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A preliminary report on DNA barcoding and phylogenetic relationships of certain public health important mosquito species recorded in rural areas of south India

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Biological research depends on proper identification and classification of species. Species were identified and classified by morphological key-based method. However, the conventional morphology-based taxonomical methods have many demerits. The inherent limitations of the conventional taxonomical identification methods indicate the need for a new and simple method of taxon identification. Mosquito remains the major contributor of causing nuisance and transmitting deadly pathogens to human. Correct identification and classification of mosquito vector is the central part to the study of vector-borne disease surveillance and control. The conventional morphology-based identification methods are time consuming and not always sufficient to identify at the species level. Therefore, a multidisciplinary approach including morphological and molecular techniques is essential.

DNA barcode technique has been found promising in the rapid description of biodiversity[1]. DNA barcoding works by amplifying a short stretch of a single gene of mitochondria of the animal kingdom. The technique has also been used in the identification of sibling species of mosquitoes[2].

The present work describes the use of DNA barcoding method in molecular identification of certain public health important mosquito species recorded in Tirupuvanam block villages of Sivagangai district, Tamil Nadu. During 2007–08, adult mosquitoes were collected from Tirupuvanam block villages of Sivagangai district, Tamil Nadu as per the method described by Pandian *et al*[3]. Adult mosquitoes were brought to the laboratory and identified as per the standard taxonomical keys[4–7]. Total genomic DNA was extracted from the whole mosquito species by using the commercially available DNA extraction kit (M/s. Genei Pvt Limited, Bengaluru) as per the manufacturer's instructions. PCR was carried out as described by Collins *et al*[8] in an PTC-100 Thermal cycler (M/s MJ Research, USA). The primer sets were selected to amplify approximately 500 base pair (bp) amplimer in the *COI* gene. The amplicon was visualized and documented in a gel documentation unit (M/s Vilber Lourmet, France) and were purified using commercially available Millipore purification kit as per the manufacturer's instructions. Custom DNA sequencing (M/s MWG, Bengaluru) was carried out for certain public health important mosquito species (*Aedes aegypti*, *Ae. albopictus*, *Culex tritaeniorhynchus*, *Cx. quinquefasciatus*). Basic local alignment of sequence test was used to find out homogenous sequences and to confirm the species. Sequence divergences among the species were studied using Kimura two-parameter distance model[9]. MEGA software was used for the phylogenetic analysis[10]. The sequences were submitted to the NCBI GenBank and the accession numbers are as follows:

FJ372982 – *COI* gene of *Ae. aegypti*
FJ372983 – *COI* gene of *Ae. albopictus*
FJ372984 – *COI* gene of *Cx. tritaeniorhynchus*
FJ372985 – *COI* gene of *Cx. quinquefasciatus*

In this study, the primers successfully produced an approximately 545 bp amplicon from all the mosquito species studied (Fig.1). DNA sequencing was carried out only for four mosquito species of public health importance, i.e. *Ae. aegypti* and *Ae. albopictus,* the vectors of dengue and chikungunya virus; *Cx. tritaeniorhynchus* the vector of Japanese encephalitis virus and the *Cx. quinquefasciatus,* vector of lymphatic filariasis. The results of the BLAST search in the Genbank data base have shown that the test DNA sequences were found to have >98% sequence homology with the representative species.

Further, phylogenetic analysis with MEGA 4 showed that the Tirupuvanam *Ae. aegypti* strain was found clustered along with ae ASAP 34503, ae ASAP 34003, ae Liverpool, Red eye strain, etc (Fig. 2). Interestingly,

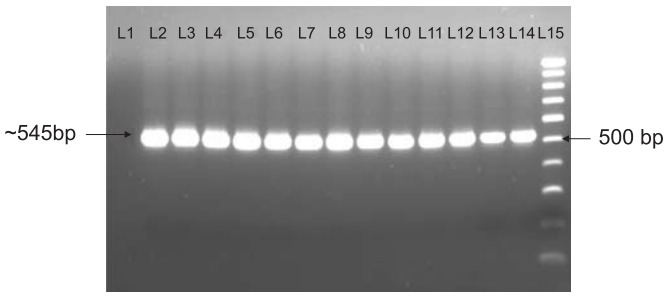

Fig. 1: PCR amplification of Cytochrome C oxidase subunit-I (*COI*) gene from various mosquitoes. L1–Negative control; L2–*Aedes aegypti*; L3–*Ae. albopictus*; L4–*Ae. vittatus*; L5–*Ae. vexans*; L6–*Culex tritaeniorhynchus*; L7–*Cx. vishnui*; L8–*Cx. infula*; L9–*Cx. gelidus*; L10–*Cx. quinquefasciatus*; L11–*Cx. pseudovishnui*; L12–*Anopheles subpictus*; L13–*An. stephensi*; L14–*Armigeres subalbatus*; and L15–Molecular weight marker.

ae strain *formosus* also clustered in the same arm. Further, studies are warranted to understand the occurrence of *Ae. aegypti formosus* in the rural areas. This may be correlated with the prevalence of dengue in the rural area since the *formosus* population has been shown to be less susceptible to dengue virus-2 infection than the aegypti population[11]. Along with the *Ae. aegypti* populations the other genus belongs to the Aedini group. *Armigeres subalbatus*' DNA sequence has been downloaded from the Genbank, and the phylogenetic relationship has been analyzed. The Tirupuvanam strain of *Ae. albopictus* was phylogenetically clustered along with strains such as aa Vikhroli, aa Karnataka, aa VCRC MM-A 10456 and aa AY729984 (Fig. 2).

