

Original Research Article

Molecular Identification of *Aedes albopictus* (Diptera: Culicidae) and Quantitative Analysis of COI gene in South-Indian species.

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ABSTRACT

The alarming fact is that the dengue fever has become endemic in the urban areas of India and especially in Hyderabad; there are about 200 confirmed and 181 suspected dengue cases were reported in the year 2012. In our study we found that *Aedes albopictus* is a major vector for dengue in Hyderabad. Rapid, accurate and timely identification of mosquitoes has become a challenging task, especially for mosquito control programme managers. The CO I gene of mitochondria is used as powerful taxon gene in molecular identification of the species. DNA barcoding is a method for the identification of species in a wide range of animal taxa, which uses the 5' region of the mitochondrial cytochrome C oxidase-I (CO-I) gene. The *Aedes albopictus* mosquito has a 600 bp length of CO I gene and shows distinct variation when compared with the other species. The CO I gene is amplified and sequenced in this study. The data has been submitted to NCBI gene bank and shown 100% similarity of COI gene with that of different geographically located *Aedes albopictus* species. The Hyderabad region species shows significant variation at amino acid percentage within Indian population of *Aedes albopictus*.

Keywords

Dengue;
Aedes albopictus;
CO I gene;
Amino acids;
Mosquito.

Introduction

The city of Greater Hyderabad has a population of 8 million in the area of 172 Sq. Km. The study area covers five circles and 150 wards, each ward having a typical slum area. The study area is endemic to Dengue, Chikungunya and Urban Malaria. A total of 381 dengue cases were reported during the year 2012 and there might have been many more unreported. The data available is collected from both public and private hospitals and information is

retrieved from the medical and health authorities of Greater Hyderabad. Malaria is another vector borne diseases spread by the mosquitoes which is on the upswing with 439 cases reported in the year 2012. In our study it is observed that the major vector for dengue is *Aedes albopictus*.

It is imperative that, different mosquito species have different breeding habitat and which attracts different approaches to

tackle them. The significance of molecular characterization is a handy tool for species identification. The traditional method of morphological species is time consuming and needs professional expertise. Through the traditional method only 10% of species were identified on earth which is known to science so far (Besansky *et al.*, 2003; Pennisi 2003). Recent studies on DNA-based approaches had shown a promising trend in the rapid description of biodiversity (Hebert *et al.*, 2003; Hebert and Gregory 2005). Hebert's approach to DNA barcode for the entire animal kingdom based on single gene could be advantageous, being economic and practicable. The analysis of the DNA sequences of this gene may yield a useful tool for species identification in different species of the Animal Kingdom. According to Saccone *et al.*, (1999), mitochondrial genome showed its merits such as maternal lineage, lack of recombination and lack of introns. Among the mitochondrial genes, cytochrome *c* oxidase subunit 1 (COI) is reported to be the most conserved gene in the amino acid sequences and hence has distinct advantage for taxonomic studies (Knowlton and Weight 1998). Hence, standardization of tools that could identify mosquito species even from a small piece of tissue from any developmental stage would be of importance for the taxonomy of mosquitoes. Construction of DNA barcodes for each species of mosquito would provide an important tool for identification of mosquito species and may enable the description of the species biodiversity of this important group of insects (Kumar *et al.*, 2007). Here, we present the barcoding of *Aedes albopictus* mosquito species prevalent in Hyderabad. Multiple specimens collected from different regions of the Hyderabad city and studied the Genetic make up of CO I.

In this study we have observed that the molecular characterization of COI gene gives scope to study the genetic variation of *Aedes albopictus* with different parts of south India at amino acid level. The Hyderabad species shows variation in amino acid percentage when compared with Pondicherry and Karnataka species.

