Spatial diversification of *Panstrongylus geniculatus* (Reduviidae: Triatominae) in Colombia

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Abstract

Background

Triatomines are responsible for the most common mode of transmission of *Trypanosoma cruzi*, the etiologic agent of Chagas disease. Although, *Triatoma* and *Rhodnius* are the vector genera most studied, other triatomines such as *Panstrongylus* can also contribute to *T. cruzi* transmission creating new epidemiological scenarios that involve domiciliation. *Panstrongylus* has at least twelve reported species but there is limited information about their intraspecific diversity and patterns of diversification. Here, we began to fill this gap, studying intraspecific variation in Colombian populations of *P. geniculatus*.

Methodology/Principal finding

We examined the pattern of diversification as well as the genetic diversity of *P. geniculatus* in Colombia using mitochondrial and ribosomal data. We calculated genetic summary statistics within and among *P. geniculatus* populations. We also estimated genetic divergence of this species from other species in the genus (*P. lignarius* and *P. megistus*), and with these samples, we obtained ML and BI topologies as well as haplotype networks. We also dated the *P. geniculatus* lineages. The total evidence tree recovered four clades within *P. geniculatus* that are consistent with genetic structure by geography with no effect of the Andes orogeny or isolation by distance. We also report the first case of heteroplasmy in *Panstrongylus* samples. These multiple haplotype in the sequences did not show major phylogenetic incongruences.

Conclusions/Significance

We found considerable divergence among *P. geniculatus* Colombian populations consistent with geography. This pattern is not linked to past geological events such as the Andean uplift nor is related to isolation by distance. Other factors including anthropogenic and eco-epidemiological effects await to be explored to explain the existence of *P. geniculatus* recent geographical lineages. Broadening the knowledge of *P. geniculatus* is necessary for the accurate development of effective strategies for vector control of Chagas disease.

Author summary

We present the first approximation of genetic diversity, phylogenetic relationships and population structure of *P. geniculatus* in Colombia. We compiled an extensive sampling to construct a phylogeny and estimate the genetic structure of the populations found. The main four clades showed a remarkably recent divergence time suggesting that geography changes are not the main factor shaping the genetic structure of *P. geniculatus*. We discuss several epidemiological consequences for the diversification of this vector and its importance for vector control programs.

Introduction

Chagas disease affects about six million people in Latin America and is caused by the parasite *Trypanosoma cruzi*, which is transmitted mainly by insects of the subfamily Triatominae (Hemiptera: Reduviidae) (1). The subfamily Triatominae is composed by 5 tribes, 15 genera and 149 described species (2) and only few genera are involved in the transmission of *T. cruzi* (3,4). In particular, the genera *Triatoma, Rhodnius* and *Panstrongylus* are the main vectors that transmit the parasite to humans due to their capacity of domiciliation (1,5). After *Triatoma* and *Rhodnius*, *Panstrongylus* is the genus with more species (currently 13), some of which appear to be involved in a domiciliation process (where at least three development life stages can be found in the domicile) (4). However, studies on *T. cruzi* transmission and control strategies have focused mainly in *Rhodnius* and *Triatoma*, and secondary vectors such as *Panstrongylus* remains unstudied. The species relationships, genetic

diversity and evolutionary trends are also understudied for this genus in contrast to what is known for *Rhodnius* and *Triatoma.*

Within *Panstrongylus*, the specie *P. geniculatus* is known to have domiciliated. The incursion of this species in the domicile is relevant due to the irruption of *T. cruzi* sylvatic strains that are more virulent compared to the domestic strains (10,12). This changes in vector behavior creates new transmission dynamics of *T. cruzi* and emerging challenges for the control of Chagas disease (2,4,6).

P. geniculatus occurs in 18 countries of Latin America, from Mexico to Argentina, where it has the widest range of habitats, including dry, humid, rainy forest and savannah (4). *P. geniculatus* is one of the primary incriminated vectors of oral Chagas Disease outbreaks in Colombia and Venezuela (7–9) with reports of higher parasitemia compared to other infection routes (2,7,10). Specifically, in Colombia, the domiciliation process of *P. geniculatus* was mainly associated with light attraction (11). Since *P. geniculatus* occurs in 25 departments of Colombia (13), the domiciliation process represents an increase of risk of new Chagas disease cases. Previous studies indicated that the infection rate of *P. geniculatus* with *T. cruzi* was higher than 80% (14) and that in seven Colombia departments, this species had the highest frequency of infection with *T. cruzi* (1).

