Genome of Rhodnius prolixus, an insect vector of Chagas disease, reveals unique adaptations to hematophagy and parasite infection

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Rhodnius prolixus, an insect vector of Chagas disease, reveals unique adaptations to hematophagy and parasitic infection


Rhodnius prolixus not only has served as a model organism for the study of insect physiology, but also is a major vector of Chagas disease, an illness that affects approximately seven million people worldwide. We sequenced the genome of R. prolixus, generated assembled sequences covering 95% of the genome (~702 Mb), including 15,456 putative protein-coding genes, and completed comprehensive genomic analyses of this obligate blood-feeding insect. Although immune-deficiency (IMD)-mediated immune responses were observed, R. prolixus putatively lacks key components of the IMD pathway, suggesting a reorganization of the canonical immune signaling network. Although both Toll and IMD effectors controlled intestinal microbiota, neither affected Trypanosoma cruzi, the causative agent of Chagas disease, implying the existence of evasion or tolerance mechanisms. R. prolixus has experienced an extensive loss of selenoprotein genes, with its repertoire reduced to only two proteins, one of which is a selenocysteine-based glutathione peroxidase, the first found in insects. The genome contained actively transcribed, horizontally transferred genes from Wolbachia sp., which showed evidence of codon use evolution toward the insect use pattern. Comparative protein analyses revealed many lineage-specific expansions and putative gene absences in R. prolixus, including tandem expansions of genes related to chemoreception, feeding, and digestion that possibly contributed to the evolution of a blood-feeding lifestyle. The genome assembled and these associated analyses provide critical information on the physiology and evolution of this important vector species and should be instrumental for the development of innovative disease control methods.

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Rhodnius prolixus is an important vector of Chagas disease, a parasitic disease that affects millions of people worldwide. The genome of R. prolixus, generated through sequencing, provides insights into the insect's adaptations to hematophagy and parasitic infection. This study highlights the unique biological features of R. prolixus that contribute to its role as a vector, including adaptations in immune and microbiome management. The findings are valuable for the development of novel control strategies for Chagas disease.
We assembled 702.6 Mb (v3.0.1, designated prolixus rpDorsal populations, as previously of the Toll pathway (17) (expression directly controls (strain DM28C) did not change D defensin A codon frequency (or, alternatively, to a novel defensin A expression, but not the Anopheles gambiae C | (29.2%), although compa- | Acyrthosiphon pisum (13), such as the cluster rpr- | and SI Ap- | use bias toward the Rhodococcus rhodnii (Fig. 2 cruzi knock- | and Tables D10 and D11 in and | which affects approximately 7 million people worldwide. This report describes the first ge- | nome sequence of a nondipteran insect vector of an important human parasitic disease. This insect has a gene repertoire substantially distinct from dipteran disease vectors, including im- | mune signaling pathways that display major departures from the canonical network. Large gene expansions related to che- | moreception, feeding, and digestion have facilitated triatome adaptation to a blood-feeding lifestyle. This study provides in- | formation about the physiology and evolution of an important disease vector that will boost understanding of transmission of a life-threatening parasite and may lead to the development of innovative control methods.

and social costs. Approximately 10,000 people die from the disease annually and 100 million people are at risk for infection (2).

Results and Discussion

Genome Landscape. We assembled 702.6 Mb (v3.0.1, designated RproC1) of the predicted 733-Mb genome size (3, 4), with mean depth coverage of 8x. The current assembly includes 27,872 scaffolds (SI Appendix, Fig. A1), with a measured GC content of 27.1% that was slightly lower than that of another hemipteran, the pea aphid, Acyrthosiphon pisum (29.2%), although compa- | ble GC content was found in protein-coding regions (5). The consensus gene prediction (VectorBase 1.3) includes 15,456 protein-coding genes and 738 RNA genes (Figs. L4 and SI Ap- | pendix, Fig. A1). We also found 25 Y-chromosome linked scaffolds, including nine Y-linked genes.

