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Multilocus genotyping reveals a polyphyletic pattern among naturally antimony-resistant *Leishmania braziliensis* isolates from Peru

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ABSTRACT

In order to understand the epidemiological dynamics of antimonial (Sb^V) resistance in zoonotic tegumentary leishmaniasis and its link with treatment outcome, we analyzed the population structure of 24 Peruvian *Leishmania braziliensis* clinical isolates with known *in vitro* antimony susceptibility and clinical phenotype by multilocus microsatellite typing (14 microsatellite loci). The genetic variability in the Peruvian isolates was high and the multilocus genotypes were strongly differentiated from each other. No correlation was found between the genotypes and *in vitro* drug susceptibility or clinical treatment outcome. The finding of a polyphyletic pattern among the Sb^V-resistant *L. braziliensis* might be explained by (i) independent events of drug resistance emergence, (ii) sexual recombination and/or (iii) other phenomena mimicking recombination signals. Interestingly, the polyphyletic pattern observed here is very similar to the one we observed in the anthroponotic *Leishmania donovani* (Laurent et al., 2007), hereby questioning the role of transmission and/or chemotherapeutic drug pressure in the observed population structure.

1. Introduction

Protozoan parasites of the genus *Leishmania* cause a broad spectrum of diseases, collectively referred to as leishmaniasis, which occur predominantly in tropical and subtropical regions. It is estimated that worldwide there is an annual incidence of 1.5–2 million new cases, with up to 350 million people at risk of infection (Murray et al., 2005). Chemotherapy is a pillar of control strategies but is jeopardized by the emergence and spreading of parasite resistance. The latter is well documented for pentavalent antimonials (Sb^V), the first-line drugs since decades in many countries.

Our group previously reported a lower response to antimonial therapy in Peruvian patients infected with *Leishmania braziliensis* (Arevalo et al., 2007; Llanos-Cuentas et al., 2008), and a high prevalence of *in vitro* Sb^V resistance among pre-treatment clinical isolates of that species (up to 85%, Yardley et al., 2006), and this independently of treatment outcome. This high frequency of primary Sb^V resistance raises a particular concern in the generally accepted zoonotic context of leishmaniasis in the *L.* (*Viannia*) subgenus. Under conditions of zoonotic transmission, humans are generally considered to be a "dead end" for transmission, and most of the parasites are in animals in which drug pressure is nonexistent. In order to investigate the way by which drug resistance is emerging and spreading in a zoonotic context, a population genetics approach focused on natural parasite populations and using highly discriminatory DNA fingerprinting methods is needed.

Microsatellite markers have proved to be the most powerful tools for population genetic studies in *Leishmania* (Botilde et al., 2006; Rougeron et al., 2009). The power of these markers is that they are abundant in the genomes of *Leishmania*, highly informative, (supposedly) neutral, and co-dominant (Schönian et al., 2011). Screening of the length polymorphism in microsatellite sequences is relatively easy to assay, and the results are reproducible and exchangeable between laboratories. So far, microsatellite loci with high discriminatory power and suitable for characterizing closely related strains have been reported for the *Leishmania donovani* complex (Bulle et al., 2002; Jamjoom et al., 2002; Ochsenreither

