**Distinct drug-resistant *Trypanosoma cruzi* clones can arise independently in a single population undergoing benznidazole-selection**

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**Abstract**

Benznidazole is the front-line drug used against *Trypanosoma cruzi*, the causative agent of Chagas disease. This pro-drug is activated within the parasite by a mitochondrial type I nitroreductase. Loss or disruption of a single copy of the gene encoding this flavoprotein is sufficient to cause resistance to benznidazole and other nitroheterocycle drugs, including nifurtimox. Here, we demonstrate that distinct cross-resistant clones can arise independently within a single population. Following selection of benznidazole-resistant parasites, we found that all clones examined had lost one of the chromosomes containing the *TcNTR* gene. Sequence analysis of the remaining *TcNTR* allele revealed the presence of three distinct mutant genes in different resistant clones, each arising from missense mutations. Expression studies showed that, unlike the enzyme from the parental sensitive strain, those from the resistants were unable to reduce benznidazole. This correlated with loss of FMN-binding. The drug-resistant phenotype could be reversed by transfection with an active copy of the *TcNTR* gene. These results provide further evidence that TcNTR is a central player in the development of resistance to the therapeutic drugs used against Chagas disease. They also demonstrate that *T. cruzi* has a propensity to readily undergo genetic changes that can lead to drug-resistance under selective pressure, a finding that has implications for future therapeutic strategies.

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**Introduction**

Chagas disease is caused by *Trypanosoma cruzi,* a flagellated protozoan parasite transmitted by blood-sucking triatomine bugs. In Latin America, 10 million people are infected, with >15,000 deaths annually (1). Due to migration, the disease is also undergoing globalisation. In the USA, there are an estimated 300,000 infected individuals (2). Chagas disease has three phases; acute, indeterminate and chronic. The acute stage is usually asymptomatic, although it can present as a febrile-like illness in children and young adults, with a fatality rate up to 5%. Most symptoms resolve within 4-6 weeks and patients enter the indeterminate stage. Usually, active disease does not proceed further. However, ~30% of individuals progress to the chronic phase, a process that can occur many years after the initial infection. This can result in serious cardiac and digestive tract pathologies, where prognosis is poor.

There is no immediate prospect of a Chagas disease vaccine and infection is life-long. Chemotherapy is therefore of major importance. For many years, benznidazole and nifurtimox have been the only drugs available (3). However, their use is characterised by toxicity and their efficacy against chronic stage disease is unreliable. In addition, cases refractory to treatment are commonly reported (4) and drug-resistant parasites can be selected in the laboratory (5, 6). Benznidazole and nifurtimox are nitroheterocyclic compounds which contain a nitro group linked, respectively, to an imidazole and furan ring (3). They are pro-drugs and require nitroreductase (NTR)-catalysed activation within the parasite to have trypanocidal effects. Two classes of NTR have been identified in trypanosomes (7). Type II NTRs are $O_2$-sensitive flavin-containing enzymes which are capable of 1-electron reduction of nitro-drugs to

generate an unstable nitro-radical. In the presence of $O_2$, this leads to the production of superoxide anions and regeneration of the parent nitro-compound, a process known as redox cycling (8, 9). Although activation of benznidazole and nifurtimox by *T. cruzi* has been associated with the formation of reactive oxygen species (ROS), and candidate reductases have been implicated, there is no direct experimental evidence of a significant correlation between drug-induced ROS and trypanocidal activity (10-15).

Type I NTRs are $O_2$-insensitive FMN-dependent enzymes that can mediate the 2-electron reduction of nitro-drugs through a nitroso, to hydroxylamine derivatives. These can react further to generate nitrenium cations and other highly electrophilic intermediates which may promote damage to DNA and other macromolecules (16, 17). Two enzymes with type I activity have been identified in *T. cruzi.* The first is prostaglandin F2$\alpha$ synthase (18), although this is only capable of mediating 2-electron reduction under anaerobic conditions. The second, for which there is now strong evidence of a central role in activating both benznidazole and nifurtimox, is a NADH-dependent mitochondrial type I NTR (5). In the case of nifurtimox, the active metabolite is an unsaturated open chain nitrile (19).

TcNTR can reduce a range of nitroheterocycles and deletion of the corresponding genes from *T. cruzi* and *T. brucei* results in loss of sensitivity (5). The first evidence that loss of TcNTR activity might be an important mechanism for causing drug-resistance came from analysis of laboratory-generated nifurtimox-resistant *T. cruzi* (5). These were found to lack one of the chromosomes containing the *TcNTR* gene and to exhibit cross-resistance to benznidazole. Consistent with this, a genome-wide RNAi screen of *T. brucei* for genes associated with nifurtimox and benznidazole

resistance by loss-of-function mechanisms identified *TbNTR* as the major candidate (20).

To investigate the capacity of *T. cruzi* to develop resistance against benznidazole, we generated resistant clones following *in vitro* selection. Here, we show that distinct drug-resistant clones can arise independently, and that in each case, resistance under selective pressure is associated with loss of TcNTR activity.

## Results

**Benznidazole-resistant *T. cruzi* lack one of the chromosome bands containing the *TcNTR* gene.** To select for benznidazole-resistance, *T. cruzi* GAL61 (Table 1) were submitted to continuously increasing drug pressure until we had established a population (61R) that grew at a comparable rate in the presence or absence of 50 μM benznidazole (Materials and Methods). This population displayed ~10-fold resistance. Six clonal lines derived from this population exhibited between 3- and 7-fold resistance, when examined independently (Fig. 1*A*). In the absence of drug, the clones grew slightly slower in culture that the parental cells (doubling times from 28–42 hours, compared to 26 hours), but otherwise displayed no obvious morphological changes. Previously, when we generated nifurtimox-resistant *T. cruzi*, we found that they were also resistant to other nitroheterocyclic drugs, including benznidazole (5). A similar cross-resistance phenomenon was observed here, with 2-fold greater resistance to nifurtimox and 4-fold to nitrofurazone (Fig. 1*B*).

