

Molecular epidemiology and evolutionary genetics of *Leishmania* parasites

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Abstract

In order to illustrate the relevance of the concepts and methods of evolutionary genetics in the understanding of the epidemiology of pathogenic agents, we develop in this paper the case of the *Leishmania*, a genus of parasitic protozoa. An extensive study of various natural populations of *Leishmania* in different countries (Old and New World) was carried out by using Multilocus Enzyme Electrophoresis (MLEE) and Random Amplified Polymorphic DNA fingerprinting (RAPD) as genetic markers. The data have been interpreted in evolutionary genetic terms. The main benefit of this approach has been to better define the concept of species in the genus *Leishmania*, on rigorous phylogenetic bases. As a matter of fact, a sound taxonomical background is a prerequisite for any epidemiological approach. Since the biological concept of species is difficult or impossible to apply for most pathogenic microorganisms, we recommend relying on criteria of both phylogenetic discreteness and of epidemiological/medical relevance to describe new species of *Leishmania*. Through this approach, for example, we have shown that the species status of *L. (V.) peruviana* can be supported. On the contrary, we have been unable to clearly distinguish *L. (V.) panamensis* from *L. (V.) guyanensis* with genetic tools. Additionally, we have shown that the epidemiological inferences based on a limited set of genetic markers can be misleading. As a matter of fact, we have demonstrated that a collection of *L. (L.) infantum* stocks identified as zymodeme 'MON 1' by other authors present additional genetic heterogeneity and do not correspond to a distinct 'Discrete Typing Unit' DTU, and are actually polyphyletic. Lastly, in the samples that were conveniently designed, we have confirmed that *Leishmania* parasites have a basically clonal population structure. As the clonal model specifies it, occasional bouts of genetic exchange remain nevertheless possible. Telling comparisons are drawn with the evolutionary genetics of other pathogens *Trypanosoma cruzi* and *Trypanosoma congolense*. © 1999 Australian Society for Parasitology Inc. Published by Elsevier Science Ltd. All rights reserved.

Keywords: Biological concept; Clinic; DTU; *Leishmania*; Phylogeny; Epidemiology; Species; Clonality

1. Introduction

In the past 20 years, genetic and molecular methods for characterising pathogen strains have taken a major place in modern approaches to the

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epidemiology of parasitic and other infectious diseases. This paper emphasises the relevance of the concepts and methods of evolutionary genetics in the understanding of the epidemiology of *Leishmania* parasites. The parasitic protozoa pertaining to the *Leishmania* genus Ross, 1903, are the causative agents of the leishmaniasis. These parasitoses are widespread over all continents, except Australia and Antarctica. Nowadays, the leishmaniasis still pose considerable problems of public health. Some recent studies [1–3] have shown the reactivation of several foci, the apparition of new epidemic situations and an underestimation of the severity of these affections and of their socio-economic impact. Moreover, the growing problem of VIH/Leishmaniasis associations (for example: VIH/*L. infantum* in Southern Europe; [4, 5]) has called the attention of health authorities from the industrial world on these parasitoses. The study of these parasites is all the more difficult since they present a very broad epidemiological and clinical diversity. Like many pathogenic microorganisms, the classification and evolutionary genetics (two tightly linked fields) of *Leishmania* parasites still need much refinement. A sound and consensual taxonomical background, based on the knowledge of the population structure and phylogenetic diversity, are indispensable prerequisites for the understanding of the epidemiology of pathogenic microorganisms. With emphasis on the specific approach of our group, this paper reviews the present state of the art in the specific case of *Leishmania* parasites, with comparisons with other pathogens. As stressed by Tibayrenc [6–9], the progress of this field of research sorely needs standardised and comparative approaches among various kinds of pathogens, not only parasites, but also fungi and bacteria, either of medical, veterinary or agronomy relevance.

2. Evolutionary genetic concepts applied to the epidemiology of pathogenic microorganisms

This question has been extensively developed through recent reviews by Tibayrenc [6–9], and will be only briefly summarised here.

According to the definition of the Centers for Disease Control (CDC) in Atlanta [10], molecular epidemiology refers to as “the various biochemical and molecular techniques used to type and subtype pathogens”. The usefulness of molecular methods for pathogen identification at the species, subspecies or strain level is now widely accepted, as well as the major role that these methods will play in the future of epidemiology of infectious diseases [11]. However, our group has advocated that the definition of the CDC is too narrow and too technology-based, and that the interpretation of molecular data in the epidemiology and taxonomy of pathogens definitely needs to be based on evolutionary genetic concepts.