Orthologous gene clustering analyses (6) classified R. prolixus genes with orthologs in other insects and outgroup species (Fig. 1B), and highlighted 630 lineage-specific expansions (LSE) in many R. prolixus gene groups related to defense mechanisms, behavior, development, and physiology. A comparison of gene families based on protein domain annotations revealed a few putative lineage- | specific reductions (LSR), where R. prolixus possessed substantially fewer genes. These LSEs and LSRs will be specifically discussed below in relation to their biological roles.

Transposable Elements and Horizontal Gene Transfer. Approximately 1,400 transposable elements (TEs), with members from most of the known TE superfamilies, comprised 5.6% of the genome; this percentage was much lower relative to Aedes aegypti (~50%) and Anopheles gambiae (~20%) but similar to other anopheles (2–11%) (7). Nearly 70% of the TEs belonged to class II (mariner TEs) and a single superfamily (DTTRP1-7), composed of seven new families, represents almost 3% of the genome (Fig. 1A and C and SI Appendix, Tables A1 and A2). Many of these sequences have full-length transposases and ORFs suggesting a recent period of increased transposition (8). Horizontal gene transfers from Wolbachia were likely the origin of 27 genes (Tables D1–D3 in Dataset S1) located in some of the 85 Wolbachia-like genomic regions. These regions span- | ned 100–2,500 bp, which were smaller and more dispersed than those reported in Glossina morsitans (two segments of ~500 Kb) (9). A previous transcriptome analysis showed that eight of these genes appear to be transcribed in the midgut (10). Codon use analysis of the horizontally transferred genes (HTG) revealed that, of the 61 amino acid codons, 38 codons showed a shift from a Wolbachia spp. use bias toward the Rhodnius codon frequency (SI Appendix, Fig. A2 and Table D4 in Dataset S1). This codon frequency shift suggests an ongoing adaptive process whereby the HTG coevolved with the insect tRNA profile for more efficient transla- | tion. The HTG also revealed two transposases (RPRC000742 and RPRC000770), one reverse transcriptase (RPRC000723), and DNA recombination/repair enzymes, including one Holliday junction helicase (RPRC004559), two DNA mismatch repair MutL proteins (RPRC010818 and RPRC011745), and one DNA polymerase I (RPRC006979). These findings suggest that extensive machinery to transpose, recombine, and repair the host DNA were likely in- | strumental for the success of Wolbachia gene transfer to R. prolixus.

RNAI Machinery and Target Genes. RNAI is a posttranscriptional gene-silencing mechanism triggered by double-stranded RNAs (dsRNAs) that degrade a target messenger RNA (mRNA) in a sequence-specific manner (11). The RNAI mechanism can also be triggered by microRNAs (miRNAs), which are small noncoding RNAs (12). We identified RNAi machinery (Table D5 in Dataset S1) as well as precursors and 87 mature miRNA sequences (SI Appendix, Figs. A3 and A4) and extensive loss of the IMD pathway comprised a complete gene-silencing pathway for 804 potential target genes (SI Appendix, Fig. A3C and Tables D10 and D11 in Dataset S1). Some miRNA clusters were similar to those described for Caenorhabditis elegans (13), such as the cluster rpr-miR-71/2a-1, which was identified in one of the introns of the phosphatase-four-like protein gene (RPRC004331). The miRNA-target gene pairs also showed conservation; for example, rpr-miR-124-3p targeting the ROCK1 protein kinase gene (RPRC007732-RA) (14) and rpr-miR-10-3p targeting the Huntington-like gene (RPRC006340-RA) (15).

