A deep insight into the sialotranscriptome of the mosquito, *Psorophora albipes*

Andrezza C Chagas1, Eric Calvo1, Claudia M Rios-Velásquez2, Felipe AC Pessoa2, Jansen F Medeiros3 and José MC Ribeiro1*

Abstract

**Background:** *Psorophora* mosquitoes are exclusively found in the Americas and have been associated with transmission of encephalitis and West Nile fever viruses, among other arboviruses. Mosquito salivary glands represent the final route of differentiation and transmission of many parasites. They also secrete molecules with powerful pharmacologic actions that modulate host hemostasis, inflammation, and immune response. Here, we employed next generation sequencing and proteome approaches to investigate for the first time the salivary composition of a mosquito member of the *Psorophora* genus. We additionally discuss the evolutionary position of this mosquito genus into the Culicidae family by comparing the identity of its secreted salivary compounds to other mosquito salivary proteins identified so far.

**Results:** Illumina sequencing resulted in 13,535,229 sequence reads, which were assembled into 3,247 contigs. All families were classified according to their in silico-predicted function/activity. Annotation of these sequences allowed classification of their products into 83 salivary protein families, twenty (24.39%) of which were confirmed by our subsequent proteome analysis. Two protein families were deorphanized from *Aedes* and one from *Ochlerotatus*, while four protein families were described as novel to the *Psorophora* genus because they had no match with any other known mosquito salivary sequence. Several protein families described as exclusive to Culicines were present in *Psorophora* mosquitoes, while we did not identify any member of the protein families already known as unique to Anophelines. Also, the *Psorophora* salivary proteins had better identity to homologs in *Aedes* (69.23%), followed by *Ochlerotatus* (8.15%), *Culex* (6.52%), and *Anopheles* (4.66%), respectively.

**Conclusions:** This is the first sialome (from the Greek sialo = saliva) catalog of salivary proteins from a *Psorophora* mosquito, which may be useful for better understanding the lifecycle of this mosquito and the role of its salivary secretion in arboviral transmission.

Background

*Psorophora* mosquitoes—commonly known as “giant mosquitoes”—belong to the subfamily Culiciniae, which includes many genera with epidemiologic importance to humans and animals such as *Aedes*, *Ochlerotatus*, *Haemagogus*, and *Culex*. Notably, members of the *Psorophora* genus are found only in the New World. *Psorophora* mosquitoes are opportunistic, having mammals and birds as the main hosts of their blood-feeding [1,2]. *Psorophora* females have been associated with transmission of equine encephalitis virus, West Nile fever virus, and other arboviruses [3-9].

The phylogeny of mosquitoes includes three subfamilies within the Culicidae: Anophelinae, Culicinae, and Toxorhynchitinae. Studies based on the morphology, behavior, biogeographic distribution, and life-history suggest the Anophelinae subfamily as monophyletic and basal into the Culicidae family. On the other hand, the Culicinae subfamily includes the majority of remaining mosquito genera distributed into ten tribes. *Psorophora* mosquitoes share the tribe Aedini together with *Aedes*, *Ochlerotatus*, and other mosquito genera, while *Culex* mosquitoes belong to the Culicinae tribe. Previous studies have supported the genera from the tribe Culiciniae as basal to genera of the tribe Aedini [10]. These results are
in agreement with the phylogeny proposed by Besansky and Fahey [11]. The Psorophora genus contains 48 species divided into three subgenera: Grabhamia (15 species), Janthinosoma (23 species), and Psorophora (10 species) [12]. Recently, morphologic and molecular studies have supported Psorophora as a sister group with Aedes/Ochlerotatus [13-15]. In contrast, studies using 18S rDNA sequence have suggested Psorophora species as a sister group to Culex and/or to Aedes/Ochlerotatus species [12,16].

The salivary glands (SGs) of hematophagous insects secrete a cocktail of biochemically active compounds [17] that interacts with hemostasis [18-21], immunity, and inflammation of their hosts [22,23]. Perhaps because of the continuous contact of mosquito salivary proteins with host immunity, salivary proteins are at a fast pace of evolution and divergence, even in closely related species [24]. In the past decade, the continuous advances in the fields of transcriptome and proteome analysis led to the development of high-throughput sialotranscriptome studies (from the Greek saliva = saliva) [23,25]. These studies resulted in a large database of secreted salivary proteins from different blood-feeding arthropod families including members of the Culicidae family.

All mosquito sialotranscriptome studies so far have targeted members of the Aedes, Ochlerotatus, Anopheles, and Culex genera [24], which are important vectors of human and animal diseases. Although some Psorophora species are known to be vectors of several arboviruses, the molecular composition of their salivary secretion remains unknown. Our primary aim was to investigate the salivary transcriptome and proteome of a member of the Psorophora genus (Psorophora albipes) to ultimately better understand the evolution of SG composition within the Culicidae family. In addition, our work makes available the first platform of salivary proteins from this mosquito genus, relevant for improving our understanding of mosquito evolution, the evolving risks in public health due to the recent expansions of Psorophora mosquitoes to the North, and for development of exposure markers to mosquito bites and to vector-borne diseases transmitted by mosquitoes.

**Methods**

**Mosquitoes**

Psorophora mosquitoes were collected in fragments of unflooded rain forest in Manacapuru municipality, Amazonas state, Brazil, using modified CDC traps. The mosquitoes were maintained with water and sugar solution and transported to Biodiversity Laboratory of Leônidas and Maria Deane Institute (Fiocruz/Manaus). The mosquitoes were identified using the taxonomic keys proposed by Forattini [12] and Consoll and Lourenco de Oliveira [26].

Dissection and RNA extraction

SGs from *P. albipes* (50 pairs) were dissected in 150 mM sodium chloride pH 7.4 and immediately transferred to 50 μl RNAlater™ solution and maintained at 4°C until the RNA extraction. SG RNA was extracted and isolated using the Micro-FastTrack™ mRNA isolation kit (Invitrogen, San Diego, CA) per manufacturer’s instructions. The integrity of the total RNA was checked on a Bioanalyser (Agilent Technologies Inc., Santa Clara, CA).

**Next-Generation Sequencing (NGS) and bioinformatic analysis**

The SG library was constructed using the TruSeq RNA sample prep kit, v2 (Illumina Inc., San Diego, CA). The resulting cDNA was fragmented using a Covaris E210™ focused ultrasonicator (Covaris, Woburn, MA). Library amplification was performed using eight cycles to minimize the risk of over-amplification. Sequencing was performed on a HiSeq 2000 (Illumina) with v3 flow cells and sequencing reagents. One lane of the HiSeq machine was used for this and two other libraries, distinguished by bar coding. A total of 135,651,020 sequences of 101 nt in length were obtained. A paired-end protocol was used. Raw data were processed using RTA 1.12.4.2 and CASAVA 1.8.2. mRNA library construction, and sequencing was done by the NIH Intramural Sequencing Center (NISC). Reads were trimmed of low-quality regions (< 10) and were assembled together with the assembly by short sequences (ABYSS) software (Genome Sciences Centre, Vancouver, BC, Canada) [27,28] using various kmer (k) values (every even number from 24 to 96). Because the ABYSS assembler tends to miss highly expressed transcripts [29], the Trinity assembler [30] was also used. The resulting assemblies were joined by an iterative BLAST and cap3 assembler [31]. Sequence contamination between bar-coded libraries were identified and removed when their sequence identities were over 98%, but their abundance of reads were > 50 fold between libraries. Coding sequences (CDS) were extracted using an automated pipeline, based on similarities to known proteins, or by obtaining CDS containing a signal peptide [32]. Coding and their protein sequences were mapped into a hyperlinked Excel spreadsheet (presented as Additional file 1, and also located at http://exon.niaid.nih.gov/transcriptome/Psorophora_albipes/Pso-s2-web.xlsx.). Signal peptides, transmembrane domains, furin cleavage sites, and mucin-type glycosylation were determined with software from the Center for Biological Sequence Analysis (Technical University of Denmark, Lyngby, Denmark) [32-35]. Reads were mapped into the contigs using blastn [36] with a word size of 25, masking homonucleotide decamers and allowing mapping to up to three different CDS if the BLAST results had the same score values. Mapping of the reads was also included.
in the Excel spreadsheet. Automated annotation of proteins was based on a vocabulary of nearly 250 words found in matches to various databases—including Swissprot, Gene Ontology, KOG, PFAM, and SMART, and a subset of the non-redundant protein database of the NCBI containing proteins from vertebrates. Further manual annotation was done as required. Detailed bioinformatics analysis of our pipeline can be found in our previous publication [31]. Sequence alignments were done with the ClustalX software package [37]. Phylogenetic analysis and statistical neighbor-joining bootstrap tests of the phylogenies were done with the Mega package [38]. Blast score ratios were done as indicated previously [39]. For visualization of synonymous and non-synonymous sites within coding sequences, the tool BWA aln [40] was used to map the reads to the CDS, producing SAM files that were joined by BWA sampe module, converted to BAM format, and sorted. The sequence alignment/map tools (samtools) package [41] was used to do the mpileup of the reads (samtools mpileup), and the binary call format tools (bcftools) program from the same package was used to make the final vcf file containing the single-nucleotide polymorphic (SNP) sites, which were only taken if the site coverage was at least 100 (−D100), the quality was 13 or better and the SNP frequency was 5 or higher (default). Determination of whether the SNPs lead to a synonymous or non-synonymous codon change was achieved by a program written in Visual Basic by JMCR, the results of which are mapped into the Excel spreadsheet and color visualized in hyperlinked rtf files within Additional file 1.

