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Soliang Manyu
Department of Zoology, Annamalai University, Annamalai Nagar, Tamil Nadu, India

C Elanchezhiyan
Department of Zoology, Annamalai University, Annamalai Nagar, Tamil Nadu, India

K Sivasankaran
Department of Zoology, Annamalai University, Annamalai Nagar, Tamil Nadu, India

P Basker
Consultant Entomologist, National Centre for Disease Control (NCDC), Ministry of Health and Family Welfare, Government of India, New Delhi, India

Corresponding Author:
Soliang Manyu
Department of Zoology, Annamalai University, Annamalai Nagar, Tamil Nadu, India

Molecular identification of *Aedes albopictus* (Skuse, 1894) of Chidambaram strain from inferred mitochondrial gene COI in dengue infested sentinel site, Tamil Nadu, India

Soliang Manyu, C Elanchezhiyan, K Sivasankaran and P Basker

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Abstract
Background: Morphological identification is the gold standard method for identifying species based on their external features. However, at times, this could lead to incomplete identification and faulty results when key features such as scales and bristles are damaged or when encountered mosquitoes with identical characteristics. Therefore, in the incumbent study, *Aedes albopictus* of Chidambaram strain was taken as a species model.
Objectives: To identify the DENV vector morphologically and genetically, creating a Genbank ID for the variant strain, and mapping the trait of the species via phylogenetic tree construction.
Methods: The specimen larvae were collected from Annamalai Nagar, DENV infected area. First, the specimen was morphologically identified using taxonomy key features, and subsequently, molecular identification was carried out using the Sanger Dideoxy sequencing method on the M1 cytochrome c oxidase subunit (COX1) gene, partial CDs; mitochondria. The sequenced nucleotides were analysed using the BLAST platform, and the evolutionary tree was inferred using the Neighbor-Joining method.
Result: The vector species model was confirmed and identified as *Aedes albopictus* morphologically as well as genetically. The sequenced gene length was 517bp and submitted to NCBI under gene bank ID: OP002091.1. The sequenced data has shown 100% similarity of the COI gene with that of different geographically located *Aedes albopictus* species. Phylogenetic tree analysis reveals its common origin with species of MZ5001505.1; MF148287.1 and MN080757.1 accession numbers.
Conclusion: Confirmation of *Aedes albopictus* in the present study is an immense asset in vector control planning and management in the region where vector borne disease are endemic. Also, the evolutionary history of the species recorded would enable better understanding of vector biology and its evolution navigation.

Keywords: *Aedes albopictus*, Chidambaram strain, molecular identification, DNA barcoding, COI maker

1. Introduction
Mosquitoes are in the limelight of insect taxa [1] due to their cosmopolitan distribution and medical significance. As of 2021, there are 3600 recognized species in the Culicidae family of mosquitoes that are distributed into subfamilies: Anophelinae and Culicinae [2]. Moreover, species belonging to the genus *Aedes, Culex,* and *Anopheles* transmit arboviruses, pathogens, and bacteria [3]. *Aedes albopictus* of the genus Aedes is observed to transmit 32 pathogenic viruses [4]. It is the DENV (Asian origin) vector that is recorded to be the fastest expanding vector both in terms of population exploration and geographical adaptation. Its occurrence in different parts of the globe is mainly due to international trade and the mobilization of individuals overseas [5]. Today, *Aedes albopictus* is popularly known to cause dengue, chikungunya, and zika virus infections [6] and is well fitted to tropical and sub-tropical climatic conditions [7, 8]. Despite relentless efforts to clean up the occurrence, their natural adaptation to breeding in human habitats has flourished, signalling a vicious threat to public health. The increasing cases of DENV infections worldwide necessitate the availability of easy and precise vector identification keys as the basis of entomological surveys and effective vector control [9]. Mosquito identification is either done morphologically using diagnostic features mentioned in

