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Origin and phylogeography of the Chagas disease main vector *Triatoma infestans* based on nuclear rDNA sequences and genome size

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Abstract

For about half of all Chagas disease cases *T. infestans* has been the responsible vector. Contributing to its genetic knowledge will increase our understanding of the capacity of geographic expansion and domiciliation of triatomines. Populations of all infestans subcomplex species, *T. infestans*, *T. delpontei*, *T. platensis* and *T. melanosoma* and the so-called *T. infestans* ''dark morph'', from many South American countries were studied. A total of 10 and 7 different ITS-2 and ITS-1 haplotypes, respectively, were found. The total intraspecific ITS-2 nucleotide variability detected in *T. infestans* is the highest hitherto known in triatomines. ITS-1 minisatellites, detected for the first time in triatomines, proved to be homologous and thus become useful markers. Calculations show that ITS-1 evolves 1.12–2.60 times faster than ITS-2. Despite all species analyzed presenting the same $n = 22$ chromosome number, a large variation of the haploid DNA content was found, including a strikingly high DNA content difference between Andean and non-Andean specimens of *T. infestans* (mean reduction of 30%, with a maximum of up to 40%) and a correlation between presence/absence of minisatellites and larger/smaller genome size. Population genetics analysis of the eight composite haplotypes of *T. infestans* and net differences corroborate that there are clear differences between western and eastern populations (60%), and little genetic variation among populations (1.3%) and within populations (40%) within these two groups with migration rates larger than one individual per generation corresponding only to pairs of populations one from each of these groups. These values are indicative either of a large enough gene flow to prevent population differentiation by drift within each geographic area or a very recent spread, the latter hypothesis fitting available data better. Phylogenetic trees support a common ancestor for *T. infestans* and *T. platensis*, an origin of *T. infestans* in Bolivian highlands and two different dispersal lines, one throughout Andean regions of Bolivia and Peru and another in non-Andean lowlands of Chile, Paraguay, Argentina, Uruguay and Brazil.
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Keywords: Chagas disease; *Triatoma infestans* subcomplex; rDNA ITS-1, 5.8S and ITS-2 sequences; DNA quantification; Flow cytometry; Population genetics analysis; Molecular clock; Phylogeography

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1. Introduction

American trypanosomiasis or Chagas disease is caused by *Trypanosoma cruzi*, a protozoan mainly transmitted by blood-sucking reduvid bugs of the subfamily Triatominae. While restricted to the Americas, Chagas disease is the health problem with the highest socio-economic impact in Latin America, where more than 90 million people are in risk (World Bank, 1993). A parasitic disease sometimes fatal and incurable due to the absence of effective drugs, control activities are mainly based on antivectorial fight (Schofield and Dias, 1998).

Chagas disease transmission is mainly related to triatomine species adapted to live within human dwellings. The understanding of the capacity of triatomines to colonize the domicile, and inherent biological and genetic processes related to this domiciliation process from the wild, becomes the central target for future research to avoid human habitat re-colonisation by the same previously existing or other sylvatic triatomine species once the effect of insecticide spraying disappears (Dujardin et al., 2000). Genetic tools, mainly DNA techniques, are able to furnish the information which can help in the understanding of the domiciliation capacity of triatomine species (Bargues et al., 2002).

Among the 137 species of Triatominae recognized up to the present (Galvão et al., 2003), *Triatoma infestans* is responsible for about half of all Chagas disease cases. This triatomine is a very good model for such studies: (i) it is the species most adapted to human dwellings; (ii) sylvatic populations are still found in rock piles in association with wild guinea-pigs, *Galea musteloides*, in Andean valleys of Cochabamba and Sucre in Bolivia (Torrico, 1946; Dujardin et al., 1987; Bermudez et al., 1993); (iii) its distribution in the mid-1980s was very wide, including vast regions of Bolivia, southern Peru, Chile, Brazil, Paraguay, Uruguay, and Argentina (Fig. 1A) (Carcavallo et al., 1999), although today substantially reduced by large-scale control interventions (Schofield and Dias, 1998).

T. infestans is included in the infestans sub-complex (which in turn is part of the infestans complex), together with *T. platensis*, *T. delpontei* and *T. melanosoma* (Dujardin et al., 2000). *Triatoma platensis*, known from Paraguay, Uruguay and Argentina, is a species almost exclusively present in nests of Furnariidae and Psittacidae birds, but may

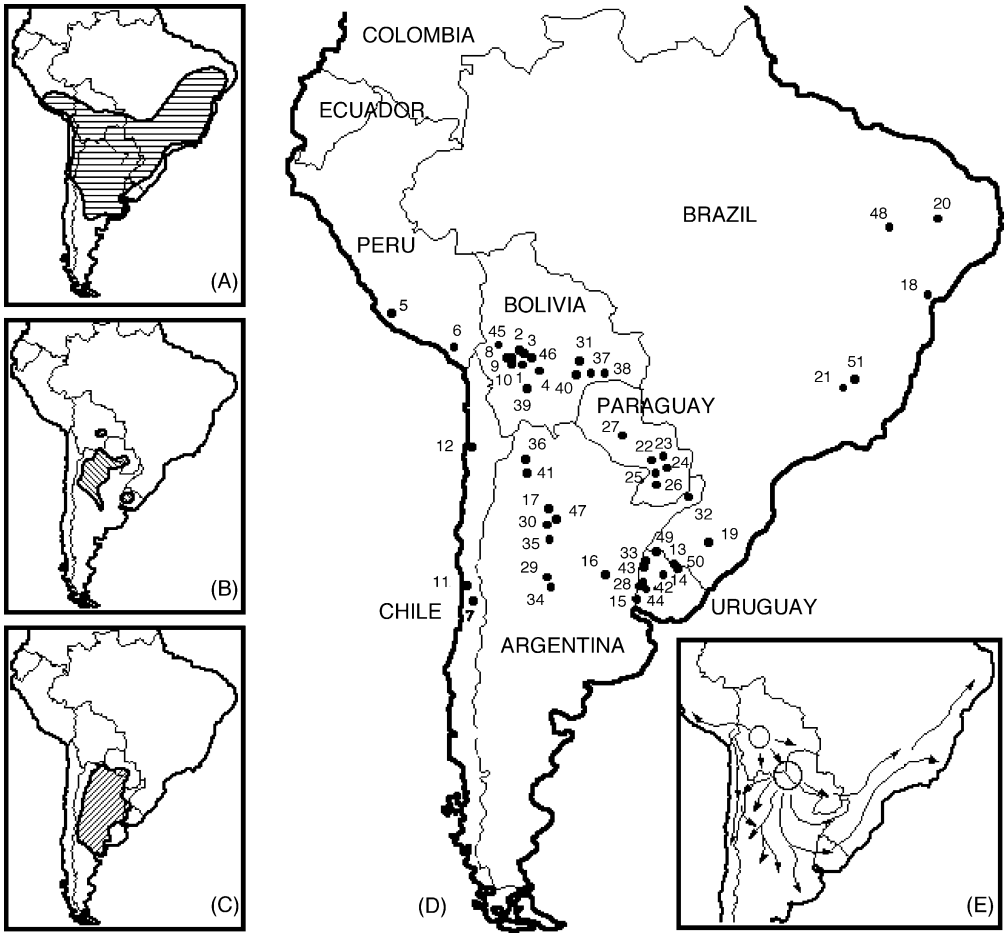

Fig. 1. Maps showing the distributions of species and localities studied: (A) geographical distribution of *T. infestans* in the 1980s; (B and C) geographical distributions of *T. delpontei* and *T. platensis*, respectively; (D) map showing localities furnishing the triatomine materials studied (compare with Tables 1 and 3); (E) map illustrating main phylogenetic results on the geography of *T. infestans* (see text for details).

also be found in chicken coops, where it is able to crossbreed with *T. infestans* (Pereira et al., 1996). *Triatoma delpontei* is another ornithophilic arboreal species without relevance for Chagas disease transmission (Salvatella, 1986, 1987). It is distributed in Bolivia, Paraguay, Uruguay and Argentina, showing a marked preference for woven stick nests of colonial monk parrots, *Myiopsitta monacha* (Noireau et al., 2000a). Despite the different bird specificity, *T. delpontei* is able to crossbreed with *T. platensis* at least under laboratory conditions (Usinger et al., 1966; Pereira et al., 1996). *Triatoma melanosoma*, was originally described as a subspecies of *T. infestans* (Martinez et al., 1987), later raised to species rank (Lent et al., 1994), and recently synonymized with *T. infestans* (Gumiel et al., 2003). It is only found in Argentina, and differs only by the black colour of its exoskeleton from *T. infestans*, with which it is interfertile, this melanic characteristic appearing to be recessive in crossbreds (Monteiro et al., 1999; Dujardin et al., 2000). A similar chromatic form, called *T. infestans* ''dark morph'', has also been described living in hollow trees from the Bolivian Chaco (Noireau et al., 1997, 2000a,b).

Recently two allopatric groups apparently related to altitude have been distinguished within *T. infestans* (Panzera et al., 2004): (a) ''Andean'', including populations presenting C-heterochromatic blocks in most of their 22 chromosomes; (b) ''non-Andean'', including populations with only 4–7 autosomes with C-bands. It has been suggested that these heterochromatin differences might be the cause of a striking DNA content decrease of about 30% from ''Andean'' to ''non-Andean'' populations. Despite all this, ''Andean'' and ''non-Andean'' individuals appear to be able to hybridise normally and give rise to fertile progeny.

