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

Vera Margarete Scarpassa and
Ronildo Baiatone Alencar

Additional information is available at the end of the chapter

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

INTECH
open science | open minds

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. brazilienesis* [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 Neartic 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**	
Leishmania chagasi[Br/A]	*Leishmania*	Visceral and cutaneous*	*Lutzomyia longipalpis*[P] *Lutzomyia cruzi*[P] *Lutzomyia evansi*[P]	*Lutzomyia* *Lutzomyia* Group *Verrucarum*	Canids (*Cerdocyon thou, Speothos venaticus, Canis familiaris*), felines (*Panthera onca, Felis concolor*), marsupials (*Didelphis marsupialis* and *D. albiventris*)
Leishmania enriettii[Br]	*Leishmania*	Not registered	*Lutzomyia monticola*[S] *Lutzomyia correalimai*[S]	Ungrouped Group *Rupicola*	Rodents (*Cavia porcellus*)
Leishmania mexicana	*Leishmania*	Cutaneous	*Lutzomyia olmeca olmeca*[P] *Lutzomyia diabolica*[S]	*Nyssomyia* *Lutzomyia*	Rodents (*Ototylomys phyllotis, Nyctomys sumichrasti, Heteromys desmarestianus, Sigmodon hispidus, Neotoma albigula*)

