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Research Article

Morphotaxonomic and DNA barcoding analyses of mosquitoes collected from Chattogram Metropolitan area

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ABSTRACT

Mosquitoes are important vectors for a wide variety of pathogens including parasites. Identification of different mosquito species are crucial for their effective control. We have used the classical morphometry and DNA barcoding approach for identification of 20 mosquitoes randomly collected from different locations of Chattogram Metropolitan area of Bangladesh. Different morphological features of several body parts of the mosquitoes were examined using compound and stereo microscope. The morphological characteristics of the mosquitoes showed high similarity with the *Aedes and Culex* species. For DNA barcoding, genomic DNA was extracted from all of the 20 samples using commercial kits and specific primers were used for amplification of partial cytochrome oxidase (COI) gene for molecular characterization. PCR products were then sequenced followed by bioinformatics analyses. Sequence similarity based BLASTn and phylogenetic analyses (MEGA6) of the sequence indicated similarity with three different species namely *A. aegypti, A. albopictus* and *C.pipiens*. The findings were complimentary with the morphological data and reliably characterized the mosquito species. The study showed the feasibility of using molecular tools for authentic characterization of different insects and vectors that can be used for year-long survey in any part or region of the country.

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1. INTRODUCTION

Various mosquito species are responsible for high nuisance and transmission of deadly pathogens including parasites. These include arboviruses, malaria and filariasis, to humans and animals (Naddaf *et al.*, 2012). In most cases, an experienced taxonomist and suitable methods are required for reliable morphological identification. Sometimes identification can be confusing or biased when the morphological features are not unique and several species have similar morphology. Classical studies indicated that most of the taxonomic keys can be limited in case of adult stage and fourth instar larvae due to unknown morphological features. These limitations may hinder

the application of taxonomic keys for reliable identification of a particular species. To overcome these limitations of morphotaxonomy, complementary approaches like molecular DNA barcoding is available which can help identify the mosquitoes at genus and species level (Chan *et al.*, 2014; Batovska *et al.*, 2016; Murugan *et al*, 2016). A unique gene, Cytochrome Oxidase I (COI) is usually used for DNA barcoding and has been reported by previous authors. Molecular study carried out on endemic Australian mosquitoes demonstrated the potential of DNA barcoding with further details on geographical distributions and genetic diversity of species (Foley *et al.*, 1998; 2007). Similar study has been reported in India as well. However in Bangladesh no study has been reported so far to employ DNA barcoding tools to identify mosquitoes. Therefore, the present study was designed to identify the mosquito specimens by observing the morphological features as well as genetic characterization through using the modern DNA barcode technique.

2. MATERIALS AND METHODS

Study area

The proposed study was conducted at CVASU while samples were collected from different parts of Chattogram Metropolitan area of Bangladesh. The samples were collected during January to June, 2019.

Sample collection and Microscopic examination

The whole mosquito or larva or pupa was collected from mosquito breeding sites. Morphological features of the mosquitoes were observed under the stereo binocular microscope at 10X, 20X and 100X magnification.

DNA extraction, PCR amplification and sequencing

Total genomic DNA was isolated from single whole mosquito sample using the tissue genomic DNA extraction mini-kit (Favorprep®, Taiwan) according to manufacturer instructions. For DNA barcode analysis, the 735 bp region of mitochondrial COI gene was targeted and amplified with the following primers: forward 5´-GGATTTGGAAATTGA TTAGTTCCTT-3´ and reverse 5´-AAAAATT TTAATTCCAGTTGG AACAGC-3´ (Kumar *et al.* 2007). The polymerase chain reaction (PCR) was carried out in a thermo cycler (Applied Biosystem Inc., USA). The 25µl PCR reaction consisted of 4µl of extracted DNA, 2µl of each primer, 12.5µl master mix (2X) and 4.5µl nuclease free water. PCR reaction conditions was as follow: An initial denaturation of 5 min (95°C) was followed by five cycles of 94°C for 40s (denaturation), 45°C for 1min (annealing), and 72°C for 1 min (extension) and 35 cycles of 94°C for 40s (denaturation), 51°C for 1 min (annealing), 72°C for 1 min (extension), final extension at 72°C for 10 min. The amplified PCR product was visualized in 1.5 % agarose gel electrophoresis. After completing of PCR reaction, it was stored at 4°C. Sequencing was performed by using ABI 3500 XL Genetic Analyser (USA) through commercial suppliers (Biotech Concern Ltd.). The sequences were trimmed and edited using Clustal W and Bio Edit v.7.2.5 and submitted to GenBank database of National Center for Biotechnology Information (NCBI) in USA.