Evolutionary genetics permits the reliable identification and delimitation of the discrete genetic units and subdivisions that are relevant for both evolution and epidemiology. ‘Discrete Typing Units’ or DTUs [8, 9] refers to as such discrete subdivisions, each identifiable by specific genetic markers or ‘tags’. In this perspective, the first task of the geneticist is to verify whether the presently-described species can be considered a reliable DTU. The second task is to look for additional, lesser DTUs within the species under study (structured species model vs nonstructured, [6]; see Fig. 1). Lesser DTUs that subdivide a given species (the structured model) can be explained by either clonal evolution or the existence of cryptic biological species [12] within the species under study. Nonstructuring of a pathogen species can be explained either by actual panmixia (the species undergoes sex in the broad sense, that is to say: genetic exchanges with a sufficient frequency to constantly shuffle its gene pool) or by ‘epidemic clonality’ (propagation of ephemeral clones, which lifetime does not exceed a few years, in a basically sexual species) [13].

Tools to identify DTUs or to reject their existence are population genetics and phylogenetic analysis. The first approach gives a ‘snapshot’ of the present population structure of a given species (individualisation of populations, gene flow, migration rate, action of natural selection). In the case of pathogenic microorganisms, it has

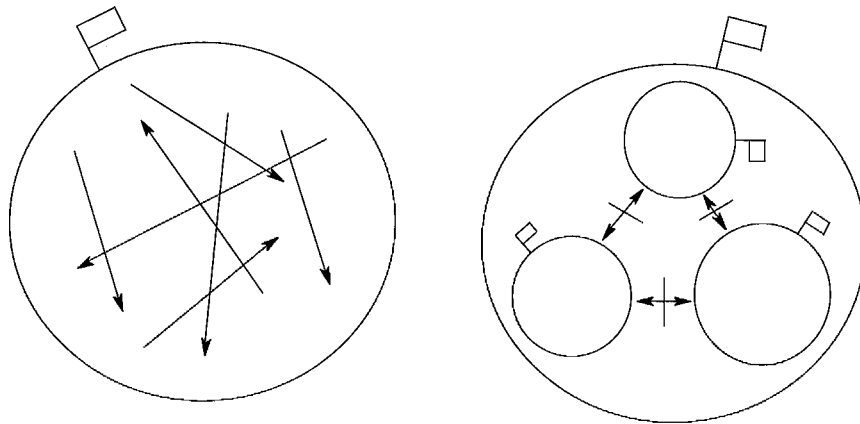


Fig. 1. Diagrammatic representation of nonstructured (left) vs structured (right) models of pathogen populations [6]. In the non-structured model, gene flow (symbolised by arrows) is frequent enough to prevent the maintenance of discrete genetic lines. The operational unit of research is the entire species only. On the contrary, in the structured model, the species is subdivided into discrete genetic entities between which gene exchange (symbolised by double arrows) is inhibited. In the two models, the species are supposed to correspond to discrete typing units or DTUs (see text), that can be specifically identified by convenient genetic markers or 'tags' (symbolised by small flags). In the structured model, the species is further subdivided into lesser DTUs, each characterized by specific 'tags'. These lesser DTUs can be taken as units of research as well as the whole species.

focused on the 'clonality–sexuality debate', and is essentially based on the statistics of linkage disequilibrium (nonrandom association of genotypes occurring at different loci; see Fig. 2). The existence of a strong linkage disequilibrium reveals obstacles to gene flow. These obstacles can be merely extrinsic (geographical distance for example), or intrinsic, biological (clonal evolution or cryptic biological speciation). Specific linkage disequilibrium statistics applicable to the study of pathogenic microorganisms have been proposed by Tibayrenc et al. [14]. Phylogenetic analysis aims at reconstructing the evolutionary past of a species at a time scale of millions of years. Obviously, in molecular epidemiology and taxonomy of pathogens (for example: delimitating *Leishmania* species), microevolutionary levels are the relevant ones to consider. As a matter of fact, at the genus and family levels, other characters (morphology) can be used. Specific phylogenetic concepts (mainly the cladistic approach) [15], statistics and softwares can be used to depict the evolutionary relationships among taxa, and to reliably identify the specific genetic characters ('tags', [8, 9]) that can identify these taxa and their lower genetic subdivisions. It is important to recall that the dendrograms that

can be computed from the mere quantification of genetic differences among strains and taxa cannot be considered as evolutionary trees in themselves. Only the use of specific phylogenetic approaches

