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ORIGINAL ARTICLE

Variant Ionotropic Receptors are Expressed in the Antennae of *Anopheles sinensis* (Diptera: Culicidae)

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Abstract

Mosquitoes transmit many harmful diseases that seriously threaten public health. The mosquito's olfactory system is of great significance for host selection. Inotropic receptors (IRs) and olfactory receptors (ORs) have been demonstrated to be capable of odorant molecular recognition. Analyzing the molecular principles of mosquito olfaction facilitates the development of prevention and therapy techniques. Advances in the understanding of IRs have been seriously inadequate compared to those of ORs. Here, we provide evidence that 35 *Anopheles sinensis* IR (AsIR) genes are expressed, 7 of which are in the antennae and 2 have expression levels that are upregulated with a blood meal. A homologous analysis of the sequences showed that AsIRs are a subfamily of ionotropic glutamate receptors (iGLURs). This is the first that time IRs have been identified in *Anopheles sinensis* in vitro. The ultrastructure of the antennae supports the theory that diverse sensilla are distributed in the antennae. The results here may facilitate the revelation of the regulation mechanism in AsIRs, which could mitigate the transmission of diseases by mosquitoes.

Keywords *Anopheles sinensis* · Disease · Ionotropic receptor · Mosquito · Olfactory system

Introduction

The evolution of an organisms' genetics is diverse and elaborate to accommodate the environment (Silva 2018). Certain genes can regulate insect resistance to pesticides, which impacts environmental protection, crops, and health. It is essential to

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develop novel transgenic crops and insecticides based on molecular understanding. Mosquitoes are generally considered to be harmful vectors of many diseases caused by viruses and parasites due to their blood-feeding behavior. *Anopheles sinensis* is a widely distributed Asiatic mosquito species that transmits some of the most prevalent human parasitic diseases, including malaria and lymphatic filariasis (Chang et al. 2014; Ree 2005). In addition, the species is highly susceptible to *P. vivax* (Lee et al. 2007) and has also increasingly been identified in a vectorial capacity in recent outbreaks of malaria in Asia (Shui et al. 2010). Traditional methods, such as insecticide-treated nets (ITNs) and indoor residual spraying (IRS), are challenged as mosquitoes develop resistance and inconstant behavior. Therefore, reducing mosquitoes contact with human hosts may provide another opportunity to control and prevent the transmission of infectious diseases. The genome of *Anopheles sinensis* was sequenced in 2014 (Dan et al. 2014), which provided a novel approach to reduce human malaria transmission.

Olfaction is a highly specific, intricate, and extraordinarily sensitive system that dominates food exploration, danger escape, and courtship of mosquitoes (Ab Majid et al. 2018; Takken 1991; Takken and Knols 1999). Hence, blocking or disrupting the mosquitoes' sense of smell is of current interest for its potential to restrict the spread of the diseases (Carey et al. 2010; Lu et al. 2007; Wang et al. 2010, 2009; Xia et al. 2008). Antennae, maxillary palps, and proboscis are the main olfactory appendages of mosquitoes, and the surface of these appendages is covered with olfactory sensilla that contain the dendrites of one or more odorant receptor neurons (ORNs) (Jacquin-Joly and Merlin 2004). Chemosensory stimuli are recognized and bound specifically by receptors on the membranes of the dendrites when diffusing through the pore channels that are located on the surface of sensilla. The stimulus signal is converted into an electrical signal and eventually passes to the nervous system to provide the appropriate instructions (Steinbrecht 1997). The receptors are considered to be the most significant molecular factor in the processes of olfactory events due to their particular role. In past decades, the role of the odorant receptors (ORs) and gustatory receptors (GRs) in defining the high selectivity of the ORN response to odor stimuli was elucidated (Pelosi et al. 2006; Su et al. 2009). Fifty functional ORs of *Anopheles gambiae* were tested against 110 odorants to elucidate how the odorant receptor repertoires of mosquitoes have adapted to suit their diverse ecological requirements. The research showed that all of the functional ORs have no signal evoke against ammonia and lactic acid, which are human kairomones (Carey et al. 2010). Several lines of evidence demonstrated that the coeloconic sensilla, a subset of olfactory sensilla without any ORs or GRs, in *Drosophila melanogaster* are tuned to acids, ammonia, and humidity. In contrast, basiconic and trichoid sensilla have been reported to express ORs (Yao et al. 2005). It is assumed that other chemoreceptors are likely responsible for olfactory sensitivity to ammonia and lactic acid (Liu et al. 2010). A novel family of chemosensory receptors called the ionotropic receptors (IRs) has been identified as being able to discern ammonia and carboxylic acids in *Anopheles gambiae* and *Drosophila melanogaster* (Benton et al. 2009; Pitts et al. 2017).

