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Cuticular hydrocarbon pattern as a chemotaxonomy marker to assess intraspecific variability in *Triatoma infestans*, a major vector of Chagas' disease

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Abstract. *Triatoma infestans* Klug (Hemiptera: Reduviidae) populations were sampled in various localities throughout most of the species' geographic range of distribution in Argentina, Bolivia, Paraguay and Peru. In order to contribute to understanding of the diversity and population structure of this major vector of Chagas' disease, cuticular hydrocarbon (CHC) profiles were analysed by capillary gas chromatography and variations evaluated by statistical methods of classification and ordination. High levels of intrapopulation variation were detected, along with low levels of variability among populations. Based on relative amounts of the major odd-numbered straight-chain hydrocarbons *n*-C27 to *n*-C33, two hydrocarbon phenotypes were evident, unequally distributed along the species' geographic range. Analysis of CHC patterns showed that *T. infestans* populations segregate into two major groups consisting of an Andean group, which comprises specimens from Peru and most parts of Bolivia, and a non-Andean group, which includes all specimens from Argentina and Paraguay, together with those from Tarija (Bolivia). Pyrethroid-resistant and -susceptible specimens were differentiated based on relative amounts of some straight and monomethyl-branched hydrocarbon components.

Key words. *Triatoma infestans*, Chagas' disease, chemotaxonomy, cuticular hydrocarbons, intraspecific variability, population structure.

Introduction

As more than 10 million people are infected and a further 25 million are at risk for infection, Chagas' disease is by far the most important parasitic disease in Latin America [World Health Organization (WHO), 2010] and is becoming a serious health issue in the U.S.A. and Europe (Guerri-Guttenberg *et al*., 2008; Reisenman *et al*., 2010). This infectious disease is caused by the protozoan parasite *Trypanosoma cruzi* Chagas, which is mainly transmitted by blood-sucking insects of the Triatominae subfamily. Among the triatomine species that are adapted to live in human habitats, and hence relevant to the transmission of Chagas' disease, *Triatoma infestans* Klug is the major vector in endemic areas in the southern cone of South America, including southern Peru, Bolivia, central and northern Argentina, and Paraguay. A multinational initiative to control *T. infestans* in this region was launched in 1992 with the implementation of residual insecticide applications. A substantial reduction in household infestation rates of the vector and a subsequent halt in disease transmission through direct contact with the vector were achieved in large parts of its distribution range, except for the Gran Chaco region, which extends to over 1.3 million square km and has a mostly rural and poorly resourced human population (Schofield & Kabayo, 2008). The lack of sustainable vector control strategies, the low efficacy of pyrethroid insecticides in peridomestic habitats and the need for new vector control methods to counter pyrethroid resistance

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in this area have driven multidisciplinary efforts to understand critical traits of *T. infestans*. Behavioural, biochemical, genetic and morphological studies are crucial to enhancing our understanding of migration between populations, re-infestation incidence (i.e. from peridomestic and sylvatic foci) and genetic variability. Understanding of the rising levels and spread of pyrethroid insecticide resistance (Santo Orihuela *et al*., 2008; Pedrini *et al*., 2009) is also needed.

Although a number of *T. infestans* chromatic morphs are known (Monteiro *et al*., 1999; Noireau *et al*., 2000), the first evidence of a clear allopatric subdivision between Bolivian and Argentinian-Brazilian populations was obtained by Monteiro *et al.* (1999) based on mitochondrial DNA and isoenzymes analyses. Two *T. infestans* cytotypes were described by Panzera *et al.* (2004), based on autosomal C heterochromatin content: an 'Andean group', which included Peruvian and western Bolivian populations, and a 'non-Andean group', which comprised insects from Argentina, Paraguay, Brazil, Uruguay and the Bolivian Chaco. Intraspecific genetic diversity was also studied by comparative analysis of rDNA internal transcribed spacer (ITS)-1 and ITS-2 sequences from several *T. infestans* populations (Bargues *et al*., 2006); five haplotypes were detected, separating the species into two main groups, in agreement with the Andean and non-Andean groupings. In 2005, a multinational research project involving studies designed to increase understanding of the diversity of *T. infestans* and its population structure along most of its distribution range (Gran Chaco and the Andean valleys of Bolivia) was developed, with the aim of contributing to vector surveillance and control. This project included cytogenetic, antennal phenotype, wing morphometry and cuticular hydrocarbon (CHC) pattern analyses among other markers, using both different body parts of single insects and different insects from the same population (Catalá *et al*., 2007). A third cytotype with heterochromatin content that located it in an intermediate position between the Andean and non-Andean groups was proposed (Panzera *et al*., 2007). Significantly higher CHC content and cuticle thickness, together with diminished deltamethrin penetration, were detected in pyrethroid-resistant insects, thus providing the first direct evidence of the relationship between the hydrocarbon surface layer and the entire cuticle with resistance to insecticide penetration (Juárez *et al*., 2007, 2010; Pedrini *et al*., 2009). Previous analysis of triatomine CHC contributed to understanding of the taxonomic relationship between species of the main genera of *Triatoma*, *Panstrongylus* and *Rhodnius* (Juárez *et al*., 2000), as well as the population structure of *Triatoma dimidiata*, an important vector of Chagas' disease in North and Central America (Calderón-Fernández *et al*., 2011). Triatomine hydrocarbons are mostly saturated, with straight and methyl-branched structures of 18 to >40 total carbons. Odd-numbered homologous series of *n*-alkanes, different isomers of terminally and internally branched mono-, di-, tri- and tetramethylalkanes, were identified in several species of the *Triatoma* genus (*T. infestans*, *Triatoma mazzottii* and *Triatoma pallidipennis*) and in *Rhodnius prolixus*, using capillary gas chromatography coupled to mass spectrometry (CGC-MS) analyses (Juárez & Calderón-Fernández, 2007).

As part of a larger study, the aim of this work was to examine the intraspecific variability and population structure of *T. infestans* using CHCs as chemotaxonomic characters, and to compare these data with those for other population markers.

