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Prevalence, Genetic Characterization, and 18S Small Subunit Ribosomal RNA Diversity of *Trypanosoma rangeli* in Triatomine and Mammal Hosts in Endemic Areas for Chagas Disease in Ecuador

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Abstract

Trypanosoma rangeli is a nonpathogenic parasite for humans; however, its medical importance relies in its similarity and overlapping distribution with *Trypanosoma cruzi*, causal agent of Chagas disease in the Americas. The genetic diversity of *T. rangeli* and its association with host species (triatomines and mammals) has been identified along Central and the South America; however, it has not included data of isolates from Ecuador. This study reports infection with *T. rangeli* in 18 genera of mammal hosts and five species of triatomines in three environments (domestic, peridomestic, and sylvatic). Higher infection rates were found in the sylvatic environment, in close association with *Rhodnius ecuadoriensis*. The results of this study extend the range of hosts infected with this parasite and the geographic range of the *T. rangeli* genotype KP1(−)/lineage C in South America. It was not possible to detect variation on *T. rangeli* from the central coastal region and southern Ecuador with the analysis of the small subunit ribosomal RNA (SSU-rRNA) gene, even though these areas are ecologically different and a phenotypic subdivision of *R. ecuadoriensis* has been found. *R. ecuadoriensis* is considered one of the most important vectors for Chagas disease transmission in Ecuador due to its wide distribution and adaptability to diverse environments. An extensive knowledge of the trypanosomes circulating in this species of triatomine, and associated mammal hosts, is important for delineating transmission dynamics and preventive measures in the endemic areas of Ecuador and Northern Peru.

Key Words: *Trypanosoma rangeli* characterization—*Rhodnius ecuadoriensis*—KP1(−)—Lineage C—SSU-rRNA—Loja Province—Manabí Province—Ecuador.

Introduction

The parasite *Trypanosoma rangeli* has been reported as being distributed extensively in Central and South America (Vallejo et al. 2009). Although it is considered nonpathogenic for humans, its medical relevance is related to its morphological and genetic similarity with *Trypanosoma cruzi*, which causes Chagas disease. *T. rangeli* is a member of the *T. cruzi* clade, a group of ≈18 species of trypanosomes that includes *T. cruzi* and its closest relatives (Cottontail et al.

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2014). Moreover, both *T. cruzi* and *T. rangeli* overlap their geographic distribution and share the same invertebrate (triatomines) and vertebrate (mammals) hosts. Mixed infection with both parasites has been reported (Vallejo et al. 1988, Pinto et al. 2006, Grijalva et al. 2011, 2012, Villacis et al. 2015). Chagas disease is listed by the World Health Organization (WHO) as one of the Neglected Topical Diseases that requires intensified research for integrated measures to improve health and social well-being of the affected populations (World Health Organization 2010). Within this scope, it is necessary to understand the host associations and genetic variation of *T. rangeli*, a parasite that could be misidentified as *T. cruzi*, resulting in confounding the diagnosis of Chagas disease (Guhl et al. 1985, Saldana et al. 2005, de Sousa et al. 2008).

T. rangeli is mainly transmitted by salivary inoculation, although oral transmission by ingestion of triatomines has also been proposed as an important epidemiological route of infection with trypanosomes (*T. rangeli* and *T. cruzi*) of mammalian hosts such as dogs (Montenegro et al. 2002, Pineda et al. 2011) and other mammals with grooming behavior. Distribution of *T. rangeli* is related to the distribution of the triatomines; however, it depends on the capacity of triatomines to harbor the infective stage of the parasite in the salivary glands (Guhl and Vallejo 2003). Most species of triatomines can transmit *T. cruzi*, but the transmission of *T. rangeli* has been more restricted to the triatomines of the genus *Rhodnius* (D'Alessandro and de Hincapie 1986, Vallejo et al. 2009). Experimental infection has been conducted in *Triatoma*, *Panstrongylus*, and *Rhodnius*, but only *Rhodnius* has demonstrated the presence of the infective forms of *T. rangeli* in the salivary glands (De Stefani Marquez et al. 2006). Natural or experimental infection has been demonstrated in 12 out of the 15 species of *Rhodnius* (Guhl and Vallejo 2003), although natural infection of the salivary glands has also been reported in the genus *Triatoma* (Marinkelle 1968).

Biochemical, immunological, and molecular characteristics have identified polymorphisms in *T. rangeli* strains isolated from different hosts (Grisard et al. 1999). However, the close association with the genus *Rhodnius* has defined the distribution of the different genotypes (Machado et al. 2001, Urrea et al. 2011). Two main groups (KP1[+] and KP1[−]) have been defined based on the presence/absence of the KP1 minicircle in the kinetoplast DNA (kDNA) (Vallejo et al. 2002). Additionally, analysis of the small subunit of ribosomal RNA (SSU-rRNA) has also identified the presence of five lineages (A, B, C, D, and E) in Central and South America (Maia Da Silva et al. 2007, 2009). Further analysis of the splice leader intergenic region (SL) confirmed that the different genotypes of *T. rangeli* circulate in association with vectors of the same evolutionary line, independently of the geographic distance (Urrea et al. 2011). Most recently, subdivision in KP1(−) has been detected by microsatellite typing (Sincero et al. 2015). Moreover, with the publication of the genome of *T. rangeli* (Stoco et al. 2014), the complexity of this parasite could be further addressed.