Besides the epidemiological importance of this species, its current classification is difficult and based exclusively on morphological characters (4). Also, morphometric analyses have revealed high morphological variability within *P. geniculatus* (4,15–17). Despite the advances in molecular methods, few studies have used DNA markers to understand the species relationships in *Panstrongylus* and the phylogenetic status of this genus in the Triatominae subfamily. Previously, studies have shown incongruence in the phylogeny of the species suggesting polyphyly (18) and paraphyly (19). Furthermore, as far as we know, there is no information about diversity levels and divergence events in each species. Herein, we began to address this lack of information using molecular data to document *P. geniculatus* genetic variability and diversification in Colombia. Our results broaden the knowledge of this vector and contribute to a better understanding of the biology of *P. geniculatus*.

Methods

Sampling

A total of 128 samples corresponding to six species (103 *P. geniculatus*, 6 *P. lignarius*, 14 *P. megistus*, 2 *Triatoma infestans*, 2 *T. pallidipennis* and 1 *Triatoma* sp.) were used in this study. *Panstrongylus* spp. were collected in nine departments of Colombia (Casanare, Arauca, Meta, Santander, North Santander, Magdalena, Córdoba, Boyacá and Amazon) and in Venezuela (Caracas). The remaining samples were collected in Brazil and Bolivia (Table S1, Fig 1). We sampled insects in three different ecotopes: sylvatic (25m away from any residence), domestic (inside houses) and in the peridomestic ecotope (10m away from any residence). Insect capture in the sylvatic ecotope was performed using two techniques: manual search and modified Noireau baited chicken traps located in palms. Insects in the domestic ecotopes were collected by hand picking. Insects were identified using standard taxonomic keys (20) and stored individually in plastic containers with 100% ethanol. Upon arrival to the microbiology laboratory at Universidad del Rosario, these containers were stored at -20ºC until processing.

Figure 1. Map showing the location of the samples included in this study. The size of the circle is not representative of the number of individuals collected at each location.

DNA extraction, amplification and sequencing

DNA was extracted from the whole body (63 samples) and from the head, leg and thorax (68 samples) using the DNeasy® Blood & Tissue kit (Qiagen) (21) but doubling the amount of Buffer ATL, proteinase K, Buffer AL and ethanol. In addition, the final elution step was performed with only 150 µL of Buffer AE. The concentration and quality of DNA was measured at 260/280 and 230/260nm using a Nanodrop 1000 spectrophotometer. The quality of the DNA was also verified in 2% agarose gels.

We used five sets of primers to amplify ribosomal (rDNA) and mitochondrial gene fragments (Table 1). We amplified 823 bp of *18s* (19) and 696 bp of *28s* (22) and for the mitochondrial loci, we amplified 630 bp of NADH dehydrogenase subunit 4 - *ND4* (23), 552 bp of *Cytochrome b* - *Cytb* (19) and 508 bp of *16s* (19). PCR reactions for all gene fragments markers were conducted in a final volume of 12.5 µl using 1.5µl of DNA template, 6.25µl of GoTaq Green Master Mix (2x), 1.25 µl (10 µM) of each primer and 2.25 µl of Nuclease Free Water. The fragments were amplified with previously reported thermal cycling conditions (see references in Table 1). For all loci, we visualized 2 µL of the PCR product in a 1.5% agarose gel stained with SYBR® Safe DNA Gel Stain to verify the success of the PCR. Samples that showed a solid band of the expect size were purified with ExoSAP-IT and then sequenced by Sanger sequencing (both strands) in MACROGEN (Seoul, South Korea) using a ABI3100 machine. The output sequences were read and assembled to contigs with Geneious 11.0.5 (24). After base calling and editing, we used the same program to obtain a consensus sequence per sample. We also used a plug-in of Geneious to construct Muscle alignments for each locus (25). The resulting alignments were manually inspected for misalignments and ambiguities. In cases where heterozygosity/heteroplasmy was present, different haplotypes for each locus were inferred with the PHASE algorithm implemented in DnaSP v5.10 (26) with 1000 iterations per simulation.

