Zoological Studies **64:**31 (2025)

Understanding the Global Dynamics of Pyrethroid Resistance-related *kdr* mutations in *Aedes* (*Stegomyia*) *aegypti* (Linnaeus, 1762) (Diptera: Culicidae)

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(Received 23 December 2024 / Accepted 29 May 2025 / Published -- 2025)
Communicated by John Wang

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Among the mechanisms of insecticide resistance, knockdown resistance (*kdr*), causes alterations in the functioning of the voltage-gated sodium channel (Na_v), which is the target site for pyrethroids (PYs) and dichloro diphenyl chloroethane (DDT). In *Aedes aegypti*, 13 *kdr* mutations associated with PYs resistance have been identified, with V410L, V1016I, V1016G, and F1534C being the most reported mutations in the literature. To assess global and temporal trends in the allelic frequencies of these V410L, V1016I/G and F1534C mutations, a PRISMA-guided systematic review was conducted to analyzed their distribution and frequency, incorporating new genotyping data from five southeastern Brazilian populations. Genotyping in these populations was performed using allele-specific PCR (AS-PCR), thereby complementing the findings of the review. The results revealed that, out of a total of 187

studies, the F1534C mutation is the most studied (144 studies) and has the widest geographical distribution (47 countries, 4 continents), followed by the V410L, V1016I, and V1016G mutations. In southeast Brazil, resistant alleles were detected both individually and in co-occurrence (*e.g.*, V410L + V1016I + F1534C), and were associated with PY resistance. These mutations alter Na_v, reducing insecticide binding affinity and leading to high-level resistance—particularly when specific genotypic combinations are present. Their global spread poses a significant threat to *A. aegypti* control efforts, as PYs remain a cornerstone of public health interventions. Urgent, systematic monitoring of *kdr* allele frequencies and their synergistic effects is essential to optimize insecticide rotation strategies and prevent operational failures. This calls for coordinated international efforts to develop adaptive control strategies.

Keywords: Insectide resistance, Vector control, F1534C, Knockdown resistance, Voltage-gated sodium channel

Citation: Valencia-Marín BS, Aguirre-Obando OA, Palacio-Cortés AM, de Freitas PL, Vink JM, Navarro-Silva MA. 2025. Understanding the global dynamics of pyrethroid resistance-related *kdr* mutations in *Aedes* (*Stegomyia*) *aegypti* (Linnaeus, 1762) (Diptera: Culicidae). Zool Stud **64:**31.

BACKGROUND

The mosquito *A. aegypti* is a widely distributed African species in tropical and subtropical regions of the world, adapted to live in urban, peri-urban, and rural environments associated with humans (Kushwaha et al. 2015). *A. aegypti* is the primary vector for the transmission of dengue virus (DENV), Zika (ZIK), chikungunya (CHIKV) (Paixão et al. 2018; Simmons et al. 2012), and urban yellow fever (Deming et al. 2016), making this species a significant focus for global healthcare systems. Currently, there is no effective polyvalent vaccine or drug for the treatment of these viral diseases (Wilder-Smith 2022). Therefore, worldwide, vector control based on chemical insecticides is the most commonly used strategy to reduce the population size of *A. aegypti* and consequently decrease epidemiological outbreaks (Marcombe et al. 2019; Zara et al. 2016). Among the insecticides used for controlling the adult stage of *A. aegypti*, PYs are the most widely used option by vector control programs and the human population due to their rapid effect and low environmental toxicity (Van Den

Berg et al. 2012). However, the continuous use of these compounds has favored the emergence of *A. aegypti* populations resistant to PYs (Moyes et al. 2017; Smith et al. 2016).

PYs and organochlorines, such as DDT (started to be banned worldwide since the 1970s), interact with the voltage-gated sodium channel (Na_v), disrupting electrical signaling in the nervous system, leading to paralysis and death of the mosquito (Narahashi 1996; Rinkevich et al. 2013). Na_v is a transmembrane protein found in neuronal axons, composed of four homologous domains (I-IV), each with six hydrophobic segments (S) (Catterall 2000). The best-characterized nonmetabolic mechanism is knockdown resistance (*kdr*), first identified in houseflies, and conferring resistance to PYs as well (Farnham 1977). The L1014F mutation in the Na_v is a key mechanism underlying *kdr* resistance. This mutation induces structural alterations in the Na_v channel through amino acid substitution, potentially conferring PY resistance via two primary mechanisms: (a) reduced binding affinity of PYs to their target domains, and/or (b) altered channel gating dynamics that limit PY. (Zhorov and Dong 2017). In *A. aegypti*, most *kdr*-associated mutations are located in the IIS6 (A1007G, I1011V/M, V1016G/I) and IIIS6 (T1520I, F1534C/L) segments (Du et al. 2016). However, additional mutations have also been identified in other regions of the Na_v channel, including the IS6 segment (V410L) (Haddi et al. 2017), as well as in the IIS5-IIS6 (S989P, L982W) (Brengues et al. 2003; Du et al. 2016), the P loop of domain IV (IVS5-IVS6; D1763Y) (Chang et al. 2009) and the IIS4-IIS5 (G923V) (Brengues et al. 2003.

The *kdr* mutations can confer resistance to PYs either individually or in combination, in some cases, increasing resistance to PYs (Chen et al. 2020). Electrophysiological studies and heterologous expression in *Xenopus laevis* oocytes have shown that *kdr* mutations alone confer resistance to PYs, and their combination enhances insensitivity (Dong et al. 2014). Thus, the V410L and V1016G mutations individually confer resistance to type I PYs (permethrin) and type II PYs (deltamethrin) (Haddi et al. 2017; Smith et al. 2016). In contrast, the F1534C mutation confers insensitivity to permethrin (type I PYs), but not to deltamethrin (type II PYs) (Chen et al. 2019; Haddi et al. 2017; Hu et al. 2011). The S989P and V1016I mutations have little or no effect on PY insensitivity (Du et al. 2013; Hirata et al. 2014).

Furthermore, the co-occurrence of two or more mutations is associated with higher levels of PYs resistance (Du et al. 2013; Fan et al. 2020; Silva et al. 2021). For example, the V1016G mutation in association with S989P mutation increases resistance levels to multiple type I (permethrin) and type II (cypermethrin and deltamethrin) PYs (Chung et al. 2019; Hirata et al. 2014; Smith et al. 2018), while the double mutation V1016I + F1534C enhances insensitivity to type I (permethrin) and type II (deltamethrin) PYs (Chen et al. 2019). Triple mutations in Na_v channel are strongly associated with the

highest levels of PY resistance (Scott 2019). For instance, the triple mutation combination S989P + V1016G + F1534C significantly reduces sensitivity to both type I PYs (e.g., permethrin) and type II PYs (e.g., deltamethrin) (Hirata et al. 2014). In contrast, a different triple mutation V410L + V1016I + F1534C confers greater resistance to type II PYs, including, deltamethrin, permethrin, flumethrin, and 1R-cis αS compared to the F1534C mutation alone. Additionally, this combination provides higher resistance to 1R-cis αS and permethrin than the dual S989P + V1016G *kdr* mutations (Silva et al. 2021).

Some studies have quantified the levels of resistance conferred by *kdr* mutations to different PYs in natural populations of *A. aegypti*. It has been observed that populations with the presence of the C_{1534} allele exhibit between 7 and 16 times more resistance to type I (permethrin) and type II (cypermethrin, deltamethrin, cyhalothrin, flumethrin) PYs compared to Rockefeller laboratory strains (Fan et al. 2020). On the other hand, strains with the double mutation S989P + V1016G confer resistance between 21 and 107 times to multiple type I and II PYs (Smith et al. 2018). In the case of strains with the triple mutant allele L_{410} + I_{1016} + C_{1534}, resistance levels between 2.8 and 57 times to multiple PYs have been observed (Smith et al. 2018).

Understanding the distribution of mutations in *A. aegypti* populations can be crucial for designing effective vector control strategies, such as insecticide rotation or the implementation of alternative methods (Silva et al. 2021). However, it is important to note that, due to the chronology and discovery of new mutations, studies in the existing literature have focused on the genotyping of specific *kdr* point mutations in specific regions or countries without considering the entire extent of Na_v. This limitation in the literature may result in a biased understanding of the global distribution and frequency of *kdr* mutation alleles (Fan et al. 2020). For instance, if a study is conducted in a country and only examines the F1534C mutation, only this mutation will be reported, potentially overlooking the presence of other mutations and/or alleles. Significant research has addressed key aspects of *kdr* mutations in *A. aegypti*, including resistance levels (Chen et al. 2019; Smith et al. 2016), temporal trends (Chen et al. 2020; Fan et al. 2020), and continental-scale distribution patterns (Moyes et al. 2017). Additional studies have explored country-specific distributions and the evolutionary origins of certain alleles (Fan et al. 2020). However, no comprehensive analysis has yet synthesized the geographical and temporal frequencies of major *kdr* genotypes across multiples scales: continents, countries, and localities. Given that the mutations F1534C, V1016I/G, S989P, and V410L are the most common (Fan et al., 2020), this work aims to compile, update, and make available, through a systematic review, the temporal and geographical information on the allelic and genotypic frequencies

of the mutations V410L, V1016I/G, and F1534C by continent, country, and locality in natural populations of *A. aegypti*, including new data for five populations on the southeastern coast of Brazil.

