PCR-RFLP analyses of *Leishmania* species causing cutaneous and mucocutaneous leishmaniasis revealed distribution of genetically complex strains with hybrid and mito-nuclear discordance in Ecuador

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Abstract

PCR-Restriction Fragment Length Polymorphism (RFLP) analyses targeting multiple nuclear genes were established for the simple and practical identification of *Leishmania* species without using expensive equipment. This method was applied to 92 clinical samples collected at 33 sites in 14 provinces of Ecuador, which have been identified at the species level by the kinetoplast cytochrome *b* (*cyt b*) gene sequence analysis, and the results obtained by the two analyses were compared. Although most results corresponded between the two analyses, PCR-RFLP analyses revealed distribution of hybrid strains between *Leishmania* (Viannia) *guyanensis* and *L. (V.) braziliensis* and between *L. (V.) guyanensis* and *L. (V.) panamensis*, of which the latter was firstly identified in Ecuador. Moreover, unexpected parasite strains having the kinetoplast *cyt b* gene of *L. (V.) braziliensis* and nuclear genes of *L. (V.) guyanensis*, *L. (V.) panamensis*, or a hybrid between *L. (V.) guyanensis* and *L. (V.) panamensis* were identified. This is the first report of the distribution of a protozoan parasite having mismatches between kinetoplast and nuclear genes, known as mito-nuclear discordance. The result demonstrated that genetically complex *Leishmania* strains are present in Ecuador. Since genetic exchanges such as hybrid formation were suggested to cause higher pathogenicity in *Leishmania* and may be transmitted by more species of sand flies, further country-wide epidemiological studies on clinical symptoms, as well as transmissible vectors, will be necessary.
Author summary

Leishmaniosis caused by intracellular protozoa of the genus *Leishmania* is a neglected tropical disease widely distributing worldwide, especially in tropical and subtropical areas. Approximately 20 species are known to be pathogenic to humans, of which eight species have been recorded as causative agents of cutaneous and mucocutaneous leishmaniasis in Ecuador. Since infecting species are the major determinant of clinical outcomes, identification at the species level is important for the treatment and prognosis. The parasite species have been identified conventionally by multilocus enzyme electrophoresis (MLEE) and recently by genetic analysis such as sequencing and genotyping. In the present study, PCR-Restriction Fragment Length Polymorphism (RFLP) targeting multiple nuclear genes was employed, and the results were compared with those obtained by kinetoplast cytochrome b (*cyt b*) gene sequence analysis, which is widely applied to species identification. Although most results corresponded between the two analyses, PCR-RFLP revealed presence of unexpected genetically complex *Leishmania* strains having characteristics of hybrid and mito-nuclear discordance. Since hybrid strains of *Leishmania* were suggested to increase disease severity and may be transmitted by a wider range of sand fly species, careful epidemiological research, including clinical courses and vector research, will be needed.

Introduction

Leishmaniasis, caused by protozoan parasites of the genus *Leishmania*, is a neglected tropical disease widely distributed worldwide, especially in tropical and subtropical areas, affecting at least 12 million people in 96 countries [1]. Approximately 20 *Leishmania* species belonging to the subgenera *Leishmania* (*Leishmania*), *Leishmania* (*Viannia*) and *Leishmania* (*Mundinia*) are pathogenic to humans [1, 2]. Since infected parasite species is known to be the major determinant of clinical outcomes in leishmaniasis [1], identification of the causative parasite is important for appropriate treatment and prognosis.

*Leishmania* species have been classified conventionally by multilocus enzyme electrophoresis (MLEE) [3, 4]. Genetic analysis of kinetoplast and nuclear targets, such as cytochrome b (*cyt b*), cysteine protease (*cpb*), heat shock protein 70 (*hsp70*) genes and the internal transcribed spacer (ITS) regions of ribosomal RNA, has commonly been used for species identification due to its sensitivity, simplicity and reliability [5–13]. In addition, a simple PCR-Restriction Fragment Length Polymorphism (RFLP), which does not require costly equipment, was developed for species identification, and the ITS region and *hsp70* gene are widely applied to epidemiological studies [11, 14–19].

In Ecuador, leishmaniasis is endemic in Pacific coast, Andean highland, and Amazonian areas, and eight species, *Leishmania* (*Leishmania*) *mexicana*, *L. (L.) amazonensis*, *L. (L.) major-like*, *L. (Viannia) guyanensis*, *L. (V.) panamensis*, *L. (V.) braziliensis*, *L. (V.) naiifi*, and *L. (V.) lainsoni*, have been recorded as causative agents of cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL) [8, 20, 21]. Of these, distribution of *L. (L.) amazonensis* and *L. (L.) major-like* have been reported to be localized, and infections by them have not been reported recently [8, 21]. Infection by *L. (V.) guyanensis* together with its closely-related species, *L. (V.) panamensis*, has been identified from CL patients in Pacific coast areas by MLEE [21–24]; however, our recent *cyt b* gene analysis revealed a wide range distribution of *L. (V.) guyanensis*, without detecting any *L. (V.) panamensis* in these areas [8]. These results suggest
that endemic species may change, or the reported results may be caused by the discordance between the MLEE analysis and kinetoplast cyt b gene analysis employed for species identification. Recently, a countrywide epidemiological study was carried out based on the cyt b sequence analysis and it identified L. (V.) guyanensis and L. (V.) braziliensis widely in Pacific coast and Amazonian areas and L. (L.) mexicana in Andean high lands as current major causative species in Ecuador [8]. Additionally, L. (V.) naiffi and L. (V.) lainsoni were recently recorded in Amazonian areas [8, 20, 25].

