FACULTAD DE POSGRADOS

PHYLOGENETIC IDENTIFICATION OF *Leishmania* spp. IN SANDFLIES (DIPTERA: PSYCHODIDAE, PHLEBOTOMINAE) FROM ECUADOR BY USING INTERNAL TRANSCRIBED SPACER 1 (ITS1) SEQUENCES.

Trabajo de Titulación presentado en conformidad con los requisitos establecidos para optar por el título de Magíster en Ciencias Biomédicas

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DECLARACIÓN DEL PROFESOR GUÍA

Declaro haber dirigido este trabajo a través de reuniones periódicas con el estudiante, orientando sus conocimientos y competencias para un eficiente desarrollo del tema escogido y dando cumplimiento a todas las disposiciones vigentes que regulan los Trabajos de Titulación.

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DECLARACIÓN DE AUTORÍA DEL ESTUDIANTE

Este trabajo de titulación es el trabajo original de su autor, que ha trabajado en equipo con otros investigadores. Una versión de este trabajo está en el proceso de publicación en la literatura científica profesional con el siguiente grupo de co-autores: Patricio Rojas, Patricio Ponce, Paúl Cárdenas y Varsovia Cevallos.

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RESUMEN

La infección natural de las especies de flebótomos por especies del protozoo *Leishmania* en las zonas endémicas de leishmaniasis es importante para la predicción del riesgo y la expansión de esta parasitosis. En este estudio, la técnica molecular de identificación de especies de *Leishmania* en flebótomos fue establecida mediante el uso de la relación filogenética de las secuencias del gen espaciador transcrito interno (ITS1). Este análisis identificó *Leishmania major, Leishmania naiffi, Leishmania mexicana, Leishmania tropica y Leishmania lainsoni* en ocho de las nueve provincias muestreadas del Ecuador. Estos resultados se discuten en relación con los resultados de otras técnicas y marcadores moleculares que fueron integrados con el fin de construir una identificación genética de cada especie de *Leishmania*.

Palabras clave: *Leishmania*, ITS1 y filogenia.

ABSTRACT

The natural infection of *Leishmania* protozoa within sand fly species in endemic areas of disease is important for prediction of the risk and expansion of leishmaniasis. In this study, a molecular technique of typing *Leishmania* species circulating within sand flies in leishmaniasis endemic areas in Ecuador was established by using relationship phylogenetic of Internal Transcript Spacer ITS gene sequences. This analysis identified *Leishmania major, Leishmania naiffi, Leishmania mexicana, Leishmania tropica* and *Leishmania lainsoni* in eight of the nine provinces in Ecuador. These findings are discussed in relation to results from other techniques and genes, which have to be integrated in order to build a genetically identification of each species of *Leishmania*.

Keywords: *Leishmania*, ITS1 and phylogeny.

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ABBREVIATIONS

DNA	Deoxyribonucleic acid
bp	Base-pair
ITS1	Internal transcribed spacer 1
BP	Bootstrap
NCBI	National Center for Biotechnology Information

1. Introduction

Leishmaniasis is an endemic disease in 98 countries and it is considered a public health problem in developing countries. It has spread by urban sprawl, deforestation, climate change and ecological tourism (Abdul-Ghani et al. 2016,Tuon et al. 2008, Schwartz et al. 2006). 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). This tropical disease is caused by an intracellular protozoan parasite of the Trypanosomatidae family and genus *Leishmania* which in America (New World) is transmitted by a sand fly of the subfamily Phlebotominae, genus *Lutzomyia* (Akhoundi et al. 2016).

There are 53 species of *Leishmania* in the world, out of which 31 species are parasites of mammals and 20 species cause the visceral, cutaneous and mucocutaneous forms of leishmaniasis in humans (Akhoundi et al. 2016). The most common form is cutaneous leishmaniasis caused by *Leishmania braziliensis, L. panamensis, L. guyanensis, L. peruviana, L. mexicana, L. amazonensis, L. venezuelensi, L. naiffi and L. lainsoni* in America(Akhoundi et al. 2016, Kato et al. 2016, Kato et al. 2013, Bañuls et al. 2002) .