MATERIALS AND METHODS

Information search and eligibility criteria

The mapping of global patterns of allelic frequency distribution of the mutations V410L, V1016I, V1016G, and F1534C in natural populations of *A. aegypti* was conducted using a systematic review approach following the PRISMA protocol (Moher et al. 2009). Searches were performed in the Scopus, Google Scholar, and PubMed databases using the following keywords: “410” OR “V410L” OR “Val410Leu” OR “1016” OR “Val1016Gly” OR “Val1016IIe” OR “V1016I” AND “*Aedes aegypti*” AND “*kdr*” AND "frequency". These searches were conducted from 2002 (the year of the first *kdr* mutation report in *A. aegypti*) through 2023 at annual intervals. Non-related or duplicate publications were excluded. Preselected articles and studies were included based on the following criteria: (1) Studies must report numerical data on allelic or genotypic frequencies of the V410L, V1016I, and F1534C mutations. (2) evaluated mosquito populations must include geographic information on their collection sites (latitude and longitude or approximate location) and (3) Studies were excluded it they exclusively featured results from mosquito populations subjected to artificial selection for insecticide resistance selection over generations.

Data analysis

From the obtained information, a database was constructed that includes the study name, publication year, country, locality (state, province, etc.), the number of individuals evaluated, collection date, geographical information of the collection, generation used, life stage of the individual (larva, adult, or pupa), allelic frequency, and genotypic frequency of each of the mutations independently or linked. Using this data, maps were created to represent the frequency of resistant alleles for mutations V410L, V1016I, V1016G, and F1534C at the local level in a global context. In addition, distribution maps were generated to visualize the combined frequencies of resistant alleles for the V1016I + F1534C mutations; the analyzed combinations included I_{1016}/C_{1534}, I_{1016}/F_{1534}, and V_{1016}/C_{1534}.

Similarly, for the V1016G + F1534C mutations, the assessed combinations were G_{1016}/C_{1534}, G_{1016}/F_{1534}, and V_{1016}/F_{1534} across global geographic regions.

The results obtained from the allelic and genotypic frequencies of the mutations V410L, V1016I, and F1534C in natural populations of *A. aegypti* from the Southeastern cities of Brazil, including Curitiba, Guaraqueçaba, Morretes, Antonina, and the port city of Paranaguá, were included in this database.

Sampling and genotyping the V410L, V1016I and, F1534C mutations from Brazil

The *A. aegypti* eggs from Curitiba, Guaraqueçaba, Morretes, Antonina, and the port city of Paranaguá were collected using oviposition traps as proposed by Fay and Eliason (1966). The eggs from Paranaguá were collected in 2017, 2018, and 2021, while those from the other cities were collected between 2020 and 2021. The eggs from each city were individually placed for hatching and reared to the adult stage under controlled conditions (25 ± 1°C, 80 ± 10% humidity, and a 12:12-hour light-dark cycle) in the Laboratory of Culicidae and Chironomidae Morphology and Physiology (LAMFIC[2]). Each mosquito was sacrificed and identified at the species level based on the taxonomic key by Forattini (1996), and stored at -80°C. For each locality, between 30 and 40 mosquitoes were randomly selected, and their genomic DNA was individually extracted using the Dneasy Blood & Tissue kit (Qiagen@) following the manufacturer's instructions. The DNA was eluted from the column in 40 µL and then quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) at 260 nm.

Genotyping of mutations V410L, V1016I and F1534C was performed using Allele-Specific Polymerase Chain Reaction (AS-PCR) with 1.0 µL of DNA (~ 25 ng), a common oligonucleotide, and two site-specific oligonucleotides (see Table 1). The PCR protocol for amplifying the V410L mutation followed the method described by (Haddi et al. 2017). Reactions were carried out in a final volume of 12 µL, containing 1.2 µL of 10X Buffer (comprising 100 mM Tris-HCl; pH 8.8; 500 mM KCl, and 0.8% Nonidet P40), 0.072 µM of site-specific oligonucleotides, 0.14 µM of common oligonucleotides, 0.2 mM dNTPs (Invitrogen®), 1.2 µL of MgCl2, and 2 U of Taq DNA Polymerase (Sigma®). PCR conditions were as follows: initial denaturation at 94°C for 3 minutes, 35 cycles of denaturation, annealing, and extension at 94°C/30 sec, 60°C/30 sec, and 72°C/60 sec, respectively. PCR for amplifying the V1016I and F1534C mutations followed the procedure described by Saavedra-Rodriguez et al. (2007). In both cases, reactions were carried out in a final volume of 12 µL, consisting of 1.2 µL of 10X Buffer (containing 100 mM Tris-HCl; pH 8.8; 500 mM KCl, and 0.8% Nonidet P40),

0.12 µM of site-specific oligonucleotides, 0.24 µM of common oligonucleotides, 0.29 mM dNTPs (Invitrogen®), 1.2 µL of MgCl2, and 2 U of Taq DNA Polymerase (Sigma®). PCR conditions for the mutation V1016I were as follows: initial denaturation at 95°C for 3 minutes, 32 cycles of denaturation, annealing, and extension at 95°C/30 sec, 54°C/40 sec, and 72°C/45 sec, respectively, with a final extension at 72°C for 5 minutes. For the 1534 site, the PCR conditions were: initial denaturation at 95°C for 3 minutes, 35 cycles of denaturation, annealing, and extension at 94°C/30 sec, 63°C/30 sec, and 72°C/30 sec, respectively, with a final extension at 72°C for 10 minutes. All PCR reactions included positive controls for the genotypes at the 410 (VV, VL, LL), 1016 (VV, VI, II), and 1534 (FF, FC, CC) sites, derived from the DNA extraction of *A. aegypti* Rockefeller (Rock) and Rock-*kdr* strains. The *A. aegypti* Rockefeller lineage is a standard of vigor and susceptibility to insecticides (Kuno 2014), while Rock-*kdr* strain is a PYs-resistant lineage, previously selected in our laboratory for the V1016I and F1534C mutations (Aguirre-Obando et al. 2015; Aguirre-Obando et al. 2016). Amplified alleles were verified using 4% agarose gel electrophoresis and a transilluminator (Kasvi 33-333).

Table 1. Primers used for the amplification of *kdr* mutations V410L, V1016I, and F1534C in *A. aegypti*

Name of Oligonucleotide	Sequence direction 5′ - 3 ′	Codon and Amino Acid detected
1016 Val+ F1	ACAAATTGTTTCCCACCCGCAC*C* GG	GTA/Valine
1016 Ile*kdr* F2	ACAAATTGTTTCCCACCCGCAC*T* GA	ATA/Isoleucine
1016 comom R	GGATGAACCGAAATTGGACAAAAGC	Both
1534 Phe+ F1	TCTACTTTGTGTTCTTCATCAT*A* TT	CTT/Phenylalanine
1534 Cys*kdr* F2	TCTACTTTGTGTTCTTCATCAT*G* TG	CTG/Cysteine
1534 comom R	TCTGCTCGTTGAAGTTGTCGAT	Both
410 F1:	TTACGATCAGCTGGACCGTG	GTA/Valine
410 F2:	ATCAGCTGGACCGTGGCA	TTA/Leucine
410 R1:	TTCCTCGGCGGCCTCTTC	Both

For each population, the genotypic and allelic frequencies of the V410L, V1016I, and F1534C mutations were calculated independently (Ayres et al. 2020; Martins et al. 2009; Yanola et al. 2011). Additionally, they were calculated as linked loci V1016I + F1534C (Linss et al. 2014; Melo-Costa et al. 2020) and V410L + V1016I + F1534C (Haddi et al. 2017; Melo-Costa et al. 2020). For the analysis of independent mutations, the frequencies of wild-type alleles (V_{410}, V_{1016}, and F_{1534}) and mutant alleles (L_{410}, I_{1016}, and C_{1534}) were calculated based on the observed genotypic frequencies—homozygous wild-type, heterozygous, and homozygous mutant—at three loci: 410 (genotypes VV, VL, LL), 1016 (VV, VI, II), and 1534 (FF, FC, CC). Allele frequencies and their corresponding 95% Highest Density Intervals (HDIs) were estimated using the adjusted Wald method (García et al. 2009) to account for

statistical uncertainty. Additionally, for each mutation, Wright's inbreeding coefficient (FIS) was estimated using the Hierfstat package in R Studio (Goudet 2005), followed by a $\chi 2$ p to test the null hypothesis, $F_{IS}=0$ with 1 degree of freedom, using the formula described by Black et al. (1985).

The calculation of allelic and genotypic frequencies for the V1016I + F1534C mutations results in the possible combination of nine genotypes: VVFF, VVFC, VVCC, VIFF VIFC, VICC, VVCC, VICC, and IICC, and 4 alleles: V_{1016}/F_{1534} (wild-type), V_{1016}/F_{1534} (single mutant at F1534C), I_{1016}/C_{1534} (double mutant) and I_{1016}/F_{1534} (single mutant at V1016I). (Linss et al. 2014). For the V410L + V1016I + F1534C mutations, there were 27 possible genotype combinations (Melo-Costa et al. 2020).

Deviation from Hardy-Weinberg expectations was assessed using the classical equation, and the null hypothesis of equilibrium was tested with a $\chi 2$ test with one degree of freedom (for each site) and three degrees of freedom (for the V1016I + F1534C mutations). Linkage disequilibrium between the three loci: 410-1016, 410-1534, and 1016-1534, was evaluated in each population using LINKDIS following Vera-Maloof et al. (2015). Briefly, the composite disequilibrium coefficient (Δij) (Cockerham and Weir 1977; Weir 1979) and the linkage disequilibrium correlation coefficient (R_{ij}) (Weir 1979) were calculated, along with a χ^2 test with 1 degree of freedom, to determine if there is a significant disequilibrium between all allele pair comparisons.

