Genetic profiling of the isoprenoid and sterol biosynthesis pathways of *Trypanosoma cruzi*

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Abbreviations: SBP, sterol biosynthesis pathway, SNP, single nucleotide polymorphism; DTU, Discrete Typing Unit; PMK, phosphomevalonate kinase; SMO, Sterol Methyl Oxidase
Abstract

**Background.** Sterols such as cholesterol, are important components of cellular membranes. But unlike mammalian cells, the main sterols found in the membranes of trypanosomes and fungi are ergosterol, and other 24-methyl sterols, which are required for growth and viability. In spite of this strict requirement, this group of organisms have evolved different strategies to produce and/or obtain sterols. *Trypanosoma cruzi* is the causative agent of Chagas Disease. In this parasite, one of the few validated targets for chemotherapeutic intervention is the sterol biosynthesis pathway. In this work we present a study of the genetic diversity observed in genes of the isoprenoid and sterol biosynthesis pathways in *T. cruzi*, and a comparative analysis of the diversity found in other trypanosomatids.

**Methodology/Principal Findings.** Using a number of bioinformatic strategies, we first completed a number of holes in the pathway by identifying the sequences of genes that were missing and/or were truncated in the draft *T. cruzi* genome. Based on this analysis we identified a non-orthologous homolog of the yeast ERG25 gene (sterol methyl oxidase, SMO) and propose that the orthologs of ERG25 have been lost in trypanosomes (but not in leishmanias). Next, starting from a set of 16 *T. cruzi* strains representative of six major evolutionary lineages, we have amplified and sequenced ~24Kbp from 18 genes of the pathway, and identified a total of 975 SNPs or fixed differences, of which 28% represent non-synonymous changes. We observed different patterns of accumulation of nucleotide changes for different genes of the pathway, from genes with a density of substitutions ranging from those close to the average (~2.5/100 bp) to some showing a high number of changes (11.4/100 bp, for a putative lathosterol oxidase gene). The majority of genes are under apparent purifying selection. However, two genes (TcPMK, TcSMO-like) have a ratio of non-synonymous to synonymous changes that is close to neutrality. None of the non-synonymous changes identified affect a catalytic or a ligand binding site residue. However, after mapping these changes on top of available structural data, we identified a number of changes that are in the close vicinity (7Å) of key residues, and that could therefore be functionally important. A comparative analysis of the corresponding *T. brucei* and *Leishmania* genes, obtained from available complete genomes highlights a high degree of conservation of the pathway, but with differences in the genes that are under apparent purifying selection in each case.

**Conclusions/Significance.** We have identified a number of genes of the sterol biosynthesis pathway that were missing from the *T. cruzi* genome assembly. Also, we have identified unequal apparent selection acting on these genes, which may provide essential information for the future of drug development studies focused on this pathway.
Introduction

*Trypanosoma cruzi*, a protozoan parasite of the order Kinetoplastida, is the causative agent of Chagas Disease, a neglected disease that is endemic in South America, affecting in excess of 8 million people (Rassi, Rassi & Marin Neto, 2016). The currently available drugs used to treat Chagas’ Disease (Nifurtimox, Benznidazole) have several drawbacks including toxicity, and the fact that they are mostly effective during the acute phase of the infection.

The *T. cruzi* species has a structured population, with a predominantly clonal mode of reproduction, with infrequent genetic exchange (Tibayrenc & Ayala, 2002, 2015). Through the use of a number of genetic markers the population has been divided into six evolutionary lineages (Barnabé, Brisse & Tibayrenc, 2000; Brisse, Barnabé & Tibayrenc, 2000; Zingales, Miles, Campbell et al., 2012). Lineages TcV and TcVI (this latter lineage includes the strain used for the first genomic sequence of *T. cruzi*, CL Brener) have a very high degree of heterozygosis. The currently favoured hypothesis suggests that these two lineages originated after one or two ancestral hybridization events (Machado & Ayala, 2001; Westenberger, Barnabé, Campbell et al., 2005; Flores López & Machado, 2011). The estimated time of divergence of these lineages is 1-4 Myr, which suggests that the diversification of *T. cruzi* was linked to the origin of its blood-sucking triatomine vectors, well before the contact with humans in South America (Flores López & Machado, 2011). This divergence was accompanied with a diversification of phenotypic and biological properties. Indeed, several investigations suggest that the observed diversity in host preference, cell tropism and drug susceptibility might be properties of different strains and/or lineages (Buscaglia & Di Noia, 2003; Vago, Andrade, Leite et al., 2000; Andrade, Machado, Chiari et al., 1999; Andrade, Galvão, Meirelles et al., 2010).

The parasite ergosterol biosynthesis pathway is one of the major routes for chemotherapeutic intervention against *T. cruzi*. Inhibitors that block sterol biosynthesis or the biosynthesis of isoprenoid precursors inhibit growth of the parasite and cause severe morphological defects (Urbina, 2009). Triazole derivatives that inhibit the parasite C14-α sterol demethylase are the most promising compounds, with proved curative activity in murine models of acute and chronic Chagas disease (Urbina, Payares, Molina et al., 1996; Silva, de Meirelles, Almeida et al., 2006; Lopesheva, Villalta & Waterman, 2011; Buckner & Urbina, 2012). And one of them (posaconazole) is undergoing a number of clinical trials. Other ergosterol biosynthesis inhibitors with good in vitro and in vivo potency, include those that target 3-hydroxy-3-methyl-glutaryl-CoA reductase, farnesyl diphosphate synthetase (Rosso, Szaalready, Malayil et al., 2011), squalene synthase (Rodrigues Poveda, González Pacanowska, Szaalready et al., 2012), lanosterol synthase (Buckner, Griffin, Wilson et al., 2001), squalene epoxidase (Gerpe, Odreman Núñez, Draper et al., 2008; Gerpe, Alvarez, Benitez et al., 2009) and 24-C sterol methyl transferase (Magaraci, Jimenez, Rodriguez et al., 2003; Lorente, Rodrigues, Jiménez Jiménez et al., 2004; Braga, Magaraci, Lorente et al., 2005), as well as compounds with dual mechanisms of action (ergosterol biosynthesis inhibition and free radical generation) (reviewed in Urbina, 2009; Urbina & Docampo, 2003).

The azoles, like the triazoles, are used extensively for the treatment of fungal infections with excellent results, though different resistant strains have appeared over time in different species. One of the main resistance mechanisms observed is based on a diminished affinity of the target enzyme for the compound; which is caused by specific mutations in the gene. Different point mutations were identified in several fungi species as responsible for thisazole resistance (reviewed in Lupetti, Danesi, Campa et al., 2002; Morio, Loge, Besse et al., 2010).