Immune Pathways and Their Effects on Intestinal Microbiota and T. cruzi. We identified most of the genes from canonical immune pathways, including the Toll, immune deficiency (IMD), and Jak/ STAT pathways, and several immune effectors, including a substantial LSE of defensins (SI Appendix, Fig. A5 and Table D12 in Dataset S1). However, some canonical components of the IMD pathway were not detected: IMD, Fas-associated protein with death domain (Fadd), death-related ced-3/Nedd2-like caspase (Dredd), and Caspar. A similar observation was reported for the brown planthopper, where a more extensive loss of the IMD pathway genes purportedly allowed the development of its obligate endo- | symbiont (16). In R. prolixus, however, some members of the IMD pathway were found, such as the peptidoglycan recognition proteins (PGRPs) and the NF-kB/Rel homolog, rprRelish (PK129556) (Fig. 24). The expression of the latter was up-regulated in both the intestinal epithelium and fat body 24 and 72 h after a blood meal (Fig. 2B and C), thereby indicating an active IMD pathway in R. prolixus. This possibility was investigated by rprRelish knock- | down (SI Appendix, Fig. A6D), which reduced expression of mimut defensin A (AA047462), whereas expression of lysozyme B (ABX11554) remained unchanged and lysozyme A expression (ABX11553) increased (Fig. 2D). These data suggest that rprRelish directly controls defensin A expression, but not the lysozymes. Silencing of rprRelish also increased the population of the symbiotic bacteria Rhodococcus rhodnii (Fig. 2E–G), thus providing further support for an active IMD pathway despite the lack of several canonical proteins. This could be explained by either the existence of unknown alternative components linking the GPR receptors to rprRelish or, alternatively, to a novel rewiring of the immune network (Fig. 2F). The role of the IMD pathway in the control of the gut microbiota led us to investigate its role in the control of T. cruzi. Unexpectedly, rprRelish-silenced insects infected with T. cruzi (strain DM25C) did not change parasite loads after 7 (SI Appendix, Fig. A6C) or 14 days post- | infection (Fig. 2H). Even more surprisingly, silencing the transcrip- | tion factor rpdorsal of the Toll pathway (17) (SI Appendix, Fig. A6D), did not change parasite levels (Figs. 2H and SI Ap- | pendix, Fig. A6C). These findings strongly indicate that either T. cruzi infection does not activate the insect’s immune system or that the parasite is not affected by antimicrobial peptides that are pro- | duced in response to parasitism or the ingestion of a blood meal. A plausible hypothesis for these data is that T. cruzi developed active evasion or tolerance mechanisms, because activation of these im- | mune pathways did not control T. cruzi populations, as previously suggested (18, 19).

Significance

Rhodnius prolixus is a major vector of Chagas disease, an illness caused by Trypanosoma cruzi which affects approximately 7 million people worldwide. This report describes the first ge- | nome sequence of a nondipteran insect vector of an important human parasitic disease. This insect has a gene repertoire substantially distinct from dipteran disease vectors, including im- | mune signaling pathways that display major departures from the canonical network. Large gene expansions related to che- |

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Selenocysteine Machinery, Selenoproteins, and Detoxification Enzymes.
Selenocysteine (Sec) is a proteinogenic amino acid that is incorporated into proteins during translation by the reassignment of specific UGA codons. We identified enzymes involved in Sec synthesis and insertion (Table D13 in Dataset S1), but only two Sec proteins were found: selenophosphate synthase 2 (RPRC0109014), which is involved in Sec synthesis, and glutathione peroxidase (GPx; RRPC011108), a major antioxidant protein. In all insect genomes sequenced to date, the known GPx genes were non-selenium, cysteine-based enzymes. It was previously hypothesized that Sec was replaced by cysteine in an ancient common ancestor of all insects. The discovery of a Sec-based GPx in an insect brings new insight into the evolution of this gene family, indicating that the Sec-GPx enzymes, together with glutathione S-transferases, can drive in one (RPRC014349) of the two R. prolixus thioredoxin reductases (Fig. 34). These results, together with the finding of a Sec-GPx in R. prolixus, indicate that insects originally had a more extensive repertoire of selenoproteins, and that Sec loss and replacement by cysteine occurred independently in different species.

A substantial LSE of enzymes involved in drug and detoxification occurred, such as in carboxylesterases and cytochrome P450 (Tables D14 and D15 in Dataset S1) (see also ref. 21). These enzymes, together with glutathione S-transferases, can drive insecticide resistance. Their identification is particularly relevant for public health surveillance, as recent reports indicated that natural triatomine populations have responded with increasingly higher levels of insecticide resistance and could threaten the successful vector control initiatives that have occurred over the last two and half decades in Latin America (22, 23).

