Introgression of the Kinetoplast DNA: An Unusual Evolutionary Journey in *Trypanosoma cruzi*

Short title: Mitochondrial introgression in *Trypanosoma cruzi*

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ABSTRACT

Phylogenetic relationships between different lineages of *Trypanosoma cruzi*, the agent of Chagas disease, have been controversial for several years. However, recent phylogenetic and phylogenomic analyses clarified the nuclear relationships among such lineages. However, incongruence between nuclear and kinetoplast DNA phylogenies has emerged as a new challenge. This incongruence implies several events of mitochondrial introgression at evolutionary level. However, the mechanism that gave origin to introgressed lineages is unknown. Here, I will review and discuss how maxicircles of the kinetoplast were horizontally and vertically transferred between different lineages of *T. cruzi*. Finally, I will discuss what we know — and what we don't — about the kDNA transference and inheritance in the context of sexual reproduction in this parasite.

Keywords: *Trypanosoma cruzi*; DTU; Evolution; Phylogeny; Hybridization; Kinetoplast; Mitochondrial introgression;

1. KINETOPLATIDS AND SEXUAL REPRODUCTION

Kinetoplastids are a group of unicellular heterotrophic eukaryotes including several species with medical or ecological importance. Although this group has some unorthodox solutions to common problems of eukaryotic cells, the main common characteristic is the single mitochondrion with its DNA organized in a really complex network [1-3]. This network is known with the term kinetoplast and its DNA as kDNA. Kinetoplastids cluster together with diplonemids in the subphylum Glycomonada of the phylum Euglenozoa [4], one of the candidates as the most ancient branch in the eukaryotic tree of life [5-6]. Although it was assumed for several years that such clades were asexual, it is currently known that sexual reproduction (meiosis + mating) is as ancient as the eukaryotes [7-8]. However, although sex may occur it does not always happen. Even some species of kinetoplastids may have lost the ability of sexual reproduction [9]. However, in most of the species, it has been proposed that sex is not an obligate step for the organism and only occur in some situations [8]. In this regard, Tibayrenc and Ayala have proposed that parasitic kinetoplastids have a predominant clonal evolution [10-11]. They argued that genetic exchange is restrained at the population level, or at least mainly occur between genetically identical organisms (selfing or inbreeding). This model was challenged by several authors and controversy is still installed [12-15]. Despite the debate on the true impact of sexual exchange to the population structure of different kinetoplastids, there are several evidences that sex may occur (because it was observed in the laboratory) [16-17] or it has already happened (because its traces in phylogenetic and population genetic analyses) [18-19].

2. SEXUAL EXCHANGE IN *TRYPANOSOMA*

Most evidences of genetic exchange have been shown for trypanosomes. The around ninety species described in the genus *Trypanosoma* infect a wide range of vertebrates and they are transmitted by blood-sucking arthropods (insects and ticks) and leeches. Few species are implicated in human disease. Although atypical infections on humans caused by *T. vivax*, *T. congolense*, *T. evansi*, *T. lewisi*, and *T. lewisi*-like have been reported [20], *T. brucei* and *T. cruzi* are the most common causes of human trypanosomiasis (sleeping sickness and Chagas disease respectively). In this regard, because the genetic exchange is a common way to disperse virulence factors or any other undesirable medical characteristic, the sexual exchange in such trypanosomes was actively looked for in the last decades. Particularly, evidence of formation of haploid

gametes and mating has been shown for *T. brucei* and several papers had success to get recombinants (reviewed in [16]). However, the finding of sexual reproduction in *T. cruzi* was really elusive. Only one paper described the formation of a genetic hybrid [21]. However, meiosis and gametes were not observed yet. Despite, there are several phylogenetic and population genetic evidences of events of genetic exchange [22-28] and even two lineages show characteristics of a meiotic F1 [29]. However, it was reported a different phenomenon of hybridization in *T. cruzi*, the mitochondrial introgression [22, 25, 30-31]. This phenomenon consists in the observation by genetic analysis of a particular hybrid which has the mitochondrial genome (kDNA in this case) of one lineage but a nuclear background from a different one. Mechanisms for the formation of such hybrids are unknown in *Trypanosoma*. Below we describe and discuss the evidence of such hybrids and how the kDNA was transferred between different lineages.

