

VOLUME 8 ISSUE 2 2022

ISSN 2454-3055



**INTERNATIONAL
JOURNAL OF
ZOOLOGICAL
INVESTIGATIONS**

***Forum for Biological and
Environmental Sciences***

Published by Saran Publications, India



International Journal of Zoological Investigations

Contents available at Journals Home Page: www.ijzi.net

Editor-in-Chief: Prof. Ajai Kumar Srivastav

Published by: Saran Publications, Gorakhpur, India



ISSN: 2454-3055

Morphological Identification and DNA Barcoding of the Tropical Bed Bug *Cimex hemipterus* Fabricius (Hemiptera: Cimicidae) from Thrissur, Kerala, India

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Received: 30th August, 2022; Accepted: 10th October, 2022; Published online: 19th October, 2022

<https://doi.org/10.33745/ijzi.2022.v08i02.070>

Abstract: Knowledge of bed bugs is scant from Kerala due to improper identification, sparse population and better sanitation. But if an infestation begins it reaches extreme proportions because of the lack of awareness. To the best of our knowledge no detailed scientific studies on the identification of the bed bug species were carried out in Kerala. This study was aimed to perform morphological and molecular identification along with the morphometric analysis of the bed bug species from two sites—Avittappally and Mulankunnathukavu. Morphological identification was made by calculating the width to length ratios of the pronotum and hind femora. DNA barcoding was performed to identify the bed bug species accurately. A total of 21 morphological characters were used to compare bed bugs from the sample and the morphometric characters were measured and analysed using a Leica S8APO stereo microscope. The results indicated that the width-to-length ratios of pronotum and hind femora were 2.2 ± 0.04 mm and 3.5 ± 0.04 mm in Avittappally specimens and 2.1 ± 0.05 mm and 3.2 ± 0.05 mm in Mulankunnathukavu specimens. The genetic distance between the 2 *COI* sequence of our study were 0.027% and the intraspecific genetic distance between the 2 *COI* sequence of this study with 5 *C. hemipterus* sequences reported from other countries was found to be 0.025, 1.227, 0.058, 0.025 and 0.024% for Mulankunnathukavu specimen and 0.001, 1.280, 0.008, 0.001 and 0.001% for Avittappally specimen, respectively. The mean values of HL, HFW, HTW, MTW, FTW between the two places were significantly different. The results of PCA showed that the total percentage of variance reached 89.073% and 93.760%. Based on Morphological and morphometric characteristics along with molecular sequencing we assert that the bed bugs collected from both places were *Cimex hemipterus*.

Keywords: *Cimex hemipterus*, Pronotum, Hind femora, *COI* gene, Morphometry

Citation: Surendran Arunnya and Binoy C.F.: Morphological identification and DNA barcoding of the tropical bed bug *Cimex hemipterus* Fabricius (Hemiptera: Cimicidae) from Thrissur, Kerala, India. Intern. J. Zool. Invest. 8(2): 560-567, 2022.

<https://doi.org/10.33745/ijzi.2022.v08i02.070>



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Introduction

Bed bugs are small parasitic insects that feed on the blood of humans and other warm-blooded

animals. The two common species are *Cimex lectularius* Linnaeus and *Cimex hemipterus*

Fabricius (Usinger, 1966; Potter, 2011). They might have evolved from cave-dwelling ectoparasites of mammals and moved from caves to houses along with humans (Harlan, 2006). They hide in cracks and crevices and come out in search of the host only for feeding. The feeding behaviour is triggered by the body temperature and Carbon dioxide released by the host animal (Saari *et al.*, 2019). Bed bug infestations have remained widespread all over the world. Beginning of the 20th century, all species of bed bugs in India were thought to be *C. lectularius* until Patton reported it similar to *Cimex rotundatus* Signoret (Girault, 1907; Patton, 1908). *Cimex hemipterus* is a synonym for *C. rotundatus* Signoret and is widely distributed in the plains of India and known as tropical and sub-tropical bed bug (Wattal and Kalra, 1961; Bhat, 1974). *Cimex lectularius* were thought to be confined to hilly regions of India before being reported from Hyderabad and coexisting with *C. hemipterus* from Alappuzha in Kerala (Bhat, 1974; Venkatachalam and Belavady, 1962; Burton, 1962).

An early study conducted in Hyderabad concluded that excessive bed bug bites and resultant blood loss acted as an aetiological factor for iron deficiency anaemia in infants and young children (Venkatachalam and Belavady, 1962). Bandyopadhyay *et al.* (2015) reported bed bug outbreak in the neonatal care unit not only affecting newborns and mothers with rashes and sleeplessness but also the healthcare professionals and families.

