Tegumentary leishmaniasis by *Leishmania braziliensis* complex in Bolivia: the presence of *L. braziliensis* outlier

Leishmania braziliensis complex in Bolivia

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ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to.

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SUMMARY

Leishmaniasis is caused by protozoans of the *Leishmania* genus, which includes more than 20 species capable of infecting humans worldwide. In the Americas, the most widespread specie is *L. braziliensis*, present in 18 countries, including Bolivia. The taxonomic position of the *L. braziliensis* complex has been a subject of controversy, complicated further by the recent identification of a particular subpopulation named *L. braziliensis* atypical or outlier. The aim of this study was to carry out a systematic analysis of the *L. braziliensis* complex in Bolivia and to describe the associated clinical characteristics. Forty-one strains were analyzed by sequencing an amplified 1245 bp fragment of the *hsp70* gene, which allowed its identification as: 24 (59%) *L. braziliensis*, 16 (39%) *L. braziliensis* outlier and one (2%) *L. peruviana*. In a dendrogram constructed, *L. braziliensis* and *L. peruviana* are grouped in the same cluster, whilst *L. braziliensis* outlier appears in a separate branch. Sequence alignment allowed the identification of five non-polymorphic nucleotide positions (288, 297, 642, 993 and 1213) that discriminate *L. braziliensis* and *L. peruviana* from *L. braziliensis* outlier. Moreover, nucleotide positions 51 and 561 enable *L. peruviana* to be discriminated from the other two taxa. A greater diversity, was observed in L. *braziliensis* outlier than in *L. braziliensis*-*L. peruviana*.

The 41 strains came from 32 patients with tegumentary leishmaniasis, among which 22 patients (69%) presented cutaneous lesions (11 caused by L. *braziliensis* and 11 by *L. braziliensis* outlier) and ten patients (31%) mucocutaneous lesions (eight caused by *L. braziliensis*, one by *L. braziliensis* outlier and one by *L. peruviana*). Nine patients (28%) simultaneously provided two isolates, each from a separate lesion, and in each case the same genotype was identified in both. Treatment failure was observed in six patients infected with *L. braziliensis* and one patient with *L. peruviana*.

Keywords: Sequencing *hsp70* gene, *Leishmania braziliensis* complex, clinical characteristics, tegumentary human leishmaniasis, Bolivia.

1. INTRODUCTION

Leishmaniasis is caused by protozoans of the *Leishmania* genus, which includes more than 20 species capable of infecting humans worldwide. The parasites are transmitted to humans and vertebrate animals by phlebotomine sand flies (Pan American Health Organization/World Health Organization, 2019a; Organización Mundial de la Salud, 2020). The most extended specie in the Americas is *L. braziliensis*, which is present in 18 countries (Pan American Health Organization/World Health Organization, 2019b). *L. braziliensis* is widespread in areas of tropical forests, where it is transmitted in wild, peridomestic and domestic cycles (Ballart et al., 2016; Campbell-Lendrum et al., 2001; Davies et al., 2000; Rojas et al., 2009). This specie is reported to cause tegumentary leishmaniasis (TL) in up to 90% of suspected cases (Garcia et al., 2007; Teles et al., 2015). Although patients with TL can suffer simultaneously from localized cutaneous (CL) and mucocutaneous leishmaniasis (MCL), more frequently the skin lesions evolve to a destructive mucosal inflammation years after their first appearance and when the CL has apparently healed (Burza et al., 2018; Kevric et al., 2015; Reithinger & Dujardin, 2007). After treatment, mucosal lesions can leave mutilating and disfiguring sequelae and even be fatal due to associated infections (Organización Panamericana de la Salud/Organización Mundial de la Salud, 2018; Pan American Health Organization/World Health Organization, 2019b). The other specie within the *L. braziliensis* complex, *L. peruviana*, is limited to regions of the inter-Andean valleys in Peru, and causes skin lesions, with rare affectation of the mucosa (Davies et al., 2000; Kato et al., 2019; Perez et al., 2007).

The taxonomic position of the *L. braziliensis* complex has been a subject of controversy, *L. braziliensis* and *L. peruviana* being considered as variants of the same species or as two distinct species within the complex (Arana et al., 1990; Bañuls et al., 2000; Chouicha et al., 1997; Valdivia et al., 2015; Van der Auwera et al., 2015; Van den Broeck et al., 2020). Investigations carried out in Peru have reported the polymorphism of *L. braziliensis* complex, observing two genotypically different groups (groups 1 and 2), and *L. peruviana* has been included in group 1 (Van der Auwera et al., 2014). Other studies have identified hybrids of *L. braziliensis/L. peruviana* (Dujardin et al., 1995; Kato et al., 2019; Koarashi et al., 2016;) that are capable of producing mucosal lesions (Nolder et al., 2007).

The application of different molecular tools, such as the amplified fragment length polymorphisms (AFLP) and the sequencing of a *hsp70* gene fragment, has revealed high genetic diversity among isolates of the *L. braziliensis* complex and allowed the identification of a particular subpopulation named *L. braziliensis*

atypical or outlier (Odiwuor et al., 2012; Van der Auwera et al., 2013). This subpopulation is apparently widespread in Latin America, being detected in Peru, Bolivia and Panama (Fraga et al., 2013; Van der Auwera et al., 2016), although few clinical or epidemiological information has been described (Fraga et al., 2013). Previous studies have shown that *L. braziliensis* atypical or outlier strains exhibit a high degree of similarity (98.9-99.7%) for the *mpi*, *mdh*, *gpi* and *6pgd* genes, with respect to the other isolates of *L. braziliensis*; however, it has several unique polymorphisms in the four genes (Tsukayama et al., 2009).

Considering the genetic variability of the *L. braziliensis* complex and the fact that atypical isolates of *L. braziliensis* have been reported in Bolivia, the aim of the present research was to carry out a systematic study of the complex in the Bolivian department of Cochabamba and identify atypical isolates of *L. braziliensis* by sequencing an *hsp70* gene fragment. Also, the clinical characteristics associated with the complex and the response to TL treatment were investigated. By updating the epidemiological situation of the disease, the results will help this pathology to be controlled in Bolivia.

2. METHODS

2.1. Study population

Thirty-two patients with cutaneous, mucosal or mucocutaneous lesions, attending the Service of Dermatology of the Tropical Medicine Center at the Faculty of Medicine, Universidad Mayor de San Simón (UMSS) (Cochabamba, Bolivia) from September 2014 to November 2015, were included. Those with suspected CL and MCL leishmaniasis were referred to the Parasitology laboratory service in the same center for the sample collection.

