Protease expression analysis in recently field-isolated strains of *Trypanosoma cruzi*: a heterogeneous profile of cysteine protease activities between TC I and TC II major phylogenetic groups

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SUMMARY

Protease expression among TCI and TCII field isolates was analysed. Gelatin-containing gels revealed hydrolysis bands with molecular masses ranging from 45 to 66 kDa. The general protease expression profile showed that TCII isolates presented higher heterogeneity compared to TCI. By utilizing protease inhibitors, we showed that all active proteases at acid pH are cysteine-proteases and all proteases active at alkaline pH are metalloproteases. However, the expression of cruzipain, the *T. cruzi* major cysteine-protease, did not reproduce a heterogeneous TCII cysteine zymogram profile. Dendogram analyses based on presence/absence matrices of proteases and cruzipain bands showed a TCI separation from the TCII group with 50–60% similarity. We suggest that the observed cysteine protease diversification contributes to differential host infection between TCI and II genotypes.

Key words: *Trypanosoma cruzi*, TCI and TCII genotypes, proteases, cruzipain, host-parasite interaction, virulence factors.

INTRODUCTION

Trypanosoma cruzi is a protozoan parasite that causes Chagas disease, a parasitosis that affects around 18 million people in Latin America (WHO, 2005). *T. cruzi* presents invertebrate and vertebrate hosts during its life cycle: Triatominae insect vectors and a wide variety of mammals. In the vector, the parasites are present as epimastigotes that differentiate into metacyclic trypomastigotes, forms infective to mammal hosts, which circulate in wild and domestic environments (Morel *et al*. 1986). Based on molecular markers derived from constitutive genes, such as the 24Sα rRNA and the mini-exon genes, two major phylogenetic groups *T. cruzi* I (TCI) and *T. cruzi* II (TCII) were defined (Fernandes *et al*. 1998), which coincide with the isoenzymatic dichotomy early proposed (Tibayrenc and Ayala, 1988; Souto *et al*. 1996). A putative association of these main genotypes with a given host, human disease or biome still needs to be confirmed. TCI has been mainly associated with the sylvatic transmission cycle, infecting *Didelphis* as well as placental mammals, and presents a broader distribution in the wild, but has also been observed in domestic cycles (Brisse *et al*. 2000). TCII parasites are proposed to be associated with the domestic transmission cycle, infecting mainly placental mammals (Tibayrenc and Ayala, 1988; Andrade, 1999; Buscaglia and Di Noia, 2003). However, the genotype TCII has already been described infecting several sylvatic mammalian species (Lisboa *et al*. 2007).

Characterizing parasite populations found in nature is crucial to the elucidation of the Chagas disease complex transmission scenario. Biological, biochemical and molecular tools have demonstrated that different strains from endemic areas might be responsible for distinct clinical manifestations and chemotherapy response (Andrade, 1999). Outbreaks of acute Chagas disease due to oral transmission culminating with human infections by *T. cruzi* wild strains (Secretaria de Vigilância em Saúde, 2005), reinforce the importance of a better understanding of the parasite transmission cycles in nature. The *in vitro* engagement of the trypomastigote metacyclic gp82 molecule onto the gastric mucosal epithelium

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Table 1. *Trypanosoma cruzi* I and II field isolates

(Utilized isolates according to *T. cruzi* phylogenetic groups (TCI or TCII), their origin hosts and biomes.)