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

Parasites		Leishmaniasis in humans	Sandfly vectors		Reservoir host
Species	Subgenus		Species	Subgenus/Group	
Leishmania guyanensis[Br/A]	*Viannia*	Cutaneous	*Lutzomyia umbratilis*[P] *Lutzomyia anduzei*[P]	*Nyssomyia* *Nyssomyia*	Xenarthrans (*Choloepus didactylus, Tamandua tetradactyla*), rodents and marsupials
Leishmania panamensis	*Viannia*	Cutaneous	*Lutzomyia trapidoi*[P] *Lutzomyia ylephiletor*[P] *Lutzomyia gomezi*[P] *Lutzomyia panamensis*[P]	*Nyssomyia* *Nyssomyia* *Lutzomyia* *Psychodopygus*	Xenarthrans (*Choloepus hoffmanni, Bradypus infuscatus* and *B. griseus*); racoons (*Bassaricyon gabbi, Nasua nasua, Poto flavus*), primates (*Aotus trivirgatus, Saguinus geoffroyi*), rodents (*Heteromys* spp.)
Leishmania lainsoni[Br/A]	*Viannia*	Cutaneous	*Lutzomyia ubiquitalis*[P] *Lutzomyia velascoi*[S]	*Trichophoromyia* *Trichophoromyia*	Rodent (*Agouti paca*)
Leishmania naiffi[Br/A]	*Viannia*	Cutaneous	*Lutzomyia ayrozai*[P] *Lutzomyia panamensis*[P] *Lutzomyia squamiventris*[P]	*Psychodopygus* *Psychodopygus* *Psychodopygus*	Xenarthrans (*Dasypus novemcinctus*)
Leishmania shawi[Br/A]	*Viannia*	Cutaneous	*Lutzomyia whitmani*[P]	*Nyssomyia*	Primates (*Cebuspaella, Chiropotes satanas*), xenarthrans (*Choloepus didactylus,Bradypus tridactylus*) and racoon (*Nasua nasua*)
Leishmania colombiensis	*Viannia*	Cutaneous	*Lutzomyia hartmanni*[P] *Lutzomyia gomezi*[P] *Lutzomyia panamensis*[P]	*Helcocyrtomyia* *Lutzomyia* *Psychodopygus*	Xenarthrans(*Choloepus hoffmanni*)
Leishmania equatorensis	*Viannia*	Not registered	*Lutzomyia hartmanni*[P]	*Helcocyrtomyia*	Xenarthrans (*Choloepus hoffmanni*) and rodent (*Sciurus grantensis*)
Leishmania lindenbergi[Br/A]	*Viannia*	Cutaneous	*Lutzomyia antunesi*[S]	*Nyssomyia*	Unknown
Leishmania utingensis[Br/A]	*Viannia*	Not registered	*Lutzomyia tuberculata*[P]	*Viannamyia*	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.
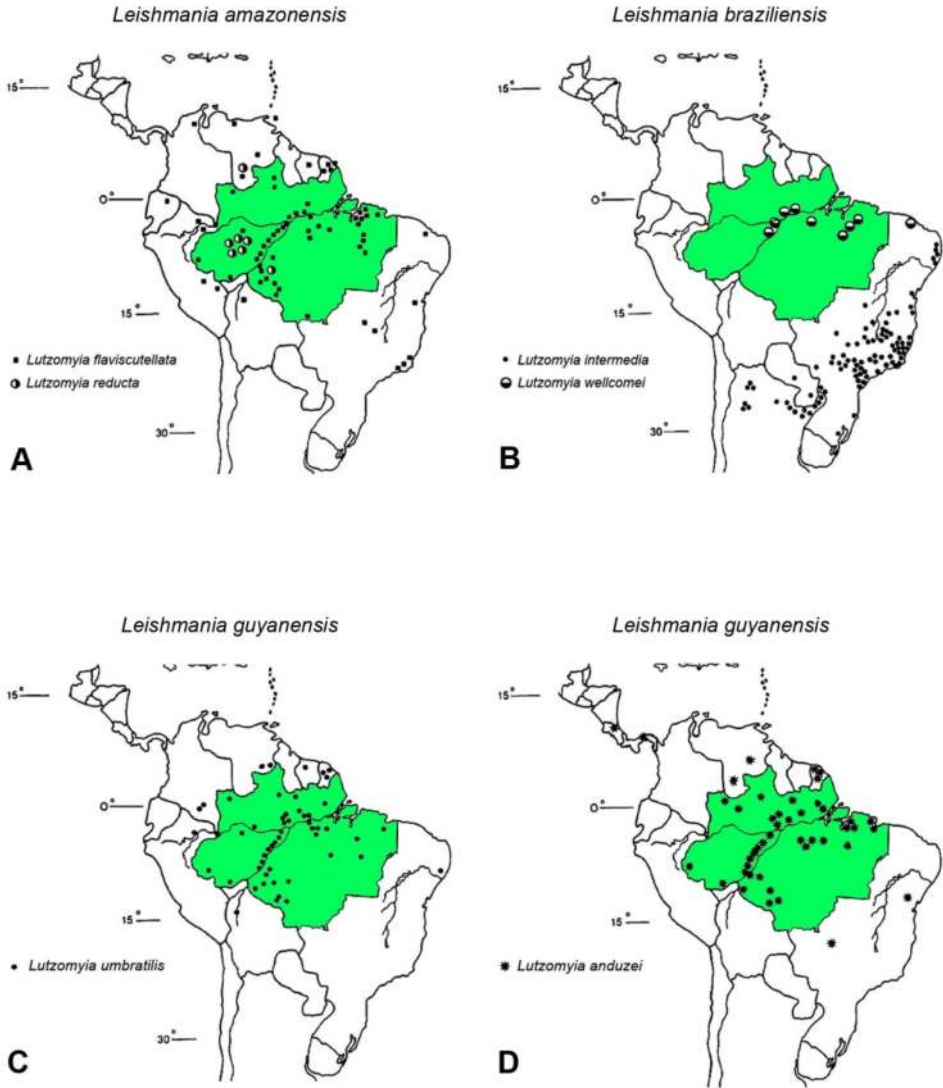
Figure 1. Distribution of the sandfly vectors of *Leishmania amazonensis* (A), *Leishmania braziliensis* (B) and *Leishmania guyanensis* (C and D). Highlight in green color corresponding to the Brazilian Amazon region. Map modified from Young and Duncan (1994) [2].
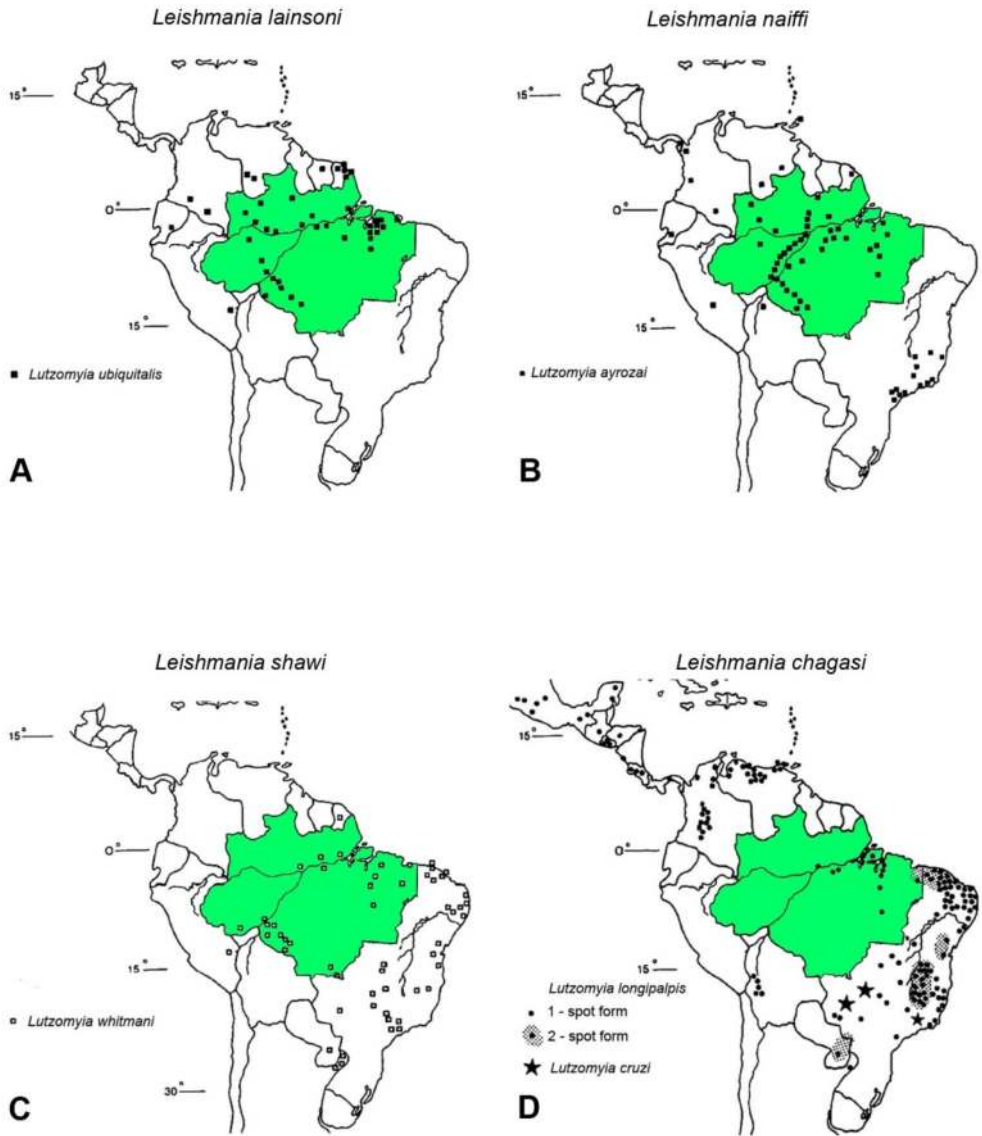
Figure 2. Distribution of the sandfly vectors of *Leishmania lainsoni* (A), *Leishmania naiffi* (B), *Leishmania shawi* (C) and *Leishmania chagasi* (D). Highlight in green color corresponding to the Brazilian Amazon region. Map modified from Young and Duncan (1994) [2].