Materials and Methods

Mosquito Sampling and Rearing

The wild type larva of *Aedes albopictus* was collected from different parts of the city and reared in the Laboratory under specific conditions according to manual for Mosquito rearing and experimental techniques published by American Mosquito Control Association (AMCA bulletin no. 5, January 1979) nude mice were used for blood meal to mosquitoes to obtain the pure cultures. This pure culture was reared again to get the F₁ generation and the 4th instar larvae of F₁ generation were used for genomic DNA extraction. The *Aedes albopictus* was identified morphologically with the help of Entomologist deposited at The National History of Museum, Department of Zoology, Osmania University with Accession No (OUNHM.ART.2011.1).

Isolation of Genomic DNA

Genomic DNA was extracted following the procedure of Co en *et al.*, (1982). The larva were thoroughly ground in 50 µl of homogenization buffer [0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl pH 9.1, 0.05 M EDTA, and 0.5% sodium dodecyl sulfate (SDS)]. The homogenate was incubated in thermal cycler at 65°C for 30 min. While still warm, 7 µl of 8 M Potassium Acetate (CH₃ COOK) was added and mixed by tapping. The tubes were then placed in ice

for 30 min. to precipitate the SDS. The homogenate was centrifuged at 17,000x g for 15 minutes, and the supernatant transferred to fresh 0.5 ml tubes. Care has been taken not to transfer any of the precipitated SDS. Ethanol (95%) was added to each tube (100 µl) and incubated for 5 min at room temperature to precipitate nucleic acids. The tubes were spun on a centrifuge at 17,000x g for 15 min. The ethanol was carefully removed and the nucleic acid pellet was washed with 100 µl 70% ethanol and 100 µl 100% ethanol. The supernatant was decanted and DNA pellet was dried in a vacuum drier. The DNA pellet was re-suspended in 50 µl of TE buffer (0.05 M Tris- HCl, EDTA, pH 8.0) for overnight at -4°C.

PCR Amplification

The desired CO I gene 5' segment of the mtDNA COI gene was amplified in thermal cycler by using appropriate primers that was designed back from 722 bp into the COI genes of *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* sequences obtained from Genebank. The designed primers for the present study were the forward primer (5'- ATT GGA TTA TTA GGA TTT ATT G - 3') which was designed to start approximately 76 bp into the COI 5' region and the reverse primer (5'- GCA GGA GGA AGA GTA TGA TAT C -3'). The total polymerase chain reaction (PCR) mixture of 25 µl was made by mixing 17.50µl millique water (H₂O), 2.50µl of complete (PCR) buffer, 1.00 µl of 2.5 mM dNTPs, and 1.00 µl of the each forward and reverse primer. 1 µl of template DNA was added to each well and placed on a thermal cycler. The plate was heated to 95°C for 5 min for denaturation. And 1.0 unit of *Taq* polymerase was added into each well. The PCR conditions were

subjected to 35 cycles of (1) 95°C for 0.1 sec. (Denaturation); (2) 55°C for 30 sec (annealing); (3) ramp to 68°C at a rate of 1°C every 30sec; and (4) 68°C for 1 min (extension). A final 68°C extension was carried out for 10 min and the temperature was held at 4°C.

CO I gene sequencing

The CO I gene amplicons submitted for sequencing to the Bio serve Pvt. Ltd, Hyderabad, India. The PCR product of CO I gene was amplified and the amplicons were sequenced by Sanger's procedure automated sequencing method

Results and Discussion

The obtained result of CO I gene was blasted with NCBI Gene Bank which had shown 100% similarity of the test specimen and the NCBI gene bank accession number is JX649856. The morphological characters of the test specimen were matched with molecular characterization and the species was confirmed as *Aedes albopictus* (Skuse). The adult specimens are deposited as voucher specimens at The National History of museum, Department of Zoology, Osmania University with Accession No (OUNHM.ART.2011.1). Though, the re reported barcode data of *Aedes albopictus* species form different geographical areas such as Pondicherry, Karnataka and Hyderabad have shown 100% similarity the CO I gene, but there was an astonishing and distinguishable variation in their amino acid percentage frequencies of the CO I gene. For example, the species reported form Pondicherry (India)(Kumar et, al., 2007) with a gene bank accession number (AY729984), Karnataka (AY834241), VCRC Specimen (GU299769) and a

Hyderabad sample with accession number is (JX6498560) has shown the variations in amino acid percentage when aligned them and studied their amino acid composition by MEGA5 (Tamura, *et al.*, 2011) as shown in figure.1.

