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Genetic diversity of the *Trypanosoma cruzi* and *Trypanosoma rangeli* mini-exon gene in two localities of Southern Ecuador

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Certifico que la Tesis de **Magister en Biología de las Enfermedades Infecciosas** del Lcdo. Jalil Maiguashca Sánchez ha sido concluida de conformidad con las normas establecidas; por lo tanto, puede ser presentada para la calificación correspondiente.

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Director de la Tesis
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LISTA DE CONTENIDOS

LISTA DE ABREVIATURAS

Abreviatura	Significado
CD	Chagas Disease
DTU	Discrete Typing Unit
NGS	New Generation Sequencing
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid

LISTA DE FIGURAS

LISTA DE TABLAS

MANUSCRITO PARA PUBLICACIÓN

Genetic diversity of the *Trypanosoma cruzi* and *Trypanosoma rangeli* mini-exon gene in two localities of Southern Ecuador

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Abstract

Trypanosoma cruzi, causative agent of Chagas Disease, and *Trypanosoma rangeli,* are both protozoan parasites endemic to Latin America, including Ecuador. *T. cruzi* shows high molecular and morphological heterogeneity and six lineages or DTU's are currently recognized. In Ecuador, TcI predominates while zymodeme III, corresponding to what is currently TcIV, was previously reported by a single study. *T. rangeli,* although nonpathogenic to humans, is a species of interest due to its sympatric distribution with *T. cruzi* and confounding effect in Chagas Disease diagnosis. In the present study, we isolated DNA of intestinal contents of triatomines belonging to two communities of Southern Ecuador. Infection status determined by kinetoplast-minicircle PCR was contrasted with results yielded by PCR amplification of the non-transcribed region of the mini-exon gene. For disambiguation of the results obtained, amplified fragments from the non-transcribed region of the mini-exon gene were sequenced using an Illumina MiSeq platform. We evaluated genetic variability in terms of richness and diversity at a population level for both parasites using Operational Taxonomic Units*. T. cruzi* genetic diversity was found to be significantly larger in adult female vectors as compared to the nymphal stages. Additionally, NGS revealed 21 mixed infections between TcI and *T. rangeli,* and the presence of DTU TcIV in 7 samples, (a novel record in Southern Ecuador), which the PCR-based methods were not able to detect. This suggests that the PCR-based methods are not reliable as molecular tools for identification of mixed infections. Furthermore, parasite populations present in only one of the two studied localities were not found, suggesting active parasite dispersal over the study area. Our results

highlight the value of NGS methodologies to clarify the population dynamics of triatomines and inform control strategies.

Author Summary

Chagas disease is a vector-borne parasitic infection affecting 6 to 7 million people worldwide, mainly in Latin America. Understanding the population dynamics and molecular heterogeneity of the causative agent, *Trypanosoma cruzi*, is crucial to inform control campaigns. We analyzed the population structure of *T. cruzi* and *Trypanosoma rangeli,* a closely related yet harmless parasite, in vectors from two localities in Southern Ecuador. We employed three molecular techniques, including New Generation Sequencing, to scrutinize parasite DNA samples isolated from the intestinal content of infected triatomine vectors. Richness, diversity, and a possible link with developmental stage of the insects were explored. At least 21 samples were infected with both parasite species, suggesting that the prevalence of the *T. rangeli* is underestimated in Ecuador. Additionally, a *T. cruzi* variant known as TcIV, previously not reported for the region, was detected in 7 of the samples. No significant genetic differences were identified among parasite populations of the two localities. Finally, higher molecular diversity and richness of *T. cruzi* were found in adult female vectors than in early developmental stages. Our results highlight the value of NGS methodologies to clarify the population dynamics of triatomines and inform control strategies.

Introduction

Chagas disease (CD) is caused by the kinetoplastid protozoan parasite *Trypanosoma cruzi* and affects 6 to 7 million people worldwide [1]. This neglected disease is endemic of Latin America, where 25 million people are at risk of infection and approximately 10 000 die every year with symptoms related to CD [2]. Transmission in endemic countries most of the times involves contact with feces of an infected triatomine of the Reduviidae family. However, other secondary mechanisms of transmission, such as blood transfusion, organ transplants, congenital transmission, consumption of infected food and laboratory accidents exist, and can cause infections in non-endemic regions where vectors are not present [3–5].

Trypanosoma rangeli, a related kinetoplastid, shows an overlapping geographical distribution with *T. cruzi* and may also be found in the same triatomine vectors and mammalian hosts; mixed infections with both species have been reported [6–8]. Despite being considered nonpathogenic for humans, *T. rangeli* has epidemiological relevance due to its morphological and genetic similarity with *T. cruzi*, which may result in false positives in microscopic and serologic diagnosis, the latter due to cross reactivity caused by shared antigenic determinants [9,10]. As with *T. cruzi*, transmission of *T. rangeli* involves the feeding behavior of triatomines, although metacyclic trypomastigotes are released from the salivary glands during the bloodmeal [11]. In Ecuador, little is known about this parasite. Ocaña-Mayorga and colleagues [12] reported the presence of *T. rangeli* in Manabí and Loja provinces. From 3606 samples, kinetoplast minicircle amplification determined that around 10% of infected triatomines and mammals were infected with *T. rangeli*, and from them, 1.25% presented mixed infections with *T. cruzi* [12].

The first report of CD in Ecuador dates from 1930 [13]. Currently, the parasite has been reported to be present in all major geographic regions of Ecuador (coast, highlands and Amazonia), with the exception of the Galapagos Islands [13–17]. As in most Latin American countries, population in rural areas are prone to infection due to several factors, including lack of an adequate healthcare, malnutrition, limited resources and deficient housing infrastructure, among other poverty features typically associated with CD [18,19].

In 2004, the Ecuadorian Ministry of Public Health launched the National Chagas Disease Control Program. For almost ten years, entomological searches, insecticide spraying, and epidemiologic surveillance took place in 11 provinces including Loja [20], where ~3.6% of the population is estimated to be seropositive for *T. cruzi* [21]. The program faced several drawbacks: control activities did not include all areas of endemicity and those included received only sporadic interventions [20]. Triatomine reinfestation was reported in Loja soon after the program ended [22] and case reports, although increased after program initiation, were believed to be largely underestimated at a national level suggesting that available data does not reflect the real situation of CD in Ecuador [23].

The subfamily Triatominae includes 150 extant species, although a reduced number are competent vectors for *Trypanosoma* [24]. Sixteen species of triatomines are found in Ecuador [13,25]. Among them, *Triatoma dimidiata, Triatoma carrioni, Panstrongylus chinai, Panstrongylus rufotuberculatus* and *Rhodnius ecuadoriensis* are epidemiologically relevant [8,22]. *T. dimidiata* and *R. ecuadoriensis* have been the target of previous research and much more is known for these two species than any other triatomine in Ecuador [26–29].

R. ecuadoriensis shows a wide distribution that includes the western lowlands, the southern provinces of Ecuador and northern Perú [30]. Moreover, it is also capable of inhabiting domestic and peridomestic environments increasing the chance of contact with humans [26,31]. In Loja, *R. ecuadoriensis* sylvatic populations are associated with rodent nests and particularly with the squirrel *Sciurus nebouxii*, while the abundance is strongly influenced by distance with human dwellings [7,31]. Considering habitat delimitation, *R. ecuadoriensis* could be targeted through fumigation, although risk of re-infestation from sylvatic environments persists.

The study of the evolution of *T. cruzi* and the persisting criterion that there may be a link between strain and clinical symptomatology of CD, triggered the examination of several molecular markers in different strains of the parasite [32]. However, *T. cruzi* displays high molecular heterogeneity, and the elucidation of strains or clusters with similar molecular patterns has proven difficult. Research performed during the last four decades revealed molecular patterns that cluster *T. cruzi* strains and isolates in zymodemes [33–36]. As the number of samples increased, so did the geographical areas covered. Coupled with inclusion of new molecular markers, this allowed for the recognition of six lineages or Discrete Typing Units (DTU´s), commonly referred as Tc!-TcVI [37–41]. In Ecuador, TcI predominates in coastal provinces [28,42] and Loja [43], with only one previous report of other parasite lineages. Garzón, et al, [44] reported the presence of parasites belonging to zymodemes II and III. These two zymodemes include strains corresponding to lineages named TcII, TcIII, TcIV and TcV in current nomenclature, although the presence of each of these has not been confirmed in Ecuador to date. The mentioned samples were isolated from chronic chagasic patients from Zamora Chinchipe and El Oro provinces (east and west from Loja, respectively) [44].

