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Review

Evolutionary genetics of *Trypanosoma* and *Leishmania*

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ABSTRACT – We review recent advances in the study of population structure and phylogenetic diversity of parasites belonging to the genera *Trypanosoma* and *Leishmania*. In all species properly analyzed, these parasites exhibit a basically clonal population structure, with occasional bouts of genetic exchange or hybridization, and a strong structuration of their populations into discrete evolutionary lineages. On an evolutionary scale, the impact of sex appears to be greater in African than in American trypanosomes. The taxonomic status of some *Leishmania* ‘species’ is questionable. © Elsevier, Paris

molecular epidemiology / population genetics / clonality / linkage disequilibrium / Chagas’ disease / pathogenesis

1. Introduction

The last 20 years have seen the advent of evolutionary genetics in the field of medical microbiology. This has considerably changed our view of the basic biology of microbes, especially of medically relevant protists. Parasites belonging to the genera *Trypanosoma* and *Leishmania* have yielded valuable results concerning their population structure and evolutionary dynamics. These results have made possible the development of paradigmatic approaches that have successfully been applied to other pathogens. Nevertheless, even for *Trypanosoma* and *Leishmania* species, there remain many unanswered questions. The current picture of their population structure is complex.

2. Setting the problem

For a decade, the question of the population structure of microbes has focused on a clonality/sexuality debate (for reviews, see [1–8]) Maynard Smith et al. [9] considerably clarified the debate by distinguishing true clonal evolution from ‘epidemic clonality’ (which refers to occasional bouts of clonal propagation in a sexual species). The matter is important, because in epidemic clonality, the clones are ephemeral and last at best only a few years. This evanescence considerably diminishes the usefulness of clones as epidemiological markers. We have pro-

posed [1–4] that the relevant distinction for the population structure of microbes is between ‘structured’ and ‘non-structured’ species (*figure 1*), rather than between clonal and sexual species. Within nonstructured species, genetic exchange is frequent enough to prevent the appearance of separate evolutionary lineages. Nonstructured species may conform either to the model of epidemic clonality, or simply to a model of panmixia (in which sexuality is common enough so that genetic exchange is only inhibited by spatial separation). Structured species are subdivided into discrete evolutionary units that correspond either to stable clonal lineages or to cryptic species, corresponding to the Biological Species Concept or BSC [10], based on the criterion of interbreeding.

3. Approaches for investigating the population structure of pathogens

The present review seeks to present the main results concerning the population structure of *Trypanosoma* and *Leishmania* species. Only the basic evolutionary genetic methodology is presented. For additional details, the reader can consult other reviews [1–6]. Two complementary approaches are used for exploring the population structure of a given species. Population genetic analysis gives a ‘snapshot’ of the present population structure of the species, whereas phylogenetic analysis aims to reconstruct its evolutionary past.

In the context of the distinction between structured and nonstructured species, population genetics seeks to identify obstacles to gene flow emerging from distinctive bio-

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Figure 1. Diagrammatic representation of nonstructured (left) vs structured (right) models of pathogen populations [1, 2]. In the nonstructured model, genetic exchange (symbolized by arrows) is frequent enough to prevent the maintenance of discrete genetic lines. The operational unit of research is the whole species only. In contrast, in the structured model, the species is subdivided into discrete genetic entities between which gene flow (symbolized by double arrows) is inhibited. In the two models, the species are assumed to correspond to discrete typing units or DTUs ([3, 4]; see text) that can be specifically identified by appropriate genetic markers or 'tags' (symbolized by small flags). In the structured model, the species is further subdivided into lesser DTUs, each characterizable by specific tags. These lesser DTUs can be taken as units of research for applied studies (epidemiological tracking, vaccine and drug designing, clinical studies), as can the whole species.