The Phlebotominae sand flies reported in Ecuador include 76 species in 17 genera (Galati 2011; Akhoundi et al. 2016). However, only *Lutzomyia gomezi* (Nitzulescu), *L. hartmanni* (Fairchild & Hertig), *Nyssomyia trapidoi* (Fairchild & Hertig), *Pintomyia maranonensis* (Galati, Caceres & Le Pont), *P. serrana* (Damasceno & Arouck) y *L. ayacuchensis* (Galati & Caceres) have been associated with leishmaniasis transmission (Alexander et al. 1992) and (K. Hashiguchi et al. 2014).

The first report of leishmaniasis in Ecuador was reported in 1920 (Calvopiña et al. 2005). Since then, 25 species of sand flies have been identified from four endemic localities (Hashiguchi et al. 1991). However, only *Lutzomyia ayacuchensis* has been implicated as a vector for *Leishmania* sp. (Kato et al.

2005). According to Kato (2016), *Leishmania guyanensis*, *L. braziliensis, L. naiffi, L. lainsoni,* and *L. mexicana* have been isolated from human samples from tropical and subtropical areas of Ecuador.

Lainson and Shaw (1987) classified *Leishmania* species based on their geographical distribution, vector origin, tropism and clinical manifestations. Due to the difficulty of identifying the species of *Leishmania* and associating species with specific clinical manifestations, modern approaches to taxonomic identification use molecular markers (Bates et al. 2015, Schönian et al. 2010, Fraga et al. 2009). Several techniques of genotypification have been used to identify *Leishmania* species. These techniques include multilocus sequence typing (MLST), multilocus microsatellite typing (MLMT), restriction fragment length polymorphism PCR-RFLP (Terayama et al. 2008) (Schönian et al. 2003) 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 cytocrome B (Oshiro et al. 2008) (Yang et al. 2013). Sequence analysis of ITS1 and ITS2 has shown to be suitable for species discrimination (Abdul-Ghani et al. 2016, Khan et al. n.d., Orlando et al. 2002).

Understanding the genetic difference among the species of the *Leishmania* genus and their phylogenetic classification would allow identification of the relationships between parasite virulence and drug resistance that might be relevant for the correct application of leishmaniasis treatments. In the present study, we determined phylogenetic relationships by analyzing the ITS1 sequences of *Leishmania* species obtained from sand flies collected at ten locations in Ecuador.

2. Materials and methods

Parasite culture

The reference strains used were *Leishmania tarentolae* LT2I (donated by Dr. Larry Simpson, UCLA) and clinical isolates *Leishmania mexicana* M379 and *Leishmania braziliensis* LB2903 (donated by Dr. Vanessa Adaui from the Instituto de Medicina Tropical "Alexander von Humboldt" Universidad Peruana Cayetano Heredia, Lima, Peru). *Leishmania* promastigotes were grown at 24ºC in Schneider's Drosophila medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, USA) and 1% penicillin – streptomycin (10000 U/mL) solution (Gibco, USA). Promastigotes were harvested on the third day of growth for genomic DNA extraction.

Sand fly sample collection

Phlebotomine sand flies were collected between 2014 and 2016 in 9 localities (Figure 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. The traps were placed at 150 m (peridomiciliar), 300 m (secondary forest) and 600 m (primary forest). Samples were sorted using a Discovery V12 stereo microscope (Zeiss, Germany). Females were separated by blood feed level (not fed and blood fed females). The abdomens of fed females were dissected and individually stored at −20 °C for further DNA extraction. The head, wings, and thorax of each specimen were cleared in 10% potassium hydroxide and mounted in a temporal fructose-Arabic gum medium for taxonomic identification.

