Revisiting the Homoploid Hybrid Speciation Process of the *Triatoma brasiliensis macromelasoma* Galvão, 1956 (Hemiptera, Triatominae) Using Cytogenetic and Molecular Markers

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Abstract. Triatomines are vectors of *Trypanosoma cruzi*, the etiologic agent of Chagas disease. Although the evolutionary process in triatomine is considered as disruptive, cryptic speciation and homoploid hybridization also are possible modes of speciation. Several analyses suggested *Triatoma brasiliensis macromelasoma* as a product of hybridization between *T. brasiliensis* and *Triatoma juazeirensis*. Thus, we analyzed genetic characteristics (chromosomal analysis, genetic distance for the mitochondrial ND1 gene, and the pattern of bands of internal transcribed spacer [ITS]-1) of these species, with emphasis on the phenomenon of homoploid hybridization. All species showed the same cytogenetic characteristics, low genetic distance for ND1 gene, and the same pattern of ITS-1 bands. We consider that these genetic characteristics, together with the large chromatic polymorphism and the viability of experimental crosses possibly are due to the processes of introgression that these species suffered during the process of homoploid hybridization.

Triatomines are hematophagous insect vectors of protozoan *Trypanosoma cruzi* (Chagas, 1909), the etiologic agent of Chagas disease.¹ This disease is an important public health problem not only in Latin America, where it is endemic in 21 countries, but it is increasingly spreading to other areas such as Europe, North America, Japan, and Australia mainly because of migration.² Around 6–7 million people are affected worldwide and approximately 7,000 deaths occur annually, making Chagas disease the major cause of death from a parasitic disease in Latin America and a significant contributor to the global burden of cardiovascular disease, with Chagas disease being the main cause of infectious cardiomyopathy in the world.^{1,3}

Currently, there are 152 species of triatomines, distributed in 18 genera and five tribes, all species being considered as a potential vector of Chagas disease.^{4,5} Although the evolutionary process in triatomine is considered as disruptive (occurs when extreme phenotypes have a fitness advantage over more intermediate phenotypes) by Dujardin et al.,⁶ cryptic speciation (morphologically identical populations that are reproductively isolated and successfully differentiated by molecular data) has been detected for some species (for example, *Triatoma sordida* [Stål, 1859]⁷ and *Triatoma dimidiata* [Latreille, 1811]⁸), and Costa et al.⁹ also suggested homoploid hybridization (derived species by hybridization without a change in chromosome number) as a possible mode of speciation in Triatominae. By means of morphometrics,⁹ morphological,⁹ ecological,¹⁰ geographic,¹⁰ isoenzymes,¹¹ cross-experimental,¹² and molecular analyses,¹³ it was suggested that *Triatoma brasiliensis macromelasoma* Galvão, 1956 could be a product of hybridization between *Triatoma brasiliensis* Neiva, 1911 and *Triatoma juazeirensis* Costa and Félix, 2007.

Recently, Costa et al.¹⁴ observed intermediate chromatic phenotypes for *T. b. macromelasoma* in various locations in

areas between the *T. brasiliensis* and *T. juazeirensis* occurrences and suggested that these data, combined with molecular analysis and experimental crosses, support the distinction of a zone of hybridization (Pernambuco state, Brazil) that gave rise to the *T. b. macromelasoma* through homoploid evolution. Thus, considering that the knowledge of more details of *T. b. macromelasoma*, *T. brasiliensis*, and *T. juazeirensis* will allow to evaluate the processes related to this important model of speciation, we analyzed genetic characteristics (chromosomal analysis, genetic distance for the mitochondrial ND1 gene, and the pattern of bands of internal transcribed spacer [ITS]-1) of these species, with emphasis on the phenomenon of homoploid hybridization.

Ten adult males of each species (*T. b. macromelasoma*, *T. brasiliensis*, and *T. juazeirensis*) were used for cytogenetics and molecular analysis (as out-group for molecular analysis we used *Triatoma petrocchia* Pinto and Barreto, 1925, the last species grouped in the *T. brasiliensis* complex¹⁵). The specimens were provided by insectarium of the FCFAR/UNESP, Araraquara, São Paulo, Brazil. The molecular cytogenetic technique CMA₃/DAPI¹⁶ (characterization of DNA rich in AT and CG in chromosomes) was performed using an Olympus BX-FLA fluorescence microscope.

For the analysis of the genetic distance between *T. b. macromelasoma*, *T. brasiliensis*, and *T. juazeirensis*, the forward and reverse primers for the ND1 gene were constructed based on the sequence of the complete mitochondrial genome of *T. dimidiata*,¹⁷ with the assistance of BioEdit 7.0.5 software, used to perform the alignments of sequences. Primer3 and ITD software were used for the construction and verification of the quality and integrity of the generated primers. The reverse primer was degenerate to encompass a greater number of species. The desired sequence was amplified using the following primers: *Forward*: 5'-TCTGAT TCACCCTCAGCAAA-3' and *Reverse*: 5'-GGAGCGTARG GTKTTTRRGTTAT-3'. The conditions for the amplification reaction of this gene were as follows: an initial denaturation at 95°C for 2 minutes, followed by 40 cycles comprising denaturation (95°C, 30 seconds), annealing (61°C, 30 seconds), and extension (72°C, 1.5 minutes), finishing by a final

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TABLE 1

Cytogenetic characteristics of *Triatoma brasiliensis macromelasoma*, *Triatoma brasiliensis*, and *Triatoma juazeirensis*

