Genetic diversity of *Leishmania infantum* field populations from Brazil

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Leishmania infantum *(syn.* Leishmania chagasi*) is the etiological agent of visceral leishmaniasis (VL) in Brazil. The epidemiology of VL is poorly understood. Therefore, a more detailed molecular characterization at an intraspecific level is certainly needed. Herein, three independent molecular methods, multilocus microsatellite typing (MLMT), random amplification of polymorphic DNA (RAPD) and simple sequence repeats-polymerase chain reaction (SSR-PCR), were used to evaluate the genetic diversity of 53* L. infantum *isolates from five different endemic areas in Brazil. Population structures were inferred by distance-based and Bayesian-based approaches. Eighteen very similar genotypes were detected by MLMT, most of them differed in only one locus and no correlation was found between MLMT profiles, geographical origin or the estimated population structure. However, complex profiles composed of 182 bands obtained by both RAPD and SSR-PCR assays gave different results. Unweighted pair group method with arithmetic mean trees built from these data revealed a high degree of homogeneity within isolates of* L. infantum. *Interestingly, despite this genetic homogeneity, most of the isolates clustered according to their geographical origin.*

Key words: *Leishmania infantum* - genetic diversity - MLMT - RAPD - SSR-PCR

Visceral leishmaniasis (VL) is a systemic disease caused by an intracellular protozoan belonging to the *Leishmania donovani* complex: *L. donovani*, *Leishmania infantum* and *Leishmania chagasi* (Lainson & Shaw 1987), which display different geographical distributions. In Brazil, the etiological agent of VL is *L. chagasi*, recently found to be derived from European strains of *L. infantum* (Kuhls et al. 2011, Leblois et al. 2011).

Clinical and laboratory manifestations of VL are well characterized and include fever, paleness, gradual weight loss, splenomegaly, hypergammaglobulinaemia and pancytopenia. However, different clinical symptoms have been associated with the etiological agent species. Postkala-azar dermal leishmaniasis, a dermal complication following recovery from a VL infection, is caused by *L. donovani*. In Europe, *L. infantum* causes VL and also cutaneous leishmaniasis, whereas in Brazil, the latter clinical symptom associated with *L. infantum* is uncommon.

Brazilian VL has intriguing epidemiological data, such as patients showing severe disease that leads to death while others have no complications, the high incidence of the disease in the Northeast Region (which accounts for 65% of cases) and the fact that 41% of cases are reported in children under five years of age (MS 2006). In this context, it raises the question of whether the variation in the pathogenicity of the parasite and clinical features of VL may be related to its genetic diversity. However, before addressing this question, it is important to analyse the genetic diversity of the parasite.

Many molecular methods have been developed to evaluate the genetic diversity of *Leishmania* parasites. These include the amplification of DNA sequences of the parasite by specific polymerase chain reaction (PCR), the random amplification of polymorphic DNA (RAPD) (Hide et al. 2001, Zemanova et al. 2004), simple sequence repeats-PCR (SSR-PCR) (Oliveira et al. 1997, de Oliveira et al. 2007), PCR of antigen-encoding genes (*gp63* and *cpb*) followed by analysis of restriction fragment length polymorphism (RFLP) (Mauricio et al. 2001, Quispe-Tintaya et al. 2004), PCR-RFLP of kinetoplast DNA (kDNA) minicircles (Morales et al. 2001, Chicharro et al. 2002) and, more recently, multilocus microsatellite typing (MLMT) (Ochsenreither et al. 2006, Alam et al. 2009).

Using these different molecular approaches, many studies have been conducted to evaluate the variability of various species of *Leishmania* (Mauricio et al. 1999, Hide et al. 2001, Ishikawa et al. 2002, Ochsenreither et al. 2006, de Oliveira et al. 2007). These studies mainly aimed to detect polymorphisms between isolates from different species, as well as within isolates from the same species, but from different geographic areas. Recently, a study was conducted to evaluate the variability of 40 Brazilian *L. infantum* strains, but all of the isolates were obtained from the same region (Alonso et al. 2010).

Therefore, considering the relevance of VL and the role that genetic polymorphisms play in the epidemiology of the disease, this study evaluated the genetic diversity of *L. infantum* isolates obtained from different endemic areas in Brazil, using three different molecular approaches: MLMT, RAPD and SSR-PCR.