taxonomic keys [10] or by molecular methods using gene COI encoding cytochrome C oxidase subunit I and internal transcribed spacer 2 (ITS2) [11]. Molecular identification using cytochrome C oxidase subunit I (COI) genes is commonly preferred over its low polymorphism existence, yielding reliable results. In addition, it is found in all eukaryotic organisms. Due to cosmopolitan habitat [12, 13], vectors are bound to develop genetically distinct populations displaying different capabilities to transmit arboviruses [14]. Many organisms, including mosquitos, exhibit morphological and genetic variations within a species (intra-specific). As a result, if these variations are not properly understood, they can have an impact on species identification, leading to the failure of measures taken [15]. A species complexion of sibling species could be puzzling morphologically but have different behavioural (Host preference, feeding/biting behaviour, resting behaviour) and physiological traits (Ability to transmit pathogens). As a result, prior knowledge of intra-specific variations is required for species identification, which serves as the foundation for accurate interpretation and implementation of the results [16]. Thus, studies on vectors that rely solely on morphological identification keys may produce incorrect results and treatments [17-21]. Therefore, prior to any investigation involving vectors, their precise identification should be the first step in the process.

Therefore, in this work, we coupled both the identification methods and barcoding was done using the M1 cytochrome oxidase subunit (COX1) gene, partial CDs; mitochondria. Furthermore, we compared the newly sequenced nucleotides to the nucleotides of BLAST to conduct a phylogenetic analysis of the identified species to determine their evolutionary history. It is the first barcoding study conducted in the region on the vector, *Aedes albopictus* of the Chidambaram strain. In general, we aimed to access greater insight of *Aedes albopictus* in terms of proper identification perspective, thereby yielding precise data for effective vector control planning in the Chidambaram.

2. Materials and methods

2.1 Collection sites

Aedes albopictus larvae were collected from Annamalai Nagar (Figure 1) on the Annamalai University campus. It is a public state university in Chidambaram, Tamil Nadu, India with a 950-acre (3.8 km^2) sprawling campus with GPS coordination of 11.3918°N and 79.7132°E. The specimen required for the study was accessed from the natural habitat in the larval stage. Larvae collection from their natural breeding habitats was done by a pipetting method using a Pasteur pipette and dipper cup with a handy magnifying glass and transferred to a plastic cup as per the guidelines given in [22, 23]. The larvae from the breeding habitat were collected in a container (~250 ml) labeled with the habitat type, and date of collection and were brought to the laboratory. From the collected larvae, a total of 70 numbers were separated in a tube containing 70% ethanol and sent to Tri-Biotech of Trichy Research Institute of Biotechnology Pvt. Ltd., Thillai-Nagar, Tamil Nadu, India for molecular identification. The rest of the collected larvae specimen was then reared in the laboratory.