Multiple studies have evaluated the genetic diversity of *T. rangeli* along Central and South America. However, none of those have included samples from Ecuador. Even though the presence of *T. rangeli* has been reported previously in triatomines and mammals from central coastal and southern Ecuador (Pinto et al. 2006, Grijalva et al. 2011, 2012, Villacis

Table 1. Infection Rates with *Trypanosoma rangeli* of Mammal Host Species from Three Orders (Chiroptera, Didelphimorphia, and Rodentia) and Triatomines Vectors in Loja Province (Southern Ecuador) and Manabí Province (Central Coastal Ecuador) 2004–2012

	Domicile		*Peridomicile*		*Sylvatic*		*Total*	
Region/host/species	*No. samples*	T. rangeli *(%)*	*No. samples*	T. rangeli *(%)*	*No. samples*	T. rangeli *(%)*	*No. samples*	T. rangeli *(%)*
Southern Ecuador (Loja)	668	12 (2)	882	42 (5)	584	92 (16)	2134	146 (7)
Chiroptera[a]	—	—	—	—	49	1 (2)	49	1 (2)
Didelphimorphia[b]	1	0 (0)	57	0 (0)	85	6 (7)	143	6 (4)
Rodentia[c]	139	0 (0)	37	0 (0)	90	17 (19)	266	17 (6)
Triatomine	528	12 (2)	788	42 (5)	360	68 (19)	1676	122 (7)
Panstrongylus chinai	149	5 (3)	33	2 (6)	—	—	182	7 (4)
Panstrongylus rufotuberculatus	12	0 (0)	—	—	—	—	12	0 (0)
Rhodnius ecuadoriensis	167	5 (3)	617	39 (6)	360	68 (19)	1144	112 (10)
Triatoma carrioni	200	2 (1)	138	1 (1)	—	—	338	3 (1)
Western Ecuador (Manabí)	106	3 (3)	591	66 (11)	775	136 (18)	1472	205 (14)
Didelphimorphia[d]	—	—	35	1 (3)	12	0 (0)	47	1 (2)
Rodentia[e]	43	0 (0)	31	0 (0)	29	0 (0)	103	0 (0)
Triatomine	63	3 (5)	525	65 (12)	734	136 (19)	1322	204 (15)
Panstrongylus howardi	7	0 (0)	74	4 (5)	1	0 (0)	82	4 (5)
Panstrongylus rufotuberculatus	18	0 (0)	17	2 (12)	14	0 (0)	49	2 (4)
Rhodnius ecuadoriensis	38	3 (8)	434	59 (14)	719	136 (19)	1191	198 (17)
Total	774	15 (2)	1473	108 (7)	1359	228 (17)	3606	351 (10)

[a]Includes species from the genera *Artibeus*, *Desmodus*, and *Glossophaga.*
[b]Includes species from the genera *Didelphis*, *Marmosa*, and *Micoureus.*
[c]Includes species from the genera *Aegialomys*, *Akodon*, *Cavia*, *Mus*, *Rattus*, *Rhipidomys*, and *Sciurus.*
[d]Includes species from the genera *Didelphis*, *Marmosa*, and *Philander.*
[e]Includes species from the genera *Handleyomys*, *Hoplomys*, *Mus*, *Proechimys*, *Rattus*, *Rhipidomys*, *Sciurus*, and *Transandinomys.*

et al. 2015), a detailed picture of its distribution and prevalence in these areas has not been assessed. The aim of this study was to contribute information about the diversity of the *T. rangeli* populations that are circulating in mammals and triatomines in the southern province of Loja and the central coastal province of Manabí, both considered as endemic areas for Chagas disease (Black et al. 2007, 2009).

Materials and Methods

This study was carried out with DNA samples previously collected as part of the Chagas disease projects of the Center for Infectious and Chronic Disease Research (CIDCR) from 2004 to 2012. Samples of this study were collected in 50 rural communities (19 counties) from central coastal Ecuador (Manabí Province) and 53 rural communities (11 counties) from southern Ecuador (Loja Province) (Table 1). DNA was obtained from intestinal contents (Grijalva et al. 2012) of triatomines and blood samples from mammals (Pinto et al. 2006). An aliquot of each sample was inoculated in culture for *in vitro* trypanosomes isolation (Chiari et al. 1989, Yeo et al. 2007), and DNA was isolated from cultures that presented growth of parasites. All samples were obtained in strict accordance with the protocol approved by the Ohio University Institutional Animal Care and Use Committee (IACUC).

Differential PCR for T. rangeli *identification*

Initial screening for detection of natural infection with *T. rangeli* was carried out on 3606 DNA samples obtained from the intestinal contents of triatomines and blood from mammals (Table 1). Samples were amplified for the conserved domain of the minicircle of kDNA, as previously described by Vallejo et al. (1999) for the samples from 2004–2010 and Virreira et al. (2006) for the samples from 2011–2012. Differential detection between *T. cruzi* and *T. rangeli* was determined based on the size of the PCR products. A band of 330 bp was expected for *T. cruzi*, whereas a band of 760 bp together with bands of 300–450 bp defined *T. rangeli*. Confirmation of *T. rangeli* infection was carried out with further amplification of the D7a divergent region of the large subunit rRNA gene, as previously described (Souto et al. 1999). A band of 210 bp was expected for *T. rangeli*, whereas *T. cruzi* presented a band of 265 bp. A negative control (without DNA) and control DNA for *T. cruzi* and *T. rangeli* were used to validate the gels. Bands were detected in a 2% agarose gel.

Selection of T. rangeli *samples for genotyping*

Genotyping was carried out in a subset of 111 samples. From them, 68 were genotyped from the DNA obtained from intestinal contents/blood samples and 43 from DNA obtained from *T. rangeli* isolates in culture. This subset is representative of the geographic origin of the samples and includes a diversity of hosts (seven species of triatomines, and four species of mammals) (Table 2). The geographic area included 12 and 27 rural communities in Loja and Manabí Province, respectively.

