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Original article

Genetic polymorphism of Algerian *Leishmania infantum* strains revealed by multilocus microsatellite analysis

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Abstract

The present study applies multilocus microsatellite typing (MLMT) for studying the polymorphism among 55 strains of *Leishmania infantum* from Algeria. These strains from different Algerian foci representing different zymodemes, hosts and clinical forms were analysed using 14 microsatellite markers. All 55 strains had individual MLMT profiles and no relationship was observed between them and different host or geographical origins. Three populations of Algerian *L. infantum* were identified by a Bayesian clustering approach implemented in STRUCTURE software and supported by genetic distance analysis. Two populations, A and B, consisted mainly of strains belonging to zymodeme MON-1, and the third population, C, mainly of MON-24 strains isolated from cutaneous leishmaniasis cases. Interestingly, a small group of strains appeared as a mixture of different populations and might be putative hybrids. Genetic migration was noticed among the two MON-1 populations, A and B, as well as between populations A and C. Due to its high discriminatory power MLMT could be also successfully applied for differentiating relapses or re-infection for patients suffering from multiple episodes of visceral leishmaniasis.

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Keywords: *Leishmania infantum*; Visceral leishmaniasis; Cutaneous leishmaniasis; Microsatellite typing; Algeria; polymorphism

1. Introduction

Leishmania (L.) infantum Nicolle, 1908, is the causative agent of visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) around the Mediterranean basin. The features of its life cycle are well known. Multilocus enzyme electrophoresis (MLEE) depicted polymorphism within this species that was reflected in insect vector and host specificity, geographical distribution and virulence. Twenty-eight zymodemes were described for *L. infantum* in the Mediterranean region so far

[1]. In Algeria, six different zymodemes, MON-1, MON-24, MON-34, MON-80, MON-77 and MON-33, are present [2], of which the first two are the most frequent ones. *L. infantum* MON-1 is the main zymodeme responsible for VL. Zymodeme MON-24 was isolated from CL cases however, was described to cause VL cases too [3]. Other zymodemes, such as MON-34 and MON-80, were also isolated from immunocompetent patients in Algeria. In immunocompromised, mainly HIV co-infected patients, *L. infantum* MON-1, MON-24, MON-33 and MON-78 were found [2]. Dogs are the main reservoir host of *L. infantum*. Beside the zymodeme MON-1, other enzymatic variants (MON-34, MON-24, MON-77) were occasionally isolated from them [2]. Sand flies belonging to the sub-genus *Larrousius*, *P. perfliewi* and *P. perniciosus*, were found infected by *L. infantum* MON-1 and MON-24 in northern Algeria [4,5].

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In Algeria, *L. infantum* parasites are associated with diverse clinical and eco-epidemiological situations. They are distributed over humid and sub-humid bioclimatic zones along the coast. The association of *L. infantum* to different clinical forms of the disease, VL and CL, to different hosts and the wide geographical distribution in Algeria raised the question about the role of parasite diversity in the clinical manifestation and epidemiology of the disease.

Molecular typing has demonstrated polymorphism of *L. infantum* parasites in Algeria [6]. Using a PCR-RFLP approach targeting two antigenic protein genes, *gp63* and *cpb*, five different genotypes were identified for Algerian strains of *L. infantum* [6]. These genotypes showed a weak correlation to geographical distribution of the strains but not to clinical manifestation of the disease or host background.

Direct comparison of different DNA-based typing approaches revealed that multilocus microsatellite typing (MLMT) and kDNA-PCR-RFLP were most discriminatory for typing strains of *L. infantum*, and especially for discrimination of MON-1 strains [7]. MLMT has the advantage over kDNA-PCR-RFLP of better reproducibility and feasibility of inter-lab comparisons. Furthermore, microsatellite markers are co-dominant and thus suited for population genetic studies in *Leishmania*.

In this study, we analysed genetic diversity among strains of *L. infantum* from different endemic foci in Algeria by applying MLMT. The endemic foci were characterized by circulation of parasites belonging to different zymodemes and sympatric occurrence of strains causing visceral or cutaneous diseases.