logical features of a particular species. Obstacles emerging from geographical or temporal separation are irrelevant. Departures from panmictic expectations are traditionally explored with two kinds of statistics: Hardy-Weinberg statistics and linkage disequilibrium measures. Hardy-Weinberg ascertains the randomness of association between alleles at a given locus. This ascertainment encounters a lot of problems in microorganisms, for the following reasons. First, the ploidy level of the organism must be known, which is not always the case or easy to ascertain. Second, the ploidy level must be different from 1, which is not the case for bacteria or for the human blood forms of *Plasmodium* species. In the case of *Trypanosoma* and *Leishmania* species, diploidy is the currently accepted working hypothesis, but the evidence is not definitive. Lastly, some kinds of molecular data fail to identify all alleles at a locus (e.g., a given band of protein in electrophoresis may correspond to one or other of several alleles). Because of these difficulties, we favor the linkage disequilibrium approach [1–5], which is not subject to the problems just listed and has, in any case, finer resolving power than the Hardy-Weinberg statistics.

Linkage disequilibrium refers to the nonrandom association between genotypes at different loci. The resolving power of the statistics can simply be increased by increasing the number of loci assayed and/or the number of individuals in the sample. Consider figure 2, which represents isoenzyme data from *Trypanosoma cruzi*. Assume for simplicity that at each of the three isoenzyme loci – *Pgm*, *Me*, and *Gpi* – and each of the two observed types – I and II – has a frequency of 0.5. If there is no linkage

Figure 2. Diagrammatic representation of the genetic variability recorded at three enzyme loci (phosphoglucosmutase, malic enzyme, and glucose phosphate isomerase) in *T. cruzi*. Two genotypes are observed at each locus. *Pgm* 1/1, *Me* 1/1, and *Gpi* 3/3 are consistently associated with one another (linkage disequilibrium); similarly mutually associated are *Pgm* 2/3, *Me* 2/2, and *Gpi* 1/2. Recombinant genotypes such as *Pgm* 1/1 with *Gpi* 1/2 have not been observed in a sample of more than 500 stocks.

disequilibrium, the expected frequency of *Pgm* I/*Me* I is the product of the frequencies of each type, or $0.5 \times 0.5 = 0.25$. The frequency actually observed is 1.0 (that is, *Pgm* I/*Me* II and *Pgm* II/*Me* I are not observed). If we now include a third locus, namely *Gpi*, in the analysis, the expected frequency of the three-locus genotype *Pgm* I/*Me* I/*Gpi* I is $0.5 \times 0.5 \times 0.5 = 0.125$. The actually observed frequency is 1.0. The departure from linkage equilibrium has become greater. In point of fact, the total association between the genotypes *Pgm*, *Me*, and *Gpi* has been corroborated on more than 500 *T. cruzi* stocks to date. It reflects the existence of two major phylogenetic subdivisions within *T. cruzi* as we shall see below. To summarize: once possible biases attributable to geographical or temporal separation have been accounted for, linkage disequilibrium provides a presumption that the particular species is structured, and the strength of this presumption increases with the level of significance of the tests.

Several tests of linkage disequilibrium have been proposed, all based on the same principle, although they explore different facets of the biological phenomenon of lack of recombination between different loci, and are therefore not redundant. Of course, as with other statistical tests, linkage disequilibrium tests depend upon the size of the population sample. The power of resolution increases with the number of loci assayed, as noted above, and the amount of variability recorded at each locus, but also with the number of individuals sampled.

It is critical to be aware of these factors for two reasons. First, if the sample size is limited, there is a great risk of statistical type II error; the null hypothesis (in this case, panmixia) is not rejected, not because it is correct, but because the test is not powerful enough to reject it owing to insufficient data. Second, when comparing different sets

Figure 3. Two unweighted pair-group method with arithmetic average (UPGMA) dendrograms [11] built from the genetic distances recorded for isoenzyme data (MLEE, right) and for RAPD (left) in *T. cruzi*. Agreement between the two dendrograms is evidence of linkage disequilibrium (or nonrandom association between genotypes at different loci), and supports the robustness of the two main DTUs and of some lower DTUs that subdivide this species [3, 4]. The symbols on the left-hand dendrogram indicate RAPD tags [3, 4] that are specific to given DTUs (after [20]).

of data (for example, two different species), the results need to be weighted for the comparison to be meaningful. For example, the phylogenetic diversity within *T. cruzi* is roughly double that within *Trypanosoma brucei* (see below). All things being equal, the opportunity to test any hypothesis related to species structure is therefore much greater in *T. cruzi* than in *T. brucei*.

