The ancient and divergent origins of the human pathogenic trypanosomes, *Trypanosoma brucei* and *T. cruzi*

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SUMMARY

This study presents new findings concerning the evolution of the human pathogens, *Trypanosoma brucei* and *T. cruzi*, which suggest that these parasites have divergent origins and fundamentally different patterns of evolution. Phylogenetic analysis of 18S rRNA sequences places *T. brucei* in a clade comprising exclusively mammalian trypanosomes of African origin, suggesting an evolutionary history confined to Africa. *T. cruzi* (from humans and sylvatic mammals) clusters with trypanosomes specific to Old and New World bats, *T. rangeli* and a trypanosome species isolated from an Australian kangaroo. The origins of parasites within this clade, other than some of those from bats, lie in South America and Australia suggesting an ancient southern super-continent origin for *T. cruzi*, possibly in marsupials; the only trypanosomes from this clade to have spread to the Old World are those infecting bats, doubtless by virtue of the mobility of their hosts. Viewed in the context of palaeogeographical evidence, the results date the divergence of *T. brucei* and *T. cruzi* to the mid-Cretaceous, around 100 million years before present, following the separation of Africa, South America and Euramerica. The inclusion in this study of a broad range of trypanosome species from various different hosts has allowed long phylogenetic branches to be resolved, overcoming the limitations of many previous studies. Moreover, *T. brucei* and the other mammalian tsetse-transmitted trypanosomes appear, from these data, to be evolving several times faster than *T. cruzi* and its relatives.

Key words: *Trypanosoma brucei*, *Trypanosoma cruzi*, evolution, phylogenetics, small subunit ribosomal RNA.

INTRODUCTION

Protozoan flagellates of the genus *Trypanosoma* are ubiquitous parasites found in all classes of vertebrates and are transmitted from host to host by blood-sucking arthropod or leech vectors. The trypanosomes are taken up with a bloodmeal and usually undergo 1 or more cycles of development and multiplication in the alimentary tract of the invertebrate before infective forms are transmitted to a new vertebrate host via saliva, contamination with faeces or ingestion of the whole vector. In this and other respects, the life-histories of the 2 human pathogenic trypanosomes are very different: *T. brucei* is transmitted by tsetse flies (genus *Glossina*) by the salivarian route, while *T. cruzi* develops in the hindgut of triatomine bugs and infective forms are excreted in the faeces to infect the new host by contamination of wounds or mucous membranes, the so-called stercorarian route (Hoare, 1972).

The human infective trypanosomes are thus very different from each other and must clearly have separate evolutionary histories. Hoare (1972) considered the stercorarian mode of development to be more primitive and ancient, since it corresponded to that in the trypanosomatid parasites of insects, e.g. *Crithidia*. He envisaged that the ancestral trypanosomes colonized vertebrate hosts as their insect hosts adopted micropredatory habits. The salivarian trypanosomes secondarily changed their mode of development from stercorarian to an anterior route as they adapted to a new vector, the tsetse fly. Nevertheless, as Baker (1963) observed, this evolutionary scenario ignores important questions. Trypanosomes other than those transmitted by tsetse take a salivarian route, notably those transmitted by leeches; *T. rangeli* may be transmitted by both anterior and posterior routes in triatomine bugs, while *T. grayi* is transmitted by tsetse, but via the posterior developmental route.

Molecular phylogenetic analyses of trypanosome evolution, mostly based on ribosomal gene sequences, are now starting to provide the sound framework needed to carry these arguments forward. Such studies have become successively more focused, moving from broad evolutionary questions (e.g. eukaryotes, Sogin, Elwood & Gunderson, 1986), through studies relating to higher taxonomic levels (e.g. kinetoplastid protozoa, Lake *et al.* 1988; Fernandes, Nelson & Beverley, 1993; Maslov *et al.* 1994), to work targeting specific questions related to the phylogeny of particular trypanosomatid groups

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(e.g. *Phytomonas* spp., Marché *et al*. 1995; *Trypanosoma* spp., Maslov *et al*. 1996; Lukes *et al*. 1997; Haag, O'Huigin & Overath, 1998; *Leishmania* spp., Croan, Morrison & Ellis, 1997). During the course of these studies, various conclusions regarding the evolutionary origins of certain groups (e.g. the genus *Trypanosoma*) have been formulated. Nevertheless, all have suffered to some extent from insufficient taxa to be able to resolve the relationships between distantly related species and, perhaps not surprisingly, this phenomenon is most marked in earlier studies, resulting in a paraphyletic origin being proposed for the genus *Trypanosoma*, relative to various other trypanosomatid genera (Fernandes *et al*. 1993; Maslov *et al*. 1994; Vickerman, 1994).

