The molecular epidemiology and phylogeography of *Trypanosoma cruzi* and parallel research on *Leishmania*: looking back and to the future

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SUMMARY

Trypanosoma cruzi is the protozoan agent of Chagas disease, and the most important parasitic disease in Latin America. Protozoa of the genus *Leishmania* are global agents of visceral and cutaneous leishmaniasis, fatal and disfiguring diseases. In the 1970s multilocus enzyme electrophoresis demonstrated that *T. cruzi* is a heterogeneous complex. Six zymodemes were described, corresponding with currently recognized lineages, TcI and TcIIa-e – now defined by multiple genetic markers. Molecular epidemiology has substantially resolved the phylogeography and ecological niches of the *T. cruzi* lineages. Genetic hybridization has fundamentally influenced *T. cruzi* evolution and epidemiology of Chagas disease. Genetic exchange of *T. cruzi in vitro* involves fusion of diploids and genome erosion, producing aneuploid hybrids. Transgenic fluorescent clones are new tools to elucidate molecular genetics and phenotypic variation. We speculate that pericardial sequestration plays a role in pathogenesis. Multilocus sequence typing, microsatellites and, ultimately, comparative genomics are improving understanding of *T. cruzi* population genetics. Similarly, in *Leishmania*, genetic groups have been defined, including epidemiologically important hybrids; genetic exchange can occur in the sand fly vector. We describe the profound impact of this parallel research on genetic diversity of *T. cruzi* and *Leishmania*, in the context of epidemiology, taxonomy and disease control.

Key words: *Trypanosoma cruzi*, Chagas disease, molecular epidemiology, pericardial effusion, phylogeography, *Leishmania*, genetic exchange, microsatellites, multilocus sequence typing, taxonomy.

INTRODUCTION

The centenary of this journal coincides with the centenary celebration of publication of one of the most remarkable discoveries in parasitology and tropical medicine. In 1909 Carlos Chagas described *Trypanosoma cruzi*, the agent of American trypanosomiasis (Chagas disease) (Chagas, 1909). This discovery was unusual because Chagas first found the organism, not in patients, but in the faeces of the insect vector, the bloodsucking triatomine bug, infesting poor quality housing in Minas Gerais state, Brazil. Marmosets exposed to infected bugs sent to Rio de Janeiro developed parasitaemia with a new trypanosome, which Chagas named *Trypanosoma cruzi*, after his mentor Oswaldo Cruz. Chagas then saw the same trypanosome in acutely ill infants living in bug-infested houses. He and his colleagues went on rapidly to describe the basic features of the life cycle, the pathology, vector species and reservoir hosts (Miles, 2006). The illustrations of pseudocysts and triatomines from those early years are beautifully drawn and still entirely valid. Carlos Chagas became an international celebrity and was twice nominated for the Nobel Prize: the history of these early years is of considerable scientific and political interest (Miles, 2004).

In this Centenary Issue of *Parasitology* the senior author looks back, with members of his current research group, at the development of research on the genetic diversity and molecular epidemiology of *T. cruzi*, focusing on some of his own work and interests from the early 1970s onwards. We also briefly examine the development of parallel interests in the genetic diversity of *Leishmania*. In the context of this historical reflection, we consider the current state-of-the-art, indicating questions that have been answered and some of those that remain to be addressed.

The lead author's interest in both Chagas disease and leishmaniasis arose from attending inspirational lectures by the late Professor Philip Marsden. Here were two fascinating and gruesome diseases that surely deserved research and public health attention. At the time two salient features of Chagas disease were enigmatic. Firstly, why did the clinical manifestations of chronic Chagas disease appear to be distinct in different geographical regions, with

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Table 1. Comparison of *Trypanosoma cruzi* zymodemes and DTUs

Zymodeme[a]	DTU[b]	Reference strains
ZI	TcI	Sylvio X10/1
ZIII	TcIIa	CanIII cl1
ZII	TcIIb	Esm cl3
ZIII/ZI ASAT	TcIIc	M5631 cl5
Bolivian ZII	TcIId	Sc43 cl1
Paraguayan ZII	TcIIe	CL Brener

[a] Based upon MLEE; (Miles *et al*. 1977, 1978, 1981*b*; Tibayrenc and Miles, 1983; Chapman *et al*. 1984; Póvoa *et al*. 1984).
[b] Based upon MLEE, RAPD and nuclear loci (Brisse *et al*. 2001).

megaoesophagus and megacolon prominent in the southern countries of South America but either absent or rare in northern South America and Central America? Secondly, why were there so few cases of Chagas disease in the vast Amazon region of South America?

A PRIMER ON NOMENCLATURE

As we shall see, *Trypanosoma cruzi*, was found to be a highly diverse species and to this day some researchers use various nomenclature systems to describe the subdivisions within the species, which have been extensively reviewed elsewhere (Momen, 1999; Campbell *et al*. 2004). For the sake of clarity in this review we shall use Miles's zymodemes, which were based on multilocus enzyme electrophoresis (MLEE) and their modern equivalents – discrete typing units (DTUs), which are supported by multiple genetic markers (Table 1). Where possible we have retrospectively applied the 6 DTU nomenclatures to some of the studies reviewed.

TRYPANOSOMA CRUZI: HETEROGENEITY AND A COMPARISON OF TRANSMISSION CYCLES IN BRAZIL AND VENEZUELA

To address the first question, regarding ostensible geographical differences in disease presentation, at the invitation of Professor Aluisio Prata in Brazil and with support from the late Dr David Godfrey in London, we applied the relatively new technique of MLEE to domestic and sylvatic isolates of *T. cruzi* from the village of São Felipe, Bahia State, Brazil. The results were immediately striking and illuminating. Heterogeneity within the species *T. cruzi* had been suspected previously, and phenotypic variability had been apparent (Miles, 1979). However, the MLEE results demonstrated that the domestic and sylvatic strains of *T. cruzi* in São Felipe were dramatically distinct, by 11 of 18 enzymes examined, far more than separated named species of *Leishmania*. This landmark paper (Miles *et al*. 1977) fundamentally changed perceptions of *T. cruzi*. The vector of the domestic genetic lineage (ZII, now TcIIb) was *Panstrongylus megistus*, and we discovered the vector of the sylvatic lineage (ZI, now TcI) to be *Triatoma tibiamaculata*, living in bromeliad epiphytes with the opossum, *Didelphis albiventris*. We described this situation as one of separate, non-overlapping domestic and sylvatic transmission cycles (Fig. 1), implying that *P. megistus* might be controlled without fear of re-invasion from sylvatic habitats.

Subsequently, in collaboration with Professor Rafael Cedillos and others, we characterized numerous *T. cruzi* isolates from various locations in Venezuela, where the principal domestic vector is *Rhodnius prolixus*. These results were again surprising and enlightening. *T. cruzi* ZI (TcI), which was sylvatic in Brazil turned out to be the domestic agent of Chagas disease in Venezuela, as well as being present in sylvatic habitats (Table 2). This large comparative study was published in 1981 (Miles *et al*. 1981*b*) but is often overlooked in the subsequent and contemporary literature. On the basis of MLEE we described the situation in Venezuela as comprising contiguous or potentially overlapping domestic and sylvatic transmission cycles (Fig. 1), implying that re-invasion by sylvatic *Rhodnius* might prejudice vector control programmes. However, we will see below that MLEE does not give full insight into the molecular epidemiology of *T. cruzi* in Venezuela; microsatellite analysis has recently been applied to both the vector and parasite and provided a higher resolution understanding of transmission dynamics.

Thus, here was a potential answer to the first of our questions. Apparent rarity of megasyndromes in Venezuela might circumstantially be linked to the radical genetic differences between the strains of *T. cruzi* that predominantly cause Chagas disease in Brazil (ZII/TcIIb) and in Venezuela (ZI/TcI).

ENZOOTIC TRANSMISSION OF *TRYPANOSOMA CRUZI* IN THE AMAZON REGION

To address the second question concerning the reasons why there were so few cases of Chagas disease in the Amazon region, attempts were made to hunt for sylvatic triatomine species and their habitats in Amazon forest, adjacent to Belém, Pará State, Brazil. Initial searches yielded virtually nothing, despite considerable effort. This led to the development of 'spool-and-line mammal tracking', initially with makeshift components. The first animal tracked, an opossum, *Didelphis marsupialis*, was released at the trap site, carrying the tracking device with the free end of the thread tied to vegetation. The next day the thread was followed through the forest for a considerable distance and the opossum recovered from its arboreal nest, which contained 2 triatomine

Table 2. Zymodemes of 316 isolates of *Trypanosoma cruzi* from Venezuela and Brazil (Miles, 1981)

	Domestic	Sylvatic
Brazil (Bahia State)*	(ZII) *T. cruzi* IIb: **107** isolates	(ZI) *T. cruzi* I: **23** isolates
	(ZI) *T. cruzi* I: **1** isolate	(ZIII) *T. cruzi* IIa: **2** isolates
Venezuela	(ZI) *T. cruzi* I: **32** isolates	(ZI) *T. cruzi* I: **18** isolates
		(ZIII) *T. cruzi* IIa: **2** isolates
Amazon Basin	N/A	(ZI) *T. cruzi* I: **113** isolates
		(ZIII) *T. cruzi* IIa or (ZIII/ZI ASAT) *T. cruzi* IIc: **18** isolates

* Also 12 *T. cruzi* I acute cases in an outbreak in the Sao Francisco valley (Luquetti *et al*. 1986).