Among molecular markers, the mini-exon genes emerge as a powerful tool since they include conserved, semiconserved and highly variable regions, all useful in phylogenetic analysis [45], discrimination between DTU´s [46] and population structure analysis [47]. The mini-exon genes comprise several copies disposed in tandem and encode the Splice Leader, a 35-nucleotide sequence translocated to the 5´ end of every newly synthesized mRNA in *Trypanosoma* [48,49], in what is referred as discontinuous transcription [50].

New Generation Sequencing (NGS) constitutes a milestone in biological and medical research. The last decade has recorded an explosion of NGS techniques and by 2010 the capacity of data generation increased by a factor of 100-1000 in comparison with Sanger-sequencing technologies [51]. In recent years, NGS has been used for clinical diagnostics, forensic science and also for the study of ecology and population genetics of microorganisms, such as bacteria [52,53]. In the case of *T. cruzi*, the technique has helped to elucidate mitochondrial heteroplasmy [54] and has also provided insights about multiclonal infections [55]. NGS offers high molecular resolution that can be exploited in pooled samples such as DNA isolated from intestinal content of triatomines.

In the present study, we aimed to evaluate the molecular diversity of *T. cruzi* and *T. rangeli* in two localities of Southern Ecuador through the analysis of the mini-exon gene. NGS data, displayed as Operational Taxonomic Units (OTUs, a cluster of sequences with a 97% homology), were used to explore the distribution of mini-exon genotypes for both species and a possible divergence of populations between two localities: Bramaderos and Bella María. With this scope, OTUs were used to measure genetic richness and diversity (Simpson Index of Diversity and the Shannon-Weaver Index). Additionally, the data set included samples isolated form different developmental stages of the vector to evaluate a potential correlation with parasite genetic diversity. Moreover, we were able to compare PCR-based genotyping tools with NGS in terms of sensitivity for discrimination between *T. cruzi* and *T. rangeli*.

Materials and methods

Sample panel and PCR typing

Archival DNA samples collected between years 2009-2013 by the Center for Research on Health in Latin America (CISeAL) were used. Details of entomological searches, intestinal content DNA extractions, and PCR amplification of the kinetoplast minicircle for determination of infection status, have been reported elsewhere with corresponding approved protocols by Institutional Review Boards and collection permits [7,22]. Kinetoplast minicircle amplification was performed using primer pairs: S35 - S36 [56] and 121 – 122 [57]. Forty-six samples from two locations from Southern Ecuador (Loja Province) were included: 31 from Bramaderos (-4.0797, -79.8244) and 15 from Bella María (-4.2115, -79.6063) (Fig 1). To evaluate a possible link between vector developmental stage and molecular diversity in the parasite´s mini-exon gene, samples obtained from 19 adult triatomines, 13 instar V, 11 Instar III and 3 Instar IV, were included.

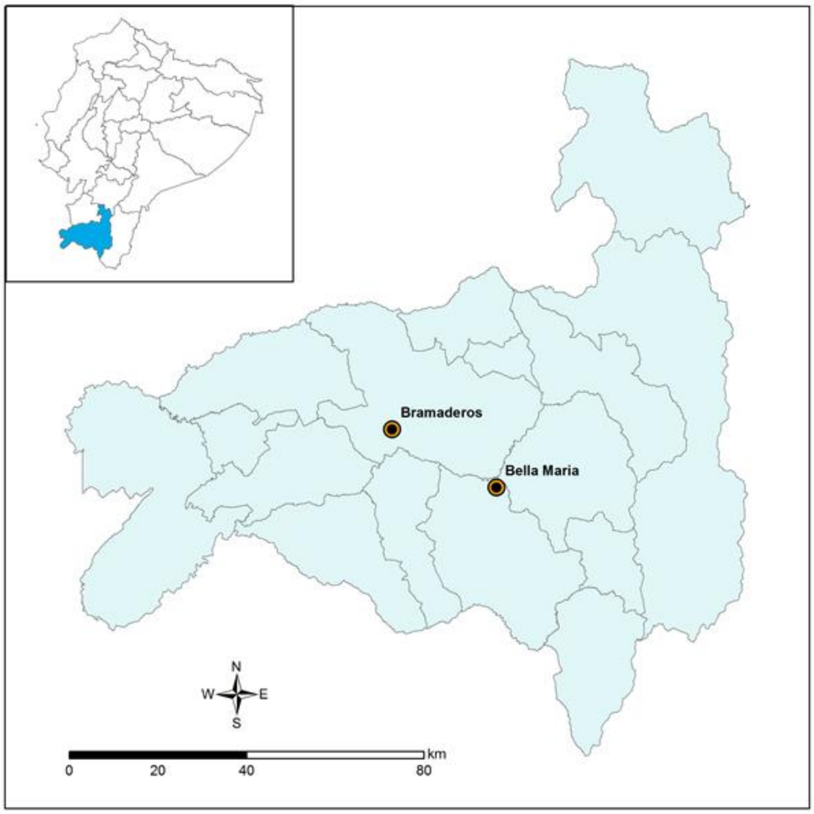

Fig 1. Study area. The two studied communities within Loja province in Southern Ecuador are depicted. They are separated by approximately 28.3km.

Multiplex PCR

PCR amplification of the non-transcribed spacer of the mini-exon gene was performed as described in [46]. Briefly, the technique uses five primers in a multiplex reaction to discriminate between three *T. cruzi* groups: zymodeme I (equivalent to DTU TcI in the current nomenclature), zymodeme II (comprising DTUs V-VI), zymodeme III (current DTUs III and IV) [41] and *T. rangeli* (Fig 2). The amplified products were electrophoresed in 2% agarose gels and visualized with SYBR green. Some samples displayed a two-band pattern (~200bp/~400bp) so gel excision and purification were performed for each fragment. Subsequently, amplicons were sequenced by NGS technology as explained below.

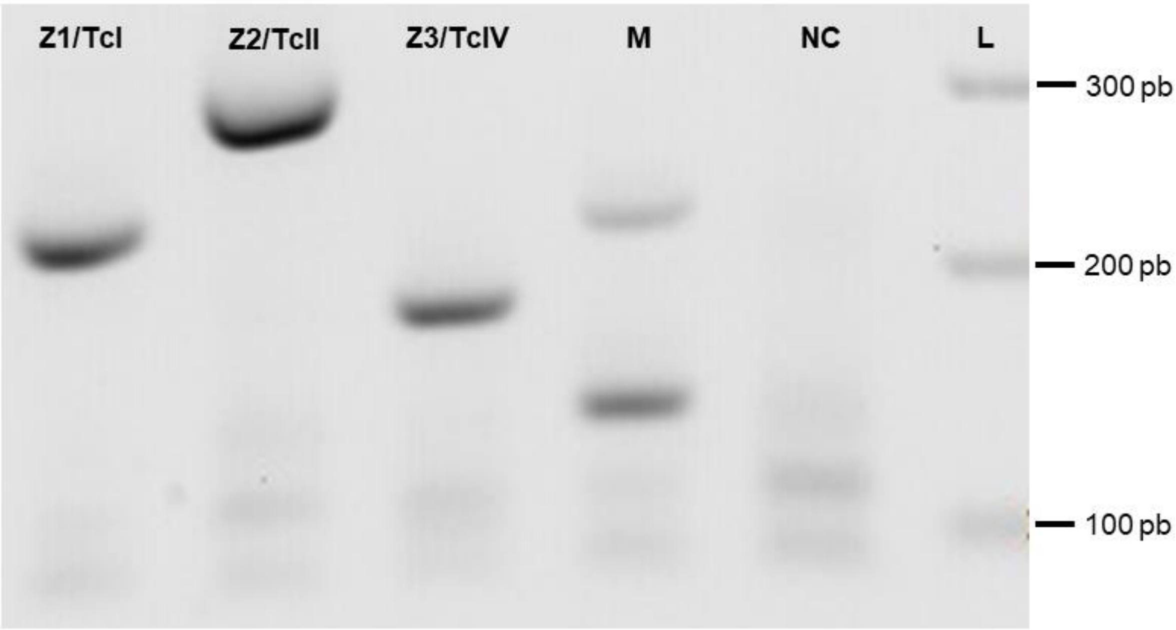

Fig 2. PCR amplification patterns for reference strains. Non transcribed spacer of the mini-exon gene was amplified as in [46], and electrophoretically separated in 2% agarose gels. Expected banding patterns are shown. TcI (formerly Z1): sample TBR1391; TcII (formerly Z2): Y strain DNA; TcIV (formerly Z3): CanIII DNA; M: *T. cruzi/T. rangeli* mixed-infection: sample TBR1413; NC: Negative control (ddH20 as template); L: molecular weight marker ladder.