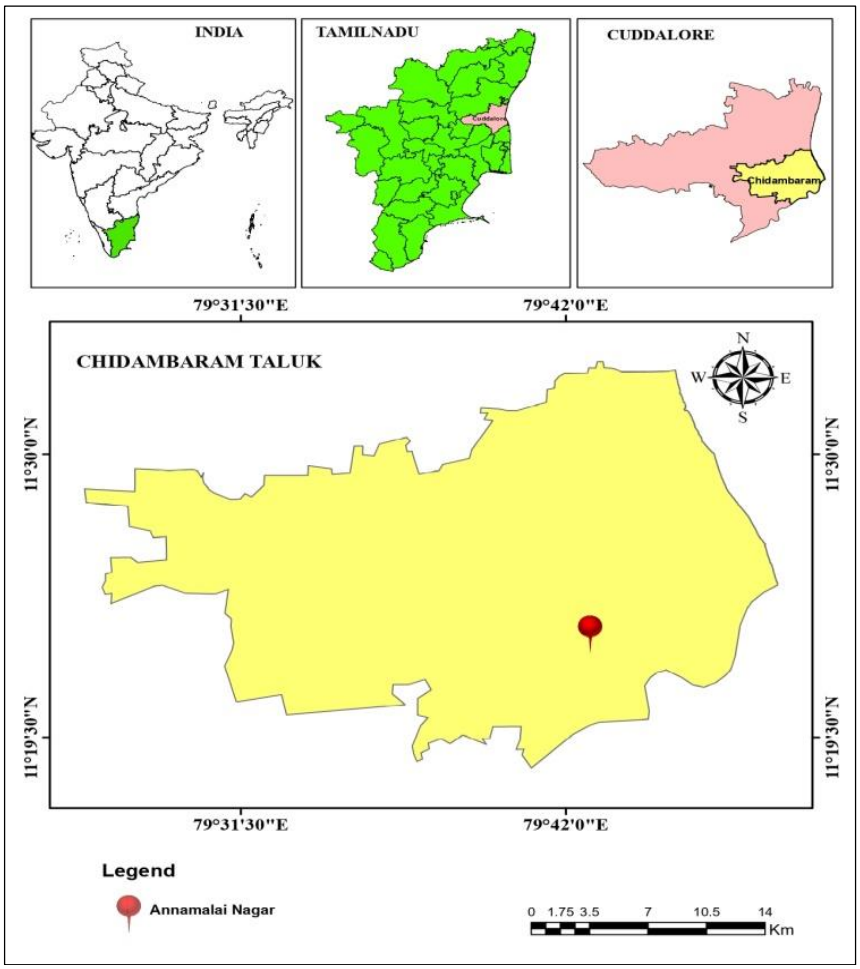

Fig 1: Presenting sampling site-Annamalai-Nagar, Chidambaram, India

2.3 Identification

2.3.1 Morphological identification

The morphological identification of collected larval and emerged adult was followed in accordance with the illustrated keys of Leopoldo M. Rueda., 2004 [24].

2.3.2 Molecular identification

Each morphologically identified specimen was placed in a 1.5 ml micro centrifuge tube containing 70% ethanol and stored at 4 °C for molecular species confirmation and further processing. The molecular identification was carried out at tri-biotech, Trichy Research Institute of Biotechnology Pvt. Ltd., Thillai-Nagar, Tamil Nadu, India.

2.3.3 DNA Isolation procedure

The test samples were placed in 1.5 mL tubes separately and then 500 µL of Solution and 10% of SDS were added. Homogenize the sample with a sterile homogenizer. And 5 µL of Proteinase K was added (20 mg/mL). The mixture was incubated at 55 °C for 2 hrs. in a water bath (with occasional mixing/quick vortex) for easy digestion. After complete digestion, the sample was kept on ice for 10 min.

To this, 250 µL of Solution 2 was added (Saturated NaCl) and inverted several times to mix. Subsequently, the samples were chilled on ice for 5 min. Then the samples were centrifuged at 8000 rpm for 15 min. After that, about 500 µL of clear supernatant was collected into a new-labelled 1.5 mL tube. Then twice the volume of 100% molecular biology grade ethanol was added to precipitate the DNA. Then the samples were centrifuged at 11, 000 rpm for 15 min. After that, the supernatant was removed and 500 µL of ice-cold 70% ethanol was added to the precipitate for washing. The sample was spun at 11000 rpm for 5 minutes. Carefully, removed the supernatant, then pipetted out excess liquid and allowed to partially dry with lid off at room temperature. Partially dried DNA was re-suspended in 100 µL of 1x TE buffer.

Quantification of DNA

The quantity of the extracted DNA was checked in a UV spectrophotometer (Labman, India) by taking the optical density (OD) at 260 nm and 280 nm. The quality was checked by measuring the ratio of absorbance at 260 nm and 280 nm (260/280). The value between 1.7 and 1.8 indicates good quality DNA without protein or RNA contamination. DNA quantification was done according to the following calculation: a sample showing 1 OD at 260 nm is equivalent to 50 µg of DNA/mL. The OD of each DNA sample at 260 nm was measured and quantified accordingly.

Agarose gel electrophoresis

The unit is set according to the manufacturer's instructions. The (0.3 g) agarose powder was soaked in 30 mL of 1X TAE buffer and boiled until it formed into a clear solution. Then it was allowed to cool, down to approximately 50 °C. Then add 1.5 µL of ethidium bromide and mix well. It was poured into a gel casting plate with an already adjusted gel comb and kept at room temperature for 1/2 hrs for solidification. The gel was soaked in 1X TAE buffer in the electrophoresis tank. 3 µL of DNA with 3 µL of gel loading dye was loaded into the wells using micropipettes. It was run at 70 V for 15 to 20 min. The orange-coloured (DNA) bands were observed in the UV gel documentation system.

