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

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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.

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

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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 *gp*63 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. *NWSP* 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 1000*g* 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-laurilsarcozyl, 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′CTTTTCTGGTCCCGCGGGTAGG 3′) and RV2 (5′CCACCTGGCCTATTTTACACCA3′). 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*	Itapecerica 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*	Reginópólis	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*	Rubinéia	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; Ochsenreither et al., 2006; Kuhls et al., 2007) and are listed in

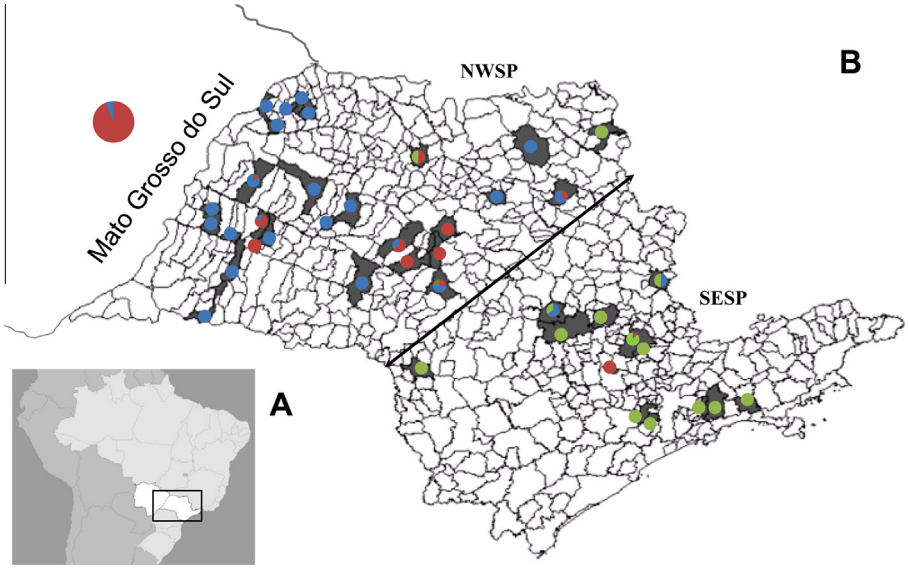

Fig. 1. Geographical distribution of the *L. infantum* genetic clusters POP-B (red), and subclusters subPOPA1 (blue) and subPOP-A2 (green). Map of Brazil indicating location of Mato Grosso do Sul and São Paulo States (A). Map of São Paulo State indicating the studied municipalities (grey) located in 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 GCACGGTCGGCATTTGTA 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 CTTTGCGTTGCGCACTAA R GCTTGTGTTCCGTGTGTGTT	1	6-FAM/0.4	5 (CA)	167
LIST7040	F GCAGAGCGAGACACACAGAC R GTGCACGTTGATGTGCTTCT	1	HEX/0.4	10 (GT)	236
LIST7029	F GCAGAGCTTCTGCTTGGATT R GCATTGCTGTTCTCATCCAC	2	HEX/0.4	17 (GT)	195
Li71-19	F CAAACCGGTGTTCTGCTTTT R CGGACATTGGCAACACACT	2	TAMRA/0.5	15 (CA)	126
LIST7039	F CTCGCACTCTTTCGCTCTTT R GAGACGAGAGGAACGGAAAA	1	6-FAM/0.4	15 (CA)	220
Li71-33	F CTCCTTTCACACCGCCTCT R GAGAGAAGACGAGCCGAAGT	1	HEX/0.3	11–15 (TG)	127–135
LIST7022	F GTCGCTCTGTCTCTGTGTGC R TCCGCATTTTCCTCTCCTT	2	HEX/0.5	15–16 (CA)	218–220
LIST7028	F CACTCCACTGCGTTGGATA R CTTTGACCGCCGTTCTTT	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 GCGCCTACAGGCATAAAGGA R CTGGCGCATCAACGGTGT	1	6-FAM/0.4	12–16 (CA)	108–116
LIST7032	F CTAGAGGCGTGCGGATGTA R TCGCAGTTTTCGGTCCA	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 TTTGCCACACACATACACTTAG 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′GTAAAACGACGGCCAGT3′), HEX (5′AGGGTTTTCCCAGTCACGA3′) 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 μl. Each reaction was composed by 1 μl of the DNA template, primers (which concentrations are described in Table 2), 1.5 mM of $MgCl_2$, 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 μM 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 μl) 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 Meeûs et al., 2007). F_{IS} is an estimate of the inbreeding of individuals resulting from the deviation from panmixia (De Meeûs 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 Meeûs 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.

