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Genetic and morphological polymorphisms of *Aedes scapularis (Diptera: Culicidae),* vector of filariae and arboviruses

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ABSTRACT

Background: Aedes scapularis is a neotropical mosquito that is competent to vector viruses and filariae. It is reputed to be highly morphologically and genetically polymorphic, facts that have raised questions about whether it is a single taxonomic entity. In the last five decades, authors have posed the hypothesis that it could actually be a species complex under incipient speciation. Due to its epidemiological importance, its taxonomic status should be determined with confidence.
Aim and method: Our objective was to investigate more deeply the polymorphism of *Ae. scapularis* to detect any evidence of incipient speciation of cryptic species. We then compared populational samples from the Southeastern, Northern and Northeastern regions of Brazil. The biological markers used in the comparison were: the complete mitochondrial DNA, the isolated mitochondrial gene cytochrome oxidase subunit I (*COI*) and wing geometry.
Results and discussion: As expected, high morphological/genetic polymorphism was observed in all *Ae. scapularis* populations, however it was not indicative of segregation or incipient speciation. There was no correlation between wing shape and the geographical origin of the populations analysed. A congruent observation resulted from the analysis of the *COI* gene, which revealed a high number of haplotypes (51) and no clusterization of populational samples according to the original biomes. In the phylogenetic analysis of the 13 mitochondrial protein-coding genes, the *Ae. scapularis* clade clustered with maximum support (100% bootstrap support and posterior probability of 1). No significant internal structure was observed in the *Ae. scapularis* clade, which was nearly a polytomy. Taken together, our results indicate that this species is not a species complex.
Conclusion: We conclude that there was no indication, in the analysed regions, of the occurrence of more than one taxon in the species *Ae. scapularis*, despite it being highly polymorphic. By ruling out the former species complex hypothesis, our phylogenetic results reinforce that *Ae. scapularis* is a single taxonomic unit and should be monitored with standardized surveillance and control methods.

1. Introduction

Aedes scapularis is a widely spread neotropical mosquito species able to vector filariae and arboviruses (Forattini, 2002; Arnell, 1976). The *Ae. scapularis* larvae grow in natural pools of water in sylvatic environments, however this mosquito is currently under incipient adaptation to urban regions (Wilke et al., 2017). The *Ae. scapularis* larvae are observed with increasing frequency in rural, semi-rural and urban regions in artificial containers such as paint cans and water tanks (Dávalos-Becerril et al., 2019). The fact that this species can adapt to the urban environment makes it a potential risk to public health, since it presents vectorial competence for etiologic agents of arboviruses (Forattini, 2002), such as

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Melao virus, Yellow Fever virus, Ilhéus and Venezuelan Equine encephalitis, in addition to the Rocio virus (Spence et al., 1962; Arnell, 1976), and filarias (Labarthe et al., 1998) such as *Wuchereria bancrofti*, (Forattini, 2002).

Among these etiologic agents, its vectorial capacity for the Rocio virus stands out. Rocio is a RNA virus of the Flaviviridae family which causes symptoms similar to those of dengue virus: headache, fever, body aches and can cause the patient's death. It is believed that *Ae. scapularis* was the most likely vector of the Rocio virus in the Vale do Ribeira area (São Paulo State, Brazil), since this species was the one most frequently found at the site, with high anthropophilia and presenting vectorial competence for this virus in the laboratory (Mitchell et al., 1984).

In addition, *Ae. scapularis* has already been found naturally infected with the Yellow Fever virus in the Brazilian states of Minas Gerais, São Paulo and Rio de Janeiro (Vasconcelos et al., 2001).This mosquito species can also act as a vector for *Dirofilaria immitis*, a parasite of dogs, and it possibly transmits the larvae of this filaria in the coastal lowlands of Rio de Janeiro (Lourenço-de-Oliveira et al., 1995).

The species *Ae. scapularis* has high genetic and morphological polymorphism. The first work focused on the polymorphism of *Ae. scapularis* was carried out in 1976 by Arnell in a population study of this mosquito. That author found differences in the number of retrodirected processes (RPs) of the apical filament of the gonocoxite on one side of the male genitalia (presence and absence of RP) between individuals of the same population of this species and differences in the size of the scale cover of the scutellum of this mosquito. Later, based on those findings, Forattini, 2002 suggested the hypothesis that such polymorphism was evidence of a complex of species under incipient evolution *Ae. scapularis*. In the papers of Petersen et al. (2015) and Petersen et al. (2018), a high genetic and morphological polymorphism (in the genitalia) was observed in populations of this mosquito in the Atlantic forest, but no evidence of a species complex was found. Currently, the hypothesis of the existence of a complex in *Ae. scapularis* has not been ruled out, because the populations of this species from other regions of Brazil had not yet been analysed.

Thus, in the present work we raised the following questions: Is there intraspecific polymorphism in *Ae. scapularis* that is discrete enough to indicate incipient speciation? Is there any cryptic species within *Ae. scapularis*? The following markers were used to answer these questions: complete mitochondrial DNA and wing geometric morphometrics. We carried out collections of *Ae. scapularis* in the Atlantic Forest (Southeast), Caatinga (Northeast) and Amazon Forest (North) regions of Brazil. We believe that by using the mitochondrial and morphological markers of the wing, it will be possible to verify if this species is solely a single taxon.

2. Material and methods

2.1. Mosquito collections

In this work, adult female mosquitoes were collected with entomological aspirators. After the collection, these mosquitoes were euthanized in a freezer at −20 °C. After this process, the wings of the individuals collected were removed to perform the wing geometric morphometric technique. After removing the wings, the same individuals were kept in a freezer at −80 °C until DNA extraction for the amplification of mitochondrial genes. The collection sites were: Ananindeua-Pará (ANA), Poço Redondo - Sergipe (PRE), locations in the city of São Paulo-SP –the garden of the Butantan Institute (BUT) and Tietê Ecological Park (PET), Pariquera-Açu - São Paulo (PAR) and Itaboraí - Rio de Janeiro (PAR) (Fig. 1).