Fig. 1. R. prolixus genome. (A) Genome overview. All scaffolds are represented in layer I and are organized clockwise from the longest to the shortest, starting at the arrowhead. The genic (layer II, red) and TEs (layer III, blue) showed opposite densities until the asterisk (*) and were similarly low from this point until the end (shorter scaffolds). The Wolbachia sp. insertions (layer IV, orange) were observed throughout the genome without a trend. The kernel picture illustrates an adult R. prolixus. (B) Gene clustering. The Venn diagram partitions 15,439 OrthoMCL gene clusters according to their species compositions for R. prolixus and three other Hemimetabola (blue), four Diptera (yellow), four other Holometabola (green), and four noninsect outgroup species (pink). The 6,993 R. prolixus genes show widespread orthology (white circle, and bars, Bottom Left) and three other Hemimetabola (blue), four Diptera (yellow), four other Holometabola (green), and four noninsect outgroup species (pink). The 6,993 R. prolixus genes show widespread orthology (white circle, and bars, Bottom Left): these are part of the 7,115 clusters that have representatives from each of the four species sets, of which a conserved core of 2,253 clusters have orthologs in all 16 species. The 5,498 R. prolixus genes show no confident orthology (bars, Bottom Right), but most of these are homologous (e-value < 1e-05) to genes from other animals or to genes in its own genome. (C) TE distribution. The inner chart represents the three main classes of TEs (LTRs, non-LTRs, and class II), and the outer shows the distribution of TE superfamilies within each class. The charts are based on the total base pairs occupied by TE-related sequences longer than 0.5 Kb, as shown in SI Appendix, Table A1.

Fig. 2. Characterization of IMD pathway and control of T. cruzi replication. Although several proteins from the IMD pathway are missing from the R. prolixus genome, the pathway is still active. (A) The rprRelish protein contains a Rel homology domain (RHD), an Ig-like fold, Plexins, a transcription factor domain (PFT), and several ankyrin (Ank) domains. (B and C) In response to the growth of the native microbiota following a blood meal, rprRelish expression is increased at 24 and 72 h postblood meal in both the midgut (B) and fat body (C). Fas: fasting. When rprRelish expression is knocked down (pink) in the gut using RNAi (SI Appendix, Fig. A6B), the expression of defensin A (Def) is greatly reduced, and the expression of lysozyme A (LizA) is increased (D). Upon knockdown of rprRelish expression (pink), the bacteria load in the anterior midgut (AM) (E) and posterior midgut (PM) (F) is increased, with the same trend observed in the rectum (R) (G). dsMal control was shown in gray. *P < 0.05. Upon knockdown of rpdorsal or rphrelish expressions (SI Appendix, Fig. A6B), T. cruzi levels are not altered in any section of the digestive tract 14 d after infection (H). dsMal control (gray), dsRelish (pink) and dsDorsal (blue). Median values are at the graphic top. (I) Representation of the IMD R. prolixus immune pathways depicting the absence of key IMD pathway components. In R. prolixus, although the Toll and Jak/STAT pathways are usually highly conserved, members of IMD pathway are missing (dashed lines shapes with red “∗”), including the IMD, Fadd, and Dredd genes and the negative modulators Pipk (poor IMD response upon knock-in) and Caspar. However, the pathway receptors, PGRPs, and a homolog of the transcription factor Relish (rprRelish) are present, suggesting that the activity of the pathway is exerted through a noncanonical mechanism.
We identified and manually curated (47), most urea cycle enzymes were (and Rhodnius (RPRC000102) and involves a set of remarkably con-
has 32 TKs and and Tables D24 and D25 in
valois saliva was previously studied for Rhodnius prolixus A. gambiae pisum and Table D22 and D23 in
All developmental stages of has 90 TK
prolixus prolixus
prolixus
prolixus
100
| PNAS
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| Triatoma dimidiata
| no. 48
| 14939
| sequences. TRIDI, several transcription factor (TF) families. Recent independent
identified forms present in triatomines are still unknown. Nevertheless, we have been identified in other insects, ironically, the identity of the JH
Wigglesworth served genes (Table D20 in
comparison,
uble TKs). This represents the smallest TK described to date; for
and cell death. The tyrosine kinome (TK) of
and several LSEs that we identified included odorant and
receptor subfamily was also missing in
highly conserved in holometabolous insects (40). The sugar re-