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.

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

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 sub-POP-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 (allele15), 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	*L. infantum* samples	Genetic cluster
1	TYPE1	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 *NWSP*	201 *NWSP*	POP-A
15	605 *NWSP*	605 *NWSP*	POP-B
16	606 *NWSP*	606 *NWSP*	POP-A
17	611 *NWSP*	611 *NWSP*	POP-A
18	617 *NWSP*	617 *NWSP*	POP-A
19	441 *NWSP*	441 *NWSP*	POP-A
20	202 *NWSP*	202 *NWSP*	POP-B
21	296 *SESP*	296 *SESP*	POP-A
22	376 *SESP*	376 *SESP*	
23	1148 *SESP*	1148 *SESP*	POP-A
24	1436 *NWSP*	1436 *NWSP*	POP-A
25	2004 *SESP*	2004 *SESP*	POP-B
26	2104 *SESP*	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 *NWSP*	42 *NWSP*	POP-B
32	168 *NWSP*	168 *NWSP*	POP-A
33	753 *NWSP*	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.

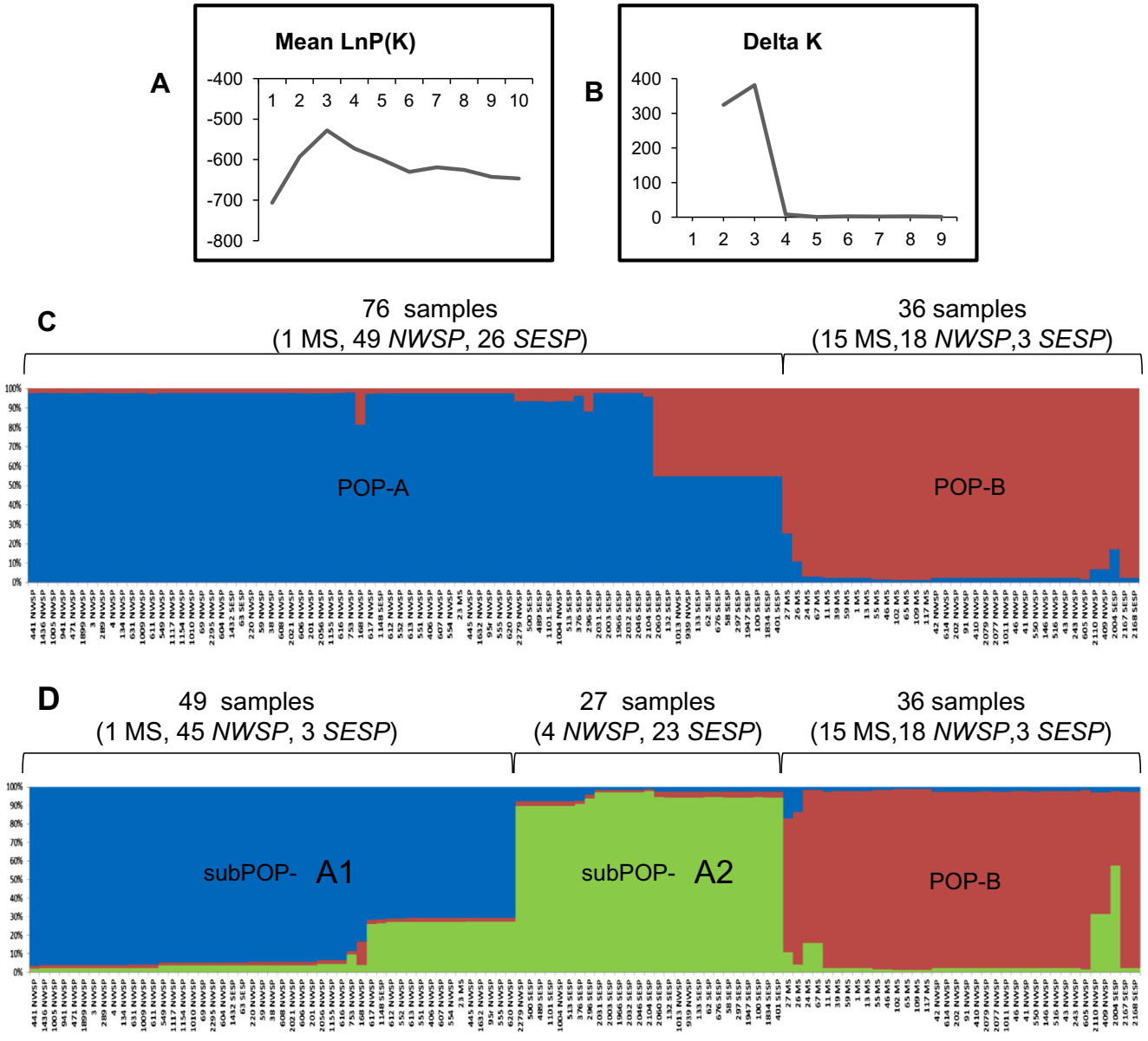

Fig. 2. Estimated population structure of *L. infantum* from Brazil (São Paulo and Mato Grosso do Sul States) obtained on the STRUCTURE program. Results are based on MLMT of 17 microsatellite markers of the 112 *L. infantum* samples. According to the ln Pr(*X*|*K*) graph (2A) and the Δ*K* (2B), the most 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.)

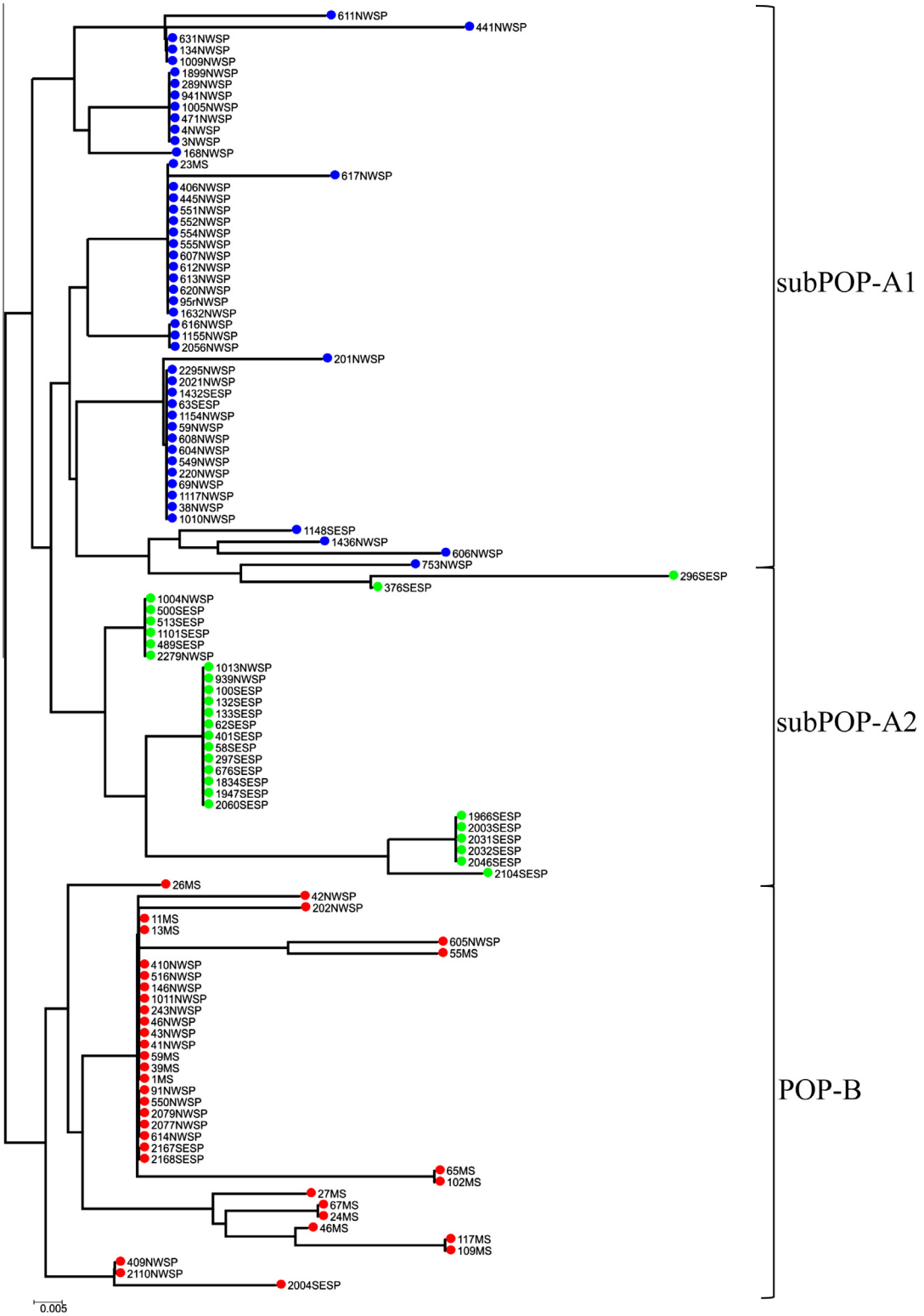

Fig. 3. Neighbor-joining tree inferred from Dc-distances calculated for the data of 17 microsatellite markers for the *L. infantum* samples from subPOP-A1 (blue), subPOP-A2 (green) and POP-B (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web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

