IDENTIFICATION OF BAT ECTOPARASITE *LEPTOCIMEX INORDINATUS* FROM BAT-DWELLING CAVE, KANCHANABURI PROVINCE, THAILAND

Rutcharin Potiwat, Sungsit Sungvornyothin, Yudthana Samung, Anon Payakkapol and Chamnarn Apiwathnasorn

Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Abstract. Bat bugs are blood-feeding insects of bats or warm blooded animals and humans. Since 2011, *Leptocimex* spp (Heteroptera: Cimicidae) has been reported in Thailand. However, microscopic examination of *Leptocimex* spp is complicated, especially when the entire body of the specimen is not available. To confirm the phenotypic identification of *L. inordinatus* from a limestone bat cave in Thailand, partial fragments of mitochondrial cytochrome c oxidase subunit 1 (COI) gene and 16S mitochondrial ribosomal DNA were PCR amplified and sequenced, which revealed 97% sequence identity with Cimicidae family members, being most similar to *Cacodminae* gen. sp. and *C. vicinus*, both bat bugs. Phylogenetic tree construction showed that *L. inordinatus* has a separate genetic lineage from that of with human bed bugs (*Cimex hemipterus* or *C. lectularius*), swallow bugs and other tick species. The presence of *L. inordinatus* in a bat-dwelling cave frequented by humans presents a potential public health problem requiring attention in particular regarding the possibility of zoonotic transmission of pathogens.

Keywords: *Leptocimex inordinatus*, Cimicidae, bat ectoparasite, bat-dwelling cave, bat bug, Thailand

INTRODUCTION

Bat bugs (Heteroptera: Cimicidae) are common ectoparasites of bats, which live and primarily feed on their bat host, and occasionally feed on warm blooded animals, including humans (Whyte *et al*, 2001; Araujo *et al*, 2009). Common names of members of Cimicidae family, which contain at least 92 species (Usinger, 1966), also include bed bugs and swallow bugs. Some Cimicidae members have been reported to feed on domestic animals (Wattal and Kalra, 1961; Usinger, 1966; Reinhardt and Siva-Jothy, 2007). In addition, there are three medically important bat bugs, namely, genera *Cimex, Cacodomus* and *Leptocimex* (Usinger, 1966). Genus *Cimex* dwells on variable host species, such as bats, birds and humans (Araujo *et al*, 2009).

Medically important human ectoparasites are *C. lectularius* (Linnaeus 1758) and *C. hemipterus* (Fabricius 1803), the former found in Europe and America and the latter in Southeast Asia (Reinhardt and

Correspondence: Chamnarn Apiwathnasorn, Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Bangkok 10400, Thailand. Tel: +66 (0) 2306 9100-4; Fax: +66 (0) 2643 5582 E-mail: chamnarn.api@mahidol.ac.th

Siva-Jothy 2007; Davies *et al*, 2012), but during the past decade *C. hemipterus* has been detected in Thailand (Tawatsin *et al*, 2011). However, the two species have very similar phenotypes and require high expertise for their identification (Usinger, 1966; Balvin *et al*, 2012; Wawrocka and Bartonicka, 2013). Recent genetic studies have revealed that *C. lectularius* originates from bat bug (Balvin *et al*, 2012; Booth *et al*, 2015).

Leptocimex spp have been reported from bat-dwelling limestone caves in Kanchanaburi Province, Thailand, and also a number of biting insect species, such as sand fly, bat fly, tick, mite and mosquito (Apiwathnasorn *et al*, 2011). Identification of bat bugs usually is based on external morphological characteristics examined under a light microscope following previously monography descriptive keys (Usinger, 1966), but such morphological appearance of bat bugs can often be confused with human bed bugs. Indeed, the bat bug, *Cimex adjunctus*, has been misidentified as being a human ectoparasite (Whyte *et al*, 2001).

