RESEARCH ARTICLE

Nuclear and kinetoplast DNA analyses reveal genetically complex *Leishmania* strains with hybrid and mito-nuclear discordance in Peru

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Abstract

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the mannose phosphate isomerase (*mpi*) gene was applied to 134 skin samples collected from patients with cutaneous leishmaniasis (CL) in Peru for identification of the infecting parasite at the species level, and the results were compared with those of cytochrome *b* (*cyt* b) gene sequencing obtained in previous studies. Although most results (121/134) including 4 hybrids of *Leishmania (Viannia) braziliensis* and *L. (V.) peruviana* corresponded to those obtained in the previous study, PCR-RFLP analyses revealed the distribution of putative hybrid strains between *L. (V.) peruviana* and *L. (V.) lainsoni* in two samples, which has never been reported. Moreover, parasite strains showing discordance between kinetoplast and nuclear genes (kDNA and nDNA), so-called mito-nuclear discordance, were identified in 11 samples. Of these, six strains had the kDNAs of *L. (V.) braziliensis* or *L. (V.) peruviana* and nDNAs of *L. (V.) guyanensis*, and three strains had the kDNAs of *L. (V.) shawi* and nDNAs of *L. (V.) braziliensis*. The rest were identified as mito-nuclear discordance strains having kDNAs of *L. (V.) braziliensis* or *L. (V.) peruviana* and nDNAs of *L. (V.) lainsoni*, and kDNAs of *L. (V.) lainsoni* and nDNAs of *L. (V.) braziliensis*. The results demonstrate that *Leishmania* strains in Peru are genetically more complex than previously considered.

Author summary

Protozoan parasites of the genus *Leishmania* are able to undergo genetic exchange during their growth. The previous description of hybrids in Peru and the recent discovery of unexpected genetically complex strains having characteristics of both hybrid and mito-

nuclear discordance in its neighbouring country (Ecuador) with a similar eco-epidemiological situation led us to consider that the genetic structure of *Leishmania* strains in Peru is more complicated than previously thought. In an effort to revise the data on *Leishmania* strain dispersion in Peru and to search for evidence of genetic recombination, the present study was conducted. A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis targeting the mannose phosphate isomerase (*mpi*) gene sequence was performed to identify the infecting parasite at the species level in 134 skin samples collected from patients with cutaneous leishmaniasis (CL) in Peru, and the results were compared with those of cytochrome *b* (*cyt* b) gene sequencing obtained in previous studies. Most results (121/134) including 4 hybrids between *L. (V.) braziliensis* and *L. (V.) peruviana* showed agreement between PCR-RFLP of the *mpi* gene and *cyt* b gene sequence analysis; however, 13 of 134 samples revealed the distribution of strains with hybrids and mito-nuclear discordance. The results demonstrate that genetically complex *Leishmania* strains are present in Peru. These findings indicate that *Leishmania* strain dispersion in Peru is genetically more complex than previously considered. Further prospective studies including larger samples and the isolation of parasite strains are required to update the available data.

Introduction

Parasitic protozoa belonging to the genus *Leishmania* show marked epidemiologic and clinical diversity, causing a wide-range of human and animal diseases extending from localized, self-limiting cutaneous lesions, and severe diffuse and destructive mucocutaneous lesions, to disseminating visceral infection that is fatal in the absence of treatment. The clinical heterogeneity, together with reservoir host spectrum and vector species compatibilities, lead to different parasite species associations, with over 20 species related to human infections. The cause of this heterogeneity, whether by continuous accumulation of mutations via mitotic cell division and/or via sexual recombination promoting admixtures of divergent genomes, is still a matter of debate [1]. Even if we are far from explaining how *Leishmania* parasites evolve and emerge in natural populations, correct species identification and searching for evidence of genetic recombination can provide key clues to the ecology and transmission patterns of these organisms. Correct diagnosis of leishmaniasis will also help to determine the clinical prognosis and an appropriate species-specific therapeutic regimen [2].

Multi-locus enzyme electrophoresis (MLEE) has been the gold standard for species characterization [3, 4]. However, this classification has been challenged based on genetic analysis of molecular targets, as they were much simpler, easier, and more practical [5–14]. Kinetoplast DNA (kDNA) is frequently used as a target for detection and typing of *Leishmania* due to its multicopy nature and high sensitivity [15, 16]. However, combining nuclear DNA (nDNA) and kDNA markers has improved the power of molecular data to detect unexpected genetically complex strains with characteristics of hybrid and mito-nuclear discordance widely distributed in Ecuador [17]. These recent findings have shown the necessity of updating the available data in its neighbouring country of Peru, with a similar eco-epidemiological situation, and searching for genetic recombination in Peruvian strains that were identified at the species level by kinetoplast cytochrome *b* (*cyt* b) gene sequence analysis in a previous study. The latter led to the identification of 7 species and 1 hybrid: *Leishmania (Viannia) braziliensis*, *L. (V.) peruviana*, *L. (V.) guyanensis*, *L. (V.) lainsoni*, *L. (V.) shawi*, *Leishmania (Leishmania) mexicana*, *L. (L.) amazonensis*, and a hybrid of *L. (V.) braziliensis/L. (V.) peruviana* [18].

Previous results on the current epidemiological situation in Peru showed that the predominant species were *L. (V.) peruviana*, *L. (V.) braziliensis*, and *L. (V.) guyanensis* in the Andean highlands, tropical lowland rainforests, and northern to central rainforest areas, respectively [19–22]. These studies also demonstrated the presence of *L. (V.) lainsoni* and *L. (L.) amazonensis* in lower-altitude rainforest areas [20–22], and the current prevalence in the same areas was confirmed [18]. The identification of hybrid forms was performed using a polymerase chain reaction-restriction fragment length polymorphism (PCR- RFLP) analysis of a short mannose phosphate isomerase (*mpi*) gene fragment. Hybrid genotypes have been rarely revealed among natural isolates of *Leishmania* parasites, which conventionally indicated that members of the genus possess the machinery for genetic exchange [17, 19]. A hybrid of *L. (V.) braziliensis* and *L. (V.) peruviana* was first reported in the Department of Huanuco [19, 23], and was recently identified in the Department of Cajamarca [18]. Additionally, just four cases of infection by *L. (V.) shawi* were detected in rainforest areas by the Departments of Junin, Madre de Dios, Cusco, and Puno, as previously reported [18, 22], and the question remains unanswered about the low rate of infection caused by this species. Verification of the full details of gene property variants will help to understand the eco-epidemiological situation in this endemic area.

The present study utilized PCR-RFLP analysis targeting the *mpi* gene sequence to identify the infecting parasite at the species level in 134 skin samples collected from patients with cutaneous leishmaniasis (CL) in Peru, and the results were compared with those of *cyt* b gene sequencing obtained in previous studies. The results demonstrate that genetically complex *Leishmania* strains are present in Peru, highlighting the need to combine both nDNA and kDNA targets to improve the validity of species identifications for full details of gene property variants.

