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GENOME SIZE DETERMINATION IN CHAGAS DISEASE TRANSMITTING BUGS (HEMIPTERA-TRIATOMINAE) BY FLOW CYTOMETRY

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Abstract. Because information about genome size in triatomines is scarce and contradictory, we performed DNA quantification by flow cytometry in 13 species belonging to five genera (*Dipetalogaster*, *Eratyrus*, *Panstrongylus*, *Rhodnius,* and *Triatoma*) to infer overall tendencies and phylogenetic associations. The results show that the haploid DNA content of the subfamily Triatominae varies nearly 4-fold, from < 0.7 pg in *Rhodnius* species (0.6×10^9 bp) to 2.7 pg in *Triatoma delpontei* (2.6×10^9 bp). Considering that triatomines present similar chromosome numbers, we suggest that genome size differences are the result of variation in the quantity of repetitive DNA sequences localized in hetero and euchromatin. Changes in heterochromatin are particularly important when considering populations or closely related species; in more distant taxa, euchromatic changes also play a role. Our analyses indicate that flow cytometry is a useful tool for population, taxonomic, and evolutionary studies in this subfamily.

INTRODUCTION

The subfamily Triatominae (Hemiptera-Reduviidae) includes 137 species of hematophagous bugs divided into 5 tribes and 17 genera,[1] most of them vectors of the protozoan *Trypanosoma cruzi*, causative agent of American Trypanosomiasis or Chagas disease. This parasitemia is the human health problem presenting the highest socioeconomic impact in Latin America, where 13 million persons are infected and > 90 million people are in risk.[2,3] Because of the absence of effective anti-trypanosomal drugs, the primary method for disease control has been vector control.[3] More than 10 species of triatomines are considered relevant targets for vector control because of their role in Chagas disease transmission.

The subfamily Triatominae has been the subject of numerous genetic studies to resolve systematic and evolutionary questions.[4,5] There is, however, scarce and contradictory information regarding genome size in this group. Genome size describes the nuclear DNA content in picograms per haploid genome and is often called the "C" value. This value has fundamental biologic significance and use for population level and taxonomic studies.[6] Genome size data are also required in detailed analyses of genome structure and evolution and for the application of molecular techniques. Knowledge of genome size of a species is essential for assessing genomic library coverage, estimating the copy number of a gene, and for developing gene cloning strategies based on genome mapping. In particular, the choice of future genome sequencing projects will be dependent on knowledge regarding the size of the genomes to be sequenced.[7]

Genome size data in triatomines have been studied primarily using Feulgen densitometry,[8,9] and only more recently using flow cytometry (Table 1).[10–12] In this study, we analyze DNA cell content of 13 triatomine species, belonging to five genera, using flow cytometry. The results are compared with those previously reported in the literature for the evaluation of interspecific variation. This analysis provides the first substantial data set for the subfamily Triatominae and highlights some areas of interest for future research regarding this important group of insects.

MATERIALS AND METHODS

Materials. A total of 77 male specimens from 13 Triatominae species, several of which are major Chagas disease vectors, were collected from natural populations. Numbers of individuals analyzed and their geographic origins are detailed in Table 1. *Rhodnius prolixus* is the principal vector of Chagas disease in Central America and several countries of South America.[5] *R. ecuadoriensis* is the most important vector in several regions of Peru and Ecuador. *Triatoma brasiliensis* and *T. pseudomaculata* are the major vectors of Chagas disease in the arid Caatinga region of Northeastern Brazil. *T. maculata* is the second most important vector in Venezuela, whereas *T. barberi* and *T. longipennis* are important vectors restricted to Mexico. *T. nitida* and *T. ryckmani* are sylvatic species from Central America, with adults occasionally colonizing human-made constructions. *Panstrongylus geniculatus* and *P. rufotuberculatus* have a widespread geographic distribution (Central and South America) and are considered secondary vectors because of their increasing ability to invade and colonize domestic habitats. *Dipetalogaster maximus* (Baja California Sur, Mexico) and *Eratyrus cuspidatus* (Mexico, Central America, Colombia) are sylvatic species, occasionally colonizing human houses.