Next Generation Sequencing

Sequencing was performed in a MiSeq Illumina platform using a 2 x 250 bp (Reagent Kit version 2) following the protocol provided by manufacturer. Amplicon datasets were processed with Sickle for trimming (-q 20) [58]. Trimmed sequences were denoised with BayesHammer [59] and overlapping of forward and reverse sequences was performed using Pandaseq [60]. Duplicated sequences, singletons and chimeras were removed from the dataset by using Usearch7 and ChimeraSlayer [61]. OTUs were generated by *de novo* tool of Usearch [62] and taxonomy was assigned with the aid of a customary reference database created with corresponding mini-exon sequences available in the NCBI.

Richness and diversity analysis

Richness and diversity analysis were performed following the tutorial provided by Dr. Umer Zeeshan Ijaz (https://docs.google.com/document/d/1BcZAk28k7Uycr7iKKAVSiZ0MB9jDs9bODpdPZtYFH3Y/pub#h.b8qk9lvbjxdy) using the “vegan” package available in R. Briefly, richness was defined as the total number of OTUs found in each sample, without consideration for amount or proportions. Simpson Index of Diversity (1-D) was calculated based on P_i (the probability of finding a given OTU in the sample) and D (ΣP_i^2) [63]. For the Shannon-Weaver Diversity Index, the Pi was also calculated and used in Σ(Piln[Pi]) [64].

Results

Genotyping by multiplex PCR and NGS

Samples selected for the study had been classified as infected exclusively by *T. cruzi* through the minicircle kinetoplast PCR-based analysis (data not published). Amplification of the mini-exon gene was performed by multiplex PCR under conditions described in [46]. In contrast with the results obtained by kinetoplast minicircle amplification, the multiplex PCR showed 18 mixed infections with *T. cruzi* (TcI) and *T. rangeli* (39.1%), 25 *T. cruzi* (TcI) infections (54.3%) and 3 samples were apparently infected exclusively by *T. rangeli*. (6.5%) (Table 1).

Table 1. Samples employed in the study and genotyping results.

CODE	VECTOR SPECIES	COMMUNITY	INSTAR	MINI-EXON MULTIPLEX PCR[a]	NGS
TBR1379	*R. ecuadoriensis*	Bramaderos	III	*T. cruzi* (TcI)	Reads <2000[b]
TBR1391	*R. ecuadoriensis*	Bramaderos	Adult/male	*T. cruzi* (TcI)	*T. cruzi* (TcI)
TBR1409	*R. ecuadoriensis*	Bramaderos	Adult/male	*T. cruzi* (TcI)/*T. rangeli*	N/A[c]
TBR1410	*R. ecuadoriensis*	Bramaderos	Adult/male	*T. cruzi* (TcI)	*T. cruzi* (TcIV)/*T. rangeli*
TBR1413	*R. ecuadoriensis*	Bramaderos	V	*T. cruzi* (TcI)/*T. rangeli*	*T. cruzi* (TcI/TcIV)/*T. rangeli*
TBR1414	*R. ecuadoriensis*	Bramaderos	V	*T. cruzi* (TcI)/*T. rangeli*	*T. cruzi* (TcI)/*T. rangeli*
TBR1422	*R. ecuadoriensis*	Bramaderos	III	*T. rangeli*	*T. cruzi* (TcI)/*T. rangeli*
TBR1432	*R. ecuadoriensis*	Bramaderos	Adult/female	*T. cruzi* (TcI)/*T. rangeli*	Reads <2000[b]
TBR1445	*R. ecuadoriensis*	Bramaderos	III	*T. cruzi* (TcI)/*T. rangeli*	*T. cruzi* (TcI/TcIV)/*T. rangeli*
TBR1446	*R. ecuadoriensis*	Bramaderos	V	*T. cruzi* (TcI)/*T. rangeli*	*T. cruzi* (TcI)/*T. rangeli*
TBR1455	*R. ecuadoriensis*	Bramaderos	V	*T. cruzi* (TcI)/*T. rangeli*	*T. rangeli*
TBR1470	*R. ecuadoriensis*	Bramaderos	Adult/male	*T. cruzi* (TcI)	Reads <2000[b]
TBR1475	*R. ecuadoriensis*	Bramaderos	Adult/male	*T. cruzi* (TcI)/*T. rangeli*	*T. cruzi* (TcI/TcIV)/*T. rangeli*
TBR1480	*R. ecuadoriensis*	Bramaderos	V	*T. rangeli*	Reads <2000 [b]
TBR1483	*R. ecuadoriensis*	Bramaderos	Adult/female	*T. cruzi* (TcI)	*T. cruzi* (TcI)/*T. rangeli*
TBR1484	*R. ecuadoriensis*	Bramaderos	III	*T. cruzi* (TcI)/*T. rangeli*	*T. cruzi* (TcI)/*T. rangeli*
TBR1485	*R. ecuadoriensis*	Bramaderos	III	*T. cruzi* (TcI)/*T. rangeli*	*T. cruzi* (TcI)/*T. rangeli*
TBR1486	*R. ecuadoriensis*	Bramaderos	III	*T. cruzi* (TcI)	*T. cruzi* (TcI)
TBR1487	*R. ecuadoriensis*	Bramaderos	IV	*T. cruzi* (TcI)	N/A[c]
TBR1489	*R. ecuadoriensis*	Bramaderos	IV	*T. cruzi* (TcI)	*T. cruzi* (TcI)/*T. rangeli*
TBR1491	*R. ecuadoriensis*	Bramaderos	V	*T. cruzi* (TcI)	*T. cruzi* (TcI)/*T. rangeli*
TBR1492	*R. ecuadoriensis*	Bramaderos	V	*T. cruzi* (TcI)/*T. rangeli*	*T. cruzi* (TcI/TcIV)/*T. rangeli*
TBR1493	*R. ecuadoriensis*	Bramaderos	Adult/male	*T. cruzi* (TcI)	*T. cruzi* (TcI)/*T. rangeli*
TBR1494	*R. ecuadoriensis*	Bramaderos	Adult/male	*T. cruzi* (TcI)/*T. rangeli*	*T. cruzi* (TcI)/*T. rangeli*
TBR1495	*R. ecuadoriensis*	Bramaderos	Adult/male	*T. cruzi* (TcI)	*T. cruzi* (TcI/TcIV)
TBR1502	*R. ecuadoriensis*	Bramaderos	V	*T. cruzi* (TcI)	Reads <2000[b]
TBR1503	*R. ecuadoriensis*	Bramaderos	V	*T. cruzi* (TcI)/*T. rangeli*	*T. cruzi* (TcI)/*T. rangeli*
TBR1505	*R. ecuadoriensis*	Bramaderos	Adult/female	*T. cruzi* (TcI)/*T. rangeli*	*T. cruzi* (TcI)/*T. rangeli*

TBR1508	*R. ecuadoriensis*	Bramaderos	V	*T. cruzi* (TcI)	Reads <2000[b]
TBR1509	*R. ecuadoriensis*	Bramaderos	Adult/male	*T. cruzi* (TcI)	*T. cruzi* (TcI)
TBR1510	*R. ecuadoriensis*	Bramaderos	Adult/female	*T. cruzi* (TcI)	*T. cruzi* (TcI)/*T. rangeli*
TBM2798	*P. chinai*	Bella Maria	V	*T. cruzi* (TcI)	*T. cruzi* (TcI)
TBM2823	*P. chinai*	Bella Maria	IV	*T. cruzi* (TcI)	*T. cruzi* (TcI)
TBM2824	*P. chinai*	Bella Maria	III	*T. cruzi* (TcI)	*T. cruzi* (TcI)
TBM2841	*R. ecuadoriensis*	Bella Maria	Adult/male	*T. cruzi* (TcI)/*T. rangeli*	N/A[c]
TBM2903	*R. ecuadoriensis*	Bella Maria	III	*T. cruzi* (TcI)	Reads <2000[b]
TBM2972	*R. ecuadoriensis*	Bella Maria	Adult/female	*T. rangeli*	Reads <2000[b]
TBM2982	*R. ecuadoriensis*	Bella Maria	V	*T. cruzi* (TcI)	Reads <2000[b]
TBM3102	*R. ecuadoriensis*	Bella Maria	Adult/female	*T. cruzi* (TcI)	*T. cruzi* (TcI)/*T. rangeli*
TBM3131	*R. ecuadoriensis*	Bella Maria	III	*T. cruzi* (TcI)	*T. cruzi* (TcI)
TBM3132	*R. ecuadoriensis*	Bella Maria	Adult/female	*T. cruzi* (TcI)	*T. cruzi* (TcI)
TBM3135	*R. ecuadoriensis*	Bella Maria	Adult/female	*T. cruzi* (TcI)	*T. cruzi* (TcI)
TBM3329	*R. ecuadoriensis*	Bella Maria	V	*T. cruzi* (TcI)/*T. rangeli*	*T. cruzi* (TcI)/*T. rangeli*
TBM3376	*R. ecuadoriensis*	Bella Maria	III	*T. cruzi* (TcI)/*T. rangeli*	N/A[c]
TBM3377	*R. ecuadoriensis*	Bella Maria	III	*T. cruzi* (TcI)	*T. cruzi* (TcI/TcIV)
TBM3405	*R. ecuadoriensis*	Bella Maria	Adult/male	*T. cruzi* (TcI)/*T. rangeli*	*T. cruzi* (TcI)/*T. rangeli*

[a] Multiplex PCR described in [46]

[b] "Reads <2000" indicates that sample did not reach the 2000 read threshold in NGS and therefore excluded from further analysis.

