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DNA barcode of COI genetic marker of the Indian *Aedes albopictus* (Skuse) (Insecta: Diptera: Culicidae)

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ABSTRACT

Aedes albopictus is an anthropophilic mosquito and an efficient vector of certain arboviral diseases such as dengue and chikungunya in India. In the recent past dengue cases were increasing steadily in urban areas and especially in Hyderabad city. In this context mosquito species composition and their density matters to note the disease burden. The recent technique of species identification through DNA barcode was unveiled and found to be novel approach in furthering evolutionary connection with other Indian species of *Aedes* mosquito. Interestingly, it was found to be divergent from the species reported within India. DNA sequence comparisons and phylogenetic genetic relationship of COI gene were carried out in order to inspect the homology of the sequences and evolutionary divergence with that of Hyderabad species. The partial COI gene sequence was submitted to NCBI-GenBank nucleic acid database with the accession number JX649856.

Key words: *Aedes albopictus*, DNA barcode, Molecular phylogeny, Mosquito and COI gene

1. INTRODUCTION

The mosquito species *Aedes albopictus* is commonly known as Asian tiger mosquito. It has been spreading the world from native tropical forests of South East Asia, Islands of the Western Pacific and Indian Ocean by human travel and international trade via dormant eggs (Benedict et al. 2007; Zhong et al. 2013). In fact, adult females of *A. albopictus*

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are anthropophilic, aggressive day time biters and efficient vectors of certain arboviruses notably dengue fever or dengue haemorrhagic fever (DHF) and chikungunya, in addition to that filarial nematodes like mirofilaria were also reported (Gratz, 2004). Epidemic outbreak of dengue in 2006 was reported in all tropical regions of the world; however India was most affected among others. In India, First dengue virus was reported in Delhi state (Gratz, 2004). Although early dengue spread had reported on *Aedes aegypti* vector (Sharma et al. 2005; Kaul et al.1998), later *A. albopictus* was predominated in transitional and rural locations of Delhi (Kumari, 2011; Ansari, 1998). Alternatively, chikungunya outbreak was observed in Kerala, India (Eapen, et al. 2010) and consequently the dengue and chikungunya were spread all over the India. Despite all *A. albopictus* look similar in morphology; their evolutionary lineage is different in different parts of the country.

There is a growing number of mosquito population distributed all parts of the India, however identification of species or sibling is difficult for even experts moreover time consuming and misdiagnosis is unavoidable. Conventional phylogenetic evolution of organisms often focused on 16s ribosomal DNA (Setti et al 2012). However, this approach is usually constrained by individuals that can discriminate inter specific diversity of taxa (Doyle, 2000). The limitations of biologists confront while identification of closely allied species and phylogenetic groups within a single species were switched to mitochondrial gene cytochrome c oxidase I (COI) sequence. The partial COI gene can distinguish inter and intra specific diversity of taxa in such short genetic marker in an organism is DNA (Hebert et al. 2003). There are several advantages with DNA barcoding. First, lucid discrimination of very close species, second, identification of invasive species, third, recognition of cryptic species (Hebert et al. 2004), and finally, molecular detection of species, for instance, prompt discrimination of mosquito species from a small piece of tissue from any developmental stage. There are few reports on DNA barcodes of *A. albopictus* from different parts of the India except Hyderabad region (Kumar et al. 2007). In the present study DNA barcode of *A. albopictus* from Hyderabad was studied. Furthermore, sequence comparisons and phylogenetic analysis were attained, in order to understand the invasive property and diversity of *A. albopictus*.

2. MATERIAL & METHODS

In the present study larvae of *A. albopictus* mosquitoes were collected from different parts of the Hyderabad city and reared in laboratory for further analysis. At least 10 larvae were collected from each specified spot and they were characterized to be *A. albopictus* based on larval features. However, to conform further they were allowed to grow into adults. To avoid the likely possibilities of specie mixture among the larvae, F1 generation was raised and used for DNA isolation. The DNA was isolated and amplified by mitochondrial partial COI gene with specific primers. Amplified product was run on Agarose gel electrophoreses and purified product was used for DNA sequencing. The sequence was submitted to National Center for Biotechnology Information (NCBI) GenBank. Further sequence alignment and phylogenetic analysis was carried out by Clustal W and MEGA tools.

