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# Gene discovery in *Triatoma infestans*

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## Abstract

**Background:** *Triatoma infestans* is the most relevant vector of Chagas disease in the southern cone of South America. Since its genome has not yet been studied, sequencing of Expressed Sequence Tags (ESTs) is one of the most powerful tools for efficiently identifying large numbers of expressed genes in this insect vector.

**Results:** In this work, we generated 826 ESTs, resulting in an increase of 47% in the number of ESTs available for *T. infestans*. These ESTs were assembled in 471 unique sequences, 151 of which represent 136 new genes for the Reduviidae family.

**Conclusions:** Among the putative new genes for the Reduviidae family, we identified and described an interesting subset of genes involved in development and reproduction, which constitute potential targets for insecticide development.

## Background

Chagas disease affects 8 million people, and ~28 million, in 21 endemic countries of Latin America, are at risk of infection [1]. The etiologic agent of Chagas disease is the parasite *Trypanosoma cruzi*, which is mainly transmitted through blood-sucking insect vectors of the Triatominae subfamily, being *Triatoma infestans*, *Rhodnius prolixus*, and *Triatoma dimidiata* the most epidemiologically important vectors. *T. infestans* is currently the main vector in the southern part of South America including regions of northern Argentina, Bolivia, Paraguay, and southern Peru. *R. prolixus*, on the other hand, is distributed in northern South America (mainly in Colombia and Venezuela) and has few foci in Central America. *T. dimidiata*, is present in Central America and Mexico, and also has limited foci in northern South America [2-4].

Due to population migration from Latin America, Chagas disease is becoming an important health issue in North America, Europe and in the western Pacific region [5]. Currently, there are >390,000 individuals infected with *T. cruzi* in non-endemic regions and, accordingly, serological monitoring of blood banks and

organ donors as well as additional controls to detect vertical transmission in newborns must be implemented to prevent the spread of the disease.

As there are currently no vaccines available against *T. cruzi* and effective treatments are limited to chemotherapies that exhibit high toxicity, large efforts are being made to implement prevention strategies, such as domestic vector control and improvement of blood bank surveillance [6]. Currently, the main method of vector control is spraying houses with residual insecticides. However, the occurrence of *T. infestans* populations resistant to pyrethroid compounds in the north of Argentina and Bolivia [7,8] poses the alternative of organophosphate insecticides. Unfortunately, these insecticides, although effective, are very toxic and less accepted by the community due to their unpleasant odor [9]. Novel methods of control will undoubtedly require detailed understanding of the molecular biology of Chagas disease vectors. This will provide unique opportunities to develop new vector control tools such as the use of transgenic vectors refractory to pathogens. The identification of trait genes linked to transposable elements could drive those alleles into wild populations and interrupt disease transmission in the long-term [10,11]. Although in the short run, this strategy is not feasible for Chagas disease control due to the need of an intense laboratory and field research, it is, nevertheless, an approach that deserves to be explored.

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The availability of complete genome sequences of medically important vectors will accelerate the identification of new target genes to advance in novel vector control strategies, such as the use of transgenic vectors [12], and the control of pathogen transmission by targeting genes responsible for host-seeking behaviors. In line with this, several initiatives to sequence vectors of infectious diseases have been launched and some have been finished [13,14]. In 2005, the NIH-National Human Genome Research Institute (NHGRI) and the Washington University Medical School Genome Sequencing Center decided to sequence the genome of *R. prolixus*, as a model for the Reduviidae family [15]. However, advancing in *T. infestans* genomics is also of major importance, as this is the main vector in many South American areas, particularly in Argentina.

As an alternative or complement of genome projects, transcriptome studies performed by sequencing cDNA libraries of Expressed Sequence Tags (ESTs) or of open reading frame ESTs (ORESTES), constitute a rapid, low-cost and effective way to obtain information of the transcriptionally active regions of any organism and to discover novel genes [16-18].

Currently, transcriptome information available for the *Triatoma* genus is very limited. In fact, only salivary gland ESTs projects have been carried out for *T. brasiliensis*, *T. infestans* and *T. dimidiata* [19-21]. The study of *T. infestans* sialome has provided a set of 1534 salivary gland cDNA sequences, 42% of which encode proteins of a putative secretory nature - most of which (55%) have been described as lipocalins [20].

With the aim to gain further insights into *T. infestans* transcriptome and to discover novel genes in this insect, we started a small project to generate ESTs from this vector. In the present work, we report the analysis of 826 ESTs and provide information of interest for the development of new drugs to target the biological cycle of the insect.