In this paper we analyze the genetic diversity present in the ergosterol biosynthesis pathway of *T. cruzi* and describe the apparent selection acting on these genes.
Results

Filling pathway holes: genes involved in sterol biosynthesis in T. cruzi

To analyze the genetic diversity of the T. cruzi sterol biosynthesis pathway (SBP) we decided to sequence all enzymes of the pathway, starting from enzymes that produce the terpenoid backbone precursors, and going down to the last enzyme that produces ergosterol as a product. Therefore, as a first step we looked for T. cruzi genes that were mapped to the corresponding KEGG metabolic pathway maps (Kanehisa, Goto, Sato et al., 2012). SBP genes in KEGG are classified in two maps: the steroid biosynthesis pathway map (TCR00100, http://www.genome.jp/kegg/pathway/tcr/tcr00100.html), and the terpenoid backbone biosynthesis pathway map (TCR00900, http://www.genome.jp/kegg/pathway/tcr/tcr00900.html). These maps contain information derived from the T. cruzi CL-Brener reference genome. From this analysis we were able to identify 15 genes mapped to these pathways. However, we also detected a number of holes in the pathway: enzymatic reactions with no enzyme mapped, and cases in which the enzymes available in KEGG were truncated (probably because of genome assembly problems). Therefore, before attempting to amplify and sequence the corresponding genes, we invested some effort in analyzing the existing sequence data to obtain a relevant complement of genes. As mentioned, in one case the corresponding genes from the reference genome were truncated, probably because of genome assembly problems. This was the case of the isopentenyl-diphosphate delta-isomerase gene (TcIDI1), which was cloned and sequenced by Dr. TK Smith (unpublished, GenBank accession number: AJ866772, 1071 bp). The corresponding genes in KEGG, mapped from the CL-Brener genome (Esmeraldo-like and non-Esmeraldo-like alleles) were both shorter, at 537 bp (TcCLB.408799.19), and 540 bp (TcCLB.510431.10). Therefore for this work we used the full-length TcIDI1 sequence obtained from GenBank (AJ866772).

To fill in other identified gaps, we used the Saccharomyces cerevisiae sterol biosynthetic pathway as a reference model. The yeast SBP has been studied extensively, and is essentially complete in pathway databases. Using the yeast genes mapped to this pathway, we looked for orthologs in T. cruzi by doing sequence similarity searches (BLAST) against the complete T. cruzi genome or using databases of orthologs compiled from complete genome data, such as the OrthoMCL database (Chen, Mackey, Stoeckert et al., 2006). As a result of this strategy, we were able to map five additional genes (see Table 1), which are the orthologs of the S. cerevisiae genes: ERG13 (3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase), ERG7 (lanosterol synthase), ERG26 (C-3 sterol dehydrogenase), ERG3, (C-5 sterol desaturase), and ERG5 (C-22 sterol desaturase). These genes were present in the T. cruzi genome, but were not mapped to the corresponding metabolic maps. In all these cases except for one (ERG26), the identification of the corresponding ortholog did not present further complications. The putative T. cruzi ortholog of the yeast ERG26 gene (TcCLB.510873.10, C3-sterol dehydrogenase) was found in the T. cruzi genome database as a truncated gene of 675 bp (224 aa). This may explain why it was not mapped to the corresponding pathway map in KEGG. Reasoning that this might be a consequence of assembly problems, as in the case of the TcIDI1 gene, we set out to identify the missing portions of this gene by performing BLAST searches against a database of unassembled genomic reads (GSS or WGS reads) from the CL-Brener genome project (data provided originally by the TIGR-SBRI-KI sequencing consortium). Starting with the truncated TcCLB.510873.10 sequence as query, we identified a number of matching sequence reads (BLASTN, E-value < 10⁻⁴⁰). These sequences were then assembled into a single contiguous sequence, which was used as a query in successive rounds of BLASTN searches, followed by reassembly. This iteration cycle was repeated until we recovered the complete (full-length) sequence of the T. cruzi putative C3-sterol dehydrogenase.
gene, as judged by its alignment against the yeast ERG26 gene. This reconstructed full-length sequence of the *T. cruzi* C-3 sterol dehydrogenase gene has 1,221 bp and has been used to design primers for amplification from *T. cruzi* DNA (see Methods). The final sequence, obtained after amplification and sequencing from CL-Brener DNA, was submitted to GenBank under the accession number JN050853.

At this point, there were still two gaps present when modeling the *T. cruzi* SBP on top of the yeast pathway. These correspond to the enzymatic reactions catalyzed by the yeast genes ERG27 (3-keto sterol reductase) and ERG25 (C-4 methyl sterol oxidase). Our failure at identifying the ortholog of the yeast ERG27 gene came as no surprise, as the enzymes performing C-3 ketoreduction in land plants and sterol synthesizing bacteria are still unknown (*Bouvier, Rahier & Camara, 2005; Desmond & Gribaldo, 2009*).

However, a number of putative homologs of ERG25 in *T. cruzi* were readily identified in sequence similarity searches. In this case, it was difficult to discern which of these were the true orthologs of the yeast ERG25 gene.

### Loss of ERG25 homologues in *T. cruzi* and *T. brucei*

A BLASTP search using the yeast ERG25p as query against kinetoplastid proteomes retrieved 5 *T. cruzi* significant hits, with 4 of them having identical length (278-279 aa, See Supplementary Excel File S1), sharing a common Pfam Domain (PF04116, Fatty Acid Hydroxylase Superfamily), and a common architecture with 3-4 predicted trans-membrane domains (the fifth gene is a truncated copy). These hits correspond to two pairs of alleles, and are currently annotated as 'C-5 sterol desaturases' in the *T. cruzi* genome database (or 'lathosterol oxidases', both are synonymous terms). Because there is detectable similarity between ERG3 and ERG25 genes, a similar search using the yeast ERG3p as query retrieves the same set of *T. cruzi* loci. Therefore, to identify which loci correspond to the true orthologs of ERG25, we carried out a detailed phylogenetic analysis between these genes, in different organisms. Using the sequences of fungi, plant, insect, human, fish and trypanosomatid enzymes, we obtained a maximum-likelihood tree with a clear segregation of ERG25 and ERG3 orthologs in separate branches (Figure 1). The tree suggests that there is one *T. cruzi* locus (containing TcCLB.473111.10 and its TcIII-like allele, TcCLB.507853.10) that is the true ortholog of the yeast ERG3 gene. Interestingly, the branch containing ERG25 orthologs does not show genes from African or American trypanosomes (the only kinetoplastid genes are from Leishmanias). The other trypanosomatid genes identified in our BLAST searches are grouped together in a third branch, carrying only trypanosomatid genes. These results are consistent with reciprocal best-hits identified in BLAST searches (see Fig S1). Reciprocal or bidirectional best hits provide support for the conjecture that the genes are equivalent orthologs (*Wolf & Koonin, 2012; Koonin, 2005*). In these searches, there is a clear drop in the BLAST score when the query sequence is ERG3 or an ERG3 homolog. This score drop is not observed when the query sequence is ERG25 or an ERG25 homolog. Apart from this evidence, there is additional support for this separate branch from the BLAST searches, in the observed reciprocity of best hits. For example when using the *L. major* gene LmjF.36.2540 as query (grouped with ERG25 orthologs in Fig 1) the best hits are the yeast ERG25 and their orthologs. And viceversa, when doing the reciprocal BLAST search using the yeast ERG25p as query, the best *L. major* hit is again LmjF.36.2540, even though in this case the scores are lower than for those obtained for ERG3 orthologs. These bidirectional hits are not observed for the group of trypanosomatid genes grouped in the middle branch in the phylogenetic tree.

