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# Cryptic speciation in *Triatoma sordida* (Hemiptera: Reduviidae) from the Bolivian Chaco

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**Summary** Eight natural Bolivian populations of two closely related species of Triatominae, *Triatoma sordida* and *T. guasayana*, were analysed by multilocus enzyme electrophoresis at 20 loci. Both species were readily separated and no natural hybrid was observed. Among the silvatic sample of *T. sordida*, strong departure from panmixia within and between loci was detected in two sites of the Chaco, suggesting two reproductively separate populations easily recognized at *Idh2* and *Mdh2* loci. Genetic distance between them was in agreement with the hypothesis of distinct species. However, the detection of 3% of putative hybrids suggested a recent evolutionary divergence.

**keywords** *Triatoma sordida*, *T. guasayana*, multilocus enzyme electrophoresis (MLEE), Hardy–Weinberg disequilibrium, linkage disequilibrium, speciation

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## Introduction

*Triatoma sordida* (Hemiptera: Reduviidae) is a blood-sucking insect vector of *Trypanosoma cruzi*, the causative agent of American trypanosomiasis. It occurs in the silvatic environment but it is often found in peridomestic habitats and can also form domestic colonies (Carcavallo *et al.* 1988; Schofield 1994). This process of domiciliation may reflect invasion of habitats from which *Triatoma infestans* has been eliminated but may also be primary, without any relation to a previous eradication of the main vector (Forattini *et al.* 1984; Noireau *et al.* 1996). *T. sordida* is widely distributed throughout central Brazil, eastern and central Bolivia, the Chaco region of Paraguay and north-western Argentina (Lent & Wygodzinsky 1979). Because of its tendency to invade domestic environment and its wide distribution, *T. sordida* is classically considered a triatomine of epidemiological importance (WHO 1991; Schofield 1994). Nevertheless, recent studies have shown that its vectorial significance could be considerably less than previously thought, especially through its inability to form large colonies in houses (Diotaiuti *et al.* 1995; Noireau *et al.* 1997).

*T. sordida* belongs to a group considered as a complex of at least three closely related species: *T. sordida*, *T. guasayana* and *T. patagonica* (Usinger *et al.* 1966). Morphometry and enzyme electrophoresis have confirmed the earlier distinction of *T. sordida* and *T. guasayana* as well-defined species (Gorla *et al.* 1993; García *et al.* 1995). However, among Brazilian and Argentine populations of *T. sordida*, cytogenetics combined with isoenzymes provided evidence for high levels of genetic differentiation that might be compatible with the existence of strong intraspecific variation in *T. sordida* (Panzera *et al.* 1997). Using multilocus enzyme electrophoresis (MLEE), we investigated the within and between-locus genetic equilibrium (Dujardin *et al.* 1996) in various natural populations of *T. sordida*, some of which were found together with *T. guasayana*. Results agreed with the existence of two sympatric biological species occurring within *T. sordida*.

## Materials and methods

### Insects

Morphological separation of *T. sordida* and *T. guasayana* adults was based upon: (i) the pattern of dark connexival markings and (ii) the ratio of rostral segments (Lent & Wygodzinsky 1979). According to these authors, an insect

presenting a rostrum with a second segment more than twice as long as the first would be *sordida*. Otherwise, it would be *guasayana*. Likewise, the presence of a narrow black line along intersegmental sutures of the connexivum dorsally would be typical of *sordida* (and its absence typical of *guasayana*).

Six natural populations of *T. sordida*, two of *T. guasayana* and one laboratory-reared colony of each species were studied. The natural populations of *T. sordida* were collected from silvatic and domestic habitats while the populations of *T. guasayana* were silvatic.

*Domestic populations*

One hundred and thirty nymphal and adult specimens of *T. sordida* were collected: (i) from localities of San Juan Bautista, Tacoigo and Guapomocito, Department of Santa Cruz (populations A, B and C, respectively) and (ii) from the locality of Huaritolo, Department of La Paz (population D).

*Silvatic populations*

In addition, 101 adult specimens of *T. sordida* (65) and *T. guasayana* (36) were collected by light trapping from two silvatic sites located in the Chaco: Izozog area (populations E and F, respectively) and Tita area, (populations G and H, respectively), Department of Santa Cruz.

*Laboratory colonies*

Twenty-one adult specimens of *T. sordida* and 32 of *T. guasayana* (fifth generation, unknown number of founders), originally collected together from a bromeliad in the Chaco, Boyuibe area, Department of Santa Cruz, were also examined (populations I and J, respectively).

Detailed data on the populations and number of individuals studied are summarized in Table 1.

