

## Population genetics

### Evolutionary genetics and molecular diagnosis of *Leishmania* species

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#### Abstract

An extensive study has been performed on various natural populations of *Leishmania* from the 'Old' and 'New Worlds' using multilocus enzyme electrophoresis and random amplification of polymorphic deoxyribonucleic acid. The data are interpreted in evolutionary genetic terms in order to give a firm basis to studies dealing with the relevant medical properties of pathogens. We confirm that *Leishmania* undergoes clonal evolution with occasional phenomena of hybridization. This suggests that the microorganism genotypes are stable in space and time and consequently have epidemiological and medical relevance. It is crucial to have a clear definition of the taxa to be identified. In the case of *Leishmania*, there is at present no firm consensus on the species concept. We propose that any new species of *Leishmania* should correspond to a 'discrete typing unit' that exhibits specific medical and/or epidemiological characters. Based on this approach, the species status of *L. peruviana* can be supported. On the contrary, *L. panamensis* cannot be clearly distinguished from *L. guyanensis*. Our studies on the genetic diversity of *Leishmania* show that a given stock is not representative of the genus, the subgenera, or any species. We suggest that the genetic polymorphism of *Leishmania* has a strong impact on the parasite's biomedical properties.

**Keywords:** leishmaniasis, *Leishmania* spp., evolutionary genetics, population structure, genetic diversity

#### Introduction

In spite of notable advances in our knowledge of the basic biology of *Leishmania*, leishmaniasis remain a serious health problem and are classified as one of the endemic diseases by the World Health Organization. Recent studies have revealed the reactivation or appearance of numerous foci, demonstrating that the severity of these diseases is underestimated (e.g., MARTINEZ *et al.*, 1998; CHIHEB *et al.*, 1999; FELICIANGELI *et al.*, 1999).

Antileishmanial drugs are toxic and the not always satisfactory, which makes their use difficult. Moreover, no vaccine is available against the leishmaniasis and the control of phlebotomine sand flies is difficult. For these reasons, the control of leishmaniasis is far from satisfactory, and considerable scientific effort is sorely needed to improve this situation. From this perspective, evolutionary genetics, the topic of the present paper, is one of the most promising avenues. Evolutionary genetics improves our basic knowledge of the taxonomy and evolution of microorganisms, and also greatly contributes to applied research in microbiology. It permits the accurate identification of pathogenic agents including *Leishmania* at the level of subspecies and strain ('molecular epidemiology'; CDC, 1994). It also provides a firm basis for all studies dealing with the relevant medical properties of pathogens, such as pathogenicity and resistance to drugs or vaccines ('downstream studies') (TIBAYRENC, 1996).

#### Population structure and strain typing

The aim of this section is to show that the study of population structure is indispensable to evaluate the epidemiological and medical relevance of strain typing. Too often, molecular markers are used for epidemiological tracking in an empirical way, which can lead to highly misleading results. It is essential to estimate the impact of genetic exchange in natural populations in order to assess the stability in space and time of the microorganisms' genotypes, and hence their epidemiological and medical relevance. The 'clonality/sexuality debate' (ØRSKOV & ØRSKOV, 1983; SELANDER *et al.*, 1987; TIBAYRENC *et al.*, 1990; WALLIKER, 1991; MAYNARD SMITH *et al.*, 1993), has revealed that many species

of pathogenic agents have sexuality, in the sense of exchange of genetic information between different cells. The rate of genetic exchange has a deep impact on the population structure and genotype stability of the species under study. If genetic exchange is very frequent, the situation tends towards panmixia, in which genetic recombination occurs at random. In this case, the pathogen's genotypes are ephemeral and can be equated to individual variants; as a consequence, multilocus typing will be misleading. At the other extreme, if sex is very rare or absent, the individual genotypes will be extremely stable and could even last thousands or millions of years (clonal evolution). Moreover, if the microorganism under study evolves clonally, all its genes are tightly linked (linkage disequilibrium, see below). Therefore, apart from the possibility of using the multilocus genotypes as epidemiological markers, it is possible to design indirect markers for genes coding for pathogenicity, clinical forms or resistance to drugs. Between these two polar situations, some species of microorganisms may adopt intermediary behaviours, such as 'epidemic clonality' (occasional bouts of short-term clonal propagation in a basically sexual species; MAYNARD SMITH *et al.*, 1993). Lastly, clonal evolution can be mimicked by cryptic speciation, the 'species' under study being actually divided into 2 or more biological species, genetically isolated from each other (MAYNARD SMITH *et al.*, 1993).

