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

Faria Farhana Rain and Abu Faiz Md. Aslam

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_. 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 et al., 2012) [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].

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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., 2003) [27]. 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′-TAAACTTCAACAAATCATAAAGATATTGG-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

<table>
<thead>
<tr>
<th>Species Name</th>
<th>Latitude, Longitude</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cimex hemipterus</td>
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<tr>
<td>1</td>
<td>23.781156N, 90.271508E</td>
<td>MG587917</td>
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<tr>
<td>2</td>
<td>23.880202N, 90.263620E</td>
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<tr>
<td>3</td>
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<td>MG572241</td>
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<td>MG572242</td>
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<td>5</td>
<td>23.851246N, 90.251618E</td>
<td>MG587910</td>
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<tr>
<td>6</td>
<td>24.095831N, 91.944143E</td>
<td>MH607404</td>
</tr>
</tbody>
</table>

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) [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

<table>
<thead>
<tr>
<th>Species name</th>
<th>Total Score</th>
<th>Query cover</th>
<th>E value</th>
<th>Identity</th>
<th>GenBank Acc. no.</th>
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<tr>
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<tr>
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<td>99%</td>
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<td>MG587910</td>
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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

<table>
<thead>
<tr>
<th>Species</th>
<th>T</th>
<th>C</th>
<th>A</th>
<th>G</th>
<th>AT</th>
<th>GC</th>
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<tbody>
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<td>30.1%</td>
<td>16.6%</td>
<td>63.3%</td>
<td>36.7%</td>
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<td>Cimex_hemipterus_2</td>
<td>33.2%</td>
<td>20.1%</td>
<td>30.1%</td>
<td>16.6%</td>
<td>63.3%</td>
<td>36.7%</td>
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<tr>
<td>Cimex_hemipterus_3</td>
<td>33.2%</td>
<td>20.1%</td>
<td>30.1%</td>
<td>16.6%</td>
<td>63.3%</td>
<td>36.7%</td>
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<tr>
<td>Cimex_hemipterus_4</td>
<td>33.2%</td>
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<td>Cimex_hemipterus_5</td>
<td>33.2%</td>
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<td>30.1%</td>
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<tr>
<td>Cimex_hemipterus_6</td>
<td>33.2%</td>
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<td>16.6%</td>
<td>63.3%</td>
<td>36.7%</td>
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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

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<td>1.C hemipterus_1</td>
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<td>5. C. hemipterus_5</td>
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<td>6.C. hemipterus_6</td>
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<td>7. C. hemipterus_USA</td>
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<td>8. C. hemipterus_Prague</td>
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<td>9.C. hemipterus_Iran</td>
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<td>10. C. hemipterus_Malaysia</td>
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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).

![Diagram](image)

Fig 1: The evolutionary relationship of sequenced bedbug species based on the Tamura-Nei model (Tamura et al., 2013) by MEGA, version 10 (Kumar et al., 2018). 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).

~ 51 ~
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 [46] 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.

6. Acknowledgement
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7. Conflict of interest disclosure
The authors declare no conflict of interest.

8. References
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