Phylogenetic group/ Isolate	Host	Biome/Brazilian states
TCI 4264	*Trychomys apereoides* (rodent)	White scrub (Caatinga)/Piauí
TCI 8612	*Rattus rattus* (rodent)	Piauí
TCI 8622	*Didelphis albiventris* (opossum)	Jaguaruana/Ceará
TCI 8628	*Rattus rattus* (rodent)	Jaguaruana/Ceará
TCI 8636	*Didelphis albiventris* (opossum)	Jaguaruana/Ceará
TCI 8659	*Didelphis albiventris* (opossum)	Jaguaruana/Ceará
TCI Gambá 05	*Didelphis aurita* (opossum)	Poço das Antas Reserve/Rio de Janeiro
TCII 750	*Leontopithecus rosalia* (primate)	Poço das Antas Reserve/Rio de Janeiro
TCII 812	*Leontopithecus rosalia* (primate)	Poço das Antas Reserve/Rio de Janeiro
TCII 832	*Leontopithecus rosalia* (primate)	Poço das Antas Reserve/Rio de Janeiro
TCII 840	*Leontopithecus rosalia* (primate)	Poço das Antas Reserve/Rio de Janeiro
TCII 860	*Leontopithecus rosalia* (primate)	Poço das Antas Reserve/Rio de Janeiro
TCII R4	*Trychomys apereoides* (rodent)	White scrub/Piauí
TCII JCT1	*Triatomae brasilienses* (triatominae)	João Costa/Piauí
TCII MLCD88	*Leontopithecus chrysomelas* (primate)	REBIO-Una/Bahia
TCII M3	*Didelphis albiventris* (opossum)	White scrub/Piauí

during invasion, associated to a gp90 glycoprotein down-modulating effect which has already been demonstrated. However, a *T. cruzi* isolate from an orally infected patient expressed high levels of gp90, and produced high parasitaemia and mortality when orally inoculated into mice (Covarrubias *et al*. 2007).

A few studies have focused on the differential gene expression of factors involved in key events of the parasite biology between TCI and TCII groups (Ruiz *et al*. 1998; Risso *et al*. 2004; Di Noia *et al*. 2002; Dutra *et al*. 2006; Mathieu-Daudé *et al*. 2007). A correlation between the expression of a sialidase-homologue gene and parasite virulence in mice (Risso *et al*. 2004) or between the expression of surface glycoproteins involved in Ca^{2+} mobilization and the ability of the parasite to enter mammalian cells (Ruiz *et al*. 1998) was observed. The trypomastigote small surface antigen (TSSA), a surface glycosylphosphatidyl inositol (GPI)–anchored mucin-like protein, revealed a dimorphism that matches the two *T. cruzi* lineages. The seroprevalence for TSSA in Chagas patients is restricted to *T. cruzi* II isoform (Di Noia *et al*. 2002). Differences in ecto-phosphatase activities (Dutra *et al*. 2006), as well as sequence differences and expression levels of Tc52, an immuno-regulatory parasitic protein (Mathieu-Daudé *et al*. 2007), were also demonstrated. Finally, we have been showing that TCI and II groups activate the complement system differentially, the latter being more resistant to complement system mediated-lysis (Cestari *et al*. 1998). Proteases have been implicated in host-parasite interactions, infectivity, pathogenicity, virulence, intracellular survival, replication, differentiation, immune evasion and nutrition (Sajid and McKerrow, 2002; Santos *et al*. 2006). *T. cruzi* contains several proteolytic activities, among them, cysteine, serine, threonine and metalloproteases (Cazzulo, 2002; Cuevas *et al*. 2003). However, the most abundant is cruzipain, a cysteine-protease expressed as a complex mixture of isoforms by the major developmental stages of the parasite. Inhibitors of cruzipain kill the parasite and cure infected mice, thus making the enzyme a very promising target for the development of drugs against Chagas disease (Meirelles *et al*. 1992). Differences in protease expression between TCI and TCII phylogenetic groups have not been investigated so far.

We have interest in understanding the biological differences between TCI and TCII phylogenetic groups. In this study we have worked with 16 *T. cruzi* low-passage field-available strains, isolated from different Brazilian states. The protease expression profile is analysed to identify molecules that can be correlated to TCI or TCII lineages and their contribution to wild isolates biological differences in competence.

MATERIALS AND METHODS

T. cruzi *strains, hosts and geographical location*

We utilized TCII CL Brener and TCI Dm28c strains (Coleção de Tripanosomatídeos, Instituto Oswaldo Cruz, Rio de Janeiro). The other 16 *T. cruzi* field-isolated strains differ according to the phylogenetic group (TCI or TCII), hosts and origin biome, as shown in Table 1.