Genomic DNA extraction

Genomic DNA extraction was conducted using abdomens of sand flies and promastigotes from *Leishmania* cultures. The samples were then processed with DNeasy Blood & Tissue Kit (Qiagen, Germany)

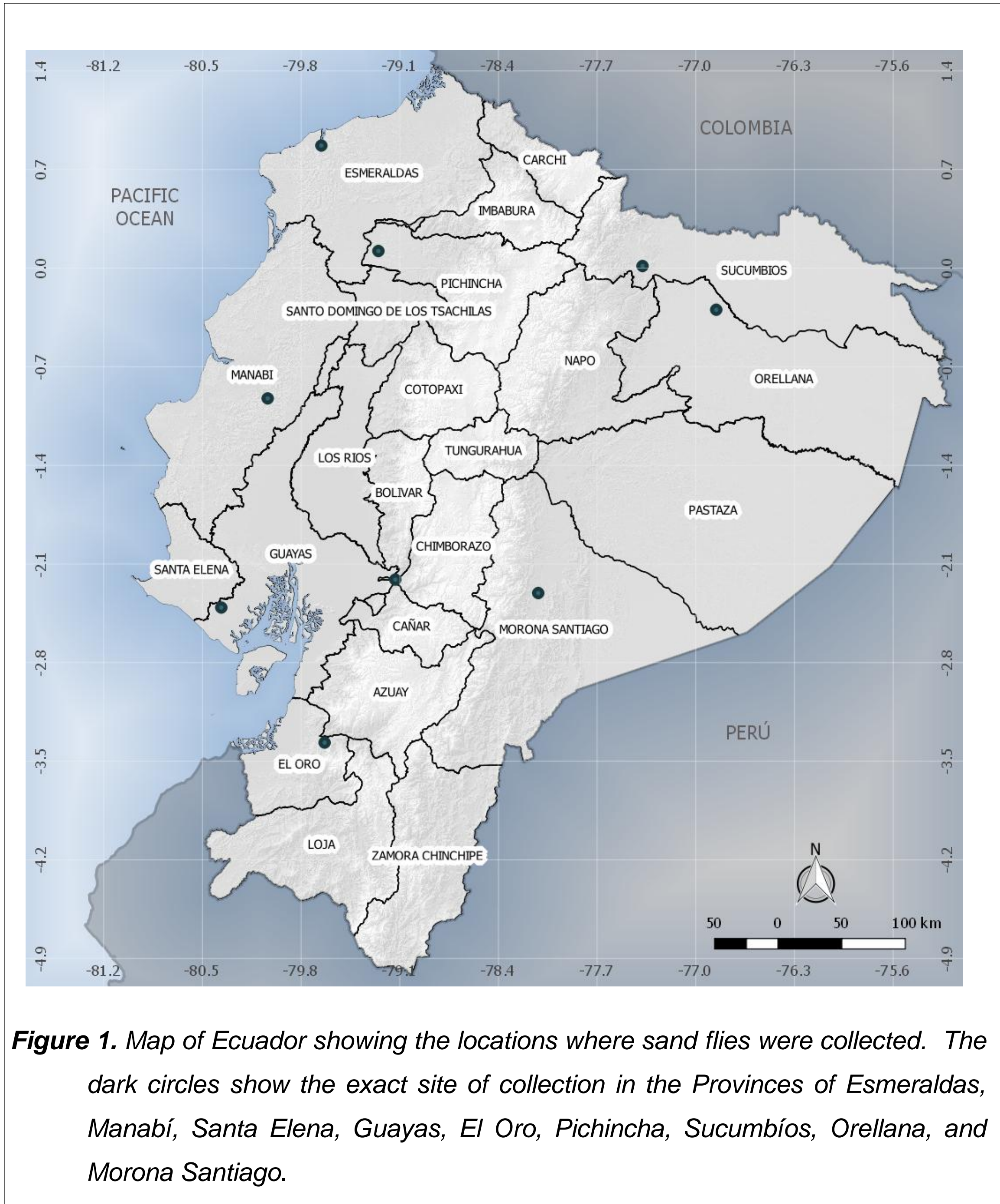

Figure 1. *Map of Ecuador showing the locations where sand flies were collected. The dark circles show the exact site of collection in the Provinces of Esmeraldas, Manabí, Santa Elena, Guayas, El Oro, Pichincha, Sucumbíos, Orellana, and Morona Santiago.*

ITS1 amplification and sequencing

PCR was performed to generate a 330 bp ITS1 amplicon using the forward primer LITSR (5´ CTGGATCATTTTCCGATG3´) and the reverse primer L5.8S (5´TGATACCACTTATCGCACTT 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). The purified PCR product was then sequenced (Macrogen, Seoul).