Population genetics through the analysis of linkage disequilibrium constitutes a powerful tool for exploring the structuration of a given species, but it gives only statistical presumptions of it and must be completed with the qualitative analysis provided by phylogenetic investigations. These approaches also are exposed in greater detail in [1–6] and in textbooks (for example [11]). We will highlight only a few important points. Phenetic approaches must be distinguished from true phylogenetic methods. Phenetic methods seek quantitative evaluation of as many characters as possible, and the organisms are then clustered according to overall similarities. In the case of molecular data, the basic character is usually the electrophoresis band (isoenzyme or DNA), which is coded as either 1 (presence) or 0 (absence). Various distance indices and computing methods are available for analyzing the

data [12]. Dendrograms are generated (see, for example, figure 3), which constitute a visual summary of the matrix of genetic distances and should not be interpreted as true phylogenetic trees, unless this interpretation has been verified by appropriate means.

True phylogenetic methods seek rather to uncover true evolutionary information conveyed by the characters under study. The best-known phylogenetic method is the cladistic method [13], which distinguishes between ancestral (plesiomorphic) and derived (apomorphic) characters. Only those derived characters that are shared by several evolutionary lines (synapomorphic characters) are informative for identifying monophyletic lineages or clades. All members of a given clade are inferred to descend from a common ancestor.

Cladistic analysis is invaluable, but must be used cautiously in the present context for two reasons. First, the cladistic method was originally designed for morphological characters, and its use for molecular data is the subject of debate [14]. Especially with electrophoresis banding patterns, the risk of homoplasy (that is, similarity arisen for reasons other than common ancestry) is high. Second, many microorganisms exhibit a complex interplay be-

tween clonal evolution and genetic recombination, which makes the use of cladistics perilous (a 'cladistic nightmare').

We have proposed the phrase 'discrete typing unit' (DTU) [3, 4] to refer to the discrete evolutionary units that may subdivide a given species, even when they do not correspond to a clade *sensu stricto* (or when this correspondence cannot be corroborated with the available data). For example, *T. cruzi* and *Leishmania* hybrid genotypes (see further) do not correspond to monophyletic lines, since they are inferred to descend from two ancestors instead of only one. Nevertheless, these hybrids are stable and behave like clades. The DTUs are identifiable by molecular characters ('tags') that are shared by all members (strains) of a given DTU [3, 4] (see figure 3). A tag can be an isoenzyme profile, a random-primer amplified polymorphic DNA (RAPD) fragment or pattern, a restriction fragment length polymorphism pattern, a specific DNA probe, or PCR diagnostic, etc. When a DTU corresponds to a true clade, the tags correspond to synapomorphic characters. A given DTU can be identified by one or more tags. Before identifying DTUs within a given species, the geneticist's first task is to ascertain that the species itself corresponds to a DTU identifiable by tags. This is not necessarily the case, since many microbial species have been described first on medical or epidemiological rather than genetic criteria. These considerations are crucial for molecular diagnosis. The tags should not be defined a priori, but rather a posteriori, after careful and complete evolutionary genetic analysis of the species under study.

Lastly, when phylogenetic analysis is sought, it becomes important to distinguish between gene phylogenies (taken from the analysis of only one gene or a few genes) and species phylogenies (that are supposed to reflect the evolution of the whole species). Species phylogenies should be based on the analysis of several genes. Multilocus analyses such as multilocus enzyme electrophoresis (MLEE) or RAPD fingerprinting (based on the use of a sufficient diversity of primers) are appropriate for this purpose.

