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MOLECULAR CHARACTERIZATION OF MAJOR VECTOR MOSQUITOES OF BANGLADESH

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Abstract

Mosquito-borne diseases are considered major contributors to vector-borne diseases, threatening more than eighty per cent of the global population. Pest management depends on proper identification techniques. The barcode region of the cytochrome oxidase subunit I gene of mitochondrial DNA has recently been proposed as a systematic tool, functional in taxonomy and evolutionary study for species definition. This work is the first attempt to identify the main vector mosquito species from Bangladesh based on the MT-COI gene. Eleven vector mosquitos were identified. AT content (69%) was found to be higher than GC content (31%) at the COI barcode region of the mosquito. The interspecific genetic divergence range of medically important mosquitoes was 0.01-0.21. Haplotype analysis revealed that *Mansonia annulifera* diverged highly from its immediate ancestor by the highest mutational steps (59). Phylogenetic analysis indicated that species belonging to the same family were in the same major clade. Overall, our findings contribute to a better method of identifying major vector mosquito species by COI genes and for implementing management measures against mosquito pests in Bangladesh.

KEY WORDS: Vector mosquito, MT-COI gene, genetic distance, haplotype, phylogenetic analysis

Introduction

Mosquitoes, belonging to the family Culicidae, are the vectors of some pathogens that cause serious diseases in humans (Becker *et al*., 2010; Dieme *et al*., 2015). Approximately 41 genera and 3,556 species and subspecies of mosquito exist worldwide (Tandina *et al*., 2018). According to Irish *et al*. (2016), 121 mosquito species are found in Bangladesh. Only a few are considered vector mosquitoes in Bangladesh (Irish *et al*.,

2016; Paul *et al*., 2018; Alam *et al*., 2018). Mosquitoes have been researched more than most other insect groups because of their role as disease vectors (José *et al*., 2009).

Mosquitoes are the most important medical pests. Their bite causes irritation by blood sucking (Karim *et al*., 2013). Mosquito vectors can spread various pathogens, including arboviruses, protozoans, and filariae, that cause infectious diseases (Becker *et al*., 2010). Mosquito-borne viruses (arboviruses) have afflicted humans for millennia and continue to cause immeasurable suffering (Powell *et al*., 2018). Viruses causing dengue infect nearly 100 million people living in 110 countries each year and spread over the world's tropical areas (Halstead, 2000). *Aedes aegypti* and *Aedes albopictus* are recognized vectors of dengue and dengue hemorrhagic fever (Lane & Crosskey, 1993; Kettle, 1995). *Aedes aegypti* is the principal vector of yellow fever, dengue, dengue hemorrhagic fever, and dengue shock syndrome (Georghiou *et al*., 1987). At present, mosquito-borne diseases are the main concern in Bangladesh (Dayaram & Pokharel, 2019). The people of Dhaka city fear this mosquito species because of the outbreak of dengue and Chikungunya (Mone *et al*., 2019). Dengue is linked to Zika virus, yellow fever, West Nile virus, and Japanese encephalitis (Cousins, 2019). More than 115,986 suspected cases of dengue and 456 deaths were reported in the Philippines in 2019 (Agrupis *et al*., 2019). *Culex quinquefasciatus* is a proven vector of Bancroftian filariasis in Dhaka and in other places of Bangladesh (Wolfe & Aslam, 1971). The female *Anopheles* mosquito is responsible for malaria (Dimopoulos, 2002; Surendran *et al*., 2013). *Armigeres subalbatus* is the vector of zoonotic infections (Muslim *et al*., 2013). *Wuchereria bancrofti,* the causative agent of lymphatic filariasis (LF), is transmitted by mosquito species belonging to the genus *Mansonia* (Ugashi *et al*., 2012). To a lesser extent, they may also transmit bacterial diseases (Dieme *et al*., 2015).