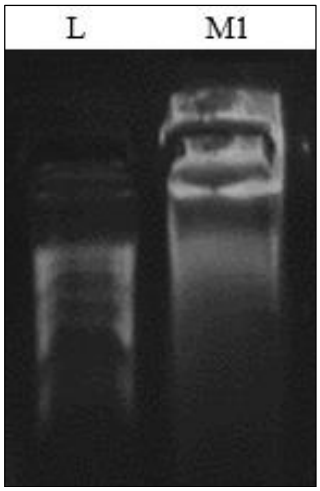

Fig 2: An agarose gel showing the presence of the band of interest denoted by the M1 code, while L denotes the ladder used

PCR amplification

The COI gene was amplified using the primers (Table.1). The amplification was conducted in a PCR thermal cycler (PCR System, Himedia). In post amplification activities, ExoSAP-IT Treatment was given, which consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications. Five microliters of PCR product are mixed with 21 of ExoSAP-IT and incubated at 37 °C for 30 minutes, followed by enzyme inactivation at 80 °C for 15 minutes.

Table 1: Primers used for the amplification of the CO1 gene, M1

Gene	Direction	Sequence (5´-3´)
CO1	Forward	TTCTCCAACCACAAAGACATTGGCAC
	Reverse	ACTTCTGGGTGGCCAAAGAATCAGAA

Sequencing using the Big Dye Terminator v3.1

The sequencing reaction was done in a PCR thermal cycler (Gene Amp PCR System 9700, Applied Bio-systems) using the Big Dye Terminator v3.1 Cycle sequencing Kit (Applied Bio-systems, USA) following manufacturer protocol. The sequencing PCR temperature profile consisted of a first cycle at 96 °C for 2 minutes followed by 30 cycles at 96 °C for 30 seconds, 50 °C for 40 seconds, and 60 °C for 4 minutes. A total of 517bp nucleosides were sequenced (Table 2).

Table 2: The sequenced nucleotides of the COI unit of mitochondria for forward (M1 F) and reverse (M1 R) directions

Sample Code	M1 Sequence results
M1 F	TTTGATAAAATAGGGTCTCCCCCTCCAATTMGGATCAAAAAAAGATGTA TTTAAATTMTCGGTCTGTTAATAATATAGTAATAGCTCCGGCTAATACGG GTAGAGAAAGAAGTAATAAAATAGCTGTAATTYACTACTGATCACACAAA TAAAGGTAGTCGATCAAGAGTAATACCAGCTGATCGTATATTAATTACAG TYGTAATAAAATYTACTGCTCCTAAAATAGATGAGATTCCCGCTAAATGT

	AAAGAAAAAATYGCTAAATCAACTGAAGCCCCAGCATGAGCTGTTCCAGA AGAAAGGGGAGGATAAACCGTTCACCCTGTTCCAGCTCCGTTTTCTACTA TAGAACTARAAAGCAGCAGTGTTAAAGAGGGGGGTAATATTCAAAAACTT ATATTATTTATTCGAGGAAAAGCTATATCAGGGGCTCCTAGTATTAAGGG TACTAGTCAGTTTCCAAATCCTCCAATTATGATAGGTATTACTATAAAAA AAATTATAATAAAAGCATGAGCAGTAACAATTACATTATAAATTTGATCA TTTCCAATAAATATACCAGGATGTCTAAGTTCAATACGAATTAAAACTCT TAGTGAAGTTCCGACTATTCCAGATCAAATACCGAAAATAAAGTATAATGTTCCAATATCTT
M1 R	CCTTACTTTCCGGTATTTGATCTGGATAGTCGGAACTTCACTAAGAGTTT TAATTCGTATTGAACTTAGACATCCTGGTATATTTATTGGAAATGATCAA ATTTATAATGTAATTGTTACTGCTCATGCTTTTATTATAATTTTTTTTAT AGTAATACCTATCATAATTGGAGGATTTGGAAACTGACTAGTACCCTTAA TACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAATAATATAAGTTTT TGAATATTACCCCCCTCTTTAACACTGCTGCTTTYTAGTTCTATAGTAGA AAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCCCTTTCTTCTGGAA CAGCTCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTA GCGGGAATCTCATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAAT TAATATACGATCAGCTGGTATTACTCTTGATCGACTACCTTTATTTGTGT GATCAGTAGTAATTACAGCTATTTTATTACTTCTTTCTCTACCCGTATTA GCCGGAGCTATTACTATATTATTAACAGACCGAAATTTAAATACATCTTT TTTTGATCCAATTGGAGGGGGAGACCCTATTTTATATCAACATTTATTTT GATTTTTTGGCAC

