

Genetic variability of populations of *Nyssomyia neivai* in the Northern State of Paraná, Brazil

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ABSTRACT

The genetic study of sandfly populations needs to be further explored given the importance of these insects for public health. Were sequenced the *NDH4* mitochondrial gene from populations of *Nyssomyia neivai* from Doutor Camargo, Lobato, Japira, and Porto Rico, municipalities in the *State of Paraná*, Brazil, to understand the genetic structure and gene flow. Eighty specimens of *Ny. Neivai* were sequenced, 20 from each municipality, and 269 base pairs were obtained. A total of 27 haplotypes and 28 polymorphic sites were found, along with a haplotypic diversity of 0.80696 and a nucleotide diversity of 0.00567. Haplotype H5, with 33 specimens, was the most common among the four populations. Only haplotypes H5 and H7 were present in all four populations. The population from Doutor Camargo showed the highest genetic diversity, and only this population shared haplotypes with those from the other municipalities. The highest number of haplotypes was sheared with Lobato which also had the highest number of unique haplotypes. This probably occurred because of constant anthropic changes that happened in the environment during the first half of the twentieth century, mainly after 1998. There was no significant correlation between genetic and geographical distances regarding these populations. However, the highest genetic and geographical distances, and the lowest gene flow were observed between Japira and Porto Rico. Geographical distance is a possible barrier between these municipalities through the blocking of haplotype sharing.

KEYWORDS: Sandflies. *Lutzomyia*. mtDNA. Phylogeny. Genetic polymorphism.

INTRODUCTION

The importance of leishmaniasis for public health is expressed by its wide distribution in 98 countries¹. The incidence of cutaneous leishmaniasis (CL) in Latin America is high, especially in Brazil, where 635,399 cases were detected from 1990 to 2013². Of these cases, 13,889 (2.2%) were reported in the South of the country, especially in the *State of Paraná* (94.9% of the total cases in the South)².

Worldwide, 988 species of sandflies have been described (961 current and 27 fossil). American species comprise 529 of these, of which 512 are current and 17 are fossil³. In Brazil, 19 species are suspected or implicated in the transmission of *Leishmania* to humans⁴. Among the small number of species of importance for the epidemiology of CL in Brazil, *Nyssomyia whitmani* (Antunes and Coutinho) and *Nyssomyia neivai* (Pinto) are prominent, and both species are widely distributed in *Paraná*⁵⁻⁸. In this state, *Ny. neivai* occurs in several ecotopes in peridomiciles, especially in domestic-animal shelters⁵⁻⁸.

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Ny. neivai and *Ny. intermedia* belong to the *Nyssomyia intermedia* complex, and can be morphologically differentiated by their spermathecae⁹. *Ny. neivai* is found in colder and drier areas in natural environments, and easily adapts to human-impacted environments¹⁰⁻¹³. This species is present on the coast and interior of several States of Brazil, including Western São Paulo, Paraná, Southern and Western Minas Gerais, Southern Goiás and Southern Pará, as well as in Argentina, Bolivia and Paraguay¹⁴.

Research on molecular markers has been used to evaluate sandfly polymorphism. Investigations on the genetic structure of populations of these insects are necessary for the understanding of the species dispersal patterns¹⁵ and for the development of control strategies. Mitochondrial DNA (mtDNA) has often been used as a molecular marker in the study of genetic variability of populations because it is maternally inherited and does not recombine. Thus, it is possible to study the genetic variability among populations of a certain species and estimate the rates of gene flow among populations by using a fragment of the mitochondrial NADH dehydrogenase subunit 4 (NDH4)^{16,17}.

Genetic variability is high among sandfly species. *Ny. intermedia* shows a greater genetic diversity in the wild than in domestic environments^{18,19}. Souza *et al.*²⁰ observed that *Ny. whitmani* has a high polymorphism and this is associated with the species' adaptability to different regions.

Studies of the genetics of sandfly populations in the Americas have been conducted with populations of *Lu. longipalpis*, *Lutzomyia cruzi*, *Ny. intermedia*, *Ny. whitmani* and *Ny. neivai* in Central and South America, and have revealed the existence of extensive haplotype diversity in these populations^{15,18-27}. In the Old World, studies of population genetics have given particular attention to *Phlebotomus papatasi*²⁸⁻³⁰.

