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Origin and distribution of AT-rich repetitive DNA families in *Triatoma infestans* (Heteroptera)

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ABSTRACT

Triatoma infestans, one of the most important vectors of *Trypanosoma cruzi*, is very interesting model, because it shows large interpopulation variation in the amount and distribution of heterochromatin. This polymorphism involved the three large pairs up to almost all autosomal pairs, including the sex chromosomes. To understand the dynamics of heterochromatin variation in *T. infestans*, we isolated the AT-rich satDNA portion of this insect using reassociation kinetics (C_0t), followed by cloning, sequencing and FISH. After chromosome localization, immunolabeling with anti-5-methylcytosine, anti-H4K5ac and anti-H3K9me2 antibodies was performed to determine the functional characteristics of heterochromatin. The results allowed us to reorganize the karyotype of *T. infestans* in accordance with the distribution of the families of repetitive DNA using seven different markers. We found that two arrays with lengths of 79 and 33 bp have a strong relationship with transposable element sequences, suggesting that these two families of satDNA probably originated from Polintons. The results also allowed us to identify at least four chromosome rearrangements involved in the amplification/dispersion of AT-rich satDNA of *T. infestans*. These data should be very useful in new studies including those examining the cytogenomic and population aspects of this very important species of insect.

1. Introduction

Heterochromatin is known as a nuclear fraction with high methylation/condensation state, low functional activity, late replication and lower susceptibility to recombination events. These characteristics are common in regions rich of repetitive DNA families, such as satDNA, and they can vary between species in size, composition and amount of motifs (Schmidt and Heslop-Harrison, 1998; Subirana and Messeguer, 2013). This diversity of heterochromatic regions can be exemplified by proximal chromosome regions of some coleopterans, whose composition of repetitive DNA families depends on the species or group of species. *Palorus subdepressus*, for instance, shows an array with a length of 72 bp, tandemly repeated in the pericentromeric region, whose sequence is also found in other coleopterans, such as *Chysolina americana* (Plohl et al., 1998; Lorite et al., 2001). In Aphididae, a family of Hemiptera with holokinetic chromosomes, three families of repetitive DNA were found: (i) an array of 3.000 bp length associated with a terminal GC-rich region in the X chromosome, (ii) a 200 bp length sequence associated with an AT-rich region of X chromosome, and (iii) a satDNA obtained after *Hae*III cleavage associated with AT-rich regions of autosomes (Mandrioli et al., 1999).

The traditional methods of chromosome banding (C-Giemsa and CMA_3/DAPI) have been useful for revealing the location, size and AT- or GC-rich composition of heterochromatic regions in the chromosomes and nucleus, and also for producing markers to compare karyotypes of different species and/or groups of insects (Schneider et al., 2006; Bressa et al., 2008; Cabral-de-Mello et al., 2010; Panzera et al., 2010; Bardella et al., 2014). Especially for Triatominae, chromosome banding allows us to identify intra- and interspecific variations in the amount and distribution of

Abbreviations: TRF, tandem repeats finder; TIRs, Terminal Inverted Repeats; SSC, saline-sodium citrate; SDS, sodium dodecyl sulfate; FITC, fluorescein isothiocyanate; DABCO, 1,4-diazabicyclo[2.2.2]octane; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TRITC, tetramethylrhodamine-5-(and-6-)isothiocyanate; FISH, fluorescence in situ hybridization; HCl, hydrochloric acid; CMA_3, chromomycin A_3; DAPI, 4′-6-diamidino-2-phenylindole; Tris, tris(hydroxymethyl)aminomethane hydrochloride; $MgCl_2$, magnesium chloride; NCBI, National Center of Biotechnology Information.

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heterochromatin (Panzera et al., 1992, 1995). An example is the intraspecific polymorphism of chromosome band patterns between natural populations of *Triatoma infestans* (Panzera et al., 1992, 1995). Panzera et al. (2004) suggested that Andean population of *T. infestans* exhibit heterochromatin in 14–20 chromosomes, and about 1.825 ± 0.49 pg per haploid nucleus. However, non-Andean populations seems to be from four to seven chromosomes carrying bands and a *C*-value with about 1.401 ± 0.111 pg. These data suggested that polymorphisms were associated with migration and adaptation of genomes during the occupation of this species in new environments in South America. Karyotype changes were also observed in the distribution of 18S rDNA in distinct population of *T. infestans*, whose hybridization signals can occur in autosomes or alosomes (Panzera et al., 2012).

Triatoma infestans has holokinetic chromosomes and $2n = 20 + XY$, like the majority of species of Triatominae (Ueshima, 1979; Panzera et al., 1996). Karyotypes of *T. infestans* are asymmetrically organized into three large pairs and the remainder smaller pairs, with the Y chromosome always larger than X in the non-Andean group (Pérez et al., 2000), because the X not have C-heterochromatin. But in Andean group, both sex chromosomes are similar in size and both of them have C-heterochromatin (Panzera et al., 2004). This species is also particularly intriguing because it belongs to a hematophagous insect group that is important to public health in Latin America, because they are the main vectors of the parasite *Trypanosoma cruzi*, responsible for Chagas disease. According to the World Health Organization (http://www.who.int/topics/chagas_disease/en/), approximately 10 million people were infected with *T. cruzi* in 2012.

To understand the origin and variation of heterochromatic regions in *T. infestans*, we used C_0t DNA reassociation to isolate and to produce a plasmid microlibrary with short DNA fragments, followed by cloning and SANGER sequencing. This strategy was useful in selecting different repetitive DNA fragments for physical mapping. The main goal of this study was to determine the mechanisms that account for the origin, distribution and diversity of AT-rich heterochromatin that occupy predominantly the terminal chromosome regions.

