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Trypanosoma cruzi: Study of the Distribution of Two Widespread Clonal Genotypes in Bolivian *Triatoma infestans* Vectors Shows a High Frequency of Mixed Infections

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BOSSENO, M.-F., TELLERIA, J., VARGAS, F., YAKSIC, N., NOIREAU, F., MORIN, A., AND BRENIÈRE, S. F. 1996. *Trypanosoma cruzi:* study of the distribution of two widespread clonal genotypes in Bolivian *Triatoma infestans* vectors shows a high frequency of mixed infections. *Experimental Parasitology* **83,** 275–282. The detection of two widespread *Trypanosoma cruzi* clonal genotypes (20 and 39) in feces of Bolivian specimens of the vector *Triatoma infestans* was performed by a combination of polymerase chain reaction and clone-specific DNA hybridization. The hybridization pattern of 186 PCR positive samples of *T. infestans* feces collected in two Bolivian departments identified clone 20 in 74.2% and clone 39 in 63.4% of the triatomine bugs. For the first time, a high percentage (mean: 43.2 ± 26%) of mixed infections (presence of both clones in a given fecal sample) in various localities was recorded. Results were in agreement with the two assumptions of independent transmission of clones 20 and 39 and of the absence of selection in the natural cycles under survey. Statistical analysis of the geographical distribution of clones 20 and 39 favored the hypotheses that the frequencies of *T. cruzi* natural clones are different among localities and that these differences are not proportional to the distances that separate the localities. The epidemiological significance of these results is discussed.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Trypanosoma cruzi; Triatoma infestans*; clones, PCR, polymerase chain reaction.

INTRODUCTION

Trypanosoma cruzi, the agent of Chagas' disease, exhibits in nature a basically clonal population structure. Isozyme analysis at 15 genetic loci made it possible to identify 43 different clonal genotypes (Tibayrenc *et al.* 1986a). Some clones appeared widespread and abundant and were named ''major clones'' (Tibayrenc and Brenière 1988). In Bolivia, major clones 19, 20, and 39 (numberings according to Tibayrenc *et al.* 1986a) are abundant in domestic cycle (Tibayrenc *et al.* 1986b). Clones 19 and 20 are genetically closely related to each other (only one allelic difference) and are both distantly related to clone 39. (Tibayrenc and Ayala 1988; Tibayrenc *et al.* 1986b; Brenière *et al.* 1989). The genotypes identified with a limited number of genetic markers should not be regarded as actual clones, but rather as families of closely related clones. The use of a broader range of genetic markers is bound to show evidence of additional variability within each of the formerly described ''clones''; the term ''clonet'' has been coined to designate the genotypes that appear to be identical for a given set of genetic markers in a clonal species (Tibayrenc and Ayala 1988; Tibayrenc *et al.* 1991). As a matter of fact, the use of 22 enzyme loci instead of 15 (Tibayrenc *et al.* 1993) gives evidence of additional variability within each of the formerly described major clones (which are ''clonets'' delimitated by 15 enzyme loci). Nevertheless, the overall phylogenetic picture formerly drawn from 15 loci remained valid. Indeed, all stocks formerly attributed to either clone 19 or clone 20 were

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still closely related, and this was also true for all stocks formerly attributed to clone 39, while the first and the second remained separated by vast evolutionary distances.

We have previously designed a molecular identification tool based on both kDNA PCR amplification and Southern hybridization with specific probes (Brenière *et al.* 1992). Probe 20 identifies stocks attributed to clonet 20 and some stocks attributed to clonet 19, while probe 39 identifies stocks attributed to clonet 39. These probes therefore identify two radically distinct groups of clonal genotypes that are abundant in Bolivia. The present work aimed at using the probes to evaluate the frequency of these two groups without undergoing the possible bias due to culture selection (Miles and Cibulskis 1986). For simplicity's sake, in the rest of the text, these two groups of clonal genotypes will be referred to as ''clonet 20'' and ''clonet 39,'' respectively, although the first group probably involves some genotypes that were previously attributed to clonet 19.

Materials and Methods

Vectors. Specimens of *Triatoma infestans* (1331) were captured from domestic and peridomestic habitats of different locations in Cochabamba and La Paz departments (Table I). These two departments are considered areas of high and low endemicity for Chagas' disease, respectively (Valencia 1990).