In view of the importance of sandflies for CL epidemiology, studies to evaluate these insects' polymorphism with molecular markers are needed to extend the knowledge of the genetic variability and patterns of species dispersal in order to support further investigations about sandflies ecology. The importance of *Ny. neivai* for CL epidemiology aroused our interest in studying the genetic variability in populations of this sandfly species.

MATERIAL AND METHODS

Sample sites

Collections of *Ny. neivai* were made in four municipalities (Doutor Camargo, Japira, Lobato and Porto Rico) (Figure 1). The climate is mesothermal humid subtropical, with more rainfall in summer and a dry winter, and mean

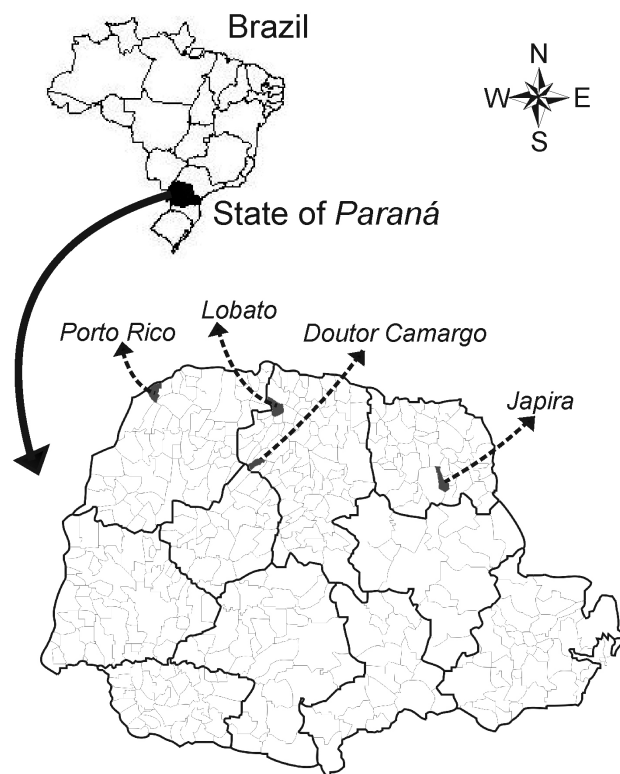


Figure 1 - Geographical location of municipalities of Doutor Camargo, Japira, Lobato, and Porto Rico, where the collection of sandflies was made, State of Paraná, Brazil.

temperatures above 22 °C in summer and below 18 °C in winter. The native vegetation is composed of semideciduous forests. The criteria for selecting the municipalities were geographical distance, environmental factors, and reported LT cases.

Doutor Camargo municipality is located in the *North Central Paraná Mesoregion* (23°33' S and 52°13' W), at 585 m above sea level and on the banks of the *Ivaí river*. Sandflies were collected in a sheep shelter and a hen house in the Recanto Marista, a recreation area that covers 57.6 ha, of which 40.8 ha is occupied by an altered forest remnant that is still inhabited by birds and wild mammals.

Japira municipality is located in the *North Pioneer Paraná Mesoregion* (23°48' S and 50°08' W). Sandflies were collected in the locality of Estrelinha in a sheep shelter, pigsty, hen house, and in a guava tree located at a small and much-altered woodlot.

Lobato municipality is located in the *North Central Paraná Mesoregion* (23°30' S and 51°54' W). Sandflies were collected on the porch of a residence and in a pigsty and a hen house on Barra Farm. The native vegetation of this municipality was replaced by permanent and temporary crops, although 700 ha of native forest remain on the farm.

Porto Rico municipality is located on the bank of the *Paraná river*, in the *Northwest Paraná Mesoregion* (22°46' S

and 53°16' W). Sandflies were collected in a hen house and pigsty on Mutum Island, within the Environmental Protection Area (APA) of Islands and Lowlands of the *Paraná river*. This island belongs to Porto Rico municipality and is approximately 15 km long and 500 to 1,000 m wide³¹.

The areas of municipalities mentioned above have been occupied since the first half of the twentieth century, which are pioneering agricultural areas of Brazil. Since 1998, in Doutor Camargo municipality efforts to control these insects have been made, such as removing organic matter accumulated on the soil (decomposing leaves and fruits), cutting of trees and other vegetation, draining the soil to reduce moisture, and spraying buildings with an insecticide¹². The monitoring of control measures has been periodic³², and changes in riparian vegetation have been favored by the occasional floods of the *Ivaí river*.