Amplified ITS1 sequences from the abdomen of sand flies were edited and multiple-aligned with reference sequences of *Leishmania naiffi, L. tarentolae, L. major, L. mexicana, L. brazilliensis, L. siamensis, L. tropica, L. laisoni* 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 accession ATCC 30220).

3. Results

A total of 11.307 sand flies were captured with CDC light traps in 9 locations of Ecuador and identified using a taxonomic key (Young and Ducan 1994). Of these, 6.764 were males, 4.200 not fed females and 343 were blood fed females. Genomic DNAs were extracted from fed females, and ITS1 gene was amplified. The ITS1 gene was obtained from 139 out of the 343 fed females. Of these 17 ITS1 sequences showed over 97% homology (Blatsn) of the NCBI with *Leishmania* species (Table 1). The rest of the ITS1 sequences showed over 86% homology with *Crithidia fasciculate* and *Tripanosoma* sp. indicating that the flagellate parasite inside the sand flies may be a different species of *Leishmania*, *Crithidia fasciculate* or *Tripanosoma* sp. because some sand flies species are reported to harbor these other parasites. These results were supported by a phylogenetic analysis using Neighbor-Joining (NJ) method (Figure 2) based on the analysis of ITS1 showed that two sequences of ITS1 were located in the clade *Leishmania major*, two in the clade *L. naiffi*, one in the clade *L. mexicana*, one in the clade *L. tropica*, two in the clade *L. lainsoni* and six in the clade *Leishmania sp. siamensis* - *Leishmania sp.*

***Table 1**. Sites of collection of sand flies species and associated Leishmania species.*

Province /Locality	Vector identification	Parasite identification	Sample code
Pichincha / Puerto Quito	*Lutzomyia* sp.	*Leishmania major*	F001
	Lutzomyia sp.	*Leishmania major*	F002
Chimborazo / Cumánda	*Lutzomyia* sp.	*Leishmania naiffi*	F003
Guayas / Progreso	*Micropygomyia cayennensis*	*Leishmania mexicana*	F004
Manabí / Bolivar	*Brumptomyia* sp.	*Leishmania* sp - *Leishmania siamensis*	F005
El Oro / Carabota	*Lutzomyia* sp.	*Leishmania* sp - *Leishmania siamensis*	F006
Morona Santiago / Macas	*Lutzomyia tortura*	*Leishmania naiffi*	F007
	Lutzomyia sp.	*Leishmania* sp - *Leishmania siamensis*	F008
	Trichophoromyia ubiquitalis		F009
	Trichophoromyia ubiquitalis	*Leishmania* sp - *Leishmania siamensis*	F010
	Trichophoromyia ubiquitalis	*Leishmania lainsoni*	F011
	Trichophoromyia ubiquitalis	*Leishmania lainsoni*	F012
	Pressatia		F013
	Pressatia		F014
Orellana / Joya de los Sachas	*Lutzomyia* sp.	*Leishmania tropica*	F015
	Lutzomyia sp.	*Leishmania sp - Leishmania siamensis*	F016
Sucumbíos / Gonzalo Pizarro	*Lutzomyia* sp.	*Leishmania sp - Leishmania siamensis*	F017