In addition, as highlighted by the work of Maslov *et al*. (1996), in which the genus *Trypanosoma* was again hypothesized to be paraphyletic, the problem of resolving evolutionary questions and obtaining the 'correct' topology for phylogenetic trees is also affected by the suitability of taxa to break up long phylogenetic branches. This is essential if problems of saturation of nucleotides which are potentially informative between distantly related taxa and the related phylogenetic phenomenon of long-branch attraction (Felsenstein, 1978; Hendy & Penny, 1989) are to be resolved. Accordingly, inclusion of additional, appropriate taxa in recent studies has supported a monophyletic origin for the genus *Trypanosoma* (Lukes *et al*. 1997; Haag *et al*. 1998). Although this question is phylogenetically important, no study to-date has included suitable taxa to investigate either the evolutionary relationship between the human pathogenic trypanosomes responsible for African sleeping sickness and Chagas disease, or compare the patterns of evolution within these disease agents.

By including a selected, broad range of trypanosome species in our phylogenetic analysis of 18S small subunit rRNA (ssu rRNA) sequences, we now present a novel hypothesis on the specific evolutionary origins of *T. brucei* and *T. cruzi*, together with a comparison of the different rates of evolution apparent within these clades.

MATERIALS AND METHODS

Trypanosomes

Trypanosome DNA was obtained from the species listed in Table 1. For most species purified DNA was used; where trypanosomes could not be grown in large numbers, a crude lysate of DNA was prepared for use as PCR template from 10^6–10^7 purified trypanosomes (Bromidge *et al*. 1993).

Phylogenetic marker

The ssu rRNA gene was selected as the marker for this study. Its inherently diverse rates of genetic evolution facilitate its use across a broad phylogenetic range, so that it is suitable for elucidating both higher evolutionary relationships and those between closely related species (Sogin *et al*. 1986). Indeed, over the course of numerous studies (Fernandes *et al*. 1993; Maslov *et al*. 1994, 1996; Marché *et al*. 1995; Lukes *et al*. 1997; Haag *et al*. 1998) the 18S rRNA gene, sometimes in combination with the 28S large subunit rRNA gene, has become the marker of choice for evolutionary analyses of the kinetoplastid protozoa.

Sequencing

The approximately 2 kb fragment containing the 18S ssu rRNA sequence was obtained from 32 *Trypanosoma* isolates by PCR using conserved primers (Maslov *et al*. 1996). The products from 8–10 separate PCR reactions were purified and pooled for automated sequencing. Fragments were sequenced in both directions at approximately 300 base pair intervals using 12 additional internal primers (Maslov *et al*. 1996) and products were run on a Perkin-Elmer ABI 377 automated sequencer. A consensus sequence was assembled from the individual internal primer sequences for each trypanosome strain using the ABI program AutoAssembler v.2.0. An additional 15 *Trypanosoma* and 8 non-*Trypanosoma* ssu rRNA sequences were taken from the EMBL/GenBank data bases (Table 1).

Outgroup

The order Kinetoplastida contains both parasitic and free-living flagellates. The suitability of free-living bodonid taxa as outgroups for phylogenetic studies of trypanosomatids has been established by a number of phylogenetic studies using a range of ribosomal and protein coding genes (Fernandes *et al*. 1993; Marché *et al*. 1995; Wiemer *et al*. 1995; Alvarez, Cortinas & Musto, 1996; Maslov *et al*. 1996; Haag *et al*. 1998). In this study, *Trypanosoma* species are compared with a range of outgroup taxa (*Trypanosoma borreli*, *Crithidia* spp., *Leishmania* spp.) and trees were rooted on *Bodo caudatus*.

Alignments

All sequences were aligned primarily to 8 *Trypanosoma* sequences downloaded from the rRNA data base maintained at the University of Antwerp (Neefs *et al*. 1990); the alignment of these 8 template sequences is based on their secondary structure. Subsections of the alignment, between 'anchor' regions of high homology were then subaligned using the program Clustal V (Higgins, Bleasby & Fuchs, 1992), before final adjustments were made by eye. Hypervariable sites where nucleotide changes were saturated and regions where it was not possible

Table 1. Isolation details of *Trypanosoma* spp. sequenced

(References provide original isolation date and details of each trypanosome stock; LSHTM: London School of Hygiene and Tropical Medicine; STIB: Swiss Tropical Institute, Basel; CTVM: Centre for Tropical and Veterinary Medicine, Edinburgh. Additional 18S sequences added from EMBL/GenBank are as follows: *T. boissoni* U39580; *T. carassii* L14841; *T. rotatorium* U39583; *T. triglae* U39584; *T. brucei brucei* M12676; *T. congolense* (kilifi-type) U22317; *T. congolense* (forest-type) U22319; *T. congolense* (savannah-type) U22315; *T. congolense* (tsavo-type) U22318; *T. simiae* U22320; *T. vivax* U22316; *T. cruzi* X53917; *T. cruzi* M31432; *T. avium* U39578; *T. scelopori* U67182; *Crithidia fasciculata* X03450; *C. oncopelti* L29264; *Leishmania amazonensis* X53912; *L. donovani* X07773; *L. guyanensis* X53913; *L. major* X53915; *Trypanoplasma borreli* L14840; *Bodo caudatus* X53910.)