Genetic studies of *T. infestans* may help us to understand the geographic expansion and domiciliation of triatomines, two phenomena closely related to Chagas disease. The aim of this paper is to study genetically numerous populations of all the species of the infestans subcomplex, by means of nuclear ribosomal DNA sequences, population genetics analyses and genome size. The second internal transcribed spacer ITS-2 has recently proved to be a useful tool for classifying species, subspecies, and populations, and inferring phylogenetic relationships in Triatominae (Bargues, 2002; Bargues et al., 2000, 2002; Marcilla et al., 2001, 2002).

2. Materials and methods

2.1. Triatomine materials

Triatomine materials originated from seven different countries (Bolivia, Peru, Chile, Brasil, Uruguay, Argentina and Paraguay). A total of 106 specimens was studied, of which 54 specimens representing 30, 10, 2, 1 and 1 populations of *T. infestans*, *T. delpontei*, *T. platensis* and *T. melanosoma* and the so-called *T. infestans* ''dark morph'', respectively, were studied for sequencing and population genetics analyses (Tables 1 and 2, Fig. 1) [note: new nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers listed in Table 1] and 52 specimens of the same species and countries for genome size measuring (Table 3, Fig. 1). For comparison purposes, two other vector species were also sequenced and their genome size measured: *T. rubrovaria* (subcomplex rubrovaria) from Santana do Livramento, Rio Grande do Sul, Brazil, and *T. sordida* (subcomplex sordida) from Terreno, Mato Grosso, Brazil. Both species were selected as they also belong to the infestans complex (Dujardin et al., 2000), *T. rubrovaria* even having been included in the infestans subcomplex until recently (Pereira et al., 1996).

2.2. Triatomine composite haplotype code nomenclature

The nomenclature here introduced for composite haplotyping (CH) considers the main rDNA regions able to furnish valuable information on intraspecific variability of triatomine species. The code follows an order according to the information capacity of the different rDNA markers, related to their evolutionary ratios and usefulness for distinguishing at species and population levels. For instance, the code CH2Badb will refer to Haplotype 2 according to the ITS-2, Haplotype B after ITS-1, and Haplotypes a, d and b after the first, second and third variable domains D1, D2 and D3 of the 28S gene, respectively (other variable domains of the 28S gene can successively be added in this way, if needed). This nomenclature may be useful in future genetic studies of triatomines, as it is already in other groups of parasites and vectors (i.e., see Bargues et al., 2001). In the present paper, the codes only refer to ITS-2 and ITS-1.

2.3. Sequencing of rDNA ITS-1, 5.8S and ITS-2

For DNA extraction, one or two legs fixed in ethanol 70% from each of the specimens from each population were used and processed individually, as previously described (Marcilla et al., 2001, 2002) and following the steps according to methods outlined before (Bargues and Mas-Coma, 1997). Total DNA was isolated by standard phenol/chloroform extraction and ethanol precipitation techniques (Sambrouk et al., 1989). The pellet was dried and resuspended in 30 μl sterile TE buffer (pH 7.6). This suspension was stored at −20 °C until use. The nuclear ribosomal DNA intergenic fragment, corresponding to the ITS-1, 5.8 S and ITS-2 region, was amplified by polymerase chain reaction (PCR) using 4–6 μl of genomic DNA for each 50 μl PCR reaction. The complete region was amplified using primers designed in conserved positions of 18S and 28S rRNA genes and previously described (Bargues and Mas-Coma, 1997; Bargues et al., 2000, 2002). Amplifications were generated in a Peltier thermal cycler (MJ Research, Watertown, MA, USA), by 30 cycles of 30 s at 94 °C, 30 s at 50–55 °C and

Table 1
Triatominae species and populations studied, including geographic origins, length and AT composition (in percentage) of the ITS-1-5.8S-ITS-2 region, and corresponding GenBank accession nos

Species genotype code	Population no.	Preliminary classification	Locality	Country	Length (bp)	% AT	GenBank accession no.
(1) *T. infestans*: 7 different sequences/31 populations studied:							
T.inf-CH1A	1	*T. infestans*	Dpto. Cochabamba	Bolivia	1304	67.4	AJ576051
	2	*T. infestans*	Sylvatic, Quillacollo, Cochabamba	Bolivia	1304	67.4	
	3	*T. infestans*	Domestic, Quillacollo, Cochabamba	Bolivia	1304	67.4	
	4	*T. infestans*	Domestic, Mataral, Chuquisaca	Bolivia	1304	67.4	
	5	*T. infestans*	Dpto. Ica, Prov. Nasca	Peru	1304	67.4	
	6	*T. infestans*	Dpto. Moquegua	Peru	1304	67.4	
	7	*T. infestans*	Santiago de Chile, lab. strain Prof. Schenone	Chile	1304	67.4	
T.inf-CH1B	8	*T. infestans*	Sylvatic, Inca wall, Quillacollo, Cochabamba ($n = 2$)	Bolivia	1323	67.3	AJ582024
T.inf-CH1C	9	*T. infestans*	Sylvatic, Quillacollo, Cochabamba	Bolivia	1330	67.6	AJ582025
	10	*T. infestans*	Domestic, Quillacollo, Cochabamba	Bolivia	1330	67.6	
T.inf-CH2A	11	*T. infestans*	Reg. IV, Viña del Mar, Los Andes	Chile	1308	67.5	AJ576054
	12	*T. infestans*	Reg. II, Antofagasta, Lascar, Toconao	Chile	1308	67.5	
	13	*T. infestans*	Dpto. Rivera, Seccional 7	Uruguay	1308	67.5	
	14	*T. infestans*	Dpto. Tacuarembó, Seccional 6	Uruguay	1308	67.5	
	15	*T. infestans*	Dpto. Colonia, Seccional 8	Uruguay	1308	67.5	
	16	*T. infestans*	Prov. Santa Fe	Argentina	1308	67.5	
	17	*T. infestans*	Prov. Santiago del Estero, Dpto. Avellaneda ($n = 2$)	Argentina	1308	67.5	
	18	*T. infestans*	Estado Bahia, Salvador	Brasil	1308	67.5	
	19	*T. infestans*	Rio Grande do Sul, Horizontina	Brasil	1308	67.5	
	20	*T. infestans*	Estado Paraiba	Brasil	1308	67.5	
	21	*T. infestans*	Estado Minas Gerais, Belo Horizonte	Brasil	1308	67.5	
	22	*T. infestans*	Dpto. Cordillera, Primero de Marzo	Paraguay	1308	67.5	
	23	*T. infestans*	Dpto. Cordillera, San Jose Obrero	Paraguay	1308	67.5	
	24	*T. infestans*	Dpto. Cordillera, Caraguatay	Paraguay	1308	67.5	
	25	*T. infestans*	Dpto. Cordillera, Caacupé	Paraguay	1308	67.5	
	26	*T. infestans*	Dpto. Paraguarí, Acahay	Paraguay	1308	67.5	
	27	*T. infestans*	Dpto. Presidente Hayes, Km 218	Paraguay	1308	67.5	
	28	*T. infestans*	Dpto. Rio Negro ($n = 2$)	Uruguay	1308	67.5	
T.inf-CH3A	29	*T. infestans*	Prov. Córdoba, Dpto. Cruz del Eje	Argentina	1310	67.5	AJ576052
T.inf-CH4A	30	*T. infestans*	Prov. Santiago del Estero, Dpto. Avellaneda	Argentina	1302	67.3	AJ576053
T.inf-CH5A 31	31	*T. infestans* ''dark morph''	Dpto. Santa Cruz, Prov. Cordillera	Bolivia	1308	67.5	AJ576055
(2) *T. melanosoma*: one sequence/one population studied:							
T.mel-CH1A	32	*T. melanosoma*	Col. Sta. Rosa, Dp. San Ignacio, Pr. Misiones ($n = 2$)	Argentina	1308	66.7	AJ576056
(3) *T. platensis*: two different sequences/two populations studied:							
T.pla-CH1A	33	*T. platensis*	Dpto. Paysandú ($n = 4$)	Uruguay	1279	67.4	AJ576061
T.pla-CH1B	34	*T. platensis*	Prov. Córdoba, Dpto. Cruz del Eje	Argentina	1279	67.5	AJ576062
(4) *T. delpontei*: 4 different sequences/10 populations studied:							
T.del-CH1A	35	*T. delpontei*	Santiago del Estero, Dpto. Avellaneda	Argentina	1273	67.5	AJ576057
	36	*T. delpontei*	Prov. Salta, Dpto Rivadavia	Argentina	1273	67.5	
	37	*T. delpontei*	Chaco, Dpto. Santa Cruz, Prov. Cordillera	Bolivia	1273	67.5	
	38	*T. delpontei*	cotorra nest, Chaco, Dpto. Sta Cruz, Prov. Cordillera ($n = 2$)	Bolivia	1273	67.5	
	39	*T. delpontei*	Potosí	Bolivia	1273	67.5	
	40	*T. delpontei*	Dpto. Santa Cruz, Prov. Cordillera	Bolivia	1273	67.5	
T.del-CH2A	41	*T. delpontei*	Prov. Salta, Dpto. Rivadavia	Argentina	1275	67.6	AJ576058
T.del-CH3A	42	*T. platensis*	Dpto. Rio Negro ($n = 3$)	Uruguay	1273	67.6	AJ576059
	43	*T. platensis*	Dpto. Paysandú	Uruguay	1273	67.6	
T.del-CH3B	44	*T. platensis*	Dpto. Rio Negro	Uruguay	1273	67.5	AJ576060

CH = composite haplotypes; numbers 1 to 5 correspond to ITS-2 haplotypes; letters A, B, C correspond to ITS-1 haplotypes. For the geographical location of populations, see numbers in map of Fig. 1D. When more than one individual was sequenced, it is noted in parentheses.

