**Kdr genotyping in Aedes aegypti from Brazil on a nation-wide scale from 2017 to 2018**

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Insecticide resistance is currently a threat to the control of *Aedes aegypti*, the main vector of arboviruses in urban centers. Mutations in the voltage gated sodium channel (Naᵥ), known as *kdr* (knockdown resistance), constitute an important selection mechanism for resistance against pyrethroids. In the present study, we investigated the *kdr* distribution for the Val1016Ile and Phe1534Cys alterations in *Ae. aegypti* from 123 Brazilian municipalities, based on SNP genotyping assays in over 5,500 mosquitoes. The alleles *Naᵥ*S (1016Val⁺+1534Phe⁺), *Naᵥ*R₁ (1016Val⁺+1534Cys*⁴⁺) and *Naᵥ*R₂ (1016Ile*⁴⁺+1534Cys*⁴⁺*⁴⁺) were consistently observed, whereas *kdr* alleles have rapidly spread and increased in frequency. *Naᵥ*S was the less frequent allele, mostly found in Northeastern populations. The highest allelic frequencies were observed for *Naᵥ*R₁, especially in the North, which was fixed in one Amazonian population. The double *kdr* *Naᵥ*R₂ was more prevalent in the Central-west and South-eastern populations. We introduce the ‘*kdr* index’, which revealed significant spatial patterns highlighting two to three distinct Brazilian regions. The 410L *kdr* mutation was additionally evaluated in 25 localities, evidencing that it generally occurs in the *Naᵥ*R₂ allele. This nationwide screening of a genetic mechanism for insecticide resistance is an important indication on how pyrethroid resistance in *Ae. aegypti* is evolving in Brazil.

The number of dengue cases in Brazil totaled over 1.9 million records between 2016 and 2018. During the same period, the recent additional chikungunya and Zika arbovirus epidemics were responsible for around 540 and 240 thousand cases, respectively, according to Ministry of Health official bulletins¹. Failure in the control of *Aedes aegypti*, so far considered the primary vector of the aforementioned arboviruses in Brazil, is considered the main reason for the increase in these records. Despite a series of studies evidencing arbovirus transmission by Brazilian *Aedes albopictus* populations, this species probably acts as a secondary urban vector, mostly significant in maintaining arbovirus circulation during inter-epidemic seasons and in rural regions²,³. The measures for controlling the density of *Aedes* mosquitoes largely rely on insecticides. However, their intense use has been, increasingly, selecting resistant populations at a global scale⁴. This is also true in Brazil, where an extensive insecticide resistance monitoring system has tracked the susceptibility status of *Ae. aegypti* populations since 1999⁵,⁶. Organophosphate application has been intensified since the 1980s, substituted by insect growth regulators and pyrethroids two decades later, against larvae and adults, respectively, given the confirmation of resistance to the larvicide temephos⁷. However, pyrethroid resistance was also confirmed a few years later⁸. Currently, the organophosphate malathion is employed by governmental campaigns in the entire country as the only alternative to pyrethroids, although the latter class of compounds is still intensively used in households and

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by private companies for mosquito control, as well as against other urban insect vectors, such as Culex, sandflies, anophelines and triatomines.

The main cause of resistance is the pyrethroid knockdown effect is an alteration in the molecular target of this chemical, namely the voltage gated sodium channel (Naᵥ, also commonly referred to as VGSC), caused by one or a few amino acid substitutions that alter the channel conformation, thus avoiding its interaction with pyrethroid molecules. These substitutions, known as knockdown resistance mutations (kdr) have been linked to pyrethroid and DDT resistance in several arthropod species. The kdr amino acid position is generally convergent throughout distinct taxa, as the Naᵥ gene is very conserved and, therefore, few variations are permissive to maintain its neurophysiological role. In several species, including Anopheles and Culex mosquitoes, the most classical kdr is a Leu to Phe substitution in the 1014 position (L1014F). In the wild-type allele Naᵥ, the Phe at this position is required for high-affinity binding of the pyrethroid agonists, whereas the Leu at this position results in a lower affinity for the agonists. Substitutions in the 1016 and 1534 sites have been consistently detected in the Naᵥ of Ae. aegypti populations worldwide. The Phe to Cys substitution in the 1534 site (F1534C) is found in populations from the Americas, Africa and Asia, whilst the wild-type Val is substituted by Ile (V1016I) in the 1016 site in American populations and by Gly (V1016G) in Asian populations.