### Isoenzyme electrophoresis

Nymphal instars and adults of both sexes were used. Alary muscles were dissected out and ground in 100 µL of an enzyme stabilizer (dithiothreitol, E-aminocaproic acid and EDTA, each at 2 mM). Extracts were stored at −70 °C until used. MLEE was performed on cellulose acetate plates (Helena Laboratories, Beaumont, TX). The following 12 enzyme systems were assayed: diaphorase (DIA, EC 1.6.2.2.); aspartate aminotransferase (GOT, EC 2.6.1.1.); glucose-6-phosphate dehydrogenase (G-6-PDH, EC 1.1.1.49.); glucose phosphate isomerase (GPI, EC 5.3.1.9.); α-glycerophosphate dehydrogenase (GPD, EC 1.1.1.8.); isocitrate dehydrogenase (IDH, EC 1.1.1.42.); leucine aminopeptidase (LAP, EC 3.4.11); malate dehydrogenase (MDH, EC 1.1.1.37.); malic enzyme (ME, EC 1.1.1.40.); aminopeptidase A (PEP-A with substrate L-Leucyl-leucyl-leucine, EC 3.4.11.); phosphoglucomutase (PGM, EC 2.7.5.1.); and 6-phosphogluconate dehydrogenase (6-PGDH, EC 1.1.1.44.). Running conditions described in Table 2 were derived from Dujardin & Tibayrenc (1985), with the following modifications: a different buffer (0.135 M Tris, 0.043 M citric acid, pH 7.0) was used for IDH, LAP and MDH; the running time was increased for G-6-PDH, IDH and ME; we used centre application of extracts for GOT and MDH. Conditions for enzyme staining were as in Ben Abderrazak *et al.* (1993).

### Sample processing

We followed two main steps. First, the whole sample (284 specimens) was subdivided into *T. sordida* and *T. guasayana* using morphological criteria applied to adults. Each morphological species was confirmed by

**Table 1** Data of *T. sordida* and *T. guasayana* populations studied

| Population | Species | Collecting site: Locality/Department | Geographic region | Ecotope | No. of specimens | Year of collection |
|---|---|---|---|---|---|---|
| Natural populations | | | | | | |
| A | *T. sordida* | San Juan Bautista, Santa Cruz | Cerrado | Domestic | 24 | 1995 |
| B | *T. sordida* | Tacoigo, Santa Cruz | Cerrado | Domestic | 37 | 1995 |
| C | *T. sordida* | Guapomocito, Santa Cruz | Cerrado | Domestic | 34 | 1995 |
| D | *T. sordida* | Huaritolo, La Paz | Sub-andean region | Domestic | 35 | 1995 |
| E | *T. sordida* | Izozog, Santa Cruz | Chaco | Silvatic | 32 | 1995 |
| F | *T. guasayana* | Izozog, Santa Cruz | Chaco | Silvatic | 9 | 1995 |
| G | *T. sordida* | Tita, Santa Cruz | Chaco | Silvatic | 33 | 1995 |
| H | *T. guasayana* | Tita, Santa Cruz | Chaco | Silvatic | 27 | 1995 |
| Laboratory colonies | | | | | | |
| I | *T. sordida* | Boyuibe, Santa Cruz | Chaco | Silvatic | 21 | 1991 |
| J | *T. guasayana* | Boyuibe, Santa Cruz | Chaco | Silvatic | 32 | 1991 |

**Table 2** Specific electrophoresis procedures for *T. sordida* and *T. guasayana*

| Enzyme | Buffer* | Soaking** | Migration*** | Voltage (V) | Time (min) | Sample application |
|---|---|---|---|---|---|---|
| DIA | A | 0.5 | 1 | 200 | 20 | Cathode |
| GOT | A | 0.5 | 1 | 160 | 30 | Centre |
| G-6-PDH | B | 0.8 | 1 | 200 | 25 | Cathode |
| GPD | C | 0.1 | 1 | 200 | 30 | Cathode |
| GPI | C | 0.1 | 1 | 200 | 10 | Cathode |
| IDH | C | 0.2 | 1 | 200 | 30 | Cathode |
| LAP | C | 0.2 | 1 | 200 | 20 | Cathode |
| MDH | C | 0.1 | 1 | 200 | 15 | Centre |
| ME | C | 0.2 | 1 | 200 | 20 | Cathode |
| PEP-A | B | 0.5 | 1 | 200 | 20 | Cathode |
| PGM | D | 0.1 | 1 | 200 | 20 | Cathode |
| 6-PGDH | C | 0.2 | 1 | 200 | 30 | Cathode |

*Stock buffer; A: 0.5 M Tris, 0.65 M boric acid, 0.016 M EDTA, pH 8.0 (Shaw & Prasad 1970); B: Tris-barbital-sodium barbital (Helena HR, ref. no. 5805), pH 9.0; C: 0.135 M Tris, 0.043 M citric acid, pH 7.0 (Shaw & Prasad 1970); D: 0.1 M Tris, 0.1 M maleic acid, 0.01 M EDTA, 0.01 M MgCl2, pH 7.4 (Kreutzer *et al* 1977); **Buffer dilution for electrophoresis – plate soaking from stock buffer; ***Buffer dilution in the electrophoresis chamber.