1 min at 72 °C, preceded by 30 s at 94 °C and followed by 7 min at 72 °C. Ten microliters of the reaction mixture were examined by 1% agarose gel electrophoresis, followed by ethidium bromide staining. Primers and nucleotides were removed from PCR products by purification on Ultra Clean™ PCR Clean-up DNA Purification System (MoBio, Solana Beach, CA, USA) according to the manufacturer's protocol and resuspended in 50 μl of 10 mM TE buffer

Table 2
Triatominae species and populations studied, including distribution of composite haplotypes by country of origin

Species	Country	Sample size	*T. infestans* composite haplotypes							*T. melanosoma* composite haplotype, 1A
			1A	1B	1C	2A	3A	4A	5A	
T. infestans	Bolivia	8	4	2	2					
T. infestans "dark morph"	Bolivia	1							1	
T. infestans	Peru	2	2							
T. infestans	Chile	3	1		2					
T. infestans	Uruguay	5				5				
T. infestans	Brasil	4				4				
T. infestans	Paraguay	6				6				
T. infestans	Argentina	5				3	1	1		
T. melanosoma	Argentina	2								2

Species	Country	Sample size	*T. platensis* composite haplotypes		*T. delpontei* composite haplotypes			
			1A	1B	1A	2A	3A	3B
T. platensis	Uruguay	4	4					
T. platensis	Argentina	1		1				
T. delpontei	Bolivia	5			5			
T. delpontei[a]	Uruguay	5					4	1
T. delpontei	Argentina	3			2	1		

For abbreviations see Table 1.
[a] Originally sampled as *T. platensis.*

(10 mM Tris–HCl, 1 mM EDTA, pH 7.6). The final DNA concentration in μg/ml and the absorbance at 260/280 nm was determined in a spectrophotometer UV–VIS (Biophotometer, Eppendorf, Hamburg, Germany). Sequencing was performed on both strands by the dideoxy chain-termination method, and with the Taq dye-terminator chemistry kit for ABI 3730 and ABI 3700 capillary system (Perkin Elmer, Foster City, CA, USA), using PCR primers. For sequence alignment, the CLUSTAL-W version 1.8 (Thompson et al., 1994) was used. Subsequently, minor corrections were manually introduced for a better fit of nucleotide correspondences in minisatellite sequence regions.

2.4. Genome size measuring

Flow cytometry was used to establish the haploid genome size, because the technical features of this analytical method are unique in obtaining a degree of biological parameter information in each single analysis (O'Connor, 1996). Flow cytometry has shown to be useful in species differentiation (Arumuganathan et al., 1999). The nuclear total DNA content was measured in gonadal cells of male individuals previously fixed in ethanol–acetic acid (3:1). Gonads from fixed insects were excized and deposited on excavated glass slides. A few drops of hypotonic DNA-staining buffer

Table 3
Haploid DNA contents (*C*-value) expressed in pg (minimum, maximum values and mean values and standard deviation), measured by flow cytometry, in different *Triatoma* species of the infestans complex

Species	Geographic origin	Pop. no.	Ind. no.	Min.	Max.	Mean	S.D.
T. infestans (Andean)	Rio Abajo in Dep. La Paz, Bolivia	45	4	1.664	1.996	1.799	0.140
	Jamachuma in Dep. Cochabamba, Bolivia	46	8	1.626	2.077	1.839	0.160
T. infestans (non-Andean)	Prov. Santiago del Estero in Dep. Moreno and	47	6	1.249	1.502	1.352	0.094
	Prov. Córdoba in Dep. Cruz del Eje, Argentina	29					
	Dep. Rivera and Dep.	13	13	1.265	1.625	1.414	0.106
	Tacuarembó, Uruguay	14					
	Estados Bahia and	18	3	1.395	1.468	1.420	0.041
	Piaui, Brazil	48					
T. infestans "dark morph"	Dep. Santa Cruz, Bolivia	31	4	1.271	1.377	1.320	0.046
T. melanosoma	Prov. Misiones, Argentina	32	4	1.310	1.486	1.406	0.079
T. platensis	Dep. Paysandu, Uruguay	33	4	1.202	1.274	1.224	0.034
T. delpontei	Prov. Salta, Argentina	36	6	2.566	2.761	2.666	0.076
	Dep. Santa Cruz and	37					
	Dep. Potosi, Bolivia	39					
T. rubrovaria	Dep. Artigas and	49	4	1.045	1.101	1.079	0.026
	Dep. Rivera, Uruguay	50					
T. sordida	Estado Minas Geraes, Brazil	51	4	0.934	1.072	1.031	0.065

Pop. no. = for the geographical location of populations, see numbers in map of Fig. 1D; ind. no. = number of individuals analyzed; S.D. = standard deviation.

(HDSB, containing 0.1% trisodium citrate, 0.1% Triton X-100, 100 μg/mL RNAase A and 50 μg/mL propidium iodide) were added to cover the tissue. Gonads were then minced using scalpel blades until homogeneous slurries were obtained. These were transferred with a Pasteur pipette to 5 mL polypropylene tubes, with the glass slides washed with additional HDSB to obtain a final volume of 2 ml. The suspensions were incubated for 30 min at 37 °C in the dark with occasional vortexing of the tubes. Before flow cytometric analysis, suspensions were filtered through 60 μm nylon mesh. For the evaluation of absolute DNA contents, the normal DNA index (Coulter Cytometry, PN 6699500) was used as reference. This reagent consists of normal human lymphocytes fixed in ethanol/acetic acid, and is a standard for the human lymphocyte genome size ($2C = 6.436$ pg) (Dolezel et al., 2003). All measurements were performed on an EPICS XL-MCL flow cytometer (Coulter Electronics, Hialeah, Florida, USA) with an air-cooled argon-ion laser tuned at 488 nm and 15 mW. Propidium fluorescence (FL3), proportional to DNA content, was collected through a 650 nm DL dichroic filter plus a 625 nm BP band-pass filter. Forward and side scatter signals were used for morphological assessment of the samples. Cell aggregates and coincident cells were excluded by analysis of the relationship between FL3 integral and peak signals. The DNA content in single cells was determined from FL3 linear histograms. The absolute DNA amount was calculated from the ratio of the mean channel of the insect haploid G0 peak to the mean channel of the human lymphocyte diploid G0 peak. For measurement standardization, the flow cytometer was calibrated every day with standard FlowSet fluorescent microspheres (Coulter Cytometry) and replicate samples of normal DNA index were run with every batch of insect gonadal cells.

2.5. Phylogenetic inference

Phylogenies were inferred using maximum-likelihood (ML) estimates with PAUP v.4.0b10 (Swofford, 2001). Maximum-likelihood parameters such as model, base frequencies, transition/transversion ratio (ti/tv), shape of gamma distribution, and proportion of invariant sites, were optimized using the hierarchical likelihood ratio test (hLRTs) implemented in Modeltest 3.06 (Posada and Crandall, 1998). The ML phylogeny was estimated using the HKY85 (Hasegawa et al., 1985) model, and then repeated while constraining the estimate to fit the molecular clock model. These estimates were used to calculate the LRT statistic as described (Felsenstein, 1988; Swofford et al., 1996) and PAUP was used to obtain the likelihood scores of each phylogeny. Starting branch lengths were obtained using least-squares method with ML distances. To provide an assessment of the reliability of the nodes of the trees, a quartet puzzling analysis was employed with 1000 puzzling steps. For comparison purposes, another method as distance through neighbor-joining (NJ) analysis (Saitou and Nei, 1987) for minimum evolution (ME) trees with different distance estimators was also used. Statistical support for each ME tree was assessed with bootstrap-resampling technique (Felsenstein, 1985) over 1000 replications, with and without removal of invariant and gapped positions. The complex model TN93 proved to be the one better fitting the data. Maximum parsimony is not included because this traditional method does not fit the data of this paper, as to accurately reconstruct relationships it requires reasonably large numbers of variable characters, which is not the case of this study. *T. rubrovaria* and *T. sordida* were used as outgroup for rooting the infestans subcomplex ingroup.

