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Speciation in the *Leishmania guyanensis* Vector *Lutzomyia umbratilis* (Diptera: Psychodidae) from Northern Brazil — Implications for Epidemiology and Vector Control

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Additional information is available at the end of the chapter

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Abstract

This chapter starts with a brief mention of the *Leishmania* species and sandflies vectors that occur in the Neotropical region, especially in the Brazilian Amazon. The main focus of this chapter is a review of the taxonomic, biologic and epidemiologic studies conducted in *Lutzomyia umbratilis*, the main vector of *Leishmania guyanensis* in the northern region of Brazil. We associated these data with the population genetics studies carried out in this sandfly vector by our research team. The genetic studies were made with six samples of *L. umbratilis* from the central region of the Brazilian Amazon, using a large fragment (1,181 bp) of the mitochondrial DNA COI gene. Also, another study was conducted in these samples using the DNA barcode region. The results revealed rather high levels of genetic variability for all samples analyzed and a pronounced genetic differentiation between samples from both banks of the Negro and Amazon rivers. The degree of differentiation found may reflect the presence of distinct species within *L. umbratilis*, suggesting that the Amazon and Negro rivers may be acting as effective barriers, preventing gene flow between populations living on the two sides. These findings have important implications for epidemiology, especially regarding vector competence, which is vital information for surveillance and vector control strategies. Furthermore, this diversification process of *L. umbratilis* represents an interesting example for speciation studies.

Keywords: Sandflies, Brazilian Amazon, Population genetics, Speciation, Cryptic species

1. Introduction

Phlebotomine sandflies (Diptera: Psychodidae) are insects of medical and veterinary importance since they are involved in transmission of various pathogens (bacteria, virus and protozoa) that cause diseases such as Bartonellosis, Arboviruses and Leishmaniasis. The latter is caused by trypanosomatids of the genus *Leishmania*, the pathogenic agent of human leishmaniasis. *Leishmania* infection is characterized by a species-specific pathology, varying from cutaneous lesions to the potentially fatal visceral form [1-3]. The distribution of this disease encompasses the tropical, subtropical and Mediterranean regions of the world and its global burden has been estimated to be approximately 500,000 cases of visceral leishmaniasis (VL) and approximately 1.1-1.5 million cases of cutaneous leishmaniasis (CL) per year [4,5]. Despite its widespread distribution, most of the leishmaniasis cases occur in only a few countries: more than 90% of the VL cases occur in Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan, and most of the CL cases occur in Afghanistan, Algeria, Brazil, Colombia, Iran, Pakistan, Peru, Saudi Arabia and Syria [5].

In the Americas, CL occurs from southern USA to northern Argentina, but its main focus is concentrated in South America, especially in Bolivia, Brazil and Peru, with approximately 90% of the recorded cases of the muco-cutaneous type [5,6]. Yet, in spite of its importance, leishmaniasis is one of the most neglected tropical diseases in the world [5].

In Brazil, there has been an expansion of this disease since 1950 [7,8]. Currently, CL has been reported in all Brazilian states, causing outbreaks in several regions of country [9], especially in the Brazilian Amazon. This situation has been correlated to several factors, such as deforestation, the construction of highways and dams, implementation of agricultural poles, migrations of human populations, new mining ventures, the emergence of villages and cities, the use of forest locations for military training, among other factors [6-8,10-16].

Leishmania displays two main morphological forms, the amastigote and the promastigote, which are found in close association with vertebrate (mammals) and invertebrate (phlebotomine sandflies) hosts, respectively, comprising the link of several transmission cycles [13,17,18]. The vertebrate hosts include a large variety of mammals, such as rodents, xenarthrans (armadillo, anteater and sloth), marsupials (opossum), canids and primates, including humans [19-21].

There are approximately 30 species of *Leishmania* described in the World and, of these, at least 20 are pathogenic in mammals [22]. In the Neotropical region 22 species were recorded; of these, 12 were reported in Brazil [23] and seven were found infecting humans in the Brazilian Amazon region [24]. The studies conducted in Brazil have found a large number of dermotropic *Leishmania* species that are proved to infect humans, such as *Leishmania amazonensis*, *Le. braziliensis*, *Le. guyanensis*, *Le. lainsoni*, *Le. naiffi*, *Le. shawi* and *Le. lindenbergi*. There are others species too, but they have been found only in their natural reservoir hosts, as follows: *Le. enriettii*, *Le. forattinii*, *Le. deanei* and *Le. utingensis* [18,20,23,25-27]. With the exception of the two species (*Le. enriettii* and *Le. forattinii*), all those listed above were reported in the Brazilian Amazon, including *Leishmania chagasi* that causes visceral leishmaniasis and whose main

vector is *Lutzomyia longipalpis* [23]. Table 1 presents all species of *Leishmania* and their respective proven and suspected sandfly vectors and reservoir hosts reported in Neotropical region.

In addition to those listed in Table 1, other species of sandflies have been observed in the Brazilian Amazon region, harboring *Leishmania* spp. such as *L. (Lutzomyia) spathotrichia* and *L. (Psathyromyia) dendrophyla* [18].

The detection and identification of the *Leishmania* spp. in phlebotomine species are important to predict the risk of the disease spreading in and around endemic areas, once these species are the main determinants of the clinical outcome in humans. Currently, the use of molecular techniques such as polymerase chain reaction (PCR) has increased the sensitivity and specificity of parasite identification [28]. Based on this technique, *L. (Evandromyia) georgii* was reported for the first time to be infected with *Leishmania* spp. in the Brazilian Amazon region [29]. Similarly, *L. (Trichophoromyia) ubiquitalis* and *L. (Psychodopygus) davisi* were found for the first time to be infected with *Le. lainsoni* in the state of Amazonas, Brazil [30]. These sandflies had already been identified as vectors of *Le. lainsoni* [31] and *Le. braziliensis* [32], respectively, in the state of Pará (Brazil).

Phlebotomine sandflies are amply distributed in all continents, except in Antarctica. Out of the six genera belonging to the subfamily Phlebotominae, only *Lutzomyia* and *Phlebotomus* harbor the main vectors of human leishmaniasis. The former is restricted to the Neotropical and Nearctic regions, where approximately 32 out of more than 500 species described [33] are implicated as vectors, whereas the latter is distributed in all the other regions of the world and comprises important vectors such as *Phlebotomus papatasi* in the Old World, which is the main vector of *Leishmania major* [34,35]. Genus *Lutzomyia* includes the subgenera *Nyssomyia* and *Psychodopygus*, which comprise the most important vectors of CL in the Neotropics, in particular in the Brazilian Amazon region (Table 1; Figures 1 and 2).