Species identification constitutes the first step in the control of mosquito-borne diseases (Osorio *et al*., 2014). Morphological identification has limitations as it identifies mosquito species based on their external characteristics (Chan *et al*., 2014). Many species of the *Culex* genus are identified based on variations in male antennae, palpus, proboscis, and genitalia, making the morphological identification of their female counterparts complex (Sirivanakarn, 1977; Rattanarithikul *et al*., 2007). These constraints limit the validity of current taxonomic keys for the identification of several mosquito species (Chan *et al*., 2014). DNA analysis, which provides a more precise method of identifying species in combination with morphological methods, has fixed some long-standing taxonomic questions (Friedheim, 2016). Herbert *et al*. (2003, 2004) proposed using the 658 base pair (bp) region of the COI gene as a universal marker to barcode animal life (Hebert). Many studies have proven that the COI gene is an efficient and valuable barcode for the identification of metazoans, including mosquitoes (Kumar *et al*., 2007; Wang *et al*., 2012; Khamis *et al*., 2012; Bourke *et al*., 2013).

The present research is the first molecular approach to identify important disease-causing mosquito species in Bangladesh. It was also decided to conduct a bioinformatic analysis of the mosquitoes to establish their molecular characterization. Sequencing the mitochondrial COI (MT-COI) of mosquito species may be used as a barcode for proper taxonomic identification.

Materials and Methods

Collection and morphological identification

The study was carried out from March 2017 to October 2019. Immature larvae were collected from various breeding sites in different locations in Bangladesh (Mirpur, Mohakhali, Jahangirnagar University, Banderban), and were then reared in a laboratory. Newly emerged adults were well-preserved in plastic vials for identification. Adult mosquitoes were also collected while biting and swarming near biting sites, cattle sheds, and human dwellings. A total of 1,150 mosquito species were collected. The preserved mosquitoes were identified up to the species level by the taxonomic keys of Bram (1967) and Ahmed (1987). Morphological

characteristics, such as scales on the wings, proboscis, chaetotaxy, wing veins, wing margins, palpi served to preliminary identify vector mosquitoes.

DNA extraction

The genomic DNA was extracted from collected tissues (legs/wings) using the Wizard® Genomic DNA Purification Kit (Promega, USA) following the manufacturer's protocols with slight modifications, as discussed by Aslam et al. (2019). Seventy-five specimens were considered for DNA barcoding. The concentration and purity of DNA were measured by a Nanodrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA) and stored at -20°C until further use.

PCR amplification, gel electrophoresis, and sequencing

Genomic DNA was subjected to PCR amplification of a 710 bp region near the 5' terminus of the COI gene in a 96-well thermal cycler (Veriti, Applied Biosystems, Thermo Fisher Scientific, USA). Forward primer LCO1490(F)-5'GGTCAACAAATCATAAAGATATTGG-3' and reverse primer HCO2198(R)5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer *et al*., 1994) were used to amplify a 710 bp segment. A 20 µl PCR reaction consisted of 10 µl of Master mix (Taq DNA polymerase, dNTPs, $MgCl_2$, and buffers) (Promega, USA), 1 µl of forward primer, 1 µl of reverse primer, 50 ng of template DNA, and nuclease-free water. COI was amplified using a PCR protocol, as follows: initial step was at 94°C for 3 min, 32 cycles of denaturation at 94°C for 30 s, annealing at 49°C for 30 s, extension at 72°C for 45 s. After thermal cycling, the amplified DNA was stored at -20°C. The amplified product was analyzed by 1% agarose gel electrophoresis. The PCR product was purified using a Promega Wizard® SV Gel and PCR clean-up system (Promega Corporation, USA). Sequencing was performed using an ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, USA) and the manufacturer's protocol of Apical scientific, Malaysia.

Sequence analysis

After editing sequences using FinchTV software, all sequences were deposited in the NCBI GenBank (BankIt) to obtain unique accession numbers (Table I).