2.6. Population genetics analysis

Genetic variation within and among populations of *T. infestans* was evaluated using Arlequin 2.000 (Schneider et al., 2000). Summary parameters include those based on the frequency of variants (haplotype number and diversity) as well as some taking genetic differences among variants into account (gene diversity, polymorphic sites). Since relationships among closely related variants are not always best represented by bifurcating trees (Posada and Crandall, 2001) we conducted an alternative analysis of *T. infestans* ITS haplotypes by constructing a minimum spanning tree (Dunn and Everitt, 1982) extended to include alternative minimal length connections, hence resulting in a minimum spanning network (Excoffier and Smouse, 1994) coupled with analysis of molecular variance (AMOVA, Excoffier et al., 1992). A hierarchical analysis of molecular variance (AMOVA) was performed with Arlequin. This analysis provides estimates of variance components and F-statistics (Wright, 1931) analogs reflecting the correlation of haplotype diversity at different levels of hierarchical subdivision. The hierarchical subdivision was made at three levels. At the top level two groups were defined based on geographical location of the sampling countries: Andean (Bolivia, Peru and Chile) and plain (Uruguay, Brazil, Paraguay and Argentina) countries. The second level corresponded to countries within each of the above two groups, and the third level corresponded to the different haplotypes found in each country. AMOVA reports components of variance at the three levels under consideration (within populations, among populations within groups, and among groups) as well as F-statistics analogs. Under the present scheme, F_{ST} is viewed as the correlation of random haplotypes within populations, relative to that of random pairs of haplotypes drawn from the whole species; F_{CT} as the correlation of random haplotypes within a group of populations, relative to that of random pairs of haplotypes drawn from the whole species, and F_{SC} as the correlation of the molecular diversity of random haplotypes within populations, relative to that of random pairs of haplotypes drawn from the group (Excoffier et al., 1992). The statistical significance of fixation indices was tested using a non-

parametric permutation approach (Excoffier et al., 1992). Genetic differentiation between pairs of populations was evaluated by means of F-statistics (Wright, 1931). Exact tests of population differentiation were performed following Raymond and Rousset (1996). We also followed Slatkin's linearized F_{ST}'s (Slatkin, 1995) procedure to obtain estimates of pairwise equilibrium migration rates.

2.7. Molecular clock estimations

To estimate whether the sequences are evolving linearly with time, it was verified that the molecular clock was not rejected by using options provided for the maximum likelihood model in PAUP. To test for molecular clocks, the likelihood ratio test (LRT) was applied using the same substitution models except for the clock or non-clock assumptions. Calculations were based on the rDNA ITS-2 molecular clock rate obtained for Triatomini from fossil history of Hemiptera, biogeographic data, and using an 18S rDNA molecular clock (Bargues et al., 2000). The ITS-2 base substitution rate showed to be of 41.1–99.4% per 100 million years (My) or 0.4–1.0% per 1 My, that is (41.4–99.4) $\times 10^{-10}$ substitutions per site per year (Bargues et al., 2000). ITS-2 rate was used to infer ITS-1 molecular clock by comparison. To obtain wider estimation ranges, chronological data were inferred from both total variable positions in alignments between the two taxa in question obtained with CLUSTAL-W and differences between taxa in the pairwise distance matrix of nucleotide divergences according to Kimura's two-parameter model furnished by PAUP, for each one of the two spacers.

3. Results

3.1. Intergenic region sequences

A total of 14 different ITS-1–5.8–ITS-2 sequences detected in the infestans subcomplex species (Table 1) have been deposited in the GenBank–EMBL, as well as those of *T. rubrovaria* (length: 1375 bp; 67.8% AT) and *T. sordida* (length: 1378 bp; 66.9% AT) (accession nos. AJ557258 and AJ576063, respectively). The 5.8S gene was 155 bp long and of a 42.6% of AT content, and presented the same sequence in all triatomines analyzed.

ITS-2: A total of 10 different ITS-2 haplotypes was found (Table 4). In *T. infestans*, five different ITS-2 sequences were found (Tables 1 and 4): (a) the haplotype T.inf-H1 refers to

Table 4
Interhaplotype sequence differences found in the rDNA ITS-1, 5.8 S and ITS-2 region of the *T. infestans* subcomplex according to one-species alignments, excepting *T. infestans* dark morph and *T. melanosoma* which were included within the *T. infestans* alignment

	ITS-1*		ITS-2														
	bp length	% AT	bp length	% AT	Nucleotide position												
					881	883	884	903	904	905	906	1054	1055	1056	1057	1141	1180
T. infestans comp. haplotypes ($n = 34$)																	
T.inf-CH1A	673	65.8	476	77.7	T	–	–	–	–	–	–	A	A	A	T	A	A
T.inf-CH1B	692	65.7	476	77.7	T	–	–	–	–	–	–	A	A	A	T	A	A
T.inf-CH1C	699	66.1	476	77.7	T	–	–	–	–	–	–	A	A	A	T	A	A
T.inf-CH2A	673	65.8	480	77.9	A	–	–	A	T	A	T	A	A	A	T	A	T
T.inf-CH3A	673	65.8	482	78.0	A	A	T	A	T	A	T	A	A	A	T	A	T
T.inf-CH4A	673	65.8	474	77.6	A	–	–	A	T	–	–	–	–	–	–	A	A
T.inf-CH5A*	673	65.8	480	77.9	A	–	–	A	T	A	T	A	A	A	T	A	A
T. melanosoma comp. haplotype ($n = 2$)																	
T.mel-CH1A	673	65.8	480	77.7	A	–	–	A	T	A	T	A	A	A	T	G	T

T. platensis comp. haplotypes ($n = 5$)	ITS-1			ITS-2	
	bp length	% AT	Nucleotide position 442	bp length	% AT
T.pla-CH1A	647	65.8	C	476	77.7
T.pla-CH1B	647	66.1	T	476	77.7

T. delpontei comp. haplotypes ($n = 13$)	ITS-1			ITS-2					
	bp length	% AT	Nucleotide position 241	bp length	% AT	Nucleotide position			
						872	873	884	1252
T.del-CH1A	644	66.0	A	474	77.8	–	–	T	G
T.del-CH2A	644	66.0	A	476	77.9	A	T	T	G
T.del-CH3A	644	66.0	A	474	78.1	–	–	A	A
T.del-CH3B	644	65.8	G	474	78.1	–	–	A	A

bp = base pairs; %AT = base composition; n = number of specimens analysed; CH = haplotypes (see Table 1); nucleotide differences listed under position number in respective alignment; * = in *T. infestans*, *T. infestans* dark morph (=T.inf-CH5A) and *T. melanosoma*, for nucleotide differences according to positions in ITS-1, see Fig. 2.

sylvatic, peridomestic and intradomiciliary populations of Bolivia, also found in Peru and a laboratory strain in Chile; (b) T.inf-H2 is the most abundant, found in Chile, Paraguay, Argentina, Uruguay and Brazil; (c) T.inf-H3 concerns the population from Cordoba, Argentina; (d) T.inf-H4 refers to one population from Avellaneda, Argentina; (e) T.inf-H5 is the ''dark morph'' from Cordillera, Bolivia.

In *T. melanosoma* one haplotype was detected (T.mel-H1). In *T. delpontei*, two differing by one microsatellite (AT) were found: Argentina and Bolivia (T.del-H1), and Salta, Argentina (T.del-H2). In *T. platensis*, one haplotype was detected (T.pla-H1). Specimens of *T. platensis* from Rio Negro and a specimen from Paysandu, Uruguay, showed a ITS-2 sequence closer (only two mutations) to that of *T. delpontei* and were consequently included in *T. delpontei* (T.del-H3) (Tables 1, 2 and 4).

In the 482 bp long alignment including the 10 different ITS-2 haplotypes, 22 nucleotide differences (4.58%) appeared, of which 10 (2.07%) were mutations (8 ti + 2 tv) and 12 (2.48%) were insertions/deletions (indels).

When comparing species (Table 5), *T. infestans* showed only a very few nucleotide differences with *T. melanosoma* and *T. platensis*, the majority being indels. Those differences were reduced to one ti between *T. infestans* H2 and *T. melanosoma*, and two ti between *T. infestans* H1 and *T. platensis*. The differences between the other *T. infestans* haplotypes (T.inf-H2, H3, H4, and H5) and *T. platensis* varied between seven, eight and nine nucleotides. The number of differences, mainly ti, increased when comparing *T. infestans* with *T. delpontei*.

The interrupted microsatellite $(AT)_nT(AT)_1AA(AT)_m$ appeared in several *T. infestans* haplotypes (T.inf-H2, H3, H4, and H5) and *T. melanosoma* (T.mel-H1), being $n = 6–7$ and $m = 7–8$. It presented a tv resulting in $(AT)_nTTT(AT)_1AA(AT)_m$ in *T. platensis* and *T. delpontei* haplotypes, as well as in *T. infestans* H1, being $n = 5$ and $m = 6–7$. Additionally, the microsatellite $(AT)_4TT(A)_n$ allowed to distinguish *T. delpontei* ($n = 1$) from *T. infestans*, *T. melanosoma* and *T. platensis* ($n = 3$).

Triatoma rubrovaria and *T. sordida* showed ITS-2 sequences of a length of 491 and 473 bp and a 78.2 and 77.8% of AT content, respectively.

