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***Leishmania* species identification using FTA card sampling directly from patients' cutaneous lesions in the state of Lara, Venezuela**

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ABSTRACT

A molecular epidemiological study was performed by using FTA card materials directly sampled from lesions of patients with cutaneous leishmaniasis (CL) in the state of Lara, Venezuela, where causative agents have been identified as *Leishmania (Viannia) braziliensis* and *L. (Leishmania) venezuelensis* in the past studies. Of the seventeen patients diagnosed with CL, *Leishmania* species were successfully identified in sixteen patients based on analyses of the cytochrome *b* and ribosomal RNA internal transcribed spacer sequences. Consistent with previous findings, seven of the patients were infected with *L. (V.) braziliensis*. However, parasites from the other nine patients were genetically identified as *L. (L.) mexicana*, which differed from results of previous enzymatic and antigenic analyses. These results strongly suggested that *L. (L.) venezuelensis* is a variant of *L. (L.) mexicana*, and the classification of *L. (L.) venezuelensis* should be reconsidered.

Key Words: Venezuela, *Leishmania venezuelensis*, *Leishmania mexicana*, *Leishmania braziliensis*, cytochrome *b*, rRNA internal transcribed spacer

1. Introduction

Leishmaniasis is caused by protozoan parasites of the genus *Leishmania*, which is further divided into two subgenera, *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*).¹ The disease is widely distributed around the world especially in tropical and subtropical areas, affecting at least 12 million people in 88 countries.² Approximately 20 *Leishmania* species are known to be pathogenic to humans, and species is the major determinant of clinical outcome (cutaneous, mucocutaneous and visceral forms).² Therefore, identification of the parasite species in given endemic areas is important for both appropriate treatment and prognosis.

The standard method for the classification of *Leishmania* species is multi-locus enzyme electrophoresis (MLEE), which requires the isolation and mass culture of the parasites.^{3,4} To overcome the disadvantages of MLEE, molecular biological methods have been developed for the detection and identification of *Leishmania* species using DNA extracted from clinical materials such as biopsy samples.⁵⁻⁷ Recently, to facilitate the sample collection and DNA extraction processes, a FTA card, a filter paper that readily lyses the spotted materials and fixes nucleic acids, was used for direct sampling of patients' materials in an epidemiological study of leishmaniasis.⁸

Cutaneous leishmaniasis (CL) is endemic in the state of Lara located in western

Venezuela, and the causative parasites have been identified as *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) venezuelensis*.⁹⁻¹² *L. (L.) venezuelensis* was originally described on the basis of distinguishable MLEE patterns as a species independent of other members of the *L. mexicana* complex^{9,10}. Further, a serodeme-based analysis using a panel of monoclonal antibodies was performed to differentiate New World *Leishmania* species including *L. (L.) venezuelensis*¹³, and then a monoclonal antibody specific for *L. (L.) venezuelensis* was developed for identification using immunological methods¹⁴ since the species is difficult to maintain in the axenic culture required for MLEE. *L. (L.) venezuelensis* occurs in western Venezuela, mainly in the state of Lara⁹⁻¹², and is not well-characterized, especially in terms of its genetics. To date, two genes, the ribosomal RNA (rRNA) internal transcribed spacer (ITS) region and the mini-exon gene, have been analyzed in the culture stock of *L. (L.) venezuelensis* strain PM-H3; however, both gene sequences showed greater homology with those from strains of *L. (L.) major* in spite of a member of *L. mexicana* complex^{15,16}, suggesting that the present culture stock of strain PM-H3 may not retain the original features. Unfortunately, other strains of *L. (L.) venezuelensis* have never been analyzed on the genetic level because of the difficulty to maintain as a culture. Moreover, no genetic analysis has been performed on parasites

collected directly from patients' lesions in endemic areas.

Here, a molecular epidemiological study was performed by using FTA card materials directly sampled from lesions of patients with CL in the state of Lara, Venezuela, and the causative species for CL in this area were genetically characterized.

