Genomic Rearrangements in Trypanosomatids: an Alternative to the "One Gene" Evolutionary Hypotheses?

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Most molecular trees of trypanosomatids are based on point mutations within DNA sequences. In contrast, there are very few evolutionary studies considering DNA (re) arrangement as genetic characters. Waiting for the completion of the various parasite genome projects, first information may already be obtained from chromosome size-polymorphism, using the appropriate algorithms for data processing. Three illustrative models are presented here. First, the case of Leishmania (Viannia) braziliensis/L. (V.) peruviana *is described. Thanks to a fast evolution rate (due essentially to amplification/deletion of tandemly repeated genes), molecular karyotyping seems particularly appropriate for studying recent evolutionary divergence, including eco-geographical diversification. Secondly, karyotype evolution is considered at the level of whole genus* Leishmania. *Despite the fast chromosome evolution rate, there is qualitative congruence with MLEE- and RAPD-based evolutionary hypotheses. Significant differences may be observed between major lineages, likely corresponding to major and less frequent rearrangements (fusion/fission, translocation). Thirdly, comparison is made with* Trypanosoma cruzi. *Again congruence is observed with other hypotheses and major lineages are delineated by significant chromosome rearrangements. The level of karyotype polymorphism within that "species" is similar to the one observed in "genus"* Leishmania. *The relativity of the species concept among these two groups of parasites is discussed.*

Key words: *Leishmania* - *Trypanosoma cruzi* - chromosome evolution

The DNA sequence and its variation – essentially by point mutations – underlie the discipline of molecular evolution. Methods of analysis and evolutionary models are robust and, according to the grade of conservation of the gene or intergenic region considered, studies at different evolutionary scales can be performed. Nevertheless, the adequacy between "one gene" trees, and "species" trees might be questioned. Indeed, only orthologous genes (homology resulting from speciation; Wiley 1981) should be used and several genes should be sequenced to confirm orthology and discard paralogous (homology resulting from gene duplication) and xenologous (result of gene transfer) genes (Larson 1994, MacIntyre 1994). An other advantage of multiple gene analysis is the possibility of exploring different metabolic pathways and thus getting a more global insight on evolution. This concept has been extensively – albeit indirectly – explored by multi-locus enzyme Electrophoresis (MLEE, Bañuls et al. 1999).

However, according to Danchin (1998), genomes are not merely collections of genes, and the map of the cell would be in the chromosome (Danchin & Hénaut 1997). In other words, rearrangement of genes can either induce or reflect evolutionary changes. This hypothesis proposed for prokaryotes (Danchin & Hénaut 1997) is documented in higher eukaryotes too and, according to Wilson et al. (1974), gene rearrangements would be more important than point mutations as sources for evolutionary changes. However, very few evolutionary studies have considered gene arrangement as molecular character in trypanosomatids and other

Financial support from the Flemish Fund for Scientific Research (FWO, Grants 346/1990 and 9.0024.90), the Swedish Agency for Research Co-operation with Developing Countries (SAREC), the Economical Union (TS2-CT90-0315, TS3-CT92-0155, TS3-CT92-0129, IC18-CT96-0123 and IC18-CT98-0256) and FONDECYT-Chile (193/1043).

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Received 13 April 2000
Accepted 15 May 2000

protozoa, despite their primordial phylogenetic position between prokaryotes and eukaryotes.