[c] N/A indicates that sample did not undergo NGS due to technical issues.

Since four samples faced technical problems, only 42 underwent NGS analysis. Most of them showed rarefaction curves that stabilized at 2000 reads. Samples with less than 2000 reads were excluded from further analysis. The “assign taxonomy” step identified OTUs from *T. cruzi* and *T. rangeli* based on corresponding sequences of both species available in GenBank. NGS results showed that one sample contained exclusively *T. rangeli* DNA, 21 samples exhibited both, *T. cruzi* and *T. rangeli*, and 11 samples were infected only by *T. cruzi* (Table 1). Notably, we found OTUs corresponding to DTU TcIV in 7 samples: TBM3377 and TBR1495 showed OTUs that matched with sequences belonging to TcI and TcIV, while samples TBR1413, TBR1445, TBR1475 and TBR1492 presented OTUs from TcI, TcIV and *T. rangeli*. Only one sample, TBR1410, showed a mixed infection between TcIV and *T. rangeli*

Richness and diversity of *T. cruzi* and *T. rangeli* in Bramaderos and Bella Maria

Results for richness for both parasites are shown in Fig 3. No statistically significant differences among sampling sites were detected.

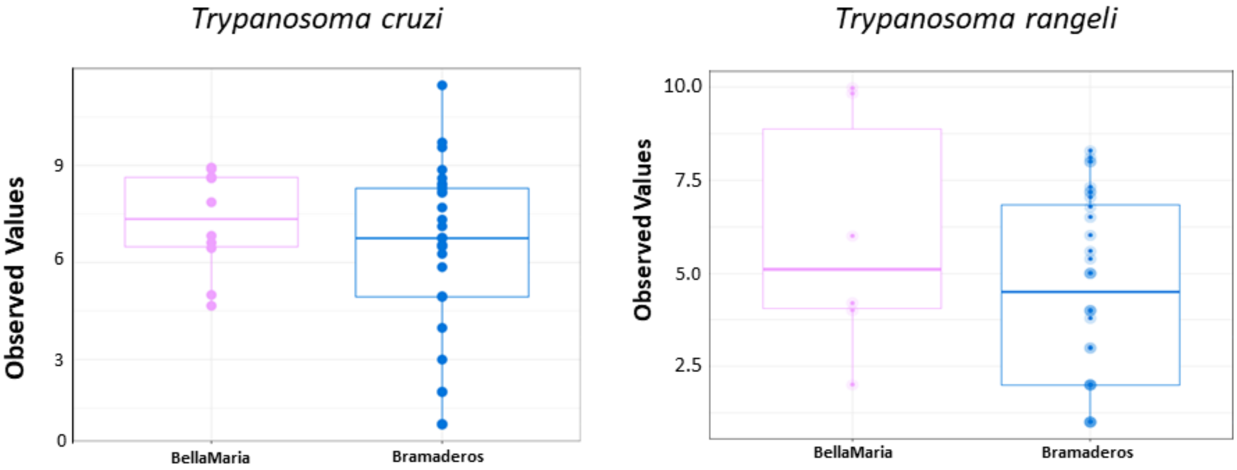

Fig 3. Richness of the mini-exon gene for *T. cruzi* and *T. rangeli* in Bramaderos and Bella María. Richness (y axis), defined as the number of OTUs present in each sample, is shown. No statistical significant difference was found between communities.

Results for diversity analysis are shown in Fig 4. Simpson and Shannon-Weaver indexes revealed no significant differences between communities.

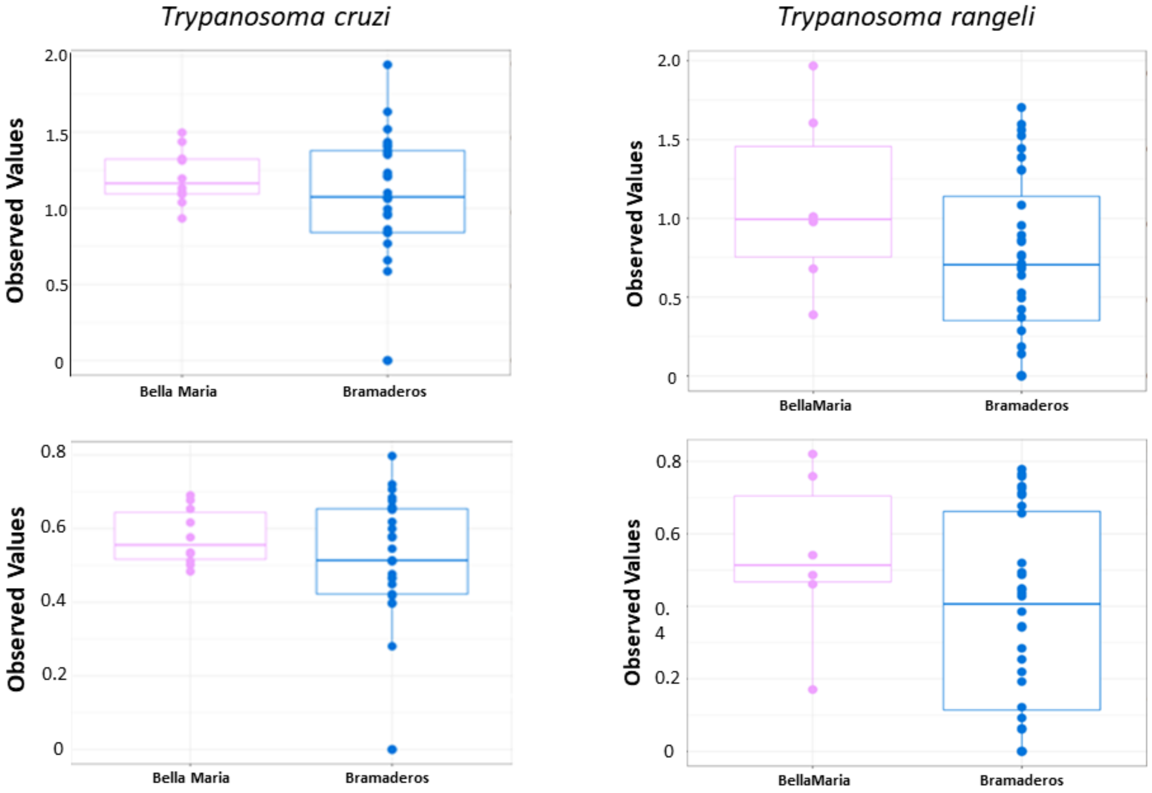

Fig 4. Diversity for *T. cruzi* and *T. rangeli* in Bramaderos and Bella María. Higher panels show Shannon-Weaver Index results for both parasites and communities. Lower panels show Simpson's index of diversity for both parasites and communities.

Molecular diversity of the mini-exon gene in *T. cruzi* and *T. rangeli* across the vector developmental stages

Results for the analysis between nymph stage of the triatomine and molecular diversity in *T. cruzi* and *T. rangeli* are shown in Fig 5. Our data suggests that in the case of *T. cruzi* richness increases in more advanced developmental stages of the vector, as shown by significant differences between female adults and nymph Instar III. *T. rangeli* showed no significant differences between adults and nymphs in terms of richness.