2. Materials and methods

2.1. Parasites

Fifty-five Algerian *L. infantum* strains cultured in Novy–MacNeal–Nicolle biphasic culture medium (NNN) or in SL medium [8] were used in this study. All parasites were characterized by isoenzyme analysis (15 enzymes) [9], and three zymodemes have been identified – MON-1, MON-24, and MON-80. Forty-two strains were isolated from humans, 27 visceral (24 of MON-1, one of MON-24 and two of MON-80) and 15 cutaneous cases (14 of non-MON-1 and one of MON-1). Some of these strains were isolated from the same patient during different episodes of the disease. Thirteen strains (all MON-1) were obtained from dogs. The WHO code, clinical manifestation, zymodeme and geographical origin of the strains are listed in Table 1.

The following strains of *L. infantum*, INF-1 (MHOM/TN/1980/IPT1), INF-10 (MCAN/TR/1996/EP16), INF-39 (MHOM/FR/1978/LEM75), INF-41 (MHOM/ES/1993/PM1), and INF-44 (MHOM/PT/2000/IMT260) were used as references in all genotyping experiments.

2.2. DNA extraction

Parasite pellets were re-suspended in one volume of lysis buffer (NaCl, 50 mM; EDTA, 10 mM; Tris–HCl, 50 mM, pH

7.4). High molecular weight genomic DNA was isolated as previously described [6].

2.3. Microsatellite analysis

Fourteen short tandem repeats (STR) or microsatellites were amplified for the 60 DNA samples in volumes of 25 μ l as previously described [10,11]. The markers were: Lm2TG, TubCA, 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, LIST7031, LIST7039 and CS20. Fragment size analysis has been performed by either MetaPhor agarose gel electrophoresis or automated fragment analysis using the Beckman Coulter CEQ 8000 automated genetic analysis system [10].

2.4. Population genetic analysis

MLMT data were analysed by a Bayesian statistics-based method implemented in the STRUCTURE program [12] to investigate population structure, and by construction of neighbor-joining (NJ) trees based on the calculation of genetic distances.

The program STRUCTURE was run using the admixture model with length of burn-in period of 20,000 iterations, followed by 200,000 of MCMC (Markov Chain Monte Carlo) repeats after burn-in. The strains were assigned to clusters with membership coefficients between 0 and 1. Based on multilocus genotype data the individuals were divided into K subpopulations with K ranging from 1 to 8. The appropriate number of populations in our sample set was determined by comparison of the log-likelihood values for K 1–8, and calculation of ΔK based on 10 runs for each K . The optimal number of populations corresponds to the peak in the ΔK graph [13].

For construction of the neighbor-joining (NJ) tree, genetic distances (Das – proportion of shared alleles) were calculated using the MSA software [14]. The distance matrix was imported into the program POPULATIONS 1.2.28 (<http://www.legs.cnrs-gif.fr/bioinfo/populations>) for calculation of the tree and the output tree file was opened with MEGA 3.1 [15].

Descriptive statistics for the populations found, n – average sample size, A – mean number of alleles per locus, observed (H_o) and expected (H_e) heterozygosity, and the inbreeding coefficient (F_{IS}), was calculated using the software Gene Data Analysis (GDA) [16]. The degree of genetic isolation of populations, with p -values (confidence test), was estimated by Wright's F -statistics [17] using the MSA software [14].

3. Results

All 14 microsatellite markers were polymorphic for the 55 Algerian strains of *L. infantum* (data not shown). A multilocus microsatellite profile summing up the repeat numbers obtained for the 14 microsatellite sequences was assigned to each strain under study and yielded 55 different genotypes. No correlation was found between a particular MLMT profile and the host