Table 1. Primers for mitochondrial and ribosomal gene fragments.

Gene fragment	**Primers**	**Region amplified (pb)**
[a] NADH dehydrogenase subunit 4 -*ND4* (23)	Primers Forward: TCAACATGAGCCCTTGGAAG	630
	Primers Reverse: ATTCGTTGTCATGGTAATG	
[a]*Cytochrome b* - *Cytb* (19)	Primers Forward: GGACG(AT)GG(AT)ATTTATTATGGATC	522
	Primers Reverse: ATTACTCCTCCTAGYTTATTAGGAATT	
[a]*16s* (19)	Primers Forward: CGCCTGTTTATCAAAAACAT	508
	Primers Reverse: CTCCGGTTTGAACTCAGATCA	

[b]*18s* (19)	Primers Forward: AAATTACCCACTCCCGGCA	823
	Primers Reverse: TGGTGUGGTTTCCCGTGTT	
[b]*28s* (22)	Primers Forward: GCGAGTCGTGTTGCTTGATAGTGCAG	696
	Primers Reverse: TTGGTCCGTGTTTCAAGACGGG	

[a]Mitochondrial marker; [b]ribosomal (rDNA) markers

Tree estimation and dating

We determined the best-fit nucleotide substitution model for each locus using the Bayesian Information Criterion (BIC) (27) in IQ-Tree (28). Phylogenetic reconstruction was performed with maximum likelihood (ML) in the same program (28), and using *Triatoma* spp. as outgroups. For ribosomal loci, we used *T. infestans* and *T. pallidipennis* as outgroups and for the mitochondrial loci, we used *T. infestans.*

For mtDNA, the ML tree was constructed using a concatenated alignment with unlinked substitution model per locus. We also evaluated the ML reconstruction for each locus (Fig S1). Afterwards, topologies were estimated with Bayesian inference (BI) using BEAST 2.5.1 (29) only for mitochondrial concatenated loci since they these revealed the presence of recent lineages in *P. geniculatus* and resolved its phylogenetic relationships with other *Panstrongylus* species (see results). We performed two independent runs with 100 million generations, sampling every 1000 generations and discarding 10% as burn-in. We used an uncorrelated lognormal relaxed clock to estimate divergence times, as this clock model allows each branch of a phylogenetic tree to have its own clock rate; we also used a fossil calibration with an age range reported for *P. hispaniolae* of 20.44-13.82 Ma (30). We analyzed the Beast output with Tracer v1.7.1 (31) to evaluate if the analysis yielded sufficient number of independent samples from the posterior distribution (effective sample size >200). To obtain the tree that best represents the posterior distribution, we used TreeAnnotator

v.1.10.4 (29) with a 10% of burning percentage. Afterwards, all trees (ML and BI) where visualized and edited using FigTree v.1.4.4 software (32).

Population genetics

We calculated networks per locus to visualize the haplotype diversity and clustering of the three *Panstrongylus* species (*P. geniculatus*, *P. lignarius*, and *P. megistus*) using algorithms of minimum distance in the program PopArt (33). The same program was used to build haplotype networks among *P. geniculatus* populations.

Genetic diversity statistics were calculated for the clusters revealed by the haplotype networks: nucleotide diversity (π), substitution rate (θ), number of segregating sites (S) and Tajima's D test. These stats were calculated with DNASP V5.10 (26). Genetic structure between species/geographical groups was estimated with F_{ST} (34), D_{XY} and Da (35). The effect of isolation by distance (IBD) in the population structure was evaluated with a Mantel test (36) using the R package vegan (37). To do so, the matrix of genetic distances was estimated by linearizing F_{ST} values (38) and the pairwise geographic distances among localities was processed using the function *distm* from R package *geosphere* (39). We also performed a linear correlation between geographic distances and genetic distances as recommended by (40) with the entire dataset and without extreme points.

