

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Spatial distribution and population genetics of *Leishmania infantum* genotypes in São Paulo State, Brazil, employing multilocus microsatellite typing directly in dog infected tissues



Gabriela Motoie^a, Gabriel Eduardo Melim Ferreira^b, Elisa Cupolillo^b, Flavio Canavez^c, Vera Lucia Pereira-Chioccola^{a,*}

^aLaboratório de Parasitologia do Instituto Adolfo Lutz, São Paulo, Brazil

^bLaboratório de Pesquisa em Leishmaniose, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, Brazil

^cGenoa Biotecnologia SA, São Paulo, Brazil

ARTICLE INFO

Article history:

Received 11 August 2012

Received in revised form 19 April 2013

Accepted 27 April 2013

Available online 9 May 2013

Keywords:

Leishmania infantum

Microsatellite markers

American visceral leishmaniasis

Clinical samples

São Paulo State – Brazil

ABSTRACT

This study investigated the genetic characteristics of *Leishmania infantum* samples from São Paulo (SP) State, Brazil in order to collaborate with information about the possible origins of the parasites, as well as, the introduction and spread of visceral leishmaniasis in this Brazilian State. Multilocus microsatellite typing (MLMT) was performed using a set of 17 microsatellite markers. DNA was extracted from 250 samples collected from dogs diagnosed with visceral leishmaniasis and 112 (45%) were genotyped: 67 from the northwest region (NWSP), and 29 from the southeast region (SESP) of SP. The results were correlated with other 16 samples from Mato Grosso do Sul State (MS) (which borders NWSP). Although, a small portion of samples was genotyped, it was possible to genotype multiple loci using small amounts of *Leishmania* DNA extracted directly from dog tissues. Despite the fact that MLMT analysis defined 33 different genotypes, a low polymorphism was detected within the parasites studied with 10 polymorphic loci. There are two main genetic clusters circulating in SP with strong genetic differentiation, one (POP-A) is composed by samples from SESP and NWSP and presented a weak signal of geographical substructure. The other, belongs to the same cluster found in the state of MS (POP-B), which was the main one. The majority (93.75%) of MS parasite genotypes belonged to POP-B, with just one sample (6.25%) grouped in POP-A. POP-B also comprised 10.34% of SESP and 26.87% of NWSP samples. Besides one sample from MS, POP-A is composed by 73.13% of NWSP and 89.66% of SESP samples. The MLMT analysis supported the idea of canine visceral leishmaniasis being introduced in the Northwest region of SP State by the traffic of humans and dogs from MS. In the southeast region of SP occurred an introduction of a new *L. infantum* genetic cluster. Probably the transmission was spread by traffic of infected dogs from other Brazilian regions, or by introduction of imported dogs from other countries. All these data together contributed to the detection of the genetic profile of *L. infantum* population in SP State.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Visceral leishmaniasis (VL) is an important infection caused by protozoan species classified in the *Leishmania donovani* complex which is transmitted by infected phlebotomine sand flies. VL is by far the most severe form of leishmaniasis and is often lethal if untreated (Desjeux, 2004; Dujardin, 2005). Its worldwide prevalence is estimated at 400,000–600,000 new cases per year. VL can be found in the Americas, Africa, Southern Europe, and Asia,

although most cases occur in India, Bangladesh, Sudan, Brazil, and Nepal (Stuart et al., 2008; WHO, 2012).

In Brazil, VL is caused by *L. infantum* (Lainson and Shaw, 1998; Alvar et al., 2012). Until the 1980s, the infection was an ancient endemic zoonotic disease considered restricted to rural areas, mainly in Northeast and Central-West Regions of the country (Deane and Deane, 1962; Alencar, 1983; MSB, 2012). Since then, a different epidemiological pattern has been observed, including cases reported in regions previously considered non-endemic such as the peri-urban and urban areas. Socioeconomic factors combined with severe periods of drought forcing a mass migration from rural endemic areas are among the main causes of the VL expansion distribution (Correa Antonialli et al., 2007). Presently, approximately 2500–5000 cases are reported per year in Brazil, and 10% of the

* Corresponding author. Address: Laboratório de Parasitologia, Instituto Adolfo Lutz, Av. Dr Arnaldo, 351 8 andar, CEP 01246-902, São Paulo, SP, Brazil. Tel.: +55 11 3068 2991; fax: +55 11 3068 2890.

E-mail address: pchioccola@gmail.com (V.L. Pereira-Chioccola).

population living in these endemic areas is at risk for acquiring the infection (Desjeux, 2001; MSB, 2012).

Since 1980, VL is highly endemic in the cities of Corumbá and Ladário in Mato Grosso do Sul State (MS). The disease reached the capital Campo Grande with the first autochthonous canine case in 2000 and the first autochthonous human case in 2002. In 1998 VL reached São Paulo (SP), when several cases were simultaneously reported in the northwestern region of the State, and in different municipalities of MS (Central-West Region of Brazil) (Correa Antonialli et al., 2007). Since the first autochthonous human cases were reported in Araçatuba, a municipality in the northwest of SP (1999), the infection has been steadily growing and spreading in this State (Costa et al., 2001). Currently, the parasite has been detected in more than 55 municipalities, with 10% mortality and the incidence in this State is approximately 600 cases per year (Camargo-Neves, 2004; Lindoso and Goto, 2006; Camargo-Neves, 2007; CVE, 2010). Since 2003, canine visceral leishmaniasis (CVL) has been detected in municipalities located in the southeastern region of SP (Camargo-Neves, 2004). These findings show the wide ranging dispersal of *L. infantum* through non-contiguous areas of SP (Camargo-Neves, 2007).

Different studies have shown genetic diversity between *L. infantum* strains, despite MON-1 zymodeme being the most prevalent in more than 30 countries worldwide, including Brazil and representing approximately 70% of all identified strains (Gallego et al., 2001; Gramiccia, 2003; Campino et al., 2006; Kuhls et al., 2011). Epidemiological studies on VL caused by *L. infantum* use techniques capable of differentiating MON-1 strains such as RAPD analyses (Bañuls et al., 1999; Hide et al., 2001), PCR-RFLP using cysteine proteinase B and *gp63* genes as target (Quispe Tintaya et al., 2004), RFLP analysis of minicircle kDNA (Cortes et al., 2006) or multilocus microsatellite typing (MLMT) (Bulle et al., 2002; Ochsenreither et al., 2006; Kuhls et al., 2008, 2011; Ferreira et al., 2012). Microsatellites are tandemly repeated stretches of short nucleotide motifs of 1–6 base pairs ubiquitously distributed in the genomes of eukaryotic organisms. Because of their high mutation rate, they can be used to develop species-specific marker sets. Sets of this kind of markers have been developed for the *L. donovani* complex (Jamjoom et al., 2004; Ochsenreither et al., 2006; Kuhls et al., 2007, 2008; Montoya et al., 2007). These studies have shown that molecular markers are suitable for population genetics and epidemiological studies (Bulle et al., 2002; Kuhls et al., 2007; Amro et al., 2009; Gelanew et al., 2010; Ferreira et al., 2012).

Despite the importance of VL for the Brazilian public health system, the genetic diversity of *L. infantum* was only recently accessed, with endemic areas that were poorly studied (Kuhls et al., 2011; Ferreira et al., 2012; Segatto et al., 2012). Thus, this study aimed to analyze genetic diversity of *L. infantum* population from SP State in order to collaborate with information about the possible origins of the parasite as well as, the introduction and spread of VL in this Brazilian State. The results corroborated with the possibility for performing MLMT directly in clinical samples without the isolation of the parasite (Bulle et al., 2002; Ochsenreither et al., 2006; Kuhls et al., 2007; Alam et al., 2009). The samples were collected in different municipalities and were analyzed by MLMT with a set of 17 microsatellite markers.

2. Material and methods

2.1. Epidemiological data, dog samples and parasite strains

Initially, 250 tissue samples of dogs with CVL were collected from Centers for Zoonosis Control in SP. The analysis of *L. infantum* polymorphism was performed in 112 of them whose were possible

to amplify the complete set of microsatellite markers. Epidemiological registers of different Centers for Zoonosis Control in SP were used to analyze dog data. All animals had strong clinical signs for VL (at least four signs) including exfoliative dermatitis, cutaneous ulcers and/or nodules, lymphadenopathy, splenomegaly, hepatomegaly, weight loss, lameness, anorexia, fever, abnormal nail growth, muscle wasting, or pale mucous membranes. The clinical samples included lymph node aspirates, liver or spleen necropsy fragments. They were collected in aseptic conditions, immediately added to tubes containing 1–2 ml of a sterile 0.85% NaCl solution with 200 µg/ml gentamicin, sent to the laboratory within 48 h, and immediately processed to confirm the clinical diagnosis.

The municipality of samples with the respective month and year of sample collection is listed in Table 1 and they were grouped by geographic origin. NW/SP group was composed of 67 DNA samples from 28 municipalities located in northwest region of SP. SESP group was composed of 29 DNA samples from 12 municipalities located in southeast regions of SP. Both groups were compared with a group that comprised 16 DNA samples collected from infected dogs in MS (Fig. 1A). The geographical localization of each region is shown in Fig. 1B. Clinical samples from dogs that came from or have visited other endemic areas were excluded from the study.

In order to check if culturing of parasites affects genetic profiles, DNA from a parasite culture isolated from dog lymph node aspirate and DNA extracted directly from this tissue were genotyped (samples 100 SESP and 1432 SESP). Promastigotes of these samples were grown at 25 °C in Eagle's medium, supplemented with 292 mg/l L-glutamine, 110 mg/l sodium pyruvate, 2.2 g/l sodium bicarbonate, 0.02% hemin, 10% heat-inactivated fetal calf serum and 200 mg/ml gentamicin (Gomes et al., 2007). In the log curve phase, 1×10^8 parasites were harvested, washed twice in phosphate-buffered saline (pH 7.2) at 1000g for 10 min. The parasite pellets were used for DNA extraction. The negative control comprised DNA samples from three non-infected dogs from non-endemic regions and the sample 100 SESP was used as positive control. This study was performed according to guidelines of the Sociedade Brasileira de Ciência em Animais de Laboratório/Colégio Brasileiro de Experimentação Animal (SBCAL/COBEA) and the institutional review board of the Ethics Committee of the Instituto Adolfo Lutz approved this study.