In this study it is noticed that the percentage of Alanine was zero in all samples except Hyderabad. Whereas the frequency of Aspartate amino acid is Zero in Karnataka and Pondicherry samples but expressed in Hyderabad and VCRC samples. The frequency of Leucine amino acid was more in Hyderabad region when compare it with two rest of regions, similarly Tryptophan frequency was Zero in Hyderabad region sample. The frequency variation of all amino acids in the different regions is shown in figure.1.

The data reveals that the Hyderabad, Pondicherry, Karnataka and VCRC samples have the progressive variation to all amino acids as shown in the above said figure.1.

Multiple sequences Alignment of CO I gene Amino acid Sequence.

The multiple sequence alignment of CO I gene amino acids was done by Clustal W (Larkin *et al.*, 2007). This alignment shows amino acid variation within the *Aedes albopictus* (figure.2) species of different geographical locations of India.

Molecular Phylogenetic analysis of *Aedes albopictus* in South India by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix- based model [Jones D.T et al]. The bootstrap consensus tree inferred from 500 replicates is taken

to represent the evolutionary history of the taxa analyzed (Felsenstein J). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein J). Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 17.6710)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 4 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 79 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura K).

This study underscores that the DNA barcoding is the technique which can be used to categorize species at molecular level. Even though, morphological identification with respect to their DNA sequence variation is noteworthy. In the present study we have selected four samples of the same species from different geographical locations of India which has shown 100% similarity with NCBI gene bank. According to Knowlton and Weight (1998), it was said that the mitochondrial genes, cytochrome *c* oxidase subunit 1 (COI) is reported to be the most conserved gene in the amino acid sequences and hence has distinct advantage for taxonomic studies could differ with the

Figure.1 The Graphical and tabular representation of amino acids percentage of CO I gene