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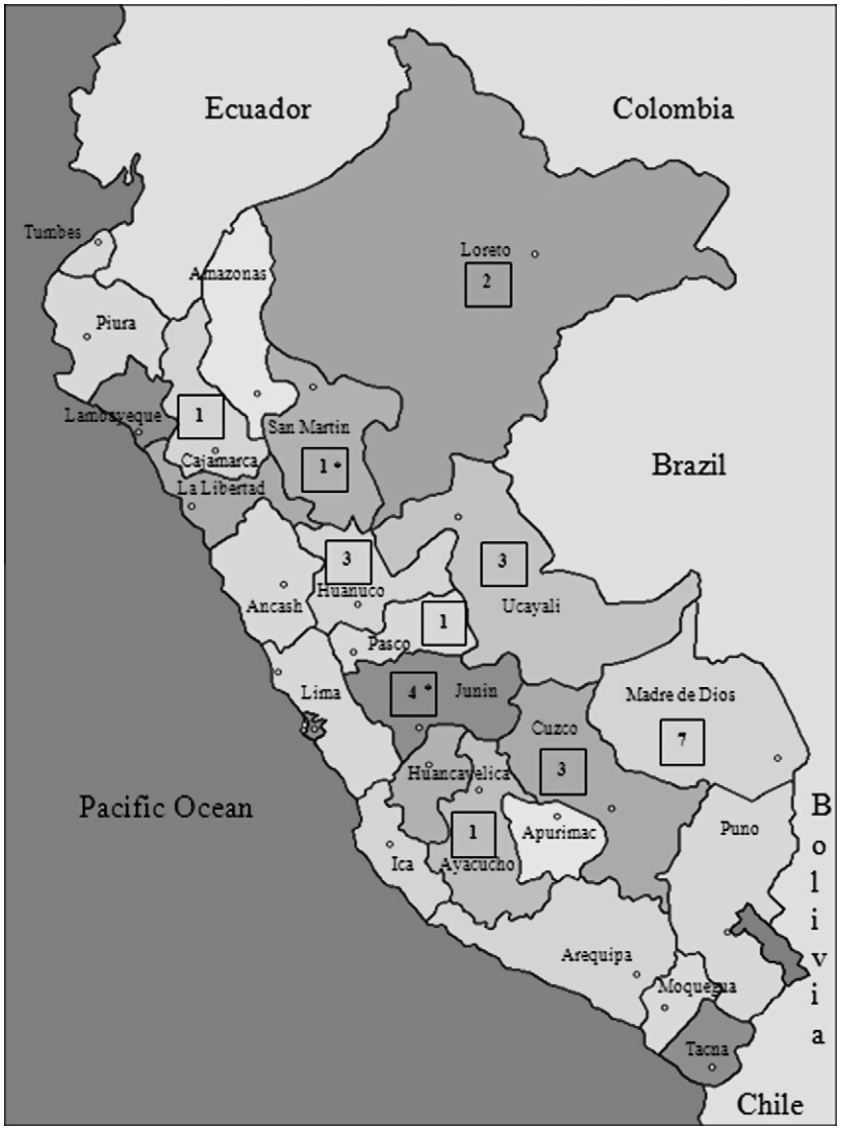

Fig. 1. Map of Peru and geographical distribution of the 26 Peruvian *L. braziliensis* isolates in this study. The number indicated within the square symbol shows the number of isolates studied from each location (Department). ∗One isolate from San Martin (PER091) and one isolate from Junin (PER231) were removed from the dataset and, thus, were not considered for the population genetic analysis.

et al., 2006), *Leishmania tropica* (Schwenkenbecher et al., 2006), *Leishmania major* (Al-Jawabreh et al., 2008) and for species of the subgenus *Leishmania* (*Viannia*) (Oddone et al., 2009; Rougeron et al., 2008; Russell et al., 1999).

We aimed here to analyze the population structure of naturally Sb^V-sensitive and Sb^V-resistant *L. braziliensis* isolates for understanding the epidemiological dynamics of drug resistance in zoonotic tegumentary leishmaniasis and its link with antimonial treatment outcome. For this, 14 microsatellite markers, polymorphic for the subgenus *L.* (*Viannia*) (Oddone et al., 2009) have been used for analyzing 24 *L. braziliensis* isolates (18 Sb^V-resistant and 4 Sb^V-sensitive) originating from tegumentary leishmaniasis patients of Peru.

2. Materials and methods

2.1. Parasite isolates and DNA samples

In this study, we used 26 isolates of *L. braziliensis* isolated between 2001 and 2003 from confirmed cutaneous or mucosal leishmaniasis patients recruited at the Institute of Tropical Medicine Alexander von Humboldt in Lima, Peru, within the framework of LeishNatDrug-R, a multicenter study on Sb^V treatment failure in leishmaniasis. The geographical origins (Fig. 1), biological and clinical features of the isolates used in this study are listed in Table 1. Isolates were essentially obtained before treatment of patients (marked as PERXYZ/0 in the WHO Code, in Table 1), typed by PCR-RFLP analysis of *hsp70* and *cpb* genes (Garcia et al., 2005) and tested as intracellular amastigotes for their *in vitro* susceptibility to Sb^V (Yardley et al., 2006). For some of the isolates, data of *in vitro* susceptibility to Sb^{III}, the reduced and active form of the drug, were also available (Yardley et al., 2006). We also added 3 reference strains: MHOM/BR/75/M2903 (*L. braziliensis* from Brazil), MHOM/BR/84/LTB300 (*L. braziliensis* from Brazil), and MHOM/SR/87/TRUUS1 (*Leishmania guyanensis* from Suriname). The reference strain MHOM/BR/75/M2903 was obtained from the cryobank of the Institute of Tropical Medicine, Antwerp, Belgium. Genomic DNA from two cloned reference strains (MHOM/BR/84/LTB300 and MHOM/SR/87/TRUUS1) was obtained from the Institute of Microbiology and Hygiene, Charité University Medicine Berlin,

Table 1
Characteristics of *Leishmania* isolates used in this study including reference strains.