## Results and discussion

### ESTs overview

To generate *T. infestans* ESTs, a total of 1881 clones were sequenced from different cDNA libraries obtained from different tissues and developmental stages (for details see Additional file 1: Table S1). The complete dataset was analyzed as a unit, independently of the library to which the sequences belonged.

After the cleaning steps, 826 sequences (i.e., 44% of all the clones sequenced) were classified as high quality ESTs (Table 1) and were uploaded into the GenBank ESTs database with accession numbers from [GenBank: HO762759 to GenBank:HO763584]. This dataset increases the number of available *T. infestans* ESTs by 47%, from 1738 to 2564.

**Table 1 Summary of *T. infestans* ESTs**

Description	Number	%
Total number of clones sequenced	1881	100
High quality ESTs	826 <sup>a</sup>	44
Contigs	119	
Singlets	352	
Assembled Unique sequences (AUS)	471	25
AUS with positive hits	185 <sup>b</sup>	39
BLASTX NR protein database	176	
InterProScan	20	
AUS with no hits	286	61
AUS with EC numbers	44	26
AUS GO annotated	123	26

<sup>a</sup>ESTs deposited in GenBank ESTs database with accession numbers [GenBank: HO762759 to GenBank:HO763584]. <sup>b</sup>See Additional file 2: Table S2 for hit details.

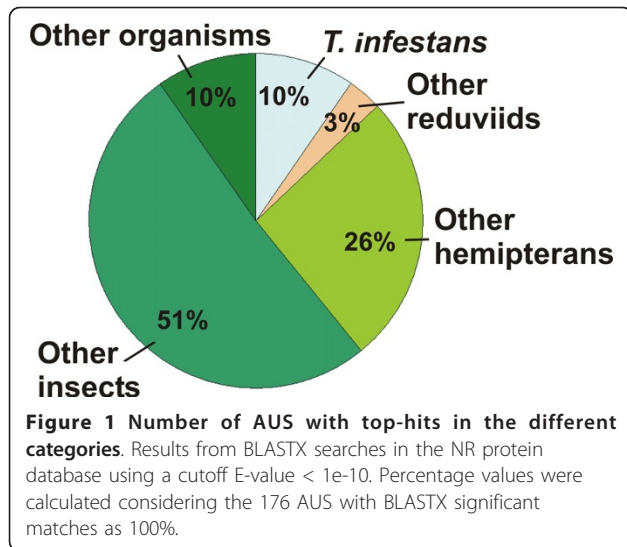
To unify overlapping high quality ESTs, eliminate redundancies and facilitate further studies, a *de novo* assembly was performed. This step grouped the high quality ESTs in 119 contigs and 352 singlets, summing up to 471 assembled unique sequences (AUS), with an average size of 358 bp, representing different putative transcripts or different parts of the same transcript of *T. infestans* (Table 1).

To know the percentage of new *T. infestans* ESTs provided by the AUS generated, BLASTN searches were performed against the EST database at NCBI. Only 10% (47) of the AUS gave positive hits against *T. infestans*, indicating that the remaining 90% (424) AUS are novel ESTs sequences for *T. infestans* (Additional file 2: Table S2).

BLASTX searches yielded 37% (176) of AUS with significant similarity to protein sequences (E-value < 1e-10) (Table 1). InterProScan searches yielded 4% (20) of AUS with similarity to a protein domain, 45% of which also had a hit in the NR protein database. As a whole, homologies against protein databases summarized 39% (185) of AUS with hits in at least one database (Table 1). Additionally, Enzyme Commission (EC) codes were assigned to 44 AUS, and Gene Ontology (GO) terms to 123 AUS (Table 1).

### Comparative analysis to other taxa

The AUS with positive BLASTX results (n = 176, see Table 1) were taxonomically classified according to the top-hit (Figure 1). Only 10% (17) of these AUS were most similar to predicted proteins from *T. infestans* and 3% (6) to predicted proteins from four members of the family Reduviidae (*R. prolixus*, *T. dimidiata*, *T. vitticeps* and *T. brasiliensis*; see Additional file 2: Table S2 for details). These results can be partially explained by the fact that at the time that this paper was written there were no complete genomes available for any triatomine. Actually, the phylogenetically closest sequenced genome



available belongs to the hemipteran *Acyrtosiphum pisum*, which has been recently released in a draft assembly status [22]. Not surprisingly, *A. pisum* is the most represented organism in the “other hemiptera” category with 20 top-hits. The most represented organism in the “other insects” category, and in the entire dataset, is the coleopteran *Tribolium castaneum*, with 29 top-hits. This last result is in agreement with the findings for the *A. pisum* genome, for which the organisms with the highest shared gene content were *T. castaneum* and *Nassonia vitripennis*, both with 53% [22].