Proteome analysis
Fifty SG pairs from female *P. albipes* were used in the proteome analysis. Briefly, the glands were sonicated and the supernatant was boiled for 10 min in reducing Laemmli gel-loading buffer and subsequently resolved on a NuPAGE 4-12% Bis-Tris precast gradient gel. Proteins were visualized with SimplyBlue stain (Invitrogen). The gel was arbitrarily sliced into 19 individual sections (coded as F1–19) that were destained and digested overnight with trypsin at 37°C. ZipTips® (Millipore, Belford, MA) were used to extract and desalt the peptides, which were resuspended in 0.1% TFA before mass spectrometry analysis (MS).

Nanoflow reverse-phase liquid chromatography coupled with tandem MS (MS/MS) was performed as described [42]. We obtained a database of the tryptic peptides identified by MS as a final product. This was used to search for matches from our transcriptome database of *P. albipes*. Additional details about the proteome procedure and analysis can be found in the methodology described in Chagas et al. [42].

Results and discussion
Exploring the sialotranscriptome of a *Psorophora mosquitio*
Assembly of 135,651,020 reads into 43,466 contigs (see assembly details in Additional file 2) allowed the extraction of 3,247 CDS (which mapped 13,535,229 reads) which in turn were classified according to their primary sequence (presence of homology to an already described sequence) into three categories: *i*) transcripts encoding for secreted (S) proteins, *ii*) transcripts encoding for housekeeping (H) proteins, and *iii*) transcripts encoding for proteins of unknown (U) function that lack homology with any functionally characterized protein from another organism (Table 1). Notice that these 3,247 CDS contain 485 similar CDS divergent by a few amino acids, which can be verified in the clusterization column grouping protein sequences with 95% similarity on 50% of their lengths. These may represent allele products, recent gene duplications, or sequencing/assembly errors.

After annotation, 7,537,805 reads (55.69% of the reads mapped to CDS) were classified as originating from transcripts encoding putative S proteins, and these were assembled into 802 contigs (24.70% of the total contigs) (Table 1). Signal peptide was detected in these contig sequences, suggesting that these contigs encode for proteins secreted in the saliva. In addition, 5,473,151 transcript reads (40.44% of the total reads) mapped to transcripts encoding H proteins, which were assembled into 1,973 contigs (60.76% of the total contigs). Another 85,213 reads (0.63% of total reads) correspond to transposable elements, and 439,060 reads (3.24% of total reads) were classified as originating from transcripts that encode for U products (Table 1).

The sequences encoding for H proteins were further classified into 26 subgroups according to their predicted function or membership to previously described protein families (Table 2). The potentially highly expressed H proteins include those associated with protein synthesis machinery (14.92% of the reads classified as H products), signal transduction proteins (5.18% of the total reads), unknown conserved—which represent highly conserved proteins of unknown function most likely related with

| Class              | Number of coding sequences | % of CDS | Number of reads | % of total reads |
|--------------------|-----------------------------|----------|-----------------|------------------|
| Secreted           | 802                         | 24.70    | 7,537,805       | 55.69            |
| Housekeeping       | 1,973                       | 60.76    | 5,473,151       | 40.44            |
| Transposable element| 38                          | 1.17     | 85,213          | 0.63             |
| Unknown product    | 434                         | 13.37    | 439,060         | 3.24             |
| Total              | 3,247                       | 100.00   | 13,535,229      | 100.00           |
cellular function (3.25% of the total reads), transporters and channels (3.18% of total reads), and proteins with a potential role in lipid metabolism (2.84% of the total reads). Because SGs are specialized in secretion, high expression of transcripts encoding for constituents of protein synthesis machinery and energy metabolism is commonly observed in similar analyses of blood-feeding arthropod sialotranscriptomes. Here energy metabolism represents only 0.86% of the total transcript reads encoding for H products.

The putative S proteins were further divided into 16 general categories (Table 3), several of which were abundantly expressed in *P. albipes* SGs at the transcriptome level. Their members had a classic secretion signal:

| Table 2 Functional classification of the housekeeping products expressed in the female *Psorophora albipes* salivary glands |
| --- |
| **Housekeeping** | **Number of contigs** | **Number of reads** | **% Reads** |
| Protein synthesis machinery | 132 | 1,711,561 | 14.92 |
| Signal transduction | 283 | 594,284 | 5.18 |
| Unknown conserved | 265 | 372,425 | 3.25 |
| Transporters and channels | 192 | 364,580 | 3.18 |
| Lipid metabolism | 90 | 326,204 | 2.84 |
| Transcription machinery | 122 | 292,742 | 2.55 |
| Protein export | 136 | 236,848 | 2.06 |
| Cytoskeletal proteins | 100 | 202,027 | 1.76 |
| Protein modification | 68 | 185,852 | 1.62 |
| Extracellular matrix | 65 | 159,217 | 1.39 |
| Storage | 14 | 149,093 | 1.30 |
| Unknown conserved with transmembrane domains | 51 | 140,420 | 1.22 |
| Energy metabolism | 76 | 98,777 | 0.86 |
| Carbohydrate metabolism | 55 | 96,997 | 0.85 |
| Proteases | 45 | 94,848 | 0.83 |
| Amino acid metabolism | 28 | 83,082 | 0.72 |
| Proteasome machinery | 85 | 76,054 | 0.66 |
| Intermediary metabolism | 10 | 60,190 | 0.52 |
| Native immunity | 21 | 27,736 | 0.24 |
| Signal transduction - apoptosis | 37 | 50,036 | 0.44 |
| Transcription factor | 26 | 33,506 | 0.29 |
| Nucleotide metabolism | 15 | 33,498 | 0.29 |
| Nuclear regulation | 22 | 31,366 | 0.27 |
| Oxidant metabolism/ Detoxification | 19 | 22,649 | 0.20 |
| Nuclear export | 6 | 16,187 | 0.14 |
| Detoxification | 10 | 12,972 | 0.11 |
| Total | 1973 | 5,473,151 | 47.71 |