Species	Subspecies/ type	Sample	Host		Location	Reference/Source	Accession number
T. avium		LSHTM 144B	Chaffinch	*Fringilla coelebs*	Czech Republic	LSHTM (1978)	AJ009140
T. brucei	*gambiense*	Tsuaa cl.G	Human	*Homo sapiens*	Nigeria	Gray (1972)	AJ009141
T. brucei	*rhodesiense*	UTRO 2509	Human	*Homo sapiens*	Uganda	Gibson & Gashumba (1983)	AJ009142
T. cobitis		LUMP 1243	Stone loach	*Noemacheilus barbatulus*	England	Letch (1979)	AJ009143
T. congolense	kilifi	WG 5	Domestic goat	*Capra* sp.	Kenya	Gashumba, Baker & Godfrey (1988)	AJ009144
T. congolense	riverine forest	CAM 22b	Domestic goat	*Capra* sp.	Cameroon	Gashumba, Baker & Godfrey (1988)	AJ009145
T. congolense	savannah	WG 81	Domestic goat	*Capra* sp.	Kenya	Gashumba, Baker & Godfrey (1988)	AJ009146
T. cruzi	Z I	Sylvio X10 cl.1	Human	*Homo sapiens*	Brazil	Miles *et al.* (1977)	AJ009147
T. cruzi	Z II	VINCH 89	Triatomine bug	*Triatoma infestans*	Chile	LSHTM (19??)	AJ009149
T. cruzi	Z III	CAN III cl.1	Human	*Homo sapiens*	Brazil	Miles *et al.* (1978)	AJ009148
T. cruzi	*marinkellei*	B7	Bat	*Phyllostomum discolor*	Brazil	Baker *et al.* (1978)	AJ009150
T. dionisii		P3	Pipistrelle bat	*Pipistrellus pipistrellus*	England	Baker & Thompson (1971)	AJ009151
T. dionisii		PJ	Pipistrelle bat	*Pipistrellus pipistrellus*	Belgium	Jadin (1971)	AJ009152
T. equiperdum		STIB 818	Horse	*Equus caballus*	China	Lun *et al.* (1992)	AJ009153
T. evansi		E 110	Capybara	*H. hydrochaeris*	Brazil	Stevens *et al.* (1989)	AJ009154
T. godfreyi		KEN 7	Tsetse fly	*G.m. submorsitans*	The Gambia	McNamara, Mohammed & Gibson (1994)	AJ009155
T. grayi		ANR 4	Tsetse fly	*G.p. gambiensis*	The Gambia	McNamara & Snow (1991)	AJ005278
T. lewisi		Molteno B3	Rat	*Rattus* sp.	England	Kilgour & Godfrey (1973)	AJ009156
T. mega		ATCC 30038	African toad	*Bufo regularis*	Africa	Lun & Desser (1995)	AJ009157
T. microti		TRL 132	Vole	*Microtis agrestis*	England	P. Dukes, unpublished (19??)	AJ009158
T. pestanai		LEM 110	Badger	*Meles meles*	France	J.-P. Dedet, unpublished (1974)	AJ009159
T. rangeli		RGB (Basel)	Dog	*Canis* sp.	Venezuela	STIB (1949)	AJ009160
T. rotatorium		B2-II	Bullfrog	*Rana catesbeiana*	Canada	Martin, Desser & Hong (1992)	AJ009161
T. simiae		KEN 2	Tsetse fly	*G.m. submorsitans*	The Gambia	McNamara & Snow (1991)	AJ009162
T. theileri		K127	Cattle	*Bos taurus*	Germany	Bose *et al.* (1993)	AJ009164
T. theileri		TREU 124	Cattle	*Bos taurus*	Scotland	CTVM (1965)	AJ009163
T. varani		V54	Monitor lizard	*Varanus exanthematicus*	Senegal	Minter-Goedbloed *et al.* (1993)	AJ005279
T. vespertilionis		P14	Pipistrelle bat	*Pipistrellus pipistrellus*	England	Baker (1974)	AJ009166
T. sp.		K&A	Leech	*Piscicola geometra*	England	W. Gibson, unpublished (1996)	AJ009167
T. sp.		H25	Kangaroo	*Macropus giganteus*	Australia	H. Noyes, unpublished (1997)	AJ009168
T. sp.		H26	Wombat	*Vombatus ursinus*	Australia	H. Noyes, unpublished (1997)	AJ009169
T. sp.		D30	Fallow deer	*Cervus dama*	Germany	Bose *et al.* (1993)	AJ009165

to produce a single reliable alignment across all 55 taxa were excluded from the analysis. Beyond this, separate alignments, representing more or less stringent subsets of the 'standard' alignment, were explored and used as the basis for the phylogenetic analyses presented in this paper; certain sites (included in less stringent alignments), while locally informative between closely related taxa, may introduce 'noise' resulting in a loss of definition (reduced bootstrap support) at higher phylogenetic levels. The 3 alignments considered included 1800 (stringent), 1809 (standard) and 1845 (least stringent) nucleotide positions and are available on request from J.R.S.