Table I. GPS position of the sampling locations and GenBank accession numbers of the sequenced mosquitoes

Species name	GPS position	Accession no
Culex pipiens	23.80231N, 90.351549E	MK333464
Culex quinquefasciatus	23.784919,90.404354	MK370091
Aedes aegypti	23.802316N, 90.351549 E	MH052647
Aedes albopictus_1	23.52228 N, 90.16414 E	MG572237
Aedes albopictus_2	23.875183 N,90.26667 E	MG913592
Anopheles subpictus	21.8002624N,92.2608249E	MK142267
Anopheles vagus	21.8002626,92.2608251	MK189164
Anopheles varuna	22.190818N,92.216574E	MK216790
Anopheles kochi	21.8002630N,92.2608255 E	MK234124
Anopheles jeyporiensis	21.800372N, 92.260149E	MK138573
Mansonia annulifera	23.811814N, 90.346886 E	MH616625
Armigeres subalbatus_1	23.873741N, 90.268192E	MH626641
Armigeres subalbatus_2	23.875183 N,90.26667 E	Mk248724

Bioinformatics analysis

COI sequences were aligned using the ClustalW algorithm with the help of MEGA X with gap opening penalty 15, gap extensions penalty 6.66, transition weight 0.5, and delay divergent cutoff of 30% (Kobayashi *et al*., 1998; Simon & Hadrys, 2013; Kumar *et al*., 2018). ClustalW aligned the most similar sequences first and the least similar sequences for global alignment. ClustalW can align any number of homologous nucleotide sequences (Kobayashi *et al*., 1998).

Multiple sequence alignment images were prepared using Jalview, version 2.9 (Waterhouse *et al*., 2009). MEGA X software was used to calculate nucleotide base components. For the calculation of genetic distances among sequences, Kimura's two-parameter method (K2P) of base substitution was used in MEGA X (Kumar *et al*., 2018). The Kimura 2 parameter model corrects multiple hits and corrects transitional and transversional substitute rates; the substitute rates do not vary among the sites. (Kumar *et al*., 2018).

The evolutionary history was inferred using the maximum likelihood method based on the Tamura-Nei model (Tamura-Nei, 1993) which corrects multiple hits and distinguishes between transitional substitution rates between purines, and the transversional substation rates between pyrimidines. The haplotype was constructed using PopART 1.7 based on the TCS network (Clement *et al*., 2000).

Results

Sequence result and BLAST analysis

During the study period, a total of 1,150 mosquito specimens were collected. Thirteen specimens were selected and sequenced based on the morphological differences. A BLAST search revealed that the analyzed sequences belonged to 11 mosquito species: *Culex quinquefasciatus*, *Cx. pipiens*, *Aedes aegypti*, *A. albopictus*, *Anopheles jeyporiensis*, *An. vagus*, *An. varuna*, *An. kochi*, *An. subpictus*, *Armigeres subalbatus* and *Mansonia annulifera*.

Nucleotide composition

Sequence analysis results using MEGA X software (Kumar et al., 2018) showed percentage of base contents in the COI region of mosquito species (Table II). The AT base content was higher than G-C base content in the MT-DNA of mosquito species. The highest AT content (69%) was found in *Cx. pipiens*, and the highest GC content (34.9%) was found in *An. vagus.* The lowest AT content (65.1%) was found in *An. vagus,* and the lowest GC content (31%) was found in *Cx. pipiens*.

Genetic distance analysis

Interspecific pairwise distance analysis was conducted using MEGA, version X (Kumar *et al.* 2018). The interspecific genetic divergence range of the medically important mosquito was 0.01-0.21 (Table III). The lowest (0.01) pairwise distance was found between *C. quinquefasciatus* and *C. pipiens*. The highest pairwise distance (0.21) was found between *M. annulifera* and *A. subalbatus.*

Table II. Nucleotide base composition of the sequenced vector mosquito species

Species	T	C	A	G	AT	GC
Aedes aegypti	38.9	17.7	28.4	15.1	67.2	32.8
Aedes albopictus_1	38.7	17.4	28.5	15.4	67.2	32.8
Aedes albopictus_2	38.9	17.2	28.4	15.6	67.2	32.8
Culex.pipiens	39.5	15.4	29.5	15.6	69.0	31.0
Culexquinquefasciatus	38.9	15.7	29.5	15.9	68.4	31.6
Anophelesjeyporiensis	38.7	16.1	29.3	15.9	68.0	32.0
Anopheleskochi	37.5	16.4	30.5	15.6	68.0	32.0
Anopheles subpictus	36.9	17.2	29.3	16.6	66.2	33.8
Anopheles vagus	36.2	18.0	28.9	16.9	65.1	34.9
Anopheles varuna	37.9	16.7	29.3	16.1	67.2	32.8
Mansonia annulifera	37.5	18.7	28.5	15.2	66.1	33.9
Armigeres subalbatus_1	39.8	17.4	28.2	14.6	68.0	32.0
Armigeres subalbatus_2	39.8	17.4	28.2	14.6	68.0	32.0