2.2. Wing morphometric analysis

The wing shape data was analysed using geometric morphometrics (similarly Vidal et al., 2012 and Petersen et al., 2015) as detailed as follows. The wings after removing the scales were assembled with Canadian balsam between slide coverslips for morphometric analysis. Images of these wings were captured using a digital camera coupled to a Leica S6 stereoscopic microscope. Approximately 30 females from each population were analysed. On these images, and with the help of the TpsDig V.1.40 software (QSC - James Rohlf), the positional coordinates of each of the 18 anatomical points on a Cartesian plane were taken. On this data, the sizes of the centroid and the relative warps, which are the

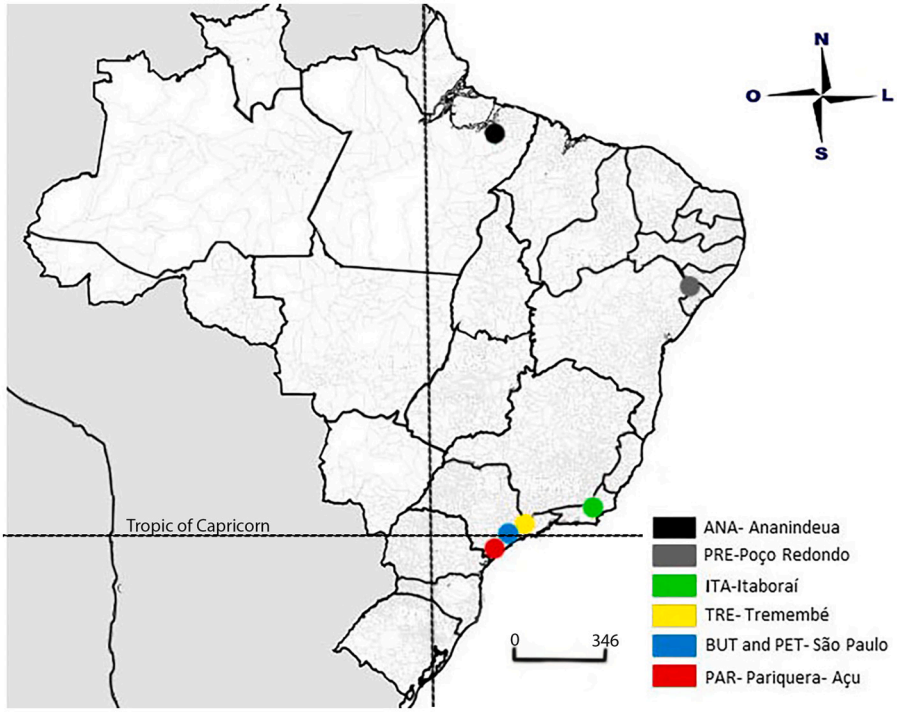


Fig. 1. Map of samples collected in Brazil: Ananindeua, Itaboraí, São Paulo, Pariquera-Açu and Tremembé.

main components of form, were computed, with the aid of the computer programs TpsUtil 1.29, TpsRelw 1.39 (James Rohlf) and Statistica 11 (StatSoft). The statistical tests used were ANOVA for size and discriminant analysis and metric disparity for shape. For the assembly of the morphological similarity phenogram we used, as an external group, a population of *Aedes aegypti* collected in São Paulo city. Morphological diversity index (*sensu* Petersen et al., 2015) was estimated using the "amount of dispersion" of individuals in the morpho-space of PCs. This dispersion was calculated (according o Petersen et al., 2015) as follows: points in the morpho-space of CPs (each corresponding to a single mosquito) were digitized using TpsDig software (as performed for the wings), in order to record their position coordinates in an imaginary Cartesian plane. The least squares of a set of individuals (the population) was calculated using TpsRelW software. Such least squares was then considered as an indicator of the morphological diversity of a population. Theoretically, the amount of dispersion of individuals (of a single set) in the morpho-space of PCs is proportional to the morphological variability of that set.

2.3. Genomic DNA extraction

Genomic DNA was extracted from each individual using the DNeasy Blood & Tissue extraction kit (Qiagen, California, USA), following manufacturer instructions. The DNA of individuals from the ANA, PRE, BUT, and ITA populations were extracted for the analysis of complete mitochondrial DNA (17 individuals) (Table 1). The total mitochondrial DNA of 17 individuals was amplified in order to arrive at a sufficient sample in order to answer the hypothesis of the occurrence of a species complex in *Ae. scapularis*. (Sufficiency sampling calculated according to Devendran et al., 2019).

2.4. Amplification and sequencing of complete mitochondrial DNA

For a 50 µl final reaction (Oliveira et al., 2016) of "Long-Range PCR", 25 µl of GoTaq Long PCR Master Mix 2 x, 1.5 mM from each primer (HPK16Saa and HPK16Sbb) was added, 500 ng of extracted DNA and 50 ml of Milli-Q® water. The following degenerate and palindromic primers were used, with the sequences: HPK16Saa 5 'ATG CTA CCT TTG CAC RGT CAA GAT ACY GCG GC 3' (Primer Foward) and HPK16Sbb 5 'CTT ATC GAY AAA AAA GWT TGC GAC CTC GAT GTT G 3 '(Primer Reverse), these primers were developed by Hwang et al. (2001). For DNA amplification, the following conditions were used: 94 °C for 2 min, 94 °C for 15 s, 65 °C for 15 min in 10 cycles, 94 °C for 15 s, 65 °C for 15 min, 65 °C for 10 s in 20 cycles and 72 °C for 10 min.

