The protozoan Trypanosoma cruzi, its mammalian reservoirs, and vectors have existed in nature for millions of years. The human infection, named Chagas disease, is a major public health problem for Latin America. T. cruzi is genetically highly diverse and the understanding of the population structure of this parasite is critical because of the links to transmission cycles and disease. At present, T. cruzi is partitioned into six discrete typing units (DTUs), TcI–TcVI. Here we focus on the current status of taxonomy-related areas such as population structure, phylogeographical and eco-epidemiological features, and the correlation of DTU with natural and experimental infection. We also summarize methods for DTU genotyping, available for widespread use in endemic areas. For the immediate future multilocus sequence typing is likely to be the gold standard for population studies. We conclude that greater advances in our knowledge on pathogenic and epidemiological features of these parasites are expected in the coming decade through the comparative analysis of the genomes from isolates of various DTUs.

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**Abbreviations:** ITS1 rDNA, internal transcribed spacer 1 of rDNA; MLEE, multilocus enzyme electrophoresis; MLST, multilocus sequence typing; NTS, non-transcribed spacer; RAPD, randomly amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; SNPs, single-nucleotide polymorphisms; SL, spliced leader; SL-IR, spliced leader intergenic sequence.

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1. Introduction

Infection with *Trypanosoma cruzi* is a complex zoonosis, transmitted by many hematophagous triatomine species and sustained by over 70 genera of mammalian reservoir hosts. *T. cruzi* has a broad endemic range that extends from the Southern United States to Argentinean Patagonia. The human infection, named Chagas disease in recognition of Carlos Chagas who first discovered American trypanosomiasis in 1909, is found mostly in South and Central America, primarily affects poor rural populations, and is considered to be the most important parasitic infection in Latin America with serious consequences for public health and national economies.

The spectrum of pathological outcomes associated with acute and chronic Chagas disease ranges from subclinical infection through the cardiac and digestive syndromes to death. Specific outcomes may be determined by a variety of non-exclusive factors including parasite genetics, host genetics, mixed infections, and cultural and geographical factors (Macedo et al., 2002, 2004; Buscaglia and Di Noia, 2003; Campbell et al., 2004).

The diversity of the *T. cruzi* genome and multiplicity of its genotypes and phenotypes is well recognized (Dvorak et al., 1982; Barba et al., 2000; Brisse et al., 2000; Devera et al., 2003; Lewis et al., 2009a). Designation of ecologically and epidemiologically relevant groups for *T. cruzi* has oscillated between a few discrete groups (Miles and Cibulskis, 1986; Souto and Zingales, 1993; Souto et al., 1996; Zingales et al., 1999) and many (Tibayrenc and Ayala, 1988). Currently, six discrete typing units (DTUs) are assigned (Brisse et al., 2000). In 2009, these DTUs were renamed by consensus as TcI–TcVI (Zingales et al., 2009). Several reviews already describe how these DTUs correspond with former nomenclatures and with prospective biological and host associations (Campbell et al., 2004; Miles et al., 2009; Sturm and Campbell, 2010; Zingales et al., 2009).

The aim of this review is to explain further the rationale for naming TcI–TcVI, with reference to their known molecular genetics, eco-epidemiological features and pathogenicity. We also summarize methods for DTU genotyping, and discuss a possible seventh *T. cruzi* branch, provisionally named Tcbat. An understanding of the *T. cruzi* DTUs and their epidemiological implications will provide new insights to guide research and future interventions against this devastating infectious disease.

2. The concept of discrete typing unit

Since the late 1970s, *T. cruzi* has become one of the models for molecular epidemiologists and population geneticists, and consequently this protozoan parasite is a pathogenic agent for which evolution and population structure are among the best studied, although not necessarily the best understood. The emerging picture is that of a typical pattern of reticulate evolution, similar to that of many plant species (Avise, 2004).

The concepts of DTUs and clonal evolution have been designed within the framework of evolutionary research on *T. cruzi*. Tibayrenc and co-workers devised descriptive concepts and terminology to make such research and its implications accessible to non-specialists, including medical professionals and epidemiologists, and to bypass certain demands of classical evolutionary biology definitions. Classical cladistic and population genetics approaches imperfectly depict the biological realities of the evolution of pathogenic microorganisms.

2.1. The clonal model of evolution in *T. cruzi*

In the framework of this model, a “clonal species” refers to all cases where descendant multilocus genotypes are virtually identical to the founding genotype. The main parameter focused on in this scenario is the inhibition of genetic recombination. The term “clone” in this context refers to the population structure of the species under study, not to its precise mating system. Different methods of propagation can generate genetic clones, including classical cell division, several cases of parthenogenesis and gynogenesis. Following this definition (Tibayrenc et al., 1990), selfing and extreme inbreeding are not alternative hypotheses to clonality (Rougeron et al., 2009), but rather a particular case of it. Selfing refers to mating between identical genotypes, which can be issued from the same clone (Tibayrenc et al., 2010). Extreme inbreeding refers to mating between extremely similar genotypes. The result is a lack or extreme limitation of genetic recombination, hence genetic clonality.

Stating that *T. cruzi* is a basically clonal species means neither that recombination is totally absent in the parasite’s natural populations, nor that it does not have an impact on the evolutionary scale, but rather that it is too rare to break the prevalent pattern of clonality. The potential for genetic exchange is still present (Gaunt et al., 2003). Moreover, some localized transmission cycles suggest that genetic recombination does occur within DTUs of *T. cruzi* (Carranza et al., 2009; Ocaña-Mayorga et al., 2010). The possibility of limited genetic exchange between DTUs is also under debate (Lewis et al., 2011). However, the species *T. cruzi* considered as a whole shows all the signs for a typical clonal population structure: departures from panmictic expectations, strong linkage disequilibrium (non-random association of genotypes at different loci) within and especially between DTUs, and division into discrete genetic clusters (see DTUs, below).

2.2. Discrete typing units and clonets

Often the genetic subdivisions identified by evolutionary studies in pathogen species do not fulfill the criteria demanded by rigorous cladistic analysis. The main reason is that even in predominantly clonal species such as *T. cruzi* there is a certain amount of genetic recombination that clouds the distinction of
phylogenetic subdivisions. By definition, a clade represents an evolutionary unit that is strictly isolated from other evolutionary units. Moreover, some pathogen lineages may have a hybrid origin: in other words, two ancestors. This is the case for several T. cruzi genetic subdivisions (Sturm and Campbell, 2010). Thus the clade concept is not applicable in the case of T. cruzi.

However, even when some genetic exchange occurs, discrete and stable subdivisions can be identified reliably in many instances. Thus the term “discrete typing unit” (Tibayrenc, 1998) was proposed to describe sets of stocks that are genetically more similar to each other than to any other stock, and are identifiable by common molecular markers sometimes referred to as “tags”. The DTUs constitute reliable units for analysis for molecular epidemiology and experimental studies of evolution. Genetic clusters within T. cruzi perfectly fit this definition, and thus the DTUs TcI–TcVI have been assigned (Brisse et al., 2000; Zingales et al., 2009).