Phylogenetic analyses

Bootstrapped maximum parsimony analysis of 55 kinetoplastid 18S ssu rRNA sequences (Table 1) was performed with 100 replicates for each of 3 alignments. The number of taxa included necessitated the use of a heuristic search strategy to find the most parsimonious trees. The default options of PAUP were used: TBR branch swapping, zero length branches collapsed and 10 random addition sequences (bootstrap analyses used simple addition).

Maximum-likelihood analysis was also performed for each of the 3 alignments; however, due to computational constraints, analyses were performed on a reduced data set of 36 taxa. Taxa were selected for inclusion in the maximum likelihood analysis so as to maximize the degree of sequence variation analysed, highly homologous sequences being excluded; starting trees were derived by both parsimony and neighbour-joining. Transition/transversion ratios were estimated from the data in preliminary runs and then set for full analyses. All analyses were performed using test version 4.0d63 of PAUP*, written by David L. Swofford.

RESULTS

Alignments

By comparing phylogenetic trees based on the 3 alignments (a representative tree based on the standard alignment is shown in Fig. 1), the effects of including and excluding faster evolving characters were explored. In the standard alignment (1809 sites; Fig. 1) definition within the terminal branches of the phylogram is supported by high bootstrap values, while an 8-way polytomy is produced at the upper level of the genus *Trypanosoma*. In the more stringent alternative alignment (1800 sites), more variable nucleotide positions were excluded, resulting in increased bootstrap support ($> 80\,\%$) for the early divergence of the clade containing the majority of *Trypanosoma* species from that containing aquatic trypanosomes (node A, Fig. 1). The increase in resolution in the upper branches of the phylogram, accompanying the reduction in phylogenetic noise (due to the removal of saturated sites), was associated with a corresponding reduction in resolution at the terminal branches between more recently diverged taxa (e.g. nodes B and C, Fig. 1), when compared to the degree of resolution observed between terminal taxa in the tree based on the less stringent alignment (1845 sites). Such a process can be likened to a sliding window of phylogenetic resolution and represents one of the significant strengths, in terms of the phylogenetic range across which it can be used, of the 18S ssu rRNA gene (Sogin *et al*. 1986).

Phylogenetic analysis

The results of the parsimony analyses were strongly supported by the maximum-likelihood analyses. The positions and branching order of all major clades were identical between methods (irrespective of starting tree), and only minor variations in the positions of certain terminal taxa were apparent.

The monophyly of the genus *Trypanosoma* is confirmed by the phylogenetic analysis and bootstrap support for the monophyly of the clade is $> 83\,\%$ (Fig. 1). Support for many other previously recognized intra-genus taxonomic groupings, however, is not apparent in this study and the clades which correspond to the salivarian group and approximately to the subgenus *Schizotrypanum* in Fig. 1 are referred to as the *T. brucei* clade and the *T. cruzi* clade, respectively. The *T. brucei* and *T. cruzi* clades (and all species therein) are defined in Fig. 1. The terms '*T. brucei* clade' and '*T. cruzi* clade' are used throughout this study to refer to the clades containing (a) all mammalian salivarian (Hoare, 1972) trypanosomes (*T. brucei* clade) and (b) trypanosomes in the subgenus *Schizotrypanum*, plus *T. rangeli* and an as yet unidentified species of trypanosome from a kangaroo (*T. cruzi* clade).

The phylogram places the human pathogenic trypanosomes, *T. brucei* and *T. cruzi*, in separate clades; bootstrap support for the monophyly of each of these clades is high ($> 96\,\%$). *T. brucei* is in a clade comprising exclusively mammalian trypanosomes and, except for *T. evansi* and *T. equiperdum*, these trypanosomes are all of African origin and transmitted by tsetse flies. Analysis of kinetoplast (mitochondrial) DNA (Borst, Fase-Fowler & Gibson, 1987) and isoenzymes (Lun *et al*. 1992; Gibson, Wilson & Moloo, 1983) points to *T. evansi* and *T. equiperdum* being comparatively recent mutants of *T. brucei*, which have been able to spread outside Africa because they no longer rely on tsetse transmission; the particularities of these 2 species need not therefore interfere with the more ancient evolution of the clade. The host exclusivity of the *T. brucei* clade suggests a distinct evolutionary history initially confined to Africa. Trypanosomes of African origin from other vertebrates are completely unrelated (e.g.

