resistance. The insect voltage-gated sodium channels (VGSCs) are the target of pyrethroids and organochlorine insecticides. Many studies have demonstrated that mutations at 1014 locus are able to confer knockdown resistance (*kdr*) in many arthropod species including Anophelines[7]. To date, in Anopheles, a total of 4 types of *kdr* mutations (L1014F, L1014C, L1014S and L1014W) that are related to insecticide resistance were reported [8–10], of which L1014F was evidenced to be the most common mutation in Africa, Asia and America [11]. Further, organophosphates (OPs) and carbamates (CBs) can target acetylcholinesterases (AChEs) of insects which are encoded by two acetylcholinesterase genes *ace-1* and *ace-2* in mosquito, but only *ace-1* has been found to be significantly associated with resistance to OPs and CBs [12]. The G119S mutation of *ace-1* gene causes a spatial shift of AChE structure, which alters the interaction between the enzyme and insecticides, hereby resulting in insecticide resistance[13]. Multiple lines of evidence have revealed that the G119S mutation occurs at high frequency in many field populations of *An. sinensis* in Asia[8, 13].

There are mainly 4 species of *Anopheles* that can transmit malaria in China, of which *Anopheles sinensis* was found to be the most concerned vector because of its wide distribution and species predominance in the country[14].Currently, the insecticide resistance level and resistance mechanism on *An. sinensis* have been widely reported in many places in China, including Sichuan Province, Guangxi Province and Chongqing that neighbor Guizhou [15–20]. However, little information on the insecticide resistance status of *An. sinensis* has been reported in Guizhou Province, southwest of China. In this study, the distribution and frequency of target-site mutation in *vgsc* and *ace-1* genes of *An. sinensis* across Guizhou were detected to reveal the molecular resistance status.

Materials And Methods

Mosquito samples

Adult mosquitoes were collected in 12 sites (Fig. 1) across Guizhou province by light trap (wave length 365 nm) in August 2017 and July-August 2018. In each site, three or four houses (with a distance > 50 m) were each equipped with a light trap. The light trap was placed in the pig pen or cattle pen, 1.5-2.0 m above the ground. The mosquitoes were collected from 1 hour before sunset to 1 hour after sunrise, for consecutive three days. The captured mosquitoes individually underwent morphological identification and

those confirmed to be *An. sinensis* adults were put into Eppendorf tubes containing 100% ethanol and kept at -4℃ until use.

Extraction of genomic DNA and species identification

The genomic DNA of mosquitoes was extracted with TaKaRa MiniBEST Universal Genomic DNA Extraction Kit Ver. 5.0, according to the manufacturer's instructions. Genomic DNA was kept at -20℃ until use. The extracted DNA was used immediately for PCR assay or stored at -20°C for later use. Molecular identification of *An.sinensis* species was performed with primers for species-specific mitochondrial DNA cytochrome oxidase subunit I (*mtDNA-COI*) gene (COI-F: 5' -GGTCAACAAATCATAAAGATATTGG – 3'; COI-R: 5'-TAAACTTCAGGGTGACCAAAAAATCA – 3') [21]. The *mtDNA-COI* gene fragment was amplified in a 15 μL reaction system containing Premix TaqTM (7.5μL) (TAKARA Bio Inc., Shiga, Japan), ddH2O (4.9 μL), DNA template (1 μL), and 10 μM primers (1μL each). PCR reactions were performed in a SimpliAmpTM Thermal Cycler (Thermo Fisher Scientific Inc., Waltham, MA, USA) under the following conditions: 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and terminated with a final extension at 72°C for 10 min. PCR products (5 μL) were identified and bi-directionally sequenced by Sangon Biotech (Shanghai, China).

Amplification and sequencing of vgsc gene

The *vgsc* gene was amplified in a 25 μL reaction system containing Premix Taq™ (12.5μL) (TAKARA Bio Inc., Shiga, Japan), ddH_2O (9.5 μL), DNA template (1 μL), 10 μM forward primer *vgsc*-F (1 μL) (5' -TGCCACTCCGTGTGTTTAGA – 3') and 10 μM reverse primer *vgsc*-R (1μL) (5' -GAGCGATGATGATCCGAAAT – 3')[15]. The reactions were performed in a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific Inc., Waltham, MA, USA) under the following conditions: 95 ℃ for 3 min, followed by 35 cycles of 95 ℃ for 30 s, 55 ℃ for 30 s, and 72℃ for 45 s, and a final extension at 72 ℃ for 10 min. PCR products (5 μL) were identified and bi-directionally sequenced by Sangon Biotech (Shanghai, China).

