Mitochondrial and Ribosomal Internal Transcribed Spacer 1 Diversity of *Cimex lectularius* (Hemiptera: Cimicidae)

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ABSTRACT Understanding genetic variation among populations of medically significant pest insects is important in studying insecticide resistance and insect dispersal. The bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), is widespread hematophagus insect pest around the world, including North America, and it has recently been identified as an emerging resurgent pest. To date, no studies have been conducted on genetic variation of this species. For this study, 136 adult bed bugs representing 22 sampled populations from nine U.S. states, Canada, and Australia were subjected to genetic analysis using polymerase chain reaction (PCR) to amplify and sequence a region of the mitochondrial DNA (mtDNA) 16S rRNA gene and a portion of the nuclear rRNA internal transcribed spacer (ITS) 1 region. For the 397-bp 16S marker, a 12 nucleotide sites in total were polymorphic, and 19 unique haplotypes were observed. Heterozygosity was observed within many of the sampled populations for the mtDNA marker. This suggests that bed bug populations did not undergo a genetic bottleneck as one would expect from insecticide control during the 1940s and 1950s, but instead, that populations may have been maintained on other hosts such as birds and bats. In contrast to the high amount of heterozygosity observed with the mitochondrial DNA marker, no genetic variation in the 589-bp nuclear rRNA marker was observed. This suggests increased gene flow of previously isolated bed bug populations in the United States, and given the absence of barriers to gene flow, the spread of insecticide resistance may be rapid.

KEY WORDS bed bug, *Cimex lectularius*, genetic variation, mitochondrial DNA

The bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), is a hematophagous insect of humans, and it has regained worldwide attention due to its recent resurgence. Although active dispersal of bed bugs can be important, passive dispersal is almost exclusively their dispersal modus operandi. Because the species is easily translocated by passive dispersal and adapts to multiple hosts (Usinger 1966, Marshall 1981, Lehane 2005) when preferred food sources are unavailable, it is extremely difficult to isolate the origins of recent infestations, even with genetic tools. However, basic molecular biology tools can be applied to elucidate the identities of hosts fed upon by bed bugs, whether for forensic or veterinary applications. Bed bugs are obligate hematophages, with both sexes feeding on blood, and bloodmeals are required before eclosion into subsequent molts (Usinger 1966).

Passive dispersal is the most important way for wingless cimicids to reach new hosts. Bed bugs can be transported by humans in clothing and luggage (Axtell 1999, Boase 2001), and they have been detected on people traveling by airplanes, trains, ships, and cars. In addition, birds and bats are hosts of bed bugs, and they are important in the dispersal of cimicids. Since the late 1970s, bed bugs have undergone a resurgence that became widespread in the late 1990s, and it seems to be global across the developed world (Reinhardt and Siva-Jothy 2007). Reasons for this resurgence may include increased long-range airline travel (Boase 2001), the ability of bed bugs to disperse locally, reduction in the use of residual insecticides around structures, and movement of bed bugs from birds and bats to humans.

Knowledge of genetic variation within medically important insect species is an essential element required for understanding vector transmission, disease epidemiology and disease control (Tabachnick and Black 1995). Although implicated, *C. lectularius* is not commonly known to transmit communicable diseases. However, mechanical transmission of infectious particles of typhus, kala-azar, anthrax, plague, relapsing fever, tularemia, Q-fever, hepatitis B virus, and human immunodeficiency virus have been documented previously (Burton 1963, Usinger 1966, Ryckman et al. 1981, Blow et al. 2001). Conducting genetic studies on *C. lectularius* can provide evidence of their dispersal patterns and population structure, and also provide insight into their movement for forensic purposes (Szalanski et al. 2006). If populations exhibit genetic

