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High voltage-gated sodium channel gene diversity in *Aedes albopictus* across Brazil

Gabriel Joventino Nascimento[1], Luciano Veiga Cosme[2], André Luiz Quintanilha Torres[1], José Bento Pereira Lima[1] & Ademir Jesus Martins[1,3]✉

The invasive *Aedes albopictus*, commonly known as the Asian tiger mosquito, has spread globally, posing public health risks because of its role as a secondary vector for arboviruses and capacity to transmit pathogens across sylvatic and urban cycles. In Brazil, where *Ae. aegypti* remains the primary vector of dengue, Zika, and chikungunya viruses; *Ae. albopictus* is being increasingly monitored because of its ecological plasticity and potential to develop insecticide resistance. Here, we analyzed the genetic diversity of voltage-gated sodium channel (*Na_V*) gene in *Ae. albopictus* populations across Brazil, in which knockdown resistance mutations (*kdr*) are associated with pyrethroid resistance. We collected *Ae. albopictus* from 46 Brazilian cities, extracted DNA from individual mosquitoes, and prepared pooled samples for next-generation sequencing. We targeted two *Na_V* segments, regions commonly associated with *kdr* in other mosquito species: IIS6 and IIIS6 segments. High-throughput sequencing and bioinformatics analysis were used to assess haplotype diversity, distribution, and phylogenetic relationships. We identified 20 IIS6 and 24 IIIS6 haplotypes, indicating high genetic diversity within the *Na_V* gene among Brazilian *Ae. albopictus* populations. No *kdr* mutations were detected despite the documented occurrence of these mutations in *Ae. albopictus* from other regions of the world. Nonetheless, we observed several synonymous polymorphisms, suggesting ancestral variation and potential for adaptive evolution. Our findings revealed substantial genetic diversity within the *Na_V* gene in Brazilian *Ae. albopictus* populations but no current evidence of pyrethroid resistance-associated *kdr* mutations. The observed diversity provides a foundation for tracking shifts in allele frequencies that may affect insecticide susceptibility and vector competence. Continuous monitoring of genetic variation is essential to preemptively address the development of resistance in *Ae. albopictus* and mitigate potential public health risks.

Keywords Asian tiger mosquito, *Aedes albopictus*, Molecular surveillance, Diversity, Vector genetics

In recent decades, *Aedes (Stegomyia) albopictus* (Skuse, 1894), commonly known as the Asian tiger mosquito, has garnered significant scientific and public health attention due to its global spread and role in arbovirus transmission[1,2]. A distinctive feature of *Ae. albopictus* is the chitinous serosa layer between the embryo and chorion eggshell, which enhances desiccation resistance and supports population expansion, particularly in urban environments[3]. Ranked among the world's most invasive species, *Ae. albopictus* exhibits a remarkable capacity for propagation, posing epidemiological risks when it establishes populations[2]. Over the past four decades, this mosquito has spread from Southeast Asia to Europe, Africa, the Middle East, and the Americas, leaving Antarctica as the only unaffected continent. Genetic studies offering insights into its evolutionary trajectory trace its origins to the islands of the Western Pacific and Indian Oceans[4]. Trade navigation and more recently, climate and environmental changes have further broadened its geographic distribution, creating conditions for the expansion of its establishment in diverse regions[5,6].

In Brazil, *Ae. albopictus* was first reported in Rio de Janeiro State[7] and has since been detected across other states, raising concerns about its rapid spread and underscoring the importance of understanding its behavior and gene flow for effective control. Although *Ae. aegypti* remains the primary vector of arboviruses such as dengue (DENV), chikungunya (CHIKV), and Zika (ZIKV) in the Americas, *Ae. albopictus* has demonstrated the ability to transmit these viruses in specific regions of the globe[4,8] and, notably, under controlled laboratory conditions[9–11].

[1]Laboratório de Biologia, Controle e Vigilância de Insetos Vetores, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil. [2]Department of Entomology, University of California, Riverside, CA, USA. [3]Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular, Rio de Janeiro, Brazil. ✉email: ademirjr@ioc.fiocruz.br

Unlike *Ae. aegypti*, *Ae. albopictus* exhibits a broader host range, feeding on various vertebrates, including reptiles and mammals, and its larvae can survive in both artificial and natural water reservoirs[12,13]. These traits suggest *Ae. albopictus* can act as a bridge for pathogen transmission between sylvatic and urban cycles[2]. Additionally, the species' potential for vertical transmission of arboviruses, combined with photoperiodic diapause in temperate regions, supports virus persistence across seasons[14–16].

Insecticide resistance presents a major global challenge for mosquito control, particularly with respect to resistance to pyrethroids, the most commonly used insecticide class for household mosquito control[17]. A primary mechanism conferring pyrethroid resistance across various insect orders involves mutations in the voltage-gated sodium channel gene (Na_V), commonly known as knockdown resistance mutations (*kdr*), which diminish susceptibility to the knockdown effect of pyrethroids[18]. The most well-characterized *kdr* mutations are single nucleotide polymorphisms (SNPs) that occur in the conserved regions of $\mathrm{Na_V}$. This channel is composed of four homologous domains (I-IV), each formed by six transmembrane segments (IS6-IVS6) that form the sodium channel pore of the excitable cells. Most *kdr* SNPs occur in the IIS6 and IIIS6 segments, generally at conserved sites. For example, substitutions at $\mathrm{Na_V}$ sites 1016 (V1016G, IIS6) and 1534 (F1534C, IIIS6) were observed in both *Ae. aegypti* and *Ae. albopictus*[17].

South America ranks among the world's highest insecticide users for agricultural and public health purposes. For example, pesticide use has surged by 120% in the last 20 years[19], with potentially serious implications for public health and the environment. Since 1999, a robust insecticide resistance monitoring program has been established in Brazil, accompanied by extensive studies on the molecular mechanisms underlying resistance[20,21], however relatively few studies have focused on *Ae. albopictus.* Given the recent introduction and rapid spread of *Ae. albopictus* in Brazil, understanding the ecological and genetic factors driving insecticide resistance is critical for the design of effective control strategies. In this study, we examined the genetic diversity of two Na_V gene segments in *Ae. albopictus* sampled from 46 localities in Brazil.