Table 1
Algerian and reference strains of *L. infantum* analysed in this study

WHO code	Origin	Pathology	Zymodeme	Population	M. coefficients		
					Pop. A	Pop. B	Pop. C
MHOM/DZ/1995/LIPA448 ^a	Setif	VL	MON-1	A	0.832	0.139	0.029
MHOM/DZ/1996/LIPA504	Boumerdes	CL	MON-24	A	0.989	0.007	0.004
MHOM/DZ/1996/LIPA529	Tizi Ouzou	VL	MON-1	A	0.888	0.052	0.059
MHOM/DZ/1998/LIPA815	Boumerdes	CL	MON-24	A	0.957	0.039	0.005
MHOM/DZ/1998/LIPA842	Ain-Defla	VL	MON-1	A	0.863	0.133	0.004
MHOM/DZ/1998/LIPA851	Blida	VL	MON-1	A	0.997	0.007	0.004
MHOM/DZ/1998/LIPA867	Alger	VL	MON-1	A	0.977	0.014	0.009
MCAN/DZ/1998/LIPA904	Alger	CanL	MON-1	A	0.989	0.008	0.004
MCAN/DZ/1998/LIPA911	Alger	CanL	MON-1	A	0.986	0.01	0.004
MHOM/DZ/1999/LIPA979	Tizi Ouzou	VL	MON-1	A	0.988	0.007	0.006
MHOM/DZ/1999/LIPA1002	Tizi Ouzou	VL	MON-1	A	0.984	0.008	0.008
MCAN/DZ/2000/LIPA1109	Alger	CanL	MON-1	A	0.989	0.007	0.004
MCAN/DZ/2000/LIPA1113	Alger	CanL	MON-1	A	0.989	0.007	0.004
MCAN/DZ/2000/LIPA1117	Alger	CanL	MON-1	A	0.981	0.01	0.009
MHOM/DZ/2001/LIPA1148	Bouira	VL	MON-1	A	0.966	0.016	0.017
MCAN/DZ/2001/LIPA1179	Alger	CanL	MON-1	A	0.986	0.011	0.004
MCAN/DZ/2001/LIPA1213	Alger	CanL	MON-1	A	0.986	0.006	0.008
MCAN/DZ/2001/LIPA1246	Alger	CanL	MON-1	A	0.984	0.012	0.004
MHOM/DZ/2002/LIPA1313	Lakhdaria	VL	MON-1	A	0.661	0.188	0.151
MCAN/DZ/2002/LIPA1341	Alger	CanL	MON-1	A	0.988	0.009	0.004
MHOM/DZ/2002/LIPA1342	Blida	VL	MON-1	A	0.989	0.008	0.004
MHOM/DZ/2002/LIPA1343	Blida	VL	MON-1	A	0.988	0.008	0.004
MHOM/DZ/1996/LIPA482 ^b	Médéa	VL	MON-1	B	0.018	0.977	0.004
MHOM/DZ/2001/LIPA1227 ^c	Bejaia	VL	MON-1	B	0.091	0.905	0.005
MHOM/DZ/2001/LIPA1233 ^c	Béjaia	VL	MON-1	B	0.055	0.94	0.005
MCAN/DZ/2002/LIPA1281	Alger	CanL	MON-1	B	0.213	0.783	0.004
MHOM/DZ/2002/LIPA1285	Bouira	CL	MON-24	B	0.087	0.905	0.008
MHOM/DZ/2002/LIPA1337	Alger	VL	MON-1	B	0.02	0.976	0.004
MHOM/DZ/2002/LIPA1338	Lakhdaria	VL	MON-1	B	0.142	0.838	0.019
MCAN/DZ/2000/LIPA1139	Alger	CanL	MON-1	A/B	0.673	0.323	0.004
MHOM/DZ/2002/LIPA1339	Ain Defla	VL	MON-1	A/B	0.625	0.369	0.006
MHOM/DZ/2002/LIPA1318	Biskra	VL	MON-1	A/B	0.407	0.588	0.005