2. Materials and methods

2.1. Sample collection

Seventeen clinical samples were included in this study. The samples were taken from cutaneous lesions of patients who visited at the Servicio de Dermatología Sanitaria Estado, Edificio de Inmunología, Hospital Central, Estado de Lara, Venezuela on February 2008 for the diagnosis and treatment of leishmaniasis. The present patients and children's parents voluntarily consented to participate in this study after receiving detailed explanation of the present procedures by a dermatologist (I.M.N.) of the hospital. The clinical form including lesion features and the detection of amastigotes in the skin lesions by smear test are shown in Table 1. All the referred patients from whom the present FTA card materials were obtained were systemically treated for 20-30 days with 10-20 mg/day/kg body weight of pentavalent antimony for each cycle. In the case of the incomplete clinical cure of lesion, another cycle of treatment was given according to the recommendation of the World Health Organization. The clinical controls and treatment protocols were exclusively conducted by our Venezuelan dermatologist (I.M.N.). Tissue materials were taken by scraping the margin of active lesions of a patient, spotted onto a FTA Classic Card (Whatman, Newton Center, MA) and stored at room temperature. Disks 2 mm in diameter were

punched out from each filter paper and washed three times with FTA Purification Reagent (Whatman) and once with Tris-EDTA buffer. The disks were air-dried and directly subjected to PCR amplification.

2.2. Identification of *Leishmania* species

Leishmania species were identified by an analysis of the cytochrome *b* (*cyt b*) gene.^{8,17,18} Amplification with a pair of specific primers, L.cyt-AS (5'-GCCGAGAGRARGAAAAGGC-3') and L.cyt-AR (5'-CCACTCATAAATATACTATA-3'), was performed with 30 cycles of denaturation (95 °C, 1 min), annealing (55 °C, 1 min) and polymerization (72 °C, 1 min). Ampdirect Plus reagent (Shimadzu Biotech, Tsukuba, Japan) was used to obtain maximum sensitivity of PCR amplification against the possible carry-over of tissue-derived inhibitors for enzymatic reaction in each sample. One microliter of the PCR product was subjected to the nested PCR with a set of inner primers, L.cyt-S (5'-GGTGTAGGTTTTAGTYTAGG-3') and L.cyt-R (5'-CTACAATAAACAATCATAATATRCAATT-3'). To further determine species of the *L. mexicana* complex, the rRNA ITS region was analyzed. Nested PCR was performed with a pair of outer primers, RI1 (5'-

GCTGTAGGTGAACCTGCAGCAGCTGGATCATT-3') and RI2 (5'-GCGGGTAGTCCTGCCAAACACTCAGGTCTG-3')¹⁹, and then with a pair of inner primers, L.ITS-S (5'-ATCATTTTCCGATGATTACA-3') and L.ITS-R (5'-CTGTAAACAAAGGTTGTCGG-3'). The products were cloned into the pGEM-T Easy Vector System (Promega, Madison, WI) and sequenced from both forward and reverse sides by the dideoxy chain termination method using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). To verify the accuracy of the sequences, FTA samples were subjected to PCR amplification with L.cyt-S and L.cyt-R primers by using a high fidelity enzyme, KOD DNA polymerase (TOYOBO, Tokyo, Japan), and the fragments were sequenced by the direct sequencing method.

L. (V.) braziliensis was differentiated from *L. (V.) peruviana* by a PCR-restriction fragment length polymorphism (RFLP) analysis of the mannose phosphate isomerase (MPI) gene as described previously.¹⁸

2.3. Phylogenetic analysis

The obtained sequences were aligned with CLUSTAL W software²⁰ and examined using the program MEGA (Molecular Evolutionary Genetics Analysis) version 4.0.²¹