Additional alterations have also been reported, such as the Pro to Ser substitution in the 989 (P989S) and Val to Leu in the 410 position (V410L), respectively, in Asian and Latin American populations.

Since the beginning of the 2000s, kdr mutations have been detected in Ae. aegypti populations from Brazil, displaying geographical expansion and increasing frequency trends. A haplotype with kdr mutation F1534C (NaᵥR1 allele) was present in all but one population among natural populations evaluated from more than 60 localities. An additional haplotype containing both V1016I and F1534C was absent or detected at very low frequencies. The kdr frequencies in Brazilian Northeast, Southeast, and South regions were obtained. All evaluated populations displayed at least one kdr allele. Six genotypes were substantially observed, all including the S, R1 and R2 (wild-type, kdr in 1534 only and kdr in 1016 + 1534, respectively) haplotypes. The other three genotypes composed by the kdr R3 haplotype (kdr in 1016 site only) accounted for 0.1% of the total samples.

The Kdr genotyping. The kdr genotypes for 5,341 individuals, distributed in 123 localities, for both the 1016 and 1534 Naᵥ sites were obtained. All evaluated population displayed at least one kdr allele. Six genotypes were substantially observed, all including the S, R1 and R2 (wild-type, kdr in 1534 only and kdr in 1016 + 1534, respectively) haplotypes. The other three genotypes composed by the kdr R3 haplotype (kdr in 1016 site only) accounted for 0.1% of the total samples.

The Kdr index and spatial analyses. The kdr index ranged from 1.75 (Jardim do Seridô, RN) to 6.21 (Corumbã, MS) (Fig. 4). The localities presenting the lowest kdr index (first quartile: 1.75 to 3.96) were predominately located in the Northeast (61.3%), South (19.4%) and North (16.1%) regions. Those beyond the last quartile (5.36–6.21) were distributed in the Southeast (48.4%), Center-West (25.8%), North (12.9%), Northeast (9.7%) and South (6.5%) regions.

The spatial distribution kdr index value analysis concerning the geographic coordinates of their respective localities revealed statistically significant spatial patterns, i.e. the existence of clearly identifiable Brazilian regions with different predicted Ae. aegypti resistance status. A total of 55 candidate models, related to different (a priori) inter-city “links” structures, were generated (see Supplementary Text S1 for methodological details and intermediary results). Figure 5 displays the most significant model, first evidencing a large scale spatial differentiation in the Northeast–Southwest direction, separating North-Eastlast and to a lesser extent, North) localities from the rest of the country (areas A11 and A12, respectively, represented in Fig. 5a and, second, a three-mode differentiation in the same direction, separating: (i) North and Northeast localities; (ii) a strip of cities oriented...
Southeast–Northwest and (iii) South localities (areas A21, A22 and A23, respectively, in Fig. 5b. The kdr index value distributions as a function of the aforementioned areas are displayed in Fig. 5c,d. Including the V410L SNP. In addition to V1016I and F1534C, the V410L SNP was also evaluated in 25 populations, mostly in the state capitals, totaling 977 genotyped samples for these three sites, which together rendered 11 genotypes, amongst which five were poorly represented (Table 2, Fig. 6). The three most prevalent genotypes were VV + VV + CC (27.8%), VL + VI + CC (26.8%) and LL + II + CC (23.9%) (in order, 410 + 1016 + 1534), composed by the kdr haplotypes VVC (kdr in the 1534 site only) and LIC (kdr in 410, 1016 and 1534 sites). Next are the genotypes formed with the wild-type haplotype (VVF): VV + VV + FC (11.1%), VL + VI + FC (11.1%), VL + VI + FC (6.0%) and VV + VV + FF (2.7%). The remaining five genotypes occurred in frequencies below 1%, which would be formed by alleles such as VIC (kdr in 1016 and 1534), LIC (kdr in 410, 1016 and 1534), VIF (kdr in 1016 only) and VIF (kdr in 1016 only). Considering only samples with the six best-represented genotypes, the haplotypic frequencies were calculated as 40.0% (VVC), 38.2% (LIC) and 10.4% (VVF), indicating that 410L was associated with 1016I.
and 1534C (NaV R2 allele) and that 1534C also occurs alone (NaV R1 allele). Nevertheless, we cannot exclude the possibility that the underrepresented genotypes could be composed by additional haplotypes circulating under low frequencies. The complete dataset displaying the numbers and frequencies for each SNP in all populations is presented in Supplementary Table S2.