MATERIALS AND METHODS

Parasite isolates - A total of 53 isolates were analysed: 49 were obtained from humans and four were obtained from dogs (Table I). Parasites were isolated from patients by bone marrow aspirates and from dogs by spleen biopsies. The samples were collected in different VL endemic areas of Brazil, including the states of Espírito Santo (ES), Maranhão (MA), Minas Gerais (MG), Paraíba (PB) and Piauí (PI) (Fig. 1). All stocks were identified as *L. infantum* by PCR-RFLP from the conserved region of the *Leishmania* kDNA minicircle, in accordance with Volpini et al. (2004). As reference strains, we included *L. infantum* (MHOM/BR/74/PP75), *Leishmania amazonensis* (MHOM/BR/73/M2269) and *Leishmania braziliensis* (MHOM/BR/75/M2903). This study was approved by the Ethical Committee of the Federal University of Espírito Santo (CEP-023/07).

Parasite culture and DNA preparation - The parasites were cultivated in liver infusion tryptose medium supplemented with 10% of heat inactivated foetal calf serum and grown at 24-26ºC until the parasite concentration reached ~10^7 parasites/mL. At this phase, 1.0 mL of the promastigote culture was washed in phosphate-buffered saline (pH 7.2) and DNA extraction was performed using the Wizard™ Genomic DNA Purification System (Promega, Madison, Wisconsin, USA), as recommended by the manufacturer.

Microsatellite typing - For microsatellite analyses, we amplified seven polymorphic loci previously described for the *L. donovani* complex Li22-35, Li23-41, Li45-24, Li71-33, Lm2TG, Lm4TA and TubCA (Ochsenreither et al. 2006). Each PCR was performed in a total volume of 15 μL containing 250 μM of each dNTP, 0.5 U Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA, USA), 20 mMTris-HCl (pH 8.0), 50 mMKCl, 2.0 mM $MgCl_2$, 0.2 μM of each primer and 3 ng of DNA template. Amplifications were performed using a step-down protocol (Hecker & Roux 1996) modified for the amplification of *L. infantum* DNA, which consisted of an initial denaturation step at 94ºC for 5 min, annealing at 58ºC for 30 s, primer extension at 72ºC for 1 min and a denaturation step at 94ºC for 30 s. After five cycles, the annealing temperature was decreased to 56ºC and after an additional five cycles to 54ºC. At the final annealing temperature, the number of cycles was increased to 23, followed by a final extension step at 72ºC for 10 min.

To determine the allele sizes, 0.8-3 μL of the fluorescent PCR products were analysed in a 6% denaturing polyacrylamide gel in an Automatic Laser Fluorescent sequencer (GE Healthcare, Milwaukee, Wisconsin, USA) and compared with fluorescent DNA fragments of 50-500 bp using the AlleleLocator software (GE Healthcare, Milwaukee, Wisconsin, USA).