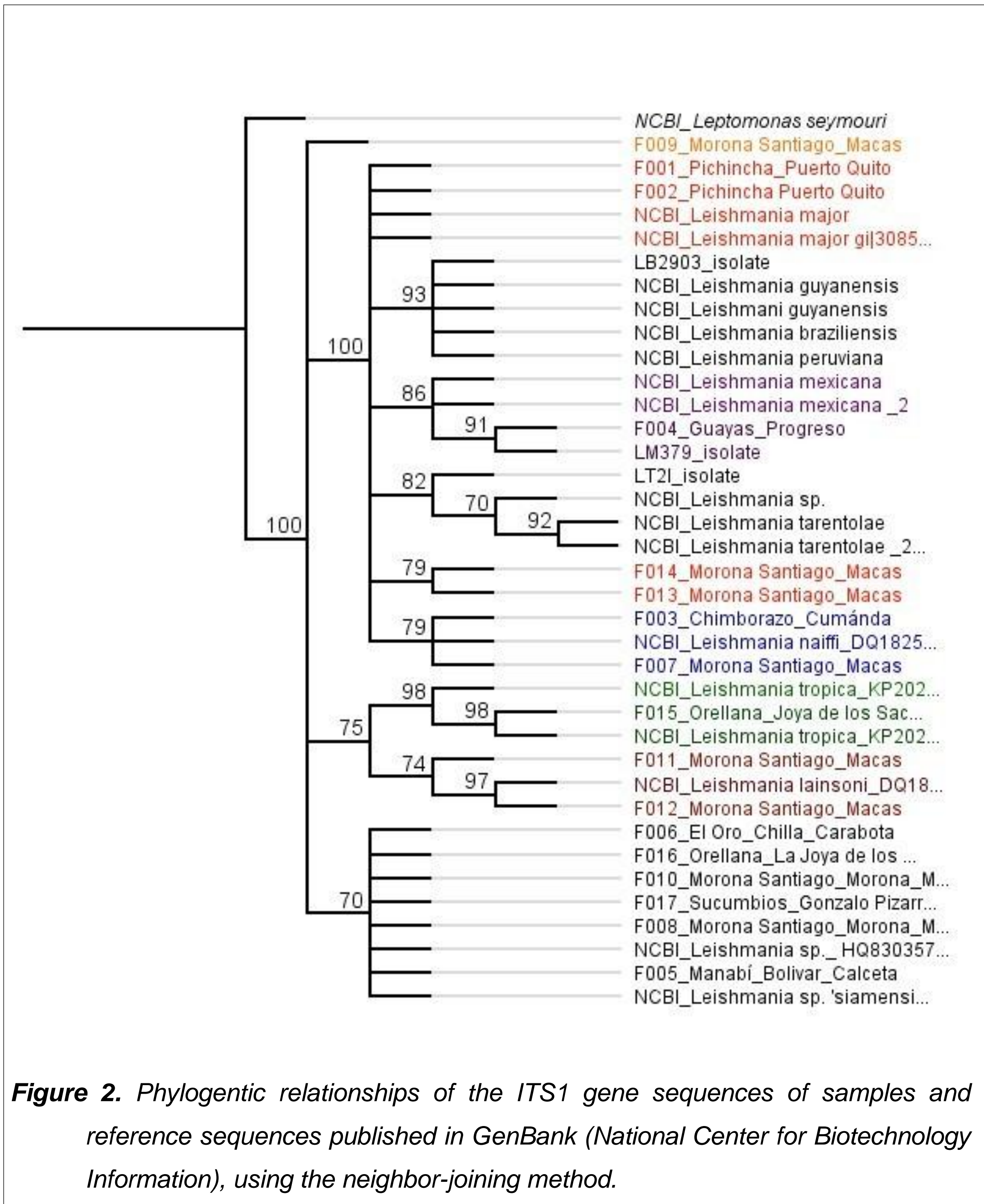

Figure 2. *Phylogentic relationships of the ITS1 gene sequences of samples and reference sequences published in GenBank (National Center for Biotechnology Information), using the neighbor-joining method.*

4. Discussion

The 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 in sand flies for observing promastigotes of *Leishmania* by microscopy has been considered the gold standard test for identifying natural infection in these vectors (Kato et al. 2005). However, for identification of the *Leishmania* species within sand flies, it is necessary to examine of a large number of sand flies, to isolation *Leishmania* parasites in culture from each dissected sand fly, and in some cases, to inoculate parasites in laboratory animals to find true positives. Given the laboriousiness of this procedure, it has been necessary to standardize molecular techniques to easily detect *Leishmania* DNA (Bates et al. 2015).