In the case of microparasites, the basic statistics for evaluating the rate of genetic exchange in natural populations rely on the analysis of linkage disequilibrium or non-random association between genotypes occurring at different loci. If linkage disequilibrium is weak or absent, genetic recombination occurs freely in the population under survey and, therefore, sex is abundant. On the contrary, if linkage disequilibrium is strong, this is taken as circumstantial evidence that the population is either clonal or subdivided into cryptic species. The biases due to physical obstacles (space and/or time), that could also generate linkage disequilibrium (the Wahlund effect), must be evaluated (TIBAYRENC, 1995).

The population structure of *Leishmania* is still a matter of debate. These pathogens have been included in the clonal model (TIBAYRENC *et al.*, 1990). However, other authors have challenged this hypothesis, and have proposed from pulse field gel electrophoresis data that some *Leishmania* populations are potentially panmictic (BASTIEN *et al.*, 1992). Powerful statistical analysis of the

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population structure in *Leishmania* is rendered difficult by the fact that many *Leishmania* species exhibit only limited genetic variability in comparison with other parasites (e.g., *Trypanosoma cruzi*, the agent of Chagas disease). All things being equal, this generates a strong risk of statistical type II error: linkage disequilibrium tests are negative, not because the population is panmictic, but because the test lacks power of resolution (TIBAYRENC, 1995). However, even with this restriction, convergent results from our team have confirmed that several *Leishmania* species exhibit strong linkage disequilibrium, which argues against the hypothesis of a panmictic structure and is in favour of clonal evolution (BAÑULS *et al.*, 1999, 2000). A particularly telling situation, that we have observed in several cases, is the statistical correlation between genetic distances generated from isoenzyme data on one hand (see Fig. 1) and from random amplification of polymorphic deoxyribonucleic acid (RAPD; WILLIAMS *et al.*, 1990) (see Fig. 2) on the other. Such agreement between different kinds of genetic markers provides strong evidence for linkage disequilibrium (see Fig. 3).

In summary, although additional studies are necessary before we are able to generalize this model to the whole genus *Leishmania*, in those cases which are adequately documented these parasites appear to undergo clonal evolution with occasional hybridization phenomena

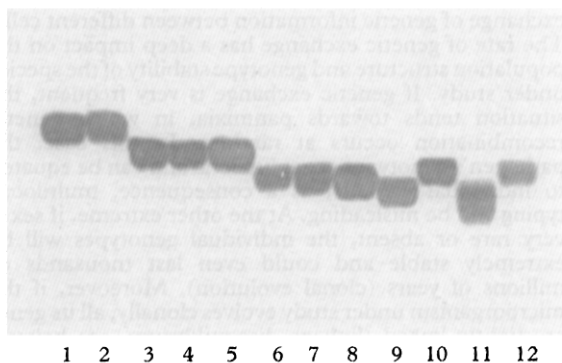


Fig. 1. Multilocus electrophoretic enzyme profiles of the enzymatic system 6-phosphogluconate dehydrogenase for different *Leishmania* species. Lanes 1–2, *L. (Leishmania) major*; lanes 3–5, *L. infantum*; lanes 6–8, 10, 12: *L. (Viannia) braziliensis*; lanes 9, 11: *L. lainsoni*.