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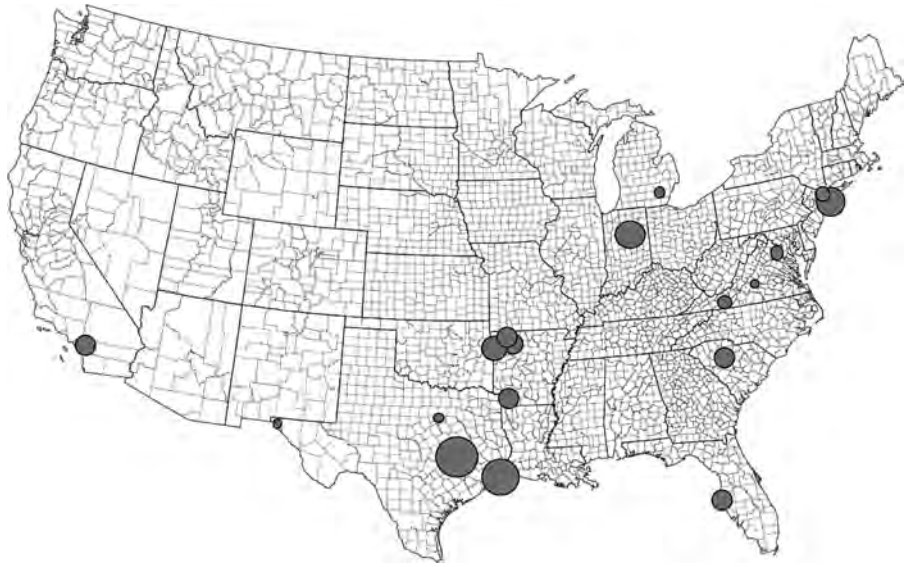

Fig. 1. Bed bug sample locations from the United States. Circle size is proportional to sample size.

distinctiveness, gene flow and bed bug dispersal may be restricted; however, if populations are not genetically distinct, there may be a high level of gene flow, which may indicate that bed bugs are frequently moving among populations. To date, no studies have been conducted on the genetic variation of *C. lectularius* by using either molecular or biochemical methods.

Because nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) are inherited by independent evolutionary means, their combined application to elucidate gene flow of bed bugs can reveal both their sexually and matrilineally derived ancestry, respectively, yielding an important genetic viewpoint to more recent explanations concerning bed bug resurgence. Therefore, the objective of this study was to determine the extent of genetic variation within and among populations of *C. lectularius* from the United States by using both mitochondrial and nuclear DNA markers.

Materials and Methods

In this study, adult and nymphal bed bugs were collected from various locations in the United States, Canada, and Australia from 2005 to 2007, and they were preserved in alcohol, dried, or frozen at −20°C. (Fig. 1; Table 1). Samples were morphologically identified using descriptions outlined by Usinger (1966),

Table 1. Source of bedbug samples

State/country	City or county	Source	Yr collected or colony established	No. bed bugs studied
Arkansas	Madison Co.	Poultry facility	2000	8
	Washington Co.	Poultry facility	2007	5
	Lafayette Co.	Poultry facility	2007	4
	Carroll Co.	Poultry facility	2007	5
Virginia	Montgomery Co.	Lab colony	2006	1
	Arlington Co.	Lab colony	2006	4
	Appomattox Co.	Lab colony	2007	3
South Carolina		Lab colony	2005	4
Michigan	Oakland Co.	Lab colony	2005	12
Indiana	Miami Co.	Lab colony	2006	2
Texas	Brazos Co.	Multidwelling 1	2006	13
	Brazos Co.	Multidwelling 2	2006	15
	El Paso Co.	Single family home	2007	1
	Tarrant Co.	Single family home	2007	2
	Jefferson Co.	Single family home 1	2007	8
	Jefferson Co.	Single family home 2	2005	11
California	Riverside Co.	Lab colony	2007	6
New York	New York	Hotel	2007	10
	Woodside	Single-family home	2005	5
Florida	Clearwater	Single-family home	2005	5
Canada	Toronto, Ontario	Shelter	2005	2
Australia	Sydney	Hospital	2006	4

Table 2. Nucleotide variation and frequency of 19 mtDNA haplotypes

Haplotype	Nucleotide site												
	49	153	161	201	208	213	215	226	256	301	313	321	*n*
1	T	C	T	T	G	C	C	G	G	A	A	A	28
2	.	.	.	.	.	.	.	.	.	.	.	T	4
3	C	.	A	.	.	T	.	A	.	.	.	.	1
4	C	.	.	.	.	.	.	.	.	.	.	.	43
5	C	T	.	.	.	.	.	.	.	.	.	.	3
6	C	.	.	.	.	T	.	.	.	.	.	.	14
7	C	T	.	.	A	.	.	.	.	.	.	.	18
8	C	.	.	A	.	T	T	.	.	.	.	.	1
9	C	.	.	.	.	T	T	.	.	.	.	.	2
10	C	T	.	.	A	.	.	.	.	.	.	T	3
11	.	.	.	.	.	.	T	.	.	.	.	T	2
12	C	.	.	.	.	.	.	.	.	.	.	T	3
13	C	.	.	A	.	.	T	.	.	.	.	.	1
14	C	.	.	.	.	T	.	.	A	.	.	.	2
15	.	.	.	.	.	T	T	.	.	.	.	.	2
16	C	.	.	.	.	.	.	.	A	T	T	T	5
17	C	.	.	.	.	.	.	.	.	.	T	T	2
18	C	.	.	.	.	T	T	.	A	.	.	.	1
19	C	.	.	.	.	.	T	.	A	.	.	.	1

and voucher specimens are deposited in the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville, AR.

