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# Molecular cloning, sequence analysis and developmental stage expression of a putative septin gene fragment from *Aedes albopictus* (Diptera: Culicidae)

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**Septins belong to GTPases that are involved in vital cellular activities, including cytokinesis. Although present in many organisms, they are yet to be isolated from *Aedes albopictus*. This study reports for the first time on a serendipitous isolation of a partial septin sequence from *Ae. albopictus* and its developmental expression profile. The *Ae. albopictus* partial septin sequence contains 591 nucleotides encoding 197 amino acids. It shares homology with several insect septin genes and has a close phylogenetic relationship with *Aedes aegypti* and *Culex quinquefasciatus* septins. The *Ae. albopictus* septin fragment was differentially expressed in the mosquito's developmental stages, with an increased expression in the adults.**

**Key words**: *Aedes albopictus*, cytokinesis, developmental stages, phylogenetic analysis, septin

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## INTRODUCTION

Septins are a group of P-loop guanosine triphosphate (GTP) binding proteins that were first discovered in the budding yeast *Saccharomyces cerevisiae* (Hartwell, 1971), with several homologues now identified in many animals and yeasts, but currently absent in higher plants (Pan *et al.*, 2007; Nishihama *et al.*, 2011). They form oligomeric filaments which polymerize into large paired septin filaments to further form higher order structures (Sirajuddin *et al.*, 2007; DeMay *et al.*, 2011). Septins are involved in cytokinesis (Hartwell, 1971), cytoskeletal regulation and membrane remodeling (Kinoshita, 2006; Berepiki & Read, 2013). Aberrations in septin genes have been implicated in several diseases such as cancer, male infertility and Parkinson disease (Peterson & Petty, 2010; Saarikangas & Barral, 2011).

Most studies on insect septins have focused on the fruit fly, *Drosophila melanogaster*, as a model organism, to understand the function of these genes. Although septins have been identified in some insects, at the time of this study none had been identified in the Asian tiger mosquito, *Aedes albopictus*. The identification of septins in this mosquito is essential due to the important roles they play; such discovery could provide useful information on the biology of this mosquito. *Aedes albopictus* is a vector of such diseases as dengue and chikungunya, and thus is a focus of chemical control. One of the ways in which it is able to survive insecticide applications is to metabolize the chemical compounds. Here, whilst trying to amplify a major insecticides metabolic enzyme, cytochrome P450 from *Ae. albopictus*, a septin gene fragment was serendipitously amplified. This gene was cloned and its phylogenetic relationship was inferred. The expression of this gene in the life stages of the mosquito was also studied. This is the first report on the cloning and expression of a septin gene fragment from *Ae. albopictus*.

## MATERIALS AND METHODS

**Extraction of total RNA and cDNA synthesis**. Total RNA was extracted from the 4th instar larvae (30 mg) of *Ae. albopictus* obtained from the Vector Control Research Unit, Universiti Sains Malaysia using the RNeasy® Mini Kit (Qiagen®). Reverse transcription of 2 μg of total RNA using the M-MuLV Reverse Transcriptase and Oligo $(dT)_{18}$ from First strand cDNA synthesis kit (Fermentas®) was done at 37°C/1 h, followed by incubation at 70°C/5 min. The manufacturers' instructions of the products used in this study were followed unless stated otherwise.

**Polymerase Chain Reaction (PCR) and cDNA cloning**. The forward (5'-GCGGTGGAAAATATGATTGCGCTG-3') and reverse (5'-TTTTTTTTTTTTTTTTTTTTTTTTGTTTCAT-3') primers were initially designed to flank the coding region of a putative cytochrome P450 gene. A 50 μl reaction mixture contained 25 μl of OneTaq®2X Master Mix with standard buffer (New England Biolabs®), 1 μl of each of the primers (10 μM), 4 μl of cDNA and 19 μl of nuclease free water. The cycling reaction was as follows: 94°C/3 min, 7 cycles of 94°C/30 s, 42°C/30 s and 72°C/1 min, and then 30 cycles at 94°C /30 s, 60°C/30 s and 72°C/ 1 min, and finally 72°C/10 min. The product was analyzed on a 1% agarose gel and the fragment of interest was excised and purified using the Gel/PCR DNA Fragments Extraction Kit (Geneaid®). The DNA purified from the gel band was ligated into a pGEM®-T Easy Vector (Promega®) and transformed into chemically competent *Escherichia coli* JM 109 cells (Chung *et al.*, 1989). The cells were plated onto Luria-Bertani (LB) agar containing 100 μg/ml ampicillin, 100 μl of 0.1 M isopropyl-β-D-thiogalactopyranoside (IPTG) and 40 μl of 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and incubated overnight at 37°C. Single white