RESULTS

Global distribution, frequency and co-occurrence of *kdr* mutations in *A. aegypti*

Figure 1 provides the flowchart illustrating the article selection process used in this review. A total of 1002 documents were initially identified, of which only 182 were selected (Table S1). From these, a comprehensive database was created, compiling records for natural populations of *A. aegypti* by continents, countries, locations, collection years, allelic and genotypic frequencies of V410L, V1016I/G, and F1534C mutations.

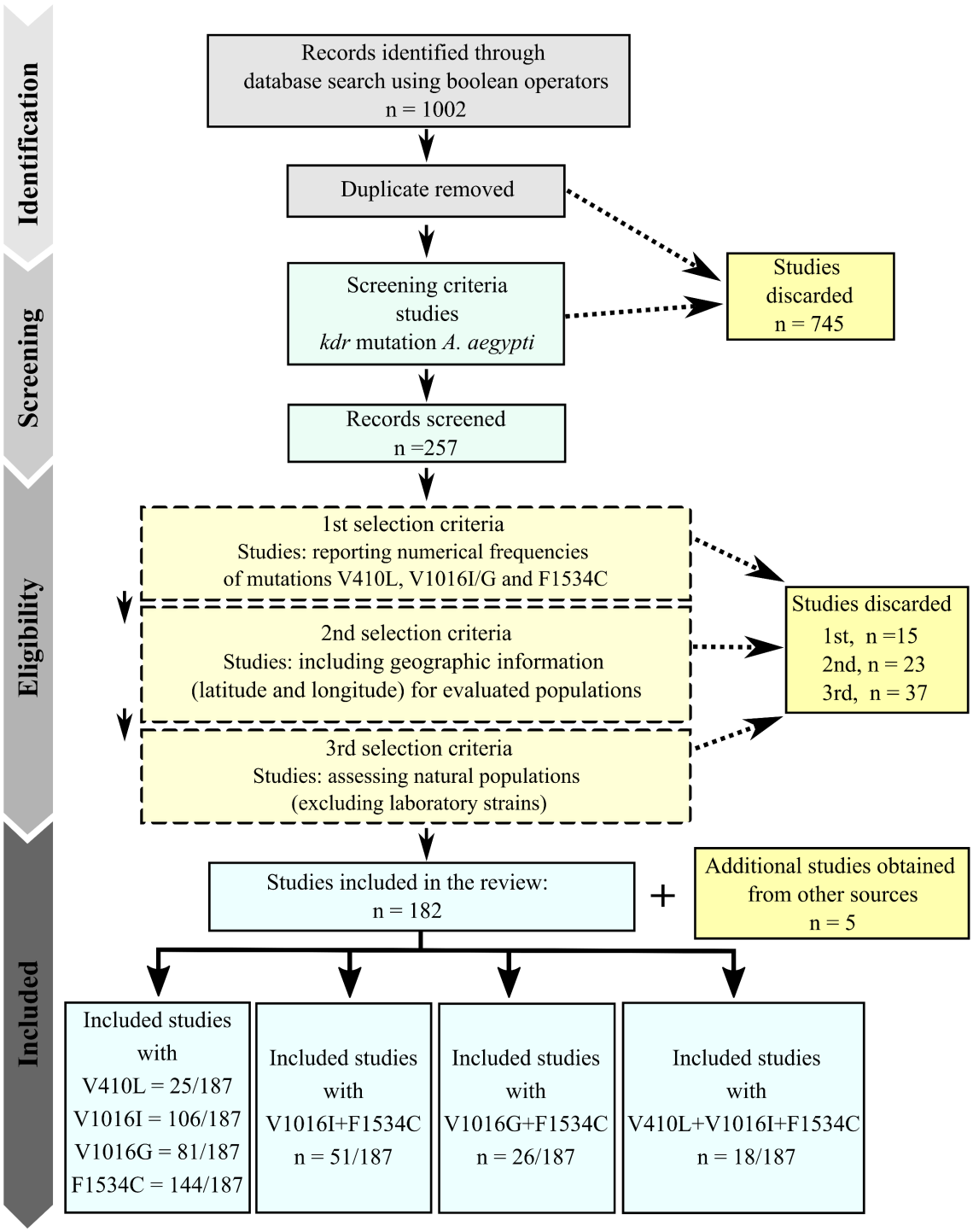

Fig. 1. PRISMA flowchart for the systematic review of *kdr* mutations V410L, V1016I, V1016G, and F1534C in natural populations of *A. aegypti.*

The V410L, V1016I, V1016G, and F1534C mutations have been reported in 13, 26, 28, and 47 countries, respectively (Fig. 2, Table S2–S5). The V410L mutation is found in the fewest number of locations, with 133 distributed across America (5 countries), Africa (6 countries), and Asia (2

countries) (Fig. 2a). The V1016I mutation is widely distributed across 563 locations, primarily in America (19 countries) and Africa (6 countries), with a single report in Asia (Vietnam) (Fig. 2b). The V1016G mutation has been reported in 244 locations in Asia and Oceania (24 countries) and Africa (3 countries), with a single report in America (Panama) (Fig. 2b). On the other hand, the F1534C mutation is the most widely distributed, reported in 651 locations across America (15 countries), Africa (9 countries), Asia, and Oceania (23 countries) (Fig. 2c). Additionally, as an indirect result obtained from our review, we present the countries and continents where the rest of the common *kdr* mutations in *A. aegypti* have been detected (Fig. 3). Within the Na_v, the following mutations have been reported across varying numbers of countries: L989W mutation has been detected in three countries, S989P (22 countries), A1007G (three countries), I1011V (10 countries), I1011M (12 countries), T1520I (four countries), F1534L (one country), and D1764Y (two countries).

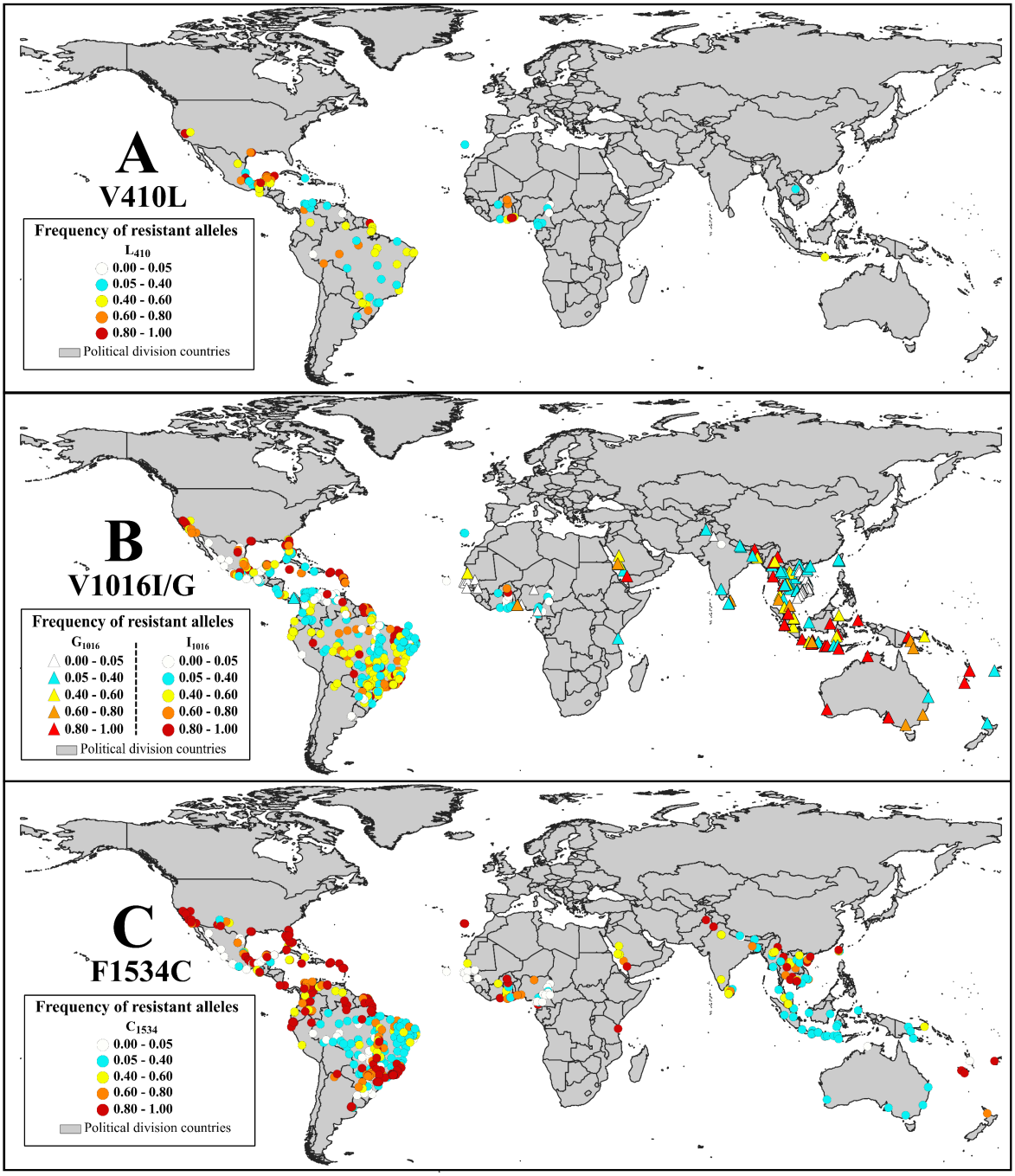

Fig. 2. Distribution of the main *kdr* mutations in *A. aegypti*. A, Distribution of V410L mutation and frequency of L_{410} allele. B, Distribution of V1016I and V1016G mutations and frequency of resistant alleles I_{1016} and G_{1016}. C, Distribution of F1534C mutation and frequency of C_{1534} allele.