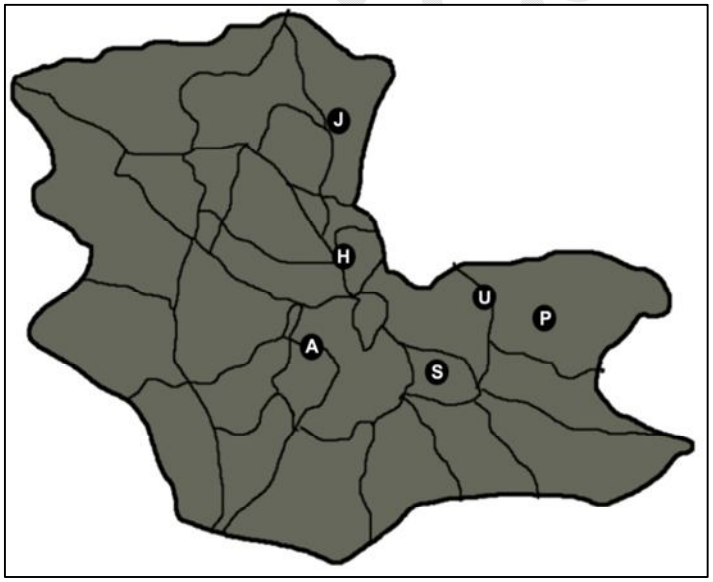

Figure 1

Map showing the distribution of A. albopictus larvae collection sites in the Hyderabad, India. The names of the sites are A: Attapur; H: Hussain Sagar; U: Uppal; S: Saroornagar; P: Parvathapuram; J: Jeedimetla

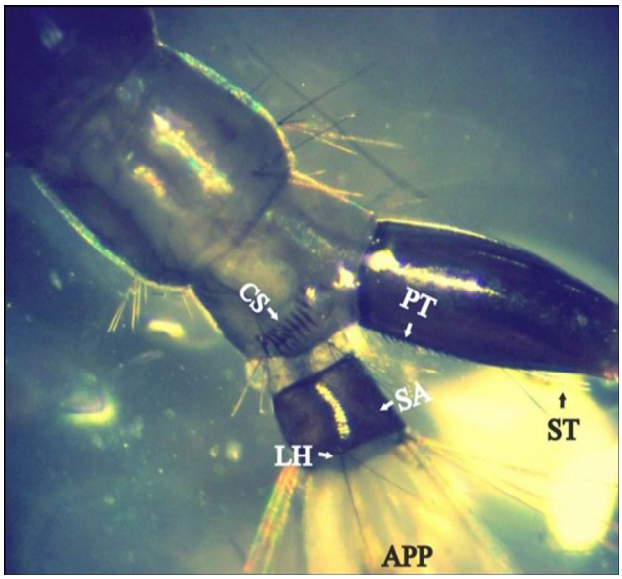

Figure 2

Lateral view of terminal segment of A. albopictus, labeled with APP (anal papilla), CS (comb scale (CS), LH (lateral hair), PT (pecten teeth) and ST (siphonal tuft)

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Table 1

List of Indian *A. albopictus* partial mitochondrial COI gene sequences: submitted to GenBank database along with their sampling GPS coordinates

S.No	Mosquito Species	Region	GPS Coordinates	GenBank Accession number
1	*A. albopictus*	Hyderabad	17° 22.31' & 78° 28.27'	JX649856
2	*A. albopictus*	Pondicherry	11° 57.09' & 79° 47.61'	AY729984
3	*A. albopictus*	Karnataka	12° 52.18' & 74° 52.10'	AY834241
4	*A. albopictus*	Maharashtra	19° 05.77' & 72° 56.43'	DQ424959
5	*A. albopictus*	Orissa	21° 35.69' & 85° 24.93'	DQ310142