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.

Lutzomyiaumbratilis, 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 ↔ 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.
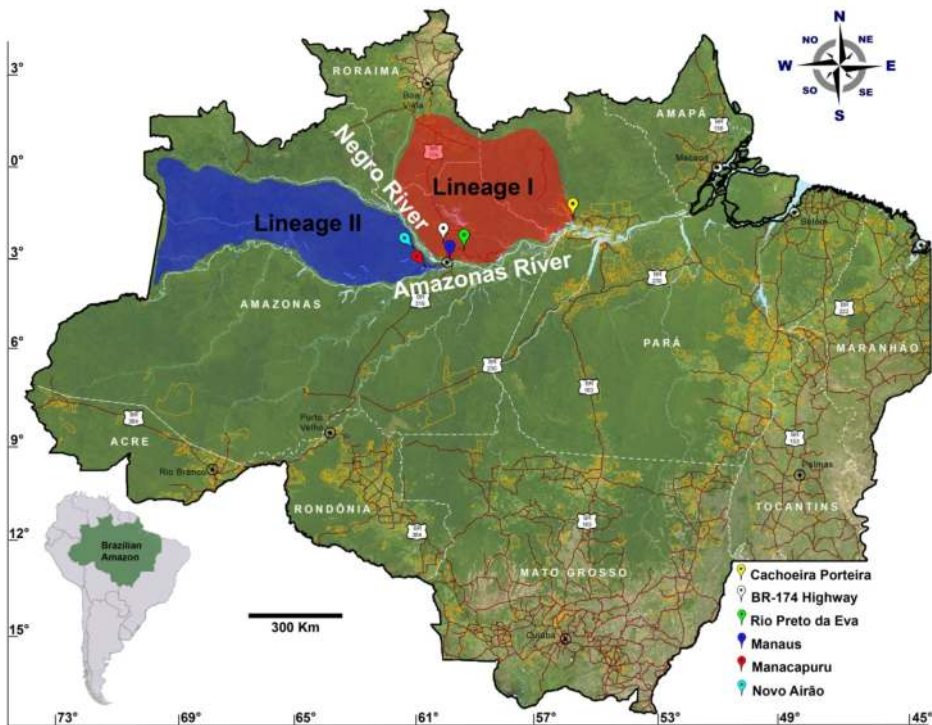

Figure 3. Collection sites of *Lutzomyia umbratilis* from the Brazilian Amazon. Geographic distribution inferred of lineage I (in red color); Geographic distribution inferred of lineage II (in blue color). Map modified from Scarpassa and Alencar (2013) [70].

Species	Localities, State	Co-ordinates	N
		Latitude; Longitude	
L. umbratilis	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.
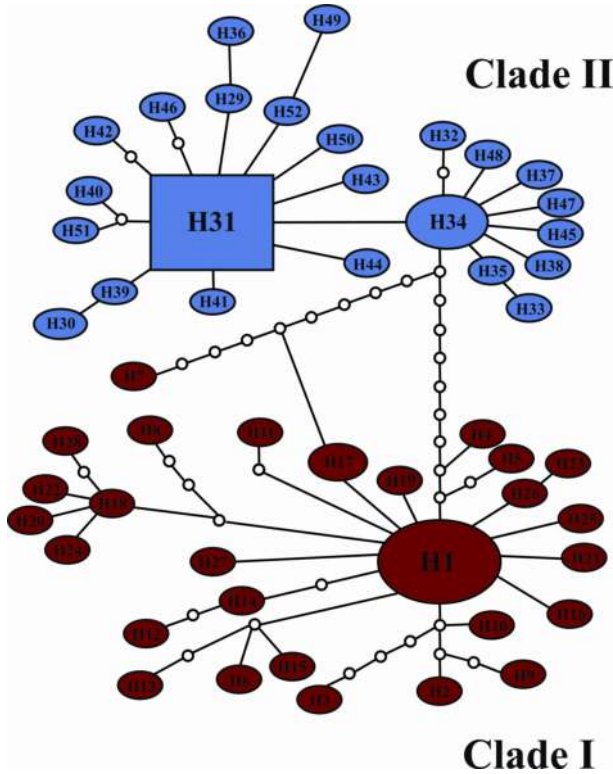

