MOLECULAR DNA BARCODING OF MOSQUITOES COLLECTED FROM CHATTOGRAM MEDICAL COLLEGE AREA.

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made

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Dedicated to

My

Parents

Contents

List of Tables

List of Figures

List of abbreviations and symbols

Abbreviation and symbols	Elaboration
%	Percentage
µg	Microgram
µl	Microlitre
DNA	Deoxy ribonucleic Acid
COI	cytochrome c oxidase
Sp	species
PCR	Polymerase chain reaction
BOLD	barcode of life database
RVF	Rift Valley fever
CVASU	Chattogram Veterinary and Animal Sciences University

Abstract

Mosquitoes are important vectors for a wide variety of diseases. For effective control of various mosquito borne diseases, identification of different species of mosquitoes is crucial. In this study DNA barcoding method was attempted to employ a complementary method alongside classical microscopy to identify species of mosquito. About 300 mosquito samples were collected from the surroundings of Chattogram Medical College Area and were included in this study. Different morphological features of head, clypeus, vertex, antenna, maxillary palp, thorax, wings, leg and abdomen of the mosquitoes were observed under the microscope. For DNA barcoding experiment, genomic DNA was extracted from the samples using commercial kits and specific primers were used for amplification of partial cytochrome oxidase (COI) genes. While morphological investigation was useful in identifying different species including *Culex quinquefasciatus* (170), *Aedes albopictus* (53), *Toxorhynchites spp.* (45) and *Aedes aegypti* (27), molecular technique was also informative. Further sequence analyses revealed successful identification of several genera of mosquitoes including *Aedes* and *Culex.* In total 163 males and 137 females mosquitoes were identified. Further study about the origin and vector potential of these mosquitoes will increase the research understanding to improve existing vector surveillance and control program.

Key words: Mosquitoes, *Aedes aegypti*, *Culex quinquefasciatus,* DNA barcoding, gene sequence

Chapter-1

Introduction

Mosquitoes are leading agents in the spread of vector-borne diseases such as; Malaria, Lymphatic filariasis, Dengue, Chikungunya, Kala-azar, Yellow fever, West Nile Fever, and Zika fever causing mortality and morbidity in humans. Mosquito borne disease is a serious public health concern in Bangladesh, a low-income country with a population of ~160 million. The global spread of recent emerging vector borne diseases such as Chikungunya and Zika fever has enforced enhanced surveillance which requires understanding of the species composition and distribution of potential dangerous mosquito. As knowledge of faunal diversity and vector control is often an important component of disease control, it is important to correctly identify and understand the species. It also allows the investigation of other aspects of biology that differs between species such as larva ecology, resting behavior and insecticide resistance that are vital to implement effective vector control. This has been the driving force in the development in mosquito identification.

Mosquitoes of a species complex are genetically distinct from each other although appear almost morphologically indistinguishable. In conventional identification method Mosquitoes are classified based on characteristic morphological features, which are used as tools for taxonomic keys to identify individual species. Sometimes morphological identification can be confusing or biased when the morphological features are faulty such as damaged scales and bristles. In addition, identifying species would be very difficult as there are little differences observed in same genus. Studies indicate that most of the taxonomic keys can be limited in case of adult species and fourth instar larvae due to unknown morphological features in all these cases. Molecular markers are a better option for identification of a vector species which is less time consuming and more reliable. Moreover, little has been documented on the species composition and diversity of all mosquito groups by use of molecular markers.

In this study, we employed the classical morphological investigations which are used as tools for taxonomic keys to identify individual species alongside molecular genetic techniques like PCR and sequencing of *Cytochrome oxidase* subunit 1 (CO1) gene to identify and characterize mosquito species (Ratnasingham and Hebert, 2007). The aim of this study is to explore and identify the type of mosquitoes recovered from selected areas of Chattogram Medical College.

General Overview

The Mosquito is a small winged insect that belongs to the order Diptera and the family Culicidae. Mosquitoes are a very large group of insects and Culicidae family is a monophyletic taxon that consists of 3,490 currently recognized species grouped in 44 genera (Haarlem and Vos, 2018). Mosquitoes generally feed on nectar from plants, but females of most species consume blood to gain nutrients and proteins needed to produce their eggs. They can feed on divers' host including most animals in higher animal kingdom (Das, 2014). Various mosquito species are responsible for high nuisance and transmission of deadly pathogens and parasites (Swan and Harding, 2017).

Morphological features

They differ morphologically in numerous ways. The genera difference is apparent and is notable with naked eye but closely related species are difficult to recognize without microscope (Clements, 1992). Differences between species are visible in different phases of the insect life, such as larvae may be identified by their abdominal segments, setae or siphon (Clements, 1992; Swan and Harding, 2017).

Breeding preference

Mosquito, go through four different life stages: egg, larva, pupa and imago (adult). Some species may have multiple offspring annually and others may have only one. Some species are able to vegetate in the egg stage, whereas others either as larvae or as adults. Eggs are laid in or near bodies of water since the larvae are aquatic (Swan and Harding, 2017).

Mosquito-borne disease

In the transmission of disease, mosquitoes act as vectors for the propagation of pathogenic organisms such as Bacteria, viruses and other parasites. Millions of deaths are caused by these propagated diseases such as West-Nile fever, Malaria, Dengue, and Yellow fever in adults. Malaria kills more than 1 million children annually. Dangue virus has expanded its range over the past several decades, following its principal vector. Aedes aegypti, back into regions from which it was eliminated in the mid 20^{th} century and causing wide spread epidemics of hemorrhagic fever.

(Michael And Tolle, 2009)

Mosquito identification

Identification of mosquitoes based on morphology is difficult since it requires expert taxonomic and entomological knowledge with limited unique identification keys, to accurately identify these species. Additionally, morphological identification can be baffling when the morphological features are damaged such as defective limbs or wings.

Molecular markers are a better option for the identification since its less time consuming and more reliable. Moreover, little has been documented on the species composition and diversity of all mosquito groups by use of molecular markers. DNA barcoding though promising, is held back by requirement of expensive equipment and specialized labs (Chan *et al.*, 2014). Molecular genetic techniques which involve PCR and sequencing of cytochrome oxidase subunit 1 (CO1) gene to identify and characterize mosquito species (Shahhosseini *et al.*, 2018). Nuclear genes have several desirable attributes relative to mitochondrial genes. Nuclear genes generally have less biased base composition and evolve more slowly than mitochondrial genes. However, nuclear genes are often more difficult to work with than mitochondrial genes since they are difficult to amplify via PCR. According to experts, there's a notable distinction between nuclear genes since they perform better compared to mitochondrial genes (Caterino *et al.*, 2000).

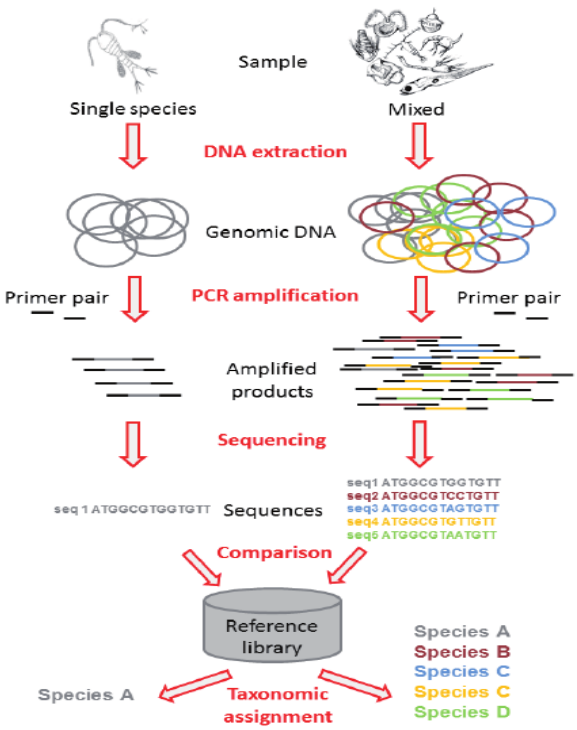

Figure 1: Schematic representation of DNA barcoding (Corell and Rodriguez-Ezpleta, 2014)

Objectives of the study

During this study an attempt was made to;

1. Identify the collected mosquito specimens by observing the morphological features as well as genetic characters using the modern DNA barcode technique.
2. Assessing the feasibility to apply DNA barcoding technology to reliably identify mosquito species

Chapter-2

Review of Literature

2.1. Vector Importance of Mosquitoes

The vector potential of mosquitoes has been well documented around the world. Experts are convinced that they remain to play a critical role in the spread of pathogenic diseases resulting in morbidity and motility, especially in third world countries, such as Bangladesh. However, a thorough in-depth study has not yet been conducted in Bangladesh to record prevalent mosquito species that may propagate life-threatening diseases.

Hawley (1988) reported that mosquitos play an important vector role in transmitting several diseases. *Aedes albopictus* is often considered as a competent laboratory vector for more than 30 different viruses. From these, only a few can infect humans, such as Eastern Equine Encephalitis, Cache Valley virus, Dengue, St. Louis and Lacrosse encephalitis viruses.

Mitchell *et al.*, (1992) reported an isolated incident in 1991 at Polk County, Florida, where *Aedes albopictus* was suspected in the transmission of *Eastern equine encephalitis*.

