Institut de recherche pour le développement

MiVEGEC

Inlasa

Instituto Nacional de Laboratorios de Salud

Universidad de San Simon

Rapport final du projet ANR TiBo

Système vectoriel émergent dû aux populations sauvages de *Triatoma infestans* : la maladie de Chagas en Bolivie

Coordinateur : Dr. François Noireau
Co - Coordinateur : Dra. Simone Frédérique Brenière

Contenu du rapport

Compte rendu de fin de projet

Annexe 16 : Publication, Roca Acevedo G, Cueto GM, Germano M, Orihuela PS, Cortez MR, Noireau F, Picollo MI, Vassena C. 2011. Susceptibility of sylvatic *Triatoma infestans* from Andeans valleys of Bolivia to deltamethrin and fipronil. J Med Entomol. 48(4):828-35.

Annexe 17 : Publication, Aliaga C, Brenière SF, Barnabé C. 2011 Further interest of miniexon multiplex PCR for a rapid typing of Trypanosoma cruzi DTU groups. Infect Genet Evol. 11, 1155-1158.

Annexe 18: Publication soumise, Brenière SF, Aliaga C, Waleckx E, Buitrago R, Salas R, Barnabé C, Tibayrenc M, Noireau F. Genetic Characterization of *Trypanosoma cruzi* DTUs in Wild *Triatoma infestans* from Bolivia: Predominance of TcI. Plos Neglec Trop Dis.

Annexe 19: Typification et génétique des populations de *Trypanosoma. cruzi* chez *Triatoma infestans.*

Annexe 20 : Publication, Barnabé C., De Meeus T., Noireau F., Bosseno MF., Monje EM., Renaud F., Brenière SF. 2011. *Trypanosoma cruzi* discrete typing units (DTUs): Microsatellite loci and population genetics of DTUs TcV and TcI in Bolivia and Peru. Infect Genet Evol 11, 1752–1760.

Annexe 21 : Publication, Waleckx E., Salas R., Huaman N., Buitrago R., Bosseno M.F., Aliaga C., Barnabé C., Rodriguez R., Zoveda F., Monje M., Baune M., Quisberth S., Villena E., Kengne P., Noireau F., Breniere, S.F., 2011. New insights on the Chagas disease main vector *Triatoma infestans* (Reduviidae, Triatominae) brought by the genetic analysis of Bolivian sylvatic populations. Infect. Genet. Evol. 11, 1045-1057.

Annexe 22, Publication, Quisberth S, Waleckx E, Monje M, Chang B, Noireau F, Brenière SF. 2011 "Andean" and "non-Andean" ITS-2 and mtCytB haplotypes of *Triatoma infestans* are observed in the Gran Chaco (Bolivia): Population genetics and the origin of reinfestation. Infect Genet Evol. 11(5):1006-14.

Annexe 23: Génétique des populations naturelles de *Triatoma infestans* sylvestres et domestiques.

AGENCE NATIONALE DE LA RECHERCHE R	**Compte-rendu de fin de projet**	

Projet ANR-AA-PPPP-000

ANR-07-SEST-022-01, conv.3624.

Acronyme et/ou nom du projet

TiBo

Programme SEST 2008

A IDENTIFICATION

Acronyme du projet	TiBo
Titre du projet	Système vectoriel émergent dû aux populations sauvages de *Triatoma infestans* : la maladie de Chagas en Bolivie
Coordinateur du projet (société/organisme)	François Noireau (décédé le 2 aout 2011) UR 016 – IRD devenue, au 1er janvier 2011, MIVEGEC (UM1 et 2-CNRS 5290-IRD 224)
Période du projet (date de début – date de fin)	1er février 2008 – 22 septembre 2011
Site web du projet, le cas échéant	

Rédacteur de ce rapport	
Civilité, prénom, nom	Mme Simone Frédérique Brenière, Mr Christian Barnabé
Téléphone	591 2 278 29 69
Adresse électronique	Frederique.Breniere@ird.fr
Date de rédaction	Décembre 2011

Si différent du rédacteur, indiquer un contact pour le projet	
Civilité, prénom, nom	
Téléphone	
Adresse électronique	

Liste des partenaires présents à la fin du projet (société/organisme et responsable scientifique)	Le projet, lors de son élaboration, prenait en considération trois équipes. Mais, du fait que ces trois équipes appartiennent à la même unité de recherche (UR 016 de l'IRD devenue au 1er janvier 2011 UM1-CNRS 5290-IRD 224) et, qu'en conséquence, elles émargent au même budget géré de manière unitaire, la division qui n'avait plus de raison d'être a été supprimée. A noter que ce projet a été réalisé dans le cadre de deux conventions de recherche en coopération entre IRD et les partenaires boliviens INLASA et IIBISMED

B RESUME CONSOLIDE PUBLIC

B.1 INSTRUCTIONS POUR LES RESUMES CONSOLIDES PUBLICS

Les résumés publics en français et en anglais doivent être structurés de la façon suivante.

Titre d'accroche du projet (environ 80 caracteres espaces compris)

Les populations sauvages de *Triatoma infestans*, un danger pour la maladie de Chagas

Rôle épidémiologique des populations sauvages de *T. infestans* - Environ 10 millions de personnes sont infectées par *Trypanosoma cruzi*, l'agent de la maladie de Chagas transmis par des punaises hématophages (Triatominae) en Amérique Latine. Les pays du Cône Sud ont été les plus durement frappés mais les opérations de lutte contre le vecteur principal *Triatoma infestans* ont été un succès. Néanmoins, en Bolivie, l'existence de foyers sauvages de *T. infestans* (étendue depuis le début du projet au Chili, à l'Argentine et au Paraguay) dont la tendance synanthropique est encore peu connue, constituent une problématique prioritaire de recherche. Le projet TiBo avait donc pour objectif de mieux connaître le rôle épidémiologique de ces populations. Il a permis de préciser la distribution géographique des populations sauvages en Bolivie puis d'évaluer le risque qu'elles représentent pour la santé humaine. La retombée principale des recherches est d'alarmer les autorités en charge du contrôle des vecteurs sur la vaste distribution de ces populations souvent très proches des habitations car elles ont la capacité de se déplacer du milieu naturel vers les habitations et sont porteuses du parasite à une fréquence particulièrement élevée.

Epidémiologie moléculaire et relevés entomologiques et parasitologiques (71)
L'effort de recherche des populations sauvages de *T. infestans* mené par un protocole systématisé sur toute l'aire d'endémie de *T. infestans,* en plus de recherches dirigées (pré information de sites potentiels), a permis de préciser la distribution géographique de ces populations et d'identifier certains de ses déterminants environnementaux. Les outils de biologie moléculaire appliqués aux vecteurs et au parasite (détermination des sources alimentaires, séquençage de gènes et analyses du polymorphisme microsatellite) ont permis d'actualiser les concepts sur l'origine de *T. infestans* et son histoire évolutive. L'étude micro-spatiale dans cinq aires « partagées » (milieux sylvestre, péri domestique et domestique) a abouti à une évaluation précise de la cinétique de la ré-infestation des maisons permettant, grâce à l'analyse spatiale, celle du comportement humain, et l'analyse de la sensibilité des insectes aux insecticides d'évaluer le risque que ces populations représentent pour la santé d'une façon plus intégrale.

Résultats majeurs du projet *(environ 600 caractères espaces compris)*

Découverte d'une large distribution de *T. infestans* sylvestres en Bolivie dans deux écorégions « Bosques Secos Interandinos » (BSIA, Andes) et le « Gran Chaco » (GC, basses terres). Argumentaire en faveur d'une nouvelle origine de *T. infestans*, d'une expansion très ancienne et d'une domestication ubiquiste. Fort taux d'infection par *T. cruzi* des populations vectorielles Andines. Persistance de la ré-infestation dans les Andes et le Gran Chaco ; implication des populations sylvestres dans les Andes, phénomènes de résistance aux insecticides dans le Gran Chaco. Ces phénomènes sont probablement transposables sur toute la zone de distribution de *T. infestans* sylvestre.

Production scientifique et brevets depuis le début du projet *(environ 500 caracteres espaces compris)*
Ne pas mettre une simple liste mais faire quelques commentaires. Vous pouvez aussi indiquer les actions de normalisation

Onze articles publiés ou proposés pour publication : - sept de génétique des populations (nouvelle origine de *T. infestans*, origine de la ré infestation dans le Chaco, validation d'un outil de typification rapide du parasite, caractérisation de *T. cruzi* I (TcI) chez les *T. infestans* sauvages, variabilité microsatellite de souches boliviennes. Trois articles sur la découverte de

nouveaux foyers sauvages et résistance aux insecticides. Un article méthodologique sur la détection de l'origine des repas sanguins des triatomes.

Illustration

Habitats naturels les plus fréquents de *T. infestans* en Bolivie: à gauche trou d'arbre (capture du morphotype obscure (« dark morph ») dans le Gran Chaco ; à droite affleurement rocheux vallée de Cochabamba, capture du morphotype habituel (BSIA).

Informations factuelles

Le projet TiBo est un projet de recherche exploratoire et de recherche fondamentale qui était coordonné par François Noireau. Il associe aussi plusieurs chercheurs de l'équipe INCHA (MIVEGEC, UM1 et 2-CNRS 5290-IRD 224) travaillant en partenariat avec l'INLASA (Intituto Nacional de Laboratorios de Salud) et IIBISMED (Instituto de Investigaciones Biomédical, Universidad de San Simon) en Bolivie. Le projet a commencé en février 2008 et a duré 43 mois. Il a bénéficié d'une aide ANR de 250 000 € pour un coût global de l'ordre de 1 199 901€.

B.2 RESUME CONSOLIDE PUBLIC EN FRANÇAIS

Environ 10 millions de personnes sont infectées par *Trypanosoma cruzi*, l'agent de la maladie de Chagas. Dans les pays du Cône Sud les opérations de lutte contre le vecteur principal *Triatoma infestans* ont été un succès. Néanmoins, l'existence de foyers sauvages de *T. infestans* dont la tendance synanthropique est encore peu connue, constituent une problématique prioritaire de recherche. Le projet TiBo avait donc pour objectif de mieux connaître le rôle épidémiologique de ces populations. Il a permis de (i) préciser la distribution géographique des populations sauvages de *T. infestans* en Bolivie, (ii) montrer que ces populations représentent un risque pour la santé. Les populations sauvages se distribuent principalement dans deux écorégions, l'une d'altitude (BSIA) et l'autre de basses terres (GC). De plus les données ont permis d'argumenter en faveur d'une nouvelle origine de *T. infestans*, d'une expansion très ancienne de l'espèce et d'une domestication ubiquiste. Le suivi

entomologique dans cinq aires « partagées » comprenant le domicile, le péri domicile et l'environnement sylvestre proche a mis en évidence la ré infestation persistante des habitations et le risque que ces populations sylvestres représentent suite à l'analyse spatiale, celle du comportement humain, celle de la sensibilité des insectes aux insecticides et celle de génétique des populations. La retombée principale des recherches est d'alarmer les autorités en charge du contrôle des vecteurs sur la vaste distribution de ces populations sylvestres souvent très proches des habitations car elles ont la capacité de se déplacer de leur milieu naturel vers les habitations et sont porteuses du parasite à une fréquence particulièrement élevée. Elles peuvent donc être la source de la persistance de la transmission vectorielle de la maladie de Chagas par *T. infestans*.

B.3 RESUME CONSOLIDE PUBLIC EN ANGLAIS

Approximately 10 million people are infected with *Trypanosoma cruzi*, the agent of Chagas disease. In the Southern Cone operations against the main vector, *Triatoma infestans*, were a success. Nevertheless, the existence of wild foci of *T. infestans*, whose synanthropic tendency is still little known, is a priority issue for research. The TiBo project was therefore designed to better understand the epidemiological role of these populations. It has, (i) specified the geographic distribution of wild populations of *T. infestans* in Bolivia, (ii) showed that these populations represent a health risk. Wild populations are distributed mainly in two ecoregions, one in altitude (BSIA) and the other in lowlands (GC). In addition, the data allowed proposing a new origin of *T. infestans*, an ancient expansion of the species and an ubiquitous domestication. The entomological monitoring in five "shared" areas, including dwellings (indoors and outdoors), and the close environment showed a persistent re infestation of dwellings and the danger of wild populations, using the spatial analysis, the human behavior, the sensitivity of insects to insecticides and population genetics. The main significance of the research is to alarm the authorities in charge of vector control on the wide distribution of wild populations often very close to dwellings because they have the ability to move from their natural habitat to the villages and, because they carry the parasite at particularly high frequency. They can be the source of the persistence of vector transmission of Chagas disease by *T. infestans*.

C MEMOIRE SCIENTIFIQUE

Maximum 5 pages. *On donne ci-dessous des indications sur le contenu possible du memoire. Ce memoire peut être accompagné de rapports annexes plus détaillés.*

Mémoire scientifique confidentiel : oui x / non
Partie des résultats originaux sur la génétique des populations de parasite et de vecteurs n'est pas encore publiée.

C.1 RESUME DU MEMOIRE

Environ 10 millions de personnes sont infectées par *Trypanosoma cruzi*, l'agent de la maladie de Chagas. Dans les pays du Cône Sud les opérations de lutte contre le vecteur principal *Triatoma infestans* ont été un succès. Néanmoins, l'existence de foyers sauvages de *T. infestans* dont la tendance synanthropique est encore peu connue, constituent une problématique prioritaire de recherche. Le projet TiBo avait donc pour objectif de mieux connaître le rôle

épidémiologique de ces populations. Il a permis de (i) préciser la distribution géographique des populations sauvages de *T. infestans* en Bolivie, (ii) montrer que ces populations représentent un risque pour la santé. Les populations sauvages se distribuent principalement dans deux écorégions, l'une d'altitude (BSIA) et l'autre de basses terres (Chaco). De plus les données ont permis d'argumenter en faveur d'une nouvelle origine de *T. infestans*, d'une expansion très ancienne de l'espèce et d'une domestication ubiquiste. Le suivi entomologique dans cinq aires « partagées » comprenant le domicile, le péri domicile et l'environnement sylvestre proche a mis en évidence la ré infestation persistante des habitations et le risque que ces populations sylvestres représentent suite à l'analyse spatiale, celle du comportement humain, celle de la sensibilité des insectes aux insecticides et celle de génétique des populations. La retombée principale des recherches est d'alarmer les autorités en charge du contrôle des vecteurs sur la vaste distribution de ces populations sylvestres souvent très proches des habitations car elles ont la capacité de se déplacer de leur milieu naturel vers les habitations et sont porteuses du parasite à une fréquence particulièrement élevée. Elles peuvent donc être la source de la persistance de la transmission vectorielle de la maladie de Chagas par *T. infestans*.

C.2 ENJEUX ET PROBLEMATIQUE, ETAT DE L'ART

La maladie de Chagas est principalement transmise par les déjections infectées de punaises hématophages appartenant à la sous-famille des Triatominae qui colonisent l'habitat et transmettent alors le parasite à l'homme. Elle concerne 21 pays d'Amérique Latine. La principale composante des différentes initiatives régionales de contrôle de la transmission de l'agent pathogène *Trypanosoma cruzi*, est la lutte antivectorielle assurée par la pulvérisation intra- et péri domicilaire d'insecticide et l'amélioration de l'habitat. Dans les pays du Cône Sud très rapidement, les opérations de lutte (initiative INCOSUR) dirigées contre *T. infestans* ont été un succès si l'on considère que, globalement, l'incidence de la maladie a chuté de 70% depuis 10 ans. Néanmoins, deux phénomènes nouveaux menacent les résultats acquis contre *T. infestans*: l'existence de foyers sauvages du vecteur et l'apparition d'une résistance aux pyréthroides de synthèse. Ainsi la clarification de la distribution géographique et de l'implication épidémiologique des populations sauvages est nécessaire pour atteindre l'objectif fixé par INCOSUR qui est le contrôle de la transmission de *T. cruzi* par *T. infestans* dans les pays du Cône Sud. Les premiers foyers sauvages de *T. infestans* décrits dans la vallée de Cochabamba étaient connus depuis plusieurs années mais quelques autres foyers découverts plus récemment en Bolivie, dans les Andes et dans le Chaco, ont fait peser une incertitude sur la réussite du contrôle des vecteurs (Noireau et al. 2000a, b, Noireau *et al.* 2005b). Une approche systématique développée dans le projet TiBo de la recherche des foyers sylvestres sur toute l'aire de distribution des populations domestiques de *T. infestans* a permis de montrer que les populations sylvestres présentent une large aire de distribution. De plus l'étude de plusieurs aires partagées et le suivi entomologique a permis de mettre clairement en évidence la persistance de populations dans les villages et celle de génétique des populations a montré que dans certaines zones les populations sylvestres pouvaient se déplacer vers les zones habitées (péri domicile et domiciles).

C.3 APPROCHE SCIENTIFIQUE ET TECHNIQUE

Etudes de terrain :

La recherche de nouveaux foyers sylvestres a bénéficié, d'une part de recherches orientées dans certaines aires par les informations données par les habitants ou les services de santé et d'autre part, d'une approche originale permettant de connaître la distribution des populations sauvages de *T. infestans* en fonction des facteurs environnementaux (annexe 1). Les zones de captures ont été choisies en continuité avec l'habitat humain, afin de prioriser l'étude du risque que les populations sylvestres peuvent représenter pour l'homme.

Sur un site sylvestre de capture, à proximité de la ville de La Paz, a été établi un suivi entomologique durant un an en appliquant une méthode originale de marquage-recapture. Par ailleurs, 5 aires partagées (espaces sylvestre, péri domiciliaire et domiciliaire) ont été étudiées par un suivi entomologique et une étude anthropologique fine (structure et organisation de l'espace, enquêtes socioéconomiques auprès des habitants), (annexe 2).

Epidémiologie moléculaire

Les outils de biologie moléculaire ont été utilisés pour déterminer l'origine des repas sanguins des triatomes et leur infection par *T. cruzi*. Le séquençage de gènes a permis de développer des études de génétique des populations et de phylogéographie de *T. infestans* (ITS-2 et Cytochrome b) et la méthode de PCR multiplex (MMPP) associée au séquençage du gène GPI (Glucose phosphate isomérase) a permis de préciser les DTUs (discrete typing unit) de *T. cruzi* infectant les triatomes. L'analyse du polymorphisme de zones microsatellites chez les populations de *T. infestans* et de *T. cruzi* a permis d'évaluer les flux génétiques et la structure des populations de vecteurs et celle des parasites. Les amorces utilisées dans toutes ces études sont présentées en annexe 3 ; un manuel en espagnol compilant les protocoles utilisés a été rédigé pour le partenaire (annexe 4).

C.4 RESULTATS OBTENUS

Distribution géographique des populations sauvages de *T. infestans* en Bolivie - La recherche systématisée de foyers sauvages dans 7 écorégions (protocole TiBoEco), concerne 47 zones de capture où 2719 pièges ont été posés. Des triatomes ont été capturés dans 18 sites dont 10 avec *T. infestans*. Soixante trois autres sites connus ou non explorés, ont été échantillonnés à l'aide de 6204 pièges ; dans 52 sites (82,5%) des triatomes ont été capturés dont 37 avec des *T. infestans*. L'annexe 5 présente un résumé des résultats et une première carte de distribution prédictive des populations de *T. infestans* sauvages publiée dans un chapitre de livre. L'annexe 6 comprend les tableaux détaillés des lieux de capture. Deux articles sont attachés à ce chapitre (annexes 7 et 8).

Dernièrement dans un souci d'améliorer le rendement des piégeages en milieu sylvestre, un nouveau piège avec un attractif artificiel « multi composite » a été évalué avec succès sur le terrain (annexe 9).

Habitat et écologie des triatomes et sources alimentaires - Les recherches ont permis de montrer que dans les Andes *T. infestans* ne vit pas seulement dans les affleurements rocheux (habitat rupicole) mais dans bon nombre d'autres écotopes (annexe 10) et de confirmer que l'habitat des *T. infestans* sauvages capturés dans le GC est arboricole.

Le suivi entomologique durant un an d'une population sylvestre distribuée dans un champs semi-anthropisé (vallée de La Paz) accompagné d'une méthode de capture-marquage-recapture a permis de montrer, la stabilité temporelle de la population, sa distribution discontinue et le déplacement notable de nymphes et d'adultes (annexe 10).

La méthode moléculaire d'analyse de l'origine des repas sanguins des triatomes a été validée par des expériences au laboratoire (publication en annexe 11). Dans les écorégions (BSIA) et Prepuna (PP) deux hôtes nourriciers de *T. infestans* sont majoritaires : *Octodontomys gliroides* (46,4%) et *Galea musteloides* (25,8%). De façon inattendue des repas sur homme ont été détectés chez plusieurs spécimens. L'information détaillée des repas identifiés chez des triatomes sylvestres et domestiques est présentée en annexe 12.

Connaissance et expérience des triatomes par les habitants – Un total de 812 personnes vivant dans les 7 écorégions étudiées a été enquêté. La grande majorité des habitants reconnait le triatome qu'ils nomment « vinchuca », la moitié signale avoir vu des vinchucas dans le milieu sylvestre et 42,5% les ont vu voler occasionnellement. L'annexe 13 présente le détail des enquêtes.

Etude des aires partagées (milieu sylvestre, péri domestique et domestique), Rancho Nuevo, San Silvestre, Quillacollo, Sapini et Thago Thago (annexe 14)

Environnements et distribution des triatomes sylvestres – Dans l'écorégion du Gran Chaco (GC) des spécimens de *T. infestans* (type « dark morph ») ont été capturés dans les environs de San Silvestre mais pas à Rancho Nuevo. Les insectes seraient très dispersés et rares car peu d'individus sont capturés sur un même piège. Dans l'écorégion BSIA l'environnement proche des habitations est extrêmement infesté par *T. infestans* : à Quillacollo 29,6% des pièges sont positifs (702 *T. infestans* capturés), à Sapini 20,2% (216 *T. infestans* capturés), et à Thago Thago 16,8% (318 *T. infestans* capturés). Par ailleurs le taux d'infection des insectes est très élevé de 40,7% à Quillacollo jusqu'à 88,5% à Sapini.

Cinétique de ré infestation - Dès décembre 2007 et jusqu'au mois de janvier 2011 les deux villages du GC ont fait l'objet d'un suivi entomologique suite à la fumigation de toutes les maisons et la mise en place d'un système de vigilance. La ré infestation a atteint des taux très importants tout au long du suivi. En janvier 2011, 37 mois après le traitement, la ré infestation s'avère encore plus sévère à Rancho Nuevo, à San Silvestre elle est moins élevée mais persistante. Cette situation peut avoir plusieurs sources, celle du développement d'une résistance aux insecticides (comme le montrent les tests insecticides) mais également un problème d'intervention dans la vigilance des villages rendue difficile par le nombre de maison non visitées lors des contrôles (maisons souvent fermées).

A Quillacollo (BSIA), zone péri urbaine, en septembre 2009, février et juin 2010 des enquêtes entomologiques ont été menées dans 176 maisons choisies au début de l'étude. Durant la période, aucun traitement insecticide n'a été appliqué par les autorités locales en charge. Les triatomes ont été exclusivement trouvés en péri domicile (infestation = 14,7%). Dès septembre et durant 9 mois il a été demandé aux habitants de collecter les triatomes qu'ils voyaient. Remarquablement ils ont alors capturé des insectes à l'intérieur de leur maison (infestation intra domiciliaire, 15 / 176 = 8,5%).

Les deux autres aires partagées sont rurales et situées à 2000 m d'altitude dans l'écorégion BSIA. A Sapini (dpt. de La Paz) une seule visite des habitations (27) a été faite en

octobre 2010. Les données antérieures du programme de contrôle et les nôtres montrent une infestation persistante des péri domiciles avec une pénétration occasionnelle à l'intérieur des maisons. A Thago Thago (dpt. de Potosi) le suivi par les habitants et les recherches actives démontrent aussi une situation analogue de persistance de l'infestation intra et péri domiciliaire.

Sensibilité des *T. infestans* a la deltaméthrine insecticide utilisé dans les campagnes de fumigation - Les analyses au laboratoire de 12 colonies issues de *T. infestans* sylvestre a permis d'identifier chez 3 d'entre elles un certain niveau de résistance à la delthaméthrine. Dans les aires partagées, seules les colonies de *T. infestans* issues des villages du GC (Rancho Nuevo et San Silvestre) présentent des niveaux significatifs de résistance à cet insecticide. L'annexe 15 résume l'ensemble des expériences qui portent sur un total de 21 colonies. Par ailleurs, la publication en annexe 16 présente les premiers résultats obtenus sur 4 populations sylvestres.

Typification et génétique des populations de *T. cruzi* chez *T. infestans* - La méthode MMPP de typification rapide et directe de *T. cruzi* dans les tubes digestifs des triatomes (sans isolement des souches) a permis de déterminer que la très large majorité des *T. infestans* sylvestres sont infectés par la DTU TcI (discrete typing unit, TcI à TcVI actuellement décrites) (annexe 17, 18, et 19). Par contre, dans les deux villages du GC au moins 5 des 6 DTUs sont présentes chez *T. infestans* collectés avant et après traitement insecticide (annexe 19).

Une première analyse de génétique des populations basée sur le polymorphisme de microsatellites (8 loci) de souches boliviennes et péruviennes isolées antérieurement et, majoritairement domestiques a posé les bases du travail (annexe 20). L'analyse récente de 6 populations (79 souches TcI au total) isolées de *T. infestans* sylvestres ne permet pas de rejeter l'hypothèse de panmixie chez ces populations et donc pose l'hypothèse d'une reproduction sexuée de *T. cruzi* en milieu sylvestre. Par ailleurs, il existe souvent une différenciation génétique forte entre populations même si elles sont géographiquement peu éloignées (annexe 19).

Génétique des populations de vecteurs

***Phylogéographie de* T. infestans -** Un premier article porte sur l'étude phylogéographique de *T. infestans* où des populations sauvages du vecteur, et non des domestiques dont l'origine est incertaine car souvent déplacées par l'homme, sont étudiées (ITS-2 et Cytb). L'étude révèle une origine andine possible de l'espèce mais plutôt dans l'écorégion du « Chaco Serrano » (CS) que dans celle de BSIA comme suggéré antérieurement ; par ailleurs les évènements de domestication de l'espèce seraient multiples et non issus d'un évènement unique dans les hautes vallées de Cochabamba (annexe 21).

***Origine de la ré infestation* -** Dans l'article publié (annexe 22) les ré infestant observés dans les deux villages des aires partagées du GC (Rancho Nuevo et San Silvestre), seraient issus de populations résiduelles (ITS-2 et Cytb). De plus on observe une forte structuration entre les villages même s'il n'existe pas de barrière géographique. Par ailleurs ces populations pourraient être composées de la réunion de populations originelles du GC et de population andines (transport passif).

Dans les 3 autres aires partagées sélectionnées dans BSIA (Sapini, Quillacollo et Thago Thago) les flux géniques ont été analysés entre populations capturées dans l'espace sylvestre et celles capturées en milieu domestique et péri domestique à l'aide de 8 marqueurs

microsatellite (annexe 23). Dans chaque aire plusieurs populations ont été prises en compte en fonction de critères de proximité et un total de 21 populations a été analysé. A Sapini les données suggèrent un mouvement important des insectes entre les deux milieux. A Quillacollo il existe une forte structuration entre milieu sylvestre et domestique avec cependant quelques individus migrants du sylvestre vers le domestique. A Thago Thago il s'agit d'une situation intermédiaire avec une migration d'individus importante entre les deux milieux.

C.5 EXPLOITATION DES RESULTATS

Pas de brevet. Voir liste des livrables et annexes.

C.6 DISCUSSION

L'intense travail de terrain à la recherche de nouveaux foyer sylvestres de *T. infestans* en Bolivie a modifié complètement la notion de foyers sylvestres supposés être limités à la vallée andine de Cochabamba, ce qui n'est pas le cas. De plus, l'étude a permis de proposer un nouveau scénario sur l'origine intermédiaire (BSIA/GC) des populations et des phénomènes multiples de domestication. Les analyses de la dynamique de ré infestation des habitations dans les aires partagées, montrent des profils très différents : (i) dans GC, ré infestation rapide, persistante malgré le traitement insecticide des villages, probablement associée à des phénomènes de résistance et des difficultés d'intervention, mais non associée aux populations sylvestres, (ii) dans BSIA, nombreux villages entourés d'abondants foyers sylvestres, persistance de l'infestation dans les villages à mettre en relation avec l'arrivée plus ou moins importante d'insectes du milieu sylvestre. L'étude aboutit (i) au constat de l'inadéquation des mesures actuelles de contrôle des vecteurs, (ii) à la nécessité de la diffusion des résultats auprès des instances sanitaires, (iii) à l'ouverture des futures recherches sur de nouvelles mesures de contrôle basées sur la participation sociale et la mise en place de nouveaux outils.

C.7 CONCLUSIONS

Le projet révèle clairement dans BSIA l'importance des populations sylvestres de *T. infestans* dans les phénomènes de ré infestation. Il montre l'efficacité des approches moléculaires à répondre à des questions d'épidémiologie. Il permet d'apporter une base scientifique qui confirme ou infirmer les hypothèses antérieurement émises sur la distribution et l'impact des populations sylvestres. Enfin, dans un contexte de changements sociétaux et environnementaux, ce projet servira de base à de futures études sur l'évolution de la situation en Bolivie.

C.8 REFERENCES

Abad-Franch & Monteiro 2005. Annals of the Brazilian Academy of Sciences, 77, 437-454.
Bacigalupo et al. 2006. Revista Médica de Chile, 134, 1230-1236.
Brenière et al. 2002. Memórias do Instituto Oswaldo Cruz, 97, 289-295.
Cortez et al. 2006. Experimental Parasitology, 114, 305-313.
Dujardin et al. 1998 Med Vet Entomol. 12(1):20-9.
Giordano et al. 2005. Memórias do Instituto Oswaldo Cruz, 100, 753-760.

Gorla 2002. Ecologia Austral, 12, 117-127.
Ceballos et al. 2011. PLoS Negl Trop Dis. 5:e1365. Epub 2011
Gurevitz et al. 2011. PLoS Negl Trop Dis. 5(10):e1349.
Gürtler et al. 2004. Bulletin of the World Health Organization, 82, 196-205.
Marcet et al. 2006. Infect Genet Evol. 6(1):32-7.
Noireau et al. 1999. Trans. R. Soc. Trop. Med. Hyg., 93, 13-14.
Noireau et al. 2005b. Trends in Parasitology, 21, 7-10.
Peterson et al. 2002. Emerging Infection Diseases, 8, 662-667.
Picollo et al. 2005. Journal of Medical Entomology, 42, 637-642.
Pojo de Rego et al. 2006. Parasite, sous presse.
Abad-Franch et al. 2010. Acta Trop. 115(1-2):44-54.
Walter 2003. Parasite, 10, 191-204.
WHO 2002. Technical Report Series no. 905, Geneva, pp. 109.
Zhu et al. 2000. Genetical Research, 76, 227-236.

D LISTE DES LIVRABLES

Quand le projet en comporte, reproduire ici le tableau des livrables fourni au début du projet. Mentionner l'ensemble des livrables, y compris les éventuels livrables abandonnés, et ceux non prévus dans la liste initiale.

Date de livraison	**N°**	**Titre**	**Nature** (rapport, logiciel, prototype, données, ...)	**Partenaires** (souligner le responsable)	**Commentaires**
	1	Distribution des populations sauvages de *T. infestans*	Données, cartes, rapport	Noireau-Brenière	Nombreux participants boliviens techniciens et étudiants
	2	Ecotopes (habitat) des populations sauvages de *T. infestans*	Données rapport	*Noireau-Brenière*	
	3	Validation de la méthode de détermination des repas sanguins de triatomes	Article international	Buitrago et al 2011	Doctorante bolivienne
	4	Détermination des repas sanguins des triatomes	Données, rapport	Buitrago	
	5	Taux d'infection des triatomes	Données rapport	Salas, Aliaga, Buitrago, Noireau Brenière	
	6	Cinétique de réinfestation des habitations	Données, histogramme	Noireau Brenière Monje	Participation des techniciens des SEDES de Santa Cruz et de La Paz
	7	Contexte anthropologique	Enquêtes	Salas, Aliaga, Brenière Sosa	
	8	Montage des SIG dans les aires partagées	cartes	Brémond, Monje, Sossa	Encadrement par Brémond (CR1-IRD) des deux collègues boliviens
	9	Tests de sensibilité aux insecticides des *T. infestans*	rapport	Depickère-Chavez	Post-doc et responsable laboratoire d'entomologie INLASA

Date de livraison	N°	Titre	Nature (rapport, logiciel, prototype, données, ...)	Partenaires (souligner le responsable)	Commentaires
	10	Séquences ITS2 *T. infestans*	Données, dépôt des séquences en banque	Waleckx, Salas, Quisberth	Post doc, technicienne, étudiant master
	11	Séquences cytochrome b de *T. infestans*	Données, dépôt des séquences en banque	Waleckx, Salas, Quisberth	Post doc, technicienne, étudiant master
	12	Fréquences microsatellite de *T. infestans*	Données rapport	Salas, Barnabé	Technicienne, IR IRD
	13	Validation de la typification rapide des DTUs par le marqueur miniexon	Article international	Aliaga, Barnabé Brenière	
	14	Détermination des DTUs de *T. cruzi* par le marqueur miniexon	Article international et rapport	Brenière, Aliaga, Perez, Barnabé, Garcia	
	15	Détermination des DTUs de *T. cruzi* par séquençage du gène GPI	Article international, dépôt des séquences en banque	Brenière, Barnabé, Buitrago Perez	
	16	Enquêtes sur connaissance et expérience des triatomes	Rapport	Salas, Aliaga, Buitrago, Brenière	
	17	Enquêtes sociologique de genre sur la perception, attitude et connaissance en relation avec la maladie de Chagas	Base de donnée (non encore analysées)	Benavides	Estudiant sociologue
	18	Manuel technique en espagnol	Livret	Brenière, Barnabé	
	19	Rapports annuels en espagnol pour le partenaire	Documents	Brenière	

E IMPACT DU PROJET.

E.1 INDICATEURS D'IMPACT

Nombre de publications et de communications (à détailler en E.2)

Comptabiliser séparément les actions monopartenaires, impliquant un seul partenaire, et les actions multipartenaires résultant d'un travail en commun.

***Attention** : éviter une inflation artificielle des publications, mentionner uniquement celles qui résultent directement du projet (postérieures à son démarrage, et qui citent le soutien de l'ANR et la référence du projet)*

Rappel : le projet est devenu monopartenaire (voir page 2)

		Publications multipartenaires	Publications monopartenaires
International	**Revues à comité de lecture**		11 (en partenariat avec les chercheurs et techniciens boliviens)
	Ouvrages ou chapitres d'ouvrage		3
	Communications (conférence)		24
France	**Revues à comité de lecture**		
	Ouvrages ou chapitres d'ouvrage		

	Communications (conférence)		
Actions de diffusion	**Articles vulgarisation**		
	Conférences vulgarisation		
	Autres		

Autres valorisations scientifiques (à détailler en E.3)

	Nombre, années et commentaires (valorisations avérées ou probables)
Brevets internationaux obtenus	0
Brevet internationaux en cours d'obtention	0
Brevets nationaux obtenus	0
Brevet nationaux en cours d'obtention	0
Licences d'exploitation (obtention / cession)	0
Créations d'entreprises ou essaimage	0
Nouveaux projets collaboratifs	ANR_Programme Blanc édition 2012 (en cours de soumission) ; Collaboration universitaire UMSA (La Paz), instances sanitaires boliviennes (SEDES), société Central des Peuples Indigènes
Colloques scientifiques	
Autres (préciser)	

E.2 LISTE DES PUBLICATIONS ET COMMUNICATIONS

Publications internationales à comité de lecture

1. Noireau F. Wild *Triatoma infestans*, a potential threat that needs to be monitored. Mem Inst Oswaldo Cruz. 2009 Jul;104 Suppl 1:60-4.
2. Buitrago R, Waleckx E, Bosseno MF, Zoveda F, Vidaurre P, Salas R, Mamani E, Noireau F, Brenière SF, **2010**. First report of widespread wild populations of *Triatoma infestans* (Reduviidae, Triatominae) in the valleys of La Paz, Bolivia. Am J Trop Med Hyg. 82(4):574-9.
3. Waleckx E., Salas R., Huaman N., Buitrago R., Bosseno M.F., Aliaga C., Barnabé C., Rodriguez R., Zoveda F., Monje M., Baune M., Quisberth S., Villena E., Kengne P., Noireau F., Breniere, S.F., **2011**. New insights on the Chagas disease main vector *Triatoma infestans* (Reduviidae, Triatominae) brought by the genetic analysis of Bolivian sylvatic populations. Infect. Genet. Evol. 11, 1045-1057.
4. Quisberth S, Waleckx E, Monje M, Chang B, Noireau F, Brenière SF. 2011 "Andean" and "non-Andean" ITS-2 and mtCytB haplotypes of *Triatoma infestans* are observed in the Gran Chaco (Bolivia): Population genetics and the origin of reinfestation. Infect Genet Evol. 11(5):1006-14.
5. Aliaga C, Brenière SF, Barnabé C. 2011 Further interest of miniexon multiplex PCR for a rapid typing of *Trypanosoma cruzi* DTU groups. Infect Genet Evol. 11, 1155-1158.

6. Buitrago R, Cupolillo E, Bastrenta B, Le Pont F, Martinez E, Barnabé C, Brenière SF. 2011. PCR-RFLP of ribosomal internal transcribed spacers highlights inter and intra-species variation among Leishmania strains native to La Paz, Bolivia. Infect Genet Evol. 11(3):557-63.
7. Barnabé C., De Meeus T., Noireau F., Bosseno MF., Monje EM., Renaud F., Brenière SF. 2011. *Trypanosoma cruzi* discrete typing units (DTUs): Microsatellite loci and population genetics of DTUs TcV and TcI in Bolivia and Peru. Infect Genet Evol 11 1752–1760.
8. Ryelandt J, Noireau F, Lazzari CR. A multimodal bait for trapping blood-sucking arthropods. Acta Trop. 2011 Feb;117(2):131-6.
9. Roca Acevedo G, Cueto GM, Germano M, Orihuela PS, Cortez MR, Noireau F, Picollo MI, Vassena C. Susceptibility of sylvatic *Triatoma infestans* from Andeans valleys of Bolivia to deltamethrin and fipronil. J Med Entomol. 2011 Jul;48(4):828-35.
10. Buitrago R, Depickère S, Bosseno MF, Patzi ES, Waleckx E, Salas R, Aliaga C, Brenière SF. Combination of cytochrome b heteroduplex-assay and sequencing for identification of triatomine blood meals. Infect Genet Evol. 2011 Sep 22. [Epub ahead of print] PubMed PMID: 21963963. In press.
11. Waleckx E, Depickère S, Salas R, Aliaga C, Monje M, Calle H, Buitrago R, Noireau F, and Brenière SF. New Discoveries of Sylvatic *Triatoma infestans* (Hemiptera:Reduviidae) Throughout the Bolivian Chaco. Am J. Trop. Med. Hyg. In press

Chapîtres de livre

1 - Noireau, F., Gorla, D. Biology of Triatominae. In : Telleria, J. and Tibayrenc M. 2010. (Ed.), American Trypanosomiasis Chagas Disease, Elsevier, Elsevier, Burlington, UK, pp. 209-223.

2 - Brenière, S.F., Aznar, C., Hontebeyrie, M. Vector transmission. 2010. (Ed.), American Trypanosomiasis Chagas Disease, Elsevier, Elsevier, Burlington, UK, pp. 523-533.

3 - Triatominae del Gran Chaco. Etienne Waleckx, Victor M. S. Soruco, Ricardo E. Gürtler, François Noireau. A paraître.

Conférences

1 - XX Congreso Latinoamericano de Parasitologia, FLAP, 27 septembre-1er octobre 2011. Bogota.

- Brenière S.F., Barnabé, C. De la genética evolutiva de triatominos y de *Trypanosoma cruzi* a los sistemas epidemiológicos de la enfermedad de Chagas. Biomedica, 31 (Suppl) 3, 180-182. Invité
- Noireau, F., Bustamante, M., Garcia, L. *Triatoma infestans* para los primeros ensayos de campo de triatoma virus. Biomedica, 31 (Suppl) 3, 298-300. Invité
- Salas, R., Buitrago, R., Brémond, P., Aliaga, C., Noireau, F., Waleckx, E., Brenière, S.F. Fluctuaciones temporales y desplazamientos de *Triatoma infestans* silvestres a una escala micro geográfica. Travaux libres, oral.

- Buitrago, R., Bosseno, M.F., Salas, R., Waleckx, E., Noireau, F., Brenière, S.F. Identificacion de fuentes alimenticias provenientes de humanos en *Triatoma infestans* (Hemiptera : Reduviidae) silvestres de Bolivia. Travaux libres, oral.
- Perez, E., Monje, M., Parrado, R., Garcia, L., Noireau, F., Brenière, S.F. Deteccion y caracterizacion de los DTUs (discrete typing units) de *Trypanosoma cruzi* circulando en comunidades del Chaco boliviano. Travaux libres, oral.
-

2 - Journées scientifiques Franco-Mexicaines sur les maladies infectieuses. 3-7 mai 2010. Veracruz Mexique.

- Brenière, S.F. Les cycles sylvestres de *Trypanosoma cruzi*, un nouveau défi, de la nécessité d'une approche intégrée de l'étude des risques épidémiologiques. (Invité)

3 - 2ème Conférence Internationale : Le climat, Durabilité" et Développement en Régions Semi-arides. 16-20 Aout 2010. Fortaleza, Brésil.

- Noireau, F. An ecosystem perspective of the process of reinfestation by Triatoma infestans in rural communities of the Gran Chaco ecoregion.

4 – 2ème Congreso Nacional de Parasitologia, 27-29 de agosto de 2009, Sucre, Bolivia.

- Brenière, S.F. Linages I y II de *Trypanosoma cruzi* presentes en Bolivia: implicaciones epidemiologias.
- Quisberth, S., Waleckx, E., Monge, M., Depickère, S., Gutierrez, T., Chang, B., Brenière, F. & Noireau, F. Estructura poblacional de Triatoma infestans (Hemiptera: Reduviidae) en comunidades del Chaco Boreal bajo control vectorial.
- Aliaga C, Bosseno MF, Baune M, Waleckx E, Salas R, Buitrago R, Noireau F, Brenière SF. Predominio del linaje *Trypanosoma cruzi* I sobre T. cruzi II en triatominos silvestres de Bolivia: *Triatoma infestans*, *Triatoma sordida* y *Triatoma guasayana*.
- Waleckx E, Salas R, Buitrago R, Bosseno MF, Baune M, Villena E, Noireau F, Brenière SF, Estructura genética y filogeografía de las poblaciones silvestres de Triatoma infestans (Hemiptera: Reduviidae) en Bolivia.
- Salas R, Charrière M, Sanjines A, Buitrago R, Bosseno MF, Waleckx E, Noireau F, Brenière SF. Distribución geográfica y hábitat de triatominos silvestres: colecta estandarizada basada sobre una estratificación del medio en eco regiones.
- Buitrago R, Waleckx E, Bosseno MF, Zoveda F, Vidaurre P, Salas R, Mamani E, Noireau F, Brenière SF.Primer reporte de poblaciones silvestres de Triatoma infestans (Reduviidae, Triatominae) detectadas en dos valles de La Paz-Bolivia.

5 – 2ème Congreso Nacional de Entomologia, 11-13 de noviembre 2009, La Paz, Bolivia.

- Waleckx E, Salas R, Buitrago R, Bosseno MF, Baune M, Villena E, Noireau F, Brenière SF. El Gran Chaco, posible centro de origen de Triatoma infestans (Hemíptera: Reduviidae).

- Quisberth, S., Waleckx, E., Monge, M., Depickère, S., Gutierrez, T., Chang, B., Brenière, F. & Noireau, F. Origen de la reinfestacion de pueblos del Gran Chaco Boliviano por Triatoma infestans vector de la enfermedad de Chagas.
- Bosseno MF, Buitrago R, Brenière SF.Determinación de fuentes alimenticias en Triatominos.
- Salas R, Charrière M, Sanjines A, Buitrago R, Bosseno MF, Waleckx E, Noireau F, Brenière SF.Búsqueda estandardizada de Triatominos silvestres vectores de la enfermedad de Chagas en Bolivia.
- Buitrago R, Waleckx E, Bosseno MF,Zoveda F, Vidaurre P, Salas R, Mamani E, Noireau F, Brenière SF.Hallazgo de extensas poblaciones silvestres Triatoma infestans (Reduviidae, triatominae) en el departamento de La Paz, Bolivia.
- Aliaga C, Bosseno MF, Baune M, Waleckx E, Salas R, Buitrago R, Noireau F, Brenière SF.*Trypanosoma cruzi* I, linaje dominante en Triatoma infestans silvestres en Bolivia.
- Huamán N, Buitrago R, Zoveda F, Noireau F, Brenière SF, Kengne P.Variabilidad genética de poblaciones silvestres y domésticas de Triatoma infestans en Bolivia.

6 - XIX Congreso Latinoamericano de Parasitología, FLAP. 22 al 24 de octubre 2009, Asunción Paraguay.

- Brenière, S.F. Los ciclos silvestres de *Trypanosoma cruzi*: hacia un enfoque integrado de los riesgos epidemiológicos. Oral
- F. Noireau, F. Los vectores de la enfermedad de Chagas en el Cono Sur de América. Oral.

7 - International Symposium on the Centenary of Chagas Disease. 08-10 juillet 2009. Rio de Janeiro, Brésil.

- Noireau F., Rojas de Arias, A., Gürtler, R. Wild *Triatoma infestans*, a potential threat that needs to be monitored. Invité.

E.3 LISTE DES ELEMENTS DE VALORISATION

La liste des éléments de valorisation inventorie les retombees (autres que les publications) décomptées dans le deuxieme tableau de la section ***Erreur ! Source du renvoi introuvable.****. On détaillera notamment :*

- *brevets nationaux et internationaux, licences, et autres éléments de propriete intellectuelle consécutifs au projet.*
- *logiciels et tout autre prototype*
- *actions de normalisation*
- *lancement de produit ou service, nouveau projet, contrat,…*
- *le developpement d'un nouveau partenariat,*
- *la création d'une plate-forme à la disposition d'une communauté*
- *création d'entreprise, essaimage, levées de fonds*
- *autres (ouverture internationale,..)*

Elle en précise les partenariats éventuels. Dans le cas où des livrables ont éte spécifiés dans l'annexe technique, on présentera ici un bilan de leur fourniture.

A part les actions de publications et présentations lors de congrès, chaque année un rapport en espagnol a été fourni aux partenaires Boliviens. Dans les mois qui viennent l'IRD assurera la traduction en espagnol du présent rapport avec ses annexes et ce document sera alors diffusé au Programme National Chagas, aux SEDES de Santa Cruz et la Paz ainsi qu'à l'INLASA et l'IIBISMED organismes collaborateurs dans le cadre de convention de coopération scientifique avec l'IRD. Pour la partie technique un manuel technique a été fourni aux partenaires.

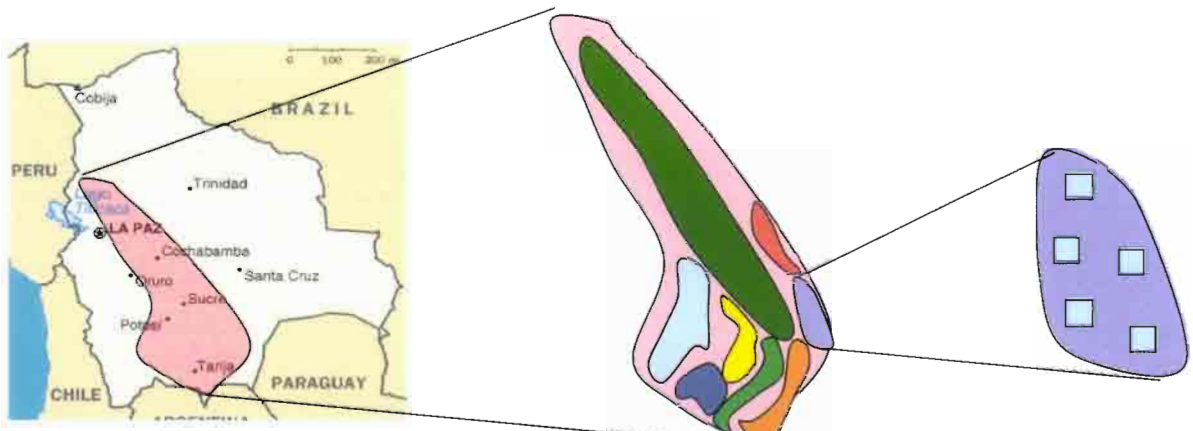
E.4 BILAN ET SUIVI DES PERSONNELS RECRUTES EN CDD (HORS STAGIAIRES)

Pas de recrutement dans le projet

Identification				Avant le recrutement sur le projet			Recrutement sur le projet				Après le projet				
Nom et prénom	Sexe H/F	Adresse email (1)	Date des dernières nouvelles	Dernier diplôme obtenu au moment du recrutement	Lieu d'études (France, UE, hors UE)	Expérience prof. Antérieure, y compris post-docs (ans)	Partenaire ayant embauché la personne	Poste dans le projet (2)	Durée missions (mois) (3)	Date de fin de mission sur le projet	Devenir professionnel (4)	Type d'employeur (5)	Type d'emploi (6)	Lien au projet ANR (7)	Valorisation expérience (8)

Annexe 1

Recherche systématisée des populations sauvages de *T. infestans*

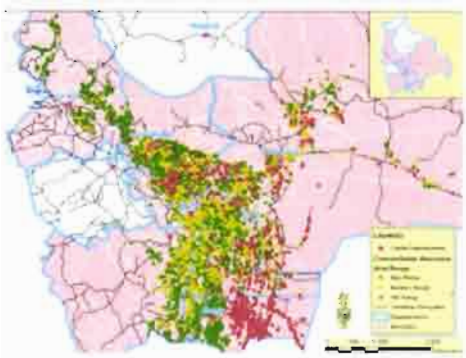


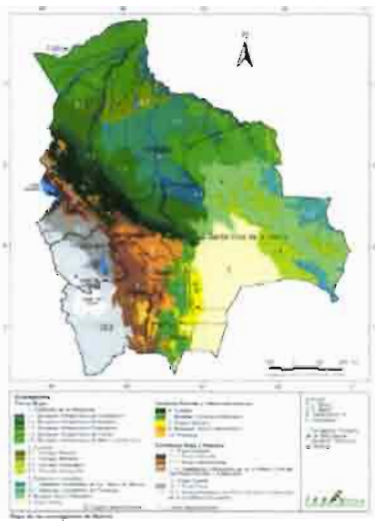
1 - Zone d'intérêt incluse dans l'aire de distribution de *T. infestans* domestique, zone d'endémie majeure de la maladie de Chagas

2 - La zone d'intérêt comprend au moins 7 écorégions (entière ou partielle)

3 - Couverture de chaque écorégion par un minimum de 3 zones de piégeage et en nombre proportionnel à la surface de l'écorégion

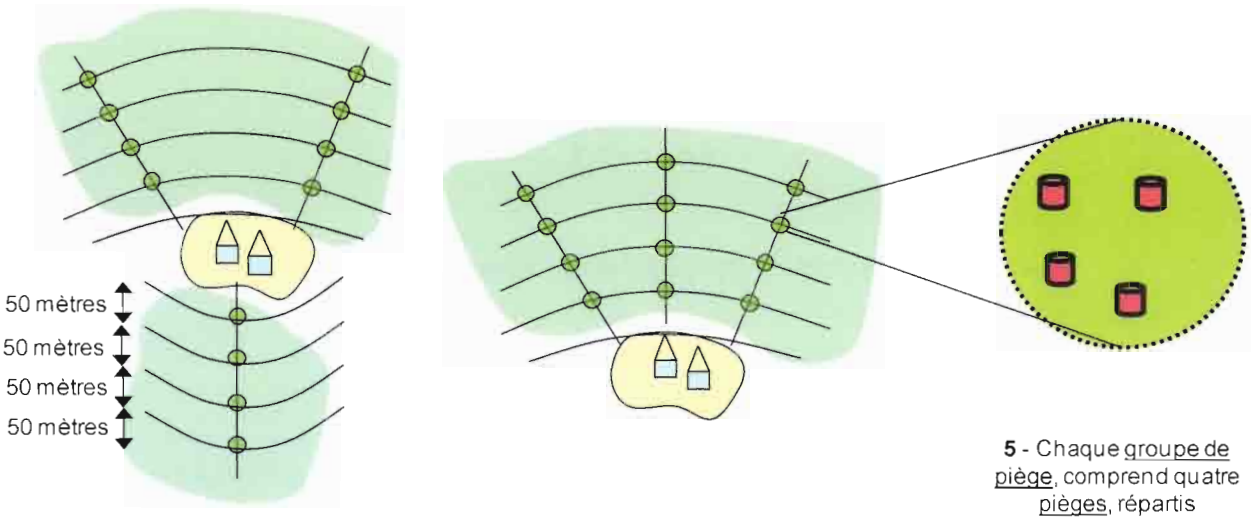
Degré d'infestation des communautés par *T. infestans* sur son aire de distribution





Carte des écorégions de Bolivie selon Ibish et Mérida, 2008

Figure 1. Nouvelle stratégie d'échantillonnage (protocole TiBoEco).



4 - Zone de piégeage: 12 groupes de piège, répartis en trois transects, selon un gradient de distance à partir de la limite du péri-domicile (50 m, 100 m, 150 m et 200 m)

5 - Chaque groupe de piège, comprend quatre pièges, répartis opportunément dans un cercle de 5 mètres de rayon

Figure 2. Organisation du piégeage dans les zones d'étude (protocole TiBoEco).

Figure 3. Pièges « Noireau » attractif avec souris

Figure 4. Capture de plusieurs larves de *T. infestans* collées sur le scotch double face.

Annexe 2

Plans des 5 aires partagées (zones sylvestre, péri domiciliaire et domiciliaire)

Localisation des aires partagées : deux villages dans l'écorégion du Gran Chaco, (GC) (San Silvestre et Rancho Nuevo) ; 2 villages (Sapini et Thago Thago) et une zone péri urbaine (Quillacollo) dans l'écorégion de « Bosque Seco Interandino » (BSIA). Les deux villages du GC sont situés dans la partie sèche du Chaco (terres basses, altitude moyenne de 300 m) le long de la rivière Parapeti et bordés de forêts généralement denses, basses et épineuses. La température moyenne annuelle est de 26°C (0° - 42°) avec une moyenne de précipitation d'environ 550 mm de décembre à avril. Le village de Rancho Nuevo comprend 128 maisons, les habitants (669) sont majoritairement des indigènes de l'ethnie Izoceño. San Silvestre comprend 23 maisons, ses habitants (78) sont de l'ethnie Karai. La forêt est plus haute autour de San Silvestre que de Rancho Nuevo.

Rancho Nuevo

San Silvestre

Les deux villages Sapini et Thago Thago sont situés dans l'écorégion de BSIA. Sapini est un petit village (27 maisons dont 19 sont habitées) de cultivateurs avec environ 60 habitants Aymara. Le village se situe au bord de la rivière Luribay à une altitude de 1880 m, il est entouré de hautes montagnes arides couvertes de très peu de basse végétation et composée majoritairement d'épineux. La température moyenne annuelle est de 19°C (8.3°C – 30.8°C), avec environ 379 mm / an de précipitation. Le village de Thago Thago comprend seulement 12 maisons avec environ 42 habitants, il est situé dans une vallée plus large à 2000 m entourée de collines arides couvertes d'une

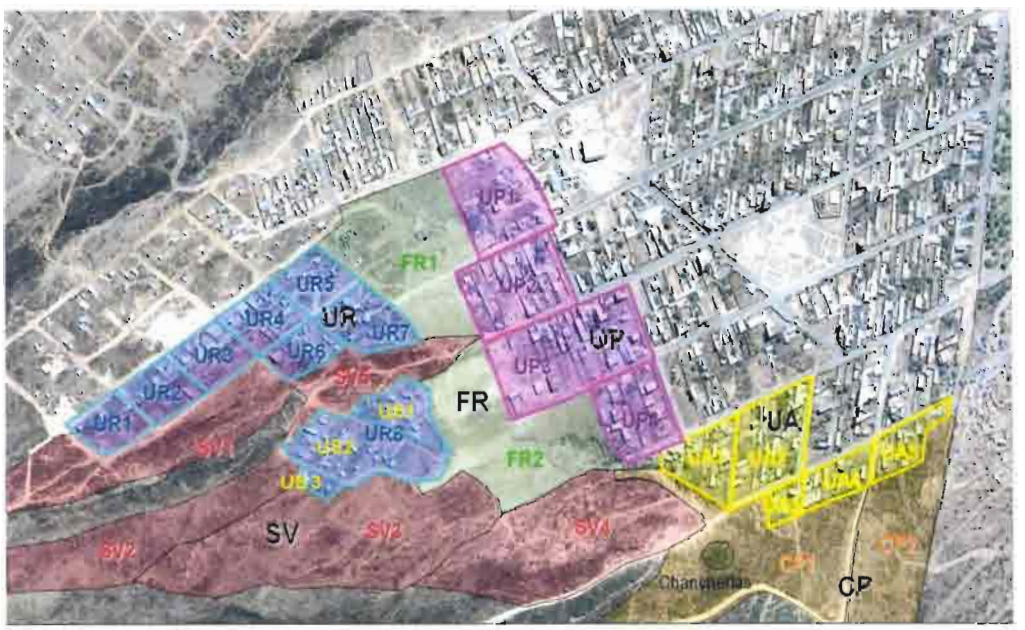
végétation basse et épineuse parsemée d'affleurement rocheux. La température moyenne annuelle est de 16°C, avec environ 939 mm / an de précipitation. Les habitants sont majoritairement des Quechua.

Sapini

Thago Thago

La dernière aire partagée est une zone péri urbaine située en bordure de la ville de Quillacollo. La ville de Quillacollo est localisée dans la large vallée de Cochabamba (15 km de la ville de Cochabamba) a 2700 m. L'étude a compris les blocs de maisons (175 maisons au total) qui sont en bordure de la zone sylvestre composée de collines où affleurent des barres discontinues de rochers. L'urbanisation est plus où moins récente et les familles sont Quechua ou Aymara. La température moyenne annuelle est de 19°C (5.7 °C– 32.0°C), avec environ 482 mm / an de précipitation.

U = zones habitées CP = zones semi anthropisée
S = zones sylvestres FR = terrains vagues



Quillacollo

Annexe 3

Liste des marqueurs génétiques utilisés dans le projet TIBO, amorces, tailles attendues et références bibliographiques

Triatoma infestans : marqueurs géniques

ITS2 : selon Marcilla et al 2001

Primer 1	5'-CTAAGCGGTGGATCACTCGG-3'	827bp
primer 2	5'-GCACTATCAAGCAACACGACTC-3'	

Cytochrome B . selon Lyman et al. 1999

Primer 1	5'-GGACAAATATCATGAGGAGCAACAG-3'	428 bp
Primer 2	5'-ATTACTCCTCCTAGCTTATTAGGAATTG-3'	

T. infestans : marqueurs microsatellite selon Garcia *et al.* 2004

TiA02	5'-GGAAACTCATGTTATGGACACG-3'	171bp-226bp
	5'-AAACCTTATTGTTAGTTCGTTTTGG-3'	
TiC02	5'-CTCTGGGGATCATCGTTCTG-3'	157bp-211bp
	5'-TTTAGGATTCATACCGCCTTT-3'	
TiC09	5'-TTTGCCACATTTACCATTTCC-3'	135bp-159bp
	5'-TCAAGAGAAGCCGTCCAACT-3'	
TiD09	5'-TGGACATAAGCCCCCTGTAA-3'	188bp-230bp
	5'-GGATCCTACTGTGCGGATGT-3'	
TiE02	5'-AGCACGGTTTGCAACTTTTC-3'	147bp-167bp
	5'-TGTGGAATTGAAGGAGCACA-3'	
TiE12	5'-CCTTTAATTTCCCTTTGCCATC-3'	301bp-323bp
	5'-CCTACACGAAATGCCCAAGT-3'	
TiF03	5'-AAAATGGCGGACAAACATTC-3'	162bp-216bp
	5'-TTCCTCAACACAAACACAAACC-3'	
TiF11	5'-TCAATAAAACGAAAATGCGACTT-3'	197bp-239bp
	5'-GCGGGATCTAAGCCAACAGT-3'	
TiG03	5'-CGGAAATGCAAAATTTTTTAGGCG-3'	200bp-254bp
	5'-ATTCTGATCGTGGCAATTTT-3'	

Trypanosoma cruzi : marqueur génique

GPI : selon Broutin et al. 2001

GPI-L	5'-CGCCATGTTGTGAATATTGG-3'	652bp
GPI-R	5'-TTCCATTGCTTTCCATGTCA-3'	

Trypanosoma cruzi : marqueur inter-génique

Miniexon : selon Fernandes et al 2001

primer TC2OF	5'-TTGCTCGCACACTCGGCTGCAT-3'	TC2OF-MEOF: 250 bp
primer MEOF	5'-TACCAATATAGTACAGAAACTG-3'	TC3OF-MEOF = 150 bp
primer TC3OF	5'-CCGCGWACAACCCCTMATAAAAATG-3'	TROF-MEOF = 100 bp
primer TC1OF	5'-ACACTTTCTGTGGCGCTGATCG-3'	

primer TROF	5'-CCTATTGTGATCCCCATCTTCG-3'	

Trypanosoma cruzi : marqueurs microsatellite selon *Oliveira *et al.* 1998 et **Gaunt *et al.* 2003

MCLE01*	5'-CTGCCATGTTTGATCCCT-3'	119bp-136bp
	5'-CGTGTACATATCGGCAGTG-3'	
MCLE08*	5'-ATGGACAACAAATGGGAG-3'	114bp-129bp
	5'-TGGGTATGCCAAATGTGAT-3'	
SCLE10*	5'-GATCCCGCAATAGGAAAC-3'	138bp-288bp
	5'-GTGCATGTTCCATGGCTT-3'	
SCLE11*	5'-ACGACCAAAGCCATCATT-3'	136bp-162bp
	5'-GATGCTAACTGCTCAAGTGA-3'	
MCLF10*	5'-GCGTAGCGATTCATTTCC-3'	181bp-193bp
	5'-ATCCGCTACCACTATCCAC-3'	
MCLG10*	5'-AGGAGTCAAATATAATGAGGCA-3'	153bp-178bp
	5'-ACGTGTGAAAGGCATCTATC-3'	
MCL03*	5'-GGAGCAAGAATGAAGGCA-3'	247bp-290bp
	5'-TCAGAAAAAGCACGCCTC-3'	
MCL05*	5'-TTAAACGACCTCTATGTCTCTC-3'	208bp-266bp
	5'-CCTGAGCAAGATACAAGGAC-3'	
A427**	5'-ACGCGCGTTACTTGTGGTAT-3'	170bp-215bp
	5'-CCAAATATGCATGTGTTTGGA-3'	
C875**	5'-CCATGTCGACTCCATGTCTC-3'	166bp-246bp
	5'-TTGTTGCTGTTGTTGGCAAT-3'	

Cytochrome B de reptiles, oiseaux et mammifères pour la détermination de l'origine des repas sanguins de *T. infestans* selon Lee *et al.* 2002

primer F	5'-CCCCTCAGAATGATATTTGTCCTCA-3'	355bp
primer R	5'-CCATCCAACATCTCAGCATGATGAAA-3'	

Broutin H, Tarrieu F, Tibayrenc M, Oury B, Barnabé C (2006) Phylogenetic analysis of the glucose-6-phosphate isomerase gene in *Trypanosoma cruzi*. Exp Parasitol 113: 1-7.

Fernandes O, Santos SS, Cupolillo E, Mendonca B, Derre R, et al. (2001) A mini-exon multiplex polymerase chain reaction to distinguish the major groups of *Trypanosoma cruzi* and *T. rangeli* in the Brazilian Amazon. Trans R Soc Trop Med Hyg 95: 97-99.

García BA, Zheng L, Pérez de Rosas AR, Segura EL (2004) Isolation and characterization of polymorphic microsatellite loci in the Chagas' disease vector *Triatoma infestans* (Hemiptera: Reduviidae). Molecular Ecology Notes, 4, 568–571.

Gaunt MW, Yeo M, Frame IA, Stothard JR, Carrasco HJ, et al. (2003) Mechanism of genetic exchange in American trypanosomes. Nature 421: 936-939.

Lee JH., Hassan H., Hill G., Cupp EW., Higazi TB., Mitchell CJ., Godsey Jr, M.S., Unnasch T.R., 2002. Identification of mosquito avian-derived blood meals by polymerase chain reaction-heteroduplex analysis. Am. J. Trop. Med. Hyg. 66, 599–604.

Lyman DF, Monteiro FA, Escalante AA, Cordon-Rosales C, Wesson DM, et al. (1999) Mitochondrial DNA sequence variation among triatomine vectors of Chagas' disease. Am J Trop Med Hyg 60: 377-386

Marcilla A, Bargues MD, Ramsey JM, Magallon-Gastelum E, Salazar-Schettino PM, et al. (2001) The ITS-2 of the nuclear rDNA as a molecular marker for populations, species, and phylogenetic

relationships in Triatominae (Hemiptera: Reduviidae), vectors of Chagas disease. Mol Phylogenet Evol 18: 136-142.

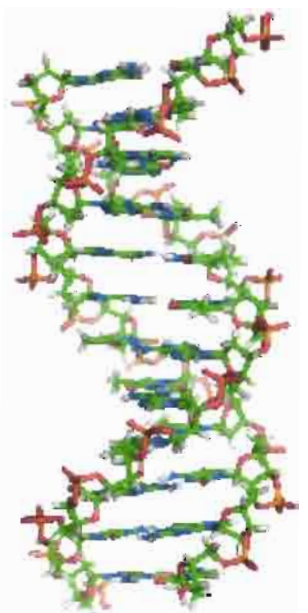
Oliveira RP, Broude NE, Macedo AM, Cantor CR, Smith CL, et al. (1998) Probing the genetic population structure of *Trypanosoma cruzi* with polymorphic microsatellites. Proceedings of the National Academy of Sciences USA 95: 3776-3780

Annexe 4

Manuel des protocoles utilisés dans le projet TiBo à l'intention du partenaire INLASA

MANUAL DE TECNICAS para la EPIDEMIOLOGIA MOLECULAR de la ENFERMEDAD DE CHAGAS Procesadas en INLASA 2008-2011

MF Bosseno-R Buitrago-R Salas-C Barnabé y SF Brenière



SUMARIO

1 - EXTRACCIONES DE ADN

EXTRACCIÓN DE ADN DE PATAS DE TRIATOMINOS

Extracción con CTAB

El material tiene que ser enjuagado (tijeras y pinzas) con lavandina al 10% y después con agua desmineralizada después de procesar cada muestra.

La extracción se procesa con insectos enteros para los 1er y 2do estadios y con 3 patas para 3ro, 4to, 5to estadios y adultos.

1. Cortar las patas en pequeños fragmentos y ponerlas en un microtubo, añadir 200 µl de CTAB al 2%.
2. Añadir 20 µl de proteinasa K a 20 mg/ml y incubar la solución en baño maría a 37ºC durante toda la noche.
3. Añadir 200 µl de cloroformo, mezclar por inversión.
4. Centrifugar 5 min a 12000 rpm.
5. Recuperar el sobrenadante y ponerlo en otro tubo.
6. Añadir 200 µl de isopropanol al sobrenadante, mezclar por inversión.
7. Centrifugar 15 min a 12000 rpm.
8. Vaciar el isopropanol, escurrir bien y añadir 200 µl de etanol al 70%.
9. Centrifugar 5 min a 12000 rpm.
10. Vaciar el alcohol.
11. Secar el sedimento (con el tubo abierto en el mesón o en la estufa a 30ºC)
12. Una vez que el sedimento este bien seco, añadir 20 µl de agua bi-destilada, dejar que se solubilice el ADN dejando el tubo toda la noche a temperatura del laboratorio.

Preparación del buffer CTAB 2%

		Concentración final
Tris-HCl 1M pH 8.0	100 ml	100 mM final
0.5 M EDTA	20 ml	10 mM
NaCL	81.4 g	1.4 M
CTAB*	20 g	2%
H2O	Hasta completar 1 Lt	

* Cetyl trimethyl ammonium bromide

Mezclar con agitador magnético y conservar a temperatura ambiente

Cuantificación del ADN:

Se procesa la lectura al espectrofotometro donde se puede medir la densidad óptica (DO) a partir de un volumen mínimo de 20 µl de ADN: diluir el ADN a 2 % sea 2 µl de ADN + 98 µl de agua y leer a 260 y 280 nm.

Información general: Para cuantificar la cantidad de ADN, las lecturas deben ser tomadas en longitudes de onda de 260 nm y 280 nm. La lectura a 260 nm permite el cálculo de la concentración de ácidos nucleícos en la muestra. Una DO de 1, corresponde a 50 mg / ml de ADN de doble cadena. La relación entre las lecturas a 260 nm y 280 nm (DO260/DO280) proporciona una estimación de la pureza del ADN: ratio igual o superior a 1,8 = ADN puro (Maniatis et al., 1989).

Para visualizar el estado del ADN se puede hacer una electroforesis de las muestras en un gel de agarosa al 1%: cargar 1 µl de ADN puro. Se visualiza una banda de alto peso molecular, si esta banda es tenue o inexistente entonces se harán las PCR con mas cuantidad de muestra.

EXTRACCIÓN DE ADN DE FUENTES ALIMENTICIAS DE TRIATOMINOS

EXTRACCIÓN CON EL KIT QUIAGEN MODIFICADA

DNeasy Blood and Tissue Kit (N° 69504): cf Manual p. 25; Adaptado para purificaciones de ADN total de sangre y de células animales.

1- Estimar el volumen de la muestra (fuente).

2- Ajustar el volumen a 200 µl con PBS.

3- Añadir 20 µl de Proteinasa K

4- Añadir 200 µl de buffer AL, mezclar con vortex e incubar a 56°C por 10 min.

5- Añadir 200 µl de etanol absoluto, mezclar por inversión.

6- Poner toda la mezcla en una columna que debe estar dentro de un tubo de colección de 2 ml.

7- Centrifugar a 8000 rpm, por 1 min, descartar el eluído y el tubo (el ADN esta en el filtro de la columna)

8- Colocar la columna en un nuevo tubo de colección y añadir 500 µl de buffer AW1.

9- Centrifugar a 8000 rpm, por 1 min, descartar el fluido y el tubo (el ADN esta en el filtro de la columna).

10- Colocar la columna en un nuevo tubo de colección y añadir 500 µl de buffer AW2.

11- Centrifugar por 4 min a 14000 rpm.

12- Remover cuidadosamente la columna, evitando todo contacto con el eluído.

13- Colocar la columna en un nuevo tubo de colección y destapar por 5 min.

14- Añadir agua bi-destilada (50 µl para un volumen de 100 µl de muestra) para eluir el ADN.

15- Incubar por 5 min a temperatura ambiente.

16- Centrifugar a 8000 rpm, por 1 minuto (el eluído obtenido es el extracto de DNA).

EXTRACCIÓN CON EL

KIT PROMEGA MODIFICADO

Wizard Genomic DNA Purification Kit (N° A1120 Promega): Adaptado para purificaciones de ADN total de sangre, levaduras, bacterias y tejidos.

Estimar la cuantidad de la muestra (fuente) y aforar hasta 100 µl con PBS, bien homogeneizar con vortex.

1- Añadir 300 µl de la solución de lisis **CELL.**

2- Mezclar por inversion.

3- Incubar 10 min al baño maria a 60°C.

4- Centrifugar 1 min en la microcentrifugadora.

5- Descartar el sobrenadante.

6- Vortersar el sediment.

7- Añadir 100 µl de la solución **NUCLEI.**

8- Mezclar por inversión

9- Añadir 33 µl de la **SOLUCION PRECIPITATION PROTEINAS** y mezclar con vortex 1 min.

10- Centrifugar 3 min

11- Transferir el sobrenadante en un tubo nuevo y añadir 100 µl de **ISOPROPANOL**, mezclar por inversión

12- Centrifugar 5 min.

13- Descartar el sobrenadante. Añadir 100 µl de **ETANOL 70%** .

14- Centrifugar 5 min.

15- Aspirar el etanol con una punta delgada cuidando de no aspirar el sedimento y dejar secar el sedimento a la temperatura ambiental (10-15 min).

16- Resuspender el sedimento con la **SOLUCION DE REHIDRATACION** durante 1 hora a 65°C o toda la noche a 4°C.

<u>Volúmenes de solución de rehidratación a utilizar:</u>

Si muestra < 10 µl ---------- ---------→ 10 µl
Si muestra < 50 µl ----------→ 20 µl
Si muestra > 50 µl < 100 µl ---------→ 4 0 µl
Si muestra > 100 µl ----------→ 50 µl

EXTRACCIÓN DE ADN TOTAL (GENÓMICO Y KDNA) DE SEDIMENTOS DE PARÁSITOS (*T. CRUZI*) DE CULTIVO CON CTAB

Las formas epimastigotes del cultivo (LIT) fueron recuperadas por centrifugación y se tomo el peso del sedimento antes de congelarlo.

A 20-100 mg de peso húmedo de parásitos se añade 600 µl solución de CTAB (ver abajo). De preferencia no se extrae pesos menores a 20 mg.

Agregar a la mezcla 200 µg / ml de proteinasa K, y incubar 2h a 56°C o a 37°C durante la noche.

Técnica de extracción:

1 - aumento de V / V la solución (A): cloroformo (24 V) y alcohol isoamílico (1 V).
2 - Mezclar suavemente por inversión, no utilizar el vortex.
3- centrifugar 10 min 12000 rpm.
4 - recuperar la fase superior y transferir a un tubo limpio y registrar el volumen.
5 - Añadir el mismo volumen de solución (A), mezclar invirtiendo varias veces.
6- Centrifugar 10 min 12000 rpm.
7 - Recuperar la fase acuosa (superior) medir cuidadosamente el volumen y añadir 1 / 10 V acetato de sodio 3M y mezclar invirtiendo el tubo.
8 - Añadir 2,5 V de alcohol absoluto frío (tubo en el congelador).
9 - Agite el tubo suavemente por inversión, vemos que el ADN precipita, coloque el tubo en hielo 15 min.
10 - Centrifugar durante 30 min a 12000 rpm.
11 - Eliminar el sobrenadante, el ADN se adhiere al tubo.
12 - Lavar el ADN con alcohol frío al 70%, añadir aproximadamente 500 µl y sacarlo enseguida suavemente.
13 - Secar el ADN a la temperatura del laboratorio.
14 - Re suspender el ADN en 100-300 µl de H2O destilada según el peso del sedimento inicial de la muestra.
15 – Cuantificación del ADN por espectrofotometría después de de una dilución de la muestra 1/100, con longitud de ondas siguientes: 260, 280, 320 nm.
Interpretación: Un ratio de las densidades ópticas DO260 nm / DO280 nm < 1.8, todavía hay proteínas en el ADN, si ratio > 2 hay presencia de ARN.
Une DO de valor 1 a 260 nm corresponde a una solución de 50 mg / ml de ADN doble cadena.

Preparación de la solución de CTAB 2%

Tris-HCL1MpH8.0	100ml	100mM final
0.5M EDTA	20ml	10mM final
NaCL	81.4g	1.4M final
CTAB*	20g	2% final
H2Oqsp	1litre	

Mezclar la solución con agitador magnético. La solución se conserva a temperatura ambiente.

*Cetyl trimethyl ammonium bromide.

2 - AMPLIFICACIONES POR PCR DE GENES

ITS-2

Las secuencias de los cebadores son:

Forward : 5'-CTA-AGC-GGT-GGA-TCA-CTC-GG-3'

Reverse : 5'-GCA-CTA-TCA-AGC-AAC-ACG-ACT-C-3'

Según Marcilla et al. (2001), The ITS-2 of the nuclear rDNA as a molecular marker for populations, species, and phylogenetic relationships in Triatominae (Hemiptera: Reduviidae), vectors of Chagas disease. Mol. Phylogenet. Evol. 18, 136–142.

El tamaño del amplificado es de 827 pb.

1 - Condiciones de amplificación con el Master Mix (solución de amplificación PCR completa; Promega, N° catalogo M7502)

	Concentración madre	Concentración Final	volumen/ tubo (µl)
Master Mix	2X	1X	15
Cebador Forward ITS-2	10 µM	0.4 µM	1.2
Cebador Reverse ITS-2	10 µM	0.4 µM	1.2
H2O			11.6
DNA	100 ng/µl		1

El volumen total de la reacción es de 30 µl

2 - Condiciones de amplificación con la polimerasa GoTaq® Flexi DNA Polymerase (Promega N° catalogo M829)

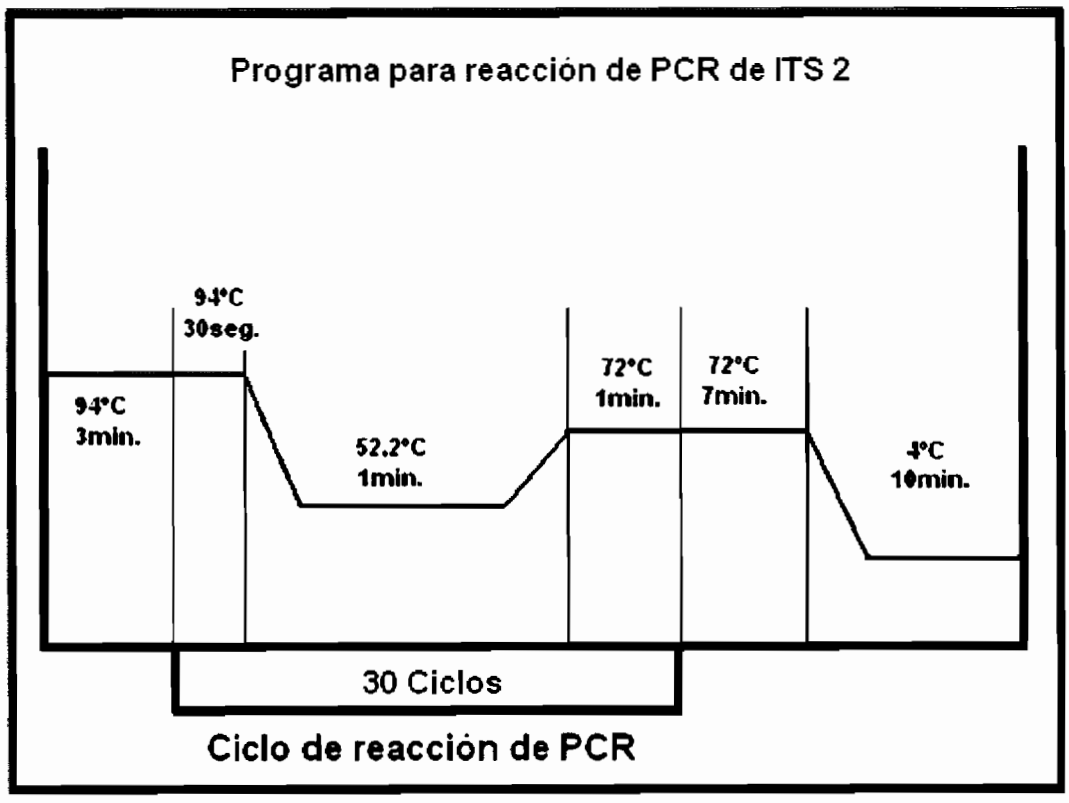
	Concentración madre	Concentración Final	volumen/ tubo (µl)
H2O			16,95
Buffer	5X	1X	6
MgCl2	25 mM	2,5 mM	3
dNTPs	10 mM each	0,16 mM each	0,5
Cebador Forward ITS-2	10 µM	0.4 µM	1.2
Cebador Reverse ITS-2	10 µM	0.4 µM	1.2
GoTaq	5 U/µl	0,025 U/µl	0,15
DNA	100 ng/µl		1

El volumen total de la reacción es de 30 µl.

3 - Condiciones de amplificación con la polimerasa Taq DNA (Promega N° catalogo M1861)

	Concentración Inicial	Concentración Final	VOLUMEN/ TUBO [µl]
H_2O			19,8
Buffer	10X	1X	3
MgCl2	25 mM	2,5 mM	3
dNTPs	10 mM each	0,16 mM each	0,5
Cebador Forward ITS-2	10 µM	0,4 µM	1.2
Cebador Reverse ITS-2	10 µM	0,4 µM	1.2
Taq	5 U/µl	0,05 U/µl	0,3
DNA	100 ng/µl		1

El volumen total de la reacción es de 30 µl



Citocromo b

Las secuencias de los cebadores son:

Forward: 5'-GGA-CAA-ATA-TCA-TGA-GGA-GCA-ACA-G-3'

Reverse: 5'-ATT-ACT-CCT-CCT-AGC-TTA-TTA-GGA-ATT-G-3'

Secuencias modificadas Según Lyman et al. 1999, Mitochondrial DNA sequence variation among triatomine vectors of Chagas' disease. Am. J. Trop. Med. Hyg. 60, 377–386.

El tamaño del amplificado es de 428 pb.

1 - Condiciones de amplificación con el Master Mix (Promega, N° catalogo M7502)

	Concentración madre	Concentración Final	volumen tubo (µl)
Master Mix	2X	1X	15
Cebador Forward Cit b	10 µM	0,4 µM	1,2
Cebador Reverse Cit b	10 µM	0,4 µM	1,2
H2O			11,6
DNA	100 ng/µl		1

El volumen total de la reacción es de 30 µl

2 - Condiciones de amplificación con la polimerasa GoTaq® Flexi DNA Polymerase (Promega N° catalogo M829)

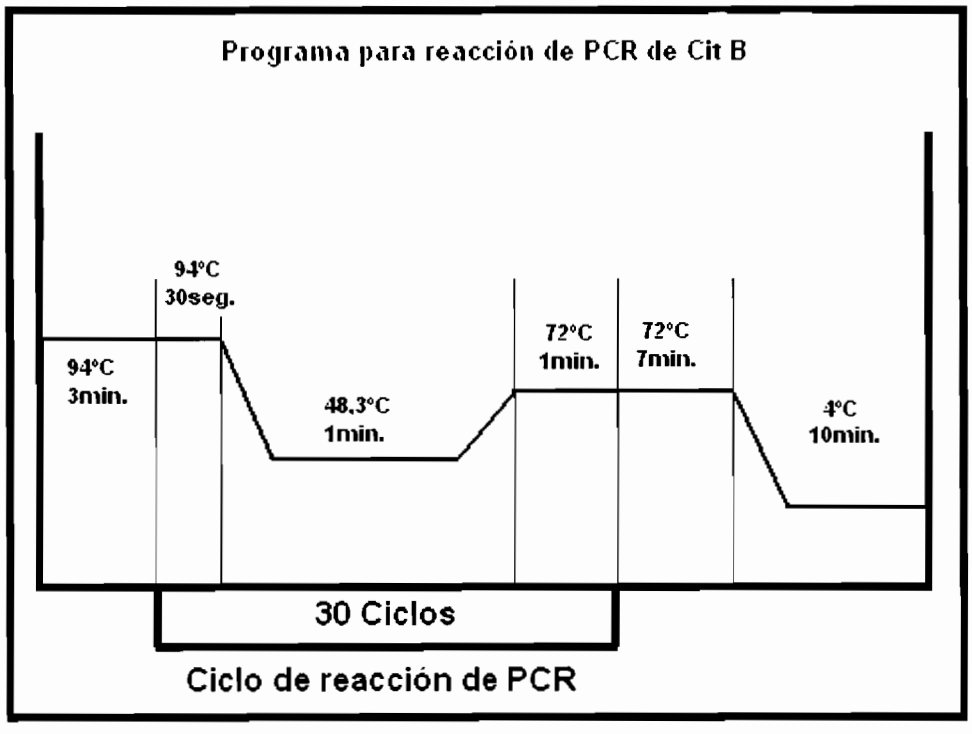
	Concentración madre	Concentración Final	volumen/ tubo (µl)
H2O			16,95
Buffer	5X	1X	6
MgCl2	25 mM	2,5 mM	3
dNTPs	10 mM each	0,16 mM each	0,5
Cebador Forward Cit b	10 µM	0,4 µM	1.2
Cebador Reverse Cit b	10 µM	0,4 µM	1.2
GoTaq	5 U/µl	0,025 U/µl	0,15
DNA	100 ng/µl		1

El volumen total de la reacción es de 30 µl

3 - Condiciones de amplificación con la polimerasa Taq DNA (Promega N° catalogo M1861)

	Concentración madre	Concentración Final	volumen/ tubo (µl)
H2O			19,8
Buffer	10X	1X	3
MgCl2	25 mM	2,5 mM	3
dNTPs	10 mM each	0,16 mM each	0,5
Cebador Forward Cit b	10 µM	0,4 µM	1.2
Cebador Reverse Cit b	10 µM	0,4 µM	1.2
Taq	5 U/µl	0,05 U/µl	0,3
DNA	100 ng/µl		1

El volumen total de la reacción es de 30 µl.



REGIÓN INTERGÉNICA DEL GEN MINIEXON DE *TRYPANOSOMA CRUZI* (TIPIFICACIÓN RÁPIDA)

Referirse a las publicaciones de:

Fernandes et al 2001. A mini-exon multiplex polymerase chain reaction to distinguish the major groups of Trypanosoma cruzi *and* T. rangeli *in the Brazilian Amazon.Trans.R.Soc. Trop. Med.Hyg.95,97–99 y*

Aliaga et al 2011, Further interest of miniexon multiplex PCR for a rapid typing of Trypanosoma cruzi *DTU groups. Infect Genet Evol. 11(5):1155-8.*

Las secuencias de los cebadores son:

TC1OF	Tamaños esperados de los fragmentos
ACACTTTCTGTGGCGCTGATCG	TC1OF-MEOF = 200 bp
TC2OF	
TTGCTCGCACACTCGGCTGCAT	TC2OF-MEOF = 250 bp
TC3OF	
CCGCGWACAACCCCTMATAAAAATG	TC3OF-MEOF = 150 bp
TROF	
CCTATTGTGATCCCCATCTTCG	TROF-MEOF = 100 bp
MEOF	
TACCAATATAGTACAGAAACTG	

1 - Condiciones de amplificación con el Master Mix (Promega, N° catalogo M7502)

	Sol. madre	Con. Final Vol./tubo
Master Mix	2X	1X 12,5 µl
Cada cebador 10 µM		5 µl
H_2O		2,5 µl
ADN		5 µl

El volumen total de reacción es de 25 µl

Los cebadores llegan liofilizados y para conservarlos a largo plazo es mejor re constituirlos a una concentración de 1 mM, luego se hace un Mix con cada cebador a 10 µM cada uno.