Parasite growth conditions

T. cruzi epimastigotes were cultivated in LIT culture medium (bovine liver infusion at 5 g/l, tryptose at 5 g/l, NaCl at 4 g/l, KCl at 0·4 g/l; Na_2HPO4 at 8 g/l; glucose at 2 g/l; pH 7·2) supplemented with hemin at 10 mg/l and bovine fetal serum 10% at 27 °C.

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Parasite extracts

Briefly, 5-day parasite cultures were harvested by centrifugation at 4000 ***g*** for 5 min, and washed 3 times with cold PBS (150 mM NaCl, 20 mM sodium phosphate buffer; pH 7,2). Cells were resuspended in 200 μl of distilled water, submitted to vortex for 10 sec and transferred to ice. Then 20 μl of SDS 10% (sodium dodecyl sulfate) were added, and cells submitted to 3 cycles, alternating vortex for 30 sec and incubation on ice for 1 min. Extracts were then centrifuged at 15 300 ***g*** for 10 min. Supernatants were transferred to clean microcentrifuge tubes and kept at −20 °C. The proteins were measured according the Lowry *et al*. (1951) method.

Protease activity assay

Proteases were assayed and characterized by electrophoresis on 10% SDS-PAGE with 0·1% co-polymerized gelatin as substrate (Heussen and Dowdle, 1980). The gels were loaded with 25 or 100 μg of protein per slot, for pH 5·5 and 10, respectively. Electrophoresis was performed at a constant current of 120 V at 4 °C and gels were then incubated at room temperature for 1 h in 10 vols of 2·5% Triton X-100. The gels were then incubated for 24 h at 37 °C in 50 mM sodium phosphate buffer, pH 5·5, supplemented with 2 mM DTT, or for 48 h in 50 mM glycine-NaOH buffer, pH 10, in the presence or absence of proteolytic inhibitors (10 mM 1,10-phenanthroline or 10 μM E-64. Sigma Chemical Co., St Louis, MO, USA). The gels were stained for 2 h in 0·2% Comassie Brilliant Blue R-250 in methanol-acetic acid-water (50 : 10 : 40), and destained in the same solvent. Molecular mass was calculated from the mobility of molecular weight standards (Hanover, MD, USA).

Immunoblotting

Expression of cruzipain among *T. cruzi* field-isolated strains were compared by Western-blotting. Protein extracts equivalent to 20 μg per slot were separated in 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked in 5% milk/PBS containing 0·5% Tween (PBS-Tween) at 4 °C overnight. Then, membranes were washed 3 times (10 min each) with the blocking solution and incubated with anti-cruzipain at 1 : 5000 dilution (kindly provided by Dr J. J. Cazzullo) for 1 h. The secondary antibody used was peroxidase-conjugated goat anti-rabbit IgG 1 : 2500 for 1 h. Immunoblots were exposed to X-ray films after reaction with ECL reagents (kit from Pierce, USA) for chemiluminescence.

Phenetic analysis

Proteases activity and cruzipain expression band patterns were collected into a matrix indicating the presence or absence of specific polypeptide bands (scored as 1 or 0, respectively). Simple matrices were obtained using a similarity coefficient, and TCI versus TCII profile dendograms were constructed using an unweighted pair group method analysis (UPGMA). For these analyses, the NTSYS software package (Version 2.02, Exeter Software, Setauket, NY, USA) was used.