Identification of T. rangeli *groups (KP1[+] and KP1[–]) by amplification of the conserved regions of the minicircles of kDNA*

The conserved regions of the minicircle of kDNA were amplified with the primers S35 (5′-AAA TAA TGT ACG GGT GGA GAT GCA TGA-3′), S36 (5′-GGG TTC GAT TGG GGT TGG TGT-3′), and KP1L (5′-ATA CAA CAC TCT CTA TAT CAG G-3′), as previously described (Vallejo et al. 2002). A band at 760 bp and fragments between 300 and 450 bp were expected in all samples. KP1(+) was identified by the presence of an additional band at 165 bp, whereas KP1(−) was identified by the absence of this fragment. Additionally, samples that could not be classified by the

Table 2. Percentage of *Trypanosoma rangeli* Samples Genotyped As KP1(−)/Lineage C Isolated from Triatomines and Mammalian Hosts in Loja Province (Southern Ecuador) and Manabí Province (Central Coastal Ecuador)

		T. rangeli *group*		T. rangeli *lineage*	
Region/host/species	*No. samples analyzed*	*KP1(−) (%)*	*N.D. (%)*	*C (%)*	*N.D. (%)*
Southern Ecuador (Loja)	49	48 (98)	1 (2)	35 (71)	14 (29)
Mammal					
Desmodus rotundus	1	1 (100)	—	—	1 (100)
Didelphis marsupialis	4	4 (100)	—	3 (75)	1 (25)
Rhipidomys spp.	3	3 (100)	—	2 (67)	1 (33)
Sciurus stramineus	3	3 (100)	—	2 (67)	1 (33)
Triatomine					
Panstrongylus chinai	5	5 (100)	—	2 (40)	3 (60)
Rhodnius ecuadoriensis	31	30 (97)	1 (3)	24 (77)	7 (23)
Triatoma carrioni	2	2 (100)	—	2 (100)	—
Western Ecuador (Manabí)	62	62 (100)	—	54 (87)	8 (13)
Mammal					
Didelphis marsupialis	1	1 (100)	—	—	1 (100)
Triatomine					
Panstrongylus howardi	3	3 (100)	—	1 (33)	2 (67)
Panstongylus rufotuberculatus	1	1 (100)	—	1 (100)	—
Rhodnius ecuadoriensis	57	57 (100)	—	52 (91)	5 (9)
Total	111	110 (99)	1 (1)	89 (80)	22 (20)

N.D., not determined.

TABLE 3. SAMPLES USED FOR MAXIMUM LIKELIHOOD PHYLOGENETIC ANALYSES AND RESPECTIVE GENBANK ACCESSION NUMBERS FOR THE 18S rRNA SEQUENCES

Isolate[a]	*Host*		*Locality*	*18S rRNA*
Lineage A				
San Augustin	Human	*Homo sapiens*	Colombia	AJ012417
Macias	Human	*Homo sapiens*	Colombia	AJ012415
H8GS	Human	*Homo sapiens*	Honduras	AY491744
SMH-03	Human	*Homo sapiens*	Guatemala	AY491739
SMH-79	Human	*Homo sapiens*	Guatemala	AY491740
MHOM/VE/99/CH-99	Human	*Homo sapiens*	Venezuela (Barinas)	AY491742
TryCC_533; MAN/VE/00/LOBITA	Dog	*Canis familiaris*	Venezuela (Barinas)	EF071572
220; AT-AEI	Monkey	*Saimiri sciureus*	Brazil (PA) Marajó	AY491747
202; AT-ADS	Monkey	*Saimiri sciureus*	Brazil (PA) Marajó	AY491746
353; Maloch-05	Monkey	*Callicebus m. cupreus*	Brazil (AC) P. de Castro	AY491750
369; ROma 01	Opossum	*Didelphis marsupialis*	Brazil (RO) Monte Negro	AY491748
382; ROma 06	Opossum	*Didelphis marsupialis*	Brazil (RO) Monte Negro	AY491749
P19	Opossum	*Didelphis marsupialis*	Brazil (MG) Uberaba	EF071573
P21	Opossum	*Didelphis marsupialis*	Brazil (MG) Uberaba	EF071574
Choachi	Triatomine	*Rhodnius prolixus*	Venezuela	AJ012414
Palma-2	Triatomine	*Rhodnius prolixus*	Venezuela	AY491741
TryCC_775; VE/9	Triatomine	*Rhodnius prolixus*	Venezuela (Barinas)	EF071575
TryCC_795; VE/3	Triatomine	*Rhodnius robustus* I	Venezuela (Trujillo)	EF071576
TryCC_677; ROR-20	Triatomine	*Rhodnius robustus* II	Brazil (RO) Monte Negro	EF071577
TryCC_701; ROR-62	Triatomine	*Rhodnius robustus* II	Brazil (RO) Monte Negro	EF071578
TryCC_704; ROR-85	Triatomine	*Rhodnius robustus* II	Brazil (RO) Monte Negro	EF071579
Lineage B				
AM80	Human	*Homo sapiens*	Brazil (AM) Rio Negro	AY491766
AM11	Human	*Homo sapiens*	Brazil (AM) Rio Negro	AY491758
207; AE-AAA	Monkey	*Cebuella pygmaea*	Brazil (AC) Rio Branco	AY491752
194; AE-AAB	Monkey	*Cebuella pygmaea*	Brazil (AC) Rio Branco	AY491753
233; 4–30	Monkey	*Saguinus l. labiatus*	Brazil (AC) Rio Branco	AY491756
238; 5–31	Monkey	*Saguinus l. labiatus*	Brazil (AC) Rio Branco	AY491754
236; 8–34	Monkey	*Saguinus f. weddelli*	Brazil (AC) Rio Branco	AY491755
205; M-12229	Monkey	*Aotus* sp.	Brazil (AM) Manaus	AY491757
416; 2495	Monkey	*Alouatta stramineus*	Brazil (AM) Rio Negro	AY491760
427; 2570	Monkey	*Callicebus lugens*	Brazil (AM) Rio Negro	AY491751
Saimiri	Monkey	*Saimiri sciureus*	Brazil (AM) Manaus	AY491768
Preguici	Sloth	*Choloepus didactylus*	Brazil (PA) Belém	AY491767
Legeri_10	Anteater	*Tamandua tetradactyla*	Brazil (PA) Belém	AY491769
Legeri_32	Anteater	*Tamandua tetradactyla*	Brazil (PA) Belém	AY491759
4176	Triatomine	*Rhodnius brethesi*	Brazil (AM) Rio Negro	EF071580
Lineage C				
PG	Human	*Homo sapiens*	Panama	AJ012416
1625	Human	*Homo sapiens*	El Salvador	AY491738
T. leeuwenhoeki	Sloth	*Choloepus didactylus*	Panama	AJ012412
RGB	Dog	*Canis familiaris*	Colombia	AJ009160
SO29	Triatomine	*Rhodnius pallescens*	Colombia (Sucre)	EF071581
G5	Triatomine	*Rhodnius pallescens*	Colombia (Sucre)	EF071582
TCE_694ci	Triatomine	*Panstrongylus chinai*	Ecuador (Coamine, Paltas) Loja	
TJQ_941ci	Triatomine	*Triatoma carrioni*	Ecuador (Jacapo, Quilanga) Loja	
TCQ_991ci	Triatomine	*Rhodnius ecuadoriensis*	Ecuador (Chaquizhca, Calvas) Loja	
MBJ_1411s	Opossum	*Didelphis marsupialis*	Ecuador (El Bejuco, Portoviejo) Manabi	
MCQ_1483k	Rodent	*Rhipidomys* spp.	Ecuador (Chaquizhca, Calvas) Loja	
MCQ_1487k	Rodent	*Sciurus stramineus*	Ecuador (Chaquizhca, Calvas) Loja	
MCQ_1516k	Opossum	*Didelphis marsupialis*	Ecuador (Chaquizhca, Calvas) Loja	
TBJ_1540ci	Triatomine	*Panstrongylus howardi*	Ecuador (El Bejuco, Portoviejo) Manabi	
TTO_2151ci	Triatomine	*Rhodnius ecuadoriensis*	Ecuador (Tablada del Algodón, Junin) Manabi	
TBJ_2239k	Triatomine	*Rhodnius ecuadoriensis*	Ecuador (El Bejuco, Portoviejo) Manabi	
TBJ_2268ci	Triatomine	*Rhodnius ecuadoriensis*	Ecuador (El Bejuco, Portoviejo) Manabi	
TCI_2307ci	Triatomine	*Rhodnius ecuadoriensis*	Ecuador (Chita, San Vicente) Manabi	
TLG_2345k	Triatomine	*Rhodnius ecuadoriensis*	Ecuador (Liguiqui, Manta) Manabi	

