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The first record of the tropical bed bug, *Cimex hemipterus* (Hemiptera: Cimicidae) from Iran

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Bed bugs are regarded as one of the most important pests in human societies. *Cimex hemipterus* (Fabricius) is a parasite of humans and bats across tropical areas of the world. In the present study, the nymph and adult specimens were collected from residential buildings in central and western parts of Iran. Samples were identified using a diagnostic key at the species level and confirmed by molecular studies. Phylogenetic analysis using *COI* gene was also carried out. This study is the first report to have confirmed the presence of C. hemipterus in Iran.

Key words: *Urban pest, Bed bugs, Molecular identification, Phylogenetic tree, COI*

INTRODUCTION

Urban insects are among the most important pests of human societies (Robinson, 2005). In fact, life and activities of these pests (which sometimes cause bites, harassment, distress, skin and systemic reactions in humans) are linked to urban life style (Dhang, 2011). Bed bugs are examples of these insect pests which are classified as true bugs (Hemiptera) from the family Cimicidae. As at now, 110 species have been identified in this family; three species associated with humans include; the common (*Cimex lectularius*) and tropical (*C. hemipterus*) bed bugs, parasites of human, bats, poultry and rarely other domestic animals throughout the world; bat bugs (*Leptocimex boueti*) are parasite of bats and humans in west Africa and south America (Usinger, 1966; Henry, 2009). Secondary infections such as impetigo, erythema, cellulitis and inflammation of the lymphangitis, caused by scratching of the skin have been reported among the side effects of bed bug bites on humans (Thomas *et al.*, 2004; Hwang *et al.*, 2018); however, there is limited information on these insects as vectors of disease agents (Doggett, 2018; Krinsky, 2002). The process of dispersal in bed bugs is believed to be almost entirely passive (Newberry *et al.*, 1991). The bed bugs can be transferred from one place to another through infested clothing, travel equipment, home furnishings and infected homes (Triplehorn *et al.*, 2005). Global commerce, international travel and misguided insect control efforts are important factors influencing the dispersal of bed bug infestations (Pereira *et al.*, 2018).

In general, *C. lectularius* is normally found in the temperate regions, whilst *C. hemipterus* species are common in the tropical regions of the world, but in some areas both species can be found in the same region, i.e., *C. lectularius* giving place to *C. hemipterus* and sometimes hybridization occurs between the two species but no progeny was be produced (Walpole & Newberry, 1988).

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Global increase in the world's infestation of hotels by *C. lectularius* is possibly one of the most considerable problems caused by bed bugs (Reinhardt & Siva-Jothy, 2007). Due to the increased number of people traveling around the world in the 1990s, the infestation rate of human dwellings had increased (Service, 2012).

There is a significant similarity between the two species, with minor morphological differences and perhaps greater tolerance to higher temperatures in *C. hemipterus* (Usinger, 1966; Anonymous, 1964; Schofield & Dolling, 1993). The combination of molecular methods in traditional taxonomy has created a major revolution in the identification of medically important pests and vectors of human diseases (Hill & Crampton, 1994). For this purpose, DNA-based technologies including; PCR-RFLP (Polymerase Chain Reaction- Restriction Fragment Length Polymorphism), sequencing, probe and others types have been used. The separation of two types of human bed bugs with molecular methods has already been done (Tawatsin *et al*., 2013). The *COI* gene is considered as a differential "bar-coding" gene that correctly distinguishes between 95 to 97 percent different species of living organisms (Hebert & Gregory, 2005).

In previous studies on bugs of Iran, only *C. lectularius* was mentioned (Safavi, 1986; Askari *et al*., 2009; Haghi *et al*., 2014; Dehghani *et al*., 2016; Ghahari *et al*., 2016). The distribution of this species has been reported from the Mazandaran, Tehran and Zanjan provinces (Ghahari *et al*., 2016), but it appears the species is currently widespred across the country. So far, taxonomic molecular studies have not been conducted on bed bugs of Iran. This study was carried out in order to identify the samples of human buildings in two districts located in Tehran (Central part of the country) and Lorestan (Western part of the country) provinces. We also confirmed the identity of species by sequencing part of the *COI* gene and comparing with the Genbank sequences.