IRs were characterized for the first time in *Drosophila melanogaster*, facilitating the exploration of IRs in other insects and crustaceans, including mosquitoes, locusts, moth, wasp, and crabs (Ahmed et al. 2016; Bengtsson et al. 2012; Groh-Lunow et al. 2014; Groh et al. 2013; Guo et al. 2013). The expression and distribution pattern of IRs have been studied in the cells of coeloconic sensilla and their function may govern animal behavior (Guo et al. 2013). IR8a and IR25a are extensively expressed in the antennae of *Drosophila melanogaster* and are deemed to be co-receptors. In addition, two or three other IR genes were shown to be coexpressed with a co-receptor in each ORN (Benton et al. 2009; Guo et al. 2013). Advances in the understanding of olfactory signal transmission and IRs of *Anopheles sinensis* are far inferior in comparison with those of *Drosophila melanogaster*, *Anopheles gambiae*, and other mosquitoes (Chen et al. 2017).

Human volatile kairomones, carbon dioxide, 1-octen-3-ol, lactic acid, and ammonia, can be recognized by IRs, which play a crucial role in the process of locating host for mosquitoes (Braks et al. 2001; Allan et al. 2014; Cook et al. 2011; Costantini et al. 1996; Dekker et al. 2005; Takken et al. 1997). The blood meal-induced dynamic expression of chemosensory receptors may bring about regulation of odor sensitivity (Siju et al. 2010). It is highly necessary to decipher the relationship among IRs, blood meal, and human kairomones.

Here, we scrutinize the antennae ultrastructures of adult female *Anopheles sinensis* in China. Coeloconic sensilla structures are categorized, and their distributions are reported here. Thirty-five *Anopheles sinensis* IR genes have been ascertained, 7 of which have been illustrated to be expressed in the female antennae. The quantitative reverse transcription PCR analyses indicate that some of the 2 IRs are immediately involved in blood meals.

Materials and Methods

Mosquito Rearing

Anopheles sinensis originated from the Center for Disease Control of Hunan Province (China). The standard mosquito-rearing process was performed as published previously (Chen et al. 2017). We used 5- to 7-day-old blood-fed and non-blood-fed females for the experiments (Choumet et al. 2012). For stock propagation, 4- to 5-day-old female mosquitoes were blood-fed for 60 min on anesthetized mice.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy (Hitachi S-4200, Japan) was performed following protocols as described (Pitts and Zwiebel 2006; Schymura et al. 2010). Briefly, the heads from 4- to 6-day-old adult *Anopheles sinensis* were fixed with 4% paraformaldehyde and 0.1% Triton X-100 in phosphate-buffered saline (PBS). Heads were then dehydrated with an ethanol series from 50 to 100% in 10% increments, followed by ethanol: hexamethyldisilazane (HMDS) at 75:25, 50:50, 25:75, and 0:100. HMDS was decanted, and the heads were dried in a fume hood. The heads were then glued onto aluminum pin mounts with colloidal silver paint and were sputter coated for

30 s with gold–palladium. The samples were viewed using SEM, and the digital micrographs of each flagellomere were collected by Quartz PCI version 6.0 image acquisition software.

Identification and Sequence Analysis of AsIRs

A detailed screening of AsIRs was conducted as described (Chen et al. 2017). Candidate IR sequences were identified in the *Anopheles sinensis* (AsIR) genome with the website of www.vectorbase.org, and *Drosophila melanogaster* IR (DmIR)and *Anopheles gambiae* IR (AgIR) amino acid sequences were analyzed as tBLASTn and BLASTp queries, respectively (Supplementary Table S1). The DmIR and AgIR amino acid sequences were cited from previous publications (Croset et al. 2010; Pitts et al. 2017). The potential exon–intron gene models were predicted based on their homology to DmIRs and AgIRs. For the AsIRs structures, a domain annotation was added according to the DmIRs definitions reported previously (Abuin et al. 2011). The selected AsIR proteins were aligned by ClustalX 7. A structural analysis of the identified AsIRs was performed by a Hidden Markov Model-based gene structure predictor (www.Softberry.com).

