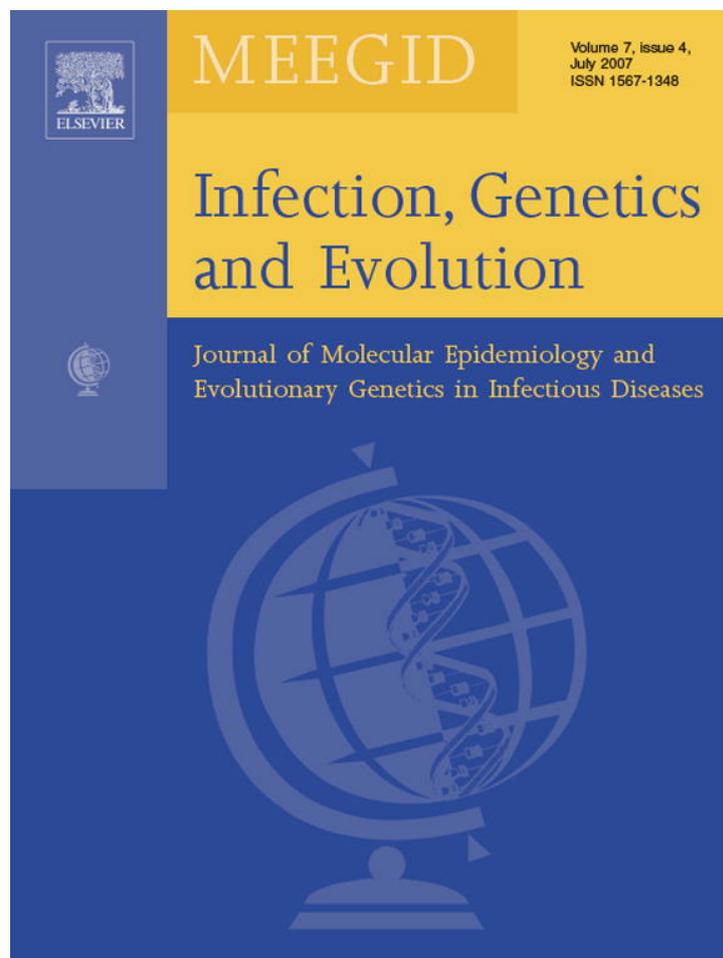


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Evolutionary relationships based on genetic and phenetic characters between *Triatoma maculata*, *Triatoma pseudomaculata* and morphologically related species (Reduviidae: Triatominae)

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Abstract

The *maculata* group currently comprises two species of Triatominae, *Triatoma maculata* and *Triatoma pseudomaculata*, which share morphologic and chromatic characteristics. In order to clarify the systematic status of these two vectors of *Trypanosoma cruzi* and to infer their evolutionary relationships, we performed an enzymatic, morphometric and cytogenetic comparison of them, also taking into account two sister species not included in the group (*T. arthurneivai* and *T. wygodzinskyi*). According to our results, *T. maculata* and *T. pseudomaculata* belong to distinct evolutionary lineages. Similarly, *T. arthurneivai* topotypes from Minas Gerais form an independent isolated group by morphometrics. Our results also support the specific status of the *Triatoma* population from São Paulo State (formerly referred to *T. arthurneivai*), and suggest the possibility that it is *T. wygodzinskyi*. Finally, we suggest that only the arbicolous *T. pseudomaculata* from northeast Brazil and the rupicolous sister species originated from São Paulo State should be classified together in the same group.

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Keywords: *Triatoma maculata*; *T. pseudomaculata*; *T. arthurneivai*; *T. wygodzinskyi*; Multilocus enzyme electrophoresis (MLEE); Morphometrics; Cytogenetics; Taxonomy; Phylogeny

1. Introduction

Triatoma maculata (Erichson, 1848) is a triatomine species found in Venezuela, Colombia, the Roraima state in Brazil, Suriname, Guyana, French Guiana and also in the Caribbean islands of Aruba, Bonaire and Curaçao (Carcavallo et al., 1999). It shares many morphologic and chromatic characteristics with *Triatoma pseudomaculata* Corrêa and Espínola (1964), a species that occurs throughout northeastern Brazil. For this

reason, *T. pseudomaculata* remained misidentified for more than a century before being described from insects collected in the Ceará State. According to the current hypothesis about their origin, *T. maculata* and *T. pseudomaculata* resulted of from the evolution of two geographic populations derived from a common ancestor by passive dispersion of nymphs associated with migratory birds (Schofield, 1988). Because both species display great similarity, they form the *maculata* group (Carcavallo et al., 2000; Dujardin et al., 2000). Two other species, *Triatoma arthurneivai* Lent and Martins, 1940 and *Triatoma wygodzinskyi* Lent, 1951, exhibit morphological similarities with *T. maculata* and *T. pseudomaculata*, which led Carcavallo et al. (1997) to considerate their relationship with the group.

