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Molecular phylogenetic analysis of *Culex sitiens* (Diptera: Culicidae) based on the mitochondrial COI sequence

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Abstract
Culex is mosquito commonly known to transmit several arboviral diseases which have a significant impact on human health. An earlier study shows the dominancy of Culicine having 57 mosquito species under 12 genera were in coastal regions of Odisha state. *Culex sitiens* Weidmann is a proficient coastal vector of the Japanese encephalitis virus (JEV). Bio-ecology of mosquito vector and their precise identification is of paramount importance to develop species specific vector control strategies. In this study, molecular identification of *Cx. sitiens* was made using mitochondrial COI after collection. Genetic divergence study was performed to analyze the intra-specific distance among the studied mosquito species. Additionally, a haplotype network was constructed based on the nucleotide differences, to determine the genealogical relationship between haplotypes. The current study shows the abundance and efficiency of use of the mt COI gene marker which strongly supports the positioning of *Cx. sitiens* by clustering with their respective group. Further, the study of population genetics illustrated the occurrence of genetic differentiation within the population and the haplotype network study suggested that the Indian *Cx. sitiens* population shows a certain level of genetic differences.

Keywords: *Culex sitiens*, molecular phylogeny, COI, India

Introduction
The family Culicidae is a huge and abundant group of mosquitoes that occurs all through tropical and temperate regions of the world. India, a country with diverse climatic conditions supports the breeding and survival of mosquito vectors. It is ranked fifth in mosquito biodiversity [1] and includes 393 species grouped between 49 genera and 41 subgenera [2] out of which an online systematic catalog of Culicidae listed 356 mosquito species in India [3]. Culex is one of the largest and important groups of mosquitoes with 770 species in 26 subgenera [4]. Among them, *Culex sitiens* mosquito is broadly dispersed throughout the Afro-Eurasian coastal region of several countries like India, Burma, Bangladesh, Iran, Oman, Saudi- Arabia, Malaysia, Vietnam, Australia, Myanmar, Cambodia, New Zealand, Thailand, Indonesia, Papua New Guinea and eastern Africa [5-8]. The presence of *Cx. sitiens* was likewise reported in the catalogue of Indian mosquitoes by Tyagi *et al*., in 2014 and Bhattacharyya *et al*., in 2014 [9, 2]. *Cx. sitiens* subgroup contains at least eight morphological species [10]. Some of its individuals engaged with the transmission of medically significant arboviruses [11]. Among them, *Cx. sitiens* Weidmann is an efficient coastal vector of Japanese encephalitis virus (JEV) [12, 13]. In 1994, *Cx. sitiens* was found to be positive for JE virus in Malaysia [14]. Henceforth, viable control of mosquito vectors requires an assortment of entomological information, including proper identification, behavior, biology and ecology of this vector. On account of the covering morphological attributes of this *Cx. sitiens* subgroup, it gets hard to recognize every individual from this subgroup depending on their morphological features.
Classically, the mosquito vector identification depends on the description and investigation of morphological structures yet the number of trained taxonomists in species identification has been consistently declining. Additionally, various mosquito species identification is challenging as in most of the cases as the key morphological features are often damaged during collection and storage and are not available in all developmental stages.

Thus, to comprehend the infection cycle of the mosquito-borne disease, it is important to recognize different species of the mosquito vector even from the damaged specimen. Therefore, the ongoing investigations indicate the necessity of continuous surveillance and identification of globally distributed mosquito species by the combined use of traditional and DNA-based techniques, to achieve a better assessment of mosquito faunal diversity.

In recent times, DNA barcoding approach employed to compare and differentiate closely related species by amplifying and sequencing a small conserved region of DNA. DNA barcoding approach is greatly facilitating and compliment the taxonomic study. This method has been utilized in various taxonomic investigations on dipteran taxa [15, 16] including mosquito vectors [17-20]. Molecular taxonomic analysis on local strains of mosquitoes was carried out in Canada [21], India [22], Pakistan [23], Persian Gulf region [24] and China [25], showing the utility of DNA barcoding in the Culicidae family. There is no current adequate information available on mosquito faunal diversity of southern districts of Odisha state and therefore, we have considered this region as our study area. The principal goal of the present study was to distinguish the Culex mosquito using mt COI gene. Adding to this, the study also focused on the analysis of genetic variation and molecular phylogeny among the *Cx. sitiens* mosquito from various regions with the mosquitoes of the studied area.