Name	WHO Code	Species	Country	Origin (state, province)	Geographical location[A]	Clinical picture	Clinical response to antimonial therapy	A.I. Sb^{V} [B]	A.I. Sb^{III} [B]
LTB300	MHOM/BR/2000/LTB300	*L. braziliensis*	Brazil	Bahia	–	MCL	nd	nd	nd
TRUUS1	MHOM/SR/1987/TRUUS1	*L. guyanensis*	Suriname	nd	–	nd	nd	nd	nd
M2903	MHOM/BR/1975/M2903	*L. braziliensis*	Brazil	Para	–	CL	nd	nd	nd
PER002	MHOM/PE/2001/PER002/0	*L. braziliensis*	Peru	Madre de Dios, Tambopata	South	CL	Unresponsive	6	2
PER005	MHOM/PE/2001/PER005/0	*L. braziliensis*	Peru	Loreto, Ucayali	North	CL	Unresponsive	1	nd
PER006	MHOM/PE/2001/PER006/1	*L. braziliensis*	Peru	Junin, Satipo	Central	CL	Unresponsive	6+	nd
PER010	MHOM/PE/2002/PER010/0	*L. braziliensis*	Peru	Cajamarca, Jaen	North	CL	Initial cure	6	nd
PER012	MHOM/PE/2001/PER012/1	*L. braziliensis*	Peru	Cusco, Calca	South	CL	Unresponsive	6+	nd
PER014	MHOM/PE/2001/PER014/0	*L. braziliensis*	Peru	Junin, Satipo	Central	CL	Unresponsive	6+	nd
PER015	MHOM/PE/2002/PER015/0	*L. braziliensis*	Peru	Ucayali, Coronel Portillo	East-Central	CL	Unresponsive	6+	2
PER016	MHOM/PE/2002/PER016/0	*L. braziliensis*	Peru	Huanuco, Puerto Inca	Central	CL	Definite cure	6+	nd
PER067	MHOM/PE/2002/PER067/0	*L. braziliensis*	Peru	Cusco, La Convencion	South	CL	Unresponsive	6+	nd
PER086	MHOM/PE/2002/PER086/0	*L. braziliensis*	Peru	Pasco, Oxapampa	Central	CL	Unresponsive	6+	0
PER091	MHOM/PE/2002/PER091/0[a]	*L. braziliensis*	Peru	San Martin, Tocache	North	CL	Unresponsive	nd	nd
PER094	MHOM/PE/2002/PER094/0	*L. braziliensis*	Peru	Huanuco, Puerto Inca	Central	CL	Definite cure	6	2
PER096	MHOM/PE/2002/PER096/0	*L. braziliensis*	Peru	Madre de Dios, Manu	South	CL	Definite cure	nd	nd
PER104	MHOM/PE/2002/PER104/0	*L. braziliensis*	Peru	Madre de Dios, Tambopata	South	CL	Unresponsive	6+	6+
PER122	MHOM/PE/2002/PER122/0	*L. braziliensis*	Peru	Madre de Dios, Tambopata	South	CL	Definite cure	6+	nd
PER130	MHOM/PE/2003/PER130/0	*L. braziliensis*	Peru	Cusco, Echarate	South	CL	Unresponsive	1	0
PER157	MHOM/PE/2003/PER157/0	*L. braziliensis*	Peru	Madre de Dios, Tambopata	South	CL	Definite cure	6+	2
PER163	MHOM/PE/2003/PER163/0	*L. braziliensis*	Peru	Huanuco, Leoncio Prado	Central	CL	Definite cure	2	0
PER164	MHOM/PE/2003/PER164/0	*L. braziliensis*	Peru	Ucayali, Coronel Portillo	East-Central	CL	Initial cure	6+	1
PER182	MHOM/PE/2003/PER182/0	*L. braziliensis*	Peru	Ayacucho, La Mar	South	CL	Definite cure	6	5
PER186	MHOM/PE/2003/PER186/0	*L. braziliensis*	Peru	Junin, Satipo	Central	CL	Definite cure	2	1
PER201	MHOM/PE/2003/PER201/0	*L. braziliensis*	Peru	Loreto, Requena	North	ML	Definite cure	6	1
PER207	MHOM/PE/2003/PER207/0	*L. braziliensis*	Peru	Madre de Dios, Tambopata	South	CL	Definite cure	nd	nd
PER215	MHOM/PE/2003/PER215/0	*L. braziliensis*	Peru	Ucayali, Coronel Portillo	East-Central	ML	Definite cure	6	2
PER231	MHOM/PE/2003/PER231/0[a]	*L. braziliensis*	Peru	Junin, Satipo	Central	ML	Definite cure	5	2
PER260	MHOM/PE/2003/PER260/0	*L. braziliensis*	Peru	Madre de Dios, Tahuamanu	South	ML	Definite cure	6	2

CL, cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis; ML, mucosal leishmaniasis. Sb^{III}, trivalent antimony; Sb^{V}, pentavalent antimony; WHO, World Health Organization; nd, not determined.

[a] Isolates were not considered for the population genetic analyses.