2.2. DNA extraction

The canine tissue samples and parasite pellets were dissolved in 10 mM Tris-HCl, pH 8.0; 10 mM EDTA; 0.5% SDS; 0.01% N-laurylsarcosyl, 100 µg/ml proteinase K and briefly vortex mixed and incubated at 56 °C until complete cell lyses. DNA molecules were extracted by QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions. DNA pellets were dissolved in ultra-pure water. DNA concentrations and purity were determined by the ratio of O. D. at 260 and 280 nm in a NanoDrop ND1000 (Thermo Scientific).

2.3. Species determination

Leishmania infantum infection in canine samples was confirmed by PCR with specific primers RV1 (5'CTTTTCTGGTCCCGGGTAGG 3') and RV2 (5'CCACCTGGCTATTTTACACCA3'). This primer set amplifies specifically a 145-bp fragment of the variable region of the minicircle kDNA of *L. donovani* complex (Le Fichoux et al., 1999; Lachaud et al., 2002). Reactions were carried out with Go Taq Green Master Mix (Promega) containing 1 µM of each primer and 5 µl of DNA in a final volume of 25 µl. Amplification conditions were made in one initial step of 95 °C for 5 min; 30 cycles of 30 s at

Table 1
Canine clinical samples genotyped in this study.

Sample code-sample collection	Group (region)	Municipality	State	Sample code-sample collection	Group (region)	Municipality	State	Sample code-sample collection	Group (region)	Municipality	State
41-05/2004	NWSP	Adamantina	SP	604-01/2008	NWSP	Mirandópolis	SP	132-04/2005	SESP	Cotia	SP
42-05/2004	NWSP	Adamantina	SP	605-01/2008	NWSP	Mirandópolis	SP	133-04/2005	SESP	Cotia	SP
43-05/2004	NWSP	Adamantina	SP	606-01/2008	NWSP	Mirandópolis	SP	62-07/2004	SESP	Espírito Santo do Pinhal	SP
46-05/2004	NWSP	Adamantina	SP	607-01/2008	NWSP	Mirandópolis	SP	63-07/2004	SESP	Espírito Santo do Pinhal	SP
168-07/2005	NWSP	Adamantina	SP	608-01/2008	NWSP	Mirandópolis	SP	513-10/2007	SESP	Itapeverica da Serra	SP
243-10/2005	NWSP	Adamantina	SP	611-01/2008	NWSP	Mirandópolis	SP	401-11/2006	SESP	Limeira	SP
2056-03/2011	NWSP	Aparecida d'Oeste	SP	612-01/2008	NWSP	Mirandópolis	SP	58-08/2004	SESP	Mogi das Cruzes	SP
3-03/2004	NWSP	Araçatuba	SP	613-01/2008	NWSP	Mirandópolis	SP	296-07/2006	SESP	Mogi das Cruzes	SP
4-03/2004	NWSP	Araçatuba	SP	614-01/2008	NWSP	Mirandópolis	SP	297-07/2006	SESP	Mogi das Cruzes	SP
471-06/2007	NWSP	Araçatuba	SP	616-01/2008	NWSP	Mirandópolis	SP	1834-03/2010	SESP	Piracicaba	SP
146-05/2005	NWSP	Bauru	SP	617-01/2008	NWSP	Mirandópolis	SP	500-09/2007	SESP	Piraju	SP
753-04/2008	NWSP	Bauru	SP	620-01/2008	NWSP	Mirandópolis	SP	2167-11/2011	SESP	Salto	SP
1004-07/2008	NWSP	Bauru	SP	441-04/2007	NWSP	Monte Alto	SP	2168-11/2011	SESP	Salto	SP
1005-07/2008	NWSP	Bauru	SP	941-07/2008	NWSP	Morro Agudo	SP	376-10/2006	SESP	Salesópolis	SP
1009-07/2008	NWSP	Bauru	SP	59-08/2004	NWSP	Penápolis	SP	1101-08/2008	SESP	São Pedro	SP
1010-08/2008	NWSP	Bauru	SP	2295-12/2011	NWSP	Pirapozinho	SP	1148-10/2008	SESP	São Pedro	SP
1011-08/2008	NWSP	Bauru	SP	202-08/2005	NWSP	Pirajuí	SP	^a 1432-05/2009	SESP	São Pedro	SP
1013-08/2008	NWSP	Bauru	SP	2077-05/2011	NWSP	Reginópolis	SP	676-03/2008	SESP	Suzano	SP
516-11/2007	NWSP	Borborema	SP	1436-05/2009	NWSP	Ribeirão Preto	SP	489-08/2007	SESP	Valinhos	SP
38-05/2004	NWSP	Brauna	SP	2021-12/2010	NWSP	Ribeirão Preto	SP	2060-03/2011	SESP	Valinhos	SP
406-11/2006	NWSP	Cafelandia	SP	2079-05/2011	NWSP	Ribeirão Preto	SP	1-06/2008	MS	Campo Grande	MS
409-11/2006	NWSP	Cafelandia	SP	1899-08/2010	NWSP	Rubineia	SP	11-06/2008	MS	Campo Grande	MS
410-11/2006	NWSP	Cafelandia	SP	2110-07/2011	NWSP	São José do Rio Preto	SP	13-06/2008	MS	Campo Grande	MS
134-04/2005	NWSP	Dracena	SP	2279-11/2011	NWSP	São José do Rio Preto	SP	23-06/2008	MS	Campo Grande	MS
201-08/2005	NWSP	Flora Rica	SP	1632-09/2009	NWSP	Santa Salete	SP	24-06/2008	MS	Campo Grande	MS
939-07/2008	NWSP	Franca	SP	631-01/2008	NWSP	Presidente Prudente	SP	26-06/2008	MS	Campo Grande	MS
445-04/2007	NWSP	Inubia Paulista	SP	289-06/2006	NWSP	Tupi Paulista	SP	27-06/2008	MS	Campo Grande	MS
1117-09/2008	NWSP	Jales	SP	1154-11/2008	NWSP	Urania	SP	39-06/2008	MS	Campo Grande	MS
91-07/2012	NWSP	Lucélia	SP	1155-11/2008	NWSP	Urania	SP	46-08/2008	MS	Campo Grande	MS
69-08/2004	NWSP	Marília	SP	1947-10/2010	SESP	Campinas	SP	55-08/2008	MS	Campo Grande	MS
220-09/2005	NWSP	Marília	SP	1966-11/2010	SESP	Campinas	SP	59-08/2008	MS	Campo Grande	MS
95r-02/2008	NWSP	Mirandópolis	SP	2003-12/2010	SESP	Campinas	SP	65-08/2008	MS	Campo Grande	MS
549-01/2008	NWSP	Mirandópolis	SP	2004-12/2010	SESP	Campinas	SP	67-08/2008	MS	Campo Grande	MS
550-01/2008	NWSP	Mirandópolis	SP	2031-01/2011	SESP	Campinas	SP	102-10/2008	MS	Campo Grande	MS
551-01/2008	NWSP	Mirandópolis	SP	2032-01/2011	SESP	Campinas	SP	109-10/2008	MS	Campo Grande	MS
552-01/2008	NWSP	Mirandópolis	SP	2046-02/2011	SESP	Campinas	SP	117-10/2008	MS	Campo Grande	MS
554-01/2008	NWSP	Mirandópolis	SP	2104-06/2011	SESP	Campinas	SP				
555-01/2008	NWSP	Mirandópolis	SP	^a 100-03/2005	SESP	Cotia	SP				

The dogs with CVL were from Mato Grosso do Sul (MS) State, and different municipalities of northwest (NWSP) and southeast (SESP) regions of São Paulo (SP) State.

^a Samples that also have culture of parasites.

95 °C, 30 s at 60 °C, 30 s at 72 °C; and a final step of 5 min at 72 °C. PCR products were electrophoresed in 2% agarose gel, stained with ethidium bromide and visualized under UV illumination. Considering that as far as we know *L. infantum* is the only species from the complex *L. donovani* that circulates in Brazil, we assumed all positive samples as infection by *L. infantum*.

2.4. Microsatellite markers, probes and PCR

The genetic polymorphism within Brazilian *L. infantum* parasites were analyzed using 17 microsatellite markers described in previous studies (Rossi et al., 1994; Jamjoom et al., 2002; Ochsneither et al., 2006; Kuhls et al., 2007) and are listed in

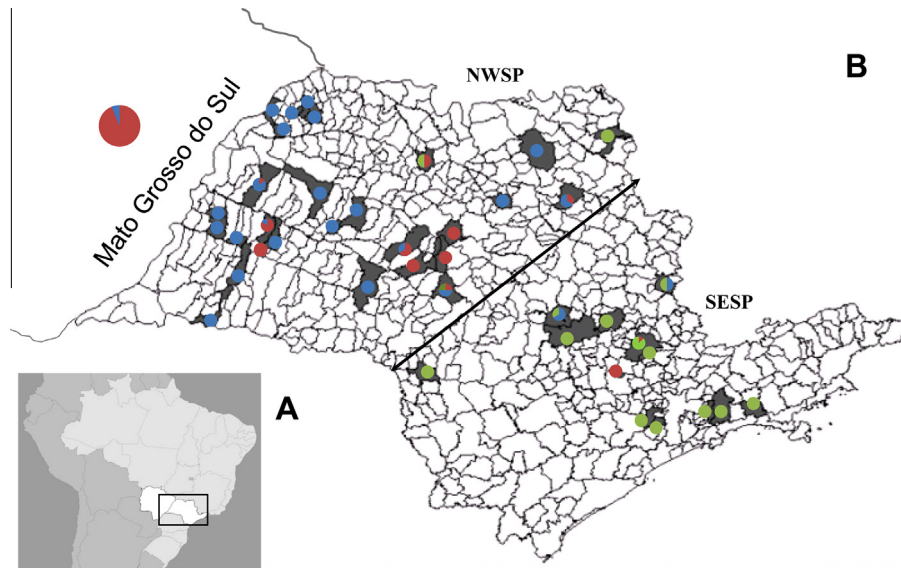


Fig. 1. Geographical distribution of the *L. infantum* genetic clusters POP-B (red), and subclusters subPOP-A1 (blue) and subPOP-A2 (green). Map of Brazil indicating location of Mato Grosso do Sul and São Paulo States (A). Map of São Paulo State indicating the studied municipalities (grey) located in Northwest (NWSP) and in Southeast (SESP) regions. The arrow divides both regions (B). Pie-charts show the proportion of each cluster in the respective region analyzed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Descriptive information and statistics of the 17 microsatellite markers analyzed.