(*continued*)

TABLE 3. (CONTINUED)

Isolate[a]	*Host*		*Locality*	*18S rRNA*
TET_2388k	Triatomine	*Panstrongylus rufotuberculatus*	Ecuador (Estero Seco, Jama) Manabi	
TGA_2552ci	Triatomine	*Rhodnius ecuadoriensis*	Ecuador (Guara, Calvas) Loja	
TCQ_2915k	Triatomine	*Rhodnius ecuadoriensis*	Ecuador (Chaquizhca, Calvas) Loja	
TCQ_3086k	Triatomine	*Rhodnius ecuadoriensis*	Ecuador (Chaquizhca, Calvas) Loja	
Lineage D				
SC58	Rodent	*Echimys dasythrix*	Brazil (SC)	AY491745
Lineage E				
TryCC_643; Tra643	Bat	*Platyrrhinus lineatus*	Brazil (MS) Miranda	EU867803
Outgroups				
Trypanosoma sp.	Bat	*Rousettus aegyptiacus*	Gabon	AJ012418
1_EA_2008; *Trypanosoma* sp.	Civet	*Nandinia binotata*	Cameroon	FM202492
P14; *Trypanosoma vespertilionis*	Bat	*Pipistrellus pipistrellus*	England	AJ009166
2_EA_2008; *Trypanosoma* sp.	Monkey	*Cercopithecus nictitans*	Cameroon	FM202493
USP; *Trypanosoma conorhini*	Rodent	*Rattus rattus*	Brazil	AJ012411

[a]Samples in bold indicate the sequences newly generated for this study.

previous markers were amplified for the specific variable region KP1 using the primers KP1U (5′-GTA GAA AGA TCC GAA AAA ATG C-3′) and KP1L (5′-ATA CAA CAC TCT CTA TAT CAG G-3′) (Vallejo et al. 1994). A band of 280 bp was visible in KP1(+) group and was absent in KP1 (−). A negative control and positive DNA for KP1(+) and KP1(−) were used to validate the gels. Bands for both molecular markers were detected in a 6% polyacrylamide gel.

Identification of T. rangeli *lineages by the intergenic-spacer of the SL gene*

T. rangeli lineages were identified by differential amplification of the SL intergenic spacer using the primers TraSL1 (5′-GAA CGG TCG TGT TCT G-3′) and TraSL2 (5′-GAC GGG ATG TGG TGC-3′), as previously described (Maia Da Silva et al. 2007). The expected band sizes for the different lineages are: lineage A (417 bp), lineage B (380 bp), lineage C (480 bp), lineage D (500 bp), and lineage E (987 bp) (Maia Da Silva et al. 2007, 2009).