In this study, a simple and practical method for the identification of Leishmania species in Ecuador was established on the basis of PCR-RFLP analyses targeting mannose phosphate isomerase (mpi) and 6-phosphogluconate dehydrogenase (6pgd) genes, and the result was compared with that obtained by the cyt b gene sequence analysis. This study demonstrated the presence of genetically complex Leishmania strains in Ecuador, and strongly suggested the importance of applying multiple target approaches to enhance the reliability of species identification and to characterize more detailed genetic properties of the parasite.

**Methods**

**Parasite specimens and clinical samples**

Frozen stocks of 24 parasite strains of five Leishmania species [L. (V.) guyanensis, L. (V.) panamensis, L. (V.) braziliensis, L. (L.) major-like, L. (L.) mexicana] that were isolated from CL patients in Ecuador and identified at the species level by MLEE [22–24] (Table 1) were spotted on an FTA Classic Card (Whatman, Newton Center, MA) and subjected to sequence analysis. Three strains of L. (V.) naiffi identified by cyt b gene analysis [25, 26] were also utilized (Table 1).

Most of the clinical samples employed in this study were collected from patients suspected of CL in the previous study [8, 20], and each 3 samples newly obtained from Provinces of Manabi and Santo Domingo de los Tsachilas, all of which were identified as L. (V.) guyanensis by the cyt b gene analysis, were included in this study. Leishmania parasites were identified on the basis of cyt b sequence analysis [8, 20]. The samples were collected at 33 sites in 14 provinces of Ecuador (S1 Fig). Residual tissue materials were spotted onto an FTA Classic Card, after taking scraped margin samples of active lesions for routine diagnosis. Two-mm-diameter disks of FTA card were punched out from each filter paper, washed three times with an FTA Purification Reagent (Whatman), and subjected to PCR amplification.

**PCR and sequence analysis**

PCR primers for amplification of cyt b, hsp70, mannose phosphate isomerase (mpi) and 6-phosphogluconate dehydrogenase (6pgd) gene fragments were designed based on the sequence regions conserved among species (Table 2). PCR amplification with a pair of outer primers was performed with 30 cycles of denaturation (95˚C, 1 min), annealing (55˚C, 1 min) and polymerization (72˚C, 2 min) using Ampdirect Plus reagent (Shimadzu Biotech, Tsukuba, Japan). Each 0.5-μl portion of the PCR product was reamplified with inner primers under the same condition described above. The products were cloned into the pGEM-T Easy Vector System (Promega, Madison, WI) and sequences were determined on both strands by the dideoxy chain termination method using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Primers for amplification of a partial sequence of the kinetoplast cytochrome oxidase subunit II-NADH dehydrogenase subunit I region (COII-ND1) were also designed based on the sequences conserved among species (Table 2). The COII-ND1 sequences were determined on both strands by direct sequencing with inner primers, L.COII-
2S and L.COII-2R. Restriction enzyme mapping was performed in silico by using BioEdit Sequence Alignment Editor to obtain species-specific RFLP patterns. Clinical samples spotted on FTA cards, in which parasites were identified by cyt b gene analysis in a previous study, were subjected to PCR-RFLP analysis. PCR amplifications targeting mpi and 6pgd were performed as described above using a high fidelity DNA polymerase, KOD plus (Toyobo, Osaka, Japan). The PCR products were digested by restriction enzymes HaeIII, HapI, and BstXI for the mpi gene and Bsp1286I and HinfI for the 6pgd gene, and resulting restriction fragment patterns were analyzed by 2% agarose gel electrophoresis. GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific, Waltham, MA) was used as a DNA size marker. The gel was stained with GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA), and DNA fragments were visualized with UV transilluminator.