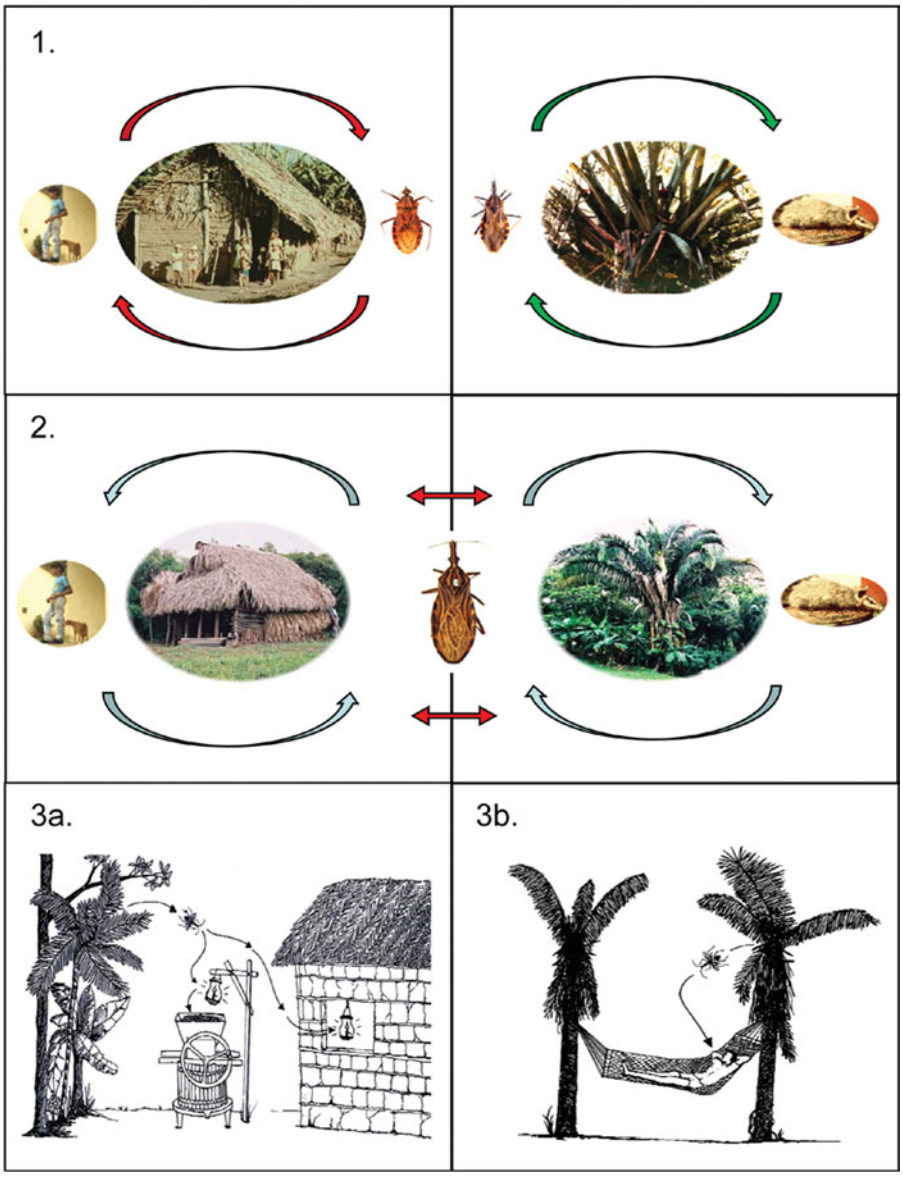

Fig. 1. Non-overlapping, overlapping and enzootic *Trypanosoma cruzi* transmission cycles. (1) Domestic transmission of TcII by *Panstrongylus megistus* in Bahia State, Brazil and separate sylvatic transmission of TcI to *Didelphis albiventris* by *Triatoma tibiamaculata*. (2) Overlapping domestic and sylvatic transmission of TcI in parts of Venezuela. (3) Sporadic enzootic transmission of TcI and occasionally TcIIa in the Amazon basin: (a) by light attraction of adult triatomine bugs to palm presses or houses and (b) by exposure of piassaba palm frond collectors to faecal contamination from *Rhodnius brethesi*.

species, one a new species record for Brazil and both species infected with *T. cruzi*. Improved tracking devices were devised, using non-rotating, precision-wound spools of thread (Miles, 1981*a*). Spool-and-line mammal tracking has become a standard and widely applied method for investigating mammal nesting sites and behaviour (Wells *et al*. 2006). In the Amazon basin, this method helped to reveal that all the local triatomines are fortunately species that do not readily adapt to colonizing houses (Miles *et al*. 1981*c*). Furthermore, this approach helped to give insight into the exquisite adaptation of several triatomine species to their particular ecological niche, including their camouflage colouration, which can give a clue to the nature of that niche (Gaunt and Miles, 2000). Thus, for example, *Panstrongylus lignarius* has nymphs in arboreal tree holes and adults perfectly camouflaged to roam on the trunks of their elected tree species. Using spool-and-line mammal tracking a new species, *Rhodnius paraensis*, was discovered in nests of the arboreal rodent, *Echimys chrysurus* (Sherlock *et al*. 1977). The sporadic cases of Chagas disease that do occur in the Amazon region are thus not due to domestic triatomine colonies but are caused by adult bugs attracted to dwellings, especially to artificial light. More than half of these cases are attributable to triatomine contamination of food and oral outbreaks, especially due to infected bugs entering juice presses, such as those used for açai palm or sugar cane (Coura *et al*. 2002; Valente *et al*. 2009; Pan American Health Organisation, 2009). Of triatomine species in the Brazilian Amazon, only *Panstrongylus geniculatus* has shown some new peridomestic adaptation to pig sties (Valente *et al*. 1998; Feliciangeli *et al*. 2004). Fortunately, peridomestic *Triatoma rubrofasciata*, which occurs in Belém and is widespread in ports around the New and Old Worlds, has a preferred animal host, the rat *Rattus rattus* and very rarely bites humans. This epidemiological situation in the Amazon region was described as one of an enzootic transmission cycle, with *T. cruzi* highly prevalent in sylvatic mammal reservoirs and vectors but without household triatomine colonies (Fig. 1). The transmission of *T. cruzi* in the USA, where triatomines are occasionally peridomestic with dogs but do not colonize houses, is analogous to that in the Amazon region. In North America there may be some risk of sporadic blood transfusion transmission if Latin American donors from endemic regions are not screened serologically for *T. cruzi* infection (Leiby *et al*. 2008). Thus, the comparative rarity of Chagas disease in the Amazon basin is due to the good fortune that local triatomine species do not readily colonize houses, and domiciliated bug species have not yet been imported from other endemic regions. However, as human disturbance continues to impact on Amazonia, active surveillance is necessary to monitor the status of domestic *T. cruzi* transmission. In recognition of this, the Intergovernmental Initiative for Surveillance and Control of Chagas Disease in the Amazon Region (AMCHA) was launched in 2004 (Aguilar *et al*. 2007).

TRYPANOSOMA CRUZI DIVERSITY IN THE AMAZON AND BEYOND

Not unexpectedly, *T. cruzi* ZI (TcI) was found to be the cause of sporadic Chagas disease in Amazonian Brazil corresponding with its sylvatic presence elsewhere in Brazil. A novel, third *T. cruzi* zymodeme (ZIII, now TcIIa) was identified from a small oral outbreak of Chagas disease in the suburbs of Belém, and this was furthermore shown to be the secondary agent of Chagas disease in Venezuela (Miles *et al*. 1978, 1981*b*). Subsequently, a fourth *T. cruzi* zymodeme (ZIII + ASATI, now TcIIc) was characterized from sylvatic cycles in Amazonian Brazil. Collaborative studies in Bolivia and Paraguay, yielded 2 further groups that were characterized by highly heterozygous novel MLEE profiles (Bolivian ZII, now TcIId and Paraguayan ZII, now TcIIe) (Tibayrenc and Miles, 1983; Chapman *et al*. 1984; Miles *et al*. 1984).

Michel Tibayrenc and his collaborators performed a further series of collaborative *T. cruzi* genotyping studies in the Americas, initially using MLEE and later supplemented with random amplification of polymorphic DNA (RAPD) (Tibayrenc *et al*. 1993). Various authors applied a plethora of different genetic markers, including kDNA fragment length polymorphisms (schizodemes), karyotype variation, PCR amplicon size polymorphisms, PCR restriction fragment length polymorphisms (PCR-RFLPs) and comparative DNA sequencing of nuclear and mitochondrial targets (Morel *et al*. 1980; Brisse *et al*. 2001; de Freitas *et al*. 2006; Rozas *et al*. 2007). These studies revealed additional diversity within each principal zymodeme. A confusing profusion of conflicting nomenclature arose in the literature, largely resolved, firstly by consensus in 1999 (Anon, 1999) to define 2 major *T. cruzi* lineages TcI and TcII and secondly by development of the DTU nomenclature system (Brisse *et al*. 2001), which is now clearly supported by all but the most conserved nuclear genetic markers (Westenberger *et al*. 2005). The original principal zymodemes thus correspond with the *T. cruzi* DTUs, as summarized in Table 1.