carbon dioxid contributes to host finding in triatomines (39). Nevertheless, the carbon di-
oxide receptor subfamily was absent in R. prolixus, as previously reported in the body louse and the pea aphid, despite being highly conserved in holometabolous insects (40). The sugar re-
ceptor subfamily was also missing in R. prolixus, but this may represent a more recent loss as it was found in the pea aphid (SI Appendix, Fig. A12). Rhodnius saliva was previously studied for its capacity to interfere with host blood clotting, platelet aggre-
and vasoconstriction (41), and also for its immunomodu-
ulaty activity. Lipocalin genes [n = 51 genes, some previously described (10, 41)] which transcribe the most abundant salivary proteins, formed large LSEs, and occurred as tandem clusters (SI Appendix, Fig. A15 and Table D28 in Dataset S1). We discovered 12 previously unidentified members of the nitrophorin clade (Fig. 3B), which encode lipocalins that carry nitric oxide in R. prolixus saliva. We also found LSEs occurring in tandem clusters for aspartic peptidases (cathepsin-D like aspartic proteases), probably reflecting a replacement of digestive serine proteases found in most insects by lysosomal proteases for blood digestion in he-
miterans (Table D29 in Dataset S1). Among the putative LSRs (Table D30 in Dataset S1), a reduced set of only seven amiloride-
sensitive sodium channels (SI Appendix, Fig. A16) may also be linked to hematophagy because amiloride-sensitive sodium channels are involved in the malpighian tubules filtration. This paucity could have evolved to limit sodium transport for the production of hypoosmotic urine and can be related to the unusually weak re-
response of R. prolixus sodium channels to amiloride (42).

Moreover, we manually annotated other gene families related to hematophagy but without displaying signals of LSE. These families included the following pathways/functions: iron/heme binding, transport and metabolism (43, 44) (Table D31 in Dataset S1); beh-
behavioral control of the blood-seeking habits (45), including loco-


dory and visual activities, memory formation, temperature and humidity detection, mechanoreception, and circadian clock control (Tables D32 and D33 in Dataset S1); energy metabolism pathways [glycolysis, gluconeogenesis, and pentose-phosphate pathways (Table D34 in Dataset S1)]; and regulatory peptides and receptors, as well as 37 neuropeptide precursors or hormone genes (46).

As reported for A. pisum (47), most urea cycle enzymes were not observed, with the exception of arginosuccinate synthetase

Hematophagy-Related Genes. All developmental stages of R. pro-
lixus, from first instar nymphs to adults of both sexes, feed ex-
nclusively on blood. Chemoreception is essential to host finding (38), and several LSEs that we identified included odorant and gustatory receptors as well as odorant and chemosensory binding proteins (SI Appendix, Figs. A11–A14 and Tables D24–D27 in
Dataset S1). Some of the recent LSEs, such as OR58-87 (SI Appendix, Fig. A11) (named “recent Rhodnius expansion”) were found in tandem arrays (Table D24 in Dataset S1). As in other blood-feeding insects, detection of carbon dioxide contributes to host finding in triatomines (39). Nevertheless, the carbon di-
oxide receptor subfamily was absent in R. prolixus, as previously reported in the body louse and the pea aphid, despite being highly conserved in holometabolous insects (40). The sugar re-
ceptor subfamily was also missing in R. prolixus, but this may

| Fig. 3. Thioredoxin reductases and nitrophorins trees. (A) The tree shows the phylogenetic relationship of thioredoxin reductases (TR) in nine Pananeoptera

genomes. Colored balls indicate the amino acid aligned to the Sec position: green (U) selenocysteine, red (C) cysteine, and gray (−) unaligned. One of the two Cys-TR in R. prolixus (RPRC014349) clusters with the Sec-TR proteins, implying a Sec to Cys conversion. (B) Nitrophorins branch of lipocalins tree exemplify a Rhodnius enriched cluster. Sequences clustered by OrthoMCL on group 691 together with unclassified sequences similar to lipocalins were aligned and a tree constructed (SI Appendix, Fig. A15). Black dots mark new Rhodnius sequences. TRIDI, Triatoma dimidiata; TRIMA, Triatoma matogrosensis. Support values based on bootstrap are included at nodes.

