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Molecular Identification of *Leishmania* spp. in Sand Flies (Diptera: Psychodidae, Phlebotominae) From Ecuador

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Abstract

The detection and identification of natural infections in sand flies by *Leishmania* protozoan species in endemic areas is a key factor in assessing the risk of leishmaniasis and in designing prevention and control measures for this infectious disease. In this study, we analyzed the *Leishmania* DNA using nuclear ribosomal internal transcript spacer (ITS) sequences. Parasite DNA was extracted from naturally infected, blood-fed sand flies collected in nine localities considered leishmaniasis-endemic foci in Ecuador.

The species of parasites identified in sand flies were *Leishmania major*-like, *Leishmania naiffi*, *Leishmania mexicana*, *Leishmania lainsoni*, and *"Leishmania* sp. *siamensis"*. Sand fly specimens of *Brumptomyia leopoldoi*, *Mycropigomyia cayennensis*, *Nyssomyia yuilli yuilli*, *Nyssomyia trapidoi*, *Pressatia triacantha*, *Pressatia dysponeta*, *Psychodopygus carrerai carrerai*, *Psychodopygus panamensis*, and *Trichophoromyia ubiquitalis* were found positive for *Leishmania* parasite. These findings contribute to a better understanding of the epidemiology and transmission dynamics of the disease in high-risk areas of Ecuador.

Key words: *leishmania*, internal transcript spacer 1, sand fly, Ecuador

Leishmaniasis is a neglected endemic disease present in 98 countries worldwide and is an important public health problem in developing countries. It has spread owing to urban sprawl, deforestation, climate change, and ecological tourism (Schwartz et al. 2006, Tuon et al. 2008). It is estimated that 12 million people are infected, 350 million people are at risk of contracting the disease, and each year about 2 million new cases are reported worldwide (Akhoundi et al. 2016). Leishmaniasis has been reported in 23 of the 24 provinces of Ecuador; the exception is Galápagos Islands (World Health Organization [WHO] 2015). This tropical disease is caused by an intracellular protozoan parasite of the genus *Leishmania* in the family Trypanosomatidae, which in the Americas (the New World) is transmitted by sand flies of several genera in the subfamily Phlebotominae (Galati 2016).

Phlebotominae sand flies reported in Ecuador include 81 species in 17 genera (Alexander et al. 1992, Galati 2016). *Lutzomyia gomezi* (Nitzulescu, 1931), *Lutzomyia hartmanni* (Fairchild & Hertig, 1957), *Nyssomyia trapidoi* (Fairchild & Hertig, 1952), *Pintomyia maranonensis* (Galati, Caceres & Le Pont, 1995), *Pintomyia serrana* (Damasceno & Arouck, 1949), and *Lutzomyia ayacuchensis* (Galati & Caceres, 1988) have been incriminated with leishmaniasis transmission (Hashiguchi et al. 2016).

There are 53 described species of *Leishmania* in the world; 31 of them have been reported as parasites of mammals and about 20 species cause the visceral, cutaneous, and mucocutaneous forms of leishmaniasis in humans (Akhoundi et al. 2016). The most common form in Ecuador is cutaneous leishmaniasis, which is caused by *Leishmania braziliensis* Vianna, 1911, *Leishmania panamensis* Lainson & Shaw, 1972, *Leishmania guyanensis* Floch, 1954, *Leishmania peruviana* Velez, 1913, *Leishmania mexicana* Biagi, 1953, *Leishmania amazonensis* Lainson & Shaw, 1972, *Leishmania venezuelensis* Bonfante Garrido, 1980, *Leishmania naiffi* Lainson & Shaw, 1989, and *Leishmania lainsoni* Silveira et al., 1987 (Bañuls et al. 2002, Akhoundi et al. 2016, Kato et al. 2016a, Ministerio de Salud Pública [MSP] 2016).

The first documented human case of leishmaniasis in Ecuador was reported in 1920 (Rodriguez 1974). Since then, 25 species of sand flies have been identified from four endemic localities (Hashiguchi et al. 1991). According to Kato et al. (2016b) and Hashiguchi et al. (2016), *Leishmania guyanensis*, *L. braziliensis*, *L. naiffi*, *L. lainsoni*, *L. panamensis*, *L. amazonensis*, *L. mexicana*, and *Leishmania major*-like have been isolated from human samples from tropical and subtropical areas of Ecuador. *Lutzomyia ayacuchensis*, *Lutzomyia trapidoi*, *Lu. gomezi*, *Lutzomyia tortura*, and *P. maranonensis* have been incriminated as the vectors for *Leishmania* spp. (Kato et al. 2005, Hashiguchi et al. 2016).