ECOLOGICAL NICHES AND PHYLOGEOGRAPHY OF THE *TRYPANOSOMA CRUZI* LINEAGES

What then is the biological, ecological and epidemiological significance of these distinct *T. cruzi* lineages or DTUs? Increasing evidence supports the idea that the 6 *T. cruzi* DTUs are historically and currently associated with distinct ecological niches, with concomitant implications for the epidemiology

Table 3. Sylvatic niche, host, vector, geographical distribution and disease associations of the major *Trypanosoma cruzi* DTUs

Genotype	Niche	Sylvatic hosts	Sylvatic vectors	Geography [Chagas disease]
TcI	***Primary* Arboreal**, lowland tropical/ semi-tropical, especially palms of the genus *Attalea*	***Primary* Arboreal**/ semi-arboreal, ***Didelphis***, primates, arboreal rodents	***Primary Rhodnius spp.***	***Primary*** Lowland tropical/ semi-tropical USA, Central & South America
	Secondary Arid rocky	***Secondary*** Terrestrial rodents	***Secondary***: *Panstrongylus Triatoma*	***Secondary***: Central Brazil and Eastern Andean foothills **[North of Amazon CHAGAS DISEASE]**
TcIIa	Arboreal primates, some terrestrial hosts	Primates, *D. novemcinctus*, bats, *N. nasua* (coati); *P. lotor* (USA)	*Rhodnius*, *Panstrongylus*, *Triatoma*	Northern South America, USA
TcIIb	? Rare in sylvatic cycles	? Atlantic forest primates; *E. sexcinctus* (Paraguay)	?	**[Atlantic/Central Brazil CHAGAS DISEASE]**
TcIIc	**Terrestrial/fossorial**, lowland arid and tropical	***D. novemcinctus***, *E. sexcinctus*, rodents, marsupials, carnivores	***P. geniculatus***, *P. lignarius*, *T. rubrovaria*	Lowland South America
TcIId	? Rare in sylvatic cycles	? Possibly *D. novemcinctus, E. sexcinctus*	?	**[Southern cone CHAGAS DISEASE]**
TcIIe	? Rare in sylvatic cycles	?	?	**[Southern cone CHAGAS DISEASE]**

of Chagas disease. The niches are not fully understood, partly due to limited sampling and genotyping of *T. cruzi* isolates. As to be expected, interaction between niches occurs, changing as ecologies are disturbed, as evidenced by mixed DTU infections in vectors and mammals, including humans (Breniere *et al*. 1989). Here we review each DTU in the context of its known or putative ecological niche associations, summarized in Table 3.

Trypanosoma cruzi *lineage* (*DTU*) *TcI*. This is the predominant agent of Chagas disease in countries North of the Amazon and in the Amazon region. This was revealed in 1981 by the comparative study mentioned above (Miles *et al*. 1981*b*), where it was also proposed that the rarity of megaoesophagus and megacolon in these regions could be ascribed to the local predominant endemicity of TcI. It was not, however, suggested that TcI infection was entirely benign, in fact we demonstrated clearly by analysis of an unusual outbreak of TcI infection in Goiania State, Brazil, associated with drought and incursion of sylvatic rodents into houses, that both TcI and TcIIb could cause severe acute Chagas disease (Luquetti *et al*. 1986). Furthermore, chagasic cardiomyopathy is commonplace in TcI endemic countries such as Venezuela, and elsewhere TcI has been associated with severe disease, including meningoencephalitis (Anez *et al*. 2004; Burgos *et al*. 2008). Nevertheless, the perception that megaoesophagus and megacolon are uncommon North of the Amazon remains (Sanchez-Guillen Mdel *et al*. 2006).

In the context of TcI pathogenesis, the lead author of this review noted, some years ago, pericardial sequestration in experimental TcI infections in immunocompromised (SCID) mice that were severely ill but had barely detectable peripheral parasitaemias (Miles, unpublished data). Cardiac puncture retrieved clear effusion with parasitaemias 100 to 1000-fold greater than that detectable in the peripheral blood of the same animals. There is a prior reference to such elevated pericardial parasitaemias in experimentally infected hamsters but the DTU involved is not clear (Ramirez *et al*. 1994). Acquatella (2007) notes the high frequency of pericardial effusion in acute Chagas disease in Venezuela, French Guiana and the Amazon region, regions endemic for TcI. In a summary of 233 acute cases of Chagas disease in the Brazilian Amazon, Pinto *et al*. (2008) observed that the most frequent echocardiographic feature was pericardial effusion. We speculate therefore that pericardial sequestration may explain the cardiomyopathy associated with low peripheral parasitaemias and be a hitherto neglected aspect of the pathogenesis of Chagas disease.

As mentioned above, the presence of TcI in both sylvatic and domestic transmission cycles in Venezuela suggested triatomine re-invasion of dwellings, not an unreasonable hypothesis, because fronds from triatomine-infested palms are used to roof rural houses. In contrast, it had been suggested that all bugs in Venezuelan palms were not *Rhodnius prolixus* but were actually *Rhodnius robustus* and, because this species does not colonize houses, are of little threat except as a cause of sporadic cases of Chagas disease from lone, short-lived adult bugs light-attracted to dwellings (Fitzpatrick *et al*. 2008). To resolve this issue we applied mitochondrial markers and a new range of microsatellites to the high resolution analysis of populations of *Rhodnius* from palms

and adjacent dwellings. The results clearly demonstrated that *bona fide Rhodnius prolixus*, the domestic vector species, was present in palms and that at some sites but not all, mitochondrial haplotypes were shared between palms and houses, and this was confirmed by the distribution of microsatellite genotypes (Fitzpatrick *et al*. 2008). The implication was that in some cases *R. prolixus* did indeed re-invade houses from sylvatic foci, whilst there was also concomitant spread of *R. prolixus* between palms and between houses.

To investigate this phenomenon of re-invasion more thoroughly, we used a large panel of polymorphic microsatellite markers optimized for genotyping of *T. cruzi* for fine-scale resolution of the geographical distribution and population structure of TcI (Llewellyn *et al*. 2009*a*). Research in Colombia and elsewhere has shown that miniexon sequencing detects diversity within TcI, which appears to be associated with type of transmission cycle, i.e. domestic, peridomestic or sylvatic (Herrera *et al*. 2007; O'Connor *et al*. 2007). Our microsatellite analysis revealed substantial diversity within TcI, potential founder events in Venezuela and Bolivia and evidence of structure between distant populations, including indications of the dispersal of TcI from South to North America, presumably facilitated by mammals crossing the Isthmus of Panama. Interestingly, in some cases notable genetic structure could be detected between nearby populations. In Bolivia, adjacent lowland and highland TcI populations – corresponding to primary and secondary TcI niches, as described below and in Table 3, showed greater subdivision than that between lowland Bolivia and Venezuelan sylvatic strains, thousands of kilometres to the north. Highland Bolivian strains were also characterized by a reduction in diversity, indicating a probable founder event. Crucial in terms of our understanding of disease transmission was detection of a potential founder event in Venezuela among most cases of chronic Chagas disease (Llewellyn *et al*. 2009*a*) (Fig. 2). Here, although we found evidence that sylvatic strains of TcI do occasionally infect humans in Venezuela, it appears that a domestic strain of TcI with restricted genetic diversity has become widespread across endemic areas. This may be explicable by the precarious contaminative transmission route of *T. cruzi* and the fact that an invading infected sylvatic adult *R. prolixus* has a low likelihood of transmitting the infection compared to the probability of its offspring transmitting an established domestic strain. In this context, it may be of interest to examine the comparable genetic diversity of *Trypanosoma rangeli*, which is transmitted by the less precarious route of inoculation from *Rhodnius* salivary glands (Miles *et al*. 1983). Alternatively or additionally, there may be selection of a particular genotype of TcI within the human population. Intriguingly, sylvatic TcI (and TcIIc) populations show an unexpectedly high level of homozygosity, which does not theoretically accord with prolonged clonal propagation, whereas the founder domestic TcI populations show a somewhat increased level of heterozygosity.

There is now a wealth of TcI isolate and genotype records (Yeo *et al*. 2005; Llewellyn *et al*. 2009*a*). These observations reinforce the fact that among arboreal mammals, the common opossum, *Didelphis marsupialis*, is by far the most abundantly recorded mammal host of TcI and that *Rhodnius* species (tribe Rhodniini), most of which are associated with palm tree ecotopes, predominantly but by no means exclusively transmit TcI. It can therefore be stated that TcI is largely associated with arboreal transmission cycles. Strict boundaries are hard to define, however, and a secondary, allopatric TcI population is also now evident from arid rocky ecotopes in Piaui (Central/Eastern Brazil) (Herrera *et al*. 2005) and around Cochabamba (Cortez *et al*. 2006). It remains to be seen how widely sylvatic TcI strains occur within these ecotopes, and their possible link(s) with domestic cycles of TcI transmission if, as was noted by Luquetti *et al*. (1986), rodents can provide a bridge by invading dwellings in times of drought.