ITS-1: A total of seven different ITS-1 haplotypes was detected (Table 4). In *T. infestans*, three different ITS-1 sequences were found (Tables 1 and 4). The majority of populations showed identical sequence (T.inf-CH1A, CH2A, CH3A and CH4A). Sequence differences were only detected in one sylvatic population from an Inca wall (T.inf-CH1B), in a population from peridomestic rocks and a domestic population (T.inf-CH1C), all from Cochabamba, Bolivia. The ''dark morph'' (T.inf-CH5A) was identical to the majority, as well as *T. melanosoma* (T.mel-CH1A). In *T. delpontei*, one haplotype was detected in Argentina and Bolivia (T.del-CH1A and CH2A). In *T. platensis*, one mutation distinguished two haplotypes: Paysandu, Uruguay (T.pla-CH1A), and Cordoba, Argentina (T.pla-CH1B). Several specimens of *T. platensis* should be included in *T. delpontei*: most from Rio Negro and one from Paysandu, Uruguay, showed an ITS-1 (T.del-CH3A) identical to *T. delpontei*, and another from Rio Negro presented an ITS-1 (T.del-CH3B) differing from *T. delpontei* by one mutation (Tables 1, 2 and 4).

In the 699 bp long alignment including the 7 different ITS-1 haplotypes, 76 nucleotide differences (10.87%) appeared, of which 16 (2.29%) were mutations (10 ti + 6 tv) and 60 (8.58%) were indels.

In the interspecific comparison (Table 5), a high number of nucleotide differences appeared. The majority were indels, substitutions being very few, even none between *T. infestans* and *T. melanosoma*. Contrarily, *T. platensis* and *T. delpontei* differed by a number of substitutions higher than that of indels.

Table 5
ITS-2 and ITS-1 sequence differences detected in pairwise comparisons between species of the *T. infestans* subcomplex

Compared species	Align. length no. bp	Nucleotide differences		Substitutions				Insertions + deletions	
		No.	%	Transitions		Transversions		No.	%
				No.	%	No.	%		
ITS-2:									
T. infestans vs. *T. melanosoma*	482	1–8	0.21–1.66	1	0.21	1–3	0.21–0.62	2–6	0.41–1.24
T. infestans vs. *T. platensis*	482	2–9	0.41–1.87	2	0.41	1–2	0.21–0.41	2–6	0.41–1.24
T. infestans vs. *T. delpontei*	482	9–17	1.86–3.52	5–7	1.03–1.45	1–3	0.21–0.62	2–8	0.41–1.66
T. melanosoma vs. *T. platensis*	480	9	1.87	3	0.62	2	0.42	4	0.83
T. melanosoma vs. *T. delpontei*	480	11–15	2.29–3.12	5–6	1.04–1.25	2–3	0.42–0.62	4–6	0.83–1.25
T. platensis vs. *T. delpontei*	476	6–7	1.26–1.47	3–4	0.63–0.84	1	0.21	2–4	0.42–0.84
ITS-1:									
T. infestans vs. *T. melanosoma*	699	28–35	4.00–5.00	0	0.00	0	0.00	28–35	4.00–5.00
T. infestans vs. *T. platensis*	699	41–69	5.86–9.87	4–5	0.57–0.71	4	0.57	33–61	4.72–8.73
T. infestans vs. *T. delpontei*	699	38–64	5.43–9.15	6–9	0.85–1.28	1–2	0.14–0.28	29–55	4.17–7.86
T. melanosoma vs. *T. platensis*	673	36–37	5.35–5.49	3–4	0.44–0.59	3	0.44	30	4.45
T. melanosoma vs. *T. delpontei*	673	39–40	5.79–5.94	8–9	1.18–1.33	2	0.29	29	4.30
T. platensis vs. *T. delpontei*	648	13–15	2.00–2.31	5–6	0.77–0.92	4–5	0.62–0.77	4	0.62

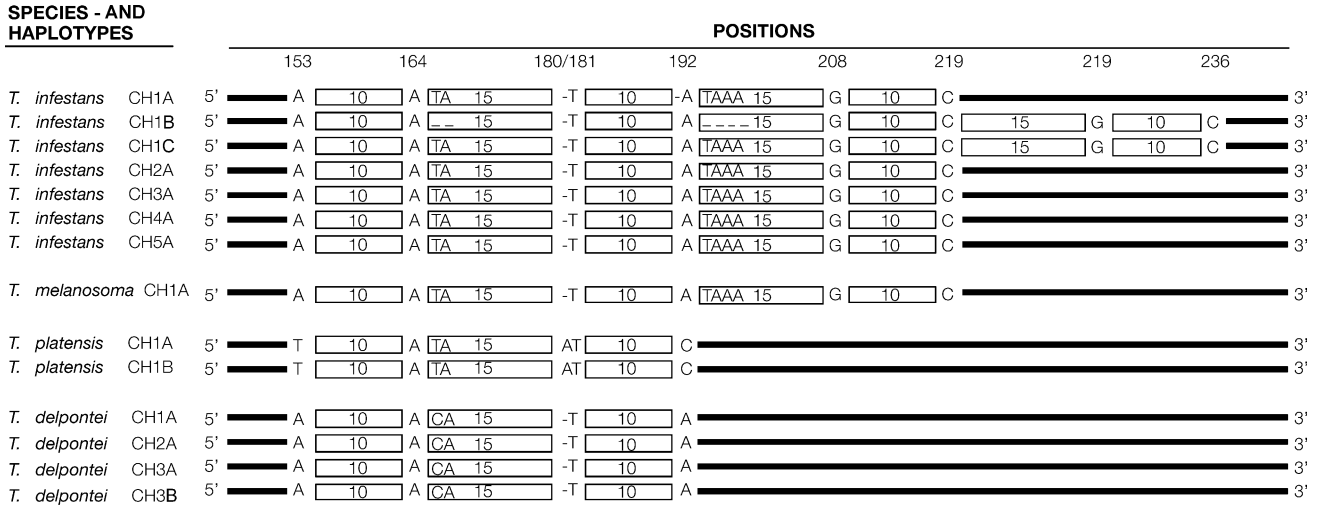

Fig. 2. Distribution of minisatellites repeats in the rDNA ITS-1 sequence of the infestans subcomplex species and haplotypes. Numbers in boxes: minisatellite 10 = CCGCAAAGAC; minisatellite 15 = TAAATAAAATAAAAA. Positions refer to nucleotides separating minisatellites in the alignment of all species and haplotypes. Thick lines represent the rest of the sequence of each haplotype in both 5′ and 3′ senses. Letters in boxes of the 15 bp minisatellite represent its first nucleotides.

Sequence repeats were of two types, minisatellites and microsatellites, both being responsible for marked differences in ITS-1 length.

There is one short minisatellite of 10 nucleotides (CCGCAAAGAC) and another longer one of 15 nucleotides (T/C-AAATAAAATAAAAA) in which the first base changes according to species (T in *T. infestans*, ''dark morph'', *T. melanosoma* and *T. platensis*; C in *T. delpontei*); moreover, in *T. infestans* CH1B the first two nucleotides of the ministatellite are lacking in its first repeat and the first four nucleotides are also lacking in its second repeat. These two minisatellites alternate regularly, always separated by one nucleotide, except in *T. platensis* CH1A and CH1B, in which two nucleotides separate the last two minisatellites. The short 10 bp minisatellite appears three–four times in *T. infestans*, ''dark morph'', and *T. melanosoma*, but only two times in *T. platensis* and *T. delpontei*. The long 15 bp minisatellite appears two–three times in *T. infestans*, ''dark morph'', and *T. melanosoma*, but only once in *T. platensis* and *T. delpontei* (Fig. 2). In both *T. rubrovaria* and *T. sordida*, the short minisatellite is present (once) but the long one is absent.

The repeat $(AT)_n$ appeared, with $n = 4$ in *T. infestans*, ''dark morph'', *T. melanosoma* and *T. delpontei*, and $n = 5$ in *T. platensis*.

T. rubrovaria and *T. sordida* showed ITS-1 sequences of a length of 729 and 750 bp and a 66.2 and 65.3% of AT content, respectively.

3.2. Genome size

T. delpontei appeared to be the species with the highest haploid DNA content, with very high values even far away from the highest detected in *T. infestans* from Bolivia. In *T. infestans*, a large range of DNA content values was found, ''Andean'' specimens from Bolivia harboring a genome size larger than that presented by domestic individuals from other countries (non-Andean). The ''dark morph'' entered the range of *T. infestans* non-Andean specimens. So did *T. melanosoma*, although with a somewhat higher value. *T. platensis* showed the smallest size within the infestans subcomplex. *T. rubrovaria* and *T. sordida* presented the lowest values among the species studied (Table 3).

3.3. Phylogenetic analyses

The ML model best fitting the intergenic region data was HKY85 when considering only variable positions and using the ti/tv ratio of 0.9152 (kappa = 2.1015), base frequencies for A, C, G, and T of 0.3361, 0.1419, 0.1787 and 0.3433, respectively, proportion of invariable sites = 0, and the equal distribution of rates at variable sites. The resulting phylogeny (−ln = 2489.81776) was evaluated using least-squares method with ML distances, with very high puzzle values supporting the reliability of the nodes of the main branches. The tree obtained shows that: (i) *T. delpontei* haplotypes group in a clade independent from all other branches including *T. infestans*, ''dark morph'', *T. melanosoma* and *T. platensis*; (ii) *T. platensis* haplotypes cluster in a branch always linked to those comprising *T. infestans*, ''dark morph'' and *T. melanosoma*; (iii) both *T. melanosoma* and ''dark morph'' haplotypes appear in the branch grouping all *T. infestans* haplotypes; (iv) *T. infestans* haplotypes from Bolivia and Peru (and also the lab strain cultured in Chile) do appear separated from the rest of *T. infestans* haplotypes from all other countries (Fig. 3).