Marker	Primer sequence (5'-3')	Multiplex	Dye labeling/ [*] μM	Repeat array	Allele size array (bp)
Li71-5/2	F GCACGGTCGGCATTGTGTA R GATAAACGAGATGGCCGC	1	6-FAM/0.4	9 (CA)	136
Li72-14	F AGAGTGTCTGCGCGTGAGTA R AAGAAAAGAAGGTGCAGCGA	2	TAMRA/0.5	10 (TG)	110
LIST7023	F CTTTGCCTTCCGCACTAA R GCCTTGTTCCTGTGTGTT	1	6-FAM/0.4	5 (CA)	167
LIST7040	F GCAGAGCGAGACACACAGAC R GTGCACGTTGATGTGCTTCT	1	HEX/0.4	10 (GT)	236
LIST7029	F GCAGAGCTTCTGCTGGATT R GCATTGTCTTCTCATCCAC	2	HEX/0.4	17 (GT)	195
Li71-19	F CAAACCGGTGTTCTGCTTTT R CGGACATTGGCAACACACT	2	TAMRA/0.5	15 (CA)	126
LIST7039	F CTCGCACTTCTTCGCTCTTT R GAGACGAGAGGAACGGAAAA	1	6-FAM/0.4	15 (CA)	220
Li71-33	F CTCCTTTACACCCGCTCT R GAGAGAAGACGAGCCGAAGT	1	HEX/0.3	11–15 (TG)	127–135
LIST7022	F GTCGCTGTCTCTGTGTGTC R TCCGCAATTTCTCTCTCT	2	HEX/0.5	15–16 (CA)	218–220
LIST7028	F CACTCCACTCGGTGGATA R CTTTGACCCCGTTCTTT	2	6-FAM/0.3	11–12 (GT)	169–171
Li71-7	F GCTGCAGCAGATGAGAAGG R GTGAGAAGGCAGGGATTCAA	1	HEX/0.3	12–13 (AC)	156–158
LIST7030	F TCTCTGCACGTCTGTGTGTG R TCTTCCTGAAGGGCGATG	1	HEX/0.4	3–10 (GT)	183–197
ISA136	F CAAAGATGGTGAAGCAGGCG R GTTGTGGTGAGCATCAGTAG	2	HEX/0.4	17–19 (GT)	246–250
Li45-24	F GCGCTACAGGCATAAAGGA R CTGGCGCATCAACGGTGT	1	6-FAM/0.4	12–16 (CA)	108–116
LIST7032	F CTAGAGGCGTGGGATGTA R TCGCAGTTTTCCGTCCA	2	6-FAM/0.3	8–17 (CA)	186–204
ST436	F CCTCACTGCCACGATAAC R GACAGGGAGCAAGTAAGG	2	HEX/0.4	8–16 (GT)	108–124
Lm4TA	F TTTGCCACACATACACTTAG R GTAGACGACATCGCGAGCAC	1	6-FAM/0.4	11–16 (TA)	91–101

^{*} Primer concentration in PCR.

Table 2. To reduce the final cost of reactions, the forward primer of each marker was synthesized with a tail in the 5' extremity complementary to a probe labeled with 6-FAM (5'-GTAAACGACGGCCAGT3'), HEX (5'-AGGGTTTTCCAGTCACGA3') or TAMRA (5'-CAGGAAACAGCTATGA3'). The reactions were carried

out in two multiplex PCRs, due to the large number of markers analyzed (Table 2). Table 2 also shows the optimal PCR conditions for each microsatellite marker as the respective primer concentration, the dye used for the subsequent labeling reaction as well as the repeat array and allele-size range.

Table 3
Allelic polymorphism of the 17 microsatellites markers analyzed for *Leishmania infantum* samples from MS, NWSP and SESP.

Markers	A	MS	NWSP	SESP
Li71-5/2	1	9 (1)	9 (1)	9 (1)
Li72-14	1	10 (1)	10 (1)	10 (1)
LIST7023	1	5 (1)	5 (1)	5 (1)
LIST7040	1	10 (1)	10 (1)	10 (1)
LIST7029	1	17 (1)	17 (1)	17 (1)
Li71-19	1	15 (1)	15 (1)	15 (1)
LIST7039	1	15 (1)	9 (1)	9 (1)
Li71-33	2	11 (1)	11 (0.993) 15^b (0.007)	11 (1)
LIST7022	2	15 (1)	15 (1)	15 (0.793) 16^c (0.207)
LIST7028	2	11 (1)	11 (0.993) 12^b (0.007)	11 (1)
Li71-7	2	13 (1)	13 (1)	12^c (0.052) 13 (0.948)
LIST7030	2	3^a (0.25) 10 (0.75)	10 (1)	10 (1)
ISA136	3	18 (0.938) 19^a (0.062)	17^b (0.015) 18 (0.985)	18 (1)
Li45-24	3	15 (1)	12^b (0.007) 15 (0.978) 16^b (0.015)	15 (1)
LIST7032	3	11 (0.5) 17 (0.5)	8 (0.007) 11 (0.522) 17 (0.471)	8 (0.034) 11 (0.517) 17 (0.449)
ST436	4	8 (0.875) 13^a (0.125)	8 (0.985) 10^b (0.007) 16^b (0.007)	8 (1)
Lm4TA	6	11 (0.03) 12 (0.813) 13 (0.063) 14 (0.094)	11 (0.007) 12 (0.254) 13 (0.06) 14 (0.336) 15 (0.276) 16^b (0.067)	12 (0.086) 13 (0.621) 14 (0.259) 15 (0.034)

Number of repeats are presented in bold and the frequency of alleles in the parenthesis; A, number of alleles per locus.

^a Private alleles from MS.

^b Private alleles from NWSP.

^c Private alleles from SESP.

The multiplex PCR was performed in a final volume of 10 μ l. Each reaction was composed by 1 μ l of the DNA template, primers (which concentrations are described in Table 2), 1.5 mM of MgCl₂, 0.2 mM of dNTP and 1 unit of *Taq* DNA polymerase (Invitrogen). Amplifications were made in a Veriti (Applied Biosystems, Foster City, CA/US) thermocycler with initial step of 95 °C for 3 min; 30 cycles of 45 s at 95 °C, 45 s at 60 °C and 60 s at 72 °C. Next, a labeled probe (6-FAM, HEX or TAMRA) complementary to 5' extremity was added in a concentration of 0.18 μ M for the respective microsatellite marker. The reaction conditions were 12 cycles of 45 s at 95 °C; 45 s at 60 °C; 60 s at 72 °C, followed by an extension step of 30 min at 72 °C. Two separate runs of multiplex 1 and 2 were made. PCR products (1 μ l) were mixed with formamide and a GENESCAN-500 ROX size standard (Applied Biosystems); and were separated by capillary electrophoresis through a POP7 denaturing polymer in an ABI3130 Genetic Analyzer and analyzed on GeneMapper ID v3.2 software (Applied Biosystems, Foster City, CA, USA). Reference strain (MHOM/BR/1974/PP75) was only included on late runs with 100 SESP sample but only six loci previously analyzed (Kuhls et al., 2011; Ferreira et al., 2012) could be standardized for number of repeats.

The number of repeats of the 11 different markers was calculated in an empirical way, based on the sequence obtained for each microsatellite marker of cultured parasites from samples 100 SESP and 1432 SESP and was used to set up a binset. Therefore, the number of repeats analyzed on GeneMapper can be used just for the internal sample set of this work because a reference strain was not used in all the runs.