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Table 1
Known distribution and some ecological traits of *T. maculata*, *T. pseudomaculata*, *T. arthurneivai* and *T. wygodzinskyi*

Species	Geographic distribution	Silvatic habitat	Trends to domesticity	References
<i>T. maculata</i>	Brazil (RR), Colombia Venezuela, Surinam, Guyana, French Guiana, Caribbean islands	Palm tree, hollow tree, bird nest, bromeliad	+	Tonn et al. (1978), Carcavallo et al. (1998), Carcavallo et al. (1999)
<i>T. pseudomaculata</i>	Brazil (AL, BA, CE, DF, GO, MG, PB, PE, PI, RN, SE, TO)	Hollow tree, bird nest	+	Carcavallo et al. (1999), Vinhaes and Dias (2000), Dias-Lima et al. (2003)
<i>T. arthurneivai</i>	Brazil (MG, PR, SP)	Rocky formation	N.R.	Forattini et al. (1968), Barretto and Ribeiro (1981), Carcavallo et al. (1999)
<i>T. wygodzinskyi</i>	Brazil (MG)	N.R.	N.R.	Lent and Wygodzinsky (1979)

N.R.: not recorded. AL (Alagoas), BA (Bahia), CE (Ceará), DF (Distrito Federal), GO (Goiás), MG (Minas Gerais), PB (Paraíba), PE (Pernambuco), PI (Piauí), PR (Paraná), RN (Rio Grande do Norte), RR (Roraima), SE (Sergipe), SP (São Paulo), TO (Tocantins).

T. arthurneivai was described from a type material collected in the Serra do Cipó, Minas Gerais State, Brazil, and was later reported in the São Paulo State where its eco-biological traits were described (Corrêa et al., 1965; Forattini et al., 1968; Barretto and Ribeiro, 1981). *T. wygodzinskyi* was described from a small number of specimens collected, on a single occasion, in the south of Minas Gerais State. The known distribution and some ecological traits of all four species are reported in Table 1.

Sylvatic *T. maculata* and *T. pseudomaculata* are known to exist in a variety of arboreal habitats while *T. arthurneivai* is found only in the cracks of stones (Table 1). The ecological traits of *T. wygodzinskyi* are unknown. *T. maculata* is considered to be a secondary vector of *Trypanosoma cruzi* to man in Venezuela and Colombia and also in the State of Roraima, Brazil, where a process of domestication is occurring (Felicangeli et al., 2003; Luitgards-Moura et al., 2005). *T. pseudomaculata* is an autochthonous species of “caatingas”, a set of xerophytic formations located in northeast Brazil which is characterized by the fall of leaves during the dry season and the abundance of cacti and bromeliads (Forattini, 1980). Additionally, *T. pseudomaculata* is typically a vector candidate which was originally restricted to wild environments and is currently reported to be invading artificial structures (Silveira and Vinhaes, 1998). Thus, *T. pseudomaculata* must be regarded

as a species that has not yet completed its transition to a domestic habitat.

The importance of phylogenetic information in interrelations of ecosystems, population dynamics, evolutionary trends and possibilities for control is recognized, particularly when disease vectors such as Triatominae are involved. Such information can provide new clues to understand the synanthropic process when sister species exhibit a marked difference. The evolutionary relationship between *T. maculata* and *T. pseudomaculata* is still a matter of debate that deserves deeper analysis as various authors hold opposing views about their lineages (Hypša et al., 2002; Sainz et al., 2004; De Paula et al., 2005). In order to further ascertain the systematic relationships of both epidemiologically important taxa and sister species (*T. arthurneivai* and *T. wygodzinskyi*), we used multilocus enzyme electrophoresis, morphometric and cytogenetic techniques.