Trypanosoma cruzi *lineage* (*DTU*) *TcIIa*. This is a relatively poorly understood group. It is a secondary cause of Chagas disease in Venezuela (Miles *et al*. 1981*b*) and was also responsible for the first recorded outbreak of presumed orally transmitted simultaneous acute cases of Chagas disease in the suburb of Canudos, Belém, Pará State Brazil (Miles *et al*. 1978); a TcIIa reference strain, one of the few isolated from humans, is from that outbreak (the CANIII strain and its clones). Nevertheless, few isolates of TcIIa are available and its sylvatic ecological niche is poorly understood. In the Amazon basin a few isolates were initially obtained from the armadillo, *Dasypus novemcinctus*, and the terrestrial opossum *Monodelphis*, suggesting an ecological niche similar to that of TcIIc, described below. However, a series of new isolates has been obtained from primates (*Saguinus, Aotus* and *Cebus*) and from *Rhodnius brethesi* and *R. robustus* in the Amazon basin, with an overlapping distribution with TcI (Marcili *et al*. 2009*c*). Understanding of the distribution and phylogeography of TcIIa is complicated by the fact that several genotyping methods fail to distinguish the lineage from others, particularly from TcIIc.

Importantly, TcIIa is known to be endemic, with TcI, in North America, and has there been provisionally associated with raccoons (Clark and Pung, 1994; Roellig *et al*. 2008). Furthermore, firstly, there is evidence that TcIIa in North America is quite distinct from TcIIa in South America (Barnabé *et al*. 2001*b*; Marcili *et al*. 2009*c*) and secondly, the presence of identical mitochondrial DNA sequences

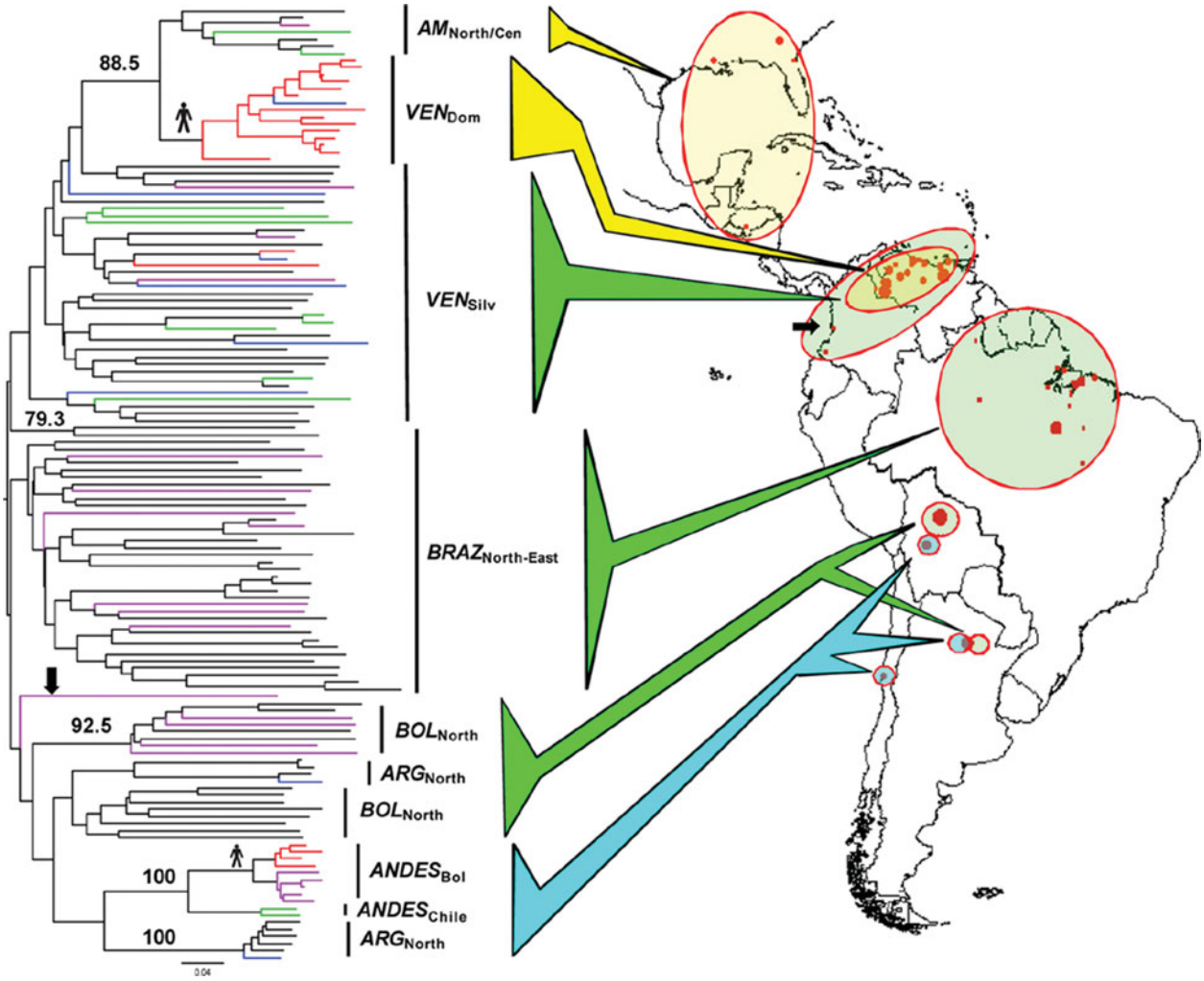

Fig. 2. Unrooted neighbour-joining D_{AS} tree showing TcI population structure across the Americas. Based on the multilocus microsatellite profiles of 135 TcI isolates. D_{AS}-based bootstrap values were calculated over 10 000 trees from 100 re-sampled datasets and those >75% are shown on major clades. Branch colour codes indicate strain origin. Black: *Didelphis* species; Purple: non-*Didelphis* mammalian reservoir; Green: silvatic triatomine; Red: human; Blue: domestic triatomine. Coloured block arrows and circles indicate broad population types. Yellow: Venezuelan domestic and North/Central American groups; green: major silvatic populations; blue: South-Western clade. Black arrow indicates Colombian outlier assigned to Brazilian population. Human symbol indicates putative genetic association with domestic transmission. Closed red circle area is proportionate to sampling density. Population codes: North and Central American ($AM_{North/Cen}$), Venezuelan silvatic (VEN_{silv}), North Eastern Brazil ($BRAZ_{North\text{-}East}$), Northern Bolivia (BOL_{North}), Northern Argentina (ARG_{North}), Bolivian and Chilean Andes ($ANDES_{Bol/Chile}$) and Venezuelan domestic (VEN_{dom}). Reproduced from Llewellyn *et al.* (2009*a*).

in North American TcIIa strains and TcI strains suggests that genetic exchange has contributed to the diversity of the *T. cruzi* strains seen in North America (Machado and Ayala, 2001; Yeo *et al.*, unpublished data). A further concerted research effort is therefore required to understand the origin of TcIIa cases of Chagas disease in Venezuela, the ecological niche of its natural populations, and its molecular epidemiology in the USA.

Trypanosoma cruzi *lineage* (*DTU*) *TcIIc*. Although, like TcIIa, TcIIc is relatively rare in domestic transmission cycles it is one of the better understood of the *T. cruzi* lineages. It is predominantly associated over a vast geographical expanse from Northern South America to Argentina, with the armadillo *Dasypus novemcinctus* and less frequently with other terrestrial burrowing animals, namely the armadillo *Euphractus sexcinctus*, skunk (*Conepatus*), *Monodelphis brevicaudata* and terrestrial rodents (Yeo *et al.* 2005; Marcili *et al.* 2009*b*). In view of its mammal host association it would be interesting to investigate whether TcIIc might also be found in North America. The implicated triatomine vector species, although not well known, are terrestrial *Panstrongylus* and *Triatoma* (tribe Triatomini). A high resolution microsatellite analysis revealed genetic diversity and spatial population substructure within TcIIc (Llewellyn *et al.* 2009*b*). Sylvatic populations showed an elevated level of homozygosity

that is not consistent with clonal propagation, although it is not clear whether this is explicable by intralineage recombination or gene conversion. TcIIc rarely causes human Chagas disease but has been recorded from domestic dogs (Chapman *et al.* 1984; Cardinal *et al.* 2008; Marcili *et al.* 2009*b*), threatening to be an emergent disease agent as transmission cycles evolve.

Trypanosoma cruzi *lineage* (*DTU*) *TcIIb*. This is, with TcIId and TcIIe, a main agent of Chagas disease in the Southern Cone region of South America, where *Triatoma infestans* is the principal domestic vector. TcIIb is a primary cause of severe acute and chronic Chagas disease in the Atlantic forest region of Brazil and central Brazil, where megaoesophagus and megacolon are recorded. It is the disease agent described from the São Felipe study in Bahia State, where domestic *Panstrongylus megistus* is the vector.