Materials and methods

Collection area and insects

Sampling sites in Argentina, Bolivia, Paraguay and Peru comprised an extensive and heterogeneous area, located between latitudes 16°25′ S and 30°14′ S and longitudes 71°30′ W and 56°49′ W. This area includes the Andean region in the west, which has altitudes in the range of 1850–2900 m a.s.l. and where the climate is dependent on altitude, and the Gran Chaco region, which has a hot and dry climate and altitudes in the range of 100–650 m a.s.l. Insects were sampled from 35 collection sites during the years 2005 and 2006, as detailed in Fig. 1 and Table 1. Adult male and female *T. infestans* were collected, and the forewings of each specimen were removed and sent to our laboratory (471 specimens). Data on the pyrethroid susceptibility of insects from collection sites in Yacuiba (Tarija, Bolivia) and Salvador Mazza, La Unión, Morillo and San Carlos (Salta, Argentina) were determined by topical application bioassays (WHO, 1994), within the framework of the project SSA/ATU INCO-CT 2004 515942 [Zerba and Picollo, Centro de Investigaciones de Plagas e Insecticidas (CITEFA-CONICET), internal report, 2007; Catalá *et al*., 2007].

Hydrocarbon analyses

Wings were washed with redistilled water to remove any water-soluble contaminants, transferred to glass vials with Teflon-lined caps, and submerged in redistilled hexane (Carlo Erba Reagents, Milan, Italy) (6 mL/g) overnight to extract total lipids. After solvent reduction in volume under nitrogen, hydrocarbons were separated from other components by adsorption chromatography on a mini-column (10 × 5 mm internal diameter) of activated Supelcosil A (Supelco, Inc., Bellefonte, PA, U.S.A.), eluting with redistilled hexane (6 mL/mg hydrocarbon). The extract was then evaporated to an appropriate volume for gas chromatography. Capillary gas chromatographic (CGC) analysis was performed using a Hewlett-Packard 6890 gas chromatograph (Hewlett Packard Co., Wilmington, DE, U.S.A.) equipped with a split/splitless injector port, fitted with a non-polar fused silica (0.25-μm) HP-5 capillary column (Hewlett Packard Co.) (30 × 0.32 mm internal diameter). The oven temperature was programmed to rise from 50 °C (hold time 2 min) to 180 °C at 20 °C/min, and then from 180 °C to 310 °C at 3 °C/min (hold time 10 min). Injection of *n*-alkane standards of 22–40 carbons was similarly performed for estimation of Kovats indices (KIs) (Kovats, 1965). The KI value of a chemical component is the chromatographic elution time normalized to the elution time of adjacently eluting alkanes; it facilitates the estimation of the carbon number and branching pattern, and provides a helpful tool supporting identification. The KIs agreed well with those published by Juárez &

Fig. 1. Map showing sites of collection of *Triatoma infestans*, [Country: province/department, locality (number on map)]: Argentina: Catamarca, Palo Blanco (1); Chaco, Pampa Avila (2), Tres Estacas (3); Salta, La Unión (4), Morillo (5), Salvador Mazza (6), San Carlos (7); Santiago del Estero, Capital (8), Puente Negro (9), Huachana (10), Silipica (11), Colonia Alcira (12), Campo Union (13), La Martona (14). Bolivia: Cochabamba, Bandorniyoc (15), Mataral (16), Quillacollo (17); Potosi, La Deseada (18), Urulica (19); Chuquisaca, Carapari (20); Tarija, Yacuiba (21), Entre Rios (22), Villa Montes (23). Paraguay: Boqueron, Jerico (24), Jope (25); Presidente Hayes, Pozo Colorado (26), Monte Lindo (27); Paraguari, Carapegua (28); Cordillera, Isla Pucu (29), Emboscada (30), San Jose Obrero (31); San Pedro, Itacurubi del Rosario (32), General Elizardo Aquino (33), San Pedro de Ycuamandiyu (34). Peru: Arequipa, Arequipa (35). Numbers correspond to localities listed in Table 1.

Blomquist (1993). Peak identity was determined by CGC-MS analysis operating the gas chromatograph in conditions similar to those described above, coupled to an Agilent 5975C VL mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.), with the detector set at 70 eV, and transfer line and quadrupole held at 320 °C and 150 °C, respectively, as previously described (Juárez *et al*., 2001; Calderón-Fernández *et al*., 2011). Shorthand nomenclature was used in the text and tables to identify the hydrocarbons: Cxx denotes the total number of carbons in the straight chain; *n*-Cxx denotes linear alkanes, and x-methyl describes the location of methyl branches for monomethyl alkanes.

Statistical analyses

Hydrocarbon peaks were considered as characters and their relative amount values as character states. The relative amount of each component was calculated by dividing the corresponding peak area by the total hydrocarbon peak area. A total of 85 CHC peaks were selected as input variables (peak area >0.2% of total hydrocarbon peak area), and their relative amount values subjected to arcsine transformation prior to multivariate analysis in order to normalize data (Sokal & Rohlf, 2001). Principal component analysis (PCA) and linear discriminant analysis (DA) were used to assess the relationships between specimens and populations, using SPSS Version 11.0 (SPSS, Inc., Chicago, IL, U.S.A.) and STATISTICA Version 6.0 (StatSoft, Inc., Tulsa, OK, U.S.A.). For DA, backward-elimination stepwise procedures were applied to select those hydrocarbons that best contribute to discrimination. In some Paraguayan areas, a low number of specimens were collected; thus these insect populations were regrouped as those from western Paraguay (Boqueron and Presidente Hayes) and eastern Paraguay (San Pedro, Paraguari and Cordillera) in order to achieve an acceptable number of insects per group. Wilks' lambda (λ) statistic (Wilks, 1932) and its statistical significance were used as discrimination significance measures. The accuracy of the discriminant function (DF) was tested by reclassifying the specimens using the cross-validation method (Lachenbruch & Mickey, 1968). An unweighted pair-group method with arithmetic average (UPGMA) dendrogram was generated from PCA case scores in order to visualize the relationships among *T. infestans* populations, using MVSP Version 3.13b (Kovach Computing Services, Anglesey, U.K.).