2.5. Data analysis

Multilocus genotype data consist of the number of repeats in each microsatellite marker for each *L. infantum* infected sample analyzed. The calculations of allelic frequencies were made with the program PowerStats, v12 (<http://www.promega.com/geneticidtools/powerstats/Default.htm>). Expected (H_e , gene diversity) and observed heterozygosity (H_o) as well as Inbreeding coefficient (F_{IS}) and mean number of alleles were estimated using the Genetic Data Analysis software (Lewis and Zaykin, 2001). According to the Hardy–Weinberg equilibrium, values of $H_e = 0$ or $H_o < H_e$ indicate homozygosity (Tibayrenc et al., 1991; De Meeüs et al., 2007). F_{IS} is an estimate of the inbreeding of individuals resulting from the

deviation from panmixia (De Meeüs et al., 2006). Values of F_{IS} may vary between -1 and 1 . Negative values correspond to an excess of heterozygotes and positive values correspond to a lack of heterozygotes. Value 1 means that all strains are homozygous and value of zero is expected under panmixia. Population structure was analyzed by the STRUCTURE software, which applies a Bayesian model-based clustering approach (Pritchard et al., 2000). This algorithm identified genetically distinct clusters based on allelic frequencies. A series of 10 runs was performed for each K value between 1 and 10 . The Markov chain Monte Carlo consisted of a burn-in period of 100,000 iterations and runs of 1,000,000 replications. Assumptions of correlated allele frequencies and admixture model for assignment of individuals were taken in the parameter setting. The most probable number of clusters was defined as suggested in the software manual by combining the analyses of the mean $\ln \Pr(X|K)$ and the calculation of ΔK , which is based on the rate of change in the log probability of data between successive values of K (Evanno et al., 2005) using the Structure Harvester v0.6.92 (Earl and vonHoldt, 2012). The software CLUMPP 1.1.2 (Jakobsson and Rosenberg, 2007) was used to align the Q values for the probable number of clusters. Phylogenetic analysis was based on genetic Chord-distance of Cavalli-Sforza and Edwards – Dc – (Cavalli-Sforza and Edwards, 1967) calculated with the software POPULATIONS 1.2.32 (available at <http://bioinformatics.org/~tryphon/populations/> accessed on June 14th, 2012). This Dc-distance follows the infinitive allele model (IAM). POPULATIONS software was also used to construct a Neighbor-joining tree, which was visualized with MEGA 5 software (Tamura et al., 2011). The Neighbor-Net (N-net), which is a phylogenetic network, was constructed in the software SplitsTree 4.12.3 (Huson and Bryant, 2006). The N-net provides good visualization of the data when it presents complex evolutionary steps or reticulate relationship among lineages (Huson and Bryant, 2006). Genetic differentiation and gene flow was assessed by calculating F_{ST} (theta) values (Weir and Cockerham, 1984) with the corresponding p -values using the MSA software (Dieringer and Schlotterer, 2003). F_{ST} is an estimate of genetic structure between populations (De Meeüs et al., 2006). Values may vary between 0 and 1 , where zero represents no genetic structure or differentiation and one represents complete differentiation (Wright, 1978). F_{ST} values can be interpreted following these guidelines: $F_{ST} < 0.05$ indicates little genetic differentiation, F_{ST} between 0.05 and 0.15 indicates moderate ge-

netic differentiation, F_{ST} between 0.15 and 0.25 indicates great genetic differentiation and $F_{ST} > 0.25$ indicates very great genetic differentiation (Wright, 1978).

3. Results

Dog samples were selected based on positive PCR results using the RV1–RV2 marker together with clinical and epidemiological data. DNA samples were collected from dogs with CVL in the Brazilian municipalities listed in Table 1 and Fig. 1. We analyzed 250 dog DNA samples that were positive for RV1–RV2, but only 112 (45%) of them were totally genotyped for the 17 microsatellite

markers. The other 138 samples were excluded, since PCR products were not amplified in all microsatellite markers.

As expected, no amplification was detected in DNA extracted from dogs without CVL (negative control) and PCR products were obtained for all markers in the positive control. The genotyping tests with DNAs obtained from the same dog (clinical sample and cultured parasite) resulted in the same profiles for samples 100 *SESP* and 1432 *SESP*. These results confirm the possibility of using DNA extracted directly from infected host to analyze *Leishmania* polymorphism by microsatellite markers without previous isolation of the parasite (Bulle et al., 2002; Ochsenreither et al., 2006; Kuhls et al., 2007; Alam et al., 2009).

All genotyped samples showed one or two peaks in the electropherogram suggesting that there is no occurrence of aneuploidy. Allele frequencies were low in most of the markers, but a considerable number of alleles (1–6 alleles) were observed. Lm4TA marker was the most polymorphic and informative, since it showed 6 alleles. On the other hand, 7 markers (Li71-5/2, Li72-14, LIST7023, LIST7040, LIST7029, Li71-19 and LIST7039) were monomorphic. Li71-33, LIST7022, LIST7028, Li71-7 and LIST7030 markers showed 2 alleles each. ISA136, Li45-24, LIST7032 showed 3 alleles and ST436 4 alleles. In spite of the low frequency values, some private alleles could be detected. Six polymorphic microsatellite markers showed private alleles in *NWSP*. They were: Li71-33 (allele 15), LIST7028 (allele 12), ISA136 (allele 17), Li45-24 (alleles 12 and 16), ST436 (alleles 10 and 16) and Lm4TA (allele 16). Three polymorphic microsatellite markers showed private alleles in *MS* samples. The markers were ISA136 (allele 19), ST436 (allele 13), and LIST7030 (allele 3). Only two polymorphic microsatellite markers showed private alleles in *SESP* samples. The markers were LIST7022 (allele 16) and Li71-7 (allele 18). *MS* and *NWSP* groups shared 21 alleles, which represent, 87.5% and 67.74% of the total number of alleles, respectively. *NWSP* and *SESP* shared 22 alleles, which represent 70.96% and 91.66% of the total number of alleles, respectively. *MS* and *SESP* shared 20 alleles, which correspond to 83.33% for both (Table 3), of the total number of alleles of each group, respectively.

Table 4 shows the variability measures of the 17 microsatellite loci, the observed and expected heterozygosity (H_o and H_e) as well as, the inbreeding coefficient (F_{IS}). The F_{IS} values for the 17 markers ranged from -0.9444 to 1. H_o ranged from 0 to 0.9722 and H_e ranged from 0 to 0.6961. All markers but one indicated a depletion of heterozygotes. The exception was LIST7032 marker, which revealed an excess of heterozygotes ($H_e < H_o$) corroborated by negative F_{IS} .

MLMT profiles of the 112 *L. infantum* presented 33 genotypes. Twenty of them are unique. The description of each genotype and the respective cluster are shown in Table 5.

As inferred by mean $\ln \Pr(X|K)$ and ΔK calculations, the sample set studied comprised two genetic clusters (Fig. 2A and B, respectively). Following STRUCTURE analysis, F_{ST} values and previous insights on population structure in Brazil (Ferreira et al., 2012) the 112 samples were split into two genetic clusters (Fig. 2C).

The first genetic cluster, POP-A, comprises 49 (from 67) samples from *NWSP*, 26 (29) from *SESP* and only one from *MS*. The second, POP-B, was composed by 36 samples and consists in most parasites of *MS* (15/16), 18 from *NWSP* and three from *SESP*. This genetic cluster structure was confirmed by distance analysis and *F*-statistics. *F*-statistics was used to estimate the genetic structure among the two genetic clusters identified by STRUCTURE. The F_{ST} values indicated very great genetic differentiation between POP-A and POP-B ($F_{ST} = 0.3258$; $p < 0.05$).

Even though the data set was considered to be composed by two clusters, analyses of samples in other *K* values revealed constant clusters. When *K* was considered to be three, which was considered the second possibility that could be inferred by mean \ln

Table 4

Descriptive statistics of the 17 microsatellite markers in *L. infantum* populations obtained from STRUCTURE analysis.

Locus	Cluster ID	A	H_e	H_o	F_{IS}
Li71-5/2	POP-A	1	0	0	0
	POP-B	1	0	0	0
	Mean	1	0	0	0
Li72-14	POP-A	1	0	0	0
	POP-B	1	0	0	0
	Mean	1	0	0	0
LIST7023	POP-A	1	0	0	0
	POP-B	1	0	0	0
	Mean	1	0	0	0
LIST7040	POP-A	1	0	0	0
	POP-B	1	0	0	0
	Mean	1	0	0	0
LIST7029	POP-A	1	0	0	0
	POP-B	1	0	0	0
	Mean	1	0	0	0
Li71-19	POP-A	1	0	0	0
	POP-B	1	0	0	0
	Mean	1	0	0	0
LIST7039	POP-A	1	0	0	0
	POP-B	1	0	0	0
	Mean	1	0	0	0
Li71-33	POP-A	2	0.0132	0.0132	0
	POP-B	1	0	0	0
	Mean	2	0.0066	0.0066	0
LIST7022	POP-A	2	0.1464	0	1
	POP-B	1	0	0	0
	Mean	2	0.0732	0.0000	1
LIST7028	POP-A	2	0.0132	0.0132	0
	POP-B	1	0	0	0
	Mean	2	0.0066	0.0066	0
Li71-7	POP-A	2	0.0261	0	1
	POP-B	2	0.0278	0.0278	0
	Mean	2	0.027	0.0139	0.4902
LIST7030	POP-A	1	0	0	0
	POP-B	2	0.2003	0.1111	0.4488
	Mean	2	0.1002	0.0556	0.4488
ISA136	POP-A	1	0	0	0
	POP-B	3	0.108	0	1
	Mean	2	0.054	0	1
Li45-24	POP-A	2	0.0261	0	1
	POP-B	2	0.0278	0.0278	0
	Mean	2	0.027	0.0139	0.4902
LIST7032	POP-A	3	0.5208	0.9605	-0.8547
	POP-B	2	0.5067	0.9722	-0.9444
	Mean	3	0.5137	0.9664	-0.8984
ST436	POP-A	3	0.0262	0.0263	-0.0033
	POP-B	2	0.1064	0	1
	Mean	3	0.0663	0.0132	0.8015
Lm4TA	POP-A	5	0.6961	0.5132	0.2641
	POP-B	4	0.1823	0.1944	-0.0675
	Mean	5	0.4392	0.3538	0.1962
Overall	POP-A	2	0.0864	0.0898	-0.0399
	POP-B	2	0.0682	0.0784	-0.1527
	Mean	2	0.0773	0.0841	-0.0892

A, the number of alleles per locus; H_e , the expected heterozygosity; H_o , the observed heterozygosity; F_{IS} , the inbreeding coefficient.

Pr(X|K) and ΔK calculations one of the clusters remain the same as POP-B (Fig. 2D), and the other two were considered as subclusters of POP-A (named subPOP-A1 and subPOP-A2; Fig. 2D). The subPOP-A1 is composed by one sample from MS, three samples from SESP and 45 from NWSP. The subPOP-A2 comprises 23 samples from SESP and only four from NWSP (Fig. 2D). The mean number of alleles for both subclusters was two and seven markers (Li71-33, LIST7022, Li7028, Li71-7, Li45-24, ST436 and Lm4TA) differed these subclusters.