2 - Condiciones de amplificación con Taq Promega

	Solución madre	Solución final	Volumen/ tubo (µl)
H2O			13,25
Buffer	10 X	1 X	2,5
MgCl2	25 mM	1,5 mM	1,5
dNTP's	10 mM	0,2 mM	0,5
Cebador 1	10 µM	0,4 µM	1
Cebador 2	10 µM	0,4 µM	1
Cebador 3	10 µM	0,4 µM	1

Cebador 4	10 µM	0,4 µM	1
Cebador 5	10 µM	0,4 µM	1
Taq Promega	5 U/µl	0,05U/µl	0,25
DNA			2

El volumen total de reacción es de 25 µl

Programa de amplificación

1 etapa inicial: 94°C 5 min
35 ciclos

94°C 30 sec

50°C 30 sec

72°C 30 sec

1 etapa final de extensión: 72°C por 7 min; 4°C por 20 min.

GEN NUCLEAR GPI (GLUCOSA FOSFATASA DESHIDROGENASA) DE *T. CRUZI*

Cebadores originales definidos por C. Barnabé et al.

Cebador 1, GPI-L: CGCCATGTTGTGAATATTGG

Cebador é, GPI-R: TTCCATTGCTTTCCATGTCA

Tamaño del fragmento esperado: 652 bp incluyendo los primers y 612 sin los primers

	Solución madre	Solución final	Volumen/ tubo (µl)
Buffer 10X	10 X	1 X	5
dNTP's	5 mM	50 µM	1,5
Cebador 1	10 µM	0,2 µM	1
Cebador 2	10 µM	0,2 µM	1
Taq Roche	5 UI/µl	1UI	0,2
H2O			41,3
DNA	50 ng/µl		2

El volumen total de reacción es de 50 µl

Programa de amplificación

1 etapa inicial: 94°C 1 min
35 ciclos

94°C 1 mn

55°C 1 mn

72°C 1mn

1 etapa final de extensión: 72°C por 5 min; 4°C por 20 min.

GEN CITOCROMO B DE REPTILES, AVES Y MAMÍFEROS A PARTIR DE FUENTES ALIMENTICIAS DE TRIATOMINOS PARA LA DETERMINACIÓN DEL ORIGEN DE FUENTES ALIMENTICIAS,

Principio: se amplifica a partir de un extracto de ADN de la fuente alimenticia de un triatomino el citocromo b con cebadores apropiados; para mas informaciones consultar el artículo : *Buitrago et al, 2011, Combination of cytochrome b heteroduplex-assay and sequencing for identification of triatomine blood meals, Infect Genet Evol, 2011 Sep 22, [Epub ahead of print] PubMed PMID: 21963963,*

Las secuencias de los cebadores son según: *Lee et al, 2002, Identification of mosquito Avian-derived blood meals by Polymerase chain reaction-heteroduplex analysis, Am, J, Trop, Med, Hyg, 66, 599-604,*

Cebador forward: F-5'CCCCTCAGAATGATATTTGTCCTCA3'
Cebador reverse: R-5'-CCATCCAACATCTCAGCATGATGAAA3'

Condiciones de amplificación con la polimerasa Taq Promega

	Solución madre	Solución final	Volumen/ tubo (µl)
H2O			32,5
Buffer	10 X	1 X	5
Cl2 Mg	25 mM	2,5 mM	5
dNTP's	10 mM	0,2 mM	1
Cebador 1	10 µM	0,4 µM	2
Cebador 2	10 µM	0,4 µM	2
Taq Promega	5 U/µl	0,05U/µl	0,5
DNA	No conocida		2

El volumen total de reacción es de 50 µl

El tamaño del fragmento es de 355 pb,

3 - AMPLIFICACIONES DE LOCI MICROSATELITES

PARA TRIATOMINOS

Principios: Los microsatélites se amplifican por PCR clásicas con un par de cebadores, el cebador "forward" de la PCR está marcado con fluorescencia con la denominación siguiente:

- 6-FAM = Azul
- NED = amarillo (aparece negro en los cromatogramas)
- VIC = Verde
- PET = Rojo

Para poder determinar los tamaños de los alelo, se añade un marcador de peso molecular llamado Genescan 500 LIZ de color naranja. Cuando el fragmento PCR pasa por el lector laser con su fluorocromo, el laser detecta la señal del fragmento como la señal del peso molecular, así se puede medir la dimensión exacta del fragmento PCR MLMT (multilocus microsatellite typing),

Condiciones de amplificación con la polimerasa Taq ROCHE (N° 11435094001)

	Concentración madre	Concentración Final	volumen tubo [µl]
H_2O			17,3 µl
Buffer	10X	1X	2,0 µl
dNTPs	10 mM	50 µM	0,1 µl
Cebador Forward	10 µM	0,1 µM	0,2 µl
Cebador Reverse	10 µM	0,1 µM	0,2 µl
GoTaq	5 UI/µl	1 UI/µl	0,2 µl
ADN	20 ng/µl		1,0 µl

Para 21 µl de reacción

Los productos de PCR están visualizados en electroforesis de agarosa 1,5% y están diluidos de 1 a 10 para se analizados por el secuenciador según la intensidad del señal.

Se prepara una solución de Hidi (formamide) + GeneScan 500 LIZ: 12 µl GeneScan para 1 ml de HiDi.

1 µl de la solución diluida de productos PCR esta añadida a 16 µl de la mezcla HiDi+GeneScan y esas soluciones están colocadas en placas PCR especiales para ser analizadas en un secuenciador.

Etapa 2 : Lectura del tamaño de los alelos

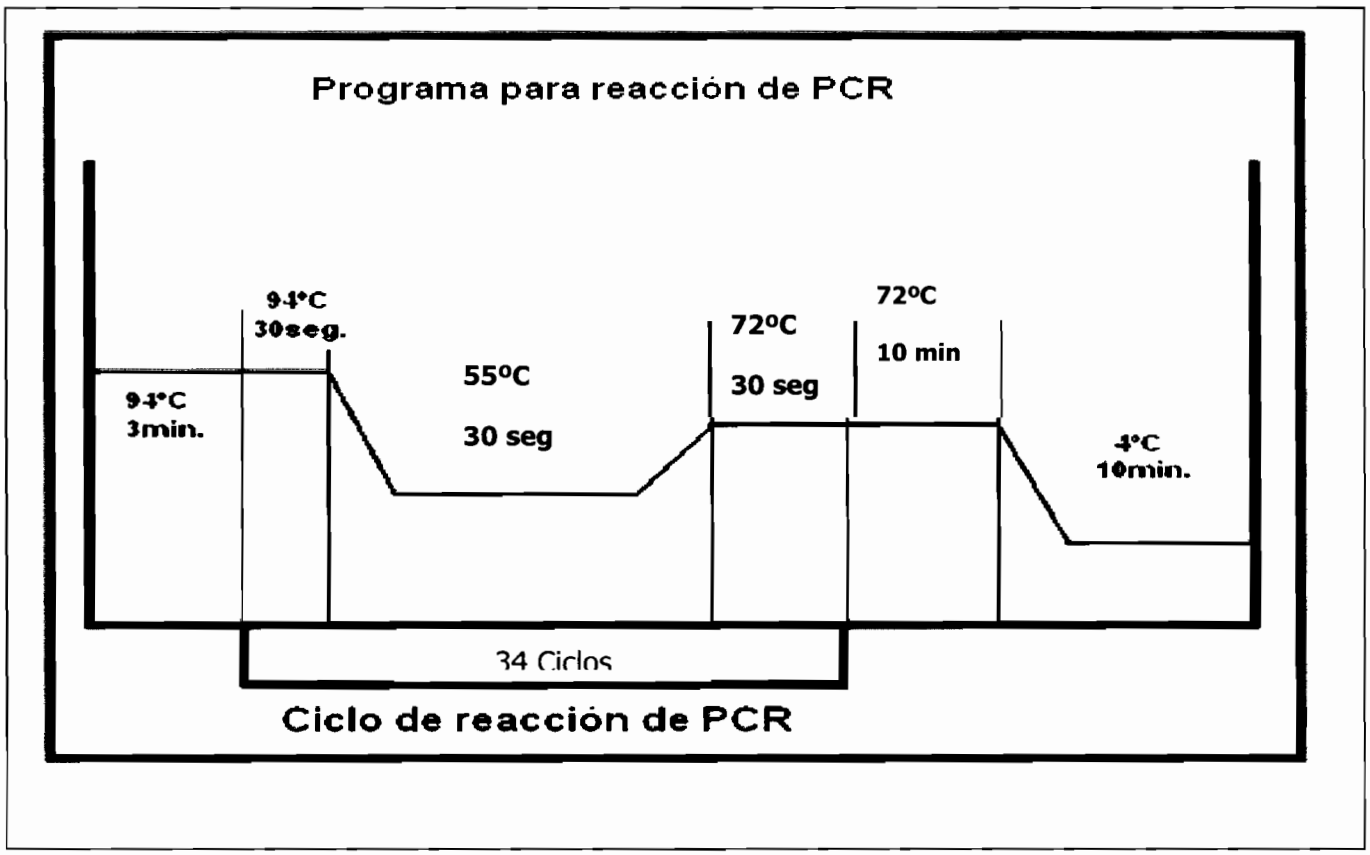
Es la etapa que utiliza el programa Genemapper (ver su manual especifico)

Referencia:

Garcia B. A., Zheng L., Perez de Rosas A. R., and Segura E. L., (2004). Isolation and characterization of polymorphic microsatellite loci in the Chagas disease vector Triatoma infestans (Hemiptera: Reduviidae). Molecular Ecology Notes pp 568-571.

Name	Sequence	Size Range of PCR Products (bp)
TiA02	GGAAACTCATGTTATGGACACG	171-226
	AAACCTTATTGTTAGTTCGTTTTGG	
TiC02	CTCTGGGGATCATCGTTCTG	157-211
	TTTAGGATTCATACCGCCTTT	
TiC09	TTTGCCACATTTACCATTTCC	135-159
	TCAAGAGAAGCCGTCCAACT	
TiD09	TGGACATAAGCCCCCTGTAA	188-230
	GGATCCTACTGTGCGGATGT	
TiE02	AGCACGGTTTGCAACTTTTC	147-167
	TGTGGAATTGAAGGAGCACA	
TiE12	CCTTTAATTTCCCTTTGCCATC	301-323
	CCTACACGAAATGCCCAAGT	
TiF03	AAAATGGCGGACAAACATTC	162-216
	TTCCTCAACACAAACACAAACC	
TiF11	TCAATAAAACGAAAATGCGACTT	197-239
	GCGGGATCTAAGCCAACAGT	
TiG03	CGGAAATGCAAAATTTTTTAGGCG	200-254
	ATTCTGATCGTGGCAATTTT	

Programación de los ciclos



PARA *TRYPANOSOMA CRUZI*

Principios: Los microsatélites se amplifican por PCR clásicas con un par de cebadores. El cebador "forward" de la PCR está marcado con fluorescencia con la denominación siguiente:

- 6-FAM = Azul
- NED = amarillo (aparece negro en los cromatogramas)
- VIC = Verde
- PET = Rojo

Para poder determinar los tamaños de los alelos, se añade un marcador de peso molecular llamado Genescan 500 LIZ de color naranja. Cuando el fragmento PCR pasa por el lector laser con su fluorocromo, el laser detecta la señal del fragmento como la señal del peso molecular, así se puede medir la dimensión exacta del fragmento PCR MLMT (multilocus microsatellite typing),

Protocolo MLMT

Se puede dividir en tres etapas principales

Etapa 1: PCR MLMT para el locus microsatelite SCLE10 por ejemplo (se aplica el mismo protocolo para los otros cebadores)

Productos	[] sol madre	[] por tubo	Volumen / tubo (µl)
Buffer 10x	10x	-	2
dNTP	10 mM	50 µM	0,1
SCLE10-fluo	10 µM	0,1 µM	0,2
SCLE10-R	10 µM	0,1 µM	0,2
Taq	5UI/µl	1 UI	0,2
H_20 qsp	20 µl -	-	17,3
ADN 20 ng/µl		2	

Los productos de PCR están visualizados en electroforesis de agarosa 1,5% y están diluidos de 1 a 10 para se analizados por el secuenciador según la intensidad del señal.

Se prepara una solución de Hidi (formamide) + GeneScan 500 LIZ: 12 µl GeneScan para 1 ml de HiDi.

1 µl de la solución diluida de productos PCR esta añadida a 16 µl de la mezcla HiDi+GeneScan y esas soluciones están colocadas en placas PCR especiales para ser analizadas en un secuenciador.

Etapa 2 : Lectura del tamaño de los alelos

Es la etapa que utiliza el programa Genemapper (ver su manual especifico)

Etapa 3 : Explotación de los datos

Ejemplo de matriz obtenida, la primera columna son los nombres de las cepas de *T. cruzi*, la secunda de las poblaciones y las siguientes representan los genotipos MLMT, por ejemplo 128128 es un homocigota 128/128, 250254 es el heterocigoto 250/254 etc ...,

	pop	MCLE01	SCLE10	SCLE11 ...
cepa1	Arequipa	128128	250254	140140
cepa2	Arequipa	128128	250254	140140
cepa3	Cbba	128128	250254	140140
cepa4	Cbba	128128	250254	140142 ...

En general, se usa el programa Fstat para obtener los resultados de tipo, Fis, Fst, desequilibrio de ligamiento, equilibrio de HW, diferentes pruebas etc ...

Los cebadores utilizados para *T. cruzi* son actualmente:

CODIGO	Cebador 1	Cebador 2
MCLE01*	CTGCCATGTTTGATCCCT	CGTGTACATATCGGCAGTG
MCLE08*	ATGGACAACAAATGGGAG	TGGGTATGCCAAATGTGAT
SCLE10*	GATCCCGCAATAGGAAAC	GTGCATGTTCCATGGCTT
SCLE11*	ACGACCAAAGCCATCATT	GATGCTAACTGCTCAAGTGA
MCLF10*	GCGTAGCGATTCATTTCC	ATCCGCTACCACTATCCAC
MCLG10*	AGGAGTCAAATATAATGAGGCA	ACGTGTGAAAGGCATCTATC
MCL03*	GGAGCAAGAATGAAGGCA	TCAGAAAAAGCACGCCTC
MCL05*	TTAAACGACCTCTATGTCTCTC	CCTGAGCAAGATACAAGGAC
A427**	ACGCGCGTTACTTGTGGTAT	CCAAATATGCATGTGTTTGGA
C875**	CCATGTCGACTCCATGTCTC	TTGTTGCTGTTGTTGGCAAT

*Oliveira, Broude et al, (1998); **Gaunt, Yeo et al, 2003

5 - ANALISIS ELECTROFORETICO DE HETERODUPLEX DEL CITOCROMO B

IDENTIFICACION DE FUENTES ALIMENTICIAS POR HETERODUPLEX

Los heteroduplex son moléculas hibridas de DNA cuyas cadenas simples provienen de dos diferentes especies: una, de la fuente alimenticia que se quiere identificar (muestra) y la otra, de un ADN conocido denominado "driver", estas moléculas usualmente migran en un campo eléctrico de manera diferenciada dependiendo del número, tipo y posición de los lugares no homólogos (los "mismatches") presentes en la molécula, la migración electroforética es específica de la combinación de la especie 1 con la especie 2.

FORMACION DE HETERODUPLEX

Una vez realizada la reacción en cadena de la polimerasa del gen citocromo b de las fuentes alimenticias, proceder de la siguiente manera:

1. En un tubo de 0,2 ml, poner 5 µl de producto de PCR de la muestra
2. Añadir 5 µl de producto de PCR de un ADN conocido (driver)
3. Añadir 8 µl de agua destilada
4. Poner el tubo en el termociclador

Programa del termociclador para que se formen los heteroduplex:

94°C	5 min.
28°C	60 min.
4°C	10 min.

Con los heteroduplex formados, hacer una corrida electroforética en gel de acrilamida.

ELECTROFORESIS EN GEL DE ACRILAMIDA

La acrilamida es un neurotóxico potente que debe ser manipulado con precaución, usando guantes y barbijo.

Condiciones de corrida:

Concentración del gel	5 %
Tamaño del gel	10 x 10 x 0,2 cm
Tampón de disolución	TBE 1X
Voltaje	100v
Amperaje	30 A
Tiempo de corrida	3 horas

Preparación de los geles (2)

Reactivo	Volumen
Acrilamida/bis-acrilamida 40% 29/1	2,5 ml
TBE 10X	2 ml
Agua destilada	15,4 ml
APS (persulfato de amonio)10%	140 µl (la solución guardar congelada)
TEMED	6 µl

Se prepara colocando el TEMED al final de todos los componentes, mezclar con vortex y vaciar 10 ml de la mezcla en cada "cassette", posteriormente colocar los peines, cuidando que no haya burbujas y dejar polimerizar por 30 minutos.

Para sembrar las muestras, mezclar los 18 µl del tubo muestra con 2 µl de EZ-Vision™ buffer y sembrar en cada pozo el volumen total.

INTERPRETACION DE LOS PERFILES

En el gel se observa 3 tipos de bandas:

1. las bandas más rápidas que corresponden a las cadenas de DNA homodúplex (cadenas homólogas).
2. Las bandas más lentas de alrededor de 1000 pares de bases que están formadas por cadenas simples de DNA.
3. Entre estos dos tipos de bandas están las dos moléculas heterodúplex

Tres tipos de perfiles de heteroduplex se pueden observar:

(i) Un perfil básico de dos bandas correspondientes a la formación de dos cadenas de ADN heterodúplex, cuando el triatomino ha comido de un solo huésped.

(ii) Un perfil más complejo (múltiples bandas), cuando los triatominos tiene múltiples comidas sanguíneas (comieron de más de un huésped).

(iii) Ausencia de formación de heteroduplex , en este caso puede tratarse de la misma especie del driver, por lo que se forma solo homoduplex y no heteroduplex, usar otro driver y comparar el perfil formado de la muestra con este nuevo driver con el perfil formado entre el primer driver y el segundo driver, si son iguales se trata de la misma especie del primer driver.

La identificación de las fuentes alimenticias se realiza por comparación de perfiles de heteroduplex, con perfiles conocidos

TAMPÓN TBE	
Tris base	108 g
Acido bórico	55 g
EDTA, 2H2O	9,3 g
Agua destiladas q.s.p.	1 litro

Annexe 5

Distribution géographique des populations sauvages de *T. infestans* en Bolivie

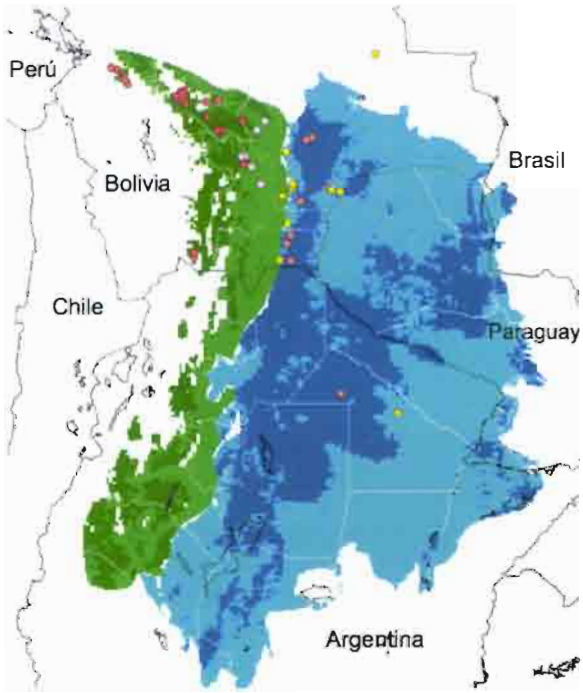
La recherche systématisée des populations de *T. infestans* sylvestres dans 7 écorégions de Bolivie montre clairement que ces populations se répartissent principalement dans deux écorégions, Bosques Secos Interandinos (BSIA, 36% des sites positifs) et Gran chaco (GC, 40% des sites positifs) (annexe 6, tableau 1). Ceci n'exclu pas d'autres écorégions en particulier celle de la Prepuna (PP), au sud de la Bolivie, de plus haute altitude que BSIA, où plusieurs sites sont positifs (annexe 6, tableau 1 et 2). Par ailleurs un seul *T. infestans* a été trouvé dans l'écorégion « Bosque Tucumano Boliviano» (BTB) intermédiaire entre les Andes et les basses terres du Chaco, cependant l'effort de recherche a été moindre dans cette écorégion que pour BSIA et GC. Les résultats montrent aussi nettement que les colonies sont plus grandes et plus abondantes dans le BSIA que dans le GC où très peu de spécimens sont généralement capturés sur un même piège (1-2). En marge des captures de *T. infestans*, beaucoup d'exemplaires du complexe *T. sordida* ont été capturés dans BSIA et GC mais plus fréquemment dans le GC. L'ensemble des activités de recherche sur le terrain ont permis d'identifier un total de 60 nouveaux sites avec *T. infestans.* Quelques curiosités sont à relever : (i) dans le site de Mataral (tableau 2) qui présente aussi des caractéristiques de l'écorégion du Chaco Cerrano (CS), 3 espèces ont été capturée sur une aire d'environ 5000 m^2 mais dans des écotopes différents , *T. infestans* dans un affleurements rocheux, *T. guasayana* dans des bromélias et *T. breyeri,* espèce non encore décrite en Bolivie, dans un pierrier. Une autre espèce *Triatoma costalimai* a été trouvée pour la première fois dans l'écorégion de Bosque Seco Chiquitano (BSC). *T.* costalimai est décrite comme endémique dans le biome du Cerrado (Brésil) comme une espèce synanthropique, étant donné qu'elle a été capturée en péri domicile dans l'état de Goais.

Figure 1. Distribution des sites sylvestres où des triatomes ont été trouvés par piégeage. En vert sites négatifs, en rouges sites avec T. infestans, *en rose sites avec* T. infestans *et espèces du complexe* T. sordida, *en bleu espèces du complexe* T. sordida.

Les captures ont été intenses autour des aires partagées choisies (Sapini, Thago Thago, Quillacollo, Rancho Nuevo et San Silvestre). Dans les trois premières aires les *T. infestans* sont facilement capturés à proximité des villages (colonies abondantes d'adultes et de nymphes) alors que *T. infestans* n'a pas été trouvé dans les environs du village de Rancho Nuevo. Cependant, lors d'une dernière mission en 2011, des nymphes supposées être *T. infestans* capturées dans les environs de San Silvestre ont été mise en élevage et les adultes issus de cette colonie sont réellement des *T. infestans*.

Etant donné l'importante distribution de ces populations, l'hypothèse de leur interférence avec les programmes de contrôle des populations domestiques (ré-invasion des aires domestiques) est à examiner avec beaucoup d'attention.

Figure 2 - Première carte prédictive de la distribution des populations sauvages de T. infestans dans les pays du Cône Sud.



Gorla & Noireau. 2010. Geographic distribution of Triatominae vectors in America. In: Telleria, J.,Tibayrenc, M. (Eds.), American trypanosomiasis Chagas disease. One hundred years of research, Elsevier, Burlington, USA. pp: 209-231.

Tableau 1: Localisation des zones de piégeage du protocole TiboEco et résultats des captures: capture de *T. infestans* en rouge, complexe *T. sordida* en bleu, *T. infestans* + *T. sordida* en rose, autres espèces ou espèce non déterminée en jaune, absence de capture en vert.

N° Ecorégion*	Ecorégion	Code	N° de zone de capture	Date de capture	Village le plus proche	Département	Province	Municipalité	Altitude (m)	Latitude	Longitude	Résultat des captures	Nb de pièges posés**	Nb de piège positif	*Triatoma infestans*	Complexe *Triatoma sordida*	Autre espèce de triatome ou espèce non déterminée	Total
4	Bosque seco Chiquitano	BSC 01	1	mai-10	Guabira	Santa Cruz	Obispo Santiestevan	Montero 1ra seccion	285	17° 17' 55,5''	63° 14' 17,3''		48 + 7S	1	0	0	1	1
4		BSC 02	2	mai-10	San Julian	Santa Cruz	Nuflo de Chavez	San Julian 4ta seccion	251	16° 54' 04,6''	62° 35' 35,3''		48	0	0	0	0	0
4		BSC 03	3	mai-10	San Ramon	Santa Cruz	Nuflo de Chavez	San Ramon 3ra seccion	353	16° 36' 32,8''	62° 25' 31,5''		48	3	0	0	3	3
4		BSC 04	4	mai-10	La Banda	Santa Cruz	Guarayos	El Puente 3ra seccion	223	16° 22' 35,5''	62° 57' 35,6''		48 + 8S	0	0	0	0	0
5	Gran Chaco	GC 01	5	oct-09	La Quinta Caiza	Tarija	Gran Chaco	Yacuiba 1ra seccion	605	21° 49' 56,00''	63° 33' 15,20''		48	0	0	0	0	0
5		GC 02	6	oct-09	Cueva de Leon	Tarija	Gran Chaco	Villamontes 3ra seccion	347	21° 22' 37,00''	63°21' 34,80''		48 + 13S	5 + 3S	1 + 3S	4 + 1S	0	0
5		GC 03	7	nov-09	San Lorenzo	Santa Cruz	Cordillera	Charagua 2da seccion	479	19° 38' 54,30''	63° 06' 46,10''		48 + 15S	5 + 1S	1	5 + 1S	0	1
5		GC 04	8	nov-09	Estancia Basilio	Santa Cruz	Andres Ibanez	La Guardia	543	18° 06' 55,90''	63° 12' 57,50''		48	0	0	0	0	0
5		GC 05	9	nov-09	Sinai	Santa Cruz	Chiquitos	Pailon	282	17° 33' 44,64''	62° 39' 21,85''		48	3	0	2	2	4
6	Yungas	YU 02	10	avr-10	Machacamarca	La Paz	Sur Yungas	Coripata	1767	16° 20' 50,96''	67° 36' 41,65''		48	0	0	0	0	0
6		YU 03	11	avr-10	Mercedes	La Paz	Sur Yungas	La asunta	842	16° 17' 36,92	67° 20' 50,06		48	0	0	0	0	0
6		YU 01	12	avr-10	Siquilini	La Paz	Sur Yungas	Chulumani	1856	16° 25' 27,08''	67° 32' 13,81''		48	0	0	0	0	0
7	Bosque Tucumano Boliviano	BTB 01	13	mai-09	Chiquiaca	Tarija	Burnet O'Connor	Entre Rios	891	21° 49' 50,4''	64° 07' 40,0''		48 + 20S	0	0	0	0	0
7		BTB 02	14	mai-09	San Josecito	Tarija	Burnet O'Connor	Entre Rios	971	21° 11' 13,5''	64° 13' 42,0''		48 + 20S	0	0	0	0	0
7		BTB 03	15	mai-09	Leon Cancha	Tarija	Mendez	San Lorenzo	2670	21° 10' 45,30''	64° 42' 50,10''		48	0	0	0	0	0
7		BTB 04	16	mai-09	La Cuevas	Chuquisaca	Azurduy	Azurduy	1170	19° 59' 37,90''	64° 26' 15,70''		48 + 11S	0	0	0	0	0
7		BTB 05	17	mars-09	Cerrillos	Chuquisaca	Hernando Siles	Monteagudo 1ra seccion	1049	19° 55' 39,20''	63° 54' 8,80''		48 + 5S	0	1***	0	0	0
7		BTB 06	18	mai-09	San Pedro	Chuquisaca	Tomina	Villa Alcala 4ta seccion	2578	19° 37' 5,80''	64° 26' 57,20''		48 + 11S	0	0	0	0	0
7		BTB 07	19	mars-09	Achiras	Chuquisaca	Belisario Boeto	Villa Serrano 1ra seccion	1870 à 1200	18° 55' 5,00''	64° 17' 26,20''		48 + 13S	3 + 3S	0	5 + 6S	0	0
7		BTB 08	20	mars-09	Valle Abajo	Santa Cruz	Florida	Samaipata	1434	18° 13' 5,80''	63° 54' 59,80''		48 + 20S	0	0	0	0	0
8	Chaco Serrano	CS01	21	mai-09	Timboy	Tarija	Burnet O'Connor	Entre Rios 1ra seccion	752	21° 09' 53,7''	64° 03' 51,7''		48 + 19S	0	0	0	0	0
8		CS02	22	mai-09	Santa Rosa	Chuquisaca	Luis Calvo	Huacaya	1088	20° 29' 26,7''	63° 31' 30,4''		48 + 19S	0	0	0	0	0
8		CS03	23	mai-09	Animbo	Chuquisaca	Hernando Siles	San Pablo de Huacareta	990	20° 34' 40,2''	64° 08' 52,9''		48 + 18S	0	0	0	0	0
8		CS04	24	mai-09	Salinas	Santa Cruz	Cordillera	Cuevo	855	20° 13' 15,8	63° 27' 57,4		48 + 19S	1 + 1S	0	2 + 1S	0	0
8		CS05	25	mai-09	Lagunillas	Santa Cruz	Cordillera	Lagunillas	967	19° 38' 56,6''	63° 41' 13,4''		48 + 13S	0	0	0	0	0
8		CS06	26	mai-09	Ipita	Santa Cruz	Cordillera	Gutierrez	930	19° 21' 09,7''	63° 30' 15,5''		48 + 10S	1S	0	1S	0	0
9		BSIA 01	27	mai-09	Juntas	Tarija	Avilez	Uriondo	1920	21° 48' 37,0''	64° 47' 59,6''		48 + 20S	0	0	0	0	0
9		BSIA 02	28	mai-09	Acchilla	Chuquisaca	Nor Cinti	San Lucas	2030	20° 19' 31,9''	64° 48' 13,7''		48 + 80S	0	0	0	0	0

9		BSIA 03	29	mars-09	Jatun Cka Cka	Chuquisaca	Yamparaez	Yamparaez 2da seccion	2281	19° 17' 41,7''	65° 00' 08,9''		48 + 4S	1	1	0	0	1
9		BSIA 04	30	mars-09	Presto	Chuquisaca	Zudanez	Presto 2da seccion	2470	18° 55' 48,4''	64° 56' 15,0''		48 + 2OS	0	0	0	0	0
9		BSIA 05	31	mars-09	Mataral	Cochabamba	Campero	Aiquile	1521	18° 36' 06,8''	65° 08' 38,0''		48 + 2OS	4	0	6	0	6
9		BSIA 06	32	mars-09	Selbas	Cochabamba	Campero	Pasorapa	2393	18° 29' 38,2''	64° 34' 25,2''		48	0	0	0	0	0
9	Bosques secos Interandinos	BSIA 07	33	mars-09	Lagunillas	Santa Cruz	Valle Grande	Tirpal 2da seccion	1770	18° 15' 37''	64° 09' 34''		48	0	0	0	0	0
9		BSIA 08	34	nov-08	Villa Granado	Cochabamba	Campero	Aiquile 1ra seccion	2157	18° 12' 29,3''	65° 01' 47,7''		48	1	0	0	1	1
9		BSIA 09	35	nov-09	Tabacal	Cochabamba	Mizque	Mizque	2195	17° 55' 54,9''	65° 23' 22,1''		48	1	2	0	0	2
9		BSIA 10	36	mars-09	Pojo	Cochabamba	Carrasco	Pojo 2da seccion	1990	17° 45' 18,3	64° 52' 00,8		48 + 68S	0	0	0	0	0
9		BSIA 11	37	nov-08	Mayca	Cochabamba	Quillacollo	Quillacollo	2564	17° 27' 50,7''	66° 18' 48''		48 + 8S	1 + 1S	11	0	0	11
9		BSIA 12	38	oct-08	Luribay	La Paz	Loayza	Luribay	2738	17° 03' 55,6''	67° 39'' 51,4''		47	11	45	0	0	45
9		BSIA 13	39	sept-08	El Palomar	La Paz	MUrillo	Mecapaca	2824	16° 42' 10,7''	68° 00' 19,4''		48	4	22	0	0	22
9		BSIA 14	40		Cotapachi	Cochabamba	Quillacollo	Quillacollo	2689	17° 25' 28,9''	66° 15' 53,0''		48 + 19S	22 + 11S	104 + 14S	1	0	1
10		PP 01	41	oct-08	Humacha	Potosi	Sur Chicha	Tupiza	3080	21° 39' 26,7''	65° 35' 02,6''		48	0	0	0	0	0
10		PP 02	42	oct-08	Estancia Pompeya (El Puente)	Tarija	Mendez	El Puente	2366	21° 18' 10, 7''	65° 12' 31,5''		48	0	0	0	0	0
10		PP 03	43	oct-08	Villa Abecia	Chuquisaca	Sud Cinti	Catamaci Villa Abecia (Camataqui)	2300	20° 58' 15,1''	65° 14' 02,9''		48	0	0	0	0	0
10	Prepuna	PP 04	44	oct-08	Tablaya Palca	Potosi	Nor Chicha	Cotagaita	2797	20° 47' 06,5''	65° 32' 34,8''		48	0	0	0	0	0
10		PP 05	45	oct-08	Orkhola	Potosi	Nor Chicha	Vitichi	3100	20° 25' 57,5''	65° 34' 36,2''		48	0	0	0	0	0
10		PP 06	46	oct-08	Urulica	Potosi	Sur Chicha 1ra seccion	Tupiza	3849	21° 35' 15,8''	65° 49' 53,6''		48	0	0	0	0	0
10		PP 07	47	oct-08	Viscachani	Potosi	Sur Chicha 1ra seccion	Tupiza	3729	21° 49' 27,7''	65° 49' 04,6''		32	2	3	0	0	3
													Total	**2719**	**89**	**208**	**35**	**7**	250

* se réfère à la carte des écorégions de l'annexe 1

** S correspond aux pièges supplémentaires

*** Cet insecte a été trouvé manuellement

Tabeau 2: Localisation des zones de piégeage supplémentaires au protocole TiBoEco; capture de *T. infestans* en rouge, complexe *T. sordida* en bleu, *T. infestans* + *T. sordida* en rose, autres espèces ou non déterminées en jaune, absence de capture en vert.

N° écorégion*	Ecorégion	Code	N° de zone de capture**	Date de capture	Village le plus proche	Département	Province	Municipalité	Altitude (m)	Latitude	Longitude	Résultat des captures	Nb de pièges posés	Nb de piège positif	*Triatoma infestans*	Complexe *Triatoma sordida*	Autre espèce de triatome ou espèce non déterminée	Total
4		PIE	48	26/11/2008	Las Piedras	Santa Cruz	Nuflo de Chavez	San Javier 2da seccion	520	16°16'22,70"	62°18'54,74"		59	2	0	0	3	3
4		LT	49	31/05/2010	Los Troncos	Santa Cruz	Nuflo de Chavez	San Julian 4ta seccion	265	17° 02' 05,4"	62° 37' 55,1"		30	1	0	0	7	7
4		SJCH	50	03/06/2010	San Jose de Chiquitos	Santa Cruz	Chiquitos	San Jose 1ra seccion	266	17° 42' 15,9"	60° 46' 51,4"		32	0	0	0	0	0
4	Bosques Secos Chiquitano	CH	51	03/06/2010	Chochis	Santa Cruz	Chiquitos	Roboré 3ra seccion	625	18° 06' 29,7"	60° 05' 18,4"		20	4	0	0	4	4

4		SCH	52	03/06/2010	Santiago de Chiquitos	Santa Cruz	Chiquitos	Robore 3ra seccion	705	18° 19' 17,1"	59° 34' 22,8"		26	0	0	0	0	0
4		FOR	53	05/06/2010	Fortin Suarez	Santa Cruz	Cordillera	Charagua 2da seccion	320	18° 44' 44,0"	60° 11' 18,1"		19	0	0	0	0	0
5		TT	54	17/03/2008	Tita	Santa Cruz	Cordillera	Charagua 2da seccion	347	18° 34' 1,20"	62° 38' 23,71"		143	8	2	7	0	9
5		TT	54	01/03/2009	Tita	Santa Cruz	Cordillera	Charagua 2da seccion	347	18° 34' 1,20"	62° 38' 23,71"		145	11	8	3	0	11
5		TLP	55	01/03/2011	Terraplen	Santa Cruz	Cordillera	Charagua 2da seccion	370	19° 08' 46,6"	62° 38' 53,3"		57	11	10	8	0	18
5		KUA	56	18/10/2008	Kuarirenda	Santa Cruz	Cordillera	Charagua 2da seccion	374	19° 10' 30,22"	62° 31' 58,73"		115	11	0	12	0	12
5		KUA	56	15/10/2010	Kuarirenda	Santa Cruz	Cordillera	Charagua 2da seccion	374	19° 10' 30,22"	62° 31' 58,73"		203	13	0	0	26	26
5		KUA_2	57	01/03/2011	Kuarirenda	Santa Cruz	Cordillera	Charagua 2da seccion	374	19° 10' 30,22"	62° 31' 58,73"		76	10	1	10	0	11
5		SS	58	04/10/2008	San Silvestre	Santa Cruz	Cordillera	Charagua 2da seccion	400	19° 17' 7,94"	62° 38' 46,10"		159	19	0	22	0	22
5		SS	58	01/03/2009	San Silvestre	Santa Cruz	Cordillera	Charagua 2da seccion	400	19° 17' 7,94"	62° 38' 46,10"		96	7	0	7	0	7
5		SS_2	59	01/03/2011	San Silvestre	Santa Cruz	Cordillera	Charagua 2da seccion	412	19° 25' 7,39"	62° 38' 24,79"		70	8	6	6	0	12
5		RN	60	14/10/2008	Rancho Nuevo	Santa Cruz	Cordillera	Charagua 2da seccion	412	19°27'12.53"	62°34'6.92"		119	10	0	10	0	10
5		TAM	61	10/10/2008	Tamachindi	Santa Cruz	Cordillera	Charagua 2da seccion	412	19° 27' 59,44"	62° 33' 59,26"		105	19	0	25	0	25
5		TAM	61	01/03/2009	Tamachindi	Santa Cruz	Cordillera	Charagua 2da seccion	412	19° 27' 59,44"	62° 33' 59,26"		59	3	0	3	0	3
5	Gran Chaco	CAB	62	13/03/2008	Cabapi	Santa Cruz	Cordillera	Charagua 2da seccion	780	19° 53' 27.96"	63° 11' 48.30"		30	6	0	6	0	6
5		Z06	63	10/11/2009	Yanahigua	Santa Cruz	Cordillera	Charagua 2da seccion	385	19° 56' 09,1"	62° 07' 35,8"		15	0	0	0	0	0
5		Z05	64	08/11/2009	17 de noviembre	Santa Cruz	Cordillera	Charagua 2da seccion	352	20° 00' 48,3"	61° 54' 11,2"		22	0	0	0	0	0
5		HGUA	65	15/03/2008	Hacienda Guarirenda	Santa Cruz	Cordillera	Camiri 6ta seccion	770	20° 6'11.27"S	63°28'36.26"O		43	0	0	0	0	0
5		Y09	66	15/10/2009	Canada	Santa Cruz	Cordillera	Charagua 2da seccion	615	20° 10' 15,9"	63° 01' 22,7"		60	11	17	1	13	31
5		Z08	67	05/11/2009	Canada	Santa Cruz	Cordillera	Charagua 2da seccion	578	20° 12' 02,3"	63° 01' 21,4"		49	7	8	0	3	11
5		SA	68	01/03/2011	San Antonio	Santa Cruz	Cordillera	Charagua 2da seccion	553	20° 13' 42,4"	62° 54' 27,4"		125	8	9	3	0	12
5		Z07	69	03/11/2009	Canada	Santa Cruz	Cordillera	Charagua 2da seccion	581	20° 15' 06,8"	62° 57' 46,5"		45	4	8	2	2	12
5		Z02	70	29/10/2009	Taiguati	Tarija	Gran Chaco	Villamontes 3ra seccion	488	21° 09' 25,3"	63° 22' 30,7"		20	1	0	1	0	1
5		ROM	71	15/03/2008	Romerillo	Chuquisaca	Luis Calvo	Machareti 3ra seccion	800	20° 38' 30.16"	63° 18' 7.92"		32	1	0	1	0	1
5		Z03	72	01/11/2009	Machareti	Chuquisaca	Luis Calvo	Machareti 3ra seccion	646	20° 49' 06,4"	63° 19' 18,8"		20	0	0	0	0	0
5		Z04	73	29/10/2009	Taiguati	Tarija	Gran Chaco	Villamontes 3ra seccion	471	21° 10' 34,0"	63° 23' 05,5"		41	2	0	2	0	2
5		Z01	74	27/10/2009	Caiza	Tarija	Gran Chaco	Yacuiba 1ra Seccion	442	21° 50' 50,6"	63° 14' 51,6"		40	4	0	1	3	4
7	Bosques Tucumano Boliviano	MAI	75	03/02/2009	Mairana	Santa Cruz	Florida	Mairana T[1] seccion	1400	18° 6'59,80"S	63°56'58,80"O		43	0	0	0	0	0

9		VIZ 01	76	08/05/2008	Aucani	La Paz	Murillo	Mecapaca	2821	16° 41' 25,47"	68° 00' 39,76"		13	2	4	0	0	4
9		VIZ 01	76	20/08/2008	Aucani	La Paz	Murillo	Mecapaca	2821	16° 41' 25,47"	68° 00' 39,76"		25	6	22	0	0	22
9		VIZ 01	76	19/05/2011	Aucani	La Paz	Murillo	Mecapaca	2821	16° 41' 25,47"	68° 00' 39,76"		35	13	40	0	0	40
9		VIZ 01	76	27/05/2011	Aucani	La Paz	Murillo	Mecapaca	2821	16° 41' 25,47"	68° 00' 39,76"		25	13	24	0	0	24
9		TUN 01	77	08/05/2008	Huayhuasi	La Paz	Murillo	Mecapaca	2757	16° 42' 25,47"	67° 59' 37,64"		30	6	46	0	0	46
9		TUN 01	77	16/07/2008	Huayhuasi	La Paz	Murillo	Mecapaca	2757	16° 42' 25,47"	67° 59' 37,64"		49	9	59	0	0	59
9		TUN 01	77	19/08/2008	Huayhuasi	La Paz	Murillo	Mecapaca	2757	16° 42' 25,47"	67° 59' 37,64"		30	8	50	0	0	50
9		RUI 01	78	10/05/2008	Tahuapalca	La Paz	Murillo	Palca 1ra seccion	2459	16° 42' 56,45"	67° 52' 13,53"		40	15	34	0	0	34
9		AGP 01	79	10/05/2008	Tahuapalca	La Paz	Murillo	Palca 1ra seccion	2380	16° 43' 30,0"	67° 52' 19,82"		23	1	2	0	0	2
9		SAP	81	12/05/2010	Sapini	La Paz	Loayza	Sapahaqui 5ta seccion	1884	16° 48' 51,1"	67° 42' 17,3"		85	38	111	0	0	111
9		SAP	81	10/10/2010	Sapini	La Paz	Loayza	Sapahaqui 5ta seccion	1884	16° 48' 51,1"	67° 42' 17,3"		273	31	62	0	0	62
9		SAP	81	01/06/2011	Sapini	La Paz	Loayza	Sapahaqui 5ta seccion	1884	16° 48' 51,1"	67° 42' 17,3"		93	22	43	0	0	43
9		TUN 02	80	11/05/2008	Tahuapalca	La Paz	Murillo	Palca 1ra seccion	2427	16° 43' 12,36"	67° 52'25,41"		29	12	37	0	0	37
9		COS 01	82	14/05/2010	Cosiraya	La Paz	Loayza	Sapahaqui 5ta seccion	1971	16° 49' 33,5"	67° 42' 18,1"		30	10	15	0	0	15
9		COS 02	83	14/05/2010	Cosiraya	La Paz	Loayza	Sapahaqui 5ta seccion	1910	16°49'50,00"	67° 42' 22,20"		19	10	74	0	0	74
9		POO	84	11/05/2010	Poopo	La Paz	Loayza	Sapahaqui 2da seccion	2020	16°51'5,70"	67° 42' 26,50"		81	3	4	0	0	4
9		TUN 03	85	18/06/2008	Khola	La Paz	Loayza	Sapahaqui 2da seccion	2095	16° 53' 12,2"	67° 42' 43,1"		25	7	21	0	0	21
9		CAC 01	86	18/06/2008	Lacayani	La Paz	Loayza	Luribay 1ra seccion	2210	16° 55' 33,2"	67° 41' 42,6"		9	0	0	0	0	0
9		VIZ 02	87	19/06/2008	Lacayani	La Paz	Loayza	Luribay 1ra seccion	2182	16° 55' 48,8"	67° 41' 32,9"		39	31	284	0	0	284
9		VIZ 02	87	02/06/2011	Lacayani	La Paz	Loayza	Luribay 1ra seccion	2182	16° 55' 48,8"	67° 41' 32,9"		92	38	75	0	0	75
9		CAC 03	88	20/06/2008	Palca	La Paz	Loayza	Luribay 1ra seccion	2356	17° 00' 30,1"	67° 39' 25,2"		49	41	263	0	0	263
9		QUE 01	89	20/06/2008	Achocara bajo	La Paz	Loayza	Luribay 1ra seccion	2159	17° 01' 54,8"	67° 40' 38,6"		40	5	7	0	0	7
9		CAC 02	90	18/06/2008	El Calvario	La Paz	Loayza	Luribay 1ra seccion	2645	17° 04' 07,7"	67° 39' 25,5"		25	5	7	0	0	7
9		TUN 07	91	18/06/2008	Pena Colorada	La Paz	Loayza	Luribay 1ra seccion	2543	17° 04' 24,2"	67° 38' 42,7"		30	1	1	0	0	1
9		TUN 06	92	18/06/2008	Catavi	La Paz	Loayza	Luribay 1ra seccion	2493	17° 04' 25,2"	67° 37' 59,7"		20	3	10	0	0	10
9	Bosques secos interandinos	LIE 01	93	19/06/2008	Cuty	La Paz	Loayza	Luribay 1ra seccion	2602	17° 04' 44,6"	67° 37' 57,0"		25	12	40	0	0	40
9		TUN 05	94	17/06/2008	Pucuma	La Paz	Loayza	Luribay 1ra seccion	2864	17°07'32"	67°35'59,5"		20	6	14	0	0	14
9		TUN 04	95	17/06/2008	Khara Yapu	La Paz	Loayza	Luribay 1ra seccion	2767	17°08'10,8"	67°35'17,9"		30	6	10	0	0	10
9		QUI	96	15/09/2009	Quillacollo	Cochabamba	Quillacollo	Quillacollo 1ra seccion	2650	17° 25' 29,0"	66° 17' 45,2"		525	185	458	3	0	461

9		QUI	96	08/03/2010	Quillacollo	Cochabamba	Quillacollo	Quillacollo 1ra seccion	2650	17° 25' 29,0"	66° 17' 45,2"		289	69	112	5	0	117
9		QUI	96	14/05/2010	Quillacollo	Cochabamba	Quillacollo	Quillacollo 1ra seccion	2650	17° 25' 29,0"	66° 17' 45,2"		175	39	54	0	0	54
9		QUI	96	10/06/2010	Quillacollo	Cochabamba	Quillacollo	Quillacollo 1ra seccion	2650	17° 25' 29,0"	66° 17' 45,2"		150	44	78	4	2	84
9		Cac 04	97	30/05/2008	Alto Cara Cara	Cochabamba	Cercado	Cochabamba seccion capital	2720	17°28'37.50"S	66° 8'16.14"O		63	9	32	0	0	32
9		PRC	98	06/02/2008	Puente Rio Caine	Cochabamba	Estebab Arze	Anzaldo 2da seccion	2040	17° 54' 3,06"	65° 51' 9,26"		33	0	0	0	0	0
9		VP	99	11/02/2008	Vina Perdida	Cochabamba	Mizque	Mizque 1ra seccion	2117	17° 54' 57.40"	65° 20' 54.10"		84	13	21	2	0	23
9		KIR	100	22/02/2009	Kirus Mayu	Potosi	Charcas	Toro Toro	2025	17° 59' 16,40"	65° 50' 11,10"		46	10	14	2	0	16
9		KIR	100	07/02/2008	Kirus Mayu	Potosi	Charcas	Toro Toro	2025	17° 59' 16,40"	65° 50' 11,10"		36	9	29	1	0	30
9		THA	101	10/09/2010	Thago Thago	Potosi	Charcas	Toro Toro	2000	18° 00' 43,02"	65° 48' 32,28"		294	27	66	3	0	69
9		THA	101	06/10/2010	Thago Thago	Potosi	Charcas	Toro Toro	2000	18° 00' 43,02"	65° 48' 32,28"		79	16	58	8	0	66
9		THA	101	10/11/2010	Thago Thago	Potosi	Charcas	Toro Toro	2000	18° 00' 43,02"	65° 48' 32,28"		83	18	27	0	0	27
9		THA	101	07/12/2010	Thago Thago	Potosi	Charcas	Toro Toro	2000	18° 00' 43,02"	65° 48' 32,28"		93	22	76	0	0	76
9		THA	101	11/01/2011	Thago Thago	Potosi	Charcas	Toro Toro	2000	18° 00' 43,02"	65° 48' 32,28"		93	24	49	1	0	50
9		THA	101	13/04/2011	Thago Thago	Potosi	Charcas	Toro Toro	2000	18° 00' 43,02"	65° 48' 32,28"		101	18	42	0	0	42
9		JUGR	102	22/02/2009	Julo Grande	Potosi	Charcas	Toro Toro	1990	18° 1' 50,90"	65° 47' 18,70"		61	21	31	0	0	31
9		JUGR	102	07/02/2008	Julo Grande	Potosi	Charcas	Toro Toro	1990	18° 1' 50,90"	65° 47' 18,70"		25	9	19	0	0	19
9		TUJ	103	22/02/2009	Tujhallana	Potosi	Charcas	Toro Toro	2560	18° 6' 21,00"	65°45'31,30"O		45	12	45	0	0	45
9		ILL_2	104	14/02/2008	Illicuni 2	Cochabamba	Campero	Omereque 3ra seccion	1641	18° 9' 7.75"	64° 51' 6.85"		30	3	4	0	0	4
9		ILL-1	105	13/02/2008	Illicuni 1	Cochabamba	Campero	Omereque 3ra seccion	1576	18° 09' 52,9"	64° 52, 0,18"		50	3	6	0	0	6
9		MAT	106	08/02/2009	Mataral	Cochabamba	Campero	Aiquile	1600	18° 36' 0,82"	65° 7' 57,46"		163	14	1	10	14*	25
9		MAT	106	16/02/2008	Mataral	Cochabamba	Campero	Aiquile	1600	18° 36' 0,82"	65° 7' 57,46"		193	16	18	0	10	28
9		ZUD	107	20/02/2008	Zudanez	Chuquisaca	Zudanez	Zudanez 1ra seccion	2489	19° 3' 3.27"	64° 47' 7.58"		41	0	0	0	0	0
10		PALQ	108	15/03/2010	Palquiza	Potosi	Sur Chichas	Tupiza 1ra seccion	2913	21° 31' 45,2"	65° 45' 04,7"		20	13	68	0	0	68
10	Prepuna	URU	109	15/03/2010	Urulica	Potosi	Sur Chichas	Tupiza 1ra seccion	3113	21° 35' 40,0"	65° 49' 55,8"		10	1	2	0	0	2
10		VIS	110	15/03/2010	Viscachani	Potosi	Sur Chichas	Tupiza 1ra seccion	2979	21° 36' 41,0"	65° 48' 21,8"		20	5	37	0	0	37
													Total	6204	1142	2775	169	87	3031

* se refere à la carte des écorégions de l'annexe 1, ** voir figure 1 en annexe 5

Am J Trop Med Hyg, 82(4), 2010, pp 574–579
doi 10 4269/ajtmh 2010 09-0325

First Report of Widespread Wild Populations of *Triatoma infestans* (Reduviidae, Triatominae) in the Valleys of La Paz, Bolivia

Rosio Buitrago,† Etienne Waleckx,† Marie-France Bosseno, Faustine Zoveda, Pablo Vidaurre, Renata Salas, Elio Mamani, François Noireau, and Simone Frédérique Brenière*

Département Société et Santé, Institut de Recherche pour le Développement (IRD), Montpellier, France, Ministerio de Salud y Deporte, Servicio Departamental de Salud La Paz (SEDES), Department of La Paz, La Paz, Bolivia, Instituto Nacional de Laboratorios de Salud (INLASA), Department of La Paz, La Paz, Bolivia

Abstract. Wild populations of *Triatoma infestans*, the main vector of Chagas disease in the Southern Cone countries, may be involved in reinfestation of human dwellings, limiting the success of vector-control campaigns in Bolivia. Knowledge of the distribution of these populations remains incomplete. We report here the detection of *T. infestans* wild populations in large areas in the department of La Paz, Bolivia. Among 18 sylvatic areas investigated, 17 were positive with *T. infestans* specimens. The infection rate of captured *T. infestans* with *Trypanosoma cruzi* was 85.7% in adult specimens. These results expand the geographical distribution of wild populations of *T. infestans*; it may be distributed throughout the Inter-Andean Dry Forest eco-region of Bolivia. The current information allows us to propose the hypothesis that a sylvatic origin of the reinfestation is located in the valleys of La Paz.

INTRODUCTION

Chagas disease is a major public health problem in Latin America. *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae), the causative agent of the disease, is transmitted by contaminated feces of triatomine bugs. In Bolivia and other countries of the Southern Cone in South America, *Triatoma infestans* (Klug 1834) remains the main vector species.

In the Southern Cone, control of Chagas disease is being pursued by elimination of *T. infestans* (principally through insecticidal spraying). Control efforts have relied on the assumption that this species was almost exclusively domestic, although a few wild populations had been reported in the Cochabamba valley in Bolivia.[1–3] More recently, other wild foci of *T. infestans* have been described, however, indicating that wild populations of *T. infestans* have a larger dispersion than previously assumed.[4–6] As new species or populations tend to invade the ecological niche released by a first species, wild populations of *T. infestans* could infest habitats free of domestic triatomine populations and jeopardize vector-control efforts.[7,8]

We found wild populations of *T. infestans* in two valleys of La Paz, Bolivia, where transmission of *Trypanosoma cruzi* persists. New hypotheses about the geographical distribution of wild populations of *T. infestans* and the epidemiological risk that they represent are suggested.

MATERIALS AND METHODS

Study areas. In conjunction with the National Program of Chagas disease (PNCH), two valleys under vector control since 2003, Rio La Paz and Rio Luribay, were chosen. Despite insecticidal spraying, locals were still suffering domiciliary reinfestation by *T. infestans*. In these valleys, 6 and 12 trapping areas between 2,159 and 2,864 m altitude were selected (Figure 1; Table 1). All areas were situated in wild environments, which will be referred as "sylvatic" in this work, at a distance varying from 20 to 500 m from human dwellings. Fields closest to rivers are dedicated to vegetable crops and maize in the two valleys. Surrounding mountains have scarce vegetation dominated by thorny plants and cacti. People also cultivate prickly pear (*Opuntia ficus-indica*) and the cochineal (*Dactylopius coccus*) insect used for food coloring and cosmetics. Both valleys are situated in the Inter-Andean Dry Forest eco-region.[9] Climate in this area is semi-arid with an average annual temperature of 18°C (range = 8.3–30.8°C), and mean relative humidity is 5.8%. Rainfall occurs between December and April, and winter and spring are very dry (June to November).

Survey of human population. During April and May 2008, 305 inhabitants from 31 different villages in these valleys were questioned about their knowledge of triatomine bugs (locally called *vinchucas*) in domestic/sylvatic environments and their localization. They were also asked if they have ever seen *vinchucas* flying.

Collection of wild triatomines and laboratory processing. Collection was carried out between May and October 2008 using mice-baited adhesive traps.[10] The traps were placed in small caves and cracks, in burrows, or under vegetation. They were set in the afternoon and inspected the next morning. More than 20 traps were placed in most of the trapping areas (Table 1). All insects caught by a trap were placed together in one tube, and they were transported alive to the laboratory. The identification of triatomines (adults and nymphs) was performed according to morphological taxonomic keys.[11] Sex and stages were also determined. Feces from each bug were examined for the presence of trypanosomatides by direct microscopical observation at 400× magnification. Bugs were then dissected under a safety hood, and legs and digestive tracks were conserved for further studies.

RESULTS

Knowledge of the triatomine bugs by the surveyed population. More than 96% of interviewed locals recognized the vector known as *vinchuca* in the two valleys (Rio La Paz = 117 interviews; Rio Luribay = 188 interviews). Triatomines have been observed in 12% of interviewees' homes within the previous 12 months, and 7% have seen them during the prior month. Moreover, 9% have seen bugs during the year in their peridomicile. In addition, 39.1% of women and 63% of men

*Address correspondence to Simone Frédérique Brenière, Institut de Recherche pour le Développement (IRD), Représentations en Bolivia, UR 016 Caractérisation et contrôle des populations de vecteurs, Avenida Hernando Siles N° 5290, Esq. Calle 7, Obrajes, C P 9214, La Paz, Bolivia. E-mail: breniere@ird fr
†These authors contributed equally to the work.

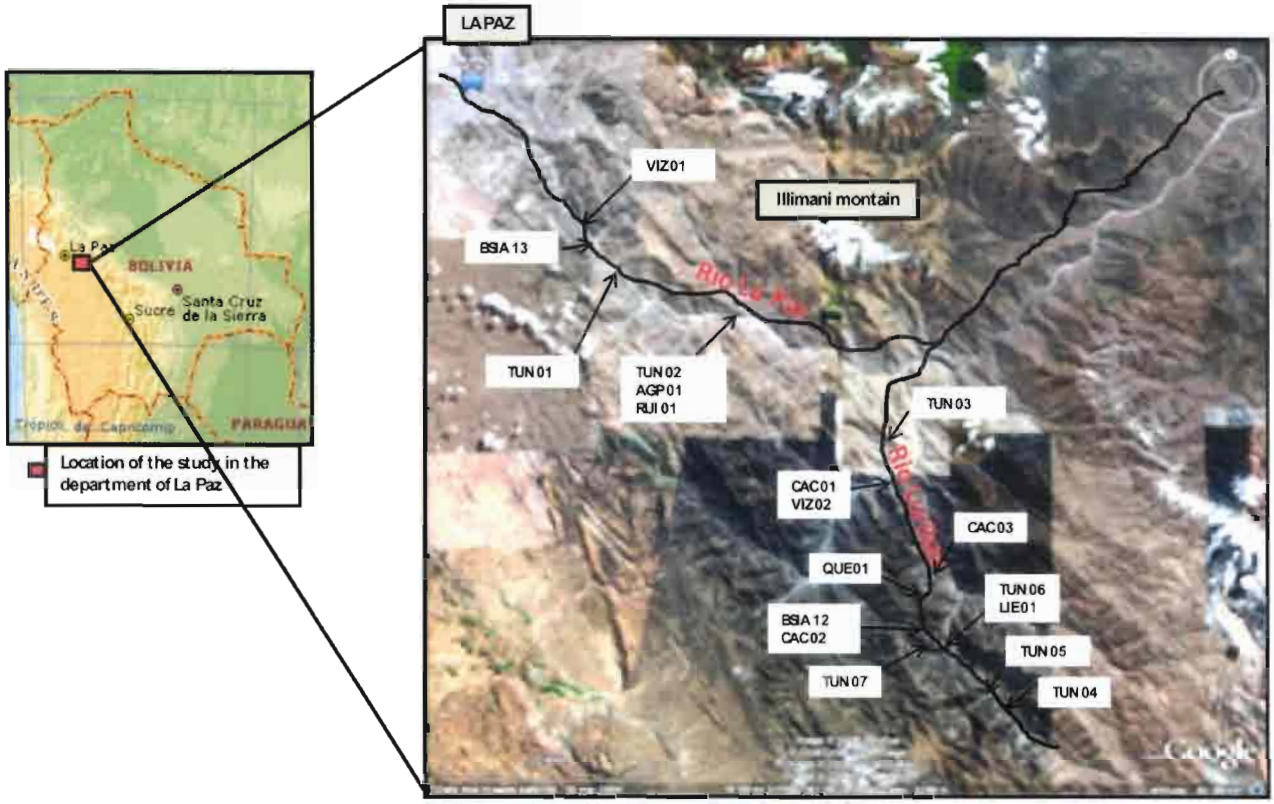


FIGURE 1. Map of Rio La Paz and Rio Luribay valleys (lines) obtained from Google Earth. All the studied areas are located between VIZ 01 (2,821 m) and RUI 01 (2,459 m) and between TUN 04 (2,767 m) and TUN 03 (2,095 m) in Rio La Paz and Rio Luribay, respectively. This figure appears in color at www.ajtmh.org.

surveyed claimed to have seen bugs in the sylvatic environment: in the hills, under rocks, in fields of prickly pear, in ruins, and in cemeteries. Finally, 30% of those surveyed reported that they have seen the bugs flying. "They fly from the hill towards the houses, they fly to the roof of the houses or to the window in the night, and we hear them arriving to the tinny roof" were expressions frequently used by the interviewees.

Areas of collection. Triatomine bugs were found in all trapping areas except one (area CAC 01) in which only nine traps have been placed (Table 1). A total of 646 traps were used during the study, and 29.6% of them caught wild *T. infestans* specimens. The proportion of positive traps in an area was variable and ranged from 3.3% in TUN 07 to 83.7% in area CAC 03, but only two areas had fewer than 5% positive. The five areas with greatest infestation (> 37.5% of traps were positive; Figure 2A–E) were wilderness areas with thorny vegetation and prickly pear fields with rock-pile boundary walls (areas TUN 02, CAC 03, and LIE 01), a ruin with mud walls providing shelter for small rodents (area RUN 01), and a mountainside composed of large rocks where excrements of *vizcachas* (rodents belonging to the Chinchillidae family) were observed (area VIZ 02).

Interestingly, in the positive area VIZ 01 (small highland), traps were placed in deep cracks formed in sediment by erosion (2–5 m) that serve as shelters for *vizcachas* (Figure 2F).

Triatomine bug collection and infection rate. A total of 978 *T. infestans* specimens were collected in 17 positive areas. The number of triatomines by positive trap ranged from 1.4 triatomines per trap in areas QUE 01 and CAC 02 to 9.16 triatomines per trap in area VIZ 02. Adult insects as well as nymphal instars were caught in all areas, and a predominance of young nymphs was observed among the total population (1st + 2nd + 3rd nymphal instars = 70.1%; Figure 3). The proportion of positive traps was significantly higher in Rio Luribay than in Rio La Paz (χ^2 $P < 0.001$), but triatomine density per positive trap was not significantly different (Mann–Whitney–Wilcoxon test, $P > 0.05$). A total of 448 *T. infestans* feces were examined, and positive samples for trypanosomatide were found in all the trapping areas (Table 2). The infection rates ranged from 3.8% in first nymphal instar to 85.7% in adult insects with no significant differences between sex (Figure 4; χ^2 $P > 0.05$). Preliminary analysis of feces by polymerase chain reaction (PCR) confirmed infection of *T. infestans* by *T. cruzi* species (data not shown).[12]

DISCUSSION

Since the first identification of a wild population of *T. infestans* in 1946, several reports have described the existence of other wild foci in the department of Cochabamba, Bolivia.[1–5] Subsequent reports have suggested a wider distribution of wild *T. infestans* in Bolivia than just the Cochabamba valley, and findings of wild *T. infestans* in Argentina (LA Ceballos and others, unpublished data) and Chile have also been reported.[8,13]

TABLE 1

Description, geographical localization, and infestation of the investigated sylvatic areas in two valleys of the department of La Paz, Bolivia

Valley	Location code	Nearest village	Description of trapping areas (distance from nearest habitat)	Latitude	Longitude	Alt (m)	Date of trapping	Number of traps placed	Number of positive traps	% of positive traps	Total number of triatomine bugs
Rio La Paz	VIZ 01	Aucani	Deep cracks in a small highland that are shelters for vizcachas (Andean small rodent) (200 m)	16°41'25.47"	68°00'39.76"	2,821	5/8/2008	13	2	15.4	4
							8/20/2008	25	6	24.0	22
	BSIA 13	El Palomar	Wilderness mountainside with sparse vegetation of mainly cacti and thorny vegetation (50–200 m)	16°42'02.75"	67°59'54.91"	2,765	9/10/2008	48	4	8.3	22
	TUN 01	Huayhuasi	Field of prickly pears adjacent to some houses outside the village (20–150 m).	16°42'25.47"	67°59'37.64"	2,757	5/8/2008	30	6	20.0	46
							7/16/2008	49	9	18.4	59
							8/19/2008	30	8	26.7	50
	AGP 01	Tahuapalca	Edges of footpaths between crop fields around the village (20–200 m).	16°43'05.03"	67°52'30.59"	2,380	5/10/2008	23	1	4.3	2
	TUN 02	Tahuapalca	Field of prickly pears overhanging the village (50–150 m).	16°43'11 53"	67°52'25.70"	2,427	5/11/2008	29	12	41.4	37
	RUI 01	Tahuapalca	Ruin of a 19th century barracks with mud walls next to a small cemetery on a highland overhanging the village of Tahuapalca (500 m).	16°42'56.45"	67°52'13.53"	2,459	5/10/2008	40	15	37.5	34
	Total Rio La Paz							287	63	22.0	276
Rio Luribay	TUN 04	Carayapu	Wilderness mountainside with sparse vegetation of mainly cacti and thorny vegetation, which is adjacent to some house of the village (50–300 m).	17°08'10.80"	67°35'17.90"	2,767	6/17/2008	30	6	20 0	10
	TUN 05	Pucuma	Wilderness mountainside and cemetery outside the village (100–300 m).	17°07'32 00"	67°35'59.50"	2,864	6/17/2008	20	6	30.0	14
	LIE 01	Cuty	Rock-pile boundary walls and wilderness field at the entrance of the village (50–100 m)	17°04'44.60"	67°37'57.00"	2,602	6/19/2008	25	12	48 0	40
	TUN 06	Catavi	Field of prickly pears adjacent to the church of the village (50–100 m).	17°04'25.20"	67°37'59.70"	2,493	6/18/2008	20	3	15.0	10
	TUN 07	Peña Colorada	Field of prickly pears outside the village (50–300 m).	17°04'24.20"	67°38'42.70"	2,543	6/18/2008	30	1	3.3	2
	CAC 02	El Calvario	Field of prickly pears adjacent to the cemetery of the village (50–150 m)	17°04'07.70"	67°39'25.50"	2,645	6/18/2008	25	5	20.0	7
	BSIA 12	Luribay	Wilderness mountainside with sparse vegetation of mainly cacti and thorny vegetation (50–200 m).	17°03'34.80"	67°39'58.40"	2,583	10/31/2008	47	11	23 4	44
	QUE 01	Achocara Bajo	Cliff of red earth along the road, forming small caves, and isolated from any village (500 m of a solitary house).	17°01'54.80"	67°40'38.60"	2,159	6/20/2008	40	5	12.5	7
	CAC 03	Palca	Field of prickly pears adjacent to some houses outside the village (20–100 m).	17°00'30.10"	67°39'25 20"	2,356	6/20/2008	49	41	83.7	263
	VIZ 02	Lacayani	Large stones at the side of the road (500 m far from an isolated house).	16°55'48.80"	67°41'32 90"	2,182	6/19/2008	39	31	79 5	284
	CAC 01	Lacayani	Wilderness valley with sparse vegetation composed mainly of cacti and thorny vegetation (500 m far from isolated houses).	16°55'33 20"	67°41'42 60"	2,210	6/18/2008	9	0	–	0
	TUN 03	Khola	Wilderness site with sparse vegetation of mainly cacti and thorny vegetation (50–500 m)	16°53'12.20"	67°42'43 10"	2,095	6/18/2008	25	7	28 0	21
	Total Rio Luribay							359	128	35.7	702
Total								646	191	29 6	978

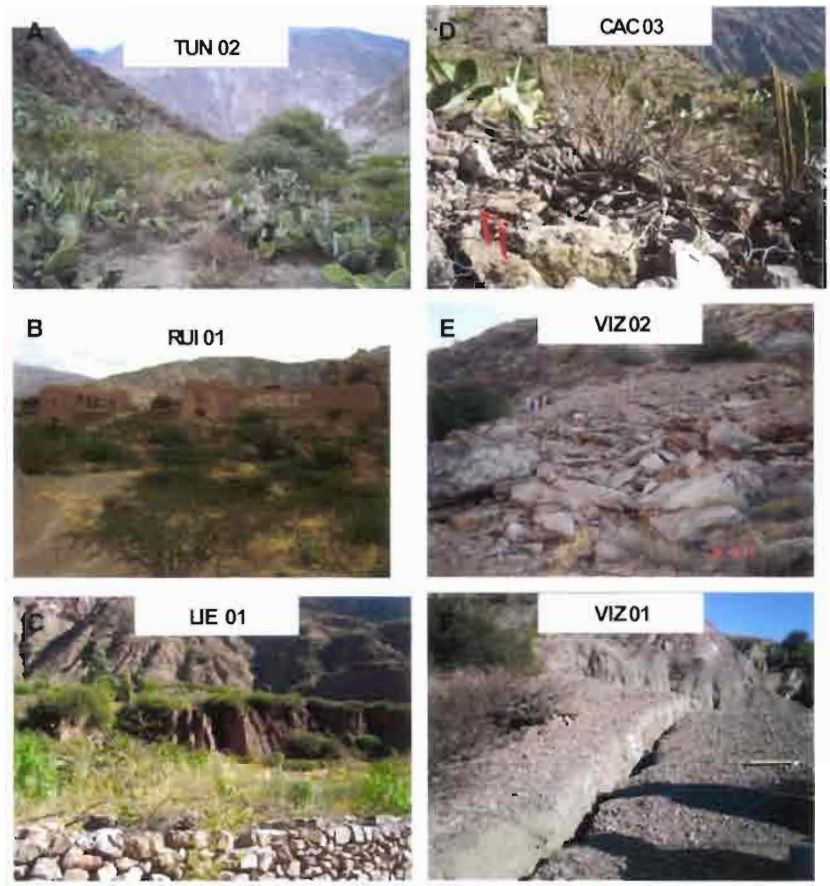


FIGURE 2. Different sampling areas. (**A**) Field of prickly pears overhanging the village of Tahuacalpa. (**B**) Ruin of 19th century barracks with mud walls next to a small cemetery on a highland overhanging the village of Tahuapalca. (**C**) Rock-pile boundary walls and wilderness field at the entrance of the village of Cuty. (**D**) Field of prickly pears adjacent to houses outside of the village of Palca. (**E**) Large stones along the driveway at 500 m from an isolated house. (**F**) Deep cracks in the ground of a small highland where vizcachas (rodents belonging to the Chinchillidae family) have been observed. This figure appears in color at www.ajtmh.org.

This fieldwork in two La Paz valleys reported here showed a wide distribution of wild populations of *T. infestans*. The sequences of cytochrome b gene of some insects confirmed the species *T. infestans*. In both valleys, wild populations were easily captured in places very near the human habitat as well as remote places, and the percentage of positive traps was similar to that reported previously in Cochabamba (~30%).[4]

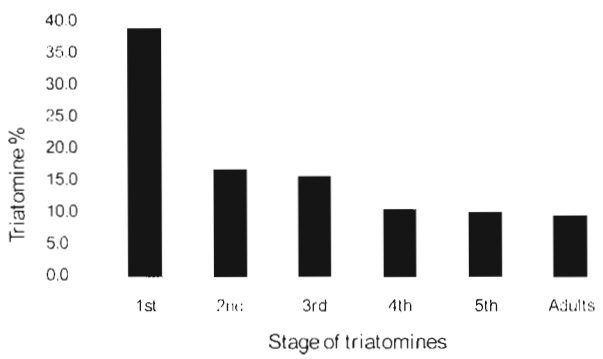


FIGURE 3. Age structure of wild *T. infestans* populations caught in the valleys of Rio La Paz and Rio Luribay.

T. infestans was found in settings never described before as habitats of this species (thorny hills, fields of prickly pear, screes, rock-pile boundary walls, ruins, and deep cracks formed by erosion). Indeed, the typical ecotope of wild *T. infestans* has been previously thought to be a rocky outcrop, except for the *T. infestans* Dark Morph found in the Gran Chaco region.[8] These new findings suggest the existence of a continuous distribution of wild populations in the valleys of La Paz. However, the distribution and abundance of these populations are little compatible with secondary wild colonization of the environment by domestic populations. However, the full distribution of *T. infestans* remains to be more accurately measured. One approach would be to investigate the different eco-regions of Bolivia where domestic *T. infestans* is endemic. Indeed, the valleys of La Paz as well as the valleys of Cochabamba, where wild populations exist, belong to the Inter-Andean Dry Forest eco-region, but the endemic area of domestic populations of *T. infestans* includes other eco regions where few data of sylvatic foci are available.

The Bolivian PNCH has expressed interest in whether or not occurrences of reinfestation by *T. infestans* have a sylvatic origin. In both valleys investigated here, the PNCH reports continuous reinfestation, and this was confirmed by local residents. Moreover, local residents reported seeing *T. infestans* in

TABLE 2
Infection rate of sylvatic *T. infestans* captured in the 17 positive areas in the department of La Paz

Area code	Number of adults			Number of nymphs			
	Infected	Not infected	% of infected adults	Infected	Not infected	% of infected nymphs	% of total infected
VIZ 01	3	0	100.0	1	2	33.3	66.6
BSIA 13	1	0	100.0	3	12	20.0	25.0
TUN 01	7	1	87.5	40	36	52.6	55.9
AGP 01	0	0	–	2	0	100.0	100.0
TUN 02	7	0	100.0	11	6	64 7	75 0
RUI 01	5	0	100.0	14	7	66.6	73 1
Total Rio La Paz	23	1	95.8	71	63	53.0	59.5
TUN 04	2	2	50.0	0	5	0.0	22.2
TUN 05	0	0	–	2	6	25.0	25.0
LIE 01	2	1	66.6	12	4	75.0	73.7
TUN 06	2	1	66.6	1	2	33.3	50.0
TUN 07	0	0	–	0	2	0.0	0 0
CAC 02	3	0	100.0	1	0	100.0	100.0
BSIA 12	8	0	100.0	6	6	50 0	70.0
QUE 01	0	0	–	1	1	50.0	50 0
CAC 03	6	3	66.6	47	72	39.5	41.4
VIZ 02	2	0	100.0	22	51	30.1	32.0
TUN 03	0	0	–	11	6	64.7	64.7
Total Rio Luribay	25	7	78.1	103	155	39.9	44.1
Total	48	8	85.7	174	218	44.4	49.5

the sylvatic environment and flying to dwellings. These data suggested the capacity of the bug to fly from their natural habitats to human-developed areas. Moreover, our work points to the utility of adding interviews with locals to help determine the presence of sylvatic triatomines: information from our interviewees was strongly corroborated in the field. To effectively assess the epidemiological role of wild *T. infestans* populations, it is necessary to better understand the mechanisms of the reinfestation and to discriminate residual populations, which can develop after insecticide spraying, from reinfestation by sylvatic populations. Therefore, population genetic analyses by different genetic markers should be useful. Also, it is necessary to bring eco-epidemiological research to understand the anthropological, sociological, and biological determinants of reinfestation.[14,15]

Received June 14, 2009. Accepted for publication December 26, 2009.

Acknowledgments: We extend our gratitude to the authorities of the Ministerio de Salud y Deporte and Mirko Rojas, head of the National Program of Chagas Control (PNCH), for their help. This study received financial support from the Institut de Recherche pour le Développement (IRD) and the Agence Nationale de la Recherche (ANR) of France.

Authors' addresses: Rosio Buitrago, Etienne Waleckx, Marie-France Bosseno, Faustine Zoveda, Renata Salas, François Noireau, and Simone Frédérique Brenière, Institut de Recherche pour le Développement (IRD), Representación en Bolivia, UR 016 Caractérisation et Contrôle des Populations de Vecteurs, Avenida Hernando Siles N° 5290, Esq. Calle 7, Obrajes, C.P. 9214, La Paz, Bolivia. Pablo Vidaurre and Elio Mamani, Servicio Departamental de Salud La Paz (SEDES La Paz), Calle Capitán Ravelo No. 2180, Zona Central, La Paz, Bolivia.

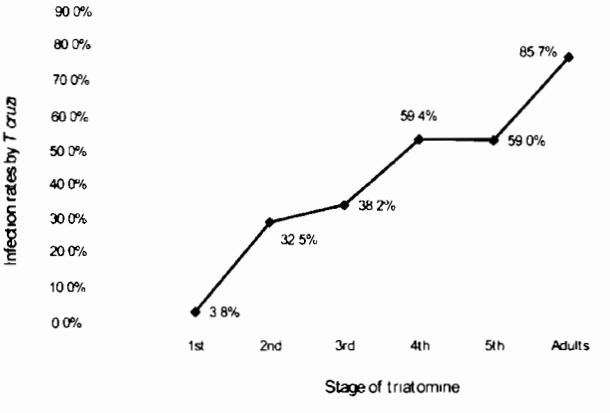


FIGURE 4. Infection rate of the different stages of wild *T. infestans* caught in the valleys of Rio La Paz and Rio Luribay.

REFERENCES

1. Torrico RA, 1946. Hallazgo de *Eratyrus mucronatus*, infestación natural de vinchucas de cerro y *Eutriatoma sordida* en Cochabamba. *An Lab Central Cochabamba 1:* 19–23.
2. Dujardin JP, Tibayrenc M, Venegas E, Maldonado L, Desjeux P, Ayala FJ, 1987. Isozyme evidence of lack of speciation between wild and domestic *Triatoma infestans* (Heteroptera, Reduviidae) in Bolivia. *J Med Entomol 24:* 40–45.
3. Bermudez H, Balderrama F, Torrico F, 1993. Identification and characterization of sylvatic foci of *Triatoma infestans* in Central Bolivia. *Am J Trop Med Hyg 49 (Suppl):* 371.
4. Noireau F, Flores R, Gutierrez T, Dujardin JP, 1997. Detection of sylvatic dark morphs of *Triatoma infestans* in the Bolivian Chaco. *Mem Inst Oswaldo Cruz 92:* 583–584.
5. Cortez MR, Pinho AP, Cuervo P, Alfaro F, Solano M, Xavier SCC, D'Andrea PS, Fernandes O, Torrico F, Noireau F, Jansen AM, 2006. *Trypanosoma cruzi* (Kinetoplastida Trypanosomatidae), ecology of the transmission cycle in the wild environment of the Andean valley of Cochabamba, Bolivia *Exp Parasitol 114:* 305–313.
6. Cortez MR, Emperaire L, Piccinali RV, Gurtler RE, Torrico F, Jansen AM, Noireau F, 2007. Sylvatic *Triatoma infestans* (Reduviidae, Triatominae) in the Andean valleys of Bolivia. *Acta Trop 102:* 47–54.
7. Krebs CJ, 1985. *Ecology: The Experimental Analysis of Distribution and Abundance*. New York: Harper and Row, 3rd ed.
8. Noireau F, Cortez MGR, Monteiro FA, Jansen AM, Torrico F, 2005. Can wild *Triatoma infestans* foci in Bolivia jeopardize Chagas disease control efforts? *Trends Parasitol 21:* 7–10.
9. Ibisch PL, Merida G, 2003 *Biodiversidad: La riqueza de Bolivia. Estado de Conocimiento y Conservación*. Santa Cruz de la Sierra: Editorial FAN.
10. Noireau F, Abad-Franch F, Valente SAS, Dias-Lima A, Lopes CM, Cunha V, Valente VC, Palomeque FS, de Carvalho-Pinto CJ,

Sherlock I, Aguilar M, Steindel M, Grisard EC, Jurberg J, 2002. Trapping triatominae in silvatic habitats. *Mem Inst Oswaldo Cruz 97:* 61–63.

11. Lent H, Wygodzinsky P, 1979. Revision of the triatominae (Hemiptera, Reduviidae), and their significance as vectors of Chaga's disease. *Bull Am Mus Nat Hist 163.* 123–520.
12. Bosseno MF, Garcia LS, Baunaure F, Gastelum EM, Gutierrez MS, Kasten FL, Dumonteil E, Brenière SF, 2006. Identification in triatomine vectors of feeding sources and *Trypanosoma cruzi* variants by heteroduplex assay and a multiplex miniexon polymerase chain reaction. *Am J Trop Med Hyg 74:* 303–305.
13. Bacigalupo BA, Segura MJA, Garcia CA, Hidalgo CJ, Galuppo GS, Cattan PE, 2006. Primer hallazgo de vectores de la enfermedad de Chagas asociados a matorrales silvestres en la Región Metropolitana, Chile. *Rev Med Chil 134:* 1230–1236.
14. Dujardin JP, Cardozo L, Schofield CJ, 1996. Genetic analysis of *Triatoma infestans* following insecticidal controlinterventions in central Bolivia *Acta Trop 61:* 263–266
15. Cerere MC, Vazquez-Prokopec DGM, Gütler RE, Kitron U, 2004. Spatio-temporal analysis of reinfestation by *Triatoma infestans* (Hemiptera: Reduviidae) following insecticida spraying in a rural community in Northwestern Argentina. *Am J Trop Med Hyg 71:* 803–810

LRH: WALECKX AND OTHERS

RRH: SYLVATIC *T. INFESTANS* IN THE BOLIVIAN CHACO

New Discoveries of Sylvatic *Triatoma infestans* (Hemiptera: Reduviidae) Throughout the Bolivian Chaco

Etienne Waleckx, Stéphanie Depickère, Renata Salas, Claudia Aliaga, Marcelo Monje, Hiber Calle, Rosio Buitrago, François Noireau, and Simone Frédérique Brenière

MIVEGEC (Maladies Infectieuses et Vecteurs : Ecologie, Génétique, Evolution et Contrôle), Université Montpellier 1 et 2 - CNRS 5290 -IRD 224, Institut de Recherche pour le Développement (IRD), La Paz, Bolivia; Instituto Nacional de Laboratorios de Salud (INLASA), Laboratorio de Entomología Médica, La Paz, Bolivia; Escuela Técnica de Salud, Cochabamba, Bolivia; IIBISMED, Facultad de Medicina, Universidad Mayor de San Simon, Cochabamba, Bolivia

Abstract. Sylvatic populations of *Triatoma infestans* might be involved in the recolonization of human dwellings. We report here the discoveries of new *T. infestans* sylvatic foci in the Bolivian Chaco. Eighty-one triatomines were caught, 38 of which were identified as *T. infestans*. *Triatoma sordida* and *Panstrongylus geniculatus* were the other species collected. One *T. infestans* and one *T. sordida* were infected with *Trypanosoma cruzi* TcI; one *T. infestans* was infected with TcII. These discoveries add to the debate on the geographic distribution of sylvatic *T. infestans* populations, the geographic origin of the species and the epidemiological role of these populations.

In South America, *Triatoma infestans* remains the main vector of *Trypanosoma cruzi*, the causative agent of Chagas disease. This species has been considered almost exclusively domestic for a long time: despite old reports of occasional findings of *T. infestans* in sylvatic areas in Argentina, Brazil and Paraguay, [1-5] it was thought that true sylvatic foci were restricted to the Andean valleys of Cochabamba in Bolivia, where sylvatic populations had repeatedly been detected. [6-8] This belief led to the hypothesis that the origin and initial domestication of the species occurred in the Bolivian Andes. [9, 10] Moreover, when the vector control programs started, the possibility of a recolonization of treated areas by sylvatic bugs was mainly discarded. Recently, it has been shown that Andean sylvatic populations in Bolivia are distributed in larger areas than the Cochabamba valleys. [11-14] In addition to the Bolivian Andean valleys, dark morphs of *T. infestans* were reported in 1997 in one sylvatic area of the Bolivian Chaco, [15] and recently, other sylvatic foci were discovered in the Argentinean and Paraguayan Chaco and in Chile, [16-19] challenging the hypothesis that the species originated in the Bolivian Andes. All of these data show that sylvatic *T. infestans* populations are much more widespread than previously thought and that their role in the recolonization of treated areas has to be considered. [13, 20] Indeed, domestic *T. infestans* persists primarily in the Bolivian Andean valleys and in the Gran Chaco, precisely where sylvatic populations have been found. [21]

Here, we report the discovery of new sylvatic foci in the Bolivian Chaco, highlighting the wide distribution of sylvatic *T. infestans* in this region and questioning the geographic origin of the species, its domestication process and the epidemiological role played by sylvatic populations.

The search for triatomines was conducted in October and November 2009, using mouse-baited adhesive traps in 14 forest sites located in 11 different sylvatic areas (Table) of the Bolivian Chaco (Figure 1). [22] Two search methods were used. In the first method, five sylvatic sites were randomly pre-selected (GC1 - GC5), and traps were placed along transects (200-500 m) into the forest, in hollows of live (35.1%) or dead (42.2%) trees, burrows (13.4%), and other locations, such as under woodpiles (9.3%). The second method focused the search on nine forest sites selected according to the information provided by inhabitants (Y01 to Y09), and traps were placed mostly in hollows of live trees (88.2%). Traps were set in the afternoon and inspected the next morning. The triatomines caught were first identified with morphological keys [23], and then after sequencing of the rDNA ITS-2 region according to previous descriptions. [14, 24]

A total of 580 traps were set. Forty-six were positive for triatomines (7.9%) in nine different forest sites (64%, Table). Eighty-one triatomines belonging to *T. infestans*, *Triatoma sordida* and *Panstrongylus geniculatus* were collected. Only five adults were found: four were morphologically and genetically identified as *T. infestans* and exhibited the dark phenotype; [15] one was identified as *T. sordida*. The other specimens were nymphs and were identified only after sequencing. Complete or partial ITS-2 sequences (386 bp at least) were resolved for 57 specimens. Thirty-eight were characterized as *T. infestans*: (i) 27 presented the ITS2Hap2 haplotype, previously found in sylvatic *T. infestans* from the Bolivian Chaco (accession n° HQ333212); [14] (ii) one, collected in GC03, presented the ITS2Hap1 haplotype (accession n° HQ333211), previously reported as the only Andean haplotype in sylvatic *T. infestans* from Bolivia; [14] and (iii) 10, for which the resolved sequences were partial, presented 99 or 100% identity with various sequences deposited for *T. infestans* (accession n° HQ333214, HQ333212, HQ333211, AY860388, AY860387, AJ582025, AJ582024, AJ576055, AJ576054, AJ576052, AJ576051). Seventeen specimens were characterized as *T. sordida*

(accession n° AJ576063, 99% identity) and two as *P. geniculatus* (AJ306543, 99% identity). *T. infestans* and *T. sordida* specimens were all collected in hollow trees (Figure 2); the two *P. geniculatus* were captured in burrows.