Table 1. *Triatoma infestans* collection sites.

Country	Department/province	*n*	Localities*	Longitude	Latitude	Habitat†
Argentina	Catamarca	38	Palo Blanco (1)	67°45′35″ W	27°20′26″ S	P
	Chaco	49	Pampa Avila (2)	61°30′44″ W	26°59′50″ S	P/D
			Tres Estacas (3)	61°35′60″ W	26°56′00″ S	P
	Salta	34	La Unión (4)	63°08′15″ W	23°45′38″ S	D
			Morillo (5)	62°53′10″ W	23°28′11″ S	D
			Salvador Mazza (6)	63°41′21″ W	22°01′24″ S	D
			San Carlos (7)	65°57′53″ W	25°48′33″ S	P
	Santiago del Estero	69	Capital (8)	64°18′08″ W	27°47′08″ S	P
			Puente Negro (9)	62°45′12″ W	28°14′25″ S	P
			Huachana (10)	64°07′13″ W	28°13′25″ S	P
			Silipica (11)	64°07′08″ W	28°06′12″ S	P
			Colonia Alcira (12)	63°09′58″ W	28°29′25″ S	P
			Campo Union (13)	62°10′36″ W	30°14′13″ S	P
			La Martona (14)	62°13′16″ W	28°50′16″ S	P
Bolivia	Cochabamba	68	Bandorniyoc (15)	64°48′39″ W	18°16′17″ S	P
			Mataral (16)	64°12′25″ W	18°07′53″ S	P
			Quillacollo (17)	66°17′25″ W	17°25′55″ S	S
	Potosi	24	La Deseada (18)	65°41′34″ W	21°31′19″ S	P
			Urulica (19)	65°43′02″ W	21°25′43″ S	P
	Chuquisaca	20	Carapari (20)	63°52′24″ W	19°26′00″ S	P
	Tarija	74	Yacuiba (21)	63°40′53″ W	22°02′22″ S	D/P
			Entre Rios (22)	64°10′24″ W	21°31′35″ S	D/P
			Villa Montes (23)	63°28′35″ W	21°15′43″ S	D
Paraguay	Boqueron	45	Jerico (24)	59°48′41″ W	22°35′46″ S	D
			Jope (25)	59°49′16″ W	23°35′02″ S	D
	Presidente Hayes	7	Pozo Colorado (26)	58°47′35″ W	23°29′43″ S	D
			Monte Lindo (27)	58°25′53″ W	23°53′13″ S	D
	Paraguari	9	Carapegua (28)	57°14′54″ W	25°46′01″ S	D
	Cordillera	8	Isla Pucu (29)	56°54′20″ W	25°18′38″ S	D
			Emboscada (30)	57°21′18″ W	25°07′24″ S	D
			San Jose Obrero (31)	56°55′05″ W	25°10′36″ S	D
	San Pedro	6	Itacurubi del Rosario (32)	56°49′37″ W	24°32′23″ S	D
			General Elizardo Aquino (33)	56°54′05″ W	24°26′27″ S	D
			San Pedro de Ycuamandiyu (34)	57°04′35″ W	24°05′19″ S	D
Peru	Arequipa	20	Arequipa (35)	71°29′58″ W	16°25′51″ S	D

*Numbers in parentheses indicate the geographic position of the collection sites in Fig. 1.
†Habitat: D, domestic; P, peridomestic; S, sylvatic.

The Mann–Whitney non-parametric *U*-test was used to evaluate differences in CHC relative amounts within populations, and to compare CHC relative amounts between pyrethroid-resistant and pyrethroid-susceptible insect populations ($\alpha = 0.05$) using SPSS Version 11.0.

Results

Hydrocarbon pattern variation

The chromatographic profiles revealed no qualitative differences between *T. infestans* populations of different geographic origins. In agreement with previous work (Juárez & Brenner, 1985, 1986), neither qualitative nor quantitative sex dimorphism was evident and thus both males and females were used for statistical analyses. The hydrocarbon pattern showed large intrapopulation variability along the species' geographic range, with considerable variation in the relative amount of the major odd-numbered chains (*n*-C27 to *n*-C33); these four components accounted for 38–57% of the total CHC fraction. Based on their variation, two CHC phenotypes were proposed *a priori* by visual observation of the chromatograms. CHC phenotype I was the most abundant (84% of the specimens collected) and had similar amounts of major odd-numbered chain *n*-alkanes (Fig. 2A). CHC phenotype II grouped specimens showing a distinctive abundance of *n*-C29 (Fig. 2B). In order to support the existence of the two phenotypes, we designed a numerical indicator (I) based on the relationship among major straight-chain CHC components: I_1 represented *n*-C29/*n*-C27 + *n*-C31, and I_2 represented *n*-C29/*n*-C31 + *n*-C33. When the relative amount of *n*-C29 doubled that of the other two major components combined (I_1 and $I_2 \geq 2$), specimens were assigned to CHC phenotype II, which differed significantly from CHC phenotype I (Table 2). Most of the specimens of phenotype II were found in Argentina (71%), Bolivia (20%), Paraguay (5%) and Peru (4%) (Table 3). Furthermore, CHC phenotype distribution analysis for each collection site showed that CHC phenotype II predominated in Catamarca, Argentina (66% of specimens collected in this