Microscopic observation. Microscopic observation was considered positive if flagellate parasites were observed in the feces of a triatome specimen during 5 min examination of a drop feces mixed with phosphate-buffered saline at a 400× magnification.

Processing of triatomine feces for PCR. A sample of 186 positive triatomes (microscopic observation) from different locations were process by PCR (Table 1). To prevent contamination, fecal samples were collected and processed in different locations. Briefly, 1 to 20 μl of triatomine feces was individually collected in sterile microtubes using forceps and gloves rinsed in bleach between each sample. Samples were then stored at −20°C. In a second room devoted to the preparation of PCR samples (free of all sources of contamination), the samples were prepared by addition of 200 μl of distilled water (free from DNA contamination) followed by two cycles of boiling (10 min) and centrifugation at 8000*g*. Negative controls included mock-treated water or feces from *T. infestans* reared in the laboratory and known as *T. cruzi* free. Ten microliters of the supernatant was used as a template in each of the PCR assays.

PCR procedure. PCR was performed as previously described (Veas *et al.* 1991a). The sequences of the oligonucleotide primers chosen to amplify the hypervariable region of kDNA minicircles (HVRm) for all *T. cruzi* stocks were CV1: 5′-GATTGGGGTTGGAGTACTAT-3′ and CV2: 5′-TTGAACGGCCCTCCGAAAAC-3′ (Genset laboratory, Paris, France). The amplification buffer has been previously described (Veas *et al.* 1991a), and the total reaction volume was 50 μl using 2.5 U of *Thermus aquaticus* DNA polymerase (Promega, Madison, WI). The PCR reagents (buffer, primers, oligonucleotides, water, and mineral oil) were prepared beforehand, aliquoted in sterile microtubes, and stored at −20°C. This ''Master mix'' was quality tested by amplification of one tube with water template (negative control) and another with *T. cruzi* DNA template (positive control). In addition, each assay included no more than 10 fecal samples. The amplification was performed on a Biometra, Trio thermoblock PCR device (Göttingen, Germany) and involved three distinct steps: (i) an initiation step: DNA denaturation (95°C, 5 min), oligonucleotide primer annealing (48°C, 2 min), elongation (72°C, 2 min); (ii) an amplification step: 30 cycles (95°C for 5 s, then 48°C for 30 s, then 72°C for 1 min), and finally (iii) a cooling step (4°C for variable times). Each run included: (i) one positive control of total DNA template of *T. cruzi* reference stock and (ii) two negative controls with water instead of DNA template, one of them having been treated identically to the fecal samples. PCR products were analyzed by electrophoresis on a 0.8% agarose gel and visualized by ethidium bromide staining.

Southern blot. One-tenth of each PCR samples was electrophoresed on a 0.8% agarose gel (alkali denaturated) (0.5 *N* NaOH, 1.5 *M* NaCl, twice for 15 min each) and then transferred onto charged Hybond N+ nylon membranes (Amersham, Buckinghamshire, UK) by vacuum blotting. For the vacuum transfer, the gel was placed on a prewet membrane, with filter paper followed by a piece of diaper below. This ''sandwich'' was covered with plastic wrap and placed on a gel dryer (Bio-Rad, Paris, France) and vacuum applied for 10 min without heat. Each membrane included a negative control (water template), two PCR positive controls corresponding to clonets 20 and 39, as well as PCR reactions from uninfected feces.

Probes. The two clone-specific probes (numbered 20 and 39) were purified from their respective HVRm DNA fragments produced by the PCR from two *T. cruzi* reference stocks, namely, TPk1, attributed to clonet 39, and S034 cl4, attributed to clonet 20 (Brenière *et al.* 1992). The DNA were digested with the restriction endonuclease *Sau*96 and *Sca*I (Promega) to eliminate part of the oligonucleotide primers selected in the conserved region of the minicircle.

Labeling and hybridization conditions. Membranes were hybridized using the enhanced chemiluminescence

gene detection system (ECL, Amersham, Buckinghamshire, UK) and the probes were labeled according to the manufacturer's recommendations for ECL. Briefly, the membranes were incubated at 42°C in hybridization buffer (0.12 ml/cm^2) for 15 min. Each of the purified probes was labeled for 10 min at 37°C. Ten nanograms of labeled probe per milliliter of hybridization buffer was added to the membranes. Hybridization was performed at 42°C overnight in a rotating oven. The membranes were washed twice under highly stringent conditions (6 *M* urea, 0.1 SSC at 42°C for 20 min) and then twice in 2× SSC at room temperature for 10 min. Two exposures were performed (1 and 30 min) on Hyperfilm-MP (Amersham, Buckinghamshire, UK).