In distance analysis, the ME tree (score of 0.8516) constructed using the distance estimator TN93 provided topology similar and bootstrap values somewhat lower than the ML tree, but with *T. infestans* CH1B appearing basal to

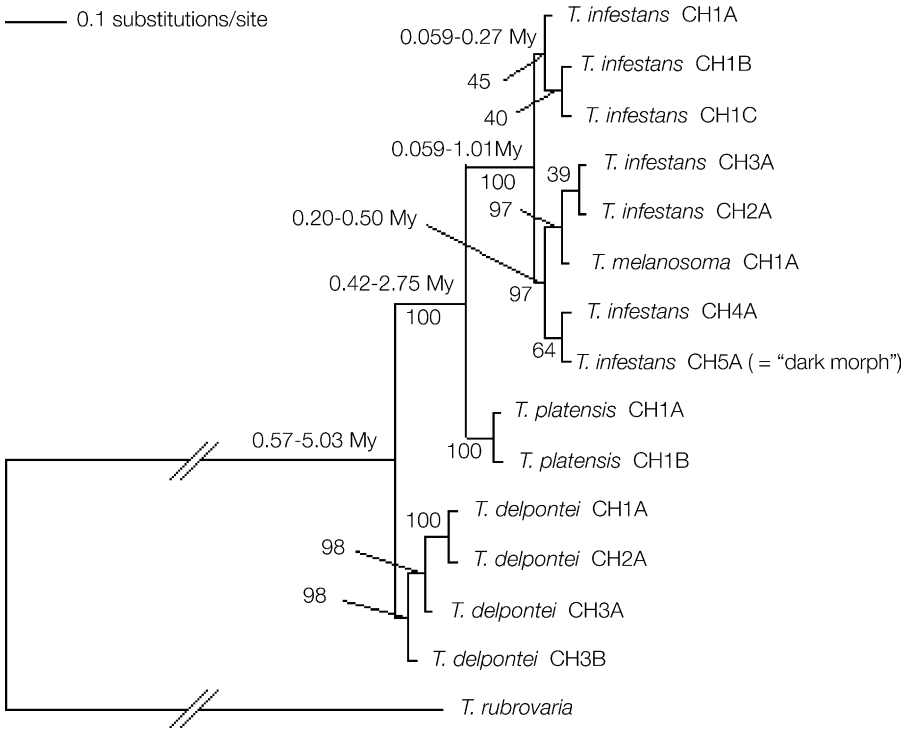

Fig. 3. Phylogenetic tree of the infestans subcomplex species and haplotypes, based on sequences of the whole intergenic region, derived from the maximum likelihood (ML HKY85) model, using the transition/transversion ratio of 0.9152 and obtained using *T. rubrovaria* as outgroup. Scale bar indicates the number of substitutions per sequence position. Numbers represent the percentage of 1000 puzzling replicates. Estimates of the times of divergence (in million years—My) between species and haplotypes are noted for the most important nodes.

the branch including the rest of *T. infestans* haplotypes (tree not shown).

Nonclocklike and clocklike trees were inferred for the data set. The LRT value obtained was 18.4 and the degrees of freedom were 13. The Chi-square critical value obtained was 22.362, indicating that a clock-like model is appropriate for our data.

3.4. Population genetics analysis

A summary of relevant population genetic parameters for *T. infestans* is presented in Table 6. Genetic variation in *T. infestans* populations is unevenly distributed, with four populations (Peru, Brazil, Paraguay and Uruguay) with only one haplotype, and the three others with up to four different haplotypes (Bolivia and Argentina). When the nature of these genetic variants was also considered, the most variable population was found to be Argentina, with an estimated gene diversity of 0.8. Variants in this population are more divergent at the nucleotide level than those found in Bolivia and Chile, since part of the variation in the latter is due to haplotypes with an extra repeat unit in the ITS-1.

Different estimates of q were obtained based on the expected heterozygosity, the expected number of alleles, and

Table 6
Summary of population genetic variation parameters from ITS composite haplotypes in *T. infestans* (*T. melanosoma* included)

Parameter	Bolivia	Peru	Chile	Uruguay	Brasil	Paraguay	Argentina
Gene copies	8	2	3	5	4	6	6
Haplotypes	4	1	2	1	1	1	4
Polymorphic sites	39	0	30	0	0	0	10
Hap. diversity	0.656	1	0.444	1	1	1	0.667
Gene diversity	0.750	0	0.667	0	0	0	0.800
Std. error	0.139	0	0.314	0	0	0	0.172
θ(Het)	2.328	0	1.519	0	0	0	3.171
S.D. θ(Het)	1.841	0	2.237	0	0	0	3.692
$\theta(k)$	2.501	0	1.414	0	0	0	4.063
95% C.I. for $\theta(k)$	[0.727, 8.440]	0	[0.268, 7.458]	0	0	0	[1.047, 16.119]
$\theta(S)$	1.157	0	1.333	0	0	0	0.876
S.D. $\theta(S)$	0.781	0	1.098	0	0	0	0.684

θ(Het) = expected heterozygosity; $\theta(k)$ = expected number of alleles; $\theta(S)$ = number of polymorphic sites; S.D. = standard deviation; C.I. = confidence interval.

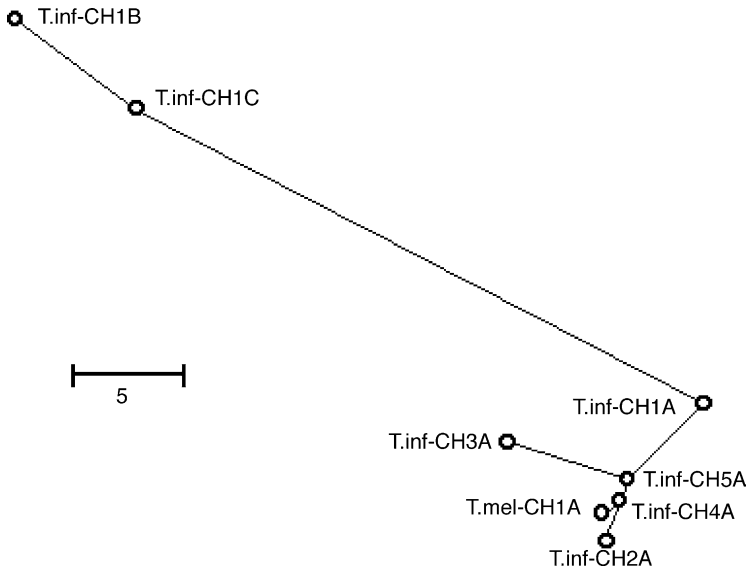

Fig. 4. Minimum spanning network for the eight different ITS composite haplotypes found in *T. infestans* populations. No alternative connections to those represented in the minimum spanning tree were found. The scale bar represents number of differences including indels.

the number of polymorphic sites. The three estimates are quite consistent for the three polymorphic populations and they agree in assigning a larger value to Argentina.

Phylogenetic relationships among the eight different composite haplotypes present in *T. infestans* were represented in a minimum spanning network (Fig. 4). There are two clearly divergent groups of variants, with most differences between them being accounted for by the previously mentioned presence of an extra repeat unit in alleles CH1B and CH1C. No alternative minimal length connections were found among the eight alleles, hence the tree-like structure resulting from this analysis.

Populations may be clearly separated on the basis of their geographical location, east and west of the Andes. This geographical divide also corresponds to a clear genetic differentiation as revealed by the analysis of molecular variance (Table 7). Differences between these two groups account for almost 60% of the total variation found in this species. Furthermore, within any of these groups there is little genetic variation among populations (1.3% of the total variation), and the remaining 40% is found in variation within populations. The three F-statistics at the species level are highly significant, especially F_{CT} which accounts for differentiation among the two previously mentioned groups.

Table 7
Summary of analysis of molecular variance for *T. infestans* populations (*T. melanosoma* included)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	1	86.546	5.13438 Va	59.14
Among populations within groups	5	19.788	0.11575 Vb	1.33
Within populations	27	92.667	3.43210 Vc	39.53
Total	33	199.000	8.68223	

: $P < 0.01$; ***: $P < 0.001$. d.f.= degrees of freedom. Fixation indices: $F_{SC} = 0.03263$; $F_{ST} = 0.60470$***; $F_{CT} = 0.59137$***.

Table 8
Population average pairwise differences in *T. infestans* (*T. melanosoma* included)

	Bolivia	Peru	Chile	Uruguay	Brasil	Paraguay	Argentina
Bolivia	18.00	12.00	17.50	18.75	18.75	18.75	8.17
Peru	3.00	0.00	20.00	8.00	8.00	8.00	7.33
Chile	−1.50	10.00	20.00	28.00	28.00	28.00	27.33
Uruguay	9.75	8.00	18.00	0.00	0.00	0.00	2.33
Brasil	9.75	8.00	18.00	0.00	0.00	0.00	2.33
Paraguay	9.75	8.00	18.00	0.00	0.00	0.00	2.33
Argentina	7.23	5.40	15.40	0.40	0.40	0.40	3.87

Above diagonal: average number of pairwise differences between populations (p_{XY}). Diagonal elements: average number of pairwise differences within population (π_X). Below diagonal: corrected average pairwise difference ($\pi_{XY} - (\pi_X + \pi_Y)/2$).