Materials and Methods

Collection and Identification of Mosquito

A survey of various mosquito breeding habitats was carried out for the updation of mosquito faunal diversity in some densely populated southern districts of Odisha state, Indian Fig- 1. Mosquitoes in their different developmental stages were collected throughout the year by using a variety of standard procedures from multiple locations and transported to the laboratory for identification. Morphological identification of all the field-collected samples was performed using the available identification keys [26, 9]. After successful identification; the mosquito samples were vouchered and stored for future study.

Fig 1: Location of the mosquito collection site

Genomic DNA isolation

DNA was extracted from the whole adult mosquito by the Bender Buffer method [27]. Further, the isolated DNA was used as a templet for the amplification of the mitochondrial COI gene. DNA amplification was carried out using previously described primer pairs [28]. The reaction mixture consisted of 1X PCR buffer, 0.5 U Taq DNA polymerase, 2.5mM $MgCl_2$, 200 µM dNTPs, 10pmol of each primer, 100 pmol template DNA, total dilution was made up to 25 µl. The thermal profile consisted of an initial cycle of 95 °C for 5min followed by 35 cycles of 95° for 30sec, 45°-55° for 30sec and 72° for 1minwith a final extension step of 7 min at 72 °C.

DNA Sequence analysis

The trace files of COI sequences were edited and assembled using Geneious version 9.0.5 (Biomatters Ltd, Auckland, NZ) (http://www.geneious.com) software and sequences of low quality were excluded at the time of data analysis. At least three specimens were sequenced for *Cx. sitiens* species and the good quality sequence was selected for further analysis in the present study. The generated nucleotide sequence was compared with barcode sequences on NCBI using nucleotide Basic Local Alignment Search Tool (BLASTn), and the final obtained sequence was submitted to NCBI to get the accession number (MW421872). Further, to resolve the genetic relationship among different species, some COI sequences representing the same and related species of Culex taxa under study were retrieved from GenBank as replicate data for evaluating the taxonomic position of our target species.

To conduct multiple sequence alignment (MSA), the Clustal W algorithm was implemented in software package MEGA X [29]. The transition/transversion (ts/tv) bias (R) and pairwise genetic sequence divergence was inferred using Tamura 3 Parameter (T_{92}) model and Kimura 2 parameter model (K_2P) respectively. The intra-population polymorphism of COI sequences was determined using DnaSp v6.12.03. The number of variable sites, nucleotide diversity, number of haplotypes, haplotype diversity was analyzed. Furthermore, the phylogenetic analysis of the COI sequence was carried out with a published set of sequences of different mosquito species. The phylogenetic tree was used for the mapping of the gene phylogenies by Maximum Likelihood (ML) with the studied COI gene of Culex mosquito species from various geographical locations using MEGA X software. The support for nodes in ML analysis was evaluated with 1000 bootstrap replicates. ML tree was used as a framework to compare sequence divergence of COI gene fragments separately and the scores for log-likelihood were calculated in MEGA X.A haplotype network was constructed based on the nucleotide differences, using a TCS network algorithm to determine the genealogical relationship between haplotypes [30].

Results

Different developmental stages of mosquitoes were collected from various sites of the study area. The larval stages were reared to the adult stage for morphological identification. All the adult *Culex sitiens* mosquitoes were identified morphologically based on the available standard identification keys. Then, to reconfirm the morphological identification, the DNA extraction was made and the COI gene sequenced. The length of the generated COI sequence of *Culex sitiens* was 687bp. The generated COI sequence was with 71% of AT content and with the GC content of 29%. The transition and transversion bias (R) of the COI gene sequence in *Cx. sitiens*

was 1.93. The number of transversions of COI sequence between A and T was 5.30 which were higher compared with the transversions of G and C which was 2.26. The number of transitions of COI sequence between A and G was same as C and T (24.44). The genetic divergence among the *Cx. sitiens* mosquito was determined and the species displayed discriminative estimations of intra-specific divergence. The intra-specific COI distance among *Cx. sitiens* ranges from 0-0.039, with an average value of 0.013 which was less than the threshold value of 2% (0.02) (Fig. 2). Further, the T_{92} + G (Tamura 3 Parameter model with gamma distribution parameter) was found to be the best-fit substitution model for the COI gene-sets based on the BIC score, for the Maximum-Likelihood (ML) analysis. The log-likelihood value of the resultant ML tree for mt COI gene-sets of *Cx. sitiens* was estimated to be -2917.07. In the resulted phylogenetic tree, most of the sequences were clustered with their respective group of species (Fig. 3).