Methods

Mosquito collections

Mosquito sampling was conducted between 2017 and 2018, as part of a previous study aimed at monitoring the resistance of *Aedes aegypti* to the insecticides pyriproxyfen and malathion across Brazil. Eggs were collected using ovitraps installed in urban centers across 146 selected cities nationwide. Once collected, the eggs were shipped to the laboratory for further processing (see details in Campos et al., 2020[21]). Upon arrival, the eggs hatched, larvae reared, and adult mosquitoes identified by species. *Ae. albopictus* was cryopreserved and used in this study. We selected a sample of 998 mosquitoes from 46 cities, including 11 cities in the north (n = 254), 11 in the northeast (n = 245), 11 in the southeast (n = 276), 10 in the Center-West (n = 175), and three in the south (n = 48). Supplemental Table S1 provides further information on the sampling locations, including city, state, geographic coordinates, and number of mosquito samples.

DNA extraction and population pool preparation

Each mosquito was placed in a 2 mL microtube containing 50 µL of buffer and two glass beads. Cells were lysed by shaking the samples vigorously for 1 min using TissueLyser II (Qiagen). Subsequent steps of the DNA extraction were performed using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's protocol. The DNA concentration of each sample was measured using a NanoDrop One C (ThermoFischer Scientific) spectrophotometer, and samples were diluted to 20 ng/µL with ultra-pure water. To create the DNA pool for each population, we combined all individual DNA samples from the same locality (from 2 to 30 samples, with an average of 22 per locality) (Supplemental Table S1). Each pool was adjusted to contain 20 ng DNA.

Na_V segments amplification

In other mosquito species, most mutations associated with knockdown resistance (*kdr*) are located in the gene exons encoding the IIS6 and IIIS6 segments. To explore polymorphism diversity in these regions, we designed specific primers to amplify these segments using Geneious Prime 2024.0.7 (http://www.geneious.com/), based on the sequence XM_029865132.1 from GenBank (NCBI) as a reference, and forward and reverse primers were designed to generate fragments of approximately 500 bp for IIIS6 segment and 400 bp for IIS6 segment. The IIS6 segment spans exons 20–21, and IIIS6 covers exons 30–31, with exon numbering based on *Musca domestica* Na_V gene conventions. Illumina adapter sequences (hangers) were added to the 5'ends of both primers according to the *16S Metagenomic Sequencing Library Preparation* (version 15,044,223-B). Amplification of each region was performed independently for each population pool using the Phusion Hot Start Flex DNA Polymerase kit (BioLabs, New England), containing a high-fidelity enzyme. Each 25 µL PCR reaction included 60 ng of genomic DNA, 2 mM $MgCl_2$, 1 × Phusion HF buffer, 200 µM dNTPs, 0.5 µM or 1 µM of each primer (for IIIS6 and IIS6, respectively), 1 U of polymerase (0.25 µL), 3% DMSO, and ultrapure water to reach the total volume. PCR cycling conditions were as follows: initial denaturation at 98 °C for 30 s; 35 cycles of 98 °C for 10 s, annealing at 56 °C (IIS6) or 60 °C (IIIS6) for 15 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 7 min. The primer sequences used are listed in Table 1.

NGS library preparation and sequencing

Amplicons from both Na_V segments of each population pool were run on 1% agarose gel electrophoresis to confirm DNA bands of approximately 400 or 500 bp using a 1.5 kb molecular weight marker (NEB). The verified bands were excised and purified using the kGeneJET Gel Extraction Kit (Thermo Scientific) according to the manufacturer's instructions. Equimolar amounts of purified amplicons from each population pool were combined. The quality and concentration of the pooled amplicons (IIS6 + IIIS6 mixture) were analyzed using an Agilent 2100 Bioanalyzer System (Agilent). A unique DNA barcode was added to each pool using the Nextera

Primer name	Sequence (5′-3′)*	Orientation	Na_V segment
Hang_4,723F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTTCAAGGGCTGGATCCAGA	Forward	IIS6
Hang_5,198R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTCGAATACTATTGCTTGTGGTCG	Reverse	IIS6
Hang_5′para3F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACAATGTGGATCGCTTCCC	Forward	IIIS6
Hang_3,398R	TCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGCGATCTTGTTCGTTTCG	Reverse	IIIS6

Table 1. Sequences of primers used to amplify the IIS6 and IIIS6 Na_V segments of Ae. *albopictus*. *The underline indicates the Illumina adapter sequences added to the 5′ tail of specific primers.

XT index kit (Illumina) in a PCR reaction with the KAPA HiFi HotStart ReadyMix Kit (Roche) according to the manufacturer's protocol. Sequencing was performed on a MiSeq platform (Illumina) at the FIOCRUZ NGS facility using the MiSeq Reagent Kit v2 (500 cycles, paired-end, 2 × 250 bp).

Data analysis
Raw sequencing data were de-multiplexed using the SeekDeep pipeline[22], which was used to trim primers and adapters, remove low-quality reads, make the sequence assembly, call haplotypes (sequences with 100% identity), and calculate haplotype frequencies within each population pool. To determine whether haplotypes were present in previous studies, each haplotype was used as a query in BLAST searches against the nucleotide (nt) database, retrieving only the best hits that covered the entire query sequence. All haplotypes were imported into Geneious Prime 2024.0.7 (http://www.geneious.com) and aligned using MUSCLE algorithm. Phylogenetic trees were constructed with IQ-TREE 2[23] using the Maximum Likelihood method and Tamura-Nei model selected by ModelFinder[24], with branch support evaluated using 500 bootstrap replications. Haplotype networks were reconstructed in PopArt using TCS[25].

Results
The DNA yield per mosquito was 1.1 ± 0.1 µg (mean ± standard deviation), which exceeded the quantity required for the molecular analyses. The remaining DNA was catalogued and stored at − 20 °C in the Brazilian Vector DNA Repository at FIOCRUZ for future research.

Sequence quality and filtering
Gel electrophoresis revealed two distinct bands at approximately 400 and 500 bp, confirming the successful amplification of the IIS6 and IIIS6 segments of the Na_V gene. High-throughput sequencing generated 14.8 million paired reads, of which 11.7 million (79.1%) had a quality score greater than 30, indicating 99.9% accuracy. After demultiplexing, filtering low-quality reads, and off-target sequences, we retained 10.05 million sequences separated into the IIS6 and IIIS6 segments. Sequence lengths, haplotype frequencies, and GenBank accession numbers are listed in Table 2.

