A 72 years of temporal analysis through geometric morphometrics detects phenotypic variation in populations of *Triatoma infestans (*Klug, 1834)

Análise temporal de 72 anos com o uso de morfometria geométrica detecta variação fenotípica em populações de *Triatoma infestans* (Klug, 1834)

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BACKGROUND

Chagas disease is caused by the protozoan *Trypanosoma cruzi* and its main form of transmission occurs through the domiciliated vector species. In this route, hematophagous insects of the Triatominae subfamily defecate during or shortly after the blood meal, eliminating the infective forms of the parasite. These enter the host organism through skin lesions or mucosal contamination. There are also other transmission routes, such as: blood transfusion, organ transplant, oral route (ingestion of food contaminated with *T. cruzi*), vertical (mother to child during pregnancy or delivery), and laboratory accidents (Coura, 2015; Silveira, 2011). Characterized as a neglected tropical disease, American Trypanosomiasis or Chagas disease remains as one of the main causes of morbidity in Latin America. It is estimated that, approximately, six to seven million people are infected with *T. cruzi* in the world, mainly in Latin America

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(Moncayo *et. al.*, 2009; WHO, 2017) Because there is no specific vaccine or antiparasitic treatment available for curing the disease in the chronic phase, the main strategies to prevent human infections are related to actions against domiciliated vectors through insecticide spraying in infested areas of the endemic countries (Coura & Viñas, 2010).

Triatoma infestans is the vector of *T. cruzi* mostly domiciled in South America, infesting several several countries: Brazil (nowadays just two residual foci), Argentina, Bolivia, Chile, Paraguay, southern Peru and Uruguay. Its domiiliation process is known to have occurred in Brazil due to human migration, during the colonization time, and also to the drastic environmental changes as a consequence of the agricultural activities (Costa & Lorenzo, 2009; Waleckx *et al.*, 2015). In Brazil, Chagas Disease control began in 1948 however, only after the finding of more than 700 municipalities infested by that vector, in 12 Brazilian States, the National Program of Chagas Disease Control was implemented (Silveira *et. al.*, 1984; Silveira & Vinhaes, 1999; Costa *et. al.*, 2009b). In 1991, Brazil joined the initiative of the southern cone countries, an international consortium formed by Argentina, Bolivia, Chile, Paraguay, Uruguay, and Peru, with the main objective to reduce the vectorial transmission by *T. infestans*, and control the blood banks (Silveira & Vinhaes, 1999). Only in 2006 Brazil was certified by the Pan-American Health Organization (PAHO) at the Southern Cone Initiative Conference as free of the vectorial transmission. Nevertheless, after the certification, residual foci were found in some states, such as Bahia and Rio Grande do Sul (Silveira, 2011; Gurgel-Gonçalves *et. al.*, 2012).

Ecological succession observed by the replacement of one species for another have resulted from control programs and from drastic environmental changes and deforestation. Data from the Chagas Disease Control Program in Minas Gerais, for the period 1979-1989, show that *Triatoma sordida* (Stal, 1859) predominated over *T. infestans* which was previously the main domiciliary vector. This fact was largely attributed to the control actions which had eliminated *T. infestans* from the domiciliary units (Diotaiuti *et al.*, 1995). The same was observed for *Triatoma rubrovaria* (Blanchard, 1843) and *T. infestans* in Rio Grande do Sul for the period 1980-1998 (Almeida *et al.*, 2000; Costa, 1999).

Studies on evolutionary biology have shown that external environmental factors may influence morphology in several aspects. In this context, the local selection may result in the emergence of different phenotypes, which subsequently, could be influenced by mechanisms such as the genetic assimilation or accommodation, which could lead to a genetic change (Dujardin *et. al.*, 2009). In Brazil, *T. infestans* has always been a strictly domiciled vector and its populations have been for many years, since 1948 (70 years), under the action of insecticides (Costa *et. al.*, 2009b) mainly in the State of Rio Grande do Sul. In this state, historically, there have been high rates of infestation recorded by that vector, therefore, it is then expected that the changes that could have occurred as a consequence of the environmental modifications and also due to the chemical control actions over time, could have had influenced the phenotype of the *T. infestans* populations, being a relevant issue to be investigated.