Parasites		Leishmaniasis in humans	Sandfly vectors		Reservoir host
Species	Subgenus		Species	Subgenus/Group	
<i>Leishmania chagasi</i> ^{Br/A}	<i>Leishmania</i>	Visceral and cutaneous*	<i>Lutzomyia longipalpis</i> ^P <i>Lutzomyia cruzi</i> ^P <i>Lutzomyia evansi</i> ^P	<i>Lutzomyia</i> <i>Lutzomyia</i> Group Verrucarum	Canids (<i>Cerdocyon thous</i> , <i>Speothos venaticus</i> , <i>Canis familiaris</i>), felines (<i>Panthera onca</i> , <i>Felis concolor</i>), marsupials (<i>Didelphis marsupialis</i> and <i>D. albiventris</i>)
<i>Leishmania enriettii</i> ^{Br}	<i>Leishmania</i>	Not registered	<i>Lutzomyia monticola</i> ^S <i>Lutzomyia correaimai</i> ^S	Ungrouped Group <i>Rupicola</i>	Rodents (<i>Cavia porcellus</i>)
<i>Leishmania mexicana</i>	<i>Leishmania</i>	Cutaneous	<i>Lutzomyia olmeca olmeca</i> ^P <i>Lutzomyia diabolica</i> ^S	<i>Nyssomyia</i> <i>Lutzomyia</i>	Rodents (<i>Ototylomys phyllotis</i> , <i>Nyctomys sumichrasti</i> , <i>Heteromys desmarestianus</i> , <i>Sigmodon hispidus</i> , <i>Neotoma albigula</i>)

Parasites		Leishmaniasis	Sandfly vectors		Reservoir host
Species	Subgenus	in humans	Species	Subgenus/Group	
<i>Leishmania pifanoi</i>	<i>Leishmania</i>	Cutaneous	<i>Lutzomyia flaviscutellata</i> ^S	<i>Nyssomyia</i>	Unknown
<i>Leishmania amazonensis</i> ^{Br/A}	<i>Leishmania</i>	Cutaneous	<i>Lutzomyia flaviscutellata</i> ^P <i>Lutzomyia o. olmeca</i> ^P <i>Lutzomyia reducta</i> ^P	<i>Nyssomyia</i> <i>Nyssomyia</i> <i>Nyssomyia</i>	Rodents (<i>Proechimys</i> spp., <i>Oryzomys</i> spp., <i>Nectomys</i> , <i>Neacomys</i> , <i>Dasyprocta</i>) Marsupials (<i>Marmosa</i> , <i>Metachirus</i> , <i>Didelphis</i> , <i>Philander</i>), fox (<i>Cerdocyon thous</i>)
<i>Leishmania aristidesi</i>	<i>Leishmania</i>	Not registered	<i>Lutzomyia olmeca bicolor</i> ^S	<i>Nyssomyia</i>	Marsupials (<i>Marmosa robinsoni</i>), rodents (<i>Poecornys semispinosus</i> , <i>Dasyprocta punctata</i>)
<i>Leishmania garnhami</i>	<i>Leishmania</i>	Cutaneous	<i>Lutzomyia youngi</i> ^S	Group <i>Verrucarum</i>	Marsupials (<i>Didelphis marsupialis</i>)
<i>Leishmania venezuelensis</i>	<i>Leishmania</i>	Cutaneous	<i>Lutzomyia olmeca bicolor</i> ^S <i>Lutzomyia rangelliana</i> ^S	<i>Nyssomyia</i> Ungrouped	Domestic cat
<i>Leishmania forattinii</i> ^{Br}	<i>Leishmania</i>	Not registered	<i>Lutzomyia ayrozai</i> ^P <i>Lutzomyia yuilli</i> ^P	<i>Psychodopygus</i> <i>Nyssomyia</i>	Rodents (<i>Proechimys inheringi</i>), marsupials (<i>Didelphis marsupialis</i>)
<i>Leishmania hertigi</i>	<i>Leishmania</i>	Not registered	Unknown		Rodent(<i>Coendou rothschildi</i>)
<i>Leishmania deanei</i> ^{Br/A}	<i>Leishmania</i>	Not registered	Unknown		Rodent (<i>Coendou p. prehensilis</i>)
<i>Leishmania braziliensis</i> ^{Br/A}	<i>Viannia</i>	Cutaneous	<i>Lutzomyia intermedia</i> ^P <i>Lutzomyia whitmani</i> ^P <i>Lutzomyia wellcomei</i> ^P <i>Lutzomyia davisi</i> ^P <i>Lutzomyia complexa</i> ^S	<i>Nyssomyia</i> <i>Nyssomyia</i> <i>Psychodopygus</i> <i>Psychodopygus</i> <i>Psychodopygus</i>	Rodents (<i>Oryzomys concolor</i> , <i>O. capito</i> , <i>O. nigripes</i> , <i>Akodon arviculoides</i> , <i>Proechimys</i> spp., <i>Rattus rattus</i> , <i>Rhipidomys leucodactylus</i> , <i>Sigmodon hispidus</i> , <i>Bolomys lasiurus</i>), marsupials (<i>Didelphis marsupialis</i>)
<i>Leishmania peruviana</i>	<i>Viannia</i>	Cutaneous	<i>Lutzomyia peruensis</i> ^S <i>Lutzomyia verrucarum</i> ^S	<i>Helcocyrtomyia</i> Group <i>Verrucarum</i>	Rodent (<i>Phyllotis andinum</i>), marsupials (<i>Didelphis marsupialis</i>) and domestic dog