Table III. Interspecific (K2P) genetic divergence of the mosquito species at the COI barcode region

	1	2	3	4	5	6	7	8	9	10	11	12	13	1
1.*Aedesaegypti*														
2.*Aedesalbopictus_1*	0.13													0.13
3.*Aedesalbopictus_2*	0.13	0.12												0.13
4.*Culexpipiens*	0.13	0.14	0.14											0.13
5.*Culex quinquefasciatus*	0.13	0.15	0.15	0.01										0.13
6.*Anohelesjeyporiensis*	0.19	0.16	0.16	0.15	0.15									0.19
7.*Anopheleskochi*	0.17	0.18	0.18	0.15	0.15	0.12								0.17
8.*Anopheles.subpictus*	0.18	0.17	0.17	0.17	0.18	0.13	0.14							0.18
9.*Anopheles_vagus*	0.19	0.19	0.18	0.18	0.19	0.13	0.14	0.14						0.19
10.*Anophelesvaruna*	0.19	0.17	0.17	0.15	0.16	0.10	0.12	0.13	0.14					0.19
11.*Mansoniaannulifera*	0.18	0.19	0.19	0.17	0.18	0.18	0.20	0.21	0.19	0.19				0.18
12.*Armigeres subalbatus_1*	0.15	0.16	0.16	0.13	0.14	0.17	0.18	0.19	0.19	0.19	0.18			0.15
13.*Armigeres subalbatus_2*	0.15	0.15	0.15	0.13	0.14	0.17	0.18	0.19	0.19	0.19	0.18	0.03		0.15

Haplotype network

Statistical parsimony (TCS) haplotype network was constructed based on the COI gene sequences of 11 medically important mosquito species. Haplotype network analysis helps determine the genetic divergence among species through their mutation rates. Results focused on the phylogenetic relationship among medically important mosquito species, indicating a high diversity among them by showing the mutational steps (Fig. 1). Both *Culex* species evolved from their immediate ancestor in 33 mutational steps. *M. annulifera* diverged from its immediate ancestor in 59 mutational steps. *A. aegypti* and *A. albopictus* were separated from their immediate ancestors in 33 mutational steps and 38 mutational steps, respectively. *A. subalbatus* diverged from its immediate ancestor in 37 mutational steps. *A. jeyporiensis* diverged from its immediate ancestor in 26 mutational steps. *A. vagus*, *A. kochi*, *A. subpictus*, and *An. varuna* diverged from their immediate ancestors in 43, 36, 44, and 26 mutational steps, respectively. According to the number of mutational steps, *M. annulifera* was the most highly diverged from its immediate ancestor (59 steps).

