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Molecular characterization of *Culex gelidus* from Hyderabad region of Telangana state, India

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Abstract
Background: *Culex* (*Cx.*) *gelidus* (Theobald, 1901) mosquito is an important primary vector for Japanese encephalitis virus (JEV). It is an outdoor biting, resting mosquito suggesting its exophagic and exophilic behavior. In absence of domestic hosts this mosquito bites voraciously and breeds in lentic oligotrophic and eutrophic waters with high concentration of organic matter. Identification of the mosquito is a cornerstone for any entomological investigations. Conventional taxonomical identification is the golden way to naming of any specimen but it requires expertise and has limitations such as identification of sibling species and identification with damaged specimens may not be possible, moreover leads to misidentifications. Maternally inherited mitochondrial cytochrome c oxidase subunit I (COI) gene has been proposed as a potential 'molecular marker' or 'barcode' for animal species identification.
Methods: In the present study mitogenomic marker (mtDNA-COI) was used to identify the mosquito species along with the phenotypic traits, which was supportive and alternate technique for classical taxonomy.
Results and conclusion: Phylogenetic tree was generated with COI gene based sequences, and results were shown that the Hyderabad *Culex gelidus* species having close relationship with Tamilnadu, Vietnam and Sri Lanka species.

Keywords: Japanese encephalitis virus (JEV), mitochondrial cytochrome C oxidase subunit I (COI), DNA barcoding and phylogenetic tree

Introduction
Culicidae family includes 3,581 mosquito species classified in two subfamilies and 113 genera. Among them the tribe Culicini (Diptera: Culicidae: Culicinae) is the second largest group of mosquitoes, with nearly 805 currently recognized species under four genera: *Culex* Linnaeus, *Deinocerites* Theobald, *Galindomyia* Stone & Barreto and *Lutzia* Theobald. *Culex* is by far the largest genus with 777 species allocated to 26 subgenera. In comparison, *Deinocerites* has 18 species, *Galindomyia* one species and *Lutzia* nine species [1, 2]. Numerous species of genus *Culex* are known vectors for many diseases. *Culex* (*Cx.*) *gelidus* (Theobald, 1901) is an important primary vector for Japanese encephalitis virus (JEV) [3, 4] in Southeast Asian countries [5] and also transmit other viruses of public health importance, *viz.* Ross River virus (RRV), Getah virus (GETV), Sindbis virus (SINV) and Tembusu virus (Bunyamwera) [6, 7, 8, 9]. Active during Crepuscular time [10] and prefers to feed throughout the night, with maximum biting frequency at just before the dawn [11, 12, 13].
Culex gelidus is an outdoor biting, resting mosquito suggesting its exophagic and exophilic behavior. Voracious biter of humans in absence of domestic animal hosts such as pig, cattle etc., [14, 15, 16, 17]. Breed in lentic oligotrophic and eutrophic waters with high concentration of organic matter, *i.e.* marshes and waste water canals, animal watering troughs, dairy and piggery wastewater ponds, sewerage overflow ponds and flooded cattle pasture [18, 19, 20].
To overcome the limitations of inherent classical taxonomical identification, micro genomic identification system is one of the best alternative techniques for the species identification [21, 22]. Small fragment of genome (marker or barcode) can diagnose biological diversity. Unlike nuclear DNA, there is usually no change in mtDNA from parent to offspring. The rapid mutation rate (in animals), maternal inheritance, inability to recombine and high copy number makes mtDNA useful for identifying different species. Insect mitogenome is a double stranded circular DNA molecule of approximately 14.5kbp to 19.5 kbp. A typical insect mitogenome encodes 37 genes of which, 13 are protein coding genes, 22 transfer RNAs and 2 ribosomal RNAs. With compare to other mitochondrial markers, COI gene is a potential barcode for species identification and phylogenetic relationships. A 658-bp region (the Folmer region) of the mitochondrial cytochrome c oxidase subunit I (COI) gene was proposed as a potential 'molecular marker' or 'barcode' for animal species identification [23]. In the present study mitogenomic marker (COI) was used to identify the mosquito species along with the phenotypic traits, which was supportive and alternate technique for classical taxonomy.

Material and Methods
Study area
Hyderabad is the financial and technological capital of the state of Telangana, and located on the Musi River. As Hyderabad is totally an urban district there are no villages but the district has one Census town (Osmania University), one Cantonment Board (Secunderabad) and One GHMC (Greater Hyderabad Municipal Corporation). Hyderabad, Telangana, India is located at India country in the cities place category with the GPS coordinates of 17^0 23'6.16" N Latitude and 78^0 29'12.02"E Longitude geo coordinates, and occupies an area of approximately 360 square kilometres.

Mosquito collection and identification

According to the WHO standard methods [24] mosquito samples were collected from Banda Cheruvu, Moula Ali, ECIL Hyd (17.459612° and 78.548788°). And the morphological characterization of the mosquito species has been performed with the help of standard keys such as [25, 26, 27].