Collection and preparation of sandflies

Specimens of *Ny. neivai* were collected with *Falcão* light traps installed in peridomicile areas, from 20:00 to 24:00 h on two or more consecutive days. The insects were killed with chloroform and placed in 1.5-mL eppendorf microtubes containing 80% ethanol for conservation and later identification. Twenty females of *Ny. neivai* from each municipality were identified, and preserved in individual 0.6-mL eppendorf microtubes containing 2.5 µL of 80% ethanol until DNA extraction. The nomenclature of sandfly species follows Galati³.

Extraction of DNA

DNA was extracted according to Loxdale and Lushai³², with modifications. The ethanol-preserved insects were macerated with a plastic pestle in an eppendorf microtube with 200 µL of a solution of 5% Chelex resin.

Amplification of NDH4 gene

PCR used 0.6 µM of each primer (forward and reverse), 2 mM MgCl₂, 1 x enzyme buffer, 0.6 mM dNTP (Invitrogen), 1.5 U Taq DNA Polymerase (Invitrogen) and 2.0 µL of the DNA sample, in a final volume of 25 µL. The primers were the same as those used by Gorrochotegui-Escalante *et al.*³³ and Paduan and Ribolla³⁴ and included the following sequences: *NDH4* forward (5'-TTGTGACCTAAGGCTCATGT-3') and *NDH4* reverse (5'-CTATTCGGCTTCGTCCATGTT-3'). The amplification was carried out in a Bio-Rad iCycler thermocycler with 3 cycles at 94 °C for 2 min., 37 °C for 2 min. and 72 °C for 1 min. followed by 35 cycles at 94 °C

for 30 sec., 50 °C for 30 sec. and 72 °C for 1 min.; and an extension at 72 °C for 5 min. The PCR products were submitted to electrophoresis in 1% agarose gel with a 100 bp molecular marker. The gels were stained with ethidium bromide for 20 min. and visualized in a Gibco BRL TFX-35M UV transilluminator.

Sequencing reaction

All the PCR products were quantified by the Nanodrop 1000 spectrophotometer (Thermo Scientific). Samples were diluted at 25 ng/µL for sequencing, using 1 µL of Big Dye Terminator v 3.1, 0.4 x dilution buffer, 1 mM of each primer (forward and reverse), and 5 µL of the sample, totaling 10 µL of reaction volume. The reaction was carried out in a Bio-Rad iCycler thermocycler: 1 cycle at 96 °C for 1 min., followed by 25 cycles at 96 °C for 10 sec., 56 °C for 5 sec. and 60 °C for 4 min., and stored at 4 °C overnight. The amplified product was purified and precipitated by adding 80 µL of 65% isopropanol to each sample. These were incubated at room temperature for 20 min. and centrifuged at 16,000 x g for 25 min. at 13 °C, and the supernatant was discarded. Afterwards, to each sample 200 µL of 70% ethanol were added and centrifuged at 16,000 x g for 10 min. at 13 °C. The supernatant was discarded and tubes were incubated at a temperature of 50 °C or at room temperature for approximately 24 hours. Next, each sample was re-suspended in 10 µL of formamide, submitted to heat shock, and sequenced in an ABI 3130 automated sequencer (Applied Biosystems).

Alignment of sequences

Sense and anti-sense strand sequences of the *NDH4* gene were examined in the Staden software package version 1.5³⁵, which produced a consensus strand for each sample. The sequences were aligned using the Clustal X program version 1.83³⁶.

Data analysis

Relationships between mtDNA haplotypes were evaluated and dendrograms were constructed using the Neighbor-Joining method by means of the 5.5-MEGA Molecular Evolutionary Genetics Analysis program³⁷. *NDH4* gene sequences of *Ny. whitmani*, *Pintomyia fischeri* and *Pintomyia pessoai* were used as outgroups (Costa-Ribeiro, unpublished data).