There is little we need to say here about the technical tools used for evolutionary genetic analysis. All techniques have advantages and drawbacks. The crucial point to keep in mind is the level of resolution desired, which is governed by the speed of evolution of the marker used (molecular clock). In order to explore high levels of phylogenetic divergence (for example, when performing a phylogenetic analysis of the whole genus *Leishmania*), a slowly evolving marker is required. If the marker is evolving too fast, it will soon get 'saturated' and provide no resolution at the higher levels of phylogenetic divergence (see below, the *Trypanosoma congolense* case). But for analysing finer levels of phylogenetic divergence (for example: identifying different strains of *Leishmania infantum*), a fast-evolving marker is necessary. Slowly evolving markers are, for example, certain domains of rRNA genes, while microsatellites are typically fast-evolving markers. MLEE and RAPDs have intermediate levels of discriminatory power. It may well be added that it is often desirable to use two or more different kinds of genetic markers in a

given study. This will increase by far the level of resolution for the purposes of i) strain typing, ii) linkage disequilibrium tests, and iii) phylogenetic analysis (see figures 3 and 4).

4. The paradigmatic example of *T. cruzi*, the agent of Chagas' disease

T. cruzi was the basis for pioneer studies on pathogen genetic diversity in the 1970s and 1980s [15–19]. Early important results were obtained by Miles et al., who described three main groupings of isoenzyme categories or 'principal zymodemes' in *T. cruzi* [15, 16] and proposed the hypothesis that these zymodemes impact the clinical diversity of Chagas' disease [17]. It was later shown by investigations explicitly formulated in population genetic terms, that the isoenzyme variability of *T. cruzi* shows all the signs of a basically clonal population structure [18, 19], mainly strong linkage disequilibrium (such as is illustrated in figure 2).

The natural clones of *T. cruzi* are distributed into two major phylogenetic lineages [1, 20, 21], each exhibiting considerable genetic variability. The robustness of these two clades is corroborated by strong agreement between isoenzyme and RAPD phylogenies [20] (figure 3). Microsatellite polymorphisms also yield a phylogenetic picture in close agreement with the isoenzyme and RAPD phylogenies [22, 23]. The consistency between genetic distances obtained with diversified kinds of genetic markers favors the hypothesis that these genetic distances reflect true evolutionary divergence (clonal evolution) rather than the individual genetic diversity of a putative, recent sexual ancestral population (epidemic clonality) [9].

The observation of RAPD synapomorphic characters within one of the two main phylogenetic lineages [20] suggested additional substructuring within it (figure 3). This has been amply corroborated by refined studies based on a larger set of stocks and on more discriminating isoenzyme and RAPD analyses, which have shown that the second main phylogenetic lineage is itself subdivided into five additional robust clusters [24]. These studies confirmed that the whole species *T. cruzi* can be considered as a distinct DTU, consistently recognizable by diverse 'tags'.

In summary: the species *T. cruzi* is a DTU, subdivided into two main DTUs, of which one is further structured into five lesser DTUs. The most parsimonious hypothesis to account for this clear structuration of *T. cruzi* into discrete evolutionary lines is predominant and long-term clonal evolution.

Consistent evidence for clonal evolution does not rule out the possibility of occasional mating [5, 18, 19]. There is fair evidence of the existence of hybrid genotypes in *T. cruzi* [24–26]. Interestingly, these hybrids appear to be stable and seem to propagate clonally. Two of these hybrids [24] actually correspond to two previously identified 'major clones' (ubiquitous clonal genotypes) [19].

One final point is that there appears to be a strong statistical linkage between *T. cruzi* phylogenetic diversity and several biological parameters explored by experi-

Figure 4. Two UPGMA dendrograms [11] built from the genetic distances recorded for isoenzyme data (MLEE, right) and for RAPD (left) in a collection of *Leishmania* stocks. Both the RAPD and MLEE dendrograms show that the *L. infantum* stocks previously identified by a different typing system as 'MON-1' do not fall into a unique cluster, and therefore, cannot be considered as a DTU [3, 4] (results after [53]).