DNA extraction and PCR amplification

Adult mosquitoes were collected with oral aspirator and brought to the laboratory [28]. The samples collected were analysed for their systematic position through morphological characters and stored in 95% ethyl alcohol at -20^{0}C for DNA extraction. Asghar *et al.,* (2015) [29] proposed methodology was used for obtaining the genomic DNA. PCR reactions were performed to amplify a 658bp fragment of COI by using forward (C1-J-1718-5'-GGAGGATTTGGAAATTGATTAGTTCC-3') and reverse (C1-N-2191-5'-CCGGTAAAATTAAAATATAAACTTC-3') primers. Standard PCR conditions were maintained (initial denaturation at 94˚C for 2 minutes, cycle denaturation at 95˚C for 30 seconds, primer annealing at 55˚C for 30 seconds, extension at 70˚C for 30 seconds, and final extension at 70˚C for 10 minutes). The amplicon was confirmed in agarose gel (1%) electrophoresis and the product was given for commercial sequencing (Bio serve Biotechnologies Pvt. Ltd.). The PCR product of COI gene was amplified and the amplicons were sequenced by Sanger's procedure automated sequencing method.

Multiple Sequence alignment and phylogenetic analysis

Multiple sequence alignment (MSA) was performed in Clustal W [30, 31] for partial COI gene sequence of 489bp with the default parameters. The pattern of the evolution of mosquito specimens was studied by phylogenetic analysis. The results of the DNA barcoding were made progressively with the phylogenetic analysis by the construction of a phylogenetic tree. For phylogeny, the available nucleotide sequences from National Center for Biotechnology Information (NCBI) were collected. In MEGA 7 tool [32, 33, 34] the analysis of the phylogeny was attained by maximum likelihood method with the deletion of gaps and missing data. Furthermore, Bootstrap replication (1000 numbers) was used to validate the tree.

Results and Discussion

Fragment of COI region was amplified by PCR, a single discrete PCR amplicon band of ~500 bp was observed when resolved on agarose (Figure-1). The obtained sequence (467bp) of CO I gene was blasted with NCBI Gene Bank which had shown 100% similarity with the test specimen and the NCBI gene bank accession number is MG686504 (Figure-2). The morphological characters of the test specimen (Pale rings present on proboscis and tarsi, lower mesepimeral setae absent, wings do not have any pale spots or pale scales, abdominal terga II-VIII with dark and pale bands or completely dark, abdominal terga II-VI with basal pale bands, anterior 0.7 of scutum covered with pure white scales, anterior surface of fore-and midfemora without speckling of pale scales, scales on prescutellar space, behind wing base, and on scutellum entirely dark, pale band on proboscis narrow and less than length of basal dark area) were compared with molecular characterization and the species was confirmed as *Culex gelidus* (Figure-3). The adult specimen was deposited as the voucher specimen at the Department of Zoology, Osmania University, Hyderabad (Accession No: OUNHMZ-2019.M7).

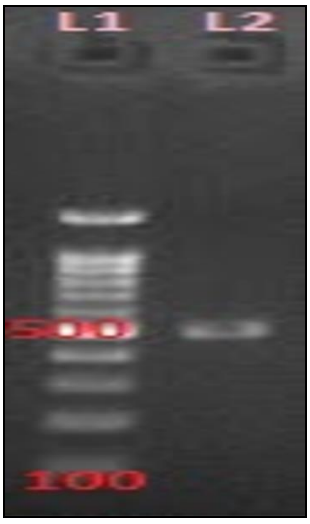

Fig 1: Gel electrophoresis of mitochondrial COI gene isolated from Hyderabad *Culex gelidus.* Lane 1: 100 bp DNA ladder; lane 2: PCR product of COI gene isolated from the Hyderabad region. The amplified product of 467bp was corresponded at 500bp.

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>MG686504.1 Culex gelidus voucher OUNHMZ.ART.2016.M7 cytochrome oxidase subunit I (COI)
gene, partial cds; mitochondrial
GATATAGCATTTCCTCGAATAAATAATATAAGTTTTTGAATACTTCCTCCTTCATTAACTTTACTACTTT
CAAGTAGTTTAGTTGAAAATGGAGCTGGAACTGGATGAACAGTTTATCCCCCTCTTTCATCAGGTACAGC
TCATGCTGGAGCTTCAGTTGATTTAGCTATTTTTTCTTTACATTTAGCTGGGATTTCATCAATTTTAGGA
GCAGTAAATTTTATTACAACAGTAATTAATATACGATCTTCAGGAATTACACTTGATCGAATACCTTTAT
TTGTTTGATCTGTAGTTATTACTGCTGTTTTATTACTCCTTTCATTACCCGTATTAGCTGGAGCTATTAC
AATATTATTAACTGATCGAAACCTAAATACTTCATTTTTTGACCCTATTGGAGGAGGAGATCCTATTTTA
TACCAACATTTATTTTGATTTTTTGGACATCCAGAAGTTTATATTTT
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Fig 2: Partial mitochondrial sequence (COI gene) of *Culex gelidus* with accession number MG686504.