Taken together, the most parsimonious interpretation of these results is that the C-4 sterol oxidase gene has been lost in the ancestor of *T. cruzi* and *T. brucei* (but not in the ancestor of
Leishmanias). The presence of Leishmanial and Trypanosomal proteins grouped in the middle branch of the tree suggests that these group of homologs have a different ancestral origin (see Discussion). Because these genes have significant sequence similarity to ERG25 we decided to call them Sterol Methyl Oxidase-like (SMO-like) in this work.

The Table 1 summarizes the results of our efforts to close pathway gaps. Once the T. cruzi SBP genes were identified, we proceeded to study the genetic diversity by re-sequencing these genes in a panel of T. cruzi strains.

Genetic diversity in the sterol biosynthesis pathway

To obtain sequence information from the selected genes we decided to use a methodology based on PCR amplification followed by direct sequencing. Therefore, it was important to reduce the possibility of amplification problems generated by polymorphisms that could prevent the annealing of the primers. A number of aspects were considered to reduce these risks: 1) we decided...
| ORF | Molecular function | Locus Identifier(s) | In KEGG? | Current annotation | Ortholog group | Gene Name |
|-----|--------------------|---------------------|----------|--------------------|----------------|-----------|
| 2.1 | N-acetyl-CoA thiolese | /uniF63A | No | Hypothetical protein | TcTAC2 | TcTAC2 |
| 2.2 | Hypothetical protein, conserved | /uniF63B | Yes | Hypothetical protein | TcTAC2 | TcTAC2 |
| 2.3 | Phenylalanine ammonia-lyase | /uniF63C | Yes | Phenylalanine ammonia-lyase | TcTAC2 | TcTAC2 |
| 2.4 | Acetyl-CoA synthetase | /uniF63D | No | Acetyl-CoA synthetase | TcTAC2 | TcTAC2 |

TcCLB.:
- TcCLB.::N
- TcCLB.::S

*Note:* Only mentioned if it differs from the yeast Putative ortholog.

Nomenclature used in this work. Some of these names were already used for the corresponding trypanosomatid genes. Only mentioned if it differs from the Yeast Putative ortholog.

The TcCLB::N-R allele is shorter, and probably truncated. The copy annotated by the genome project is truncated due to genome assembly problems. The full-length gene is deposited in GenBank as No:5083.
to focus our analysis on coding sequences where possible, as they are generally less variable than non-coding sequences; ii) we used the T. cruzi SNP database (TcSNP, http://snpa.tcruzi.org) (Ackermann, Carmona & Agüero, 2009), to select the best regions for primer design, avoiding regions with candidate SNPs; and iii) we also limited the size of amplification products, to optimize the quality of the final sequence (see Methods). In this latter case, an optimal size was set to ~ 750–800 bp, which would allow us to get complete sequence coverage, with good quality on both strands. Depending on the size of each gene, one or more overlapping amplification products had to be analyzed (the number of amplification products per gene is listed in Table 2).

All amplification products were analyzed with a software package (PolyPhred, see Methods) that allows the identification of heterozygous peaks in the chromatograms. Therefore, for all selected genes, we identified two types of sequence polymorphisms: i) allelic variation within a strain/clone (heterozygous peaks identified by Polyphred); and ii) variation between strain/clones (identified in a multiple sequence alignment of sequenced products). For every gene, we identified the position of the variant sites, and the type of change introduced (synonymous or non synonymous). The complete information for each gene is available as supplementary material (Table S2), and a table summarizing these results is included herein (Table 2). Using this strategy, we generated ~ 24 Kb of sequence, from 16 strains covering the 6 major T. cruzi evolutionary lineages. Overall we attained a coverage of ~ 90% of the total coding sequence for the selected genes. From this analysis we identified 975 polymorphic sites, producing 978 codon changes, which generate 692 synonymous changes (72%) and 273 non-synonymous changes (28%, see Table 2). The genes Tc24SMT, TcHMGR and TcNSDHL had the lowest SNP density (2.68, 2.74 and 2.50 every 100bp respectively), while TcSC5D had the highest SNP density (11.39 every 100bp). This density is at least two times higher than that found in other SBP genes. However, at the same time, this gene had one of the lowest ratios of non-synonymous SNPs over synonymous SNPS (NS/S ratio). Upon further investigation of this gene, we noticed a number of informative SNPs that could be exploited in a lineage typing assay. Therefore, we performed a separate re-sequencing experiment for this gene, in an expanded panel of strains, and developed two alternative typing assays based on this locus (published separately, Cosentino & Agüero, 2012).

Other genes with low NS/S ratios were TcHMGS and Tc14DM (0.15), indicating a high degree of conservation in the panel of strains analyzed. On the other extreme, the genes TcSMO-like and TcPMK had the highest NS/S ratios (0.93 and 0.94 respectively) and together with TcMVD have the highest non-synonymous SNP density (between 1.8 and 2.5 non synonymous SNPs every 100bp). The average NS/S ratio in our dataset was 0.4, and in no case was this ratio higher than 1, indicating the relatively high degree of conservation of T. cruzi SBP genes.