DNA was extracted from individual insects using the Puregene DNA isolation kit D-5000A (Gentra Systems, Inc., Minneapolis, MN). Extracted DNA was resuspended in 50 μl of Tris-EDTA and stored at −20°C. Polymerase chain reaction (PCR) was conducted with two primer sets. Primers LR-J-13007 (5′-TTA CGC TGT TAT CCC TAA-3′) (Kambhampati and Smith 1995) and LR-N-13398 (5′-CGC CTG TTT ATC AAA AAC AT-3′) (Simon et al. 1994) amplify a 428-bp fragment of the insect mtDNA 16S rRNA gene. The thermocycler profile was 35 cycles of 94°C for 45 s, 46°C for 45 s, and 72°C for 60 s. For the internal transcribed spacer (ITS) 1 maker, a 3′ portion of 18S rDNA, all of ITS-1, and the 5′ portion of 5.8S was first amplified using the primers rDNA2 (5′-TTGATTACGTCCCTGCCCTTT-3′) (Vrain et al. 1992) and rDNA 1.58S (5′-GCCACCTAGTGAGCCGAGCA-3′) (Cherry et al. 1997) with a thermal cycler profile consisting of 40 cycles of 94°C for 45 s, 53°C for 1 min, and 72°C for 1 min as described by Szalanski and Owens (2003). Due to the large size of the PCR product, ≈1,500 bp, a 3′ primer in the ITS1 region was designed using DNA sequence alignments. This new primer, ITS1-R (5′-TCTCTTTCCGTGTGTTTAAAATGT-3′), combined with rDNA2 results in a 589-bp amplicon. The PCR reactions were conducted with 2 μl of the extracted DNA (Szalanski et al. 2003). The PCR products were resolved on 1% agarose gels.

Amplified DNA from individual bed bugs was purified, concentrated using Microcon-PCR Filter Units (Millipore Corporation, Billerica, MA), and it was sent to University of Arkansas Medical Sciences DNA Sequencing Core Facility (Little Rock, AR) for direct sequencing in both directions. DNA sequences for each unique haplotype were submitted to GenBank, with accession numbers EU126949 to EU126968.

For population genetics analysis, DNA sequences were aligned using Clustal W (Thompson et al. 1994) using BioEdit (Hall 1999). Haplotype distribution between populations, number of haplotypes, number of unique haplotypes, haplotype diversity, and average number of pairwise differences were calculated using DNaSP version 3.51 (Rozas and Rozas 1999). To test for neutral mutation, the D statistics of Tajima (1989)) and Fu and Li (1993) were calculated using DnaSP. Genealogical relationships among haplotypes were constructed using TCS (Clement et al. 2000).

For the phylogeographic analysis of mtDNA haplotyes, DNA sequences from each haplotype were compared with a *C. lectularius* sequence (GenBank AY252754) and a *Cimex* sp. sequence (GenBank AY252702). The sequences were aligned using Clustal W (Thompson et al. 1994), and *Rhodnius prolixus* (Stål) (GenBank AF324519) was used as the outgroup taxon. The best-fitting nucleotide substitution model was chosen according to the general time reversible + gamma (GTR+G) model among 64 different models by using the ModelTest version 3.7 (Posada and Crandall 1998) and PAUP* 4.0b10 (Swofford 2001) programs. Phylogenetic analysis was conducted using maximum parsimony (MP) analysis with the best-fitting evolutionary model as implemented in PAUP*. Bootstrapping was performed using neighbor joining or MP (1,000 replicates) to determine the reliability of the obtained topologies. Phylogenetic trees also were obtained using Bayesian inference with the GTR+G model by using Bayesian Evolutionary Analysis Sampling Trees (BEAST) version 1.4.2 software (Drummond and Rambaut 2003). For Bayesian inference, four Markov chains run for 10^6 generations with a burn-in of 2×10^4 were used to reconstruct the consensus tree.