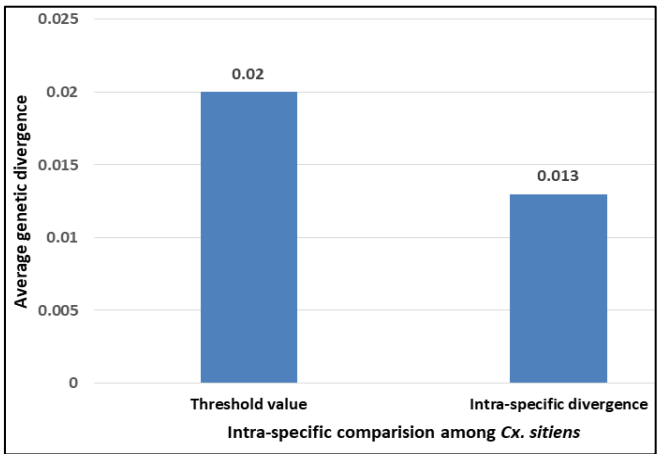

Fig 2: Estimation of Intra-species genetic divergence of the COI gene marker in *Cx. sitiens* mosquito species

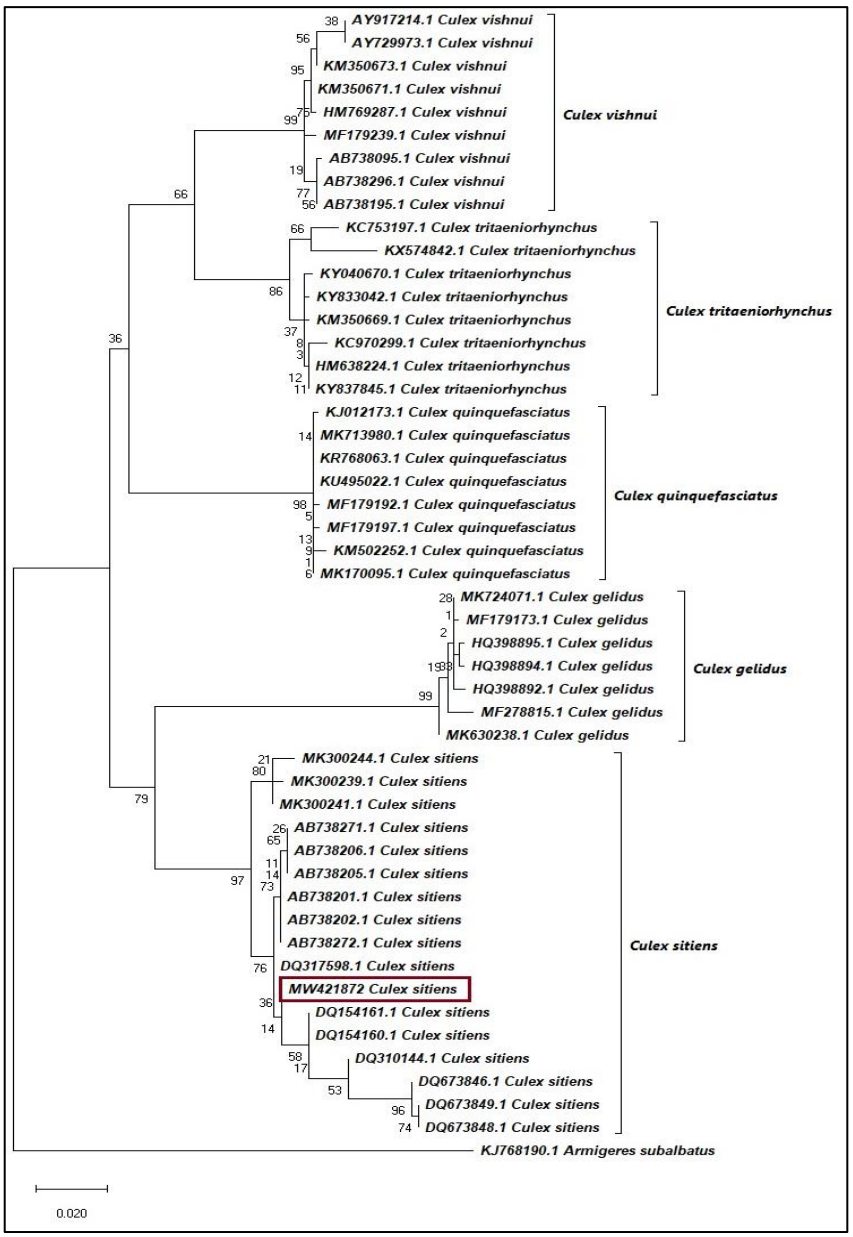

Fig 3: Phylogenetic positioning of *Culex sitiens* Maximum-Likelihood subtree of Culex obtained from the alignment of COI genes. A sequence of *Armigeres subalbatus* was used to root the tree. All the sequences are tagged with GenBank accession no. and the sequence within the box indicates the generated sequence

Haplotype diversity (Hd) and nucleotide diversity (Pi) were used to study the degree of haplotype differentiation and nucleotide sequence variation within the species. In *Culex sitiens*, the total number of mutations and the number of haplotypes were found to be 12 and 7, respectively. The haplotype diversity (Hd), nucleotide differences (k) and nucleotide diversity (Pi) were found to be 0.809, 3.250 & 0.009 respectively.

