D2 Region of the 28S RNA Gene: A Too-Conserved Fragment for Inferences on Phylogeny of South American Triatomines

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Abstract. The *brasiliensis* complex is composed of five triatomine species, and different approaches suggest that *Triatoma lenti* and *Triatoma petrochiae* may be the new members. Therefore, this study sought to analyze the phylogenetic relationships within this complex by means of the D2 region of the 28S RNA gene, and to analyze the degree of polymorphism and phylogenetic significance of this gene for South American triatomines. Phylogenetic analysis by using sequence fragments of the D2 domain did not allow to perform phylogenetic inferences on species within the *brasiliensis* complex, because the gene alignment composed of a matrix with 37 specimens exhibited only two variable sites along the 567 base pairs used. Furthermore, if all South American species are included, only four variable sites were detected, reflecting the high degree of gene conservation. Therefore, we do not recommend the use of this gene for phylogenetic reconstruction for this group of Chagas disease vectors.

Triatomines are bloodsucking insects of great importance to public health because they transmit the protozoan *Trypanosoma cruzi*, the etiologic agent of Chagas disease. Since 1966, triatomines have been grouped into complexes and specific subcomplexes.^{1,2} Although the use of complexes and subcomplexes do not follow the International Code of Zoological Nomenclature, there is a consensus that these groupings should reflect the phylogeny.³

Using the morphological characteristics and geographical distribution mainly, Schofield and Galvão² grouped most South American triatomine species within the *infestans* complex. This complex comprises the *brasiliensis*, *infestans*, *maculata*, *matogrossensis*, *rubrovaria*, and *sordida* subcomplexes.

With the exception of *Triatoma melanocephala*, *Triatoma vitticeps*, and *Triatoma tibiamaculata*, which exhibit fragmentation of the sex chromosome X, all *infestans* complex species have 22 chromosomes.^{4–7} Using mitochondrial genes (*16S*, *COI*, *COII*, and *Cytb*) and nuclear genes (*18S* and *28S*), Justi and others³ analyzed the phylogenetic relationships of 64 species within the Triatominae subfamily and suggested that *infestans* and *rubrovaria* subcomplexes are monophyletic. The nonmonophyly of the *brasiliensis* subcomplex was then evidenced because *T. melanocephala*, *T. vitticeps*, and *T. tibiamaculata* were clustered separately, and they were then excluded via cytogenetic analysis.^{5,6} With these exclusions, the *brasiliensis* complex is indeed a monophyletic group composed of *Triatoma brasiliensis*, *Triatoma juazeirensis*, *Triatoma melanica*, and *Triatoma sherlocki*, and subspecies *Triatoma brasiliensis macromelasoma*.^{8–10} However, it is important to note that *Triatoma lenti* and *Triatoma petrochiae* were not used in the phylogenetic analyses mentioned previously, possibly due to their rarity. Cytogenetic data,^{6,11} experimental laboratory crossings,¹² morphological analysis,² and geographic information² have also suggested that *T. lenti* may be a member of the complex. *Triatoma*

petrochiae has cytogenetic characteristics similar to those of *brasiliensis* complex.⁶

The nuclear D2 region of the 28S RNA gene is frequently used in phylogenetic studies in the tribe Rhodniini,^{13–15} and was recently analyzed in the *brasiliensis* complex.³ This gene consists of core segments that are highly conserved, and others useful for taxonomic purposes due to some variable regions, which are described as divergent D domains or expansion segments.¹⁶ Hillis and Dixon¹⁶ emphasized that the coexistence of variability and conserved regions along the 28S gene make this region suitable for estimating phylogenetic relationships among species, since sequence variation provides phylogenetic information, whereas the conserved structure makes it easier to identify homology.

Therefore, this study aimed to analyze the phylogenetic relationships that *T. lenti* and *T. petrochiae* share with other members of the *brasiliensis* complex using the nuclear D2 region of the 28S RNA gene. In addition, a comparative analysis including other species of the subcomplexes grouped within the *infestans* complex was conducted to address the degree of polymorphism and the phylogenetic significance of this gene among South American triatomines.

Six adult specimens of each species in the *brasiliensis* complex (with the exception of *T. melanica*, with only two samples) and six adult *T. infestans* specimens (the outgroup) were used in the present study, comprising a matrix with 37 sequences. The specimens were provided by the Triatomine Insectarium of the College of Pharmaceutical Sciences of São Paulo State University, Araraquara Campus, Brazil. The triatomines were dissected and genetic material was extracted from the legs using the DNeasy Blood and Tissue Kit (QIAGEN, São Paulo, Brazil) according to the manufacturer's instructions. Polymerase chain reaction (PCR) reactions were performed for amplification of the nuclear D2 variable region of the 28S RNA gene according to Porter and Collins,¹⁷ with the forward sequence: 5'-GCGAGTCCG TGTTCCTTGATAGTGCAG-3' and the reverse sequence: 5'-TTGGTCCGTGTTTCAAGACGGG-3'. The conditions for the amplification reactions of this gene were as follows: an initial cycle at 95°C for 2 minutes, followed by 40 cycles comprising denaturation (95°C, 30 seconds), annealing (68°C, 30 seconds)

