Journal of Phylogenetics & Evolutionary Biology

Mithilesh Kajla et al., J Phylogen Evolution Biol 2015, 3:4
DOI: 10.4172/2329-9002.1000160

Research Article Open Access

Identification of an *Anopheles* Lineage-Specific Unique Heme Peroxidase HPX15: A Plausible Candidate for Arresting Malaria Parasite Development

Mithilesh Kajla[1], Kuldeep Gupta[1], Parik Kakani[1], Rini Dhawan[1], Tania Pal Choudhury[1], Lalita Gupta[1], Surendra K Gakhar[2] and Sanjeev Kumar[1]*

[1]*Molecular Parasitology and Vector Biology Laboratory, Department of Biological Sciences, Birla Institute of Technology and Sciences, Pilani (BITS-Pilani), Pilani-333 031, Rajasthan, India*
[2]*Department of Life Sciences, Maharashi Dayanand University, Rohtak, Haryana, India*

Abstract

Background: Human malaria parasite *Plasmodium falciparum* is transmitted by several species of *Anopheles* mosquito. The advancement of drug-resistant parasites and insecticide resistance in mosquito vectors are major hurdles in the malaria control. Alternatively, the manipulation of mosquito immunity is also an ideal way to block *Plasmodium* development inside the insect host. This approach demands the identification of key mosquito molecules that regulate anti-plasmodial immunity. Our previous findings revealed that the silencing of *Anopheles gambiae* heme peroxidase 15 (AgHPX15, AGAP013327) induced mosquito innate immunity and drastically suppressed the development of human and rodent malaria parasites. Further, we aim to characterize HPX15 orthologs in Indian malaria vectors and other worldwide-distributed anophelines to understand the novelty of this molecule as a plausible target to block *Plasmodium* development.

Method: AgHPX15 orthologs were cloned from major Indian malaria vectors *A. stephensi* and *A. culicifacies* and their conserve domains were determined by CDD search tool. The sequence homology and phylogenetic relationship of these clones with other heme peroxidases was analysed using Mega 5.2 software.

Results and conclusion: We found that *A. stephensi* AsHPX15 and *A. culicifacies* AcHPX15 clones are close orthologs of *A. gambiae* AgHPX15. The phylogenetic relationship of these anopheline HPX15 with other animal and plant heme peroxidases revealed that they form a separate lineage-specific cluster and their orthologs are not found in human, nematodes or other related arthropods such as, *Drosophila, Aedes* and *Culex* mosquitoes. However, their putative orthologs are present in 16 other globally distributed anophelines and exhibit a highly conserved amino acids identity in the range of 70-99%. Based on these findings we propose that the anopheline-specific and evolutionary conserved heme peroxidase HPX15 may serve as a unique target for designing transmission-blocking strategies to block *Plasmodium*-mosquito cycle. These findings will generate new frontiers in the field of malaria research and disease control.

Keywords: Heme peroxidase; HPX15; *Anopheles* mosquito; Malaria transmission; Ortholog; Innate immunity; *Plasmodium*

Abbreviations: HPX: Heme Peroxidase; As: *Anopheles stephensi;* Ac: *Anopheles culicifacies*; Ag: *Anopheles gambiae;* WHO: World Health Organization

Introduction

Malaria is a major health problem in tropical and subtropical countries of the world. In spite of different control measures, approximately 3.2 billion people are at the risk of malaria throughout the world. WHO reported 198 million cases of malaria and 584,000 deaths world widely in 2013 [1]. In the South East Asian region, India alone contributes nearly 1.5 million cases of malaria [2]. Malaria is caused by *Plasmodium* (a protozoan), which is transmitted among humans by the female *Anopheles* mosquito. In India, there are approximately 58 anopheline mosquito species and amongst them, six are potent malaria vectors [3]. *Anopheles culicifacies* and *A. stephensi* are major vectors of rural and urban malaria, respectively [4].

Human race pays a heavy toll to malaria in terms of death and economic loss. This warrants an attention to develop novel effective methods that can control *Plasmodium* development either in human or mosquito host. Such strategies are synthesis of effective vaccine/drugs against *Plasmodium* and the application of synthetic mosquitocidal compounds [5]. Currently there is no licensed vaccine against malaria [6] and the drug resistance in *Plasmodium* is also a major challenge in this field [7]. Moreover, disease vectors developed resistance against many synthetic mosquitocidal compounds as well as their non-biodegradability is also a serious issue [8,9]. On the other hand, a potent method of controlling malaria may be impeding parasite development inside the mosquito through transmission blocking strategies. However, this requires exploring the molecular interactions of *Plasmodium* and mosquito immune system and identifying potent targets to manipulate mosquito immunity.

So far, major studies regarding *Plasmodium*-mosquito interactions are carried in African mosquito *A. gambiae*. In these mosquitoes numerous anti-bacterial and/or -plasmodial immune molecules have been identified, which effectively kill *Plasmodium* at different stages of development [10-17]. Some of these immune molecules successfully control the growth of *Plasmodium berghei* (mouse malaria) however, they are incompetent to regulate *P. falciparum* (human malaria) development [18,19]. These facts demand for the discovery of potent mosquito immune molecules that can broadly regulate the development of major human malaria parasites.

***Corresponding author:** Sanjeev Kumar, Department of Biological Sciences, Birla Institute of Technology and Science (BITS), Pilani Campus, Pilani, Rajasthan, India, Tel: + 91 1596 515 670; E-mail: sanjeev@pilani.bits-pilani.ac.in

Received October 12, 2015; **Accepted** November 16, 2015; **Published** November 20, 2015

Citation: Kajla M, Gupta K, Kakani P, Dhawan R, Choudhury TP, *et al.* (2015) Identification of an *Anopheles* Lineage-Specific Unique Heme Peroxidase HPX15: A Plausible Candidate for Arresting Malaria Parasite Development. J Phylogen Evolution Biol 3: 160. doi:10.4172/2329-9002.1000160

Recent studies identified *A. gambiae* heme peroxidases (HPXs) as major regulators of anti-*P. falciparum* immunity [20,21]. One of the *A. gambiae* heme peroxidase named AgHPX15 (AGAP013327) catalyzes protein cross-linking to form a physical barrier on the luminal surface of midgut epithelial cells. This barrier suppresses the recognition of blood bolus antigens by the mosquito immune system. The barrier-based mechanism maintains a 'low immunity' zone in this area to support the growth of endogenous bacteria in the blood fed midguts. *Plasmodium* takes the advantages of this situation to support its own development. Interestingly, the silencing of AgHPX15 gene suppressed the formation of midgut barrier and induced mosquito immunity against bacterial flora and *P. facliparum* [20]. Thus, AgHPX15 may be a 'potent candidate' that can be targeted to manipulate mosquito immunity to block *Plasmodium* development.

In this study, we characterized AgHPX15 orthologs in major Indian malaria vectors, *A. stephensi* and *A. culicifacies* and analyzed their evolutionary relationships. Our results demonstrate that HPX15 is an *Anopheles* lineage-specific gene and evolutionary conserved among nineteen different species of *Anopheles* mosquitoes. These findings may help us to design common strategies for blocking the transmission of human malaria by targeting a key central molecule that regulates *Plasmodium* development.

Materials and Methods

Rearing of mosquitoes

Anopheles stephensi mosquitoes were reared in insectory at 28°C, 80% relative humidity (RH) and 12 h light: dark cycle as described before [18]. Larvae were fed on a 1:1 mixture of dog food (Pet Lover`s crunch milk biscuit, India) and fish food (Gold Tokyo, India). Adults were regularly maintained at 10% sucrose solution ad libitum. For colony propagation, adult females were fed on anesthetized mice and their eggs were collected in moist conditions. *A. culicifacies* has a complex of five sibling species named as A, B, C, D and E. All these sibling species, except B, are good vectors for human malaria (*P. falciparum* and *P. vivax*) [22,23]. The sibling species A was reared in an insectory at Maharshi Dayanand University (MDU), Rohtak under the similar conditions as mentioned above. In addition, for rearing *A. culicifacies* the insectory was also equipped with simulated dusk and dawn system with a 14h light and 10h dark cycle as described before [24].