Sequencing was carried out by the company "Genotyping" Botucatu, São Paulo. Sequencing was carried out as follows: a) Quantification of total DNA: in this step, the kit "Qubit® dsDNA BR Assay Kit was used in Qubit 2.0 Fluorometer equipment; b) Tagmentation; c) PCR amplification: In this stage, the following cycling was performed: stage 1 was 72 °C for 3 min, in the following stages, 12 cycles were performed. Stage 2 was 95 °C for 30 s, stage 3 was 95 °C for 10 s, stage 4 was 55 °C for 30 s and stage 5 was 72 °C for 30 s. Stage 6 was 72 °C for 5 min, stage 7 was 10 °C. The amplification reactions were carried out in a Veriti ™ Thermal Cycler thermocycler (Applied Biosystems); d) Quantification of DNA by Real Time PCR: the reagent used was KAPA-KK4824 (Library Quantification Kit-Illumina / Universal); e) Sequencing of the libraries in a Illumina MiSeq instrument.

2.5. Amplification, purification and sequencing of the cytochrome oxidase subunit I gene

The primers used in the amplification of the coding gene for the enzyme Cytochrome Oxidase Subunit I are universal (LCO1490: 5′-GGTCAACAAATCATAAAGATATTGG-3′ for the forward primer and HC02198: 5′- TAAACTTCAGGGTGACCAAAAAATCA-3′ for the reverse primer), as published by Folmer et al. (1994) and employed by Petersen et al. (2015). PCR to COI reactions were carried out according to the general guidelines of Sambrook & Russell (2006). A reaction containing a final volume of 25 ml was used: PCR buffer 10 X (Invitrogen), 3 mM MgCl2 (25 mM) (Invitrogen) was added), 0.4 mM dNTP (25 mM) (KapaBiosystems), 10 pmol of each primer, 0.5 U of Platinum ® Taq DNA Polymerase (5 U / µl) (Invitrogen) and 500 ng of extracted DNA sample. The amplification cycle consisted of: Initial temperature of 94 °C for 3 min, followed for 40 cycles 1 min of 94 °C, 55 °C for 1 min, 72 °C for 1 min, with a final extension cycle of 72 °C for 7 min for 40 cycles. Amplification was checked using 1% agarose gel electrophoresis. The PCR product was purified using the DNA PureLink® PCR Purification kit (Invitrogen, California, USA). The sequencing reaction used 2 µl of BigDye, 1 µl of 5× buffer, 5 µM of primer and 40 ng of purified PCR product. The sequencing reaction occurred as follows: 96 °C for 1 min, 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. The sequencing of the *COI* genes was carried out in an ABI 3730 sequencer, according to the protocol used by the "Human Genome Center of the University of São Paulo".

2.6. Assembly and annotation of mitochondrial genes

The mitochondrial DNA sequences were assembled using Mira 4 and MITObim Assembly software (Hahn et al., 2013). To check the quality of the assembled sequences, it was visualized in the Tablet software. Mitochondrial genes were noted in online MITOs Web Service software (). The protein coding genes (PCGs) were verified in the software Wise 2 and ExpASy (Artimo et al., 2012).

2.7. Test of correlation between markers

We tested the correlation between the variables genetic distances (p-distance) and morphological distances (Procrustes distance) using the linear regression test. The same procedure was used to verify the correlation between haplotype diversity and morphological diversity. We also tested the correlation between geographic and morphological (Mahalanobis) distances. The Statistica 13.5 (StatSoft) software was used to perform these linear regression analyses.

Table 1-
Data from seven collected samples (municipalities, locations, number of individuals used in morphological and genetic analysis) in different cities in Brazil: São Paulo, Itaboraí, Pariquera-Açu, Poço Redondo and Tremembé.

Code	Municipality	Locality	wing shape	mtDNA	complete mtDNA	Geographic coordinates
ANA	Ananindeua	Evandro Chagas Institute	33	14	4	1°22′24.5″S 48°23′09.0″W
BUT	São Paulo	Butantan Institute Garden	39	10	6	23°34′04.4″S 46°43′08.4″W
PET	São Paulo	Parque Ecológico do Tietê	29	34	–	23°29′42.9″S 46°31′11.2″W
ITA	Itaboraí	Pasture	30	36	5	22°44′03.8″S 42°51′21.7″W
PAR	Pariquera-Açu	Experimental Farm	25	21	–	24°42′48.3″S 47°52′56.4″W
PRE	Poço Redondo	Grota do angico	28	18	3	640 44″67′S 890 33″ 45′O
TRE	Tremembé	City outskirts	30	29	–	22°58′07.3″S 45°33′23.5″W

3. Results

3.1. Wing geometry

The interpopulation analysis of canonical variables derived from the wing geometric morphometry method of the ANA, PRE, PAR, ITA, TRE, PET, and BUT populations revealed a low population structure, as there is an intersection between the seven studied samples. Despite the apparent low population structure in the morpho-space (Fig. 2), it is possible to better observe it through the values of the Mahalanobis distances, where these distances were significant among the seven populations in this study. The greatest morphological distances occurred between the populations of ITA and BUT (5.6241) and the smallest between PRE and PET (2.1225). Observing the phenogram constructed using the Neighbor Joining method (Fig. 2), based on the Mahalanobis distances to verify the inter-population morphological similarities analysed, it was not possible to associate the population grouping to its geographical location. To test for the presence of a correlation between the variables geographic location and phenetic distance (Mahalanobis distance), a regression analysis was performed between these variables, but the correlation found was not statistically significant, with a value of $p = 0.5$. The populations with the most morphological diversity were TRE, ITA, and PET, all of which occur in the Atlantic forest biome and the population with the least morphological diversity was ANA (Fig. 3).