Within these DTUs T. cruzi stocks that share profiles for a given panel of molecular markers are not necessarily genetically identical, and can often be distinguished with additional markers. Thus strains within DTUs should be considered as families of closely related clones, not as a single clone. Tibayrenc and Ayala (1991) coined the term ‘clonet’ to refer to sets of stocks that appear to be indistinguishable with a given set of genetic markers in a basically clonal species, such as T. cruzi. The clonets are relevant units of analysis for molecular epidemiology, for example, with multilocus sequence types (MLSTs) or karyotypes derived from pulsed-field gel electrophoresis. However, it is crucial to keep in mind that the most recent common ancestor of a given clonet can be either a few weeks or hundreds of years old, depending on the marker’s power of resolution and rate of evolutionary change (molecular clock). The latter parameter cannot be known a priori and may have considerable epidemiological relevance.

3. Two major models for the origin of hybrid DTUs

T. cruzi is predominantly diploid (El-Sayed et al., 2005) and the known cell replication method is binary fission, i.e. it is an asexual process. Under the clonal model (above) new DTUs evolve with the accumulation of discrete mutations, unaffected by rare events of genetic exchange. However, consistent with the caveat that some genetic exchange events may occur, evidence for T. cruzi heterozygosity in nature emerged through the study of individual genes (Chapman et al., 1984; Bogliolo et al., 1996; Carrasco et al., 1996; Souto et al., 1996; Brisse et al., 1998). The pronounced heterozygosity observed in natural isolates of TcV and TcVI suggested that these DTUs are hybrids and derived from TcI and TcII (Sturm et al., 2003; Sturm and Campbell, 2010). The remaining DTUs, TcI, TcII, TcIII, TcIV and Tcbat, see below) show substantial allelic homozygosity. While the tenet of the clonal theory may still explain the common mode of T. cruzi population expansion, newer models incorporate hybridization events to explain the extant population structure that includes hybrid DTUs (Westenberger et al., 2005; Freitas et al., 2006).

The ‘Two-Hybridization’ model (Westenberger et al., 2005) and the ‘Three Ancestor’ model (Freitas et al., 2006) both incorporate two hybridization events (Fig. 1). In the Three Ancestor model the two recent genetic exchange events between TcI and TcII yield TcV and TcVI. The Two-Hybridization model invokes an ancient genetic exchange event between TcI and TcII, with loss of heterozygosity among progeny to produce TcIII and TcIV, followed by a second more recent hybridization event between TcI and TcII to yield both TcV and TcVI.

Analysis of single-nucleotide polymorphisms (SNPs) among the six DTUs by multilocus sequence typing (MLST) revealed four rather than six distinct DNA sequence classes, termed haplogroups (Machado and Ayala, 2001; Sturm et al., 2003; Brouin et al., 2006). Two of the four haplogroups were always present in TcV and TcVI, confirming the predominantly heterozygous nature of their alleles. Despite being similar by most standards TcV and TcVI are distinguishable by isoenzyme electrophoresis (Chapman et al., 1984; Barnabé et al., 2000), ribosomal RNA markers (Souto et al., 1996), restriction fragment length polymorphism (RFLP) assays (Roza et al., 2008), some MLST markers (Yeo et al., 2011), and microsatellite analysis (Lewis et al., 2011). The nucleotide patterns in each TcV/TcVI haplogroup closely resemble TcI and TcII alleles (Machado and Ayala, 2001; Brisse et al., 2003; Westenberger et al., 2005; Freitas et al., 2006; Yeo et al., 2011; Lewis et al., 2011), confirming that TcI and TcII as the most likely parental types of TcV and TcVI.

However, different MLST data indicated that the TcII parental cells included characters derived from TcI and TcII (Sturm et al., 2003; Elias et al., 2005; Tomazi et al., 2009). Thus, TcIII, as well as TcV and TcVI, could be the product of a hybridization event (Westenberger et al., 2005; Ienne et al., 2010).

The major difference between the Two Hybridization and the Three Ancestor models is therefore whether TcV and TcVI are progeny from a single hybridization event incorporating TcIII alleles acquired via TcIII (Westenberger et al., 2005) or progeny of two

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**Fig. 1.** Comparison of (A) the Two-Hybridization and (B) the Three Ancestor models for the roles of genetic exchange during the clonal evolution of T. cruzi. Rectangles indicate the distinct DTUs. Fusion of two cells and genetic exchange is indicated by the ovals, with parental contribution indicated by the red arrows. The mitochondrial clades are shown by fill colors: Blue = clade A; Green = clade B; orange = clade C.
hybridization events excluding TcI (Freitas et al., 2006) (Fig. 1). The participation of TcI in the generation of the extant heterozygous lines is supported by maxicircle sequences (Westenberger et al., 2006a; Ruvalcaba-Trejo and Sturm, 2011), MLST data (Tomazi et al., 2009), RFLP data (Rozas et al., 2008), and 195-bp satellite DNA sequences and distribution (Elias et al., 2005; Ienne et al., 2010). Conversely, maxicircle-encoded genes cytochrome oxidase subunit II and NADH dehydrogenase subunit 1 (Machado and Ayala, 2001; Freitas et al., 2006), microsatellite analyses (Freitas et al., 2006) and some other nuclear markers (Machado and Ayala, 2002; Rozas et al., 2007) did not detect participation of TcI. The comparison of the new genome sequence of the Sylvio X10/1 strain (Franzen et al., 2011), representative of TcI, with CL Brener, a TcVI hybrid that encompasses both TcII and TcIII genomes (El-Sayed et al., 2005), has confirmed that TcIII has an average higher genetic similarity at the genome sequence level with TcI than TcII. This observation is not inconsistent with the previous conclusion that TcIII may be the product of an ancient hybridization between TcI and TcII, as depicted in the Two Hybridization model of Fig. 1 (Elias et al., 2005; Westenberger et al., 2005). However, the possibility