Designing of *A. stephensi* heme peroxidase degenerate and gene-specific primers

Initially at the starting of this study genome sequences were not available thus, degenerate primers approach was used to clone *A. stephensi* HPX15 (ortholog of *A. gambiae* AgHPX15). Degenerate primers were designed based on the conserved regions of different insect peroxidase proteins as illustrated in Figure S1. The sequences of degenerate primers (5' to 3') were following:

Forward (degF): TACTRBGARTGGYTGCCVATY,

Reverse (degR): GCCARWCCRTGRTCVCGRKYRCGCTG.

Later on with the availability of *A. stephensi* genome (taxid: 30069), the AsHPX15 gene sequence was identified in contig 5285 (recently super contig KB665221, Ensembl gene identifier ASTE008179 in annotated genome) based on the nucleotide blast with the clone obtained by degenerate primers. The nucleotide sequence of this putative AsHPX15 gene was aligned with known nucleotide sequence of AgHPX15 to design gene-specific primers as illustrated in Figure S2 and their sequences (5' to 3') are following:

Forward 2 (F2): GAGAAGCTTCGCACGAGATTA,

Reverse 2 (R2): GAATGTCGATTGCTTTCAGGTC;

Forward 3 (F3): AGTGCAACAGCTTGCGTACC,

Reverse 3 (R3): CCTTTAGTCCATGAGTGTTGTCA.

Mosquito tissue collection and RNA isolation

A. stephensi and *A. culicifacies* females were allowed to feed on an anesthetized mouse. After 24 h of blood feeding the midgut (Mg) and the rest of the body parts (carcass, CC) were collected from the pool of mosquitoes in RNAlater and stored at -80°C. Total RNA was isolated from these samples using RNAeasy mini kit from Qiagen (Cat no. 74104) following manufacturer's instructions.

cDNA preparation and RT-PCR

The first-strand cDNA was synthesized using Quantitech reverse transcription kit (Qiagen Cat no. 205311). RT-PCR was performed with midgut and carcass cDNA using above mentioned primers. PCR with degenerate primers followed the first cycle at 94°C for 3 min, 55°C for 1 min, 72°C for 3 min, 94°C for 1 min, 42°C for 1 min, 72°C for 3 min. Then another cycle at 94°C for 1 min, 52°C for 1 min, 72°C for 3 min was repeated 34 times with a final extension at 72°C for 10 min.

Cloning and sequencing of AsHPX15 and AcHPX15 heme peroxidases

The resulting PCR product (428 bp, Figure 1A) with degenerate primers was cloned into PCR2.1-TOPO TA vector (Invitrogen). The clone was sequenced in an automated sequencer at the commercial sequencing facility. The sequence identity was confirmed through Mega blast against the nucleotide sequences database at NCBI. Further, this degenerate clone nucleotide sequence was blasted against *A. stephensi* genome to identify AsHPX15 putative gene as discussed above.

The primer set F3R3 amplified ~ 1.1 kb PCR product from *A. stephensi or A. culicifacies* midgut cDNA templates and we termed them AsHPX15 and AcHPX15, respectively (Figure 1B and 1C). Further, the sequence identity of AsHPX15 and AcHPX15 was confirmed and submitted to NCBI. The sequence accession numbers [GenBank: KP223285] for *A. stephensi* AsHPX15 and [GenBank:KP299257] for *A. culicifacies* AcHPX15 were obtained. The primer set F2R2 was used to confirm the identity of all these clones through PCR (data not shown).

Analysis of conserved domains in AsHPX15 and AcHPX15 sequences

Signature domains in cloned AsHPX15 and AcHPX15 were identified using the Conserved Domain Database (CDD) search tool available online at NCBI [25]. These results were validated after comparing them to the CDD results of *A. gambiae* heme peroxidase AgHPX15 and other anopheline peroxidases.

Selection of heme peroxidases for phylogenetic analysis

The evolutionary relationship of cloned anopheline peroxidases (AsHPX15 and AcHPX15) was analyzed with selected 74 heme peroxidase protein sequences from various organisms (as shown in Table S1). These sequences were downloaded from NCBI and VectorBase databases and their overhanging sequences were trimmed based on predicted protein of AsHPX15 clone. Importantly, for these analyses, we selected heme peroxidase protein sequences from blood

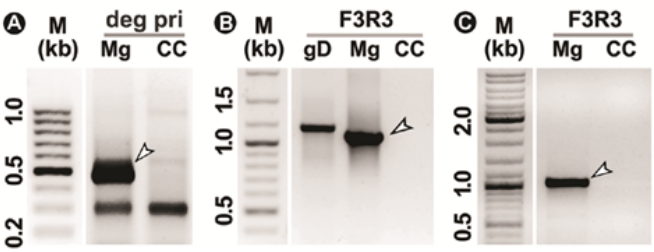

Figure 1: Cloning of HPX15 gene from Indian malaria vectors, *A. stephensi* and *A. culicifacies*.
A) 24 h post blood fed *A. stephensi* midgut (Mg) and carcass (CC) cDNA templates were PCR amplifies using degenerate primers (deg pri) as depicted in Figure S1. These primers amplified a desired product of 428 bp from the midgut (Mg) cDNA.
B) PCR amplification of *A. stephensi* midgut (Mg) and carcass (CC) cDNA using gene-specific primers (F3R3) as depicted in Figure S2. These primers amplified a single product of ~ 1100 bp exclusively from Mg cDNA. The genomic DNA (gD) template served as a control for the PCR reactions.
C) *A. culicifacies* cDNA templates were PCR amplifies using F3R3 primers. The desired band of ~1100 bp is seen only in Mg cDNA. The leftmost lane in all the cases represents the standard DNA molecular marker (M) reference for identifying the size of amplified DNA fragments. Numbers on the left side indicate standard DNA size in kb. PCR products indicated by arrowheads were used for cloning and sequencing.

feeding (*Pediculus humanus, Aedes aegypti, A. gambiae* and *Culex quinquefasciatus*) as well as non blood feeding (*Tribolium castaneum, Apis mellifera* and *Drosophila. melanogaster*) arthropods. In addition, *Caenorhabditis elegans, Arabidopsis thaliana* and *Homo sapiens* heme peroxidases were also included to understand their phylogenetic relationship and advancements during evolution. In some analyses, we also included non heme peroxidase protein sequences from *A. gambiae* (Table S1).

To retrieve putative HPX15 peroxidases from additional 16 different anophelines, we blasted nucleotide sequence of AsHPX15 clone against their whole genome shotgun (WGS) sequences available at NCBI. The contig with best match was obtained and aligned with AsHPX15 clone by ClustalW algorithm to trim the overhanging regions. The predicted proteins for these trimmed sequences are mentioned in Table 1. Peroxidases from other organisms were also trimmed in the same way. The phylogenetic tree was constructed using the full length or trimmed protein sequences of these peroxidases. The tree topology was same for full length as well as trimmed sequences thus, the results with trimmed sequences are presented in this manuscript.

Phylogenetic tree construction and analysis

Phylogenetic trees were constructed from selected peroxidases using the maximum likelihood (ML) and neighbor-joining (NJ) methods implemented in MEGA 5.2 program as described before [26]. We aligned all selected protein sequences by ClustalW algorithm in the MEGA 5.2 program as before [27]. Following criteria were selected for the phylogenetic analysis: 'WAG' option was selected as the model for amino acid substitution as this model best fits to our data. For gaps and missing data, we used 'all sites' option. The ML tree was generated using nearest neighbor interchange (NNI) tree search algorithm. Branching pattern reliability was tested for both ML and NJ tree by 1000 bootstrap replicates. The resulting phylogenetic tree was analyzed based on clusters and nodes formed. Because the topologies of phylogenetic tree obtained by ML and NJ methods were similar, therefore, only the ML tree for respective analyses is presented in this study.

Results

Characterization of AgHPX15 orthologs in *A. stephensi* and *A. culicifacies*

To identify AgHPX15 orthologs in major Indian malaria vectors (*A. stephensi* and *A. culicifacies*), we cloned HPX15 from these mosquito species. Initially at the starting of this study *A. stephensi* genome sequences were not available thus, degenerate primers approach was used to clone HPX15 as discussed in Materials and Methods and Figure S1. A PCR product of 428 bp from *A. stephensi* midgut cDNA was cloned and sequenced (Figure 1A). The nucleotide blast results revealed its closest match with *A. gambiae* AgHPX15 (77% identity and E value 6e-84). This clone was named degAsHPX15.