Amplification and sequencing of ace-1 gene

The *ace-1* gene was amplified in a 25 μL reaction system containing Premix Taq™ (12.5 μL) (TAKARA Bio Inc., Shiga, Japan), ddH_2O (8.5 μL), DNA template (2 μL), 10 μM forward primer *ace-1*-F (1 μL) (5' -GCGCGACCATGTGGAACC – 3') and 10 μM reverse primer *ace-1*-R (1μL) (5' -ACCACGATCACGTTCTCCTC-3'). The reactions were perfromed in a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific Inc., Waltham, MA, USA) under the following conditions: 94 ℃ for 3 min, followed by 35 cycles of 95 ℃ for 30 s, 55 ℃ for 30 s, and 72℃ for 45s, and a final extension at 72 ℃ for 10 min [8]. PCR products (5 μL) were identified and bi-directionally sequenced by Sangon Biotech (Shanghai, China).

Data analysis

The sequencing data were manually checked and cleaned. All confirmed DNA sequences were aligned with the sequence at locus 1014 in *vgsc* gene or locus 119 in *ace-1* gene in the MEGA 7.1 software[22].

The nucleotide and amino-acid sequences of *vgsc* (Genbank ID:KP763810.1) and *ace-1* (Genbank ID: KM875636.1) of *An. sinensis* were used as the reference for alignment. Data were imported into Excel 2019 to construct the database for analysis.

Results

The distribution and frequency of mutations at 1014 locus of kdr gene in Anopheles sinensis

A total of 548 samples of *An.sinensis* representing 12 field populations were collected in Guizhou province. They were genotyped and six different alleles (TTG/L, TTT/F, TTC/F, TGT/C, TGC/C and TCG/S) were found at the locus 1014 of *vgsc* gene in *An.sinensis* (Fig. 2, Table 2). The TGC/C allele was found for the first time and in only one sample of the TZ population. The total frequency of *kdr* mutation alleles was 27.4%, of which the TTT/C (F) allele had a highest mutation frequency of 22.5%. The frequency of *kdr* mutation alleles ranged from 2.00% (LP) to 56% (TZ) among the 12 field populations.

Table 1
Collection information of field-population of *Anopheles sinensis* in Guizhou Province

Collection sites	Longitude and latitude	Altitude	Collection date	Population code
PingBa County, AnShun City	26.554 N,106.341 E	1253 m	2017.07	PB
CeHeng County, QianXinan Prefecture	24.994 N,105.771 E	706 m	2017.08	CH
LuoDian County, QianNan Prefecture	25.226 N,106.682 E	389 m	2017.08	LD
HuaXi District, GuiYang City	26.399 N,106.591 E	1170 m	2018.07	HX
ZhiJin County, BiJie City	26.694 N,105.835 E	1270 m	2018.07	ZJ
LiPing County, QianDongNan Prefecture	26.202 N,108.951 E	594 m	2018.07	LP
DuYun City, QianNan Prefecture	26.292 N,107.439 E	842 m	2018.07	DY
SanDu County, QianNan Prefecture	25.572 N,107.933 E	485 m	2018.07	SD
DeJiang County, TongRen City	28.319 N,108.142 E	560 m	2018.08	DJ
XiShui County, ZunYi City	28.156 N,106.348 E	402 m	2018.08	XS
TongZi County, ZunYi City	28.115 N,106.595 E	1018 m	2018.08	TZ
XingRen City, QianXinan Prefecture	25.411 N,105.230 E	1341 m	2018.08	XR

Table 2
Distribution and frequency of seven alleles at 1014 locus of *kdr* gene in *Anopheles sinensis* from Guizhou Province, China