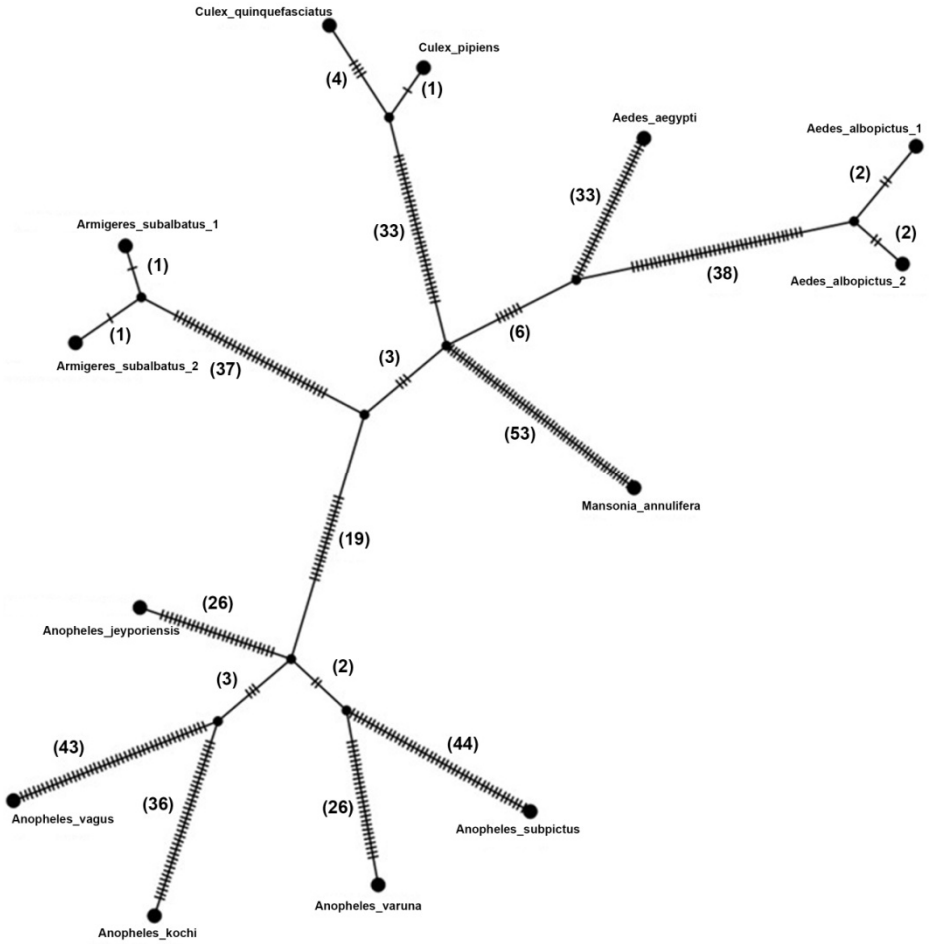

Figure 1. Haplotype network of the different mosquito species constructed by PopART 1.7, based on the TCS network. Large circles represent the haplotypes, and their sizes are proportional to the number of specimens with them; small circles represent the intermediate haplotype.

Phylogenetic analysis

Using the Tamura-Nei model, a phylogenetic tree was constructed for analysis with MEGA X (Kumar *et al*., 2018). In the maximum-likelihood tree, 11 mosquito species were grouped into two distinct clades. *Cx. pipiens*, *C. quinquefasciatus*, *A. subalbatus*, *A. aegypti*, *M. annulifera* and *A. albopictus* were in one clade, and *A. subpictus*, *A. vagus*, *A. kochii*, *A. jeyporiensis*, and *A. varuna* were in another (Fig. 2).

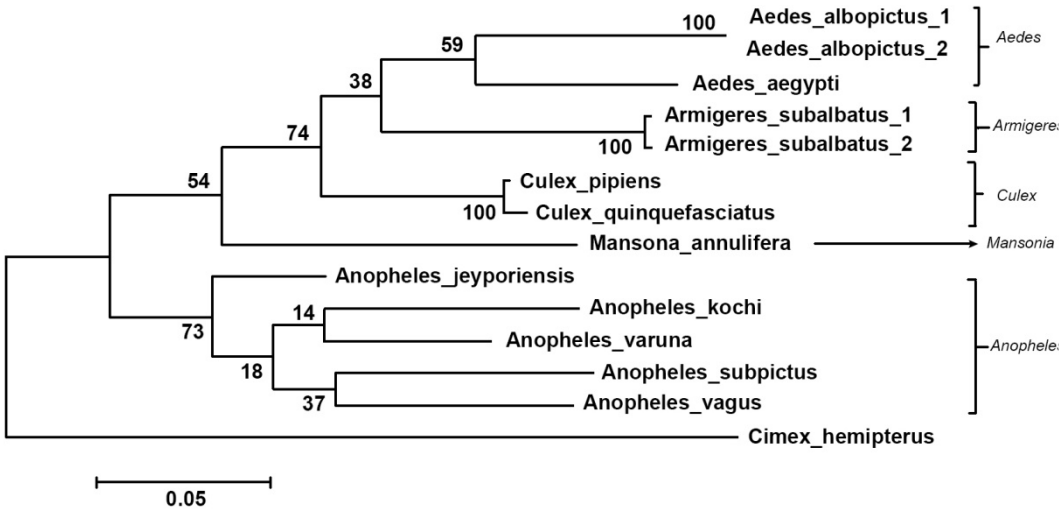

Figure 2. Evolutionary relationship among eleven mosquito species was constructed using MEGA X (Kumar *et al*., 2018). The tree with the highest log likelihood = -3567.6735. The bar at the bottom is a scale denotes the genetic change. *Cimex hemipterus* was considered an outgroup in the maximum-likelihood tree.