Nifurtimox-resistance in both *T. cruzi* and *T. brucei* has been associated with down-regulation or loss of a type I *NTR* gene (5, 20). We therefore examined the benznidazole-sensitive and resistant cells for changes in copy number at this locus. In the sensitive parental cells (61S), *TcNTR* is a single copy gene located on chromosome homologues of 1.1 and 0.85 Mb. With the resistant parasites however, the 0.85 Mb band was missing in clonal and polyclonal populations (Fig. 1*C*, lanes 2 and 3). There were no other apparent changes to the chromosome profile. To determine if drug-resistance was associated with loss of *TcNTR*, rather than another gene located elsewhere on the missing chromosome, we re-introduced an active copy

of *TcNTR* into 61R clone 2 (21, Fig. 1*D*). When the transformed cells were assessed, we found that benznidazole-sensitivity had been restored.

**The remaining *TcNTR* allele in each benznidazole-resistant clone encodes an inactive protein.** We next investigated if the remaining chromosomal copy of *TcNTR* in the benznidazole-resistant 61R parasites had altered. Genes from the 6 resistant clones were amplified and sequenced. Missense mutation(s) were identified in each case. In clones 1, 2, 4 and 5, there was C/T transition at position 374, compared with the *TcNTR* gene amplified from sensitive clones. In the protein, this would result in replacement of the evolutionarily conserved Pro-125 with leucine (Fig. 2). With clone 6, in addition to the mutation at position 374, we also identified a missense mutation at nucleotide 460 (C/G), giving rise to the conversion of Pro-154 to alanine. For clone 3, there was a single missense mutation resulting in C/G transversion at nucleotide 477, leading to the replacement of Phe-159 with leucine. No other mutations were observed in the *TcNTR* genes isolated from the resistant clones. In the $O_2$-insensitive *E. coli* nitroreductase *nfsB*, most mutations associated with nitrofuran resistance are located in the corresponding region to those in TcNTR (22, 23; Fig. 2).

To determine if the TcNTR mutations had perturbed activity, we amplified a fragment encoding the catalytic region of the enzyme using DNA from 61S and 61R clones 3, 4 and 6. TcNTR is mitochondrial and previous attempts to express active full length enzyme had been unsuccessful. Activity was only detectable when the amino terminal domain was excluded from the recombinant protein (Fig. 2; 5). After sequence confirmation, the expressed histidine-tagged proteins were purified on nickel columns (Materials and Methods; Fig. 3*A*). Fractions containing recombinant protein derived

from the 61S *TcNTR* gene were yellow, as expected of a flavoprotein. Those containing enzyme derived from the resistant clones were colourless.

The capacity of the recombinant enzymes to reduce benznidazole and nifurtimox was established from double reciprocal plots of 1/TcNTR activity against 1/[drug], at a fixed NADH concentration (100 μM) (Materials and Methods, Fig. 3*B, C*). For the enzyme derived from the sensitive clone, we established apparent $K_m$ values of 26 μM for benznidazole and 18 μM for nifurtimox. Further analysis gave apparent $V_{max}$ values of 1933 and 389 nmol NADH oxidised $min^{-1}$ $mg^{-1}$ for benznidazole and nifutimox, respectively. When each of the mutant TcNTRs were analysed, no activity could be detected, even when 10 times as much recombinant protein was used. We then investigated the mutant proteins for flavin binding (Fig. 3*D*) using fluorescent detection under neutral and acidic conditions (Materials and Methods). At neutral pH and using an excitation wavelength of 450 nm, the FMN standard and 61S TcNTR-derived co-factor both gave a fluorescence profile that peaked at 535 nm, a signal which was quenched under acidic conditions. By contrast with FAD, the 535 nm peak occurs at pH2 and is quenched at pH7. No flavin fluorescence was detected with mutant TcNTR protein (Fig. 3*D*).

**The infectivity of benznidazole-resistant parasites.** To investigate the scope for drug-resistance in the field to result from loss/inactivation of *TcNTR* genes, we examined the effects of these events on infectivity. First, we generated heterozygous parasites to test for haploid insufficiency. One *TcNTR* allele in the 61S genome was disrupted by targeted integration (Fig. S1). The 61S *TcNTR*+/- epimastigotes grew at the same rate in culture as homozygotes, and to the same density. When these

heterozygotes were examined for benznidazole-resistance, there had been a 4-fold increase (Fig. 4*A*). These parasites were used to infect rat myoblast L6 cells. No differences were observed in the ability of the heterozygotes to develop into infective metacyclic trypomastigotes, to invade cells (Fig. 4*B*), to grow as intracellular amastigotes (Fig. 4*C*) and subsequently to differentiate into bloodstream trypomastigotes. Therefore, drug-resistance that arises through loss of one copy of *TcNTR* is not associated with a reduction in infectivity *in vitro*.

The infective phenotype of the 61R resistant clones, which contain a single inactive copy of *TcNTR*, was also examined. In culture, epimastigotes differentiated into metacyclic trypomastigotes at a level similar to sensitive clones. When culture-derived trypomastigotes were used to initiate infections, all the resistant clones tested (clones 3, 4 and 6) were able to develop through the intracellular cycle as amastigotes and differentiate into bloodstream trypomastigotes, which were released following host cell lysis. At two levels however, we observed a reduction in virulence. When Vero cells were used (Fig. 4*D*), the number infected by resistant clones was significantly less than the level observed with the parental sensitive parasites (Fig. 4*E*) and the average number of amastigotes per infected cell was reduced (Fig. 4*F*). When L6 cells were infected with drug-resistant metacyclics, although released trypomastigotes could be observed, their numbers were too few for a quantifiable infection assay to be carried out. This compares to an infection rate of ~25% in the case of the 61S *TcNTR* heterozygotes and homozygotes (Fig. 4*B*). These experiments therefore suggest that functional loss of both *TcNTR* genes, by the mechanisms identified here, is associated with a reduction in virulence that would reduce the capacity of highly drug-resistant parasites to spread within the population.

