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DNA barcoding and evolutionary relationship of medically important bed bugs of Bangladesh

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Abstract
Recently, DNA barcoding has become an effective method for the accurate identification of species. This work is the first attempt to identify the bed bug species based on the MT-COI gene from Bangladesh. Nucleotide composition analysis revealed that A-T base content was higher than G-C base contents in mtDNA of *C. hemipterus*. The intraspecific genetic divergence range of *C. hemipterus* was 0.000-0.002. Phylogenetic analysis revealed that all sequences of *C. hemipterus* were clustered in the same major clade. Single nucleotide polymorphism was found in 53 positions of nucleotide sequences of *C. hemipterus*_4. Therefore, our results suggest that COI barcodes can contribute to the exact identification of bed bugs which can aid in pest management programs.

Keywords: Bed bug, COI gene, Genetic distance, SNP and phylogenetic analysis

1. Introduction
The bed bugs (Order: Hemiptera, Family: Cimicidae) are considered household pests for more than 3300 years (Haghi *et al*. 2014; Bandyopadhyay *et al*., 2015) [1-2]. They are blood-feeding ectoparasites of humans, chickens, bats and rarely domesticated animals (Robinson, 2004) [3]. The family Cimicidae comprises more than a hundred species of which only two have succeeded to feed on humans (Service, 1996) [4]. *Cimex lectularius* is temperate species, whereas *Cimex hemipterus* is subtropical and tropical in nature (Zorrilla *et al*., 2015; Balvin *et al*., 2012) [5-6]. In Bangladesh, only *Cimex hemipterus* is found in both rural and urban conditions (Ahmed and Begum, 1992) [7].

The bed bugs are gregarious and live under crowded and uncared for living conditions and often associated with army barracks, labor and prison camps and similar situations where they may readily contact a variety of hosts (Metcalf and Flint, 1973) [8]. As bed bugs have been detected in aircraft, boats, train and hotels, travelers are also at risk of infection (Delaunay, 2012) [9].

Bed bug infestations are considered as a significant socio-economic burden and a major concern to public health (Lai *et al*., 2016) [10]. Bed bug bites may take place mainly around the ankles, face, neck, shoulders, arms, and hands (Doggett *et al*., 2012; Goddard and deShazo, 2009) [11-12]. Humans who are regularly bitten by bed bugs may suffer from nervousness, constant agitation and sleeplessness (Abott, 2002; Adelman *et al*., 2013; Doggett *et al*., 2009) [13-15]. They are suspected to be competent vectors for *Bartonella quintana* and *Trypanosoma cruzi*, the cause of trench fever and chagas disease, respectively in humans (Leulmi *et al*., 2015; Salazar *et al*., 2015; Lai *et al*., 2016) [16-18].

Bed bugs have been reported to carry more than 40 microorganisms in the stomach, feces, exoskeletons, and saliva (Delaunay *et al*., 2011) [19]. Bedbugs feces have been found to contain disease agents and to be infective to livestock animals in oriental sore, chagas disease, anthrax, tularemia, brucellosis, paratyphoid fever, yellow fever, smallpox, and lymphocytic choriomeningitis (Shortt and Swaminath, 1924; Epstein *et al*., 1936; Braun and Caspari, 1938; Milzer, 1944; Caspari and Kann, 1989) [20-24].

Accurate identification is mandatory factor for any type of research with insects (Dantas-Torres *et al*., 2013) [25]. Classical identification depends on morphological study which is not sufficient to identify the insect species accurately. Molecular identification has become a quick and reliable method as an alternative of the traditional taxonomic keys (Ball and Armstrong, 2006) [26]. DNA barcoding has become an efficient molecular technique for species identification (Hebert *et al*., 2003a; 2004a; Hebert *et al*., 2004b; Valentini *et al*., 2008; Smith and Fisher, 2009) [27-31].

A 658-base pair fragments of the mitochondrial cytochrome c oxidase sub unit I (COI) gene is considered as standard barcode fragment (Hebert *et al*., 2004; Hebert *et al*., 2003) [27-29]. DNA barcodes have turned into a vital and increasingly used tool as part of an integrative taxonomy in recent species descriptions (Hendrich and Balke, 2011; Butcher *et al*., 2012; Riedel *et al*., 2013) [32-34].