| Table 3 Functional classification of transcripts coding for putative secreted proteins in female *Psorophora albipes* salivary glands |
| --- |
| **Secreted** | **Number of contigs** | **% Secreted reads** |
| **Enzymes** |  |  |
| Glycosidases | 7 | 681,837 | 9.05 |
| S’ nucleotides/Apyrase family | 6 | 41,550 | 0.55 |
| Serine proteases | 5 | 40,574 | 0.54 |
| Cathepsins | 3 | 36,397 | 0.48 |
| Serine-type carboxypeptidases | 2 | 20,779 | 0.28 |
| Mosquito lipases | 1 | 9,608 | 0.13 |
| Alkaline phosphatases | 2 | 8,087 | 0.11 |
| Adenosine deaminase family | 1 | 6,885 | 0.09 |
| Endonucleases | 4 | 6,281 | 0.08 |
| Ribonucleases | 3 | 2,417 | 0.03 |
| Destabilase family | 1 | 842 | 0.01 |
| Hylauronidases | 1 | 372 | 0.00 |
| **Ubiquitous protease inhibitor domains** |  |  |
| Serpin family | 15 | 169,094 | 2.24 |
| Kazal domain-containing peptides | 13 | 46,712 | 0.62 |
| TIL domain family found in mosquitoes | 8 | 38,268 | 0.51 |
| Metalloproteinase inhibitors | 1 | 2,029 | 0.03 |
| Schistocerca protease inhibitor | 1 | 2,023 | 0.03 |
| Cystatins—may be housekeeping |  |  |
| **Immunity-related proteins** |  |  |
| Fred/Ficolin domain-containing proteins | 9 | 65,681 | 0.87 |
| Lysozymes | 7 | 59,845 | 0.79 |
| C type lectins | 7 | 58,303 | 0.77 |
| Peptidoglycan recognition domains | 7 | 16,505 | 0.22 |
| Gram-negative binding proteins | 1 | 5,968 | 0.08 |
| ML domains | 3 | 4,480 | 0.06 |
| Gambicin | 2 | 4,360 | 0.06 |
| Leucine-rich proteins | 1 | 1,249 | 0.02 |
| Cecropins | 1 | 277 | 0.00 |
| Defensin | 1 | 275 | 0.00 |
| Galectin—may be housekeeping | 1 | 173 | 0.00 |
| **Mucins** |  |  |
| Mucin I mosquito family | 22 | 1,866,771 | 24.77 |
| gSG5 mucin protein family | 8 | 60,819 | 0.81 |
| Aedes-specific mucins | 7 | 30,550 | 0.41 |
| Other mucins | 8 | 24,225 | 0.32 |
mucin I mosquito family (24.77% of total reads classified as S products), similar to OT-19 containing HH repeats family (10.25% of total reads), glycosidases (9.05% of total reads), HHH peptide family (peptides containing a His triad) (7.65% of total reads), 30.5-kDa family (4.35% of total reads), long-D7 mosquito family (3.95% of total reads), Antigen-5 family (3.42% of total reads), Aegyptin family (2.62% of total reads), Serpin family (2.24% of total reads), Culicine short-D7 protein family (2.08% of total reads), Aedes 5-kDa family (1.67% of total reads), 41-kDa canonical family (1.45% of total reads), and Hyp8.2 Culicine family (1.30% of total reads) (Table 3).

Additionally, eight novel protein families were described in *P. albipes* with either no significant matches to any sequence deposited in the NCBI database, or matching mosquito hypothetical proteins not previously described in sialotranscriptomes; these were named Psor 4.7 kDa, Psor 4.2 kDa, Psor 12 kDa, Psor 6.3 kDa, Psor 4.01 kDa

### Table 3 Functional classification of transcripts coding for putative secreted proteins in female *Psorophora albipes* salivary glands (Continued)

| Protein families exclusive of bloodsucking Nematocera | 18 | 576,548 | 7.65 |
|--------------------------------------------------------|----|---------|------|
| HHH peptide family                                     | 4  | 327,913 | 4.35 |
| hyp8.2 Culicine family                                 | 6  | 98,361  | 1.30 |
| 9.7-kDa family                                          | 17 | 63,969  | 0.85 |
| Aedes W-rich peptides                                   | 1  | 51,783  | 0.69 |
| Aedes 6.5–8.5 protein family                           | 3  | 43,900  | 0.58 |
| Aedes 62-kDa family                                     | 6  | 41,700  | 0.55 |
| Basic tail mosquito family                             | 6  | 32,687  | 0.43 |
| 34-kDa Aedes family                                    | 8  | 30,082  | 0.40 |
| Aedes/Anopheles darlingi 14–15 family                  | 6  | 25,350  | 0.34 |
| GQ-rich Culicine family                                | 3  | 8,203   | 0.11 |
| 23.5 kDa Culicine family                               | 4  | 8,099   | 0.11 |
| gSG8 family                                            | 1  | 3,237   | 0.04 |
| Hyp6.2 family                                          | 3  | 2,291   | 0.03 |
| Culex WRP/16-kDa family                                | 2  | 2,138   | 0.03 |
| Salivary protein 16 family                             | 2  | 1,778   | 0.02 |
| HHH family 2                                           | 3  | 1,492   | 0.02 |
| Anopheline SG1 family                                  | 1  | 273     | 0.00 |

### Table 3 Functional classification of transcripts coding for putative secreted proteins in female *Psorophora albipes* salivary glands (Continued)

| Protein families specific to mosquitoes | 18 | 576,548 | 7.65 |
|----------------------------------------|----|---------|------|
| 30 kDa/Aegyptin family - Mosquitoes and black flies | 5  | 197,279 | 2.62 |
| Canonical                              | 8  | 109,171 | 1.45 |
| Mucin II mosquito family               | 2  | 9,705   | 0.13 |

### Protein families specific to mosquitoes

| Specific to mosquitoes | Specific to mosquitoes | Specific to mosquitoes | Specific to mosquitoes | Specific to mosquitoes | Specific to mosquitoes |
|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|
| HHH peptide family     | 18                     | 576,548                | 7.65                   |                        |                        |
| 30.5-kDa protein       | 4                      | 327,913                | 4.35                   |                        |                        |
| hyp8.2 Culicine family | 6                      | 98,361                 | 1.30                   |                        |                        |
| 9.7-kDa family         | 17                     | 63,969                 | 0.85                   |                        |                        |
| Aedes W-rich peptides  | 1                      | 51,783                 | 0.69                   |                        |                        |
| Aedes 6.5–8.5 protein  | 3                      | 43,900                 | 0.58                   |                        |                        |
| Aedes 62-kDa family    | 6                      | 41,700                 | 0.55                   |                        |                        |
| Basic tail mosquito    | 6                      | 32,687                 | 0.43                   |                        |                        |
| 34-kDa Aedes family    | 8                      | 30,082                 | 0.40                   |                        |                        |
| Aedes/Anopheles darlingi 14–15 family | 6  | 25,350 | 0.34 | |
| GQ-rich Culicine family | 3    | 8,203   | 0.11 | |
| 23.5 kDa Culicine family | 4   | 8,099   | 0.11 | |
| gSG8 family            | 1                      | 3,237                  | 0.04                   |                        |                        |
| Hyp6.2 family          | 3                      | 2,291                  | 0.03                   |                        |                        |
| Culex WRP/16-kDa family| 2                      | 2,138                  | 0.03                   |                        |                        |
| Salivary protein 16 family | 2   | 1,778   | 0.02 | |
| HHH family 2           | 3                      | 1,492                  | 0.02                   |                        |                        |
| Anopheline SG1 family  | 1                      | 273                    | 0.00                   |                        |                        |
ultrashort-D7 family, Psor 12.8 kDa novel mosquito peptide family, Psor 4.69 kDa weakly similar to Aedes, and Psor 20.44 kDa weakly similar to Culicine. These new protein families account for 1% of all the transcripts reads of P. albipes SG transcriptome. A summary and details related to the transcript annotation encoding for S proteins can be found in Table 3 and in Additional file 1.

Proteomics analysis of P. albipes SGs

We employed a proteomics analysis to investigate protein expression in SGs of P. albipes. After Coomassie staining, five bands were revealed as strongly stained at approximate molecular weight (MW) near 191 kDa, 64 to 51 kDa, between 51 to 39 kDa, between 39 to 28 kDa, and one last band with an estimated MW of 28 kDa. Other bands with lesser stain intensity were also revealed in the gel (Figure 1). The NuPage gel was arbitrary cut into 19 fractions and submitted to MS/MS analysis. Contigs showing up two or more tryptic peptides were identified by using the P. albipes transcriptome database. Table 4 presents the details of all secreted contigs identified in the P. albipes SG proteome.