Sequencing and Phylogenetic Analysis

Freely available Chromastool (https://technelysium.com.au/wp/chromas/) was used for analyzing the sequencing data that was confirmed through BLAST searching. The COI sequences of *Aedes* and *Culex* isolates submitted by other investigators were retrieved from the NCBI database. The Clustal omega platform (http://www.clustal.org/omega/) was used for the alignment of DNA sequences. Sequence divergences were determined among the individual species by using of Kimura two parameters (K_2P) distance model. The neighbor-joining (NJ) method in MEGA 6 was used for estimating of average evolutionary divergence. As a number of base substitutions per site by averaging over all sequence pairs within and between each group, the average evolutionary divergence was estimated.

3. RESULTS

The body length of most adult mosquito is about 2.9 to 7 mm. Males are comparatively smaller than females. In case of male mosquito, palps are small and tipped with silver or white scales as well as plumose antennae (Fig 1). But in female, sparse short hairs are present on antennae. In male, modified mouth parts are observed under microscope that can be used for nectar feeding organ where as the mouthparts are used as blood feeding organ in female. Other notable features were dark proboscis, clypeus with two clusters of white scales, the dorsal part of the thorax has white scales which forms a lyre or violin shape.

The adults of *Culex* species are usually unicolorous mosquitoes. Some species of *Culex* subgenus possesses markings on legs as well as pale spots on their wings. The distinct pulvilli and the absence of prespiracular setae as well as post spiracular setae are the

Figure 1. Larval stage of *Aedes albopictus* (a), *Culex sp* (b) and adult mosquito of *Aedes aegypti* (c) In figure (a) and (b) arrows indicate siphon which is comparatively larger in *Culex sp* than *Aedes sp.* In figure (a) arrowhead indicates the single row of anal comb at the last segment. In figure (c) Two straight lines bordered by curved lyre-shaped lines on the side is the distinguishing feature of *Aedes aegypti.*

identifiying characteristics. The characteristic thorax of *A. aegypti* is larger in female than male. The average length and width of thorax in female is near about 0.5±0.08mm and 0.35±0.07 mm but in case of male is near about 0.41±0.06mm and 0.29±0.02 mm. The thoracic region of *A. aegypti* is dark brown or black in color and thorax has three segments such as pro, meso and meta which consists of wings, legs and halters although white scale patches are present in both sexes. There is a lyre shaped white scales marking on dorsum and two longitudinal lines between the marking. Identification of adult *A. albopictus* mosquitoes were confirmed by observing the distinct silver white scales and bold black shiny scales on the palpus and tarsi (Hawley 1988). *A. albopictus* is a medium-sized mosquito ranging from 2.0 to 10.0 mm with a striking white and black pattern (Huang, 1968). The antennae of the male are plumose and they have modified mouthparts for nectar feeding. The dark scales covered the abdominal tergites. The black legs have white basal scales on each tarsal segment. The larva has single row of comb scale (Figure 1).

