# Chapter-1

## Introduction

Various mosquito species are responsible for high nuisance and transmission of deadly pathogens and parasites. These include arboviruses, malaria and filariasis, to humans and animals (Naddaf et al., 2012). Different mosquito species possess characteristic morphological features, which are used as tools for taxonomic keys to identify individual species. In most cases, an experienced taxonomist and suitable method are required for reliable morphological identification and the procedures is highly time consuming for the researchers. 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 indicates 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. These limitations may hinder the application of taxonomic keys for reliable identification of a particular species. To overcome these limitations, complementary approachs like molecular DNA barcoding is available which can help identify the mosquitoes at genus and species level (Abigail Chan et al., 2014).

Until now only 10% of different mosquito species have been identified by studying the morphological characteristics throughout the world. To overcome these limitations of morphotaxonomy, molecular markers is a better option for identification of a vector species which is less time consuming and more reliable than the morphological features study. One molecular study carried out on endemic Australian mosquitoes the investigators 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.

In molecular analyses, Cytochrome Oxidase I (COI) is called the ‘Universal’ or ‘Folmer’ region which possesses 5′ segment of the mitochondrial gene that is commonly used for barcode region for animals. This region is considered as the standard marker as online platform for collating and curating DNA barcoding information over the world that chosen by the Barcode of life Database (BOLD)

(Ratnasingham and Hebert 2007). Although the majority of mosquito barcoding studies use this region, some of studies are using a different region of COI. Sometimes both mitochondrial and nuclear genes are used instead of using only one marker for distinguishing the species (Lin and Danforth 2004). In case of mosquito barcoding studies, there is a variety of nuclear markers have been used that includes elongation factor-1 alpha (EF 1$\alpha$), acetylcholinesterase 2 (ace 2), alpha amylase, zinc finger, and internal transcribed spacer subunit 2 (ITS2) (Foley et al., 2007; Hasan et al., 2009; Hemmerter et al., 2009; Puslednik et al., 2012).

Previously there were 36 various endemic mosquito species detected by a mosquito surveillance program in Victoria, Australia (Lynch et al., 2015 unpublished data). Among these species, only ten have COI sequences published in GenBank and BOLD database.

**Objectives of the study**

During this study an attempt was made to identify the collected mosquito specimens by studying the morphological features as well as genetic characters using the modern DNA barcode technique.

# Chapter-2

# Review of Literature

## 2.1. Vector Importance of Mosquitoes

Mosquito-borne pathogens remain an important source of morbidity and mortality in throughout the world. Several authors have reported the vector potential of mosquitoes and in Bangladesh, a comprehensive study has not yet been conducted to record all available species.

Hubalek and Haluzka, 1999 described that mosquitoes are the most important single group of insects that is well known for their public health importance and it acts as the vector for many tropical and subtropical diseases as like as dengue fever, yellow fever, malaria, filariasis and encephalitis of different types including, Japanese encephalitis.

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

Tiawsirisup and Nithiuthai, 2006 found that there are some vector competence research focuses on a specific mosquito on a specific disease. *Aedes aegypti* mosquito becomes well known as a vector of filariasis.

Hawley, 1988 reported the mosquitoes are very important as vector different diseases. The mosquitoes can transmit some important diseases. *Aedes albopictus* is considered as a competent laboratory vector of more than 30 viruses. From these 30 only a few are considered for affecting humans as like as Eastern Equine Encephalitis, Cache Valley virus, Dengue, St. Louis and Lacrosse encephalitis viruses.

CDC, 2001 considered the *Aedes albopictus* has been implicated in the transmission of dengue. *Aedes albopictus* is known as Asian tiger mosquito that is considered as a

maintenance vector and which is occasionally involved with dengue transmission in Asia.

Lounibos, 2002 in Mexico reported after epidemic, dengue virus was isolated from the vector *Aedes albopictus.*

Moore and Mitchell, 1997 reported that there is one isolated incidence in Polk County, Florida where *Aedes albopictus* was implicated in the transmission of Eastern equine encephalitis in 1991.

Gerhardt et al., 2001 isolated La Crosse virus from the field collected *Aedes albopictus* in North Carolina. The implications of these findings are that this mosquito should be monitored for disease activity, but at this time should not be considered a public health threat.

Stoops et al., 2007 and Vezzani et al., 2006 are told that malaria caused by Anopheles mosquitoes and filariasis caused by Aedine, Anopheline and Culicine.

Masuduzzaman, 2011., Bourgeade and Marchou, 2003., Knox et al., 2003 were told that chikungunya, dengue and yellow fever caused by *Aedes aegypti*.

**2.2. Mosquitoes of Banglaedesh**

Masuduzzaman, 2011 from Chittagong, Bangladesh reported some important mosquito vectors in animals. The study was conducted at Chittagong University where morphotaxonomy was applied.

Haque et al., 2014 reported a number of diseases in Bangladesh that are transmitted by mosquitoes. Malaria is one of the most important causes of morbidity and mortality specially in Hill tracts area of Chittagong and the border area of Bangladesh.

Khan, 1980 described *Aedes aegypti* as commonly known mosquito while the skin of human, animals and birds is considered as the predilection site.

Hafiz et al., 2015 demonstrated that lymphatic filariasis is a common problem, despite multiple rounds of mass drug administration.

Dusfour et al., 2007 told on his study that *An. epiroticus* is most likely the species present in coastal areas of Bangladesh.

## 2.3. Morphotaxonomy of Mosquitoes

### *2.3.1. Aedes aegypti*

**Stephens, 1829** reported that the *Aedes aegyti* is very slender shaped mosquitoes. The length is about 2-10 mm and they possess elongated piercing proboscis without any ocelli where as the palpi is stiff not pendulous. They have spherical heads and long legs with 14-15 segmented conspicuous antennae. The female has pilose where as the male has densely plumose. The wings are long and fingered with scales along posterior margin and veins. They have a U- shaped labium which is consist of paired maxillae, mandibles and a hypopharynx. In case of non-parasitic male, maxillae and mandibles reduced or absent. The roof of proboscis is formed by labrum.

Masuduzzaman, 2011 found the characteristic ornamentation; 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 usually only about one quarter of 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 the 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 length of abdomen of *Aedes albopictus* is 2.63 mm, the wings 2.7 and the proboscis was 1.88 mm. Some other morphological features are described by different investigators.

Hawley, 1988 reported that the mosquito has silver white scales and bold black shinny scales on the palpus and tarsi.

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

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

Walker, 2007 described as the females are 20% larger than the male but morphologically similar. In case of male maxillary palps are longer than proboscis but in females, maxillary palps are shorter. In case of males, the tarsus of hind legs is silvery. Tarsomere IV of male is 75% silver but in female 60% is silver. 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 proboscis of *Aedes albopictus* is dark colored. There is a silvery scales that covered the upper surface of the end segment of the palps and the labium does not feature a light line on its underside. The compound eyes are clearly separated from each other. The dorsal portion of thoracic segment is black that alongside the characteristic white midline. The scutellum, on the side of the thorax and the abdomen are numerous spots that is covered in white-silvery scales. This type of white-silvery scales may also be noticed on the tarsus, specially, on the hind leg which is suspended in the air. A ring shaped white scales is present on the bases of tarsomeres I through IV that making 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 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 abdomen are dark which have a triangular silvery-white marking on the base and there is no aligned with the silvery bands of scales on the ventral side of the abdomen. A triangular marking and a silvery band are aligned on abdominal segment VII.

### *2.3.3. Culex pipiens*

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

## 2.4. DNA barcoding of mosquitoes

Merget et al., 2012 described the main concept of this method is that every species has an unique genetic identity. A DNA barcode is a short standardized sequence of DNA which may be used as a genetic maker for species identification.

Webster et al., 2012 reported that DNA barcoding have used the nuclear internal transcribed spacer 2, cytochrome oxidase, 12S rRNA and nicotinamide adenine dinucleotide dehydrogenase (NADH) as target genes.

Hebert et al., 2003 told that the mitochondrial cytochrome c oxidase subunit 1 (*COI*) gene has become more popular day by day, primarily because of the easy 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 intra-species level. *COI* gene-based DNA barcoding is, therefore, an alternative species identification method that can easily be standardized to obtain comparable results from different sources.

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

Reid et al., 1968 observed a differentiation observed in the wing venation of field-caught and laboratory colonized *A. sinensis* adults in Singapore.

Frezal et al., 2008 noticed in his study, 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 polymorphic nature of wing venation which can be impede taxonomic identification. Cytochrome oxidase barcoding enabled us to confirm that those specimens were indeed *A. sinensis*. The findings suggested that Cytochrome oxidase based DNA barcode can effectively be used when morphological traits of certain species.

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

# Chapter-3

## Materials and Method

**3.1. Study area:** The proposed study was conducted at CVASU while samles were collected from different parts of Chittagong Metropolitan area of Bangladesh.

**3.2. Study period:** The proposed study was conducted in January, 2018 to June, 2018.

**3.3. Collection of sample:** Samplee were collected from different parts of Chittagong Metropolitan area, where the mosquitoes are available. The whole mosquito or larva or pupa was collected from mosquito breeding sites.

