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Identification of One Putative D7 Related Gene Specifically Expressed in Female *Anopheles anthropophagus*

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Abstract: *Anopheles anthropophagus* is an important mosquito vector transmitting malaria and *Brugia malayi*. Due to differences in feeding behaviors between the female and male adult *An. anthropophagus*, female *An. anthropophagus* plays a more important role in transmitting pathogens than male ones. The present study identified a gene specifically expressed in female *An. anthropophagus* from a subtractive cDNA library for female *An. anthropophagus* and its full-length cDNA was amplified by 3' and 5' Rapid Amplification of cDNA Ends (RACE) technique. This gene was named fsyz and further analyzed by using Northern blot and bio-informatics analyses. It was shown that the fsyz could encode a D7 related salivary protein and was female-specifically expressed and not characterized previously worth further studies.

Key words: *Anopheles anthropophagus*, RT-PCR, RACE, Northern blot, sequence analysis, salivary protein

INTRODUCTION

Mosquitoes transmit many human diseases including malaria, filariasis, dengue fever, encephalitis and others. Among these, the malaria was estimated to kill at least 1-2 million persons year^{-1} (Tangpukdee *et al*., 2009). Mosquito-borne diseases represent an important global public health problem.

Mosquito control, through environmental perturbation and pesticide application used to be the primary strategy for controlling mosquito-borne diseases (Roberts *et al*., 2000; Phillips-Howard *et al*., 2003; Lengeler, 2004; Mabaso *et al*., 2004). However, these traditional approaches have been limited by environmental and human health concerns as well as the development of pesticide resistance (Hemingway *et al*., 2002; Gong *et al*., 2005; Tao *et al*., 2006). Therefore, the tools of molecular biology and genomics are being applied to explore new avenues of research in vector biology such as constructing genetically modified mosquitoes (Moreira *et al*., 2002; Benedict and Robinson, 2003; Fu *et al*., 2007).

Anopheles anthropophagus is an important mosquito vector transmitting malaria and *Brugia malayi*. A recent molecular study indicates that *An. anthropophagus* in China is a synonym of *Anopheles lesteri* in Japan (Hwang *et al*., 2006).

For convenience and consistency with previous studies, researcher used *An. anthropophagus* in the present study. Due to differences in feeding behaviors between the female and male adult *An. anthropophagus*, female *An. anthropophagus* plays a more important role in transmitting pathogens than male ones. Studying gender-specific genes in *An. anthropophagus* could help us to reveal the genetic background behind these mosquitoes' pathogen-transmitting mechanisms and further explore more effective approaches for controlling mosquito-borne diseases.

In the previous study, a subtractive cDNA library for female *An. anthropophagus* was constructed by using Suppression Subtractive Hybridization (SSH) and 139 valid ESTs were obtained. Of these ESTs, six were firstly identified by using semi-quantitative Reverse Transcription-coupled Polymerase Chain Reaction

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(RT-PCR) but no further characterization of genes represented by these six ESTs were documented (Geng *et al*., 2009). In the present study, one EST (GenBank Accession No. EX916955) was chosen and the gene it represented was cloned by 3' and 5' Rapid Amplification of cDNA Ends (RACE) technique and it was further characterized by Northern blot, bio-informatic and phylogenetic analyses.

MATERIALS AND METHODS

Mosquito colony and their maintenance: *An. anthropophagus* mosquitoes were obtained from a laboratory colony maintained at the Zhengzhou Center for Disease control and prevention, Henan province, China. The colony was maintained in a rearing room at 25-26°C, 60-70% humidity with a 12 h light/dark cycle. Adults were maintained on 10% sucrose solution and females were blood-feed on rats which were fixed on a board.

RNA extraction: Total RNA was prepared from adult females and males separately by using the Trizol reagent (Invitrogen) by following the manufacturer's protocols.