Hubalek and Halouzka (1999) described the great significance of mosquitoes in the eyes of public health, especially as it relates to the vector propagation of tropical and sub-tropical epidemics such as yellow fever, filariasis, malaria,dengue fever and encephalitis of different types including, japanese encephalitis.

Walton *et al.*, (1999) discuss the relative merits of three different approaches to species identification, all of which use amplification of the ribosomal FWA genes by the polymerase chain reaction. They include; restriction fragment length polymorphism in the Anopheles maculipennis complex from the UK, allele-specific amplification in the An. dim complex from Thailand; and single strand conformational polymorphism in the An. minimus complex from Thailand. The application of these methods is considered in the context of recent data on intra-specific genetic variation, geographic population structure and genetic introgression.

Gerhardt *et al.*, (2001) document findings of isolated *La Crosse virus* from *Aedes albopictus* in North Carolina. The conclusions of this study deemed it necessary to monitor the mosquitoes for further activity, however shouldn't be deemed a public health threat yet.

Lounibos (2002) reported findings of the dengue virus from the vector *Aedes albopictus* following an epidemic in Mexico.

Tiawsirisup and Nithiuthai (2006) discovered findings that indicate vector competence of specific mosquito species against specific diseases. *Aedes aegypti* is well established vector of *filariasis.*

Dennett *et al.* (2007) noticed that the major urban vectors of malaria, dengue and lymphatic filariasis are *Anopheles stephensi, Aedes aegypti* and *Culex quinquefasciatus,* which are capable of transmitting diseases to human beings. There is a correlation between the presence of specific mosquitoes and mosquito-borne disease. However, vector competence research is needed to confirm this relationship, usually by means of laboratory experiments. Some of researches report a correlation between specific diseases such as WNV and mosquito species.

Ricci *et al.,* (2011) has reviewed the recent reports of interdependent association between *Wickerhamomyces anomalus (Pichia anomala)* and several mosquito vector species as potential methods to implement control of mosquito-borne diseases.

Murugan *et al.,* (2015) this research highlighted that seaweed-borne compounds are highly effective against larval populations of the filarial vector *C. quinquefasciatus,* and can be used as an effective reducing agent for the synthesis of mosquitocidal silver nano particles.

Reeves *et al.,* (2016) described the preservation method, storage time and post-feeding time to all significantly impact PCR amplification success. Filter papers and, to a lesser extent, 95% ethanol, were the most effective methods for the maintenance of host DNA templates. Amplification success of host DNA preserved in cold storage at -20 °C and desiccation was poor. Conclusions: Our data suggests that, of the methods tested, host DNA template integrity was most stable when blood meals were preserved using filter papers. Filter paper preservation is effective over short- and long-term storage, whilst ethanol preservation is only suitable for short-term storage. Cold storage at -20 °C, and desiccation of blood meal specimens, even for short time periods, should be avoided.

Swan and Harding (2017) conducted a survey on mosquitoes at 84 diverse locations across Tongatapu Island to record the distribution and occurrence of mosquito larvae. Nine mosquito species were collected. They were also able to generate an identification key for the mosquito larvae species of Tonga.

2.2. Mosquitoes of Bangladesh

Ubydul *et al.*, (2009) reported that Bangladesh has hypoendemic malaria with *P. falciparum* being the dominant parasite species. The malaria situation in the five north-eastern districts of Bangladesh in particular warrants urgent attention. Detailed maps of the baseline malaria prevalence and summaries of the data collected are provided along with the survey results in full, in the supplemental information.

Masuduzzaman (2011) reported crucial mosquito vectors in animals using morpho taxonomy from Chattogram, Bangladesh reported some important mosquito vectors in animals. This study was conducted at Chattogram University (CU), Bangladesh.

Haque *et al.*, (2014) documented several diseases being transmitted by mosquitoes in Bangladesh. The most prominent being Malaria due to its morbidity and motility, especially in the border areas and hill tracts of Chattogram, Bangladesh.

Haque *et al.*, (2014) reported a number of diseases in Bangladesh that are transmitted by mosquitoes. They showed that *P. falciparum* malaria continues to be hypoendemic in the Chattogram Hill Districts of Bangladesh, which is highly seasonal, and much more common in certain geographically limited hot spots and among certain occupations.

Khatun *et al.*, (2015) suggested that rural Bangladeshi populations are at risk for emerging mosquito-borne diseases, such as Chikungunya. Efforts to improve surveillance and identify outbreaks more quickly could provide an opportunity for public health action to reduce transmission, such as mosquito control. This investigation provides further evidence that Chikungunya virus has become an emerging public health problem in Bangladesh.

Irish *et al*., (2016) demonstrated that despite multiple rounds of mass drug administration, *lymphatic filariasis* was still found to be a common problem in Bangladesh.

Afroza *et al.*, (2017) reported *Aedes aegypti* as a common prominent mosquito in Bangladesh which had a predilection for the skins of humans, animals and birds.

2.3. Morphotaxonomy of mosquitoes

2.3.1. Aedes aegypti

Masuduzzaman (2011) mentioned characteristic ornamentation in *Aedes aegyti*; the adults take rest by using its body angled and its abdomen directed towards surface. Female-silvery-white flat scales in middle vertex of head, continued downwards between eyes; similar scales on tori; two small silvery-white dots on clypeus; palps usuallyonly about one quarter the length of proboscis; flat silvery-white scales on all lobes of scutellum of thorax; mid-femur of legs, when viewed from front, with a white longitudinal line running from the base for nearly whole length but not continued quit to knee. The tarsi of fore and mid-legs which possess comparatively more conspicuous white basal rings on 1-4 segments, segment number 4 is widest than other segments and the segment number 5 is entirely white. The color of abdomen tergites is brownish to black that contains some narrow dull white basal bands on II-VI which possess two small silvery-white dots. Lateral basal silvery-white patch is not well developed on I-VII in dorsal view. Male- ornamentation is similar with female. The long segment of palpi has two white rings and the last two segments at base have white marks on undersides.

2.3.2. Aedes albopictus

The average abdomen length of *Aedes albopictus* is 2.63 mm, with the wings being 2.7 mm and the proboscis being 1.88 mm. Some other morphological features are described by various investigators.

Belkin (1962) reported that, in the case of a male, modified mouthparts and plumose antennae are present. The leg is black and each tarsal segment contains the white basal scales. The abdominal tergites are covered by dark scales.

Huang (1968) found the color of scutum to be black that also contains a characteristic distinguished white stripe down the center, beginning at the dorsal surface of the head that continues along the thorax. The length of this mosquito is about 2.0-10 mm with a striking black and white pattern.

Hawley (1988) reported bold black shinny scales and silvery white scales on the tarsi and palpus

Walker (2007*)* described the females to be comparatively 20% larger to its male counterpart but morphologically similar. There is a single silvery-white line of tight scales present between the eyes and it continues down the dorsal side of the thorax. The compound eyes are clearly separated from each other. Male maxillary palps are longer than their proboscis whereas the opposite is true for females where the maxillary palps are shorter. Furthermore, Males' hind legs tarsus is silvery. Tarsomere IV of malesare 75% silver but in females its 60% silver instead. The proboscis of *Aedes albopictus* is dark colored. There is a silvery scale that covers the upper surface of the end segment of the palps whilst the labium does not feature a light line on its underside. The dorsal portion of the thoracic segment is black alongside the characteristic white midline. The scutellum, on the side of the thorax and the abdomen are numerous spots that are covered in white-silvery scales. This type of white-silvery scales may also be noticed on the tarsus, especially, on the hind legs which are suspended in the air. A ring-shaped white scale is present on the bases of tarsomeres I through IV which makes the appearance of black and white rings. In the fore- and middle legs, only the first three tarsomeres contain the ring of white scales but in the case of hind legs, tarsomere V is completely white. The femur of each leg is black and the end of the knee contains white scales. There is no silver line on the base of the upper side of the femora of middle legs but in case of hind legs, it contains short white lines. The base of tibia is black and there is no white scale. The terga on segments II through VI of the abdomens are dark, having a triangular silvery-white marking on the base. A triangular marking and a silvery band are aligned on abdominal segment VII. There are no aligned silvery bands of scales on the ventral side of the abdomen.

2.3.3. Culex pipiens

Irish *et al.* (2016) described the *Culex pipiens* as being miniscule in length ranging from 3-6 mm. They are easily identifiable by observing their long proboscis, which are projecting forwards from the head. The scale is present on their wing veins with a fringe of scales along the posterior margin of the wing. Wing venation is a characteristic feature with the second, fourth and fifth longitudinal veins being branched (Goma, 1966). Females and males may be identified by the form of their respective antennae. Females have only a few short hairs whereas the male antennae are plumose. Maxillary palp is longer than proboscis in males whereas in females, the maxillary palps is shorter. They are holometabola with the first stage differing completely from the last one in terms of form, structure and habits.

2.4. DNA barcoding of mosquitoes

Longbottom *et al.*, (1901) reported that DNA barcodes allow taxonomists to re-confirm the reference voucher specimens. *COI*-based molecular characterization has immense potential to be used as a complementary tool for the identification of mosquito species.

Harrison *et al.*, (1975) noticed that, when field-caught *A. sinensis* mosquitoes were subsequently colonized in the laboratory, an interesting phenomenon was observed. *An. sinensis* is identified according to morphological characteristics such as apical pale bands on hind tarsomeres and the wing venation.

