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Evolution of the *Leishmania braziliensis* species complex from amplified fragment length polymorphisms, and clinical implications

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ABSTRACT

In order to get more insight into its evolution and geographical distribution, we investigated the *Leishmania* (*Viannia*) *braziliensis* species complex using amplified fragment length polymorphisms and sequencing of a heat-shock protein 70 gene fragment. Previously, several assays had alluded to the high genetic diversity of the group, and single-locus assays typically identified two species, i.e. *L. braziliensis* and *Leishmania peruviana*, with occasional genetic signatures of both in the same strain. By analysis of 53 parasite isolates from Peru, and eight additional ones from other countries, we identified an atypical *L. braziliensis* cluster, and confirmed the origin of *L. peruviana* from the *L. braziliensis* cluster during the colonization of the western Andean coastal valleys. We discuss the clinical and taxonomical implications of our findings in relation to currently used species typing assays.

1. Introduction

Leishmania braziliensis (Vianna, 1911) and *Leishmania peruviana* (Velez, 1913) are two closely related species united in the *L. braziliensis* complex, the most important dermotropic *Leishmania* group in the Americas (Grimaldi et al., 1989). In Peru, they are differentially distributed within a zone where the Brazilian Amazon rain forest transitions into the Pacific coastal ecosystem through the Peruvian Andes (Lumbreras and Guerra, 1985), with local areas of sympatric occurrence (Dujardin et al., 1993b,c, 1995b; Nolder et al., 2007). The distribution is characterized by populations relatively separated within specific bio-geographical units demarcated by physical barriers such as rivers, deserts and mountains (Lamas, 1982).

L. peruviana causes human cutaneous leishmaniasis (CL), locally referred to as "uta" (Lainson and Shaw, 1987; Lumbreras and Guerra, 1985), and has been isolated from both dogs and small peridomestic animals such as mice and opossums, suggesting it is a zoonosis (Llanos-Cuentas et al., 1999). The species is endemic in Peru, where it occurs at altitudes between 800 and 3000 m above sea level (a.s.l.) on the western slopes of the Andes and in the inter-Andean valleys in the lower (500–1500 m a.s.l.) and upper (1500–3500 m a.s.l.) montane forests. The departments of Piura and Ayacucho are the northern and southern limits respectively. *L. braziliensis* on the other hand has a much wider distribution within Central and South America, both as a native anthropozoonosis (Grimaldi et al., 1989; Oddone et al., 2009) or as incidental cases imported by human migration (mier-David et al., 1993). Circulation is sylvatic, and it is encountered mainly on the eastern slopes of the Peruvian Andes, stretching into the Brazilian jungle lowlands at altitudes below 800 m a.s.l. (Lumbreras and Guerra, 1985). Contrary to *L. peruviana*, human CL caused by *L. braziliensis* is characteristically known for its ability to progress to the severe mucocutaneous form also known as "espundia" or Amazonian leishmaniasis (Lainson and Shaw, 1987; Lumbreras and Guerra, 1985).

The two species were originally separated by their differences in genomic and kinetoplast DNA buoyant density and by

Abbreviations: AFLP, amplified fragment length polymorphism; a.s.l, above sea level; CL, cutaneous leishmaniasis; *cpb*, cysteine proteinase B gene; *hsp*70, heat-shock protein 70 gene; ML, mucocutaneous leishmaniasis; MLEE, multilocus enzyme electrophoresis; MLST, multilocus sequence typing; *mpi*, mannose phosphate isomerase gene; MPI, mannose phosphate isomerase; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism.

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serological typing (Lainson and Shaw, 1987). Different reports either treat them as a single heterogeneous species (Arana et al., 1990; Grimaldi et al., 1987), as a single group with *L. peruviana* being a subspecies of *L. braziliensis*, or as two distinct taxa (Bañuls et al., 2000; Dujardin et al., 1993a, 1998; Garcia et al., 2005; Victoir et al., 1998). A global multilocus enzyme electrophoresis (MLEE) classification of *Leishmania* was performed by Rioux et al. (1990), who treated *L. peruviana* as a zymodeme within the *L. braziliensis* complex. Other MLEE analyses showed *L. braziliensis* and *L. peruviana* to have similar electrophoretic profiles for all standard enzymes except mannose phosphate isomerase (MPI) (Arana et al., 1990; Bañuls et al., 2000; Chouicha et al., 1997). Further analysis at sequence level by multilocus sequence typing (MLST) revealed the weak genetic support underlying the separation of the two species, as only three discriminatory single nucleotide polymorphisms, one in the *mpi* gene (Tsukayama et al., 2009; Zhang et al., 2006) and two within the aspartate dehydrogenase gene (Tsukayama et al., 2009), were revealed. Both parasites have been separated as well by molecular karyotyping (Dujardin et al., 1993c, 1995a), random amplified polymorphic DNA (RAPD, Bañuls et al., 2000), glycoprotein 63 (Victoir et al., 2003), and cysteine proteinase B (*cpb*, Garcia et al., 2005). A global phylogeny based on heat-shock protein 70 gene (*hsp*70) sequences (Fraga et al., 2010) suggested a subspecies status of *L. peruviana* within the larger and more diverse *L. braziliensis*.

Apart from the debatable status of *L. peruviana* as either a species or a subspecies, taxonomy is further complicated by the apparent existence of intermediate forms between both parasites. Several years of molecular characterization of parasite isolates of clinical samples from Peru have revealed variants that show both *L. peruviana* and *L. braziliensis* alleles in either *cpb*, *mpi*, and/or *hsp*70, the evolutionary implications and clinical relevance of which are as yet unclear. Also previous MLEE and RAPD-based studies have identified parasites with mixed enzyme profiles (Bañuls et al., 2000; Dujardin et al., 1995a; Nolder et al., 2007). In order to get more insight into the relationships and phylogeographic patterns of the *L. braziliensis* species complex, we undertook a broad genetic study essentially in Peru, using amplified fragment length polymorphisms (AFLP, Vos et al., 1995) as a random genome screening method. To this end we applied a protocol validated on the *Leishmania donovani* species complex (Odiwuor et al., 2011).

2. Material and methods

2.1. Parasite selection

DNA was prepared from cultures of parasites originally isolated from cutaneous and mucocutaneous leishmaniasis patients in Peru, Bolivia, and Panama (one sample) for the projects LeishNatDrug-R (a multicenter study on pentavalent antimonials), LeishBolPe (an epidemiological study in Bolivia and Peru), and LeishEpinetSA (an epidemiological study in South-America). *L. braziliensis* and *L. peruviana* isolates from these studies were selected on the basis of species determination by one or a combination of assays based on three different loci (Table 1): species-specific PCRs of *mpi* (Boggild et al., 2010; Zhang et al., 2006); restriction fragment length polymorphism (RFLP) analysis of *cpb* (Garcia et al., 2005); and *hsp*70 RFLP with enzymes *Hae*III and *Rsa*I (Montalvo et al., 2010). MLEE-typed (Rioux et al., 1990) reference strains of *L. peruviana* (MHOM/PE/LCA08, Bañuls et al., 2000) and *L. braziliensis* (MHOM/BR/75/M2903) were also included. From some isolates, cultured parasite clones were analyzed instead of the original isolate to exclude the possibility of mixed isolates. All isolates, strains, and clones are listed in Table 1.