3' and 5' RACE: The SMART™ RACE cDNA Amplification kit (Clontech) was used to perform 3' and 5' RACE for the EST348 expressed specifically in female *An. anthropophagus*. For 3' RACE, the first-strand cDNA was synthesized from 1 μg total RNA by using the MMLV reverse transcriptase and 3'-CDS primer A provided by the manufacturer. Target cDNA was then amplified by using the Universal Primer A Mix (UPM) contained in the kit (Long primer: 5'-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT-3', short primer: 5'-CTA ATA CGA CCA CTA TAG GGC-3') and a forward gene-specific primer (5'-GTC GAC AAG GAT GGC CGA GGA GAT T-3') based on the sequence of the female-specific EST348. The PCR amplification conditions were 5 cycles of 94°C for 30 sec, 72°C for 3 min; 5 cycles of 94°C for 30 sec, 70°C for 30 sec, 72°C for 3 min; 29 cycles of 94°C for 30 sec, 68°C for 30 sec, 72°C for 3 min. For 5' RACE, first-strand cDNA was synthesized from 1 μg total RNA by using the MMLV reverse transcriptase, 5'-CDS primer A and SMART II A oligo provided by the manufacturer. Target cDNA was then amplified by using the UPM and a reverse gene-specific primer (5'-GAA CGA CTC TGC CGA ATT GGA CTG C-3'). The PCR amplification conditions were as above. After that the 3' and 5' RACE products were ligated into pGEM®-T Easy vector (Promega), transformed into competent *Escherichia coli* strain DH5α and four positive clones representing each 3' and 5' RACE products were sequenced by Shanghai Sangon bio-technology using the Applied bio-systems 3730 DNA analyzer.

Northern blot analysis: The female-specific gene forward primer 5'-CGA TAG TCG CCA TTG TGC-3' and reverse primer 5'-CGG CTG CCT TCA TTT GGT-3' were designed to amplify a 301 bp fragment. The *actin* gene was chosen as control because of its high intensity and consistency of expression in all mosquitoes tested (Salazar *et al*., 1994) and the forward primer 5'-CTC ACG CTG AAA TAC CCG AT-3' and reverse primer 5'-TGT GGT GGT GAA CGA GTA GC-3' were designed according to the *actin* gene sequence of *Anopheles gambiae* (Accession No. U02930) to amplify a 417 bp fragment. Using them, the Digoxigenin (DIG)-labeled cDNA probes were prepared according to the DIG DNA Labeling and detection kit (Roche) following the manufacturer's recommendations.

Total RNA extracted from adult females and males was separately mixed with 2×RNA loading dye (95% formamide, 0.025% SDS, 0.025% bromophenol blue, 0.025% xylene cyanol FF, 0.025% ethidium bromide, 0.5 mM EDTA). The mix was heated for 10 min at 70°C then placed on ice for 3 min. Total 40 μg of the denatured total RNA were separated by electrophoresis on a 1% denature agrose gel (0.41 M formaldehyde, 1×Mops-EDTA-sodium acetate). Then the RNA was transferred for 4 h onto a 0.45 μm Immobilon-Ny+charged Nylon membrane (Millipore) with upward capillary action in 20×SSC followed by immersion in 6×SSC and UV cross-linking (Spectroline).

Hybridization was carried out overnight at 50°C with constant rotation (UVP) and was followed by stringent washes and immunological detection by using the DIG DNA Labeling and Detection kit (Roche) according to manufacturer's protocol.

Sequence analyses and phylogenetic reconstruction: The sequences of 5' and 3' RACE products were edited and assembled using DNAStar. Similar sequences were identified by performing BLAST searches online (http://www.ncbi.nlm.nih.gov/BLAST). The deduced amino acid sequence was further analyzed and aligned with similar sequences by using Clustal X (Thompson *et al*., 1997). Phylogenetic relationship of mosquitos was reconstructed by Neighbor Joining (NJ) within the MEGA 4.0 (Tamura *et al*., 2007) using Jukes-Cantor as substitution model. Branch supports in NJ tree was estimated by bootstrap analysis of 1000 replicates. *Drosophila melanogaster* was used as outgroup.