For molecular identification, mtCOI region was amplified by using the protocols described earlier. The

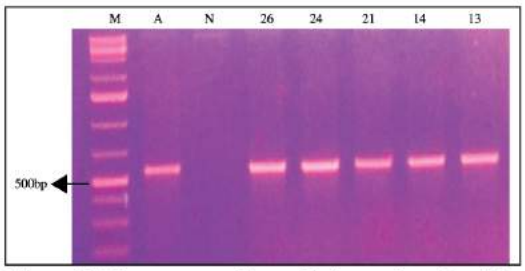

Figure 2. The corresponding gel electrophoresis of the PCR analyses showing positive bands at 520 bp region. M: DNA Ladder, A: Positive control, N: Negative control, Remaining lanes contain isolates from respective samples.

PCR product of 520 bp fragment (Fig. 2) of COI gene was sent for sequencing through commercial suppliers. To know their nucleotide identity, COI sequences were checked by BLASTn analysis which confirmed them as *A. aegypti*. Partial COI sequences have been submitted to NCBI for future references (GenBank Accession no. MH836623, MH885495, MH885496). BLAST analyses confirmed the identity of the mosquito species as *A. aegypti, A. albopictus* and *C. pipiens* as the available species. Among 5 samples only three were *A. aegypti* (CVASU-21, CVASU-24 and CVASU-26), one was *A. albopictus* (CVASU-14) and one was *C. pipiens* (CVASU-13).

To know the phylogenetic relationships amongst *A. aegypti* isolates, COI sequences was aligned with their respective counterparts from different region of the world. The sequences of seven globally isolated *A. aegypti* were selected for constructing evolutionary tree by NJ method using K_2P model with 1000 bootstrap value. In case of COI, the evolutionary divergence among Indian strains of *A. aegypti* and global strains was analyzed separately by using phylogenetic tree. However, globally it comes in first clade along with Thailand, Brazil and Martinique isolates. Using MEGA 6, different number of sequences used to produce three different phylogenetic tree (Figure 3,4,5) using Neighbour joining method and being studied are compared and tentative measures of similarity is derived, represented by distance matrix. The branching patterns of these trees are used to determine the most closely related pair of sequence. These phylogenetic trees represented two main branches of the phylogenetic relationship between sequences. The final nodes (leaves of the tree) represent existing sequences where as internal nodes represent hypothetical ancestor. Despite similarity