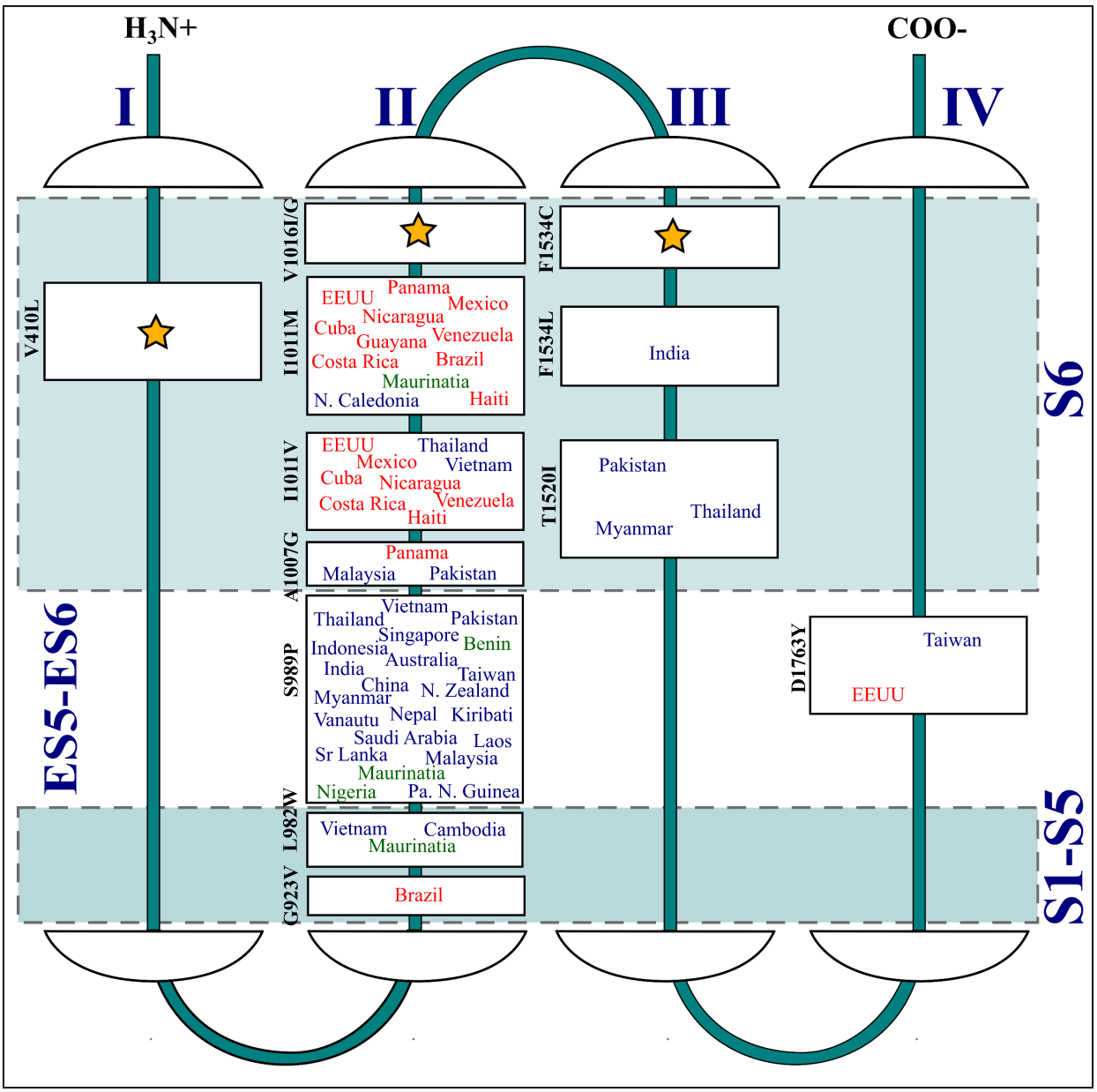

Fig. 3. Scheme of known *kdr* mutations in *A. aegypti* and their distribution by country up to 2023. The distribution of mutations containing a yellow star is illustrated in figure 2. Countries in red, blue, and green correspond to those located in the Americas, Asia - Oceania, and Africa, respectively.

Mutations occurring in combination, such as V1016I + F1534C and V1016G + F1534C, have been reported in countries across Africa, the Americas, Asia, and Oceania. The allelic and genotypic frequencies and their distribution by country and location are shown in tables S6 and S7. In Africa, the V1016I + F1534C mutations has been reported in 5 countries, and the summed frequency of resistant alleles combined I_{1016}/C_{1534}, I_{1016}/F_{1534}, and V_{1016}/C_{1534} in the evaluated locations tends to be greater than 0.4, except in Ghana, where only a single double mutant mosquito has been detected (Fig. 4a). The

kdr allele V_{1016}/C_{1534} has the highest frequency, followed by I_{1016}/C_{1534}, while the susceptible V_{1016}/F_{1534} allele is absent on the island of Madeira (Fig. 4b). In Africa, the V1016G + F1534C mutations has only been reported in Mauritania and the island of Zanzibar. In the case of Zanzibar, the V_{1016}/F_{1534} allele being the most frequent, followed by G_{1016}/C_{1534} and V_{1016}/C_{1534} (Fig. 4c). In Asia and Oceania, the V1016G + F1534C mutations has been reported in 22 countries (Fig. 5a). The allele frequencies of the V1016G + F1534C mutations varies between countries and temporal samples. In countries with extensive temporal data, such as Papua New Guinea, Saudi Arabia, and Thailand, the V_{1016}/C_{1534} and G_{1016}/C_{1534} alleles increase in frequency over time. However, in the case of Australia and Indonesia, the C_{1016}/F_{1534} and V_{1016}/F_{1534} alleles prevail with greater frequency over time (Fig. 5b). In the Americas, the co-occurrence of V1016I + F1534C mutations has been reported in 9 countries, with the summed frequency of resistant alleles I_{1016}/C_{1534}, I_{1016}/F_{1534}, and V_{1016}/C_{1534} being high (greater than 0.8) in most locations (Fig. 6a). Mexico, Colombia, and Brazil have the most evidence of the presence of the V1016I + F1534C mutations. the frequency of the susceptible wild-type V_{1016}/F_{1534} allele declines over time, while the resistant alleles I_{1016}/C_{1534} and V_{1016}/C_{1534} tend to increase in frequency (Fig. 6b). The triple mutation V410L + V1016I + F1534C has been reported in the Americas (5 countries) and Africa (5 countries) (Table S8). Figure 7 displays the distribution and frequency of the homozygous triple mutant genotype LLIICC in the locations where it has been observed.

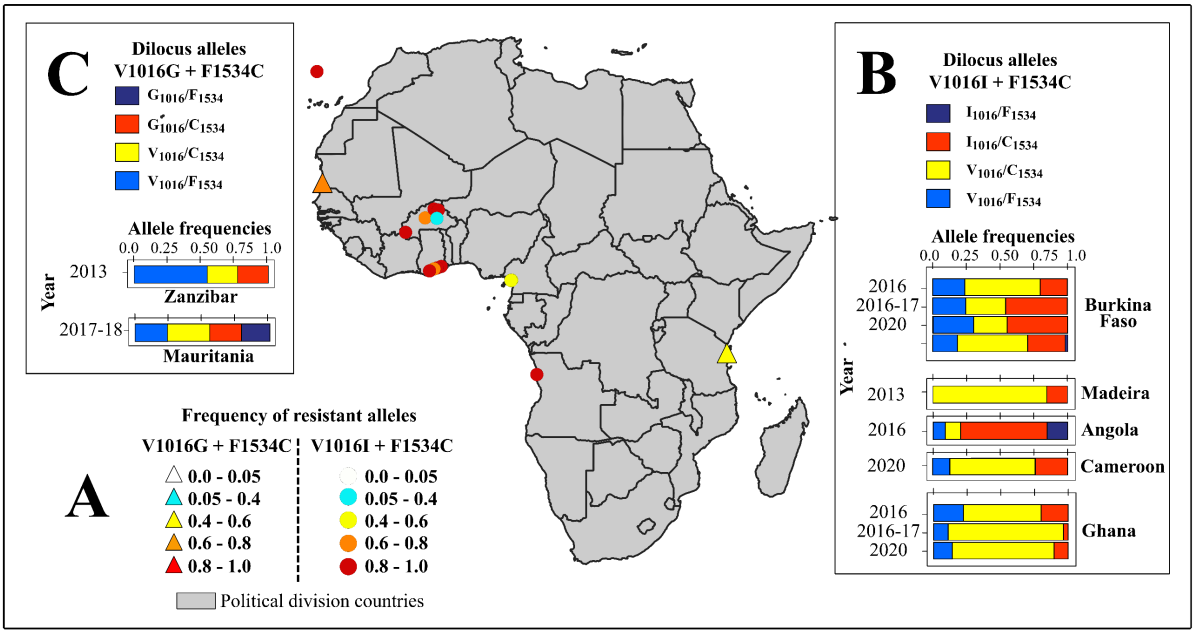

Fig. 4. Distribution and temporal frequency of two-locus mutations V1016I + F1534C and V1016G + F1534C in Africa. A, Distribution and frequency of the sum of resistant alleles V_{1016}/C_{1534}, I_{1016}/C_{1534},

and I_{1016}/F_{1534} (V1016I + F1534C); and V_{1016}/C_{1534}, G_{1016}/C_{1534}, and G_{1016}/F_{1534} (V1016G + F1534C). B, Temporal frequency by country of alleles V_{1016}/F_{1534}, V_{1016}/C_{1534}, I_{1016}/C_{1534}, and I_{1016}/F_{1534} (V1016I + F1534C). C, Temporal frequency by country of alleles V_{1016}/F_{1534}, V_{1016}/C_{1534}, G_{1016}/C_{1534}, and G_{1016}/F_{1534} (V1016G + F1534C).