Table 1. Genotypes of 123 Aedes aegypti populations from Brazil, considering the V1016I and F1534C SNPs in the NaV gene. a Number of samples with their respective genotypes, b Minimum and c Maximum observed frequencies.

| PCR results | Genotypes | Number (frequency) | Median | Min | Max |
|-------------|------------|--------------------|--------|-----|-----|
| VV + FF     | SS         | 255 (0.048)        | 0.022  | 0   | 0.523 |
| VV + FC     | SR1        | 652 (0.122)        | 0.098  | 0.511 |
| VV + CC     | R1R1       | 1,054 (0.197)      | 0.136  | 1.000 |
| VI + FC     | SR2, R1R3  | 524 (0.098)        | 0.089  | 0.333 |
| VI + CC     | R1R2       | 1,548 (0.290)      | 0.279  | 0.591 |
| II + CC     | R2R2       | 1,301 (0.244)      | 0.222  | 0.810 |
| VI + FF     | SR3        | 3 (0.006)          | 0.000  | 0.024 |
| II + FC     | R2R3       | 2 (0.004)          | 0.000  | 0.022 |
| II + FF     | R3R3       | 2 (0.004)          | 0.000  | 0.023 |

Figure 2. Distribution of the kdr allelic frequencies in Aedes aegypti populations from Brazil considering the V1016I and F1534C variations in the voltage gated sodium channel. Localities are represented by numbers in the legend, exhibited on the map according to their respective geographical coordinates, grouped within the five Brazilian macro-regions. The background map is the “OSM TF Landscape” product (Maps Thunderforest, Data OpenStreetMap contributors, under license CC-BY-SA 2.0). Map was generated with the free and open source software (GNU General Public License) QGIS version 3.12.3 (developed by the Open Source Geospatial Foundation Project, http://qgis.org).
Discussion

The molecular nation-wide monitoring of the main pyrethroid resistance marker in 123 *Aedes aegypti* populations from Brazil, a country comprising 8.5 million km², where the 26 federal states are infested with this vector, is presented herein. To the best of our knowledge, this is the largest *kdr* survey performed using samples collected in the time span of one year. The regionalized profile of allelic frequency distribution indicates that this sample size is enough to represent the whole country.

The frequency of *kdr* alleles was very high in Brazil (median of 83.1%), representing an increase of 27.8% since a previous survey was performed, evaluating 29 localities where mosquitoes were collected between 2009 and 2012\(^1\). The *kdr* NaVR1 allele (1016Val + 1534Cys\(\text{kdr}\)) was present in all Brazilian populations evaluated here, and fixed in one of them. This was probably the first *kdr* allele to spread in Latin America\(^2\) and is present in at least two haplotypes of independent phylogenetic origins\(^1\). *kdr* NaVR2 (1016Ile\(\text{kdr}\) + 1534Cys\(\text{kdr}\)) confers a higher level of resistance to pyrethroids compared to NaVS and *kdr* NaVR1 alleles, as observed in *Ae. aegypti* populations from São Paulo\(^9\).

It is interesting to note that, out of the 17 populations where the wild-type NaVS was absent, 12 were from the Amazonian region. In many of these localities, chemical control is not only exerted to combat *Ae. aegypti*, but also targeting other important vectors, such *Anopheles* mosquitoes, triatomines and phlebotomines, against which pyrethroids are the main choice inside houses and in the peri-domicile area. Therefore, the scenario observed herein may be explained by this constant selection pressure.

We also presented the application of a ‘*kdr index*’ which considers the *kdr* genotype frequencies weighed by their respective knockdown time resistance ratio (*KdT RR\(_{95})*). The lowest *kdr* indexes were predominately found in populations from the Northeast, where the *kdr* NaVR2 allele was almost absent from the previous survey\(^2\) and is now present in all states in that region, with frequencies ranging from 10.2% (Jardim do Seridó, RN) to 83.8% (Itabaiana, SE). In fact, in a previous study assessing resistance to the pyrethroid deltamethrin amongst the 13 populations with pyrethroid resistance ratios (*RR\(_{95}\)*) below 10, reported that seven were located in the Northeast region. In addition, only two populations in the Northeast were amongst the 24 highly resistant populations (*RR\(_{95}\) > 10)\(^6\).

