

# Increasing putative vector importance of *Trichophoromyia* phlebotomines (Diptera: Psychodidae)

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Despite some phlebotomines being well recognised as vectors of leishmaniasis agents, vector importance of those belonging to the genus *Trichophoromyia* has not been extensively studied. The present study provides evidence regarding the putative vector role played by some species of *Trichophoromyia* on leishmanine enzootics, based on literature reports and findings obtained from field experiments conducted in the ecotopes of Pará State, Brazil. The species *Th. ubiquitalis*, *Th. velascoi*, *Th. auraensis*, *Th. ininii* and *Th. brachippyga* possess minimal criteria to be included in the list of suspected leishmanine vectors. However, knowledge on man-biting behavior, substantiation of vector competence and determination of epidemiological implications are limited for all of the above mentioned species. Published studies together with present data draw attention to prioritize these phlebotomine species in entomological surveillance programs and studies on experimental susceptibility to *Leishmania* spp. infection.

Key words: leishmaniasis - psychodidae - attention - vector

Phlebotomines (Diptera: Psychodidae: Phlebotominae) are a group of insects of medical importance. Their blood-feeding females are proven vectors of protozoan leishmanine species, mainly from the *Leishmania* genus (Euglenozoa: Trypanosomatidae: Leishmaniinae), the causative agents of a group of neglected zoonotic diseases, known as the leishmaniasis.<sup>(1,2)</sup>

In the New World, life cycle of leishmanine parasites is maintained by a complex mosaic of vector/reservoir interactions, resulting in a variety of ecological arrangements with worrisome consequences in clinical manifestations, namely - American visceral leishmaniasis (AVL) and American cutaneous leishmaniasis (ACL).<sup>(3,4)</sup> In the Americas, a total of 541 species - 524 current and 17 fossil species - have been described, and they are grouped into supra-specific categories (genera, subgenera, groups, and series of species).<sup>(5,6)</sup> Among these, almost more than 20 species are considered vectors of leishmaniasis in the New World. This relatively small number of vectors is the function of a number of ecological, physiological and biochemical factors inherent to both insects and parasites. These factors influence the close contact between insect/parasite binomial, survival of the parasites in the digestive tract of the insect, and their multiplication and transformations that result in the development of infective forms, influencing their transmission to the vertebrate host.<sup>(3)</sup>

*Trichophoromyia* was originally established as a subgenus of *Lutzomyia* França, 1924 by Barretto<sup>(7)</sup> and later upgraded to genus level.<sup>(8)</sup> Its type-species is *Th. ubiquitalis* (Mangabeira, 1942) (formerly *Phlebotomus ubiquitalis*). To date, 44 species of *Trichophoromyia* have been reported in the New World.<sup>(6)</sup> Females of several *Trichophoromyia* species are markedly similar, and therefore, identification is usually based on male morphology. The geographical overlapping of the species usually complicates the diagnosis.<sup>(5,9)</sup>

For many years, an apparently null medical importance of *Trichophoromyia* may have contributed to this phlebotomine genus to be understudied. *Trichophoromyia* was considered to have limited geographical distribution, predominantly in the Amazon basin, with unknown human biting behavior, and circumstantial history of natural infection by flagellates; obtained only when sharing same ecotope of *Leishmania* vector/reservoir systems, thus, not likely playing an important role as vectors of leishmaniasis agents to humans, limiting them to the zoological interest.<sup>(9)</sup> However, since the 1990s, some species have been found to be closely associated with ACL foci and have started garnering attention. Currently, five species of *Trichophoromyia* have been identified naturally infected using microscopic analysis and/or have been found to be *Leishmania* DNA positive based on the results of polymerase chain reaction (PCR) analyses, which is the first essential criterion to be regarded as suspected vectors.<sup>(1)</sup> Data available on these species are summarised in Table and further discussed in the context of ACL epidemiology. In addition, the present study also includes our recent findings on entomological studies conducted in locations from the Brazilian State of Pará, as the mineral province of Carajás (municipality of Canaã dos Carajás), the Belo Monte hydroelectric system (municipality of Vitória do Xingu), the Bosque Rodrigues Alves - Jardim Botânico da Amazônia and the Mosqueiro Island (municipality of Belém).

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### **Trichophoromyia species with putative vector importance**

*Trichophoromyia ubiquitalis* - In 1983, during entomological investigations of the river Parapanema region, in the foothills of the Serra dos Carajás (Pará State, Brazil), Lainson, Shaw and Ready found a specimen of *Th. ubiquitalis* naturally infected, and the *Leishmania* isolate was designed, at that time, as an unnamed parasite of the subgenus *Viannia*.<sup>(10)</sup> Years later, after re-examination, the isolate was recognized as *L. (Viannia) lainsoni* Silveira, Shaw, Braga & Ishikawa, 1987.