TABLE I

Leishmania infantum isolates used in this study

Laboratory code	WHO code	Geographic area (city - state)
PP75	MHOM/BR/74/PP75	ATCC
ES01	MHOM/BR/96/ES01	Pancas - ES
ES02	MHOM/BR/96/ES02	Pancas - ES
ES03	MHOM/BR/95/ES03	Pancas - ES
ES04	MHOM/BR/06/ES04	Brejetuba - ES
ES05	MHOM/BR/96/ES05	Pancas - ES
MG01	MHOM/BR/05/MG01	Francisco Sá - MG
MG02	MHOM/BR/05/MG02	São Francisco - MG
MG03	MHOM/BR/05/MG03	Montes Claros - MG
MG04	MHOM/BR/05/MG04	Montes Claros - MG
MG05	MHOM/BR/05/MG05	Montes Claros - MG
MG06	MHOM/BR/05/MG06	Pirapora - MG
MG07	MHOM/BR/05/MG07	Montes Claros - MG
MG08	MHOM/BR/05/MG08	Montes Claros - MG
MG09	MHOM/BR/05/MG09	Montes Claros - MG
MG10	MHOM/BR/05/MG10	Porteirinha - MG
MG11	MHOM/BR/05/MG11	Montes Claros - MG
MG12	MHOM/BR/05/MG12	Montes Claros - MG
MG13	MHOM/BR/05/MG13	São Francisco - MG
MG14	MHOM/BR/05/MG14	Bocaiúva - MG
MG15	MHOM/BR/05/MG15	Montes Claros - MG
MG16	MHOM/BR/05/MG16	Bocaiúva - MG
MG17	MHOM/BR/05/MG17	Porteirinha - MG
MG18	MHOM/BR/05/MG18	Montes Claros - MG
MG19	MHOM/BR/05/MG19	Catuni - MG
MG20	MHOM/BR/05/MG20	Montes Claros - MG
MA01	MHOM/BR/06/MA01	Paraibano - MA
MA02	MHOM/BR/05/MA02	Codó - MA
MA03	MHOM/BR/06/MA03	Timon - MA
MA04	MHOM/BR/06/MA04	Codó - MA
MA05	MHOM/BR/05/MA05	Timon - MA
MA06	MHOM/BR/06/MA06	Timon - MA
MA07	MHOM/BR/06/MA07	Caxias - MA
PB01	MHOM/BR/00/PB01	Itabaiana - PB
PB02	MHOM/BR/00/PB02	Marcação - PB
PB03	MHOM/BR/00/PB03	João Pessoa - PB
PB04	MHOM/BR/00/PB04	João Pessoa - PB
C01	MCAN/BR/99/C01[a]	João Pessoa - PB
C02	MCAN/BR/99/C02[a]	João Pessoa - PB
C03	MCAN/BR/99/C03[a]	João Pessoa - PB
C04	MCAN/BR/99/C04[a]	João Pessoa - PB
PI01	MHOM/BR/06/PI01	José de Freitas - PI
PI02	MHOM/BR/06/PI02	Valença do Piauí - PI
PI03	MHOM/BR/06/PI03	Cabeceiras - PI
PI04	MHOM/BR/05/PI04	Valença do Piauí - PI
PI05	MHOM/BR/06/PI05	Valença do Piauí - PI
PI06	MHOM/BR/05/PI06	Nossa Senhora dos Remédios - PI
PI07	MHOM/BR/06/PI07	Piripiri - PI
PI08	MHOM/BR/05/PI08	Altos - PI
PI09	MHOM/BR/05/PI09	Nova Santa Rita - PI
PI10	MHOM/BR/06/PI10	Teresina - PI
PI11	MHOM/BR/06/PI11	José de Freitas - PI
PI12	MHOM/BR/05/PI12	Lima Campos - PI
PI13	MHOM/BR/06/PI13	Santa Cruz dos Milagres - PI

a: canine isolate; ATCC: American Type Culture Collection; ES: Espírito Santo; MA: Maranhão; MG: Minas Gerais; PB: Paraíba; PI: Piauí; WHO: World Health Organization.
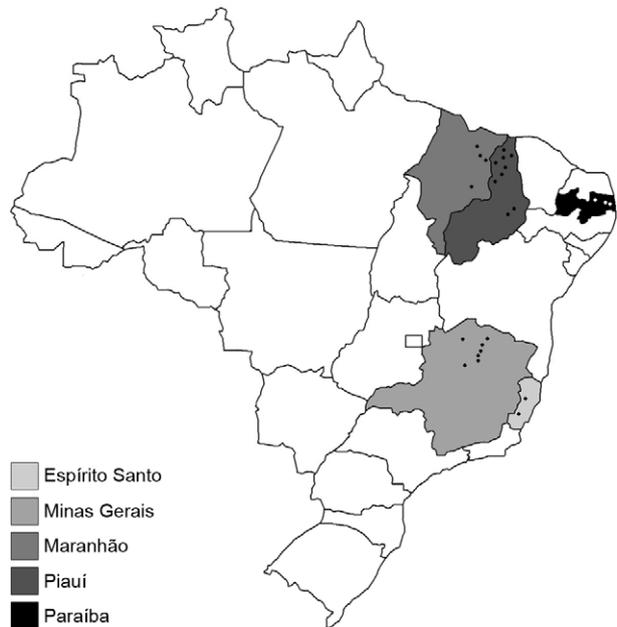
Espírito Santo
Minas Gerais
Maranhão
Piauí
Paraíba

Fig. 1: map of Brazil and geographical distribution of the Brazilian *Leishmania infantum* isolates used in this study.