It is noteworthy that despite the elimination of *T. infestans* in most of the Brazilian territory, there are residual foci requiring attention, especially in the border areas with other countries, where the results of the chemical control actions were not effective. The persistence of the residual foci of this vector in Rio Grande do Sul led to the need for morphometric studies to investigate possible morphological changes over time, to check the influences of the environment on the main *T. cruzi* vector species. This information is of utmost importance to understand the evolutionary processes of *T. infestans* that may be correlated with its domiciliation capacity, for monitoring residual infestations and, to better understand aspects of the vectorial potentiality. In this 72 years temporal study, the relationships of *T. infestans* populations collected since 1942, before the control actions implemented by the Southern Cone Initiative and, populations collected in 2014, in the residual foci, were for the first time compared and analyzed. The persistence of the residual foci of this vector in Rio Grande do Sul led to the need for morphometric studies to investigate possible morphological changes over time. This information is of great importance to understand the evolutionary processes of *T. infestans* that may be correlated with its domiciliation capacity, bringing relevant information for monitoring residual infestations and, to better understand aspects of the vectorial potentiality. In this 72 years temporal study, the relationships of *T. infestans* populations collected since 1942, before the control actions implemented by the Southern Cone Initiative and, populations collected in 2014, in the residual foci, were compared and analyzed.

METHODS

Origin of the studied insects — Specimens of *T. infestans* were from: different locations of Rio Grande do Sul state, collected by the technicians of the Health Surveillance Department (SVS- RS); specimens deposited in the Entomological Collections of Instituto Oswaldo Cruz, under the responsibility of the Laboratory of Entomological Biodiversity (FIOCRUZ); and specimens of colonies kept in the insectaries of the National and International Reference Laboratory in Triatomine Taxonomy.

In total, 199 specimens of *T. infestans* were identified according to LENT & WYGODZINSKY (1979) and analyzed: 94 males and 105 females. From these, 98 specimens were obtained from the field, captured in different periods: 1942, 1950 and 2014. It is noteworthy that, in this group, there are rare specimens with records of 1942, obtained in the collection under the responsibility of LABE and of 1950 granted by SVS-RS. In 2014, specimens were collected in a residual foci in Santa Rosa, Rio Grande do Sul, and some of these insects were granted by SVS-RS (18 females and 14 male) and by LIVDIH (10 female and 22 male). From the remaining 101 specimens, 21 are from colonies reared in the insectary of LINIRTT, located in the State of Rio de Janeiro, and 80 specimens are from the insectary of the SVS-RS Laboratory (Table 1) (Fig. 1). Males of the 2006 population could not be included in the analyses given the low number of specimens (n=2).

WINGS PREPARATION — The left anterior wings (hemelytron) of males and females were used. The wings eventually damaged and which did not show the same number of reference anatomical points were excluded. All the wings were classified according to the sex, collection, date, and geographical origin. Wings of the insects reared in the insectary, were excised using tweezers, assembled in plates with alcohol 70 %, covered with glass slide and subsequently photographed, with scale and

Fig. 2. Photograph of the left wing of the male of *Triatoma infestans* (Klug, 1834), showing the conformation of the Reference Anatomic Points (PAR 1-8) selected for the analysis of the triatomine populations coordinates. Photo: Letícia Paschoaletto.