Lainson and Shaw (1987) classified *Leishmania* species based on their geographical distribution, vector origin, tropism, and clinical manifestations. Owing to the difficulty of identifying the species of *Leishmania* and associating them with specific clinical manifestations, modern approaches to taxonomic identification use molecular markers (Fraga et al. 2009, Schönian et al. 2010, Bates et al. 2015). Several techniques of genotyping and molecular characterization have been used to identify *Leishmania* species (Van der Auwera and Dujardin 2015). These techniques include multilocus-sequence typing (MLST; Van der Auwera and Dujardin 2015), multilocus-microsatellite typing (MLMT), restriction-fragment length polymorphism PCR-RFLP (Schönian et al. 2003, Terayama et al. 2008), and sequencing of specific genetic markers such as Hsp70 (Fraga et al. 2009), ribosomal DNA (rDNA), and kinetoplast DNA (kDNA; Orlando et al. 2002), internal transcript spacer ITS, and cytochrome B (Asato et al. 2008, Yang et al. 2013). Sequence analysis of ITS1 and ITS2 have been shown to be suitable for species discrimination of *Leishmania* parasites (Orlando et al. 2002, Mahdy et al. 2016, Nazma et al. 2016). There are between 150 and 200 copies of these rRNA genes in *Leishmania* genome (Berzunza-Cruz et al. 2002). Several authors have used the ITS region to determine the polymorphism of clinical samples and the genetic variability of strains of the *Leishmania* species complex with different PCR methods (Schönian et al. 2001, Toz et al. 2013, Maia et al. 2015).

The detection and identification of *Leishmania* parasites in sand flies may help in understanding the transmission dynamics of the parasite species in high transmission areas among vectors and human and other animal reservoirs. In the present study, we use ITS1 sequences to identify the *Leishmania* species from engorged sand flies collected in nine localities of Ecuador.

Materials and Methods

Sand Fly Sample Collection

Phlebotomine sand flies were collected between 2014 and 2016 in nine tropical and subtropical localities, five in the Pacific lowlands and three in the Amazon basin of Ecuador (Fig. 1). Collections were made during both the rainy and dry seasons using CDC light traps (John W. Hock USA). Traps were placed in the forest at three different distances from inhabited houses: at 150 m (peridomiciliary), 300 m (forest), and 600 m (forest). All light traps operated from 1800 to 0600 hours for three consecutive nights, during each of the dry and rainy seasons. Samples were sorted using a Discovery V12 stereo microscope (Zeiss, Germany). Females were separated as to whether blood-fed or unfed. Blood-fed females were identified using the taxonomic keys of Young and Ducan 1994 and Galati 2016. The abdomens of fed females were dissected and individually stored at −20 °C for DNA extraction. The head, wings, and thorax of each specimen were cleared in 10% potassium hydroxide and mounted in a temporary fructose-gum Arabic medium for taxonomic identification. The natural infection rates (NIRs) for the identified species of sand flies were calculated including only the total number of engorged females (Pinheiro et al. 2010).

Positive Control Cultures and Genomic DNA Extraction

Three reference strains of *Leishmania* were used as positive controls in the molecular studies. The species used were *Leishmania tarentolae* Wenyon, 1921 (LT2I UCLA) and clinical isolates of *L. mexicana* LM379 and *L. braziliensis* LB2903 (Instituto de Medicina Tropical "Alexander von Humboldt" Universidad Peruana Cayetano Heredia, Lima, Peru). *Leishmania* promastigotes were grown at 24 °C in Schneider's *Drosophila* medium (Gibco USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) and 1% penicillin–streptomycin (10,000 U/ml) solution (Gibco USA). Promastigotes were harvested on the third day of growth for genomic DNA extraction and posterior-sequence comparison with the DNA from the parasites extracted from the sand flies.