**Table 1. Leishmania strains isolated in Ecuador.**

| Species                  | Strains         |
|--------------------------|-----------------|
| L. (V.) guyanensis       | MHOM/EC/05/EC4  |
| L. (V.) guyanensis       | MHOM/EC/05/EC6  |
| L. (V.) guyanensis       | MHOM/EC/05/EC7  |
| L. (V.) guyanensis       | MHOM/EC/05/EC8  |
| L. (V.) guyanensis       | MHOM/EC/05/EC9  |
| L. (V.) guyanensis       | MHOM/EC/05/EC11 |
| L. (V.) guyanensis       | MHOM/EC/05/EC12 |
| L. (V.) guyanensis       | MHOM/EC/05/XPEA1|
| L. (V.) panamensis       | MHOM/EC/05/EC1  |
| L. (V.) panamensis       | MHOM/EC/05/EC2  |
| L. (V.) panamensis       | MHOM/EC/05/EC8  |
| L. (V.) panamensis       | MHOM/EC/05/EC9  |
| L. (V.) panamensis       | MHOM/EC/05/EC11 |
| L. (V.) panamensis       | MHOM/EC/05/EC12 |
| L. (V.) panamensis       | MHOM/EC/05/EC13 |
| L. (V.) panamensis       | MHOM/EC/05/EC14 |
| L. (V.) panamensis       | MHOM/EC/05/EC15 |
| L. (V.) panamensis       | MHOM/EC/05/EC16 |

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2S and L.COII-2R. Restriction enzyme mapping was performed in silico by using BioEdit Sequence Alignment Editor to obtain species-specific RFLP patterns.

**PCR-Restriction Fragment Length Polymorphism (RFLP) analysis**

Clinical samples spotted on FTA cards, in which parasites were identified by cyt b gene analysis in a previous study, were subjected to PCR-RFLP analysis. PCR amplifications targeting mpi and 6pgd were performed as described above using a high fidelity DNA polymerase, KOD plus (Toyobo, Osaka, Japan). The PCR products were digested by restriction enzymes HaeIII, HapI, and BstXI for the mpi gene and Bsp1286I and HinfI for the 6pgd gene, and resulting restriction fragment patterns were analyzed by 2% agarose gel electrophoresis. GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific, Waltham, MA) was used as a DNA size marker. The gel was stained with GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA), and DNA fragments were visualized with UV transilluminator.

Differentiation between L. (V.) guyanensis and L. (V.) panamensis was performed by restriction enzyme-digestion of the hsp70 gene fragment [27]. Briefly, the hsp70 gene fragment was amplified by a nested PCR using sets of outer primers (L.HSP-Ty1S and L.HSP-OR) and inner primers (L.HSP-Ty2S and L.HSP-IR2) (Table 2). The amplicons were digested with a
restriction enzyme, BccI, and resulting fragment patterns were analyzed by 3% agarose gel electrophoresis.

**Ethics statement**

Clinical samples were collected by local physicians and well-trained laboratory technicians of health centers of the Ministry of Health, Ecuador. For routine parasitological diagnosis, scratching smear samples of skin lesions were taken from suspected leishmaniasis patients at health centers. In this study, only residual tissue materials were collected after the routine procedure to minimize the burden on patients. Signed consent was obtained from the adult subjects and from the children’s parents or guardians, prior to the diagnostic procedures at each health center of the Ministry, providing information on the process of diagnosis and Leishmania species analysis, following the guidelines of the Ethics Committee of the Ministry. The subjects studied were volunteers in routine diagnosis/screening and treatment programs promoted by the Ministry. All routine laboratory examinations were carried out free of charge, and treatment with specific drug, meglumine antimoniate (Glucantime) was also offered free of charge at each health center. The study was approved by the ethics committee of the Graduate School of Veterinary Medicine, Hokkaido University (approval number: vet26-4) and Jichi Medical University (approval number: 17–080) [8].

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**Table 2. Primer sequences used in this study.**

| Target gene                  | Primer     | Primer sequence (5’ to 3’) | Expected amplicon size (bp) |
|------------------------------|------------|-----------------------------|----------------------------|
| cytochrome b                 | outer      | L.cyt-AS                    | GCGGAGAGRARGAAAAGGC         | 978 |
| (cyt b)                      | inner      | L.cyt-S                     | GGTGATGGTTTATAGTTAGG         | 866 |
| cytochrome oxidase           | outer      | L.C0II-1S                   | AACATAGTCTCATTAGAGA         | 954 |
| subunit II—NADH              | inner      | L.C0II-2S                   | AATGCAACATGCAGTTATWA        | 736 |
| dehydrogenase subunit 1      | outer      | L.C0II-1R                   | ACAMCGRCCAGTTCTCTAC         |     |
| (COII-ND1)                   | inner      | L.C0II-2R                   | AATGAAATGTAACACATCAAC       |     |
| heat shock protein 70        | outer      | L.HSP-OS                    | GGGCACGAGTACCTGTTGCG        | 1,931|
| (hsp70)                      | inner      | L.HSP-IS2                   | CGTCTGAGTTCGTTGTTCAC        | 1,735|
| outer*                       | L.HSP-Ty1S | AATAATGATACACATCTAC         | 847 |
| inner*                       | L.HSP-Ty2S | AGTCGACCTCTTCGACCTTG        | 468 |
| mannose phosphate            | outer      | L.MPI-OS2                   | GGTGGGGAAGGAGATGGCG         | 1,214|
| isomerase                    | inner      | L.MPI-IS2                   | CGTCCAXCTTTTCGAGC           | 1,130|
| (mpi)                        | inner      | L.MPI-IR2                   | CTCAAAGCTGTTTTCGAGC         |     |
| 6-phosphogluconate           | outer      | L.6PGD-0S                   | GACACGATCTGTTGACCT          | 1,346|
| dehydrogenase                | inner      | L.6PGD-0R                   | GACACGATCTGTTGACCTG         |     |
| (6pgd)                       | inner      | L.6PGD-IR                   | CGTGTACATATGGGAGTGT         | 1,272|