**3.4. Microscopic examination:** The morphological features of the mosquitoes were observed under the stereo binocular microscope (3D microscope) at 22X, 28X and 108X (Entovision®) magnification in the laboratory of Zoology department, University of Chittagong (Fig 1)

Fig-1: Entovisioin Microscopes (3D Microscope) used during this study

**3.5. DNA barcoding:** The DNA barcoding method has been proposed recently for using as a tool for identification of the species in many species diverse groups of animals. The efficacy of this tool for mosquitoes remains unexplored. A study was undertaken to construct DNA barcodes for many species of mosquitoes prevalent in India that included major vector species. The DNA barcode method is based on DNA sequences of mitochondrial cytochrome oxidase gene sequences can identify the species, in confirmation with the conventional taxonomy (Pradeep Kumar et al., 2007). DNA barcoding of mosquitoes evidenced the utility of this tool for species identification even from a very small portion of the specimen.

**3.6. DNA extraction**

DNA was extracted by using the tissue genomic DNA extraction mini-kit (Favorprep™ Tissue Genomic DNA Extraction Mini Kit) according to manufacturer instructions. At first, we took the mosquito samples from the eppendorf tube by a clean and sterile forceps. The samples were poured into ethanol in the eppendorf tube. Then we kept the samples in open air for air drying to remove the ethanol from the samples. After air drying, we took the whole mosquito or some organs of mosquito such as legs or wings into another new eppendorf tube by the forceps. Then 200 µl binding lysis solution was added into the eppendorf tube. After adding binding lysis solution properly, the mixture was vortexed for sometimes for proper mixing. Then 20µl proteinase-k was added into the eppendorf tube. Again the mixture was properly mixed by pulse vortexing for sometimes. Then the mixture was incubated at 60° C for 15 minutes. After incubating, 200 µl concentrate ethanol (96-100%) was added into the mixture and properly vortex for mixing. Then we have done a little bit spin (centrifuge) for 30 seconds. After spinning, we have transferred it into a spin column and centrifuged at 8000 rpm for 1 minute again. After centrifuging, we have discarded the lower part of the mixture (discarded the drops from the inside of the lid). Then 500 µl wash buffer-I into the tube. Again the mixture was centrifuged at 8000 rpm for 1 minute. After centrifuging, the lower part was discarded. Then again 500 µl wash buffer-II was added. After adding of wash buffer-II, the mixture was centrifuged at 8000 rpm for 1 minute. After centrifuging, the lower part of the mixture was again discarded. Empty spin column centrifuged at maximum speed at 13000 rpm for 3 minutes for removing of ethanol. Then 100 to 200 µl elution buffer was added. After adding of elution buffer, incubated the mixture at room temperature for 1 minute.

Again centrifuged the mixture at 13000 rpm for 2 minutes and collected the DNA into a new eppendorf tube. Finally the extracted DNA was stored at -20° C until PCR perform.

### 3.7. Amplification of Cox-I gene and DNA sequencing

A 520 bp region flanking the mitochondrial Cox-I gene was amplified by polymerase chain reaction (PCR) using following primers (Table 2)

**Table-1: Primer name**

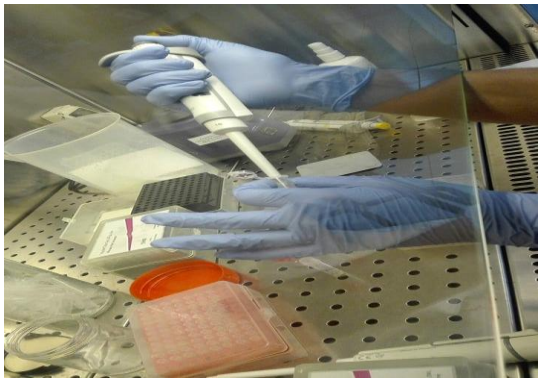
| Primer | Primer sequence | Reference |
|---|---|---|
| Forward | 5'- GGATTTGGAAATTGATTAGTTCCTT - 3' | Kumar et al., 2007 |
| Reverse | 5'- AAAAATTTTAATTCCAGTTGGAACAGC- 3' | |

The 25µl PCR reaction consisted of 4µl of extracted DNA, 2µl of each primer, 12.5µl master mix (2X) and 4.5µl double distilled water or nuclease free water.

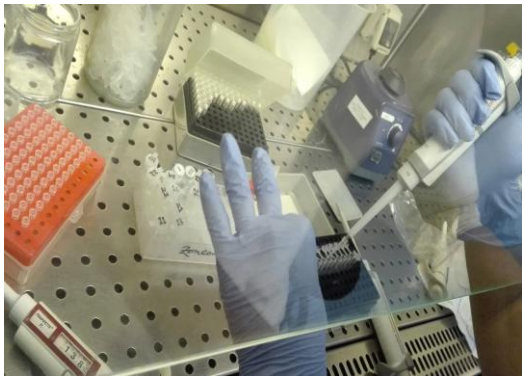
PCR reaction conditions was followed: An initial denaturation of 5 min (95° C) was followed by five cycles of 94° C for 40 s (denaturation), 45° C for 1min (annealing), and 72° C for 1 min (extension) and 35 cycles of 94° C for 40 s (denaturation), 51° C for 1 min (annealing), 72° C for 1 min (extension), final extension at 72° C for 10 min. After completing of PCR reaction, it was store at 4° C (Find the figure -2 to figure-4).

### 3.8. Gel electrophoresis

1.5% agarose gel was used for gel electrophoresis. At first, 0.75 gm agarose powder was taken in the conical flax. Then 50ml 1X TAE buffer (Tris, Acetic acid and EDTA) was added and mixed thoroughly. After mixing, the mixture was heated in the oven for 2 minutes. Then 5µl ethidium bromide was added into the mixture. Ethidium bromide is very much carcinogenic, so it was handled with extra care. Finally, the mixture / gel was poured on gel tray and waiting for half an hour. 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. After completion of gel electrophoresis, finally the bands were visualized using the gel documentation system (UV illuminator).



(A)



(B)

**Fig-2: Preparation of PCR reaction (A and B)**

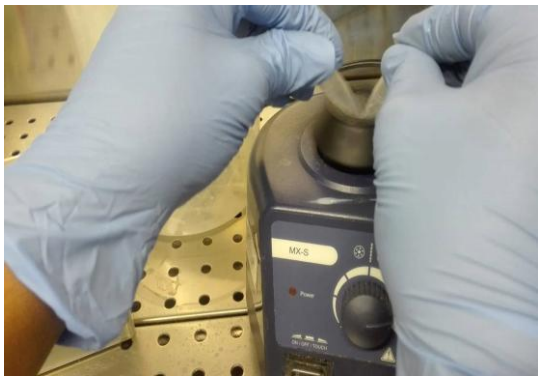


**Fig-3: Vortexing of PCR reaction**

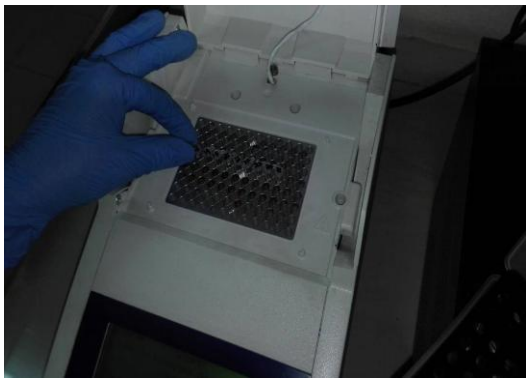


**Fig-4: Denaturation of PCR reaction by five cycles**

**Fig-5: Preparation of gel**

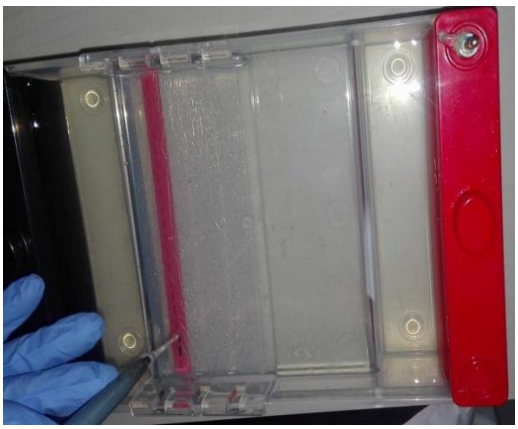


**Fig-6: Adding of PCR reaction into the gel**

# Chapter-4

# Results

## 4.1. Morphology study

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. But in case of female, sparse short hairs are present on antennae. In case of 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. 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. There are VIII abdominal segment of setae 9-12 M.T with well developed spine and comb scales with strong subapical spines.