The study of the spatial distribution of resistance markers can aid in identifying underlying resistance emergence processes in surrounding areas. In fact, these determinants can exhibit significant “spatial patterns”, due to their strong dependency to space, climate, land cover and land use, active and passive mosquito mobility between connected cities and regional strategies for vector control, among others. These patterns may display spatial variations at different scales. As a consequence, a spatial distribution of the resistance level results of the mixture of these different multi-scale spatial patterns is observed. The decomposition of the observed level of resistance into various independent and significant spatial patterns can, consequently, not only exhibit spatial clusters of cities that present significantly high (or low) resistance levels, but also highlight spatial patterns typical of phenomena that may, therefore, be considered as potential determinants. In the present study, we tested a series of analyses considering the spatialized *kdr* index values, revealing two to three well defined clustered...
populations in the country. This evidenced that the genetic trend of higher or lower pyrethroid resistant (the kdr index) is not random in Brazil. The populations placed in the A12 or A22 areas, in the models with two or three clusters, respectively, are more likely to be resistant to pyrethroids than the rest of the country, considering the target site mechanism. On the other hand, specific conditions may lead to diverse insecticide resistance patterns, including distinct kdr allelic frequencies, as observed in Ae. aegypti populations from five neighborhoods in Merida, Mexico.

New studies including spatial patterns details will further knowledge on the dynamics of insecticide resistance distribution and expansion in natural populations.

The most frequent genotype in Ae. aegypti from Brazil was R1R2, which was heterozygous for the alleles NaVR1 and NaVR2, in 28.9% of all evaluated samples. The second most frequent, at 24.4%, was the double kdr R2R2, homozygous for NaVR2, followed by R1R1 in 19.9%, homozygous for NaVR1. The sum of all “resistant genotypes” indicated that 73.2% of all Ae. aegypti from Brazil evaluated herein would be resistant to pyrethroid, at least considering the target-site resistance mechanism. It is worth noting, however, that metabolic resistance might also contribute, alone or in synergism with kdr, to confer pyrethroid resistance in the natural populations of this country.

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kdr mutations, or at least the kdr NaVR2 allele, have a high fitness cost and tend to decrease in insecticide-free environments. In a very practical example, the first Ae. aegypti lineage infected with Wolbachia released in Rio de Janeiro was actually a result of backcrosses between an original infected Australian lineage and a natural Brazilian population, therefore resulting in a Wolbachia-infected lineage resistant to pyrethroids. However, during several generations in the laboratory awaiting governmental licenses for release, kdr frequencies decreased and the lineage became susceptible. As residents were spraying pyrethroid-based aerosols in the study area,
Wolbachia dispersion did not occur as expected and the first release failed. Thereafter, a new lineage was again backcrossed with field mosquitoes in order to guarantee similar conditions to the well-adapted field population, which then allowed Wolbachia to expand and stabilize in the study area. The kdr frequency is now monitored in the laboratory lineage and in the target field population in the World Mosquito Program32. The kdr SNP V410L was first detected in Ae. aegypti samples from Northern and Southeastern Brazil29, also observed in samples from Mexico since 200221, where it is currently highly disseminated35. The V410L SNP was detected in several of our evaluated samples, with strong association of the mutant variation (410Leu) with 1016Ile (and, therefore, with 1534Cys), i.e. the triple kdr allele. In a few samples however, 410L was dissociated from these other kdr mutations, as evidenced by individuals genotyped as LL + VI + FC, VL + VV + CC and VL + II + CC (all in the order 410  + 1016 + 1534), which account for only 1.1% of the total. Similar results were

Figure 5. Results of the kdr index value distribution spatial analysis. (a) and (b) indicate the spatial patterns and the corresponding regions identified from the kdr index value spatial distributions in Brazil for Ae. aegypti. These patterns correspond to the most explanatory eigenvectors of a principal coordinates analysis of a given neighbor matrix (see Supplementary Text S1). White and black squares consist of negative and positive eigengenon component values, respectively, and square size is proportional to the absolute value of the vector components. These patterns were selected because they present a significant Moran’s I spatial autocorrelation and explain 51.6% (29.6% and 22.0% for A and B, respectively) of the total variance of the observed kdr index values, by their inclusion as explanatory variables in a multiple linear regression model. (c) and (d) display the kdr index value distributions as a function of the identified regions. The background map is the “OSM TF Landscape” product (Maps Thunderforest, Data OpenStreetMap contributors, under license CC-BY-SA 2.0). Map was generated with the free and open source software (GNU General Public License) QGIS version 3.12.3 (developed by the Open Source Geospatial Foundation Project, http://qgis.org).