Feces of 43 bugs were microscopically observed, and no flagellates were found. The infection was then determined for 24 bugs (11 *T. infestans*, 11 *T. sordida*, and the two *P.geniculatus*) using the MMPCR method after DNA extraction from the digestive tracts, [25] and the genetic characterization of parasites was completed by sequencing of the GPI gene. Only three bugs were positive: two *T. infestans* (one male and one 4th instar captured in Y08 and GC02, respectively) and one *T. sordida* (4th instar captured in GC05). The *T. infestans* male was infected with TcII. The two other bugs were infected with TcI. The present report is the first to document the presence of TcII in a sylvatic *T. infestans* specimen.

Sylvatic *T. infestans* were captured in three areas (3, 6, and 9) farther south (130-320 km) than Tita, the only area with sylvatic *T. infestans* previously recorded in the Bolivian Chaco (Figure 1). [15] These discoveries, and the recent finding of sylvatic foci in the Argentinean and Paraguayan Chaco, [17, 19] highlight the wide distribution of sylvatic *T. infestans* in the Gran Chaco. Interestingly, the Y01 and Y08 sites were distant from human habitat (≈ 8 km). As in the Tita area, this supports the primary occurrence of sylvatic populations in the Gran Chaco and makes the hypothesis of a secondary colonization of the sylvatic environment by bugs derived from domestic populations unlikely. Different studies have recently shown that the Andean origin of *T. infestans* was not unequivocally supported and that a Gran Chaco origin could not be rejected. [14, 26, 27] Among the arguments used to support the hypothesis of an Andean origin is the scarcity of *T. infestans* sylvatic foci in the Gran Chaco; this hypothesis is now weakened given the new foci reported here and those reported in the Argentinean and Paraguayan Chaco. [17, 19]

In this study, several forest sites positive for *T. infestans* were close to human habitat (< 500 m), supporting the possibility of incursion events of sylvatic specimens into houses. The traditional hypothesis to explain the current distribution of domestic *T. infestans* in South America puts forward an initial and major vector domiciliation in the Bolivian Andes, followed by a recent passive human-mediated spread. [9, 10] This hypothesis has been further supported by the genetic similarities between domestic and sylvatic *T. infestans* populations in the Bolivian Andes. [7, 28] In fact, the same genetic argument can be applied to the Bolivian Chaco, where ITS-2 and mtCytB haplotypes are shared between domestic and sylvatic *T. infestans*. [14, 24] The diversity of eco-regions and ecotopes in which *T. infestans* sylvatic populations have been found to date and the propensity of this species to diversify feeding sources suggest a long evolutive process that allowed it to acquire a strong adaptive ability and to survive in a great variety of environments. Consequently, domestication could be an opportunity rather than a costly adaptive change. In this way, new human settlements intruding into the sylvatic environment would provide an opportunity for *T. infestans* to feed on new mammal species (humans, domestic animals) that are more stable (an easy feeding source) than sylvatic mammals. Easy domestication of *T. infestans* sylvatic populations suggests that their epidemiological role has to be considered carefully because they can infest dwellings after vector control and new human settlements in sylvatic environments.

In the current study, the infection rate of sylvatic *T. infestans* was low (18.2%) but not negligible. In contrast, in the Andes, sylvatic *T. infestans* populations have a high infection rate. [13] The low infection reported here agrees with previous results in the Gran Chaco. [17, 29] It might be related to the main ecotope investigated (hollow trees), perhaps occupied by birds (which are not *T. cruzi* reservoirs). This result might strengthen the hypothesis of an ornithophilic feeding behavior of these populations. Nevertheless, most of the hollow trees in which *T. infestans* were found during the current fieldwork did not seem to be inhabited by

birds. Consequently, other blood sources (lowly infected small mammals, reptiles) and the occurrence of haemolymphagy cannot be discarded. Future studies determining the blood sources of *T. infestans* in the Gran Chaco will clarify the *T. infestans* ecology in this region.

Extensive searches for sylvatic *T. infestans* in the Gran Chaco must be pursued to determine the distribution of sylvatic populations in this eco-region more accurately. Future genetic studies of these sylvatic populations with Andean populations will help determine the geographical origin and dispersion routes of the species. Other molecular studies comparing the genetic characteristics of sylvatic and domestic bugs are also needed to clarify the epidemiological role of sylvatic populations and to improve our understanding of the domestication processes of *T. infestans*.

Acknowledgments: We thank Carlos Chura and Marcelo Claure (IRD) for their valuable help in the field.

Financial support: This study was supported by the ANR (Agence Nationale de la Recherche, France), the IRD (Institut de Recherche pour le Développement, France), the UNICEF/UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases (TDR, N° A70596), and the IDRC (International Development Research Centre, Canada, N° 103696-012).

Authors' addresses: Etienne Waleckx, Stéphanie Depickère, Renata Salas, Claudia Aliaga, Marcelo Monje, Hiber Calle, Rosio Buitrago, François Noireau, and Simone Frédérique Brenière, Representación IRD en Bolivia, Avenida Hernando Siles #5290, esquina Calle 7 de Obrajes, CP 9214 – 00095, La Paz, Bolivia, Telephone: +591 (2) 278 29 69, Fax: +591 (2) 278 29 44, E-mail: etienne.waleckx@ird.fr

REFERENCES

1. Mazza S, 1943. Comprobaciones de *Triatoma platensis*, *Eutriatoma oswaldoi*, *Panstrongylus seai* y *Psammolestes coreodes* en la Provincia de Santiago del Estero, todos ellos sin infestación y de *Eutriatoma sordida* con infestación por *S. cruzi*. Otros datos sobre infestación esquizotripanósica natural silvestre de *Triatoma infestans*. *Prensa Med Arg 30:* 1-23.

2. Velasquez CJ, González G, 1959. Aspectos de la enfermedad de Chagas en Paraguay. *Rev Goiana Med 5:* 357-373.

3. Barretto MP, Siqueira AF, Corrêa FMA, 1963. Estudos sôbre reservatórios e vetores silvestres do *Trypanosoma cruzi*. I. Encontro do *Triatoma infestans* em ecótopos silvestres. *Rev Inst Med Trop Sao Paulo 5:* 289-293.

4. Bejarano JFR, 1967. Estado selvático de *T. infestans* y otros aspectos a tener en cuenta para la eliminación de la enfermedad de Chagas. *2da Jorn Entomoepidemiol Arg 3:* 171-196.

5. Cichero JA, Gimenez AL, Martinez A, 1984. Estudio de los vectores de la enfermedad de Chagas en ambientes silvestres, peridomésticos y domésticos. *Chagas 1:* 33-37.

6. Torrico RA, 1946. Hallazgo de *Eratyrus mucronatus*, infestación natural de vinchucas de cerro y *Eutriatoma sordida* en Cochabamba. *An Lab Central Cochabamba 1:* 19-23.

7. Dujardin JP, Tibayrenc M, Venegas E, Maldonado L, Desjeux P, Ayala FJ, 1987. Isozyme evidence of lack of speciation between wild and domestic *Triatoma infestans* (Heteroptera, Reduviidae) in Bolivia. *J Med Entomol 24:* 40-45.

8. Bermudez H, Balderrama F, Torrico F, 1993. Identification and characterization of sylvatic foci of *Triatoma infestans* in Central Bolivia. *Am J Trop Med Hyg 49 (Suppl):* 371.

9. Usinger RL, Wygodzinsky P, Ryckman RE, 1966. The biosystematics of Triatominae. *Annu Rev Entomol 11:* 309-330.

10. Schofield CJ, 1988. Biosystematics of the Triatominae. Service MW, ed. Biosystematics of Haematophagous Insects. Oxford, UK: Clarendon Press, 284-312.

11. Noireau F, Cortez MR, Monteiro FA, Jansen AM, Torrico F, 2005. Can wild *Triatoma infestans* foci in Bolivia jeopardize Chagas disease control efforts? *Trends Parasitol 21:* 7-10.

12. Cortez MR, Emperaire L, Piccinali RV, Gurtler RE, Torrico F, Jansen AM, Noireau F, 2007. Sylvatic *Triatoma infestans* (Reduviidae, Triatominae) in the Andean valleys of Bolivia. *Acta Trop 102:* 47-54.

13. Buitrago R, Waleckx E, Bosseno MF, Zoveda F, Vidaurre P, Salas R, Mamani E, Noireau F, Brenière SF, 2010. First report of widespread wild populations of *Triatoma infestans* (Reduviidae, Triatominae) in the valleys of La Paz, Bolivia. *Am J Trop Med Hyg 82:* 574-579.

14. Waleckx E, Salas R, Huamán N, Buitrago R, Bosseno MF, Aliaga C, Barnabé C, Rodriguez R, Zoveda F, Monje M, Baune M, Quisberth S, Villena E, Kengne P, Noireau F, Brenière SF, 2011. New insights on the Chagas disease main vector *Triatoma infestans* (Reduviidae, Triatominae) brought by the genetic analysis of Bolivian sylvatic populations. *Infect Genet Evol 11:* 1045-1057.

15. Noireau F, Flores R, Gutierrez T, Dujardin JP, 1997. Detection of sylvatic dark morphs of *Triatoma infestans* in the Bolivian Chaco. *Mem Inst Oswaldo Cruz 92:* 583-584.

16. Bacigalupo A, Segura JA, Garcia A, Hidalgo J, Galuppo S, Cattan PE, 2006. First finding of Chagas disease vectors associated with wild bushes in the Metropolitan Region of Chile. *Rev Med Chil 134:* 1230-1236.

17. Ceballos LA, Piccinali RV, Berkunsky I, Kitron U, Gurtler RE, 2009. First finding of melanic sylvatic *Triatoma infestans* (Hemiptera: Reduviidae) colonies in the Argentine Chaco. *J Med Entomol 46:* 1195-1202.

18. Bacigalupo A, Torres-Perez F, Segovia V, Garcia A, Correa JP, Moreno L, Arroyo P, Cattan PE, 2010. Sylvatic foci of the Chagas disease vector *Triatoma infestans* in Chile: description of a new focus and challenges for control programs. *Mem Inst Oswaldo Cruz 105:* 633-641.

19. Rolón M, Vega MC, Román F, Gómez A, Rojas de Arias A, 2011. First report of colonies of sylvatic *Triatoma infestans* (Hemiptera: Reduviidae) in the Paraguayan Chaco, using a trained dog. *PLoS Negl Trop Dis 5:* e1026.

20. Noireau F, 2009. Wild *Triatoma infestans*, a potential threat that needs to be monitored. *Mem Inst Oswaldo Cruz 104:* 60-64.

21. Cortez MR, Monteiro FA, Noireau F, 2010. New insights on the spread of *Triatoma infestans* from Bolivia - implications for Chagas disease emergence in the Southern cone. *Infect Genet Evol 10:* 350-3.

22. Noireau F, Flores R, Vargas F, 1999. Trapping sylvatic Triatominae (Reduviidae) in hollow trees. *Trans R Soc Trop Med Hyg 93:* 13-14.

23. Lent H, Wygodzinsky P, 1979. Revision of the triatominae (Hemiptera, Reduviidae), and their significance as vectors of Chaga's disease. *Bull Am Mus Nat Hist 163:* 123-520.

24. Quisberth S, Waleckx E, Monje M, Chang B, Noireau F, Brenière SF, "Andean" and "non-Andean" ITS-2 and mtCytB haplotypes of *T.infestans* are observed in the Gran Chaco (Bolivia): population genetics and the origin of reinfestation. *Infect Genet Evol 11:* 1006-1014.

25. Fernandes O, Santos SS, Cupolillo E, Mendonca B, Derre R, Junqueira AC, Santos LC, Sturm NR, Naiff RD, Barret TV, Campbell DA, Coura JR, 2001. A mini-exon multiplex polymerase chain reaction to distinguish the major groups of *Trypanosoma cruzi* and *T. rangeli* in the Brazilian Amazon. *Trans R Soc Trop Med Hyg 95:* 97-99.

26. Torres-Perez F, Acuna-Retamar M, Cook JA, Bacigalupo A, Garcia A, Cattan PE, 2011. Statistical phylogeography of Chagas disease vector *Triatoma infestans*: Testing biogeographic hypotheses of dispersal. *Infect Genet Evol 11:* 167-174.

27. Piccinali RV, Marcet PL, Ceballos LA, Gürtler RE, Dotson EM, 2011. Genetic variability, phylogenetic relationships and gene flow in *Triatoma infestans* dark morphs from the Argentinean Chaco. *Infect Genet Evol 11:* 895-903.

28. Monteiro FA, Perez R, Panzera F, Dujardin JP, Galvao C, Rocha D, Noireau F, Schofield CJ, Beard CB, 1999. Mitochondrial DNA variation of *Triatoma infestans* populations

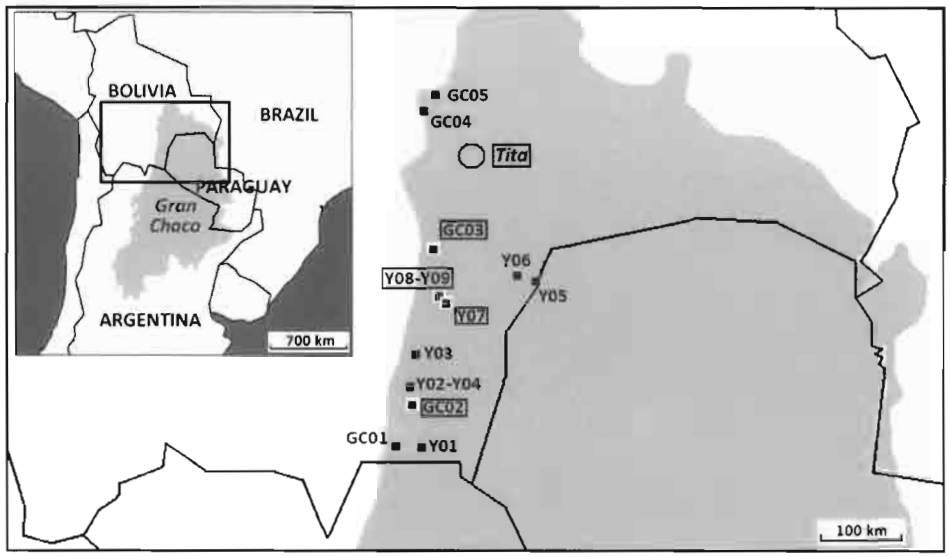
and its implication on the specific status of *T. melanosoma*. *Mem Inst Oswaldo Cruz 94:* 229-238.

29. Noireau F, Flores R, Gutierrez T, Abad-Franch F, Flores E, Vargas F, 2000. Natural ecotopes of *Triatoma infestans* dark morph and other sylvatic triatomines in the Bolivian Chaco. *Trans R Soc Trop Med Hyg 94:* 23-27.

Figure captions

FIGURE 1. Forest sites in which the search for sylvatic triatomines was carried out in the Bolivian Chaco (see also the Table). Sylvatic areas: 1 = Y01; 2 = GC01; 3 = GC02; 4 = Y02 + Y04; 5 = Y03; 6 = Y07 + Y08 + Y09; 7 = Y05; 8 = Y06; 9 = GC03; 10 = GC04; 11 = GC05; Tita = only one with sylvatic *T. infestans* previously recorded in the Bolivian Chaco. [15] Circled names: positives for sylvatic *T. infestans*.

FIGURE 2. Hollow tree of the Bolivian Chaco, positive for *T. infestans*. Typical ecotope in which sylvatic triatomines were collected in the current fieldwork.



Forest sites in which the search for sylvatic triatomines was carried out in the Bolivian Chaco (see also the Table). Sylvatic areas: 1 = Y01; 2 = GC01; 3 = GC02; 4 = Y02 + Y04; 5 = Y03; 6 = Y07 + Y08 + Y09; 7 = Y05; 8 = Y06; 9 = GC03; 10 = GC04; 11 = GC05; Tita = only one with sylvatic *T. infestans* previously recorded in the Bolivian Chaco. 15 Circled names: positives for sylvatic *T. infestans*.
250x146mm (96 x 96 DPI)

Hollow tree of the Bolivian Chaco, positive for *T. infestans*. Typical ecotope in which sylvatic triatomines were collected in the current fieldwork.
147x197mm (96 x 96 DPI)

Table: Geographical localization of the investigated sylvatic areas in the Bolivian Chaco and triatomine captures

Sylvatic area	Department	Nearest village	Forest site code	Distance to the nearest human habitat (m)	Latitude (S)	Longitude (W)	Altitude (m)	Number of traps	Number of positive traps (%)	Number and species of caught triatomines				
										T. infestans	*T. sordida*	*P. geniculatus*	Undetermined species[a]	Total
1	Tarija	Caiza	Y01	6850	21° 50' 50.6"	63° 14' 51.6"	442	40	4 (10.0)		1		3	**4**
2	Tarija	La Quinta - Caiza	GC01	850	21°49' 56.0"	63°33'15.2"	605	48	0 (0.0)					**0**
3	Tarija	Cueva de Leon	GC02	810	21° 22' 37.0"	63° 21' 34.8"	347	61	8 (13.1)	4	4		1	**9**
4	Tarija	Taiguati	Y04	570	21° 10' 34.0"	63° 23' 05.5"	471	41	2 (4.9)		2			**2**
			Y02	760	21° 09' 25.3"	63° 22' 30.7"	488	20	1 (5.0)		1			**1**
5	Chuquisaca	Machareti	Y03	2840	20° 49' 06.4"	63° 19' 18.8"	646	20	0 (0.0)					**0**
6	Santa Cruz	San Antonio	Y07	110	20° 15' 06.8"	62° 57' 46.5"	581	45	4 (8.9)	8	2		2	**12**
			Y08	8555	20° 12' 02.3"	63° 01' 21.4"	578	49	7 (14.3)	8			3	**11**
			Y09	1500	20° 10' 15.9"	63° 01' 22.7"	615	60	11 (18.3)	17	1		13	**31**
7	Santa Cruz	17 de noviembre	Y05	170	20° 00' 48.3"	61° 54' 11.2"	352	22	0 (0.0)					**0**
8	Santa Cruz	Yanahigua	Y06	260	19° 56' 09.1"	62° 07' 35.8"	385	15	0 (0.0)					**0**
9	Santa Cruz	San Lorenzo	GC03	450	19° 38' 54.3"	63° 06' 46.1"	697	63	6 (9.5)	1	5		1	7
10	Santa Cruz	Estancia Basillo	GC04	2200	18° 06' 55.9"	63° 12' 57.5"	543	48	0 (0.0)					**0**
11	Santa Cruz	Sinai	GC05	1200	17° 56' 24.0"	62° 65' 6.07"	272	48	3 (6.3)		1	2	1	**4**
Total								**580**	**46 (7.9)**	**38**	**17**	**2**	**24**	**81**

[a] all the triatomines in this column were nymphs whose species has not been genetically confirmed

Acta Tropica 117 (2011) 131–136

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

A multimodal bait for trapping blood-sucking arthropods

Julien Ryelandt[a], François Noireau[b,c], Claudio R. Lazzari[a,*]

[a] Institut de Recherche sur la Biologie de l'Insecte, UMR CNRS 6035 - Université François Rabelais, Tours, France
[b] Institut de Recherche pour le Développement (IRD), UR016, Montpellier, France
[c] IIBISMED, Facultad de Medicina, Universidad Mayor de San Simon, Cochabamba, Bolivia

ARTICLE INFO

Article history:
Received 17 March 2010
Received in revised form 7 November 2010
Accepted 10 November 2010
Available online 27 November 2010

Keywords:
Lure
Trap
Monitoring
Haematophagous
Triatominae
Ticks

ABSTRACT

Artificial baits constitute important tools for the detection and sampling of blood-sucking arthropods, in particular those that are vectors of parasites affecting human health. At present, many different devices have been proposed to attract blood-sucking arthropods, mostly based on the attractiveness of particular chemicals or blends. However, most of them revealed themselves as unpractical (e.g. they require an electrical supply), expensive (e.g. gas bottles) or not efficient enough. On the other hand, the use of living baits is as effective but it has practical constraints and/or raises ethical questions. We present here a multimodal lure to attract blood-sucking arthropods designed taking into account both practical constraints and costs. The main characteristics of our bait are: (1) artificiality (no living-host); (2) multimodality (it associates heat, carbon dioxide and chemical attractants); (3) independency from any energy source; (4) no need for gas bottles; (5) easy to prepare and use in the field; (6) low cost. We tested the ability of the bait to attract blood-sucking arthropods in the laboratory and in the field, using capture sticky-traps. Our bait evinced to be almost as efficient as live hosts (mice) for the capture of Chagas disease and *Borrelia* vectors in Bolivia. The multimodal lure here presented is a generalist bait, i.e. effective for attracting different haematophagous species.

1. Introduction

The detection and capture of disease vectors in both, domestic and wild habitats, constitute main strategies for the study and control of the diseases that they transmit.

Different methods based on the exploitation of behavioural responses of blood-sucking insects are being used for a long time ago (Lumsden, 1958). Some of them are physical devices providing refuge (e.g. Gomez-Nuñez, 1965; Wisnivesky-Colli et al., 1987; Vazquez-Prokopec et al., 2002), whereas others employ different types of lures, either living or artificial (e.g. Guerenstein et al., 1995; Lorenzo et al., 1998; Noireau et al., 1999; Lourenço-de-Oliveira et al., 2008; Anderson et al., 2009). Refuge-like sensors reveal as very useful for long-term surveillance and do not need any kind of maintenance. Insects are not attracted, but when they encounter the device, they may use it as a refuge or leave traces of their passage inside (e.g. excrements, exhuviae). Baited devices are more useful for rapid detection, due to their ability to attract insects by means of chemical or physical lures, or a combination of both. They are usually more complex than the first one; their source of attractants remains active for relatively shorter periods and they need in some cases an energy source.

Triatoma infestans and *Rhodnius prolixus* are the main vectors of *Trypanosoma cruzi*, the causative agent of Chagas disease, in Latin America. To capture them, different types of traps are used, in some cases including live hosts, e.g. mice, rats, rabbits, guinea pigs, chicks, hens and chickens, as bait (Rabinovich et al., 1976; Tonn et al., 1976; Carcavallo, 1985; Noireau et al., 1999). Numerous studies showed that haematophagous arthropods, including triatomine bugs, find their hosts detecting the emission of heat, carbon dioxide and odours emitted by their bodies (see reviews by Guerenstein and Lazzari, 2009 and Lazzari, 2009). In the present work, we describe a multimodal bait, delivering different potentially attractive signals for haematophagous arthropods. In order to assess the effectiveness of this bait, we tested its ability to capture triatomine bugs, which are vectors of Chagas disease, in the laboratory and in wild natural environment.

2. Materials and methods

2.1. Lure description

The lure consisted on a combination of sources of heat, carbon dioxide and volatiles. Heat production was obtained by means of iron oxidation (exothermic reaction) by mixing 5 g of iron powder,

* Corresponding author at: Institut de Recherche sur la Biologie de l'Insecte, Faculté des Sciences et Techniques, Avenue Monge, Parc Grandmont, 37200 Tours, France.
E-mail address: claudio.lazzari@univ-tours.fr (C.R. Lazzari).

0001-706X/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.actatropica.2010.11.005

steel wool or workshop sawdust (oxidation substrate), 1 g activated charcoal (to increase the oxidation surface), 5 g vermiculite or sand (heat conservation), 3 g NaCl (table salt, to slow down oxidation) and 2.5 g tap water. Five grams of the mixture were put into a pocket made of aluminium foil, which constituted the heat source. By making a few holes with a needle in the aluminium foil just before use, air entry initiated iron oxidation. The temperature and duration of heat production could be adjusted by changing the total amount and the proportion of each individual component, as well as their granulometry. Provided that the adequate amounts depend on the employed components, users should conduct some tests to find out the right proportions.

The CO_2 was produced by means of a simple acid–base reaction combining citric acid and sodium bicarbonate in a proportion of 1:3, in the presence of water. A soaked piece of filter paper was separated from the powder by an aluminium foil previously pierced with a needle. This provides a humid environment for the reaction to occur, but assure a slow CO_2 release. The concentration of CO_2 was maintained above the perception threshold of triatomines, which is 300–400 ppm over the environment (Barrozo and Lazzari, 2004a).

As volatiles, we employed a compound mixture, which combined with CO_2 revealed as highly attractive for triatomines (composition in Barrozo and Lazzari, 2004b). The mixture included L-lactic, valeric, butyric and propionic acid. Three ml of the mixture were put inside a 15 ml glass recipient, which was closed with a small cotton ball to allow a slow evaporation of volatiles for many hours.

Exact amounts can be adjusted according to the characteristics of the products employed (i.e. powders granulometry, volatile concentration), as well as the environmental temperature, in order to obtain the desired delivery period.

2.2. Traps

The traps used for testing the bait were the same described by Noireau et al. (1999), associated either with a living mouse, or with the artificial bait. In some experiments, empty traps (no bait) served as negative controls.

2.3. Laboratory tests

Two species of triatomine bugs were used. *Rhodnius prolixus* were maintained and tested in our laboratory in Tours, France, under a 12:12 h L:D illumination regime, at 26 C and 30–50% RH. Bugs were fed weekly on heparinised sheep blood using an artificial feeder (Núñez and Lazzari, 1990; Núñez et al., 1996). *Triatoma infestans* were reared and tested in Cochabamba, Bolivia, under a 12:12 h L:D illumination regime, at 25 C and 30–50% RH, and fed weekly on live hens.

Tests on *R. prolixus* were conducted in an experimental arena (100 length × 60 wide × 20 cm height), in a room kept at 25 ± 1 C under darkness.

In order to better simulate a natural situation, fifth-instar larvae and adults from both sexes were used in each tests. Eight bugs were placed in an artificial shelter made of cardboard in the centre of the arena and left 24 h for familiarization. After this period, two traps were placed at opposite ends of the arena at the evening. The experiment lasted for one night and the number of bugs captured by each trap recorded. Every experiment was repeated 10 times ($n = 8$; $k = 10$; $N = 80$). Three experimental series were done: (1) Living bait vs. Control; (2) Artificial bait vs. Control; and (3) Living vs. Artificial bait.

Laboratory tests on *T. infestans* were conducted in a similar way, but the experimental arena consisted in a 50 cm side cube made of plastic supports and tissue walls. A total of 11 replications using 8 bugs at a time ($n = 8$; $k = 11$; $N = 88$). Again, to match natural conditions, different larval instars and adults of both sexes were tested together. One experimental series was performed, comparing Artificial bait vs. Control.

2.4. Field tests

2.4.1. Sites

The two study areas were rocky outcrops made of large blocks located in the Cochabamba valley in the central zone of the eastern cordillera in the Department of Cochabamba (Bolivia). The first, situated some 100 m from the nearest house, is named "Inca wall" by the local population after its resemblance to human constructions. The second study area, which was quite close to a house, is named here as "peridomestic outcrop". These two zones were described by Cortez et al. (2007) as areas of high *T. infestans* density (Inca wall: summer: 45.1%, winter: 20.5% of positive mouse baited traps; and peridomestic zone: summer: 69.7%, winter: 29.4%). All the assays were conducted during May 2009 (beginning of the winter).

We placed two types of traps, i.e. baited either with our artificial lure or with a living mouse. The traps were installed around 06:00 pm (early night) and recovered at the following morning, around 09:00 am. The traps were deployed at random, in potential *T. infestans* habitats, in pairs (mouse, artificial bait) per type of environment, but not side by side.

2.5. Statistical analyses

Numbers of bugs captured in the experimental arenas were compared by means of a Wilcoxon test in paired experiments or with Fisher's exact test of proportion for independent samples.

Field captures by the different types of baits were compared by means of a Mann–Whitney test.

3. Results

3.1. The artificial bait

By adjusting the proportions of the different components, we have obtained a production of heat for many hours. The thermal pad containing the mixture had a size similar to that of a mouse and kept a constant temperature of around 30 C for at least 15 h (Fig. 1(a)). Changes in the proportions of the components allowed for a variation of both, the temperature and the duration of heat production. With this procedure, temperatures up to 90 C could be obtained, but the higher the temperature, the shorter the duration.

Concerning carbon dioxide production, the amount produced and the duration were adjusted by modifying, in a similar way as for heat production, the composition and the mixture humidity. Concentrations above the triatomines detection threshold (300–400 ppm over the environmental background) could be maintained for at least 9 h (Fig. 1(b)).

The release of volatiles was not measured; we just incorporated the attracting cues such as previously described (Barrozo and Lazzari, 2004b).

3.2. Laboratory experiments

We found significantly more bugs captured by baited (living or artificial) than by control traps in the experimental arenas (Figs. 2 and 3) for the two species tested, *T. infestans* (Fig. 2) and *R. prolixus* (Fig. 3(b) and (c)) (Wilcoxon test $p < 0.01$ in all cases). When both living and artificial baits were presented simultaneously, more bugs were captured by the living-baited trap than by the artificially-baited trap (Fig. 3(a), Wilcoxon test, $p < 0.05$). However, when tested

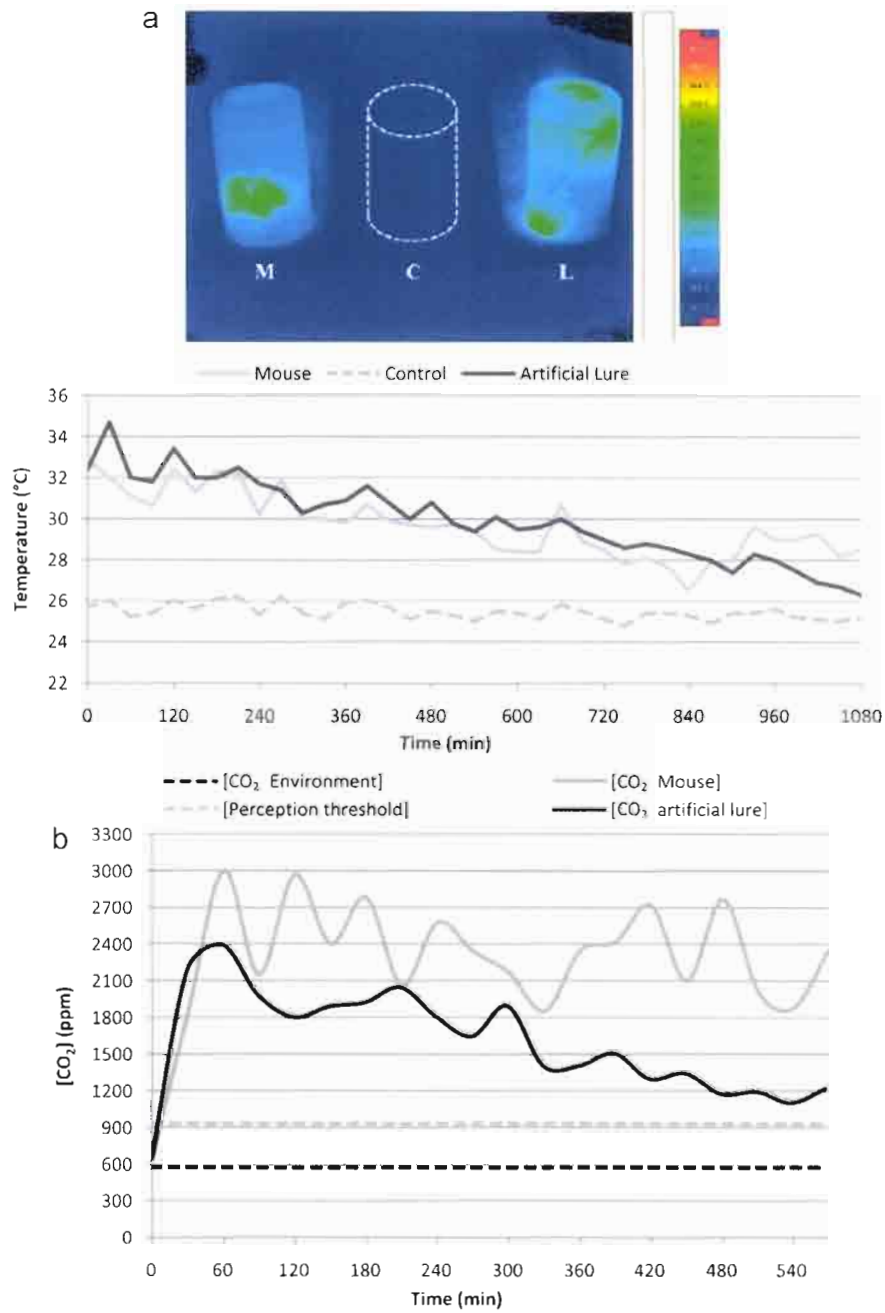


Fig. 1. Dynamics of the release of two attractive signals by a living and an artificial lure. (a) Thermographic image (PYROVIEW 380L compact camera) and variation of the surface temperature of traps associated either to the artificial lure (L), to a living mouse (M) or empty (C) as a function of the time. (b) Carbon dioxide release from traps baited either with a living mouse or the artificial lure. Dotted lines represent the environmental concentration (black line) and the perception threshold of triatomines (grey line). The gradual decrease in the temperature of the mouse can be attributed to the habituation to confinement (decreasing activity), but also to its normal resting period.

separately against a non-baited control, their efficiency was similar (Fig. 3(b) vs. Fig. 3(c), Fisher's exact test of proportion for independent samples, n.s.; *t*-test for independent samples of capture rate, n.s.).

3.3. Field experiments

In the field, mouse and artificially-baited traps were similar in capturing *T. infestans* (Mann–Whitney test, n.s.), however, the number of captured bugs was low compared to previous captures at the same time of the year (Cortez et al., 2007; Fig. 4). It should be said that the environmental temperature during the study period was also lower than usual, i.e. minimal temperatures around 5 C, which could have decreased bug's activity. Table 1 shows the proportion of instars and sex of insects captured by both kinds of traps. No bias was observed in the baits efficiency of capture.

In addition to the capture of larvae and adults of *T. infestans*, both kinds of trap captured ticks of the family Argasidae. The species has been identified as *Carios talaje* (genus *Alectorobius* or *Ornithodoros*, depending on the author), potential vector of *Borrelia mazzottii*, the causative agent of tick-borne relapsing fever (TBRF) (Goodman et al., 2005). The artificial bait was significantly more efficient than the living one for capturing this species (Fig. 4; Mann–Whitney test, n.s. for *T. infestans* and $p = 0.04$ for *C. talaje*).

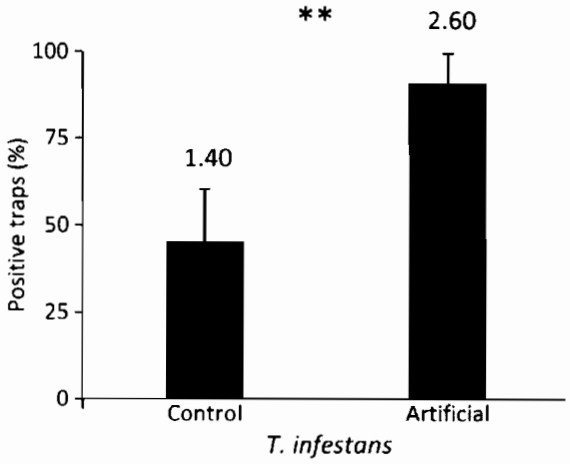


Fig. 2. Capture of *Triatoma infestans* by control and artificially baited traps tested in an experimental arena. Numbers over the bars indicate the average of captured individuals by each trap. Wilcoxon-test, $p = 0.0053$.

4. Discussion

By using a simple, low-cost bait (combining heat, carbon dioxide and attractive odours), we were able to capture three species of haematophagous arthropods under laboratory controlled conditions (*R. prolixus* and *T. infestans*) and in natural environment (*T. infestans* and *C. talaje*).

Indirect efficiency comparison of the living and the artificial baits showed that they have a similar capacity for attracting blood-sucking arthropods. When tested in competition, a better performance of the living bait was obtained. This result indicates that when a living hosts is available, our bait would be less preferred (Fig. 3). So, when used for detecting bugs, the living bait would be more efficient if no host is in the close proximity.

Field sampling using the artificial bait only allowed us to capture a low number of bugs. Nevertheless, similar low efficiency capture was also observed for the living bait (no significant difference, Fig. 4). It should be noted that field experiments were conducted during the month of May (middle autumn), when the weather was particularly cold, affecting the sampling of bugs. In fact, in the Andean region at more than 2500 m, nights are cold and the temperature can decrease below 0 C. Some studies showed that the spontaneous activity of *T. infestans* is maximal around 25 C and much lower at 20 C (Lazzari, 1992). This dependence of activity on the environmental temperature has been related with reduced capture *T. infestans* at about 18 C in Argentina (Lorenzo et al., 1998), even though bugs are able to feed on warm-blooded hosts at relatively low temperatures in confined habitats (Gürtler et al., 2009). This could be related to the microclimate in the proximity of hosts where heat-radiation might warm-up the surroundings. Small rodents (mice) or our bait, emit much less heat than chicken

Table 1
Proportion of bugs of each instar and sex captured by traps associated to the artificial and the live bait

	Chemical	Mouse
First-instar	0	0
Second-instar	0.2	0
Third-instar	0.2	0.375
Fourth-instar	0.2	0
Fifth-instar	0 2	0.125
Males	0 2	0.25
Females	0	0.25
Total	1	1

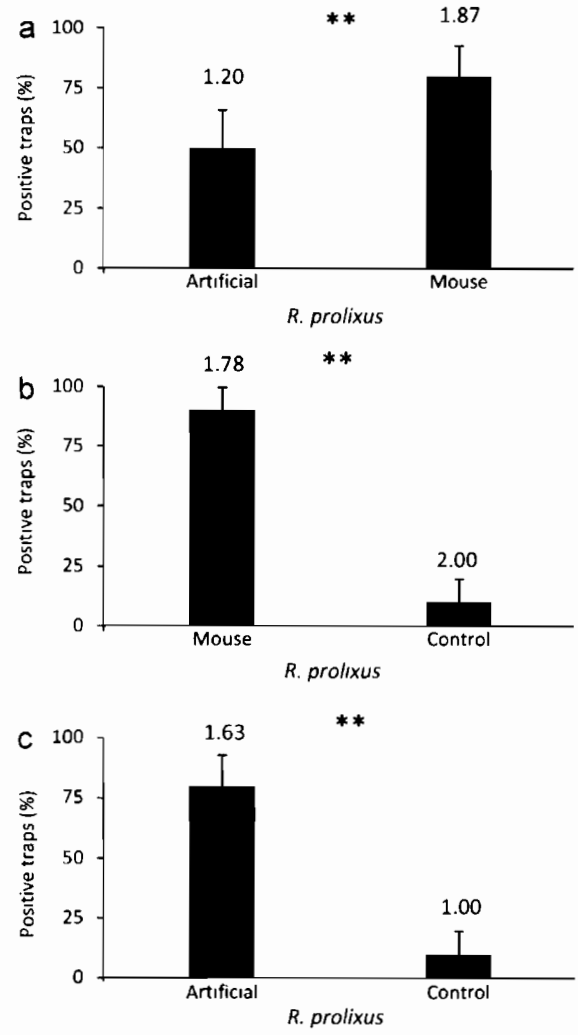


Fig. 3. Comparative tests of the efficiency of artificially- and living-baited traps for capturing *Rhodnius prolixus*. (a) Presented together, the living-baited trap revealed as more efficient than the artificial one (Wilcoxon-test, $p = 0.03$). Tested separately against a non-baited trap, both types of baited traps were significantly attractive for bugs (b, living bait, $p = 0.008$, c, artificial bait, $p = 0.008$). When the attractiveness of the baited traps against a control is compared, both revealed to behave similarly (Fisher's exact test of proportion for independent samples, n.s.; *t*-test for independent samples of capture rate, n.s.) Numbers over the bars indicate the average of captured individuals by each trap.

or dogs and are therefore not capable of increasing the air temperature around them enough to allow bugs to remain active. The radiation should be sufficient, however, to attract those bugs that are relatively close or able to display some activity despite low temperatures. Previous experiments showed that capture rates around 20–30% of positive traps could be obtained in the same seasons as in this study but on warmer years (Cortez et al., 2007).

Despite the low number of captures, both traps were similar in capturing either nymphs or adults of *T. infestans*. However, some instars, like the first one, were not captured on any trap. Given the generalist character of the stimuli offered by the artificial bait trap compared to the complexity of a live host, it is highly improbable that the ability capture of the artificial bait was biased to any particular instar or sex. Besides bugs, both, the artificial and living bait traps were able to capture ticks. These were not the aimed target of our tests, but their capture confirms the attractiveness of our bait for blood-sucking arthropods and reveals its potential as a "generalist" lure for different species of haematophagous arthropods.

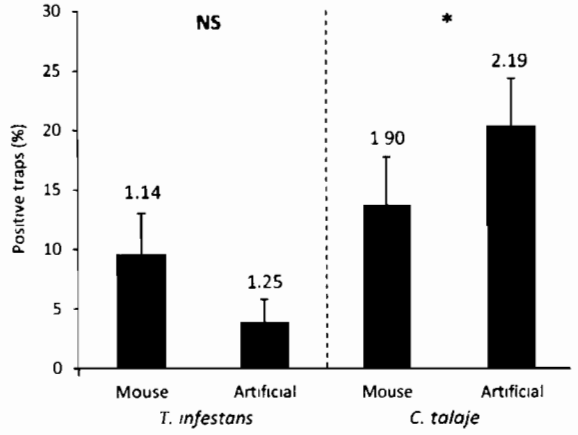


Fig. 4. Comparative efficiency of living- and artificially-baited traps for the capture of *Triatoma infestans* and *Carios talaje* in wild environments. Mann–Whitney test, n.s. for *T. infestans* and $p = 0.04$ for *C. talaje*. Numbers over the bars indicate the average of captured individuals by each trap

The capture of different arthropod species is not completely surprising, because we have chosen for composing our artificial bait not only the most powerful, but also the most ubiquitous components perceived by different vector species. The design of the trap, however, should be adapted to the specific kind of blood-sucking arthropod intended to be captured (e.g. pitfall traps, gluing surfaces, etc.). In any case, however, it is important to adapt them to a relevant behavioural context. By combining attractive elements acting in the same biological context (in our case, host-search), synergic effects and multimodal convergence should increase the attractiveness of the stimuli complex (Lazzari, 2009). Combining cues that are attractive in different behavioural contexts (e.g. host-search, refuge search, dispersion), the risk, is the loss of attractiveness due to the lack of a biological meaning of the combination for insects (Lazzari, 2009).

Of course, the utilization of a live host as a bait renders a trap as highly attractive, and in particular, the use of mice allows for quick implementation in field campaigns. Nevertheless, it requires rearing facilities or the maintenance of hosts in the laboratory. In addition, their transport over long distances or long periods imposes constraints and practical difficulties for providing the adequate care conditions. Our artificial bait represents a good alternative for replacing living hosts. It is also cost-effective in the sense that it solves adequately the trade-off between making an effective bait, but keeping it simple to prepare and utilize, as well as relatively cheap.

On the other hand, the use of living hosts imposes a minimal size and particular form to the traps, in order to keep them comfortable enough. The artificial bait presented here can be adapted to any size, form and position of traps, allowing their use in places not suitable for host-baited traps.

Summarizing, artificial baits constitute important tools for the detection and sampling of blood-sucking arthropods, in particular of disease vectors. Different models have been proposed, where suitability and cost are highly variable. Our bait has been designed not only for optimizing attractiveness, but taking also into account practical and budgetary constraints for potential users, as well as a potential suitability for a large spectrum of haematophagous targets. The constraints that we imposed for our bait were to: (1) be fully artificial (no living-host); (2) be multimodal; (3) include a heat source independent of any electric energy source; (4) produce carbon dioxide in an autonomous fashion (no gas bottle); (5) be easy to prepare and to use in the field; (6) which cost should be kept as reduced as possible; and (7) be effective for attracting different blood-sucking arthropods. Even if more laboratory and field tests are necessary to fully assess its potential, the bait presented here appears as a cost-effective artificial alternative. Other multimodal artificial baits have been proposed (e.g. Anderson et al., 2009), which revealed to be as effective for capturing haematophagous insects as ours. Nevertheless, they request to be plugged to the electric line and/or depend on the use of gas bottles, which significantly increase their cost and limit their utilization, particularly in the field or in houses where electricity is not available, such as in many areas where Chagas disease is endemic.

Acknowledgments

The authors deeply acknowledge C. Labrousse, M. Bustamante, V. Sossa and H. Calle for their valuable technical assistance during the laboratory and field work, to Philippe Parola for the identification of ticks and to J. Crespo, C. Vinauger and C. Lahondère for critical reading the manuscript and improving the English. This work was supported by the Agence Nationale de la Recherche (ANR), the Centre National de la Recherche Scientifique (CNRS), and the University of Tours (France).

References

Anderson, J.F., Ferrandino, F.J., McKnight, S., Nolen, J., Miller, J , 2009 A carbon dioxide, heat and chemical lure trap for the bedbug, *Cimex lectularius* Med. Vet. Entomol. 23, 99–105.

Barrozo, R.B., Lazzari, C.R., 2004a. The response of the blood-sucking bug *Triatoma infestans* to carbon dioxide and other host odours. Chem. Senses 29, 319–329.

Barrozo, R.B., Lazzari, C.R , 2004b. Orientation behaviour of the blood-sucking bug *Triatoma infestans* to short-chain fatty acids: synergistic effect of L-lactic acid and carbon dioxide. Chem Senses 29, 833–841.

Carcavallo, R.U., 1985. Técnicas de estudio de triatominos en ambiente silvestre. In: Carcavallo, R.U., Rabinovich, J.E., Tonn, R.J. (Eds.), Factores Biológicos y Ecológicos en la Enfermedad de Chagas, vol. 1. OPS-ECO/MSAS-SNCH, Buenos Aires, pp. 49–52

Cortez, M.R., Emperaire, L., Piccinali, R.V., Gurtler, R.E., Torrico, F., Jansen, A.M., Noireau, F., 2007. Sylvatic *Triatoma infestans* (Reduviidae, Triatominae) in the Andean valleys of Bolivia. Acta Trop. 102, 47–54.

Gomez-Nuñez, J.C., 1965. Development of a new method for the evaluation of house infestation by *Rhodnius prolixus*. Acta Cient. Venez. 16, 26–31.

Goodman, J.L., Dennis, D.T., Sonenshine, D E., 2005. Tick-Borne Diseases of Humans. ASM Press, xv + 401 pp.

Guerenstein, P.G., Lazzari, C.R., 2009. Host-seeking: how triatomines acquire and make use of information to find blood. Acta Trop. 110, 148–158.

Guerenstein, P.G., Lorenzo, M.G., Núñez, J.A , Lazzari, C.R., 1995. Baker's yeast, an attractant for baiting traps for Chagas' disease vectors. Experientia 51, 834–837.

Gurtler, R.E., Ceballos, L.A., Ordóñez-Krasnowski, P., Lanati, L.A., Stariolo, R., Kitron, U., 2009. Strong host-feeding preferences of the vector *Triatoma infestans* modified by vector density: implications for the epidemiology of Chagas disease. PLoS Negl. Trop. Dis. 3 (5), e447, doi:10.1371/journal.pntd.0000447.

Lazzari, C.R., 1992. Circadian organization of locomotion activity in the hematophagous bug *Triatoma infestans*. J. Insect Physiol. 38, 895–903.

Lazzari, C.R., 2009. Orientation towards hosts in haematophagous insects: an integrative perspective. Adv. Insect Physiol. 37, 1–58.

Lorenzo, M.G., Reisenman, C.E., Lazzari, R., 1998. *Triatoma infestans* can be captured under natural climatic conditions using yeast-baited traps. Acta Trop. 70, 277–284.

Lourenço-de-Oliveira, R., Lima, J.B.P., Peres, R., da Costa Alves, F , Eiras, A.E., Torres Codeço, C., 2008. Comparison of different uses of adult traps and ovitraps for assessing dengue vector infestation in endemic areas J Am. Mosq. Contr. 24, 387–392.

Lumsden, W.H.R., 1958. A trap for insects biting small vertebrates. Nature 181, 819–820.

Noireau, F , Flores, R., Vargas, F., 1999. Trapping sylvatic Triatominae (Reduviidae) in hollow trees. Trans. R Soc. Trop. Med. Hyg. 93, 13–14.

Núñez, J.A , Lazzari, C.R., 1990. Rearing of *Triatoma infestans* Klug (Het Reduviidae) in the absence of a live host. 1. Some factors affecting the artificial feeding. J Appl. Entomol. 109, 87–92.

Núñez, J.A., Insausti, T.C., Lazzari, C.R., 1996. Rearing of *Triatoma infestans* Klug (Het. Reduviidae) in the absence of a live host. 2. Egg yolk as a diet supplement. J. Appl. Entomol. 120, 541–547.

Rabinovich, J.E., Carcavallo, R.U., Barreto, M.P., 1976. Ecological methods: marking, trapping and sampling for vector studies in the field. In: New Approaches in

American Trypanosomiasis Research. PAHO Scientific Publication 318, Washington, pp. 16–20.
Tonn, R.J., Otero, M A., Jimenez, J., 1976. Comparación del método hora-hombre con la trampa Gomez-Nuñez en la búsqueda de *Rhodnius prolixus*. Bol. Dir. Malar. Saneam. Ambiental 16, 269–275.
Vazquez-Prokopec, G.M., Ceballos, L.A., Salomon, O.D., Gurtler, R.E., 2002 Field trials of an improved cost-effective device for detecting peridomestic populations of *Triatoma infestans* (Hemiptera: Reduviidae) in rural Argentina. Mem. I. Oswaldo Cruz 97, 971–977.
Wisnivesky-Colli, C., Paulone, I., Perez, A., Chuit, R., Gualtieri, J., Solarz, N , Smith, A., Segura, E.L., 1987. A new tool for continuous detection of the presence of triatomine bugs, vectors of Chagas disease, in rural households Medicina-Buenos Aires 47, 45–50.

Annexe 10

Habitat et écologie des triatomes et sources alimentaires

Habitats - Lors de la recherche systématisée de *T. infestans* sauvages dans les 7 écorégions, la description de l'environnement (essentiellement couverture du sol) a été faite au niveau du paysage de chaque site de travail, de chaque transect, de chaque groupe de pièges et enfin pour chaque site où les pièges ont été posés. Dans le cas des variables relevées au niveau des pièges, l'analyse discriminante (programme R) permet d'évaluer les différences d'habitat potentiel en fonction des écorégions. La figure 1 montre que les habitats de l'écorégion du Gran Chaco (5) sont très nettement différenciés des autres écorégions. De plus les habitats des écorégions andines « Bosque Seco Interandino et Prepuna (9 et 10) où les populations sylvestres sont abondantes, sont relativement semblables entre elles et différentes des autres écorégions. Les analyses fines à différentes échelles des facteurs liés à la distribution des insectes restent cependant à faire.

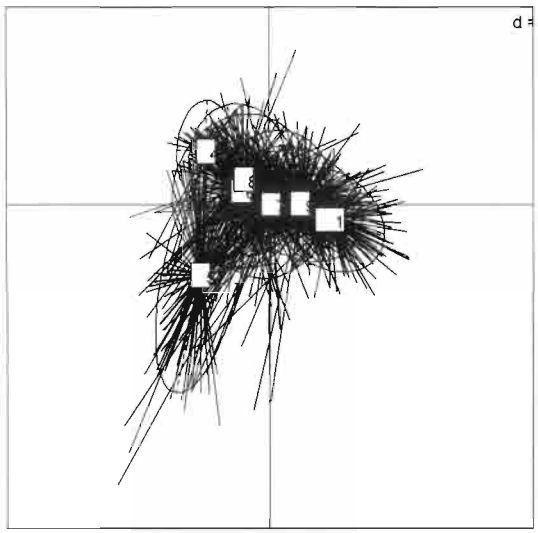


Figure 1 : Les étiquettes représentent le barycentre des ellipses de probabilité de chaque écorégion.

Des nouveaux habitats naturels et artificiels de *T. infestans* sauvages ont été identifiés dans la partie andine et sont présentés ci-dessous (Figures 2 et 3).

Falaises sédimentaires; Sapini, vallée de Sapahaqui du dpt. de La Paz

Champs de figues de Barbarie; Pena Colorada, vallée de Luribay dpt. de La Paz

Figure 2. Nouveaux habitats naturels de T. infestans *dans les vallées andines du département de La Paz*

Pierriers naturels; Huayhuasi, vallée Luribay Lacayani, Rio La Paz dpt. La Paz

Crevasses profondes dans sédiments; Aucani, vallée du Rio La Paz du dpt. de La Paz

Il est particulièrement important de signaler que dans l'espace sylvestre mais aussi dans les villages, l'homme procure aux triatomes de nouveaux habitats en accumulant des tas de cailloux débarrassés des champs de culture.

Tas de pierres entreposés dans les champs et dans les villages

Murs de pierres séparant les champs

Ancienne caserne en terre du 18ème siècle

Figure 3. Habitats artificiels de T. infestans *en milieu sylvestre aménagés par l'homme*

Stabilité et déplacement des populations sylvestres – Le suivi entomologique durant un an de la population sylvestre dispersée dans un champ semi-anthropisé proche du village de Huayhuasi (la Paz), par la méthode de capture-marquage-recapture (25 temps de piégeage), a permis de montrer que l'infestation est importante (figure 4), qu'au cours de l'année la population est relativement stable et que les colonies s'établissent dans certains sites durant toute l'année comme le montre la fréquence de capture positive au cours des essais (figure 5). La méthode de capture-marquage-recapture a permis d'identifier des déplacements de nymphes comme d'adultes.

Figure 4. *Terrain expérimental à flanc de montagne ; Huayhuasi, vallée du Rio La Paz, Dpt. La Paz ; d'aout 2009 à aout 2010, 25 essais de piégeage ont été effectués.*

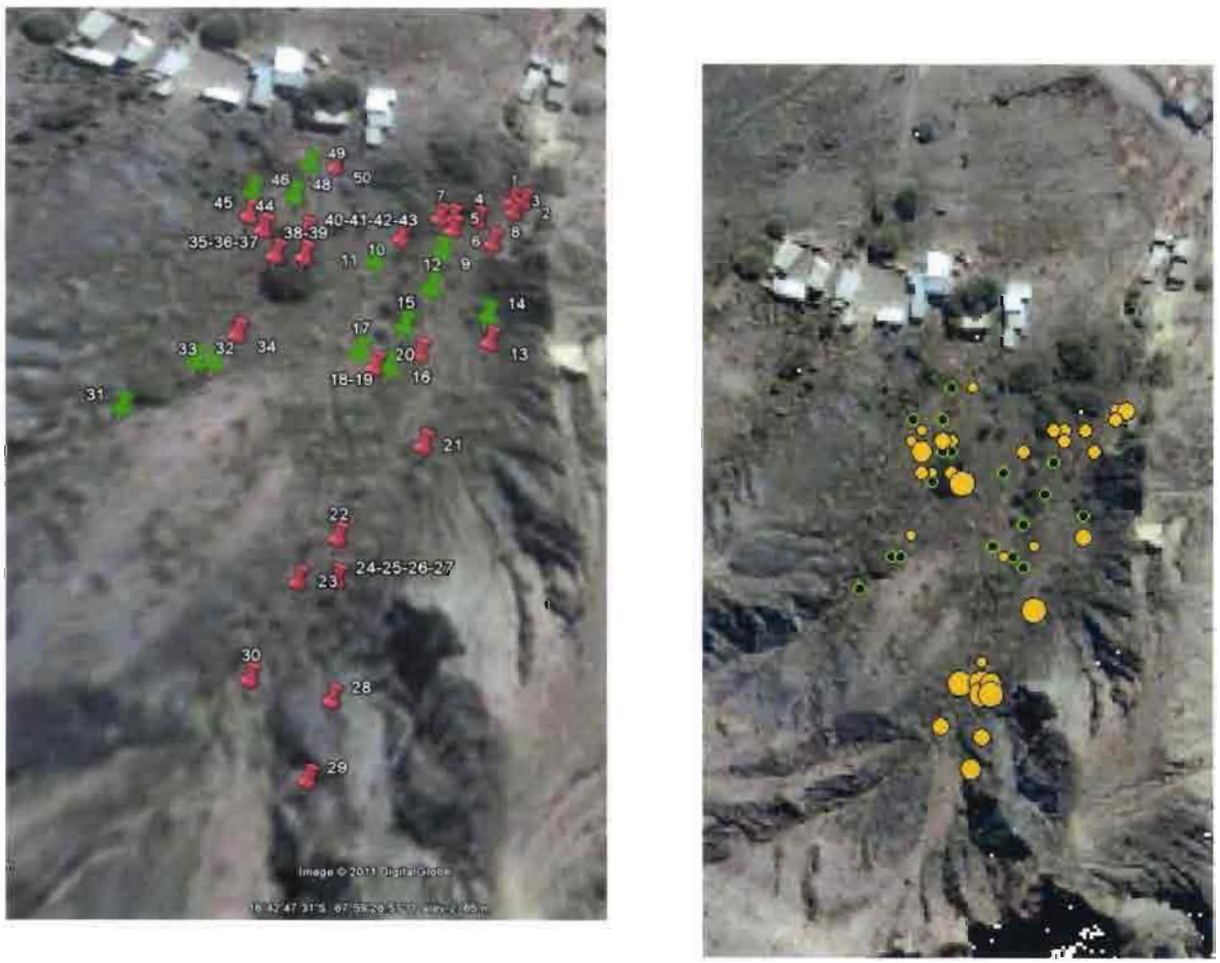


Figure 5. Au cours de chaque essai, 50 pièges sont positionnés dans 50 sites choisis au début de l'étude à gauche : en rouge, les sites au moins une fois positif (76%); en vert, les sites toujours négatifs. à droite : les cercles en jaune sont proportionnels aux nombres d'essais positifs dans chaque site.

Au cours des 25 essais un total de 138 recaptures de triatome a été obtenu. Le tableau 1 montre que les nymphes comme les adultes se déplacent sur la zone d'étude. Etant donné que les adultes capturés lors d'un essai ont été remis après marquage personnalisé sur le site de leur capture, certains spécimens ont été recapturés plusieurs fois au cours de l'année. Ainsi la figure 6 illustre les déplacements record d'une femelle qui a été recapturée 7 fois s'étant déplacée vers la partie haute de la zone (pierrier) puis ayant redescendu dans la partie basse au bout de 318 jours.

Tableau 1. Capture-marquage-recaptures de *T. infestans* sylvestres au cours de 25 essais de piégeage effectués durant un an dans un champ semi anthropisé de la vallée de Mecapaca (Dpt. La Paz)

	Stades de développement					
	3	**4**	**5**	**Femelle**	**Mâle**	**total**
Nb d'insectes marqués	93	67	73	40	28	301
Nb de recaptures	32	30	30	33	13	138
Nb de recaptures dans un même site	24	24	26	15	7	96
Nb de recaptures dans un site différent	8	6	4	18	6	42

Figure 6. Déplacement d'une femelle recapturée 7 fois ; 2 fois dans le même site, 5 fois dans un site différent. La dernière recapture dans la zone basse du champ d'étude a été effectuée 318 jours après son marquage.

Infection, Genetics and Evolution xxx (2011) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Combination of cytochrome b heteroduplex-assay and sequencing for identification of triatomine blood meals

Rosio Buitrago [a,b,*], Stéphanie Depickère [a,b], Marie-France Bosseno [a,b], Edda Siñani Patzi [b], Etienne Waleckx [a,b], Renata Salas [a,b], Claudia Aliaga [a,b], Simone Frédérique Brenière [a,b]

[a] MIVEGEC (Maladies Infectieuses et Vecteurs: Ecologie, Génétique, Evolution et Contrôle), Université de Montpellier 1 et 2, CNRS 5290, IRD 224, Institut de Recherche pour le Développement (IRD), Representation in Bolivia, Av. H. Siles # 5290, CP 9214, La Paz, Bolivia
[b] Instituto Nacional de Laboratorios de Salud (INLASA), Laboratorio de Entomología Médica, Rafael Zubieta #1889, Miraflores, Casilla M 10019, La Paz, Bolivia

ARTICLE INFO

Article history:
Received 9 July 2011
Received in revised form 30 August 2011
Accepted 8 September 2011
Available online xxxx

Keywords:
Blood meal
Heteroduplex
Triatomines
Cytochrome b
Sequencing

ABSTRACT

The identification of blood meals in vectors contributes greatly to the understanding of interactions between vectors, microorganisms and hosts. The aim of the current work was to complement the validation of cytochrome b (*Cytb*) heteroduplex assay (HDA) previously described, and to add the sequencing of the *Cytb* gene of some samples for the identification of blood meals in triatomines. Experimental feedings of reared triatomines helped to clarify the sensitivity of the HDA. Moreover, the sequencing coupled with the HDA, allowed the assessment of the technique's taxonomic level of discrimination. The primers used to produce DNA fragments of *Cytb* genes for HDA had a very high sensitivity for vertebrate DNAs, rather similar for mammals, birds and reptiles. However, the formation of heteroduplex depended on blood meal's quality rather than its quantity; a correlation was observed between blood meals' color and the positivity of HDA. HDA electrophoresis profiles were reproducible, and allowed the discrimination of blood origins at the species level. However, in some cases, intraspecific variability of *Cytb* gene generated different HDA profiles. The HDA based on comparison of electrophoresis profiles is a very useful tool for screening large samples to determine blood origins; the subsequent sequencing of PCR products of *Cytb* corresponding to different HDA profiles allowed the identification of species whatever the biotope in which the vectors were captured.

1. Introduction

Triatomines are hematophagous insects of epidemiological importance, because most of them are vectors of *Trypanosoma cruzi*, the agent of Chagas disease. The identification of blood meal origins in triatomines contributes to the understanding of their feeding habits, both in natural and human habitats (Carneiro Freitas et al., 2005; Bosseno et al., 2006; Caranha et al., 2006). It gives an insight into vector-host interactions in relation to transmission cycles of the parasite (Brenière et al., 2004). In the 1980's immunological techniques were developed to identify blood meals in hematophagous vectors, such as the complement fixation test based on the detection of host antibodies (Staak et al., 1981) and enzyme-linked immunosorbent assay (ELISA) that specifically detects host immunoglobulins, through the development of specific conjugates of potential host genus or species (Chow et al., 1993). These techniques are laborious and need the elimination of undesirable cross-reactions (Hunter and Bayley, 1996). Moreover, they have low applicability in studies with wild vectors because of the difficulty of obtaining specific conjugates for wild host species. More recently, several tools based on DNA analysis techniques have been developed to identify blood meals origins. Many of them consist in multiplex-PCR that can rapidly discriminate between potential hosts by molecular weights of PCR products (Mota et al., 2007). Nevertheless, these techniques assume the development of specific primers for species, genera or other taxonomic levels based on known DNA sequences. Other techniques such as cytochrome b heteroduplex-assay (*Cytb*-HDA) (Boakye et al., 1999; Lee et al., 2002; Kirstein and Gray, 1996) or partial sequencing of cytochrome oxidase I (*COI*) and *Cytb* genes (Townzen et al., 2008), have the advantage of being exhaustive. Previous studies showed that the *Cytb*-HDA was very useful to discriminate blood meals (Njiokou et al., 2004; Bosseno et al., 2006) because it detects differences between DNA sequences of species closely related or not (Tang and Unnasch, 1995). Moreover, using this technique and subsequent cloning and sequencing of PCR products, Bosseno et al. (2009) identified multiple blood meals in triatomines.

In the present study we further studied the *Cytb*-HDA combined with the sequencing of PCR products to identify blood meals of triatomines collected in any environment. The specificity and sensitivity of the technique were investigated. Experiments with artificial feeding of triatomines also helped to clarify the limits of

* Corresponding author. Address: Representation IRD in Bolivia, avenida Hernando Siles #5290, esquina Calle 7 de Obrajes, CP 9214, 00095 La Paz, Bolivia. Tel.: +591 2 278 29 69; fax: +591 2 278 29 44.
E-mail address: rosiob2002@yahoo.com (R. Buitrago).

1567-1348/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.meegid.2011.09.005

the method. The interpretation of HDA profiles was strongly highlighted by the analysis of current sequences with GenBank ones to examine the taxonomic level of HDA discrimination. Also recommendations are suggested for the processing of triatomines in the field to facilitate these molecular analyses in the laboratory.

2. Materials and methods

2.1. Harvesting blood meals

Blood meals were obtained in different ways: (i) by abdomen dissection and removing the entire digestive track with forceps, (ii) abdominal pressure to get the blood contents, after cutting the terminal part of the abdomen when the bugs are fully engorged. Instruments (scissors, forceps) must be cleaned with a 10% sodium hypochlorite solution and rinsed with distilled water between each sample. Samples were stored at −20 °C or diluted V/V with a solution of 6 M Guanidine-HCl, 200 mM EDTA for storage at room temperature or at 4 °C.

2.2. DNA extraction and polymerase chain reaction (PCR)

The extraction of DNA from blood meal samples was performed with the QIAamp DNA mini kit (Quiagen, Courtaboeuf, France), according to the recommended protocol for blood samples with minor modifications: a maximum volume of 200 μl of blood meal was processed and samples <200 μl were diluted in PBS to a final volume of 200 μl. In the final step, DNA was eluted with distilled water in a final volume of 50 or 30 μl for blood meal samples <20 μl. DNAs from animal blood or tissue were extracted using the same kit according to the manufacturer's recommendations. The quantification and purity of DNA was performed by measuring the absorbance at 260 and 280 nm, using a Biomate 3 spectrophotometer (Thermo Electron Corporation, Madison, WI, USA). A first PCR amplification of a 355 bp *Cytb* fragment (PCR1-*Cytb*) was achieved with the set of primers previously described (Lee et al., 2002): F-5′-CCCCTCAGAATGATATTTGTCCTCA-3′ and R-5′-CCATCCAACATCTCAGCATGATGAAA-3′, in 50 μl of reaction mixture containing 50 mM of Tris–HCl (pH 8.5), 50 mM of NaCl, 1.5 mM of $MgCl_2$, 200 μM of each dNTP, 0.2 μM of each primer, 2.5 U of Taq polymerase (Gotaq Flexi, Promega, Madison, Wisconsin, USA), and 5 μl of DNA template. Amplification was performed on a Mastercycler (Eppendorf, Hamburg, Germany) under the following conditions: starting step of 95 °C for 3.5 min, 36 cycles (95 °C, 30 s; 55 °C 50 s; 72 °C, 40 s) followed by an extension step at 72 °C for 5 min. The PCR products were then used for HDA. A second multiplex PCR amplification of *Cytb* (PCR2-*Cytb*) proposed to discriminate human (315 pb) and other mammal (420 pb) blood feedings was applied to animal DNAs to test their sensitivity and specificity with the following set of primers: DC-*Cytb*-UP 5′-CRT GAG GMC AAA TAT CHT TYT-3′, DC-*Cytb*-DW 5′-ART ATC ATT CWG GTT TAA TRT-3′ y H-*Cytb*-DW 5′-AGG AGA GAA GGA AA GAA AT-3′ (Mota et al., 2007) under the same conditions previously described for PCR1-*Cytb*. Five microliter of each PCR product were analyzed by electrophoresis in a 1% agarose gel using EZ-Vision™ as staining solution and visualized under UV light. The intensity of each amplified products was also estimated.

2.3. Sensitivity and specificity of PCR

To evaluate the sensitivity and specificity of the PCR assays, different amounts of DNA templates (1 fg to 100 ng) obtained from several vertebrate species, mammal, birds, reptiles and triatomines species (*Triatoma infestans* and *Triatoma sordida*), were tested.

2.4. Heteroduplex-assay (HDA)

The heteroduplex DNA chains were generated by mixing 5 μl of PCR1-*Cytb* products of each sample with 5 μl of PCR1-*Cytb* products obtained from known DNA chosen as a driver (human DNA), and 8 μl of distilled water. The heteroduplex formation was performed either by using a thermocycler Mastercycler (Eppendorf, Hamburg, Germany) under the following conditions: 94 °C for 5 min, 28 °C for 1 h and 4 °C for 10 min, or by DNA denaturation (5 min boiling at 94 °C) and cooling at laboratory temperature (below 25 °C). The heteroduplex products were diluted in EZ-Vision™ buffer (2 μl) and separated on a pre-cast NuPAGE 5% Bis–Tris acrylamide gel in Tris-Borate EDTA buffer according to the manufacturer's instructions (Invitrogen, Carslbad, California, USA). The electrophoresis conditions were 100 V for 3 h and the banding was examined under UV light. Blood meal origins were identified by comparing the DNA patterns between them and with those formed with the DNAs of known vertebrate species.

2.5. Sequencing of PCR1-Cytb products

The PCR products were directly sequenced by the company Macrogen (Seoul, Korea). Then Blast n-searches in GenBank permitted the identification of the level of identity with known sequences, and subsequently the species corresponding to the blood meals.

2.6. Experimental feeding of reared *T. infestans*

Colonies of *T. infestans* were reared in the insectarium under controlled conditions (26 °C and 60–70% humidity). A first protocol was designed to assess the minimum amount of blood meal that can be identified by testing the PCR1-*Cytb*-HDA. Thirty 5th nymphal instars of *T. infestans* were fed on mouse (*Mus musculus*) during 1–49 min after 45 days of fasting. The bugs numbered from 1 to 20 had received partial feedings ranging between 10 and 70 mg of blood. The ten others (21–30) were left on the mice until they no longer wanted to eat and got 182–524 mg of blood. Eight days post feeding, all bugs were processed.

A second protocol was designed to evaluate how long after feeding blood meals can be identified by PCR1-*Cytb*-HDA. Thirty 5th nymphal instars of *T. infestans* were fed on mouse for partial meals during 5–10 min. Twenty, 30 and 45 days post feeding, 10 specimens were processed in each group. For each specimen, the volume of blood ingested was estimated by the difference between the weight before and after feeding. Similarly, before processing each bug was weighed and the color of the food source was registered.

2.7. Origin of natural populations of triatomines

A total of 258 blood meals of *T. infestans* captured in domestic (151) and wild environments (107) in various Bolivian departments were processed by PCR1-*Cytb* and by HDA for PCR positive samples. The details of the results (geographic origin of the samples, relationship between ecotopes where the bugs were collected and blood meal origins) of the total sample will be exposed elsewhere; here 2–5 PCR1-*Cytb* products corresponding to the different HDA profile detected among the total sample were sequenced in order to analyze the relationship between HDA patterns and blood meal origins.

2.8. Data analysis

Correlations between HDA results (positive/negative) and the insects' weights before feeding, weight increase after feeding expressed by the ratio weight after feeding/weight before feeding, and weight at sacrifice expressed by the ratio weight at sacrifice/

Please cite this article in press as: Buitrago, R., et al. Combination of cytochrome b heteroduplex-assay and sequencing for identification of triatomine blood meals. Infect. Genet. Evol. (2011), doi:10.1016/j.meegid.2011.09.005

weight before feeding, were tested the Mann and Whitney non-parametric *U* test which do not rely on assumptions that the data are drawn from a given probability distribution as normality. All data analyses were performed using SPSS v.10.0 software (SPSS, Inc., Chicago, IL).

3. Results

3.1. Specificity and sensitivity of PCRs

Serial dilutions of DNA samples purified from several mammals, reptiles, triatomines and one bird were tested using the PCR1-*Cytb* set of primers. A band of 355 bp was observed for all DNAs (data not shown). However, the sensitivity of the PCRs was variable according to the DNAs (Table 1). The PCR1-*Cytb* had a similar sensitivity of 10 fg of DNA for all tested mammals except for donkeys for which it was 100 fg. For the bird tested (*Gallus gallus*) the sensitivity was rather similar. For reptiles, the PCR was only positive with templates of 1–10 ng of DNA. The positivity of PCR with DNAs from triatomines was surprising; nevertheless, the sensitivity was clearly lower than for vertebrates (50–100 ng). The primers were aligned with different GenBank sequences in order to evaluate their complementarities (Table 2). The forward and reverse PCR1-*Cytb* primers presented only one mismatch with the human complementary regions, 1–4 mismatches with those of other vertebrates except for *Alsophis rufiventris* where 7 mismatches were observed with the reverse primer. For *T. infestans*, 3 and 7 mismatches were observed; for *T. sordida* the homologous *Cytb* fragment was not available in GenBank.

The same set of DNAs was tested by the multiplex PCR2-*Cytb* (Table 1). As expected, one band of 315 bp was obtained with human DNA and one band of 420 bp with other mammal DNA. Nevertheless, similar bands (420 bp) were obtained for the bird, reptiles and triatomines species. The sensitivity of the reaction was similar for the different DNAs (range: 1 ng to100 pg). The primers were also aligned with GenBank sequences. The forward primer presented an absence of mismatch or only 1 in vertebrates and 2 mismatches in triatomine, while the reverse primer showed 1–3 mismatches in vertebrates and only 2 in triatomine (Table 2). The HDA was not applied with PCR2-*Cytb* products because the sensitivity was equal with vertebrates and triatomine DNAs.

3.2. Experimental feeding of reared T. infestans and blood meal detections by Cytb-HDA

On the 8th day of fasting PCR1-*Cytb* was positive for all samples, being the evidence of one intense band of 355 bp as expected. The HDA results using as driver human-*Cytb* DNA are summarized in Fig. 1 and a typical acrylamide gel shows the different HDA patterns (Fig. 2). In general terms, the faster bands correspond to single DNA chains; the slower bands of about 1000 bp correspond to homoduplex formed by homologous DNA chains from a single species; between these bands are the two heteroduplex molecules formed by the combination of the single chains of homologous sequences from 2 different species (one from the driver, the other from the blood sample). Usually heteroduplex chains migrate differently depending on the number, type and position of the mismatches present in the heteroduplex molecule; their electrophoresis' migration is specific to the combination of species 1 with species 2. In the present experiment, all HDA patterns were identical and the blood origin of a mouse except for two samples for which an absence of heteroduplex formation was observed (Figs. 1 and 2); these samples corresponded to two bugs with small blood meals of 10 mg (Fig. 1). The detection of the blood meal origin was possible in the large majority of the bugs even if, after 8 days of fasting, some of them had a weight rather similar to that before feeding (Fig. 1). Remarkably, all processed blood meals were reddish except the 2 ones with a negative result in HDA which had a light brown and white color, respectively. The two PCR products giving negative HDA results were sequenced, and the sequences best aligned with *T. infestans* (Accession No. EF639038, 98% identity).

Another group of 30 *T. infestans* nymphs of 5th instar was fed less than 10 min on mice to obtain partial meals and then processed after 20, 30 or 45 days of fasting. After 20 and 30 days of fasting, all blood meal samples were PCR1-*Cytb* positive (intense band of 355 bp). After 45 days, variable intensity of banding was observed, 4 out of 10 samples presented weak bands, and the others were intense bands. Among the overall sample, only 30% of HDA were positive (Fig. 3). The percentage of positive HDA was not significantly different between the groups under 20, 30 and 45 days of fasting conditions (Fig. 3, $X^2 = 0.95$, df = 2, $p = 0.62$). Similarly to the above result, 5 PCR products corresponding to negative HDA and randomly selected were sequenced. The sequences best aligned with *T. infestans* (Accession No. EF639038, 90–97% identity). In this experiment, no correlation was observed between the HDA result and, (i) the weight of the insects before feeding (Mann–Whitney *U-test:* $U = 77$, $N = 30$, $p = 0.43$), (ii) the increase of weight after feeding (Mann–Whitney *U-test:* $U = 90.5$, $N = 30$, $p = 0.86$) and (iii), the weight at sacrifice (Mann–Whitney *U-test:* $U = 51$, $N = 30$, $p = 0.051$). The observation of the blood meal color at the time of dissection showed that for the samples with positive HDA the blood meals were reddish (1 sample) or dark brown (8 samples). For the negative HDA samples the blood meals had a color ranging from light brown to white. The correlation observed between HDA results and the color of blood meals (Mann–Whitney *U-test:* $U = 0$, $N = 30$, $p < 0.001$) indicated that the changing of color was related to DNA degradation (digestion process). This experiment showed that under conditions of partial feeding, most of the bugs had digested all their blood meal after 20 days, but some

Table 1
Sensitivity of PCRs-Cytb using vertebrate and triatomine DNAs.

	DNA templates and results of PCR1-*Cytb*/PCR2-*Cytb*									
	100 ng	50 ng	25 ng	10 ng	1 ng	100 pg	1 pg	100 fg	10 fg	1 fg
Human (*Homo sapiens*)	+/+	+/+	+/+	+/+	+/+	+/+	+/−	+/−	+/nd	−/nd
Mouse (*Mus musculus*)	+/+	+/+	+/+	+/+	+/+	+/+	+/−	+/−	+/nd	−/nd
Wild guinea pigs (*Galea musteloides*)	+/+	+/+	+/+	+/+	+/+	+/+	+/−	+/−	+/nd	−/nd
Camelid (*Lama glama*)	+/+	+/+	+/+	+/+	+/+	+/+	+/−	+/−	+/nd	−/nd
Donkey (*Equus asinus*)	+/+	+/+	+/+	+/+	+/+	+/−	+/−	+/−	−/nd	−/nd
Hen (*Gallus gallus*)	+/+	+/+	+/+	+/+	+/+	+/+	+/−	+/−	−/nd	−/nd
Lizard (*Tropidurus oreadicus*)	+/+	+/+	+/+	+/+	−/+	−/+	nd/−	nd/−	nd/nd	nd/nd
Snake (*Acontias gracilicauda*)	+/+	+/+	+/+	+/+	−/+	−/+	nd/−	nd/−	nd/nd	nd/nd
Snake (*Alsophis rufiventris*)	+/+	+/+	+/+	+/+	+/+	−/+	nd/−	nd/−	nd/nd	nd/nd
Triatoma infestans	+/+	+/+	−/+	−/+	nd/+	nd/+	nd/−	nd/−	nd/nd	nd/nd
Triatoma sordida	+/+	−/+	−/+	−/+	nd/+	nd/+	nd/−	nd/−	nd/nd	nd/nd

nd: Not done; (+) PCR positive result; (−) PCR negative result.

Please cite this article in press as: Buitrago, R., et al. Combination of cytochrome b heteroduplex-assay and sequencing for identification of triatomine blood meals. Infect. Genet. Evol. (2011), doi:10.1016/j.meegid.2011.09.005

Table 2
Identification of mismatch numbers between PCR1 and PCR2-Cytb primers and GenBank sequences.

GenBank sequences		Number of mismatches			
		PCR1-*Cytb* primers		PCR2-*Cytb* primers	
Species	Accession No.	Forward	Reverse	Forward	Reverse
Human (*Homo sapiens*)	NC012920	1	1	0	1
Mouse (*Mus musculus*)	NC006914	1	2	0	2
Wild guinea pigs (*Galea musteloides*)	AY466605	2	2	0	1
Camelid (*Lama glama*)	U06429	1	3	0	1
Donkey (*Equus asinus*)	X97337	2	4	0	3
Hen (*Gallus gallus*)	NC001323	1	2	1	2
Lizard (*Tropidurus oreadicus*)	EF616030	2	3	0	2
Snake (*Acontias gracilicauda*)	DQ249093	1	3	*	*
Snake (*Alsophis rufiventris*)	FJ416730	3	7	*	*
Triatoma infestans	EF639038	3	7	2	2

*The homologous *Cytochrome b* fragments are not available in GenBank.

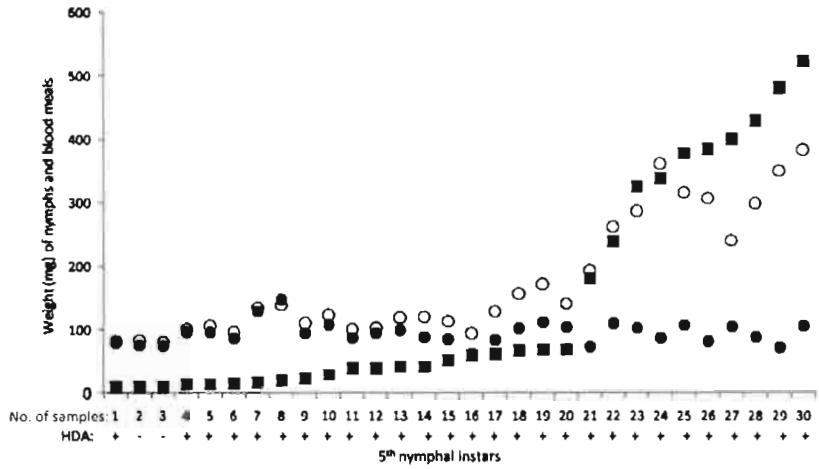


Fig. 1. Results of HDA detection of blood meals after 8 days of fasting of reared *T. infestans* bugs fed on mice (*Mus musculus*) according to the quantity of meal ingested. Estimate of bug weights before feeding, black circles; estimate of bug weights after 8 days of fasting, white circles; weight of blood meals, black squares. HDA: (+) for positive results (formation of heteroduplex), (−) for negative result (absence of heteroduplex formation).

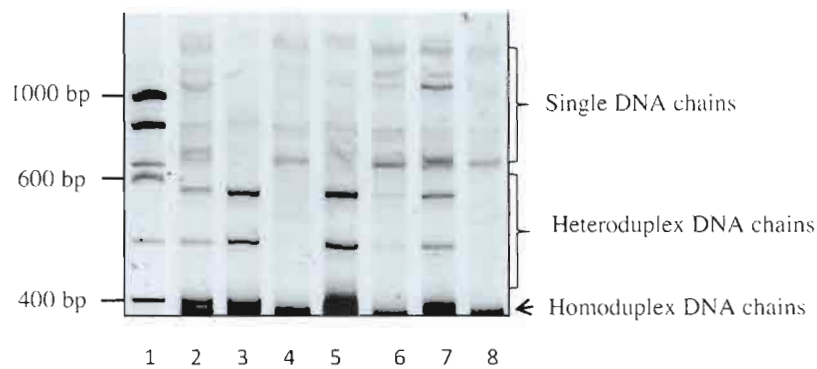


Fig. 2. Example of acrylamide electrophoresis patterns of PCR1-*Cytb*-HDA samples obtained from mice blood meals of reared *T. infestans* after 8 days of fasting. Lane 1, MW (Smart ladder); lane 5, standard pattern corresponding to a *Mus musculus* blood meal; lanes 2, 3, 6 and 7, similar heteroduplex patterns corresponding to *Mus musculus* blood meals; lanes 4 and 10, absence of heteroduplex formation.

bugs are "energy efficient" and can save some food resource even after 45 days of fasting.

3.3. HDA patterns obtained from natural populations of triatomine and interpretation

Three kinds of HDA patterns were observed when blood meals of natural populations of *T. infestans* were processed (Fig. 4): (i) a basic pattern of two bands corresponding to the formation of two heteroduplex DNA chains, when the triatomine received a single blood meal, (ii) a more complex pattern (multi banding) when the triatomines received blood meals from different sources and (iii) a pattern without bands corresponding to the heteroduplex DNA chains.

In order to further analyze the relationship between HDA patterns and blood meal origins, several PCR1-*Cytb* products

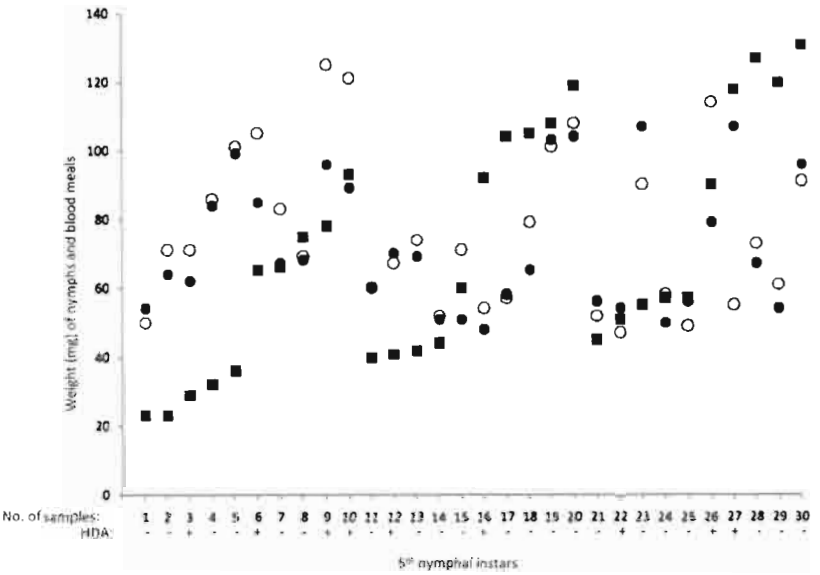


Fig. 3. Results from HDA detection of blood meals after 20 (sample No. 1–10), 30 (sample No. 11–20) and 45 (sample No. 21–30) days of fasting of reared *T. infestans* fed on mice (*Mus musculus*); estimate of bug weight before feeding, black circles; estimate of bug weight after 20, 30 and 45 days of fasting, white circles; weight of blood meals black squares. HDA: (+) for positive results (formation of heteroduplex), (−) for negative results (absence of heteroduplex formation).

corresponding to the 8 different patterns obtained from the current sample were sequenced and the results are summarized in Table 3. The sequences corresponding to the same patterns were aligned and the identity between them was always very high (99–100%). Samples with identical HDA patterns always gave sequences that best aligned with the same species. However, samples with different HDA patterns could have sequences that best aligned with a same species (Fig. 5, Table 3): this is the case of patterns A and F that corresponded to *Octodontomys gliroides* and patterns B and G that corresponded to *Galea musteloides*. In the first case the sequences of A and F HDA patterns differed between them by only one mutation and by 3–4 mutations with the sequence of *O. gliroides* deposited in GenBank. For B and G HDA patterns, the corresponding sequences differed between them by 22 mutations; the blast results showed that sequences corresponding to the G pattern best aligned with *Galea musteloides* (Accession No. GU067530), while that corresponding to the B pattern, best aligned with another sequence of *Galea musteloides* (Accession No. GU067494).

