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Genetic diversity evaluation on Portuguese *Leishmania infantum* strains by multilocus microsatellite typing

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ABSTRACT

Leishmania infantum is the main etiological agent of zoonotic visceral leishmaniasis in the Mediterranean region, including Portugal, but, given its low isoenzyme diversity in this country, the population structure is poorly known. A set of 14 polymorphic microsatellite markers was studied on 136 Portuguese *Leishmania* strains isolated from different hosts, geographic regions and different clinical forms. A total of 108 different genotypes were found, which is a degree of genetic diversity comparable to other regions, even within zymodeme MON-1. A single most common genotype was detected in 1:5 of all strains, which, with a greater number of multi-strain genotypes found in the Lisbon Metropolitan Region, particularly for human strains, was suggestive of the occurrence of clonal transmission. In addition, a high re-infection rate was found among HIV+ patients. Model based analysis by STRUCTURE uncovered two main populations (populations A and B, composed of MON-1 and non-MON-1 strains, respectively), with great genetic diversity between them, and two MON-1 sub-populations (A1 and A2). High inbreeding coefficients were found in these populations, although strains with mixed ancestry were identified, suggesting that recombination also plays a role in the epidemiology of this species in Portugal. Some but limited geographical differentiation was observed, with groups of strains from the same regions clustering together, particularly those from canine origin.

Our results show that *L. infantum* isolates from Portugal present microsatellite diversity comparable to other regions and that different transmission models play a role in its epidemiology, from clonal transmission to recombination. In addition, although Portugal is a small country, mobility of people and animals is high and *Leishmania* can be probably easily disseminated between infected hosts throughout the country, two instances of seemingly local restricted transmission were identified.

1. Introduction

Leishmania is a genus of flagellated protozoa, which are the causative agents of several clinical forms of the leishmaniases and are transmitted by the bite of infected phlebotomine sand flies. In the Mediterranean basin, *Leishmania infantum* is the main etiological agent of canine and human visceral leishmaniasis (VL), with dogs considered to be the main domestic reservoir. Less frequently reported, the same species is also responsible for cutaneous leishmaniasis (CL). According to Pratlong et al. (2001) 31 *L. infantum* zymodemes have been described so far by isoenzyme analysis, although three have since then been considered as *Leishmania donovani* (Lukes et al., 2007; Schönian et al., 2010a,b). In total 25 *L. infantum* zymodemes have been found around the Mediterranean basin, being MON-1 the most prevalent zymodeme (Chicharro et al., 2003; Aït-Oudhia et al., 2011).

In Portugal, three endemic foci of VL were identified in the 1980s: the Alto Douro Region (ADR) in the north, the Lisbon

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Metropolitan Region (LMR) in the centre, and the Algarve Region in the south (Campino et al., 2006). As in other southern European countries, VL was initially considered a childhood disease in Portugal, but in the last decades, while the number of cases in children has decreased, there has been an increase in adults, mostly associated with HIV/AIDS (Campino et al., 1997). Conversely, CL is barely known in Portugal, although it is no longer a rare disease (Campino and Abranches, 2002). About 97% of the Portuguese *L. infantum* isolates belong to the MON-1 zymodeme, regardless of the disease form, host or vector species and immune status (Campino et al., 2006). However, other zymodemes have been isolated not only from immunocompromised patients (MON-24, MON-29 and MON-80) but also immunocompetent patients (MON-24 from a VL case and MON-29 from a CL case) without a travel history to other *L. infantum* endemic countries, as well as from the vector *Phlebotomus ariasi* (MON-24) and from one dog (MON-98). Furthermore, three *L. infantum*/*Leishmania major* hybrids have also been identified in autochthonous Portuguese human leishmaniasis (Ravel et al., 2006) and recently, *L. major* DNA was found in a *Sergentomyia minuta* sand fly collected in Portugal, in the Algarve Region (Campino et al., 2013).

Multi-locus enzyme electrophoresis (MLEE) (Rioux et al., 1990) has been considered the gold standard technique for identification of *Leishmania* at species and sub-specific levels and for epidemiological studies during the past 25 years. More recently MLEE started to be complemented, and will probably be replaced, by more powerful molecular approaches for strain discrimination (Schönian et al., 2008, 2010a,b), such as analysis of restriction fragment length polymorphism of kDNA minicircles (kDNA-PCR-RFLP), multilocus microsatellite typing (MLMT) (Botilde et al., 2006) and multilocus sequence typing (Mauricio et al., 2006; Zemanova et al., 2007; El Baidouri et al., 2013). These markers have been extensively used in epidemiological and genetic studies of leishmaniasis in the past few years (Schönian et al., 2010a,b). Specifically, microsatellite markers, have high discriminatory power, are neutral, co-dominant and results are reproducible and comparable between different laboratories.

Through molecular methods such as kDNA-PCR-RFLP, we have previously shown that Portuguese strains, within the prevalent MON-1 zymodeme, present substantial genetic diversity (Cortes et al., 2006), which was supported in a recent MLMT analysis of 107 European strains of *L. infantum* MON-1 (Kuhls et al., 2008), which included 42 Portuguese strains.

In this study, microsatellite markers were used to analyze more comprehensively the genetic diversity within *L. infantum* parasites from different regions of Portugal, using the largest sample size to date, and, in particular, to investigate its population structure and any association with clinical forms of the disease, vertebrate and invertebrate hosts and geographical origin that could inform control strategies. Genetically diverse populations of *L. infantum* were identified in Portugal, possibly due to the existence of local independent transmission cycles, as well as a high reinfection rate among HIV+ patients (i.v. drug users).

2. Materials and methods

2.1. Parasites

A total of 136 Portuguese *Leishmania* strains from the IHMT cryobank were included in this analysis. These included 58 human strains, of which 49 were from VL cases, five CL and also four VL with associated cutaneous lesions, which will be hereinafter designated as viscerocutaneous leishmaniasis (VCL). Among the human strains, 38 belong to immunocompromised patients (HIV+), all intravenous drug users. In 12 HIV+ cases, two to three strains per patient were isolated before and after treatment, with intervals of 1 month to 2 years, corresponding to 15 courses of treatment among all patients. The time between strain isolation was divided into three intervals: $\leqslant$1 month, 1 month to 1 year or >1 year. The sample from reservoir hosts, included 69 canine and four vulpine strains, in addition to five sand fly strains (one *Phlebotomus perniciosus* and four *Phlebotomus ariasi*).

Concerning geographical distribution, strains were from Alto Douro Region (ADR) ($n = 28$), Lisbon Metropolitan Region (LMR) ($n = 92$), Alentejo ($n = 8$) and Algarve ($n = 8$). Strains of vector and canine origin had been isolated in previous epidemiological surveys and human strains from samples sent for diagnosis by pathologists and clinicians at local hospitals. Strains were isolated between 1982 and 2004, with most strains (83%) isolated after 1994. However, all isolates from foxes and from sand flies (except one sand fly isolate) had been obtained before 1990. Laboratory and WHO codes, zymodemes, clinical manifestations and geographical origin of strains are listed in Table 1. Q3

Twenty-five non-Portuguese additional *L. infantum* and *L. donovani* DNA samples were used as references and outgroup, respectively, and were obtained from: London School of Hygiene and Tropical Medicine, UK; Centre National de Référence des *Leishmania*, France; Laboratory of Molecular Parasitology, Hellenic Pasteur Institute, Greece; and Universidade Federal de Minas Gerais, Brazil (Table 2).

2.2. DNA extraction

DNA was extracted from parasites grown in Schneider's medium (Sigma, USA) supplemented with 20% fetal bovine serum (Lonza, Switzerland), or directly from clinical samples (bone marrow or skin) using a commercial extraction kit (High Template DNA Preparation Kit, Roche, Germany) according to the manufacturer's instructions.