Later on the unannotated genome of *A. stephensi* (taxid: 30069) was available at NCBI. The nucleotide blast of degAsHPX15 and AgHPX15 against *A. stephensi* genome retrieved contig 5285 (recently super contig KB665221, Ensembl identifier ASTE008179 in annotated genome), which was used to design gene-specific primers (Figure S2). PCR with F3R3 primers amplified a fragment of ~ 1100 bp exclusively from *A. stephensi* midgut cDNA and no product was observed when carcass cDNA was used as template (Figure 1B). The PCR with other primers F2R2 amplified a desired (329 bp) product from these clones (data not shown) and we used it to confirm the identity of our clones. We further continued with the larger (~ 1100 bp) PCR product obtained by F3R3 primers. This PCR product was sequenced (size 1075 bp), named as AsHPX15 and its sequence were submitted to NCBI [GenBank: KP223285]. The nucleotide or predicted protein blast of AsHPX15 identified its closest match to *A. gambiae* AgHPX15. NCBI nucleotide blast of obtained sequences disclosed 89% query coverage, 76% identity and E value 3e-168 with *A. gambiae* HPX15 (AgHPX15).

Interestingly, the *A. stephensi* AsHPX15 specific F3R3 primers also amplified a similar size PCR product when *A. culicifacies* midgut cDNA was used as template (Figure 1C). The obtained clone was named as AcHPX15 and its sequence (1077 bp) was submitted to NCBI [GenBank: KP299257]. NCBI nucleotide blast of AcHPX15 sequences revealed first hit as AgHPX15 with 81% query coverage, 77% identity and E value 0.0. Predicted proteins for AcHPX15 and AsHPX15 also have a close orthology to each other (79% identity and 86% similarity). These results revealed that AgHPX15, AsHPX15 and AcHPX15 are identical orthologs.

AgHPX15, AsHPX15 and AcHPX15 have identical domains

To understand details regarding the sequence identity, structure and functional relationships, AgHPX15, AsHPX15 and AcHPX15 proteins were subjected to conserved domain database (CDD) analysis. Results depicted in Figure 2A revealed that all these peroxidases exhibit characteristics identity similar to the peroxinectin-like conserved domain of animal heme peroxidases superfamily. Peroxinectins are secreted as cell-adhesive and opsonic arthropod proteins that play crucial role in invertebrate immunity and interact with integrin family of transmembrane receptors [28]. Human myeloperoxidase (MPO) is also a member of this vast family and interacts with integrins [29,30]. Animal heme peroxidases and related proteins superfamily contains a diverse group of enzymes, including peroxidases from metazoans and their members are also found in fungi, plants and bacteria [31,32]. We also observed identical binding sites in the conserved domain of these three anopheline peroxidases. These sites are 10 heme binding, 14 putative substrate binding and 2 homodimer interface sites (Figure 2B).

Vectorial capacity	*Anopheles* spp. (abbreviation)	Ensembl identifier or gene bank identity	Retrieved contig	% nucleotide query coverage with AsHPX15 (KP223285)	% nucleotide Identity with AsHPX15 (KP223285)	Nucleotides (Amino acids) in trimmed sequence
Major vectors	*A. arabiensis* (Aar)	AARA008901	1.3480	96	75	1011 (337)
	A. atroparvus (Aat)	AATE013459	1.2435	90	70	934 (311)
	A. darling (Ada)	ADAC006384	7077	77	71	985 (335)
	A. dirus (Adi)	ADIR011596	1.4912	83	72	909 (303)
	A. farauti (Afa)	AFAF010327	2.1809	93	73	1015 (337)
	A. funestus (Afu)	AFUN008618	1.6688	98	76	995 (331)
	A. maculatus (Ama)	AMAM004556	1.2526	98	83	986 (328)
	A. sinensis (Asi)	Not known	012260	96	69	1009 (336)
	A. nili (Ani)	Not known	19203	84	71	915 (304)
	A. gambiae (Ag)	AGAP013327	-	89	76	1005 (335)
	A. culicifacies (Ac)	KP299257	1.17233	84	79	1059 (353)
	A. stephensi (As)	KP223285	5285	100	100	1075 (358)
Minor vectors	*A. albimanus* (Aal)	AALB010446	1.835	78	71	1050 (323)
	A. epiroticus (Aep)	AEPI000890	1.174	93	73	980 (326)
	A. melas (Amel)	AMEC000291	2.2419	96	74	995 (331)
	A. merus (Amer)	AMEM005191	2.5072	96	74	994 (331)
	A. minimus (Ami)	AMIN007008	1.3585	98	83	1009 (336)
Non-vectors	*A. quadriannulatus* (Aq)	AQUA003275	1.8077	96	75	1011 (337)
	A. christyi (Ach)	ACHR005516	1.2278	97	75	986 (328)

Table 1: List of putative HPX15 peroxidases retrieved from different species of *Anopheles* mosquitoes.
The putative HPX15 peroxidase contig (or Ensembl identifier) from the genome of different anophelines were retrieved using nucleotide sequences of AsHPX15 clone (GenBank:KP223285) as query. The overhanging sequences of the contigs were trimmed based on AsHPX15 sequences as mentioned in the Materials and Methods. The blast query coverage, identity of corresponding HPX15 contig with AsHPX15 clone and the nucleotides and total amino acids in trimmed sequences are presented in the table. The abbreviation for individual mosquito is also mentioned in parenthesis.

HPX15 is a unique anopheline-specific heme peroxidase

The CDD analysis revealed that the domain structure of AgHPX15, AsHPX15 and AcHPX15 is identical and they all are designated heme peroxidases (Figure 2). In vertebrates and invertebrates heme peroxidases catalyze protein crosslinking, which is generally a crucial process to stabilize the extracellular matrix [20,31]. The basic mechanism of peroxidase-mediated protein crosslinking is evident in *A. gambiae* [20]. Thus, we believe that AsHPX15 and AcHPX15 may also demonstrate a similar mechanism in Indian malaria vectors. Therefore, these heme peroxidases were further analyzed to understand their common features.

As we discussed before that the nucleotide and predicted protein general blasts of AsHPX15 and AcHPX15 revealed *A. gambiae* AgHPX15 as closest match. However, they do not have a considerable match with any peroxidase from other organisms. Thus, we hypothesized that HPX15 is a unique type of heme peroxidase and its orthologs may be present only in the genus *Anopheles*. To understand the novelty of anopheline HPX15 peroxidases, we used WAG model to reconstruct their phylogenetic relationship with other peroxidases as explained in Material and Methods. For this analysis, we selected heme peroxidases from numerous blood feeding and non blood feeding insects, human and plant (as mentioned in Table S1). Results presented in Figure 3 revealed that these peroxidases appear in two major clades. Each clade defines separate lineage for plants and animal heme peroxidases. The clade for animal heme peroxidases is further divided into 11 subclades that are designated based on *A. gambiae* peroxidase representative (this nomenclature was adopted from VectorBase database) in the cluster of that particular subclade. For example, AgHPX4, AgHPX3, AgDBLOX, AgHPX6, AgHPX5, AgHPX7, AgHPX8, AgHPX1, AgHPX16 and AgDUOX (Figure 3).

Among 16 *A. gambiae* heme peroxidases, AgHPX4 and AgDUOX reveal orthology to a wide range of animal heme peroxidases that include human EPO and MPO and peroxidases from *Caenorhabditis elegans*, mosquitoes and other insects. This shows the ubiquitous nature of these genes that have been conserved during the evolution through lower to higher organisms. Interestingly, AgHPX3 orthologs are present only in arthropod as well as *C. elegans*. AgHPX1, AgHPX5, AgHPX6, AgHPX7 and AgDBLOX are arthropod-specific heme peroxidases (Figure 3) and might have conserved function across insect species irrespective of their blood feeding or non blood feeding behaviors as observed in case of other genes [33]. These peroxidases might have been selected by gene duplications that occurred prior to the most recent common ancestor (MRCA) of Hemimetabolous (e.g., *Pediculus humanus,* Ph) and Holometabolous (e.g. mosquitoes) insects. AgHPX16 has no ortholog in other mosquitoes such as, *Aedes* and *Culex* however, its ortholog is present in *Drosophila.* AgHPX2 and AgHPX8 are mosquito-specific heme peroxidases and their orthologs are present in *Aedes* and *Culex* mosquitoes (Figure 3).