MATERIAL AND METHODS

Collection and identification of specimens

The nymph and adult specimens were collected from residential buildings located in Tehran province, Tehran (anonymous place) and Lorestan province, Khorramabad city (Posht-e Bazar) using forceps, between 2016 and 2017. So, the samples were placed inside glass containers with their specifications and later transferred to the Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences laboratory. Then, samples were identified using the CDC diagnostic key (Anonymous, 1964) at the species level and typical samples were photographed. Of the identified samples, two were selected for the confirmation of morphological identification by molecular methods.

DNA extraction, PCR amplification and sequencing

DNA was extracted using CTAB (cetyl trimethylammonium bromide) method, according to Doyle & Doyle (1987) and this was used in PCR amplification, after measuring quantity and quality. Then a 524-bp fragment of cytochrome oxidase subunit 1 (*COI*) of mitochondrial genome of *Cimex* specimens was amplified by polymerase chain reaction (PCR), using the primers designed by Simon *et al.* (1994), including C1-J-1718: 3′- GGA GGA TTT GGA AAT TGA TTA G -5′ and C1-N-2191: 3′- CCC GGT AAA ATT AAA ATA TAA ACT TC -5′ with minor modification in C1-J-1718 as follow: deletion of –TTCC– from end 5′. PCR reactions for *COI* gene were carried out by the following touchdown temperature profile; 4 min. at 95 ˚C, 11X (50 s. at 94 ˚C, 60 s. at 60-50 ˚C, 60 s. at 72 ˚C), followed by 25X (60 s. at 94 ˚C, 50 s. at 50 ˚C, 60 s. at 72 ˚C) and a final 5 min. at 72 ˚C. The following reagents were used in each PCR (as quantity, final concentration in 25 µl): Taq DNA polymerase enzyme SinaClon® (Iran) Bioflux®-5 U/µl (0.3 µl, 1.5 U); PCR buffer Bioflux®-10x (2.5 µl, 1 mM); $MgCl_2$ Bioflux®-50 mM (1 µl that can be increased up to 2 µl, 2 mM); dNTPs Bioflux®-10

mM (0.5 µl, 200 µM); each of the forward and reverse primers was 10 µM (1 *µl*, 0.4 µM); template DNA (50-100 ng/*µl*) and 14.8 *µl* sterile water. The PCR products were visualized using 1% agarose gel electrophoresis. So, the positive products were purified using GeneJET® gel extraction kit (Fermentas®, Lithuania), then the purified PCR products were sequenced by a third-party service provider for sequencing (Faza-Biotech® Inc., Iran). The sequences were edited by FinchTV software (Ver. 1.4), then BLASTed via Basic Local Alignment Search Tool (BLAST) in Genbank NCBI and accession numbers (MG702197, KY560443) were assigned.

Phylogenetic studies

First, 52 *COI* sequences of *Cimex* taxon including 16 *C. hemipterus* (JX826468-79, KF018754-55, KY560443 (this study), MG702197 (this study), and 36 *COI* sequences of *C. lectularius* (HQ105554, JX826480-82, KF018756, KJ937979-92, KR002571-83, KR002585, KR035952, KR044731, KU874630) of the Genbank were used to construct the *COI* phylogenetic tree. Out-group taxon was chosen according to Smith (1994) and Wenzel (2002). They suggested that out-group could be selected from sister groups as well as successively more distant lineages. Thus, the species *Triatoma infestans* (HQ437704) was examined as an out-group. All sequences were aligned using SeaView4 software (Ver. 4.6.2) (Gouy *et al.* 2010), and the genetic distances, as the index of intra- and inter-species variations among the sequences calculated using Kimura 2 parameter (K_2P) model in the MEGA software (Ver. 7) (Kumar *et al.*, 2016). The BEAST software (Ver. 1.8.2) (Drummond *et al.*, 2012) according to the Bayesian Inference (BI) method was used to construct *COI* phylogenetic tree. The clades of constructed phylogenetic tree were reorganized, based on posterior probability value criterion of each *Cimex* clade and genetic distance within and among clade members. After constructing the *COI* phylogenetic tree, the position of the out-group (*Triatoma*), the polytomy of the tree, the robustness of the main clade *C. hemipterus*, based on the posterior probability value and finally, the relationship of the taxa were compared with each other.

RESULTS

Insect identification

Three nymphs and five adults were identified, based on morphological characteristics of *C. hemipterus* (Fig. 1). The Lorestan samples consisted of two adult and one nymph specimens and Tehran samples included three adults and two nymphs.