Adult bed bugs are oval, flattened 3/16-inch long and reddish-brown in colour (Potter, 2012). The pronotum width-to-length ratio is less than 2.5 in *C. hemipterus* and greater than 2.5 in *C. lectularius*. The hind femora width to length ratio is 2.9-3.5 in *C. hemipterus* and 3.4-4.1 in *C. lectularius* (Usinger, 1966) which marks the key differentiation in taxonomy. Bed bugs are obligate hematophagous, an adequate volume of one blood meal is required by the adults for nutrition and reproduction. Each active instar molts into another after receiving a complete meal and may

feed multiple times if hosts are readily available (Harlan, 2006).

There has been evidence of Indian researchers misidentifying *C. hemipterus* as *C. lectularius* which emphasizes the need to precisely identify the bed bug throughout the country (Siddiqui and Raja, 2015; Rao and Rao, 2018). In the present study, the collected sample of bed bugs was identified using morphological characters and molecular identification tools. In addition, morphometric analysis was also done to elaborate the morphological characters.

Materials and Methods

Insect collection:

Adult bed bugs were collected from a house in Avittappally and Government Medical College, Mulankunnathukavu, Thrissur, Kerala, India. Specimens were transferred into plastic bottles of 6.5 cm x 5.5 cm using a brush. Paper tents were placed in the bottles as harborage. The bottles were then placed in a bed bug box of 30 cm x 20 cm x 17.5 cm, 25±2°C temperature and relative humidity of 70±10%.

Morphological identification:

Adult bed bug specimens collected were examined under a dissecting microscope. Ten bed bugs (5 males, 5 females) each from both places were studied. The pronotum width to length ratio as well as hind femora width to length ratio were calculated (Usinger, 1966). Bed bug photos were taken using Leica S8APO stereo microscope.

Molecular identification:

To validate the morphological features, genomic DNA was extracted from the hind legs of bed bugs collected from both places. Genomic DNA was isolated from the tissues using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions. Amplification of *COI* gene was conducted using LepF1 (5'-ATTCAACCAATCATAAAGATATTGG-3') LepR1 (5'-TAAACTTCTGGATGTCCAAAAAATCA-3') (Hebert *et al.*, 2004). Big Dye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) was

used for the sequencing reaction inside the PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). The cleaned air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems). The quality of the sequence was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond *et al.*, 2010). Further editing of the sequence was done using Sequencher 4.10.1. The sequences were submitted in GenBank with accession numbers OM108444 and OM991736.

In addition to the sequences generated in this research, *COI* sequences of five *C.hemipterus* (GenBank MH607404-Bangladesh, KY561678-Canada, KT851505-Malaysia, MN915235-China, KF018754-Czech Republic) submitted from five different countries in GenBank were included in genetic distance analysis. All sequence were aligned in MEGA 11 (Tamura *et al.*, 2021).

Morphometric analysis:

The bed bugs examined for morphometric analysis were preserved in 70% ethanol. The preserved adults were observed, dissected and studied for 21 morphometric characters using Leica S8APO stereo microscope.

Data Analysis:

The statistical analysis was performed to compare 21 morphometric characters for bed bugs collected from two different study locations of Thrissur district. The data were analyzed using SPSS version 25. The descriptive statistical measures (mean and SEM) were calculated for ratios of pronotum l/w, hind femora l/w between the two study locations and for all cephalic and thoracic morphological features of bed bugs. The independent t-test was performed to compare the mean values of morphological characteristics between the two places. P values <0.05 was considered statistically significant. Principal component analysis (PCA) was carried out using SPSS and PAST software to study population variation within species from two places. The

morphometric variation in bed bug samples was examined using PCA based on covariance.

Results and Discussion

Morphological identification:

The pronotum of bed bugs (Fig. 1) collected from Avittappally and Mulankunnathukavu were not broadly expanded and the lateral margins were not upturned. The mean ($\pm$ SE) values of pronotum width-to-length ratio was found as 2.2 ± 0.04 mm in Avittappally specimens and 2.1 ± 0.05 mm in Mulankunnathukavu specimens. Hind femora width-to-length ratio was 3.5 ± 0.04 mm in Avittappally specimens and 3.2 ± 0.05 mm in Mulankunnathukavu specimens. The mean measurements of pronotum length and width and hind femora length and width are given in Table 1.

