

## Predominance of *Leishmania (Leishmania) amazonensis* DNA in *Lutzomyia longipalpis* sand flies (Diptera: Psychodidae) from an endemic area for leishmaniasis in Northeastern Brazil

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### ABSTRACT

Leishmaniasis is a serious public health concern in the Northeastern region of Brazil, where the sand fly fauna is well studied, although few species have been identified as competent vectors. The detection of *Leishmania* spp. parasites in wild-caught sand flies could help sanitary authorities draw strategies to avoid the transmission of the parasites and, therefore, the incidence of leishmaniasis. We detected *Leishmania* DNA in wild-caught sand flies and correlated that data with aspects of sand fly ecology in the Caxias municipality, Maranhão State, Brazil. The sand flies were sampled in the peridomicile (open areas in the vicinity of human residences) and intradomicile (inside the residences) from July/2019 to March/2020. *Leishmania* DNA was detected in females, targeting a fragment of the Internal Transcribed Spacer (ITS1) from ribosomal DNA. Among the fourteen species of sand flies identified, five (*Lutzomyia longipalpis*, *Nyssomyia whitmani*, *Evandromyia evandroi*, *Micropygomyia trinidadensis*, and *Micropygomyia quinquefer*) harbored DNA of *Leishmania (Leishmania) amazonensis*. The most abundant species in rural (*Ny. whitmani*: 35.2% and *Ev. evandroi*: 32.4%) and urban areas (*Lu. longipalpis*: 89.8%) are the permissive vectors of *L. (L.) amazonensis*, especially *Ny. whitmani*, a known vector of causative agents of cutaneous leishmaniasis. Although *Lu. longipalpis* is the vector of *L. (L.) infantum*, which was not detected in this study, its permissiveness for the transmission of *L. (L.) amazonensis* has been reported. We suspect that visceral leishmaniasis and cutaneous leishmaniasis are caused by *L. (L.) amazonensis*, and the transmission may be occurring through *Lu. longipalpis*, at least in the urban area.

**KEYWORDS:** Phlebotomines. Infectious parasitic diseases. Parasites. Molecular biology.

### INTRODUCTION

Leishmaniasis are neglected tropical diseases that affect numerous people annually; caused by protozoans from the genus *Leishmania* (Kinetoplastida, Trypanosomatidae), transmitted among vertebrate hosts by sand flies (Diptera: Psychodidae). Traditionally, leishmaniasis are divided according to the causative agent, disease progression, and symptoms into visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL), depending on the species of the pathogen<sup>1</sup>.

Leishmaniasis are found in the Americas from South-Central Texas to Central and South America, except for Chile<sup>2</sup>. The North and Northeast regions of Brazil have the most cases of visceral and cutaneous leishmaniasis<sup>3</sup>. In Maranhão State,

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Northeastern Brazil, where both diseases are endemic, more than 90 sand fly species are distributed throughout several ecosystems<sup>4</sup>. Molecular surveys have shown the occurrence of DNA from at least seven *Leishmania* species in sand flies sampled in Maranhao<sup>5-9</sup>. Such diversity of sand flies and parasites is determined by the intersection of three biomes in Maranhao State: the Amazon Rainforest, the Cerrado (savannah-like vegetation), and the Caatinga (seasonal dry forest), which promotes the formation of several ecotones<sup>4</sup>.

In Caxias, a municipality located in the Cerrado biome of Maranhao, 27 species of sand flies have already been recorded<sup>4</sup>. The most medically relevant species are: i) *Lutzomyia longipalpis* (Lutz and Neiva, 1912), the main vector of *Leishmania (Leishmania) infantum*, which has large populations in urban areas; and ii) *Nyssomyia whitmani* (Antunes and Coutinho, 1939), the competent vector of *Leishmania (Viannia) braziliensis* and a putative vector of *L. (V.) shawi*, and usually found in rural zones of this region<sup>10,11</sup>.

The vegetation cover of the municipality of Caxias has been severely deforested by the real estate business, with the construction of large popular housing estates, malls, roads, and other ventures<sup>12</sup>. This process occurs in peri-urban areas (suburbs), thus contributing to a higher density and adaptation of vector species in these environments, primarily *Lu. longipalpis* and *Ny. whitmani*, thereby promoting and increasing the number of leishmaniasis cases<sup>10</sup>. In the last four years (2017 to 2020), forty-four confirmed cases of VL, twenty-nine cases of CL in humans, and 1,001 cases of seropositive dogs with VL were reported.