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DNA cell content measurements. The total nuclear DNA content of 13 species was measured from gonadal cells of male specimens (Table 1). Testes were dissected from live individuals and fixed in ethanol-acetic acid (3:1). Part of the fixed tissue was excised and deposited on excavated glass slides with a few drops of hypotonic DNA-staining buffer (HDSB, containing 0.1% trisodium citrate, 0.1% Triton X-100, 100 μg/mL RNase A, and 50 μg/mL propidium iodide). Tissues were minced using scalpel blades until homogeneous slurries were obtained. These suspensions were filtered through 60-μm nylon mesh and transferred to polypropylene tubes, and the glass slides washed with additional HDSB to a final volume of 2 mL. The suspensions were incubated for 30 minutes at 37°C in the dark with occasional vortexing. After this incubation period, and within a maximum period of 1 hour, measurement of cell DNA content was performed on an EPICS XL-MCL Flow cytometer (Coulter Electronics, Hialeah, FL) with an air-cooled argon-ion laser tuned at 488 nm and 15 mW. Propidium fluorescence (FL3), proportional to DNA content, was collected through a 650-nm DL dichroic filter fitted with a 625-nm BP band-pass filter. Forward and side scatter signals were used for morphologic assessment of the samples. Cell aggregates and coincident cells were excluded by analysis of the relationship between FL3 integral and peak signals. The DNA content in single cells was determined from FL3 linear histograms. The occurrence of a particular peak depends on the relative amount of the corresponding cell population in the tissue. For each sample, information for a minimum 10,000 nuclear events was acquired using the System II software program (Beckman-Coulter).

For the evaluation of absolute DNA content, a sample of normal human lymphocytes fixed in ethanol/acetic acid was used as internal reference. Lymphocytes were mixed and stained together with gonadal cells to standardize the procedure. To translate relative DNA cell content into picograms of DNA, the human lymphocyte control was calculated to have 6.436 pg of DNA per diploid nucleus (2C). This value is based on the number of base pairs reported by the International Human Genome Sequencing Consortium.[13] The absolute DNA amount was calculated from the ratio of the mean channel of the insect haploid peak to the mean channel of the human lymphocyte diploid G_0/G_1 peak. Base pair number was calculated using the equivalency of 1 pg of DNA = 0.978×10^9 bp.[14] The flow cytometer was calibrated daily with standard FlowSet fluorescent microspheres (Coulter Cytometry). An individual of *T. delpontei*, a species previously analyzed with flow cytometry,[12] was also measured in this study as an internal control for Triatominae.

RESULTS

A DNA flow cytometric profile was obtained for each sample, although only a representative group of histograms is presented in Figure 1. These histograms are characterized by the presence of fluorescence peaks corresponding to cell populations with different amount of DNA: 1C (haploid value) from spermatids and spermatozoids; 2C (diploid value) from cells in G_0/G_1 phase (before DNA duplication) and meiotic cells in second division; and 4C from mitotic and meiotic cells in G_2 phase (after DNA duplication). The occurrence of a particular peak depends on the relative amount of the corresponding cell population in the piece of testis used. All species had at least a haploid peak (1C), whereas all other fluorescence peaks corresponded to 2C, 4C, or control values (Figure 1A, C, E, and F). We found that the interspecific range of the haploid genome size varies from 0.66 (Figure 1A) to 2.67 pg of DNA (Figure 1B). *R. ecuadoriensis* contained the lowest haploid DNA amount of all species (C = 0.67 pg; Figure 1A), whereas *T. longipennis* had the lowest DNA content for the genus *Triatoma* (C = 0.84 pg; Figure 1D). *T. delpontei* had the highest haploid DNA content (C = 2.67 pg) of all species analyzed (Figure 1B).

Table 1 summarizes the haploid DNA content (C value) expressed in picograms (mean values and SD) measured in 13 triatomine species analyzed herein, along with 18 species or populations previously studied.[8–12] For comparative purposes, the diploid chromosome number (2n) and the quantity of autosomal heterochromatin for each species are included in Table 1 (F. Panzera and others, unpublished data).[9,10,15–17]