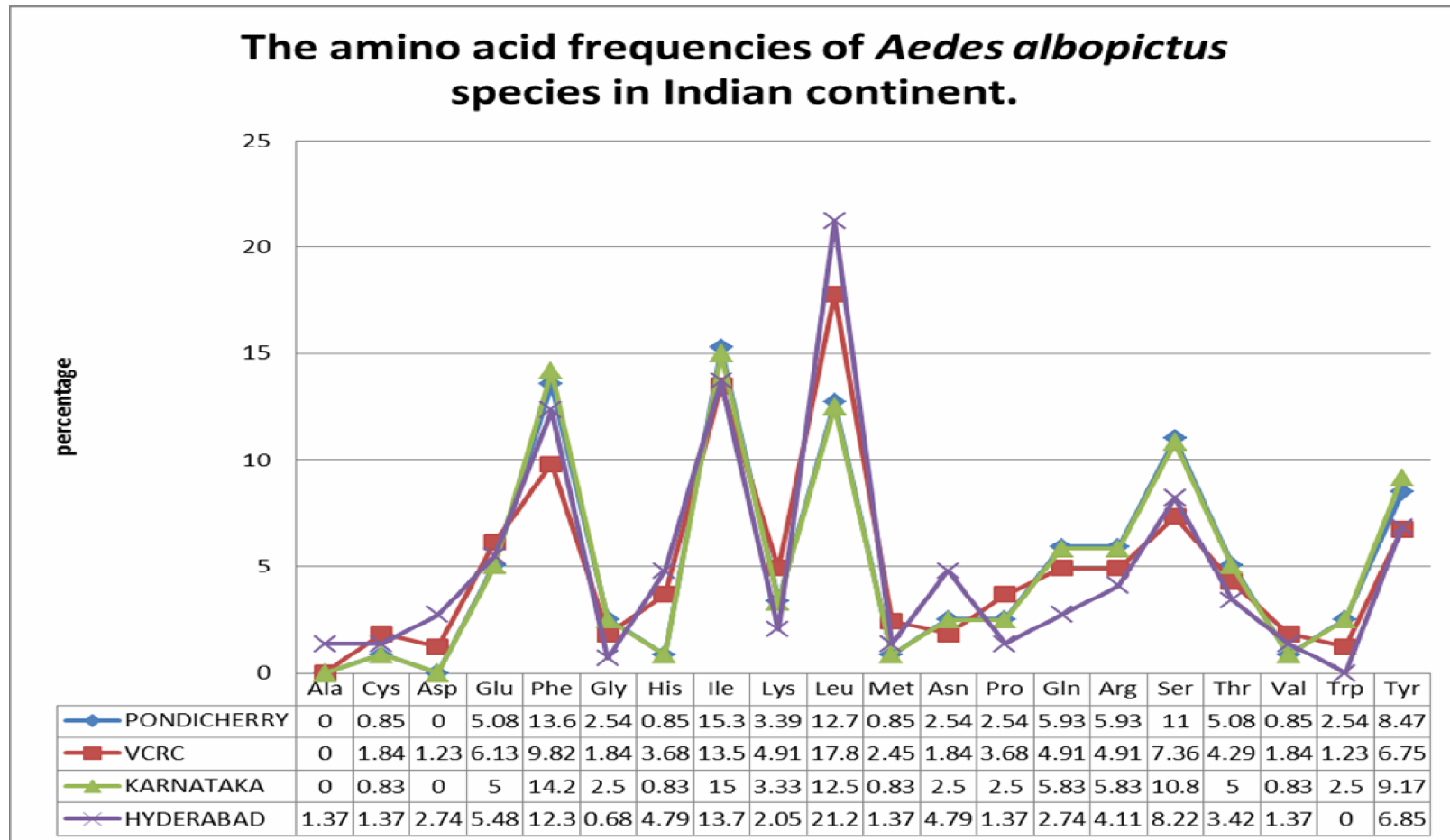


Figure.2 CLUSTAL 2.1 multiple sequence alignment

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gi | AY729984.1 | CTATAGTAGAAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCCCTTCTTCTGGAA
gi | AY834241.1 | CTATAGTAGAAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCCCTTCTTCTGGAA
gi | GU299769.1 | GTATAGTAGAAAATGGGGCAGGAACAGGTTGAACTGTTTATCCTCCTCTTTCATCTGGAA
gi | JX649856.1 | -----ATTAAAATT---TTTAGTTGATTAGCTACTCTTCATGGAACACAATTA
          *: . . . . : : : . * ***: * * : . * * * . : : * *

gi | AY729984.1 | CAGCTCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTTACATTTAGCGGGAATCT
gi | AY834241.1 | CAGCTCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTTACATTTAGCGGGAATCT
gi | GU299769.1 | TTGCACATGCAGGAGCTTCTGTAGATTTAGCCATTTTCTCTTTTACATTTAGCAGGATTT
gi | JX649856.1 | CATATAGCCCTGCACCTTTTATGATCTTTAGGATTTGTTTTTTTTTATTTACAGTTGGGGGAT
          : . : . . * : * . * * : : . * * * * . : * * * * * * * * * * : * : * * * * . *

gi | AY729984.1 | CATCTATTTTAGGAGCAGTAAATTTT---ATTACAACGTAATTAAT-ATACG-ATCAGC
gi | AY834241.1 | CATCTATTTTAGGAGCAGTAAATTTT---ATTACAACGTAATTAAT-ATACG-ATCAGC
gi | GU299769.1 | CATCTATTTTAGGAGCAGTAAATTTT---ATTACTACAGTTATTAAT-ATACG-ATCCCC
gi | JX649856.1 | TAACCGGAGTAATTTTAGCTAATTCATCAATTGATATTGTACTTCACGATACATATTATG
          * : * . : * * . : * * : * * * * : * * * . : * : * * : . * * * * . * * .