**TcNTR diversity and benznidazole-sensitivity in the field**

To explore possible relationships between natural susceptibility to benznidazole and TcNTR, we sequenced the gene from 28 Colombian strains of different biological and geographical origins and with a range of benznidazole-sensitivities ($IC_{50}$ 1.5 - 35 μM) (Table 1). *TcNTR* length varied between 939 - 951 nucleotides in these strains, mainly due to changes in the copy number of a trinucleotide $(ATC)_{5-9}$ located between residues 210 - 238. This region of the protein is not required for enzyme activity (5). Excluding this repeat, we identified 42 polymorphisms, 25 of which were nonsynonymous. These amino acid differences were restricted to 7 strains, all but one of human origin (Fig. S2, S3). None of the polymorphisms were located in the region of TcNTR where we had identified mutations associated with benznidazole-resistance. Most were located in the amino terminal extension (Fig. S3). The major amino acid haplotype group encompassed 21 strains of various biological and geographical origins. Importantly, these had a wide range of benznidazole-sensitivities ($IC_{50}$ 4 - 35 μM) (Table 1). This extensive natural variation is therefore independent of TcNTR sequence and must be due to other factors. This suggests that resistance arising from changes to TcNTR is an acquired trait that requires selective pressure.

**Discussion**

Despite being the front line drug against *T. cruzi* infections for >40 years, benznidazole has drawbacks (24, 25). It can have serious side effects, requires long term administration (30-60 days), and its efficacy against chronic stage disease is inconsistent. Treatment failures are widely reported, although the extent to which this is an acquired trait, or reflects diversity in the level of susceptibility within natural parasite populations is unknown (26). As shown here and elsewhere (5, 27, 28), laboratory selection of drug-resistant *T. cruzi* is readily achievable, but in the case of benznidazole and nifurtimox, it is only recently that a mechanism has been identified (5). Activation of these pro-drugs by the trypanosome type I NTR, an enzyme absent from mammals, is central to their mode of action and explains why they are more toxic to the parasite than to the host. The 61R benznidazole-resistant *T. cruzi* clones that we investigated were characterised by loss of a 0.85 Mb chromosome band containing *TcNTR*. Genome plasticity is a common phenomenon in trypanosomes (29). Confirmation that reduced *TcTNR* expression caused this resistance was provided by reversion of the phenotype following re-introduction of the gene. Unexpectedly, we also found that in each of the 61R clones examined, the *TcNTR* gene on the 1.1 Mb chromosome homologue had acquired missense mutation(s) which rendered the expressed product enzymatically inactive (Fig. 2,3).

The most parsimonious explanation for our data is that drug pressure led initially to selection of benznidazole-resistance due to loss of the *TcNTR*-containing 0.85 Mb chromosome. Continued treatment then resulted in selection, from within this population, of distinct lineages in which mutation(s) had inactivated the remaining *TcNTR* gene. The acquisition of two distinct missense mutations in *TcNTR* of clone 6

(nucleotides 374 and 460) implies consecutive events. This two-step process is reminiscent of *E. coli*, where increased nitrofuran-resistance resulted from consecutive mutations in the type I NTR genes *nfsA* and *nfsB* (23). The mutant TcNTR proteins were found to be deficient in FMN-binding. In the NTR group of enzymes, location of the flavin-binding is highly conserved within the overall structure (22). All of the mutations in TcNTR were restricted to a region (residues 125-159, Fig. 2), which in the *E. coli* enzyme, contains residues that interact with the isoalloxazine O2, N3, O4 face of FMN (22). The mutation of residue 125 resulted in conversion of an evolutionarily conserved proline to a leucine (clones 1, 2, 4, 5 and 6). At position 154 in clone 6, proline was converted to alanine. Both changes would be expected to perturb structure. In clone 3, the mutation associated with disruption of FMN-binding involved conversion of phenylalanine 159 to leucine. Phenylalanine is present at the corresponding position in *E. coli* and *T. cruzi* NTRs (Fig. 2), suggesting a functionally conserved role.

The ability of distinct TcNTR-deficient *T. cruzi* clones to arise independently in a single population is strong additional evidence that the drug-activating properties of this enzyme (5) are central to the trypanocidal mechanism. The *TcNTR* single-knockouts were 4-fold less susceptible to benznidazole (Fig. 4), a level of resistance that is significant in the context of this drug, where the therapeutic window is limited (3). The virulence properties *in vitro* were also indistinguishable from *TcNTR* homozygotes. This potential for benznidazole-resistance by a straightforward mechanism, coupled with the absence of haploid insufficiency, may explain some of the observed treatment failures. The inability of the 61S strain to produce a patent infection in mice has restricted us from investigating this further. Complete loss of

TcNTR activity in the 61R resistant clones did however have a detrimental effect on infectivity *in vitro* (Fig. 4). This implies that *in vivo* there will be a limit to the extent of benznidazole-resistance achievable by mechanisms involving TcNTR (approximately 4-fold), since parasites need to retain a residual level of enzyme activity. When we investigated possible relationships between susceptibility to benznidazole and TcNTR sequence in a diverse group of parasites (Table 1), we found no correlation. These data suggest that natural variation in sensitivity does not involve mutations in *TcNTR* and that resistance by this mechanism may be a trait that arises only after selective pressure. Currently, there is no information on the extent to which treatment failures reflect natural or acquired resistance.

An observation, which has wider implications for treatment of Chagas disease, is the ease with which drug-resistance can arise. In a single experiment, we identified two distinct mechanisms, chromosome loss and point mutation, which acted to reduce TcNTR activity. In the latter case, three distinct, independently-acquired mutations were identified. *T. cruzi* is extremely diverse, with a genome characterised by extensive and highly variable surface antigen gene families (30). This antigenic diversity may have arisen in response to selective immune pressure during evolution which acted to limit the proof-reading ability of DNA polymerase and/or DNA repair mechanisms. As a consequence, the parasite may have acquired an ability to readily develop drug-resistance by mutational mechanisms such as those described here. This is an important consideration which should inform drug development strategies for Chagas disease.