Data analysis. Statistical analysis was performed using the programs 4F and CA of BMDP (Biomedical package). The earlier versions of BMDP were developed at the National Institute of Health and at UCLA in the 1960s and were initially devoted to biomedical applications. The association between rates of triatomine infection and mixed infections was tested by the correlation coefficient of Spearman (Schwartz 1963). The random association of the two clones in triatomines was tested in the whole population and in each region by the χ^2 test applied between observed and expected sizes of mixed infections; the expected sizes were calculated according to the formula: $N \times (\% 20 \times \% 39)$, where N = number of studied samples and % 20 and % 39 = observed percentage of fecal samples hybridized by probes 20 and 39, respectively. Differences in *T. cruzi* clonet frequencies in triatomine bugs among localities were analyzed using the program 4F, which handles frequency tables and analyzes two-way and multiway frequency tables: we could simply examine cross-tabulations and their percentages, or compute measures of association such the χ^2, or fit loglinear models to multiway contingency tables. We calculated similarity coefficients of clonet distribution among localities using the Phi2 index and the dendogram of distances between localities constructed using the unweighted paired-group method of averaging (UPGMA).

RESULTS

Following PCR amplification, 186 positive fecal samples were examined by hybridization with the two probes obtained from two reference *T. cruzi* stocks attributed to clonets 20 and 39, respectively. Figure 1a shows the PCR results after ethidium bromide staining from several fecal samples and controls. Figures 1b and 1c show the patterns obtained after Southern blotting on nylon membrane and hybridization with each of the two probes. All positive PCR samples showed a major band of 270 bp previously demonstrated to be specific for the *T. cruzi* species (Veas *et al.* 1991b). Hybridization of the positive PCR products revealed three different hybridization patterns: (i) samples positive with either probe 20 or probe 39 (lanes 6, 7, 8, 9, 10, and 11); (ii) samples positive with both probes (mixed infections, lane 12); (iii) samples negative with both probes (lane 13). The 28 fecal samples from laboratory-reared *T. infestans* and the water template (controls) were all PCR negative, as expected (Fig. 1a). Moreover, no hybridization with either probe to these controls was observed. The same result was obtained with 10 *T. infestans* fecal samples from the field that yielded no PCR amplification products (Figs. 1a, 1b and 1c). The hybridization results are summarized in Table I. Subsequently we have analyzed (i) the rates of triatomine bug infection by each clonet; (ii) the rate of mixed infections; and (iii) the geographical distribution of the clones.

The majority of *T. cruzi* positive bugs captured in both departments were infected by clonet 20, or clonet 39, or a mixture of both clonets. Clonet 20 was present in 74.2% of triatomines and clonet 39 in 63.4%. A low percentage of samples (8.0%) was not recognized by either probe 20 or 39. These remaining samples were probably infected by clonal genotypes different from both clonets 20 and 39. Their phylogenetic position remains to be determined. The rate of triatomine infections (microscopic observation) in the different locations is not associated with the respective rates of clones (clone 20, $\rho = 0.76$; clone 39, $\rho = 0.91$; $n = 9$).

The percentage of mixed infections ranged from 7.7 to 85.7% according to the localities, with an average of 43.2 ± 26.0%. The null hypothesis of independent transmission and absence of interaction of the two groups of clones identified by probes 20 and 39 was taken to perform a χ^2 statistical test on the whole population: the result ($\chi^2 = 0.04$, $P > 0.05$) did not make it possible to reject this null hypothesis. The same analysis gave similar results for each province: Mizque, $\chi^2 = 0.06$, $P > 0.05$; Campero, $\chi^2 = 0.04$, $P > 0.05$; Capinota, $\chi^2 = 0$, $P > 0.05$; Caranavi,