Recently, Guimarães-e-Silva *et al.*<sup>8</sup> detected the DNA of *Leishmania* spp. belonging to seven distinct sand fly species from urban and rural areas of the municipality of Caxias. This surprising result motivated us to obtain more consistent information about these parasites circulating in the city. Therefore, we monitored, through molecular biology approaches, the circulation of different *Leishmania* species in sand flies from urban and rural areas of Caxias, where cases of both forms of leishmaniasis occur.

## MATERIAL AND METHODS

### Characterization of the sample collection sites and ethical considerations

Sand flies were collected in two localities of the Caxias municipality, Maranhao State, Brazil: Povoado Mulata/rural area (04°54'55" S and 43°23'15" W) and Volta Redonda/urban area (04°52'57" S and 43°21'00" W). These localities were chosen based on the presence of domestic animals within the residences and the record of human leishmaniasis cases during 2018 and 2019.

The Povoado Mulata includes approximately ninety-seven residences; it is characterized by unpaved streets, mud-built houses (the majority), large green and shaded areas, yards with fruit trees, and secondary vegetation located close to the houses. The Volta Redonda neighborhood contains 2,002 residences, high human density, paved streets, an intense flow of people, commercial areas, and several environmental alterations caused by the man, surrounded by the presence of secondary vegetation (Figure 1).

The Research Ethics Committee from the Universidade Estadual do Maranhao - CEP/UEMA approved this study under protocol N° 3.893.983. The field studies were conducted on private properties. The landowners authorized the installation of traps in their peridomicile areas (yards and open areas next to the residence, but still within the property) and intradomicile areas (living rooms or kitchens), which did not involve endangered or protected species or contact with people.

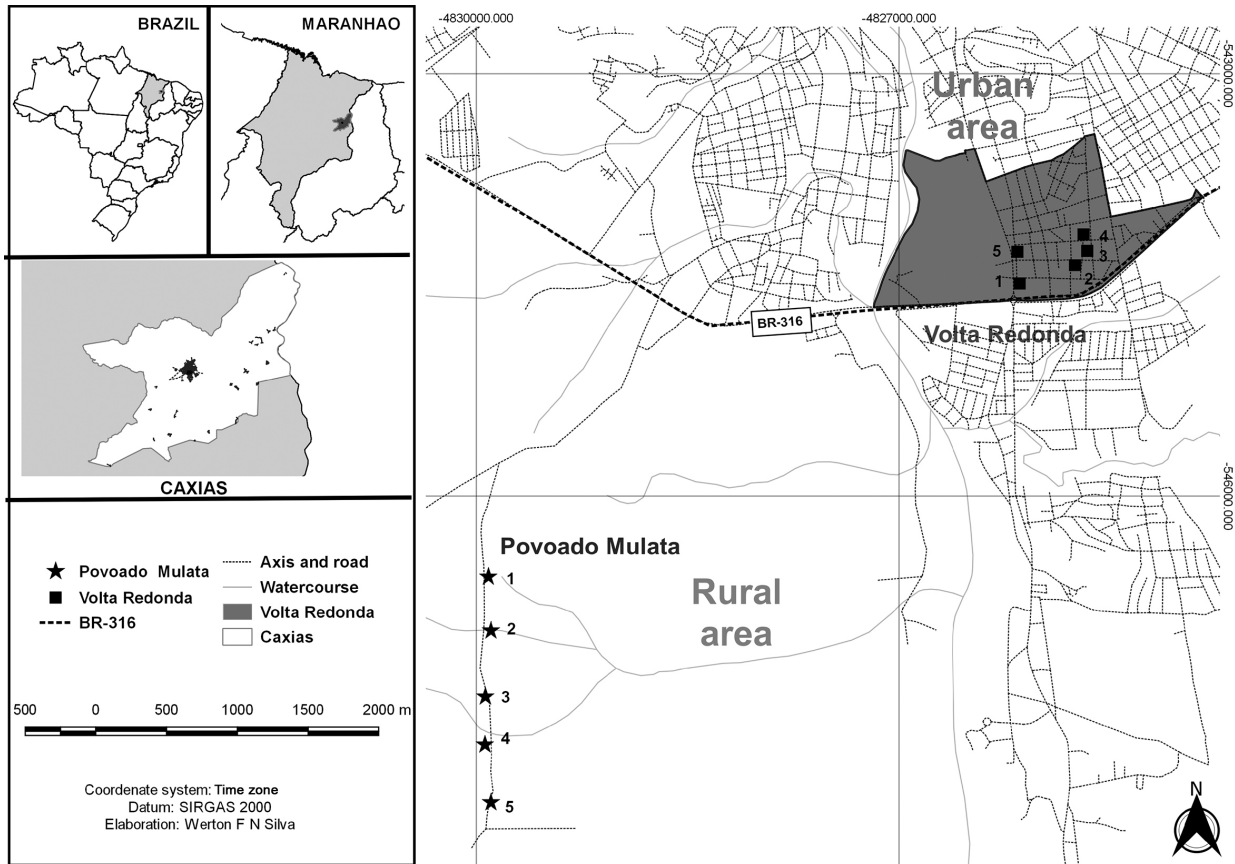
### Sampling and identification of the sand flies