3.2. COI mitochondrial gene analysis

The *COI* gene of 162 individuals from BUT (Butantan 10 samples), ITA (Itaborai 36 samples), PET (Parque ecológico do Tietê 34 sample), TRE (Tremembé 29 samples), PAR (Pariquera-Açu 21 samples), PRE (Poço Redondo 18 samples), and ANA (Ananindeua 14) populations was analysed (Table 1). About 600 bp from this gene were amplified and 68 haplotypes were found; of this total, only 19 haplotypes were shared between at least 2 individuals. The total haplotypic diversity was 0.98, the most polymorphic population considering the *COI* gene was TRE (Fig. 2), and the least polymorphic populations were BUT and PAR. It was not possible to find population structure or evidence of a complex of species using this gene, because in the analysis of the haplotype network (Fig. 4) no cluster formation occurred between any of the seven studied populations of *Ae. scapularis*.

The genetic distance analysis (p-distance) performed using the *COI* gene found that the most genetically similar populations are those of PAR and BUT, while the TRE and ITA populations are the most genetically differentiated from the PRE population. As observed in the dendrogram of phenetic distances, there was no relationship between genetic distances and the biomes of origin of each species (Table 2).

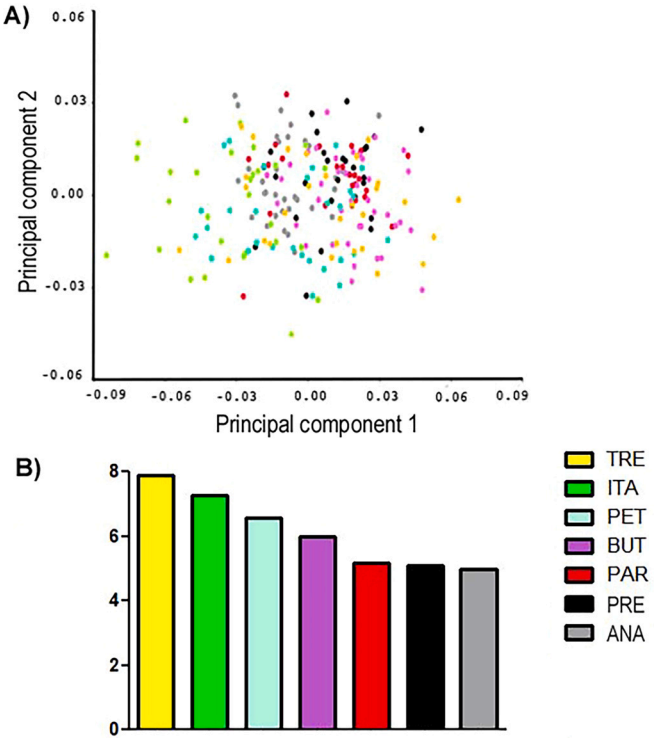


Fig. 3. A) Principal components of the seven *Ae. scapularis* populations studied: from Tietê Ecological Park (PET), Itaboraí (ITA), Tremembé (TRE), Pariquera-Açu (PAR), Butantan (BUT), Poço Redondo (PRE), and Ananindeua (ANA). B) Morphological diversity of *Ae. scapularis* populations.

3.3. Molecular markers X morphological markers

We found a statistically significant correlation between the haplotypic diversity and morphological diversity markers ($p < 0.005$).

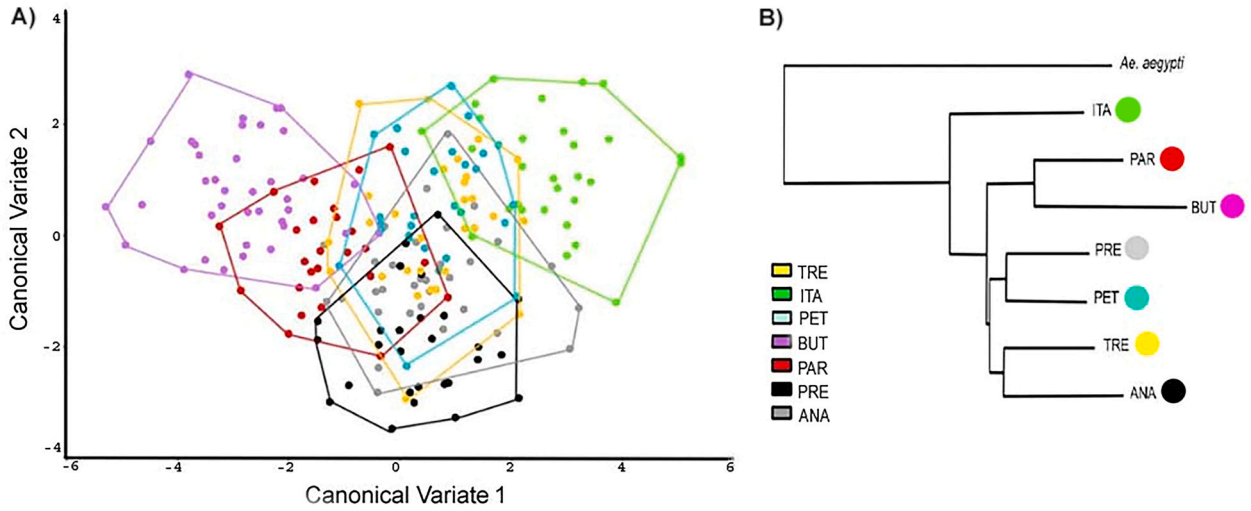
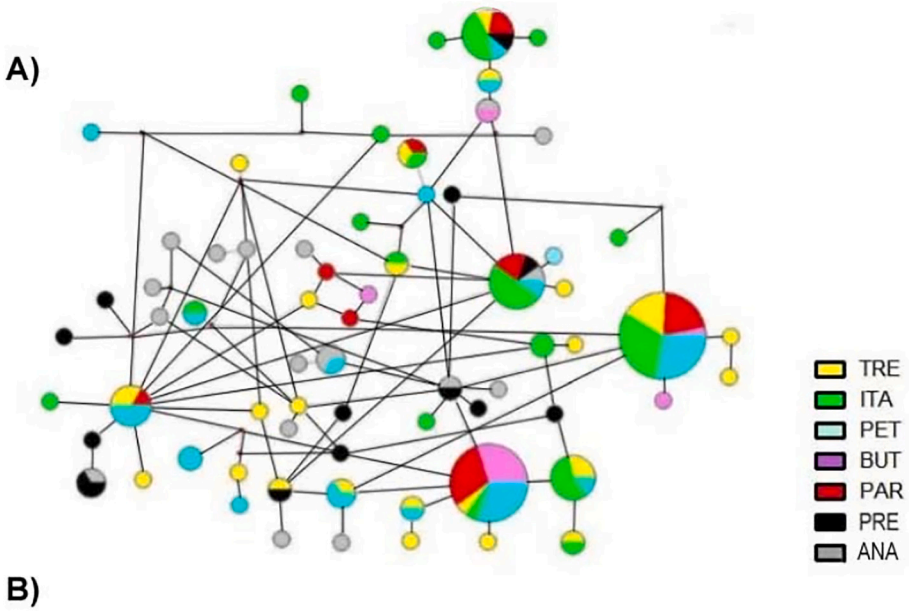