There is no report on the DNA barcoding of bed bug species in Bangladesh. Therefore, the present study was undertaken to identify the medically important bed bug species and also to understand the nucleotide substitution among the bed bug species, to construct the phylogenetic relationship within bed bug species using COI gene and estimate the divergence from the common ancestor.

2. Materials and methods

2.1 Collection and identification

Adult bedbugs were collected from infested mattresses in different areas of Bangladesh. The bed bugs were collected with the help of a soft brush and kept in a micro centrifuge tube. The specimens were tried to identify up to the species level using the taxonomic keys given by Ahmed and Begum 1992; Zorrilla *et al*. 2015 [5, 7].

2.2 Molecular identification

2.2.1 DNA extraction: The genomic DNA was extracted from unfed adult bed bugs using Wizard® Genomic DNA Purification Kit, (Promega, USA) following the manufacturer's protocol. The concentration and purity of DNA was measured by using Nanodrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA) and stored at -20 °C until further use.

2.2.2 PCR amplification, Gel electrophoresis and Sequencing: The genomic DNA extract was subjected to PCR amplification of a 658 bp region near the 5′ terminus of the COI gene in a thermal cycler 96 well plates (Veriti, Applied biosystems by Thermo fisher Scientific, USA). COI gene was amplified using PCR protocol, as follows: Initial step: 94 °C for 3 minutes, 32 cycles of the following profile: Denaturing step: 94 °C for 30 seconds, Annealing step: 49 °C for 30 seconds, Extending step: 72 °C for 45 seconds. Forward primer LCO1490 (F)-5'GGTCAACAAATCATAAAGATATTGG-3' and reverse Primer HCO2198 (R) 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer *et al*., 1994) [35] which amplify a 650 bp segment were used. The amplified product was analyzed on a 1% agarose gel electrophoresis. The PCR product was cleansed using Promega Wizard® SV Gel and PCR clean up system (Promega Corporation, USA). Sequencing reaction was sent to Apical Scientific, Malaysia and performed using ABI PRISM 3730 xl Genetic Analyzer (Applied Biosystems, Germany).

2.2.3 Sequence analysis: After proper editing of sequences using Finch TV software, all the sequences were deposited in the NCBI GenBank (Bank It) to obtain the accession numbers for all these sequences (Table 1). Some sequences were downloaded from NCBI GenBank for bioinformatics analysis. During the study, out of 63 collected bed bug specimens from different region of Bangladesh, barcoding of 6 specimens were carried out based on their morphological differences (Table 1).

Table 1: GPS position of the sampling locations and GenBank accession number of the sequenced bedbug

Species Name	Latitude, Longitude	Accession Number
*Cimex hemipterus*_1	23.875116N, 90.271508 E	MG587917
*Cimex hemipterus*_2	23.880202N, 90.263620 E	MG552132
*Cimex hemipterus*_3	23.874852N, 90.271494E	MG572241
*Cimex hemipterus*_4	23.874966N, 90.283546E	MG572242
*Cimex hemipterus*_5	23.851246N, 90.251618E	MG587910
*Cimex hemipterus*_6	24.695813N, 91.944143E	MH607404

2.2.4 Bioinformatic analysis: COI gene sequences were aligned using Clustal W algorithm with the help of MEGA tools (version 10) with gap opening penalty 15, gap extensions penalty 6.66, transition weight 0.5 and delay divergent cutoff 30% (Kobayashi *et al*., 1998; Simon and Hadrys, 2013) [36-37]. For calculation of nucleotide base components, MEGA X software was used. For calculation of genetic distances among sequences, Kimura's two parameter method (K2P) of base substitution was used in MEGA X (Kumar *et al*., 2018) [38]. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Kumar *et al*., 2018; Tamura and Nei, 1993) [38-39].

3. Results

During the study period, total of 63 bed bug specimens were collected. Based on morphological differences, selected 6 bed bug specimens were sequenced. BLAST search homology analysis was carried out to check the homology between the retrieved sequences and sequences of the database. Based on BLAST search analysis, the analyzed sequences were identified as belonging to one species named *Cimex hemipterus* (Table 2).