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**Abbreviations**: LB, Luria-Bertani; PCR, polymerase chain reaction; RT-qPCR, real time-quantitative PCR

colonies were selected and cultured overnight at 37°C, 180 rpm in 3 ml LB broth containing 100 μg/ml ampicillin. Plasmids were extracted using the High-Speed Plasmid Mini Kit (Geneaid®) and digested with EcoRI (Promega®) to confirm the insert DNA. Undigested plasmids from clones with confirmed inserts were sequenced with SP6 and T7 universal primers.

**Sequence and phylogenetic analysis**. The nucleotide sequences were translated using the ExPASy translate tool (http://web.expasy.org/translate/) and used for Basic Local Alignment Search Tool (BLAST®) searches in the National Center for Biotechnology Information (NCBI) database. Phylogenetic analysis was done with comparison to other insect septins in Molecular Evolutionary Genetics Analysis version 5 (MEGA5) (Tamura *et al.*, 2011).

**Quantification using Real Time-quantitative PCR (RT-qPCR)**. Expression of the septin gene fragment in the life stages of the mosquito (the four larval stages, pupae and 2-day old adults) was studied to observe the stage-specific patterns. All the adult mosquitoes were non-blood fed. Total RNA was extracted as described earlier with the RNeasy® Mini Kit (Qiagen®) and DNase-treated using the RNase-free DNase set (Qiagen®). cDNA for RT-qPCR was synthesized based on 1 μg of total RNA obtained from each life stage, using the iScript™ Reverse Transcription Supermix (Bio-Rad®). Target-specific primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and analyzed with OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html). The primers: 5'-AGATCCGCGAGTTGGAAGAC-3' (forward) and 5'-AAGTGACGCGGTCTCTTTGC-3' (reverse) were used to amplify a 134 bp septin amplicon. The reference genes: *β-actin* (GenBank accession no. DQ657949) and ribosomal protein l8 (*rpl8*) (GenBank accession no. M99055), amplification condition and detection procedures were as described in Avicor *et al.* (2014). A 120 bp *β-actin* amplicon was amplified using 5'-AGAAGGAAATCACCGCCCTG-3' and 5'-GCTGGAAGGTGGATAGCGAG-3', whilst a 187 bp *rpl8* was amplified with 5' TTGGGGGTGTTTTGGATCGC-3' and 5'-GGCTCCTCGGGAAAGAACAC-3' as forward and reverse primers respectively (Avicor *et al.*, 2014). Quantification was conducted in a CFX96™ Real Time system (Bio-Rad®) with a reaction solution containing 5 μl of iQ™ SYBR Green Supermix (Bio-Rad®), 0.5 μl of each of the 10 μM gene specific primers, 1 μl of cDNA and 3 μl of nuclease free water. The cycling conditions were as follows: 95°C/3 min, 40 cycles of 95°C/15 s and 57°C/30 s, with a plate read at the end of each amplification cycle (Avicor *et al.*, 2014). Immediately after the entire amplification reaction, a melting curve analysis was conducted ranging from 55–95°C with an increase of 0.5°C/10 s per step.

Serial dilution of cDNA was used to construct standard curves for assessing the reaction efficiency for each of the gene specific primer pairs. The relative transcription ratio was compared to the control calibrator (4th instar larvae) and computed (Pfaffl, 2001) after normalization with the reference genes. Fourth instar larvae were used as the control because the gene was cloned from this stage of the mosquito's development. At least three independent biological and technical replicates were done for each sample. The data was analyzed with a one-way analysis of variance using Genstat® Release 9.2 (Payne *et al.*, 2006) and means were separated using the least significant difference.