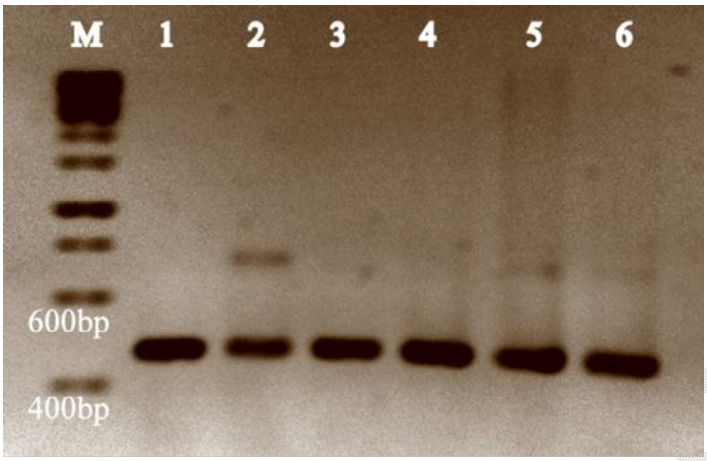

Figure 3

PCR products of mitochondrial COI partial sequences. Lane M: 200bp DNA ladder; lane 1- 6: sample from Attapur, Hussain Sagar, Uppal, Saroornagar, Parvathapuram, Jeedimetla of Hyderabad, India respectively; corresponded between 400bp and 600bp: 500bp

2.1. Mosquito sampling and Morphological characterization

A. albopictus larvae were collected from different parts of the Hyderabad city (Figure 1). Indeed, the larvae are usually abundant in monsoon season June to October, thus larvae were collected in this season from discarded tires, coconut shells and stagnant water bodies. Morphological characterization of larval features of *A. albopictus* was identified with the spotlight on terminal segment under microscope enables to point out further characteristics such as anal papilla (APP), comb scale (CS), lateral hair (LH), pecten teeth (PT), siphon, saddle, and siphonal tuft (Farajollahi et al.2013), ss shown in figure 2. The larvae were grown to adult as specified in the manual for mosquito rearing and experimental techniques, AMCA, Bulletin number 5 (Eugene, 1970), and adult morphology was identified after complete growth. Further, pure culture of F1 generation was used for molecular studies.

2.2. DNA extraction

The total DNA was isolated from the 4th instar larvae. The larvae were finely ground in 50µl of homogenization buffer: 0.1M NaCl, 0.2M sucrose, 0.1M tris, 0.05M EDTA, and 0.5%SDS. The homogenate was incubated in thermal cycler for 30 minutes at 65^{o}C and immediately, 7µl of 8M CH_3COOK was added. The tubes were incubated on ice for 30 minutes, centrifuged for 15 minutes at maximum speed; supernatant was separated in fresh tube, added 100µl of 95% ethanol and incubated for 15 minutes to precipitate the DNA. To collect the DNA, it was centrifuged for 15 minutes at maximum speed; supernatant was decanted. DNA pellet was air dried and suspended in tris buffer for further analysis and stored at -20^{o}C.

2.3. COI partial sequence Amplification and Sequencing

The total DNA that was isolated from *A. albopictus* larvae of six different regions were further specifically amplified by polymerase chain reaction (PCR). The mitochondrial partial COI gene, ~ 500bp was amplified by forward primer: 5' ATT GGA TTA TTA GGA TTT ATT G 3', and reverse primer: 5' GCA GGA GGA AGA GTA TGA TAT C 3'. The primers for the partial COI gene were designed based on *Aedes aegypti, Anopheles stephensi,* and *Culex quinquefasciatus,* spanning ~700bp. Each 25µl of PCR reaction mixture contained 10-50ng of template DNA, 1X assay buffer, 200µM of each dNTP, 5pmol of each primer, and1U of Taq DNA polymerase. The reaction mixture was amplified with cycling profile of 94°C for 2 minutes: initialization, followed by 35 cycles at 95°C for 30 seconds: denaturation of template DNA, 55°C for 30 seconds: primer annealing, 70°C for 30 seconds: extension, and 70°C for 10 minutes: final extension. Finally, amplified sequences were run on agrose gel electrophoresis and purified sequences were given for commercial sequencing (Bioserve Biotechnologies Pvt. Ltd.)