Materials and methods

Sample collection

The clinical samples employed in this study were collected from patients suspected of having CL from 58 sites in 38 provinces administered by 17 departments in Peru using FTA cards (Whatman, Newton Center, MA, USA) and Giemsa-stained smears in previous studies [18, 22] (S1 Fig). Only identified specimens in previous studies were used in this study to avoid selection bias. The preparation of FTA Card samples was done as previously described [18]. Briefly, two-mm-diameter disks were punched out from each filter paper. The disks were then transferred to separate tubes, washed two times with FTA Purification Reagent (Whatman) and one time with Tris-EDTA buffer, and then air dried. The filter paper disks were directly subjected to PCR amplification. DNA extraction from Giemsa-stained smears obtained from skin lesions (ulcers and/or nodules) on CL patients was performed as previously described [18]. Briefly, 50 μL of DNA extraction buffer [150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.1% sodium dodecyl sulfate (SDS)] containing 100 μg/mL of proteinase K was spotted onto each smear sample and mixed well. The samples were transferred to 1.5-mL tubes and incubated at 37˚C for 12 hours. After being heat-inactivated at 95˚C for 5 min, 0.5-μL portions were directly used as the templates for PCR amplification.

PCR and sequence analysis

Multiple targets including *mpi*, 6-phosphogluconate dehydrogenase (*6pgd*), heat shock protein 70 (*hsp70*), and cytochrome oxidase subunit II-NADH dehydrogenase subunit I (*COII-ND1*) gene fragments were used to confirm species identifications for full details of gene property variants. The primers and PCR conditions were described previously [17] except for the *mpi* gene fragment, where new primers were designed to amplify a shorter fragment (S2 and S3

Figs). For the *mpi* gene fragment, PCR amplification with a pair of specific primers, L.MPI-AS (5'- TCGATTCGCACGGCTCTGTC-3') and L.MPI-OR (5'-CTCAAGTCGTTGGTCGAC GC-3'), was performed with 30 cycles of denaturation (95˚C, 1 min), annealing (55˚C, 1 min), and polymerization (72˚C, 1 min) using Ampdirect Plus reagent (Shimadzu Biotech, Tsukuba, Japan) and high-fidelity DNA polymerase, KOD plus (Toyobo, Osaka, Japan), or PrimeSTAR HS (Takara Bio, Shiga, Japan). Each 0.5-μL portion of the PCR product was reamplified with L.MPI-IR2 (5'-GCCGTACGGYACCGCAAAGC-3') and L.MPI-BS (5'-AACCACAAGCCW GAGCTCAT-3').

PCR-RFLP analysis

PCR products of the *mpi* gene fragment were digested with the restriction enzymes *Hae*III, *Vpa*K11BI, and *Bst*XI. The restriction enzymes were selected with the support program BioEdit. The digested samples were separated by electrophoresis in 2% agarose gel (STAR Agarose RSV-AGRP from Rikaken, Aichi, Japan) for *Vpa*K11BI and *Bst*XI to separate longer fragments and 3% gel for *Hae*III to separate shorter fragments in order to produce DNA fragment patterns using the GeneRuler 100 bp Plus DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA) as molecular size markers. The gel was stained with GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA), and DNA fragments were visualized with a UV transilluminator.

Ethics statement

Informed consent was obtained from all participating adults; for children under the age of 18 years, consent was also obtained from their parents or guardians. Verbal explanations and written information sheets were provided, following the guidelines of the Ethics Committee of the Ministry of Health, Peru. Clinical samples were collected by well-trained local doctors, biologists, and laboratory technicians from health posts and centers 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 a specific drug, sodium stibogluconate (Pentostan), 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) [17, 18]. A case report form (CRF) was collected for each sample and unique codes were assigned to ensure confidentiality. All subjects and guardians consented to lesions being photographed anonymously. Permission was obtained from the Ministry of Health and from community leaders in Peru.

Results

Identification of *Leishmania* species using PCR-RFLP

In a recent study, PCR-RFLP analyses of a long *mpi* gene fragment (1,130 bp) using the restriction enzymes *Hae*III and *Hpa*I differentiated *Leishmania* species in Ecuador, except for two very closely-related species, *L. (V.) guyanensis* and *L. (V.) panamensis* [17]. In addition, using a short *mpi* gene fragment (460 bp), 4 *Leishmania* species, *L. (V.) braziliensis*, *L. (V.) peruviana*, *L. (V.) guyanensis*, and *L. (V.) lainsoni*, and a hybrid of *L. (V.) braziliensis* and *L. (V.) peruviana*, could be differentiated in the Department of Huanuco, Peru [23]. In the present study, new primers were designed to amplify a 807 bp-*mpi* fragment (S2 Fig), and the PCR-RFLP technique was optimized using three diagnostic restriction enzymes (*Hae*III, *Vpa*K11BI, and *Bst*XI) for species identifications in the studied area. The RFLP patterns were predicted according to the sequences obtained from the GenBank database, and were tested on the
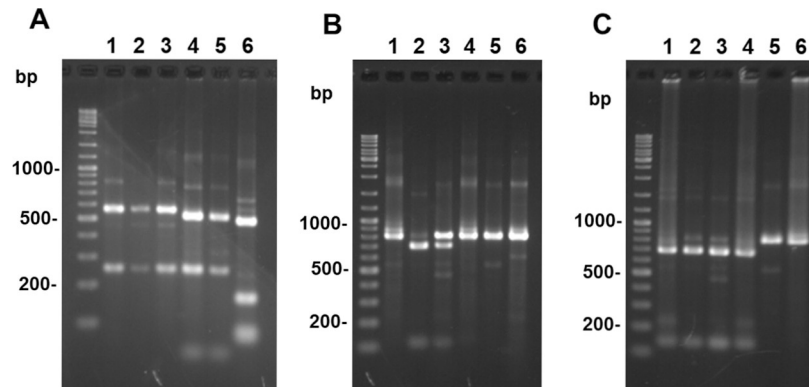
Fig 1. PCR-RFLP analyses of *mpi* gene fragments from 6 *Leishmania* species in Peru. PCR amplification was performed with leishmanial mannose phosphate isomerase (*mpi*) gene-specific primers, and PCR products were digested with *Hae*III (A), *Vpa*k11BI (B), or *Bst*XI (C), and resulting restriction fragment patterns were analyzed by agarose gel electrophoresis. 1. *L. (V.) braziliensis*, 2. *L. (V.) peruviana*, 3. a hybrid of *L. (V.) braziliensis/L. (V.) peruviana*, 4. *L. (V.) guyanensis*, 5. *L. (V.) lainsoni*, 6. *L. (L.) amazonensis*.

Leishmania species listed in S1 Table (Fig 1). The application of PCR-RFLP to 134 skin samples collected from patients with CL in Peru (Fig 2) identified 5 *Leishmania* species [*L. (V.) braziliensis*, *L. (V.) peruviana*, *L. (V.) guyanensis*, *L. (V.) lainsoni*, and *L (L.) amazonensis*] and two putative hybrids [*L. (V.) braziliensis/L. (V.) peruviana* and *L. (V.) peruviana/L. (V.) lainsoni*] (Table 1). However, an unexpected RFLP pattern appeared in one sample, in which a single polymorphism nucleotide prevented the enzymes from cutting the target position, corresponding to *L. (V.) braziliensis* based on sequence analysis.