RESULTS AND DISCUSSION

Obtaining the full length gene sequence by 3' and 5' RACE-PCR: As the EST348 was just a part of the gene, obtaining the whole gene sequence was very important for further studying its biological function. RACE technique was proved to be a classic method to generate

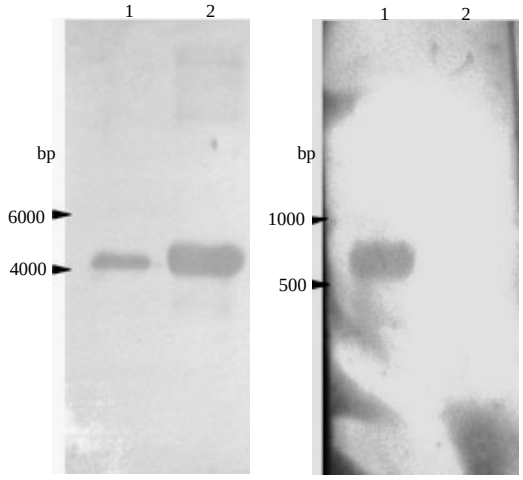

Fig. 1: Northern blot analysis of *Anopheles anthropophagus* female-specific *fsyz* gene (right) and *actin* gene (left). Lanes 1 and 2 represent the total RNA from female and male *An. anthropophagus*, respectively

full cDNA and widely applied in various organisms (Park *et al*., 2001; Bellatin *et al*., 2002; He *et al*., 2010; Lu *et al*., 2010). By using RACE-PCR, the 3' and 5' RACE products for the EST348 were amplified. Sequencing revealed that the 5' RACE sequence was 465 bp in length while the 3' RACE sequence was 442 bp in length. Then the 3' and 5' RACE sequences were edited and assembled and the full-length mRNA was composed of 629 nucleotides including a 36 nt 5' Untranslated Region (UTR), a 92 nt 3' UTR and a 501 nt Open Reading Frame (ORF) which encoded 166 amino acids with a calculated molecular weight of 18.632 kDa and a predicated isoelectric point (pI) of 8.37. The full-length nucleotide sequence was deposited in the GenBank™ database and an accession number FJ907237 was obtained. The gene it represented was named fsyz.

Northern blot: Northern blot analysis was carried out to characterize the female-specific nature of the *fsyz* gene expressed in female *An. anthropophagus*. The result showed that the fsyz cDNA probe hybridized specifically to total female RNA only with blot position between 500-1000 bp while the actin cDNA probe hybridized to both male and female total RNA with blot position of 4000-6000 bp (Fig. 1).

Sequence comparison and phylogenetic analysis: Similar sequences were identified by performing BLAST searches in the GenBank™. A mutiple sequence alignment of the deduced amino acids of the *fsyz* gene ORF with other relevant sequences was shown in Fig. 2. The translated ORF of *An. anthropophagus fsyz* gene had 66, 62, 60, 60, 56, 56, 55, 52, 50, 42, 37, 30 and 20% identities with the short form D7 salivary protein 1 of *Anopheles funestus*, the short form D7 salivary protein D7r4 of *An. funestus*, the D7-related 1 protein of *An. gambiae*, the short form D7r1 salivary protein of *Anopheles arabiensis*, the short form D7clu5 salivary protein of *Anopheles stephensi*, the contact-activation-inhibitor protein hamadarin of *An. stephensi*, the D7-related 1 protein of *An. stephensi*, the short form D7clu4 salivary protein of *An. stephensi*, the D7r4 protein of *An. gambiae*, the D7-related 3.2 protein of *Anopheles darlingi*, the D7r2 salivary protein of *Anopheles dirus* B., the D7 protein of *Culex quinquefasciatus* and the D7 protein, putative of *Aedes aegypti*, respectively (Fig. 2). For further analyzing, the evolutionary relationships of the *fsyz* gene with other D7 related proteins of Anopheles mosquitos, Aedes mosquitos and Culex mosquitos, phylogenetic reconstruction was performed. Researchers observed that three main mosquito branches could be distinguished and the fsyz was more closely-related to the short form D7 salivary protein 1 of *An. funestus*, the D7-related 1 protein of *An. gambiae*, the short form D7clu5 salivary protein of *An. Stephensi* and the short form D7r1 salivary protein of *An. Arabiensis* than to other D7 proteins (Fig. 3).