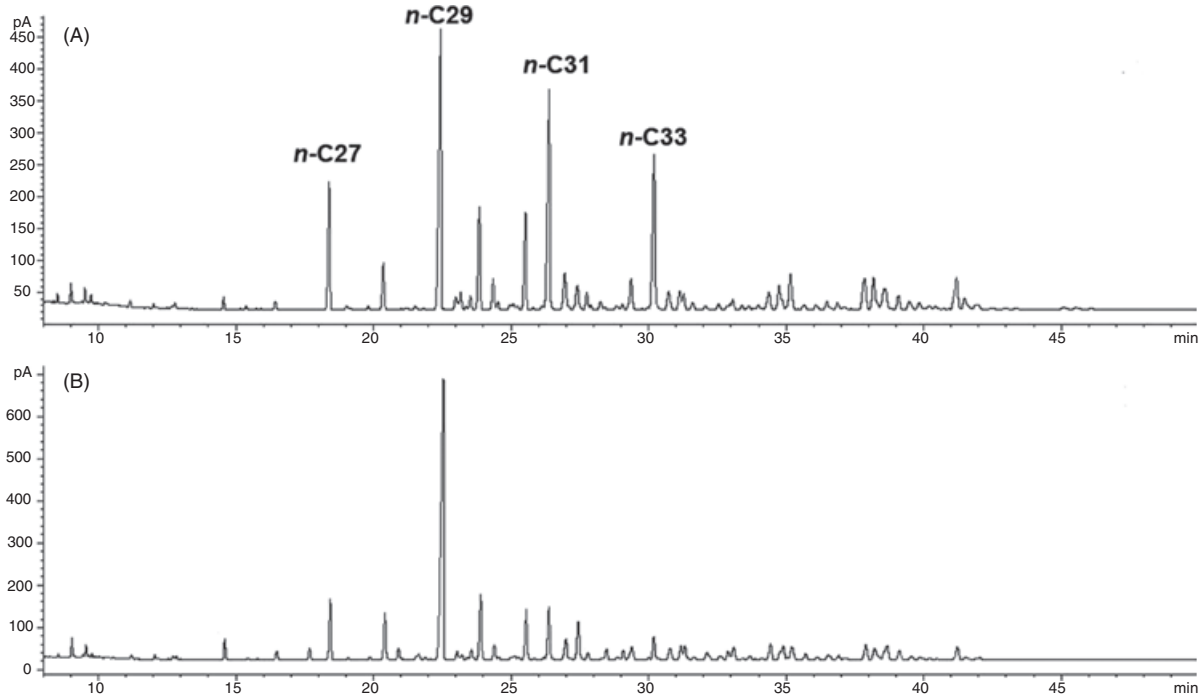

Fig. 2. Capillary gas chromatography profiles of *Triatoma infestans* cuticular hydrocarbon (CHC) phenotypes (A) I and (B) II. In CHC phenotype I, none of the major odd-numbered *n*-alkanes is clearly predominant; by contrast, *n*-C29 was the predominant straight-chain component in phenotype II. The four major odd-numbered straight-chain hydrocarbons KI 2700 (*n*-C27), KI 2900 (*n*-C29), KI 3100 (*n*-C31) and KI 3300 (*n*-C33) are marked for better comparison.

Table 2. Relative amount of major straight odd-chain hydrocarbons of phenotypes I and II.

	Phenotype I	Phenotype II
CHC	Mean ± SD	Mean ± SD
n-C27	7.24 ± 3.04	4.78 ± 1.32
n-C29	24.62 ± 5.07	31.45 ± 5.97
n-C31	12.85 ± 3.44	7.32 ± 2.68
n-C33	6.25 ± 2.55	4.84 ± 2.00

CHC, cuticular hydrocarbon; SD, standard deviation.
The relative amounts of the four hydrocarbons differed significantly between the phenotypes (Mann–Whitney *U*-test, $P < 0.000$); the number of specimens was 396 and 75 for phenotype I and phenotype II, respectively.

province), varied from 2% to 22% in most locations, and was not detected in some areas of Bolivia and Paraguay (Table 3). To exclude the intrapopulation variance component related to the existence of two hydrocarbon phenotypes, only insects of the most abundant phenotype, CHC I, were used in the multivariate analyses (396 specimens).

Population structure

Using the backward-elimination method, relative amount values for 13 CHCs (KIs 2700, 2830, 2900, 2940, 3159, 3174, 3200, 3207, 3258, 3300, 3355, 3534 and 3954) were retained for DA, which yielded a statistically significant Wilks' λ of 0.02 ($P < 0.001$). Ten DF-values were obtained, the first three of which accounted for 89% of the total sample variance. The percentages of insects correctly classified in the original (78.2%) and cross-validated (72.6%) classifications were very close, thus supporting the usefulness of the DF to assess the species population structure (Table 4). The partial classification results (Table 4) revealed a variable degree of mixing among specimens from Potosi, Cochabamba, Chuquisaca and Arequipa, but little or no overlap between these samples and the other populations under study. By contrast, a large degree of merging was detected among insects from Tarija, Catamarca, Chaco, Salta, Santiago del Estero, and western and eastern Paraguay, reflecting the high level of similarity in their hydrocarbon pattern. The UPGMA dendrogram based on PCA shows that *T. infestans* populations segregate into two major groups (Fig. 3). Cuticular hydrocarbon compounds with KIs 3174, 3159 and 3355 differentiate an Andean group, comprising populations from Arequipa (Peru), Potosi, Cochabamba and Chuquisaca (Bolivia), and a non-Andean group, which includes one Bolivian population (Tarija) and those from Argentina and Paraguay. In the Andean group, hydrocarbons with KIs of 3258 and 3300 separate the Arequipa and Potosi populations from those from Cochabamba and Chuquisaca; in the non-Andean group, the hydrocarbon component with KI 2700 differentiates two clusters, of which one includes Argentinean populations from Santiago del Estero, Chaco and Catamarca, and the other encompasses specimens from Paraguay, Salta (Argentina) and Tarija (Bolivia). Further details on population clustering by CHC profiles are presented in Fig. 3.

Table 3. Frequency of cuticular hydrocarbon phenotypes.