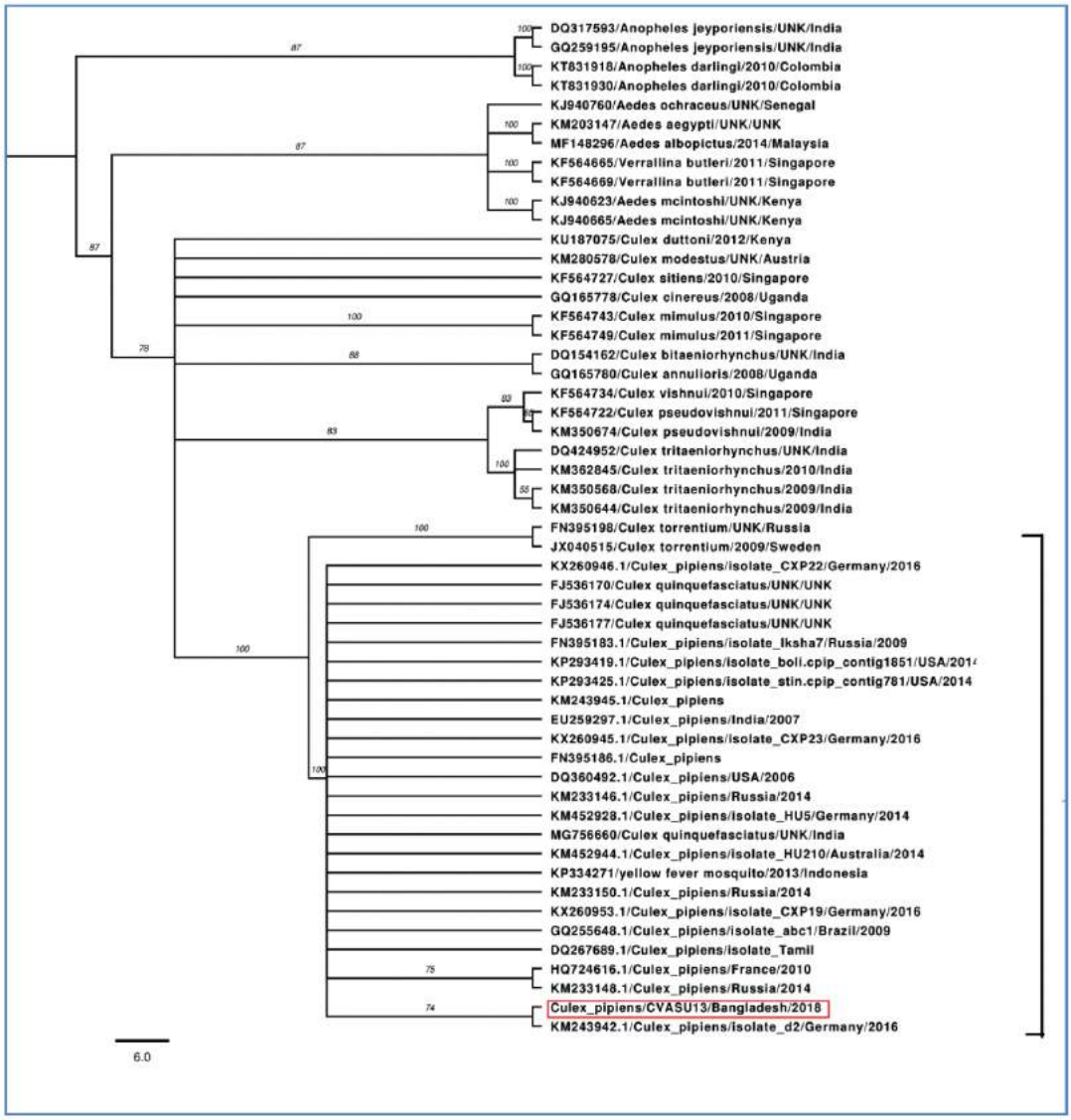

Figure 3. Phylogenetic tree based on partial nucleotide sequences of the COX-I gene of *C. pipiens* from Chattogram Metropolitan Area. The numbers of adjacent each branch represents the value of consensus support (of 100 replicates) for the right of the node. The isolates (tree taxa) from this study indicated by colored boxes. For all other taxon level, annotations were presented as Accession No. /Species Name/Country of collection/year of collection.