Sand flies were sampled from 6:00 p.m. to 6:00 a.m., on two consecutive nights, once a month, from July 2019 to March 2020. Two light traps of the CDC (Center of Disease Control) type were set 1.5 meters above ground level in five residences in each area: one in the peridomicile (near animal shelters such as pigsties, hen houses, and stables) and another in the intradomicile (installed in the living room or the kitchen), for a total of ten traps per night in each location. The capture effort was 2 traps x 12h x 2 nights x 5 residences x 2 locations x 9 months = 4,320h. The locations of the residences were georeferenced using the Global Positioning System (GPS) with the consent of the residents.

After transportation to the laboratory, the insects were stored at -20 °C in 1.5 mL plastic microtubes. The specimens were clarified and prepared for identification following the technique described by Vilela *et al.*<sup>13</sup>, and identified morphologically according to the taxonomy and classification of Galati<sup>14</sup>. The female specimens' thorax and abdomen were properly conditioned in a -20 °C freezer for molecular assays.

### Molecular assays

Total DNA from each specimen was extracted by Phenol:Chloroform:Isoamyl Alcohol (25:24:1), according to Michalsky *et al.*<sup>15</sup>. The DNA of *Leishmania* spp. was detected by Polymerase Chain Reaction (PCR), with amplification of a fragment of approximately 300 to 350 base pairs (bp) of the Internal Transcribed



**Figure 1** – Location of the capture sites of phlebotomine sand flies in Povoado Mulata and Volta Redonda, municipality of Caxias, Maranhão State, Brazil.

Spacer (ITS1) ribosomal DNA, using the primers LITSR (5'-CTGGATCATTTCGGATG-3') and L5.8S (5'-TGATACCACTTATCGCACTT-3'), according to Schonian *et al.*<sup>16</sup>. The Personal Cycler Thermal Cycler (Biometra™, Jena, Germany) was used for sample amplification, and the PCR reaction mixture (26µL) was prepared with a final concentration of 1µM of each primer; 0.2mM dNTP (Promega®, Madison, WI, USA), 1U of Taq DNA Polymerase (Promega®), 3mM of MgCl<sub>2</sub>, enzyme buffer and 3µL of total DNA (about 30 ng/µL for each sample). The amplified products were stored at -20 °C until analysis.

For each PCR assay, a negative control (reaction mixture + 3µL sterile water) and a positive control (reaction mixture + 90 ng of DNA from *L. (L.) amazonensis* (IFLA/BR/1968/PH8), extracted from cultures) were used. Amplicons were stained with GelRed™ (Nucleic Acid Gel Stain - Biotium, Hayward, CA, USA) and visualized under UV light after electrophoresis through a 1% agarose gel. All PCR products that showed the same band patterns as the positive control were purified with the commercial ExoSAP-IT PCR Product Cleanup Reagent kit (Applied Biosystems™, Carlsbad, CA, USA) according to the manufacturer's instructions.

Subsequently, 1µL of a purified sample (30-40 ng/µL), 1µL (4.5 µM) of the LITSR primer, and 4µL of nuclease-free water were added to sterile microtubes of 0.2 mL, dried and shipped to the sequencing service by the ACTGene (Analises Moleculares Ltda, Brazil). After our analysis, the DNA sequences were deposited on the GenBank platform of the National Center for Biotechnological Information (NCBI) website, under the access numbers available in [Supplementary Table S1](#).

### Sequence identification

The identification of *Leishmania* species was based on the identity of the ITS1 sequences obtained by PCR from the total DNA of sand flies. The sixty-two sequences obtained were preliminarily aligned by the ClustalW algorithm in Bioedit v7.2.5 software<sup>17</sup>. Sixty of those were checked preliminary for identity using the NCBI's online BLAST (Basic Local Alignment Search Tool) tool<sup>18</sup>. Only fifty sequences presented an acceptable size (at least 230 base pairs) for further phylogenetic analysis, which resembles sequences from other studies deposited in the NCBI GenBank (final alignment of 260 base pairs), representing different species

and complexes within the genus *Leishmania*, and sequences from *Trypanosoma cruzi* as an outgroup (Supplementary Table S2). For haplotype identification, we used the software DnaSP v6 (Barcelona, Spain).

## Statistical analyses

An F-test (Quasi-Poisson model with log-link function) was used to verify whether the frequencies of the sand fly species found in the areas (rural and urban) were equal or different from each other. The Chi-square ( $\chi^2$ ) test was used to verify this difference by type of environment (intradomicile and peridomicile). The tests were performed with the R software package version 2020 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria).