2.2. Definitions

Clinical forms: Patients presenting only cutaneous lesions in any area of the body were classified as CL, and those with mucosal lesions in mouth and/or nostrils, or concomitant cutaneous and mucosal lesions, were classified as MCL.

Treatment failure: Patients who had a relapse due to treatment failure were identified from historical clinical data (Ballart et al., 2021). Treatment failures were considered as CL and MCL patients who had previously

119 received a complete anti-leishmanial treatment in the past, regardless of its duration, and were not cured. In
120 the case of MCL, patients presented an absent or incomplete scarring of lesion(s) and/or persistence of
121 inflammation around the initial lesion, and/or clinical regression of a healed lesion and/or the presence of
122 new mucosal lesion(s). In the case of CL, the previous treatment had to be directed to the same lesion(s)
123 identified during our study.

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125 **2.3. *Leishmania* isolation**

126 *Leishmania* isolates were obtained by aspiration of the edge of the lesions (Torrico and Zubieta, 2010) and
127 cultured in TSTB media at 26-27ºC (Bermudez et al., 2005). Isolates were cryopreserved at -80˚C in the
128 laboratory of Parasitology at the UMSS in Bolivia and then sent to the Parasitology Laboratory at the
129 University of Barcelona (UB), Spain. Promastigotes were recovered by thawing isolates in a water bath at
130 37ºC and cultured in parallel with NNN medium and Schneider's medium (Sigma-Aldrich) supplemented
131 with 20% fetal bovine serum (Life Science Production) and 1% of sterile human urine. When the exponential
132 growth stage was reached, cultures were washed with PBS, and the pellet resuspended in 1 ml of PBS to
133 proceed with the DNA extraction.

134

135 **2.4. DNA extraction, amplification, purification and gene sequencing**

136 DNA extraction was performed using the commercial QIAmp DNA Mini Kit (Qiagen) from 200 µl of
137 culture in PBS that was treated with 20 µl of proteinase K following the manufacturer's instructions. The
138 extracted DNA was eluted in 100 µl of AE buffer and stored at -20ºC until use. Subsequently, the DNA was
139 quantified in the EpochTM Multi-Volume Spectrophotometer System (BioTek) reader, and the extractions in
140 which the DNA ratio 260/280 was equal to or less than 2 were processed.
141 The amplification of the 1245bp *hsp70* gene was performed by using two PCRs that together cover this
142 fragment: PCR-N (552bp) and PCR-T (723bp), according to a previously described protocol (Van der
143 Auwera et al., 2013), with the following modifications: for each reaction were used 1 µM of buffer + $MgCl_2$,
144 200 µM dNTPs, 1 µM of each primer, 1.5 U of DreamTaq DNA polymerase (Thermo Scientific), 5 µL of the
145 extraction product and sterile distilled water to adjust the final volume to 50 µL. The PCR products, together
146 with a negative control, were assessed by 1% agarose gel electrophoresis, 0.5% TBE buffer and ethidium

147 bromide at 100V for 45 minutes. The fragments were identified in comparison with the DNA Molecular
148 Weight Marker VIII (Roche).
149 Amplicons were enzymatically purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo
150 Scientific), in a ratio of 2:5 (4 µl of ExoSAP in 10µl amplified product). Double-strand DNA was sequenced
151 by the Sanger method. The sequencing was carried out at the Scientific and Technologic Centers of the UB
152 (Spain).
153
154 **2.5. Sequence alignment, identification and polymorphism analysis**
155 The sequences obtained were analyzed and edited with the MEGA 7.0.26 program and submitted to the
156 GenBank databases under the accession numbers MW507486-MW507526 (http://www.ncbi.nlm.nih.gov).
157 Minor errors such as undefined or mismatched nucleotide positions were manually corrected.
158 Chromatogram positions with two overlapping nucleotide peaks were considered heterozygous and
159 were corrected according to the IUPAC ambiguity codes (IUPAC, 2020). The alignment of the
160 forward and reverse sequences of each fragment and the subsequent alignment of both fragments
161 was performed with the SerialCloner 1.3.11 program to obtain the consensus sequence of the *hsp70*
162 gene. To identify the strains sequenced, the BLAST (Basic Local Alignment Search Tool) program
163 was used, which compares the entered sequence with those published in the GenBank database,
164 allowing its identification when regions of similarity are found and the statistical significance
165 calculated.
166 Only the strains characterized within the *L. braziliensis* complex were taken into account for sequence
167 alignment, population study and polymorphism analysis, in order to describe their genetic and phenotypic
168 characteristics. The sequences obtained were analyzed together with additional sequences of 27 selected
169 reference strains of the *L. braziliensis* complex (*L. braziliensis, L. peruviana, L. braziliensis* outlier) and
170 other New World species (*L. lainsoni, L. naiffi, L. guyanensis, L. panamensis*) from GenBank
171 (www.ncbi.nlm.nih.gov) (Supplement Table 1) with the aim of evaluating the location of the studied
172 *Leishmania* strains in the dendrogram. These reference strains were selected with the following criteria: they
173 were published, covered at least 1245 bp of the *heat-shock protein 70* (*hsp70*) gene, and were preferably

isolated from humans in Bolivia or neighboring countries. Alignment was done with the ClutalW function of the MEGA 7.0.26 program. The ends of the sequences of the reference strains were trimmed to obtain a consensus length of 1245 bp of the *hsp70* gene in the studied Bolivian strains.

Dendrograms were built and the evolutionary relationship of the sequences was calculated using the Neighbor-joining statistical method. All nucleotides were duplicated in order not to lose the information of the heterozygous positions, and the clustering analysis was done using the MEGA 7.0.26 and BioNumerics 7.6.3 (Applied Maths) programs. As external group, a *Trypanosoma cruzi* sequence (KC960000.1) obtained from GenBank was added. The monophyletic groups were calculated with a bootstrap of 1000 replicates. Evolutionary distances were calculated using the p-distance method. In parallel, Neighbour-nets (NN) were constructed using Splitstree version 4.14.8 software (Huson and Bryant, 2006) from hsp70 datasets using the uncorrected p method and an equal angles representation.