[A] Isolates were assigned to the respective geographical location in Peru (North, Central-including East- Central- and South).

[B] The *in vitro* Sb^{V} or Sb^{III} susceptibility of a tested isolate was expressed as an 'activity index' (A.I.), i.e. as the ratio of the ED_{50} (50% effective dose) of that tested isolate to the ED_{50} of the WHO reference *L. braziliensis* strain MHOM/BR/75/M2903. Isolates with an A.I. of 0–2 were considered sensitive to Sb^{V} or Sb^{III} (0, more sensitive than the reference strain M2903), while isolates with an A.I. of 3 or higher were considered resistant. Data shown were reported in Yardley et al. (2006).

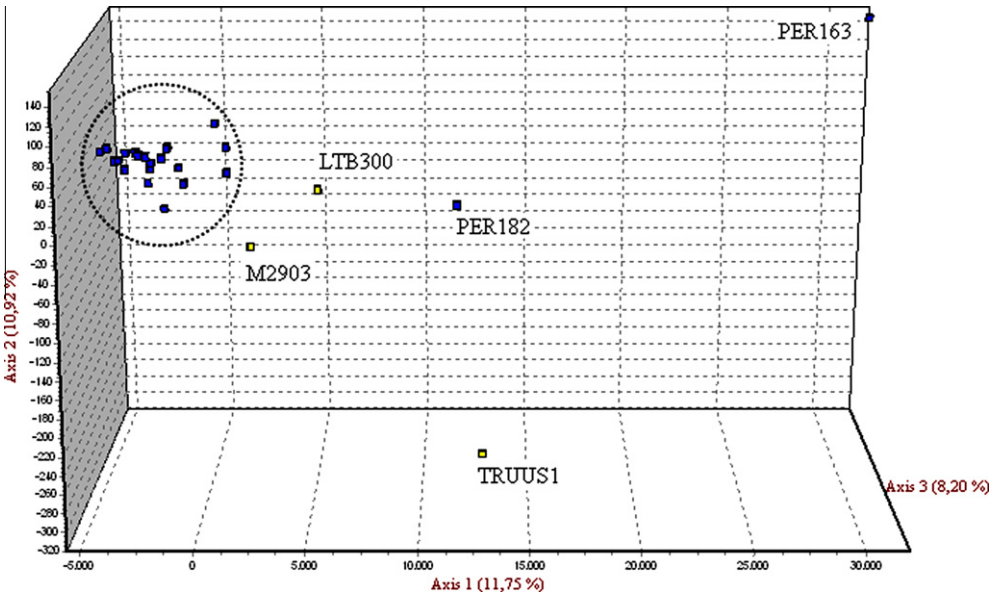

Fig. 2. Factorial correspondence analysis (FCA) plot including all isolates. The MLGs from 24 Peruvian *L. braziliensis* isolates (dark symbols) and the 3 reference strains (M2903, LTB300 and TRUUS1) (light symbols) are shown. Isolates PER163 and PER182 are clearly different from the rest of the Peruvian isolates.

Germany. Promastigote forms were grown as previously described (Adaui et al., 2011a), harvested by centrifugation and washed twice in phosphate buffered saline. DNA was extracted by the QIAamp DNA mini kit (Qiagen) and stored at 4 °C.

2.2. Multilocus microsatellite typing (MLMT)

For this population genetic study, we used a standard set of 15 microsatellite markers (*CSg46, CSg47, CSg48, CSg53, CSg55, CSg59, 7GN, 11H, 11C, 6F, 10F, B6F, B3H, AC01R, AC16R*), polymorphic for *Leishmania* species of the subgenus *L.* (*Viannia*), previously described elsewhere (Oddone et al., 2009). The 15 markers are located on 13 chromosomes. Markers *CSg46* and *10F* are both located on chromosome 18, and markers *CSg53* and *11H* are both located on chromosome 21, albeit far enough apart to be considered independent (Oddone et al., 2009). Forward primers of the targeted loci were fluorescently labeled using the dyes 6-FAM or HEX (Applied Biosystems). PCR amplifications were performed according to Oddone et al. (2009). Twelve microsatellite markers were analyzed in six duplex reactions (no loss of alleles was observed compared to single reactions). The remaining 3 markers were analyzed in single reactions. The precise size of the amplicons was determined using an automated capillary ABI3730XL DNA sequencer (by Genoscreen, Lille, France) and GeneScan™ 1200LIZ® as Size Standard. Amplicon sizes were manually verified using the GENEMAPPER software v4.0 (Applied Biosystems) and automatically binned using TANDEM v1.07 (Matschiner and Salzburger, 2009). The sizes of the amplified fragments (and thus the number of repeats) were compared to the fragment size from the cloned strains *L. braziliensis* MHOM/BR/84/LTB300 and *L. guyanensis* MHOM/SR/87/TRUUS1 for which the microsatellite sizes for the 15 loci had been determined by sequencing (Oddone et al., 2009). Each isolate is represented by a multilocus genotype (MLG), which is the diploid genotype containing the allelic information at all amplified loci.