2.2. Amplified fragment length polymorphism analysis

AFLP was done with 12 primer pairs, and the data were analyzed using the BioNumerics software (Applied-Maths, Sint-Martens-Latem, Belgium), exactly as described in Odiwuor et al. (2011). Bands were scored as either present, absent or uncertain, whereby the latter were excluded from the analysis. A Neighbor-net clustering (Bryant and Moulton, 2004) based on Jaccard distance coefficients (Jaccard, 1908) was constructed as implemented in the program SplitsTree (Huson and Bryant, 2006). Confidence in the clusters was estimated by bootstrap analysis of 2000 replicates. The population structure of the *L. braziliensis* complex was inferred using the program Structure (Pritchard et al., 2000), which assigns individuals to different populations (K) on the basis of allele frequencies. K was estimated according to Evanno et al. (2005), which is based upon the rate of change in the probability between successive values of K.

2.3. Hsp70 sequence analysis

A partial *hsp*70 nucleotide sequence of 1380 base pairs (Fraga et al., 2010) was determined from 10 strains (seven of which were included in the AFLP analysis), and these were aligned with sequences available from EMBL/GenBank. Sequence-based clustering was performed using the software package MEGA4 (Tamura et al., 2007), on the basis of *p*-distances and the Neighbor-Joining method (Saitou and Nei, 1987), thereby excluding gaps and ambiguous nucleotides from the analysis in a pair-wise fashion. Two thousand bootstrap replicates were run to assess the confidence of the groups. Sequence ambiguities in the EMBL/GenBank entries of the new sequences do not reflect uncertainties, but indicate the presence of multiple alleles.

3. Results

3.1. AFLP typing

Twelve AFLP runs generated 1350 markers forming the basis for clustering and population analyses of the isolates listed in Table 1. Fig. 1 shows a Neighbor-net graph, in which three main groups can be distinguished. The majority of the isolates is united in group 1, and seems to originate from the center of the network without any apparent structured relationships, apart from a few exceptions of parasites clustering with more than 99% bootstrap support. Group 2 is supported by a 99.3% bootstrap value and also originates from the center of the network, but within this group more resolution is apparent. We recognize the bootstrap supported entities 2b and 2c, and refer to the remaining isolates as 2a. Finally, the bootstrap supported group 3 represents the most distant isolates. Two parasites split off from the line connecting group 3 with the rest of the parasites, and hence cannot be assigned to any of the three groups: CU00181 and LH2538.

Results from Structure population analysis (Fig. 2) were congruent with Neighbor-net clustering. With three major populations identified, group 1 parasites were found to derive 85–100% of their genetic repertoire from one parasite population. Group 2a parasites represent a second population, while groups 2b and 2c contain a mix of alleles from groups 1 and 2a. Group 3 represents the remaining population, with isolates CU00181 and LH2538 combining alleles from populations 1 and 3.

The parasites included in the AFLP analysis were originally isolated between 1975 and 2008 (Table 1), and this could introduce a time bias into the observed genotypes. This was found not to be the case, as groups 1, 2, and 3 all cover at minimum the parasite isolation period 1991–2007, which proves all these groups existed

Table 1
List of isolates and reference strains used in AFLP analysis.

	Name[a]	*hsp*70 GenBank ID[b]	Country	Year of isolation	Department	Disease[c]	AFLP[d]	*hsp*70[e]	*mpi*[e]	*cpb*[e]
1	CUM555 [A]	FR872760*	Bolivia	2001	–	CL	3	B	–	–
2	CUM663 [A]	FR872761*	Bolivia	2002	–	CL	3	B	–	–
3	CUM623 [B]		Bolivia	2002	–	CL	1	B	–	–
4	CUM700 [B]		Bolivia	2002	–	CL	1	B	–	–
5	M2903	M87878	Brazil	1975	–	CL	1	B	B	B
6	LH2182	FN395040	Peru	2001	Loreto	CL	1	B	–	–
7	LH2210		Peru	2002	Cajamarca	CL	1	B	–	–
8	LH2217		Peru	2001	Cusco	CL	1	B	–	–
9	LH2033		Peru	2001	Junín	CL	1	B	–	–
10	LH2309		Peru	2002	Cusco	CL	1	B	–	–
11	LH2356		Peru	2002	Huánuco	CL	1	B	–	–
12	LH2382	FR715989	Peru	2002	Madre de Dios	CL	1	B	–	–
13	LH2419		Peru	2002	Madre de Dios	CL	1	B	–	–
14	LH2442		Peru	2003	Cusco	CL	1	B	–	–
15	LH2501		Peru	2003	Madre de Dios	CL	1	B	–	–
16	LH2512		Peru	2003	Ucayali	CL	1	B	–	–
17	LH2863		Peru	2003	Loreto	ML	1	B	–	–
18	LH2875		Peru	2003	Madre de Dios	CL	1	B	–	–
19	LH2981		Peru	2003	Madre de Dios	ML	1	B	–	–
20	CU00131		Peru	2006	Cusco	CL	1	B or P	B	B
21	CU00185		Peru	2006	Madre de Dios	CL	1	B or P	B	B
22	LH3550		Peru	2006	Lima	CL	1	B or P	B	B
23	LH3597		Peru	2006	Madre de Dios	CL	1	B or P	B	B
24	LH3663		Peru	2006	Huánuco	CL	1	B or P	B	B
25	LH3751		Peru	2007	Cusco	CL	1	B or P	B	B
26	LH3589		Peru	2006	Madre de Dios	CL	1	B or P	B	B + P
27	LH3660		Peru	2006	Ucayali	CL	1	B or P	–	B + P
28	LH3821		Peru	2007	Cusco	CL	1	B or P	B	B + P
29	LH3828		Peru	2007	Lima	CL	1	–	B	B + P
30	LH3857		Peru	2007	Ucayali	CL	1	B or P	B	B + P
31	LH3864		Peru	2007	Lima	CL	1	B or P	B	B + P
32	CU00181	FR872764*	Peru	2006	Cusco	CL	H	B or P	B	B + P
33	AM001		Peru	2008	Huánuco	CL	2c	–	B + P	B + P
34	LC1407.cl5	FR872766*	Peru	1991	Huánuco	CL	2c	B or P	B + P	B + P
35	LC1419		Peru	1991	Huánuco	CL	2c	B or P	B + P	B + P
36	LH2542		Peru	2003	Junín	CL	1	B + P	–	–
37	CUM57 [C]		Bolivia	1994	–	ML	1	B + P	–	–
38	CUM505 [C]		Bolivia	2000	–	ML	1	B + P	–	–
39	LH2140	FR715987	Peru	2001	Junín	CL	1	B + P	–	–
40	LH2224		Peru	2002	Huánuco	CL	1	B + P	–	–
41	LH2887		Peru	2003	Ucayali	ML	1	B + P	–	–
42	LH2920		Peru	2003	Junín	ML	1	B + P	–	–
43	LH2332		Peru	2002	Pasco	CL	1	B + P	–	–
44	LH2538	FR715988	Peru	2003	Ayacucho	CL	H	B + P	–	–
45	LH2206		Peru	2002	Ucayali	CL	1	B + P	–	–
46	LH2162	FR715986	Peru	2001	Madre de Dios	CL	1	B + P	–	–
47	LH2358		Peru	2002	Madre de Dios	CL	1	B + P	–	–
48	LH2511	FR715990	Peru	2003	Huánuco	CL	3	B	–	–
49	HB22	FR872765*	Peru	1990	Piura	–	2b	P	P	P
50	HB86.cl4		Peru	1990	Piura	–	2b	P	P	P
51	LCA08.cl2	EU599089	Peru	1990	Ayacucho	–	2a	P	P	P
52	LH3596		Peru	2006	Ancash	CL	2a	B or P	P	P
53	LH3630		Peru	2006	Lima	CL	2a	B or P	P	P
54	LH3631		Peru	2006	Lima	CL	2a	B or P	P	P
55	LH3644		Peru	2006	Lima	CL	2a	B or P	P	P
56	LH3807		Peru	2007	Lima	CL	2a	B or P	P	P
57	LH3853		Peru	2007	Ancash	CL	2a	B or P	P	P
58	LH3855		Peru	2007	Lima	CL	2a	B or P	P	P
59	LH3650	FR872762*	Panama	2006	El Darien	CL	3	B or P	B	P
60	LH3657		Peru	2006	Ayacucho	ML	1	B or P	B	P
61	LH3851	FR872763*	Peru	2007	Cusco	CL	3	B or P	B	P