In the present study, 5 species of *Leishmania* were identified in sand flies by phylogenetic relationships of ITS1 gene sequences.

Leishmania major

Leishmania major is one of the causative agents of cutaneous leishmaniasis in the Old World which is transmitted by *Phlebotomus papatasi* and *P. duboscqi* (Ayari et al. 2015, Wu et al. 2015). In several countries of South America, including Brazil and Ecuador, *L. major* parasites have been identified, but in this region *Leishmania major* is called *Leishmania like major*. Brazil has developed for studies in isoenzym electrophoretic mobility patterns, RAPD and SSR-PCR, that found different biochemical and genetic patterns between these *Leishmania major* and *Leishmania like major*. (Wu et al. 2015, de Oliveira Silva et al. 2009).

In this study, the ITS1 sequences F001 and F002 are clustered with *Leishmania major* (BP= 100; Figure 2). This species called from the Old World that belongs to the subgenus *L. (Leishmania)* has already been reported previouslyreported previously in America. In 2002, *L. major* was reported in clinical isolates in Mexico, as detected by RFLP studies, and that has suggested the recent introduction of this species in the New World (Berzunza-

Cruz et al. 2002). In Ecuador, studies by Hashiguchi & Landires (1991) and Calvopiña et al. (2005) have reported epidemiological records of the presence of *Leishmania like-major* in clinical isolates of patients with cutaneous leishmaniasis. However, in Ecuador there are no records of natural infection in sandflies by this species. This study could not compare the ITS1 sequences with the GenBank sequences that are registered as *Leishmania like -major* because there are no deposited ITS1 sequences of this species. The vector of this species was identified only at the genus level *Lutzomyia* sp. Future analysis will allow ensuring identification of these sand flies to the level of species using molecular tools (Kato et al. 2005).

Leishmania lainsoni

The sequences of ITS1 for samples F011 and F012 of sand flies collected in Macas, Morona Santiago province, were grouped with *Leishmania lainsoni* (BP = 80; 100, Fig 2). In Ecuador, *L. lainsoni* was identified for the first time in the province of Sucumbíos in a clinical sample. However, they couldn't determine the natural infection in *Lutzomyia* species collected in this study (Kato, et al. 2016).

In Bolivia the infection of *L. lainsoni* was registered in *Lutzomyia nuneztovari* (Bastrenta et al. 2002) *L. auraensis,* in Peru (Valdivia et al. 2012) and in Brazil in *Trichophoromya ubiquitalis* (Lainson et al. 1992). The latest record is consistent with the identification of *T. ubiquitalis* of sand flies F011 and F012 part in this study. This first record of natural infection in sand flies allows us to establish the vector incrimination of *L. lainsoni* species in the vector *T. ubiquitalis* and identification possible considerations of the distribution of the disease and its vector.

Leishmania braziliensis, Leishmania tarentolae and unidentificable Leishmania sp.

The LB2903 reference strain was related phylogenetically (BP=89) to sequences of ITS1 from *Leishmania peruviana, Leishmania braziliensis* and *Leishmania guyanensis* deposited in the GenBank. These species have been

reported in the subgenus *L*. (*Viannia)*, complex *Leishmania braziliensis* by Hsp70 (Fraga et al. 2009).

The *Leishmania tarentolae* LT2I isolate used in this study was grouped in the clade of *Leishmania* sp. and *Leishmania tarentolae* of the subgenus *Sauroleishmania*. This is located closer to the subgenus *L*. *(Viannia)* than subgenus *L*. *(Leishmania)* (Figure 2). This result is consistent with that reported by Yang et al. (2013) but it differs from other studies where the subgenus *L. (Sauroleishmania)* is related to the Old World species of the subgenus *L. (Leishmania)* (Fraga et al. 2009, Croan et al. 1997, Zelazny et al. 2005).
This could indicate that there is a genetic similarity between species of subgenus *L.*(*Viannia)* and *L. (Sauroleishmania).* Thus, *Leishmania tarentolae* species is geographically in the New World.