DISCUSSION

Genome size data in Triatominae. Genome size is a species-specific characteristic that has fundamental biologic significance, and the knowledge of which has many uses and bears on areas of study as different as physiology, developmental biology, and ecology.[6] Information regarding the genome size of triatomines has been published in only five previous reports that included 12 of the 137 species belonging to the subfamily.[8–12] Despite the restricted number of studies and species analyzed, conflicting DNA values have been reported for the same species (Table 1) using different methodological approaches: traditional Feulgen densitometry and flow cytometry. DNA values obtained with densitometry are always less than, and even half of, that obtained by flow cytometry (see *R. prolixus*, *T. delpontei*, *T. pseudomaculata,* and *T. rubrovaria* in Table 1). In organisms with small C values, similar to triatomines, Feulgen staining methods generally give lower values than flow cytometry.[18] Striking variation of DNA content values for the same species determined by different methods have been reported in species of the genus *Arachis* and *Cajanus*.[19,20] Critical points in the technical procedure (particularly the quantitative Feulgen reaction, including hydrolysis and post-hydrolysis steps) and the accuracy of the knowledge of the reference DNA standard used in the experiments may also contribute to the observed differences.[20] It is recommended that the reference standard of choice had 2C and 4C nuclear DNA content peaks similar to, but not overlapping with, the 2C and 4C peaks of the target species.[21] For flow cytometry, we chose human lymphocytes as a reference standard, because of its closer DNA value to triatomines, instead of the calibrations standards previously used in densitometry (*Bufo paracnemis* and *Allium cepa*). However, despite the discrepancies in the absolute DNA values, both methods yielded similar DNA relative values and showed the same tendencies: the genomes of *T. pseudomaculata* and *T. rubrovaria* have similar size, the genome of *T. infestans* (non-Andean countries) is twice that of *Rhodnius*, and *T. delpontei* always has almost double the DNA content of *T. infestans*, independently of the DNA method used.

Our research group has applied both methodologies in triatomines and observed that both seem to be valid to provide relative values. However, as observed in other organisms,