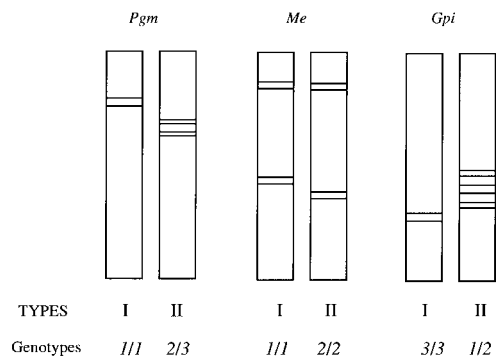


Fig. 2. Diagrammatic representation of the genetic variability recorded at three enzyme loci (phosphoglucosmutase, malic enzyme, and glucose phosphate isomerase) in *Trypanosoma cruzi*, the agent of Chagas' disease. Two distinct genotypes are observed at each locus. *Pgm* 1/1, *Me* 1/1 and *Gpi* 3/3 are constantly associated with one another (linkage disequilibrium); similarly mutually linked are *Pgm* 2/3, *Me* 2/2 and *Gpi* 1/2. Recombinant genotypes such as *Pgm* 1/1 with *Gpi* 1/2 have never been observed in a sample of more than 500 stocks.

can ascertain that such dendrograms do depict actual phylogenetic relationships.

A crucial notion to keep in mind while performing population genetic or phylogenetic analyses is the "molecular clock" (speed of evolution of a given genetic marker, conditioned by the rate of mutation of the genes involved). A fast molecular clock gives a marker that is more relevant to explore lower levels of evolutionary divergence (for example: at the strain level), whereas a slow molecular clock gives a marker that is better adapted to higher levels of phylogenetic divergence (at the species or the genus level). Molecular studies are not a goal in themselves, and while selecting the marker to be used, it is important to think about the question under study. For exploring the whole genetic diversity of the entire genus *Leishmania*, and its phylogenetic relationships with related genera, a slow marker such as rRNA genes is well-fitted. When typing different strains for a fine epidemiological survey, fast markers such as microsatellites are better.

3. Evolutionary genetics of *Leishmania*

In our laboratory, an extensive survey of many *Leishmania* stocks (mostly from Latin America) has been conducted with two main techniques: Multilocus Enzyme Electrophoresis (MLEE) on cellulose acetate gels and Random Primed Amplified Polymorphic DNA or RAPD. Our MLEE technique relies on the analysis of 15 enzyme loci with cellulose acetate gels, using the protocols described by Ben Abderrazak et al. [16] with slight modifications. The RAPD technique, first described by Williams et al. [17] and Welsh and McClelland [18] has been used by Tibayrenc et al. [19] to explore the genetic polymorphism of several parasitic protozoan genera, of which *Leishmania*. We have screened an extensive set of 120 primers, and have selected the 10 primers that give the more readable and the more reproducible profiles. Among different *Leishmania* species, these 10 primers may be the same ones. The following main questions have been explored in the course of this study: (i) Exploring the mat-

ing system, population structure and main mode of evolution of *Leishmania*; (ii) Evaluating the genetic validity of some *Leishmania* species; (iii) Clarifying the intraspecific variability of some *Leishmania* species.

3.1. *Leishmania* and the clonality/sexuality debate

The clonality/sexuality debate is relevant for the epidemiology of pathogenic microorganisms for the following reasons. If the species is sexual, its multilocus genotypes are unstable due to genetic recombination, and cannot be properly used as epidemiological markers. If the species is clonal, its genotypes propagate themselves like 'genetic photocopies' and therefore, can be used as reliable epidemiological markers. Moreover, if the impact of sex is very limited, parasite clones will accumulate more and more divergent mutations (including for genes that govern relevant medical properties such as pathogenicity or resistance to drugs), till they give birth to discrete evolutionary lines or DTUs [8,9]. *Leishmania* mating system and populations structure still is under debate. Tibayrenc et al. [14], by analysing published data in population genetic terms, have found a strong linkage disequilibrium in several *Leishmania* species, of which *L. (Leishmania) infantum*, and have proposed that these parasites and other ones are basically clonal. This hypothesis has been corroborated by Jimenez et al. [20] for *L. (L.) infantum* populations isolated from AIDS patients. The clonal model in *Leishmania* has been challenged by Bastien et al. [21] and Blaineau et al. [22], who have proposed, after Pulse Field Gel Electrophoresis data, that some *L. (L.) infantum* populations are panmictic. In our study, we have tried to select as small an evolutionary unit as possible, and as sympatric a population as possible, to test the clonal/sexual alternative. We have surveyed: (i) A *L. (Viannia) peruviana* population from Central Peru; (ii) Two *L. (V.) braziliensis* populations, one from Peru, one from Bolivia. These three populations have shown strong statistical departures from panmictic expectations through the analysis of linkage disequilibrium. The Peruvian *L. (V.) braziliensis* population exhibited an especially telling indi-