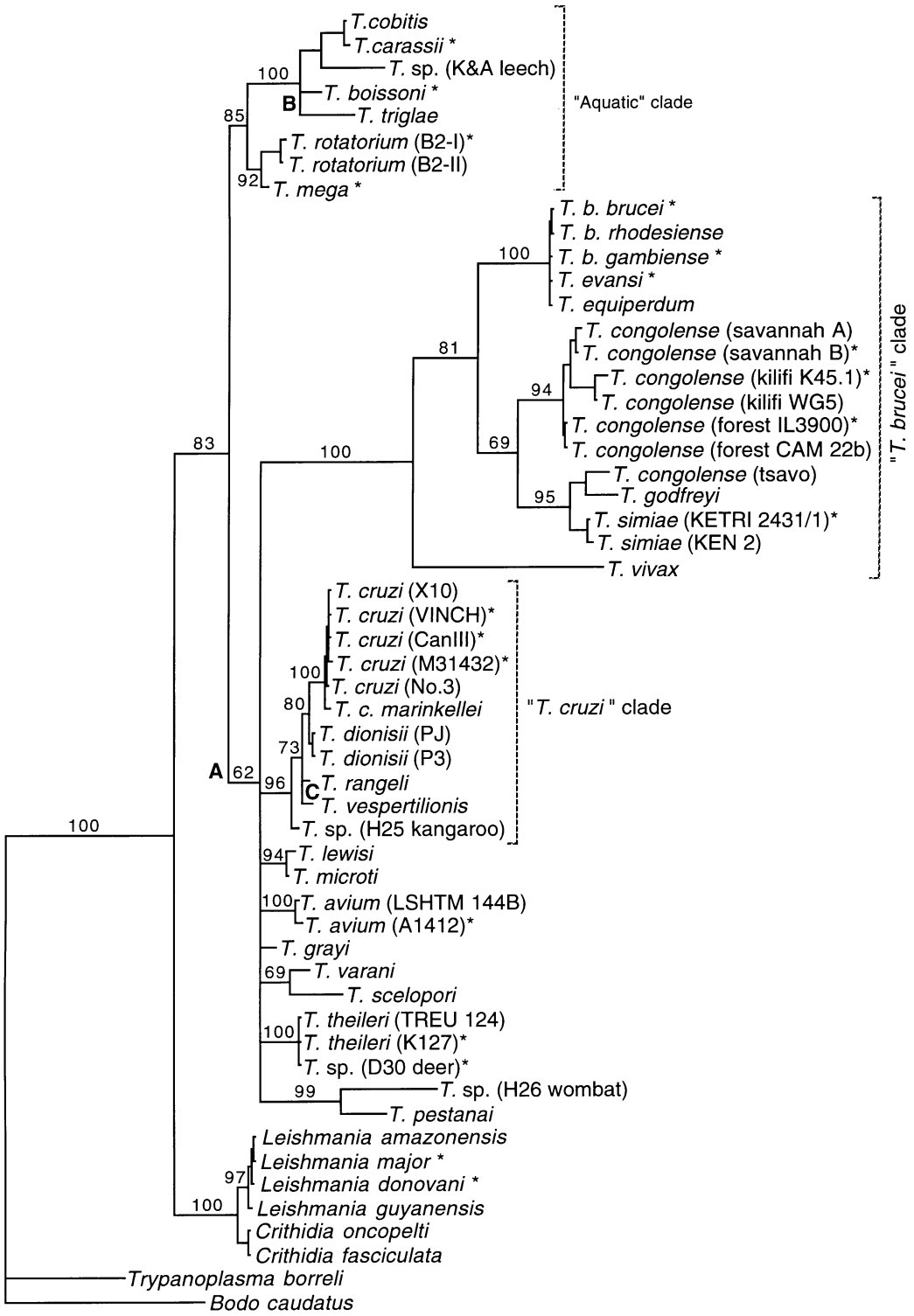

Fig. 1. Phylogram constructed by bootstrapped (100 replicates) maximum parsimony analysis of 55 kinetoplastid 18S ssu rRNA sequences. The tree is derived from 2 most parsimonious trees of length = 1103 (RI = 0·8421, CI = 0·5956), based on the standard alignment of 1809 nucleotide sites. Bootstrap values for all major nodes are given and all branches receiving bootstrap support values > 50% are shown; relationships failing to achieve this level of support are shown as polytomies (i.e. branch points at which 3 or more branches arise from the ancestral line). Certain clades, referred to in the text, are defined by dashed brackets. Certain nodes at which bootstrap support and/or tree topology varies depending on alignment are labelled A, B and C (see Results section – *Alignments*). The 19 taxa not included in the maximum-likelihood analyses are marked *. Full details of all taxa are given in Table 1.