*Sequencing of the SSU-rRNA (*18S rRNA*) gene*

From the samples that were able to be characterized by both the conserved region of the minicircle of kDNA and the intergenic-spacer of the SL gene, a representative subset of samples ($n = 17$), which represents the geographic and host diversity, were selected for sequencing of a fragment of the SSU-rRNA gene (Table 3). PCR amplifications were conducted with the newly designed primers SSU4_F (5′-GTG CCA GCA CCC GCG GTA AT-3′) and 18Sq1R (5′-CCA CCG ACC AAA AGC GGC CA-3′), using a touchdown PCR profile (Murphy and O'Brien 2007). The PCR products were cleaned with ExoSAP-IT (Affymetrix, Santa Clara, CA), and sequencing reactions for both ends were performed with the ABI BigDye chemistry (Applied Biosystems, Inc., Foster City, CA) for the external primers mentioned above and the internal primers SSU561F (5′-TGG GAT AAC AAA GGA GCA-3′) and SSU561R (5′-CTG AGA CTG TAA CCT CAA AGC-3′) (Noyes et al. 1996, 1999).

Sequencing of DNA fragments was conducted on an ABI 3730xl DNA Analyzer automatic sequencer (Applied Biosystems, Inc., Foster City, CA). Contigs for each trypanosome sample were assembled using Geneious version 8.0.3 (Biomatters 2014) with untrimmed sequences with a percentage of high quality bases (HQ%) higher than 50%, resulting in trimmed sequences with a HQ% higher than 90%; consensus sequences were extracted from each contig. The sequences obtained were aligned with *T. rangeli* representatives of lineages A to E (Maia Da Silva et al. 2007, 2009) and selected species of the *T. cruzi* clade as outgroups (Table 3) using the MUSCLE (Edgar 2004) plugin in Geneious. The alignment was checked manually for misplacements and trimmed to 816 bp to ensure completeness of 100% of the sequence length of representatives of all *T. rangeli* lineages. A phylogenetic analysis using maximum likelihood was performed in RAxML v. 8 (Stamatakis 2014) using the GTRGAMMA model and 1000 bootstrap replicates.

Results

Diversity and distribution of hosts infected by T. rangeli

A total of 3606 samples from triatomines and mammals were screened for the presence of trypanosomatids. Of those, 351 (10%) presented infection for *T. rangeli*, and from them, 1.25% corresponded to mixed infections with *T. cruzi*. A higher infection rate was found in Manabí Province than in Loja Province, 14% and 7%, respectively (Table 1) (Fig. 1A, B). *T. rangeli* had a wide distribution in both regions and was present in more than 60% of the counties included in this study. Along this geographic distribution, mixed infections with *T. cruzi* were also reported (Fig. 2).