**Table-2: Morphological characteristics of mosquitoes**

| Name of Mosquitoes | Morphological Characteristics |
|---|---|
| *Aedes aegypti* | **Head:** The head of *Aedes aegypti* is globular in shape dorsally. They are 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 is silvery white flat scales in vertex of male and female that is extended to the interocular area between compound eyes. Round vertex scales are present in male where as the oval in female. The dorsal surface of the head is covered by a dark scales. The head capsule is not same in case of male and female. The antero-posterior length and width of head capsule is about 0.55±0.09 mm and 0.81±0.13 mm where as 0.53±0.06 mm and 0.73±0.11 mm in female.<br>**Proboscis:** The proboscis is dark, long and straight. The length of proboscis is 0.76±0.04 mm in male |

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|---|---|
| *Aedes aegypti* | where as 0.66 ±0.03 mm in female.<br>**Maxillary palp:** There is a pair of maxillary palps in mouth parts of *Aedes aegypti.* There are 5 white scale bands in maxillary palps and the length is about 0.77±0.06 mm in male where as 0.08 ±0.01mm in female.<br>**Antenna:** The antenna of *Aedes aegypti* arise from its globular pedical. There are 13 flagellar segments in the antenna and the hairs of antenna form a whorl fashion. In case of male, the length is near about 0.57±0.03 mm where as 0.52±0.07 mm in female. Bushy and plumose antennal hairs are found in male but comparatively smaller and less dense in female.<br>**Thorax:** In case of female, the thorax is larger 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 *Aedes aegypti* is dark brown or black in color and it has three segments such as pro, meso and meta which consists of wings, legs and halters. 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. Three lobes is found in scutellum and the lobes contain silvery white scale patches in both sexes. The mesothoracic spiracle and the meta thoracic spiracle are oval shaped.<br>**Wing:** The wings are flat, narrow and membranous in *Aedes aegypti,* both in male and female. There is no white scales in wing membrane but specific venation is present which contains flat scales. The tip of wing is oval, the middle part is broad and the base is narrower. Although the anterior margin is linear as |

| | |
|---|---|
| *Aedes aegypti* | well as flat, the posterior margin contains erect fringe scales.<br>**Leg*:*** There are three pairs of legs in *Aedes aegypti* which consists of coxa, trochanter, tibia, femur and tarsal segments. Only the last tarsal segment has claws. The coxa is attached the thorax with trochanter and the segment contains white flat patches. The fore and hind coxa has a scale patch where as the mid coxa contains two scale patches. Trochanter is attached with femur.<br>**Abdomen:** The abdomen has 8 segments which is covered by dark and white scales in both sexes. In case of female, there is a pale scale patch in $1^{st}$ segment which possesses dark brown tergites where is the $8^{th}$ segment is highly reduced. There are transverse white bands present in the upper portion of abdominal segments from II to VII. In case of male, the abdominal tip is narrow posteriorly and the VI and VII segments are terminal which possess white scale patches dorsolaterally, where as it is broad and rounded shaped in female. The abdominal size of male is larger than female. The length is 3.03a±0.18 mm and width is 0.51a±0.07 mm in male where as the female length is 2.94a±0.20 mm and width is 0.41a±0.06 mm. |
| *Aedes albopictus* | Identification of adult *Aedes albopictus* are very easy by observing the distinct silver white scales and bold black shiny scales on the palpus and tarsi (Hawley 1988). The scutum is black which has a distinguishing white stripe down the center that is beginning at the dorsal surface of the head and it is continuing along the thorax. *Aedes albopictus* is a medium-sized mosquito that is ranging from 2.0 to |

| | |
|---|---|
| *Aedes albopictus* | 10.0 mm with a striking white and black pattern (Huang, Y. M. *1968)*. The antennae of the male are plumous 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. Although the males are 20% smaller than females but morphologically they are very similar. The proboscis is shorter than the maxillary palps of the males but in case of females, maxillary palps are very shorter. In case of males, the tarsus of hind legs is silvery. Tarsomere IV of male is 75% silver but in female 60% is silver. 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 proboscis of *Aedes albopictus* is dark colored. There is a silvery scales that covered the upper surface of the end segment of the palps and the labium does not feature a light line on its underside. The compound eyes are clearly separated from each other. The dorsal portion of thoracic segment is black that alongside the characteristic white midline. The scutellum, on the side of the thorax and the abdomen are numerous spots that is covered in white-silvery scales. This type of white-silvery scales may also be noticed on the tarsus, specially on the hind legs which is suspended in the air. There is a ring of white scales is present on the bases of tarsomeres I through IV that making the appearance of white and black rings. In case of fore and middle legs, only the first three trsomeres contains the ring of white scales bur in tarsomer V on hind leg is completely white. The femur of each leg is black and the end of the knee contains white scales. |

| | |
|---|---|
| *Aedes albopictus* | 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 shortwhite lines. The base of the tibiae are black and there is no white scales. The terga on segments II through VI of the abdomen are dark which have a triangular silvery-white marking on the base and there is no aligned with the silvery bands of scales on the ventral side of the abdomen. There is a triangular marking as well as silvery band are only aligned on abdominal segment VII. 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 (Walker, *2007*). |
| *Culex pipiens* | The adults of *Culex* species are usually drab and unicolorous mosquitoes but in case of 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 is the identifiying characteristics. The larvae of *Culex*, the palatal brushes normal, not developed for grasping prey; mandible normal, without lateral lobe at base; maxillary brush present, well developed; seta 12-I and comb always present; siphon with three or more pairs of prominent setae (seta 1-S); pecten normally present; saddle usually complete, sometimes incomplete and greatly reduced but never divided into dorsal and ventral sclerites or longer than the siphon; ventral brush (seta 4-X) usually with three or more pairs of setae. The length of adult *Culex* mosquito can be measured from 4-10 mm. The adult *Culex* contains the head, thorax and |

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|---|---|
| *Culex pipiens* | abdomen which is well defined. The two wings are held horizontally over the abdomen at the time of rest. The second pair of wings is reduced which is modified into tiny and the halters are inconspicuous. In case of females, the palps are shorter and they have clear wings. *Culex* larvae float with head low and only the siphon at the tail held at the surface. |

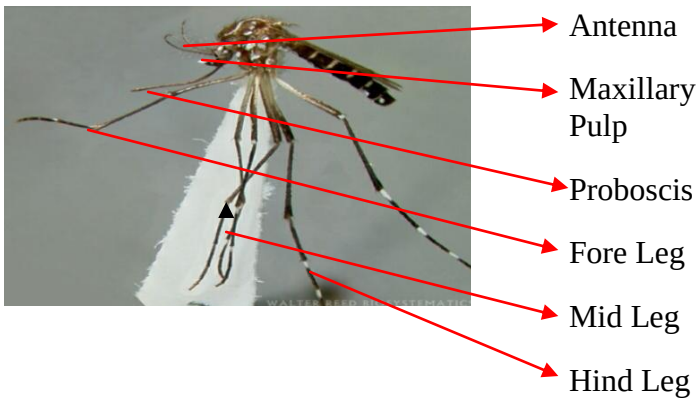
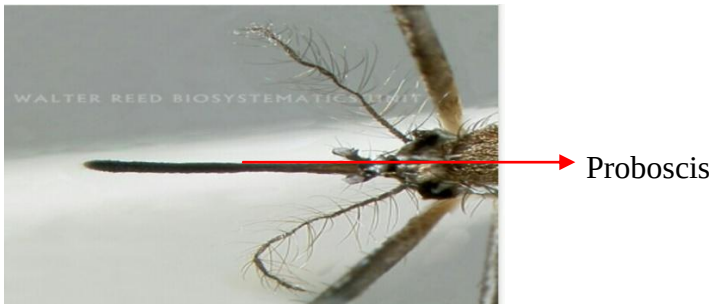