Results

Heterozygous sites and evidence of heteroplasmy

We observed ambiguous bases in the chromatograms in multiple *P. geniculatus* samples and in all mitocondrial loci amplified (Table 2, Fig S2). In contrast, the samples of the other species of Panstrongylus evaluated in this study did not present heteroplasmy.

Table 2. Genetic marker, ID, number of nucleotide ambiguities per sequence of *P. geniculatus*

Genomic marker	Sequence ID	W	M	Y	R
16s	26-MetaPg	-	1/508	-	-
	122-SNSMPg	1/508	-	-	-
Cytb	31-MetaPg	-	-	11/550	2/550
	84-CordobaPg	-	-	1/550	2/550
	130-Leticiag	-	-	1/550	-
ND4	23-MetaPg	-	-	4/630	1/630
	29-MetaPg	-	-	1/630	-
	39-MetaPg	-	-	4/630	1/630
	42-MetaPg	-	-	-	1/630
	45-MetaPg	-	-	6/630	4/630
	54-CasanarePg	-	-	6/630	3/630
	59-CasanarePg	-	-	7/630	6/630
	61-CasanarePg	-	-	3/630	3/630
	70-AraucaPg	-	1/630	-	-
	72-AraucaPg	-	-	2/630	1/630
	76-AraucaPg	-	-	-	2/630
	79-AraucaPg	-	-	1/630	3/630
	121-SNSMPg	-	-	2/630	-

Nucleotide ambiguity codes based on IUPAC designations: W = A/T; M = A/C; Y = A/C and R = A/G. Numbers in each cell represents the number of sites with the IUPAC code in the total length of the sequence for each gene.

Molecular phylogenetics and divergence times

For the ML phylogeny reconstruction, the most suitable models were TIM2+F+I+G4 for *Cytb*, TN+F+R3 for *ND4*, HKY+F+G4 for *16s*, K2P for *18s* and HKY+F+I for *28s*. The two ribosomal loci showed little phylogenetic resolution (Fig. 2). Specifically, the 28s gene fragment revealed *P. lignarius* and *P. megistus* as sister species, although but this node is not well supported (Fig 2) while the *18s* showed the three species intermixed (Fig 2). Two samples of *T. pallidipennis* are closely related to *Panstrongylus* in both genes (Fig 2).

Fig 2. Phylogenetic trees (top) and haplotype networks (bottom) inferred with rDNA. Numbers on the nodes are the bootstrap support values. Each tick on the haplotype network represents a mutational step.

In contrast, mitochondrial gene fragments resulted much more informative ML and BI topologies were completely concordant and revealed three highly supported clades corresponded to the *Panstrongylus* species, namely *P. geniculatus, P. megistus* and *P. lignarius* (Fig 3). Within *P. geniculatus*, we recovered four clades associated, to some extent, with geography: 1) East of the Eastern Cordillera of the Colombian Andes clade (BS=58, PP=0.99) that include the departments Arauca, Casanare, Meta, Amazonas, Córdoba and some individual of Santander, Boyacá and Magdalena, 2) West of the Eastern Cordillera of the Colombian Andes clade (BS=100, PP=1) which includes the rest of Santander and Boyacá samples, 3) North of Santander clade (NSant; BS=100, PP=1) that only includes samples collected there and 4) Magdalena (Sierra Nevada of Santa Marta - SNSM) with Venezuela (SNSM-Ven; BS= 100, PP=1) includes all individuals from Venezuela and almost all of the samples of Magdalena except for one that was recovered in the East clade.

Figure 3. Consensus phylogenetic tree inferred with mitochondrial DNA. Numbers in the nodes are the support values of bootstrap/posterior probabilities.

Figure 4. Consensus phylogenetic ML (left side) and BI (right side) tree inferred with mitochondrial DNA. Numbers on the nodes are the support values (Bootstrap for ML and Posterior probability for BI). Color highlighted individuals indicate where they were collected based on the color used for each clade. For BI, branch labels are posterior probabilities and node labels are estimated divergence times. Each blue bar corresponds to the 95% HDP interval. Tip label of the East clade correspond to the identifiers (IDs) described in Table S1.