**Lineage-specific and intra-lineage differences.**

All the collected data on sequence diversity was also analyzed in the context of the current separation of T. cruzi in discrete evolutionary lineages. For this analysis we considered three types of polymorphisms or fixed differences: i) lineage specific polymorphisms (LSPs) (these are the polymorphic sites that could differentiate one lineage from all the others; ii) intra-lineage polymorphisms (ILP) (differences between strains of the same lineage); and iii) heterozygous sites (those that differ between alleles of a single diploid individual). In this comparative analysis we found that the lineage TcI showed more than one LSP every 200 bp, while in the other lineages, the LSP density was at least four times lower (data not shown). This data agrees with the hypothesis that TcI is one of the ancestral lineages Flores López & Machado (2011); Zingales, Miles, Campbell et al. (2012). Analyzing the ILP distribution, it was found that the TcIV lin-
Table 2: Comparative analysis of the quantity, density and type of SNPs identified in the sterol biosynthesis pathway of Trypanosoma cruzi. SNP counts marked with * indicate totals that do not result from the sum of synonymous + non-synonymous substitutions, in all cases because of the presence of two substitutions in the same codon.

| Genes               | Polymorphisms (SNPs) | Length (bp) |
|---------------------|----------------------|-------------|
|                     | Synonymous ($)       | Non-synonymous (NS) | Ratio | Total | CDS bp | Resequenced | PCR Fragments |
| TcACAT              | 35 (73.00)           | 11 (25.00) | 1.09 | 0.33 | 48 | 1188 | 1100 (92.59) | 2 |
| TcHMGS              | 37 (86.05)           | 6 (13.95)  | 0.43 | 0.16 | 43 | 1497 | 1390 (92.85) | 2 |
| TcHMGR              | 27 (79.41)           | 7 (20.59)  | 0.56 | 0.26 | 34 | 1356 | 1240 (91.45) | 2 |
| TcMK                | 31 (79.49)           | 12 (30.77) | 1.24 | 0.39 | 50* | 1086 | 970 (89.32)  | 2 |
| TcPMK               | 35 (53.85)           | 25 (50.77) | 2.50 | 0.94 | 69* | 1428 | 1320 (92.44) | 2 |
| TcMVD               | 32 (62.75)           | 18 (37.25) | 1.83 | 0.59 | 51 | 1140 | 1040 (91.23) | 2 |
| TcFPSS              | 22 (66.67)           | 2.27 | 11 (33.33) | 1.13 | 0.50 | 36 | 1275 | 970 (76.08)  | 2 |
| TcD1I               | 25 (78.13)           | 7 (21.88)  | 0.70 | 0.28 | 32 | 1071 | 1000 (93.37) | 2 |
| TcSQS               | 32 (69.57)           | 14 (30.43) | 1.26 | 0.44 | 46 | 1212 | 1110 (91.48) | 2 |
| TcSQLE              | 50 (72.46)           | 21 (30.43) | 1.29 | 0.42 | 73* | 1716 | 1630 (94.99) | 3 |
| TcOSC               | 61 (66.30)           | 2.53 | 31 (37.70) | 1.29 | 0.51 | 92 | 2706 | 2410 (89.06) | 4 |
| Tc14DM              | 47 (82.04)           | 7 (12.96)  | 0.51 | 0.15 | 54 | 1443 | 1360 (94.25) | 2 |
| Tc14SR              | 33 (61.46)           | 19 (36.54) | 1.48 | 0.58 | 52 | 1371 | 1280 (93.16) | 2 |
| TcSMO-like          | 14 (51.85)           | 13 (48.15) | 1.94 | 0.93 | 27 | 837  | 670 (80.05)  | 1 |
| TcNSDHL             | 21 (70.00)           | 9 (30.00)  | 0.75 | 0.43 | 30 | 1221 | 1200 (98.28) | 2 |
| Tc24SMT             | 17 (72.27)           | 5 (22.73)  | 0.61 | 0.29 | 22 | 1050 | 820 (78.10)  | 1 |
| Tc8SI               | 18 (78.26)           | 5 (21.74)  | 0.88 | 0.28 | 23 | 654  | 570 (87.16)  | 1 |
| Tc5C4D              | 70 (89.74)           | 11 (14.10) | 1.53 | 0.16 | 82* | 834  | 720 (86.33)  | 1 |
| Tc5C2ZD             | 29 (69.05)           | 10 (30.95) | 0.94 | 0.45 | 42 | 1518 | 1390 (91.57) | 2 |
| Tc5C24R             | 53 (75.71)           | 7 (24.29)  | 1.21 | 0.32 | 70 | 1467 | 1400 (95.43) | 2 |
| Total/Avg           | 692 (71.71)          | 273 (28.29) | 1.16 | 0.39 | 975 | 26070 | 23590 (90.50) | 39 |

**Potentially important non-synonymous changes.**

In many cases, variations in susceptibility or resistance to a drug are associated to mutations that occur near the interaction site of a substrate or an inhibitor. To analyze the potential relevance of the non-synonymous SNPs found, we gathered relevant information from the literature on the selected targets, their PFAM domains, PROSITE motifs, and the available crystallographic structures in the Protein Data Bank (PDB, [http://www.rcsb.org](http://www.rcsb.org)) for the *T. cruzi* enzyme or their orthologs (see Methods). Starting with these crystal structures, we first identified residues near the co-crystallized ligands (substrate, inhibitor or co-factor). For this we established a maximum distance limit of 7Å from each corresponding ligand. This analysis revealed a number of potential targets for further study.
potentially important changes in the Tc14DM (TcCYP51), and TcMK genes.

In the azole target gene, Tc14DM (TcCYP51), we identified only 5 non-synonymous substitutions, none of which affect key residues of the enzyme (see Figure 2). However, after mapping these substitutions on top of the available TcCYP51 structure (Chen, Leung, Guilbert et al., 2010), we identified a number of potentially important substitutions that lie just next to important residues, or are within 7Å of the co-crystallized ligand. One of these is a A117S substitution that sits just next to a tyrosine (Y116) that is within 7Å of the ligand, and that has been shown to be involved in the generation of resistance to azoles in C. albicans (Kelly, Lamb & Kelly, 1999; Delye, Laigret & Corio Costet, 1997). In trypanosomatids, residue 117 marks the start of a short helix in the structure, that is uniquely found in trypanosomatids (Lepesheva, Park, Hargrove et al., 2010). Alanine at residue 117 is present in T. brucei and was found in strains from the TcIII lineage (former Tcruzi Iic) in homozygosis and in heterozygosis in hybrid strains, while Serine at this position was found in homozygosis in strains from lineages TcI, TcII, and TcIV (former Tcruzi I, Iia and Iib).