cation for linkage disequilibrium, that is to say a strong correlation between MLEE and RAPD data, verified by statistical tests (the *g* test of Tibayrenc, [6]), giving very similar clustering patterns on the corresponding MLEE and RAPD evolutionary trees. These results are in favour of the clonal model, and do reject the hypothesis that the populations surveyed are panmictic. Nevertheless, they must be confirmed on other *Leishmania* species. Moreover, even a strong linkage disequilibrium means only that sex is not frequent, and does not rule out occasional bouts of genetic exchange.

3.2. Hybrids between different *Leishmania* species

The hypothesis that certain *Leishmania* genotypes correspond to hybrid genotypes between different species has been first proposed by Evans et al. [23] in the Old World. In the New World, evidence for hybridisation events has been brought by Darce et al. [24] and Belli et al. [25].

In the framework of the present study, we have found clear indications for hybridisation between *L. (V.) braziliensis* and either *L. (V.) panamensis* or *L. (V.) guyanensis* (see further) in Ecuador [26]. The evidence is taken from both MLEE and RAPD data (see Figs. 3 and 4). Three MLEE loci proved to be at a heterozygous state which alleles correspond to the ones of the putative parents, whereas four other loci showed homozygous profiles corresponding to either one of the two parents. The rest of the loci showed profiles that were different from the parents profiles. RAPD profiles showed a combination of the parental profiles (Fig. 4). Although these data are more parsimoniously explained by a hybridisation event, they are not compatible with the hypothesis that the hybrids correspond to a F1 offspring of the parents, since most loci are not at a heterozygous state, and even, several of them have alleles that are different from both parents. Two hypotheses, not mutually exclusive, can account for these results. First, the putative parents may not be the actual parents, but rather, genotypes that are closely related to them. Second, the hybridisation event could be ancient, and the hybrids could have propagated clonally

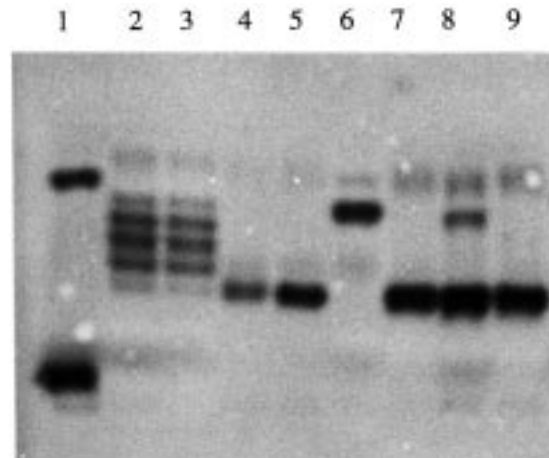


Fig. 3. MLEE profile obtained for the NHI enzymatic system, showing the existence of putative hybrids between *Leishmania (Viannia) guyanensis/panamensis* and *L. (V.) braziliensis*. The *L. (V.) guyanensis* putative parental profile is represented by sample 6 and the *L. (V.) braziliensis* parent is represented by samples 4, 5, 7 and 9. The heterozygous patterns (putative hybrids; samples 2 and 3) exhibit five bands, which is a characteristic profile for a tetrameric enzyme. Sample 8 is composed of a mixture of the two species, *L. (V.) guyanensis* and *L. (V.) braziliensis*.

since then, and could have accumulated divergent mutations. The hybridisation hypothesis does not support the panmictic model and shows only that occasional bouts of genetic exchange can occur even in a basically clonal organism.