The nucleotide sequences and frequencies of each haplotype (synonymous and non-synonymous regions), the mean number of nucleotide differences (K), haplotype

diversity (Hd), genetic distance, gene flow³⁸ and random evolution of *Ny. neivai* were estimated using the DnaSP program, version 5^{39,40}. The relationship between genetic haplotypes was evaluated using the Network program, version 4.6.10, based on the Median Joining method (Fluxus Technology Ltd.). The genetic distance (Fst) was analyzed using the formula $Fst=1/2Nm+1$, and $Nm=$ gene flow. The geographical distance between localities was determined using the Google Earth program, version 6.0⁴¹. The correlation between genetic distance (Fst) and geographical distance was analyzed by the Pearson correlation test.

RESULTS

Distribution and frequency of haplotypes

Sequencing of *Ny. neivai* specimens from Doutor Camargo, Japira, Lobato and Porto Rico resulted in 80 sequences of 269 bp. The average composition of nucleotides was 40.1% thymine, 6.3% cytosine, 32.5% adenine and 21.1% guanine.

Table 1 shows the 27 haplotypes (H) found; 22 specimens were represented by only one haplotype (H1, H2, H3, H6, H9, H11, H12, H14, H15, H16, H18, H19, H20, H21, H22, H23, H24, H25, H26, H27). Haplotype H5 appeared in the largest number of specimens (N = 33), followed by haplotype H7 (N = 12), both of which were shared by all four populations. The third most common haplotype was H17, shared by five specimens from Japira and Lobato. Haplotypes H4, H10, H8 and H13 were present in Doutor Camargo and were shared with Porto Rico, Japira and Lobato. The largest number of exclusive haplotypes was detected in Doutor Camargo (H1, H2, H3, H6, H9, H11, H12, H14 and H15), followed by Lobato (H19, H20, H21, H22 and H23), Porto Rico (H24, H25, H26 and H27) and Japira (H16 and H18).

Table 2 shows the 28 polymorphic sites (S), a haplotype diversity (Hd) of 0.80696 and a nucleotide diversity (Pi) of 0.00567. Sandflies from Doutor Camargo and Lobato showed the largest number of haplotypes (H), the greatest haplotype diversity (Hd), the highest nucleotide diversity (Pi), and the largest number of polymorphic sites (S). The highest number of single variations (singletons) was observed in the population from Doutor Camargo (19), followed by Lobato (7), Porto Rico (3) and Japira (3). Tajima’s test was significant in Doutor Camargo.

Table 3 shows that H12 had the largest number of nucleotide mutation sites (3, 21, 108, 114, 189, 195, 222 and 267). The majority of haplotypes were distinct in sandflies by only two nucleotide sites. H4 and H17 differed in only one site (position 267) as did H10 and H20 (position 246).

Table 1 - Haplotype distribution of *Nyssomyia neivai* populations in the municipalities of Doutor Camargo, Japira, Lobato and Porto Rico, State of Paraná, Brazil.

Haplotypes/ Municipality	D	J	L	P	Total of specimens
H1	1	0	0	0	1
H2	1	0	0	0	1
H3	1	0	0	0	1
H4	1	0	0	1	2
H5	5	11	6	11	33
H6	1	0	0	0	1
H7	2	4	4	2	12
H8	1	0	1	0	2
H9	1	0	0	0	1
H10	1	1	0	0	2
H11	1	0	0	0	1
H12	1	0	0	0	1
H13	1	0	1	0	2
H14	1	0	0	0	1
H15	1	0	0	0	1
H16	0	1	0	0	1
H17	0	2	3	0	5
H18	0	1	0	0	1
H19	0	0	1	0	1
H20	0	0	1	0	1
H21	0	0	1	0	1
H22	0	0	1	0	1
H23	0	0	1	0	1
H24	0	0	0	2	2
H25	0	0	0	2	2
H26	0	0	0	1	1
H27	0	0	0	1	1
Total	20	20	20	20	80

Doutor Camargo=(D), Japira=(J), Lobato=(L), Porto Rico=(P).

H4 and H5 differed in 2 sites (positions 201 and 267) as did H7 and H8 (positions 9 and 21).

The circle size was proportional to the number of individuals found in each haplotype (Figure 2). The haplotype network has shown ancestrality among *Ny. neivai* populations, and probably the haplotype H5 has a close genetic relationship with other haplotypes because of its 7 derived groups, including exclusive haplotypes from Doutor Camargo (H1, H2, H14, H15), Lobato (H19, H20, H21, H23) and Porto Rico (H24, H25, H26). H7 has originated exclusively haplotypes from Japira (H16, H18), Doutor Camargo (H11) and Lobato (H22).