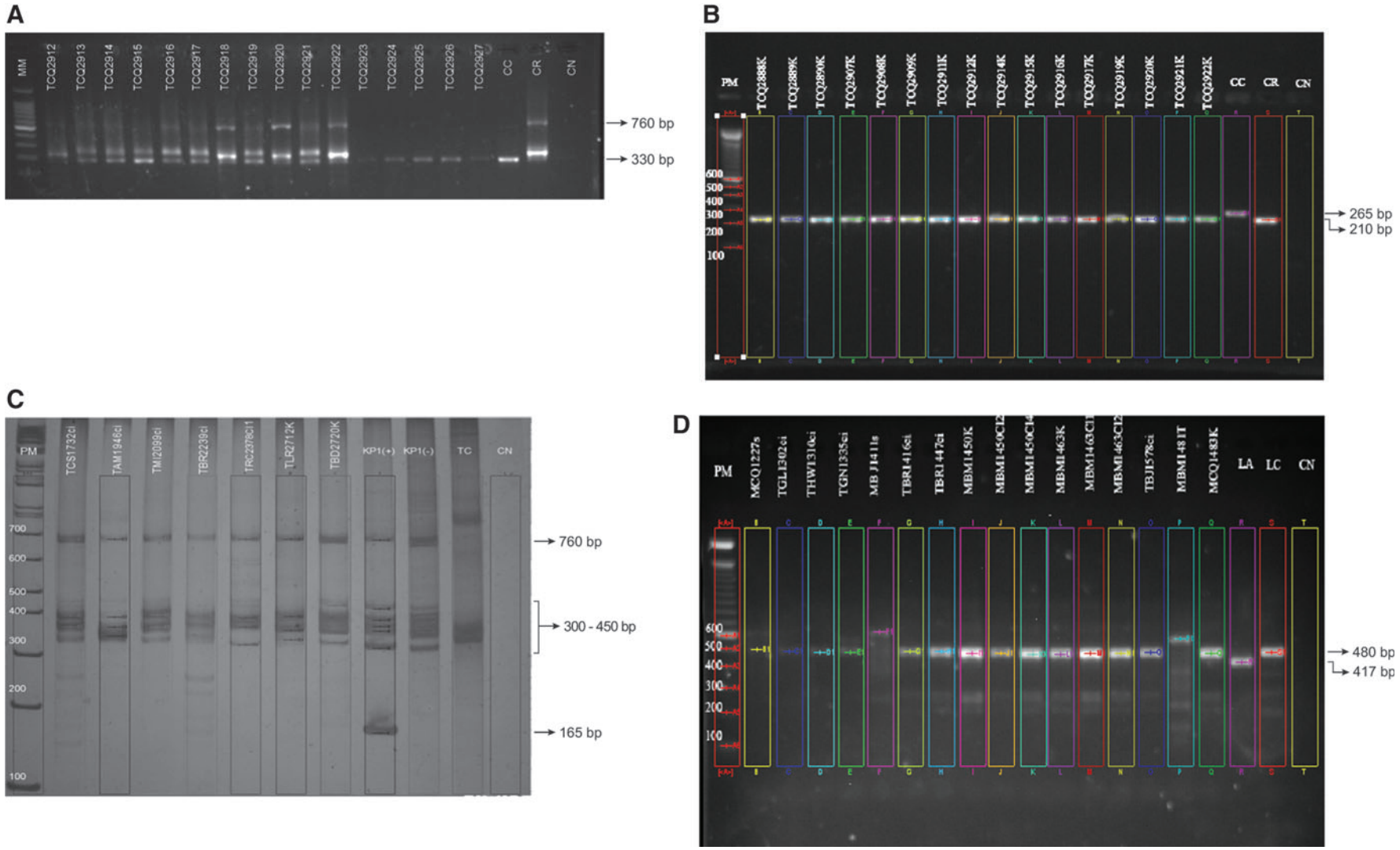

FIG. 1. Differential detection of *T. cruzi* and *T. rangeli* infection of triatomine's intestinal contents by (**A**) amplification of the conserved regions of the minicircle of kDNA and (**B**) D7a divergent region of the large subunit ribosomal RNA gene. CC, *T. cruzi* control; CR, *T. rangeli* control; CN, negative control. Genotyping of *T. rangeli* was carried out by (**C**) the amplification of the conserved regions of the minicircle of kDNA to identify among the groups KP1(+) and KP1(−) and (**D**) the intergenic spacer of the splice leader (SL) gene to characterize the lineage. TC, *T. cruzi* control; LA, lineage A control of *T. rangeli*; LC, lineage C control of *T. rangeli*; CN, negative control. Color images available online at www.liebertpub.com/vbz

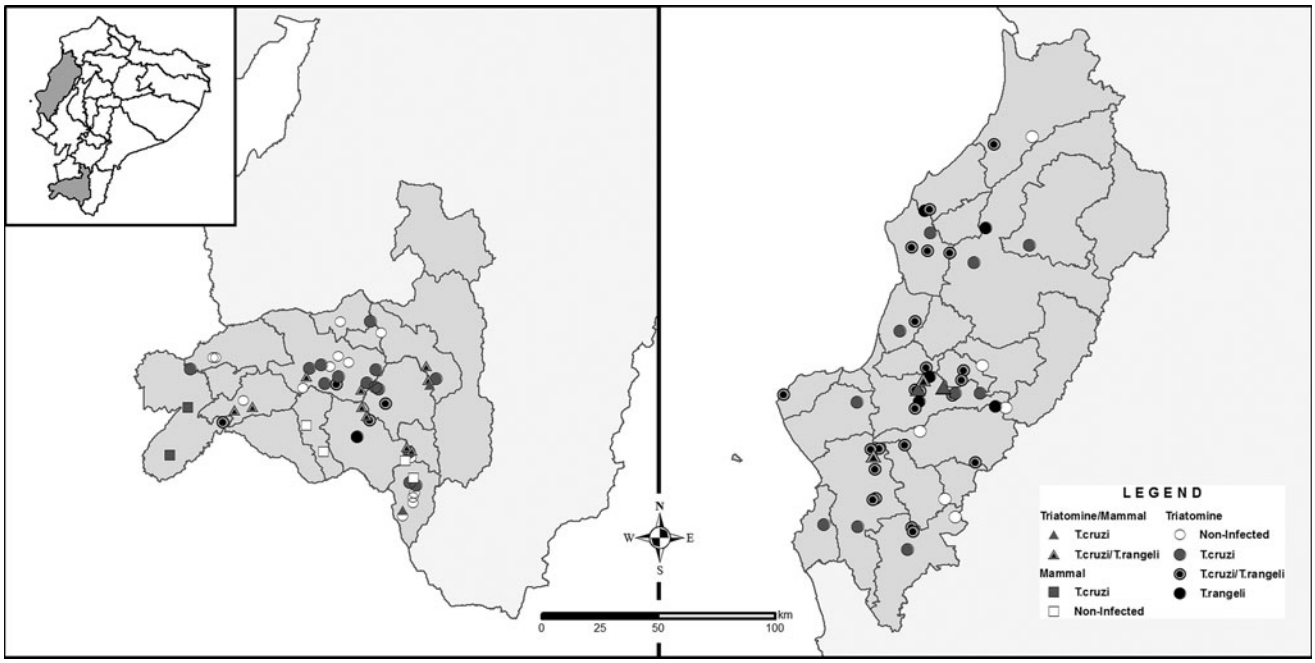

FIG. 2. Geographic distribution of *T. rangeli* and *T. cruzi* infection in invertebrate (triatomines) and vertebrate (mammal) hosts in southern Ecuador, Loja Province (*left*), and central coastal Ecuador, Manabí Province (*right*). Lines indicate county division.

Regarding the mammalian hosts, 18 genera from the order Chiroptera (*Artibeus, Desmodus, Glossophaga*), Didelphimorphia (*Didelphis, Marmosa, Micoureus, Philander*), and Rodentia (*Aegialomys, Akodon, Cavia, Mus, Rattus, Rhipidomys, Sciurus, Handleyomys, Hoplomys, Proechimys, Transandinomys*) were found to be infected with *T. rangeli*. However, infection rates were <6% (Table 1). With respect to the triatomine hosts, five species were found infected: *Panstrongylus chinai, P. rufotuberculatus*, *P. howardi, Triatoma carrioni*, and *Rhodnius ecuadoriensis*. The species *R. ecuadoriensis* showed the highest infection rates in both regions (10% and 17%), whereas the other species presented infection rates <5% (Table 1).

T. rangeli was found circulating in all the three habitats (domicile, peridomicile, and sylvatic). Nevertheless, a notably higher infection rate was found in the sylvatic environment (17%) compared with the peridomicile and domestic environments, which presented 7% and 2%, respectively (Table 1).

Genotyping of T. rangeli *circulating in vertebrate and invertebrate host*

From the 351 samples that were determined to be positive for *T. rangeli* infection, a representative subset of 111 was characterized by the conserved region of the minicircle of kDNA and the intergenic-spacer of the SL gene. Ninety-nine percent of the samples were classified as KP1(−), independent from the geographic or host origin (Fig. 1C). It was not possible to genotype one sample from *R. ecuadoriensis* due to the presence of nonspecific bands (Table 2). On the other hand, 80% ($n = 89$) of the samples (including triatomines and mammals) were determined as lineage C (Fig. 1D), while 20% ($n = 22$) did not amplify any of the expected bands or amplified bands of not expected size (Table 2).