Like TcIId and TcIIe, TcIIb has rarely been recorded from sylvatic cycles and its natural ecological niche is yet to be defined. A few isolates have been reported from opossums in the Atlantic forest and from sylvatic primates, which led to the suggestion that such primates might be the primary original mammalian host of TcIIb (Lisboa *et al.* 2004). Accordingly, we investigated whether primates in the Amazon region might be a hitherto undiscovered reservoir of TcIIb. However, we found that Amazonian primates mainly carried TcI and to some extent TcIIa, with no isolate of TcIIb recorded (Marcili *et al.* 2009*c*). The original primary host of TcIIb thus seems not to be primates and its ecological niche has yet to be ascertained. In a separate study we obtained a single isolate of TcIIb from *E. sexcinctus* in the Paraguayan Chaco and, in this context, it could be important to isolate more *T. cruzi* from terrestrial opossums and from edentates in the Atlantic forest region (Yeo *et al.* 2005).

Trypanosoma cruzi *lineage* (*DTUs*) *TcIId and TcIIe*. These two appear to be the main causes of severe acute and chronic Chagas disease in the greater Gran Chaco region and neighbouring countries, namely Bolivia, Chile, northern Argentina, Paraguay and parts of southern Brazil, where they are present almost exclusively in domestic transmission cycles, transmitted by domestic *Triatoma infestans* (Chapman *et al.* 1984; Miles *et al.* 1984; Bosseno *et al.* 1996; Barnabe *et al.* 2000, 2001*a*; Virreira *et al.* 2006*b*; Cardinal *et al.* 2008). It is important to note, however, that mixed lineage infections may occur in single patients, for example in Bolivia and Argentina, with local transmission of TcI occurring, also by *T. infestans* (Breniere *et al.* 1989; Bosseno *et al.* 1996; Cardinal *et al.* 2008; Valadares *et al.* 2008). TcIId and TcIIe are associated with megaoesophagus and megacolon (Carranza *et al.* 2009). TcIId is also linked to congenital transmission of Chagas disease in Bolivia, although this may only be a reflection of the local abundance of that lineage (Virreira *et al.* 2006*a*; Corrales *et al.* 2009). In contrast, however, congenital Chagas disease appears to be rare in both TcI and TcIIb endemic regions.

Fascinatingly, TcIId and TcIIe are inter-lineage hybrids derived from hybridization between TcIIb and TcIIc. They are also exceedingly rare in sylvatic transmission cycles, raising questions about their origin. The circumstances that led to the evolution of TcIId and TcIIe are the focus of current research in our laboratory and the large number of microsatellite markers that we have developed should allow a deeper understanding of these important disease agents.

NEW TOOLS, OLD QUESTIONS

As highlighted above, a number of knowledge gaps exist with respect to the geographical distribution of the *T. cruzi* DTUs as well as their mammalian hosts and vectors. This is largely due to inadequate sampling but also because genotyping methods lacking sufficient discriminatory power have often been applied to characterize isolates. For example, isolates have frequently been described simply as TcII (or various equivalent terms) without assignment into one of the 5 DTUs encompassed by this grouping. This situation has made interpretation of data and comparison of different studies problematic and led to confusion over the geographical distribution of the lineages. To overcome this problem, we have recently proposed the adoption of a standardized, simple combination of 3 genotyping assays that can reliably distinguish between all 6 known lineages. The protocol comprises the combination of the popular LSU rDNA PCR assay (Souto *et al.* 1996) with PCR-RFLP assays targeted to the *GPI* and *HSP60* loci (Lewis *et al.* 2009).

In order to answer questions regarding the phlyogeography of the *T. cruzi* lineages, and to resolve intra-lineage relationships, a different set of genetic markers is required to that used for simple DTU assignment. In the modern era 3 techniques potentially provide the requisite level of resolution: multilocus sequence typing (MLST), multilocus microsatellite typing (MLMT) and comparative genomics. The development and application of MLMT in our laboratory has already been discussed in the previous sections; a brief overview of MLST and comparative genomics now follows.

MULTILOCUS SEQUENCING TYPING (MLST) AS A POPULATION GENETIC, TAXONOMIC AND PHYLOGENETIC TOOL

Multilocus sequence typing (MLST) is a typing approach in which short regions of 7 or more genes are targeted, amplified and sequenced to provide a

highly resolutive and reproducible typing system. Originally developed for bacterial epidemiology and evolutionary phylogenetics, chosen targets are usually 'housekeeping genes' subject to stabilizing selection, avoiding false evolutionary associations in genes subject to diversifying selection. In most MLST typing schemes the SNPs (single nucleotide polymorphisms) from chosen genes produce a ratio of non-synonymous to synonymous amino acid changes (dN/dS) of below 1·0 (Odds and Jacobsen, 2008). The global spread of clonal populations can be monitored and the lack of contiguity between individual gene trees has led to the detection of genetic recombination among bacterial populations previously thought to be clonal. MLST has also been applied to an increasing number of diploid organisms and is applicable to *T. cruzi* and other protozoa, with the complication that such organisms are not haploid but minimally diploid and carry at least 2 alternative alleles at each locus. Heterozygous loci are usually detected as split peaks from direct sequencing and allelic phase determined by cloning, allele specific PCR or by haplotype reconstruction algorithms. Sequences from multiple loci are typically concatenated to produce a diploid sequence type (DST) for each isolate. A basic MLST approach, comparing incongruence between two individual phylogenetic trees, was in effect initiated by Machado and Ayala in their study of the genetic recombination in natural *T. cruzi* populations (Machado and Ayala, 2001) and this is now being expanded by others to additional targets (Subileau *et al*. 2009). We have investigated 5 single-locus MLST targets, encoding proteins with varying functional roles. We find that some MLST targets are relatively conserved, whereas others have high resolute power, emphasizing the importance of a multilocus approach, although not equivalent to that of microsatellites. Not only can MLST resolve the lineages but we find some evidence of intra-lineage genetic recombination, population structuring and discrete host associations and the data may enable us to infer the evolutionary origins of the hybrid lineages TcIId and TcIIe (Yeo *et al*. manuscript in preparation).

Until superseded by high throughput comparative genomics of many *T. cruzi* genomes, MLST has the potential to provide a substantial contribution to the understanding of the epidemiology, transmission and phylogenetics of *T. cruzi*, particularly if a standardized MLST protocol can be adopted and data deposited in easily accessible databases such as MLST.net (Aanensen and Spratt, 2005), which contains typing schemes for a growing number of pathogens.

COMPARATIVE GENOMICS

To date, only 1 *T. cruzi* genome sequence has been generated, for the CL Brener strain of the TcIIe lineage, with an imperfect assembly that has been complicated by the hybrid nature of the strain and the abundance of repetitive sequences (El-Sayed *et al*. 2005). A new attempt has been made to assemble the CL Brener genome sequence but this assembly is apparently still incomplete (Weatherly *et al*. 2009). The application of second generation sequencing technologies to trypanosomatid genomes should imminently improve the situation with respect to coverage of the diversity of the species and for assembly accuracy. Indeed, new genome sequencing initiatives are under way, notably for a reference strain of the TcI lineage (Andersson *et al*., in progress) and for multiple *T. cruzi* reference strains isolated from diverse cases of Chagas disease. These genome sequences will be incisively informative on characteristics that may relate to pathogenesis, drug susceptibility and evolution of the different DTUs, although considerable skill will be required in this context to elucidate differential gene expression and regulation. Furthermore, although these new sequencing technologies can quickly generate a vast amount of data, the resolution of multigene families, which are characterized by highly repetitive regions and are thus potentially consolidated in genome sequence output, may require supplementary approaches. Nevertheless, the fundamental value of comparative genomics can be seen from the impact on understanding of other organisms, for example *Candida* (Butler *et al*. 2009).

NOMENCLATURE AND TAXONOMIC CONSIDERATIONS

Questions have been raised as to whether the current nomenclature for *T. cruzi* lineages or DTUs should be updated and modified. Should TcI and TcII be named as different species? No, at least not until we have the wealth of new data that will soon emerge from comparative genomics, more widespread availability of simple methods to distinguish them, and clarification of which species definition should apply. Additionally, it is clear from numerous sequencing studies (Machado and Ayala, 2001; Brisse *et al*. 2003; Westenberger *et al*. 2005; Broutin *et al*. 2006) that TcII does not represent a monophyletic group (TcIIc for example is, at most loci, more genetically similar to TcI than to TcIIb). Should TcIIa and TcIIc be merged? Not at the moment, TcIIc seems to have the most discrete ecology of all the lineages and consistently clusters separately; knowledge of TcIIa is still rudimentary and distinct TcIIa populations have been found in the USA. Should the hybrid lineages TcIId and TcIIe be merged, or renamed because they are derived from TcIIb and TcIIc? Probably not, partly because these hybrids have fundamental epidemiological significance and it would require an unnecessary new round of adjustment in the literature but also because it is not

yet clear whether they arose from discrete hybridization events (Westenberger *et al.* 2005; de Freitas *et al.* 2006). Although we are currently in favour of maintaining the status quo, it should be borne in mind that the current nomenclature needs to be dynamic, new lineages and lineage interactions will certainly emerge as sampling improves, and vector and reservoir species have yet to be fully explored, notably the Chiroptera (bats) (Marcili *et al.* 2009*a*).