Amplification of the AsIR Genes

The total RNA was extracted from frozen female antennae using TRIzol reagent (Invitrogen, Germany), and the cDNA was synthesized using the Smarter Race cDNA Amplification Kit (Takara, Japan) according to the instruction booklet. The antennal cDNA was used as a template in the PCR as previously described (Fox et al. 2001). PCR primers specific for AsIRs were designed by DNA MAN (Supplementary Table S2). The amplified fragments were inserted into a plasmid pMD-18T (Invitrogen, Germany) and were sequenced by Takara to guarantee their authenticity.

Quantitative Reverse Transcription PCR

Approximately forty female mosquitoes were divided into two groups, and were fed with either anesthetized mice or glucose for 1 h. Fifteen pairs of antennae from each group were collected for RNA extraction 5 h later. cDNA preparation and reverse transcription were accomplished as mentioned above. The success of qRT-PCR likely counted on the appropriate primers designed by DNA MAN (Supplementary Table S3). We chose β-Actin as a reference so that relative quantification was possible. LightCycler 480 SYBR Green I Master (Roche, Germany) was used to execute qRT-PCR with LightCycler 480. qRT-PCR was performed with three parallel samples for three group replicates. The results of the analysis were obtained by LightCycler 480 software 1.5 with a $2^{-\Delta\Delta CT}$ method. The relative significant differences were revealed by Student's *t* test ($p < 0.05$).

Results

Scanning Electron Microscopy (SEM)

At present, the research on mosquitoes has mainly concentrated on morphology, living habit, hazard, prevention, and control. There are scarcely any reports about the sensilla of the antennae of *Anopheles sinensis*. We observed the antennae by SEM to make explicit distribution and styles of the sensory receptor, which will allow the elucidation of relevance between the receptors and olfactory behavior (Fig. 1). *Anopheles sinensis* generally has three kinds of sensory organs: antenna, maxillary palp, and proboscis (Fig. 1a). Antenna, the primary olfactory chemoreceptor, is filamentous with 13 flagella. The antennae sensilla are composed of chaetica, trichoid, basiconic, and coeloconic sensilla (Fig. 1b, c).The length of trichoid sensilla ranges approximately between 400 μm and 500 μm, while the length of basiconic sensilla is approximately 100 μm. The coeloconic sensilla are considered to be the uppermost site for AsIRs (Fig. 1d).

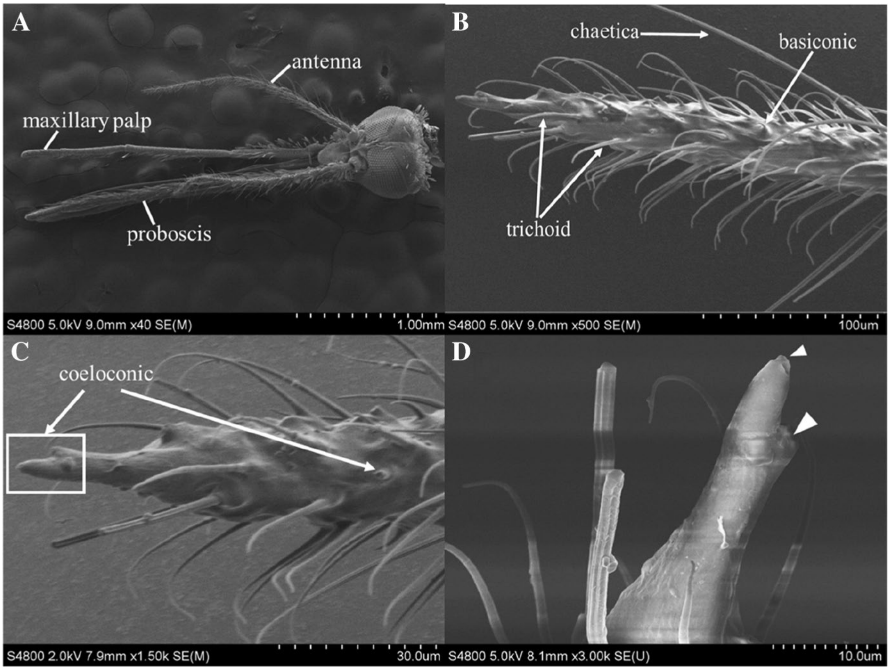

Fig. 1 Tactile organ morphologies of *Anopheles sinensis* by SEM. Each tactile system consists of an antenna, maxillary palp and proboscis. Chaetica, trichoid, basiconic, and coeloconic sensilla are the four main types of sensilla in the antenna. Coeloconic sensilla that contain AsIRs are shown in C and D. Scale bars, 10 μm