Parasites		Leishmaniasis in humans	Sandfly vectors		Reservoir host
Species	Subgenus		Species	Subgenus/Group	
<i>Leishmania guyanensis</i> ^{Br/A}	<i>Viannia</i>	Cutaneous	<i>Lutzomyia umbratilis</i> ^P <i>Lutzomyia anduzei</i> ^P	<i>Nyssomyia</i> <i>Nyssomyia</i>	Xenarthrans (<i>Choloepus didactylus</i> , <i>Tamandua tetradactyla</i>), rodents and marsupials
<i>Leishmania panamensis</i>	<i>Viannia</i>	Cutaneous	<i>Lutzomyia trapidoi</i> ^P <i>Lutzomyia ylephiletor</i> ^P <i>Lutzomyia gomezi</i> ^P <i>Lutzomyia panamensis</i> ^P	<i>Nyssomyia</i> <i>Nyssomyia</i> <i>Lutzomyia</i> <i>Psychodopygus</i>	Xenarthrans (<i>Choloepus hoffmanni</i> , <i>Bradypus infuscatus</i> and <i>B. griseus</i>); racoons (<i>Bassaricyon gabbi</i> , <i>Nasua nasua</i> , <i>Poto flavus</i>), primates (<i>Aotus trivirgatus</i> , <i>Saguinus geoffroyi</i>), rodents (<i>Heteromys</i> spp.)
<i>Leishmania lainsoni</i> ^{Br/A}	<i>Viannia</i>	Cutaneous	<i>Lutzomyia ubiquitalis</i> ^P <i>Lutzomyia velascoi</i> ^S	<i>Trichophoromyia</i> <i>Trichophoromyia</i>	Rodent (<i>Agouti paca</i>)
<i>Leishmania naiffi</i> ^{Br/A}	<i>Viannia</i>	Cutaneous	<i>Lutzomyia ayrozei</i> ^P <i>Lutzomyia panamensis</i> ^P <i>Lutzomyia squamiventris</i> ^P	<i>Psychodopygus</i> <i>Psychodopygus</i> <i>Psychodopygus</i>	Xenarthrans (<i>Dasyopus novemcinctus</i>)
<i>Leishmania shawi</i> ^{Br/A}	<i>Viannia</i>	Cutaneous	<i>Lutzomyia whitmani</i> ^P	<i>Nyssomyia</i>	Primates (<i>Cebuspaella</i> , <i>Chiropotes satanas</i>), xenarthrans (<i>Choloepus didactylus</i> , <i>Bradypus tridactylus</i>) and racoon (<i>Nasua nasua</i>)
<i>Leishmania colombiensis</i>	<i>Viannia</i>	Cutaneous	<i>Lutzomyia hartmanni</i> ^P <i>Lutzomyia gomezi</i> ^P <i>Lutzomyia panamensis</i> ^P	<i>Helcocyrtomyia</i> <i>Lutzomyia</i> <i>Psychodopygus</i>	Xenarthrans (<i>Choloepus hoffmanni</i>)
<i>Leishmania equatorensis</i>	<i>Viannia</i>	Not registered	<i>Lutzomyia hartmanni</i> ^P	<i>Helcocyrtomyia</i>	Xenarthrans (<i>Choloepus hoffmanni</i>) and rodent (<i>Sciurus grantensis</i>)
<i>Leishmania lindenbergi</i> ^{Br/A}	<i>Viannia</i>	Cutaneous	<i>Lutzomyia antunesi</i> ^S	<i>Nyssomyia</i>	Unknown
<i>Leishmania utingensis</i> ^{Br/A}	<i>Viannia</i>	Not registered	<i>Lutzomyia tuberculata</i> ^P	<i>Viannamyia</i>	Unknown

Br/A=Brazil, including Amazon; Br=Brazil, except Amazon; P=proven vector; S=suspect vector. *In Costa Rica, the infection occurs mostly as non-ulcerative skin lesions; Honduras and Nicaragua, the infection is much visceral as skin. Information compiled from Lainson (2010) [23].

Table 1. *Leishmania* species with their respective proven and suspect vectors (phlebotomine sandflies) and natural reservoirs (mammals) reported for the Neotropical region.

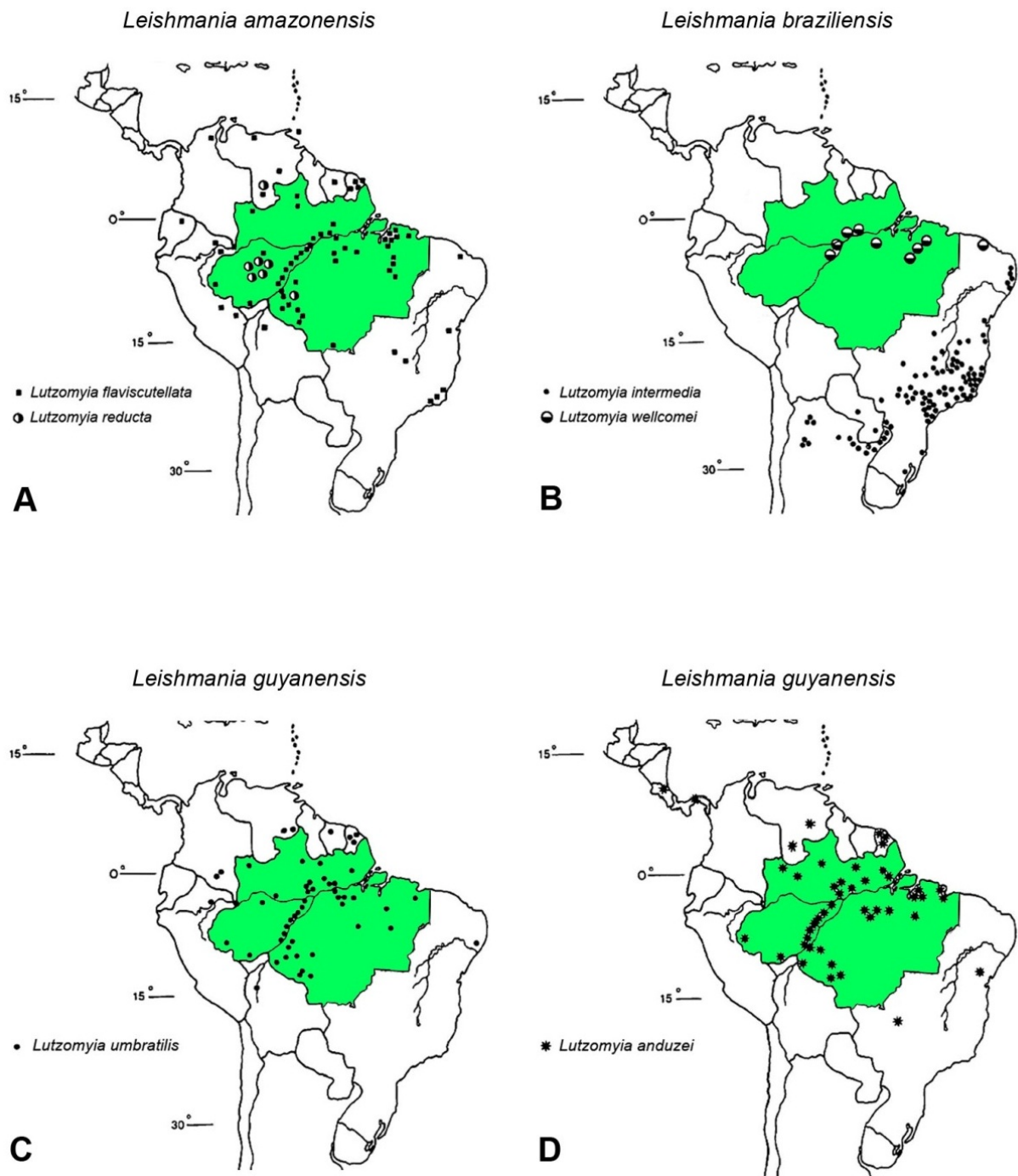


Figure 1. Distribution of the sandfly vectors of *Leishmania amazonensis* (A), *Leishmania braziliensis* (B) and *Leishmania guyanensis* (C and D). Highlight in green color corresponding to the Brazilian Amazon region. Map modified from Young and Duncan (1994) [2].