MECHANISMS OF GENE REARRANGEMENT AND APPROACHES FOR THEIR ANALYSIS

A first, direct approach to gene rearrangements can be considered, in which the organisation of specific genes is compared in different taxa. This concept has been applied to the study of a large genomic region containing glucose transporter genes in *Trypanosoma brucei, T. congolense, T. vivax, T. cruzi* and *Leishmania donovani* (Bringaud et al. 1998). Gene organisation was shown to be identical in the three Salivarian trypanosomes, but in *T. cruzi* and *Leishmania*, insertion of additional genes was observed. Phylogenetic reconstruction based on glucose transporters was in agreement with the monophyly of genus *Trypanosoma* and the early separation of *T. vivax* from the other Salivarian trypanosomes. Another example concerns the α- and β-tubulin genes which are linked and organised in alternated tandem repeats in *T. cruzi* (Maingon et al. 1988, Cano et al. 1995) and *T. brucei* (Tomashow et al. 1983), while they are unlinked in *Leishmania* and *Sauroleishmania* (Dujardin 1995, Wincker et al. 1996, Britto et al. 1998). Furthermore, within *Leishmania*, three types of β-tubulin gene organisation have been encountered, with gene copies on (i) 3 chromosomes (8/29, 21 and 32 in New World subgenus *Leishmania*, or NWL and, 8, 21 and 32 in Old World subgenus *Leishmania*, or OWL; Wincker et al. 1996, Britto et al. 1998), and (ii) 4 chromosomes (1, 8, 21, and 32 in subgenus *Viannia*, or NWV; Dujardin 1995, Britto et al. 1998). As globins in mammals (Dover et al. 1982, Jeffreys 1982), it is likely that tubulin genes arose from a single copy gene that duplicated, diverged towards α- and β-tubulin genes, and then began to spread differentially among the genome of the different trypanosomatids. The tracking of this spread among key trypanosomatid taxa would pave the way to new evolutionary hypotheses. However, as for DNA sequence analysis, "one gene rearrangement" trees might also be questioned, and rearrangement of different genes should be studied. This should be feasible in a near future through completion of the different projects of parasite genome sequencing.

A second, indirect approach to gene rearrangement is provided by molecular karyotyping and analysis of chromosome-size variability. This approach infers that chromosome size-polymorphism is reflecting gene rearrangements and it allows to explore the whole nuclear genome. Within genus *Leishmania* and within *T. cruzi*, there is conservation of most linkage groups (Henriksson et al. 1995, Wincker et al. 1996, Britto et al. 1998), and chromosome-specific probes are available for identifying homologous chromosomes. This allowed to visualise chromosome-size differences, the extent of which varies according to the underlying mechanisms, their frequency and possibly their functional consequences. As such, three distinct molecular mechanisms have been described so far as being responsible for chromosome size-variation in trypanosomatids: (1) expansion/contraction of telomeric repeats are responsible for small size-variations (up to 35 kb) and they are reported to happen at a very high frequency, reaching an amplitude of 10 bp/division in *T. brucei* (Bernards et al. 1983). Functional significance of these phenomena is unknown, but in African trypanosomes, telomere exchange, does play a role in VSG gene switching (Rudenko et al. 1996); (2) amplification-deletions among tandemly repeated genes cause larger size-differences (up to 400 kb) as illustrated in *Leishmania* (Victoir et al. 1995, Inga et al. 1998, Kebede et al. 1999) and *T. cruzi* (Wagner & So 1990, Campetella et al. 1992, Aslund et al. 1994, Henriksson et al. 1995). As for telomeric sequences, size-variation is progressive, but its frequency is lower. In *Leishmania*, we found rearrangement among the gp63 gene locus (leading to chromosome size-polymorphism) in a strain cultivated over four years (Victoir et al. 1995), while in *Sacharomyces cerevisiae* the frequency of amplification of rDNA was evaluated at about $5x10^{-3}$ copies/generation (Szostak & Wu 1980). Considering the importance of the rearranged genes, functional consequences might be expected through (i) gene dosage (Ashburner 1989), (ii) deletion of unique interspersed genes (Bourke et al. 1996), and (iii) effect on intergenic regulatory sequences (Ramamoorthy et al. 1995); (3) fusion/fission events are responsible for the most significant size-differences. For instance in *Leishmania*, there is a difference of about 1,200 kb between the 8/29 fused chromosome of NWL and the individual chromosome 8 of OWL and NWV (as calculated from data of Britto et al. 1998). In contrast to previous mechanisms, fusion/fission is not characterised by stepwise size-variation. Functional consequences are unknown, but considering their low frequency (three in the whole genus *Leishmania*; Britto et al. 1998), they most probably reflect major evolutionary events.