Discussion

During the present research, an extensive survey was done to collect the mosquito species throughout Bangladesh. A total of 1,150 mosquito specimens were collected, and the vector mosquitoes were preliminarily identified based on morphological keys. The sequenced mosquito species identified 11 species: *A. aegypti*, *A. albopictus, C. quinquefasciatus, C. pipiens, A. subalbatus, M. annulifera, A. subpictus, A. vagus, A. kochi, A. varuna*, and *A. jeyporiensis.*

Based on morphological keys, Irish *et al*. (2016) recorded 121 mosquito species in Bangladesh, among which 7 *Anopheles* species were responsible for malaria: 1. *Culex* species were responsible for filariasis, 2. *Aedes* species for dengue, Zika, and chikungunya diseases (Irish *et al*., 2016; Paul *et al*., 2018; Alam *et al*., 2018). Thirty-six *Anopheles* (Diptera: Culicidae) species were identified in Bangladesh, only seven of which were recognized as important vectors of malaria (Irish *et al*., 2016). Four of these species, *A. baimaii*, *n. philippinensis*, *A. sundaicus*, and *A. minimus* were considered as vector species (Renshaw *et al*., 1996; Alam *et al*., 2012; Al-Amin *et al.,* 2015). *A. aegypti* and *A. albopictus* are the known vectors of dengue virus and other arboviruses, including chikungunya, yellow virus, and Zika virus (Kraemer *et al*., 2015). These species are very efficient vectors of arboviruses and live close to humans (Scott & Takken, 2012). *Cx. quinquefasicatus* is a potential vector of filariasis (Karlekar & Andrew, 2015). Ilahi & Suleman (2013) recorded a total of 21 mosquito vector species from Pakistan from five genera: *Culex*, *Anopheles*, *Armigeres*, and *Aedes*. Suhashini & Sammaiah (2014) recorded seven mosquito vector species belonging to 3 genera: *A. culicifacies, A. stephensi, A. annularis, C. quinquefasciatus, C. tritaeniorhynchus, A. aegypti* and *A. albopictus*, which were collected and identified in the urban area of Warangal, India. Anandh & Sevarkodiyone (2017) recorded twelve vector mosquito species in India: *A. aegypti, A. albopictus, A. scatophagoides, A. barbirostris, A. pallidus, A. peditaeniatus, A. stephensi, A. vegus, A. subalbatus, C. tritaeniorhynchus, C. quinquefasciatus*, and *C. vishuni.*

A few studies carried out molecular identification of mosquito species worldwide. Chan *et al*. (2014) sequenced 128 adult mosquito specimens belonging to 45 species of 13 genera in Singapore. Batovska *et al*. (2016) obtained cytochrome oxidase I (COI) sequences for 113 morphologically identified mosquito specimens of Australia, representing 29 species and 12 genera. In Bangladesh, this is the first study to identify medically important mosquito species based on the COI gene.

The nucleotide analysis showed that AT base content was higher than GC base content in the MT-DNA of mosquito species (Table II). The GC bond was more stable since it had three hydrogen bonds, compared to the AT bond, which only has two hydrogen bonds. Soni *et al*. (2018) found the total nucleotide base composition of the COI fragment varied across the selected mosquito specimens with an expected AT bias. The sequences were highly AT-rich, ranging from 66.7 to 67.5% and GC content from 32.5% to 33.3%.

The interspecific genetic divergence range of the medically important mosquito was 0.01-0.21 (Table III). The lowest (0.01) pairwise distance was found between *C. quinquefasciatus* and *C. pipiens*. The highest pairwise distance (0.21) was found between *M. annulifera* and *A. subalbatus*. It is worth mentioning that the findings of Taira *et al*. (2012) showed medically important vector species in a less than 0.02 range with 00.15 divergences.