![Figure 2: Alignment of T. cruzi, T. brucei, M. tuberculosis and human Lanosterol 14-α demethylases, showing the non-synonymous changes identified in this work (red arrows). Important residues either in Tc14DM or in the CYP51 family are noted (Lepesheva, Zaiteva, Nes et al., 2006; Lepesheva & Waterman, 2011), as well as residues associated with resistance to azoles in C. albicans and U. necator (Kelly, Lamb & Kelly, 1999; Delye, Laigret & Corio Costet, 1997). P500086 is the Prosite Cytochrome 450 motif (cysteine-heme-iron ligand signature) in the case of the TcMK gene, we identified 12 non-synonymous changes. One of these is a H29Y change, located in a conserved region of the sequence, close to a group of residues that are involved in substrate binding at <7Å of the ligand (mevalonate) in the L. major structure (1LmjE3:0560, PDB: 2Hfu) (Soria, Smith & Hunter, 2007). Histidine is encoded by the gene
from strains M5631 and X109/2, both from lineage TcIII, while tyrosine is encoded in all other
strains, including M6241, also of lineage TcIII. The second possibly important change in TcMK
is a G287A substitution, also located in a very conserved motif of the MK gene, that is part of
the GHMP kinase family domain (Pfam PF08544). That particular motif, contains a number of
highly conserved glycines, some of which are within 7Å of the ligand in the L. major MK. In
this polymorphic position strains from lineage TcV (Sc43 and Mn) encode glycine and alanine
in heterozygosis, while all the other strains encode glycine in homozygosis.

For other targets, crystallographic structures were not available, and so we resorted to ana-
lyze non-synonymous substitutions in the context of functionally important motifs or domains.
In the case of the squalene epoxidase gene (TcSQLE), we identified two changes (S306G and
I307L) that stand between highly conserved proline residues (at 95% and 94% identity within
the squalene epoxidase domain, PF08491). The I307L substitution is apparently conservative,
as these are the two most frequent residues in this position in the Pfam domain. In contrast,
the S306G substitution introduces a glycine that is completely absent at this position in the 429
sequences currently available for this protein family in the Pfam database. Serine is the second
most frequent residue in this position, and is present in 14% of the sequences, all from fungi
(glutamine is the most frequent residue, in 50% of the sequences). The Ser residue occurs in lin-
eage TcII in homocysis, and in lineages TcV and TcVI in heterocysis, and its conservation
in fungi suggests that this is the ancestral character at this position. Finally in the C-14 sterol
reductase gene (Tc14SR), we identified two consecutive substitutions (P280H, V289F) in strains
9122102R and Dog Theis (lineage TcIV), that fall within the sterol reductase family signature
motif (PS01917). However the substitutions are conservative, because they are both described
by the motif pattern.

The phosphomevalonate kinase of T. cruzi is not under strong purifying
selection

The phosphomevalonate kinases (PMKs) of pathogenic bacteria, fungi, and trypanosomes are
attractive targets for the design of selective inhibitors, because the same phosphorylation reaction
in humans and other animals is catalyzed by a non-orthologous enzyme (Houten & Wa-
terham, 2001). In the first group of organisms, phosphorylation of mevalonate is performed by
orthologs of the yeast ERG8 gene, while in animals it is performed by a group of orthologs of
the human PMK gene (hPMK). These two groups of enzymes differ in a number of kinetic, bi-
ophysical properties, and in the ATP-binding motifs (ERG8-like kinases contain a protein kinase
motif, while orthologues of the human enzyme have a P-loop or “Walker A” motif) (Houten &
Waterham, 2001; Chang, Yan, Gu et al., 2008). Analysis of the TcPMK gene in different strains
of T. cruzi showed that the gene has accumulated 68 changes (35 synonymous, 33 non-synony-
mous, see Table 2), and 1 non-sense substitution (see below) in these independently evolving
lineages. With a ratio of non-synonymous to synonymous differences close to 1 (0.94), the sub-
stitutions can be considered largely neutral, and together with the TcSMO-like gene, these are
the only two genes in the analyzed pathway that are not under apparent purifying selection.
Moreover, an interesting substitution was observed at codon 136 (407bp) in the T. cruzi strain
IVV (TcII). In this strain, one allele encoded a Serine (TCA), as in all other strains, whereas the
second allele encoded a premature STOP codon (TAA). All ERG8-like PMKs are composed of
two GHMP kinase domains: an N-terminal domain (Pfam PF00288) located in the middle of
the protein (starting at residue 160 in T. cruzi), and a C-terminal domain (PF08544) closer to
the C-termini. In T. cruzi, the nonsense mutation in the IVV strain is located upstream of this
first domain (PF00288). Considering that the next possible translational start codon is located
downstream of this domain, the IVV strain is therefore probably producing a very short non-functional protein from this allele, or two truncated proteins, both devoid of this domain. Thus, the most plausible hypothesis is that the IVV strain carries only one functional PMK allele, and can be considered naturally hemizygous for the PMK gene.

**Comparative analysis of genetic diversity in kinetoplastid sterol biosynthesis pathways**

Although phylogenetically related, kinetoplastid parasites have evolved different adaptations to their host environments. This is particularly evident in the case of ergosterol dependency: both *T. cruzi* and *Leishmania* have an essential requirement for ergosterol and/or other 24-methylsterols, and are unable to survive by salvaging cholesterol from the host (*Roberts, McLeod, Rice et al., 2003*). Sterol biosynthesis in *Trypanosoma brucei*, is apparently suppressed in bloodstream forms, relying instead on receptor-mediated endocytosis of host low-density lipoproteins carrying cholesterol (*Coppens & Courtoy, 2000*). Apart from differences in their strict requirement for *de novo* synthesis of sterols, there are also differences in the exact type and abundance of synthesized sterols, which may be explained by differences in the complement of genes and/or their activities or regulation. As an example, it has been described that *T. cruzi* amastigotes lack Δ⁵,⁷ sterols, suggesting the lack of Δ⁵ desaturase activity in this stage (*Liendo, Visbal, Piras et al., 1999; Roberts, McLeod, Rice et al., 2003*). Based on these premises, we decided to investigate the accumulation of nucleotide changes (fixed differences) in genes from the sterol biosynthesis pathway in African Trypanosomes, and Leishmanias, reasoning that the selection acting on these genes could be different in each case. For this we identified the corresponding orthologs of the yeast and *T. cruzi* genes used in this work (see Table 1) in currently available kinetoplastid genomes (see Methods). The available information includes that of *T. brucei brucei* (2 strains) and *T. brucei gambiense*; and that of 4 species of Leishmanias (major, infantum, braziliensis and mexicana). Using this information we proceeded to analyze the nucleotide substitutions observed in each group. A summary of this analysis is presented in Table 3.

Because the genetic space explored in each case is different, it would be perhaps incorrect to compare the number of nucleotide changes observed for each gene between the three groups of organisms. However, it is still possible to analyze and compare the information on sequence diversity within each group (e.g. column-wise in Table 3). When looking at data in this way, a number of observations can be made: even though the genetic diversity in African trypanosomes is the lowest observed (in number of substitutions per 100 bp), the ratios of non-synonymous to synonymous changes are higher than those observed in Leishmania and *T. cruzi* in 13 of 16 cases with data. The genes accumulating less changes in African trypanosomes are the C-24 sterol methyl transferase (24SMT, ortholog of ERG6), and the HMGR gene (HMG CoA reductase). In Leishmanias, although we observe higher densities of substitutions, the ratios of Non-syn to Syn are the lowest overall, suggesting that the pathway is under purifying selection. However, interestingly the two genes with the highest NS/S ratios are the Leishmanial SMO-like and ERG25 orthologs.