Table 2: BLAST search of *Cimex hemipterus*

Species name	Total Score	Query cover	E value	Identity	GenBank Acc. no.
Cimex hemipterus	1136	100%	0.0	100%	MH607404
Cimex hemipterus	1136	100%	0.0	100%	MG770889
Cimex hemipterus	1136	100%	0.0	99.84%	MG739322
Cimex hemipterus	1136	99%	0.0	100%	MG696803
Cimex hemipterus	1136	99%	0.0	100%	MG587910

Homology of the other 5 sequences of bed bug samples was also checked (Between the retrieved sequences and the database of sequences).

3.1 Nucleotide base contents

Nucleotide composition analysis revealed that A-T base content was higher than G-C base contents in mtDNA of *C. hemipterus*. Highest AT was 63.3% and lowest GC was 36.5% (Table 2).

Table 2: Nucleotide base contents of sequenced bed bug of Bangladesh

Species	T	C	A	G	AT	GC
*Cimex_hemipterus*_1	33.2%	20.1%	30.1%	16.6%	63.3%	36.7%
*Cimex_hemipterus*_2	33.2%	20.1%	30.1%	16.6%	63.3%	36.7%
*Cimex_hemipterus*_3	33.2%	20.1%	30.1%	16.6%	63.3%	36.7%
*Cimex_hemipterus*_4	33.2%	20.1%	30.3%	16.4%	63.1%	36.5%
*Cimex_hemipterus*_5	33.2%	20.1%	30.1%	16.6%	63.3%	36.7%
*Cimex_hemipterus*_6	33.2%	20.1%	30.1%	16.6%	63.3%	36.7%

3.2 Genetic distance analysis

Intraspecific genetic divergence range of *C. hemipterus* was

0.000-0.002. The highest pairwise distance (0.002) found between *Cimex_hemipterus*_2 and *Cimex_hemipterus*_4. The interspecific pairwise distance range was 0.00-0.99. The highest pairwise distance found between *Cimex hemipterus* and *Cimex lectularius* (Table 3).

Table 3: K2P sequence divergence of the sequenced bed bug species at the COI barcode region

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1.*C. hemipterus*_1																
2.*C. hemipterus*_2	0.00															
3.*C. hemipterus*_3	0.00	0.00														
4.*C. hemipterus*_4	0.00	0.02	0.00													
5.*C. hemipterus*_5	0.00	0.00	0.00	0.00												
6.*C. hemipterus*_6	0.00	0.00	0.00	0.00	0.00											
7.*C. hemipterus*_USA	0.00	0.00	0.00	0.00	0.00	0.00										
8.*C. hemipterus* Prague	0.00	0.00	0.00	0.00	0.00	0.00	0.00									
9.*C. hemipterus* Iran	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00								
10. *C. hemipterus* Malaysia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00							
11.*C. hemipterus* Thailand	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00						
12.*C. lectularius*_Canada_1	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99					
13.*C. lectularius*_Canada_2	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.98	0.97	0.97	0.00				
14. *C. lectularius*_Canada_3	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.98	0.97	0.97	0.00	0.00			
15. *C.lectularius*_Canada_4	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.00	0.00	0.00		
16. *C. lectularius*_Canada_5	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.00	0.00	0.00	0.00	

3.3 Phylogenetic analysis

Phylogenetic analysis through the construction of phylogenetic tree was performed by MEGA, Version 10 (Kumar *et al*. 2018). The COI gene sequences of *C. hemipterus* of available subcontinents (USA, Prague, Malaysia, Iran, and Thailand) and *C. lectularius* from NCBI database were considered for proper comparison. In maximum likelihood tree, the total seventeen (17) sequences of *C. hemipterus* and *C. lectularius* were grouped into two distinct clades. All sequences of *C. hemipterus* were clustered in the same major clade that means there were no genetic differences among them (Fig 1).