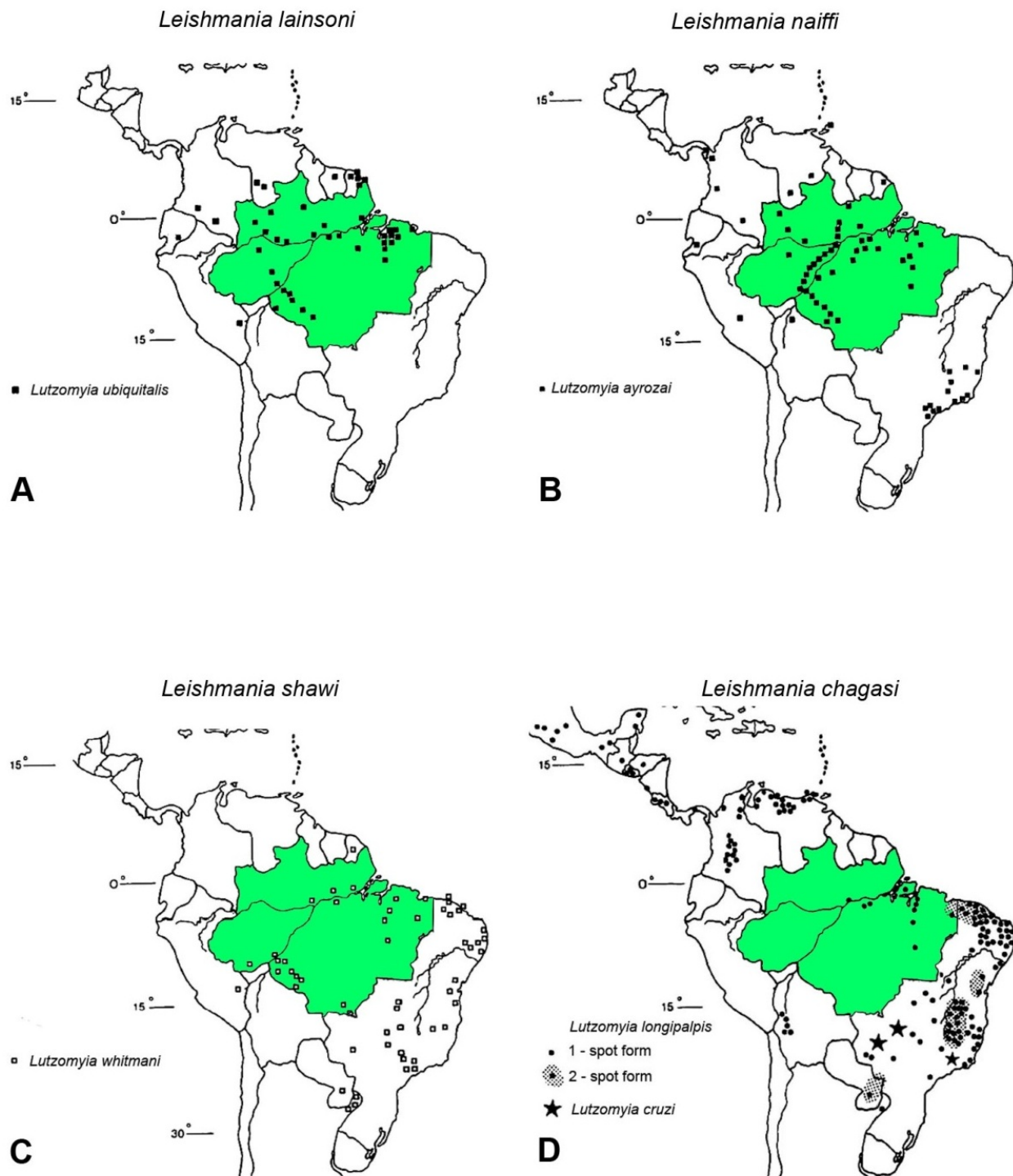


Figure 2. Distribution of the sandfly vectors of *Leishmania lainsoni* (A), *Leishmania naiffi* (B), *Leishmania shawi* (C) and *Leishmania chagasi* (D). Highlight in green color corresponding to the Brazilian Amazon region. Map modified from Young and Duncan (1994) [2].

Similar to other insect groups, the Brazilian Amazon hosts a large diversity of sandfly species likely because of the great variety of ecological niches available [36] which are favorable for survival and reproduction. For example, in a single hectare of forest 50 sandfly species were captured [37]. This high level of diversity of insect vectors and also of reservoirs permits the simultaneous circulation of several species of *Leishmania* and is particularly interesting for the dynamic transmission studies of CL in this region [38,39].

In northern South America, in particular in the Brazilian Amazon region, the transmission of CL is associated to *Lutzomyia umbratilis* Ward and Fraiha and *Lutzomyia anduzei* Rozeboom, implicated as principal and secondary vectors of *Le. guyanensis*, respectively. The *Le. guyanensis* cycle is completed in several species of mammals, especially in xenarthrans, the two-toed sloth (*Choloepus didactylus*), considered the main reservoir, and marsupials such as the opossum (*Didelphis marsupialis*) (*Didelphis marsupialis*) [40-43].

2. Distribution, biological aspects and population genetics of *Lutzomyia umbratilis*

In the last years, as genetic molecular markers became available, the number of studies on population genetics and evolutionary genetics in sandfly species has significantly increased [44-49], and the results have revealed large intra-population genetic variation, genetically populations structured, genetic lineages and cryptic species complexes. In the case of vector species, the knowledge of the genetic structure of populations and the processes responsible for the differentiation distribution is important for the identification of the disease transmission heterogeneity patterns. Such patterns are often produced by the presence of cryptic species, structured populations and/or genetic lineages, which may show variation in the degrees of anthropophily, susceptibility of females to infection by the pathogen, infection rates and females longevity. The identification of these factors is of paramount importance for developing effective management and vector control strategies.

The diversification patterns (structured populations, lineages, complete speciation) observed in sandfly species have generally been associated to multiple factors, such as climate barriers (or climate events in the past), geographic distances, differences in latitude or altitude, habitat modification, landscape fragmentation caused by anthropogenic actions and others, vegetation type or geographic barriers (rivers, mountains). These factors can reduce the dispersal capacity of sandflies, leading them to become isolated populations and causing loss of genetic diversity and increase of differentiation among the populations, as discussed by Ready et al. [50] with regard to *Lutzomyia whitmani*, by Mukhopadhyay et al. [51] for *Lutzomyia shannoni*, by Uribe-Soto et al. [52] for *L. longipalpis* and by Pech-May et al. [45] for *Lutzomyia cruciata*. Additionally, the low flight capacity of this group of insects which seldom spread over more than 1 km, and the breeding soil type are also factors that may contribute even more to the population isolation and then favor the process of divergence and speciation events.