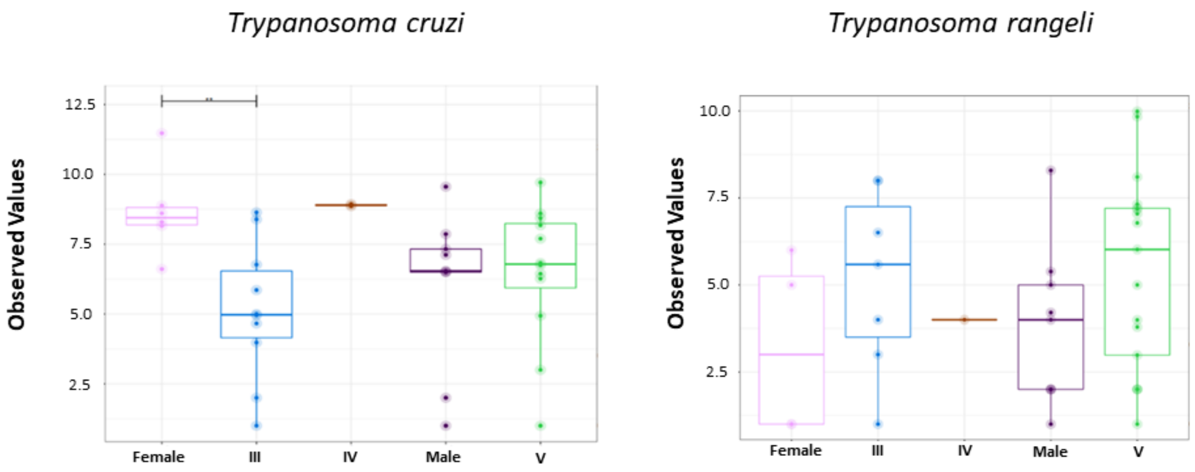

Fig 5. Molecular richness of the mini-exon gene in *T. cruzi* and *T. rangeli* evaluated at different developmental stage of the vector. The adult female of *T. cruzi* is richer than Instar III nymphs. No statistical differences were observed for *T. rangeli*.

Simpson's index of diversity and Shannon-Weaver index suggest a higher diversity of *T. cruzi* in adults that in earlier developmental stages of the vector. Both analysis show significant differences between female adults and nymphs Instar V. In contrast, no linkage between diversity and vector developmental stage was found in *T. rangeli* (Fig 6).

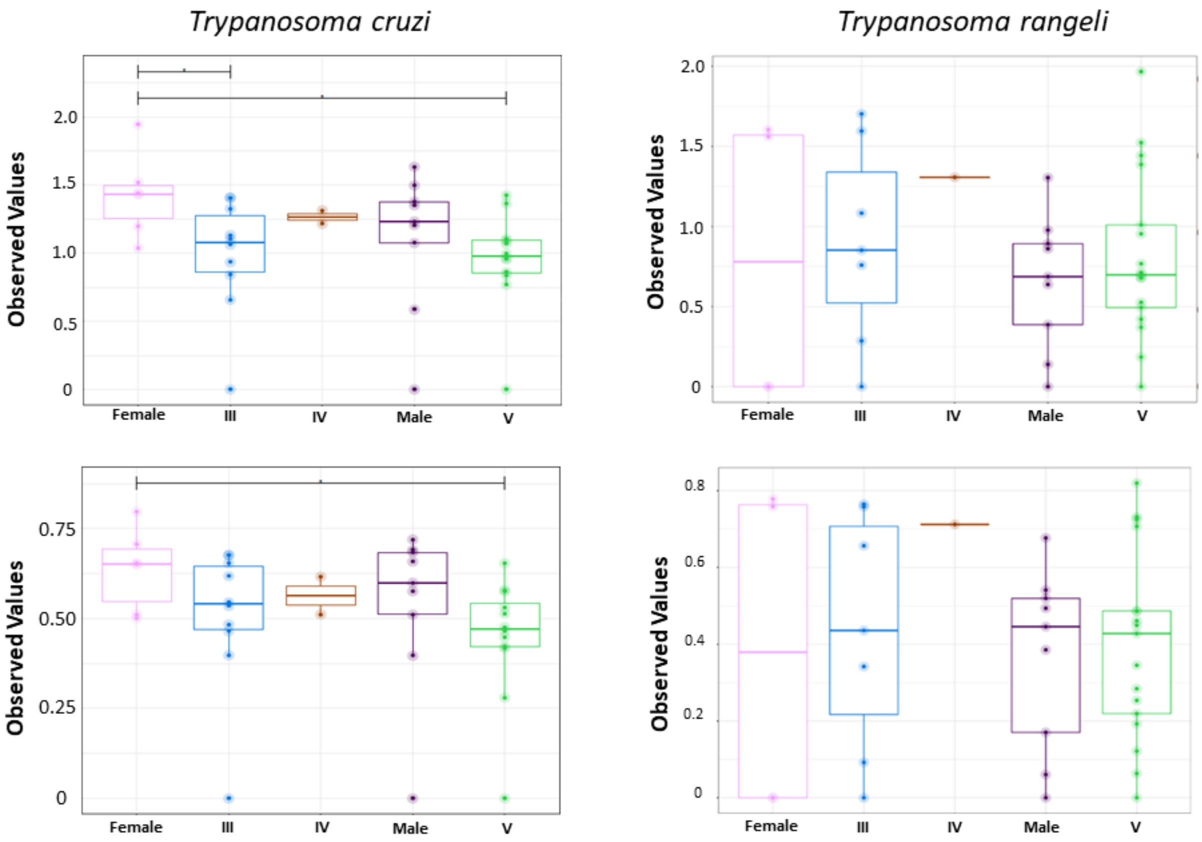

Fig 6. Diversity for *T. cruzi* and *T. rangeli* in different developmental stages of the vector. Higher panels show Shannon-Weaver Index. Lower panels show Simpson's index of diversity.

Discussion

Chagas disease is recognized as one of 20 neglected tropical diseases (NTDs) posing a heavy toll over the most economically disadvantage segments of the population of Latin American countries [2], and it is considered to be the most important parasitic disease in the region [65]. Over the last decade, vector control has been the center of CD control efforts in Ecuador [20]. However, the interventions applied have shown only limited success in vector control, number of reported cases and inclusion of endemic areas [22,23]. Altogether, new research approaches are required to provide information to control programs.

Mixed infections of *T. cruzi* and *T. rangeli* in vector and mammalian hosts are frequently encountered [6–8,66] and several groups have reported lack of specificity in minicircle kinetoplast PCR-based diagnostic methods widely used to discriminate between parasites [66–69]. Bearing in mind that previous reports estimated *T. rangeli* infection in 10% of sampled triatomine and mammals, and from them, 1.25% showed mixed infections with *T. cruzi* [12], we contrasted available genotyping data (data not published) with results obtained with multiplex PCR and NGS.

We studied the mini-exon gene sequences for parasites present in the intestinal content of triatomine vectors (*R. ecuadoriensis* and *P. chinai*) collected in two rural communities of Loja, Ecuador. Through minicircle kinetoplast PCR, multiplex PCR and NGS we were able to characterize the heterogeneous population of parasites present in the vector posterior digestive tract, and to determine the presence of both *T. cruzi* and *T. rangeli*. We exploited the capacity of NGS for detailed examination of pooled samples derived from heterogeneous populations to disambiguate the cases of co-infection with these two species of kinetoplastids, since large differences were observed between results of minicircle kinetoplast amplified products and multiplex PCR of the mini-exon genes.

Our study exemplifies the discordant results which many times are obtained by different PCR protocols for the analysis of the kinetoplast genes. Three samples, which based on previous kinetoplast PCR analysis suggested the presence of *T. cruzi* exclusively (TBM2972, TBR1422 and TBR1480), displayed *T. rangeli* patterns when amplified by multiplex PCR.

PCR sensitivity and specificity for *T. cruzi* genotyping (DTU discrimination) has been reported to be affected by several factors: quality of input DNA, sample conservation, technique of isolation, reagents, thermocycling conditions, etc. [70]. Our findings suggest that these factors may also affect *T. cruzi*-*T. rangeli* discrimination through PCR-based assays. Previous reports show that PCR amplifications using primers S35-S36 for *T. cruzi/T. rangeli* discrimination may be misleading when dealing with mixed infections. Amplified fragments ranging from 300bp to 450bp from *T. rangeli* could generate confusion with the expected 330bp fragment for *T. cruzi* [67]. Moreover, when Vargas and colleagues [66] used a mixture of DNA of both species in a 1:1 proportion as input for PCR reaction using S35-S36, a bias was detected and *T. cruzi* was preferentially amplified. Similar results were reported by yet another group which artificially infected *Rhodnius prolixus* with *T. cruzi* and *T. rangeli*: the latter was detected in less than 10% of samples with mixed infections while *T. cruzi* was detected in 100% of them [69]. Presumably, inter-specific differences in the molecular organization of the minicircle include a higher copy number of the target sequence in *T. cruzi*, which in turn would favor primer annealing in this species [66,69].