2. Materials and methods

2.1. Triatominae

Specimens from three of the four triatomine species analyzed in this paper were obtained from laboratory colonies and used for multilocus enzyme electrophoresis (MLEE) and cytogenetics. *T. wygodzinskyi*, which is unavailable as a

Table 2
Characteristics of the different samples analyzed

Species identification	Code ^a	Geographic origin	Latitude, longitude	Year of collecting	No. of individuals studied by		
					MLEE	Morphometrics	Cytogenetics
<i>T. maculata</i>	Tm-RR	Boa Vista, RR	2°49'N,60°40'W	2001	27	13	4
	Tm-VN	Venezuela	–	–	–	20	–
<i>T. pseudomaculata</i>	Tps-CE	Sobral, CE	3°42'S,40°21'W	2001	4	5	5
	Tps-PI	João Costa, PI	3°53'S,42°07'W	2002	10	9	–
	Tps-BA	Curaçá, BA	9°28'S,39°44'W	2002	27	35	4
	Tps-PE	Caruaru, PE	8°16'S,35°58'W	2001	–	7	–
<i>T. arthurneivai</i>	Ta-SP	E. S. de Pinhal, SP	22°11'S,46°45'W	2001	19	8	4
	Ta-MG	Serra do Cipó, MG	19°11'S,43°22'W	1940	–	9	–
<i>T. wygodzinskyi</i>	Tw-MG	Sta Rita de Caldas, MG	22°01'S,46°19'W	1951	–	3	–

^a A symbol code is used to identify the samples. The first symbol represents the species (Tm for *T. maculata*, Tps for *T. pseudomaculata* . . .) and the second one the geographic origin after a hyphen (BA: Bahia, CE: Ceará, MG: Minas Gerais, PE: Pernambuco, PI: Piauí, RR: Roraima, SP: São Paulo, VN: Venezuela). With the exception of Venezuela, all the geographic origins are situated in Brazil.

laboratory colony given the extreme rarity of collected specimens, could not be analyzed by these genetic markers. For morphometric analysis, we used the same laboratory colonies and also included the topotypes of *T. arthurneivai* and *T. wygodzinkyi* (both from Minas Gerais, Brazil) conserved in the entomological collection of Oswaldo Cruz Institute. Data on populations and number of individuals studied by MLEE, morphometrics and cytogenetics are summarized in Table 2.

2.2. Multilocus enzyme electrophoresis

Nymphal instars and adults of both sexes were used. Thoracic muscles were dissected out and ground in 100 μ l of an enzyme stabilizer (dithiothreitol, *E*-aminocaproic acid and EDTA, each at 2 mM). Extracts were stored at -70°C prior to use. Multilocus enzyme electrophoresis (MLEE) was performed on cellulose acetate plates (Helena Laboratories, Beaumont, TX). The following 16 enzyme systems were assayed: aconitate hydratase (ACON, EC 4.2.1.3); diaphorase (DIA, EC 1.6.2.2); fructose-1, 6-diphosphatase (FDP, EC 3.1.3.11); fumarate hydratase (FUM, EC 4.2.1.2); glutamate dehydrogenase (GDH, EC 1.4.1.3); aspartate aminotransferase (GOT, EC 2.6.1.1); glycerol-3-phosphate dehydrogenase (GPD, EC 1.1.1.8); glucose phosphate isomerase (GPI, EC 5.3.1.9); glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49); hexokinase (HK, EC 2.7.1.1); isocitrate dehydrogenase (IDH, EC 1.1.1.42); malate dehydrogenase (MDH, EC 1.1.1.37); malic enzyme (ME, EC 1.1.1.40); mannose-phosphate isomerase (MPI, EC 5.3.1.8); phosphoglucomutase (PGM, EC 2.7.5.1); and 6-phosphogluconate dehydrogenase (6-PGDH, EC 1.1.1.44). Electrophoresis and enzyme staining were performed as described previously by Ben Abderrazak et al. (1993) and Noireau et al. (1998). Genotype frequencies were obtained by direct genetic interpretation of gel banding patterns. Genetic variability was estimated by the percentage of polymorphic loci (*P*) and the mean expected heterozygosity (*He*). Nei's standard genetic distance (Nei, 1987) was used to compare gene frequency differences between species or populations.