ments, such as growth rate in in vitro culture, infectivity to cell cultures, pathogenicity in mice, in vitro antichagasic drug sensitivity, and transmissibility through *Triatoma infestans*, one of the main vector species [27–30]. The variability pattern with respect to these parameters indicates that different stocks pertaining to a given clonal genotype exhibit substantial biological diversity. Nevertheless, the statistical differences between sets of stocks pertaining to different clonal genotypes are highly significant, despite large standard deviations within a given clonal genotype. An interesting phenomenon has been recorded in experiments with mixtures of clonal genotypes in the same host [28]. In some cases, there seems to be interaction between the clonal genotypes, in the sense that the mixture behaves differently from a simple juxtaposition of the biological properties of each clonal genotype. Accordingly, Macedo and Pena [31] have recently proposed a 'clonal-histotropic model' for *T. cruzi*, which states that each clonal genotype has a distinct tissue tropism and that different clones infect different organs in the same host.

5. African trypanosomes

T. brucei sensu lato, the agent of sleeping sickness, has been the material for many evolutionary genetic studies. Nevertheless, its population structure remains controver-

sial. *T. brucei* has traditionally been subdivided into three 'subspecies': *T. brucei gambiense*, the agent of sleeping sickness in West Africa; *T. brucei rhodesiense*, the agent of sleeping sickness in East Africa; and *T. brucei brucei*, the agent of 'nagana', a cattle disease. Based on isoenzyme variability recorded in field isolates, Tait [32] first proposed that *T. brucei* is capable of sexual recombination. This hypothesis has been fully corroborated by experiments [33]. Nevertheless, the actual impact of sex on *T. brucei* population structure remains uncertain. A panmictic model has been definitely rejected by population genetic analyses, according to which we have proposed that *T. brucei*, like many other parasitic protozoa, has a pre-vaillingly clonal population structure [5, 34, 35]. Cibulskis [36] and Maynard Smith et al. [9] have cast doubt on the long-term stability of *T. brucei* clones, and have suggested that this parasite corresponds to the 'epidemic clonality' model. Hide et al. [37] have proposed that the respective impact of genetic recombination and clonal propagation on *T. brucei* population structure is dependent upon a particular ecogeographical cycle (wild versus domestic, East versus West Africa).

When estimated with the same techniques and the same statistical tools, *T. brucei sensu lato* is genetically less diverse than *T. cruzi* [34], a circumstance that proportionally reduces the possibility of demonstrating clonality and structuration (statistical type II error [1]). In spite of this

limitation, there is no doubt that, in the present state of the art, the existence of clear-cut genetic subdivisions within *T. brucei* remains less certain than in the case of *T. cruzi*. According to our results, the only reliable DTU within *T. brucei* corresponds to the so-called *T. brucei gambiense* 'group 1' [38], a relatively homogeneous group of parasites identifiable by two 'tags', a heterozygous genotype for the malic enzyme locus [34], and a kinetoplast DNA probe [39]. The population structure of *T. brucei* is a question now being reconsidered with new molecular tools (multiprimer RAPD typing) and the new statistical approaches developed by our research group.

T. congolense is a cattle parasite of high economic importance in Africa. Pioneer studies based on isoenzymes have shown that this species is subdivided into three major genetic groups, called savannah, Kilifi, and forest, according to their main zone of ecogeographic origin [40, 41]. Statistical analysis of the published data led us to propose that *T. congolense* has a clonal population structure [5]. Analyses by both MLEE and multiprimer RAPD fingerprinting of a broad sample of stocks fully confirmed the subdivision of *T. congolense* into discrete phylogenetic lineages, which made it possible to gather an ample harvest of 'tags' (mainly RAPD overall profiles and individual fragments) for each of the three subdivisions, and gave preliminary results in agreement with the clonal hypothesis [42]. An interesting result was the impossibility of coalescing the three *T. congolense* subdivisions into a unique (monophyletic) clade. This difficulty could be due either to polyphyly or to the fact that MLEE and RAPD have too fast a molecular clock for ascertaining high levels of phylogenetic divergence. Ribosomal RNA gene sequencing suggests that the second hypothesis is correct [43].