Comparative analysis of nDNA and kDNA

The results obtained by PCR-RFLP analyses were compared with those obtained by *cyt* b gene sequence analysis. In the previous studies, *L. (V.) braziliensis* was differentiated from *L. (V.) peruviana* by PCR-RFLP of the 460bp *mpi* gene fragment (S2 Fig) since the two species cannot be differentiated by kDNA sequence analysis [18,22]. Most results including 4 hybrids between *L. (V.) braziliensis* and *L. (V.) peruviana* agreed with previous ones obtained by *cyt* b gene sequence analysis; however, 13 of 134 samples showed discordance between the present and previous analyses (Tables 1 and 2) [18, 22]. In detail, of the 100 samples identified as *L. (V.) braziliensis* or *L. (V.) peruviana* by *cyt* b gene sequence analysis, 43, 44, and 4 samples were identified as *L. (V.) braziliensis*, *L. (V.) peruviana*, and a hybrid between *L. (V.) braziliensis* and *L. (V.) peruviana* by PCR-RFLP analyses of the *mpi* gene, corresponding to previous studies [18, 22]. On the other hand, six samples from wide areas of Peru and one sample from the north area, all of which were identified as *L. (V.) braziliensis* or *L. (V.) peruviana* by the *cyt* b gene sequence analysis, were *L. (V.) guyanensis* and *L. (V.) lainsoni*, respectively, by PCR-RFLP analyses of the *mpi* gene (Table 1). Sequence analyses of the *mpi* gene fragment supported the results of PCR-RFLP. In addition, two samples identified as *L. (V.) peruviana* using sequence analysis of the *cyt* b gene and PCR-RFLP analysis of the short *mpi* gene fragment in the previous studies showed hybrid patterns with *L. (V.) lainsoni* using PCR-RFLP analyses targeting 807bp-*mpi* gene fragments in the present study (Fig 3A–3C). Direct sequencing of the *mpi* fragments identified double peaks at the corresponding positions (Figs 3D and S3). According to our knowledge, this is the first reported evidence of putative hybridization between *L. (V.) peruviana* and *L. (V.) lainsoni*. On the other hand, of the 16 samples identified as *L. (V.) lainsoni* by *cyt* b gene analysis, 15 samples were identified as *L. (V.) lainsoni* whereas one sample
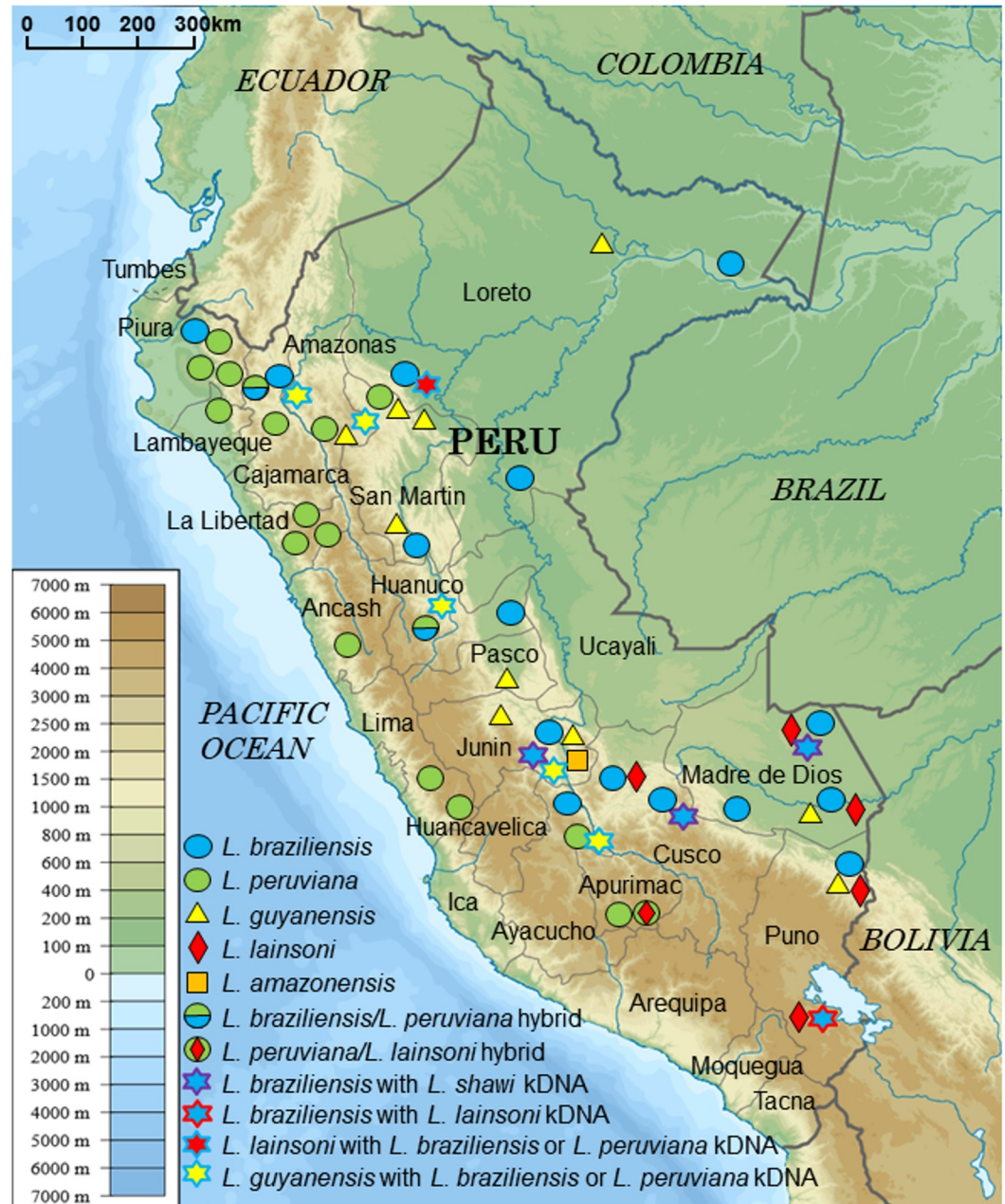
Fig 2. Geographic distribution of *Leishmania* species in Peru identified by PCR-RFLP of *mpi* and sequence analyses of kinetoplast and nuclear DNAs. (Adapted from a map available at https://commons.wikimedia.org/wiki/File%3APeru_physical_map.svg).

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was *L. (V.) braziliensis* by PCR-RFLP analyses of the *mpi* gene. In the same way, all three samples identified as *L. (V.) shawi* by sequence analysis of the *cyt* b gene were identified as *L. (V.) braziliensis* by PCR-RFLP analysis of the *mpi* gene. The mito-nuclear discordances having kDNA of *L. (V.) braziliensis* or *L. (V.) peruviana* and nDNA of *L. (V.) lainsoni*, and kDNA of *L. (V.) braziliensis* or *L. (V.) peruviana* and nDNA of *L. (V.) shawi* have not been reported in Peru or elsewhere. This is also the first reported evidence of mito-nuclear discordance having kDNA of *L. (V.) braziliensis* or *L. (V.) peruviana* and nDNA of *L. (V.) guyanensis* in Peru.