2.3. Cytogenetics

Testes were removed from freshly killed adults, fixed in an ethanol–acetic acid mixture (3:1) and stored at -20°C . Air-dried chromosome preparations were made by squashing gonads in 50% acetic acid, freezing them in liquid nitrogen and removing the coverslip with a razor blade. The C-banding technique was performed as reported by Pérez et al. (1997) in order to observe the distribution and behavior of C-heterochromatin during mitosis and meiosis. Observations were carried out with a Nikon Microphot FX microscope (Nikon, Tokyo, Japan). The photographs in the bright field microscope were made with Ilford 50 film (Ilford Ltda, Cheshire, U.K.).

2.4. Morphometrics

Measurements were made on the head and thorax of each specimen by the same investigator according to Casini et al.

(1995). The nine head measurements were: inner distance between the eyes, inner distance between ocellae, ante-ocular distance, post-ocular distance excluding the neck, length of the antenniferous tubercle, head length, and length of the 1st, 2nd and 3rd rostral segments. The four measurements performed on the thorax were: width of the collar of the thorax, thorax partial width at the intersection of the fore and median lobes, thorax total width between the humerus, and the total length of the thorax excluding the scutellum. Head log-transformed characters, together with thorax log-transformed characters, were scaled for size by subtracting row means and submitted to a principal component analysis (Darroch and Mosimann, 1985). To preserve an acceptable number of variables relative to the smallest group, the seven first components were retained and used as input for a so-called “size-free discriminant analysis” on the four groups. The three specimens of *T. wygodzinkyi* were classified on the basis of their Mahalanobis distance to each group centroid and projected onto the factorial map of the first two discriminant factors. For morphometric analyses and their graphical display, we used the PAD and related morphometric software modules freely available at <http://www.mpl.ird.fr/morphometrics>.

3. Results

3.1. Isoenzyme electrophoresis

A single zone of enzymatic activity or locus was scored for ACON, DIA, FDP, FUM, GDH, GOT, GPI, IDH, MDH, MPI, PGM and 6PGDH, while two loci were scored for ME. The HK and G6PD enzymes were excluded from the analysis because they could not be reliably scored, and the GPD enzyme because it was dependent of the insect stage. Thus, the studied set of enzymes represented a total of 14 gene loci. Three polymorphic loci ($P = 0.21$) were found for the *T. pseudomaculata* from Piauí (Tps-PI), two ($P = 0.14$) for *T. maculata* (Tm-RR) and *T. pseudomaculata* from Bahia (Tps-BA). For the other populations (Tps-CE and Ta-SP), the different loci were monomorphic. The estimate of gene diversity (or mean expected heterozygosity, *He*) was 0.07 (Tm-RR), 0.06 (Tps-PI) and 0.05 (Tps-BA), respectively. The genotype proportions for the polymorphic loci showed no significant departures from the expectations of the Hardy–Weinberg equilibrium (calculated by fixation index *F*; $P > 0.05$). Only two loci (*Dia* and *Me2*) did not present intergroup variation. Eleven loci (*Gdh*, *Fdp*, *Fum*, *Gdh*, *Got*, *Gpi*, *Idh*, *Mdh*, *Mpi*, *Pgm* and *6Pgdh*) and 12 loci (*Gdh*, *Fdp*, *Fum*, *Gdh*, *Got*, *Gpi*, *Idh*, *Mdh*, *Me1*, *Mpi*, *Pgm* and *6Pgdh*) allowed us to differentiate *T. maculata* from *T. pseudomaculata* and *T. arthurneivai*, respectively. One or two diagnosis loci (*Me1* and *6Pgdh*) were found between *T. arthurneivai* and *T. pseudomaculata*, depending on the geographic origin of these last samples (Table 3). *T. pseudomaculata* populations and *T. arthurneivai* were separated by genetic distances >0.10 . Higher genetic distances (from 1.48 to 1.51) were observed between *T. maculata* and the *T. pseudomaculata* populations (Table 3).

Table 3
Number of isoenzyme diagnosis loci (above diagonal) and Nei genetic distance (below diagonal) between the samples analyzed

	Tm-RR	Tps-CE	Tps-PI	Tps-BA	Ta-SP
Tm-RR	–	11	11	11	12
Tps-CE	1.50	–	0	0	2
Tps-PI	1.51	0.01	–	0	1
Tps-BA	1.48	0.09	0.06	–	1
Ta-SP	1.91	0.15	0.11	0.11	–

Tm: *T. maculata* (RR: Roraima); Tps: *T. pseudomaculata* (CE: Ceará, PI: Piauí, BA: Bahia); Ta: *T. arthurneivai* (SP: São Paulo).