RAPD - The RAPD procedure was performed essentially as described by Steindel et al. (1993). One nanogram of total DNA was amplified in a final volume of 25 μL containing 1 U of *Taq* DNA polymerase, 200 μM of each dNTP, 10 mM Tris-HCl (pH 8.6), 50 mMKCl, 2 mM $MgCl_2$ and 0.5 μM of each primer. We selected the five primers showing the highest polymorphism in a previous report: A1, B4, B10, F10 and R15 (Hide et al. 2001). The amplification program consisted of an initial denaturation step at 95ºC for 5 min, followed by two cycles of denaturation at 95ºC for 30 s, annealing at 30ºC for 2 min and extension at 72ºC for 1 min; this was followed by 30 cycles in which the annealing step temperature was increased to 40ºC. The final extension was for 5 min at 72ºC. Amplification products were analysed on 6% polyacrylamide gels after ethidium bromide staining. PCRs were repeated at least twice and only the consistent bands present in both gels were taken into account to build a reproducible profile of each isolate. The reproducibility was assessed using different concentrations of DNA obtained from different isolates, including the reference strain *L. infantum* (MHOM/BR/74/PP75). Every step was performed under strict and standardized conditions, including the enzyme and primer source and high-quality DNA at an adequate concentration.

SSR-PCR - The SSR-PCR amplification procedure was carried out as described by Oliveira et al. (1997). Each reaction was performed in a final volume of 10 μL, containing 10 mM Tris-HCl (pH 8.6), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of dNTPs, 2% formamide (v/v), 2.5 μM of the primer $(CA)_8RY$ (Zietkiewicz et al. 1994), 1 U of *Taq* DNA polymerase and 1 ng of DNA. The amplification cycles consisted of an initial denaturation step at 94ºC for 3 min, followed by 26 cycles at 94ºC for 30 s, 52ºC

for 45 s and 72ºC for 1 min. The final extension was done at 72ºC for 7 min. The amplification products were analysed on 6% polyacrylamide gels after ethidium bromide staining. PCR reactions were performed at least twice and amplifications were carried out on different days.

Data analysis - The multilocus microsatellite genotype data, consisting of the allele sizes or repeat numbers, were analysed by two different approaches: a Bayesian model-based clustering method using STRUCTURE software (Pritchard et al. 2000) and a distance-based method. For inferring population structure, the admixture model was assumed, which correlated allele frequencies among populations, independent of a particular mutation model. For each isolate, the fraction of its genotype that belongs to each population is measured and then genetic clusters are constructed from a collection of individual genotypes. Markov chain Monte Carlo searches consisted of a burn-in length of 50,000 iterations followed by a run of 250,000 replications for each setting of *K*. The complete set of individual isolates was divided into *K* sub-populations, with *K* ranging from 1-10 with 10 replicate runs of each. The appropriate number of populations in our sample test was determined by comparison of the log-likelihood values for *K* 1-10 and the calculation of Δ*K* (Evanno et al. 2005). The intra-population diversity was measured using the statistic expected heterozygosity (H_E), as implemented in Arlequin 3.5 software (Excoffier et al. 2005).

Genetic distances D1 (average square distance), which follows the stepwise mutation model, were calculated for the repeat numbers within each microsatellite locus by using MICROSAT software (Stanford University Press, Stanford, CA). A tree based on these distances was constructed by the algorithm of the unweighted pair group method with arithmetic averages (UPGMA) using PHYLIP 3.67 package version 3.5. (University of Washington, Seattle, USA) and visualized with TreeView1.6.6 (Roderic 1996).

The band patterns obtained for each isolate resulting from RAPD and SSR-PCR procedures were scored by eye. In order to guarantee the quality control of inter-gel comparisons, the gels were aligned using the standard molecular weight marker and the profile of reference strain PP75, included in every PCR reaction and electrophoresis. Only well-defined bands were used to construct a binary table. Each amplification band was numbered and scored as present (1) or absent (0) and for each isolate genetic distances among them were calculated using the Nei coefficient (Nei 1978). A phylogenetic tree was constructed based on the genetic distance matrices obtained through UPGMA using Treecon software program version 1.3b (Van de Peer & De Wachter 1994). The robustness of the internal branches was tested by bootstrap analysis from 1,000 replicates.

RAPD and SSR-PCR amplicons were analysed as dominant markers. To estimate genetic diversity from these data, a Hardy-Weinberg equilibrium was assumed among the populations and the allele frequencies were calculated based on the frequencies of the null alleles. A locus was considered polymorphic when a specific band present at least in one isolate was not observed in

all other strains. H_E and percentage of polymorphic loci were estimated using the Tools for Population Genetic Analyses program version 1.3 (Department of Biological Sciences, Flagstagg, AZ, USA).