[a] Pairs of strains indicated by [A], [B], and [C] respectively were collected from the same patients at different episodes of the disease. The name followed by ".cl" indicates a parasite clone number.
[b] Accession numbers from the sequences included in Fig. 3. Those indicated by * were sequenced in this study.
[c] CL: cutaneous leishmaniasis (Uta); ML: mucocutaneous leishmaniasis (Espundia).
[d] Groups as defined by AFLP (Figs. 1 and 2). H: Presumed hybrid between groups 1 and 3.
[e] Results from single locus typing assays: *mpi*, mannose phosphate isomerase species-specific PCRs; *cpb*, cysteine proteinase B PCR-RFLP; *hsp*70, heat-shock protein 70 PCR-RFLP. Outcome of assays: B, *L. braziliensis*; P, *L. peruviana*; B + P, mixed alleles from both species; –, not done. "B or P" indicates that *hsp*70 was analyzed with restriction endonuclease *Hae*III, which does not allow separating both species, as opposed to a digest with *Rsa*I (Montalvo et al., 2010).

simultaneously. This takes into account LEM2222 not included in the AFLP analysis, but clearly from group 3 in the *hsp*70 dendrogram (Fig. 3, see below). As for groups 2a, 2b, and 2c, these all contain at least one isolate from the period 1990–1991, again showing these parasites circulated simultaneously. Groups 2a and 2c moreover contain parasites isolated in the period

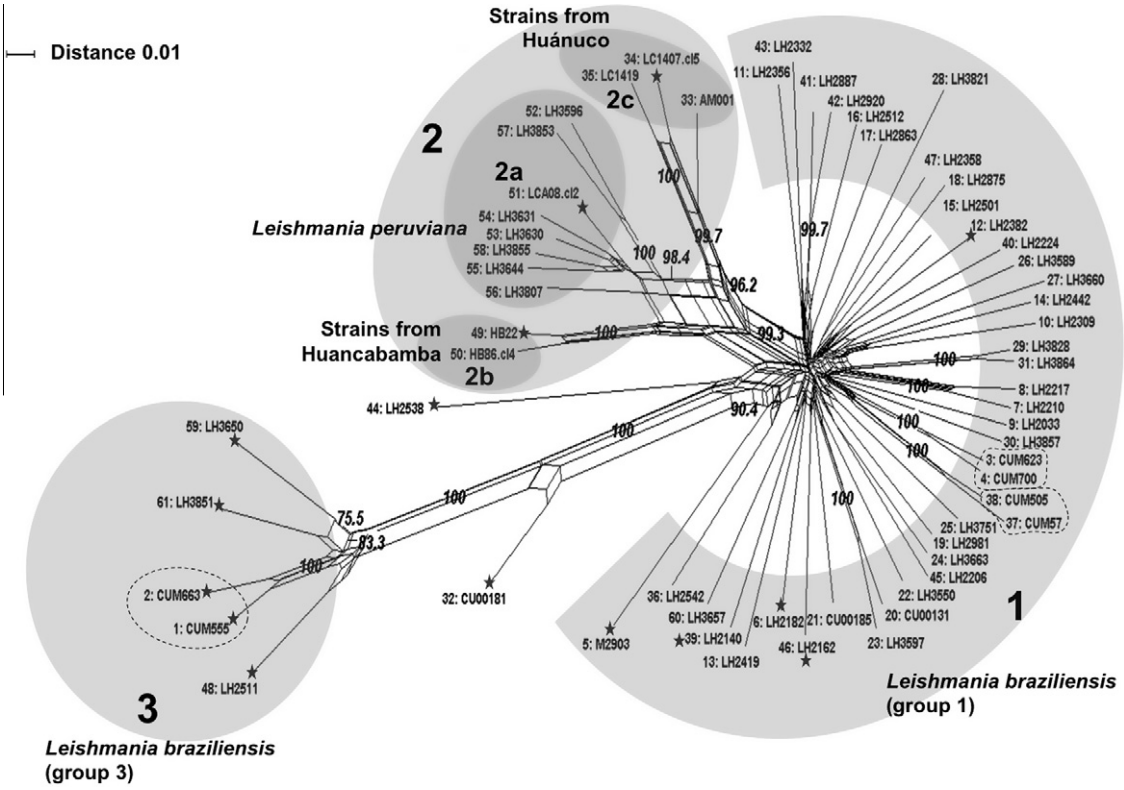

Fig. 1. Neighbor-net cluster based on Jaccard distance coefficients of AFLP data from the 61 strains studied (Table 1, whereby the strains are numbered accordingly). The different recognized groups are numbered as referred to in the text, and also the species and in some cases the geographic origin is mentioned to aid the discussion. Bootstrap values of 2000 replicates ⩾ 70% are shown on specific edges, in percentages. Pairs of strains from the same patient are circled by dotted lines. Strains that were analyzed with *hsp*70 sequences (Fig. 3) are indicated with ★. The distance scale is given on the top left.