3.2. Cytogenetics

The males of the three species had the same diploid chromosome number ($2n = 22$), constituted by 20 autosomes and a pair of sex chromosomes (XY). The analysis of the C-banding patterns in gonial mitosis and the meiotic chromosome behavior showed that the three species presented different amounts and distribution of autosomal heterochromatin. Moreover, all individuals of each species presented only one C-banding pattern. These differences in the heterochromatin content were clearly observed during early meiotic prophase (Fig. 1). *T. arthurneivai* consisted completely of euchromatic autosomes (i.e. without heterochromatin) and only the Y chromosome was heterochromatic. During early meiotic prophase, there was one heteropycnotic chromocenter formed only by the association of the XY sex chromosomes (arrow Fig. 1a). In *T. maculata* most autosomes presented terminal and small C-heterochromatic dots that appeared scattered throughout the nucleus (Fig. 1b). In *T. pseudomaculata* only three or four autosomes appeared with C-regions, but some of them were closely associated with the sex chromosomes (arrowheads Fig. 1c). The number of C-heterochromatic dots was higher in *T. maculata* than in *T. pseudomaculata* (compared Fig. 1b with c). The C-banding patterns detected in gonial mitosis (data not shown) were in agreement with the ones observed during meiotic prophase (Fig. 1).

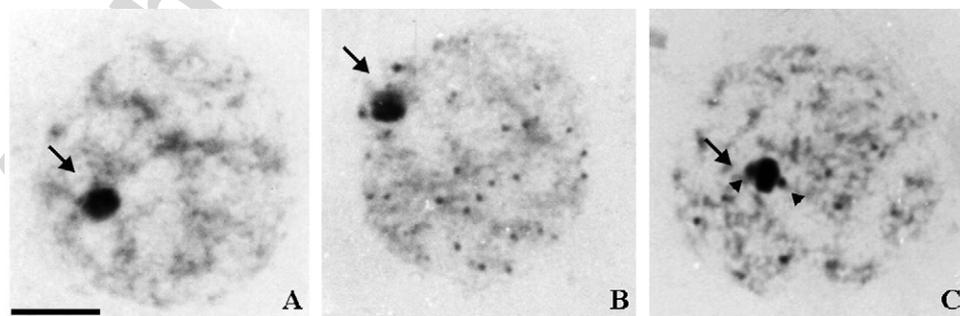


Fig. 1. Representative C-banding patterns observed at meiotic prophase (diffuse stage) in male specimens of *Triatoma arthurneivai*, *T. maculata* and *T. pseudomaculata*: (A) *T. arthurneivai* from São Paulo: the association of the XY sex chromosomes constituted one heterochromatic chromocenter (arrow). All autosomal bivalents were euchromatic while that the Y chromosome is heterochromatic. (B) *T. maculata*: almost all autosomes had small heterochromatic C-dots that appear scattered throughout the nucleus. (C) *T. pseudomaculata*: this species had a chromocenter (arrow) formed by the association of the heterochromatic regions of one autosome (arrowheads). This species presented fewer heterochromatic C-dots than *T. maculata*.