Country	Department/province	Phenotype I		Phenotype II		Total Insects, *n*
		Insects, *n* (%)	RD, %∗	Insects, *n* (%)	RD, %∗	
Peru	Arequipa	17 (85.0)	4.3	3 (15.0)	4.0	20
Bolivia	Tarija	69 (93.2)	17.4	5 (6.8)	6.7	74
	Potosi	24 (100)	6.0	– (0)	—	24
	Cochabamba	58 (85.3)	14.6	10 (14.7)	13.3	68
	Chuquisaca	20 (100)	5.0	– (0)	—	20
Paraguay	Boqueron	44 (97.8)	11.1	1 (2.2)	1.3	45
	Presidente Hayes	6 (85.7)	1.5	1 (14.3)	1.3	7
	San Pedro	6 (100)	1.5	– (0)	—	6
	Cordillera	8 (100)	2.0	– (0)	—	8
	Paraguari	7 (77.8)	1.8	2 (22.2)	2.7	9
Argentina	Salta	27 (79.4)	6.8	7 (20.6)	9.3	34
	Catamarca	13 (34.2)	3.3	25 (65.8)	33.3	38
	Chaco	43 (87.7)	10.8	6 (12.3)	8.0	49
	Santiago del Estero	54 (78.3)	13.6	15 (21.7)	20.0	69
Total		396 (84.1)		75 (15.9)		471

∗Relative distribution (RD) of each phenotype: number of specimens in a department or province/number of specimens along the geographic range studied.

Table 4. Classification of *Triatoma infestans* populations.

Population	Predicted population membership, %										
	Arequipa	Potosi	Cochabamba	Chuquisaca	Tarija	W Paraguay	E Paraguay	Salta	Catamarca	Chaco	S del Estero
Arequipa	87.50	6.25	0	0	6.25	0	0	0	0	0	0
Potosi	0	100.00	0	0	0	0	0	0	0	0	0
Cochabamba	1.85	7.41	79.63	11.11	0	0	0	0	0	0	0
Chuquisaca	0	0	40.00	60.00	0	0	0	0	0	0	0
Tarija	0	0	0	0	84.06	7.25	2.90	2.90	2.90	0	0
W Paraguay	0	0	0	0	14.29	75.51	0	4.08	0	4.08	2.04
E Paraguay	0	0	0	0	10.00	0	55.00	5.00	0	25.00	5.00
Salta	0	0	0	0	44.00	8.00	0	24.00	8.00	12.00	4.00
Catamarca	0	0	0	0	23.08	0	0	7.69	61.54	7.69	0
Chaco	0	0	0	0	2.38	4.76	11.90	4.76	4.76	71.43	0
S del Estero	0	0	0	0	2.44	0	0	0	0	29.27	68.29

Cross-validated classification results based on discriminant analyses performed in *T. infestans* populations; 78.2% of the original cases were correctly classified; 72.6% of cases were correctly classified by cross-validation.
W Paraguay, western Paraguay; E Paraguay, eastern Paraguay; S del Estero, Santiago del Estero.

Pyrethroid-resistant vs. pyrethroid-susceptible populations

Pyrethroid-resistant *T. infestans* from Salvador Mazza (Salta) and the neighbouring city of Yacuiba (Tarija) were compared with pyrethroid-susceptible specimens from other collection sites in Salta (La Unión, Morillo and San Carlos) (Fig. 1, Table 1). Both populations were differentiated based on the relative amount of 10 CHCs of straight (KIs 3000 and 3300) and monomethyl-branched (KIs 2950, 3258 3652, 2830, 3030, 3131, 3228 and 3534) chains (Table 5).

Discussion

This is the first report on the variation in hydrocarbon patterns in *T. infestans* across most of its geographic range. Our results showed quantitative rather than qualitative differences between populations. Quantitative differences in CHC patterns (i.e. the same compounds occurring in different proportions) are usually associated with intraspecific variability, whereas qualitative differences (i.e. some CHC components of different structures are present in each taxa) are expected to occur between different species (Juárez & Blomquist, 1993; Juárez & Calderón-Fernández, 2007). High intraspecific variability in CHC patterns was found, with most variation recorded within populations. By contrast, intrapopulation variability was much lower and interpopulation variation higher in putative *T. dimidiata* samples, probably representing several subspecies and cryptic species (Dorn *et al*., 2009; Calderón-Fernández *et al*., 2011). In addition, two CHC phenotypes were detected, unequally distributed along the species' geographic range. CHC phenotype I predominated in almost all the collection sites, except the Argentinean province of Catamarca (where CHC phenotype II accounted for 66% of the sample). Different CHC phenotypes probably evolved after mutation events affecting the corresponding biosynthetic enzymes. Small sequence changes can alter the activity range of

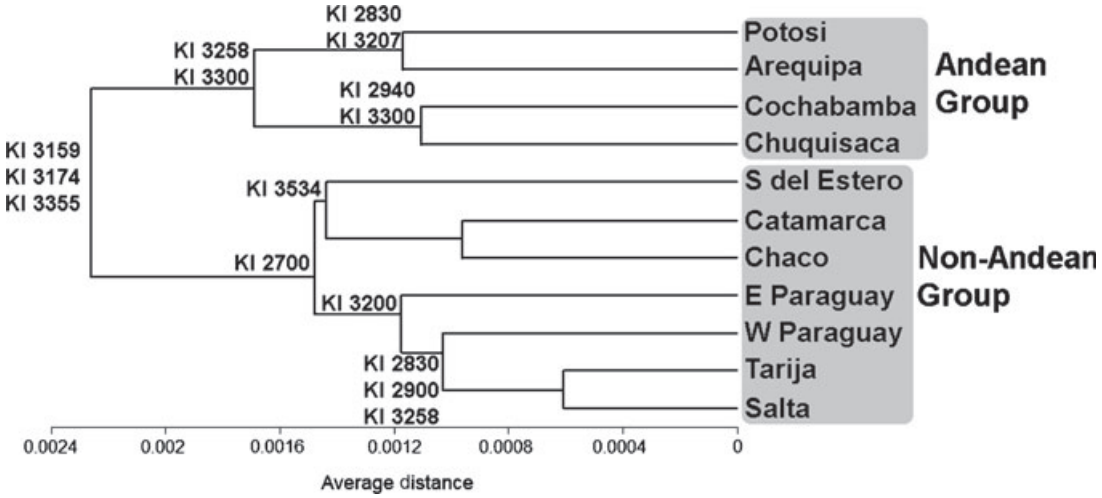

Fig. 3. *Triatoma infestans* population structure based on cuticular hydrocarbon (CHC) analyses. The unweighted pair-group method with arithmetic average dendrogram is based on specimen scores in the first 26 principal components, accounting for 96% of the total sample variance. CHC components contributing to each branch are shown. E Paraguay, eastern Paraguay; S del Estero, Santiago del Estero; W Paraguay, western Paraguay.