2.3. Quality control on the MLMT dataset

To identify error-prone loci, 11/29 samples (i.e. 38%) were re-genotyped yielding a total of 22 replicates. The Mean Error Rate per Locus (MERL) was quantified using the formula $e_l = m_l/nt$, with m_l the number of single-locus genotypes including at least one allelic mismatch, and nt the number of replicated single-locus genotypes (Pompanon et al., 2005). If a sample did not amplify for $\geqslant 3$ loci (i.e. >20% of tested loci), it was excluded from the analyses. If a sample contained more than two alleles at (at least) one locus, it was defined as a mixed infection and excluded from the analyses.

2.4. Population genetic analysis

Microsatellite-based genetic distances were calculated with MSA software v4.0 (Dieringer and Schlötterer, 2003) and POPULATIONS software v1.2.28 (http://bioinformatics.org/~tryphon/populations/) by using the Chord distance (Cavalli-Sforza and Edwards, 1967) and D_{AS} (D_{ps}) distance measures (based on the proportion of shared alleles, Bowcock et al., 1994). Both methods follow the infinite allele model (IAM). Neighbor-Joining (NJ) trees were constructed based on both distance matrices using POPULATIONS and MEGA v3.1 (Kumar et al., 2004), following bootstrap analysis (1000 replications).

A factorial correspondence analysis (FCA) implemented in GENETIX (Dawson and Belkhir, 2001) was performed to plot multilocus genotypes in three dimensions, without *a priori* assumptions about grouping, using each allele as an independent variable. As FCA is sensitive to missing data, we repeated the analysis after removing all the markers for which at least 1 sample did not amplify, resulting in a dataset of 8 loci.

The genetic diversity was assessed for a reduced dataset (excluding identical MLGs and excluding outliers identified by the FCA) by calculating the numbers of alleles (A) and observed heterozygosities (H_o) for each locus using the GDA software (http://www.eeb.uconn.edu/people/plewis/software.php).

Linkage disequilibrium between pairs of loci (non-random association of alleles at different loci) was assessed using the reduced dataset with a likelihood ratio test using the ARLEQUIN software v3.1 (http://cmpg.unibe.ch/software/arlequin3). Briefly, the likelihood of the sample evaluated under the hypothesis of no association between loci (linkage equilibrium) is compared to the likelihood of the sample when association is allowed. The signifi-