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TABLE 1
Jukes–Cantor distance between species of the *infestans* complex studied

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
<i>Triatoma infestans</i> 1																	
<i>Triatoma platensis</i>	0.000																
<i>T. infestans</i>	0.002	0.002															
<i>Triatoma wygodzinski</i>	0.004	0.004	0.006														
<i>Triatoma sordida</i>	0.004	0.004	0.006	0.000													
<i>Triatoma guasayana</i>	0.004	0.004	0.006	0.000	0.000												
<i>Triatoma costalimai</i>	0.004	0.004	0.006	0.000	0.000	0.000											
<i>Triatoma circummaculata</i>	0.004	0.004	0.006	0.000	0.000	0.000	0.000										
<i>Triatoma baratai</i>	0.004	0.004	0.006	0.000	0.000	0.000	0.000	0.000									
<i>Triatoma brasiliensis</i>	0.006	0.006	0.007	0.004	0.004	0.004	0.004	0.004	0.004								
<i>T. brasiliensis</i> 2	0.004	0.004	0.006	0.002	0.002	0.002	0.002	0.002	0.002	0.002							
<i>Triatoma lenti</i> 1	0.004	0.004	0.006	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000						
<i>Triatoma brasiliensis macromelasoma</i> 1	0.004	0.004	0.006	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000					
<i>Triatoma juazeirensis</i> 1	0.004	0.004	0.006	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000				
<i>Triatoma petrochiae</i> 1	0.004	0.004	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.002	0.002	0.002	0.002			
<i>Triatoma sherlocki</i> 1	0.004	0.004	0.006	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.002		
<i>Triatoma melanica</i> 1	0.004	0.004	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.002	0.002	0.002	0.002	0.000	0.002	0.000

and extension (72°C, 1 minute), and a final single cycle at 72°C for 5 minutes. After electrophoresis, the amplified fragments were purified using the GFX PCR DNA and Gel Band kit (GE Healthcare and Life Technology, São Paulo, Brazil) according to the manufacturer's instructions.

Direct sequencing was applied to the purified PCR products. The samples were sent to the Human Genome and Stem Cell Research Center, of the University of São Paulo, Brazil. Sequences generated were analyzed using the Bio-Edit software, version 7.0.5 (Ibis Biosciences, Carlsbad, CA), and a consensus sequence was obtained for each DNA segment. The sequences were aligned using the ClustalW. Subsequently, the MEGA 6.0 program for phylogenetic analysis was used using the maximum likelihood method as a distance criterion. The bootstrapping resampling method was applied to assess support for the individual nodes (1,000 pseudoreplications). The Jukes–Cantor model was used to calculate genetic distances pairwise within each member of the complexes (Table 1) in the MEGA 6.0 software.

In addition, the nuclear D2 variable region of the 28S RNA gene sequences deposited in GenBank for species from the *infestans*, *maculata*, *matogrossensis*, *rubrovaria*, *sordida*, and *brasiliensis* subcomplexes were used (Supplemental Table 1).

The nuclear D2 variable region of the 28S RNA gene did not allow to perform inferences on phylogenetic relationships among species within the *brasiliensis* complex due to the highly conserved nature of the fragments, with only two variable sites of 567 base pairs sequenced (Table 2 and Supplemental Figure 1). Furthermore, when aligned with the species of the *infestans* complex, the gene also proved to be extremely conserved, with only four variable sites (Table 3).

Phylogenetic analyses of the tribe Rhodniini showed that this gene had 9% of variable sites.¹³ For species within the *brasiliensis* complex, however, the gene had only 0.35% of variable sites, and for the species in the *infestans* complex, the gene had 0.70% of variable sites. It is natural for the nuclear genes that are less polymorphic than mitochondrial genes that have a rate of evolution by mutating five to ten times higher than a single copy nuclear gene.¹⁸ However, the Intergenic Spacer Region-2 nuclear intergenic region was

found to have 22% of variable sites between *T. infestans* and the *phyllosoma* complex and 21.2% of variable sites between *T. infestans* and *Dipetalogaster maxima*.¹⁹ This region was also found to have many polymorphic sites compared with the tribes Triatomini and Rhodniini (85.8% variable sites).¹⁹

TABLE 2
Polymorphic site determinants of the members of the *brasiliensis* complex, concerning the fragment of 567 base pairs of the nuclear D2 region of the 28S RNA gene