Identification of IRs from the *Anopheles sinensis*

HMMER of VectorBase was utilized to identify the unknown insect ionotropic glutamate receptor genes. As used in previous research to reveal *Aedes albopictus* IRs, the most effective evaluations were obtained with a combination of HMMER, BLASTp, and tBLASTn (Chen et al. 2017). We used *Drosophila melanogaster* IR (DmIR) and *Anopheles gambiae* IR (AgIR) protein sequences as queries with an E value less than 0.01. Thirty-five putative IRs were identified in total (Supplementary Table S1), of which two co-receptors, AsIR8a and AsIR25a, existed. The normative nomenclature method was applied to name these AsIRs (Croset et al. 2010; Liu et al. 2010). IR gene names consisted of a 4-letter species abbreviation, of which an uppercase first letter of the genus came to be the initial one. The three lowercase letters of species served as the rest (*Anopheles sinensis* = *Asin*). We referred to the *Anopheles sinensis* ionotropic receptors (*AsinIRs*) to AsIRs for convenience. The AsIRs, orthologues with DmIRs, would be named the same. A point and a number would be added when there were several copies of an orthologue gene. There was no novel species-specific AsIR observed in our study. Same numbers and different final letters featured the multiple paralogues of DmIRs.

Molecular Phylogenetic Tree of IRs

Molecular phylogenetic tree of IRs was constructed to demonstrate the evolutionary progress among *Drosophila melanogaster*, *Anopheles gambiae*, and *Anopheles sinensis* (Fig. 2). We used the neighbor joining method with a 1000 bootstrap value by MEGA 6 (Tamura et al. 2011). Most of the 35 IRs showed sequence diversity, with the exception of the highly conserved IR8a and IR25a.

Expression and Structural Characteristics of AsIRs in *Anopheles sinensis* Antennae

We designed specific primers for these 35 putative AsIRs, which ensured that the PCR products lengths were approximately 500 bp with an intron region included. Non-specific amplification of the genomes can be eliminated by sequence. The RT-PCR data and DNA agarose gel electrophoresis indicated that 7 (AsIR8a, AsIR25a, AsIR140, AsIR143, AsIR144, AsIR7l, and AsIR75k) of the 35 putative AsIRs could be expressed in female antenna (Fig. 3a).

The amino acid structures of the putative 7 AsIRs were predicted and aligned by ClustalX 7 and the Hidden Markov Model-based gene structure predictor (www.Softberry.com). Similar to other olfactory receptors, insect IRs conform to the topology of an extracellular N-terminus and intracellular C-terminus. AsIR amino acids sequences contain an extracellular ligand binding domain (LBD) and a transmembrane domain (TM) that consists of 4 highly conserved hydrophobic regions: M1, a pore loop (P), M2, and M3 (Fig. 3b). These extraordinary structures allow AsIRs to