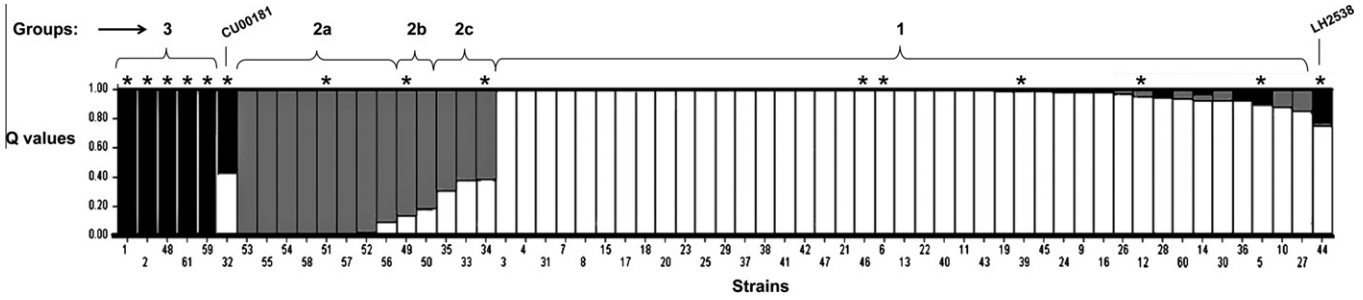

Fig. 2. Structure analysis of the strains in Table 1 for an estimated number of populations $K = 3$. The fraction of genotype attributed by each population (Q) is shown for every strain, numbered as in Table 1 and Fig. 1 (black: *L. braziliensis* group 3; gray: *L. peruviana*; white: *L. braziliensis* group 1). Groups are shown as in Figs. 1 and 3. Strains not belonging to any of these groups are indicated separately with their respective names. Isolates and clones included in the *hsp*70 analysis (Fig. 3) are indicated by ★. Population parameters were estimated by 200,000 Markov Chain Monte Carlo iterations after a burn-in of 100,000 simulations, using the options of admixture and correlated allele frequencies.

2006–2008, indicating that the genetic separation of these groups persists over time.

3.2. AFLP alleles

From the 1350 alleles, 324 (24%) were common to all strains studied, the rest (76%) were polymorphic. No diagnostic markers could be identified for any of the groups identified by Neighbor-net and Structure, except for 34 markers diagnostic for group 3. In addition, no markers could be identified that were correlated with the disease profile. The average number of presence alleles in the different groups is given in Table 2, and serves as an estimation of genome complexity as AFLP markers are dominant.

3.3. Single locus typing

The relationship of group 3 (Figs. 1 and 2) to other *L.* (*Viannia*) species was assessed using *hsp*70 clustering of available and newly determined sequences. The result is depicted in Fig. 3, also listing all accession numbers. The groups indicated in this figure are derived from the AFLP analysis. In the sequences belonging to groups 1 and 3, up to four sequence ambiguities were identified in each sequence, indicative of at least 2 *hsp*70 alleles. Sequence LC1407.cl5 (group 2c) showed 4 ambiguities, CU00181 and LH2538 (intermediate between groups 1 and 3) showed 7 and 4 ambiguities respectively. The sequences from groups 2a and 2b were all identical, and contained no ambiguities.