The above phylogenetic analysis also indicated the presence of lineage-specific mosquito heme peroxidases. Our findings revealed that there is a cluster of unique *Anopheles* heme peroxidases, which includes AgHPX10, AgHPX11, AgHPX12, AgHPX14 and AgHPX15. These *A. gambiae*-specific peroxidases do not have any ortholog in other arthropods that we analysed. Interestingly, AgHPX10 and AgHPX15 have their own paralogs named AgHPX11 and AgHPX14, respectively. However, the heme peroxidase AgHPX12 has no paralog even in *A. gambiae* (Figure 3). These unique peroxidases might have some crucial role in biology, physiology, geographical distribution or environmental adaptations of *Anopheles* mosquito as reported in case of other genes [33]. We emphasize that these peroxidases may be considered unique targets to synthesize blockers for regulating the biological activities of the malaria vector. However, the involvement of these peroxidases in the regulation of *Plasmodium* development needs further investigations and our group is actively engaged in that direction.

It is also evident from the phylogenetic analysis that AsHPX15, AcHPX15 and AgHPx15 are evolutionary closer orthologs (Figure 3). Thus, overall this data represents that HPX15 is a novel peroxidase

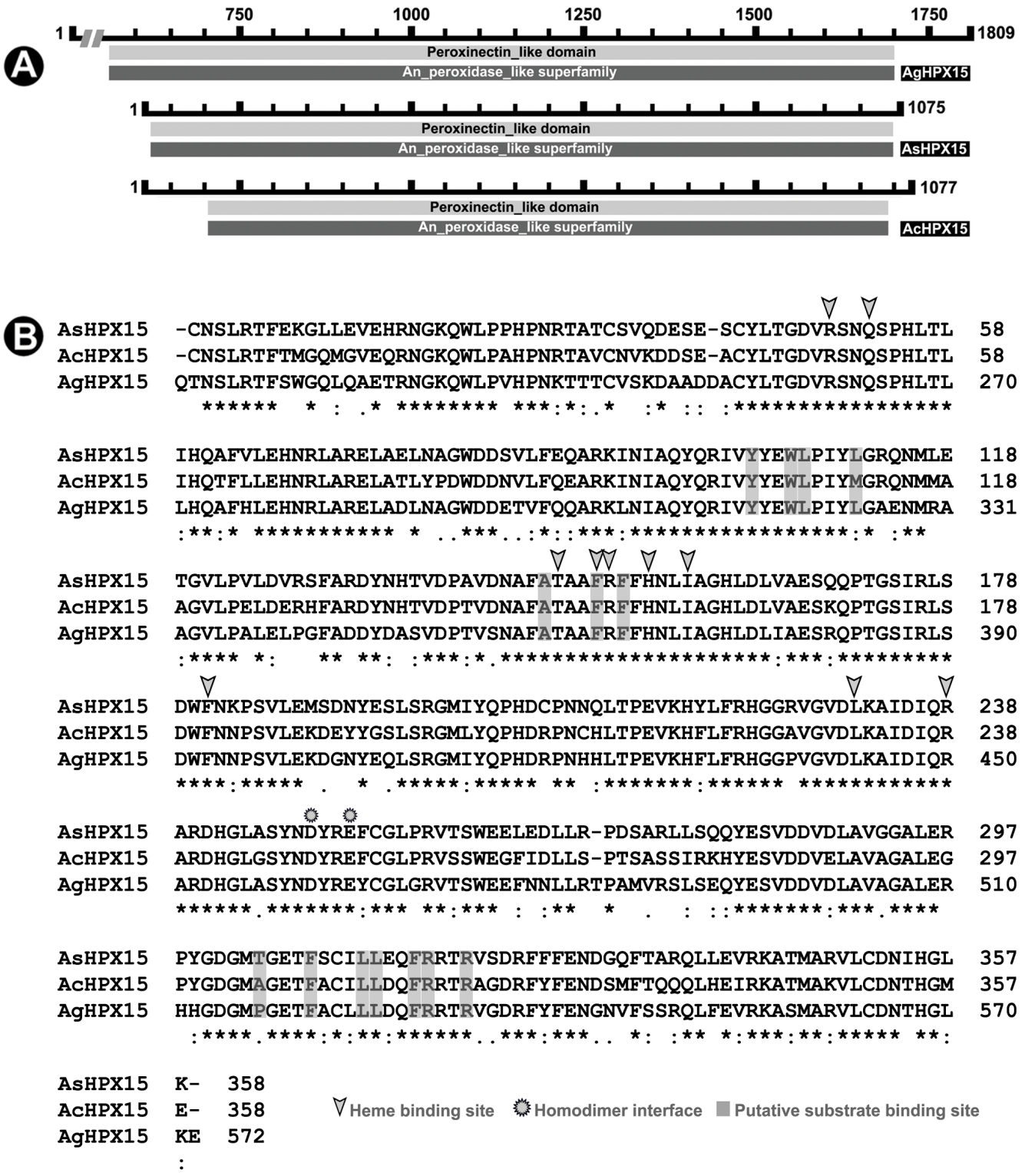

Figure 2: Analysis of conserve domains in AgHPX15, AsHPX15 and AcHPX15.
A) Signature domains in protein sequences of three heme peroxidases were determined through the Conserved Domain Database (CDD) search tool available online at NCBI. The number scale indicates amino acids in corresponding peroxidase proteins. Thick gray and black bars depict the peroxinectin-like conserved domain and animal heme peroxidases superfamily, respectively.
B) Numerous CDD predicted binding sites such as heme binding, putative substrate binding and homodimer interface sites are represented by the characteristic symbols in the aligned amino acids sequences of AgHPX15, AsHPX15 and AcHPX15.

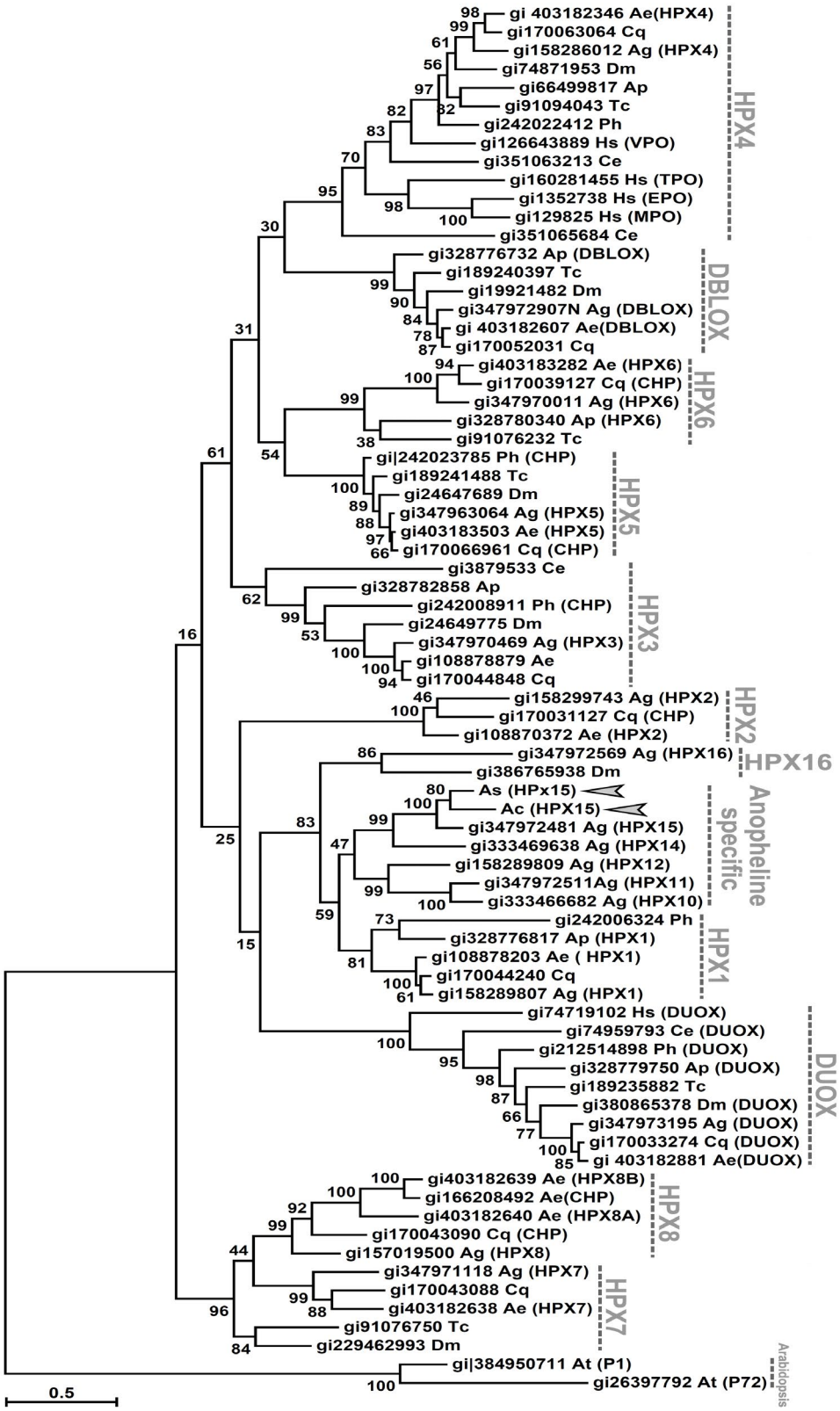

Figure 3: Phylogenetic analysis of selected heme peroxidases from various organisms