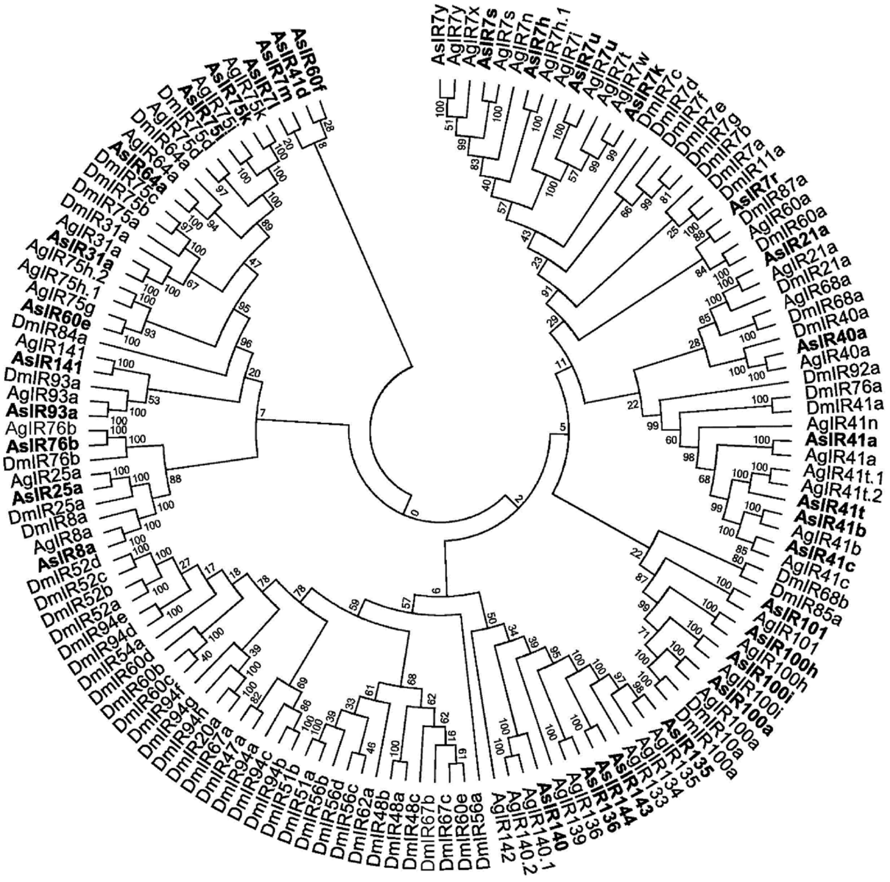

Fig. 2 A phylogenetic tree of IRs among *Drosophila melanogaster*, *Anopheles gambiae*, and *Anopheles sinensis*. The bold items are 35 putative AsIRs that share a high level of homology with AgIRs and a distant homology with DmIRs. The tree was constructed using neighbor joining with 1000 bootstrap replicates

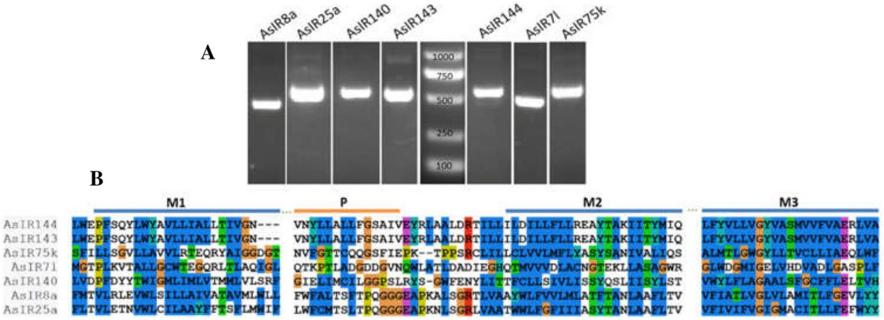

Fig. 3 Gene and amino acid sequence analysis of 7 AsIRs. **a** DNA agarose gel electrophoresis detection of 7 AsIR genes. The bright bands indicate specific expression. **b** All 7 AsIRs have 4 hydrophobic domains, M1, P, M2, and M3, which carry out the function of ion channels

function as ion channels. IR8a and IR25a were highly conserved among those three species while other IRs were more diverse.

qRT-PCR

The factors that affect the ability of mosquitoes to find the host have always been attractive in the insect research field. ORs and IRs are considered to be the most significant proteins in recognizing and binding odorant molecules. One of the purposes in this study is to illuminate the molecular mechanism of the AsIR gene expression regulation by a blood meal that is closely related to the host orientation. We acquired the AsIR expression profiles in female antennae before and after blood feeding (Fig. 4). The expression levels of two AsIRs (AsIR140 and AsIR75k) were demonstrated to be apparently upregulated, while the other AsIRs were stable after blood feeding. AsIR140 and AsIR75k might play crucial roles in finding the blood meal host and oviposition place. The expression levels of co-receptors AsIR8a and AsIR25a were not affected by the blood meal, which was in agreement with previous studies (Rinker et al. 2013).

We demonstrated that a blood meal can upregulate the expression level of some AsIRs, which provided novel thoughts for the principles of blood meal host selection.

Discussion

IRs, a subfamily member of iGLUR, can regulate the insect olfactory system to control blood meal host selection and oviposition. We identified 35 IRs in *Anopheles sinensis* by sequence alignment with *Drosophila melanogaster* and *Anopheles gambiae*.