**Discussion**

The main goal of our work was to study the genetic diversity present in the isoprenoid and sterol biosynthesis pathway (SBP) of *T. cruzi*. These pathways are validated chemotherapeutic targets for Chagas Disease, with a number of compounds currently undergoing clinical trials. Because
Table 3: Comparative analysis of the genetic diversity present in the sterol biosynthesis pathway of different kinetoplastids. The table shows the density of synonymous or non-synonymous nucleotide changes (substitutions per 100 bp) observed in the comparison of T. brucei (Tbr), Leishmania (Leish) and T. cruzi (Tcr) genes from different strains and/or species. The lowest (*) and highest (†) figures in each column are marked. NA = sequence not available; DBZ = Division by Zero. In some cases (§) sequences were not available for all strains.

| Yeast gene, Std symbol | Syn SNPs / 100 bp | Non-Syn SNPs / 100 bp | Total SNPs / 100 bp | Ratio Non-Syn/Syn |
|------------------------|-------------------|-----------------------|---------------------|-------------------|
|                        | Tbr    | Leish   | Tcr    | Tbr    | Leish   | Tcr    | Tbr    | Leish   | Tcr    | Tbr    | Leish   | Tcr    | Tbr    | Leish   | Tcr    |
| ERG16 / ACAT           | NA     | NA      | 3.27   | NA     | NA      | 1.09   | NA     | 4.36    | NA     | NA     | 0.33   |
| ERG13 / HMGS           | 0.60   | 9.96    | 2.81   | 0.33   | 2.59   | 0.43   | 0.93   | 12.55   | 3.24   | 0.56   | 0.28   | 0.15†  |
| HMGI / HMGR            | 0.08   | 10.50   | 2.18   | 0.07   | 3.60   | 0.56   | 0.08   | 14.10   | 2.74   | 0.04   | 0.34   | 0.26   |
| ERG12 / MK             | 0.10   | 16.77   | 3.20   | 0.20   | 3.56   | 1.24   | 0.30   | 22.32   | 5.15   | 2.00†  | 0.33   | 0.39   |
| ERG8 / PMK             | 0.56   | 11.47   | 2.65   | 0.42   | 6.54   | 2.50†  | 0.99   | 18.01   | 5.23   | 0.79   | 0.57   | 0.94‡  |
| ERG9 / MVD             | 0.26   | 14.93   | 3.08   | 0.44   | 5.90   | 1.83   | 0.70   | 20.83   | 4.90   | 1.67   | 0.40   | 0.59   |
| IDE1 / IDI             | 0.19   | 12.61   | 2.50   | 0.09   | 3.08   | 0.70   | 0.28   | 15.69   | 3.20   | 0.50   | 0.24†  | 0.28   |
| ERG10 / EPPS           | 0.63†  | 9.55‡  | 2.27   | 0.45†  | 3.67   | 1.13   | 1.09   | 13.22   | 3.71   | 0.73   | 0.38   | 0.50   |
| ERG6 / FDTF, SQS       | 0.15   | 10.92   | 2.88   | 0.32   | 5.62   | 1.26   | 0.29   | 16.55   | 4.14   | 1.00   | 0.51   | 0.44   |
| ERG1 / S0L             | 0.40   | 12.16   | 3.07   | 0.23   | 6.49   | 1.29   | 0.63   | 18.65   | 4.48   | 0.57   | 0.53   | 0.42   |
| ERG7 / LSS, OSC        | 0.40   | 12.00   | 2.53   | 0.33   | 6.98†  | 1.29   | 0.74   | 18.98   | 3.82   | 0.82   | 0.58   | 0.51   |
| ERG11 / 14DM, CYP51     | 0.41   | 10.07   | 3.46   | 0.28   | 2.78   | 0.51   | 0.69   | 12.85   | 3.97   | 0.67   | 0.28   | 0.15†  |
| ERG24 / 14SR, TM7SF2   | 0.65   | 10.65   | 2.58   | 0.29   | 6.39   | 1.48   | 0.94   | 17.05   | 4.06   | 0.44   | 0.60   | 0.58   |
| ERG25 / SMO / SC4MOL†  | —      | 12.01   | —      | 9.39   | —      | 21.40  | —      | 0.78†   | —      | —      | —      | —      |
| — / SMO-like           | 0.27   | 10.55   | 2.09   | 0.27   | 6.55   | 1.94   | 0.54   | 17.09   | 4.03   | 1.00   | 0.63   | 0.93   |
| ERG26 / NSDLH          | 0.08   | 10.16   | 1.75†  | 0.16   | 3.66   | 0.75   | 0.25   | 13.83   | 2.50†  | 2.00†  | 0.36   | 0.43   |
| ERG27 / JKSR           | NA     | NA      | NA     | NA     | NA      | NA     | NA     | NA      | NA     | NA     | NA     | NA     |
| ERG6 / 2aSMT           | 0.10   | 10.45   | 2.07†  | 0.16   | 6.12   | 0.61†  | 0.25   | 16.57   | 2.68†  | DBZ    | 0.59   | 0.29   |
| ERG2 / 8SI             | 0.30   | 12.80   | 3.16   | 0.45†  | 6.85   | 0.88   | 0.75   | 19.64   | 4.04   | 1.50   | 0.53   | 0.28   |
| ERG3 / SC5D            | 0.12   | 12.54   | 4.72†  | 0.12   | 4.84   | 1.53   | 0.25   | 17.38   | 11.39† | 1.00   | 0.39   | 0.16   |
| ERG5 / SC22D           | NA     | 12.18   | 2.09   | NA     | 4.85   | 0.94   | NA     | 17.03   | 3.02   | NA     | 0.40   | 0.45   |
| ERG4 / SC24R           | NA     | 11.47   | 3.79   | NA     | 6.44   | 1.21   | NA     | 17.91   | 5.00   | NA     | 0.56   | 0.32   |

Average: 0.31 11.67 3.06 0.25 5.18 1.26 0.56 16.86 4.27 0.81 0.44 0.38
Std deviation: 0.21 1.80 1.66 0.15 1.50 0.53 0.34 2.69 1.87 0.57 0.13 0.32

The T. cruzi SBP pathway appeared to be incomplete in metabolic pathway databases such as KEGG when we started this work, and because the annotation of the SBP genes was also incomplete, we had to perform a small-scale bioinformatics analysis to fill in the gaps in available sequence and annotation. This task was performed primarily based on the well studied S. cerevisiae ergosterol biosynthesis pathway. As a result of this strategy, the majority of the genes of the pathway have now been identified, with the exception of the orthologs of the yeast gene ERG27. This gene encodes a 3-keto sterol reductase. As mentioned, the gene(s) responsible for this activity in land plants and sterol synthesizing bacteria have not been identified yet (Desmond & Gibraldo, 2009). It is therefore highly likely that the trypanosomatid 3-keto sterol reductase is phylogenetically closer to the plant enzymes, and that once this elusive gene is identified it will be readily identified in trypanosomatids.