2.3. Microsatellite analysis

Fourteen microsatellite markers were amplified as previously described (Ochsenreither et al., 2006; Kuhls et al., 2007, 2008): Lm2TG, Lm4TA, Li 41–56, Li 46–67, Li 22–35, Li 23–41, Li 45–24, Li 71–33, Li 71–5/2, Li 71–7, kLIST7031, kLIST7039, TubCA and CS20. Amplification products were analyzed through polyacrylamide gel electrophoresis (PAGE), high resolution 4% MetaPhor™ agarose (Lonza, Switzerland) gel electrophoresis or automated capillary electrophoresis in a Beckman Coulter CEQ 8000 automated genetic analysis system, as described by Kuhls et al. (2008). Fragment size was determined by comparison to a 10 bp ladder (Invitrogen, USA) and reference strains, with known fragment sizes, as previously established through automated capillary electrophoresis.

Amplification products of the reference strains and a subset of 44 Portuguese samples were analyzed by both PAGE, MetaPhor agarose electrophoresis and automated capillary electrophoresis, in order to confirm reproducibility of results.

2.4. Data analysis

The population structure was analyzed using the programme STRUCTURE, version 2.3.4 (Pritchard et al., 2000), which applies a Bayesian model-based clustering method for multilocus genotype data to infer population structure and assign individuals to populations. The following parameters were used: burn-in period of 20,000 iterations following 200,000 Markov Chain Monte Carlo (MCMC) iterations. The most appropriate (or likely) number of populations was determined by calculation of Delta K (ΔK), which is based on the rate of change in the log probability of data

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Table 1
Portuguese *L. infantum*[a] strains included in this study.

Lab code	Zymodeme MON[b]	Geographic origin	Host	Disease	WHO code	Genotype (G)	Population assignment all sample set ($K = 2$)	Sub-population assignment MON-1 ($K = 2$)
H1	1	LMR	Human	VL/HIV+	MHOM/PT/2004/IMT364	**1**	**A**	**A1**
H2	1	LMR	Human	VL/HIV+	MHOM/PT/2001/IMT266b	**6**	**A**	**A1A1A2**
H3	1	LMR	Human	VL/HIV+	MHOM/PT/1993/IMT190	**7**	**A**	**A1A2**
H3a	1	LMR	Human	VL/HIV+	MHOM/PT/1993/IMT192	**5**	**A**	**A1**
H3b	Li/Lm	LMR	Human	VL/HIV+	MHOM/PT/1994/IMT208	NA	NA	NA
H6	1	LMR	Human	VL/HIV+	MHOM/PT/1998/IMT242	**8**	**A**	**A2**
H6a	1	LMR	Human	VCL/HIV+	MHOM/PT/1999/IMT242b	**8**	**A**	**A2**
H8	1	LMR	Human	VL/HIV+	MHOM/PT/2002/IMT290	**2**	**A**	**A1**
H8a	1	LMR	Human	VL/HIV+	MHOM/PT/2002/IMT290a	**2**	**A**	**A1**
H10	1	LMR	Human	VL/HIV+	MHOM/PT/2000/IMT262	**2**	**A**	**A1**
H10a	1	LMR	Human	VL/HIV+	MHOM/PT/2000/IMT262a	**2**	**A**	**A1**
H12	1	LMR	Human	VL/HIV+	MHOM/PT/2001/IMT269	**4**	**A**	**A2**
H12a	1	LMR	Human	VL/HIV+	MHOM/PT/2001/IMT269b	**4**	**A**	**A2**
H14	1	LMR	Human	VL/HIV+	MHOM/PT/1999/IMT249	**9**	**A**	**A1**
H14a	1	LMR	Human	VL/HIV+	MHOM/PT/2001/IMT249c	**5**	**A**	**A1**
H19	1	LMR	Human	VL/HIV+	MHOM/PT/1996/IMT223	**2**	**A**	**A1**
H19a	1	LMR	Human	VL/HIV+	MHOM/PT/1999/IMT223b	**2**	**A**	**A1**
H19b	1	LMR	Human	VL/HIV+	MHOM/PT/2003/IMT223c	**2**	**A**	**A1**
H22	1	LMR	Human	VL/HIV+	MHOM/PT/2001/IMT271	**2**	**A**	**A1**
H22a	1	LMR	Human	VL/HIV+	MHOM/PT/2003/IMT271b	**5**	**A**	**A1**
H26	1	LMR	Human	VCL/HIV+	MHOM/PT/2004/IMT362	**10**	**A**	**A2**
H27	29	LMR	Human	VCL/HIV+	MHOM/PT/1997/IMT227	**11**	**B**	NA
H28	29	LMR	Human	VCL/HIV+	MHOM/PT/1997/IMT228	**12**	**B**	NA
H30	24	LMR	Human	VL/HIV+	MHOM/PT/1992/IMT181	**13**	**B**	NA
H32	1	LMR	Human	VL/HIV+	MHOM/PT/1989/IMT163	**14**	**A**	**A1**
H33	1	LMR	Human	VL/HIV+	MHOM/PT/2002/IMT296	**2**	**A**	**A1**
H34	1	LMR	Human	VL/HIV+	MHOM/PT/2002/IMT293	**10**	**A**	**A2**
H34a	1	LMR	Human	VL/HIV+	MHOM/PT/2003/IMT293b	**10**	**A**	**A2**
H36	1	LMR	Human	VL/HIV+	MHOM/PT/2003/IMT299	**2**	**A**	**A1**
H37	1	LMR	Human	VL/HIV+	MHOM/PT/1993/IMT184	**4**	**A**	**A2**
H37a	Li/Lm	LMR	Human	VL/HIV+	MHOM/PT/1994/IMT211	NA	NA	NA
H48	1	LMR	Human	VL/HIV+	MHOM/PT/2004/IMT364	**1**	**A**	**A1**
H52	1	LMR	Human	VL/HIV+	MHOM/PT/1999/IMT250	**15**	**A**	**A1A2A2**
H52a	1	LMR	Human	VL/HIV+	MHOM/PT/2000/IMT250b	**16**	**AAB**	**A2**
H25	1	LMR	Human	CL/HIV+	MHOM/PT/2002/IMT294	**17**	**A**	**A1**
H24	1	LMR	Human	VL	MHOM/PT/2000/IMT264	**18**	**A**	**A1**
H42	1	LMR	Human	VL	MHOM/PT/1988/IMT151	**2**	**A**	**A1**
H43	1	LMR	Human	VL	MHOM/PT/2001/IMT274	**5**	**A**	**A1**
H44	1	LMR	Human	VL	MHOM/PT/2002/IMT291	**19**	**A**	**A2**
H45	1	LMR	Human	VL	MHOM/PT/2004/IMT358	**20**	**A**	**A2**
H55	1	LMR	Human	CL	MHOM/PT/2000/IMT260	**21**	**A**	**A2**
C1	1	LMR	Canine	CanL	MCAN/PT/2003/IMT300	**22**	**A**	**A1**
C2	1	LMR	Canine	CanL	MCAN/PT/2003/IMT304	**3**	**A**	**A1**
C3	1	LMR	Canine	CanL	MCAN/PT/2003/IMT331	**23**	**A**	**A2**
C4	1	LMR	Canine	CanL	MCAN/PT/2003/IMT314	**24**	**A**	**A2**
C5	1	LMR	Canine	CanL	MCAN/PT/2003/IMT315	**3**	**A**	**A1**
C6	1	LMR	Canine	CanL	MCAN/PT/2003/IMT316	**3**	**A**	**A1**
C7	1	LMR	Canine	CanL	MCAN/PT/2003/IMT318	**25**	**A**	**A1**
C8	1	LMR	Canine	CanL	MCAN/PT/2003/IMT321	**2**	**A**	**A1**
C9	1	LMR	Canine	CanL	MCAN/PT/2003/IMT305	**26**	**A**	**A1**
C10	1	LMR	Canine	CanL	MCAN/PT/2003/IMT310	**27**	**A**	**A1**
C11	1	LMR	Canine	CanL	MCAN/PT/2003/IMT312	**28**	**A**	**A2**
C12	ND	LMR	Canine	CanL	MCAN/PT/2003/IMT306	**29**	**A**	NA
C13	1	LMR	Canine	CanL	MCAN/PT/2003/IMT319	**30**	**A**	**A1**
C14	1	LMR	Canine	CanL	MCAN/PT/2003/IMT320	**31**	**A**	**A2**