However, it is expected that more closely related orthologs will begin to appear as genome projects of phylogenetically closer organisms are finished and annotation information becomes available, in particular from the in-progress genome project of *R. prolixus* [23]. Today, this is reflected when using our AUS as queries in BLASTN searches against the WGS database at NCBI, where 56% (265) of the top-hits are from *R. prolixus* whole-shotgun genome sequences (Additional file 2: Table S2). Consequently, our study will contribute with gene prediction and annotation in the aforementioned genome project.

#### Gene Ontology annotation

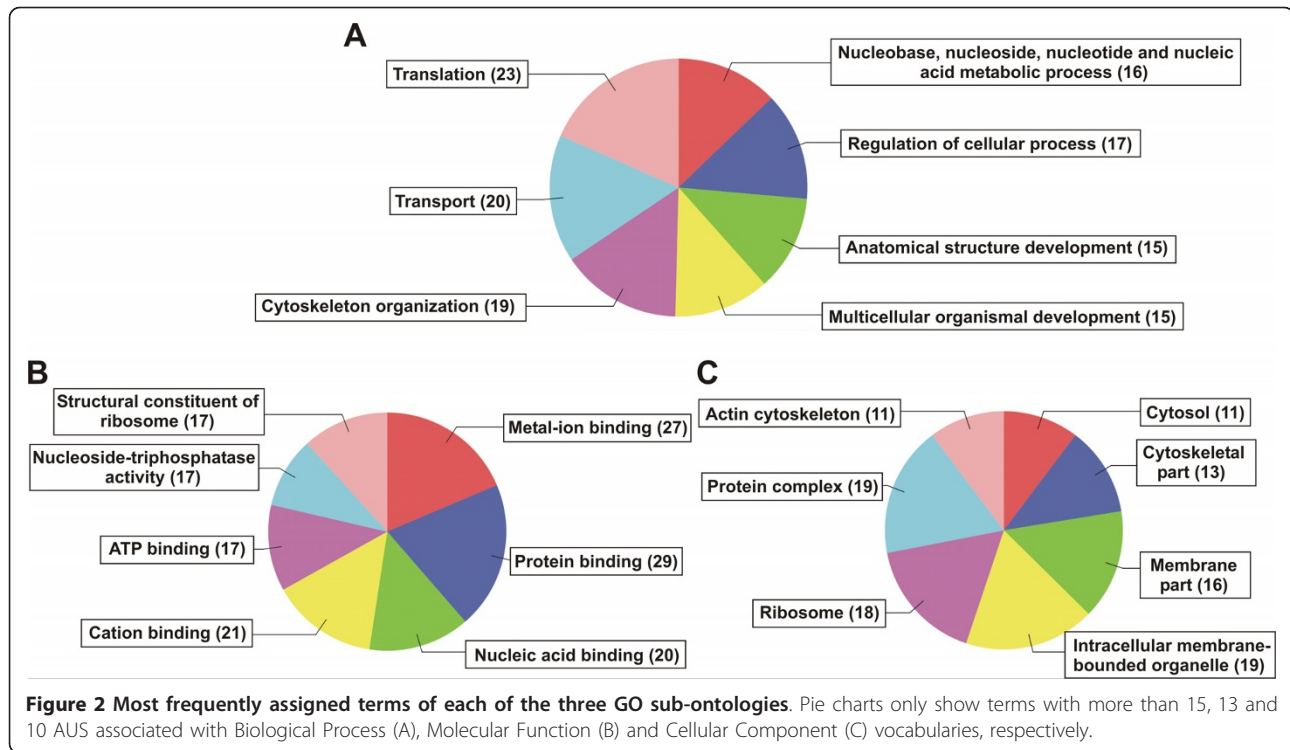
The GO project is a bioinformatics initiative aiming to standardize the representation of gene and gene product attributes across species [24]. GO provides three controlled vocabularies to describe a gene product in terms of: the biological process (BP) in which it is involved, its molecular function (MF), and the cellular component (CC) where it acts. The assignment of GO terms to our dataset with the default parameters resulted in the annotation of 123 AUS. Among them, 95 were classified according to BP, 110 to MF and 78 to CC, and 60 had