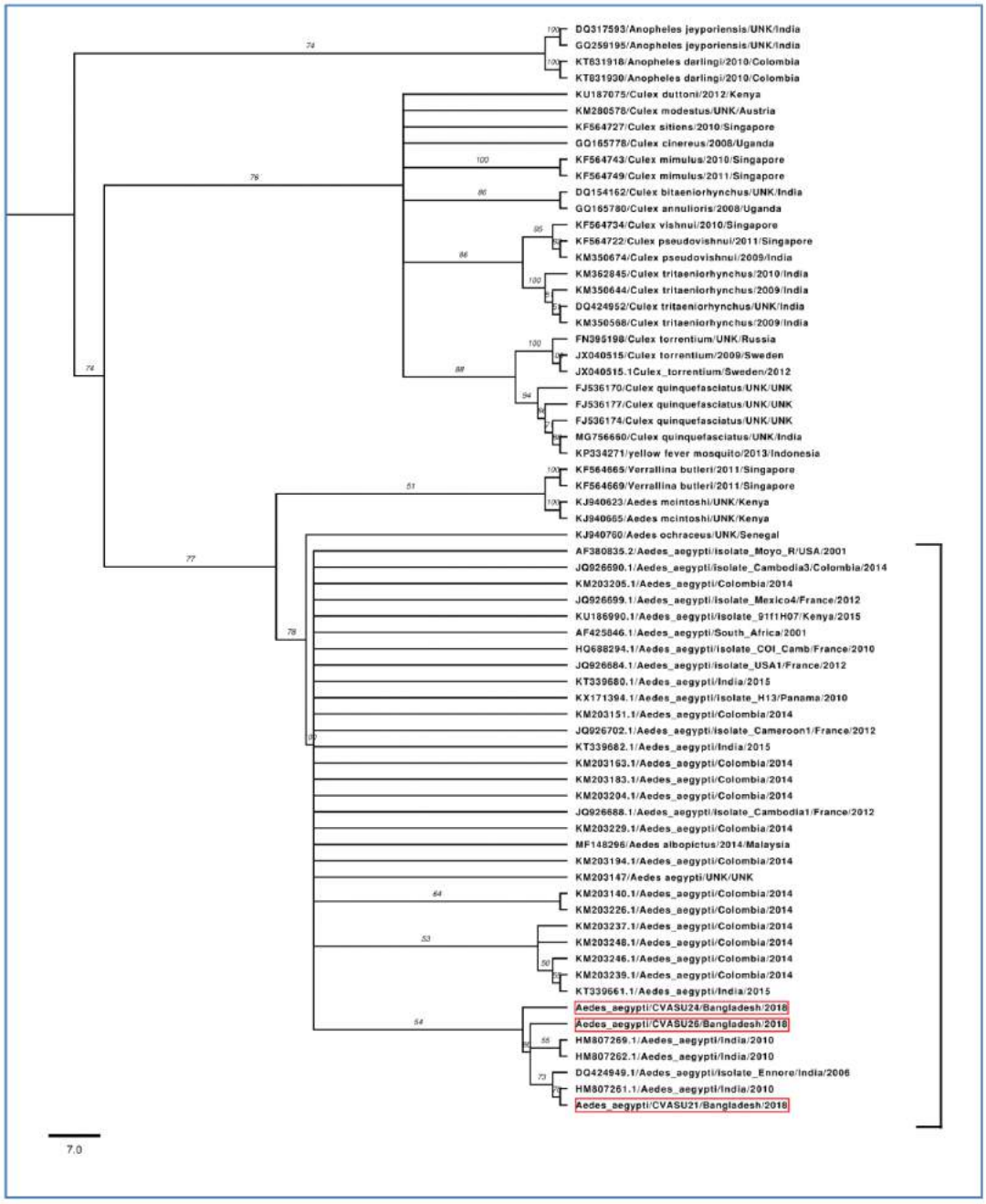

Figure 4. Phylogenetic tree based on partial nucleotide sequences of the COX-I gene of *A. aegypti* collected from Chattogram Metropolitan area.