The Maximum likelihood (ML) method was used to construct the phylogenetic tree for 76 heme peroxidase (HPX) proteins selected from different organisms. The gene ID and abbreviations of organisms' scientific names have been mentioned in Table S1. Arrowheads indicate AsHPX15 and AcHPX15 clones. Vertical broken gray lines indicate specific clusters, which are named after the representative *A. gambiae* HPX. The scale bar represents base substitutions per site. The numbers on the branches represent the % of 1000 bootstrap.

that is present at least in three different anopheline mosquitoes (*A. gambiae, A. stephensi and A. culicifacies*) and does not have ortholgs in any other dipterans. Further, we analyzed the phylogenetic relationship of AsHPX15 and AcHPX15 with all heme peroxidase proteins of related mosquitoes (all belonging to family Culicidae). For that, we selected total 42 HPX proteins from the genome of *A. aegypti*, *C. quinquefaciatus* and *A. gambiae* (as mentioned in Table S1). These sequences were trimmed as mentioned before in the Materials and Methods and reconstructed a phylogenetic tree using the WAG model (Figure 4). Interestingly, the topology of this phylogenetic relationship is broadly similar to that of the corresponding subclade in the previously constructed phylogenetic tree (comparing Figure 3 and Figure 4). This phylogenetic analysis clearly demonstrated that out of 16 *A. gambiae* heme peroxidases 10 have their orthologs in *Aedes* and Culex mosquitoes. These heme peroxidases are AgHPX1, AgHPX2, AgHPX3, AgHPX4, AgHPX5, AgHPX6, AgHPX7, AgHPX8, AgDUOX and AgDBLOX. This topology supports that these orthologs are selected by gene duplications that occurred prior to the most recent common ancestor (MRCA) of family *Culicidae*. Importantly, 5 heme peroxidases that further exhibited anophelines lineage specificity are AgHPX10, AgHPX11, AgHPX12, AgHPX14 and AgHPX15 (Figure 4). AgHPX16 seems to be the most divergent *A. gambiae* heme peroxidase and does not have any known paralog. However, we observed its ortholog in *Drosophila* (Figure 3). In addition, the anophelines heme peroxidases also experienced a lineage-specific expansion as reported in other insects [33]. The phylogenetic relationship among 16 *A. gambiae* heme peroxidases and 8 non heme peroxidases revealed that both types of peroxidases appear in two different clades and have diverged from each other a long before (Figure S3). It is also noteworthy to mention that gene expansion and duplication is dominant in *A. gambiae* heme peroxidases. For example, AgHPX15 and HPX14 are in same cluster (99% bootstarp value), HPX12 is forming cluster with HPX10-HPX11 (98% bootstarp value), HPX7-HPX8 and HPX4-HPX5 are also appearing in their own clusters, respectively (Figure S3). These clusters represent gene duplication (origin of paralogs) in heme peroxidases and explain the expansion of HPX family in *A. gambiae*. However, AgHPX1, AgHPX2, AgDUOX, AgHPX16 and AgDBLOX do not reveal any characteristic duplication pattern (Figure S3).

HPX15 orthologs are present in the malaria vector and non vector species of *Anopheles* mosquitoes

Our previous phylogenetic analyses revealed that HPX15 orthologs are present at least in three major malaria transmitting species of *Anopheles* mosquito such as, *A. gambiae*, *A. stephensi* and *A. culicifacies* (Figures 3 and 4). The recent availability of whole genome shotgun sequences of 16 additional species of anopheline mosquitoes drew our attention to understand the universality of HPX15 in these worldwide-distributed *Anopheles* mosquitoes. Nucleotide blast of AsHPX15 clone against these shotgun sequences provided us the matching contigs (recently named as ensemble identifier as mentioned in Table 1). We found that all of these 16 species of *Anopheles* mosquitoes also have the putative HPX15 gene in their genome. Interestingly, in this comprehensive analysis of 19 above-mentioned anophelines (see Table 1) there are twelve major, five minor and two non malaria vector species as defined by others [34]. Thus, the presence of HPX15-like peroxidases in the genome of vectors and non vector species may not reveal its direct association with their vectorial capacity. We believe that HPX15 might have a general physiological role in these blood feeding vectors or non vector species of *Anopheles* mosquitoes and managing the phenomenon of 'immune balance in midgut' during food digestion as we reported before in case of *A. gambiae* [20]. This further warrants the detailed investigations of these mechanisms in other anophelines.

HPX15 proteins are highly conserved among anophelines

We compared the predicted protein sequences of all the sixteen HPX15 peroxidases to analyze their relative identity with AgHPX15, AsHPX15 and AcHPX15 proteins. The amino acids identity among these total 19 HPX15 peroxidases was found to be highly conserved (70-99%, Table 2). This range of percentage identity, confirmed that all these peroxidases are orthologs as described in case of other proteins [35].

In addition, we also analyzed the evolutionary relationship among these 19 species-specific peroxidase proteins. Results presented in Figure 5 revealed that the phylogenetic relationship of these nineteen HPX15 proteins follows a pattern similar to the evolutionary classification of these mosquito species as described by other researchers (Figure 1 of [34]). These findings explain that HPX15 is conserved among anopheline mosquitoes and might have evolved the same way as different species of *Anopheles* evolved. In parallel, the nucleotide based phylogenetic tree of these 19 peroxidases (using Tamura Nei model) also revealed similar branching pattern (data not shown).

In conclusion, HPX15 is a highly conserved anopheline lineage-specific peroxidase and present in the genome of globally distributed major/minor malaria vectors. These findings have great advantage to the society in terms of blocking the activity of this single molecule might interrupt the malaria cycle in those vectors.

Discussion

Heme peroxidases (HPXs) are found almost in all living organisms and generally catalyze the one- and two-electron oxidation of numerous organic and inorganic substrates. The redox cofactor is heme b or post translationally modified heme that is ligated to either histidine or cysteine residue. Four heme peroxidase superfamilies (peroxidase-catalase, peroxidase-cyclooxygenase, peroxidase-chlorite dismutase and peroxidase-peroxygenase) have differences in their enzymatic activities and also evolved independently [36].

In general, heme peroxidases play an important role in development and immunity. In vertebrates and invertebrates, heme peroxidases catalyze protein crosslinking that is a crucial process to stabilize the extracellular matrix. This crosslinking is a fundamental feature of basal membranes and essential as an elemental mechanism of tissue biogenesis [37,38]. Heme peroxidases are also extensively involved in the evolution and adaptations to the environment [33]. Thus, HPXs superfamily is considered to be a group of highly diversified members. Our phylogenetic analysis of the heme peroxidases (HPXs) from different phyla revealed the existence of two separate lineages of highly diversified HPXs, namely animals and plants (Figure 3). These results are supported by the previous reports where heme peroxidases are classified into two major families, namely the animal and non-animal peroxidases that include fungal and protist heme peroxidases [32].