Silveira et al.<sup>(11)</sup> performed entomological studies in the forested outskirts of Belém in Pará State, Brazil (recognised as the type locality of *L. (V.) lainsoni*, and the presumed infection site of ACL patients) and in the ecologically related forest of Utinga in Belém metropolitan region. Out of the 375 *Th. ubiquitalis* specimens captured, they found nine females to be highly infected with flagellates, and eight of these parasites were identified to be *L. (V.) lainsoni*. Under laboratory conditions, 71/83 specimens successfully bit humans, 48 h after capture. However, under field conditions, they did not find *Th. ubiquitalis* to be anthropophilic. Lainson et al.<sup>(12)</sup> provided further observations on the laboratory and field behaviors of *Th. ubiquitalis* captured from the mineral province of Carajás, Pará State, Brazil. In their study, only one specimen attempted to bite a professional during field capture. In order to establish a colony, 64/80 flies previously confined in a nylon cage were fed on a volunteer at a time varying from 24-48 h after capture; strengthening the hypothesis that *Th. ubiquitalis* can be triggered to actively attack humans under unusual conditions. In an agricultural settlement of Mosqueiro Island in the outskirts of Belém, we recently investigated an ACL presumed infection site where *L. (V.) lainsoni* has been isolated from the cutaneous lesion of a dweller. Light traps installed in the intradomiciliary environment captured several *Th. ubiquitalis*, providing evidence for the potential indoor behavior of this species.

Other ecotopes distinct from those of the State of Pará, have also been recorded with *L. (V.) lainsoni/Th. ubiquitalis* association. During an entomological survey in Tefé municipality of Amazonas State, Brazil, Pereira Junior et al.<sup>(13)</sup> found 7/60 pooled *Th. ubiquitalis*, captured in dry land forest environment, to be DNA positive for the 70 kilodalton heat shock protein (*hsp 70*) target, with sequences of *L. (V.) lainsoni*. In contrast, Silva et al.<sup>(14)</sup> found *Th. ubiquitalis* DNA positive for *L. (L.) amazonensis* and *L. (Viannia)* sp. Quiroga et al.<sup>(15)</sup> found *L. (V.) lainsoni* DNA in two peridomiciliary pooled samples of nine specimens of *Th. ubiquitalis* from Macas, Morona Santiago province, Ecuador, during the rainy season. In Porto Velho, Rondônia State, Brazil, two pools of *Th. ubiquitalis* tested positive for *Leishmania* sp. DNA, suggesting the putative vector role of this species in that municipality.<sup>(16)</sup>

Currently, there are strong evidences on the putative vector role of *Th. ubiquitalis* with respect to *L. (V.) lainsoni* transmission from distinct ecoregions of Brazil and Ecuador. In addition, spatiotemporal congruence of this phlebotomine species and ACL cases due to this para-

site in other regions/countries, even with no findings of natural infection during entomological surveys, consubstantiate its vector candidacy. Intriguing findings about the southward expansion of *L. (V.) lainsoni* were recently reported in Paraguay;<sup>(17)</sup> however, raise concerns on the existence of alternative vectors for this parasite, distinct from *Th. ubiquitalis* or other *Trichophoromyia* species. Furthermore, it is noteworthy to mention that *Pintomyia nuneztovari* (Ortiz, 1954) has already been found with *L. (V.) lainsoni* DNA in an ACL focus from the sub Andean region of Bolivia.<sup>(18)</sup>

*Trichophoromyia velascoi* (Le Pont & Desjeux, 1992) - During investigation on the first ACL case caused by *L. (V.) lainsoni* in Bolivia, several females of *Th. velascoi* were found to be infected naturally at the end of the rainy season (May) in Caranavi province; however, the parasites were not isolated at that time.<sup>(19)</sup> Nonetheless, based on the spatiotemporal congruence of human/phlebotomine infections, the authors highlighted the need of further studies on putative vector importance of *Th. velascoi* in the context of ACL transmission in that region.

