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Guns, germs and dogs: On the origin of *Leishmania chagasi*

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ABSTRACT

The evolutionary history of *Leishmania chagasi*, the aetiological agent of visceral leishmaniasis in South America has been widely debated. This study addresses the problem of the origin of *L. chagasi*, its timing and demography with fast evolving genetic markers, a suite of Bayesian clustering algorithms and coalescent modelling. Here, using 14 microsatellite markers, 450 strains from the *Leishmania donovani* complex, we show that the vast majority of the Central and South American *L. chagasi* were nested within the Portuguese *Leishmania infantum* clade. Moreover, *L. chagasi* allelic richness was half that of their Old World counterparts. The bottleneck signature was estimated to be about 500 years old and the settlement of *L. chagasi* in the New World, probably via infected dogs, was accompanied by a thousand-fold population decrease. Visceral leishmaniasis, lethal if untreated, is therefore one more disease that the Conquistadores brought to the New World.

1. Introduction

The origin of *Leishmania chagasi*, the aetiological agent of visceral leishmaniasis in the Americas is associated with contrasting scenarios and intimately linked with our past history (Killick-Kendrick et al., 1980). This protozoan flagellate is a member of the *Leishmania donovani* complex that encompasses two other species from the Old World (*Leishmania infantum* and *L. donovani*). Until recently, based on surface proteins, glycoconjugate ligands and radiorespirometry, *L. chagasi* was considered as a "clear" species (Lainson et al., 1987) indigenous in the New World. However, this situation did not hold longer once polymorphic genetic markers replaced the phenotypic data and different authors suggested that *L. chagasi* is in fact a *L. infantum* subpopulation that arose from imported European strains (Kuhls et al., 2008; Lukes et al., 2007; Mauricio et al., 2000; Momen and Cupolillo, 2000). Though this scenario convinced the "molecularists", there is still an ongoing controversy concerning the age and geographic origin of this pathogen. Moreover, some specialists cast some doubts that the Old World protozoan might have encountered the right vector in the New World (Lainson et al., 1987). This study addresses the problem of the origin of *L. chagasi*, its demography and evolutionary timing with fast evolving genetic markers by applying Bayesian methods, coalescent modelling and phylogenetics. Using data from a set of 14 microsatellite markers (Kuhls et al., 2007), we examined strains from the Mediterranean area, Asia, Africa, South and Central America ($n = 450$) (Kuhls et al., in press). Our results confirm the Old World origin of *L. chagasi*, dramatically improve the detection of the source population and determine a temporal frame for this transcontinental transfer. Finally, we unravel the parallel evolutionary histories and demographies of humans and one of their pathogens.

2. Materials and methods

2.1. Sampling and genotyping

To infer the *L. donovani* complex evolutionary history, we used a sample of 450 strains, from European, African and Asian countries, representative of the species complex diversity. More specifically, according to the topic of this communication we genotyped 106 *L. chagasi* strains from Honduras, Panama, Costa Rica, Colombia, Venezuela, Paraguay and Brazil. Sources, designation, geographical origins, MLEE identification, if known, are provided in Table S1. Most of the samples were of clinical origin; only few were isolated from proven and suspected reservoir animals and from sand fly vectors. DNA was isolated using proteinase K-phenol/chloroform extraction or the Wizard™ Genomic DNA Purification System (Promega, Madison, WI, USA) according the manufacturer's

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protocol, suspended in TE-buffer or distilled water and stored at 4 °C until use. The parasites were genotyped using 14 microsatellite markers as previously described (Kuhls et al., 2007; Ochsenreither et al., 2006). We focused on these nuclear markers because they currently provide the most powerful and discriminative method for strain differentiation and population genetics in this species complex. PCRs were performed with fluorescence-conjugated forward primers. Screening of length variations of the amplified markers was done by automated fragment analysis using capillary sequencers. PCR products from amplified microsatellites were analysed either with the fragment analysis tool of the CEQ 8000 automated genetic analysis system (Beckman Coulter, USA) or the ABI PRISM GeneMapper (Applied Biosystems, Foster City, CA). Microsatellites allelic profiles are available upon request.