IIS6 segment Na_V haplotypes
Haplotype analysis identified 20 distinct IIS6 haplotypes, with lengths ranging from 314 to 334 bp (Table 2, Fig. 1). The haplotypes showed greater than 90% identity with the reference sequence XM_029865132.1, confirming primer specificity. Multiple alignments revealed 60 polymorphic sites, of which 27 were informative. Twelve polymorphic sites within the coding region, eight in exon 20 and four in exon 21, all resulted in synonymous substitutions, indicating the absence of *kdr* mutations in our sampling. The remaining 48 polymorphic sites were located in intron 20 and included insertions and deletions (indels).

Five unique synonymous substitutions were identified in exon 20 at codons 962 (GAC/GTA, 2s6.07), 968 (TTC/TTT, 2s6.16), 980 (GTG/GTA, 2s6.15), 993 (TGC/TGT, 2s6.07), and 1001 (TGT/TGC, 2s6.05), according to *Musca domestica* Na_V codon numbering. Additional synonymous substitutions were shared among two or three haplotypes, specifically at codons 981 (CGG/CGA, 2s6.00 and 2s6.03), 1,006 (TTG/TTA, 2s6.08, and 2s6.12), and 993 (TGC/TGT, 2s6.00, 2s6.03, 2s6.16, and 2s6.07). The Maximum likelihood phylogenetic tree (Fig. 2) and the haplotype network (Supplementary Fig. S1) of IIS6 haplotypes of *Ae. albopictus* suggest that the substitution at codon 1006 shared an ancestral origin, while those at 981 and 993 codons appeared to have emerged independently, given their presence in haplotypes with unrelated ancestors. The Maximum likelihood phylogenetic tree did not reveal distinct clade groupings, unlikely that reported for *Ae. aegypti*[26].

Haplotype 2s6.00 was found in 37 populations, making it the most frequent with 580,564 sequences (35.97%) (Table 2). The highest frequencies (73–98%) were observed in eight populations: four from the Center-West (Iporá—GO, Coxim—MS, Goiania—GO, and Dourados—MS), three from the Southeast (Belo Horizonte—MG, Angra dos Reis—RJ, and São Sebastião—SP), and one from the North (Redenção—PA, though where only two mosquitoes were sampled, limiting significance). In the other 29 populations, 2s6.00 ranged from moderate to low frequency (2.9–67%). The second most frequent haplotype, 2S6.02, represented by 355,901 sequences (20.5%), was found in 25 populations and was identical to OP940459.1 and KC152045 (GenBank accession numbers) reported in Chinese and Malay populations. It was prevalent in the northeast and absent from populations in the south and lower mid-west (Fig. 3). The third most frequent sequence was 2s6.01, with 145,155 sequences (9.64%) distributed across 32 populations. Similar to 2s6.00, was distributed countrywide; however, it was scarcely found in northeastern populations, except in Nossa Senhora da Glória (SE) and Brumado (BA). The fourth most frequent haplotype, 2s6.3, with 134,723 sequences (7.66%), was identical to OP941111.1, previously

Haplotype	Submission ID	GenBank accession ID	Na_V segment	Sequence length (bp)	Total frequence/ Na_V segment	Number of used reads	Number of populations where found
2s6.00	BankIt2831600	PP842849	IIS6	314	0,36	580.564	37
2s6.01	BankIt2831600	PP842850	IIS6	331	0,10	145.155	32
2s6.02	BankIt2831600	PP842851	IIS6	334	0,20	355.901	25
2s6.03	BankIt2831600	PP842852	IIS6	325	0,08	134.723	17
2s6.04	BankIt2831600	PP842853	IIS6	326	0,05	66.466	17
2s6.05	BankIt2831600	PP842854	IIS6	334	0,04	66.494	13
2s6.06	BankIt2831600	PP842855	IIS6	331	0,03	48.794	11
2s6.07	BankIt2831600	PP842856	IIS6	333	0,01	18.372	11
2s6.08	BankIt2831600	PP842857	IIS6	334	0,03	30.845	7
2s6.09	BankIt2831600	PP842858	IIS6	334	0,01	12.118	7
2s6.10	BankIt2831600	PP842859	IIS6	331	0,02	26.102	6
2s6.11	BankIt2831600	PP842860	IIS6	325	0,01	24.935	5
2s6.12	BankIt2831600	PP842861	IIS6	334	0,01	8.857	5
2s6.13	BankIt2831600	PP842862	IIS6	325	0,02	29.951	4
2s6.14	BankIt2831600	PP842863	IIS6	317	0,01	24.705	3
2s6.15	BankIt2831600	PP842864	IIS6	332	0,01	19.311	3
2s6.16	BankIt2831600	PP842865	IIS6	314	0,01	20.223	3
2s6.17	BankIt2831600	PP842866	IIS6	326	0,00	4.695	3
2s6.18	BankIt2831600	PP842867	IIS6	330	0,01	15.973	2
2s6.19	BankIt2831600	PP842868	IIS6	328	0,00	8.057	2
3s6.00	BankIt2795736	PP737830	IIIS6	415	0,68	626.082	44
3s6.01	BankIt2795736	PP737829	IIIS6	416	0,10	91.610	31
3s6.02	BankIt2795736	PP737828	IIIS6	431	0,05	42.791	29
3s6.03	BankIt2795736	PP737827	IIIS6	415	0,06	47.730	20
3s6.04	BankIt2795736	PP737826	IIIS6	430	0,02	13.915	11
3s6.05	BankIt2795736	PP737825	IIIS6	433	0,02	15.748	10
3s6.06	BankIt2795736	PP737824	IIIS6	430	0,03	26.081	9
3s6.07	BankIt2795736	PP737823	IIIS6	417	0,01	8.490	6
3s6.08	BankIt2795736	PP737822	IIIS6	431	0,01	4.523	6
3s6.09	BankIt2795736	PP737821	IIIS6	431	0,01	3.362	5
3s6.10	BankIt2795736	PP737820	IIIS6	416	0,00	4.490	5
3s6.11	BankIt2795736	PP737819	IIIS6	415	0,00	2.860	5
3s6.12	BankIt2795736	PP737818	IIIS6	433	0,00	2.810	4
3s6.13	BankIt2795736	PP737817	IIIS6	415	0,00	2.353	4
3s6.14	BankIt2795736	PP737816	IIIS6	428	0,00	4.169	3
3s6.15	BankIt2795736	PP737815	IIIS6	431	0,00	2.297	3
3s6.16	BankIt2795736	PP737814	IIIS6	422	0,00	1.969	3
3s6.17	BankIt2795736	PP737813	IIIS6	416	0,00	1.927	3
3s6.18	BankIt2795736	PP737812	IIIS6	418	0,00	1.200	3
3s6.19	BankIt2795736	PP737811	IIIS6	416	0,00	3.078	2
3s6.20	BankIt2795736	PP737810	IIIS6	418	0,00	2.286	2
3s6.21	BankIt2795736	PP737809	IIIS6	415	0,00	1.852	2
3s6.22	BankIt2795736	PP737808	IIIS6	431	0,00	2.042	2
3s6.23	BankIt2795736	PP737807	IIIS6	431	0,00	1.068	2