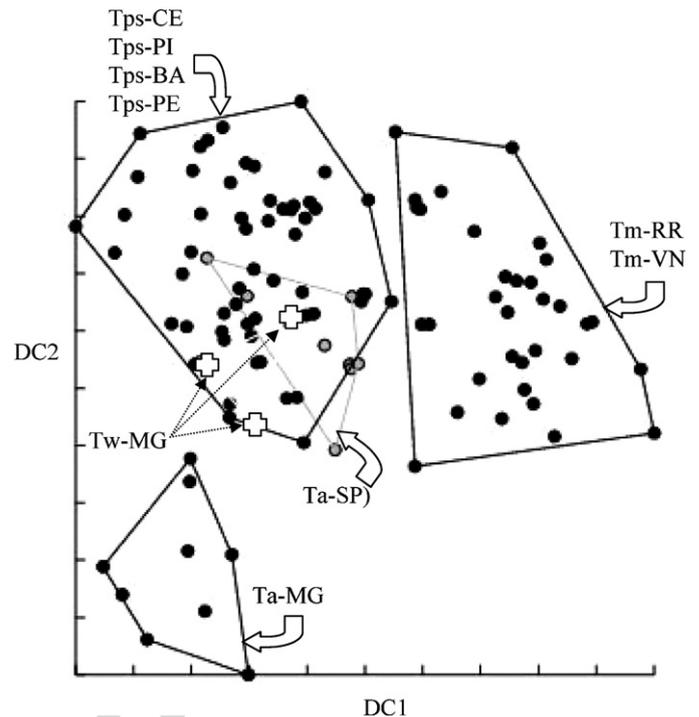


Fig. 2. Factorial map based on the measurements of head and thorax of adults of *Triatoma maculata* (Tm-RR and Tm-VN), *T. pseudomaculata* (Tps-CE, Tps-PI, Tps-BA, Tps-PE), *Triatoma arthurneivai* from São Paulo (Ta-SP), and *T. arthurneivai* (Ta-MG). The three *T. wygodzinskyi* specimens were introduced as supplementary data after completion of the discriminant analysis, and projected onto the factor map (Tw-MG, white cross). DC1 and DC2 are first and second discriminant function derived from size-free components.

3.3. Morphometrics (Fig. 2)

The first discriminant function derived from size-free components completely separated *T. maculata* from the remaining species. There was an obvious subdivision within the *T. arthurneivai* taxon, one group (Ta-SP) overlapping with *T. pseudomaculata*, the other one (Ta-MG) behaved as an independent, isolated group. The projected specimens of *T. wygodzinskyi* were compatible with either *T. arthurneivai* from São Paulo or *T. pseudomaculata*.

4. Discussion

With the exception of *T. maculata* that occurs in Venezuela and some regions of adjacent countries, all the other species are Brazilian and spread from the northeastern (*T. pseudomaculata*) to central Brazil (*T. arthurneivai* and *T. wygodzinskyi*). *T. maculata* and *T. pseudomaculata* are arboricolous and allopatric, being separated by the Amazon forest (Carcavallo et al., 1999). Because *T. maculata* is commonly found indoors and infected with *T. cruzi*, it is considered the second most important Chagas disease vector in Venezuela (Lent and Wygodzinsky, 1979; Feliciangeli et al., 2003). As for *T. pseudomaculata*, it is often found in peridomestic areas and can occasionally invade human dwelling (Silveira and Vinhaes, 1998). It should be considered to be a vector candidate and thus be monitored. On the other hand, *T. arthurneivai* and *T. wygodzinskyi* are exclusively sylvatic. *T. arthurneivai* is rupicolous while the habitat of *T. wygodzinskyi* is unknown. Both species occur in sympatry in southern Minas Gerais (Carcavallo et al., 1999).

Specific taxonomic status and population structuring/relationships were first assessed by isoenzyme electrophoresis. Isoenzyme analysis is a classic technique that has remained a valuable tool to address the correct level of phylogenetic divergence in several organisms, including Triatominae (Hartl and Dykhuisen, 1984; Dujardin et al., 2000). In order to obtain reliable estimates of genetic distance, it is important to examine a large number of loci rather than a large number of individuals (Richardson et al., 1986; Nei, 1987). With a total of 14 gene loci examined and sample sizes ranging from 8 to 20 individuals by population (except for the Tps-CE population originated from the type locality, with only 4 individuals), our work allows a reliable analysis on evolutionary relationships. Although the apparently null or very low levels of genetic variation in populations analyzed (with H_e ranging from 0 to 0.085) might be attributed to the small sample sizes and the colony origin of the specimens, it is in agreement with other studies that indicate low isoenzyme variability in Triatominae (Dujardin et al., 2000). In several groups of triatomines, allozyme electrophoresis was successfully used for the distinction of cryptic species and the determination of the correct status of dubious populations (Panzera et al., 1995; Noireau et al., 1998). According to Noireau et al. (1998) and Dujardin et al. (2000), values of genetic distance higher to 0.10 are supposed to indicate specific rank in triatomines.

For morphometrics, the removal of size was intended to allow partitioning of environmental differences (size related) from evolutionary influences (Hutcheson et al., 1995). This statistical processing of initial measurements has been used successfully to ascertain evolutionary relationships in Triatominae (Dujardin et al., 1999). Seven shape components were retained for species/populations characterization and discrimination, and these seven variables contributed to 93% of the total shape variation. Out of the two discriminant functions, the first one represented 84% of the variation, to which size was slightly (7%) contributing. Thus, on the basis of this model, the three specimens of *T. wygodzinskyi* were projected, and their

tentative attribution obtained by comparing Mahalanobis distances with the three species.