A NEW METHOD FOR THE ANALYSIS OF CHROMOSOME-SIZE VARIATION: aCSDI

Interpretation of the extensive chromosome size-polymorphism among natural populations of parasites remains a main problem. Classically, processing of molecular data and building of trees are based on disjunctive encoding of all the character states (here the size of the different homologues), followed

by phenetic or cladistic analyses (Gower 1984). By doing so, any chromosome size-difference has the same weight in the analysis. However, as a consequence of the different mechanisms described above, the evolutionary importance of genomic rearrangements seems to vary proportionally to the extent of chromosome-size differences. The only theoretical exception is size-conservative rearrangements such as inversions, which – to our knowledge – are not described so far in trypanosomatids. We may thus assume that phenetic analysis of chromosome size-polymorphism should be based best on the weighing of size-difference, rather than on the disjunctive encoding. Therefore, we developed the measure of the absolute chromosome size difference index (aCSDI; Dujardin et al. 1995), in which the genomic distance between two organisms is simply the sum of the absolute size-differences between their homologous chromosomes. The formulation fits with the diploid state assumed for most chromosomes of trypanosomatids (Henriksson et al. 1990, Bogliolo et al. 1996, Britto et al. 1998), and is very close to the 'absolute genetic distance' of Gregorius (1984), which is considered as having one of the best mathematical properties (Katz 1988). After calculation of aCSDI, agglomeration may be performed by any algorithm like UPGMA (Sneath & Sokal 1973) or Fitch-Margoliash (Felsenstein 1984). This leads to significantly structured dendrograms in contrast to the ones built-up from disjunctively encoded data (Dujardin et al. 1998). We present hereafter the application of the aCSDI method to three evolutionary models.

INFRA-SPECIFIC EVOLUTION

The first application of aCSDI concerns analysis at low level of evolutionary divergence, in particular at inter- and intra-specific levels. *L. (V.) braziliensis*, one of the most aggressive species of Latin America, is endemic in the whole Amazonian basin (Guerra 1988). *L. (V.) peruviana* is a rather benign species, endemic only on the Pacific slopes of the Peruvian Andes and in some inter-Andean valleys, mostly in xerophytic environments (Guerra 1988). Despite these extensive phenotypic differences, the two parasites were shown to differ by only one out of 16 enzymatic loci (Bañuls et al. 2000), an indication of recent divergence (less than 1.5 Myrs, the estimated divergence time between *L. (L.) infantum* and *L. (L.) donovani*, two species differing by two enzymatic loci; Moreno et al. 1986). This even led some authors to question the validity of the distinction of the two species (Chouicha et al. 1997).