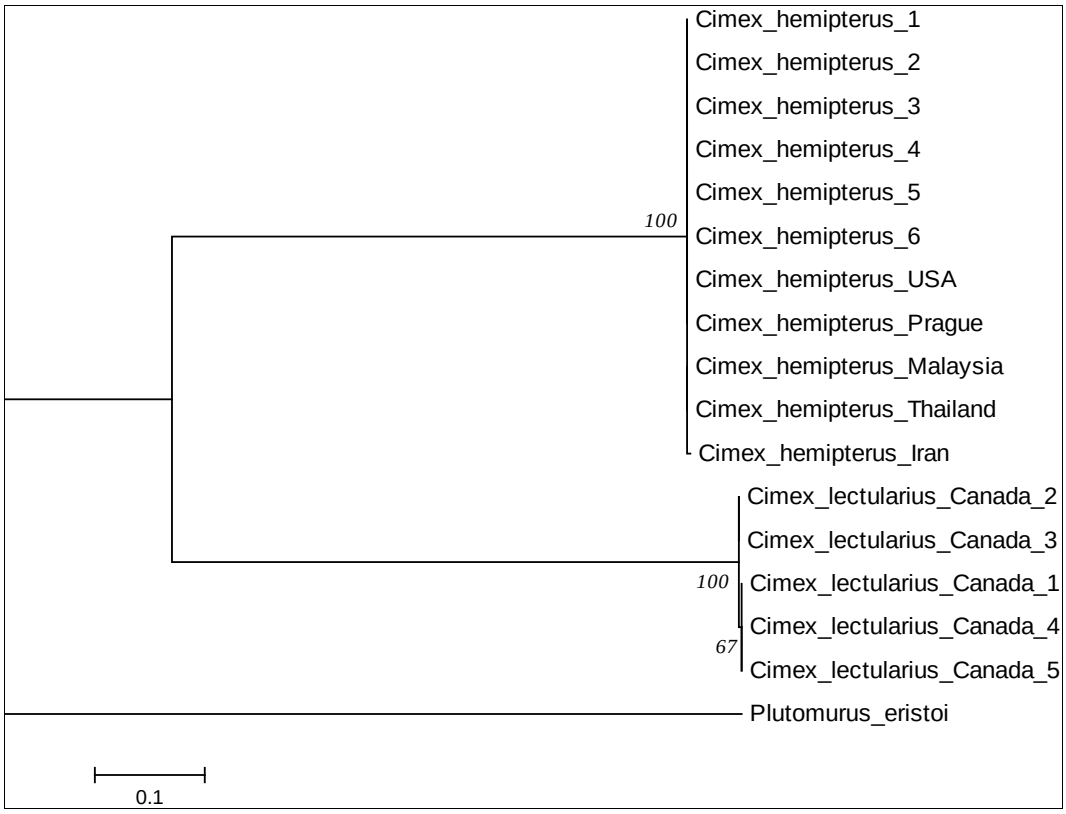
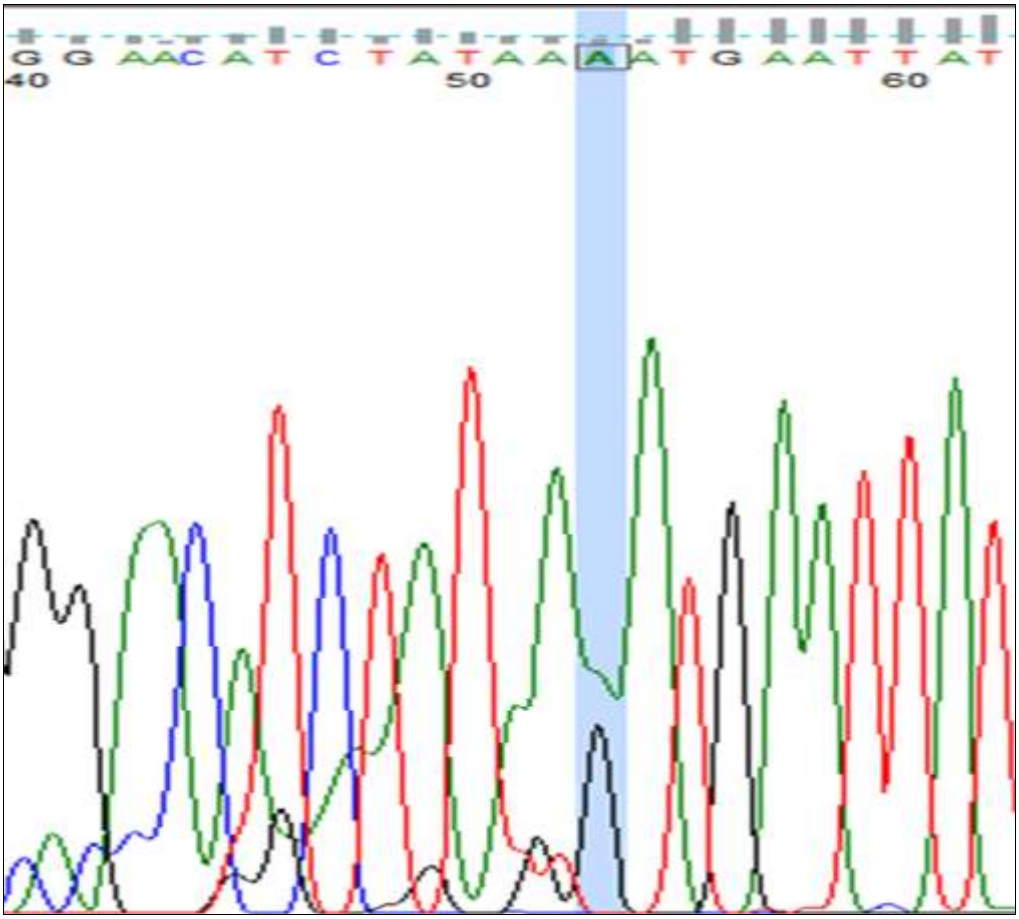