Our phylogenetic analysis of 74 animal heme peroxidases (16 from *A. gambiae* and 58 from other animals) reveal that they appear in 11 different clades. Some of the *A. gambiae* heme peroxidases have their orthologs in other organisms, including human (Figure 3). For example, an extensively conserved HPX4 and DUOX clusters, which are originated by gene duplication events that occurred during the evolution of the kingdom Animalia. In addition, some of the *A. gambiae* heme peroxidases (e.g., HPX1, HPX3 and HPX5) are more close relatives of other arthropods and emerged by gene duplications that occurred prior to the most recent common ancestor (MRCA) of hemimetabola and holometabola in the late carboniferous period,

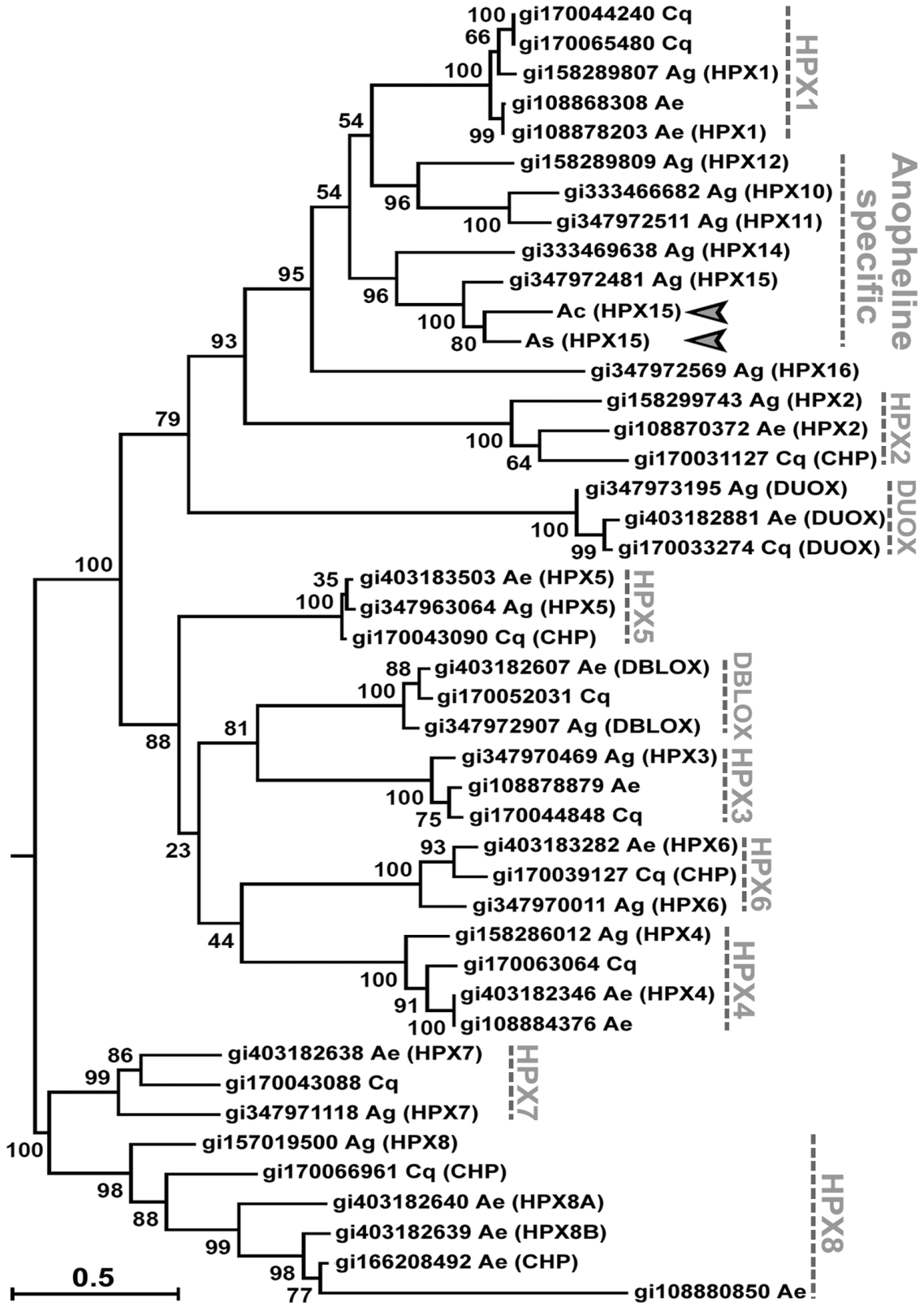

Figure 4: Phylogenetic analysis of mosquito's heme peroxidases. The ML method was used to construct the phylogenetic tree of 44 heme peroxidases selected from *Aedes aegypti, Culex quinquefaciatus* and *Anopheles gambiae* as discussed in Materials and Methods. The gene ID and organisms details are mentioned in Table S1. Arrowheads indicate AsHPX15 and AcHPX15 clones. Vertical broken gray lines indicate specific clusters, which are named after the representative *A. gambiae* HPX. The scale bar represents base substitutions per site. The numbers on the branches represent the % of 1000 bootstrap.

		AA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	**AarHPX15**	337		98.81	99.10	97.58	97.28	84.76	72.46	69.97	74.28	72.54	74.67	77.89	76.05	72.24	79.04	77.34	78.96	76.88	75.46
2	**AqHPX15**	337	98.81		99.10	97.58	97.28	84.76	73.19	70.27	74.28	71.94	74.01	77.56	76.35	71.64	79.34	77.64	78.66	76.58	74.54
3	**AgHPX15**	335	99.10	99.10		97.89	97.58	85.37	73.55	70.87	74.92	72.37	74.01	77.56	77.11	72.97	80.18	78.25	79.57	77.48	74.85
4	**AmerHPX15**	331	97.58	97.58	97.89		96.68	84.36	72.46	69.91	73.63	71.73	73.36	77.56	76.83	72.95	79.64	77.51	78.96	77.20	74.54
5	**AmelHPX15**	331	97.28	97.28	97.58	96.68		85.89	72.83	70.21	73.95	72.04	74.01	77.23	77.13	73.25	80.55	78.72	79.27	78.12	74.85
6	**AchHPX15**	328	84.76	84.76	85.37	84.36	85.89		75.82	72.87	75.32	73.78	73.42	76.33	77.06	74.09	80.18	80.79	79.38	76.22	79.57
7	**AalHPX15**	323	72.46	73.19	73.55	72.46	72.83	75.82		91.34	74.28	74.28	70.65	71.64	74.64	71.01	72.83	72.83	71.38	69.20	67.03
8	**AdaHPX15**	335	69.97	70.27	70.87	69.91	70.21	72.87	91.34		73.95	72.97	70.72	72.28	72.59	65.77	70.57	71.00	69.51	67.87	66.26
9	**AatHPX15**	311	74.28	74.28	74.92	73.63	73.95	75.32	74.28	73.95		87.46	77.96	76.79	76.45	70.42	74.60	73.31	73.31	72.67	67.85
10	**AsiHPX15**	336	72.54	71.94	72.37	71.73	72.04	73.78	74.28	72.97	87.46		76.97	77.23	75.22	66.67	72.54	72.21	73.48	71.77	67.18
11	**AniHPX15**	304	74.67	74.01	74.01	73.36	74.01	73.42	70.65	70.72	77.96	76.97		77.85	76.32	71.71	72.37	73.03	72.70	72.37	70.07
12	**AdiHPX15**	303	77.89	77.56	77.56	77.56	77.23	76.33	71.64	72.28	76.79	77.23	77.85		85.15	76.24	77.89	76.90	77.56	73.60	71.62
13	**AfaHPX15**	337	76.05	76.35	77.11	76.83	77.13	77.06	74.64	72.59	76.45	75.22	76.32	85.15		69.94	76.42	76.36	75.54	74.40	71.08
14	**AcHPX15**	337	72.24	71.64	72.97	72.95	73.25	74.09	71.01	65.77	70.42	66.67	71.71	76.24	69.94		85.12	79.76	81.40	75.08	71.78
15	**AmiHPX15**	336	79.04	79.34	80.18	79.64	80.55	80.18	72.83	70.57	74.60	72.54	72.37	77.89	76.42	85.12		86.71	85.98	81.08	73.31
16	**AfuHPX15**	331	77.34	77.64	78.25	77.51	78.72	80.79	72.83	71.00	73.31	72.21	73.03	76.90	76.36	79.76	86.71		82.62	80.66	72.70
17	**AmaHPX15**	336	78.96	78.66	79.57	78.96	79.27	79.38	71.38	69.51	73.31	73.48	72.70	77.56	75.54	81.40	85.98	82.62		87.50	73.01
18	**AsHPX15**	333	76.88	76.58	77.48	77.20	78.12	76.22	69.20	67.87	72.67	71.77	72.37	73.60	74.40	75.08	81.08	80.66	87.50		70.25
19	**AepHPX15**	326	75.46	74.54	74.85	74.54	74.85	79.57	67.03	66.26	67.85	67.18	70.07	71.62	71.08	71.78	73.31	72.70	73.01	70.25	

Table 2: Percentage amino acids identity among HPX15 peroxidases from nineteen different anophelines. The percentage identity among 19 different HPX15 peroxidase proteins was analyzed through their ClustalW alignments. The total amino acids (AA) used for analysis and the name of individual peroxidases (1 to 19) are also mentioned in the table. The protein sequences, gene ID and abbreviations of anophelines' scientific names are same as mentioned in Table 1.