3. *TRYPANOSOMA CRUZI* kDNA: A BRIEF DESCRIPTION OF THE TRAVELLER PROFILE

The kDNA structure is composed by several thousand of circular DNA molecules which are concatenated in a complex network (see [32] for a detailed review kDNA in kinetoplastida). There are two different DNA types in this network: maxicircles and minicircles. There is a few dozen of maxicircles in the kDNA and they code for different mitochondrial proteins. In *T. cruzi*, the maxicircles have 20 genes [33]. Particularly, nine out of them are cryptogenes because their DNA sequences are very different from their mature mRNA. Such cryptogenes are transcribed and the immature mRNA needs a complex system of edition in order to be a fully functional transcript (reviewed in [34]). The edition of mRNA is guided by short RNAs called guide RNA or gRNA and most of such gRNA are coded by minicircles. There are 20,000-30,000 minicircles in the kinetoplast of *T. cruzi* and each minicircle has four constant regions flanked by hyper-variable sequences [35]. The last ones code for gRNAs. Consequently, there are several thousands of different gRNA in a single parasite. Such gRNA can be sorted in different classes according the sequence they edit. If a class of minicircles is lost, the target mRNA cannot be edited and thus a functional protein cannot be synthetized. Consequently, it is important to correctly duplicate and segregate minicircles during the kDNA division. Basically, each minicircle is released of the network, then it is replicated and finally both are linked to new networks in antipodal sites [36]. However, the system may fail to correctly distribute minicircles between

both new kDNAs [37]. Consequently, minicircles and maxicircles are subjected to a certain degree of genetic drift. Although replication and segregation of the kDNA was broadly studied, the behaviour of such complex network in sexual reproduction is mainly unknown. In *T. brucei* hybrids, kDNA is bi-parentally inherited which probably implies fusion of the parental mitochondria [38]. However, genetic drift homogenises maxicircle sequences in few generations [38]. Consequently, inheritance is just apparently uniparental for maxicircles. In *T. cruzi*, maxicircles from hybrid DTUs TcV and TcVI are similar to maxicircles from the parental TcIII [31, 33, 39-40]. However, it was not addressed if maxicircles of TcII parental were not inherited or they were lost by homogenization as observed in *T. brucei*. In addition, minicircle inheritance was not addressed yet.

4. NUCLEUS AND KINETOPLAST DO NOT TRAVEL TOGETHER PART I: THE NUCLEAR JOURNEY

Currently, *T. cruzi* is divided into six discrete typing units (DTUs) called TcI to TcVI [41-42]. The phylogenetic relationships among them were extensively studied. There is strong evidence supporting that two DTUs (TcV and TcVI) have their origin in nuclear hybrids between TcII and TcIII [31, 39, 43-44]. Additional evidences of genetic exchange in *T. cruzi* have been inferred by detecting incongruence in the phylogeny for different genes or markers. When two genes have different evolutionary stories, it means that genetic exchange was implied. Consequently, phylogenetic incongruence between different genes or genomic regions is an indicium of genetic exchange. If incongruence is detected between nuclear and mitochondrial genes but not between different nuclear genes the term mitochondrial introgression is used. Consequently, confident phylogenies of nuclear and mitochondrial markers are required to get a confident evidence of mitochondrial introgression. The nuclear phylogeny of different DTUs was controversial for several years and different models for relationships among different DTUs are shown in Figure 1.