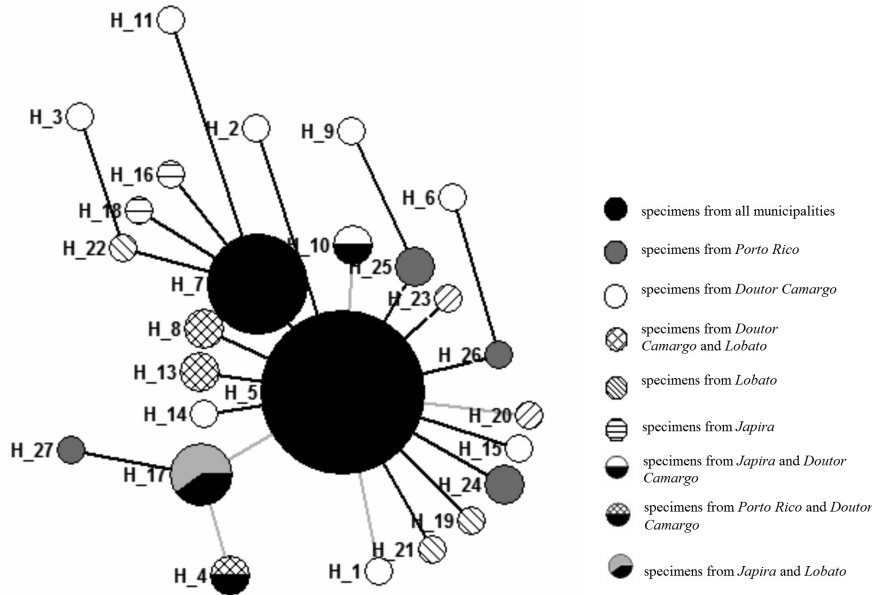


Figure 2 - Haplotype network of *Nyssomyia neivai* populations from municipalities of Doutor Camargo, Japira, Lobato, and Porto Rico, *State of Paraná*, Brazil.

Analysis by means of dendrograms

The dendrograms showed a strong similarity between individuals of *Ny. neivai* populations from the four municipalities. The dendrogram branches were clearly defined and form a single group, even in Doutor Camargo and Lobato, which had the highest haplotype diversity, showing a low interspecific genetic variability among *Ny. neivai* specimens from each locality (Figure 3).

Genetic variation among populations

Table 4 shows that the largest genetic distance (Fst) occurred between Japira and Porto Rico populations (Fst=0.02477), and the lowest between Doutor Camargo and Porto Rico (Fst=-0.00428). The number of nucleotide substitutions per site (Dxy) was the highest in Doutor Camargo and Lobato (Dxy=0.00759 and Da=-0.00009) and the lowest in Japira and Porto Rico (Dxy=0.00379 and Da=0.00009). *Ny. neivai* populations from Japira and Lobato showed an intense gene flow (39.64). The lowest gene flow (12.60) occurred between Japira and Porto Rico populations.

The Pearson correlation test between genetic distance (Fst) and geographical distance (Km) of populations from the four municipalities showed that there was no correlation between these two factors ($p = 0.2246$).

DISCUSSION

A total of 27 haplotypes of the *NDH4* gene were detected from 80 female *Ny. neivai*. This number of haplotypes is

similar to that detected in another study of the *NDH4* gene of *Lu. longipalpis* populations²⁶. Thymine and adenine were the most frequent, followed by guanine and cytosine, as also detected by Soto *et al.*²⁶ who sequenced the *NDH4* gene of mtDNA from 46 specimens of *Lu. longipalpis*.

H5, with 33 specimens, and H7, with 12, were the most frequent haplotypes and the only ones present in all four populations. Ribolla *et al.*¹⁵ detected 13 haplotypes by analyzing mtDNA of 133 specimens of *Lu. longipalpis* and seven of *Lu. cruzi*, and found a major haplotype (H2) composed of 111 individuals that represented 89% of three populations of the *State of São Paulo*, 90% of two populations of the *State of Mato Grosso do Sul*, and 38% of one population of the *State of Piauí*. Arrivillaga *et al.*²¹ detected 18 haplotypes in 343 *Lu. longipalpis* specimens collected in 12 localities of 5 countries (Brazil, Costa Rica, Colombia, Honduras and Venezuela). They did not identify any common haplotype among these populations, probably due to the large geographical distance and possible reproductive isolation followed by a dispersal period, causing the separation of species.