Some HDA profiles had more than two bands between those of homoduplex and single DNA chains. It was previously reported that these patterns corresponded to multi blood meal origins as it was demonstrated by cloning of PCR products (Bosseno et al., 2009). Here, the artificial mixture of PCR1-*Cytb* products obtained from DNAs of different species and from blood meals with different HDA always showed a multi banding pattern (Fig. 6).

4. Discussion

The purpose of this study was to further study and validate the *Cytb*-HDA already described (Bosseno et al., 2006, 2009), for its application to triatomines. Moreover, the direct sequencing of PCR products (*Cytb* fragment) used in the HDA was proposed for some samples corresponding to the different HDA profiles identified, in order to characterize the origin of blood meals of triatomines captured in any biotope without prior knowledge of the food hosts.

The first step was to test the sensitivity and specificity of the primers used to amplify fragments of *Cytb* (Boakye et al., 1999; Lee et al., 2002), later used to form heteroduplexes (PCR1-*Cytb* primers). The primers proposed to discriminate human from mammal blood meals were also tested (PCR2-*Cytb* primers) (Mota et al., 2007).

The first set of primers was previously applied to identify mammals and birds species (Boakye et al., 1999; Lee et al., 2002), but we showed that it can also amplify *Cytb* from reptile DNAs with a rather similar sensitivity, confirming results previously reported (Farikou et al., 2004). The ubiquity of these primers in vertebrates gives an advantage to the method since it is also possible to detect

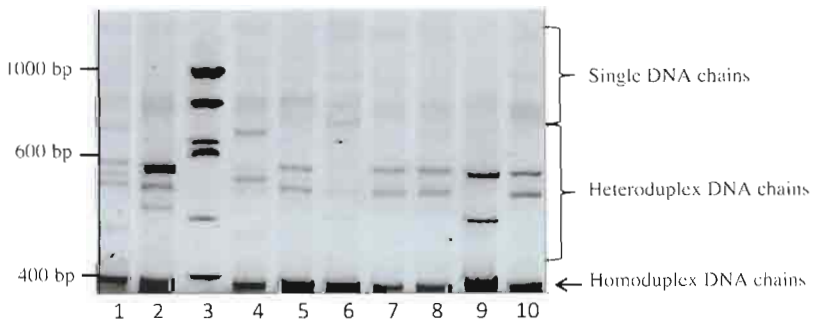


Fig. 4. Examples of acrylamide electrophoresis patterns of PCR1-*Cytb*-HDA samples obtained from natural *T. infestans*; lanes 4, 5, 7, 8, 9, and 10 show formation of heteroduplex banding (single blood meal); lanes 1 and 2 show formation of multi banding (blood meals from multiple sources); lane 6, absence of heteroduplex formation; lane 3, MW (Smart ladder).

Please cite this article in press as: Buitrago, R., et al. Combination of cytochrome b heteroduplex-assay and sequencing for identification of triatomine blood meals. Infect. Genet. Evol. (2011), doi:10.1016/j.meegid.2011.09.005

Table 3
Sequencing of PCR1-Cytb products from *T. infestans* blood meals with different HDA patterns and identification of blood sources.

HDA pattern	*T. infestans*		Size of *Cytb* sequenced fragment	Identity between pairwise sequences giving the same HDA pattern	Blast		
	Biotope origin	*N*			Species	Accession No.	Identity
A	Wild	5	289 bp	100%	*Octodontomys gliroides*	AF370706	98%
F	Wild	5	290 bp	99–100%	*Octodontomys gliroides*	AF370706	99%
G	Wild	3	264 bp	100%	*Galea musteloides*	GU067530	100%
B	Wild	2	264 bp	99%	*Galea musteloides*	GU067494	99–100%
C	Wild	4	221 bp	100%	*Homo sapiens*	NC012920.1	99%
C	Peridomiciliar	1	221 bp	100%	*Homo sapiens*	NC012920.1	99%
D	Wild	3	255 bp	100%	*Lagidium viscacia*	AY 254887	99%
E	Peridomiciliar	4	281 bp	100%	*Gallus gallus*	EU839454	100%
H	Peridomiciliar	5	288 bp	100%	*Sus scrofa*	GU135833	100%
K	Peridomiciliar	2	295 bp	100%	*Canis lupus*	EU408266	99%

HDA = heteroduplex-assay; N = number of bugs.

meals on vertebrates other than mammals. Mammals are the principal feeding hosts of triatomines, but birds, and more rarely reptiles, are also a blood source for triatomines (Gajate et al., 1996), thereby helping to maintain the colonies in peridomestic (Breniere et al., 2004) and in wild environments. Surprisingly, the set of primers also amplified the *Cytb* from triatomine DNAs (*T. infestans and T. sordida*), but with a much lower sensitivity (from 50 to 100 ng of DNA template) compared to vertebrates (1 ng to 10 fg). Indeed, the analysis of the complementarity of the primers with sequences of *Cytb* genes of mammals, birds, reptiles and *T. infestans* obtained in GenBank, showed the lowest complementarity with triatomine DNAs, a data fitting with the lower sensitivity of the PCR with triatomine DNAs. In this study, the different experiments showed that when the DNA template is extracted from the total digestive tube of the triatomine, the *Cytb* of the blood meal is amplified in priority if the blood DNA is not degraded; otherwise it is the *Cytb* of the triatomine which is amplified. Nevertheless, our results showed that the color of blood meal before processing seems to be a good indicator of the success of the HDA detection: the positivity of the HDA was correlated with reddish and dark brown blood meals. The second set of primers described for the identification of blood meal in triatomines did not show a

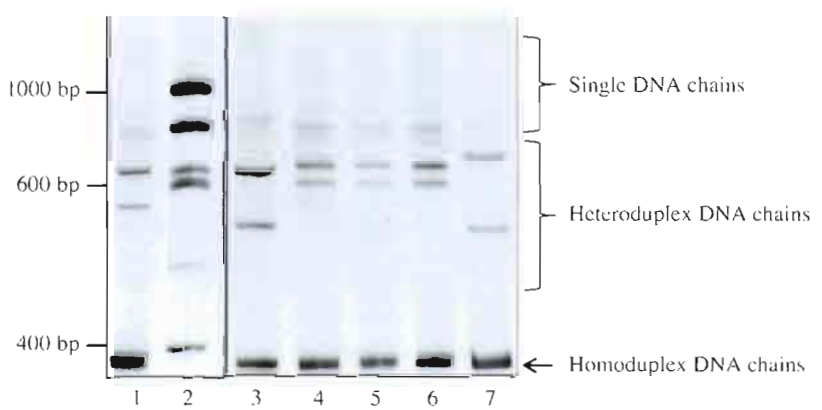


Fig. 5. Acrylamide electrophoresis patterns of PCR1-*Cytb*-HDA samples obtained from natural *T. infestans*; different patterns corresponding to blood meals from *Galea musteloides*; lane 1, pattern B; lanes 4, 5 and 6, pattern G; different patterns corresponding to blood meals from *Octodontomys gliroides* species; lane 3, pattern A; lane 7, pattern F; lane 2, MW (Smart ladder).

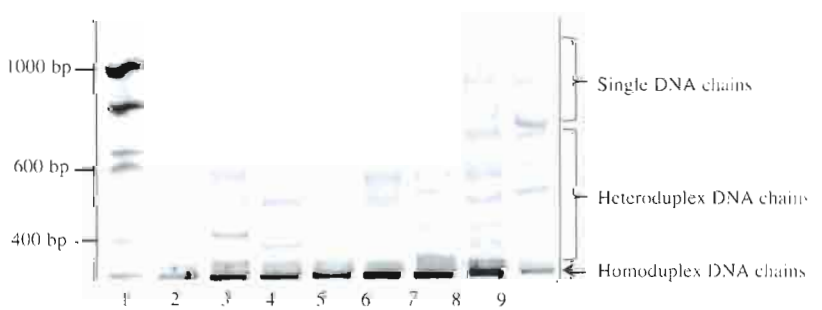


Fig. 6. Acrylamide electrophoresis of HDA using artificial mixtures of PCR1-*Cytb* products corresponding to different hosts. Samples were obtained from natural *T. infestans*: lane 1, MW (Smart ladder); lane 2, mixture of *Octodontomys gliroides*, *Gallus gallus* and *Mus musculus*; lane 3, mixture of *Octodontomys gliroides*, *Galea musteloides* and *Gallus gallus*; lane 4, mixture of *Octodontomys gliroides*, *Mus musculus* and *Sus scrofa*; lane 5, mixture of *Galea musteloides*, *Sus scrofa* and *Mus musculus*; lane 6, mixture of *Galea musteloides*, *Gallus gallus* and *Mus musculus*; lane 7, mixture of *Gallus gallus*, *Sus scrofa* and *Mus musculus*; lane 8, mixture of *Galea musteloides* and *Mus musculus*; lane 9, multi banding obtained from a natural *T. infestans*.

Please cite this article in press as: Buitrago, R., et al. Combination of cytochrome b heteroduplex-assay and sequencing for identification of triatomine blood meals. Infect. Genet. Evol. (2011), doi:10.1016/j.meegid.2011.09.005

satisfactory specificity, since with the same sensitivity, it amplified the Cytb of DNAs from vertebrates and triatomines. However, this system can detect human blood meals with certainty, because the PCR2-*Cyt b* product from human DNA has a different molecular weight (315 bp) compared to other vertebrates and triatomines (420 pb). When the working DNA template is extracted from the digestive tube of a triatomine which was fed on another vertebrate than human, a fragment of 420 bp is expected, but this product can be composed of the *Cytb* of the feeding host and also of the triatomine. For this reason, we did not apply the HDA to the *Cytb* products obtained after using this second set of primers.

Previous studies reported the HDA detection of food sources as a result of fasting as long as 30 days (Bosseno et al., 2006) or even after 10 weeks (Mota et al., 2007). In the present study, the results showed that after 8 days of fasting, 93% of the blood meals were identified whatever the volume of the blood meal, but after 20 and 45 days of fasting only 40% and 30%, respectively, were detected. The discrepancy between our results and previous ones could be due to variations in experimental conditions, as the use of different species of triatomine, stages, feeding host, and physical conditions in rearing. In our experimental conditions various parameters were analyzed: (i) the weight of the triatomine before feeding, (ii) the quantity of blood ingested by the triatomine during feeding, (iii) the duration of fasting (8, 20, 30 and 45 days), (iv) the weight of the triatomine after fasting and (v) the color of the food source at the time of dissection. The only parameter linked to the positivity of HDA, was the color of blood meals. Reddish and dark brown blood colors could correspond to intact DNA, while the lighter colors could correspond to degraded DNA generating a negative HDA.

The second step of the study was to evaluate the discriminative power of the HDA. Previous studies indicate that HDA is very discriminative; Tang and Unnasch (1997) proposed this technique for the identification of insect species even for population genetics and detection of new allele in a species. It was also used to detect a mutation in the cystic fibrosis gene that is the result of a single base substitution (White et al., 1992). Moreover, Lee et al. (2002) had already suggested the existence of some intraspecific polymorphism in *Cytb* sequences of vertebrates, detected by this method. The current results showed that the HDA always discriminates different species but an intraspecific variation was also observed. We obtained two cases of intraspecific variation: in the first case, 2 different HDA profiles corresponded to the same species *Octodontomys gliroides* although only one mutation was observed between the sequences, and in this case allelic variation can be inferred to explain the different HDA patterns. In the second case, two HDA patterns for *Galea musteloides* were observed; the corresponding sequences had 22 mutations between them, representing about 10% of divergence, and they aligned with around 100% of identity with two different sequences of *Galea musteloides* (Accession Nos. GU067530 and GU067494). In fact, a 10% sequence divergence is more likely to suspect different species and not intraspecific variation. Solmsdorff et al. (2004) described a new species in *Galea* genus, *Galea monasteriensis*, corresponding to the GU067494 sequence. Another study also based on *Cytb* gene, suggests that *G. monasteriensis* should be considered as a junior synonym of *G. musteloides boliviensis* and the GU067530 sequence would correspond to *Galea musteloides leucoblephara* (Dunnum and Salazar-Bravo, 2010). There is still no taxonomic consensus within the genus *Galea*, but these data suggest the possible detection of two different species in the sample under study.

5. Conclusion

The PCR1-*Cytb*-HDA combined with sequencing is a powerful, useful and cheap tool in epidemiological studies. The detection of blood meals with this method provides various advantages, giving valuable information with regards to triatomine movements between different ecotopes. This method can also identify potential reservoirs after the detection of *Trypanosoma cruzi* in the same blood samples, and allows the analysis of the relationships between vectors, mammals, and parasite genetic variants.

Acknowledgments

We are particularly grateful to the direction of the INLASA (Instituto de Laboratorios de Salud, La Paz Bolivia), Dr. José Raphael Gutiérrez and Dr. Walter Agreda to have hosted this work in the Department of Medical Entomology directed by Dr. Tamara Chavez. This study received financial supports from the ANR (Agence Nationale de la Recherche, France), and the IRD (Institut de Recherche pour le Développement, France).

References

Boakye, D.A., Tang, P., Truc, A., Merriweather, A., Unnasch, T.R., 1999. Identification of bloodmeals in haematophagous Diptera by cytochrome B heteroduplex analysis. Med. Vet. Entomol. 13, 282–287.

Bosseno, M.F., Garcia, L.S., Baunaure, F., Gastelum, E.M., Gutierrez, M.S., Kasten, F.L., Dumonteil, E., Breniere, S.F., 2006. Identification in triatomine vectors of feeding sources and *Trypanosoma cruzi* variants by heteroduplex assay and a multiplex miniexon polymerase Chain reaction. Am. J. Trop. Med. Hyg. 74, 303–305.

Bosseno, M.F., Barnabé, C., Sierra, M.J., Kengne, P., Guerrero, S., Lozano, F., Ezequiel, K., Gastélum, M., Brenière, S.F., 2009. Wild ecotopes and food habits of *Triatoma longipennis* infected by *Trypanosoma cruzi* lineages I and II in Mexico. Am. J. Trop. Med. Hyg. 80, 998–991.

Brenière, F., Pietrokovsky, S., Magallón, E., Bosseno, M.F., Soto, M.M., Ouaissi, A., Lozano, F., Wisneveski-colli, C., 2004. Feeding patterns of *Triatoma longipennis* Usinger (Hemiptera Reduviidae) in Peridomestic Habitats of Rural Community in Jalisco State. Mexico J. Med. Entomol. 4, 1015–1020.

Caranha, L., Seixas, E., da Silva Rocha, D., Jurberg, J., e Galvao, C., 2006. Estudo das fontes alimentares de *Pastrongylus lutzi* (Neiva y Pinto 1923) (Hemiptera Reduviidae Triatominae) no Estado de Ceará. Rev. Soc. Bras. Med. Trop. 39, 347–351.

Carneiro Freitas, S., Seixas, E., Silva Rodriguez, D., Carneiro Freitas, A., Monte Goncalves, T., 2005. Fontes alimentares de *Triatoma pseudomaculata* no Estado do Ceará Brasil. Rev. Saúde Pública 39 (1), 27–32.

Chow, E., Wirtz, R.A., Scott, T.W., 1993. Identification of bloodmeals in *Aedes aegypti* by antibody sandwich enzyme-linked inmunosorbent assay. Am. Mosq. Control Assoc. 9, 196–205.

Dunnum, J., Salazar-Bravo, J., 2010. Phylogeny, evolution, and systematics of the *Galea musteloides* complex (Rodentia: Caviidae). J. Mammal. 91, 243–259.

Farikou, O., Njiokou, F., Simo, G., Tazoacha, A., Cuny, G., Geiger, A., 2004. Tsetse fly blood meal modification and trypanosome identification in two sleeping sickness foci in the forest of southern Cameroon. Acta Trop. 116, 81–88.

Gajate, P.P., Bottazzi, M.V., Pietrokovsky, S.M., Wisnivesky-Colli, C., 1996. Potential colonization of the peridomicile by *Triatoma guasayana* (Hemiptera: Reduviidae) in Santiago del Estero. Argent. J. Med. Entomol. 33, 635–639.

Hunter, F.F., Bayly, R., 1991. ELISA for identification of bloodmeal source in black flies (Diptera Simuliidae). J. Med. Entomol. 28, 527–532.

Kirstein, F., Gray, J.S., 1996. A molecular marker for the identification for the zoonotic reservoirs of Lime borreliosis by analysis of the blood meal in the European vector *Ixodes ricinus*. Appl. Environ. Microbiol. 62, 4060–4065.

Lee, J.H., Hassan, H., Hill, G., Cupp, E.W., Higazi, T.B., Mitchell, C.J., Godsey Jr, M.S., Unnasch, T.R., 2002. Identification of mosquito avian-derived blood meals by polymerase chain reaction-heteroduplex analysis. Am. J. Trop. Med. Hyg. 66, 599–604.

Mota, J., Chacon, J.C., Gutierrez-Cabrera, A.E., Sánchez-Corderon, V., Wirtz, R.A., Ordoñez, R., Panzera, F., Ramsey, J.M., 2007. Identification of blood meal source and infection with *Trypanosoma cruzi* of chagas disease vectors using a multiplex cytochrome b polimerase chain reaction assay. Vector Borne Zoonotic Dis. 7, 617–627.

Njiokou, F., Simo, G., Mbida Mbida, A., Truc, P., Cuny, G., Herder, S., 2004. A study of host preference in tsetse flies using a modified heteroduplex PCR- based method. Acta Trop. 91, 117–120.

Staak, C., Allmang, U.K., Mehlitz, D., 1981. The complement fixation test for the species identification of bloodmeals from tsetseflies. Tropenmed. Parasitol. 32, 97–98.

Solmsdorff, K., Kock, D., Hohoff, C., Sachser, N., 2004. Comments on the genus *Galea* Meyen 1833 with description of *Galea monasteriensis* n. sp. from Bolivia (Mammalia, Rodentia, Caviidae). Senckenbergiana Biologica 84, 137–156.

Tang, J., Unnasch, T.R., 1995. Discriminating PCR artifacts using DHDA (directed heteroduplex analysis). BioTechniques 19, 902–905.

Townzen, J.S., Brower, A.v.Z., Judd, D.D., 2008. Identification of Mosquito Blood meals using mitochondrial cytochrome oxidase subunit I and cytochrome b gene sequences. Med. Vet. Entomol. 22, 386–393.

White, M.B., Carvalho, M., Derse, D., O'Brien, S.J., Dean, M., 1992. Detecting single base substitutions as heteroduplex polymorphisms. Genomics 12, 301–306.

Please cite this article in press as: Buitrago, R., et al. Combination of cytochrome b heteroduplex-assay and sequencing for identification of triatomine blood meals. Infect. Genet. Evol. (2011), doi:10.1016/j.meegid.2011.09.005

Annexe 12

Hôtes nourriciers de triatomes sylvestres et domestiques

Dans l'écorégion de « Bosques Secos Interandinos » (BSIA) un total de 97 repas sanguins de *T. infestans* sylvestres a été déterminé. Deux hôtes (petit rongeurs endémiques dans les Andes d'Amérique du sud) sont majoritaires : *Octodontomys gliroides* (46,4%) et *Galea musteloides* (25,8%) avec des variations relatives entre région; dans les vallées du département de La Paz *Octodontomys gliroides* serait plus abondant. Quatre autres espèces de petits rongeurs ont été détectées, de rares animaux domestiques (âne et chat) ainsi que de quelques oiseaux et de reptile. De plus, 5 repas sanguins sur *Lagidium viscacia* ont été identifiés; ce petit mammifère endémique en Amérique du Sud pourrait être un nouveau réservoir du parasite. Plus étonnant a été la détection de repas humains (10,3%) chez plusieurs spécimens collectés dans les mêmes sites de capture que les autres insectes. Dans l'écorégion "Prepuna" (PP) au sud des Andes, les mêmes hôtes majoritaires en BSIA ont été identifiés, *Galea musteloides* (73,0%) et *Octodontomys gliroides* (27%). Aucun repas sanguin n'a pu être identifié chez les *T. infestans* capturés dans le Gran Chaco (GC). Le tableau ci-dessous résume ces résultats.

Un total de 184 sources alimentaires a été identifié chez les *T. infestans* capturés en péri domicile. Bien évidemment, la source alimentaire dépend de la structure dans laquelle les insectes sont capturés, un poulailler, une porcherie ou une remise. Il est cependant intéressant de constater que chez quelques insectes des repas sur animaux sauvages comme *G. domurus*, *G. musteloides*, *L. viscacia* et *O. gliroides* sont détectés. Deux hypothèses sont possibles, les insectes proviennent du milieu sauvage ou alors les mammifères sauvages circulent dans le péri domicile.

Peu de données sont encore disponibles sur des insectes capturés à l'intérieur des maisons dans la mesure où ils sont rares et souvent ramassés par les habitants qui les conservent un certain temps dans leur maison ; les repas sanguins sont alors difficilement indentifiables. Cependant les premiers résultats sont plutôt en faveur d'une incursion des insectes depuis l'extérieur de la maison car ils se sont alimentés sur des animaux qui ne vivent pas dans la maison (*E. asinus*, *G. galus*, *Leiolopisma sp.*).

Tableau 1 - Hôtes nourriciers de *T. infestans* sylvestres

		BSIA										PP		
Département		La Paz					Cochabamba					Potosi		
Municipalités		Luribay	Mecapaca	Sapahaqui			Palca	Cochabamba	Quillacollo	Aiquile	Toro Toro	Tupiza	Total	%
Galea musteloides		8							17			19	25	25,8%
Octodontomys gliroides		15	11	16			1		2			7	45	46,4%
Phyllotis xanthopygus chileansis		1		1									2	2,1%
Akodon glaucinus									3				3	3,1%
Graomys domorum									1		1		2	2,1%
Phyllotis wolffsohni									1				1	1,0%
Lagidium viscacia		5											5	5,2%
Felis catus				1									1	1,0%
Equus asinus								1					1	1,0%
Homo sapiens		6	1				1		2				10	10,3%
Sublegatus modestus				1									1	1,0%
Tropidurus oreadicus											1		1	1,0%

Tableau 2 - Hôtes nourriciers de *T. infestans* capturés en péri domicile

		Bosques Secos Interandinos												Gran Chaco					
	Département	La Paz					Potosi			Santa Cruz				Tarija					
	Municipalités	Sapahaqui	Irupana	Quilliacollo			Toro Toro	Villa Abecia	Cotagaita	Charagua	San Lucas	Caiza		La Guardia	Villa montes		Total	%
Gallus gallus		5		79			7			5		8		1			105	58,7%
Meleagris gallopavo				1													1	0,6%
Capra hircus							1										1	0,6%
Canis lupus familiaris				2													2	1,1%
Sus scrofa				9			1		7		1						18	10,1%
Oryctolagus cuniculus							1										1	0,6%
Rattus rattus				9			1								5		10	5,6%
Mus musculus		1															1	0,6%
Graomys domarus		1						1									2	1,1%
Galea musteloides		1		20				2									23	12,8%
Lagidium viscacia			1	3													4	2,2%
octodontomys gliroides		1					1										2	1,1%
Homo sapiens		6		3													9	5,0%

Tableau 3 - Hôtes nourriciers de *T. infestans* capturés à l'intérieur des maisons

Municipalités		Irupana	Quillacollo	Villa Abecia	Charagua	Total	%
Equus asinus				1		1	6,7%
Galus galus					2	2	13,3%
Homo sapiens			4		7	11	73,3%
Leiolopisma sp.		1				1	6,7%

Annexe 13

Connaissance et expérience des triatomes par les habitants

Parallèlement à la recherche des populations sylvestres de triatomes, les habitants ont été interrogés afin de connaître quelle était leur expérience des triatomes. L'interrogatoire c'est fait en présentant une boîte où étaient montés tous les stades de développement de *T. infestans*. Le Tableau 1 résume les principaux résultats.

Un total de 812 personnes vivant dans les 7 écorégions étudiées a été enquêté (les effectifs variant de 19 dans les « Yungas » (YUN) à 426 dans l'écorégion « Bosques Seco Interandinos » (BSIA). La très large majorité de la population quelque soit l'écorégion reconnait l'insecte (82,35% « Bosque Seco Chiquitano » (BSC) à 100% pour YUN et le nomme « vinchuca » (94,2%).

A la question s'ils ont vu des « vinchucas » dans leur maison cette année (à l'intérieur comme dans le péri domicile) seulement 32,4% répondent affirmativement. Remarquablement, dans l'écorégion Gran Chaco (GC) les réponses affirmatives atteignent 72,5% alors que celles des autres écorégions sont toutes entre 20 ,7% et 34%. De plus, un pourcentage non négligeable d'habitants (18% en moyenne) avec un maximum de 37,5% dans le GC, ont observé des triatomes à l'intérieur de leur maison. Actuellement le GC est l'écorégions la plus fortement touchée par les phénomènes de ré infestation et les chiffres observés reflètent bien cette situation.

A la question s'ils voyaient des triatomes antérieurement, 78,2% répondent affirmativement ce qui traduit clairement l'effet positif (exception faite de GC) de la campagne nationale de control des vecteurs entreprise dès 2003.

A la question s'ils ont observé des triatomes dans la campagne, en moyenne 51% répondent oui avec un maximum de 65% dans le GC et 42,5% disent avoir vu voler les insectes occasionnellement. Exception faite de l'écorégion YUN où seulement 3 zones de piégeage ont été explorées, dans les autres écorégions des triatomes ont été capturés dans la campagne (milieu sylvestre, voir annexe 6). Parmi les réponses positives peu donnent une information précise ; la plupart disent les avoir vu dans la campagne (« cerro », « monte », « campo ») ou sur leur lieu de travail dans les champs (« potrero », « chaco », « Chacra »), cependant certains donnent une information plus précise (tableau 2) ; dans la BSIA les réponses indiquent majoritairement la présence de triatomes sous des pierres ou des rochers et dans les champs de figues de Barbarie et cactus qui sont caractéristiques des vallées andines (48,8% des réponses spécifiques). Curieusement, plusieurs réponses signalent la présence de triatomes dans les cimetières (BSIA); de fait dans cette écorégion les cimetières sont à l'extérieur des villages, non clôturés, peu entretenus mais souvent visités par les habitants. Plusieurs réponses précisent dans les tas de bois mort surtout dans le Bosque Tucumano Boliviano (BTB). Certains habitants signalent le bois de chauffe qui peut être la cause de l'introduction de triatomes dans la maison.

Tableau 1. Principales réponses des habitants concernant leur connaissance et expérience des triatomes

Réponses positives sur 812 personnes enquétées

	Ecorégions							
Questions	Bosque Seco Chiquitano 4	Gran Chaco 5	Yungas 6	Bosque Tucumano Boliviano 7	Chaco Serrano 8	Bosques Secos Interandinos 9	Prepuna 10	Total
Connaissez-vous cet insecte?	82,4%	92,5%	100,0%	97,6%	95,7%	97,2%	98,0%	96,1%
A la question comment il s'appelle, % qui disent vinchuca	58,8%	82,5%	100,0%	97,6%	95,7%	97,7%	97,0%	94,2%
L'avez-vous vu cette année dans votre domicile?	31,4%	72,5%	31,6%	25,3%	20,7%	34,0%	26,7%	32,4%
L'avez-vous vu cette année à l'intérieur de la maison?	17,6%	37,5%	21,1%	16,9%	12,0%	17,6%	17,8%	18,0%
L'avez-vous vu avant dans votre domicile?	66,7%	80,0%	89,5%	81,9%	75,0%	79,6%	75,2%	78,2%
L'avez-vous vu dans la campagne?	43,1%	65,0%	42,1%	50,6%	54,3%	54,5%	33,7%	51,0%
L'avez-vous vu voler?	41,2%	65,0%	68,4%	45,8%	57,6%	37,3%	34,7%	42,5%

Tableau 2 : Réponses à la question "où avez-vous vu des triatomes dans la campagne"

	Ecorégions								
Réponses	Bosque Seco Chiquitano 4	Gran Chaco 5	Yungas 6	Bosque Tucumano Boliviano 7	Chaco Serrano 8	Bosques Secos Interandinos 9	Prepuna 10	Total	%
Lieux non spécifiques									
cerro				1	1	40	6	48	14,5%
monte				3	3	10		16	4,8%
campo	4	15		3	9	3	4	38	11,5%
potrero				2	4	1		7	2,1%
chaco	7	1		2				10	3,0%
chacra						2		2	0,6%
Lieux spécifiques									0,0%
Sous les pierres ou rochers				8		58	5	71	21,5%
Champs de figues de Barbarie				1		32		33	10,0%
Bois mort		3		6	4	2	1	16	4,8%
Cimetière						13	2	15	4,5%
Champs de cactus						11	3	14	4,2%
Champs cultivés			8	4				12	3,6%
Arbres		3			4	3		10	3,0%
Bois de chauffe		3				6	1	10	3,0%
Broméliacées				1		6		7	2,1%
Maison abandonnée						5		5	1,5%
Enclos bétail					1		3	4	1,2%
Plantes					1	3		4	1,2%
Présence de vaches						2		2	0,6%
Feuilles mortes				1				1	0,3%
Paille				1				1	0,3%
Nid de rat					1			1	0,3%
Où vivent les "Viscachas" (*Lagidium viscacia*)						1		1	0,3%
Entrepos de mais					1			1	0,3%
Attaqué par des tnatomes					1			1	0,3%
Total	15	30	14	40	38	207	35	330	1

Annexe 14

Environnement sylvestre et cinétique d'infestation et ré infestation des villages sélectionnés dans les aires partagées

1 - Ecorégion du Gran Chaco département de Santa Cruz: Rancho Nuevo et San Silvestre

a) Stratification de l'espace

Les premières aires potentiellement partagées par l'homme et les populations sauvages de *T. infestans* sont dans le Gran Chaco les communautés de Rancho Nuevo (RN, 128 maisons initialement) et San Silvestre (SS, 23 maisons), *Capitania* d'Izozog. L'habitat est composé de petites maisons assez dispersées avec un péri domicile sans clôture. L'espace a été divisé en zone habitée et périphérie où a été effectuée la recherche de triatomes sylvestres (voir annexe 5). Les 154 habitations ont été géo référencées (GPS) afin de procéder ultérieurement à l'analyse spatiale des données.

b) Piégeage en milieu sylvestre

La recherche de *T. infestans* sylvestres (type « dark morph ») a été infructueuse en octobre 2008 dans les alentours de Rancho Nuevo et San Silvestre malgré le nombre important de pièges posés. Seul des spécimens du complexe *T. sordida* ont été capturés.

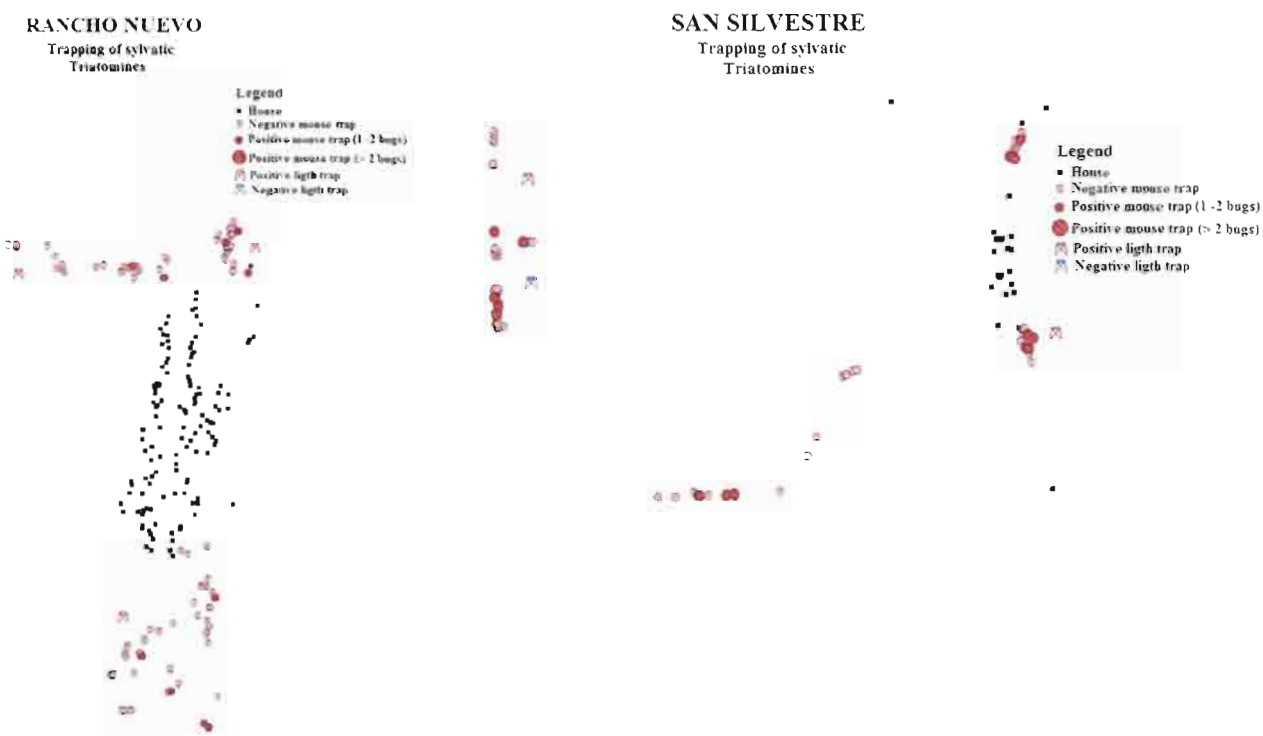


Figure 1. Captures de triatomes à l'aide de pièges dans les environs de Rancho Nuevo et San Silvestre en octobre 2008. Les points roses, pièges négatifs, points rouges pièges positif, carrés noirs, habitations ; absence de T. infestans *et capture de spécimens du complexe* T. sordida.

A Rancho Nuevo 13 triatomes ont été capturés (119 pièges posés, 8,4% des pièges positifs,) et identifiés comme *T. sordida* et *T. guasayana,* deux espèces fréquemment échantillonnées dans le Gran Chaco (Figure 1). A San Silvestre 22 triatomes ont été capturés mais de la même manière seules ces deux espèces étaient identifiées (159 pièges posés, 11,9% positifs) (Figure 1). Malgré ces premiers résultats d'autres explorations ont été menées dans les alentours de San Silvestre car le milieu environnant est moins anthropisé qu'à Rancho Nuevo procurant plus de chance de trouver des *T. infestans*. En mars 2009 une nouvelle recherche est restée infructueuse mais un an plus tard (mars 2011), 13 insectes ont été capturés dont un adulte femelle de *T. infestans* et 5 nymphes ; sept autres triatomes ont été identifiés comme *T. sordida (*Figure *2)*. Une colonie de laboratoire de *T. infestans* a pu être développée et de fait l'espèce pleinement confirmée.

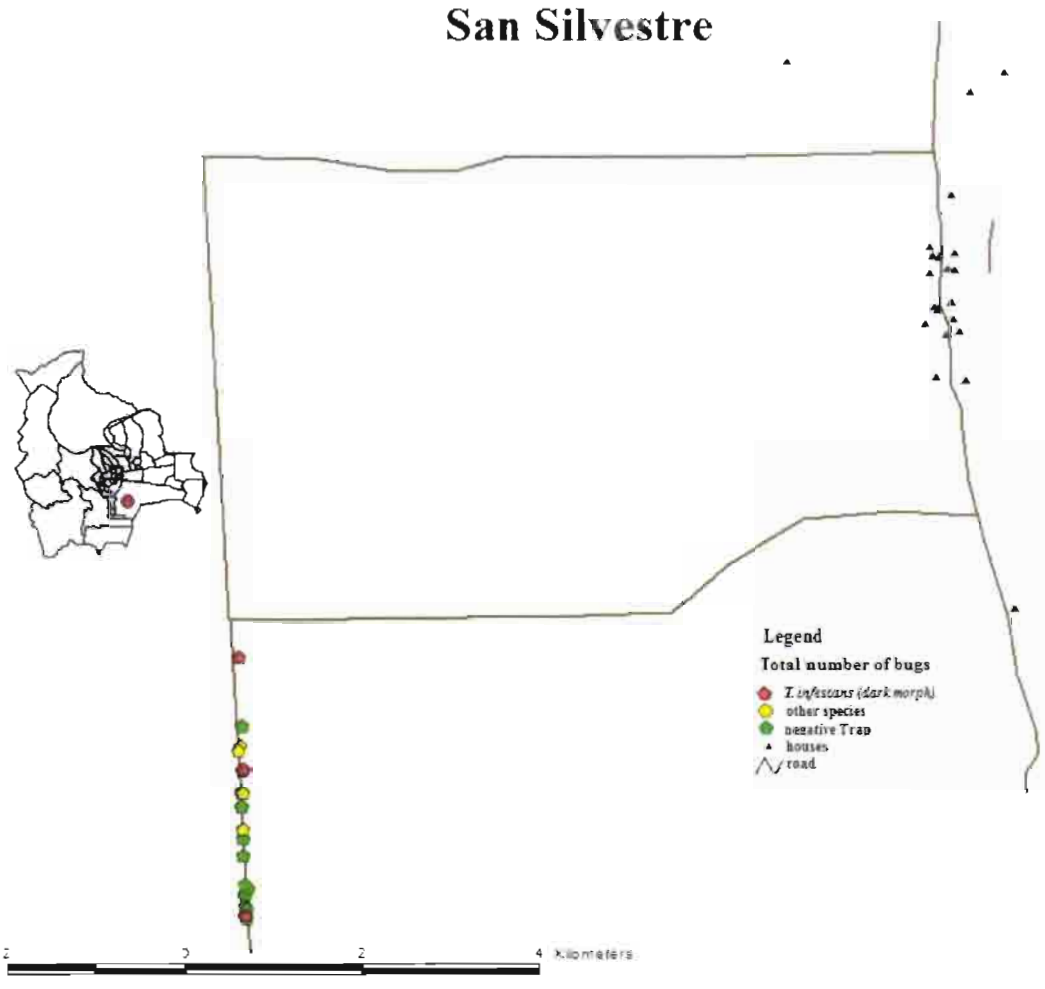


Figure 2. Sites de capture de triatomes à l'aide de pièges dans les environs de San Silvestre en mars 2011 : présence de T. infestans (rouge), site avec autre espèce (jaune) et sites négatifs (vert).

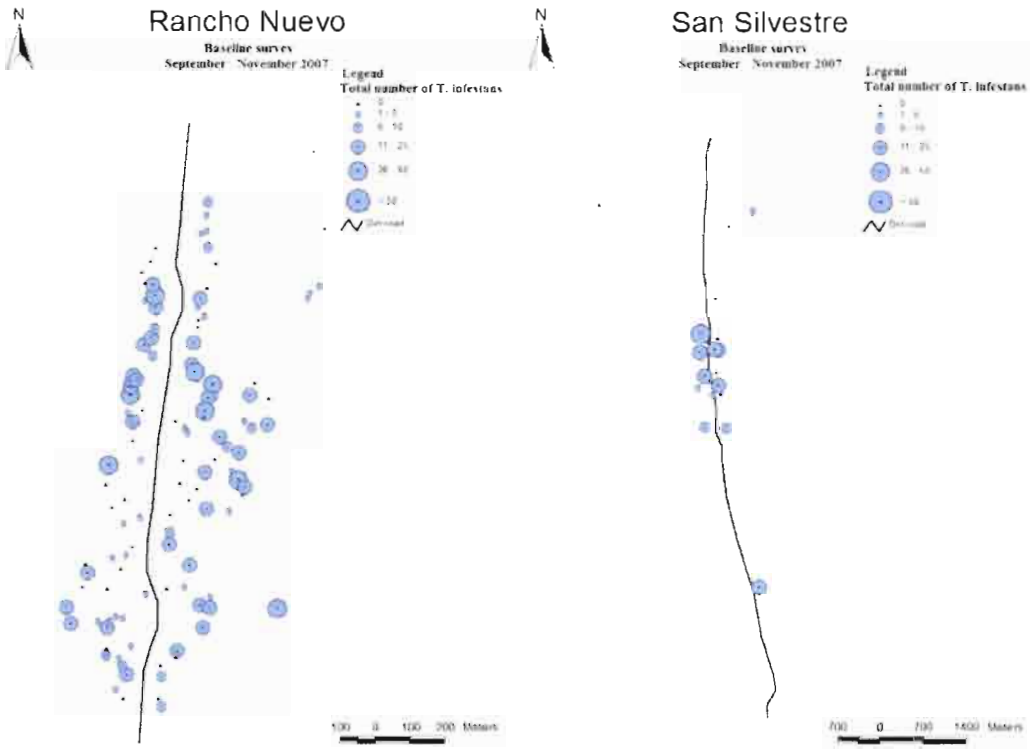
c) Zone habitée

L'étude entomologique correspondant à la ligne de base a été réalisée en novembre 2007 puis la totalité des maisons a été traitée par l'alpha-cyperméthrine (30-50 mg a.i./m^2). De nouvelles enquêtes entomologiques ont été effectuées après 5, 9, 13, 17, 21, 27, 33 et 38 mois afin d'étudier la ré infestation. Les résultats de la recherche active des triatomes dans les maisons mettent en

évidence une très forte infestation par *T. infestans* avant la fumigation qui atteint 68,2% des maisons à RN (Tableau 1, Figure 3).

Tableau 1. Résultats entomologiques de la ligne de base dans 2 villages d'Izozog (septembre - novembre 2007)

	Rancho Nuevo	San Silvestre	Total
No. de maisons	128	23	368
No. (%) de maisons infestées, intérieur*	88 (68.7)	13 (56.5)	271 (73.6)
No. (%) de maison infestées, extérieur*	11 (8.6)	5 (21.7)	78 (21.2)
No. de *T. infestans* collectés	1016	170	4054
No. (%) avec des triatomes d'invasion**	4 (3.1)	1 (4.3)	46 (12.5)



* infestation par *Triatoma infestans* ** par *Triatoma sordida* et/ou *T. guasaya*

Figure 3. Distribution des T. infestans *capturés dans les maisons (intérieur et extérieur) par recherche active avant traitement insecticide*

Le même protocole de recherche a été appliqué au cours des 8 contrôles post-fumigation en traitant de nouveau les maisons positives entre les contrôles. Ce suivi entomologique montre la diminution très nette de l'infestation intra domiciliaire mais en général des petites colonies persistent et par exemple l'infestation atteint, 19 mois après le début de l'étude (aout 2009), 6,9% à RN et 20% à SS avec un total de 52 et 4 insectes collectés dans ces villages (Figure 4). Au cours de l'évaluation de janvier 2011 (37 mois après fumigation de la totalité des villages) la ré infestation s'avère nettement plus sévère à RN même si les colonies identifiées sont plus petites (44/117, 37,6% de re infestation intra domiciliaire) ; à SS la ré infestation moins élevée reste cependant persistante (2/12, 16,6% de re infestation intra domiciliaire). Cette situation peu avoir plusieurs sources, celle du développement d'une résistance aux insecticides mais également un problème dans le processus de vigilance des villages et du traitement focalisé des maisons ré infestées car le nombre de maisons non visitées lors des contrôles (maisons fermées) est important. Beaucoup de changements ont également été notés (maisons qui deviennent déshabitées et construction de nouvelles maisons (20 à RN et 1 à SS) rendant plus difficile le suivi mais il est remarquable que dans certaines nouvelles maisons des insectes ont pu être capturés. Les figures 5A et 5B illustrent les résultats des captures tout au long de l'étude à RN et SS.

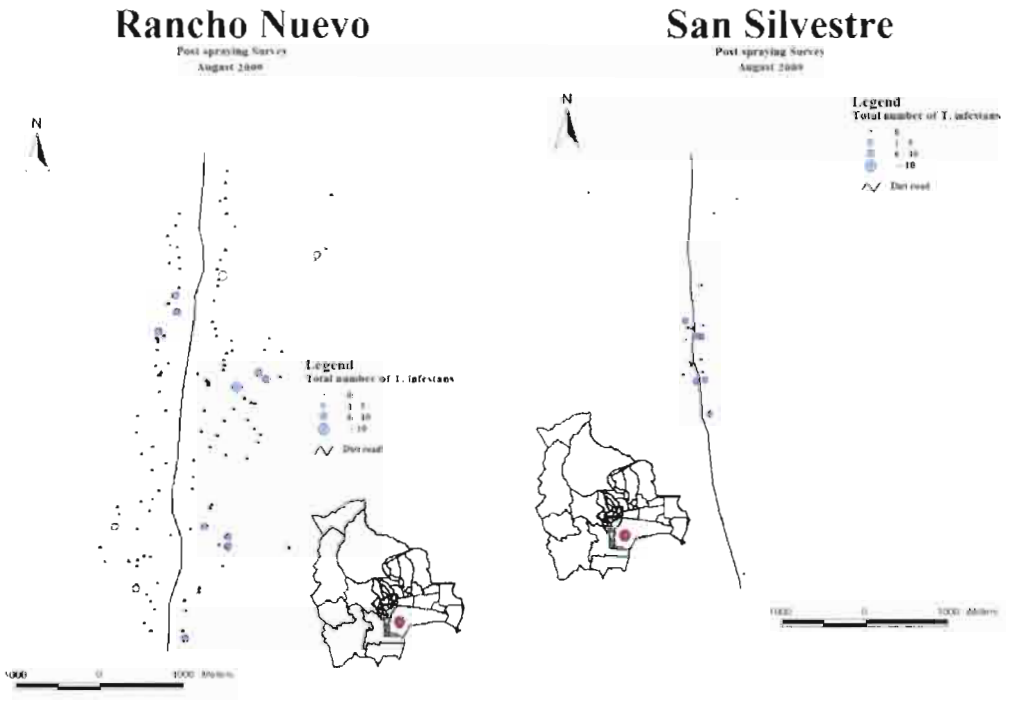
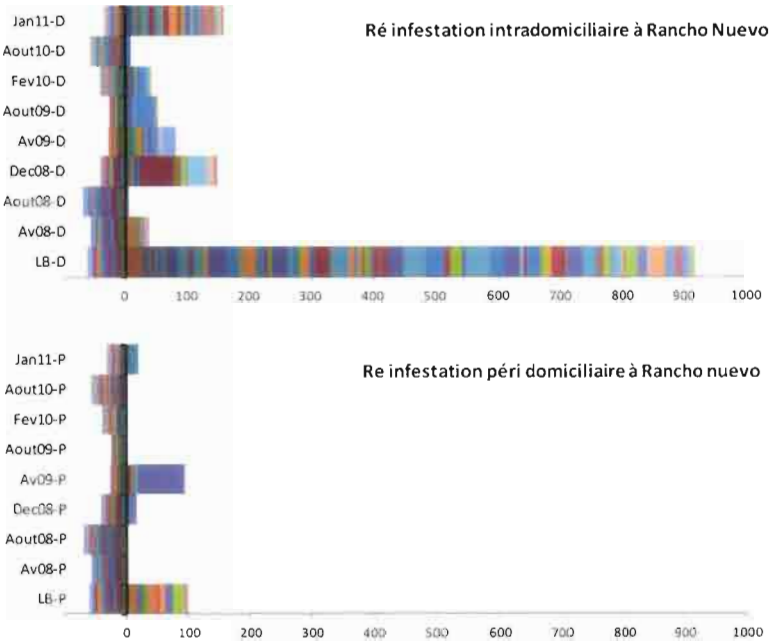


Figure 4. Distribution des T. infestans *capturés dans les maisons (intérieur et extérieur) par recherche active 19 mois après de traitement insecticide initial et ceux focalisés effectués au cours de la vigilance.*

A



B

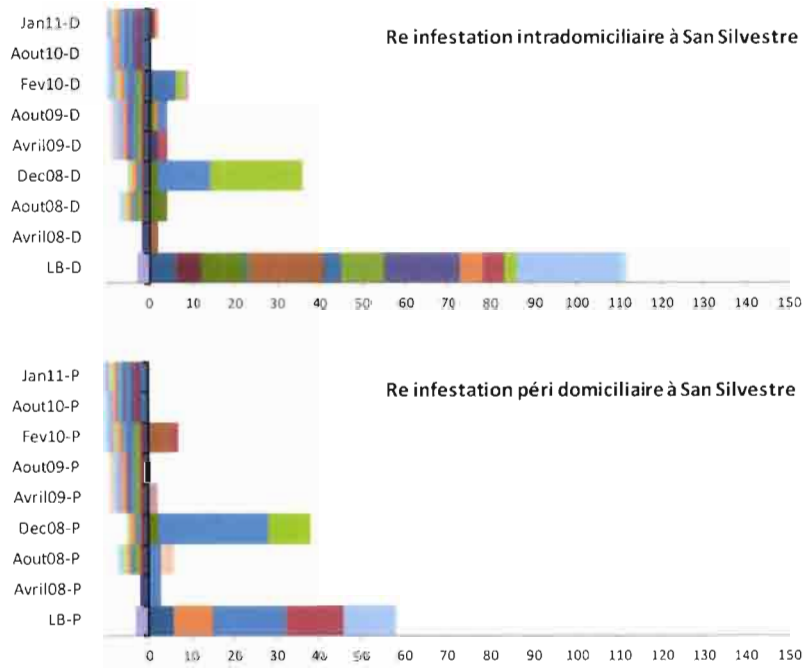


Figure 5 : Cinétique de ré infestation par T. infestans intra et péri domiciliaire à Rancho Nuevo et San Silvestre. Abscisse : en positif, chaque rectangle représente une maison infectée par des triatomes et la longeur du rectangle est proportionnelle au nombre de triatomes trouvés ; en négatif, sont représentées les maisons non visitées et les maisons non encore construites au moment du contrôle. En ordonnée sont représentés les différents temps de contrôle ; LB = ligne de base, D = intra domicile, P = péri domicile.

L'ensemble des structures permanentes et temporaires composant l'espace utilisé par chaque habitant autour de sa maison a été caractérisé. Ces données ont été complétées par une enquête sociologique auprès des habitants des deux communautés en avril 2009. La majorité des données ont

été saisies dans une base de données (septembre-octobre 2009). L'analyse préliminaire des facteurs de risques de l'infestation avant fumigation (analyse de corrélation bi factorielle) basée sur 50 variables identifie seulement 6 facteurs significatifs et l'analyse multi factorielle montre que l'infestation est associée : aux maisons de plus de 5 ans (OR = 4,34; *P* = 0,004), les maisons où les habitants sont préoccupés par les triatomes (OR = 4,70; *P* = 0,009), l'absence d'armoire pour ranger les vêtements (OR = 3,16; *P* = 0,023) et la pénétration des animaux dans la maison (OR = 4,31; *P* = 0,004). L'analyse multi factorielle des risques de ré infestation identifie un seul facteur qui est l'infestation de l'habitat avant la fumigation (ligne de base).

2 - Ecorégion Bosques Secos Interandinos (BSIA) département de Cochabamba: Quillacollo aire péri urbaine

a) Stratification de l'espace

L'annexe 2 présente la zone d'étude habitée qui est bordée par l'espace sylvestre. Ce quartier peri urbain de la ville de Quillacollo est composé de blocs de maisons (« manzanos ») de construction ancienne et aussi récente (19 manzanos au total). Les 176 habitations qui ont été inclues dans l'étude ont été géo référencées (GPS) afin de procéder ultérieurement à l'analyse spatiale des données.

b) Piégeage en milieu sylvestre

En septembre 2009, 525 pièges ont été répartis le long des barres rocheuses de manière régulière (environ tous les 5-6 m). Au total, 35,2% des pièges était positifs avec une moyenne de 2,5 *T. infestans* par piège positif (Figure 6, Tableau 4). La très large majorité des insectes étaient de l'espèce *T. infestans* et seulement sur 3 des pièges positifs à *T. infestans*, 3 spécimens de *T. sordida* (1 adulte et 2 N3) ont été capturés. De plus, toutes les barres rocheuses étaient positives.

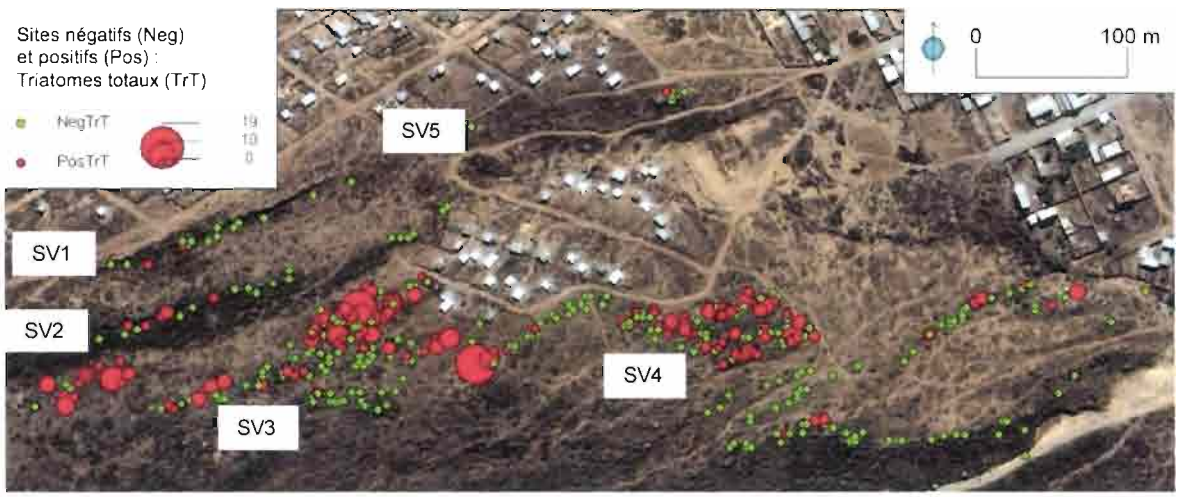


Figure 6. Distribution spatiale des pièges positifs (cercles rouges) et négatifs (cercles verts) en septembre 2009. La taille des cercles rouges est proportionnelle au nombre de triatomes capturés par chaque piège.

Tableau 4: Captures de *T. infestans* et *T. sordida* dans les barres rocheuses des zones SV1 à SV5 en septembre 2009

Zones sylvestres	Nombre de pièges	Pièges positifs	N° *T. i* et *T.s* capturés	N° *T.i*/piège positif
SV1	55	20,0%	14	1,3
SV2	172	52,9%	248	2,7
SV3	210	32,4%	170	2,5
SV4	78	17,9%	28	2,0
SV5	10	10,0%	1	1,0
Total	**525**	**35,2%**	**461***	**2,5**

T. i. = *Triatoma infestans;T. s = T. sordida ; * inclus 3 T. sordida*

Trois autres explorations de l'espace sylvestre ont été réalisées en mars, mai et juin 2010. A ces dates, toutes les barres rocheuses n'ont pas systématiquement été explorées mais a titre indicatif, en mars 23,9% des pièges étaient positifs (112 *T. infestans* et 5 *T.sordida*), en mai 22,3% (54 *T.infestans*) et en juin 29,3% (78 *T. infestans*). Le pourcentage de pièges positifs est assez comparable entre les périodes de capture mais la densité de *T. infestans* capturés par piège positif (1,38 à 1,7) est inférieur à celle observée en septembre (époque la plus chaude). Le calcul global du taux d'infection des *T. infestans* collectés en mars, mai et juin atteint 40,7%, un chiffre très supérieur au taux évalué chez les spécimens capturés en péridomicile (2,2%, voir paragraphe suivant).

c) *Enquêtes entomologiques dans la partie urbaine en septembre 2009 et février et juin 2010*

En septembre 2009, février et juin 2010 des enquêtes entomologiques ont été menées dans les 176 maisons choisies au début de l'étude (Tableau 5). Durant la période, aucun traitement insecticide n'a été appliqué par les autorités locales en charge car elles n'avaient plus le personnel recruté nécessaire. Des triatomes ont été exclusivement trouvés en péri domicile (infestation = 14,7%) en 39 occasions, répartis dans 11/19 blocs de maisons différents (Tableau 5) ; les colonies détectées n'étaient pas toujours de petite taille marquant une colonisation focalisée (cases en rouge). La collecte de spécimens du complexe *T. sordida* (espèce d'incursion) est plus élevée en juin indiquant probablement la saison où ces espèces se dispersent. Le taux d'infection des insectes capturés aux trois périodes est faible puisqu'il n'atteint que 2,2%. Des souches de *T.*

Tableau 5. Captures de triatomes par recherche active dans les domiciles et péri domiciles de la zone de Quillacollo (176 habitations)

		Septembre 2009		Février 2010		Juin 2010	
		Nombre d'insectes					
Bloc de maisons	Code maison	*T. infestans*	Complexe *T. sordida*	*T. infestans*	Complexe *T. sordida*	*T. infestans*	Complexe *T. sordida*
UA4	R119			48		17	
UA4	R118					1	
UA4	R120					1	
UA5	R114				3		
UA5	R116	2					
UP1	V356	1					
UP3	V209					4	5
UP4	V165			13			
UR1	V072	7		3		1	
UR1	V073			4			
UR2	V063			8		11	1
UR2	V069			1			
UR3	V060			3		11	2
UR3	V059	143		45			
UR4	V051			1			2
UR4	V053			7			1
UR4	V052						2
UR7	V079			14			2
US1	V095		3				3
US1	V100						1
US1	V101			7			
US1	V097		1				
US2	V113					1	
US2	V118			1			
US2	V121			5			
US3	V125	4					

cruzi appartenant aux DTUs (discrete typing unit) TcI et TcIII ont été identifiées. Le relevé de toutes les structures composant les habitations a été effectué et permettra d'examiner quels peuvent être les facteurs associés à la présence de triatomes dans cet espace.

d) Vigilance entomologique par les habitants dans la partie urbaine

Suite à l'enquête active par l'équipe de professionnel en septembre 2009, il a été demandé aux habitants de capturer les insectes qu'ils voyaient dans leur domicile et péri domicile jusqu'au mois de juin 2010. La collecte des triatomes a été faite lors de 8 passages (octobre- novembre 2009 et Janv-Juin 2010) en demandant maison par maison s'ils avaient capturés des triatomes. Le tableau 4 résume les résultats. Contrairement aux recherches actives faite dans les péri domiciles et intra domiciles en septembre février et juin, les habitants capturent des triatomes à l'intérieur de leur maison (Tableau 6). Toutefois de 52 *T. infestans* examinés aucun ne présentait de parasites dans les fèces.

Tableau 6. Captures des triatomes par les habitants durant 9 mois

	Oct-2009	Nov-2009	Dec-2009	Jan-2010	Fev-2010*	Mar-2010	Avr-2010	Jun-2010*	Total
Maison visitée	109	55	23	57	166	52	37	150	176
Maison avec triatomes	4	2	1	3	7	1	3	10	31
Maison avec des triatomes en intradomicile	3	0	1	2	3	0	2	5	16
N° total de *T.infestans*	3	2	6	4	29	1	18	10	73
N° total *T. sordida*	3	0	0	0	0	0	0	0	3

* A ces dates la majorité des maisons ont été visitées car parallèlement il y a eu l'enquête entomologique par recherche active des triatomes

La dévolution de triatomes correspondait à 26 maisons différentes (26/176 = 14,7%), dont 1 en 3 occasions, 3 en 2 occasions et les autres une seule fois (Tableau 7). En 16 occasions les habitants ont collecté ces triatomes à l'intérieur de leur domicile, correspondant à 15 maisons différentes (infestation intra domiciliaire, 15/176 = 8,5%). Les triatomes capturés dans l'intra domicile étaient tous des *T. infestans* sauf 1 *T. sordida* (espèce d'incursion). Le nombre d'insectes capturés dans l'intra domicile/maison variait de 1-2 exceptions faite de la remise de 15 insectes par un habitant qui les avait trouvés dans une pièce utilisée comme remise. De même le nombre d'insectes capturés en péri domicile / maison était de quelques insectes exception faire d'une occasion où 18 insectes ont été remis marquant la présence de colonies réellement installées dans le péri domicile ; cette donnée coïncide avec les résultats des recherches actives. Les habitants ont seulement capturé 3 exemplaires du complexe *T. sordida* les autres triatomes étant *T. infestans*. Les triatomes sont issus de nombreux bloc de maisons (15/19). Le bilan des captures sur la période et leur distribution sur la zone étudiée (nombre de maison positive/bloc de maison) montre que les triatomes sont plutôt rarement identifiés dans les blocs de maisons UP1, UP2 et UP3 séparés de l'espace sylvestre par des terrains vagues sans formation rocheuse ni couverture végétale. Comme attendu l'infestation péri domiciliaire est plus importante que l'intra domiciliaire.

Tableau 7. Capture de triatomes par les habitants dans la zone de Quillacollo (176 habitations)

		Oct 2009		Nov 2009	Déc 2009	Jan 2010	Fév 2010	Mars 2010	Avr 2010	Jun 2010		
		Nombre d'insectes										
Bloc de maisons	Code maison	Complexe *T. i*	*T.s**	*T. i*	*T. i*	*T. i*	*T. i*	*T. i*	*T. i*	*T. i*	N° de fois positive	Ecotope
UA1	V158					1					1	P
UA1	V161									1	1	I
UA2	V138	2									1	P + I
UA2	V143									1	1	P
UA2	V144									1	1	
UA3	V149						1				1	I
UA4	R118									1	1	I
UA5	R114								1		1	P
UA5	R115									1	1	I
UP3	V211									1	1	P
UP3	U206						2				1	I
UP4	V168							1			1	P
UP4	T168						1				1	I
UP4	V165			1			4				2	P
UP4	V169			1						1	2	P
UR1	V073						2				1	P
UR1	V071						1		1	1	3	I
UR3	V058						18				1	P
UR4	V051		1								1	P
UR4	V052	1									1	I
UR4	V053					2					1	I
UR5	V039				6						1	P + I
UR6	V090									1	1	I
UR7	V084					1				1	2	I
US1	V101		2								1	P + I
US2	V120								16		1	P + I

P = péri domicile, I = intérieur de la maison

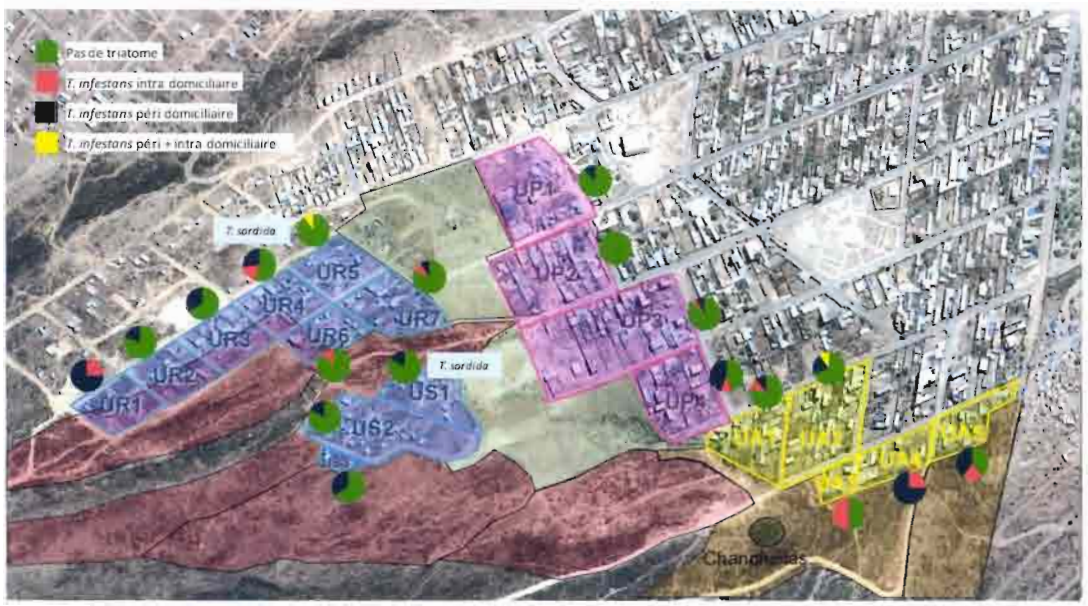


Figure 7. Pour chaque bloc de maisons les cercles indiquent la proportion relative des maisons trouvées infestées durant le suivi de la zone de Quillacollo.

3 - *Ecorégion Bosques Secos Interandinos (BSIA) département de la Paz: Sapini, aire rurale*

a) Stratification de l'espace

Sapini est un petit village composé de 27 maisons géo-référencées en octobre 2010 et dont les péri domiciles sont non clôturés. Il se trouve au bord de la rivière « Luribay » sur une élévation et est bordé, coté montagne, de falaises sédimentaires. L'annexe 2 présente la zone d'étude.

b) Piégeage en milieu sylvestre

En mai 2010, 85 pièges ont été répartis le long des falaises et dans des tas de pierres accumulés dans les champs de culture. Au total, 44,7% des pièges était positifs avec une moyenne de 2,9 *T. infestans* par piège positif (Figure 8). Aucune autre espèce n'a été identifiée. Deux autres explorations ont été effectuées en octobre 2010 (11,35% des pièges positifs) et en mai 2011 (23,6% des pièges positifs). Le taux d'infection des *T. infestans* collectés en mai et octobre est extrêmement élevé puisqu'il atteint 88,1% (42 spécimens examinés, principalement des stades 3, 4, et 5 de développement).

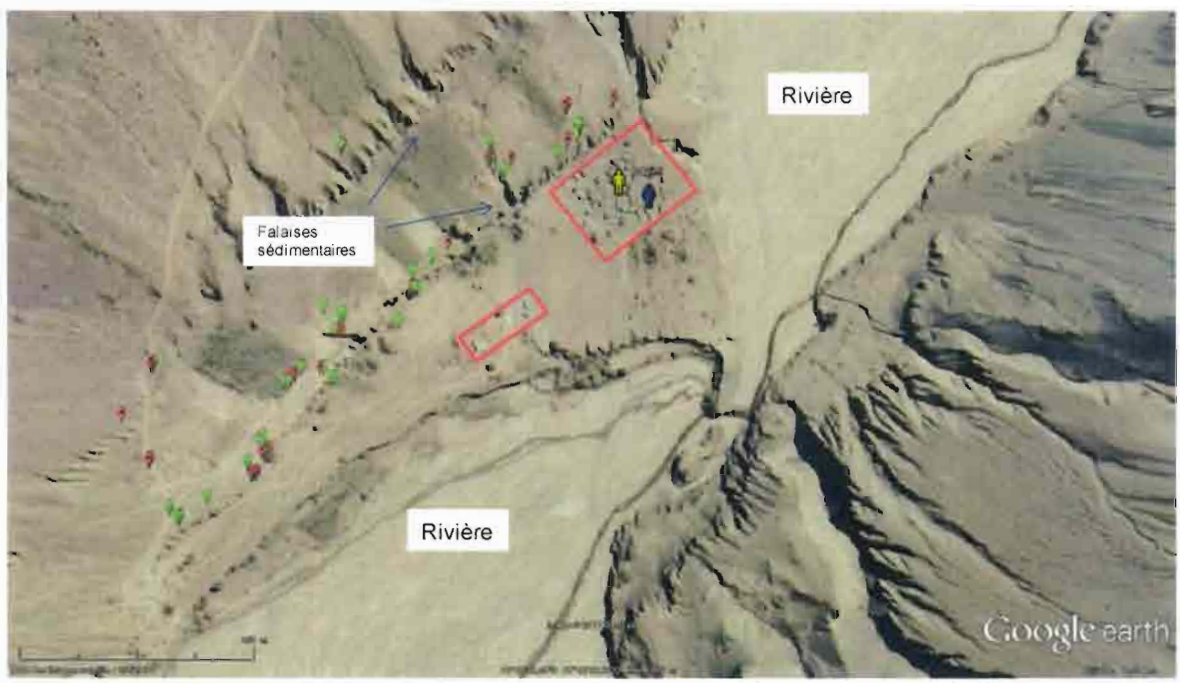


Figure 8. *Distribution spatiale des pièges positifs avec* T. infestans *(en rouge) et négatifs (en vert). En encadré rouge les habitations de Sapini.*

c) *Enquêtes entomologiques dans la partie habitée en octobre 2010*

Tableau 8 : Evaluations du taux d'infestation des maisons à Sapini par les autorités départementales sanitaires.

Année	% d'infestation par *T. infestans*
2000	100,0
2003	3,7
2004	11,1
2005	5,3
2006	20,0
2007	13,3
2009	25,0
2010	9,1

En 2000 le service départemental de La Paz en charge des évaluations des taux d'infestation des villages constate une infestation de 100% des habitations. Toutes les habitations sont alors traitées par insecticide et la vigilance entomologique assurée environ chaque année par le service départemental ; a chaque contrôle les maisons positives sont a nouveau traitées. Le tableau 8 montre la baisse importante du taux d'infestation mais aussi la persistance de cette infestation durant toutes ces années.

En octobre 2010 l'équipe a pu visiter 20 maisons les 7 autres étant fermées ou abandonnées. Des spécimens de *T. infestans* (N = 16) ont été trouvés dans 6 maisons (taux d'infestation = 30%) dont 7 spécimens en intra domicile (4 maisons). Le taux d'infection de ces insectes a été de 25% (2/8 examinés). Etant donné qu'entre les péri domiciles sont accumulés de nombreux tas de pierres, l'évaluation de leur infestation a été réalisée par piégeage comme dans l'espace sylvestre.

Figure 8. Tas de pierres qui sont retirées des terrains afin de pouvoir construire ou cultiver.

De 12 tas de pierres explorés 4 sont positifs, parmi ces tas positifs plusieurs sont extrêmement proche d'une habitation (Figure 9).

Figure 9. Village de Sapini où sont localisés les tas de pierres explorés à l'aide de pièges (en rouge présence de T. infestans*, en vert absence)*

4 - *Ecorégion Bosques Secos Interandinos (BSIA) département de Potosi: Thago Thago aire rurale*

a) Stratification de l'espace

Thago Thago est un hameau composé de 10 maisons le long d'une route où, des deux cotés, les collines sont infestées par des triatomes (Figure 10). Pour assuré un suivi entomologique de la zone de septembre 2010 à avril 2011 (7 mois), la partie sylvestre a été échantillonnées en même temps que la partie habitée qui l'a été par deux méthodes appliquées en parallèle : la recherche active des triatomes dans chaque maison et la vigilance par les habitants comme il a été fait à Quillacollo.

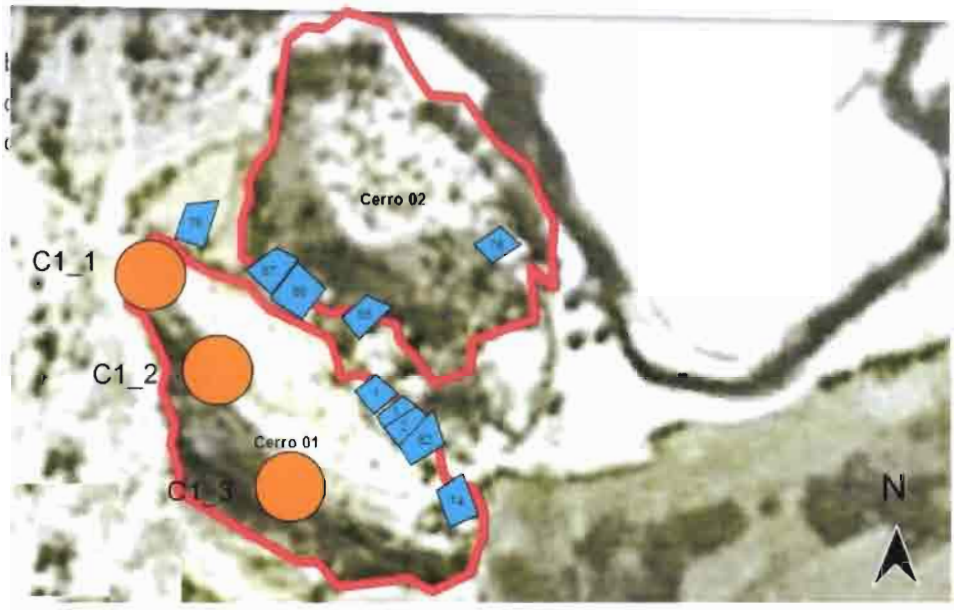


Figure 10. Aire partagée de Thago Thago : les maison sont indiquées par des carrés bleus, les cercles oranges indiquent une concentration d'affleurements rocheux dans l'espace sylvestre ; cerro = colline

b) Comparaison des captures en milieu sylvestre et habité durant 7 mois

La figure 11 exprime les taux d'infestation dans les deux milieux au cours des 6 explorations. Dans le milieu sylvestre le pourcentage de pièges positifs ne varie pas beaucoup au cours de la période de même que le nombre de maison où les habitants capturent des triatomes, par contre la recherche active a été plus fructueuse durant les mois de septembre et avril. Les habitants ont pu collecter un total de 147 insectes et la recherche active a permis de collecter 115 insectes. De même qu'à Quillacollo, la recherche active n'a permis de collecter que des insectes dans les péri domiciles, alors que les habitants ont capturé des insectes dans leur maison en 16 occasions ; sur la période, 8/10 maisons ont présenté au moins un triatome à l'intérieur de la maison. Sur l'ensemble des triatomes capturés environ 10% sont des *T. sordida* et les deux espèces sont trouvées en intra domicile par les habitants. Souvent les habitants collectent

seulement 1 ou 2 spécimens adultes, *T. sordida* ou *T. infestans*, à l'intérieur de leur maison ce qui permet de penser que ce sont des insectes d'incursion et non des insectes correspondant à des colonies établies à l'intérieur de la maison ; en effet dans 16 occasions les habitants collectent des triatomes à l'intérieur de leur maison et dans 11 cas, seulement des adultes.

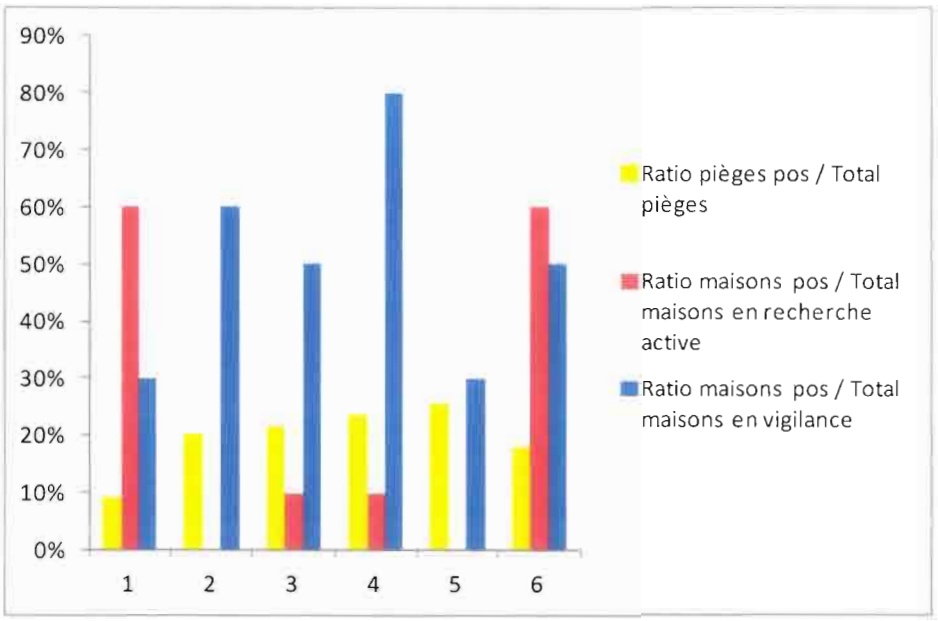


Figura 11. Pourcentage des pièges positifs posés dans le milieu sylvestres (histogramme jaune), pourcentage des maisons avec au moins 1 triatome suite aux recherches actives (histogramme rouge) à la vigilance par les habitants (histogramme bleu) ; 1, 2, 3 et 4 correspond aux mois de septembre, octobre, novembre y décembre 2010; 5 et 6 de janvier et avril 2011.

Annexe 15

Sensibilité à la deltaméthrine de souches de *T. infestans* sylvestres, péri domestiques et intra domiciliaires

Le tabeau 1 présente les tests à la dose diagnostic, c'est-à-dire au double de la dose de deltaméthrine qui tue 100% des insectes de la souche de référence sensible (souche CIPEIN) : (i) deux souches sylvestres localisées à proximité de l'aire partagée de Quillacollo et deux souches isolées de péri domiciles, sont sensibles à la deltaméthrine il n'y aurait donc pas de résistance dans cette zone ; (ii) de la même manière une souche sylvestre et une souche péri domestique de Cutty, village situé dans la vallées de Luribay où de nombreux foyers sylvestres ont été échantillonnés sont sensibles.

Tableau 1. Sensibilité à la deltraméthrine (dose diagnostic) de souches sylvestres de *T. infestans*, péri domestiques et intra domiciliaires isolées de sites à proximité les uns des autres

Dpt. (province)	Code localisation*	Localité la plus proche	Ecotope	Site de capture	% mortalité (n° d'insectes testés)
Cochabamba (Quillacollo)	BSIA 14	Cotapachi	S	Affleurement rocheux, 100-150m d'une maison isolée	100% (46)
	QUI	Quillacollo**	S	Affleurement rocheux, 100-250 m de la zone péri urbaine	100% (80)
	QUI_CV59		P	Tas de briques entreposées dans la zone péri urbaine	100% (34)
	QUI_CV165		P	Tas de pierres dans la zone péri urbaine	100% (70)
La Paz (Loayza)	LIE 01	Cutty	S	Muret de pierres sèches, 100 m du village	100% (49)
	CUTTY	Cutty	D	Intra domicile	100% (37)

* Voir l'annexe 6

Le tableau 2 présente les résultats des tests à dose diagnostic de plusieurs populations sylvestres de *T. infestans* isolées (i) dans les vallées de Mécapaca (TUN 01, RUIN 01 et TUN 02) et de Luribay (QUE 01Tun 06, VIZ02 et CAC 03) du département de La Paz où de nombreux foyers sylvestres de *T. infestans* ont été échantillonnés (voir annexe 6). Ces souches sont toutes sensibles excepté QUE 1 pour laquelle un faible niveau de résistance n'est pas exclu. La souche de Mataral présente un résultat similaire. Celle de Julo Grande est considérée comme résistante. Ces résultats montrent que chez ces populations sylvestres on peut observer un certain niveau de résistance naturelle qui pourrait être dû à l'emploie intensif d'autres insecticides pour traiter les cultures environnantes. Cette situation pourrait avoir des conséquences néfastes quand ces populations sylvestres colonisent les villages car elles ne seraient pas complètement sensibles au traitement insecticide appliqué actuellement.

Tableau 2. Sensibilité à la deltraméthrine de souches sylvestres de différentes zones géographiques

Dpt. (province)	Code localisation*	Localité la plus proche	Ecotope	Site de capture	% mortalité (n° d'insectes testés)
La Paz (Murillo)	TUN 01	Huayhuasi	S	Terrier et pied de figuier de Barbarie, 50-100 m de quelques maisons isolées	100% (49)
	RUIN 01	Tahuapalca	S	Terrier dans une caserne di 18ème siècle en ruine, 500 du village	100 (32)
	TUN 02	Tahuapalca	S	Entre des pierres dans champ de figues de Barbarie, 50-150 m du village	100% (38)
La Paz (Loayza)	QUE 01	Achocara bajo	S	Falaise sédimentaire, 500 m d'une maison isolée	98% (58)
	Tun 06	Catavi	S	Champs de figues de Barbarie, 50-100 m de maison isolée	100% (9)
	VIZ02	Lacayani	S	Pierriers formé de gros blocs, 500 m d'une maison isolée	100% (43)
	CAC 03	Palca	S	Entre des pierres, 20-100 m du village	100% (42)
Potosi (Charas)	JUGR	Julo Grande (PO)	S	Falaise sédimentaire, 200 m du village	79% (85)
Cochabamba (Campero)	MAT	Mataral (CO)	S	Pierrier formé de petits blocs, 1 km du village	96% (56)

*Se réfère à l'annexe 6

Le Tableau 3 présente les tests à dose diagnostic et la mesure de la dose létale de deltaméthrine appliqués à des souches de *T. infestans* isolées dans l'écorégion du Gran Chaco. Cette région est depuis des années celle où les mesures de contrôle des vecteurs dans les villages sont en échec. Les résultats des tests montrent clairement que les souches des villages de Rancho Nuevo et San Silvestre (aires partagées) étudiés au cours du projet et ceux de Tamachindi et Kuarirenda situés à proximité des précédents sont résistantes. Les deux autres souches sont isolées dans les villages de Estancia Basilio et Taiguati proches des sites GC 04 et GC 02 (voir annexe 5 et 6) à partir de colonies importantes installées dans des poulaillers ; leur niveau de résistance est très important et là tout traitement insecticide de ces structures par la deltaméthrine ne peut qu'échouer.

Tableau 3. Sensibilité à la deltraméthrine de souches sylvestres de *T. infestans* isolées de péri domiciles et intra domiciles dans l'écorégion du Gran Chaco

Localité (Departement)	% de mortalité à la dose diagnostic (n° d'insectes testés)	"Probit line" n tested insects	Pente (erreur standard)	Chi 2 (df) P	LD50 (95% CI)	RR50 (95% CI)
CIPEIN (Souche sensible de référence)	100	739	2.73 (0.21)	1.29 (4) P=0.86	0.24 (0.22-0.26)	-
Tamachindi (SC)	90 (150)	-	-	-	-	-
Kuarirenda (SC)	94 (154)	203	3.34 (0.16)	1.37 (3) P=0.71	0.91 (0.00-1.71)	3.80 (0.00-6.58)
Rancho Nuevo (SC)	80 (306)	274	3.53 (0.10)	0.99 (3) P=0.80	1.98 (1.78-2.24)	8.25 (8.09-8.61)
San Silvestre (SC)	74 (117)	213	4.21 (0.03)	0.08 (3) P=0.99	1.99 (0.77-2.45)	8.29 (3.50-9.42)
Estancia Basilio (SC)	88 (26)	-	-	-	-	-
Taiguati (TA)	0 (25)	328	3.41 (0.36)	5.36 (3) P=0.15	196.38 (166.95-262.60)	818 (758-1010)

Dose létale (LD50), taux de résistance à LD50 (RR50). LD et RR sont exprimés avec 95% d'intervalle de confiance. SC = Santa Cruz, TA = Tarija

Infection, Genetics and Evolution 11 (2011) 1155–1158

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Short communication

Further interest of miniexon multiplex PCR for a rapid typing of *Trypanosoma cruzi* DTU groups

Claudia Aliaga [a,b,*], Simone Frédérique Brenière [a,b], Christian Barnabé [a,b]

[a] Institut de Recherche pour le Développement (IRD), Society and Health Department, MIVEGEC (University of Montpellier - CNRS 5290 - IRD 224), Representation in Bolivia, Av Hernando Siles N 5290, CP 9214, La Paz, Bolivia

[b] Instituto Nacional de Laboratorios de Salud (INLASA), Laboratorio de Entomología Médica,14 Rafael Zubieta #1889, Miraflores, Casilla M-10019, La Paz, Bolivia

ARTICLE INFO

Article history:
Received 6 September 2010
Received in revised form 23 November 2010
Accepted 23 November 2010
Available online 19 January 2011

Keywords:
Trypanosoma cruzi
Discrete typing units
Miniexon
PCR multiplex

ABSTRACT

In order to validate a rapid typing of *Trypanosoma cruzi* DTUs, the miniexon multiplex PCR was tested for the first time, on a large and diversified sample of 70 strains belonging to all current DTUs (TcI to TcVI). Three DTU groups have been distinguished by specific PCR molecular weight, TcI (200 bp), TcII, V, VI (250 bp) and TcIII and IV (150 bp) with no incorrect grouping. These groups are epidemiologically and genetically relevant; moreover the method is easy and cheap and allows direct identification of parasites from triatomine faeces.

Trypanosoma cruzi, the causative agent of Chagas disease, is widely distributed in Latin America exhibiting considerable genetic polymorphism (Tibayrenc and Ayala, 1988). For many years, numerous genetic markers have been employed to depict the population structure of *T. cruzi* and its mode of reproduction. Clonality was found to be dominant, nevertheless at the evolutionary scale, scarce events of hybridization have occurred and have been the source of new genetic sub-groups presently identified (Barnabé et al., 2000; Brisse et al., 2000; Westenberger et al., 2005; de Freitas et al., 2006). Biological and biochemical analyses (biodemes and zymodemes) (Miles and Cibulskis, 1986) as well as DNA analyses such as RAPD (Brisse et al., 2000), RFLP of kinetoplast DNA (Solari et al., 1990) and multigene sequencing (Subileau et al., 2009) have lead to a consensus nomenclature into six DTUs, now called TcI to TcVI (Zingales et al., 2009). For rapid typing of *T. cruzi* and in order to discriminate *Trypanosoma rangeli* (non-pathogenic parasite that share common vectors and hosts with *T. cruzi*), from *T. cruzi*, Fernandes et al. (2001) developed a multiplex PCR based on amplification of the non-transcribed spacer region of the miniexon gene. Designing appropriate primers the authors proposed to distinguish by different molecular weights of the PCR products "*T. cruzi* I "that can be equate to TcI, "*T. cruzi* II" equivalent to TcII and "*T. cruzi* Z3" equivalent to TcIV, and *T. rangeli*. Although this tool has already been applied to several samples of strains (Monteiro et al., 2010; Santos-Mallet et al., 2008; Lisboa et al., 2009) and to blood meals of triatomines for direct parasite typing (Bosseno et al., 2006, 2009; Breniere et al., 2007), a formally evaluation of this miniexon multiplex PCR among a large sample of strains belonging to all current DTUs (TcI to TcVI) has never been done.