<i>MHOM/DZ/1995/LIPA454^b</i>	<i>Médéa</i>	<i>VL</i>	<i>MON-1</i>	<i>A/C</i>	<i>0.432</i>	<i>0.11</i>	<i>0.459</i>
<i>MHOM/DZ/1996/LIPA466^b</i>	<i>Médéa</i>	<i>VL</i>	<i>MON-1</i>	<i>A/C</i>	<i>0.465</i>	<i>0.127</i>	<i>0.408</i>
<i>MHOM/DZ/1996/LIPA487</i>	<i>Boumerdes</i>	<i>VL</i>	<i>MON-1</i>	<i>A/C</i>	<i>0.336</i>	<i>0.103</i>	<i>0.561</i>
<i>MHOM/DZ/1996/LIPA509^c</i>	<i>Sétif</i>	<i>VL</i>	<i>MON-1</i>	<i>A/C</i>	<i>0.481</i>	<i>0.032</i>	<i>0.487</i>
<i>MHOM/DZ/1999/LIPA992</i>	<i>Ain Defla</i>	<i>CL</i>	<i>MON-1</i>	<i>A/C</i>	<i>0.408</i>	<i>0.01</i>	<i>0.583</i>
<i>MHOM/DZ/1999/LIPA1057</i>	<i>Alger</i>	<i>CL</i>	<i>MON-80</i>	<i>A/C</i>	<i>0.323</i>	<i>0.014</i>	<i>0.663</i>
<i>MHOM/DZ/1999/LIPA1067</i>	<i>Tizi Ouzou</i>	<i>VL</i>	<i>MON-80</i>	<i>A/C</i>	<i>0.562</i>	<i>0.042</i>	<i>0.397</i>
<i>MHOM/DZ/2002/LIPA1319</i>	<i>Tipaza</i>	<i>VL</i>	<i>MON-80</i>	<i>A/C</i>	<i>0.412</i>	<i>0.019</i>	<i>0.57</i>
MHOM/DZ/1996/LIPA477	Ain Defla	CL	MON-24	C	0.003	0.005	0.992
MHOM/DZ/1998/LIPA881	Tizi Ouzou	VL	MON-24	C	0.092	0.038	0.87
MCAN/DZ/1998/LIPA882	Tizi Ouzou	CanL	MON-1	C	0.004	0.004	0.992
MHOM/DZ/1999/LIPA977	Tizi Ouzou	CL	MON-24	C	0.021	0.024	0.954
MHOM/DZ/1999/LIPA1058	Lakhdaria	CL	MON-80	C	0.004	0.005	0.99
MHOM/DZ/1999/LIPA1066	Alger	CL	MON-24	C	0.004	0.004	0.992
MHOM/DZ/2000/LIPA1086	Boumerdes	CL	MON-24	C	0.003	0.004	0.993
MHOM/DZ/2000/LIPA1087	Béjaia	VL	MON-1	C	0.005	0.005	0.99
MCAN/DZ/2000/LIPA1118	Alger	CanL	MON-1	C	0.004	0.005	0.991
MHOM/DZ/2001/LIPA1140	Boumerdes	CL	MON-24	C	0.004	0.005	0.992
MHOM/DZ/2001/LIPA1226	Alger	CL	MON-24	C	0.003	0.007	0.99
MHOM/DZ/2002/LIPA1323	Tizi Ouzou	VL	MON-1	C	0.009	0.019	0.972
MHOM/DZ/1995/LIPA 440	Alger	CL	MON-24	C	0.053	0.008	0.939
MHOM/DZ/1995/LIPA452	Boumerdes	CL	MON-24	C	0.059	0.012	0.929
MHOM/DZ/1995/LIPA459	Lakhdaria	CL	MON-24	C	0.004	0.004	0.991
Reference strains from Tunisian and European foci							
MHOM/TN/1980/IPT1	Tunisia	VL	MON-1	A	0.964	0.009	0.027
MCAN/TR/1996/EP16	Turkey	CanL	MON-1	B	0.166	0.827	0.006
MHOM/FR/1978/LEM75	France	VL	MON-1	B	0.007	0.989	0.004
MHOM/ES/1993/PM1	Spain	VL	MON-1	B	0.009	0.977	0.013
MHOM/PT/2000/IMT260	Portugal	CL	MON-1	B	0.007	0.99	0.004

VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; CanL, canine leishmaniasis. A, B and C represent populations A, B and C, respectively, according to STRUCTURE. M. coefficient, membership coefficient for each strain in each population as calculated by STRUCTURE. ^{a,b,c}Samples from patients a, b and c. Putative hybrids between two populations are highlighted in bold and italic.

background of strains (canine vs. human) however, intrazymodeme diversity was observed within MON-1 as well as within MON-24/MON-80.

To estimate the optimal number of populations, we used a Bayesian model-based clustering algorithm implemented in the software STRUCTURE which indicated the existence of three populations in our data set (Fig. 1). These three populations of Algerian *L. infantum* were: Population (A) formed by 23 strains (21 of MON-1, and two of MON-24) and including the *L. infantum* WHO reference strain from Tunisia (MHOM/TN/1980/IPT1), population (B) consisting of seven Algerian, all but one MON-1, and four European MON-1 strains which were added for comparison, and population (C) comprising 10 strains of MON-24, four of MON-1 and one of MON-80. Furthermore, three MON-1 strains had mixed A/B genotypes; and eight strains, five MON-1 and three MON-80, mixed A/C genotypes as inferred by their membership coefficients (Table 1). Fig. 2 shows origin and geographical distribution of the strains corresponding to these genetic groups.

To understand the partition and the dynamics of population structure we have tested the clustering for K values from 1 to 4 (Fig. 1). At $K=2$, the majority of MON-1 strains was assigned to one population, whereas most MON-24 and MON-80 strains formed the other one. At $K=3$, the MON-1 group was divided into two populations, A and B. At $K=4$ (data not shown), the grouping of the strains and the number of strains showing mixed membership were the same as in previous partitions except for two strains, MHOM/DZ/1995/LIPA440 and MHOM/DZ/1995/LIPA 452, which formed a single group, because of some unique alleles in few markers.

MLMT profiles were also used for calculating genetic distances and constructing a NJ tree. This tree clearly showed three main clusters corresponding to the three populations obtained by STRUCTURE (Fig. 3). Interestingly, the strains with mixed A/C genotypes appeared in the NJ tree as intermediate cluster between the two populations. The three strains with A/B mixed genotypes were dispersed among populations A and B.

F -statistics was used to estimate the genetic isolation between the three populations identified by STRUCTURE. Strains showing mixed memberships in different clusters were excluded from this analysis. The F_{ST} values were 0.338 for populations A and B, 0.417 for populations B and C, and 0.523 for populations A and C, indicating clear genetic differentiation between them. Significance of these estimates was confirmed by p -values which were all 0.001 (data not shown).

Gene flow between populations was inferred by STRUCTURE with strains of mixed MLMT profiles included (Fig. 4). Gene flow was evident between populations A and C, as well as A and B, respectively. No contact was, however observed between populations B and C.

Measures for genetic diversity have been calculated for the three populations of *L. infantum* (strains of mixed A/B and A/C genotypes have been excluded) circulating in Algeria (Table 2). The mean number of alleles per locus, MNA, varied between 2.929 and 4.643 and the proportion of polymorphic loci, P , was high for all populations. The values for H_e were ranging from 0.262 to 0.591 and H_o varied between 0.14 and 0.34. Population C was most variable showing the highest MNA, H_e and H_o values. The lowest inbreeding coefficient F_{IS} was found for population A. Analysis of the A/C group showed very high H_e and H_o values (0.595 and 0.754, respectively) and no indication for inbreeding (data not shown).

We have also included strains isolated from the same patient during different episodes of the disease (Table 1). The strains MHOM/DZ/1995/LIPA448 and MHOM/DZ/1996/LIPA509, isolated from the same patient (a) within 2 years, had different genotypes and clustered into two different populations. Of the three isolates obtained from patient (b), MHOM/DZ/1995/LIPA454 and MHOM/DZ/1996/LIPA466 have shown mixed genotypes between population A and C with identical MLMT profiles (with the exception of an additional band in a single marker), while MHOM/DZ/1996/LIPA482 grouped with population B. Strains MHOM/DZ/2001/LIPA1227 and MHOM/DZ/2001/LIPA1233, isolated from patient (c) in 1 year, grouped together in population B, moreover, they showed identical MLMT profiles with an

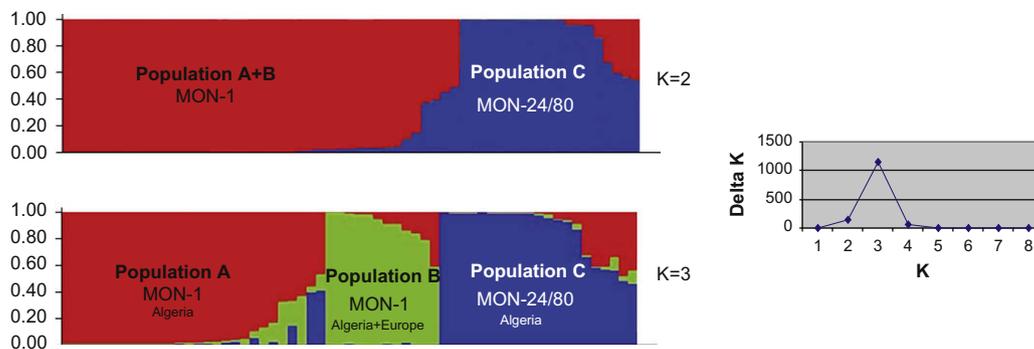


Fig. 1. Estimated population structure for *L. infantum* from Algeria as inferred by STRUCTURE software on the basis of data for 14 microsatellite markers obtained for 60 strains. Each of the strains is represented by a single vertical line divided into K colors, where K is the number of populations assumed. Each color represents one population, and the length of the colors segment shows the strain's estimated proportion of membership in that population. On the right side the derived graph for ΔK shows a peak at $K=3$, indicating the existence of three populations in the investigated strain set.

