Original Article

The Phylogenetic Analysis of *Cimex hemipterus* (Hemiptera: Cimicidae) Isolated from Different Regions of Iran Using Cytochrome Oxidase Subunit I Gene

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Abstract

Background: Bedbugs are blood feeding ectoparasites of humans and several domesticated animals. There are scarcity of information about the bed bugs population throughout Iran and only very limited and local studies are available. The aim of this study is to assess the phylogenetic relationships and nucleotide diversity using partial sequences of cytochrome oxidase I gene (COI) among the populations of tropical bed bugs inhabiting Iran.

Methods: The bedbugs were collected from cities located in different geographical regions of Iran. After DNA extraction PCR was performed for COI gene using specific primers. Then DNA sequencing was performed on PCR products for the all 15 examined samples.

Results: DNA sequencing analysis showed that the all *C. hemipterus* samples were similar, despite the minor nucleotide variations (within the range of 576 to 697bp) on average between 5 and 10 Single nucleotide polymorphisms (SNPs**)**. Subsequently, the results were compared with the database in gene bank which revealed close similarity and sequence homology with other *C. hemipterus* from other parts of the world.

Conclusion: In conclusion, this study has demonstrated the ability of the COI gene to differentiate between the *C. hemipterus* populations from a few different locations in Iran. The current research is the first report of phylogenetic and genetic species diversity analysis conducted on *C. hemipterus* in Iran. These results provided basic information for further studies of molecular epidemiology, public health and pest control operators in Iran.

Keywords: Bed bug; *Cimex hemipterus*; COI; Phylogenetic analysis; Iran

Introduction

"Bed bugs" is a term often applied to the approximately 90 species within the Cimicidae; of these, only *Cimex lectularius*, the common bed bug, and *C. hemipterus*, the tropical bed bug, show a strong host preference for humans (1). *Cimex hemipterus*, is considered as the common tropical bedbug whereas *C. lectularius* is common in temperate climates (2). It has been well documented that bed bugs harbor at least 40 human pathogens, but there is no tangible evidence regarding the ability of routine mechanical transmission any of them (1, 3-6). All the daytime, bed bugs disappear and hide in inaccessible places such as: cracks and crevices in beds, wooden furniture, floors, and walls and reappear at night to feed from their preferred host, humans (7).

Apart from the discomfort caused by the bite, bedbugs have been known to cause secondary infections and psychological disorders (4). Chronic infestation can cause nervousness, lethargy, pallor, diarrhea, and even iron deficiency (8-10). Bedbugs infest any kind of temporary accommodations, especially hotels, serviced apartments, and cause severe problems. A resurgence of bed bugs has been reported

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from the United States, Canada, Australia, Europe, and some Asian countries during the past 15 years (11-16). The reasons for the explosion have not yet been clarified; however, several factors such as increased rate of international travel, reduced use of residual insecticides indoors, and insecticide resistance may play a role (17).

Few studies have been undertaken on these bugs in Iran. Dehghani et al. examined an outbreak of these bugs in 1998 in villages west of Kashan. Out of 495 houses in 10 villages there was 6.7% contamination1 (18). In that year, the National Association of Managing World Pests announced the contamination in New York at 6.7%. Shahraki et al. in 2000, examined an outbreak of bugs in university dormitories among 180 boys and 145 girls in Yasuj, western Iran. They reported 28.9% contamination which approximated the current study after taking into consideration that the numbers of individuals were not close to our study (19). Haghi et al. reported that in Bahman Amir, Mazandaran, Iran most bugs were found in bedrooms (56.54%), living rooms (31.25%), and kitchens (8.59%) (17). Also, from the 182 examined containers in Polour, Mazandaran Province, 164 (approximately 90.1%) had evidence of contamination by bed bugs (20).

Genetic analysis of medically important insect species is absolutely required, because it provides useful information about vector transmission, disease epidemiology and disease control (21). There is scarcity of information about the bed bug population throughout Iran and only very limited and local studies are available (17, 20). Taxonomic and morphologic identification of bed bugs require highly experienced person and appropriate samples. Nowadays, molecular techniques such as nucleotide sequence analysis and phylogenetic tree have been developed for taxonomic identification (22).