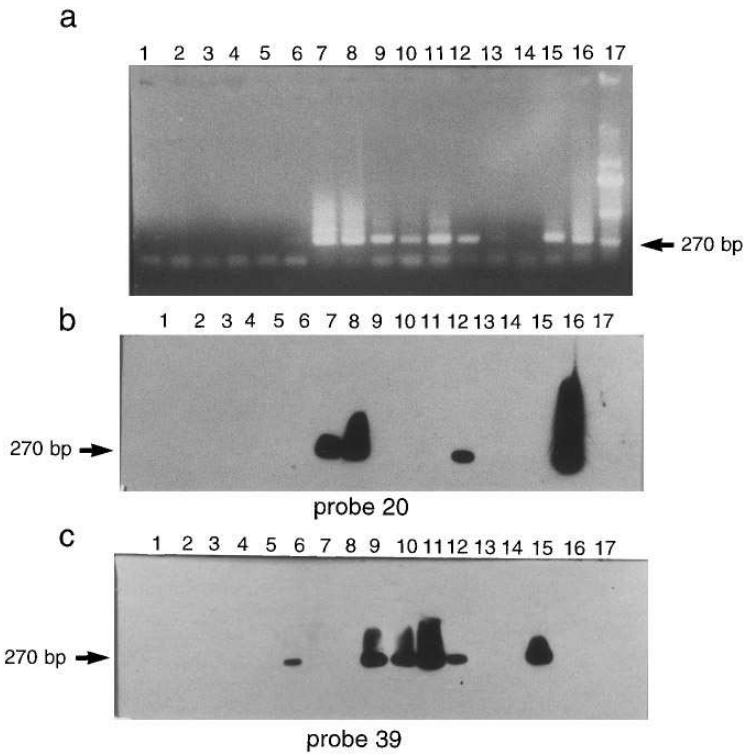

FIG. 1. (a) Ethidium bromide-stained 0.8% agarose gel containing polymerase chain reaction products from *Triatoma infestans* feces. (b and c) Hybridization patterns of these products with clone-specific probes corresponding to natural clones 20 and 39, respectively lanes 1 to 3, control samples (using feces from laboratory-reared *T. infestans*); lanes 4 and 5, negative controls (using distilled water as the template); lanes 6 to 13, positive *T. infestans* feces samples; lane 14, negative *T. infestans* feces sample; lane 15 and 16, positive controls (using 10 ng of total DNA from the reference *T. cruzi* stocks pertaining to natural clones 39 and 20, respectively, as the template); lane 17, DNA size marker *Rsa*I digest of pUC 19. The arrows indicate the major amplified band (270 bp).

$\chi^2 = 0.20$, $P > 0.05$; North Yungas, $\chi^2 = 0.12$ $P > 0.05$. We obtained a significant correlation between rates of triatomine infection in the different locations (microscopic observation) and mixed infections ($\rho = 0.38$, $n = 9$, $P < 0.05$).

Moreover, clonet distribution among localities was tested by taking the null hypothesis that this repartition was homogeneous (triatomes from ''other'' localities in the department of La Paz were excluded). Four different possible hybridization patterns were considered: (i) fecal samples recognized by probe 20 only; (ii) samples recognized by probe 39 only; (iii) samples recognized by both probes; (iv) fecal samples not recognized by any of the two probes. The null hypothesis was rejected ($\chi^2 = 38.65$, $df = 21$, $\rho < 0.01$). The analysis of likelihood ratio χ^2 showed that various localities (Quiroga, Capinota, Caranavi, and Pararani) presented exceptional patterns contributing to the rejection of the hypothesis of geographical homogeneity. At Pararani and Caranavi, we observed a high percentage of fecal samples not recognized by either of the two probes. At Quiroga, samples recognized by probe 20 were constantly recognized by probe 39 also. At Capinota, an exceptionally high percentage of triatomines with only clone 20 were observed. We used an analysis of Phi2 distances to summarize the differences of distri-

TABLE I
Detection of *T. cruzi* Clones 20 and 39[a] in *T. infestans* from Two Bolivian Departments