Fig. 2. A) Morpho-space of canonical variables (discriminant analysis) from data referring to the wing shape of the populations of the Parque Ecológico do Tietê (PET), Itaboraí (ITA), Tremembé (TRE), Pariquera-Açu (PAR), Butantan (BUT), Poço Redondo (PRE), and Ananindeua (ANA) from females of *Ae. scapularis*. B) Phenogram constructed with Neighbor joining method from Mahalanobis distances from the seven populations of Ae. scapularis: park Tietê Ecological (PET), Itaboraí (ITA), Tremembé (TRE), Pariquera-Açu (PAR), Butantan (BUT), Poço Redondo (PRE) and Ananindeua (ANA).



B)

Samples	N	Unique Haplotypes	H	H(SD)
PET	34	5	16	0.8841
ITA	36	8	18	0.9238
TRE	29	12	23	0.9704
PAR	21	3	9	0.8476
BUT	10	3	5	0.6667
PRE	18	12	18	0.9940
ANA	14	8	14	1.0000

Fig. 4. A) Haplotype network based on COI gene in five populations of *Ae. scapularis* from Brazil. The size of each circle or circular sector is proportional to the number of individuals sharing that haplotype; the smallest circle corresponds to a single individual.B) Summary polymorphisms analysis of seven *Ae. scapularis* populations. N = Number of sequences used; H = Number of haplotypes, h (SD) = Haplotype diversity; π (SD) = Nucleotide diversity.

Table 2
Genetic distance of *COI* sequences from 7 *Ae. scapularis* populations (Calculation of mean pairwise distances within groups of taxa). PRE = Poço Redondo, ANA = Ananindeua, BUT = Butantan, PAR = Pariquera Açu, TRE = Tremembé, ITA = Itaboraí, PET = Parque Ecológico do Tietê.

	PRE	ANA	BUT	PAR	TRE	ITA	PET
PRE	–						
ANA	0.010						
BUT	0.009	0.008					
PAR	0.009	0.008	0.006				
TRE	0.0011	0.009	0.007	0.008			
ITA	0.011	0.010	0.008	0.008	0.010		
PET	0.010	0.009	0.006	0.007	0.009	0.009	–

However, the same was not seen when we correlated genetic distances (p-distance) and morphological distances (Procrustes Distance) in which the p value was not significant ($p \geq 0.005$).

3.4. Phylogenetic analyses

With the nearly complete mitochondrial DNA of 17 individuals of the *Ae. scapularis* species available, all 13 protein coding genes were used for the phylogenetic analysis: *ATP6*, *ATP8*, *COB*, *COI*, *COII*, *COIII*, *NAD1*, *NAD2*, *NAD3*, *NAD4*, *NAD5*, *NAD6* and *NADl*. In this phylogeny, the following species of culicids were used as an outgroup: *Ochlerotatus fulvus* (MK575476.1), *Haemagogus janthinomys* (MK575481.1), *Ochlerotatus vigilax* (MK575484.1), *Aedes koreicus* (NC_046946.1), *Culex quinquefasciatus* (MK57544), *Culex pipiens pallens* (KT851543.1), *Culex tritaeniorhynchus* (KT851544.1), *Anopheles braziliensis* (NC_037791.1), *Anopheles darlingi* (MK575478.1), *Ae. aegypti* (NC_035159.1), and *Aedes albopictus* (NC_006817) (Fig. 5).

The 7 populations (PET, TRE, PAR, BUT, ITA, PRE and ANA) *of Ae. scapularis* formed a monophyletic clade with a Bayesian probability of 1. However, low probability values were found in the nodes within this clade, which indicates that this species is probably not a complex of species. The *Ochlerotatus fulvus* was the closest species to *Ae. scapularis*, with support in the clade was 1 (as in most of the other nodes in the tree). The species *An. braziliensis* and *An. darlingi* were the most genetically differentiated from the species *Ae. scapularis*.

4. Discussion

4.1. Wing morphometrics

We found low population structure among the seven populations analysed: BUT (Butantan), ITA (Itaboraí), TRE (Tremembé), PAR (Pariquera-Açu), PRE (Poço Redondo), and ANA (Ananindeua). There are morphological intersections between all these populations. The presence of intersections in population groups in the morpho-space of canonical variables also demonstrates that possibly this species is not a complex of species, at least with regard to these biological markers. Individuals of different species, even if phylogenetically close, form a cluster with little or no intersection in the morpho-space, as observed by Lorenz et al. (2015) in a study involving populations of morphologically similar mosquitoes of the species *Anopheles bellator*, *Anopheles homunculus*, and *Anopheles cruzii*.