Population	Sample size	Allelic genes frequency(%)						Mutant alleles frequency(%)
		TTG(L)	TTT(F)	TTC/F	TCG(S)	TGT(C)	TGC(C)	
DJ	50	56	40	2	0	2	0	44
TZ	50	44	46	4	0	5	1	56
XS	26	45	39	6	0	10	0	55
ZJ	50	66	30	2	0	2	0	34
PB	50	88	8	0	1	3	0	12
HX	50	77	13	1	1	8	0	23
DY	50	65	26	0	0	9	0	35
SD	49	65	34	0	0	1	0	35
LD	24	90	4	0	4	2	0	10
CH	48	84	8	0	0	8	0	16
XR	51	93	4	0	1	2	0	7
LP	50	98	2	0	0	0	0	2
average		72.6	21.2	1.3	0.6	4.3	0.1	27.4

Three homozygous genotypes (1014LL (TTG), 1014FF (TTT), and 1014CC (TGT)) and seven heterozygous genotypes (1014LF (TTG/TTT), 1014FF (TTT/TTC), 1014LF (TTG/TTC), 1014FC (TTT/TGT), 1014FC (TTT/TGC), 1014LC (TTG/TGT), and 1014LS (TTG/TCG)) of *vgsc* gene were detected (Fig. 1), with a frequency ranging 0.17–56.87%. The wild-type homozygous genotype 1014LL had the highest frequency (56.87%), followed by genotypes 1014LF(TTG/TTT/C) (24.47%) and 1014FF(TTT/TTT/C) (9.1%).

The polymorphism of *kdr* mutation genotypes varied significantly among the 12 populations. The TZ and HX populations had highest diversity of genotypes for *vgsc* gene (Fig. 1). The top 3 mutation alleles frequency were from the populations of XS(55%), TZ(56%) and DJ(44%), north Guizhou.

The distribution and frequency of mutations at locus 119 of ace-1 **gene in** An. sinensis

Three alleles (GGC/G, GGT/G and AGC/S) were identified at the locus 119 of *ace-1* gene in 551 samples of *An. sinensis* (Fig. 2). The GGT/G allele was found for the first time and in only one sample of the ZJ

population. The total frequency of the resistance allele (AGC/S) was 50.36%. Specifically, the frequency of the resistance allele AGC/S ranged 6% (LP)–77% (CH) among the 12 populations (Table 3).

Table 3
Distribution and frequency of three alleles at 119 locus in *ace-1* gene in *Anopheles sinensis* from Guizhou Province, China

Population	Sample size (n)	Alleles Frequency(%)			Mutant alleles frequency(%)
		GGC/G	GGT/G	AGC/S	
DJ	50	43	0	57	57
TZ	50	50	0	50	50
XS	26	46.15	0	53.85	53.85
ZJ	50	43	2	55	55
PB	50	55	0	45	45
HX	50	52	0	48	48
DY	50	65	0	35	35
SD	49	38.78	0	61.22	61.22
LD	25	30	0	70	70
CH	50	33	0	77	77
XR	51	32.35	0	67.65	67.65
LP	50	94	0	6	6
Total	551	49.47	0.17	50.36	50.36

The frequency of mutation genotypes (119GG, 119GS) was over 66% in all 12 field populations, except for the LP population in which the frequency was 12% (Fig. 1). The populations of CH, LD, and SD ranked top in the frequency of mutation genotypes (100%), which were from the once highly epidemic areas of malaria.

Discussion

Guizhou Province is located in the Yunnan-Guizhou Plateau with complex landforms, humid climate, diverse natural environment and abundant mosquito species. As a once prominent malaria-endemic province, no indigenous cases were reported since 2016 when a long-term strategy on malaria elimination in Guizhou was implemented [23]. In spite of this, the imported malaria infection cases reported every year become the new concern[24]. In this study, 12 field populations were selected across Guizhou Province, according to the previous distribution feature of malaria epidemics. After morphological identification and

molecular identification, the distribution and frequencies of *kdr* and *ace-1* mutations were detected for the first extensive survey.