Hebert *et al.*, (2003) reported that the mitochondrial cytochrome c oxidase subunit 1 (*COI*) gene has become more and more popular day by day, primarily because of the ease of using a universal set of primers to amplify the gene and its ability to provide a higher sequence variation at inter-species than at the intra-species level. *COI* gene-based DNA barcoding is therefore used as an alternative species identification method that can easily be standardized to obtain comparable results from different sources.

Cywinska *et al.*, (2006) A short fragment of mt DNA from the cytochrome c oxidase 1 (CO1) region was used to provide the first CO1 barcodes for 37 species of Canadian mosquitoes. In summary, this study has provided the first CO1 barcodes for Canadian mosquitoes and has established their effectiveness in discriminating species of mosquitoes recognized through prior taxonomic work. Specimens of single species formed barcode clusters with tight cohesion that were usually clearly distinct from those of allied species.

Frezal and Leblois (2008) noticed that the previous report showed that almost 75% of *A. sinensis* specimens had pale fringe spots at vein CuA. All *COI* barcode sequences of *A. sinensis* specimens clustered together in the phylogenetic tree regardless of their morphological differences at the end of vein CuA. Instead of the polymorphic nature of wing venation which can impede taxonomic identification, COI barcoding enabled us to confirm that those specimens were indeed *A.sinensis*. The findings suggested that COI-based DNA barcode can effectively be used when morphological traits of certain species were unverifiable or difficult.

Merget *et al*. (2012) described that the main concept of this method is fundamentally becayse every species has a unique genetic identity. A DNA barcode is a short-standardized sequence ofDNAwhich may be used as a genetic maker for species identification.

Webster *et al.*, (2012) reported that DNA barcoding have usednicotinamide adenine dinucleotide dehydrogenase (NADH), the nuclear internal transcribed space, cytochrome b oxidase, and 12S rRNA as target genes.

Chiang *et al.*, (2014) demonstrated that mitochondrial COI gene-based DNA barcoding was comparable to morphological identification for the differentiation of 45 mosquito species analysed. In their analyses, COI barcode was even able to differentiate several mosquito species that were difficult to distinguish morphologically.

Hoyos-López (2018) reports that DNA barcode is a methodology that allows the identification of species using a short fragment of cytochrome oxidase I and library sequences stored in thebarcode of life database (BOLD), which acts as an alternative tool for mosquito identification in areas epidemiologically active for arbo viruses, protozoa and bacteria. In our study, we collected 114 adult mosquitoes in a rural area in the municipality of La Pintada (Antioquia, Colombia), which were then separated for genus and species using morphological keys. Two legs were taken of specimens mounted, and these were used for DNA extraction, amplification of COI-Barcode through PCR and sequencing.38 sequences were characterized of seven mosquito species and used in BOLD for molecular identification, subsequent characterization of genetic distances intra/interspecies, and MOTUs grouping by neighbor-joining analyses.

Shahhosseini *et al.*, (2018) reports identifying different morphologically indistinguishable culex species and biotypes using a multiplex real-time PCR. The method was used to analyze 21,170 Culex specimens from a recent mosquito-borne virus surveillance program in Iran. The results indicate the existence of *C. pipiens* complex and *C. torrentium* in northern Iran, as well as, the sympatric occurrence of Culex spp. biotype *pipiens* and biotype *molestus*. Considering the low prevalence of biotype molestus within the investigated regions, it can be assumed that crossbreeding between the two Culex spp biotypes are a rare event.

2.5. Parasites and vectors

The most important pest and vector species belong to the genera *Anopheles, Culex, Aedes, Psorophora, Mansonia, Haemagogus and Sabethes. Aedes* species are important vectors of yellow fever, dengue, West Nile virus and many other arboviruses. *Anopheles* species, as well as transmitting malaria, are vectors of filariasis and a few arboviruses. Some *Culex* species also transmit *Wuchereria bancrofti* as well as several arboviruses. Mansonia species transmit *Brugia malayi* and sometimes *Wuchereria bancrofti* and a few arboviruses. Haemagogus and Sabethes mosquitoes are vectors of yellow fever and a few other arboviruses. Many mosquitoes which are not vectors can nevertheless be troublesome because of the serious biting nuisances they cause.

Petersen *et al.,* (2013) heightened that the severity of infection varies significantly and the fever develops in nearly 25% of those infected. Neuroinvasive disease carries a fatality rate of 10%. About Two-thirds of the individuals had significant weakness in the limbs affected with paralysis. The operations to control the West Nile virus appear negligible.

Roth *et al.,* (2014) Reported that Pacific Region has 28 recent documented outbreaks since Jan 2012 and flow of dengue, chikungunya and Zika virus and is more likely that this is only the early stages of a wave that will continue for numerous years. More investigation and control measures are needed to lower the burden on island health systems and prevent it from spreading.

Linthicum *et al.,* (2016) reviewed the presents of significant threats of Rift Valley fever {(RVF), which is a mosquito borne viral infection} to public health and agriculture in Africa and the Middle East. Over past 60 years RVFV caused devastating periodic epizootics and epidemics in Africa. With severe and nutritional and economic impacts on humans from illness and livestock loss.

Plourde and Bloch (2016) conducted a survey according to which**,** Zika virus infections are subclinical or mild influenza-like illness sometimes with severe manifestations of Guillain-Barre syndrome and microcephaly. There is no effective treatment nor a vaccine is available. Public health response focuses mainly on preventing infection, predominantly in pregnant women.

Table 1: Mosquito borne diseases and their respective vectors:

Mosquitoes	Vectors	Parasite	Reference
Malaria	Anopheles	Plasmodium	Bradlei *et al.* 2018.
Yellow Fever	Aedes aegypti		Mutebi *et al.* 2004.
Chikungunya	Aedes	Chikungunya virus	Khatun *et al.* 2015.
West Nile Virus (WNV)	Culex	West Nilevirus	Peterson *et al.*2013.
Eastern Equine Encephalitis (EEE)	Culiseta melanura	EEE virus	Armstrong and Andreadis. 2013.
Zika Virus	Aedes aegypti	Zika virus	Ciota *et al.* 2017.
Dengue	Aedes aegypti	Dengue virus	Das, 2014.
Filariasis	Aedine, Anopheline and Culicine	Filaria	Samarawickrema *et al.*, 1992
Kala-azar	Phlebotomus spp	Leishmania donovani	Galati *et al.* 2017.
Japanese B Encephalitis	Culex	Flavivirus	Longbottom *et al.* 2017.

Chapter-3

Materials and Method

3.1. Study area: The proposed study was conducted at CVASU while samples were collected from different parts of Chattogram Medical College Area.

3.2. Study period: The proposed study was conducted during January to June 2019.

3.3. Collection of samples: Samples were collected from different parts of Chattogram where the mosquitoes are available. The whole mosquito or larva was collected from mosquito breeding sites. Adult mosquito specimens were collected using light traps and human baited net traps. Larval samples were also collected manually from logged water.

3.4. Microscopic examination: The morphological features of the mosquitoes were observed and recorded under the stereo binocular microscope in the laboratory of Pathology and Parasitology Department, CVASU

3.5. DNA barcoding

The recently developed DNA-based molecular method lets us to complement the taxonomical identification of mosquito species. Our analysis included *Culex quinquefasciatus, Aedes albopictus, Toxorhynchites spp* and *Aedes aegypti*.

3.6. DNA extraction

From mosquito specimens, DNA was extracted by using the tissue genomic DNA extraction mini-kit (Favorprep™ Tissue Genomic DNA Extraction Mini Kit) according to manufacturer instructions. The extracted DNA was stored at −20 °C until further analysis. The mosquito samples were taken in eppendorf tubes and labeled accordingly. Ethanol was added in the eppendorf tube. The samples were air-dried to remove the ethanol. After air drying, whole mosquito body or some parts such as legs or wings were added in new eppendorf tubes and 200µl binding lysis solution was added into it. After adding binding lysis solution properly, the mixture was vortexed. Then 20µl proteinase-k was added into the eppendorf tube. Again, the mixture was properly mixed by pulse vortex. Mixture was incubated at 60°C for 15 minutes. After incubating, 200 µl concentrate ethanol (96-100%) was added into the mixture then vortexed and Spin centrifuged for 30 seconds. Mixture was transferred to a spin column and centrifuged at 8000 rpm for 1 minute again. After centrifuging, we have the discarded the lower part of the mixture (discardrd the drops from the inside of the lid). Then 500 µl wash buffer-I was added into the tube and centrifuged at 8000 rpm for 1 minute. This process was repeated twice. Then empty spin column was centrifuged at maximum speed at 13000 rpm for 3 minutes to remove ethanol. Then 100 to 200 µl elution buffer was added and incubated at room temperature for 1 minute. Later, mixture was centrifuged at 13000 rpm for 2 minutes and DNA was collected into new eppendorf tube. Finally, the DNA product was preserved at -20° C for PCR.