The greatest morphological distances regarding the wing were observed between the populations ITA and BUT, also suggesting an absence of correlation between genetic and geographic distance, which can be better visualized in the dendrogram constructed from the Mahalanobis distances. We can see in the cladogram constructed by the neighbor joining method that the BUT and PET populations, both from the municipality of São Paulo with only 40 km of geographical distance

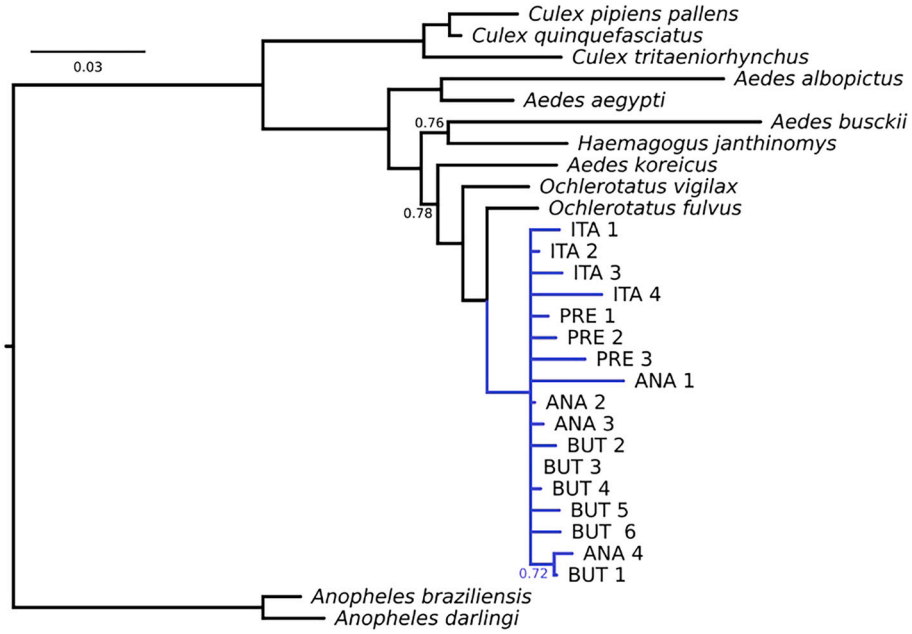


Fig. 5. Bayesian topology generated from the analysis of the 13 mitochondrial protein coding genes in the species *Aedes scapularis*, *Ochlerotatus fulvus*, *Haemagogus janthinomys*, *Ochlerotatus vigilax*, *Aedes koreicus*, *Culex quinquefasciatus*, *Culex pipiens pallens*, *Culex tritaeniorhynchus*, *Anopheles braziliensis*, *Anopheles darlingi*, *Aedes aegypti* e *Aedes albopictus*. The Bayesian probability values were omitted when they had values equal to 1 with the exception of within the Ae. scapularis clade (depicted in blue), which had values below 0.5 except where noted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

between two, are separated in this graph, while geographically distant populations such as PET and PRE are in the same branch. The absence of a correlation between geographical distance and morphological distance from the wing was confirmed by a regression test performed between these two variables. Similar results to this absence of correlation between wing morphology and geographic distance were observed in Atlantic Forest populations studied by Petersen et al. (2015); Motoki et al. (2012) also found no correlation between these two variables in *An. darlingi* populations from five Brazilian ecoregions. The same was observed by Vidal et al. (2012) working with populations of *Ae. aegypti* in the state of São Paulo.

We also did not observe a correlation between morphological similarity and biome of origin for the populations analysed, for example, the TRE mosquitoes collected in a degraded Atlantic Forest area are more similar in wing morphology to mosquitoes from the ANA area, which is from the Amazon Forest environment and is about 3000 km away from TRE. This lack of correspondence between phenotype and geographical distance between populations may have resulted from the action of evolutionary convergence. Another phenomenon that would explain the similarity of the wing shape between populations separated by hundreds or thousands of kilometers is the retention of an ancestral form (Donnelly et al., 2002). However, we do not rule out the possibility that this equivalence between the wing forms between distant populations is caused by an evolutionary convergence, probably due to these populations experiencing similar environmental pressures, even in biomes with xerophytic conditions such as the population of the Caatinga PRE.

Regarding the morphological diversity studied among the seven samples of *Ae. scapularis*, the populations that showed the least diversity were the ANA (Ananindeua-PA) and PRE (Poço Redondo-SE) populations. The low morphological variability of ANA and PRE is probably not due to bottleneck effects, as these populations are genetically diverse according to our mitochondrial gene results.

The wing morphometry discriminates interspecific variations in the wing shape of species and populations of culicids, however, when assessing the presence of a species complex, this analysis must be combined with additional biological markers, especially molecular markers, as done in the present study. An approach similar to the present study was carried out foi Gómez et al. (2013) using wing morphometrics and the *COI* gene to evaluate the members of the *Albitarsis* complex. Those authors found no evidence of more than one species in the analysed populations.

The high morphological polymorphism found in *Ae. scapularis* drew the attention of Forattini, 2002, to the possible existence of a species complex. However, the data of the present study strongly suggests that this polymorphism is a generic characteristic of the species. This high variability, which is also pronounced in genetic markers, might enable the mosquito to adapt to new environments in the urban regions. The interpopulation similarities concerning the shape of the wings by the analysis of Canonical Variables and by the Neighbor Joining method in the seven populations of *Ae. scapularis* indicate that the populations in the present study are not a species complex.

4.2. Cytochrome oxidase subunit I

We found high polymorphism in the *COI* gene, with 68 haplotypes observed in 162 individuals analysed. The network of haplotypes built with the *COI* gene did not show a cluster of individuals from the same populations analysed, indicating low population structure. This high nucleotide variability was observed by Petersen et al. (2015), in which 46 haplotypes were found in 130 individuals analysed. Similarly, Lorenz et al. (2014) had previously observed that populations of *An. cruzii* collected in the plain region and in the higher altitude region also had a large number of haplotypes: 68 haplotypes in only 96 individuals analysed. Despite the high polymorphism found in these *Anopheles* populations, structure was observed in the populations of *An. cruzii* in the plains in relation to mosquitoes collected in the higher regions.