(*continued on next page*)

Table 1 (*continued*)

Lab code	Zymodeme MON[b]	Geographic origin	Host	Disease	WHO code	Genotype (G)	Population assignment all sample set ($K = 2$)	Sub-population assignment MON-1 ($K = 2$)
C15	1	LMR	Canine	CanL	MCAN/PT/2003/IMT307	**32**	**A**	**A2**
C16	1	LMR	Canine	CanL	MCAN/PT/2002/IMT283	**33**	**AAB**	**A2**
C19	1	LMR	Canine	CanL	MCAN/PT/2003/IMT324	**1**	**A**	**A1**
C20	1	LMR	Canine	CanL	MCAN/PT/2003/IMT325	**2**	**A**	**A1**
C21	1	LMR	Canine	CanL	MCAN/PT/2003/IMT327	**34**	**A**	**A1**
C22	1	LMR	Canine	CanL	MCAN/PT/2003/IMT329	**35**	**A**	**A1**
C23	1	LMR	Canine	CanL	MCAN/PT/2003/IMT330	**36**	**A**	**A1A1A2**
C24	1	LMR	Canine	CanL	MCAN/PT/2003/IMT331	**37**	**A**	**A1**
C25	1	LMR	Canine	CanL	MCAN/PT/2003/IMT335	**2**	**A**	**A1**
C26	1	LMR	Canine	CanL	MCAN/PT/2003/IMT338	**38**	**A**	**A1**
C27	1	LMR	Canine	CanL	MCAN/PT/2003/IMT339	**39**	**A**	**A2**
C28	1	LMR	Canine	CanL	MCAN/PT/1997/IMT229	**2**	**A**	**A1**
C30	1	LMR	Canine	CanL	MCAN/PT/2003/IMT334	**40**	**A**	**A2**
C31	1	LMR	Canine	CanL	MCAN/PT/2003/IMT340	**1**	**A**	**A1**
C32	1	LMR	Canine	CanL	MCAN/PT/2003/IMT341	**41**	**A**	**A1**
C33	1	LMR	Canine	CanL	MCAN/PT/2003/IMT322	**2**	**A**	**A1**
C34	1	LMR	Canine	CanL	MCAN/PT/2003/IMT323	**2**	**A**	**A1**
C35	1	LMR	Canine	CanL	MCAN/PT/2003/IMT326	**42**	**A**	**A1**
C37	1	LMR	Canine	CanL	MCAN/PT/2003/IMT342	**43**	**A**	**A2**
C38	1	LMR	Canine	CanL	MCAN/PT/2004/IMT343	**43**	**A**	**A2**
C39	1	LMR	Canine	CanL	MCAN/PT/2003/IMT344	**38**	**A**	**A1**
C40	1	LMR	Canine	CanL	MCAN/PT/2003/IMT346	**2**	**A**	**A1**
C41	1	LMR	Canine	CanL	MCAN/PT/2003/IMT347	**2**	**A**	**A1**
C42	1	LMR	Canine	CanL	MCAN/PT/2003/IMT349	**43**	**A**	**A2**
C43	1	LMR	Canine	CanL	MCAN/PT/2003/IMT350	**4**	**A**	**A2**
C44	1	LMR	Canine	CanL	MCAN/PT/2003/IMT351	**2**	**A**	**A1**
C50	1	LMR	Canine	CanL	MCAN/PT/2003/IMT354	**44**	**A**	**A2**
C53	1	LMR	Canine	CanL	MCAN/PT/2003/IMT352	**2**	**A**	**A1**
C54	1	LMR	Canine	CanL	MCAN/PT/2004/IMT361	**2**	**A**	**A1**
C56	1	LMR	Canine	CanL	MCAN/PT/2004/IMT366	**45**	**A**	**A2**
C57	1	LMR	Canine	CanL	MCAN/PT/2003/IMT309	**46**	**A**	**A2**
C58	1	LMR	Canine	CanL	MCAN/PT/2003/IMT307	**47**	**A**	**A2**
C59	1	LMR	Canine	CanL	MCAN/PT/2003/IMT308	**48**	**A**	**A2**
R1	1	LMR	Fox	Unknown	VUL/PT/1982/IMT108	**2**	**A**	**A1**
R2	1	LMR	Fox	Unknown	VUL/PT/1984/IMT129	**2**	**A**	**A1**
R3	1	LMR	Fox	Unknown	VUL/PT/1983/IMT116	**2**	**A**	**A1**
R4	1	LMR	Fox	Unknown	VUL/PT/1984/IMT128	**2**	**A**	**A1**
H16	ND	ADR	Human	VL	NA	**5**	**A**	NA
H17	1	ADR	Human	VL	MHOM/PT/2002/IMT288	**49**	**A**	**A2**
H40	1	ADR	Human	VL	MHOM/PT/2002/IMT279	**50**	**AB**	**A2**
H41	1	ADR	Human	VL	MHOM/PT/2001/IMT267	**51**	**A**	**A2**
H46	1	ADR	Human	VL	MHOM/PT/2004/IMT359	**2**	**A**	**A1**
H47	1	ADR	Human	VL	MHOM/PT/2004/IMT360	**52**	**AAB**	**A2**
H54	1	ADR	Human	VL	MHOM/PT/2000/IMT259	**53**	**A**	**A2**
H56	1	ADR	Human	VL	MHOM/PT/2000/IMT261	**53**	**A**	**A2**
H57	1	ADR	Human	VL	MHOM/PT/2001/IMT267	**51**	**A**	**A2**
H29	1	ADR	Human	CL	MHOM/PT/2003/IMT337	**54**	**A**	**A1A2A2**
H39	ND	ADR	Human	CL	NA	**55**	**B**	NA
C29	1	ADR	Canine	CanL	MCAN/PT/1989/IMT161	**56**	**A**	**A1A2**
C45	1	ADR	Canine	CanL	MCAN/PT/1989/IMT160	**57**	**AAB**	**A2**
C47	1	ADR	Canine	CanL	MCAN/PT/1989/IMT162	**58**	**A**	**A2**
C60	ND	ADR	Canine	CanL	NA	**59**	**A**	NA
C61	ND	ADR	Canine	CanL	NA	**60**	**A**	NA
C62	ND	ADR	Canine	CanL	NA	**61**	**A**	NA
C63	1	ADR	Canine	CanL	MCAN/PT/1999/Leish	**62**	**A**	**A2**
C64	ND	ADR	Canine	CanL	NA	**63**	**A**	NA