We discovered 5 *kdr* mutation alleles at the locus 1014 of *vgsc* gene as a result of three amino acid replacements (namely 1014F/C/S) in 548 samples of 12 *An. sinensis* populations. Of the three replacements at the amino acids level, the highest allele frequency was found for 1014F (22.5%), implying that 1014F is a predominant mutation allele in Guizhou Province, similarly to the previous studies [9, 10, 16, 19, 25, 26].1014C is also a common variant in southeast Asia and China. In addition to the reported variant, TGC/C allele was found for the first time in this study which indicated it had the most variants in Guizhou. It might be related to the environment variety in Guizhou. Though 1014C was demonstrated to be the predominant in northeast of Guangxi[15], a low frequency was detected in most of places in China, including Guizhou. Guizhou is located in north of Guangxi, and the low frequency of L1014C suggests the geographical barriers limiting gene flow imposed by the mountainous landscapes of Guizhou. 1014S has been widely reported in *Anopheles* in the Greater Mekong Subregion and Africa[25, 26]. However, to date it is only detected in *An. sinensis* in some provinces of South China, such as Guangdong[26], Sichuan[16], Guangxi[15] and Guizhou.

The frequency of *kdr* mutation has been evidenced to be significantly positively correlated with the resistance phenotype to Pyrethroids and DDT in Anopheles[29–31]. In this study, distinct distribution patterns for *kdr* mutation genotypes were observed in the 12 field populations across Guizhou. The frequency for *kdr* mutation genotypes including homozygotes and heterozygotes in north part of Guizhou was higher than that in other locates, which warn it should be strengthened to surveil the pyrethroids resistance in north Guizhou. A high frequency of *kdr* mutation were also observed in *An. sinensis* from Sichuan Province[16] and Chongqing[19], the neighbors next to north part of Guizhou, which indicates it is possible that migration of *An. sinensis* population occurs from Sichuan to north Guizhou.

Previous genotyping results on *ace-1* gene have revealed a modest-to-high frequency of 119S resistance allele in *An. sinensis* populations in Guangxi province (80%) [32], at the China-Vietnam border in Guangxi province (73%)[8], Hainan Island (45 ~ 75%)[33], Sichuan province (56%) [16], and Yunnan (38.5%) and Anhui (58.9%) provinces[20]. Here we found a modest frequency of the 119S resistance allele (53.6%) in *An. sinensis* across Guizhou (Table 1). These results suggest that the insecticide resistance to OPs and CBs may be widely distributed in *An. sinensis* populations in China, including Guizhou province. It is well known that OPs have been used for pest control in China since 1950s and the high frequency of mutation genotypes may be a consequence of the long-term use of OPs in agriculture industry[19]. It is worth to note that 100% frequency mutation genotypes (119GS, 119SS) occurred in 3 regions in Guizhou: Ceheng, Luodian and Sandu, which were previously considered as the highest endemic malaria regions in Guizhou. It further indicates a strong risk of resistance to OPs and CBs in these regions.

In general, mutation and migration are the main driving forces for the occurrence of a novel resistance allele in a given population[34]. It is noted that the LP population had lowest mutation frequency for *kdr*

and *ace-1* gene among all 12 populations, suggesting that this population faces low insecticide selection pressure and has no migration force.

Conclusion

In this study, we investigated the mutation and distribution of two target-site resistance genes in 12 field populations of *Anopheles sinensis* across Guizhou Province. We discovered 5 different *kdr mutation* alleles at the locus 1014 and 4 different *ace-1* alleles at the locus 119 in these samples, of which the TGG/C allele of *kdr* gene and TTG/G allele of *ace-1* were found for the first time. The results showed a diverse genetic mutations in *An. sinensis* in Guizhou. A high frequency of kdr mutation (> 44 %) in DeJiang, TongZhi, and Xishui indicates a risk of resistance to pyrethroids and DDT in *An. sinensis* in these areas. The G119S mutation is widespread in *An. sinensis* in Guizhou, especially in the previously identified high-risk malaria endemic areas which implicate that cautions should be paid when using OPs and CBs as control agents for vectors. Continued monitoring of insecticide resistance and the genotypic diversity of resistance genes may assist to formulate effective region-customized resistance management strategies before implementing malaria control strategies.

Declarations

Ethics approval and participant consent

All the field studies on mosquito were approved by the Institutional Animal Care and Use Committee of Guizhou Medical University (China).

Consent for publication

No applicable.

Availability of data and material

No.

Competing interests

The authors declare that they have no competing interests.