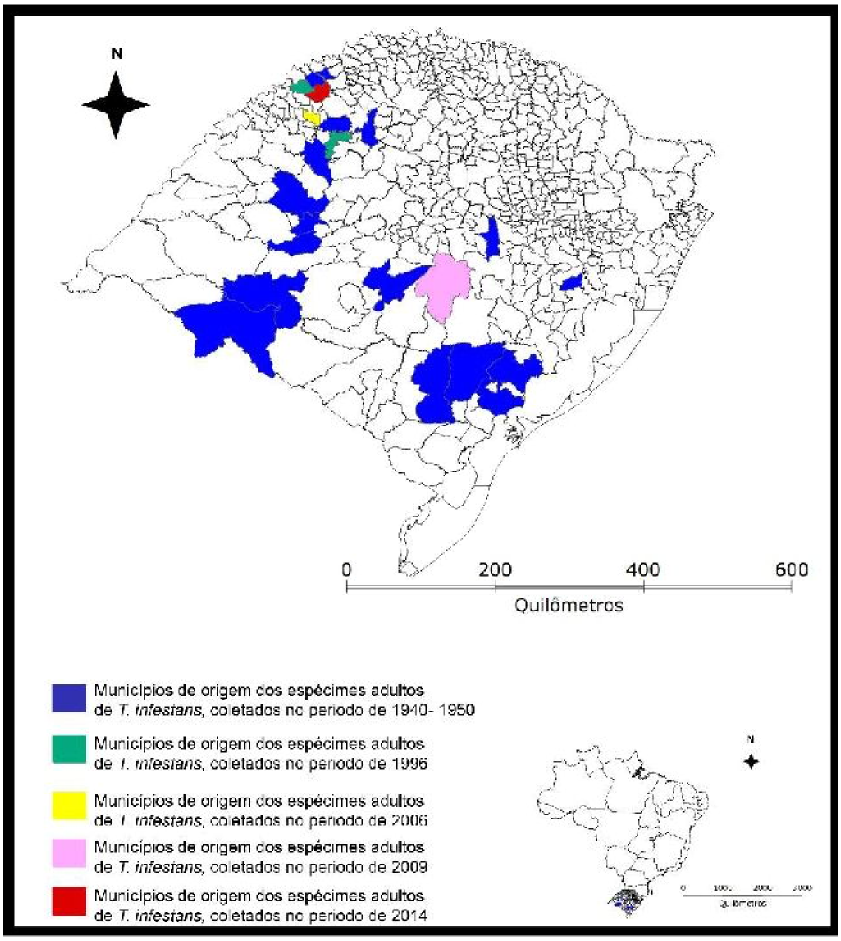

Fig. 1. Map showing the municipalities of the State of Rio Grande do Sul, Brazil, and the different locations correlated to the year of origin of the specimens of *Triatoma infestans* (Klug, 1834) used for the temporal analysis (1942 – 2014) through geometric morphometrics.

identification. The camera used was a Sony model DSC-070, coupled to the stereo microscope of Leica model MZ125 with an increase of x 0.8. The wings of the insects that were deposited in the CEIOC were photographed without removing them from the insect body, because they are very old and rare samples.

DATA COLLECTION — Reference Anatomic Points (PAR) were selected clockwise from the base to the apex of the wing and its homology (BOOKSTEIN, 1991), and were identified from the digital photos of each

Table 1. Origin and number of the left wings of females and males specimens of *Triatoma infestans* used for the geometric morphometics analysis, organized in chronological order.

Analysis Groups	Year	Origin	State	City	Female (n)	Total	Male (n)	Total
	1942	Laranja Collection (CEIOC)	Rio Grande do Sul	Cariguçu	2		0	
	1942	Laranja Collection (CEIOC)		Guaíba	0		1	
	1942	Laranja Collection (CEIOC)		Caçapava	0		1	
	1942	Laranja Collection (CEIOC)		Pelotas	0		2	
	1942	Laranja Collection (CEIOC)		São Miguel	0		1	
	1942	Laranja Collection (CEIOC)		São Tiago	0		1	
	1942	Laranja Collection (CEIOC)		Santo Angelo	0		1	
	1942	Laranja Collection (CEIOC)		São Vicente	0		1	
	1942	Laranja Collection (CEIOC)		Piratini	2	n= 17	0	n= 17
1942-1950	1942	Laranja Collection (CEIOC)		Rosário	0		1	
	1942	Laranja Collection (CEIOC)		Pinheiro Machado	1		1	
	1942	Laranja Collection (CEIOC)		Santa Cruz	1		2	
	1942	Laranja Collection (CEIOC)		São Sepé	2		0	
	1950	SVS-RS		Fundo Alegre	2		0	
	1950	SVS-RS		Tucunduva	0		1	
	1950	SVS-RS		Santana do Livramento	3		2	
	1950	SVS-RS		São Roque	4		2	
	1996	Insectary (LINIRTT)		Entre Ijuís	4	n= 8	6	n= 8
1996	1996	Insectary (LINIRTT)		Santo Cristo	4		2	
	2006	Insectary (LINIRTT)		Guarani das Missões	5	n= 5	0	Excluded
2006	2009	Insectary (SVS-RS)		Cachoeira do Sul	47	n= 47	33	n= 33
	2014	SVS-RS and LIVDH		Santa Rosa	28	n= 28	36	n= 36
2009					105		94	