The haplotype diversity observed in the *NDH4* gene of *Ny. neivai* is considered high (0.80696), although lower than that detected by Soto *et al.*²⁶ for the same gene of *Lu. longipalpis*. Depaquit *et al.*²⁷ observed in 11 haplotypes of the *NDH4* gene of *Phlebotomus papatasi* a greater diversity than that observed in the present study, indicating that diversity is independent of the number of haplotypes. Ribolla *et al.*¹⁵ found higher diversities (0.672 and 0.545) in populations of *Lu. longipalpis* in Teresina (*Piauí*), with six exclusive haplotypes, and Araçatuba (*São Paulo*), with

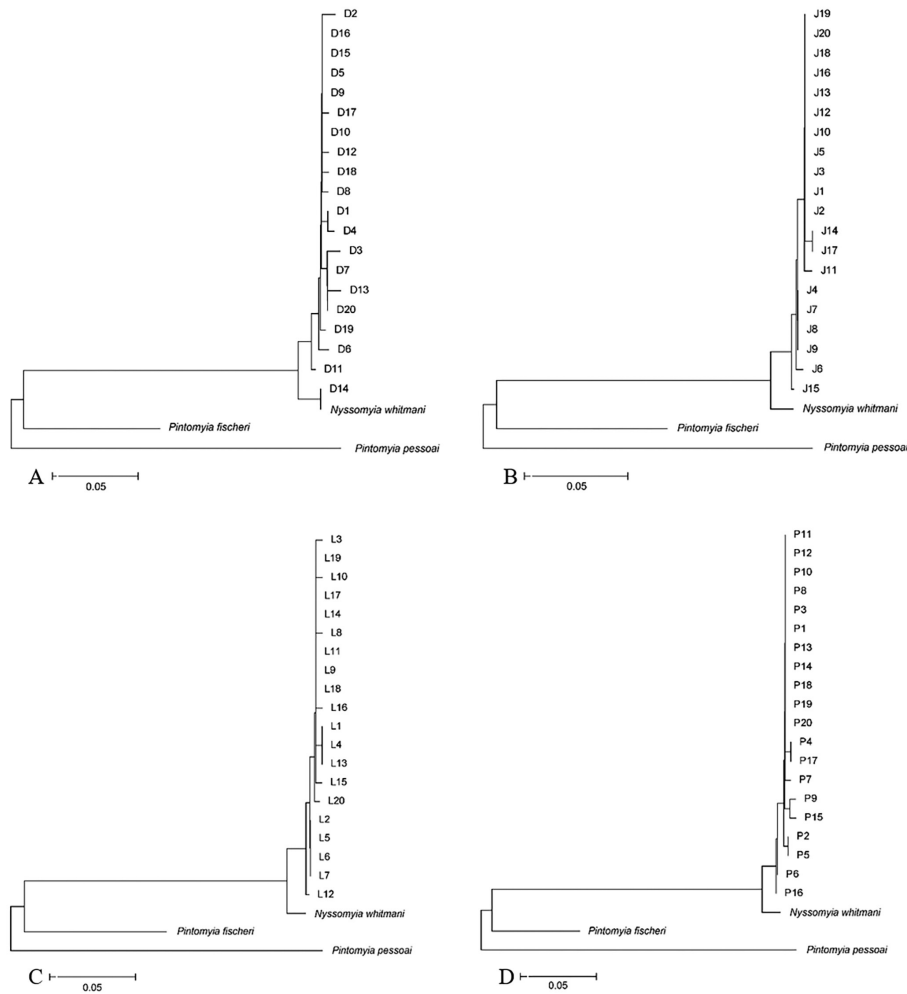


Figure 3 - Dendrogram of *Nyssomyia neivai* populations from (A) Doutor Camargo, (B) Japira, (C) Lobato, and (D) Porto Rico, State of Paraná, Brazil.

Table 4 - Genetic differentiation (Fst) of *Ny. neivai* populations from municipalities of Doutor Camargo, Japira, Lobato, and Porto Rico, State of Paraná, Brazil.