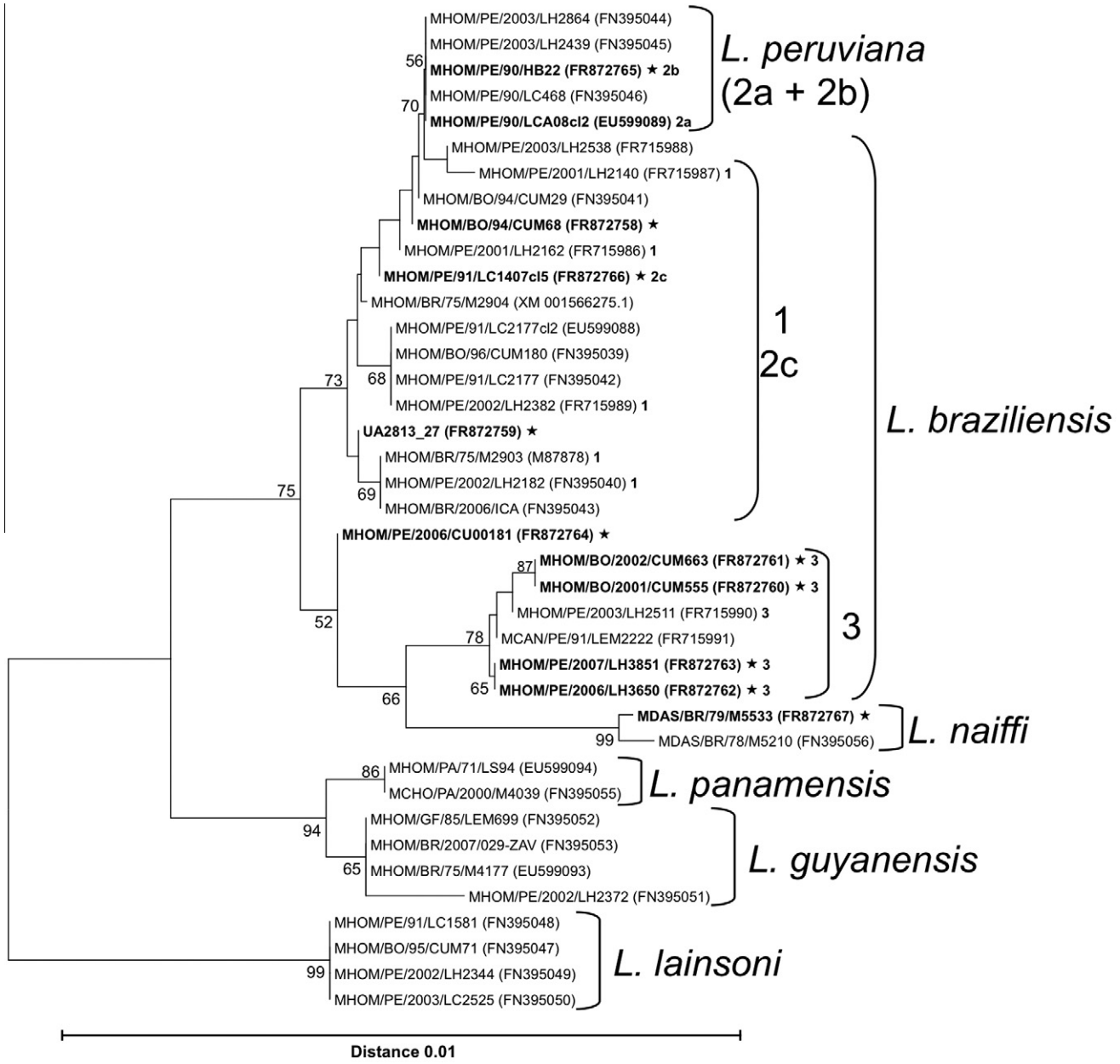

Fig. 3. Neighbor-Joining tree of all currently available partial *hsp*70 sequences (1380 bp) of the subgenus *Leishmania* (*Viannia*). The dendrogram is based on *p*-distances, and bootstrap values from 2000 replicates are shown in percentages at the internodes when exceeding 50%. The position of the root was determined in an analysis including the subgenera *L.* (*Leishmania*) and *L.* (*Sauroleishmania*). The distance scale is shown at the bottom, and refers to the horizontal branches only. Isolate, clone, and strain names are followed by the sequence GenBank ID between brackets, whereby those shown in bold and indicated with ★ were determined for this study. Groups 1, 2a, 2b, 2c, and 3 are indicated as in Figs. 1 and 2, and are again explicitly mentioned behind the strain names for those parasites included in the AFLP analysis. Species are indicated as currently typed in common molecular and MLEE assays.

Table 2
AFLP alleles in different groups.

Group[a]	Mean[b]	Standard deviation	Minimum	Maximum
1	715	14	671	760
2a	662	8	652	671
2b	680	2	678	681
2c	702	4	697	704
3	700	23	666	720
CU00181	749			
LH2538	791			

[a] Groups as defined in Figs. 1 and 2. Two strains intermediate between groups 1 and 3 are shown separately.
[b] For the individual isolates CU00181 and LH2538, the number of alleles is given.

The presence of multiple alleles was also observed in the single locus typing assays used to select the strains for inclusion in the AFLP analysis, and which are based upon *hsp*70, *cpb*, and/or *mpi* genes (Table 1). Each assay identified *L. braziliensis* and *L. peruviana* strains, but also mixed genotypes composed of signatures from both species pointing to the presence of multiple alleles. In group 1, all strains had either an *L. braziliensis* or mixed genotype signature, the exception being LH3657 with an *L. peruviana cpb* genotype and an *L. braziliensis mpi* genotype. In groups 2a and 2b, all strains were identified as *L. peruviana*, while in group 2c mixed genotypes were apparent. Parasites from group 3 were found to contain different species signatures in different genes.

4. Discussion

4.1. Evolution of the L. braziliensis species complex

When looking at the relationships of *L. braziliensis* parasites (Fig. 1), it is striking that there is no apparent evolutionary path in groups 1 and 3 that could indicate how these parasites gradually evolved from their respective ancestral types. Within both groups, they seem to have originated from a central point, having been separated by a history of independent evolution since. Moreover, there is a striking absence of a link with their geographical distribution (Fig. 4): both types of parasites are scattered, and appear to be sympatric. There are a few marked exceptions in the absence of structure, namely seven couples of strains that are well supported by bootstrap values over 95% (CUM57/CUM505, CUM555/CUM663, CUM623/CUM700, CU00131/LH3597, LH2217/LH2210, LH2887/LH2920, LH3828/LH3864). The first three couples of parasites were isolated from the same patient, so they can hardly be considered independent observations. The fact that they are not identical may relate to the error rate seen in AFLP, which can be up to 6% (Odiwuor et al., 2011). Three other couples unite parasites from quite different localities, and only LH3828 and LH3864 are both from the Lima department, which may represent imported cases from other areas. The observed phylogeographic distribution indicates a relatively rapid spread of both *L. braziliensis* groups 1 and 3, followed by a history of independent evolution, possibly linked to the existence of so-called biogeographical units that minimize floral and faunal interactions (Hausdorf, 2002; Lamas, 1982). How groups 1 and 3 originated and were introduced into the area is currently pure speculation, as common typing assays identify