Table 1. Distribution of *Leishmania* species by department in Peru.

Department	*Leishmania* species[*]							Total
	Lb	Lp	Lg	Ll	La	Lb/Lp	Lp/Ll	
Amazonas	0	1	3 (2[#])	0	0	0	0	4
Ancash	0	1	0	0	0	0	0	1
Apurimac	0	1	0	0	0	0	2	3
Ayacucho	2	1	2[#]	0	0	0	0	5
Cajamarca	1	1	0	0	0	3	0	5
Cusco	4 (1[#])	0	0	1	0	0	0	5
Huanuco	3	0	1[#]	0	0	1	0	5
Junin	2 (1[#])	0	3 (1[#])	0	1	0	0	6
La Libertad	0	8	0	0	0	0	0	8
Lambayeque	0	18	0	0	0	0	0	18
Lima	0	2	0	0	0	0	0	2
Loreto	3	0	1	1[#]	0	0	0	5
Madre de Dios	24 (1[#])	0	1	2	0	0	0	27
Pasco	0	0	2	0	0	0	0	2
Piura	1	10	0	0	0	0	0	11
Puno	6 (1[#])	0	1	12	0	0	0	19
San Martin	1	1	6	0	0	0	0	8
Total	47	44	20	16	1	4	2	134

[*]Parasite species were identified by the PCR-RFLP analysis targeting 807bp *mpi* gene fragments (S2 Fig) with restriction enzymes *Hae*III, *Vpa*K11BI, and *Bst*XI. Lb, *L. (V.) braziliensis*; Lp, *L. (V.) peruviana*; Lg, *L. (V.) guyanensis*; Ll, *L. (V.) lainsoni*; La, *L. (L.) amazonensis*; Lb/Lp, a putative hybrid of *L. (V.) braziliensis* and *L. (V.) peruviana*; Lb/Ll, a putative hybrid of *L. (V.) peruviana* and *L. (V.) lainsoni*

[#]Mito-nuclear discordance was determined by the comparative analysis of nuclear (*mpi*, *hsp70*, and *6pgd*) and kinetoplast (*cyt* b and *COII-ND1*) DNA fragments.

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Eleven samples showing discordance between *mpi* gene and *cyt* b gene analyses were systematically analyzed using multiple targets including *mpi*, *hsp70*, *6pgd*, and *COII-ND1* genes to confirm mito-nuclear discordance (Tables 1 and 2). Sequence analysis confirmed the discordance

Table 2. Comparison of *Leishmania* species identification in Peru targeting kinetoplast and nuclear DNAs.

Target DNA[1]	Identification[2]: numbers						
kDNA	Lb or Lp[3]: 100			Lg: 14	Ll: 16	Ls: 3	La: 1
nDNA	Lb: 43	Lp: 44	Lb/Lp: 4	Lg: 14	Ll: 15	Lb: 3	La: 1
	Lg: 6	Ll: 1	Lp/Ll: 2		Lb: 1		
kDNA + nDNA	Lb: 43	Lp: 44	Lb/Lp: 4	Lg: 14	Ll: 15	Ls-Lb: 3	La: 1
	Lb,p-Lg: 6	Lp/Ll: 2			Ll-Lb: 1		
	Lb,p-Ll: 1						

[1]kDNA: kinetoplast DNA, nDNA: nuclear DNA. Parasite species were identified by the sequencing analysis of *cyt* b gene (kDNA) and by the PCR-RFLP analysis of *mpi* gene (nDNA) fragments with restriction enzymes *Hae*III, *Vpa*K11BI, and *Bst*XI. Mito-nuclear discordance was further determined by the comparative analysis of nDNA (*hsp70*, and *6pgd*) and kDNA (*COII-ND1*) fragments.

[2]Lb: *L. (V.) braziliensis*, Lp: *L. (V.) peruviana*, Lg: *L. (V.) guyanensis*, Ll: *L. (V.) lainsoni*, Ls: *L. (V.) shawi*, La: *L. (L.) amazonensis*, Lb/Lp: a putative hybrid of *L. (V.) braziliensis* and *L. (V.) peruviana*, Lp/Ll: a putative hybrid of *L. (V.) peruviana* and *L. (V.) lainsoni*, Lb,p-Lg: *L. (V.) guyanensis* with kDNAs of *L. (V.) braziliensis* or *L. (V.) peruviana*, Lb,p-Ll: *L. (V.) lainsoni* with kDNAs of *L. (V.) braziliensis* or *L. (V.) peruviana*, Ll-Lb: *L. (V.) braziliensis* with *L. (V.) lainsoni* kDNA, Ls-Lb: *L. (V.) braziliensis* with *L. (V.) shawi* kDNA

[3]*L. (V.) braziliensis* and *L. (V.) peruviana* cannot be differentiated by kDNA sequence analysis.

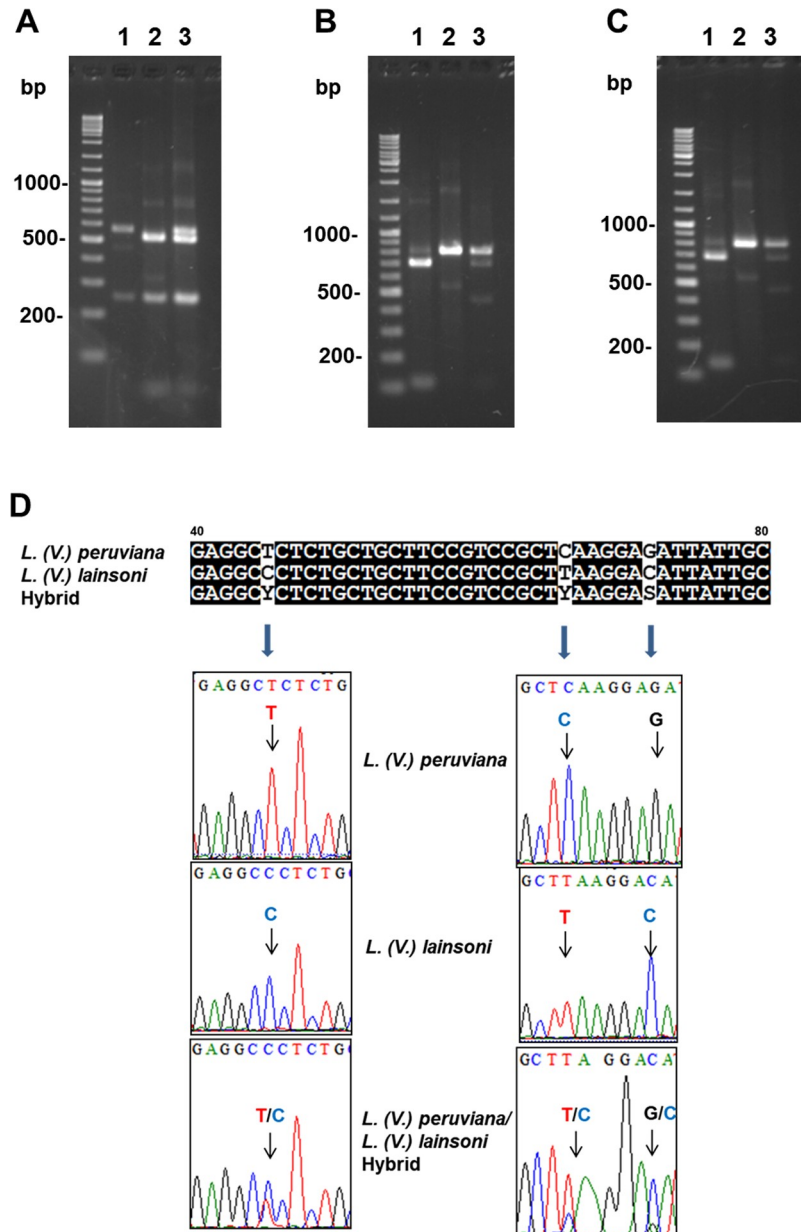https://doi.org/10.1371/journal.pntd.0008797.t002