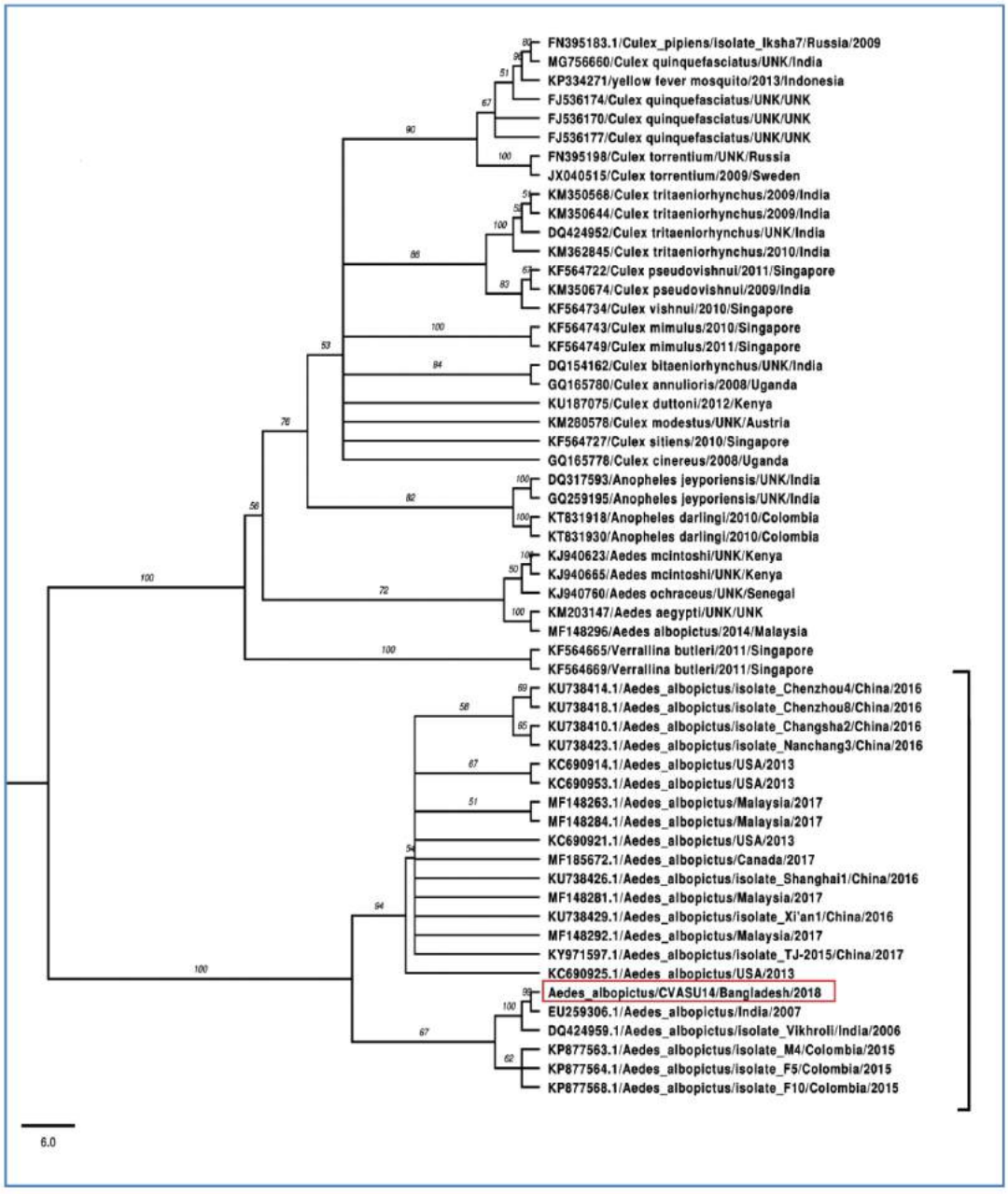

Figure 5. Phylogenetic tree based on partial nucleotide sequences of the COX-I gene of *A. albopictus* from Chattogram Metropolitan area.

4. DISCUSSION

Mosquito remained as an important vector of different infectious diseases of viral or bacterial pathogens. Identification and epidemiological investigation require trustworthy tools with complementary information for their surveillance. Until now no organized effort has been initiated to identify different vectors in Chattogram. The present study was first of its type to use modern molecular tools for characterization of available mosquito species in this cosmopolitan city.