T. grayi, *T. varani* from African reptiles; *T. mega* from an African toad). A similar result was reported by Haag *et al*. (1998).

T. cruzi isolates from humans, sylvatic and domestic mammals, including bats and opossums, cluster with trypanosomes specific to Old and New

World bats, *T. rangeli* and a trypanosome species from an Australian kangaroo. The origins of parasites within the *T. cruzi* clade thus lie largely in South America and Australia; the only trypanosomes from this clade representing the Old World are those infecting bats.

The taxonomic and evolutionary status of *T. rangeli* (generally classified as subgenus *Herpetosoma*) remains controversial (D'Alessandro & Saravia, 1992). In the current study *T. rangeli*, albeit only a single isolate, is classified firmly within the *T. cruzi* clade (bootstrap 96%). Similarly, while morphological analysis of culture-form trypanosomes from a wombat suggested an *Herpetosoma* species, the phylogenetic analysis revealed a close relationship with *T. pestanai* (subgenus *Megatrypanum*; Hoare, 1972) from a European badger. Interestingly, morphological analysis of the culture forms of the kangaroo trypanosome, which is unequivocally classified by the phylogenetic analysis as a member of the *T. cruzi* clade, suggested it to be a *Megatrypanum* species. The remaining *Herpetosoma* species included in the analysis, *T. lewisi* and *T. microti*, both of which are confined mainly to rodents, cluster together.

The current study does not provide support for the monophyly of the subgenus *Megatrypanum* (represented here by *T. theileri*, *T.* sp. (D30) from a deer, *T. pestanai* and possibly *T.* sp (H25) from a kangaroo; Table 1). Classically, the subgenus *Megatrypanum* comprises a diverse assortment of trypanosomes grouped primarily on the basis of their large size. Results of the current study suggest that this somewhat arbitrarily defined character is polyphyletic and is thus unsuitable as the basis on which to define a phylogenetic (or taxonomic) grouping. This result highlights the limitations of morphometric characters for taxonomic purposes and underlines the need to review the phylogenetic validity of other established taxonomic categories in trypanosomes.

A third major clade within the *Trypanosoma* (Fig. 1) comprises trypanosomes of fish and amphibia (see also Maslov *et al*. 1996; Lukes *et al*. 1997). The tree suggests an early divergence of the aquatic trypanosome clade (bootstrap support 85%) from other *Trypanosoma* spp. (bootstrap support 62%), although this relatively low bootstrap value suggests that alternative hypotheses might also be considered. Nevertheless, the diverse hosts of trypanosomes within the aquatic clade all appear to be linked by their environment and it seems probable that the common evolutionary factor may well be an aquatic vector, e.g. a leech.

The evolutionary and taxonomic status of trypanosomes of reptiles remains unclear. According to the phylogram, they do not appear to have diverged any earlier or evolved any faster than the majority of mammalian and bird trypanosomes, indicating a similar point of origin. Such a result is particularly unexpected considering the ancient origins of crocodiles, the natural hosts of *T. grayi*.

For the majority of apparently unrelated *Trypanosoma* clades it is not possible to determine the exact order of clade divergence on the basis of these data and an 8-way polytomy is apparent at the upper level of the genus *Trypanosoma* (Fig. 1). This may be due to the limitations of the ssu gene over this time-scale and/or to a possibly explosive divergence of trypanosome taxa over a very short period, some time prior to 100 million years before present (mybp). Nevertheless, the inclusion of more taxa has enabled elucidation of the complex relationships of the human infective trypanosomes and, while saturation of some variable regions within the ssu gene may preclude the accurate determination of branching order at more ancient levels, considerable support for the 'correctness' of the phylogenetic relationships represented in the tree is provided by the logical placement of the outgroup trypanosomatids, *Leishmania* spp. and *Crithidia* spp., which are well separated from the *Trypanosoma* (Fig. 1).

Comparative rates of sequence evolution

The phylogenetic analysis provides evidence of very different rates of evolution within the *T. brucei* and *T. cruzi* clades. Comparison of deeper branch lengths suggest a difference in intra-clade evolution rate of approximately 8-fold. Such differences in evolutionary rates between trypanosome clades are in keeping with results from previous studies (Maslov *et al*. 1996). However, in the current study, while the exact extent to which the rapid evolution of the *T. brucei* clade may have distorted the topology of the tree (and hence the final estimation of rate differences) is unknown, our tree appears sufficiently robust to have avoided this clade being drawn towards the outgroup taxa by long-branch attraction, a problem encountered in many previous studies.