Additionally, the TCS network was formed based on the similarity and divergence of *Cx. sitiens* COI sequences (Fig. 4). The TCS network comprises seven groups based on the haplotype identity, which were represented as S1-S7. The identical sequences clustered in S1, S2, S4, S7 were with three, seven, two, two haplotypes, respectively. The S2 and S7 clusters contain Indian haplotypes. Our studied sequence clustered in S2 with the Indian population. The largest S2 cluster neighbored by S1 and S3 which were differed from S2 by a single mutational step. A bifurcation from S3 cluster was observed. The S6 cluster was differed from the S3 by five mutational steps and S7 by six mutational steps. Similarly, the S4 cluster differs from S3 by a single mutational step and S5 by three steps from the S4 cluster.

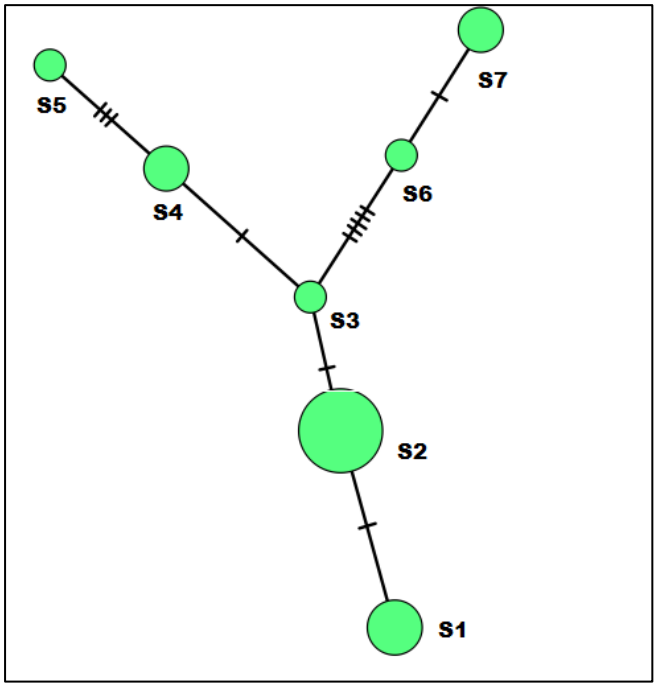

Fig 4: TCSnetwork constructed based on mitochondrial COI gene of *Culex sitiens* population. Each sphere indicates the identical haplotypes, the size of the sphere is proportional to the number of haplotypes