Morphological parameters are important since many years as classical tools of taxonomy. During this study we observed the morphological characteristics of the mosquitoes such as their size and pattern of head, proboscis, maxillary palp, antenna, thorax, wings, legs and abdomen etc. using conventional microscopy. The head of *A. aegypti* is laterally convex as well as round towards the occiput. There are two silvery white dots in clypeus of female but in case of male, there is no dots. There are 5 white scale bands in maxillary palps. The antenna of *A. aegypti* arise from its globular pedicel and has 13 flagellar segments. The thoracic region of *A. aegypti* is dark brown or black in color having three segments such as pro, meso and meta which consists of wings, legs and halters. There are three pairs of legs in *A. aegypti* which consists of coxa, trochanter, tibia, femur and tarsal segments. These all characters were clearly observed and compared with previous reports towards their eventual identification as *A. aegypti.*

Another species found during this study was *A. albopictus* having white scales and bold black shiny scales on the palpus and tarsi. The proboscis of *A. albopictus* is dark colored. A silvery scales covered the upper surface of the end segment of the palp. The dorsal portion of thoracic segment is black that alongside the characteristic white midline. There is a white spots on the base of the costae of the transparent wings. In case of older mosquito specimens, the scales could be partially worn off, making these characteristics not stand out as much (Spain, 2009). All these patterns were considered for taxonomic identification during this study.

The adults of *Culex* species are usually unicolorous mosquitoes that possesses markings on legs as well as pale spots on their wings. Absence of prespiracular setae and post spiracular setae and the distinct pulvilli are the main identifying characteristics. The adult mosquito has well defined head, thorax and abdomen. *Culex* larvae float with head low and only the siphon at the tail held at the surface. The length of the adult mosquitoes are usually 4-10 mm. The characteristic differences among these three different species were then compared with the molecular data during this study.

The epidemiological significance of mosquitoes greatly depends on its geographical origins. From previous studies, a close association among the geographical origins of vectors were recorded with different traits such as vector competence and insecticide resistance. Mosquito species collected from different parts of Chattogram Metropolitan Area was phylogenetically compared based on COI gene sequences reported by other researchers in different countries all over the world. The information will be useful to identify the epidemiological factors associated with vector distribution and dispersion over time (Spatio-temporal analyses). In addition this study can increase our understanding about their distribution pattern and prediction about any specific lineage that each species is originating from.

DNA barcoding is a novel approach that complement classical morphotaxonomic identification of any species. During this study, based on sequence similarity searching, mosquito isolates CVASU 21, CVASU 24 and CVASU 26 were identified as *A. aegypti*. The BLAST search result showed that COX-I region of these mosquitoes showed intra-species variation in the sequences. All of these three were exhibited 84% similarity with thatreported from India (NCBI accession number HM807261.1 and HM807269.1; Kumar *et al.* 2010). Homology based BLAST searching also revealed CVASU 13 as *C. pipiens*. It was very interesting that isolate CVASU 13 exhibited almost 100% similarity with that collected from Germany (Aceession number KM243942.1) and India (Accession number of EU259297.1 and DQ267689.1). The isolate CVASU 14 was identified as *A. albopictus*. Interestingly isolate CVASU 14 exhibited 99.4% similarity with the collected from India (accession number DQ424959.1). The result of this study showed that the mosquitoes collected from Chattogram Metropolitan area have significant genomic variation and this might be responsible for differences in virulence of infection. Further genetic analyses using next generation sequencing tools will enable better understanding of these important vectors and their comprehensive molecular characterization.

5. CONCLUSIONS

The present study attempted to identify the randomly collected mosquitoes from urban Chattogram by observing their morphological characteristics and

analysed the utility of DNA barcoding approach in vector surveillance through generating a barcode library for mosquitoes found in Bangladesh. With the well-known limitations of morphotaxonomy, DNA barcoding method could be the most reliable tool for identifying different species. The ability to identify species from any life stage, including eggs, means DNA barcoding is not only useful in surveillance programs but also bio-security operations. Future applications of this approach should involve barcoding more species and adding other genetic markers that increase the discriminatory power of this identification method. DNA barcoding could also be utilized with next generation sequencing to identify large numbers of mosquitoes at one time (i.e., bulk samples), thereby significantly lowering the processing time involved in species identification and nationwide surveillance. The present study was first of its type and shows the suitability of modern biotechnology tools to explore vector research in Bangladesh.

6. ACKNOWLEDGEMENTS

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