Fig 3. Differentiation between *L. (V.) peruviana* and *L. (V.) lainsoni* by PCR-RFLP and direct sequence analyses of *mpi* gene fragment. A, B, C. PCR amplification was performed with *mpi* gene-specific primers and the PCR products were digested with *Hae*III (A), *Vpa*k11BI (B), or *Bst*XI (C). 1. *L. (V.) peruviana*, 2. *L. (V.) lainsoni*, 3. a hybrid of *L. (V.) peruviana* and *L. (V.) lainsoni*. D. Direct sequence analysis showing three among several species-specific polymorphic sites of the *Leishmania mpi* gene fragment.

between nDNAs and kDNAs in these samples. The nucleotide sequence data reported were deposited in GenBank databases under the accession numbers LC517842-LC517879.

Discussion

Comparative analysis of nuclear and kinetoplast DNA fragments revealed the occurrence of 5 *Leishmania* species in the study areas: *L. (V.) braziliensis*, *L. (V.) peruviana*, *L. (V.) guyanensis*, *L. (V.) lainsoni*, and *L. (L.) amazonensis*, and two putative hybrids: *L. (V.) braziliensis/L. (V.)*

peruviana and *L. (V.) peruviana*/*L. (V.) lainsoni*, of which the latter was firstly identified in Peru. We also identified mito-nuclear discordances having kDNAs of *L. (V.) braziliensis* or *L. (V.) peruviana* and nDNAs of *L. (V.) lainsoni*, and kDNAs of *L. (V.) braziliensis* or *L. (V.) peruviana* and nDNAs of *L. (V.) shawi*, which have never been reported in Peru or elsewhere. In addition, we reported here the first evidence of mito-nuclear discordance having kDNAs of *L. (V.) braziliensis* or *L. (V.) peruviana* and nDNAs of *L. (V.) guyanensis* in Peru. Based on the geographic distribution, kDNAs of these mito-nuclear discordance strains probably originate from *L. (V.) braziliensis* since *L. (V.) peruviana* is prevalent mostly in Andean highland areas. Unexpectedly, approximately 10% of the samples were identified as strains with mito-nuclear discordance or hybrids caused by genetic exchange, indicating that the genetic structure of *Leishmania* strains in Peru is more complicated than previously recorded [18].

Of the 134 studied samples, 2 and 4 samples were identified as putative hybrids of *L. (V.) peruviana*/*L. (V.) lainsoni* and *L. (V.) braziliensis*/*L. (V.) peruviana*, respectively. Regarding its capacity to identify hybrids of these species, the PCR-RFLP method should be considered as a fundamental tool to be applied. Here, we report the first putative hybrid between *L. (V.) peruviana* and *L. (V.) lainsoni*. Natural hybrids were reported to occur episodically [17, 24–33]. In this context, it is important to note that most hybridization reported was either intra-specific or involved very closely related species: *L. (V.) braziliensis* and *L. (V.) peruviana*, although hybrids of more distant species have also been reported, such as *L. (L). major*/*L. (L.) infantum* [33] and *L. (L.)* major/*L. (L.)* arabica [31]. The compatibility of different species to construct hybrids is unknown, and our finding of a putative *L. (V.) peruviana*/*L. (V.) lainsoni* hybrid expands the known natural compatibility between species. Both species are quite different with respect to these characteristics, and the impact of hybridization between such divergent species is currently unknown. Although the possibility of co-infection cannot be excluded completely, both alleles were proportionately amplified in PCR-RFLP analysis, strongly suggesting infection by hybrid strains in these patients. It is important to note here that isolation of putative hybrid strains as a culture is required for further detailed characterization of these parasites, since the possibility of co-infection cannot be excluded completely. *Lutzomyia ayacuchensis* is a proven vector of *L. (V.) peruviana* in the Andean area of southern Peru [34], whereas no transmission of *L. (V.) lainsoni* by sand fly species has been reported in these areas. Further investigation in relation to isolation of natural hybrids strains may be necessary for basic parasitological research on how and where genetic exchange occurs among *Leishmania* species. The genetic exchange may affect vector susceptibility, and hybrid strains may be transmitted by both vectors of parental species/strains [35]. In addition, using an animal model, hybrids between *L. (V.) braziliensis* and *L. (V.) peruviana* have been suggested to increase the disease severity when compared with the parental strains [36]. The pathological impact of recombinant genotypes in human infections associated with virulence, transmissibility, and drug susceptibility warrants further investigation in relation to improving methods of identification and isolation of natural hybrid strains.

Our results are the first reported evidence of mito-nuclear discordance among *Leishmania* species from Peru, highlighting the importance of combining both nDNA and kDNA to improve the validity of species identification for full details of gene property variants. Here, we report the first evidence of mito-nuclear discordance having kDNAs of *L. (V.) braziliensis* or *L. (V.) peruviana* and nDNAs of *L. (V.) guyanensis* in Peru, which was reported in Ecuador [17]. In this context, it is important to note that the most mito-nuclear discordant strains were found in the species identified as *L. (V.) braziliensis* or *L. (V.) peruviana* using *cyt* b gene analysis. The present study showed the occurrence of mito-nuclear discordance having kDNAs of *L. (V.) braziliensis* or *L. (V.) peruviana* and nDNAs of *L. (V.) shawi*, never detected in Peru or elsewhere. In this context, it is important to note that just four cases infected by *L. (V.) shawi*