On the other hand, multiplex PCR of the mini-exon genes identified many instances of *T. cruzi*-*T. rangeli* mixed infections undetected by the kinetoplast PCR. However, NGS sequencing detected an even larger number of mixed infections, suggesting that *T. cruzi* DNA may also be preferentially amplified by the mini-exon-PCR method. This is consistent with previous observations where *T. cruzi* amplification is favored in mixed infections by this method. By mixing DNA input belonging to *T. cruzi* and *T. rangeli* in varying ratios, it was demonstrated that excess of starting material is a determinant factor for detection of a given species in mixed infections (and omission for the DNA species in lesser amount) [68]. The same authors also mention that the technique showed a higher sensitivity in detection of *T. cruzi*. Therefore, neither of the PCR-based methods is ideal for molecular epidemiology analysis.

Additionally, cases of incongruence between the PCR-based assays may be resolved through NGS analysis. One of our samples (TBR1455) showed a *T. cruzi* profile in the kinetoplast minicircle amplification with S35-S36, while the multiplex PCR suggested a mixed infection. In contrast, NGS exclusively detected *T. rangeli*-derived OTUs. This could be explained by the gel excision step, where TBR1455 was one of the samples that showed a two-band pattern in agarose gels and two amplicons were treated separately. Unfortunately, the larger fragment was lost due to technical problems. Therefore, we assume that the infection observed by solely *T. rangeli* is rather the result of mishandling instead of incongruence among techniques. We must point out that this was the only case where a sample with two amplicons showed NGS results for only one of the fragments.

The sample panel in this study was biased toward *R. ecuadoriensis* (43 out of 46 DNA samples in the analysis), one of the two most important vectors of CD in Ecuador [29]. A close association between *T. rangeli* and *Rhodnius* has been established in the past, where 12 out of 15 species of the genus have shown vectorial capacity [71] and two distinct lineages of the parasite (defined by the presence or absence of KP1 minicircle in the kinetoplast DNA) are transmitted selectively by different species of *Rhodnius* [72,73]. Our results suggest that current reports regarding *T. rangeli* infection in *R. ecuadoriensis* may underestimate the presence of this parasite in Loja. Indeed, from 33 samples analyzed by NGS, 21 were characterized as mixed infections and only 11 samples presented *T. cruzi* related OTUs exclusively. Therefore, we consider a wider distribution of *T. rangeli* in Loja and perhaps the rest of the country, which may have been overlooked in the past due to lack of sensitivity in molecular tools.

The presence of TcIV in samples from both communities is to our knowledge the first report of this DTU in the Loja province. There is only one similar record for the country with samples collected from the coastal region [44]. This remarkable finding poses a new insight into the distribution of the parasite in Ecuador. The occurrence of mixed infections (TcI/TcIV and TcI/TcIV/*T. rangeli*) has been suggested to have profound repercussions in vector parasite interactions [74], symptomatology onset and clinical outcome in human infections [75]. Moreover, as mentioned for *T. rangeli*, and taking into account that 7 samples out of 33 that went through NGS (21.2%), we consider that the presence of TcIV may have been overlooked in the past due to the lack of high resolution techniques, in a scenario where this DTU may have a wider distribution throughout the country than previously foreseen.

Altogether, our results show that genotyping by PCR amplification of kinetoplast minicircle regions using primers S35 and S36 is not recommended as a diagnostic tool in triatomines with mixed infections. Similarly, multiplex PCR of mini-exon genes is not recommended for diagnosis because it favors *T. cruzi* amplification rather than showing the presence of both parasites. NGS on the other hand, shows to be a powerful, though expensive and labor intensive, method for disambiguation.

The apparent homogeneity among localities in terms of richness and diversity may be the result of parasite dispersal between communities. Infected livestock, small mammals, passive transportation of triatomines or movement of infected humans should be considered. Both communities are only 28.3 km apart, so all the mentioned possibilities may be factors in this matter. Indeed, a previous work regarding *T. cruzi* in 16 communities in Loja demonstrated the existence of two discrete parasite populations: a cluster from sylvatic foci and another one represented by a domestic and peridomestic foci; no spatial genetic variation was recorded in the latter and a rapid parasite dispersal was suggested [43]. Similarly, our results are consistent in regard of homogeneity although NGS revealed several community-specific OTUs. Low sample number in Bella Maria may be the underlying cause of the lower number of OTUs recorded for both parasites which are exclusive for this locality, where *T. cruzi* and *T. rangeli* show 2 and 3 specific OTUs, respectively, that were not shared with Bramaderos. Clearly, no discrete populations exist within these two communities.

Resource-limited local control programs may improve their cost-efficiency by targeting specific areas of endemicity defined by more precise molecular tools. In this context, although non-pathogenic to humans, the numbers of *T. rangeli* infected vectors we encountered may confound efforts to map the distribution of *T. cruzi* and to unravel its transmission cycles in the country. Therefore, the distribution of *T. rangeli* in the region needs to be further explored.

Richness and diversity analysis were also performed to compare the parasite populations in different nymphal stages and adult individuals of the vectors. We hypothesized that the parasite population in late nymphal stages would evidence higher richness and diversity due to greater chance of exposure to potentially infected bloodmeals. Indeed, this was the case for *T. cruzi,* where adult females presented significantly higher richness than Instar III samples. Similarly, adult females exhibit significantly higher diversity values than Instars III and V. In contrast, *T. rangeli* did not evidenced significant differences neither for richness nor diversity among the different stages analyzed. This result may be obscured by lower number of *T. rangeli* samples in comparison with *T. cruzi*, although it has been previously reported that in *T. rangeli* natural infection was higher in nymphs than in adults [7]. *T. rangeli* has been proven pathogenic to several triatomine species [76] but no information is available in this matter for *R. ecuadoriensis*. The fact that no significant differences were found in richness nor diversity analysis suggests that the parasite sustains infection through nymphal stages to the adulthood without detrimental effects on the insect. It must also be considered that most of our samples were not infected with *T. rangeli* exclusively, so the interaction between the immune system of the triatomine was not confined to one parasite only, allowing *T. rangeli* to uphold the infection. Scarce information regarding mixed infections of *T. cruzi* and *T. rangeli* in *R. ecuadoriensis* is available, and a deep insight is required to clarify this situation in this vector with epidemiological importance.

R. ecuadoriensis is one of the two most important vectors of CD in Ecuador. From 2004 to 2015, the National Chagas Program for Chagas disease control targeted vector populations through insecticide spraying in selected regions. The efficiency of such initiatives is still under debate and several approaches aiming to understand the ecology of the vector have provided new insights into the epidemiology of CD. Here we suggest a wider distribution of *T. rangeli* in the Loja province and we report the molecular identification of DTU TcIV for the first time in Ecuador. NGS proves to be a high-resolution alternative for parasite scrutinization and we suggest that PCR based techniques are not reliable for detection of mixed infections due to a lack of sensibility. Our results show a wider view regarding population dynamics and structure of *T. cruzi* and *T. rangeli* in Southern Ecuador. Altogether, this information is of value in the elucidation of the dynamics of Chagas disease in Ecuador.

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INSTRUCCIONES PARA AUTORES

Revista PLOS Neglected Tropical Diseases
Instrucciones para autores obtenidas de:
http://journals.plos.org/plosntds/s/submission-guidelines

PLOS Neglected Tropical Diseases publishes original research articles of importance to the NTDs community and the wider health community. We will consider manuscripts of any length; we encourage the submission of both substantial full-length bodies of work and shorter manuscripts that report novel findings that might be based on a more limited range of experiments.

The writing style should be concise and accessible, avoiding jargon so that the paper is understandable for readers outside a specialty or those whose first language is not English. Editors will make suggestions for how to achieve this, as well as suggestions for cuts or additions that could be made to the article to strengthen the argument. Our aim is to make the editorial process rigorous and consistent, but not intrusive or overbearing. Authors are encouraged to use their own voice and to decide how best to present their ideas, results, and conclusions.

PLOS Neglected Tropical Diseases is committed to the highest ethical standards in medical research. Accordingly, we ask authors to provide specific information regarding ethical treatment of research participants, patient consent, patient privacy, protocols, authorship, and competing interests. We also ask that reports of certain specific types of studies adhere to generally accepted standards. Our requirements are based on the Uniform Requirements for Manuscripts Submitted to Biomedical Journals, issued by the International Committee for Medical Journal Editors.

Style and Format

File format

Manuscript files can be in the following formats: DOC, DOCX, RTF or PDF. Microsoft Word documents should not be locked or protected.

LaTeX manuscripts must be submitted as PDFs. Read the LaTeX guidelines.

Length

Manuscripts can be any length. There are no restrictions on word count, number of figures, or amount of supporting information.

We encourage you to present and discuss your findings concisely.