Although the populations of *Ae. scapularis* and An. *cruzii* (Lorenz et al., 2014) have shown a high number of haplotypes, this polymorphism is not very common in mosquitoes non-urban and mainly in exotic urban species. Kang et al. (2012) observed only 59 haplotypes in 305 individuals of the species *Anopheles sinensis*, collected in 20 locations. Gutiérrez et al. (2010) also found a low number of haplotypes in populations of *Ae. darlingi* in five locations in Colombia: 12 haplotypes in 88 individuals. In a study by Paupy et al. (2012), only 8 haplotypes were found in 127 individuals of the species *Ae. aegypti* collected in different metropolitan areas of Bolivia. We found a high number of haplotypes in the *Ae. scapularis*, although this mosquito is naturally infected by the bacteria *Wolbachia*, and this bacterium generally decreases the number of haplotypes in its hosts, as was documented by Morais et al. (2012) in a study of *Cx. quinquefasciatus*.

The haplotype network of the *COI* gene did not show any clustering between the populations analysed, so there was no evidence of the existence of a species complex in *Ae. scapularis* using this marker. Unlike our results, Lehr et al. (2015), in an investigation of a species complex using the mitochondrial gene *COI*, managed to identify four species within the *Albitarsis* complex: *An. albitarsis* ss, *An. albitarsis* B, *An. marajoara*, and E. The authors Scarpassa and Conn (2006) in a study of *Anopheles* (*Nyssorhynchus*) *oswaldoi* populations, found 25 haplotypes in the *COI* gene and none shared among the studied populations. These authors observed four subgroups in this species, which they suspected belonged to the species *An. oswaldoi* S., *An. konderi* and the other two subgroups to the lineages or species within the *Oswaldoi* Complex. Although the *COI* molecular marker does not indicate the existence of complex species in *Ae. scapularis*, this marker once again showed high polymorphism. The results found in the analyses performed with the molecular marker *COI* corroborate the results found in Petersen et al. (2015), and despite the expansion of the collection areas in the present work in relation to those carried out in the article by Petersen et al. (2015), for biomes not previously explored, high polymorphism and haplotypic richness were found in populations in the expanded areas, as occurred with populations collected in the Atlantic Forest (PET, PAR, BUT, ITA and TRE). The greatest genetic distances occurred between the populations of ANA and PRE and the smallest between PRE and PET. The genetic distances were not correlated with the morphological distances of the wing because the greatest morphlogical distances occurred between the populations of ITA and BUT and the smallest between PRE and PET.

In the populations ANA (region of the Amazon Forest) and PRE (Caatinga), haplotypic diversity values of 0.99 and 1.00 were found, respectively, which are similar to those found in populations collected in regions of the Atlantic Forest where the highest values varied from 0.84 to 0.97. This polymorphism found in different Brazilian regions in the three different biomes (Atlantic forest, Caatinga and Amazon region) may be due to the fact that the species *Ae. scapularis* is close to its evolutionary center or origin in South America. The centers of origin are where species originate, and in these places they have high genetic diversity, according to, the centers of origin "act as evolutionary radiation centers". The species *Ae. scapularis* is present in South, North and Central America, so possibly its center of origin is in Brazil or in a region nearby, because even in regions with low rain and with high temperatures like the Caatinga, the PRE population of this location showed high polymorphic value. Probably, this population of mosquitoes (PRE) that inhabits an environment of extreme conditions is quite plastic to adapt to such conditions. Another interpretation that this high polymorphism suggests is that although *Ae. scapularis* lives in a habitat of fragmented forest that has suffered anthropic action or environments with high temperatures and scarcity of rain, apparently this mosquito has not suffered a recent "bottleneck" effect.

The *COI* genetic marker did not show the presence of a species complex, however through the results obtained using this marker it was possible to observe that the species *Ae. scapularis* is equally polymorphic in Atlantic Forest environments and in Caatinga and Amazon biomes.

4.3. Complete mitochondrial DNA - investigation of possible species complex

Sequences of complete mtDNA were and deposited in GenBank at the provisional accession number BankIt2514679. In the phylogenetic analyses constructed by the Bayesian Method using the 13 PCGs was observed the formation of clades of the 4 populations of *Ae. scapularis* analysed. It occurred cluster formation related to the population origin of individuals of this species, the only exception was 1 individual from the ANA population. The bayesian probability of 1 present in the clade of the populations of *Ae. scapularis* in relation to the other species analysed suggests that this mosquito is not a complex, but a single taxon. Allied to this fact, we observed low values of bootstraps among individuals from different populations of *Ae. scapularis* analysed. In addition we also found that the genetic distances between individuals in the populations of clade *Ae. scapularis* is smaller than at the genetic distances between species *Oc. fulvus*, *Hg. janthinomys*, *Oc. vigilax, Ae. koreicus*, *Cx.quinquefasciatus*, *Cx. pipiens pallens*, *Cx. tritaeniorhynchus*, *An. braziliensis*, *An. darlingi*, *Ae. aegypti* and *Ae. albopictus*. This fact also weakens the hypothesis of existence of cryptic species t species complex in *Ae. scapularis*. According to what could be observed in phylogeny, the sister species of *Ae. scapularis* is the mosquito *Oc. fulvus*, while the species of *Anopheles* were the most genetically distant from *Ae. scapularis*.

Mitogenome analysis is an important tool in solving the phylogeny of some unresolved species using one or a few genetic markers of nuclear or mitochondrial origin. In work developed for Wang et al. (2017), it was possible to separate sister species and cryptic species using the Maximum Likelihood, Parsimony Analysis and Bayesian methods, using mitochondrial genes. Williams et al. (2014) demonstrated that it was possible to resolve the phylogenetic relationships within the phylum Gastropoda using the Maximum likelihood and Bayesian probability analyses of mitochondrial genes.