RESULTS

The analysis of data obtained by the microsatellite assay showed that most of the isolates had profiles that differed from each other by only one locus. For the seven analysed microsatellite loci, the number of different alleles detected ranged from 1-4 (Table II). The most polymorphic microsatellite locus was Lm4TA with four alleles. In contrast, the TubCA locus was monomorphic. The Li23-41 marker showed two alleles, but one was exclusively found in the canine isolate C02. Among the 51 Brazilian isolates evaluated, 18 different genotypes (multilocus microsatellite profiles) could be detected and they were closely related to one another. Most of the isolates had homozygous profiles for the seven loci tested, whereas eleven isolates showed heterozygosis in one locus (Table II). Three or more peaks, suggestive of aneuploidy or a mixture of heterozygous strains, were not observed. None of the seven microsatellite markers used here for the *L. donovani* complex were able to amplify the *L. braziliensis* strain and only two of them (Lm4TA and TubCA) amplified the *L. amazonensis* reference strain. However, the allele sizes were not compatible with those obtained from *L. infantum* isolates (data not shown). MLMT profiles were used to calculate genetic distances and to construct a UPGMA tree. As shown in Fig. 2A, no correlation was found between MLMT profiles, geographical origin or the host of isolates (human vs. canine).

Measures of genetic diversity within each population were low (Table III). The mean number of alleles per locus ranged from 1.0-1.7. The percentage of polymorphic loci was low for all populations. The H_E, representing the probability of an individual to be heterozygous over the loci tested, varied between 0.097-0.193, with an exception for ES isolates ($H_e = 0.355$). One in five ES isolates had an allelic variant for the Li22-35 locus.

Based on the data from seven microsatellite loci, an estimative population structure for Brazilian *L. infantum* was inferred from the STRUCTURE software, which indicated the existence of five ancestral populations. However, these genetic groups did not correlate with the geographical distribution of the isolates.

More interesting results were obtained with the RAPD and SSR-PCR techniques. Our results showed that both techniques were able to generate complex band patterns. However, these profiles were very similar regardless the approach used demonstrating the genetic homogeneity amongst the *L. infantum* isolates (Supplementary data). The RAPD amplifications of all isolates resulted in reproducible patterns, each primer giving characteristic and distinct band profiles. A total of 144 bands were analysed, with molecular weight varying from 400-1,200 bp. Isolate MG03 did not amplify with the A1 and B10 primers, even after several PCR assays.

The SSR-PCR approach yielded 38 reproducible bands (ranging from 400-1,200 bp). Fifty out of 53 *L. infantum* isolates showed amplified products with the primer $(CA)_8RY$. Multiple attempts to isolate DNA and amplify SSR from isolates MG05, PI13 and ES05 were

TABLE II

Multilocus microsatellite typing genotypes of the Brazilian *Leishmania infantum* isolates based on the number of repeats of the microsatellite markers

Genotype	Isolates (n)	Repeat units (n)						
		Li22-35	Li23-41	Li45-24	Li71-33	Lm2TG	Lm4TA	TubCA
1	13	16	18	14	12	26	13	8
2	2	16	18	14	12	26	12/13	8
3	2	16	18	14	12	26	13/14	8
4	3	16	18	14	12	26	14	8
5	1	16	18	14/15	12	26	13	8
6	3	16	18	15	12	26	13	8
7	1	16	18	15	10/12	27	13	8
8	3	16	18	15	12	27	13	8
9	1	16	18	14	11/12	27	13	8
10	1	16	18	14	12	27	13/14	8
11	11	16	18	14	12	27	13	8
12	1	16	18	14/18	12	26	13	8
13	1	16	18	14	12	25	13	8
14	1	16	18	15	12	25	13	8
15	1	16	18	15	10/12	26	13	8
16	4	19	18	14	12	26	13	8
17	1	16	18	14	12	25	11	8
18	1	16	17	14	12	25	11/14	8
Alleles (n)		2	2	3	3	3	4	1

unsuccessful. However, unlike RAPD, identical profiles were generated for some of the different isolates evaluated. For both methodologies different profiles were generated for *L. amazonensis*, *L. braziliensis* and *L. infantum* (data not shown).