Table 2. Characterization of IIS6 and IIIS6 segment haplotypes in the Na_V gene of *Aedes albopictus* populations from Brazil.

reported in China, and was predominantly found in the north, upper Center-West (Brasília and Água Boa), and southeast coasts (Rio de Janeiro and Espírito Santo) (Fig. 3).

Sixteen additional haplotypes had frequencies < 5%. Among them, five (2s6.6, 2s6.9, 2s6.11, 2s6.13, and 2s6.15) were identical to the haplotypes identified in China, suggesting that multiple migration events may have introduced them into the Brazilian gene pool. The 2s6.11 was restricted to the north, while the other four were distributed across multiple regions (Fig. 3). Interestingly, haplotypes 2s6.14, 2s6.16, and 2s6.17 were found exclusively in the state of Espírito Santo. This pattern suggests that these haplotypes either originated locally or

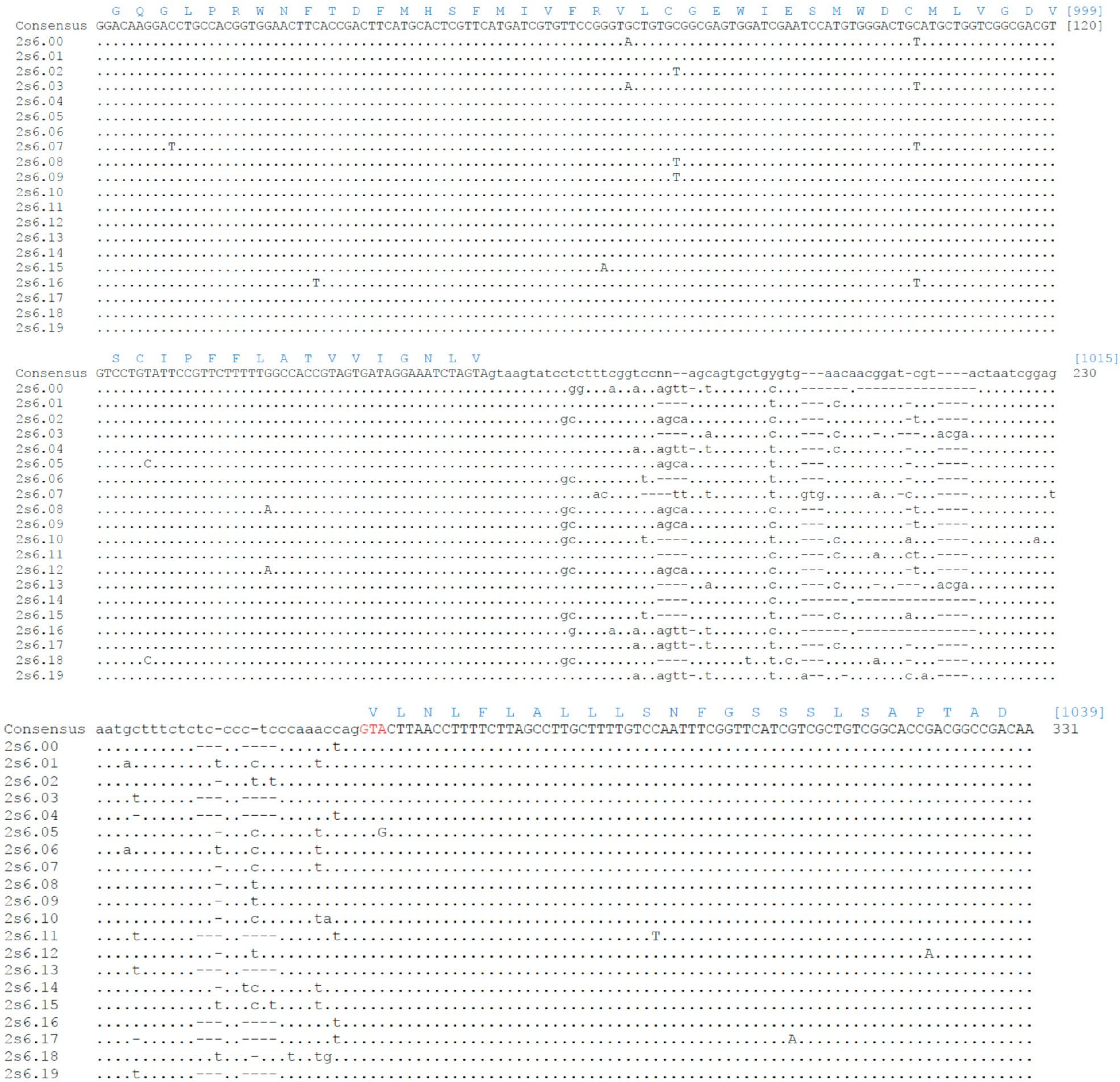


Fig. 1. Nucleotide diversity of the voltage-gated sodium channel gene, IIS6 segment haplotypes, found in *Aedes albopictus* populations from Brazil. Geneious alignment among haplotypes found in this study (2s6.00-2s6.19), and consensus sequence generated by the software. Uppercase nucleotides correspond to the coding region (exons 20 and 21), while lowercase nucleotides refer to the intron, with alignment numbering at the top of each block. Invariable sites are indicated with dots, polymorphic sites with the alternative nucleotide, and gaps with (-). Codons in red represent codons 1016 where *kdr* mutations were previously described in *Ae. albopictus*. GenBank accession numbers of each haplotype are presented in Table 2.

were introduced into the gene pool through recent migration. Using the Maximum Likelihood tree, haplotype 2s6.17 shares the same ancestor with 2s6.04, and haplotype 2s6.16 with 2s6.00 (Fig. 2), both of which are widely distributed (Table 2, Supplemental Fig. S1).