The phylogenetic tree revealed that all mosquito species were separated into two different clades. Species belonging to the same genera were in the same clade (Fig. 2). Phylogenetic analysis revealed that the same species of the same family were grouped into the same clade. As they belong to the same clade, that means they have some similarity among them. If a control measure for one species works, then the control measure would be effective for other species of the same clade. According to Wang *et al*. (2012), specimens of the same species always grouped closely together, regardless of the collection site, and except for some species, no noticeable geographic differences in sequences within the same species were found. Paramasivan *et al*. (2013) found that *A. aegypti*, and *A. albopictus* were in the same clade. *A. subalatus* was closely related to *A. albopictus*. *Culex* species were in the same cluster. Chan *et al*. (2014) constructed trees for *Aedes*, *Anopheles*, *Culex,* and other mosquitoes, and specimens of the same species were always grouped closely together. All the above results agree with our findings.

Weeraratne *et al.* (2018) identified 14 mosquito species from Sri Lanka using the COI gene for the first time. The interspecific genetic divergence range was 0.2-1.4, just like our findings. Phylogenetic analysis revealed that each species was represented by well-supported clades, confirming the morphological identification of 14 species. All species were highly genetically diverged, just like our findings. The present study highlights the importance of molecular characterization in the recognition of mosquito species, which can be effectively combined with the future development and implementation of vector control strategies.

Conclusion

Mosquitoes are considered serious, medically important pests that transmit many pathogenic viruses and cause serious health problems in humans. In the present study, 11 vector mosquito species were sequenced through the COI gene and identified. COI barcode can differentiate several mosquito species that were hard to identify morphologically. The phylogeny-assisted DNA barcode analyses enabled us to refine the taxonomic identification. The accuracy of DNA barcoding as a species identification tool makes it an essential part of vector surveillance. Conventional morphological identification failed to identify the egg and juvenile stages, missing or damaged specimens, and indistinguishable features of mosquito species. In the DNA barcoding method, only a small amount of DNA is needed to identify mosquito species at their egg and juvenile stage. If we can detect vector species at their early stages, control measures can be taken immediately. Vector control programs should be planned carefully, as the same control measures may not be equally effective for

genetically diverse species. Genetically divergent species can be confirmed by genetic distance, phylogenetic analysis, and haplotype analysis. Genetic identification is possible by assessing mitochondrial DNA sequences derived from an unidentified specimen against a reference database of DNA profiles of known species. The effectiveness of DNA barcoding as an identification tool will continue to improve as the reference database is further expanded. Thus, the present results may be an important contribution to future management programs against mosquito species through the correct identification of mosquito species.

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МОЛЕКУЛАРНА КАРАКТЕРИЗАЦИЈА ГЛАВНИХ ВЕКТОРСКИХ ВРСТА КОМАРАЦА БАНГЛАДЕША

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Извод

Болести које преносе комарци сматрају се главним фактором који доприносе болестима које се преносе векторима, угрожавајући више од осамдесет процената глобалне популације. Контрола штеточина зависи од правилних техника идентификације. Баркод регион гена подјединице I цитокром оксидазе митохондријалне ДНК недавно је предложен као систематски алат, функционалан у таксономији и еволуционој студији за дефиницију врсте. Овај рад је први покушај да се идентификују главне векторске врсте комараца из Бангладеша на основу гена MT-COI. Идентификовано је једанаест векторских комараца. Утврђено је да је садржај AT (69%) већи од садржаја GC (31%) у COI баркод региону комарца. Опсег генетске дивергенције између врста медицински важних комараца био је 0,01-0,21. Анализа хаплотипа је открила да се *Mansonia annulifera* у великој мери одваја од свог непосредног претка највишим степеном мутације (59). Филогенетска анализа је показала да су врсте које припадају истој породици биле у истој главној клади. Наши налази доприносе бољем методу идентификације главних векторских врста комараца помоћу COI гена као и за спровођење мера контроле против штетних комараца у Бангладешу.

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