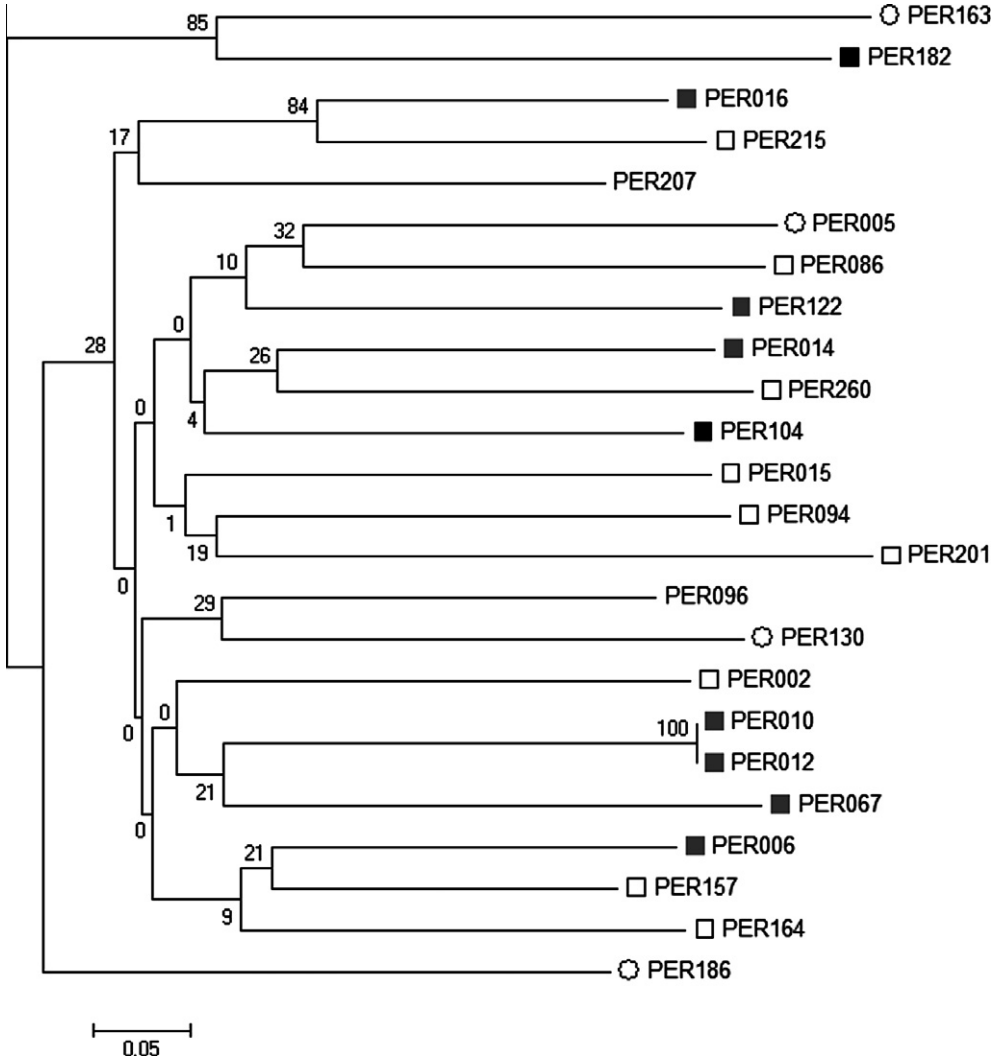

Fig. 3. Neighbor-Joining tree inferred from the Chord distances calculated for 14 microsatellite markers genotyped in 24 Peruvian *L. braziliensis* isolates. Bootstrap values were calculated from 1000 iterations and are shown at the nodes. Midpoint rooting was applied since no outgroup has been used. The isolates are labeled according to their *in vitro* antimony susceptibility (*white circle*: sensitive isolates; *white square*: Sb^V-resistant/Sb^{III}-sensitive isolates; *black square*: Sb^V-resistant/Sb^{III}-resistant isolates; *grey square*: Sb^V-resistant/Sb^{III}:not done).

cance of the observed likelihood ratio is found by computing the null distribution of this ratio under the hypothesis of linkage equilibrium, using a permutation procedure. Because this procedure was repeated on all pairs of loci, we applied the sequential Bonferroni correction (Holm, 1979) to the *P* values.

3. Results

3.1. Dataset

Locus *CSg48* (located in chromosome 20) was removed from the dataset as it showed significant 1 base pair shifting among samples, which made correct scoring impossible. Based on the re-genotyped samples, only locus *AC01R* and locus *B3H* showed an error rate of 5% while all the other loci resulted in 0% MERL. Of the 26 *L. braziliensis* isolates analyzed, PER091 did not amplify for 7 of the 14 tested loci. Three to four peaks were detected at four loci in one isolate, namely PER231. While mixed infection is the most likely explanation for the latter case, the possibility of aneuploidy cannot be excluded (Sterkers et al., 2011; Downing et al., unpublished results). Isolates PER091 and PER231 were removed from the dataset. The remaining Peruvian *L. braziliensis* isolates ($n = 24$) displayed only one or two peaks at each microsatellite locus. In total, 24 MLGs containing 14 loci were retained for the genetic analyses. The proportion of missing data was only 3.6%; 14 samples had all loci successfully scored, whereas 8 samples had 1 locus missing and 2 samples 2 loci missing. Thus, the highest proportion of missing data per sample was 14%.

3.2. Genetic analysis

Out of 24 MLGs, 23 distinct MLGs were identified in the 24 *L. braziliensis* isolates; the isolates PER010 and PER012 had an identical MLG. As the isolates originated from distant localities (Cajamarca and Cusco; Table 1 and Fig. 1) this might suggest a recent migration of one of the patients. Based on the FCA plot (Fig. 2), isolates TRUUS1 (the outgroup *L. guyanensis* strain from Suriname), PER163 and PER182 were the most distinct from all other isolates, followed by LTB300 and M2903 (both from Brazil). Pairwise D_{AS} distances among the Peruvian isolates ranged between 36% and 100% (mean 65%), highlighting the diversity of the isolates. The three reference strains (TRUUS1, LTB300 and M2903) were removed from further analyses, as they were not the focus of this study. The NJ trees constructed based on the Chord and D_{AS} dis-