The antenna is the primary sensillum for insects. This study shows the ultrastructure of antennae of *Anopheles sinensis* in China, which is in accordance with

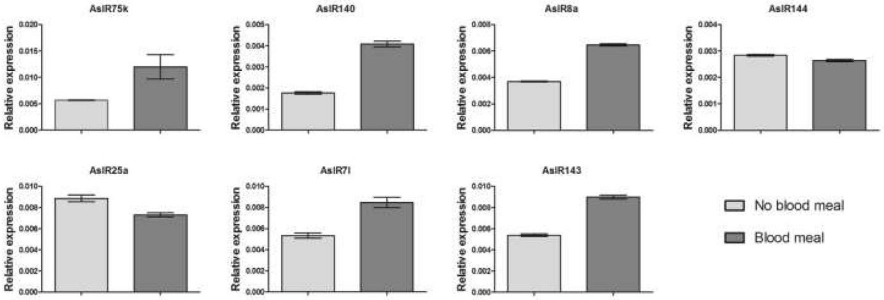

Fig. 4 Quantitative reverse transcription PCR analyses of the expression of 7 AsIRs with or without a blood meal. The gray column is the expression level without a blood meal; the black column is the expression level with a blood meal. AsIR75k and AsIR140 are significantly upregulated, whereas the others maintain stability after treatment with a blood meal

previous study. (Hempolchom et al. 2017). AsIR proteins are mainly expressed in coeloconic sensilla, which is consistent with reports on *Aedes aegypti* by Gingl and his colleagues, which proved that thermoreceptive neurons are distributed in coeloconic sensilla (Gingl et al. 2005). In addition, trichoid and basiconic sensilla are also widely expressed in antennae. *Anopheles gambiae and Anopheles quadriannulatus* have extra ampullaceal sensilla, in comparison to *Anopheles sinensis*, which is probably derived from a phylogenetic process (Pitts and Zwiebel 2006). There is little difference in the appearance and density of sensilla among those three species, which indicates that IRs may be more significant to host selection.

Taking into consideration incomplete gene annotation, tissue-specific expression, and temporal specificity, this study documents the expression and identification of 7 AsIRs that are in accordance with the characteristics of iGLUR. Among them, AsIR8a and AsIR25a are co-receptors similar to ORCO in *Drosophila melanogaster*, and carry high homology between the two species (Benton et al. 2009). IRs and ORs in *Drosophila melanogaster* and *Anopheles gambiae* have been intensively studied, while studies of these receptors are limited in *Anopheles sinensis* (Liu et al. 2010). The results revealed that AsIR8a and AsIR25a were expressed extensively in the whole antenna with a stable level of expression.

qRT-PCR has been implemented to elucidate the expression level with or without the stimulus of a blood meal. Indeed, host selection-related gene expression can be upregulated or downregulated after blood meal (Bonizzoni et al. 2011; Taparia et al. 2017). DmIR75a and DmIR75d were demonstrated to be able to detect propionic acid. The olfactory sensory neurons containing DmIR75b and DmIR75c were shown to respond to propanol and propionic acid (Benton et al. 2009). The function of IR140 has not been elucidated before. It is the first report to characterize AsIR140 and AsIR75k in vitro in *Anopheles sinensis*. Our results suggested that these two upregulated genes may regulate blood-induced physiological response. The knockout or overexpression of AsIR140 and AsIR75k is likely to disturb the insect and prevent malaria and dengue disease. Furthermore, responsive inotropic receptors can be employed in olfactory bionic sensors. A potential therapeutic strategy could be explored based on these IRs. Further experiments are needed to characterize the functional domain and three-dimensional structure to unveil the molecular mechanism of these AsIRs.

Conclusions

The antennae of female *Anopheles sinensis* in China have four typical sensilla, chaetica, trichoid, basiconic, and coeloconic, that carry similar morphology to sensilla in other mosquitoes. In summation, this study demonstrated that there was a significant ≥ twofold upregulation in AsIR140 and AsIR75k, while no significant difference occurred in the other 5 AsIRs, which mean the two AsIRs may regulate blood meal-related physiological response of mosquitoes. Moreover, this work established a systematic protocol for the screening and analysis of blood meal-related genes in various mosquitoes. Our analysis of the IRs expressed in *Anopheles sinensis* may support further exploration of IRs and their potential application in controlling the transmission of malaria. Here, we lay the foundation for further epidemic prevention and impetus research in olfaction-related sensors.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflicts of interest.

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