Table 5. Relative amount of cuticular hydrocarbon contributing to differentiate pyrethroid-resistant and pyrethroid-susceptible *Triatoma infestans.*

	R	S	
KI	Mean ± SD	Mean ± SD	*P*-value∗
2830	0.14 ± 0.21	0.03 ± 0.04	0.002
2950	0.17 ± 0.07	0.22 ± 0.08	0.017
3000	2.63 ± 0.74	2.28 ± 0.55	0.037
3030	0.37 ± 0.19	0.51 ± 0.18	0.005
3131	2.37 ± 1.16	3.18 ± 1.40	0.027
3228	0.19 ± 0.09	0.27 ± 0.10	0.007
3258	1.60 ± 0.58	1.29 ± 0.46	0.024
3300	7.34 ± 2.37	5.98 ± 2.28	0.031
3534	0.83 ± 0.34	1.05 ± 0.37	0.030
3652	0.75 ± 0.90	0.43 ± 0.25	0.034

∗Statistical significance (Mann–Whitney *U*-test). Pyrethroid-resistant (R) and pyrethroid-susceptible (S) populations numbered 46 and 21 insects, respectively. Resistance ratios for R and S insects were ≥130 and ≤5, respectively (Zerba and Picollo SSA/ATU INCO-CT 2004 515942 internal report, 2007; Catalá *et al*., 2007).
KI, Kovats index; R, pyrethroid-resistant; S, pyrethroid-susceptible; SD, standard deviation.

enzymes; thus, a 16-bp deletion in the desaturase gene (*desat2*) was suggested to probably underlie CHC polymorphism in *Drosophila melanogaster* (Diptera: Drosophilidae) (Dallerac *et al*., 2000). In the same fly, a low CHC type derived from an ancestral high CHC type was identified based on the 5,9 heptacosadiene : 7,11 heptacosadiene ratio (Takahashi *et al*., 2001). In *T. infestans* phenotype II, subtle changes in genes involved in hydrocarbon biosynthesis, more specifically those coding for the C30acyl-CoA ligase in the fatty acyl-CoA elongating system, might favour the release of C30-CoA–a precursor to *n*-C29–from the elongating system. *Triatoma infestans* CHC phenotype II (with marked abundance of C29) seems to be mostly associated with the most arid regions of the species' geographic range, in which average annual rainfall is <400 mm. Although we might be tempted to speculate that the chromatographic profile of phenotype II reflects a longterm adaptation to dry climatic conditions, CHC phenotype II seems to be absent in the arid areas of Potosi and Chuquisaca (Bolivia) (Table 3). The best-known function of insect CHCs is to protect against environmental stress and prevent lethal desiccation (Blomquist *et al*., 1987). Increased total amounts of CHC have been shown to correlate with desiccation resistance (Noorman & Den Otter, 2002; Urbanski *et al*., 2010). Insecticide resistance was also associated with similar changes in total CHC content in *T. infestans* (Pedrini *et al*., 2009). However, there are no conclusive studies on how climate can affect the CHC pattern in insects (Gibbs & Pomonis, 1995; Woodrow *et al*., 2000). In triatomines, different *T. dimidiata* genotypes living in sympatry in the Yucatán Peninsula (exposed to the same environmental conditions) showed quite distinct CHC profiles (Calderón-Fernández *et al*., 2011). Similarly, two sympatric *Triatoma sordida* genotypes from Izozog, Bolivia (Noireau *et al*., 1998) showed different CHC phenotypes (Calderón-Fernández & Juárez, unpublished data, 2009). The results of a more comprehensive analysis of *T. infestans* phenotypic and genetic variability in relation to environmental heterogeneity, performed in the context of the international project mentioned earlier in this paper, will be presented elsewhere.

In the present study, both principal component and discriminant analyses revealed that *T. infestans* populations segregate into two main groups, named after Panzera *et al.* (2004):

- Andean group, including Peruvian and Bolivian populations (Tarija excluded). This group is largely heterogeneous and is clearly structured into populations from western (Arequipa, Peru and Potosi, Bolivia) and eastern (Cochabamba and Chuquisaca, Bolivia) regions. The

differentiation of these subgroups is based mostly on the relative amount of two hydrocarbons, KI 3258 (4-methyl C32) and KI 3300 (*n*-C33).

- Non-Andean group, formed by Argentinean and Paraguayan populations together with Bolivian specimens from Tarija. The CHC with KIs 3159 and 3355 (internal dimethyl isomers of C31 and C33), together with the component with KI 3174 (3-methyl C31) are largely responsible for differentiating this group from the former. Similarity in CHC profiles, together with high intrapopulation variability, resulted in a variable degree of mixing between Argentinian and Paraguayan populations. The inclusion of the population from Tarija in this group suggests that the groups probably segregate in Bolivian territory.