We confirmed expression of 20 of 83 (24.09%) S protein families described in the sialotranscriptome. The three strongly stained bands of the gel apparently match to: F9 (glycosidase family), F11 (apyrase, adenosine deaminase), F15 (long-D7 mosquito family, 30-kDa Aegyptin family, Antigen-5). To conclude, six of ten protein families described as highly expressed in our P. albipes SG transcriptome (glycosidases, 30.5-kDa family, long-D7 mosquito family, 30-kDa Aegyptin-like family, Serpin family, and Culicine D7 mosquito family, respectively) were confirmed to be present in the salivary proteome of P. albipes based on our subsequent proteomics analysis. Furthermore, seven families (35% of the total families confirmed by proteome) described in the transcriptome as specific for mosquitoes (basic tail mosquito, Aedes 62 kDa, 9.7-kDa family, Hyp8.2 Culicine, 30.5-kDa family, 23.5-kDa family, Aedes 34 kDa) were also confirmed by our proteome analysis. Additionally, the proteomics analysis confirmed the presence of the newly described protein family named as “Psor-4 kDa ultrashort-D7 family—Contig Psor-9075.” More details about contigs/families found in the proteome of Psorophora can be seen in Figure 1 and Table 4. Tryptic peptides were assigned to several contigs encoding for H proteins (Additional file 1) such as a P. albipes Sphingomyelin phosphodiesterase that shows 55% amino acid identity to the homolog/ortholog from Culex quinquefasciatus. Previous proteomic studies using mosquito SGs identified some abundant protein families in Aedes aegypti such as long-D7 protein, adenosine deaminase, serpin, and 30-kDa Aegyptin [43]. Members of all these families were similarly identified in our P. albipes proteome. Additionally, members of the two mosquito-specific families—known as 34-kDa and 32-kDa families—were identified in our Psorophora proteome; members of this family were described as immunogenic in the proteome study of Ae. aegypti saliva [43]. Also, the antigen-5 protein was confirmed in the Psorophora proteome, and members of this family have been previously described as a SG-secreted product in Culex [44]. Many of the identified proteins have homologs/orthologs in other mosquitoes that have been described as related to blood feeding.

Figure 1 Salivary gland proteins from the mosquito Psorophora albipes. The left gel lane shows the protein standards with their molecular weights (kDa). The right gel lane shows the P. albipes salivary proteins (Coomassie stained). The grid at the right (F1–F19) shows the area of the gel slices submitted for tryptic digest and tandem mass spectrometry identification.
Table 4 Putative secreted proteins identified in the sialotranscriptome of *Psorophora albipes* and confirmed by our proteomic studies

| Description | Protein name | Fraction | Number of Tryptic peptides* |
|-------------|--------------|----------|-----------------------------|
| 5'-nucleotidase/apyrase | Psor-13556[F11→8, Psor-17515[F11→8, Psor-22761[F11→8, Psor-17516[F11→7, Psor-12600[F11→5, Psor-17320[F11→4 | | |
| Adenosine deaminase | Psor-13638[F11→163 | | |
| Endonuclease | Psor-34082[F12→18, Psor-34081[F12→18, Psor-34084[F12→8 | | |
| Glycosidas | Psor-12511[F9→84 | | |
| Serpin | Psor-18383[F12→594, Psor-18379[F12→562, Psor-18389[F12→504, Psor-18380[F12→473, Psor-20123[F12→263, Psor-20135[F12→239, Psor-18402[F12→207, Psor-20121[F12→159, Psor-18262[F12→151, Psor-12755[F12→19 | | |
| Lysozyme | Psor-13510[F19→7, Psor-14008[F19→7, Psor-12935[F19→7, Psor-29999[F19→7 | | |
| gSG5 mucin | Psor-13808[F7→34, Psor-12515[F7→29, Psor-13816[F7→24, Psor-21012[F7→23, Psor-12520[F7→16, Psor-16372[F14→3 | | |
| Aedes-specific mucin | Psor-24903[F14→8 | | |
| Long-D7 mosquito | Psor-34191[F15→29, Psor-34198[F15→28, Psor-34194[F15→28, Psor-34190[F15→22, Psor-34202[F15→17, Psor-32651[F15→9, Psor-16454[F14→26, Psor-14130[F14→22, Psor-27735[F14→22, Psor-22249[F14→22, Psor-21941[F14→10, Psor-14244[F14→5, Psor-22962[F14→5, Psor-16516[F14→4, Psor-16517[F14→4, Psor-3299[F14→2 | | |
| Short-D7 Culicine | Psor-24290[F19→11, Psor-14799[F19→8, Psor-24280[F19→8, Psor-14821[F19→8, Psor-24282[F19→8, Psor-24283[F19→7, Psor-11438[F15→5, Psor-33959[F19→3 | | |
| Antigen-5 | Psor-12020[F16→22, Psor-14194[F16→5, Psor-12762[F15→7, Psor-17302[F15→3 | | |
| 30-kDa/Aegyptin family | Psor-20191[F15→39, Psor-18075[F14→19, Psor-18076[F14→18, Psor-18880[F14→10, Psor-19455[F14→7 | | |
| Basic tail mosquito | Psor-13880[F19→3 | | |
| Aedes 62-kDa | Psor-23962[F18→7, Psor-23027[F12→4, Psor-18121[F12→2, Psor-15772[F12→2, Psor-15774[F12→2 | | |
| 9.7-kDa family | Psor-19686[F19→5, Psor-19685[F19→5, Psor-19523[F19→3, Psor-12072[F19→3 | | |
| Hyp82 Culicine | Psor-31485[F19→2, Psor-31484[F19→2, Psor-31419[F19→2 | | |
| 305-kDa | Psor-14979[F15→4 | | |
| 235-kDa | Psor-16925[F17→2 | | |
| 34-kDa Aedes | Psor-15738[F14→2 | | |
| Psor-4 kDa ultrashort-D7 | Psor-9075[F19→2 | | |

*Summary in the form of F11→18 | F12→18 | F13→2 | indicates that 18 fragments were found in Fraction 11, while 18 and 2 peptides were found in fractions 12 and 13, respectively. This summary included protein identification only when two or more peptide matches to the protein were obtained from the same gel slice.

Insight into the *P. albipes* Secreted Sialome

The following highlights are related to the secreted sialome of *P. albipes* compared with others from blood-sucking Nematocera.

Ubiquitous protein families

**Enzymes**

Members of the 5'-nucleotidase/apyrases, adenosine deaminase, ribonuclease, endonuclease, alkaline phosphatase, serine proteases, lipase, de Stafford/lipoyzyme, hyaluronidase, and glycosidas were identified. Cathepsins and serine-type carboxypeptidase are also noted but could be of H functions. These enzymes have all been found before in mosquito sialotranscriptomes, and their role in blood and sugar feeding has been reviewed [24]. Notably in the case of *Psorophora*, however, is the finding of both endonuclease (identified by MS/MS in band 12) and hyaluronidase, which were previously restricted to *C. quinquefasciatus* [24] and sand flies, but not found in *Aedes* or *Anopheles* sialotranscriptomes. This enzyme combination may help decrease skin-matrix viscosity and diffusion of salivary components, as well as breaking down neutrophil extracellular traps [45]. Apyrase, adenosine deaminase, and glycosidas were found by MS/MS in fraction 10, consistent with their expected sizes. Transcripts encoding for sphingomyelin phosphodiesterases (SMases)—some of which are highly transcribed with coverages higher than 500—is an unusual finding in mosquito sialotranscriptomes. Although lacking the initial methionine, Psor-15064 matches at position 6 and 18 fragments were found in Fraction 11, while 18 and 2 peptides were found in fractions 12 and 13, respectively. This summary included protein identification only when two or more peptide matches to the protein were obtained from the same gel slice.
ceramide in response to cellular stresses [46]. Tryptic peptides originating from SMase were found in fractions 11 and 12 of the NuPage gel in our proteomic analysis. The high expression of this enzyme suggests it may be secreted.