*The primer sets were used for the PCR-RFLP analysis to differentiate *L. (V.) guyanensis* from *L. (V.) panamensis.*
Results

Sequence analysis of cyt b, hsp70, mpi and 6pgd genes from Leishmania strains

Leishmania cyt b, hsp70, mpi and 6pgd partial gene sequences were amplified from 27 strains of 6 species isolated in Ecuador. Sequences of these fragments showed high degrees of homology (88–100%, 82–100%, 83–100% and 94–100% in cyt b, mpi, 6pgd and hsp70 genes, respectively) with corresponding leishmanial genes registered in GenBank. The restriction enzyme mapping was performed in silico to see if species-specific enzyme sites could be found in cyt b, mpi, 6pgd and hsp70 gene fragments obtained in this study. Species-specific RFLP patterns could not be obtained for the cyt b gene because of intraspecies genetic variations through the sequences. On the hsp70 gene, restriction enzymes to differentiate Leishmania species were found; however, RFLP patterns including several smaller fragments (< 300 bp) were similar among species. Therefore, it seems difficult to identify the species based on RFLP patterns of hsp70 using agarose gel electrophoresis in some cases because of the resolution. On the other hand, restriction enzyme sites that can differentiate Leishmania species in Ecuador were identified in mpi and 6pgd genes, except for two very closely-related species, L. (V.) guyanensis and L. (V.) panamensis. Different RFLP patterns were obtained in L. (V.) guyanensis/L. (V.) panamensis, L. (V.) braziliensis/L. (V.) naiffi, L. (L.) major-like and L. (L.) mexicana for digested mpi gene fragments with a restriction enzyme HaeIII (Fig 1A). Although an RFLP polymorphism was observed in one (strain PT27) of seven L. (L.) mexicana strains, it did not affect species identification (Table 3). L. (V.) braziliensis and L. (V.) naiffi, showing the same RFLP patterns as HaeIII digestion, were differentiated by HpaI digestion (Table 3, Fig 1B). Although L. (V.) lainsoni, a recently reported species in the Ecuadorian Amazon [20], showed the same RFLP patterns as L. (V.) guyanensis/L. (V.) panamensis when digested with HaeIII and HpaI, BstXI digestion successfully differentiated it from L. (V.) guyanensis/L. (V.) panamensis, as reported in Peruvian strains (S2 Fig) [28].

Digestion of the 6pgd gene with Bsp1286I resulted in distinct gene fragment patterns of L. (V.) guyanensis/L. (V.) panamensis, L. (V.) braziliensis, L. (V.) naiffi, L. (L.) major-like and L. (L.) mexicana; however, the patterns between L. (V.) guyanensis/L. (V.) panamensis and L. (V.) naiffi were similar and difficult to discriminate because of only about a 50 bp difference in a

![Fig 1. PCR-RFLP analyses of mpi gene fragments from 6 Leishmania species in Ecuador.](https://doi.org/10.1371/journal.pntd.0007403.g001)
The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank databases under the accession numbers LC468908-LC468956.

Identification of genetically complex Leishmania strains in Ecuador

Identification of Leishmania species in clinical samples by PCR-RFLP

The results of the species identification obtained by the two nuclear genes always agreed with each other. The identification by PCR-RFLP analyses completely matched fragment of approximately 1 kbp (Fig 2A). The two species were successfully differentiated by digesting with HinfI (Fig 2B).

Although L. (V.) guyanensis and L. (V.) panamensis were not discriminated by PCR-RFLP of mpi and 6pgd genes, PCR-RFLP of the hsp70 gene with a restriction enzyme, BcI, successfully differentiated the two species as reported previously (Fig 3) [27].