**Materials and Methods**

**Parasites.** *T. cruzi* MRAT/COL/Gal61 (Table 1; 31) were cultivated in supplemented RPMI-1640 medium at 28°C (32). Clones were derived by limiting dilution. Transformed *T. cruzi* were maintained at 10 μg $ml^{-1}$ blasticidin or 50 μg $ml^{-1}$ G418. Amastigotes were grown in African green monkey kidney (Vero) or rat skeletal myoblast L6 cells cultured in RPMI-1640/10% FBS at 37°C in 5% $CO_2$. Intact *T. cruzi* chromosomes were extracted using an agarose-embedding technique (29) and fractionated by contour-clamped homogenous field electrophoresis (CHEFE) using a BioRad CHEFE Mapper. For analysis of natural benznidazole-sensitivity, *TcNTR* from 28 *T. cruzi* strains from different regions of Colombia was amplified and sequenced.

To generate benznidazole-resistance, epimastigotes were seeded at the $IC_{50}$ and subcultured for several weeks under selective pressure. The drug concentration was then doubled and the process repeated. This was continued until a resistant population was established (61R) at 50 μM, the reported level of therapeutic resistance (33). $IC_{50}$s were determined by an enzymatic micromethod (34). $2x10^6$ epimastigotes $ml^{-1}$ were cultured with different drug concentrations for 72 hours at 28°C in 96-well microtitre plates. The plates were then incubated with 10 mg $ml^{-1}$ 3(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) for 90 min and MTT reduction to formazan crystals measured at 595 nm.

**Construction of vectors.** For expression of TcNTR, a 708 bp fragment corresponding to the catalytic domain of the protein was amplified using DNA from sensitive and resistant clones (5). Fragments were digested with BamHI/HindIII, ligated into the

vector pTrcHis-C (Invitrogen), and the resulting constructs used to transform *E. coli* BL-21. To express active protein in benznidazole-resistant *T. cruzi*, the full-length *TcNTR* gene (939 bp) was amplified from 61S DNA and ligated into the BamHI/HindIII site of the vector pTEX (21). Parasites were electroporated and transformants selected with G418. To generate *TcNTR* heterozygotes from 61S parasites, we used gene disruption with a construct containing a blasticidin-resistance cassette (5). All constructs were confirmed by sequencing.

**Biochemical analysis.**

*E.coli* transformed with pTrcHis-TcNTR were treated with IPTG to induce expression of recombinant histidine-tagged proteins, which were purified on Ni-NTA columns (5, 35). Fractions were analyzed by SDS-PAGE and protein concentrations determined by the BCA assay (Pierce). TcNTR activity was measured by following the changes in absorbance at 340 nm due to NADH oxidation (5).

The TcNTR flavin co-factor was established by determining the fluorescence spectrum in acidic and neutral buffers (36). Purified protein (0.5 mg) was desalted and boiled for 5 min. Clarified supernatant (90 µl) was then mixed with 10 µl 50 mM $NaH_2PO_4$ pH7.6 or 1 M HCl (final pH = 2.2) and the fluorescence profile measured with a Gemini Fluorescent Plate Reader (Molecular Devices). The mean fluorescence values (excitation $\lambda$= 450 nm; emission $\lambda$=535 nm) was determined and compared to FMN and FAD standards.

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**Fig. 1.** Properties of *T. cruzi* clones derived by benznidazole selection. (A) Benznidazole concentration that inhibited growth of resistant parasites (61R non-cloned population and clones 1-6) and parental (61S) cells by 50% ($IC_{50}$) (Materials and Methods). (B) The 61R cells are cross-resistant to the other nitroheterocycles nifurtimox and nitrofurazone. (C) *T. cruzi* chromosomal DNA separated by CHEFE and hybridized with a *TcNTR* gene probe. Left panel, ethidium bromide stained gel; right panel, autoradiograph of gel after Southern blotting. Lane 1, parental 61S; lane 2, 61R clone 3; lane 3, 61R (non-cloned population). (D) Re-introduction of an active copy of *TcNTR* into benznidazole-resistant parasites (61R clone 2) using the pTEX vector reverses the drug-resistance phenotype. Growth inhibition data are the mean of 3 experiments $\pm$ standard deviation. An autoradiograph (right) shows BamHI-digested DNA from parental 61R clone 2 (lanes 1, 2) and pTEX-TcNTR transformed cells (lanes 3, 4) hybridized with a *TcNTR* gene probe.

**Fig. 2.** Mutations in TcNTR from benznidazole-resistant *T. cruzi*. The TcNTR schematic identifies the amino terminal extension (excluded from recombinant proteins) and the location of putative FMN-binding regions inferred by analogy with *E. coli* nfsB (22). Full-length copies of *TcNTR* from 61R resistant clones were amplified and sequenced. Differences in the amino acid sequence compared to the parental TcNTR (61S) were restricted to a single region and are highlighted in red. Several 61S clones were sequenced, but no differences were identified. The sequence in this region of 61S TcNTR (residues 112-162) is identical to that in the genome strain CL Brener (accession no. XP_810645). Mutations in the corresponding region of *E. coli* nfsB which confer nitrofurantoin-resistance are indicated by asterisks (23).