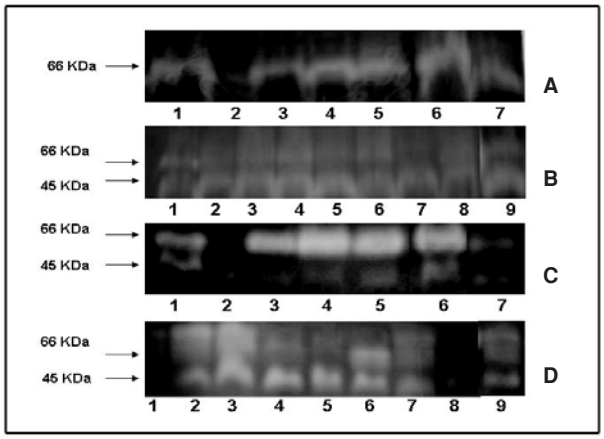

Fig. 1. Zymogram of *Trypanosoma cruzi* isolates. Proteolytic enzymes in *T. cruzi* field-isolated TCI (A, C: lanes:1- 4264; 2- 8612; 3- 8622; 4- 8628; 5- 8636; 6- 8659; 7- Gambá 05) and TCII (B, D: lanes: 1- 750; 2- 812; 3- 832; 4- 840; 5- 860; 6- R4; 7- JCT1; 8- MLCD88; 9- M3) isolates analysed on gelatin-SDS-PAGE. Gels were incubated for 24 h at 37 °C in 50 mM sodium phosphate, pH 5·5 supplemented with 2 mM DTT (C, D) or for 48 h in glycine-NaOH (50 mM), pH 10·0 (A, B).

RESULTS

Protease expression profile in T. cruzi *field-isolated strains*

In order to compare the protease general expression patterns among the 16 field-isolated TCI and TCII strains, epimastigote total cellular extracts were submitted to gelatin-SDS-PAGE.

At pH 10·0, almost all TCI isolates presented active proteases around 66 kDa (Fig. 1A). Gambá 05 isolate presented a second protease of 45 kDa (Fig. 1A, lane 7). On the other hand, all TCII isolates presented 2 proteases around 66 and 45 kDa (Fig. 1B). Active proteases at pH 5·5 were also investigated (Fig. 1C, D). All TCI isolates presented 2 protease bands around 66 and 45 kDa (Fig. 1C), while TCII isolates exhibited different patterns with 2 (860 and MLCD88 isolates), 3 (750, 840, R4 and M3 isolates) or 4 (812, 832 and JCT1 isolates) protease activities/bands (Fig. 1D).

TCI 8628 and TCII M3 isolates were analysed by gelatin-SDS-PAGE, at different points of culture growth: pre-log, log and stationary phase epimastigotes as well as *in vitro* differentiated metacyclic

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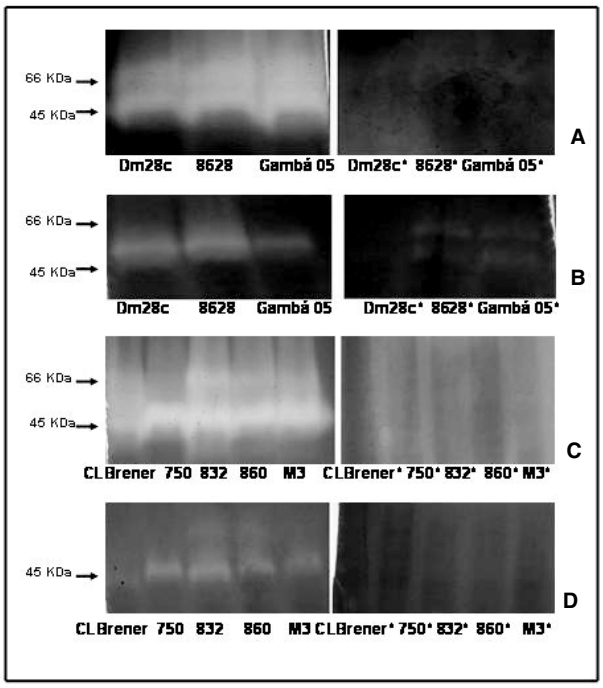

Fig. 2. Zymogram of *Trypanosoma cruzi* isolates with proteolytic inhibitors. Gelatin-SDS-PAGE showing the modulation of proteolytic activity from *T. cruzi* I (A, B) and II (C, D) strains, when gels were incubated in the absence (control) or the presence (*) of proteolytic inhibitors: 10 μM E-64 at pH 5·5 (A, C) or 10 mM 1,10-phenanthroline at pH 10 (B, D).

trypomastigotes. Their respective patterns of 2 (8628) and 3 (M3) bands at pH 5·5 were maintained at all culture time-points, indicating that differential TCI and TCII protease expression is independent of culture growth phase (not shown). Blood-stage trypomastigotes and intracellular amastigotes were not investigated in this study.