Phylogenetic trees were constructed by the neighbor-joining method with the distance algorithms in the MEGA package. Bootstrap values were determined with 1,000 replicates of the data sets. The database for phylogenetic analyses consisted of *cyt b* gene sequences from *L. (L.) infantum* (GenBank accession no. AB095958), *L. (L.) donovani* (AB095957), *L. (L.) major* (AB095961), *L. (L.) tropica* (AB095960), *L. (L.) amazonensis* (AB095964), *L. (L.) mexicana* (AB095963, EF579915, EF579906, EU499922, AB558217, AB558218, AB558219, AB558220, AB558221), *L. (L.) garnhami* (AB095965), *L. (L.) pifanoi* (AB434679), *L. (L.) aristidesi* (AB434678), *L. (V.) panamensis* (AB095968), *L. (V.) guyanensis* (AB095969), *L. (V.) braziliensis* (AB095966), *L. (V.) peruviana* (AB433282), *L. (V.) lainsoni* (AB433280), *L. (V.) naiffi* (AB433279) and *L. (V.) shawi* (AB433281), and rRNA ITS sequences from *L. (L.) major* (AJ000310), *L. (L.) tropica* (AJ000301), *L. (L.) amazonensis* (DQ300194), *L. (L.) mexicana* (AF466383, AJ000312, AJ000313, AF466382, AF466381, AF466380, AB558238, AB558239, AB558240, AB558241, AB558242) and *L. (L.) venezuelensis* (AF339752). The sequences from *L. (L.) major* and *L. (L.) tropica* were used as out-groups for the phylogenetic analysis of *L. mexicana* complex.

3. Results

A total of 17 samples (V-1 – V-17) were collected from patients diagnosed with CL at the Dermatologia Sanitaria Estado in the state of Lara, Venezuela. The clinical features of each patient are listed in Table 1. No patient with mucocutaneous lesions was observed. The samples scratched from cutaneous lesions and spotted onto FTA cards were subjected directly to PCR targeting the leishmanial *cyt b* gene. Consequently, *cyt b* gene fragments were obtained from 16 patients, and the nucleotide sequences analyzed. Nine samples (V-2, V-3, V-4, V-9, V-11, V-13, V-14, V-15 and V-17) had a greater degree of homology with *L. (L.) mexicana* (99.1-99.6 %) than with other species (88.3-97.1 %). The other seven specimens (V-1, V-5, V-6, V-7, V-10, V-12 and V-16) had greater homology with *L. (V.) braziliensis* (99.8-100 %) and *L. (V.) peruviana* (99.5-99.8 %) than any of the others (88.4-98.9 %). A phylogenetic tree showed that the nine samples divided into the clade of *L. (L.) mexicana*, while the other seven specimens had a closer relationship with *L. (V.) braziliensis* and *L. (V.) peruviana* (Fig. 1). The latter seven specimens were further differentiated by a PCR-RFLP-based analysis of the MPI gene targeting a single nucleotide polymorphism between the two species.^{8,18} The RFLP patterns of all seven samples corresponded to that of *L. (V.) braziliensis* not *L. (V.) peruviana* (data not shown).

The causative agents of CL in this area have been identified as *L. (V.) braziliensis* and *L. (L.) venezuelensis*.⁹⁻¹² Since *L. (L.) venezuelensis* is classified into the *L. mexicana* complex, the *cyt b* genes from the nine samples showing the greatest homology with *L. (L.) mexicana* were further compared with those of *L. mexicana* complex species including WHO reference strains of *L. (L.) mexicana* isolated from Mexico and Belize, and isolates of *L. (L.) mexicana* from Ecuador. The phylogenetic analysis showed that the parasites from the present Venezuelan patients were located in the clade of WHO reference strains of *L. (L.) mexicana* isolated from Mexico (NAN1) and Belize (M379 and BEL21), and different clades of *L. (L.) mexicana* from a patient with diffuse CL in a subtropical area (DCL An) and from a patient with CL (Huigra1) and sand flies (Hu1-7G, 1-11B and 2-5F) in Andean highland areas of Ecuador^{18,22,23} (Fig. 2A). All the parasites identified as *L. (L.) mexicana* were separated from other *L. mexicana* complex species, *L. (L.) garnhami*, *L. (L.) aristidesi* and *L. (L.) amazonensis* (Fig. 2A). The results of the *cyt b* gene analysis strongly suggested that the parasite species from Venezuelan patients are *L. (L.) mexicana*. Genetic information on *L. (L.) venezuelensis* is very scarce, and at present, the only nucleotide sequences available on GenBank are the rRNA ITS region and mini-exon gene, both determined from strain PM-H3 isolated in the same study area.^{15,16} ITS regions are