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prolixus

(RPPC013927) (Table D35 in Dataset S1). The uricosylation pathway, which is present in mosquitoes (48), is putatively absent in R. prolixus. Absence of both pathways explains the lack of elimination of nitrogen as urea, also originally described by Wigglesworth, even in the second instar (3). As is the case for most cekaryotes, the R. prolixus genome does not encode several pathways for essential amino acid synthesis (47) (Table D36 in Dataset S1), but the lack of almost all urea cycle enzymes (Table D35 in Dataset S1) also precludes the de novo synthesis of arginine. This is noteworthy because arginine is the precursor for nitric oxide, an important salivary vasodilator for blood-feeding insects. We postulate that adaptation to a diet rich in amino acids, such as blood, allowed for relaxation of the constraints in the arginine biosynthetic pathway.

This first analysis of the R. prolixus genome generated novel insights regarding multiple adaptive mechanisms that likely contributed to a strict blood-feeding lifestyle, and thus provided new working hypotheses that will drive future research in the biology of triatomines and Chagas disease. Some of the unique features of R. prolixus, including its numerous lineage-specific gene family expansions, the peculiar immune network, and a “silent” relationship between the insect host and the trypanosome parasite, also provide a new starting point for further understanding its evolutionary adaptations. In summary, our data illustrate the important role of the midgut.

Materials and Methods

All candidate colonies were genotyped to assure R. prolixus identity before extracting genetic material from ovaries or testes. Whole-genome shotgun sequencing using Sanger and 454 technologies produced 8x genome coverage. After end-sequencing a BAC library, we assembled all data by implementing the default parameters in CABOG (49).

We predicted gene sequences using ab initio and similarity-based approaches that were merged to construct the final set of gene models. We performed orthology-based clustering using OrthoMCL (60) and gene family counts based on conserved protein domain annotations from InterProScan (51) for 16 species. We used three different methods for identifying TEs. First, we identified class II and nonlong-terminal repeats (non-LTRs) with RPS-blast (52). Second, we identified LTRs using homology-based approaches (53, 54). The third method, used to discover MITEs, relied upon RepeatFinder (55) and a repeat library produced with RepeatScout (56). We assessed the genomic coverage of all TE using BLAT (57). We identified genomic duplicated regions using BLASTN (52) of all scaffolds against each other with 5-Kb cut-off to avoid TE detection.

miRNA precursors and mature sequences were retrieved using Envistdexcept (58) and filtered. Their target genes were predicted using miRanda (59). Selenoprotein genes were identified using Selenoprotfiles (60) and Sebastian (61). We also identified SEC5 elements using SECSearch (61). We used tRNAscan-SE (62) to detect tRNA-Sec. Selenoprotein multiple sequence alignments used T-Coffee (63) and then performed phylogenetic analyses by reconstructing trees with maximum likelihood using the best-fitting evolutionary model (64).

Wolbachia similar regions were searched using BLASTN (52) to compare the Rhodnius genome with 16 Wolbachia genomes. The gene models present in these regions were selected and grouped with those having a Wolbachia protein as best hit in a BLASTP (52). This gene group had their codon-use compared with Rhodnius and Wolbachia backgrounds by Expander Tool (65).

Immunogenic genes were searched in expressed sequence tags, gene prediction, assembled genome, and genomic unassembled raw reads to assure reliability of the absence; all searches used BLAST (52) and homologous genes from closely related taxa. The rpPrelEl gene was manually assembled using contigs of a previously published transcriptome (10). For confirmation, rpPrelEl was cloned and sequenced. Insects were immunologically challenged through blood meal as it increases endosymbiont (R. rhodni) population in the gut. Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer’s instructions. Real-time PCR (quantitative RT-PCR) was used to assess the transcript abundance and silencing efficiency of the genes of interest. 7T Megascript kit (Ambion) was used to generate and purify dsRNA from PCR-amplified genes following the manufacturer’s instructions. The dsRNA in sterile water was introduced into the thorax of adult females by injection. The microbiota of R. prolixus digestive tract was analyzed by counting colony forming units after plating homogenates of the different sections of the midgut.

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