Polymorphism analysis was carried out manually, identifying the nucleotide positions with variability between isolates, which allowed us to discriminate them at the species level and to identify differences in the nucleotide positions between species and sub-species within the *L. braziliensis* complex.

The genetic diversity of sequences and the diversity of haplotypes for the *hsp70* gene was calculated using the program DnaSP v.5.10.01.

The clinical data collected was related to the typified species and subspecies.

2.6. Ethical aspects

This research was carried out with isolates from patients with suspected TL attending the LABIMED, as part of a collaborative research project between ISGlobal (Barcelona, Spain) and CEADES Foundation (Cochabamba. Bolivia). The study protocol was approved by the Ethics Committees of the *Hospital Clínic de Barcelona* in Spain (HCB/2014/0582); *CEADES Salud y Medio Ambiente* and the *Facultad de Medicina* UMSS both in Bolivia. All suspected cases of leishmaniasis provided written informed consent (parents or guardians in case of patients under 18 years old) before participating to the study. All leishmaniasis suspects were diagnosed for free and CL and MCL confirmed cases were referred for treatment.

3. RESULTS

3.1. Identification of the *Leishmania braziliensis* complex

A total of 41 Bolivian strains of the *L. braziliensis* complex (obtained from 32 patients) were analyzed. On the basis of the BLAST results, 24 (59%) were identified as *L. braziliensis* and 16 (39%) as atypical forms of *L. braziliensis* (outlier). The percentage of similarity of these strains with respect to the GenBank reference strains was 99.76-100% (Table 1). One strain (MHOM/BO/2015/CUM-1372) presented the same percentage of similarity (99.92%) to both *L. peruviana* and *L. braziliensis* reference strains, but was finally characterized as *L. peruviana*.

3.2. Population study

The dendrogram obtained with the alignment of the 68 sequences (41 strains of the present study, plus the 26 reference strains of *Leishmania* obtained from GenBank and one sequence of *T. cruzi* obtained from the GenBank used as external group) is shown in Figure 1. Two clusters are observed, one corresponding to the subgenus *Viannia* and the other to the subgenus *Leishmania* (*L. mexicana* and *L. amazonensis* reference strains). Within the *Viannia* subgenus, the *L. lainsoni* strains are separate from the others in their own branch. The *L. guyanensis* and *L. braziliensis* complexes bifurcate from the other branch. *L. braziliensis* and *L. peruviana* appear grouped in a cluster, whilst *L. braziliensis* outlier is grouped in a separate branch together with *L. naiffi* (bootstrap values of 98 and 70, respectively). In the branch of *L. peruviana*, the Bolivian strain CUM-1372 is found in a sub-branch together with a reference strain of *L. braziliensis*.

The Neighbor net analysis identifies three distinct genetic clusters showing that *L. brazilienis* outlier is clearly separated from *L. braziliensis* and the presence of possible hybrids of *L. braziliensis*/*L. peruviana* (CUM 1343, CUM 1352) located between *L. brazilienis* and *L. peruviana* clusters (Figure 2).

3.3. Polymorphism analysis

The alignment of the *hsp*70 gene fragment of 1245 bp allowed the identification of three species of the *L. braziliensis* complex: *L. braziliensis* (24 strains), *L. peruviana* (1 strain) and *L. braziliensis* outlier (16 strains), which differ in seven nucleotide positions (Table 2). Five non-polymorphic nucleotide positions (288, 297, 642, 993 and 1213) allow *L. braziliensis* outlier to be discriminated from *L. peruviana* and *L. braziliensis*. Nucleotide positions 51 (A) and 561 (A) enable to distinguish *L. peruviana* from the other two taxa. This study revealed 16 genotypes (1-16) among the Bolivian strains (six genotypes of *L. braziliensis*,

nine of *L. braziliensis* outlier, and one of *L. peruviana*), plus six different genotypes found among the reference strains (17R-22R) (two genotypes of *L. braziliensis,* three of *L. braziliensis* outlier and one of *L. peruviana)*.

Twenty-seven per cent of the strains (n=11; 4/24 *L. braziliensis*, 1/1 *L. peruviana* and 6/16 *L. braziliensis* outlier) showed heterozygosity in any of the nucleotide polymorphic positions analyzed. Traces of genetic exchange between *L. braziliensis* and *L. peruviana* were observed in some of the strains (genotypes 4, 5, 6).

The genetic diversity analysis of the *hsp70* gene fragment performed by the DnaSP v.5.10.01 program, including the *L. braziliensis-L. peruviana* group and the *L. braziliensis* outlier sequences, showed four polymorphic sites and four mutations in both groups analyzed (Table 3). A greater genetic diversity (Hd = 0.8) and nucleotide diversity (π = 0.00124) were observed in *L. braziliensis* outlier compared to *L. braziliensis-L. peruviana.*

3.4. Clinical characteristics of Bolivian *Leishmania braziliensis* complex strains

The 41 strains were obtained from 32 patients, 23 (69%) of which provided a single isolate. In the nine patients (31%) providing two isolates, each from a separate lesion and taken on the same day, the same genotype was identified for both isolates in each case. The clinical characteristics associated with the identified species are presented in Table 4. *L. braziliensis* outlier was identified as a causal agent of mucosal involvement in one case (CUM-1307), and was not associated with treatment failure. One third of the patients (6 out of 19 patients, 32%) infected by *L. braziliensis* suffered treatment failure: four had skin lesions (one to three lesions) with an average evolution of 4 months (from 2 to 8 months); the single patient with a chronic skin lesion did not remember the time of onset, nor did the one patient with chronic mucosal lesions. The *L. peruviana* strain was isolated from a patient suffering from mucosal lesions and with treatment failure (CUM-1372).

The CUM-1372 strain, which was identified as *L. peruviana*, was isolated from a 71-year-old male patient living in Puerto Zudañes (Chapare province, Cochabamba), who had two scars, one on the face and one on an upper limb, and active lesions in the nostrils. The patient referred that the skin lesions were active approximately 5 years ago (around 2010) and were healed with one or two ampoules of meglumine antimoniate (Glucantime®) purchased from local pharmacies and applied repeatedly. In addition, the patient reported that in 2010 he already had mucosal lesions, and in 2015 he traveled to the city of Cochabamba to

request medical attention being confirmed the diagnostic of MCL in our laboratory. He first received a complete treatment with Glucantime® but did not improve. After several months, he was hospitalized for three months to receive a complete treatment with amphotericin B, which led to an apparently complete recovery. In 2015, the mucosal lesions reactivated and he returned to Cochabamba to receive another full treatment with amphotericin B.