Genomic DNA extraction was conducted using abdomens of engorged field-collected sand flies and promastigotes from *Leishmania* cultures (positive controls). All the samples were then processed using DNeasy Blood & Tissue Kit (Qiagen, Germany)

ITS1 Amplification and Sequencing

Polymerase chain reaction was performed to generate a 330-bp ITS1 amplicon using the forward primer LITSR (5′-CTGGATCATTTT CCGATG-3′) and the reverse primer L5.8S (5′-TGATACCACTTAT CGCACTT-3′ (Schönian et al. 2003). The PCR protocols for amplification were: 94 °C for 3 min followed by 40 cycles of 94 °C for 40 s, 53 °C for 45 s, and 72 °C for 60 s, followed by a final elongation step at 72 °C for 10 min. The amplified products were purified on a 2.0% agarose gel stained with SYBR safe (Invitrogen USA). T he purified PCR product was then sequenced (Macrogen, Seoul).

Polymerase chain reactions included DNA from the reference strains as positive controls (*L. tarentolae* LT2I, *L. mexicana* M379, and *L. braziliensis* LB2903) and a negative control (water).

Amplified ITS1 sequences from the abdomen of sand flies were edited and multiple-aligned with reference sequences of *Leishmania major* Yakimov & Schockov, 1914, *L. naiffi*, *L. tarentolae*, *L. mexicana*, *L. brazilliensis*, "*Leishmania* sp. *siamensis*," *L. tropica*, *L. lainsoni*, and *Leishmania* sp. retrieved from GenBank using Geneious v 9.0 software (Biomatters Ltd., New Zealand).

Phylogenetic analysis was conducted in Geneious v 9.0 software using the neighbor-joining (NJ) method, 100-replicates and 70 bootstrap (BP) test. For species identification of *Leishmania*, ITS1 sequences from studied isolates were multiple-aligned using ClustalW-Geneious v 9.0 software (Biomatters Ltd, New Zealand). In the analysis, a sequence of *Leptomonas seymouri* was included as an outgroup (GenBank ATCC 30220).

Results

In total, 11,307 sand flies were captured with CDC light traps in nine locations of Ecuador. There were 6,764 males, 4,200 unfed females, and 343 blood-fed females. The total capture effort per location was six traps per night (total = 18 traps in three nights) in each season (dry and rainy). The capture effort at each collecting ecotope was two traps per night (total = six traps in three nights).

Genomic DNA was extracted from fed females, and ITS1 gene was amplified. The ITS1 gene was obtained from 139 of the 343 fed females. Specimens of the genera *Brumptomyia*, *Lutzomyia*, *Mycropygomyia*, *Nyssomyia*, *Pressatia*, *Psychodopygus*, and *Trichophoromyia* (14 specimens) were found positive for