---

**Table 1**

Summary of ecotope, host, vector and disease associations of T. cruzi DTUs.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Ecotope/niche</th>
<th>Silvatic hosts</th>
<th>Silvatic vectors</th>
<th>Geographyb</th>
<th>Chagas disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>TcI</td>
<td>Primary: arboreal; palms (e.g. Attalea), tree holes; Secondary: arid, rocky; terrestrial in Amazonia</td>
<td>Primary: arboreal, semi-arboreal; especially Didelphis, other didelphids, arboreal rodents, primates, Tamandua</td>
<td>Primary: Rhodnius species; Secondary: Panstrongylus Triatoma, Erythrurus</td>
<td>South, Central and North America</td>
<td>North of the Amazon, sporadic in Southern Cone Cardiomyopathy</td>
</tr>
<tr>
<td>TcII</td>
<td>Incompletely known; rare in silvatic cycles</td>
<td>Incompletely known: Atlantic forest primates, didephids, Euphractus (Paraguay)</td>
<td>Incompletely known: Triatoma, P. geniculatus</td>
<td>Southern Cone, sporadic further North</td>
<td>Atlantic and Central Brazil. Cardiomyopathy, megasyndromes</td>
</tr>
<tr>
<td>TcIII</td>
<td>Terrestrial, fossorial</td>
<td>Armadillos, especially Dasypus, Chaetophractus, Euphractus, Didelphis, Monodelphis</td>
<td>(Triatomini)</td>
<td>South America</td>
<td>Rare in humans (also domestic dogs). Acute cases in Amazonian Brazil. Clinical presentation poorly known</td>
</tr>
<tr>
<td>TcIV</td>
<td>Arboreal, and some terrestrial hosts in North America</td>
<td>Primates, Nasua nasua, Rhodnius, Panstrongylus, Triatoma</td>
<td>Incompletely known: Dasypus, Euphractus, Octodon</td>
<td>North and South America</td>
<td>Secondary cause of Chagas disease in Venezuela, sporadic elsewhere in South America</td>
</tr>
<tr>
<td>TcV</td>
<td>Rare in silvatic cycles</td>
<td>Incompletely known: Dasypus, Euphractus, Octodon</td>
<td>Incompletely known:</td>
<td>Southern Cone, greater Gran Chaco, extreme South of Brazil Southern Cone, greater Gran Chaco</td>
<td>Southern Cone Cardiomyopathy, megasyndromes, Vector borne</td>
</tr>
<tr>
<td>TcVI</td>
<td>Rare in silvatic cycles</td>
<td>Incompletely known</td>
<td>Incompletely known</td>
<td>Southern Cone, greater Gran Chaco</td>
<td>Southern cone Cardiomyopathy, megasyndromes</td>
</tr>
</tbody>
</table>

---

*a* Ecotope host and vector associations are not exclusive.

*b* See map in Fig. 2.
that characteristics shared by TcI and TcII are ancestral states that have diverged in TcI may need further consideration.

### 4. Phylogeography of the DTUs

Setting aside the theoretical origins of the DTUs, divergent geographical and biological characteristics are apparent, as is their relevance to understanding of the eco-epidemiology of Chagas disease (Fig. 2).

It is not surprising that, given the current level of sampling, the ecological history for all T. cruzi DTUs cannot yet be fully discerned. Relationships have been obscured by massive changes, from mammal migrations between the Americas, to climate induced retraction and expansion of habitats, and dramatic recent habitat destruction and urbanization by humans. A summary of ecotone, host, and vector associations of T. cruzi DTUs is given in Table 1, as has been extensively reviewed elsewhere (Miles et al., 2009). Here we will provide primarily more information on the phylogeography and the extensive genetic diversity of TcI, for which there has been recent rapid knowledge progress. In terms of propensity to cause severe Chagas disease, all six DTUs are known to be infective to humans, and clinical aspects are described in more detail in Section 6.

#### 4.1. TcI and its extensive genetic diversity

TcI is the most abundant and widely dispersed of all the T. cruzi DTUs in the Americas. It is found throughout the range of triatomine vector distribution, and can be associated with silvatic and domestic cycles. Human infection with TcI is concentrated in the north of South and Central America, and is associated with chagasic cardiomyopathy. There are only disparate reports of infection and disease south of the Amazon basin. Wild TcI isolates exist from the United States (Roellig et al., 2008) at 32° North, to Limari, Chile (Apt et al., 1987) at 30° South. Review of the literature reveals 52 mammalian genera naturally infected with this DTU, with representatives from Marsupialia, Rodentia, Primata, Chiroptera, Xenarthra, Carnivora, and Artiodactyla in order of abundance, as well as all major genera of triatomine bugs (Llewellyn, unpublished records, updated 05/01/2010). The divergence date between TcI and TcII is ill defined, estimated between 88 and 37 million years ago, based on small subunit rDNA (Briones et al., 1999; Kawashita et al., 2001) and between 16 and 3 million years ago, based on dihydrofolate reductase-thymidylate synthase and trypanothione reductase genes (Machado and Ayala, 2001). Unsurprisingly for a parasite so ancient and dispersed, significant genetic diversity has accumulated within TcI. The earliest recognition of TcI heterogeneity is manifest in the isoenzyme clonet typing schemes proposed by Tibayrenc and co-workers whereby 25 genotypes were defined, estimated 28 and 37 million years ago, based on small subunit rDNA (Briones et al., 1999; Kawashita et al., 2001) and between 16 and 3 million years ago, based on dihydrofolate reductase-thymidylate synthase and trypanothione reductase genes (Machado and Ayala, 2001). Unsurprisingly for a parasite so ancient and dispersed, significant genetic diversity has accumulated within TcI. The earliest recognition of TcI heterogeneity is manifest in the isoenzyme clonet typing scheme proposed by Tibayrenc and co-workers whereby 25 genotypes are assigned to TcI, with a much lower number assigned to any other of the other five DTUs (Tibayrenc et al., 1986). Sample size may have represented an early confounder, and we now know that substantial diversity has also accumulated within other lineages (Machado and Ayala, 2001; Westenberger et al., 2006b; Llewellyn et al., 2009a; Marcili et al., 2009a). Access to new, high-resolution genotyping techniques has seen a resurgence of interest in the delineation of TcI intra-DTU diversity.

Saravia et al. (1987) examined genetic diversity among 54 Colombian T. cruzi isolates collected from silvatic and domestic localities at several foci in Meta, Casanare and Cundinamarca provinces using 13 isoenzyme markers. Of those isolates examined, most were TcI (‘21-like’). Furthermore, among TcI isolates, marked genetic subdivision was observed between strains from domestic and silvatic transmission cycles, largely independent of geographic origin. This biological observation is now supported by spliced leader (SL, also known as mini-exon) intergenic region (SL-IR) sequence data from western Colombia (Herrera et al., 2007, 2009; Falla et al., 2009). Several other molecular methods have been applied to study TcI heterogeneity in Colombia, including molecular karyotypic analysis (Triana et al., 2006), SL probe hybridization (Triana et al., 2006) and minicircle random RFLP (Jaramillo et al., 1999). More recently, low-stringency single primer PCR (Rodriguez et al., 2009) provided important insight into the dynamics of TcI transmission among several communities in the Sierra Nevada de Santa Marta. Outside Colombia, multilocus enzyme electrophoresis (MLEE) and randomly amplified polymorphic DNA (RAPD) were used to reveal putative hybrid and parental strains at a focus of silvatic TcI transmission in Caraíjas, Pará State, Brazil (Carrasco et al., 1996). Using the same parental strains, an extant capacity for genetic exchange was demonstrated in vitro (Gaunt et al., 2003). Polymorphic microsatellites can resolve T. cruzi inter-specific variability (Oliveira et al., 1998), and Llewellyn et al. (2009a) demonstrated their efficiency in revealing TcI intra-DTU diversity at a continental scale. Crucially, the use of a multilocus typing system permits inference of linkage disequilibrium between markers within parasite populations and the extent of clonal vs. sexual reproduction. Thus, when the same markers were employed to analyze the molecular epidemiology of TcI transmission at restricted geographic foci in Ecuador (Ocaña-Mayorga et al., 2010), the first population genetic evidence for genetic exchange in TcI was uncovered among isolates not subdivided in space or time.