**Protease inhibitor domains:** Serpins were well expressed, with 2.24% of the reads of the S class, and were identified by MS/MS in gel fraction 12. The protein encoded by Psor-18383 is 44% identical with the FXa-directed anticoagulant precursor of *Aedes albopictus*. Phylogenetic analysis indicated the presence of at least five distinct gene families (Roman numerals in Figure 2), of which clades I, II, and III are found in both culicines and anophelines (clade III also having a sand fly member), but clades IV and V are exclusively Aedine; clade IV includes the salivary Xase clotting inhibitor of *Ae. aegypti* [47]. The targets of serpins from clades I, II, III, and V remain to be identified. It is to be noted that the salivary anticoagulant of anophelines is not a serpin but rather a novel protein family of antithrombins [48,49]. TIL and Kazal domain-containing peptides may be related to additional anticoagulant proteins [50] or antimicrobials. A metalloproteinase inhibitor represents the first such finding in Nematocera sialomes: Psor-25577 is 85% and 78% identical to their *Ae. aegypti* and *C. quinquefasciatus* homologs, respectively. Psor-21372 codes for a pacifastin homolog, which may be an H protein. A poorly expressed cystatin may also be an H protein, but tick salivary cystatins are secreted and poorly expressed and could have immunosuppressive function [51,52].

**Immunity-related proteins**
Lysozyme, gambicin, cecropin, and defensins were found among antimicrobial agents. Pathogen recognition proteins of the ML domain, Fred/ficolin, Gram negative binding, peptidoglycan recognition, leucine-rich, galectin, and C-type lectin families were identified. Of these, lysozyme was identified in gel fraction 19 by MS/MS.

**Yellow protein family**
The yellow gene in *Drosophila* is responsible for tanning of the cuticle, and the mosquito homolog was shown to have a dopachrome oxidase function [53,54]. This protein family is specific to insects, the royal jelly protein being a member of the superfamily [55]. Interestingly, sand flies—but no other insect sialotranscriptomes—have two members of this family recently shown to be a scavenger of serotonin [56-58]. The *P. albipes* sialotranscriptome revealed two members of this family, probably alleles, relatively well expressed, assembled with over 200 × coverage. This is the first description of a yellow family member in mosquito sialotranscriptomes; however, these results derive from a high-coverage mosquito sialotranscriptome, and it may be possible that members of this family may be found in species previously studied if higher transcript coverage is attained.

**Mosquito-specific protein families**
1,319,744 reads (18% of the total reads classified as S products) mapped to transcripts encoding proteins that can be classified according to their sequence similarity to 18 different protein families (21.68% of the total S protein families described in this transcriptome) previously described as unique to mosquitoes, i.e., they are not recognized in any other organism apart from mosquitoes [24]. A total of 69.23% of these mosquito-specific contigs had their best matches originating from *Aedes*, followed by 8.15% best matching to *Ochlerotatus*, 6.52% to *Culex*, and 4.66% to *Anopheles*. A previous review of Nematocera sialomes [24] proposed that some of these mosquito-specific families appear to be spread in all mosquito genera (studied so far), while others show specific distributions to a certain mosquito subfamily and/or genus. Accordingly, we conceptually divided our discussion regarding the mosquito-specific protein families present in *Psorophora* sialomes into four groups: i) mosquito-specific protein families common to Culicines and Anophelines, ii) mosquito-specific protein families thus far found only in Culicines, iii) mosquito-specific protein families unique to *Aedes/Ochlerotatus*, and iv) mosquito-specific protein families unique to *Culex*.

**Mosquito-specific protein families common to Culicines and Anophelines**
Nine of the 12 protein families previously known as common to Culicine and Anopheline were described in the *P. albipes* transcriptome: the HHH peptide family, the HHH peptide family 2, the mosquito basic tail family, the salivary protein 16 family, the *Aedes/Anopheles darlingi* 14–15 family, the gSG8 family, the Hyp6.2 family, the *Aedes* 62 kDa family, and the Anopheline SG1 family. Although commonly found in mosquito SG transcriptome analyses, no member of these families has been functionally characterized so far. Moreover, studies based on RT-PCR have assigned to some of these family members a tissue and/or sex specificity in their expression that suggests a role in the physiology of *Ae. albopictus* SGs [24].

Among them, the HHH peptide family was previously suggested to play a role in antimicrobial defense because of its His richness as Zn ion chelators [24,59]. Here, this family was revealed as the fourth most abundantly expressed, with 7.65% of the total reads (Table 3). This family appears to be expanded in *Psorophora*, with a possible total of at least six genes (Figure 3). The abundant expression of this protein family suggests this protein as a good candidate for exposure marker to mosquito bites. Alignment of *Psorophora* transcripts
encode two HHH repeats separated by NGTS amino acids, while one repeat was seen in the homologs from Aedes, Ochlerotatus, and Culex (Figure 3A); 35% to 55% identity is observed to the Ae. aegypti and Ae. albopictus homologs. The phylogram obtained after alignment of all HHH peptide genes found in mosquitoes shows at least five distinct clades with strong bootstrap support (Figure 3C). Two clades contain solely Psorophora transcripts (in several subclades). The remaining clades are specific to Culex, An. darlingi, Ochlerotatus, and Aedes (Figure 3C).

Mosquito basic tail proteins contain a Lys dipeptide tail (Figure 3B) and have been suggested as binding to negatively charged phospholipids found in cell membranes such as in the surface of platelets [60]. They may also be associated with plasminogen activation [61,62]. In the Psorophora transcriptome, six contigs (0.43% of the total contigs classified as S products) match mosquito basic tail peptides with 50% identity to Ae. albopictus family members (Additional file 1). Three tryptic peptides in our proteome analysis match contig Psor-13880, which encodes for a member of this family. Phylogenetic analysis of the basic tail mosquito family supports divergence of Culicine salivary proteins from the Anopheline family members (Figure 3D) where Anopheline and Culicine proteins are grouped in distinct clades (Figure 3D). Although Anopheles lack the basic tail, they have a conserved backbone (Figure 3B). In the Culicine clade, we observe that all Psorophora proteins are isolated in a genus-specific branch, separated from the other Culicine proteins with strong bootstrap support (Figure 3D).

Family Hyp6.2, represented with three truncated-sequences, is approximately 45% identical to the homologs from Ochlerotatus (Additional file 1). Additionally, all the contigs found in P. albipes transcriptome from the mosquito-specific families HHH family-2, salivary protein 16 family, Aedes/An. darlingi family, gSG8 family, and Aedes 62-kDa family have as their best matches
Figure 3 (See legend on next page.)
the homologs from *Ae. aegypti*, with identities varying from 80% to 42% (Additional file 1). Proteome analysis revealed tryptic peptides originating from *Psorophora* family members showing higher similarities to the *Aedes* 62-kDa family (Additional file 1).

### Mosquito-specific protein families thus far found only in Culicides

To date, five protein families found in the *P. albipes* sialotranscriptome are unique to Culicides. Two of these (9.7-kDa and Hyp8.2 Culicine protein families) may play a role in blood feeding, as they are abundantly expressed in female *Ae. albopictus* SGs [63]. The 30.5-kDa and 23.5-kDa protein families appear to be involved in mosquito sugar feeding due to their reported expression in male and female SGs [63]; however, the tissue specificity of the fifth protein family—namely, the GQ-rich Culicine family—is still unknown [63,64]. So far, no member from these families has been functionally characterized.

Two abundantly expressed families in our transcriptome analysis are represented by the 30.5-kDa (4.35% of total reads encoding for S products) and Hyp8.2 Culicine families (1.30% of total reads encoding for S products). The first family was also within the 50 most-expressed families in this transcriptome (Table 3). Expression of these two families in *Psorophora* SGs was confirmed by our proteome analysis (Figure 1 and Table 4). Overall, they share 53% amino acid identity with the family member from *Ae. albopictus* (Additional file 1). The *Psorophora* 9.7-kDa and 23.5-kDa families had *Ae. aegypti* proteins as their best BLAST similarity matches; tryptic peptides were found in our proteome analysis identifying these family members. In contrast, members of the GQ-rich Culicine family revealed 58% identity to its homologs from *C. quinquefasciatus* (Additional file 1).

Here we performed phylogenetic analysis of the 9.7-kDa family (Figure 4A), the 30.5-kDa family (Figure 4B), the 23.5-kDa family (Figure 4C), and GQ-rich family (Figure 4D). Overall, all four phylograms show *Psorophora* proteins phylogenetically far from *Culex* proteins. The phylogenetic tree of the 9.7-kDa family (Figure 4A) shows at least four different transcript clusters in *Psorophora* (one cluster in the branch named *Psorophora*-I and three in *Psorophora*-II). Also, several gene duplications can be found in each cluster. This phylogeny shows that *Aedes* family members are closer to *Psorophora* family members, while *Culex* proteins appear as an outgroup (Figure 4A).