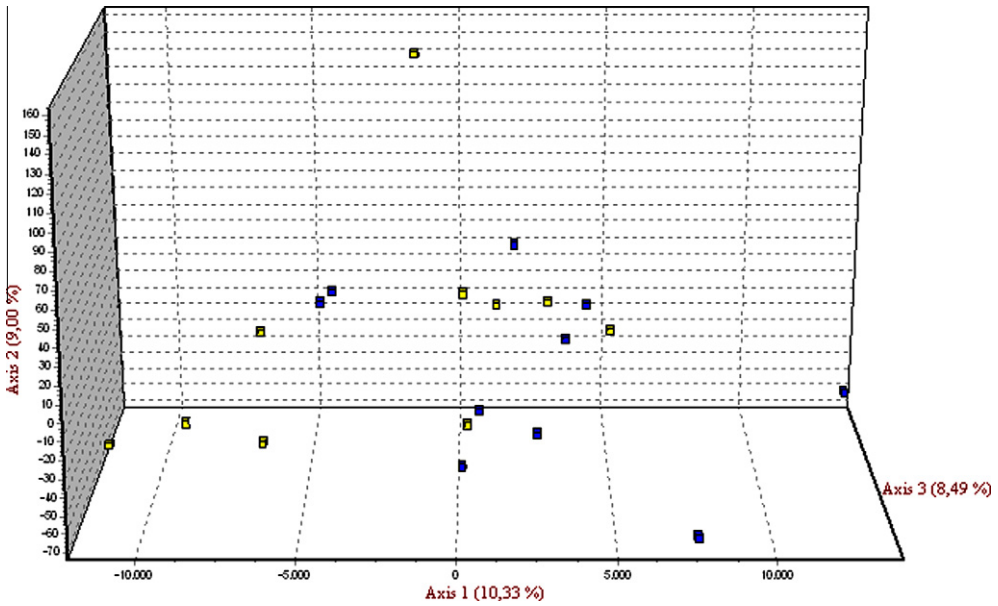

Fig. 4. FCA plot of MLGs from Peruvian *L. braziliensis* isolates in relation to the clinical phenotypes. The outliers PER163 and PER182 were not considered. The remaining MLGs (Fig. 2, dashed circle) were plotted and are labeled according to the clinical treatment outcome in the respective patients. Dark symbols: MLGs of isolates from patients showing clinical unresponsiveness ($n = 10$); light symbols: MLGs of isolates from patients with clinical definite cure ($n = 10$).

tance measures had a similar topology, with most nodes having little bootstrap support. Only the isolates PER163 and PER182, and the isolates PER016 and PER215 clustered strongly together (85% and 84% bootstrap support respectively; Fig. 3).

In Fig. 3 we also examined the topology of the tree in relation to the *in vitro* susceptibility to antimonials of the studied isolates. The Sb^V-sensitive ($n = 4$) and Sb^V-resistant ($n = 18$) isolates were scattered amongst the tree, i.e. there was no clustering of isolates in relation to the *in vitro* phenotypes. Next, we examined the topology of the tree in relation to the clinical treatment outcome. There was no grouping of clinical definite cure ($n = 10$) or treatment failure ($n = 10$) isolates (not shown). There was also a lack of clustering in relation to the clinical phenotypes in the factorial correspondence analysis (Fig. 4). No geographic structure could be detected in either the FCA plot or the dendrogram.

After removing the outliers (PER163, PER182) and one identical MLG (PER012), we treated the remaining Peruvian MLGs as a single population (Fig. 2, dashed circle) for the estimation of genetic diversity and linkage disequilibrium. Genetic diversity was quantified by the number of alleles per locus, which ranged from 2 alleles for *CSg55* to 19 alleles for *CSg47*, and the mean observed heterozygosity (H_o) (0.597 ± 0.223; Table 2). Linkage disequilibrium was analyzed between pairwise combinations of loci and found to be significant for 4 of the 91 pairs of loci (4.4%), which is similar to the 5% expected by chance. After sequential Bonferroni correction, none of the pairwise combinations appeared significant, i.e. the alleles at different loci associated randomly.

4. Discussion

The epidemiological dynamics of Sb^V resistance has been previously addressed in the context of anthroponotic visceral leishmaniasis due to *L. donovani* in Nepal (Laurent et al., 2007). Herein, we aimed to assess the situation for zoonotic tegumentary leishmaniasis due to *L. braziliensis* in Peru, by investigating the genetic polymorphism at 14 microsatellite loci on a sample of isolates (i) originating from patients with different antimonial treatment outcome and (ii) showing different susceptibility to Sb^V. The genetic variability of the parasites was very high, but there was no clear genetic structure in the dataset and no association was found between the genotypes and drug susceptibility or clinical treatment outcome. These findings, together with the polyphyletic pattern shown by Sb^V-resistant *L. braziliensis* parasites in the NJ tree, might theoretically have two non-exclusive explanations.