Identification of Leishmania species in clinical samples by PCR-RFLP

PCR-RFLP analyses of mpi gene with restriction enzymes, HaeIII and HpaI, and 6pgd gene with Bsp1286I and HinfI were applied to 92 clinical samples collected at 33 sites in 14 provinces of Ecuador. PCR-RFLP analysis of the hsp70 gene with a restriction enzyme, BcI, was used for differentiation between L. (V.) guyanensis and L. (V.) panamensis. The results obtained by PCR-RFLP analyses were compared with those obtained by the cyt b gene sequence analysis. The results of the species identification obtained by the two nuclear genes always agreed with each other. The identification by PCR-RFLP analyses completely matched...
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with that obtained by the *cyt b* gene sequence analysis in all of *L. (V.) naiffi* (2 samples) and *L. (L.) mexicana* (3 samples) (Table 4). Of the 73 samples identified as *L. (V.) guyanensis* by *cyt b* gene analysis, 72 samples were identified as *L. (V.) guyanensis* by PCR-RFLP analyses, whereas one sample from a Pacific coast area showed a hybrid pattern of *L. (V.) guyanensis* and *L. (V.) panamensis* based on the PCR-RFLP of the *hsp70* gene (Figs 3 and 4). The sequence of the *hsp70* gene fragment was analyzed by direct sequencing, and a single nucleotide polymorphism was confirmed, showing “C” in *L. (V.) guyanensis* but “T” in *L. (V.) panamensis*, whereas a sample having a hybrid RFLP pattern had both “C” and “T” peaks at the corresponding position (S3 Fig), indicating the presence of a hybrid strain of *L. (V.) guyanensis* and *L. (V.) panamensis* in Ecuador. On the other hand, of the 14 samples identified as *L. (V.) braziliensis* by *cyt b* gene analysis, only 6 samples were identified as *L. (V.) braziliensis* by RFLP analyses (Table 4). In the other 8 samples identified as *L. (V.) braziliensis* by the *cyt b* gene analysis, three samples showed hybrid patterns in PCR-RFLP analyses of both the *mpi* and *6pgd* genes (Fig 5A and 5B). The sequences of *mpi* and *6pgd* gene fragments were analyzed by direct sequencing, and a single nucleotide polymorphism was confirmed, showing “C” in *L. (V.) guyanensis* but “T” in *L. (V.) braziliensis* of the *mpi* gene, and “T” in *L. (V.) guyanensis* but “C” in *L. (V.) braziliensis* of the *6pgd* gene. On the other hand, the *mpi* and *6pgd* genes from the three samples with hybrid RFLP patterns had both “C” and “T” peaks at the corresponding position (S4 Fig). From these results, the parasite species of these three samples were identified as a hybrid of *L. (V.) braziliensis* and *L. (V.) guyanensis* (Table 4, Fig 4). In the remaining 5 samples identified as *L. (V.) braziliensis* by sequence analysis of the *cyt b* gene, PCR-RFLP analyses showed that one sample from a Pacific coast area was *L. (V.) guyanensis*, three samples from the northern Pacific coast and Amazonian areas were *L. (V.) panamensis*, and one sample from a northern Pacific coast area had a hybrid pattern of *L. (V.) guyanensis* and *L. (V.) panamensis* (Table 4, Fig 4). The sequence analyses of *mpi*, *6pgd*, and *hsp70* gene fragments corresponded to PCR-RFLP analyses, indicating the presence of a mismatch between kinetoplast and nuclear genes, known as mito-nuclear discordance, in *Leishmania* distributing in Ecuador (Table 4, Fig 4). To further confirm the mito-nuclear discordance, partial sequences of the COII-ND1 region were analyzed as another target of kinetoplast genes in samples showing a mismatch between kinetoplast *cyt b* gene and nuclear *mpi*, *6pgd* and *hsp70* genes. The
sequences were compared to each two corresponding sequences obtained from *L. (V.) braziliensis* and *L. (V.) guyanensis* in this study since this region has not been well-analyzed in subgenus *Viannia* species. The sequences from parasites with mito-nuclear discordance showed 98.9–99.1% and 98.5–98.9% identities with those of *L. (V.) braziliensis* and *L. (V.) guyanensis*, respectively (accession numbers: LC475135-LC475142). When partial COII gene sequences in the obtained COII-ND1 region sequences were analyzed on the GenBank database, the sequences from parasites with mito-nuclear discordance showed 99.5% and 98.9% identities with those of *L. (V.) braziliensis* and *L. (V.) guyanensis*, respectively. This result strongly suggested that the kinetoplast genes of these parasites originated from *L. (V.) braziliensis*, corresponding to the result of cyt b gene analysis.

Fig 3. Differentiation between *L. (V.) guyanensis* and *L. (V.) panamensis* by PCR-RFLP of the *hsp70* gene fragment. PCR amplification was performed with *hsp70* gene-specific primers and the PCR products were digested with *BclI*. 1. *L. (V.) guyanensis*, 2. *L. (V.) panamensis*, 3. a hybrid of *L. (V.) guyanensis* and *L. (V.) panamensis*. https://doi.org/10.1371/journal.pntd.0007403.g003
In the present study, PCR-RFLP analyses were employed for the identification of Leishmania species distributing in Ecuador in order to develop a simple and practical way for species identification independent of expensive equipment such as a genetic analyzer. As a result, mpi and 6pgd genes, for which encoding enzymes have been widely used as the gold standard of species identification, were identified as suitable targets for this purpose in the tested samples. The results obtained by the PCR-RFLP analyses of multiple nuclear targets were compared to those of cyt b gene sequence analysis [7, 8, 29–36]. Although most results corresponded between the two analyses, PCR-RFLP revealed distribution of hybrid and mito-nuclear discordant Leishmania strains, which could not be identified only by cyt b gene sequence analysis. The results indicated that Leishmania strains distributing in Ecuador are genetically more complex than previously thought.