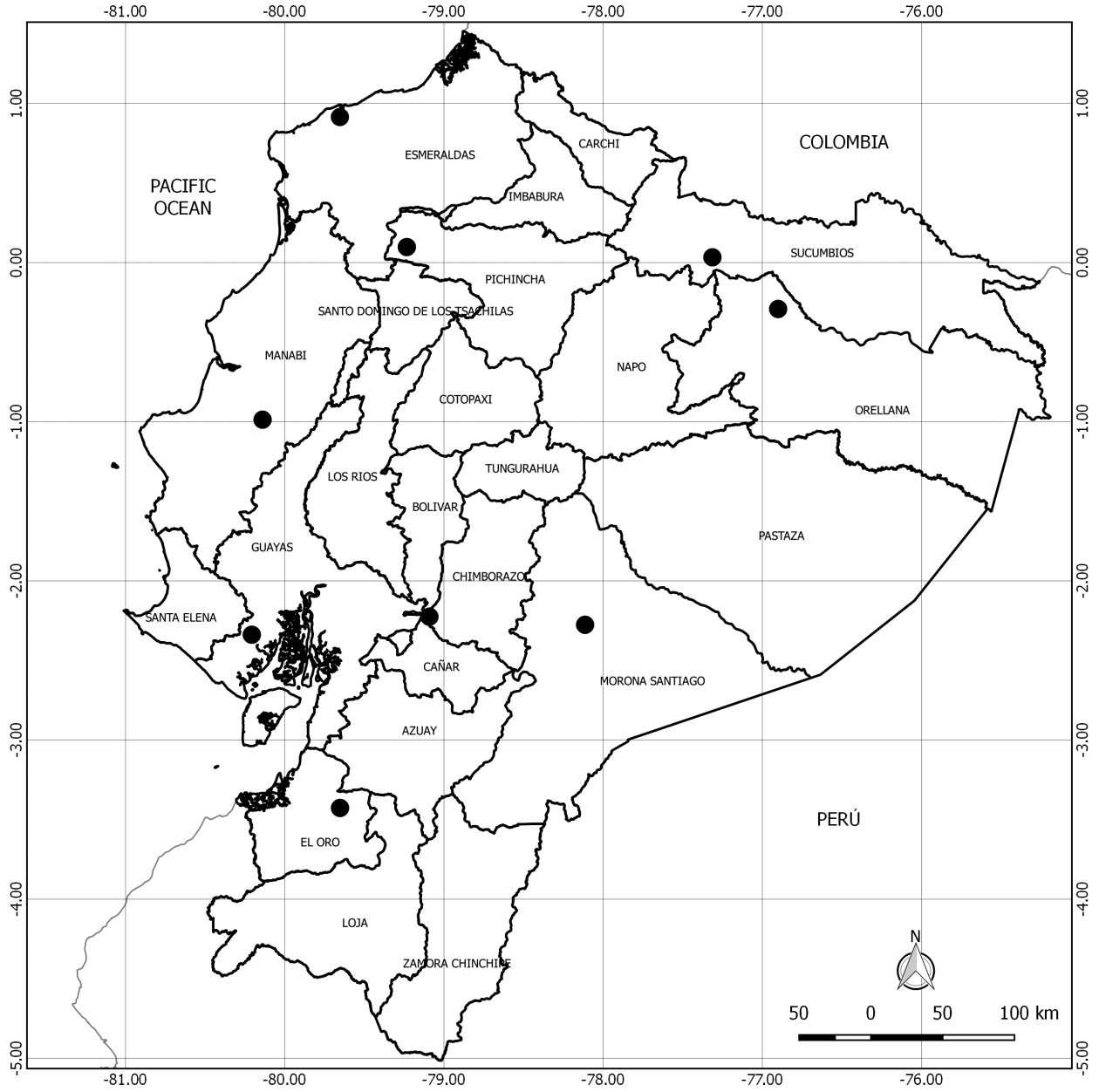

Fig. 1. Map of Ecuador showing the locations where sand flies were collected. The dark circles show the sites of collection in the Provinces of Esmeraldas, Manabí, Guayas, El Oro, Pichincha, Chimborazo, Sucumbíos, Orellana, and Morona Santiago, Qgis v2.18.

Leishmania. The infected sand flies of *Nyssomyia yuilli yuilli* (Young & Porter, 1972), *Ny. trapidoi*, *Psychodopygus carrerai carrerai* (Barreto, 1946), *Trichophoromyia ubiquitalis* (Mangabeira, 1942), and *Pressatia triacantha* (Mangabeira, 1942) were collected in the Amazonian lowlands, while those of *Brumptomyia leopoldoi* (Rodriguez, 1953), *Psychodopygus panamensis* Shannon, 1926, *Presatia dysponeta* (Fairchild & Hertig, 1952), and *Mycropygomyia cayennensis* (Floch & Abonnenc, 1941) were collected in the Pacific coastal localities. There was also one sand fly infected with *Crithidia fasciculata* and two with *Trypanosoma* sp. Sand flies from the province of Esmeraldas were negative for *Leishmania* parasites.

The peridomiciliary ecotope showed higher diversity of sand flies, higher number of infected sand flies, and higher NIRs than the forest ecotope. Natural infection rate showed values that ranged from 2.9% to 50% in the dry season, and from 6.3% to 12.5% during the rainy season (Table 1). "*Leishmania* sp. *siamensis*" was detected in five different species of sand flies from five localities. *Leishmania major*-like was only detected in *Psychodopygus panamensis* from Puerto Quito-Pichincha. The sequence that clustered with *L. tropica* was detected in *Nyssomyia trapidoi* collected in Joya de los Sachas-Orellana. *Leishmania mexicana* was detected in *Micropygomyia cayennensis* from El Progreso in the province of Guayas, in the coastal region (Table 1).