The central observation of almost all analyses of TcI diversity in northern South America is the apparent subdivision between domestic and silvatic cycles of transmission (Saravia et al., 1987; Herrera et al., 2007; Falla et al., 2009; Llewellyn et al., 2009a,b; Ocaña-Mayorga et al., 2010). However, compiling data from these studies is frustrated by constraints of the different genotyping techniques employed. Certain types of population genetic data, especially those derived from microsatellites and isoenzyme loci, are difficult to standardize between studies. Sequence data, on the other hand, are a more versatile population genetic currency. Cura et al. (2010) demonstrated this in an ambitious multi-centric study using the SL-IR, and corrected the erroneous use of the term ‘haplotype’ in the context of these data (Cura et al., 2010). On the basis of this dataset, the authors delineated several discrete TcI groups, some widely dispersed, as well as instances of mixed infections of genotypes in humans and vectors.

Whilst the SL-IR represents an accessible marker as it is diverse, easy to amplify directly from biological samples, and straightforward to sequence with no internal primers required, there are several limitations associated with its use. First, it is a multicyclic gene. Non-identical copies are tandemly repeated hundreds of times throughout the T. cruzi genome, and orthology between samples is impossible to ascertain. Second, polymorphic microsatellites located at the 5’ end introduce numerous ambiguous alignments, with an adverse effect on phylogenetic stability (Tomassini et al., 2011). Third, significant insertions and/or deletions (indels) in this region with respect to other T. cruzi DTUs (Souto et al., 1996) prohibit the identification of a suitable outgroup. Perhaps the most important criticism, however, is not intrinsic to the SL-IR per se. Gene trees are not genome trees. The use of a single genetic locus to describe genetic diversity in an organism limits conclusions that can be drawn, especially in the context of genetic recombination, which may occur not only in TcI (Ocaña-Mayorga et al., 2010) but also other DTUs.

Typing strategies must be improved and standardized if further progress is to be made. Sequence data are the ideal genotypic format for swift comparison. However, new, low copy number, highly discriminatory markers must be identified, aided by the publication of the Sylvio X10/1 genome (Franzen et al., 2011). Furthermore, the T. cruzi mitochondrial genome, used for bar-coding so many other
species, should not be ignored (Machado and Ayala, 2001; Freitas et al., 2006; Spotorno et al., 2008; Carranza et al., 2009), although some incongruence between nuclear and mitochondrial markers is likely due to introgression events. Accordingly, and if intra-DTU genetic recombination is common, the delineation of fixed genetic groups within TcI represents at best a distraction. Studies targeted at TcI diversity must be designed with a specific biological or epi-

mological hypothesis in mind, not undertaken merely to categorize diversity for its own sake. Irrespective of the genotyping system in-

volved, studies like those of Saravia et al. (1987), Falla et al. (2009), Rodriguez et al. (2009) and Ocaña-Mayorga et al. (2010) all provide significant insight into the epidemiology of local transmission. Thus their conclusions inform future interventions to the benefit of the communities involved.

4.2. TcIII and TcIV

TcIII is mostly associated with the silvatic cycle in Brazil and adjacent countries, and documented human infections are rare. Silvatic TcIII is associated with the terrestrial niche and with Dasypus novemcinctus, over a vast range from western Venezuela to the Argentine Chaco (Llewellyn et al., 2009a; Marcili et al., 2009a). TcII is also isolated occasionally from domestic dogs (Chapman et al., 1984; Cardinal et al., 2008).

TcIV shows a similar pattern of distribution in South America to TcIII, with the exception of the Chaco, where it appears to be ab-
sent. Unlike TcII, TcIV occurs fairly frequently in humans and is a secondary cause of Chagas disease in Venezuela (Miles et al., 1981). Five new isolates of TcV from primates and eight from Rhodnius brethesi in the Amazon basin were recovered (Marcili et al., 2009b), confirming earlier indications (Yeo et al., 2005) that TcV can have an arboreal ecotope. Evidence is accumulating that TcVI is split into distinct South and North American lineages (Lewis et al., 2009b; Marcili et al., 2009b). Further research is required to understand the history of TcVI and these complex ecological associations.

4.3. TcII, TcV and TcVI

TcII is found predominantly in the southern and central regions of South America, but its true extent is not yet clear. Within its main geographic distribution TcII is associated with cardiac mani-

festations, and concomitant megaesophagus and megacolon may be present. It has been isolated mostly from domestic transmission cycles. The natural hosts and vectors of TcII have proven elusive and most of the reported isolations have been made in remaining fragments of the Atlantic forest of Brazil, from primates and sporadically from other mammal species (Fernandes et al., 1999; Zin-
gales et al., 1999; Lisboa et al., 2007).

TcV and TcVI are two similar hybrid DTUs associated with Chagas disease in southern and central South America. Even more so than TcII, TcV and VI are virtually unknown as silvatic isolates. Comparative molecular genetics have proven that TcV and TcVI are hybrids of TcII and TcIII (see above). Until recently genetic markers have not been of sufficient resolution to determine firstly, whether TcV and TcVI are the products of independent hybridiza-
tion events (Freitas et al., 2006) or a single hybridization event fol-

lowed by clonal divergence, and secondly, whether these hybridization(s) were evolutionarily ancient (Tibayrenc and Ayala, 2002; Briste et al., 2003) or recent events (Machado and Ayala, 2001; Westenberger et al., 2005). Two recent studies have at-
ttempted to address these issues: Flores-López and Machado (2011) analyzed the evolution of 31 nuclear genes to show the hybridization events occurred less than 1 million years ago, con-

cluding a single event prior to arrival of humans in the Americas. Lewis et al. (2011) analyzed higher resolution maxicircle sequences and multiple microsatellite loci and found evidence for two independent events dated to within the last 100,000 years, concluding that hybridization may conceivably have occurred as a result of human activities. TcII and TcIII may have met and hybrid-
ized as co-infections emerged in humans, peridomestic mammals, or domestic Triatoma infestans.

Nevertheless, understanding of the ecology of TcII, V and VI is as yet vulnerable to the limited sampling of silvatic hosts and vectors. The paradigms may change. It has been suggested that TcII, V and VI are more widespread geographically than currently understood and might be found much further North (Zafra et al., 2008). If the known TcV and TcVI hybrids are also found in Central and North America it will most likely imply recent migration with humans or other carriers; if genetically distinct TcII/TcIII hybrids are ob-
served, hybridization may be an ongoing phenomenon where such mixed infections occur. Indeed in some endemic areas, notably parts of Bolivia, mixed DTU infections are common. Microsatellite analysis reveals that even within a single mammal, there may be a remarkable range of mixed genotypes (Llewellyn et al., 2011).