Figure 4. Parsimony haplotypes network of the 52 haplotypes observed in *Lutzomyia umbratilis*. H1 to H52, haplotypes. The haplotype circle sizes are proportional to number of individuals observed in each haplotype. Clade I is in red color. Clade II is in blue color. Empty smaller circles represent mutational events. **Source:** Scarpassa and Alencar (2012) [48].
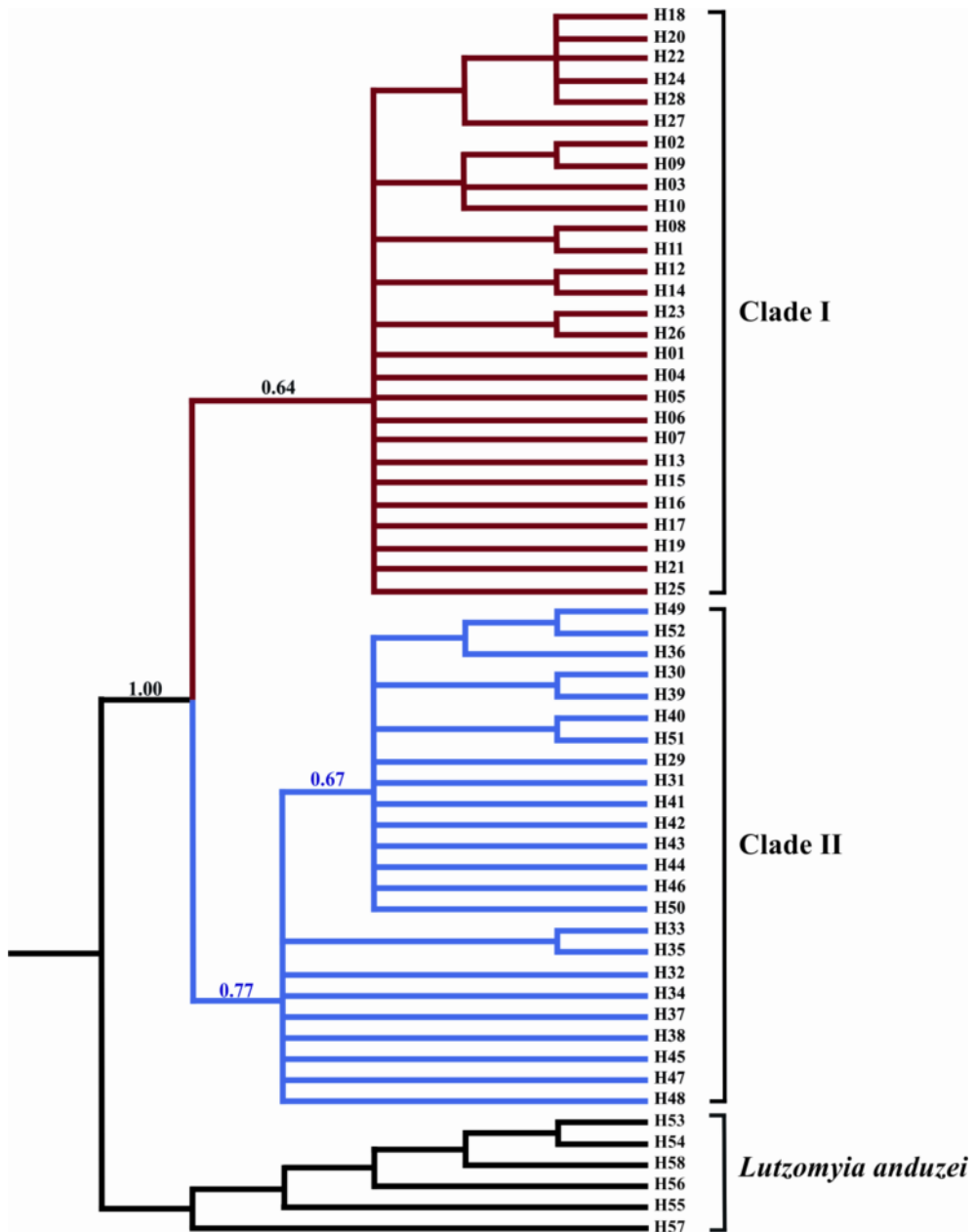
Figure 5. Bayesian Inference (BI) topology tree of the 52 haplotypes of *Lutzomyia umbratilis* inferred under the TIM1+I evolutionary model. Numbers above branch represent posterior probabilities obtained in the BI. *Lutzomyia anduzei* was used as outgroup. **Source:** Scarpassa and Alencar (2012) [48].

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 ± 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 interfluves 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.

Acknowledgements

Author details

Vera Margarete Scarpassa[1] and Ronildo Baiatone Alencar[2]

*Address all correspondence to: vera@inpa.gov.br

1 Laboratório de Genética de Populações e Evolução de Vetores de Malária e Dengue. Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Amazonas, Brazil

2 Universidade do Estado do Amazonas (UEA), Manaus, Amazonas, Brazil

References

[1] Lane RP. Sandflies. *In* Medical insects and arachnids. Edited by Lane RP, Crosskey RW. London: Chapman and Hall 1993; 78-119.