The 14 ITS1 sequences obtained showed over 97% homology (Blatsn) with *Leishmania* species at the NCBI (Fig. 2). One group of six sequences (F005, F006, F008, F010, F016, and F017) showed over 99% homology with "*Leishmania* sp. *siamensis*" and with *Leishmania* sp. (Fig. 2). The analysis of ITS1 showed that two sequences of ITS1 (F001 and F002) were placed in the cluster with *L. major*, two (F003 and F007) in the cluster with *L. naiffi*, one (F004) in the cluster with *L. mexicana*, one (F015) in the cluster

Table. 1. *Leishmania* species detected in sand flies collected in two ecotopes in eight localities of Ecuador during the rainy and dry seasons.

Year	Province/Locality	Ecotope	Vector	Parasite identification	Sample code	GenBank	Natural Infection Rate (NIR)	Total no. of specimens (*n*)	Season
2014	Puerto Quito/Pichincha	Peridomicilliary	*Psychodopygus panamensis*	*Leishmania major*-like	F001, F002	KY769941	11.1%	18	Dry
2014	Joya de los Sachas/Orellana	Forest	*Nyssomyia trapidoi*	*Leishmania tropica*	F015		6.3%	16	Rainy
2014		Peridomicilliary	*Nyssomyia trapidoi*	*Leishmania* sp. *siamensis*	F016		6.3%	16	Rainy
2015	Cumánda/Chimborazo	Forest	*Pressatia dysponeta*	*Leishmania naiffi*	F003	KY769937	2.9%	35	Dry
2015	Macas/Morona Santiago	Peridomicilliary	*Psychodopygus carrerai carrerai*	*Leishmania naiffi*	F007		9.1%	11	Rainy
		Peridomicilliary	*Psychodopygus carrerai carrerai*	*Leishmania* sp. *siamensis*	F008		12.5%	8	Rainy
		Peridomicilliary	*Nyssomyia yuilli yuilli*	*Leishmania* sp. *siamensis*	F010	KY769938	9.1%	11	Rainy
		Peridomicilliary	*Trichophoromyia ubiquitalis*	*Leishmania lainsoni*	F011, F12	KY769939	11.1%	9	Rainy
2015	Gonzalo Pizarro/Sucumbíos	Forest	*Pressatia triacantha*	*Leishmania* sp. *siamensis*	F017		16.7%	6	Dry
2016	Progreso/Guayas	Peridomicilliary	*Micropygomyia cayennensis*	*Leishmania mexicana*	F004	KY769940	12.5%	8	Dry
2016	Bolívar/Manabí	Peridomicilliary	*Brumptomyia leopoldoi*	*Leishmania* sp. *siamensis*	F005		50.0%	2	Dry
2016	Carabota/El Oro	Peridomicilliary	*Psychodopygus panamensis*	*Leishmania* sp. *siamensis*	F006		12.5%	8	Dry

NIR—Natural infection rate of engorged female sand flies.

with *L. tropica*, and two (F011 and F012) in the cluster with *L. lainsoni*. The rest of the ITS1 sequences showed over 86% homology with *Crithidia fasciculata* and *Trypanosoma* sp. (F009, F013, and F014; Fig. 2).

Discussion

One step in vector incrimination for leishmaniasis is to determine the occurrence of natural infection in sand fly populations. The dissection of the digestive tract of sand flies for observing *Leishmania* promastigotes by light microscopy has been considered the gold standard test for detecting natural infections in these vectors (Kato et al. 2005). However, identification of *Leishmania* parasites within sand flies requires examination of a large number of sand flies and isolation and culture of *Leishmania* parasites from each dissected fly. In some cases, it is necessary to inoculate parasites into laboratory animals to find true positives. Owing to the laboriousness of this procedure, molecular techniques have been standardized to easily detect *Leishmania* DNA in sand flies (Bates et al. 2015).

Leishmania tropica

One ITS1 sequence from a sand fly collected in Joya de los Sachas-Orellana corresponded to 98% homology with the *Leishmania tropica* cluster (BP = 98). Although there are cases of visceralizing *L. tropica*, this parasite is more commonly the causative agent of cutaneous leishmaniasis, such as *Leishmaniasis recidivans* (Akhoundi et al. 2016, Sarkari et al. 2016). In Ecuador and South America, there are no epidemiological records of visceral leishmaniasis caused by *L. tropica*. It is known that *L. tropica* is genetically very heterogeneous (Schönian et al. 2001, Schnur et al. 2004) and for this reason further studies should include a larger number of samples and genetic markers, along with studies of clinical samples in the province of Orellana. On the other hand, our analysis includes *L. tropica* and *L. naiffi* in the same cluster, implying a sequence similarity that requires further analysis of different alleles to differentiate between these two species.