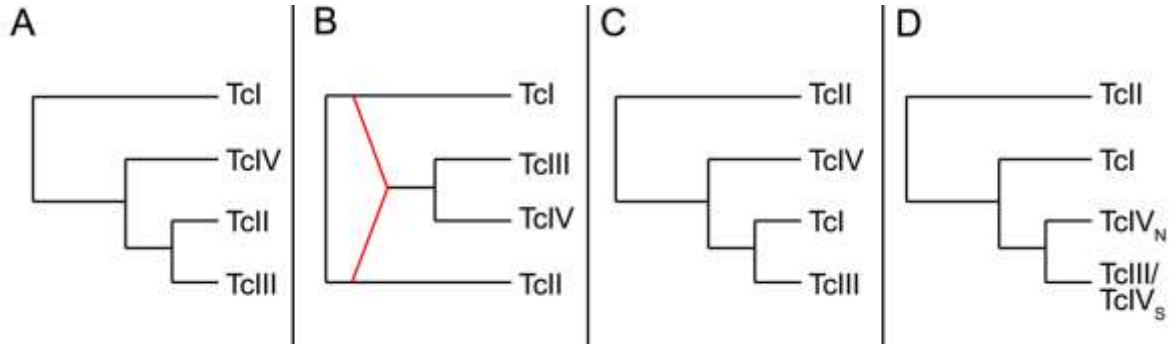

Figure 1. Different models of nuclear or kDNA relationships among DTUs TcI, TcII, TcIII and TcIV. A, the model proposes that TcIII and TcIV clusters with TcII according nuclear markers; B, the model proposes that TcIII and TcIV are hybrids between TcI and TcII according nuclear markers; C, the model proposes that TcIII and TcIV clusters with TcI according nuclear markers; D, The model proposes that TcIII and TcIV clusters with TcI, and TcIII and TcIV are not monophyletic groups according kDNA markers.

Brisse and coworkers proposed the first model for relationships of DTUs and the current division into six DTUs [45]. This first model was based on Multilocus Enzyme Electrophoresis (MLEE) and Random Amplified Polymorphic DNA (RAPD) for 49 stocks of *T. cruzi*. MLEE is based on electrophoretic patterns of enzymes codified by housekeeping genes whereas RAPD gets a random overview of the genome by using a PCR with short random primers. The model proposed two main clusters: TcI and TcII-TcIII-TcIV (this last group also included hybrids TcV and TcVI but they were not included here for simplicity). However, other papers that analysed sequence data questioned this model [39, 43, 46-47]. In a previous paper, I proposed that the clustering observed by Brisse and coworkers was biased by the inclusion of hybrid DTUs TcV and TcVI in the analysis [23]. I made a simple simulation of MLEE data based on multi-locus sequences showing that tree topology by MLEE data is strongly modified by inclusion of hybrids. Although the analysis of the simulated MLEE data without including the hybrids showed the cluster TcIII-TcI-TcIV, the inclusion of the hybrids biased the analysis and clustered instead TcIII with TcII.

The second model about DTU relationships (Figure 1B) proposed an ancient hybridization between TcI and TcII as the origin of TcIII and TcIV [46-48]. The observation of inconsistencies of sequence data against the model A supported this model. Initially, the hypothesis of the hybrid origin of TcIII and TcIV was based on the analysis of nine loci in representative strains of different DTUs [46-47]. Six out of such nine loci corresponded to histones and heat shock proteins which have multiple copies in the genome. It was observed

that certain SNPs of TcIII and TcIV were shared with TcII whereas others were shared with TcI. Consequently, the authors proposed that such sequences of TcIII and TcIV were mosaics of TcI and TcII. However, such mosaicism is only apparent because the authors did not included an outgroup in their analyses. In phylogenetic analysis, a character shared by a group of taxa is only evidence of a common ancestor if such character is not shared with an outgroup (synapomorphy). Instead, if the character is shared with the outgroup, it is probably an ancestral feature (plesiomorphy). Most of the SNPs that apparently clustered TcIII or TcIV with TcII were also shared with the outgroup (plesiomorphy) and consequently they do not support such clustering (see [23]). Figure 2 shows an example of apparent mosaicism that is solved by inclusion of an outgroup. In addition, the authors proposed that four loci showed shorter distances between TcIII/TcIV and TcII [46]. However, despite the shorter distances, including an outgroup did not support the clustering of TcIII/TcIV with TcII [23].