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C65	98	ADR	Canine	CanL	MCAN/PT/1999/BOBI	**64**	**A**	NA
C66	ND	ADR	Canine	CanL	NA	**65**	**A**	NA
C67	ND	ADR	Canine	CanL	NA	**66**	**A**	NA
C68	ND	ADR	Canine	CanL	NA	**67**	**A**	NA
C69	ND	ADR	Canine	CanL	NA	**68**	**A**	NA
FI2	1	ADR	*Phlebotomus*	NA	IARI/PT/1989/IMT169	**69**	**AB**	**A2**
FI3	1	ADR	*Phlebotomus*	NA	IARI/PT/1989/IMT170	**70**	**ABB**	**A2**
FI4	24	ADR	*Phlebotomus*	NA	IARI/PT/1989/IMT171	**71**	**B**	NA
FI5	24	ADR	*Phlebotomus*	NA	IARI/PT/1989/IMT172	**72**	**B**	NA
H58	24	Alentejo	Human	VL	MHOM/PT/2004/IMT369	**73**	**ABB**	NA
H18	24	Alentejo	Human	VL	MHOM/PT/2004/IMT363	**74**	**B**	NA
C36	1	Alentejo	Canine	CanL	MCAN/PT/2003/IMT328	**2**	**A**	**A1**
C18	1	Alentejo	Canine	CanL	MCAN/PT/1995/IMT205	**4**	**A**	**A2**
C46	1	Alentejo	Canine	CanL	MCAN/PT/2004/IMT357	**75**	**A**	**A1A2A2**
C51	1	Alentejo	Canine	CanL	MCAN/PT/2004/IMT355	**9**	**A**	**A1**
C52	1	Alentejo	Canine	CanL	MCAN/PT/2004/IMT356	**37**	**A**	**A1**
C55	1	Alentejo	Canine	CanL	MCAN/PT/2004/IMT345	**76**	**A**	**A1**
H49	1	Algarve	Human	VL/HIV+	MHOM/PT/1999/IMT248	**77**	**A**	**A1**
H49a	1	Algarve	Human	VL/HIV+	MHOM/PT/1999/IMT248b	**78**	**A**	**A2**
H49b	1	Algarve	Human	VL/HIV+	MHOM/PT/1999/IMT248c	**8**	**A**	**A2**
H31	29	Algarve	Human	CL	MHOM/PT/1994/IMT202	**79**	**B**	NA
C17	1	Algarve	Canine	CanL	MCAN/PT/1993/IMT193	**80**	**A**	**A2**
C48	1	Algarve	Canine	CanL	MCAN/PT/1993/IMT193	**81**	**A**	**A1**
C49	1	Algarve	Canine	CanL	MCAN/PT/1994/IMT204	**82**	**A**	**A2**
FI1	1	Algarve	*Phlebotomus*	NA	IPERN/PT/1993/IMT189	**83**	**ABB**	**A2**

Li/Lm, *L. infantum/L. major* hybrid; VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; CanL, canine leishmaniasis; VCL, viscero-cutaneous leishmaniasis; LMR, Lisbon Metropolitan Region; ADR, Alto Douro Region; ND, not done; NA, not applicable; Population assignments: to one population if $K \geqslant 0.75$ (P_1 or P_2, respectively A or B and A1 or A2), to $P_1P_1P_2/P_1P_2P_2$ if 0.749–0.600, and to P_1P_2 if 0.599–0.399.

[a] Samples H3b and H37a had been previously identified as *L. infantum/L. major* hybrids (Ravel et al., 2006).

[b] Strain identification according to isoenzyme analysis (Rioux et al., 2004).

between successive *K* values. Strains were assigned to populations according to their calculated ancestry, according to the following scheme: $\geqslant 0.750$ to one population (P_1 or P_2, respectively A or B and A1 or A2), $P_1P_1P_2/P_1P_2P_2$ if 0.749–0.600, P_1P_2 if 0.599–0.399.

Phylogenetic analyzes were done based on microsatellite profiles. Microsatellite Analysis (MSA) software (Dieringer and Schlotterer, 2002) was used to calculate genetic distances (Dps – proportion of shared alleles) (Bowcock et al., 1994), with bootstrapping done for 1000 replicates. The distance matrix was used by the programme POPULATIONS (http://www.legs.cnrs-gif.fr/bioinfo/populations) to build a Neighbor-Joining (NJ) tree that was edited with the programme MEGA5 (Tamura et al., 2011). In addition, NeighborNet (NN) networks were constructed with the same distance matrix using Splits Tree4 (Huson and Bryant, 2006).

Descriptive population statistics were obtained with the programme Gene Data Analysis (GDA) version 1.0 (Lewis and Zaykin, 2001): average sample size (n), proportion of polymorphic *loci* (P), mean number of alleles per locus (MNA), observed (H_o) and expected (H_e) heterozygosity and inbreeding coefficient (F_{IS}) under Hardy–Weinberg equilibrium. The fixation index (F_{ST}) was calculated using MSA to evaluate population differentiation and was interpreted according to the guidelines of Wright (1978).

Chi-square statistical test was applied with the software "Statistical Package for Social Sciences" version 21.0 (SPSS®), using a significance level of 5% ($p < 0.05$), in order to evaluate associations between STRUCTURE MON-1 populations and geographical distribution or immune status.

For comparison of results regarding the number of genotypes obtained with other studies using the same or subsets of the microsatellites used here, it was determined a genotype frequency value – the ratio number of genotypes/number of strains/number of markers (*loci*).

3. Results

In total, 161 samples were typed with 14 microsatellite markers (Supplementary file, Table S1). All markers were polymorphic for all samples studied. Markers Lm2TG, Li 22–35, Li 23–41, Li45–24 and kLIST7039 were the most polymorphic, with 16, 14, 14, 10 and 10 alleles each, respectively. Li 46–67, Li 71–5/2 and TubCA were the least polymorphic with only four different alleles (Fig. 1).

A total of 108 different genotypes (G) were identified from the 161 strains, with 83 different genotypes found in the 136 Portuguese strains (Tables 1 and 2), for the 14 *loci*, giving a genotype frequency of 0.043 (genotypes per strain per *locus*) for the Portuguese *L. infantum*. All but 13 genotypes (12%) were unique in the total sample set. The non-unique genotypes were zymodeme MON-1 and only identified here in the Portuguese strains (Table 3). Twelve of the non-unique genotypes were found in a small number of isolates (two to five isolates) but one genotype (G2) was found in 28 Portuguese strains, corresponding to 20.5% of all Portuguese strains and 43% of strains with non-unique genotypes. The vast majority of G2 strains (26) were from the LMR, but found in different vertebrate hosts (human, canine and vulpine), and two were from the same human HIV+ patient. Five of the 13 non-unique genotypes were only found in the LMR and two (G51 and G53) only in the ADR, although 11 in total had strains from LMR. Four non-unique genotypes were found only in isolates from dogs (G3, G37, G38 and G43) and five only in humans (G5, G8, G10, G51 and G53). Of the nine non-unique genotypes with two or more strains from humans, seven included HIV+ patients. Strains from HIV+ patients represented 72% (21/29) of samples from non-unique genotypes, excluding strains from the same patient with the same genotype (probable treatment fails), was higher than the overall percentage of strains from HIV+ patients in the sample set (65.5%, 38/58).

Table 2
Leishmania DNA used as reference group in this study.