3.7. Amplification of COI gene

Each individual mosquito or its parts were homogenized and DNA extracted using the procedure as described above. For PCR, amplification was conducted with primers targeting the mitochondrial COI gene (520 bp region) using following primers (Table 2). The Reactions were made 25µl volume containing 4µl of extracted DNA, 2µl of each primer, 12.5µl master mix (2X) and 4.5µl double distilled water or nuclease free water. The run conditioned was an initial denaturation step for 5 mins at 95˚C followed by 5 cycles of 94˚C

for 40 s, 45˚C for 1 min for annealing and 72˚C for 1 min extension, and 35 cycles of 94° C for 40s (denaturation), 51° C for 1 min for annealing, 72° C for 1 min (extension), final extension at 72° C for 10 min. After completing of PCR reaction, it was store at 4° C.

Table-2: Primer pair used for DNA barcoding in this study (Kumar *et al*., 2007)

Cox-I gene	**Name of Primer**
Forward	5'- GGATTTGGAAATTGATTAGTTCCTT - 3'
Reverse	5'- AAAAATTTTAATTCCAGTTGGAACAGC- 3'

3.8. Gel Electrophoresis

An aliquot of 5-6 µl of each PCR product was subjected to electrophoresis on a 1.5% agarose gel. 0.75 gm agarose powder was added to 50ml 1X TAE buffer (Tris, Acetic acid and EDTA) was and mixed thoroughly. The mixture was then heated in the oven for 2 minutes. Then 5µl ethidium bromide was added into the mixture. Finally, the mixture / gel was poured on gel tray. Then 5-6µl PCR product was added into the gel tray. After adding of PCR product, run the gel electrophoresis and waiting for minimum 40 minutes. The bands were visualized using the gel documentation system (UV illuminator).

Chapter-4

Results

4.1 Microscopic and Molecular Study

4.1.1 Microscopic and molecular study

Following classical morphological procedures, microscopic features were recorded and used for immediate identification of different mosquito species. Various distinctive characteristics were assessed and finally each genus and species of mosquitoes were identified with expert opinion from qualified entomologist. Several genus were successfully identified through this approach that include were *Culex quinquefasciatus* (n=175), *Aedes albopictus* (n=53), *Toxorhynchites sp* (n=45) and *Aedes aegypti* (n=27).

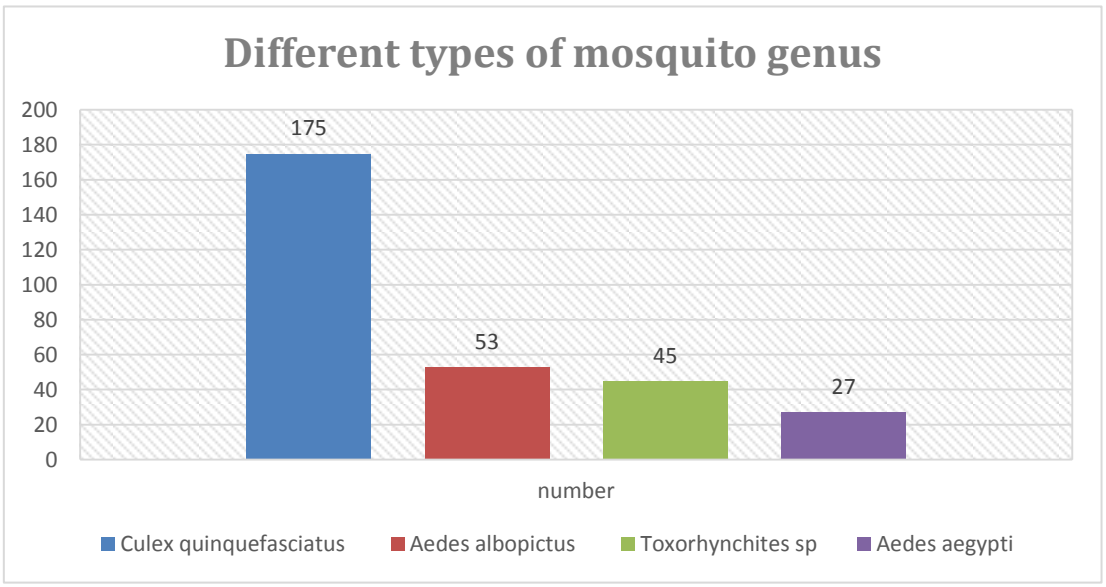

Figure 2: Frequency distribution of mosquito species identified among all specimens. These were found through microscopy at different objectives (X4, X40, X100)

When the sex of the mosquito specimens were considered, it revealed the males were the most prominent (n=161). Females were found in all four species (n=53).

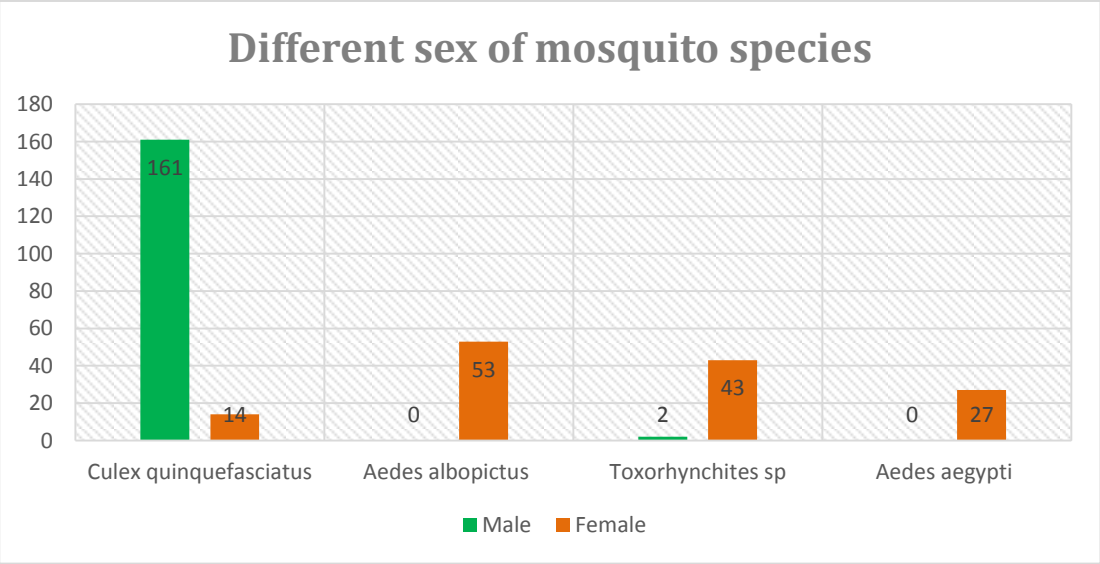

Figure 3: Frequency number of male and female mosquito species

Characterization of mosquitoes based on morphological characteristics:

During careful microscopic examination, different distinctive features from different mosquito specimens were recorded and considered for their identification. Some of the common features were very clear and easy to differentiate. Some of the characteristic pictures are mentioned in the photo gallery. The thoracic region of *Aedes aegypti* is dark brown or black in color. *Aedes aegypti* has two white lyre-shaped bands on its thorax (back), whereas *Aedes albopictus* has a white central band on its thorax. Identification of adult *Aedes albopictus* are the distinct silver white scales and bold black shiny scales on the palpus and tarsi. *Culex quinquefasciatus* is a golden-brown mosquito with a dark proboscis. *Toxorhynchites* are easily identified by their huge size and sturdily bent proboscis.

4.2. Molecular Study

4.2.1. Sequence Analysis

The collected mosquitoes from the field were morphologically examined under the microscope for observing their unique characteristics that was described in the key guides of *Aedes aegypti* mosquitoes. For molecular identification, mt COI region was amplified by using of AePL-2 gDNA. This was done based on previously reported PCR assay. PCR product of 520 bp of COI gene was sent for sequencing through commercial suppliers. To know their nucleotide identity, COI sequences were checked by BLASTN analysis which confirmed that selected pure-line belongs to *Aedes aegypti*. Complete COI sequences have been reported to NCBI (GenBank) with accession number. Thus in combination with morphological features of the mosquitoes that were collected from the field, molecular markers based analysis further confirmed the identity of the mosquito species were *Aedes aegypti, Aedes albopictus* and *Culex quinquefasciatus.*

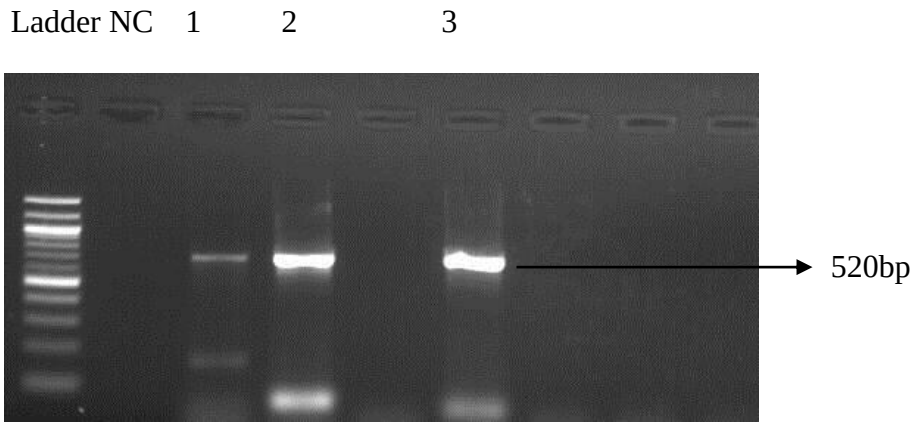

Fig: 4. Representative photo of PCR amplification of COI gene (520 bp) after 1% agarose electrophoresis. 1, 2 and 3 indicates samples used during this study. NC: Negative control

4.2.2. DNA Sequencing and BLAST alignment

Freely available Chromas software was used for analyzing the sequencing data that was confirmed through blast search. COI sequence of *Aedes aegypti* mosquito isolates that was submitted by others were retrieved from the NCBI. The clustal omega platform was used for the alignment of DNA sequences. Sequence divergences were determined among the individual species by using of Kimura two parameters (K2P) distance model. BLAST sequence alignment was used to identify the genus and species of mosquito specimen.