[2] Young DG, Duncan NA. Guide to the identification and geographic distribution of *Lutzomyia* sandflyies in Mexico, the West Indies, Central and South America (Diptera: Psychodidae). Memoirs of the American Entomological Institute (Gainesville) 1994; 54 1-881.

[3] WORLD HEALTH ORGANIZATION - WHO. Control of the leishmaniasis. Report of a meeting of the WHO Expert Committee on the Control of Leishmaniasis, 22-26 March, Geneva 2010; 5-88.

[4] Reithnger R. Leishmaniases' burden of disease: ways forward for getting from speculation to reality. PLoS Neglected Tropical Diseases 2008; 2 e285. doi:10.1371/ journal.pntd.0000285.

[5] WORLD HEALTH ORGANIZATION - WHO. Leishmaniasis. 2015. Available in: http://www.who.int/tdr/publications/publications/pdf/prl/pr17/leishmaniasis.pdf. Accessed at February 15, 2015.

[6] Gontijo B, Carvalho MLR. Leishmaniose tegumentar americana. Revista Brasileira de Medicina Tropical. 2003; 36(1) 71-80.

[7] Vale ECS, Furtado T. Leishmaniose tegumentar americana: revisão histórica da origem, expansão e etiologia. Anais Brasileiro de Dermatologia 2005; 80(4) 421-428.

[8] Guerra JAO, Ribeiro JAS, Coelho LIARC, Barbosa MGV, Paes MG. Epidemiologia da leishmaniose tegumentar na Comunidade São João, Manaus, Amazonas, Brasil. Caderno de Saúde Pública 2006; 22 2319-2327.

[9] MS/SVS - Ministério da Saúde/Secretaria de Vigilância em Saúde. Manual de Vigilância da Leishmaniose Tegumentar Americana 2010. 2ª ed. – Brasília: Editora do Ministério da Saúde, 180 pp. – (Série A. Normas e Manuais Técnicos).

[10] Marzochi MC. As Leishmanioses no Brasil: as leishmanioses tegumentares. Jornal Brasileiro de Medicina 1992; 63 82-104.

[11] Ministério da Saúde – MS/Fundação Nacional de Saúde – FUNASA. Manual de Controle da Leishmaniose Tegumentar Americana 2000. 5ª ed., Brasília, 62pp.

[12] Basano SA, Camargo LMA. Leishmaniose Tegumentar Americana: histórico, epidemiologia e perspectiva de controle. Revista Brasileira de Epidemiologia 2004; 7(3) 328-337.

[13] Lainson R. The American Leishmaniasis: some observations on their ecology and epidemiology. Transactions of the Royal Society of Tropical Medicine and Hygiene 1983; 77 569-596.

[14] Barrett TV, Senra MS. Leishmaniasis in Manaus, Brazil. Parasitology Today 1989; 5(8) 255-257.

[15] Sá SMA, Sá EV, Diniz AM. Saúde e Qualidade de Vida na Amazônia. *In*: Furtado, L.G. (Org.). *Amazônia, Desenvolvimento, Sociodiversidade e Qualidade de Vida*, UFPA, Belém 1997; 135-145.

[16] Guerra JAO, Talhari S, Paes MG, Garrido M, Talhari JM. Aspectos clínicos e diagnósticos da leishmaniose tegumentar americana em militares simultaneamente expostos à infecção na Amazônia. Revista da Sociedade Brasileira de Medicina Tropical 2003; 36 587-590.

[17] Lainson R, Shaw JJ. Leishmaniasis in Brazil. I. Observations on enzootic rodent leishmaniasis – incrimination of *Lutzomyia flaviscutellata* (Mangabeira) as the vector in the lower Amazonian basin. Transactions of the Royal Society of Tropical Medicine and Hygiene 1968; 62 385-395.

[18] Freitas RA, Naiff RD, Barrett TV. Species Diversity and Flagellate Infections in the Sand Fly Fauna near Porto Grande, State of Amapá, Brazil (Diptera: Psychodidae. Ki-

netosplatida: Trypanosomatidae). Memórias do Instituto Oswaldo Cruz 2002; 97(1) 53-59.

[19]   Lainson R, Shaw JJ. Leishmaniasis in Brazil: III. Cutaneous leishmaniasis in an opossum, *Marmo samurina* (Marsupialia, Didelphidae) from the lower Amazon region. Transactions of the Royal Society of Tropical Medicine and Hygiene1969; 63(6) 738-740.

[20]   Lainson, R, Shaw JJ. Leishmaniasis in Brazil: V. Studies on the epidemiology of cutaneous leishmaniasis in Mato Grosso State, and observations on two distinct strains of *Leishmania* isolated from man and forest animals. Transactions of the Royal Society of Tropical Medicine and Hygiene 1970; 64(5) 654-667.

[21]   Naiff RD, Talhari S, Barrett TV. Isolation of *Leishmania guyanensis* of the nasal mucosa. Memórias do Instituto Oswaldo Cruz 1988; 83(4) 529-530.

[22]   Center for Food Security and Public Health (CFSPH). Leishmaniasis (cutaneous and visceral). Iowa State of University, College of Veterinary Medicine, Iowa 2009; 1-10.

[23]   Lainson, R. Espécies neotropicais de *Leishmania*: uma breve revisão histórica sobre sua descoberta, ecologia e taxonomia. Revista Pan-Amazônica de Saúde 2010; 1 13-32.