**Fig. 3.** Biochemical analysis of TcNTR from benznidazole-sensitive and resistant *T. cruzi.* (A) Purification of recombinant TcNTR. Upper image, wild type (61S) enzyme; lower, clone 4. Protein expression was induced by IPTG (Materials and Methods) and a clarified fraction (lane 1) was loaded onto a Ni-NTA column and the flowthrough collected (lane 2). The column was washed with 50 then 100 mM imidazole (lanes 3 and 4) and the recombinant protein eluted with 500 mM imidazole, 1% triton X-100 (lane 5). The 32 kDa TcNTR band is highlighted by an arrow. Recombinant protein from each of the resistant clones was purified in a similar manner. (B) TcNTR activity was monitored (340 nm) by following oxidation of NADH (100 μM) in the presence of wild type or mutant (P125L, clone 4) enzyme (0.2 μg) and benznidazole (100 μM). (C) Activity (v) of the wild type enzyme (nmol NADH $min^{-1}$ $mg^{-1}$) was established by this assay with a fixed concentration of NADH (100 μM) in the presence of different levels of benznidazole (BNZ) (10-100 μM). (D) Florescence (excitation λ=450nm; emission λ=535nm) of the TcNTR co-factor (wild type and P125L) and FMN and FAD controls under acidic and neutral conditions (Materials and Methods). (E) Activity of wild type and mutant TcNTRs.

**Fig. 4.** Loss of one copy of *TcNTR* does not reduce infectivity. Targeted disruption of *TcNTR* in 61S epimastigotes was achieved using a construct which confers blasticidin-resistance (Fig. S1). (A) *TcNTR* heterozygotes are benznidazole-resistant. 61S *TcNTR* homozygotes (+/+) and heterozygotes (+/-) were tested to establish their $IC_{50}$. Data are the mean of 3 experiments $\pm$ standard deviation. (B) Heterozygotes are not deficient in infectivity. Cell-derived trypomastigotes were added to an L6 cell monolayer at a ratio of 1:5 cells:parasites. Infected cells were counted after 72 hours, (experiment performed in triplicate). (C) Heterozygotes are not deficient in their

ability to replicate in mammalian cells. Infections were carried out and monitored as described above. (D) Infection of Vero cells with 61S sensitive and 61R resistant parasites, stained with Giemsa. (1) 61S; (2) 61R clone 3; (3) 61R clone 4; (4) 61R clone 6. Arrows indicate intracellular amastigotes 48 hours post-infection. (E) Benznidazole-resistant clones are deficient in their ability to infect Vero cells. Values shown were from 5 experiments ($P<0.05$). (F) Benznidazole-resistant clones are less able to replicate in Vero cells ($P<0.05$).

**Table 1.** Natural sensitivity to benznidazole is not associated with TcNTR sequence. *TcNTR* genes were amplified from DNA of 28 Colombian *T. cruzi* strains and sequenced.

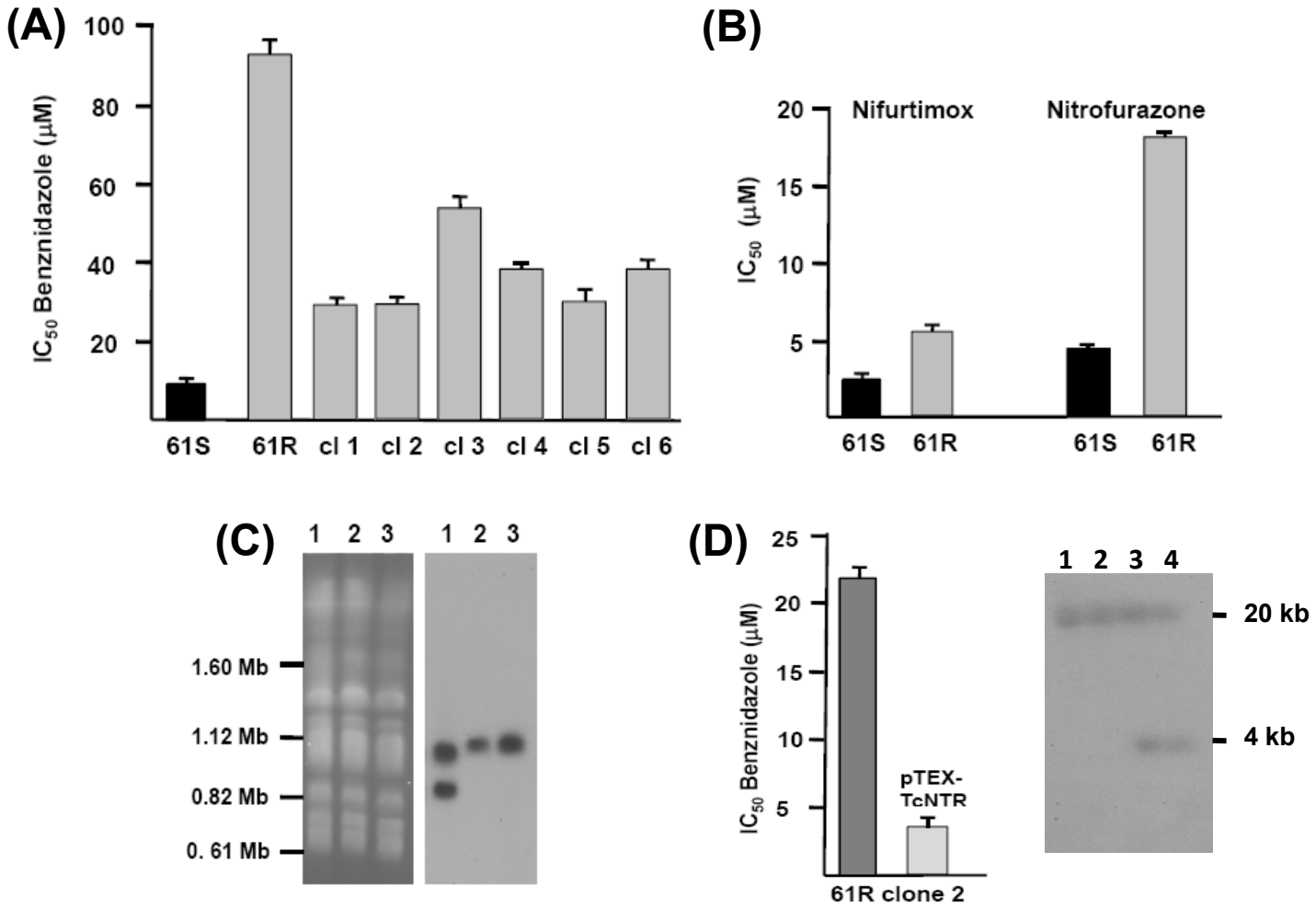

(A)
IC50 Benznidazole (μM)
100
80
60
40
20
61S
61R
cl 1
cl 2
cl 3
cl 4
cl 5
cl 6
(B)
IC50 (μM)
20
15
10
5
Nifurtimox
Nitrofurazone
61S
61R
61S
61R
(C)
1 2 3
1 2 3
1.60 Mb
1.12 Mb
0.82 Mb
0. 61 Mb
(D)
IC50 Benznidazole (μM)
25
20
15
10
5
pTEX-TcNTR
61R clone 2
1 2 3 4
20 kb
4 kb