In this study, ITS1 sequences isolated from sandflies F006, F016, F008, F010, F017 and F005 corresponded to 98% and 100% homology to *Leishmania* sp. and *Leishmania* sp*. siamensis* respectively, according to Blastn analysis. However, these isolates grouped in the clade *Leishmania* sp. *and Leishmania* sp. *"siamensis"* (BP = 71, Figure 2) did not group in subgenus *Sauroleishmania* as it was expected according to the classification proposed by (Fraga et al. 2009). A detailed phylogeny of species of *Leishmania* sp. requires a greater number of studies using genes isolated from parasites in different geographic ranges to determine the degree of polymorphisms and to clarify about the evolution and epidemiology of *L tarentolae, L siamensis* sp. and other Leishmania sp. (Berzunza-Cruz et al. 2002, Yang et al. 2013).

Leishmania naifii

Previous studies in the province of Orellana, located in the Amazon region of Ecuador, determined the vector incrimination of *Lutozmyia yuilli* and *L. torture* as vectors of *Leishmania naiffi*, and identified the species of parasite in clinical samples from patients with cutaneous leishmaniasis in the same geographical location (Kato et al. 2013, Kato et al. 2005). Sample F007, collected in Morona Santiago province, was identified as *Lutzomyia torture* and its ITS1 sequence

was located into the *Leishmania naiffi (*BP =89) group. However, this clade is related to the same value (BP =89) with subgenus *Sauroleishmania* and *Leishmania braziliensis* complex (Figure 2). A further analysis with other genes may allow species differentiation between these clades.

Leishmania mexicana

The ITS1 amplicon of F004 samples collected in the province of Guayas grouped in the clade *Leishmania mexicana* and the samples were isolated from *Micropygomyia cayennensis*. The genus *Micropygomyia* 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 Venezuela that registered the natural infection of *Micropygomyia cayennensis* (Feliciangeli 1987), and in Brazil *Micropygomyia quinquefer* has been associated with *Leishmania braziliensis* (Rocha et al. 2010).

Hashiguchi and colleagues (1991) reported possible vector incrimination of *Lutzomyia ayacuchensis* and *Leishmania mexicana* was established. However, Kato and colleagues (2005) registered the presence of *Lutomyzia ayacuchensis* only in the provinces of Manabí, Pichincha, Chimborazo and Azuay. Similarly, the analysis of the geographical distribution of cases of cutaneous leishmaniasis by the phylogenetic relationships based on cytochrome b found the presence of *Leishmania mexicana* in the area of the Andes of Ecuador (Kato, et al. 2016). To validate this study with other studies, it will be necessary to amplify the sequence analysis with other molecular markers such as cytochrome b, and ITS2 to compare polymorphisms between species and determine their distribution in the country.

Leishmania tropica

Leishmania tropica is determined as one of the causal species of visceral leishmaniasis in humans in the Old World (Sarkari et al. 2016, Akhoundi et al. 2016). In our study, the ITS1 sequence of sample F015 corresponded with 98% homology to the *Leishmania tropica* clade (BP = 100). In Ecuador and South America there are no epidemiological record cases of visceral leishmaniasis due to *L. tropica* therefore our results require validation by studies involving a larger number of sandflies and clinical samples in the province of Orellana. On the other hand, our phylogenetic tree indicates a grouping of *Leishmania tropica* clade and *L. naiffi* implying a sequence similarity that requires an analysis of polymorphisms between these two types of species.

5. Conclusions

The distribution and identification of *Leishmania* species require standardization of methodologies to discriminate between DNA isolated from clinical samples and sand fly vectors. Similarly, the correct vector incrimination requires the right identification of the vectors; the wide variety of species of sand flies did not allow to relate vectors and parasites of all species of *Leishmania* including the nonpathogenic for humans. Therefore, only the whole genome sequencing of the isolated parasite would generate consensus regions and establish target nucleotide sequences of (mitochondrial or nuclear genes) for inter-species difference.

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