MLEE and analysed for genetic equilibrium and variability parameters. The genetic equilibrium tests were performed for each species in each locality or collection site. Since the disequilibrium detected within *T. sordida* was associated with the presence of two genotypically distinct forms (groups 1 and 2, see below), we further subdivided the *T. sordida* sample according to these genotypes.

In a second step, we explored the genetic variability and equilibrium within these genotypically distinct groups of *T. sordida* and estimated their level of genetic differentiation (see genetic distances below).

### Statistical methods

A locus was defined as polymorphic if the frequency of the rarest allele was $\geq 0.02$ in at least one of the samples. Genetic variability was estimated by the rate of polymorphism (P), the mean number of alleles per locus (A) and the expected mean heterozygosity or He (Nei 1987). Departures from panmixia were looked for using both single locus analysis (fixation index F) and multilocus analysis (linkage disequilibrium computed using Fisher's exact test). For the latter calculations, only two possible alleles were taken into account: the most frequent one, and all other ones plotted together as a unique allele. The standard genetic distance (Ds) of Nei (1987) was used to compare gene frequency differences between species, geographical areas or groups, and converted into an unweighted pair group method arithmetical average (UPGMA) dendrogram.

## Results

### Isoenzyme electrophoresis

A single zone of enzymatic activity or band was scored for DIA, GOT, G-6-PDH, GPI, PGM and 6-PGDH, while two bands interpreted as two distinct loci were scored for GPD, LAP, MDH and ME. In *T. sordida*, two bands were scored for IDH whereas one only in *T. guasayana*. Four bands corresponding to four loci were scored for PEP-A. Thus, the studied set of enzymes represented a total of 20 gene loci for *T. sordida* and 19 for *T. guasayana*.

### Accuracy of morphological identification

Using isoenzymes, we checked the accuracy of separating the sympatric adults of *T. sordida* and *T. guasayana* originating from the Chaco (populations E, F, G and H; 101 specimens) by means of the pattern of dark connexival markings and the ratio of rostral segments. The specific identification of insects was based upon the banding profiles for GOT, GPI, IDH, LAP, ME, PEP-A and PGM (see below).

The morphological separation based upon the pattern of dark connexival markings showed a 96.0% agreement with enzymatic pattern. A higher score was obtained by the ratio of rostral segments (99.0%).

### First step of the sample processing

Zymograms for enzymes GOT, GPI, IDH, LAP, ME, PEP-A and PGM presented clear differences between *T. sordida* and *T. guasayana* (Table 3). Seven loci (*Got*, *Gpi*, *Idh1*, *Lap2*, *Me1*, *Pep3* and *Pgm*) showed fixed alleles and could be

**Table 3** Allelic frequencies of enzymatic loci in population samples of *T. sordida* and *T. guasayana*

| | | *T. sordida* | | | | | | | | | | | *T. guasayana* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Group 1 | | | | | | | Group 2 | | Putative hybrids | | | | |
| Locus | Allele | A | B | C | D | E1 | G1 | I | E2 | G2 | E12 | G12 | F | H | J |
| *Dia* | 1 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| *Got* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.67 | 0.83 | 0.69 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.33 | 0.17 | 0.31 |
| | 3 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0 | 0 | 0 |
| *G-6-pdh* | 1 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| *Gpd1* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.11 | 0 | 0 |
| | 2 | 0.37 | 0.16 | 0.29 | 0.57 | 1.00 | 1.00 | 0.71 | 1.00 | 1.00 | 1.00 | 1.00 | 0.89 | 1.00 | 0.78 |
| | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.22 |
| | absent | 0.63 | 0.84 | 0.71 | 0.43 | 0 | 0 | 0.29 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Gpd2* | 1 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.88 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.12 |
| *Gpi* | 1 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.00 | 1.00 | 1.00 |
| *Idh1* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.37 | 0.36 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.30 | 0.52 | 0 | 0 | 0 | 0 | 0 |
| | 3 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.33 | 0.12 | 1.00 | 1.00 | 0 | 0 | 0 |
| | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.00 | 0.98 | 0.97 |
| | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.02 | 0.03 |
| *Idh2* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.98 | 1.00 | 0.50 | 0.50 | | | |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.02 | 0 | 0 | 0 | | absent | |
| | 3 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0 | 0 | 0.50 | 0.50 | | | |
| *Lap1* | 1 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| *Lap2* | 1 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.00 | 1.00 | 1.00 |
| *Mdh1* | 1 | 0 | 0.07 | 0.06 | 0 | 0 | 0.04 | 0.05 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0.96 | 0.69 | 0.78 | 0.48 | 0.83 | 0.73 | 0.90 | 0.20 | 0.14 | 1.00 | 1.00 | 0.17 | 0.44 | 0.16 |
| | 3 | 0.04 | 0.24 | 0.16 | 0.52 | 0.17 | 0.23 | 0.05 | 0.80 | 0.86 | 0 | 0 | 0.72 | 0.56 | 0.84 |
| | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.11 | 0 | 0 |
| *Mdh2* | 1 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0 | 0 | 1.00 | 1.00 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.00 | 1.00 | 0 | 0 | 1.00 | 1.00 | 1.00 |
| *Me1* | 1 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.00 | 1.00 | 1.00 |
| *Me2* | 1 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| *Pep1* | 1 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| *Pep2* | 1 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| *Pep3* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.00 | 1.00 | 1.00 |
| | 2 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0 | 0 | 0 |
| *Pgm* | 1 | 0 | 0 | 0.03 | 0 | 0.33 | 0 | 0 | 0 | 0 | 0.50 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0.12 | 0 | 0 | 0.09 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 3 | 1.00 | 0.99 | 0.85 | 0.99 | 0.50 | 0.91 | 1.00 | 0.04 | 0.05 | 0 | 1.00 | 0 | 0 | 0 |
| | 4 | 0 | 0.01 | 0 | 0.01 | 0.17 | 0 | 0 | 0.92 | 0.93 | 0.50 | 0 | 0 | 0 | 0 |
| | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04 | 0.02 | 0 | 0 | 0 | 0 | 0 |
| | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.00 | 0.91 | 1.00 |
| | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.09 | 0 |