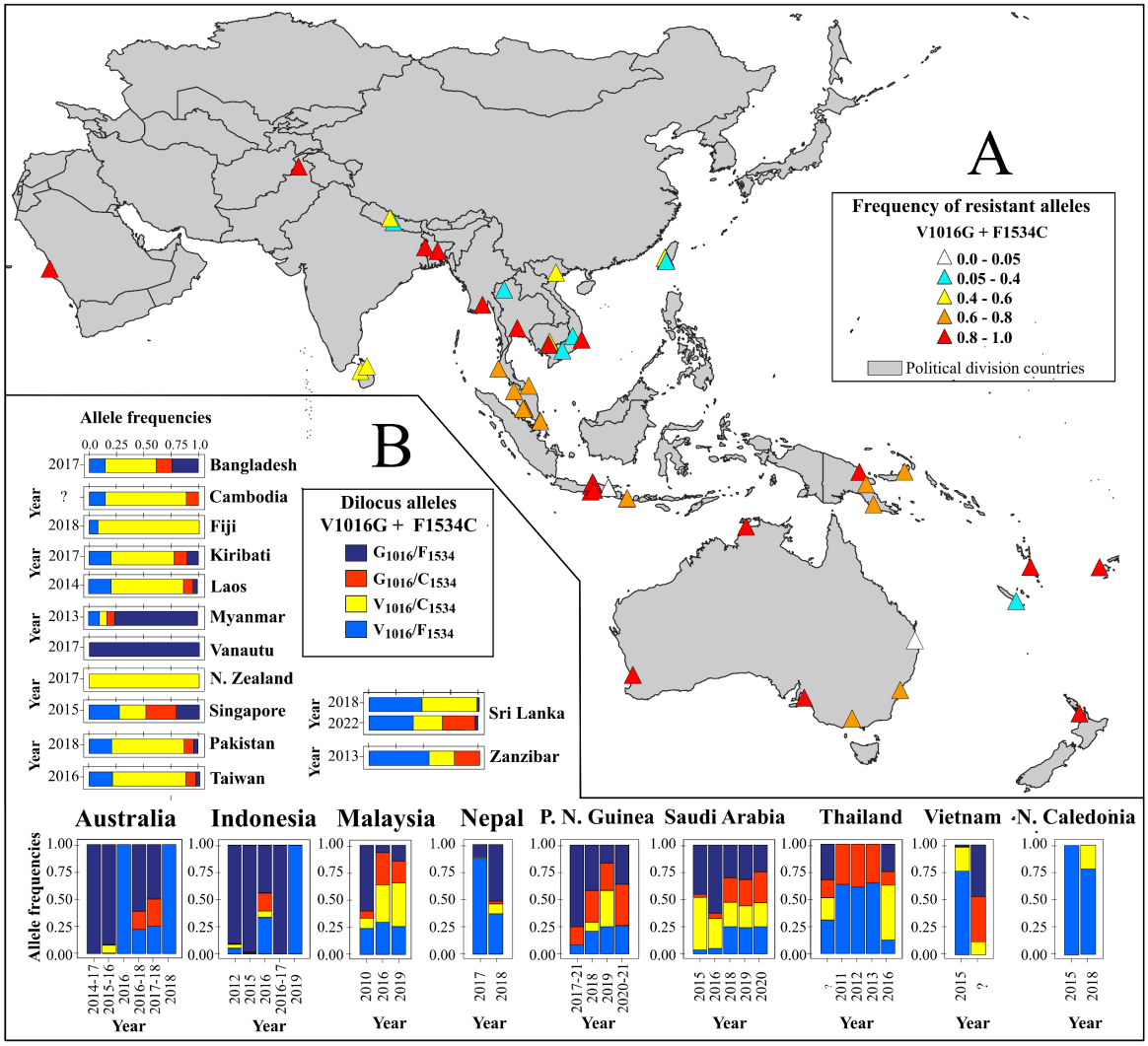

Fig. 5. Distribution and temporal frequency of two-locus mutation V1016G + F1534C in Asia. A, Distribution and frequency of the sum of resistant alleles V_{1016}/C_{1534}, G_{1016}/C_{1534}, and G_{1016}/F_{1534} (V1016G + F1534C). B, Temporal frequency by country of alleles V_{1016}/F_{1534}, V_{1016}/C_{1534}, G_{1016}/C_{1534}, and G_{1016}/F_{1534} (V1016G + F1534C).

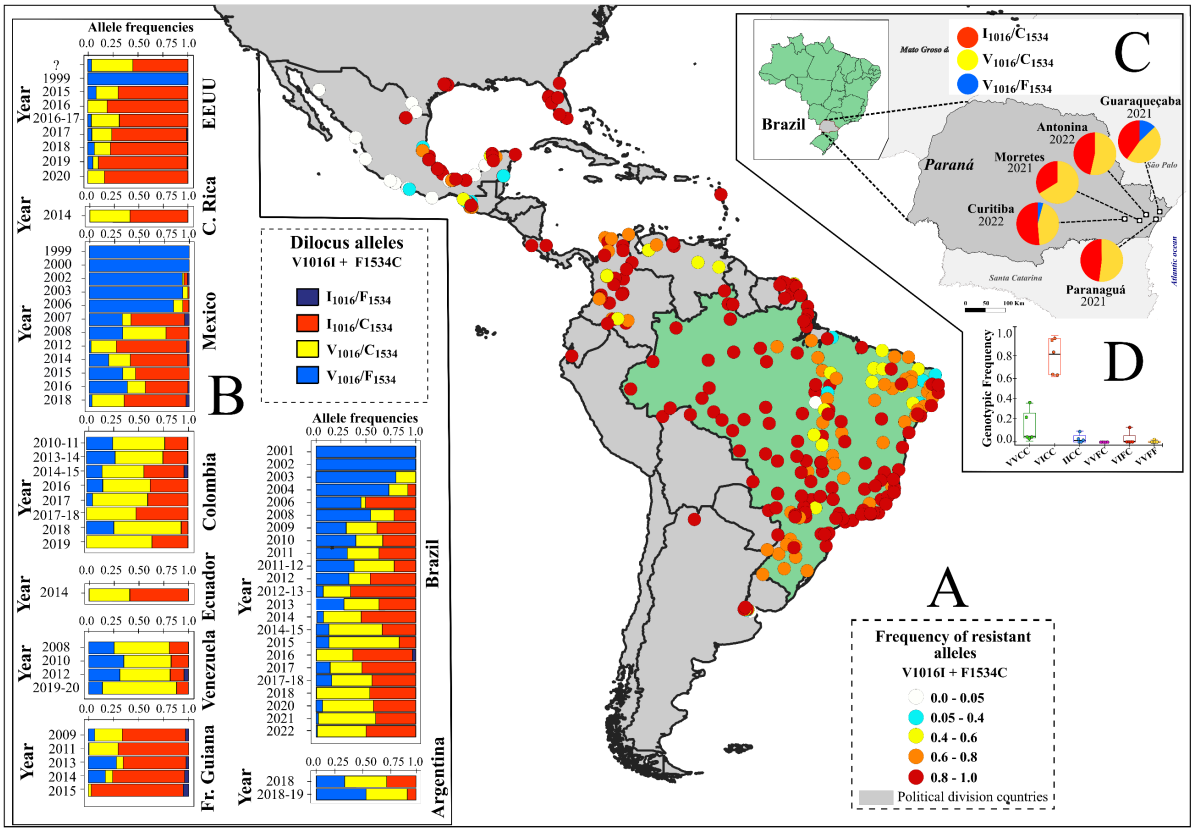

Fig. 6. Distribution and temporal frequency of two-locus mutations V1016I + F1534C in America and Brazilian Southeast. A, Distribution and frequency of the sum of resistant alleles V_{1016}/C_{1534}, I_{1016}/C_{1534}, and I_{1016}/F_{1534} (V1016I + F1534C). B, Temporal frequency by country of alleles V_{1016}/F_{1534}, V_{1016}/C_{1534}, I_{1016}/C_{1534}, and I_{1016}/F_{1534} (V1016I + F1534C). C, Frequency of V1016I + F1534C genotypes in the Brazilian southeast. D, Frequency of alleles V_{1016}/F_{1534}, V_{1016}/C_{1534}, and I_{1016}/C_{1534} in 5 populations of the Brazilian southeast.