Population genetics

Mitochondrial networks clustered haplotypes by species with multiple mutational steps among them (Fig 5). Consistently, we found high genetic differentiation between the *P. geniculatus*, *P. lignarius* and *P. megistus* (Table 4). *P. geniculatus* nucleotide diversity was higher in comparison with the other species (Table 3), suggesting an increased variability in this species, we cannot rule out a sample size effect. A close up of the *P. geniculatus* haplotype networks confirmed the geographic groups previously described (Fig 6) that are genetically differentiated (Table 4). However, there are some shared haplotypes between groups (Fig 6; Fig 4); the East group shared haplotypes with West and SNSM-Ven groups (Fig 6). Isolation by distance was ruled out as a causal factor contributing to the geographical genetic structure (Table 5, Fig 7) and even though we found significant correlation between geographic and genetic distances in some cases, the adjust of the lineal model is extremely poor and the Mantel test was non-significant.

Figure 5. Haplotype networks for mitochondrial loci grouped by species. Each tick on branches represents a mutational step.

Figure 6. Haplotype networks for mitochondrial loci grouped by geographic location. Each tick on branches represents a mutational step.

Figure 7. Isolation by distance plots for mitochondrial loci. Two tests of correlation were carried out: with all the data (top) and without extreme points (bottom)

Table 3. Genetic summary statistics for the three species used in this study and for the Eastern, Western, North Santander (NSant) and Magdalena-Venezuela (SNSM-Ven) groups for each mitochondrial locus.

Genomic marker	Population	n	*h*	S	π± SD	D
Cytb	***P. geniculatus***	84	43	134	0.039±0.004	-0.671
	P. megistus	13	2	21	0.020±0.003	2.539
	P. lignarius	1	1	0	0	----------
	East	61	30	54	0.022±0.002	0.185
	NSant	2	1	0	0	---------
	SNSM-Ven	7	3	17	0.014±0.005	0.775
	West	14	10	99	0.066±0.010	0.734
ND4	***P. geniculatus***	92	60	145	0.045±0.003	-0.26792
	P. megistus	12	7	62	0.031±0.012	-0.28969
	P. lignarius	6	2	20	0.012±0.007	-1.49247
	East	65	44	76	0.028±0.002	0.081
	NSant	4	4	3	0.003±0.001	2.012
	SNSM-Ven	12	6	23	0.013±0.003	-0.050
	West	10	7	92	0.054±0.011	0.039
16s	***P. geniculatus***	92	35	48	0.014±0.002	-0.84369
	P. megistus	14	4	4	0.003±0.001	1.05159
	P. lignarius	6	2	2	0.001±0.001	-1.13197
	East	65	18	16	0.006±0.001	-0.185
	NSant	2	1	0	0	---------
	SNSM-Ven	7	6	1	0.007±0.001	1.381

	West	18	11	34	0.027±0.002	1.400

n: number of sequences; h: number of haplotypes; S: number of segregating sites; π: nucleotide diversity; D: Tajima's D. None of the loci showed Tajima's D values that departed from neutral expectations.

Table 4. Measures of population structure between species and *P. geniculatus* geographical clusters for mtDNA loci.

		Cytb				***ND4***				***16s***			
Population 1	**Population 2**	**F_{ST}**	**D_{XY}**	**Da**	**P-value**	**F_{ST}**	**D_{XY}**	**Da**	**P-value**	**F_{ST}**	**D_{XY}**	**Da**	**P-value**
P. geniculatus	***P. megitus***	0.82133	0.16425	0.13489	0.00001***	0.75562	0.15771	0.11917	0.00001***	0.91053	0.0842	0.07667	0.00001***
P. geniculatus	***P. lignarius***	---------	---------	---------	---------	0.82312	0.15882	0.13073	0.00001***	0.91003	0.0949	0.08636	0.00001***
P. megitus	***P. lignarius***	---------	---------	---------	---------	0.86254	0.15546	0.13409	0.00001***	0.96861	0.075	0.07264	0.00001***
SNSM-Ven	**West**	0.4626	0.0749	0.0347	0.0000***	0.6406	0.0851	0.0569	0.00001***	0.4173	0.0293	0.0122	0.00001
SNSM-Ven	**NSant**	0.8828	0.0614	0.0542	0.045*	0.8737	0.0609	0.0532	0.002**	0.7564	0.0150	0.0113	0.037*
SNSM-Ven	**East**	0.6891	0.0588	0.0405	0.00001***	0.5503	0.0447	0.0246	0.00001***	0.4564	0.0126	0.0057	0.00001***
West	**NSant**	0.5991	0.0824	0.0493	0.028*	0.7013	0.0927	0.0650	0.006**	0.5394	0.0247	0.0081	0.005**
West	**East**	0.1570	0.0523	0.0082	0.00001***	0.5379	0.0876	0.0471	0.00001***	0.3281	0.0247	0.0081	0.00001***
NSant	**East**	0.8527	0.0752	0.0641	0.001**	0.7435	0.0617	0.0459	0.00001***	0.7129	0.0111	0.0079	0.00001***