Because its morphology is very similar to *T. maculata*, *T. pseudomaculata* has remained misidentified for a long time before being ranked as a distinct species based on experimental crossings (Corrêa and Espínola, 1964). Our results obtained with MLEE (11 diagnostic loci and genetic distance ≈ 1.50), cytogenetics (the two species exhibit differences in heterochromatin content) and morphometrics strongly support that *T. maculata* and *T. pseudomaculata* do not exhibit close phylogenetic affiliation and belong to distinct lineages. This result is consistent with the proposal of Hypša et al. (2002) and De Paula et al. (2005) that made use of mitochondrial rDNA sequences (16S), but disagrees with the analysis of mitochondrial DNA sequences performed by Sainz et al. (2004), suggesting that *T. maculata* and *T. pseudomaculata* are closely related species. This discordance can be attributed to the misidentification of the specimens of *T. maculata* used by Sainz et al. (2004). These authors analyzed specimens from Sergipe (Brazil), which is the geographic location of *T. pseudomaculata* but not of *T. maculata*. In conclusion, the current opinion suggested by Schofield (1988) that both species originated as allopatric populations derived from a recent common ancestor can be discarded.

The existence of diagnostic loci (1 or 2) and genetic distances ≥ 0.11 between *T. pseudomaculata* populations and *T. arthurneivai* from São Paulo (Ta-SP) were consistent with the hypothesis of distinct species. This assumption is also supported by cytogenetic findings. The difference in heterochromatin content reported here (Fig. 1) reveals genetic differences between the species analyzed in a level similar to the ones observed among other triatomine species (Panzera et al., 1995; Pérez et al., 2002). Surprisingly, morphometrics shows a clear differentiation between the two geographic populations of *T. arthurneivai*: the specimens from SP are grouped with *T. pseudomaculata* and *T. wygodzinskyi*, while the topotypes Minas Gerais (Ta-MG) form an independent and isolated group. Consequently, our results support the hypothesis that the population from SP should be regarded as a distinct species closely related to *T. pseudomaculata* and *T. wygodzinskyi*. It is likely that the field works dedicated to *T. arthurneivai* from the SP State had involved this *Triatoma* sp. (Corrêa et al., 1965; Forattini et al., 1968; Barretto and Ribeiro, 1981). Similarly, the phylogenetic analyses using rDNA sequences, which clustered *T. pseudomaculata* and *T. arthurneivai*, involved individuals from SP (*Triatoma* sp.) and not *T. arthurneivai* topotypes from MG (Hypša et al., 2002; De Paula et al., 2005).

Because morphometrics shows the projection of the three *T. wygodzinskyi* specimens over or close to *Triatoma* sp. from SP (Fig. 2) and both populations occur in the same region of southeastern Brazil, we put forward the hypothesis that they belong to the same species, i.e. *T. wygodzinskyi*. Nevertheless, the fact to compare topotypes of *T. arthurneivai* and *T. wygodzinskyi*, deposited in entomological collection for more than 50 years, with *Triatoma* specimens from SP freshly collected requires caution. New collecting and further studies

are necessary before establishing the taxonomic status of *Triatoma* sp. from SP. Lastly, the close evolutionary relationships between *T. pseudomaculata* and *Triatoma* sp. from SP (formerly *T. arthurneivai*) are clearly demonstrated by our isoenzyme study, which is in agreement with the molecular analyses of HYPŠA et al. (2002) and De Paula et al. (2005). The low genetic distances (0.11–0.15) (Table 3) indicate that both species belong to the same evolutionary lineage.

The current classification of Triatominae in species groups is based mainly on extensive phenotypical similarities rather than their evolutionary relationships and behavioral/ecological shared traits (Usinger et al., 1966; Carcavallo et al., 2000). If we support our classification only in morphological traits, the *maculata* group has to include all the species studied here. However, if we consider that the species composing a group must also share genetic traits, the existence of a *maculata* group is questionable. Only the arboreal *T. pseudomaculata* and the rupicolous sister *Triatoma* sp. from the São Paulo State should be classified in a same evolutionary group.

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