at least one GO term of each vocabulary. The most frequently assigned terms in each vocabulary are presented in Figure 2. For the BP category, the three most common terms were “translation” (23), “transport” (20) and “cytoskeleton organization” (19) (Figure 2A). For the MF category, the four most common terms were “protein binding” (29), “metal ion binding” (27), “cation binding” (21) and “nucleic acid binding” (20) (Figure 2B); and for the CC category, the most common terms were “protein complex” (19), “intracellular membrane-bounded organelle” (19) and “ribosome” (18) (Figure 2C). Interestingly, when we performed a similar analysis upon the *T. infestans* ESTs already in the EST database, in the BP vocabulary, the term “evasion or tolerance of host defense response” was by far the most represented (data not shown). This result is directly associated with the fact that the *T. infestans* ESTs available up to now are significantly enriched in sequences coding for salivary lipocalins. Thus, we think that the heterogeneity of our ESTs will enlarge and complement the data already available, particularly with regard to genes involved in diverse biological processes.

#### Description of genes of interest

In this work we identified 151 AUS that did not match with protein sequences of *T. infestans* or other reduviids and which represent 136 novel genes for the Reduviidae family (Table 2 and Additional file 3: Table S3). A detailed analysis of the putative genes identified is not within the scope of this work and will certainly be carried out by researchers interested in the field. However, a number of interesting matches with sequences from non-reduviid organisms were observed in the category “development and reproduction” (Additional file 3: Table S3). Among them, we found several transcripts encoding proteins related to the juvenile hormone (JH) metabolism such as vitellogenin, hexamerins and JH esterase. The JH is a sesquiterpene essential for development and reproduction in insects [25]. Interestingly, it has been shown that a compound that interferes with the metabolism of the JH induce biomorphological alterations in three species of the Triatominae subfamily, including *T. infestans* [26], suggesting that the JH could be a new target to control the vector population.

ESTs belonging to Contig114 and Contig118, and the singlets Tinf\_ail\_1\_i4 and Tinf\_am\_4\_a4, have significant matches to different parts of the vitellogenin protein (Additional file 3: Table S3). This hemolymph lipoprotein, a predominant yolk protein precursor, is synthesized mainly in adult female fat body, secreted into the hemolymph and taken up by the growing oocytes. This biological process called vitellogenesis allows embryonic development outside the maternal body and is regulated by the JH in most insects [27].



Although vitellogenin has been purified and described biochemically for *T. infestans* [28,29], its gene sequence has not been determined yet, therefore our data will enable and facilitate obtaining the entire gene sequence.

Contig15, Contig51, Tinf\_4itla\_3\_a16 and Tinf\_4its\_12\_A11 putatively code for different hexamerin proteins or different parts of the same hexamerin protein (Additional file 3: Table S3). These proteins are

**Table 2** Putative new genes for the Reduviidae family in each category

New genes	Category
16	Membrane traffic regulation and signal transduction (16 AUS)
22	Nucleic Acid metabolism (25 AUS)
10	Development and reproduction (16 AUS)
14	Protein metabolism (14 AUS)
14	Ribosomal proteins (14 AUS)
6	Cytoskeleton (9 AUS)
8	Muscle (9 AUS)
7	Cuticle/exoskeleton (9 AUS)
4	Mitochondrial proteins (6 AUS)
20	Others (20 AUS)
13	Hypothetical, conserved, unknown (13 AUS)

Classification was made taking into account the sequence description of the 151 AUS with BLASTX hits from non-reduviid organisms. For further category details see Additional file 3: Table S3.

synthesized by the fat body and secreted into the hemolymph where they reach extraordinary concentrations prior to metamorphosis, a process in which they serve as a source of amino acids. They also may function as JH-binding proteins [30,31]. The biosynthetic processes related to the production of the very high density lipoprotein (VHDL), a *T. infestans* hexamerin, have been described by Rimoldi et al. (1997) [32]. In addition, hexamerins have also been described in *R. prolixus* [33] and in *Riptortus clavatus* [34], both members of the panheteroptera hemipteran clade. However, the hemipteran *A. pisum* apparently lacks these proteins [22].