4.4. An enigmatic T. cruzi genotype from bats (Tcbat)

Silvatic cycles of T. cruzi transmission are numerous and com-

plex. DTUs circulate in relatively independent cycles with particu-

lar ecological niches and preferentially or opportunistically determined mammals and vectors. However, members of the same DTU can infect mammals of distinct species and orders, indicating that host-switching may be common among sympatric hosts (Gaunt and Miles, 2000; Yeo et al., 2005; Marcili et al., 2009a,b; Miles et al., 2009).

Several species of the genus Trypanosoma occur in species of Chirop tera throughout the world, with more than 30 trypanosomae species recorded from more than 100 species of bats. Insectivorous bat species are infected more frequently and can harbor stercorarian (subgenera Herpetosoma, Schizotrypanum and Megatrypanum) and salivarian (Trypanosoma evansi of the subgenus Trypanozoon) trypanosomes. An extensive summary of the prevalence of bat try-

panosomae worldwide is available (Cavazzana et al., 2010). The strong association between bats and all Schizotrypanum spp. except T. cruzi suggests a long shared evolutionary history. However, the evolutionary processes that have led to the current phylogenetic structure of Schizotrypanum trypanosomes are understood poorly. Phylogenetic studies of chiropteran stercorarian species can enhance understanding of host-parasite interactions and reconstruc-
tion of T. cruzi evolutionary history (Stevens et al., 2001; Barnabé et al., 2003; Cavazzana et al., 2010).

Brazilian bats infected with T. cruzi are reported from the Ama-
zonian rainforest to urban areas of Central, Northeast and South-
east Brazil (references cited in Marcili et al., 2009c). To date, most in vitro adapted isolates from bats belong to the subgenus Schizotrypanum. Identification of T. cruzi from bats requires careful analysis; Schizotrypanum species are morphologically indistin-
guishable and generally named as T. cruzi-like. However, T. cruzi can be confirmed by the ability to infect mice. Since T. cruzi isolates from wild mammals may induce very low parasitemias in mice, as is the case for bat isolates, infections must be evaluated using immunocompromized mice and sensitive parasitological methods such as PCR and multiple haemocultures.

Analysis of SSU rDNA, gCAPDH and cytochrome b sequences allows separation of T. cruzi from other trypanosomes infecting Bra-
zilian bats, including Trypanosoma cruzi-marinkellei, Trypanosoma dionisi-like and Trypanosoma rangeli (Maia da Silva et al., 2009; Marcili et al., 2009c; Cavazzana et al., 2010). Traditional genotyping methods based on the SL (Fernandes et al., 2001) and LSU rDNA (Souto et al., 1996) markers placed four bat isolates from Amazonia within Tcl. However, 11 bat isolates from other Brazilian regions
yielded a new combination of genotypes, with a TclI-SL pattern and a novel LSU rDNA product (Marcili et al., 2009c). This group of isolates earned the provisional title of 'Tcbat' and awaits further characterization for definitive DTU assignment (Marcili et al., 2009c), potentially as a seventh DTU: TcVII.

Tcbat is distinguished from the six DTUs by PCR-RFLP analysis of the internal transcribed spacer 1 of rDNA (ITS1 rDNA) (Marcili et al., 2009c). The method of fluorescent fragment length barcoding, developed to identify species of trypanosomes on the basis of polymorphisms of regions of the rDNA locus, when applied to T. cruzi DTUs showed a unique barcoding pattern for Tcbat (Ham-ilton et al., 2011). Karyotype (Marcili et al., 2009c) and sequence analyses of SL gene repeats (D.A.C. and N.R.S., unpublished results) corroborated that Tcbat diverges from the known DTUs. All phylo-genetic analyses using sets of molecular markers point to the placement of Tcbat in a distinct cluster, closer to Tcl, but clearly separated from clusters comprising all the other DTUs (Marcili et al., 2009c; Cavazzana et al., 2010). MLST analysis corroborates some affinity of Tcbat to Tcl (Teixeira and Yeo, unpublished observations).

Tcbat develops within mammalian cells in vitro, similar to other T. cruzi DTUs (Marcili et al., 2009c). Unlike isolates of the six DTUs, Tcbat does not develop in the commonly available triatomine species reared in laboratory colonies: T. infestans, Rhodinus prolixus and Panstrongylus megistus. The Tcbat insect vector is unknown. Possible vectors are triatomine species encountered in bat refuges, or cimicids, vectors of T. dionisii in Europe, or bat ectoparasites (Cavazzana et al., 2010).

Some other T. cruzi isolates also display unusually complex combinations of molecular markers (Lewis et al., 2009b; Marcili et al., 2009a,b,c). Thus, Tcbat is but one indicator that the complexity of T. cruzi is higher than currently defined, and will require revision of DTU relationships as more silvatic isolates are genotyped. Ideally, criteria for establishing new DTUs could be considered, for example using MLST data and a specified degree of divergence from existing DTUs.

5. Standardizing genotyping for identification of the six T. cruzi DTUs

The standardized nomenclature for the six T. cruzi DTUs will improve scientific communication and guide future research on comparative epidemiology and pathology. To achieve this aim a straightforward and reproducible genotyping strategy is required for DTU identification, manageable in any laboratory and adopted by the T. cruzi research community.

Over the years, numerous approaches have been used to characterize the biochemical and genetic diversity of T. cruzi isolates. No single genetic target allows complete DTU resolution, and reliance on a single target is also inadvisable because of the potential influence of genetic exchange.

A PCR assay system based on the amplification of particular regions of the SL gene and 245Sr rDNA (Souto and Zingales, 1993; Souto et al., 1996) and 18S rDNA (Clark and Pung, 1994) was proposed (Brisse et al., 2001) in which the size polymorphisms of the amplification products were suitable for T. cruzi assignment into each of the six DTUs (Table 2). However, assignments based on the absence rather than the presence of PCR products are problematic, thus an alternative set of criteria is preferable for a gold standard typing method.

A multilocus PCR-RFLP analysis of genetic polymorphism of 12 loci was proposed for DTU genotyping (Rozas et al., 2007), several of which demonstrated inter-DTU differences, and a combination of one, two or three of these assays allowed identification of the complete DTU set. The major limitation of this strategy is the complexity of the analysis.

A three-marker sequential typing strategy (Fig. 3A) was proposed (Lewis et al., 2009b) consisting of PCR amplification of the 245Sr rDNA (Souto and Zingales, 1993; Souto et al., 1996) and PCR-RFLP of the heat shock protein 60 (HSP60) and glucose-6-phosphate isomerase (GPI) loci (Westenberger et al., 2005). The combined application of the three PCR-RFLP markers was sufficient to discriminate the six DTUs in 45 out of 48 analyzed strains (Lewis et al., 2009b).

Another three-step assay (D’Avila et al., 2009) is outlined in Fig. 3B. PCR-RFLP analysis of the COII gene (Freitas et al., 2006) allows discrimination of Tcl and TclII from the other DTUs; amplification of the non-transcribed spacer (NTS) of SL genes (Burgos et al., 2007) of the unclassified strains defines two distinct clusters, one formed by TclII and TclIV and another by TclV and TclVI; amplification of 245Sr rDNA (Souto et al., 1996) then resolves the four DTUs.