Leishmania major-Like

The analysis of ITS1 sequences clustered two samples with sequences of *L. major* reported from the Old World. *Leishmania major* is one of the causative agents of cutaneous leishmaniasis in the Old World, which is transmitted by *Phlebotomus papatasi* and *Phlebotomus duboscqi* (Ayari et al. 2015, Wu et al. 2015). Thus, the strong relationship of our sequence with *L. major* is remarkable. However, isoenzyme electrophoretic mobility patterns using RAPD, SSR-PCR, and enzyme electrophoresis (zymodeme), and digestion profiles of kinetoplast DNA with restriction enzymes (schizodeme) showed that parasites from the Americas, including those from Paraguay, Brazil, and Ecuador, are different and have been denominated as *L. major*-like (Yamasaki et al. 1994, De Oliveira Silva et al. 2009, Wu et al. 2015). Berzunza-Cruz et al. (2002) reported *L. major* in clinical isolates using RFLP studies in Mexico, and it has been suggested that this species may have been introduced to the New World. In Ecuador, several studies also reported *L. major*-like in clinical isolates from patients with cutaneous leishmaniasis from Andean highland areas (Hashiguchi et al. 1991, Calvopiña et al. 2005, Kato et al. 2016a). This is the first recorded natural infection of *L. major*-like parasites in sand flies of the genus *Psychodopygus (Ps. panamensis)* in Ecuador (from Puerto Quito-Pichincha). The comparison of ITS1 sequences obtained of *L. major*-like was not possible because there are not registered sequences for this strain in

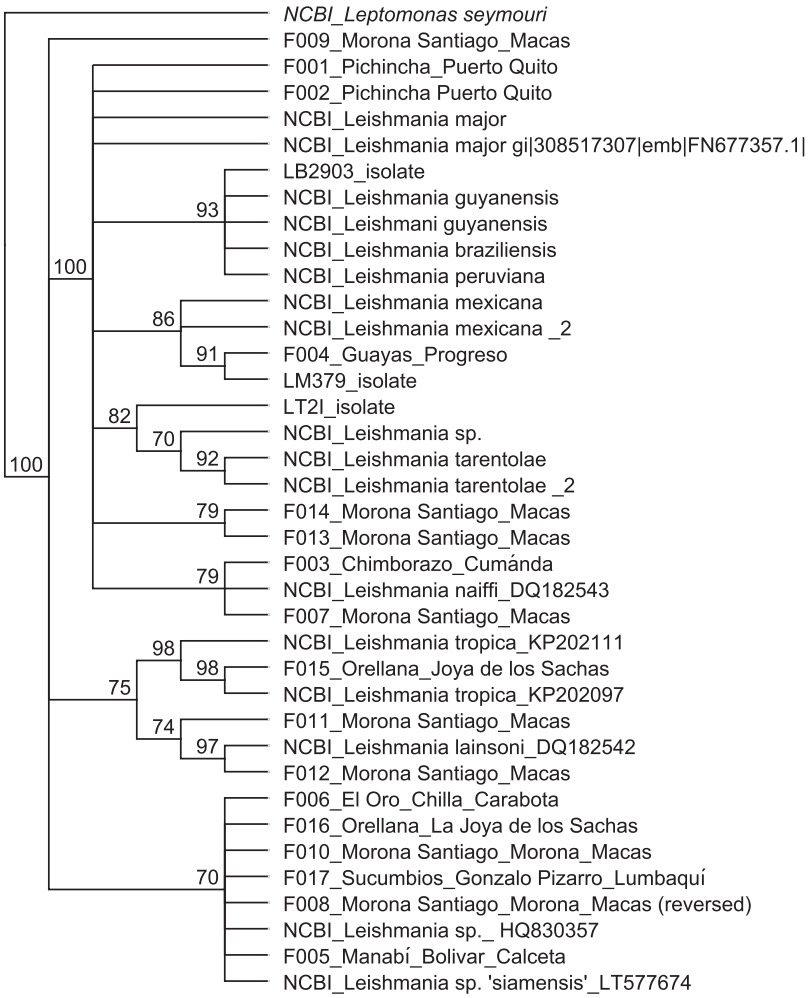

Fig. 2. Phylogenetic relationships of the ITS1 gene sequences of samples and reference sequences published in GenBank (National Center for Biotechnology Information), using the neighbor-joining method.

the GenBank. Further sampling and analysis from the same area are needed to determine the identity and relationships of this group of *Leishmania* parasites and their vectors.