## RESULTS

### Sand fly fauna

A total of 2,244 sand flies belonging to fourteen species and seven genera were collected: *Lutzomyia* (França, 1924), *Nyssomyia* (Barreto, 1962), *Evandromyia* (Mangabeira, 1941), *Micropygomyia* (Barreto, 1962), *Sciopemyia* (Barreto, 1962), *Psathyromyia* (Barreto, 1962) and *Brumptomyia* (França & Parrot, 1921). The most abundant species were *Ny. whitmani* (31.4%), *Lu. longipalpis* (23.4%), *Ev. evandroi* (Costa Lima & Antunes, 1936) (28.1%), *Ev. lenti* (Mangabeira, 1938) (9.63%) and *Mi. trinidadensis* (Newstead, 1922) (5.84%) (Table 1).

### Association with rural and urban environments

The abundance of sand flies was higher in rural areas (86.4%) than in urban areas (13.6%) ( $F = 8,8494$ ,  $p < 0.0001$ ). In addition, the rural area also showed higher species diversity ( $n = 11$ ) than the urban area ( $n = 8$ ). Seven species were only found in the rural area whereas three exclusively in the urban area. Four were sampled in both areas (Table 1). In the rural area, the dominant species were *Ny. whitmani* (35.2%), *Ev. evandroi* (32.4%), *Lu. longipalpis* (12.9%) and *Ev. lenti* (11.1%). In the urban area, *Lu. longipalpis* was predominant (89.9%) (Table 1).

### Association with intra- and peridomicile environments

In both rural (53.7%;  $X^2 = 147.8571$   $p < 0.05$ ) and urban (59.1%;  $X^2 = 25.28571$   $p < 0.05$ ) areas, abundance was higher intradomicile (Table 2). In the rural area, the prevalent intradomicile species were *Ev. evandroi* (44.6%) and *Ny. whitmani* (20.9%), and the prevalent peridomicile species were *Ny. whitmani* (51.8%) and *Lu. longipalpis* (19.1%). In the urban area, *Lu. longipalpis* was the prevalent species in both environments, intradomicile (95.6%) and peridomicile (81.6%) (Table 2).

### Identification of *Leishmania* spp. DNA sequences in phlebotomine sand flies

The presence of *Leishmania* spp. DNA was investigated in a total of 123 individually prepared samples of female

**Table 1** - Distribution of phlebotomine sand fly species collected from July 2019 to March 2020 according to the area, rural (Povoado Mulata) and urban (Volta Redonda), and gender (male and female) of the municipality of Caxias, Maranhao State, Brazil.

Species	Rural area			Urban area			Overall Total	
	♂	♀	♂/♀	♂	♀	♂/♀	N	%
<i>Lu. longipalpis</i>	168	83	251	212	63	275	526	23.4
<i>Ny. whitmani</i>	453	230	683	16	6	22	705	31.4
<i>Ev. evandroi</i>	326	303	629	1	1	2	631	28.1
<i>Ev. lenti</i>	134	82	216	0	0	0	216	9.63
<i>Mi. trinidadensis</i>	39	90	129	0	2	2	131	5.84
<i>Mi. oswaldoi</i>	9	3	12	0	0	0	12	0.53
<i>Mi. quinquefer</i>	4	5	9	0	0	0	9	0.40
<i>Ev. termitophila</i>	0	3	3	1	0	1	4	0.18
<i>Br. avellari</i>	3	0	3	0	0	0	3	0.13
<i>Sc. sordellii</i>	1	1	2	0	0	0	2	0.09
<i>Ev. cortelezii</i>	0	0	0	2	0	2	2	0.09
<i>Ny. intermedia</i>	1	0	1	0	0	0	1	0.045
<i>Ps. hermanlenti</i>	0	0	0	1	0	1	1	0.045
<i>Ev. sallesi</i>	0	0	0	1	0	1	1	0.045
TOTAL	1,138	800	1,938	234	72	306	2,244	100
%	58.7	41.3	100	76.5	23.5	100	100	

*Lu.* = *Lutzomyia*; *Ny.* = *Nyssomyia*; *Ev.* = *Evandromyia*; *Mi.* = *Micropygomyia*; *Sc.* = *Sciopemyia*; *Ps.* = *Psathyromyia*; *Br.* = *Brumptomyia*; ♂ = male; ♀ = female; N = absolute number; % = percentage.

**Table 2** - Distribution of phlebotomine sand fly species collected from July 2019 to March 2020 according to intra- and peridomicile environments in rural and urban areas, in the Caxias municipality, Maranhao State, Brazil.