Results

Analysis of 16S Sequences. A 397-bp region of the mtDNA 16S gene was sequenced from a total of 136 bed bugs collected (Table 1). There was a total of 12 polymorphic sites, of which two were singleton vari-

Table 3. Summary statistics for mtDNA genetic variation[a]

Sample	n	h	Hd	$\pi(k)$	θ_s	θ_g	D^{+}**	F^{+}**	D*
Texas	53	14	0.808 ± 0.045	0.006 (2.30)	0.006	2.204	0.77NS	0.62NS	0.03NS
Total	136	19	0.861 ± 0.019	0.006 (2.19)	0.006	2.292	0.199NS	0.10NS	−0.12NS

* $P < 0.05$; ** $P < 0.02$.

[a] n is number of sequences, h is number of haplotypes, Hd is haplotype diversity ± SD), π is nucleotide diversity, k is mean number of pairwise nucleotide differences, θ_s is theta per site, θ_g is theta per gene, D^{+} and F^{+} are statistics as per Fu and Li (1993), and D is Tajima (1989) statistic.

able sites and 10 were parsimony informative sites (Table 2). The average number of pairwise nucleotide differences was 2.19. The 12 polymorphic sites defined 19 haplotypes. Of these, five were uniquely represented (singletons). Nucleotide diversity, π, was 0.006, and the mean number of pairwise nucleotide differences between haplotypes, k, was 2.19 (Table 3). Fu and Li (1993) D+ and F+, as well as Tajima (1989) D tests of neutrality of mutations against excess of recent mutations were not significant (Table 3). Similar results were observed for the 53 sampled bed bugs from Texas (Table 3). The most common haplotype, designated as "4," was found in 14 locations, and it represented 32% of all sampled individuals (Table 4). Haplotype 4 was not only widespread in the United States, but it was also the only haplotype observed from the Canadian and Australian samples (Table 4). Because it is very common and widespread, haplotype 4 may be the ancestral haplotype (Castelloe and Templeton 1994) among the bed bugs used in this study. This is supported by the 95% parsimony network for the 19 haplotypes (Posada and Crandall 2001; Fig. 2), which revealed that haplotype 4 was the root haplotype for all of the 19 observed haplotypes. Among the four poultry facilities sampled in Arkansas, three haplotypes were observed with haplotype 1 being the most common (Table 4). Haplotype 2 was only found in poultry facilities located in Madison and Washington counties. Bayesian phylogenetic analysis of the 19 *C. lectularius* haplotypes revealed several distinct clades (Fig. 3), however, the posterior bootstrap values were low (0.13–0.57) for these clades, indicating a lack of support.

Table 4. Mitochondrial DNA16S rRNA haplotype frequencies for all *C. lectularius* samples

State/country (sample size)	City or county	Haplotype (frequency)
Arkansas (22)	Madison Co.	1(4), 2(3), 4(1)
	Washington Co.	1(4), 2(1)
	Lafayette Co.	4(4)
	Carroll Co.	1(4), 4(1)
Virginia (8)	Montgomery Co.	3(1)
	Arlington Co.	4(2), 5(1), 12(1)
	Appomattox Co.	4(1), 9(1), 12(1)
South Carolina (7)		4(2), 5(2)
Michigan (12)	Oakland Co.	7(12)
Indiana (2)	Miami Co.	8(1), 9(1)
Texas (53)	Brazos Co. (multidwelling 1)	1(2), 4(7), 11(1), 13(1), 15(2)
	Brazos Co. (multidwelling 2)	4(7), 6(4), 11(1), 12(1), 14(2)
	El Paso Co.	4(1)
	Tarrant Co.	6(2)
	Jefferson Co. (1)	4(1), 6(3), 7(1), 10(3)
	Jefferson Co. (2)	4(3), 6(1), 7(2), 16(4), 18(1), 19(1)
New York (15)	New York	1(10)
	Woodside	4(2), 7(3)
California (6)	Riverside Co.	1(4), 6(2)
Florida (5)	Clearwater	4(1), 6(4)
Canada (2)	Toronto, Ontario	4(2)
Australia (4)	Sydney	4(4)

Analysis of ITS1 Sequences. The ITS1 amplicon was 589 bp, of which, 140 bp was from the 18S rRNA gene, and the remaining 449 bp was from the ITS1 region. From the 136 bed bugs subjected to DNA sequencing for this marker, no polymorphic nucleotide sites were observed.