**Fig-7: *Aedes aegypti***

**(**Picture link: http://www.wrbu.org/mqID_medspc/AD/AEaeg_hab.html)

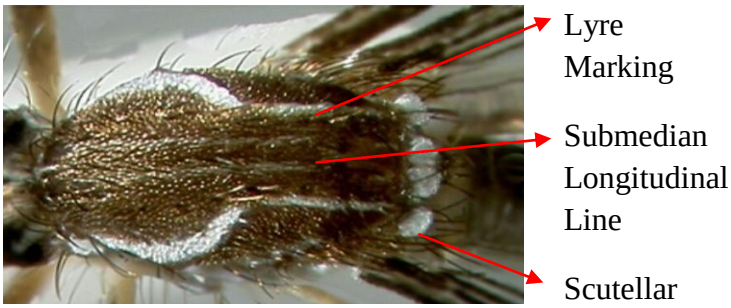



Dorsal View


Lateral View

**Fig-8: Head of *Aedes aegypti***

**(**Picture link: http://www.wrbu.org/mqID_medspc/AD/AEaeg_hab.html)




Dorsal View

Lateral View

**Fig-9: Thorax of *Aedes aegypti***

**(**Picture link: http://www.wrbu.org/mqID_medspc/AD/AEaeg_hab.html)

**Fig-10: Wing of *Aedes aegypt***

**(**Picture link: http://www.wrbu.org/mqID_medspc/AD/AEaeg_hab.html)

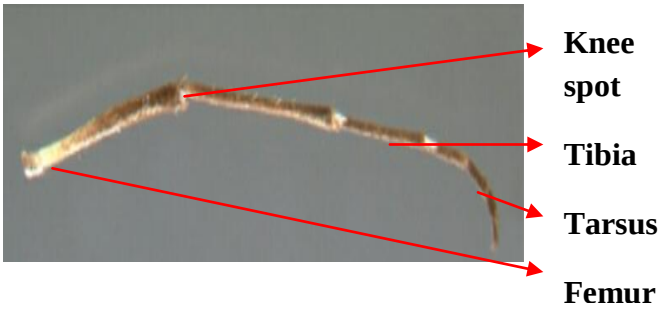



**Fig-11: Fore leg of *Aedes aegypti***

**(**Picture link: http://www.wrbu.org/mqID_medspc/AD/AEaeg_hab.html)

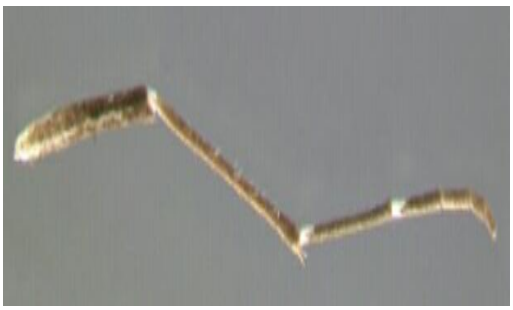


**Fig-12: Mid leg of *Aedes aegypti***

**(**Picture link: http://www.wrbu.org/mqID_medspc/AD/AEaeg_hab.html)

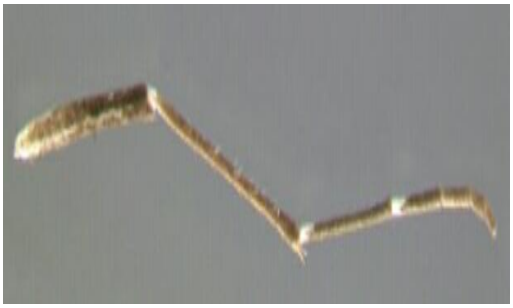


**Fig-13: Hind leg of *Aedes aegypti***

**(**Picture link: http://www.wrbu.org/mqID_medspc/AD/AEaeg_hab.html)

**Fig-14: Hind tarsi of *Aedes aegypti***

**(**Picture link: http://www.wrbu.org/mqID_medspc/AD/AEaeg_hab.html)

Dorsal View

Lateral View

**Fig-15: Abdomen of *Aedes aegypti***

**(**Picture link: http://www.wrbu.org/mqID_medspc/AD/AEaeg_hab.html)

**Fig-16: *Aedes albopictus***

**(**Picture link: http://www.wrbu.org/mqID_medspc/AD/AEaeg_hab.html)

**Fig-17: *Culex pipiens***

**(**Picture link: http://www.wrbu.org/mqID_medspc/AD/AEaeg_hab.html)

**Fig-18: Head of *Culex pipiens***

**(**Picture link: http://www.wrbu.org/mqID_medspc/AD/AEaeg_hab.html)

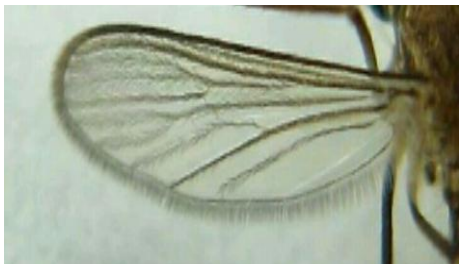


**Fig-19: Wing of *Culex pipiens***

**Fig-20: Abdomen of *Culex Pipiens***

(Picture link: http://www.wrbu.org/mqID_medspc/AD/AEaeg_hab.html)

### 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, mtCOI region was amplified by using of AePL-2 gDNA.

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 (Table-4). 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 pipens* . Among 5 samples only three were *Aedes aegypti* (CVASU-21, CVASU-24 and CVASU-26), one was *Aedes albopictus* (CVASU-14) and one was *Culex pipiens* (CVASU-13, NCBI accession no. MH836623).

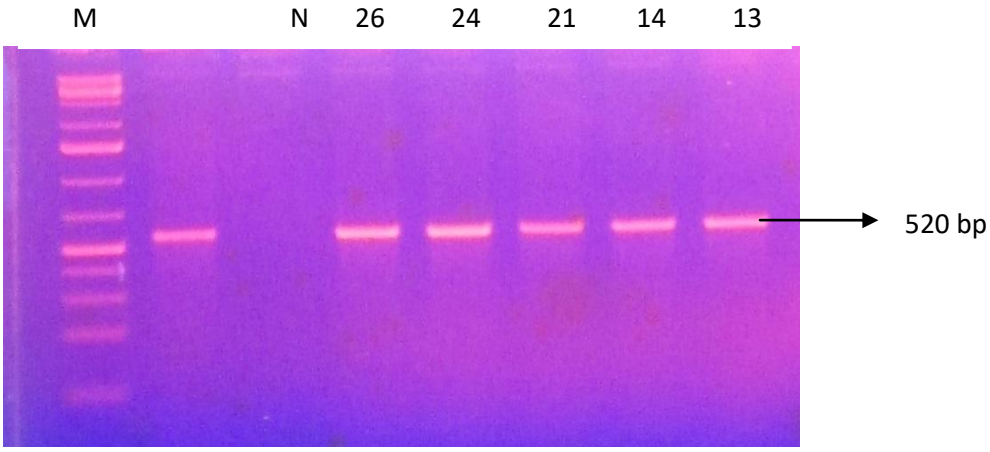



**Fig-21: PCR amplification of Cox-I gene (520 bp) after 1% agarose electrophoresis (CVASU-13, CVASU-14, CVASU- 21, CVASU-24, CVASU26)**

### 4.2.2. Sequencing and Phylogenic Analysis

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. 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. According to the pair wise analysis of known sequences of COI from other reported isolates (Table: 4), all results were formed. Gaps and missing data from all positions were eleiminated (Complete deletion option) from the data set. By MEGA 6 software using NJ method with 1000 bootstrap value and partial deletion option was used to construct the phylogenic tree.

### 4.2.3. Phylogenetic Analysis of *Aedes aegypti* , *Aedes albopictus* and *Culex pipens*

To know the phylogenetic relationships amongst Ae. aegypti isolates COI sequences was aligned with their respective counterparts from different region. Indian isolates of *Aedes aegypti* have never been identified using both COI and ITS-2 together; this is the first report where the species is identified using both nuclear and mitochondrial molecular markers. ITS-2 sequences of seven global isolated *Aedes aegypti* were

selected for constructing evolutionary tree by NJ method using K2P model with 1000 bootstrap value. As expected from sequence analysis, phylogeny showed a close proximity with Saudi Arabia strain and significant diversity with France isolate. In case of COI, the evolutionary divergence among Indian strains of *Ae. aegypti* and global strains was analyzed separately by using phylogenetic tree. Phylogenetic tree with Rajasthan COI (GenBank: KJ862124) revealed close resemblance with Andhra Pradesh P2 isolate. However, globally it comes in first clade along with Thailand, Brazil and Martinique isolates, which is also according to the lesser sequence variability that exists among these continental species of subtropical and tropical origin.

**Table-3: Similarities of our identified species with the species of different parts of the world**

| Sample No. | Identified Species | Accession Number | Variants | Similarity (%) |
|---|---|---|---|---|
| CVASU-13 (MH836623) | *Culex pipens* | KP293425.1 | USA (2014) | 100 |
| | | KP293419.1 | USA (2014) | 100 |
| | | KM233148.1 | Russia (2014) | 100 |
| | | FN395183.1 | Russia (2009) | 100 |
| | | KM233150.1 | Russia (2014) | 100 |
| | | KM233146.1 | Russia (2014) | 100 |
| | | FN395186.1 | Russia (2009) | 100 |
| | | GQ255648.1 | Brazil (2009) | 99.2 |
| | | KX260953.1 | Germany (2016) | 100 |
| | | KX260949.1 | Germany (2016) | 100 |
| | | KX260947.1 | Germany (2014) | 100 |
| | | KM243945.1 | Germany (2014) | 100 |
| | | KM243942.1 | Germany (2014) | 100 |
| | | KM452944.1 | Australia (2014) | 100 |
| | | EU259297.1 | India (2007) | 100 |
| | | KX260946.1 | Germany (2016) | 100 |
| | | KX260945.1 | Germany (2016) | 100 |
| | | DQ267689.1 | India (2005) | 100 |

| Sample No. | *Identified Species* | Accession Number | Variants | Similarity (%) |
|---|---|---|---|---|
| CVASU-14 (MH885495) | *Aedes albopictus* | DQ424959.1 | India (2006) | 99.4 |
| | | KP877568.1 | Colombia (2015) | 98.9 |
| | | KP877564.1 | Colombia (2015) | 98.9 |
| | | EU259306.1 | India (2007) | 99.1 |
| | | MF148263.1 | Malaysia (2017) | 98.7 |
| | | KP877563.1 | Colombia (2015) | 98.7 |
| | | MF148292.1 | Malaysia (2017) | 98.6 |
| | | MF148284.1 | Malaysia (2017) | 98.6 |
| | | KU738429.1 | China (2016) | 98.6 |
| | | KC690953.1 | USA (2013) | 98.6 |
| | | KC690925.1 | USA (2013) | 98.6 |
| | | KC690921.1 | USA (2013) | 98.6 |
| | | KC690914.1 | USA (2013) | 98.6 |
| | | MF185672.1 | Canada (2017) | 98.4 |
| | | KY971597.1 | China (2017) | 98.4 |
| | | MF148281.1 | Malaysia (2017) | 98.4 |
| | | KU738426.1 | China (2016) | 98.4 |
| | | KU738423.1 | China (2016) | 98.4 |
| | | KU738418.1 | China (2016) | 98.4 |
| | | KU738414.1 | China (2016) | 98.4 |
| | | KU738410.1 | China (2016) | 98.4 |
| | | FN395183. | Russia(2009 | 86.7 |