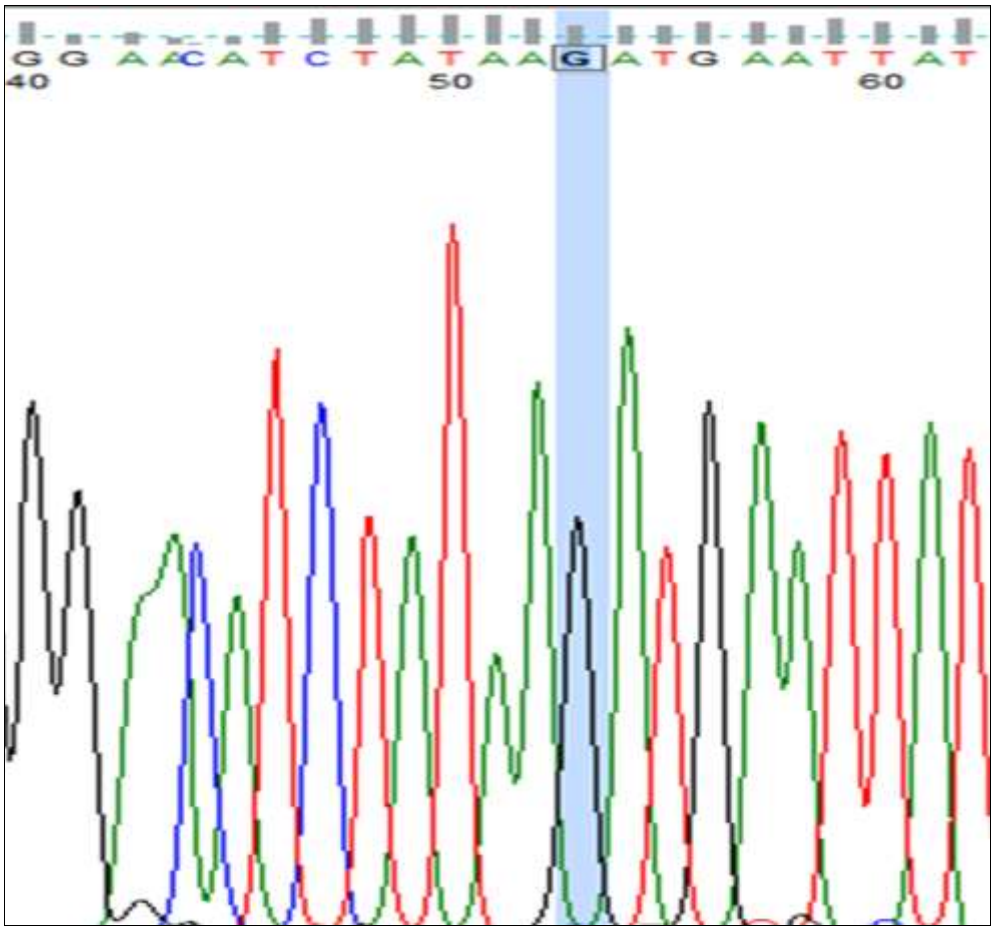
Fig 1: The evolutionary relationship of sequenced bedbug species based on the Tamura-Nei model (Tamura *et al*., 2013) [39] by MEGA, version 10 (Kumar *et al*., 2018) [38]. In Maximum-Likelihood tree, the tree with the highest log likelihood= -1399.5284. The bar at the bottom 0.1 was a scale for genetic change.