IIIS6 segment Na_V haplotypes

We identified 24 haplotypes in the IIIS6 Na_V segment of *Ae. albopictus* samples (Fig. 4), corresponding to the exons 30 and 31 flanking the intron 30, and sequences ranging from 415 to 431 nucleotides, given indels in the intron. The most prevalent haplotype, 3s6.00, accounted for 67.8% of the IIIS6 sequences (626,082 reads) and was widely distributed across all populations, excluding Brumado (BA), likely because of the small sample size from this location (two individuals). Following, haplotype 3s6.01 was found at a frequency of 9.5% and present in 31 populations, and 3s6.02, with a frequency of 4.52%, was identified in 29 populations. The remaining 17

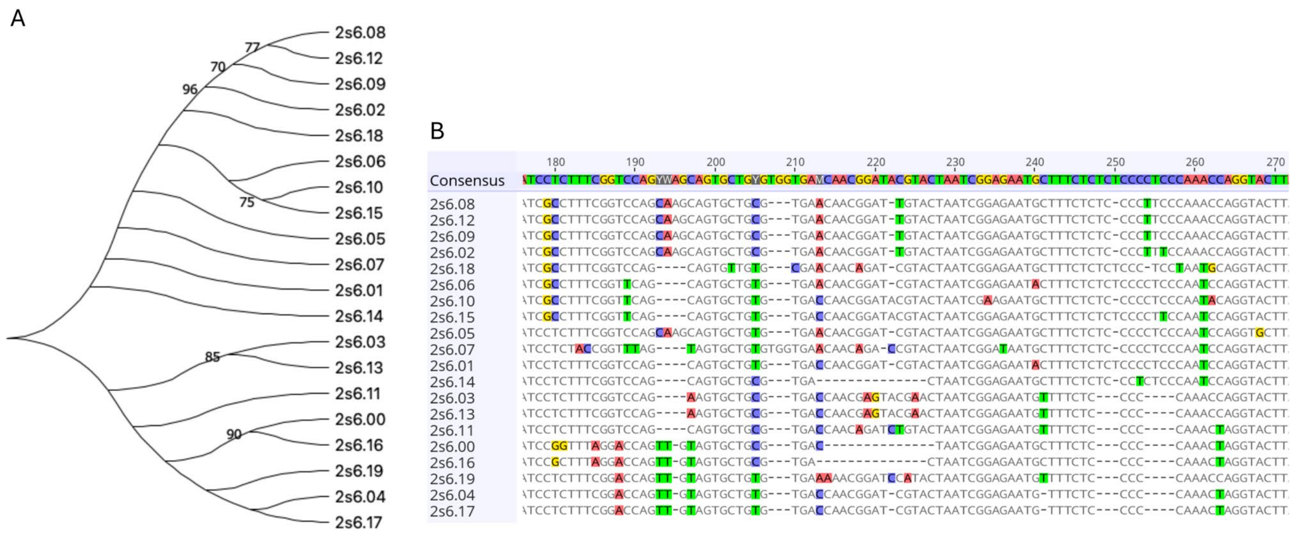
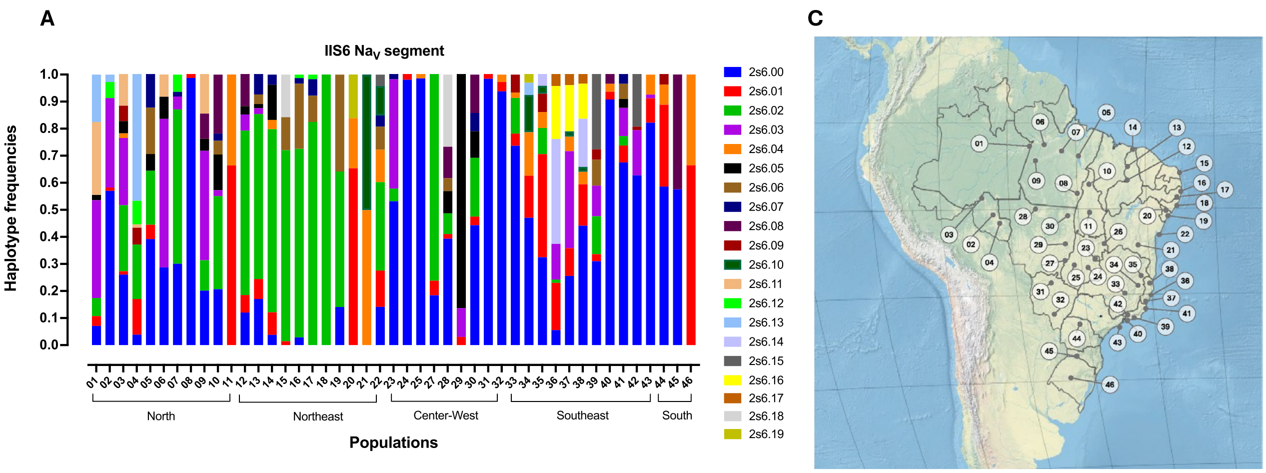


Fig. 2. Phylogenetic relationship of the IIS6 haplotypes identified in *Aedes albopictus* populations from Brazil. Panel **A**–Maximum likelihood tree with bootstrap values exceeding 70%. Panel **B**–Alignment of intron 20, highlighting a region with greater sequence variation. Haplotypes were arranged in the same order as in the phylogenetic tree.