wing (Fig. 2) using the COO_39 software. This software is part of the CLIC software package developed by Dujardin (2010). Eight PARS were determined for the analysis (Fig. 2).

Numerical Analysis — The analyses were performed in the PAR setting, through the MOGwin_82 PAD_91 software included in the CLIC ("General Public License" <https://xyom-clic.eu/)>.

Using the algorithm of the GPA, Generalized Procrustes Analysis, the x and y coordinates were superimposed. The Procrustes analysis is defined as the sum of the squared distances between the PAR of each object. The reference setting was minimized by the translation, rotation and size effects (Rohlf & Slice, 1990), making it possible to work separately the conformation and the overall size called centroid size (Bookstein, 1991).

Regarding the conformation, the GPA variables were used for the analysis in the Principal Components through the COV software, included in the CLIC (Dujardin, 2010). This analysis is based on the main components of the shape variables, where the Euclidean distances between the analyzed groups are obtained. The discriminant analysis was performed using the PAD software, also included in the CLIC (Dujardin, 2010, Rolf & Slice, 1990) where the Mahalanobis distances were calculated between the samples from the main components extracted from the conformation variables. This analysis also verified the validated simple reclassification of the individuals among the groups.

The statistical significance of the centroid size among the groups was tested by ANOVA analysis, and between pairs by the Tukey test. In the discriminant analysis, the significance was tested through the Lambda of Wilks.

RESULTS

Analysis of Female Size — The results of the centroid size analysis evidenced significant population differences among all groups (Fig. 3).

Females that belong to the 2014 group, recently collected specimens, and the females from 1942-1950 were the ones that showed larger wings (Fig. 2). In group of 1996, the centroid size of the wings was the smallest observed. This population is from the insectary of LINIRTT/RJ, collected in 1996 in the city of Santo Cristo (RS). The ANOVA was significant ($p<0.001$) regarding the other analyzed populations.

Tukey test showed that the differences were statistically significant among the groups of males: 1942-1950 and 1996, as well as between 1942-1950 e 2009. The population groups of females, where the differences were also significant among them, are: 2014 and 2009, and

Table 2. Percentage of the validated simple reclassification of females of *Triatoma infestans* according to the location and chronology.

POPULATIONS	No. OF RECLASSIFIED INDIVIDUALS	%
FEMALES 1942-1950	12/17	70.6
FEMALES 1996	08/08	100.0
FEMALES 2006	05/05	100.0
FEMALES 2009	34/47	72.3
FEMALES 2014	28/28	100.0

between 2014 and 1996. Among the groups that were not from colonies the differences were not significant (Tabela 2).

Analysis of Male Size — The populations coming directly from the field, 1942-1950 and 2014 were the ones with larger wings, and the specimens of 2014 are the group with the larger size of alar structures. The groups of 1996 and 2006 were those with smaller wings (Fig. 4). Through the ANOVA analysis it was found that the difference of centroid size of the male's wings is significant ($p<0.001$).

Tukey test showed that the differences were statistically significant only among the groups: 1942-1950 and 2009 and between 2009 and 2014.

Analysis of the Females Conformation — The factorial map prepared from a canonical analysis showed a separation between the female populations of *T. infestans,* where a separation of the specimens from the 2006 population was observed. The other populations are structured with overlapping areas (Fig. 5).