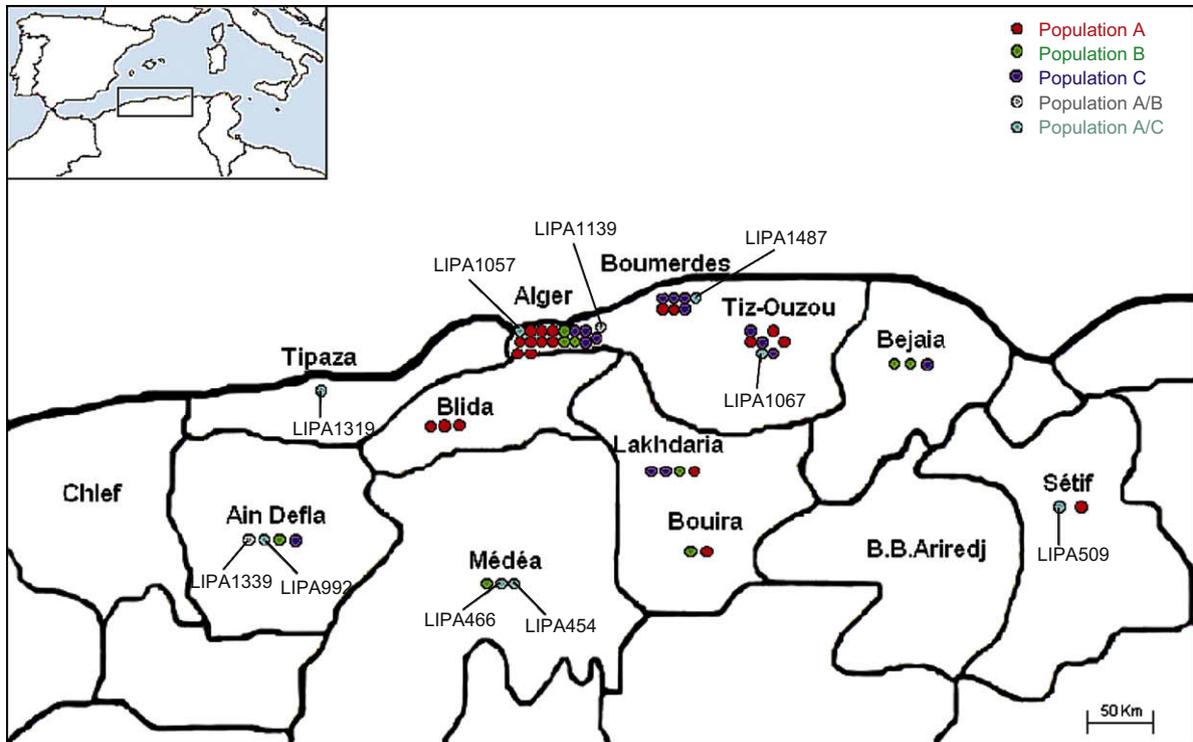


Fig. 2. Geographical distribution of the strains according to the populations identified by STRUCTURE analysis and the NJ tree.

exception in a single marker, where a weak additional band was detected in one of the strains.

4. Discussion

This molecular epidemiology study of leishmaniasis in Algeria using 14 microsatellite markers demonstrated high

variability (55 genotypes) in 55 strains of *L. infantum* isolated from VL and CL cases. The three genetic clusters identified for Algerian *L. infantum* correlated largely, however not completely, with isoenzyme typing. Interestingly, population A consists only of Algerian MON-1 strains and the single Tunisian MON-1 strain, but population B comprises MON-1 strains from both Algeria and Europe. This might be an effect of the close relationship between people from South Europe and North Africa and frequent migration between these places. Population C was genetically more heterogeneous as the two populations of MON-1 strains. MLMT results did not correlate to host origin as strains from humans and dogs are grouped together in populations A and B. The majority of strains isolated from dermatropic cases, mainly caused by MON-24 in Algeria [3], grouped in population C, whilst population A and B comprised most of the visceral cases. More strains from different clinical forms of the disease need to be investigated, and the role of host susceptibility towards infections by *L. infantum* and of sand fly factors needs to be elucidated in order to better understand the importance of genetic differences between parasites causing VL and CL in Algeria.