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Authors` Contribution

WJH designed the whole study, genetic analysis and wrote the manuscript. CJZ and LQG performed mosquito collection, genotyping and data process. YX and WYM performed housefly collection and data processing. TWL proofread the manuscript. All authors read and approved the final manuscript.

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Figures

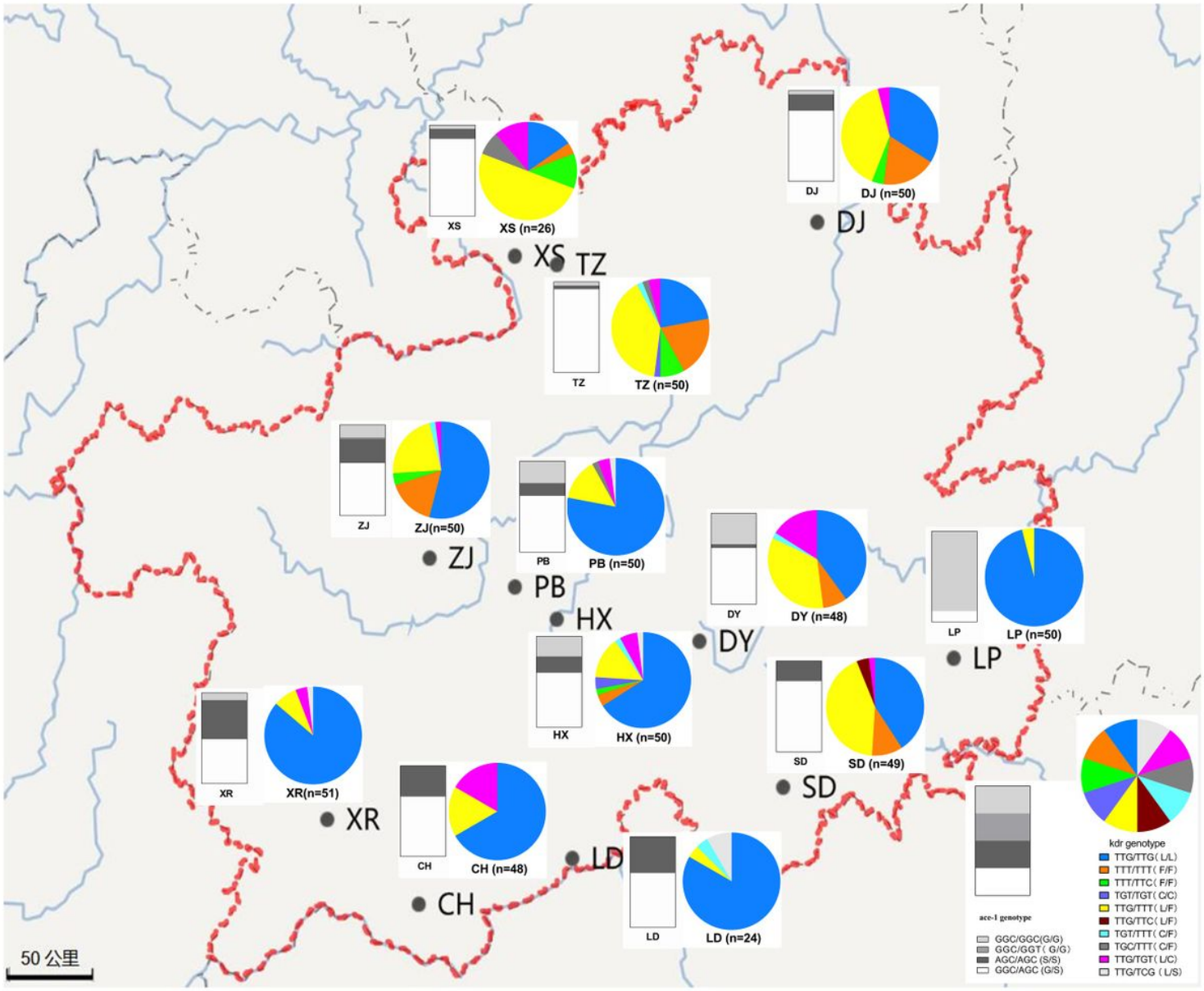

Figure 1