## RESULTS AND DISCUSSION

### Analysis of partial septin gene sequence

After the pioneering work of Hartwell (1971), septins have been identified from diverse eukaryotes, with the exception of higher plants (Pan *et al.*, 2007; Nishihama *et al.*, 2011), although prior to this work there was no septin identified in *Ae. albopictus*. The primers used in this study were designed to amplify a cytochrome P450 gene from *Ae. albopictus* but fortuitously amplified a septin gene fragment which has been deposited at the GenBank database (accession no. KF483526). The *Ae. albopictus* partial septin gene sequence is 591 nucleotides long, encodes 197 amino acid residues (Fig. 1), and contains the consensus GTPase G1 motif sequence GXXXXGK[S/T] (Pan *et al.*, 2007). It shares a high nucleotide identity with septins from other insects, notably the *Aedes aegypti* (94%) (GenBank accession no. XM_001653540.1) and *Culex quinquefasciatus* (87%) (GenBank accession no. XM_001841948.1) mosquitoes, and *D. melanogaster* fruit fly (75%) (GenBank accession no. NM_165597.1). It is also homologous to proteins of insect septins, sharing 97% amino acid identity with an *Ae. aegypti* partial septin sequence (GenBank accession no.XP_001653590.1), and 94% with a *Cx. quinquefasciatus* septin (GenBank accession no. XP_001842000.1), as well as 69–80% identical with septin proteins from *D. melanogaster* (GenBank accession nos. AAA19603.1 and NP_477064.1) and the *Acromyrmex echinatior* (GenBank accession no. EGI59097.1), *Camponotus floridanus* (GenBank accession no. EFN62648.1) and *Harpegnathos saltator* (GenBank accession no. EFN75751.1) ants.

### Phylogenetic tree

The phylogenetic relationship of the *Ae. albopictus* partial septin sequence was compared with other insect septins which were downloaded from the GenBank database and aligned by ClustalW (Gap opening penalty 10, Gap extension penalty 0.2, Delay divergent cutoff 30%), and the alignments were used to draw a neighbor-joining tree (Saitou & Nei, 1987) with 1 000 bootstraps (Felsenstein, 1985) (Fig. 2). The phylogenetic tree showed that the partial septin sequence of *Ae. albopictus* and septins of *Ae. aegypti* (partial gene sequence) and *Cx. quinquefasciatus* were closely related. It is inferred from the tree that

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cggaacatggttatccggacgcacctgcaggacctcaaggatgtcaccaacaacgttcac  60
 R  N  M  V  I  R  T  H  L  Q  D  L  K  D  V  T  N  N  V  H   20
tacgaaaactatcgctgtcgaaagctcgccggtttgggtaacgacggaaagaccaaactg 120
 Y  E  N  Y  R  C  R  K  L  A  G  L  G  N  D  G  K  T  K  L   40
agcaacaagaacccactggcccagatggaggaggaaaagcgcgggcacgaatccaagatg 180
 S  N  K  N  P  L  A  Q  M  E  E  E  K  R  G  H  E  S  K  M   60
aagaaaatggaagcagagatggaacaggtgttcgagatgaaagtcaaggaggagaagcag 240
 K  K  M  E  A  E  M  E  Q  V  F  E  M  K  V  K  E  E  K  Q   80
aaactgaaggactccgaagccgaactcaccagaaggcacgaggagaggaaaaaggctttg 300
 K  L  K  D  S  E  A  E  L  T  R  R  H  E  E  R  K  K  A  L  100
gagtttcagatccgcgagttggaagaccgccgaaaagcgttcgaaatcgagaaggccgag 360
 E  F  Q  I  R  E  L  E  D  R  R  K  A  F  E  I  E  K  A  E  120
tgggaacaacagaacggagtcacgctcgaagagctccggcgtaagagcctcgaggcgaac 420
 W  E  Q  Q  N  G  V  T  L  E  E  L  R  R  K  S  L  E  A  N  140
agcaaagagaccgcgtcacttgcatcaagaagttccgatgagtccaagggcaggcgcgtg 480
 S  K  E  T  A  S  L  A  S  R  S  S  D  E  S  K  G  R  R  V  160
tttggatcgctgctgcgtaggcacacgagcttcggggcccccgataccatgcgggcttcg 540
 F  G  S  L  L  R  R  H  T  S  F  G  A  P  D  T  M  R  A  S  180
ggaccttccactactaccactactaccagcgcaatcatattttccaccgca          591
 G  P  S  T  T  T  T  T  T  S  A  I  I  F  S  T  A           197
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**Figure 1. Nucleotide and deduced amino acid sequences of putative partial septin gene sequence from *Aedes albopictus*.** The G1 motif is in bold and underlined.