What is certainly required is the more uniform adoption of nomenclature in the scientific literature, with vigilant referees excluding both presumptive conclusions based on inadequate identification of the lineages and throwbacks to historical terms. This will be aided by the transfer to endemic areas of technologies to allow the simple identification of all the known lineages. Issues of biased sampling, mixed infections and *in vitro* selection of genotypes can also not be ignored.

MOLECULAR EPIDEMIOLOGY IN THE CONTEXT OF CONTROL OF CHAGAS DISEASE

What then has the study of the genetic diversity of *T. cruzi* to do with the control of Chagas disease? This has been addressed in a previous review (Miles *et al.* 2003), but can be summarized in 5 key points. (1) As we have seen, the application of molecular methods has shown that *T. cruzi* is not one organism but a fascinating heterogeneous complex, which will inevitably have diverse phenotypes; (2) we have shown that the molecular epidemiology unravels the different types of transmission cycle and this is important to devising vector control strategies and understanding limitations; (3) genotyping demonstrates that the *T. cruzi* lineages have an historical and current ecological framework that broadly makes biological sense, although all the details are not yet clear; (4) surveillance measures are facilitated by genotyping methods because they allow the identification and molecular tracking of lineages, such as TcIIc and TcIIa, that at present rarely cause human disease but represent emergent risks as they infiltrate peridomestic and domestic transmission cycles; (5) an understanding of the genetic lineages and access to representative strains provides a basis for broad-ranging fundamental phenotypic, genetic and genomic comparisons relevant to pathogenesis and therapy. Ideally, the conundrum of whether infecting genotypes, as well as host factors, govern clinical manifestations (Carranza *et al.* 2009) can be addressed, although this hope is yet to be fully realised and may require innovative technical approaches to reveal the *T. cruzi* genotypes present in blood and internal organs (Vago *et al.* 2000; Valadares *et al.* 2008) and to resolve the patient's prior history of exposure to such genotypes.

THE CLONALITY PARADIGM

In the 1990s a prominent theory proposed to explain population structures of parasitic protozoa, including *T. cruzi*, was that of clonality, with genetic exchange considered to be absent or rare and of little consequence (Tibayrenc and Ayala, 1991). At this juncture, our group embarked upon a new series of *T. cruzi* studies, convinced that recombination may well occur, based on the MLEE and RAPD profiles seen among TcI in the Amazon basin, and on the heterozygosity of TcIId and TcIIe. A renewed study in Serra das Carajas, Pará State, Brazil, displayed MLEE profiles circumstantially compatible with some genetic recombination within the local sylvatic TcI populations (Carrasco *et al.* 1996). Consequently, research was undertaken to investigate experimentally whether *T. cruzi* had an extant capacity for genetic exchange.

SEX *IN VITRO*

We took advantage of new methods that allowed trypanosomatid protozoa to be genetically manipulated to express selectable marker genes. A pair of putative parental biological clones of TcI chosen from the Carrasco *et al.* study (Carrasco *et al.* 1996) was transformed with plasmids bearing drug resistance markers such that each of the parents was resistant to a different antibiotic. The drug-resistant putative parents were then co-passaged through mammalian cells, through mice and through triatomines. Double drug selection was applied to isolate potential recombinants from among the recovered populations. Six double drug-resistant TcI clones were recovered from parental pairs passaged through mammalian cell cultures. Although the plasmid vectors were episomal and not integrated into the parental chromosomes, characterization of the double drug-resistant clones (Stothard *et al.* 1999) demonstrated that they were indeed hybrids that carried MLEE, RAPD and karyotype markers inherited from both parents. Subsequent microsatellite and DNA sequence analysis provided additional evidence of hybridization, and showed that the maxicircle kDNA genotypes of the hybrids had been uniparentally inherited (Gaunt *et al.* 2003). When the pattern of inheritance was examined in the hybrids it was immediately clear that they had not acquired parental alleles in a Mendelian fashion. In fact, at almost all the loci examined every parental allele was found to be present, with the exception of one microsatellite locus (L660) and the tryparedoxin locus where parental alleles were absent. This indicated that the hybrids were most likely to have been formed by diploid-diploid fusion, creating a tetraploid intermediate, which subsequently underwent a limited degree of genome erosion. Measurement of the DNA content of the hybrids using flow cytometry supported this conclusion: when compared

to the parents, the hybrids had profiles consistent with an aneuploid DNA content mid-way between triploidy and tetraploidy (Lewis *et al*. 2009). This aneuploid state was found to be relatively stable, even after passage through a mammalian host model, and in response to stressful growth conditions. Strikingly, the passage of the subtetraploid experimental hybrids through mice demonstrated that they retained full competence to establish infection, including clear parasitism of both cardiac and skeletal muscle tissues (Lewis and Miles, unpublished data). We are currently investigating the comparative pathogenesis of hybrid and non-hybrid populations in more detail.

This series of experiments has therefore proved for the first time that *T. cruzi* has an extant capacity for genetic exchange and furthermore demonstrated that the mechanism involved hybridization through diploid fusion and genome erosion, similar to the parasexual processes of fungi (Heitman, 2006). Nevertheless, until more data become available the occurrence of orthodox meiosis in *T. cruzi* cannot be excluded, nor can the additional presence of genetic exchange within triatomine vectors.

SEX IN NATURE

The heterozygous MLEE profiles of TcIId and TcIIe led to occasional speculation that they could be consistent with some level of genetic exchange in natural transmission cycles. Sequencing of a number of genes has now proven that TcIId and TcIIe strains do indeed have recombinant genotypes and are the products of one or more hybridization events between a TcIIb parent and a TcIIc parent (Machado and Ayala, 2001; Brisse *et al*. 2003; Gaunt *et al*. 2003; Westenberger *et al*. 2005; de Freitas *et al*. 2006). Analysis of polymorphisms in additional targets led to the proposal that TcIIa and TcIIc may be the products of a more ancient hybridization event between TcI and TcIIb (Westenberger *et al*. 2005), however the far greater maxicircle sequence identity of TcIIa and TcIIc, as compared to their nuclear sequence divergence (de Freitas *et al*. 2006) has yet to be reconciled with a model of ancient hybridization.

The finding that TcIId/IIe are the products of hybridization, and the emerging evidence of some degree of genetic exchange on an evolutionary timescale has made it clear that recombination has had a profound influence on the evolution of natural *T. cruzi* populations. The 6 *T. cruzi* DTUs do exhibit strong linkage disequilibrium and their characteristic multi-locus genotypes are found across vast geographical distances (Tibayrenc and Ayala, 1991). This can only be explained by predominantly inter-DTU clonality. However, the frequency of genetic exchange within natural transmission cycles, its epidemiological significance, and whether the mechanism in nature corresponds with that in our laboratory are still being resolved.

FREQUENCY OF GENETIC EXCHANGE AND ITS EPIDEMIOLOGICAL SIGNIFICANCE

Virtually all protozoan pathogens previously considered to be clonal have now been shown to posess the capacity for genetic exchange, the latest being *Giardia* and *Leishmania* (Miles *et al*. 2009). Early studies of genetic exchange in *T. cruzi* were drastically constrained, because the isolates examined were few, collected from distant geographical sites and represented distinct genetic lineages. Whilst this eventually led to the detection of the inter-lineage hybrids TcIId and TcIIe, there was little or no capacity to detect the more likely presence of intra-lineage recombination, which has still not been adequately studied. This requires much more intensive sampling of natural populations, similar to that in the first study in São Felipe and to recent studies of *Trypanosoma congolense* (Morrison *et al*. 2009) and *Trypanosoma gambiense* (Koffi *et al*. 2009). The frequency of genetic exchange in natural *T. cruzi* populations is therefore unknown, as are the precise range of genetic mechanisms (see following section), the possible locations of genetic exchange and the stimuli involved. The extensive linkage disequilibrium that characterizes *T. cruzi* populations analysed so far does suggest, however, that if it does occur it is most likely to be between closely related individuals. Unfortunately the more pronounced this putative phenomenon is, the more difficult it becomes to detect. Levels of microsatellite homozygosity provide a clue that intra-lineage genetic recombination in some undisturbed natural transmission cycles might be commonplace, although it is not yet clear if this is explicable by gene conversion (Llewellyn *et al*., unpublished data). The fundamental importance of genetic exchange to the evolutionary history of *T. cruzi* and to the current epidemiology and distribution of Chagas disease is profound and beyond doubt. One has only to look at the endemic range of the hybrid lineages TcIId and TcIIe and associated severe disease to appreciate this. There are also parallels with the actual or potential distribution of hybrid strains of *Leishmania*, as indicated below.

GENETIC EXCHANGE IN *TRYPANOSOMA CRUZI*: HOW MANY MECHANISMS?