Font

Use a standard font size and any standard font, except for the font named “Symbol”. To add symbols to the manuscript, use the Insert → Symbol function in your word processor or paste in the appropriate Unicode character.

Headings

Limit manuscript sections and sub-sections to 3 heading levels. Make sure heading levels are clearly indicated in the manuscript text.

Layout and spacing

Manuscript text should be double-spaced.

Do not format text in multiple columns.

Page and line numbers

Include page numbers and line numbers in the manuscript file. Use continuous line numbers (do not restart the numbering on each page).

Footnotes

Footnotes are not permitted. If your manuscript contains footnotes, move the information into the main text or the reference list, depending on the content.

Language

Manuscripts must be submitted in English.

You may submit translations of the manuscript or abstract as supporting information. Read the supporting information guidelines.

Abbreviations

Define abbreviations upon first appearance in the text.

Do not use non-standard abbreviations unless they appear at least three times in the text.

Keep abbreviations to a minimum.

Reference style

PLOS uses “Vancouver” style, as outlined in the ICMJE sample references.

See reference formatting examples and additional instructions below.

Equations

We recommend using MathType for display and inline equations, as it will provide the most reliable outcome. If this is not possible, Equation Editor or Microsoft's Insert→Equation function is acceptable.

Avoid using MathType, Equation Editor, or the Insert→Equation function to insert single variables (e.g., “$a^2 + b^2 = c^2$”), Greek or other symbols (e.g., β, Δ, or ′ [prime]), or mathematical operators (e.g., x, ≥, or ±) in running text. Wherever possible, insert single symbols as normal text with the correct Unicode (hex) values.

Do not use MathType, Equation Editor, or the Insert→Equation function for only a portion of an equation. Rather, ensure that the entire equation is included. Equations should not contain a mix of different equation tools. Avoid “hybrid” inline or display equations, in which part is text and part is MathType, or part is MathType and part is Equation Editor.

Nomenclature

Use correct and established nomenclature wherever possible.

Units of measurement: Use SI units. If you do not use these exclusively, provide the SI value in parentheses after each value. Read more about SI units.

Drugs: Provide the Recommended International Non-Proprietary Name (rINN).

Species names: Write in italics (e.g., Homo sapiens). Write out in full the genus and species, both in the title of the manuscript and at the first mention of an organism in a paper. After first mention, the first letter of the genus name followed by the full species name may be used (e.g., H. sapiens).

Genes, mutations, genotypes, and alleles: Write in italics. Use the recommended name by consulting the appropriate genetic nomenclature database (e.g., HUGO for human genes). It is sometimes advisable to indicate the synonyms for the gene the first time it appears in the text. Gene prefixes such as those used for oncogenes or cellular localization should be shown in roman typeface (e.g., v-fes, c-MYC).

Allergens:The systematic allergen nomenclature of the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee should be used for manuscripts that include the description or use of allergenic proteins. For manuscripts describing new allergens, the systematic name of the allergen should be approved by the WHO/IUIS Allergen Nomenclature Sub-Committee prior to manuscript publication. Examples of the systematic allergen nomenclature can be found at the WHO/IUIS Allergen Nomenclature site.

Manuscript Organization

Most manuscripts should be organized as follows. Instructions for each element appear below.

- Title
- Authors and Affiliations
- Abstract
- Author Summary
- Introduction
- Methods
- Results
- Discussion
- Acknowledgments
- References
- Supporting information Captions

Uniformity in format facilitates the experience of readers and users of the journal. To provide flexibility, however, the Results and Discussion can be combined into one Results/Discussion section.

Other elements

Figure captions are inserted immediately after the first paragraph in which the figure is cited. Figure files are uploaded separately.

Tables are inserted immediately after the first paragraph in which they are cited.

Supporting information files are uploaded separately.

Please refer to our downloadable sample files to ensure that your submission meets our formatting requirements:

Viewing Figures and Supporting Information in the compiled submission PDF

The compiled submission PDF includes low-resolution preview images of the figures after the reference list. The function of these previews is to allow you to download the entire submission as quickly as possible. Click the link at the top of each preview page to download a high-resolution version of each figure. Links to download Supporting Information files are also available after the reference list.

Parts of a Submission

Title

Include a full title and a short title for the manuscript.

Full title: 250 characters, specific, descriptive, concise, and comprehensible to readers outside the field.

Ex:

Impact of cigarette smoke exposure on innate immunity: A *Caenorhabditis elegans* model.

Solar drinking water disinfection (SODIS) to reduce childhood diarrhoea in rural Bolivia: A cluster-randomized, controlled trial

Short title: 70 characters, state the topic of the study. Cigarette smoke exposure and innate immunity.

Titles should be written in sentence case (only the first word of the text, proper nouns, and genus names are capitalized). Avoid specialist abbreviations if possible. For clinical trials, systematic reviews, or meta-analyses, the subtitle should include the study design.

Author list

Authorship requirements

All authors must meet the criteria for authorship as outlined in the authorship policy. Those who contributed to the work but do not meet the criteria for authorship can be mentioned in the Acknowledgments.

The corresponding author must provide an ORCID iD at the time of submission by entering it in the user profile in the submission system.

Author names and affiliations

Enter author names on the title page of the manuscript and in the online submission system.

On the title page, write author names in the following order:

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Middle name (or initials, if used)

Last name (surname, family name)

Each author on the list must have an affiliation. The affiliation includes department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country. Authors have the option to include a current address in addition to the address of their affiliation at the time of the study. The current address should be listed in the byline and clearly labeled “current address.” At a minimum, the address must include the author’s current institution, city, and country.

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Author names will be published exactly as they appear in the manuscript file. Please double-check the information carefully to make sure it is correct.

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Only one corresponding author can be designated in the submission system, but this does not restrict the number of corresponding authors that may be listed on the article in the event of publication. Whoever is designated as a corresponding author on the title page of the manuscript file will be listed as such upon publication. Include an email address for each corresponding author listed on the title page of the manuscript.

Consortia and group authorship

If a manuscript is submitted on behalf of a consortium or group, include the consortium or group name in the author list. The consortium or group name should be listed in the manuscript file only, and not included in the online submission form. You may include the full list of members in the Acknowledgments or in a supporting information file. Please be aware that as of October 2016, the National Library of Medicine's (NLM) policy has changed and PubMed will only index individuals and the names of consortia or group authors listed in the author byline itself. Individual consortium or group author members need to be listed in the author byline in order to be indexed, and if included in the byline, must qualify for authorship according to our criteria.

Author contributions

Provide at minimum one contribution for each author in the submission system. Use the CRediT taxonomy to describe each contribution. Read the policy and the full list of roles.

Contributions will be published with the final article, and they should accurately reflect contributions to the work. The submitting author is responsible for completing this information at submission, and we expect that all authors will have reviewed, discussed, and agreed to their individual contributions ahead of this time.

Cover letter

Upload a cover letter as a separate file in the online system.

The cover letter should address the following questions:

Why is this manuscript suitable for publication in PLOS Neglected Tropical Diseases?

Why will your study inspire the NTDs community, and how will it drive the understanding of NTD pathobiology, epidemiology, prevention, treatment, control, or policy?

If your study addresses an infection that is outside our detailed scope, you must first send a presubmission inquiry indicating why you consider the infection to be a neglected tropical disease.

The cover letter will be available to the editors and to any external peer reviewers, so please send anything confidential directly to the journal office.

Title page

The title, authors, and affiliations should all be included on a title page as the first page of the manuscript file.

Abstract

The Abstract comes after the title page in the manuscript file. The abstract text is also entered in a separate field in the submission system.

The Abstract succinctly introduces the paper. It should not exceed 250–300 words. It should mention the techniques used without going into methodological detail and summarize the most important results with important numerical results given.

The Abstract is conceptually divided into the following three sections with these headings: Background, Methodology/Principal Findings, and Conclusions/Significance.

Do not include any citations in the Abstract. Avoid specialist abbreviations.

Author Summary

We ask that all authors of research articles include a 150- to 200-word non-technical summary of the work, immediately following the Abstract. Subject to editorial review and author revision, this short text is published with all research articles as a highlighted text box.

Distinct from the scientific abstract, the Author Summary should highlight where the work fits in a broader context of life science knowledge and why these findings are important to an audience that includes both scientists and non-scientists. Ideally aimed to a level of understanding of an undergraduate student, the significance of the work should be presented simply, objectively, and without exaggeration.

Authors should avoid the use of acronyms and complex scientific terms and write the author summary using the first-person voice. Authors may benefit from consulting with a science writer or press officer to ensure that they effectively communicate their findings to a general audience.