In recent years, the application of high-resolution molecular markers has provided important new insight into the population genetic structure and infestation dynamics of many insect pest species of public health concern (23-25). New molecular tools now make it feasible to not only accurately identify the number of populations actively infesting a building (24, 26) but also to elucidate dynamics and characteristics essential for understanding infestation patterns and history, e.g., levels of genetic diversity (a measure often associated with population health (27), temporal stability of populations after pest control efforts (28), and the presence or absence of genetic mutations associated with insecticide resistance (29).

Population genetics studies on bed bugs have been completed using nuclear rRNA, mt DNA genes, and microsatellite loci as markers (25, 30-34). The aim of the first of these studies (Szalanski et al. 2008) shed light on the dispersal patterns of bed bugs during their recent global resurgence. All of the studies on bed bugs thus far have found genetic diversity within human associated population to be low, resulting from a great deal of inbreeding (25, 30-32). Despite the importance of bed bugs in Iran, genetic evaluation has not yet been studied. Therefore, we aimed at this shortcoming and analyzed DNA sequences of *C. hemipterus* gathered from different parts of Iran, using partial sequences of cytochrome oxidase I gene (COI) and evaluated the genetic relationship between them.

Materials and Methods

Sample collection

All procedures in this study were carried out in accordance with the guidelines of the Animal Ethics Committee of Faculty of Veterinary Medicine, Urmia University (AECVU) and supervised by authority of Sample collection Urmia University Research Council (UURC).

Geographically, there are 4 different zones in Iran, known as region 1: Caspian Sea (temperature: 8–26 °C, annual rainfall: 400–1,500 mm), region 2: Mountainous area (temperature: -5–29 °C, annual rainfall: 200–500mm), region 3: Persian Gulf (temperature: 12.6–35 °C, annual rainfall: 200–300mm), and region 4: the Central Desert (temperature: -4–44 °C, rainfall: less than 100mm). The pattern of bed bug species distribution for the 4 different areas was determined according to the method of Skerman and Hillard (35). Considering 10% prevalence, 95% confidence level and 5% error rate, 138 bed bugs collected and were used in this study. That way, adult bed bugs were collected from various locations such as infested hotels, residential houses and industrial buildings during May 2016 to August 2017, with the help of pest control companies. The following cities were selected from each region: Region 1: Sari and Rasht, region 2: Tehran, Isfahan, Urmia, Tabriz, Shiraz, Saghez, Sanandaj, Kermanshah and Hamadan, region 3: Ahvaz and Bandar Abbas, and region 4: Semnan. These locations were mapped by collecting the locality data via Google Earth (Fig. 1).

Individual samples were collected by using forceps then stored in a sample collection bottle and preserved in 95% alcohol and stored at -20 °C until analysis onset. The insects were transferred to the laboratory of parasitology division, faculty of veterinary medicine, Urmia University and identified under a stereo microscope (Olympus SZ61, Olympus Corporation, Tokyo, Japan), using morphological keys described by Usinger (1) and Walpole (36). The pronotum which is the most distinguishing feature used to identify the two bedbugs species. The pronotum of *C. lectularius* is wider than that of *C. hemipterus* because of an upturned lateral flange on the margin of the pronotum on the thorax of *C. lectularius* which is absent in *C. hemipterus* (1). The dorsal and ventral sides of the Bedbug pronotum were observed to get a distinct image of the pronotum because the projecting edge of some were not very clear dorsally.

DNA extraction

Genomic DNA from Individual tropical bed bug of each collection site was extracted using DNA isolation kit, MBST (Molecular and Biological system transfer, Tehran, Iran) following the manufacturer's instructions. For this, samples were grinded by pestle and placed into 1.5ml micro centrifuge tube and total DNA was eluted in 100µl of elution buffer. DNA quality and concentration from each specimen was determined spectrophotometrically using the NanoDrop (Thermo Scientific 2000c, United States) and stored in -20 °C for further procedures. We control the contaminations by check the 260/230 ratio because a poor ratio may be the result of a contaminant absorbing at 230nm or less. Also we check the wavelength of the trough in the spectra; this should be at 230nm.