2. Materials and methods

2.1. Biological material

Ten males and eight females of *T. infestans* from Andean regions of Peru, South America, were obtained from the insectarium of the Faculty of Pharmaceutical Sciences, Department of Biological Sciences, UNESP, Araraquara, Brazil. The insects were obtained from a colony that was founded from seven nymphs in August 1983. Samples were used for the removal seminiferous tubules and DNA extraction. Gonads were dissected out and the tubules were directly fixed in a freshly prepared solution of methanol:acetic acid (3:1, v:v) and then stored at −20 °C. Genomic DNA was extracted from the leg muscles of males and females using the DNeasy Blood & Tissue Kit (Qiagen).

2.2. Obtaining the C_0t fraction and microlibrary construction

Repetitive DNA was isolated using renaturation kinetics (Zwick et al., 1997), with modifications, using a pool of males and females of *T. infestans*. About 10 μg DNA dissolved in 0.3 M NaCl were autoclaved at 1.4 atm (120 °C) for 15 min, and the size of fragments was checked by electrophoresis in 1% agarose. The sample was denatured at 95 °C for 10 min, placed on ice for 10 s and transferred to a water bath at 65 °C for 10 min. The sample was then incubated at 37 °C for 8 min with S1 nuclease and the reaction stopped by the addition of liquid nitrogen. DNA was purified using phenol–chloroform (1:1, v:v), and subsequently cloned using the Blunt-Ended PCR Cloning Kit (GE Healthcare Life Sciences), with *Escherichia coli* TOP10 competent cells. Six hundred fifty-three clones were used in a pre-selection by PCR with the primers M13 F (5′-GTAAAACGACG GCCAG-3′) and M13 R (5′-CAGGAAACAGCTATGAC-3′) to obtain fragments larger than 200 bp.

Inserts were sequenced on a 3500 × L automatic sequencer (Applied Biosystem), according to the manufacturer's procedures. The quality of sequences was tested with Phred-PhrapConsed software. After the identification and removal of parts of the vectors using Vector Screen (NCBI), the consensus sequences were contrasted against the NCBI (http://www.ncbi.nlm.nih.gov/blast), Flybase (http://flybase.org) and RepeatMasker (http://www.girinst.org/censor/index.php) gene banks. To get repetitive stretches, the sequences were tested with RepFind (http://cagt.bu.edu/page/REPFIND_submit) and Tandem Repeats Finder (TRF) (http://tandem.bu.edu/trf/trf.html) software.

2.3. Cytogenetics

Seminiferous tubules were incubated in 60% acetic acid for 10 min, and then minced and squashed. Coverslips were removed by freezing in liquid nitrogen. For chromosome banding (Sumner, 1982, with small modifications) all the seminiferous tubules of six males were used. Slides were aged at room temperature and also incubated in 0.2 M HCl for 10 min at room temperature, 5% barium hydroxide for 2 min at 60 °C and 2 × SSC, pH 7.0, for 60 min at 60 °C. The samples were dehydrated in an alcohol series, air dried and stained with fluorochromes: 0.5 mg/mL chromomycin A_3 (CMA_3) for 1.5 h and 2 μg/mL 4'-6-diamidino-2-phenylindole (DAPI) for 30 min. Slides were mounted with a medium composed of glycerol/McIlvaine buffer (pH 7.0) 1:1, plus 2.5 mM $MgCl_2$. After image acquisition, slides were treated with ethanol:acetic acid (3:1, v:v) for 15 min and stained with 2% Giemsa.

For FISH, the slides were prepared as previously described, and probes were labeled with biotin or digoxigenin using nick translation (Invitrogen). 18S rDNA, which was isolated from *Antiteuchus tripterus* (Bardella et al., 2013) was also labeled by nick translation, and used as control for FISH. Preparations were treated with a mixture composed of 100% formamide (15 μL), 50% polyethylene glycol (6 μL), 20 × SSC (3 μL), 100 ng sonicated calf thymus DNA (1 μL), 10% SDS (1 μL), and 100 ng probe (4 μL each). This mixture was denatured at 70 °C for 10 min, immediately chilled on ice for 5 min, and added to the slide. Chromosome denaturation/hybridization was done at 90 °C for 10 min, 48 °C for 10 min, and 38 °C for 5 min, using a thermal cycler (MJ Research, Inc., USA). Samples were incubated in a humidified chamber at 37 °C overnight. Post-hybridization washes were carried out in SSC, with 80% stringency. Probes were detected with avidin-FITC or anti-digoxigenin-rhodamine. The post-detection washes were performed in 4 × SSC/0.2% Tween 20, all at room temperature. Slides were mounted with 25 μL of a solution composed of glycerol (90%), DABCO (2.3%), 20 mM Tris–HCl, pH 8.0 (2%), 2.5 mM $MgCl_2$ (4%), and distilled water (1.7%), plus 1 μL of 2 μg/mL DAPI.

2.4. Immunolabeling

For detection of hypermethylated chromatin, seminiferous tubules were fixed in methanol–acetic acid (3:1, v:v), washed in 1 × PBS buffer, dissected in a drop of 60% acetic acid and squashed. After removing the coverslips by freezing, slides were placed in 1 × PBS for 5 min. The material was blocked in a solution of 3% BSA in 1 × PBS plus 0.2% Tween 20 (w/v) at room temperature for 10 min. Afterwards, samples were incubated with a 1:100