The validated reclassification values of the female specimens of *T. infestans* were generally considered from excellent to moderate (100-50%), and excellent (100%) for the populations of 1996, 2006 and 2014; high (80-70 %) for the populations of 2009 and 1942-1950; and moderate (50-66 %) (Table 3).

Analysis of males conformation — The populations overlapping was also observed. The data corroborate those observed in females (Fig. 6).

Table 3. Percentage of the validated simple reclassification of males of *Triatoma infestans* according to the location and chronology.

POPULATIONS	No. OF RECLASSIFIED INDIVIDUALS	%
MALES 1942-1950	8/17	47.1
MALES 1996	4/8	50.0
MALES 2009	23/33	69.7
MALES 2014	25/36	69.4

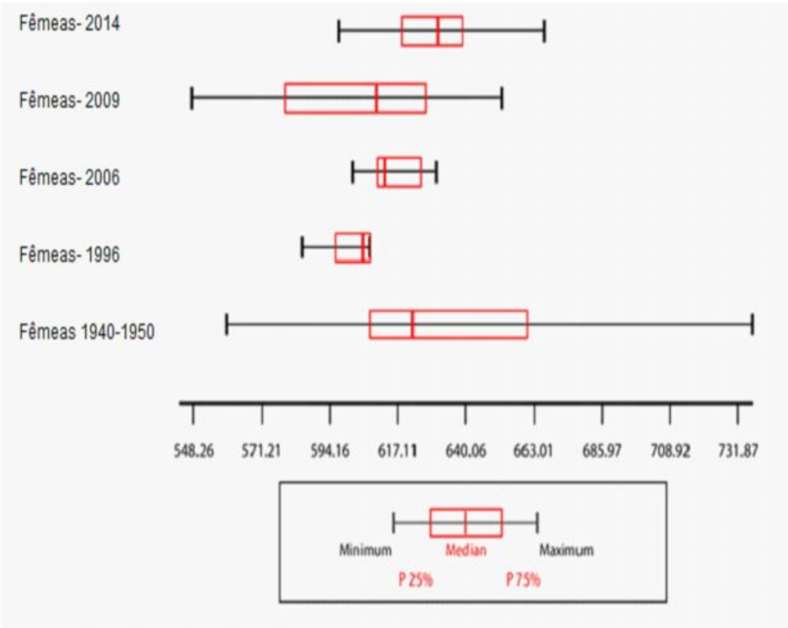

Fig. 3. Centroid size of the left alar structures of *Triatoma infestans* females from the Entomological Collection of Instituto Oswaldo Cruz, from insectary and from field captures in municipalities of Rio Grande Sul of different periods (1942-2014).

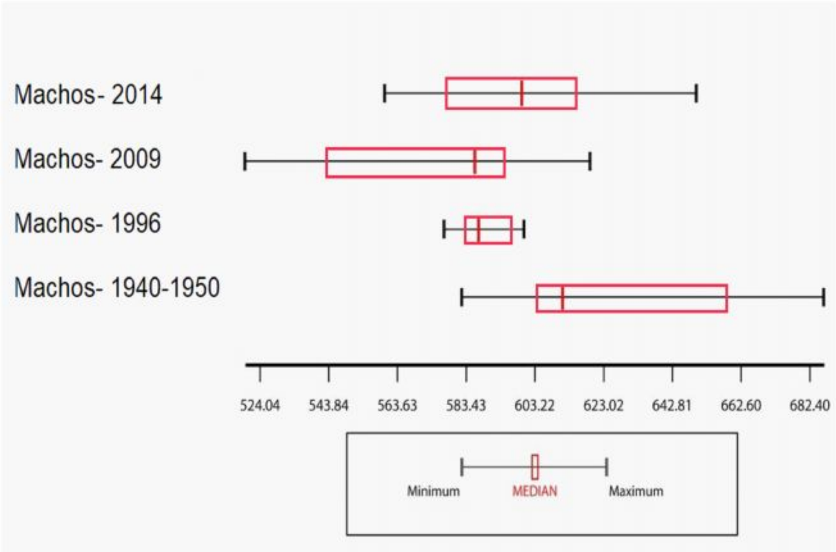

Fig. 4. Centroid size of the left alar structures of *Triatoma infestans* males from the Entomological Collection of Instituto Oswaldo Cruz, from insectary and from field captures in cities of Rio Grande Sul of different periods (1942-2014).