Lab code	Species	Zymodeme MON[a]	Geographic origin	Host	Disease	WHO code	Genotype	Population assignment whole sample set ($K = 2$)	Sub-population assignment MON-1 ($K = 2$)
ESP1[b]	*L. infantum*	1	Spain	Human	VL/ HIV+	MHOM/ES/93/PM1	**84**	**A**	**A2**
ESP2[b]	*L. infantum*	1	Spain	Human	CL	MHOM/ES/86/BCN16	**85**	**AAB**	**A2**
ESP3	*L. infantum*	1	Spain	Human	VL	MHOM/ES/2001/LLM-981	**86**	**A**	**A2**
ESP4	*L. infantum*	1	Spain	Canine	CanL	MCAN/ES/2001/LLM-1007	**87**	**A**	**A2**
ESP5	*L. infantum*	1	Spain	Canine	CanL	MCAN/ES/2002/LLM-1139	**88**	**AAB**	**A2**
FR1[b]	*L. infantum*	1	France	Human	CL	MHOM/FR/97/LSL29	**89**	**A**	**A1A2**
FR2[b]	*L. infantum*	1	France	Human	VL	MHOM/FR/78/LEM75	**90**	**A**	**A2**
FR3	*L. infantum*	11	France	Human	CL	MHOM/FR/80/LEM189	**91**	**B**	NA
GR1	*L. infantum*	1	Greece	Canine	CanL	MCAN/GR/2002/GR9	**92**	**B**	**A2**
GR2	*L. infantum*	1	Greece	Canine	CanL	MCAN/GR/2003/GR12	**93**	**AB**	**A1A2A2**
GR3	*L. infantum*	98	Greece	Human	VL	MHOM/GR/2003/GR19	**94**	**ABB**	NA
GR4	*L. infantum*	98	Greece	Human	VL	MHOM/GR/2004/GR17	**95**	**AAB**	NA
GR5	*L. infantum*	1	Greece	Human	VL	MHOM/GR/2002/GR26	**96**	**ABB**	**A1A2A2**
BR1[b]	*L. infantum*	1	Brazil	Human	VL	MHOM/BR/74/PP75	**97**	**A**	**A1**
BR2	*L. infantum*	1	Brazil	Human	VL	MHOM/BR/2001/RR071	**98**	**A**	**A1**
BR3	*L. infantum*	1	Brazil	Human	VL	MHOM/BR/2001/RR072	**99**	**A**	**A1A2A2**
BR4	*L. infantum*	1	Brazil	Human	VL	MHOM/BR/1998/RR055	**100**	**A**	**A1**
BR5	*L. infantum*	1	Brazil	Canine	CanL	MCAN/BR/2002/BH400	**101**	**A**	**A1**
BR6	*L. infantum*	1	Brazil	Canine	CanL	MCAN/BR/2001/BH401	**102**	**A**	**A1**
BR7	*L. infantum*	1	Brazil	Canine	CanL	MCAN/BR/2002/BH402	**103**	**A**	**A1**
ET1[b]	*L. donovani*	82	Ethiopia	Human	VL	MHOM/ET/72/GEBRE 12	**104**	NA	NA
ET2[b]	*L. donovani*	31	Ethiopia	Human	VL	MHOM/ET/00/HUSSEN	**105**	NA	NA
ET3	*L. donovani*	18	Ethiopia	Human	VL	MHOM/ET/67/LV9	**106**	NA	NA
SD1[b]	*L. donovani*	30	Sudan	Human	VL	MHOM/SD/82/GILANI1	**107**	NA	NA
SD2	*L. donovani*	81	Sudan	Human	VL	MHOM/SD/62/3S	**108**	NA	NA

VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; CanL, canine leishmaniasis; NA, not applicable. *L. donovani* strains were not included in population structure analysis as they were set as out group. Population assignments: to one population if $K \geqslant 0.75$ (P_1 or P_2, respectively A or B and A1 or A2), to $P_1P_1P_2/P_1P_2P_2$ if 0.749–0.600, and to P_1P_2 if 0.599–0.399.

[a] Strain identification according to isoenzyme analysis (Rioux et al., 1990).

[b] Strains with known repeat numbers, used as references in all microsatellite amplifications.

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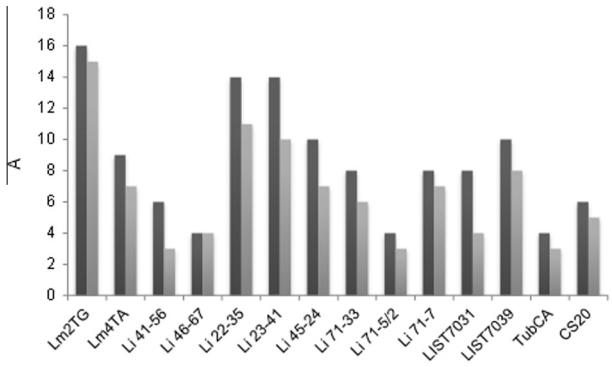

Fig. 1. Number of alleles (A) per microsatellite *locus* (marker) for the whole sample set and for the Portuguese sample set studied. Dark gray bars correspond to all dataset and light gray bars to Portuguese samples.

From the 12 sets of samples from HIV+ patients with more than one episode of leishmaniasis, six (50%) presented identical MLMT profiles. In two patients (H14/H14a and H22/H22a), with two episodes each, the second isolate differed from the first in only one marker and by one repeat unit (Li71-5/2 and kLIST7031, respectively) (Supplementary file, Table S1). In another patient, also with two clinical episodes (H52/H52a), the second strain differed from the first in five markers. In a patient, with three different clinical episodes (H49/H49a/H49b), the second and third isolates had almost indistinguishable profiles except for a null allele in H49a, but distinct from the first isolate at five markers. All these previous sets of samples were zymodeme MON-1 except two sets (H3/H3a/H3b and H37/H37a), in which the third and second isolates, H3b and H37a, respectively, had already been identified by isoenzyme typing and multilocus sequence typing as *L. infantum*/*L. major* hybrids (Ravel et al., 2006). These hybrids differed from the previous isolates (all MON-1) in 13 of the 14 markers studied, with heterozygous alleles in all markers (H3b) or atypical homozygous alleles (H37a), for five markers (Supplementary file, Table S1).

Strains obtained from HIV+ patients before and after one or more courses of treatment were compared. Eight isolates obtained post-treatment were indistinguishable from their respective pre-treatment isolates and a further three post-treatment isolates were distinct at only one marker. No correlation was observed between the time between strain isolation and similarity of profiles for the pairs of strains.

In order to infer the population structure of the data set, a Bayesian model-based clustering algorithm was used with the software STRUCTURE. According to ΔK, the most probable number of populations for the dataset of 154 strains (*L. donovani* strains and hybrids excluded) was two ($\Delta K = 2$), corresponding to MON-1 and non-MON-1 groups (populations A and B, respectively) (Fig. 2a). A further analysis of the MON-1 population (A), excluding admixed strains with population B (15 samples), subdivided it into two populations (A1 and A2) (Fig. 2b).

The NJ tree (Supplementary file, Fig. S1) produced from genetic distances based on the MLMT profiles of all strains, had low bootstrap values (<80%), so, a NeighborNet (NN) network was generated for all strains (Fig. 3). In both, NJ tree and NN networks, substantial genetic diversity was observed. Non-MON-1 strains branched off close to the outgroup, but as a paraphyletic group. The five MON-24 and three MON-29 strains were dispersed among other zymodemes and mostly at the end of long branches. As in the STRUCTURE analysis, the Greek MON-1 and MON-98 strains formed a sub-cluster between the MON-1 and non-MON-1 groups, and did not cluster with the Portuguese MON-98. The STRUCTURE MON-1 group (population A) strains formed a star shaped network without clear clusters, and interspersed European and Brazilian strains.

A NN network only with strains assigned to population A, MON-1 group (Fig. 4), showed four clusters (A–D). Most isolates assigned to STRUCTURE subpopulation A1 formed small sub-clusters, although several isolates were dispersed. Strains from LMR showed a tendency to cluster in relation to ADR isolates. The non-unique genotypes with only human or only canine isolates were mostly found in cluster A and in cluster C, respectively. Cluster B only included unique genotypes.

The proportion of polymorphic *loci* was high in both STRUCTURE populations A and B, being 100% in population B (non-MON-1) (Table 4), excluding samples with mixed membership ($n = 15$), and the mean number of alleles per locus (MNA) was similar between the two populations (mean = 4.571).

The F_{ST} index for populations A and B was 0.399 ($p < 0.001$), which corresponds to very great genetic differentiation according to Wright (1978). The inbreeding coefficient (F_{IS}) was high (>0.7), and very close to 1 in population A (Table 4).

Within population A, the proportion of polymorphic *loci* was also high, >70% in A1 and 100% in A2, excluding 11 admixed membership samples, and the mean number of alleles/locus (MNA) was much lower for population A1 (Table 5). The F_{ST} index for populations A1 and A2 was 0.176 ($p < 0.001$), corresponding to great genetic differentiation. F_{IS} was also very high (>0.8), being very close to 1 in population A1.