Some private alleles for each genetic cluster could be detected. Five markers showed private alleles in the subcluster subPOP-A1: Li45-24 (allele 16), Li71-33 (allele 15), Lm4TA (alleles 15 and 16), Li7028 (allele 12) and ST436 (alleles 10 and 16). In subPOP-A2 just the marker LIST7022 presented a private allele (allele 16). In the cluster POP-B, it could be observed private alleles in five markers:

LIST7030 (allele 3), Li45-24 (allele 12), Lm4TA (allele 12), ISA136 (alleles 17 and 19) and ST436 (allele 13).

A Neighbor-joining tree inferred from Dc-distance matrix presents the same genetic cluster pattern as STRUCTURE (Fig. 3). The Dc-distance matrix generated by POPULATIONS software was used to generate a N-net on SplitsTree software. The N-net shows two main clusters corresponding to POP-A (subPOP-A1 and subPOP-A2) and POP-B obtained by STRUCTURE (Fig. 4).

The clusters of subPOP-A1 and subPOP-A2 observed on STRUCTURE are not distant defined on the Neighbor-joining tree (Fig. 3) and N-net (Fig. 4) which is concordant with low but significant F_{ST} ($F_{ST} = 0.0460$; $p = 0.0001$).

Table 4 shows the descriptive analyses per cluster. The H_o values per locus within the clusters ranged from 0 to 0.9722, and H_e ranged from 0 to 0.6961.

Table 5

Assignment of strains and genetic clusters to each genotype.

Genotype	Type	<i>L. infantum</i> samples	Genetic cluster
1	TYPE 1	102 MS, 65 MS	POP-B
2	TYPE 2	109 MS, 117 MS	POP-B
3	TYPE 3	11 MS, 13 MS, 1 MS, 39 MS, 59 MS, 41 NWSP, 43 NWSP, 46 NWSP, 243 NWSP, 1011 NWSP, 146 NWSP, 516 NWSP, 410 NWSP, 91 NWSP, 550 NWSP, 614 NWSP, 2077 NWSP, 2079 NWSP, 2167 SESP, 2168 SESP	POP-B
4	TYPE 4	23 MS, 406 NWSP, 445 NWSP, 551 NWSP, 552 NWSP, 554 NWSP, 555 NWSP, 607 NWSP, 612 NWSP, 613 NWSP, 620 NWSP, 95r NWSP, 1632 NWSP	POP-A
5	TYPE 5	24 MS, 67 MS	POP-B
6	TYPE 6	3 NWSP, 4 NWSP, 471 NWSP, 1005 NWSP, 941 NWSP, 289 NWSP, 1899 NWSP	POP-A
7	TYPE 7	1004 NWSP, 500 SESP, 513 SESP, 1101 SESP, 489 SESP, 2279 NWSP	POP-A
8	TYPE 8	1009 NWSP, 134 NWSP, 631 NWSP	POP-A
9	TYPE 9	1010 NWSP, 38 NWSP, 1117 NWSP, 69 NWSP, 220 NWSP, 549 NWSP, 604 NWSP, 608 NWSP, 59 NWSP, 1154 NWSP, 63 SESP, 1432 SESP, 2021 NWSP, 2295 NWSP	POP-A
10	TYPE 10	1013 NWSP, 939 NWSP, 100 SESP, 132 SESP, 133 SESP, 62 SESP, 401 SESP, 58 SESP, 297 SESP, 676 SESP, 1834 SESP, 1947 SESP, 2060 SESP	POP-A
11	TYPE 11	409 NWSP, 2110 NWSP	POP-B
12	TYPE 12	616 NWSP, 1155 NWSP, 2056 NWSP	POP-A
13	TYPE 13	1966 SESP, 2003 SESP, 2031 SESP, 2032 SESP, 2046 SESP	POP-A
14	201	201 NWSP	POP-A
15	NWSP	605 NWSP	POP-B
16	606	606 NWSP	POP-A
17	611	611 NWSP	POP-A
18	617	617 NWSP	POP-A
19	441	441 NWSP	POP-A
20	202	202 NWSP	POP-B
21	296	296 SESP	POP-A
22	376	376 SESP	
23	1148	1148 SESP	POP-A
24	1436	1436 NWSP	POP-A
25	2004	2004 SESP	POP-B
26	2104	2104 SESP	POP-A
27	26 MS	26 MS	POP-B
28	27 MS	27 MS	POP-B
29	46 MS	46 MS	POP-B
30	55 MS	55 MS	POP-B
31	42	42 NWSP	POP-B
32	168	168 NWSP	POP-A
33	753	753 NWSP	POP-A

4. Discussion

One of the most important risk factors in the increase of VL worldwide has been the migration of people from endemic regions (WHO, 2012). MLMT has shown to be a preferential method for *Leishmania* genotyping for species and strains, and an excellent tool for epidemiological studies (Botilde et al., 2006; Ochsenreither et al., 2006; Schonian et al., 2011). This methodology has been used to analyze population structure within *Leishmania* species complexes indicating genetic differentiation between populations (Kuhls et al., 2008, 2011).

This study investigated the population structure of *L. infantum* parasites, through the microsatellite loci profiles, from different municipalities of SP and one from MS. These areas are endemic

for VL and the canine infection occurrence has been growing and spreading rapidly in different SP regions (Lindoso and Goto, 2006).

Although, clinical samples have a small amount of parasites when compared with culture isolates, 45% of them were genotyped for all 17 loci. According to the results, this study demonstrated the possibility of performing MLMT using small amounts of *Leishmania* DNA from host tissues. Both, DNA extracted from *Leishmania* culture and DNA extracted from the lymph node aspirates obtained from the same dog presented the same profiles in MLMT analysis. Thus, these data suggest the possibility of genotyping *Leishmania* populations with the analysis of DNA extracted directly from clinical samples. This information is important since in a large number of laboratories there are no conditions to isolate and culture parasites from clinical samples.

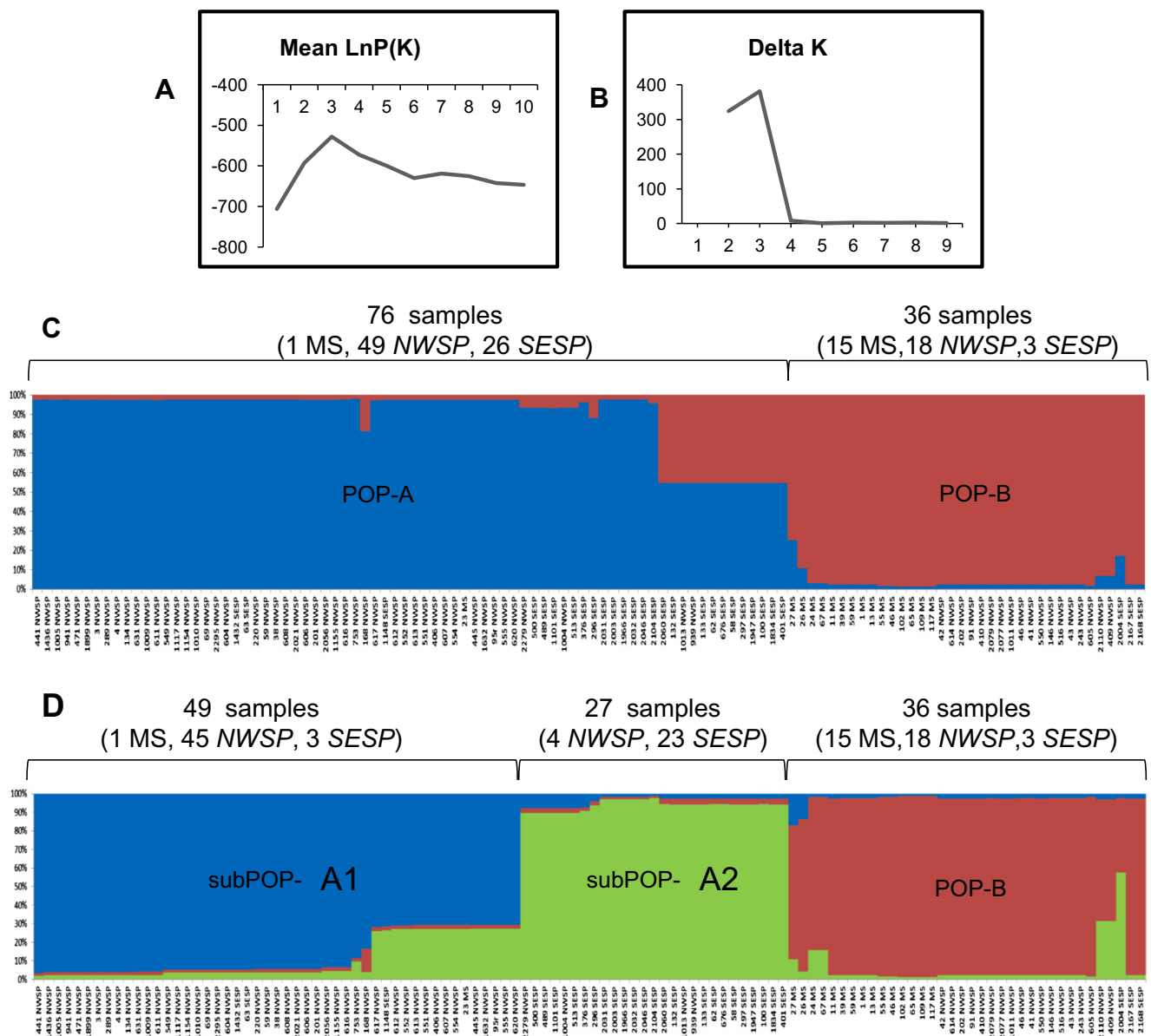


Fig. 2. Estimated population structure of *L. infantum* from Brazil (São Paulo and Mato Grosso do Sul States) obtained on the STRUCTURE program. Results are based on MLMT of 17 microsatellite markers of the 112 *L. infantum* samples. According to the $\ln Pr(X|K)$ graph (2A) and the ΔK (2B), the most probable number of genetic clusters is two. Based on previous insights, two main genetic clusters were defined corresponding to POP-A (blue) and POP-B (red) (2C). POP-A presented a low substructure observed between subPOP-A1 (blue) and subPOP-A2 (green) (2D). In the barplots each sample is represented by a single vertical line divided into K colors, where K is the number of populations assumed. Each color represents one cluster, and the length of the colors segment shows the estimated proportion of membership in that cluster. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