3.3. The validity of the presently-described *Leishmania* species in the light of evolutionary genetics

Thirty *Leishmania* species have been described to date. Most of them were originally described on the basis of clinical, epidemiological and biological features. The validity of these species were often supported by genetic criteria, using the phylogenetic species concept or PSC [27], although some inconsistencies were noted. For example, genetic studies demonstrated that *L. (L.) pifanoi* and *L. (L.) garnhami* cannot be differentiated from *L. (L.) mexicana* and *L. (L.) amazonensis*, respectively [28–30]. It is probable that the number of currently-described *Leishmania* species is excessive, as also suggested

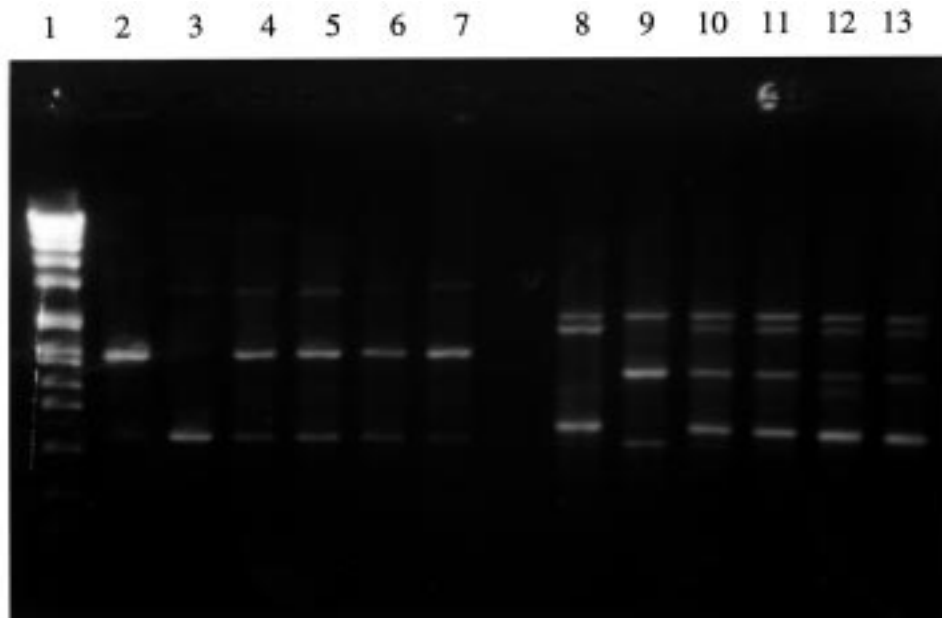


Fig. 4. RAPD profiles obtained with two different primers, showing evidence for hybridisation between *L. (V.) guyanensis/panamensis* and *L. (V.) braziliensis*. Lane 1: DNA molecular weight ladders. Lanes 2 and 8: *L. (V.) guyanensis/panamensis*; lanes 3 and 9: *L. (V.) braziliensis*; lanes 4–7 and 10–13: four putative hybrids.

by comparison of the phylogenetic diversity of *Leishmania* with that of related parasites. A direct comparison performed in our laboratory (hence with the same techniques and the same statistical analyses) shows that the level of phylogenetic diversity recorded within either *T. cruzi* or *T. congolense* corresponds to the level of genetic divergence between the *Viannia* subgenus and *Leishmania* subgenus (see Fig. 5). This clearly shows that *Leishmania* specialists had a higher tendency to describe new species than the specialists of other parasites. It is at least debatable that the description of all these species is justified by clear epidemiological or clinical specificities. Moreover, with the rapid improvement of molecular techniques, the number of species could increase by far. In order to clarify the present taxonomy of the genus, and to restrict the description of an excessive number of species, it is necessary to settle rigorous and consensual criteria for the description of new species.

Since the predominant mode of evolution of *Leishmania* parasites is probably clonal [14] (and our present results), they should be considered as

'agamospecies', like many other species of pathogenic microorganisms, since the biological species concept or BSC [12] is not applicable to asexual organisms. We recommend relying on criteria of both phylogenetic discreteness (PSC, [27]), and of epidemiological/medical relevance to validate a pre-existent species or to describe new species. The first prerequisite (genetic discreteness) demands that the species can be equated to a DTU, identifiable by one or several specific genetic markers or 'tags' [8,9]. This phylogenetic validation must be based on a convenient sample, representative of the whole ecogeographical range and phylogenetic diversity of the species under verification or description, and including related species taken as 'outgroups'. The validity and robustness of the DTU as an individual genetic unity is conveniently ascertained by the joint use of two or more genetic markers (the g test) [6]. We present thereafter three cases that are illustrative of this approach.