The trees constructed based on RAPD and SSR-PCR data are shown in Fig. 2B, C, respectively. A geographical structure was observed in the RAPD tree: 12/13 PI isolates and 7/7 MA isolates were found in the same branch, isolates from ES were grouped with one isolate from MG (MG20), 18/19 MG isolates were grouped together in two branches and 7/8 isolates from PB were clustered together. The results obtained by SSR-PCR also showed the existence of clusters composed of neighbouring populations. When a tree was built by combining the RAPD and SSR-PCR data, four clusters were evident in which the isolates were grouped according to their geographic origin (Fig. 2D). Isolates from ES were grouped into a specific branch, within a major cluster composed of isolates from MG. Isolates from MA and PI were clustered together in a single branch without a clear distinction between them. PB isolates were clustered together in a fourth branch. We did not observe any specific clusters in relation to the host, as both human and canine isolates were found in the same cluster. In general, the UPGMA trees revealed a small genetic distance detected between the isolates.

Descriptive statistics of RAPD and SSR-PCR are shown in Table IV. The H_E varied between 0.121-0.222. The percentage of polymorphic loci ranged from 68.42 (SSR-PCR marker) to 86.96 (RAPD primer A1), with an average of 78.16 for all populations. According to this last parameter, RAPD assays were more discriminative than the SSR-PCR assay.

DISCUSSION

In this study we analysed the genetic polymorphisms of *L. infantum* isolates from different endemic areas of Brazil via MLMT, RAPD and SSR-PCR strategies. The UPGMA tree built from both RAPD and SSR-PCR data showed clustering of the isolates according to their geographical origin, with one cluster including isolates from MA and PI, a second cluster including isolates from MG and ES, which are neighbouring states, and a third cluster including only isolates from PB.

In accordance with our results, previous studies have demonstrated geographic structuring of *Leishmania* populations using different molecular markers. Cupolillo et al. (2003) used PCR-RFLP analysis for *L. braziliensis* and showed that most of the genotypes were associated with specific geographic areas. These results corroborate data from other studies, which demonstrated that DNA fingerprinting and RAPD analysis could separate strains of *L. braziliensis* and *L. donovani* on the basis of their geographic origin (Gomes et al. 1995, Zemanova et al. 2004). In the Mediterranean region, the distribution of *L. infantum* strains from southern Spain, characterized by RAPD analysis, was also found to be related to their geographic origin (Toledo et al. 2002). Similar results were observed by Guerbouj et al. (2001) when strains from southern France were analysed on the basis of chromosomal size. On the other hand, many authors

did not find an association between isolate variability and their geographic distribution (Cortes et al. 2006, de Oliveira et al. 2007, Seridi et al. 2008). The same result was found in our study using MLMT analysis.

MLMT analysis showed 18 different genotypes, but most of the isolates shared an identical profile or differed only by one locus. According to the microsatellite typing, *L. infantum* from Brazil is a homogeneous species and the geographic correlation observed for RAPD and SSR-PCR was not detected. These different results can be attributed to the fact that we evaluated only seven loci for the microsatellite assay vs. 182 bands analysed for RAPD and SSR-PCR analyses.

Even though many studies have demonstrated that highly polymorphic microsatellite markers are useful for strain typing and population genetic studies in the *L. donovani* complex (Ochsenreither et al. 2006, Kuhls et al. 2007), this is not the first time that MLMT reveals high genetic homogeneity within a *Leishmania* species (Alam et al. 2009, Alonso et al. 2010). Because this method was unable to discriminate between different *L. infantum* isolates, it suggests that this methodology is not suitable for epidemiological studies of closely related *Leishmania* strains. Nevertheless, high-resolution electrophoresis is required and a set of polymorphic markers has to be analysed to achieve the highest discrimination amongst strains, which dramatically increases the cost of the assay.

Although the *L. infantum* isolates have been grouped according to their geographical origin using RAPD and SSR-PCR analyses, their genetic profiles were similar, suggesting that parasite populations of different regions from Brazil are homogenous. This similarity was supported by the low genetic distance detected between the isolates and the low estimated heterozygosity rates in the populations studied.

Despite the homogeneity detected in *L. infantum*, each isolate had a unique RAPD profile. In contrast to our results, the study performed by Alonso et al. (2010) using kDNA PCR-RFLP methodology demonstrated that more than 60% of the *L. infantum* isolates (26 of 40) showed an identical genetic profile. These authors attributed the observed homogeneity to the maintenance of the parasites in culture, which might select one or a few parasite lineages and for this reason, the genetic variability could be underestimated. However, in our study, all isolates were also obtained by culture. Therefore, it seems that RAPD analysis provided greater discriminatory power than did kDNA PCR-RFLP.