**Table 3** *continued*

| | | *T. sordida* | | | | | | | | | | | *T. guasayana* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Group 1 | | | | | | | Group 2 | | Putative hybrids | | | | |
| Locus | Allele | A | B | C | D | E1 | G1 | I | E2 | G2 | E12 | G12 | F | H | J |
| *6-Pgdh* | 1 | 0 | 0 | 0 | 0 | 0 | 0.09 | 0.26 | 0.11 | 0.26 | 0.50 | 0 | 0 | 0 | 0 |
| | 2 | 0.40 | 0.63 | 0.54 | 1.00 | 1.00 | 0.41 | 0.67 | 0.43 | 0.40 | 0.50 | 0 | 0.44 | 0.48 | 0.84 |
| | 3 | 0.60 | 0.37 | 0.46 | 0 | 0 | 0.50 | 0.07 | 0.46 | 0.34 | 0 | 1.00 | 0.56 | 0.52 | 0.16 |
| | *n* = | 24 | 37 | 34 | 35 | 3 | 11 | 21 | 28 | 21 | 1 | 1 | 9 | 27 | 32 |

*Population samples A, B, C, D, F, H, I and J: see Table 1; E1 and G1: individuals pertaining to E and G populations and showing homozygosity for *Idh2-3* and *Mdh2-1* attributed to the group 1 of *T. sordida*; E2 and G2: individuals pertaining to E and G populations and showing *Idh2-1* or *Idh2-2* and homozygosity for *Mdh2-2* attributed to one cryptic species of *T. sordida* (group 2); E12 and G12: putative hybrid individuals between groups 1 and 2; *n* = number of specimens; 0 = null frequency; the *Pep4*, which was not amenable to a reliable allelic interpretation, was not considered.

considered as diagnostic, in addition to *Idh2* which seems to be a *sordida* specific locus (40% of 'fixed differences', *sensu* Richardson *et al.* 1986).

### Second step of the sample processing

*T. sordida* exhibited three well-separated alleles for *Idh2* and two for *Mdh2*. Only one heterozygous form *Idh2–1.3* was detected in each *T. sordida* population from the Chaco (E and G) though both alleles were present (Table 3). At the locus *Mdh2*, no heterozygous form was detected in the same populations though both alleles were present. The virtual lack of heterozygotes at these loci allowed us to subdivide these populations according to the observed genotypes (Figures 1 and 2): those specimens that exhibited the slowest migrating allele at *Idh2* and a null mobility allele at *Mdh2*