gi | AY729984.1 | TGGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACAGCTATTTT
gi | AY834241.1 | TGGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTACAGCTATTTT
gi | GU299769.1 | CGGAATCACATTAGACCGTATACCATTATTTGTATGATCAGTAGTAATTACAGCCATTTT
gi | JX649856.1 | TAGTAGCCCATTT--TCATTATGTATATCTATAGGAGCAGTATTGCTATTATAGCAGG
          . * : * . * : * * : * . : : : * * * * * * * . * * * * * * * : . * * : . . :

gi | AY729984.1 | ATTACTTCTTTCTCTACCCGTATTAGCCGGAGCTATTACTATATTATTAACAGACCGAAA
gi | AY834241.1 | ATTACTTCTTTCTCTACCCGTATTAGCCGGAGCTATTACTATATTATTAACAGACCGAAA
gi | GU299769.1 | ATTATTACTATCCCCTCCTGTATTAGCTGGAGCAATTACTATATTACTTACAGATCGAAA
gi | JX649856.1 | ATTTATTCATTGATATCCATTATTAACAGGAATAATTA-TAAATCCTTCAT-GATTAAAA
          * * * : * : * : * * : * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

gi | AY729984.1 | TTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCATTTTATATCAACATTT
gi | AY834241.1 | TTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCATTTTATATCAACATTT
gi | GU299769.1 | TTTAAATACATCTTCTTTTGACCCAGCAGGAGGAGATCCTATTTTATATCAACATCT
gi | JX649856.1 | GCTCAATTTACAATAATAT----TTATCGGAGTTAATTTAACTTTTTTCTCAACATTT
          * . * * * : * . : * : * * : . * * * * . . : : . * * : * * * * : . * * * * * * *

gi | AY729984.1 | ATTTTGATTTTTTTGGT-CATCCAGAAGTTTATATTTTAA-----
gi | AY834241.1 | ATTTTGATTTTTTTGGT-CATCCAGAAGTTTATATTTTATTTTT-----
gi | GU299769.1 | ATTTTGATTTTTTTGGA-CACCCAGAAGTTTACATTTTAAATTTTACCTGGATTGGGAATAA
gi | JX649856.1 | TTTAGGATTAGCCGGAATACCTCGACGTT--ACTCTGATTTT-CCAGATAGTTATTTAA
          : * * : * * * * : * * : * * . * * * * * * * * * * * * * *