There is a paucity of genetic characterization of *Leptocimex* spp reported from Thailand (Apiwathnasorn *et al*, 2011). *L. inordinatus* is a bat ectoparasite found in bat-dwelling caves in Kanchanaburi Province. Identification of *L. inordinatus* depends on morphology and karyotyping. The female *L. inordinatus* is holotype and male allotype. The species genetically is close to *L. vespertilionis* Ferris and Usinger, but differs by the absence of a row of bristles on inner posterior face and fore femora. The first report of *L. inordinatus* was from Matale District, Sri Lanka in 1966 (Ueshima, 1968), but has not been discovered in any other country except Thailand. Molecular identification of *L. inordinatus* has not been conducted, but provide a more definitive confirmation of its identification based on morphological criteria. Thus, this study employed both molecular typing and phylogenetic analysis based on partial nucleotide sequences of 16S mitochondrial ribosomal (mtr)DNA and mitochondrial cytochrome c oxidase subunit 1 (COI) gene (Whyte *et al*, 2001; Szalanski *et al*, 2011) to confirm the morphological identification of *L. inordinatus*.

MATERIALS AND METHODS

Study area

The study sites were bat limestone caves located near the temple, Wat Tham Phromlok Khaoyai (14°12′14.2″N 99°07′54.3″E), Kanchanaburi Province, Thailand containing numerous inner chambers and a horizontal extension of approximately 50 meters. The biggest chamber (40 x 60 x 15 m) has access to sunlight. There are several groups of insectivorous bats in each chamber and also biting insects, *viz* bat fly, tick, mite and bat bug, the latter usually found in the crevices of the caves.

Sample collection

Individual bat bugs were collected using fine forceps and rapidly fixed in 70% ethanol until used. The specimens were characterized and identified according to species as previously described (Usinger, 1966; Apiwathnasorn *et al*, 2011).

PCR-based assay

All specimens were washed three times with phosphate-buffered saline to remove any contamination prior to DNA extraction using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA samples were stored at -20°C until used. *L. inordinatus* specific primers of COI gene used were 5′-GGTCAACAAATCATAAAGATATTGG-3′ and 5′-TAAACTTCAGGGTGACCAAAAAAT-

CA-3') (Folmer *et al*, 1994) and those of 16S mtrDNA were LR-J-13007 (5'-TTACGCT-GTTATCCCTAA-3') (Kambhampati and Smith, 1995) and LR-N-13398 (5-CGCCT-GTTTATCAAAAACAT-3) (Szalanski *et al*, 2008, 2011). PCR was conducted in 50 μl solution containing 0.25 μl of TaKaRa Ex Taq™ DNA polymerase (5 U/μl) (Takara Shuzo, Shiga, Japan), 5.0 μl of 10X buffer, 20 mM Mg^{2+}, 4.0 μl of 200 μM dNTPs, 0.2 μM each specific primer pair and 200 ng of DNA template. Thermocycling was performed in an Eppendorf Mastercycler® pro with vapo.protect (USA Scientific, Orlando, FL) as follows: 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds (for COI gene primers) or 46°C for 45 seconds (16S mtrDNA primers) and 72°C for 60 seconds. Amplicons were analyzed by 2% agarose gel-electrophoresis, visualized by ethidium bromide staining, then purified using QIAquick PCR Purification Kit (Qiagen) and sequenced (Pacific Science, Singapore). As positive control, *C. hemipterus* laboratory strain, maintained at the Arthropod Insectarium of Medical Entomology Department, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand was used (Tawatsin *et al*, 2011). COI gene sequence was submitted to GenBank, accession nos. KT 380159.

Nucleotide sequence and phylogenetic analysis

Nucleotide sequences were compared with available sequences of human bed bugs (*C. hemipterus* and *C. lectularius*), swallow bugs, ticks and mites deposited in GenBank using BLASTn (version 2.2.30) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic relationship was evaluated using K2P parameter (Tamura *et al*, 2011) with 1,000 bootstrap replications, and the neighbor-joining (NJ) and Maximum Likelihood (ML) tree was generated with MEGA (version 6.06) program (Tamura *et al*, 2007). Non-biting fruit fly, *Drosophila melanogaster* (KC750827.1), was used for the out of group.

RESULTS

Characterization of bat bugs collected

L. inordinatus samples were identified based on the following criteria (Usinger, 1966; Ueshima, 1968;): (i) mouthpart not extending beyond the base of front legs, (ii) fore femora without a row of short and stiff bristles on inner posterior face, (iii) antennal segments IV shorter than antennal segment III, and (iv) fringe of setae along lateral margin of pronotum longer than the width of compound eyes. In addition, other external characteristics of *L. inordinatus* include a gray to brownish color, three segments of antenna, wingless, fusion of head and thorax part, oval body shape with flat dorsal-ventral part. Thirty samples were collected from bat cave and used for morphology identification under a light microscope (Fig 1).