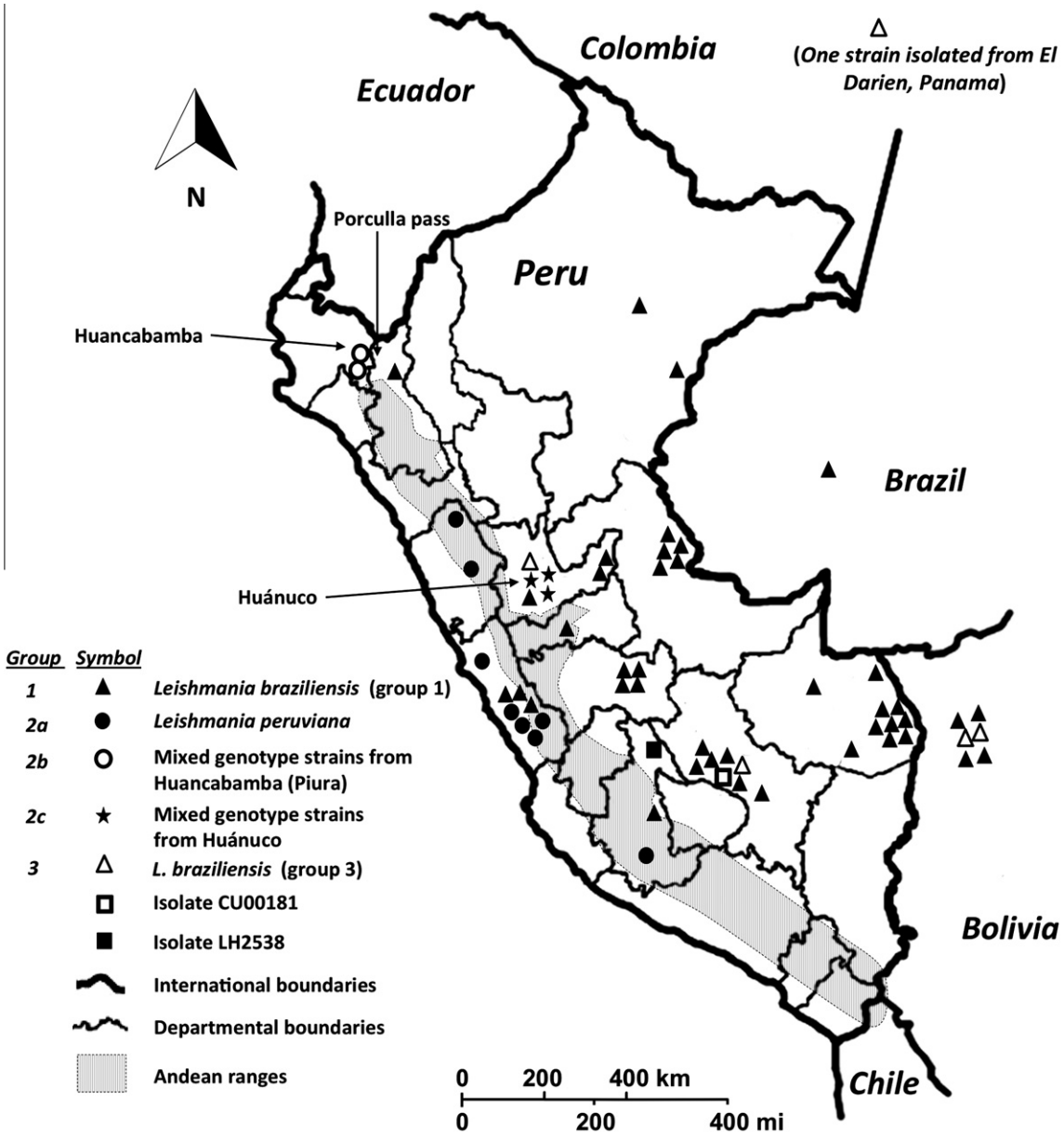

Fig. 4. Map of Peru showing the geographical distribution of the groups depicted in Figs. 1–3. Geographical origins are shown at the department level, and are mapped within each department according the village or region of origin as accurately as possible with the available information. Strains isolated in neighboring countries are also shown, but without indication of specific regions. One strain from El Darien (near the border between Colombia and Panama) is indicated at the top right corner. The Porculla pass separates the Northern and Southern Andes.

both as *L. braziliensis*. Consequently, any data on the epidemiology or possible role of differential transmission cycles, hosts, or vectors, are lacking.

Contrary to groups 1 and 3, the relationships in group 2 are much more resolved, and can shed light on the evolutionary history of *L. peruviana*. It has been suggested on the basis of molecular karyotyping that *L. peruviana* originated from *L. braziliensis* by adaptation from the Amazon ecosystem to the coastal ecosystem of the western Andean valleys (Dujardin et al., 1993c, 1995b; Nolder et al., 2007). In this scenario, *L. braziliensis* would have crossed the Andean ranges via the Porculla Pass in the North of Peru, which is considered a biological gateway for many species, and separates the Southern from the Northern Andes. Molecular karyotyping further revealed a southbound genome reduction, and a gradient-wise decline of virulence possibly related to the loss of genome fragments (Bañuls et al., 2000; Dujardin et al., 1993b,c, 1995a, 2000; Gamboa et al., 2008). Such hypothesis is entirely compatible with our AFLP observations, which support the link (bootstrap 98.4%) between *L. peruviana* groups 2a and 2b, the latter potentially representing an earlier separation from this lineage, being closer to *L. braziliensis* group 1 as also evidenced from Structure analysis. Indeed, both strains of group 2b were isolated in Huancabamba, close to the Porculla Pass (Fig. 4), while the strains from group 2a have a more southern origin. Moreover, LH3596 and LH3853 are grouped in the network (Fig. 1), and they represent the two most northern strains of group 2a, giving a clear geographical direction to the analysis. When looking at the genetic complexity of all these strains, as evidenced by the average number of AFLP alleles present (Table 2), there is an apparent reduction from group 1 over group 2b to group 2a, again compatible with earlier karyotype analyses. Finally, even though based on a too limited amount of data, the *hsp*70 sequences back up the reduction in complexity, as contrary to groups 1, 2c, and 3, only one allele was detected in sequences from groups 2a and 2b.

Based essentially on data from MLEE and karyotyping, strains in group 2c have previously been identified as *L. peruviana*/*L. braziliensis* hybrids (Dujardin et al., 1995a; Nolder et al., 2007). This finding was supported by molecular analysis identifying both *L. peruviana* and *L. braziliensis* alleles in *mpi* and *cpb* (Table 1), and by the fact that both species are sympatric in the region of origin of 2c, Huánuco. These data are essentially compatible with AFLP, but it should nevertheless be pointed out that the position of 2c is comparable to that of the Huancabamba group 2b. First, the network equally well supports the grouping 2a–2c (96.2%) as it does 2a-2b (98.4%). Second, both 2b and 2c derive part of their genotype from *L. braziliensis* group 1 (Fig. 2), even though 2c has a higher contribution from group 1. The genetic complexity of group 2c falls well within the *L. braziliensis* group 1 range, even though at the lower end of spectrum (Table 2). Taken together, group 2c may equally well represent a transition form between both species rather than a hybrid. Hence, our analysis cannot discriminate between a scenario whereby *L. braziliensis* was transitioned into *L. peruviana* via the Porculla pass, to reunite later on down south to form the Huánuco hybrids, and an alternative theory whereby the transition took place via the Huánuco region, to form hybrids later on in Huancabamba (group 2b). The only AFLP data favoring the former hypothesis is the fact that the group 2b isolates have a reduced genetic complexity as evidenced by less AFLP alleles, which would not be expected from a hybrid parasite unless another reduction took place after the hybridization event. Also, the Huánuco region is an important transport route between the Amazon jungle and the coast, facilitating parasite transfer primarily through human migration, which makes it more plausible that *L. peruviana* was more recently introduced into these areas to recombine with *L. braziliensis*.

Finally, two isolates occupy an intermediate position between *L. braziliensis* groups 1 and 3. The fact that CU00181 originates from a region where both groups are sympatric (Fig. 4), it has a markedly high number of alleles (Table 2), it occupies an intermediate position in Fig. 3, and in each of its seven *hsp*70 sequence ambiguities combines alleles from both groups, it can be concluded that this strain is a hybrid form of groups 1 and 3. Also in LH2538, ambiguities combine alleles from groups 1 and 3, and as it has by far the highest number of AFLP alleles (Table 2), it too can be considered a hybrid isolate. As our analysis included one parasite clone only from groups 2a, 2b, and 2c (Table 1), we cannot exclude the possibility that some of the other isolates identified as hybrids could in fact originate from mixed parasite cultures.

4.2. Taxonomical and clinical implications

Previous studies, both based on nucleotide as well as MLEE data, have suggested that *L. peruviana* should be considered a subgroup of the *L. braziliensis* species, rather than a separate entity. This is confirmed by our analysis, in which group 2 as well as group 1 isolates seem equidistant from the center of the group 1 network. In addition, our *hsp*70 analysis (Fig. 3) positions *L. peruviana* as one of the branches in the cluster defined by groups 1 and 2. Given the lack of clearly defined boundaries, we would consider groups 2a (including the reference strain LCA08, Bañuls et al., 2000) and 2b as *L. peruviana*. In the three single-locus assays, only one allele was detected in each case by PCR, RFLP, and sequencing, which could be considered an *L. peruviana* signature. Moreover, all *hsp*70 sequences from 2a and 2b are identical. Also, none of these parasites were isolated from mucocutaneous leishmaniasis (ML) patients, even though data are lacking for group 2b. As the clinical interest of discriminating *L. peruviana* from *L. braziliensis* lies in the observation that only the latter can cause ML, our definition would also be a practical one, certainly given the fact that currently no markers are available for ML strains, and neither could they be found in our data set. All ML parasite isolates included in our study are found in group 1, in which the *cpb* and *hsp*70 single-locus assays identify what could be called an *L. braziliensis* allele, often in combination with the *L. peruviana* allele, in line with the higher variability and complexity of *L. braziliensis* as compared to *L. peruviana*. The exception is *mpi*, which seems to separate *L. braziliensis* and *L. peruviana* as here described, even though many data are lacking (Table 1). Also on the basis of MLEE, MPI was the only enzyme separating both species (Arana et al., 1990; Bañuls et al., 2000; Chouicha et al., 1997).