The sequence data generated through this study is given below-

Sequence 1

TAGTAGAAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCCCTTTCTTCTGGAACA
GCTCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCGGGAATCTCATCTATTTTAG
GAGCAGTAAATTTTATTACAACTGTAATTAATATACGATCAGCTGGTATTACTCTTGATCGACTACCTTT
ATTTGTGTGATCAGTAGTAATTACAGCTATTTTATTACTTCTTTCTCTACCCGTATTAGCCGGAGCTATT
ACTATATTATTAACAGACCGAAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCTATTT
TATATCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTCTGCCAGGATTTGGAAT
AATTTCTCATATTATTACACAAGAAAGAGGAAAAAAGGAAACTTTTGGTACTTTAGGAATAATTTATGCT
ATATTAACAATTGGTTTATTAGGATTTATTGTATGAGCTCATCATATATTCACAGTTGGTATAGATGTTG
ATACTCGAGCTTATTTTACGTCTGCAACTATAATTATTGCTGTTCCAACTGGAAT

Sequence 2

TCAGGAATTACTTTAGATCGACTACCTTTATTTGTTTGATCTGTAGTTATTACAGCTATCTTATTACTTC
TTTCTCTTCCTGTTTTAGCTGGAGCTATTACTATGTTATTAACAGACCGAAACTTAAATACATCTTTCTT
TGATCCAATCGGAGGAGGAGATCCTATTTTATACCAACACTTATTCTGATTCTTTGGACACCCAGAAGTT
TATATTTTAATTTTACCCGGATTTGGAATAATTTCTCATATTATTACTCAAGAAAGTGGAAAAAAGGAAA
CATTTGGAACTTTAGGAATAATTTATGCTATATTAACAATTGGATTATTGGGATTTATTGTTTGAGCTCA
TCACATATTTACAGTAGGTATAGACGTAGATACTCGAGCTTATTTTACTTCAGCAACTATAATTATTGCT

Sequence 3

GAATACTACCTCCTTCATTGACACTACTACTTTCAAGTAGTTTAGTAGAAAATGGAGCT
GGGACTGGATGAACAGTGTATCCCCCTCTTTCATCTGGAACAGCTCATGCTGGAGCTTCAGTAGACTTAG
CTATTTTTTCTTTACATTTAGCAGGAATTTCATCAATTTTAGGTGCAGTAAATTTTATTACAACAGTAAT
TAATATACGATCTTCAGGAATTACTCTTGATCGAATACCTTTATTTGTTTGATCAGTAGTAATTACTGCA
GTTTTATTACTTCTTTCTTTACCTGTTTTAGCTGGTGCTATTACTATGTTATTAACAGATCGAAATTTAA
ATACTTCATTCTTTGATCCAATTGGAGGAGGAGATCCAATTTTATATCAACATTTATTTTGATTCTTTGG
ACATCCAGAAGTTTATATTTTAATTCTTCCAGGGTTTGGAATAATTTCTCATATTATTACTCAAGAAAGA
GGAAAAAAGGAAACATTTGGAACTTTAGGAATAATTTATGCTATATTAGCTATTGGTTTATTAGGGTTTA
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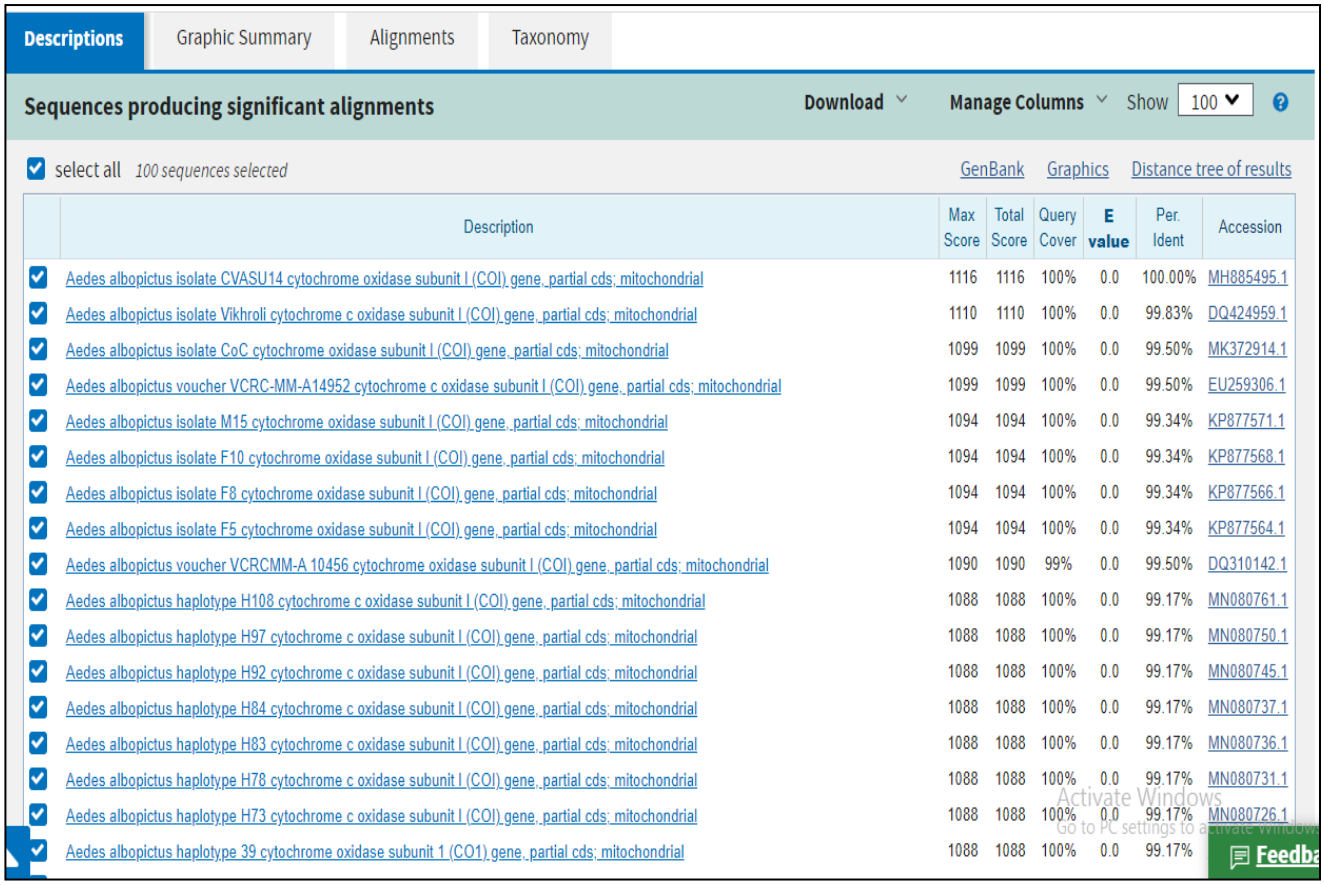
AATAATTATTGCTGTTCCAACTGGAAT



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download Manage Columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Aedes albopictus isolate CVASU14 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	1116	1116	100%	0.0	100.00%	MH885495.1
Aedes albopictus isolate Vikhroli cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1110	1110	100%	0.0	99.83%	DQ424959.1
Aedes albopictus isolate CoC cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	1099	1099	100%	0.0	99.50%	MK372914.1
Aedes albopictus voucher VCRC-MM-A14952 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1099	1099	100%	0.0	99.50%	EU259306.1
Aedes albopictus isolate M15 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	1094	1094	100%	0.0	99.34%	KP877571.1
Aedes albopictus isolate F10 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	1094	1094	100%	0.0	99.34%	KP877568.1
Aedes albopictus isolate F8 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	1094	1094	100%	0.0	99.34%	KP877566.1
Aedes albopictus isolate F5 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	1094	1094	100%	0.0	99.34%	KP877564.1
Aedes albopictus voucher VCRCMM-A 10456 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	1090	1090	99%	0.0	99.50%	DQ310142.1
Aedes albopictus haplotype H108 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1088	1088	100%	0.0	99.17%	MN080761.1
Aedes albopictus haplotype H97 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1088	1088	100%	0.0	99.17%	MN080750.1
Aedes albopictus haplotype H92 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1088	1088	100%	0.0	99.17%	MN080745.1
Aedes albopictus haplotype H84 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1088	1088	100%	0.0	99.17%	MN080737.1
Aedes albopictus haplotype H83 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1088	1088	100%	0.0	99.17%	MN080736.1
Aedes albopictus haplotype H78 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1088	1088	100%	0.0	99.17%	MN080731.1
Aedes albopictus haplotype H73 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1088	1088	100%	0.0	99.17%	MN080726.1
Aedes albopictus haplotype 39 cytochrome oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	1088	1088	100%	0.0	99.17%	