The phylogram of the 30.5-kDa (Figure 4B) and 23.5-kDa (Figure 4C) families confirm the same pattern seen for the 9.7-kDa family (Figure 4A) in the sense that *Psorophora* proteins are grouped in the same clade with *Aedes* proteins (Figure 4A–C). The GQ-rich family shows *Psorophora* members grouped within the same clade containing *Ochlerotatus* proteins (Figure 4D). Although previous studies using 18S rDNA sequence suggested *Psorophora* species as a sister group to *Culex* and/or a sister group to the *Aedes/Ochlerotatus* species [12,16], our results suggest—based on the composition of the salivary proteins—that *Psorophora* is much closer to *Aedes* than to *Culex*.

### Mosquito-specific protein families unique to *Aedes/Ochlerotatus* mosquitoes

Three protein families—*Aedes* 6.5–8.5-kDa family (Figure 5A), *Aedes* W-rich peptides family (Figure 5B), and *Aedes* 34-kDa family (Figure 5C)—previously described as exclusive to *Aedes/Ochlerotatus* were found in the *Psorophora* genus. Previous studies showed that these families are female- and SG-specific [63,64], but their function still remains unknown. Alignment of the transcripts found in *Psorophora* encoding for *Aedes* 6.5–8.5-kDa (Figure 5A) and *Aedes* W-rich peptides (Figure 5B) families reveal higher identity in their amino acid sequences to *Ae. albopictus* (55%) and to *Ochlerotatus triseriatus* (62%), respectively (Additional file 1). Here, only the 34-kDa family was confirmed as present in the *Psorophora* SG proteome (fraction F14; Figure 1, Table 4). Alignments of 34-kDa family members showed 29–37% identity to their homologous proteins from *Aedes* mosquitoes (Figure 5C and Additional file 1). The phylogram shows all *Psorophora* proteins of the 34-kDa family grouped in the same clade, while the second clade of the phylogram contains all the *Aedes/Ochlerotatus* gene products (Figure 5C).
Protein family so far found only in Culex

The Culex W-rich protein (WRP)/16-kDa family is a salivary protein family so far uniquely found in Culex sialotranscriptomes, where nearly 20 genes coding for this family are known, subdivided into different subfamilies varying in their number of cysteine residues [44,65]. Although highly expressed and specific to Culex, the function of the WRP/16-kDa family remains still unclear. Here we report for the first time members of this family originating from a non-Culex mosquito.

A total of 2,138 reads were found grouped into two contigs, Psor-32363 and Psor-32364, the latter being a truncated variant of the first with a few amino acid changes. The mature MW of the encoded polypeptides is approximately 24 kDa with an estimated isoelectric point of 7.1 and amino acid sequences that are W rich (Additional file 1). Interestingly, the Psorophora protein best matches two putative Ae. aegypti proteins never previously described in sialotranscriptomes.

Alignment of the P. albipes sequence with Aedes and selected Culex sequences shows three conserved tryptophan residues among a total of 8 identities and 22 similarities, with a total similarity of only 14% (Figure 6A). Phylogenetic analysis (Figure 6B) groups the Psorophora and Aedes sequences with 100% bootstrap support within a clade of four Culex proteins having 99% bootstrap support (Clade III in Figure 6B). These results suggest that Culicines shared a common ancestor of a gene coding for this protein family that expanded in Culex but not in Aedes, indicating this family was not a Culex “invention.” The Psorophora member of the family thus helped us to partially understand the evolution of this family in Culex by providing a link between Culex and Aedes sequences.

Figure 4 Phylogram of salivary protein families derived from Psorophora albipes sialotranscriptome that are exclusively found in Culicine mosquitoes. A: 9.7-kDa family. B: 30.5-kDa family. C: 23.5-kDa family. D: GQ-rich family. The phylogram derives from the alignment of Psorophora proteins (indicated by Psor- and their contig number) and their comparison with their best matches in the non-redundant database. The three first letters indicate the genus name, followed by the three first letters of the species name, followed by the NCBI accession numbers. Numbers on the tree bifurcations indicate the percentage of bootstrap support above 75%. The bar at the bottom represents 10% amino acid substitution. Scale sequences were aligned by the ClustalW program, and the phylogram was made with the Mega package after 10,000 bootstraps with the neighbor-joining algorithm.
Figure 5 Phylogenetic analyses of salivary *Psorophora* protein families previously found exclusively in *Aedes/Ochlerotatus* mosquitoes. Clustal alignment of all *Aedes* 6.5–8.5-kDa proteins (A) and *Aedes* W-rich peptides (B) derived from *Psorophora* salivotranscriptome. Symbols above the alignment indicate (*) identical sites, (:) conserved sites, and (.) less-conserved sites. *Aedes* 34-kDa family alignment and bootstrapped phylogram (C). The numbers on the tree bifurcations indicate the percentage bootstrap support above 75%. The scale bar at the bottom represents 10% amino acid substitution. Sequences were aligned by the ClustalW program, and the dendrogram was made with the Mega package after 10,000 bootstraps with the neighbor-joining algorithm.
Other putative secreted proteins

Two putative S protein sequences match black fly proteins previously thought to be unique to *Simulium* sialomes. Three previously thought to be orphan proteins of *Aedes* and *Ochlerotatus* (*Aedes* 7-kDa and 5-kDa families and *Ochlerotatus* OT-19 family) were deorphанизed. Eight novel salivary protein families were found in the *Psorophora* sialotranscriptome, four of which appear unique to *Psorophora*, while the others have matches to mosquito hypothetical proteins not previously described in sialotranscriptomes.

We additionally identified 372 transcripts sequences encoding for secreted polypeptides, most of which have no relevant matches to any sequence deposited thus far.
in the NR database. Two of these were identified by proteome analysis. All details of these proteins are in the hyperlinked Excel spreadsheet available in Additional file 1.

**P. albipes similarities to other mosquito species**

The availability of the genomes of *Ae. aegypti*, *C. quinquefasciatus*, and *Anopheles gambiae* [66-68] allows for comparisons of the protein sequences of *Psorophora* to those deduced from the three mosquito genomes. We have determined (Additional file 1) the BLAST score ratios of each protein for the three genomes by dividing the BLAST score found for the blastp result against one of the three mosquito proteomes by the BLAST score of the *Psorophora* protein blasted against itself [39]. The comparisons indicate that *Aedes* is the closest related mosquito to *Psorophora*, followed by *Culex* and *Anopheles* (Figure 7). It also shows that the S class of proteins has the lowest ratio of all, while those for proteasome machinery, nuclear regulation, and cytoskeletal are among the most conserved (Figure 7). In the S class computed above, those 372 proteins indicated as “Other putative secreted peptides” were not included, as they are of uncertain nature and would further decrease the score ratios. This divergence of salivary proteins in mosquitoes has been previously reported for other taxa [22,24,63-65,69-71].

**Polymorphism of *P. albipes* coding sequences inferred from the RNAseq data**

RNAseq data produces high contig coverage. In our CDS set, 1,822 had average coverage of 100 or larger per nucleotide site, allowing reliable identification of SNPs using the tools BWA and Samtools (see Methods). From this CDS set we excluded the 372 proteins indicated in the previous section, plus all of the unknown class, and all CDS having a similar protein sequence with >95% similarity (to avoid overrepresentation of alleles), producing a set of 1,100 CDS. When comparing the number of synonymous sites per 100 codons among different functional classes, the protein syntheses and secreted classes have the smallest value, while the proteasome and immune categories have the highest (Figure 8A and Table 5). When the values of the number of non-
synonymous SNPs are compared, the figure reverses (Figure 8B), the secreted category having the highest value, to near 0.33 per 100 codons (Table 5). The overall non-synonymous to synonymous rate also shows the secreted class to have the highest ratio (Table 5). This increased non-synonymous polymorphism is not an artifact resulting of increased read coverage of the contigs of the secreted class because the protein synthesis class of contigs has an even higher read coverage but has the second smallest non-synonymous polymorphism index. It is possible that this high value of non-synonymous polymorphism observed for the secreted class may result from chimeric assembly of coding sequences originating from multiple recently duplicated genes coding for very similar proteins. At any rate, this high polymorphism may underlie the mechanisms leading to accelerated evolution of salivary proteins observed in bloodsucking arthropods.