2.2. Genetic diversity estimation

The number of alleles (allelic richness) in Old and New World populations was estimated and sample sizes were corrected by the rarefaction procedure using Hp-rare (Kalinowski, 2005). Comparison tests as well as *P*-values were estimated using the Statistica 6.1 package.

2.3. Phylogenetic inferences

Cavalli-Sforza chord distance (Cavalli-Sforza and Edwards, 1967) was used to construct a population tree using a neighbor-joining algorithm (Saitou and Nei, 1987) as implemented in the software POPULATIONS v.1.2.30 (http://bioinformatics.org). Sup-

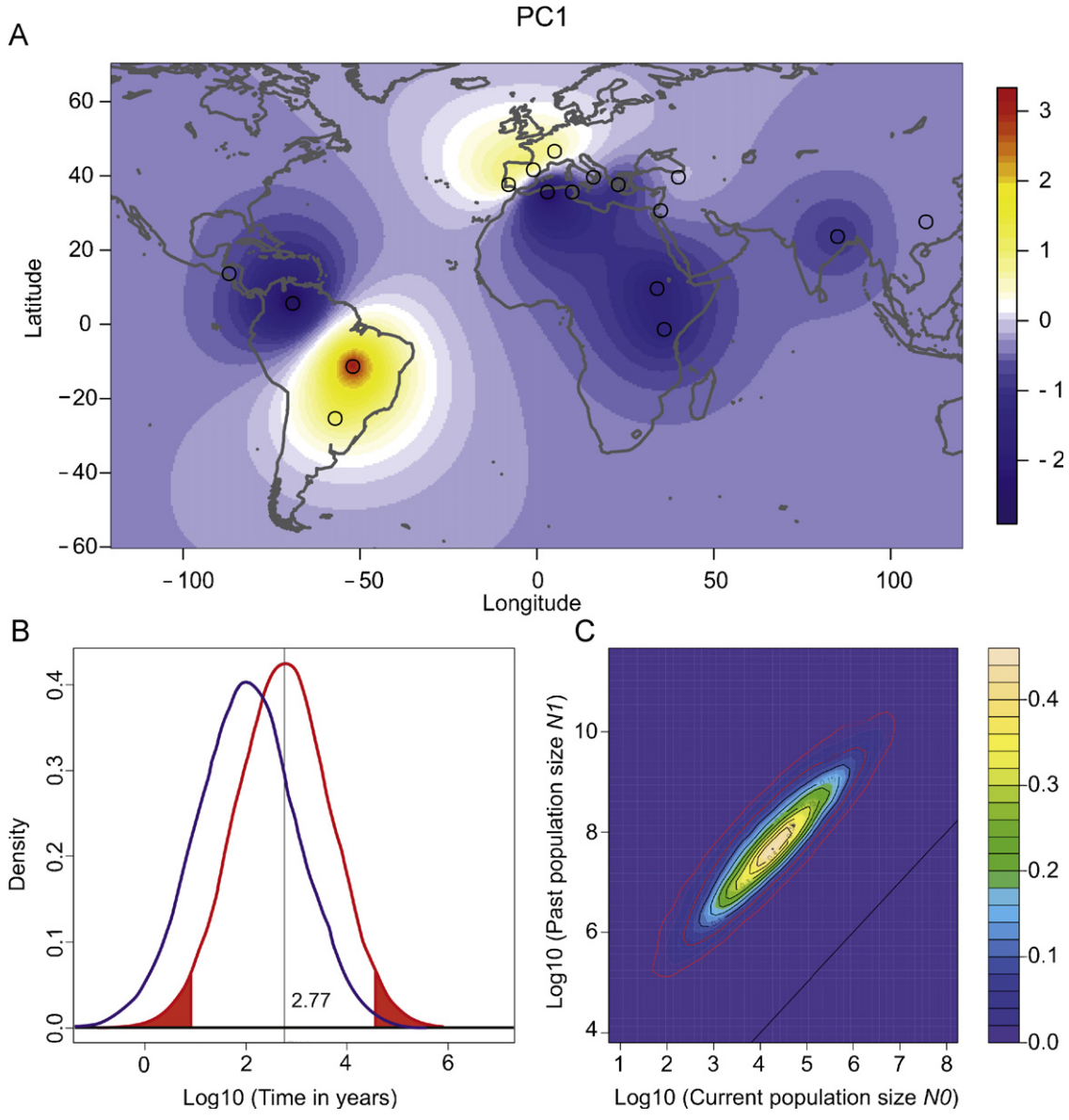

Fig. 1. (A) Synthetic map of the first principal component for *L. donovani* complex populations based on the 14 microsatellite polymorphisms. The first PC accounts for about 99% of the total genetic variation (PC2 accounts for 0.1% and contains the vast majority of the Old World samples). The PC1 map exhibits maximal values for western European and southern American samples confirming the shared ancestry of these populations. (B) Detection of a recent contraction in the *L. chagasi* lineage using the Bayesian methods MsVar. Posterior (red) and prior (blue) distributions of the elapsed time since *L. chagasi* population declined, including the 95% credibility intervals of the posterior distribution (between the two red areas). Time is expressed in years on a log scale and point estimate (mode of the posterior distribution) is indicated with the vertical thin line. (C) Two-dimensional density plot of the marginal posterior distribution of $\log(N_0)$ and $\log(N_1)$, where N_0 is the current number of individuals and N_1 is the number of individuals before contraction. Red isolines represent 95% and 99% credibility intervals. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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port for the tree nodes was assessed by bootstrapping over individuals (100 iterations).

2.4. Inferring population structure

Inference of population structure was first performed using principal component analysis (PCA) implemented in the "prcomp" function of the R statistical package on the normalized genotypic matrix. Principal component analysis is a tool for exploring multilocus population genetic data (Cavalli-Sforza et al., 1994; Patterson et al., 2006). The results of the PCA can be visualized using "synthetic maps" that describe how each principal component