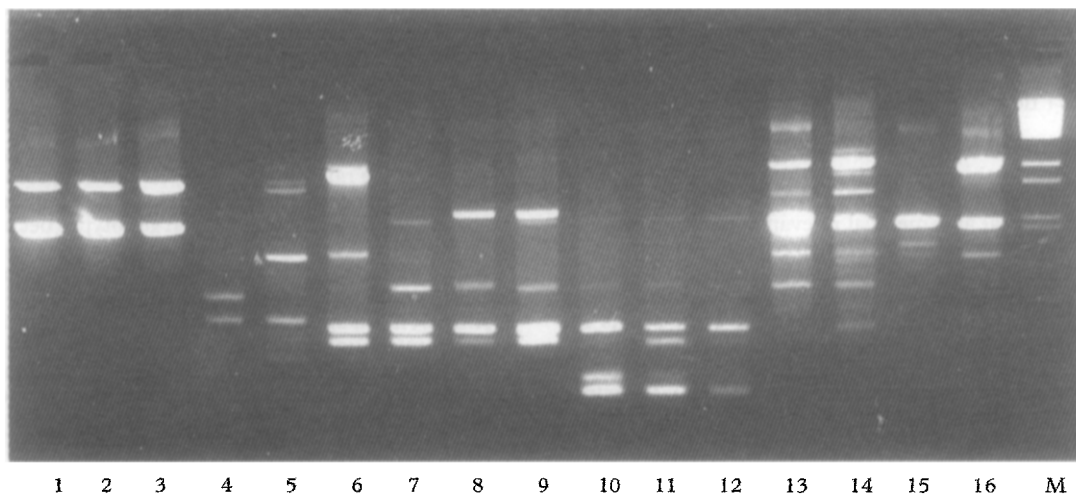


Fig. 2. Random amplification of polymorphic DNA profiles (WILLIAMS *et al.*, 1990) obtained with primer A10 for *Leishmania* species. Lane 1, *L. (Leishmania) infantum*; lane 2, *L. (L.) chagasi*; lane 3, *L. (L.) donovani*; lane 4, *L. (L.) tropica*; lane 5, *L. (L.) major*; lanes 6–9, *L. (Viannia) braziliensis*; lanes 10–12, *L. (V.) peruviana*; lane 13, *L. (V.) guyanensis*; lane 14, *L. (V.) panamensis*; lane 15, *L. (V.) lainsoni*; lane 16, *L. (L.) amazonensis*; lane M, molecular mass standards.

(EVANS *et al.*, 1987; BELLI *et al.*, 1994; BAÑULS *et al.*, 1997). This result implies that multilocus enzyme electrophoretic characterization (MLEE) of *Leishmania* strains, considering their stability in space and time due to their clonal propagation, is a powerful tool for epidemiological tracking. However, one has to keep in mind that the 'clones' identified by a limited set of genetic markers are not real clones but, rather, families of closely related clones. TIBAYRENC & AYALA (1991) have proposed the term clonets to refer to a set of genotypes that appear identical with a given set of molecular markers in a clonal species. If a marker with a higher power of resolution is used, a clonet may prove to be genetically heterogeneous. As an example, a sample of *L. (Leishmania) infantum* identified as zymodeme MON-1 by MLEE on starch gel revealed additional genetic variability when examined by MLEE on cellulose acetate and by RAPD (see Fig. 3). It is also probable that the clonets characterized by either isoenzymes or RAPD will show additional genetic heterogeneity if their microsatellites (short tandem repeats of deoxyribonucleic acid [DNA]) are examined, as these are markers with a faster molecular 'clock'. Multilocus characterization of *Leishmania* stocks using microsatellites should therefore prove to be a powerful epidemiological marker (RUSSELL *et al.*, 1999).