Leishmania lainsoni

The ITS1 sequences from two sand fly specimens collected in Macas, Morona Santiago province, were grouped with *Leishmania lainsoni* (BP = 74; 97). This species has already been reported from a human lesion collected in the province of Sucumbíos, Ecuador (Kato et al. 2016a). *Leishmania lainsoni* has been detected in *Lutzomyia nuneztovari* (Young & Duncan 1994) in Bolivia (Bastrenta et al. 2002), in *Lutzomyia auraensis* in Perú (Valdivia et al. 2012), and in *Trichophoromyia ubiquitalis* in Brazil (Silveira et al. 1991, Lainson et al. 1992). The latter report is consistent with the identification of *L. lainsoni* in the sand fly *Th. ubiquitalis* in our study.

Leishmania naiffi

Psychodopygus carrerai carrerai collected in Macas-Morona Santiago province and *Presatia dysponeta* from Cumandá-Chimborazo were infected with parasites placed in the *Leishmania naiffi* group (BP = 79). This is the first record for Ecuador of these sand fly species naturally infected with *L. naiffi*. This species of *Leishmania* is known to occur widely in South America and the Caribbean, where it causes cutaneous leishmaniasis (CL) in humans (Pratlong et al. 2002). Previous studies in the province of Orellana, located in the Amazon region of Ecuador, detected *Leishmania naiffi* in clinical samples from patients with cutaneous leishmaniasis and *Lutzomyia yuilli* and *Lu. tortura* as the vectors (Kato et al. 2005, Kato et al. 2013). Azpurua et al. (2010) found *Lu. trapidoi* and *Lu. gomezi* infected with *L. naiffi* in Barro Colorado Island in Panamá.

The analysis of the gene sequences placed our sample in the cluster that is related to the subgenus *Sauroleishmania* and *Leishmania braziliensis* complex with the same BP value (79; Fig. 2). A further analysis with other genes may allow species differentiation in the cluster.

Leishmania mexicana

The sand flies collected in Progreso, province of Guayas, were identified as *Micropygomyia cayennensis* and the parasites extracted from them grouped in the cluster with *Leishmania mexicana*. Sand flies of the genus *Micropygomyia* are known to feed on cold-blooded animals, which does not correspond to the habitat requirements of the *Leishmania* parasite (Rêgo et al. 2015). However, this result is consistent with findings reported in Colombia and Venezuela, where *Mi. cayennensis* was reported infected with *Leishmania* (Feliciangeli 1987, Alveiro et al. 2015). In Colombia, this sand fly attacks humans and is very common inside homes (Cochero et al. 2007, Cortés and Fernández 2008). Detection of *L.*

mexicana in *Mi. cayennensis* expands the distribution of the parasite in the lowlands along with a new vector. Hashiguchi et al. (1991) reported the possible vector incrimination of *Lutzomyia ayacuchensis* with *Leishmania mexicana*. Kato et al. (2005) registered the presence of *Lutzomyia ayacuchensis* in the provinces of Manabí, Pichincha, Chimborazo, and Azuay, and Gomez et al. (2014) report this species at altitudes >650 m. The analysis of the geographic distribution of cases of cutaneous leishmaniasis by phylogenetic relationships based on cytochrome b revealed the presence of *Leishmania mexicana* in the Andes of Ecuador (Kato et al. 2016a). However, Calvopiña et al. (2006) reported an isolate of *L. mexicana* from a human clinical case in the Pacific lowlands. Owing to the wide distribution of the parasite that includes the highlands and the lowlands, it is necessary to identify the parasite using other molecular markers such as cytochrome b and ITS2, and also determine the polymorphisms.