Distribution and frequency of genotypes in Anopheles sinensis in Guizhou

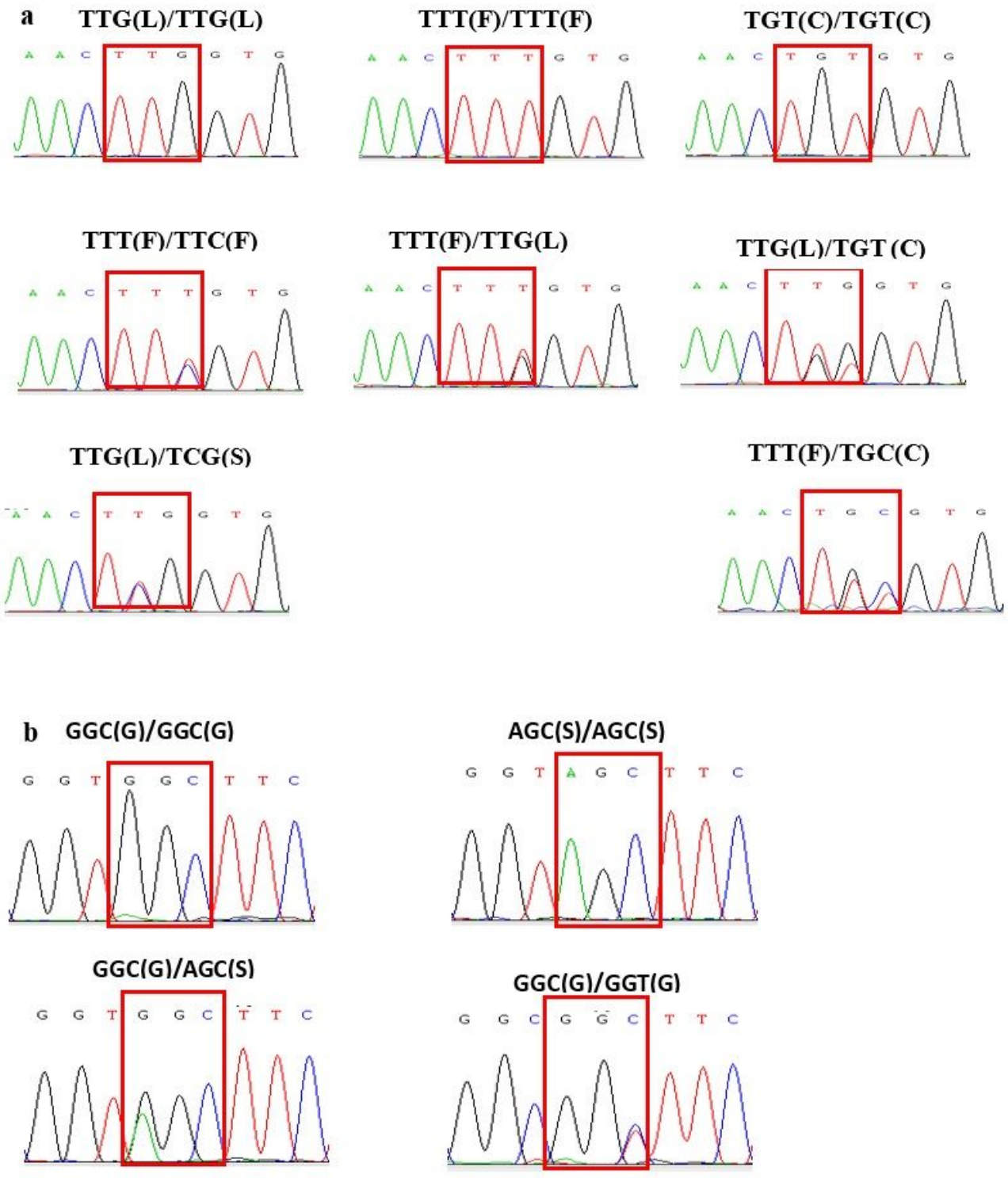

Figure 2

Example of nucleotide sequence chromatograms of kdr and ace-1 genotypes detected in Anopheles sinensis from Guizhou. a: Eight genotypies of kdr. The position at codon 1014 of the para-type sodium channel gene is indicated by a rectangle box. b: four genotypies of ace-1. The position at codon 119 of ace-1 gene is indicated by a rectangle box.

Supplementary Files

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