The CHC-based groups corresponded closely with the Andean and non-Andean genotypes, respectively, differentiated by both cytogenetic and molecular markers. Analyses of CHCs in distinct *T. dimidiata* groups have led to suggestions of the existence of several subspecies (Calderón-Fernández *et al*., 2005, 2011); similarly, the level of differentiation between the Andean and non-Andean specimens suggests that these could be assigned subspecific rankings in agreement with cytogenetic and molecular results (Panzera *et al*., 2004; Bargues *et al*., 2006). Our study shows that insects from Santiago del Estero exhibit the highest degree of differentiation within the non-Andean group, associated mostly with variation in the relative amount of components with KIs 3534, 3630, 3733 and 3932, corresponding to monomethyl isomers of 35–39 carbon atoms in the straight-chain skeleton. Coincidently, similar results were obtained both with rDNA ITS-2 and mitochondrial cytochrome c oxidase subunit I (*COI*) gene analyses (Bargues *et al*., 2006; Piccinali *et al*., 2009), showing high variability in specimens from this area. Previous isoenzyme and molecular analyses separated populations collected from western and eastern Paraguay (Marcilla *et al*., 2000; Acosta *et al*., unpublished data, 2001). This separation is clearly supported here by CHC analysis. Specimens from western Paraguay (Boqueron, Presidente Hayes) showed higher relative amounts of CHCs, with KIs 3200 (*n*-C32) and 3300 (*n*-C33) contributing to this differentiation. In addition, the partial classification results showed no merging between the groups (Table 4), which is evidence of a clear distinction between them.

An 'intermediate' *T. infestans* cytotype, with heterochromatin content intermediate between those of the Andean and non-Andean groups, was proposed by Panzera *et al.* (2007) for populations collected in Yacuiba (Tarija) and Salvador Mazza (Salta). In our CHC analysis, insects from this region were included in the non-Andean group, although they clustered separately (Fig. 3). Analysis of antennal sensilla patterns in the same *T. infestans* populations clearly resolved the Andean and non-Andean groups by discriminant analyses, but did not differentiate the intermediate group. However, re-analysis of these data grouping populations in accordance with the Panzera cytotypes partially separated the intermediate group from the non-Andean group (Hernández *et al*., 2008). Further studies with all available molecular and phenotypic markers are needed in order to elucidate the putative existence of an intermediate group, as well as to advance understanding of the evolutionary relationship between Andean and non-Andean populations.

Pyrethroid-resistant specimens from Salvador Mazza were recently shown to contain significantly larger amounts of CHCs than pyrethroid-susceptible insects (Pedrini *et al*., 2009). Present results also show significant differences in specific CHCs of straight and monomethyl-branched chains between pyrethroid-resistant and -susceptible insects (Table 5). Additional studies would be helpful to establish the usefulness of CHCs as markers of pyrethroid-resistance.

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References

Bargues, M.D., Klisiowicz, D.R., Panzera, F. *et al.* (2006) Origin and phylogeography of the Chagas' disease main vector *Triatoma infestans* based on nuclear rDNA sequences and genome size. *Infection, Genetics and Evolution*, **6**, 46–62.

Blomquist, G.J., Nelson, D.R. & de Renobales, M. (1987) Chemistry, biochemistry, and physiology of insect cuticular lipids. *Archives of Insect Biochemistry and Physiology*, **6**, 227–265.

Calderón-Fernández, G., Juárez, M.P., Ramsey, J., Salazar Schettino, P.M., Monroy, M.C., Ordoñez, R. & Cabrera, M. (2005) Cuticular hydrocarbon variability among *Triatoma dimidiata* (Hemiptera: Reduviidae) populations from Mexico and Guatemala. *Journal of Medical Entomology*, **42**, 780–788.

Calderón-Fernández, G.M., Girotti, J.R. & Juárez, M.P. (2011) Cuticular hydrocarbons of *Triatoma dimidiata* (Hemiptera: Reduviidae): intraspecific variation and chemotaxonomy. *Journal of Medical Entomology*, **48**, 262–271.

Catalá, S., Gorla, D., Juárez, P. *et al.* (2007) Analytical appraisal, Southern Cone—biological and environmental causes of the spatial structuration in *Triatoma infestans* and the implications for vector control programmes. *Proceedings of the SSA/EC American Trypanosomiasis Update Workshop*, Asunción, pp. 13–18.

Dallerac, R., Labeur, C., Jallon, J.-M., Knipple, D.C., Roelofs, W.L. & Wicker-Thomas, C. (2000) A Δ9 desaturase gene with a different substrate specificity is responsible for the cuticular diene hydrocarbon polymorphism in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 9449–9454.

Dorn, P.L., Calderón, C., Melgar, S. *et al.* (2009) Two distinct *Triatoma dimidiata* (Latreille, 1811) taxa are found in sympatry in Guatemala and Mexico. *PLoS Neglected Tropical Diseases*, **3**, e393.

Gibbs, A. & Pomonis, J.G. (1995) Physical properties of insect cuticular hydrocarbons: the effects of chain length, ethyl-branching and unsaturation. *Comparative Biochemistry & Physiology B*, **112**, 243–249.

Guerri-Guttenberg, R.A., Grana, D.R., Ambrosio, G. & Milei, J. (2008) Chagas cardiomyopathy: Europe is not spared! *European Heart Journal*, **29**, 2587–2591.

Hernández, L., Abrahan, L., Moreno, M., Gorla, D. & Catalá, S. (2008) Phenotypic variability associated to genomic changes in the main vector of Chagas' disease in the southern cone of South America. *Acta Tropica*, **106**, 60–67.

Juárez, M.P. & Blomquist, G.J. (1993) Cuticular hydrocarbons of *Triatoma infestans* and *Triatoma mazzottii*. *Comparative Biochemistry and Physiology, Part B*, **106**, 667–674.

Juárez, M.P. & Brenner, R.R. (1985) The epicuticular lipids of *Triatoma infestans*, II. Hydrocarbon dynamics. *Comparative Biochemistry and Physiology, Part B*, **82**, 793–803.

Juárez, M.P. & Brenner, R.R. (1986) Biochemistry of the evolution of *Triatoma infestans*. IX. Composition of cuticular hydrocarbons compared to other Triatominae. *Acta Physiologica Pharmacologica Latinoamericana*, **36**, 47–57.

Juárez, M.P. & Calderón-Fernández, G.M. (2007) Cuticular hydrocarbons of triatomines. *Comparative Biochemistry and Physiology, Part A*, **147**, 711–730.

Juárez, M.P., Fernández, R., Schofield, C.J. & Dujardin, J.P. (2000) Intergeneric comparison of epicuticular hydrocarbons in Triatominae. *Research and Reviews in Parasitology*, **60**, 121–127.