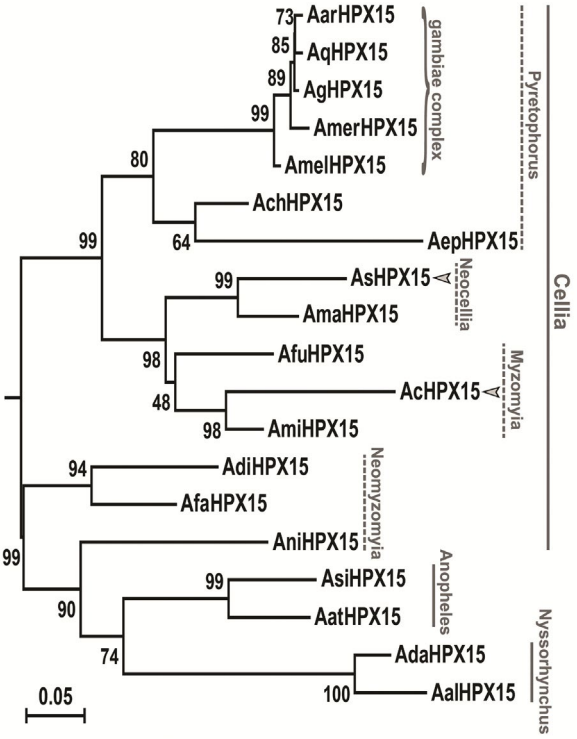

Figure 5: Phylogenetic analysis of putative HPX15 peroxidases from different species of *Anopheles* mosquitoes.
ML tree was constructed using heme peroxidase protein sequences from 19 different species of *Anopheles* mosquitoes. Details regarding HPXs nomenclature and anopheline species are mentioned in Table 1. Arrowheads indicate AsHPX15 and AcHPX15. Gray color solid vertical lines represent three sub genera (*Cellia, Anopheles* and *Nyssorhynchus*) of the genus *Anopheles*. Broken vertical lines represent series (Neocellia, Myzomyia, Neomyzomyia and Pyretophorus) of sub genus *Cellia*. The gambiae complex is the part of series Pyretophorus. The scale bar represents base substitutions per site. The numbers on the branches represent the % of 1000 bootstrap.

318-300 million years ago [39,40]. Thus, it is clearly evident that gene duplication event in heme peroxidase superfamily is crucial in the evolution of biological complexity and offers raw material that diverged under positive selection [41]. These events are critical during evolution, speciation and the birth of new life forms.

In *A. gambiae* there are 16 heme and 8 non heme peroxidases, which are evolutionary separated from each other (Figure S3). This indicates that in *A. gambiae* there is a large expansion of heme peroxidases and that may be associated with their geographical distribution or biological behaviors as reported in other organisms [33]. It is also noticeable that some lineage-specific heme peroxidses are present only in anophelines and do not have any ortholog in other animals including *Aedes* and *Culex* mosquitoes. These unique *Anopheles* heme peroxidases are HPX10, HPX11, HPX12, HPX14 and HPX15 (Figure 3). In this group, HPX12 has no paralog in *A. gambiae*. However, HPX10-HPX11 and HPX14-HPX15 are paralogs.

As we have mentioned before that heme peroxidases are important to perform numerous biological functions in living organisms including insects. *Anopheles* mosquito is a known vector for human malaria therefore; we were interested to identify those novel heme peroxidases in this insect that regulate *Plasmodium* development. Among these above-mentioned unique *A. gambiae* peroxidases, AgHPX15 demonstrated a general physiological role in the blood fed mosquitoes, which indirectly supports *Plasmodium* development [20]. Thus, we believe that AgHPX15 might be considered a potent target to block the malaria cycle in *A. gambiae*.

The advocacy of AgHPX15 as a potent candidate for blocking malaria parasite development in mosquito requires that two important issues should be addressed. Whether, HPX15 is also present in all the known species of *Anopheles*, which are considered to be major/minor malaria vectors. So this single molecule may be a central point of interest to target all these vector species. Secondly, how conserved this molecule is so that a common approach to block its activity will be enough to control *Plasmodium* development in all worldwide-distributed malaria vectors. To address the first issue, we provided evidences that AgHPX15 orthologs are present in major Indian malaria vectors, *A. stephensi* and *A. culicifacies* (Figure 1, 3 and 4). In addition, our phylogenetic analysis with the retrieved sequences from 16 additional vector as well as non-vector anophelines also proved that the putative HPX15 gene is present in all these species (Figure 5). It is interesting to note that HPX15 is present in both vector and non vector anophelines. This may be due to the reason that all these mosquitoes are hematophagous in nature and thus, HPX15 may be required to crosslink the midgut barrier around the ingested blood in a way similar to *A. gambiae* [20]. However, it warrants further analysis and understanding the correlation between the HPX15 expression and blood feeding in these mosquito species individually.

For developing blocking strategies, the polymorphism in the target molecules is also a hurdle as reported in other systems [42]. Thus, it is important to understand the genetic diversity in HPX15 orthologs as a general rule. The conserved domain (CD) analysis of AgHPX15, AsHPX15 and AcHPX15 provided the details about their identity and functional relationships (Figure 2). Moreover, we also found the same conserved domain architectures when we analyzed other 16 anopheline putative HPX15 protein sequences (data not shown). A further comparison of amino acids among total 19 HPX15 peroxidases also revealed 70-99% identity (Table 2). These observations strengthen our hypothesis that HPX15 can be a central plausible target and a common strategy may be applied to block its functioning to regulate the vectorial capacity of worldwide-distributed malaria vectors. Our previous double stranded (ds) RNA-mediated gene silencing experiments add in proof to this concept. Here the dsAgHPX15 RNA, prepared from *A. gambiae* cDNA, could silence HPX15 gene in both *A. gambiae* and *A. stephensi*. Moreover, the silencing-mediated effects on *Plasmodium* development were same in these mosquitoes (Figures 2 and S6 of [20]. These effects were probably due to the high (~ 75%) sequence identity between AgHPX15 and AsHPX15, which is evident now from the present study.

Recently a remarkable role of *A. gambiae* AgHPX15 gene is also reported in a study. Here this research group demonstrated that AgHPX15 induction in female spermathica is regulated by sexually transferred 20-hydroxyecdysone (20E), which is essential to preserve the functionality of stored sperm and long-term fertility [43]. Thus, targeting AgHPX15 will disrupt the reproductive cycle and numbers of mosquitoes in the field. These findings indicate that blocking the function of HPX15 will be beneficial in many ways and can be easily achieved as reported previously for other mosquito targets [44,45]. In these studies, a midgut-specific monoclonal antibody demonstrated a dose-dependent blocking effect against *P. yoelii* development in *A. stephensi*. Thus, we propose that the evolutionary conserved HPX15 protein in several anopheline mosquitoes can also be targeted in a similar way and may certify this molecule as a unique candidate to block mosquito cycle of *Plasmodium* development.

Conclusion

A lineage-specific heme peroxidase HPX15 is present in several globally distributed species of *Anopheles* mosquito. The highly conserved nature of HPX15 reveals that this molecule can be a potent target for blocking *Plasmodium* development. These findings may be a great help to fight against malaria, one of the world's deadliest diseases.

Acknowledgement

The authors wish to thank the laboratory staff for maintaining the insectory and central animal facilities (CAF) for providing all the supports. Special thanks to Divya Vaswani for providing critical comments. MK and KG are recipients of Basic Science Research (BSR) fellowship from the University Grants Commission (UGC), India. This work was supported by grants from the Department of Science and Technology (DST), Government of India (grant number SR/SO/HS-0131/2010).