PCR, sequencing and BLAST

Of the two adult *C. hemipterus*, from Tehran and Lorestan, which were subjected to PCR, the target partial *COI* gene was amplified from the relevant samples. Of the 2 extracted DNA from adult *C. hemipterus*, which were subjected to PCR, the target partial *COI* gene was amplified from the relevant samples. Two samples of positive PCR products were successfully sequenced. The results of sequences BLAST in the Genbank showed that both sequences of the present study belong to the *C. hemipterus* species with a similarity of 97 to 99% to Genbank sequences.

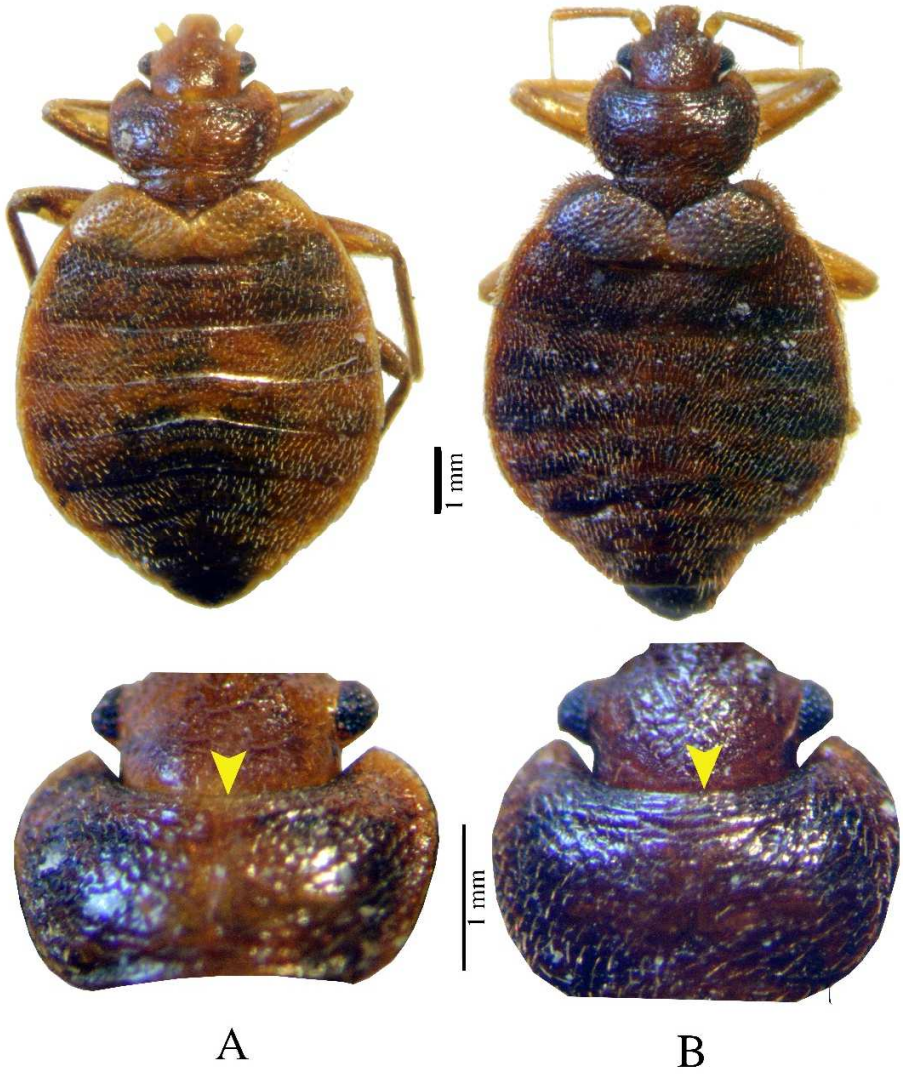

FIGURE 1. Dorsal view of adult *Cimex hemipterus* with pronotum characteristics as the main distinguishing feature of identification between *C. hemipterus* and *C. lectularius*. A: Tehran specimen, B: Lorestan specimen. The pronotum with anterior margin moderately excavated (*C. hemipterus*) and pronotum with anterior margin deeply excavated (*C. lectularius*).