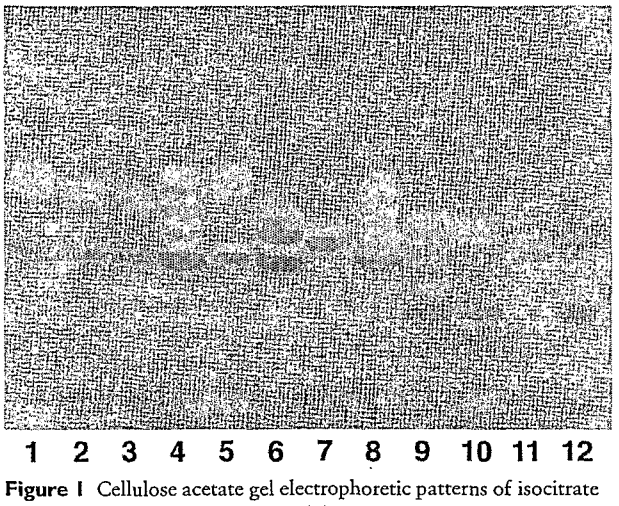


**Figure 1** Cellulose acetate gel electrophoretic patterns of isocitrate dehydrogenase (IDH) from *T. sordida* and *T. guasayana* tissues. *T. sordida* group 1: lanes 10 and 12; *T. sordida* group 2: lanes 1, 2, 3, 4, 5, 6 and 8; *T. sordida* putative hybrid: lane 9; *T. guasayana*: lanes 7 and 11. *Idh1*: lane 1, phenotype 1.2; lanes 2 and 3, phenotype 2.2; lanes 4 and 8, phenotype 1.3; lane 5, phenotype 1.1; lanes 6, 9, 10 and 12, phenotype 3.3; lanes 7 and 11, phenotype 4.4. *Idh2*: lanes 1, 2, 3, 4, 5, 6 and 8, phenotype 1.1; lanes 10 and 12, phenotype 3.3; lane 9, phenotype 1.3; lanes 7 and 11, no band detected.

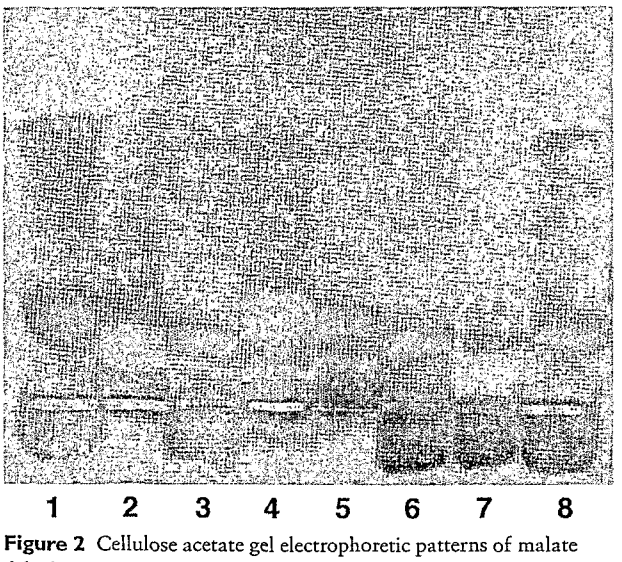


**Figure 2** Cellulose acetate gel electrophoretic patterns of malate dehydrogenase (MDH) from *T. sordida* and *T. guasayana* tissues. *T. sordida* group 1: lanes 2 and 5; *T. sordida* group 2: lanes 3, 6 and 7; *T. sordida* putative hybrid: lane 4; *T. guasayana*: lanes 1 and 8. *Mdh1*: lane 1, phenotype 2.3; lanes 2 and 4, phenotype 1.2; lanes 3, 6, 7 and 8, phenotype 3.3; lane 5, phenotype 2.2. *Mdh-2*: lanes 1, 3, 6, 7 and 8, phenotype 2.2; lanes 2, 4 and 5, phenotype 1.1.

were scored as group 1 (3 specimens from E and 11 from G), while the remaining ones exhibiting the fastest migrating alleles at *Idh2*, together with and an allele migrating toward the cathode at *Mdh2*, were scored as group 2 (28 specimens from E and 21 from G). We have considered the two insects which exhibited the heterozygous pattern *Idh2–1.3* as putative hybrids. The allelic distribution at *Idh2* and *Mdh2* (Table 3) assigned the remaining *T. sordida* populations (A, B, C, D and I) to the group 1.

### Isoenzyme variability

Of the 19 loci analysed in *T. guasayana*, seven (*Got*, *Gpd1*, *Gpd2*, *Idh1*, *Mdh1*, *Pgm* and *6-Pgdh*) were polymorphic ($P = 0.37$). *T. sordida* showed seven polymorphic loci (*Idh1*, *Idh2*, *Mdh1*, *Mdh2*, *Pep4*, *Pgm* and *6-Pgdh*) over the 20 analysed ($P = 0.35$). The obvious polymorphism at *Pep4* in *T. sordida* was not amenable to a reliable allelic interpretation (heterozygote pattern not clearly defined) and this locus was not considered for further genetic interpretation. The estimate of gene diversity (or expected heterozygosity, He) was $0.120 \pm 0.044$ in *T. sordida* and $0.089 \pm 0.038$ in *T. guasayana*, while the mean number of alleles per locus (A) was 1.68 and 1.42, respectively.

The genetic variability of *T. sordida* Group 1 was apparently lower than in Group 2 ($P = 0.20$, $He = 0.050 \pm 0.031$, $A = 1.32$ and $P = 0.30$, $He = 0.065 \pm 0.033$, $A = 1.42$, respectively).