#### The species concept in *Leishmania*

In order to design reliable diagnostic tools, it is crucial to agree on a clear definition of the taxa to be identified. In the case of *Leishmania*, the notion of species has been particularly confusing. There is presently no firm consensus on the species concept to be used for these parasites. About 30 species of *Leishmania* have been described. Initially, species were defined exclusively on the basis of extrinsic criteria (biological, epidemiological and clinical characteristics). After 1970, with the development of genetic tools, 17 new species were described. According to the literature, the status of some of these species appears to be questionable. There is a risk that a non-codified use of refined molecular tools will lead to an uncontrolled and useless increase of *Leishmania* species. It is therefore urgent to agree on the definition of a species based on reliable and consensual concepts. For *Leishmania*, as for most pathogenic microorganisms, due to the scarcity or absence of genetic exchange, the biological species concept (BSC; MAYR, 1963), which is based on the interbreeding criterion, is difficult or impossible to apply. Species of asexual organisms were referred to as agamospecies by CAIN (1954).

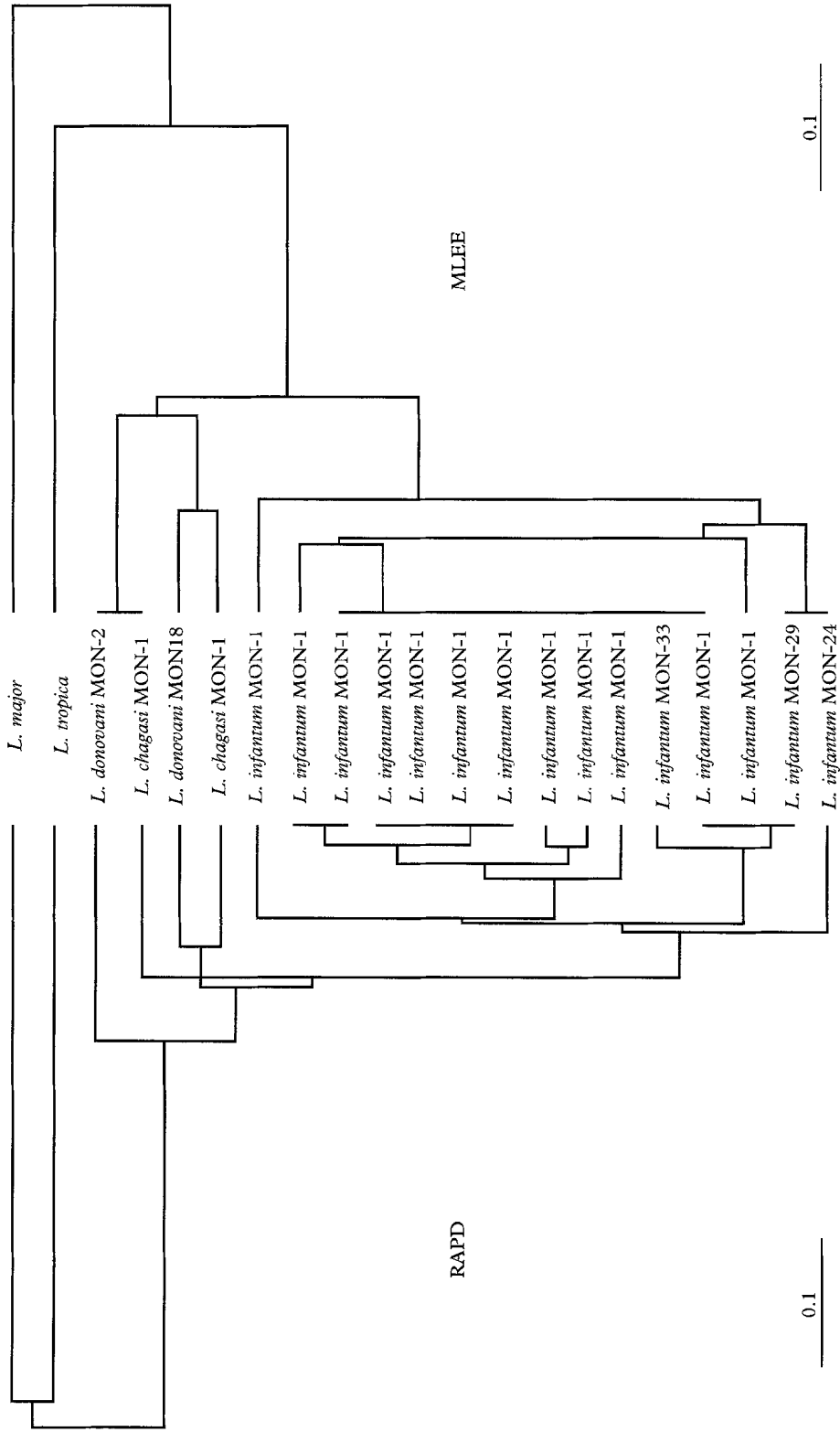


Fig. 3. Dendrograms derived from genetic distances obtained by multilocus enzyme electrophoresis (MLEE) (right) and random amplification of polymorphic DNA (RAPD) (left). The degree of agreement between the two dendrograms is evidence of linkage disequilibrium. These results reveal additional genetic diversity within the zymodeme MON-1 of *L. (Leishmania) infantum*.

The delimitation of such agamospecies is only a matter of convenience. When the BSC is not applicable, species can be described on the basis of phylogenetic individualization (phylogenetic species concept or PSC; CRAFT, 1983). In the case of pathogenic microorganisms, there is a consensus that the PSC should be supported by criteria of medical or epidemiological relevance (TIBAYRENC, 1993). The PSC requires that species can be equated to discrete evolutionary lines. In the case of pathogenic microorganisms, a purely cladistic approach is sometimes difficult, for 2 reasons. First, as stated above, even when clonal evolution is dominant in a given species, there is always the risk that the individualization of discrete phylogenetic lines has been clouded by occasional bouts of sex. Second, as cladistic methods were designed for the analysis of morphological characters, their use with molecular data is debatable (RICHARDSON *et al.