The three populations of Algerian *L. infantum* overlap geographically, as shown in Fig. 2, and no clear trend for geographical distribution was noticed. This was in contrast to the polymorphisms in the *cpb* gene, which indicated a trend for a West/East cline in the distribution of strains of *L. infantum*, from ancient to new foci in Algeria, being an indication of a founder effect [6]. There was no association between the population structure based on the 55 MLMT profiles and the five different genetic groups identified by *gp63/cpb* PCR-RFLP. This could be due to the fact that

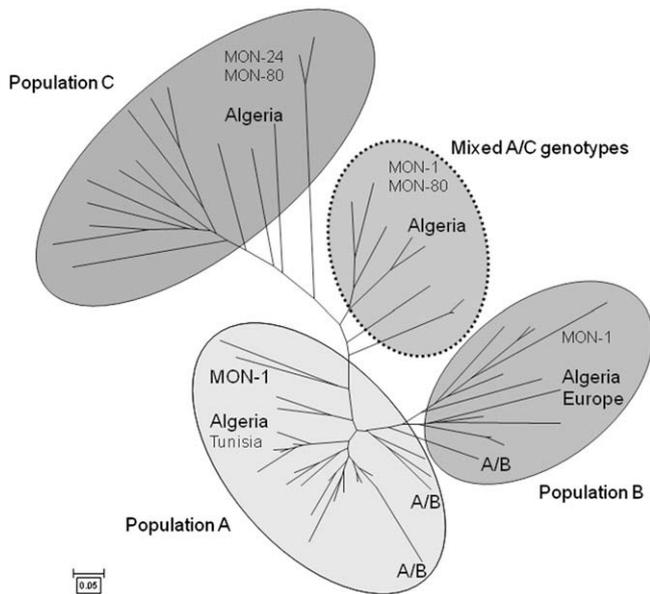


Fig. 3. Neighbor-joining tree (unrooted) inferred from the Das distances calculated for the data of 14 microsatellites markers for 60 strains. Three main populations A (MON-1), B (MON-1), and C (MON-24, MON-80) and one group representing the mixed A/C genotype isolates were identified. Isolates with mixed A/B genotype are also shown.

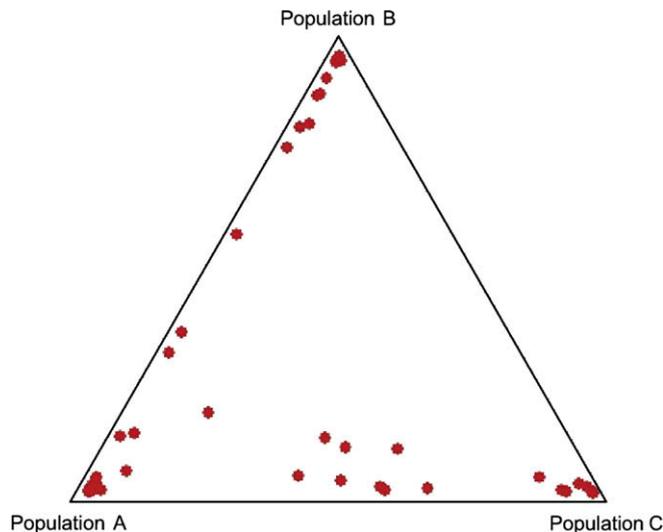


Fig. 4. Illustration of gene flow between populations as inferred by STRUCTURE. Each data point corresponds to a single strain whose proportion of ancestry from each of the three sources is represented by its proximity to the corresponding corner of the triangle.

microsatellites are neutral single copy markers, whereas selective pressure is acting on the multicopy antigen-coding genes *gp63* and *cpb*.