Isoenzyme polymorphism was independent of the insect stage except for the *Gpd1*. Indeed, *T. sordida* showed an activity at this locus in 90.7% of adult stages *vs.* only 1.3% of nymphs ($P < 0.001$). Interestingly, all the adult specimens which did not exhibit the *Gpd1* activity were originated from domestic or laboratory populations (D and I).

### Genotypic equilibrium

For *T. sordida* from the Chaco (and excepting the *Mdh1 and 6-Pgdh* loci for which results were consistent with panmixia), significant departures from Hardy–Weinberg expectations and from linkage disequilibrium were detected in Izozog and Tita when groups 1 and 2 were pooled (Tables 4 and 5). When each group was considered separately at each site (E1 and G1 for group 1, E2 and G2 for group 2), no departure from panmictic expectations was observed. On the other hand, other *T. sordida and T. guasayana* populations were compatible with genetic equilibrium (results not shown).

### Genetic distances

*T. sordida* groups 1 and 2 were separated by more than 10 times the average genetic distance within group 1 and 50 times the genetic distance within group 2 (0.271). A greater genetic distance of 0.769 was observed between *T. sordida* and *T. guasayana*. Nei's standard genetic distance separating the populations within *T. sordida* group 1, *T. sordida* group 2 or *T. guasayana* ranged between 0.005 and 0.024 (Figure 3).

## Discussion

### *T. sordida* and *T. guasayana*

Geographical records have shown that *T. sordida* and *T. guasayana* distributions overlap throughout northern Argentina and parts of the Chaco region in Bolivia and Paraguay (Lent & Wygodzinsky 1979; Schofield 1994). Both species are morphologically very similar and occupy a comparable variety of silvatic ecotopes. They frequently enter peridomestic habitats and dwellings where they form small domestic colonies (Forattini *et al.* 1984; Wisnivesky-Colli *et al.* 1993). These common features suggest that *T. sordida* and *T. guasayana* are closely related species (Usinger *et al.* 1966). Nevertheless, studies of microdistribution carried out in areas of overlapping are hindered by the difficulty of reliably distinguishing them on the basis of external morphology. The morphological separation of *T. sordida* and *T. guasayana* adults based on connexival markings or

**Table 4** Fixation indices at five loci in *T. sordida* from Izozog and Tita, Bolivia*

| | Izozog E1E2 ($n = 32$) | Tita G1G2 ($n = 33$) | Izozog E2 ($n = 28$) | Tita G1 ($n = 11$) | Tita G2 ($n = 21$) |
|---|---|---|---|---|---|
| *Idh1* | 0.482‡ | 0.692§ | 0.345 | / | −0.348 |
| *Idh2* | 0.840† | 0.933§ | −0.018 | / | / |
| *Mdh1* | 0.382 | 0.292 | 0.208 | −0.375 | −0.167 |
| *Mdh2* | 0.840† | 0.933§ | / | / | / |
| *Pgm* | 0.349 | 0.812§ | −0.037 | −0.100 | −0.077 |
| *6-Pgdh* | 0.062 | 0.311 | 0.282 | 0.091 | 0.111 |

*The value of the fixation index (F) at each locus was computed according to Nei (1987): $F = (4n_{11}n_{22} - n^2_{12})/(2n_{11} + n_{12})(2n_{22} + n_{12})$, with $n_{11}$ and $n_{22}$ being the number of homozygotes and $n_{12}$ the number of heterozygotes. The deviation of F from 0 was tested by $\chi^2 = nF^2$ with one degree of freedom, and $n = n_{11} + n_{12} + n_{22}$. E1, individuals from the population E and pertaining to the group 1 of *T. sordida*; E2, individuals from the the population E and pertaining to the group 2 of *T. sordida*; G1, individuals from the the population G and pertaining to the group 1 of *T. sordida*; G2, individuals from the population G and pertaining to the group 2 of *T. sordida*; E1E2, individuals E1 and E2 grouped together; G1G2, individuals G1 and G2 grouped together; *n*, number of specimens; /, monomorphic locus. †$P < 002$; ‡$P < 0.01$; §$P < 0.001$.

**Table 5** Linkage disequilibrium of enzymatic loci in *T. sordida* from Izozog and Tita, Bolivia*

| | Izozog E1E2 ($n = 32$) | Tita G1G2 ($n = 33$) | Izozog E2 ($n = 28$) | Tita G1 ($n = 11$) | Tita G2 ($n = 21$) |
|---|---|---|---|---|---|
| *Idh1 Idh2* | 0.009 | 0.000 | / | / | / |
| *Idh1 Mdh1* | NS | 0.000 | NS | / | NS |
| *Idh1 Mdh2* | 0.009 | 0.000 | / | / | / |
| *Idh1 Pgm* | 0.026 | 0.000 | NS | / | NS |
| *Idh1 6-Pgdh* | NS | NS | NS | / | NS |
| *Idh2 Mdh1* | 0.004 | 0.000 | NS | / | / |
| *Idh2 Mdh2* | 0.000 | 0.000 | / | / | / |
| *Idh2 Pgm* | 0.004 | 0.000 | NS | / | / |
| *Idh2 6-Pgdh* | 0.013 | NS | NS | / | / |
| *Mdh1 Mdh2* | 0.004 | 0.000 | / | / | / |
| *Mdh1 Pgm* | NS | 0.000 | NS | NS | NS |
| *Mdh1 6-Pgdh* | NS | NS | NS | NS | NS |
| *Mdh2 Pgm* | 0.001 | 0.000 | / | / | / |
| *Mdh2 6-Pgdh* | NS | NS | / | / | / |
| *Pgm 6-Pgdh* | NS | NS | NS | / | NS |