Discussion

Until now, no studies have been focused on the genetic structure of *C. lectularius* populations. For resolving intraspecific phylogeography, mtDNA has often been the tool of choice (Avise 1994). *C. lectularius* is a highly variable species with respect to mtDNA. We observed 19 haplotypes among the 136 samples examined. Variation was high even within several local populations. Bayesian phylogenetic analysis along with TCS spanning tree analysis did not show strong support for any mtDNA lineages, suggesting that these populations have been recently intermixed. Tajima's D statistic (Table 3) does not indicate an excess of recent or rare haplotypes. This gives support to the conclusion that intermixing as opposed to a sweep or expansion of haplotypes has occurred. The high level of mtDNA diversity found in *C. lectularius* is not typical of insect species with interbreeding populations that have undergone a genetic bottleneck due to insecticide selection pressure. Chlorinated hydrocarbon and other synthetic insecticides were widely used in the 1940s and 1950s (Romero et al. 2007), and bed bugs became so uncommon in the United States that pest control professionals rarely encountered them (Potter 2005). This provides evidence that the source of current bed bugs populations are either 1) introduced to the United States from areas of the world that did not have bed bug insecticide control, or 2) bed bug populations in the United States were maintained by isolated populations found on nonhuman hosts such as birds and bats. Hypothesis 2 is supported by the fact that bed bugs are an important pest of poultry (Usinger 1966, Axtell and Arends 1990, Steelman 2000) and that two of the three of the mtDNA haplotypes observed in the poultry facilities were commonly found in human structures.

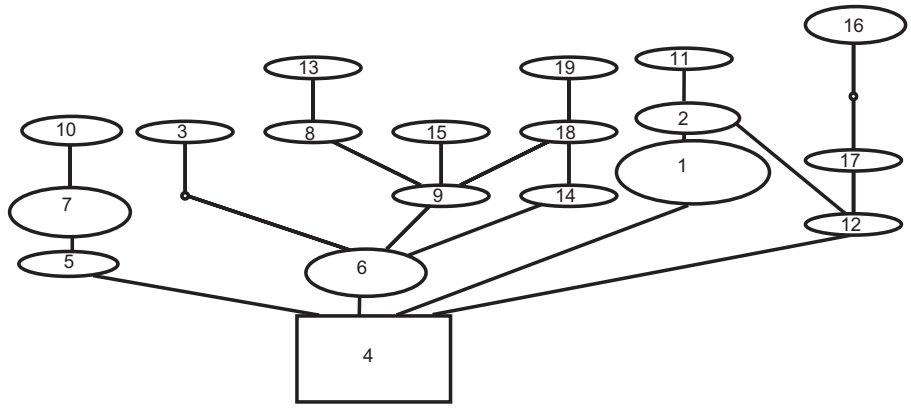

Fig. 2. Genealogical relationship among 19 haplotypes of *C. lectularius* estimated by TCS (Clement et al. 2000). A unit branch represents one mutation and small ovals indicate haplotypes that were not observed. Circle and square size is proportional to sample size.