The association between strains from LMR to population A1 (53/82, 64.6%) and ADR strains to population A2 (12/13, 92.3%) was statistically significant ($p < 0.001$) (Table 6). Subpopulation A1 also had a statistically significant higher number of samples from HIV+ patients than subpopulation A2 (19/23, 0.82 *vs* 11/21, 0.52, $p < 0.001$) (Table 7).

Table 3
Genotypes found in more than one strain (non-unique genotypes).

Genotype (G)	Number of strains	Zymodeme (MON)	Region	Strains from HIV+ patients	Host
1	4	1	LMR	2	2H, 2C
2	28	1	26 LMR, 1 ADR, 1 Alentejo	10*	12H, 12C, 4F
3	3	1	LMR	n.a.	3C
4	5	1	4 LMR, 1 Alentejo	3*	3H, 2C
5	5	1	4 LMR, 1 ADR	3	5H
8	3	1	2 LMR, 1 Algarve	3*	3H
9	2	1	1 LMR, 1 Alentejo	1	1H, 1C
10	3	1	LMR	3*	3H
37	2	1	1 LMR, 1 Alentejo	n.a.	2C
38	2	1	LMR	n.a.	2C
43	3	1	LMR	n.a.	3C
51	2	1	ADR	0	2H
53	2	1	ADR	0	2H

* Two samples were strains from the same patient; H, human; C, canine; F, fox; n.a., not applicable.

4. Discussion

Visceral leishmaniasis, a zoonotic disease caused by *L. infantum*, is endemic in Portugal, a country in south-western Europe, with at least three main endemic regions, but shown to be spread across the country (Campino et al., 2006). The most prevalent zymodeme in Portugal is MON-1, although other zymodemes have also been found, which had hindered population structure analyzes, even using RAPD (I. Mauricio, data not shown). In this study, we used MLMT, a useful molecular tool for population genetic studies of

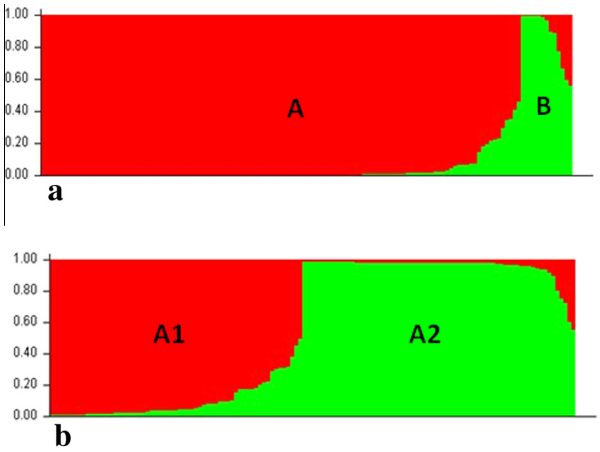

Fig. 2. Population structure of *L. infantum* samples inferred by STRUCTURE based on 14 microsatellite markers. (a) Bar plot for $K = 2$ with the total of 154 samples (Portuguese hybrid samples and *L. donovani* samples (outgroup) were excluded from this analysis); (b) Bar plot for $K = 2$ with the 129 samples of population A previously found in (a), excluding samples assigned to both A and B populations ($n = 15$). Each sample is represented by a single vertical line. Each color represents one population, and the length of the colors segment shows the strain's estimated proportion of membership in that population. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Leishmania, including the *L. donovani* complex (Schönian et al., 2010a,b), to analyze an extensive set of 136 strains isolated in Portugal. Portuguese *L. infantum* were shown to be genetically diverse, with a total of 83 genotypes and, thus, a genotype frequency of 0.043. While the genotype frequency was similar to other populations in Greece (0.037; Gouzelou et al., 2013) and Brazil (0.049; Segatto et al., 2012), it was about twice that found in a region of neighboring Spain (0.019; Montoya et al., 2007), which was geographically and host (dogs) restricted and only used two markers in common. These results highlight the genetic diversity of the Portuguese *L. infantum* strains, despite the overwhelming presence of MON-1 zymodeme strains.

Most strains presented unique genotypes, although non-unique genotypes were suggestive of clonal propagation. One genotype (G2), though, was particularly common and found in 20% of all Portuguese *L. infantum* strains and corresponded to kDNA-PCR-RFLP genotype A (Cortes et al., 2006), suggesting that they are indeed part of a single clade. This is in contrast with reports where two genotypes were found to be the most common and with similar frequencies in Spain (Montoya et al., 2007) and in Brazil (Segatto et al., 2012), but similar to mainland Greece (Gouzelou et al., 2013) and in another study in Brazil (Ferreira et al., 2012).

The highest number of non-unique genotypes found in the LMR could be due to a higher frequency of clonal propagation in this region, although sampling bias cannot be discarded. The host restricted nine non-unique genotypes (four in dogs and five in humans) could suggest host restricted transmission cycles, but the small number of samples in each does not allow to discard sampling bias. Moreover, the high percentage of strains from HIV+ patients in non-unique genotypes (72%) suggested human to human transmission, such as the artificial cycle previously proposed in intravenous drug users (Campino et al., 1994; Cruz et al., 2002).

Estimation of relapse and reinfection rates is important to evaluate the presence of drug resistance and transmission dynamics, respectively. Here, we detected probable cases of treatment failure in HIV+ patients (indistinguishable MLMT profiles), unclear cases (differences at only one marker) and probable re-infections (differences at five markers). The strains pairs with only one distinct marker may be due to reinfection with a similar strain, possibly from the same source of the initial infection, or selection of a genetically diverse clone from the initial population, although calling errors