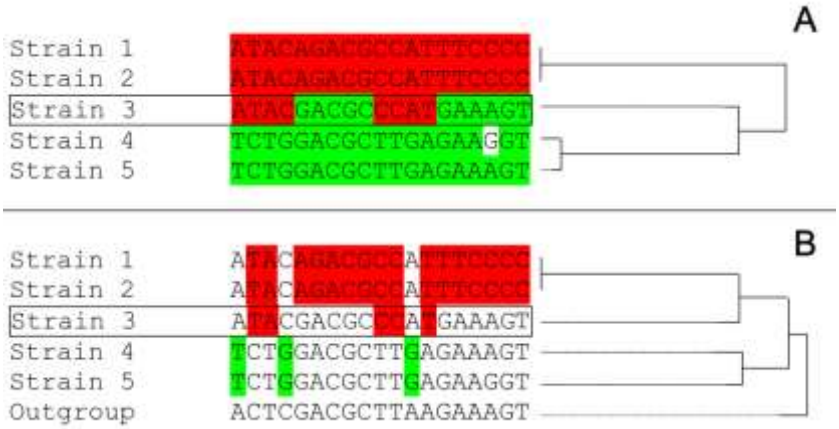

Figure 2. Example of a false mosaic sequence caused by plesiomorphies. A, alignment of sequences with a potential recombinant sequence (Strain 3). Note that the midpoint-rooted Neighbour Joining tree cluster such sequence with strains 4 and 5 (distance from strain 3 to strains 4 and 5 is shorter than distance to strains 1 and 2). B, the same alignment than in A but including an outgroup strain. Note that only sinapomorphies are highlighted and there is no SNP supporting the clustering of strain 3 with strains 4 and 5. Instead, there are five SNPs supporting the clustering of Strain 3 with strains 1 and 2. In addition, a rooted Neighbour Joining tree (right) show the clustering of Strain 3 with strains 1 and 2.

Recent papers also used more sophisticated methods of analysis of mosaicism such as BOOTSCAN [49] to demonstrate recombination [50-51]. However, I observed serious methodological concerns. For example,

149 Franco and co-workers [50] showed a mosaic sequence in the gene for the ABCG-like transporter for a TcIII
150 strain with TcI and TcII as putative parentals. However, the authors used the "close relative" option in RDP
151 software which is only recommended when putative parentals are more similar between them than with the
152 recombinant (it is not the case here). In addition, they did not inform *p* values for each potential
153 recombination event (bootstrap value is only for detection of potential recombinant regions, a binomial test
154 should be used to evaluate significance in order to avoid false positives according [49]). I have repeated the
155 analysis with their sequences in the same conditions used by the authors and no statistically significant events
156 were detected using the binomial test with or without a Bonferroni correction. Similar concerns are detected
157 in [51]. Other papers that support the ancient hybridization between TcI and TcII were based on networks
158 using the neighbour-net analysis and showing reticulate patterns [52-53]. However, reticulate pattern in
159 neighbour-net only shows character inconsistency which may be caused by different phenomena (i.e.
160 homoplasy, paralogy, etc) instead of recombination [54]. In addition, supported splits of phylogenetic
161 networks shown in such papers are fully compatible with the third model described below (See figure 2 in
162 [53] supporting the TcI-TcIII cluster according the 195 bp satellite sequences with high bootstrap support).
163 Moreover, the analysis of the CL-Brener genome, a representative strain of the hybrid TcVI revealed very few
164 sequences (less than 1% of the core genome) as candidates for mosaicism in the TcIII-derived counterpart of
165 the genome [55]. Other papers presented data proposing that sequences similar to TcI in the genome of CL-
166 Brener are evidence of the ancient hybrid origin of TcIII [56-57]. However, such data are not conclusive
167 because they are also compatible with the third model that proposes TcI and TcIII are closely related. This
168 relatedness between TcI and TcIII may explain the similarity between TcI and CL-Brener sequences.