To achieve this goal, a wide variety of strains, previously characterized by different genetic markers, from different geographic and host origins, emphasizing strains isolated from wild cycle, were selected (Table 1). All the stocks have been previously characterized, not necessarily for the first time, by Barnabé et al. (2000); except for TCMB3 (Barnabé et al., 2003), RGB (Dereure et al., 2001), PalDacl9 (O'Connor et al., 2007) and Tev91 (Diosque et al., 2003). DNA amplifications from 70 *T. cruzi* strains, 1 *T. rangeli* and 1 *T. cruzi marinkellei* were performed according to previous work (Fernandes et al., 2001) by using three primers from the intergenic region of *T. cruzi* miniexon, namely Tc1: 5′-ACACTTTCTGTGGCGCTGATCG; Tc2: 5′-TTGCTCGCACACTCGGCTG-CAT and TC3: 5′-CCGCGWACAACCCCTMATAAAAATG, one primer from *T. rangeli* Tr: 5′-CCTATTGTGATCCCCATCTTCG and a common oligonucleotide downstream was selected in the most conserved part of the miniexon gene Me: 5′-TACCAATATAGTACAGAAACTG. The strain DNAs were obtained from parasites (epimastigote forms) cultured at 28 °C in LIT liquid medium. PCR conditions were according to Fernandes et al. (2001) with slight modifications. DNA was amplified in 25 μl reaction volume containing 1.5 mM $MgCl_2$, 50 μM of each nucleotide, 0.2 μM of each primer, 0.5 UI of Taq DNA polymerase (Roche Applied Science, Penzberg, Germany) and 20 ng of DNA template. The amplification was performed in a thermocycler (Eppendorf, Hamburg, Germany), with conditions

* Corresponding author. Tel.: +591 2 278 2969; fax: +591 2 278 29 44.
E-mail address: aliagaclau@yahoo.es (C. Aliaga).

1567-1348/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.meegid.2010.11.013

Table 1
Origin, genetic characterization of the 70 stocks of *Trypanosoma cruzi*, 1 *T. cruzi marinkellei* and 1 *Trypanosoma rangeli* and results of the miniexon multiplex polymerase chain reaction (MMPCR).

Stock	Host/vector	Country	Species/DTUs	MMPCR (bp)
MPHY/BR/00/TCMB3	*Phyllostomus discolor*	Sao Paulo, Brazil	*T. c. marinkellei*	Absent
MCAN/VE/00/RGB	*Canis familiaris*	Caracas, Venezuela	*T rangeli*	100
MDAP/BR/00/Cutiacl1	*Dasyprocta aguti*	Espiritu Santo, Brazil	TcI	200
MDID/BR/88/G-38-01	*Didelphis albiventris*	Camalau, Brazil	TcI	200
MDID/ARG/00/Paldacl9	*Didelphis albiventris*	El Palmar, Chaco, Argentina	TcI	200
MDID/BO/85/85/818	*Didelphis marsupialis*	Alto Beni, Bolivia	TcI	200
MDID/US/93/93070103P	*Didelphis marsupialis*	Fort Stewart Bryan Co, USA	TcI	200
MDID/CO/XX/FX18	*Didelphis marsupialis*	Galeras, Colombia	TcI	200
MDID/CO/86/361TA	*Didelphis marsupialis*	Inguapi del Guadal, Colombia	TcI	200
MDID/US/93/93041401P	*Didelphis marsupialis*	Statesboro Bulloch Co, USA	TcI	200
MDID/MX/00/Z17	*Didelphis marsupialis*	Yucatán, Mexico	TcI	200
MHOM/PE/00/PERU	Human	Peru	TcI	200
MHOM/BO/83/P209cl93	Human	Sucre, Bolivia	TcI	200
MHOM/VE/77/OPS21cl1	Human	Venezuela	TcI	200
MHOM/MX/00/H10	Human	Yucatán, Mexico	TcI	200
MPHI/GF/86/A269	*Philander opossum*	Cacao, French Guiana	TcI	200
MPHI/BR/00/CUICAcl1	*Philander opossum*	Riberao Preto, Brazil	TcI	200
MPOT/CO/87/458	*Potus flavus*	Bajo Calima, Colombia	TcI	200
IRHO/BO/XX/PB3cl2	*Rhodnius pictipes*	Alto Beni, La Paz, Bolivia	TcI	200
MSAI/VE/00/Saimiri4A	*Saimiri sciureus*	Venezuela	TcI	200
ITRI/HN/83/Davis3	*Triatoma dimidiata*	Tegucigalpa, Honduras	TcI	200
ITRI/CHI/XX/V120	*Triatoma infestans*	San Pedro, Chile	TcI	200
ITRI/ARG/00/Tev91	*Triatoma infestans*	Tres Estacas, Chaco, Argentina	TcI	200
ITRI/PE/00/SABP3	*Triatoma infestans*	Vitor, Peru	TcI	200
ITRI/CHI/XX/SP31	*Triatoma spinolai*	Flor del Valle, Chile	TcI	200
MHOM/BR/00/CP2T3	Human	Campinas, Goias, Brazil	TcII	250
MHOM/CHI/00/MVBcl8	Human	Cogoti, Chile	TcII	250
MHOM/CHI/00/MCVII	Human	Illapel, Chile	TcII	250
MHOM/CHI/98/IVVcl4	Human	Región IV, Chile	TcII	250
MHOM/BR/77/Esmcl3Ref	Human	Sao Felipe, Brazil	TcII	250
MHOM/BR/00/HC13884T1	Human	Sao Paulo, Brazil	TcII	250
MHOM/CHI/91/CBBcl2	Human	Tulahuen, Chile	TcII	250
ITRI/BO/98/Tu18cl93	*Triatoma infestans*	Bolivia	TcII	250
MCAN/PY/84/X109/2	*Canis familiaris*	Makthlawaiya, Paraguay	TcIII	150
MDAS/BR/81/M5631cl5	*Dasypus novemcinctus*	Selva Terra, Brazil	TcIII	150
MHOM/BR/00/M6251cl6	Human	Belem, Brazil	TcIII	150
MCAN/US/00/DogTheis	*Canis familiaris*	USA	TcIV	150
MHOM/BR/78/CanIIIRef	Human	Belem, Brazil	TcIV	150
MMAC/US/00/TexasTheis	*Macaca mulatta*	USA	TcIV	150
MPRC/US/00/93071502R	*Procyon lotor*	Georgia, USA	TcIV	150
MPRC/US/00/92122102R	*Procyon lotor*	Georgia, USA	TcIV	150
MPRC/US/00/STC13Rcl3	*Procyon lotor*	Georgia, USA	TcIV	150
MPRC/US/00/STC9Rcl4	*Procyon lotor*	Georgia, USA	TcIV	150
MHOM/BO/85/P255	Human	Chuquisaca, Bolivia	TcV	250
MHOM/CL/00/JMJ	Human	Combarbala, Chile	TcV	250
MHOM/CL/00/XHCH56	Human	Monte Patria, Chile	TcV	250
MHOM/CL/00/FCV	Human	Moquella, Chile	TcV	250
MHOM/PE/00/SAXP5b	Human	Peru	TcV	250
MHOM/CL/00/ALD	Human	Pica, Chile	TcV	250
MHOM/CL/00/MNcl2	Human	Regino IV, Chile	TcV	250
MHOM/CL/00/NRcl3	Human	Salvador, Chile	TcV	250
MHOM/BO/80/92.80cl1	Human	Santa Cruz, Bolivia	TcV	250
0000/BO/00/C815	nk	Cochabamba, Bolivia	TcV	250
0000/BO/00/86-1	nk	Santa Cruz, Bolivia	TcV	250
0000/00/00/Kundera	nk	nk	TcV	250
ITRI/BO/82/CA34	*Triatoma infestans*	Camiri, Bolivia	TcV	250
ITRI/CL/00/VGM	*Triatoma infestans*	Pica, Chile	TcV	250
ITRI/BOL/86/SO3cl5	*Triatoma infestans*	Potosi, Bolivia	TcV	250
ITRI/BR/96/Bug2148cl1	*Triatoma infestans*	Rio Grande do sul, Brazil	TcV	250
ITRI/BR/00/Bug2149cl10	*Triatoma infestans*	Rio Grande do sul, Brazil	TcV	250
ITRI/BO/83/SC43cl1Ref	*Triatoma infestans*	Santa Cruz, Bolivia	TcV	250
MCAN/PY/00/X154/7	*Canis familiaris*	Makthlawaiya, Paraguay	TcVI	250
MCAN/PY/00/X57/3	*Canis familiaris*	Makthlawaiya, Paraguay	TcVI	250
MDID/BO/86/86/2036	*Didelphis marsupialis*	Bolivia	TcVI	250
MHOM/BO/85/P251	Human	Cochabamba, Bolivia	TcVI	250
MHOM/CL/00/Tulacl2	Human	Tulahuen, Chile	TcVI	250
0000/CO/00/CALI	nk	Colombia	TcVI	250
ITRI/BO/82/CA15	*Triatoma infestans*	Camiri, Bolivia	TcVI	250
ITRI/PY/00/P63cl1	*Triatoma infestans*	Makthlawaiya, Paraguay	TcVI	250
ITRI/PY/00/P42/8	*Triatoma infestans*	Makthlawaiya, Paraguay	TcVI	250
ITRI/BR/98/CLBrener	*Triatoma infestans*	Rio Grande do sul, Brazil	TcVI	250
ITRI/CL/00/Vincho195	*Triatoma infestans*	San Pedro, Chile	TcVI	250

nk: not known.

already described (Fernandes et al., 2001). PCR products were separated on 3% agarose gel using molecular weight marker Smart Ladder (Eurogentec, Angers, France) and visualized under UV with Ez-vision (Amresco, OH, USA).

All the PCR products from the 21 strains of *T. cruzi* I showed a 200 bp molecular weight while there were isolated in 11 different countries covering a large part of the endemic area from southern USA to Argentina. It is worth noting that most of these strains were isolated from mammalian reservoirs that maintain sylvatic cycles of *T. cruzi*. Indeed TcI is the most widely distributed in sylvatic cycles where it exhibits a very high genetic diversity (Lewicka et al., 1995). The strict correlation between previous genetic characterization of TcI strains based on mutilocus methods and the multiplex PCR used here (a single band of 200 bp for all the TcI) shows that this 200 bp band can be considered as a useful synapomorphic character for TcI. In the same way the 250 bp multiplex PCR molecular weight was observed for all the strains belonging to *T. cruzi* II, V and VI. These three DTUs are dominant in domestic cycles. It is interesting to note that TcV and TcVI are hybrid DTUs between TcII and TcIII (Brisse et al., 2000; Westenberger et al., 2005), however all PCR multiplex profiles showed a single band of 250 bp and not a double band because the genome of hybrid DTUs just contains mini-exon copies related to TcII, so double bands are not expected. Strains belonging to TcIII and TcIV showed one multiplex PCR band of 150 bp. Indeed a common insertion in the intergenic region of the miniexon gene was previously observed for two strains (Can III and M5631) belonging to TcIV and TcIII, respectively and the primer TC3 was selected in this insertion (Fernandes et al., 2001). The current analysis of a larger sample of these DTUs confirms the presence of this insertion in both groups. These DTUs are clearly associated to sylvatic cycles but they are more scarcely sampled than TcI although they have a wide geographic distribution (Barnabé and Breniere, 1999). The reference strain of *T. rangeli* presented one band of 100 bp as expected for this species. Moreover, none PCR product was observed with *T. cruzi marinkellei* DNA template. All these results showed that this method of PCR multiplex allows the characterization of *T. cruzi* strains for three DTUs groups which are epidemiologically and genetically relevant. Indeed, TcI clusters strains initially attributed to an accurate genetic lineage previous called *T. cruzi* I while the other DTUs formed the second genetic lineages called *T. cruzi* II itself divided into 5 subgroups corresponding to the current TcII to TcVI. Nevertheless this dichotomy observed with markers such as MLEE or RAPD is now questioned by other molecular markers such as MLST (Subileau et al., 2009). Consequently, the new consensual classification of *T. cruzi* has retained the division of *T. cruzi* into six subgroups (TcI to TcVI) with no assumption of clustering in distinct lineages. The group of TcIII and TcIV are two highly polymorphic DTUs separated from the other DTUs by great genetic distances. The character 250 bp, regroups the two hybrid DTUs (TcV and TcVI) with one of their parents (TcII) and this group is mostly associated with domestic cycles while TcIII–IV is mainly sylvatic. Moreover, the tandem repeated nature of the target sequences allowed a sensitive detection of the different groups of at least 20 pg of DNA and it is even more sensitive for TcI, TcV and *T. rangeli* (200 fg to 2 pg).

So, this method based on multiplex PCR, validated by the present work, is highly efficient to quickly separate *T. cruzi* strains into three relevant groups (TcI, TcII–V–VI and TcIII–IV). Because of its high sensitivity this tool, of easy application, allows characterizing parasites directly in triatomines faeces avoiding the step of the parasite isolation with the only disadvantage of not identifying all the six DTUs; also the method remains to be tested for direct detection in blood reservoir mammals and in patients. The multiplex PCR is an efficient marker to detect a mixture of DTU's directly in triatomines faeces. The method procures an interesting first step of genetic screening before more elaborated studies more time consuming and costly.

Acknowledgements

This investigation received financial support from the "Institut de Recherche pour le Développement, IRD" and the "Agence Nationale de la Recherche (ANR), France". We extend our gratitude to the authorities of the "Instituto Nacional de Laboratorios de Salud (INLASA)", Department of Entomology, La Paz, Bolivia, where the PCR work was done.

References

Barnabé, C., Breniere, S.F., 1999. Eco-distribución de los clones de *Trypanosoma cruzi*. In: Alfred Cassab, J.R., Noireau, F., Guillen, G. (Eds.), Chagas: La Enfermedad en Bolivia, Conocimientos Cientificos al Inicio del Programa de Control (1998–2002). Editiones Graficas "E.G.", La Paz, pp. 209–215.

Barnabé, C., Brisse, S., Tibayrenc, M., 2000. Population structure and genetic typing of *Trypanosoma cruzi*, the agent of Chagas disease: a multilocus enzyme electrophoresis approach. Parasitology 120, 513–526.

Barnabé, C., Brisse, S., Tibayrenc, M., 2003. Phylogenetic diversity of bat trypanosomes of subgenus *Schizotrypanum* based on multilocus enzyme electrophoresis, random amplified polymorphic DNA, and cytochrome b nucleotide sequence analyses. Infect. Genet. Evol. 2, 201–208.

Bosseno, M.F., Garcia, L.S., Baunaure, F., Gastelum, E.M., Gutierrez, M.S., Kasten, F.L., Dumonteil, E., Breniere, S.F., 2006. Identification in triatomine vectors of feeding sources and *Trypanosoma cruzi* variants by heteroduplex assay and a multiplex miniexon polymerase chain reaction. Am. J. Trop. Med. Hyg. 74, 303–305

Bosseno, M.F., Barnabe, C., Sierra, M.J., Kengne, P., Guerrero, S., Lozano, F., Ezequiel, K., Gastelum, M., Breniere, S.F., 2009. Wild ecotopes and food habits of *Triatoma longipennis* infected by *Trypanosoma cruzi* lineages I and II in Mexico. Am. J. Trop. Med. Hyg. 80, 988–991.

Breniere, S.F., Bosseno, M.F., Magallon-Gastelum, E., Castillo Ruvalcaba, E.G., Gutierrez, M.S., Montano Luna, E.C., Basulto, J.T., Mathieu-Daude, F., Walter, A., Lozano-Kasten, F., 2007. Peridomestic colonization of *Triatoma longipennis* (Hemiptera, Reduviidae) and *Triatoma barberi* (Hemiptera, Reduviidae) in a rural community with active transmission of *Trypanosoma cruzi* in Jalisco state, Mexico. Acta Trop. 101, 249–257.

Brisse, S., Barnabé, C., Tibayrenc, M., 2000. Identification of six *Trypanosoma cruzi* phylogenetic lineages by random amplified polymorphic DNA and multilocus enzyme electrophoresis. Int. J. Parasitol. 30, 35–44.

de Freitas, J.M., Augusto-Pinto, L., Pimenta, J.R., Bastos-Rodrigues, L., Goncalves, V.F., Teixeira, S.M., Chiari, E., Junqueira, A.C., Fernandes, O., Macedo, A.M., Machado, C.R., Pena, S.D., 2006. Ancestral genomes, sex, and the population structure of *Trypanosoma cruzi*. PLoS Pathog. 2, e24.

Dereure, J., Barnabé, C., Vie, J.C., Madelenat, F., Raccurt, C., 2001 Trypanosomatidae from wild mammals in the neotropical rainforest of French Guiana. Ann. Trop. Med. Parasitol. Mar 95, 157–166.

Diosque, P., Barnabé, C., Padilla, A.M., Marco, J.D., Cardozo, R.M., Cimino, R.O., Nasser, J.R., Tibayrenc, M., Basombrio, M.A., 2003. Multilocus enzyme electrophoresis analysis of *Trypanosoma cruzi* isolates from a geographically restricted endemic area for Chagas' disease in Argentina. Int. J. Parasitol. 33, 997–1003.

Fernandes, O., Santos, S.S., Cupolillo, E., Mendonca, B., Derre, R., Junqueira, A.C., Santos, L.C., Sturm, N.R., Naiff, R.D., Barret, T.V., Campbell, D.A., Coura, J.R., 2001. A mini-exon multiplex polymerase chain reaction to distinguish the major groups of *Trypanosoma cruzi* and *T. rangeli* in the Brazilian Amazon. Trans. R. Soc Trop. Med. Hyg. 95, 97–99.

Lewicka, K., Brénière-Campana, S F., Barnabé, C., Dedet, J.P., Tibayrenc, M., 1995. An isoenzyme survey of *Trypanosoma cruzi* genetic variability in sylvatic cycles from French Guiana. Exp. Parasitol. 81, 20–28.

Lisboa, C.V., Xavier, S.C., Herrera, H.M., Jansen, A.M., 2009 The ecology of the *Trypanosoma cruzi* transmission cycle: dispersion of zymodeme 3 (Z3) in wild hosts from Brazilian biomes. Vet. Parasitol..

Miles, M.A., Cibulskis, R.E., 1986. Zymodeme characterization of *Trypanosoma cruzi*. Parasitol. Today 2, 94–97.

Monteiro, W.M., Magalhaes, L.K., Santana Filho, F.S., Borborema, M., Silveira, H., Barbosa, M.D., 2010. *Trypanosoma cruzi* TcII/Z3 genotype as agent of an outbreak of Chagas disease in the Brazilian Western Amazonia. Trop. Med. Int. Health.

O'Connor, O., Bosseno, M.F., Barnabe, C., Douzery, E.J., Breniere, S.F., 2007. Genetic clustering of *Trypanosoma cruzi* I lineage evidenced by intergenic miniexon gene sequencing. Infect. Genet. Evol..

Santos-Mallet, J.R., Silva, C S., Gomes, S.A., Oliveira, D.L., Santos, C.L., Sousa, D.M., Pinheiro, N.L., Junqueira, A.C., Goncalves, T.C., 2008. Molecular characterization of *Trypanosoma cruzi* sylvatic isolates from Rio de Janeiro, Brazil. Parasitol. Res. 103, 1041–1045.

Solari, A., Muñoz, S., Venegas, J., Wallace, A., 1990. Characterization of Chilean and other south American *T. cruzi* population by RFLP of Kinetoplast DNAs. Mem. Inst. Oswaldo Cruz 85, 55.

Subileau, M., Barnabe, C., Douzery, E.J., Diosque, P., Tibayrenc, M., 2009. *Trypanosoma cruzi* new insights on ecophylogeny and hybridization by multigene sequencing of three nuclear and one maxicircle genes. Exp. Parasitol..

Tibayrenc, M., Ayala, F.J., 1988. Isozyme variability in *Trypanosoma cruzi*, the agent of Chagas' disease: genetical, taxonomical, and epidemiological significance. Evolution 42, 277–292.

Westenberger, S.J., Barnabé, C., Campbell, D.A., Sturm, N.R., 2005. Two hybridization events define the population structure of *Trypanosoma cruzi*. Genetics 171, 527–543.

Zingales, B., Andrade, S.G., Briones, M.R., Campbell, D.A., Chiari, E., Fernandes, O., Guhl, F., Lages-Silva, E., Macedo, A.M., Machado, C.R., Miles, M.A., Romanha, A.J., Sturm, N.R., Tibayrenc, M., Schijman, A.G., 2009. A new consensus for *Trypanosoma cruzi* intraspecific nomenclature: second revision meeting recommends TcI to TcVI. Mem. Inst. Oswaldo Cruz 104, 1051–1054.

Glossary

DTU: discrete typing unit
RAPD: random amplification of polymorphic DNA
RFLP: restriction fragment length polymorphism

Susceptibility of Sylvatic *Triatoma infestans* From Andeans Valleys of Bolivia to Deltamethrin and Fipronil

GONZALO ROCA ACEVEDO,[1,2] GASTÓN MOUGABURE CUETO,[1] MÓNICA GERMANO,[1] PABLO SANTO ORIHUELA,[1] MIRKO ROJAS CORTEZ,[3] FRANÇOIS NOIREAU,[4,5] MARÍA INÉS PICOLLO,[1] AND CLAUDIA VASSENA[1,6]

J. Med. Entomol. 48(4): 828–835 (2011); DOI: 10.1603/ME10208

ABSTRACT We describe the susceptibility to deltamethrin and fipronil of four sylvatic *Triatoma infestans* populations from the Andean valleys of Bolivia. Fifty percent lethal doses were determined from topical application of insecticide on first instars, and mortality was assessed after 24 h for deltamethrin and 48 h for fipronil. In comparison with a reference strain from Argentina, the Bolivian populations showed deltamethrin 50 percent lethal dose ratios ranging from 1.9 to 17.4. In the case of fipronil, an insecticide never used for control of *T. infestans*, the Bolivian populations showed even higher variation in toxic response, with relative susceptibilities ranging from 0.5 to 139.2. However, although the sylvatic *T. infestans* toxicological profiles differ from each other and from those of the domiciliary population studied in this work, there were no significant differences in the activities of P450 mono-oxygenases and pyrethroid esterases between the reference strain and the studied populations.

KEY WORDS *Triatoma infestans*, insecticide susceptibility, sylvatic populations

Chagas disease, because of infection with *Trypanosoma cruzi*, is endemic to the American continent, mainly transmitted to humans by blood-sucking triatomine bugs (Hemiptera, Reduviidae, Triatominae). In Argentina and Bolivia, ≈9 million people are currently infected (Schofield et al. 2006), and the main vector of *T. cruzi* is *Triatoma infestans* (Klug, 1834).

Domestic infestations of *T. infestans* are being successfully controlled in much of the Southern Cone of South America, by spraying infested dwellings with residual pyrethroid insecticides (Dias et al. 2002, Schofield et al. 2006). However, only limited success has been achieved in the Gran Chaco of Argentina, Bolivia, and Paraguay, even in areas under intensive vector control efforts (Gürtler et al. 2007). This region is thought to represent the origin of *T. infestans* and it is there where it shows highest genetic variability (Bargues et al. 2006, Torres-Pérez et al. 2011) and emerging resistance to pyrethroid insecticides (Gürtler et al. 2004, Picollo et al. 2005, Lardeux et al. 2010, Moncayo and Yanine 2006, Cecere et al. 2006, Toloza et al. 2008, Santo Orihuela et al. 2008, Ceballos et al. 2009).

Characterization of the resistance developed north of Salta (Argentina) and south and center of Bolivia has shown important toxicological differences (Toloza et al. 2008, Germano et al. 2010). Populations near the Argentinian and Bolivian border had high levels of resistance to deltamethrin, but were susceptible to fipronil, a phenylpyrazole insecticide. Populations of the Andean areas of Bolivia are of particular interest because they show different patterns of resistance in eggs and first instars than in the north of Salta, showing intermediate levels of resistance to deltamethrin and very high levels to fipronil. (Toloza et al. 2008, Germano et al. 2010).

Research on resistance to insecticides in *T infestans* has been focused on domiciliary populations, but recent reports provide evidence that sylvatic populations of *T. infestans* are much more widespread than previously thought (Noireau et al. 2005, Noireau 2009). The objective of the current study was to determine whether the susceptibility of sylvatic *T. infestans* populations from Bolivia is similar to that of domiciliary populations.

Materials and Methods

Insects. *T. infestans* were collected in 2007 from domicilliary (D) areas in the Department of Cochabama, Bolivia (Mataral–D), and from a nearby sylvatic (S) area ≈2 km distant (Mataral–S). Additional sylvatic bugs were collected in February 2008 from other

[1] Centro de Investigaciones de Plagas e Insecticidas (CITEFA-CONICET), Juan Bautista de la Salle 4397 (B1603ALO), Villa Martelli, Provincia de Buenos Aires, Argentina

[2] Corresponding author, e-mail: gonzalora@conicet.gov.ar

[3] Programa Nacional de Control de Chagas, Ministerio de Salud, La Paz, Bolivia

[4] Institut de Recherche pour le Développment, Montpellier, France

[5] Instituto de Investigaciones Biomédicas e Interacción Social, Universidad Mayor de San Simón, Cochabamba, Bolivia.

[6] Instituto de Investigación e Ingeniería Ambiental, 3iA, de la Universidad Nacional de San Martín, Buenos Aires, Argentina.

0022-2585/11/0828–0835$04.00/0

Table 1. Samples of sylvatic (S) and domiciliary (D) populations of *T. infestans* analyzed according to the collecting site in Bolivia

Site of collection	Location (city/province)	Latitude/Longitude	Altitude meters above sea level	No. of specimens collected in the field
NFS[a]	Susceptible reference strain	–	–	–
Mataral—D	Alquile/Cochabamba	18°35′44,08″ S/65°08′58,74″ W	1,750	10
Mataral—S	Alquile/Cochabamba	18°36,190 S/65°07,117 W	1,750	18
Kirus Mayu—S	Toro Toro/Potos	17°59,302 S/65°50,281 W	2,070	30
Ilicuni—S	Omereque/Cochabamba	18°09,502 S/64°51,943	1,580	10
20 de Octubre—S	Cochabamba/Cochabamba	17°29,057 S/66°06,741 W	2,596	32

[a] Susceptible reference strain.

sites in Cochabamba (Ilicuni–S and 20 de Octubre–S) and Potosi (Kirus-Mayu–S) (Table 1, Fig. 1).

The sylvatic *T. infestans* were collected from rockpiles using mouse-baited sticky traps (Noireau et al. 1999). They were reared in Bolivia, and eggs of the descendent populations were transported to the laboratory in Argentina, where further generations were bred.

For comparison, we used a susceptible reference strain (NFS) derived from a domestic population collected in December 2004, from Santiago del Estero, Argentina, in an area where insects have since been successfully controlled with deltamethrin.

For the susceptibility tests, first instars of each population were kept in enclosed boxes (30 × 30 × 30 cm) at 28 ± 1°C, 50–60% RH, and a photoperiod of 12:12 (L:D) h. A pigeon was weekly provided as a blood meal source (WHO 1994).

Chemicals. Technical grade deltamethrin (99.0%) and fipronil (95.5%) were obtained from Ehrestorfer (Augsburg, Germany). Analytical grade acetone was purchased from J. T. Baker (Edo. de Mex., Mexico). The 7-ethoxycoumarin (7-EC) and 7-hydroxycoumarin (7-OHC) (umbelliferone) were purchased from Sigma-Aldrich (St. Louis, MO). The *cis-trans* (43.8% *cis;* 56.2% *trans*)-permethrinic acid was supplied by Chemotecnica (Buenos Aires, Argentina), and thionyl chloride (Cl_2SO; 99%) and triethylamine (+99%) were purchased from Aldrich Chemical (Milwaukee, WI). The 7-coumaryl permethrate (7-CP) was synthesized in our laboratory by the method of Santo Orihuela et al. (2006).

Topical Application Bioassays. Lethal doses were determined according to the World Health Organization protocol (WHO 1994). *T. infestans* first instars (5–7 d old; mean weight 1.3 ± 0.2 mg), unfed since emergence, were selected for the toxicity tests. Bioassays consisted of topical application on the dorsal abdomen with 0.2 μl of the insecticide diluted in acetone, using a 10 μl Hamilton syringe with automatic dispenser. Control groups received only pure acetone.

Three replicates of at least four doses in a range that produced between 10 and 100% mortality were conducted. Mortality was evaluated after 24 h by placing the insects at the center of a circular filter paper, 11 cm diameter; only those nymphs able to walk to the border of the filter paper were considered alive.

Statistical Analysis. Mortality data were analyzed using the POLO Plus software (LeOra Software 1987).

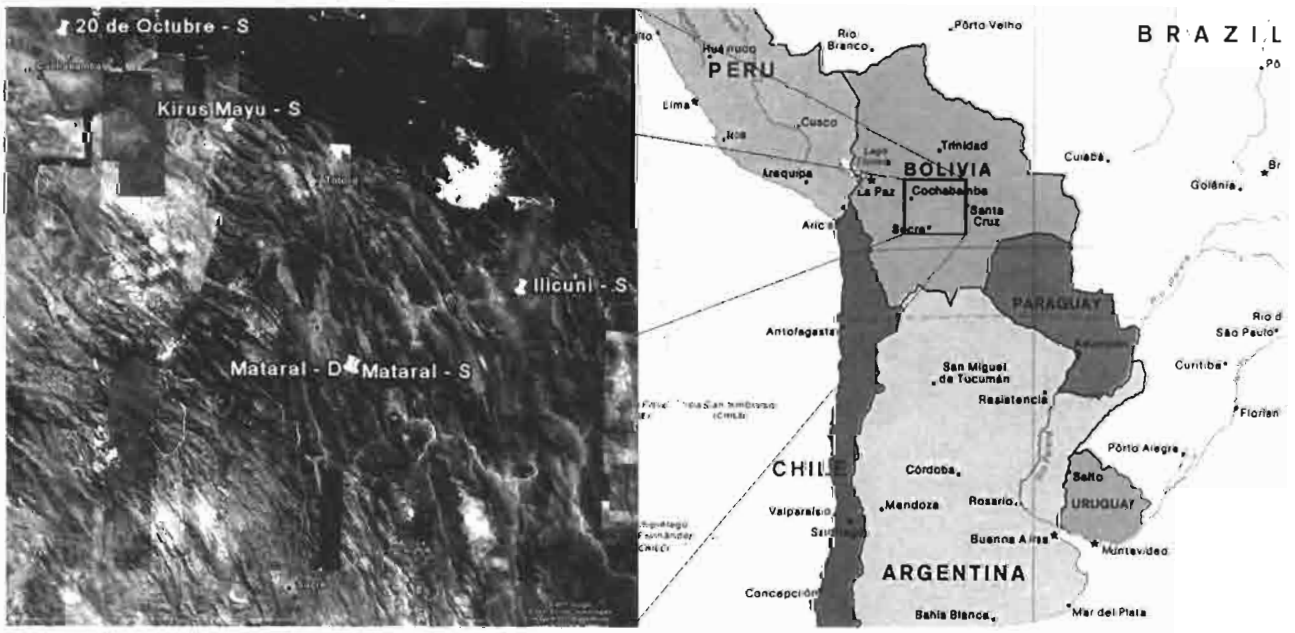


Fig. 1. Map showing study sites where *T. infestans* samples were collected. The populations collected in Bolivia were as follows: Mataral (domiciliary and sylvatic), Ilicuni (sylvatic), 20 de Octubre (sylvatic), and Kirus Mayu (sylvatic).

Table 2. Toxicity of topically applied deltamethrin to *T. infestans* first instars of a susceptible reference strain, sylvatic (S), and domiciliary (D) field populations collected from the Andean valleys of Bolivia

Population	n^a	Slope ± SE	χ^2	LD_{50} ng/insect (95% CL)	LDR_{50} (95% CL)	LD_{95} ng/insect (95% CL)	LDR_{95} (95% CL)
NFS[b]	120	3.10 ± 0.26	0.96	0.13 (0.11–0.15)	–	0.44 (0.58–0.36)	
Mataral-D[c]	186	1.11 ± 0.10	5.17	2.25 (0.28–4.80)	17.4 (11.88–25.43)	68.31 (24.82–2,220.54)	156.5 (78.548–311.70)
Mataral-S	90	1.55 ± 0.10	51.34	1.53 (0.53–3.34)	11.9 (9.43–14.93)	17.46 (6.52–385.42)	39.9 (5.08–312.96)
Kirus-Mayu-S	120	1.42 ± 0.08	34.72	0.95 (0.49–1.66)	7.4 (5.78–9.25)	13.82 (6.33–60.50)	31.6 (20.73–48.16)
20 de Octubre-S	120	1.50 ± 0.15	25.2	0.88 (0.08–1.84)	6.8 (4.98–9.26)	10.91 (4.59–393.10)	24.9 (15.95–38.93)
Ilicuni-S	120	1.33 ± 0.10	4.33	0.25 (0.14–0.39)	1.9 (1.44–2.56)	4.22 (2.09–13.82)	9.7 (5.74–16.25)

CL, confidence limit; LD_{95}, 95% lethal dose; LDR_{50}, 50% LDR; LDR_{95}, 95% LDR (calculated following Robertson et al. [2007]).

[a] Number of insects used for bioassays

[b] Reference strain.

[c] Data from Toloza et al. 2008

Dose-mortality data were subject to probit analysis (Litchfield and Wilcoxon 1994) to estimate the lethal dose (nanograms per insect) required to kill 50% of treated individuals (50% lethal dose [LD_{50}]). Lethal dose ratios (LDR) and 95% confidence limits of each population were calculated, as described by Robertson et al. (2007), by comparison of the dose-response curves between the studied populations and the reference strain. Studied populations were considered resistant if the LDR confidence limits did not include the number 1.

A nonparametric test (Kruskal-Wallis) was used to compare the enzymatic activities (P450 mono-oxygenases and pyrethroid esterases) of the studied populations.

Cytochrome P450 Mono-Oxygenase. Enzymatic activities were measured for individual abdomens using 7-EC-*O*-deethylation (Bouvier et al. 1998, González Audino et al. 2005). Fluorescence of 7-OHC was determined using a microplate fluorescence reader (Packard Fluorocount, Meriden, CT) with 400 nm excitation and 440 nm emission filters.

First-instar abdomens were placed individually into wells of a 96-well microplate containing 100 μl of 0.05 M phosphate buffer and 4 mM 7-EC. The reaction was stopped after incubation (4 h at 30°C) by adding 100 μl of glycine buffer (10^{-4} M), pH 10.4. Microplates were centrifuged at 2,000 × *g* for 30 s in a refrigerated centrifuge for microplates (4237 R, ALC International SRL, Cologna Monzese, Italy) before and after the incubation of the enzymatic reaction at 30°C. For each population, similar wells receiving glycine buffer previous to incubation were used for blanks. The relative fluorescence units were all corrected for background hydrolysis, nonspecific fluorescence of substrate, and transformed to picomoles per minute (activity units) by using a calibration curve per replicate with dilutions of 7-OHC (60.19, 117.50, 172.50, and 224.33 total pmol/well).

Esterase Activity. Esterase activity was determined by the hydrolysis of 7-CP, a new fluorescent substrate appropriate for determining pyrethroid hydrolysis activity on individual insects (Santo Orihuela et al. 2006). For this, the insects were cooled and each nymph was homogenized in 220 μl of phosphate buffer (pH 7.2, 0.05 M) using a plastic mortar and pestle. Reaction was initiated by adding 10 μl of 7-CP (3.5 mM, 2-methoxy ethanol) to 190 μl of each homogenate. Incubation was performed at 25°C for 33 min, at pH 7.2. The fluorescence was measured using a microplate fluorescence reader (Packard Fluorocount), and results were analyzed with Fluorocount and Excel 2000 software. Assays were conducted at 25°C in black 96-well polystyrene flat-bottom microtiter plates (Packard, Meriden, CT). Production of 7-OHC was monitored with excitation wavelength at 400 nm and emission at 440 nm. Activity was measured each 3 min for 33 min, such that assay was linear over the reported time. The relative fluorescence units were all corrected for background hydrolysis, nonspecific fluorescence of substrate, and transformed in picomoles per minute (activity units) using a calibration curve per duplicate with dilutions of 7-OHC (68.5, 342.69, 685.44, and 1370.8 total pmol/well).

Results

Toxicology. The sylvatic Mataral-S population showed a LD_{50} similar to that of the domiciliary insects (Mataral-D) from the same area. Kirus-Mayu-S and 20 de Octubre-S populations showed LD_{50} lower than Mataral insects, but higher than those previously reported for susceptible populations (Table 2).

Susceptibility values (LD_{50} and 95% lethal dose), slopes of regression lines, and LDR to fipronil in the studied population are shown in Table 3. Mataral-D, Mataral-S, and Kirus-Mayu-S populations showed LDR (50%) between 23.4 and 139.2, values that differ significantly from 1, indicating that those populations were resistant to fipronil. The Ilicuni-S population showed a lower level of resistance, whereas the 20 de Octubre-S population showed susceptibility to fipronil.

As the slopes of dose-response curve of the studied populations were steeper than that of the reference strain, the LDR calculated at 95% mortality levels were higher than those calculated at 50% mortality. Because the toxicity values at 50% mortality have the lowest statistical error, the discussion of results is based on LDRs obtained at that level.

We had observed that sylvatic *T. infestans* tend to be larger than domiciliary *T. infestans*, but even expressing the deltamethrin and fipronil activity as ng insec-

Table 3. Toxicity of topically applied fipronil to *T. infestans* first instars of a susceptible reference strain, sylvatic (S), and domiciliary (D) field populations collected from the Andean valleys of Bolivia

Population	n^a	Slope ± SE	χ^2	LD_{50} ng/insect (95% CL)	LDR_{50} (95% CL)	LD_{95} ng/insect (95% CL)	LDR_{95} (95% CL)
NFS[b]	90	1.15 ± 0.11	11.55	2.12 (1.6–3.2)	–	56.86 (31.14–131.01)	–
Mataral–D	90	0.75 ± 0.09	3.98	296.0 (160.2–414.8)	139.2 (77.05–251.46)	4.62×10^4 (1.83×10^4–16.7×10^4)	819.1 (169.72–3,952.90)
Mataral–S	120	0.49 ± 0.05	13.43	49.8 (10.2–256.0)	23.4 (12.07–45.54)	10.62×10^1 (0.34×10^1–3.32×10^6)	1,900.9 (324.98–11,118.72)
Kirus-Mayu–S	120	0.78 ± 0.06	8.16	96.86 (32.0–332.0)	45.6 (26.67–77.80)	1.23×10^1 (0.31×10^1–12.6×10^1)	217.7 (72.41–654.43)
20 de Octubre–S	90	0.53 ± 0.09	5.05	1.0 (0.5–2.1)	0.5 (0.21–1.13)	1,294.9 (350.45–7,927.64)	23.2 (2.27–237.97)
Illicuni–S	90	1.16 ± 0.14	0.96	11.8 (8.6–17.0)	5.5 (3.39–8.97)	30.4 (14.0–1,048)	5.4 (1.65–17.41)

CL, confidence limit. LD_{95}, 95% lethal dose; LDR_{50}, 50% LDR; LDR_{95}, 95% LDR (calculated following Robertson et al. [2007]).

[a] Number of insects used for bioassays.

[b] Reference strain.

ticide per insect weight unit (Table 4), their lower susceptibility is apparent.

The frequency distribution of 7-OHC activities among domiciliary and sylvatic *T. infestans* populations is shown in Fig. 2. The frequency distribution of pyrethroid esterase activity is shown in Fig. 3. In these histograms, vertical lines are marked at values of 1.44 pmol/min for mono-oxygenases and 30.7 for pyrethroid esterase activity, which represent the corresponding threshold of enzyme activity containing the majority of insects (>75%) in susceptible populations.

There are no significant differences between the domiciliary, sylvatic, and reference populations in pyrethroid esterase and mono-oxygenase activity levels.

Discussion

This work presents the first study on toxicological profiles of sylvatic *T. infestans*. Those profiles included the susceptibility values to deltamethrin and fipronil, and the activities of detoxifying enzymes (pyrethroid esterases and mono-oxygenases), which were compared with those of a domiciliary population from the same Bolivian area and a reference colony. The toxicological profiles were different both among the Bolivian populations and between reference and Bolivian populations. All populations except one (20 de Octubre–S) were less susceptible than the NFS reference colony for the two insecticides, with the susceptibility to fipronil less than to deltamethrin. The domiciliary population, Mataral–D, was the least susceptible to both insecticides, followed by Mataral–S and Kirus-Mayu–S, whereas the 20 de Octubre–S and Illicuni–S populations were the most susceptible to both insecticides. These differences in the susceptibilities could not be explained by the activities of detoxifying enzymes given that there was no association between enzymatic activities and the susceptibility ratios. These comparative results support the idea that *T. infestans* from different geographic areas have different toxicological profiles determining differences in their susceptibility to insecticides (Germano et al. 2010).

The populations collected from sylvatic habitats had not been targeted by chemical control interventions. In addition, current evidence does not support a consistent flow of Andean *T. infestans* between sylvatic refuges and domestic habitats (Richer et al. 2007). In consequence, the toxicological profiles shown by sylvatic populations are unlikely to result from selection pressure with insecticide or gene flow from controlled areas, but those profiles would represent wild toxicological phenotypes. As those wild profiles were different, this study demonstrates that *T. infestans* from different geographic areas have different toxicological profiles.

As the Andean valleys in Bolivia are believed to represent the center of origin and dispersal of *T. infestans* (Bargues et al. 2006, Cortez et al. 2010), it is possible to speculate that the toxicological profiles of the sylvatic populations from Cochabamba could represent the ancestral toxicological profile of *T. infestans*. Nevertheless, phylogenetic evidence does not unequivocally support the hypothesized Andean origin

Table 4. Toxicity of deltamethrin against sylvatic (S) and domiciliary (D) populations of *T. infestans* from Andean valleys of Bolivia

Population	Weight (mg) (95% CL)	DL_{50} (ng/μg) (95% CL) deltamethrin	LDR	DL_{50} (ng/μg) (95% CL) fipronil	LDR	DL_{95} (ng/μg) (95% CL) deltamethrin	LDR	DL_{95} (ng/μg) (95% CL) fipronil	LDR
NFS[a]	1.31 (0.07)	0.10 (0.09–0.11)	–	1.52 (1.22–2.44)	–	0.34 (0.44–0.27)	–	43.40 (23.77–100)	–
Mataral–D	1.94 (0.22)	0.86 (0.14–2.47)	8.7	152.58 (82.58–213.81)	100.38	35.21 (5.72–1,144.61)	104.83	2.38×10^4 (9.43×10^3–5.60×10^4)	548.36
Mataral–S	2.31 (0.04)	0.66 (0.23–1.45)	6.70	21.56 (4.41–110.82)	14.18	7.56 (2.82–166.85)	22.24	4.60×10^3 (1.47×10^3–1.44×10^4)	1,059.90
Kirus-Mayu–S	2.19 (0.41)	0.43 (0.22–0.76)	4.38	44.24 (14.61–151.60)	29.10	6.31 (2.89–27.63)	18.56	5.61×10^3 (1.41×10^3–5.75×10^4)	129.26
20 de Octubre–S	2.33 (0.10)	0.37 (0.03–0.79)	3.81	0.43 (0.21–0.90)	0.28	4.68 (1.97–168.71)	13.76	555.75 (150.41–3,402.42)	12.81
Ilicani–S	1.95 (0.05)	0.13 (0.07–0.20)	1.30	6.05 (4.41–8.72)	3.98	2.16 (1.07–7.09)	6.35	155.90 (71.80–537.44)	3.59

Activity expressed as ng of insecticide per insect weight unit (ng/μg). CL, confidence level.
[a] Susceptible reference strain.

of *T. infestans*, and instead suggests an origin in the Gran Chaco (Torres-Pérez et al. 2011). Future studies should be conducted to test those evolutionary and toxicological hypotheses.

The situation is different for the domiciliary population, which showed the lowest susceptibility to both deltamethrin and fipronil. This result is in accordance with Toloza et al. (2008) and Germano et al. (2010), who detected a low susceptibility to fipronil in domiciliary populations with moderate resistance to deltamethrin in the center and south of Bolivia. The toxicological profile of those populations can be interpreted in terms of resistance. As the pyrethroids alphacypermethrin and deltamethrin have been widely used for control of domestic *T. infestans* during the last decade, the evolution of pyrethroid resistance could be the consequence. However, Germano et al. (2010) discussed the possibility of cross-resistance between fipronil and lindane or dieldrin due to the early use of these compounds in Bolivia.

The higher resistance level to fipronil than that to deltamethrin in the domiciliary population of Mataral could be explained in terms of the alleged evolutionary relationship between domiciliary and sylvatic populations of *T. infestans* from that area. As yet there is no evidence for recolonization of sylvatic habitats from a domiciliary population, so the hypothesis that domiciliary *T. infestans* have derived from sylvatic populations is favored. The sylvatic populations from Mataral would express the wild toxicological profile of *T. infestans* from that area, as follows: low susceptibility to deltamethrin and fipronil, with lower fipronil than deltamethrin susceptibility. In this context, it is possible to hypothesize that the toxicological baseline of domiciliary Mataral *T. infestans* was the wild toxicological profile of Mataral. Similarly, because the sylvatic Andean *T. infestans* showed lower susceptibility than the reference colony from Argentina, it may be that the toxicological profile of the reference susceptible population could be representing a wild toxicological profile of *T. infestans* from Argentina. This would imply the existence of different wild profiles for Argentina and Bolivia.

Toxicological differences between Argentinean and Bolivian populations were demonstrated in previous studies. Toloza et al. (2008) showed that deltamethrin resistance is shown in nymphs from Yacuiba (Bolivia), but not in eggs, whereas both the eggs and nymphs from Salvador Mazza (Argentina) express resistance to this insecticide. Toloza et al. (2008) and Germano et al. (2010) detected fipronil resistance in Bolivia, but not in Argentina. Santo Orihuela et al. (2008), studying the same populations, showed that the esterase activity was increased only in the population of Salvador Mazza. Those differences could have resulted from selection pressure with insecticides on populations of *T. infestans* with different wild toxicological profiles.

The susceptibility and biochemical differences may relate to the genetic structure of the *T. infestans* populations. Several studies confirmed that populations of *T. infestans* from Argentina and Bolivia are highly structured, both at macrogeographical and microgeo-

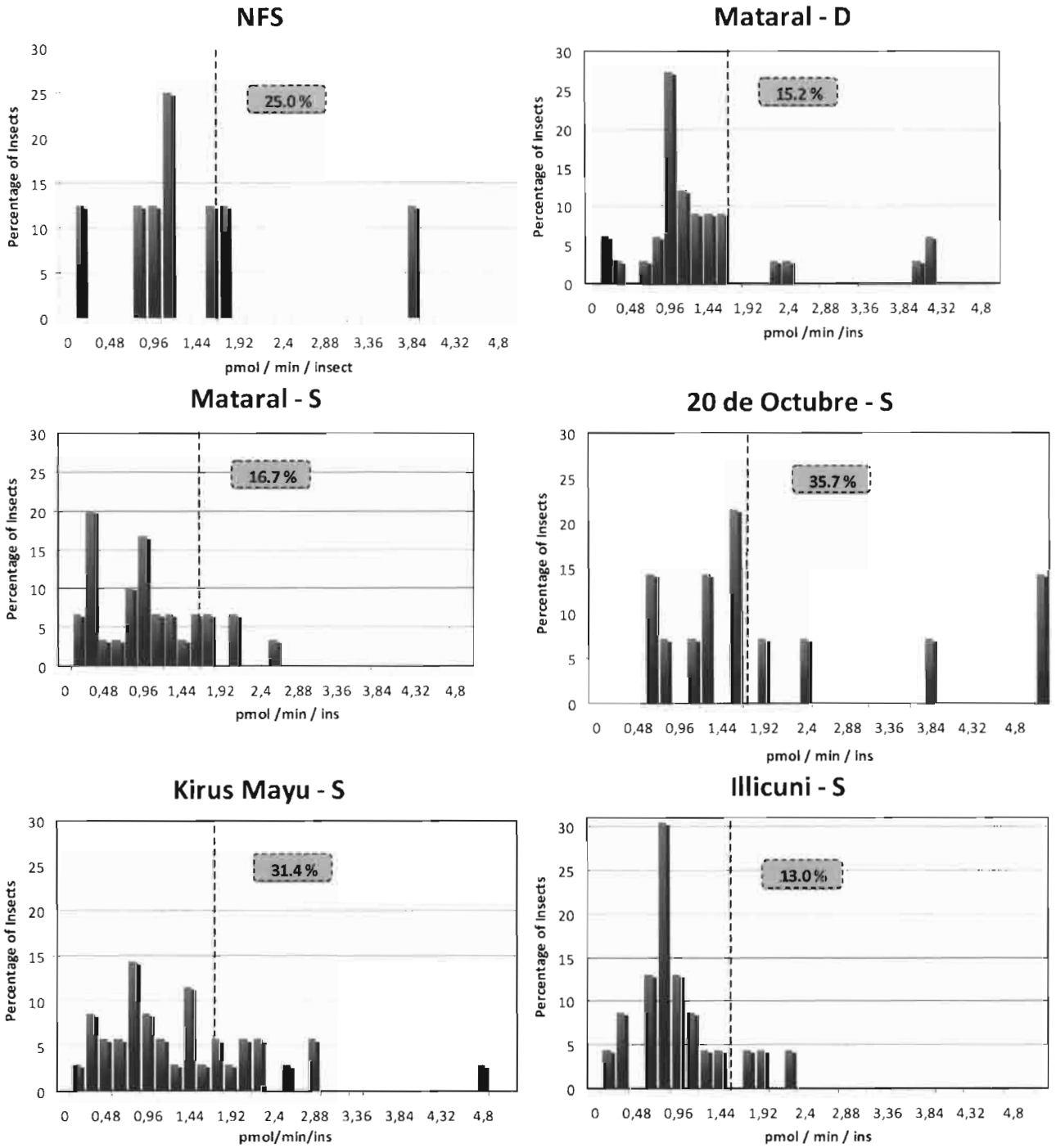


Fig. 2. Frequency distribution of P450 mono-oxygenase activity among sylvatic (S) and domiciliary (D) *T. infestans* from Andean valleys of Bolivia. Vertical lines are marked at values of 1.44 pmol/min, which represent the corresponding threshold of enzyme activity containing the majority of insects (>75%) in susceptible populations. Enzyme activity as picomoles of 7-OHC per minute.

graphical levels (Pérez de Rosas et al. 2007, 2008; Marcet et al. 2008). Furthermore, recent research demonstrated that Bolivian and Argentinian populations are part of different haplotype clusters (Monteiro et al. 1999, Piccinali et al. 2009). In toxicological bioassays, the slopes of the dose-response relationships are indicators of the population's phenotypic variation, e.g., high slopes indicate little phenotypic variation. If environmental variation were constant, the slope would be an indicator of genetic variation, with high slopes related to low genetic variation, and this would occur in populations fully susceptible or fully resistant. By contrast, populations in process of selection would have greater genetic variation, and therefore the slopes will be less steep (Chilcutt and Tabashnik 1995). This relationship seems to be observed in the susceptible population because they show higher slope values to both deltamethrin and fipronil. Field populations show a lower slope (except for Ilicuni–S and fipronil), suggesting greater genetic variability.

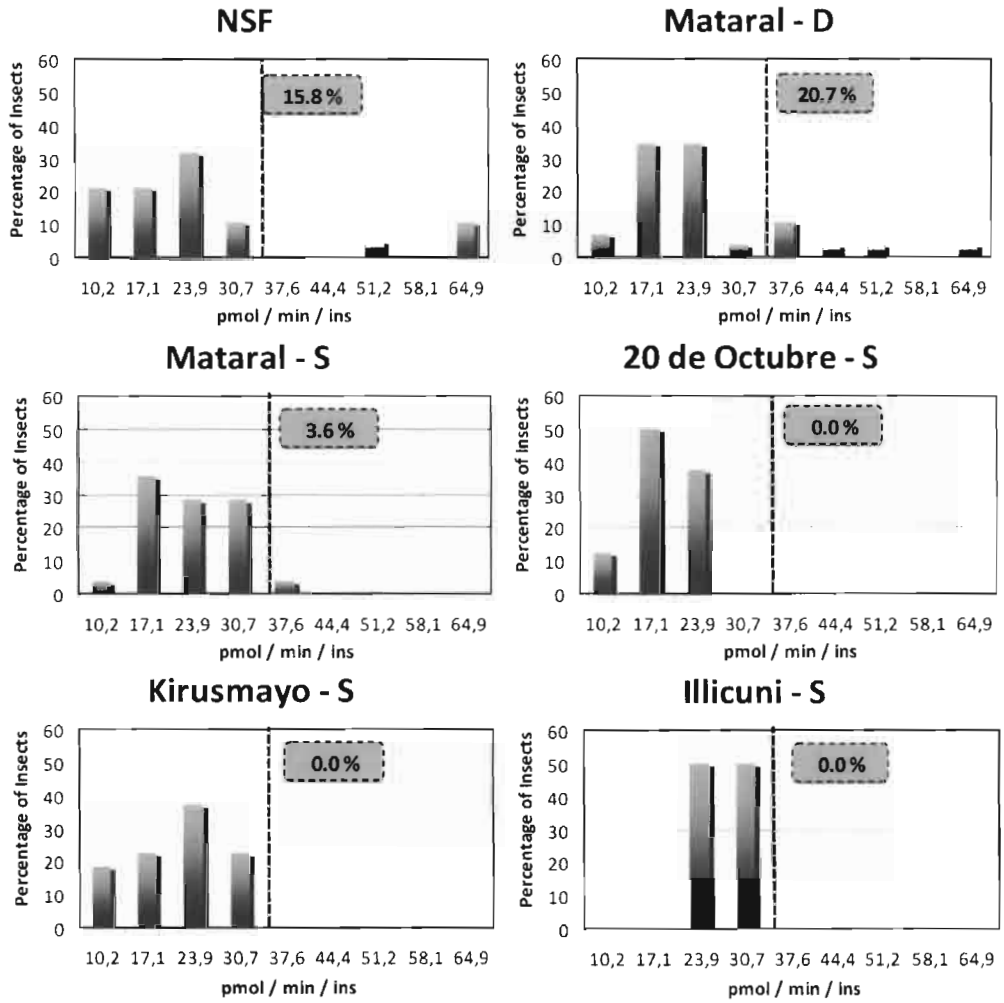


Fig. 3. Frequency distribution of pyrethroid esterase activity among sylvatic (S) and domiciliary (D) *T. infestans* from Andean valleys of Bolivia. Vertical lines are marked at values of 30.7 pmol/min, which represent the corresponding threshold of enzyme activity containing the majority of insects (>75%) in susceptible populations. Enzyme activity as picomoles of 7-OHC per minute.

Acknowledgments

This work was supported by the Red Latinoamericana de Control de Vectores (RELCOV), United Nations Children's Fund/United Nations Development Program/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases, and the Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina. We thank C. Schofield for helpful comments and editorial assistance.

References Cited

Bargues, M. D., D. R. Klisiowicz, F. Panzera, F. Noireau, A. Marcilla, R. Perez, M. G. Rojas, G. E. O'Connor, F. Gonzalez-Candelas, C. Galvão, J. Jurberg, R. U. Carcavallo, J. P. Dujardin, and S. Mas-Coma. 2006. Origin and phylogeography of the Chagas disease main vector *Triatoma infestans* based on nuclear rDNA sequences and genome size. Infect. Genet. Evol. 6: 46–62.

Bouvier, J. C., A. Cuany, C. Monier, V. Brosse, and B. Sauphanor. 1998. Enzymatic diagnosis of resistance to deltamethrin in diapausing larvae of the codling moth, *Cydia pomonella* (L.). Arch. Insect Biochem. Physiol. 39: 55–64.

Ceballos, L. A., R. V. Piccinali, I. Berkunsky, U. Kitron, and R. E. Gürtler. 2009. First finding of melanic sylvatic *Triatoma infestans* (Hemiptera: Reduviidae) colonies in the Argentine Chaco. J. Med. Entomol. 46: 1195–1202.

Cecere, M. C., G. M. Vazquez-Prokopec, R. E. Gürtler, and U. Kitron. 2006. Reinfestation sources for Chagas disease vector, *Triatoma infestans*, Argentina. Emerg. Infect. Dis. 12: 1096–1102.

Chilcutt, C. F., and B. E. Tabashnik. 1995. Evolution of pesticide resistance and slope of the concentration-mortality line: are they related? J. Econ. Entomol. 88: 11–20.

Cortez, M. R., F. A. Monteiro, and F. Noireau. 2010. New insights on the spread of *Triatoma infestans* from Bolivia: implications for Chagas disease emergence in the Southern Cone. Infect. Genet. Evol. 10: 350–353.

Dias, J.C.P., A. C. Silveira, and C. J. Schofield. 2002. The impact of Chagas disease control in Latin America: a review. Mem. Inst. Oswaldo Cruz 97: 603–612.

Germano, M. D., G. Roca Acevedo, G. A. Mougabure Cueto, A. C. Toloza, C. V. Vassena, and M. I. Picollo. 2010. 2010. New findings of insecticide resistance in *Triatoma infestans* (Heteroptera: Reduviidae) from the Gran Chaco. J. Med. Entomol. 47: 1077–1081.

González Audino, P., S. Barrios, C. V. Vassena, G. A. Mougabure Cueto, E. Zerba, and M. I. Picollo. 2005. Increased monooxygenase activity associated with resistance to permethrin in *Pediculus humanus capitis*

(Anoplura: Pediculidae) from Argentina. J. Med. Entomol. 42: 342–345.

Gürtler, R. E., D. M. Canale, C. Spillmann, R. Stariolo, O. D. Salomon, S. Blanco, and E. L. Segura. 2004. Effectiveness of residual spraying of peridomestic ecotopes with deltamethrin and permethrin on *Triatoma infestans* in rural western Argentina: a district-wide randomized trial. Bull. W.H.O. 82: 196–205.

Gürtler, R. E., U. Kitron, M. C. Cecere, E. L. Segura, and J. E. Cohen. 2007. Sustainable vector control and management of Chagas disease in the Gran Chaco, Argentina. Proc. Natl. Acad. Sci. USA 104: 16194–16199.

Lardeux, F., S. Depickère, S. Duchon, and T. Chávez. 2010. Insecticide resistance of *Triatoma infestans* (Hemiptera, Reduviidae) vector of Chagas disease in Bolívia. Trop. Med. Int. Health. 15: 1037–1048.

LeOra Software. 1987. POLO-PC: a user's guide to probit or logit analysis. LeOra Software, Berkeley, CA.

Litchfield, J., and F. Wilcoxon. 1994. A simplified method of evaluating dose-effect experiments. J Pharmacol. Exp. Ther. 96: 99–113.

Marcet, P. L., M. S. Mora, A. P. Cutrera, L. Jones, R. E. Gürtler, U. Kitron, and E. M. Dotson. 2008. Genetic structure of *Triatoma infestans* populations in rural communities of Santiago del Estero, northern Argentina. Infect. Genet. Evol. 8: 835–846.

Moncayo, A., and M. I. Ortiz Yanine. 2006. An update on Chagas disease (human American trypanosomiasis). Ann. Trop. Med. Parasitol. 100: 663–677.

Monteiro, F. A., R. Perez, F. Paenza, J. P. Dujardin, C. Galvao, D. Rocha, F. Noireau, C. Schofield, and C. B. Beard. 1999. Mitochondrial DNA variation of *Triatoma infestans* populations and its implication on the specific status of *T. melanosoma*. Mem. Inst. Oswaldo Cruz 94 (Suppl. 1): 229–238.

Noireau, F. 2009. Wild *Triatoma infestans*, a potential threat that needs to be monitored. Mem. Inst. Oswaldo Cruz 104 (Suppl. 1): 60–64.

Noireau, F., R. Flores, and F. Vargas. 1999. Trapping sylvatic Triatominae (Reduviidae) in hollow trees. Trans. R. Soc. Trop. Med. Hyg. 93: 13–14.

Noireau, F., M. R. Cortez, F. A. Monteiro, A. M. Jansen, and F. Torrico. 2005. Can wild *Triatoma infestans* foci in Bolivia jeopardize Chagas disease control efforts? Trends Parasitol. 21: 7–10.

Noireau, F., P. Diosque, and A. M. Jansen. 2009. *Trypanosoma cruzi*: adaptation to its vectors and its hosts. Vet. Res. 40: 26.

Pérez de Rosas, A., E. Segura, and B. García. 2007. Microsatellite analysis of genetic structure in natural *Triatoma infestans* (Hemiptera: Reduviidae) populations from Argentina: its implication in assessing the effectiveness of Chagas disease vector control programmes. Mol. Ecol. 16: 1401–1412.

Pérez de Rosas, A., E. Segura, L. Fichera, and B. García. 2008. Macrogeographic and microgeographic genetic structure of the Chagas disease vector *Triatoma infestans* (Hemiptera: Reduviidae) from Catamarca, Argentina. Genetica 133: 247–260.

Piccinali, R. V., P. L. Marcet, F. Noireau, U. Kitron, R. E. Gürtler, and E. M. Dotson. 2009. Molecular population genetics and phylogeography of the Chagas disease vector *Triatoma infestans* in South America. J. Med. Entomol. 46: 796–809.

Picollo, M. I., C. V. Vassena., P. Santo Orihuela, S. Barrios, and E. N. Zerba. 2005. High resistance to pyrethroid insecticides associated with ineffective field treatments in *Triatoma infestans* (Hemiptera, Reduviidae), form northern Argentina. J. Med. Entomol. 42: 637–642.

Richer, W., P. Kengne, M. Rojas Cortez, M. M. Perrineau, A. Cohuet, D. Fontenille, and F. Noireau. 2007. Active dispersal by wild *Triatoma infestans* in the Bolivian Andes. Trop. Med. Int. Health 72: 759–764

Robertson, J. L., R. M. Russell, H. K. Preisler, and N. E. Savin. 2007. Bioassays with Arthropods. 2nd ed. CRC, Boca Raton, FL.

Santo Orihuela, P. L., M. I. Picollo, P. González Audino, S. Barrios, E. Zerba, and H. Masuh. 2006. 7-Coumaryl permethrate and its *cis*- and *trans*-isomers as new fluorescent substrates for examining pyrethroid-cleaving enzymes. Pest Manag. Sci. 62: 1039–1044.

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

Schofield, C. J., J. Jannin, and R. Salvatella. 2006. The future of Chagas disease control. Trends Parasitol. 12: 583–588.

Toloza, A. C., M. Germano, G. Mougabure Cueto, C. V. Vassena, E. Zerba, and M. I. Picollo. 2008. Differential patterns of insecticide resistance in eggs and first instars of *Triatoma infestans* (Hemiptera: Reduviidae) from Argentina and Bolivia. J. Med. Entomol. 45: 421–426.

Torres-Pérez, F., M. Acuna-Retamar, J. A. Cook, A. Bacigalupo, A. García, and P. E. Cattan. 2011. Statistical phylogeography of Chagas disease vector *Triatoma infestans*. testing biogeographic hypotheses of dispersal. Infect. Genet. Evol. 11: 167–174.

[WHO] World Health Organization. 1994. Protocolo de evaluación de efecto insecticida sobre triatominos Acta. Toxicol. Argent. 2: 29–32.

Received 21 September 2010, accepted 8 March 2011.

Short Title: Predominance of TcI in wild *Triatoma infestans*

Genetic Characterization of *Trypanosoma cruzi* DTUs in Wild *Triatoma infestans* from Bolivia: Predominance of TcI

Simone Frédérique Brenière[1,2,*], Claudia Aliaga[1,2], Etienne Waleckx[1,2], Rosio Buitrago[1,2], Renata Salas[1,2], Christian Barnabé[1,2], Michel Tibayrenc[1,3], François Noireau[1,4]

[1] MIVEGEC (Université de Montpellier 1 et 2, CNRS 5290, IRD 224), Maladies Infectieuses et Vecteurs: Ecologie, Génétique, Evolution et Contrôle, Institut de Recherche pour le développement (IRD), Representation in Bolivia, Av Hernando Siles No. 5290, Esq Calle 7 Obrajes, CP 9214 La Paz, Bolivia

[2] Instituto Nacional de Laboratorios de Salud (INLASA), Laboratorio de Entomologia Médica, 14 Rafael Zubieta #1889, Miraflores, Casilla M-10019, La Paz, Bolivia

[3] Universidad Mayor de San Andrés, Facultad de Farmacia y Bioquímica, Servicios de Laboratorio de Diagnóstico e Investigaciones en Salud (SELADIS), Av. Saavedra, Miraflores, La Paz, Bolivia

[4] IIBISMED, Facultad de Medicina, Universidad Mayor de San Simon, Avenida Aniceto Arce 21 #371, Cochabamba, Bolivia

* corresponding author

Abstract

We characterized 234 stocks of *Trypanosoma cruzi* from wild *Triatoma infestans* collected from north to south of the endemic area of Chagas disease in Bolivia, combining mini-exon multiplex PCR (MMPCR) and sequencing of the glucose phosphate isomerase (*Gpi*) gene. Of the six genetic lineages ("Discrete Typing Units"; DTU) (TcI-VI) presently recognized in *T. cruzi*, TcI was overpredominant on TcIII in wild Andean *T. infestans*. In the lowlands (Bolivian Chaco), one TcI and one TcII stocks were identified in *T. infestans* "dark morph", which exhibited a lower *T. cruzi* infection rate than Andean specimens. Together with exploring at large scale which DTUs are infecting *T. infestans* wild populations, this study opens the discussion on the origin of TcI and TcV DTUs, that are predominant in domestic Bolivian cycles.

Key words: Chagas disease, molecular epidemiology, population genetics, parasite.

Introduction

Trypanosoma cruzi, the agent of Chagas disease, represents a serious health problem in the Americas, being the highest disease burden in Latin American with 8-9 million people infected and 25-90 million at risk [1-3]. This parasite, which belongs to the order Kinetoplastida, is mainly transmitted by blood-sucking bug vectors (Hemiptera, Reduviidae, Triatominae), but also by blood transfusion and oral transmission. Moreover, newborns can be infected through congenital transmission. There are over 140 species of triatomines, but only a few of them, belonging to three genera (*Triatoma, Rhodnius* and *Panstrongylus*) may be considered as important vectors of Chagas disease. With the exception of one species (*T. rubrofasciata*), all Triatominae have populations living in natural habitats in contact with wild mammals, birds or reptiles [4-7]. *T. cruzi* is found in two different, although overlapping, ecosystems. One is related to wild environment and involves wild triatomines and mammals (sylvatic cycle), and the other depends on human dwelling and surrounding artificial structures, involving domestic/peridomestic vector populations, humans, domestic and synanthropic animals (domestic cycle).

Population genetics analyses have shown that *T. cruzi* has a predominantly clonal mode of evolution, and exibits a considerable phenotypic and genetic diversity [8]. This population genetics model refers to genetic clonality, that is to say: limited or absent genetic recombination with persistence of durable multilocus associations, whatever be the cytological mechanism of reproduction [9]. Six distinct genetic lineages or "discrete typing units" (DTUs; [10] have been described [11, 12]. They have been recently validated by a committee of experts and labeled TcI to TcVI [13]. TcI is ubiquitous, and prevalent in different sylvatic cycles. It exhibits a considerable genetic diversity. It is responsible of the large majority of human infections in the Amazon basin and more northern countries, and part of the infections in South America. It is found with a notable frequency in domestic cycles. TcII, V, and VI are mainly associated with domestic cycles and prevalent in human infections in the Southern Cone countries; TcV and TcVI are hybrid genotypes, which putatives ancestors are TcII and TcIII [14, 15]. Finally, TcIII and IV are more scarcely sampled over all the endemic area and seem to be specific to sylvatic cycles, with few reports of human infection.

In Bolivia, *Triatoma infestans* (Hemiptera: Reduviidae) still remains the main domestic vector of *T. cruzi*. It is the target of the National Control Program based on house spraying with residual insecticides. Wild populations of *T. infestans* are now seriously considered as a problem to keep the villages free of triatomines [16-18]. Sylvatic populations of the vector have been described in different Andean valleys in Bolivia [16, 17, 19, 20]. Moreover, the detection of wild foci of *T. infestans* in the Bolivian Chaco has extended the distribution of wild populations to the lowlands of Bolivia [21, 22].

Two main genotypes belonging to TcI and TcV were previously identified in domestic cycle in regions where *T. infestans* was the main vector [23-29]. Moreover, these genotypes had been identified in strict sympatry in the same host [23, 30]. In contrast, few data are available on DTUs circulating in sylvatic *T. infestans*. Dujardin et al. [31] found that wild *T. infestans* were infected with the same *T. cruzi* genotypes than domestic *T. infestans* (TcI and TcV), with the same frequencies. They took it as additional evidence for lack of speciation between wild and domestic *T. infestans*. Another work identified TcI as the unique DTU in a wild focus located in the valley of Cochabamba [20].

Among the genetic markers that can identify the different *T. cruzi* groups and differentiate this parasite from *T. rangeli*, a non pathogenic trypanosome which causes diagnosis problems, the non-transcribed spacer region of the mini-exon gene was previously proposed to discriminate *T. cruzi* I (now TcI), *T. cruzi* II (now TcII), *T. cruzi* zymodeme 3 (now TcIV), and *T. rangeli* through the mini-exon multiplex PCR (MMPCR) [32]. Recently, the MMPCR was tested over a large sample of stocks belonging to the six DTUs, and three relevant groups of DTUs were identified in *T. cruzi* (group 1, now TcI, group 2, now TcII, TcV, and TcVI, and group 3, now TcIII and TcIV), and one in *T. rangeli* [33]. This method was also successfully applied for rapid DTU identification in triatomine digestive tracts [4, 34]. Moreover, among housekeeping genes, the glucose-6-phosphate isomerase (*Gpi*), a single copy nuclear gene, presented a sequence polymorphism valuable for DTUs characterization [14, 35].

In this study, we applied the MMPCR and *Gpi* sequencing for the characterization of *T. cruzi* DTUs directly in digestive tract of wild *T. infestans* collected in Bolivia.

Materials and methods

Origin of *T. infestans* populations

The triatomines were sampled in sylvatic environment from April to November 2009. Collections were carried out by using mice-baited adhesive traps [36] in different ecotopes such as under bush and bromeliads, rocks, burrows, hollow trees, and stone walls. The bugs were transported alive to the laboratory for species confirmation using morphological taxonomic keys [37]. Table 1 summarizes the geographical and ecotope origin of the collected *T. infestans* according to the ecoregions defined by Ibisch and Mérida [38]. Before dissection, feces from each bug were examined for the presence of trypanosomatides by direct microscopic observation at 400 × magnification. Bugs were then dissected under a safety hood, and the digestive tracts stored at -20°C.

Mini-exon multiplex PCR (MMPCR)

DNA was extracted from triatomine digestive tracts of with the QIAamp DNA mini kit (Quiagen, Courtaboeuf, France), according to the proposed blood sample protocol. The multiplex primers set was as previously described: 3 oligonucleotides derived from the hypervariable region of *T. cruzi* mini-exon repeats, and a common downstream oligonucleotide, corresponding to sequences present in the most conserved region of mini-exon gene used as opposing primer in the multiplex reaction. PCR conditions were according to [32], with slight modifications. DNA was amplified in a 25 µl reaction volume containing 1X reaction buffer, 1.5 mM $MgCl^2$, 50 µM of each nucleotide, 0.2 µM of each primer, 0.5 UI of Taq polymerase (Roche Applied Science, Penzberg, Germany). The amplifications were performed in a thermocycler (Eppendorf, Hamburg, Germany), with conditions already described [32]. PCR products were separated on 3% agarose gel using the molecular weight marker Smart Ladder (Eurogentec, Angers, France) and visualized under UV with Ez-vision (Amresco, OH, USA).

PCR of the *T. cruzi* glucose-6-phosphate isomerase fragment

A fragment of 652 bp was amplified with a set of primers, forward (*Gpi*-L) starting at the position 591 of the gene (5'-CGCCATGTTGTGAATATTGG-3') and reverse (*Gpi*-R) 5'–TTCCATTGCTTTCCATGTCA-3' starting at the position 1246, from each DNA sample. DNA was amplified in a 25 µl reaction volume containing 0.**75** mM $MgCl^2$, 0.2 mM of each nucleotide, 0.4 µM of each primer, 2.5 UI of Taq DNA polymerase (Roche Applied Science, Penzberg, Germany), and 20 ng of DNA template. The amplification was performed in a thermocycler (Eppendorf, Hamburg, Germany), with the following cycle conditions: 94°C for 3 min; 94°C for 1 min, 58°C for 1 min, 72°C for 1 min (35 cycles); 72°C for 5 min.

Purification and direct sequencing of both strands of DNA amplicons were performed by the company MACROGEN (Seoul, South Korea). Sequences were aligned and corrected by using the BioEdit software v. 7.0.9 [39], and a 458 bp partial sequence was resolved for each sequence (from nucleotide site 691 to 1148).

Results

Mini-exon multiplex PCR (MMPCR) analysis

A total of 333 DNA samples from digestive tracts of wild *T. infestans* were processed in MMPCR for DTU identification. Before dissection, we examined the bug feces (85.0% of the total sample) by microscopy. The parasite infection rate was 71.7% in Andean specimens while no positive insect was found among the 17 specimens from the Chaco (GC ecoregion). The identification of the three DTUs was assessed by determining the molecular weight of the MMPCR products for each sample (Table 1). The results showed that the large majority (98.3%) of the 234 wild *T. infestans* was infected by TcI (PCR products of 200 bp). Only one sample from an adult *T. infestans* ("dark morph" type) collected in the site 32 (GC ecoregion) gave a MMPCR product of 250 bp corresponding to either TcII or TcV or TcVI). Three other samples (sites 23, 25, and 29) gave a MMPCR products of 150 bp corresponding to either TcIII or TcIV. The MMPCR product of the specimen of this last group captured in site 29 was sequenced and the DNA fragment (64 bp) matched the TcIII reference stock named M5631 (accession No AF050521.1 and AY367126.1, 98% identity).

Sequence variability of the *Gpi* gene from *T. cruzi* infecting wild *T. infestans*

Partial sequence of the *Gpi* obtained from 15 samples (13 of the set corresponding to TcI, one of the set corresponding to either TcII or TcV or TcVI , and one of the set corresponding to either TcIII or TcIV) were sequenced in order to explore the variability within TcI and to discriminate the DTUs within the other sets. The 458 bp partial sequences (starting at site 691 and ending at 1148 of the entire gene of stock CL Brener, accession no. XM815802.1) were aligned with the sequences corresponding to *T. cruzi* reference stocks belonging to the 6 DTUs previously deposited in GenBank (Table 2). Without ambiguities, each sequence under study had been attributed to a DTU. Within TcI, 3 haplotypes were observed: the most frequent (11 stocks) presented an identity of 100% with the two identical sequences from TcI reference stocks (OPS21 and P/209) deposited in GenBank, the two other sequence exhibited

one single mutation. Remarkably, the Vis01 stock identified in a triatomine bug captured at site 27, presented a heterozygous pattern at nucleotide position 940. The sequence of the sample Char09 of the second set (corresponding to either TcII or TcV or TcVI), detected in a "dark morph" (site 32), presented 100% of identity with two identical TcII reference stocks (Tu18cl2 and CBBcl3). For the sample of the last set corresponding to either TcIII or TcIV (Tor05 from site 25), the sequence presented 100% of identity with two identical TcIII reference stocks (M6241cl6 and X110/8).

Discussion

Recently, active search for new foci of wild *T. infestans* in Bolivia enabled us to show that their distribution was broader than initially described [16, 40]. Also, few data on genetic characterization of *T. cruzi* stocks infecting these vector populations were available apart from the work by Dujardin et al. [31], performed by Multilocus Enzyme Electrophoresis, and the detection of the only TcI at Cotapachi at 15 km west of Cochabamba city (Andean area) [20]. In the present context, where wild *T. infestans* might enter houses and recolonize them after eliminating domestic populations by insecticide spraying, it is important to know what *T. cruzi* DTUs are carried by the vectors. In this work, 234 *T. cruzi* stocks isolated from wild *T. infestans* were characterized. The vectors came from several areas mainly situated in two ecoregions of Bolivia, the "Inter-Andean Dry Forest" and the Gran Chaco where the "dark morph" was found. In the overall sample, the DTU TcI is widely dominant, but in the Andean and intermediate areas TcIII (group III) stocks were detected. In the lowlands, only TcI and TcII have been characterized in "dark morph" specimen.

Interestingly, the DTU distribution in wild *T. infestans* is very different from that reported in domestic *T. infestans* before the campaigns of vector control undertaken at large-scale in Bolivia from 2003 [27]. At the same time, TcV was mostly detected in patients during the chronic phase of the infection while both TcI and TcV were detected in younger patients with early infection [24, 41]. Concerning the vectors, it was suggested that the domestication of *T. infestans* has taken place in high Andean valleys, and that the dispersal of domestic *T. infestans* to other areas had occurred by human transport [42, 43]. The current observations do not fit these hypotheses, since the only TcI (and to a lesser extent TcIII) would then have been introduced into domestic cycles but not TcV, unless it is assumed that TcV has disappeared from the wild *T. infestans* cycle in the Andes valleys after its domestication.

Among the 6 *T. cruzi* DTUs , TcV and TcVI are composed of stocks that appear to be recent hybrids between TcII and TcIII [14]. So, they probably originate from an area where the putative parents coexist. Moreover, this hybridization event is still considered to have occurred much earlier than human colonization in South America [44]. Consequently, parental and hybrid DTUs are likely to coexist in sylvatic cycle in a putative geographical area in South America. Lately, the Andean origin of *T. infestans* was challenged by the hypothesis of a Chaquean origin [21, 40, 45, 46]. If parental and hybrid DTUs are not found in the sylvatic cycle in the Andes, an alternative might be the Gran Chaco region. Available data of *T. cruzi* genetic characterization of wild cycle in the Bolivian lowlands are not available except a report of a TcVI stock from a *Didelphis marsupialis* captured on the Amazon slope [47]. In the Paraguayan Chaco, TcII, TcIII and TcV have been identified in different wild mammal species [48]. In spite of fairly scarce data, the hypothesis of a Chaco origin of hybrid DTUs should be considered, especially considering the detection of all DTUs except for TcIV in the domestic cycle in the Bolivian Gran Chaco (unpublished data). The search of DTUs circulating in wild cycles will give more valid information about the evolution of *T. cruzi* than studies carried out in domestic cycles where the geographical distribution of the DTUs is skewed by passive transport of parasites (human migration, transport of triatomines) and by the selection of specific DTUs by hosts considering that host diversity is more reduced in domestic cycle than in wild ones.

Acknowledgments

We are particularly grateful to the direction of the Inlasa (Instituto de Laboratorios de Salud, La Paz Bolivia), Dr. Jose´ Raphael Gutiérrez and Dr. Walter Agreda to have hosted this work in the Department of Entomology directed by Dr. Tamara Chavez. This work was funded by the National Agency for Research (ANR, France), the UNICEF/UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases (TDR, No. A70596), the IDRC Canada Grant No. 103696-012, and the IRD France.

Authors Contributions

Conceived and designed the experiments: SFB CB FN. Performed the field research: SFB, CA, EW, RB, RS, FN. Performed the experiments: CA. Analyzed the data: SFB, AC, CB. Wrote the paper: SFB, CA, CB, MT, FN.

References

1. Schmunis GA, and Yadon ZE (2010) Chagas disease: a Latin American health problem becoming a world health problem. Acta Trop 115: 14-21.
2. Hotez PJ, Bottazzi ME, Franco-Paredes C, Ault SK, Periago MR (2008) The neglected tropical diseases of latin america and the Caribbean: a review of disease burden and distribution and a roadmap for control and elimination. PLoS Negl Trop Dis 2: e300.
3. WHO 2007. American trypanosomiasis and neglected diseases. http://www.who.int/neglected_diseases/en/ (accessed 27.7.2009).
4. Bosseno MF, Barnabé C, Sierra MJ, Kengne P, Guerrero S, Lozano F, Magallon-Gastelum E, Brenière, SF (2009) Wild ecotopes and food habits of *Triatoma longipennis* infected by *Trypanosoma cruzi* lineages I and II in Mexico. Am J Trop Med Hyg 80: 988-91.
5. Salvatella R, Calegari L, Puime A, Basmadjian Y, Rosa R, Guerrero J, Martinez M, Mendaro G, Briano D, Montero C et al. (1994) Feeding pattern of *Triatoma rubrovaria* (Blanchard, 1843) (Hemiptera, Triatominae) in peridomiciliary habitats, of a rural area of Uruguay. Rev Inst Med Trop Sao Paulo 36: 311-20.
6. Salvatella R, Rosa R, Basmadjian Y, Puime A, Calegari L, Guerrero J, Martinez M, Mendaro G, Briano D, Montero C et al. (1995) Ecology of *Triatoma rubrovaria* (Hemiptera, Triatominae) in wild and peridomestic environments of Uruguay. Mem Inst Oswaldo Cruz 90: 325-328.
7. Freitas SP, Lorosa ES, Rodrigues DC, Freitas AL, Goncalves TC (2005) Feeding patterns of *Triatoma pseudomaculata* in the state of Ceara, Brazil. Rev Saude Publica 39: 27-32.
8. Tibayrenc M, Ward P, Moya A, Ayala FJ (1986) Natural populations of *Trypanosoma cruzi*, the agent of Chagas disease, have a complex multiclonal structure. Proc Natl Acad Sci USA 83: 115-119.
9. Tibayrenc M, Barnabé C, Telleria J. (2010) in "American trypanosomiasis: Chagas disease. One hundred years of research" (Telleria J and Tibayrenc M, Eds.), Elsevier, Burlington.
10. Tibayrenc M (1998) Integrated genetic epidemiology of infectious diseases: the Chagas model. Mem Inst Oswaldo Cruz 93: 577-580.
11. Barnabé C, Brisse S, Tibayrenc M. (2000) Population structure and genetic typing of *Trypanosoma cruzi*, the agent of Chagas disease: a multilocus enzyme electrophoresis approach. Parasitology 120: 513-526.
12. Brisse S, Barnabé C, Tibayrenc M (2000) Identification of six *Trypanosoma cruzi* phylogenetic lineages by random amplified polymorphic DNA and multilocus enzyme electrophoresis. Int J Parasitol 30: 35-44.
13. Zingales B, Andrade SG, Briones MR, Campbell DA, Chiari E, Fernandes O, Guhl F, Lages-Silva E, Macedo AM, Machado CR, Miles MA, Romanha AJ, Sturm NR, Tibayrenc M, Schijman AG (2009) A new consensus for *Trypanosoma cruzi* intraspecific nomenclature: second revision meeting recommends TcI to TcVI. Mem Inst Oswaldo Cruz 104: 1051-1054.
14. Broutin H, Tarrieu F, Tibayrenc M, Oury B, Barnabé C. (2006) Phylogenetic analysis of the glucose-6-phosphate isomerase gene in *Trypanosoma cruzi*. Exp Parasitol Exp Parasitol 113: 1-7.
15. Westenberger SJ, Barnabé C, Campbell DA, Sturm NR (2005) Two hybridization events define the population structure of *Trypanosoma cruzi*. Genetics 171: 527-543.
16. Buitrago R, Waleckx E, Bosseno MF, Zoveda F, Vidaurre P, Salas R, Mamani E, Noireau F, Brenière SF (2010) First report of widespread wild populations of *Triatoma infestans* (Reduviidae, Triatominae) in the valleys of La Paz, Bolivia. Am J Trop Med Hyg 82: 574-579.
17. Noireau F, Cortez MG, Monteiro FA, Jansen AM, Torrico F (2005) Can wild *Triatoma infestans* foci in Bolivia jeopardize Chagas disease control efforts? Trends Parasitol 21: 7-10.
18. Noireau F (2009) Wild *Triatoma infestans*, a potential threat that needs to be monitored. Mem Inst Oswaldo Cruz 104 Suppl 1 : 60-64.
19. Cortez MR, Emperaire L, Piccinali RV, Gurtler RE, Torrico F, Jansen AM, Noireau F (2007) Sylvatic *Triatoma infestans* (Reduviidae, Triatominae) in the Andean valleys of Bolivia. Acta Trop 102/ 47-54.

20. Cortez MR, Pinho AP, Cuervo P, Alfaro F, Solano M, Xavier SC, D'Andrea PS, Fernandes O, Torrico F, Noireau F, Jansen AM (2006) *Trypanosoma cruzi* (Kinetoplastida Trypanosomatidae): ecology of the transmission cycle in the wild environment of the Andean valley of Cochabamba, Bolivia. Exp Parasitol 114/ 305-313.
21. Noireau F, Flores R, Gutierrez T, Dujardin JP (1997) Detection of sylvatic dark morphs of *Triatoma infestans* in the Bolivian Chaco. Mem Inst Oswaldo Cruz 92: 583-584.
22. Waleckx E, Depickère S, Salas R, Aliaga C, Monje M, Calle H, Buitrago R, Noireau F, Brenière SF (2011) New Discoveries of Sylvatic *Triatoma infestans* Throughout the Bolivian Chaco. Am J Trop Med Hyg. In press.
23. Flores-Chavez M, Bosseno MF, Bastrenta B, Dalenz JL, Hontebeyrie M, Revollo S, Brenière SF (2006) Polymerase chain reaction detection and serologic follow-up after treatment with benznidazole in Bolivian children infected with a natural mixture of *Trypanosoma cruzi* I and II. Am J Trop Med Hyg 75: 497-501.
24. Bosseno MF, Torrico F, Telleria J, Noireau F, Brenière SF (1995) Polymerase chain reaction: detection and characterization of *Trypanosoma cruzi* strains in chagasic children. Medicina (B Aires) 55: 277-279.
25. Brenière SF, Bosseno MF, Noireau F, Yacsik N, Liegeard P, Aznar C, Hontebeyrie M (2002) Integrate study of a Bolivian population infected by *Trypanosoma cruzi*, the agent of Chagas disease. Mem Inst Oswaldo Cruz 97: 289-295.
26. Brenière SF, Lopez J, Vargas F, Barnabé C (1997) Genetic variability and microdistribution of *Triatoma infestans* genotypes and *Trypanosoma cruzi* clones in Arequipa region (Peru). Mem Inst Oswaldo Cruz 92: 401-408.
27. Brenière SF, Bosseno MF, Telleria J, Carrasco R, Vargas F, Yaksic N, Noireau F (1995) Field application of polymerase chain reaction diagnosis and strain typing of *Trypanosoma cruzi* in Bolivian triatomines. Am J Trop Med Hyg 53: 179-184.
28. Brenière SF, Carrasco R, Revollo S, Aparicio G, Desjeux P, Tibayrenc M (1989) Chagas' disease in Bolivia: clinical and epidemiological features and zymodeme variability of *Trypanosoma cruzi* strains isolated from patients. Am J Trop Med Hyg 41: 521-529.
29. Brenière SF, Llanos B, Tibayrenc M, Desjeux P (1985) Isoenzymic studies and epidemiological data of *Trypanosoma cruzi* from Arequipa (Peru), Pacific side. Ann Soc Belg Med Trop 65 Suppl 1 : 63-66.
30. Bosseno MF, Telleria J, Vargas F, Yaksic N, Noireau F, Morin A, Brenière SF (1996) *Trypanosoma cruzi*: study of the distribution of two widespread clonal genotypes in Bolivian *Triatoma infestans* vectors shows a high frequency of mixed infections. Exp Parasitol 83: 275-282.
31. Dujardin JP, Tibayrenc M, Venegas E, Maldonado L, Desjeux P, Ayala FJ (1987) Isozyme evidence of lack of speciation between wild and domestic *Triatoma infestans* (Heteroptera: Reduviidae) in Bolivia. J Med Entomol 24: 40-45.
32. Fernandes O, Santos SS, Cupolillo E, Mendonca B, Derre R, Junqueira AC, Santos LC, Sturm NR, Naiff RD, Barret TV, Campbell DA, Coura JR (2001) A mini-exon multiplex polymerase chain reaction to distinguish the major groups of *Trypanosoma cruzi* and *T. rangeli* in the Brazilian Amazon.Trans R Soc Trop Med Hyg 95: 97-99.
33. Aliaga C, Brenière SF, Barnabé C (2011) Further interest of miniexon multiplex PCR for a rapid typing of *Trypanosoma cruzi* DTU groups. Infect Genet Evol 11: 1155-1158.
34. Bosseno MF, Garcia LS, Baunaure F, Magallon-Gastelum E, Gutierrez MS, Lozano-Kasten F, Dumonteil E, Brenière SF (2006) Identification in triatomine vectors of feeding sources and *Trypanosoma cruzi* variants by heteroduplex assay and a multiplex miniexon polymerase chain reaction. Am J Trop Med Hyg 74: 303-305.
35. Llewellyn MS, Lewis MD, Acosta N, Yeo M, Carrasco HJ, Segovia M, Vargas J, Torrico F, Miles MA, Gaunt MW (2009) *Trypanosoma cruzi* IIc: Phylogenetic and Phylogeographic Insights from Sequence and Microsatellite Analysis and Potential Impact on Emergent Chagas Disease. PLoS Negl Trop Dis 3: e510.