"Leishmania sp. *siamensis"*

ITS1 sequences isolated from sand flies corresponded to 100% homology with "*Leishmania* sp. *siamensis*" and *Leishmania* sp. According to Akhoundi et al. (2016), this species has been reported in both Old and New World. However, the name "*Leishmania siamensis*" is not a valid taxon and is used with quotation marks (Akhoundi et al. 2016). This species has been reported in Central Europe, Thailand, and United States, where it causes visceral and cutaneous leishmaniasis (Sukmee et al. 2008, Bualert et al. 2012, Reuss et al. 2012, Kanjanopas et al. 2013, Akhoundi et al. 2016).

The isolates are grouped in the cluster *Leishmania* sp. and "*Leishmania siamensis*" (BP = 70), but they did not group with the subgenus *Sauroleishmania* as was expected according to the classification proposed by Fraga et al. (2009). A detailed phylogeny of the genus *Leishmania* is required with a greater number of genes from parasites in different geographic ranges to determine the degree of polymorphisms to clarify the evolution and epidemiology of the genus (Berzunza-Cruz et al. 2002, Yang et al. 2013).

Our study has confirmed the presence of *Leishmania* infection in the sand flies *Brumptomyia leopoldoi*, *Micropygomyia cayennensis*, *Nyssomyia yuilli yuilli*, *Ny. trapidoi*, *Pressatia triacantha*, *Pr. dysponeta*, *Psychodopygus carrerai carrerai*, *Ps. panamensis*, and *Trichophoromyia ubiquitalis*.

The sequences from samples corresponded to 98% homology with "*Leishmania* sp. *siamensis*." The sand flies hosting these parasites were identified as *Br. leopoldoi* (F005), *Ps. panamensis* (F006), *Ps. carrerai carrerai* (F008), *Ny. yuilli yuilli* (F010), *Ny. trapidoi* (F016), and *Pr. triacantha* (F017). Valdivia et al. (2012) reported *Pressatia* sp. sand flies infected with *Leishmania* sp. for the first time in Perú. Araujo-Pereira et al. (2017) also reported *Pressatia* sp. infected with *Leishmania brazilienzis*. These findings, along with ours, underscore the importance of further investigations to determine the species involved as the potential vectors of leishmaniasis. *Leishmania naiffi* has been found infecting *Lutzomyia trapidoi* and *Lu. gomezi* (Azpurua et al. 2010), whereas the transmission of *Leishmania lainsoni* is associated with the sand fly *Trichophoromyia ubiquitalis*. Our results indicate that under these conditions, humans potentially can become infected with more than one *Leishmania* species. This is the first record of natural infection of *T. ubiquitalis* with *L. lainsoni* in Ecuador, which may help to establish the distribution of the parasite and its vector. According to (Forattini 1973), *Brumptomyia* species do not have epidemiological importance in the transmission of *Leishmania* spp. to humans, and some species are known for feeding on other mammals like armadillos.

The high number of sand fly species and engorged females found in the peridomiciliary ecotope may be related to the availability of blood sources. It has already been reported that the feeding behavior of sand flies in this ecotope is highly diversified and includes blood from humans, chickens, cows, and dogs (Anaguano et al. 2015). However, it is essential to determine if the domesticated animals are reservoirs of the disease, as the risk for humans would be higher in these areas than in the forest.

Crithidia fasciculata (Kinetoplastida: Trypanosomatidae) found in some of the sand flies is a known exclusive parasite of several orders of insects, including Diptera (Wallace 1966). *Trypanosoma* sp. detected in some sand flies may be parasites of anurans and reptiles reported in other studies in the neotropics (Viola et al. 2008).

Determining the distribution and identify of *Leishmania* species requires standardization of methodologies to identify DNA isolated from clinical samples and sand fly vectors. Therefore, only whole-genome sequencing of the isolated parasite would generate consensus regions and establish target nucleotide sequences of mitochondrial or nuclear genes for interspecies difference. Similarly, the correct vector incrimination requires the right identification of the collected sand fly specimens.

The high diversity of sand flies and high rates of *Leishmania*-infected insects, especially in the peridomiciliary ecotope, suggests a very complex epidemiology of the disease. Consequently, determination of vector–parasite–reservoir relationships is needed to quantify the risk in these areas and implement preventive and control strategies.

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