varies across geographic space. In this representation, each PC is interpolated and displayed on a separate map (Cavalli-Sforza et al., 1994). PCA results were spatially interpolated using the Kriging method and displayed on geographic maps. Two genetic clustering algorithms were run: (i) TESS 2.3 (Chen et al., 2007; Durand et al., 2009) was used by analysing 100 runs of 50,000 iterations for each value of the number of clusters, K, from 2 to 4 using a burn-in period of 2000 sweeps. Admixture coefficients where then averaged over the 10 runs with the smallest values of the deviance information criterion and the values for each cluster were displayed on separate maps. (ii) We also implemented the spatial model of GENELAND 3.1.4 (Guillot et al., 2008) with the Dirichlet model for allelic frequencies for $K = 2$ (first split) and $K = 3$. We used 10 long runs of 10^7 iterations with a thinning of 500 and a burn-in of 50% under the spatial and correlated allelic frequency model.

2.5. Coalescence, TMRCA and demography

We used a Markov chain Monte Carlo Bayesian approach (Beaumont, 1999) that assumes a stepwise mutation model for the microsatellite markers and estimates the posterior probability distributions of the genealogical and demographic parameters under a model of a single population of variable size. This method permits to infer important biological parameters like the past (N_1) and present (N_0) effective population sizes and the time, in years, that has elapsed since the last demographic change (decline or expansion) began (T) (Wirth et al., 2008). The software MSVAR 1.3 (Beaumont, 1999) provides separate estimates of those parameters and was run on the 106 *L. chagasi* strains. The analyses were performed assuming exponential demographic change. A prior mean mutation rate of 10^{-4} per replication was considered based on prokaryotes and *Saccharomyces cerevisiae* experiments (Henderson and Petes, 1992; Vogler et al., 2006, 2007; Wierdl et al., 1997), and uninformative prior means of 10^2 were considered for time and population size parameters. The generation time was set on one day (Chakraborty and Das Gupta, 1962). Three chains of 8×10^8 iterations with a thinning of 20,000 were run for each analysis to confirm the convergence of the analyses. Contraction signatures assessed with a burn-in of 50% were robust and were confirmed with additional runs where an expansion was assumed as a prior.