were detected in rainforest areas by the Departments of Junin, Madre de Dios, Cusco, and Puno, as reported previously [18, 22] and the question remains unanswered about the rarity of infection caused by this species. *L. (V.) shawi* was originally identified as a parasite of wild animals, and sand fly species that transmit it may prefer to feed on animals rather than humans, resulting in a steady rate of infections in wild animals via sand fly bites and a lower risk of infection for humans. However, further investigations using both and nDNA and kDNA genes may provide more clarifications about its epidemiological roles and geographical distributions including mito-nuclear discordance, as reported in the present work. Another unexpected finding was the identification of mito-nuclear discordant strains having kDNAs of *L. (V.) braziliensis* or *L. (V.) peruviana* and nDNAs of *L. (V.) lainsoni*, and kDNAs of *L. (V.) lainsoni* and nDNAs of *L. (V.) braziliensis*, never reported in Peru or elsewhere. Only one mito-nuclear discordant strain was identified in the species identified as *L. (V.) lainsoni* by *cyt* b gene analysis, which may confuse the previous results on identifications [18]. Further investigations are required to elucidate the mechanism of mito-nuclear discordance in protozoa. Several mechanisms have been shown to cause mito-nuclear discordance: incomplete lineage sorting, sex-biased asymmetries, introgression, natural selection, and genetic sweeps mediated by *Wolbachia* infection [37]. Mito-nuclear discordance was firstly reported in systems where early genetic tools were more developed compared with other taxa such us mitochondrial introgression between two species of fruit fly (*Drosophila pseudoobscura* and *D. persimilis*) and two species of mouse (*Mus domesticus* and *M. musculus*) [38, 39]. The number of cases continued to increase slowly until 2001. Following these early discoveries, methods for assaying numerous individuals for their nuclear genotype became more widely available to researchers and, subsequently, the number of cases increased markedly in animals such as mammals, birds, reptiles, amphibians, fish, and insects [37]. More recent studies revealed that mito-nuclear discordance occurs in helminth parasites [40–44].

In the present study, PCR- RFLP targeting leishmanial *mpi* gene fragments was performed, and the results were compared with those obtained by kinetoplast *cyt* b gene sequence analysis. The PCR-RFLP method was shown to be practical for the identification of *Leishmania* species in Peru, revealing unexpected genetically complex *Leishmania* strains with characteristics of hybrid and mito-nuclear discordance. Since hybrid strains were proposed to aggravate the severity of disease and may be transmitted by a larger range of sand fly species, many questions have been raised about the occurrence and frequency of such cross-species genetic exchange under natural conditions, modalities of hybrid transmission, their long-term maintenance, and the consequences of these transfers on phenotypes such as drug resistance or pathogenicity. Further country-wide epidemiological studies will be needed to reveal the characteristics of hybrid and mito-nuclear discordance and provide further insight into the mechanism of genetic exchanges of these parasites.

Supporting information

S1 Fig. The locations where one or more *Leishmania* specimens were collected. 1, Utcubamba, Amazonas; 2, Rodriguez de Mendoza, Amazonas; 3, Luya, Amazonas; 4, Huarmey, Ancash; 5, Aymaraes, Apurimac; 6, La Mar, Ayacucho; 7, Huanta, Ayacucho; 8, Jaen, Cajamarca; 9, Cutervo, Cajamarca; 10, Calca, Cusco; 11, La Convención, Cusco; 12, Puerto Inca, Huanuco; 13, Huanuco, Huanuco; 14, Leoncio Prado, Huanuco; 15, Chanchamayo, Junin; 16, Satipo, Junin; 17, Otuzco, La Libertad; 18, Santiago de Chuco, La Libertad; 19, Gran Chimú, La Libertad; 20, Lambayeque, Lambayeque; 21, Yauyos, Lima; 22, Huarochirí, Lima; 23, Alto Amazonas, Loreto; 24, Maynas, Loreto; 25, Mariscal Ramón Castilla, Loreto; 26, Ucayali, Loreto; 27, Tahuamanu, Madre de Dios; 28, Tambopata, Madre de Dios; 29, Manu, Madre de

Dios; 30, Oxapampa, Pasco; 31, Ayabaca, Piura; 32, Huancabamba, Piura; 33, Morropon, Piura; 34, Sandia, Puno; 35, Puno, Puno; 36, Moyobamba, San Martin; 37, Lamas, San Martin; 38, Tocache, San Martin. (Adapted from a map available at http://english.freemap.jp/).
(TIF)

S2 Fig. Schematic presentation of the coding region of the mannose phosphate isomerase (*mpi*) gene and location of primers for amplification of *mpi* gene fragments [17, 23]. Triangles denote primers used for amplification of each *mpi* gene fragment, and nucleotide positions of each primer are in parentheses. An asterisk at nucleotide position 1,082 shows a polymorphic nucleotide between *L. (V.) peruviana* and *L. (V.) braziliensis*.
(TIF)

S3 Fig. Direct sequence analysis showing all species-specific polymorphic sites of the *Leishmania mpi* gene fragment.
(TIF)

S1 Table. "Fragment size of leishmanial mpi gene generated by digestion with selected restriction enzymes".
(DOCX)

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References

1.   Rougeron V, De Meeus T, Banuls AL. A primer for *Leishmania* population genetic studies. Trends in parasitology. 2015; 31(2):52–9. https://doi.org/10.1016/j.pt.2014.12.001 PMID: 25592839

2. Berman JD. Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. Clin Infect Dis. 1997; 24:684–703. https://doi.org/10.1093/clind/24.4.684 PMID: 9145744

3. Cupolillo E, Grimaldi G Jr, Momen H. A general classification of New World *Leishmania* using numerical zymotaxonomy. Am J Trop Med Hyg. 1994; 50:296–311. https://doi.org/10.4269/ajtmh.1994.50.296 PMID: 8147488

4. Rioux JA, Lanotte G, Serres E, Pratlong F, Bastien P, Perieres J. Taxonomy of *Leishmania*. Use of iso-enzymes. Suggestions for a new classification. Ann Parasitol Hum Comp. 1990; 65:111–125. https://doi.org/10.1051/parasite/1990653111 PMID: 2080829

5. Dedet JP. Current status of epidemiology of leishmaniases. Farrell JP, ed. *Leishmania*, World-Class Parasites: Volume 4. Boston, MA: Kluwer Academic Publishers, 2002;1–10.

6. Laurent T, Van der Auwera G, Hide M, Mertens P, Quispe-Tintaya W, Deborggraeve S, De Doncker S, Leclipteux T, Bañuls AL, Büscher P, Dujardin JC. Identification of Old World *Leishmania* spp. by specific polymerase chain reaction amplification of cysteine proteinase B genes and rapid dipstick detection. Diagn Microbiol Infect Dis. 2009; 63:173–181. https://doi.org/10.1016/j.diagmicrobio.2008.10.015 PMID: 19097841

7. da Silva LA, de Sousa C, dos S, da Grac GC, Porrozzi R, Cupolillo E. Sequence analysis and PCR RFLP profiling of the *hsp70* gene as a valuable tool for identifying *Leishmania* species associated with human leishmaniasis in Brazil. Infect Genet Evol. 2010; 10:77–83. https://doi.org/10.1016/j.meegid.2009.11.001 PMID: 19913112

8. Kato H, Caceres AG, Mimori T, Ishimaru Y, Sayed AS, Fujita M, Iwata H, Uezato H, Velez LN, Gomez EA, Hashiguchi Y. Use of FTA cards for direct sampling of patient's lesions in the ecological study of cutaneous leishmaniasis. J Clin Microbiol. 2010; 48:3661–3665. https://doi.org/10.1128/JCM.00498-10 PMID: 20720027