Fig. 1

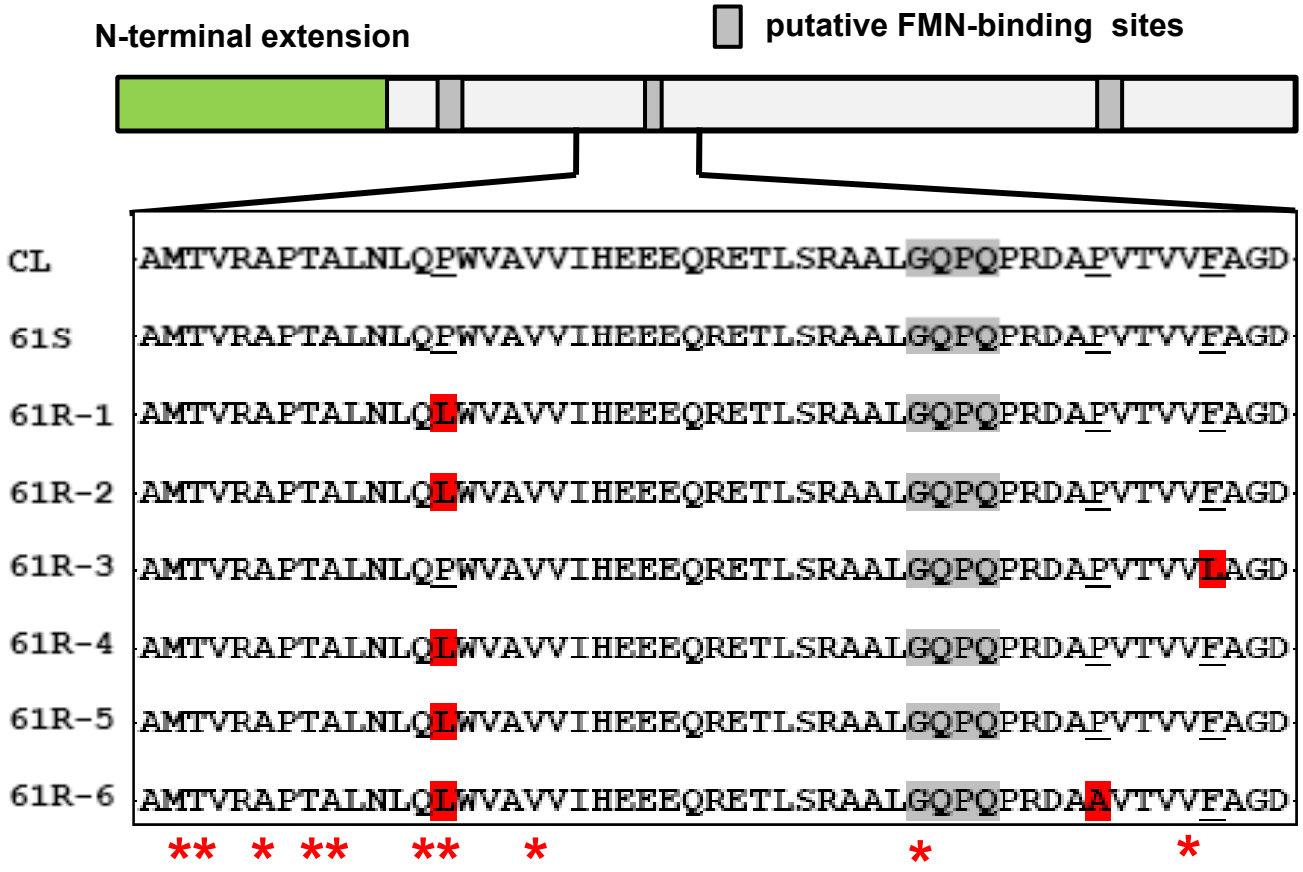
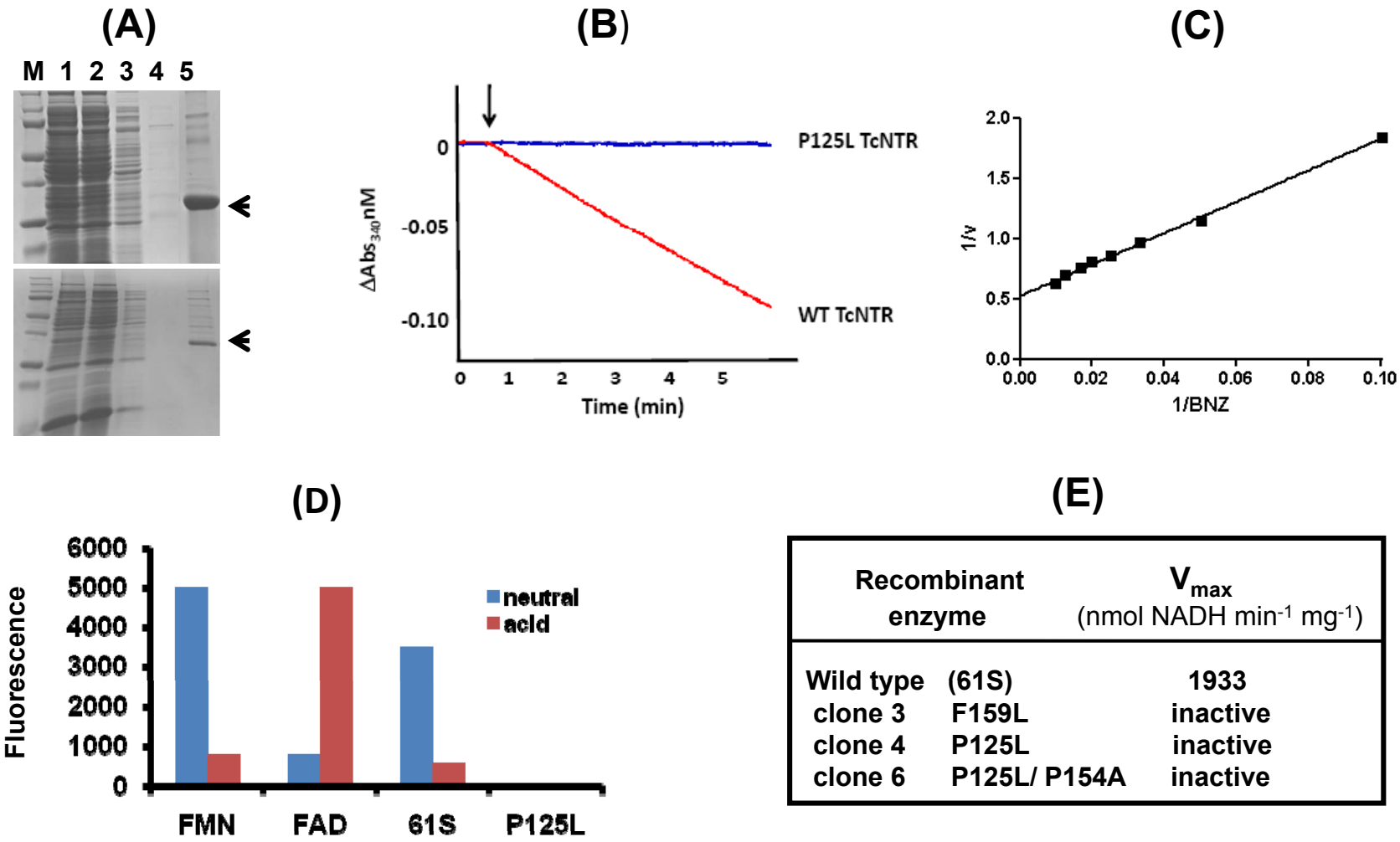