suitable for the characterization of closely related species because of the less conservation between species.²⁴ Therefore, ITS sequences were analyzed in the specimens from the nine Venezuelan patients to compare them with those from *L. (L.) venezuelensis* PM-H3 and strains of *L. (L.) mexicana*. The specimens of Venezuelan patients located in the clade of *L. (L.) mexicana* of the WHO reference strain (M379) and isolates from Mexico (INDRE NBO, SOLIS, UNAM RR, SET GS and GO22) and Ecuador (DCL An), and the genetic distance between Venezuelan specimens and the reference strain was shorter than distances between the reference strain and *L. (L.) mexicana* from Andean highland areas of Ecuador (Huigra1, Hu1-7G, Hu1-11B and Hu2-5F) (Fig. 2B). In addition, the isolates from the Venezuelan patients were located some distance from *L. (L.) venezuelensis* PM-H3, which unexpectedly occurred in a clade of *L. (L.) major* (Fig. 2B).¹⁵ Based on these results, the parasites from the nine Venezuelan patients detected in this study area were genetically identified as *L. (L.) mexicana*.

4. Discussion

In the present study, using FTA cards as direct sampling media, an epidemiological study of leishmaniasis was performed in the state of Lara, Venezuela. Of the 17 specimens, 16 were successfully identified based on analyses of the *cyt b*, *MPI*, and *ITS* genes. A case (V-8) with positive for smear-test but negative for PCR was probably caused by an unsuccessful sampling onto FTA card after scratching. There is no longer any doubt about the diagnostic value of molecular biological methods for leishmaniasis. For the classification of *Leishmania* species, several genetic markers have been studied.^{15,17,25-31} FTA cards were recently used for the less invasive dry collection of specimens from patients with CL.⁸ The card readily lyses the spotted cells and pathogens and fixes the nucleic acids, resulting in protection from nuclease, oxidative, and UV damage, and prevention of the growth of bacteria and other microorganisms. In addition, the extraction and purification of DNA can be skipped and the minimum processes for the preparation of PCR templates pose little risk of contamination among samples. In the present study, using the cards for direct sampling of patients' specimens from the state of Lara, Venezuela, causative agents of CL were genetically identified as *L. (V.) braziliensis* and *L. (L.) mexicana*. However, *L. (L.) venezuelensis*, which was described as a separate species in the *L. mexicana*

complex species, was not observed in the clinical samples though the species has been reported as a principal pathogen in this endemic area.⁹⁻¹² The grouping of *L. mexicana* complex species is still controversial. By conventional classification using MLEE, at least six species, *L. (L.) mexicana*, *L. (L.) amazonensis*, *L. (L.) garnhami*, *L. (L.) pifanoi*, *L. (L.) aristidesi*, and *L. (L.) venezuelensis* have been differentiated as valid in the *L. mexicana* complex. A recent study suggested that *L. (L.) mexicana*, *L. (L.) amazonensis*, and *L. (L.) garnhami* could not be distinguished as a monophyletic clade based on the phylogenetic analysis of *hsp70* sequences and *L. (L.) mexicana* be recognized as a single species.³¹ However, analyses of the SSU rRNA and *cyt b* genes suggested that the *L. mexicana* complex can be divided into two subgroups; one consisting of *L. (L.) mexicana* and *L. (L.) pifanoi*, and the other including *L. (L.) amazonensis*, *L. (L.) garnhami*, and *L. (L.) aristidesi*.^{17,28,30}