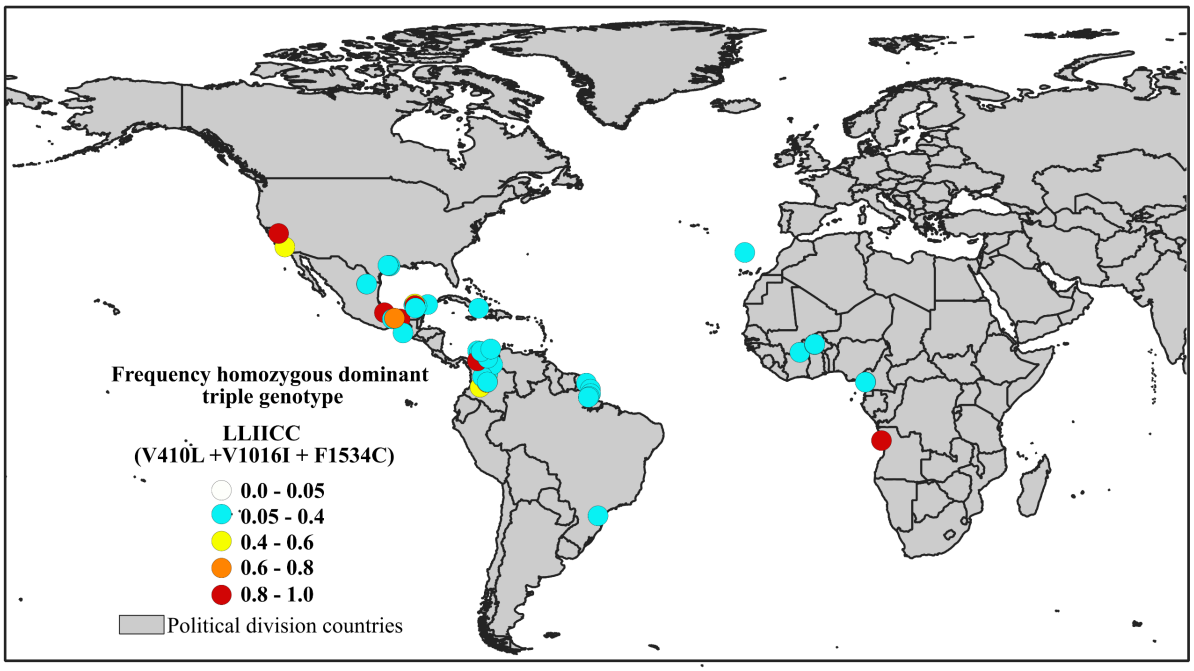

Fig. 7. Worldwide distribution and frequencies of the LLIICC genotype of the triple mutation V410L + V1016I + F1534C.

Allelic, genotypic and combined *kdr* mutation profiles of *A. aegypti* in southeastern Brazil (Paraná)

In the locations we evaluated in southeastern Brazil, the allelic and genotypic frequencies, as well as the F_{IS} statistic for the V410L, V1016I, and F1534C mutations analyzed individually, are shown in table 2. The V410L mutation was present in four out of the five analyzed populations, and the frequencies of the mutant allele L_{410} ranged from 0.275 (Curitiba) to 0.066 (Guaraqueçaba), except for Morretes, where it was absent. In the case of the mutant alleles I_{1016} and C_{1534}, they were found in all populations. The frequency of the mutant allele I_{1016} ranged from 0.512 (Curitiba) to 0.337 (Morretes), while the frequency of the C_{1534} allele ranged from 1.0 (Paranaguá and Antonina) to 0.907 (Guaraqueçaba). None of the five populations were in Hardy-Weinberg equilibrium for the V410L, V1016I, and F1534C mutations, except for Morretes. For the V410L mutation, the cities of Curitiba, Paranaguá, Antonina, and Guaraqueçaba showed a significant excess of homozygotes (F_{IS} = 1). In the case of Morretes, the entire population exhibited the wild-type genotype. For the V1016I mutation, there was a deficit of homozygotes in all populations, with FIS values ranging from -0.94 (Guaraqueçaba and Antonina) to -0.39 (Morretes). On the other hand, the F1534C mutation in Curitiba and Morretes indicated an excess of homozygotes (F_{IS} = 1). Pairwise linkage disequilibrium analysis

showed significant associations between V410L and F1534C mutations in four of the five populations analyzed. For positions 1016-1534 and 410-101, all populations were in linkage equilibrium (Table 3).

Table 2. Genotypes and allele frequencies of V410L, V1016I and F1534C mutations in five collections from Paraná, Brazil. The tablet includes site, year, sample size, genotype frequency, allele frequency for the L_{410}, I_{1016}, C_{1534}, 95% high density intervals (HDI) and inbreeding coefficients (F_{IS})

Locality	Year	V410L Genotypes				L_{410} allele Frequency and 95% HDI			Hw Disequilibrium	
		n	VV	VL	LL	Freq	Lower	Upper	F_{IS}	*P*-Value
Curitiba	2022	40	29	0	11	0.28	0.19	0.38	1.00	0.00
Paranágua	2021	40	36	0	4	0.10	0.05	0.19	1.00	0.00
Antonina	2022	30	25	0	5	0.13	0.09	0.29	1.00	0.00
Guaraquecaba	2021	30	28	0	2	0.07	0.02	0.17	1.00	0.00
Morretes	2021	40	40	0	0	0.00	-0.01	0.06	-	0.00
Locality	Year	V1016I Genotypes				I_{1016} allele Frequency and 95% HDI			Hw Disequilibrium	
		n	VV	VI	II	Freq	Lower	Upper	F_{IS}	*P*-Value
Curitiba	2022	40	3	33	4	0.51	0.40	0.62	-0.65	0.00
Paranágua	2021	40	2	38	0	0.48	0.37	0.59	-0.91	0.00
Antonina	2022	30	1	29	0	0.48	0.36	0.61	-0.94	0.00
Guaraquecaba	2021	30	6	23	1	0.42	0.30	0.55	-0.57	0.00
Morretes	2021	40	14	25	1	0.34	0.24	0.45	-0.40	0.01
Locality	Year	F1534C Genotypes				C_{1534} allele Frequency and 95% HDI			Hw Disequilibrium	
		n	FF	FC	CC	Freq	Lower	Upper	F_{IS}	*P*-Value
Curitiba	2022	40	1	0	39	0.98	0.91	1.00	1.00	0.00
Paranágua	2021	41	0	0	40	1.00	0.94	1.00	-	-
Antonina	2022	30	0	0	30	0.93	0.93	1.00	-	-
Guaraquecaba	2021	30	0	4	26	0.91	0.83	0.98	-0.05	0.70
Morretes	2021	40	1	0	39	0.98	0.91	1.00	1.00	0.00

Table 3. Linkage disequilibrium coefficients (Rij), χ^2 and associated probabilities between loci 410-1016, 410-1534 and 1016-1534

Site	Year	410 - 1016			410 - 1534			1016 -1534		
		Rij	χ^2	P	Rij	χ^2	P	Rij	χ^2	P
Curitiba	2022	0.18	0.83	0.3542	1,00	173.36	0.0001	0.37	3,14	0.0764
Paranágua	2021	0.08	0.04	0.8331	-	-	-	-	-	-
Antonina	2022	0.08	0.02	0.8822	1,00	130.61	0.0001	0.08	0,02	0.8997
Guaraquecaba	2021	0.10	0.26	0.6087	0,94	129.25	0.0001	0.03	0,01	0.9080
Morretes	2021	-	-	-	-	-	-	0.10	0,51	0.4753

Table S6 shows the genotypic and allelic frequencies for the V1016I + F1534C mutations. In general, the VICC genotype was the most frequent in all populations, ranging from 0.967 (Antonia) to 0.60 (Guaraqueçaba) (Fig. 6d), in contrast to the VVFC genotype, which was absent in all evaluated locations. The resistant genotypes VVCC, IICC, and VICC together reached an average global frequency of 0.747. In Paranaguá, Antonina, and Morretes, exclusively the three resistant genotypes were found. The locations with the highest frequency of resistant genotypes were Paranaguá, Antonina,

and Morretes (1.0), and the lowest was Guaraqueçaba (0.933). Among all mosquitoes, 4 alleles were found: $V_{1016}/F_{1534,}$ $V_{1016}/C_{1534,}$ I_{1016}/F_{1534} and I_{1016}/C_{1534} (Fig. 6c). In the case of the I_{1016}/F_{1534} allele, only one individual was found, so it was excluded, leading to adjustments in the calculation for the other alleles. In the study area, southeastern Brazil, Paraná state, the average frequency of the most common allele V_{1016}/C_{1534} was 0.487, followed by I_{1016}/C_{1534} 0.475. The double wild-type allele V_{1016}/F_{1534}, had the lowest frequency (0.02).

Among the locations, Curitiba and Paranaguá had a higher frequency of the I_{1016}/C_{1534} allele compared to V_{1016}/C_{1534}. Meanwhile, the wild-type allele V_{1016}/F_{1534} was found in the locations of Curitiba, Guaraqueçaba, and Paranagúa (except in 2021) with frequencies below 0.14. In the case of the port city of Paranaguá, there is a temporal reduction in the frequency of the V_{1016}/F_{1534} allele in samples from 2017 to 2021 (Table S9). In all cases, the genotypic frequencies in the five locations deviated from the assumption of Hardy-Weinberg equilibrium (Table S10).