N-terminal extension
putative FMN-binding sites
CL AMTVRAPTALNLQPWVAVVIHEEEQRETLSRAALGQPQPRDAPVTVVFAGD
61S AMTVRAPTALNLQPWVAVVIHEEEQRETLSRAALGQPQPRDAPVTVVFAGD
61R-1 AMTVRAPTALNLQLWVAVVIHEEEQRETLSRAALGQPQPRDAPVTVVFAGD
61R-2 AMTVRAPTALNLQLWVAVVIHEEEQRETLSRAALGQPQPRDAPVTVVFAGD
61R-3 AMTVRAPTALNLQPWVAVVIHEEEQRETLSRAALGQPQPRDAPVTVVLAGD
61R-4 AMTVRAPTALNLQLWVAVVIHEEEQRETLSRAALGQPQPRDAPVTVVFAGD
61R-5 AMTVRAPTALNLQLWVAVVIHEEEQRETLSRAALGQPQPRDAPVTVVFAGD
61R-6 AMTVRAPTALNLQLWVAVVIHEEEQRETLSRAALGQPQPRDAAVTVVFAGD
** * ** ** * * *

Fig. 2




(E)

| Recombinant enzyme | | $V_{max}$ (nmol NADH $min^{-1}$ $mg^{-1}$) |
|---|---|---|
| Wild type | (61S) | 1933 |
| clone 3 | F159L | inactive |
| clone 4 | P125L | inactive |
| clone 6 | P125L/ P154A | inactive |