*Linkage disequilibrium was tested using a Fisher's exact test, with each cell containing the number of individuals with a particular phenotype (locus 1) × phenotype (locus 2) combination. E1, individuals from the population E and pertaining to the group 1 of *T. sordida*; E2, individuals from population E and pertaining to group 2 of *T. sordida*; G1, individuals from population G and pertaining to group 1 of *T. sordida*; G2, individuals from population G and pertaining to group 2 of *T. sordida*; E1E2, individuals E1 and E2 grouped together; G1G2, individuals G1 and G2 grouped together; *n*, number of specimens; /, monomorphic locus; NS, not significant.

ratio of rostral segments is not totally reliable, nor are other chromatic differences as examined by Gorla *et al.* (1993). Consequently, the enzyme-based species distinction applied to field-collected populations is recommended in the area of overlapping.

MLEE is a powerful tool to detect biological species and was applied in systematics of Triatominae by various authors (Dujardin *et al.* 1987; Harry *et al.* 1992; Pereira *et al.* 1996). In addition to *Idh2* only detected in *T. sordida*, seven loci were found to distinguish *T. sordida* from *T. guasayana*: *Got*, *Gpi*, *Idh1*, *Lap2*, *Me1*, *Pep3* and *Pgm*. Consequently, the zymograms corresponding to these loci offer a means for reliable identification of *T. sordida* and *T. guasayana*, especially in nymphal instars which are not obviously distinguishable at the morphological level. No hybrid was observed in nature during the present study and among 246 *T. sordida* and *T. guasayana* occurring in sympatry and examined by MLEE (Noireau *et al.* unpublished data), which contrasts with laboratory studies (Usinger *et al.* 1966) where reproductive isolation was only partial.

### Sample processing within *T. sordida*

Silvatic populations of *T. sordida* and *T. guasayana* were caught by light trapping in two distinct capture sites within repeated 3-h time periods. Knowing the relatively reduced dispersive flight capacity of Triatominae (Schofield 1994), this way of sampling ensures that *T. sordida* specimens collected in each site were living in sympatry. The scarce natural hybrids which exhibited the heterozygous pattern *Idh2–1.3* represented only 3% (2/65) of the pooled populations E and G. Although we have contemplated them as putative hybrids, they might also be scarce genotypes from the group 1 (they exhibited the null mobility allele at *Mdh-2*). Nevertheless, we chose the unfavourable hypothesis to test the genetic equilibrium.

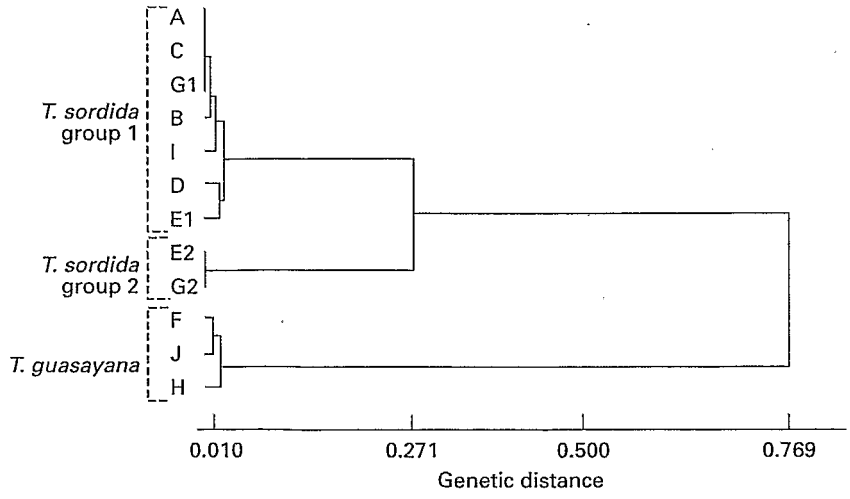


**Figure 3** UPGMA dendrogram derived from Nei's standard genetic distances (Ds) between cryptic species of *T. sordida* and *T. guasayana* and within *T. guasayana* and cryptic species of *T. sordida*. Populations A, B, C, D, F, H, I and J: see Table 1; E1, individuals from the population E and pertaining to group 1 of *T. sordida*; E2, individuals from population E and pertaining to group 2 of *T. sordida*; G1, individuals from population G and pertaining to group 1 of *T. sordida*; G2, individuals from population G and pertaining to group 2 of *T. sordida*.