Probability obtained by the Hudson Permutation test with 1000 replicates. *, 0.01<P<0.05; **, 0.001<P<0.01; ***, P<0.001. "--------": some statistics could not be calculated due to low number of haplotypes per population.

Table 5. Isolation by distance analyses. Mantel and correlation test were performed with: all samples and without extreme points.

	Entire Data				**Excluding extreme points**			
Locus	**R**	**R^2**	**P value**	**Mantel r**	**P-value**	**R**	**R^2**	**P-value**
16s	0.3121	0.0974	0.0046**	0.1442	0.2548	0.8107	0.6572	0.5143
Cytb	0.1935	0.0374	0.0538	0.0672	0.3373	0.2025	0.0410	0.0616
ND4	0.2422	0.0587	0.0294*	0.0998	0.2328	0.0813	0.0066	0.5164

P-value: *, 0.01<P<0.05; **, 0.001<P<0.01; ***, P<0.001

Discussion

Increasingly evidence suggest that some of the species of *Panstrongylus* should be consider as primary vectors for Chagas disease. Despite of this, few studies are known about the genetic diversity of these species. Specifically, few representatives of this genus were used to establish the phylogenetic relationships of the Triatominae subfamily (19,41), and a seminal study using ITS-2 sequences from six species showed that they are polyphyletic (18). In particular, *P. geniculatus* has become relevant over the years mainly for three reasons: its geographical distribution, record of domiciliation and its association with oral outbreaks mainly in Colombia and Venezuela. Phenotypic variability in this species has been studied at the morphological level (15,16,42) and as far as we know, only one karyotype is known for *P. geniculatus* (42). Intraspecific karyotypic and morphological diversity observed coupled with *P. geniculatus* wide distribution let to these authors to propose that this specie must be a complex of species (42). Yet, this asseveration awaits to be tested at the molecular level.

Heteroplasmy has been documented in other invertebrate organisms including *T. cruzi* (43,44). This is the first report to our knowledge of heteroplasmy in *Panstrongylus*. Remarkably, most examples of heteroplasmy come from experimental hybrids and natural hybrid zones (45) and rarely from natural populations as the one evaluated in this study. As shown in Table 2, all mitochondrial loci evaluated exhibit ambiguous bases which could lead to health problems to the organism (46). These problems could be associated with neurodegenerative complications (47) that may affect the transmission cycle. Further studies are required to test this premise. Moreover, heteroplasmy could cause ambiguities in the interpretation of phylogenetic, phylogeographic and population genetic data (48–50). In this study, even though we did find ambiguities in the sequences (Table 2, Fig S2), they did not reflect in phylogeny reconstruction. Some samples vary the relation found within the clade they belong but none of the haplotypes of the individuals with heteroplasmy were recovered in different clades (Fig 4).

We did not find genetic evidence for the existence of geographical lineages inside *P. geniculatus* in our rDNA data set (Fig 3 & 6), which can be explained by differences in coalescence times and effective population sizes between rDNA and mtDNA (51–53). Also, ML phylogeny reconstruction for the rDNA loci differed in the number of species of *Panstrongylus* recovered (Fig 2). Despite both *18s* and *28s* are rDNA fragments, each gene has different mutation rates that could be modified by different selection pressure that may be modeling de divergence rate of each gene (54,55). These gene fragments did not resolve intraspecific relations within *P. geniculatus* (Fig 2). The slow substitution of nucleotides in these fragments amplified was not informative to elucidate the relationships of the populations of *P. geniculatus* evaluated. Within the polytomy of *P. geniculatus*, both markers recovered the samples of *T. pallidipennis* as part of this unresolved clade. This may be explained by the other species used as outgroup -*T. infestans*-.