*Sequencing of the SSU-rRNA (*18S rRNA*) gene*

Of the 89 samples that were able to be characterized as KP1(−)/lineage C, a subset of 17 samples were sequenced. Within this subset, we included samples that could not be characterized by PCR of the kDNA (TCQ991) or SL (MBJ1411, TCE694). All samples in this study were identical, and the phylogenetic analysis grouped the sequences with lineage C of *T. rangeli* (Fig. 3). In the phylogenetic tree, lineage E does not differentiate from lineage A, and there is little differentiation between lineages D and A (Fig. 3).

Discussion

Manabí and Loja Provinces are considered as endemic areas for Chagas disease in Ecuador, and active transmission due to the presence of the parasite *T. cruzi* and its hosts (triatomines vectors and mammal hosts) has been confirmed (Grijalva et al. 2003, 2005, Pinto et al. 2006, Black et al. 2009). Moreover, the presence of the nonpathogenic *T. rangeli* has also been reported as overlapping *T. cruzi* distribution (Pinto et al. 2006, Grijalva et al. 2011, 2012, Villacis et al. 2015). This study confirmed the presence of both species of trypanosomes in 1.25% of the samples. However, it is important to consider that the infection rate reported may be underestimated due to the fact that screening of *T. rangeli* was carried out in intestinal contents and not in salivary glands or hemolymph. Nevertheless, a broad delineation of the distribution and genetic diversity of *T. rangeli* circulating in these areas has not been reported until now.

Infection of *T. rangeli* has been detected in mammals of at least five orders (Didelphimorphia, Chiroptera, Rodentia, Pilosa, and Primates) (Ramirez et al. 2002, Maia da Silva et al. 2004a, b, Cabrine-Santos et al. 2009, Maia da Silva et al. 2009, Ramirez et al. 2014). This study confirmed the

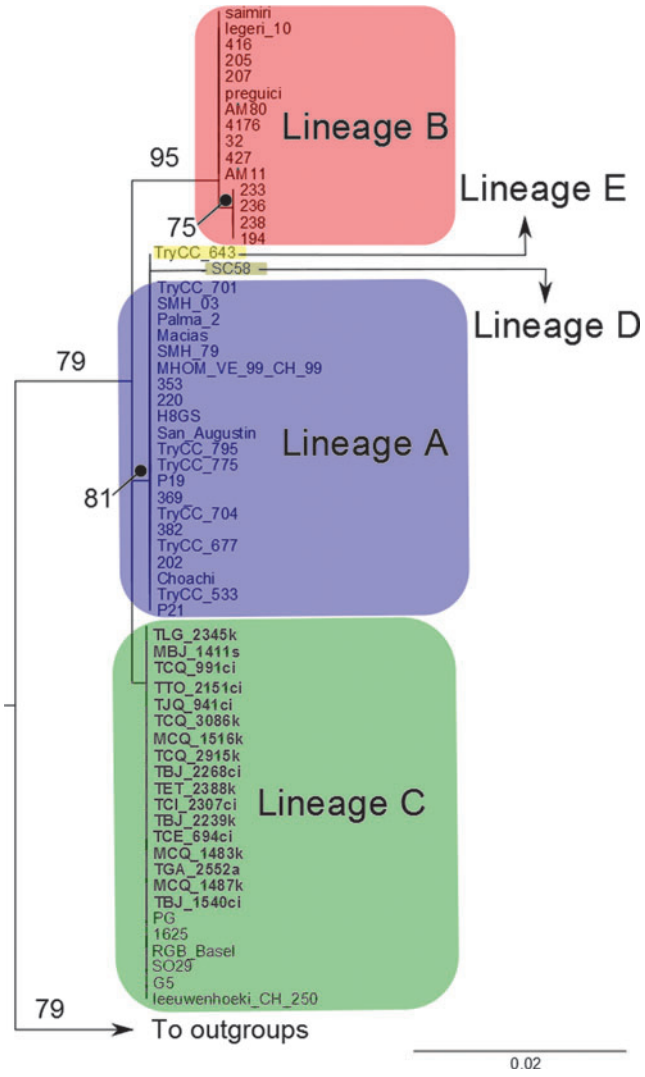

FIG. 3. Maximum likelihood tree of isolates of *T. rangeli* with representatives of five lineages (**A–E**). Names of isolates in bold represent the Ecuadorian isolates sequenced in this study. Numbers on the branches indicate bootstrap support values. Color images available online at www.liebertpub.com/vbz

presence of *T. rangeli* in three mammalian orders: Didelphimorphia, Chiroptera, and Rodentia, and in 18 genera, thus extending the report of host diversity for this parasite (Maia da Silva et al. 2004a, b, Pinto et al. 2006, Gurgel-Goncalves et al. 2012). However, infection rates reported in this study are low compared with others previously reported in Didelphimorphia and Chiroptera (Ramirez et al. 2002, Ramirez et al. 2014).

Extensive research of the vector–parasite relationships between *T. rangeli* and triatomine insects supports that the distribution of *T. rangeli* is closely related to the distribution of triatomines of the genus *Rhodnius* (Gaunt and Miles 2000, Machado et al. 2001, Urrea et al. 2005, Vallejo et al. 2007, Pulido et al. 2008, Salazar-Anton et al. 2009, Vallejo et al. 2009, Garcia et al. 2012). Even though the species of the genus *Rhodnius* are particularly susceptible to infection of salivary glands by *T. rangeli*, such infection has been also reported in *Triatoma dimidiata* (Marinkelle 1968, D'Alessandro-Bacigalupo and Gore Saravia 1991). However, the vector capacity due to the presence of infective stages of *T. rangeli* has only been proven in *Rhodnius* (Guhl and Vallejo 2003). While other triatomines species of the genera *Triatoma* and *Panstrongylus* have also reported to be naturally infected with *T. rangeli*, these reports are restricted to infection in the intestines (D'Alessandro-Bacigalupo and Gore Saravia 1991, Vallejo et al. 2002, Villacis et al. 2015). This study extends the species of triatomines naturally infected with *T. rangeli* to *P. chinai, P. rufotuberculatus*, and *T. carrioni*. Although, finding other species than *Rhodnius* infected with this parasite is feasible, our results do not imply the capacity of transmission of these species because the samples came from intestinal contents isolates and not from salivary glands.