3.4 Single nucleotide polymorphism

Single nucleotide polymorphism was found in *C. hemipterus*_4. Other nucleotide sequences of *Cimex hemipterus* have same codon structure. In *Cimex hemipterus*_1, codon A was present in 53 position but in *Cimex hemipterus*_4 codon G was found in 53 position (Fig 2).



a) *Cimex hemipterus* (MG587917)



b) *Cimex hemipterus* (MG572242)

Fig 2: Single Nucleotide Polymorphism analysis of (a) *Cimex hemipterus* (MG587917) and (b) *Cimex hemipterus* (MG572242).

4. Discussion

During the present study, a total of 63 bed bug specimens were collected from different regions of Bangladesh. Considering the morphological differences, six (6) bed bug specimens were sequenced. The result showed that all the sequenced bed bugs belong to a single species, *Cimex hemipterus*. This study provides the first report on molecular data of bed bug, *Cimex hemipterus* of Bangladesh. Khan and Rahman (2012) [40] identified only one bed bug species from Bangladesh.

For precise identification of several taxonomic group, DNA barcoding is the most promising method that utilizes molecular data as an alternative of morphological data (Blaxter, 2003) [41]. The benefit of molecular tactic in establishing phylogenetic relations over the more typical approaches is that the variations can be determined readily. To recognize the close relationship among organisms, sequences alignment is very essential technique in bioinformatics (Kashmeera and Shudhikumar, 2015) [42].

It was reported that the AT base contents were found higher than the GC base contents of bed bug's mitochondria (Table 2). This may be caused by A-T bond, which has a non-coding region that has a further evolution rate compared to the coding region. The composition of the mitochondrial sequence of the COI gene in the present research was expectedly AT biased and this was generally detected in several former studies (Zhang *et al*., 2007) [43]. The intraspecific genetic distances for six COI genes of *C. hemipterus* was 0.000-0.002. The interspecific pairwise distance range was 0.00-0.99. The highest pairwise distance found between *Cimex hemipterus* and *Cimex lectularius* (Table 3). The intraspecific divergence was higher enough to discriminate between the individuals. The divergences among intra species was closely associated. The intraspecific divergences are rarely greater than 0.02 and most are less than 0.01, and higher genetic divergences generally, include taxonomic ambiguity and imply recognition of new species (Avise, 2000) [44].

One SNP was found in 53 nucleotide position (Fig 2). SNPs are absolutely a product of chemical reactions guiding to base substitutions/removal in DNA fragments. The mutation and repair these two forces counteract but the balance is shifted a little bit on the mutational side of the interaction so some mutations (SNPs) can survive.

The purpose of the study was to evaluate the phylogenetic relationship among *C hemipterus* species. For understanding of phylogeny, we constructed Maximum-Likelihood tree. All sequences of *Cimex hemipterus* were clustered in one single clade (Fig. 1). Balvin *et al*. 2012 [6] reported molecular identification of *Cimex lectularius* and found large morphological differences between the groups of bed bug specimens feeding on human and bats. Jung *et al*. (2010) [45] tested the effectiveness of a COI barcode to identify true bugs from 139 species collected from Korea and adjacent regions. DNA barcoding identified one probable new species of true bug and disclosed identical or very newly divergent species that were clearly differentiated by morphological characteristics. These results suggest that COI barcodes can contribute to the exact identification of the bugs.

5. Conclusion

Cimex hemipterus causes some health problems for its human host. In present study, 63 bed bug species were collected from different regions of Bangladesh. Based on morphological differences, total 6 bed bug specimens were sequenced. This is the first attempt of identifying bed bug, *Cimex hemipterus* of Bangladesh based on mitochondrial COI gene sequences. Bioinformatics analysis was also done to know the molecular characterization *i.e.* nucleotide composition, genetic distance, phylogenetic analysis, single nucleotide composition of bed bug, *Cimex hemipterus*. This research would be very effective for attempting any control program of a bed bug, *Cimex hemipterus* through accurate identification.

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7. Conflict of interest disclosure

The authors declare no conflict of interest.

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