Species	384	514
<i>Triatoma brasiliensis</i> 2	T	T
<i>T. brasiliensis</i> 4	T	T
<i>T. brasiliensis</i> 5	T	T
<i>T. brasiliensis</i> 6	C	T
<i>T. brasiliensis</i> 7	C	T
<i>T. brasiliensis</i> 1	T	T
<i>Triatoma lenti</i> 1	T	T
<i>T. lenti</i> 2	T	T
<i>T. lenti</i> 3	T	T
<i>T. lenti</i> 4	T	T
<i>T. lenti</i> 5	T	T
<i>Triatoma brasiliensis macromelasoma</i> 1	T	T
<i>T. b. macromelasoma</i> 2	T	T
<i>T. b. macromelasoma</i> 3	T	T
<i>T. b. macromelasoma</i> 5	C	T
<i>T. b. macromelasoma</i> 6	C	T
<i>T. b. macromelasoma</i> 7	C	T
<i>Triatoma juazeirensis</i> 1	T	T
<i>T. juazeirensis</i> 3	C	T
<i>T. juazeirensis</i> 4	T	T
<i>T. juazeirensis</i> 5	T	T
<i>T. juazeirensis</i> 6	C	T
<i>T. juazeirensis</i> 7	T	T
<i>Triatoma petrochiae</i> 1	C	T
<i>T. petrochiae</i> 3	C	T
<i>T. petrochiae</i> 4	C	T
<i>T. petrochiae</i> 5	C	T
<i>T. petrochiae</i> 6	C	T
<i>T. petrochiae</i> 7	T	T
<i>Triatoma sherlocki</i> 1	T	T
<i>T. sherlocki</i> 2	T	T
<i>T. sherlocki</i> 3	T	T
<i>T. sherlocki</i> 4	T	T
<i>T. sherlocki</i> 5	T	T
<i>T. sherlocki</i> 7	C	T
<i>Triatoma melanica</i> 1	C	T
<i>T. melanica</i> 2	C	T

TABLE 3

Polymorphic site determinants of the members of the *infestans* complex, concerning the fragment of 567 base pairs of the nuclear D2 region of the 28S RNA gene

Species	179	384	468	514
<i>Triatoma infestans</i> GQ853397			C	
<i>Triatoma brasiliensis</i> CQ853395	C	T		T
<i>Triatoma wygodzinskyi</i> KC249222		C		T
<i>Triatoma sordida</i> KC249209		C		T
<i>Triatoma guasayana</i> KC249163		C		T
<i>Triatoma costalimai</i> KC249149		C		T
<i>Triatoma circummaculata</i> KC249147		C		T
<i>Triatoma baratai</i> KC249143		C		T
<i>T. brasiliensis</i> 2		T		T
<i>T. brasiliensis</i> 4		T		T
<i>T. brasiliensis</i> 5		T		T
<i>T. brasiliensis</i> 6		C		T
<i>T. brasiliensis</i> 7		C		T
<i>T. brasiliensis</i> 1		T		T
<i>Triatoma lenti</i> 1		T		T
<i>T. lenti</i> 2		T		T
<i>T. lenti</i> 3		T		T
<i>T. lenti</i> 4		T		T
<i>T. lenti</i> 5		T		T
<i>Triatoma brasiliensis macromelasoma</i> 1		T		T
<i>T. b. macromelasoma</i> 2		T		T
<i>T. b. macromelasoma</i> 3		T		T
<i>T. b. macromelasoma</i> 5		C		T
<i>T. b. macromelasoma</i> 6		.		T
<i>T. b. macromelasoma</i> 7		C		T
<i>Triatoma juazeirensis</i> 1		T		T
<i>T. juazeirensis</i> 3		C		T
<i>T. juazeirensis</i> 4		T		T
<i>T. juazeirensis</i> 5		T		T
<i>T. juazeirensis</i> 6		C		T
<i>T. juazeirensis</i> 7		T		T
<i>T. petrochiae</i> 1		C		T
<i>Triatoma petrochiae</i> 3		C		T
<i>T. petrochiae</i> 4		C		T
<i>T. petrochiae</i> 5		C		T
<i>T. petrochiae</i> 6		C		T
<i>T. petrochiae</i> 7		T		T
<i>Triatoma sherlocki</i> 1		T		T
<i>T. sherlocki</i> 2		T		T
<i>T. sherlocki</i> 3		T		T
<i>T. sherlocki</i> 4		T		T
<i>T. sherlocki</i> 5		T		T
<i>T. sherlocki</i> 7		C		T
<i>Triatoma melanica</i> 1		C		T
<i>T. melanica</i> 2		C		T

Recently, Justi and others³ stated that DNA barcoding (COI) relies on a gene that does not separate the triatomines from South America, as observed by at least one intraspecific distance greater than the interspecific distances for species in the *infestans* complex. Initially, four *T. brasiliensis* populations were separated based on genetic distance.²⁰ Later, these populations were defined as *T. melanica*, *T. juazeirensis*, *T. brasiliensis brasiliensis*, and *T. b. macromelasoma*. The analysis of the distance matrix for the *infestans* complex species is in agreement with this study,²⁰ because the nuclear D2 region of the 28S RNA gene exhibited low variation (Table 1). This finding once again reflects the high degree of conservation of the gene. Our analysis confirms that the nuclear D2 region of the 28S RNA gene is not a good molecular marker for triatomine species from South America.

Thus, the nuclear D2 region of the 28S RNA gene did not allow for the confirmation of the phylogenetic relationships of *T. lenti* and *T. petrochiae* in comparison with the species

from the *brasiliensis* complex. This domain proved to be extremely conserved in the South American triatomine species, thus demonstrating the fact that it is not a good molecular marker for phylogenetic studies on these vectors.

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