*, 1986). TIBAYRENC (1998, 1999) has proposed the concept of 'discrete typing unit' (DTU) to refer to a collection of microbial genotypes that are more related to each other than to any other genotype and that share one or more genetic characters (tags). From this perspective, we may propose that a new species of *Leishmania* should correspond to a DTU that exhibits specific medical and/or epidemiological characters. In the light of this proposal we have reconsidered the species status of *L. (Viannia) peruviana*, and the specific distinction between *L. (V.) guyanensis* and *L. (V.) panamensis*. We propose that *L. (V.) peruviana* deserves species status (BAÑULS *et al.*, 2000), since it corresponds to a DTU that has specific epidemiological and medical properties (geographical range, pathogenicity). On the contrary, in spite of the use of a broad range of genetic markers (16 isoenzyme loci and 13 RAPD primers), we were unable to confirm a clear phylogenetic separation between *L. guyanensis* and *L. (V.) panamensis*. The epidemiological/medical distinction between these 2 taxa is also far from clear. The species status of these 2 taxa therefore remains debatable (BAÑULS *et al.*, 1999c). This is the case, too, for other species that demand extensive additional phylogenetic studies (*L. (L.) pifanoi*, *L. (L.) garnhami*, *L. (L.) chagasi*, *L. (L.) donovani*).

The DTUs and tags are individualized by a pragmatic use of phylogenetic methods. Most of them are available as convenient software, of which the most widely used is the 'phylip' package of Joe Felsenstein. It is crucial to base the phylogenetic analysis on a representative set of stocks sampled in diversified ecoepidemiological conditions. The goal is to avoid an 'iceberg bias' (TIBAYRENC, 1999); that is, to identify artefactual phylogenetic subdivisions that merely result from examining limited subsamples of the total phylogenetic diversity of the populations under study (for example: geographical populations, or collections of genotypes specialized in a given host or vector). Even if one is very careful in choosing representative samples, it is not possible to guarantee, in the case of *Leishmania*, that iceberg bias has been entirely avoided. Our knowledge of these parasites' transmission cycles probably needs much refinement, and one cannot rule out the possibility that entire populations of a given species remain unsampled.