Discussion

Culex sitiens, a vector of Japanese encephalitis, is native to different coastal regions since it utilizes salt and brackish water as its larval habitats [6, 7]. Therefore, the unequivocal identification of mosquito specimens is one of the backbones for mosquito vector surveillance programs and major attention should be given to their phylogenetic and genetic diversity study. A prior study shows the morphological changes in the wing pattern of *Cx. sitiens* from the coastal region of Thailand and their biology and ecology such as biting activity and breeding habitat of *Cx. sitiens* were studied by Chaiphongpachara *et al*. in 2019 [8] and by Prummongkol *et al*. in 2011[31]. Earlier, Jansen *et al*. in 2013 used both morpho-taxonomy and PCR-based technique using ITS1 region for reliable identification of *Cx. sitiens* subgroup in Australia [11]. The use of genetic markers provides an idea about the population structure, genetic differentiation and gene flow of species, which is a fundamental component to design control strategies of vector-borne disease [32].

DNA-based approaches to identify mosquito [33-35], phylogenetic analysis [36] and genetic diversity [37] have gained increasing adoption in recent years as it is faster to perform and more reliable. Phylogenetic trees utilized for analysis of gene duplication, estimating rates of diversification, polymorphism, recombination, population dynamics and inferring organismal phylogenies by combining it with other publicly available data sources. Earlier studies have proved the use of the mitochondrial COI marker in finding more biodiversity and increasing species richness than traditional taxonomic approaches by uncovering undescribed and cryptic species [38-40]. This COI gene as a molecular marker is used to infer the phylogeny of various dipteran taxa including the Culex mosquitoes [41]. In the current study, we utilized the mtCOI gene for *Cx. sitiens* identification. As mentioned earlier, the studied species was confirmed by comparing those publicly available barcode sequences on NCBI using BLASTn tool. We observed that the composition of the generated COI sequence was AT-rich which is similar to the findings of Cywinska *et al* (2006); Rivera and Currie (2009) [21, 42] on dipterans.

The transition and transversion bias (R) were determined to understand the pattern of DNA sequence evolution, genetic distance estimation and phylogenetic reconstruction. In this study, the R-value of the COI gene of *Cx. sitiens* was found to be 1.93 which was greater than the threshold value (0.5) which indicates there is no bias in transitional and transversional substitution and both can be equally probable. Based on COI sequence set, the pairwise genetic divergence of 2-3% has been considered as the threshold value to differentiate two species [38, 43] and even for the two closely related mosquito species [22, 44, 45]. Here, the intra-specific K_2P genetic divergence was found to be 0.013 (1.3%) which is less than the threshold value (2-3%). Furthermore, to confirm the morphological identification and to obtained a taxonomic position of the selected taxa, the phylogenetic analysis was carried out. In the ML tree, the mtCOI based analysis strongly supports the positioning of *Cx. sitiens* by forming a distinct cluster with their respective group. Based on the automatically generated initial tree, a final ML tree with the highest log-likelihood value with a well-supported bootstrap value provides a greater probability. The computed ML tree was in general agreement with the morpho-taxonomy as reported previously [38, 43, 46].

Study on the population genetic study illustrated the occurrence of genetic divergence within the population. The haplotype diversity, nucleotide diversity and genetic distance are all indicators to reflect the genetic diversity of the population. In the TCS Network diagram, our studied *Cx. sitiens* sequence grouped with the S2 cluster with the Indian populations. Additionally, this examination suggested that the Indian population shows a specific level of genetic divergence, possibly due to a variety of climatic regions ranging from temperate to tropical.

In conclusion, our study proved the abundance of *Culex sitiens* in the southern district of Odisha and also the efficiency of the utility of COI genetic marker in the DNA

barcoding technique for mosquito vector identification. The use of the standard mtCOI gene is believed to be effective and rapidly finds more diversity of species. This study also adds valuable information about the systematics and molecular biology of *Cx. sitiens* acting as a vector for Japanese encephalitis in various Asian continents. Furthermore, the generated COI sequence possibly be used as a reference nucleotide sequence of the respective haplotypes in future mosquito identification studies and will facilitate the con-specific comparison to reveal the appropriate reason for high intra-specific divergence.

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Conflict of interest
The authors declare no potential conflict of interest with respect to the research, authorship and/or publication of this article.

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