Fig. 5. Screenshot from BLAST search page for sequence 1

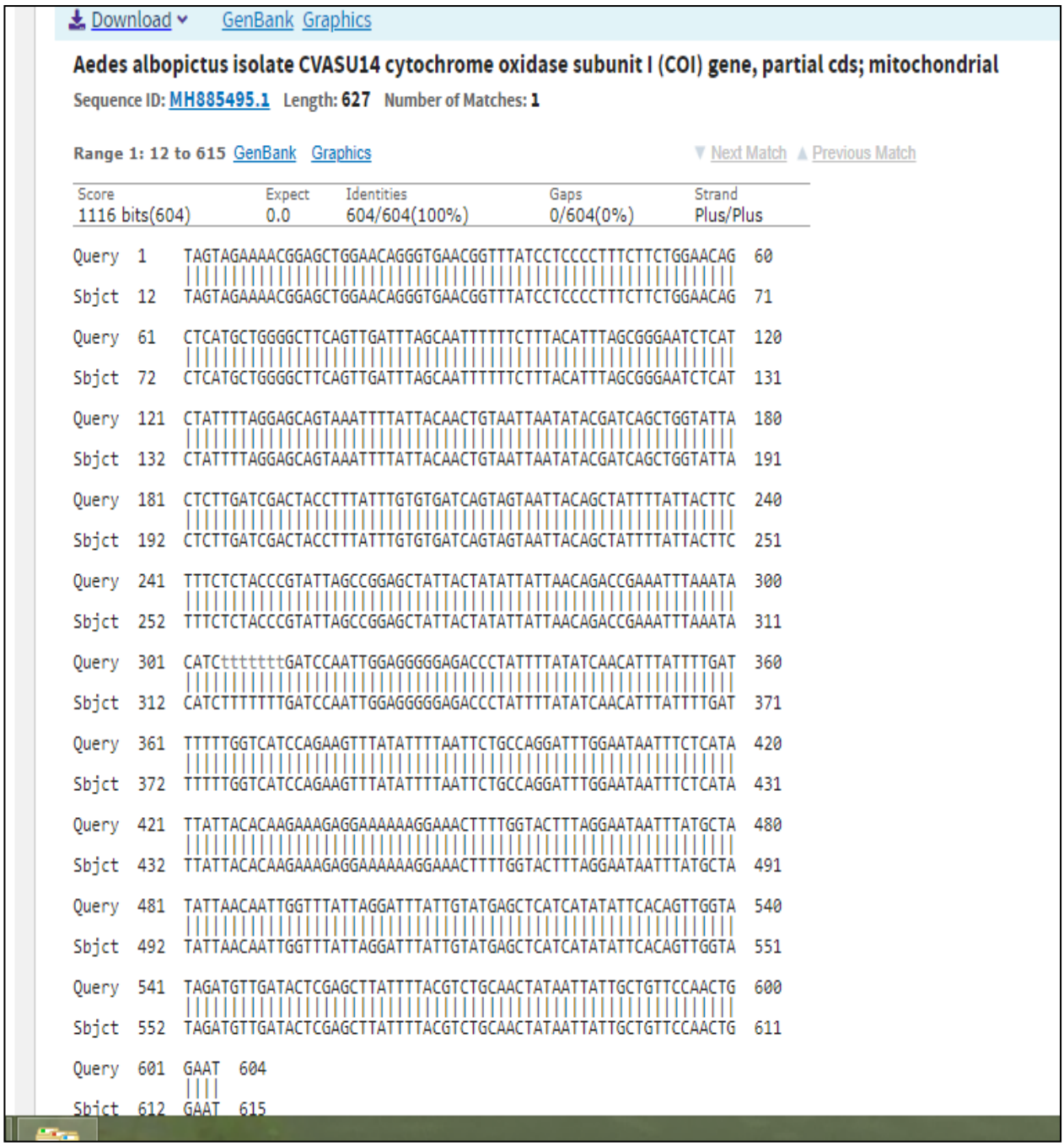
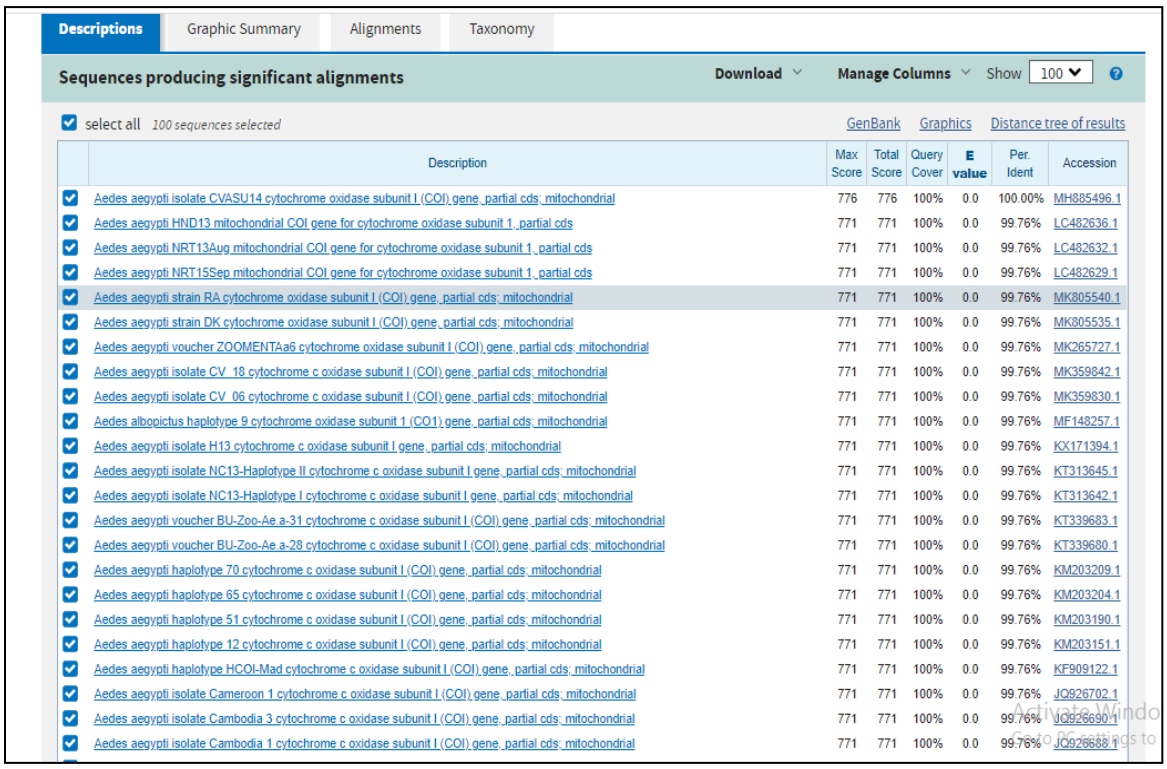


Figure 6. Screenshot from BLAST homology search page indicating 100% similarity with *Aedes albopictus.*



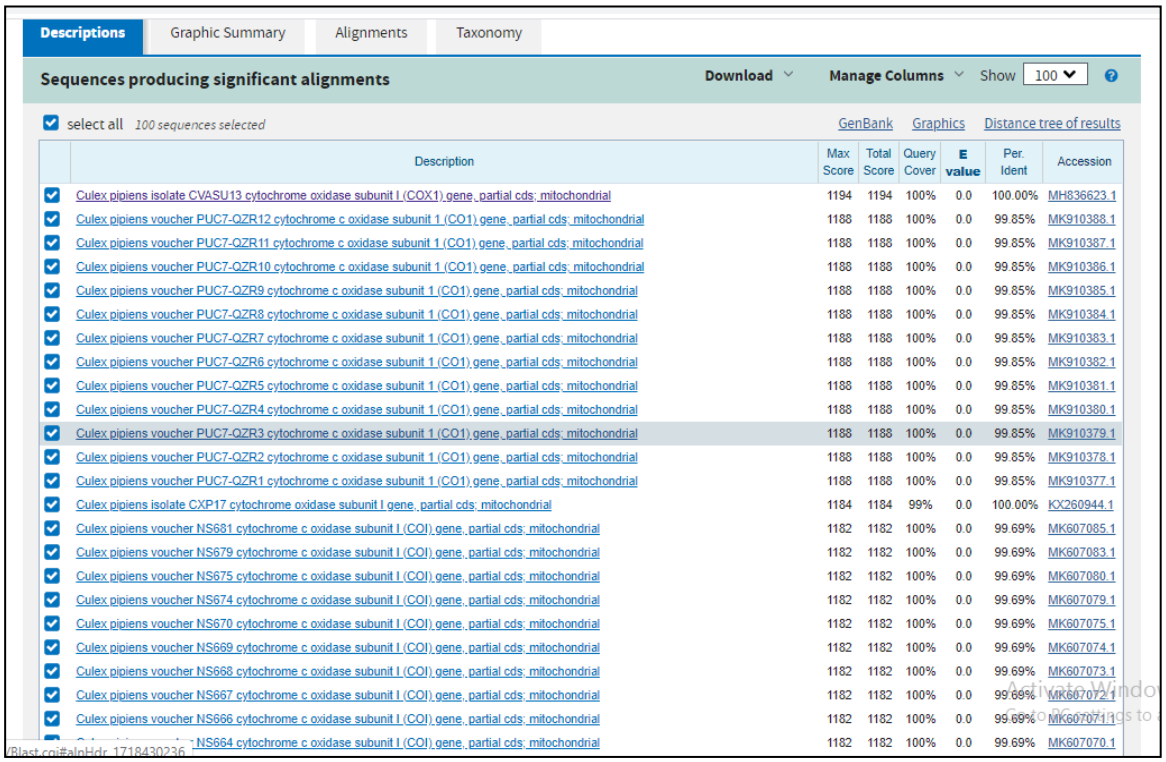
Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Manage Columns — Show 100