The *L. (V.) peruviana* species was described in 1913 by Velez, on the basis of extrinsic characters. Indeed, this parasite presents pathological

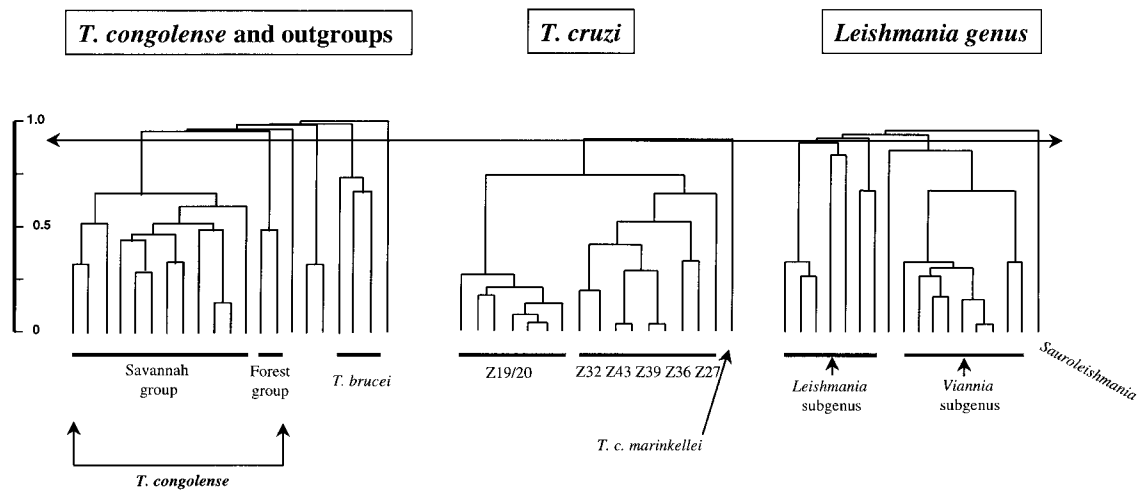


Fig. 5. Comparison of the phenetic diversity recorded in three different parasitic protozoa with the same markers (RAPD) and the same statistical methods (Unweighted Pair-group Method with Arithmetic Averages (UPGMA) dendrograms [44]) (Brisse, Sidibé and Bañuls, unpublished data). The results show that the total level of phenetic diversity recorded between several distantly related *Leishmania* species is comparable to that observed within each of the two species *Trypanosoma congolense* and *T. cruzi* (see horizontal arrow).

and geographical specific characteristics. It is responsible for a strictly cutaneous clinical form (Andean leishmaniasis, AL or uta) and its distribution is limited to Peru (on the Pacific coast of the Andean valleys and in the inter-Andean valleys). The biochemical and molecular analysis of *L. (V.) peruviana* showed a very high genetic similarity with *L. (V.) braziliensis* [31,32]. Therefore Grimaldi et al. [33] have suggested that *L. (V.) peruviana* may simply be a variant of *L. (V.) braziliensis* and not a valid species. In our study, the phenetic and phylogenetic analysis of MLEE and RAPD data showed that *L. (V.) peruviana* does correspond to a DTU, distinct from all *L. (V.) braziliensis* stocks analysed. Fig. 6 presents the phylogenetic tree obtained by the Wagner method after a bootstrap test on the MLEE data. Moreover, *L. (V.) peruviana* can be conveniently identified by specific MLEE and RAPD markers ('Tags', [8,9]). The individualisation of *L. (V.) peruviana* is confirmed by the karyotypical data [34] and the analysis on the Gp63 gene [35]. The genetic individualisation demonstrated by these analyses based on various genetic markers on a broad sample, together with the specific epidemiological and clinical properties of

L. (V.) peruviana, support the view that this parasite deserves the rank of a distinct species [36].

The second example deals with the two species *L. (V.) guyanensis* and *L. (V.) panamensis*, which both belong to the *guyanensis* 'complex'. These species were originally defined mainly according to their geographical localisation as suggested by their name [37,38]. At present, the coexistence of the two species is reported in some geographical areas [39]. Moreover, no clear-cut clinical expression distinguishes these two taxa. In the literature, only one enzyme locus (*6pgd*) was identified as discriminative between *L. (V.) guyanensis* and *L. (V.) panamensis*, each species being characterised by a specific one-banded pattern [40]. The MLEE characterisation of an Ecuadorian *Leishmania* sample did not permit us to confirm the specificity of the *6pgd* locus, since a high polymorphism was recorded for this locus, with no specific pattern for each of the two species [41]. Moreover, the phenetic and phylogenetic analysis performed on the MLEE and RAPD data did not show a clear structuring between the two species *L. (V.) panamensis* and *L. (V.) guyanensis*. All the results infer that *L.*