Lutzomyia umbratilis, the main vector of *Le. guyanensi*, that causes Cutaneous Leishmaniasis (CL), occurs in northern South America, including Bolivia, Brazil, Colombia, French Guyana,

Peru, Suriname and Venezuela [2,53]. In Brazil, *L. umbratilis* has been reported in all states of the northern region, the states of Mato Grosso and Mato Grosso do Sul (Southwest), besides the state of Maranhão and an isolated population in the state of Pernambuco, both in the northeastern region [2, 54,55]. Thus, populations of this species are spread over vast areas, separated by geographic barriers such as the largest rivers, the Amazon and the Negro, in the Brazilian Amazon region. Additionally, sandfly species have very limited dispersal capabilities, usually no more than 1 km [56,57], which favors geographic isolation of the populations. Thus, considering the vast geographic area, with discontinuous distribution, along with the low flight capacity of this insect group, *L. umbratilis* populations could be more susceptible to evolve into differentiated populations, incipient species and, ultimately, reproductively isolated species.

Lutzomyia umbratilis has been implicated in the transmission of *Le. guyanensis* in several countries of northern South America, including northern Brazil, and French Guiana and Suriname [58-60]. In the Brazilian Amazon, this species has shown to be highly anthropophilic and has been appointed as the main *Le. guyanensis* vector in the states of Pará [58-60], Amazonas [42,61-63] and Amapá [18] and is probably involved in the transmission in the states of Acre [64] and Rondônia [65]. Moreover, according to the hypothesis of Arias and Freitas [40], the susceptibility of this vector to *Leishmania* seems to vary in the central Brazilian Amazon region. *Lutzomyia umbratilis* populations naturally infected with *Le. guyanensis* have been observed east of the Negro River and north of the Amazonas River; however, there is no report of natural infections by *Leishmania* in this species south of the Amazon River system. Arias and Freitas [40] suggested that the fluvial system formed by the Amazon, Solimões and Negro Rivers may act as a barrier to the *Le. guyanensis* transmission cycle, where *L. umbratilis* populations display distinct degrees of vector competence between the opposite sides of these rivers, suggesting that these populations might represent a species complex or incipient speciation event.

Despite its importance as vector and the probable existence of a cryptic species complex, only few studies have tested the role of the rivers barrier in the genetic subdivision of *L. umbratilis*. A biological study conducted with two *L. umbratilis* populations from Manaus and Manacapuru (left and right banks of the Negro River, respectively) in the Brazilian Amazon region, revealed significant differences in the life cycle, fecundity, fertility, emergence degree and adult longevity between these populations, reflecting intrinsic biological differences [66]. Subsequently, a study that combined morphology, chromosome and isozymes analyses of four *L. umbratilis* populations from this fluvial system showed significant differences in the bristle lengths of 4th instar larvae and in the number and size of the spines of the female genital atrium armature [67]. The latter has been a useful marker for distinguishing closely related species of sandflies [68]. Unfortunately, polytene chromosome analysis was not possible, but the metaphase karyotype was $2n=6$. Isozymes did not reveal any differences among the populations [67]. This result may be due to the slow evolution rate, negative selection and the amino acid codon wobble effect. Consequently, isozymes are not informative markers for detecting incipient or recently diverged species. Therefore, the taxonomic status of *L. umbratilis* remains unclear.

Lutzomyia umbratilis was described by Ward and Fraiha [69], based on specimens captured in the Jari River region, state of Pará, Brazil. Because of the high morphological similarity between *L. umbratilis* and *L. anduzei*, the former has been wrongly identified as *L. anduzei* in the past. In

fact, most of *L. anduzei* specimens found to be naturally infected with *Leishmania* before this date (1977) could actually be *L. umbratilis*. After this date, it has been possible distinguish *L. umbratilis* and *L. anduzei* morphologically, based on the internal and external genitalia of males and females [2,70]. Currently, they can also be identified molecularly by using DNA barcode sequences from *COI* of mitochondrial DNA [70]. The phylogenetic analysis of this dataset found two strongly supported monophyletic clades, although the genetic distances between them, based on the Kimura 2 Parameters (K2P) model, were very small (4.4%), suggesting that these species are very closely related (sister species) [70]. These species have been found infected naturally with *Le. guyanensis* in the Brazilian Amazon, although the studies revealed much higher infection rates in *L. umbratilis* than in *L. anduzei* females, consequently, the former has been recognized as principal vector of this parasite [18,63,71,72].

L. umbratilis adults are generally found in the rainforest (primary forest) of the Brazilian Amazon region, with its high humidity and dim light; therefore, the species has been recognized as ombrophilous, as expressed in its name, *L. umbratilis*. This species is further recognized as dendrobatic, because it is associated to tree trunks during the daytime. In the field, its density may vary, depending on the location and of these characteristics, but it seems to be denser in the central Amazon region, tending to reduce its density towards the edges of this region (Alencar, R. B., personal information). *L. umbratilis* adults are captured using aspirators on the bases of tree trunks during daytime and with CDC (Center for Disease Control) miniature light traps at ground level and in the forest canopy at night. These methods have been employed efficiently throughout Brazilian Amazon region [73].

In addition to the isozyme studies mentioned above, the most recent population genetics analyses were performed on the six *L. umbratilis* populations from the two opposite banks of the Amazon and Negro rivers (Table 2; Figure 3) by using a large fragment (1,181 bp) of the *COI* gene (the 3' end fragment of *COI*) [48] and the Barcode region (663 bp) [70], both from mitochondrial DNA. The aim of these analyses was to assess whether the populations of the opposite banks of these rivers consist of incipient or distinct species. In the study of Scarpassa and Alencar [48], 111 specimens were sequenced and the results revealed 52 haplotypes, reflecting a very large genetic variability for most of the samples examined, except one (Rio Preto da Eva). The genealogical relationships of the haplotypes were accessed using the TCS program [74] at the 95% confidence level. This analysis showed two haplotype groups (lineages), separated by ten mutational steps, but all connected in the network (Figure 4). Similarly, phylogenetic analysis using Bayesian Inference (BI) and inferred under the TIM1+I model, generated two distinct evolutionary lineages (probably clades), with probability support from moderate to slightly high (0.64 and 0.77; Figure 5), suggesting two monophyletic clades. These lineages can be separated by one fixed mutation at position 933 (A \leftrightarrow G) of the dataset, and the estimated sequence divergence between them was 1%. Lineage I consisted of four samples from the left bank of the Amazon and Negro rivers, whereas lineage II comprised two samples from the right bank of the Negro river (Figure 3). No haplotypes were shared between samples of the two lineages. Samples from the same clade (within-clades) exhibited low to moderate genetic differentiation ($F_{ST} = -0.0390-0.1841$), whereas samples from different clades (between clades) exhibited extremely high and significant differentiation ($F_{ST} =$