Fig. 3

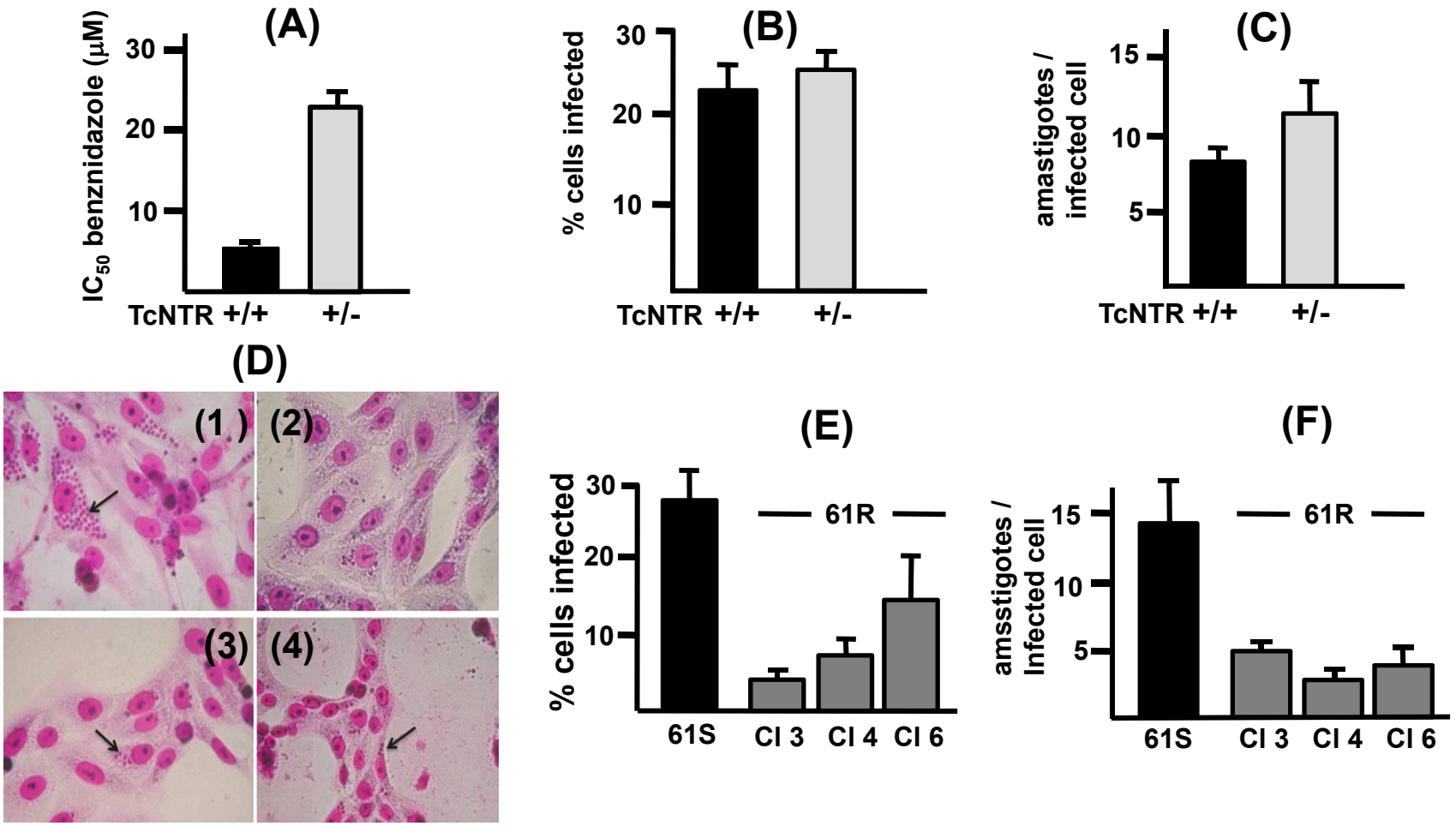

(A)
IC50 benznidazole (μM)
30
20
10
TcNTR +/+
+/-
(B)
% cells infected
30
20
10
TcNTR +/+
+/-
(C)
amastigotes / infected cell
15
10
5
TcNTR +/+
+/-
(D)
(1 )
(2)
(3)
(4)
(E)
% cells infected
30
20
10
61R
61S
Cl 3
Cl 4
Cl 6
(F)
amsstigotes / Infected cell
15
10
5
61R
61S
Cl 3
Cl 4
Cl 6

Fig. 4

| Strain | GenBank accession N° | Biological Origin | Geographical Origin | Phylogentic Group | $IC_{50}$ (μM) | |
|---|---|---|---|---|---|---|
| AC17 | JN043349 | *Rhodnius pallescens* | Chocó | I | 6.53 ± 1.12 | |
| AMP07 | JN043351 | *Panstrongylus geniculatus* | Antioquia | I | 17.6 ± 0.3 | |
| B114 | JN043353 | *Triatoma dimidiata* | Córdoba | I | 18.7 ± 1.4 | |
| B138 | JN043352 | *T. dimidiata* | Córdoba | I | 17.6 ± 0.7 | |
| B51 | JN043354 | *R. pallescens* | Córdoba | I | 20.6 ± 1.1 | |
| CAS18 | JN043345 | *D. marsupialis* | Casanare | I | 3.90 ± 0.78 | |
| CG | JN043336 | *Homo sapiens* | Caquetá | II | 4.61 ± 0.35 | |
| GAL52 | JN043347 | *Didelphis marsupialis* | Sucre | I | 9.07 ± 2.06 | |
| GAL61 | JN043346 | *Rattus rattus* | Sucre | I | 5.85 ± 1.51 | |
| HA | JN043337 | *H. sapiens* | Casanare | I | 4.66 ± 0.61 | |
| LB53 | JN043358 | *T. dimidiata* | Sucre | I | 17.0 ± 0.7 | |
| MG | JN043339 | *H. sapiens* | Arauca | I | 4.90 ± 0.33 | **Haplotype 1** |
| MG10 | JN043356 | *T. dimidiata* | Magdalena | I | 14.9 ± 0.89 | |
| OV1 | JN043359 | *P. geniculatus* | Sucre | I | 17.4 ± 0.76 | |
| OV17 | JN043355 | *P. geniculatus* | Sucre | I | 22.2 ± 2.4 | |
| SN3 | JN043361 | *Rhodnius prolixus* | La Guajira | I | 34.6 ± 1.9 | |
| SN5 | JN043360 | *R. prolixus* | La Guajira | I | 24.2 ± 1.3 | |
| SN6 | JN043357 | *R. prolixus* | La Guajira | I | 16.9 ± 0.9 | |
| SP | JN043342 | *H. sapiens* | Casanare | I | 6.41 ± 0.75 | |
| SPR | JN043341 | *H. sapiens* | Casanare | II | 5.32 ± 1.08 | |
| STP33 | JN043350 | *R. prolixus* | Tolima | I | 11.3 ± 1.0 | |
| AF1 | JN043348 | *P. geniculatus* | Antioquia | II | 4.69 ± 1.87 | **Haplotype 2** |
| JEM | JN043340 | *H. sapiens* | Putumayo | I | 5.19 ± 0.65 | |
| DA | JN043344 | *H. sapiens* | Boyacá | I | 32.8 ± 3.3 | **Distinct Haplotypes** |
| FCH | JN043334 | *H. sapiens* | N.de Santander | II | 1.50 ± 0.51 | |
| MR | JN043338 | *H. sapiens* | Cesar | II | 4.71 ± 0.29 | |
| W3534 | JN043343 | *H. sapiens* | Sucre | I | 14.0 ± 1.3 | |
| YLY | JN043335 | *H. sapiens* | Putumayo | I/II | 4.38 ± 0.24 | |