2.4. Sequence Alignment

The Hyderabad *A. albopictus* mitochondrial partial COI sequence of 501bp was aligned with other sequences that were reported from different parts of the India (Kumar et al. 2007) as shown in Table 1. The multiple sequence alignment studies were attained in Clustal W (Larkin et al. 2007) with default parameters. Consequently, similarities and differences were noticed in sequence alignments in order to confer the sequence homology, conservation, and insertions and deletions (INDELS) among Indian *A. albopictus* mosquitoes.

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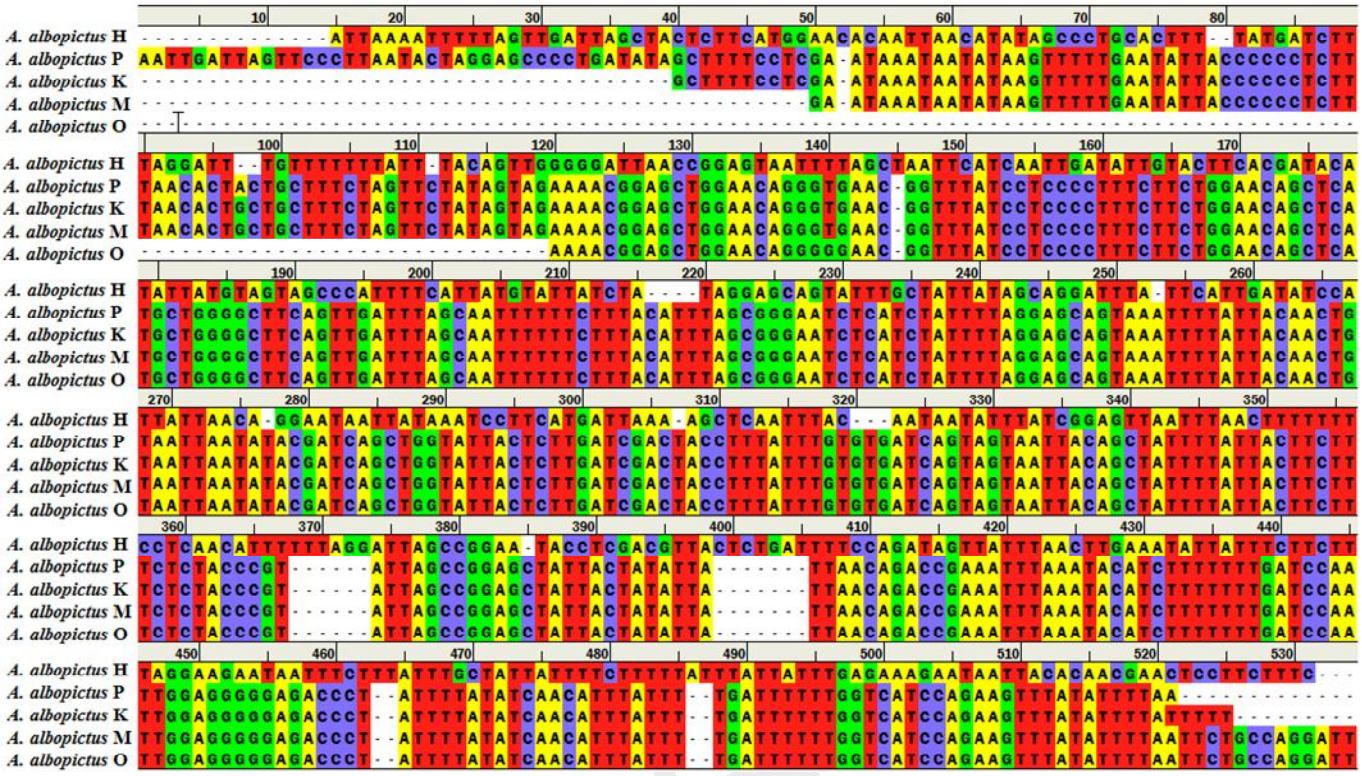

Figure 4

Multiple sequence alignment of mitochondrial partial COI gene from *A. albopictus*. Hyderabad species was found to be more divergent. H: Hyderabad; P: Pondicherry; K: Karnataka; M: Maharashtra; O: Orissa