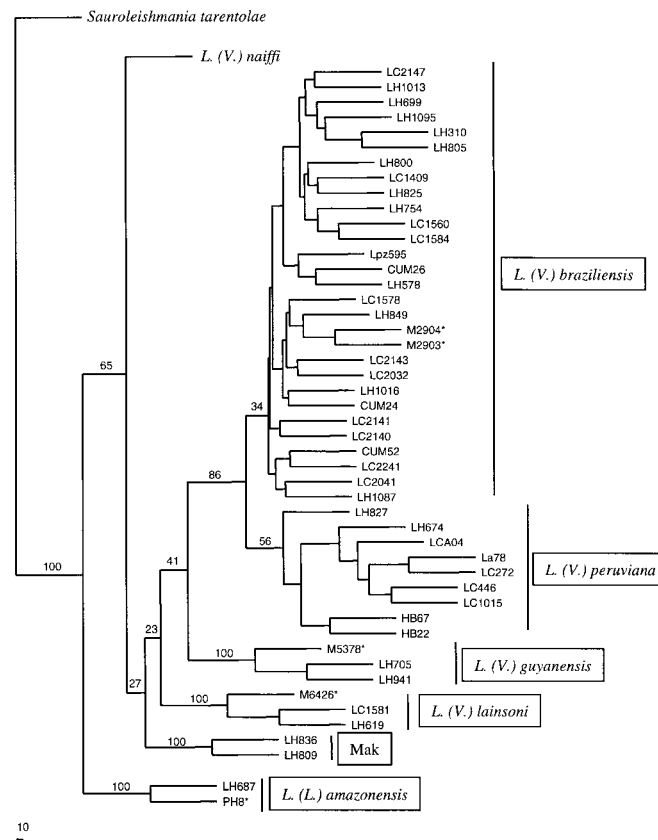


Fig. 6. Phylogenetic tree constructed by bootstrapped (100 replicates) parsimony analysis of Wagner of a *Leishmania* sample. Bootstrap values for all major nodes are given. This tree shows the individualization between *L. (V.) peruviana* and *L. (V.) braziliensis*. The two samples corresponding to the new genetic group called 'Mak' are clearly separated from all other *Leishmania* species included in this analysis.

(V.) panamensis and *L. (V.) guyanensis* do not correspond to two distinct DTUs. Therefore, the epidemiological, clinical and phylogenetic studies do not confirm the status of *L. (V.) panamensis* and *L. (V.) guyanensis* as distinct species.

The third example deals with a distinct DTU we have identified both in Peru and Bolivia. This group of genotypes, which we have temporarily called 'Mak', is clearly distinct from any known *Leishmania* species (Fig. 6). Moreover, it does correspond to an individual DTU with specific tags. On the other hand, in the present state of knowledge, it has no ecoepidemiological or clinical specificity by comparison with *L. (V.) braziliensis*. This group of genotypes therefore meets only the criterion of genetic discreteness, and its

status as a distinct species is debatable. The problem raised by such a result is that a genetic marker (tag) specific to *L. (V.) braziliensis* would be unable to identify this group of parasites, which apparently exhibits a comparable pathological risk.

3.4. Exploring the variability of *Leishmania* at the subspecific level with evolutionary genetic concepts.

The implications of the DTU concept are very clear. Only DTUs can be considered reliable evolutionary units identifiable by specific 'genetic common denominators' or tags [8, 9]. It is therefore desirable, when studying a given species, to look for the smallest DTUs that can subdivide

this species, in order to design as many robust units of research as possible for epidemiological tracking. However, a problem of resolution comes very quickly. When one considers very low levels of phylogenetic divergence, for which by definition, the genetic variability becomes very limited, as stressed by Tibayrenc [6], both population genetic and phylogenetic approaches are subject to a considerable ‘statistical type II error’ (impossibility to reject the null hypothesis—here: lack of structuring—not because it is true, but because data are not sufficient). A possible solution is to improve the level of resolution of the markers used. We have applied the DTU analysis based on MLEE and RAPD to the interesting case of the zymodeme ‘MON1’ of *L. (L.) infantum*. This zymodeme, characterised by a highly

standardised set of 15 enzyme loci analysed by starch gel electrophoresis [42], is considered of strong epidemiological and medical relevance. As it is widespread over the whole Mediterranean basin, and is responsible for most cases of visceral leishmaniasis. The goal of our study was to look for possible additional genetic variability among stocks previously attributed to MON1 and to see whether the stocks attributed to MON1 correspond to a DTU, which would validate their epidemiological and medical relevance. To address these questions, we have analysed a set of *L. (L.) infantum* stocks, of which some had been previously attributed to MON1. We have added other species as outgroups. The results are clearly illustrated by Fig. 7. A notable genetic variability was shown within the MON1