Species	Rural area				Urban area			
	Intradomicile		Peridomicile		Intradomicile		Peridomicile	
	N	%	N	%	N	%	N	%
<i>Lu. longipalpis</i>	80	7.68	171	19.1	173	95.6	102	81.6
<i>Ny. whitmani</i>	218	20.9	465	51.8	4	2.21	18	14.4
<i>Ev. evandroi</i>	464	44.6	165	18.4	0	0	2	1.60
<i>Ev. lenti</i>	152	14.6	64	7.13	0	0	0	0
<i>Mi. trinidadensis</i>	104	9.99	25	2.79	0	0	2	1.60
<i>Mi. oswaldoi</i>	10	0.96	2	0.22	0	0	0	0
<i>Mi. quinquefer</i>	7	0.67	2	0.22	0	0	0	0
<i>Ev. termitophila</i>	3	0.29	0	0	1	0.55	0	0
<i>Br. avellari</i>	0	0	3	0.33	0	0	0	0
<i>Sc. sordellii</i>	2	0.19	0	0	0	0	0	0
<i>Ev. cortelezzii</i>	0	0	0	0	2	1.10	0	0
<i>Ny. intermedia</i>	1	0.10	0	0	0	0	0	0
<i>Ps. hermanlenti</i>	0	0	0	0	0	0	1	0.80
<i>Ev. sallesi</i>	0	0	0	0	1	0.55	0	0
Total	1,041	100	897	100	181	100	125	100

*Lu.* = *Lutzomyia*; *Ny.* = *Nyssomyia*; *Ev.* = *Evandromyia*; *Mi.* = *Micropygomyia*; *Sc.* = *Sciopemyia*; *Ps.* = *Psathyromyia*; *Br.* = *Brumptomyia*; N= absolute number; % = percentage.

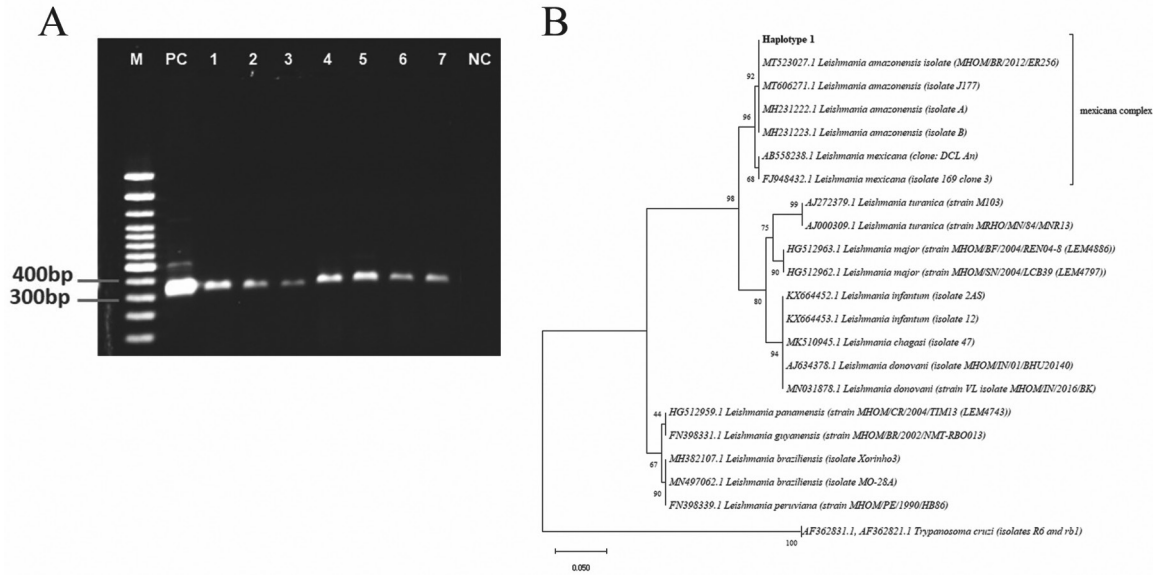
sand flies (68 rural and 55 urban) belonging to eight species (Table 3). The ITS1 fragment was successfully amplified from sixty-two females belonging to five species, found in both, intradomicile and peridomicile environments, in rural and urban areas. The species *Lu. longipalpis* showed the highest positivity (44%), followed by *Ny. whitmani* (27%), *Ev. evandroi* (13%), *Mi. trinidadensis* (13%) and *Mi. quinquefer* (3%) (Table 3 and Figure 2A).