3. Results and discussion

3.1. Leishmania chagasi origin and genetic structure

We applied three complementary approaches, principal component analysis (PCA), as well as two clustering algorithms, TESS 2.3 (Chen et al., 2007; Durand et al., 2009) and GENELAND 3.1.4 (Guillot et al., 2008). The two Bayesian programs infer population genetic structure based on multilocus genotypes and individual spatial coordinates. TESS infers individual admixture proportions in K ancestral populations, whereas GENELAND tries to assign individuals to K random mating groups. The first PC explained about 99% of the variation in the entire data set and suggested a clear link between *L. infantum* strains from southwest Europe and *L. chagasi* strains from South America (Fig. 1A). The abilities of principal components to estimate admixture proportions have been recently investigated by several studies (Patterson et al., 2006; Francois et al., 2010). Here the PC analysis was in complete agreement with the TESS analysis (Fig. S1), confirming the shared ancestry of the lineages. Moreover, using a hierarchical approach, we investigated the first split ($K = 2$) that could be detected using the spatial model implemented in Geneland (Fig. S2). Interestingly, the vast majority of the *L. chagasi* strains (94 out of 106; 88.7%) clustered with the Portuguese *L. infantum* sample. For $K = 3$, the *L. donovani* complex split accordingly to the accepted nomenclature (*L. donovani*, *L. infantum* with the exception of Portugal, and *L. chagasi*). This result was confirmed when a phylogenetic reconstruction using the Cavalli-Sforza chord distance was implemented (Fig. 2). Indeed, the *L. chagasi* and Portugal lineages were supported by strong bootstrap support (99%) and formed one sister-group within the European *L. infantum* clade. Twelve strains of *L. chagasi* differed from the main pool (data shown elsewhere in Kuhls et al., in press), half of them belonged to Venezuela, and they likely reflect independent minor import events from Spain or Southern-Europe. Those strains differed from the others by their non-MON-1 genotype, confirming a different evolutionary origin. They could not be clearly assigned to their source population due to a lack of statistical power (i.e. sample size to small for accurate allelic frequencies estimates and source population assignment).

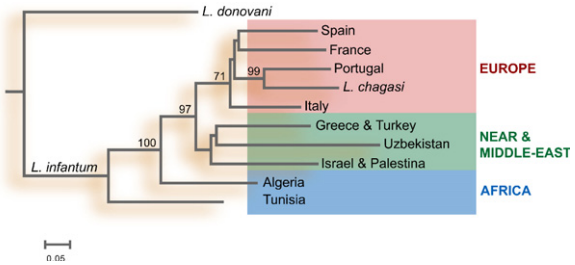

Fig. 2. Neighbor-joining (NJ) phenogram summarizing Cavalli-Sforza & Edwards' (1967) chord distances, D_{CE}, among 11 populations of *Leishmania infantum* strains collected in Europe, the Near and Middle East and in Africa. When the number of strains was too small (<10), we pooled geographically closely related samples in order to avoid statistical discrepancies due to strong allelic variance. The *L. donovani* strains were used as an out-group. Values on the nodes represent the percentage of bootstrap replicates over loci ($n = 100$). Branch lengths are proportional to the genetic distance between the taxa. The scale bar represents a distance D_{CE} of 0.05.

3.2. Demogenetics and dating

Once validated, the status of *L. chagasi* as an emerging pathogen in the New World might leave specific signatures, like a loss of diversity accompanying a founding event. This is exactly what we observed as the mean allelic richness of *L. chagasi* was significantly lower ($P < 0.001$) when compared to the source *L. infantum* populations (Fig. 3). Moreover, the mean number of private alleles in *L. chagasi* was close to 1, achieving a six-fold decrease when compared to the *L. infantum* samples. High levels of genetic diversity are a surrogate indication of ancestral origins as illustrated in African human populations. To estimate and confirm the prior mutation rate, we used Msvar (Beaumont, 1999), a coalescent based method. Indeed a mutation rate of 10^{-4} seems to be realistic since all posterior estimates converged near this initial value. The algorithm indicated that *L. chagasi* populations had undergone a severe and recent decline (Fig. 1C). Inferred current population sizes were extremely small (~15,000) whereas ancestral population sizes were estimated to be close to fifty

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Table 1
Bayesian estimates for the actual and ancestral population sizes, the time when the exponential decrease in population size started and the mean mutation rate of the nine loci considered. The analysis was done using the MsVar method; population sizes are expressed as a number of individuals and time in generations. Point estimates correspond to the mode of each marginal posterior distribution and corresponding 95% credibility intervals are given in brackets.