169 The third model proposes that the *T. cruzi* ancestor diverged into two main groups (TcII and TcI-TcIII-TcIV).
170 Posteriorly, TcI-TcIII-TcIV was divided into two groups TcI-TcIII and TcIV. Finally, TcIV diverged into
171 $TcIV_S$ (from South America) and $TcIV_N$ (from North America) nearly at the same time that TcI and TcIII
172 diverged into two different DTUs. The model is supported by the analysis of nuclear sequences from 13
173 single-copy housekeeping genes in 18 strains [23, 58]. In addition, the same phylogeny was also supported by
174 analysing sequences for thirty-two protein coding regions in seven strains [43]. Other papers analysing few
175 loci also get evidence of this model [31, 59].

Finally, although a single hybridization event was proposed for the origin of TcV and TcVI [40] as the most parsimonious hypothesis, more recent papers support two independent hybridization events [23, 31].

5. NUCLEUS AND KINETOPLAST DO NOT TRAVEL TOGETHER PART II: THE kDNA JOURNEY

Phylogenetic relationships of maxicircle sequences of different DTUs were not as controversial as the nuclear phylogeny. Basically, three main clades were observed (TcI, TcII and TcIII-TcIV) which are incongruent with nuclear clustering (Fig 1D) and constitutes evidence of mitochondrial introgression. Machado and Ayala were the first to describe three different kDNA clades based on the sequence of two maxicircle genes (NADH dehydrogenase subunit 1 and Cytochrome Oxidase subunit II) [39]. The three kDNA clades were also observed with an additional maxicircle gene (Cytochrome b) and in a more extensive number of strains [40].

In addition, a phylogenomic analysis of the entire maxicircle sequences of Sylvio x10 (TcI), Esmeraldo (TcII), CL-brener (TcVI which inherited maxicircles only from TcIII) corroborated that clades TcI and TcIII-TcIV are joined in a major clade [33]. More recent papers showed that TcIII-TcIV may be divided into two main clades $TcIV_N$ and $TcIV_S$-TcIII showing that both DTUs are not monophyletic for kDNA [23, 25, 31, 60].

Joining nuclear and maxicircle phylogenies gives information about kDNA transfer among different DTUs (Figure 3). An ancient separation between TcII and TcI-TcIII-TcIV is supported by both nuclear and maxicircle genes. Posteriorly, TcIV separated from TcI-TcIII according to nuclear data. Finally, TcIV separated into $TcIV_N$ and $TcIV_S$ whereas TcI-TcIII diverged in the current DTUs. Incongruence between nuclear and maxicircle sequences may be explained by kDNA transfer from one DTU to another one. Consequently, two alternative hypotheses are possible according the direction of the kDNA transfer: from TcIII to TcIVs or from TcIVs to TcIII. Considering that TcI-TcIII and $TcIV_S$-$TcIV_N$ are monophyletic clades, if TcIII transferred its kDNA to $TcIV_S$ it would be expected that TcIII-$TcIV_S$ clustered with TcI in the kDNA phylogeny. Instead, TcIII-$TcIV_S$ clusters with $TcIV_N$ suggesting the alternative way: $TcIV_S$ transferred its kDNA to TcIII. In a recent paper we proposed that such transference should have occurred several times in the history of TcIII (at least three times always from $TcIV_S$ to TcIII) [23]). Finally, TcIII transferred this TcIVs kDNA to hybrids TcV and TcVI in two independent hybridization events [23]. Finally, these

evolutionary events of mitochondrial introgression are also supported by the observation of several recent events between TcIV and TcI [22, 25, 30].