Blast Analysis

The RBCL region sequence was used to carry out the BLAST database. The first ten sequences were selected and aligned using the multiple alignment software program Clustal. Based on the extreme identity score program Clustal W. A distance matrix was generated and the phylogenetic tree was constructed using MEGA7.

Table 3: The BLAST result of OP002091.1

Sl. No.	Description	E-Value	% IDENT.	Accession
1.	Isolate 68#VB1 Haplogroup A1b2a1 mitochondria	0.0	100.00%	MH587214.1
2.	Isolate 67#CAM3 Haplogroup A1b2a1 mitochondria	0.0	100.00%	MH587213.1
3.	Isolate 66#CAM2 Haplogroup A1b2a1 mitochondria	0.0	100.00%	MH587212.1
4.	Isolate 61#Rdj4 Haplogroup A1b1 mitochondria	0.0	100.00%	MH587211.1
5.	Isolate 62#Rdj3 Haplogroup A1b1 mitochondria	0.0	100.00%	MH587210.1
6.	Isolate 60#Rdj2 Haplogroup A1b1 mitochondria	0.0	100.00%	MH587209.1
7.	Isolate 59#Rdj1 Haplogroup A1b1 mitochondria	0.0	100.00%	MH587208.1
8.	Isolate VTE 111 cyto. C oxidase subunit 1 (COX1)	0.0	100.00%	MW526719.1
9.	Isolate VTE 109 cyto. C oxidase subunit 1 (COX1)	0.0	100.00%	MW526717.1
10.	Isolate VTE 108 cyto. C oxidase subunit 1 (COX1)	0.0	100.00%	MW526716.1

Phylogenetic tree analysis

The phylogenetic tree was inferred using the neighbour joining tree method to discern the evolutionary history of the present confirmed species. Evolutionary analyses were performed through the software MEGA5 [25]. The optimal tree is shown (Figure 2). Species discrimination using DNA barcodes was performed by comparing the nucleotide sequences on the NCBI gene bank database.

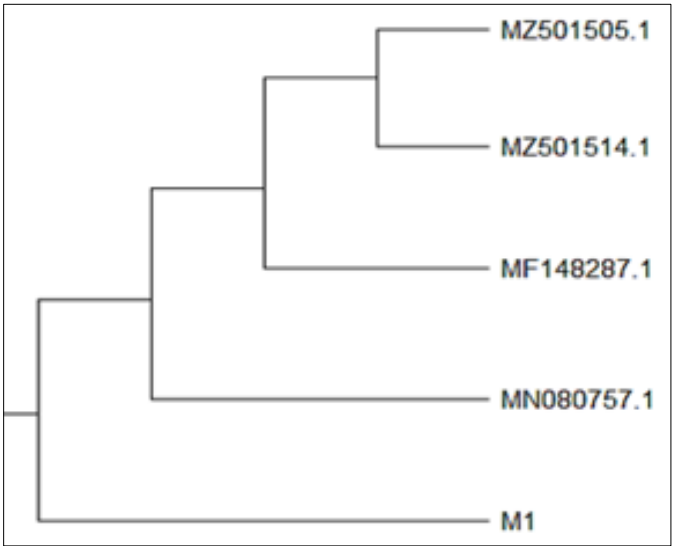

Fig 3: Phylogenetic relationship of *Aedes albopictus* of the Chidambaram strain in Tamil Nadu, India with accession id OP002091

Result

Morphological identification result: Morphological features like a stout siphon, setae and comb scales in larvae, a longitudinal white strip on the scutum, a V-shaped white strip on the mesepimeron and the absence of a whiter strip on the anterior mid femur and clypeus accounted for the key features of M. Rueda., 2004 [24] for *Aedes albopictus*. Hence, morphologically, the vector model is confirmed to be *Aedes albopictus*.