Table 2. Genotypes of 25 Ae. aegypti populations from Brazil, considering the V410L, V1016I and F1534C SNPs in the NaV gene. a Number of samples with their respective genotypes, bMinimum and cMaximum observed frequencies.

| PCR results (410 + 1016 + 1534) | Genotypes   | Number (frequency)a | Median | Minb | Maxc |
|-----------------------------|-------------|---------------------|--------|------|------|
| VV + VV + FF                | VVF/VVF (SS)| 26 (0.027)          | 0      | 0    | 0.250|
| VV + VV + FC                | VVF/VVC (SR1)| 108 (0.111)        | 0.089  | 0    | 0.356|
| VV + VV + CC                | VVC/VVC (R1R1)| 272 (0.278)      | 0.227  | 0.044 | 1.000|
| VL + VI + FC                | VVF/LIC (SR2), VVC/LIF (R1R3)| 59 (0.060) | 0.034  | 0    | 0.182|
| VL + VI + CC                | VVC/LIC (R1R2)| 262 (0.268)      | 0.244  | 0    | 0.489|
| LL + II + CC                | LIC/LIC (R2R2)| 233 (0.239)      | 0.227  | 0    | 0.583|
| VL + VI + CC                | VVC/VIC     | 2 (0.002)          | 0      | 0    | 0.022|
| VL + VI + FC                | VVF/VIC, VVC/VIF | 4 (0.004) | 0      | 0    | 0.067|
| VL + VV + CC                | VVC/LVC     | 2 (0.002)          | 0      | 0    | 0.038|
| LL + VI + FC                | LYF/LIC, LVC/LIF | 6 (0.006) | 0      | 0    | 0.044|
| VL + II + CC                | LIC/LIC     | 3 (0.003)          | 0      | 0    | 0.047|

Wolbachia dispersion did not occur as expected and the first release failed. Thereafter, a new lineage was again backcrossed with field mosquitoes in order to guarantee similar conditions to the well-adapted field population, which then allowed Wolbachia to expand and stabilize in the study area. The kdr frequency is now monitored in the laboratory lineage and in the target field population in the World Mosquito Program32. The kdr SNP V410L was first detected in Ae. aegypti samples from Northern and Southeastern Brazil29, also observed in samples from Mexico since 200221, where it is currently highly disseminated35. The V410L SNP was detected in several of our evaluated samples, with strong association of the mutant variation (410Leu) with 1016Ile (and, therefore, with 1534Cys), i.e. the triple kdr allele. In a few samples however, 410L was dissociated from these other kdr mutations, as evidenced by individuals genotyped as LL + VI + FC, VL + VV + CC and VL + II + CC (all in the order 410 + 1016 + 1534), which account for only 1.1% of the total. Similar results were
also observed in samples from Mexico, where mutations in 410 and 1016 were always associated, or, otherwise, dissociated in very low frequencies. In order to save consumables in a broad surveillance in *Ae. aegypti* in Latin American countries, where resources are limited, we recommend that the 1016 site may be genotyped first, and for samples genotyped as 1016 V/V or V/I, a second reaction for the 1534 SNP should be performed. On the other hand, when the samples are detected as 1016 I/I, their genotype are likely to be R2R2, i.e. homozygous for the three *kdr* mutations. Thus, it would not be necessary to genotype the 410 site at all. For academic purposes however, it would be interesting to track possible fluctuations in the frequency of these rare genotypes over time.