An. anthropophagus is important in the transmission of malaria and *Brugia malayi* in China and several other Asian countries. Despite efforts of previous studies on this mosquito, the knowledge of this mosquito relating to its transmission of malaria and/or *Brugia malayi* remains little. That only female *An. anthropophagus* sucks blood and transmits pathogens suggests that studying female-specific genes in *An. anthropophagus* could help us reveal the genetic background underlying this mosquito's pathogen-transmitting mechanism. SSH technique was a simple and efficient method to generate substractive cDNA libraries of differentially expressed genes (Park *et al*., 2001; Bellatin *et al*., 2002; Luo *et al*., 2004; Raibaud *et al*., 2006). Using this technique, the colleagues identified 139 ESTs expressed in female *An. anthropophagus* but no one was further characterized. Here, we characterized one of these ESTS, namely the EST348 and we obtained its full length gene sequence which was essential for further studying its biological function and we named it as fsyz.

In the present study, the whole cDNA sequence of the *fsyz* gene was obtained by using RACE. Both previous semi-quantitative RT-PCR and Northern blot analyses herein confirmed that the *fsyz* gene was expressed specifically in female *An. anthropophagus*. Besides, Northern blot also indicated the length of fsyz mRNA was

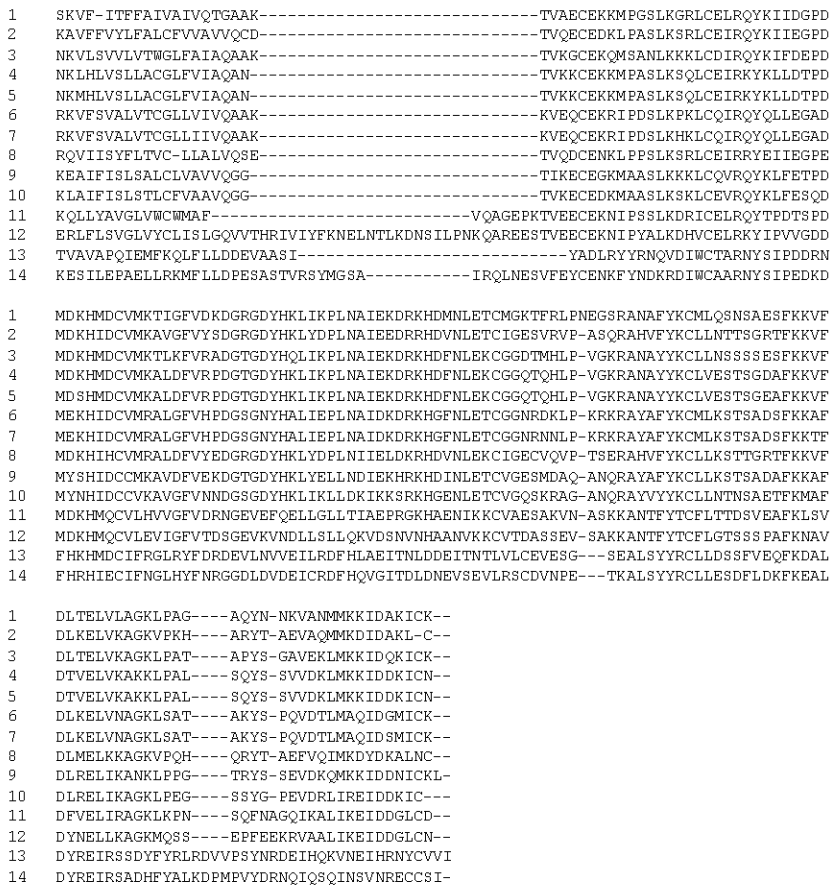

Fig. 2: Alignment of deduced amino acid sequence of ORF of the *Anopheles anthropophagus* female-specific *fsyz* gene with those of related sequences available in the GenBank™. Number 1 represents the fsyz protein, numbers 2-14 represent the short form D7 salivary protein D7r4 of *A. funestus* (ABI83753), the short form D7 salivary protein 1 of *A. funestus* (ABI83754), the D7-related 1 protein of *A. gambiae* (CAB39727), the short form D7r1 salivary protein of *A. arabiensis* (AAL16039), the short form D7clu5 salivary protein of *A. stephensi* (AAL16045), the contact-activation-inhibitor protein hamadarin of *A. stephensi* (AAM12343), the D7r4 protein of *A. gambiae* (CAC35524), the D7-related 1 protein of *A. stephensi* (CAC70633), the short form D7clu4 salivary protein of *A. stephensi* (AAL16044), the D7-related 3.2 protein of *A. darlingi* (AAL29441), the D7r2 salivary protein of *A. dirus* B. (AAP68775), the D7 protein of *C. quinquefasciatus* (XP_001848430) and the putative D7 protein of *A. aegypt*i (XP_001662172), respectively