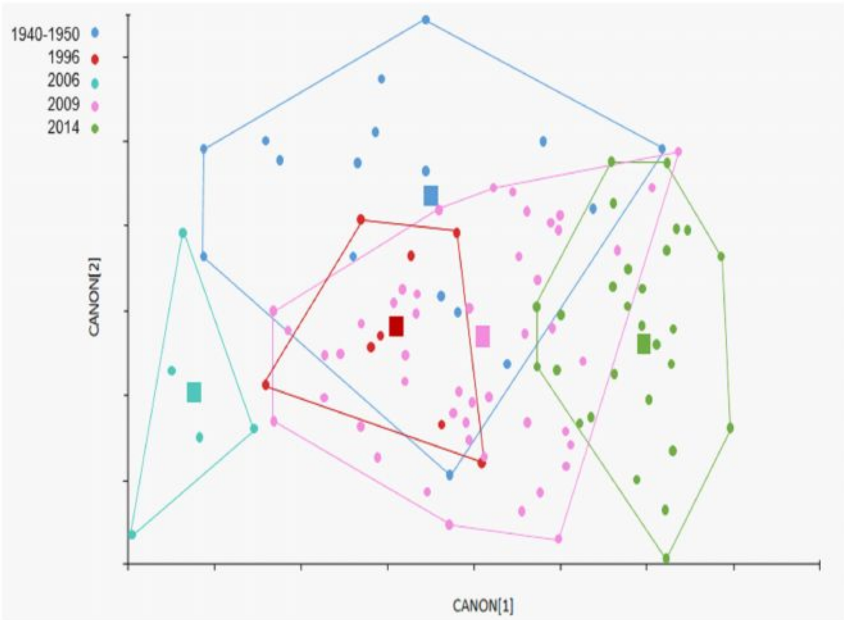

Fig. 5. Factorial map made from Mahalanobis distances showing the conformation influence in females of *Triatoma infestans* from the cities of Rio Grande Sul, from insectary and from the Entomological Collection of Instituto Oswaldo Cruz of different periods.

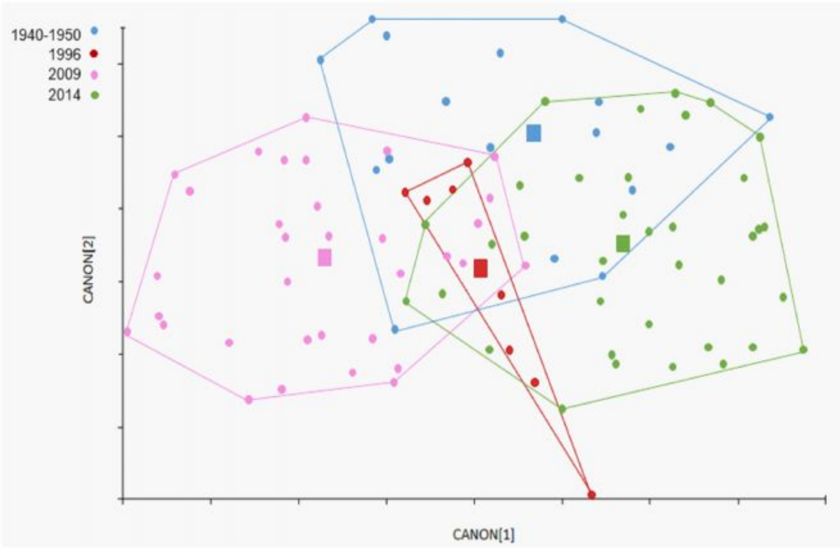

Fig. 6. Factorial map made from Mahalanobis distances showing the conformation influence in males of *Triatoma infestans* from municipalities of Rio Grande Sul, from insectary and from the Entomological Collection of Instituto Oswaldo Cruz of different periods.