36. Noireau F, Flores R, Vargas F (1999) Trapping sylvatic Triatominae (Reduviidae) in hollow trees. Trans R Soc Trop Med Hyg 93: 13-14.
37. Lent H, Wygodzinsky P (1979) Revision of the Triatominae (Hemiptera, Reduviidae), and significance as vectores of Chagas' disease. Bull Am Museum Nat Hist 163: 125-520.
38. Ibisch PL, Beck SG, Gerckmann B, Carretero A (2008) in "Biodiversidad: la riqueza de Bolivia. Estado de conocimiento y conservacion" (Ibisch PL and Mérida G, Eds.), FAN Bolivia, Santa Cruz de la Sierra.
39. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic acids symposium series 41: 95-98.
40. Waleckx E, Salas R, Huaman N, Buitrago R, Bosseno MF, Aliaga C, Barnabé C, Rodriguez R, Zoveda F, Monje M, Baune M, Quisberth S, Villena E, Kengne P, Noireau F, Brenière SF (2011) New insights on the Chagas disease main vector *Triatoma infestans* (Reduviidae, Triatominae) brought by the genetic analysis of Bolivian sylvatic populations. Infect Genet Evol 11:1045-1057.
41. Brenière SF, Bosseno MF, Telleria J, Bastrenta B, Yacsik N, Noireau F, Alcazar JL, Barnabé C, Wincker P, Tibayrenc M (1998) Different behavior of two *Trypanosoma cruzi* major clones: transmission and circulation in young Bolivian patients. Exp. Parasitol 89: 285-295.
42. Schofield CJ (1988) Biosystematics of the Triatominae, In: Service, M.W. (Ed.), Biosystematics of Haematophagous Insects, Clarendon Press, Oxford.
43. Cortez MR, Monteiro FA, Noireau F (2010) New insights on the spread of *Triatoma infestans* from Bolivia, implications for Chagas disease emergence in the southern cone. Infect Genet Evol 10: 350-353.
44. Machado CA, Ayala FJ (2001) Nucleotide sequences provide evidence of genetic exchange among distantly related lineages of *Trypanosoma cruzi*. Proc Natl Acad Sci USA 98: 7396-7401.
45. Ceballos LA, Piccinali RV, Berkunsky I, Kitron U, Gurtler RE (2009) First finding of melanic sylvatic *Triatoma infestans* (Hemiptera: Reduviidae) colonies in the Argentine Chaco. J Med Entomol 46: 1195-1202.
46. Quisberth S, Waleckx E, Monje M, Chang B, Noireau F, Brenière SF (2011) "Andean" and "non-Andean" ITS-2 and mtCytB haplotypes of *Triatoma infestans* are observed in the Gran Chaco (Bolivia): population genetics and the origin of reinfestation. Infect Genet Evol 11:1006-1014.
47. Valette E, Brenière SF, Le Pont F, Desjeux P (1988) Zymodemes of *Trypanosoma cruz*i isolated from wild mammals in Bolivia. Mem Inst Oswaldo Cruz 83: 139-140.
48. Yeo M, Acosta N, Llewellyn M, Sanchez H, Adamson S, Miles GA, Lopez E, Gonzalez N, Patterson JS, Gaunt MW, de Arias AR, Miles MA (2005) Origins of Chagas disease: Didelphis species are natural hosts of *Trypanosoma cruzi* I and armadillos hosts of *Trypanosoma cruzi* II, including hybrids. Int J Parasitol 35: 225-233.

Table 1: Geographical origin, biotopes of wild triatomines and *T. cruzi* identification

Geographical origin						Ecotopes[b]	No. of specimens[c]	DTU groups of *T cruzi*[d]		
N° of site	Ecoregion[a]	Latitude (S)	Longitude (W)	Altitude (m)	Bolivian area			Tc I	Tc II, TcV, TcVI	Tc III, Tc IV
1	BSIA	16°41'21.0"	68°00'40.9"	2679	Andean	1	5	5		
2	BSIA	16°42'08.0"	68°00'16.3"	2821	Andean	2	2	1		
3	BSIA	16°42'42.9"	67°59'25.3"	2732	Andean	3, 4	23	21		
4	BSIA	16°42'56.3"	67°52'13.6"	2459	Andean	3, 5	6	5		
5	BSIA	16°43'11.1"	67°52'26.5"	2380	Andean	3, 6, 7	11	9		
6	BSIA	16°48'51.5"	67°42'18.3"	1873	Andean	1, 6, 7, 8	19	19		
7	BSIA	16°49'43.3"	67°42'17.0"	1957	Andean	1, 7, 9	19	19		
8	BSIA	16°53'12.2"	67°42'43.1"	2095	Andean	7	13	13		
9	BSIA	16°55'48.8"	67°41'32.9"	2182	Andean	4, 8	18	16		
10	BSIA	17°00'30.1"	67°39'25.2"	2356	Andean	4, 6, 7	47	43		
11	BSIA	17°01'54.8"	67°40'38.6"	2159	Andean	2	2	1		
12	BSIA	17°03'55.6"	67°39'51.4"	2619	Andean	1, 3, 5, 7	5	5		
13	BSIA	17°04'07.7"	67°39'25.5"	2645	Andean	4, 7	5	5		
14	BSIA	17°04'24.2"	67°38'42.7"	2543	Andean	7	1	1		
15	BSIA	17°04'25.2"	67°37'59.7"	2493	Andean	6, 7, 10	5	5		
16	BSIA	17°04'44.6"	67°37'57.0"	2602	Andean	6, 7, 9	17	16		
17	BSIA	17°07'32"	67°35'59.5"	2864	Andean	7, 11	6	3		
18	BSIA	17°08'10.8"	67°35'17.9"	2767	Andean	3, 4, 6, 9	6	4		
19	BSIA	17°42'45.2"	66°29'38.8"	2583	Andean	7	9	9		
20	BSIA	17°25'39.2"	66°15'32.1"	2576	Andean	4, 7	11	11		
21	BSIA	17°27'45.5"	66°18'51.0"	2543	Andean	7	3	3		
22	BSIA	17°28'37.5"	66°08'16.14'	2710	Andean	7	14	4		
23	BSIA	17°59'16.4"	65°50'11.1"	2059	Andean	7, 8	10			1
24	BSIA	18°00'44.5"	65°48'32.6"	2025	Andean	7	4	3		
25	BSIA	18°01'50.9"	65°47'18.7"	1968	Andean	8	11			1
26	BSIA	65°47'18.7"	65°45'31.3"	2571	Andean	8	5			
27	PP	21°37'16.8"	65°48'46.0"	2963	Andean	6	7	7		
28	PP	21°44`51``	65°49`26``	3080	Andean	4	30			
29	CS	18°35'52.6"	65°07'33.4"	1754	Intermediate	3, 7	1			1
30	BTB	19°55'39.2"	63°54'08.8"	1039	Intermediate	uk[e]	1	1		
31	GC	19°21`20.86``	62°34`10.19``	398	Non-Andean (lowlands)[g]	12, uk[f]	3			
32	GC	20°11'10.1"	63°61'21.7"	614	Non-Andean (lowlands)[g]	12	2		1	
33	GC	20°15'09.1"	62°58'26.9"	573	Non-Andean (lowlands)[g]	12	6			
32	GC	21°9'25.30"	63°22'30.70	488	Non-Andean	12	1			
35	GC	21°22'37.00"	63°21'34.80	351	Non-Andean (lowlands)[g]	12	2	1		
36	GC	21°50'50.60"	63°14'51.60"	443	Non-Andean (lowlands)[g]	12	3			
						Total	333	230	1	3

[a] The ecoregions are according to Ibisch et al. (2008), BSIA Bosque Seco Inter Andino, PP Prepuna, CS Chaco Serrano, BTB Bosque Tucumano Boliviano, GC Gran Chaco; [b] 1 crack, 2 cliff, 3 under vegetation, 4 burrow, 5 adobe wall, 6 stone wall, 7 under stones, 8 cave, 9 hollow ground, 10 bird nest, 11 graves, 12 hollow tree, uk unknown; [c] Total number of specimens tested by MMPCR; [d]DTU Discrete Typing Unit were identified by MMPCR, the undetermided samples were MMPCR negative; [e]specimen captured on the exterior wall of a house; [f]captured by light traps. [g] Between Andean and non-Andean areas.

Table 2. Variable sites of glucose phosphate isomerase gene of *T. cruzi* indentified in wild *T. infestans* compared with reference stocks

Name of reference and current stocks	Acession no	DTU[a]	No of current stock	Country	Bolivian area	Nucleotide position																		
						715	739	784	805	811	828	829	830	831	832	856	859	863	898	913	940	945	1030	1051	1108
OPS21	AY484472	TcI		Venezuela		A	A	A	T	T	G	T	G	A	G	G	C	C	A	G	T	C	G	C	T
P/209	AY484473	TcI		Bolivia				.				.					.								
Arq02[b]		TcI	1	Bolivia	Andean																C				
Lur 112[b c]		TcI	11	Bolivia	Andean																		.		
Vis01[b]		TcI	1	Bolivia	Andean												.		.		Y		.	.	
Tu18cl2	AY484477	TcII		Brazil			T		C	C	A						T	T	G	.		A		G	
CBBcl3	AY484476	TcII		Chile			T		C	C	A	.				.	T	T	G			A		G	
Char09[b]		TcII	1	Bolivia	Non-Andean (lowlands)		T		C	C	A	.		.			T	T	G			A		G	
M6241cl6	AY484478	TcIII		Brazil			.									A						.			
X110/8	AY484479	TcIII		Paraguay												A	.		.						
Tor05[b]		TcIII	1	Bolivia	Andean				.		.		.			A						.			.
CanIIIcl11	AY484474	TcIV		Brazil		.		C									.		G	A	.		T	T	C
EP272	AY484475	TcIV		Colombia				C				.				.			G	A		.	T	T	
MNcl2	AY484480	TcV		Chile		.	W		Y	Y	R	K	R	R	K	.	Y		G			M		S	
Bug2148cl11	AY484481	TcV		Brazil		.	W	.	Y	Y	R		R	R			Y		G			M		S	
ClBrener	AY484482	TcVI		Brazil		R	W		Y	Y		K	R	R	K	.		.	R	.				.	
TulaCl2	AY484483	TcVI		Chile		R	W		Y	Y	R	K	R	R	K			.	G			M		S	.

[a]DTU, Discrete Typing Unit, [b] Samples under study, [c] 10 other samples had identical sequence, they were from Northern Andean area

Table
Click here to download Table Tables_Breniere_PNTD xlsx

Table 1 Geographical origin, ecotopes of wild triatomines and *T. cruzi* identification

Geographical origin								DTU groups of *T cruzi*[d]		
N° of site	Ecoregion[a]	Latitude (S)	Longitude (W)	Altitude (m)	Bolivian area	Ecotopes[b]	No of specimens[c]	Tc I	Tc II, TcV, TcVI	Tc III, Tc IV
1	BSIA	16°41'21 0"	68°00'40 9"	2679	Andean	1	5	5		
2	BSIA	16°42'08 0"	68°00'16 3"	2821	Andean	2	2	1		
3	BSIA	16°42'42 9"	67°59'25 3"	2732	Andean	3, 4	23	21		
4	BSIA	16°42'56 3"	67°52'13 6"	2459	Andean	3, 5	6	5		
5	BSIA	16°43'11 1"	67°52'26 5"	2380	Andean	3, 6, 7	11	9		
6	BSIA	16°48'51 5"	67°42'18 3"	1873	Andean	1, 6, 7, 8	19	19		
7	BSIA	16°49'43 3"	67°42'17 0"	1957	Andean	1, 7, 9	19	19		
8	BSIA	16°53'12 2"	67°42'43 1"	2095	Andean	7	13	13		
9	BSIA	16°55'48 8"	67°41'32 9"	2182	Andean	4, 8	18	16		
10	BSIA	17°00'30 1"	67°39'25 2"	2356	Andean	4, 6, 7	47	43		
11	BSIA	17°01'54 8"	67°40'38 6"	2159	Andean	2	2	1		
12	BSIA	17°03'55 6"	67°39'51 4"	2619	Andean	1, 3, 5, 7	5	5		
13	BSIA	17°04'07 7"	67°39'25 5"	2645	Andean	4, 7	5	5		
14	BSIA	17°04'24 2"	67°38'42 7"	2543	Andean	7	1	1		
15	BSIA	17°04'25 2"	67°37'59 7"	2493	Andean	6, 7, 10	5	5		
16	BSIA	17°04'44 6"	67°37'57 0"	2602	Andean	6, 7, 9	17	16		
17	BSIA	17°07'32"	67°35'59 5"	2864	Andean	7, 11	6	3		
18	BSIA	17°08'10 8"	67°35'17 9"	2767	Andean	3, 4, 6, 9	6	4		
19	BSIA	17°42'45 2"	66°29'38 8"	2583	Andean	7	9	9		
20	BSIA	17°25'39 2"	66°15'32 1"	2576	Andean	4, 7	11	11		
21	BSIA	17°27'45 5"	66°18'51 0"	2543	Andean	7	3	3		
22	BSIA	17°28'37 5"	66°08'16 14"	2710	Andean	7	14	4		
23	BSIA	17°59'16 4"	65°50'11 1"	2059	Andean	7, 8	10			1
24	BSIA	18°00'44 5"	65°48'32 6"	2025	Andean	7	4	3		
25	BSIA	18°01'50 9"	65°47'18 7"	1968	Andean	8	11			1
26	BSIA	65°47'18 7"	65°45'31 3"	2571	Andean	8	5			
27	PP	21°37'16 8"	65°48'46 0"	2963	Andean	6	7	7		
28	PP	21°44`51``	65°49`26``	3080	Andean	4	30			
29	CS	18°35'52 6"	65°07'33 4"	1754	Intermediate	3, 7	1			1
30	BTB	19°55'39 2"	63°54'08 8"	1039	Intermediate	uk[e]	1	1		
31	GC	19°21`20 86 ``	62°34`10 19 ``	398	Non-Andean (lowlands)[g]	12, uk[f]	3			
32	GC	20°11'10 1"	63°61'21 7"	614	Non-Andean (lowlands)[g]	12	2		1	
33	GC	20°15'09 1"	62°58'26 9"	573	Non-Andean (lowlands)[g]	12	6			
32	GC	21°9'25 30"	63°22'30 70	488	Non-Andean	12	1			
35	GC	21°22'37 00 "	63°21'34 80	351	Non-Andean (lowlands)[g]	12	2	1		
36	GC	21°50'50 60 "	63°14'51 60 "	443	Non-Andean (lowlands)[g]	12	3			
						Total	333	230	1	3

[a] The ecoregions are according to Ihisch et al (2008), BSIA Bosque Seco Inter Andino, PP Prepuna, CS Chaco Serrano, BTB Bosque Tucumano Boliviano, GC Gran Chaco, [b] 1 crack, 2 cliff, 3 under vegetation, 4 burrow, 5 adobe wall, 6 stone wall, 7 under stones, 8 cave, 9 hollow ground, 10 bird nest, 11 graves, 12 hollow tree, uk unknown, [c] Total number of specimens tested by MMPCR, [d]DTU Discrete Typing Unit were identified by MMPCR, the undetermided samples were MMPCR negative, [e]specimen captured on the exterior wall of a house, [f]captured by light traps [g] Between Andean and non-Andean areas

Table 2. Variable sites of glucose phosphate isomerase gene of *T. cruzi* indentified in wild *T. infestans* compared with reference stocks

Name of reference and current stocks	Acession no.	DTU[a]	No. of current stock	Country	Bolivian area	Nucleotide position														
						715	739	784	805	811	828	829	830	831	832	856	859	863	898	913
OPS21	AY484472	TcI		Venezuela		A	A	A	T	T	G	T	G	A	G	G	C	C	A	G
P/209	AY484473	TcI		Bolivia		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Aiq02[b]		TcI	1	Bolivia	Andean	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Lur 112[b, c]		TcI	11	Bolivia	Andean	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Vis01[b]		TcI	1	Bolivia	Andean	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Tu18cl2	AY484477	TcII		Brazil		.	T	.	C	C	A	.	.	.	.	.	T	T	G	.
CBBcl3	AY484476	TcII		Chile		.	T	.	C	C	A	.	.	.	.	.	T	T	G	.
Char09[b]		TcII	1	Bolivia	Non-Andean (lowlands)	.	T	.	C	C	A	.	.	.	.	.	T	T	G	.
M6241cl6	AY484478	TcIII		Brazil		.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
X110/8	AY484479	TcIII		Paraguay		.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
Tor05[b]		TcIII	1	Bolivia	Andean	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
CanIIIcl11	AY484474	TcIV		Brazil		.	.	C	.	.	.	.	.	.	.	.	.	.	G	A
EP272	AY484475	TcIV		Colombia		.	.	C	.	.	.	.	.	.	.	.	.	.	G	A
MNcl2	AY484480	TcV		Chile		.	W	.	Y	Y	R	K	R	R	K	.	Y	.	G	.
Bug2148cl11	AY484481	TcV		Brazil		.	W	.	Y	Y	R		R	R		.	Y	.	G	.
ClBrener	AY484482	TcVI		Brazil		R	W	.	Y	Y		K	R	R	K	.	.	.	R	.
TulaCl2	AY484483	TcVI		Chile		R	W	.	Y	Y	R	K	R	R	K	.	.	.	G	.

[a]DTU, Discrete Typing Unit; [b] Samples under study; [c] 10 other samples had identical sequence, they were from Northern Andean area

940	945	1030	1051	1108
T	C	G	C	T
.	.	.	.	.
C	.	.	.	.
.	.	.	.	.
Y	.	.	.	.
.	A	.	G	.
.	A	.	G	.
.	A	.	G	.
.	.	.	.	.
.	.	.	.	.
.	.	.	.	.
.	.	T	T	C
.	.	T	T	.
.	M	.	S	.
.	M	.	S	.
.	.	.	.	.
.	M	.	S	.

Annexe 19

Typification et génétique des populations de *T. cruzi* chez *T. infestans*

1 – Typification rapide des souches par la méthode MMPP (Miniexon Multiplex PCR)

Cette méthode a permis de déterminer chez *T. infestans* les groupes de DTUs des parasites (*Trypanosoma cruzi*) infectant les insectes suite à la validation de l'outil moléculaire sur 70 souches de parasites appartenant aux différentes DTUs (TcI à TcVI, annexe 17 publication de Aliaga et al. 2011).

a) Résultats MMPP chez *T. infestans* sylvestre

Un total de 234 stocks de *T. cruzi* ont été caractérisés directement dans les tubes digestifs de *T. infestans* sylvestre par la méthode MMPP et pour certains d'entre eux par le séquençage direct (post-PCR) du gène *Gpi* (annexe 18 article proposée pour publication, Brenière et al.). La très grande majorité des stocks ont été identifiés TcI (produit PCR de 200 bp). Un seul stock isolé dans l'écorégion GC (Gran Chaco) a montré un produit MMPP de 250 bp et a été identifiés par le séquençage de la *Gpi* TcII. Trois autres stocks de *T. cruzi* ont montré une bande MMPP de 150 bp qui correspond à TcIII ou TcIV ; un stock a été identifié TcIII par le séquençage de la *Gpi*.

b) Résultats MMPP chez *T. infestans* domestique et péri domestique

La méthode MMPP a également été appliquée à la détermination des groupes de DTUs de *T. cruzi* en cycle domestique dans les villages de Rancho Nuevo et San Silvestre (GC), avant et après le traitement insecticide des maisons (Tableau 1). Il est intéressant de noter que (i) les taux moyens d'infections ne baissent pas après traitement insecticide, probablement parce que la ré infestation est très rapide et donc les hôtes réservoirs infectés demeurent présents, (ii) contrairement au milieu sylvestre les trois groupes de DTUs sont présents en milieu domestique avec une prédominance de *Tc*II, *Tc*V ou *Tc*VI et (iii) la présence non négligeable de mélanges de DTUs. De manière générale, la diversité génétique de *T. cruzi* est remarquable dans le GC quand on la compare à d'autres, avec la présence importante des DTUs hybrides TcV et TcVI.

Table 1: Taux d'infection de *T. infestans* et détermination des groupes de DTUs de *T. cruzi* dans les villages de Rancho Nuevo et San Silvestre (GC) par la méthode Miniexon Multiplexe PCR (MMPP)

	T. infestans	*T. cruzi* MMPP						
			Groupes de DTUs (DTUs)					
Villages	Nb.	Positifs (%)	Group 1 (*Tc*I)	Group 2 (*Tc*II, *Tc*V, *Tc*VI)	Group 3 (*Tc*III, *Tc*IV)	Groupes 1 et 2	Groupes 1 et 3	Groupes 2 et 3
Avant traitement insecticide								
San Silvestre	38	2 (5,3%)	0	1	1	0	0	0
Rancho Nuevo	84	24 (28,6%)	3	16	5	0	0	0
Total	122	26 (21,3%)	3	17	6	0	0	0
Après traitement insecticide								
San Silvestre	16	3 (18,7%)	0	1	1	0	1	0
Rancho Nuevo	46	30 (65,2)	5	25	0	0	0	0
Total	62	33 (53,2%)	10	53	6	1	2	3

La distribution des DTUs par maison est présentée dans la figure 1, et dans une des maisons de Rancho Nuevo on observe la présence de plusieurs DTUs.

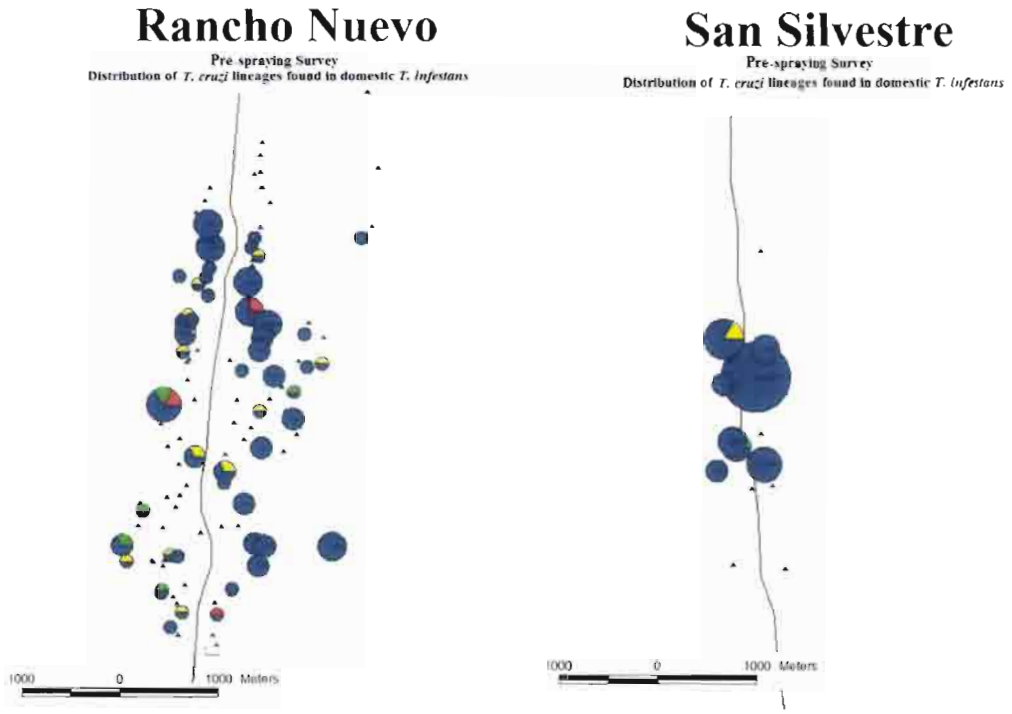


Figure 1: Distribution des groupes de DTUs de T. cruzi chez T. infestans *à Rancho Nuevo et San Silvestre. Chaque cercle est proportionnel au nombre de triatomes analysés : en bleu, PCR négative, en rouge TcI, en jaune TcII-V-VI, en vert TcIII-IV.*

2 – Génétique des populations de *T. cruzi* isolées de *T. infestans* sylvestres

La méthode MLMT (Multilocus Microsatellite Typing) sur 8 loci a été utilisée pour étudier le polymorphisme fin de 79 souches sylvestres de *T. cruzi* après isolement et leur identification *TcI* par la méthode MMPP. Ces souches ont été choisies selon des critères géographiques et distribuées en 6 populations : 2 populations proches de la zone de Quillacollo (département de Cochabamba), 4 populations dans les vallées andines du département de La Paz dont deux très proches (Figure 2).

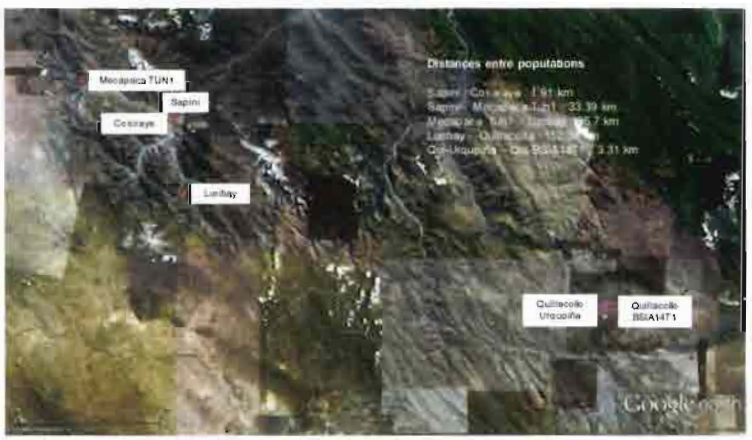


Figure 2. Localisation géographique et distances entre les 6 populations de T. cruzi

Les génotypes de ces souches ont été comparés à 21 souches de référence TcI issues de zones géographiques diversifiées. Certaines souches de référence ont montré des génotypes génétiquement très éloignés des génotypes des souches sylvestres boliviennes étudiées. L'analyse de génétique des populations a portée sur les souches sylvestres de notre étude afin de composer de réelles populations naturelles (potentialité de panmixie). L'objectif des analyses étaient d'apprécier le niveau de panmixie et l'échelle d'isolement géographique des populations.

Le tableau 2 présente pour chaque population les indices de variabilité génétique. La diversité génétique et la richesse allélique ne sont pas significativement différentes entre les populations. Le résultat le plus surprenant concerne l'analyse des F*is* et de l'équilibre de Hardy-Weinberg. En effet, tous les F*is* des six populations ne diffèrent pas significativement de 0, ce qui suggère un équilibre de Hardy-Weinberg dans toutes les populations sylvestres étudiées (Figure 3). L'intervalle de confiance 95% du F*is* général (tous locus et pops confondus) est égal à -0.28 <-> +0.39. De plus il n'y pas de déséquilibre de liaison entre paires de locus. Tous ces éléments ne nous permettent pas de rejeter l'hypothèse de panmixie de *T. cruzi* chez ces populations sylvestres.

Tableau 2: Indices de variabilité des 6 populations de *T. cruzi* isolées de *T. infestans* sylvestre et étudiées au moyen de huit loci microstellites.

Localité	Luribay	Mecapaca	Quillacollo		Sapini	
Nom des populations	Luribay	Tun1	Urqupiña	BSIA 14T1	Sapini	Cosiraya
N° des populations	pop1	pop2	pop3	pop4	pop5	pop6
Effectif	6	11	12	4	27	19
Diversité génétique	0,30	0,41	0,20	0,33	0,52	0,40
Richesse allélique	1,91	1,97	1,45	2,04	2,27	1,95
F*is* moyens	0,24	0,20	-0,24	0,25	-0,04	0,15
Intervalles de confiance à 95% des Fis						
valeur mini	-0,17	-0,11	-0,69	-0,29	-0,18	-0,08
valeur maxi	0,41	0,30	-0,07	0,33	0,06	0,31
Significativité des F*is (NS = non significatif)*	NS	NS	NS	NS	NS	NS

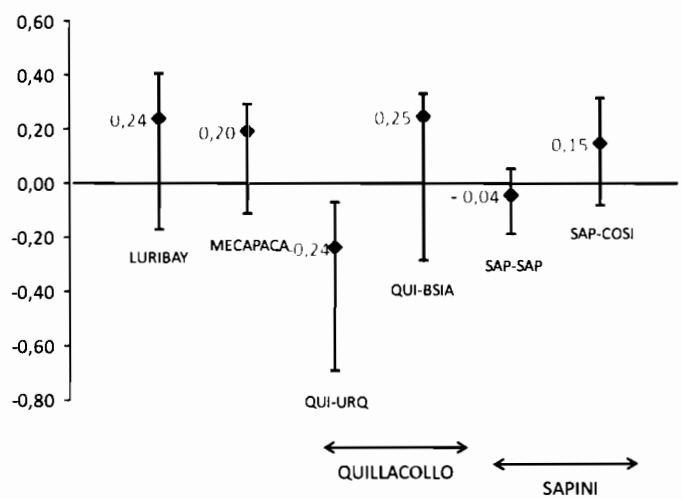


Figure 3. Intervalles de confiance à 95% des valeurs de Fis des 6 populations de T. cruzi *étudiées. Les valeurs de* Fis *en rouge ne sont pas significativement différentes de 0.*

Les valeurs de Fst entre couples de populations sont la plupart du temps significatives, cependant les arbres construits à partir des Fst ou des distances génétiques de Cavalli-Sforza entre populations ne présentent aucune valeur de bootstrap significative (Figure 4). Ce qui signe l'absence de structure géographique et invalide le modèle d'isolement génétique par la distance géographique. Toutefois il les 6 populations sont entre elles fortement différenciée (voir matrice des Fst, figure 4).

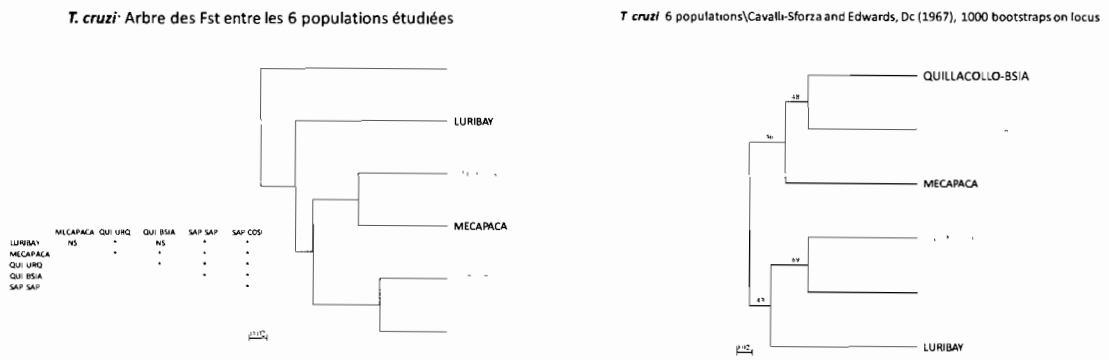


Figure 4 : Arbres construits à partir des distances Fst et à partir des distances de Cavalli-Sforza entre populations

L'analyse par le logiciel « Structure » qui permet d'inférer une structure en identifiant des populations génétiquement distinctes et en assignant chaque individu à ces populations, nous a montré un nombre de populations théoriques (k) variant de 2 à 4 en fonction des modèles utilisés. Le modèle « admixture » (les individus peuvent provenir de deux populations différentes semble plus approprié à notre jeu de données que le modèle « no admixture » (les individus proviennent tous de la même population) compte-tenu de la proximité des 2 populations de Sapini et des 2 populations de Quillacollo. L'option « prior location » est conseillée en cas de populations qui sont faiblement structurées.

La figure 5 montre dans le cas du modèle « admixture » sans l'option « prior location » un k = 2. Une forte identité génétique est identifiée entre les deux populations de Sapini et la population de Luribay, toutes 3 situées dans la même vallée et les autres populations font parti de la deuxième population proposée par le programme malgré leur distance (cas de Quillacollo et Mecapaca distantes de 150 km). Quand l'option « prior location » est appliquée, le modèle propose k = 4. Les deux populations de Sapini sont clairement différenciées avec cependant quelques individus de Sapini-Sap plus apparentés à la population de Sapini-Cosi, ce qui pourrait marquer une certaine circulation des parasites entre les deux sites. La population de Luribay se différencie des deux autres avec des individus qui seraient attribués à deux populations différentes, l'une correspondant à Sapini-Sap et l'autre à Mecapaca. La population de Quillacollo_URQ est différenciée de toutes les autres ; celle de Quillacollo-BSIA apparait présenter des individus qui sont plus semblables à la population de Mécapaca.

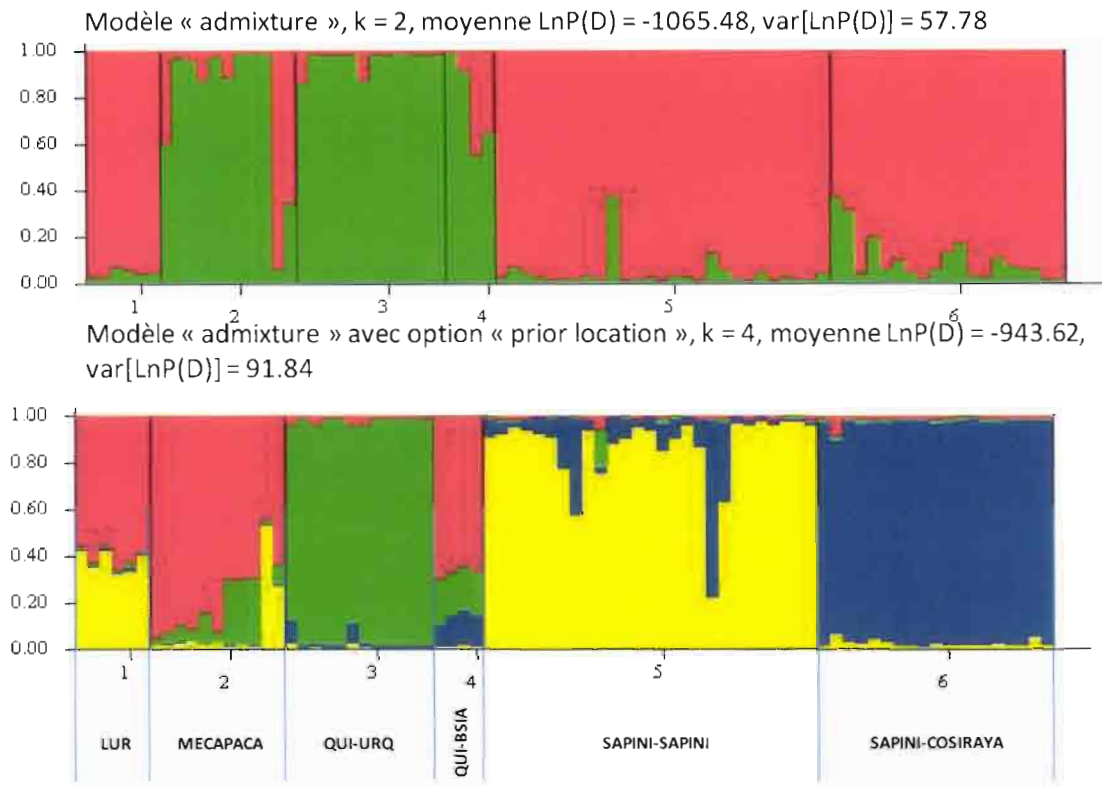


Figure 5. Représentation graphique de l'origine ancestrale estimée des 79 souches de T. cruzi *isolées de* T. infestans *sylvestres dans 6 sites géographiques. Le coefficient d'appartenance de chaque souche aux populations inférées par chaque modèle d'analyse est représenté par une seule ligne verticale divisée en fragments de différentes couleurs qui correspondent aux populations inférées.*

Infection, Genetics and Evolution 11 (2011) 1752–1760

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Trypanosoma cruzi discrete typing units (DTUs): Microsatellite loci and population genetics of DTUs TcV and TcI in Bolivia and Peru

Christian Barnabé [a,*], Thierry De Meeûs [b,c], François Noireau [a], Marie-France Bosseno [a], Eric Marcelo Monje [a], François Renaud [a], Simone Frédérique Brenière [a]

[a] MIVEGEC (Université de Montpellier 1 et 2 – CNRS 5290 – IRD 224), Maladies Infectieuses et Vecteurs: Ecologie, Génétique, Evolution et Contrôle, Institut de Recherche pour le Développement (IRD), Representation in Bolivia, Av Hernando Siles 85290, CP 9214 La Paz, Bolivia

[b] UMR 177 IRD – CIRAD, Interactions hôtes-vecteurs-parasites dans les infections dans les trypanosomatidae, Centre International de Recherche-Développement sur l'Elevage en zone Subhumide (CIRDES), 01 BP 454, Bobo-Dioulasso 01, Burkina Faso

[c] CNRS, Délégation Languedoc-Roussillon, 1919, route de Mende, 34293 Montpellier cedex 5, France

ARTICLE INFO

Article history:
Received 13 April 2011
Received in revised form 9 July 2011
Accepted 12 July 2011
Available online 21 July 2011

Keywords:
Trypanosoma cruzi
Multilocus microsatellite typing
Discrete typing units
Populations
Genetics
Reproductive systems

ABSTRACT

Trypanosoma cruzi, the agent of Chagas disease, is usually subdivided into six discrete typing units (DTUs), TcI to TcVI, among which TcI and TcV are most common in human infections in Bolivia. Multilocus microsatellite typing (MLMT) was selected to further explore the structure of the natural populations belonging to these DTUs. The analysis showed that microsatellite clustering does not fully match the six DTUs, but it is relevant for the within DTUs analyses. Population genetics analysis was conducted on 11 relevant subsamples of stocks from Bolivia and Peru, belonging to TcI (6) and TcV (5), defined by four criterions: DTU, vector species, geographic origin, and date of isolation. Most TcV strains presented the same multilocus genotype over all subsamples with the puzzling characteristic that five loci were heterozygous and the other five homozygous. In TcI, four clusters were defined according to the vector species. Most of them appeared in agreement with clonal propagation (stocks isolated from *Triatoma infestans* and *Triatoma sordida*), while a few highly homozygous stocks (e.g. those isolated from *Rhodnius stali*) suggested that scarce sex events can occur. The poor role played by spatio-temporal factors in describing the observed genetic diversity suggested that ecology, in particular as regard to host played a significant role. These results highlight the extreme heterogeneity of *T. cruzi* and suggest that further population genetics surveys will need to target the most possible precise spatio-temporal and ecological scales.

1. Introduction

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, is classified as neglected disease in Latin America by the WHO although 5–6 million individuals are infected, 25 millions are at risk of contracting the infection and there is no vaccine and no efficient treatment against the disease (http://whqlibdoc.who.int/trs/WHO_TRS_905.pdf). *T. cruzi* can be transmitted orally, congenitally and by blood transfusion (Schmunis, 1999), and by the feces of blood sucking insects belonging to the Reduviidae family and customarily classified into five tribes and 15 genera (Lent and Wygodzinsky, 1979) of which five are relevant for *T. cruzi* transmission (*Rhodnius* Stal 1859, *Dipetalogaster* Usinger 1939, *Eratyrus* Stal 1859, *Panstrongylus* Berg 1879, and *Triatoma* Laporte 1832).

From the pioneering studies of multilocus enzymes electrophoresis (MLEE) (Miles et al., 1977; Tibayrenc and Ayala, 1988) to the most recent speculations about hybridization events within *T. cruzi* (Venegas et al., 2009), a consequent amount of data has been gathered on population genetics. Authors in the field agree with the vision of a highly polymorphic species, structured into six discrete typing units (DTUs) (Barnabé et al., 2000; Brisse et al., 2000). A new consensus recommends the notation TcI to TcVI for these DTUs (Zingales et al., 2009). TcI is ubiquitous, highly variable, present in both domestic and sylvatic cycles, and apparently weakly structured. TcII, TcV, and TcVI are mainly domestic, and less polymorphic than TcI. TcV and TcVI are clearly "recent" hybrids between TcII and TcIII (Broutin et al., 2006). TcIII and TcIV are more scarcely sampled, and specific to sylvatic cycles. TcI and TcV were mostly identified in Bolivian and Peruvian domestic cycle (Brenière et al., 1985, 1989, 1997); these DTUs could be identified in strict sympatry in the same host and vector (Bosseno et al., 1996; Flores-Chavez et al., 2006). Although genetic exchanges may exist and have undoubtedly participated to the evolution of the taxon (Gaunt et al., 2003; Westenberger et al., 2005), *T. cruzi* is

* Corresponding author. Address: Institut de Recherche pour le Développement (IRD), Representation in Bolivia, Av Hernando Siles No. 5290, Esq Calle 7 Obrajes, CP 9214 La Paz, Bolivia. Tel.: +591 2 278 29 69; fax: +591 2 278 29 44.
E-mail address: Christian.Barnabe@ird.fr (C. Barnabé).

1567-1348/$ - see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.meegid.2011.07.011

Table 1
Origin, genetic discrete typing units (DTU) and subsamples of the 94 *Trypanosoma cruzi* used for the study.

Code	Country	Department/state	Province	Community	Host	Year	DTU[a]	No subsample	No. MLG
54 stocks distributed into 11 subsamples according to the DTU, geographic area, host and date of isolation									
PNC02	Bolivia	Cochabamba	Campero	Peña Colorada	*T. infestans*	1992	TcI	1	17
QRA03	Bolivia	Cochabamba	Campero	Quiroga	*T. infestans*	1992	TcI	1	26
QRA05	Bolivia	Cochabamba	Campero	Quiroga	*T. infestans*	1992	TcI	1	29
MIZ02	Bolivia	Cochabamba	Campero	Mizque	*T. infestans*	1993	TcV	2	36
MIZ04	Bolivia	Cochabamba	Campero	Mizque	*T. infestans*	1993	TcV	2	34
MIZ05	Bolivia	Cochabamba	Campero	Mizque	*T. infestans*	1993	TcV	2	34
CHUL21	Bolivia	Cochabamba	Capinota	Chulpani	*T. infestans*	1992	TcI	3	17
CHUL24	Bolivia	Cochabamba	Capinota	Chulpani	*T. infestans*	1992	TcI	3	20
IRP03	Bolivia	Cochabamba	Capinota	Irpairpa	*T. infestans*	1992	TcI	3	17
SIC08	Bolivia	Cochabamba	Capinota	Sicaya	*T. infestans*	1992	TcI	3	17
SIC12	Bolivia	Cochabamba	Capinota	Sicaya	*T. infestans*	1992	TcI	3	17
SIC18	Bolivia	Cochabamba	Capinota	Sicaya	*T. infestans*	1992	TcI	3	18
SIC29	Bolivia	Cochabamba	Capinota	Sicaya	*T. infestans*	1992	TcI	3	17
YAT07	Bolivia	Cochabamba	Capinota	Yatamoco	*T. infestans*	1992	TcI	3	17
YAT26	Bolivia	Cochabamba	Capinota	Yatamoco	*T. infestans*	1992	TcI	3	18
CHUL16	Bolivia	Cochabamba	Capinota	Chulpani	*T. infestans*	1992	TcV	4	34
CHUL23	Bolivia	Cochabamba	Capinota	Chulpani	*T. infestans*	1992	TcV	4	34
IGA04	Bolivia	Cochabamba	Capinota	Igasani	*T. infestans*	1992	TcV	4	34
KOR19	Bolivia	Cochabamba	Capinota	Korata	*T. infestans*	1992	TcV	4	34
R37	Bolivia	La Paz	Caranavi	Caranavi	*R. stali*	1993	TcI	5	5
R39	Bolivia	La Paz	Caranavi	Caranavi	*R. stali*	1993	TcI	5	1
R45	Bolivia	La Paz	Caranavi	Caranavi	*R. stali*	1993	TcI	5	2
CAR61	Bolivia	La Paz	Caranavi	Col Alto Lima	*T. infestans*	1992	TcV	6	34
CAR64	Bolivia	La Paz	Caranavi	Col.SanLorenzo	*T. infestans*	1992	TcV	6	34
CAR76	Bolivia	La Paz	Caranavi	Col.Taypiplaya	*T. infestans*	1992	TcV	6	34
AUQ13	Bolivia	La Paz	Nor Yungas	Nor Yungas Auquisamana	*T. infestans*	1992	TcI	7	23
PARA20	Bolivia	La Paz	Nor Yungas	Pararani	*T. infestans*	1992	TcI	7	25
TP06	Bolivia	La Paz	Nor Yungas	Trinidad Pampa	*T. infestans*	1992	TcI	7	17
TP27	Bolivia	La Paz	Nor Yungas	Trinidad Pampa	*T. infestans*	1992	TcI	7	17
TP28	Bolivia	La Paz	Nor Yungas	Trinidad Pampa	*T. infestans*	1992	TcI	7	20
AUQ22	Bolivia	La Paz	Nor Yungas	Auquisamaña	*T. infestans*	1992	TcV	8	34
PARA02	Bolivia	La Paz	Nor Yungas	Pararani	*T. infestans*	1992	TcV	8	34
PARA04	Bolivia	La Paz	Nor Yungas	Pararani	*T. infestans*	1992	TcV	8	34
PARA05	Bolivia	La Paz	Nor Yungas	Pararani	*T. infestans*	1992	TcV	8	34
CAJ01	Bolivia	La Paz	Inquisivi	Cajuata	*T. infestans*	1992	TcV	9	35
CAJ02	Bolivia	La Paz	Inquisivi	Cajuata	*T. infestans*	1992	TcV	9	34
CAJ15	Bolivia	La Paz	Inquisivi	Cajuata	*T. infestans*	1992	TcV	9	34
CBB33	Bolivia	Santa Cruz	Velasco	Cochabambito	*T. sordida*	1995	TcI	10	9
CBB34	Bolivia	Santa Cruz	Velasco	Cochabambito	*T. sordida*	1995	TcI	10	10
CeR29	Bolivia	Santa Cruz	Velasco	Cerrito	*T. sordida*	1995	TcI	10	14
CeR31	Bolivia	Santa Cruz	Velasco	Cerrito	*T. sordida*	1995	TcI	10	15
CeR37	Bolivia	Santa Cruz	Velasco	Cerrito	*T. sordida*	1995	TcI	10	12
COT27	Bolivia	Santa Cruz	Velasco	Cotoca	*T. sordida*	1995	TcI	10	15
COT34	Bolivia	Santa Cruz	Velasco	Cotoca	*T. sordida*	1995	TcI	10	15
COT38	Bolivia	Santa Cruz	Velasco	Cotoca	*T. sordida*	1995	TcI	10	16
GUA18	Bolivia	Santa Cruz	Velasco	Guapomocito	*T. sordida*	1995	TcI	10	11
GUA19	Bolivia	Santa Cruz	Velasco	Guapomocito	*T. sordida*	1995	TcI	10	13
GUA20	Bolivia	Santa Cruz	Velasco	Guapomocito	*T. sordida*	1995	TcI	10	13
1_5	Peru	Arequipa	Arequipa	Sta Rita de Sigua	*T. infestans*	1993	TcI	11	17
2_53	Peru	Arequipa	Arequipa	Sta Rita de Sigua	*T. infestans*	1993	TcI	11	20
3_35	Peru	Arequipa	Arequipa	Sta Rita de Sigua	*T. infestans*	1993	TcI	11	17
5_75	Peru	Arequipa	Arequipa	Sta Rita de Sigua	*T. infestans*	1993	TcI	11	17
7_61	Peru	Arequipa	Arequipa	Sta Rita de Sigua	*T. infestans*	1993	TcI	11	17
7_65	Peru	Arequipa	Arequipa	Sta Rita de Sigua	*T. infestans*	1993	TcI	11	17
22 stocks not distributed in subsamples									
86_2021	Bolivia	La Paz	Sur Yungas	nk	Porcupine	1985	TcI	–	3
PB3cl2	Bolivia	La Paz	Sur Yungas	Palos Blanco	*R. stali*	nk	TcI	–	8
CA34	Bolivia	Santa Cruz	Cordillera	Camiri	*T. infestans*	1982	TcV	–	37
CA15	Bolivia	Santa Cruz	Cordillera	Camiri	*T. infestans*	1982	TcVI	–	44
PNC07	Bolivia	Cochabamba	Campero	Peña Colorada	*T. infestans*	1993	TcVI	–	44
TintinS1	Bolivia	Cochabamba	Campero	Tintin	*T. infestans*	1995	TcV	–	34
CAR77	Bolivia	La Paz	Caranavi	Col.Perigrinos	*T. infestans*	1992	TcI	–	4
P259	Bolivia	Chuquisaca	Oropeza	Sucre	Human	1985	TcV	–	34
P188	Bolivia	Cochabamba	Cercado	Cochabamba	Human	1983	TcI	–	19
P263	Bolivia	Cochabamba	Cercado	Cochabamba	Human	1985	TcV	–	34
VM37	Bolivia	Tarija	Gran Chaco	Villamontes	*T. infestans*	1992	TcV	–	39
VM09	Bolivia	Tarija	Gran Chaco	Villamontes	*T. infestans*	1992	TcVI	–	45
P0PP	Bolivia	nk	nk	nk	Human	nk	TcI	–	17
SO30	Bolivia	Potosi	Nor Chichas	Calcha	*T. infestans*	nk	TcI	–	30
SO34cl4	Bolivia	Potosi	Nor Chichas	Toropalca	*T. infestans*	nk	TcI	–	22
13379cl7	Bolivia	Santa Cruz	Andres Ibañez	Santa Cruz	Human	1979	TcI	–	6
1979cl7	Bolivia	Chuquisaca	Oropeza	Sucre	Human	1979	TcI	–	27
TPA1	Bolivia	La Paz	Nor Yungas	Trinidad Pampa	*T. infestans*	1986	TcI	–	17

(*continued on next page*)

Table 1 (*continued*)

Code	Country	Department/state	Province	Community	Host	Year	DTU[a]	No. subsample	No. MLG
P275	Bolivia	Potosi	Sur Chichas	Tupiza	Human	1985	TcV	-	38
P217	Bolivia	La Paz	Nor Yungas	nk	Human	1984	TcI	-	20
MMX8	Peru	Arequipa	Arequipa	Moquegua Matalaque	*T. infestans*	1993	TcI	-	21
3_1	Peru	Arequipa	Arequipa	Sta Rita de Sigua	*T. infestans*	1993	TcIV	-	47
18 T. cruzi reference stocks									
Cutiacl1	Brazil	Espiritu-Santo		Colatina	Agouti	nk	TcI	-	24
OPS21cl11	Venezuela	Cojedes		Macuayas	Human	1977	TcI	-	7
P209cl93	Bolivia	Chuquisaca	Oropeza	Sucre	Human	1983	TcI	-	28
IVVcl4	Chile	Region-IV		Cuncumen	Human	nk	TcII	-	40
MAS1cl1	Brazil	Ferderal district		Brasilia	Human	nk	TcII	-	42
TU18cl93	Bolivia	Potosi	Sur Chichas	Tupiza	*T. infestans*	nk	TcII	-	41
M5631cl5	Brazil	Para		Selva Terra Islank	Armadillo	nk	TcIII	-	48
M6241cl6	Brazil	Para		Belem	Human	nk	TcIII	-	43
X109_2	Paraguay	Presidente Hayes		Makthlawaiya	Dog	nk	TcIII	-	46
92122102R	USA	Georgia		Statesboro Bulloch Co	Raccoon	1992	TcIV	-	31
CANIIIcl1Z3	Brazil	Para		Belem	Human	nk	TcIV	-	33
DogTheis	USA	nk	nk	nk	Dog	nk	TcIV	-	32
Bug2148cl1	Brazil	Rio Grande do Sul		Rio Grande do Sul	*T. infestans*	nk	TcV	-	34
MNcl2	Chile	Region-IV		Illapel	Human	nk	TcV	-	36
SC43cl1	Bolivia	Chuquisaca	Andres Ibañez	Santa Cruz	*T. infestans*	1981	TcV	-	35
CLbrener	Brazil	Rio Grande do Sul		Rio Grande do Sul	*T. infestans*	1963	TcVI	-	44
P63cl1	Paraguay	Presidente Hayes		Makthlawaiya	*T. infestans*	nk	TcVI	-	44
Tulacl2	Chile	Region-IV		Tulahuen	Human	nk	TcVI	-	45

[a] DTU identified on the basis of previous results from Brenière et al. (1997, 1998), Bosseno et al (1996), and Barnabé et al. (2000). nk, not known; MLG, multilocus genotype.

considered as mainly clonal (Tibayrenc et al., 1990). The current six-DTUs classification is likely to poorly reflect actual organization of *T. cruzi* because of probable hidden structure not yet evidenced by the markers generally used, particularly in TcI (Lewicka et al., 1995) where recent works have evidenced genetic structuring (Herrera et al., 2007; O'Connor et al., 2007; Falla et al., 2009; Llewellyn et al., 2009b). Microsatellites markers present many advantages. They are ubiquitous in the genome, in principle not affected by selection, co-dominant and hence exploitable in terms of population genetics. They also avoid certain disadvantages of other markers previously widely used, such as dominance (RAPDs), low discriminatory power (MLEE), more expense (MLSTs, multilocus sequence typing). Early studies showed some correlation between the clusters identified by MLMT (multilocus microsatellite typing) analysis and those obtained with other genetic markers, but these studies did not include all the DTUs (Oliveira et al., 1998; Macedo et al., 2001). More recent works analyzed MLMT polymorphism within TcI and TcIII, highlighting spatial and ecological structuring (Llewellyn et al., 2009a,b; Ocaña-Mayorga et al., 2010).

First, the present study analyzed the MLMT structuring among reference stocks belonging to the known DTUs (TcI to TcVI). Then, *T. cruzi* stocks from Bolivia and Peru, previously classified in DTUs with other markers, were surveyed for the first time with microsatellites. We also analyzed subsamples defined according to four criterions (DTU, geography, host and date of isolation) to tried to assess the reproductive system.

2. Material and methods

2.1. Parasites, culture, DNA extraction and genotyping

Origins of the 94 *T. cruzi* stocks under study are summarized in Table 1. All these stocks were previously characterized by MLEE (Tibayrenc and Ayala, 1988; Brenière et al., 1997, 1998, 2003; Barnabé et al., 2000) for DTU assignment. The whole sample included two kinds of stocks: (i) 18 references stocks representing the diversity of the parasite (3 for each DTU), selected from all the endemic area of Latin America; (ii) 76 Bolivian and Peruvian stocks. Fifty-four stocks from Bolivia and Peru (48 and 6, respectively) were distributed into 11 relevant subsamples defined according to their DTU, geographical origin, host species and date of isolation (Table 1 and Fig. 1). All stocks were thawed, cultured in LIT medium supplemented with 10% fetal calf serum. DNA was extracted with a conventional phenol-chloroform method and the solutions diluted to 20 ng/μl before use.

We used 10 previously described dinucleotide microsatellite loci (Oliveira et al., 1998; Gaunt et al., 2003). One primer of each pair was fluorescent labeled by a particular dye and all the PCRs assays followed the same procedure: 2 μl buffer 10×, 0.12 μl dNTP 5 mM, 0.12 μl of each primer 10 μM, 0.6 Ul Taq-DNA Polymerase (Roche Applied Sciences, France), sterile water qsp 20 μl, submitted to PCR amplification (initial denaturing 94 °C, 2 mn followed by 35 cycles of 94 °C, 30 s, 53 °C, 30 s, 72 °C, 30 s, and a final extension of 72 °C, 2 mn). Electrophoreses of the fluorescent labeled PCR products, diluted and denatured in 20 μl of HiDi formamide, were conducted on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, UK), with the Genescan 500 LIZ internal size standard.

2.2. Data analyses

Hierarchical Neighbor Joining (NJ) trees of individuals or subsamples, based on Cavalli-Sforza and Edwards chord genetic distance matrices (Cavalli-Sforza and Edwards, 1967) with bootstrap over loci, were built under the program Populations (Populations, 1.2.30 Copyright© 1999, Olivier Langella, CNRS UPR9034). We used Figtree (BEAST Software, http://beast.bio.ed.ac.uk/FigTree) to draw the trees. Wright's *F*-statistics (Wright, 1965) were estimated with Weir and Cockerham's unbiased estimators (Weir and Cockerham, 1984). Two fixation indices were principally used: F_{IS}, which is a measure of the level of inbreeding of individuals relative to inbreeding of subsamples they come from, and F_{ST} which is a measure of inbreeding within subsamples relative to inbreeding overall subsamples due to population subdivision. Hence F_{IS} also represents a measure of deviation from random union of gametes within putative subpopulations, and F_{ST} also represents a measure of genetic differentiation between putative subpopulations. Genetic diversity was estimated by Nei's unbiased estimator of genetic diversity (H_s) (Nei and Chesser, 1983). Confidence intervals of F_{IS} were estimated by bootstrapping over loci. For F_{IS}, significant devi-

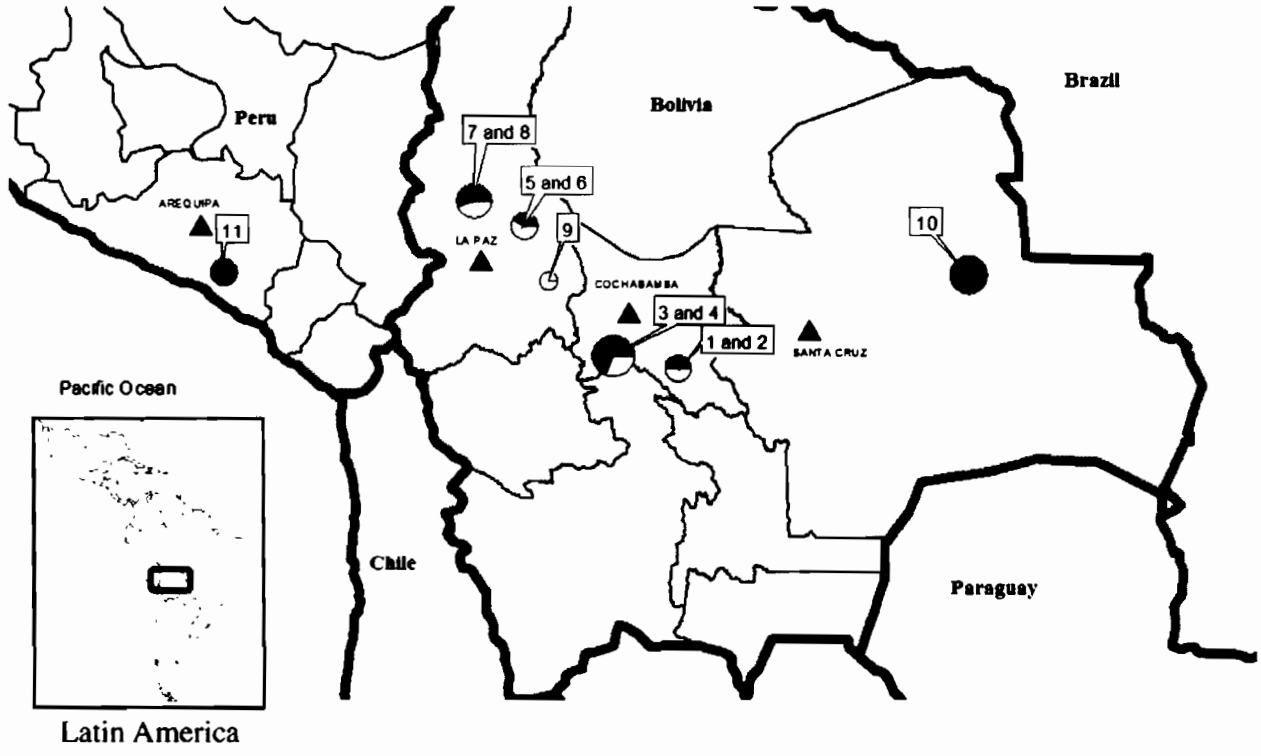


Fig. 1. Map of Bolivia and Peru showing the different origins of the 11 *Trypanosoma cruzi* subsamples under study as expounded in Table 1. Surfaces of the circles are proportional to the sample sizes: in black TcI and in white TcV. Box = map of Latin America with the localization of the region where the samples are from (small rectangle).

ation from 0 was tested by randomizing alleles within subsamples (10,000 randomizations), and the statistic used was the unbiased estimator itself. The significance of genetic differentiation was tested by randomizing individuals among subsamples and the statistic used was the log-likelihood ratio G (Goudet et al., 1996). Comparison of genetic diversities and heterozygote deficits across relevant subsamples were undertaken through multi-way analyses of variances (ANOVA) with R. The model was always of the form Stat ~ Vector + Locus + Vector: Locus where "Stat" was either H_s or F_{IS}, "Vector" the vector species," Locus" the microsatellite locus and ":" the interaction. When possible, we estimated a proxy for clonal population sizes with the formula $N_c = (-1 - F_{IS})/(4uF_{IS})$ (Simo et al., 2010), where u is the mutation rate that was set to 10^{-3} or 10^{-4}, the classical range for microsatellite loci (Hellegren, 2000). Linkage disequilibrium (nonrandom association between genotypes at different loci) was tested between pairs of loci by random association of paired loci within each subsample (nominal level for multiple tests 0.05, 9900 permutations) and using the log-likelihood ratio based G statistics summed overall subsamples, shown to be the most powerful test across subsamples (De Meeus et al., 2009). There are potentially as many tests as locus pairs $k = L(L-1)/2$ (where L is the number of different loci). We thus tested if the proportion of significant tests at level $\alpha = 0.05$ k' in the k tests series was significantly higher than 5% with an exact binomial test with k' successes, k attempts and mean 0.05 under R (R Development Core Team, 2011).

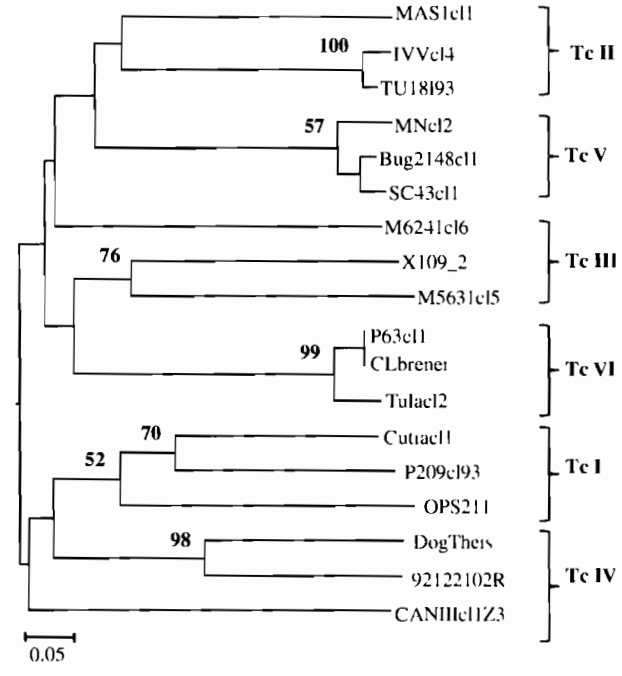


Fig. 2. NJ tree of 18 *Trypanosoma cruzi* reference stocks representative of the six DTUs identified in *T. cruzi* species. The Cavalli-Sforza and Edwards genetic distance between stocks was used. The bootstrap values higher than 50 are indicated at the nodes of the tree The names of the leaves are the codes of the stocks.

3. Results

3.1. Genetic variation and clustering among T. cruzi reference stocks

Absence of amplification was very scarce within the 18 *T. cruzi* reference stocks (only 1 of 180 PCR, stock 92122102R at locus MCLE08). Fig. 2 shows the NJ tree built from these stocks with 17 distinct multilocus genotype (MLG) separated by high genetic distances. Only TcVI stocks were clustered together with a significant bootstrap value of 99%. Stocks from TcII, though clustered to-

gether were not strongly bootstrap supported and other stocks were much less well classified.

3.2. Overall genetic diversity and clustering among Bolivian and Peruvian stocks

The NJ tree clustering of the 76 *T. cruzi* stocks from Bolivia and Peru belonging to four DTUs: TcI, TcIV, TcV and TcVI, with sample sizes of 49, 1, 23 and 3 respectively, is shown in Fig. 3. Thirty-six MLGs were identified. Twenty-nine MLGs were represented by only one stock whereas seven MLGs were sampled from 2 to 18 times. Among the seven repeated MLGs, 3 of them (No. 17, 20 and 34) were ubiquitous (Fig. 3 and Table 1). Interestingly, the only two clusters supported by high bootstrap values included all the hybrids stocks of TcV and TcVI (Fig. 3).

3.3. Populations genetic analyze of TcV and TcI

TcV stocks widely spread over South America, all isolated from *T. infestans* (except three stocks from humans), displayed extremely

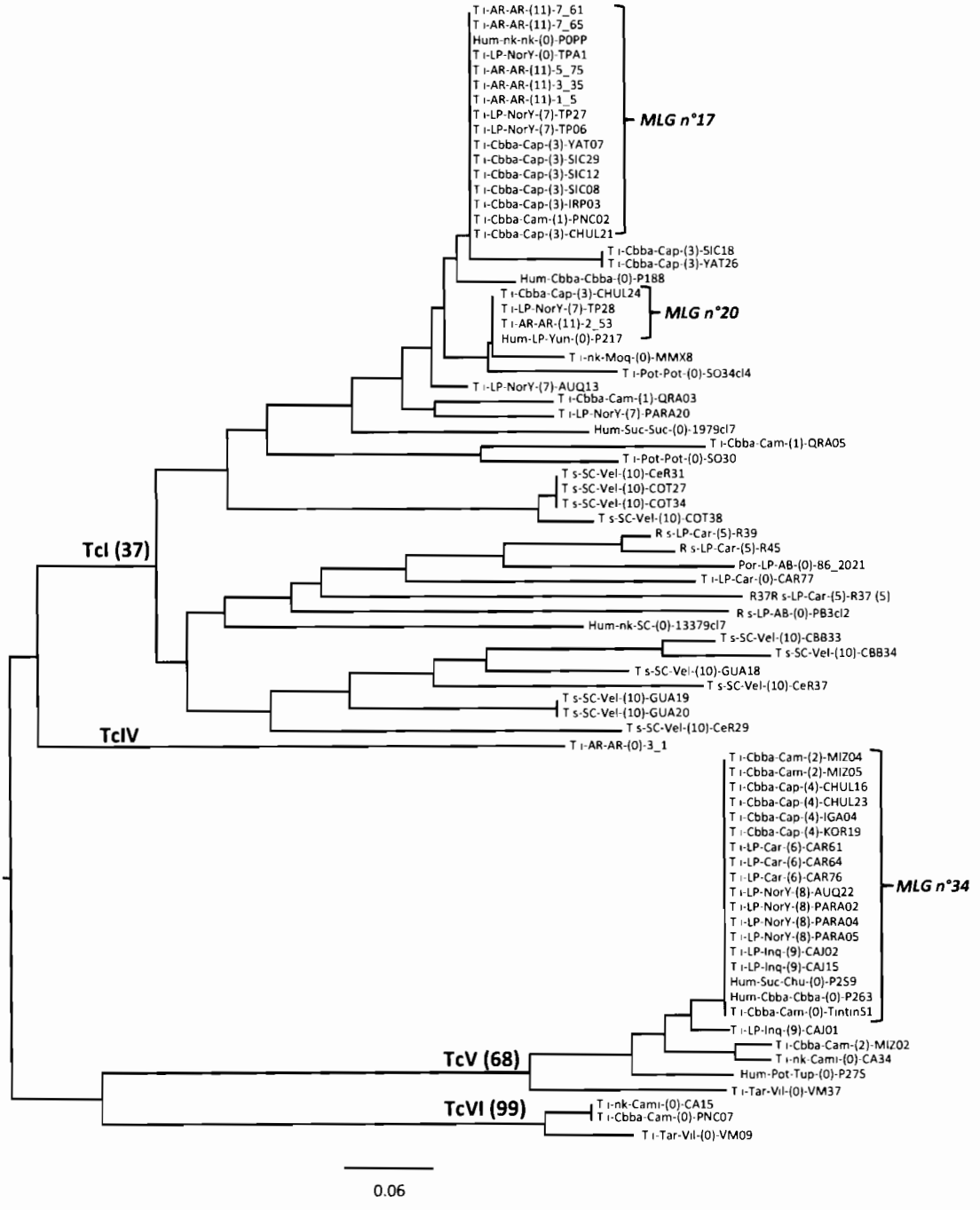


Fig. 3. NJ tree of the 76 *T. cruzi* from Bolivia and Peru. The Cavalli-Sforza and Edwards genetic distance between individuals was used. The three main ubiquitous multilocus genotypes (MLG, No. 17, 20 and 34) are indicated. DTUs are indicated at the upper branching level, and relevant bootstrap values are notated at the nodes in brackets The names of the leaves are composed of abbreviations of vector (T.i, *T. infestans*; R.s, *R. stali*, T.s, *T. sordida*), department (Cbba, Cochabamba; LP, La Paz; SC, Santa Cruz; AR, Arequipa), province (Cam, Campero; Cap, Capinota; Car, Caranavi; Nor Y, Nor Yungas; Inq, Inquisivi; Vel, Velasco; AR Arequipa) and code of the stock, the number in brackets is the No. of the subsample to which the stock belongs.

low diversity (Fig. 3). In fact, all stocks were represented by the only MLG No. 34, except six stocks differing from the others at one or exceptionally three loci (one stock). Stock P275 is 199/199 at locus A427 instead of 181/181 (double mutant), MNcl2 and MIZ02 both are 127/129 at locus MCL01 instead of 126/128 (+1 nucleotide at each allele), CAJ01 and SC43cl1 are 179/179 at locus C875 instead of 173/179 (one allele missing), VM37 is 280/280 at locus MCL03 instead of 278/278 (double mutant), 212/212 at locus MCL05 instead of 224/224 (double mutant) and is blank for locus C875 (two missing alleles) and finally CA34 is 129/129 at locus MCL01 instead of 126/128 (double mutant). Given the very low probability of occurrence of such genotypes through classical mutation or recombination these genotypes are probably due to technical problems (drop outs and/or null alleles and/or stuttering) or maybe homopolymeric tracts, or gene conversion. Anyway, it can be assumed that all TcV stocks are all recently issued from a single individual that have clonally propagated across South America. Five loci are fixed heterozygous while the five others are fixed homozygous. This odd pattern can be alternatively explained knowing that TcV is a hybrid between two other DTUs according two mechanisms. When parental alleles are different at all loci and if this hybridization was immediately followed by a selfing event and the clonal propagation of a single MLG and if these events occurred sufficiently recently so that no mutation could restore a heterozygous state at some of the homozygous fixed loci, then we would indeed expect about half the loci to be heterozygous and the other half homozygous; nevertheless when some alleles are shared between the parental strains, this selfing event is not necessary to explain such a situation.

TcI stocks presented strong differentiations between subsamples from *T. infestans* and the two other vector species with strong F_{ST} val-

Table 2
Differentiation, as measured by Weir and Cockerham's θ (F_{ST} unbiased estimator), between the different subsamples of TcI.

	No. of subsamples	*T. infestans*				*R. stali*
		1	3	7	11	5
T. infestans	3	0.1440 (0.0833)				
T. infestans	7	−0.0001 (0.5667)	0.0188 (0.2867)			
T. infestans	11	0.1182 (0.1300)	0.0056 (0.4800)	0.0021 (0.5200)		
R. stali	5	0.3891 (0.1100)	0.7027 (**0.0067**)	0.6186 (**0.0133**)	0.7067 (**0.0233**)	
T. sordida	10	0.2643 (**0.0033**[a])	0.3878 (**0.0033**[a])	0.3336 (**0.0033**[a])	0.3745 (**0.0033**[a])	0.2722 (**0.0100**[a])

The vector from which stocks were isolated is indicated; more information of subsamples is in Table 1. The *P*-value of G-based differentiation tests are indicated in brackets, in bold, significant *P*-values

[a] Significant *P*-value after sequential Bonferroni correction.

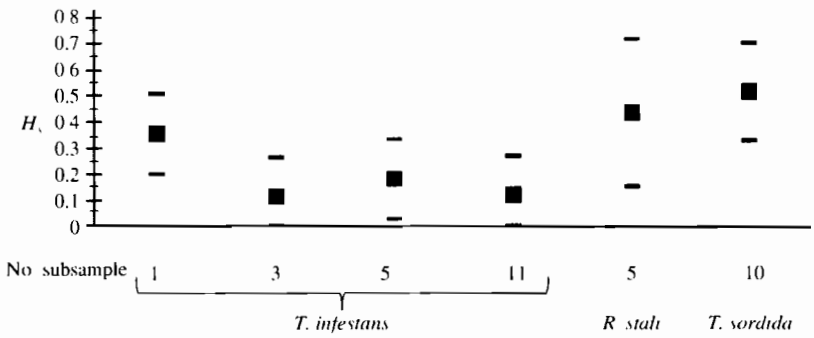


Fig. 4. Unbiased genetic diversity H_s for the different *T. cruzi* subsamples of TcI with 95% confidence intervals. The vector from which stocks were isolated is indicated; more information of subsamples is in Table 1.

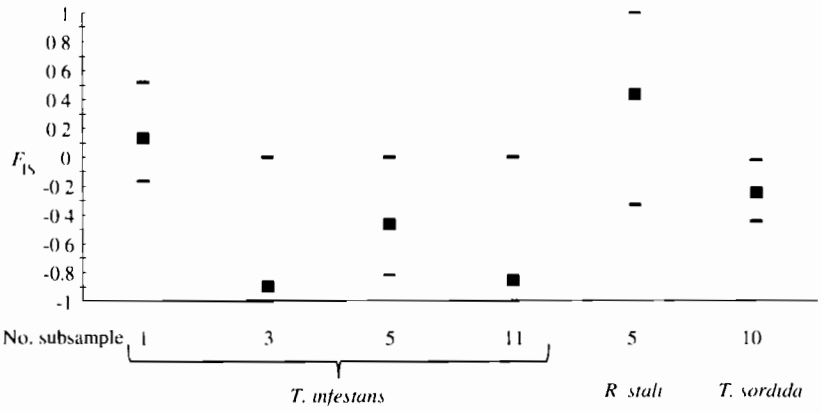


Fig. 5. Relative inbreeding F_{IS} for the different *T. cruzi* subsamples of TcI with 95% confidence intervals obtained after bootstrap over loci. The vector from which stocks were isolated is indicated; more information of subsamples is in Table 1.

ues and eight significant *P*-values (five at sequential Bonferroni level) (Table 2). Given the fact that sample from *R. stali* contained only three individuals we can assume that differentiation is indeed strong between stocks isolated from this vector species and the two others. Between subsamples isolated from *T. infestans*, no differentiation test was significant, whatever the geographic or temporal distance between it. This suggests that the genetic structure of TcI is associated with vector species which are known to be geographically structured. It is thus probable that an ecological specialization is responsible for this structure. These subsamples also differed as regard to genetic diversity (Fig. 4). All parameters (subsample, locus and interaction) had a highly significant importance in the ANOVA (all *P*-values < 0.0026) and explained 82% of the variation. Globally, most stocks from *T. infestans* displayed very low genetic diversities while *R. stali* and particularly *T. sordida* displayed intermediate levels of genetic diversity. Relative inbreeding varied greatly across subsamples (Fig. 5). The ANOVA gave a significant contribution for loci and vector species (*P*-values ~ 0.03) to this variation but it can be seen from Fig. 5 that variations are also observed between *T. infestans* subsamples. In terms of reproductive modes, we used De Meeus et al. (2006) criteria and assumed diploidy and no gene conversion. The moderately positive and moderately variable F_{IS} would translate into a significant contribution of sexual recombination for the stocks isolated from *T. infestans* in Campero (subsample No. 1), though the size of the subsample cannot provide certainty. With extremely negative F_{IS} observed, an absence or extreme rarity of sexual recombination and small clonal subpopulation size (around 100 or 1000 for $u = 10^{-3}$ and 10^{-4}, respectively) can be assumed for the other stocks isolated from *T. infestans* in Bolivia and Peru. For stocks from *R. stali*, given the very small sample size, the importance of the confidence intervals is difficult to interpret. Nevertheless, the strongly positive mean value over the 10 loci is a signature of a possible role played by sexual recombination for those stocks. Finally, the moderately negative F_{IS} observed for *T. cruzi* TcI stocks from *T. sordida* and its modest variation across loci can be interpreted as pure clonality and much bigger clonal subpopulation size (around 800 or 8000 for $u = 10^{-3}$ and 10^{-4}, respectively). This last result also provides some support for the probable limited role of aneuploidy and/or gene conversion in our results.

Cavalli-Sforza distances between all TcI stocks under study were computed and used to draw the NJ tree shown in Fig. 6. This confirms the subdivision according to vector species, though stocks from *T. sordida* might be subdivided into two supplementary lines that will require further investigations. The tree nevertheless confirms that the differences in genetic diversity and F_{IS} found between the subsample No. 1 (isolated from Campero province in 1992) and the other *T. infestans* subsamples is probably due to the fact that this subsample presents only three stocks that are remarkably distributed across the variability domain defined by the strains from this vector species. The distribution of repeated MLGs is also in line with the strong clonality and small subpopulation sizes of *T. infestans* strains and strong clonality and moderate sized populations for *T. sordida* strains. It is noteworthy that most stocks isolated from human hosts gather with the cluster defined by *T. infestans*. On the contrary, the two groups defined with strains from *T. sordida* contain no human or mammal strains from the available sampling. The cluster (so to speak) containing the TcI stocks isolated from *R. stali* also contain two human strains, one isolated from porcupine and an atypical strain from *T. infestans*. This group is also so heterogeneous that we hardly can consider it as an actual cluster. Finally, it appears clearly that, with the available data of the present study, geography explains very little of TcI genetic structure.

Over TcV, there is a major MLG (No. 34) and the linkage is thus total for this taxon. Over TcI subsamples, no pair of loci was found in significant linkage except in *T. sordida*. There, 14 of 36 tests appeared significant at 5% level (locus MCL05 could not be tested). This is far above what is expected under the null hypothesis (*P*-value < 0.0001). The probability that a locus is involved in a significant pair is $P_s = (14/36) * (8/36) = 0.086$. The locus the most frequently found in a significant pair is MCLE01, which was found six times in significant linkage with another locus (*P*-value = 0.086, unilateral exact binomial tests).

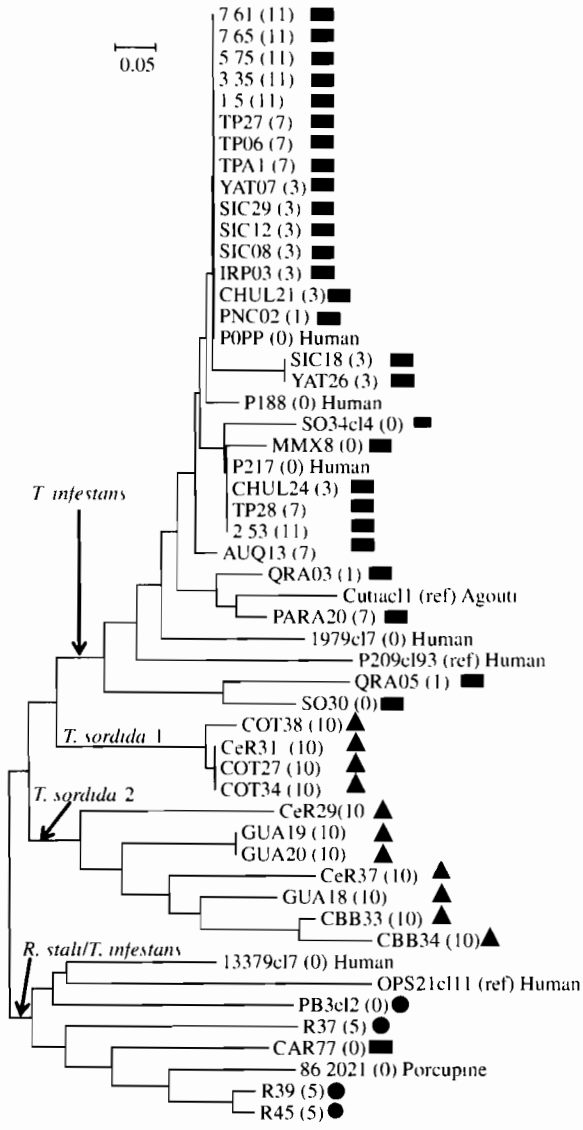


Fig. 6. NJ tree of all stocks belonging to TcI. The Cavalli-Sforza and Edwards genetic distance between populations was used. The names of the leaves correspond to the code of the stocks, the number in brackets is the No. of the subsample to which the stock belongs, the hosts from which the stocks were isolated is indicated, black rectangle for *T. infestans*, black triangle for *T. sordida*, and black circle for *R. stali*; more information of subsamples is in Table 1.

4. Discussion

4.1. Relevance and limits of MLMT method for T. cruzi

Between stocks under study (references and others) with microsatellite marker, genetic distances are of two classes: high distances within TcI, TcII, TcIII and TcIV, and low distances within TcV and VI, known to be recent hybrid DTUs. Such distances within

DTUs were previously observed with RAPD and isoenzymes (Barnabé et al., 2000; Brisse et al., 2000). Nevertheless, clusters found with the polymorphism at the ten microsatellite loci investigated here were in variance with the widely accepted actual DTU classification of *T. cruzi*. This discrepancy could be due to the homoplasic nature of the markers used; indeed the weak polymorphism found for these microsatellite loci could come from a very limited number of possible allelic states. It could also be due to more complex molecular biology processes as conserved genomic zones, where DTU markers would be, and more permissive genomic zones for microsatellite loci that could be more easily exchanged during recurrent hybridization events between DTUs. Whether the first or the second hypothesis better reflect reality remains to be investigated further.

4.2. *T. cruzi* genotypes, domestic versus sylvatic, and epidemiological implications

TcI and TcV were mostly represented here because they are the principal DTUs in the domestic Bolivian cycles as previously shown (Brenière et al., 1995, 2002). This is why the present analysis focused on TcI and TcV stocks only. The *T. cruzi* stocks used in the present study have mostly a domestic origin (*T. infestans* widely represented). Other stocks belonging to TcI were isolated from two other vectors *R. stali* and *T. sordida*, which are primarily sylvatic but can invade and colonize houses.

As expected, TcV appeared extremely homogeneous and is probably composed of few clones derived from a single clone over all South America. Over all stocks, only one TcV isolate (VM37) differed at more than one locus (three loci). For all other isolates, only five isolates diverged at one locus (and sometimes at a single allele) over sites and over years.

For TcI, three to four different groups seemed to cluster according to the vector species (Fig. 6). Stocks from *T. infestans* cluster displayed little polymorphism and were extremely homogeneous over space and time as a probable result of small subpopulation sizes and strong impact of clonal reproduction, though the smallness of sample sizes and weakness of polymorphism cannot lead to definitive conclusions so far. One notable exception was presented by isolate QRA05 that appeared far from the other stocks isolated with it (isolated from Campero province in 1992). This explains why the subsample appeared different from the other *T. infestans* isolates. Stocks from cluster *T. sordida* appeared in two fairly divergent clusters, both highly clonal. Finally, the cluster containing all stocks from *R. stali*, one "miscasted" stock from *T. infestans*, two from humans and one from a porcupine appeared far too heterogeneous for any sustainable speculation to be advanced. The fact that geography or date of isolation poorly explained the genetic structure of the available stocks is a strong signature that the diversity observed is probably more affected by ecological factors such as host species and/or environment. This will thus require further attention if real population genetics surveys are to be undertaken.

In Bolivia, significant parasite diversity has been observed (Brenière et al., 1989; Barnabé et al., 2000), but drastic selection events have likely occurred during domestication process of *T. infestans*, probably due to better fitness of few genotypes leading to a lower diversity in domestic cycle. Nevertheless, we cannot exclude stochastic mechanism such as bottleneck during domestication process. Hence, ubiquity of successful clones could be explained by the recent expansion of *T. infestans* by passive transport associated with human migration (Schofield, 1988; Cortez et al., 2010). Both the adaptive success of these genotypes and their stability over space and time due to a clonal propagation (Tibayrenc et al., 1990) could explain such a situation. Alternatively, the larger diversity observed in *T. sordida* and *R. stali* parasites, could be related to the long lived and high biological diversity found in the tropical forest where these vectors are living.