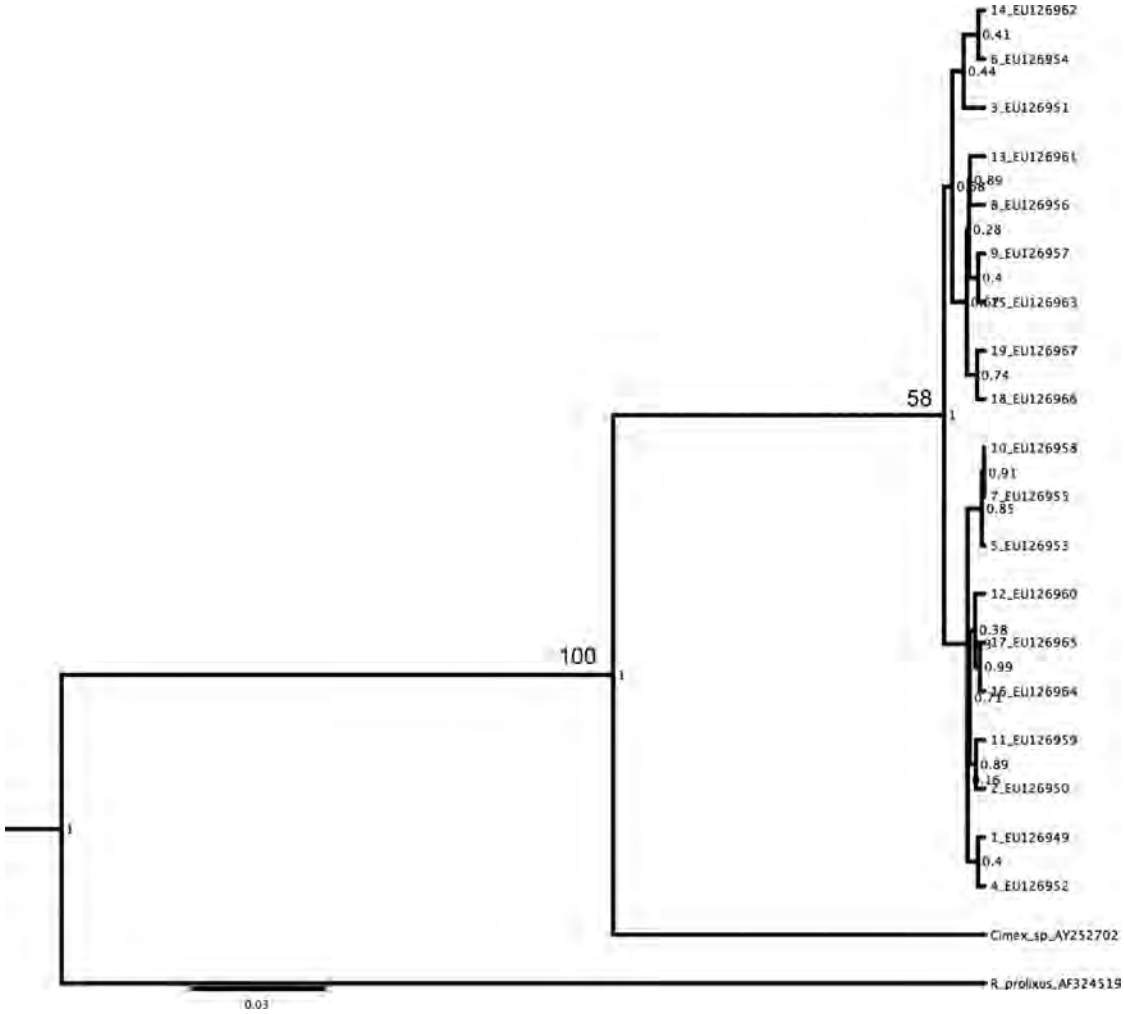

Fig. 3. Phylogenetic relationship of *C. lectularius* mtDNA 16S haplotypes. Numbers at the tree nodes indicate Bayesian posterior probabilities, and numbers above nodes indicate bootstrap values obtained from 1,000 replicates using MP analysis.

Each side of a broiler–breeder house typically has a wooden slatted platform over which the feeders and waters are hung. These wooden slats provide an ideal environment for bed bugs (Fletcher and Axtell 1993) and the number of bed bugs in a single poultry facility can number into the tens of thousands (C.D.S., unpublished data).

In poultry production, bed bugs are transported from infested facilities by human shoes, clothes, egg boxes, production equipment, and vehicles of all types and sizes (Steelman 2000) In addition, wild and domesticated birds and bats are often hosts of *C. lectularius* and are important in their dispersal. Since the late 1970s, bed bugs have undergone a resurgence within the poultry industry that became widespread in the late 1990s, and it seems to be global across the developed world (Reinhardt and Siva-Jothy 2007). One of the most plausible explanations for bed bug resurgence lies in this group's adaptive ability to alternate hosts (Myers 1928; Kemper 1936; Wendt 1939, 1941; Ryckman 1958; Overal and Wingate 1976; Stelmaszyk 1986) and their ability to phoretically translocate with workers in poultry facilities (Jacobs 2005) where they can amass in large numbers (Lyon 1995). The potential of poultry facilities to serve as reservoirs of bed bugs to urban dwellings via poultry workers could be verified by conducting molecular analysis on bed bug bloodmeals to determine host species, and it is currently under development in our laboratory. Furthermore, the ability to readily feed from mixed blood sources (by bed bugs) creates the need to evaluate what potential domestic and nondomestic animals living in proximity to urban dwellings serve to perpetuate endemic resurgences of bed bugs (e.g., in the absence of human hosts what role do pets serve as alternative food sources?).