Only sixty ITS1 sequences presented more than 90% similarity with sequences from *Leishmania* spp. and more than 97% similarity with sequences of *Leishmania (L.) amazonensis* stored in the GenBank database (Supplementary Table S1). Among them, the fifty sequences of an acceptable size for phylogenetic analysis belonged to a single haplotype (Figure 2B). The sequences of two PCR-positive samples were noticeably short, preventing us

**Table 3** - Species of phlebotomine sand flies analyzed, and the number of females positive for *Leishmania* DNA captured in the intra- and peridomicile of rural and urban areas of Caxias municipality, Maranhao State, Brazil.

Species	Rural area			Urban area			Total samples analyzed	Total positive samples	
	Intra.	Peri.	Positive samples	Intra.	Peri.	Positive samples		N	%
<i>Lu. longipalpis</i>	8	7	9	28	17	18	60	27	44
<i>Ny. whitmani</i>	4	14	14	0	6	3	24	17	27
<i>Ev. evandroi</i>	10	4	8	0	1	0	15	8	13
<i>Mi. trinidadensis</i>	11	3	7	2	1	1	17	8	13
<i>Mi. quinquefer</i>	1	2	2	0	0	0	3	2	3
<i>Ev. lenti</i>	2	0	0	0	0	0	2	0	0
<i>Ev. termitophila</i>	1	0	0	0	0	0	1	0	0
<i>Sc. sordellii</i>	1	0	0	0	0	0	1	0	0
Total	38	30	40	30	25	22	123	62	100

*Lu.* = *Lutzomyia*; *Ny.* = *Nyssomyia*; *Ev.* = *Evandromyia*; *Mi.* = *Micropygomyia*; *Sc.* = *Sciopemyia*; N = absolute number; % = percentage; Intra.= Intradomicile; Peri.= Peridomicile.



**Figure 2** – Identification of *Leishmania (Leishmania) amazonensis* DNA sequences in phlebotomine sand flies (A): Agarose gel (1%) showing amplified products of 300-350 bp with the *Leishmania* ITS1 primers. PC: positive control; NC: negative control; 1-7: positive phlebotomine sand fly samples. (B) Phylogenetic tree of ITS1 DNA sequences of *Leishmania* spp. amplified from phlebotomine sand flies captured in two areas, rural and urban, in the Caxias municipality, Maranhao State, reconstructed by the Neighbor-Joining method. The percentage of replicate trees in which the associated taxons clustered by a bootstrap test (1000 replicates) is shown next to the nodes. Branch lengths are in the same units as the evolutionary distances used to infer the phylogenetic tree. Haplotype 1, highlighted in bold, represents the only haplotype identified in all ITS-1 sequences (all positive samples). The brackets indicate sequences from the Mexican complex (*Leishmania (L.) amazonensis* and *Leishmania (L.) mexicana*). *Trypanosoma cruzi* is the external group.

from checking their identity, and 10 sequences were just about long enough just for preliminary species checking by BLAST, although not for a phylogeny confirmation.

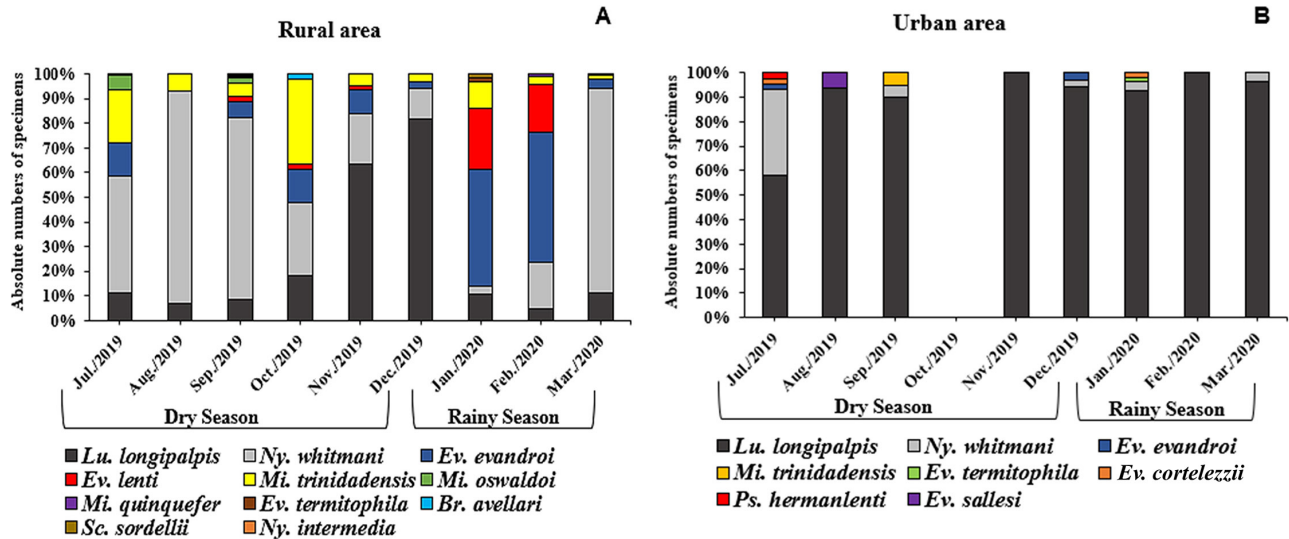
Monthly distribution of sand flies

In the rural area, *Ny. whitmani* and *Lu. longipalpis* was predominant and showed opposite trends regarding