| Sample No. | Identified Species | Accession Number | Variants | Similarity (%) |
|---|---|---|---|---|
| CVASU-21 (MH885496) | *Aedes aegypti* | HM807261.1 | India (2010) | 92 |
| | | DQ424949.1 | India (2006) | 91 |
| | | KU186990.1 | Kenya (2015) | 91 |
| | | KM203204.1 | Colombia (2014) | 91 |
| | | KM203151.1 | Colombia (2014) | 91 |
| | | JQ926702.1 | France (2012) | 91 |
| | | JQ926690.1 | Colombia (2014) | 91 |
| | | JQ926684.1 | France (2012) | 91 |
| | | HQ688294.1 | France (2010) | 91 |
| | | MF148262.1 | Colombia (2014) | 91 |
| | | KM203244.1 | Colombia (2014) | 91 |
| | | KM203242.1 | Colombia (2014) | 91 |
| | | KM203239.1 | Colombia (2014) | 91 |
| | | KM203237.1 | Colombia (2014) | 91 |
| | | KM203232.1 | Colombia (2014) | 91 |
| | | KM203229.1 | Colombia (2014) | 91 |
| | | KM203226.1 | Colombia (2014) | 91 |
| | | KM203224.1 | Colombia (2014) | 91 |
| | | KM203220.1 | Colombia (2014) | 91 |
| | | KM203214.1 | Colombia (2014) | 91 |
| | | KM203210.1 | Colombia (2014) | 91 |
| | | KM203205.1 | Colombia (2014) | 91 |
| | | KM203198.1 | Colombia (2014) | 91 |
| | | KM203194.1 | Colombia (2014) | 91 |
| | | KM203187.1 | Colombia (2014) | 91 |
| | | KM203180.1 | Colombia (2014) | 91 |

| Sample No. | Identified Species | Accession Number | Variants | Similarity (%) |
|---|---|---|---|---|
| CVASU-24 | *Aedes aegypti* | HM807261.1 | India (2010) | 99 |
| | | KU186990.1 | Kenya (2015) | 99 |
| | | JQ926702.1 | France (2012) | 99 |
| | | JQ926690.1 | Colombia (2014) | 99 |
| | | JQ926684.1 | France (2012) | 99 |
| | | HQ688294.1 | France (2010) | 99 |
| | | MF148262.1 | Colombia (2014) | 99 |
| | | KM203244.1 | Colombia (2014) | 99 |
| | | KM203242.1 | Colombia (2014) | 99 |
| | | KM203239.1 | Colombia (2014) | 99 |
| | | KM203237.1 | Colombia (2014) | 99 |
| | | KM203232.1 | Colombia (2014) | 99 |
| | | KM203229.1 | Colombia (2014) | 99 |
| | | KM203224.1 | Colombia (2014) | 99 |
| | | KM203220.1 | Colombia (2014) | 99 |
| | | KM203214.1 | Colombia (2014) | 99 |
| | | KM203210.1 | Colombia (2014) | 99 |
| | | KM203205.1 | Colombia (2014) | 99 |
| | | KM203198.1 | Colombia (2014) | 99 |
| | | KM203187.1 | Colombia (2014) | 99 |
| | | KM203180.1 | Colombia (2014 | 99 |

| Sample No. | Identified Species | Accession Number | Variants | Similarity (%) |
|---|---|---|---|---|
| CVASU-26 | *Aedes aegypti* | HM807261.1 | India (2010) | 92 |
| | | DQ424949.1 | India (2006) | 91 |
| | | KM203204.1 | Colombia (2014) | 91 |
| | | KM203151.1 | Colombia (2014) | 91 |
| | | JQ926702.1 | France (2012) | 91 |
| | | JQ926690.1 | Colombia (2014) | 91 |
| | | JQ926684.1 | France (2012) | 91 |
| | | HQ688294.1 | France (2010) | 91 |
| | | MF148262.1 | Colombia (2014) | 91 |
| | | KM203244.1 | Colombia (2014) | 91 |
| | | KM203242.1 | Colombia (2014) | 91 |
| | | KM203239.1 | Colombia (2014) | 91 |
| | | KM203237.1 | Colombia (2014) | 91 |
| | | KM203232.1 | Colombia (2014) | 91 |
| | | KM203229.1 | Colombia (2014) | 91 |
| | | KM203226.1 | Colombia (2014) | 91 |
| | | KM203224.1 | Colombia (2014) | 91 |
| | | KM203220.1 | Colombia (2014) | 91 |
| | | KM203214.1 | Colombia (2014) | 91 |
| | | KM203210.1 | Colombia (2014) | 91 |
| | | KM203205.1 | Colombia (2014) | 91 |
| | | KM203198.1 | Colombia (2014) | 91 |
| | | KM203194.1 | Colombia (2014) | 91 |
| | | KM203187.1 | Colombia (2014) | 91 |
| | | KM203180.1 | Colombia (2014 | 91 |

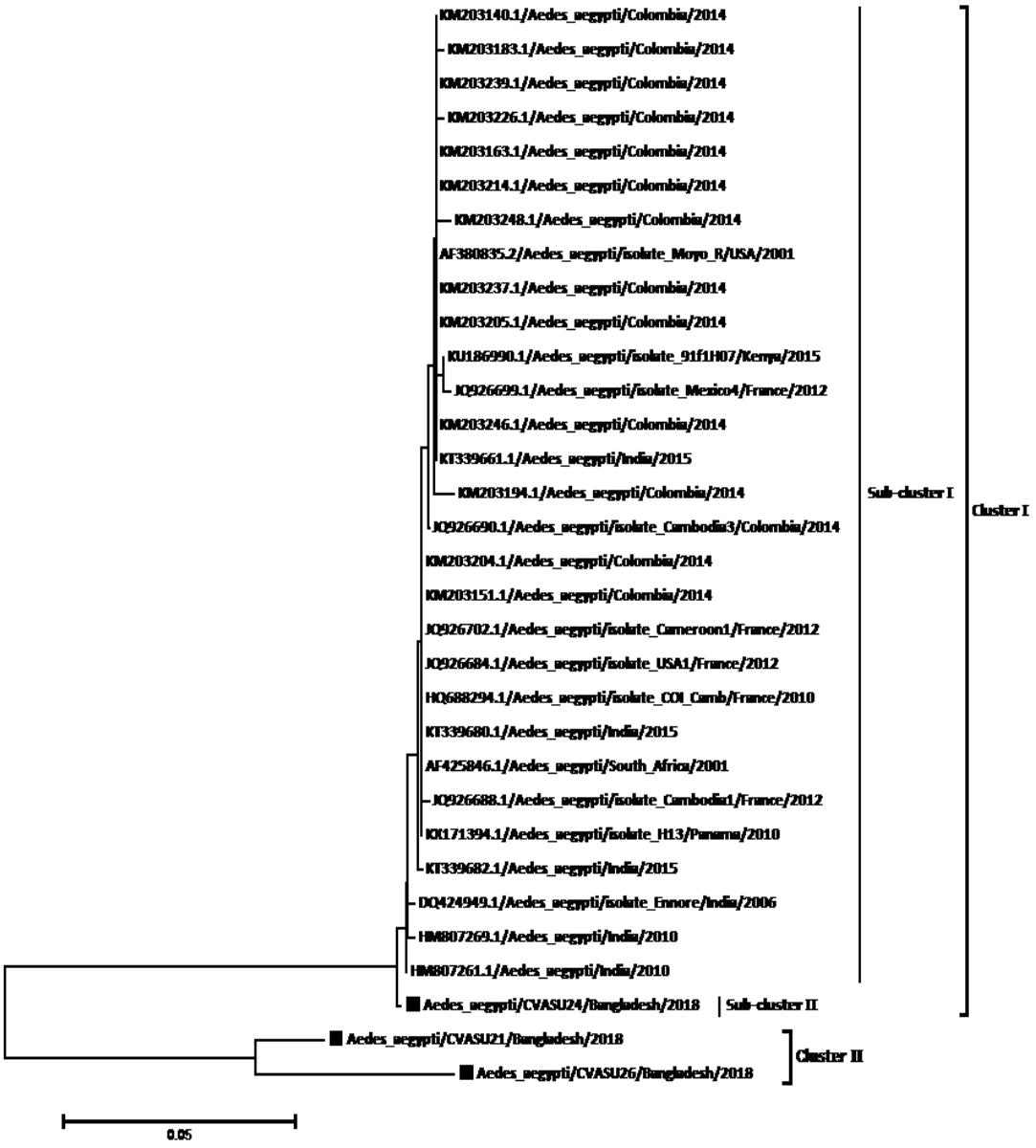



**Figure-22: Evolutionary relationships of taxa**

Phylogenetic tree build by Neighbor-joining method based on partial nucleotide sequences of the COI gene of *Aedes aegypti* from Chittagong Metropolitan area. The numbers of adjacent to the node represents the value of bootstrap support (of 1000 replicates) for the right of the node. Caption "■" indicates the isolates of our study. For each taxon level, the following data are noted as *Aedes aegypti*/CVASU21/Bangladesh/2018, *Aedes aegypti*/CVASU24/Bangladesh/2018, *Aedes aegypti*/ CVASU 26/Bangladesh/2018.