Did the hybridization event(s) that generated TcIId and TcIIe involve the same diploid-diploid fusion mechanism that we described for our TcI experimental hybrids? One approach to understanding the relevance of our experimental intra-lineage TcI hybrids to the natural *T. cruzi* inter-lineage hybrid populations TcIId and TcIIe, is to compare their

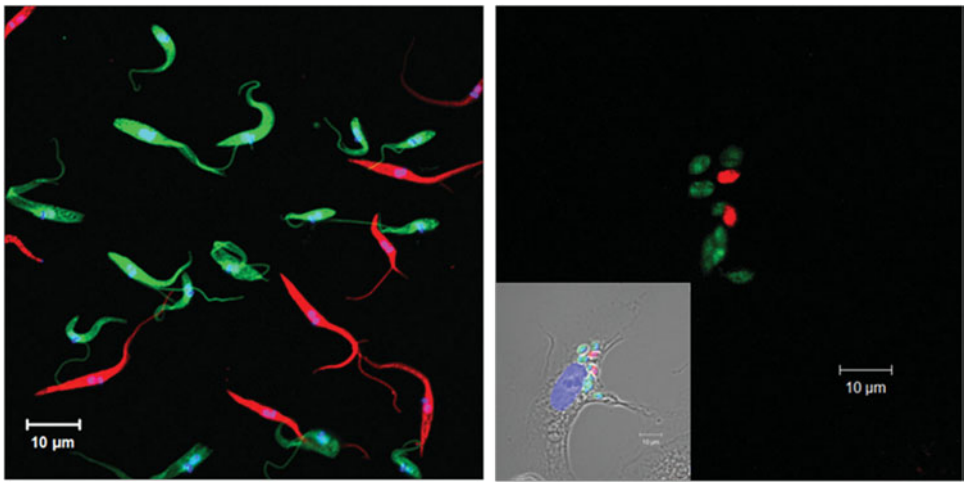

Fig. 3. Transgenic *Trypanosoma cruzi* expressing GFP or DsRed. Left: mixed epimastigote culture. Right: mixed infection of a Vero cell with amastigotes.

DNA contents. This approach was pioneered by James Dvorak who used flow cytometric analysis of *T. cruzi* to show that DNA content varied dramatically between *T. cruzi* stocks (Dvorak *et al.* 1982). As a basis of this comparative work we prepared a cohort of approximately 50 biological clones of *T. cruzi* representing all the known genetic lineages and including well-established reference strains. DNA content analysis of this reference panel confirmed an extraordinarily wide variation in DNA content within *T. cruzi* of up to 47·5% (Lewis *et al.* 2009). In particular, TcI strains had a significantly lower DNA content than the other DTUs. Importantly, the hybrid lineages TcIId and TcIIe had DNA contents that were equivalent to those found in their parental lineages, TcIIb and TcIIc. It was therefore concluded that the natural hybrid lineages were basically of diploid constitution. This was in marked contrast to the subtetraploid experimental TcI hybrids. Microsatellite analysis of TcIId/IIe using 8 loci supported the conclusions drawn from DNA content measurements, since only 1 case of allelic aneuploidy was identified (Lewis *et al.* 2009). Again, this was in contrast to the experimental TcI hybrids which exhibited >2 alleles at multiple loci (Gaunt *et al.* 2003).

Thus, there are fundamental differences between the naturally occurring hybrid DTUs and the experimental hybrids generated in our laboratory. It is not clear whether these differences reflect the operation of 2 different mechanisms of genetic exchange or whether the requisite triggers that would cause the experimental hybrids to return to diploidy are absent under the laboratory conditions so far tested. Likewise it is not known how the diploid state was reached by TcIId/IIe. The relevant hybridization event(s) could theoretically have occurred through orthodox meiosis, diploid fusion followed by either meiotic reduction (i.e. loss of complete haploid sets of chromosomes) or parasexual reduction (i.e. by random loss of individual chromosomes), or other mechanisms. The proximate triggers that precipitate genetic exchange *in vitro* have not been identified, nor what processes govern hybridization in natural populations. By analogy with other organisms, such as pathogenic fungi, it has been suggested that initiation of genetic exchange might occur in response to particular selective pressures (Heitman, 2006).

TRANSGENIC ORGANISMS FOR STUDIES OF RECOMBINATION AND PATHOGENESIS

Several attempts were made prior to our experimental production of hybrids to discover genetic recombination in *T. cruzi*. For example we gave triatomine bugs interrupted feeds on opossums and armadillos, known to be carrying different *T. cruzi* lineages and then looked for recombinant biological clones (Miles, 1982). However, only the ability to transform trypanosomatid parasites to carry selective drug-resistant markers gave such experiments sufficient power to detect and select recombinants. Without genetic transformation and the consequent ability to select double drug-resistant organisms, the *T. cruzi* experimental hybrids would certainly never have been recovered (Stothard *et al.* 1999).

The availability of transgenic trypanosomatids carrying fluorescent proteins, such as GFP and DsRed, adds a new dimension to research on recombination and pathogenesis. Single organisms of different lineages, carrying distinct fluorescent and drug-resistant markers, can now be visualized to look for co-infection and interaction (Fig. 3). This allows renewed investigation of the presence and frequency of genetic recombination and the mechanisms involved, throughout the entire life cycle. In addition, the efficiency of this approach allows multiple vector and host interactions to be explored. For example, there are approximately 140 different triatomine species and our present appreciation of the diversity

of vector parasite interactions may be simplistic. Furthermore, co-infection of individual experimental animals with transgenic strains carrying different fluorescent markers will allow the competitive virulence and pathogenesis of such strains to be explored. This approach circumvents the need for large comparative cohorts of experimental animals, and it will be aided by the development of powerful *in vivo* imaging techniques.

PARALLEL RESEARCH ON THE GENETIC DIVERSITY OF *LEISHMANIA*

Along with *T. cruzi*, the authors have followed a parallel interest in the genetic diversity of *Leishmania* that has become mutually beneficial and informative. The naming of *Leishmania* species has followed an entirely different route from *T. cruzi*, and it was primarily based on associated clinical presentation, whether visceral (VL) or cutaneous (CL) with potential for diffuse (DCL) and metastatic mucocutaneous (MCL) presentations. Two subgenera, *Leishmania* and *Viannia*, were distinguished by the distribution of their development in the sand fly vector, *Viannia* being found in the hindgut but also being confined to the New World and cutaneous (CL) or mucocutaneous (MCL) presentations. In addition the clinical features were, where possible, associated with the presence of particular vectors and reservoirs and with zoonotic or anthroponotic transmission (Ashford, 2000).

Genetic diversity of Leishmania

As with *T. cruzi*, pioneering work on the genetic diversity of *Leishmania* was based on the application of MLEE (Miles *et al.* 1981*d*; Rioux *et al.* 1990; Cupolillo *et al.* 1994). It became clear that several named *Leishmania* species were less divergent than the *T. cruzi* lineages TcI and TcII and that there was a tendency to 'split' *Leishmania* into multiple species not only based on the criteria briefly explained in the above section, but often on minor enzyme polymorphisms. The introduction of a wide range of molecular markers has broadly validated the taxonomic approach to *Leishmania* (Mauricio *et al.* 1999, 2004, 2006, 2007; Lukes *et al.* 2007). However, it has revealed interesting cases that call for revision of *Leishmania* taxonomy. The analysis of the *L. donovani* complex, for example, showed that some isolates had been misclassified as *L. infantum*, revealed a hidden extent of genetic diversity but also demonstrated that some species names are certainly not valid. *Leishmania archibaldi* turned out to be synonymous with *L. donovani* and *Leishmania infantum* synonymous with *L. chagasi* (also referred to as *L. infantum chagasi*). *L. archibaldi* strains are simply heterozygous for the gene coding for aspartate amino transferase, but are otherwise indistinguishable from strains of the Sudanese genetic group. The case of *L. infantum* and *L. chagasi* is quite interesting, as most studies used to justify their separation were based on 1 strain of each, whereas all studies in which a large number of strains were used showed that Latin American strains could not be distinguished from *L. infantum*, even with the most polymorphic markers available (Mauricio *et al.* 2000, 2001; Kuhls *et al.* 2007). The future status of *L. infantum* is also uncertain, given that this named species is simply a genetic group of *L. donovani*, giving *Leishmania* researchers another dilemma between prolificacy and conservatism. Several similar cases are emerging in the literature, such as for *L. tropica* and *L. killicki* (Schwenkenbecher *et al.* 2006). Thus, in the case of *Leishmania* there is a strong case for progressive taxonomic revision as more advanced molecular methods are extensively applied (Lukes *et al.* 2007). Maintenance of the status quo seems to confuse researchers who use species names indiscriminately and even erroneously, while the acutely needed revision will provide a basis for more in depth comparative studies of virulence, pathogenesis, host specificity and epidemiology, leading to an overall better understanding of leishmaniasis. Nevertheless, a balanced approach to taxonomic nomenclature is required, for example if striking phenotypic differences or a unique clinical presentation should be proven to depend on minor genetic diversity, unrelated to host confounders.