Introduction

The Introduction should put the focus of the manuscript into a broader context. As you compose the Introduction, think of readers who are not experts in this field. Include a brief review of the key literature. If there are relevant controversies or disagreements in the field, they should be mentioned so that a non-expert reader can delve into these issues further. The Introduction should conclude with a brief statement of the overall aim of the experiments and a comment about whether that aim was achieved.

Methods

This section should provide enough detail for reproduction of the findings. Protocols for new methods should be included, but well-established protocols may simply be referenced. Detailed methodology or supporting information relevant to the methodology can be published on our web site.

This section should also include a section with descriptions of any statistical methods employed. These should conform to the criteria outlined by the Uniform Requirements, as follows:

Describe statistical methods with enough detail to enable a knowledgeable reader with access to the original data to judge its appropriateness for the study and to verify the reported results. When possible, quantify findings and present them with appropriate indicators of measurement error or uncertainty (such as confidence intervals). Avoid relying solely on statistical hypothesis testing, such as P values, which fail to convey important information about effect size and precision of estimates. References for the design of the study and statistical methods should be to standard works when possible (with pages stated). Define statistical terms, abbreviations, and most symbols. Specify the statistical software package(s) and versions used. Distinguish prespecified from exploratory analyses, including subgroup analyses.

Submit detailed protocols for newer or less established methods. Well-established protocols may simply be referenced. Protocol documents for clinical trials, observational studies, and other non-laboratory investigations may be uploaded as supporting information.

We recommend and encourage you to deposit laboratory protocols in protocols.io, where protocols can be assigned their own persistent digital object identifiers (DOIs).

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1. Describe your step-by-step protocol on protocols.io
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Results

The Results section should include all relevant positive and negative findings. The section may be divided into subsections, each with a concise subheading. The Results section should be written in past tense.

PLOS journals require authors to make all data underlying the findings described in their manuscript fully available without restriction, with rare exception.

Large data sets, including raw data, may be deposited in an appropriate public repository. See our list of recommended repositories.

For smaller data sets and certain data types, authors may provide their data within supporting information files accompanying the manuscript. Authors should take care to maximize the accessibility and reusability of the data by selecting a file format from which data can be efficiently extracted (for example, spreadsheets or flat files should be provided rather than PDFs when providing tabulated data).

For more information on how best to provide data, read our policy on data availability. PLOS does not accept references to “data not shown.”

As outlined in the Uniform Requirements, authors that present statistical data in the Results section should do the following:

Give numeric results not only as derivatives (for example, percentages) but also as the absolute numbers from which the derivatives were calculated, and specify the statistical significance attached to them, if any. Restrict tables and figures to those needed to explain the argument of the paper and to assess supporting data. Use graphs as an alternative to tables with many entries; do not duplicate data in graphs and tables. Avoid nontechnical uses of technical terms in statistics, such *as “random” (which implies a randomizing device), “normal,” “significant,” “correlations,” and “sample.”*

Discussion

The Discussion should be concise and tightly argued. It should start with a brief summary of the main findings. It should include paragraphs on the generalizability, clinical relevance, strengths, and limitations of your study.

You may wish to discuss the following points also:

How do the conclusions affect the existing knowledge in the field?
How can future research build on these observations and what are the key experiments that must be done?

Acknowledgments

Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution.

Authors are responsible for ensuring that anyone named in the Acknowledgments agrees to be named.

References

Any and all available works can be cited in the reference list. Acceptable sources include:

- Published or accepted manuscripts
- Manuscripts on preprint servers, providing the manuscript has a citable DOI or arXiv URL. Read the Preprint Policy.

Do not cite the following sources in the reference list:

- Unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., “unpublished work,” “data not shown”). Instead, include those data as supplementary material or deposit the data in a publicly available database.
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References are listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, cite the reference number in square brackets (e.g., “We used the techniques developed by our colleagues [19] to analyze the data”). PLOS uses the numbered citation (citation-sequence) method and first six authors, et al.

Do not include citations in abstracts or author summaries.

Make sure the parts of the manuscript are in the correct order before ordering the citations.

Formatting references: PLOS uses the reference style outlined by the International Committee of Medical Journal Editors (ICMJE), also referred to as the “Vancouver” style. Example formats are listed below. Additional examples are in the ICMJE sample references.

Journal name abbreviations should be those found in the National Center for Biotechnology Information (NCBI) databases.

Published articles

Hou WR, Hou YL, Wu GF, Song Y, Su XL, Sun B, et al. cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (Ailuropoda melanoleuca). Genet Mol Res. 2011;10: 1576-1588.

Devaraju P, Gulati R, Antony PT, Mithun CB, Negi VS. Susceptibility to SLE in South Indian Tamils may be influenced by genetic selection pressure on TLR2 and TLR9 genes. Mol Immunol. 2014 Nov 22. pii: S0161-5890(14)00313-7. doi: 10.1016/j.molimm.2014.11.005.

Note: A DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers. When providing a DOI, adhere to the format in the example above with both the label and full DOI included at the end of the reference (doi: 10.1016/j.molimm.2014.11.005). Do not provide a shortened DOI or the URL.

Accepted, unpublished articles

Same as published articles, but substitute “Forthcoming” for page numbers or DOI.

Online articles

Huynen MMTE, Martens P, Hilderlink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available from: http://www.globalizationandhealth.com/content/1/1/14.

Books

Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.

Book chapters

Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28.

Deposited articles (preprints, e-prints, or arXiv)

Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity; 1991. Preprint. Available from: arXiv:1403.3301v1. Cited 17 March 2014.

Published media (print or online newspapers and magazine articles)Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 29 Jan 2014. Available from:

http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html Cited 17 March 2014.

New media (blogs, web sites, or other written works)

Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 -. [about 2 screens]. Available from: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/.

Masters' theses or doctoral dissertations

Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc. Thesis, The University of Sheffield. 1999. Available from: http://cumincad.scix.net/cgi-bin/works/Show?2e09.

Databases and repositories (Figshare, arXiv)

Roberts SB. QPX Genome Browser Feature Tracks; 2013 [cited 2013 Oct 5]. Database: figshare [Internet]. Available from: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214.

Multimedia (videos, movies, or TV shows)

Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM.

Supporting Information

Authors can submit essential supporting files and multimedia files along with their manuscripts. All supporting information will be subject to peer review. All file types can be submitted, but files must be smaller than 10 MB in size.

Authors may use almost any description as the item name for a supporting information file as long as it contains an “S” and number. For example, “S1 Appendix” and “S2 Appendix,” “S1 Table” and “S2 Table,” and so forth.

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The file number and name are required in a caption, and we highly recommend including a one-line title as well. You may also include a legend in your caption, but it is not required.

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Data reporting

All data and related metadata underlying the findings reported in a submitted manuscript should be deposited in an appropriate public repository, unless already provided as part of the submitted article.

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To support data sharing and author compliance of the PLOS data policy, we have integrated our submission process with a select set of data repositories. The list is neither representative nor exhaustive of the suitable repositories available to authors. Current repository integration partners include Dryad and

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Accession numbers

All appropriate data sets, images, and information should be deposited in an appropriate public repository. See our list of recommended repositories.

Accession numbers (and version numbers, if appropriate) should be provided in the Data Availability Statement. Accession numbers or a citation to the DOI should also be provided when the data set is mentioned within the manuscript.

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As much as possible, please provide accession numbers or identifiers for all entities such as genes, proteins, mutants, diseases, etc., for which there is an entry in a public database, for example:

Ensembl
Entrez Gene
FlyBase
InterPro
Mouse Genome Database (MGD)
Online Mendelian Inheritance in Man (OMIM)
PubChem

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Manuscripts reporting new and unpublished three-dimensional structures must include sufficient supporting data and detailed descriptions of the methodologies used to allow the reproduction and validation of the structures. All novel structures must have been deposited in a community endorsed database prior to submission (please see our list of recommended repositories).

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Authors reporting X-Ray crystallographic structures of small organic, metal-organic, and inorganic molecules must deposit their data with the Cambridge Crystallographic Data Centre (CCDC), the Inorganic Crystal Structure Database (ICSD), or similar community databases providing a recognized validation functionality. Authors are also required to include the relevant structure reference numbers within the main text (e.g. the CCDC ID number), as well as the crystallographic information files (.cif format) as Supplementary Information, along with the checkCIF validation reports that can be obtained via the International Union of Crystallography (IUCr).

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Authors reporting novel macromolecular structures must have deposited their data prior to submission with the Worldwide Protein Data Bank (wwPDB), the Biological Magnetic Resonance Data Bank (BMRB), the Electron Microscopy Data Bank (EMDB), or other community databases providing a recognized validation functionality. Authors must include the structure reference numbers within the main text and submit as Supplementary Information the official validation reports from these databases.

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