In order to better understand the evolutionary relationships between both parasites, an allopatric sampling was performed in their territory of endemism. Our sampling key was the bio-geographical units (BGUs) described in Peru on the basis of the endemism of butterfly species (Lamas 1982). Our working hypothesis was that eco-geographical factors responsible for structuring of butterfly species were also responsible for structuring among sand fly vectors and their parasites. Our assumption was supported by the known relationships existing between sand fly and environment (Cameron & Davies 1993). Five chromosomes (out of 35 in subgenus *Viannia*, Britto et al. 1998) were selected for their significant size-polymorphism and, analysed using the aCSDI method (Dujardin et al. 1995). All *L. (V.) braziliensis* isolates grouped together in a small cluster, at distance from *L. (V.) peruviana* (Fig. 1). The latter displayed a much higher chromosome size-polymorphism and its populations were structured according to their BGU of origin along a north-south cline. Northern *L. (V.) peruviana* isolates presented a higher karyotype similarity with *L. (V.) braziliensis* than with southern *L. (V.) peruviana*. Interestingly, the BGU where the northern *L. (V.) peruviana* isolates were collected is close to the pass of Porculla, the only natural pass in the Peruvian Andes between the Amazonian forest and the Pacific slopes. Considering a divergence time of *L. (V.) peruviana* and *L. (V.) braziliensis* inferior to 1.5 Myrs (see above), both species likely diverged after uplifting of the Andes (achieved 3 Myrs ago, Van der Hammen 1982). Accordingly, we think that this dynamic picture of karyotype variation reflects the evolution of *L. (V.) peruviana* from *L. (V.) braziliensis*, in the course of its colonisation of the Pacific slopes of the Andes, through the Porculla pass. Through North-South migration and isolation, *L. (V.) peruviana* would have increased its genetic and genomic differentiation during its journey through the various Pacific BGUs and their respective sand fly species (Davies et al. 1993, Villaseca et al. 1993, Caceres et al. submitted). This trend is corroborated by an important eco-epidemiological argument: evolution from sylvatic (reservoir: rodents and edentates for *L. (V.) braziliensis*; Guerra 1988) to domestic transmission (reservoir: dog for *L. (V.) peruviana*; Llanos-Cuentas et al. 1999). Furthermore, this trend was supported recently by genomic evidences: a set of *L. (V.) braziliensis*-specific genes (gp63, Victoir et al. 1998) was found to lack in *L. (V.) peruviana*. Considering the phylogenetic proximity of both species (Chouicha et al. 1997), deletion in *L. (V.) peruviana* is easier to explain than acquisition by sequence divergence in *L. (V.) braziliensis*.

Further studies established that the above-described chromosome size-polymorphism was indeed associated with significant rearrangements (at

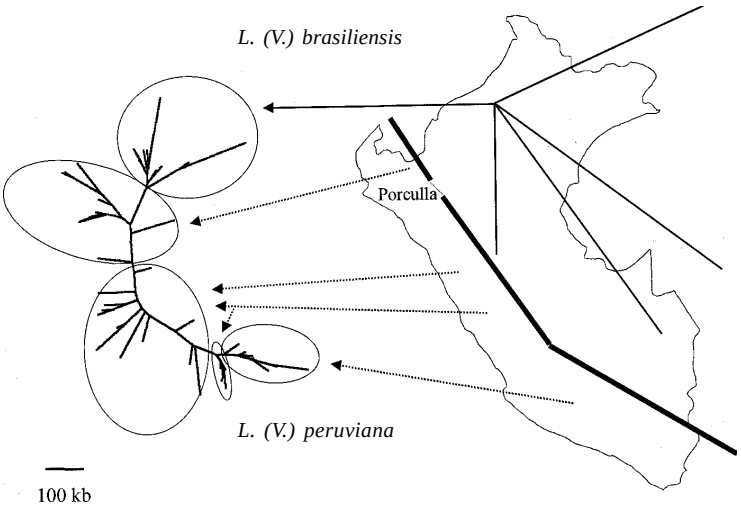

Fig. 1: size-polymorphism of chromosomes 2, 10, 11, 27 and 134Sg in Peruvian *Leishmania (V.) braziliensis* and *L. (V.) peruviana* isolates. Agglomeration by the Fitch-Margoliash method after aCSDI calculation. Porculla: natural pass between forest and Pacific slopes of the Andes (thick line)

least in four out five chromosomes studied) among important tandemly repeated genes. First, halving of gp63 gene copy number and deletion of a specific isogene were found to discriminate *L. (V.) peruviana* from *L. (V.) braziliensis* (Victoir et al. 1995). Secondly, decrease in copy number of rDNA (Inga et al. 1998), mini-exon (Kebede et al. 1999) and cystein proteinase b (Polet 1999) was shown to be involved in the North-South chromosome-size decrease observed within *L. (V.) peruviana*.