PCR-RFLP analysis has been employed for species identification of Leishmania species, and its utility is widely accepted [34]. The rRNA internal transcribed spacer 1 (ITS-1) region and hsp70 gene are mostly used as suitable target genes, of which the former is applied mainly in the Old World [6, 11, 12, 14, 17, 19, 27, 34, 37–41]. Although the hsp70 gene is one of the most valuable genetic markers for PCR-RFLP-based species identification, intraspecific polymorphism of RFLP patterns and very similar RFLP profiles among species, which affect species identification, have been reported in some Leishmania species [42]. In this study, other nuclear genes, mpi and 6pgd genes, for which encoding enzymes have been used for MLEE, were shown to be alternative useful targets for classification by PCR-RFLP analysis. Of these, the mpi gene was reported to be the only genetic marker that can distinguish two very closely-related species, L. (V.) braziliensis and L. (V.) peruviana [7, 43, 44]. In addition, a recent study demonstrated that PCR-RFLP of the shorter mpi gene fragment (approximately 500 bp) can differentiate 4 Leishmania species [L. (V.) braziliensis, L. (V.) peruviana, L. (V.) guyanensis, and L. (V.) lainsonii] and a hybrid of L. (V.) braziliensis and L. (V.) peruviana circulating in the Department of Huanuco, Peru [28]. In the present study, PCR-RFLP analyses of longer mpi and 6pgd gene fragments (>1000bp) were successfully established and applied to 92 clinical samples in Ecuador. Although a polymorphic RFLP pattern, which does not affect the identification, was detected in the mpi of one L. (L.) mexicana strain, the variant RFLP pattern was not detected in the present clinical samples identified as L. (L.) mexicana. Further sample analyses from different areas and different countries will be important to confirm the utility of this

| Target gene (analysis) | Identification * (numbers) |
|------------------------|-----------------------------|
| cyt b (cloning and sequencing) | L.g (73) L.b (14) L.n (2) L.mex (3) |
| mpi, 6pgd, and hsp70 (PCR-RFLP) | L.g (72) L.p/L.p (1) L.b (6) L.g/L.b (3) L.g’ (1) L.p’ (3) L.g/L.p’ (1) L.n (2) L.mex (3) |

*L.g: L. (V.) guyanensis, L.p: L. (V.) panamensis, L.b: L. (V.) braziliensis, L.n: L. (V.) naiffi, L.mex: L. (L.) mexicana, L.g/L.p: a hybrid of L. (V.) guyanensis and L. (V.) panamensis, L.g/L.b: a hybrid of L. (V.) guyanensis and L. (V.) braziliensis
*mito-nuclear discordance

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Discussion

In the present study, PCR-RFLP analyses were employed for the identification of Leishmania species distributing in Ecuador in order to develop a simple and practical way for species identification independent of expensive equipment such as a genetic analyzer. As a result, mpi and 6pgd genes, for which encoding enzymes have been widely used as the gold standard of species identification, were identified as suitable targets for this purpose in the tested samples. The results obtained by the PCR-RFLP analyses of multiple nuclear targets were compared to those of cyt b gene sequence analysis [7, 8, 29–36]. Although most results corresponded between the two analyses, PCR-RFLP revealed distribution of hybrid and mito-nuclear discordant Leishmania strains, which could not be identified only by cyt b gene sequence analysis. The results indicated that Leishmania strains distributing in Ecuador are genetically more complex than previously thought.

PCR-RFLP analysis has been employed for species identification of Leishmania species, and its utility is widely accepted [34]. The rRNA internal transcribed spacer 1 (ITS-1) region and hsp70 gene are mostly used as suitable target genes, of which the former is applied mainly in the Old World [6, 11, 12, 14, 17, 19, 27, 34, 37–41]. Although the hsp70 gene is one of the most valuable genetic markers for PCR-RFLP-based species identification, intraspecific polymorphism of RFLP patterns and very similar RFLP profiles among species, which affect species identification, have been reported in some Leishmania species [42]. In this study, other nuclear genes, mpi and 6pgd genes, for which encoding enzymes have been used for MLEE, were shown to be alternative useful targets for classification by PCR-RFLP analysis. Of these, the mpi gene was reported to be the only genetic marker that can distinguish two very closely-related species, L. (V.) braziliensis and L. (V.) peruviana [7, 43, 44]. In addition, a recent study demonstrated that PCR-RFLP of the shorter mpi gene fragment (approximately 500 bp) can differentiate 4 Leishmania species [L. (V.) braziliensis, L. (V.) peruviana, L. (V.) guyanensis, and L. (V.) lainsonii] and a hybrid of L. (V.) braziliensis and L. (V.) peruviana circulating in the Department of Huanuco, Peru [28]. In the present study, PCR-RFLP analyses of longer mpi and 6pgd gene fragments (>1000bp) were successfully established and applied to 92 clinical samples in Ecuador. Although a polymorphic RFLP pattern, which does not affect the identification, was detected in the mpi of one L. (L.) mexicana strain, the variant RFLP pattern was not detected in the present clinical samples identified as L. (L.) mexicana. Further sample analyses from different areas and different countries will be important to confirm the utility of this
analysis, although polymorphic RFLP profiles may be detectable in these genes. Since polymorphism was also reported in the hsp70 gene of several Leishmania species [42], PCR-RFLP analyses of multiple target genes, rather than single nuclear or kinetoplast genes, will result in