Department and provinces	Localities	No. of triatomes	No. and % recognized			
			By probe 20 only	By probe 39 only	Neither probe 20 nor .39	By probes 20 and 39
Cochabamba						
Mizque	Mizque	32	9 (28.1)	6 (18.7)	2 (6.3)	15 (46.9)
Campero	Ailquile	39	9 (23.0)	6 (15.4)	1 (2.5)	23 (59.0)
	Quiroga	14	0	2 (14.3)	0	12 (85.7)
Capinota	Near Capinota	22	11 (50.0)	2 (9.1)	2 (9.1)	7 (31.8)
Total		107	29 (27.1)	16 (14.9)	5 (4.7)	57 (53.3)
La Paz						
Caranavi	Near Caranavi	12	3 (25.0)	2 (16.7)	3 (25.0)	4 (33.3)
North Yungas	Trinidad-Pampa	32	9 (28.1)	4 (12.5)	3 (9.4)	16 (50.0)
	Pararani	13	6 (46.1)	2 (15.4)	4 (30.8)	1 (7.7)
	San Juan	9	1 (11.1)	2 (22.2)	0	6 (66.6)
	Others	13	5 (38.5)	7 (53.8)	0	1 (7.7)
Total		79	24 (30.4)	17 (21.5)	10 (12.6)	28 (35.4)
Cochabamba and La Paz	Entire population	186	53 (28.5)	33 (17.8)	15 (8.0)	85 (45.7)

[a] *T. cruzi* clones 20 and 39 are defined by the genetic classification of Tibayrenc and Ayala (1988)

bution among localities of the four hybridization patterns above exposed. The corresponding dendogram indicated two groups of localities and three localities. The first group was constituted by the localities of Mizque, Trinidad Pampa, and Caranavi; the second group was constituted by the localities of San-Juan and Ailquile. The three other localities showed distributions of hybridization patterns different from each other and from those of the two groups (Fig. 2).

DISCUSSION

The present results confirm to a large extent previous data reported by Tibayrenc *et al.* (1986b) dealing with isozyme identification of 419 *T. cruzi* stocks from Bolivia. Indeed, these authors give evidence for the presence in Bolivia of three abundant and widespread ''clonets,'' namely, clonets 19, 20, and 39. Clonets 19 and 20 on the one hand and clonet 39 on the other hand represent two groups of clonal genotypes within which genetic diversity remains limited and which are separated from each other by vast evolutionary distances. Probes 20 and 39 used in the present study, which identify specifically stocks attributed to each of these two groups of clonal genotypes, made it possible to reach more precise results and to consider with a better resolution the relevant problem of mixtures of clones in a given individual host.

In the La Paz and Cochabamba departments, previous isoenzyme typing found only one stock that was radically different from clonets 20 and 39 (2.8%) and only one mixed infection (Tibayrenc *et al.* 1986b). The rate of mixed infections in triatomine bugs for the whole of Bolivia, as estimated by isozyme analysis, was 10% (Tibayrenc *et al.* 1986b). In contrast, the present study made it possible to reach the following results: (i) Several PCR products which are not recognized by either of the two probes were scored (8%). They probably correspond to clonal genotypes that are different from both clonets 20 and 39 and whose phylogenetic positions remain to be clarified. (ii) High frequencies of mixed infections were recorded (more than 50% in various

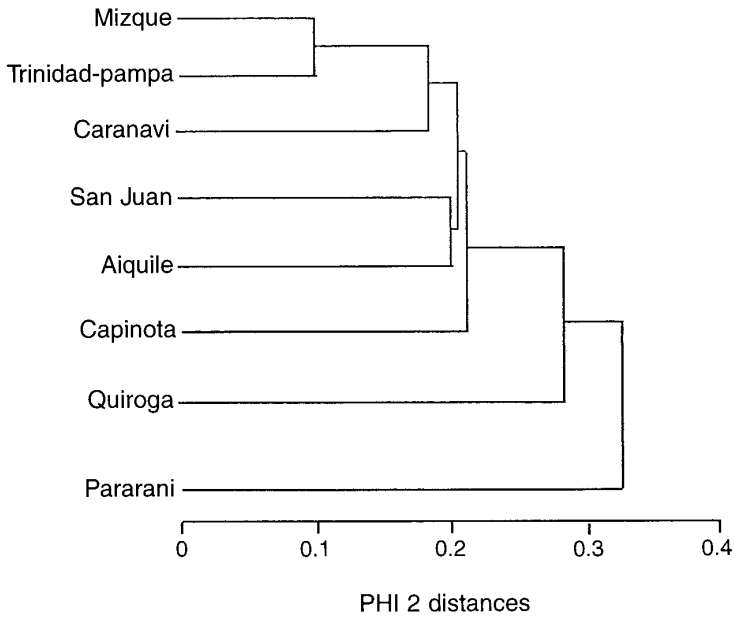

FIG. 2. Dendogram constructed using the unweighted pair-group method of averaging (UPGMA) from Phi2 similarity coefficient, based on distribution of *T. cruzi* natural clones 20 and 39 in feces of *T. infestans* insects.

localities). It is worth noting that the rate of mixed infections evidenced by our PCR/hybridization method is probably an underestimation, because only the mixed infections of clones 20 and 39 were considered in the present study. The rates of mixed infection found were variable according to the locations and correlated to rate of triatomine infections. If triatomes present a high probability of infection, higher levels of mixed infection are expected since an insect needs several meals for its development.