Molecular identification results: The COI-based DNA barcoding succeeded in identifying the mosquito species. The total length of the 517bp CO1 gene of the M1 specimen was sequenced using Sanger dideoxy sequencing (Table 2). The newly determined nucleotide sequences of the COI gene were compared with those in the BLAST database. The BLAST result confirmed the sequenced CO1 gene of 517bp to be the *Aedes albopictus* with 100% similarity to its database (Table 3). The sequenced CO1 gene is registered in Genbank with accession id: OP002091.

The Phylogenetic tree analysis results: The CO1-based phylogenetic analyses {Figure 3), showed that confirmed *Aedes albopictus* of the Chidambaram strain was congregated with *Aedes albopictus* of MF148287.1, MZ501514.1, MZ501505.1 and MN080757.1.

4. Discussion

Urbanisation has always caused either the extinction of the invaded species or their evolution into the human-defined territory. The phenomenon became disastrous when warm-blooded feeding insects were encountered. This led to a decrease in proximity and humans became the most preferable and stable nutrient source, resulting in the loss of 7 million lives annually. So from a public health perspective, precise identification of medically important vectors is extremely significant in vector control programming and study. In the present study, we investigated the identification of *Aedes albopictus* through classical taxonomy and DNA barcoding using the COI gene. Rapid and accurate identification of mosquito species [26] is essential to determine the real and potential risk of arboviruses or parasite transmission and to implement vector surveillance and control programs. There are 3,600 species in the family Culicidae, which are classified into the subfamilies Anophelinae and Culicinae [27] and identifying the targeted vector correctly from such a pool of species could be an extremely challenging task. The majority of mosquito species have been identified using traditional taxonomy that seeks skilled taxonomists, detailed documented features, and regional knowledge experts. In the present study as well, morphology identification was conducted following the guidelines of Leopoldo M. Rueda (2004) [24] and the species was confirmed to be Aedes albopictus. However, when it comes to the identification of indistinguishable cryptic species or morphologically identical sibling species, then classical taxonomy identification is not a reliable method [28, 29]. Also, some mosquitoes exhibit distinguishable morphological features, making it tough for morphological-based identification to succeed [30-32]. To mitigate this gap, molecular identification using DNA has been proposed, which applies to all developmental stages and both sexes precisely [33]. Therefore, morphologically identified *Aedes albopictus* was processed for molecular identification using mitochondrial gene CO1, which confirmed the species using the database of BLAST. The barcoding of *Aedes albopictus* has been reported from varying strains within the country [34]. All these studies have identified the same species, but the major difference in all these results is that the sequenced genes are not the same. Therefore, identical studies of different strains are not reliable to generalise the obtained data due to genetic polymorphism. So, the current study is reasonable and necessary in the course of vector study. The study also demonstrates the potential of DNA barcoding in connecting the genetic diversity of species. However, the result represents only a small minority of the total number of mosquito species in Chidambaram.

5. Conclusion

The accuracy and versatility of DNA barcoding as a species identification tool is an essential component of vector surveillance. It has seen rapid growth due to a surge in interest in studying biodiversity on a larger scale. For the first time, the DNA barcoding method has been employed on *Aedes albopictus* of the Chidambaram strain. The study created a DNA barcoding database of the Chidambaram strain of the regionally targeted dengue vector *Aedes albopictus*, hereby confirming the occurrence of the DENV vector in the region, which is an immense asset for vector control and surveillance. The study has also analyzed the evolutionary history of the vector, which would permit the navigation of genetic polymorphism or evolution.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgements

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