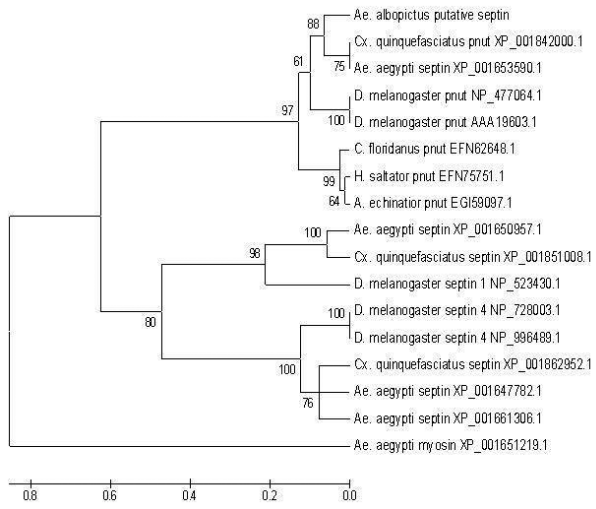


**Figure 2. Phylogenetic relationship of *Aedes albopictus* partial septin gene sequence with other insect septin genes (accession no. on the extreme right).**
The phylogenetic tree was inferred using the neighbor-joining method. *Aedes aegypti* myosin was used as an outgroup. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

the *Ae. albopictus* partial septin sequence is evolutionary closer to *pnut* septin gene than the other septin genes. The *pnut* gene was the first *Drosophila* septin gene to be discovered and is involved in cytokinesis (Neufeld & Rubin, 1994). Defects in this gene have lethal consequences (Neufeld & Rubin, 1994; Adam *et al.*, 2000). In *Drosophila*, *pnut* null mutant larvae had a significantly reduced cell number, with multinucleated cells in such tissues like the imaginal discs and brain, and died after pupation (Neufeld & Rubin, 1994). Mutant embryos without maternal and zygotic *pnut* also developed abnormal actin cytoskeleton during the cellularization stage of embryogenesis and morphological defects during gastrulation (Adam *et al.*, 2000).

## Constitutive expression pattern

Septin expression in the life stages of the mosquito was significantly different, with an increased expression in the adult stages when compared to the immature stages ($p < 0.05$, Fig. 3). The expression pattern among the immature stages was not significantly different. The highest expression of the septin gene fragment was detected in the 2-day old female, and this was not significantly different from the 2-day old male ($p > 0.05$, Fig. 3). Detection of the septin fragment in the developmental stages of *Ae. albopictus* is not surprising due to their well known cell division activities (Neufeld & Rubin, 1994), which could be involved in transiting from one developmental stage to the other, particularly in the production of new cells associated with their metamorphic processes. The increased expression in the adult could be linked to adult-stage related functions of septin genes, such as oogenesis and spermatogenesis (Hime *et al.*, 1996; O'Neill & Clark, 2013). Since septins play important developmental and structural roles in eukaryotic organisms, and are associated with physiological and morphological abnormalities when altered, the identification and elucidation of their roles in individual species could provide valuable biological information for the development of less fit individuals to control mosquito population.

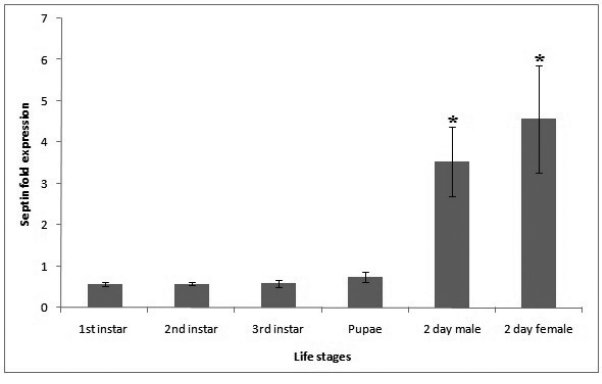


**Figure 3. Relative expression (± standard error) of septin fragment in *Aedes albopictus* at different life stages.**
The expression was normalized against β-actin and rpl8 and compared to the control (4th instar larvae). Asterisks represent a significant increase in the expression of the gene.

In conclusion, a partial septin gene sequence was isolated from *Ae. albopictus* and its phylogeny with other insect septins was inferred. This gene was differentially expressed in the developmental stages of *Ae. albopictus,* with an elevated expression in the adult stage, which was not sex-specific. This is the first report on the isolation of a putative septin gene fragment from *Ae. albopictus*. It is a major step that could be used for further studies to elucidate the cellular and physiological roles of *Ae. albopictus* septins and their impact on mosquito biology and control.

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