New methods for controlling *Ae. aegypti* or at least making the species “immune” to arbovirus infections are being tested or have already been implemented as actual control methods. The use of insecticides however may be maintained, due to their potential of rapidly decreasing the density of a targeted population, unless resistance to the applied compound is present. New products with active neonicotinoid-class ingredients have been recently approved by WHO PQT-VC, namely clothianidin, as an indoor residual spray (IRS), and imidacloprid, as an ultra-low volume (ULV) compound. Although these neonicotinoids are slower-acting than pyrethroids and organophosphates, resistant populations to these insecticides seem to not present cross-resistance, indicating them as promising alternative adulticides. In Brazil, the most recent nation-wide *Ae. aegypti* IR monitoring detected several populations resistant to malathion. The Brazilian MoH is currently planning the implementation of an alternative compound comprising a mixture of pyrethroids and clothianidin to be sprayed inside houses, in addition to another compound comprising a mixture of pyrethroids and imidacloprid as an ULV for outdoor areas. On the other hand, a deeper discussion should consider environmental issues regarding neonicotinoids in non-target organisms. In meanwhile, new arbovirus case records in 2019 in Brazil consisted of over 1.5 million dengue cases, 130 thousand chikungunya cases and 10 thousand Zika cases. In Brazil, as well as in several other countries with similar climate and urban landscape conditions, the implementation of new vector control tools is paramount.

**Material and methods**

Collections were performed in the context of the national Brazilian insecticide resistance monitoring program for *Ae. aegypti* between 2017 and 2018. Field agents installed eggtraps randomly distributed according to the total number of houses in each municipality, as follows: 100, 150, 200 or 300 traps for < 50, 50–200, 200–500 and > 500 thousand houses, respectively. The installation and collection methodology of the eggtraps is described elsewhere. A presential meeting was organized with field-personnel representatives of each state and a video with step-by-step procedures was launched (https://www.youtube.com/watch?v=2w89kagSOKM) in order to ensure that collections were made as homogeneously as possible. The palettes were sent to the laboratory, where the eggs were stimulated to hatch and larvae were raised up to adults in order to produce an F1 colony of each locality, as previously described. After 4–5 days in the cages, enough time for copulation, a sampling of this F0 generation of each population, around 45 insects, preferentially males, were removed and saved for *kdr* genotyping. These mosquitoes were maintained in 80% ethanol solution or cryopreserved prior to DNA extraction. The DNA was extracted from single mosquitoes using the FastDNA Spin (MP Biomedicals) or Nucleo Spin Tissue (Macherey–Nagel) kits, according to their manufacturers’ protocol. The eluted DNA was diluted to 10 ng/µL in extra-pure water and cryopreserved until use.

We performed independent genotyping reactions for each *kdr* site based on a qPCR approach using the Custom TaqMan SNP Genotyping Assay (ThermoFisher) (see Table 3 for the primer and probe sequences for each assay). Reactions consisted of 1X TaqMan Genotyping Master Mix (ThermoFisher), 1X of the respective Custom TaqMan SNP Genotyping Assay, 20 ng of DNA and ultra-pure water q.s. 10 µL, run in a QuantStudio

![Figure 6. Frequency of *kdr* genotypes in *Aedes aegypti* from Brazil, considering the 410, 1016 and 1534 sites in the voltage gated sodium channel. Dots represent the allelic frequency of each population and bars indicate the median and quartile distribution of the respective allele.](https://www.scientificreports.com/content/full/10/13267/fig6.png)
6 Flex (Applied Biosystems), under standard conditions: 45 cycles with a DNA denaturation step (95 °C for 15 s) and primer and probe annealing, followed by DNA polymerization (60 °C for 1 min). The genotypes were obtained by the online software Genotype Analysis Module V3.9 (Applied Biosystems, ThermoFischer cloud platform). The kdr sites 1016 (V1016I) and 1534 (F1534C) were evaluated in all populations. For 25 out of the 123 populations, mostly from state capitals, the 410 SNP (V410L) was also assessed.

Considering that the evaluated SNPs are linked in the same gene, the genotype of each individual contemplated the results of each site, 1016 (VI, II) and 1534 (FC, CC), resulting in the total combination of nine possible genotypes, composed by four possible haplotypes, S, R1, R2 and R3, as displayed in Fig. 7. For the 25 populations in which the 410 site (VV, VL, LL) was also genotyped, a total of 27 combined genotypes and, consequently, eight haplotypes: VVF, VVC, VIC, VIF, LVF, LVC, LIC and LIF, were possible.