As for group 2c, these parasites are characterized by the presence of *mpi* and *cpb* alleles from both species. Given the fact that they carry a significant *L. braziliensis* signature in their genome, the safer option is probably to treat them as *L. braziliensis*, even though all strains in our analysis are from CL patients. As for group 3, this poses additional problems. First of all, group 3 forms a well-supported entity that is less related to group 1 *L. braziliensis* as is *L. peruviana*. This makes it uncertain whether group 3 should be regarded as genuine *L. braziliensis*, even though in view of the past tendency to invent a new *Leishmania* species each time a new cluster was identified, we currently do not promote yet an additional one. As no other *L.* (*Viannia*) species were included in our AFLP analysis, we further investigated its position on the basis of *hsp*70 sequences (Fig. 3). This analysis confirmed the separation from groups 1 and 2, and placed group 3 with *L. naiffi*, even though this latter relationship tends to shift depending on the sequences included in the analysis (not shown). Nonetheless, isolate LEM2222 in group 3 (Fig. 3) is an MLEE characterized type strain of *L. braziliensis*. Species-specific PCRs and RFLP analyses type this group as *L. braziliensis*, except for *cpb* which characterizes it as *L. peruviana*, making it a problematic entity in clinical practice. As no common isolates were analyzed, it is also unclear whether this group corresponds to other atypical *L. braziliensis* groups identified

in multilocus sequence and protein analyses, where some parasites were found to cause ML, as opposed to our group 3 (Cupolillo et al., 1994; de Brito et al., 1993; Lucas et al., 1998; Revollo et al., 1992; Saravia et al., 1984; Tsukayama et al., 2009). The two strains intermediate between groups 1 and 3 are also found to contain *L. braziliensis* and/or *L. peruviana* alleles, and CU00181 was found in an intermediate position in Fig. 3, which further complicates the situation.

5. Conclusions

Our analysis supports the evolution of *L. peruviana* from *L. braziliensis* following its invasion of the Andean coastal ecosystem, and the degradation of the species to subspecies status of *L. peruviana*. Molecular assays to discriminate the atypical group 3 *L. braziliensis* are needed, as well as an assessment of its clinical profile. Given the past proliferation of species names in the *Leishmania* genus, many of which are not clearly defined, we await additional analyses in order not to introduce further unfounded complications. Our findings stress the need for a continued epidemiological monitoring of circulating species and strains, as entities with a potential clinical relevance may otherwise escape detection.

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References

Arana, M., Evans, D.A., Zolessi, A., Llanos-Cuentas, A., Arevalo, J., 1990. Biochemical characterization of *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Viannia*) *peruviana* by isoenzyme electrophoresis. Trans. Roy. Soc. Trop. Med. Hyg. 84, 526–529.

Bañuls, A.L., Dujardin, J.C., Guerrini, F., De Doncker, S., Jacquet, D., Arevalo, J., Noel, S., Le Ray, D., Tibayrenc, M., 2000. Is *Leishmania* (*Viannia*) *peruviana* a distinct species? A MLEE/RAPD evolutionary genetics answer. J. Eukaryot. Microbiol. 47, 197–207.

Boggild, A.K., Valencia, B.M., Espinosa, D., Véland, N., Ramos, A.P., Arevalo, J., Llanos-Cuentas, A., Low, D.E., 2010. Detection and species identification of *Leishmania* DNA from filter paper lesion impressions for patients with American cutaneous leishmaniasis. Clin. Infect. Dis. 50, e1–e6.

Bryant, D., Moulton, V., 2004. Neighbor-net: an agglomerative method for the construction of phylogenetic networks. Mol. Biol. Evol. 21, 255–265.

Chouicha, N., Lanotte, G., Pratlong, F., Cuba, C.A., Velez, I.D., Dedet, J.P., 1997. Phylogenetic taxonomy of *Leishmania* (*Viannia*) *braziliensis* based on isoenzymatic study of 137 isolates. Parasitology 115, 343–348.

Cupolillo, E., Grimaldi Jr., G., Momen, H., 1994. A general classification of New World *Leishmania* using numerical zymotaxonomy. Am. J. Trop. Med. Hyg. 50, 296–311.

de Brito, M.E., Brandao, S.P., Salles, N.R., Cupolillo, E., Grimaldi Jr., G., Momen, H., 1993. Human cutaneous leishmaniasis due to a new enzymatic variant of *Leishmania* (*Viannia*) *braziliensis* occurring in Pernambuco, Brazil. Mem. Inst. Oswaldo Cruz. 88, 633–634.

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

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

Dujardin, J.C., Llanos-Cuentas, A., Càceres, A., Arana, M., Dujardin, J.P., 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. 87, 335–347.

Dujardin, J.C., Bañuls, A.L., Llanos-Cuentas, A., Alvarez, E., De Doncker, S., Jacquet, D., Le Ray, D., Arevalo, J., Tibayrenc, M., 1995a. Putative *Leishmania* hybrids in the Eastern Andean valley of Huanuco, Peru. Acta Trop. 59, 293–307.

Dujardin, J.C., Dujardin, J.P., Tibayrenc, M., Timperman, G., De Doncker, S., Jacquet, D., Arevalo, J., Llanos-Cuentas, A., Guerra, H., Bermudez, H., 1995b. Karyotype plasticity in neotropical *Leishmania*: an index for measuring genomic distance among *L.* (*V.*) *peruviana* and *L.* (*V.*) *braziliensis* populations. Parasitology 110, 21–30.

Dujardin, J.C., Bañuls, A.L., Dujardin, J.P., Arevalo, J., Tibayrenc, M., Le Ray, D., 1998. Comparison of chromosome and isoenzyme polymorphism in geographical populations of *Leishmania* (*Viannia*) *peruviana*. Parasitology 117, 547–554.

Dujardin, J.C., Henriksson, J., Victoir, K., Brisse, S., Gamboa, D., Arevalo, J., Le Ray, D., 2000. Genomic rearrangements in trypanosomatids: an alternative to the "one gene" evolutionary hypotheses? Mem. Inst. Oswaldo Cruz 95, 527–534.

Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. 14, 2611–2620.

Fraga, J., Montalvo, A.M., De Doncker, S., Dujardin, J.C., Van der Auwera, G., 2010. Phylogeny of *Leishmania* species based on the heat-shock protein 70 gene. Infect. Genet. Evol. 10, 238–245.

Gamboa, D., Torres, K., De Doncker, S., Zimic, M., Arevalo, J., Dujardin, J.C., 2008. Evaluation of an in vitro and in vivo model for experimental infection with *Leishmania* (*Viannia*) *braziliensis* and *L.* (*V.*) *peruviana*. Parasitology 135, 319–326.