6. *Leishmania*

The genus *Leishmania* comprises an extremely heterogeneous group of parasites of high medical and veterinary consequence. Like the American and African trypanosomes, *Leishmania* species have stimulated pioneering work on molecular evolution of pathogens, with the particularity that *Leishmania* species were the subject of sophisticated cladistic analyses as early as the 1980s [44]. Many *Leishmania* 'species' were first described on the grounds of ecoepidemiological and clinical criteria. The validity of these taxa was then confronted with genetic criteria. At present, it is often not clear which species concept (biomedical, phylogenetic, mixiologic) some authors have in mind while describing a new *Leishmania* species.

The population structure of the *Leishmania* species appears from a population genetic perspective to be basically clonal [5], although the matter remains controversial [45, 46]. There are clear indications of the existence of hybrid genotypes between different pairs of species [47–49]. Despite these indications of occasional bouts of sexuality, and the still-pending debate on the population structure of *Leishmania* species, it is most likely that these parasites do not recombine randomly. The

panmictic concept of species can, therefore, hardly be applied to them. Accordingly, it seems advisable to describe *Leishmania* species in the light of the DTU concept, on the basis of both medical and phylogenetic criteria.

Several results recently obtained by our group are informative on this matter. The convergence of MLEE and RAPD data, and phylogenetic analysis, shows that *Leishmania peruviana* and *L. braziliensis* correspond to distinct DTUs [50] (figure 5). In contrast, it has been impossible to obtain a similar distinction between *L. panamensis* and *L. guyanensis*, which cannot be differentiated from each other with genetic markers [51]. An additional issue concerns the validation of 'zymodeme', rather than of a species. The term zymodeme refers to a collection of stocks that share the same isoenzyme profile (for a given set of isoenzyme markers). This notion is purely descriptive, for it does not explicitly include any population genetic implications. Nevertheless, international, standardized typing systems tend to be established that rely on the implicit notion that the zymodemes (and, actually, any collection of pathogen stocks that share the same profile with a given set of molecular markers) correspond to discrete and stable genetic entities (DTUs), even if this has not been verified by appropriate phylogenetic analysis. In the field of *Leishmania* research, the 'MON' (for Montpellier, France) typing system, based on 15 standardized isoenzyme loci analyzed by starch gel electrophoresis, is considered authoritative. In the species *L. infantum* (an agent of visceral leishmaniasis), the MON-1 zymodeme is ubiquitous, and is responsible for many cases of visceralizing leishmaniasis cases [52]. Our results show that i) stocks identified as MON-1 are heterogeneous when analyzed with more discriminating methods, which was expected; and ii) MON-1 is not monophyletic, and cannot therefore be considered as a DTU. As a matter of fact, about two-thirds of the MON-1 stocks fall into a distinct cluster, while the rest cluster with non-MON-1 *L. infantum* stocks, a result ascertained by both MLEE and RAPD data (figure 4) [53]. This result shows that the collection of stocks identified as MON-1 does not correspond to a discrete genetic entity and that the typing system used gives only an indication that stocks pertaining to the same MON zymodeme correspond to closely related genotypes.

7. Conclusions and perspectives

Evolutionary genetics has considerably refined our understanding of the ecology and epidemiology of trypanosomiasis and leishmaniasis. Although some debates persist, the state of the art is now ripe enough to provide a reliable framework for 'downstream' studies dealing with pathogenicity, immunology, and drug and vaccine design. Moreover, the maturity of this field of research in the case of *Trypanosoma* and *Leishmania* species has moved us to propose a new avenue of research, the 'integrated genetic epidemiology of infectious diseases', or IGEID [3, 4], in which the impacts on the transmission and pathogenicity of infectious diseases, of the host's, the pathogen's, and the vector's genetic diversity, as well as their possible interactions (coevolution phenomena), are to be considered jointly by means of evolutionary genetic methods.

Figure 5. An UPGMA dendrogram based on isoenzyme data, showing that the species *L. braziliensis* and *L. peruviana*, which exhibit specific clinical and epidemiological properties, correspond to two distinct clusters of genotypes (DTUs) [3, 4], identifiable by specific tags (here, distinct alleles for the enzyme locus mannose phosphate isomerase) (after [50]).

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