A third scheme using nested-hot-start PCR assays is technically more demanding but has the potential advantage of allowing direct DTU typing in biological (Cardinal et al., 2008; Marcet et al., 2006) and clinical (Burgos et al., 2007, 2010) samples (Fig. 3C), and has been improved by the use of four sequential multiplex Real Time PCR assays using TaqMan probes (Duffy et al., 2010). The first NTS-SL based PCR employs primers recognizing regions flanking a 50-bp insertion characteristic of TclII and TclIV intergenic regions. The second NTS-SL based PCR is a hemi-nested reaction using TCC-TC1 and TCC-TC2 primers (Souto et al., 1996). The A10 PCR uses primers (Burgos et al., 2007) that recognize a dimorphic region within the A10 nuclear fragment (Brisse et al., 2000). At present the revised assay is being tested on biological samples.

An approach based on fluorescent-labeled fragment barcoding that detects PCR products from four rDNA domains has also been devised (Hamilton et al., 2011). This technique was able to differentiate many trypanosome species from South American mammals. Some T. cruzi DTUs (including Tcbat) could be clearly identified, however, TclV and TclVI could not be distinguished from TclII and TclI, respectively.

6. Comparative experimental pathology of the DTUs

Since the discovery of Chagas disease in 1909, heterogeneity of parasite strains has been considered one factor implicated in different clinical presentations of the disease. Andrade (1974) attempted to discriminate several distinct T. cruzi morphobiological and behavioral phenotypes in murine models using the criteria of virulence (capacity of multiplication in the host) and pathogenicity (ability to produce tissue lesions and immunological responses). These studies defined three main strain phenotypes (Andrade, 1974; Andrade et al., 1983), subsequently designated as ‘biodemes’ I–III (Andrade and Magalhães, 1997), as follows:

Biodeme type I: strains with rapid multiplication rates, maximum parasitemia, and mortality from 7 to 11 days after infection; predominance of slender forms, and macrophagotropism during the early phase of infection. Neuronal alterations are more
frequent and intense in biodeme type I infections. Zymodeme patterns of this biodeme correspond to zymodeme Z2b strains, a variant of Z2.

Biodeme type II: strains with slow multiplication rates and irregular parasitemia peaks 12–20 days after infection, when mortality rates reach a maximum; predominance of broad forms, myotropism, with predominant myocardial involvement. Zymodeme patterns of this biodeme type correspond to zymodeme Z2 strains. According to the 1999 revised nomenclature, this biodeme should be classified into the major group \textit{T. cruzi} II (Anonymous, 1999).

Biodeme type III: slow multiplication strains with late and high parasitemia peaks 20–30 days following infection and late mortality, usually from day 30 after infection; predominance of broad forms and myotropism, with myocardial and skeletal muscle involvement. Biodeme type III corresponds to zymodeme Z1 strains and to the major group \textit{T. cruzi} I (Anonymous, 1999).

Due to the intense chronic myocarditis, strains of biodeme type III are considered the most pathogenic in mice (Andrade, 1974). Nevertheless, the degree of strain virulence may vary within the same biodeme and between clones of the same strain (Andrade, 1974; Postan et al., 1987). Further studies in murine experimental models support and expand these observations (Andrade et al., 1985) and clearly indicate that both the parasite and host genotypes are important in determining the tissue distribution, physiopathology and eventual outcome of \textit{T. cruzi} infection. For example, simultaneous infection of four mouse lineages with the Colombiana C-17G2 clone (TcI) and the JG clone (TcII) showed identical tissue distributions in chronic phase infections of BALB/c and DBA-2 mice, but very different distributions in C57BL/6 (H-2b) and outbred Swiss mice (Andrade et al., 1999, 2002). Inoculation of Sylvio X10/4 clone (TcI) into the C3H/HePAS mouse strain caused intense cardiac inflammatory lesions, whereas in A/J mice chronic inflammatory lesions were found in the liver and skeletal muscle without detectable cardiac pathology (Marinho et al., 2004). Huge differences in virulence in experimental infections were reported for TcI isolates from Chile (Andersson et al., 2003),
Mexico (Espinoza et al., 2010) and the United States (Roellig and Yabsley, 2010).

Tibayrenc and co-workers undertook long-term comparative studies on the association between T. cruzi subspecific genetic diversity and the parasite’s biological properties, including behavior in axenic and mammalian cell culture, drug sensitivity in vitro, transmissibility through the insect vector and pathogenicity in mice (for example, see Laurent et al., 1997; de Lana et al., 1998; Revollo et al., 1998). The general pattern is that different DTUs exhibit statistically different biological properties, but with some overlap between different DTUs. Interestingly, in several cases mixtures of two clones behaved differently from a simple summation of their behavior, suggesting interaction between the genotypes (Pinto et al., 1998).

As one approach to the underlying molecular basis of biological differences between DTUs, Telleria et al. (2010) performed phylogenetic analysis of gene expression (proteomic diversity) between the six T. cruzi DTUs. The authors found a correlation between genetic distances measured by various markers (MLEE, RAPDs, MLST) and proteomic differences between DTUs, showing a tight correlation between genetic evolution and proteic divergence, and they identified several proteins with DTU-specific expression.

Overall these studies indicate extensive intra-DTU phenotypic diversity, complicating the identification of genetic determinants of pathogenesis and virulence and requiring higher resolution intra-DTU genetic markers or methods to cross experimentally strains with different virulence and to analyze the genotypes and phenotypes of resultant progeny.

7. T. cruzi DTUs and human Chagas disease

7.1. Clinical presentations

T. cruzi is transmitted to humans mainly by triatomine insect vectors, blood transfusion, infected mothers during pregnancy, and oral infection by consumption of food contaminated with triatomines or their feces. Following infection, a short acute phase is recognized only in 1–2% of the infected individuals, characterized by an abundant parasitemia and mild symptoms that spontaneously decline after 4–8 weeks. The disease proceeds to a chronic phase with scarce parasitemia and an unpredictable clinical course. Most of the chronic individuals are asymptomatic and show no electrocardiographic or radiologic alterations in the heart, esophagus or colon. The individuals present positive serological tests for T. cruzi infection and in many the xenodiagnosis and PCR results may be repeatedly positive for many years. These persons with the “indeterminate” form will remain asymptomatic for decades, if not the rest of their lives. Each year approximately 3% will develop lesions in the heart or gastrointestinal tract (Dias, 2006). Chronic cardiomyopathy, or chronic Chagas heart disease is the most common and severe manifestation in humans, affecting approximately 30% of the patients. In endemic areas, it represents the main cause of disability and mortality. The basic lesions of chronic Chagas heart disease are focal or extensive myocardial fibrosis, which result from myocardial cell destruction due to direct parasite action, inflammatory response, and neuronal involvement. The gastrointestinal manifestations consist of progressive enlargement of the esophagus or colon caused by chronic inflammation and destruction of parasympathetic neurons. Great regional diversity of Chagas disease severity and the nature of the chronic infection has been reported, attributed to a set of complex interactions among the genetic make-up of the parasite, the host immunogenetic background, and environmental factors (reviewed by Campbell et al., 2004; Macedo et al., 2004). A goal of T. cruzi taxonomic studies is to identify links between the infecting DTUs and the clinical presentation of disease. No proven associations are evident at present. The search for these associations will drive the ultimate criterion for defining the clinically meaningful number of biological subdivisions within this species.