Our results thus clearly advocate at inter- and infra-specific levels that chromosome size-polymorphism (i) is reflecting gene rearrangements with a potential adaptive significance, (ii) allows a sensitive monitoring of genetic variation (if weighed by aCSDI), and (iii) may be used for inferring novel evolutionary hypotheses.

EVOLUTION WITHIN THE GENUS *LEISHMANIA*

In the *L. (V.) braziliensis-L. (V.) peruviana* model, species divergence is thought to be recent and consequences of gene rearrangements are still visible. Next question was to evaluate if this might still be valid at higher evolutionary level, or if the signal would become buried in the evolutionary noise. Therefore, we processed the data from the karyotypes of major *Leishmania* species published by Wincker et al. (1996) and Britto et al. (1998), and we calculated the size of each chromosome. It had been shown that, out of 36 chromosomes, 31 linkage groups were preserved across the genus, while five were the object of fusion/fission events (Britto et al. 1998).

We calculated aCSDI between each species, for the 31 "conserved" chromosomes. The ensuing dendrogram (Fig. 2) clearly showed a structuring into three clusters, corresponding to the three major taxonomical categories described by MLEE analysis within genus *Leishmania*: OWL, NWL and NWV. Interestingly, the genomic distance separating NWL from the two other clusters was quite high (about 1,400 kb). After analysis of individual chromosomes, it appeared that this distance corresponds essentially to a significantly lower size for 11 NWL chromosomes. Such a result should be confirmed by the analysis of additional stocks but, like in the *L. (V.) braziliensis-L. (V.) peruviana* model described above, it obviously raises the question about the nature of the sequences implicated in these size-differences. Considering the higher divergence time between NWL and OWL/NWV than between *L. (V.) braziliensis* and *L. (V.) peruviana*, a larger number of significant gene rearrangements might be expected. Accordingly, a specific attention should be paid on the gene content of these 11 chromosomes, as it might give further clues to the functional differences existing between the three major groups of *Leishmania*. This illustrates the feedback contribution of chromosome evolutionary studies for comparative genomics.

Thus, there is a good qualitative agreement between major evolutionary groups as defined by chromosome size-polymorphism and sequence polymorphism (as inferred by MLEE analysis). Quantitatively, however, relative distances between the three groups are not similar. Indeed, chromosome size analysis positioned NWL as the most

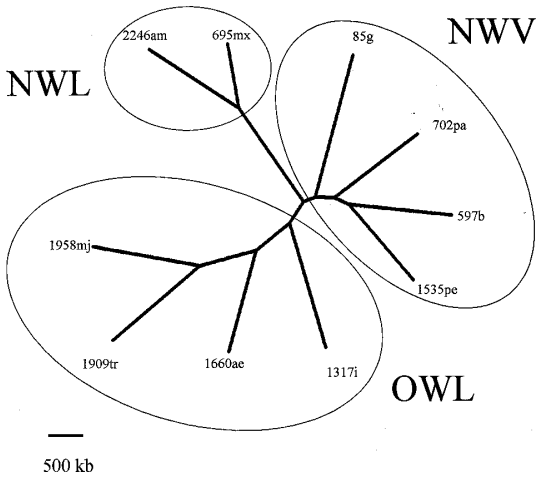

Fig. 2: size-polymorphism of 31 conserved chromosomes in genus *Leishmania* (from data of Britto et al. 1998 and Wincker et al. 1996) Agglomeration by the Fitch-Margoliash method after aCSDI calculation. NWV, subgenus *Viannia* (g, *L. (V.) guyanensis* ; pa, *L. (V.) panamensis*; b, *L. (V.) braziliensis*; pe, *L. (V.) peruviana*); NWL, New World subgenus *Leishmania* (am, *L. (L.) amzonensis* ; mx, *L. (L.) mexicana*); OWL, Old World *Leishmania* (mj, *L. (L.) major*; tr, *L. (L.)tropica*; ae, *L. (L.) aethiopica*; i, *L. (L.) infantum*); numbers correspond to LEM codes of Montpellier.