Concerning *L. (L.) venezuelensis*, the rRNA ITS region and mini-exon gene have been analyzed in the culture stock of strain PM-H3 isolated from a patient in the same study area of Venezuela.^{15,16} As far as we know, strain PM-H3 is the only culture stock of *L. (L.) venezuelensis* available at present. Unexpectedly, nucleotide sequences of the ITS region from *L. (L.) venezuelensis* PM-H3 were almost identical to those from strains of *L. (L.) major*.¹⁵ Similarly, the mini-exon gene from PM-H3

showed greater homology with that of *L. (L.) major* (97.7 %) than those of other species (59.3-74.8 %) including *L. (L.) mexicana* (67.4 %) and *L. (L.) amazonensis* (65.8 %).¹⁶ These results suggested that PM-H3 is the species of *L. (L.) major*-like but not *L. mexicana* complex. On the other hand, consistent with the results of the *cyt b* gene analysis, all the ITS sequences of the parasites from the nine Venezuelan patients in the present study showed greatest homology with those of *L. (L.) mexicana* (98.1-99.3 %) and relatively high homology with those of *L. (L.) amazonensis* (88.7-93.8 %) compared to those from other species (67.2-84.9 %) including *L. (L.) venezuelensis* PM-H3 (80.5-81.1 %). At present, the discrepancy between our results and previous findings remains to be elucidated. It is conceivable that PM-H3 has particular characteristics, possibly acquired after long-term cultivation, but usually this is not probable. Alternatively, the possible contamination of the strain with *L. (L.) major* after long-years cultivation may be taken into consideration.

L. (L.) venezuelensis has been shown to have several different characteristics from *L. (L.) mexicana* in MLEE and serodeme-based analyses^{10,13,14}, and unlike the common *L. (L.) mexicana*, is difficult to maintain as an axenic culture.¹⁴ Therefore, the parasite species endemic in the area may have particular characteristics in terms of enzymatic activity, antigenicity, and metabolism when compared to *L. (L.) mexicana*

from different areas. However, the present genetic analyses definitely demonstrated the presence of two species, *L. (L.) mexicana* and *L. (V.) braziliensis*, without *L. (L.) venezuelensis*, a species previously reported as a causative agent prevalent in the study area, strongly suggesting that *L. (L.) venezuelensis* is a variant of *L. (L.) mexicana*. Further extensive study, therefore, will be required to clarify the discrepancy between the present and previous observations. In addition, since genetic information on each *Leishmania* species is accumulating, the classification of *Leishmania* species, especially the New World species, should be reconsidered on the basis of genetic, enzymatic and antigenic characteristics.^{17,28,30-33}

Authors' contributions: HK and YH conceived the work; JW and IMN collected samples, and analyzed and interpreted the clinical data; HK and MK conducted and interpreted the laboratory work; HK prepared the first draft and all the authors contributed to the revision of the manuscript and read and approved the final manuscript.

HK is guarantor of the paper.

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Conflicts of interest: None declared.

Ethical approval: The patients visited the Central Hospital of Lara State, the Ministry

of Health, Venezuela, for the diagnosis and treatment of leishmaniasis, having routine dermatological, parasitological, and epidemiological examinations. The present patients and/or their parents voluntarily consented to participate in this study, under the approval of the Ethical Committee of the Central Hospital for the FTA card-collecting procedures employed, after receiving detailed explanation of the present procedures by a dermatologist (I.M.N.) of the Servicio de Dermatología Sanitaria Estado, Edificio de Inmunología, the Central Hospital of Lara State, Venezuela. All the patients were systemically treated with pentavalent antimony, and the clinical controls and treatment protocols were exclusively conducted by our Venezuelan dermatologist (I.M.N.). In compliance with national guidelines, permission to compile patient data was obtained from the Central Hospital of Lara State, Venezuela.