7.2. Acute Chagas disease

The control of vector and blood transfusion transmission in several Latin American countries has promoted the steady reduction of acute infections. In recent years, most of these cases are linked to oral and congenital transmission, blood transfusion or laboratory accidents (Coura, 2006, 2007). Several acute cases were documented in the Amazon region, most caused by Tcl and, less frequently, by TcII and TcIV (Coura, 2007). In northern countries of South America Tcl is also the major cause of human acute cases (Miles et al., 1981; Añez et al., 2004; Llewellyn et al., 2009b and cited references).

Acute cases resulting from oral contamination have been documented for outbreaks in different localities, most frequently in the Amazon region. Most of these cases were due to Tcl, with rare cases due to TcII and TcIV (Coura, 2007; Marcili et al., 2009a,b; Valente et al., 2009), with Tcl in Venezuela and French Guiana (Alarcón de Noya et al., 2010; Cura et al., 2010) and TcII in southern Brazil (Steindel et al., 2008). The morbidity and mortality may vary depending on the parasite burden and parasite genotype ingested.

The incidence of congenital transmission is estimated at more than 15,000 cases annually in the Americas and is one of the main modes of transmission in non-endemic countries. The risk factors determining transmission of the parasite to the fetus are largely unknown. Cases of congenital infection with all DTUs except TcIV were reported in Argentina, Bolivia, Chile, Colombia, and Paraguay (for examples, see García et al., 2001; Svoboda et al., 2005; Virreira et al., 2006; Burgos et al., 2007; Corrales et al., 2009; Del Puerto et al., 2010). The prevalence of specific DTUs among congenital cases appears to be in accordance with their presence in the infected population. However, there seems to be a disparate prevalence of congenital cases in endemic regions, with few cases reported, for example from Venezuela and Brazil, with the exception of southern Brazil (Carlier and Truyens, 2010).

7.3. Chronic Chagas disease

Tcl is implicated with human disease in Amazonia, the Andean region, Central America, and Mexico (Bossoo et al., 2002; Montilla et al., 2002; Añez et al., 2004; Higo et al., 2004; Sánchez-Guillén et al., 2006). Clinical presentations of Tcl include chagasic cardiomyopathy and in immunocompromized hosts severe cases of meningoencephalitis. In the Southern Cone region, where T. infestans is the main vector, Tcl, TcV and TcVI are the main causes of Chagas disease. Tcl predominates in eastern and central Brazil, TcV in Argentina, Bolivia, and Paraguay, and TcVI in the Gran Chaco (Chapman et al., 1984; Zingales et al., 1999; Brenière et al., 2002; Diosque et al., 2003; Higo et al., 2004; Coronado et al., 2006; Burgos et al., 2007; Cardinal et al., 2008; Carranza et al., 2009; Del Puerto et al., 2010). Throughout the Southern Cone region chagasic cardiomyopathy can be severe, and a proportion of cases may develop megaesophagus and megacolon (Luqueti et al., 1986; Freitas et al., 2005; Lages-Silva et al., 2006). The disparate geographical distribution of the megasymphomines may reflect the divergent phylogeographies of T. cruzi DTUs (Miles et al., 1981), a hypothesis supported by circumstantial evidence. Chagasic megaesophagus and megacolon are considered rare in northern South America and Central America (Miles et al., 2009).
As mentioned above, TcIII is virtually absent in chronic infections, although it is found occasionally in domestic dogs in Paraguay and Brazil and in peridomestic *Triatoma rubrofasciata* in Rio Grande do Sul, Brazil (Yeo et al., 2005; Marcili et al., 2009a; Miles et al., 2009; Câmara et al., 2010). Consequently, DTU TcIII may yet become another source of human Chagas disease. TcIV is the secondary cause of Chagas disease in Venezuela (Miles et al., 1981), and has been identified in oral transmission outbreaks (Ramirez et al., 2010). Comparisons of the clinical histories for TcI and TcIV infections are required in Venezuela, where both DTUs are endemic and sympatric. A summary of the geographic distribution of DTUs associated with human Chagas disease and the characteristics of the prevalent clinical manifestations is included in Table 1.

7.4. Chagas disease reactivation due to immunosuppression

Co-infection with HIV/AIDS and immunosuppressant therapies can bring about acute and unusual clinical manifestations of Chagas disease, such as cutaneous lesions, involvement of central nervous system, and/or serious cardiac lesions. Genotyping of parasites recovered from the blood of Chagas disease patients with HIV and in both blood and tissue lesions from patients presenting clinical reactivation due to AIDS revealed differential tissue tropism of the infecting DTUs (Burgos et al., 2005, 2008; Bisió et al., 2009). In 18 Argentinean patients, TcV was found in almost all blood samples, in agreement with previous findings in this region. In two cases, mixed infections by TcV and TcI were observed and in one of these patients the cerebrospinal fluid sample amplified only TcI (Burgos et al., 2008).

A more complex scenario was seen in late-stage Chagas heart disease patients undergoing heart transplants, manifesting TcI, TcV, or TcVI in the bloodstream, in endomyocardial biopsies of the implanted heart and in skin tissues, provoking myocarditis and skin reactivation after immunosuppressive post-transplantation treatment, respectively (Burgos et al., 2010).

Conclusions and comparisons of clinical manifestations and parasite genotype are complicated for Chagas disease for several reasons. Isolates from blood do not necessarily reveal the full complement of infecting parasite lineages in individual patients, as one or several distinct *T. cruzi* strains may be sequestered in the tissues (Vago et al., 2000; D’Avila et al., 2009; Burgos et al., 2010; Câmara et al., 2010). Asymptomatic patients may have sub-clinical cardiac or digestive alterations detectable only by imaging studies. Additionally, parasite selection may occur during isolation procedures for genetic analysis due to the preferential proliferation of certain clones. A theoretical solution to these problems is to design peptides or recombinant proteins for DTU-specific serology that could be used to provide a current and historical profile of all the *T. cruzi* DTUs infecting an individual patient. DTU-specific serology would greatly facilitate comparisons of virulence and pathogenesis (Bhattacharya et al., 2010; Risso et al., 2011).

8. *T. cruzi* genomics

When the TriTryp genome projects published their initial findings in 2005, researchers were just coming to terms with the latest hitch in *T. cruzi* genetics. Contrary to prevailing expectations, *T. cruzi* DTUs TcV and TcVI are both largely heterogeneous in their nuclear content (Westenberger et al., 2005). The strain CL Brener chosen to represent *T. cruzi* (Zingales et al., 1997; El-Sayed et al., 2005) is a member of DTU TcVI, derived from *T. infestans*. This complication resulted in several consequences: (1) the final coverage level for CL Brener was 7-fold in depth, versus the expected target 15-fold; (2) the heterozygous genome could not be accurately assembled, and thus was presented in its fragmented state; (3) the Esmeraldo strain from DTU TcVII was sequenced at 2.5× coverage to aid in the CL Brener assembly as a representative of a ‘parental’ contributor to TcVI heterogeneity.