T. cruzi is primarily transmitted by domestic vectors. Throughout the endemic area, the main DTUs that infect humans are TcI, TcII and TcV. The compared pathogenicity of each DTU remains debatable. Interestingly, these DTUs do not exhibit the same geographical distribution, in the northern Amazon TcI largely predominates (in domestic and sylvatic cycles), whereas in the Southern countries, TcI, TcII and TcV are circulating sympatrically in domestic cycles. However, the different pathologies of Chagas disease concern all endemic countries. Multiple infections are dependent of the frequency of human-vector contacts, being likely the rule in Bolivia where the density of vector colonies in houses is extremely high (Bosseno et al., 1996; Flores-Chavez et al., 2006). Another novelty of the epidemiology of Chagas disease is the emergence of transmission cases by wild triatomines, carrying a wide variety of parasites (new genotypes), which were until now restricted to zoonotic cycles. This is to be related to the possible invasion of wild triatomines after control of domestic populations and also to new human settlements mainly in tropical forests (Amazon) where numerous wild triatomines are present.

4.3. Clonal versus non-clonal propagation in Bolivian *T. cruzi* natural populations

Respective levels of clonality vs. genetic exchanges within *T. cruzi* have occupied numerous debates (Tibayrenc et al., 1986, 1991, 1993; Tibayrenc, 2003; Zhang et al., 1988; Ayala, 1993; Brisse et al., 2003) as well as the impact of clonality on the biological properties of the clones (Laurent et al., 1997; Revollo et al., 1998; Toledo et al., 2002; Tibayrenc, 2003; Macedo et al., 2004). Indeed, if *T. cruzi* spreads strictly clonally, each clone conveys its own biological properties without any ability to exchange it with other clones. On the contrary, if genetic exchange happens, then biological properties could be exchanged, this has important epidemiology consequences. Here, it appears that TcV is strictly clonal and composed of a main clone and that TcI was composed of four divergent entities. TcI *T. infestans* cluster corresponded to highly clonal stocks from small subpopulations. TcI *T. sordida* cluster 1 stocks behaved more as small, and cluster 2 as much bigger totally clonal subpopulations. The remaining stocks (containing those isolated from *R. stali*) corresponded more to heterogeneous, more homozygous groups. The precise description of the reproductive strategies displayed by the different lineages of *T. cruzi* will obviously require more precise investigations.

One question remains as to what are the relevant *T. cruzi* subsamples (or populations) for studying the population genetics of the different entities that compose this heterogeneous taxon. Here, subdividing the available stocks into subsamples according to the DTU, host, location and date not only resulted in small, and sometimes very small, subsample sizes, but also highlighted new levels of heterogeneity. Consequently, further studies will need to focus on much more localized geographical, temporal and ecological scales.

Acknowledgments

We thank the authorities of the Instituto Boliviano de Biología de Altura (IBBA) and the Bolivian and Peruvian colleagues which participate to field work and laboratory isolation of stocks during 1992–1995. Data used in this work were produced through molecular genetic analysis technical facilities of the SFR "Montpellier Environment Biodiversité". This work was funded by the National Agency for Research (ANR, France), and the IRD France.

References

Ayala, F.J., 1993. *Trypanosoma* and *Leishmania* have clonal population structures of epidemiological significance. Biol Res. 26, 47–63.

Barnabé, C., Brisse, S., Tibayrenc, M., 2000. Population structure and genetic typing of *Trypanosoma cruzi*, the agent of Chagas disease: a multilocus enzyme electrophoresis approach. Parasitology 120, 513–526.

Bosseno, M.F., Telleria, J., Vargas, F., Yaksic, N., Noireau, F., Morin, A., Brenière, S.F., 1996. *Trypanosoma cruzi*: study of the distribution of two widespread clonal genotypes in Bolivian *Triatoma infestans* vectors shows a high frequency of mixed infections. Exp Parasitol. 83, 275–282.

Brenière, S.F., Llanos, B., Tibayrenc, M., Desjeux, P., 1985. Isoenzymic studies and epidemiological data of *Trypanosoma cruzi* from Arequipa (Peru), Pacific side. Ann. Soc. Belge Med. Trop. 65 (Suppl. 1), 63–66.

Brenière, S.F., Carrasco, R., Revollo, S., Aparicio, G., Desjeux, P., Tibayrenc, M., 1989. Chagas' disease in Bolivia: clinical and epidemiological features and zymodeme variability of *Trypanosoma cruzi* strains isolated from patients. Am. J. Trop. Med. Hyg. 41, 521–529.

Brenière, S.F., Bosseno, M.F., Telleria, J., Carrasco, R., Vargas, F., Yaksic, N., Noireau, F., 1995. Field application of polymerase chain reaction diagnosis and strain typing of *Trypanosoma cruzi* in Bolivian triatomines. Am. J Trop. Med. Hyg. 53, 179–184

Brenière, S.F., Lopez, J., Vargas, F., Barnabé, C., 1997. Genetic variability and microdistribution of *Triatoma infestans* genotypes and *Trypanosoma cruzi* clones in Arequipa region (Peru). Mem Inst. Oswaldo Cruz 92, 401–408.

Brenière, S.F., Morochi, W., Bosseno, M.F., Ordonez, J., Gutierrez, T., Vargas, F., Yaksic, N., Noireau, F., 1998 *Trypanosoma cruzi* genotypes associated with domestic *Triatoma sordida* in Bolivia. Acta Trop. 71, 269–283.

Breniere, S.F., Bosseno, M.F., Noireau, F., Yacsik, N., Liegeard, P., Aznar, C., Hontebeyrie, M., 2002. Integrate study of a Bolivian population infected by *Trypanosoma cruzi*, the agent of Chagas disease Mem. Inst. Oswaldo Cruz 97, 289–295.

Brenière, S.F., Barnabé, C., Bosseno, M.F., Tibayrenc, M., 2003. Impact of number of isoenzyme loci on the robustness of intraspecific phylogenies using multilocus enzyme electrophoresis: consequences for typing of *Trypanosoma cruzi*. Parasitology 127, 273–281.

Brisse, S., Barnabé, C., Tibayrenc, M., 2000. Identification of six *Trypanosoma cruzi* phylogenetic lineages by random amplified polymorphic DNA and multilocus enzyme electrophoresis. Int. J. Parasitol. 30, 35–44.

Brisse, S., Henriksson, J., Barnabé, C., Douzery, E.J., Berkvens, D., Serrano, M., De Carvalho, M.R., Buck, G.A., Dujardin, J.C., Tibayrenc, M., 2003. Evidence for genetic exchange and hybridization in *Trypanosoma cruzi* based on nucleotide sequences and molecular karyotype. Infect. Genet. Evol. 2, 173–183.

Broutin, H., Tarrieu, F., Tibayrenc, M., Oury, B., Barnabé, C., 2006. Phylogenetic analysis of the glucose-6-phosphate isomerase gene in *Trypanosoma cruzi*. Exp. Parasitol 113, 1–7.

Cavalli-Sforza, L.L., Edwards, A.W., 1967. Phylogenetic analysis. Models and estimation procedures. Am. J. Hum. Genet. 19, 233–257.

Cortez, M.R., Monteiro, F.A., Noireau, F., 2010. New insights on the spread of Triatoma infestans from Bolivia–implications for Chagas disease emergence in the southern cone. Infect. Genet. Evol. 10, 350–353.

De Meeus, T., Lehmann, L., Balloux, F., 2006. Molecular epidemiology of clonal diploids: a quick overview and a short DIY (do it yourself) notice. Infect. Genet. Evol. 6, 163–170.

De Meeus, T., Guegan, J.F., Teriokhin, A.T., 2009. MultiTest V, 1.2, a program to binomially combine independent tests and performance comparison with other related methods on proportional data BMC Bioinformatics 10, 443.

Falla, A., Herrera, C., Fajardo, A., Montilla, M., Vallejo, G.A., Guhl, F., 2009. Haplotype identification within *Trypanosoma cruzi* I in Colombian isolates from several reservoirs, vectors and humans. Acta Trop. 110, 15–21.

Flores-Chavez, M., Bosseno, M.F., Bastrenta, B., Dalenz, J.L., Hontebeyrie, M., Revollo, S., Brenière, S.F., 2006 Polymerase chain reaction detection and serologic follow-up after treatment with benznidazole in Bolivian children infected with a natural mixture of *Trypanosoma cruzi* I and II. Am. J. Trop. Med. Hyg. 75, 497–501.

Gaunt, M.W., Yeo, M., Frame, I.A., Stothard, J.R., Carrasco, H.J., Taylor, M.C., Mena, S.S., Veazey, P., Miles, G.A., Acosta, N., De Arias, A.R., Miles, M.A., 2003. Mechanism of genetic exchange in American trypanosomes. Nature 421, 936–939.

Goudet, J., Raymond, M., de Meeus, T., Rousset, F., 1996. Testing differentiation in diploid populations. Genetics 144, 1933–1940.

Hellegren, H., 2000. Microsatellite mutations in the germline: implications for evolutionary inference. Trends Genet. 16, 551–558.

Herrera, C., Bargues, M.D., Fajardo, A., Montilla, M., Triana, O., Vallejo, G.A., Guhl, F., 2007. Identifying four *Trypanosoma cruzi* I isolate haplotypes from different geographic regions in Colombia. Infect. Genet. Evol 7, 535–539

Laurent, J.P., Barnabé, C., Quesney, V., Noel, S., Tibayrenc, M., 1997. Impact of clonal evolution on the biological diversity of *Trypanosoma cruzi*. Parasitology 114, 213–218.

Lent, H., Wygodzinsky, P., 1979. Revision of the Triatominae (Hemiptera, Reduviidae), and their significance as vectors of Chagas' disease. Bull. Am. Mus. Nat. Hist. 163, 123–520.

Lewicka, K., Brénière-Campana, S.F., Barnabé, C., Dedet, J.P., Tibayrenc, M., 1995. An isoenzyme survey of *Trypanosoma cruzi* genetic variability in sylvatic cycles from French Guiana. Exp. Parasitol. 81, 20–28.

Llewellyn, M.S., Lewis, M.D., Acosta, N., Yeo, M., Carrasco, H.J., Segovia, M., Vargas, J., Torrico, F., Miles, M.A., Gaunt, M.W., 2009a. *Trypanosoma cruzi* IIc: phylogenetic and phylogeographic insights from sequence and microsatellite analysis and potential impact on emergent Chagas disease. PLoS Negl. Trop. Dis. 3, e510.

Llewellyn, M.S., Miles, M.A., Carrasco, H.J., Lewis, M.D., Yeo, M., Vargas, J., Torrico, F., Diosque, P., Valente, V., Valente, S.A., Gaunt, M.W., 2009b. Genome-scale multilocus microsatellite typing of *Trypanosoma cruzi* discrete typing unit I reveals phylogeographic structure and specific genotypes linked to human infection PLoS Pathog. 5, e1000410.

Macedo, A.M., Pimenta, J.R., Aguiar, R.S., Melo, A.I., Chiari, E., Zingales, B., Pena, S.D., Oliveira, R.P., 2001. Usefulness of microsatellite typing in population genetic studies of *Trypanosoma cruzi*. Mem. Inst. Oswaldo Cruz 96, 407–413.

Macedo, A.M., Machado, C.R., Oliveira, R.P., Pena, S.D., 2004. *Trypanosoma cruzi*: genetic structure of populations and relevance of genetic variability to the pathogenesis of chagas disease. Mem. Inst. Oswaldo Cruz 99, 1–12.

Miles, M.A., Toye, P.J., Oswald, S.C., Godfrey, D.G., 1977. The identification by isoenzyme patterns of two distinct strain-groups of *Trypanosoma cruzi*, circulating independently in a rural area of Brazil. Trans. Royal Soc. Trop. Med. Hyg. 71, 217–225.

Nei, M., Chesser, R.K., 1983. Estimation of fixation indices and gene diversities. Ann. Hum. Genet. 47, 253–259.

Ocaña-Mayorga, S., Llewellyn, M.S., Costales, J.A., Miles, M.A., Grijalva, M.J., 2010. Sex, subdivision, and domestic dispersal of *Trypanosoma cruzi* lineage I in Southern Ecuador. PLoS Negl. Trop. Dis. 4, e915.

O'Connor, O., Bosseno, M.F., Barnabé, C., Douzery, E.J., Brenière, S.F., 2007. Genetic clustering of *Trypanosoma cruzi* I lineage evidenced by intergenic miniexon gene sequencing. Infect. Genet. Evol 7, 587–593.

Oliveira, R.P., Broude, N.E., Macedo, A.M., Cantor, C.R., Smith, C.L., Pena, S.D., 1998. Probing the genetic population structure of *Trypanosoma cruzi* with polymorphic microsatellites. Proc. Natl. Acad. Sci. USA 95, 3776–3780.

R-Development-core-team (2011). R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org, ISBN 3-900051-07-0.

Revollo, S., Oury, B., Laurent, J.P., Barnabé, C., Quesney, V., Carriere, V., Noel, S., Tibayrenc, M., 1998. *Trypanosoma cruzi*: impact of clonal evolution of the parasite on its biological and medical properties Exp. Parasitol. 89, 30–39.

Schmunis, G.A., 1999. Prevention of transfusional *Trypanosoma cruzi* infection in Latin America. Mem. Inst. Oswaldo Cruz 94, 93–101.

Schofield, C.J., 1988. Parasitology Today readership survey. Parasitology Today (personal ed 4), pp. 153–155.

Simo, G., Njiokou, F., Tume, C., Lueong, S., De Meeûs, T., Cuny, G., Asonganyi, T., 2010. Population genetic structure of Central African *Trypanosoma brucei gambiense* isolates using microsatellite DNA markers. Infect. Genet. Evol. 10, 68–76.

Tibayrenc, M., Ward, P., Moya, A., Ayala, F.J., 1986. Natural populations of *Trypanosoma cruzi*, the agent of Chagas disease, have a complex multiclonal structure. Proc. Natl Acad. Sci. USA 83, 115–119.

Tibayrenc, M., Ayala, F.J., 1988. Isozyme variability in *Trypanosoma cruzi*, the agent of Chagas' disease: genetical, taxonomical, and epidemiological significance. Evolution 42, 277–292.

Tibayrenc, M., Kjellberg, F., Ayala, F.J., 1990. A clonal theory of parasitic protozoa: the population structures of *Entamoeba*, *Giardia*, *Leishmania*, *Naegleria*, *Plasmodium*, *Trichomonas*, and *Trypanosoma* and their medical and taxonomical consequences. Proc Natl Acad Sci USA 87, 2414–2418.

Tibayrenc, M., Kjellberg, F., Arnaud, J., Oury, B., Brénière, S.F., Darde, M.L., Ayala, F.J., 1991. Are eukaryotic microorganisms clonal or sexual? A population genetics vantage. Proc. Natl. Acad. Sci. USA 88, 5129–5133.

Tibayrenc, M., Neubauer, K., Barnabé, C., Guerrini, F., Skarecky, D., Ayala, F.J., 1993. Genetic characterization of six parasitic protozoa: parity between random-primer DNA typing and multilocus enzyme electrophoresis. Proc. Natl. Acad. Sci. USA 90, 1335–1339.

Tibayrenc, M., 2003. Genetic subdivisions within *Trypanosoma cruzi* (Discrete Typing Units) and their relevance for molecular epidemiology and experimental evolution. Kinetoplastid Biol. Dis. 2, 12.

Toledo, M.J., de Lana, M., Carneiro, C.M., Bahia, M.T., Machado-Coelho, G.L., Veloso, V.M., Barnabé, C., Tibayrenc, M., Tafuri, W.L., 2002. Impact of *Trypanosoma cruzi* clonal evolution on its biological properties in mice. Exp. Parasitol. 100, 161–172.

Venegas, J., Conoepan, W., Pichuantes, S., Miranda, S., Jercic, M.I., Gajardo, M., Sanchez, G., 2009. Phylogenetic analysis of microsatellite markers further supports the two hybridization events hypothesis as the origin of the *Trypanosoma cruzi* lineages. Parasitol Res. 105, 191–199.

Weir, B.S., Cockerham, C.C., 1984. Estimating F-Statistics for the analysis of population-structure. Evolution 38, 1358–1370.

Westenberger, S.J., Barnabé, C., Campbell, D.A., Sturm, N.R., 2005. Two hybridization events define the population structure of *Trypanosoma cruzi*. Genetics 171, 527–543

Wright, S., 1965. The interpretation of population structure by F-statistics with special regard to systems of mating. Evolution 19, 395–420.

Zhang, Q., Tibayrenc, M., Ayala, F.J., 1988. Linkage disequilibrium in natural populations of *Trypanosoma cruzi* (flagellate), the agent of Chagas' disease. J Protozool. 35, 81–85.

Zingales, B., Andrade, S.G., Briones, M.R., Campbell, D.A., Chiari, E., Fernandes, O., Guhl, F., Lages-Silva, E., Macedo, A.M., Machado, C.R., Miles, M.A., Romanha, A.J., Sturm, N.R., Tibayrenc, M., Schijman, A.G., 2009. A new consensus for *Trypanosoma cruzi* intraspecific nomenclature: second revision meeting recommends TcI to TcVI. Mem. Inst. Oswaldo Cruz 104, 1051–1054.

Infection, Genetics and Evolution 11 (2011) 1045–1057

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

New insights on the Chagas disease main vector *Triatoma infestans* (Reduviidae, Triatominae) brought by the genetic analysis of Bolivian sylvatic populations

Etienne Waleckx[a,b,*], Renata Salas[a,b], Nerida Huamán[a], Rosio Buitrago[a,b], Marie-France Bosseno[a,b], Claudia Aliaga[a,b], Christian Barnabé[a,b], Roberto Rodriguez[c], Faustine Zoveda[a,b], Marcelo Monje[a], Marianne Baune[a,b], Sergio Quisberth[a,b,d], Erick Villena[e], Pierre Kengne[a], François Noireau[a,f], Simone Frédérique Brenière[a]

[a] MIVEGEC (Maladies Infectieuses et Vecteurs: Ecologie, Génétique, Evolution et Contrôle, IRD 224 – CNRS 5290 – Université Montpellier 1 – Université Montpellier 2), Institut de Recherche pour le Développement (IRD), 911 Avenue Agropolis, BP 64501, 34394 Montpellier, France
[b] Instituto Nacional de Laboratorios de Salud (INLASA), Laboratorio de Entomología Médica, Rafael Zubieta #1889, Miraflores, Casilla M-10019, La Paz, Bolivia
[c] Escuela Técnica de Salud, Avenida Aniceto Arce #440, Cochabamba, Bolivia
[d] Universidad Mayor de San Andrés, Facultad de Ciencias Farmacéuticas y Bioquímicas, Avenida Saavedra #2224, Miraflores, La Paz, Bolivia
[e] Programa Regional de Chagas Tupiza, Ministerio De Salud y Deportes, Hospital Eduardo Eguia, Calle Bení, Tupiza, Bolivia
[f] IIBISMED, Facultad de Medicina, Universidad Mayor de San Simon, Avenida Aniceto Arce #371, Cochabamba, Bolivia

ARTICLE INFO

Article history:
Received 26 October 2010
Received in revised form 10 March 2011
Accepted 26 March 2011
Available online 2 April 2011

Keywords:
Triatoma infestans
Sylvatic
Cytochrome b
Internal transcribed spacer
Population genetics
Phylogeography

ABSTRACT

Triatoma infestans is the main and most widespread vector of Chagas disease in South America. For the first time, a large sample of sylvatic populations of *T. infestans* was analyzed by ITS-2 and mtCytB sequencing. ITS-2 showed a low level of polymorphism but revealed a dichotomy between the Andean and non-Andean sylvatic populations. On the contrary, mtCytB sequences showed a high polymorphism (19 haplotypes determined by 35 variable sites) revealing a strong structuring between most of the sylvatic populations and possible ancient isolation and bottleneck in the Northern Andes. The dichotomy Andean vs. non-Andean populations was not observed with this marker. Moreover, mtCytB haplotype genealogies showed that the non-Andean haplotypes would have derived from the Andean ones, supporting somewhat an Andean origin of the species. Nevertheless, a non-Andean origin could not be discarded because a remarkable genetic diversity was found in the non-Andean sample. The comparison of the sylvatic haplotypes with the domestic ones from GenBank suggested multiple events of *T. infestans* domestication in Andean and non-Andean areas, instead of a major and unique domestication event in the Bolivian Andes, as previously proposed.

1. Introduction

Triatoma infestans (Reduviidae, Triatominae) is the main and most widespread vector of *Trypanosoma cruzi*, the causative agent of Chagas disease, in South America. This species is, among the Triatominae, the best adapted to the domestic environment, living principally in dwellings and peridomestic structures. It is consequently the main target of large-scale campaigns of vector control based on insecticide spraying.

In the mid-1980s, the vector distribution included vast regions of Argentina, Bolivia, Brazil, Chile, Paraguay, Southern Peru, and Uruguay, while current estimates show that the distribution has been markedly reduced, reflecting the success of the control interventions (Schofield et al., 2006). Nowadays, domestic *T. infestans* mostly persists in the Andean valleys of Bolivia and in the Gran Chaco ecoregion (Cortez et al., 2010).When control efforts started, the expected success in the interruption of Chagas disease transmission relied on the assumption that *T. infestans* was almost exclusively domestic. Indeed, at that time, only a few sylvatic populations had been reported in the Bolivian Andean valleys of Cochabamba (Torrico, 1946; Dujardin et al., 1987). Mainly on this base, the Bolivian Andean valleys are traditionally believed to represent the center of origin and dispersal of *T. infestans* throughout South America. The species would have been introduced to the domestic environment in these valleys and a recent expansion by passive transport associated with human migrations would have occurred (Usinger et al., 1966; Schofield, 1988; Cortez et al., 2010). More recent findings of sylvatic populations of *T. infestans* in different Andean valleys in Bolivia (Bermudez et al.,

* Corresponding author at: Representación IRD en Bolivia, Avenida Hernando Siles #5290, esquina Calle 7 de Obrajes, CP 9214 – 00095, La Paz, Bolivia. Tel.: +591 2 278 29 69; fax: +591 2 278 29 44.
E-mail addresses: etienne.waleckx@ird.fr, etienne.waleckx@gmail.com, etiennewalex@yahoo.fr (E. Waleckx).

1567-1348/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.meegid.2011.03.020

1993; Noireau et al., 2005; Cortez et al., 2006, 2007; Buitrago et al., 2010) could be viewed as additional data supporting the traditional hypothesis on the dispersal model. However, the discovery of a sylvatic focus of *T. infestans* in the Bolivian Chaco in 1997, challenged for the first time the traditional view that the Andean mesothermic valleys in Bolivia were the area of origin of *T. infestans* (Noireau et al., 1997). In this sense, Carcavallo et al. (2000) suggested that *T. infestans* populations from the subtropical Chaco forest (South of Bolivia, Northern Paraguay and Argentina) may be the most ancient ones. Indeed, the Andean hypothesis does not fit with the absence in the Andes of the closely related species *Triatoma platensis* and *Triatoma delpontei*, which are restricted to the Chaco forest. These hypotheses about the geographic origin of the species (Andes or Chaco), the domestication process and dispersal model have also been discussed in works based on different genetic tools: multi-locus enzyme electrophoresis (Dujardin et al., 1998), cytogenetics (Panzera et al., 2004; Bargues et al., 2006), nuclear ribosomal DNA sequencing (Bargues et al., 2006), and mitochondrial DNA sequencing (Giordano et al., 2005; Piccinali et al., 2009). All these works are almost in agreement with the Andean origin of the species. However, the samples analyzed were, in all cases, constituted by *T. infestans* specimens mainly collected in human dwellings (domestic and peridomestic bugs), and different phenomena such as passive transport by humans and insecticide spraying are part of the recent evolutionary history of domestic populations, which can be different from sylvatic population history. Consequently, ancient evolution of the species would be best elucidated by analyzing sylvatic populations *a priori* native of the collecting sites. Recently, new sylvatic foci of *T. infestans* have been detected out of Bolivia, in Chile and in the Argentinean Chaco (Bacigalupo et al., 2006, 2010; Ceballos et al., 2009). This indicates that sylvatic populations of *T. infestans* have a wider geographic distribution than previously assumed and that the questions about *T. infestans* origin, domestication process and dispersal routes need to be further investigated by analyzing domestic and sylvatic populations together.

In the current work, for the first time, a large sample of sylvatic *T. infestans* collected throughout Bolivia, from the Andean valleys to the Gran Chaco region, was analyzed by ITS-2 and mtCytB sequencing. Population genetics, phylogenetic analyzes, and a comparison with GenBank sequences obtained from domestic bugs allowed discussing the different hypotheses about the origin, domestication and migratory routes of the species. This work also provides an important sequence database of sylvatic *T. infestans* for further studies.

2. Materials and methods

2.1. Triatomine bugs

A total of 223 *T. infestans* specimens collected by using mice-baited adhesive traps (Noireau et al., 1999) in 20 sylvatic sites from different areas of Bolivia between 2008 and 2009 were included in the study (Fig. 1 and Table 1). Additionally, two reared *T. delpontei* specimens originating from the Bolivian Chaco and obtained from the "Escuela Técnica de Salud" in Cochabamba, Bolivia, and 2 sylvatic *Triatoma sordida* specimens (captured in sampling sites 1 and 9, see Fig. 1) were used as outgroups for phylogenetic studies.

Note that an important phenotypic variability was observed among the sample of sylvatic *T. infestans*. Specimens collected in Tita had a dark phenotype, a chromatic variant previously described in this site and recently in the Argentinean Chaco (Noireau et al., 1997; Ceballos et al., 2009); those collected in Mataral (sampling site 15) had other distinct morphologic characteristics previously described (Cortez et al., 2007). All other specimens had a "standard" phenotype.

2.2. DNA extraction and quantification

Adult and nymph legs (or entire insect for first instars nymphs) were used for DNA extraction, using the CTAB–chloroform method proposed by Edwards (1998), with slight modifications. Quantification and purity determination of DNAs were performed by measuring the absorbance at 260 and 280 nm, using a Biomate 3 spectrophotometer (Thermo Electron Corporation, Madison, WI, USA).

2.3. DNA amplification, sequencing, and alignment

A nuclear DNA fragment of approximately 900 bp containing the ITS-2 sequence was PCR-amplified using primers previously described by Marcilla et al. (2001). Primers CytobtF (5′-GGA-CAA-ATA-TCA-TGA-GGA-GCA-ACA-G-3′) and CytobtR (5′-ATT-ACT-CCT-CCT-AGC-TTA-TTA-GGA-ATT-G-3′) were used to amplify a fragment of approximately 400 bp of the mtCytB gene. These primers were slightly different from those previously described by

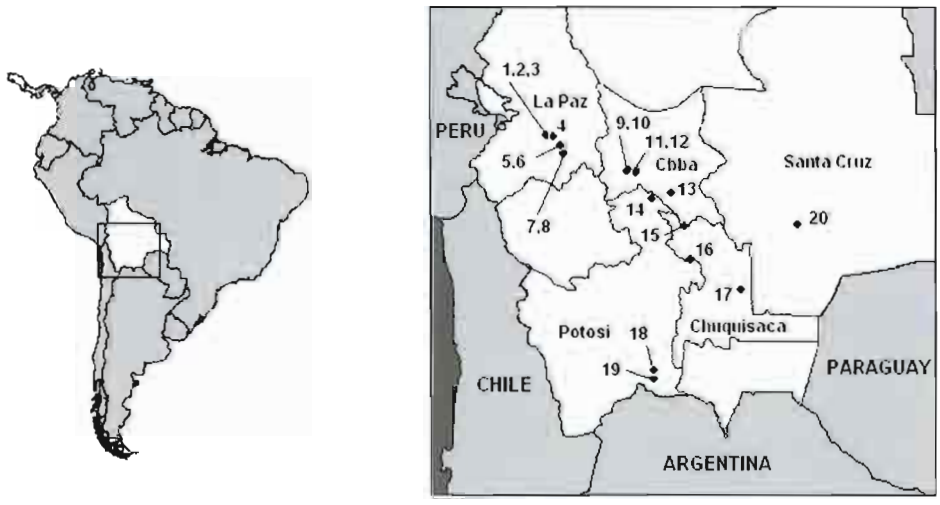


Fig. 1. Sampling sites in Bolivia of the sylvatic populations of *Triatoma infestans* (see additional information in Table 1). Bolivian department names are indicated. Cbba = department of Cochabamba.

Table 1
Bolivian sylvatic populations of *Triatoma infestans* studied, including geographic origins and sample sizes.

Population name	Sampling site (population number)	Geographic origin								Number of bugs used for mtCytB and ITS-2 sequencing
		Department	Province	Municipality	Nearest village	Latitude (S)	Longitude (W)	Altitude (m)	Bolivian area	
MEC1	1	La Paz	Murillo	Mecapaca	Aucani	16° 41′ 21.0″	68° 00′ 40.9″	2679	Northern Andean	8
MEC2	2	La Paz	Murillo	Mecapaca	El Palomar	16° 42′ 08 0″	68° 00′ 16 3″	2821	Northern Andean	10
SMEC[b]	3	La Paz	Murillo	Mecapaca	Huayhuasi	16° 42′ 42.9″	67° 59′ 25 3″	2732	Northern Andean	11
PAL	4	La Paz	Murillo	Palca	Tahuapalca	16° 43′ 11.1″	67° 52′ 26.5″	2380	Northern Andean	12
SAP	5	La Paz	Loayza	Sapahaqui	Khola	16° 53′ 12.2″	67° 42′ 43 1″	2095	Northern Andean	10
LUR1	6	La Paz	Loayza	Luribay	Lacayani	16° 55′ 48.8″	67° 41′ 32 9″	2182	Northern Andean	10
CAC003	7	La Paz	Loayza	Luribay	Palca	17° 00′ 30.1″	67° 39′ 25.2″	2356	Northern Andean	25
LUR2	8	La Paz	Loayza	Luribay	Luribay	17° 03′ 55 6″	67° 39′ 51.4″	2619	Northern Andean	10
QUI	9	Cochabamba	Quillacollo	Quillacollo 1ra seccion	Cotapachi	17° 25′ 28.9″	66° 15′ 53.0″	2689	Central Andean	10
AIQ	10	Cochabamba	Quillacollo	Sipe Sipe 2da seccion	Amiraya	17° 27′ 45.5″	66° 18′ 51 0″	2543	Central Andean	10
COC	11	Cochabamba	Cercado	Cochabamba	Alto Cara Cara	17° 28′ 37 5″	66° 08′ 16.14″	2710	Central Andean	30
20OCT	12	Cochabamba	Cercado	Cochabamba	20 de Octubre	17° 29′ 03 4″	66° 06′ 44.5″	2596	Central Andean	25
MIZ[a]	13	Cochabamba	Mizque	Mizque 1ra seccion	Tabacal	17° 56′ 01.0″	65° 23′ 06.5″	2182	Central Andean	2
TOR	14	Potosi	Charcas	Toro Toro	Julo Grande	18° 01′ 50.9″	65° 47′ 18.7″	1968	Central Andean	10
MAT	15	Cochabamba	Campero	Aiquile	Mataral	18° 36′ 09.0″	65° 07′ 07.0″	1788	Central Andean	10
JAT[a]	16	Chuquisaca	Yamparaez	Yamparaez	Jatun Cka Cka	19° 17′ 41.8″	65° 00′ 09 8″	2281	Central Andean	1
CER[a]	17	Chuquisaca	Hernando Siles	Monteagudo	Cerrillos	19° 55′ 39 2″	63° 54′ 08.8″	1039	Intermediate[c]	1
VIS	18	Potosi	Sur Chichas	Tupiza	Viscachani	21° 37′ 16 8″	65° 48′ 46 0″	2963	Southern Andean	10
TAL	19	Potosi	Sur Chichas	Tupiza	Talina	21° 44′ 51 0″	65° 49′ 26″	3080	Southern Andean	10
Tita	20	Santa Cruz	Cordillera	Charagua	Tita	18° 34′ 31 0″	62° 40′ 05.0″	300	Gran Chaco (non-Andean)	8
Total										223

[a] Not included in population genetic analyzes because of the low sample size
[b] No ITS-2 sequencing performed.
[c] Between Andean and non-Andean areas.

Lyman et al., 1999. PCR amplifications were carried out in a Mastercycler Gradient machine (Eppendorf, Hamburg, Germany) in a volume of 30 μl containing 100 ng of genomic DNA, 12 pmol of each primer, and 15 μl of PCR MasterMix 2X (Promega, Madison, WI, USA). PCR conditions were: 94 °C for 3 min; 94 °C for 1 min, 52.2 °C (ITS-2) and 48.3 C (mtCytB) for 1 min, 72 °C for 1 min (30 cycles); 72 C for 7 min. Purification and direct sequencing of both strands of PCR products were performed by the company Macrogen in Seoul, Korea. Sequences of both strands were aligned using CLUSTAL W (Thompson et al., 1994) provided in BioEdit version 7.0.9.0 (Hall, 1999), and corrected in case of any discrepancy by analyzing the corresponding chromatograms. The Corrected sequences were then aligned and fragments of 388 bp for mtCytB and 454 or 456 bp for ITS-2 were used for further analyzes.

2.4. Data analyzes

The aligned DNA sequences were imported into MEGA4 version 4.0.2 (Tamura et al., 2007) to analyze the base composition and compute basic statistics. MEGA4 was as well used to calculate a matrix of genetic distances between the sylvatic *T. infestans* populations, expressed as the average number of nucleotide differences between pairs of populations, using the mtCytB sequences, and to build a Neighbor-Joining tree (Saitou and Nei, 1987) from this matrix.

DnaSP version 5.10.00 (Librado and Rozas, 2009) was used to calculate the following genetic diversity indices: Hd (Haplotype diversity, Nei, 1987), π (nucleotide diversity expressed as the average number of nucleotide differences per site between two sequences, Nei, 1987), k (average number of nucleotide differences

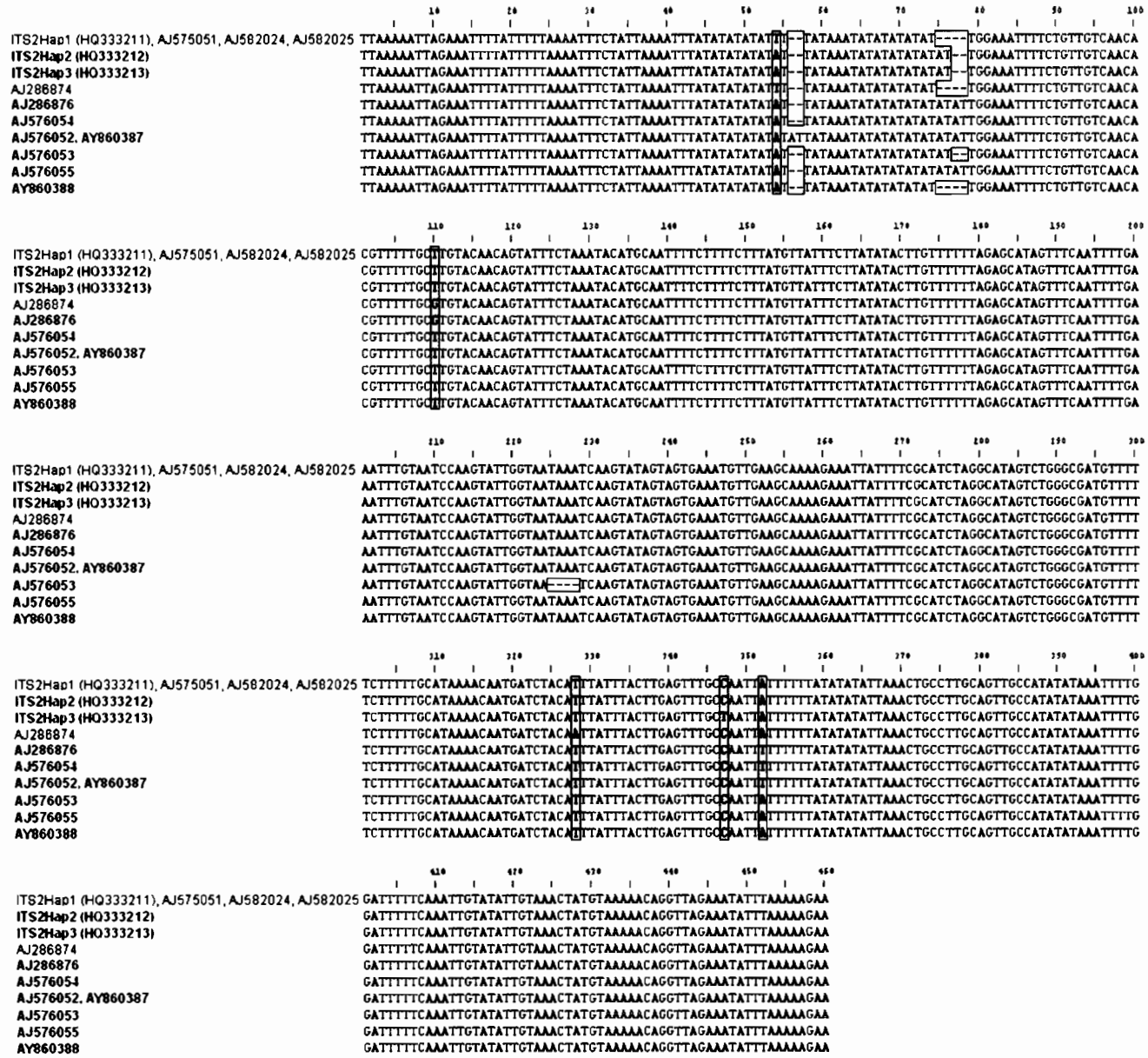


Fig. 2. Alignment of the ITS-2 haplotypes detected in the Bolivian sylvatic *Triatoma infestans*, with the ITS-2 haplotypes deposited in GenBank. Sequences names in bold: obtained from *T. infestans* specimens collected in non-Andean areas. Other sequences were obtained from specimens collected in Andean areas. In light gray: indels, in dark gray: variable nucleotide positions.

between sequences, Tajima, 1983) and S (number of segregating sites).

F_{ST} estimations, a measure of genetic differentiation between populations (Weir and Cockerham, 1984), and the analyzes of molecular variance (AMOVAs) were carried out using Arlequin version 3.11 (Excoffier et al., 2005). F_{ST} values were calculated using the average number of nucleotide differences between pairs of populations. The significance of all F_{ST} estimates was tested by haplotype permutation procedure (10,000 permutations) and corrected following Bonferroni's criteria for multiple comparisons. Arlequin software was as well used to perform Tajima's D (Tajima, 1989) and Fu's F_s (Fu, 1997) neutrality tests. The first neutrality test is based on mutations frequencies while the latter is based on haplotype frequencies.

Genetix version 4.05.2 (developed by Belkhir et al. and downloaded at http://www.univ-montp2.fr/~genetix/detsel/detsel.html) was used to perform Mantel tests and detect patterns of isolation by distance.

The phylogenetic analysis of the mtCytB haplotypes was firstly performed with a Maximum Likelihood (ML) approach using PAUP version 4.0b10 (Swofford, 2001). Maximum-likelihood parameters and the evolutionary model best fitting our dataset were determined using the Akaike Information Criterion (Akaike, 1974; Posada and Buckley, 2004) implemented in jModeltest vesion 0.1.1 (Posada, 2008). The best ML tree was found via a heuristic search with stepwise addition of taxa, using 100 random input orders and tree bisection–reconnection (TBR) branch swapping. Statistical support for clades in the phylogenetic tree was assessed by the bootstrap method (Felsenstein, 1985) with 1000 replications, using simple stepwise addition of taxa and TBR branch swapping options. The tree was rooted using the outgroup method.

To complete the phylogenetic analysis, a networking approach was performed, using Network version 4.5.1.6 (Copyright 2004–2010, Fluxus Technology Ltd.). The median-joining (MJ) network method was used, and all maximum parsimony (MP) networks were generated modifying the epsilon value according to the dataset. The post-processing option (MP calculation) was then used to simplify the network. This option removes unnecessary median vectors and links (cleaning procedure). A first network was built using only the mtCytB sequences obtained from the sylvatic bugs in this work, and a second network was built adding mtCytB haplotypes obtained from domestic bugs of different geographical origins and previously deposited in GenBank: 7 from the "Central Andean area" of Bolivia (Department of Chuquisaca, GenBank accession no. AY702018–AY702024), 3 from Brazil (non-Andean, GenBank accession no. AY062165, EF639038 and AF045721) and 41 recently deposited (GenBank accession no. GU807558–GU807598) including 7 from the department of Tarija (South of Bolivia), 3 from the province of Salta in Argentina (non-Andean), and 31 from different Brazilian States (non-Andean).

3. Results

3.1. ITS-2 and mtCytB sequence analysis

A total of 193 ITS-2 sequences of 454 or 456 bp were obtained from the sylvatic *T. infestans* specimens. Base composition was clearly biased to A+T content (mean 78.4%), as previously reported (Bargues et al., 2006). The sequence alignment revealed the existence of only 3 haplotypes named ITS2Hap1, ITS2Hap2 and ITS2Hap3 (GenBank accession no. HQ333211, HQ333212, and HQ333213). The sequence of ITS2Hap1 was identical to that found in 3 different ITS1-5.8S-ITS2 composite haplotypes previously reported for *T. infestans* (Fig. 2). ITS2Hap2 and 3 were detected for the first time. The main variation was observed in a microsatellite region constituted mostly of (AT) repetitions between positions 44 and 79. ITS2Hap2 and 3 differed together from ITS2Hap1 in 1 nucleotide (position 54) as well as in 1 insertion (AT) in the microsatellite region. ITS2Hap3 presented an additional substitution in position 347. In the full alignment, 5 nucleotide positions appeared variable, and 3 indel regions were observed (Fig. 2).

	Variable sites				
	111	1111222222	2223333333	33333	
Haplotypes	44579034	6777023778	8990133445	66677	Accession numbers
	1938186585	8147727065	8179239257	06928	
CytBHapA	CTTGGACCTT	ATCCTTCTCA	AATTATATCT	AAATA	HQ333215
CytBHapB	.CC.A.T.C.	...TC..C..	.GCC.....	.G..G	HQ333216
CytBHapC	....A.....	....	.G....G...	.....	HQ333217
CytBHapD	....A.....	..T.....A.	....	...C.	HQ333218
CytBHapE	....A.....	....	....	.....	HQ333219
CytBHapF	....	...T......	....	.....	HQ333220
CytBHapG	.CC.A.T.C.	...TCC.C..	.GCC.....	.G..G	HQ333221
CytBHapH	.CC.A.T.CC	...TC..C..	.GCC.....	.G..G	HQ333222
CytBHapI	.CCAA.T.C.	...TC..C..	.GCC.....	.G..G	HQ333223
CytBHapJ	....A.....	....	....G.....	.....	HQ333224
CytBHapK	....A.....	..T..CTCA.	.....C....	...C.	HQ333225
CytBHapL	.......T..	....	G.........	.....	HQ333226
CytBHapM	....A.....	.C........	.......C.C	.....	HQ333227
CytBHapN	T...AG....	..T.....A.	....	...C.	HQ333228
CytBHapO	....A.....	.C.......G	.......C..	.....	HQ333233
CytBHapP	....A.....	..T....CA.	.....C....	...C.	HQ333229
CytBhapQ	....A.....	.C........	....	.....	HQ333230
CytBHapR	....	G.........	....	..G..	HQ333231
CytBHapS	....A.....	.C.......G	.......CT.	G....	HQ333232

Fig. 3. Variable sites and haplotypes of mtCytB detected in the Bolivian sylvatic *Triatoma infestans*. Dot = identity with nucleotide in first haplotype

Concerning the mtCytB gene, a total of 211 fragments of 388 bp were obtained from the sylvatic *T. infestans* specimens. The overall nucleotide composition was 32.4% A, 24.3% C, 11.7% G, and 31.6% T, corresponding to a high A + T rich composition (64.0%), as expected for insect mitochondrial DNA (Simon et al., 1994). Higher variability was found in the mtCytB sequences than in the ITS-2 sequences (Fig. 3). Of the 388 nucleotide positions, 35 (9%) were variable, of which 7 were singletons (i.e., presented a substitution in a single specimen). As expected for a protein-coding gene, third codon positions were the most variable (91.4%), followed by first (8.6%). No second codon position variability was detected. All the substitutions were synonymous, and all were transitions, except 1 transversion (A–C). The sequence alignment revealed the existence of 19 different haplotypes that differed between them in a number of substitutions ranging from 1 to 18 (Fig. 3). Of them, 15 were detected for the first time, and 4 were identical to fragments of longer mtCytB sequences previously reported for *T. infestans*: CytBHapA (100% identity with GenBank accession no. AY702020), CytBHapC (100% identity with GenBank accession no. AY702018), CytBHapD (100% identity with GenBank accession no. EF639038 and AY062165), and CytBHapE (100% identity with GenBank accession no. AY702021).

3.2. Haplotype distribution and genetic diversity within and overall populations

Dealing with the ITS-2 marker, ITS2Hap1 was the unique haplotype found in all the Andean populations (monomorphic populations) while it was not found in the Gran Chaco (Pop 20, Tita). ITS2Hap2 and 3, were specifically identified in Tita, unique polymorphic population with this marker. The genetic diversity indices in Tita were: Hd = 0.476, π = 0.0010, k = 0.476, S = 1.

The distribution of the mtCytB haplotypes per population is shown in Table 2. Of the 19 recorded haplotypes, only four (CytBHapA, B, C and D) were shared between at least 2 populations. CytBHapA, the major haplotype, found in 48.3% of the specimens, was shared only among the populations located further north, from

Table 2
MtCytB variability, results of neutrality tests, and haplotype distribution in the Bolivian sylvatic populations of *T. infestans*.

	Populations																				Overall sample
	MEC1 (Pop 1)	MEC2 (Pop 2)	SMEC (Pop 3)	PAL (Pop 4)	SAP (Pop 5)	LUR1 (Pop 6)	CAC003 (Pop 7)	LUR2 (Pop 8)	QUI (Pop 9)	AIQ (Pop 10)	COC (Pop 11)	20OCT (Pop 12)	MIZ[a] (Pop 13)	TOR (Pop 14)	MAT (Pop 15)	JAT[a] (Pop 16)	CER[a] (Pop 17)	VIS (Pop 18)	TAL (Pop 19)	Tita (Pop 20)	
N	8	10	11	11	10	10	21	10	10	9	27	24	2	9	10	1	1	10	10	7	211
h	1	1	3	2	1	2	2	4	3	1	2	2	1	1	1	1	1	2	1	6	19
CytBHapA	8	10		8		9	14	1	7	9	3	22	2	9							102
CytBHapB			5	3	10	1	7	3													29
CytBHapC									1		24										25
CytBHapD																	1	1	10		12
CytBHapE															10						10
CytBHapF																		9			9
CytBHapG			5																		5
CytBHapH								3													3
CytBHapI								3													3
CytBHapJ																					2
CytBHapK									2											2	2
CytBHapL																					2
CytBHapM												2								1	1
CytBHapN																				1	1
CytBHapO																				1	1
CytBHapP																				1	1
CytBHapQ																	1				1
CytBHapR			1																		1
CytBHapS																				1	1
Hd	0.000	0.000	0.636	0.436	0.000	0.200	0.467	0 800	0.510	0.000	0 205	0.159	–	0 000	0.000	–	–	0.200	0.000	0 952	0.727
π	0 0000	0.0000	0.0084	0.0146	0.0000	0 0067	0.0156	0.0091	0.0032	0 0000	0.0016	0.0008	–	0.0000	0.0000	–	–	0 0026	0 0000	0.0177	0 0140
S	0	0	16	13	0	13	13	15	4	0	3	2	–	0	0	–	–	5	0	15	35
k	0 000	0.000	3.273	5 673	0 000	2.600	6.067	3 533	1 222	0.000	0.615	0.319	–	0.000	0 000	–	–	1.000	0.000	6 857	5.437
D	–	–	−1.787*	1.217	–	−1 976**	2.433	−1.537	−0.521	–	−0.508	−0.890	–	–	–	–	–	−1 741*	–	0 663	−0.246
F_s	–	–	3 630	8.678	–	4.861	12.440	1 975	0.875	–	2 008	0 725	–	–	–	–	–	2.197	–	−0 524	0.778

h, Number of haplotypes; *N*, number of sequences obtained (sample size); Hd, haplotype diversity (Nei, 1987); π, nucleotide diversity (average number of nucleotide differences per site between two sequences, Nei, 1987); *S*, number of segregating sites; *k*, average number of nucleotide differences between two sequences (Tajima, 1983); *D*, Tajima's neutrality test (Tajima, 1989); F_s, Fu's neutrality test (Fu, 1997).

* $p < 0.05$.

** $p < 0.01$.

[a] Not considered as "real" population because of the low sample size, but specimen(s) included for overall calculations

sampling sites 1–14 (Fig. 1). It was detected in 12 of the 14 populations captured between these two sites (Pop 1, 2, 4, and 6–14) and had a frequency of 59.3% in this area; in four populations it was the only haplotype found (Pop 1, 2, 10, and 14). CytBHapB, the second most frequent haplotype (13.7%), was only found in the Andean valleys of La Paz (Pop 3–8). CytBHapC (11.8%) was found in two populations close to the city of Cochabamba (Pop 9 and 11). CytBHapD (5.7%) was specifically found in the 3 populations located further south (Pop 17–19). All remaining haplotypes were detected exclusively in single populations (private haplotypes). The Andean populations contained up to two private haplotypes while the only population from the Gran Chaco (Pop 20, Tita, non-Andean area) exhibited six private haplotypes. It is worth noting that a group of divergent haplotypes, constituted by CytBHapB, G, H and I, was found among the sylvatic bugs. These haplotypes differed between them in 1 or 2 nucleotides, while they diverged from all others haplotypes by 12–18 nucleotide differences. They were only found in populations collected in the Andean valleys of La Paz, and occurred together with the major haplotype CytBHapA in most populations.

The overall and within population nucleotide and haplotype diversity indices found for mtCytB are summarized in Table 2. All the indices presented a very large range. For example, k ranged from 0.000 (monomorphic population) to 6.857. The monomorphic populations were seven and originated from distinct geographic regions. The most diversified population was Tita, the non-Andean one (higher values for k, π and Hd, Table 2). However, some Andean populations collected in the valleys of La Paz (Pop 3, 4, 7, and 8) presented also fairly high genetic diversity.

3.3. Neutrality tests

Two neutrality tests (Tajima's D test, and Fu's F_s test) were applied on each polymorphic population using the mtCytB sequences, in order to detect a possible deviation to the neutrality (Table 2). The D statistic obtained by Tajima's test was found negative and significant for 3 populations (Pop 3, 6 and 18), while the Fu's F_s test was found not significant for each population.

3.4. Population structure

According to above analyzes, the ITS-2 sequences, which exhibited low polymorphism, discriminated only the population from the Gran Chaco (Tita, Pop 20) from all others. Thus, only the mtCytB sequences were used to further explore the population structure.

A single-level AMOVA (without grouping populations) including 17 populations (those containing one or two specimens were not considered according to Table 1), showed highly significant differentiation among populations ($F_{ST} = 0.676$, $p < 10^{-5}$) that explained most of the total genetic variation (67.61%). The remaining variation (32.39%) was attributed to variation within populations.

Pairwise F_{ST} values were calculated between the 17 populations (Table 3). Of 136 values, 102 (75%) were significant, and 80 (59%) remained significant after Bonferroni's correction, indicating genetic differentiation between most of the populations. Genetic distances between the 17 populations can be visualized on the Neighbor-Joining tree presented in Fig. 4. Pop 3, 5 and 8, found in the Andean valleys of La Paz, constituted the group of populations the more distant from all others because they were composed of a majority of divergent haplotypes belonging to the group described above; an AMOVA performed grouping these 3 populations apart from all others confirmed this differentiation (% of variation among groups = 73.1%, $F_{CT} = 0.731$, $p = 0.002$). A second group of genetically related populations (Pop 1, 2, 6, 9, 10, 12, 14 and 18) was

Table 3
Pairwise F_{ST} values between the Bolivian sylvatic populations of *T. infestans*.

	MEC1 (Pop1)	MEC2 (Pop 2)	SMEC (Pop 3)	PAL (Pop 4)	SAP (Pop5)	LUR1 (Pop 6)	CAC003 (Pop 7)	LUR2 (Pop 8)	QUI (Pop 9)	AIQ (Pop 10)	COC (Pop 11)	200CT (Pop 12)	TOR (Pop 14)	MAT (Pop 15)	VIS (Pop 18)	TAL (Pop 19)	Tita (Pop 20)
MEC1 (Pop 1)	-																
MEC2 (Pop 2)	0.000	-															
SMEC (Pop3)	**0.848***	**0.862***	-														
PAL (Pop 4)	0.154	0 186	**0.531**	-													
SAP (Pop 5)	**1.000***	**1.000***	**0.088**	**0.688**	-												
LUR1 (Pop 6)	−0.024	0 000	**0.741***	−0 004	**0.889***	-											
CAC003 (Pop7)	0.190	0.213	**0.449**	−0.065	**0.567**	0.064	-										
LUR2 (Pop 8)	**0.841***	**0.856***	0 023	**0.510**	**0.070**	**0.728**	**0.429**	-									
QUI (Pop 9)	0 096	0.127	**0.815***	0.135	**0.953***	0.005	0.178	**0.806***	-								
AIQ (Popl0)	0.000	0 000	**0.856***	0.171	**1.000***	−0 011	0.203	**0.849**	0.112	-							
COC (Pop 11)	**0.828***	**0.837***	**0.881***	**0.526***	**0.963***	**0.647***	**0.456***	**0.878***	**0.672***	**0.832***	-						
200a (Pop 12)	−0 027	−0.010	**0.900***	**0.305**	**0.983***	0.073	**0.307**	**0.897***	**0.177**	−0.018	**0.832***	-					
TOR (Pop 14)	0.000	0 000	**0.856***	0.171	**1.000***	−0.011	0.203	**0.849***	0.112	0 000	**0.832***	−0 018	-				
MAT (Pop 15)	**1.000***	**1.000***	**0.853***	**0.276**	**1.000***	**0.381***	**0.260**	**0.846***	**0.444**	**1.000***	**0.774***	**0.813***	**1.000***	-			
VIS (Pop 18)	**0.583**	**0.615***	**0.817***	**0.214**	**0.959***	**0.250**	**0.224**	**0.808***	**0.427***	**0.600***	**0.807***	**0.619***	**0.600***	**0.762***	-		
TAL (Popl9)	**1.000***	**1.000***	**0.883***	**0.581***	**1.000***	**0.745***	**0.516***	**0.878***	**0.851***	**1.000***	**0.909***	**0.946***	**1.000***	**1.000***	**0.889***	-	
Tita (Pop 20)	**0.455***	**0.499***	**0.697***	**0.293**	**0.824***	**0.356***	**0.321**	**0.686***	**0.386***	**0.478***	**0.662***	**0.636***	**0.478***	**0.387***	**0.470***	**0.321***	-

In bold significant at the 5% level; in bold and *: significant at the 5% level after Bonferroni's correction ($p < 0.0003$)

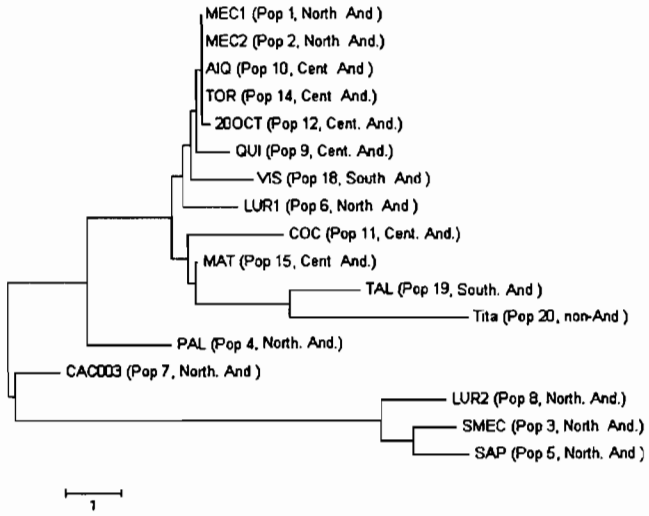


Fig. 4. Neighbor-Joining (NJ) tree representing the genetic distances between the Bolivian sylvatic populations of *T. infestans*. The genetic distances are expressed as the average number of nucleotide differences between pairwise populations, using the mtCytB haplotypes (388 bp). The scale bar indicates the length that equals 1 nucleotide difference. North. And. = Northern Andean area, Cent. And. = Central Andean area, South. And. = Southern Andean area, non-And. = non-Andean area.

observed; these populations presented mostly either the major haplotype CytBHapA, either a closely related haplotype (CytBHapF for Pop 18). Furthermore, note that in the overall sample, some populations were not significantly differentiated although they were geographically distant (e.g., Pop 1 and 14, $F_{ST} = 0.000$), while others populations geographically close were significantly differentiated (e.g., Pop 2 and 3, $F_{ST} = 0.862$, $p < 0.0003$).

To test for a genetic structure linked to the geographic origin of the populations, a two-level hierarchical AMOVA was applied grouping the populations according to 3 geographic origins: (a) "Northern Andean area" (Pop 1–8); (b) "Central Andean area (Pop 9–12, 14–15), and (c) "Southern Andean area" (Pop 18 and 19). For this analysis, Pop 20 was removed as it could not be assigned to one of these areas. As in previous analyzes, Pop 13, 16 and 17 were not considered because of the low sample sizes. Significant genetic differentiation among areas, that explained 25.46% of the total genetic variability, was observed ($F_{CT} = 0.255$, $p = 0.033$), but major part of the genetic variation (46.92%) was explained by a significant differentiation among populations within groups ($F_{SC} = 0.629$, $p < 10^{-5}$). A Mantel test showed that the observed genetic structure did not fit with a model of genetic differentiation by distance ($r = 0.025$, $p = 0.397$).

3.5. Phylogenetic analysis of the Bolivian sylvatic *T. infestans* mtCytB haplotypes

The ML model best fitting the mtCytB haplotype dataset was found to be HKY with a *ti/tv* ratio of 38.21 (kappa = 80.82), base frequencies for A, C, G, and T of 0.3166, 0.2480, 0.1269 and 0.3086, respectively, a proportion of invariable sites = 0, and an equal distribution of rates at variable sites. *T. delpontei*, species previously used as outgroup (Piccinali et al., 2009), was discarded because the number of mutations between the *T. delpontei* mtCytB sequences currently found (GenBank accession no. HQ333240 and HQ333241) and some *T. infestans* mtCytB haplotypes was lower than between some *T. infestans* mtCytB haplotypes. Consequently, *T. sordida* was selected as outgroup to build the ML tree, presented in Fig. 5. The mtCytB sequences obtained from the two sylvatic *T. sordida* used were deposited (GenBank accession no. HQ333242 and HQ333243).

Forty-nine mutations were observed between *T. infestans* and *T. sordida*. Within *T. infestans*, a cluster significantly supported (bootstrap value = 79%), grouped the divergent haplotypes found in the Andean valleys of La Paz (CytBHapB, G, H and I). Another cluster, with bootstrap value of 61%, grouped haplotype CytBHapQ, found in the "Central Andean area", with CytBHapM, S and O (Gran Chaco, non-Andean area). Finally, a cluster, not supported by high bootstrap value (55%), grouped all haplotypes apart from the four most basal haplotypes CytBHapD (found in the "Southern Andean

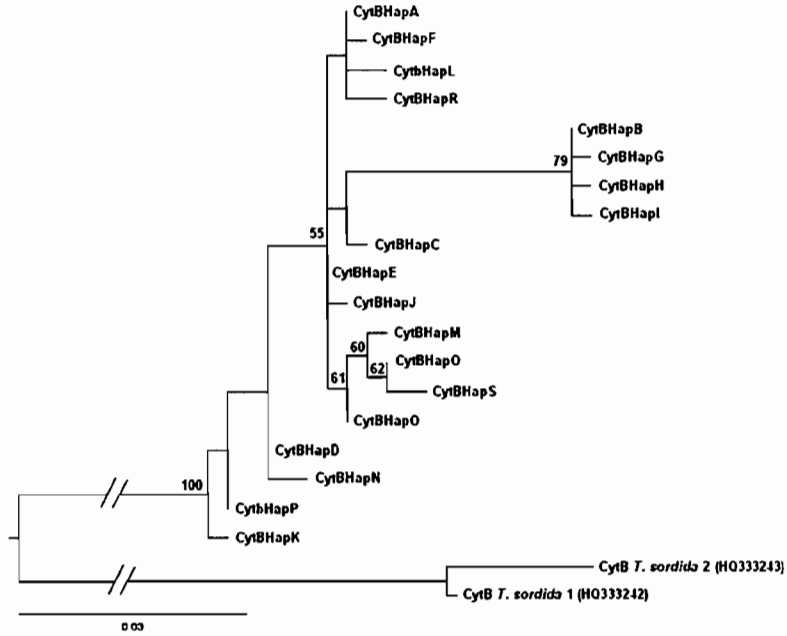


Fig. 5. Maximum Likelihood (ML) tree of the Bolivian sylvatic *Triatoma infestans* mtCytB haplotypes. The tree was built using HKY model with a transition/transversion ratio of 38.21, and rooted using 2 *T. sordida* specimens as outgroups. Root was midpointed. The scale bar indicates the number of substitutions per nucleotide position. Numbers above the branches are 50% or higher bootstrap values as percentages of 1000 replicates

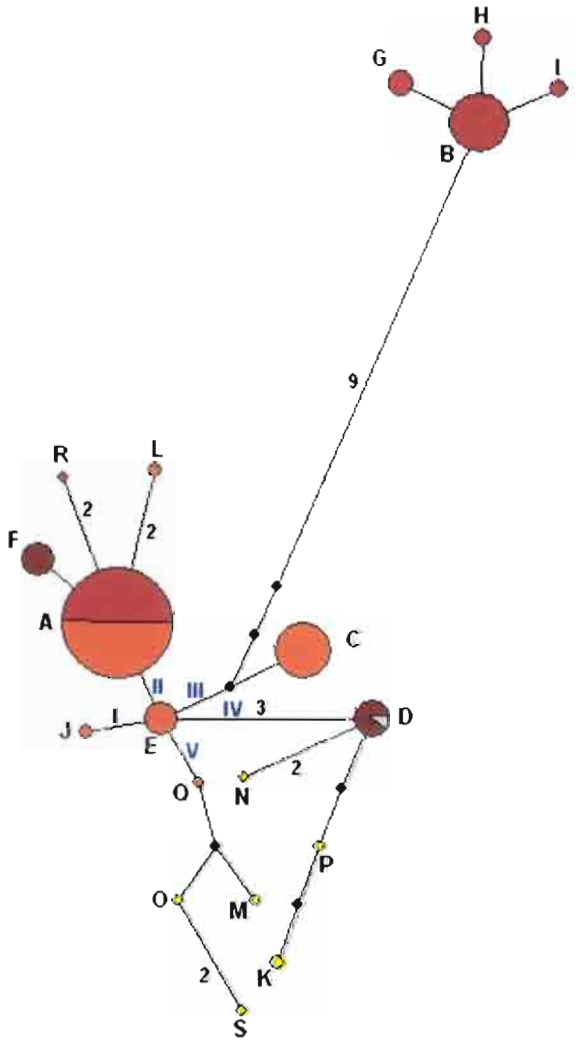


Fig. 6. Median-Joining (MJ) network for the Bolivian sylvatic *Triatoma infestans* mtCytB haplotypes. Nodes represent haplotypes and their area is proportional to the sample size. Lines between haplotypes represent mutational steps. More than one mutational step is indicated by black numbers. The haplotypes are indicated by letters: A, B, C, ..., and S = CytBhapA, CytbHapB, CytBHapC, ..., and CytBHapS, respectively. Branches connected to the central node (E) are numerated with blue Roman numerals. Node colors: Red = North Andean area; Orange = Central Andean area; Brown = Southern Andean area; Yellow = Gran Chaco (non-Andean); Gray = intermediate area between Andean and Gran Chaco areas. Small black-filled nodes represent hypothesized haplotypes not present in the sample (median-vectors). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

area" and the "Intermediate area" between Andean and non-Andean areas) and CytBHapN, P and K (Gran Chaco, non-Andean area).

The haplotype network, using the same dataset (Fig. 6), showed one principal internal node constituted by CytBHapE ("Central Andean area"). From this node, 5 branches of derived haplotypes were observed. Three of them (branches I–III) connected haplotypes found mostly in the "Northern and Central Andean areas" including the major one CytBHapA. The group of divergent haplotypes described above appeared to have derived from CytBHapE. The two other branches (IV and V) linked to the principal internal node the haplotype CytBHapQ ("Central Andean area"), and CytBHapD ("Southern Andean area" and "Intermediate area"), respectively. All the haplotypes identified in Tita (Pop 20, non-Andean area) appeared to have derived from these two last haplotypes.

An additional network analysis was done including the mtCytB haplotypes available in GenBank and obtained from domestic bugs of different geographical origins, as described in Section 2 (Fig. 7). The pattern of relationship between the sylvatic *T. infestans* mtCytB haplotypes kept unchanged. However, with the introduction of the domestic bug haplotypes, instead of one, two principal internal nodes were observed, constituted by CytBHapD and CytBHapE. CytBHapD was identical to two haplotypes from Brazil (GenBank accession no. AY062165 and EF639038), and CytBHapE was identical to a haplotype described in the Bolivian department of Chuquisaca (GenBank accession no. AY702021). All the haplotypes from Brazil and Argentina, one haplotype from Tarija, and part of the haplotypes identified in Tita (non-Andean area) were directly linked to the first node CytBHapD. To the second node CytBHapE were linked haplotypes exclusively found in Bolivia; all were identified in the Andean area except 3 haplotypes identified in Tita. Finally, between the two principal nodes, 4 other haplotypes identified in Tarija (South of Bolivia) were connected.

4. Discussion

This work provides clarification of the evolutionary history, population structure, and patterns of *T. infestans* dispersal. Its main originality is the genetic study of a large sample of sylvatic, therefore native *T. infestans* populations, discarding the distortion brought by introduced populations. Nuclear and mitochondrial markers were used to provide more reliable information. ITS-2 and mtCytB sequences had previously been used for intraspecific studies of *T. infestans*, but never together.

4.1. ITS-2 polymorphism within sylvatic *T. infestans*

The ITS-2 marker showed a low level of polymorphism within the current sample, limiting population genetics analyzes. However, some significant genetic features clearly appeared.

Firstly, the low polymorphism and the alignment of the 3 ITS-2 haplotypes currently obtained with those previously deposited in GenBank for *T. infestans* (see Fig. 2) allowed identifying the sylvatic specimens as *T. infestans*, despite of the phenetic variability observed and described in Section 2. Moreover, a genetic dichotomy between the Andean and non-Andean sylvatic populations was found. Indeed, all the specimens collected from north to south of the Andean area of Bolivia exhibited an unique ITS-2 haplotype. The two other haplotypes, identified only in the population from the Gran Chaco (Tita, Pop 20), had few differences compared to the Andean haplotype, but allowed distinguishing the non-Andean triatomines from the Andean ones. Previous cytogenetic analysis also revealed an obvious dichotomy between mainly domestic Andean populations from Bolivia and Peru, and non-Andean populations from Brazil, Uruguay, and Bolivian, Paraguayan and Argentinean Chaco, linked to significant differences of DNA content and C-heterochromatin banding (Panzera et al., 2004). This genetic differentiation between Andean and non-Andean populations had subsequently been confirmed by phylogenetic studies based on the nuclear markers ITS-1 and 2 (Bargues et al., 2006).

Finally, the data currently obtained combined with previous ones (Marcilla et al., 2001; Bargues et al., 2006; Martinez et al., 2006) revealed more polymorphism of ITS-2 in the non-Andean areas than in the Andean areas, in sylvatic as well as in domestic environments (see Fig. 2). Within the sylvatic populations, the monomorphy of this marker in all the Bolivian Andean area is surprising, and may suggest a more recent spreading of sylvatic populations throughout this area. Another hypothesis would be the occurrence of selection mechanisms maybe linked to altitude.

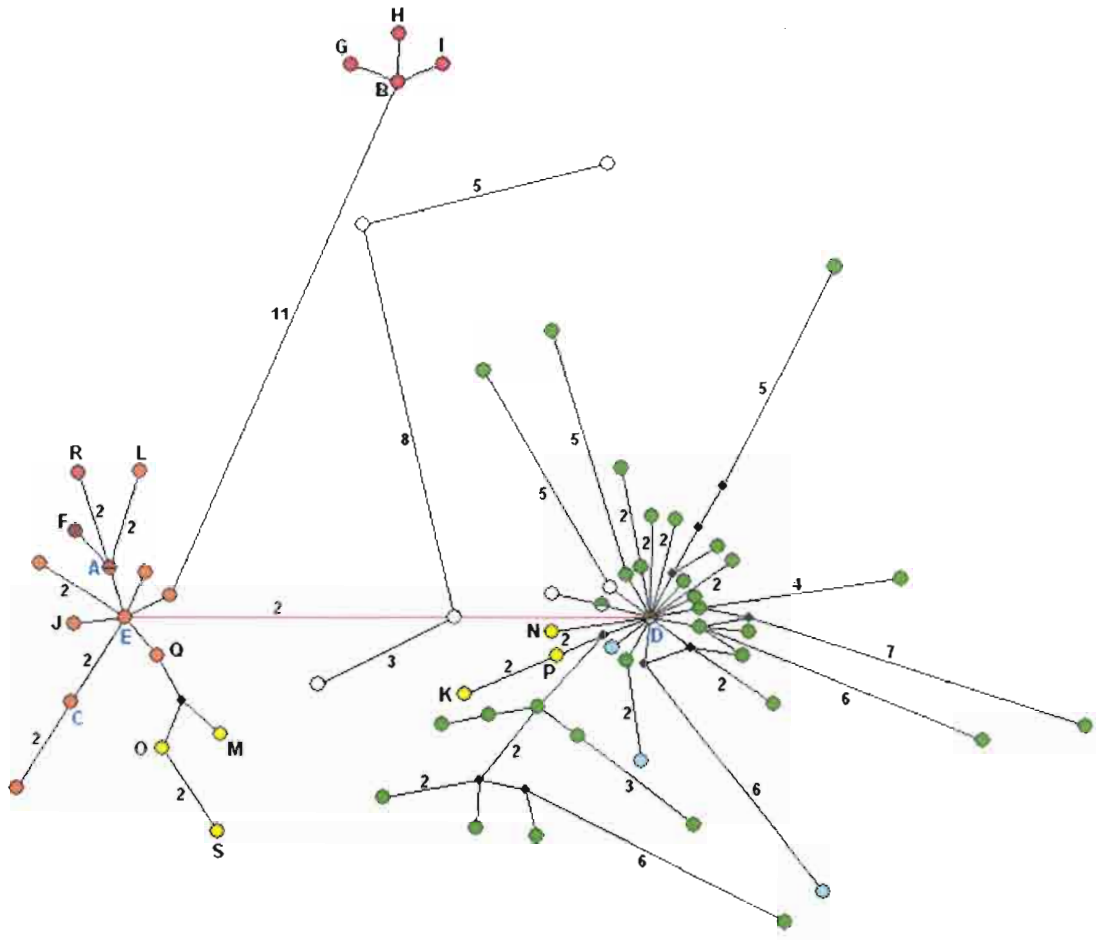


Fig. 7. Median-Joining (MJ) network for sylvatic and domestic *Triatoma infestans* mtCytB haplotypes. Nodes represent haplotypes. Lines between haplotypes represent mutational steps. More than one mutational step is indicated by numbers. Lines are proportional to mutational steps, except red lines, which have been voluntarily extended to enhance the view.The sylvatic *T. infestans* haplotypes are indicated by letters: A, B, C, . . ., and S = CytBhapA, CytbHapB, CytBHapC, . . ., and CytBHapS, respectively. Sylvatic haplotypes indicated by blue letters are as well found in domestic environment. Node colors: Red = Bolivian North Andean area; Orange = Bolivian Central Andean area; Brown = Bolivian Southern Andean area; Yellow = Bolivian Gran Chaco; Gray = Bolivian intermediate area between Andean and Gran Chaco areas; Blue = Argentina; Green = Brazil; White = Tarija. Small black-filled nodes represent hypothesized haplotypes not present in the sample (median-vectors). Haplotypes from Tarija were represented by a different color (white) because this Bolivian department contains Andean, Gran Chaco, and Intermediate areas but exact origin of haplotypes was not available. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Nevertheless, the latter hypothesis is unlikely since ITS-2 is known to be a neutral marker.

The correlation between geographic origins and genetic characteristics of the populations, whether sylvatic or domestic, suggests a separate evolution of Andean and non-Andean populations of *T. infestans*.

4.2. mtCytB polymorphism and genetic structure of sylvatic T. infestans

Among the sylvatic populations of *T. infestans*, the mtCytB marker showed a high polymorphism and appeared useful for further exploration of the population structure.

The preliminary observation of haplotype frequencies and their geographic distribution suggested a strong structuring of the sylvatic populations. The major haplotype (CytBHapA) appeared to have largely spread throughout the "Central and Northern Andean areas". The other haplotypes had generally a more restricted localization and most of them were private haplotypes. The single-level AMOVA analysis and the F_{ST} values confirmed the strong structuring of the populations. The dichotomy observed with the ITS-2 marker between the Andean and non-Andean populations was not observed with mtCytB, although the population from the Gran Chaco (Tita, pop 20) was differentiated from all others. Previous studies performed with mitochondrial markers agree with these results as an absence of dichotomy between Andean and the non-Andean haplotypes was as well observed (Monteiro et al., 1999; Giordano et al., 2005; Piccinali et al., 2009). Indeed, in these works, the haplotypes found in the non-Andean populations from Bolivia were more closely related with those found in the Bolivian Andean populations than with those found in the non-Andean populations from Argentina and Brazil.

In fact, in the current work, the highest genetic distance was found between a group of populations of the "Northern Andean area" (Pop 3, 5, and 8, see Fig. 4) and all other populations. This special group was mostly composed of divergent haplotypes, not sampled in other areas. Moreover, no intermediate haplotypes between these divergent haplotypes and their founder node (Fig. 6) were observed. The area where the divergent haplotypes were found corresponds to valleys belonging to the "Inter Andean Dry Forest" ecoregion, naturally highly fragmented and heterogeneous, with a high rate of endemic species and extreme climatic conditions (Ibisch and Merida, 2008). These particular characteristics could have favored both genetic isolation and population

bottlenecks and could explain the occurrence of the divergent haplotypes. Moreover, the co-occurrence, in these valleys, of the major haplotype CytBHapA with the divergent haplotypes might indicate a relatively recent secondary contact of different populations.

Genetic structuring was observed between the Northern, Central and Southern Andean areas (AMOVA). This may be explained by the fact that most haplotypes were not common to the different areas and by the high number of private haplotypes. However, a major genetic variation was observed within the areas, reflecting the occurrence of high genetic differentiation even between populations geographically close. Significant F_{ST} values observed between most of the populations, including geographically close ones, confirmed this observation, and reflected the absence of important historical gene flow between the sylvatic populations and the low dispersal capacity of the species. Consequently, the sylvatic populations, found in large areas, may be the result of an expansion process over a very long time.

Finally, the neutrality tests showed that most of the sylvatic populations were in mutation-drift equilibrium. This reveals that these are currently not submitted to selection pressures and are in demographic equilibrium, contrary to most of domestic populations where bottlenecks and population growths have been described after insecticidal spraying (Piccinali et al., 2009). Only for Pop 3, 6 and 18, the *D* statistic was found negative and significant. It can be the result of different phenomena, such as population growth, directional selection, purifying selection, or population admixture. Population growth was discarded because the Fu's F_s test, stronger than Tajima's test to detect population growth (Ramos-Onsins and Rozas, 2002), was found not significant for each population. For Pop 3 and 6, a recent secondary contact of different populations (admixture process), as suggested above, is probably the main reason of the observed departures. Indeed, Pop 6 was composed of 9 specimens exhibiting CytBHapA, and 1 specimen exhibiting CytBHapB (belonging to the group of divergent haplotypes), thus creating an excess of singletons. In the same way, Pop 3 was composed only of divergent haplotypes, except for 1 specimen.

4.3. Phylogenetic relationships of mtCytB haplotypes among the sylvatic T. infestans populations and putative geographic origin of the species

With the Maximum Likelihood approach performed to analyze the phylogenetic relationships between the mtCytB haplotypes, intraspecific interpretation remained very limited. Indeed, no significant bootstrap values were obtained, except for the group of divergent haplotypes (CytBHapB, G, H and I, see Fig. 5). Because of the low bootstrap values, probably reflecting the low divergence between conspecific individuals (Piccinali et al., 2009), and of the presence of zero length branches, most likely due to the persistence of ancestral haplotypes in the populations (Posada and Crandall, 2001), a networking approach was as well performed. Indeed, as explained by Posada and Crandall (2001), the relationships between genes at the intraspecific level are not hierarchical, and the networking approach can be more relevant for studies at this level. In network representations, nodes correspond to haplotypes. Internal nodes are supposed to be ancestral while terminal nodes are supposed to be recent.

The results obtained with this approach provided information worthy of discussion in light of the hypotheses proposed in the literature on the geographic origin of *T. infestans*. In the traditional hypothesis, the Bolivian Andean valleys are believed to represent the center of origin and dispersal of *T. infestans* throughout South America, but the recent discovery of sylvatic *T. infestans* in the Gran Chaco challenges this hypothesis.

The current networks suggested that the private haplotypes found in the population from the Gran Chaco (Tita, pop 20) were derived from CyBHapE ("Central Andean area") and D ("Southern Andean area" and "Intermediate area"). This result is somewhat in favor of an Andean origin of *T. infestans*. However, a non-Andean origin cannot be excluded, because the sample from the Gran Chaco was limited to a single population and consequently, the existence of these ancestral haplotypes in non-Andean areas cannot be discarded. In this sense, the fact that CytBHapD was as well found in the "Intermediate area" between Andean and non-Andean areas (Pop 17) shows that this haplotype has a wide distribution and could be found in the Gran Chaco. Moreover, the great genetic variability found in only one population from the Gran Chaco (ITS-2 and mtCytB) in comparison with that found in all the Bolivian Andean valleys suggest that the Gran Chaco region would be the one with the greatest genetic diversity. According to the evolutionary theory, higher genetic diversity is expected in the center of origin and dispersal of a species; this could be an additional argument for a non-Andean origin of *T. infestans*. Nevertheless, higher genetic diversity can also be the product of a higher historical effective population size. If sylvatic populations from the Gran Chaco are really derived from Andean ones, then biotic and environmental factors, such as a higher biodiversity in the Gran Chaco and a greater degradation of environment in the Bolivian Andes would explain the maintenance of a high diversity in the first area and its reduction in the second by selective and/or demographic processes.

Therefore, additional analyzes of sylvatic populations from the "Central and Southern Andean areas", as well as from the Gran Chaco, are needed to conclude about the origin of *T. infestans*. Recently, a new focus of sylvatic *T. infestans* has been reported in the Argentinean Chaco (Ceballos et al., 2009), and we are currently suspecting other foci in the Bolivian Chaco. Actually, it appears probable that the distribution of sylvatic *T. infestans* may not be restricted to small areas of the Gran Chaco, but may extend to all of this biogeographic region (Noireau, 2009).

4.4. Domestication and migratory routes of T. infestans

The second network (Fig. 7) allowed extending the analysis with domestic *T. infestans* mtCytB haplotypes identified in Andean and non-Andean areas and to analyze their relationships with the sylvatic ones. The unchanged pattern of the sylvatic haplotype relationships compared to the first network (Fig. 6) and the low number of alternative connections reflected its relevance. This network provided interesting information about the domestication process and migratory routes of *T. infestans*.

The traditional hypothesis puts forward an initial vector-domiciliation process in the Bolivian Andean valleys, followed by a recent passive human-mediated spread to explain the actual distribution of domestic *T. infestans* in South America (Usinger et al., 1966; Schofield, 1988; Cortez et al., 2010). Among the seven domestic haplotypes identified in the Bolivian department of Chuiquisaca (Giordano et al., 2005) located in the "Central Andean area", three of them were identical to sylvatic haplotypes identified in the corresponding area (CytBHapA, C and E, see Fig. 7). The concurrent presence of these haplotypes in domestic and sylvatic environments suggests the occurrence of dwelling colonization by sylvatic populations in the Bolivian Andes.

Sylvatic *T. infestans* populations have never been reported from Brazil, and domestic *T. infestans* populations in this country appear to have been imported throughout human activities or migrations. It is, in fact, well established that *T. infestans* arrived in Northern Uruguay in the beginning of the 20th century, and reached the Northeast of Brazil in the 1970s (Panzera et al., 2004). The high genetic diversity of the Brazilian haplotypes, the numerous

substitutions accumulated between several Brazilian haplotypes, and the absence of common domestic haplotypes between Brazil and the Andean areas (Fig. 7) suggest that Brazilian *T. infestans* would have been imported from non-Andean areas where *T. infestans* has highly diversified independently from the Andean populations. This agrees with the recent insight that the Gran Chaco region should be the center of dispersal of *T. infestans* through the non-Andean countries (Panzera et al., 2004; Bargues et al., 2006), and with the detection of a remarkable genetic diversity in the Argentinean domestic populations from the Gran Chaco (Perez de Rosas et al., 2007; Piccinali et al., 2009). The divergence time between *T. infestans* from the Gran Chaco and the Andes has previously been estimated to at least 59,000 years (Bargues et al., 2006). The genetic diversities reported in the current work in the Andean area as well as in the non-Andean area, the differentiation between the sylvatic populations from both areas, the apparent absence of common haplotypes between these populations, combined with this estimated divergence time, support the existence of *T. infestans* in both areas before human peopling (according to Guhl, 2005, human is generally thought to have arrived and peopled North and South America about 15,000 years ago). Consequently, to fit with all above data, multiple events of domestication in different areas are much more likely to have happened than a major and unique event in the Bolivian Andes. In this sense, a recent study performed in our laboratory about domestic populations of *T. infestans* from the Gran Chaco allowed the detection, in rural dwellings, of *T. infestans* haplotypes identical to the sylvatic haplotypes found in the neighboring site of Tita (Pop 20), suggesting events of domestication in this area, and strengthening the above hypothesis (Quisberth et al., 2011).

Acknowledgments

This work was developed within the Medical Entomology Laboratory of the INLASA (Instituto Nacional de Laboratorios de Salud, La Paz, Bolivia) led by Tamara Chavez. We extend our gratitude to the SEDES (Servicos Departamentales de Salud) of La Paz, Santa Cruz, and Potosí. We would like to thank Marinely Bustamante, Hiber Calle, Abdul Castillo, Mario Charrière, Carlos Chura, Marcelo Claure, Ana Mery Lopez, Elio Mamani, Ricardo Ovando, Adriana Sanjines, Fernando Uzqueda and Pablo Vidaurre for their help in the field. This study received financial support from the ANR (Agence Nationale de la Recherche, France), the IRD (Institut de Recherche pour le Développement, France), the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), and the IDRC (International Development Research Center, Canada).

References

Akaike, H., 1974. A new look at the statistical model identification. IEEE Trans. Autom. Control 19, 716–723.

Bacigalupo, A., Segura, J.A., García, A., Hidalgo, J., Galuppo, S., Cattan, P.E., 2006. First finding of Chagas disease vectors associated with wild bushes in the metropolitan region of Chile. Rev. Med Chile 134, 1230–1236.

Bacigalupo, A., Torres-Pérez, F., Segovia, V., García, A., Correa, J P., Moreno, L., Arroyo, P., Cattan, P.E., 2010. Sylvatic foci of the Chagas disease vector *Triatoma infestans* in Chile: description of a new focus and challenges for control programs. Mem. Inst. Oswaldo Cruz 105, 633–641.

Bargues, M.D., Klisiowicz, D.R., Panzera, F., Noireau, F., Marcilla, A., Perez, R., Rojas, M.G., O'Connor, J E., Gonzalez-Candelas, F., Galvão, C., Jurberg, J., Carcavallo, R.U., Dujardin, J.P., Mas-Coma, S., 2006. Origin and phylogeography of the Chagas disease main vector *Triatoma infestans* based on nuclear rDNA sequences and genome size. Infect. Genet. Evol. 6, 46–62.

Bermudez, H., Balderrama, F., Torrico, F., 1993. Identification and characterization of sylvatic foci of *Triatoma infestans* in Central Bolivia. Am. J. Trop. Med. Hyg. 49 (Suppl.), 371.

Buitrago, R., Waleckx, E., Bosseno, M F., Zoveda, F., Vidaurre, P., Salas, R., Mamani, E., Noireau, F., Breniere, S.F., 2010. First report of widespread wild populations of *Triatoma infestans* (Reduviidae, Triatominae) in the valleys of La Paz, Bolivia Am. J. Trop. Med. Hyg. 82, 574–579.

Carcavallo, R U , Jurberg, J., Lent, H., Noireau, F., Galvão, C., 2000. Phylogeny of the Triatominae (Hemiptera: Reduviidae). Proposals for taxonomic arrangements. Entomol. Vector 7 (Suppl 1), 1–99.

Ceballos, L.A., Piccinali, R.V., Berkunsky, I., Kitron, U., Gurtler, R.E , 2009. First finding of melanic sylvatic *Triatoma infestans* (Hemiptera: Reduviidae) colonies in the Argentine Chaco. J. Med. Entomol. 46, 1195–1202.

Cortez, M.R., Pinho, A.P., Cuervo, P., Alfaro, F., Solano, M., Xavier, S.C.C., D'Andrea, P.S., Fernandes, O., Torrico, F., Noireau, F., Jansen, A.M., 2006. *Trypanosoma cruzi* (Kinetoplastida Trypanosomatidae) Ecology of the transmission cycle in the wild environment of the Andean valley of Cochabamba, Bolivia Exp Parasitol 114, 305–313.

Cortez, M.R., Emperaire, L., Piccinali, R.V., Gurtler, R.E., Torrico, F., Jansen, A.M., Noireau, F., 2007 Sylvatic *Triatoma infestans* (Reduviidae, Triatominae) in the Andean valleys of Bolivia. Acta Trop. 102, 47–54

Cortez, M.R., Monteiro, F.A., Noireau, F., 2010. New insights on the spread of *Triatoma infestans* from Bolivia – implications for Chagas disease emergence in the Southern Cone. Infect Genet Evol. 10, 350–353.

Dujardin, J.P., Tibayrenc, M., Venegas, E., Maldonado, L., Desjeux, P., Ayala, F.J., 1987. Isozyme evidence of lack of speciation between wild and domestic *Triatoma infestans* (Heteroptera, Reduviidae) in Bolivia. J. Med. Entomol. 24, 40–45.