select all 100 sequences selected — GenBank | Graphics | Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Aedes aegypti isolate CVASU14 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	776	776	100%	0.0	100.00%	MH885496.1
Aedes aegypti HND13 mitochondrial COI gene for cytochrome oxidase subunit 1, partial cds	771	771	100%	0.0	99.76%	LC482636.1
Aedes aegypti NRT13Aug mitochondrial COI gene for cytochrome oxidase subunit 1, partial cds	771	771	100%	0.0	99.76%	LC482632.1
Aedes aegypti NRT15Sep mitochondrial COI gene for cytochrome oxidase subunit 1, partial cds	771	771	100%	0.0	99.76%	LC482629.1
Aedes aegypti strain RA cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	MK805540.1
Aedes aegypti strain DK cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	MK805535.1
Aedes aegypti voucher ZOOMENTAa6 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	MK265727.1
Aedes aegypti isolate CV_18 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	MK359842.1
Aedes aegypti isolate CV_06 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	MK359830.1
Aedes albopictus haplotype 9 cytochrome oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	MF148257.1
Aedes aegypti isolate H13 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	KX171394.1
Aedes aegypti isolate NC13-Haplotype II cytochrome c oxidase subunit I gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	KT313645.1
Aedes aegypti isolate NC13-Haplotype I cytochrome c oxidase subunit I gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	KT313642.1
Aedes aegypti voucher BU-Zoo-Ae.a-31 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	KT339683.1
Aedes aegypti voucher BU-Zoo-Ae.a-28 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	KT339680.1
Aedes aegypti haplotype 70 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	KM203209.1
Aedes aegypti haplotype 65 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	KM203204.1
Aedes aegypti haplotype 51 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	KM203190.1
Aedes aegypti haplotype 12 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	KM203151.1
Aedes aegypti haplotype HCOI-Mad cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	KF909122.1
Aedes aegypti isolate Cameroon 1 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	JQ926702.1
Aedes aegypti isolate Cambodia 3 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	JQ926690.1
Aedes aegypti isolate Cambodia 1 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	771	771	100%	0.0	99.76%	JQ926688.1

Figure 7. Screenshot from BLAST search page for sequence 2



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Manage Columns — Show 100

select all 100 sequences selected — GenBank | Graphics | Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Culex pipiens isolate CVASU13 cytochrome oxidase subunit I (COX1) gene, partial cds; mitochondrial	1194	1194	100%	0.0	100.00%	MH836623.1
Culex pipiens voucher PUC7-QZR12 cytochrome c oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	1188	1188	100%	0.0	99.85%	MK910388.1
Culex pipiens voucher PUC7-QZR11 cytochrome c oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	1188	1188	100%	0.0	99.85%	MK910387.1
Culex pipiens voucher PUC7-QZR10 cytochrome c oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	1188	1188	100%	0.0	99.85%	MK910386.1
Culex pipiens voucher PUC7-QZR9 cytochrome c oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	1188	1188	100%	0.0	99.85%	MK910385.1
Culex pipiens voucher PUC7-QZR8 cytochrome c oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	1188	1188	100%	0.0	99.85%	MK910384.1
Culex pipiens voucher PUC7-QZR7 cytochrome c oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	1188	1188	100%	0.0	99.85%	MK910383.1
Culex pipiens voucher PUC7-QZR6 cytochrome c oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	1188	1188	100%	0.0	99.85%	MK910382.1
Culex pipiens voucher PUC7-QZR5 cytochrome c oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	1188	1188	100%	0.0	99.85%	MK910381.1
Culex pipiens voucher PUC7-QZR4 cytochrome c oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	1188	1188	100%	0.0	99.85%	MK910380.1
Culex pipiens voucher PUC7-QZR3 cytochrome c oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	1188	1188	100%	0.0	99.85%	MK910379.1
Culex pipiens voucher PUC7-QZR2 cytochrome c oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	1188	1188	100%	0.0	99.85%	MK910378.1
Culex pipiens voucher PUC7-QZR1 cytochrome c oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	1188	1188	100%	0.0	99.85%	MK910377.1
Culex pipiens isolate CXP17 cytochrome oxidase subunit I gene, partial cds; mitochondrial	1184	1184	99%	0.0	100.00%	KX260944.1
Culex pipiens voucher NS681 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1182	1182	100%	0.0	99.69%	MK607085.1
Culex pipiens voucher NS679 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1182	1182	100%	0.0	99.69%	MK607083.1
Culex pipiens voucher NS675 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1182	1182	100%	0.0	99.69%	MK607080.1
Culex pipiens voucher NS674 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1182	1182	100%	0.0	99.69%	MK607079.1
Culex pipiens voucher NS670 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1182	1182	100%	0.0	99.69%	MK607075.1
Culex pipiens voucher NS669 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1182	1182	100%	0.0	99.69%	MK607074.1
Culex pipiens voucher NS668 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1182	1182	100%	0.0	99.69%	MK607073.1
Culex pipiens voucher NS667 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1182	1182	100%	0.0	99.69%	MK607072.1
Culex pipiens voucher NS666 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1182	1182	100%	0.0	99.69%	MK607071.1
NS664 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1182	1182	100%	0.0	99.69%	MK607070.1

Figure 8. Screenshot from BLAST search page for sequence 3

Chapter-5

Discussion

Mosquito remained as an important vector of different infectious diseases of viral or bacterial pathogens. Reliable 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. There are numerous mosquito-borne viruses transmitted by *Aedes aegypti* and *Aedes albopictus* such as; Dengue virus, chikungunya, and they have been reported in Bangladesh (Afroza *et al.*, 2017). Since only a handful of mosquito species play an important role in disease transmission in Bangladesh, precise mosquito identification and monitoring is essential for risk assessment and implementation of preventative strategies in the context of Bangladesh. During this study we observed the morphological characteristics of the mosquitoes based on their shape, size and pattern such as, head, proboscis, maxillary palp, antenna, thorax, wings, legs and abdomen etc. by means of conventional microscopy (Haarlem and Vos, 2018).

Most of the mosquito genus has distinct feature unique to their group. Likewise, *Aedes aegypti* mosquitoes are visually distinct because they have black and white markings on their bodies and legs. The head is globular in shape dorsally and laterally convex as well as round towards the occiput. The proboscis is dark, long and straight. There are two silvery white dots in clypeus of female but absent male. There is a silvery white flat scale in vertex of male and female that is extended to the interocular area between compound eyes. The thoracic region of *Aedes aegypti* is dark brown or black in color. Although the anterior margin is linear as well as flat, the posterior margin contains erect fringe scales. Legs of *Aedes aegypti* consists of coxa, trochanter, tibia, femur and tarsal segments respectively. Tarsal segment has claws. The coxa is attached to the thorax with trochanter and the segment contains white flat patches. These all characters were clearly observed and compared with previous reports towards their eventual identification as *Aedes aegypti*. All the above features were found identical with the specimens examined during this work. The morphological data were then compared with molecular data and assessment were done for the feasibility and effectiveness of DNA based techniques (Das, 2014).

Again, *Aedes albopictus* mosquitoes has conspicuous pattern of white and black shiny scales on the palpus and tarsi. The scutum contain a distinguish white stripe down the center. In case of male, they have bushy haired antennae compared to females. The abdominal tergites are covered by dark scales. Their tarsal segment is black with white basal scales. The proboscis of are dark colored. The palp has a silvery scale covering the upper surface. The compound eyes are clearly separated from each other. The thoracic segment towards the dorsal is black alongside the characteristic white midline. This type of white-silvery scales are noticeable on tarsus, specially on the hind legs. There is a ring of white scales is present on tarsomeres I through IV that making the appearance of white and black rings. The first three tarsomeres of fore and middle legs contains the rings of white scales but in V tarsomere of hind leg is completely white. Femur is black colored and white scales are present in the end of knee. On abdominal segment VII there is a triangular marking were noticeable. All the above features were found similar in a number of specimens and were identified accordingly. Further molecular DNA barcoding data supported this classification (Walton *et al.,* 1999).

Toxorhynchites were easily identified by their huge size and sturdily bent proboscis. Their body is covered with shimmery scales and on tufts on posterior abdomen. The scutellum is consistently rounded and the posterior margin of the wing is distinctly emarginated opposite the termination of vein CuA. The abdominal setae occur in groups of three to five on common sclerites and a comb and pecten are both absent. We did not sequenced any of these group of mosquitoes during this study (due to limited time and funding) and therefore molecular data could not be generated to assess the effectiveness of DNA barcoding tools for their actual characterization (Haarlem and Vos, 2015).