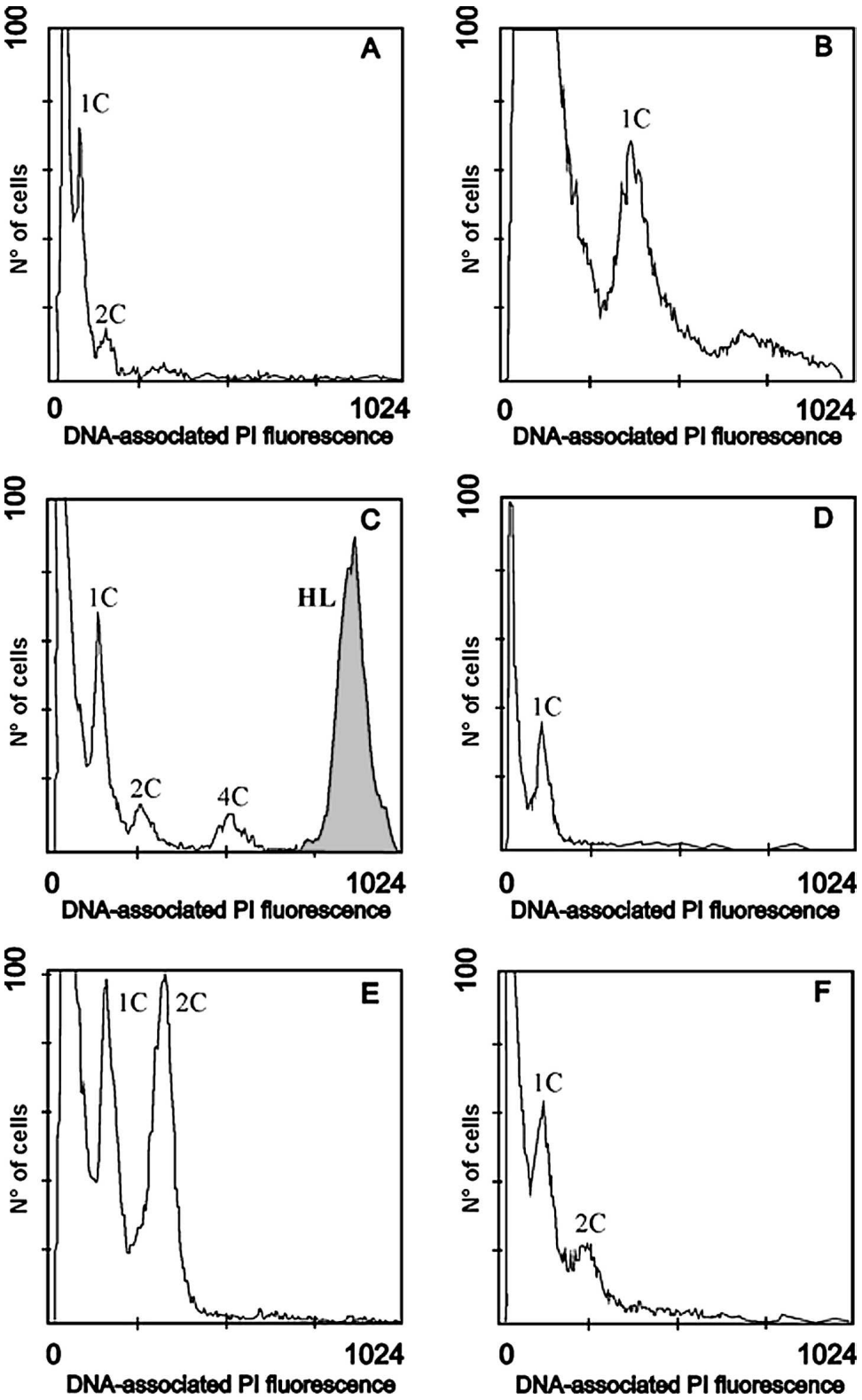

FIGURE 1. DNA flow cytometric histograms showing the distribution of testis cells from different Triatominae species. Relative intensity (in arbitrary units) of DNA associated PI fluorescence are shown on the x-axis. The corresponding number of cells is displayed on the y-axis. **A,** *R. ecuadoriensis*: smallest value of haploid DNA content observed in Triatominae species (C = 0.67 pg). **B,** *T. delpontei*: largest value of haploid DNA content observed in this subfamily (C = 2.67 pg). **C,** *T. brasiliensis*: three peaks are observed corresponding to 1C, 2C, and 4C DNA values (C = 1.00 pg) and the G_0 human diploid lymphocytes (HL, gray peak) used as an internal reference (2C = 6.436 pg). **D,** *T. longipennis*; this species has the smallest DNA value observed in *Triatoma* species (C = 0.84 pg). **E,** *D. maximus* (C = 1.19 pg). **F,** *T. maculata* (C = 0.99 pg).

TABLE 1
Summary of the haploid DNA content (C value) reported for species within the Triatominae, expressed in picograms