remote group within genus *Leishmania*, while MLEE analysis situated NWL and OWL close to each other (Thomaz-Soccol et al. 1993). This remote position of NWL was reinforced by counting the minimal number of fusion/fission events among the five chromosomes not considered by aCSDI analysis. While one event only was separating NWV and OWL, two and three events did separate NWL from OWL and NWV respectively (Britto et al. 1998). Two factors, inconstant mutation rates and/or selection might explain this relative incongruence. The Fitch-Margoliash dendrograms here presented do not consider a molecular clock hypothesis, but even when considering it (Kitch option in Phylip package), the remote position of NWL remained. Unfortunately, the test for molecular clock hypothesis as described by Felsenstein (1984) could not be performed because distances are not independent (on a same gel, chromosome sizes are evaluated from the same molecular marker). Selection was previously shown to play an important role in the modulation of chromosome size-variation in the *L. (V.) braziliensis-L. (V.) peruviana* model (Dujardin et al. 1998), and this hypothesis should be further explored at genus level by analysis of chromosome size-variants genetic content.

Our results thus show that, despite an extensive karyotype plasticity, chromosome size analysis does allow long range evolutionary studies at genus level. Potential outgroups such as *Sauroleishmania, Endotrypanum* or *Crithidia* should now be added, in order to better understand the evolutionary relationships within genus *Leishmania*; among others, this might allow to further document the Paleotropical (Kerr 2000) or Neotropical (Noyes 1998) origin of this genus. Further work is also required to understand the significance of the large genomic differences existing between major groups and to test their eventual adaptive value.

EVOLUTION WITHIN *T. CRUZI*

In the next step, our approach was applied to another organism, *T. cruzi*. This species has been extensively studied by MLEE, RAPD, and natural populations were found to be heterogeneous and highly structured (Brisse et al. 2000). Two major lineages were described, both being very heterogeneous. First lineage (clade 1) was relatively less heterogeneous, and the second one was further subdivided into five smaller lineages (designated clades 2a to 2e). Our aim was thus to evaluate whether chromosome size-polymorphism would also reflect this structuring. Therefore, seven chromosomes recognised by specific probes were analysed in representative stocks of the six *T. cruzi* major clades, and data were processed by aCSDI (Henriksson et al. in preparation). On the Fitch-Margoliash dendrogram (Fig. 3), all isolates (without exception) clustered according to their clade of origin. Furthermore, like with MLEE/RAPD, clade 1 was more homogeneous and, consistently with RAPD, quite distant from the other ones. Chromosome discrimination of the six clades lineages might have important biological or medical significance.

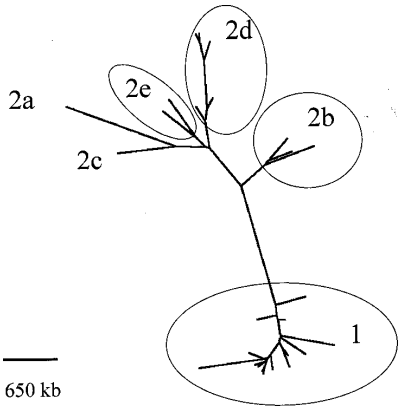

Fig. 3: size-polymorphism of seven chromosomes (recognised by probes 1F8, cruzipain, FFAg6, Tc2, CA7.12, CA7.32 and P19; Henriksson et al. 1995) in *Trypanosoma cruzi*. Agglomeration by the Fitch-Margoliash method after aCSDI calculation. Numbering of clades after Brisse et al. (2000)

Indeed, significant differences in clinically relevant biological parameters have been reported between isolates belonging to some of these clades (Montamat et al. 1996, Laurent et al. 1997, Revollo et al. 1998, De Lana et al. 1998). Identification of the genes involved in respective chromosome rearrangements will help exploring the molecular bases of these phenotypic differences.