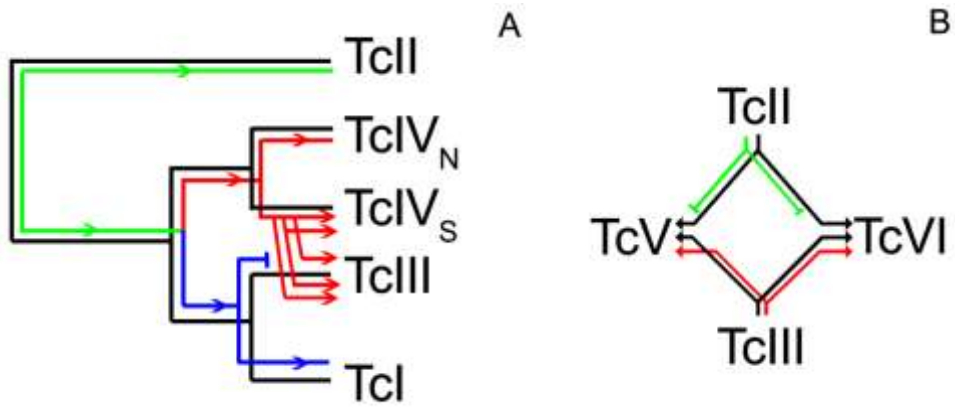

Figure 3. kDNA (maxicircle) journey in the evolution of different DTUs of *Trypanosoma cruzi*. Nuclear (black lines) and maxicircle (coloured lines) phylogenies are overlapped. Different colours in kDNA phylogeny represent the three different major kDNA clades, and arrows represent how kDNA is transmitted. **A**, nuclear and maxicircle phylogenies are mainly congruent with the exception of TcIII clustering with TcIVs (kDNA phylogeny) instead of TcI (nuclear phylogeny). This incongruence is explained by the transference of the kDNA from $TcIV_S$ to TcIII. In addition, note that TcIII and $TcIV_S$ are not monophyletic clades according to kDNA which may be explained by several events of introgression (at least three events, see [23]). Note that the ancestral TcIII kDNA was lost (interrupted blue line). **B**, schematic network showing two independent TcII/TcIII hybridizations. Note that TcII and TcIII contribute with their nuclear genomes to hybrids but maxicircles are only from TcIII. Maxicircles from TcII (green lines) were not transferred or they were lost by drift after the hybridization.

6. DO WE KNOW THE MECHANISMS OF HYBRIDIZATION?

Gaunt and coworkers proposed a mechanism of genetic exchange in *T. cruzi* several years ago [21]. The model proposes the formation of a tetraploid hybrid and posterior chromosome loss to return to a state near to the diploidy. This model was based on the observation of a tetraploid hybrid in mammal cell cultures infected with two different strains of *T. cruzi*. However, if a random loss of chromosomes (or genes) occurs to return diploidy, it is expected that some genes will lost both copies of the same parental. Consequently, a homozygous state would be expected just by random in 1/3 of the genome [29]. However, this expectancy is far away from the observed heterozygosis in the genomic data of CL-Brener strain and other strains from hybrid DTUs [58, 61]. These DTUs resembles a typical F1 after meiosis. Although meiosis and gametes were not yet observed for *T. cruzi*, both are not unlikely because they were observed in experimental crosses in the

229 relative *T. brucei*. In addition, the genome of *T. cruzi* has conserved the whole machinery for homologous
230 recombination required in meiosis [44].