Amplification of the mitochondrial COI and sequencing

For PCR amplification of the COI, the 658bp amplicon, the forward and the reverse primers LEP-F (5′-ATT CAA CCA ATC ATA AAG ATA TNG G- 3′) and LEP-R (5′-TAW ACT TCW GGR TGTCCR AAR AAT CA-3′) were used (32). The PCR was conducted in 25µl total volume, each containing 2.5µl 10x PCR buffer, 2µL 50mM MgCl2, 0.5µl dNTPs, 3µl DNA template, 10 picomole forward and reverse primers (0.5µl for each), 0.5µl Taq DNA polymerase (Sinaclon, Iran) and 15.5µl ddH_2O. The PCR cycling conditions set in the program were as follows: initial denaturation at 94 °C for 5min followed by 35 cycles of 94 °C for 30sec (denaturation), 42 °C for 30sec (annealing), 72 °C for 45sec (extension) and a final extension step of 72 °C for 2min. PCR products were analyzed by electrophoresis in 1.5% agarose gel to confirm that the samples con-

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tained a single band. Then, stained with safe DNA stain gel and visualized with UV-Transilluminator (BTS-20M, Japan). Finally, Purified PCR products were sent to SinaClon Company (Tehran, Iran) for sequencing.

Nucleotide Diversity and Phylogenetic Tree Construction

The nucleotide sequences of each species from various regions were aligned for variation positions. Sequences were uploaded on NCBI to search for the most similar reference sequences, and positions of the COI were determined with the help of BLAST, available at NCBI. A total of eleven COI sequences belong to *C. hemipterus* available in the Gene Bank were used to phylogenetic analysis, including 3, 3, 2, 1, 1 and 1 sequence related to Malaysia, Bangladesh, Thailand, Czech Republic, USA and Iran, respectively. The *Triatoma dimidiata* (Hemiptera: Reduviidae), accession number JQ575031, was used as an out group. The alignment was manually edited to remove any alignment errors using the aligning tool Clustal W (37) and exported as MEGA and FASTA format files. All the obtained nucleotide sequences were deposited in the GenBank with the assigned accession numbers (Table 1). Subsequently, phylogenetic relationship was examined and constructed by Maximum-likelihood method (ML) using the Molecular Evolutionary Genetics Analysis (MEGA), version 6.0. The reliability of an inferred tree was tested by 1000 bootstrap. The DNA sequence polymorphism analyses for determining nucleotide diversity were estimated using BioEdit Version 7, 0, 1 and Blastn software (38).

Results

The average size of COI fragment obtained from the amplified *C. hemipterus* was found to be 655bp which was at the expected PCR product size (approximate length 658 bp) for the all 15 examined samples, within the range of 576 to 697bp. The accepted COI sequences found in NCBI GenBank database showed that the percentage identity ranged from 97 to 100. Nucleotide sequences of COI obtained from this study were submitted to NCBI GenBank and then accession numbers of MG770888 to MG77089, MG739319 to MG 739326, MG737714 and MG696803 were assigned to them (Table 1).

After some processing, for example, deleting and aligning sequences using Mega 6 Molecular Software, 362bp of partial COI from 15 sequences of *C. hemipterus* were obtained successfully. Construction of phylogenetic tree is done based on mitochondrial COI sequencing by maximum likelihood (ML) method. The sequences obtained from the present study were compared with sequences of *C. hemipterus* and *C. lectularius* from different parts of the world. As the tree shows, the samples were classified in three major clusters which confirm the genetic variation among different species of *Cimex*. All our isolates and those of other parts of the world were placed in one clustered together (G1) showing no significant difference between various regions despite the minor nucleotide variations.

Consistently, they were far from *C. lectularis* and *T. dimidiata* clusters (G2 and G3, respectively). Our isolates were further clustered into three subgroups (Fig. 2). The first one (SG1) contained 9 isolates which are shown in the phylogenetic tree in Fig. 2. Sequences from this subgroup clustered together with the *C. hemipterus* reference sequences containing three nucleotide sequences from Bangladesh (MG552132, MG572242, MG 587917), three nucleotide sequences from Malaysia (KT851503 to KT851505), two nucleotide sequences from Thailand (JX 826469, JX826470), two nucleotide sequences from Czech Republic (KF018754, GU 985538), one nucleotide sequences from Iran (KY560443) and one nucleotide sequences

from USA (JQ782821). The second one (SG2) contained two isolates collected from Kermanshah (MG770888) and Esfahan (MG 739326). These sequences showed a significant nucleotide similarity with each other and were distinct from the both subgroups SG1 and SG3, and there is no reference sequence in GenBank that corresponds to this subgroup. Our third subgroup (SG3) contained four isolates collected from Hamedan (MG739319), Bandar Abbas (MG739324), Tehran north (MG739321) and Semnan (MG739325). Analysis of these sequences showed a significant nucleotide similarity between Hamedan and Bandar Abbas and also between Tehran north and Semnan.