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Table 1. Clinical features of patients with cutaneous leishmaniasis in the state of Lara, Venezuela

Patient No.	Sex	Age	No. of lesion	Type of lesion*	Evolution time**	Smear	Size of lesion (mm)
V-1	M	69	6	U, N	7 m	+	6x6, 9x9, 8x8, 8x8, 21x10, 3x4
V-2	M	12	2	U	2 m	+	12x12, 15x18
V-3	M	77	1	U	5 m	+	10x40
V-4	F	26	3	U	12 d	+	18x45, 4x3, 2x3
V-5	F	9	1	U	3 m	+	15x20
V-6	F	20	1	U	6 m	+	18x33
V-7	M	25	nd	nd	nd	nd	nd
V-8	F	48	1	U	11 m	+	46x55
V-9	F	86	2	U	2 m	+	20x20, 20x23
V-10	M	56	1	U	5 m	+	32x35
V-11	M	5	1	U	1 m	+	8x8
V-12	F	29	1	U	4 m	+	14x16
V-13	M	38	15	U	2 m	+	17x20, 4x5, 15x15, 10x15, 20x25 and 10 papules
V-14	F	79	7	U, N	3 y	+	nd
V-15	M	10	5	U, N	3 m	+	8x9, 6x6, 6x8, 6x8, 2x2
V-16	F	15	nd	nd	nd	nd	nd
V-17	F	12	nd	nd	nd	nd	nd

*U, ulcer; N, nodule. **d, days; m, months; y, years. nd, no data.

Figure legends

Figure 1. Phylogenetic tree of *cyt b* gene sequences among species. The *cyt b* genes of the parasites were amplified from material collected from the lesions of Venezuelan patients with cutaneous leishmaniasis and spotted onto FTA cards (V-1, V-2, V-3, V-4, V-5, V-6, V-7, V-9, V-10, V-11, V-12, V-13, V-14, V-15, V-16 and V-17), and sequenced. Analyses were performed by the neighbor-joining method with sequences from 14 *Leishmania* species. The scale bar represents 0.01 % divergence. Bootstrap values are shown above or below branches. The nucleotide sequence data will appear in the GenBank with the accession nos. AB558222-AB558237.

Figure 2. Phylogenetic tree of *cyt b* gene (A) and ITS (B) sequences among species of the *L. mexicana* complex. The *cyt b* genes and ITS regions of the parasites were amplified from material collected from the lesions of Venezuelan patients with cutaneous leishmaniasis and spotted onto FTA cards (V-2, V-3, V-4, V-9, V-11, V-13, V-14, V-15 and V-17), and sequenced. (A) *Cyt b* sequences were analyzed by the neighbor-joining method together with sequences from species of the *L. mexicana* complex, *L. (L.) mexicana* [WHO reference strains isolated from Mexico (NAN1) and

Belize (M379 and BEL21) and isolates from a patient with diffuse CL in a subtropical area (DCL An) and from a patient with CL (Huigra1) and sand flies, *Lutzomyia ayacuchensis* (Hu1-7G, 1-11B and 2-5F) in Andean highland areas of Ecuador], *L. (L.) garnhami*, *L. (L.) pifanoi*, *L. (L.) aristidesi*, and *L. (L.) amazonensis*, and out-group species, *L. (L.) major* and *L. (L.) tropica*. The scale bar represents 0.005 % divergence. (B) ITS sequences were analyzed together with those from the *L. mexicana* complex, *L. (L.) mexicana* [a WHO reference strain (M379), isolates from Mexico (INDRE NBO, SOLIS, UNAM RR, SET GS and GO22) and isolates from a patient with diffuse CL in a subtropical area (DCL An) and from a patient with CL (Huigra1) and sand flies, *Lutzomyia ayacuchensis* (Hu1-7G, 1-11B and 2-5F) in Andean highland areas of Ecuador], *L. (L.) amazonensis*, and *L. (L.) venezuelensis* PM-H3, and out-group species, *L. (L.) major* and *L. (L.) tropica*. The scale bar represents 0.01 % divergence. Bootstrap values are shown above or below branches. The nucleotide sequence data will appear in the GenBank with the accession nos. AB558243-AB558251.