Number	n samples	City	State	Region
01	16	Parintins	Amazonas	North
02	30	Jaru	Rondônia	
03	30	Porto Velho	Rondônia	
04	30	Cacoal	Rondônia	
05	30	Tucuruí	Pará	
06	30	Santarém	Pará	
07	16	Altamira	Pará	
08	2	Redenção	Pará	
09	30	Itaituba	Pará	
10	30	Araguaina	Tocantins	
11	10	Palmas	Tocantins	
12	30	Floriano	Pauí	Northeast
13	30	Teresina	Pauí	
14	30	Parnaíba	Piauí	
15	14	Natal	Rio Grande do Norte	
16	30	Recife	Pernambuco	
17	30	Palmares	Pernambuco	
18	30	Maceió	Alagoas	
19	26	Aracaju	Sergipe	
20	7	NSra da Glória	Sergipe	
21	2	Brumado	Bahia	
22	16	Salvador	Bahia	

Number	n samples	City	State	Region
23	9	Brasília	Distrito	Ceter-West
24	29	Goiânia	Goiás	
25	8	Iporá	Goiás	
26	30	Posse	Goiás	
27	13	Barra das Garças	Mato Grosso	
28	30	Alta Floresta	Mato Grosso	
29	10	Água Boa	Mato Grosso	
30	30	Confresa	Mato Grosso	
31	8	Coxim	Mato Grosso	
32	8	Dourados	Mato Grosso do Sul	
33	30	Belo	Minas Gerais	Southeast
34	27	Governador Valadares	Minas Gerais	
35	30	Teófilo Otoni	Minas Gerais	
36	12	Vitória	Espírito Santo	
37	30	Cachoeira do Itapemirim	Espírito Santo	
38	29	Nova Venécia	Espírito Santo	
39	30	Rio de Janeiro	Rio de Janeiro	
40	30	Angra dos Reis	Rio de Janeiro	
41	17	Campos dos Goytacazes	Rio de Janeiro	
42	30	Volta Redonda	Rio de Janeiro	
43	11	São Sebastião	São Paulo	
44	16	Londrina	Paraná	South
45	30	Chapecó	Santa	
46	2	Santa Maria	Rio Grande do Sul	

Fig. 3. Haplotype frequencies of IIS6 and IIIS6 segments in the Na_V gene of *Aedes albopictus* populations across Brazil. IIS6 (Panel **A**) and IIIS6 (Panel **B**) haplotype frequencies across populations and geographical regions. The populations are labelled with numbers matching the Brazilian map in Panel **C**.

Fig. 4. Nucleotide diversity in the voltage-gated sodium channel gene, IIIS6 segment, in *Aedes albopictus* populations from Brazil. Alignment among haplotypes found in this study (3s6.00-3s6.23) and consensus sequence generated by Geneious software. Uppercase nucleotides correspond to the coding region (Exons 30 and 31), while lowercase nucleotides refer to the intron, with alignment numbering at the top of each block, and primer position sequences underlined. Invariable sites are indicated with dots, polymorphic sites with the alternative nucleotide, and gaps with (-). Codons in red represent codons 1520, 1532, and 1534 where *kdr* mutations were found in *Ae. albopictus* from elsewhere. The Genbank accession numbers of each haplotype sequence are available in Table 2.

haplotypes were rare in our sample, with a frequency of < 1%. Of particular note, haplotype 3s6.23 (0.09%) was exclusive to populations in Rio de Janeiro State, while haplotype 3s6.16 was found only in populations from Espírito Santo State, supporting the hypothesis of unique evolutionary dynamics in *Ae. albopictus* populations from this region, potentially involving diverse origins and new introductions.

The populations displayed varied haplotype profiles, ranging from one haplotype in Santa Maria (RS, South) to nine haplotypes in Posse (GO, Center-West) (Fig. 3). Compared to the available *Ae. albopictus* IIIS6 Na_V sequences from other countries, the haplotype 3s6.15, observed in Porto Velho, Cacoal (RO), and Londrina (PR), was identical to that of KC152046 (GenBank accession number) from a sample in Malaysia. The low bootstrap values in the phylogenetic tree analysis (Fig. 5) prevented clear division of IIIS6 haplotypes into distinct groups. In addition, the haplotype network exhibited a complex structure (Supplemental Fig. S2).

We did not detect a *kdr* mutation nor any other non-synonymous substitution in the IIIS6 Na_V segment. However, eight polymorphic codons were identified within exons 30 and 31 of Na_V. The polymorphism at codon 1,505 (GAT/GAC) in exon 30 was present in haplotypes 3s6.07, 3s6.16, and 3s6.18. In exon 31, polymorphism at codon 1,516 (CCG/CCA) was found in haplotypes 3s6.06, 3s6.08, 3s6.12, 3s6.16, and in the most derivate haplotype 3s6.20. Codon 1,528 polymorphism (TTC/TTT) was shared among haplotypes 3s6.01, 3s6.02, 3s6.07, 3s6.09, 3s6.14, and 3s6.21. Polymorphism at codon 1,514 (AAG/AAA) was identified in haplotype 3s6.02 (Figs. 3, 4, and 5).

Phasing IIS6 and IIIS6 haplotypes

As we sequenced pooled DNA samples, we determined which IIS6 and IIIS6 haplotypes were phased (i.e., present on the same chromosome) only in populations where at least one segment was monomorphic. This criterion applies only to the Maceió (with the IIS6 segment monomorphic for haplotype 2s6.00) and Santa Maria (with the IIIS6 segment monomorphic for haplotype 3s6.00) populations. Based on these data, we propose that the six phased IIS6 + IIIS6 haplotypes may be present in Brazilian *Ae. albopictus* populations: (1) 2s6.00_B + 3s6.00, (2) 2s6.00_B + 3s6.01, (3) 2s6.00_B + 3s6.05, (4) 2s6.00_B + 3s6.07, (5) 2s6.01_A + 3s6.00, and (6) 2s6.04_B + 3s6.00 (Fig. 6). Therefore, the phased combination of haplotypes 2s6.00_B + 3s6.00 is likely the most common phased haplotype circulating within *Ae. albopictus* populations in Brazil.