Development of multilocus sequence (*MLST*) *and microsatellite* (*MLMT*) *typing for* Leishmania

Many different typing methods have been developed and different targets used over the years to address *Leishmania* genetic diversity, recently reviewed by Botilde *et al.* (2006); and Schonian *et al.* (2008). Common and important drawbacks regard lack of portability, mainly for PCR-RFLPs, and capacity to detect genetic diversity, mainly for MLEE. Two new methodologies have emerged recently that, combined, should allow the entire *Leishmania* genus to be addressed from the subpopulation to the genus level. Sequence and microsatellite typing, used as multilocus approaches and developed in the same context as for *T. cruzi*, have provided new insight on population genetics, taxonomy and evolutionary history of *Leishmania*. At the moment there are 10 published MLST targets available for the *L. donovani* complex (Mauricio *et al.* 2006; Zemanova *et al.* 2007), most of which are also readily applicable to other Old World *Leishmania* (unpublished observations). In addition to the published 4 targets for the sub-genus *Leishmania* (*Viannia*) (Tsukayama *et al.* 2009) we and our collaborators are developing up to 11 more, which should allow better discrimination at a population level. Preliminary results show that MLST can be used for the same

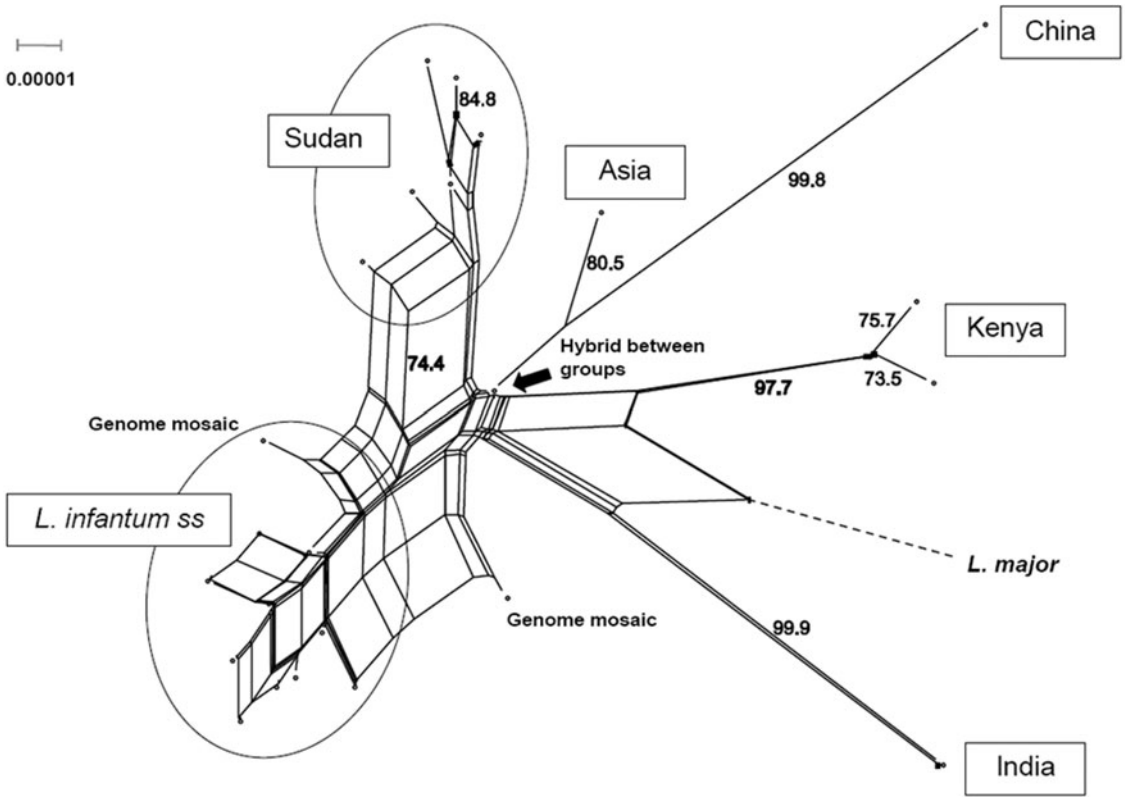

Fig. 4. *Leishmania donovani* complex MLST network suggests the importance of recombination through genetic mosaic structure and a hybrid between populations. Network built with Neighbor–Net using complete DNA sequences for *asat*, *gpi*, *nh1*, *nh2* and *pgd* coding regions, with 1000 bootstrap replicates. IUPAC codes for two bases were used for heterozygous sites. Distances were calculated using the Kimura-2-parameter. All strains were included and haplotypes were used where possible. The tree is rooted by *Leishmania major* Friedlin genome sequences (branch not to scale). Adapted from Mauricio *et al*. (2006).

targets across the *Leishmania* genus, which will enable comparisons not only of distances between species but also of the degree of genetic diversity within species. It will be possible to make more informed decisions about species validity and to compare species diversity, as well as investigate deeper evolutionary events. MLST has also emerged as a powerful tool to explore recombination between and within species and populations, as seen for the *L. donovani* complex (Mauricio *et al*. 2006).

Simultaneously, microsatellite typing has been developed for *Leishmania* strains by different groups. Microsatellite typing has beautifully shown the genetic structures of the *L. donovani* complex (Kuhls *et al*. 2008), *L. tropica* (Schwenkenbecher *et al*. 2006) and *L. major* (Al-Jawabreh *et al*. 2008) but also the finer population structure of South European *L. infantum* (Kuhls *et al*. 2008) and hybridization events in North Africa (Seridi *et al*. 2008).

Both of these techniques produce data that are easily loaded into databases and shared between researchers around the world. Even with the advent of cheap and high throughput genome sequencing, they have the capacity to resolve several questions on the molecular epidemiology of *Leishmania* at a fraction of the cost.

GENETIC RECOMBINATION IN *LEISHMANIA*

Similarly to *T. brucei* and *T. cruzi*, *Leishmania* were initially thought to be clonal, although occasional hybrids had been found between species (Evans *et al*. 1987; Kelly *et al*. 1991; Belli *et al*. 1994; Dujardin *et al*. 1995; Delgado *et al*. 1997; Ravel *et al*. 2006; Nolder *et al*. 2007). In our first studies of the *L. donovani* complex, particularly using MLST, however, we were able to show that a strain previously characterized by multilocus enzyme electrophoresis was, in fact, a hybrid between *L. donovani* genetic groups and that recombination played an important role in the genetics of this complex of species (Mauricio *et al*. 2006) (Fig. 4). Mosaic genotypes were seen in strains from local populations with high prevalence and diversity, such as Sudanese *L. donovani* and Spanish *L. infantum*.

In parallel, MLMT (recently supported by MLST, unpublished) uncovered a widespread and successful lineage of *L. tropica* that seems to have been the product of a single recombination event (Schwenkenbecher *et al*. 2006), mirroring the hybrid lineages of *T. cruzi* TcIId/e. In our group, (Nolder *et al*. 2007) also found MLMT evidence for the emergence and epidemiological significance of

multiple *L. braziliensis*/*L. peruviana* hybrids in Peru. A preliminary study of Sudanese populations of *L. donovani* suggests that at least one population may be in Hardy-Weinberg equilibrium (Baleela *et al.*, unpublished observations) in contrast with other Sudanese *L. donovani* and *L. infantum*. If *Leishmania* are mainly clonal they should show high heterozygosity due to accumulation of random mutations, but heterozygosity could be lowered by a high rate of gene conversion. On the other hand, high rates of inbreeding could explain the high homozygosity seen in most *Leishmania* populations, as found for *L. braziliensis* (Rougeron *et al.* 2009).

A long quest to produce the first laboratory hybrids of *Leishmania*, was meanwhile, underway. The first successful crosses of *L. major* were finally achieved, in the sand fly vector, at the end of 2008 (Akopyants *et al.* 2009). In addition, the *L. major* crosses suggest that meiosis, although not seen in *Leishmania*, must play a role, given that the recovered clones were either diploid or triploid. The mechanism of recombination in *Leishmania* thus seems to mirror *T. brucei*, but contrasts with *T. cruzi* (see above).

In future, experimental crosses, if routinely established, should enable genome mapping of determinants of important phenotypic traits, such as drug resistance, pathogenesis and virulence.

Genetic exchange may be crucial to survival and expansion of parasites that happily undergo clonal expansion in an ideal environment (Heitman, 2006). Through genomic re-assortment, some recombinant lineages may be able to adapt to new niches (vectors, mammalian hosts and climate) and may lead to future epidemics. A sobering reminder of the potential epidemic spread of *Leishmania* hybrids is the fact that *L. infantum*/*L. major* hybrids become transmissible by the widespread sand fly vector *Phlebotomus papatasi*, which is normally only competent to transmit *L. major* (Volf and Sadlova, 2009).

HOST SUSCEPTIBILITY

As succinctly mentioned for Chagas disease by Carranza *et al.* (2009), an important dimension has intentionally been omitted from this review, that of the host variation in susceptibility. It is abundantly clear, however, that in addition to genetic diversity of the disease agent, host susceptibility, whether due to genetic differences, age, immunocompetence, presence of co-infection, nutrition or hormonal status, plays a major role in the outcome of infection by either *Leishmania* or *T. cruzi*. A simple example in this historical context is the lead author's first paper on *T. cruzi* in this journal, showing differences in male and female susceptibility and in female susceptibility dependent on pregnancy (Miles, 1972). However, this and several other interesting aspects of Chagas disease and leishmaniasis lie beyond the scope, and space, for this review.

In conclusion, as reviewed here, molecular epidemiology and phylogeography, combined with incisive laboratory experiments, have transformed our perception of *Trypanosoma cruzi* and *Leishmania* and their corresponding diseases. Fascinating questions remain to be answered but will surely fall to this rational approach.

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