*Trichophoromyia auraensis* (Mangabeira, 1942) and/or *Th. ruifreitasi* Oliveira, Teles, Medeiros, Camargo & Pessoa, 2015 - Using a fluorescence resonance energy transfer-based real-time polymerase chain reaction, Valdivia et al.<sup>(20)</sup> found four *Trichophoromyia auraensis* pools with DNA of *L. (V.) lainsoni* and *Leishmania (V.) braziliensis* in Madre de Dios, Peru, with a minimum detection rate estimated to be 0.6%. Moreover, based on the studies conducted in Puno, Peru, anthropophilic behavior has been observed in *Th. auraensis*,<sup>(21)</sup> although early hypothesis on misidentification of the particular *Trichophoromyia* taxon have been raised for this Peruvian department and reported elsewhere.<sup>(22)</sup>

Ogawa et al.<sup>(23)</sup> detected *Leishmania* sp. DNA within three pools of *Th. auraensis* captured from caves in Rondônia State, Brazil, using kinetoplast DNA (kDNA) PCR. However, none of the samples were positive in the *hsp70* region for sequencing. Furthermore, Teles et al.<sup>(24)</sup> published an epidemiological study conducted on approximately seven thousand phlebotomines collected from Assis Brasil in Acre State, Brazil. The female phlebotomines were grouped by pools and examined for the DNA tracks of *Leishmania*. Among the positive samples, specimens of *Th. auraensis/Th. ruifreitasi* (ambiguously identified females) contained the DNA tracks of *Leishmania* from the *L. (V.) braziliensis* complex at high concentrations, with a minimal detection rate of 2.05%. It was regarded as the first report on the infection of *Th. auraensis/Th. ruifreitasi* by *L. (V.) guyanensis* and *L. (V.) braziliensis* in Brazil. Interestingly, Araújo-Pereira et al.<sup>(25)</sup> found 9/96 individually processed and unambiguously identified *Th. auraensis* positive to *Leishmania* kDNA, with three of them revealed as *L. (V.) braziliensis*. Resadore et al.<sup>(26)</sup> found *Th. auraensis* DNA positive for *Leishmania* sp. (minimal detection rate of 8.33%) in Rondônia State, Brazil. Consistent with the complementary roles described in these previous studies, there is no doubt to regard *Th. auraensis* as a putative vector.<sup>(27)</sup>

TABLE  
*Trichophoromyia* phlebotomine species associated with *Leishmania* spp.

<i>Trichophoromyia</i> species	Diagnosis method	<i>Leishmania</i> species	Reference
<i>Th. ubiquitatis</i>	Microscopically + MLEE	<i>L. (V.) lainsoni</i>	Silveira et al. <sup>(11)</sup>
	Microscopically + MLEE	<i>L. (V.) lainsoni</i>	Lainson et al. <sup>(12)</sup>
	Microscopically + RNA POLIILS RFLP-PCR	<i>L. (V.) lainsoni</i>	Present work
	ITSI PCR + sequencing	<i>L. (Vianna)</i> sp.; <i>L. (L.) amazonensis</i>	Silva et al. <sup>(14)</sup>
	kDNA PCR + <i>hsp70</i> PCR RFLP/sequencing	<i>L. (V.) lainsoni</i>	Pereira Junior et al. <sup>(13)</sup>
	ITSI PCR + sequencing kDNA PCR	<i>L. (V.) lainsoni</i> <i>Leishmania</i> sp.	Quiroga et al. <sup>(15)</sup> Resadore et al. <sup>(16)</sup>
<i>Th. velascoi</i>	Microscopically	n.i.	Martinez et al. <sup>(19)</sup>
<i>Th. auraensis</i>	kDNA PCR + FRET-based real time-PCR	<i>L. (V.) lainsoni</i>	Valdivia et al. <sup>(20)</sup>
	kDNA PCR	<i>Leishmania</i> sp.	Ogawa et al. <sup>(23)</sup>
	kDNA-PCR + <i>hsp70</i> sequencing	<i>L. (V.) braziliensis</i>	Araújo-Pereira et al. <sup>(25)</sup>
	kDNA PCR	<i>Leishmania</i> sp.	Resadore et al. <sup>(26)</sup>
<i>Th. auraensis/Th. ruifreitasi</i>	PCR-based	<i>L. (V.) braziliensis</i> ; <i>L. (V.) guyanensis</i>	Teles et al. <sup>(27)</sup>
<i>Th. ininii</i>	SSU rRNA qPCR + RFLP PCR	<i>Leishmania</i> sp.	Kent et al. <sup>(28)</sup>
	microscopically	<i>Leishmania</i> sp.	Fouque et al. <sup>(29)</sup>
	kDNA-PCR + <i>hsp70</i> sequencing	<i>L. (V.) braziliensis</i>	Vasconcelos dos Santos et al. <sup>(31)</sup>
<i>Th. brachipyga</i>	Microscopically + RNA POLIILS RFLP-PCR	<i>L. (V.) lainsoni</i>	Sánchez Uzcátegui et al. <sup>(33)</sup>
	Microscopically + RNA POLIILS RFLP-PCR	Microscopically + RNA POLIILS RFLP-PCR	Present work
<i>Th. brachipyga/Th. adelsonsouzai</i>	Microscopically	n.i.	Present work