We ranked the populations in relation to their predicted level of resistance to pyrethroids, based on the kdr index, consisting of the sum of kdr genotype frequencies weighed with their respective resistance ratios, which were previously obtained in a knockdown time test (KdT RR95, Brito et al.27. The kdr index of a population is:

Table 3. Primer and probe sequences for the SNPs V410L, V1016I and F1534C kdr in Aedes aegypti.

| Na+, site | Assay ID* | Variation | Primers | Probes |
|-----------|-----------|-----------|---------|--------|
| 410       | AN2XAPW   | GTA/TTA   | For: GTGGCAATGCTCTTCTCTATT | Val: VIC-TGGTTTACCTTGTTAATT-NFQ |
|           |           | (Val/Leu)| Rev: GGCGAATGGCCAAGATC | Leu: FAM-TGGTTTACCTTTTAAATT-NFQ |
| 1016      | AHS1DL6   | GTA/ATA   | For: CGTGCTAACCAGCAAATTGTTC | Val: VIC-CCGCACAGTACTTA-FAM |
|           |           | (Val/Ile)| Rev: GACAAAAAGCCAAAGCTAAAGATGC | Ile: FAM-CCGCACAGTACTTA-NFQ |
| 1534      | AHWSL61   | TTC/TGC  | For: TCCGCGACACACATCTAGT | Phe: VIC-AACCGGGAAGTGA-NFQ |
|           |           | (Phe/Cys)| Rev: GATGATGACCCGATGAAAGAT | Cys: FAM-ACCCGCGAGATG-NFQ |

Figure 7. Representation of the kdr genotype based on PCR reactions for the 1016 (Val+, Ile-kdr) and 1534 (Phe+, Cys-kdr) variations in the voltage gated sodium channel of Aedes aegypti.

6 Flex (Applied Biosystems), under standard conditions: 45 cycles with a DNA denaturation step (95 °C for 15 s) and primer and probe annealing, followed by DNA polymerization (60 °C for 1 min). The genotypes were obtained by the online software Genotype Analysis Module V3.9 (Applied Biosystems, ThermoFischer cloud platform). The kdr sites 1016 (V1016I) and 1534 (F1534C) were evaluated in all populations. For 25 out of the 123 populations, mostly from state capitals, the 410 SNP (V410L) was also assessed.

Considering that the evaluated SNPs are linked in the same gene, the genotype of each individual contemplated the results of each site, 1016 (VI, II) and 1534 (FC, CC), resulting in the total combination of nine possible genotypes, composed by four possible haplotypes, S, R1, R2 and R3, as displayed in Fig. 7. For the 25 populations in which the 410 site (VV, VL, LL) was also genotyped, a total of 27 combined genotypes and, consequently, eight haplotypes: VVF, VVC, VIC, VIF, LVF, LVC, LIC and LIF, were possible.

We ranked the populations in relation to their predicted level of resistance to pyrethroids, based on the kdr index, consisting of the sum of kdr genotype frequencies weighed with their respective resistance ratios, which were previously obtained in a knockdown time test (KdT RR95, Brito et al.27. The kdr index values for the genotypes were S5 (1), SR1 (2.4), SR2 (1.7), R1R1 (4.6), R1R2 (5.4) and R2R2 (6.7). The formula for the kdr index of a population is:

Table 3. Primer and probe sequences for the SNPs V410L, V1016I and F1534C kdr in Aedes aegypti.

*Identification of the customized TaqMan SNP Genotyping Assay (ThermoFischer).
In order to test the existence of significant multi-scale spatial patterns (according to Moran’s Index of spatial autocorrelation) regarding the spatial distribution of the $kdr$ frequencies, we considered the aforementioned $kdr$ index and the geographic coordinates of each locality in a series of spatial statistic tests, applying a principal coordinate analysis of neighbor matrices and Moran’s eigenvector maps \cite{35}, as detailed in Supplementary Text 1.

**Data availability**

A table with the genotyping data of all populations can be accessed in the supplementary files. Further data and materials are available from the corresponding author upon reasonable request.

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Author contributions

A.J.M., K.B.C. and J.B.P.L. conceived the idea and designed the study. C.M.R. and D.F.B. organized the sample collection and funds administration. M.M.C. and L.P.B. were responsible for the DNA extraction, qPCR reactions and analyses. E.R. designed and performed the spatial analyses. A.J.M., K.B.C. and J.B.P.L. conceived the idea and designed the study. C.M.R. and D.F.B. organized the sample collection and funds administration. M.M.C. and L.P.B. were responsible for the DNA extraction, qPCR reactions and analyses. E.R. designed and performed the spatial analyses. A.J.M. compiled the data. All authors wrote, reviewed and discussed and are in accordance with the final version of this manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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