4. DISCUSSION

Identification of *the Leishmania braziliensis* complex and polymorphism analysis

Several techniques (biochemical, molecular and proteomic) have been used to identify the *Leishmania* species, responsible for different clinical forms of leishmaniasis around the world (Akhoundi et al., 2017; Arana et al., 1990; Lachaud et al., 2017; Van der Auwera et al., 2013; 2014). PCR and sequencing of conserved gene amplified products are currently widely employed. The genes encoding the *hsp*70 proteins were among the first to be used in kinetoplastid characterization, as they are highly conserved and present in various copies in tandem (Folgueira et al., 2007). They have been useful for phylogenetic and taxonomic studies of *Leishmania* in both the New and Old Worlds (Conter et al., 2019; Odiwuor et al., 2012; Van der Auwera et al., 2013; Van der Auwera & Dujardin, 2015) and have proved to be suitable and sensitive targets for the typing of neotropical *Leishmania* species from tissues (Garcia et al., 2004) giving reproducible results.

In the present study, PCR and sequencing of a 1245bp amplified fragment of the *hsp70* gene allowed the phylogenetic analysis of *L. braziliensis* complex strains isolated in Bolivia, as already carried out in other countries in Latin America (Fraga et al., 2010; Van der Auwera et al., 2014; Van der Auwera & Dujardin, 2015). Using the GenBank BLAST online program, the obtained sequences were identified as 59% (24/41) *L. braziliensis,* 2% (1/41) *L. peruviana* and 39% (16/41) *L. braziliensis* outlier (similarity between 99.76 and 100% with the GenBank reference strains). The identification of *L. braziliensis* outlier has epidemiological and clinical relevance, as no systematic studies of this kind with TL-causing *Leishmania* strains have been carried out previously in Bolivia, or in other Latin American countries, despite the fact that several authors have reported atypical species in Peru, Panama and Bolivia (Fraga et al., 2013; Odiwuor et al., 2012). This group of parasites have been named "atypical *L. braziliensis*" (Fraga et al., 2013), *"L. braziliensis* type 2"

(Van der Auwera et al., 2014), *"L. braziliensis* type 3" (Odiwuor et al., 2012) and "*L. braziliensis* outlier" (Van der Auwera et al., 2013), Indeed, there is not yet a consensus in their taxonomic name neither position, despite being more prevalent and easier to discriminate than *L. peruviana*. A more detailed phylogenetic study is needed to clarify its taxonomic status (Van der Auwera et al., 2015).

Based on the findings of this study, the CUM-1372 strain was identified as *L. peruviana.* Although it shares a similar genetic profile (99.92%) with both *L. peruviana* (MHOM/PE/01/PER006/1 GenBank reference strain) and *L. braziliensis* (MHOM/PE/1990/HB86 GenBank reference strain), this strain has the discriminative nucleotide positions 51A and 561A found in all *L. peruviana* GenBank reference strains and clusters with the *L. peruviana* group in the dendrogram (see Table 2 and Figure 1)**.** These results suggest that the reference strain MHOM/PE/01/PER006/1 (MHOM/PE/01/LH2140), characterized as *L. braziliensis* (GenBank accession number FR715987.1) (Adaui et al., 2011), may also belong to *L. peruviana* (genotype 19R of our study). In fact, this reference strain is reported to have mixed alleles from both species, clustering with *L. peruviana* in some studies and with *L. braziliens*is in others (Odiwuor et al., 2012; Van der Auwera et al., 2013).

Our study as well demonstrated the genetic diversity of the *hsp70* gene among the *L. braziliensis* complex, as shown in the dendrogram (see Figure 1). The *L. braziliensis* complex is divided into two clearly differentiated clusters. The first cluster corresponds to the strains of the so-called *L. braziliensis* "outlier" (bootstrap 70), genetically different from *L. braziliensis*, and grouped together with the reference strains of *L. naiffi* (bootstrap 100). The second cluster corresponds to *L. braziliensis* (bootstrap 98), where the Bolivian and reference strains of *L. peruviana* are also located (bootstrap 54). Similar results have been obtained in studies using AFLP of the *hsp70* gene or MLST analysis of four markers (*hsp70* gene, 7SLRNA gene, rDNA ITS1 and miniexon) (Odiwuor et al., 2012; Van der Auwera et al., 2014). However, in some cases, despite clustering separately, *L. braziliensis* outlier and *L. braziliensis* were considered to be sister clades, *L. peruviana* being grouped with the latter (Van der Auwera et al., 2013).

L. braziliensis is known to be the predecessor of *L. peruviana*, which evolved in adaptation to the different ecosystem in Peru (Dujardin et al., 1995), with hybrids of *L. braziliensis*/*L. peruviana* being reported (Kato et al., 2016; 2019; Nolder et al., 2007; Odiwuor et al., 2012). However, nothing is known about the evolution of *L. braziliensis* outlier. A geographical origin could be ruled out, as *L. braziliensis* and *L. braziliensis* outlier are both widely distributed in Latin America, namely in Peru, Panama and Bolivia (Fraga et al.,

2013), and it seems that the two groups of parasites are sympatric. Due to parasites included in this study were isolated during two consecutive years, the identified genotypes existed simultaneously and the possibility of a time bias can be excluded (Odiwuor et al., 2012).

The polymorphism analysis of the 1245 bp sequence of the *hsp70* gene allowed the identification of seven positions capable to discriminate among *L. braziliensis*, *L. peruviana* and *L. braziliensis* outlier: five positions (288, 297, 642, 993 and 1213) distinguish *L. braziliensis* outlier from the others, and two positions (51 and 561) are characteristic of *L. peruviana*. In addition, the finding of 16 genotypes within the *L. braziliensis* complex (6/24 *L. braziliensis* strains, 9/16 *L. braziliensis* outlier strains and 1/1 classified here as *L. peruviana* (genetic profile 99.92% similar to *L. peruviana*), indicates a high degree of polymorphism in the *hsp*70 gene, especially among *L. braziliensis* outlier strains.