abundance throughout the sampling period. During the rainy season, *Ev. evandroi* and *Ny. whitmani* predominated, also with opposite trends (Figure 3A). *Lu. longipalpis* was abundant throughout the period in urban areas (Figure 3B).

DISCUSSION

Sand flies are widely distributed in urban and rural areas



**Figure 3** – Monthly distribution of phlebotomine sand fly species according to the collection area, rural (A), and urban (B).

in the Caxias municipality<sup>7,10</sup>, maybe as a result of human population growth and subsequent deforestation of areas previously inhabited by sand fly fauna. Meanwhile, a sizable proportion of the population in these areas keeps domestic animals (such as dogs, hens and pigs) in their backyards<sup>8</sup>. Domestic animals and their shelters play a significant role in attracting sand flies, especially when backyards are not clean<sup>19</sup>.

Based on the distribution pattern of the sand flies, it is possible that some species such as *Lu. longipalpis*, *Ny. whitmani*, *Ev. evandroi*, and *Ev. lenti* are well integrated into the Povoado Mulata (rural area), abundantly invading the peridomiciles and easily entering human dwellings, where large numbers accumulate. These species have anthropophilic habits and adapt to different environments<sup>20</sup>. The adjoining area has suffered from the impact of human activity and, consequently, has experienced outbreaks of leishmaniasis. This study aimed to evaluate the composition, abundance, species richness and seasonal distribution of sand flies in the region and to determine the constancy of the insect population. Methods: The survey was conducted at three sites located in the municipalities of Barreirinhas and Santo Amaro between September 2012 and August 2013. Sampling was performed monthly using automatic light traps installed 1.5 m above the soil adjacent to 13 randomly selected rural dwellings. At each site, one trap was placed in the peridomicile near to animal enclosures and another (extradomicile). Therefore, the infestation in the rural locality of Povoado Mulata by sand fly species such as *Ny. whitmani* and *Lu. longipalpis*, on its own, justifies the occurrence of CL and VL, respectively, since those sand flies are competent vectors for the parasites that cause these diseases<sup>8</sup>. Despite occurring in very low density, *Ny. intermedia* deserves attention since it has considerable epidemiological importance, being identified as a vector of parasites that cause CL<sup>21</sup>, as well as *Mi. quinquefer*, which has been found harboring *L. (L.) infantum* DNA in Puerto Iguazu, Argentina<sup>22</sup>.

In this study, we found that, unlike other sand flies, *Lu. longipalpis* appeared abundantly in the urban environment. This vector is highly adapted to different degrees of urbanization and shows opportunistic feeding habits compared to other sand flies, feeding on the blood of humans and of a wide range of domestic animals<sup>23</sup>. The high abundance of *Lu. longipalpis* in the Volta Redonda urban neighborhood warrants the occurrence of VL in this environment, corroborating previous observations and presenting a risk for the maintenance and expansion of *L. (L.) infantum* or, maybe, *L. (L.) amazonensis*<sup>7</sup>. However, the species related to the transmission of causative agents of CL, like *Ny. whitmani*, had a low abundance. In this case, it

is interesting to note the predominance of *Lu. longipalpis* in the sampling. According to previous reports, this vector is permissive for the infection by *Leishmania* species that cause CL, such as *L. (L.) amazonensis*<sup>8,24,25</sup>.

Among the sand fly species found in the municipality of Caxias, several are identified as vectors of CL, while *Lu. longipalpis* is the only vector of *L. (L.) infantum*, which causes VL. Nevertheless, we detected *Leishmania* DNA in only five species, and they all only had *L. (L.) amazonensis* DNA fragments. Interestingly, in previous studies conducted in the same city, but in the urban neighborhood of Salobro and the Bom Jardim rural village, DNA fragments of at least seven species of these parasites were detected: *L. (L.) infantum*, *L. (V.) shawi*, *L. (L.) mexicana*, *L. (V.) braziliensis*, *L. (V.) guyanensis*, *L. (L.) amazonensis*, *L. (V.) lainsoni*, and *L. (V.) naiffi*, including putative mixed infections in sand flies<sup>8</sup>.