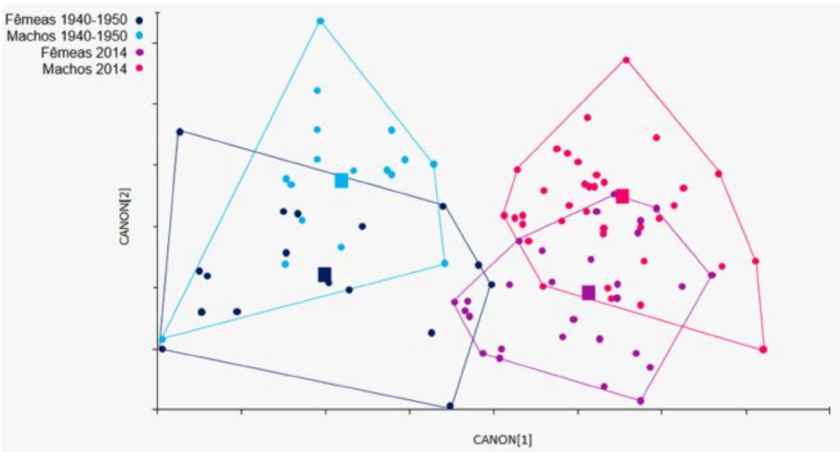

Fig. 7. Factorial map made from Mahalanobis distances showing the conformation influence in females and males of *Triatoma infestans* from municipalities of Rio Grande Sul, from insectary and from the Entomological Collection of Instituto Oswaldo Cruz of different periods, througho

The validated reclassification values of male specimens of *T. infestans* were generally considered from moderate to low (69 %-30 %), as moderate (69 %-50 %) for the populations of 2009 and 2014, and low for the population of 1942-1950 and 1996 (49 %-30 %) (Table 3).

Analysis of the Males and Females Conformation — The factorial map derived from a discriminant analysis revealed a structuring between males and females (Fig. 7).

DISCUSSION

Geometric morphometrics analyses have generated very significant results in the Triatominae subfamily, allowing the understanding of evolutionary processes and the speciation in this group (Dujardin *et al.*, 2009), especially among the species of the *T. brasiliensis* complex, presenting convergent results with the molecular tools showing high precision and resolution for systematics and phylogenetic analysis (Costa *et. al.*, 2009a; 2013; Monteiro *et. al.*, 2004; Oliveira *et. al.*, 2017).

Triatoma infestans is the main domiciliated vector in South America and in this study, unprecedented analyses were carried out using the geometric morphometrics technique to evaluate and understand possible evolutionary aspects related over 72 years. Insects studied were collected in the field and from laboratory colonies allowing broad comparisons on the *T, infestans* phenotype.

Until 1983, 36 % of the Brazilian territory was considered endemic, or at risk, for Chagas disease and more than 50 % of all Brazilian municipalities presented domiciliary triatomine infestation. In 20 years, Chagas disease transmission decreased dramatically. The number of municipalities infested by *T. infestans* (Klug, 1834) was reduced from 30.4 % in 1983 to 7.6 % in 1993 (Silveira & Rezende 1994). Despite the positive results achieved with the control actions against *T. infestans*, the few residual foci require the monitoring of the houses infestation by this vector and also, studies on the responses of this species regarding the years of insecticide purges to impair the transmission of the Chagas Disease in Brazil (Silveira, 2011).

Triatomines reared under laboratory conditions tend to show a decrease in size, a data found in the literature and this fact was also observed in the study (Dujardin *et. al.*, 1998; 1999). The reduction in size might occur due to genetic drift events and/or environmental conditions that may influence the insect adaptation to specific environmental conditions. In the insectary colonies, at a given time, the gene exchange is limited, as a consequence of endogamy, generating a decrease in the genetic diversity. Therefore, the intercrossing between individuals of the insectary generates individuals with convergent genotypes, culminating in the insects size decrease because there is no genetic flow (FITZPATRICK, 2008). In this sense, the population that showed the smallest size (population of 1996) might have been exposed to different conditions even before it was taken to the insectary in 1996, or even, it might have been influenced by the laboratory environment during the time reared in the insectary (10 years), leading to a decrease in the phenotypic variability that was evident in the size comparison with the other populations.