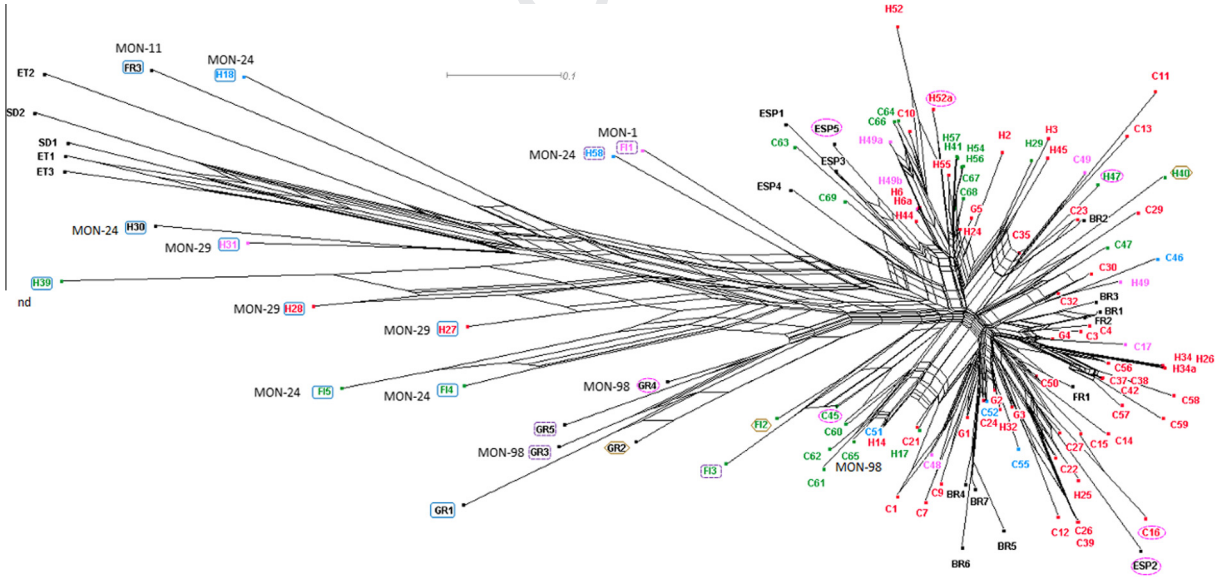

Fig. 3. NeighborNet network constructed with SplitsTree software employing the matrix of genetic distances between individuals based on proportion of shared alleles (Dps), of all strains ($n = 161$). Colored labels represent geographical origin of isolates: red for LMR, green for ADR, pink for the Algarve, blue for Alentejo and black for non-Portuguese strains. Dark blue boxes are population B (non-MON-1), purple (broken line) boxes are admixed isolates with a majority component from population B, pink (broken line) ovals are admixed isolates with a majority component from population A, brown hexagons are admixed isolates AB. All other isolates, with the exception of outgroup (SD and ET) samples, belong to population A (MON-1). C, canine; H, human; FL, phlebotomine; R, vulpine; BR, Brazil; ESP, Spain; ET, Ethiopia; FR, France; GR, Greece; SD, Sudan. G1: H1, H48, C19, C31 (humans, VL, HIV+, and canine from LMR); G2: H8, H8a, H10, H10a, H19, H19a, H19b, H22, H33, H36, H42, H46, C8, C20, C25, C28, C33, C34, C36, C40, C41, C44, C53, C54, R1, R2, R3, R4 (humans, VL, HIV+, and canine from LMR, except H46 from ADR); G3: C2, C5, C6 (canine from LMR); G4: H12, H12a, H37, C18, C43 (humans, VL, HIV+, and canine from LMR and Alentejo) G5: H3a, H14a, H16, H22a, H43 (humans, VL, HIV, from LMR). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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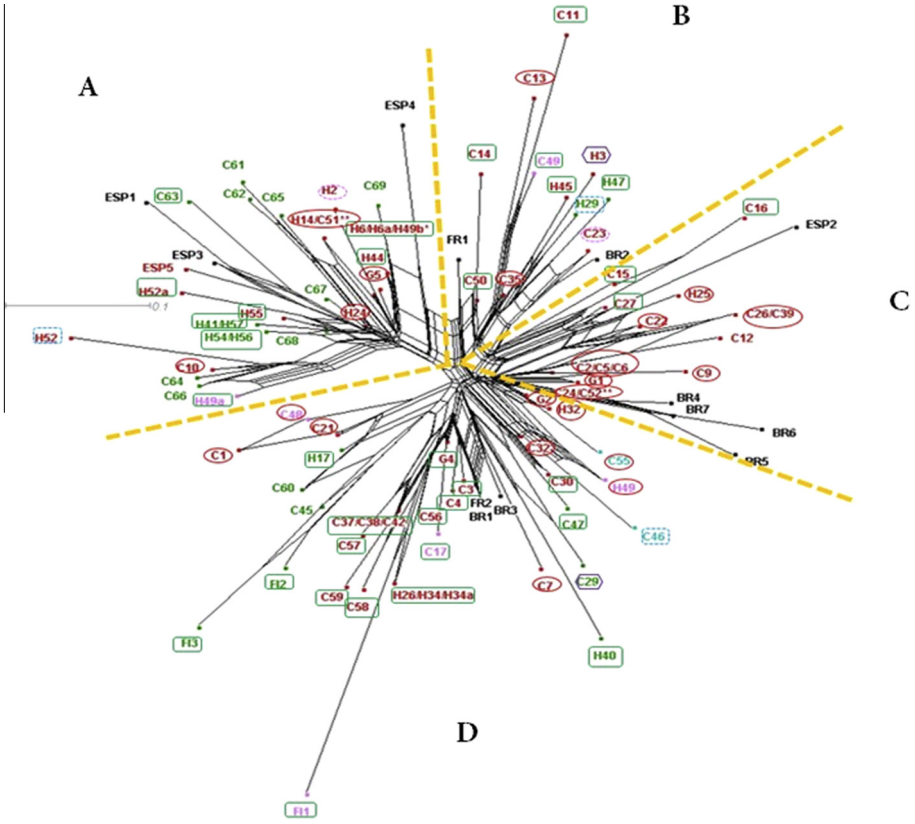

Fig. 4. NeighborNet network constructed on SplitsTree software employing the matrix of genetic distances between individuals based on proportion of shared alleles (Dps) of sub-population A strains. Colored labels represent geographical origin of isolates: red for LMR, green for ADR, pink for the Algarve, blue for Alentejo and black for non-Portuguese strains. Broken yellow lines separate observed main clusters. Red ovals are population A1, green boxes are population A2, pink (broken line) boxes are admixed isolates with a majority component from population A1, blue (broken line) boxes are admixed isolates with a majority component from population A2 and purple hexagons are admixed isolates A1A2. C, canine; H, human; FL, phlebotomine; R, vulpine; BR, Brazil; ESP, Spain; FR, France. G1: H1, H48, C19, C31(humans, VL, HIV+, and canine from LMR); G2: H8, H8a, H10, H10a, H19, H19a, H19b, H22, H33, H36, H42, H46, C8, C20, C25, C28, C33, C34, C36, C40, C41, C44, C53, C54, R1, R2, R3, R4 (humans, VL, HIV+, and canine from LMR, except H46 from ADR); G3: C2, C5, C6 (canine from LMR); G4: H12, H12a, H37, C18, C43 (humans, VL, HIV+, and canine from LMR and Alentejo) G5: H3a, H14a, H16, H22a, H43 (humans, VL, HIV, from LMR). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 4
Descriptive statistics of the inferred populations for the whole ($K = 2$) dataset (139 samples).[a]

Populations	n	P	MNA	H_e	H_o	F_{IS}	F_{ST}
A	129	0.857	4.428	0.212	0.009	0.955	0.399[b]
B	10	1.000	4.714	0.720	0.171	0.773	
Mean		1	4.571	0.466	0.090	0.817	

n, number of samples; P, proportion of polymorphic *loci*; MNA, mean number of alleles; H_e, expected heterozygosity; H_o, observed heterozygosity; F_{IS}, inbreeding coefficient; F_{ST}, fixation index.
[a] Samples assigned to both populations (15) as well as the two hybrid samples and the 5 *L. donovani* samples were excluded.
[b] $p = 0.0001$.

Table 5
Descriptive statistics for the $K = 2$ MON-1 sub-populations dataset (120 samples).[a]

Populations	n	P	MNA	H_e	H_o	F_{IS}	F_{ST}
A1	55	0.714	1.857	0.097	0.004	0.957	0.176[b]
A2	65	1.000	4.357	0.257	0.029	0.886	
Mean		0.857	3.107	0.177	0.016	0.905	

n, number of samples; P, proportion of polymorphic loci; MNA, mean number of alleles; H_e, expected heterozygosity; H_o, observed heterozygosity; F_{IS}, inbreeding coefficient; F_{ST}, fixation index.
[a] Samples assigned to both populations ($n = 11$) were excluded.
[b] $p = 0.0001$.

cannot be ruled out as the fragments differed by only one repeat unit.

The relapse rate was, thus, estimated at 53% to 73% in HIV+ patients, corresponding to a much higher re-infection rate than reported in Spain using PCR-RFLP of kDNA (Morales et al., 2002), Q4 which could be due to MLMT being more discriminatory or to the origin of infection. In the Spanish study most patients were intravenous drug users who could have been re-infected from the same source and thus with the same strain, but interpreted as a relapse. However, here all HIV+ patients were also intravenous drug users. Identification of putative relapses and re-infections did not seem to be related to time between isolations, although the sample analyzed was small. In any case, the possibility of selection of minority lineages within cryptic mixed infections, giving the impression of re-infections, cannot be discarded. Strain "evolution"

Table 6
Number of samples from the different Portuguese regions (107 samples) according to the MON-1 sub-populations inferred by STRUCTURE.