	Karyotype	Prophase	Heterochromatin	CMA ₃ /DAPI
<i>T. brasiliensis</i>	2n = 22 (20A + XY) ¹⁹	XY + 2A ¹⁹	Y and both ends of all A ¹⁹	X both ends of all A (CMA ⁺); Y (DAPI ⁺) ²⁰
<i>T. juazeirensis</i>	2n = 22 (20A + XY) ¹⁹	XY + 2A ¹⁹	Y and both ends of all A ¹⁹	X both ends of all A (CMA ⁺); Y (DAPI ⁺)
<i>T. b. macromelasoma</i>	2n = 22 (20A + XY) ¹⁹	XY + 2A ¹⁹	Y and both ends of all A ¹⁹	X both ends of all A (CMA ⁺); Y (DAPI ⁺)

A = autosomes; X = X sex chromosome; Y = Y sex chromosome.

extension at 72°C for 5 minutes. After electrophoresis, the amplified fragments were purified using the GFX PCR DNA & Gel Band Kit (GE Healthcare and Life Technology), according to the manufacturer's instructions. The purified polymerase chain reaction (PCR) products were subjected to direct sequencing, so the samples were sent to the Research Center on the Human Genome and Stem Cells, USP/São Paulo, Brazil. The sequences of all individuals were analyzed by using BioEdit software v.7.0.5 and a consensus sequence was obtained for each DNA segment. The sequences were aligned using the ClustalW tool. Subsequently, we used the MEGA v.6 software for constructing a phylogenetic tree using the maximum likelihood method as a distance criterion and the nucleotide substitution model Hasegawa-Kishino-Yano + Gamma 0.006. Bootstrap resampling was applied to assess support for the individual nodes (1,000 repetitions). The genetic distance matrix was also constructed through the MEGA v.6 using the Tamura-Nei model for estimation of evolutionary divergence between sequences.

For the characterization of the ITS-1 band pattern, the genomic DNA was extracted and the PCR for the amplification of the ribosomal DNA ITS-1 intergenic spacer was performed, based on the oligonucleotide primers described by Tartarotti and Ceron¹⁸ (Forward: 5'-CGACGCAAGAAGTAGCTG-3' and Reverse: 5'-CGTAACAAGGTTTCCGTAGG-3'). After amplification, the fragments were separated by electrophoresis in 1% agarose gel at a constant voltage of 90 V for approximately 1:30 hours, and then the band pattern of the species was analyzed.

All species analyzed showed the same cytogenetic characteristics (Table 1). This homogeneity observed in the chromosomal characteristics of *T. b. macromelasoma*, *T. brasiliensis*, and *T. juazeirensis* is an essential phenomenon for the homoploid hybridization, because they are an evolutionarily close species that present distinct chromosomal patterns,⁷ and the number of chromosomes, for example, is a factor that prevents the formation of hybrids in triatomines (personal communication).

The ND1 gene showed low genetic distance between *T. b. macromelasoma*, *T. brasiliensis*, and *T. juazeirensis*, i.e., 0.006 for *T. b. macromelasoma* and *T. brasiliensis*, 0.006 for *T. juazeirensis* and *T. brasiliensis*, and 0.002 for *T. b. macromelasoma* and *T. juazeirensis*. This mitochondrial gene belonging to the family of NADH dehydrogenase 1 (with an important role in oxidative phosphorylation) is the most conserved among the seven NADH dehydrogenase-coding genes and usually fits well for studies of closely related species.²¹ However, the genetic distance observed for the three species analyzed was extremely low when compared with the outgroup (0.085 for *T. petrocchia* and *T. brasiliensis*, 0.079 for *T. petrocchia* and *T. b. macromelasoma*, and 0.079 also for *T. petrocchia* and *T. juazeirensis*). Furthermore, although the ITS-1 band patterns made it possible to differentiate triatomine species of the genus *Triatoma*, *Rhodnius* e *Panstrongylus*,¹⁸

because the ITSs (ITS-1 and ITS-2) are important tools for demonstrating associations between closely related taxa that have diverged less than 50 million years ago,²² *T. b. macromelasoma*, *T. brasiliensis* and *T. juazeirensis* also presented the same pattern of ITS-1 bands (800 pb) and differed only from *T. petrocchia* (700 pb) (Figure 1).

The genotypic characteristics analyzed could suggest that *T. b. macromelasoma*, *T. brasiliensis* and *T. juazeirensis* are the same species, as already occurred for other triatomines. However, we consider that the chromosome homogeneity, low genetic distance, and absence of polymorphism in the ITS-1 bands, together with the large chromatic polymorphism¹⁴ and the viability of experimental crosses,¹² possibly are because of the processes of introgression that these species suffered during the process of homoploid hybridization.

FIGURE 1. Electrophoretic band patterns of internal transcribed spacer-1 for 1) *Triatoma brasiliensis* (800 pb), 2) *Triatoma brasiliensis macromelasoma* (800 pb), 3) *Triatoma juazeirensis* (800 pb) and 4) *Triatoma petrocchia* (700 pb).

Thus, we offer new genetic evidence on the relationship of *T. b. macromelasoma*, *T. brasiliensis*, and *T. juazeirensis* with the homoploid hybridization process. In addition, we suggest that complementary studies, such as analysis of transposable elements and characterization of the genome or transcriptome of *T. b. macromelasoma*, *T. brasiliensis*, and *T. juazeirensis* and their experimental hybrids may contribute with new information about this important phenomenon of speciation present in Triatominae.

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