Population	Fst	Dxy	Da	Nm	Distance (Km)
D e J	-0,00955	0,00681	-0,00007	23,43	217
D e L	-0,01140	0,00759	-0,00009	24,53	61,5
D e P	-0,00428	0,00708	-0,00003	20,77	137
J e L	-0,02673	0,00415	-0,00011	39,64	206
J e P	0,02477	0,00379	0,00009	12,60	346
L e P	0,00777	0,00454	0,00004	16,43	138

Doutor Camargo=(D), Japira=(J), Lobato=(L), Porto Rico=(P). **Dxy**= mean number of nucleotide substitutions per site between two populations, **Da**= total number of nucleotide substitutions between two populations, **Nm**= number of migrants per generation.

five exclusive haplotypes, respectively.

Tajima's D neutrality tests were significant for all the samples ($p > 0.05$), indicating that the genetic polymorphism is according to the model of neutral mutations, excepting for the population from Doutor Camargo.

Genetic diversity varied among these four populations.

Sandflies from Doutor Camargo had higher genetic diversity than those collected in the other municipalities. This probably occurred because of constant anthropic changes that happened in the environment during the first half of the twentieth century⁴², mainly after 1998, when efforts to control these insects began^{12,31}.

In Lobato, where the second-highest diversity was found, anthropic changes have been also constantly occurring, although less pronounced than in Doutor Camargo⁷. In contrast, the lowest haplotype diversity of *Ny. neivai* occurred in Japira and Porto Rico, where anthropic changes have been less intense than in Doutor Camargo and Lobato (Teodoro, personal communication). In municipalities where *Ny. neivai* populations showed high genetic diversities, Monteiro *et al.*⁴³ found increased incidences of LT.

The haplotype network demonstrated that there is a sequence of ancestry between haplotypes, evidencing that H5 has a genetic relationship with a large number of haplotypes, as seven groups of haplotypes were derived from H5.

The dendrograms show that the interspecific genetic variability is rather low in the populations of *Ny. neivai* from these municipalities. Balbino²⁴ found a moderate genetic divergence among populations of *Lu. longipalpis* in seven municipalities in Northeastern Brazil, while, Souza *et al.*²⁰ found that *Ny. whitmani* populations of four localities in the municipality of Corte de Pedra, State of Bahia, were more homogeneous than sandfly populations of *Ilhéus* (Bahia), *Serra de Baturité* (Ceará), and Martin Campos (Minas Gerais).

In the present study, the greatest genetic distance occurred between populations of Japira and Porto Rico ($F_{st}=0.02477$), as well as the lowest gene flow ($Nm=12.60$) and the lowest number of nucleotide substitutions per site ($D_{xy}=0.00379$). Geographical distance is a possible barrier between these municipalities, blocking haplotype sharing and suggesting the hypothesis that the genetic isolation between these two populations may reflect the geographical distance (346 km) between the two municipalities. Soto *et al.*²⁶ found that gene flow among populations of Guatemala and Honduras was almost nonexistent, because of mountains that act as geographical barrier between the two countries, hampering the haplotype sharing. However, populations of *Lu. longipalpis* from Costa Rica and Honduras are genetically similar despite a geographical distance that is greater than that between Honduras and Guatemala²⁶. It is noteworthy that most of Nicaragua, which is located between Costa Rica and Honduras, lies at altitudes less than 500 m.

Ribolla *et al.*¹⁵ studied populations of *Lu. longipalpis* in municipalities in the States of *São Paulo*, Mato Grosso do Sul and *Piauí*, and reported that gene flow occurs independent of the geographical distance. Populations of *Ny. neivai* from Doutor Camargo and Lobato are closer and share more haplotypes; however, there was no significant correlation between genetic ($F_{st}=-0.01140$) and

geographical distances. Local and independent factors can favor the diversity of *Ny. neivai* in each locality.

Sandflies in Doutor Camargo municipality showed the highest haplotype diversity and the largest number of exclusive haplotypes. Only the *Ny. neivai* population of Doutor Camargo shares haplotypes with populations from Lobato, Japira and Porto Rico. The largest genetic and geographic distances, and the lowest gene flow were found between Japira and Porto Rico; however, the dendrograms showed that there is interspecific homogeneity between the specimens from populations of these municipalities. Anthropic and natural environmental changes and geographical barriers are important and may be reflected in the genetic diversity of *Ny. neivai* populations.

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