Four types of ambiguities were identified (R, Y, K, M), with up to three ambiguities in the strains from genotype 6 (CUM-1343 and CUM-1352) corresponding to possible hybrids of *L. braziliensis*/*L. peruviana*. This finding is clinically relevant because hybrids can potentially cause mucosal leishmaniasis (Nolder et al., 2007). In our study, only the CUM-1343 strain was associated with a chronic mucocutaneous lesion with evolution time unknown (> 12 months), whereas the CUM-1352 strain was associated with skin lesion with three months of evolution. None of these two strains responded to the treatment with meglumine antimoniate (Glucantime®).

A previous investigation carried out with isolates from Peru, Panama and Bolivia, based on the analysis of a 1380 bp sequence of the *hsp70* gene, also showed ambiguities: up to four in group 1 (*L. braziliensis)* and group 3 (*L. braziliensis* atypical), and as many as seven in intermediate isolates between group 1 and 3 (Odiwuor et al., 2012), which may be due to *L. braziliensis*/*L braziliensis* outlier hybrids (Van der Auwera et al., 2013). The presence of numerous ambiguities could be related to genetic recombination events among parasite populations, as shown in a study of the *L. braziliensis* complex, or to genetic exchange, as demonstrated in *L. donovani* (Boité et al., 2012; ; Fernández-Arévalo et al., 2020; Lukeš et al., 2007). The presence of *L. braziliensis* outlier with three nucleotide ambiguities suggest low level of genetic recombination in the strains circulating in Bolivia or possibly clonal and occasional sexual reproduction (Cupolillo et al., 1998). More extended genetic analysis, such as sequencing a greater number of genes, could allow the typing of highly heterogeneous complexes. For instance, the sequencing analysis of four genes (*mpi, mdh, gpi,* and *6pgd*) with atypical patterns in MLEE, has allowed the characterization of New

World *Leishmania* species. Moreover, as a high number of single nucleotide polymorphisms in these genes are found on different chromosomes, it is thought that the variation is distributed throughout the genome, indicating that the divergence of this group of atypical parasites did not occur recently (Tsukayama et al., 2009).

On the other hand, the CUM-1372 strain (identified as *L. peruviana*) presented a single ambiguity (Y) in position 978, which is the only difference with the sequences of the *L. peruviana* reference strains used in this study. Thus, this could represent the first report of *L. peruviana* in Bolivia, or a possible hybrid of *L. braziliensis*/*L. peruviana*, as it has the genetic profile of both species.

A previous study with complete genome sequencing including 67 strains of the *L. braziliensis* complex from Peru suggested that deforestation in the last 150,000 years has influenced the speciation and diversity of parasites, and whole genome analysis demonstrated a meiotic-like recombination between Andean and Amazonian *Leishmania* species, resulting in a full-genome hybrid (Van den Broeck et al., 2020). The identification of possible hybrids in Bolivia is therefore another reason to suspect that *L. peruviana* could also be circulating in this country.

The genetic diversity of the *L. braziliensis* complex was demonstrated here by analyzing the *hsp70* gene sequence; the genetic diversity index showed that *L. braziliensis* outlier has greater diversity than *L. braziliensis*, both haplotype (Hd = 08) and nucleotide (π = 0.00124). These data support that *L. braziliensis* outlier diverges from *L. braziliensis*, which can also be observed in both the Neighbor-joining dendrogram, where *L. braziliensis* outlier separates from the branch of *L. braziliensis* (bootstrap 80), and in the Neighbor-Net analysis.

Clinical characteristics of Bolivian *Leishmania braziliensis* complex strains

The *L. braziliensis* complex was identified as responsible for TL in 85.4% (n = 41) of the isolates obtained in the region of Cochabamba in Bolivia. Our results agree with those described in other endemic areas of TL in Bolivia, such as 93% in Chapare, as well department of Cochabamba, and 65.5% in the department of La Paz (Bilbao-Ramos et al., 2017). Similar results have been obtained in other Latin American countries (Davies et. al., 2000; Montalvo et al., 2016; Teles et al., 2015).

In our study, nine of the 32 (28%) patients presented chronic mucosal lesions with more than 12 months of evolution and involvement of the oral and nasal mucosa: one patient by *L. braziliensis* outlier, one by *L.*

peruviana and seven by *L. braziliensis*. According to previous reports, from 1 to 10% of skin lesions caused by *L. braziliensis* tend to develop serious and disfiguring mucocutaneous lesions, and can even lead to pneumonia (Burza et al., 2018; Pan American Health Organization/World Health Organization, 2019b).

In the present study, *L. braziliensis* outlier (n = 16) was isolated from skin lesions more frequently (87.5%). Two strains of *L. braziliensis* outlier were isolated simultaneously from chronic mucosal lesions (oral and nasal > 12 months of evolution) corresponding to the same patient. So far, few publications have described the clinical and epidemiological characteristics of infections with *L. braziliensis* outlier in other countries; it has been isolated from patients with skin lesions (Odiwuor et al., 2012), whereas in a study that typified five strains as *L. braziliensis* outlier, three were obtained from mucocutaneous lesions and two from skin lesions (Tsukayama et al., 2009). In addition, hybrids of *L. braziliensis/L. braziliensis* outlier have also been reported, but without a description of the clinical lesions (Van der Auwera et al., 2013).

We identified *L. peruviana* in a patient with mucosal lesions in Bolivia without a history of traveling to Peru, and who works in agriculture in the Chapare region (Cochabamba). The distribution of *L peruviana* was thought to be restricted to endemic areas in the rural Andean and inter-Andean valleys of Peru (between 1000 and 3000 m above sea level) (Arevalo et al., 2007; Koarashi et al., 2016; Kato et al., 2019; Lucas et al., 1998), but it has also been reported from a lowland area and other ecoregions of Peru, including the Amazonian jungle (Arevalo et al., 2007). *L. peruviana* usually causes a benign form of cutaneous leishmaniasis known as uta (Laison & Shaw, 1987; Van den Broeck et al., 2020), but cases of mucosal and disseminated leishmaniasis associated with this species have been reported (Espinoza-Morales et al., 2017; Lucas et al., 1998; Organización Mundial de la Salud., 2010), as well as with *L. guyanensis*, *L. panamensis*, etc., depending on the immunological status of the patient (Organización Mundial de la Salud., 2010; Pan American Health Organization/World Health Organization, 2019b).