Fig 4. Geographic distribution of Leishmania species in Ecuador identified by PCR-RFLP analyses targeting multiple nuclear genes. The dark gray areas show the Andean plateau (>1,000 m altitude), and the light gray areas show highland jungle or Andean slopes (400–1,000 m elevation). (Adapted from a map available at http://english.freemap.jp/).

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Fig 5. Differentiation between L. (V.) guyanensis and L. (V.) braziliensis by PCR-RFLP of mpi and 6pgd gene fragments. A, B. PCR amplification was performed with mpi gene- or 6pgd gene-specific primers and the PCR products were digested with HaeIII (A) or Bsp1286I (B), respectively. 1. L. (V.) guyanensis, 2. L. (V.) braziliensis, 3. a hybrid of L. (V.) guyanensis and L. (V.) braziliensis.

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more accurate species identification and disclose more detailed genetic characteristics of the parasite.

Several samples showing hybrid RFLP patterns were identified as hybrid strains rather than mixed infection of different Leishmania species. It is due to the following reasons: 1) It is little or no chance to be infected by more than one parasite in a cutaneous lesion because the lesion is typically developed at the site bitten by a sand fly transmitting specific Leishmania species, 2) Even if mixed infection occurs, either parasite becomes dominant in the lesion, resulting in the presence of dominant allele by the genetic analysis. However, both alleles were comparably amplified as observed in the PCR-RFLP analysis, which is indicative of a putative hybrid strain. In addition, similar results were obtained on electrograms of the direct sequencing, showing comparable fluorescence intensities of polymorphic nucleotides derived from both species. 3) The presence of hybrid strain has been reported in the same area as described below [45]. Isolation of putative hybrid strains as a culture is necessary for further detailed characterization of these parasites.