Despite the high population structure that could be observed in the phylogenetic tree of PCGs, this tree corroborates the phenetic dendrogram of the wings constructed with Mahalanobis distances and with the genetic distances (p-distance) in the sense that there is no relationship between biome or locality geographic proximity with the genetic proximity of the populations analysed. Possibly among populations of different biomes, but genetically similar, such as ITA (Itaboraí) and ANA (Ananindeua), where the first is from the Atlantic Forest environment and the second from the Amazon Forest environment, the genetic similarity observed, can be related to the pattern of immigration, occurrence of ancestral retention or evolutionary coincidence.

Based on the results of the phylogenetic relationships of the populations of *Ae. scapularis* from different biomes analysed in relation to the other species of culicidae, and on the joint interpretation of all data from the mitochondrial genes and wings so far, this mosquito appears to be a single taxon.

4.4. COI, complete DNA mitocondrial and wing geometric morphometric

The objective was to verify whether the intra-population structure of *Ae. scapularis* presents incipient speciation magnitude. To achieve this goal, we used the population markers *COI*, wing morphometrics and 13 PCGs. This different population markers used in this study indicated different levels of population structure. The *COI* genetic marker did not reveal any population structure, with the geometric morphometrics it was possible to observe a slight population structure and already with Bayesian phylogenetic analysis of the 13 PCGs, there was a low population structure in four studied populations, but revealed high structuring of individuals of the *Ae. scapularis* species in relation to the other mosquitoes studied. Despite these differences related to the degree of specificity or evolutionary stability of these population markers used in the present study, these three markers showed a high polymorphism in the species *Ae. scapularis*. These different responses of the genetic and morphological markers that occurred in this study are common, as the evolutionary reasons between the markers may be different. The wing geometric morphometric is sensitive to microevolutionary processes, and can be considered as a preliminary marker of population structure, being frequently used in population characterization studies (Louise et al., 2015; Petersen et al., 2015). The 13 PCGs were effective in highlighting the population structure of the *Ae. scapularis*, possibly due to its wealth of information.

Molecular and genetic markers revealed high internal variability among individuals of the *Ae. scapularis* analysed. The high variability found in *Ae. scapularis* is consistent to what is expected being in mind ecology of the species: generalist in blood feeding, with wide geographical distribution, adaptability and eclectic vector competence. This species has a wide preference for its blood-hosts, because it feeds on

humans, birds, equine and cattle (Forattini et al., 1989). In addition, the ecological generalism of this species is illustrated by its ability to explore either urban, sylvatic and semi-sylvatic areas in Brazil (). Lately it has been increasingly common to find them in parks and urban centers (Costa-da-Silva et al., 2017). It is important to consider its comprehensive role as a vector, since *Ae. scapularis* has a vector capacity for arboviruses and filariae (Cunha et al., 2020; Labarthe et al., 1998). The genetic variability is somehow correlated to the morphological polymorphism present in this species. As shown, *Ae. scapularis* wing shape variation is pronounced in the different studied populations. Another polymorphic feature of this species is the silver spot present in the scutellum of this mosquito, whose size may vary intraspecifically. In addition to these variatiable characters, namely, wing shape and scutellar spot size, the retrorse process in the genitalic claspette filaments presents meristic variability, with number varying from 0 to 4 (Petersen et al., 2018).

High polymorphism was expected in Atlantic Forest populations, even if fragmented, as this is the natural habitat of this mosquito, however high polymorphism was found in populations from the Amazon and Caatinga areas. These findings regarding the polymorphism present in all studied biomes (Atlantic Forest, Caatinga and Amazon Forest) observed in the analysis of the markers of geometric morphometric, *COI* and PCGs corroborate the hypothesis that Brazil is the center of origin of this species or that we are close to the center of origin. The high polymorphism characteristic of the species *Ae. scapularis* possibly gives this mosquito plasticity to develop in environments with extreme environmental conditions as in the Caatinga biome. In addition, possibly its plasticity is helping to adapt it from the wild or rural environment to the urban environment. In the urban environment, this mosquito in contact with the virus can transmit the arboviruses that circulate in this environment, if it has vectorial capacity and competence. As this mosquito can move between rural and urban environments, it can carry the yellow fever virus, for example, since this species has already been found naturally infected with this virus (Abreu et al., 2019).

The population markers used in this study did not suggest or evidence the presence of a species of complex in *Ae. scapularis* in the Atlantic Forest, Caatinga and Amazon Forest biomes. The greatest evidence that there is no species complex in *Ae. scapularis* in this study could be seen in the robust results resulting from the analysis carried out with mitochondria PCGs, as the clades showed high support values for the populations of this mosquito in relation to the other culicids: *Oc. vigilax*, *Hg. janthinomys*, *Ae. aegypti*, *Ae. albopictus*, *Cx quinquefasciatus*. Furthermore, up until now, the genetic and phenetic distances observed between populations are not significant enough to make us believe that there are more species under the name *Ae. scapularis*.

5. Conclusions

We observed through molecular and morphological markers that there is no evidence of speciation or of entities compatible with morphospecies in the mosquito *Ae. scapularis*. Our interpretation is limited to the size sample ($n = 162$ for mtDNA and 214 for wing shape), but even though, if a cryptic species was present among the samples, the sensitive biological markers used would probably expose it. Hitherto, there is no evidence that *Ae. scapularis* populations are geographically distinct enough to require regionalised mitigation protocols.

Declaration of Competing Interest

The authors Vivian Petersen, Micael Santana, Lincoln Suesdek and J. Marcelo P. Alves declare that have no competing interests.

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VP, JMPA and LS designed the study. VP performed morphometric analyses. VP and MS were responsible for mitochondrial genome amplification. JMPA performed the genetic and phylogenetic analyses. All authors wrote the manuscript.

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