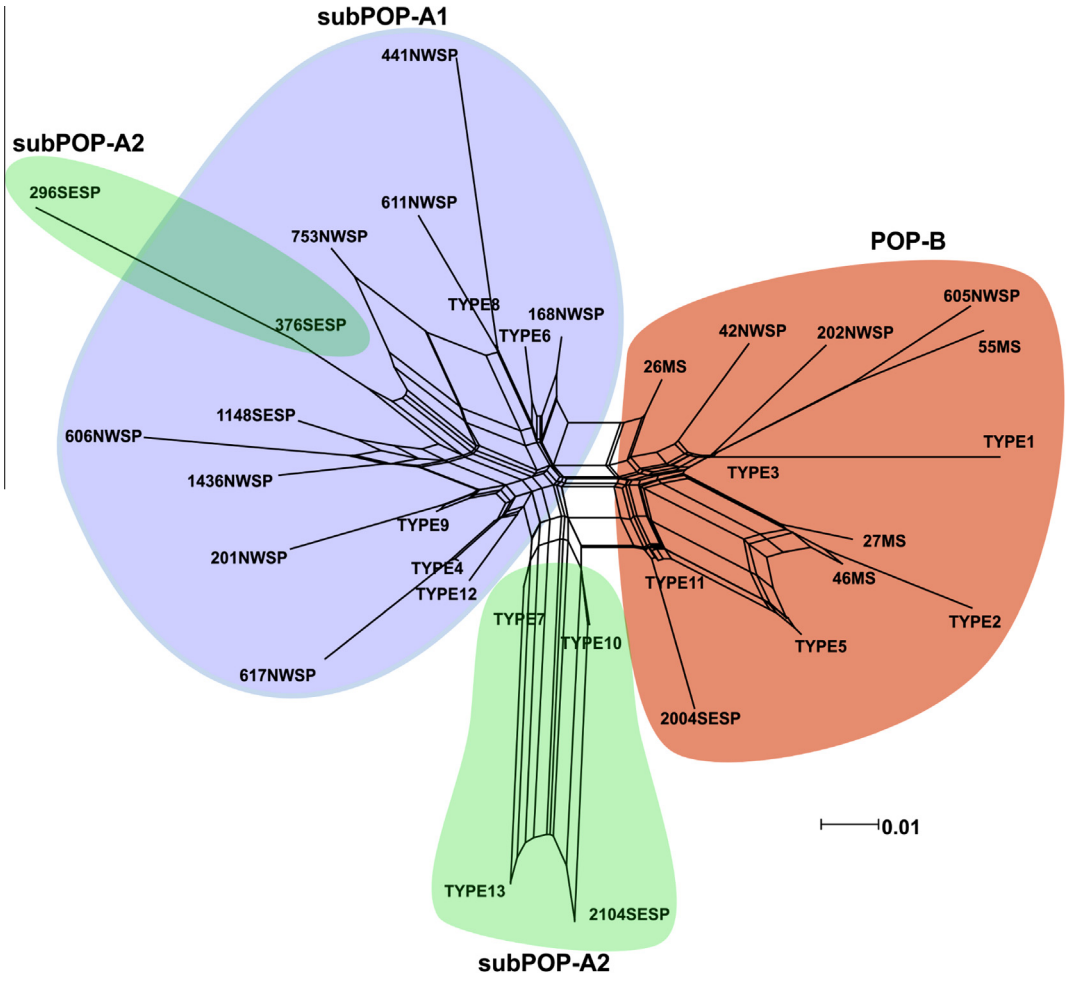

Fig. 4. Neighbor-net inferred with SplitsTree software using Dc-distances among 33 *L. infantum* genotypes from the MLMT profiles of 17 microsatellite markers. Blue spot represents genotypes from subPOP-A1, green from subPOP-A2 and red from POP-B. Genotypes with multiple samples are described in Table 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, Itapecerica 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.

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