[24]   Rotureau B. Ecology of the *Leishmania* species in the Guianan eco-region complex. American Journal of Tropical Medicine and Hygiene 2006; 74 81-96.

[25]   Silveira FT, Ishikawa EA, De Souza AAA, Lainson R. An outbreak of cutaneous leishmaniasis among soldiers in Belém, Pará State, Brazil, caused by *Leishmania (Viannia) lindenbergi* n. sp. A new leishmaniasis parasite of man in the Amazon region. Parasite 2002; 9 43-50.

[26]   Genaro O, Marques MJ, Reis AB, Silva AFF, Michalik MSM, Costa CA, Mayrinc W, Dias M. Leishmaniose Tegumentar Americana. *In*: Neves DP, Melo AL, Genaro O, Linardi PM. Parasitologia Humana 2003; 36-53. ATHENEU, São Paulo-SP.

[27]   Rangel EF, Lainson R. Flebotomíneos do Brasil, Fiocruz, Rio de Janeiro 2003; 367 pp.

[28]   Cabrera OL, Munstermann LE, Cardenas R, Gutierrez R, Ferro C. Definition of appropriate temperature and storage conditions in the detection of *Leishmania* DNA with PCR in phlebotomine flies. Biomédica (Bogotá) 2002; 22 296-302.

[29]   Albuquerque MIC. Fauna de flebotomíneos (Diptera: Psychodidae) e taxa de infecção natural por *Leishmania* spp. (Kinetoplastida: Trypanosomatidae) da Reserva Biológica de Campina – INPA da BR 174, Manaus, Amazonas, Brasil. Masters Thesis 2009; 74 pp.

[30]   Pereira Júnior AA. Fauna de flebotomíneos (Diptera: Psychodidae) e taxa de infecção natural por *Leishmania* Ross (Kinetoplastida: Trypanosomatidae) em ambientes de

várzea e de terra firme do município de Tefé, Amazonas, Brasil. Master Thesis 2014; 81 pp.

[31] Silveira FT, Souza AAA, Lainson R, Shaw JJ, Braga RR, Ishikawa EAY. Cutaneous leishmaniasis in the Amazon Region: natural infection of the sandfly *Lutzomyia ubiquitalis* (Psychodidae: Phlebotominidae) by *Leishmania lainsoni* in Pará State, Brazil. Memórias do Instituto Oswaldo Cruz 1991; 86(1) 127-30.

[32] Souza AAA, Silveira FT, Lainson R, Barata IR, Silva MGS, Lima JAN, Pinheiro MSB, Silva FMM, Vasconcelos, LS, Campos, MB, Ishikawa, EAY. Fauna flebotomínica da Serrados Carajás, Estado do Pará, Brasil, e sua possível implicação na transmissão da leishmania tegumentar americana. Revista Pan-Amazônica de Saúde 2010; 1(1) 45-51.

[33] Shimabukuro PHF, Galati, EAB. Checklist dos Phlebotominae (Diptera: Psychodidae) do Estado de São Paulo, Brasil, com comentários sobre sua distribuição greográfica. Biota Neotropical 2011; 11 1-20. http://www. biotaneotropica.org.br/v11n1a/pt/abstract?inventory +bn0361101a2011.

[34] Dantas-Torres F. The role of dogs as reservoirs of *Leishmania* parasites, with emphasis on *Leishmania* (*Leishmania*) *infantum* and *Leishmania* (*Viannia*) *braziliensis*. Veterinary Parasitology 2007; 149 139-146.

[35] Kakarsulemankhel JK. Leishmaniasis in Pak-Afghan region: a review. International Journal of Agriculture and Biology 2011; 13 611-620.

[36] Aguiar GM, Medeiros WM. Distribuição regional de habitats das espécies de flebotomíneos do Brasil. *In*: Rangel EF, Lainson R (eds). Flebotomíneos do Brasil 2003; 207-256.

[37] Young DG, Arias JR. Flebotomíneos vectores de leishmaniasis en las Americas. Organização Pan-Americana da Saúde. Caderno Técnico 1992; (3).

[38] Grimaldi G Jr, Tesh RB, McMahon-Pratt D. A review of the geographic distribution and epidemiology of leishmaniasis in the New World. American Journal of Tropical Medicine and Hygiene 1989; 41 687-725.

[39] Lainson R, Shaw JJ, Silveira FT, Sousa AAA, Braga RR, Ishikawa EAY. The dermal leishmaniasis of Brazil, with special reference to the eco-epidemiology of the disease in Amazonian. Memórias do Instituto Oswaldo Cruz 1994; 89 435-443.

[40] Arias JR, Freitas RA. Sobre os vetores de leishmaniose cutânea na Amazônia central do Brasil. 2: incidência de flagelados em flebótomos selváticos. Acta Amazônica 1978; 8 387-396.

[41] Arias JR, Freitas RA. On the vectors of cutaneous leishmaniasis in the Central Amazon of Brazil. 4. Sand fly emergence from a "terra firme" forest floor. Acta Amazônica 1982; 12 609-611.

[42] Arias JR, Naiff RD. The principal reservoir host of cutaneous leishmaniasis in the urban area of Manaus, Central Amazon of Brazil. Memórias do Instituto Oswaldo Cruz 1981; 76 279-286.