Molecular identification of *L. inordinatus* and phylogenetic relationship

Employing primers specific to *L. inordinatus* COI gene and 16S mtrDNA, amplicon of 700 and 420 bp, respectively was generated from DNA extracted from 30 individual samples of *L. inordinatus* specimens (data not shown), and amplicons from 5 randomly chosen samples were sequenced. The partial COI gene sequences were identical and had 97% sequence identity with Cimicidae family members, being most similar to *Cacodminae gen.* sp. and *C. vicinus*. Phylogenetic analysis based on partial COI gene sequences revealed that *L. inordinatus* from this study shares a distinct genetic clade with other cimicid members, and also has an evolutionary history independent from other hematophagous insects, such

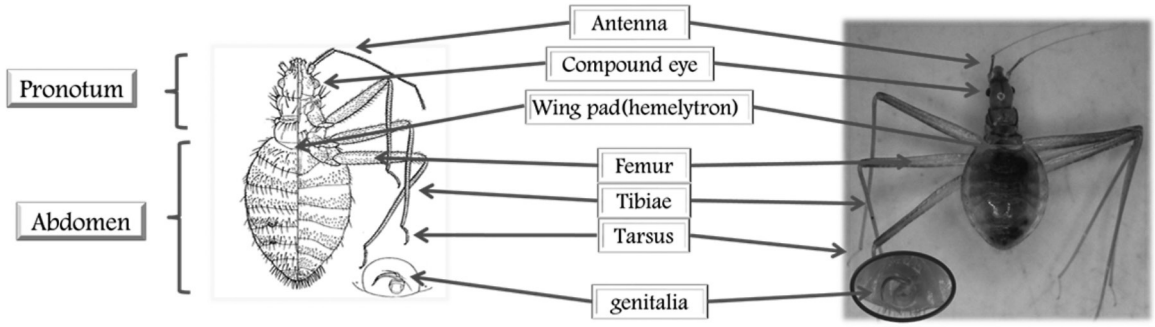

Fig 1–Morphological characteristics of adult male *Leptocimex inordinatus*.