Tags are convenient markers specifically to identify given DTUs corresponding to either distinct species or additional within-species subdivisions. Provided that their specificity is based on a sufficient sample, they may correspond to various genetic or biochemical characters. Although their specificity is the first criterion for selecting them, it is desirable that they should also be sensitive, easy to use, and cheap. Another desirable property is that they should be usable without prior cultivation *in vitro*, which is fastidious and costly and introduces an additional bias. Since a stock isolated from a given host may correspond to a mixture of different genotypes, there is a risk that cultivation selects given genotypes, so that the stock characterized after culturing is different from the original stock.

### Genetic diversity: a neglected character in *Leishmania*?

Convergent results based on various genetic markers show clearly that the phylogenetic diversity of the whole genus *Leishmania* is considerable. At such a level of phylogenetic divergence, slowly evolving markers such as ribosomal ribonucleic acid genes are indispensable to provide a reliable picture of the various phylogenetic subdivisions within this genus. It is advisable, therefore, not to limit research efforts to only one strain of only one species of *Leishmania*. As an example, the Friedlin strain of the species *L. (L.) major*, the only stock which has been sequenced in the *Leishmania* genome project, cannot be taken as representative of the genus *Leishmania*, the subgenus *Leishmania*, or the species *L. (L.) major* (see BAÑULS *et al.*, 1999b). When the species level is considered, it is apparent that most species of *Leishmania* exhibit considerable genetic diversity, although from this point of view they cannot match *T. cruzi* (see TIBAYRENC, 1995; BAÑULS *et al.*, 1999a).

*Leishmania* species, at least in general terms, have specific pathogenic properties. However, within the species level, there is a diversity of pathogenicity too. Clear examples are *L. (V.) braziliensis* and *L. (L.) infantum*. *L. (V.) braziliensis* is responsible for cutaneous forms of disease, but a significant number of cases develop to severe mucocutaneous infection (espundia). *L. (L.) infantum* can cause purely cutaneous disease as well as visceral leishmaniasis. The respective roles of environmental factors and the host's and the parasite's genetic factors in this pathogenic diversity are still a matter for debate. However, it is an unwarranted hypothesis to consider that the parasite's genetic diversity within the species level has no impact on the clinical diversity of leishmaniases. So far, at an intraspecific level, efforts to establish relationships between clinical forms of the disease and given genotypes of the parasite have not yielded clear-cut results. Indeed, some associations between zymodemes or RAPD markers and clinical forms have been recorded only in the species *L. (L.) infantum* (see PRATLONG *et al.*, 1994, 1995) and *L. (V.) peruviana* (see BAÑULS *et al.*, 2000). However, the use of more discriminative markers such as microsatellites gives a hope of achieving clearer results (RUSSELL *et al.*, 1999). Even though the working hypothesis that within-species genetic diversity of *Leishmania* has a significant effect on the clinical diversity of the disease has not yet been clearly confirmed, it would be at best tentative to consider that only one strain is representative of a given species. This reasoning can be extended to all relevant biomedical properties related to these parasites, not only clinical diversity but also immunological properties, resistance to drugs and to vaccines, and transmissibility through phlebotomine sand flies. We have shown that the phylogenetic diversity of natural clones of *T. cruzi* has a strong impact on this parasite's biomedical properties such as growth *in vitro*, infectivity to Vero cells, susceptibility to antitrypanosomal drugs, pathogenicity in mice (LAURENT *et al.*, 1997; DE LANA *et al.*, 1998; REVOLLO *et al.*, 1998). It is quite possible that what is true for *T. cruzi* is true for *Leishmania* too. From this perspective, evolutionary genetics of *Leishmania*, apart from its considerable basic scientific interest, is not only a convenient approach to the design of reliable molecular epidemiology tools; it is, even more, the rational framework for all applied studies dealing with these parasites.

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