[43] Arias JR, Miles MA, Naiffi RD, Póvoa MM, Freitas RA, Biancardi CB, Castellón EG. Flagellates infections of Brazilian sandflies (Diptera: Psychodidae): Isolation in vitro and biochemical identification of *Endotrypanum* and *Leishmania*. American Journal of Tropical Medicine and Hygiene 1985; 34 1098-1108.

[44] Zapata S, Mejía L, Le Pont F, León R, Pesson B, Ravel C, Bichaud L, Charrel R, Cruaud C, Trueba G, Depaquit J. A study of a population of *Nyssomyia trapidoi* (Diptera: Psychodidae) caught on the Pacific coast of Equator. Parasites & Vectors 2012; 5 144.

[45] Pech-May A, Marina CF, Vázquez-Domínguez E, Berzunza-Cruz M, Rebollar-Télles EA, Narváez-Zapata JA, Moo-LIanes D, Ibánez-Bernal S, Ramsey JM, Becker I. Genetic structure and divergence in populations of *Lutzomyia cruciata*, a phlebotomine sand fly (Diptera: Psychodidae) vector of *Leishmania mexicana* in southeastern Mexico. Infection, Genetics and Evolution 2013; 16 254-262.

[46] Yamamoto K, Cáceres AG, Gomez EA, Mimori T, Iwata H, Korenaga M, Sakurai T, Katakura K, Hashiguchi Y, Kato H. Genetic diversity of the mitochondrial cytochrome b gene in *Lutzomyia* spp., with special reference to *Lutzomyia peruensis*, a main vector of *Leishmania* (*Viannia*) *peruviana* in the Peruvian Andes. Acta Tropica 2013; 126 156-163.

[47] Valderrama A, Tavares MG, Andrade-Filho JD. Phylogeography of the *Lutzomyia gomezi* (Diptera: Phlebotominae) on the Panama Isthmus. Parasites & Vectors 7: 9.

[48] Scarpassa VM, Alencar RB. *Lutzomyia umbratilis*, the main vector of *Leishmaniaguyanensis*, represents a novel species complex? *PLoS ONE* 2012; 7(5) e37341.

[49] Scarpassa VM, Figueiredo AS, Alencar RB. Genetic diversity and population structure in the *Leishmania guyanensis* vector *Lutzomyia anduzei* (Diptera, Psychodidae) from the Brazilian Amazon. Infection, Genetics and Evolution 2015; 31 312-320.

[50] Ready PD, De Souza AA, Rebelo JC, Day J, Silveira FT, Campbell-Lendrum D, Davies CR, Costa JML. Phylogenetic species and domesticity of *Lutzomyia whitmani* at the southeast boundary of Amazonian Brazil. Transaction of the Royal Society Tropical Medicine and Hygiene 1998; 92 159–160.

[51] Mukhopadhyay J, Ghosh K, Ferro C, Munstermann LE. Distribution of phlebotomines and by genotypes (*Lutzomyia shannoni*, Diptera: Psychodidae) across a highly heterogeneous landscape. Journal of Medical Entomology 2001; 38 260–267.

[52] Uribe-Soto SI, Lehmann T, Rowton ED, Vélez IDB, Porter CH. Speciation and population structure in the morphospecies *Lutzomyia longipalpis* (Lutz &Neiva) as derived

from the mitochondrial *ND4* gene. Molecular Phylogenetics and Evolution 2001; 18(1) 84-93.

[53] Azevedo ACR, Lainson R, Souza AA, Fé NF, Feliciangeli DM, Meneses CRV, Rangel EF. Comparative studies of populaitons of *Lutzomyia umbratilis* (Diptera: Psychodidae) in Brazil and Venezuela. Journal of Medical Entomology 2002; 39 587-600.

[54] Forattini, OP. Notas sobre *Phlebotomus* do Território do Amapá, Brasil (Diptera, Psychodidae). Studia Entomologica1960; 11(1-2) 467-480.

[55] Balbino VQ, Marcondes CB, Alexander B, Luna LKS, Lucena MMM, Mendes AC, Andrade PP. First report of *Lutzomyia* (*Nyssomyia*) *umbratilis* Ward & Frahia, 1977 outside of Amazonian region, in Recife, State of Pernambuco, Brazil (Diptera: Psychodidae: Phlebotominae). Memórias do Instituto Oswaldo Cruz 2001; 96 315-317.

[56] Dye C, Davies CR, Lainson R. Communication among phlebotomine sandflies: a field study of domesticated *Lutzomyia longipalpis* populations in Amazonian Brazil. Animal Behaviour 1991; 42 183–192.

[57] Morrison AC, Ferro C, Morales A, Tesh RB, Wilson ML. Dispersal of the sandfly *Lutzomyia long*ipalpis (Diptera: Psychodidae) at an endemic focus of visceral leishmaniasis in Colombia. Journal of Medical Entomology 1993; 30 427–435.

[58] Lainson R, Ward RD, Shaw JJ. Cutaneous Leishmaniasis in north Brazil: *Lutzomyia anduzei* as a major vector. Transaction Royal Society Tropical Medicine and Hygiene 1976; 70(2) 171-172.

[59] Lainson R, Ready PD, Shaw JJ. *Leishmania* in phlebotomid sandflies. VII. On the taxonomic status of *Leishmania peruviana*, causative agent of Peruvian 'uta', as indicated by its development in the sandfly, *Lutzomyia longipalpis*. Proceedings of the Royal Society London Biological Science 1979; 206(1164) 307-318.