Discussion

Ae. albopictus is an invasive species first recorded in Brazil in 1986. National entomological surveillance and insecticide resistance (IR) monitoring by the Brazilian government primarily targets *Ae. aegypti*, the recognized

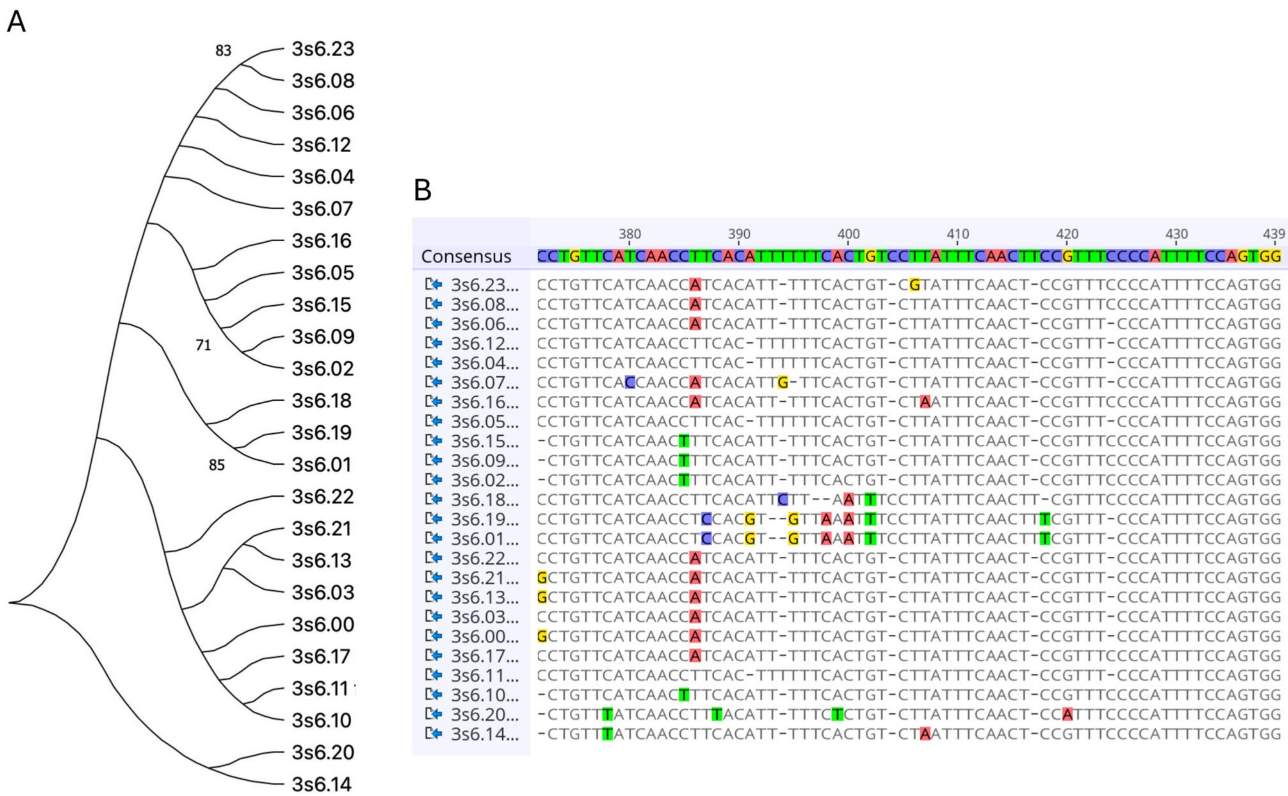


Fig. 5. Phylogenetic relationship of the IIIS6 haplotypes identified in *Aedes albopictus* populations from Brazil. Panel **A**–Maximum likelihood tree with bootstrap values exceeding 70%. Panel **B**–Alignment of intron 30, highlighting a region with greater sequence variation. Haplotypes were arranged in the same order as in the phylogenetic tree.

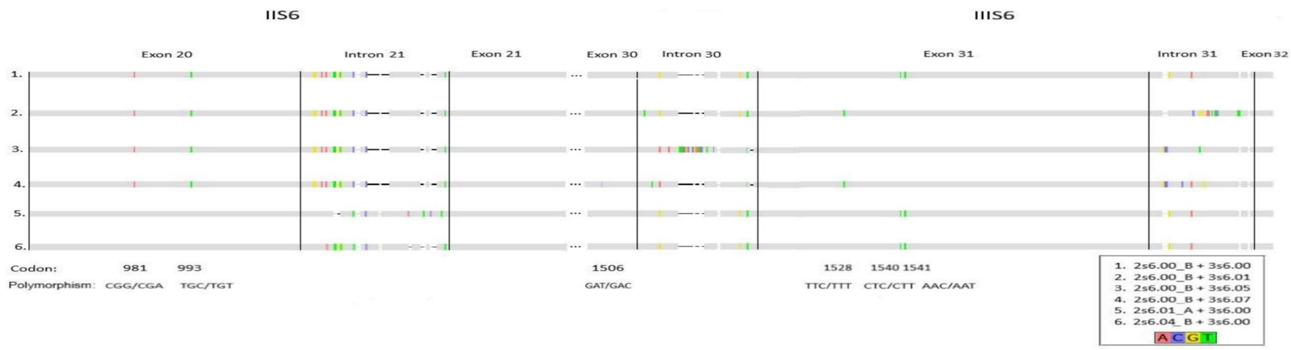


Fig. 6. Phased haplotypes of IIS6 and IIIS6 segments of the Na_V gene in *Aedes albopictus* populations from Brazil. Six distinct phasings were identified when at least one segment was monomorphic in a population. Bars represent alignments of the IIS6 and IIIS6 sequences, highlighting the polymorphisms. Synonymous codon changes are indicated.

vector of dengue, chikungunya, and Zika viruses[27,28]. Consequently, research on *Ae. albopictus* biology, physiology, and genetics have been comparatively limited, in contrast to the extensive studies on *Ae. aegypti*. In terms of IR, *Ae. aegypti* has been systematically monitored in Brazil since 1999, with studies detailing the phenotypic profiles, molecular mechanisms of resistance, and fitness effects associated with resistance selection[20,29]. Molecular surveillance of *kdr* mutations, a key mechanism related to pyrethroid resistance, has provided substantial data regarding the distribution of resistance alleles in *Ae. aegypti* populations in Brazil. Over 130 *Ae. aegypti* populations from Brazil[30–35] have been assessed for *kdr* frequencies, whereas only a few studies have evaluated the IR phenotype in *Ae. albopictus*[17]. To date, there are no records of *kdr* mutations in this species in Brazil.