0.7100-0.8497; $P < 0.0001$) and fixed differences ($S_f = 1$ to 7) (Table 3). Curiously, the samples from Manacapuru *versus* the samples from the BR-174 Highway, Rio Preto da Eva and Manaus, which are separated by smaller geographic distances (from 59.43 to 96.01 km), displayed more fixed differences ($S_f = 6$ to 7) and no shared polymorphism ($S_s = 0$), whereas, the samples from Manacapuru *versus* the samples from Cachoeira Porteira, which are separated by a larger geographic distance (449.22 km), exhibited less fixed differences ($S_f = 3$) and more shared polymorphisms ($S_s = 2$). Taken together, the evidence of absence of gene flow associated with the high levels of genetic differentiation may be an indicator of genetic discontinuity between these lineages, so they could represent incipient or distinct species. The separation time calculated between these lineages falls in the middle Pleistocene (0.22 Mya), coinciding with the more recent formation of the Amazon and Negro rivers [75], appointed as the most probable evolutionary force. This vicariant event, along with the low dispersal rate of the sandflies, and the amenable environmental conditions for adaptation and also drift are likely to have contributed to the great genetic differentiation between the populations of the opposite banks.

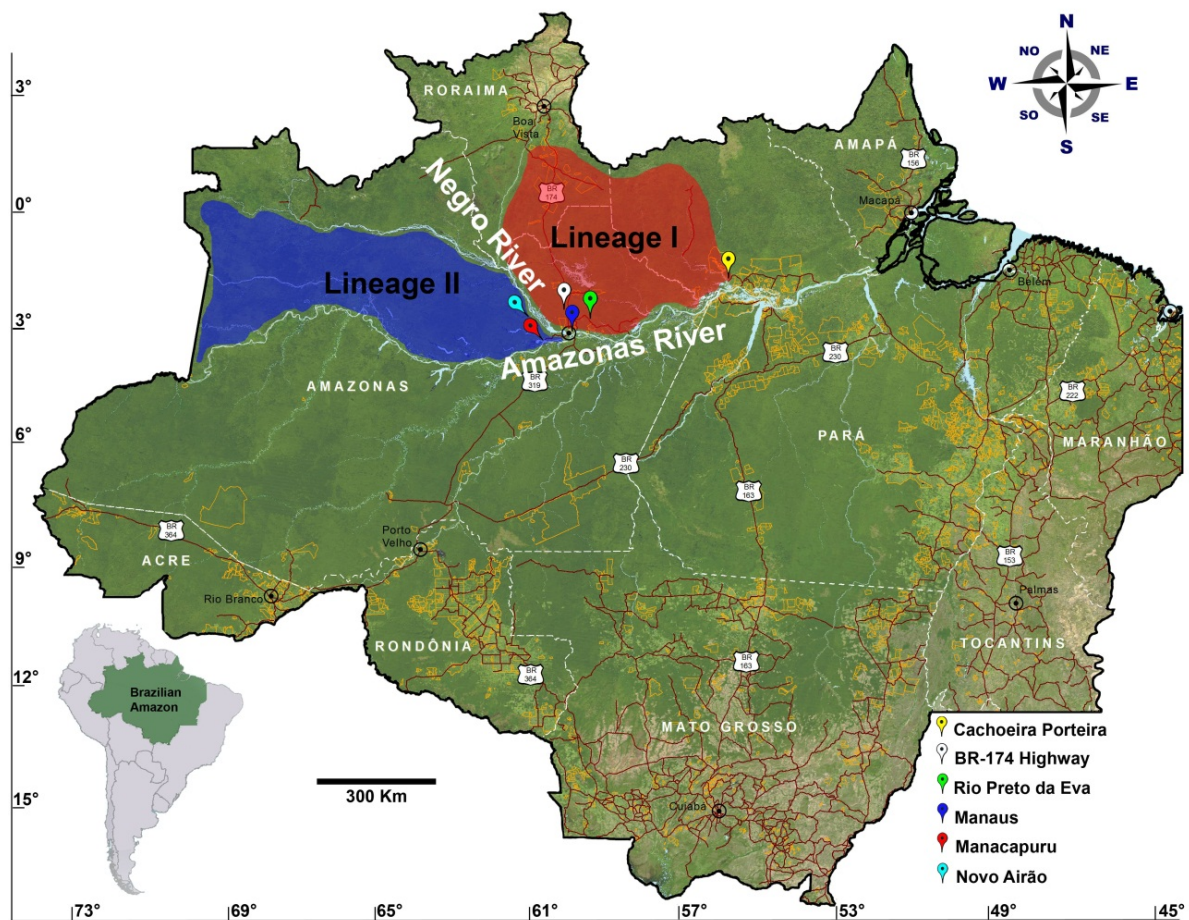


Figure 3. Collection sites of *Lutzomyia umbratilis* from the Brazilian Amazon. Geographic distribution inferred of lineage I (in red color); Geographic distribution inferred of lineage II (in blue color). Map modified from Scarpassa and Alencar (2013) [70].

Species	Localities, State	Co-ordinates	N
		Latitude; Longitude	
<i>L. umbratilis</i>	Cachoeira Porteira, Oriximiná, Pará	1° 28' S; 56° 22' W	18
	BR-174 Highway, Amazonas	2° 36' S; 60° 02' W	15
	Rio Preto da Eva, Amazonas	2° 43' S; 59° 47' W	15
	Manaus, Amazonas	3° 04' S; 59° 57' W	4
	Manacapuru, Amazonas	3° 14' S; 60° 31' W	24
	Novo Airão, Amazonas	2° 47' S; 60° 55' W	35

N: sample size. **Source:** Scarpassa and Alencar (2012) [48]

Table 2. Collection sites and sample sizes of *Lutzomyia umbratilis* from the Brazilian Amazon.