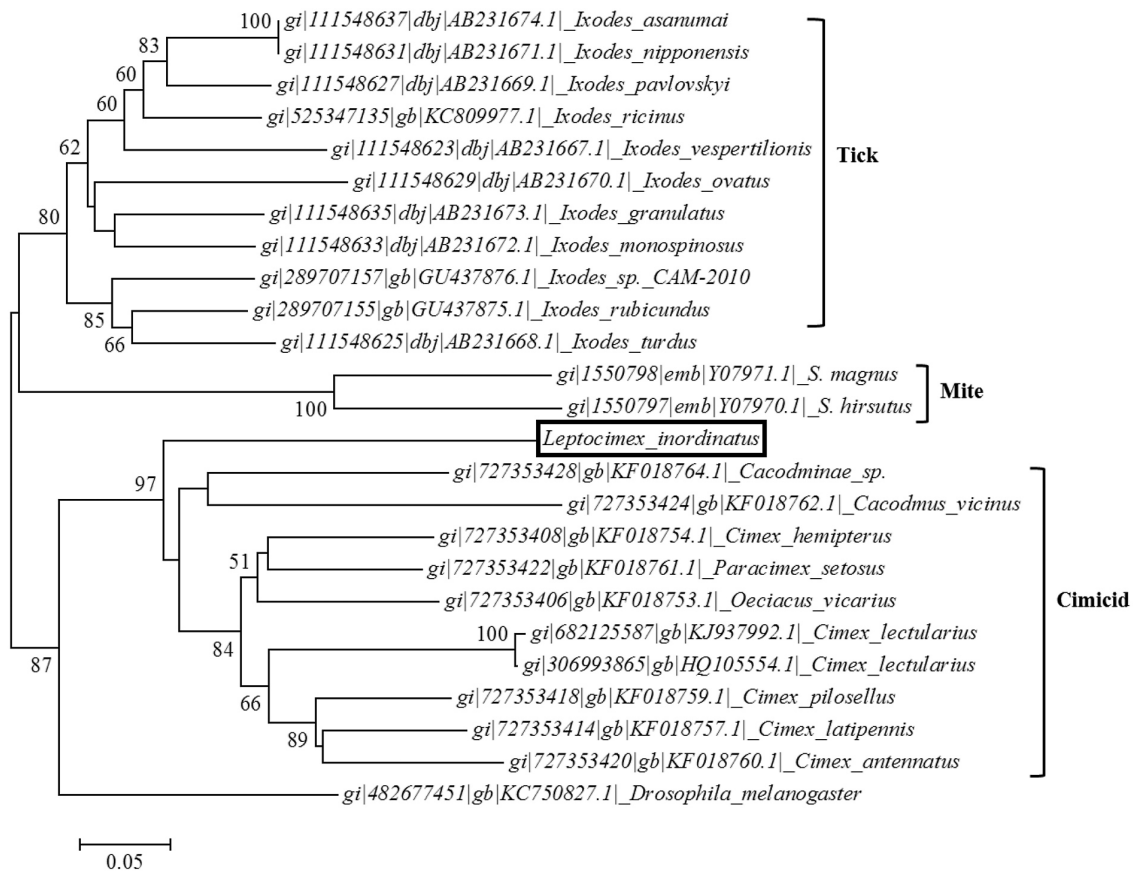

Fig 2–Phylogenetic relationship of *L. inordinatus* (from this study) with other hematophagous ectoparasites and *Cimicidae* members. Neighbor-joining consensus tree was constructed from partial COI gene sequences (from GenBank) using MEGA (V. 6.06) program. The tree is based on 1,000 bootstrap replicates, with homology values < 50% not shown. Number at branch site indicates percent homology. Box designate specimen from the study, GenBank accession number KT 380159.

as ticks and mites (Fig 2). Phylogenetic tree construction based on partial 16S mtrDNA sequences was in agreement with that produced from CO the partial I gene sequences (data not shown).

DISCUSSION

In Thailand, more than 119 bat species has been surveilled and recorded (Bumrungsri *et al*, 2006). *Pteropus* spp, which are natural fruit bats, are considered to be hosts to Hendra and Nipah viruses in Australia and Asia (Hughes, 2014; Sherrini and Chong, 2014; Plowright *et al*, 2015). The wrinkle-lipped bat (*Tadarida plicata* Buchannan) collected in 1976 from a bat cave in Lopburi Province, central Thailand carried two members of Cimicidae family, namely, *Stricticimex parvus* and *Cimex insuetus* (Williams *et al,* 1976). In addition, these ectoparasites were infected with Kaeng Khoi virus, implicating their possible role as vector of this virus (Williams *et al.* 1976). The limestone bat caves of interest in this study often are occupied by monks from a nearby temple meditation practices and some caves are open to tourists (Wacharapluesadee *et al*, 2013). Thus there is a need to determine the presence of bat bugs that could be mistaken for human bed bugs, so that appropriate measures can be taken to prevent their infection and dispersal to the population at large.

Molecular techniques have proven to be a valuable approach to confirm the identification of *L. inordinatus* specimens based on visual examination of morphological characteristics. Mitochondrial COI gene was chosen as one of the target for analysis as its sequence diversity has been shown to be capable of distinguishing insects of closely related species (Hebert *et al*, 2003). Such molecular procedures should be useful in identifying immature *L. inordinatus*, which is similar in body size and color to an immature spider (Usinger, 1966; Apiwathnasorn *et al*, 2011).

Genetic information about *Leptocimex* spp is not available as yet. This study confirmed that *L. inordinatus* from Thailand is a member of the Cimicidae family and its genetic lineage is separated from that of the tropical bed bug *C. hemipterus*. Although our analysis employing short sequence fragments of COI gene and 16S mtrDNA showed that *L. inordinatus* is most similar to *Cacodminae gen.* sp. and *Cacodmus vicinus,* both being bat bugs, other molecular methods for species typing will be needed to confirm these findings (Szalanski *et al*, 2011).

The ability to differentiate bat bugs from other biting insects is important from a public health perspective. Studying bat bug ectoparasites that bite both humans and bats is fundamentally important in advancing our understanding of the diseases transmitted by them. However, bat bug control in Thailand will require many approaches and should be focused on prevention by removal of bat bugs from bat roosting caves. Pathogens, in particular viruses, carried by *L. inordinatus* need to be identified especially from caves frequently visited by humans. Bat bugs will continue to pose a risk to humans and pest management should attempt to learn more about their habitat and biology in the context of prevention, management and control strategies. Distinguishing bat bugs from bed bugs is important also in the study of insecticide resistance (Davies *et al*, 2012).

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