[60] Lainson R, Shaw JJ, Póvoa MM. The importance of edentates (sloths and anteaters) as primary reservoirs of *Leishmania braziliensis guyanensis*, causative agent of "pian-bois" in North Brazil. Transaction of the Royal Society Tropical Medicine and Hygiene 1981; 75(4) 611-612.

[61] Arias JR, Freitas RA. On the vectors of cutaneous leishmaniasis in the Central Amazon of Brazil. Acta Amazônica 1977; 7 507-527.

[62] Nery LCR, Lorosa ES, Franco AMR. Feeding preference of the sand flies *Lutzomyia umbratilis* and *L. spathotrichia* (Diptera: Psychodidae: Phlebotominae) in an urban forest patch in the city of Manaus, Amazonas, Brazil. Memórias do Instituto Oswaldo Cruz 2004; 99 571-574.

[63] Pinheiro FG, Luz SLB, Franco AMR. Infecção natural por tripanosomatídeos (Kinetoplastida: Trypanosomatidae) em *Lutzomyia umbratilis* (Diptera: Psychodidae) em áreas de Leishmaniose Tegumentar Americana no Amazonas, Brasil. Acta Amazônica 2008; 38 165-172.

[64] Azevedo ACR, Costa SM, Pinto MCG, Souza JL, Cruz HC, Vidal J, Rangel EF. Studies on the sandfly fauna (Diptera: Psychodidae: Phlebotominae) from transmission areas of American Cutaneous Leishmaniasis in state of Acre, Brazil. Memórias do Instituto Oswaldo Cruz 2008; 103 760-767.

[65] Gil LHS, Araújo MS, Villalobos JM, Camargo LMA, Ozaki LS, Fontes CJF, Ribolla PEM, Katsuragawa TH, Cruz RM, Silva AA, Silva LHP. Species structure of sand fly (Diptera: Psychodidae) fauna in the Brazilian western Amazon. Memórias do Instituto Oswaldo Cruz 2009; 104 955-959.

[66] Justiniano SCB, Chagas AC, Pessoa FAC, Queiroz RG. Comparative biology of two populations of *Lutzomyia umbratilis* (Diptera: Psychodidae) of central Amazonia, Brazil, under laboratory conditions. Brazilian Journal of Biology 2004; 64 227-235.

[67] Justiniano SCB. Biologia comparada de populações de *Lutzomyia umbratilis* (Diptera: Psychodidae) da Amazônia Central Brasileira. Doctoral Thesis. Instituto Nacional de Pesquisas da Amazônia 2004; 151pp.

[68] Valenta DT, Anez N, Tang Y, Killick-Kendrick R. The genital atrium as a good taxonomic character to distinguish between species of phlebotomine sandflies (Diptera: Psychodidae) from Venezuela. Annual of Tropical Medicine and Parasitology 1999; 93 389–399.

[69] Ward RD, Fraiha H. *Lutzomyia umbratilis*, a new species of sandfly from Brazil (Diptera: Psychodidae). Journal of Medical Entomology 1977; 14 313–317.

[70] Scarpassa VM, Alencar RB. Molecular taxonomy of the two *Leishmania* vectors *Lutzomyia umbratilis* and *Lutzomyia anduzei* (Diptera: Psychodidae) from the Brazilian Amazon. Parasites & Vectors 2013; 6 258. http://www.parasitesandvectors.com/content/6/1/258

[71] Arias JR, Freitas RA. On the vectors of cutaneous leishmaniasis in the Central Amazon of Brazil. Acta Amazônica 1977; 7 507-527.

[72] Silva MNT, Castellón EB. Sandflies (Diptera: Psychodidae) naturally infected by *Trypanosoma* (Kinetoplastida: Trypanosomatidae) in urban forest fragments in Manaus city, Amazonas, Brazil. Revista Colombiana de Ciencia Animal 2012; 4(1) 121-129.

[73] Ready PD, Lainson R, Shaw JJ, Ward R. The ecology of *Lutzomyia umbratilis* Ward & Fraiha (Diptera: Psychodidae) the major vector to man of *Leishmania braziliensis guyanensis* in north-eastern Amazonian Brazil. Bulletin of Entomology Resources 1986; 76 21-40.

[74] Clement M, Posada D, Crandall KA. TCS: a computer program to estimate gene genealogies. Molecular Ecology 2000; 9 1657-1660.

[75] Figueiredo J, Hoorn C, Van der Ven P, Soares E. Late Miocene onset of the Amazon River and the Amazon deep-sea fan: Evidence from the Foz do Amazonas Basin. Geology 2009; 37 619–622.

[76] Alencar RB. Emergência de flebotomíneos (Diptera: Psychodidae) em chão de floresta de terra firme na Amazônia Central do Brasil: Uso de um modelo modificado de armadilha de emergência. Acta Amazônica 2008; 37(2) 287-292.

[77] Alencar RB, Queiroz RG, Barrett TV. Breeding sites of phlebotomine sand flies (Diptera: Psychodidae) and efficiency of extraction techniques for immature stages in terra-firme forest in Amazonas State, Brazil. Acta Tropica 2011; 118(3) 204-208.