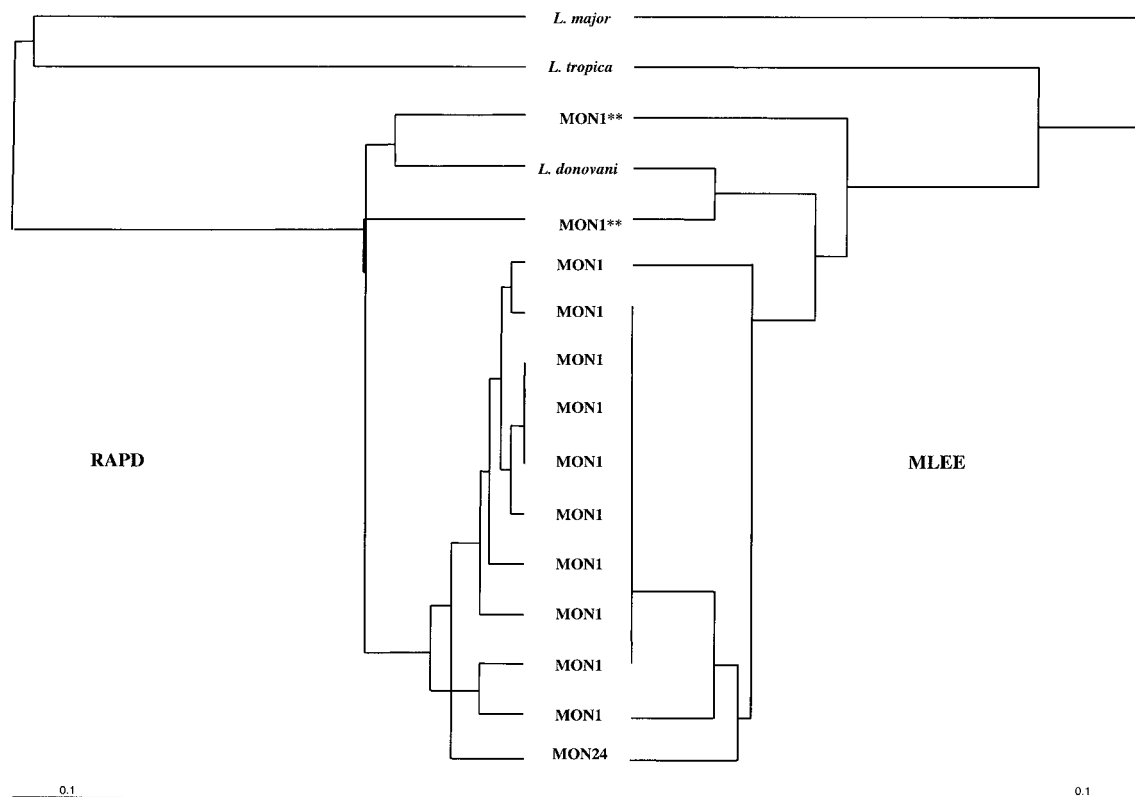


Fig. 7. Unweighted Pair-Group Method with Arithmetic Averages (UPGMA) dendrograms [44] built from genetic distances calculated on the basis of the RAPD data (left) and MLEE (right). These trees show the genetic diversity observed within *L. (L.) infantum*, and especially in the MON1 group. These analyses suggest that MON1 is not genetically individualised from the non-MON1 *L. (L.) infantum* stocks. ***L. (L.) chagasi*.

stocks by both MLEE and RAPD typing. Moreover, it is clear that MON1 stocks do not correspond to a DTU. As a matter of fact, the MON1 stocks do not fall into a specific cluster, and other stocks previously attributed to other MON zymodemes are intercalated among them, including a *L. donovani* stock. The robustness of this branching is supported by the strong agreement between MLEE and RAPD dendrograms, and statistically verified by a non parametric Mantel test [43], with $P < 10^{-4}$. MON1 stocks therefore appear as a polyphyletic group, and do not correspond to a distinct DTU identifiable by specific tags. In other words, some MON1 stocks are actually more related to some non-MON1 stocks than to other MON1 stocks. These results challenge the epidemiological relevance of the zymodeme MON1.

These few examples illustrate the power of evolutionary genetics to reconsider the taxonomy of *Leishmania* parasites on firm bases. Demanding approaches and strict criteria for the description of new species are all the more indispensable, since with the coming progresses of the surveys, it is probable that much more phylogenetic diversity will be identified within this genus (as already suggested by the example of our new DTU 'Mak'), with the risk of an unmanageable taxonomy.

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