R. ecuadoriensis was the species of triatomine with the highest infection rates. Although this study reports *T. rangeli* infecting intestinal contents, the natural and experimental infection of salivary glands has been previously reported in this species of triatomine (Cuba 1975, Guhl and Vallejo 2003, Urrea et al. 2005). However, its capacity to transmit *T. rangeli* has not been evaluated yet. Recent studies have demonstrated that *R. ecuadoriensis* is an important threat for Chagas disease transmission due to its wide geographical distribution, high infection rates with *T. cruzi*, good vector efficiency, and capacity of adaptation to different habitats (Villacis et al. 2008, Grijalva and Villacis 2009, Suarez-Davalos et al. 2010, Grijalva et al. 2012, 2014). In this regard, the presence of *R. ecuadoriensis*, infected with *T. rangeli*, constitutes an important element to consider when the distribution of trypanosomes is evaluated in Chagas disease endemic areas. Although the infection rates in the domestic and peridomestic environments were low (<7%), the high infection rates of *T. rangeli* in *R. ecuadoriensis* from sylvatic environments (17%) and the proven synanthropic tendency of the sylvatic populations of this species (Grijalva and Villacis 2009, Suarez-Davalos et al. 2010) indicate a risk for an increase of *T. rangeli* in domestic/peridomestic environments with its well-known consequences of being a confounding factor for Chagas disease diagnosis (Saldana and Sousa 1996a, b). The characteristics of *R. ecuadoriensis* and its important percentage of infection with *T. rangeli,* together with the morphological and genetic similarities that it shares with the pathogenic *T. cruzi*, need to be addressed when the biological and epidemiological scenarios are assessed, especially considering that infections of *T. rangeli* in humans have been previously reported in South America (Guhl and Vallejo 2003, Saldana et al. 2005).

Only *T. rangeli* KP1(−)/lineage C was found, even though the samples came from a variety of host species (triatomines and mammals) and two geographical regions that are ecologically different. This study complements the geographic range for KP1(−) in the Andean region, which has been previously described in Colombia and Peru (Vallejo et al. 2009, Urrea et al. 2011) and also for lineage C, which has been previously reported in Central America and Colombia (Maia da Silva et al. 2004a, b). It was not possible to identify the lineage of 22 out of 111 samples with the SL gene. However, two of these samples (MBJ1411 and TCE694) were included in the subset for sequencing, and they were characterized as lineage C. We relate this gap of information with the source of DNA. These samples were obtained directly from the hosts (intestinal contents or blood), and the amount of *T. rangeli* DNA was difficult to assess. Although

some previous attempts have been carried out to detect *Trypanosoma* from field samples, especially *T. cruzi* (Hamano et al. 2001, Marcet et al. 2006), all of them rely on the highly repetitive regions as the kDNA.

The distribution of the different genotypes of *T. rangeli* is affected by the trypanolytic capability of the species of *Rhodnius* (Pulido et al. 2008, Vallejo et al. 2009). Indeed, *T. rangeli* distribution is not associated with the vertebrate host but with the biogeographical distribution of *Rhodnius* species, even in areas where sympatric *Rhodnius* species coexist (Machado et al. 2001, Salazar-Anton et al. 2009, Urrea et al. 2011). The presence of the genotype *T. rangeli* KP1(−) associated with *R. ecuadoriensis* is in total accordance with what has been previously described for this species of triatomine (Urrea et al. 2005, 2011) and with what is expected regarding lineage C (Vallejo et al. 2009). Thus, the predominantly presence of *R. ecuadoriensis* in the study area also explains the presence of *T. rangeli* KP1(−)/lineage C in the samples from mammal hosts.

A phenotypic analysis of antennas and wings from *R. ecuadoriensis* demonstrated differences between the triatomine population of Loja and Manabí that suggest a speciation process (Villacis et al. 2010), especially considering that both geographic areas are separated by the Andes mountains, which constitute a natural barrier. An effect of triatomine subdivision was not evident in the *T. rangeli* samples of this study with the SSU-rRNA gene. However, detection of population structure among the *T. rangeli* KP1 (−/lineage C isolates with other molecular markers is possible, especially in light of the recent subdivision found by microsatellite typing on *T. rangeli* KP1(−) that was related to geographical location or co-evolution of parasites and hosts (Sincero et al. 2015). The access to the recently published complete genome of *T. rangeli* (Stoco et al. 2014) will provide new tools for elucidating the complex biology of this parasite and its interaction with its hosts.

Conclusions

This work reports the natural infection by *T. rangeli* in species of three mammalian orders (Didelphimorphia, Chiroptera, Rodentia) and four triatomine insect species (*P. chinai, P. rufotuberculatus*, *R. ecuadoriensis*, and *T. carrioni*) in southern (Loja) and central coastal (Manabí) Ecuador. Despite its presence in all the three habitats (domestic, peridomestic, and sylvatic), a higher infection rate was found in the sylvatic habitat and related to the presence of *R. ecuadoriensis*. A representative subset of *T. rangeli* that includes samples from a wide geographic area and a variety of host species (triatomines and mammals) was classified as KP1(−) and to the lineage C. No diversity was found among the SSU-rRNA gene, even though the samples came from ecologically different areas where subdivision of *R. ecuadoriensis* populations has been detected by phenotypic analysis.

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Author Disclosure Statement

No competing financial interests exist.

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