Actual population size (N_0)	Ancestral population size (N_1)	Time when bottleneck started (in years)	Mutation rate/year
15,500 [302; 1.4×10^6]	43,650,000 [0.8×10^6; 3.7×10^6]	537 [7.9; 33,900]	3.8×10^{-7} [5.8×10^{-9}; 2.5×10^{-5}]
Actual population size, θ_0	Ancestral population size, θ_1	Time when bottleneck started $T/2$, N_0	PopSize ratio, N_1/N_0
0.040 [0.007; 0.0930]	74.1 [25; 224]	0.0148 [0.010; 0.023]	3.89×10^{-4} [6.46×10^{-5}; 1.66×10^{-3}]

Settings: Bayes factor bottleneck = infinity, point estimate = mode; [*XX*; *XX*] = 95% credibility intervals; starting values: $N_0 = 1.0 \times 10^6$, $N_1 = 1.0 \times 10^4$, $\mu = 1.0 \times 10^{-8}$, $T = 500$. Thinning 20,000 and 800,000,000 iterations.

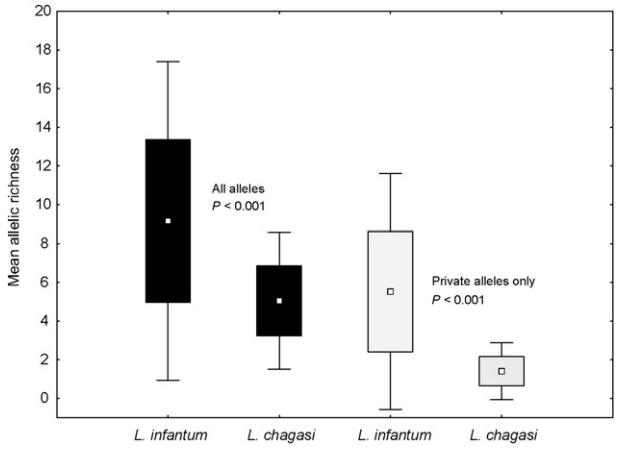

Fig. 3. Genetic variability in the different *L. infantum* lineages. Microsatellite mean allelic richness in *L. infantum* (Old World) and *L. chagasi* (New World) was computed for all alleles and for private alleles only, using the rarefaction algorithm HPrare. Notice that the Central and South American genetic diversity is significantly lower than the African and European genetic diversity ($P < 0.001$).

millions (Table 1). According to Msvar the timing of the population decline occurred 500 years ago (Fig. 1B). However, these numbers must be taken with caution, due to the rather large confidence intervals produced by the method. The differences observed between the PCA/Tess and the Geneland/NJ analyses are probably due to the assumptions of the models and the rather small samples sizes in Central America. The latter models rely on a non-recombining model whereas the two others infer admixture ancestry. Yet the reproductive system of this parasite is intermediate between those models and seems to combine moderately frequent recombinants with clonal expansions (Volf and Sadlova, 2009).

4. Conclusions

From the evidence we cumulate in this report, it is now clear that *L. chagasi* is de facto a *L. infantum* sub-population that emerged from a Portuguese population which had crossed the Atlantic Ocean most probably in the XVIth century via infected dogs and reached Central America and Brazil. The vast majority of the strains came through a first invasion wave, but secondary limited introductions also occurred. Thus, visceral leishmaniasis, lethal if untreated, is one more disease that the Portuguese Conquistadores brought in the New World, like flue and smallpox (Diamond, 1997). This study combines historical, demographic and genetic information to unravel the evolutionary history of an important tropical disease, and illustrates the benefits of Bayesian statistics and multifactorial analyses in the study of non-model pathogens. This study is yet another illustration of how human-bugs can unravel host migrations and origins (Morelli et al., 2010; Wirth et al., 2004, 2005; Wolfe et al., 2007).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2011.04.004.

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