Similarly, *Cx. quinquefasciatus* strain grouped in a cluster with Tamil Nadu as well as cpq AY729977. Though the existence of the vector is mapped in the area, the prevalence of clinical filariasis cases has not been documented. *Culex tritaeniorhynchus* of Tirupuvanam is clustered along with the Pondicherry and Marvakkadu strains. The phylogenetic tree depicted the overall relationship of the four species studied and has clearly shown

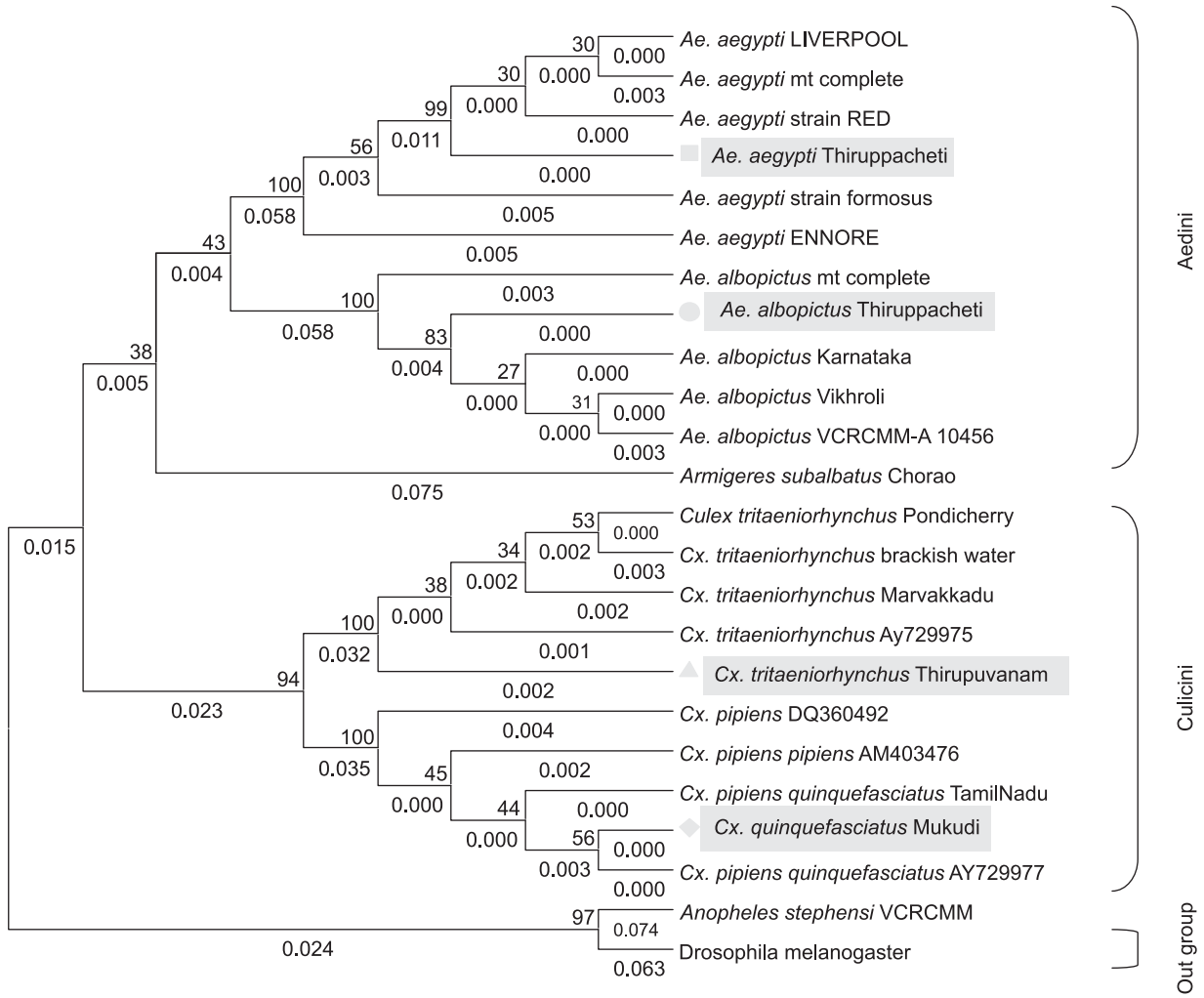

Fig. 2: Phylogenetic tree showing the relationship of *Ae. aegypti*, *Ae. albopictus*, *Cx. tritaeniorhynchus* and *Cx. quinquefasciatus* based on partial sequence of *CO1* gene.

the clustering of each species. Cywinska *et al*[12], demonstrated a higher degree (20 times) of COI sequence divergence among the congeneric species than for members of species by amplifying a short fragment of *COI* gene region of 37 species of Canadian mosquitoes. In another study, 63 species belonging to 15 genera of Indian mosquitoes were subjected to barcode analysis[10] and the DNA-based method was found correlating with the conventional taxonomical methods while confirming the mosquito species. On the other hand, the technique failed to distinguish the closely related mosquito species *Ochlerotatus portonovoensis* and *O. wardi,* since the genetic divergence was found to be negligible. This further cautions the use of *COI* gene-DNA barcoding-based identifying the closely related mosquitoes.

In conclusion, the DNA-based barcoding would really be a simple and very useful method in identifying the mosquitoes. The method could distinguish mosquitoes. Nevertheless, the DNA-based species identification technique would immensely complement the conventional morphology-based taxonomical procedures.

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