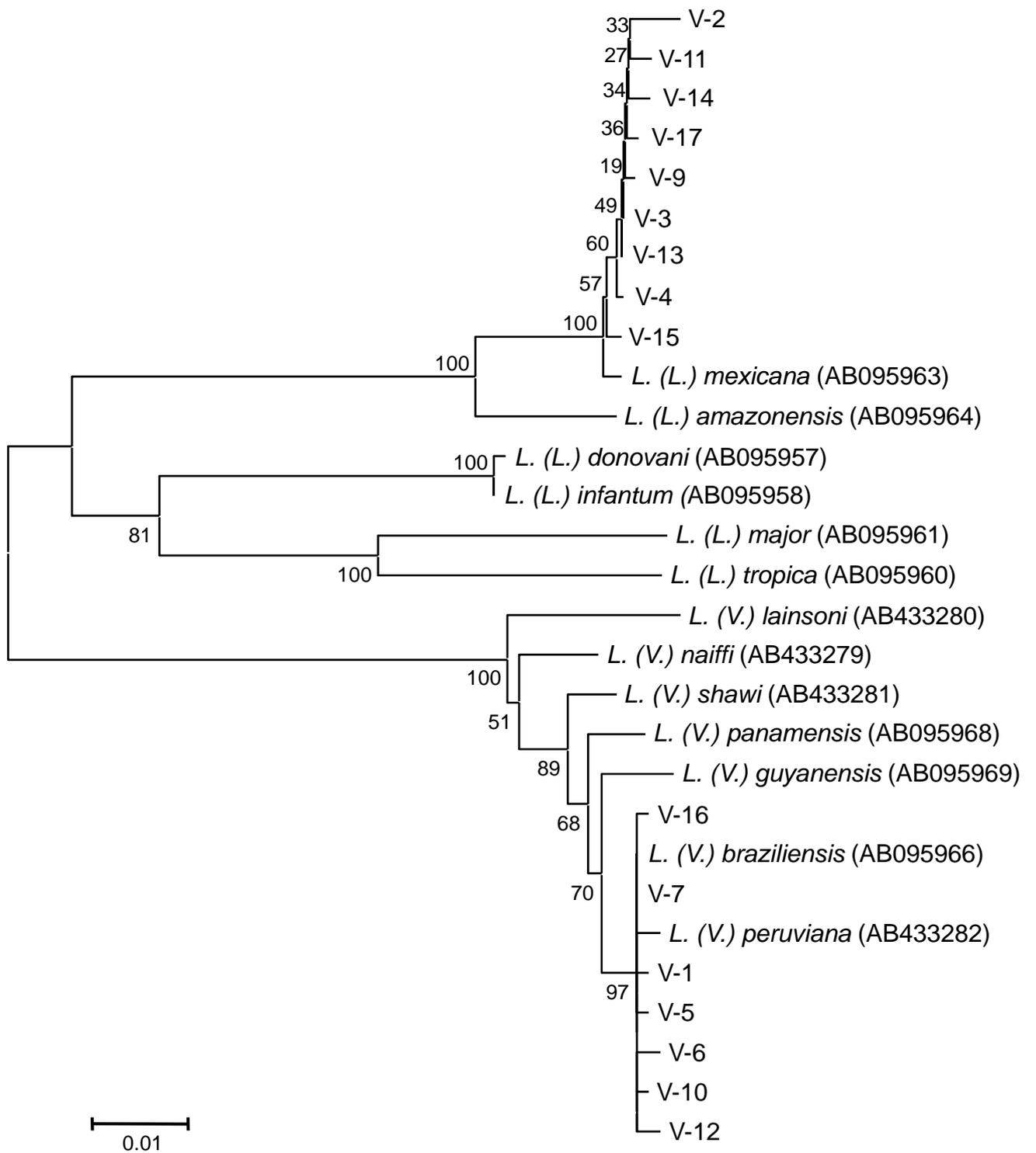


Fig. 1