Regarding the results of the females' conformation, the population of the Guarani das Missões city (group of 2006) showed significant differences compared to other populations. Similar to Santo Cristo, Guarani das Missões is also a city near the Argentina-Brazil border; a possible cause for this difference in conformation can be the human migrations (generating the passive transport of different individuals from one distant location to another), leading the insect to different types of environmental exposures and also due to the insecticides, which began to have continuous and effective application as of 1991, both in Brazil and Argentina. In both countries, as the main vector is the *T. infestans*, the main objective was to eliminate the vectorial transmission of Chagas Disease through the treatment with insecticides (SILVEIRA *&* VINHAES, 1999). Therefore, even before it was reared in the insectary in 2006, *T. infestans* was already exposed to several external factors, which may have influenced the phenotype variation that culminated in its differentiation compared to other populations. However, in this group, only 5 specimens were analyzed, and it was not possible to get a robust statistical result, which makes impossible to draw robust conclusions in this specific case.

The results obtained with the geometric morphometrics regarding the males show that the environmental conditions over time seem to have no influence in their phenotypic variability.

In the view of the historical dispersion of the *T. infestans* throughout several countries of Latin America, and the knowledge that the drastic environmental changes such as deforestation may propitiate triatomine species to get adapted into new areas and ecotopes, it is highly important preserve the natural environment as well as to study and monitor the *T. cruzi* vector species to avoid new Chagas disease cases. In addition, educational activities are also recommended to impair the transmission of the disease.

SUMMARY

Triatoma infestans is the vector of *Trypanosoma cruzi* mostly domiciled in South America. In Brazil, residual foci of this insect are currently found in Bahia and Rio Grande do Sul states. The geometric morphometrics is a tool that detects the influences of external factors on an individual or population. In this context, this study aims to verify the possible phenotypic changes in specimens of *T. infestans* collected in the State of Rio Grande do Sul, Brazil, on a temporal scale. The geometric morphometrics of the wings was performed based on the anatomical reference points, which were analyzed through the software included in the CLIC package. The results showed that, both in males and females, the wings of the field populations had greater size compared to the insectary populations. Regarding the wing conformation, the females collected in 2006, in the city of Guarani das Missões, in Rio Grande do Sul (from insectary) showed a separation from of other populations. And in males, their overlaping was evidenced. This is the first temporal analysis of *T. infestans* populations. The morphometric analysis of specimens collected 23 years ago showed differences in the wing conformation suggesting a genetic response due to environmental factors.

KEYWORDS: morphology; Chagas disease; vectors; wings; geometric morphometrics.

SUMÁRIO

Triatoma infestans é o vetor de *Trypanosoma cruzi* mais domiciliado na América do Sul. Atualmente, no Brasil, focos residuais desse inseto são encontrados nos estados da Bahia e Rio Grande do Sul. A morfometria geométrica é uma ferramenta que detecta as influências de fatores externos em um indivíduo ou população. Nesse contexto, este estudo tem como objetivo verificar as possíveis alterações fenotípicas em espécimes de *T. infestans* coletados no Estado do Rio Grande do Sul, Brasil, em escala temporal. A morfometria geométrica das asas foi realizada com base nos pontos de referência anatômicos analisados através do software incluído no pacote CLIC. Os resultados mostraram que, tanto nos machos quanto nas fêmeas, as asas das populações coletadas no campo tiveram maior tamanho em comparação às populações de insetos criados em laboratório. Em relação à conformação das asas, as fêmeas coletadas em 2006, na cidade de Guarani das Missões, no Rio Grande do Sul (de insetário), mostraram uma separação de outras populações. E nos machos, a sobreposição foi evidenciada. Esta é a primeira análise temporal das populações de *T. infestans.* A análise morfométrica de espécimes coletados há 23 anos mostrou diferenças na conformação da asa, sugerindo uma resposta genética devido a fatores ambientais.

PALAVRAS-CHAVE: morfologia; doença de chagas; vetores; asas, morfometria geométrica.

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