Juárez, M.P., Blomquist, G.J. & Schofield, C.J. (2001) Hydrocarbons of *Rhodnius prolixus*, a Chagas' disease vector. *Comparative Biochemistry and Physiology, Part B*, **129**, 733–746.

Juárez, M.P., Calderón-Fernández, G., Girotti, R. & Mijailovsky, S. (2007) Epicuticular hydrocarbons in *Triatoma* sp.: their usefulness for chemical taxonomy. *Proceedings of the SSA/EC American Trypanosomiasis Update Workshop*, Asunción, pp. 97–104.

Juárez, M.P., Pedrini, N., Girotti, J.R. & Mijailovsky, S.J. (2010) Pyrethroid resistance in Chagas' disease vectors: the case of *Triatoma infestans* cuticle—review. *Resistant Pest Management Newsletter*, **19**, 59–61.

Kovats, E. (1965) Gas chromatographic characterization of organic substances in the retention index system. *Advances in Chromatography*, **1**, 229–247.

Lachenbruch, P. & Mickey, A. (1968) Estimation of error rates in discriminant analysis. *Technometrics*, **10**, 1–11.

Marcilla, A., Canese, A., Acosta, N., López, E., Rojas de Arias, A., Bargues, M.D. & Mas-Coma, S. (2000) Populations of *Triatoma infestans* (Hemiptera: Reduviidae) from Paraguay: a molecular analysis based on the second internal transcribed spacer of the rDNA. *Research and Reviews in Parasitology*, **60**, 99–105.

Monteiro, F.A., Pérez, R., Panzera, F. *et al.* (1999) Mitochondrial DNA variation of *Triatoma infestans* populations and its implication on the specific status of *T. melanosoma*. *Memórias do Instituto Oswaldo Cruz*, **94**, 229–238.

Noireau, F., Gutierrez, T., Zegarra, M., Flores, R., Brenière, F., Cardozo, L. & Dujardin, J.P. (1998) Cryptic speciation in *Triatoma sordida* (Hemiptera: Reduviidae) from the Bolivian Chaco. *Tropical Medicine & International Health*, **3**, 364–372.

Noireau, F., Bastrenta, B., Catalá, S. *et al.* (2000) Sylvatic population of *Triatoma infestans* from the Bolivian Chaco: from field collection to characterization. *Memórias do Instituto Oswaldo Cruz*, **95**, 119–122.

Noorman, N. & Den Otter, C.J. (2002) Effects of relative humidity, temperature and population density on the production of cuticular hydrocarbons in the housefly *Musca domestica* L. *Journal of Chemical Ecology*, **28**, 1819–1829.

Panzera, F., Dujardin, J.-P., Nicolini, P. *et al.* (2004) Genomic changes of Chagas' disease vector, South America. *Emerging Infectious Diseases*, **10**, 438–446.

Panzera, F., Ferrandis, I., Calleros, L., Lucero, C., Ferreiro, M.J., Panzera, Y. & Pérez, R. (2007) Recent contributions of cytogenetics to the study of vectors of Chagas' disease. *Proceedings of the SSA/EC American Trypanosomiasis Update Workshop*, Asunción, pp. 79–84.

Pedrini, N., Mijailovsky, S.J., Girotti, J.R., Stariolo, R., Cardozo, R.M., Gentile, A. & Juárez, M.P. (2009) Control of pyrethroid-resistant Chagas' disease vectors with entomopathogenic fungi. *PLoS Neglected Tropical Diseases*, **3**, e434.

Piccinali, R.V., Marcet, P.L., Noireau, F., Kitron, U., Gürtler, R.E. & Dotson, E.M. (2009) Molecular population genetics and phylogeography of the Chagas' disease vector *Triatoma infestans* in South America. *Journal of Medical Entomology*, **46**, 796–809.

Reisenman, C.E., Lawrence, G., Guerenstein, P.G., Gregory, T., Dotson, E. & Hildebrand, J.G. (2010) Infection of kissing bugs with *Trypanosoma cruzi*, Tucson, Arizona, U.S.A. *Emerging Infectious Diseases*, **16**, 400–405.

Santo Orihuela, P.L., Vassena, C.V., Zerba, E.N. & Picollo, M.I. (2008) Relative contribution of monooxygenase and esterase to pyrethroid resistance in *Triatoma infestans* (Hemiptera: Reduviidae) from Argentina and Bolivia. *Journal of Medical Entomology*, **45**, 298–306.

Schofield, C.J. & Kabayo, J.P. (2008) Trypanosomiasis vector control in Africa and Latin America. *Parasites and Vectors*, **1**, 24.

Sokal, R.R. & Rohlf, F.J. (2001) *Biometry*, 3rd edn. W.H. Freeman & Co., New York, NY.

Takahashi, A., Tsaur, S.C., Coyne, J.A. & Wu, C.I. (2001) The nucleotide changes governing cuticular hydrocarbon variation and their evolution in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 3920–3925.

Urbanski, J.M., Benoit, J.B., Michaud, M.R., Denlinger, D.L. & Armbruster, P. (2010) The molecular physiology of increased egg desiccation resistance during diapause in the invasive mosquito, *Aedes albopictus*. *Proceedings of The Royal Society B*, **277**, 2683–2692.

Woodrow, R.J., Grace, J.K., Nelson, L.J. & Haverty, M.I. (2000) Modification of Cuticular Hydrocarbons of *Cryptotermes brevis* (Isoptera: Kalotermitidae) in Response to Temperature and Relative Humidity. *Environmental Entomology*, **29**, 1100–1107.

World Health Organization (1994) Protocolo de evaluación de efecto insecticida sobre Triatominos. *Acta Toxicológica Argentina*, **2**, 29–32.

World Health Organization (2010) Fact sheet No. 340: Chagas' disease (American trypanosomiasis). http://www.who.int/mediacentre/factsheets/fs340/en/index.html [Accessed 2 December 2010].

Wilks, S.S. (1932) Certain generalizations in the analysis of variance. *Biometrika*, **24**, 471–494.

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