The possible reduction in the diversity of circulating *Leishmania* spp. may be explained by the fact that our study was conducted in different areas (Povoado Mulata and Volta Redonda) from those in the previous survey. It is unlikely that several *Leishmania* species disappeared by competitive exclusion in such a brief period (three years), leaving only *L. (L.) amazonensis* to be transmitted by the sand fly populations. It is more feasible to assume that *Leishmania* species may segregate along the municipality area due to differences in local environmental characteristics: hosts, vectors, circulating parasites, backyard sanitary conditions, presence or absence of natural and cultivated vegetation, among others.

The presence of *L. (L.) amazonensis* could be related to the occurrence of twelve cases of VL during the study period, considering the absence of *L. (L.) infantum* in the results of this research, albeit in rural areas, where *Ny. whitmani* was abundant and could be an associated vector. However, it could not explain the urban cases or the occurrence of ten cases of VL. As mentioned, *L. (L.) infantum* DNA was not found in any sand fly in the area and period of this study. Although the clinical manifestation of VL is traditionally associated only with *L. (L.) infantum*, studies point to the participation of *L. (L.) amazonensis*<sup>26,27</sup>. This parasite has been often recovered from sources in urban environments<sup>28,29</sup> infecting dogs<sup>30,31</sup>, synanthropic rodents<sup>28</sup>, and humans<sup>27,32-34</sup>; however, its known vector, *Bichromomyia flaviscutellata*, is rarely found in this environment.

It has also been shown, by an experimental study, that a healthy hamster (*Mesocricetus auratus*) showed clinical manifestation of VL three months after being bitten by *Lu. longipalpis* infected with *L. (L.) amazonensis*<sup>26</sup>, and that this sand fly is capable of sustaining infection of

metacyclic forms of *L. (L.) amazonensis* inside its gut<sup>25</sup>. Furthermore, *L. (L.) amazonensis* is already known to be genetically diverse in different areas of Northeastern Brazil and is associated with VL cases in humans<sup>35</sup>. Moreover, recently, Ribeiro-da-Silva *et al.*<sup>36</sup> demonstrated that *Lu. longipalpis* was able to transmit *L. (L.) amazonensis* to an immunosuppressed Balb/c mouse (*Mus musculus*), which developed clinical symptoms of diffuse cutaneous leishmaniasis. In this context, the data suggest that *L. (L.) amazonensis* cannot be disregarded as a causative agent of CL and VL in the urban area of our study.

Hence, we suspect the participation of *Lu. longipalpis* as a vector of *L. (L.) amazonensis* and the possible involvement of this parasite in the production of CL and VL for the following reasons:

- 1) *Lu. longipalpis* was found with *L. (L.) amazonensis* DNA;
- 2) it constitutes the only sand fly highly abundant in the urban area during nine months of entomological survey;
- 3) occurrence of urban cases of CL in the absence of other sand fly species;
- 4) the notification of VL cases during the study period in the absence of circulating *L. (L.) infantum* in the vector;
- 5) *Lu. longipalpis* is known to be a permissive vector for *L. (L.) amazonensis* transmission. Similarly, we suspect the participation of *L. (L.) amazonensis* as the etiologic agent of the reported cases of CL and VL due to: 1) the absence of evidence of other *Leishmania* spp., including *L. (L.) infantum*, in the sand flies we studied.

## CONCLUSION

To better elucidate our findings and demonstrate whether *L. (L.) amazonensis* is predominating in previous endemic regions of *L. (L.) infantum*, we suggest that surveys (through integrative approaches) of the causative agents of leishmaniasis infecting dogs, humans and synanthropic reservoirs be carried out. Considering that in the Caxias municipality the diagnosis of dogs positive for leishmaniasis is performed only by serology – a non-specific method for identifying *Leishmania* species – and by microscopy of smears in human patients, molecular surveys, along with a genomic characterization of the parasites from those sources, must be performed. In addition to deeper ecological studies about the sand fly fauna, different sampling methods must be used in an integrative manner to verify whether some species, like *Bi. flaviscutellata*, are rare or just undersampled, as in surveys based on CDC-type traps, *Lu. longipalpis* is often the predominant sand fly in the sampling.

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## AUTHORS' CONTRIBUTIONS

RCS, RCRS, JMMR, ASGS, and VCSP: conceptualization; ASGS and VCSP: project administration and funding acquisition; RCS, RCRS, MSO, LNPDC, PMA, and ASGS: sample collection and methodology; RCS, ASGS, and VCSP: formal analysis; RCS, RCRS, JMMR, ASGS, and VCSP: writing original draft preparation; RCS, ASGS, and VCSP: writing review and editing. All authors have read and agreed to the published version of the manuscript.

## CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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