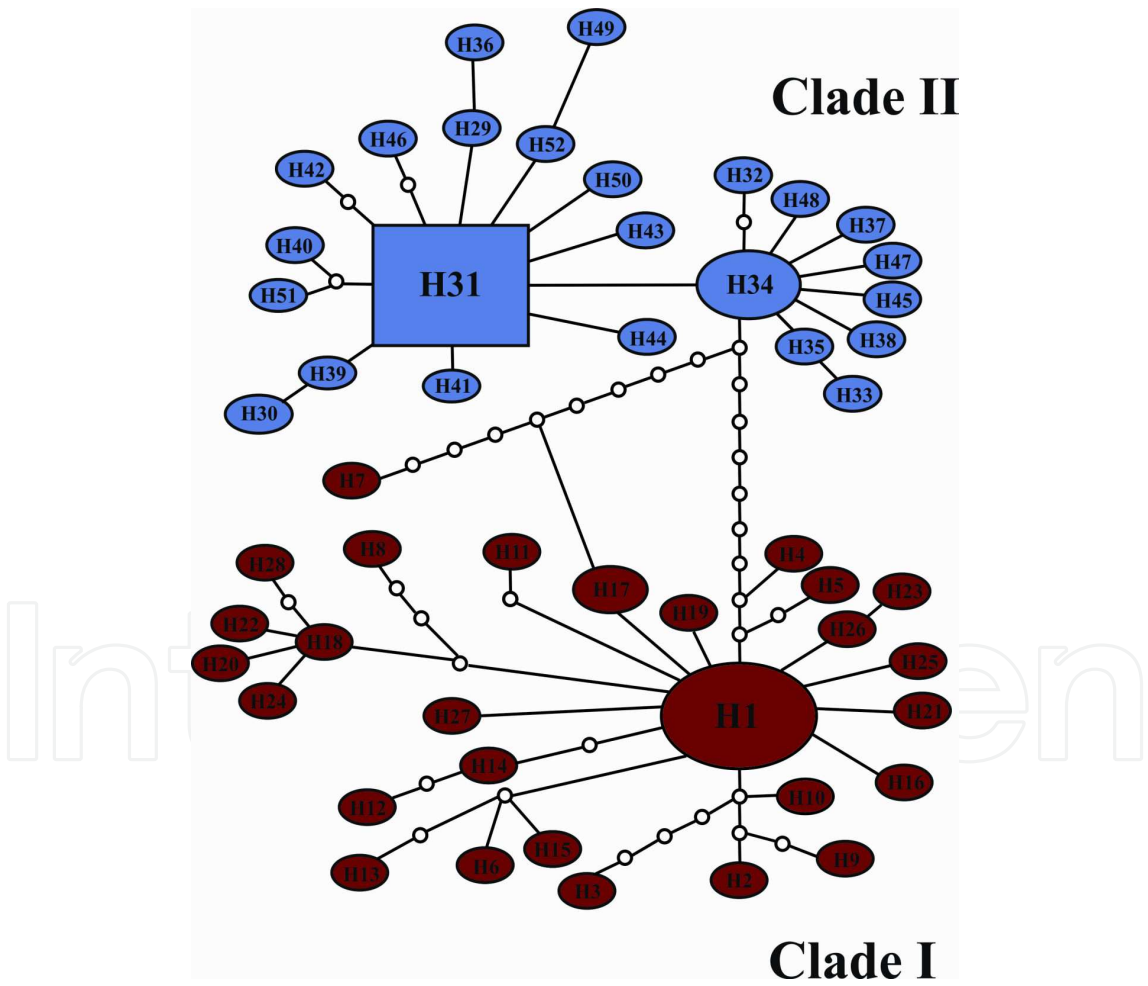


Figure 4. Parsimony haplotypes network of the 52 haplotypes observed in *Lutzomyia umbratilis*. H1 to H52, haplotypes. The haplotype circle sizes are proportional to number of individuals observed in each haplotype. Clade I is in red color. Clade II is in blue color. Empty smaller circles represent mutational events. **Source:** Scarpassa and Alencar (2012) [48].

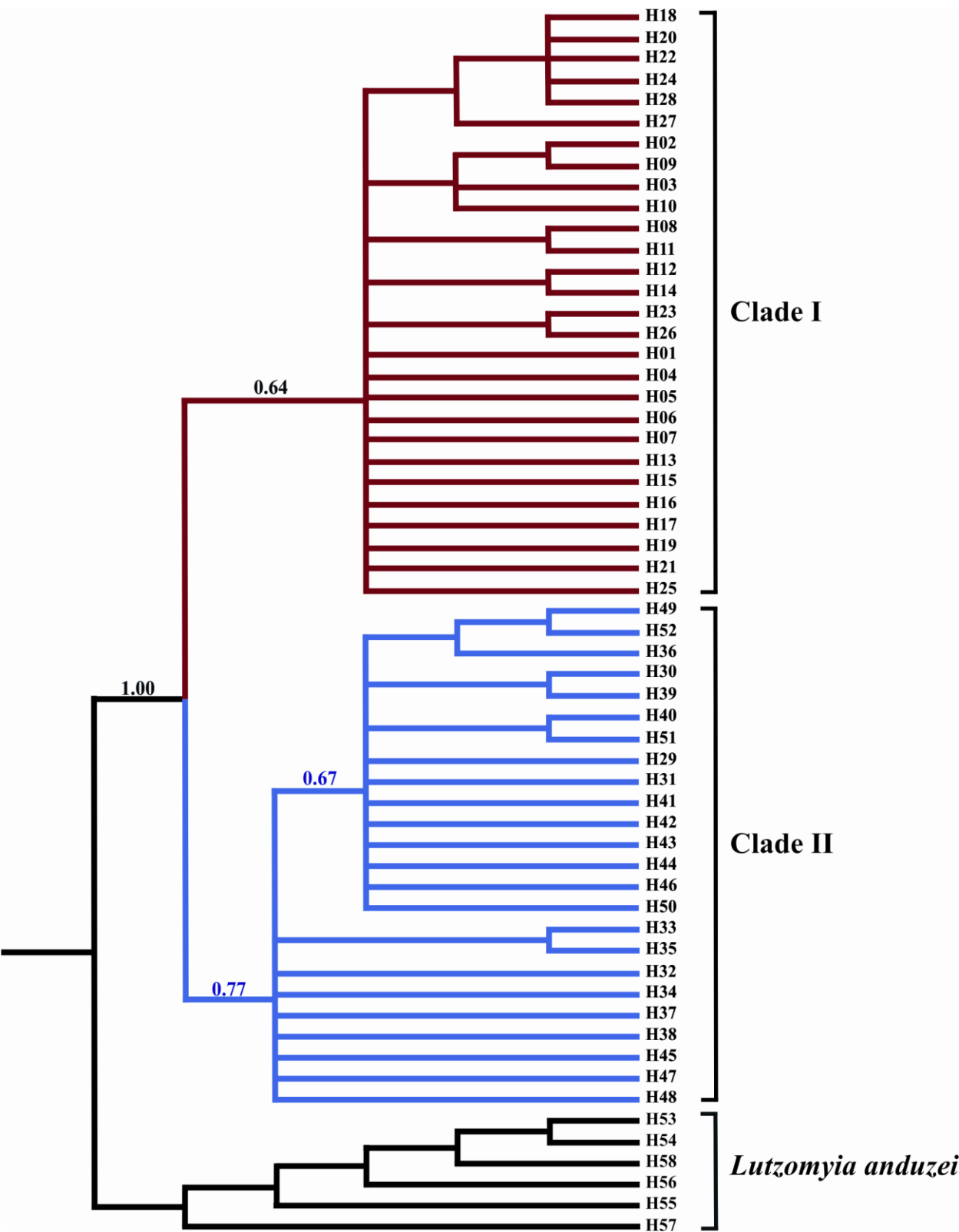


Figure 5. Bayesian Inference (BI) topology tree of the 52 haplotypes of *Lutzomyia umbratilis* inferred under the TIM1+I evolutionary model. Numbers above branch represent posterior probabilities obtained in the BI. *Lutzomyia anduzei* was used as outgroup. **Source:** Scarpassa and Alencar (2012) [48].