Figure 8 Number of polymorphic sites per 100 codons in *Psorophora albipes* proteins. Bars represent the average and standard errors of synonymous (A) and non-synonymous (B) sites per 100 codons in the indicated functional categories.
Conclusions

The sialotranscriptome of P. albipes as described here is the first—or among the first—to use solely Illumina sequences for its assembly, in the absence of a reference genome. Over 3,000 coding sequences were recovered, 1,790 of which were submitted to GenBank. This is also the first transcriptome of a member of the Psorophora genus. As expected, the protein sequences presented more similarities to Aedes, followed by Culex and Anopheles proteins. Despite this more Aedine nature, P. albipes presented some Culex characters—such as the presence of endonuclease and hyaluronidase—common in sand flies and black flies but so far uniquely found in Culex. A Psorophora protein similar to the WRP/16-kDa family also unique so far to Culex allowed the discovery of a “missing link” between this Culex family and hypothetical Ae. aegypti proteins, indicating this gene family is ancestral in all Culicines but poorly or not expressed in Aedes SGs. Orphan protein families from Aedes and Ochlerotatus were deorphanized, and several new families of proteins were identified, four of which appear unique to Psorophora, supporting the idea that sialotranscriptomes of new bloodsucking genera yield at least two novel protein families [72]. However, these novel sequences may result from misassemblies or chimeras. Further sequencing of other Psorophora species may clarify this area. Unique to Psorophora is also the finding of SMase, not previously found in mosquito sialomes.

Because the sample derived from 50 field-collected mosquitoes, we also were able to derive an estimate of SNPs and the rate of synonymous and non-synonymous mutations in this data set.

Availability of supporting data

All data from the transcriptome and proteome analysis of P. albipes SGs are disclosed in Additional file 1, a hyperlinked Excel spreadsheet available at http://exon.niaid.nih.gov/transcriptome/Psorophora_albipes/Pso-s2-web.xlsx. Raw reads were deposited in the SRA of the NCBI under bioproject numbers PRJNA208524 and 208958 and raw data file SRR908278. One thousand seven hundred and ninety coding sequences have been publicly deposited in the Transcriptome Shotgun Assembly project at DDBJ/EMBL/GenBank under accession GALA00000000. The version described in this paper is the first version, GALA01000000, ranging from GALA01000001 to GALA01001790.

Additional files

Additional file 1: Hyperlinked Excel spreadsheet with protein coding sequences and added information.

Additional file 2: Assembly information.

Abbreviations

ABySS: Assembly by short sequences software; bcftools: Binary call format tools; CDS: Coding sequences; H: Housekeeping proteins; MS: Mass spectrometry; MG/MS: Tandem MS; MW: Molecular weight; NCBI: National Center for Biotechnology Information; NGS: Next-generation sequencing;

Table 5

| Class                  | Average (Syn /Codon) x 100 | SE         | Average (NS /codon) x 100 | SE         | NS/Syn | Average coverage | SE | N   |
|------------------------|-----------------------------|------------|-----------------------------|------------|--------|-------------------|----|-----|
| Nuclear regulation      | 1.4343                      | 0.5854     | 0.0217                      | 0.0207     | 0.0152 | 278.6             | 48.3| 11  |
| Protein synthesis       | 0.4095                      | 0.1637     | 0.0885                      | 0.0389     | 0.2161 | 2981.7            | 243.9| 96  |
| Protein export          | 1.5323                      | 0.1254     | 0.1188                      | 0.0491     | 0.0963 | 340.0             | 34.0| 65  |
| Cytoskeletal            | 0.9455                      | 0.1606     | 0.1271                      | 0.0722     | 0.1344 | 322.5             | 48.0| 54  |
| Proteasome              | 2.6511                      | 0.4884     | 0.1487                      | 0.0550     | 0.0561 | 189.0             | 20.4| 35  |
| Transcription           | 1.1524                      | 0.2376     | 0.1531                      | 0.0785     | 0.1329 | 410.2             | 99.3| 47  |
| Signal transduction     | 1.0220                      | 0.1283     | 0.1604                      | 0.0385     | 0.1570 | 365.9             | 69.9| 143 |
| Transporters            | 1.6399                      | 0.3186     | 0.1997                      | 0.0638     | 0.1218 | 400.2             | 61.0| 89  |
| Detoxification          | 1.5145                      | 0.3318     | 0.2194                      | 0.0739     | 0.1448 | 198.5             | 31.2| 16  |
| Transcription factor    | 0.9573                      | 0.3958     | 0.2243                      | 0.1351     | 0.2343 | 237.2             | 30.2| 12  |
| Immunity                | 2.4690                      | 0.9928     | 0.2398                      | 0.1077     | 0.0971 | 224.7             | 47.6| 11  |
| Protein modification     | 1.1421                      | 0.2421     | 0.2514                      | 0.0877     | 0.2201 | 370.1             | 86.2| 56  |
| Metabolism              | 1.5898                      | 0.1807     | 0.2515                      | 0.0447     | 0.1582 | 389.2             | 72.1| 147 |
| Extracellular matrix     | 1.7871                      | 0.3776     | 0.3044                      | 0.0994     | 0.1703 | 280.6             | 39.9| 29  |
| Unknown conserved       | 1.6564                      | 0.1961     | 0.3244                      | 0.0626     | 0.1958 | 372.9             | 44.6| 127 |
| Secreted                | 0.5885                      | 0.0849     | 0.3308                      | 0.0498     | 0.5621 | 2729.7            | 288.8| 334 |

Total 1100

1 Number of synonymous polymorphism per 100 codons.
2 Number of non-synonymous polymorphism per 100 codons.
3 Average read coverage of coding sequences.
NISC: NIH Intramural Sequencing Center; S: Secreted proteins; samtools: Sequence alignment/map tools; SG: Salivary gland; SMase: Sphingomyelin phosphodiesterase; SNP: Single-nucleotide polymorphism; SRA: Sequence read archives; U: Proteins of unknown function; WRP: W-rich protein.

**Competing interests**
The authors declare that they have no competing interests.

**Authors’ contributions**
ACC, EC, and JMCR contributed to experimental design, bioinformatics analysis, and writing of the manuscript. CMRV, FACP, and JFM contributed to insect collection, dissections, and taxonomic identification of mosquitoes. All authors read and approved the final manuscript.

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**Reinert J, Harbach R, Kitching I:** Phylogeny and classification of Aedini (Diptera: Culeidae), based on morphological characters of all life stages. Zool J Linn Soc 2004, 142:289–368.

**Shepard JJ, Andreadis TG, Vosbrinck CR:** Molecular phylogeny and evolutionary relationships among mosquitoes (Diptera: Culeidae) from the northeastern United States based on small subunit ribosomal DNA (18S rDNA) sequences. J Med Entomol 2006, 43:434–454.

**Ribeiro JMC:** Role of arthropod saliva in blood feeding. Ann Rev Entomol 1987, 32:463–478.

**Carmo A, Dupuy E, Bellucci S, Calvo F, Blykort MC:** Human platelet-tumor cell interactions vary with the tumor cell lines. Invest-Metastasis 1986, 63:321–334.

**Calvo E, Mans B, Ribeiro JM, Andersen JF:** Multifunctionality and mechanism of ligand binding in a mosquito antiinflammatory protein. Proc Natl Acad Sci USA 2009, 106(10):3728–3733.

**Calvo E, Mizurini D, Sa-Nunes A, Ribeiro JM, Mans B, Monteiro R, Kotsyfáksis M, Francischetti I:** Alboserin, a factor Xa inhibitor from the mosquito vector of yellow fever, binds heparin and membrane phospholipids and exhibits anticoagulant activity. J Biol Chem 2011, 286(2):799–8010.

**Calvo E, Tokusamu F, Mizurini D, McPhe P, Narum D, Ribeiro JM, Monteiro R, Francischetti I:** Aegyptins displays high-affinity for the von Willebrand factor binding site (QQQQVGMG) in collagen and inhibits carotid thrombus formation in vivo. FEBS J 2010, 277(2):413–427.