This phylogenetic tree is also supported by a mean pair-wise distance that is calculated at 0.005, suggesting that all of the *C. hemipterus* populations studied are clustered together, showing no significant variance between different regions despite minor nucleotide variations, on average between 5 and 10 Single nucleotide polymorphisms (SNPs).

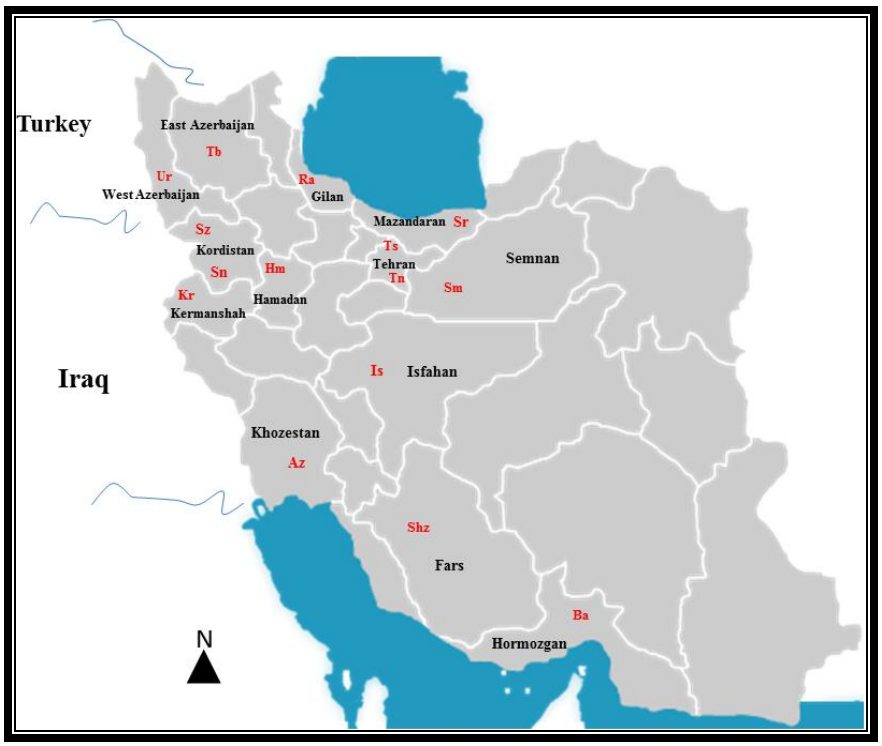

Fig. 1. Map of Iran showing study locations of *Cimex hemipterus* collected for the present study. Abbreviations are listed in Table 1

Table 1. Collection site, isolated code, collection date and accession number of each bed bug in this study

Isolated code	Isolation source	State	Collection date	Length /bp	Longitude and Latitude	Genbank Accession number
Hm	Hamadan	Hamedan	20 June 2016	585bp	34.7989° N, 48.5150° E	MG739319
Az	Ahvaz	Khozestan	25 June 2016	577bp	31.3183° N, 48.6706° E	MG739320
Tn	Tehran north	Tehran	15 May 2016	608bp	35.6892° N, 51.3890° E	MG739321
Ra	Rasht	Gilan	28 June 2017	669bp	37.2682° N, 49.5891° E	MG739322
Sr	Sari	Mazandaran	10 June 2017	706bp	36.5659° N, 53.0586° E	MG739323