FRET-based: fluorescence resonance energy transfer-based; *hsp 70*: 70 kilodalton heat shock protein; ITSI: internal transcribed spacer I; kDNA: kinetoplast DNA; MLEE: multilocus enzyme electrophoresis; n.i. not identified; PCR: polymerase chain reaction; RFLP: restriction fragment length polymorphism; RNA POLIILS: RNA polymerase II largest subunit; SSU rRNA qPCR: small-subunit ribosomal RNA.

In an ACL endemic area of Rio Branco, Acre State, where *Th. auraensis* was the numerically dominant *Trichophoromyia* species, engorged females of this genus were found positive for *Gallus gallus* blood through *cytochrome b* PCR analysis. Although birds do not play a direct role in the ACL epidemiology, their presence may influence on the population dynamics of phlebotomines with increasing population density in the peridomestic environment, which could contribute to increase the risk of the transmission of *Leishmania* to humans.<sup>(28)</sup>

*Trichophoromyia ininii* Floch & Abonnenc, 1943 - During a study conducted to identify changing patterns in ACL transmission in French Guiana, Fouque et al.<sup>(29)</sup> found *Th. ininii* as the most common species among the captured phlebotomines, where one specimen from Montsinery and one from Cacao were positive for flagellates under microscopic observation. Molecular typing indicated *Leishmania* genus, but the species was not identified. In order to broaden the knowledge on vectors involved in ACL transmission in Suriname, Kent et al.<sup>(30)</sup> isolated a pooled sample of *Th. ininii* from Sabajo hills as *Leishmania* DNA positive. They were unable to identify the parasites due to insufficient amount of DNA. Studying an environmentally impacted area of Oiapoque outskirts, Amapá State, Brazil, we recently detected, within the phlebotomine composition of the forest border of an anthropized environment, two pooled samples of ten *Th. ininii* specimens were found to be DNA positive for *Leishmania* (*Viannia*) sp.; one sample allowed characterisation at species level, with sequence compatible with *L. (V.) braziliensis*.<sup>(31)</sup>

*Trichophoromyia brachipyga* Mangabeira, 1942 and/or *Th. adelsonsouzai* Vasconcelos dos Santos, Silva, Barata, Andrade & Galati, 2013 - In the Belo Monte hydroelectric system affected area in Pará State, Brazil, a program monitoring ACL transmission has been implemented since 2012, providing substantial knowledge on ecology and taxonomy of phlebotomines. In addition to the findings, it rendered description of *Th. adelsonsouzai* based on males and females captured in Vitória do Xingu municipality.<sup>(32)</sup> Interestingly, two females of *Trichophoromyia* were found infected with suprapylarian *Leishmania*-like promastigotes. Although *Th. adelsonsouzai* has been the dominant species in the infection site, representing more than 60% of phlebotomines captured on light traps, the coexistence of few males of *Th. brachipyga* has also been noted. Due the ambiguous taxonomic determination of closely related females of *Th. adelsonsouzai/Th. brachipyga*, and failure of *Leishmania* species characterisation, this putative ACL life cycle remains unclear.

Sánchez Uzcátegui et al.<sup>(33)</sup> investigated phlebotomines from an urban park of Belém, the Bosque Rodrigues Alves - Jardim Botânico da Amazônia, where one specimen of *Th. brachipyga* was found to be moderately/weakly (++) parasitised with a peripylarian flagellate. After successful *in vitro* isolation, the restriction fragment length polymorphism - PCR (RFLP - PCR) characterisation showed a profile identical to that of *L. (V.) lainsoni* reference strain. Although in that site, *Th. ubiquitous* was

also present and expected to be infected by *L. (V.) lainsoni*, been recorded as the second most frequent species (14%), followed by *Th. brachipyga* (10.4%), no infection was diagnosed in this phlebotomine through microscopic examination. This was the first report of *Th. brachipyga* naturally infected by *L. (V.) lainsoni*.