Previously, the M3 isolate had been typed as TCI (Herrera *et al*. 2005). However, when compared to TCI isolates, it showed the TCII protease expression pattern at both pH 5·5 and 10 (Fig. 1). Gambá 05 TCI isolate also showed the TCII protease expression pattern, but only at pH 10 (Fig. 1). To test the hypothesis that M3 and Gambá 05 presented mixed TCI/TCII populations, after limiting dilution cloning, both isolates and their individual clones were molecularly re-typed (utilizing the mini-exon gene marker). *T. cruzi* isolate Gambá 05 was confirmed as corresponding to the TCI genotype, while M3 was re-classified and corresponded to TCII genotype (data not shown).

Identification of protease classes differentially expressed between T. cruzi *I and II field-isolated strains*

We observed that TCII isolates present a more complex protease expression pattern at pH 5·5 and 10 than TCI isolates. With the goal of identifying

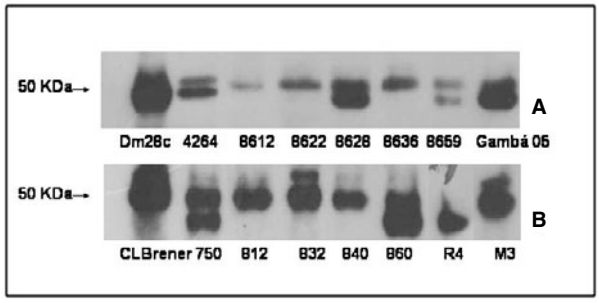

Fig. 3. Immunoblotting of *Trypanosoma cruzi* isolates. Cruzipain expression at the expected size of 50 kDa (arrow) by Western-blotting in TCI (A) and TCII (B) field isolates. Twenty μg of protein per slot were separated in SDS-PAGE, transferred to nitrocellulose membranes, and then incubated with anti-cruzipain at 1 : 5000 followed by labelling with goat anti-rabbit-peroxidase at 1 : 2500. Immunoblots were exposed to X-ray after reaction with ECL reagents.

which protease classes the TCI and TCII groups express, activity gels were incubated with cysteine and metalloprotease inhibitors (E-64 or 1,10-phenanthroline, respectively). In these assays, we utilized the following representative strains: CL Brener (TCII) and Dm28c (TCI) strains as controls; TCII 860 (2 bands at pH 5·5), TCII 750 (3 bands at pH 5·5), TCII 832 (4 bands at pH 5·5); TCI 8628 (representing all TCI isolates, once they have the same active protease patterns at pH 5·5 and pH 10). We also utilized TCI Gambá 05, for being an exception, exhibiting 2 bands at pH 10, instead of 1 (Fig. 1A), and TCII M3, once it had been previously typed as TCI.

As observed in Fig. 2, after incubation of protease activity gels at pH 5·5 buffer supplemented with E-64, all TCI and TCII proteolytic activities were inhibited (Fig. 2A and C). The same was observed when gels were incubated with pH 10 buffer supplemented with 1,10-phenantroline (Fig. 2B and D). However, E-64 had no effect at pH 10, and 1,10-phenantroline had no inhibitory effect at pH 5,5 at the tested concentrations (data not shown). These results suggest that in all the *T. cruzi* strains utilized, the active proteases at acidic pH are cysteine-proteases, and all active proteases at alkaline pH are metalloproteases.

The quantity of parasites needed to detect metalloprotease activities in *T. cruzi* epimastigotes isolates was 4 times higher than that for cysteine-protease activities (25 μg × 100 μg proteins/ slot, respectively), as shown in Figs 1 and 2.