Dujardin, J.P., Schofield, C.J., Tibayrenc, M., 1998. Population structure of Andean *Triatoma infestans*: allozyme frequencies and their epidemiological relevance. Med. Vet. Entomol. 12, 20–29.

Edwards, J.K., 1998. Miniprep procedures for the isolation of plant DNA. In: Karp, A., Issac, P.G., Ingram, D.S. (Eds.), Molecular Tools for Screening Biodiversity. Chapman & Hall, London.

Excoffier, L.G., Laval, G., Schneider, S., 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis Evol Bioinform. 1, 47–50.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using bootstrap. Evolution 39, 783–791.

Fu, Y.X., 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147, 915–925

Giordano, R , Cortez, J.C.P., Paulk, S., Stevens, L., 2005. Genetic diversity of *Triatoma infestans* (Hemiptera: Reduviidae) in Chuquisaca, Bolivia based on the mitochondrial cytochrome b gene. Mem Inst. Oswaldo Cruz 100, 753–760.

Guhl, F., 2005. ADN fossil: Arqueoparasitología en América. Rev. Acad Colomb Cienc. 29, Número 111 – Junio de 2005.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Ibisch, P.L., Merida, G., 2008 Biodiversidad: La riqueza de Bolivia. Estado de conocimiento y conservación. Editorial FAN, Santa Cruz de la Sierra.

Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452.

Lyman, D.F., Monteiro, F A., Escalante, A.A., Cordon-Rosales, C., Wesson, D M , Dujardin, J.P., Beard, C.B., 1999. Mitochondrial DNA sequence variation among triatomine vectors of Chagas' disease. Am. J. Trop. Med. Hyg. 60, 377–386.

Marcilla, A , Bargues, M.D., Ramsey, J.M , Magallon-Gastelum, E., Salazar-Schettino, P.M., Abad-Franch, F., Dujardin, J.P., Schofield, C.J., Mas-Coma, S., 2001. The ITS-2 of the nuclear rDNA as a molecular marker for populations, species, and phylogenetic relationships in Triatominae (Hemiptera: Reduviidae), vectors of Chagas disease. Mol. Phylogenet. Evol. 18, 136–142.

Martinez, F.H , Villalobos, G.C., Cevallos, A.M., De la Torre, P., Laclette, J.P., Alejandre-Aguilar, R., Espinoza, B., 2006. Taxonomic study of the *Phyllosoma* complex and other triatomine (Insecta: Hemiptera: Reduviidae) species of epidemiological importance in the transmission of Chagas disease: using ITS-2 and mtCytB sequences. Mol. Phylogenet. Evol. 41, 279–287

Monteiro, F.A., Perez, R., Panzera, F., Dujardin, J.P., Galvão, C., Rocha, D., Noireau, F., Schofield, C.J., Beard, C.B., 1999. Mitochondrial DNA variation of *Triatoma infestans* populations and its implication on the specific status of *T. melanosoma* Mem. Inst. Oswaldo Cruz 94, 229–238.

Nei, M., 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.

Noireau, F., Flores, R., Gutierrez, T., Dujardin, J.P., 1997. Detection of sylvatic dark morphs of *Triatoma infestans* in the Bolivian Chaco. Mem. Inst. Oswaldo Cruz 92, 583–584.

Noireau, F., Flores, R., Vargas, F., 1999. Trapping sylvatic Triatominae (Reduviidae) in hollow trees. Trans. R. Soc. Trop. Med. Hyg. 93, 13–14.

Noireau, F., Cortez, M.G.R., Monteiro, F.A., Jansen, A.M., Torrico, F., 2005. Can wild *Triatoma infestans* foci in Bolivia jeopardize Chagas disease control efforts? Trends Parasitol. 21, 7–10.

Noireau, F., 2009. Wild *Triatoma infestans*, a potential threat that needs to be monitored. Mem. Inst. Oswaldo Cruz 104, 60–64.

Panzera, F., Dujardin, J.P., Nicolini, P , Caraccio, M.N., Rose, V., Tellez, T , Bermudez, H., Bargues, M.D., Mas-Coma, S., O'Connor, J.E., Perez, R., 2004. Genomic changes of Chagas disease vector, South America. Emerg. Infect. Dis 10, 438–446.

Perez de Rosas, A.R., Segura, E.L., Garcia, B.A., 2007. Microsatellite analysis of genetic structure in natural *Triatoma infestans* (Hemiptera: Reduviidae) populations from Argentina: its implication in assessing the effectiveness of Chagas' disease vector control programmes. Mol. Ecol. 16, 1401–1412.

Piccinali, R.V., Marcet, P L., Noireau, F., Kitron, U., Gurtler, R.E., Dotson, E.M., 2009. Molecular population genetics and phylogeography of the Chagas disease vector *Triatoma infestans* in South America J. Med. Entomol. 46, 796–809.

Posada, D., Buckley, T.R., 2004. Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests Syst. Biol. 53, 793–808.

Posada, D., Crandall, K.A., 2001. Intraspecific gene genealogies: trees grafting into networks. Trends Ecol. Evol. 16, 37–45.
Posada, D., 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25, 1253–1256.
Quisberth, S., Waleckx, E., Monje, M., Chang, B., Noireau, F., Brenière, S.F., 2011. "Andean" and "non-Andean" ITS-2 and mtCytB haplotypes of T. infestans are observed in the Gran Chaco (Bolivia): population genetics and the origin of reinfestation. Infect. Genet Evol. 11, 1006–1014
Ramos-Onsins, S.E., Rozas, J., 2002. Statistical properties of new neutrality tests against population growth. Mol Biol. Evol. 19, 2092–2100.
Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
Schofield, C.J., 1988. Biosystematics of the Triatominae. In: Service, M.W. (Ed.), Biosystematics of Haematophagous Insects. Clarendon Press, Oxford, pp. 284–312
Schofield, C.J., Jannin, J., Salvatella, R., 2006. The future of Chagas disease control. Trends Parasitol. 22, 583–588.
Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P., 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene-sequences and a compilation of conserved polymerase chain-reaction primers. Ann. Entomol. Soc. Am. 87, 651–701.
Swofford, D.L., 2001. PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods). Sinauer Associates, Inc. Publishers, Sunderland, MA (Version 4, Computer Program Distributed by the Smithsonian Institution).
Tajima, F., 1983. Evolutionary relationship of DNA sequences in finite populations. Genetics 105, 437–460.
Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585–595.
Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL-W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680
Torrico, R.A., 1946. Hallazgo de *Eratyrus mucronatus*, infestación natural de vinchucas de cerro y *Eutriatoma sordida* en Cochabamba. An Lab Central Cochabamba 1, 19–23.
Usinger, R.L., Wygodzinsky, P., Ryckman, R.E., 1966. The biosystematics of Triatominae. Annu. Rev. Entomol. 11, 309–330.
Weir, B.S., Cockerham, C.C., 1984. Estimating F-statistics for the analysis of population structure. Evolution 38, 1358–1370.

Infection, Genetics and Evolution 11 (2011) 1006–1014

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

"Andean" and "non-Andean" ITS-2 and mtCytB haplotypes of *Triatoma infestans* are observed in the Gran Chaco (Bolivia): Population genetics and the origin of reinfestation

Sergio Quisberth [a,b,c], Etienne Waleckx [a,c], Marcelo Monje [a], Boris Chang [d], François Noireau [a,e], Simone Frédérique Brenière [a,c,*]

[a] MIVEGEC (Université de Montpellier, CNRS 5290, IRD 224), Maladies Infectieuses et Vecteurs: Ecologie, Génétique, Evolution et Contrôle, Institut de Recherche pour le développement (IRD), Representation in Bolivia, Av Hernando Siles No. 5290, Esq Calle 7 Obrajes, CP 9214 La Paz, Bolivia
[b] UMSA, Universidad Mayor de San Andrés, Facultad de Farmacia, Av. Saavedra, Miraflores, La Paz, Bolivia
[c] Instituto Nacional de Laboratorios de Salud (INLASA), Laboratorio de Entomología Médica, 14 Rafael Zubieta #1889, Miraflores, Casilla M-10019, La Paz, Bolivia
[d] Servicio Departamental de Salud (SEDES), Programa de Control de Chagas, Av. Pedro Ribera No. 510, Santa Cruz de la Sierra, Bolivia
[e] IIBISMED, Facultad de Medicina, Universidad Mayor de San Simon, Avenida Aniceto Arce 21 #371, Cochabamba, Bolivia

ARTICLE INFO

Article history:
Received 9 November 2010
Received in revised form 17 March 2011
Accepted 22 March 2011
Available online 30 March 2011

Keywords:
Triatoma infestans
Gran Chaco
Bolivia
Reinfestation
Population
Genetics

ABSTRACT

In Bolivia, the Gran Chaco ecoregion suffers from serious problems of house reinfestation with *Triatoma infestans* despite vector control by insecticides spraying. In order to identify the origin of the triatomines collected after spraying, the genetic structure of *T. infestans* populations collected in four neighboring villages, before and after spraying, was analyzed using ITS-2 and mtCytB sequencing. Before spraying, only the mtCytB marker detected genetic differentiation among the 4 populations. After spraying, the mtCytB analysis of the populations from two of the studied villages supported the hypothesis in favor of a local origin for the triatomines in each village. Surprisingly, ITS-2 and mtCytB haplotypes previously found only in Andean areas were also present with high frequencies in the studied populations; these domestic populations of the Gran Chaco seem to be the result of a mixture of "Andean" and "non-Andean" triatomines probably generated by the human passive transport of triatomines from the Andes to the Gran Chaco.

1. Introduction

In Southern Cone countries, *Triatoma infestans* (Hemiptera: Reduviidae) was and still remains the main domestic vector of *Trypanosoma cruzi*, the causative agent of Chagas disease. It is the target of control programs based on house spraying with residual insecticides (Dias, 2007). However, in different regions of the Southern Cone, the recolonisation of treated areas is a recurrent phenomenon. Such occurrence is particularly fast and severe in the Gran Chaco, a semi arid lowland region shared among northern Argentina, eastern Bolivia, and Paraguay and part of the Brazilian state of Mato Grosso (Cecere et al., 2004; Marcet et al., 2008) which represents a substantial challenge for disease control. Thus, the present study concerned populations of *T. infestans* collected in villages of the Gran Chaco where infestation is persistent after insecticide treatments.

Previous studies carried out in Argentina and Central Bolivia reported that reinfestation originated from residual populations (Dujardin et al., 1996, 1997; Perez de Rosas et al., 2007, 2008; Pizarro et al., 2008). It was observed that peridomestic structures were rapidly reinvaded by triatomines, and acted like primary source of reinfestation of the dwellings (Cecere et al., 2006). Moreover, the detection of insecticide resistance in various *T. infestans* populations seem to be one of the reasons for insecticide spraying failure (Picollo et al., 2005; Toloza et al., 2008; Lardeux et al., 2010).

From the 1980s, several markers have been used for the genetic analysis of *T. infestans*. Pioneer works on allozyme variability pointed out the low variability of *T. infestans* across its area of distribution and concluded that a massive founder effect might explain the overall similarity in different countries (Dujardin et al., 1998). In addition, a structuring between localities that fit with the isolation by distance model was observed (Dujardin et al., 1998; Piccinali et al., 2009). Within several localities, sub-structuring into smaller units such as individual dwellings or chicken coops was observed, disputing the previous conclusion that the locality could be

* Corresponding author at: Institut de Recherche pour le Développement (IRD), Representation in Bolivia, Av Hernando Siles No. 5290, Esq Calle 7 Obrajes, CP 9214 La Paz, Bolivia. Tel.: +591 2 278 29 69; fax: +591 2 278 29 44.
E-mail address: Frederique.Breniere@ird.fr (S.F. Brenière).

1567-1348/$ – see front matter
doi:10.1016/j.meegid.2011.03.014

considered the likely panmictic unit (Brenière et al., 1998; Dujardin et al., 1998). Subsequently, cytogenetic analysis revealed a dichotomy between "Andean" (from Bolivia, Peru, Chile) and "non-Andean" populations (from the lowlands of Bolivia, Paraguay, Brazil, Argentina, and Uruguay), linked to significant differences in heterochromatin amounts and C-banding patterns (Panzera et al., 2004). A phylogenetic study using nuclear rDNA (ITS-1 and ITS-2) corroborated this dichotomy (Bargues et al., 2006). The occurrence of two geographic populations was also suggested with mitochondrial markers, but the "dark morph" *T. infestans* from the Bolivian Gran Chaco (lowlands) discovered by Noireau et al. (1997) was clustered with the "Andean" populations and not with the "non-Andean" (Monteiro et al., 1999; Piccinali et al., 2009).

Surprisingly, in the Bolivian Gran Chaco, genetic study of domestic *T. infestans* has never been performed, even if vector-mediated transmission persists because of continual reinfestation of houses. To examine the structure of the populations, the origin of domestic reinfestation, and the relationships of these populations with those previously analyzed, we analyzed the genetic variation of ITS-2 and mtCytb sequences in *T. infestans* domestic populations collected in 4 neighboring villages of the Bolivian Gran Chaco.

2. Materials and methods

2.1. Areas studied

Triatomes were collected in 4 villages (2093 inhabitants in total) located in the region of Izozog (Gran Chaco, Bolivia), 300 kms southeast of the city of Santa Cruz (Fig. 1) and around 70 kms from the foothills of the Andes. They are from North to South along the Parapeti river: Kuarirenda (19°10′37.88″S; 62°31′36.17″W), San Silvestre (19°21′20.86″S; 62°34′10.19″W), Rancho Nuevo (19°26′21.59″S; 62°34′04.60″W) and Tamachindi (19°28′41.21″S; 62°33′49.46″W). They belong to an indigenous territory mainly populated by Guaranies and Creoles. The region is part of the Boreal Chaco, with an average altitude of 300 m, an average annual temperature of 26 °C (0–42 °C), and annual rainfall of around 550 mm from December to April. It is the driest part of the Chaco, covered by xeric forest vegetation generally dense, low, and thorny. Most of human dwellings have unplastered walls made of mud mixed with straw and are roofed with metal-sheets. They are separated from each other (range of distances between neighboring houses = 10–200 m) and have few peridomestic structures.

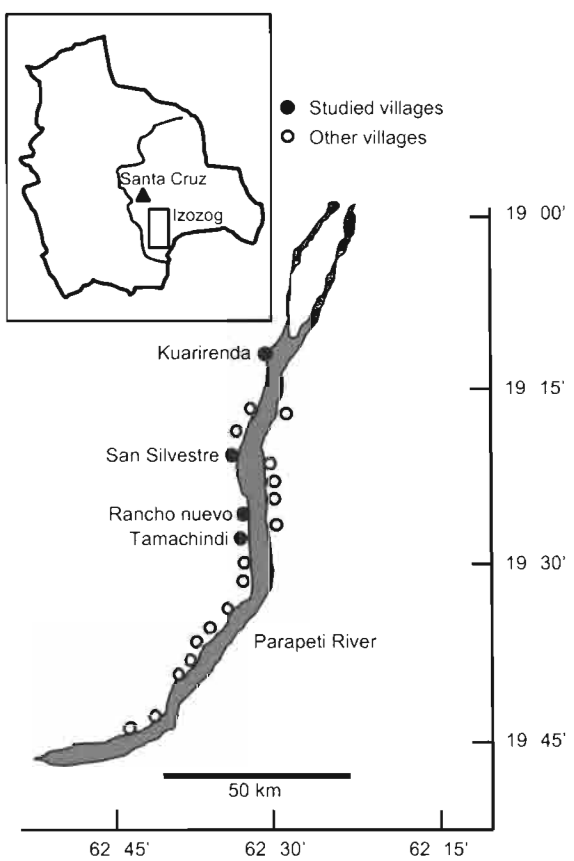


Fig. 1. Geographic origin of the domestic *T. infestans* populations under study collected in 4 villages located along the Parapiti River in the region of Izozog (Gran Chaco, Bolivia). Box = map of Bolivia with the localization of the Izozog region (small rectangle).

2.2. Triatomine populations

Before and after spraying all the *T. infestans* specimens were manually collected during the day with flashlights in domestic and peridomestic areas, with no insect dislodging agent. Before spraying part of the bugs collected from October 22 to November 9, 2007 were analyzed: about 15 bugs from each village were selected from different dwellings to get a relevant spatial representation at the village scale. Five and nine months after the initial spraying (alpha-cypermethrin 50 mg a.i./m^2) the triatomines collected in two villages (Rancho Nuevo and San Silvestre), were analyzed. All positive dwellings for triatomines at 5 months were again sprayed with insecticide. The bugs collected on both occasions were grouped and all the collected bugs were included in the analysis. In the laboratory, the legs of each bug were removed and preserved in 70% ethanol for DNA extraction.

2.3. DNA extraction

DNA was extracted from legs according to the previous description with some modifications (Edwards, 1998). Briefly, legs were dried, crushed, and incubated with 200 μl of 2% CTAB (Cetyl Trimethyl Ammonium Bromide) and 30 μl of proteinase K (20 mg/ml) overnight at 37 °C. After chloroform extraction, the final DNA pellet was diluted in 20 μl of distilled water. Concentration and purity determinations of DNAs were performed by spectrophotometry at 260 and 280 nm readings.

2.4. DNA amplification and sequencing

A nuclear DNA fragment of approximately 900 bp containing the ITS-2 sequence was PCR-amplified by using primers previously described (Marcilla et al., 2001). A fragment of approximately 400 bp of the mtCytB gene was PCR-amplified by using a set of primers previously described with slight modification (Lyman et al., 1999): CytobtF 5′-GGA-CAA-ATA-TCA-TGA-GGA-GCA-ACA-G-3′ and CytobtR 5′-ATT-ACT-CCT-CCT-AGC-TTA-TTA-GGA-ATT-G-3′). PCR were done using a Mastercycler Gradient (Eppendorf, Hamburg, Germany) in reaction mixtures of 30 μl containing 15 μl of PCR MasterMix 2X (Promega, Madison, Wisconsin, USA), 12 pmol of each primer, and 100 ng of DNA template. The cycle conditions were: 94 °C for 3 min; 94 °C for 1 min, 52.2 °C (ITS-2) and 48.3 °C (mtCytB) for 1 min, 72 °C for 1 min (30 cycles); 72 °C for 7 min. Purification and direct sequencing of both strands of DNA amplicons were performed by the company MACROGEN in Seoul, South Korea.

2.5. Data analysis

Sequences were aligned and corrected using the BioEdit software version 7.0.9 (Hall, 1999). DnaSP version 5.10.00 (Librado and Rozas,

Table 1
Variable sites and ITS-2 haplotypes detected in *T. infestans* from villages of the Izozog region (Gran Chaco, Bolivia) and GenBank available sequences.

Haplotypes identified in the study	GenBank haplotypes			Nucleotide position													
	Name[a]	Accession no.	Source	54	55	56	74	75	76	77	110	225	226	227	228	328	352
ITS2Hap1 (HQ333211)	GT1A, GT1B, GT1C	AJ576051, AJ582024 and AJ582025	(18)	T							T	T	A	A	A	T	A
ITS2Hap2 (HQ333212)				A			A	T			T	T	A	A	A	T	A
ITS2Hap4 (HQ333214)	GT2A	AJ576054	(18)	A			A	T	A	T	T	T	A	A	A	T	T
	GT3A, ITInf72	AJ576052, AY860387	(18,28)	A	T	A	A	T	A	T	T	T	A	A	A	T	T
	GT4A	AJ576053	(18)	A			A	T			T					T	A
	GT5A	AJ576055	(18)	A			A	T	A	T	T	T	A	A	A	T	A
	Bolivia La Paz	AJ286874	(22)	T							G	T	A	A	A	A	A
	Triatoma infestans	AJ289876	(22)	A			A	T	A	T	G	T	A	A	A	T	T
	ITInf74	AY860388	(28)	A							T	T	A	A	A	T	A

[a] Sequence names given by authors in GenBank.

2009) was used to determine the indexes measuring sequence variation within populations (average number of nucleotide differences, number of haplotypes, nucleotide and haplotype diversities, and mutation index. Genetic differentiation among (AMOVA test) and between pairwise populations (F_{ST} values) and neutrality tests (Tajima's *D* and Fu's *F*) were estimated by using the Arlequin version 3.5.1.2 (Excoffier et al., 2005). The Mantel test was computed with GENETIX version 4.05.2 (Belkhir et al., 2001) to test the hypothesis of isolation by distance model. Phylogenetic relationships between populations and haplotypes were inferred by the neighbor-joining (NJ) method (Saitou and Nei, 1987). To build the trees, Mega4 was used with the Nucleotide Maximum Composite Likelihood as genetic distance. The reliability of the inferred trees was tested by the bootstrap resampling technique with 10,000 replicates. To complete phylogenetic analysis, a networking approach was performed, using Network version 4.5.1.6. (Copyright 2004–2010, Fluxus Technology Ltd.) for phylogenetic relationship reconstruction between mtCytB haplotypes with the median-joining (MJ) network method. All maximum parsimony networks were generated by fixing an epsilon value of 10. The post processing option was then used to remove unnecessary median vectors and links (cleaning procedure). For the complex network, the star contraction pre-processing option was applied. This option has the advantage of identifying demographic expansions, contracting stars with their founder node.

3. Results

3.1. Sequence analysis

ITS-2 sequences were obtained for a total of 102 *T. infestans* specimens (55 before spraying and 47 after spraying). Three ITS-2 haplotypes (454–458 bp) presenting 2 single nucleotide polymorphisms (SNPs) and one indel of 1–2 (AT) repetitions in a microsatellite region were found (Tables 1 and 2). Two of them had been previously described: ITS2Hap1 aligned with an identity of 100% three ITS1-5. 8S-ITS2 composite sequences that presented an identical ITS-2 region, and ITS2Hap4 aligned with an identity of 100% another haplotype (Table 1). ITS2Hap2 was detected for the first time; later, it was also detected by our team in sylvatic *T. infestans* ("dark morph") from the same area of the Chaco (E. Waleckx et al., submitted). ITS2Hap4 was most represented in the overall sample (74.5%) and had been previously identified in the "non-Andean" group, while ITS2Hap1 (19.6%) was previously identified in the "Andean" group (Table 2) (Bargues et al., 2006).

MtCytB partial sequences of 388 bp were resolved for 106 *T. infestans* specimens (56 before spraying and 50 after spraying). A total of 15 SNPs were observed leading to 8 different haplotypes (Table 3). Only one mutation (site 188, found in CytBHapW) was not silent (Thr → Met). CytBHapU and D were haplotypes previously described in GenBank belonging to bugs from the department of Chuquisaca, Bolivia (Andean area) and to sequences obtained from Brazilian *T. infestans* (non-Andean area), respectively (Giordano et al., 2005; Assumpcao et al., 2008). All the other haplotypes were new. In the overall sample, the major haplotypes were CytBHapU and D with frequencies of 38% and 35%, respectively (Table 4); the frequencies of the other haplotypes were <11%. It is worth noting that CytBHapD and O have recently been detected in sylvatic Andean and non-Andean areas respectively (E. Waleckx et al., submitted).

3.2. Genetic diversity within and overall populations and haplotype distribution

Before spraying the 3 ITS-2 haplotypes were distributed in the 4 villages except ITS2Hap2 which was not detected in Tamachindi

Table 2
ITS-2 variability and haplotype distribution in *T. infestans* populations collected in villages of the Izozog region (Gran Chaco, Bolivia) before and after insecticide spraying.

Indices and haplotypes	Total sample	Populations before spraying				Populations after spraying	
		KUA	SS	RN	TAM	SS	RN
N	102	12	14	14	15	13	34
k^a	0.702	0.939	0.505	0.626	0.686	0.846	0 742
k^b	2 106	2.818	1.516	1.879	2.057	2.538	2.225
π	0.00155	0.00207	0.00111	0.00138	0 00151	0.00186	0.00163
Hd	0.407	0.621	0.385	0.385	0.343	0.500	0.371
θ_w	0.00085	0.00146	0.00139	0.00139	0.00135	0.00142	0.00108
h	3	3	3	3	2	3	2
ITS2Hap1	20	3	1	2	3	3	8
ITS2Hap2	6	2	2	1	0	1	0
ITS2Hap4	76	7	11	11	12	9	26

KUA, Kuarirenda; SS, San Silvestre; RN, Rancho Nuevo; TAM, Tamachindi; *N*, sample size; *k*, average number of nucleotide differences; π, nucleotide diversity; Hd, haplotype diversity; θ_w, mutation rate; *h*, number of haplotypes.
[a] Value excluding indel.
[b] Value including indel.

Table 3
Variable sites and cytochrome b haplotypes detected in *T. infestans* from villages of the Izozog region (Gran Chaco, Bolivia) and corresponding sequences in GenBank.

Haplotypes identified in the study	GenBank haplotypes			Nucleotide position													
	Name*	Accession no.	Source	54	78	87	171	174	180	188	270	276	285	294	336	342	366	372
CytBHapT (HQ333234)				A	A	C	T	C	G	C	C	C	G	A	A	T	A	T
CytBHapU (HQ333235)	Haplotype B	AY702019	(29)			T			A		T		A					
CytBHapD (HQ333218)	TinfcBra; *T. inf* clone TI-61	AY062165; EF639038	Harris and Beard; (30)				T	A		T	A	A					C	
CytBHapV (HQ333236)					G			T	A		T	A	A	G	G		G	C
CytBHapW (HQ333237)								T	A	T	T	A	A					C
CytBHapX (HQ333238)					G			T	A		T	A	A					C
CytBHapY (HQ333239)				G				T	A		T	A	A					C
CytBHapO (HQ333233)							C	–	A		T					C		

* Sequence names given by authors in GenBank.

Table 4
Cytochrome b genetic variability and haplotype distribution in *T. infestans* populations collected in villages of Izozog region (Gran Chaco, Bolivia) before and after insecticide spraying.

Indexes and haplotypes	Total population	Populations before spraying				Populations after spraying	
		KUA	SS	RN	TAM	SS	RN
N	106	15	14	14	13	14	36
K	2.972	2.343	1.758	3.451	3.667	1.451	1 366
π	0.00766	0.00604	0.00453	0.00889	0.00945	0.00374	0.00349
Hd	0 725	0.562	0.440	0.791	0.833	0.363	0.627
θ_w	0.00738	0.00555	0.00324	0.00973	0.00747	0.00324	0.00373
h	8	3	2	6	5	2	4
CytBHapT	6	5	0	1	0	0	0
CytBHapU	40	9	10	2	4	11	4
CytBHapD	37	1	4	6	3	3	20
CytBHapV	3	0	0	3	0	0	0
CytBHapW	4	0	0	1	0	0	3
CytBHapX	11	0	0	1	1	0	9
CytBHapY	2	0	0	0	2	0	0
CytBHapO	3	0	0	0	3	0	0

KUA, Kuarirenda; RN, Rancho Nuevo; SS, San Silvestre; TAM, Tamachindi; *N*, sample size; *k*, average number of nucleotide differences; π, nucleotide diversity; Hd, haplotype diversity; θ_w, mutation rate, *h*, number of haplotypes.

(Table 2). ITS2Hap4 was most represented in each village (range 58–80%). Low variability was observed within the populations (k); however, the k value was trebled when indels were included. The Watterson (θ_w) index showed that the mutation rate was lower in the total population than in each one. After spraying, the 3 ITS-2 haplotypes were identified in San Silvestre, however ITS2Hap2 was no longer detected in Rancho Nuevo. ITS2Hap4 was, as in previously sprayed populations, most represented in both populations; in general, nucleotide and haplotype diversities were slightly increased (Table 2).

MtCytB sequences were analyzed before and after spraying in the same villages (Table 4). Before spraying, only CytBHapU and D were present in the four villages; CytBHapT and X were found in two of them; CytBHapV and W were private haplotypes in Rancho Nuevo and CytBHapY and O in Tamachindi. The indexes of genetic variability (k, π, Hd and θ_w) were generally higher than those estimated for the ITS-2 (Table 4). After spraying, the genetic diversity was slightly reduced in the Rancho Nuevo population, and similar in San Silvestre when compared with the respective previously sprayed populations.

3.3. Population differentiation

The AMOVA computed across all populations with ITS-2 sequences showed that the total variation was assigned to differences within populations, and that the populations were not significantly differentiated (Va = −0.01230, % of variation = −3.48, $F_{ST} = -0.03483$, $p > 0.85$, df = 5). The hierarchical AMOVA performed between the four populations collected before spraying and the two populations after spraying confirmed the absence of structuration (Va = 0.00214, % of variation = 0.20, $F_{CT} = 0.00204$, $p > 0.40$, df = 1). Similarly, the exact test performed between pairwise populations did not detect differentiation between populations ($p = 0.37 \pm 0.01$ with 100,000 Markov steps done).

Different results were obtained for mtCytB. The AMOVA showed significant differentiation between populations, with 33.8% of the total variability assigned to differences between populations, and the remaining variability attributed to differences within populations (Va = 0.537, %, $F_{ST} = 0.337$ $p < 10^{-4}$, df = 5). Genetic differentiations between populations were estimated calculating pairwise F_{ST} (Table 5), and were represented by a NJ tree (Fig. 2). The topology of the tree showed a first branch comprising the populations of Rancho Nuevo before and after

Table 5
Pairwise differentiation between *T. infestans* populations from villages of the Izozog region (Gran Chaco, Bolivia) before and after spraying based on mtCytB sequencing and expressed by F_{ST} index.

	KUA-BS	SS-BS	SS-RE	RN-BS	RN-RE	TAM-BS
KUA-BS	–	**	***	*	*	**
SS-BS	0.144*	–	*	**	**	NS
SS-RE	0.136	−0.062	–	***	***	***
RN-BS	0.410***	0 303**	0.373***	–	NS	*
RN-RE	0.612***	0.498***	0.569***	0.046	–	*
TAM-BS	0.194***	0.085	0.142*	0.108	0.266***	–

BS, population before spraying, RE, reinfesting population; KUA, Kuarirenda; RN, Rancho Nuevo; SS, San Silvestre, TAM, Tamachindi; below the diagonal, F_{ST} values; above the diagonal, Exact test; NS, no significant.

* $p < 0.05$

** $p < 0.01$.

*** $p < 0.001$.

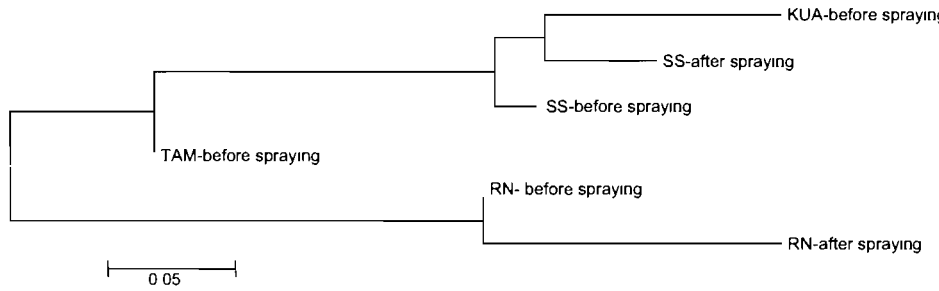


Fig. 2. Evolutionary relationships between Bolivian populations of *T. infestans* collected in Izozog (Gan Chaco) before and after spraying was inferred using the NJ method based on Fst values as genetic distances. The optimal tree has the sum of branch length = 0.514. The tree is drawn to scale.

spraying that did not exhibit significant differentiation between them (F_{ST} value and exact test no significant, Table 5). The second branch comprised the two populations of San Silvestre (before and after spraying) and Kuarirenda (before spraying). These populations exhibited some differentiations (Table 5), but they were more related between them than with the other populations. A third branch comprised the population of Tamachindi. The model of isolation by distance was also tested and the result did not fit with the hypothesis ($r = 0.231$, $p = 0.25$).

3.4. Phylogenic relationships between the present haplotypes and those available in GenBank

The alignment of the three haplotypes described here with all GenBank available *T. infestans* sequences showed a total of 9 different ITS-2 haplotypes (Table 1) (Marcilla et al., 2001; Bargues et al., 2006; Martinez et al., 2006). These haplotypes were differentiated by 4 SNPs, 2 indels of (AT) repetitions in microsatellite regions and 1 another indel of 4 nucleotides.

Genetic relationships between ITS-2 haplotypes were visualized through the construction of a NJ tree (Fig. 3). At the *T. infestans* intraspecific level, the bootstrap values of the different branches were low (≤70%): a first branch comprised one haplotype identified in the populations studied (ITS2Hap1) and in the valley of Cochabamba (Andean area, GT1A, GT1B, GT1C), another branch was formed by a haplotype corresponding to one specimen collected in the Department of La Paz (Andean area, Bolivia), and a third branch comprised ITS2Hap2 and 4 identified in the present study, and other haplotypes identified in bugs collected in non-Andean area in Argentina, Brazil, Paraguay and Bolivian Chaco (as GT5A obtained from a "dark morph" specimen). This "non-Andean" group included most of the haplotypes identified up to now in the sample.

The NJ tree constructed from the haplotypes identified in the populations under study, by using mtCytb sequences of *T. sordida* and *T. braziliensis* as outgroups, identified two clusters within *T. infestans* (Fig. 4). The first cluster comprised CytBHapU, T and O (bootstrap value of 70%), and the second one comprised the other haplotypes (bootstrap value of 89%). As it was indicated above, CytBHapU and D were haplotypes previously described in Andean and non-Andean areas respectively (see Section 3.1).

The 8 haplotypes were then aligned with 51 sequences available in GenBank (AY702018 to AY702024; GU807558 to GU807598; AY062165, EF639038 and AF045721), and a total of 54 different mtCytB haplotypes were detected (85 SNPs). The NJ tree (data not shown) showed that the monophyly of the *T. infestans* mtCytB haplotypes was strongly supported by the bootstrap value of 99%, but within *T. infestans* the two upper branches which divide the taxon were not further supported by high bootstrap values (40% and 35%). The group formed by CytBHapU, T and O haplotypes (Fig. 4), belonged to the same branch as haplotypes detected in the departments of Chuquisaca (Andean area of Bolivia, AY702018 to AY702024) and Tarija at the south of Bolivia (GU807594 and GU807597). The second group of haplotypes (CytBHapD, V, W, X, Y) described in Fig. 4, belonged to the same branch as haplotypes identified today in Brazil and 3 others in the department of Tarija (GU807592, GU807593 and GU807595).

A phylogenetic median network was constructed from the same mtCytB haplotypes data set. The star contraction option was applied before construction of the network, and allowed the identification of 2 principal nodes of expansion N1 and N2 (Fig. 5). The first founder node (N1) was CytBHapD (previously reported in

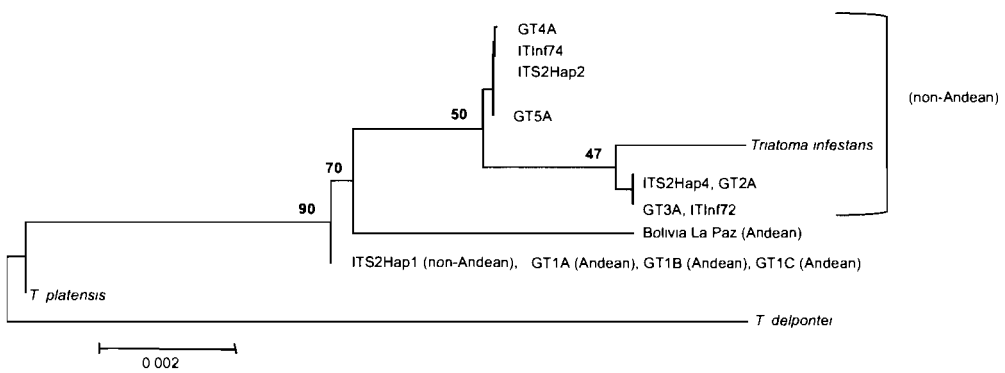


Fig. 3. Phylogenetic relationships of 9 existing ITS-2 haplotypes of *T. infestans* (new ones and GenBank haplotypes) inferred using NJ method. The tree is drawn to scale, with branch lengths in the unit of the evolutionary distance (number of base substitutions per site) computed using the Maximum Composite Likelihood method. All positions containing gaps were eliminated in each pairwise comparison. Bootstrap values were estimated with 10,000 replicates. The haplotype names refer to Table 1 and the geographical origin is indicated in brackets. Sequences of outgroups *Triatoma delponteri* and *Triatoma platensis* are from accession nos. AJ576057 and AJ576061.

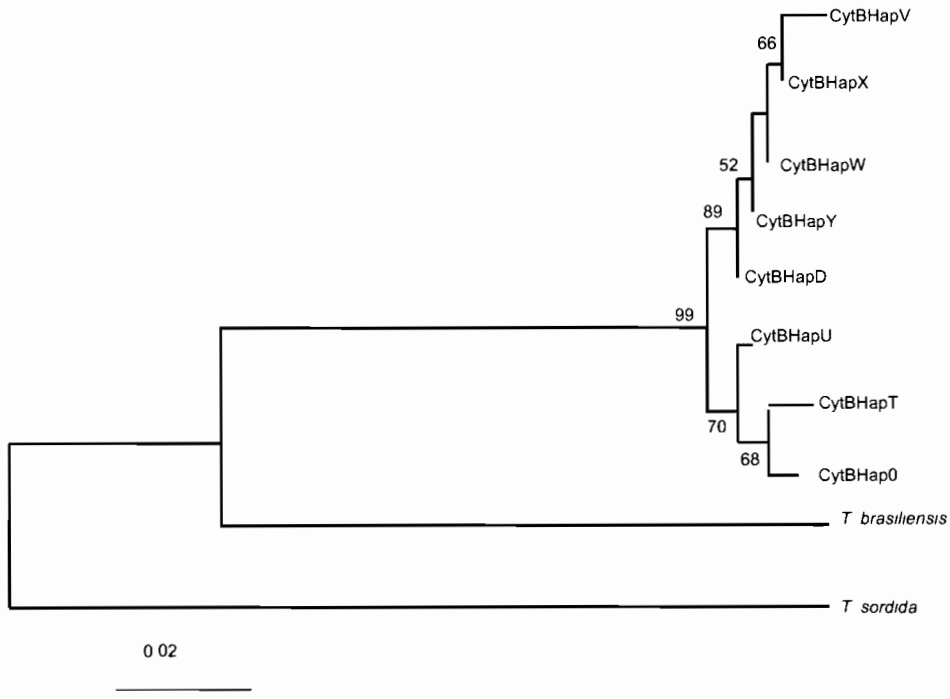


Fig. 4. Phylogenetic relationships of the 8 mtCytB haplotypes of *T. infestans* identified using neighbor joining method (NJ).The tree is drawn to scale, with branch lengths in the unit of the evolutionary distance (number of base substitutions per site) computed using Maximum Composite Likelihood method. Bootstrap values were estimated with 10,000 replicates. The haplotype names refer to Table 3 Sequences of outgroups *Triatoma brasiliensi* and *Triatoma sordida* are from accession nos. AY494161 and AF045730.

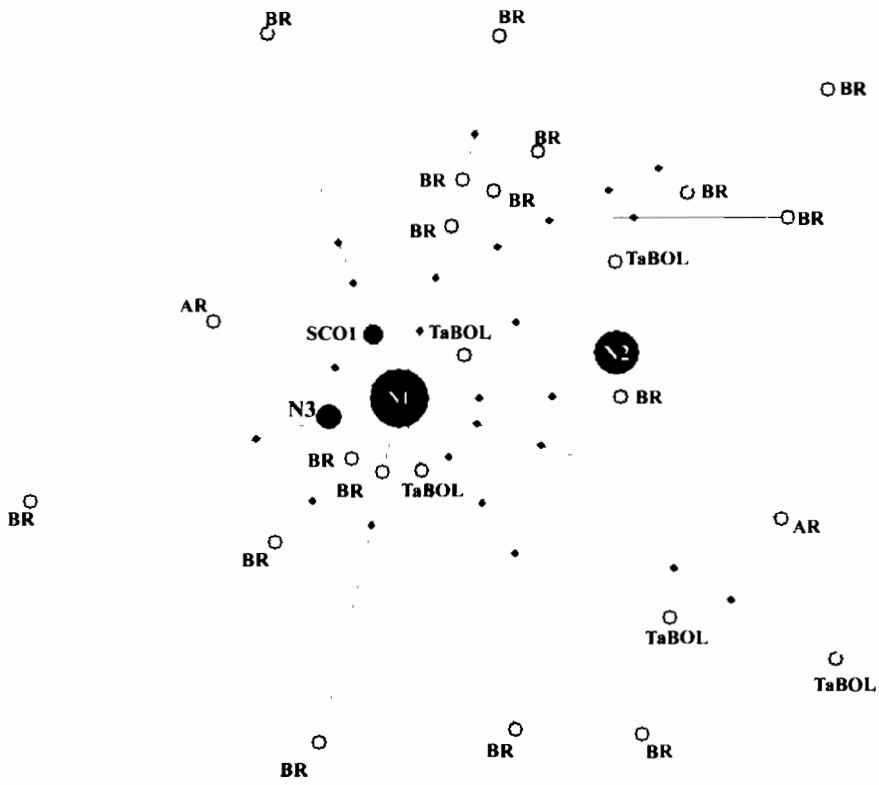


Fig. 5. MtCytB haplotype median network is resolved from 106 sequences of Izozog and 63 other GenBank of *T. infestans*. Network 4.5.1.6. is used for the reconstruction of the tree applying the star contraction option before construction with median-joining option (MJ). Cleaning procedure is done with the postprocessing option The contracting stars with their founder node are N1, N2, N3 and SCO1 (large black circles, size proportional to the number of contracted haplotypes); the founder node SCO1 is a hypothetical haplotype not found in the sample The geographical origin of the contracted haplotypes can be seen in the Results. The small grey circles are unique haplotypes identified in the sample; small black circles are median vectors that are hypothesized sequences not found in the sample and required to connect the existing haplotypes. BR = haplotype identified in Brazil, TaBol = haplotypes identified in the department of Tarija (South of Bolivia), AR = haplotypes identified in Argentina.

Table 6
ITS-2 and cytochrome b composite haplotypes of *T. infestans* collected in villages of the Izozog region (Gran Chaco, Bolivia).

MtCytB haplotypes		ITS-2 haplotypes			Total
		"Adean"[a]	"Non-Andean"[a]		
		ITS2Hap1	ITS2Hap2	ITS2Hap4	
"Andean"[a]	CytBHapT	1	2	2	5
	CytBHapU	7	1	28	36
	CytBHapO	2	0	1	3
"Non-Andean"[a]	CytBHapD	4	3	28	35
	CytBHapV	0	0	2	2
	CytBHapW	1	0	3	4
	CytBHapX	3	0	7	10
	CytBHapY	0	0	2	2
Total		18	6	73	97

[a] The classification of the haplotypes referred to the NJ tree topologies in Figs. 3 and 4.

Brazil, Table 3), and represented the contraction of the present haplotypes CytBHapV, W, X and Y with 13 others found in Brazil and 1 found in Argentina. The second node (N2) was a haplotype previously identified in the department of Chuquisaca (Andean area of Bolivia, AY702021), and represented the contraction of the present haplotypes CytBHapO, U and T with 4 others found in the department of Chuquisaca. All other haplotypes derived from the node N1 and consisted of haplotypes identified in Brazil, in Argentina, and in the department of Tarija. It is important to note that the department of Tarija has an Andean area to the west and non-Andean area to the east as it includes part of the Gran Chaco. The accurate geographical origin of the bugs corresponding to haplotypes of Tarija described in the bank remains unknown.

The link between ITS-2 and mtCytB haplotypes was analyzed after classification of the haplotypes in "Andean" and "non-Andean" haplo-groups according to the NJ trees (Table 6). The statistical analysis did not reveal a significant association: the bugs with "Andean" ITS-2 (ITS2Hap1) were carrying either an "Andean" or "non-Andean" mtCytB and vice versa ($Chi2 = 0.36$, $df = 1$, $p > 0.05$).

4. Discussion

4.1. Origin of reinfestation revealed by population genetics

A quick recolonization of treated areas, or even a persistence of domestic and peridomestic triatomine colonies, occur in Izozog region as throughout the Gran Chaco (Cecere et al., 2004; Zu Dohna et al., 2009). Therefore, in the 4 studied villages an early dwelling reinfestation was detected 5 and 9 months post-spraying (12.2% and 7.7% of reinfested houses, respectively; unpublished data). The populations after insecticide treatment may have an exogenous or local origin. In the case of an exogenous origin, two phenomena can be conferred: the intrusion of sylvan insects from the surrounding environment where triatomines are associated with wild mammals, and the introduction of domestic insects transported by man from other villages not well controlled. A local origin of the reinfestation derives from residual populations because of the reduced effectiveness of pyrethroids in peridomestic and/or domestic habitats.

In such a context, population genetics is one way of assessing the origin of populations after spraying because *T. infestans* is generally highly structured (see Section 1). At the present geographical (neighboring villages) scale, an absence of genetic differentiation was expected for ITS-2 because this marker presents low variability related to a slow molecular clock (Bargues et al., 2006). Likely, no genetic differentiation was observed between the villages. On the contrary, the mtCytB sequence analysis provided a significant genetic differentiation between the four studied villages under study. However, at this small geographical scale, the genetic differentiation did not fit a model of isolation by distance as shown at larger geographical scales (Brenière et al., 1998; Dujardin et al., 1998; Piccinali et al., 2009). A strong structuring has already been found among *T. infestans* populations of nearby villages (less than 2 km) in extremely rugged areas in the Bolivian Andes, where many geographical barriers are likely to isolate populations (Brenière et al., 1998). Also, several studies have shown that the panmictic unit could be smaller than a village (Cecere et al., 2004; Perez de Rosas et al., 2007; Pizarro et al., 2008). All these results show that, in *T. infestans*, interbreeding is restricted by short active dispersal capacity. This explains the structure of populations in the villages of the Izozog area even though no apparent geographical barriers exist. These results also suggest that the possible human mediated passive transport of triatomines from one village to another would not be sufficient to homogenize the population between the villages studied. The passive transport of triatomines should not be a frequent phenomenon, occurring only in special circumstances, and allowing the expansion in stages of *T. infestans* to new regions (Piccinali et al., 2010).

The two populations after spraying showed significant genetic differentiation, each one being more similar to their respective population before spraying than to other populations. This result supports the hypothesis of a local origin from residual specimens as previously found (Dujardin et al., 1996, 1997; Perez de Rosas et al., 2007, 2008; Marcet et al., 2008; Pizarro et al., 2008). One of the mechanisms by which *T. infestans* specimens recur in the villages is the development of a pyrethroid resistance (Picollo et al., 2005; Orihuela et al., 2008; Toloza et al., 2008; Lardeux et al., 2010). However, other factors such as the reduced effectiveness of pyrethroids in peridomestic environment (lack of insecticide stability caused by climatic conditions, intricate peridomestic structures) or the incomplete covering of the village leaving uncontrolled/untreated dwellings, may play a role (Gürtler, 1999). Our results do not show significant modifications of the genetic diversity in the vector populations before and after the spraying as previously observed (Perez de Rosas et al., 2007), and the neutrality tests show that the populations are in equilibrium (data not shown). On the contrary, it was recently reported that recurrent insecticide spraying could result in populations not in equilibrium, and bottlenecks followed by possible population growths were observed (Piccinali et al., 2009). The absence of post-spraying genetic modifications in the studied villages suggests that part of the population was not reached by insecticides, and that additional factors to bug resistance are involved. Local evaluation of the level of resistance as well as of other possible mechanisms responsible for the reemergence of *T. infestans* remains to be performed in these villages.

4.2. Relationships between the present and GenBank Haplotypes

Previous analysis of composite ITS-1-ITS-2 haplotypes indicated a differentiation between western ("Andean") and eastern ("non-Andean") populations of *T. infestans* (Bargues et al., 2006). In the current study, we report the co-occurrence in villages of the Gran Chaco of an "Andean" haplotype (ITS2Hap1), with another one (ITS2Hap4) similar to a "non-Andean" haplotype. The third haplotype is new (ITS2Hap2), and closely related to "non-Andean" haplotypes found in Argentina, Brazil, and in sylvan *T. infestans* "dark-morph" from the Izozog region (Fig. 3). The haplotype ITS2Hap4 was the most frequent (74.5%) but the "Andean" haplotype (ITS2Hap1) was fairly frequent (~20%). The topology

of the present NJ tree constructed with all ITS-2 haplotypes seems to confirm the existence of two groups of haplotypes, "Andean" and "non-Andean", but the bootstrap values are low. Low values occur whatever the traditional phylogenetic method used, e.g., the Maximum Likelihood, (PHYML program) method give a bootstrap value slightly higher than that obtained by the NJ method (Fig. 3) for the "non-Andean" group (53%). The same occurred in the previous analysis with composite ITS1-ITS2 sequences (Bargues et al., 2006). The low number of informative sites in these sequences and the assumption of a recent expansion of *T. infestans* could explain the absence of a strong structuring and an ambiguous clustering of "Andean" and "non-Andean" populations. In fact, a tentative estimate of divergence time between "Andean" and "non-Andean" groups did not allow to confirm a recent expansion (Bargues et al., 2006). Assuming an earlier divergence, the ITS-2 marker appears to have a mutation rate too low for strong structuring of *T. infestans* species.

The likely dichotomy of *T. infestans* suggested by ITS-2 haplotypes is in part supported by the analysis of mtCytB sequence variability. Indeed, this marker identified two clusters in the studied set of haplotypes (NJ tree, Fig. 4). However, the two main genetic groups seen in the NJ tree built from all the available haplotypes in GenBank are not support by strong bootstrap values. Network construction has been proposed to explore intraspecific phylogeny instead of traditional approaches based on hierarchical tree construction developed for interspecific relationships (Posada and Crandall, 2001). The present topology of the median network clarifies the relationships between the mtCytB haplotypes, highlighting two principal nodes of expansion. From the first node, which includes the major haplotype CytBHapU, derive haplotypes exclusively sampled in non-Andean areas and some sampled in the department of Tarija whose geographic origin was unknown (Andean or non-Andean part of the department). This feature is consistent with the hypothesis of a recent transport of *T. infestans* by human to Brazil, probably from the Gran Chaco region (Silveira et al., 1984; Panzera et al., 2004). The second expansion node originated from a haplotype identified in the department of Chuquisaca (Bolivian Andes), comprises several haplotypes from this department, and others from the Izozog villages. Human mediated passive transport between domestic areas could explain the arrival in the Chaco (domestic area) of *T. infestans* populations from the Andes. Moreover, the analysis of the Izozog specimens shows no linkage between the different ITS-2 and mtCytB haplotypes, suggesting that the domestic populations of the villages studied could be the result of interbreeding between local populations and migrants from the Andes.

It was proposed that "Andean" and "non-Andean" populations have diverged at least 59,000 years ago, even as early as to 200,000 to 400,000 years ago (Bargues et al., 2006). This suggests that, before human colonization, each population would have undergone a long process of evolution leading to genetic diversification in each area. After human colonization, *T. infestans* would have easily adapted to human habitats in both regions because of its high behavioral plasticity (*T. infestans* has a wide variety of habitat, and feeding hosts in its natural environment). The close genetic relationship between ITS2Hap2 (domestic area) and a haplotype previously detected in a sylvan "dark morph" specimen (collected more than 50 kms from any dwelling in Izozog region), shows that the domestic population of Izozog could also have a local origin. Moreover, ITS2Hap2 and mtcytBHapO also detected in the sylvatic site above-mentioned further support a local domestication event (E. Waleckx et al., submitted). The high genetic diversity and the large number of haplotypes observed in the Brazilian sample of *T. infestans* is somewhat surprising but it could be related to a massive importation of triatomines from an area with high genetic diversity caused by a long-lasting evolution. The high mtCytB diversity observed in a small area of the Gran Chaco (present data), and the great genetic diversity recently observed in domestic Argentinean *T. infestans* populations (Gran Chaco region) by using another mitochondrial marker (Piccinali et al., 2009) suggest that this region could be the Gran Chaco. Moreover, according to the feature of the present network, the mtCytB haplotypes previously indentified in the Andes (Pizarro et al., 2008) are all contracted together (N2), and derived from the node N1 (non-andean haplotypes). This last observation did not support an Andean origin of the Brazilian haplotypes.

Acknowledgments

We thank Roberto Vargas and Nestor Suarez (SEDES, Chagas program, Santa-Cruz), Teresa Gutierrez (FAN, Santa-Cruz), Zaira Barja and Arnoldo Roca (CENETROP, Santa-Cruz). We are particularly grateful to the direction of the Inlasa (Instituto de Laboratorios de Salud, La Paz Bolivia), Dr. José Raphael Gutiérrez and Dr. Walter Agreda to have hosted this work in the Department of Entomology directed by Dr. Tamara Chavez. This work was funded by the National Agency for Research (ANR, France), the UNICEF/UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases (TDR, No. A70596), the IDRC Canada Grant No. 103696-012, and the IRD France.

References

Assumpcao, T.C., Francischetti, I.M., Andersen, J.F., Schwarz, A., Santana, J.M., Ribeiro, J.M., 2008. An insight into the sialome of the blood-sucking bug *Triatoma infestans*, a vector of Chagas' disease. Insect Biochem. Mol. Biol. 38, 213–232.

Bargues, M.D., Klisiowicz, D.R., Panzera, F., Noireau, F., Marcilla, A., Perez, R., Rojas, M.G., O'Connor, J.E., Gonzalez-Candelas, F., Galvao, C., Jurberg, J., Carcavallo, R.U., Dujardin, J.P., Mas-Coma, S., 2006. Origin and phylogeography of the Chagas disease main vector *Triatoma infestans* based on nuclear rDNA sequences and genome size. Infect. Genet Evol. 6, 46–62.

Belkhir, K., Borsa, P., Goudet, J., Chikhi, L., Bonhomme, F., 2001 GENETIX 4.02, logiciel sous WindowsTM pour la génétique des populations. Laboratoire Génome, Populations, Interactions CNRS UMR 5000. Université de Montpellier II, Montpellier (France)

Brenière, S.F., Bosseno, M.F., Vargas, F., Yaksic, N., Noireau, F., Noel, S., Dujardin, J.P., Tibayrenc, M., 1998. Smallness of the panmictic unit of *Triatoma infestans* (Hemiptera: Reduviidae). J. Med. Entomol. 35, 911–917.

Cecere, M.C., Vasquez-Prokopec, G.M., Gurtler, R.E., Kitron, U., 2006. Reinfestation sources for Chagas disease vector, *Triatoma infestans*, Argentina. Emerg. Infect. Dis. 12, 1096–1102.

Cecere, M.C., Vazquez-Prokopec, G.M., Gurtler, R.E., Kitron, U., 2004. Spatio-temporal analysis of reinfestation by *Triatoma infestans* (Hemiptera: Reduviidae) following insecticide spraying in a rural community in northwestern Argentina. Am. J. Trop Med Hyg. 71, 803–810.

Dias, J.C., 2007. Southern Cone Initiative for the elimination of domestic populations of *Triatoma infestans* and the interruption of transfusional Chagas disease. Historical aspects, present situation, and perspectives. Mem. Inst. Oswaldo Cruz 102 (Suppl. 1), 11–18.

Dujardin, J.P., Bermudez, H., Schofield, C.J., 1997. The use of morphometrics in entomological surveillance of sylvatic foci of *Triatoma infestans* in Bolivia. Acta Trop. 66, 145–153.

Dujardin, J.P., Cardozo, L., Schofield, C., 1996. Genetic analysis of *Triatoma infestans* following insecticidal control interventions in central Bolivia. Acta Trop. 61, 263–266.

Dujardin, J.P., Schofield, C.J., Tibayrenc, M., 1998. Population structure of Andean *Triatoma infestans*: allozyme frequencies and their epidemiological relevance. Med. Vet. Entomol. 12, 20–29.

Edwards, J.K., 1998. In: Karp, A., Issac, P.G., Ingram, D.S. (Eds.), Miniprep Procedures for the Isolation of Plant DNA. Chapman & Hall, London.

Excoffier, L.G., Laval, G., Schneider, S., 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol. Bioinform. 1, 47–50.

Giordano, R., Cortez, J.C., Paulk, S., Stevens, L., 2005. Genetic diversity of *Triatoma infestans* (Hemiptera: Reduviidae) in Chuquisaca, Bolivia based on the mitochondrial cytochrome b gene. Mem. Inst. Oswaldo Cruz 100, 753–760.

Gurtler, R.E., 1999. Control campaigns against *Triatoma infestans* in a rural community of northwestern Argentina. Medicina 59, 47–54.

Hall, T.A., 1999. BioEdit a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Lardeux, F., Depickere, S., Duchon, S., Chavez, T., 2010. Insecticide resistance of *Triatoma infestans* (Hemiptera, Reduviidae) vector of Chagas disease in Bolivia. Trop. Med. Int. Health.

Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452.
Lyman, D F, Monteiro, F.A., Escalante, A.A., Cordon-Rosales, C, Wesson, D.M., Dujardin, J.P., Beard, C.B., 1999. Mitochondrial DNA sequence variation among triatomine vectors of Chagas' disease. Am. J. Trop. Med. Hyg. 60, 377–386.
Marcet, P.L., Mora, M.S., Cutrera, A.P., Jones, L., Gurtler, R.E., Kitron, U., Dotson, E.M., 2008. Genetic structure of *Triatoma infestans* populations in rural communities of Santiago del Estero, northern Argentina. Infect. Genet. Evol. 8, 835–846.
Marcilla, A., Bargues, M.D., Ramsey, J.M., Magallon-Gastelum, E., Salazar-Schettino, P.M., Abad-Franch, F., Dujardin, J.P., Schofield, C.J., Mas-Coma, S., 2001. The ITS-2 of the nuclear rDNA as a molecular marker for populations, species, and phylogenetic relationships in Triatominae (Hemiptera: Reduviidae), vectors of Chagas disease. Mol Phylogenet Evol. 18, 136–142.
Martinez, F.H., Villalobos, G.C., Cevallos, A.M, Torre Pde, L., Laclette, J.P., Alejandre-Aguilar, R., Espinoza, B., 2006. Taxonomic study of the Phyllosoma complex and other triatomine (Insecta: Hemiptera: Reduviidae) species of epidemiological importance in the transmission of Chagas disease using ITS-2 and mtCytB sequences. Mol Phylogenet. Evol. 41, 279–287
Monteiro, F.A., Perez, R., Panzera, F., Dujardin, J.P., Galvao, C., Rocha, D., Noireau, F., Schofield, C., Beard, C.B., 1999. Mitochondrial DNA variation of *Triatoma infestans* populations and its implication on the specific status of *T. melanosoma*. Mem. Inst. Oswaldo Cruz 94 (Suppl 1), 229–238.
Noireau, F., Flores, R., Gutierrez, T., Dujardin, J.P., 1997. Detection of sylvatic dark morphs of *Triatoma infestans* in the Bolivian Chaco. Mem. Inst. Oswaldo Cruz 92, 583–584.
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. J. Med. Entomol. 45, 298–306.
Panzera, F., Dujardin, J.P., Nicolini, P., Caraccio, M.N., Rose, V., Tellez, T., Bermudez, H., Bargues, M.D., Mas-Coma, S., O'Connor, J.E., Perez, R., 2004. Genomic changes of Chagas disease vector, South America. Emerg. Infect. Dis. 10, 438–446.
Perez de Rosas, A.R., Segura, E.L., Fichera, L., Garcia, B.A., 2008. Macrogeographic and microgeographic genetic structure of the Chagas' disease vector *Triatoma infestans* (Hemiptera: Reduviidae) from Catamarca, Argentina. Genetica 133, 247–260.
Perez de Rosas, A.R., Segura, E.L., Garcia, B.A., 2007. Microsatellite analysis of genetic structure in natural *Triatoma infestans* (Hemiptera: Reduviidae) populations from Argentina: its implication in assessing the effectiveness of Chagas' disease vector control programmes Mol Ecol. 16, 1401–1412.
Piccinali, R.V., Canale, D M., Sandoval, A.E., Cardinal, M.V., Jensen, O., Kitron, U., Gurtler, R.E., 2010. *Triatoma infestans* bugs in Southern Patagonia Argentina Emerg. Infec. Dis. 16, 887–889.
Piccinali, R.V., Marcet, P.L., Noireau, F., Kitron, U., Gurtler, R.E., Dotson, E.M., 2009. Molecular population genetics and phylogeography of the Chagas disease vector *Triatoma infestans* in South America. J. Med. Entomol. 46, 796–809.
Picollo, M.I., Vassena, C., Santo Orihuela, P., Barrios, S., Zaidemberg, M., Zerba, E., 2005. High resistance to pyrethroid insecticides associated with ineffective field treatments in *Triatoma infestans* (Hemiptera: Reduviidae) from Northern Argentina. J. Med. Entomol. 42, 637–642.
Pizarro, J.C., Gilligan, L.M., Stevens, L., 2008. Microsatellites reveal a high population structure in *Triatoma infestans* from Chuquisaca, Bolivia. PLoS Negl. Trop. Dis. 2, e202.
Posada, D., Crandall, K A, 2001. Intraspecific gene genealogies: trees grafting into networks. Trends Ecol. Evol. 16, 37–45.
Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
Silveira, A.C., Ramos Feitosa, V., Borges, R., 1984. Distribuçäo de Triatomíneos capturados no ambiente domiciliar, no período 1975/83, Brasil. Rev. Bras. Malariol. Doencas Trop. 36, 15–312.
Toloza, A.C., Germano, M., Cueto, G.M., Vassena, C., Zerba, E., Picollo, M.I., 2008. Differential patterns of insecticide resistance in eggs and first instars of *Triatoma infestans* (Hemiptera: Reduviidae) from Argentina and Bolivia. J. Med. Entomol. 45, 421–426.
Zu Dohna, H., Cecere, M.C., Gurtler, R.E, Kitron, U., Cohen, J.E., 2009. Spatial re-establishment dynamics of local populations of vectors of Chagas disease PLoS Negl. Trop. Dis. 3, e490.

Annexe 23

Génétique des populations naturelles de *Triatoma infestans* sylvestres et domestiques

1- Génétique des populations de *T. infestans* dans trois localités de Bolivie

La méthode MLMT (Multilocus Microsatellite Typing) a permis d'étudier la génétique des populations naturelles de *T. infestans* sylvestres et domestiques de trois localités, Sapini, Quillacollo et Tagho Tagho. Au total, 292 insectes distribués en 21 populations, (11 domestiques et 10 sylvestres) ont été étudiés en utilisant 8 locus microsatellites.

Les trois groupes de populations géographiques apparaissent séparées dans les arbres construits à partir des F*st* comme dans les arbres utilisant les distances génétiques de Cavalli-Sforza (figure 1), néanmoins les valeurs de bootstrap aux nœuds regroupant les populations des localités ne sont pas significatives.

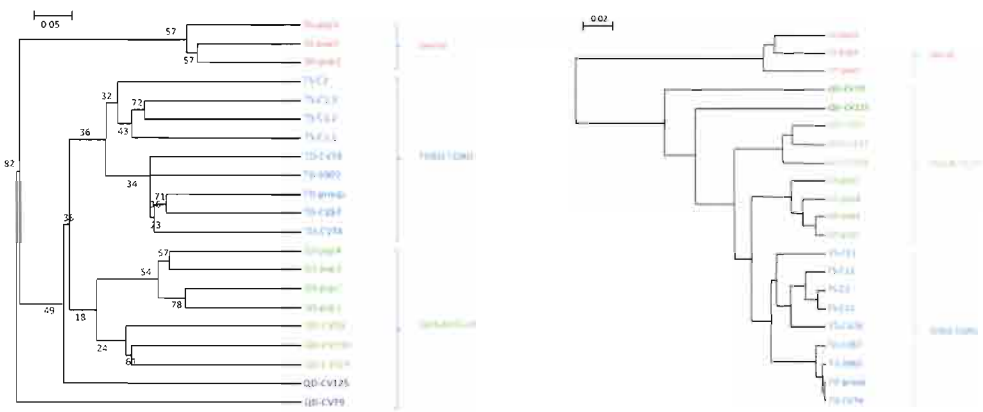


Figure 1 : A gauche arbre des 21 populations de T. infestans *étudiées, construit à partir des distances de Cavalli-Sforza avec les valeurs de bootstrap aux nœuds ; à droite arbre construit à partir des* Fst *entre paires de populations.*

La diversité génétique moyenne est très haute dans les deux cycles (*Hs* domestique = 0,71, *Hs* sylvestre = 0,68), voir Table 1 ci-dessous.

Table 1 Indices de variabilité des 21 populations de *Triatoma infestans* étudiées au moyen de huit loci microsatellites

Localité	Quillacollo									Sapini			Toro Toro								
Cycle	Domestique					Sylvestre				Domestique	Sylvestre		Domestique					Sylvestre			
Nom des populations	CV119UP4	CV125UR8	CV134UA	CV59UR3	CV79UR7	pop1	pop2	pop3	pop4	pop1	pop2	pop3	CV74	CV78	CV87	group	SN02	C1-1	C1-2	C1-3	C2
N° des populations	pop1	pop2	pop3	pop4	pop5	pop6	pop7	pop8	pop9	pop10	pop11	pop12	pop13	pop14	pop15	pop16	pop17	pop18	pop19	pop20	pop21
Effectif	16	9	11	19	12	15	14	22	12	19	9	10	12	16	15	15	16	15	16	14	5
Diversité génétique	0,66	0,67	0,73	0,76	0,72	0,69	0,72	0,74	0,72	0,45	0,43	0,37	0,78	0,79	0,77	0,77	0,75	0,79	0,74	0,73	0,77
Richesse allélique	4,00	3,95	4,48	4,77	5,32	4,20	4,11	4,53	4,44	2,67	2,77	2,05	4,77	5,20	4,69	4,78	4,57	4,80	4,27	4,45	4,25
Fis moyens	0,29	0,12	0,36	0,25	0,20	0,02	0,05	0,12	0,16	0,43	0,25	0,29	0,35	0,37	0,30	0,38	0,29	0,33	0,23	0,24	0,29
Intervalles confiance 95%																					
valeur mini	0,16	-0,14	0,14	0,14	0,03	-0,13	-0,11	-0,01	0,01	0,29	0,07	0,03	0,14	0,19	0,15	0,20	0,17	0,08	0,07	0,08	-0,10
valeur maxi	0,36	0,20	0,52	0,28	0,24	0,09	0,08	0,18	0,20	0,53	0,32	0,52	0,50	0,47	0,34	0,44	0,34	0,40	0,31	0,31	0,35
Significativité des Fis	S	NS	S	S	S	NS	NS	NS	NS	S	NS	NS	S	S	S	S	S	S	S	S	NS

La majorité des valeurs de F*is* sont positives et significativement différentes de 0 dans les cycles domestiques, c'est le contraires dans les cycles sylvestres (figure 2) ; ceci suggère plus de structuration génétique en cycle domestique qu'en cycle sylvestre, soit davantage de panmixie en cycle sylvestre qu'en cycle domestique. La valeur F*is* du groupe domestique (F*is* = 0,31) versus sylvestre (F*is*= 0,18) est d'ailleurs la seule variable significativement différente entre les deux cycles. L'explication la plus probable est un effet Wahlund fort en cycle domestique généré par la superposition de micro-effets fondateurs dans ces populations générant ainsi des microstructures locales (cohabitation de plusieurs colonies d'origines différentes).

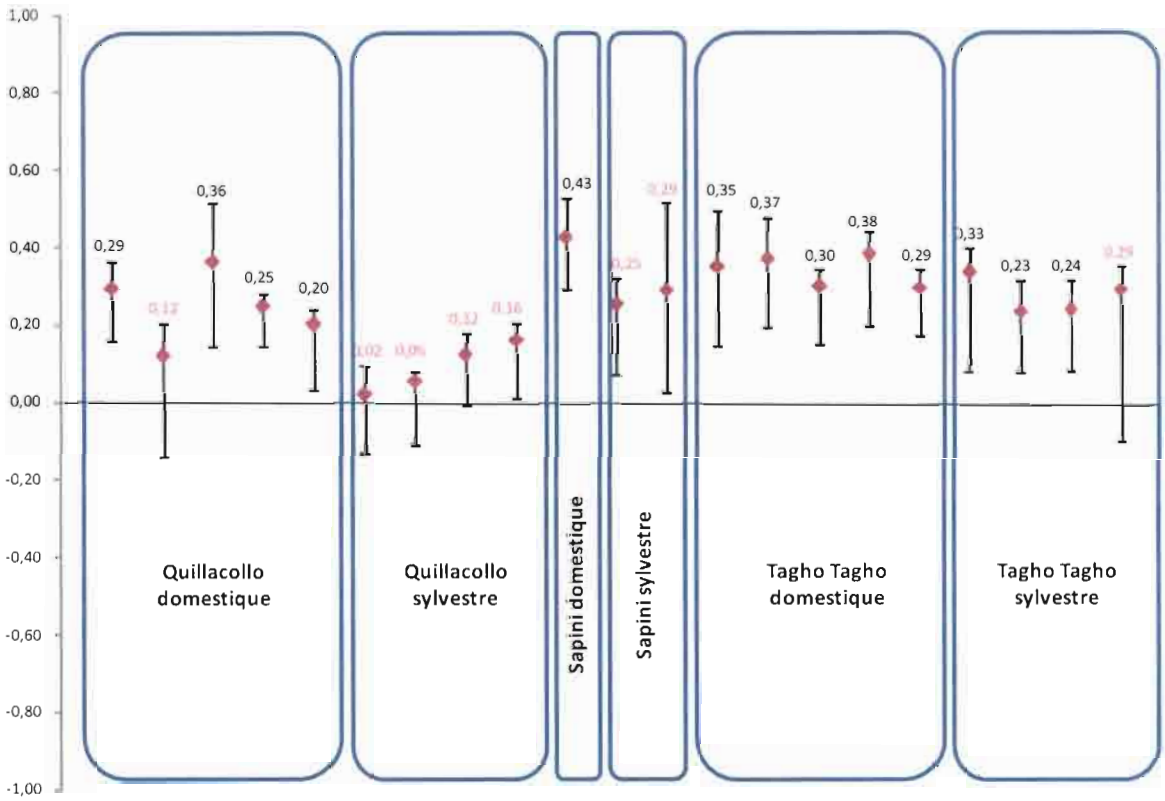
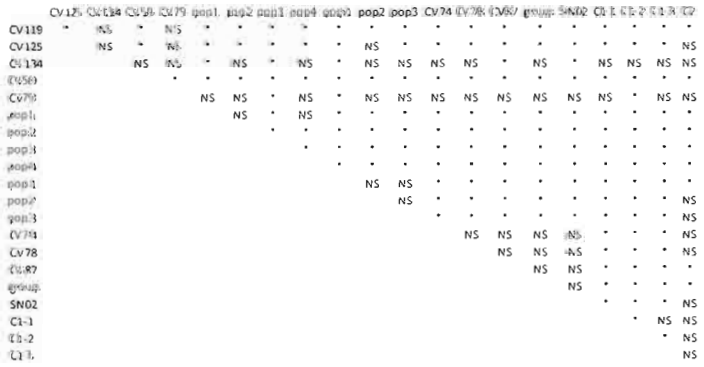


Figure 2. Intervalles de confiance à 95% des valeurs de Fis des 21 populations de Triatoma infestans *étudiées. Les valeurs de Fis en rouge ne sont pas significativement différentes de zéro.*



L'analyse de la matrice des F*st* entre paires de populations a révélé un haut niveau de différentiation entre populations puisque seulement 26,7% des valeurs de F*st* sont non significatives entre paires de populations contre 73,3% significatives (Table 2).

Table 2. Matrice des Fst *entre paires de populations sur les 21 populations de* T. infestans *étudiées. NS = valeur non significative, * = valeur significative.*

2. Génétique des populations de *T. infestans* à Sapini

Trois populations ont été étudiées, une domestique (N = 19) et deux sylvestres (N = 10 et N = 9), cf. Table 1. Les arbres de F*st* et de Cavalli-Sforza montrent une séparation entre l'unique population domestique et les deux populations sylvestres, mais cette structure n'est pas significative. La valeur F*is* de la pop domestique est positive et significative (figure 2), les F*is* des deux pops sylvestres ne sont pas significativement différentes de 0. Ceci illustre le cas général évoqué plus haut de panmixie en milieu sylvestre et de structure cachée en milieu domestique.

Le logiciel « Structure » nous a montré que le nombre de populations théoriques attendues (k) varie de 3 à 4 en fonction des modèles utilisés. Quel que soit le modèle utilisé (« admixture » *i. e. les individus peuvent provenir génétiquement de deux populations différentes* ou « no admixture » *i. e. les individus proviennent génétiquement que d'une population unique*), les 3 ou 4 types de génomes sont présents dans les trois populations (domestique et sylvestres, voir figure 3), ce qui démontre une absence de structure « domestique versus sylvestre » et par conséquent des échanges génétiques intensifs dus à des mouvements fréquents d'insectes entre les deux milieux dans cette localité.

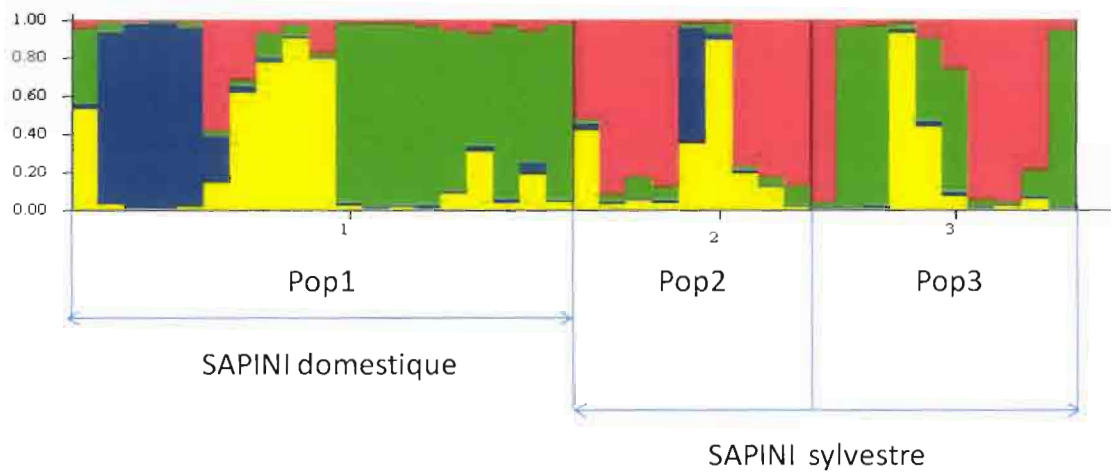


Figure 3 : Représentation graphique de l'origine génétique estimée des 38 T. infestans *récoltés à Sapini. Les probabilités d'appartenance de chaque insecte aux populations inférées (ici k = 4 soit 4 populations) par le modèle d'analyse (ici « admix ») est représenté par une ligne verticale divisée en fragments de différentes couleurs qui correspondent aux populations inférées.*

3. Génétique des populations de *T. infestans* à Quillacollo

Ici 130 insectes ont été étudiés distribués en 9 populations, 5 domestiques et 4 sylvestres (voir Table 1 pour le détail des effectifs). Contrairement à Sapini, les arbres de F*st* ou de distance de Cavalli-Sforza ne séparent même pas les populations domestiques des populations sylvestres. Les populations domestiques issues des unités CV79 et CV125 sont très différentes des autres pops domestiques et sont le résultat probable d'effets fondateurs (figure 4).

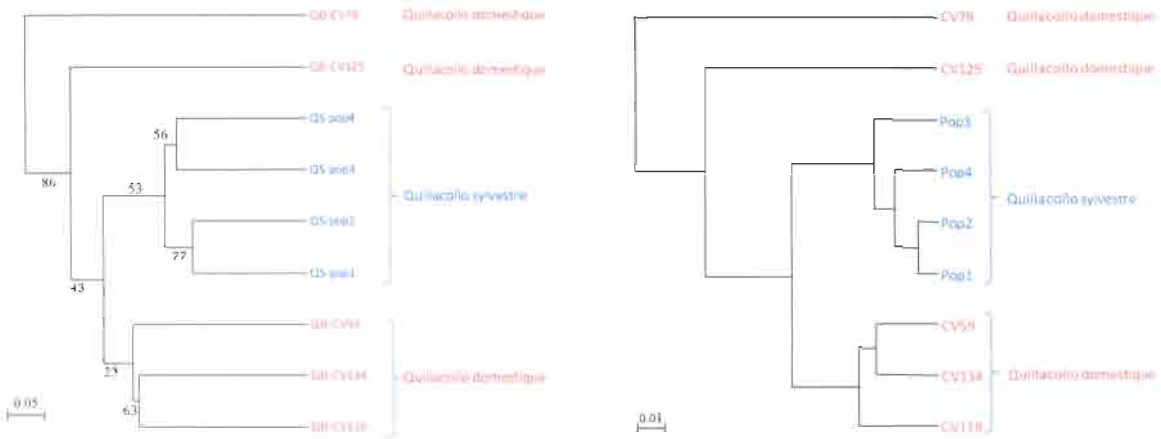


Figure 4 : A gauche arbre des 9 populations de T. infestans *étudiées à Quillacollo construit à partir des distances de Cavalli-Sforza avec les valeurs de bootstrap aux nœuds ; à droite même arbre construit à partir des valeurs de Fst entre paires de populations.*

Les valeurs de *Fis* des populations domestiques sont toutes significativement positives (sauf pour CV125), c'est le contraire pour les populations sylvestres (figure 2), ceci confirme le cas général de panmixie en milieu sylvestre et de forte structuration génétique en milieu domestique (voir figure 2).

Le nombre de populations théoriques attendues (k du logiciel « Structure ») varie de 2 à 3 en fonction des modèles utilisés. Le modèle le moins contraint « admix » avec ou sans l'option « prior location » qui peut aider dans le cas de populations faiblement structurées nous a proposé le même nombre de populations théoriques (k=3), voir figure 5.

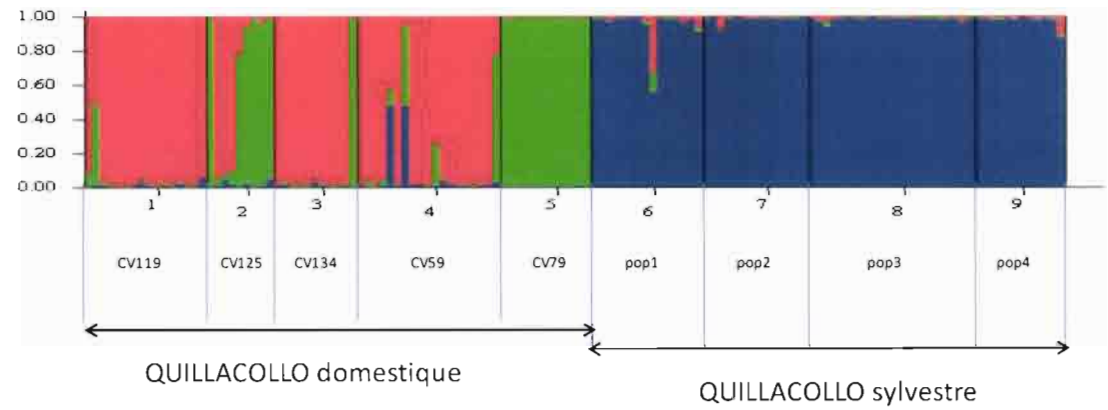


Figure 5 : Représentation graphique de l'origine génétique estimée des 130 T. infestans *récoltés à Quillacollo. Les probabilités d'appartenance de chaque insecte aux populations inférées (k = 3) par le modèle d'analyse (« admix » et « admix » avec option «prior location ») est représenté par une ligne verticale divisée en fragments de différentes couleurs qui correspondent aux populations inférées.*

Cette figure montre une structuration importante entre sylvestre et domestique et au sein du domestique une autre séparation entre deux types de génomes, l'un présent dans les populations CV119, CV134 et CV 59 et l'autre présent dans CV79 et CV125. A noter que la

population CV125 est un conglomérat de ces deux génomes, ce qui explique qu'elle soit séparée de CV79, plus homogène génétiquement. A noter aussi la présence de deux génomes hybrides entre domestique et sauvage dans la population CV59, ce qui montre que même dans ce cas de séparation entre sylvestre et domestique, quelques individus peuvent migrer du sylvestre vers le domestique. Il est important de souligner la différence avec la situation à Sapini qui montrait un intense brasage entre domestique et sylvestre, ici ce n'est pas le cas, il y a une forte structure domestique vs. sylvestre accompagnée de quelques rares passages d'individus sylvestres vers le domestique.

4. Génétique des populations de *T. infestans* à Tagho Tagho

Ici 124 insectes ont été analysés, distribués en 9 populations, 5 domestiques et 4 sylvestres (voir Table 1 pour le détail des effectifs).

L'arbre construit à partir des distances de Cavalli-Sfrorza sépare les populations domestiques de sylvestres mais de manière non significative. En revanche l'arbre construit à partir des F*st* ne sépare pas domestique de sylvestre. La population domestique CV78 apparait comme un intermédiaire entre les deux groupes (figure 6).

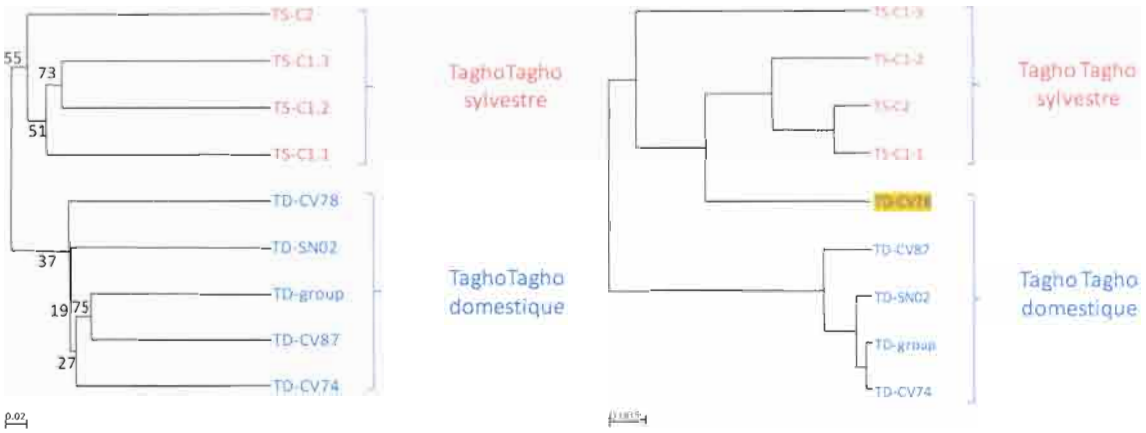


Figure 6 : A gauche arbre des 9 populations de T. infestans *étudiées à Tagho Tagho construit à partir des distances de Cavalli-Sforza avec les valeurs de bootstrap aux nœuds ; à droite même arbre construit à partir des valeurs de* Fst *entre paires de populations.*

Comme pour les autres localités les F*is* des populations sylvestres sont en équilibre de Hardy-Weinberg (ne différent pas significativement de 0) alors que les F*is* des populations domestiques montrent des F*is* significativement positifs, montrant ainsi une structure cachée comme précédemment évoqué.

Les deux modèles de populations théoriques utilisés par « Structure » avec ou sans option « prior location » ont montré un nombre de populations théoriques identiques (k = 3), c'est-à-dire la présence de trois grands groupes de génotypes dans ces populations. Ces trois types sont représentés dans les deux cycles bien qu'il y ait une prédominance de l'un des génotypes dans le cycle sylvestre. Ceci montre des échanges fréquents entre cycle sylvestre et domestique dans cette localité. La population sylvestre la plus homogène est la pop C1-2. A noter la présence d'individus domestiques dans la population sylvestre C1-1 ainsi que la présence d'individus sylvestres dans les pops domestiques CV74 et CV78 (Figure 7).

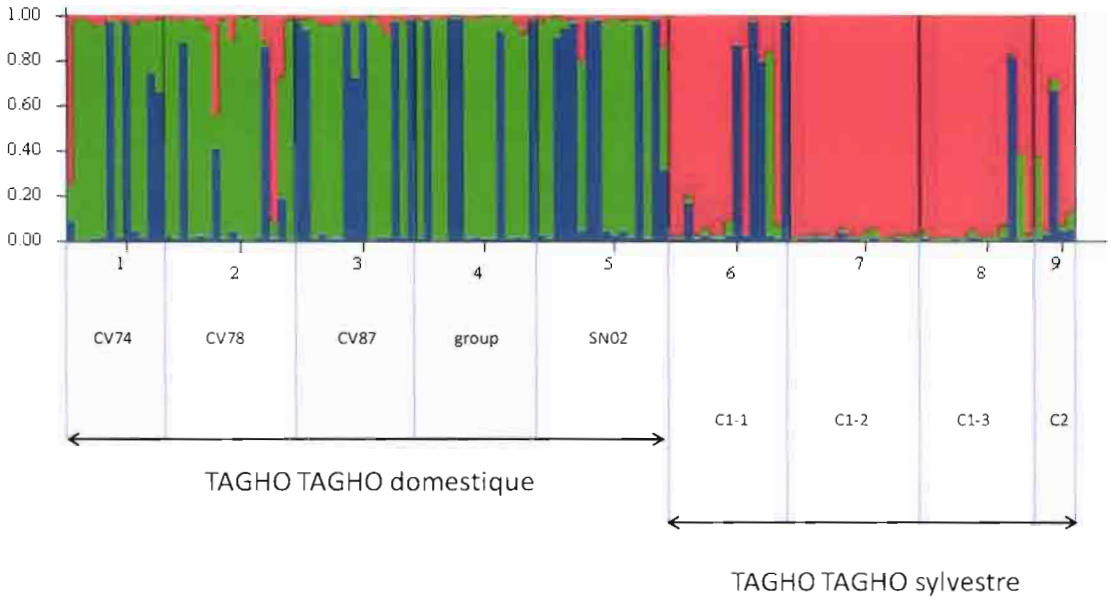


Figure 7 : Représentation graphique de l'origine génétique estimée des 124 T. infestans *récoltés à Tagho Tagho. Les probabilités d'appartenance de chaque insecte aux populations inférées (k = 3) par les deux modèles d'analyse (« admix » et « admix » avec ou sans option « prior location ») est représenté par une ligne verticale divisée en fragments de différentes couleurs qui correspondent aux populations inférées.*