Genetic distances and phylogenetic tree

Genetic distances which are indices of intra- and inter-species variations of different populations of *Cimex hemipterus*, *C. lectularius* and *Triatoma infestans,* based on partial *COI* gene sequence are presented in

. A *COI* phylogenetic tree of the present study and the Genbank sequence data are presented in **Error! Reference source not found.** 2. *Cimex lectularius* were close to *C. hemipterus*, and *T. infestans* as out-group species, and were placed into the phylogenetic tree. Also, the phylogenetic tree was created as a fully-resolved tree, i.e., the monophyly of the genus *Cimex*, the dichotomy and accuracy (posterior probability value) with a complete score (100%) in the node places.

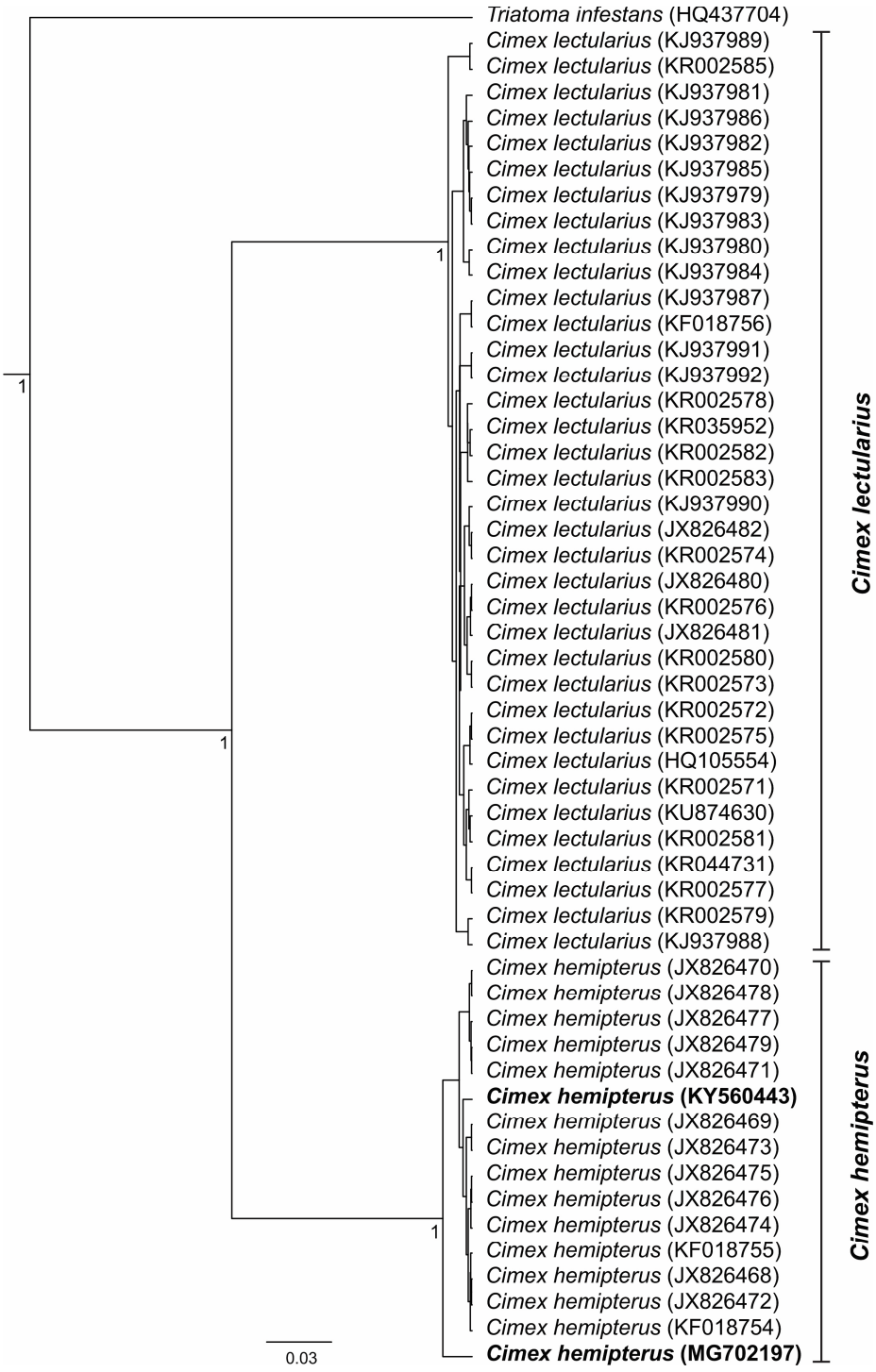

FIGURE 2. Phylogenetic tree of *Cimex* bugs inferred from *COI* sequence data constructed using Bayesian Inference (BI) method with 10 million reiterations; main clades in right side of tree for *C. hemipterus* and *C. lectularius*. Taxa are as species name following Genbank accession numbers, taxon of

the present study is indicated in bold letters. Nodes are indicated with posterior probability values. Branch lengths are proportional to evolutionary changes. The taxa sequence of *Triatoma infestans* is indicated as an out-group.