More support for a recent spread and interbreeding of bed bug populations is a high level of genetic variation observed with mtDNA and a lack of genetic variation observed with nuclear ITS1 sequence data. Two possible explanations for this difference between nuclear and mitochondrial DNA are that 1) gene flow has been maintained among populations by males, or more likely that 2) the introduction and movement of bed bugs by humans during the last 30 yr has augmented gene flow between previously isolated populations. Ueshima (1967) suggests that the loss of supernumerary chromosomes from disjunct populations of *C. lectularius* and its subspecies *C. columbarius* Jenyns from the Old World (where there are more supernumaries) to the New World (where there are fewer) among cimicids, imply a recent evolutionary event that seems to also support our genetic interpretation of the populations evaluated herein. Although active dispersal may play some role in the movement of cimicids to potential hosts (Johnson 1941; Mellanby 1938, 1939), passive dispersal is the most important way for wingless cimicids to reach new hosts (Newberry et al. 1991). Bed bugs can be transported by humans in clothing and luggage (Axtell 1999, Boase 2001), and they have been detected on people traveling by airplanes, trains, ships, cars, and from brief visits in hotels and homeless shelters (Doggett et al. 2004, Hwang et al. 2005, Ryan et al. 2005). In addition birds and bats are hosts of bed bugs and are important in the dispersal of cimicids. Reasons for this resurgence may include increased long-range airline travel (Boase 2001), the ability of bed bugs to disperse locally, reduction in the use of residual insecticides around structures, and movement of bed bugs from birds and bats to humans. This movement between hosts is a remarkable ability for an obligate hematophage such as *C. lectularius* as many other cimicids have a relatively narrow host range, and this likely contributes to the inability to eradicate local populations with any given control measure. Novel calcium- or magnesium-activated plasma membrane-bound enzymes (apyrases), or both, which prevent blood clotting during feeding, have been detected for *C. lectularius* (Valenzuela et al. 1998) and may be one of the reasons for their flexible host range.

High levels of mitochondrial DNA diversity and a lack of nuclear genetic variation is not uncommon, and they has been observed in other arthropods, including the primary screwworm, *Cochliomyia hominivorax* (Coquerel) (Taylor et al. 1996), the mosquito *Anopheles funestus* Giles (Mukabayire et al. 1999), and the twospotted spider mite, *Tetranychus urticae* Koch (Navajas and Boursot 2003). An important consideration in interpreting the ITS1 sequence data are the experimental approach of determining "consensus" sequences by direct sequencing. Implicit in this approach is the assumption that the pace of concerted evolution would be sufficiently rapid to erase mutations shared through recent common ancestry, an assumption that may be flawed on both theoretical and empirical grounds (Dover 1993). It is important to note that *C. lectularius* ITS1 electropherograms were never ambiguous. This suggests that bed bug ITS1 sequences are predominately composed of one consensus type, but it does not rule out one or more rare variants. Although direct sequencing erases information about the sequence and frequency of rare variants and therefore entails a loss of analytical power, it is unlikely to be positively misleading in the patterns it reveals. Also, the ITS1 region in *C. lectularius* may simply be a region of low genetic mutation in this species, possibly due to concerted evolution in ITS1 sequences (Hillis et al. 1991).

Additionally, another possible explanation for high mtDNA diversity between populations with high levels of nuclear gene flow is the presence of different symbiont strains that maintain mtDNA haplotype structure (Hurst and Jiggins 2005). The endosymbiont *Wolbachia* is prevalent in *C. lectularius* (Sakamoto and Rasgon 2006); however, the specific effect of *Wolbachia* on bed bug biology is unknown. Future studies on population genetic structure of *C. lectularius* should involve molecular markers that have the potential to evolve at higher rates than rDNA ITS1 or mtDNA sequences, such as microsatellites.

In conclusion, we show that large genetic differences do not exist between populations of *C. lectularius* applying nDNA sequence analysis, with mod-

erate nucleotide diversity observed applying mtDNA sequence analysis, as would be expected from suppositions supporting a relatively recent resurgence scenario. Especially among geographically disjunct regions, a moderate level of mtDNA variation was observed. This suggests that gene flow among regions is likely moderate to high, which is supported by the lack of isolated mtDNA haplotypes for any given region. Although highly diverged populations still interbreed in the laboratory, the lack of a fixed haplotype for any given geographic location supports hypotheses that imply rapid travel opportunities facilitate population mixing.

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