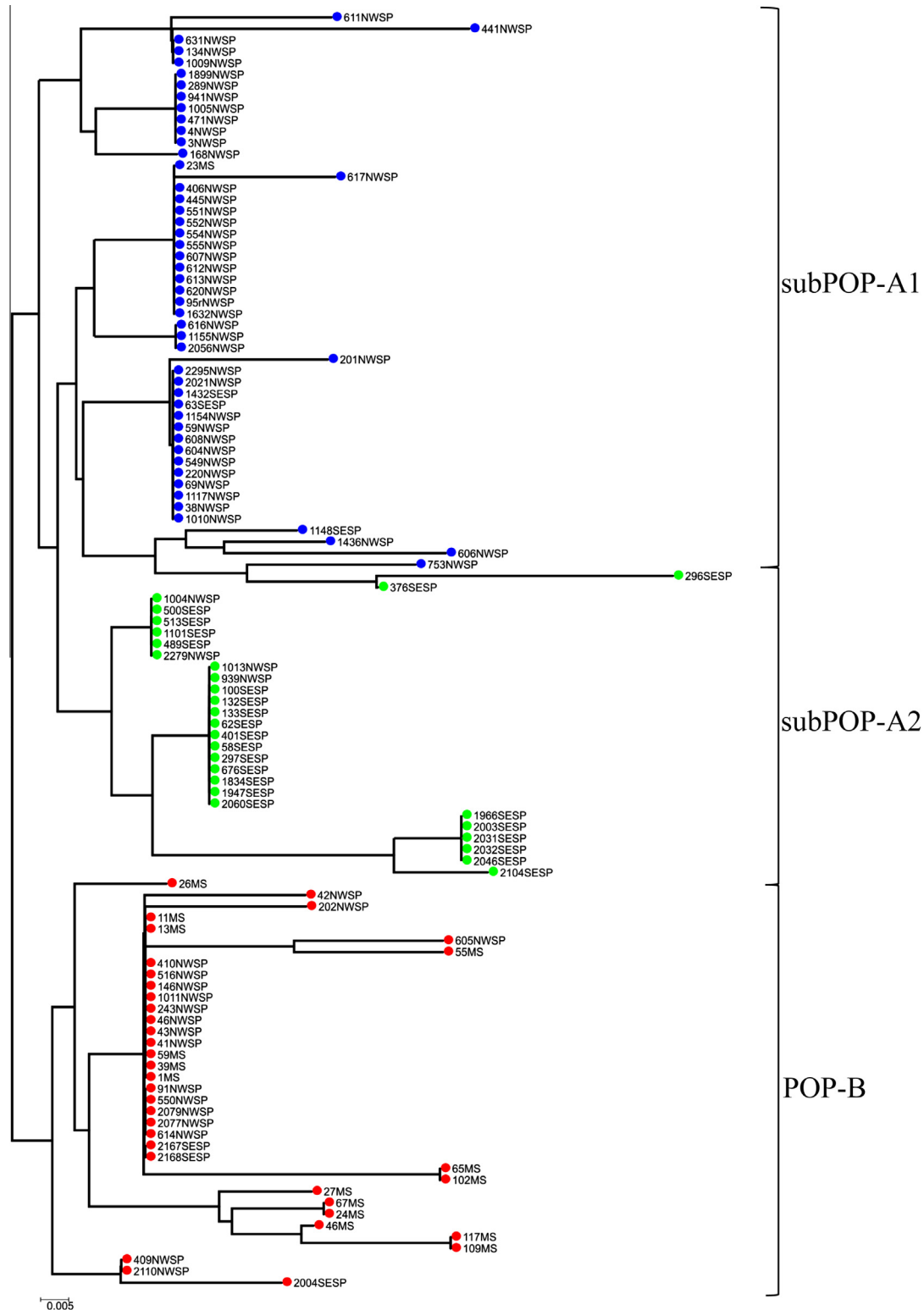


Fig. 3. Neighbor-joining tree inferred from Dc-distances calculated for the data of 17 microsatellite markers for the *L. infantum* samples from subPOP-A1 (blue), subPOP-A2 (green) and POP-B (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

According to F_{IS} , H_o and H_e values, the majority of the microsatellite markers indicated that most samples are homozygous. A high level of heterozygotes was observed only in the marker LIST7032. These data could suggest a low frequency of mating in these *L. infantum* populations and deficiency of heterozygotes. Although the reproduction of *Leishmania* remains controversial (Akopyants

et al., 2009; Miles et al., 2009; Rougeron et al., 2009), these results are incompatible with clonal populations, which should exhibit negative values of F_{IS} (Balloux et al., 2003; Rougeron et al., 2009). The heterozygote deficiency found in the studied samples could result from a population division such as the Wahlund effect. Similar patterns were shown in other Brazilian *L. infantum* (Kuhls et al.,

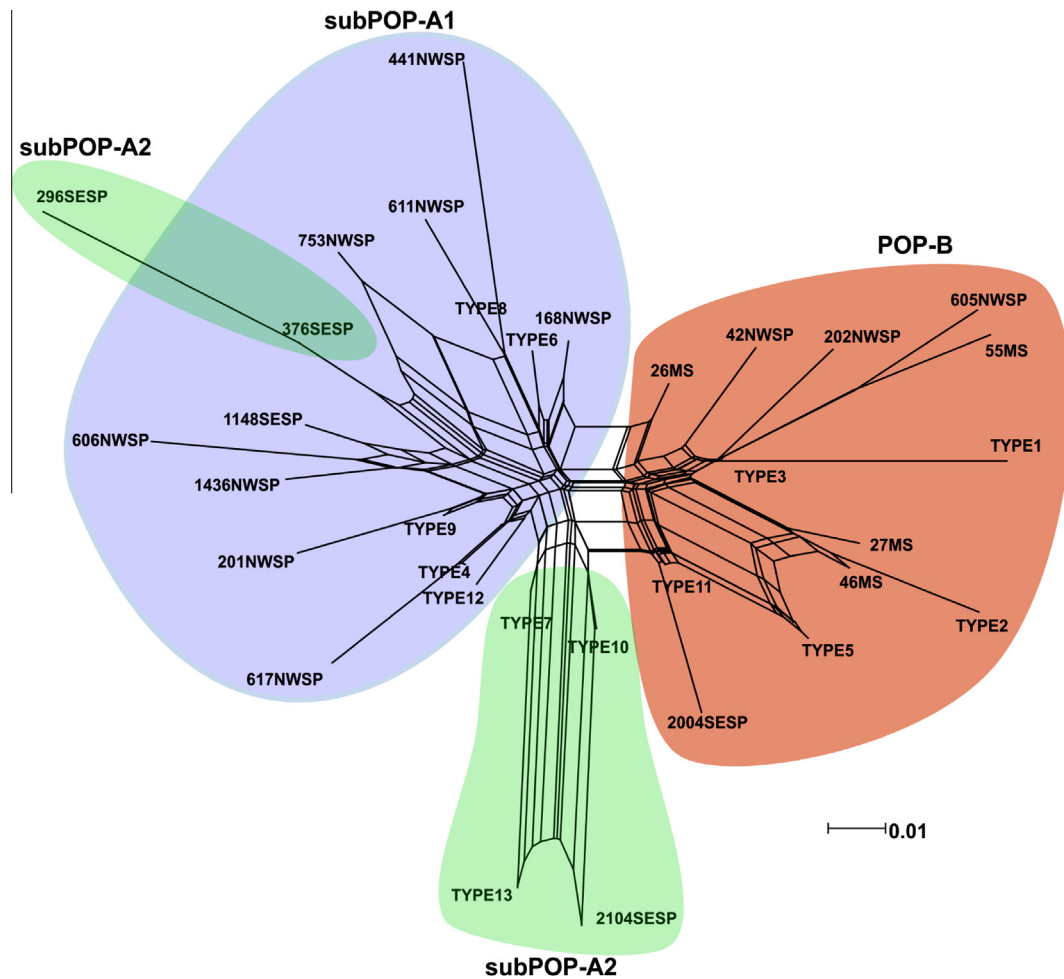


Fig. 4. Neighbor-net inferred with SplitsTree software using Dc-distances among 33 *L. infantum* genotypes from the MLMT profiles of 17 microsatellite markers. Blue spot represents genotypes from subPOP-A1, green from subPOP-A2 and red from POP-B. Genotypes with multiple samples are described in Table 5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2011; Ferreira et al., 2012) and *L. braziliensis* (Rougeron et al., 2009) populations.

Despite of the fact that MLMT analysis defined 33 different genotypes, a low polymorphism was detected within the parasites studied. The low degree of polymorphism in Brazilian *L. infantum* strains has been previously shown (Kuhls et al., 2011; Ferreira et al., 2012). As shown by *F*-statistics, the genetic variation seems well structured between POP-A and POP-B.

The majority (93.75%) of MS parasite genotypes belonged to POP-B, with just one sample (6.25%) found in POP-A. POP-B also comprised 10.34% of *SESP* and 26.87% of *NWSP* samples. Besides one sample from MS, POP-A is composed by 73.13% of *NWSP* and 89.66% of *SESP* samples. The presence of 26.87% of the *NWSP* samples in POP-B might be an effect of the proximity between both regions, MS and *NWSP* (Table 5). These data suggest that samples from POP-B in *NWSP* might be a result of expansion and dissemination of *L. infantum* from MS corroborating a previous study (Correa Antonialli et al., 2007). The expansion of VL through MS started at the same time and direction, from west to east as the Bolivia–Brazil gas pipeline construction route. This project demanded migration of workers that are considered a very important risk factor for the dissemination of the disease (Correa Antonialli et al., 2007).