Tinf\_4its\_8\_B10 has significant homology to the JH esterase (JHE) (Additional file 3: Table S3). This carboxylase enzyme, along with the JH epoxide hydrolase, is responsible for JH degradation. It is synthesized mainly by the fat body and released into the hemolymph where its high levels are associated with low levels of circulating JH [35]. Interestingly, recent studies have shown that transgenic silkworms overexpressing JHE from embryonic stages undergo precocious larval-pupal metamorphosis [36]. Due to its key role in the regulation of JH titers during insect development and reproduction, JHE has been the target for the investigation of selective drugs [37,38]. Therefore, this protein seems to be also a promising drug target to control Chagas disease vectors by targeting the biological cycle of the insect.

We also found interesting transcripts representative of proteins involved in development and reproduction but



without a direct relationship with JH. For example, the encoded protein in Contig91 is similar to the lingerer protein (Additional file 3: Table S3); the lingerer gene (*lig*) from *Drosophila* has been shown to be involved in the initiation and completion of copulation [39]. Males carrying a hypomorphic mutation in *lig* cannot withdraw their genitalia at the end of copulation. In addition, a severe reduction of the *lig* dosage causes repeated mating attempts without success, and complete loss of *lig* function causes lethality during early pupal stage [39].

Other interesting matched proteins in other insects are the protein Dumpy (Contig72), which is essential for proper wing formation in *Drosophila* [40], and a growth factor (Contig50), which belongs to the family of imaginal disc growth factors (IDGFs). In *Drosophila*, these polypeptide growth factors are expressed most strongly in the embryonic yolk cells and in the fat body of the embryo and larva, and cooperate with insulin to stimulate the proliferation, polarization and motility of imaginal disc cells [41].

## Conclusions

In this work, we generated 826 ESTs from *T. infestans*, which were assembled in 471 unique sequences, 151 of which represent 136 new genes for the Reduviidae family. These data, already available to researchers interested in the field, might provide new insights into the biology of *T. infestans*, and potential targets for future rational drug design to control *T. infestans*, an important vector of Chagas disease in South America.

## Methods

### *T. infestans* specimens, tissue samples and mRNA isolation

Fourth instar specimens were provided by the Center of Vector Reference, Córdoba, Argentina, and by the National Institute of Parasitology “Dr. Mario Fatała Chabén”, Buenos Aires, Argentina. Muscle tissue samples from adult specimens were obtained from the Department of Biochemistry and Molecular Biology, Faculty of Medicine, National University of Córdoba, Argentina. Gut tissue samples from adult specimens were provided by the Department of Microbiology, Parasitology and Immunology, Faculty of Medicine, National University of Buenos Aires, Argentina.

In all cases, total RNA was isolated using TRIzol reagent (Invitrogen) and residual genomic DNA was removed by DNase treatment using RNase-free DNase I (Ambion). Both procedures were performed according to the manufacturer's instructions.

The absence of residual DNA was tested by PCR, using the following primers: CytB\_Fwd 5'- GGACAAATAT-CATGAGGAGCAACAG and CytB\_Rev 5'- ATTACTC CTCCTAGCTTATTAGGAATTG, targeted to the

mitochondrial cytochrome B gene of *T. infestans* [42], with a final concentration of 3 mM MgCl<sub>2</sub>. PCR conditions were: 94°C 2 min - (94°C 30 sec - 47°C 40 sec - 72°C 50 sec) 30 cycles - 72°C 10 min. DNase untreated samples were included as PCR positive controls. The integrity of the total RNA was assessed by agarose electrophoresis together with the A260/A280 ratio.

Poly(A)<sup>+</sup> RNA was purified using the MicroPoly(A) Purist™ Kit (Ambion) or the PolyAtract® mRNA Isolation System IV (Promega), both according to the manufacturer's instructions.

### cDNA synthesis and library construction

Three different strategies were applied for cDNA library construction: i) a conventional library was constructed by using the SMART cDNA Synthesis Kit (Clontech), following the manufacturer's instructions; ii) a second set of conventional libraries was constructed using the PCR-Select™ cDNA Subtraction Kit (Clontech) as unsubtracted libraries, which implies ligating the sample with both adaptors at the same time, and not performing the hybridization steps; and iii) ORESTES libraries were constructed as described by Dias-Neto et al. (2000), with minor modifications [43]. Libraries ii) and iii) were ligated into pGEM-T Easy vector (Promega). All libraries were transformed into *E. coli* DH5- $\alpha$  cells.

*T. infestans* specimens, tissue samples and cDNA library construction strategies are summarized in Additional file 1.