Samples	F_{ST} (Km)	K	D_{xy}	D_a	S_s	S_f
Cachoeira Porteira x BR-174 Highway	0.0522 (368.40)	3.52	0.00297	0.00017	4	0
Cachoeira Porteira x Rio Preto da Eva	0.0569*** (353.67)	2.99	0.00248	0.00017	2	0
Cachoeira Porteira x Manaus	0.0230 (394.02)	3.92	0.00332	0.00025	1	0
BR-174 Highway x Rio Preto da Eva	0.0189 (30.46)	1.48	0.00125	0.00002	3	0
BR-174 Highway x Manaus	-0.0390 (56.29)	2.20	0.00187	-0.00012	3	0
Rio Preto da Eva x Manaus	0.1841 (45.35)	1.87	0.00158	0.00008	2	0
Manacapuru x Novo Airão	0.0548 (58.74)	1.81	0.00153	0.00008	4	0
Cachoeira Porteira x Manacapuru	0.7100*** (449.22)	10.19	0.00863	0.00599	2	3
BR-174 Highway x Manacapuru	0.8157*** (87.11)	9.78	0.00833	0.00673	0	6
Rio Preto da Eva x Manacapuru	0.8497*** (96.01)	8.98	0.00765	0.00653	0	6
Manaus x Manacapuru	0.8249*** (59.43)	10.42	0.00887	0.00699	0	7
Cachoeira Porteira x Novo Airão	0.7337 *** (477.49)	10.55	0.00899	0.00625	6	1
BR-174 Highway x Novo Airão	0.8197*** (107.97)	10.21	0.00869	0.00705	4	4
Rio Preto da Eva x Novo Airão	0.8439 *** (130.14)	9.43	0.00803	0.00687	1	4
Manaus x Novo Airão	0.8269*** (108.76)	10.84	0.00924	0.00731	2	5
Clade I x Clade II	0.7776***	9.99	0.00850	0.00660	8	1

F_{ST} : pair-wise genetic differentiation; K : average number of nucleotide differences between populations; D_{xy} : average number of nucleotide substitutions per site between populations; D_a : number of net nucleotide substitutions per site between populations; S_s : number of shared polymorphisms between pairs of populations; S_f : number of fixed differences between pairs of populations. The geographic distance (in km) between localities is represented inside the parentheses. *** $P = 0.00000 \pm 0.0000$, after the Bonferroni correction. **Source:** Scarpassa and Alencar (2012) [48].

Table 3. Genetic differentiation among samples and haplotype clade of *Lutzomyia umbratilis*.

Another study was conducted subsequently on these *L. umbratilis* populations, using the Barcode region (663bp) [70]. In the 72 specimens sequenced, 32 haplotypes were observed. In line with the results of the previous study [48], no haplotype was shared between lineages I and II. The genetic distance between the lineages, based on the K2P model, was rather small (0.009 to 0.010); however, they could be identified by one fixed mutation (T ↔ C transition at position 21).

The genetic differentiation observed in these studies supports the biological and morphological differences reported by Justiniano [67] and Justiniano et al. [66]. These results strongly

indicate that *L. umbratilis* represents a species complex with recent evolutionary history. Taken together, these findings might explain possible differences in the vector competence of these sandflies, a hypothesis raised by Arias and Freitas [40]. On the other hand, these results do not support the isozyme data, which showed genetic homogeneity among populations. These inconsistencies between markers could be attributed to incomplete lineage sorting, due to recent divergence between *L. umbratilis* lineages (or distinct species) and/or distinct evolution rates of the markers used; for instance, isozymes evolve at a slower rate than mitochondrial DNA and are not informative markers for detecting incipient or recently diverged species.

Little is known about the natural breeding sites of *L. umbratilis* and, consequently, about its biology. This knowledge is important for application in any attempt to create and maintain colonies in laboratory conditions. The maintenance of *L. umbratilis* colonies could be the key to testing the mechanisms of reproductive isolation [66,76,77], as well as the assortative mating features between populations separated by the Negro and Amazon rivers, hypothesized as distinct species. It is particularly important because species that have diverged very recently are expected to share ancestral variation at high proportions, a situation that may confound their phylogenetic reconstruction. In addition, it is likely that in young species, with a recent divergence process, there are fixed differences only in genes involved in the speciation process. The maintenance of *L. umbratilis* colonies in the laboratory would also be important to assess the level of vector competence, based on tests of experimental infection between populations from the opposite river banks.

Another interesting approach could be genomic population studies using multilocus analysis, especially using loci which are involved in the different biologic aspects of *L. umbratilis*. This approach will permit distinguishing the effects of natural selection from those of genetic drift. The importance of this approach resides in the fact that genomic analyses provide more reliable information on historic and demographic events. The effect of a specific locus (outlier locus) helps identifying signs of natural selection in genes involved in the most variable adaptability process, such as those related to vector competence and (or) vector capacity, thus allowing a better understanding of vector status in distinct areas from the Brazilian Amazon.

3. Conclusion

The two genetic lineages of *L. umbratilis* found in these studies may represent an advanced speciation process, indicating incipient or distinct species. This suggests that the Amazon and Negro rivers may be acting as effective barriers, as observed in *L. cruciata* [45], preventing gene flow between populations of opposite banks. Such findings have important implications for epidemiology, especially those related to vector competence, which are vital information for surveillance and vector control strategies in northern Brazil. Furthermore, this information may also provide a better knowledge of the evolutionary history of this species complex, as well as *L. umbratilis* represents an interesting example for speciation studies.

Finally, further studies of these populations using other molecular genetic markers, as well as additional sampling along the river banks and within interfluvies in the Brazilian Amazon, are

clearly needed to allow a more precise estimate of the differentiation, number of clades or distinct species. Studies of this kind are currently under way in our laboratory.

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