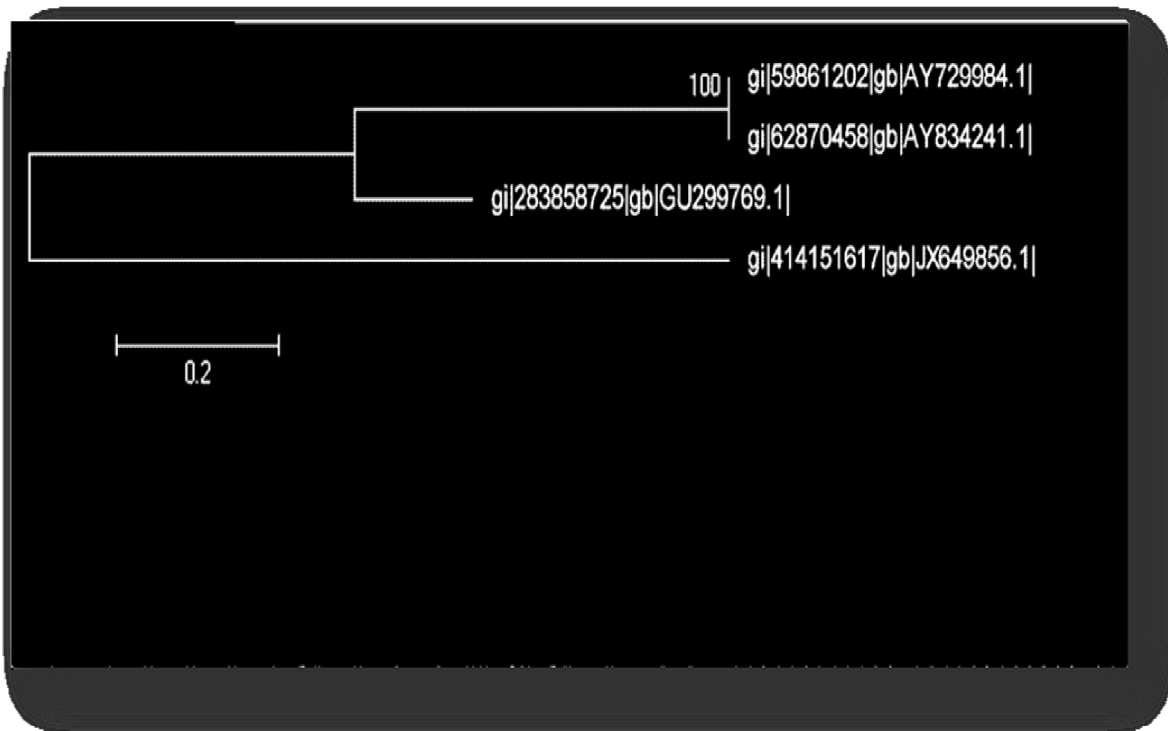
gi | AY729984.1 | -----
gi | AY834241.1 | -----
gi | GU299769.1 | TTTCTCATATTATTACTCAAGAAAGAGGTAAAAAGGAAACATTTGGAAATTTAGGAATAA
gi | JX649856.1 | CTTGAAATATTATTTCTTCTTTAGGAAGAATAA-----TTTCTTTATTTGCTATTA

gi | AY729984.1 | -----
gi | AY834241.1 | -----
gi | GU299769.1 | TTTATGCTATATTAGCAATTGGGTTGTTAGGATTCATTGTTTGAGCTCATCACATATTTA
gi | JX649856.1 | TTTTCTTTTTTATTTATTTTGGAGAAAGAATAATTACACAACGAACTCCTTCTTTC----

gi | AY729984.1 | ---
gi | AY834241.1 | ---
gi | GU299769.1 | CTG
gi | JX649856.1 | ---

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Figure.3 Phylogenetic relationship of *Aedes albopictus* (Hyderabad) species within South India



present study. As the samples of our study are shows variation at their respective amino acid levels as shown in figure.1. It presumes that the genotypic characterization of CO I gene may not have influenced the phenotypic characteristics of *Aedes albopictus*.

Gene expression is the most fundamental level at which the genotype gives rise to the phenotype. The genetic code stored in DNA is "interpreted" by gene expression, and the properties of the expression give rise to the organism's phenotype. Such phenotypes are often expressed by the synthesis of proteins that control the organism's shape and structure or act as enzymes catalyzing specific metabolic pathways characterizing the organism habit and habitat. Even though, species are morphologically identical and identified

based on the morphology, but they show variation within the species at their genetic makeup. The variation at nucleotide level of the CO I gene was observed in the present study. The CO I gene conserved in the amino acid sequences that has distinct advantage for taxonomic studies but it is species specific. The phylogenetic study was constructed to understand the variation of these barcodes which reported in south India.

This reveals that the *Aedes albopictus* is morphologically similar in all regions but they are different at molecular level. It is presumed that, CO I gene may slightly differ at amino acid level of respective species which can be classified on the basis of DNA Barcoding, but the overall phenotypic characteristics has not changed or if at all it might have not noticed so far.

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