Previous molecular studies that include these species, have reported *T. pallidipennis* as part of the Phyllosoma subcomplex and *T. infestans* as part of the Infestans subcomplex (56–58). Phylogenetic reconstructions that include these species as well as other Triatominae species reveal that *P. geniculatus* is more closely related to *T. pallidipennis* than to *T. infestans* (19,59). This study also revealed paraphyly between *Triatoma* and *Panstrongylus* suggesting a close relationship among species and a possible shared ancestry (19). Closely related species of *Triatoma* and *Panstrongylus* shared a similar distribution and therefore *Panstrongylus* could have diverged from a *Triatoma* ancestor and have not completely diverged (56). These assumptions require further studies testing this hypothesis.

At a broad scale, we also were able to recover in mtDNA the relationships for the *Panstrongylus* species included here. *P. lignarius* was sister to *P. megistus*, and this clade was sister to *P. geniculatus* (Fig 3 & 5). This result contrast with the previous lack of resolution obtained with ITS-2 (18). Within *P. geniculatus*, even though genetic differences are emerging between geographical populations of this species, their genetic divergence is low to be considered as a complex of species (Table 3 & 4).

Although the genetic haplotype geographical distribution of *P. geniculatus* is suggestive of an Andean orogeny effect in its diversification, as observed in other Triatominae (41,59–61), we found that *P. geniculatus* originated approximately 50 thousand years ago, which is outside of the lapse of time of the Andean uplift (Eastern Cordillera achieve its final elevation ~2.5 Mya during the Pliocene (62)). A better geographical sampling at intra and interspecific levels is needed to confirm this result. A similar pattern has been observed in *Triatoma dimidiata* another species with high variance in phenetic and genetic diversity (23,63–65). In this study three genetic differentiated populations from three eco-geographical regions were described: Sierra Nevada of Santa Marta, Inter Andean Valleys and Caribbean Plains (65). Genetic structure in this case was correlated with eco-epidemiological and morphological traits and not with geographic events (65). Although the Andes uplift seems unimportant in shape the *P. geniculatus* geographical pattern observed here, mountains can also promote diversification through processes like niche partitioning, altitude gradients, climate variation and long-distance dispersal among others (22,66–68). Additional eco-epidemiological factors involving vector adaptability can also be shaping divergence within populations (67,69).

As previously described, *P. geniculatus* could be considered as a eurythermal species due to its wide adaptability to several life zones (4). The rapid uplift of the Andes created a broad dimension of new ecological niches with opportunities for colonization (62). Interestingly, shared haplotypes coupled with no evidence of isolation by distance among some of these regions, account for long dispersal gene flow (Table 5, Fig 6 and Fig 7). Thus, long-distance dispersal coupled with niche colonization could facilitate the admixture between *P. geniculatus* populations occurring at opposite sides of the Andes. It is possible that dispersion and gene interchange could be facilitated by incomplete lineage sorting since the divergence of these geographical lineages is recent. However, shared variation could be also caused by vehicles (e. g. passively through humans migration or through the vertebrate host) (70) and therefore, allowing admixture between populations. Previous studies have reported the domiciliation of *P. geniculatus* principally due to their attraction to light, and having into account that

this species is principally a terrestrial triatomine (71,72), we suggest that the dispersion of individuals from different regions may be mediated by human migration. This could be promoted by flight dispersion of this triatomines near human settlements. Although previous reports have documented that adults generally fly poorly, it also has been suggested that fly capacity is enough to get this insect inside dwellings (6,69), however further studies are required to validate this premise.