Comparison of cruzipain expression among T. cruzi *I and II filed-isolated strains*

The expression of cruzipain among TCI and TCII field strains was evaluated by Western blotting, as shown in Fig. 3 (A, B). Single or double bands

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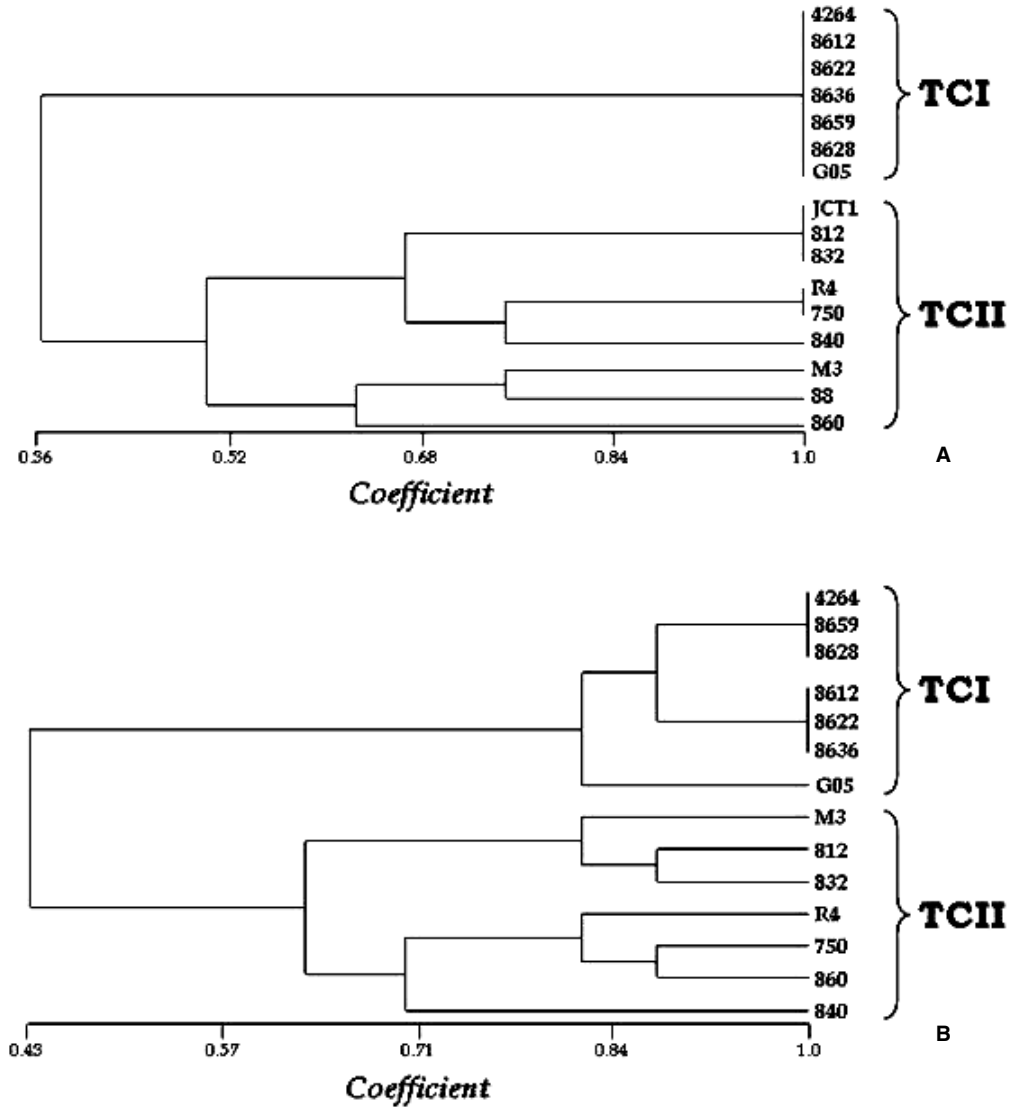

Fig. 4. Dendogram (UPGMA) of TCI and TCII-proteases. The phylogenetic tree shows a separation between TCI and TCII isolates based on cysteine-protease activity bands (A), or cruzipain expression and cysteine and metalloproteases activity bands (B). The x-axis represents the coefficient of similarity.

around 50 kDa were detected, as previously described (Campetella *et al*. 1990), independent of the phylogenetic TCI or TCII background (Fig. 3A, B).