The independent t-tests results showed that the mean values of pronotum length, pronotum width, hind femora width, hind femora l/w ratio between the two places were significantly different ($P<0.05$). However, the pronotum l/w ratio of the bed bug between the two places was not statistically significant ($P>0.05$). The pronotum width (1.102 mm), pronotum length (0.522 mm) and hind femora width (0.352 mm) were significantly higher in Mulankunnathukavu specimens than Avittappally specimens ($P<0.05$). The hind femora l/w ratio (3.460 mm) was significantly higher in Avittappally site ($P<0.05$).

Usinger (1966) reported that in *C. lectularius* pronotum is 2.5 times as wide as long, sides are broadly expanded especially anteriorly, while in *C. hemipterus* pronotum is more than twice as wide as long, sides more rounded anteriorly than middle, not broadly expanded. Hind femora is over 3 times long as broad. Compared to *C.hemipterus*, *C.lectularius* has a more upturned lateral margin (Ghauri, 1973). In the study conducted by Zhang *et al.* (2021) the mean pronotum width-to-length ratios of Guangzhou and Foshan specimens were 2.6 and 2.4. Ten adult hemipterus collected from Florida showed mean ratio of pronotum as 2.3 (Campbell *et al.*, 2016). The mean protonum



Fig. 1: (a) Pronotum (b) Hind femora of *C. Hemipterus*.

Table 1: Morphological characteristics from 2 different places (n=10) (AVT- Avittappally and MGKAVU- Mulankunnathkavu)

Morphological characteristics	Sites	Mean	Std. Deviation	t value	P- Value
Pronotum Width	AVT	1.025	0.083	-2.539	0.021
	MGKAVU	1.102	0.047		
Pronotum Length	AVT	0.468	0.041	-2.840	0.011
	MGKAVU	0.522	0.044		
Pronotum l/w ratio	AVT	2.194	0.138	1.051	0.307
	MGKAVU	2.122	0.167	-	
Hind Femora Length	AVT	1.075	0.064	-1.714	0.104
	MGKAVU	1.127	0.071		
Hind Femora Width	AVT	0.311	0.019	-4.276	0.000
	MGKAVU	0.352	0.024		
Hind Femora l/w ratio	AVT	3.460	0.144	3.713	0.002
	MGKAVU	3.202	0.166	-	

width-to-length ratios of Avittappally and Mulankunnathukavu specimens in this study were 2.2 and 2.1. According to Usinger (1966), the hind femora of *Cimex adjunctus* Barber is more than 2.5 times as long as wide and that of *C. hemipterus* is 3 times as long as wide. Based on the difference in the pronotum width-to-length ratio of *C. lectularius* and hind femora width-to-length ratio of *Cimex adjunctus* by the keys of Usinger (1966), the species was considered to be *Cimex hemipterus*.

Molecular identification:

After sequence editing, *COI* sequences of 654 and 659 bp were obtained. Based on Tamura 3-parameter model, the genetic distance between the 2 *COI* sequence of this study were 0.027% and the intra-specific genetic distance between the 2 *COI* sequence of this study with 5 *C. hemipterus*

sequences reported from other countries were found to be 0.025, 1.227, 0.058, 0.025 and 0.024% for Mulankunnathukavu specimen and 0.001, 1.280, 0.008, 0.001 and 0.001% for Avittappally specimen, respectively. The less of genetic distance between other specimens indicates that present species is *C. hemipterus*.

Zhang *et al.* (2021) found the genetic distances of their specimens with *C. hemipterus* and *C. lectularius* to be 0–0.2% and 22.2–22.6%. Morphological and molecular studies on the basis of four genes i.e. mitochondrial gene *COI*, *16S rDNA*, and ribosomal genes *28S rDNA*, *ITS2* indicated that the genetic differences of intra-species DNA sequences were less than 2%, and differences of inter-species DNA were more than 2% (Liu *et al.*, 2018).



Fig. 2: (A) Male *Cimex hemipterus* (B) Female *Cimex hemipterus* (C) Head (D) Legs.

Morphometric analysis:

Bed bug (male and female) and their head and legs are shown in Figure 2. Morphometric analyses were performed to find out variation within *C. hemipterus* collected from two different study locations and to compare with *C. hemipterus* measurements recorded in other studies. The mean values and standard error means, t and p values of 21 morphometric characters are shown in Table 2.