Species and geographic origin (department/state, country, and habitat)	C value (mean ± SD)	No. cells per sample	No. specimens analyzed	Methodology used	Reference	2n	Percent autosomal C-heterochromatin
Dipetalogaster maximus Baja California Sur, Mexico, S.	1.19 ± 0.09	10.000	5	FC	This report	20A + XY	0
Eratyrus cuspidatus Santander/Sucre, Colombia, S.	1.16 ± 0.07	10.000	5	FC	This report	20A + X_1X_2Y	0
*Panstrongylus herreri**	0.61	25	1	FD	(8)	20A + X_1X_2Y	?
P. geniculatus, Antioquia, Colombia, P.	1.31 ± 0.06	10.000	5	FC	This report	20A + X_1X_2Y	0
*P. megistus**	0.59	25	1	FD	(8)	18A + X_1X_2Y	?
P. rufotuberculatus, Antioquia/Santander, Colombia, S.	1.32 ± 0.07	10.000	5	FC	This report	20A + X_1X_2Y	16–20
Rhodnius ecuadoriensis, La Libertad, Peru, I.	0.66 ± 0.08	10.000	3	FC	This report	20A + XY	0
*R. prolixus** Santander, Colombia, I.	0.69 ± 0.05	10.000	6	FC	This report	20A + XY	0
Triatoma barberi, Queretaro/Oaxaca, Mexico, I.	1.13 ± 0.03	10.000	6	FC	This report	20A + X_1X_2Y	30–35
T. brasiliensis, Ceará, Brazil, S, P, I.	1.00 ± 0.07	10.000	12	FC	This report	20A + XY	25–32
T. delpontei, Argentina and Uruguay	1.80 ± 0.17	100	2	FD	(9)	20A + XY	45
Bolivia	2.67 ± 0.08	10.000	6	FC	(12)	20A + XY	45
T. dimidiata, Mexico, Guatemala, Colombia and El Salvador	0.99 ± 0.06	10.000	62	FC	(11)	20A + X_1X_2Y	5–10
Yucatán (Mexico)	0.84 ± 0.03	10.000	6	FC	(11)	20A + X_1X_2Y	0
Petén (Guatemala)	0.90 ± 0.06	10.000	6	FC	(11)	20A + X_1X_2Y	0
*T. infestans**	0.82	25	3	FD	(8)	20A + XY	?
Uruguay	1.03 ± 0.13	100	2	FD	(9)	20A + XY	24–30
Non-Andean countries	1.40 ± 0.11	10.000	30	FC	(10)	20A + XY	24–30
Andean countries	1.82 ± 0.15	10.000	12	FC	(10)	20A + XY	46–56
T. longipennis, Nayarit, Mexico, P.	0.84 ± 0.06	10.000	7	FC	This report	20A + X_1X_2Y	0
T. maculata, Magdalena, Colombia, S.	1.00 ± 0.07	10.000	6	FC	This report	20A + XY	5–10
T. melanosoma, Argentina	1.41 ± 0.08	10.000	4	FC	(12)	20A + XY	24–30
T. nitida, Quiché, Guatemala, I.	1.24 ± 0.07	10.000	8	FC	This report	18A + X_1X_2Y	25–30
*T. platensis**	0.70	25	3	FD	(8)	20A + XY	?
Uruguay	0.87 ± 0.12	100	2	FD	(9)	20A + XY	12
Uruguay	1.22 ± 0.03	10.000	4	FC	(12)	20A + XY	12
*T. pseudomaculata**	0.57	25	1	FD	(8)	20A + XY	?
Ceará, Brazil, P.	1.04 ± 0.07	10.000	4	FC	This report	20A + XY	5–10
T. rubrovaria, Uruguay	0.54 ± 0.08	100	2	FD	(9)	20A + XY	0
Uruguay	1.08 ± 0.03	10.000	4	FC	(12)	20A + XY	0
T. ryckmani, El Progreso, Guatemala, S.	1.01 ± 0.07	10.000	5	FC	This report	20A + X_1X_2Y	0
T. sordida, Brazil	1.03 ± 0.06	10.000	4	FC	(12)	20A + XY	30–35
*T. vitticeps**	0.89	25	1	FD	(8)	20A + $X_1X_2X_3Y$	?

We included the geographic origin and habitat of the insects studied in this paper.
Different internal references were used: *Bufo paracnemis* (2C = 7.57 pg)[8], *Allium cepa* (2C = 33.60 pg)[9], and *Homo sapiens* (2C = 6.436 pg)[10–12] and this report.
* The geographic origin of the material analyzed by Schreiber and others is uncertain.
S, sylvatic; P, peridomestic; I, intradomestic; FD, Feulgen densitometry; FC, flow cytometry with propidium iodide; 2n, diploid chromosome number in males; A, autosomes.

flow cytometry has the following advantages for DNA measurement according to Ciudad and others[22]: 1) it permits the analysis of a large number of nuclei within a few minutes, thereby providing statistically reliable results in a short period of time (in triatomines, the number of cells and individuals analyzed by flow cytometry is much higher than that used in Feulgen studies); 2) flow cytometry has high precision and objectivity, which increases reproducibility of results (SDs for DNA content measurements reported herein are lower than those obtained using the traditional Feulgen densitometry; see Table 1); and 3) standardization of operating procedures is easier using flow cytometry.

Although flow cytometry can be used with any cell type, gonadal cells are the type of choice, because both haploid and diploid peaks in the same profile lead to a more accurate estimate of DNA content (Figure 1A, C, E, and F).

DNA variation within the Triatominae subfamily. The causes and consequences of cellular DNA content variation is highly significant and, in recent years, new focuses are providing insights into the possible mechanisms associated with an increase or decrease in genome size.[6] Analysis of the 20 species studied to date using flow cytometry, standardized procedures, and internal controls (Table 1) indicates that the haploid genome in the Triatominae varies nearly 4-fold, from 0.66 pg in the *Rhodnius* species to 2.67 pg in *T. delpontei*. Although this range is relatively small when comparing all species of Heteroptera (0.3–5.4 pg), the mean haploid genome size for the Triatominae (1.17 ± 0.07 pg) is very similar to the value proposed for all Heteroptera (1.18 ± 0.18 pg).[6]