In a study using *hsp70* PCR RFLP, a Bolivian *L. braziliensis* strain isolated from a mucocutaneous lesion (CUM-29, FN395041) also showed an *L. peruviana* profile (Montalvo et al., 2010). Therefore, the authors considered that more intra-species studies with a higher number of isolates of the *L. braziliensis* complex need to be carried out to explain this behavior. In addition, we identified possible hybrids of *L. braziliensis*/*L. peruviana* (CUM-1343 and CUM-1352), which are also suspected in Colombia (Montalvo et al., 2016). Hybrids of *L. braziliensis*/*L. peruviana* have been identified in humans, dogs and sand flies in Huánuco, Peru, where both species are endemic, and are potentially causative of mucosal lesions (Dujardin

et al., 1995; Nolder et al., 2007; Kato et al., 2016). Likewise, the hybridization of strains could have clinical implications related to the behavior of the parasite, adaptation to the vector and response to treatment (Hamad et al., 2011).

In Bolivia, leishmaniasis is generally treated with meglumine antimoniate for skin lesions and amphotericin B for mucosal lesions; miltefosine, ketoconazole, and itraconazole, among others, are also used with variable results (Ministerio de Salud de Bolivia, 2015). In our study, 22% (7/32) of the patients infected with the *L. braziliensis* complex presented treatment failure; six patients infected with *L. braziliensis* failed to respond to meglumine antimoniate (two with cutaneous lesions and two with mucocutaneous lesions), whereas only the single patient infected with *L. peruviana* did not improve with amphotericin B. Previous studies in Bolivia reported treatment failure with pentavalent antimonials, miltefosine and even amphotericin B deoxycholate, resulting in the use of combined treatment strategies (Rojas Cabrera et al., 2017) or perilesional treatment (Rojas Cabrera et al., 2019).

Another study in Brazil found an effectiveness of 53.8% in patients infected with *L. braziliensis* treated with meglumine antimoniate whereas a study in Peru reported therapeutic failure in 30.4% of patients infected with *L. braziliensis* and 24.5% for *L. peruviana* (Arevalo et al., 2007). Likewise, it was reported that lesions <5 weeks of evolution, multiple lesions and infection by *L. braziliensis* have the highest risk of therapy failure with sodium stibogluconate (Pentostan®) (Llanos-Cuentas et al., 2008). Comparing these results with ours with meglumine antimoniate, similarities can be observed in the group: multiple lesions (in our study, two and three), chronic evolution (> eight weeks) and the species, as all were typified as *L. braziliensis sensu stricto.*

In our sample, none of the patients infected with *L. braziliensis* outlier (n=16) presented treatment failure during the time of study, including the patient with mucosal lesions. There are no scientific reports referring to the clinical and epidemiological characteristics of *L. braziliensis* outlier infection, possibly because the typing techniques routinely used cannot discriminate the species within the *L. braziliensis* complex (Odiwuor et al., 2012). Thus, more molecular studies on species discrimination and their clinical and epidemiological relevance are required (Tsukayama et al., 2009).

An important observation in our study, not previously reported in Latin America, is that a patient with chronic mucosal lesions (isolate CUM-1372, *L. peruviana*) failed to respond to treatment with both meglumine antimoniate and amphotericin B deoxycholate. Amphotericin B is not usually recommended

due to its toxicity (renal, hepatic, etc.), and is only used as an alternative in cases of treatment failure with first-line drugs or in special situations (Organización Panamericana de la Salud, 2013; Rodríguez Galviset al., 2020). Treatment failure is known to be more likely in mucosal lesions (Ministerio de Salud de Bolivia, 2015), possibly because this clinical manifestation is related to the immunocompromised status of the patient (perhaps due to difficulty in feeding), a factor that could contribute to the severity of the infection as well as the treatment failure of amphotericin B (Nweze et al., 2020; Van Griensven et al., 2014).

In conclusion, this first systematic genetic analysis of *L. braziliensis* complex isolates from Bolivia has revealed the presence of all the species, *L. braziliensis*, *L. braziliensis* outlier *L. peruviana,* at least in the department of Cochabamba. Regarding their associated clinical characteristics, *L. braziliensis* outlier was frequently isolated in skin lesions, in one case with mucosal involvement. Unlike *L. braziliensis*, no phenotype of treatment failure was observed for infection with this atypical species. The study of populations and the analysis of polymorphisms showed that both groups presented nucleotide ambiguities indicative of genetic recombination processes. In addition, the detection of nucleotide positions allowed groups of the complex to be differentiated. This study demonstrates the feasibility of performing similar interventions that are required in other endemic areas in Bolivia. Also, emphasize the relevance of determining the genetic characteristics, geographical distribution and clinical impact of the *L. braziliensis* complex in order to obtain more knowledge about the epidemiology of tegumentary leishmaniasis and its control.

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709 TABLE 1. Identification of Bolivian strains of the *Leishmania braziliensis* complex by hsp70 gene sequencing.

WHO CODE MHOM/BO/…	**GenBank reference strain**	**ID GenBank/species**	**Coverage (% of identity)**	**Species Identification**
2014/CUM-1272	MCAN/PE/91/LEM2222	FR715991.1/*L.b.*o	1245/1245(100%)	*L. braziliensis* outlier
2014/CUM-1275[1]	MHOM/BR/75/M2904	LS997627.1/*L.b*	1245/1245(100%)	*L. braziliensis*
2014/CUM-1276[1]	MHOM/BR/75/M2904	FR799003.1/*L.b*	1245/1245(100%)	*L. braziliensis*
2014/CUM-1279	MHOM/PE/02/LH2182	FN395040.1/*L.b*	1245/1245(100%)	*L. braziliensis*
2014/CUM-1281[2]	MHOM/BR/75/M2904	FR799003.1/*L.b*	1244/1245(99.92%)	*L. braziliensis*
2014/CUM-1282[2]	MHOM/BR/75/M2904	FR799003.1/*L.b*	1245/1245(100%)	*L. braziliensis*
2014/CUM-1284	IWHI/BR/86/M10187	HF586369.1/*L.b*	1245/1245(100%)	*L. braziliensis*
2014/CUM-1286	MHOM/BO/--/CUM180	FN395039.1/*L.b*	1245/1245(100%)	*L. braziliensis*
2014/CUM-1288[3]	IWHI/BR/86/M10187	HF586369.1/*L.b*	1245/1245(100%)	*L. braziliensis*
2014/CUM-1289[3]	IWHI/BR/86/M10187	HF586369.1/*L.b*	1245/1245(100%)	*L. braziliensis*
2014/CUM-1292	MHOM/BR/75/M2904	LS997627.1/*L.b*	1245/1245(100%)	*L. braziliensis*
2015/CUM-1293	MHOM/PE/--/LH3851	FR872763.1/*L.b.*o	1244/1245(99.92%)	*L. braziliensis* outlier
2015/CUM-1294[4]	MHOM/PE/03/PER163/0	FR715990.1/*L.b.*o	1243/1245(99.84%)	*L. braziliensis* outlier
2015/CUM-1297	MHOM/PE/02/LH2182	FN395040.1/*L.b*	1245/1245(100%)	*L. braziliensis*
2015/CUM-1298	MHOM/BR/75/M2904	FR799003.1/*L.b*	1242/1245(99.92%)	*L. braziliensis*
2015/CUM-1307	MHOM/BO/--/CUM180	FN395039.1/*L.b*	1245/1245(100%)	*L. braziliensis*