9. Kato H, Gomez EA, Martini-Robles L, Muzzio J, Velez L, Calvopiña M, Romero-Alvarez D, Mimori T, Uezato H, Hashiguchi Y. Geographic distribution of *Leishmania* species in Ecuador based on the cytochrome *b* gene sequence analysis. PLoS Negl Trop Dis. 2016a; 10:e0004844. https://doi.org/10.1371/journal.pntd.0004844 PMID: 27410039

10. Talmi-Frank D, Nasereddin A, Schnur LF, Schönian G, Töz SO, Jaffe CL, Baneth G. Detection and identification of Old World *Leishmania* by high resolution melt analysis. PLoS Negl Trop Dis. 2010; 4: e581. https://doi.org/10.1371/journal.pntd.0000581 PMID: 20069036

11. de Almeida ME, Steurer FJ, Koru O, Herwaldt BL, Pieniazek NJ, da Silva AJ. Identification of *Leishmania* spp. by molecular amplification and DNA sequencing analysis of a fragment of rRNA internal transcribed spacer 2. J Clin Microbiol. 2011; 49:3143–3149. https://doi.org/10.1128/JCM.01177-11 PMID: 21752983

12. Fraga J, Veland N, Montalvo AM, Praet N, Boggild AK, Valencia BM, Arévalo J, Llanos-Cuentas A, Dujardin JC, Van der Auwera G. Accurate and rapid species typing from cutaneous and mucocutaneous leishmaniasis lesions of the New World. Diagn Microbiol Infect Dis. 2012; 74:142–150. https://doi.org/10.1016/j.diagmicrobio.2012.06.010 PMID: 22819605

13. Montalvo AM, Fraga J, Maes I, Dujardin JC, Van der Auwera G. Three new sensitive and specific heat-shock protein 70 PCRs for global *Leishmania* species identification. Eur J Clin Microbiol Infect Dis. 2012; 31:1453–1461. https://doi.org/10.1007/s10096-011-1463-z PMID: 22083340

14. Chaouch M, Fathallah-Mili A, Driss M, Lahmadi R, Ayari C, Guizani I, Ben Said M, Benabderrazak S. Identification of Tunisian *Leishmania* spp. by PCR amplification of cysteine proteinase B (*cpb*) genes and phylogenetic analysis. Acta Trop. 2013; 125:357–365. https://doi.org/10.1016/j.actatropica.2012.11.012 PMID: 23228525

15. Jara M, Adaui V, Valencia BM, Martinez D, Alba M, Castrillon C, Cruz M, Cruz I, Van der Auwera G, Llanos-Cuentas A, Dujardin JC, Arevalo J. 2013. Real-time PCR assay for detection and quantification of *Leishmania* (*viannia*) organisms in skin and mucosal lesions: exploratory study of parasite load and clinical parameters. J. Clin. Microbiol. 2013; 51: 1826e1833.

16. Kato H, Uezato H, Katakura K, Calvopiña M, Marco JD, Bar- roso PA, Gomez EA, Mimori T, Korenaga M, Iwata H, Nonaka S, Hashiguchi Y. Detection and identification of *Leishmania* species within naturally infected sand flies in the Andean areas of Ecuador by a polymerase chain reaction. Am J Trop Med Hyg. 2005; 72: 87–93. PMID: 15728872

17. Kato H, Gomez EA, Seki C, Furumoto H, Martini-Robles L, Muzzio J, Calvopiña M, Velez L, Kubo M, Tabbabi A, Yamamoto DS, Hashiguchi Y. 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. PLoS Negl Trop Dis. 2019; 13(5):e0007403. https://doi.org/10.1371/journal.pntd.0007403 PMID: 31059516

18. Kato H, Cáceres AG, Seki C, Silupu García CR, Holguín Mauricci C, Castro Martínez SC, Moreno Paico D, Castro Muniz JL, Troyes Rivera LD, Villegas Briones ZI, Guerrero Quincho S, Sulca Jayo GL,

Tineo Villafuerte E, Manrique de Lara Estrada C, Arias FR, Passara FS, Ruelas Llerena N, Kubo M, Tabbabi A, Yamamoto DS, Hashiguchi Y. Further insight into the geographic distribution of *Leishmania* species in Peru by cytochrome *b* and mannose phosphate isomerase gene analyses. PLoS Negl Trop Dis. 2019; 13(6):e0007496. https://doi.org/10.1371/journal.pntd.0007496 PMID: 31220120

19. Dujardin JC, Banuls AL, Llanos-Cuentas A, Alvarez E, DeDoncker S, Jacquet D, et al. Putative *Leishmania* hybrids in the Eastern Andean valley of Huanuco, Peru. Acta Trop. 1995; 59(4):293–307. https://doi.org/10.1016/0001-706x(95)00094-u PMID: 8533665

20. Lucas CM, Franke ED, Cachay MI, Tejada A, Cruz ME, Kreutzer RD, Barker DC, McCann SH, Watts DM. Geographic distribution and clinical description of leishmaniasis cases in Peru. Am J Trop Med Hyg. 1998; 59:312–317. https://doi.org/10.4269/ajtmh.1998.59.312 PMID: 9715953

21. Arevalo J, Ramirez L, Adaui V, Zimic M, Tulliano G, Miranda-Vera stegui C, Lazo M, Loayza-Muro R, De Doncker S, Maurer A, Chappuis F, Dujardin JC, Llanos-Cuentas A. Influence of *Leishmania (Viannia)* species on the response to antimonial treatment in patients with American tegumentary leishmaniasis. J Infect Dis. 2007; 195:1846–1851. https://doi.org/10.1086/518041 PMID: 17492601

22. Kato H, Caceres AG, Mimori T, Ishimaru Y, Sayed AS, Fujita M, Iwata H, Uezato H, Velez LN, Gomez EA, Hashiguchi Y. Use of FTA cards for direct sampling of patients' lesions in the ecological study of cutaneous leishmaniasis. J Clin Microbiol. 2010; 48:3661–3665. https://doi.org/10.1128/JCM.00498-10 PMID: 20720027

23. Koarashi Y, Cáceres AG, Saca FMZ, Flores EEP, Trujillo AC, Alvares JLA, Yoshimatsu K, Arikawa J, Katakura K, Hashiguchi Y, Kato H. Identification of causative *Leishmania* species in Giemsa-stained smears prepared from patients with cutaneous leishmaniasis in Peru using PCR-RFLP. Acta Trop. 2016; 158:83–87. https://doi.org/10.1016/j.actatropica.2016.02.024 PMID: 26943992

24. Banuls AL, Guerrini F, Le Pont F, Barrera C, Espinel I, Guderian R, Echeverria R, Tibayrenc M. Evidence for hybridization by multilocus enzyme electrophoresis and random amplified polymorphic DNA between *Leishmania braziliensis* and *Leishmania panamensis/guyanensis* in Ecuador. J Eukaryot Microbiol. 1997; 44:408–411. https://doi.org/10.1111/j.1550-7408.1997.tb05716.x PMID: 9304809