This dispersion could also be mediated by vertebrate host as previously described (73). *P. geniculatus* is commonly associated with opossums, armadillos and bats (72) but a more recent study revealed that this species has a wide range of feeding sources (1). *P. geniculatus* was reported as one of the vectors with the greatest variety of feeding source with 18 hosts, which includes armadillos, opossums, bats, rodents, canines, rabbits and primates (74). As previously described, passive dispersion by human activity and carriage on animals is important over long distances migration (69). The incursion of *P. geniculatus* into human colonized territory, human agricultural activities and the great amount of feeding sources could be favoring the possibility of dispersion through of different regions of Colombia. The survival of *P. geniculatus* in a wide range of habitats, creates new ecological factors that could promote the diversification of this species (69). The broad spectrum of life zones coupled with the great amount of feeding sources and considering the high level of diversity shown (Table 3 & 4), increases the possibility of niche specialization and therefore divergence between populations. This is quite relevant because changes in the interaction between vector and host, have an impact in the dynamics of the disease (75).

If *P. geniculatus* is going through a diversification process, there will be new epidemiological challenges to upfront for the effective control of Chagas disease. The dispersion of the species have a high relevance due to the incursion of *T. cruzi* sylvatic strains, which have been reported as more virulent compared to domestic strains (10,12). This species has been reported with mixed infection of TcI, TcIII and TcIV strains which are associated with sylvatic foci (6) and there also have been reports of infection with the rest of the DTUs (1). This is associated with the feeding sources. As *P.*

geniculatus invade human territories and start feeding from domestic hosts (1,74,76) (e.g. *Canis lupus familiaris*) the variability among DTUs found in this vector has increased.

Even though, until now there is not enough evidence of complex of species within *P. geniculatus*, broadening the knowledge of the biology of this Triatominae is relevant for the development of effective mechanisms in the control of Chagas disease. It is important to mention the association of *P. geniculatus* with oral outbreaks (6) in different countries such as Brazil, Venezuela, Bolivia, Ecuador and Colombia. Oral outbreaks of Chagas disease have been associated with high rates of parasitemia due to ingestion of food contaminated with triatomines or their feces (77,78). This has an impact on the population in the risk of becoming infected, and that the people at risk cover all the states of endemic areas of the disease contrary of vector transmission, to which it refers mainly to the regions of poverty predominates (45). The high rates of infection of this species and its high colonizing capacity and adaptability (79) generate an increase in the probability of transmission of the parasite that causes Chagas disease. This information about *P. geniculatus* generates useful data for the development of vector control strategies that have been proposed until now (7,10,12,45). Most of them include *Triatoma dimidiata* and *Rhodnius prolixus* as vectors, and none include *P. geniculatus* despite its epidemiological relevance (2,5,19).

Vector control programs aim to reduce the prevalence of the vector. This requires an accurate identification and incrimination of suspected vector species (80) as well as understanding factors linked to transmission. These factors include biological diversity, population dynamics and spatial extent of the populations (75). Specifically, the divergence of a species with population structure can modify the disease transmission and therefore alter the disease dynamics (75). *P. geniculatus* showed several vector populations that could explode several host populations. This study is a preliminary research to elucidate the biology of *P. geniculatus* and therefore aid in the effective development of strategies that should vary with the vector implicated (75).

To the best of our knowledge, we conducted the first approximation to elucidate the intraspecific relationships within *P. geniculatus* using molecular data. We found four clades genetical differentiated with no clear association of being caused by geographic events that shaped Colombian demography (e.g. the uplift of the Eastern Cordillera of Colombian Andes). Further studies broadening the sample location used coupled with vector adaptability factors are needed to broaden the knowledge of the pathogen-vector interaction. This information should be taken into consideration for the design of vector control strategies aimed to reduce the prevalence of Chagas disease.

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Supporting files

Table S1. Sampling used in this study. Individuals 123-126 correspond to the outgroups used in this study. ID last two letters correspond to species: Pg: *Panstrongylus geniculatus*, Pl: *P. lignarius*, Pm: *P. megistus*, Ti: *Triatoma infestans*, Tp: *T. pallidipennis* and Tr: *Triatoma* sp.

Figure S1. Phylogenetic trees inferred with mitochondrial DNA per loci evaluated. Numbers in the nodes are the support values of bootstrap after 5000 bootstrap replicates.

Figure S2. *16s, Cytb* and *ND4* chromatograms of heteroplasmic loci. ID name correspond to sample reported in Table S1.

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