References

1. World Health Organization. World malaria report (2014) Geneva: World Health Organization
2. National Vector Borne Disease Control Program (2013) National Drug Policy, NVBDCP, India.
3. Nagpal BN, Sharma VP (1995) Indian anophelines. New Delhi: Oxford & IBH Publishing Co. Pvt. LTD.
4. Subbarao SK (1998) Anopheline species complexes in South-East Asia. World Health Org Tech Pub 18: 82.
5. Shiff C (2002) Integrated approach to malaria control. Clin Microbiol Rev 15: 278-293.
6. Crompton PD, Moebius J, Portugal S, Waisberg M, Hart G, et al. (2014) Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. Annu Rev Immunol 32: 157-187.
7. Klein EY (2013) Antimalarial drug resistance: a review of the biology and strategies to delay emergence and spread. Int J Antimicrob Agents 41: 311-317.
8. Kelly-Hope L, Ranson H, Hemingway J (2008) Lessons from the past: managing insecticide resistance in malaria control and eradication programmes. Lancet Infect Dis 8: 387-389.
9. Mrema EJ, Rubino FM, Brambilla G, Moretto A, Tsatsakis AM, et al. (2013) Persistent organochlorinated pesticides and mechanisms of their toxicity.

Toxicology 307: 74-88.

10. Sreenivasamurthy SK, Dey G, Ramu M, Kumar M, Gupta MK, et al. (2013) A compendium of molecules involved in vector-pathogen interactions pertaining to malaria. Malar J 12: 216.
11. Barillas-Mury C, Kumar S (2005) *Plasmodium*-mosquito interactions: a tale of dangerous liaisons. Cell Microbiol 7: 1539-1545.
12. Dimopoulos G, Christophides GK, Meister S, Schultz J, White KP, et al. (2002) Genome expression analysis of *Anopheles gambiae*: responses to injury, bacterial challenge, and malaria infection. Proc Natl Acad Sci U S A 99: 8814-8819.
13. Luckhart S, Li K, Dunton R, Lewis EE, Crampton AL, et al. (2003) *Anopheles gambiae* immune gene variants associated with natural *Plasmodium* infection. Mol Biochem Parasitol 128: 83-86.
14. Rivero A (2006) Nitric oxide: an antiparasitic molecule of invertebrates. Trends Parasitol 22: 219-225.
15. Gupta L, Molina-Cruz A, Kumar S, Rodrigues J, Dixit R, et al. (2009) The STAT pathway mediates late-phase immunity against *Plasmodium* in the mosquito *Anopheles gambiae*. Cell Host Microbe 5: 498-507.
16. Meister S, Kanzok SM, Zheng XL, Luna C, Li TR, et al. (2005) Immune signaling pathways regulating bacterial and malaria parasite infection of the mosquito *Anopheles gambiae*. Proc Natl Acad Sci U S A 102: 11420-11425.
17. Povelones M, Waterhouse RM, Kafatos FC, Christophides GK (2009) Leucine-rich repeat protein complex activates mosquito complement in defense against *Plasmodium* parasites. Science 324: 258-261.
18. Jaramillo-Gutierrez G, Rodrigues J, Ndikuyeze G, Povelones M, Molina-Cruz A, et al. (2009) Mosquito immune responses and compatibility between *Plasmodium* parasites and anopheline mosquitoes. BMC Microbiol 9: 154.
19. Molina-Cruz A, DeJong RJ, Ortega C, Haile A, Abban E, et al. (2012) Some strains of *Plasmodium falciparum*, a human malaria parasite, evade the complement-like system of *Anopheles gambiae* mosquitoes. Proc Natl Acad Sci U S A 109: E1957-1962.
20. Kumar S, Molina-Cruz A, Gupta L, Rodrigues J, Barillas-Mury C (2010) A peroxidase/dual oxidase system modulates midgut epithelial immunity in *Anopheles gambiae*. Science 327: 1644-1648.
21. Oliveira Gde A, Lieberman J, Barillas-Mury C (2012) Epithelial nitration by a peroxidase/NOX5 system mediates mosquito antiplasmodial immunity. Science 335: 856-859.
22. Barik TK, Sahu B, Swain V (2009) A review on *Anopheles culicifacies*: from bionomics to control with special reference to Indian subcontinent. Acta Trop 109: 87-97.
23. Subbarao SK, Adak T, Vasnatha K, Joshi H, Raghavandra K, et al. (1988) Susceptibility of *Anopheles culicifacies* species A and B to *Plasmodium vivax* and *Plasmodium falciparum* as determined by immunoradiometric assay. Trans Royal Soc Trop Med Hyg 82: 394-397.
24. Adak T, Kaur S, Singh OP (1999) Comparative susceptibility of different members of the *Anopheles culicifacies* complex to *Plasmodium vivax*. Trans R Soc Trop Med Hyg 93: 573-577.
25. Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu S, Chitsaz F, et al. (2015) CDD: NCBI's conserved domain database. Nucleic Acids Res 43: D222-226.
26. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425.
27. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731-2739.
28. Sritunyalucksana K, Wongsuebsantati K, Johansson MW, Söderhäll K (2001) Peroxinectin, a cell adhesive protein associated with the proPO system from the black tiger shrimp, *Penaeus monodon*. Dev Comp Immunol 25: 353-363.
29. Hampton MB, Kettle AJ, Winterbourn CC (1996) Involvement of superoxide and myeloperoxidase in oxygen-dependent killing of *Staphylococcus aureus* by neutrophils. Infect Immun 64: 3512-3517.
30. Persson T, Andersson P, Bodelsson M, Laurell M, Malm J, et al. (2001) Bactericidal activity of human eosinophilic granulocytes against *Escherichia coli*. Infect Immun 69: 3591-3596.
31. Dunford HB (1999) Heme peroxidases. New York: John Wiley and Sons Inc.
32. Passardi F, Bakalovic N, Teixeira FK, Margis-Pinheiro M, Penel C, et al. (2007) Prokaryotic origins of the non-animal peroxidase superfamily and organelle-mediated transmission to eukaryotes. Genomics 89: 567-579.
33. Shi GQ, Yu QY, Zhang Z (2012) Annotation and evolution of the antioxidant genes in the silkworm, *Bombyx mori*. Arch Insect Biochem Physiol 79: 87-103.
34. Neafsey DE, Christophides GK, Collins FH, Emrich SJ, Fontaine MC, et al. (2013) The evolution of the *Anopheles* 16 genomes project. G3 (Bethesda) 3: 1191-1194.
35. Peterson ME, Chen F, Saven JG, Roos DS, Babbitt PC, et al. (2009) Evolutionary constraints on structural similarity in orthologs and paralogs. Protein Sci 18: 1306-1315.
36. Zámocký M, Hofbauer S, Schaffner I, Gasselhuber B, Nicolussi A, et al. (2015) Independent evolution of four heme peroxidase superfamilies. Arch Biochem Biophys 574: 108-119.
37. Péterfi Z, Geiszt M (2014) Peroxidasins: novel players in tissue genesis. Trends Biochem Sci 39: 305-307.
38. Kumar S, Barillas-Mury C (2005) Ookinete-induced midgut peroxidases detonate the time bomb in anopheline mosquitoes. Insect Biochem Mol Biol 35: 721-727.
39. Wiegmann BM, Trautwein MD, Kim JW, Cassel BK, Bertone MA, et al. (2009) Single-copy nuclear genes resolve the phylogeny of the holometabolous insects. BMC Biol 7: 34-47.
40. Hughes AL (2012) Evolution of the heme peroxisases of Culicidae (Diptera). Psyche 146387.
41. Ohno S (1970) Evolution by gene duplication. New York: Springer-Verlag.
42. Mahajan RC, Farooq U, Dubey ML, Malla N (2005) Genetic polymorphism in *Plasmodium falciparum* vaccine candidate antigens. Indian J Pathol Microbiol 48: 429-438.
43. Shaw WR, Teodori E, Mitchell SN, Baldini F, Gabrieli P, et al. (2014) Mating activates the heme peroxidase HPX15 in the sperm storage organ to ensure fertility in *Anopheles gambiae*. Proc Natl Acad Sci U S A 111: 5854-5859.
44. Dinglasan RR, Fields I, Shahabuddin M, Azad AF, Sacci JB Jr (2003) Monoclonal antibody MG96 completely blocks *Plasmodium yoelii* development in *Anopheles stephensi*. Infect Immun 71: 6995-7001.
45. Shahabuddin M, Cociancich S, Zieler H (1998) The Search for Novel Malaria Transmission-blocking Targets in the Mosquito Midgut. Parasitol Today 14: 493-497.