We continued conducting studies in the mineral province of Carajás in Pará State, Brazil, and captured phlebotomines using light traps in a forest environment of Canaã dos Carajás municipality. In the field campaign of April 2019, we identified four *Th. ubiquitous* and two *Th. brachipyga* naturally infected with moderate to luxuriant-growing promastigotes. The gut contents were inoculated into culture media and hind-feet of golden hamsters (ethical approval under protocol CEUA/IEC/SVS/MS n.10/2014). All strains were successfully isolated from one of these two described methods and thereafter characterised by PCR-RFLP technique. Digestion profiles of *Leishmania* strains from both phlebotomine species were found to be similar and compatible with *L. (V.) lainsoni*. By including observations made by Lainson et al.<sup>(12)</sup> for Carajás, the vector role of *Th. ubiquitous* with respect to *L. (V.) lainsoni* is thus, well established. These novel findings for *Th. brachipyga* corroborate with the results reported by Sánchez Uzcátegui et al.<sup>(33)</sup> It is speculated that this *Trichophoromyia* species may act as an alternative vector, probably sharing its role with *Th. ubiquitous* in some ecotopes.

*Concluding remarks* - The present study assembled data suggesting the possibility of occurrence of leishmanine emerging transmission patterns, where some species of *Trichophoromyia* may act as putative vectors of ACL agents, and probably also share roles with each other, while co-habiting the same ecotope. In nature, *Leishmania* species may have enzootic and zoonotic cycles as well, that may geographically overlap, maintained by different, but closely related phlebotomine species. As stated by Ready,<sup>(34)</sup> putative vectors in previously unexplored *foci* can be targeted simply by their close taxonomic relationship with known vectors, using presumed evolutionary relationships in which *taxa* of parasites and vectors are linked together by unique behavioral or molecular phenotypes of epidemiological importance. As *Nyssomyia* and *Psychodopygus*, the most medically important phlebotomine genera for the New World, concentrate several vector species of *Leishmania*, *Trichophoromyia* may also include species possessing common conditions suitable for parasite development. Circumstantial ingestion of parasites for some of these species, however, is also likely. In this regard, long-term surveys are required to strength evidences on the ability of *Trichophoromyia* to sustain a natural cycle in sites where these apparently occasional infections have been recorded.

The number of new *Trichophoromyia* species described has been increased in the last years.<sup>(6)</sup> Most of these descriptions were provided from specimens collected in environmentally impacted ecotopes of the Amazon Basin, where ACL is considered a prioritised disease to be monitored in vector surveillance programs. Numerical dominance in light-trap captures, permis-



siveness for *Leishmania* infection and spatiotemporal congruence with ACL epidemiology are usually variables considered for drawing attention to the possible involvement of *Trichophoromyia* in ACL enzootics.

The raising question on the putative vector importance of *Trichophoromyia* is also likely to be biased toward the marked increase in the general list of suspected vectors of leishmaniasis in the New World,<sup>(1,3,4)</sup> which has been supported in the last years, mainly due to the results of PCR-based techniques for diagnosing *Leishmania* DNA targets within pooled or individually processed phlebotomines. Integrative molecular methodological approaches let us to include suspected vector species from phlebotomine genera with little or even null association with *Leishmania*, such as *Pintomyia*,<sup>(35)</sup> *Pressatia*, *Evandromyia*,<sup>(25,31)</sup> *Martinsmyia*, and *Micropygomyia*,<sup>(36)</sup> in our view, care must be taken on interpreting these intriguing results. Even considering established natural infection, quantification of the parasitic load and/or demonstration of infective forms would certainly present a further step toward improving knowledge on putative *Leishmania* vectors inferred exclusively through DNA-based findings.

In fact, records of natural *Leishmania* spp. infections in *Trichophoromyia* phlebotomines are increasing but these facts, alone, do not necessarily correlates with an epidemiological or medical issue on leishmaniasis. Low man-biting behavior of *Trichophoromyia* may contribute to the sporadically ACL due to their related *Leishmania* species, as occur with the epidemiology of *L. (V.) lainsoni*. Furthermore, studies on the knowledge of blood-feeding behavior, including attraction to bit man and *Leishmania* reservoir hosts, ecological association with ACL determinants, experimental susceptibility for *Leishmania* infection/ transmission, and mathematical modeling of vector/disease dynamics, must be improved to better comprehend vector-parasite interactions and their transmission cycles in ecotopes where these *Trichophoromyia* species are supposed to be transmitting *Leishmania* parasites.

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#### AUTHORS' CONTRIBUTION

TVS - Conceptualisation, writing (original draft, review, and editing) and visualisation; FTS - supervision, writing (review and editing) and visualisation. The authors declare no conflict of interest concerning to this manuscript.

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