The analysis in the five populations for the V410L + V1016I + F1534C trihybrid mutations, suggests eight genotypes. The three most common genotypes globally were VVVICC (0.71), followed by VVVVCC (0.13), and LLVICC (0.10) (Fig. 8a). The genotypes with lower frequencies were LLIICC, VVVIFF, and VVVVFF, each with a frequency of 0.005. The results of genotypic frequencies by location are shown (Fig. 8b). The VVVVCC genotype was observed in all five populations, with Guaraqueçaba having the highest frequency for this allele (0.166). On the other hand, the wild-type genotype for all three sites, VVVVFF, was only found in Curitiba with a frequency of 0.025. The VVVICC genotype was the most frequent, ranging in frequency from 0.87 (Paranaguá) to 0.57 (Curitiba) (Fig. 8b), while the VVVIFC and LLVVCC genotypes were only observed in Guaraqueçaba with frequencies of 0.133 and 0.033, respectively. In the case of the resistant LLVICC genotype, it was found in four locations, except for Morretes, with frequencies ranging from 0.25 to 0.03 in Curitiba and Guaraqueçaba, respectively. The homozygous mutant genotype for all three positions, LLIICC, was only detected in low frequency in Curitiba (0.025).

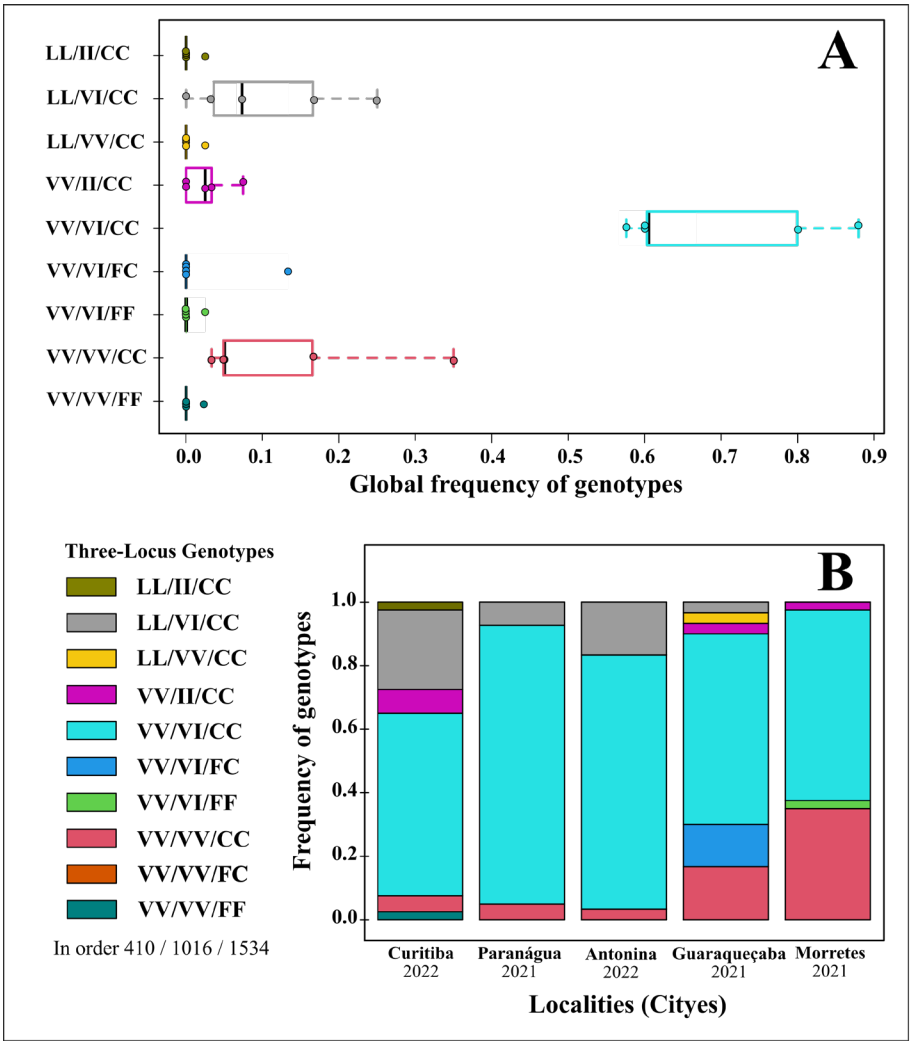

Fig. 8. Three-locus mutation genotype frequencies V410L + V1016I + F1534C in natural populations of *A. aegypti* in the Brazilian southeast. **a.** Global frequency of three-locus genotypes in Paraná. **b.** Three-locus genotypes frequency for City: Curitiba, Paranaguá, Antonina, Guaraqueçaba, Antonina, and Morretes.

DISCUSSION

In *A. aegypti*, 13 mutations within the Na_v gene have been identified and evaluated both individually and in two-locus or three-locus combinations. In some cases, the entire Na_v has been sequenced (Chen et al. 2020). Our results regarding the distribution of individual and linked mutations indicate that there are fewer reports and studies for two-locus co-occurring mutations (n = 43; V1016I + F1534C or n = 22; V1016I + F1534C) or three-locus co-occurring mutations (n = 12; V410L + V1016I + F1534C) compared to individual mutations (n = 18; V410L, n=95; V1016I, n = 71; V1016G,

n = 124; F1534C). This may be attributed to what Fan et al. (2020) mentioned, that the history of discovery of different mutations means that most studies only report on part of the potentially existing mutation landscape within their samples, resulting in a biased view.

Our results contribute to enhancing our understanding of the distribution of the most common *kdr* mutations and alleles in *A. aegypti*, which vary from locality to locality, country to country, and continent to continent. This review reveals that individual mutations V1016G and F1534C are found in countries across Asia, Oceania, Africa, and the Americas. Mutation V410L has been observed in Oceania, Africa, and the Americas, while mutation V1016I is present in Asia, Africa, and the Americas. Mutation V1016G has been reported in 71 studies and across 309 localities in 28 countries. The frequency of the mutant allele G1016 varies between countries and sampling years, ranging from a minimum of 0.00 to a maximum of 0.99. This mutation was first reported in 2002 in Thailand (Brengues et al. 2003). Since then, it has remained prevalent in Asia and Oceania (Chen et al. 2020). However, in 2019 and 2020, it was detected in the Americas (Panama) and Africa (Western and Eastern regions), respectively (Djiappi-Tchamen et al. 2021; Kampango et al. 2022; Murcia et al. 2019). On the other hand, mutation V1016I has been reported in 95 studies across 26 countries. It was first detected in Latin America (Saavedra-Rodriguez et al. 2007) and subsequently observed in multiple countries in the Americas, Africa, and Asia (Vietnam) (Fernando et al. 2018; Linss et al. 2014; Sombié et al. 2019; Zardkoohi et al. 2020). The origin of V1016I/G mutations in African populations of *A. aegypti* is still not well understood (Kampango et al. 2022).

We found that the most frequently reported and geographically widespread mutation in *A. aegypti* is F1534C, which has been reported in 124 studies across 43 countries. It was first detected in Asia (Vietnam) (Kawada et al. 2009) and subsequently in multiple countries across Oceania, Africa, and the Americas (Cosme et al. 2020; Kushwah et al. 2015; Kushwah et al. 2020; Plernsub et al. 2016; Sombié et al. 2019; Yanola et al. 2011; Zardkoohi et al. 2020). The *kdr* allele C1534 is recurrent both temporally and geographically in all regions of the world, with two independent evolutionary origins suggested for this mutation (Cosme et al. 2020). In the populations of the Americas, the origin of F1534C has been postulated by Fernando et al. (2018) in mosquito samples dating back to 2000 and more recently with genomic data (Love et al. 2023). The F1534L allele, an alternative substitution at the same position, has only been found in India (Kushwah et al. 2020). The V410L mutation has been observed in 18 studies across 13 countries, first detected in Brazilian populations (Haddi et al. 2017), and subsequently in other populations across the Americas, and more recently in Africa, including populations where it was previously absent (Ayettey et al. 2023). In our results, the frequency of this mutation varies from country to country and is commonly observed in conjunction with the double

mutation V1016I + F1534C (Ayres et al. 2020; Melo-Costa et al. 2020; Saavedra-Rodriguez et al. 2018; Toé et al. 2022). However, V410L has been found in the absence of V1016I and F1534C (Granada et al. 2021; Haddi et al. 2017).

Mutations in co-occurrence, V1016I + F1534C and V1016G + F1534C, have been reported in countries across the Americas, Africa, Asia, and Oceania. In the case of the double mutation V1016I + F1534C, it has been reported in a total of 16 countries in Africa and the Americas. This double mutation was first detected in North America (Mexico) (Aponte et al. 2013) and South America (Brazil) (Linss et al. 2014). However, it was subsequently found in various regions and countries in the Americas (Chen et al. 2020). In Africa, it was first detected in Ghana (Kawada et al. 2016) and later in Burkina Faso (Toé et al. 2022), Cameroon (Yougang et al. 2022), Angola, and Madeira Island (Ayres et al. 2020).