		Sub-population ($K = 2$)	
		A1 ($n = 60$)	A2 ($n = 47$)
Geografic regions	LMR[a] ($n = 82$)	53	29
	ADR[a] ($n = 13$)	1	12
	Other regions[a,b] ($n = 12$)	6	6

LMR, Lisbon Metropolitan Region; ADR, Alto Douro Region.
[a] Samples with mixed populations ($n = 7$) were excluded.
[b] Alentejo and Algarve.

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Table 7
Number of Portuguese human samples assigned to the MON-1 sub-populations A1 and B1 inferred by STRUCTURE, according to immune status (HIV+ and HIV− patients).

		Sub-populations ($K = 2$)	
		A1 ($n = 23$)	A2 ($n = 21$)
Immunodeficiency status[a]	HIV+ ($n = 33$)	19	11
	HIV− ($n = 15$)	4	10

[a] Human samples with mixed populations ($n = 4$) were excluded.

cannot be ruled out as causing apparent relapses, but stability of these microsatellite markers had previously been confirmed (Kuhls et al., 2008). Analyzes of more markers, including genomic sequencing, or cloning of the initial isolate would be useful.

A STRUCTURE analysis uncovered two main clusters for the *L. infantum* strains, MON-1 (population A) and non-MON-1 (population B), similarly to previous MLMT analyzes of European and South American *L. infantum* strains (Kuhls et al., 2008). Upon phylogenetic analyzes, division into those two groups was also observed but the non-MON-1 population emerged as paraphyletic. In addition, the splits in the NN network supported the hypothesis of some genetic recombination between *L. donovani* complex groups (Mauricio et al., 2006). As previously observed (Zemanova et al., 2007; Kuhls et al., 2008), the Greek samples analyzed here were genetically distinct from the Western European isolates and showed various ancestries, as previously suggested (Gouzelou et al., 2013). However, the Portuguese MON-98 sample was clearly related to Portuguese MON-1, thus showing marked polyphyly of this zymodeme.

European MON-24 and MON-29 were also here found to be polyphyletic, in the largest analysis of these zymodemes in Europe to our knowledge, as most strains in published studies have been of North African origin (Chargui et al., 2009). In addition, two MON-24 strains were found to be close to the outgroup and three to the MON-1 cluster, confirming that MON-24 strains belong to at least two distinct populations, one closer to *L. donovani* East African strains and another closer to *L. infantum* strains (Haralambous et al., 2007). Only two MON-24 sand fly samples were closely related, suggesting that they were part of the same transmission cycle, which was expected as they had been isolated from the same region and in the same year. However, they are sufficiently distant to indicate that the focus is genetically diverse, and the splits observed between the two strains are compatible with recombination occurring in this focus.

Two putative admixed strains were identified, as had also been observed in European, Algerian and Tunisian strains (Kuhls et al., 2008; Seridi et al., 2008; Chargui et al., 2009). The two strains, H58 (MON-24) and FL1 (MON-1), presented heterozygous alleles, characteristic of both MON-1 and non-MON-1 populations, and because they formed a NN cluster, are suggestive of recombination between MON-24 and MON-1 strains in nature. These results also clearly show that zymodeme typing does not reflect genetic relationships between *L. infantum* strains and that epidemiological surveillance should rely instead on markers such as MLMT.

STRUCTURE population A (MON-1 strains) was further divided in two subpopulations (A1 and A2), with F_{ST} indicating great genetic differentiation. In contrast, four clusters were observed in a NN network, with limited correspondence to STRUCTURE results, as only cluster C was composed of A1 strains. These results suggest that population subdivision within Portuguese MON-1 is not strong, or that the STRUCTURE division into two populations was too conservative. Genome sequencing might better clarify the population structure.

Other MON-1 European strains (Spain and France) were dispersed between Portuguese and Brazilian strains in the MON-1 cluster, as expected from recent larger studies using microsatellite analysis and other molecular markers that support an Old World (including Portugal) origin of Brazilian *L. infantum* strains (Kuhls et al., 2008, 2011; Leblois et al., 2011; Alvarenga et al., 2012).

Geographical correlation was observed for subpopulations A1 and A2, with LMR and ADR, respectively, but also on the NN network for clusters A (ADR, particularly for dogs) and C (LMR). As strains in Cluster C were from the LMR and sub-population A1, and mostly from dogs, it can be raised the hypothesis that this cluster represents a geographically restricted zoonotic cycle of no or limited importance in the immunocompetent human population. Local transmission cycles were suggested in Cluster A by the presence of strains of the same genotype, and related genotypes, from the same region. Cluster A seemed to represent a cycle geographically restricted to ADR and the few strains found in humans in LMR may have been acquired in ADR. The other main clusters (B and D) are more heterogeneous regarding the geographical origin of strains and may represent widespread lineages, but showed smaller clusters with strains from the same region. It is, thus likely that in Portugal *L. infantum* is transmitted in small geographically restricted foci, but further investigation would require more detailed geographical and chronological data, as well as more discriminatory genotyping methods, such as whole genome sequencing and genome-wide SNP typing (Downing et al., 2012).

The same parasite population was found to circulate in both domestic and sylvatic cycles, as samples obtained from sylvatic animals (foxes) had genotypes indistinguishable from both domestic dogs and humans from the same geographic regions.

Although *Leishmania* are known to propagate clonally or with high inbreeding, occasional natural recombination events have been identified (Bañuls et al., 2002; Mauricio et al., 2006). Three out of the five MON-1 strains isolated from the sand fly vector (FL1, FL2 and FL3) had heterozygous alleles and/or less frequent alleles for several *loci*, and were identified as having mixed ancestry by STRUCTURE. These results are consistent with previous observations of genetic recombination in the sand fly vector, stressing the role that vectors may sustain genetic diversity (Volf et al., 2007). It is not clear, however, if such recombinant lineages will be successfully transmitted by the vector, although recent work has shown that hybrid strains from *L. braziliensis* and *L. peruviana* presented higher plasticity and phenotypic diversity than the putative parental species, with potential eco-epidemiological implications (Cortes et al., 2012).

In total, 15 strains were identified here as having mixed ancestry. However, in both populations A and B observed heterozygosity was lower than expected heterozygosity, leading to high inbreeding coefficients especially in MON-1 population (0.955), which is indicative of high inbreeding or clonal propagation.

In addition, although not included in further analyzes, the two Portuguese strains (H3b and H37a), which had already been identified as *L. infantum*/*L. major* hybrids by MLEE and MLST (Ravel et al., 2006), presented heterozygous microsatellite alleles for all markers (H3b) or atypical homozygous alleles (H37a). It is possible that these lineages were generated by different hybridization events or that they have evolved differently, one by either keeping a 4n genome or accommodating a 2n genome with one chromosome from each species, and the other with extensive gene conversion probably leading to a truly homologous 2n genome. These possibilities are currently under investigation.

5. Conclusion

The present analysis of 136 *L. infantum* strains using MLMT showed that *L. infantum* isolates from Portugal presents substantial

genetic diversity, comparable to other endemic regions, even within zymodeme MON-1, confirming previous analyzes with other markers (Cortes et al., 2006). Paraphyly or polyphyly of non-MON-1 zymodemes was detected for MON-24, MON-29 and MON-98 strains analyzed, showing the inadequacy of MLEE for epidemiological surveillance. We found evidence suggestive of the existence of local independent transmission cycles, and statistical association with geographical origin, No statistical association was found of genotype with host, geographic distribution, clinical presentation or immune status. A combined analysis of different molecular markers or genome sequencing might uncover more substantial associations and genetic relationships.

This was, so far, the most extensive population analysis of *L. infantum* conducted in Portugal. It showed the presence of substantial genetic diversity, not previously identified by MLEE, and its results will enable to evaluate epidemiological changes in Portugal and prepare further in depth studies.

Ethics

Not required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2014.04.023.

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