Table 9
Summary of differentiation tests for *T. infestans* populations (*T. melanosoma* included) based on ITS composite haplotypes

	Bolivia	Peru	Chile	Uruguay	Brasil	Paraguay	Argentina
Bolivia	–	∞	∞	0.635	0.740	0.562	0.876
Peru	−0.053	–	0.857	0.000	0.000	0.000	0.347
Chile	−0.074	0.368	–	0.170	0.211	0.144	0.250
Uruguay	0.440**	1.000*	0.746*	–	∞	∞	3.271
Brasil	0.403*	1.000	0.704*	0.000	–	∞	5.524
Paraguay	0.471***	1.000*	0.777*	0.000	0.000	–	2.417
Argentina	0.363*	0.591	0.666	0.133	0.083	0.171	–

The lower hemimatrix corresponds to pairwise F_{ST} values for the corresponding populations. Significance values (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$) were derived by the exact differentiation test with a Markov chain of length 10000 steps. The upper hemimatrix presents estimates of pairwise equilibrium migration rates (as migrant individuals per generation) derived from F_{ST} values using Slatkin's (1995) method.

Further indications of the differences between pairs of populations can be obtained from the analysis of population differentiation summarized in Table 8. Net differences between population pairs larger than five are always found between the eastern and western populations, with values lower than this in comparisons within each group except for the Peru–Chile comparison. A similar result is obtained in the analysis of pairwise F_{ST} values, with all significant differentiation tests corresponding to eastern–western population pairs (Table 9). From the linearized F_{ST} values we have obtained estimates of pairwise population migration rates.

3.5. Molecular clock dating

The rDNA ITS-1 base-substitution rate was inferred from ITS-2 molecular clock estimates on the times of divergence between the most appropriate species according to the number of nucleotide differences detected in both markers. The divergence of *T. rubrovaria* from *T. sordida*, estimated at 5.91–14.30 and 1.43–3.46 Mya from the rDNA ITS-2 molecular clock, according to total and PAUP differences, respectively, may be used to estimate the ITS-1 molecular clock rate, with 49 total nucleotide differences and 26 differences between these taxa in the pairwise distance matrix of PAUP in the 751 bp long ITS-1 alignment including both species obtained with CLUSTAL-W (6.52 and 3.46%, respectively). Similarly, the divergence of *T. rubrovaria* and *T. delpontei*, and that of *T. sordida* and *T. delpontei*, may also be used for the same purpose. Calculations based on those three ITS-2 divergences furnish a base substitution rate for the rDNA ITS-1 of 0.46–2.58% per 1 My according to total differences and 0.52–2.42% per 1 My according to PAUP, that is $46–258 \times 10^{-10}$ substitutions per site per year. This means that, in Triatomini, ITS-1 evolves 1.12–2.60 times faster than ITS-2.

Based on both ITS-2 and ITS-1 molecular clocks, the dating ranges were obtained for the divergences that appeared in the phylogenetic tree (Fig. 3). Estimates obtained by computing PAUP differences may be preferred, because of estimation inexactitudes induced by ITS-2 microsatellites (mainly concerning ITS-2 estimates related to *T. infestans* H1-5) and ITS-1 minisatellites (in ITS-1 estimates related to *T. infestans* HB and HC) when computing total differences.

4. Discussion

4.1. ITS-2

Second spacer length range and base composition biased to A + T content agree with values previously found in other triatomines (Marcilla et al., 2001, 2002).

Interspecific nucleotide differences are very scarce (Table 5), as expected from species so close to one another. Lower ITS-2 sequence divergences have been found in species of the Mexican phyllosoma complex (Bargues et al., 2002). For those Mexican species it was concluded that a subspecies status would fit best (Bargues et al., 2002), as such a low number of differences are typical in organisms able to hybridize (Mas-Coma, 1999).

ITS-2 genetic distances and sequence differences clearly differentiated *T. infestans* and *T. delpontei*. Concerning *T. infestans* and *T. platensis*, on one side *T. infestans* H2-5 and *T. platensis* differed by only seven to nine nucleotide differences, the majority indels. The weight of substitutions is pronouncedly greater than that of indels. It is known in other organisms that the coexistence of many indels with very few mutations usually indicates active evolving divergence processes (Bargues et al., 2001). On the other side, the detection of only two ti between *T. infestans* H1 from Bolivia and Peru and *T. platensis* from Uruguay is surprising. Isoenzymatic studies also showed a large genetic exchange potential by crossbreeding in places of sympatry, indicating that the status of *T. infestans* and *T. platensis* as distinct species is almost entirely based upon their ecological separation. Although *T. platensis* is clearly able to invade human dwellings and peridomestic habitats by feeding on poultry, their interfertility and the probable natural gene flow between both species could modify this situation in some cases, lowering their actual genetic separation (Pereira et al., 1996).

The two chromatic forms, *T. melanosoma* and ''dark morph'', differed from the most dispersed *T. infestans* H2 by only one ti and one tv, respectively. Similarly as mtDNA results (Monteiro et al., 1999), this ITS-2 mononucleotide difference supports the recently proposed synonymy of *T. melanosoma* with *T. infestans* (Gumiel et al., 2003), although an argument may be made for *melanosoma* as a

subspecies owing to its melanic phenotype and geographical sylvatic isolation in humid subtropical forest (Galvão et al., 2003). The ''dark morph'' has always been considered a chromatic form of *T. infestans*, despite differences in morphometrics, antennal pattern, chromosome banding and RAPD profiles when compared to domestic Bolivian *T. infestans* (Noireau et al., 1997, 2000a,b; Monteiro et al., 1999; Gumiel et al., 2003).

ITS-2 results support the differentiation of *T. delpontei* and *T. platensis*, at least at the lower species limit. Although their hybridization is possible in the laboratory (Usinger et al., 1966), no natural hybrids have been observed. If hybrids occur, they must be very rare and gene flow would in any case be reduced by 50% due to the sterility between *T. delpontei* males and *T. platensis* females. Thus, their morphological similarity would be mainly the consequence of a convergence related to a highly specialized adaptation to bird nests and not of common ancestry (Pereira et al., 1996). The phylogenetical results here obtained do not discard a common ancestry.

At intraspecific level, microsatellites may be useful for species differentiation, such as *T. delpontei* from all others. In *T. infestans*, two mutations differentiate the populations of Bolivia and Peru from those of Chile, Paraguay, Argentina, Uruguay and Brazil, except for H4 from Avellaneda, Argentina, and H5 from Santa Cruz, Bolivia, which only presented one mutation difference. Two peculiar *T. infestans* haplotypes (H3, H4) were detected in Argentina besides the widely distributed one (H2). Whereas H3 from Cruz del Eje differed from H2 by only one additional AT repeat, a lower number of the AT repeat, deletions and a tv singularized H4 (Table 4). A genetic variability of *T. infestans* in Argentina has recently been detected by partial mtDNA 12S and 16S sequences (Garcia et al., 2003) and C-banding patterns (Panzera et al., 2004).

In *T. delpontei*, two mutations differentiate the populations from Argentina and Bolivia from those from Uruguay (Table 4) whose morphology led to an erroneous *T. platensis* preliminary classification (Tables 1 and 2).

The total intraspecific variability (absolute nucleotide differences) detected in *T. infestans* (13:482 = 2.70%) is not only very high but the highest hitherto known, and that of *T. delpontei* (4:476 = 0.84%) is close to that known in Triatomini species distributed through widely separated countries: 0.85% in *Panstrongylus rufotuberculatus*; 0.98% in *Panstrongylus geniculatus*; 2.10% in *Triatoma dimidiata* (Marcilla et al., 2001, 2002).

4.2. ITS-1

This is the first time that an ITS-1 sequence has been obtained in triatomines, proving to be markedly longer than ITS-2. Its length range fits in the 40–1100 bp range found in other organisms, and its biased AT composition is lower than in ITS-2 of triatomines and even somewhat lower than in other organisms (Mas-Coma, 1999).

Interspecific nucleotide differences appear to be more numerous in ITS-1 than in ITS-2, concerning both substitutions and indels, although mainly due to the high amount of insertions (Table 5). Without considering indels because of their weight less than that of mutations (Bargues et al., 2001), the higher number of ti and tv in ITS-1 than in ITS-2 makes species differentiation by means of the ITS-1 easier.

A higher number of the two minisatellite repeats in *T. infestans*, ''dark morph'', and *T. melanosoma* explain why their ITS-1 is markedly longer than in *T. platensis* and *T. delpontei* (Fig. 2). Interestingly, the ITS-1 in *T. rubrovaria* and *T. sordida* is pronouncedly longer despite the absence of those repeats.

In contrast to the tandemly 2–5 bp repeated DNA units called microsatellites (Jarne and Lagoda, 1996), minisatellites are tandemly repeated DNA regions with repeat units of 9–100 bp (Li, 1997) and are found in triatomines for the first time. Results suggest that minisatellites may be useful markers furnishing statistically significant information in triatomines, as they prove to be homologous and not homoplasic according to the location of the haplotypes bearing additional minisatellite repeats in the phylogenetic trees here obtained (Olsen, 1999). Minisatellites might enable not only species differentiation, but also the distinction of different haplotypes within species, as in *T. infestans*. A different number of a minisatellite repeat leads to a marked length difference, easier to detect by simple banding than in the case of microsatellites. Further studies with more numerous individuals and populations are needed.