DISCUSSION

Phylogenetic analysis of variation in ssu rRNA genes of 47 trypanosome specimens places the 2 human pathogens, *T. brucei* and *T. cruzi*, unequivocally in 2 early diverging clades. The time of divergence can be estimated by consideration of palaeo- and biogeographical data (Smith, Smith & Funnell, 1994; Cox & Moore, 1993). Mammalian trypanosomes are found in several clades within the phylogram; however, the tsetse-transmitted trypanosomes of African mammals group together in an exclusive clade (including *T. evansi* and *T. equiperdum*, which are probably recently derived from *T. brucei* – see comment in Results section). Trypanosomes of African origin from other vertebrate classes, notably African reptiles and amphibia, are outside this

clade. This indicates a distinct and isolated evolutionary history for the *T. brucei* clade, associated with 3 key elements: mammalian hosts, tsetse flies and Africa. The likely scenario is that the ancestors of *T. brucei* arose in Africa, perhaps parasitizing early mammals which became isolated when Africa split off from the other continents. This dates the origin of the clade in the mid-Cretaceous, approximately 100 mybp, when Africa separated from the South American and Euramerican continents. A similar evolutionary scenario was arrived at independently by Lambrecht (1980) considering only palaeoecological data, while Lake *et al.* (1988) obtained a similar estimate of divergence time based on their analysis of 9S and 12S mitochondrial rRNA genes.

At this time, the first mammals were present, but had not yet begun major diversification; it is not clear whether tsetse flies were present (Cockerell, 1907; Jordan, 1993). Eventually, ancestral *T. brucei* clade trypanosomes adapted to large mammal hosts and transmission by tsetse vectors. The first ungulates probably entered Africa from Eurasia in the Palaeocene or early Eocene, 54 mybp, as the first wave of ungulate radiation was occurring across the northern hemisphere. A second group of African mammals, including insectivores, rodents and now extinct relatives of the suids, the Anthracotheriidae, appeared in the mid-Eocene (around 45 mybp), presumably having migrated from Eurasia along island chains. Non-ruminant artiodactyls, living examples of which include pigs and hippopotami, appear to have predominated in Africa until the Miocene (ca. 23 mybp). Subsequently, the proximity of Eurasia and the development of a permanent land bridge between Africa and Asia ca. 17 mybp allowed for a huge increase in biotic exchange between the two regions. Thus, much of the evolutionary history of the *T. brucei* clade was probably shared with suids. In this context, it is interesting that several present-day trypanosome species are specific to pigs (*T. simiae*, *T. suis*, *T. godfreyi*) and that analysis of tsetse bloodmeals indicates suids to be a favourite food source (Weitz, 1963), while the ecological requirements of tsetse and pigs (forest and thickets) are also very similar.

An alternative scenario is that an ancestor of the *T. brucei* clade entered Africa with migrating ungulates. If so, we would expect *T. brucei*-related trypanosomes to occur outside Africa. In fact, the related trypanosomes, *T. evansi*, *T. equiperdum* and *T. vivax*, which do not rely on tsetse-transmission, do occur outside Africa, but all are considered to be comparatively recent exports. Moreover, their presence outside Africa demonstrates that transmission is easily maintained in the absence of tsetse, suggesting that *T. brucei*-related trypanosomes should still be found in present-day ungulates worldwide, if they had originally evolved in these animals. Ungulates certainly carried other trypanosome species with them, since *T. theileri* and related trypanosomes have a cosmopolitan distribution today. Thus, paradoxically, the presence of *T. evansi* etc. outside Africa supports an African origin of the *T. brucei* clade.

If large grazing and browsing ungulates were able to migrate into Africa, could the *T. brucei* clade and tsetse flies have moved northwards? Support for a once wider distribution of these trypanosomes is linked to somewhat limited evidence relating to the distribution of their tsetse fly vector. Fossil tsetse have been found in shale deposits in Colorado (Cockerell, 1907), suggesting the presence of ancestral tsetse flies (and, thus, perhaps trypanosomes) in North America during the Oligocene era, up to 36·5 mybp (Jordan, 1993). Presumably global climate change eliminated tsetse from northern regions, confining them to Africa and its characteristic fauna. Certainly, present day tsetse distribution appears severely limited by climate and, in the last 3–4000 years, by the additional barrier of the Sahara desert.