The independent t-test results for comparing the mean morphological characteristics of bed bugs from two places revealed that the mean values of HL, HFW, HTW, MTW, FTW between the two places were significantly different ($P < 0.05$). However, the rest of the morphological characteristics did not differ significantly between the two places ($P > 0.05$). Studies by Khan and

Rahman (2012) stated that the adult *Cimex hemipterus* average length and width to be 5.5 mm and 2.5 mm compared to 4.12 ± 0.05 mm and 2.3 ± 0.05 mm in Avittappally specimens and 4.5 ± 0.16 mm and 2.4 ± 0.07 mm in Mulankunnathukavu specimens.

Principal component analysis:

Measurements were made for 20 bed bugs collected from Avittappally and Mulankunnathukavu (10 bed bugs from each location). Each bed bug were measured for 21 potentially important characters. Rotation Method used is Varimax with Kaiser Normalization (Kaiser, 1958).

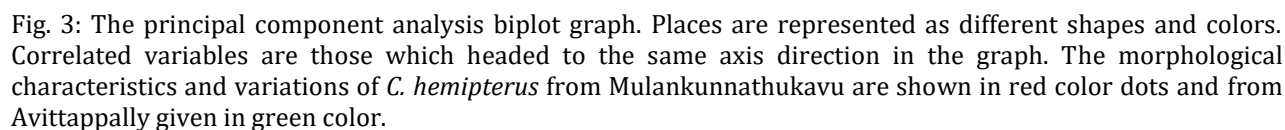
The principal component analysis (PCA) (Fig. 3) of the species from the Avittappally location was found to be 5 components which were

Table 2: Morphological characteristics of bed bug from 2 different places (n=10).

Body Regions	Morphological variables	Avittappally		Mulankunnathukavu		t Value	P Value
		Mean	Std. Error	Mean	Std. Error		
Cephalic Region	Body length (BL)	4.119	0.047	4.499	0.161	-2.269	0.036
	Body width (BW)	2.322	0.048	2.452	0.070	-1.537	0.142
	Head length (HL)	0.497	0.023	0.593	0.018	-3.273	0.004
	Head width (HW)	0.794	0.016	0.825	0.018	-1.240	0.231
	Distance between eyes (DBE)	0.551	0.012	0.572	0.012	-1.259	0.224
	Antennal length (AL)	1.656	0.027	1.659	0.037	-0.061	0.952
Thoracic Region	Hind femur length (HFL)	1.087	0.032	1.134	0.019	-1.277	0.218
	Hind femur width (HFW)	0.306	0.007	0.344	0.012	-2.679	0.015
	Hind tibial length (HTL)	1.471	0.034	1.436	0.024	0.835	0.414
	Hind tibial width (HTW)	0.097	0.004	0.114	0.004	-2.926	0.009
	Hind tarsus (HT)	0.440	0.015	0.445	0.015	-0.234	0.818
	Mid femur length (MFL)	0.967	0.030	0.982	0.021	-0.411	0.686
	Mid femur width (MFW)	0.279	0.007	0.296	0.015	-1.042	0.311
	Mid tibial length (MTL)	1.030	0.034	1.058	0.020	-0.709	0.487
	Mid tibial width (MTW)	0.086	0.003	0.101	0.003	-3.064	0.007
	Mid tarsus (MT)	0.382	0.014	0.364	0.012	0.979	0.341
	Fore femur length (FFL)	0.903	0.030	0.933	0.021	-0.804	0.432
	Fore femur width (FFW)	0.254	0.011	0.282	0.012	-1.720	0.103
	Fore tibial length (FTL)	0.961	0.025	0.907	0.018	1.724	0.102
	Fore tibial width (FTW)	0.075	0.002	0.098	0.004	-4.859	0.000
	Fore tarsus (FT)	0.343	0.012	0.334	0.012	0.512	0.615

explained from 21 morphological features and total % of Variance covered 89.073%. The PCA of the species from the Mulankunnathukavu location was found to be 6 components with a total % of Variance 93.760%. The results of the factor

analysis by Kalangi *et al.* (2017) showed three groupings of morphological characters of bed bugs from Sitaro and Manado and the results of cluster analysis formed four groupings based on similarities of morphometry.



Knowledge on proper identification of bed bugs and to differentiate it with other bugs and the usage of tools for early detection of infestation will help in the timely management of the pests. The above study also helps to distinguish *C. hemipterus* from its other related species.

We thank CSIR-HRDG, New Delhi for the research fellowship, RGC, Trivandrum for sequencing,

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