One explanation for the geographic structuring and low variability found for *L. infantum* in Brazil could be the recent introduction of this species to the New World (Mauricio et al. 1999, Kuhls et al. 2011). Another reason could be the fact that the transmission of this species in Brazil is associated with one major vector, *Lutzomyia longipalpis* (Lainson & Rangel 2005) and that the degree of variability of *Leishmania* species has been related to the number of vectors and/or animal reservoir involved in the transmission of these parasites (Cupolillo et al. 2003).

The reproductive mode of a species can also affect its genetic polymorphisms. In the case of *Leishmania*,
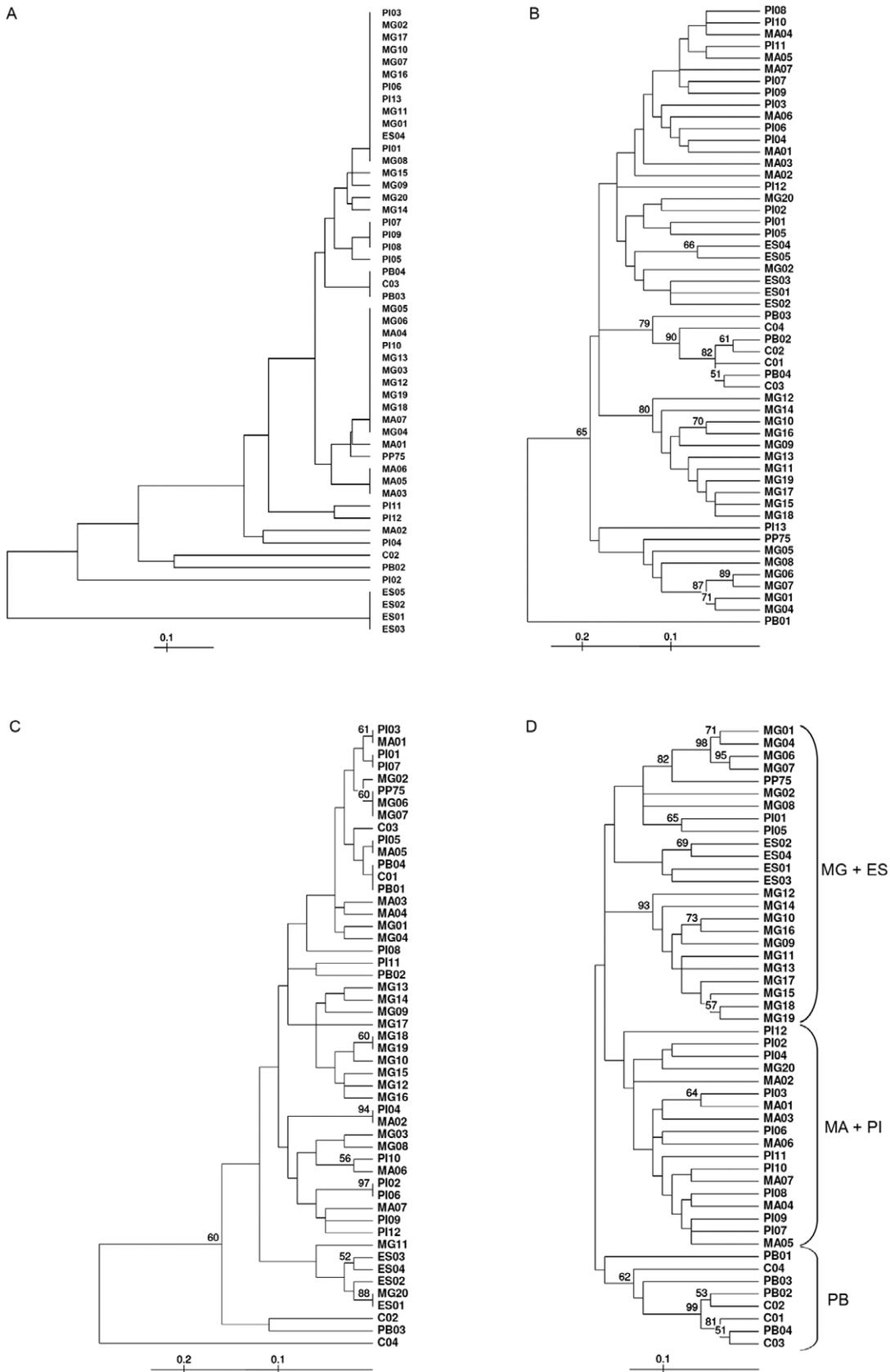
Fig. 2: unweighted pair group method with arithmetic mean tree of *Leishmania infantum* isolates built-up from data of multilocus microsatellite typing (A), random amplification of polymorphic DNA (RAPD) (B), simple sequence repeats-polymerase chain reaction (SSR-PCR) (C) [dogs strains from Paraíba (PB)] and RAPD and SSR-PCR together (D). Bootstraps values above 50 are indicated. PP75: *L. infantum* reference strain (MHOM/BR/74/PP75). ES: Espírito Santo; MA: Maranhão; MG: Minas Gerais; PI: Piauí.