Garcia, A.L., Kindt, A., Quispe-Tintaya, K.W., Bermudez, H., Llanos, A., Arevalo, J., Bañuls, A.L., De Doncker, S., Le Ray, D., Dujardin, J.C., 2005. American tegumentary leishmaniasis: antigen-gene polymorphism, taxonomy and clinical pleomorphism. Infect. Genet. Evol. 5, 109–116.

Grimaldi Jr., G., David, J.R., Mahon-Pratt, D., 1987. Identification and distribution of New World *Leishmania* species characterized by serodeme analysis using monoclonal antibodies. Am. J. Trop. Med. Hyg. 36, 270–287.

Grimaldi Jr., G., Tesh, R.B., Mahon-Pratt, D., 1989. A review of the geographic distribution and epidemiology of leishmaniasis in the New World. Am. J. Trop. Med. Hyg. 41, 687–725.

Hausdorf, B., 2002. Units in biogeography. Syst. Biol. 51, 648–652.

Huson, D.H., Bryant, D., 2006. Application of phylogenetic networks in evolutionary studies. Mol. Biol. Evol. 23, 254–267.

Jaccard, P., 1908. Nouvelles recherches sur la distribution florale. Bull. Soc. Vaudoise Sci. Nat. 44, 223–270.

Lainson, R., Shaw, J.J., 1987. Evolution, classification and geographical distribution. In: Peters, W., Killick-Kendrick, R. (Eds.), The Leishmaniases in Biology and Medicine. Academic Press, London, UK, pp. 1–120.

Lamas, G., 1982. A Preliminary Zoogeographical Division of Peru, Based on Butterfly Distributions (*Lepidoptera papilionoidea*). In: Prance, G.T. (Ed.), Biological Diversity in the Tropics. Columbia University Press, New York, pp. 336–357.

Llanos-Cuentas, E.A., Roncal, N., Villaseca, P., Paz, L., Ogusuku, E., Perez, J.E., Caceres, A., Davies, C.R., 1999. Natural infections of *Leishmania peruviana* in animals in the Peruvian Andes. Trans. Roy. Soc. Trop. Med. Hyg. 93, 15–20.

Lucas, C.M., Franke, E.D., Cachay, M.I., Tejada, A., Cruz, M.E., Kreutzer, R.D., Barker, D.C., McCann, S.H., Watts, D.M., 1998. Geographic distribution and clinical description of leishmaniasis cases in Peru. Am. J. Trop. Med. Hyg. 59, 312–317.

Lumbreras, H., Guerra, H., 1985. Leishmaniasis in Perú. In: Chang, K.-P., Bray, R.S. (Eds.), Leishmaniasis. Elsevier Science Publishers B.V. (Biomedical Division), Amsterdam, pp. 297–311.

mier-David, L., David, C., Munoz, M., Vargas, F., Bustillos, R., Valda, L., Dedet, J.P., 1993. Epidemiological, clinical and biological features of mucocutaneous leishmaniasis in Bolivia after a 221 patient sample. Bull. Soc. Pathol. Exot. 86, 106–111.

Montalvo, A.M., Fraga, J., Monzote, L., Montano, I., De Doncker, S., Dujardin, J.C., Van der Auwera, G., 2010. Heat-shock protein 70 PCR-RFLP: a universal simple tool for *Leishmania* species discrimination in the New and Old World. Parasitology 137, 1159–1168.

Nolder, D., Roncal, N., Davies, C.R., Llanos-Cuentas, A., Miles, M.A., 2007. Multiple hybrid genotypes of *Leishmania* (*Viannia*) in a focus of mucocutaneous Leishmaniasis. Am. J. Trop. Med. Hyg. 76, 573–578.

Oddone, R., Schweynoch, C., Schönian, G., de Sousa, C.S., Cupolillo, E., Espinosa, D., Arevalo, J., Noyes, H., Mauricio, I., Kuhls, K., 2009. Development of a multilocus microsatellite typing approach for discriminating strains of *Leishmania* (*Viannia*) species. J. Clin. Microbiol. 47, 2818–2825.

Odiwuor, S., Vuylsteke, M., De Doncker, S., Maes, I., Mbuchi, M., Dujardin, J.C., Van der Auwera, G., 2011. *Leishmania* AFLP: paving the way towards improved molecular assays and markers of diversity. Infect. Genet. Evol. 11, 960–967.

Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of population structure using multilocus genotype data. Genetics 155, 945–959.

Revollo, S., mier-David, L., David, C., Lyevre, P., Camacho, C., Dedet, J.P., 1992. Isoenzyme characterization of *Leishmania braziliensis braziliensis* isolates

obtained from Bolivian and Peruvian patients. Trans. Roy. Soc. Trop. Med. Hyg. 86, 388–391.
Rioux, J.A., Lanotte, G., Serres, E., Pratlong, F., Bastien, P., Perières, J., 1990. Taxonomy of *Leishmania*. Use of isoenzymes. Suggestions for a new classification. Ann. Parasitol. Hum. Comp. 65, 111–125.
Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
Saravia, N.G., Gemmell, M.A., Nance, S.L., Anderson, N.L., 1984. Two-dimensional electrophoresis used to differentiate the causal agents of American tegumentary leishmaniasis. Clin. Chem. 30, 2048–2052.
Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.
Tsukayama, P., Lucas, C., Bacon, D.J., 2009. Typing of four genetic loci discriminates among closely related species of New World *Leishmania*. Int. J. Parasitol. 39, 355–362.
Velez, L.R., 1913. Uta e espundia. Bull. Soc. Pathol. Exot. 6, 545.
Vianna, G., 1911. Sôbre uma nova especie de *Leishmania* (Nota Preliminar). Bras. Med. 25, 11.
Victoir, K., Bañuls, A.L., Arevalo, J., Llanos-Cuentas, A., Hamers, R., Noel, S., De Doncker, S., Le Ray, D., Tibayrenc, M., Dujardin, J.C., 1998. The gp63 gene locus, a target for genetic characterization of *Leishmania* belonging to subgenus *Viannia*. Parasitology 117, 1–13.
Victoir, K., De Doncker, S., Cabrera, L., Alvarez, E., Arevalo, J., Llanos-Cuentas, A., Le Ray, D., Dujardin, J.C., 2003. Direct identification of *Leishmania* species in biopsies from patients with American tegumentary leishmaniasis. Trans. Roy. Soc. Trop. Med. Hyg. 97, 80–87.
Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23, 4407–4414.
Zhang, W.W., Miranda-Verastegui, C., Arevalo, J., Ndao, M., Ward, B., Llanos-Cuentas, A., Matlashewski, G., 2006. Development of a genetic assay to distinguish between *Leishmania viannia* species on the basis of isoenzyme differences. Clin. Infect. Dis. 42, 801–809.