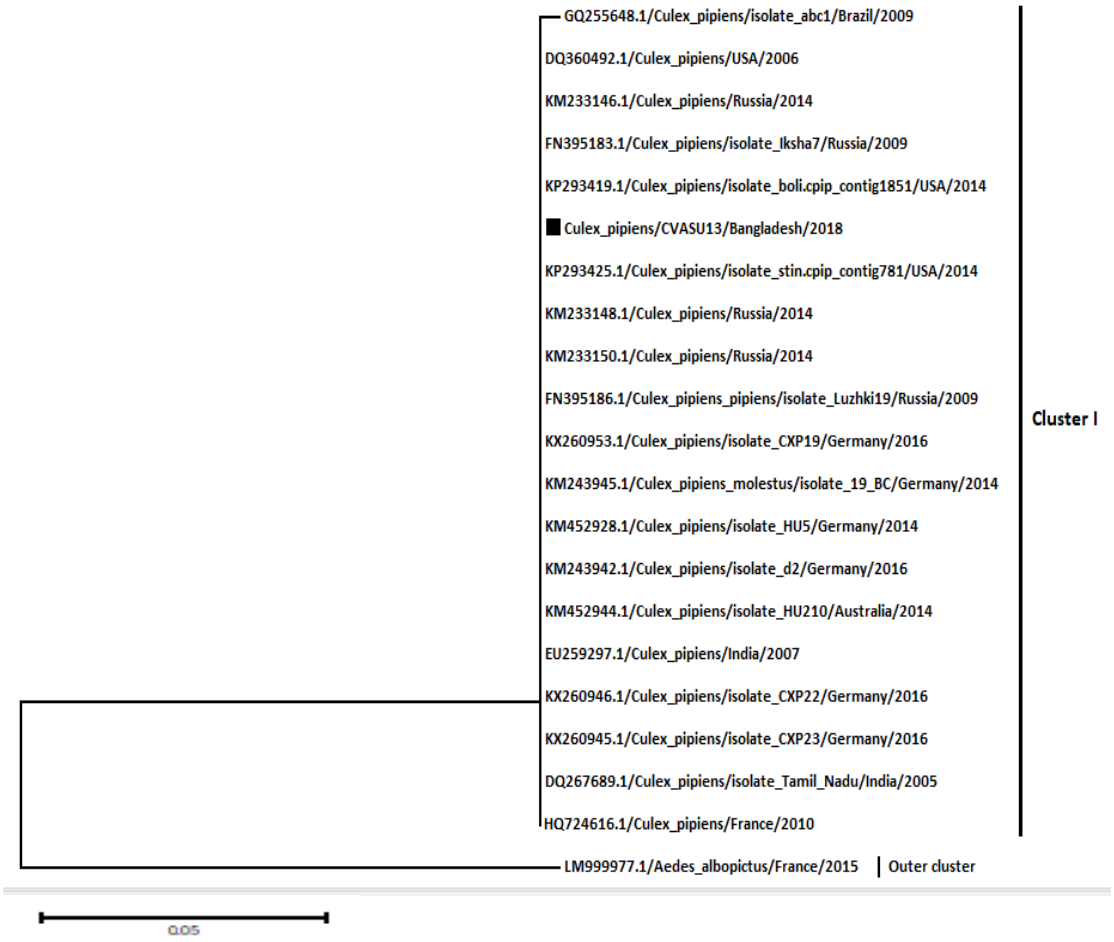



**Figure-23: Evolutionary relationships of taxa**

Phylogenetic tree build by Neighbor-joining method based on partial nucleotide sequences of the COI gene of *Culex pipiens* from Chittagong Metropolitan area. The numbers of adjacent to the node represents the value of bootstrap support (of 1000 replicates) for the right of the node. Caption"■" indicates the isolates of our study. For each taxon level, the following data are noted as *Culex pipiens*/CVASU13/Bangladesh/2018

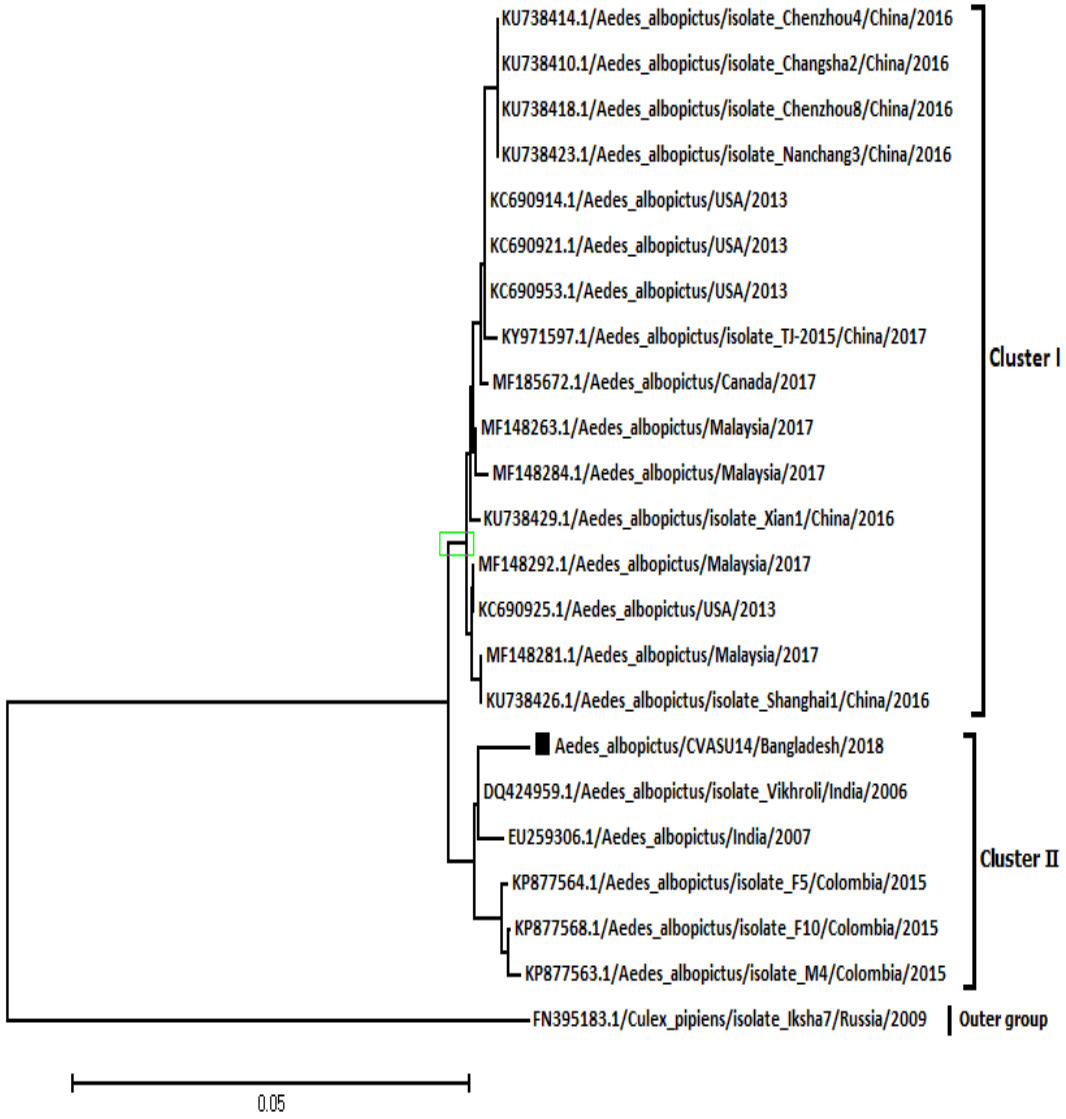



**Figure-24: Evolutionary relationships of taxa**

Phylogenetic tree build by Neighbor-joining method based on partial nucleotide sequences of the COI gene of *Aedes albopictus* from Chittagong Metropolitan area. The numbers of adjacent to the node represents the value of bootstrap support (of 1000 replicates) for the right of the node. Caption"■" indicates the isolates of our study. For each taxon level, the following data are noted as *Aedes albopictus*/CVASU14/Bangladesh/2018.

### 4.2.4. Bioinformatic analyses

The phylogenetic tree analysis revealed the two clusters in the tree, cluster-I and cluster-II. One of our study isolate CVASU-24 (CVASU-24 means sample no. 24) grouped in the cluster-I and CVASU-21, CVASU-26 (CVASU-21, CVASU-26 means sample no. 21, sample no. 26) grouped with the cluster-II. It was very interesting that CVASU-21 and CVASU-26 isolates were most unique which formed an individual cluster-II. In the similarity analysis, it was demonstrated that CVASU-21, CVASU-24 and CVASU-26 isolates showed variation in the nucleic acid similarity. Isolate CVASU-21 showed maximum homology with isolate CVASU-26 which was 94% and 84% with CVASU-24. It was very interesting that all other isolate exhibited 84% similarity which was collected from all over the world publicly available in NCBI.