The data from MS, *NWSP* and *SESP* were submitted to a clustering analysis on STRUCTURE for the six coincident loci together with genotypes from many endemic areas in Brazil and Paraguay (Ferreira et al., 2012). Even using only six loci it was possible to see quite

similar clusters. POP-A clustered with strains from POP1 (the widespread population; see Ferreira et al., 2012) while POP-B was in the same cluster of strains from POP3 (the main cluster in MS; Ferreira et al., 2012) (data not shown).

Once POP-B, which seems to represent the POP3 from Ferreira et al. (2012), was found in the Northwest region of SP where *Lutzomyia cruzi* was not registered, the persistence and dispersion of this parasite cluster may not be dependent on this secondary vector. On the other hand, it supports the idea of west–east dispersion of VL in MS and SP states (Correa Antonialli et al., 2007; Ferreira et al., 2012). An atypical situation has been seen in the *SESP* region where the incidence of the canine infection has increased rapidly in the recent years, despite no occurrence of the natural vector, *Lutzomyia longipalpis* in some municipalities such as Cotia, Mogi das Cruzes, Embu das Artes, Itapeccerica da Serra and Suzano (CVE, 2010, 2011). These data suggest that the subcluster subPOP-A2 could be related to a different transmission cycle or could have spread in the *SESP* region from other route than *NWSP*. In fact, previous analysis of four *L. infantum* strains from Embu das Artes has shown that they do not belong to the most common population in MS (Ferreira et al., 2012) which is in agreement with the hypothesis of more than one introduction route of VL in SP. Probably, immigration of infected dogs from other Brazilian endemic areas or from other endemic countries has an important role once they frequently come to these regions, whether for exhibitions, for marketing or even following human immigration. Thus, it is possible

that these dogs have been infected by another *Leishmania* population and subsequently spread the infection in the region.

Our results confirm the existence of genetic structure of *L. infantum* in Brazil (Ferreira et al., 2012). Furthermore, our results corroborated the possibility of genotyping *L. infantum* directly from clinical samples (Bulle et al., 2002; Ochsenreither et al., 2006; Kuhls et al., 2007; Alam et al., 2009). Our data also supports the hypothesis of a previous study (Correa Antonialli et al., 2007), where they suggest that VL was disseminated due to Bolivia–Brazil gas pipeline construction, that demanded intense migration of workers. According to this hypothesis, VL might be introduced in NWSP region of SP State by the traffic of humans and dogs from MS, which is adjacent to SP. In the southeast region another *L. infantum* population was also introduced. Probably the transmission was spread by traffic of infected dogs from other Brazilian regions, or by introduction of imported dogs from other countries. Collectively, these data contributed to the detection of the genetic profile of *L. infantum* populations in SP State.

Acknowledgments

We wish to thank: Dra. Suely Aparecida Correa Antonialli (Laboratório Central de Saúde/Secretaria de Estado da Saúde de Mato Grosso do Sul) and Dr. Jose Eduardo Tolezano (Centro de Parasitologia e Micologia, Instituto Adolfo Lutz), who kindly provided the DNA samples from Mato Grosso do Sul. Jim Hesson of Academic English Solutions for editing the English. This study was supported by grants from FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil. (Proc 2011/13939-8) and Genoa Biotecnologia SA.

References

- Akopyants, N.S., Kimblin, N., Secundino, N., Patrick, R., Peters, N., Lawyer, P., Dobson, D.E., Beverley, S.M., Sacks, D., 2009. Demonstration of genetic exchange during cyclical development of *Leishmania* in the sand fly vector. *Science* 324, 265–268.
- Alam, M.Z., Kuhls, K., Sundar, S., Rijal, S., Shamsuzzaman, A.K., Raju, B.V., Raju, B.V., Salotra, P., Dujardin, J.C., Schönian, G., 2009. Multilocus microsatellite typing (MLMT) reveals genetic homogeneity of *Leishmania donovani* strains in the Indian subcontinent. *Infect. Genet. Evol.* 9, 24–31.
- Alencar, J.E., 1983. Expansão do Calazar no Brasil. *Ceará Méd.* 5, 86–102.
- Alvar, J., Vélez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M., the WHO Leishmaniasis Control Team, 2012. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* 7 (5), e35671.
- Amro, A., Schonian, G., Al-Sharabati, M.B., Azmi, K., Nasereddin, A., Abdeen, Z., Schnur, L.F., Baneth, G., Jaffe, C.L., Kuhls, K., 2009. Population genetics of *Leishmania infantum* in Israel and the Palestinian authority through microsatellite analysis. *Microbes Infect.* 11, 484–492.
- Balhou, F., Lehmann, L., Meeüs, T., 2003. The population genetics of clonal and partially clonal diploids. *Genetics* 164, 1635–1644.
- Bañuls, A.L., Hide, M., Tibayrenc, M., 1999. Molecular epidemiology and evolutionary genetics of *Leishmania* parasites. *Int. J. Parasitol.* 29, 1137–1147.
- Botilde, Y., Laurent, T., Quispe Tintaya, W., Chicharro, C., Canavate, C., Cruz, I., Kuhls, K., Schonian, G., Dujardin, J.C., 2006. Comparison of molecular markers for strain typing of *Leishmania infantum*. *Infect. Genet. Evol.* 6, 440–446.
- Bulle, B., Millon, L., Bart, J.M., Gállego, M., Gambarelli, F., Portús, M., Schnur, L., Jaffe, C.L., Fernandez-Barredo, S., Alunda, J.M., Piarroux, R., 2002. Practical approach for typing strains of *Leishmania infantum* by microsatellite analysis. *J. Clin. Microbiol.* 40, 3391–3397.
- Camargo-Neves, V.L.F., 2004. A leishmaniose visceral Americana no Estado de São Paulo. *Bol. Epidemiol. Paul.* [serial on the internet] 2007 [cited 2011 January]; 6 Available from <http://www.cve.saude.sp.gov.br/agencia/bepa6_lva.htm>.
- Camargo-Neves, V.L.F., Grupo de Estudos em Leishmanioses. Classificação epidemiológica dos municípios para a leishmaniose visceral Americana. *Bol. Epidemiol. Paul.* [serial on the internet] 2007 [cited 2011 January]; 4, pp. 27–39. Available from: <http://www.cve.saude.sp.gov.br/agencia/bepa39_lva.htm>.
- Campino, L., Pratlong, F., Abranches, P., Rioux, J.A., Santos-Gomes, G., Alves-Pires, C., Cortes, S., Ramada, J., Cristovão, J.M., Afonso, M.O., Dedet, J.P., 2006. Leishmaniasis in Portugal: enzyme polymorphism of *Leishmania infantum* based on the identification of 213 strains. *Trop. Med. Int. Health* 11, 1708–1714.
- Cavalli-Sforza, L.L., Edwards, A.W., 1967. Phylogenetic analysis. Models and estimation procedures. *Am. J. Hum. Genet.* 19, 233–257.
- Correa Antonialli, S.A., Torres, T.G., Paranhos Filho, A.C., Tolezano, J.E., 2007. Spatial analysis of American visceral leishmaniasis in Mato Grosso do Sul State, central Brazil. *J. Infect.* 54, 509–514.
- Cortes, S., Mauricio, I., Almeida, A., Cristovão, J.M., Pratlong, F., Dedet, J.P., Campino, L., 2006. Application of kDNA as a molecular marker to analyze *Leishmania infantum* diversity in Portugal. *Parasitol. Int.* 55, 277–283.
- Costa, C.H.N., Gomes, A.C.G., Costa, J.M.L., Vieira, J.B.F., Lima, J.W.O., Dietz, R., 2001. Changes in the control program of visceral leishmaniasis in Brazil. *Rev. Soc. Bras. Med. Trop.* 34, 223–228.
- CVE – Centro de Vigilância Epidemiológica do Estado de São Paulo: Comitê de Leishmaniose Visceral Americana. Secretaria de Estado da Saúde. São Paulo SP, Brasil. 2010 Epidemiologic classification of municipalities according to the American Visceral Leishmaniasis Surveillance and Control Program in the State of São Paulo, updated in May, 2010. Bepa: 7 (77). Available from: <http://www.cve.saude.sp.gov.br/agencia/bepa77_lva.htm>.
- CVE – Centro de Vigilância Epidemiológica do Estado de São Paulo: Comitê de Leishmaniose Visceral Americana. Secretaria de Estado da Saúde. São Paulo SP, Brasil. 2011 Bepa Boletim Epidemiológico Paulista, vol. 7, (96). Available: <<http://www.cve.saude.sp.gov.br>>.
- Deane, L.M., Deane, M.P., 1962. Visceral leishmaniasis in Brazil: geographical distribution and transmission. *Rev. Inst. Med. Trop.* 4, 198–212.
- De Meeüs, T., Lehmann, L., Balhou, F., 2006. Molecular epidemiology of clonal diploids: a quick overview and a short DIY (do it yourself) notice. *Infect. Genet. Evol.* 6, 163.
- De Meeüs, T., McCoy, K.D., Prugnolle, F., Chevillon, C., Durand, P., Hurtrez-Boussès, S., Renaud, F., 2007. Population genetics and molecular epidemiology or how to “débusher la bête”. *Infect. Genet. Evol.* 7, 308–332.
- Desjeux, P., 2001. Worldwide The increase in risk factors for leishmaniasis. *Trans. R. Soc. Trop. Med. Hyg.* 95, 239–243.
- Desjeux, P., 2004. Leishmaniasis: current situation and new perspectives. *Comp. Immunol. Microbiol. Infect. Dis.* 27, 305–318.
- Dieringer, D., Schlotterer, C., 2003. Microsatellite analyzer (MSA): a platform independent analysis tool for large microsatellite data sets. *Mol. Ecol. Notes* 3, 167–169.
- Dujardin, J.C., 2005. Risk factors in the spread of leishmaniasis: towards integrated monitoring? *Trends Parasitol.* 22, 4–6.
- Earl, D.A., vonHoldt, B.M., 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Resour.* [serial on the internet] [cited 2011 June] 4(2), pp. 359–361. Available from: <http://taylor0.biology.ucla.edu/structureHarvester/>.
- 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.
- Ferreira, G.E., dos Santos, B.N., Dorval, M.E., Ramos, T.P., Porrozzi, R., Peixoto, A.A., Cupolillo, E., 2012. The genetic structure of *Leishmania infantum* populations in Brazil and its possible association with the transmission cycle of visceral leishmaniasis. *PLoS One* 7, e36242.
- Gállego, M., Pratlong, F., Fisa, R., Riera, C., Rioux, J.A., Dedet, J.P., Portús, M., 2001. The life cycle of *Leishmania infantum* MON-77 in the Priorat (Catalonia, Spain) involves humans, dogs and sand flies; also literature review of distribution and hosts of *L. infantum* zymodemes in the old world. *Trans. R. Soc. Trop. Med. Hyg.* 95, 269–271.
- Gelanew, T., Kuhls, K., Hurissa, Z., Weldegebreal, T., Hailu, W., Kassahun, A., Abebe, T., Hailu, A., Schonian, G., 2010. Inference of population structure of *Leishmania donovani* strains isolated from different Ethiopian visceral leishmaniasis endemic areas. *PLoS Negl. Trop. Dis.* 4, e889.
- Gomes, A.H., Ferreira, I.M., Lima, M.L., Cunha, E.A., Garcia, A.S., Araujo, M.F., Pereira-Chioccola, V.L., 2007. PCR identification of *Leishmania* in diagnosis and control of canine leishmaniasis. *Vet. Parasitol.* 144, 234–241.
- Gramiccia, M., 2003. The identification and variability of the parasite causing leishmaniasis in HIV-positive patients in Italy. *Ann. Trop. Med. Parasitol.* 97 (1), 65–73.
- Hide, M., Bañuls, A.L., Tibayrenc, M., 2001. Genetic heterogeneity and phylogenetic status of *Leishmania (Leishmania) infantum* zymodeme MON-1: epidemiological implications. *Parasitology* 123, 425–432.
- Huson, D.H., Bryant, D., 2006. Application of phylogenetic networks in evolutionary studies. *Mol. Biol. Evol.* 23, 254–267.
- Jakobsson, M., Rosenberg, N.A., 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* 23, 1801–1806.
- Jamjoom, M.B., Ashford, R.W., Bates, P.A., Kemp, S.J., Noyes, H.A., 2002. Towards a standard battery of microsatellite markers for the analysis of the *Leishmania donovani* complex. *Ann. Trop. Med. Parasitol.* 96, 265–270.
- Jamjoom, M.B., Ashford, R.W., Bates, P.A., Chance, M.L., Kemp, S.J., Watts, P.C., Noyes, H.A., 2004. *Leishmania donovani* is the only cause of visceral leishmaniasis in East Africa; previous descriptions of *L. infantum* and “*L. archibaldi*” from this region are a consequence of convergent evolution in the isoenzyme data. *Parasitology* 129, 399–409.
- Kuhls, K., Keilonat, L., Ochsenreither, S., Schaar, M., Schweynoch, C., Presber, W., Schönian, G., 2007. Multilocus microsatellite typing (MLMT) reveals genetically isolated populations between and within the main endemic regions of visceral leishmaniasis. *Microbes Infect.* 9, 334–343.
- Kuhls, K., Chicharro, C., Canavate, C., Cortes, S., Campino, L., Haralambous, C., Soteriadou, K., Pratlong, F., Dedet, J.P., Mauricio, I., Miles, M., Schaar, M., Ochsenreither, S., Radtke, O.A., Schonian, G., 2008. Differentiation and gene flow among European populations of *Leishmania infantum* MON-1. *PLoS Negl. Trop. Dis.* 2, e261.
- Kuhls, K., Alam, M.Z., Cupolillo, E., Ferreira, G.E., Mauricio, I.L., Oddone, R., Feliciangeli, M.D., Wirth, T., Miles, M.A., Schönian, G., 2011. Comparative