The sequencing effort revealed that CL Brener genome contains ~22,000 protein-encoding genes, and that over 50% is represented by repetitive sequences, consisting mostly of large gene families of surface proteins, retrotransposons, subtelomeric repeats (El-Sayed et al., 2005) and the *T. cruzi*-specific 195-bp satellite DNA (Martins et al., 2008). Putative function could be assigned to approximately half of the predicted protein-coding genes on the basis of significant similarity to previously characterized proteins or known functional domains. Thus around 6,000 proteins hold promise for new areas of investigation.

The decision to use a shotgun sequencing approach for the gathering of genomic data in *T. cruzi* led to the accumulation of linked 400–900 bp sequences. In the assembly process, elements repeated over a specific threshold were excluded, leading to the absence of the maxicircle genome in the initial report. Both the CL Brener and Esmeraldo maxicircles were reconstructed independently in their entirety from the primary sequence reads, each with a coverage of approximately 50× (Westenberger et al., 2006a). The size of the sequence reads and the depth of coverage allowed the assembly of the non-coding variable region of the mitochondrial genomes that are comprised of highly repetitive sequence motifs. A relatively low number of minicircle fragments was also found among the primary sequence reads (Thomas et al., 2007). To fulfill the complement of mitochondrial genomes, the Sylvio X10/1 strain maxicircle DNA sequence was assembled through an ordered amplification strategy (Ruvalcaba-Trejo and Sturm, 2011).

The many multicopy nuclear genes common in *T. cruzi* were relegated to the same fate as the maxicircle, resulting either in their exclusion or compression within the assembly, and skewing their initial representation. Analysis of the repeated nuclear protein-coding genes nearly doubles the total number of genes emerging from the *T. cruzi* genomic analysis (Arner et al., 2007), highlighting another level of genetic complexity in this ancient pathogen. A few multicopy RNA gene families have been studied individually, as represented by the SL RNA genes (Thomas et al., 2005) and SS rRNA genes (Westenberger et al., 2006b). The accurate assembly of any large tandem array is problematic, even among those of smaller periodicities such as the SL RNA gene array in *Leishmania major*.

Currently an assembled version of CL Brener is available to the community through TriTrypDB (Weatherly et al., 2009). A steady-state transcriptome analysis has been performed (Minning et al., 2009). At the protein level, multiple studies are emerging to complement the initial proteome study (Atwood et al., 2005) that appeared alongside the TriTryp genomes, including foci on organelles (Ferella et al., 2008) and ribosomes (Ayub et al., 2009).

Most of the available genome, transcriptome and proteomic data have been obtained for the CL Brener strain. As discussed previously, several experimental lines indicate that *T. cruzi* DTUs display differential virulence and pathogenic characteristics, however no genetic markers are linked with the severity of the infection. TcI, TcII, TcV and TcVI are the main agents of human Chagas disease in the Americas, and all are capable of causing cardiomyopathies, however, only DTUs TcI, TcV and TcVI have been so far associated to chronic digestive syndromes. The comparative analysis of the genomes from isolates of various DTUs may shed light on pathogenic and epidemiological features of these parasites, and promote the development of new DTU-specific diagnostic tests. The sequence of the TcI reference strain Sylvio X10/1 has recently been published (Franzen et al., 2011), and a second TcI sequence (JR cl4) and TcII (Esmeraldo cl3) sequence have also entered the public domain, via the TriTryp database.
9. Concluding remarks and perspectives

The revised specific nomenclature for *T. cruzi* (Zingales et al., 2009) recognized that *T. cruzi* strains should be assigned to one of six DTUs. The important change in the new nomenclature was that TcII was no longer divided into five subgroups (TcIIa-e) (Brisse et al., 2000) but each of those subgroups became independent DTUs (TcII–VI). The rationale for this change provides the underlying theme for the above review and is abundantly clear from several aspects.

The apparent affinities between TcI and TcII and TcIV were first described decades ago on the basis of MLEE: in particular overlapping isoenzyme profiles between what are now designated as TcI and TcII (Miles et al., 2009). These affinities have been confirmed repeatedly by other molecular markers and phylogenetic analysis, perhaps most notably by MLST studies and now by comparisons of the genome sequences of the hybrid TcVI (CL Brener strain) and Tc (Sylvio X10/1 strain), as cited above. Furthermore, the known phyleogeographical and eco-epidemiological associations of TcII and TcV and perceptions of their evolutionary origins, also described above, do not sit comfortably with them being subdivisions of TcII. The revised nomenclature therefore provides a more suitable and valuable framework for future research.

The decade between the two meetings on the nomenclature of *T. cruzi* has seen major advances in the understanding of this important pathogen, at many levels. Consistent with the theme of this review, here we have focused on population structure, genotyping, emergent comparative genomics, and the association of DTU with features of natural and experimental populations. Mechanisms of parasite-host interactions and immune responses to infection have not been addressed here. It is now proven experimentally that *T. cruzi* has an extant capacity for genetic exchange. The extent and mechanisms of genetic exchange in natural populations are not understood. TcV and TcVI are of special interest, because they are recent, rapidly spreading and epidemiologically important inter-DTU hybrids of TcII and TcIII (Lewis et al., 2011). There are indications, from inter-DTU mitochondrial introgression and apparent panmixia within localized populations of TcI, that genetic exchange is more widespread than hitherto appreciated. However, the broad integrity of the DTUs and their validity for population and eco-epidemiological studies is not disrupted. Nevertheless, the DTU nomenclature is inevitably a dynamic structure as research progresses and more discoveries are made. More intense and widespread sampling of *T. cruzi* isolates is required from silivatic populations, and repeated isolation from individual hosts to resolve multiclonoality.

As described above, straightforward genotyping methods to identify the DTUs are now available for widespread use in endemic areas, and research is in progress to optimize sensitivity and simplify techniques so that they may more easily be applied directly to clinical and biological samples. For the immediate future MLST, which provides reproducible and transferable data, is likely to be the gold standard for population studies. Microsatellites provide valuable high-resolution markers to produce less robust datasets that have specialized population genetic objectives. Identification of the genetic determinants of pathogenesis is extraordinarily challenging and they remain elusive. However, current rate of progress with new generation low cost sequencing technologies is astonishing and relevant. Although problems remain with assembly of highly repetitive regions, which are so abundant in *T. cruzi*, it is now realistic to obtain genome sequence from many *T. cruzi* isolates simultaneously, which will certainly illuminate the biology of this important pathogen. Fundamental to such rapid research progress is the ability of the scientific community to collaborate effectively, particularly between molecular biology laboratories and field research in endemic regions. An integrated but focused approach, not neglecting drug discovery, is important for improvement of control strategies that may reduce the public health burden of Chagas disease.

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