25. Belli AA, Miles MA, Kelly JM. A putative *Leishmania panamensis/Leishmania braziliensis* hybrid is a causative agent of human cutaneous leishmaniasis in Nicaragua. Parasitology. 1994; 109:435–442. https://doi.org/10.1017/s0031182000080689 PMID: 7800411

26. Cruz AK, Titus R, Beverley SM. 1993. Plasticity in chromosome number and testing of essential genes in *Leishmania* by targeting. Proc Natl Acad Sci USA. 1993; 90:1599–1603. https://doi.org/10.1073/pnas.90.4.1599 PMID: 8381972

27. Delgado O, Cupolillo E, Bonfante-Garrido R, Silva S, Belfort E, Grimaldi JG, Momen H. Cutaneous leishmaniasis in Venezuela caused by infection with a new hybrid between *Leishmania (Viannia) braziliensis* and *L. (V.) guyanensis*. Mem Inst Oswaldo Cruz. 1997; 92:581–582. https://doi.org/10.1590/s0074-02761997000500002 PMID: 9566221

28. Dujardin JC, Arevalo J, Llanos-Cuentas A, Caceres A, Le Ray D. From pulsed field to field: contribution of molecular karyotyping to epidemiological studies on New World leishmaniasis. Arch Inst Pasteur Tunis. 1993a; 70:505–515. PMID: 7802508

29. Dujardin JC, Gajendran N, Arevalo J, Llanos-Cuentas A, Guerra H, Gomez J, Arroyo J, De Doncker S, Jacquet D, Hamers R. Karyotype polymorphism and conserved characters in the *Leishmania (Viannia) braziliensis* complex explored with chromosome-derived probes. Ann Soc Belg Med Trop. 1993b; 73:101–118. PMID: 8368887

30. Dujardin JC, Llanos-Cuentas A, Caceres A, Arana M, Dujardin JP, Guerrini F, Gomez J, Arroyo J, De Doncker S, Jacquet D. 1993c. Molecular karyotype variation in *Leishmania (Viannia) peruviana*: indication of geographical populations in Peru distributed along a north-south cline. Ann Trop Med Parasitol. 1993b; 87:335–347. https://doi.org/10.1080/00034983.1993.11812777 PMID: 8250624

31. Kelly JM, Law JM, Chapman CJ, van Eys GJ, Evans DA. 1991. Evidence of genetic recombination in *Leishmania*. Mol Biochem Parasitol. 1991. 46:253–263. https://doi.org/10.1016/0166-6851(91)90049-c PMID: 1656255

32. Nolder D, Roncal N, Davies CR, Llanos-Cuentas A, Miles MA. Multiple hybrid genotypes of *Leishmania (Viannia)* in a focus of mucocutaneous leishmaniasis. Am J Trop Med Hyg. 2007; 76:573–578. PMID: 17360886

33. Ravel C, Cortes S, Pratlong F, Morio F, Dedet JP, Campino L. First report of genetic hybrids between two very divergent *Leishmania* species: *Leishmania infantum* and *Leishmania major*. Int J Parasitol. 2006; 36:1383–1388. https://doi.org/10.1016/j.ijpara.2006.06.019 PMID: 16930606

34. Caceres AG, Villaseca P, Dujardin JC, Banuls AL, Inga R, Lopez M, Arana M, Le Ray D, Arevalo J. 2004. Epidemiology of Andean cutaneous leishmaniasis: incrimination of *Lutzomyia ayacuchensis* (Diptera: psychodidae) as a vector of *Leishmania* in geographically isolated, upland valleys of Peru. Am J Trop Med Hyg. 2004; 70:607–612. PMID: 15211000

35. Volf P, Benkova I, Myskova J, Sadlova J, Campino L, Christophe Ravel C. Increased transmission potential of *Leishmania major/Leishmania infantum* hybrids. Int J Parasitol. 2007; 37:589–593. https://doi.org/10.1016/j.ijpara.2007.02.002 PMID: 17376453

36. Cortes S, Esteves C, Mauricio I, Maia C, Cristovao JM, Miles M, Campino L. 2012. *In vitro* and *in vivo* behaviour of sympatric *Leishmania (V.) braziliensis L. (V.) peruviana* and their hybrids. Parasitology. 2012; 139:191–199. https://doi.org/10.1017/S0031182011001909 PMID: 22054424

37. Toews DPL, Brelsford A. The biogeography of mitochondrial and nuclear discordance in animals. Mol Ecol. 2012; 21:3907–3930 https://doi.org/10.1111/j.1365-294X.2012.05664.x PMID: 22738314

38. Powell JR. Interspecific cytoplasmic gene flow in the absence of nuclear gene flow: evidence from *Drosophila*. Proc Natl Acad Sci USA. 1983; 80:492–495. https://doi.org/10.1073/pnas.80.2.492 PMID: 6300849

39. Ferris SD, Sage RD, Huang CM, Nielsen JT, Ritte UR, Wilson AC (1983) Flow of mitochondrial DNA across a species boundary. Proc Natl Acad Sci USA.1983; 80:2290–2294. https://doi.org/10.1073/pnas.80.8.2290 PMID: 6300907

40. Lawton SP, Bowen LI, Emery AM, Majoros G. Signatures of mito-nuclear discordance in *Schistosoma turkestanicum* indicate a complex evolutionary history of emergence in Europe. Parasitology. 2017; 144:1752–1762. https://doi.org/10.1017/S0031182017000920 PMID: 28747240

41. Yanagida T, Carod JF, Sako Y, Nakao M, Hoberg EP, Ito A. Genetics of the pig tapeworm in Madagascar reveal a history of human dispersal and colonization. PLoS One. 2014; 9:e109002. https://doi.org/10.1371/journal.pone.0109002 PMID: 25329310

42. Yamane K, Suzuki Y, Tachi E, Li T, Chen X, Nakao M, Nkouawa A, Yanagida T, Sako Y, Ito A, Sato H, Okamoto M. Recent hybridization between *Taenia asiatica* and *Taenia saginata*. Parasitol Int. 2012; 61:351–355. https://doi.org/10.1016/j.parint.2012.01.005 PMID: 22301089

43. Yamane K, Yanagida T, Li T, Chen X, Dekumyoy P, Waikagul J, Nkouawa A, Nakao M, Sako Y, Ito A, Sato H, Okamoto M. Genotypic relationships between *Taenia saginata*, *Taenia asiatica* and their hybrids. Parasitology. 2013; 140:1595–1601. https://doi.org/10.1017/S0031182013001273 PMID: 24112449

44. Sato MO, Sato M, Yanagida T, Waikagul J, Pongvongsa T, Sako Y, Sanguankiat S, Yoonuan T, Kounnavang S, Kawai S, Ito A, Okamoto M, Moji K. *Taenia solium, Taenia saginata, Taenia asiatica*, their hybrids and other helminthic infections occurring in a neglected tropical diseases' highly endemic area in Lao PDR. PLoS Negl Trop Dis. 2018; 12:e0006260. https://doi.org/10.1371/journal.pntd.0006260 PMID: 29420601