In this study, we analyzed the diversity of sequences from the IIS6 and IIIS6 segments of the voltage-gated sodium channel (Na_V) of *Ae. albopictus* populations across Brazil using high-throughput sequencing on a national scale. Our analysis revealed high genetic diversity within the Na_V gene of Brazilian *Ae. albopictus*, with 20 different IIS6 and 24 different IIIS6 haplotypes identified. These segments harbor key *kdr* mutations in several insect species, including *Ae. albopictus*[36–38]. Although we did not detect any *kdr*-related mutations, we observed significant haplotype diversity, detailing their phylogenetic relationships, frequencies, and geographic distributions. These data provide valuable information regarding the evolutionary dynamics of this invasive species in Brazil.

We observed that nucleotide diversity in Na_V homologous sequences was higher in *Ae. albopictus* than *Ae. aegypti*, considering the Brazilian[26] and Pakistani[39] populations despite the broader range of *kdr* mutations and larger available Na_V sequences reported in *Ae. aegypti*[40]. The F1,534C *kdr* mutation is prevalent in *Ae. aegypti* populations across America, Africa, and Asia have also been found in *Ae. albopictus* in Singapore[41], Greece[42], and India[43]. Interestingly, several other substitutions at codons 1534 were identified only in *Ae. albopictus*, including F1,534S (China and USA[37]), F1,534L (USA[44], Italy, and China[38]), and F1,534W and F1,534R (China[45]). The V1,016G *kdr* mutation commonly observed in *Ae. aegypti* populations from Asia have also been found in *Ae. albopictus* populations in China[46], Vietnam and Italy[47].

Previous studies on *Ae. albopictus* Na_V diversity examined the portions of domains II, III, and IV of this channel from samples collected in various countries. This study identified 29 synonymous substitutions with no changes at the homologous *kdr* sites (989, 1011, and 1016) observed in *Ae. aegypti*. Instead, the mutation I1532T was found in samples from Italy, F1534S in China and the USA, F1534L in China, and F1534C in Greece[37]. Interestingly, we did not observe any non-synonymous substitutions despite examining *Ae. albopictus* samples from 46 urban centers in Brazil with an average sequencing depth coverage of 119.5X. It is noteworthy, however, that while *Ae. albopictus* populations in Brazil may not be resistant to pyrethroids, and IR and *kdr* mutations are widespread in sympatric *Ae. aegypti* populations[33], suggesting similar environmental selection pressure. Phenotypic characterization of insecticide susceptibility and resistance to insecticides is required for *Ae. albopictus* from Brazil.

In Brazil, at least two *kdr* alleles are widespread in *Ae. aegypti* populations: *kdr* R1 (F1534C only) and the *kdr* R2 (V410L + V1016I + F1534C)[26,33,35]. Despite the high diversity of Na_V haplotypes in *Ae. albopictus*, we found no *kdr* mutation. A similar pattern was observed in Asian populations of both species, *Ae. albopictus* lacks kdr mutations, whereas *Ae. aegypti* showed mutations such as T1,520I and F1,534C in Pakistan[39] and V1,1016G and F1,534C in Malaysia[48]. The pyrethroid-resistant phenotype of *Ae. albopictus* without *kdr* mutations has been attributed to metabolic resistance mechanisms, particularly through the overexpression of cytochrome P450 genes, such as *CYP6P12* in Malaysian[49] and Cameroonian[50] populations.

The introduction of *Ae. albopictus* in Brazil likely occurred in the 1980s, possibly through iron trade ships from Japan that arrived at the ports of Espírito Santo. Multiple additional introductions may have subsequently occurred[51–53]. The presence of numerous Na_V haplotypes, including some unique to localities in Espírito Santo (e.g., 2s6.16, 2s6.17, and 3s6.16), supports the hypothesis of multiple introductions. Additionally, haplotype 3s6.15 (identical to the Malaysian sequence KC152046 in GenBank) was detected in both the northern (Roraima) and southern (Paraná) regions of Brazil, which are separated by over 3,100 km. If this haplotype is not widely dispersed in Brazil, it may suggest independent introduction from Asia at different times. Further studies using neutral markers will help to clarify the origins and spread of these haplotypes.

Even if *Ae. albopictus* populations in Brazil are not currently resistant to pyrethroids; the genetic diversity observed in this species provides a potential reservoir for selecting variants that are favorable to resistance. Moreover, the continuous introduction of *Ae. albopictus* from regions with established resistance, new alleles could be introduced. This highlights the need for sanitary authorities to monitor the IR and genetic diversity in *Ae. albopictus*. Although this species is not officially recognized as a major arbovirus vector in Brazil, it has the potential to serve as a bridge vector between sylvatic and urban pathogens owing to its ecological plasticity[2,10]. Thus, the continuous monitoring of *Ae. albopictus* populations in Brazil are warranted to detect emerging genetic profiles that may favor either insecticide resistance or enhanced vector competence[54].

Conclusion

This study used NGS to reveal substantial diversity in the IIS6 and IIIS6 Na_V segments of *Ae. albopictus* populations across Brazil, identifying 20 and 24 haplotypes, respectively, with no evidence of *kdr* mutation. Certain haplotypes were unique to specific regions, suggesting a limited gene flow. Overall, these findings provide valuable insights into the genetic diversity and structure of *Ae. albopictus* in Brazil, which is essential for the development of effective vector control strategies. Continuous monitoring is recommended to track potential changes in genetic profiles that may be related to public health risks such as insecticide resistance and vector competence.

Data availability

The datasets generated and analysed during the current study are freely available in the Zenodo repository at https://doi.org/https://doi.org/10.5281/zenodo.14624090. The haplotype consensus sequences have been deposited in GenBank (NCBI, NIH) and can be accessed at https://www.ncbi.nlm.nih.gov/genbank/. The accession numbers of each haplotype are listed in Table 2.

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Author contributions
AJM, GJN, JBPL and LVC contributed to the design and implementation of the research, GJN, ALQT to the analysis of the results, GJN, AJM, JBPL, LVC, ALQT to the writing and revision of the manuscript. AJM conceived the original and supervised the project.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical compliance
The Institute of Oswaldo Cruz has received accreditation for carrying out experiments with wild and urban mosquitoes kept as colonies in insectaries. This study was approved by the Institutional Ethics Committee on Animal Use (CEUAIOC-License LO28/18) of the Oswaldo Cruz Institute, FIOCRUZ.

Additional information
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Correspondence and requests for materials should be addressed to A.J.M.