For *C. pipiens*, the phylogenetic tree analysis revealed a cluster in the tree cluster-I. Our study isolate CVASU-13 (CVASU-13 means sample no. 13) grouped in the cluster-I. CVASU-13 isolate was almost similar to all other isolates which formed cluster in cluster-I. In the similarity analysis, it was demonstrated that CVASU-13 showed almost no variation in the nucleic acids of other isolates except GQ255648.1/*Culex pipiens*/isolate_abc1/Brazil/2009 isolate which showed 99.2%. It was also very interesting that isolate CVASU-13 exhibited almost 100% similarity with the other isolates collected from all over the world publicly available in NCBI.

For *A. albopictus*, the phylogenetic tree analysis revealed the two clusters in the tree, cluster-I and cluster-II. Our study isolate, CVASU-14 (CVASU-14 means sample no. 14) which grouped in the cluster-II. CVASU-14 isolate was distantly clustered from cluster-I. In the similarity analysis, it was demonstrated that CVASU 14 isolates showed very low variation in the nucleic acid with others. CVASU-14 showed maximum homology with DQ424959.1/*Aedes_albopictus*/isolate. Vikhroli/India/2006 isolated which was 99.4%. It was also very interesting that all other isolates exhibited near about 99% similarities which were collected from all over the world publicly available in NCBI.

# Chapter-5

# Discussion

Chittagong is the second largest city in Bangladesh with high population density. 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 this city. 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 *Aedes aegypti* is globular in shape dorsally. They are 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 is a silvery white flat scale in vertex of male and female that is extended to the interocular area between compound eyes. The proboscis is dark, long and straight. There is a pair of maxillary palps in mouth parts of *Aedes aegypti*. There are 5 white scale bands in maxillary palps. The antenna of *Aedes aegypti* arise from its globular pedicel. There are 13 flagellar segments in the antenna and the hairs of antenna form a whorl fashion. The thoracic region of *Aedes aegypti* is dark brown or black in color and it has three segments such as pro, meso and meta which consists of wings, legs and halters. Although the anterior margin is linear as well as flat, the posterior margin contains erect fringe scales. There are three pairs of legs in *Aedes aegypti* which consists of coxa, trochanter, tibia, femur and tarsal segments. Only the last tarsal segment has claws. The coxa is attached 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*.

Another species found during this study was *Aedes albopictus* which has white scales and bold black shiny scales on the palpus and tarsi. The black scutum contains a distinguish white stripe down the center beginning the dorsal surface of the head. In case of male, they have plumous antennae and modified mouthparts. The abdominal

tergites are covered by a dark scales. Their lega are black which has white basal scales on each tarsal segment. The proboscis of *Aedes albopictus* is dark colored. A silvery scales covered the upper surface of the end segment of the palp. The compound eyes are clearly separated from each other. The dorsal portion of thoracic segment is black that alongside the characteristic white midline. The scutellum, on the side of the thorax and the abdomen are numerous spots that is covered in white-silvery scales. This type of white-silvery scales may also be noticed on the tarsus, specially on the hind legs which is suspended in the air. There is a ring of white scales is present on the bases of 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 are black colored and white scales are present in the end of knee. There is a triangular marking as well as silvery band are only aligned on abdominal segment VII. 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 (Walker, 2007). All these patterns were considered for taxonomic identification during this study.

The adults of *Culex* species are usually drab and unicolorous mosquitoes but in case of some species of *Culex* subgenus 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. The wings of the mosquitoes are held horizontally over the abdomen at the time of rest. The second pair of wings is reduced which is modified into tiny and the halters are inconspicuous. In case of females, the palps are shorter and they have clear wings. *Culex* larvae float with head low and only the siphon at the tail held at the surface. The length of 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 Chittagong Metropoliton was phylogenetically analysed by performing of sequences of modern molecular markers of COI gene and compared with their counterparts in different

countries all over the world such as India, Colombia, Malaysia, Russia, Kenya, USA and Germany. It would be possible to identify the epeidemiological factors associated with vector distribution and therefore during this study phylogenetic analyses was attempted to explore their distribution pattern and any specific lineage.

DNA barcoding is a novel approach to complement classical morphotaxonomy. The isolated CVASU 21, CVASU 24 and CVASU 26 were identified as *Aedes aegypti*. The result showed that COI region of isolated CVASU 21, CVASU 24 and CVASU 26 showed variation in the nucleic acid similarly where as the CVASU 21 showed maximum homology with CVASU 26 which was 94% and 84% with CVASU 24. All of these three were exhibited 84% similarity with the collected from India as accession number of HM807261.1 and HM807269.1 (Kumar et al. 2010), Colombia as accession number of KM203204.1, KM203239.1, KM203237.1, KM203226.1, KM203163.1, KM203140.1 (Jaimes-Duenez et al., 2015) and JQ926702.1, JQ926690.1, JQ926684.1, JQ926699.1 (Paupy et al., 2012) ( Fig: 22).

The isolated CVASU 13 was identified as *Culex pipiens*. It was very interesting that isolated CVASU13 exhibited almost 100% similarity with the collected from India as accession number of EU259297.1 and DQ267689.1 (Prodeep Kumar et al., 2007), USA as accession number of KP293425.1 (Liu et al., 2014), KP293419.1 (Chandler et al., 2015), DQ360492.1 (Robich et al., 2007), Russia as accession number of KM233148.1, KM233150.1 (Shaikevich et al., 2014), FN395183.1, FN395186.1 (Shaikevich et al., 2009), Germany as accession number of KX260953.1, KX260946.1, KX260945.1 (Zotzmann et al., 2016), KM452928.1 (Zittra et al., 2014), KM243942.1 (Werblow et al., 2014), Australia as accession number of KM452944.1 (Zittra et al., 2014), KM243945.1 (Werblow et al., 2014) and France as accession number of HQ724616.1 (Atyame et al., 2010) (Fig: 23).

The isolated CVASU 14 was identified as *Aedes albopictus.*It was also very interesting that isolated CVASU 14 exhibited 99.4% similarity with the collected from India as accession number of DQ424959.1 (Kumar et al., 2006), 99.1% as EU259306.1 (Prodeep Kumar et al., 2015), 98.9% with Colombia as accession number of KP877568.1, KP877564.1 (Hoyos-Lopez et al., 2015), 98.7% with Colombia as KP877563.1 (Hoyos-Lopez et al., 2015), 98.6% with Malaysia as MF148292.1, MF148284.1 (Adilah-Amrannudin et al., 2017), with Colombia as

KU738429.1 (Hu et al., 2016) 98.6% with USA as KC690953.1, KC690925.1,KC690921.1, KC690914.1, 98.4% with Malaysia as MF148281.1 (Adilah-Amrannudin et al., 2017) and China as KU738426.1, KU738423.1, KU738418.1, KU738414.1, KU738410.1 (Hu et al., 2016) (Fig: 24). The result of this study showed that the collected mosquitoes from Chittagong Mmetropoliton area have significant genomic variation and it may responsible for different rate of infection. Further genetic analyses using next generation sequencing tools will enable better understanding of these important vectors and their comprehensive molecular characterization.

# Chapter-6

# Conclusion

The present study attempted to identify the randomly collected mosquitoes from urban Chittagong 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. Data from this study can help to develop national database of mosquitoes and their vector potential through screening the different pathogenic virus or bacteria in their gut or larval stages. 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 accuracy and potentiality of DNA barcoding as a species identification tool makes it an essential part of vector surveillance and will continue to grow in future as further barcode libraries and resources are developed. The present study was first of its type and shows the suitability of modern biotechnology tools to explore vector research in Bangladesh.