2.5. Phylogenetic Analysis

DNA barcode reveals the evolutionary relationship among allied species. However that can be apparent by phylogenetic analysis. Although morphological data could fetch to classify related species, but it often fails to classify sibling species and closely related species. The limitations inherent in morphology based classifications can be overthrown by molecular sequencing data. Therefore, in this study phylogenetic analysis of mitochondrial partial COI genes of *A. albopictus* were used for phylogenetic tree construction (Table 1). The phylogenetic analysis was attained in MEGA 5 (Tamura et al. 2011). Nevertheless, maximum likelihood tree was constructed with Tamura 3-parameter model. Gaps and missing data were completely deleted. Furthermore, to evaluate the tree accuracy boot strap test value of 500 was assigned.

3. RESULTS AND DISCUSSION

In the recent past *A. albopictus* was invaded throughout the world by transport and international travel. However it is epidemiologically important because of its role in transmission of many viral pathogens. In this context, quick identification and control strategies of this species is pivotal. For this reason, the study was primarily focused on molecular identification of the species by partial mitochondrial COI gene for DNA barcode and then sequence comparisons and phylogenetic analysis were carried out. The isolated DNA was amplified and run on agarose gel as shown in the figure 3. Each lane includes sample from different spot along with 200bp DNA marker. The amplicons were fairly corresponded between 400bp and 600bp of DNA marker. Consequently, the PCR products were purified and subjected to commercial sequencing. Interestingly, the sequence was found to be novel and same was deposited in NCBI-GenBank nucleotide database; the accession number JX649856 was assigned. As an overview, this sequence is identified and discriminate Hyderabad specific *A. albopictus* species with that of species reported from other parts of India.

In addition, multiple sequence alignment facilitated sequence homology, conservation, and INDELS among Indian *A. albopictus* mosquitoes. Noticeably, deletions: single base deletion at 229, 252, 281 positions, three base deletions at 293position, and four base deletions at 196 position; insertions: two base insertion at 432 position, six base

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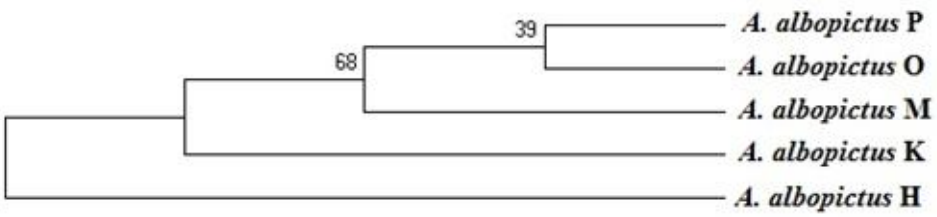

Figure 5

Molecular phylogeny of mitochondrial partial COI gene from *A. albopictus*. Maximum likelihood tree was extrapolating the evolutionary status of the Hyderabad species. However, it was found to be distinct and diverged as out group. H: Hyderabad; P: Pondicherry; K: Karnataka; M: Maharashtra; O: Orissa

insertion at 338, and seven base insertion at 368 position, were observed with frequent mismatches as shown in the figure 4. Finally, the evolutionary connection among Indian *A. albopictus* was depicted in figure 5. The ML tree infers that Pondicherry, Orissa and Maharashtra species in one cluster with separated Karnataka species. However, the Hyderabad species was diverged from others and shown as out group in the tree. From this study it is observed that, conceptually Hyderabad species is distinct from rest of the species.

4. CONCLUSION

Timely identification and control strategy of mosquitoes is global concern. To date, different parts of the Indian *A. albopictus* species partial mitochondrial COI gene sequences were reported except Hyderabad. Therefore, in this study, DNA barcode of Hyderabad species was uncovered and consequently DNA sequence was submitted to NCBI-GenBank nucleotide database. In addition, sequence alignment studies as well as phylogenetic analysis studies were attained to understand sequence divergence and evolutionary connection of the species. In summary, DNA barcode of Hyderabad species was reported and it can be used to identify *A. albopictus* species, further evolutionary connection with other Indian species was illustrated and found to be distinct.

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