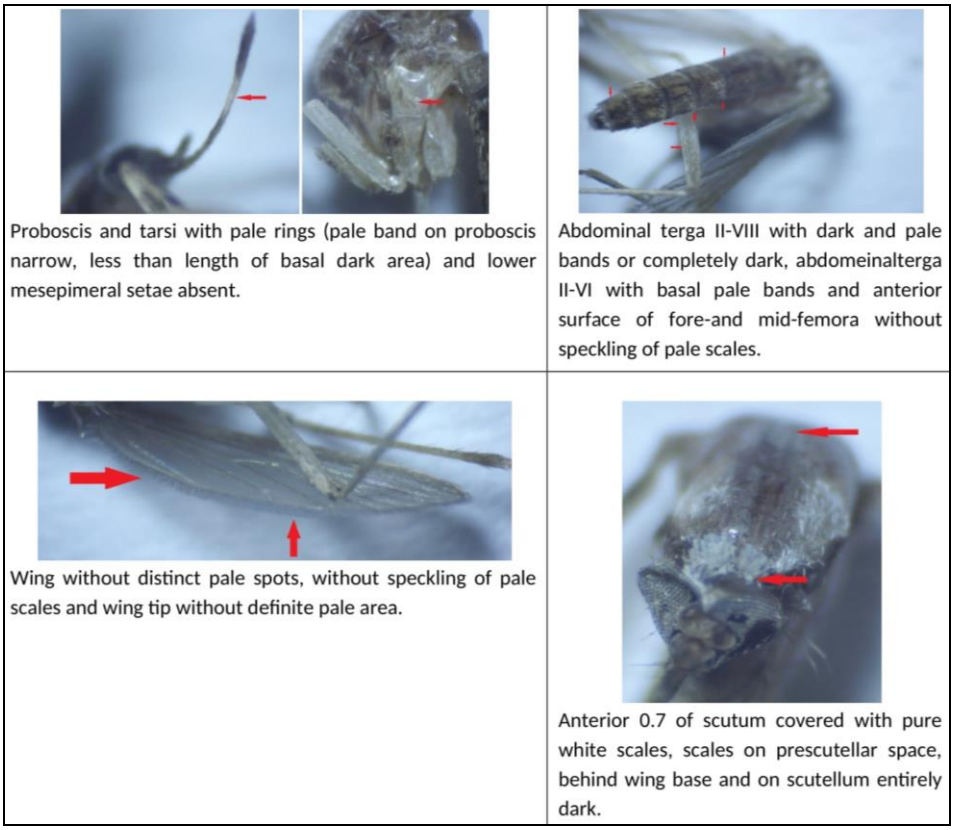

Fig 3: Morphologic identification characteristic features of *Culex gelidus.*

Kiefferulus tainanus [NCBI accession number DQ648225 (Diptera: Chironomidae)] was used as out group for assessing the phylogenetic relationship. Boot strap method with 1000 boot strap replications was used to validate the tree.

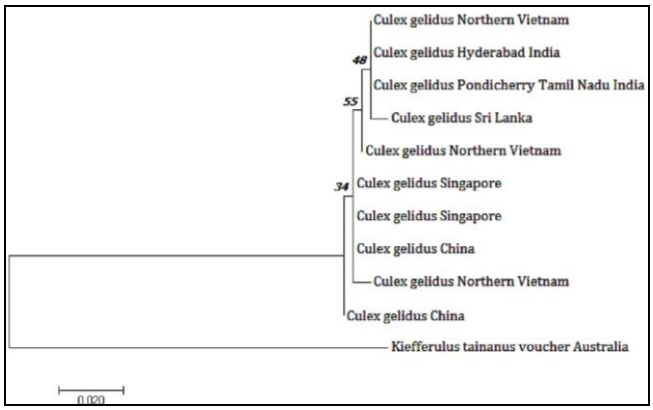

Fig 4: Molecular phylogenetic analysis of *Culex gelidus* by maximum likelihood method (Conducted in MEGA7).

Phenetic system of classification based on morphological traits is an outstanding method for identifying the any organism at species level. In some exceptions it has few limitations which may leads to miss identification of particular species. DNA barcoding technique is an emerging alternative technique for such inherent taxonomical miss identifications. 658bp mitogenome is a potential region for studying the taxonomy and phylogentic relationship of animal diversity. Phylogenetic tree has been generated with COI gene based sequences, and results showing that the Hyderabad *Culex gelidus* species having close relationship with Tamilnadu, Vietnam and Sri Lanka species. Maximum likelihood tree is showing that, the Hyderabad, Tamil Nadu, Sri Lanka and Vietnam mosquito species are in one cluster, which means all are having close relationship and Hyderabad species is sister species for other mosquito species (Figure-4).

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