Considering the differential heterogeneity amongst the TCI and TCII isolate protease patterns, a similarity analysis was applied, with the construction of UPGMA-based dendograms. Markers were defined as the bands present or absent after protease activity gels, and cruzipain Western blotting analyses, detected in the present investigation, according to Table 2. The results were expressed as dendograms that separated TCI from TCII genotypes (Fig. 4A, B). When only the cysteine-protease activities were considered, the 2 major groups TCI and TCII were separated at about 50% similarity (Fig. 4A). In this tree, we observed, as expected, that the TCI group is homogenous, since all these isolates exhibited 2 cysteine-protease activity bands. The TCII group, on the other hand, consisted of subdivided groups, as a consequence of heterogeneous cysteine-proteases activity. There was a variation amongst TCII isolates with 2–4 bands, and not necessarily the same bands were present in each group (2, 3 or 4 bands) (Fig. 4A). When all protease (cysteine and metallo) activity and cruzipain bands were used to generate a matrix of absence or presence, the resulting tree also showed a separation between the TCI and TCII groups at about 60% similarity (Fig. 4B). Nevertheless, in this case, the TCI isolates presented a low-level heterogeneity. This can be explained because, in spite of their homogenous 2 cysteine-protease activity bands, they showed 1–2 cruzipain expression band variation, as well as TCII isolates (Fig. 4B). In the case of metalloprotease activities, the separation of *T. cruzi* major phylogenetic groups would be expected, since all TCII have 2 bands, and all TCI have 1 band, expect for Gambá 05, with 2 activities. Interestingly,

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Table 2. Cysteine-proteases and cruzipain bands amongst TCI and TCII isolates

(*Trypanosoma cruzi* field-isolated strains captured in different biomes in Brazil: analysis of protease expression (1- presence or 0- absence of bands in their approximate kDa).)

		Cruzipain			Cysteine-protease				Metalloprotease	
Isolates	Genotype	45 kDa	50 kDa	52 kDa	40 kDa	45 kDa	50 kDa	55 kDa	50 kDa	65 kDa
4264	TCI	0	1	1	1	0	1	0	0	1
8612	TCI	0	0	1	1	0	1	0	0	1
8622	TCI	0	0	1	1	0	1	0	0	1
8636	TCI	0	0	1	1	0	1	0	0	1
8659	TCI	0	1	1	1	0	1	0	0	1
8628	TCI	0	1	1	1	0	1	0	0	1
G05	TCI	0	1	1	1	0	1	0	1	1
JCT1	TCII	ND[a]	ND[a]	ND[a]	1	1	1	1	1	1
M3	TCII	0	1	1	1	1	0	1	1	1
R4	TCII	1	0	0	0	1	1	1	1	1
750	TCII	1	1	0	0	1	1	1	1	1
812	TCII	0	1	0	1	1	1	1	1	1
832	TCII	0	1	1	1	1	1	1	1	1
840	TCII	0	1	0	0	1	1	1	1	1
860	TCII	1	1	0	0	1	0	1	1	1
MLCD88	TCII	ND[a]	ND[a]	ND[a]	1	0	0	1	1	1
Total n = 16	2									

[a] ND = not done.

Gambá 05 is in a separated branch from other TCI isolates at about 82% similarity (Fig. 4B).

We intend to purify additional TCII active cysteine-proteases and evaluate their role in interaction with vertebrate and invertebrate hosts.

DISCUSSION

As mentioned above, *T. cruzi* isolates typed as TCII present more complex protease activity patterns in comparison with TCI. Based on isoenzymes and RAPD analysis tools, other authors (Brisse *et al.* 2000) have reported the genotype TCII as a more diverse group. It can be partitioned into 5 sub-lineages (IIa–e) or discrete typing units (DTUs). The different DTUs display distinct geographical and ecological variations. In contrast, TCI can be no longer subdivided according to these criteria (Brisse *et al.* 2000), despite being subdivided into 3 groups by clustering the intergenic mini-exon gene sequences (O' Connor *et al.* 2007).