**Calvo E, Pharm VM, Mariotti O, Andersen JF, Ribeiro JM:** The salivary gland transcriptome of the neotropical malaria vector Anopheles darlingi reveals accelerated evolution of genes relevant to hematophagy. BMC Genomics 2009, 10:57.

**Ribeiro JM, Francischetti IM:** Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. Annu Rev Entomol 2003, 48:73–88.

**Ribeiro JM, Mans B, Arca B:** An insight into the sialome of blood-feeding Nematocera. Insect Biochem Mol Biol 2010, 40(11):767–784.

**Valenzuela JG:** High-throughput approaches to study salivary proteins and genes from vectors of disease. Insect Biochem Mol Biol 2002, 32(10):1199–1209.

**Consoli RAGB, de Oliveira RL:** Principais mosquitos de importância sanitária no Brasil. Rio de Janeiro: Editora Fiocruz; 1994.

**Brol I, Jackman SD, Nielsen GB, Qian JQ, Vahrol H, Stazky G, Morin RO, Zhao Y, Hirst M, Schein JE, et al:** De novo transcriptome assembly with Abyss. Bioinformatics (Oxford, England) 2009, 25(21):2872–2877.

**Simpson JT, Wong K, Jackman SD, Schein JE, Jones SJ, Brol I:** Abyss: a parallel assembler for short read sequence data. Genome Res 2009, 19(6):1117–1123.

**Zhao QF, Wang Y, Yong YM, Luo D, Li X, Hao P:** Optimizing de novo transcriptome assembly from short-read RNA-Seq data: a comparative study. BMC Bioinformatics 2011, 12(Suppl 14):S2.

**Grabher MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, et al:** Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol 2011, 29(7):644–652.

**Karim S, Singh P, Ribeiro JM:** A deep insight into the sialotranscriptome of the Gulf Coast tick, Amblyomma maculatum. PLoS ONE 2011, 6(12):e28325.

**Nielsen H, Brunak S, von Heijne G:** Machine learning approaches for the prediction of signal peptides and other protein sorting signals. Protein Eng 1999, 12(13–9).

**Duckert P, Brunak S, Blom N:** Prediction of proprotein convertase cleavage sites. Protein Eng Des Sel 2004, 17(1):107–112.
34. Sonnhammer EL, von Heijne G, Krogh A: A hidden Markov model for predicting transmembrane helices in protein sequences. Proc Int Conf Intell Syst Mol Biol 1998, 6:175–182.
35. Julenius K, Molgaard A, Gupta R, Brunak S: Prediction, conservation analysis, and structural characterization of mammalian mucin-type O-glycosylation sites. Glycobiology 2003, 13(2):153–164.
36. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997, 25(17):3389–3402.
37. Thompson JD, Gibson TJ, Pilewskie F, Jeanmougin F, Higgins DG: The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997, 25(24):4876–4882.
38. Kumar S, Tamura K, Nei M: MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform 2004, 5(2):150–163.
39. Rasko DA, Myers GS, Ravel J: Visualization of comparative genomic analyses by BLAST score ratio. BMC Bioinformatics 2005, 6:22.
40. Li H, Durbin R: Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics (Oxford, England) 2010, 26(5):898–905.
41. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R: Genome Project Data Processing S: The sequence alignment/ map format and SAMtools. Bioinformatics (Oxford, England) 2009, 25(16):2078–2079.
42. Chagas AC, Calvo E, Pimenta PF, Ribeiro JM: An insight into the sialome of Simulium guineense (OPITERA-SIMULIDAE), the main vector of River Blindness Disease in Brazil. BMC Genomics 2011, 12:612.
43. Waspiniamyongk L, Patramool S, Lupelton L, Surasombattapattana P, Doucoure S, Mouchet F, Seveno M, Demettre E, Brizard J, et al: Blood-feeding and immunogenic Aedes aegypti saliva proteins. Proteomics 2010, 10(15):2906–1916.
44. Ribeiro JM, Chafata R, Pham VM, Garfield M, Valenzuela JG: An insight into the salivary transcriptome and proteome of the adult female mosquito Culex pipiens quinquefasciatus. Insect Biochem Mol Biol 2004, 34(5):543–558.
45. Warth F, Beiter K, Norman K, Heniques-Normark B: Neutrophil extracellular traps: casting the NET over pathogenesis. Curr Opin Microbiol 2007, 10(1):52–56.
46. Hannun Y, Obeid L: The Ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind. J Biol Chem 2002, 277(29):25847–25850.
47. Stark KR, James AA: Isolation and characterization of the gene encoding a novel factor Xa-directed anticoagulant from the yellow fever mosquito, Aedes aegypti. Proc Natl Acad Sci USA 1999, 96(26):15155–15160.
48. Han Q, Collin N, Gomes R, Teixeira C, Reynoso D, My Pham V, Elnaïm DE, Kamhawi S, et al: Structure and function of a “yellow” protein from saliva of the sand fly Lutzomyia longipalpis that confers protective immunity against Leishmania major infection. J Med Entomol 2011, 48(6):3233–3239.
49. Loomans HI, Hahn BL, Li QQ, Suhnhle PG: Histidine-based zinc-binding sites and the antimicrobial activity of calprotectin. Infect Dis 1998, 171(7):812–814.
50. Andersen JF, Guiderdoni NP, Franchiatti IM, Valenzuela JG, Ribeiro JM: Recognition of anionic phospholipid membranes by an anthemostatic protein from a blood-feeding insect. Biochemistry 2004, 43(22):6987–6994.
51. Castellino FJ, McCance SG: The kringle domains of human plasminogen. Curr Opin Hematol 1997, 4(2):146–60. discussion 60–45.
52. Mosesson MW, Siebenlist KR, Neih DA: The structure and biological features of fibrogenin and fibrin. Ann N Y Acad Sci 2001, 936:1–30.
53. Arca B, Lombardo F, Franchetti IM, Pham VM, Marest-Simon M, Andersen JF, Ribeiro JM: An insight into the sialome of the adult female mosquito Aedes albopictus. Insect Biochem Mol Biol 2007, 37(2):107–127.
54. Ribeiro JM, Arca B, Lombardo F, Calvo E, Pham VN, Chandra PK, Wikel SK: An annotated catalogue of salivary gland transcripts in the adult female mosquito Aedes aegypti. BMC Genomics 2007, 8:116.
55. Calvo E, Sanchez-Vargas I, Favreau AJ, Barbian KD, Pham VM, Olson KE, Ribeiro JM: An insight into the sialotranscriptome of the West Nile mosquito vector, Culex tarsalis. BMC Genomics 2010, 11(1):515.
56. Arensburger P, Megy K, Waterhouse RM, Abrudan J, Arnedo P, Aroteo B, Bartholomay L, Bidwell S, Caler E, Camara F, et al: Sequencing of Culex quinquefasciatus establishes a platform for mosquito comparative genomics. Science New York, NY 2003, 300(5626):86–88.
57. Nene V, Worman JR, Lawson D, Haas B, Kodira C, Wu Z, Loheit B, Xi Z, Megy K, Grabher M, et al: Genome sequence of Aedes aegypti, a major arbovirus vector. Science New York, NY 2007, 316(5832):1718–1723.
58. Holt RA, Subramanian GM, Halpern A, Sutton GG, Charlab R, Nusskern DR, Winckler P, Clark AG, Ribeiro JM, Wides R, et al: The genome sequence of the malaria mosquito Anopheles gambiae. Science New York, NY 2002, 298(5591):119–149.
59. Calvo E, Sanchez-Vargas I, Kotsyfakis M, Favreau AJ, Barbian KD, Pham VM, Olson KE, Ribeiro JM: The salivary gland transcriptome of the eastern tree hole mosquito, Ochlerotatus triseriatus. J Med Entomol 2010, 47(3):376–386.
60. Calvo E, Dao A, Pham VM, Ribeiro JM: An insight into the sialotranscriptome and proteome of the Anopheles stephensi mosquito. Insect Biochem Mol Biol 2003, 33(7):717–732.
61. Ribeiro JM, Arca B: From sialomes to the silavorese: An insight into the salivary potion of blood feeding insects. Adv Insect Physiol 2000, 29:579–118.

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