In contrast, the present day distribution of the *T. cruzi* clade is far wider and more complex than that of the *T. brucei* clade, both geographically and in terms of host range. While *T. cruzi* and *T. rangeli* are found in South and Central America, where they infect a broad range of sylvatic and domestic mammalian hosts, including humans (WHO, 1997), 2 other species (*T. dionisii*, *T. vespertilionis*) are specific to bats and are found throughout the Americas and Europe. Although the biological similarity of *T. cruzi* and bat trypanosomes has been recognized for some time (these trypanosomes are in the same subgenus, *Schizotrypanum*), their present-day distribution is puzzling. Two opposing hypotheses may be put forward: either *T. cruzi* and relatives (including *T. rangeli*) evolved from bat trypanosomes or vice versa. Clearly, the ability of bats to disperse over long distances and particularly across water barriers, is consistent with either hypothesis. Bats may well have evolved by the presumed time of divergence of the *T. cruzi* and *T. brucei* clades (ca. 100 mybp); a recognizable, well-developed bat fossil ((*Icaronycteris index*) has been reported from the early Eocene, around 50 mybp (Jepsen, 1966), suggesting that the earliest bats may have originated 70–100 mybp. However, we propose that the occurrence of *T. cruzi* clade trypanosomes in both placental and marsupial mammal hosts, and in Australia as well as South America, points to a southern continent origin; at the time of isolation of the African continent in the mid-Cretaceous, South America, Antarctica and Australia were joined in a southern 'super-continent'. The early evolution of this clade was perhaps initially associated with the dominant marsupial fauna of this region. Indeed, the opossum, *Didelphis* sp., a not so distant relative of the Australian kangaroos (Flannery, 1989), is a

particularly important natural reservoir of *T. cruzi* in South America and can maintain a patent parasitaemia throughout its life, with no apparent clinical symptoms. Intriguingly, *T. cruzi* is reported to undergo morphological development into epimastigote 'insect forms' in the anal glands of the opossum (Deane, Lenzi & Jansen, 1986).

Due primarily to its developmental cycle, the taxonomic and evolutionary status of *T. rangeli* has long been debated (D'Alessandro & Saravia, 1992); this human infective species exhibits most of the characteristics of the subgenus *Herpetosoma* (Hoare, 1972) and yet it is apparently transmitted by both stercorarian and salivarian routes (D'Alessandro & Saravia, 1992). A limited study based on β-tubulin gene sequences (Amorim, Momen & Traub-Cseko, 1993) suggested *T. rangeli* to be more closely related to *T. brucei* than to *T. cruzi*. While this result cannot be disputed, it is widely recognized (Swofford *et al.* 1996) that studies including limited numbers of taxa spanning disparate levels of relatedness are highly prone to artifactual effects. Certainly, in the current study *T. rangeli* is placed firmly within the *T. cruzi* clade, indicating it to be more closely related to *T. cruzi* and other *T.* (*Schizotrypanum*) species.

The vectors of *T. cruzi* and its relatives are triatomine bugs, those of the bat trypanosomes are a range of ectoparasites (e.g. ticks, mites and Nycteribiid flies), while the vector of the kangaroo trypanosome is unknown. This contrasts with the *T. brucei* clade, which has clearly co-evolved with tsetse flies. Triatomine bugs are presently found throughout South and Central America, Southeast Asia and India. In contrast to the apparently ancient origins of the *T. cruzi* clade, triatomine bugs represent a recent evolutionary change from predatory reduviid bug ancestors (Schofield, 1988) to a haematophagous life-style, possibly as late as the Quaternary (1·5 mybp) and probably on more than one occasion (Gorla, Dujardin & Schofield, 1997). Thus, the transmission of *T. cruzi* and *T. rangeli* by triatomine bugs has evolved relatively recently. Certainly, the absence of endemic triatomines in Australia (Gorla, Dujardin & Schofield, 1997) suggests that they were not present on the ancient southern super-continent of South America/Antarctica/Australia.

From the separate evolutionary histories of *T. brucei* and *T. cruzi* constructed from the phylogenetic evidence, we can deduce that their pathogenicity to humans developed on different time scales. In Africa, the first hominids evolved 5–15 mybp, the genus *Homo* 3 mybp (Johanson & Taieb, 1976) and *Homo sapiens* not earlier than 300000 years bp, presumably in continuous contact with tsetse flies and trypanosomes. It may not be by chance that humans are innately resistant to infection with most species of tsetse-transmitted trypanosome by virtue of a trypanolytic factor in the serum; this trait is shared with baboons, the other primates of the African plains (Hawking, Ramsden & Whytock, 1973). In contrast, human contact with *T. cruzi* could not have occurred prior to human migration into the Americas, which is generally dated no earlier than 30–40000 years bp. Moreover, there is no evidence for contact earlier than 3000 years bp when the first permanent settlements were made by previously nomadic cultures (Rothhammer *et al.* 1985) at which time humans would have become infected as a simple addition to the already extensive host ranges of *T. cruzi* and *T. rangeli* (Hoare, 1972). Thus, while *T. brucei* has effectively co-evolved with hominids in Africa, *T. cruzi* has evolved largely in the absence of humans; both, however, remain highly pathogenic to humans, despite their very different evolutionary histories. To what extent these factors have influenced the markedly different rates of evolution observed between the *T. brucei* and *T. cruzi* clades remains to be explored.

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