### Isoenzyme variability

In our study, values of genetic variability for *T. guasayana* were apparently lower than those reported by Garcia *et al.* (1995) who used starch gel electrophoresis ($P = 0.37$ *vs.* 0.58; He = 0.089 *vs.* 0.169). Because of the different technique used by these authors, we were unable to link their analysed population of *T. sordida* with one of our genetically defined groups. The proportion of polymorphic loci they reported for *T. sordida* ($P = 0.57$) was higher than our values for groups 1 or 2 ($P = 0.20$, $P < 0.05$ or $P = 0.30$, $P > 0.05$, respectively). On the other hand, the He value reported by Garcia *et al.* (1995) for *T. sordida* (0.062) was similar to our values (0.050 and 0.065 for the groups 1 and 2, respectively). As a whole, the calculated values assessing the genetic variability for *T. sordida* and *T. guasayana* corroborate the trend towards low isoenzymatic polymorphism amongst Triatominae (Dujardin & Tibayrenc 1985; Harry *et al.* 1992; Pereira *et al.* 1996). Nevertheless, these values may be regarded as higher than those reported for other species in the genus *Triatoma* (Frías & Kattan 1989; Pereira *et al.* 1996; Dujardin *et al.* 1998). This relatively high genetic variability observed for *T. sordida* and *T. guasayana* may reflect a lack of ecological specificity as observed in nature for these species (Schofield 1994).

The *Gpd1* activity was apparently stage-related in *T. sordida*. It was absent in nymphal instars and seemed to appear in adult or just before adult moulting (the only nymph presenting an activity was a fifth instar). As the GPD enzyme takes a prominent part in the flight activity of insects (Zebe & McShan 1957; Pereira *et al.* 1996), we assume that *T. sordida* adults lacking a *Gpd1* activity would have a different level of flight activity. The fact that such specimens only occurred among our sample in domestic or laboratory populations suggested this could be linked to a reduced flight activity.

### Genotypic equilibrium

The use of both within and between loci disequilibrium as a way to detect biological species was illustrated in sand flies by Dujardin *et al.* (1996). This approach applied to silvatic *T. sordida* from the Chaco (populations E and G) revealed that mating was not random within the morphological species. The within and between loci disequilibrium we observed could be attributed either to the presence of undetected null alleles, to a Wahlund effect (spatial or temporal subdivision), to strong selection against heterozygotes or to cryptic speciation. The existence of null alleles was discarded because no specimen showed a complete lack of activity at either *Idh2* or *Mdh2* loci. Our conditions of capture ensured the insects were living in probable sympatry, which helped to discard a Wahlund effect. An unknown selecting factor acting against heterozygous forms at the same three loci (*Idh1, Idh2* and *Mdh2*) and inducing the same linkage desequilibrium at two distant capture sites (120 km) would be unlikely. These arguments, and the absence of genetic disequilibrium detected within *T. sordida* groups 1 and 2 lend support the hypothesis of two biological species showing a similar external morphology, i.e. two sibling species within *T. sordida*.

### Genetic distances

The genetic distance observed between *T. sordida* (whole population) and *T. guasayana* was high (0.769) and very similar to the value reported by Garcia *et al.* (1995). We have no doubt that both taxons are separate species. Nei's standard genetic distance between the *T. sordida* groups 1 and 2 was 0.271. This value even in sympatric conditions was more than 9 times higher than the values observed between the two geographical populations (0.030 between E1 and G1, 0.005 between E2 and G2, respectively). It greatly exceeded 0.10, a threshold beyond which no case of conspecific populations was recorded in Anophelinae (Estrada-franco *et al.* 1993), sand flies (Kreutzer *et al.* 1990; Dujardin *et al.* 1996) or Triatominae (Dujardin *et al.* 1998). The genetic distance between groups 1 and 2 was larger than or comparable to the values found between closely related triatomine species such as *T. infestans*, *T. platensis* and *T. delpontei* (Pereira *et al.* 1996). It was thus in full agreement with the hypothesis of two distinct sympatric species in *T. sordida*. However, the morphological similarity between them and the occurrence of possible scarce hybrids in nature (3% of the whole populations) could support the hypothesis of a recent evolutionary divergence within *T. sordida*.

These results bring some insight into the recent cytogenetic survey of Brazilian and Argentine populations of *T. sordida* showing an unexpected degree of genetic differences between them (Panzera *et al.* 1997). More importantly, they could help in understanding the trend to domiciliation observed in Bolivia for *T. sordida*. Indeed, of the two biological species of *T. sordida* identified by this work, only one was found in the domestic structures. Further studies will have to use the diagnostic systems IDH and MDH when examining vectorial capacity or biological traits of *T. sordida*.

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