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Table 1: Continued …

Ba	Bandar abbas	Hormozgan	23 July 2017	683bp	27.1832° N, 56.2666° E	MG739324
Sm	Semnan	Semnan	20 July 2016	683bp	35.2256° N, 54.4342° E	MG739325
Is	Isfahan	Isfahan	15 July 2017	734bp	32.6546° N, 51.6680° E	MG739326
Kr	Kermanshah	Kermanshah	14 August 2016	669bp	34.3277° N, 47.0778° E	MG770888
Sn	Sanandaj	Kordistan	10 August 2017	721bp	35.3219° N, 46.9862° E	MG770889
Shz	Shiraz	Fars	23 May 2016	717bp	29.5918° N, 52.5837° E	MG770890
Ur	Urmia	West Azerbaijan	22 May 2016	684bp	37.5498° N, 45.0786° E	MG770891
Tb	Tabriz	East Azerbaijan	19 August 2017	722bp	38.0962° N, 46.2738° E	MG770892
Ts	Tehran south	Tehran	17 May 2017	587bp	35.6892° N, 51.3890° E	MG737714
Sz	Saghez	Kordistan	16 May 2017	705bp	36.2389° N, 46.2780° E	MG696803

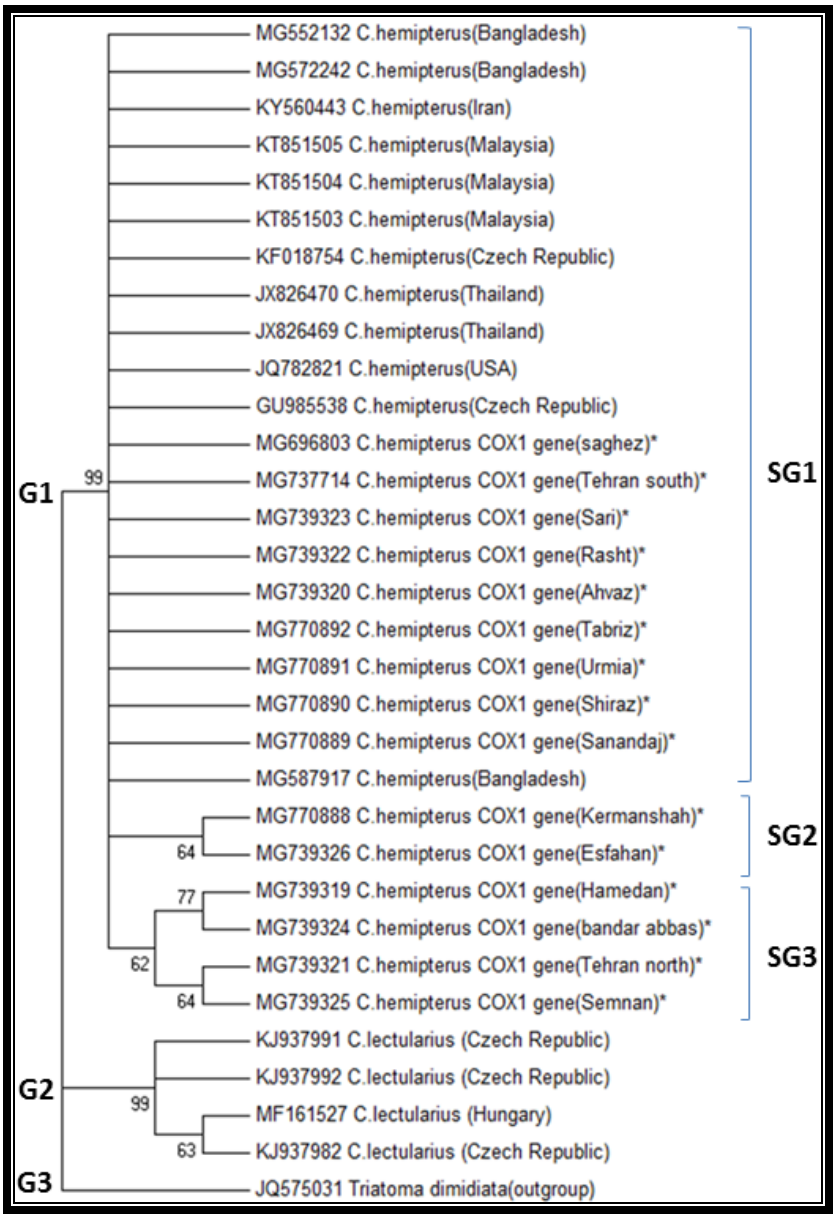

Fig. 2. Maximum likelihood (ML) tree inferred from sequences of the mitochondrial COI gene for 15 *Cimex hemipterus* populations collected in Iran (*sign) and one outgroup (*Triatoma dimidiata* accession no. JQ575031.), Numbers at nodes indicate bootstrap values (%) of ML replicates, obtained by 1,000 replications