At intraspecific level, the lower number of ITS-1 haplotypes compared to ITS-2 haplotypes was unexpected. In *T. infestans*, all populations seem to have the same ITS-1 sequence (including the ''dark morph'' and *T. melanosoma*), except populations of haplotypes 1B and 1C from the original area of Bolivia (Fig. 1). In *T. delpontei*, nucleotide differences (one) detected between the two ITS-1 haplotypes were less than those (four) detected between the three ITS-2 haplotypes (Table 4).

4.3. Genome size

Despite all species analyzed in this paper, including outgroup species, presenting the same $n = 22$ chromosome number, a large variation of haploid DNA content was found. These differences are mainly due to differences in the quantity of C-heterochromatin (Panzera et al., 1995). As observed with Feulgen stain, *T. delpontei* presents a very high value of DNA content. This, together with their chromosome meiotic behaviour and the distribution of C-heterochromatin, are characteristics very different from those observed in the other species of the infestans subcomplex. Concerning *T. infestans*, the flow cytometry results confirm the strikingly high DNA content difference between Andean and non-Andean specimens previously

detected (Panzera et al., 2004): (i) Andean populations from Bolivia and Peru with a high total DNA content, and (ii) non-Andean populations comprising the three biogeographic regions of Chaco (Bolivian and Paraguayan Boreal Chaco, and Austral Chaco of Argentina), Pampeana (Uruguay), and Caatinga (Brazil), with less DNA content per cell (mean reduction of 30%, with a maximum of up to 40%).

A relationship between total DNA content per cell, C-heterochromatin revealed by C-banding of chromosomes, and highly repetitive DNA sequences has been suggested (Panzera et al., 2004). There does not appear any relationship between total DNA content and the length of the intergenic region, ITS-2 or ITS-1. However, the correlation between the presence or absence of minisatellites and genome size is worth mentioning. In Bolivia, both the higher number of minisatellite repeats and the intraspecific variability in the ITS-1 length related to minisatellites in *T. infestans* CH1A, CH1B and CH1C agree with the higher total DNA content and the intraspecific variability of genome size detected in Bolivian populations (''dark morph'' included), which appeared to be larger than the variability found in the numerous populations studied from all other countries. Although the number of copies of the rDNA operon present in triatomines remains unknown, several hundreds may at least be supposed, as detected in other eukaryotes (Long and Dawid, 1980). Thus, a higher number of minisatellite repeats can represent a relative increase of DNA content.

4.4. Phylogeographic and population genetics analysis: origin and spread of T. infestans

In spite of the complete interfertility between *T. platensis* and *T. infestans*, and only partial sterility between *T. platensis* and *T. delpontei* (Abalos, 1948; Franca, 1985), it had been concluded that *T. platensis* and *T. delpontei* were more closely related to each other than to *T. infestans*, according to morphological, physiological and behavioural characteristics and the absence of intermediate populations in nature (Usinger et al., 1966). Later, however, cytogenetic studies showed more similarity between *T. platensis* and *T. infestans* than between *T. platensis* and *T. delpontei* (Panzera et al., 1995). Multilocus enzyme electrophoresis did not support the hypothesis of a common ancestor for *T. delpontei* and *T. platensis* and suggested that *T. infestans* and *T. platensis* might have diverged recently from each other or from a common ancestor, or their actual phylogenetic divergence may be minimized by persistent gene flow in the field (Pereira et al., 1996). The trees here obtained support the later hypotheses.

Concerning *T. infestans*, differences in divergence among composite haplotypes and their distribution in the different populations account for the pattern of genetic variation found in this species. Results of the population genetics analysis show clear differences between western (Andean) and eastern (non-Andean) populations with high values (Tables 8 and 9) indicative either of a large enough gene flow to prevent population differentiation by drift within each geographic area or a very recent spread. The latter hypothesis fits better, as most populations studied come from localities too far away one another taking into account that *T. infestans* is a bad flyer which mainly depends on its vertebrate hosts for dispersal (Galvão et al., 2001).

Thus, with sylvatic populations only known in a region of Bolivia, *T. infestans* should be considered a recent species. Dispersal from this region involved first a domiciliation process and later a passive spread related to humans throughout South America. Isoenzymatic studies do also support a discrete Bolivian origin for domestic *T. infestans* (Dujardin and Tibayrenc, 1985; Dujardin et al., 1998).

The domiciliation of *T. infestans* is a very recent adaptation phenomenon, as humans entered the New World only about 12000 years ago or perhaps some time before (Ruiz-Linares et al., 1999; Gonzalez-Jose et al., 2003). It has been suggested that the large spread over vast regions of Bolivia, southern Peru, Chile, Brazil, Paraguay, Uruguay, and Argentina (Schofield, 1988; Carcavallo et al., 1999) took place in two steps (Panzera et al., 2004). Initially, pre-Incaic and Incaic tribes may have facilitated a first passive dispersal throughout Andean regions, associated with early settlements and the domestication of wild rodents for human food. The oldest human record of *T. cruzi* infection is in 4000-year-old pre-Hispanic mummies belonging to the Chinchorro culture, in the northern coast of Chile, an area inhabited at least for 7000 years, first by hunters, fishers and gatherers, and afterwards by more permanent settlements (Guhl et al., 2000). The main expansion most probably occurred in post-Colombian times, after the 17th century, as pioneers migrated to the interior of the continent and settled into subsistence agricultural activities (Schofield, 1988). This spread continued almost to the present day, as the *T. infestans* arrival in northern Uruguay dates from the beginning of the 20th century, in Bahia during the early 1970s, and in the north-eastern Brazilian states only from the 1980s (Panzera et al., 2004).

The trees obtained support a two-wave dispersal (Fig. 1E). *T. infestans* CH1A, CH1B and CH1C from Bolivia and Peru appear separated from haplotypes of other countries. Interestingly, CH1B found in an Inca wall appears basal to all other *T. infestans* haplotypes in NJ and ML trees and population genetics analysis, suggesting that it may be a direct descendant from the populations, which initiated the peridomiciliation adaptation in the Inca period. CH1A and CH1C were found in human dwellings and may thus be probable descendants from old populations able to inhabit domiciles, their finding in peridomestic (CH1C) and sylvatic (CH1A) habitats being assumable because of living in the original area where *T. infestans* perfectly adapts to sylvatic conditions. CH1A, the only haplotype found in Peru and Bolivian Chuquisaca, outside the original Cochabamba area, may represent the one fitting the requirements for Andean highland dispersal.

The widespread *T. infestans* CH2A is the haplotype best adapted to human dwellings and responsible for the large colonization of Chile, Paraguay, Argentina, Uruguay and Brazil. The lack of variability in all those countries agrees with the low variability detected in isoenzymatic studies (Dujardin and Tibayrenc, 1985; Dujardin et al., 1998) and indicates a rapid and very recent geographical dispersal.

Variability in CH3A and CH4A may be due to local isolation phenomena after a dispersal throughout Argentina before colonization of other countries, as suggested by mtDNA sequences (Garcia et al., 2003) and C-banded karyotypes (Panzera et al., 2004). Similarly, recent isolation phenomena in Bolivia and Argentina may also explain the chromatic forms of the ''dark morph'' (CH5A) and *T. melanosoma*, respectively. The northern part of Argentina has even been suggested as having played the role of a spreading center for the non-Andean lowland regions (Panzera et al., 2004).

The molecular clock analysis indicates that the evolution of the infestans subcomplex is recent, within the last 5.0 My and most probably in less than 3.3 My. The evolutionary lineage of *T. infestans* originated in less than 1.0–2.7 My, as a derivation from *T. platensis* ancestors. Data suggest a divergence of domestic *T. infestans* lowland populations from Argentina, Chile, Paraguay, Uruguay and Brazil, and *T. infestans* highland populations from Bolivia and Peru of at least 59000 years, although most data support a greater divergence of more than 200000 or 400000 years. This chronological estimation does not fit the archaeological and historical information available which suggests a much more recent passive dispersal of domestic *T. infestans* populations thanks to human activities. This could not have taken place before the arrival of humans in South America (Ruiz-Linares et al., 1999; Gonzalez-Jose et al., 2003) and most probably not before pre-Incaic and Incaic settlements of 7000 years ago (Guhl et al., 2000). Moreover, dating suggests that the spread throughout Andean highlands may perhaps have occurred simultaneously or even perhaps later than dispersal in non-Andean lowlands, although most probably the spread throughout the highlands included less environment change enabling more haplotype conservation than in the very wide lowland expansion. Among the latter, data support an initial spread from Bolivia highlands into Bolivia and Argentina lowlands, the period elapsed since the colonization of these lowlands having been sufficient as to originate new haplotypes by isolation events developed in very recent times.

In very recent times, base-substitution rates in *T. infestans* might perhaps have been accelerated, the intradomicile adaptation phenomenon giving rise to the main haplotype CH2A and its wide geographical spread and subsequent isolation of several populations (CH3A and CH4A in Argentina), sometimes even including chromatic divergences (CH5A = ''dark morph'' in Bolivia; *T. melanosoma* in Argentina). Similar accelerated genetic divergence phenomena in animals more or less linked to humans are known in other domestic and peridomestic species (Mas-Coma, 2002). This accelerated evolution might also be at the base of the very high reduction of genome size from Andean to non-Andean *T. infestans* populations.

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