Lowdes *et al.* (1996) comparing different strains of *T. cruzi* epimastigote and metacyclic trypomastigote forms, showed metalloprotease heterogeneity, but highly conserved cysteine-protease expression patterns (Lowdes *et al.* 1996), corroborated by other data (Campetella *et al.* 1990). We showed a homogeneous expression pattern of both metallo and cysteine-proteases in TCI isolates, and a heterogeneous expression of cysteine-proteases in TCII field-isolated epimastigotes. Metalloprotease expression in TCII isolates is characterized by an additional activity in relation to TCI isolates, but is a homogeneous intra-TCII group. Cysteine-protease activities are stronger in *T. cruzi* isolates than metalloprotease activities, since higher amounts of parasites are needed in the latter case for activity detection. Cuevas *et al.* (2003) showed that *T. cruzi* metalloprotease activities are also weaker when compared to *Leishmania mexicana mexicana* promastigotes.

These wild recently-isolated TCI and TCII parasites investigated in this study had not previously been biologically characterized, except for 2 isolates (750 and R4, both typed as TCII) that caused parasitaemia with mortality in Swiss mice (Lisboa *et al.* 2007). The diversification imparted by protease expression heterogeneity presented by TCII isolates in contrast to the homogenous TCI pattern may contribute to their adaptation to differential hosts. It is noteworthy that TCI and TCII infect the same number of host orders (Lisboa *et al.* 2004).

In addition to cruzipain, a 30 kDa cathepsin B-like cysteine-protease expression increased after *T. cruzi* treatment with the Z-(SB_z)Cys-Phe-CHN_2 inhibitor, probably compensating the cruzipain inhibition and expression decrease (Yong *et al.* 2000). The active cysteine-proteases detected in TCI and TCII isolates at the present study exhibit a molecular mass range of 40–55 kDa.

Cruzipain is a *T. cruzi* cysteine-protease with an important role in many parasitic processes, including host infection (Meirelles *et al.* 1992; Aparício *et al.* 2004). The pattern observed in cysteine-protease activity gels was complex, where all TCI isolates exhibited 2 activities and TCII isolates exhibited from 2–4 proteolytic activities. Other groups

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described only 2 cysteine-proteases from different *T. cruzi* strains in activity assaying gels (Campetella *et al*. 1990; Lowdes *et al*. 1996). It is possible that the additional activities observed in substrate-containing gels for wild isolates in this work, correspond to other cysteine-proteases rather than cruzipain. We intend to purify additional TCII active cysteine-proteases and evaluate their roles in interaction with vertebrate and invertebrate hosts.

These wild isolates had never been studied, and these extra cysteine-protease activities may have a role in the interaction with hosts in the environment where they circulate. Parasites may lose these protease activities as a consequence of selective pressures exerted by long-lasting maintenance in laboratory conditions. We showed that protease activities did not change between epimastigote and metacyclic trypomastigote forms among wild isolates; however, it would be interesting to investigate intracellular amastigotes and blood-stage trypomastigotes. By comparing different *T. cruzi* strains, a correlation between the level of cruzipain secreted by trypomastigotes and the capacity of invading cells was reported (Aparício *et al*. 2004). *T. cruzi* G strain presented reduced levels of secreted cruzipain, being less infective, when compared to the Dm28c and X10/6 strains. Cruzipain-rich Dm28c trypomastigote supernatants enhanced G strain invasion capacity (Aparício *et al*. 2004).

We also intend to investigate proteases in natural mixed infections by TCI and TCII genotypes, isolated from wild mammals. Little is known about these mixed infections, for example, whether there is competition or cooperation between these populations in host infections. Indeed, mammals (including humans) are more probably exposed to consecutive and variable inocula through distinct infection routes, rather than a single inoculum as is usually performed in the laboratory. Our goal is to associate protease expression and activity with the ecological relations of mixed parasite populations, and their outcome in successful infections in their hosts.

Proteases proved to be good markers for separating TCI isolates from TCII isolates, as shown by dendogram analyses.

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