### Nucleotide Sequencing

Independent colonies were picked and transferred into 384- or 96-well culture plates containing: 80  $\mu$ l of 2xTY medium supplemented with 10% of HMFEM anti-freezing medium [44] and 0.1% of ampicillin (10 mg/ml) in each well. Colonies were grown at 37°C overnight and stored at -70°C until use. Each colony was assigned with a clone number consisting of the name of the library, followed by the culture plate number and well position.

An aliquot of 15  $\mu$ l of each culture was transferred into a 96-well PCR microplate, and incubated for 5 minutes at 100°C. A colony-PCR reaction was performed in a new 96-well PCR microplate, using universal M13 forward and reverse primers, and 1  $\mu$ l of the boiled culture. PCR conditions were: 94°C 2 min - (94°C 30 sec - 55°C 30 sec - 72°C 40 sec) 30 cycles - 72°C 5 min. All PCR reactions were carried out in a T-Professional thermocycler (Biometra).

The amplification products were visualized in a 1.5% agarose gel with ethidium bromide. The reactions that had only one band greater than 300 or 400 bp, depending on the library, were selected for clean-up and sequencing. For clean-up, 3 - 5  $\mu$ l of the PCR product

was incubated with 5 U of Exonuclease I (Fermentas) and 0.5 U of Shrimp Alkaline Phosphatase (Fermentas) (30 min at 37°C and then 15 min at 85°C). Single-pass sequencing was performed on each cleaned template in an ABI 3130 sequencer (Applied Biosystems) using T7 or Sp6 primers, or the primer pDNR-LIB\_Forward-53: 5' - TATCAGTCGACGGTACC in the case of the vector pDNR-LIB.

#### Data cleaning and assembly

The raw traces obtained were base-called using the Phred program [45] with quality cut-off set at 20. Lucy2 [46] was used to remove the vector and adaptor sequences, as well as to trim low quality regions and poly-A/T tails (>14 contiguous bases). Sequences of less than 100 bp were excluded.

The BLASTN program was used to search for similar sequences through the non-redundant (NR) nucleotide database at National Centre for Biotechnology Information (NCBI) with an E-value < 1e-3. Sequences with significant similarity to *Triatoma* virus sequences, rRNA or mtDNA genes of any organism were removed. The remaining high quality sequences were assembled with the Contig Assembly Program 3 (CAP3) [47] using the default parameters of 40 bp of overlap length and 80% of overlap identity. Phred, Lucy2 and CAP3 steps were performed with the web-based pipeline for ESTs, EST-piper [48].

#### Analysis and annotation of ESTs

The assembled unique sequences (AUS) were used as queries in BLASTN searches against the NCBI Whole-genome shotgun reads (WGS) database with a E-value < 1e-3, and against the EST database with an E-value < 1e-3. Positive *T. infestans* matches in the EST database were considered to be the same sequence when the E-value was lower than 10e-13. All BLASTN searches were carried out with the blastcl3 program, which allows blasting in batch.

To annotate each AUS, homology searches in protein sequence and protein domain databases, EC numbers assignation and GO term mapping were carried out using the Blast2GO software [49] (Additional file 2: Table S2). The BLAST step was carried out using BLASTX with an E-value cutoff of < 1e-10, and a minimal high-scoring segment pair (HSP) alignment length of 20 amino acids. The BLAST description annotation function was based on the first 20 hits that fulfill the E-value cutoff. InterProScan function (from the European Bioinformatics Institute [50]) was used to search in the following protein domain databases: HMM-Pfam, HMM-Smart, HMM-Tigr, ProfileScan, PatternScan, Superfamily and HMM-Panther. AUS were mapped to Enzyme Commission (EC) numbers [51] and to the three Gene Ontology (GO) vocabularies [52] (Biological Process (BP),

Molecular Function (MF) and Cellular Component (CC)) and were annotated using default settings, including default Evidence Codes values. For a better interpretation of data, multi-level pie charts were generated for the BP, MF and CC vocabularies, filtering by 15, 13 and 10 sequences respectively.

#### Additional material

**Additional file 1: Table S1.** Summary of *T. infestans* libraries.

**Additional file 2: Table S2.** *T. infestans* AUS analyses.

**Additional file 3: Table S3.** Putative novel genes for the Reduviidae family.

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#### Authors' contributions

DOS design the experiments. MLA, GM, SN, VT and DOS performed the experiments. JB, EML, MMS, NGB contributed with *T. infestans* specimens. MLA did the bioinformatic analysis and wrote the first version of the manuscript. MLA, VT and DOS revised and finalized the manuscript. All authors revised and approved the final version of the manuscript.

#### Competing interests

The authors declare that they have no competing interests.

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