- microsatellite typing of new world *Leishmania infantum* reveals low heterogeneity among populations and its recent old world origin. *PLoS Negl. Trop. Dis.* 5, e1155.
- Lainson, R., Shaw, J.J., 1998. New world leishmaniasis. The neotropical Leishmaniasis species. In: Collier, L., Balows, A., Sussman, M. (Eds.), *Topley and Wilson's Microbiology and Microbial Infectious Diseases*, ninth ed. vol. 5, London, Arnold, pp. 241–266.
- Lachaud, L., Marchegui-Hammami, S., Chabbert, E., Dereure, J., Dedet, J.P., Bastien, P., 2002. Comparison of six PCR methods using peripheral blood for detection of canine visceral leishmaniasis. *J. Clin. Microbiol.* 40, 210–215.
- Le Fichoux, Y., Quaranta, J.F., Aufeuve, J.P., Lelièvre, A., Marty, P., Suffia, I., Rousseau, D., Kubar, J., 1999. Occurrence of *Leishmania infantum* parasitemia in asymptomatic blood donors living in an area of endemicity in southern France. *J. Clin. Microbiol.* 37, 1953–1957.
- Lewis, P.O., Zaykin, D., 2001. Genetic data analysis: computer program for the analysis of allelic data. Version 1.0 (d16c). Free program distributed by the authors over the internet. Available from <http://lewis.eeb.uconn.edu/lewis/home/software.html>.
- Lindoso, J.A.L., Goto, H., 2006. Leishmaniose visceral: situação atual e perspectivas futuras. *BolEpidem Paul [serial on the internet]*. [cited 2011 January] 26, pp. 7–11. Available from: http://www.cve.saude.sp.gov.br/agencia/bepa26_lva.htm.
- Miles, M.A., Yeo, M., Mauricio, I.L., 2009. Genetics: *Leishmania* exploit sex. *Science* 324, 187–189.
- Montoya, L., Gallego, M., Gavignet, B., Piarroux, R., Rioux, J.A., Portus, M., Fisa, R., 2007. Application of microsatellite genotyping to the study of a restricted *Leishmania infantum* focus: different genotype compositions in isolates from dogs and sand flies. *Am. J. Trop. Med. Hyg.* 76, 888–895.
- MSB (Ministério da Saúde do Brasil). Manual de vigilância e controle da leishmaniose visceral. [cited 2012 April] Available from: <http://portal.saude.gov.br/portal/svs>.
- Ochsenreither, S., Kuhls, K., Schaar, M., Presber, W., Schönian, G., 2006. Multilocus microsatellite typing as a new tool for discrimination of *Leishmania infantum* MON-1 strains. *J. Clin. Microbiol.* 44, 495–503.
- Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of population structure using multilocus genotype data. *Genetics* 155, 945–959.
- Quispe Tintaya, K.W., Jing, X., Dedet, J.P., Rijal, S., De Bolle, X., Dujardin, J.C., 2004. Antigen genes for molecular epidemiology of leishmaniasis: polymorphism of cysteine proteinase B and surface metalloprotease glycoprotein 63 in the *Leishmania donovani* complex. *J. Infect. Dis.* 189, 1035–1043.
- Rossi, V., Wincker, P., Ravel, C., Blaineau, C., Pagés, M., Bastien, P., 1994. Structural organisation of microsatellite families in the *Leishmania* genome and polymorphisms at two (CA)_n loci. *Mol. Biochem. Parasitol.* 65, 271–282.
- Rougeron, V., de Meeûs, T., Hide, M., Waleckx, E., Bermudez, H., Arevalo, J., Llanos-Cuentas, A., Dujardin, J.C., De Doncker, S., Le Ray, D., Ayala, F.J., Bañuls, A.L., 2009. Extreme inbreeding in *Leishmania braziliensis*. *Proc. Natl. Acad. Sci. USA* 106, 10224–10229.
- Schonian, G., Kuhls, K., Mauricio, I.L., 2011. Molecular approaches for a better understanding of the epidemiology and population genetics of *Leishmania*. *Parasitology* 38 (4), 405–425.
- Segatto, M., Ribeiro, L.S., Costa, D.L., Costa, C.H., Oliveira, M.R., Carvalho, S.F., Macedo, A.M., Valadares, H.M., Dietze, R., Brito, C.F., Lemos, E.M., 2012. Genetic diversity of *Leishmania infantum* field populations from Brazil. *Mem. Inst. Oswaldo Cruz* 107, 39–47.
- Stuart, K., Brun, R., Croft, S., Fairlamb, A., Gurtler, R.E., McKerrow, J., Reed, S., Tarleton, R., 2008. Kinetoplastids: related protozoan pathogens, different diseases. *J. Clin. Invest.* 118, 1301–1310.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739.
- Tibayrenc, M., Kjellberg, F., Arnaud, J., Oury, B., Brenière, S.F., Dardé, M.L., Ayala, F.J., 1991. Are eukaryotic microorganisms clonal or sexual? A population genetics vantage. *Proc. Natl. Acad. Sci. USA* 88, 5129–5133.
- Weir, B.S., Cockerham, C.C., 1984. Estimating F statistics for the analysis of population structure. *Evolution* 38, 1358–1370.
- Wright, S., 1978. *Evolution and the Genetics of Populations. Variability within and Among Natural Populations*. University of Chicago Press, Chicago.
- WHO (World Health Organization). [cited 2012 april] Available from: <http://www.who.int/leishmaniasis/en/>.