# Chapter-7

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# Appendix-I

**Table-4: NCBI retrieved sequences of COI of *Ae. aegypti* from different parts of the world**

| Variants | Sources | Accession Number |
|---|---|---|
| India (2007) | Mitochondrian *Aedes aegypti* | DQ424949.1 |
| Kenya (2015) | Mitochondrian *Aedes aegypti* | KU186990.1 |
| France (2012) | Mitochondrian *Aedes aegypti* | KJQ926702.1 |
| Colombia (2015) | Mitochondrian *Aedes aegypti* | KM203204.1 |
| Colombia (2012) | Mitochondrian *Aedes aegypti* | JQ926690.1 |
| France (2012) | Mitochondrian *Aedes aegypti* | JQ926699.1 |
| Colombia (2012) | Mitochondrian *Aedes albopictus* | JQ926684.1 |
| Colombia (2015) | Mitochondrian *Aedes aegypti* | KM203239.1 |
| Colombia (2015) | Mitochondrian *Aedes albopictus* | KM203237.1 |
| Colombia (2015) | Mitochondrian *Aedes albopictus* | KM203226.1 |
| Colombia (2015) | Mitochondrian *Aedes aegypti* | KM203163.1 |
| India (2016) | Mitochondrian *Aedes aegypti* | HM807261.1 |
| Colombia (2012) | Mitochondrian *Aedes aegypti* | KM203151.1 |
| India (2016) | Mitochondrian *Aedes aegypti* | HM807269.1 |
| India (2016) | Mitochondrian *Aedes aegypti* | DQ424949.1 |
| Colombia (2015) | Mitochondrian *Aedes aegypti* | KM203140.1 |
| France (2010) | Mitochondrian *Aedes aegypti* | HQ688294.1 |
| India (2015) | Mitochondrian- *Aedes aegypti* | KT339680.1 |
| South Africa (2002) | Mitochondrian *Aedes aegypti* | AF425846.1 |
| USA (2001) | Mitochondrian *Aedes aegypti* | AF380835 |
| India (2016) | Mitochondrian *Aedes aegypti* | KX171394.1 |

# Appendex-II

## DNA sequence of our stuy sample

**>Culex_pipiens/CVASU13/Bangladesh/2018**

GTAGGGCCAAGAGCAGCGCAGGCTCACTGCTTTTATTATTATATAAGATTT
TTGAATACTACCTCCTTCATTGACACTACTACTTTCAAGTAGTTTAGTAGA
AAATGGAGCTGGGACTGGATGAACAGTGTATCCCCCTCTTTCATCTGGAA
CAGCTCATGCTGGAGCTTCAGTAGACTTAGCTATTTTTTCTTTACATTTAGC
AGGAATTTCATCAATTTTAGGTGCAGTAAATTTTATTACAACAGTAATTAA
TATACGATCTTCAGGAATTACTCTTGATCGAATACCTTTATTTGTTTGATCA
GTAGTAATTACTGCAGTTTTATTACTTCTTTCTTTACCTGTTTTAGCTGGTG
CTATTACTATGTTATTAACAGATCGAAATTTAAATACTTCATTCTTTGATCC
AATTGGAGGAGGAGATCCAATTTTATATCAACATTTATTTTGATTCTTTGG
ACATCCAGAAGTTTATATTTTAATTCTTCCAGGGTTTGGAATAATTTCTCA
TATTATTACTCAAGAAAGAGGAAAAAAGGAAACATTTGGAACTTTAGGAA
TAATTTATGCTATATTAGCTATTGGTTTATTAGGGTTTATTGTTTGAGCTCA
TCATATATTTACAGTTGGAATAGATGTTGATACACGAGCTTATTTTACATC
TGCTACAATAATTATTGCTGTTCCAACTGGAATTAAAAATTTTTAAGGGGA
AAAGGGGGGGGGGGTTTT

**>Aedes_albopictus/CVASU14/Bangladesh/2018**

TCGAAAAGAGGACTGTTAATGCTTTAATAATATATATATTAAACAGATATT
TGAATTTTTACCCCCTCTTTAACACTGCATGCTTTCTAGTTCTATAATAGAA
AACGGAGCTGGAACAGGGTGAACGGTTTATCCTCCCCTTTCTTCTGGAAC
AGCTCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCG
GGAATCTCATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAAT
ATACGATCAGCTGGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCA
GTAGTAATTACAGCTATTTTATTACTTCTTTCTCTACCCGTATTAGCCGGAG
CTATTACTATATTATTAACAGACCGAAATTTAAATACATCTTTTTTTGATCC
AATTGGAGGGGGAGACCCTATTTTATATCAACATTTATTTTGATTTTTTGG
TCATCCAGAAGTTTATATTTTAATTCTGCCAGGATTTGGAATAATTTCTCA
TATTATTACACAAGAAAGAGGAAAAAAGGAAACTTTTGGTACTTTAGGAA
TAATTTATGCTATATTAACAATTGGTTTATTAGGATTTATTGTATGAGCTC
ATCATATATTCACAGTTGGTATAGATGTTGATACTCGAGCTTATTTTACGT
CTGCAACTATAATTATTGCTGTTCCAACTGGAATTAAAAATTTTTAAAAAA
AAAAAAAAAGGGGGGGGAAA

**>Aedes_aegypti/CVASU21/Bangladesh/2018**

GTGGACGTACTGCCGTGCACTGTCATTTCCTTTTTTCTCTATCGGTTGTTTG
AGCTACCACTCCCGTTCTTGTCCCCTGGAGTATCAATTCCTCCTCTATAAA
GAGTCTTGGGAACAGGGGGAACTCTTTGTCCTCCTCTCTCTTCACGAACAT
TTTCGCTGGGTATTCCGTTGAATTACCAATTATTTCTGTTCATTATCTGAGA
TCTTCCCCTGTTTTTTGAGTCATTCATTGTTCTACCTGTGATTTTAATTTTTG
ATCGTCAGGAATTACTTTAGATCGACTACCTTTATTTGTTTGATCTGTAGTT
ATTACAGCTATCTTATTACTTCTTTCTCTTCCTGTTTTAGCTGGAGCTATTA
CTATATTATTAACAGACCGAAACTTAAATACATCTTTCTTTGATCCAATCG
GAGGAGGAGATCCTATTTTATACCAACACTTATTCTGATTCTTTGGACACC
CAGAAGTTTATATTTTAATTTTACCCGGATTTGGAATAATTTCTCATATTAT
TACTCAAGAAAGTGGAAAAAAGGAAACATTTGGAACTTTAGGAATAATTT
ATGCTATATTAACAATTGGATTATTGGGATTTATTGTTTGAGCTCATCACA
TATTTACAGTAGGTATAGACGTAGATACTCGAGCTTATTTTACTTCAGCAA
CTATAATTATTGCTGTTCCAACTGGATTTTAAAATTTTTTAAGGGGAAGGG
GGGGGGGGGGGGGGGGGGG

**>Aedes_aegypti/CVASU24/Bangladesh/2018**

CATAAATGGACGCTGAACCAGGCGTGAAAAAAAAAAGAACATATAAGTT
TTTGAATACTACCTCCTTTCATTTGACTCTTCTATTATCAAGCTCAATAGTA
GAAAATGGGGCAGGAACTGGGTGAACAGTTTATCCTCCTCTCTCTTCAGG
AACAGCTCATGCTGGAGCTTCTGTTGATTTAGCTATTTTTTCTCTTCATTTA
GCTGGAATTTCCTCAATTTTAGGGGCAGTAAATTTTATTACAACTGTAATT
AATATACGATCGTCAGGAATTACTTTAGATCGACTACCTTTATTTGTTTGA
TCTGTAGTTATTACAGCTATCTTATTACTTCTTTCTCTTCCTGTTTTAGCTG
GAGCTATTACTATGTTATTAACAGACCGAAACTTAAATACATCTTTCTTTG
ATCCAATCGGAGGAGGAGATCCTATTTTATACCAACACTTATTCTGATTCT
TTGGACACCCAGAAGTTTATATTTTAATTTTACCCGGATTTGGAATAATTT
CTCATATTATTACTCAAGAAAGTGGAAAAAAGGAAACATTTGGAACTTTA
GGAATAATTTATGCTATATTAACAATTGGATTATTGGGATTTATTGTTTGA
GCTCATCATATATTTACAGTAGGTATAGACGTAGATACTCGAGCTTATTTT
ACTTCAGCAACTATAATTATTGCTGTTCCAACTGGAATTAAAAATAAAATA
TTTTTAAGAAAAAGGAGAGAAGAAAAAA

**>Aedes_aegypti/CVASU26/Bangladesh/2018**

GGGGAGGAGGGACAGGACGAATAAGCCACAGATTTTATGTTGATTGAGC
GACCACTCCCGTAATTGACTCTTGGTGTATCATGTCTTCCTCTATAAGGAG
TCTTGGAAACAGGGGTCCGTCAATGTAACTCTCTCTCTTCACAGTACATTT
TCGCTGGGTATTCCGTTGAATTACCTATTATTTCTGTTCATTTTCTGAGATC
CTCCCTTGTTTTTTGTGTCATTCACTGTTCCACCTGTAATTTTAATTTTTAAT
CGTCAGGAATTACTTTAGATCGACTACCTTTATTTGTTTGATCTGTAGTTAT
TACAGCTATCTTATTACTTCTTTCTCTTCCTGTTTTAGCTGGAGCTATTACT
ATGTTATTAACAGACCGAAACTTAAATACATCTTTCTTTGATCCAATCGGA
GGAGGAGATCCTATTTTATACCAACACTTATTCTTGATTCTTTGGACACCC
AGAAGTTTATATTTTAATTTTACCCGGATTTGGAATAATTTCTCATATTATT
ACTCAAGAAAGTGGAAAAAAGGAAACATTTGGAACTTTAGGAATAATTTA
TGCTATATTAACAATTGGATTATTGGGATTTATTGTTTGAGCTCATCATAT
ATTTACAGTAGGTATAGACGTAGATACTCGAGCTTATTTTACTTCAGCAAC
TATAATTATTGCTGTTCCAACTGGAATTTAAAATTTTATAATTTATATTTTT
GGGGGTGGGTGGGGGGGGGG

# Appendex-III

## Table-5: The GenBank accession numbers of nucleotide sequences provided by NCBI

| Sample No. | Accession Number |
|---|---|
| CVASU-13 | MH836623 |
| CVASU-14 | MH885495 |
| CVASU-21 | MH885496 |