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 lengths 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. *Culex quinquefasciatus* has light brown head with the thorax, proboscis, tarsi and wings darker than the rest of the body. The antennae and the proboscis are about the same length. The scales of the thorax are slim and bent. The abdomen has narrow, light, smooth-edged bands. The second pair of the wings is minute and has

inconspicuous halters. In case of females, the palps are shorter and they have clear wings (Murugan *et al.,* 2015).

DNA barcoding is a novel approach that complements classical morphotaxonomic identification of any species. During this study, based on sequence similarity searching, several mosquito specimens were identified. 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 100% similarity with that reported from other countries and previous researchers. Homology based BLAST searching also revealed all three species very effectively and these were matched with the morphological analyses. The result of this study showed that the mosquitoes collected from Chattogram Medical College 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 (Chan *et al.,* 2014).

Chapter-6

Conclusion

The present study attempted to identify the randomly collected mosquitoes from urban Chattogram by observing their morphological characteristics and analyzed 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 unique attempt and shows the suitability of modern biotechnology tools to explore vector research in Bangladesh.

Chapter-7

Recommendation and Future Perspective

Following recommendations can be made from the results and discussion of the present study:

1. Further study about identifying epidemiological factors associated with vector distribution will improve existing vector surveillance and control program.
2. Further genetic analysis using next generation sequencing tools will enable better understanding of these important vectors and their comprehensive molecular characterization.
3. And also phylogenetic analysis could be attempted to explore their distribution pattern and any specific lineage.

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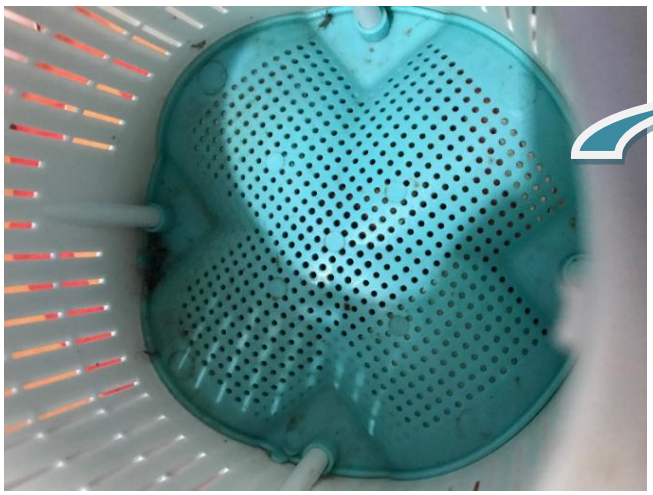
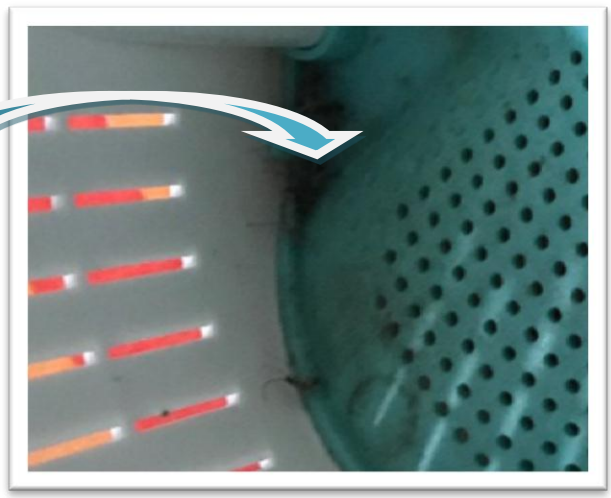
Photo Gallery

larvae of *Aedes species*

(a)

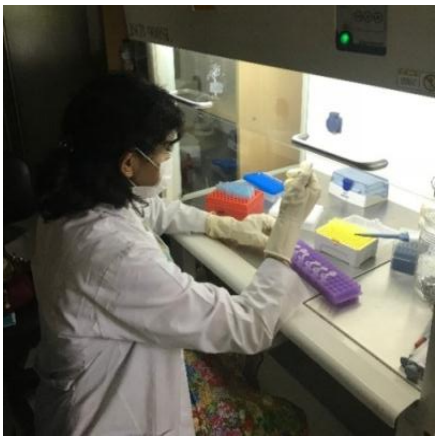


(b)

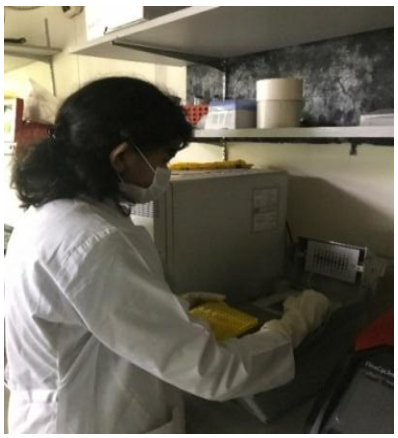


(c)

The trap used to collect mosquitoes (a, b and c) during this study



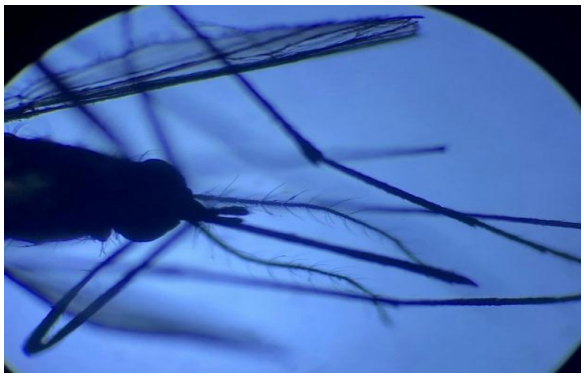
DNA extraction



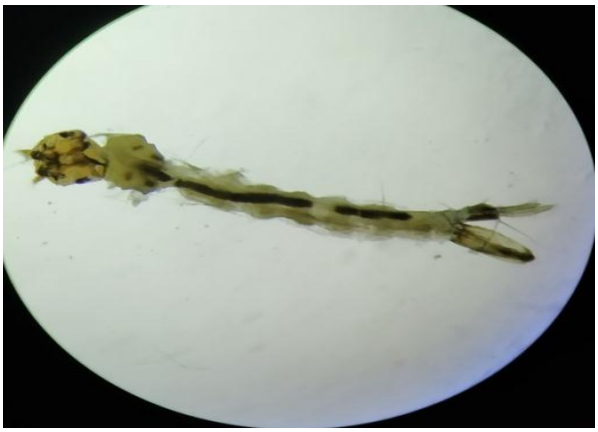
Conventional PCR

Gel electrophoresis

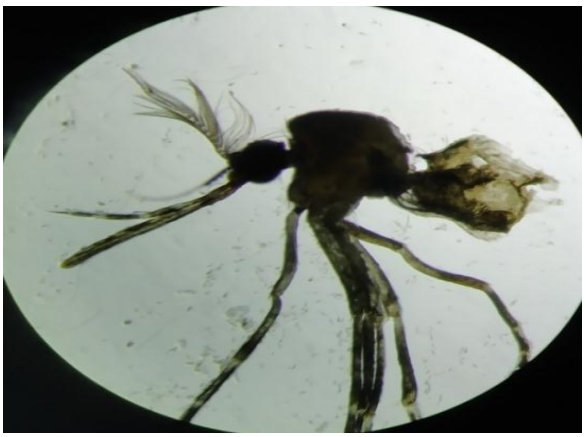
Antenna of *Culex (male) sp*



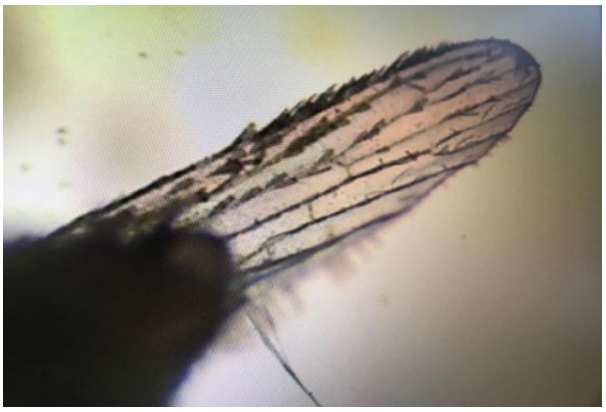
Antenna of *culex (female) sp*



larvae of *culex sp*



Adult Mosquitoes of *culex sp*



wing of *Aedes aegypti*

Dorsal view of *Aedes aegypti*

Microscopic identification of *Aedes aegypti*

Bibliography of Nibedita Devi

I Dr. Nibedita Devi, daughter of Advocate Chandra Shekhar Nath and Prof. Bijoy Lakshmi Devi. I was born on 15th October 1978 in Chattogram, Bangladesh. I have passed Secondary School Certificate (SSC) examination in 1993 from Dr. Khastagir's Govt. Girls High School and Higher Secondary Certificate (HSC) examination in 1995 from Govt. Hazi Mohammad Mohsin College. I have completed MBBS from USTC in 2001. I was a MPH fellow of One Health Institute of CVASU from 2018 to 2019. I am doing my thesis work which is compulsory for degree of Masters of Public Health (MPH). In future my immense of interest toward the higher studies as well as research in the field of Public Health.