The magnitude of genome size variation in the Triatominae contrasts somewhat with the chromosome number stability within this subfamily.[5] Because chromosome number is basically constant, we can discard the existence of correlation between both parameters. However, there seems to be a positive correlation between genome size and chromosome size. *Rhodnius* spp. have low DNA values and reduced chromosome size. Species with large chromosomes, such as those within the *Panstrongylus*, *Dipetalogaster*, *Eratyrus,* and *Triatoma* genera, have higher DNA values. This divergence is probably caused by the polyphyletic origin of the subfamily.[23]

As previously described for some *Triatoma* species, genome size heterogeneity seems to be strongly associated with the presence of heterochromatic blocks detected by C-banding techniques.[9–11] These heterochromatic regions, com-

posed of both moderate and highly repetitive sequences, are a major component responsible for the increase in genome size. *T. delpontei* has the largest DNA content and quantity of heterochromatin among all the Triatominae (45% of the autosomal complement). Other species with large heterochromatic blocks also have high DNA content (i.e., *T. nitida* and *T. barberi*). The relationship between heterochromatin and genome size explains most of the differences among populations and within some groups of species. This can be clearly observed in species of the infestans subcomplex (i.e., *T. infestans*, *T. platensis*, *T. melanosoma,* and *T. delpontei*) and protracta complex (including *T. barberi* and *T. nitida*). Among these species, changes in the amount and distribution of heterochromatin are not only the main source of karyologic differentiation but also the likely cause of variation in their DNA genome size.[5–9]

Considering all species and populations studied by flow cytometry until now, there exists a strongly positive correlation between DNA and heterochromatin content (correlation coefficient = 0.70). However, as observed in other heteropteran species,[24] the consistent association between heterochromatin and DNA content is not a general rule, particularly when the taxa are not so closely related. Genomic size changes in distantly related species of triatomines (i.e., between species from different genera or among species of the same genus but belonging to different complexes) seem to involve variation in both heterochromatin and euchromatin content.

In more phylogenetically distant *Triatoma* species with the same chromosome number and no heterochromatin in the autosomes, such as *T. longipennis* and *T. ryckmani* (see Table 1), genome size variations must involve changes in the euchromatin. As described for other organisms, changes in the euchromatic portion of the genome can be primarily attributed to modulation of the noncoding, repetitive DNA content.[6]

Prospects for future research of flow cytometry in Triatominae. Three major areas of future research are likely to be especially successful to the application of flow cytometry in Triatominae. First, genome size data can be used for species identification, a particularly relevant goal for a subfamily with multiple taxonomic uncertainties that involve species with important epidemiologic roles as Chagas disease vectors. Our results reveal that species that are difficult to differentiate using morphology and DNA analyses (i.e., *T. platensis* and *T. delpontei*) have very different DNA contents (1.22 and 2.67 pg, respectively). These results encourage similar studies for species groups that have systematic uncertainties, such as the sordida, phyllosoma, protracta, and brasiliensis complexes.[5] Recently, we identified sibling or cryptic species in *T. dimidiata* with differences in DNA and heterochromatin contents. This DNA variation could be detected by flow cytometry even though they involve only ~10% of total DNA.[11]

Second, flow cytometry can be applied to detect intraspecific genomic variations. An excellent example is observed in *T. infestans*, the vector responsible for about one half of the 13 million cases of Chagas disease reported worldwide. This species exhibits striking differences in heterochromatin and genome size that can be used to identify populations having different ecological characteristics.[10] This type of variation is expected in species with a widespread distribution such as *P. geniculatus*, in which the occurrence of an extensive C-heterochromatic polymorphism is potentially indicative of DNA variation.[17]

It would be worth considering whether there is any association between genome size variation and other parameters such as ecological niche, geographic distribution, phenotypic characteristics, development rate, and phylogeny.[6] Some of these parameters are particularly relevant in triatomines, because they are related to the bug's capacity to invade and colonize human dwellings, thereby increasing their potential role as Chagas disease vectors. It is noted that genome size variations have been reported among populations with different vectorial capacities,[10,11] but more detailed studies are necessary to achieve conclusive associations. Finally, the determination of genome size in more triatomine species and their comparison with other reduviids will provide additional insights about the debate of monophyly or polyphyly of the Triatominae subfamily.[23]

In conclusion, we believe that the use of flow cytometry to measure genome size in the Triatominae is a powerful tool applicable to population, taxonomic, and evolutionary studies. Moreover, its use will advance our understanding of the large-scale organization of triatomine genomes.

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