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Discussion

There is a paucity of data and analysis regarding phylogenetic relationships below infraorder Cimicomorpha (39, 40). The proposed evolutionary relationships of the taxa within superfamily Cimicoidea and the family Cimicidae are based on morphological characters, chromosome numbers, and host associations (1, 32). Previous studies have associated the Cimicidae with other families within the superfamily Cimicoidea using both morphological and molecular characters (39, 41). A total evidence analysis using 16S, 18S, 28S and COI DNA sequence data and 73 morphological characters (39) has determined 13 infraorder Cimicomorpha and superfamily Cimicoidea are both monophyletic. The same study reported that the families Cimicidae, Polyctenidae and Curaliidae form a monophyletic clade (39).

In this work, we studied the phylogeny of *C. hemipterus* populations come from different regions of Iran, using the mitochondrial COI gene sequences. The COI gene is a part of the mitochondrial DNA genes and has been used as a potent marker for molecular phylogenetic studies, because it is species specific and appropriate for analyses of intra specific variations. The rate of evolvement in the COI gene is also relatively rapid which allow distinction at the species level and the identification of obscure species (42, 43). Despite the vast outbreak of bedbug in various rural and urban regions of Iran, there are very limited and unsatisfactory reports about the prevalence (17, 20). Probably due to resurgence and propagation of bed bugs in other countries, the population also has increased in Iran (44, 45). Limited public awareness, increase in internal and international travels, increase in utilization of second-hand furniture and resistance to pesticides may contribute to this resurgence (46, 47). Therefore, the resurgence and subsequent problems inspired us to perform phylogenetic analysis, as well as study genetic diversity and population dynamics.

The perceived near extirpation of bed bugs from many areas around the world suggests a genetic bottleneck would have occurred, which would be reflected in low genetic diversity across current bed bug populations (25, 30-34). However, all of the studies completed thus far have found a relatively high genetic diversity between populations in different locations. Such high diversity across populations is atypical for species that have undergone a recent and single founder event (30). Szalanski et al. (30) focused on genetic variation among various bed bug populations in USA, Canada and Australia. They examined a partial sequence of the mitochondrial (mt) 16S rRNA gene and nuclear rRNA ITS-1 region of 136 adult bed bugs sampled from 22 populations and found a relatively high genetic diversity in the 16S gene, and low diversity in the ITS-1 gene.

The current research is the first report of phylogenetic and genetic species diversity analysis conducted on *C. hemipterus* in Iran. Our analysis showed one main cluster in phylogenetic tree. Therefore, from the present study it can be concluded that *C. hemipterus* in Iran is an arthropod with low genetic diversity with a potentially high capability for raise the levels of inbreeding. The previous study, done by Booth et al. (25) regarding molecular markers of bed bug infestation dynamics within apartment buildings supported the same pattern of genetic diversity in *C. lectularius* as they reveal restricted genetic diversity. Similar researches also have conducted in other countries. Seri-Masran and Majid (45) studied genetic diversity of bed bugs in Malaysia. They considered 22 selected infested structures and consistent to our findings, they observed one main monophyletic clade. Another study was performed in Thailand and one main cluster of *C. he-*

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mipterus was obtained when it was compared with *C. lectularis* isolates. Both of the aforementioned studies support our results.

Given that the current phylogenetic and taxonomic relationships within the family Cimicidae are based on host relationships and morphology, it is possible that the inclusion of molecular data could cause a restructuring of the systematic relationships (1, 32). In addition to COI genes, the DNA sequences of the entire mitochondrial genome of *C. hemipterus* may be useful for population genetics studies. There are several reports in the country using molecular methods for species identification of insects (48-58).

Conclusion

These results provided basic information for further studies of molecular epidemiology and control of *C. hemipterus* infestation to the public, medical association, entomologists and pest control operators in Iran.

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