231 The mechanism of mitochondrial introgression is also unknown and several questions arise from the
232 observation of such phenomenon. The first question is how kDNA is introgressed. There are two possible
233 mechanisms for introgression: successive backcrosses or mitochondrial exchange. The first is the mechanism
234 observed in superior organisms. As a hypothetical example of the introgression of kDNA from TcIVs to
235 TcIII, the first step would be the occurrence of a meiotic hybrid TcIII/$TcIV_S$. This hypothetical hybrid
236 inherited the kDNA from $TcIV_S$. Posteriorly, successive backcrosses with TcIII would have reduced the
237 proportion of $TcIV_S$ nuclear genome on the hybrids although $TcIV_S$ kDNA was maintained. It is important to
238 note that the proportion of $TcIV_S$ nuclear genome in the hybrid would be in average 0.5^n where n is the
239 number of backcrosses. Consequently, only 10 backcrosses of the TcIII-$TcIV_S$ hybrids with TcIII will reduce
240 the proportion of $TcIV_S$ genome to less than 0.1%. The main drawback of this hypothesis about the
241 mechanism of introgression is that it requires relatively frequent events of meiotic sexual exchange in the past
242 and this was not detected in *T. cruzi*. The alternative method, mitochondrial exchange is only hypothetical and
243 it simply consists in the exchange of mitochondria or their kDNA between parasites. It is based on the
244 observation mitochondrial transfer between mammal cells [62]. This phenomenon is able to rescue deleterious
245 mitochondrial genotypes in some cells if they are surrounded by cells with normal genotypes. Here is
246 important to note that the mitochondrion of the *T. cruzi* is candidate to suffer the Muller's ratchet because of
247 the asexual mode of reproduction of this organelle [63]. The Muller's ratchet hypothesis proposes that an
248 asexual population will undoubtedly extinct because the accumulation of deleterious mutation (sexual
249 reproduction allows escaping the ratchet). Although multiple copies of maxicircles and minicircles may help
250 to avoid the Muller's ratchet [64], it may be not enough and mitochondrial or kDNA exchange may help to
251 the parasite to avoid the ratchet.

252 The second question is related to the asymmetrical transference of the kDNA. Why the kDNA was transferred
253 in the same direction several times (from $TcIV_S$ to TcIII) and why both hybrid DTUs TcV and TcVI inherited
254 such kDNA in independent hybridizations? Is there an evolutionary advantage in the kDNA of $TcIV_S$? In a
255 previous paper I proposed that such asymmetrical introggression from $TcIV_S$ to TcIII may also be explained

by neutral demographic models (i.e. selective advantage is not implied). The model proposes that when a species invades an area already occupied by a related species, asymmetrical introgression may occur mainly from the local species towards the invader [65]. Such asymmetrical mitochondrial introgression was observed for several animal and plant species [65] and even in algae [66]. However, the major drawback of such a model is the requirement of frequent genetic exchange between $TcIV_S$ and TcIII at least in the front of the expansion wave.

The third question about introgression is related to inheritance of the kDNA, because biparental inheritance cannot be discarded. In this regard, although there is evidence that TcIII received maxicircle sequences from $TcIV_S$, it is not clear if whole minicircle sequences in TcIII came also from TcIVs.

CONCLUSION / FUTURE PERSPECTIVES

Mitochondrial introgression (at least maxicircle introgression) has occurred in the evolutionary history of *T. cruzi*. The transference of kDNA between different DTUs is shown in Figure 3. However, the mechanism and biological importance of such transference is completely unknown. Understanding the inheritance of minicircles in the hybrid DTUs (is it uniparental or biparental?) will help to understand if mitochondrion fusion is possible. The main problem in the analysis of minicircle phylogeny is the high variability and the high number of copies which makes it difficult to address the question with conventional tools. However, next generation sequencing methods may help in order to get data about populations of minicircles in strains of different DTUs. Finally, looking for other mechanisms of nuclear genetic exchange than the previously observed in the laboratory is still relevant to understand the mechanism of introgression and to explain why TcV and TcVI are mainly heterozygous.

CONFLICT OF INTEREST

The author declares no conflict of interest. This work has been supported by PICT-2014-2449 ANPCyT.

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