Although multiple PCR-RFLP and cyt b sequence analyses showed corresponding results in most clinical samples, the present study revealed the distribution of several unexpected strains in Ecuador, including hybrid and mito-nuclear discordance strains. Since hybrid strains cannot be identified by the cyt b gene analysis after molecular cloning, this is another advantage of identifying parasite species by PCR-RFLP. Distribution of a hybrid strain of "L. (V.) guyanensis/panamensis" complex and "L. (V.) braziliensis" was reported in Zumba, a province of Zamora-Chinchipe in a southern part of Ecuador by using MLEE and random amplified polymorphic DNA (RAPD) [45]. The present study confirmed the presence of the hybrid strain in Zumba, and also in another area in the same province, Palanda. In addition, a hybrid of "L. (V.) guyanensis" and "L. (V.) panamensis" was detected in northern Pacific areas of Ecuador. This is the first report of the presence of a hybrid strain of "L. (V.) guyanensis" and "L. (V.) panamensis" in Ecuador. "L. (V.) guyanensis" and its closely related "L. (V.) panamensis" have been reported to be endemic in northern Pacific areas of Ecuador by MLEE; however, only "L. (V.) guyanensis" was identified in the same areas by cyt b gene analysis in recent studies [8, 21, 46]. The present study confirmed that "L. (V.) guyanensis" is dominantly present in these areas, suggesting that endemic species may change, or that there may be discordance between MLEE and genetic analysis. However, the identification of a hybrid of "L. (V.) guyanensis" and "L. (V.) panamensis" as a minor population suggests that parental "L. (V.) panamensis" may still be present in some of these areas. Another unexpected finding was identification of mito-nuclear discordant strains of Leishmania species in northern Pacific and Amazonian areas. Interestingly, mito-nuclear discordant strains were identified only in the species identified as "L. (V.) braziliensis" by cyt b gene analysis. This finding supports a recent study using cyt b gene analysis reporting increasing cases of "L. (V.) braziliensis" infection in Pacific coast areas when compared to previous studies using enzymatic MLEE analysis [8]. The hybrid strain of "L. (V.) braziliensis" and "L. (V.) peruviana" was suggested to increase disease severity when compared to parental species in an animal model [47]. Therefore, careful investigation is needed to clarify the presence of hybrid strains, including mito-nuclear discordance, and their effects on clinical courses. In addition, hybrid strains may increase the range of transmissible sand fly species if they have a potential to be transmitted by both vector species of parental parasites. Continuous vector research is important in these endemic areas, as well as parasitological and clinical studies. Further, basic parasitological research on how genetic exchange and mito-nuclear discordance occur among Leishmania species would be another interesting subject [48–51]. Mito-nuclear discordance is reported in various animals such as mammals, birds, reptiles, amphibians, fish and insects, and is inferred to result from various processes: 1) adaptive introgression of mitochondrial DNA, 2) demographic disparities, 3) sex-biased asymmetries, 4) hybrid zone movement, 5) an
intracellular bacteria, *Wolbachia* infection in insects, and 6) human actions [52]. It provides deeper insights into the phylogenetic relationship, population structure, and evolutionary signature of these animals. Mito-nuclear discordance is also reported in helminth parasites: trematodes *Schistosoma turkestanicum* between populations [33], and cestodes *Taenia solium* between lineages [54], and between *T. saginata* and *T. asiatica* [55–57]. This is the first report of mito-nuclear discordance in protozoan parasites. Mito-nuclear discordance is speculated to be resulted from the similar process as hybridization of nuclear genes in protozoa. Further study is needed to disclose the mechanism of mito-nuclear discordance formation in protozoa. In addition, association of mito-nuclear discordance with the pathogenicity and vector competency of the parasites is important issues to be clarified. In this study, we established a novel PCR-RFLP-based genotyping approach to identify *Leishmania* species in Ecuador. Although the present PCR-RFLP analyses was shown to be practical for identification of *Leishmania* species in Ecuador, further study focusing on other *Leishmania* species and clinical samples from different countries will be needed to enhance the utility of this approach. PCR-RFLP analyses of clinical samples and subsequent comparison with kinetoplast *cyt b* sequence analysis revealed the distribution of genetically complex *Leishmania* strains having genetic characteristics of hybrid and mito-nuclear discordance. Although intraspecies genetic variation observed in the *cyt b* gene resulted in this gene as an unsuitable target for RFLP analysis, there is no doubt about the utility of *cyt b* gene sequence analysis for species identification and phylogenetic analysis since distinct interspecies genetic diversity of this gene overcomes the disadvantage of the intraspecies variation. However, the present study points to the importance of applying multiple target approaches as the combination of *cyt b* and the PCR-RFLP assays presented here, enhancing the reliability of species identification and characterization of genetic properties including hybrid and mito-nuclear discordance. Further studies are needed to reveal the parasitological characteristics of hybrid and mito-nuclear discordance, clinical outcomes caused by these parasites, and the range of vector species of these parasites. In addition, studies on mito-nuclear discordance in *Leishmania* and other protozoa may provide further insights into the mechanism of genetic exchanges of these parasites.

**Supporting information**

**S1 Fig. Sample collection sites in Ecuador.** The dark gray areas show the Andean plateau (>1,000 m altitude), and the light gray areas show highland jungle or Andean slopes (400–1,000 m elevation). 1. San Lorenzo, 2. Esmeraldas, and 3. Atacames, Province of Esmeraldas; 4. Pedernales, 5. Montalvo, and 6. Pedro Pablo Gomez, Province of Manabi; 7. Cielo Verde, Province of Imbabura; 8. Puerto Quito, 9. Pedro Vicente Maldonado, 10. Los Bancos, 11. Nangalito, 12. Pachijal, and 13. Quinche, Province of Pichincha; 14. Valle Hermoso, Province of Santo Domingo; 15. Balsapamba, Province of Bolivar; 16. Chanchan, Province of Chimborazo; 17. La Troncal, Province of Canar; 18. El Triunfo, 19. Naranjal, and 20. Balao, Province of Guayas; 21. Santa Rosa, Province of El Oro; 22. Cascales, 23. Lago Agrio, and 24. Palma Roja, Province of Scumbios; 25. Coca, 26. Shangrila, 27. La Joya de los Sachas, 28. Pompeya, 29. Union Milagrena, and 30. Loreto, Province of Orellana; 31. Puyo, Province of Pastaza; 32. Palanda, and 33. Zumba, Province of Zamora-Chinchipe. (Adapted from a map available at [http://english.freemap.jp/](http://english.freemap.jp/)) (TIF)

**S2 Fig. PCR-RFLP analysis of *mpi* gene fragments from *L. (V.) guyanensis*, *L. (V.) panamensis*, and *L. (V.) lainsoni*.** PCR amplification was performed with *mpi* gene-specific primers and the PCR products were digested with *Bst*XI. 1. *L. (V.) guyanensis*, 2. *L. (V.) panamensis*, 3. *L. (V.) lainsoni*. (TIF)
panamensis, 3. L. (V.) lainsoni.

S3 Fig. Direct sequence analysis showing a species-specific polymorphic site of *Leishmania hsp70* gene fragments.

S4 Fig. Direct sequence analysis showing a species-specific polymorphic site of *Leishmania mpi* gene (A) or *6pgd* gene (B) fragments.

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