TABLE 1. Intra- and inter-species variation among *COI* sequences of the present study and Genbank sequence data**.**

Species	*Cimex hemipterus*	*Cimex lectularius*	*Triatoma infestans*
*Cimex hemipterus**•			
Cimex lectularius•	23		
Triatoma infestans	41	40	

* A consensus sequence related to partial 236-bp of *COI* gene, includes two sequences of the present study

• Intra-species variation of *C. hemipterus* and *C. lectularius* up to 1%

DISSCUSSION

Public health importance of *C. hemipterus*

The global problem of bed bug infestation, including that which is caused by *Cimex hemipterus* in human dwellings is common in developed countries (Hwang et al., 2005; Masetti & Bruschi, 2007; Stucki & Ludwig, 2008; Wang & Wen, 2011; Gounder et al., 2014; Majid & Zahran, 2015). Also, *C. hemipterus* insects are serious pests of poultry barns (Rosen *et al*., 1987). The tropical bed bug, *C. hemipterus*, is a prominent parasitic hematophagous cimicid, which had not been reported in Iran. Thus, according to available data, the present study is the first report on the occurence of this species in Iran. We detected found this species in two locations within the Tehran (capital Iran) and Lorestan (western Iran) provinces of Iran. Bed bug infestations in other parts of Iran are attributed to the common bed bug, *C. lectularius* (Askari *et al*., 2009; Haghi *et al*., 2014; Dehghani *et al*., 2016). The accurate diagnosis of these species is of great importance in designing effective control program involving insecticide selection, because of the reported cases of insecticide resistance (Karunaratne *et al*., 2007). This species was established in some localities which were far from their primary distribution area (Hixson, 1943). Since *C. hemipterus* had not been previously reported in Iran, it seems the species was introduced to the country through foreign travels. The report on tropical bed bug in Tehran may be due to the high rate of foreign travels and the possibility of transferring this bug through clothing and household appliances. In South Africa, the general increase of *C. hemipterus* is thought to be due to the influx of foreign migrants into the country (Newberry & Mchunu, 1989). The present report on the occurrence of *C. hemipterus* in Iran can contribute to the effective planning of the country's health system. It is also necessary to study real distribution and biology of species in order to apply proper control techniques.

Phylogeny of *C. hemipterus*

There was no genetic diversity between different populations of *Cimex hemipterus*, based on *COI* gene sequences (Seri Masran & Ab Majid, 2017; Talbot *et al*., 2017). However, a minor genetic difference (intra-specific variation) was found in *C. hemipterus* (Tawatsin *et al*., 2013). Also in this study, two *COI* sequences of *C. hemipterus* showed no genetic differences. A phylogenetic clade of *C. hemipterus*, associated with the only tropical humans was detected based on *COI* and *EF1a* genes (Talbot *et al*., 2017). In the present study, one clade, representing *C. hemipterus* was constructed with a considerable genetic distance (23%). According to a study conducted by Talbot *et al.* (2017), it is likely that the

role of *C. hemipterus* bites in human health may be more important than that of *C. lectularius*, based on specific host lineages of the phylogenetic tree. They reported 25% difference in genetic distance between two *C. hemipterus* and *C. lectularius* species, according to *COI* gen fragment. There are four traditional morphologically-defined species groups of the genus *Cimex*, namely; *C. hemipterus*, *C. lectularius*, *C. pipistrelli* and *C. pilosellus* (Usinger, 1966). The *C. hemipterus* group is characterized by the existence of narrow lateral lobes on the pronotum, cleft and bristled paragenital sinus and consists of *C. hemipterus* and a bat-associated *C. insuetus* species, from Thailand (Balvín *et al.*, 2015). In the present study, a phylogenetic tree was created with the monophyly of the genus, *Cimex*. This monophyly was identified atthe sub-family level (Cimicinae) by Balvín *et al.* (2015). The report on tropical bed bug in Iran is important because, it has the tendency of providing more accurate and clear management approaches and control strategies for this pest.

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