On one hand, our results might reveal independent events of drug resistance emergence among the natural populations of *L. braziliensis*. This hypothesis of a pleomorphic adaptive response of *L. braziliensis* to drug pressure is supported by our recent work. We found that expression profiles of genes involved in Sb^V metabolism and oxidative stress varied among the isolates, which suggested different molecular adaptations of the Sb^V-resistant parasites (Adaui et al., 2011a, 2011b). On the other hand, the absence of correlation between the microsatellite-based genetic data and the drug resistance of the parasites might be explained by genetic recombination. Indeed, a recent population genetics study has shown evidence of recombination events in *L. braziliensis* from Peru and Bolivia (Rougeron et al., 2009). Even if our dataset was not ideal for recombination tests (small population size and large geographical distribution), we applied a series of tests that further supported this hypothesis. The lack of distinct subgroups in the NJ tree (Fig. 3) and FCA (Fig. 2), together with the near absence of identical MLGs, the high genetic variability across loci and the lack of significant linkage disequilibrium between pairs of loci are inconsistent with a strictly clonal reproduction. This suggests that genetic exchange could have contributed to dissociate the linkage between neutral microsatellite markers and the mutations underlying drug resistance. Considering the extensive changes in ploidy which were recently observed in natural populations of *Leishmania* (Downing et al., unpublished results), further work would also be required to test the hypothesis of pseudo-sexuality (Dujardin et al., 2007). Indeed, heterozygous genetic markers present on trisomic or tetrasomic chromosomes could mimic signals of recombination during random return to disomy. It should also be taken into account, however, that only a limited set of microsatellite markers on 12 of the 35 chromosomes present in *L. braziliensis* has been used here.

A remarkable observation is that a polyphyletic pattern was encountered among Sb^V-resistant parasites both in a zoonotic (*L.*

Table 2
Characteristics of the 14 microsatellite markers used for population genetic analysis of Peruvian *L. braziliensis* isolates[A].

Marker	Chromosome number[B]	No. of isolates	Repeat array	Fragment size (bp)	*A*	H_o
CSg46	18	18[a]	(AC) 6–25	71–109	14	0.611
CSg47	29	21	(TG) 8–35	87–141	19	0.857
CSg53	21	21	(AC) 7–14	84–98	3	0.333
CSg55	10	21	(TG) 11–15	93–101	2	0.048
CSg59	25	20[b]	(TC) 5–8	92–98	3	0.350
7GN	35	21	(AC) 9–20	108–130	10	0.667
11H	21	21	(GT) 8–11	88–94	4	0.476
11C	33	20[c]	(TG) 6–12	92–104	6	0.650
6F	27	19[d]	(AC) 7–21	83–111	9	0.737
10F	18	21	(CA) 13–16	93–99	4	0.667
B6F	16	19[e]	(AC) 6–22	79–111	14	0.842
B3H	28	19[f]	(AC) 6–23	63–97	10	0.737
AC01R	19	21	(CA) 8–16	99–115	9	0.714
AC16R	11	21	(TG) 12–21	91–109	8	0.667
Mean		20.2			8.2	0.597

A, number of alleles; H_o, observed heterozygosity.
Missing data: a, isolates PER006, PER014, PER016; b, isolate PER201; c, isolate PER094; d, isolates PER015, PER122; e, isolates PER015, PER086; f, isolates PER014, PER157.
[A] Data are shown for 21 *L. braziliensis* isolates (PER163, PER182 and PER012 have been excluded).
[B] The chromosome number stands for the specific chromosome on which the microsatellite marker is located and corresponds to the *L. braziliensis* strain MHOM/BR/1975/M2904, whose genome has been completely sequenced. These data were reported by Oddone et al. (2009).

braziliensis, this study) and an anthroponotic (*L. donovani*, Laurent et al., 2007) transmission. First, this could mean that transmission is not that different as expected, as indicated by recent reports on the domestication and possible anthropisation of the *L. braziliensis* cycle (Garcia et al., 2007; Vergel et al., 2006), and on the possible involvement of an animal reservoir in *L. donovani* transmission (Bhattarai et al., 2010). Secondly, it is possible that drug pressure does not play the most important role in structuring the parasite population. Cross-resistance of *Leishmania* to antimonials and nitric oxide (NO) has been reported (Carter et al., 2005; Holzmuller et al., 2005); hence, a portion of the Sb^V resistance measured here could have originated from a NO pressure to which *Leishmania* parasites are normally confronted in the host. In addition, co-factors present in the natural environment of *Leishmania* (Ait-Oudhia et al., 2011), like arsenic contamination (Perry et al., in press), could play a role in shaping antimony susceptibility and should be explored.

Whatever the mechanism explaining the structure observed here, our results highlight the important adaptive capacity of *Leishmania* and stress the need to monitor the evolution of parasite populations at a larger scale. The type of emerging Sb^V resistance likely depends on the genetic background of the parasite itself and the environment of the parasite (with a particular selective pressure), which is determined by both the host and the parasite. Further studies looking at genome-wide signatures of selection under a more controlled environment are required to assess the genetic basis and associated impact of the different Sb^V-resistant phenotypes on the spreading of Sb^V resistance or on *in vivo* treatment efficacy.

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