about 500-1000 bp which was accordant with the result of RACE-PCR. Sequence comparison and phylogenetic analysis revealed the *fsyz* gene was a previously uncharacterized gene in *An. anthropophagus* and it is very likely that this gene encodes a D7 related salivary protein. D7 salivary protein family is abundantly expressed in blood-feeding Diptera and two subfamilies of the long and short D7 proteins exist in mosquitoes. It was revealed that D7r1-D7r4 from *An. gambiae* and long D7 from *A. aegypt* were found to bind the biogenic amines serotonin, norepiephrine and histamine but D7r5 from *An. gambiae* did not bind biogenic amines (Calvo *et al*., 2006). Because the process of blood clotting, the maintenance of vascular tone and inflammatory responses are all regulated to some extent by biogenic amines, it will interfere the immune reaction of hosts by injecting D7 related proteins which counteract the biogenic amines when biting and this will facilitate the blood-feeding. So, does the predicated protein fsyz have the ability of binding biogenic amines?

By further amino acids sequence comparison and phylogenetic analysis, the fsyz was found to be more closely-related to the short form D7 salivary protein 1 of *A. funestus* with 66% identity and the D7-related 1 protein of *A. gambiae* with 60% identity. This indicates that it may have the ability of binding biogenic amines. Functional characterization of the *fsyz* gene is warranted in the future studies.

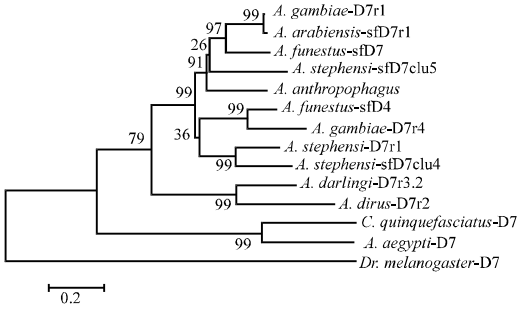

Fig. 3: Inferred phylogenetic relationship among mosquitoes reconstructed based on sequences of D7 related gene using Neighbour Joining (NJ) method. The number along branches indicates bootstrap values. *Drosophila melanogaster* was used as outgroup

CONCLUSION

In this study, using RACE-PCR, Northern Blot and bio-informatics analyses, the present study cloned a gene (*fsyz*) specifically expressed in female *An. anthropophagus*. The *fsyz* gene may encode a D7 related salivary protein which is very important in the process of blood feeding by facilitating the blood feeding, worth further studies.

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