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2015/CUM-1309[4]	MHOM/PE/03/PER163/0	FR715990.1/*L.b.*o	1242/1245(99.76%)	*L. braziliensis* outlier
2015/CUM-1313[5]	MHOM/PE/03/PER163/0	FR715990.1/*L.b.*o	1242/1245(99.76%)	*L. braziliensis* outlier
2015/CUM-1314[5]	MHOM/PE/03/PER163/0	FR715990.1/*L.b.*o	1242/1245(99.76%)	*L. braziliensis* outlier
2015/CUM-1318	MHOM/BR/75/M2904	FR799003.1/*L.b*	1245/1245(100%)	*L. braziliensis*
2015/CUM-1319	MCAN/PE/91/LEM2222	FR715991.1/*L.b.*o	1244/1245(99.92%)	*L. braziliensis* outlier
2015/CUM-1320[6]	MHOM/BR/75/M2904	FR799003.1/*L.b*	1245/1245(100%)	*L. braziliensis*
2015/CUM-1321[6]	IWHI/BR/86/M10187	HF586369.1/*L.b*	1245/1245(100%)	*L. braziliensis*
2015/CUM-1330	IWHI/BR/86/M10187	HF586369.1/*L.b*	1245/1245(100%)	*L. braziliensis*
2015/CUM-1339[7]	MHOM/BR/75/M2904	FR799003.1/*L.b*	1244/1245(99.92%)	*L. braziliensis*
2015/CUM-1343[7]	MHOM/BO/--/CUM68	FR872758.1/*L.b*	1244/1245(99.92%)	*L. braziliensis*
2015/CUM-1347	IWHI/BR/86/M10187	HF586369.1/*L.b*	1245/1245(100%)	*L. braziliensis*
2015/CUM-1352	MHOM/BO/--/CUM68	FR872758.1/*L.b*	1244/1245(99.92%)	*L. braziliensis*
2015/CUM-1355	MHOM/BO/--/CUM555	FR872760.1/*L.b.*o	1243/1245(99.84%)	*L. braziliensis* outlier
2015/CUM-1361	IWHI/BR/86/M10187	HF586369.1/*L.b*	1245/1245(100%)	*L. braziliensis*
2015/CUM-1362[8]	MHOM/PE/--/LH3851	FR872763.1/*L.b.*o	1244/1245(99.92%)	*L. braziliensis* outlier
2015/CUM-1364	MCAN/PE/91/LEM2222	FR715991.1/*L.b.*o	1245/1245(100%)	*L. braziliensis* outlier
2015/CUM-1365	MHOM/PE/03/PER163/0	FR715990.1/*L.b.*o	1244/1245(99.92%)	*L. braziliensis* outlier
2015/CUM-1366	MHOM/PE/03/PER163/0	FR715990.1/*L.b.*o	1244/1245(99.92%)	*L. braziliensis* outlier

2015/CUM-1367[9]	MHOM/PE/03/PER163/0	FR715990.1/*L.b.*o	1243/1245(99.84%)	*L. braziliensis* outlier
2015/CUM-1368[9]	MHOM/PE/03/PER163/0	FR715990.1/*L b.*o	1244/1245(99.92%)	*L. braziliensis* outlier
2015/CUM-1370[8]	MCAN/PE/91/LEM2222	FR715991.1/*L.b.*o	1244/1245(99.92%)	*L. braziliensis* outlier
2015/CUM-1372	MHOM/PE/01/PER006/1	FR715987.1/*L.b*	1244/1245 (99.92%)	*L. peruviana*
	MHOM/PE/1990/HB86	LN907845.1/*L.p*	1244/1245(99.92%)	
2015/CUM-1373	IWHI/BR/86/M10187	HF586369.1/L.b	1244/1245(99.92%)	*L. braziliensis*
2015/CUM-1374	MHOM/PE/03/PER163/0	FR715990.1/*L.b.*o	1244/1245(99.92%)	*L. braziliensis* outlier
2015/CUM-1376	IWHI/BR/86/M10187	HF586369.1/*L.b*	1245/1245(100%)	*L. braziliensis*

L.b: *L. braziliensis*, *L.b.*o: *L. braziliensis* outlier, *L.p*: *L. peruviana*

The isolates from the same patient (nine patients with more than one isolate) are marked with a superscript 1,2,3,4, …9 in the WHO code of the strain.

TABLE 2. Genotypes and nucleotide characteristics of the hsp70 gene (1245pb) in *L. braziliensis* complex strains isolated in Bolivia and reference strains of GenBank. Discriminative nucleotide positions are shaded and heterozygote positions are in bold.

Species	Genotypes	Nº of Strain Study/ Ref	Nucleotide characters at the polymorphic sites													
			51	**186**	**288**	**297**	**561**	**642**	**978**	**993**	**1119**	**1171**	**1200**	**1213**	**1223**	**1237**
L. braziliensis Study 24/Ref.6	1	8/2	G	G	G	C	G	G	C	C	G	T	G	T	A	G
	2	2/1	G	G	G	C	G	G	C	C	G	T	G	T	A	A
	3	8/1	G	G	G	C	G	G	C	C	G	T	G	T	A	**R**
	4	3/0	G	G	G	C	**R**	G	C	C	G	T	G	T	A	G
	5	1/0	G	G	G	C	**R**	G	C	C	G	T	G	T	A	**R**
	6	2/0	**R**	G	G	C	**R**	G	**Y**	C	G	T	G	T	A	G
	17R	0/1	**R**	G	G	C	**R**	G	C	C	G	T	G	T	A	G
	18R	0/1*	A	G	G	C	A	G	T	C	G	T	G	T	A	G
L. peruviana Study 1/Ref 5	7	1/0	A	G	G	C	A	G	**Y**	C	G	T	G	T	A	G
	19R	0/5	A	G	G	C	A	G	C	C	G	T	G	T	A	G
L. b. outlier Study 16/Ref. 5	8	2/0	G	G	A	T	G	A	C	A	G	T	G	A	A	G
	9	1/0	G	G	A	T	G	A	C	A	G	G	G	A	A	G
	10	3/0	G	G	A	T	G	A	C	A	G	G	T	A	C	G