Our local results for Southeast Brazil show that the allele frequencies for the two-locus mutations V1016I + F1534C are high (0.975). Alleles V_{1016}/C_{1534} and I_{1016}/C_{1534} were present in four out of the five populations. Concerning the V_{1016}/C_{1534} allele, it has been suggested to be the first *kdr* allele to spread in Latin America (Kawada et al. 2009) and may have at least two independent phylogenetic origins (Cosme et al. 2020). We observed that the population in Curitiba had a higher frequency of the I_{1016}/C_{1534} allele compared to the V_{1016}/F_{1534} and V_{1016}/C_{1534} alleles. The I_{1016}/C_{1534} allele confers a higher level of resistance to PY compared to V_{1016}/C_{1534}. Temporal results by country obtained from the systematic review show that the resistant I_{1016}/C_{1534} allele tends to increase over time, while the susceptible V_{1016}/F_{1534} allele decreases. This pattern was observed in Colombia, Guyana, the USA, Mexico, and Brazil. In the case of Mexico and Brazil, this pattern has been previously reported in multiple studies and populations (Linss et al. 2014; Macoris et al. 2018; Vera-Maloof et al. 2015). This indicates that resistant alleles increase in frequency over time in populations under PYs selection pressure, even in regions where these alleles were previously absent (De Araújo et al. 2019; Melo-Costa et al. 2020).

On the other hand, the double mutation V1016G + F1534C has been assessed in at least 22 studies across 21 countries. The double mutant allele GGCC has been observed in at least 16 countries in Asia, Oceania, and Africa. This double mutation was first reported in Vietnam in 2009 (Kawada et al. 2009) and has subsequently been widely detected in various Asian and Oceanian countries (Chen et al. 2020). Recently, the double mutation V1016G + F1534C was first detected in Africa, along with S989P, in Benin (Tokponnon et al. 2023).

The triple mutation V410L + V1016I + F1534C has been reported in 11 countries in the Americas and 5 in Africa. This mutation was first detected in *A. aegypti* populations in northern Brazil

and has since been widely reported in Mexico, the USA, and Colombia. Regarding the triple mutant genotype LLIICC, in our local results, we found this genotype in low frequency in the Curitiba population. However, in Brazil, this genotype has been observed at high frequency and widely distributed in a study that included information from 25 natural A. aegypti populations in Mexico (Melo-Costa et al. 2020), Colombia, and the USA, this allele has been reported at high frequencies and distribution (Fan et al. 2020; Saavedra-Rodriguez et al. 2018).

In various studies, the impact of specific mutations on resistance to PYs has been extensively evaluated in bioassays and electrophysiological studies (Chen et al. 2020; Smith et al. 2016; Smith et al. 2018). This knowledge is crucial for predicting how mosquito survival in regions subjected to PYs control may be affected by mutations in the Na_V. An illustrative example is the V410L mutation, for which it has been suggested that it alone does not provide complete protection against knockdown but rather facilitates the mosquito's recovery once the PYs dissociates from the ion channel and is metabolized and excreted (Saavedra-Rodriguez et al. 2021). However, when the V410L mutation coexists with the V1016I + F1534C mutations, elevated levels of resistance to type II PYs, such as deltamethrin, flumethrin, and 1R-cis αS cypermethrin, are observed, compared to other mutations like F1534C or S989P + V1016G (Silva et al. 2021). The interaction between different mutations and their effects on PYs underscores the complexity of this phenomenon and the ongoing need for further research to address the challenges posed by mosquito vector resistance to insecticides.

Our indirect results show the distribution of the remaining mutations in *A. aegypti*. Starting with the S989P mutation, which is commonly found in Asia, this mutation occurs individually or in co-occurrence with the V1016G or F1534C mutations (Amelia-Yap et al. 2019; Fernando et al. 2018; Plernsub et al. 2016; Smith et al. 2018). The S989P mutation was recently detected in Africa (Tokponnon et al. 2023) and in co-occurrence with F1534C (Fagbohun et al. 2022) and with V1016G in *A. aegypti* imported at airports in Australia and Oceania (Schmidt et al. 2019). The G923V and L982W mutations were only detected in 2003 in Brazil and Vietnam, respectively (Brengues et al. 2003). Recently, L982W was detected again in *A. aegypti* populations in Vietnam and Cambodia (Kasai et al. 2022).

The L1011M mutation has been reported in 10 countries in the Americas, initially detected in co-occurrence with the G923V mutation (Brengues et al. 2003). Subsequent studies identified L1011M in various locations in the Americas (Saavedra-Rodriguez et al. 2007), particularly in Brazil (Brito et al. 2018; De Araújo et al. 2019; Garcia et al. 2018; Martins et al. 2013) and in a single report in a French overseas territory (Dusfour et al. 2015). In 2019, the L1011M mutation was observed in co-occurrence with V1016I in a mosquito in Panama (Murcia et al. 2019). Another mutation in the same

segment and position, I1011V, has been observed in 8 countries in the Americas and in two locations in Asia: Nha Trang, Vietnam, and Thailand (Rajatileka et al. 2008).

As for the D1763Y mutation, it has been reported in 4 studies in mosquito populations in Asia (Taiwan). This mutation was first reported in 2009 by Chang et al. (2009) and has been commonly observed in co-occurrence with V1016G and V1016G + F1534C (Biduda et al. 2019; Chang et al. 2009; Chung et al. 2019). It was recently detected in low frequency in the United States (Florida) (Kosinski et al. 2022).

Other mutations that have been observed at low frequency in *A. aegypti* populations are T1520I and A1007G. In the case of T1520I, it has only been observed in four Asian countries, with its first record in India (Kushwah et al. 2015). This mutation has been observed in co-occurrence with the F1534C mutation (Fan et al. 2020; Kushwah et al. 2015; Rahman et al. 2021) and in multiple combinations with the F1534C + V1016G + S989P mutations (Naw et al. 2022). On the other hand, the A1007G mutation has only been detected in 2 Asian countries, initially found in Vietnam (Lien et al. 2018) and later in Malaysia (Zuharah and Sufian 2021).

CONCLUSIONS

Resistance to pyrethroids in *A. aegypti* populations represents a globally heterogeneous and dynamic challenge, shaped by the uneven geographic distribution of *kdr* mutations and influenced by local selection pressures. Our systematic review, complemented by new genotypic data from five populations in Paraná, Brazil, confirms the widespread presence of key resistance-associated alleles (V410L, V1016I, F1534C), both individually and in multi-locus combinations. Notably, the detection of the triple mutant genotype L410L + I1016I + C1534C (LLIICC) in Curitiba, alongside elevated resistant allele frequencies in agricultural and urban coastal municipalities (Paranaguá, Morretes, and Antonina), underscores *A. aegypti*'s capacity to rapidly adapt to insecticide-intensive environments. In contrast, lower resistance levels in more isolated and less anthropogenically influenced areas like Guaraqueçaba suggest that reduced selection pressure corresponds with lower mutation frequencies, reinforcing the link between human activity and resistance evolution. These regional disparities emphasize the importance of local context in resistance monitoring and management.

Critically, *kdr* mutations may synergize with metabolic resistance mechanisms, such as cytochrome P450-mediated detoxification, thereby amplifying resistance phenotypes and complicating control efforts. This interaction, demonstrated in heterologous systems (*Drosophila* and/or *Culex*), can

drastically diminish insecticide efficacy and accelerate resistance spread in natural populations. To confront this multifactorial threat, we strongly advocate for integrated and standardized surveillance systems that combine insecticide susceptibility testing, *kdr* genotyping, and metabolic resistance profiling. Such approaches are essential to guide adaptive vector control strategies—including insecticide rotation, the use of synergists, and habitat management—tailored to local resistance profiles. Moreover, international collaboration is crucial to harmonize methodologies, facilitate data sharing, and promote coordinated research on the interactions between resistance mechanisms. In summary, this study highlights the urgent need for proactive, regionally informed, and globally coordinated responses to preserve the long-term effectiveness of vector control tools and sustain efforts in the fight against *A. aegypti*-borne diseases.

Acknowledgments: We express our gratitude to the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) for their support, specifically for granting scholarships to BSVM (CNPq, process # 163293/2021).

Author contributions: BSVM: Methodology, Data Curation, Writing – original draft and Visualization. OAAO: Conceptualization, Writing - Review & Editing and Validation. AMPC: Methodology, Writing - Review & Editing. PLF & JMV: Resources. MANS: Supervision, Writing - Review & Editing.

Conflict of interests: The authors declare that there is no conflict of interest.

Availability of data and materials: -

Consent for publication: -

Ethics approval consent to participate: -

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Supplementary materials

Table S1. Na_v mutations detected in studies and populations of *A. aegypti* resistant to PYs. (download)

Table S2. Database of the global and temporal compilation of the distribution and frequency by countries and locations of the V410L mutation. (download)

Table S3. Database of the global and temporal compilation of the distribution and frequency by countries and locations of the V1016I mutation. (download)

Table S4. Database of the global and temporal compilation of the distribution and frequency by countries and locations of the V1016G mutation. (download)

Table S5. Database of the global and temporal compilation of the distribution and frequency by countries and locations of the F1534C mutation. (download)

Table S6. Database of the global and temporal compilation of the distribution and frequency by countries and locations of the two-locus mutation V1016I + F1534C. (download)

Table S7. Database of the global and temporal compilation of the distribution and frequency by countries and locations of the two-locus mutation V1016G + F1534C. (download)

Table S8. Database of the global and temporal compilation of the distribution and frequency by countries and locations of the three-locus mutation V410L + V1016I + F1534C. (download)

Table S9. Distribution and temporal frequency of alleles of two-locus mutations V1016I + F1534C between 2017–2021 in natural populations *of A. aegypti* in the port city of Paranaguá. (download)

Table S10. Number and frequency of two-locus genotypes and alleles V1016I + F1534C in five natural populations of *A. aegypti* in southeastern Brazil.