Analysis of individual chromosomes showed a particular size-distribution. Indeed, in most chromosomes, a bi-modal distribution was observed, with important size-differences between the respective modes (Fig. 4). The stepwise size-variation observed within each mode is suggestive of amplification/deletion among tandemly repeated genes, as shown for *Leishmania*. In contrast, the transition from one mode to the other was not stepwise, and should correspond to discrete but less frequent evolutionary events, like translocation or deletion of large chromosome arms. The occurrence of such mechanisms was supported by the linkage of two markers (CA7.12 and 7.32) on a same chromosome in clade 1, but not in the other clades (Henriksson et al. 1995).

Chromosome analysis of *T. cruzi* allowed to highlight the relativity of taxonomical subdivisions in trypanosomatids. Indeed, in *T. cruzi*, the maximal aCSDI value measured (for seven chromosomes) between the two major lineages was 3,500 kb (average of 500 kb/chromosome). In the whole genus *Leishmania*, the maximal aCSDI value (for 31 chromosomes) was of 4,750 kb (average of 150 kb/chromosome). This result can be accounted for by either a higher mutation rate or a higher frequency of large rearrangements in the species *T. cruzi*, than in genus *Leishmania*. However, when other genetic characters were considered, like RAPD and MLEE, the same picture was encountered (Bañuls et al. 1999). It appears thus more likely that species definition should be questioned in both cases. In the absence of strict applicability of the sexual exchange criterion (Mayr 1969) to trypanosomatids because of their essentially clonal structure (Tibayrenc et al. 1990), species definition is basically operational only. Our results clearly show that chromosome analysis contributes significantly to the definition of the major evolutionary groups among trypanosomatids, and thus could contribute with other genetic characters for re-definition of corresponding taxonomic units.

CONCLUSIONS AND PROSPECTS

Trypanosomatids are characterised by extensive genomic plasticity. With the appropriate algorithm (aCSDI), it is possible to rely on chromosome size-polymorphism to infer hypotheses at different evolutionary levels. Qualitative congruence with hypotheses built-up from DNA sequence polymorphism (as inferred here from MLEE and RAPD data) contributed to validate our approach. At low evolutionary level chromosome size-polymorphism involves rearrangement of key genes, with important adaptive value. The same is occurring likely at higher evolutionary levels, where dramatic chromosome size-variations are observed. This should stimulate interaction between evolutionary studies like here presented and parasite genome sequencing projects. On one hand, identification of major evolutionary groups should call for peculiar attention to the genic content of chromosomes responsible for major structuring, and to their potential relationship with biological (including medical) differences. This might be one of the major application of evolutionary studies. On the other hand, definition of linkage groups should be used for performing direct gene rearrangement studies. This is important for further validating indirect studies based on chromosome size-polymorphism, but also for inferring hypotheses at higher evolutionary levels, where chromosome size might not be applicable anymore. It is too early to propose – like in prokaryotes – that the map of trypanosomatid cells is in their chromosomal organisation. However, our results clearly demonstrate that chromosomes together with gene sequences offer useful maps for finding our way in trypanosomatids' evolution.

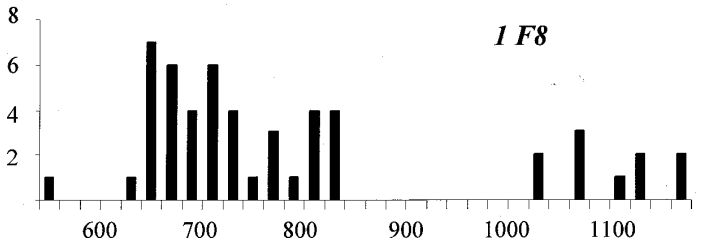

Fig. 4: bi-modal size distribution of 1F8 chromosome in a sample of *Trypanosoma cruzi* stocks representing the six major clades. Size is expressed in kb.

ACKNOWLEDGEMENTS

To Dr Jamie Stevens who organised The Trypanosome Evolution Workshop and stimulated the writing of present review.

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