We selected 21 genes from this pathway to build a genetic diversity profile from representative strains of the six major evolutionary lineages of T. cruzi. For this analysis we used at least 3 strains for each evolutionary lineage (DTU) therefore effectively sampling a large genetic space. Although it is certainly likely that other SNPs or fixed differences can be discovered when sequencing these loci from new isolates, most probably these new mutations will correspond to changes that are unique to the new isolate (e.g. introduced during the clonal expansion of this particular isolate). In our experience, when expanding our re-sequencing analysis of the TcSC5D gene to 15 additional strains, 10 new polymorphic sites were identified (see Fig S1 in Cosentino & Aguiro, 2012, 4 of which came from a new lineage/DTU (TcBat/TcVII Marcili, Lima, Cavazzana et al. (2009)) that was not included in this analysis, while 4 others came from a TcIV strain
(see discussion above about intra-lineage diversity of TcIV). This is a highly polymorphic gene, so for the other genes we believe we have sampled a significant genetic space.

The strategy employed consisted in the obtention of overlapping amplification products of approximately 750bp (with 100bp of overlap) for each gene, followed by direct sequencing of amplification products in both strands. As a result, the majority of the sequenced bases were read at least twice. The primers were designed based on the CL-Brener genome sequence, and the majority of them were designed against the corresponding coding sequence to reduce the possibility of amplification problems (under the hypothesis that coding sequences are much less polymorphic than non-coding sequences). Moreover, when designing primers we avoided SNPs already identified from sequences in the public domain by checking against the TcSNP database. This strategy enabled the amplification and sequencing of all the selected gene fragments in strains from all the lineages, except for the first amplification product of the TcMK gene, that could not be amplified initially from lineage I. These strains carry a SNP that is specific for TcI and that mapped exactly at the 3’-end of the forward primer (see Table S3). We fixed this primer after recent genomic data was made available for a number of Tcruzi I strains (Sylvio X10 (Franzén, Ochaya, Sherwood et al., 2011), JR cl4 (accessed trough TriTrypDB (Aslett, Aurrecoechea, Berri- man et al., 2010)), and TcAdriana (Westegaard G and Vazquez M, unpublished results)).

TcSC5D was the most polymorphic gene with 11 SNPs/100bp (for a total of 82 SNPs in 720bp analyzed), a SNP density that is at more than twofold larger than those observed for other re- sequenced genes. However, the TcSC5 gene is apparently under strong purifying selection, with one of the lowest ratios of NS/S SNPs in the panel (together with the Tc14DM and TcHMGCoA genes, with values of 0.16, 0.15 and 0.15 respectively (See Table 2). Tc14DM and TcHMGCoA have also the lowest non synonymous SNP density of the panel, with only 0.51 and 0.43 non synonymous SNPs every 100bp, so at least from the genetic evidence alone, these genes would be the best candidates for drug development.

The TcSMO-like genes of T. cruzi are not under strong purifying selection

As described above, when looking for the T. cruzi orthologs of the ERG25 (C4-methyl oxidase) gene, we identified two homologs (two pairs of allelic variants from the hybrid genome), which are members of the Fatty Acid Hydroxylase superfamily (Pfam Domain PF04116). Based on best-reciprocal BLAST hits and careful examination we concluded that these genes (TcCLB.511339.20, TcCLB.509235.20) are not orthologous to ERG25, and are probably divergent homologs of a different ancestral gene. In any case, apart from the divergence noticed when analyzing this group of homologs across different organisms (see Figs 1, and S1) in this work we also observed that these genes are also diverging within the T. cruzi species, as revealed by the accumulation of non-synonymous nucleotide changes in different T. cruzi strains/lineages. This could indicate an ongoing process of neo-functionalization of the encoded protein. Interestingly, when performing BLASTP searches against fungal genomes, we noticed a number of SUR2 (Sphinganine C4-hydroxylase) homologs among the significant hits (see Fig S1). In yeast, SUR2 (also a member of the FA hydroxylase superfamily) catalyzes the conversion of sphinganine to phytosphingosine in sphingolipid biosynthesis. Recently, the presence of phytosphingosine in trypanosomes was demonstrated by mass spectrometry (Vacchina, Tripodi, Escalante et al., 2012). In this article the authors also show that the biosynthesis of phytosphingosine is driven by bifunctional hydroxy- lase/desaturase enzymes. However, the trypanosomatid genes identified as responsible for this activity are not orthologs of yeast SUR2 (see Fig 2 in Vacchina, Tripodi, Escalante et al., 2012). In the reciprocal BLAST searches, using the yeast SUR2 protein as query against trypanosomatid genomes, we always retrieve the same set of ERG3 orthologs, and SMO-like genes. Therefore it
is tempting to speculate that in trypanosomes the orthologs of both SUR2 and ERG25 have been
lost, and that the SMO-like genes grouped in the middle branch in Figure 1 could represent an
ancestral hydroxylase/desaturase that has adjusted (or is still adjusting) to a new functional niche
in these organisms (cellular localization or time/stage of expression, etc). Interestingly the ex-
pression of this gene is higher in amastigotes and trypomastigotes, the two life cycle stages that
occur in the mammalian host (data from Minning, Weatherly, Atwood et al., 2009).

The protein sequences encoded by these genes show the three canonical conserved histi-
dine boxes (HxxxH, HxxHH, and HxHH) present in all FA_hydroxylase family members. The
distribution of the accumulated changes is shown in Figure S1. None of the non-synonymous
changes affect these highly conserved motifs, and at least a third of these (depending on the
membrane topology prediction) are predicted to be exposed (not embedded in the membrane).
This is important because, as reviewed in Sperling, Ternes, Zank et al., the evolution of new re-
gioselectivities in these enzymes would not involve the active site, but adjacent sequences. How-
ever, the failure to predict a reasonable topology (see Fig S1) points to the need to do an in-depth
study of the membrane topology of the protein (similar to that performed by Diaz, Mansilla, Vila
et al., 2002).