When used to characterize strains isolated during different episodes of the disease from three patients, MLMT revealed different scenarios. For one patient the strains from two successive episodes were identical and a relapse of the infection is most likely. For another patient a completely different, hybrid microsatellite pattern was obtained for the strains from the second episode, possibly as result of re-infection with this new strain. Alternatively, the first infection might have been caused by two strains, of which one was not detected during the first episode. The hybrid pattern of the second strain could be due to an isolation of two strains during the second episode or result from hybridization between the two original strains. For the third patient the first two episodes were due to a relapse (identical MLMT profiles) whereas the third episode was a clear re-infection.

Both the Bayesian clustering approach and the analysis of genetic distances revealed the existence of strains having intermediate membership coefficients between two populations, between the two MON-1 populations on one side

Table 2

Measures of genetic diversity for the three populations of *L. infantum* identified by STRUCTURE

Population	<i>N</i>	<i>P</i>	MNA	<i>H_e</i>	<i>H_o</i>	<i>F_{IS}</i>
A	23	0.786	3.214	0.262	0.172	0.347
B	11	0.786	2.929	0.381	0.140	0.643
C	15	1.000	4.643	0.591	0.340	0.400
Mean	16.3	0.857	3.595	0.411	0.217	0.463

The hybrid genotypes have been excluded. MNA, mean number of alleles/locus; *P*, proportion of polymorphic loci; *N*, sample size; *A*, number of alleles; *H_o*, observed heterozygosity; *H_e*, expected heterozygosity; *F_{IS}*, inbreeding coefficient.

and between population A (MON-1) and C (MON-24) on the other. These strains had mixed genotypes with alleles characteristic for two different populations. The A/B mixed genotypes probably represent genetic migration among different MON-1 populations similarly to what has been recently described for European populations of *L. infantum* MON-1 [24]. In the case of the A/C genotype between eight and 13 biallelic loci, characteristic for both populations A and C were detected. Interestingly, three of the four strains typed as MON-80 had mixed A/C genotypes and the other ones had been typed as MON-1. At the moment we cannot exclude that heterozygous alleles are due to mixed infections or eventual bias occurring during cultivation of parasites as shown for several *Leishmania* species in the new [18] and the old world [19–21]. For this it would be needed to clone isolates and re-type a representative number of clones. Heterozygosity can also be caused by mutation in only one of the alleles which would be a likely explanation for single heterozygous sites.

Since genome mosaic structure or mixed genotypes as identified in this study present multiple heterozygous loci, recombination between strains with different alleles seems to be the most parsimonious explanation [22]. The sympatric distribution of the A, B and C strains in Algeria could create the opportunity for genetic exchange between them. The demonstration of considerable gene flow (Fig. 4) between populations A and B, as well as A and C, respectively, is supporting this hypothesis. Why genetic exchange was not observed between B and C remains to be established.

Genetic recombination is the main origin of the genetic variability in species and the consequence of the sexual reproduction. So far, *Leishmania* species are considered to reproduce asexually because sexual reproduction has not been demonstrated experimentally and not yet in nature. Although most molecular epidemiological studies have confirmed a primarily clonal population structure in different species [23–26] they all pointed to probability of occasional recombination events in *Leishmania*. There is growing evidence for the existence of hybrid strains resulting from genetic exchange between different populations and even between different species of *Leishmania* [22,24,27–29]. This study adds the identification of putative hybrids between two populations of MON-1 strains but also between strains of MON-1 and MON-24/80 in Algeria. Interestingly, natural *L. infantum/L. major* hybrids isolated from HIV co-infected patients in Portugal [28] seem to have enhanced transmission potential and fitness since they were found to survive in *Phlebotomus papatasi*, the specific vector for *L. major* [30]. For the putative hybrids detected in this study the hybrid status needs to be confirmed by cloning and repeating MLMT on these clones. Confirmed hybrids should then be tested for changes in their biological features such as virulence and fitness in different hosts. Although mechanisms of genetic exchange and its frequency in different natural settings still remain unclear, we agree that the role of recombination in *Leishmania* is probably underestimated and has had at least historical importance in this protozoan parasite [22,26].

Finally, this study demonstrated the usefulness of MLMT for strain typing and population genetic analysis in *L. infantum* foci. Three genetically different populations, two comprising most of the MON-1 strains, and one most of the MON-24 strains were found to be distributed sympatrically in Algeria. Gene flow between them and putative hybrids were detected warranting further investigation. MLMT was also of high value for differentiating relapses or re-infections for patients suffering from multiple episodes of the disease.

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