The lower rate of mixed infection scored by isozyme analysis can be attributed to two factors, not mutually exclusive: (i) isozyme typing could have a lower resolution for evidencing such mixtures and (ii) the culture step required for isozyme analysis could tend to eliminate, or at least lower the abundance of, one of the clones entering the mixture.

Comparisons of results obtained years apart should be tentative. Nevertheless, in the present study, we were able to directly compare the results obtained from either isozyme analysis or PCR-hybridization. Thirty-eight triatomine bugs were submitted to PCR-hybridization, while the trypanosome stocks of these bugs were isolated, cultured, and analyzed by isozyme typing. Only 1 mixture was shown by this last method, while PCR-hybridization showed the presence of 18 mixtures. This last result does confirm that PCR-hybridization has a higher level of resolution than isozyme analysis for showing the presence of these mixtures. Similarly, recent characterization by PCR-hybridization of *T. cruzi* stocks in blood of chagasic patients during acute and chronic phases recorded particularly high rates of mixed infections during the chronic phase (Bosseno *et al.* 1995; no published data), while former isozyme studies had detected only 10% of mixed infections in Bolivian chronic chagasic patients (Brenière *et al.* 1989).

The comparison of the clone 20 and 39 frequences in vectors and patients previously obtained by isoenzyme studies (Tibayrenc *et al.* 1986b; Brenière *et al.* 1989) with our present data is limited by the size of the previous samples and the unknown precise origin of strain

isolated before. Nevertheless, we note in vectors higher actual frequences of both clones which depend on the best detection of mixed infections.

This new approach allows the detection of a high percentage of mixed infections in vectors. The expected number of patients presenting mixed infections in the areas studied should be very important since mixed infections can be aquired in two ways: (i) infection by fecal samples presenting a mixed infection and (ii) reinfection by another *T. cruzi* clonal genotype. The human mixed infections could reach almost the all of the patients. The clinical, pathological, and immunological consequences of the presence of various clones in a single human infection is unknown. Experimental studies of mouse infections have throughly demonstrated the variability of biological properties of *T. cruzi* stocks according to their genetic characteristics (Andrade 1990; Marsden *et al.* 1979; Tanuri *et al.* 1985). Finally, various authors have shown the high virulence of stocks belonging to clonet 20, in contrast to the low virulence observed for clonet 39 (Sanchez *et al.* 1990). Nevertheless, data of experimental co-infections remain scare and roughly show different interactions between clones according to the schedule of infections, as does the work of Deane *et al.* (1984).

The null hypothesis of independent transmission and lack of interaction between the two groups of clones identified by our probes could not be rejected by a χ^2 test. This result suggests an absence of natural selection during the cycle through the vectors and mammalian host. In addition, the absence of a significant correlation between infection rates and the prevalence of any clone sustains these results. These results conflict with some studies showing selection of clones by mice (Sanchez *et al.* 1990; Deane *et al.* 1984). However, selection of clones during human infection is not reported.

We have compared the geographic distribution of clonets 20 and 39. The results show that the frequencies of the clonets are different among localities and that these differences are not proportional to the distances that separate the localities. The two groups shown by the dendrogram in Fig. 2 (Mizque–Trinidad Pampa and San Juan–Ailquile) are located in the departments of La Paz and Cochabamba, respectively, and are at least 400 km apart. It is worth noting that other clonal genotypes (neither clonet 20 nor clonet 39) were present in Pararani but were not recorded in San Juan, while the two localities are only 29 km apart. This favors the hypothesis that clonet exchanges between different localities are limited.

Population genetic studies of vectors (Dujardin *et al.* 1994) strongly suggest that exchanges of *T. infestans* populations are limited between different localities. *T. cruzi* clonet repartition, as shown in the present study, fits this picture and could suggest that clonet repartition is partly or mainly governed by the structure and dynamics of triatomine bugs. Work is under way to test this hypothesis on a finer geographical scale.

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