TABLE III

Measures of genetic diversity for the five *Leishmania infantum*
populations studied according to multilocus microsatellite typing data

Population	Isolates (n)	Proportion of polymorphic loci	Mean number of alleles	$H_E \pm$ SD
Espírito Santo	5	0.143	1.142	0.355 ± 0.000
Maranhão	7	0.428	1.429	0.116 ± 0.193
Minas Gerais	20	0.258	1.429	0.097 ± 0.187
Paraíba	5	0.428	1.429	0.193 ± 0.247
Piauí	13	0.428	1.714	0.145 ± 0.229
Mean	-	0.343	1.400	0.181 ± 0.171

H_E: expected heterozygosity; SD: standard deviation.

TABLE IV

Characteristics of the random amplification of polymorphic DNA and simple sequence repeats-polymerase chain reaction markers
used and measures of genetic diversity for the five *Leishmania infantum* populations studied according to their data

Primers	Number of analyzed bands (n = 182)	Polymorphic loci (%)	Expected heterozygosity					
			MG	PI	ES	PB	MA	Mean per locus
A1	23	86.956	0.182	0.252	0.142	0.142	0.193	0.300
B4	26	84.615	0.292	0.244	0.093	0.103	0.116	0.287
B10	30	73.333	0.225	0.218	0.188	0.107	0.191	0.311
F10	26	84.615	0.286	0.184	0.135	0.144	0.155	0.331
R15	38	71.052	0.200	0.185	0.179	0.073	0.085	0.269
$(CA)_8RY$	38	68.421	0.149	0.179	0.037	0.159	0.127	0.255
Mean	30.333	78.165	0.222	0.210	0.129	0.121	0.144	-
Polymorphic loci within each population (%)			58.69	58.13	30.32	28.90	37.13	-

ES: Espírito Santo; MA: Maranhão; MG: Minas Gerais; PB: Paraíba; PI: Piauí.

reproduction has often been assumed to be strictly clonal, but this remains undetermined. Herein, we found a substantial heterozygote deficiency amongst the isolates studied and positive values were found for the inbreeding coefficient per microsatellite locus (data not shown), which could indicate high levels of inbreeding. These observations are inconsistent with a predominantly clonal mode of reproduction, which implies excess heterozygosity (Meselson effect). Moreover, as there is small genetic heterogeneity between populations within regions and no evidence of a strong population structure, this high number of homozygotes cannot be explained as the result of a population subdivision (Wahlund effect). A working hypothesis for these findings is that parasites alternate clonal and sexual reproduction with recombination events between individuals of similar genotypes (Kelly et al. 1991, Butlin 2002, Ravel et al. 2006, Nolder et al. 2007). Recently, it was reported that the invertebrate stages of *Leishmania* are capable of having a sexual cycle consistent with a meiotic process (Akopyants et al. 2009). However, the mechanism of recombination, at what frequency it occurs and whether it occurs in the mammalian host, remains to be addressed.

Our results also demonstrate that *L. infantum* isolates from human and dogs have the same profile, although we have only analysed a small number of isolates from dogs. These findings are in agreement with Chicharro et al. (2002) who worked with RAPD and intergenic ribosomal typing and could not discriminate amongst human and dog strains from the island of Majorca. Because the domestic dog has been incriminated as the main reservoir of VL, the fact that a strain isolated from humans displays a similar pattern to that of a canine stock fits well with the zoonotic cycle of *L. infantum* (MS 2006).

In conclusion, our results demonstrate that although the Brazilian *L. infantum* isolates are genetically homogeneous, there is a correlation between the isolates and their geographic origin. However, the development of methods with higher discriminatory power is still necessary to enhance our understanding of the molecular epidemiology of VL in Brazil.

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