Conclusion

Methods

T. cruzi stocks and strains.

Strains used in this study (and the corresponding current lineage classification) were: Sylvio
X10 ch and Dm28 (TcI); MAScl, TU18 ch93, IVV cl4 (TcII); M6241 cl6, M5631 cl5 and X109/2
(TcIII); CanIII cl1, Dog Theis and 92122102R (TcIV); Sc43 ch9 and MN cl2 (TcV); Tulahuen cl2,
CL Brener and P65 ch (TcVI).

Oligonucleotides and gene identifiers.

For each selected gene, a number of primers were designed for PCR-amplification. Taking into
account that in the direct sequencing of PCR products the chromatogram quality is optimal
in the range from 50 to 700 bp, a desirable length of the amplification products was set around
750bp. This length would also maximize our ability to sequence both strands of the amplification
product, with good quality. Depending on the size of each selected gene, one or more overlap-
ping amplification products were obtained. The list of the designed primers for each gene and
the size of the corresponding amplification product is shown in Table S3. In only one case we
had to design a separate primer (Tc-Mev-kinase26-fw) to amplify a fragment in one lineage (TcI)
because there was a SNP at the 3’ end of the primer that was absent in the release 1 of the TcSNP
database.

Amplification and sequencing.

Selected fragments were amplified by PCR using Taq polymerase (Invitrogen) in a Biometra
T Professional Gradient 96 cycler. Amplification mixtures contained 10 pmol of each primer,
PCR buffer (Invitrogen), 1.5 mM MgCl2, 50 ng of genomic DNA, 200 µM dNTPs, 2.5 U Taq
polymerase (Invitrogen), and water to a final volume of 25 µL. After denaturing at 94°C for 2
minutes, thermal cycling was performed for 35 cycles at 94°C for 30 seconds, followed by 30
SNP identification and scoring.

Gene fragments were PCR-amplified from every strain of the panel and sequenced in both strands. Base calling of chromatograms, assembly of sequences, detection of polymorphisms and manual inspection of assembled sequences and polymorphisms was done using a software package composed of Phred (version 0.020425.c) (Ewing, Hillier, Wendl et al., 1998; Ewing & Green, 1998), Phrap (version 0.9909329), Polyphred (version 5.04) (Nickerson, Tobe & Taylor, 1997) and Consed (version 15.0) (Gordon, 2003). Base calling of chromatograms was done by Phred. Sequences were then assembled by Phrap. Polyphred was used to process phrap assemblies to detect polymorphic sites. All candidate SNPs identified by PolyPhred (score >70/99), including heterozygous peaks, were visualized with Consed. A few false positives, and false negatives were removed/added after this manual inspection.

Other sequences used in this study

For the comparative analysis of SBP genes in kinetoplastid genomes, we have obtained the corresponding protein sequences for each of the yeast and/or T. cruzi orthologs used in this study from the TriTrypDB database (Aslett, Aurrecoechea, Berriman et al., 2010). A BLAST search using the corresponding SBP gene as query was used to identify the corresponding ortholog. This has been cross-checked by inspection of ortholog clusters at the OrthoMCL database (Chen, Mackey, Stoeckert et al., 2006). The sequences used belong to the following species/strains: T. brucei brucei strains TREU927 (Berriman, Ghedin, Hertz Fowler et al., 2005) and Lister 427 (George Cross, unpublished), Trypanosoma brucei gambiense (Jackson, Sanders, Berry et al., 2010); L. major (Ivens, Peacock, Worthy et al., 2005), L. infantum (Peacock, Seeger, Harris et al., 2007), L. braziliensis (Peacock, Seeger, Harris et al., 2007), and L. mexicana (Wellcome Trust Sanger Institute Pathogen Sequencing Unit, unpublished).

Mapping substitutions on three dimensional structures

Crystallographic structures of enzymes of the sterol biosynthetic pathway were obtained from the Protein Data Bank (PDB, http://www.rcsb.org). Using the molecular graphics viewer VMD (version 2.8.6) (Humphrey, Dalke & Schulten, 1996) together with the multiple sequence alignment plugin for VMD (Eagarle, Wright & Luthey Schulten, 2006) we mapped sequences from different strains on top of the reference sequence from which the structure was obtained. Atomic distances from each residue to the co-crystallized ligand were measured using standard tools implemented in VMD. Structures used in this work (and their source organism, and T. cruzi
homolog) were: 1w15 (human, TcACAT); 2fa3 (Brassica juncea, TcHMGS); 3bgl (human, TcHMGR); 2hfu (L. major, TcMK); 2hke (T. brucei, TcMVD); 1yhm (T. cruzi, TcFPSS); 1ezf (human, TcSQS); 1w6k (human, TcOSC); 3khm, 3k10, 3ks (T. cruzi, TcCYP51).

**Data deposition**

The sequences reported in this paper have been deposited in the GenBank database under the following accession numbers: JN050313-JN050853, and KF290395-KF290460. Heterozygous sequence polymorphisms have been submitted as ambiguities in the sequence using standard IUPAC notation. Sequence polymorphisms identified between different strains/clones will be available in a future release of the TcSNP database (http://snps.tcruzi.org, (Ackermann, Carmona & Agüero, 2009)).

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**Supplementary Material**

**Table S1:** Supplementary Excel File. The file contains a summary of BLASTP searches against fungal and kinetoplastid protein databases. BLAST searches using the yeast ERG3/ERG25 protein sequences as query, were run at the TriTrypDB BLAST server against a database of Kinetoplastid proteomes from reference and draft genomes. BLAST searches using a number of putative *T. cruzi* orthologs of these yeast genes were run at the SGD Fungal BLAST Server, against a database containing a selection of fungal genomes. Each BLASTP search is shown in a separate tab in the Excel workbook. File: Table-S1.xls

**Table S2:** List of nucleotide changes (SNPs, fixed differences) identified for each analyzed gene. The excel file contains one spreadsheet per gene with information on the location of each SNP relative to the start codon, the PolyPhred score for the SNP, and the character state of the SNP in each strain/lineage. File: Table-S2.xls

**Table S3:** List of oligonucleotide primers and amplification products analyzed in this study. File: Table-S3.xls

**Figure S1:** Supplementary PDF Figure. Distribution of observed SNPs in the TcSMO-like genes of *T. cruzi*. Based on the prediction of trans-membrane spanning domains (see TMHMM probability plot at the bottom), we created two alternative representations, following Sperling, Ternes, Zank et al.. The distribution of synonymous and non-synonymous SNPs is shown according to these models. The representations differ in the presence/absence of the second (non-predicted) trans-membrane domain. In these two representations the location of the 3rd histidine box always lies on the opposite side of the membrane. Both topologies may be wrong and an in-depth study (similar to the one performed by Diaz, Mansilla, Vila et al., 2002) may be required to establish the correct topology of these proteins. File: Figure-S1.pdf