11	2/1	G	**R**	A	T	G	A	C	A	G	T	G	A	**M**	G
12	2/0	G	G	A	T	G	A	C	A	G	T	G	A	**M**	G
13	1/0	G	G	A	T	G	A	C	A	G	**K**	G	A	**M**	G
14	1/0	G	G	A	T	G	A	C	A	G	**K**	G	A	C	G
15	3/0	G	G	A	T	G	A	C	A	G	**K**	**K**	A	C	G
16	1/0	G	G	A	T	G	A	C	A	G	G	**K**	A	C	G
20R	0/2	G	**R**	A	T	G	A	C	A	**K**	G	G	A	A	G
21R	0/1	G	**R**	A	T	G	A	C	A	G	T	G	A	A	G
22R	0/1	G	G	A	T	G	A	C	A	G	**K**	**K**	A	**M**	G

Study: Strains analyzed in the present study (Bolivian strains), R/Ref.: reference strains (GenBank). Sequence variants are noted according to Den Dunnen et al., (2016) and the International Union of Pure and Applied Chemistry (IUPAC, 2020) nomenclature.

*Reference strain characterized as *L. braziliensis* in the GenBank (FR715987.1) but considered to be a sequence of *L. peruviana* by us and some other authors (Odiwuor et al., 2012; Van der Auwera et al., 2013).

TABLE 3. Genetic diversity parameters of *Leishmania braziliensis* complex *hsp*70 gene sequence

Specie	N	Region	S	Eta	HN	Hd	π	K
L. braziliensis-L. peruviana	25*	1245	4	4	5	0.598	0.00075	0.932
***L. b.* outlier**	16	1245	4	4	6	0.8	0.00124	1.544

N: Number of sequences, S: Number of polymorphic sites, Eta: Total number of mutations, HN: Number of haplotypes, Hd: Haplotype diversity, π: Nucleotide diversity, K: Average number of nucleotide differences

*In *L. braziliensis* are included the 24 isolates of *L. braziliensis* and 1 isolate of *L. peruviana* from Bolivia grouped in a cluster.

TABLE 4. *Leishmania braziliensis* complex strains analyzed and their relationship with clinical characteristics.

Species	N° of isolates	N° of patients	N° lesion per patient	Patients with CL	Patients with MCL	Patient whit treatment failure
L. braziliensis	24	19	1 – 6	11	8	6*
L. peruviana	1	1	1	0	1	1
***L. braziliensis* outlier**	16	12	1 – 3	11	1	0
Total	41	32	-----	22	10	7

CL: cutaneous leishmaniosis, MCL: mucocutaneous leishmaniosis

* Four patients with localized cutaneous lesions and two patients with mucocutaneous lesions.

744 SUPPLEMENT TABLE 1: GenBank reference strains used in the study

Code GenBank	Reference strain	Species
EU599090.1	MHOM/BR/73/M2269	*Leishmania amazonensis*
EU599091.1	MNYC/BZ/62/M379	*Leishmania mexicana*
FN395048.1	MHOM/PE/91/LC1581	*Leishmania lainsoni*
FN395047.1	MHOM/BO/95/CUM71	*Leishmania lainsoni*
HF586373.1	MHOM/GF/97/CRE88	*Leishmania naiffi*
FN395056.2	MDAS/BR/78/M5210	*Leishmania naiffi*
FN395053.1	MHOM/BR/2007/029-ZAV	*Leishmania guyanensis*
FN395051.1	MHOM/PE/02/LH2372	*Leishmania guyanensis*
HF586366.1	MHOM/PA/--/P7	*Leishmania panamensis*
HF586359.1	MHOM/CR/2004/TIM13	*Leishmania panamensis*
FR872760.1	MHOM/BO/--/CUM 555	*L. braziliensis* outlier
FR872761.1	MHOM/BO/--/CUM 663	*L. braziliensis* outlier
FR715991.1	MCAN/PE/91/LEM2222	*L. braziliensis* outlier
FR872763.1	MHOM/PE/--/LH3851	*L. braziliensis* outlier
FR715990.1	MHOM/PE/03/PER163/0	*L. braziliensis* outlier
HF586372.1	MHOM/PE/90/FY	*Leishmania braziliensis*
HF586369.1	IWHI/BR/86/M10187	*Leishmania braziliensis*
FR872758.1	MHOM/BO/--/CUM68	*Leishmania braziliensis*
FR715987.1	MHOM/PE/01/PER006/1	*Leishmania braziliensis*
FN395040.1	MHOM/PE/02/LH2182	*Leishmania braziliensis*
FR799003.1	MHOM/BR/75/M2904	*Leishmania braziliensis*
FN395044.1	MHOM/PE/03/LH2864	*Leishmania peruviana*
FR872765.1	MHOM/PE/90/HB22	*Leishmania peruviana*
EU599089.1	MHOM/PE/90/LCA08CL2	*Leishmania peruviana*
FN395045.1	MHOM/PE/03/LH2439	*Leishmania peruviana*
FN395046.1	MHOM/PE/90/LC468	*Leishmania peruviana*
KC960000.1	Sp104 cl1	*Trypanosoma cruzi*

749 FIGURE 1. Neighbor joining tree based on the hsp70 gene sequences. The bootstrap values are represented
750 in the nodes. The bottom scale represents the proportional distance to the differences between the
751 alignments.
752 ◆*L. peruviana* strain identified in the study, with genetic profile of *L. peruviana* and *L. braziliensis*.
753 ◇Strains identified in the study as possible *L. braziliensis*-*L. peruviana* hybrids
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757 FIGURE 2. Genetic distance evaluation of heat-shock protein 70 (hsp70) by Neighbor-net (NN) in
758 *Leishmania braziliensis* complex. Bootstrap values (1,000 replicates) are shown on the edges (percentages).